of the coactivator TRAP220

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Abstract

Nuclear hormone receptor-specific interactions of the coactivator TRAP220

The interaction of coactivator proteins with the ligand-binding domain of nuclear hormone receptors (NRs) is mediated by amphipathic α-helices containing the signature LXXLL motif. The TRAP220 subunit of the metazoan mediator complex contains two LXXLL motifs (LXM1 and LXM2) that are required for its ligand-dependent interaction with NRs. Transient transfection experiments revealed that whilst over-expression of TRAP220 in mammalian cells resulted in enhanced transcriptional activity of the class II NR, thyroid hormone receptor β (TRβ), TRAP220 had little effect on the transcriptional activity of the class I NR, estrogen receptor α (ERα). Subsequent detailed analyses of the NR-binding properties of TRAP220 revealed that it interacts weakly with class I NRs, whereas interaction with class II NRs is strong. By contrast SRC1 bound strongly to both class I and class II NRs. Hence TRAP220 displays NR-class specific binding properties. Interaction assays using LXXLL core motifs (9 amino acids) derived from SRC1 and TRAP220 showed no discriminatory NR-binding preferences. However an extended TRAP220 LXM1 sequence containing amino acids –4 to +9 (where the first conserved leucine of the LXXLL motif is +1) showed selective binding to TRβ and reduced binding to ERα. Taken together this suggested that the amino acids immediately adjacent to the core LXM1 sequence were contributing to the NR-binding selectivity of TRAP220. Mutational analyses revealed that exchange of either TRAP220 extended LXXLL sequences (13 amino acids) with the SRC1 extended LXM2 sequence, strongly enhanced interaction with ERα, and that amino acids within and flanking the LXM1 core sequence cooperated to achieve this change in NR-binding specificity. In contrast, a mutation that increased the spacing between TRAP220 LXM1 and LXM2 had little effect on the binding properties of the nuclear receptor interaction domain (NID). Thus by swapping extended LXXLL sequences, but not by increasing spacing, it is possible to change the NR-binding properties of TRAP220.

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Happy reading.....

Contents

A	adenine
aa	amino acid
AAD	acidic activation domain
ACTR	Activator of Thyroid Receptor
AD	activation domain
ADB	antibody dilution buffer
ADH	alcohol dehydrogenase
AF-1	activation function-1
AF-2	activation function-2
AIB1	Amplified In Breast cancer 1
APS	ammonium persulphate
AR	androgen receptor
ARA	androgen receptor associated protein
ARC	SREBP-interacting complex
ATP	adenosine triphosphate
AT-RA	all-*trans*-retinoic acid
bHLH	basic helix-loop-helix
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
°C	degree Celsius
CARM1	Coactivator-Associated Arginine Methyltransferase-1
CAT	chloramphenicol acetyltransferase
CBP	CREB Binding Protein
ChIP	chromatin-immunoprecipitation
CIAP	calf intestinal alkaline phosphatase
CMV	cytomegalovirus
CNS	central nervous system
COS-1	African green monkey kidney cells
COUP-TF	Chicken ovalbumin upstream promoter-transcription factor
CREB	cAMP response element binding protein
CRSP	cofactor required for Sp1
CSM-X	complete supplement mixture minus X (X represents any aa)
CTD	C-terminal domain (of RNA polymerase II)
C-terminal	carboxy-terminal

dATP	deoxyadenosine 5'-triphosphate
DAX-1	deleted or mutated in X-linked adrenal hypoplasia
DBD	DNA binding domain
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DR1	direct repeat spaced by 1 nucleotide
DR4	direct repeat spaced by 4 nucleotides
DRIP	VDR interacting protein
ds	double-stranded
DTT	dithiothreitol
dTTP	deoxythymidine 5'-triphosphate
E	*Escherichia*
E_2	17β-estradiol
EDTA	diaminoethanetetra-acetic acid
ER	estrogen receptor
EtBr	ethidium bromide
ETS	E-twenty-six (E26) specific sequence
ext	extended
F	phenylalanine
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FTZ-F1	Fushi tarazu receptor
g	centrifugal force relative to gravity
G	guanine
GCN5	general control nonrepressed protein 5
GFP	green fluorescent protein
Glu	glutamic acid
GR	glucocorticoid receptor
GRIP1	GR interacting protein 1
GST	glutathione S-transferase
h	human
HA	haemagglutinin
HAT	histone acetyltransferase

HBS	HEPES-buffered saline
HDAC	histone deacetylase
HeLa	Henrietta Lacks
HMT	histone methyltransferase
HNF-4	Hepatocyte nuclear factor-4
hr	hour(s)
HRE	nuclear hormone receptor response element
HRP	horseradish peroxidase
hsp	heat shock protein
IgG	immunoglobulin class G
IMS	industrial methylated spirit
IPTG	isopropyl-β-D-thiogalactopyranoside
IR	inverted repeat
kb	kilobase(s)
K	lysine
kDa	kilodalton
L	leucine
LB	Luria Bertani broth
LBD	ligand-binding domain
leu	leucine
LUC	luciferase
LXM	LXXLL motif
M	molar
mCi	millicurie
mM	millimolar
MAP	mitogen-activated protein
MAPK	MAP kinase
MED	Mediator
MEF	mouse embryonic fibroblast
mg	milligram
min	minute
ml	millilitre
mm	millimetre
MR	mineralocorticoid receptor
mRNA	messenger RNA
msec	millisecond(s)
mut	mutant

mw	molecular weight
NAT	negative regulator of activated transcription
NcoA	nuclear receptor coactivator
NcoR	nuclear receptor corepressor
NETN	NP-40-, EDTA-, Tris-, NaCl-containing buffer
NF-κB	Nuclear factor-κB
ng	nanogram
NGFI-B	Nerve growth factor induced factor-B
NID	nuclear receptor interaction domain
NLS	nuclear localisation signal
nm	nanometre
nmol	nanomolar
NMR	nuclear magnetic resonance
NR	nuclear hormone receptor
NRF-1	Nuclear respiratory factor-1
NRIF3	Nuclear receptor interacting factor 3
NSD1	NR-binding SET-domain containing protein-1
N-terminal	amino-terminal
nTRE	negative TRE
Oct-2	Octamer-binding protein
OD	optical density
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PAS	Period/Aryl hydrocarbon receptor/single-minded
PBP	PPARγ binding protein
PBS	phosphate buffered saline
PC	positive cofactor
PCR	polymerase chain reaction
pCAF	p300/CBP associated factor
pCIP	p300/CBP co-integrator associated protein
PEG	polyethylene glycol
PELP-1	proline-, glutamic acid-, leucine-rich protein-1
PERC	PGC-1 related ER coactivator
PGC-1	PPARγ coactivator-1
PGK	phosphoglycerate kinase
PIC	pre-initiation complex

PML	promyelocytic leukaemia
Pol II	RNA polymerase II
PPAR	peroxisome proliferator activated receptor
PR	progesterone receptor
PRMT	protein methyltransferase
Q	glutamine
RAC3	Receptor associated coactivator 3
RAR	retinoic acid receptor
RARE	RAR response element
rGH	rat growth hormone
RGR1	repressor of glucose-regulated genes
RIPA	radioimmunoprecipitation buffer (for cell lysis)
RIP140	Receptor interacting protein 140 kDa
RNA	ribonucleic acid
RPD3	reduced potassium dependency 3
rpm	revolutions per minute
RRM	RNA recognition motif
RSV	Rous Sarcoma virus
RXR	retinoid-X receptor
S	svedberg unit
[^{35}S]	sulphur-35
SDS	sodium dodecyl sulphate
sec	second(s)
SET	suppressor of variegation, enhancer of Zeste and Trithorax
SID	SRC1 interaction domain
SIR2	silent information regulator 2
SMCC	SRB/MED-containing cofactor complex
SMRT	Silencing mediator for retinoic acid and thyroid hormone receptor
SNF	sucrose non-fermenting
SNURF	small nuclear RING finger protein
SOH1	suppressor of hpr1
Sp1	specificity protein 1
SRB	Suppressor of RNA polymerase B
SRC1	Steroid receptor coactivator 1
SREBP	sterol regulatory element binding protein
ss	single-stranded
SUN-CoR	Small ubiquitous nuclear corepressor

SUR	suppressor of ras
SV40	Simian virus 40
SWI	mating type SWItching
T	thymine
T_3	3,3',5-Triiodo-$_L$-Thyronine
TAFs	TBP-associated factors
TBE	Tris-borate EDTA
TBP	TATA-binding protein
TC	tissue culture
TE	Tris EDTA
TF	transcription factor
TFB	bacterial transformation buffer
TIF1	Transcription intermediary factor 1
TIF2	Transcription intermediary factor 2
Tip60	Tat-interacting protein 60
TK	thymindine kinase
TR	thyroid hormone receptor
TRAM1	Thyroid receptor activator molecule 1
TRAP	Thyroid hormone receptor associated protein
TRBP	thyroid receptor binding protein
TRE	TR response element
Trip	Thyroid receptor interacting protein
TRITC	tetramethyl rhodamine isothiocyanate
trp	tryptophan
TRUP	Thyroid receptor uncoupling protein
TSHβ	thyroid stimulating hormone β
USA	upstream stimulatory activity
V	volt(s)
VDR	Vitamin D3 receptor
VDRE	VDR response element
VP16	virus protein 16
v/v	ratio of volume to volume
W	tryptophan
wt	wildtype
w/v	ratio of weight to volume
X	used to represent any amino acid
YPG	yeast-peptone-glucose media

μF	microFarad
μg	microgram
μl	microlitre
μM	micromolar
9c-RA	9-*cis*-retinoic acid

CHAPTER 1
INTRODUCTION

1.1 Gene Expression: Eukaryotic Transcription

Organisms are continually exposed to changes in both their internal and external environments. In order to survive they must respond appropriately to these changes and this is achieved by the modulation of expression of their genes. The process of gene expression, whereby the genetic information encoded by DNA is used to produce specific proteins, is regulated at multiple stages including transcription, RNA processing, RNA transport (from the nucleus to the cytoplasm) and translation. However, this study concentrates upon gene expression at the level of transcription and hence the other processes involved in gene expression, and its regulation, will not be discussed here. Eukaryotic gene transcription involves copying the protein coding DNA into an RNA transcript. A plethora of proteins are involved in transcription and its regulation, and often function in response to specific cellular stimuli (Roeder, 1996 and Kaiser *et al.,* 1996). Hence depending on the stimuli, different cell types can actively transcribe or repress different gene networks and thus become specialised for their particular role in the functioning of the organism.

Transcription of a particular gene is dependent on an array of DNA regulatory sequences primarily located near the transcription start site, and known as the gene promoter Lee *et al.,* 2000). Many eukaryotic protein-encoding genes contain the canonical TATA sequence in their promoters, which specific proteins are able to recognise and bind, to facilitate transcription. The TATA box is often positioned at –25 (relative to the transcription start point) and has the consensus 'TATATAATA'. Promoter sequences are responsible for determining the basal level of transcription of the gene and its response to specific stimuli. A multitude of proteins, collectively known as transcription factors (TF), are able to bind to specific sequences within the gene promoter and either activate or repress transcription. Transcription factors can be largely divided into two broad classes, namely basal transcription factors and regulatory transcription factors. The latter serve to modulate transcription and will be discussed in later sections of this study. The basal transcription factors are typically defined as the minimum complement of proteins that are required to reconstitute accurate transcription from a minimal promoter such as a TATA box.

1.2 Transcription Initiation

In order for basal transcription of protein-encoding genes to occur, RNA polymerase II and the basal transcription factors must assemble on the gene promoter, thus forming a

transcription pre-initiation complex (PIC) (Figure 1.1.). Initially, the TATA-binding protein (TBP) subunit of the basal factor TFIID complex, recognises and binds to the TATA box within the promoter region of the target gene. Some of the other subunits of the TFIID complex are referred to as TBP-associated factors (TAFs), since they are required to mediate the activation of transcription. Next TFIIA binds directly to the TBP, thus stabilising the TFIID-TATA complex and forming a committed complex. TFIIB then binds directly to the TFIIA/TFIID-TATA complex via direct association with TBP and the backbone of the DNA and is thought to act as a bridging factor between RNA polymerase II and the TFIIA/TFIID-TATA complex. The N-terminal end of TFIIB extends downstream from the TATA box and is thought to determine the transcription start site, whilst the remainder of the TFIIB molecule provides landing sites for TFIIF, RNA polymerase II and additional TAFs. Finally, TFIIE, TFIIH and TFIIJ bind to this complex, prior to the RNA polymerase II moving away to begin transcribing the target gene. TFIID and TFIIA remain bound to the TATA box ready to permit further rounds of transcription. The TFIIH subunit of the PIC possesses kinase activity that is activated by TFIIE and permits phosphorylation of the C-terminal domain (CTD) of the RNA polymerase II. The phosphorylated form of RNA polymerase II binds less strongly to the PIC and hence is allowed to move away to catalyse the elongation phase of transcription.

Although the basal factors can assemble on the promoter in a stepwise manner *in vitro*, there is evidence to suggest that many of the TF interactions can occur in the absence of DNA and that some factors may pre-assemble into an RNA polymerase II 'holoenzyme' (for review see Greenblatt, 1997 and Roberts, 2000). Indeed it has been reported that following the binding of TFIID and TFIIA to the core promoter element, a pre-assembled RNA polymerase holoenzyme complex may bind, thus reducing the number of steps required to form a functional PIC (Kim *et al.*, 1998).

1.3 Control of Gene Expression

1.3.1 Chromatin Structure

The genome of eukaryotes is highly compacted into the nucleus of the cell by folding in a complex with specific nuclear proteins into a structure known as chromatin. These specific nuclear proteins are called histones and they have a high proportion of positively charged amino acids that can neutralise the net negative charge of the DNA, thus allowing folding to occur (for review see Lee *et al.*, 2000).

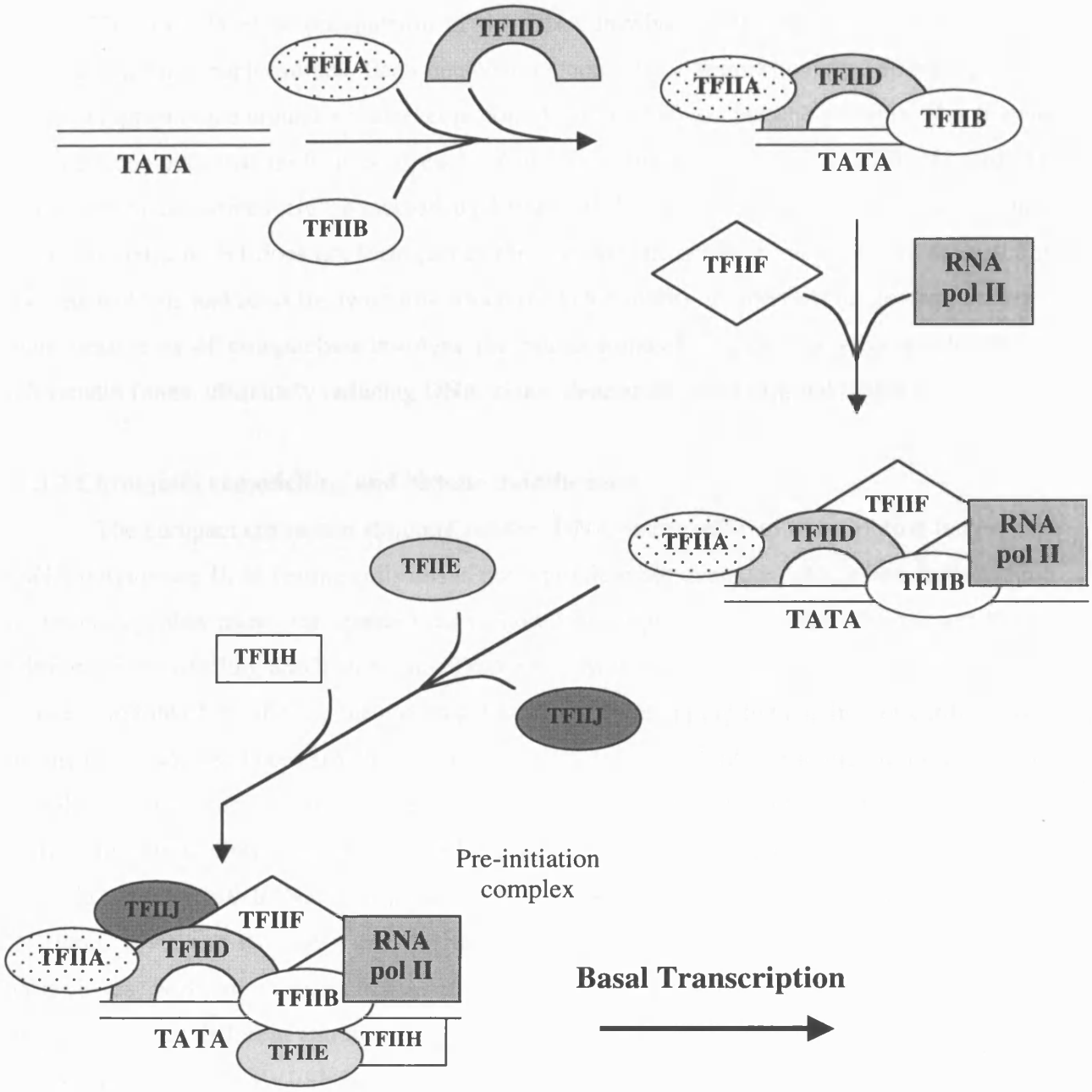


Figure 1.1 Stepwise assembly of the basal transcription factors and RNA pol II required for the initiation of transcription. Additional TAFs have been omitted for clarity. Following recruitment of TFIID and TFIIA to the promoter, the formation of the pre-initiation complex occurs via the sequential recruitment of TFIIB, PolII/TFIIF and TFIIH/TFIIE/TFIIJ. Phosphorylation of the CTD of RNA polymerase II, by TFIIH, releases it from the pre-initiation complex and initiates transcription. Adapted from Roberts, 2000.

The first level of compaction of the DNA involves wrapping the DNA around the histones to form nucleosomes. Each nucleosome consists of approximately 146 base pairs of DNA wrapped twice around a central core complex of eight basic histone proteins. This histone octamer contains two molecules of each of the four core histones, H2A, H2B, H3 and H4. Adjacent nucleosomes are connected by linker DNA, thus creating a 'beads on a string' structure. Histone H1 does not form part of the core histone octamer but is present at one copy per nucleosome and seals the two turns which the DNA makes around the core histone octamer. The next level of compaction involves the nucleosomes being further wound into 30 nm chromatin fibres, ultimately reducing DNA to one thousandth of its original length.

1.3.2 Chromatin remodelling and histone modification

The compact chromatin structure renders DNA inaccessible to transcription factors and RNA polymerase II. In resting cells this is not a problem but in active cells, where transcription is necessary, this raises the question as to how transcription is initiated. The answer lies in chromatin remodelling and histone modifications. Chromatin remodelling is an ATP-dependent process and involves altering the chromatin structure by changing the location or conformation of the nucleosomes. These structural changes do not involve covalent modifications and can be involved in both transcriptional repression and activation. Many different ATP-dependent chromatin remodelling complexes have been identified, but the two best characterised classes include the yeast SWI/SNF family and the ISWI-based family (for reviews see Kingston and Narlikar, 1999, and Kornberg and Lorch, 1999). These two classes of chromatin remodelling complexes perform similar types of ATP-dependent remodelling reactions but their mechanisms are different and they may exert their remodelling actions on different portions of the nucleosome. The SWI/SNF family can alter the histone-DNA contacts, such that the path of the DNA around the histone octamer is rearranged, thus exposing specific DNA sequences, or alternatively the SWI/SNF family can physically transfer histones from one section of DNA to another. The ISWI family of complexes function by loosening the histone-DNA contacts in such a way as to permit the nucleosome to move or 'slide' on the 'beads on a string' chromatin structure. Thus chromatin remodelling complexes assist in making the DNA more accessible to transcription factors.

A diverse array of post-translation modifications, including acetylation, phosphorylation, methylation and ubiquitination, can occur on the N-terminal 'tails' of the

histones (for reviews see Strahl *et al.*, 2000, Berger *et al.*, 2002 and Kouzarides, 2002). Of these modifications, histone acetylation is the best characterised, involving the ε-amino group of specific lysine residues within the N-terminal tails of the core histones being acetylated. This results in a neutralisation of the positive charge of the lysine residue and is thought to weaken the interaction between the histone tails and the DNA, thus creating an open chromatin structure that is more accessible to transcription factors (Figure 1.2). Histone acetylation is catalysed by a family of enzymes known collectively as histone acetyltransferases (HAT) and the reverse of this reaction, histone deacetylation, is catalysed by a family of enzymes called the histone deacetylases (HDAC). HATs transfer the acetyl moiety from the acetyl coenzyme A to the ε-amino groups of lysine residues and HDACs remove this acetyl group in the opposing deacetylation reaction. Thus histone acetylation/deacetylation is a reversible process and is maintained in dynamic equilibrium by the actions of HATs and HDACs. Several transcription regulators have been found to possess intrinsic HAT and HDAC activities, thus suggesting histone acetylation and deacetylation plays a role in regulating transcription (Ito *et al.*, 2002a). There is evidence to suggest that increased histone acetylation (hyperacetylation) results in increased gene transcription, whereas a decrease in histone acetylation (hypoacetylation) results in a reduced rate of transcription (Workman and Kingston, 1998).

In recent years advances have been made in our understanding of histone methylation and its role in the regulation of transcription (for review see Kouzarides, 2002). A number of methyltransferase enzymes have been discovered, including lysine methyltransferases and arginine methyltransferases. The first histone methyltransferase (HMT) to be characterised was SUV39 (suppressor of position effect variegation 39), which specifically methylates lysine 9 (K9) of histone H3 (Rea *et al.*, 2000). Subsequently other lysine HMTs were discovered including those which specifically methylate lysine 4 (K4) of histone H3. All lysine HMTs contain a SET (Suppressor of variegation, Enhancer of Zeste and Trithorax) domain that is necessary but not sufficient for lysine HMT activity. Cysteine-rich sequences flanking the SET domain are additionally required to permit activity. Studies in yeast revealed that K9 of H3 is methylated in condensed inactive chromatin, whereas K4 methylation occurs in actively transcribed regions (Norma *et al.*, 2001). Hence depending on the specific lysine residue involved, histone methylation is associated with both transcriptional activation (K4 of H3) and repression (K9 of H3). The exact mechanisms of repression and activation due to histone methylation are not yet clearly understood.

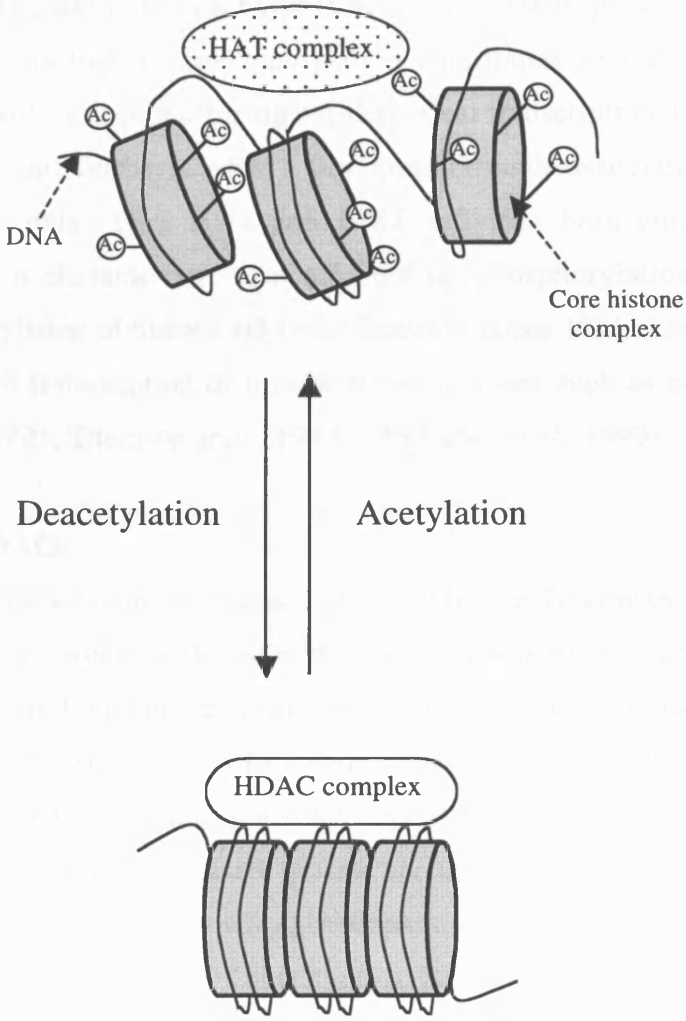


Figure 1.2 Acetylation of core histone proteins as a mechanism of transcriptional regulation. Acetylation of specific lysine residues of the core histone proteins, by HAT complexes, reduces their positive charge resulting in a destabilisation of the DNA/histone interaction. Thus transcription factors and RNA polymerase II gain access to the DNA and transcription can occur. Conversely, HDAC complexes catalyse deacetylation of the core histone proteins, retaining the compact chromatin structure and hence reducing/preventing access to the DNA. Adapted from Ito *et al.*, 2002.

The discovery of the nuclear receptor coactivator interacting protein, CARM1 (Coactivator-Associated arginine Methyltransferase-1), which possesses arginine-specific, histone H3-selective methyltransferase (HMT) activity (Chen *et al.,* 1999b) provided further evidence to support the hypothesis that histone methylation contributes to transcriptional activation. CARM1 HMT activity is required for ligand-dependent transcriptional activation by nuclear hormone receptors and further CARM1 functions through association with coactivators possessing HAT activity. Thus HAT and HMT activities both contribute to increasing chromatin fluidity, a characteristic also affected by phosphorylation of histone tails. In particular, phosphorylation of histone H3 (specifically at serine 10) has been directly correlated with the initiation of transcription of immediate-early genes such as c-*jun*, c-*fos* and c-*myc* (Mahadevan *et al.,* 1991, Thomson *et al.,* 1999 and Chadee *et al.,* 1999).

1.3.3 HATs and HDACs

HATs can be grouped into two classes (for review see Workman and Kingston, 1998). These are type A HATs, which are found in the nucleus and acetylate nucleosomal histones and type B HATs, which are found in the cytoplasm and acetylate free cytoplasmic histones prior to chromatin assembly. The first type B-HAT gene to be cloned encoded the yeast HAT1 protein (Kleff *et al.,* 1995). HAT1 represents the major type B-HAT activity in the cytoplasm. Several type A HATs have been characterised to date including the GCN5 family in yeast, with homologues in *Tetrahymena, S. cerevisiae*, Drosophila and human (Brownell *et al.,* 1996, Kuo *et al.,* 1996 and Yang *et al.,* 1996). Other type A HATs include CBP/p300 (Ogryzko *et al.* 1996) (see section 1.7.1), human $TAF_{II}250$ and its homologues in yeast, $TAF_{II}130$, and Drosophila, $TAF_{II}230$ (Mizzen *et al.,* 1996), the MYST family (named after its founding members, MOZ (monocytic leukaemia zinc finger protein), YBF2/SAS3 (something about silencing), SAS2 and Tip60 (Tat-interacting protein)) and members of the p160 family of nuclear receptor coactivator proteins (see section 1.7.3). $hTAF_{II}250$ and its homologues are the largest subunit of the TAF complex and are thought to serve as a scaffold for the entry of other TAFs to TFIID. Thus their intrinsic HAT activity may allow TFIID to gain access to the promoter via the modification of nucleosomal TATA elements (Mizzen *et al.,* 1996).

HDAC proteins are grouped into three classes according to their homology to yeast HDACs. Class I HDACs include mammalian HDAC1 and HDAC2, which are homologous to the yeast Rpd3 protein (Taunton *et al.,* 1996) and have been found to be components of the

Sin3 corepressor complex (see section 1.5.6). Class II HDACs are related to the yeast Hda1 protein and include mammalian HDAC4, HDAC5 and HDAC6. Class III HDACs are homologous to the yeast protein Sir2 and recently two complexes have been identified which contain class III HDACs (Yang *et al.*, 2002).

1.4 Regulation of Transcription

Whilst the PIC is essential for transcription, it is only capable of facilitating transcription at a low rate, often referred to as the basal level of transcription. Upstream of the TATA box there are a number of other conserved sequences that play important roles in controlling the rate of transcription. Regulatory transcription factors, distinct from the basal transcription factors, are able to recognise and bind to these conserved sequences and exert either a negative (repressors) or positive (activators) influence over the level of transcription. Indeed the balance between binding of transcriptional activators and repressors will determine the rate of transcription of a specific gene in any particular situation. Most promoters contain several of these conserved sequences upstream of the TATA box allowing the rate of transcription of a particular gene to be varied in an exquisitely sensitive manner.

1.4.1 Repression of Transcription

Although it was originally thought that most eukaryotic regulatory transcription factors positively activated transcription, it is now clear that a wide range of transcription factors exert an inhibitory effect on transcription (for review see Clark *et al.*, 1993). Transcriptional repression is frequently brought about by a transcriptional repressor protein interfering with the activity of a positively acting transcription factor, thus blocking its stimulatory effect on transcription. The repressor protein may interfere with an activating factor by inhibiting its binding to DNA. For example, the repressor and activating transcription factors may have overlapping DNA binding sites, thus competition between the two proteins for DNA binding would lead to attenuation of transcription (Figure 1.3A). Also the activator and repressor proteins may form a complex in solution, thereby preventing the activator binding to the DNA (Figure 1.3B). Alternatively, the repressor may interfere with the activation of transcription mediated by the activator, by binding to the DNA-bound activating factor and masking its activation domain (discussed in section 1.4.2), thus preventing interaction with the basal transcription machinery and its associated factors (Figure 1.3C).

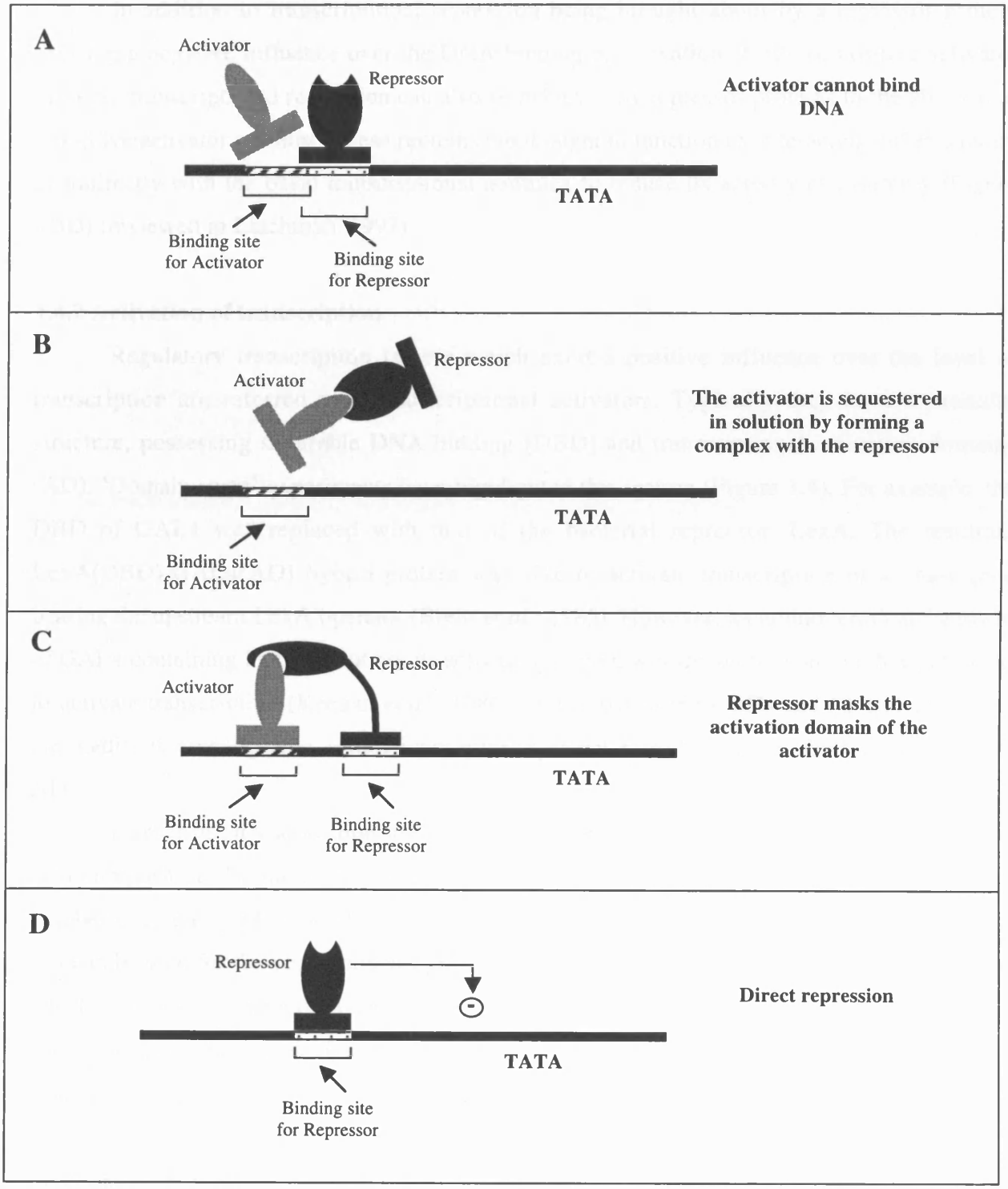


Figure 1.3 Mechanisms of Transcriptional Repression by Transcription Factors. (A) Activator and repressor proteins compete for binding to overlapping regulatory DNA sequences, (B) activator is sequestered into solution, (C) repressor binds the DNA-bound activator and blocks its AD, (D) repressor directly inhibits transcription in the absence of activator protein.

In addition to transcriptional repression being brought about by a repressor protein exerting a negative influence over the DNA binding or activation ability of positive activator proteins, transcriptional repression can also be achieved by repressor proteins in the absence of a positive activator proteins. These proteins are thought to function by interacting either directly or indirectly with the basal transcriptional complex to reduce its activity or assembly (Figure 1.3D) (reviewed in Latchman, 1997).

1.4.2 Activation of transcription

Regulatory transcription factors which exert a positive influence over the level of transcription are referred to as transcriptional activators. Typically they have a modular structure, possessing separable DNA binding (DBD) and transcriptional activation domains (AD). 'Domain swap' experiments have highlighted this feature (Figure 1.4). For example, the DBD of GAL4 was replaced with that of the bacterial repressor, LexA. The resultant LexA(DBD)-GAL4(AD) hybrid protein was able to activate transcription of a yeast gene bearing the upstream LexA operator (Brent *et al.,* 1985). However, an amino terminal fragment of GAL4 containing its DBD but not its activating region, was shown to bind to DNA but failed to activate transcription (Keegan *et al.,* 1986). Hence whilst the DBDs and ADs of activators are readily interchangeable, a functional transcriptional activator requires both a DBD and an AD.

Transcriptional activation domains are grouped into classes according to their amino acid composition. Initial studies revealed that many activation domains contain a significant number of acidic residues and hence have a net negative charge (Ma *et al.,* 1987). Examples of this can be seen for the yeast activator protein, GCN4, which has a net charge of –16 and GAL4 which contains two negatively charged regions (of ~100 amino acids each), either of which is capable of activating transcription when tethered to DNA. Similarly, the herpes simplex virus protein, VP16, which is responsible for activating transcription of immediate early genes in virally infected cells, contains a highly acidic amino acid sequence within its C-terminus which functions as a potent activating region (Sadowski *et al.*, 1988). These acidic domains were predicted to form amphipathic α-helices, where the acidic residues align on one face of the α-helix and hydrophobic residues align on the other face (Giniger *et al.,* 1987 and Hope *et al.,* 1988).

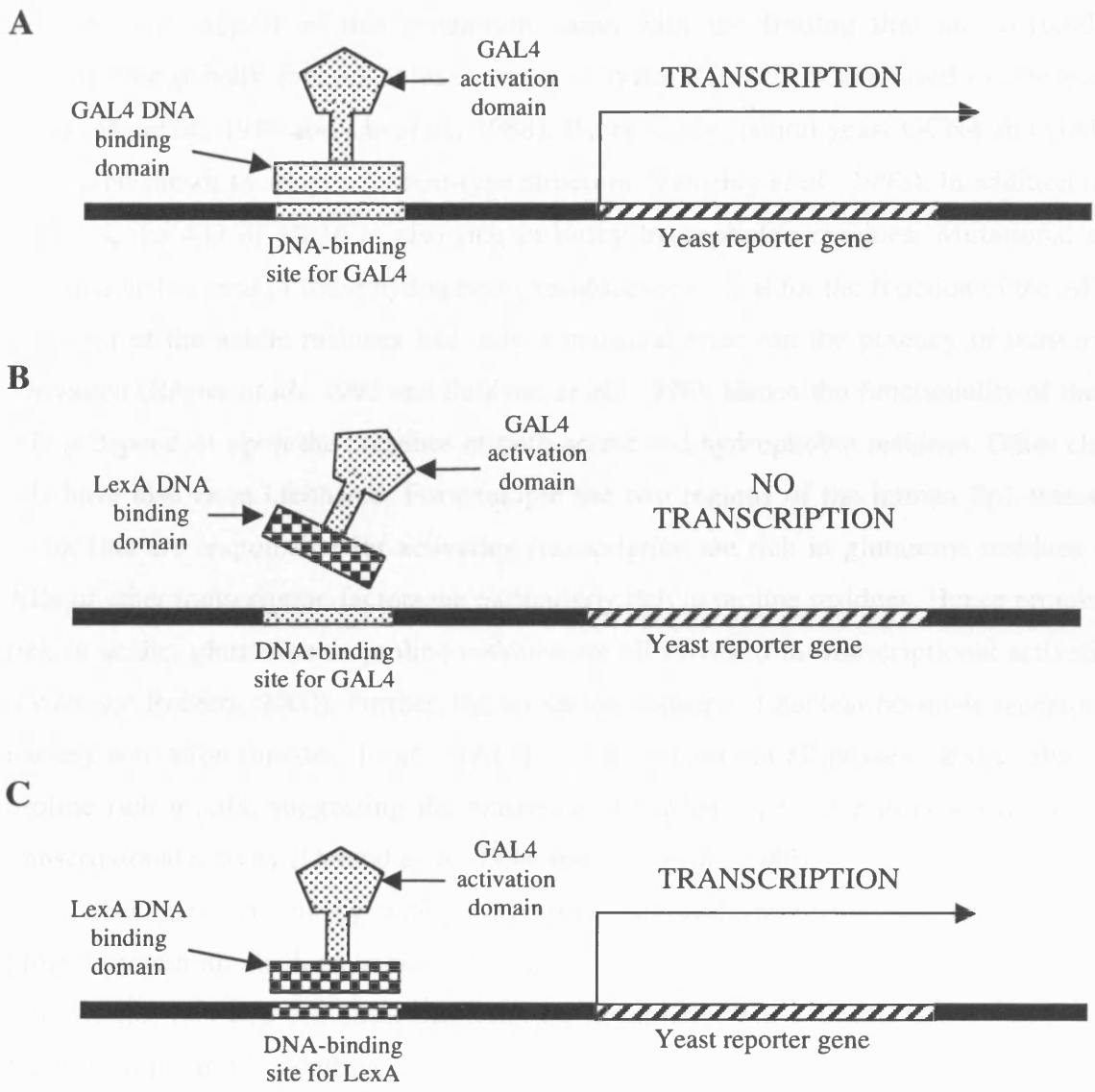


Figure 1.4 A 'Domain swap' experiment illustrating the separable nature of the DNA-binding (DBD) and activation domains (AD) of transcription factors. (A) The wildtype GAL4 protein is able to bind to the GAL4 DNA binding site and activate transcription of the yeast reporter gene, whilst the chimeric transcription factor (B), consisting of the DBD of LexA and the AD of GAL4, is unable to bind to the GAL4 DNA binding site and hence transcription is not activated. (C) The chimeric transcription factor is able to bind to the LexA DNA binding site and hence transcription is activated.

Evidence in support of this prediction came with the finding that an artificial acidic amphipathic α-helix functioned as a potent activation domain when fused to a heterologous DBD (Ma *et al.*, 1988 and Lin *et al.*, 1988). However the natural yeast GCN4 and GAL4 ADs have been shown to adopt a β sheet-type structure (Van Hoy *et al.*, 1993). In addition to acidic residues, the AD of VP16 is also rich in bulky hydrophobic residues. Mutational analysis revealed that several of these hydrophobic residues are critical for the function of the AD, while mutation of the acidic residues had only a marginal effect on the potency of transcriptional activation (Regier *et al.*, 1993 and Sullivan *et al.*, 1998). Hence the functionality of this acidic AD is dependent upon the presence of both acidic and hydrophobic residues. Other classes of AD have also been identified. For example the two regions of the human Sp1 transcription factor that are responsible for activating transcription are rich in glutamine residues and the ADs of other transcription factors are particularly rich in proline residues. Hence protein motifs rich in acidic, glutamine or proline residues are all involved in transcriptional activation (for review see Roberts, 2000). Further, the activation domains of nuclear hormone receptors (NR), namely activation function 1 and 2 (AF-1 and AF-2), do not all possess acidic, glutamine or proline rich motifs, suggesting the existence of further types of motifs which can support transcriptional activity (Durand *et al.*, 1994 and Tora *et al.*, 1989).

The mechanisms by which activators stimulate transcription are dependent upon protein-protein interactions between the AD of the activator and other activators, coactivators (see section 1.7) and the basal transcription machinery. For example activators are able to recruit chromatin remodelling activities to the promoter via their AD, thus destabilising local nucleosomes and facilitating the further assembly of transcriptional regulators and recruitment of the basal transcription machninery. Studies of several transcriptional activators have identified interactions between ADs and many basal transcription factors including TFIIA, TBP, TFIIB, TFIIF and TFIIH (Triezenberg *et al.*, 1995). Although all of these interactions may not be physiologically relevant, it is probable that activator interactions with many different components of the RNA polymerase II transcription machinery can enhance transcription. Experiments utilising fusion proteins have shown that increasing the concentration of the transcriptional machinery at the promoter results in increased transcription. For example, direct fusion of TBP to a heterologous DBD produces high levels of transcription from promoters with the appropriate DNA binding sites (Chatterjee *et al.*, 1995). Artifical recruitment of several other basal transcription factors to the promoter also leads to activated

levels of transcription (for review see Ptashne *et al.*, 1997). Hence many activators exert a positive effect on transcription by increasing the affinity of the transcription machinery for the promoter without otherwise modifying its activity. Interaction with an activator has also been found to promote a conformational change that enhances binding of the transcriptional machinery to the promoter. Studies of the interaction of the TFIID and the yeast transcription factor GAL4 on a promoter containing both a TATA box and binding sites for GAL4, have shown that whilst the binding of TFIID to the TATA box is not altered in the presence of GAL4, its conformation is. In the absence of GAL4, TFIID binds only to the TATA box but in the presence of GAL4, TFIID binding was detected over both the TATA box and the transcription start site, thus stimulating transcriptional activity (Horikoshi *et al.*, 1988).

1.4.3 Regulation of Transcription Factors

The regulation of transcription factors is necessary to maintain the precise expression patterns of genes, whether they be ubiquitiously expressed house-keeping genes or genes expressed in a tissue-specific manner or in response to a particular signal. Transcription factors are regulated on two levels: (1) by controlling their synthesis and (2) by controlling their activity. For example, a particular transcription factor can be synthesized in certain cell types but not in others, thus modulating transcription in a tissue-specific manner. The transcription factor Oct-2 (Octamer-binding protein) provides a good example of this type of regulation. Oct-2 is involved in the stimulation of immunoglobulin gene expression in B cells and is absent from all other cell types not expressing immunoglobulin genes. However artificial over-expression of Oct-2 in HeLa cells (which do not normally express immunoglobulin genes) was found to result in the transcription of immunoglobulin genes, confirming Oct-2 induces transcription of these genes in a tissue-specific manner (Muller *et al.*, 1988).

Regulation of transcription factors by modulating their activity can occur via a number of different mechanisms including ligand binding, alterations in protein-protein interactions, transcription factor phosphorylation and proteolytic cleavage. A good example of transcription factor activity modulation can be seen for the steroid hormone receptors, which act as ligand-inducible transcription factors. Steroid hormone receptors are also referred to as class I nuclear hormone receptors and will be discussed in more detail in subsequent sections. In the absence of an appropriate ligand, steroid hormone receptors remain bound to heat-shock proteins in the cytoplasm and hence are unable to mediate transcription. However upon steroid binding, the

steroid receptor dissociates from the heat-shock proteins and moves to the nucleus where it activates gene transcription. Thus the activity of a pre-existing transcription factor has been modulated in response to a specific signal.

1.5 Nuclear hormone receptors

1.5.1 Classification of Nuclear hormone Receptors

Nuclear hormone receptors (NRs) comprise a superfamily of eukaryotic transcription factors that regulate transcription in response to their cognate ligands. The human genome contains around 50 genes encoding members of this superfamily and analysis of the *C. elegans* genome has revealed that it contains over 200 members, thus establishing the superfamily of NRs as the single largest class of eukaryotic transcription factors. The NRs function in combination with other transcription factors to regulate the expression of specific genes involved in a diverse array of biological processes including cell growth and development, apoptosis, homeostasis, inflammation, lipid metabolism and the reproductive cycle. The superfamily is subdivided into classes according to their ligand binding, DNA binding and dimerisation properties (Mangelsdorf *et al.*, 1995). Class I comprises the steroid hormone receptors, including the estrogen (ER), androgen (AR), progesterone (PR), glucocorticoid (GR) and mineralocorticoid (MR) receptors, which function as homodimers and bind to DNA hexameric half-sites organised as inverted repeats (Figure 1.5A). Class II is the largest class and its members function as heterodimers with the retinoid-X receptor (RXR) and bind to directly repeated, inverted or everted DNA hexameric half-sites (Figure 1.5B). Members of class II include receptors for retinoic acid (RAR), thyroid hormone (TR), vitamin D3 (VDR) and peroxisome proliferators (PPAR). The third class of NRs comprises the orphan NRs (reviewed in Sladek and Giguere, 2000), whose natural ligands have yet to be identified e.g. COUP-TF (Chicken Ovalbumin Upstream Promoter-Transcription Factor) and HNF-4 (Hepatocyte Nuclear Factor-4) and homodimeric receptors e.g. RXR, which bind to directly repeated DNA hexameric half-sites (Figure 1.5C). Finally, class IV comprises orphan NRs which bind their cognate DNA response elements as monomers, e.g. NGFI-B (Nerve Growth Factor Induced Factor-B) and FTZ-F1 (Fushi Tarazu receptor).

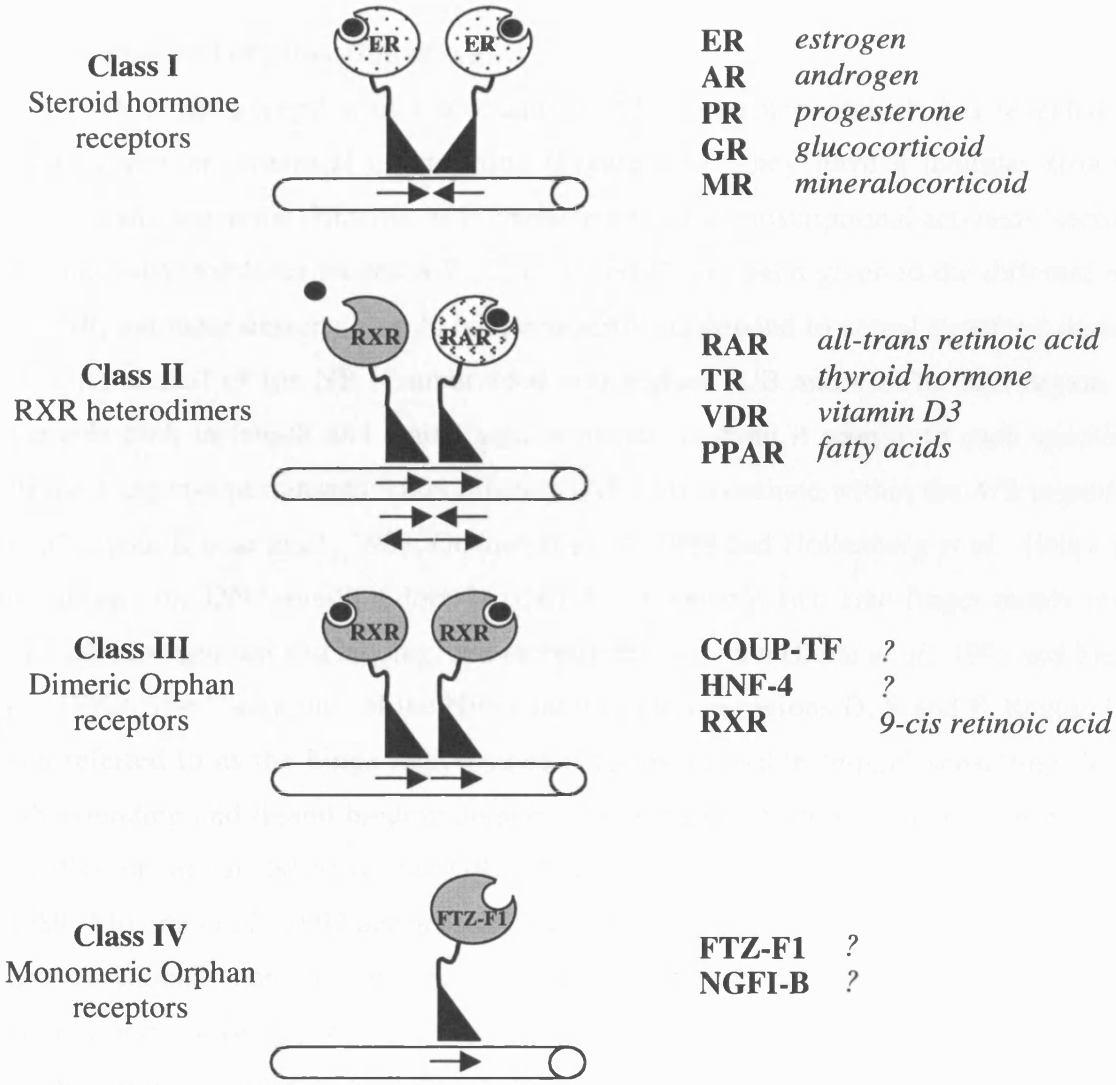


Figure 1.5 Classification of Nuclear hormone receptors is determined by ligand binding, DNA binding and dimerisation properties of the individual NR. Black circles represent receptor cognate ligand and an arrow represents a single hexameric DNA half-site. Examples of NRs belonging to each class are indicated, along with their cognate ligand, if it is known. Adapted from Mangelsdorf *et al.*, 1995.

1.5.2 Structural organisation of NRs

With the exception of a few unusual NRs, sequence analysis has revealed that NRs share a similar structural organisation (Figure 1.6). They have a modular structure with functionally separable domains, as is characteristic of a transcriptional activator (section 1.4.2). Traditionally, the letter names A/B, C, D, E and F have been given to the different regions of the NR, but these designations do not necessarily correspond to actual structural domains. The N-terminal half of the NR is subdivided into regions A/B and C. The A/B region is highly variable both in length and amino acid sequence, making it unique to each specific NR. A ligand-independent transactivation function (AF-1) is contained within the A/B region (Giguere *et al.,* 1986, Kumar *et al.,* 1987, Godowski *et al.,* 1988 and Hollenberg *et al.,* 1988). Region C constitutes the DNA-binding domain (DBD) and contains two zinc-finger motifs responsible for DNA recognition and binding, and receptor dimerisation (Luisi *et al.,* 1991 and Freedman *et al.,* 1992). The C-terminus of the NR is subdivided into regions D, E and F. Region D is short and referred to as the hinge region since it forms a flexible 'hinge' separating the globular DNA-binding and ligand binding domains (Figure 1.6). Within this region there is a nuclear localisation signal (NLS) for the GR, PR, ER and AR (Picard *et al.,* 1987, Guiochon *et al.,* 1989, Ylikomi *et al.,* 1992 and Jenster *et al.,* 1993). The ligand binding domain (LBD) (region E) is functionally complex, responsible for many functions including ligand binding, heat-shock protein association (in the case of class I NRs), dimerisation, nuclear localisation, ligand-dependent transactivation (AF-2) and transcriptional repression (in the TR, RAR and COUP-TF) (for review see Tsai and O'Malley, 1994). Some, but not all, NRs also have a C-terminal F region, of unknown function, although recent reports suggest it has an involvement in specific coactivator recruitment (Warnmark *et al.,* 2001). This domain organisation is conserved amongst the members of the NR superfamily, with the DBD showing the highest level of conservation.

1.5.3 The DNA binding domain (DBD)

The DBD of NRs consists of approximately 70 amino acids that fold into two zinc-finger motifs. Each of these zinc-finger motifs contains four highly conserved cysteine molecules coordinating binding of a central zinc atom (Figure 1.7).

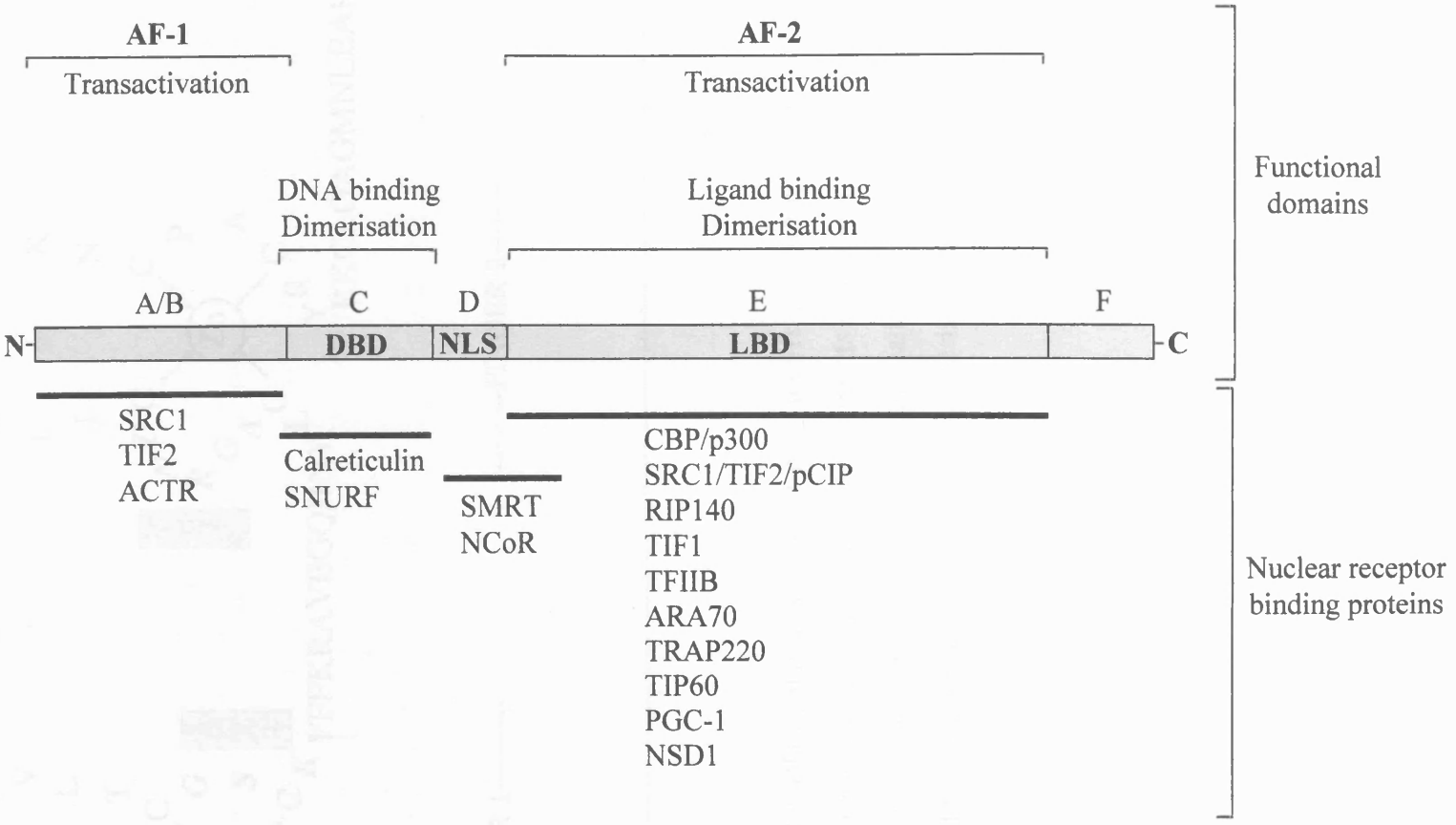


Figure 1.6 Domain organisation of nuclear hormone receptors. Different regions of the NR have been designated letter names A-F and the functions of each domain are indicated. A selection of NR-interacting proteins have also been included and the regions of the NR to which they bind are shown. Adapted from D. Heery lecture material.

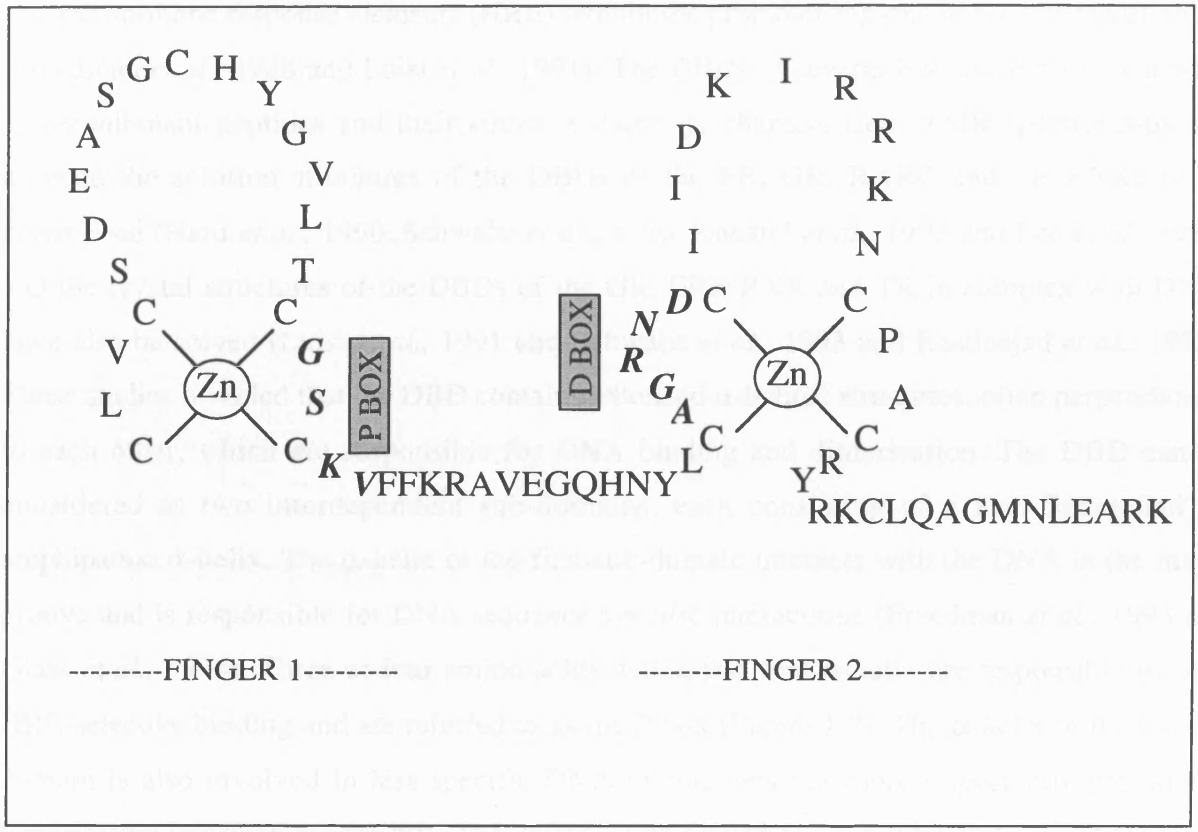


Figure 1.7 The NR DBD contains two zinc-finger motifs that are essential for DNA binding. The amino acid sequence of the zinc-finger motifs of the human glucocorticoid receptor are shown as a representative example. Bold, italicised letters represent those amino acids essential for HRE selectivity (P-box) and dimerisation (D-box).

The resultant tertiary structure is able to recognise and bind to specific DNA sequences, termed nuclear hormone response elements (HRE), within the promoter regions of the NR target genes (Freedman *et al.*, 1988 and Luisi *et al.*, 1991). The DBDs of several NRs have been expressed as recombinant peptides and their structure carefully characterised. NMR spectroscopy has allowed the solution structures of the DBDs of the ER, GR, RARβ and the RXRs to be determined (Hard *et al.*, 1990, Schwabe *et al.*, 1990, Knegtel *et al.*, 1993 and Lee *et al.*, 1993) and the crystal structures of the DBDs of the GR, ERs, RXR and TR in complex with DNA, have also be solved (Luisi *et al.*, 1991 and Schwabe *et al.*, 1993 and Rastinejad *et al.*, 1995). These studies revealed that the DBD contains extended α-helical structures, often perpendicular to each other, which are responsible for DNA binding and dimerisation. The DBD can be considered as two interdependent sub-domains, each consisting of a zinc-finger and an amphipathic α-helix. The α-helix of the first sub-domain interacts with the DNA in the major groove and is responsible for DNA sequence-specific interactions (Freedman *et al.*, 1993 and Glass *et al.*, 1994). Three or four amino acids within this first α-helix are responsible for this HRE-selective binding and are referred to as the P-box (Figure 1.7). The α-helix of the second domain is also involved in less specific DNA interactions but more importantly provides a dimerisation interface for the NR. The amino acids around the second zinc-finger that permit specific protein-protein interactions, and hence promote dimerisation, are termed the D-box (Figure 1.7).

1.5.4 Nuclear hormone receptor response elements (HRE)

Hormone response elements (HRE) are the conserved DNA sequences to which NRs bind, and consist of a single hexameric sequence (half-site) if the NR binds as a monomer or two copies of a hexameric half-site if the NR binds as a dimer. Typically, a six base pair (hexamer) sequence is the minimal requirement to permit NR-DBD recognition and conforms to the consensus AGAACA for the GR, MR, PR and AR or AG(G/T)TCA for the ER, TR, RAR, RXR, VDR and PPAR. However it should be noted that the hexameric half-site sequences found in naturally occurring genes usually exhibit some variation compared to these consensus sequences. In the case of NR dimers, the two hexameric half-sites required for binding can be arranged as direct repeats, inverted repeats or everted repeats, and additionally the nucleotide spacing between the two half-sites varies depending on NR dimer for which the HRE is specific. For example the class I NRs recognise and bind to an HRE consisting of two

hexameric half-sites arranged as an inverted repeat with a spacing of three nucleotides (Figure 1.8B). The class II NRs typically heterodimerise with RXR and these heterodimers recognise and bind to response elements consisting of a direct repeat, everted repeat or inverted repeat of the consensus sequence AG(G/T)TCA (Yu *et al.,* 1991, Kliewer *et al.,* 1992, Leid *et al.,* 1992, Zhang *et al.,* 1992a and Marks *et al.,* 1992) (Figure 1.8C). An un-spaced, inverted repeat of the AGGTCA consensus sequence has been shown to function as a response element for the TR (Glass *et al.,* 1988), the RAR (Umesono *et al.,* 1988) and the RXR (Mangelsdorf *et al.,* 1990). Similarly, an everted repeat response element found in the chicken lysozyme gene (Baniahmad *et al.,* 1990) acts as a response element for both the RAR and TR. In the case of directly repeated configuration response elements, the spacing between the half-sites determines NR-heterodimer binding specificity. For example, a direct repeat spaced by 3 nucleotides (DR3) is a VDRE, by 4 nucleotides (DR4) is a TRE and by 5 nucleotides (DR5) is a RARE (Umesono *et al.,* 1991). Hence the "3-4-5" rule of NR-heterodimer DNA response element recognition was devised. Subsequent demonstration that a DR1 serves as an RXR and PPAR response element and DR2 is a second RARE, led to the expansion of this rule to the "1-to-5" rule. Moreover, on direct repeat response elements, RXR occupies the 5' half-site whilst its heterodimeric partner, VDR, TR or RAR, occupies the 3' half-site. An exception to this rule is the PPAR/RXR heterodimer, in which PPAR occupies the 5' half-site and RXR occupies the 3' half-site.

NGFI-B (Wilson *et al.,* 1991) and the *Drosophila* transcription factor FTZ-F1 (Ueda *et al.,* 1992) are just two examples of the increasing number of orphan nuclear receptors that bind response elements consisting of a single hexameric half-site, as monomers (Figure 1.8A). The ability of these transcription factors to bind DNA response elements with high affinity as monomers appears to be due to the use of amino acids C-terminal to the conserved DBD that contact base pairs upstream of the conserved hexameric half-site and hence stabilise the protein/DNA interaction (Wilson *et al.,* 1992 and Ueda *et al.,* 1992). As a consequence of this, the minimal nucleotide sequence necessary to facilitate binding is extended from 6 nucleotides to 8 nucleotides in the case of NGFI-B or 9 nucleotides in the case of FTZ-F1.

The response elements described so far have been positive response elements to which NRs bind and activate transcription of target genes, in the presence of their cognate ligands. Negative response elements also exist which repress transcription when bound by NRs in the presence of their cognate ligand. One such example of a negative response element for the TR is found in the promoter of the mouse thyroid stimulating hormone β (TSHβ) gene, which is

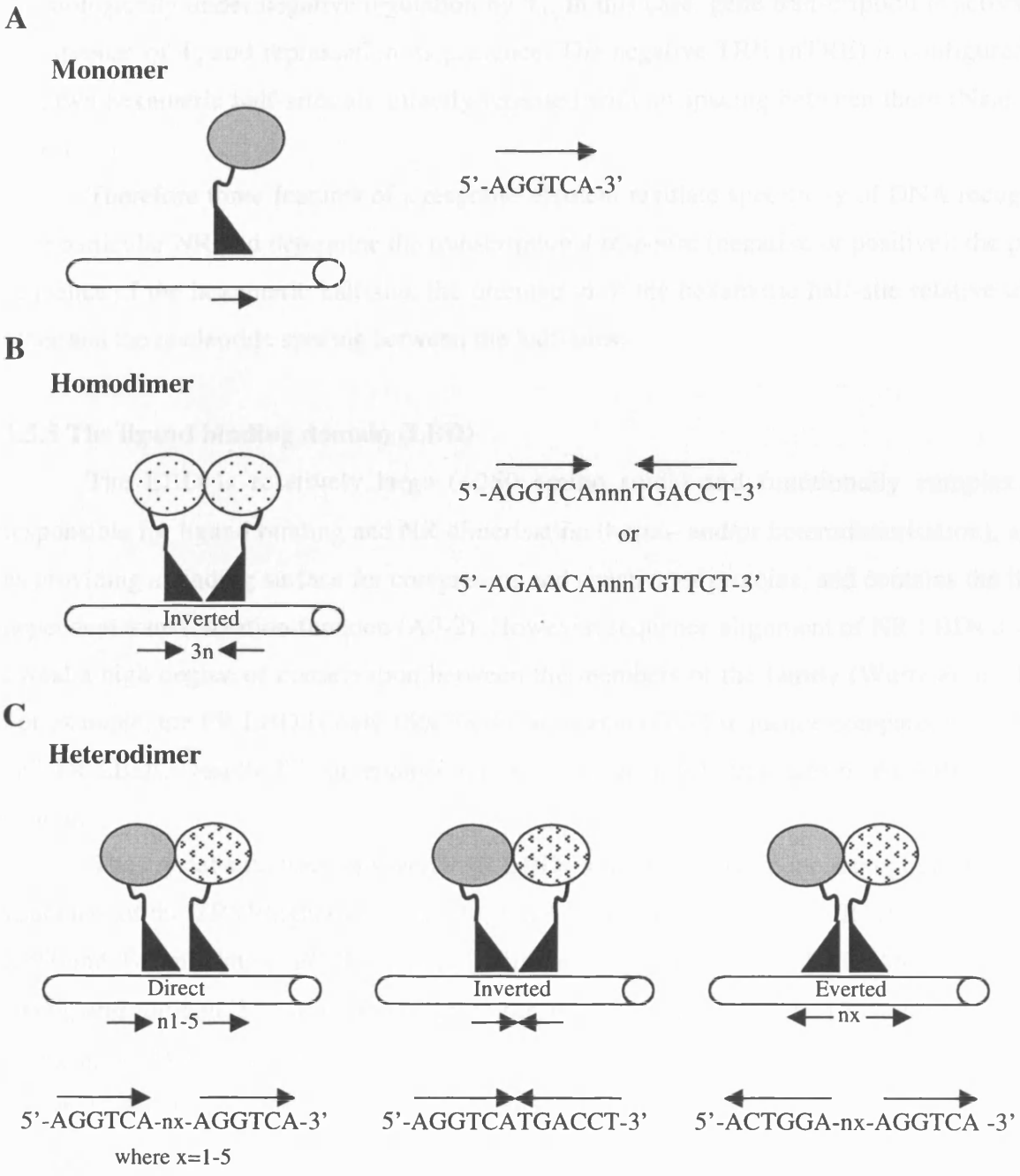


Figure 1.8 Mechanisms of DNA binding by NRs. (A) Monomers bind to DNA response elements consisting of a single hexameric repeat, (B) homodimers bind to inverted repeats spaced by 3 nucleotides and (C) heterodimers bind to direct repeats, inverted repeats and everted repeats, with variable spacing as indicated.

physiologically under negative regulation by T_3. In this case, gene transcription is activated in the absence of T_3 and repressed in its presence. The negative TRE (nTRE) is configured such that two hexameric half-sites are directly repeated with no spacing between them (Naar *et al.*, 1991).

Therefore three features of a response element regulate specificity of DNA recognition by a particular NR and determine the transcriptional response (negative or positive): the precise sequence of the hexameric half-site, the orientation of the hexameric half-site relative to each other and the nucleotide spacing between the half-sites.

1.5.5 The ligand binding domain (LBD)

The LBD is relatively large (~250 amino acids) and functionally complex. It is responsible for ligand binding and NR dimerisation (homo- and/or heterodimerisation), as well as providing a binding surface for corepressor and coactivator proteins, and contains the ligand-dependent transactivation function (AF-2). However, sequence alignment of NR LBDs does not reveal a high degree of conservation between the members of the family (Wurtz *et al.*, 1996). For example, the PR LBD is only 15% identical in amino acid sequence compared with the ER and TR LBDs. Despite this divergence the three-dimensional structures of the LBDs are very similar.

The crystal structures of several NR LBDs have been solved, including liganded (holo) structures of the TR (Wagner *et al.*, 1995), RAR (Renaud *et al.*, 1995), ER (Brzozowski *et al.*, 1997 and Tanenbaum *et al.*, 1998), PR (Williams *et al.*, 1998) and the PPARγ (Nolte *et al.*, 1998), and unliganded (apo) structures of the RXR (Bourguet *et al.*, 1995) and the PPARγ (Nolte *et al.*, 1998). These structures have revealed that the overall folds of the different LBDs are very similar. The NR LBD typically consists of 11-12 α-helices and one β-turn between helix 5 and 6, arranged as an anti-parallel α-helical 'sandwich' in a three layer structure (Figure 1.9). However some variation exists, with RARα and TR lacking a helix 2 and PPARγ possessing an additional short helix 2 (H2'). Liganded LBD structures are a lot more compact than unliganded LBD structures probably due to the conformational changes within the LBD induced upon ligand binding. Indeed upon ligand binding helix 11 is repositioned in the continuity of helix 10 and dramatic repositioning of helix 12 (H12) allows the Ω-loop between helix 2 and 3 to flip over (Figure 1.9). H12 has been shown to project away from the LBD in the unliganded structure (Bourguet *et al.*, 1995) but undergoes a rotation of nearly 180° to pack

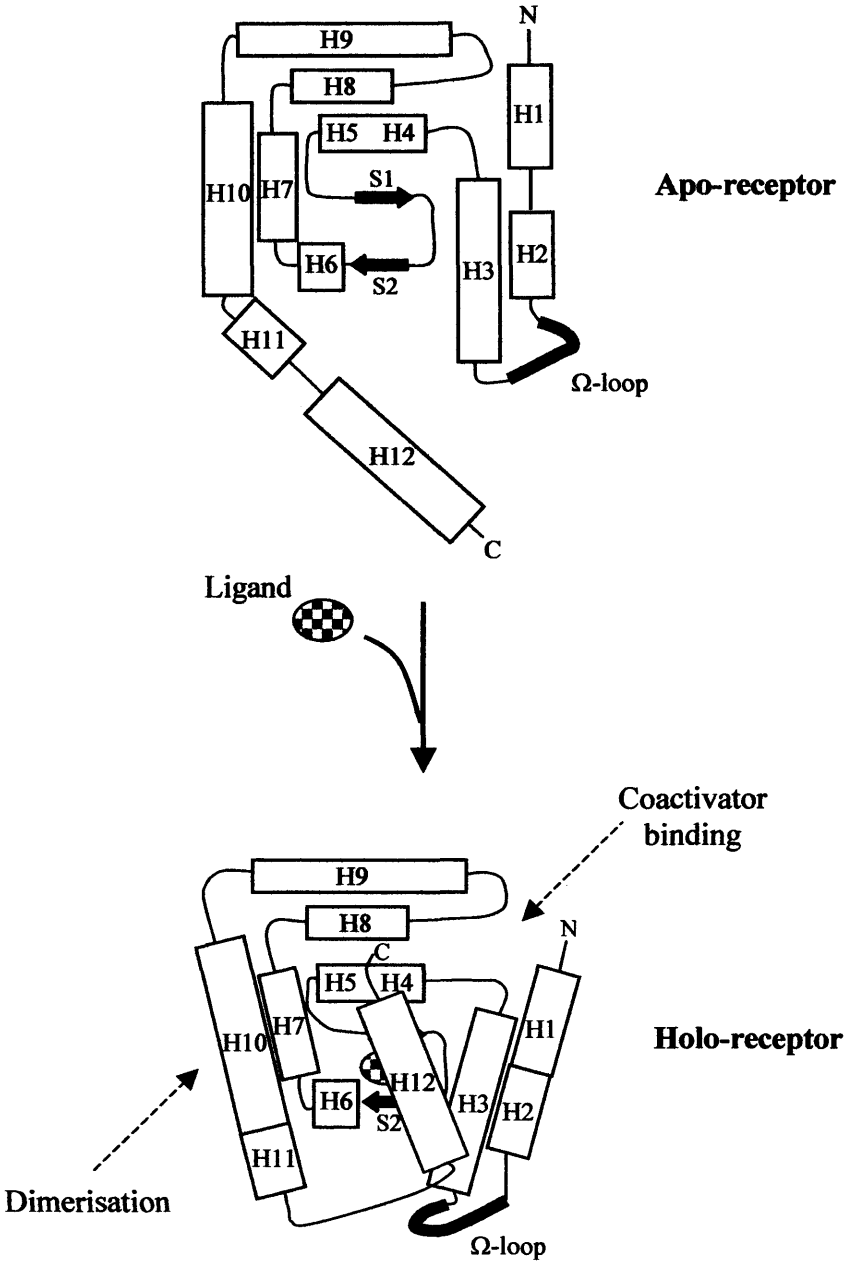


Figure 1.9 The NR LBD undergoes a conformational change upon ligand binding. A generalised schematic of the LBD is shown and the helices are numbered. The configuration of the apo-receptor permits corepressor binding whilst the holo-receptor presents a much more compact structure as compared to the apo-receptor and allows coactivator binding. Adapted from Kumar *et al.*, 1999 and D. Heery lecture material.

tightly against the LBD upon ligand binding. Thus H12 forms a 'lid' over the hydrophobic ligand binding pocket formed by the conserved residues of the LBD. In all liganded crystal structures the ligand is buried deep within the hydrophobic core of the LBD (Renaud *et al.*, 1995, Wagner *et al.*, 1995, Brzozowski *et al.*, 1997) and the specific ligand bound determines the exact conformational change induced in the LBD structure. For example, the ER antagonist raloxifene, binds to the same region of the LBD as the ER agonist, 17β-estradiol, but the large piperidine extension of raloxifene prevents H12 from sealing the ligand binding pocket as it would in the 17β-estradiol-bound ER. Thus H12 is not positioned correctly to provide a binding surface for coactivator proteins, resulting in no activation of the NR. Indeed H12 is a crucial helical component of the LBD since its ligand-induced repositioning provides the surface for coactivator interaction and thereby generates the transcriptional activity of the NR.

Certain residues within the LBD have been shown to be required for transactivation but not ligand binding. This region is known as the AF-2 and is well conserved across members of the NR family. In LBD structures, these residues cluster on a discreet LBD surface after ligand binding and correspond to H12. This helix therefore is often referred to as the AF-2 activation helix and indeed it provides a hydrophobic surface for interactions with other molecules necessary for transcriptional activation i.e. coactivators, upon ligand binding (Wagner *et al.*, 1995, Renaud *et al.*, 1995, Brzozowski *et al.*, 1997, Williams *et al.*, 1998, Nolte et al., 1998, Shiau *et al.*, 1998 and Darimont *et al.*, 1998). In the absence of ligand, the structure of the LBD is configured in such a way as to provide a binding surface for factors involved in the silencing activity of the NRs i.e. corepressors (Horelein *et al.*, 1995, Kurokawa *et al.*, 1995 and Chen *et al.*, 1995). In particular, mutational analysis has implicated helix 11 as being directly involved in corepressor binding (Horlein *et al.*, 1995). Thus the LBD can be viewed as a molecular switch, that upon ligand binding enables the NR to activate transcription but in the absence of ligand represses NR-mediated transactivation.

Finally, LBDs have an intrinsic ability to form dimers. Indeed the ER, RXR, RAR, PR and PPARγ have all been crystallised as dimers. The dimer contacts, except for the PR, are made predominately through H11, which also contacts the ligand. Mutational analysis of residues in H11 has shown that they are important in solution based dimerisation for some NRs (Fawell *et al.*, 1990 and Au-Fliegner *et al.*, 1993).

1.5.6 Transcriptional Repression by Nuclear Hormone Receptors

NRs have either a neutral (class I) or silencing activity (class II, III and IV) in the absence of their cognate ligand. Upon ligand binding they become positive regulators of transcription, although certain genes can be repressed if the HRE is configured appropriately (see section 1.5.4). In the absence of ligand, class I NRs associate with heat-shock proteins e.g. hsp90 and hsp70, which prevent dimerisation of the NR and retain it in the cytoplasm (for review see Pratt and Toft, 1997) (Figure 1.10). Conversely, the other classes of NRs have not been shown to interact with heat-shock proteins and can bind DNA in the absence of ligand and silence basal promoter activity. *In vitro* assays have established that unliganded TR can strongly repress transcription (Fondell *et al.,* 1995), by inhibiting the formation of the PIC. This inhibition was proposed to be due to *in vitro* interaction of the C-terminal half of the TR LBD with the zinc-finger of TFIIB in the absence of ligand. Thus the TR would block the interaction of the TFIIB with other components of the PIC, preventing its assembly and silencing transcription. However the observation that other NRs can interact with TFIIB in the absence of ligand but cannot repress transcription suggested that other factors may be involved in this repression. This led to the discovery of the NR corepressors NCoR (Nuclear hormone receptor Corepressor) (Horlein *et al.,* 1995 and Kurokawa *et al.,* 1995) and SMRT (Silencing Mediator for Retinoic acid and Thyroid hormone receptors) (Chen and Evans, 1995). The exact mechanism of repression by these corepressors is unclear but they have been shown to interact with the mammalian homologues of the yeast Sin3 protein, which is capable of mediating transcriptional repression (Alland *et al.,* 1997, Heinzel *et al.,* 1997 and Nagy *et al.,* 1999). The yeast and mammalian Sin3 proteins are components of corepressor complexes that also contain HDACs. Thus the corepressors, SMRT and NCoR, associate with NRs in the absence of ligand and are able to recruit complexes with intrinsic HDAC activity. Hence as described in section 1.3.2, the chromatin is retained in its compact transcriptionally repressive state by the actions of the HDACs (Figure 1.11).

1.5.7 Transcriptional Activation by Nuclear Hormone Receptors

In the presence of ligand, NRs generally act as positive regulators of transcription. Binding of ligand to the LBD of class I NRs results in the dissociation of heat-shock proteins, homodimerisation and translocation to the nucleus, where they bind their HRE (Figure 1.10). Binding of ligand to the DNA-bound class II, III and IV NRs results in a dissociation of the

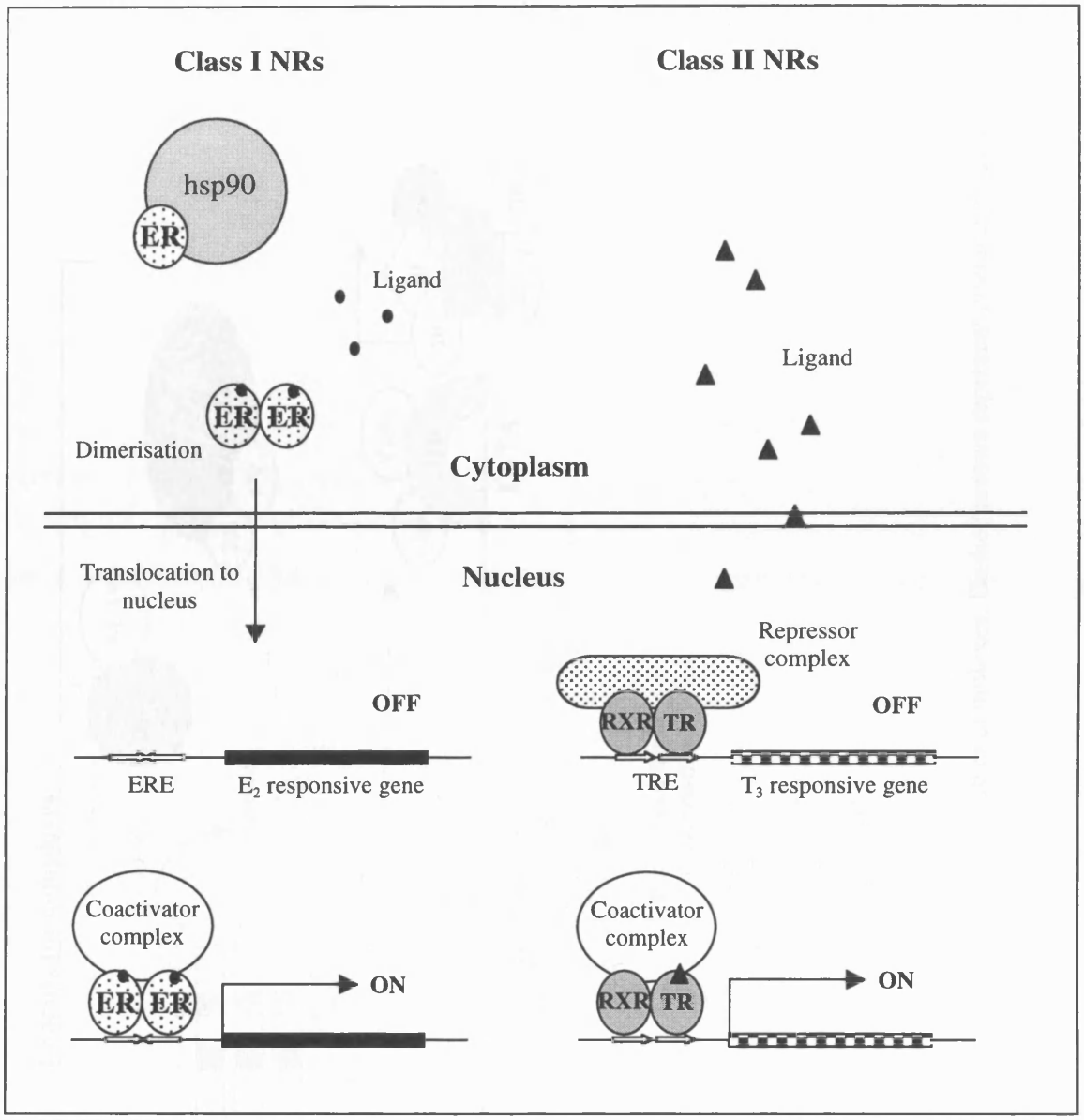


Figure 1.10 Transcriptional repression and activation by NRs. The ER and TR are used as examples of class I and class II NRs, respectively. In the absence of ligand class I NRs are held in the cytoplasm in complex with hsps, whilst class II NRs bind to their HRE and associate with corepressors to actively repress transcription. In the presence of ligand, class I NRs dissociate from the hsp complex, homodimerise, translocate to the nucleus and bind their HRE, whereas class II NRs dissociate from the corepressor complex. Thus transcriptional repression is relieved. Liganded NRs then recruit coactivators and transcription is activated.

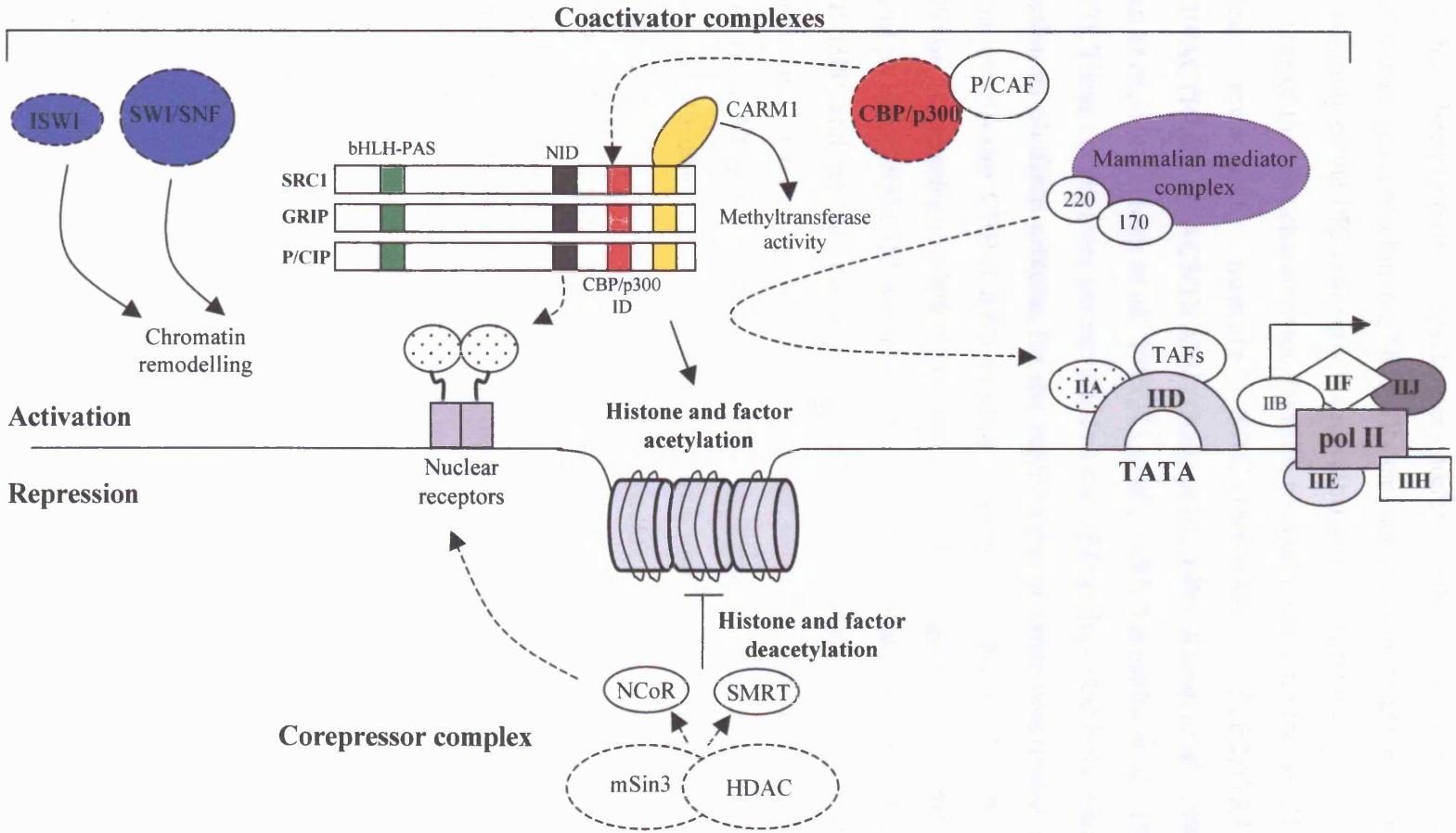


Figure 1.11 Regulation of NR activity by multiple coactivator and corepressor complexes. Dashed arrows represent interactions and solid arrows represent functions. Adapted from Glass *et al.*, 2000.

corepressor proteins and association of coactivator proteins (Figure 1.10). This coregulator exchange is brought about by a ligand induced conformational change in the NR LBD. Whilst direct interactions between the basal factor TFIIB and NRs such as the TRβ (Baniahmad *et al.*, 1993) have been described, coactivators preferentially mediate the interaction of NRs with the basal transcription machinery. Thus NRs can activate transcription by promoting or stabilising the assembly of the PIC indirectly via recruitment of coactivators.

One of the best characterised families of coactivators are the SRC1 family, which include three members namely SRC1/NCoA-1, TIF2/GRIP1/NCoA-2 and p/CIP/ACTR/AIB1/RAC3/TRAM1 (Onate *et al.*, 1995, Kamei *et al.*, 1996, Anzick *et al.*, 1997, Chen *et al.*, 1997, Hong *et al.*, 1997, Li *et al.*, 1997, Takeshita *et al.*, 1997 and Torchia *et al.*, 1997). These coactivators are recruited to the LBD of liganded NRs where they predominately function as platform proteins for the recruitment of other coactivator proteins including the global coactivator CBP (CREB Binding Protein) (Torchia *et al.*, 1997 and McKenna *et al.*, 1999) or its homologue p300 (Eckner *et al.*, 1994) (Figure 1.11). CBP/p300 in turn is able to recruit p/CAF (p300/CBP Associated Factor) (Yang *et al.*, 1996 and Blanco *et al.*, 1998). Both CBP/p300 and p/CAF possess intrinsic HAT activity. Other chromatin modifying and remodelling coactivators are also recruited to the liganded NR/coactivator complex e.g. CARM1, ISWI and SWI/SNF (Figure 1.11). Thus ligand-induced coactivator recruitment to NRs leads to chromatin modification which increases transcription factor accessibility to specific DNA sequences. In addition, the recently discovered mammalian mediator complex, TRAP/SMCC/Mediator (see section 1.9), is also recruited to liganded NRs but lacks any detectable chromatin modifying or remodelling activities. Instead it is thought to function as a bridging factor between the NR and the basal transcription machinery (Figure 1.11). Hence liganded NRs activate transcription by assembling an array of coactivator proteins, with diverse functions, on the promoter of target genes.

1.6 Corepressors

Transcriptional repression refers to the ability of class II NRs to lower basal promoter activity in the absence of ligand. This repression is imposed by the unliganded NR recruiting an array of negative coregulators, termed corepressors, whose function it is to create an environment that is incompatible with proper assembly of the PIC. NCoR is one such example of a corepressor and it was originally discovered in a yeast two-hybrid screen using unliganded

TR and RAR as bait (Horlein *et al.,* 1995). This 270 kDa protein contains domains capable of silencing transcription at heterologous promoters. Indeed fusion of NCoR to the GAL4-DBD results in strong repression at a promoter containing GAL4 DNA binding sites (Horlein *et al.,* 1995). NCoR contains two NR-interacting domains in its C-terminus which are essential for NR binding (Seol *et al.,* 1996) and it possesses three repression domains (RI, RII and RIII) in its N-terminus. The corepressor SMRT was also isolated using a yeast two-hybrid screen but in this case the bait was RXR (Chen *et al.,* 1995). Significant sequence homology exists between SMRT and NCoR, but SMRT lacks two of the three repression domains found in the N-terminus of NCoR. Similar to NCoR, SMRT possesses two NR-interacting domains in its C-terminus. These NR-interacting domains contain the conserved sequence LXX(I/H)IXXX(I/L) (where X represents any amino acid), often termed the CoRNR box (Perissi *et al.,* 1999 and Nagy *et al.,* 1999). This motif is predicted to form an extended α-helix that is essential and sufficient to bind to the unliganded LBDs of NRs. However whilst this corepressor α-helix occupies a similar region of the hydrophobic binding pocket of the NR LBD as the coactivator molecules, it can only do so in the absence of ligand when the LBD adopts a conformation favourable for corepressor binding. Additionally the corepressor interaction does not require the charge clamp that is necessary for coactivator binding (see section 1.8.1). Hence the liganded and unliganded NR LBD conformations discriminate between the NR-interacting motifs of corepressor and coactivator proteins.

As well as mediating the silencing activity of unliganded class II NRs, recent studies have also implicated NCoR in mediating the silencing activity of members of the orphan NR family, including RevErb (Zamir *et al.,* 1996), DAX-1 (Crawford *et al.,* 1998) and COUP-TFs (Shibata *et al.,* 1997). Additionally, the corepressors NCoR and SMRT, have been shown to interact with antagonist bound class I NRs (Lavinsky *et al.,* 1998). Taken together it would appear that corepressors such as NCoR and SMRT, mediate the silencing activity of a wide range of NRs. However, there is evidence for differential usage of NCoR and SMRT by NRs. For example, DAX-1 is able to interact with NCoR but not SMRT (Crawford *et al.,* 1998) and similarly, RevErb exclusively requires NCoR, but not SMRT, to effect transcriptional repression (Zamir *et al.,* 1997a).

In addition to NCoR and SMRT, other corepressors have been identified including TRUP (Thyroid Receptor-Uncoupling Protein) (Burris *et al.,* 1995) and SUN-CoR (Small Ubiquitous Nuclear Corepressor) (Zamir *et al.,* 1997b). SUN-CoR is able to enhance the transcriptional

silencing activity of TR and RevErb and interacts with NCoR, suggesting it is a component of a functional corepressor complex. TRUP is an unusual corepressor, able to diminish both the ligand-dependent transcriptional activity and the silencing activity of the TR. These activities are thought to be due to TRUP decreasing the ability of RXR/TR heterodimers to bind their cognate HREs. Thus TRUP presents a distinct mode of control over NR-mediated transcriptional activity, as opposed to the NCoR/SMRT class of corepressors.

1.7 Coactivators

1.7.1 CBP/p300

CBP (CREB Binding Protein) was originally isolated on the basis of its interaction with CREB (cAMP response element binding protein), in response to cAMP signalling (Chrivia *et al.,* 1993) and subsequently its close homologue, p300, was purified as a cellular binding protein of the adenoviral protein E1A (Eckner *et al.,* 1994). CBP/p300 are ubiquitous, evolutionary conserved nuclear phosphoproteins that function as coactivators for a diverse array of transcriptional activators including, p53 (Avantaggiata *et al.,* 1997), Nuclear factor-κB (NF-κB) (Perkins *et al.,* 1997) and NRs (Kamei *et al.,* 1996, Chakravarti *et al.,* 1996 and Fronsdal *et al.,* 1998). CBP/p300 possess conserved motifs and functional domains including a bromodomain, three cysteine-histidine (C/H)-rich regions (C/H1, C/H2 and C/H3), a glutamine (Q)-rich region, an intrinsic acetyltransferase activity, an SRC1 interaction domain (SID) and a CREB binding domain (KIX). The bromodomain recognises and binds to the acetylated lysine residues of the histone tails and is therefore necessary for the direct interaction of CBP/p300 with chromatin (Manning *et al.,* 2001). C/H3 provides a surface for the interaction of several different transcription-related factors including p/CAF (p300/CBP associated factor) (Yang *et al.,* 1996), the adenoviral oncoprotein E1A (Yang *et al.,* 1996) and RNA polymerase II complexes (Nakajima *et al.,* 1997). The glutamine-rich region contains the SID (Kamei *et al.,* 1996), which has been recently mapped to include a 72 amino acid sequence (Sheppard *et al.,* 2001). The SID permits the reported interactions of CBP with members of the p160 family of coactivators (Kamei *et al.,* 1996, Voegel *et al.,* 1998, Torchia *et al.,* 1997 and Sheppard *et al.,* 2001) and is necessary for NR-mediated transcriptional activity. The N-terminal fragment of CBP is responsible for direct NR interactions (Kamei *et al.,* 1996). However, recent reports have shown that direct CBP/p300-NR interactions are weak in comparison to NR-SRC1

interactions (Li *et al.*, 2000 and Sheppard *et al.*, 2001) and the NR-CBP/p300 interaction is preferentially mediated by SRC1 family members.

The intrinsic acetyltransferase activity of CBP/p300 is essential for its activity as a coactivator as *in vitro* transcription assays have shown. Mutations that disrupt the HAT activity of CBP/p300 abrogate their ability to enhance transcription mediated by ER (Kraus *et al.*, 1999) or RXR/TR (Li *et al.*, 2000) on reconstituted chromatin templates *in vitro*. In addition to histone acetylation, CBP/p300 has also been shown to acetylate the SRC1 family member, ACTR (Activator of the Thyroid and RA receptor), which resulted in its dissociation from the NR LBD (Chen *et al.*, 1999a). Hence, as well as enhancing NR-mediated transcriptional activity, CBP/p300 could also attenuate it, by promoting the disassembly of the NR-SRC1-CBP complex.

Based on the multiple interactions of CBP/p300 with many transcription factors, CBP is often referred to as a 'co-integrator' or 'global coactivator'. Thus CBP/p300 serves to integrate a diverse array of signalling pathways to modulate gene expression.

1.7.2 p/CAF

p/CAF (p300/CBP associated protein) is the mammalian homologue of the yeast GCN5 protein (Yang *et al.*, 1996) and it possesses intrinsic HAT activity. The C-terminus of p/CAF is highly homologous to GCN5 and contains the HAT domain responsible for the acetylation of free histones H3 and H4, and nucleosomal H3 (Yang *et al.*, 1996). The N-terminus of p/CAF however, is unique and extended, as compared to GCN5, and mediates the interaction of p/CAF with the C/H3 domain of CBP/p300, NRs and members of the SRC1 family (Korzus *et al.*, 1998, Chen *et al.*, 1997 and Blanco *et al.*, 1998). Recent studies have shown that a block in ligand-dependent activation of NRs, due to microinjection of anti-p/CAF antibodies into living cells, can be overcome by co-injecting with a p/CAF expression vector (Korzus *et al.*, 1998). Hence, together with its intrinsic HAT activity and its ability to interact with components of the transcriptional machinery i.e. CBP, p/CAF has been classified as a *bona fide* coactivator of NR-mediated transactivation.

1.7.3 The SRC1 family

The SRC1 family is also referred to as the p160 family of coactivators since all family members are approximately 160 kDa. To date, three distinct but related SRC1 family members

have been identified. These are Steroid Receptor Coactivator 1 (SRC1)/Nuclear receptor Coactivator-1 (NCoR-1) (Onate *et al.*, 1995 and Kamei *et al.*, 1996), Transcription Intermediary Factor-2 (TIF2)/Glucocorticoid receptor Interacting Protein 1 (GRIP1)/Nuclear receptor Coactivator-2 (NCoR-2) (Voegel *et al.*, 1996, Hong *et al.*, 1996) and p300/CBP co-integrator associated protein (p/CIP)/Activator of Thyroid Receptor (ACTR)/Receptor Associated coactivator-3 (RAC3)/Amplified In Breast cancer-1 (AIB1)/Thyroid Receptor Activator molecule-1 (TRAM1) (Torchia *et al.*, 1997, Chen *et al.*, 1997, Li *et al.*, 1997, Anzick *et al.*, 1997 and Takeshita *et al.*, 1997). The SRC1 family members share a common domain structure, with 40% sequence similarity between the three proteins. The N-terminus is the most highly conserved and contains a bHLH/PAS (basic helix-loop-helix/Period-Aryl hydrocarbon receptor-Single-minded) domain (Yao *et al.*, 1996 and Kamei *et al.*, 1996). The bHLH/PAS domain is also present in other transcription factors and mediates protein/protein interactions. Following the bHLH/PAS domain is the centrally located NR-interacting domain (NID), which is essential for ligand-dependent interactions with the AF-2 of NRs. The NID contains three LXXLL motifs (where L represents leucine and X represents any amino acid). These LXXLL motifs and NR/coactivator interactions will be discussed in more detail in section 1.8. The C-terminus contains two transcriptional activation domains (AD1 and AD2), which retain their activity when tethered to a heterologous DBD (Voegel *et al.*, 1996, Hong *et al.*, 1996, Voegel *et al.*, 1998 and Onate *et al.*, 1998). The AD1 domain colocalises with the CBP interaction domain (Chen *et al.*, 1997, Torchia *et al.*, 1997, Voegel *et al.*, 1998 and Kalkhoven *et al.*, 1998) and the potent transcriptional activity of AD1 is CBP-dependent. Recent studies have precisely mapped the core CBP-binding domains (AD1) of SRC1 and ACTR (Chen *et al.*, 1997 and Sheppard *et al.*, 2001). The second activation domain (AD2) resides close to the C-termini of SRC1 family members and has recently been shown to bind CARM1 (Chen *et al.*, 1999b). A glutamine-rich sequence residing between AD1 and AD2 has recently been implicated in binding the ligand-independent AF-1 domains of NRs (Webb *et al.*, 1998, Bevan *et al.*, 1999 and Ma *et al.*, 1999). Thus together with its ligand-dependent NID, SRC1 could functionally link the AF-1 and AF-2 domains of NRs. The C-terminus of SRC1 and ACTR have also been reported to possess HAT activity (Spencer *et al.*, 1997 and Chen *et al.*, 1997). This activity has been observed to be much weaker than the HAT activity of CBP/p300 and p/CAF (Sheppard *et al.*, 2001). Voegel *et al.*, (1998) were unable to detect any HAT activity associated with TIF2. Further, in contrast to the HAT domains of CBP and p/CAF, the sequence encoding the

proposed HAT domain of SRC1 did not activate transcription when fused to a Gal4-DBD (Kalkhoven *et al.*, 1998), nor does it appear to contain features that correspond to the acetyl CoA binding site of the p/CAF or GCN5 HAT domains (Clements *et al.*, 1999 and Trievel *et al.*, 1999), suggesting novel HAT activities for SRC1 family members.

1.7.4 Other coactivators

In addition to CBP/p300, p/CAF and the SRC1 family, there are many other coactivators that are able to stimulate the activity of NRs. For the sake of brevity, the following describes just a few examples of NR-coactivators.

CARM1 was originally isolated in a yeast two-hybrid screen using the C-terminal domain of GRIP1 (Chen *et al.*, 1999b) (also see section 1.3.2) and has extensive homology to the PRMT (protein methyltransferase) family of arginine specific methyltransferases. CARM1 is able to interact with the C-terminal AD2 domain of all three members of the SRC1 family and possesses intrinsic histone methyltransferase activity (HMT) *in vitro*. *In vivo* CARM1 is able to further enhance GRIP1 coactivation of AR, TR and ER activities and this coactivation has been shown to be dependent upon three amino acids located in the region critical to methyltransferase activity. This suggests that CARM1's HMT activity is necessary to enhance NR function. Further, in the absence of GRIP1, CARM1 has no effect on NR function, suggesting SRC1 coactivators are necessary to recruit CARM1 to the NR complex.

PGC-1 (PPARγ coactivator-1) is another example of a NR-associated coactivator. It was originally isolated in a yeast two-hybrid screen using PPARγ as the bait and was demonstrated to interact with several members of the NR superfamily (Puigserver *et al.*, 1998). PGC-1 exhibits tissue-specific expression that is induced in response to specific signals including cold, fasting and exercise. Similar to the SRC1 family of coactivators, PGC-1 has a domain structure, possessing an AD, an NID, an 'inhibitory' domain (that represses the function of the N-terminal AD), an arg/ser (R/S)-rich region and a putative RNA recognition motif (RRM). PGC-1 has been shown to be a transcriptional coactivator of many NRs, including PPARγ, TR (Puigserver *et al.*, 1998), PPARα (Vega *et al.*, 2000) and other transcription factors like NRF-1 (Nuclear respiratory factor-1) (Wu *et al.*, 1999), that play critical roles in regulation of oxidative metabolism and adaptive thermogenesis. PGC-1 has little, or no, HAT activity but it can recruit SRC1 coactivators and CBP/p300 via its transcriptional AD located in its N-terminus.

RIP140 (receptor interacting protein-140) was identified using far-western blotting with GST-ER-AF2 (Cavailles *et al.,* 1994). RIP140 specifically interacts with the AF-2 of ER *in vitro* and *in vivo*, and modulates its transcriptional activity in the presence of estrogen. However, whilst modest stimulation of ER AF-2 activity was observed with low concentrations of RIP140, higher concentrations of RIP140 were observed to completely abolish estrogen-dependent transcription (Cavailles *et al.,* 1995). The reason for the inhibition of ER activity by RIP140 is unclear, as is the precise role of RIP140 in NR-mediated transcriptional activation.

1.8 Nuclear hormone Receptor-Cofactor interactions

1.8.1 The LXXLL motif

Coactivators interact with agonist-bound NR-LBDs via a signature LXXLL motif (LXM) (where L represents leucine and X represents any amino acid) (Heery *et al.,* 1997 and Torchia *et al.,* 1997). The crystal structures of several agonist-bound NR-LBDs in complex with LXM-containing peptides have been described (Nolte *et al.,* 1998, Darimont *et al.,* 1998 and Shiau *et al.,* 1998) revealing that the LXXLL motif forms a two-turn amphipathic α-helix, with the conserved leucine residues comprising a hydrophobic surface on one face of the α-helix (Figure 1.12). This α-helix interacts with the AF-2 domain of the agonist-bound NR via a hydrophobic groove made from residues in receptor helices 3, 4, 5 and 12, due to a conformational change induced upon ligand binding (Figure 1.9). In addition, both AF-2 surfaces, in all of the LBD-homodimer crystal structures solved, have been shown to be occupied by a single LXXLL α-helix (Figure 1.13). The crystal structure of the agonist-bound LBD of PPARγ in complex with an 88 amino acid fragment of SRC1, containing LXM1 and LXM2, revealed that two charged residues that are highly conserved among LBDs (a glutamic acid residue in helix 12 and a lysine residue in helix 3) and are critical for AF-2 function, form a 'charge clamp' which holds the LXXLL α-helix in position on the NR-LBD (Nolte *et al.,* 1998). Figure 1.13 shows a cartoon of the crystal structure of the ERα-LBD-homodimer complexed with two LXXLL containing α-helices. The glutamic acid and lysine residues forming the 'charge clamp' are highlighted.

The integrity of the LXXLL motif has been shown to be essential for ligand-dependent NR-coactivator interactions, since mutation of any of the conserved leucine residues abolishes this interaction (Heery *et al.,* 1997 and Torchia *et al.,* 1997).

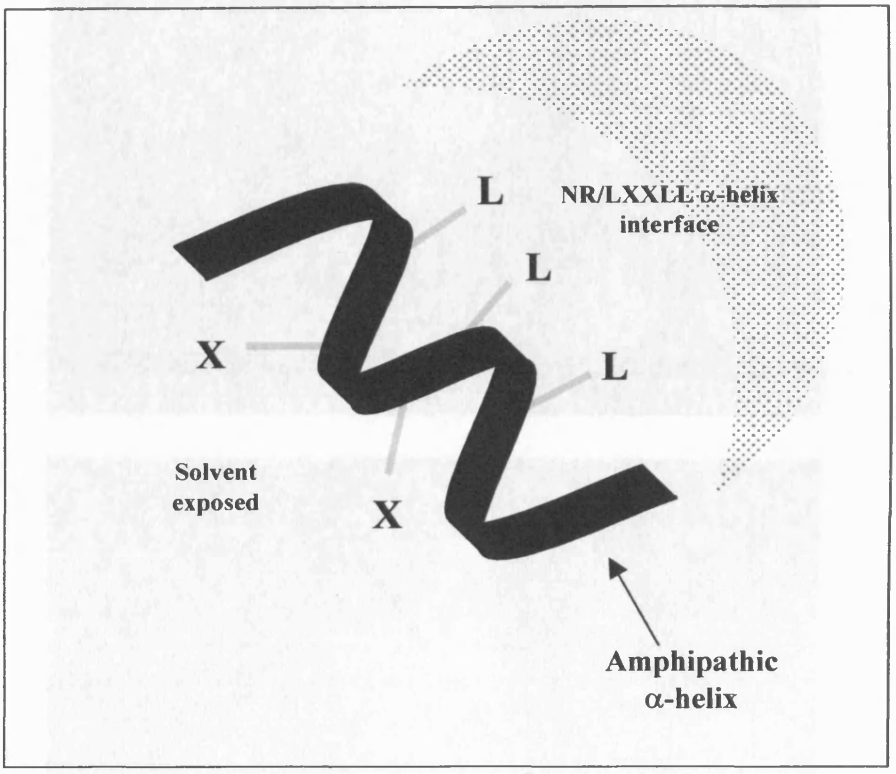


Figure 1.12. Simplified diagram of the LXXLL α-helix. The LXXLL motif forms a two-turn amphipathic α-helix, with the conserved leucine resides aligning on one face of the α-helix to form a hydrophobic surface. The amino acids occupying the +2 and +3 positions ('XX") align on the other face of the α-helix and project away from the NR-LBD. This LXXLL α-helix interacts with the hydrophobic groove of the NR-LBD (represented here by a shaded crescent).

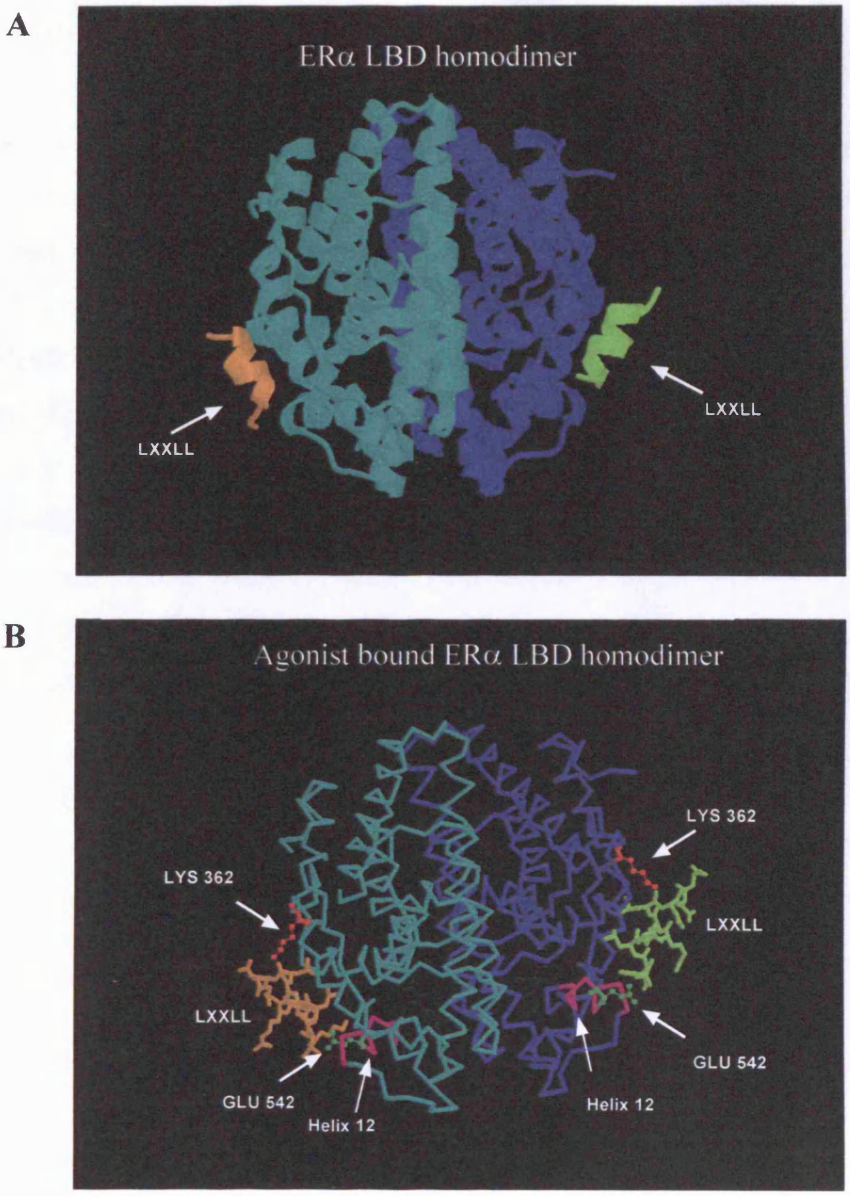


Figure 1.13 A single LXXLL α-helix interacts with the hydrophobic groove of the NR-LBD and is held in place by hydrophobic attractions and the 'charge clamp'. (A) A simplified ribbon diagram of the ERα-LBD homodimer in complex with two LXXLL α-helices. (B) The agonist-bound ERα-LBD homodimer is shown in complex with two LXXLL α-helices (orange and green). The 'charge clamp', made up of Lys 362 (red) and Glu 542 (green) is highlighted in each LBD, as well as helix 12 (AF-2 helix) (violet). (Shiau *et al.,* 1998)

Further, the minimum sequence, encompassing the LXXLL motif, that can bind the AF-2 surface (the LXXLL core motif) is contained within 8 amino acids (-1 to +7, where the first conserved leucine of the LXXLL motif is numbered +1) (Heery *et al.*, 2001). However, the number and sequence of LXXLL motifs varies considerably among coactivators (Table 1.1). For example, the SRC1 family of coactivators have a nuclear receptor interaction domain (NID) containing three LXXLL motifs. These sequences and spacing between them are highly conserved and they have been shown to mediate high affinity binding to NRs (Heery *et al.*, 1997, Torchia *et al.*, 1997, Voegel *et al.*, 1998, Kalkhoven *et al.*, 1998 and McInerney *et al.*, 1998). RIP140 contains nine functional LXXLL motifs, which show variable affinity for the LBD of the ERα (Heery *et al.*, 1997) and PELP-1 (proline-, glutamic acid-, leucine-rich protein-1) also contains nine LXXLL motifs (Vadlamudi *et al.*, 2001). Other coctivators, including Tip60 (Gaughan *et al.*, 2001), TIF1α (Le Douarin *et al.*, 1996), PGC-1 (Puigserver *et al.*, 1998), Fushi tarazu (Ftz) (Suzuki *et al.*, 2001) and NcoA6 (NcoA6 was previously designated ASC-2/RAP250/TRBP/PRIP/NRC/AIB3 (Lee *et al.*, 1999)), contain a single functional LXXLL motif that facilitates NR interactions. The NID of TRAP220 (thyroid receptor associated protein 220 kDa) contains two LXXLL motifs (Yuan *et al.*, 1998). TRAP220 will be discussed in more detail in section 1.9.

In addition to the classical LXXLL motif, variants of this motif exist which mediate ligand-dependent interactions with NR-LBDs. For example, NRIF3 (nuclear receptor-interacting factor 3) has a N-terminal LXXLL motif and a C-terminal LXXIL motif. Whilst this variant LXXIL motif is essential for NR interactions, its classical LXXLL motif is not but it is important for optimum NRIF3-NR interactions (Li *et al.*, 1999 and Li *et al.*, 2001). Another variant LXXLL motif can be found in the NID of NSD1 (NR-binding SET-domain containing protein-1). In this case an FXXLL motif is responsible for mediating ligand-dependent interactions with NRs (Huang *et al.*, 1998). The variant FXXLF motif is also found in AR-associated proteins (ARA), such as ARA70 (Yeh *et al.*, 1996), ARA55 (Fujimoto *et al.*, 1999) and ARA54 (Kang *et al.*, 1999). Additionally, the antagonist bound crystal structure of the ERα-LBD has revealed that helix 12 contains a LXXML sequence that mimics the LXXLL α-helix of coactivator proteins and is able to bind to and occlude the coactivator binding site of the ERα-LBD (Shiau *et al.*, 1998). Not all NR LBDs possess this variant LXXLL motif but sequence analysis shows that residues in this region of helix 12 in most NRs are hydrophobic in

SRC1a	Q	T	S	H	K	**L**	V	Q	**L**	**L**	T	T	T	A	E	628-642
	E	R	H	K	I	**L**	H	R	**L**	**L**	Q	E	G	S	P	685-699
	K	D	H	Q	L	**L**	R	Y	**L**	**L**	D	K	D	E	K	744-758
	Q	Q	K	S	L	**L**	Q	Q	**L**	**L**	T	E				1430-1441
TIF2	K	G	Q	T	K	**L**	L	Q	**L**	**L**	T	T	K	S	D	636-650
	E	K	H	K	I	**L**	H	R	**L**	**L**	Q	D	S	S	S	685-699
	K	E	N	A	L	**L**	R	Y	**L**	**L**	D	K	D	D	T	740-754
ACTR	K	G	H	K	K	**L**	L	Q	**L**	**L**	T	C	S	S	D	626-640
	E	K	H	R	I	**L**	H	K	**L**	**L**	Q	N	G	N	S	690-704
	E	N	N	A	L	**L**	R	Y	**L**	**L**	D	R	D	D	P	743-757
RIP140	I	V	L	T	Y	**L**	E	G	**L**	**L**	M	H	Q	A	A	16-30
	Q	D	S	T	L	**L**	A	S	**L**	**L**	Q	S	E	S	S	128-142
	Y	A	S	S	H	**L**	K	T	**L**	**L**	K	K	S	K	V	180-194
	V	A	C	S	Q	**L**	A	L	**L**	**L**	S	S	E	A	H	262-276
	A	N	N	S	L	**L**	L	H	**L**	**L**	K	S	Q	T	I	375-389
	Q	K	V	T	L	**L**	Q	L	**L**	**L**	G	H	K	N	E	495-509
	E	R	R	T	V	**L**	Q	L	**L**	**L**	G	N	P	K	G	708-722
	S	K	N	G	L	**L**	S	R	**L**	**L**	R	Q	N	Q	D	814-828
	K	S	F	N	V	**L**	K	Q	**L**	**L**	L	S	E	N	C	931-945
CBP	S	K	H	K	Q	**L**	S	E	**L**	**L**	R	G	G	S	G	64-78
	L	I	Q	Q	Q	**L**	V	L	**L**	**L**	H	A	H	K	C	352-366
	I	S	P	S	A	**L**	Q	D	**L**	**L**	R	T	L	K	S	2063-2077
p300	S	K	H	K	Q	**L**	S	E	**L**	**L**	R	G	S	S	P	76-90
	V	I	Q	Q	Q	**L**	V	L	**L**	**L**	H	A	H	K	C	338-352
	V	S	Q	Q	A	**L**	Q	N	**L**	**L**	R	T	L	R	S	2046-2060
TRAP220	S	Q	N	P	I	**L**	T	S	**L**	**L**	Q	I	T	G	N	599-613
	K	N	H	P	M	**L**	M	N	**L**	**L**	K	D	N	P	A	640-654
PELP-1	S	S	G	P	R	**L**	R	L	**L**	**L**	L	E	S	V	S	28-42
	P	H	L	P	G	**L**	M	C	**L**	**L**	R	L	H	G	S	64-78
	F	E	G	L	C	**L**	T	S	**L**	**L**	V	G	E	S	P	106-120
	L	A	V	A	V	**L**	R	D	**L**	**L**	R	Y	A	A	Q	150-164
	I	S	M	N	H	**L**	P	G	**L**	**L**	T	S	L	L	G	172-186
	S	W	E	Q	E	**L**	H	S	**L**	**L**	A	S	L	H	T	259-273
	S	L	L	A	S	**L**	H	T	**L**	**L**	G	A	L	Y	E	266-280
	S	G	E	A	L	**L**	T	H	**L**	**L**	S	D	I	S	P	599-613
	S	T	T	A	N	**L**	L	G	**L**	**L**	S	R	P	S	V	847-861
Tip60	H	E	R	A	M	**L**	K	R	**L**	**L**	R	I	D	S	K	488-502
PGC-1	E	E	P	S	L	**L**	K	K	**L**	**L**	L	A	P	A	N	137-151
TIF1α	Y	P	R	S	I	**L**	T	S	**L**	**L**	L	N	S	S	Q	718-732
NcoA6	L	T	S	P	L	**L**	V	N	**L**	**L**	Q	S	D	I	S	882-896
Ftz	E	R	P	S	T	**L**	R	A	**L**	**L**	T	N	P	V	K	107-122
NRIF3	K	R	S	L	K	**L**	D	G	**L**	**L**	E	E	N	S	F	4-18
	D	S	Y	E	F	**L**	K	A	I	**L**	N					167-177
PERC	D	E	L	S	L	**L**	Q	K	**L**	**L**	L	A	T	S	Y	151-165
	A	E	F	S	I	**L**	R	E	**L**	**L**	A	Q	D	V	L	338-352
Trip230	E	R	I	E	E	**L**	E	N	**L**	**L**	Q	Q	G	G	S	262-277
	E	K	Q	D	V	**L**	Q	K	**L**	**L**	K	E	K	D	L	1400-1414
	G	K	T	G	E	**L**	N	Q	**L**	**L**	N	A	V	K	S	1520-1534
	S	A	G	V	V	**L**	K	D	**L**	**L**	K	Q				1967-1978
Trip3	G	E	S	A	T	**L**	R	S	**L**	**L**	L	N	P	H	L	93-107
Trip4	E	R	Q	D	R	**L**	A	V	**L**	**L**	P	G	R	H	P	32-46
Trip5	I	Y	N	E	E	**L**	F	D	**L**	**L**	N	P	S	S	D	163-177
	E	R	E	Q	E	**L**	H	N	**L**	**L**	E	V	V	S	Q	840-854
Trip8	E	Q	G	S	T	**L**	R	D	**L**	**L**	T	T	T	A	G	32-46
Trip9	Q	H	E	P	F	**L**	D	F	**L**	**L**	G	F	S	A	G	69-83
	Q	A	A	D	V	**L**	E	L	**L**	**L**	R	A	G	A	N	252-266
	R	P	N	P	I	**L**	A	R	**L**	**L**	R	A	H	G	A	284-298
ARA267	E	L	S	A	A	**L**	P	G	**L**	**L**	S	D	K	R	D	721-735
	Q	N	C	E	K	**L**	G	E	**L**	**L**	L	C	E	A	Q	1278-1292
ARA70	Q	Q	A	Q	Q	**L**	Y	S	**L**	**L**	G	Q	F	N	C	87-101
ARA54	P	G	S	P	C	**F**	N	R	**L**	**F**	Y	A	V	D	V	449-463
ARA55	T	C	R	R	D	**F**	L	Q	**L**	**F**	A	P	R	C	Q	316-330
NSD1	E	P	D	Y	K	**F**	S	T	**L**	**L**	M	M	L	K	D	798-812

Table 1.1 An alignment of LXXLL and variant LXXLL motif sequences derived from transcriptional coregulators. See subsequent page for full legend to table 1.1.

Table 1.1 The signature LXXLL motif binds the NR-LBD in a ligand-dependent manner. Alignment of the LXXLL sequences derived from SRC1 (Onate *et al.,* 1995), TIF2 (Voegel *et al.,* 1996), ACTR (Chen *et al.,* 1997), RIP140 (Cavailles *et al.,* 1994), CBP (Chivia *et al.,* 1993), p300 (Eckner *et al.,* 1994), TRAP220 (Yuan *et al.,* 1998), PELP-1 (Vadlamudi *et al.,* 2001), Tip60 (Gaughan *et al.,* 2001), PGC-1 (Puigserver *et al.,* 1998), TIF1α (Le Douarin *et al.,* 1996), NcoA6 (Lee *et al.,* 1999), Ftz (Suzuki et al., 2001), NRIF3 (Li *et al.,* 1999), PERC (Kressler *et al.,* 2002), Trip230 (Chang *et al.,* 1997), the Trip proteins (Lee *et al.,* 1995), ARA267 (Wang *et al.,* 2001), ARA70 (Yeh *et al.,* 1996), ARA54 (Kang *et al.,* 1999), ARA55 (Fujimoto *et al.,* 1999) and NSD1 (Huang *et al.,* 1998). The conserved leucine residues of the classical LXXLL motifs are boxed in addition to the phenylalanine and isoleucine residues of the FXXLF, FXXLL and LXXIL variant motifs. The amino acid numbers of each LXXLL (or variant) containing sequence are shown.

nature (Wurtz *et al.*, 1996) and thus could possibly act as an intramolecular inhibitor of coactivator binding.

1.8.2 NR-binding Specificity

Several coactivators have been found to display binding preferences for NR subclasses. For example, Tip60 has been reported to bind class I NRs but displayed little interaction with VDR, TR or RXR (Gaughan *et al.*, 2001). Similarly, PERC (PGC-1 related estrogen receptor coactivator) has been shown to specifically interact with ERα (Kressler *et al.*, 2002) and NRIF3 specifically interacts with TR and RXR (Li *et al.*, 2001). By contrast the SRC1 family of coactivators interact with a wide range of NRs, although the individual LXXLL motifs derived from these proteins do display differential binding to NRs (Needham *et al.*, 2000, Heery *et al.*, 2001). Further, the LXXLL sequences derived from CBP and RIP140 have been shown to exhibit NR-binding selectivity (Heery *et al.*, 2001). Hence, whilst LXXLL motifs (or variants such as FXXLL, FXXLF and LXXIL) are essential for ligand-dependent NR interactions, there appears to be a NR specific code, where different NRs prefer different LXMs for interaction with coactivators (Leers *et al.*, 1998, Ding *et al.*, 1998, Darimont *et al.*, 1998 and McInerney *et al.* 1998). Several studies have highlighted this NR specific code. For example, *in vitro* peptide competition experiments have shown that a peptide encompassing GRIP1 LXM3 was able to effectively block the interaction between GRIP1 and GR, whilst a peptide encompassing GRIP1 LXM2 more effectively blocked the interaction between GRIP1 and TRβ, suggesting the multiple LXMs of GRIP1 are differentially utilised by NRs (Darimont *et al.*, 1998). Similarly, yeast two-hybrid assays were used to demonstrate that mutations in TIF2 LXM2 had the most deleterious effects on interactions with PPARα, whilst LXM1 mutation had the greatest effect on TIF2-RXR interactions (Leers *et al.*, 1998). This differential usage of LXMs has also been investigated in terms of transcriptional coactivation *in vivo*. Using site-directed mutagenesis and microinjection assays, McInerney and co-workers (1998), were able to demonstrate that microinjection of wildtype SRC1 into SRC1-immunodepleted cells could rescue transactivation of reporter genes. However, microinjection of certain SRC1 mutants, where one or more of the LXMs had been mutated to abolish NR interaction, could not rescue transactivation. Mutations in LXM2 of SRC1 prevented rescue of ER transcriptional activity in SRC1-immunodepleted cells but mutation of LXM2 and LXM3 prevented rescue of RAR and TR activity, and mutation in LXM1 and LXM2 prevented rescue of PR activity (McInerney *et al.*, 1998).

Although sequences encompassing the LXXLL motifs are sufficient for NR-coactivator interactions, amino acids N-terminal and C-terminal to the LXXLL motif appear to make additional contacts with the LBD (Darimont *et al.*, 1998 and McInerney *et al.*, 1998), and it has been proposed that these residues play a role in determining the NR-binding specificity of coactivators. Heery *et al.*, (2001), showed that the LXXLL motifs derived from SRC1 and RIP140, which showed high affinity binding with NR LBDs, had a hydrophobic residue at the –1 position (where the first conserved leucine of the LXM is numbered +1) but the three LXXLL motifs derived from CBP did not have a hydrophobic residue at this position and indeed showed relatively weak interactions with NR LBDs. This perhaps suggests that a hydrophobic residue at the –1 position is favourable for NR interactions. Studies using chimeric peptides of LXXLL motifs derived from SRC1 family members, have highlighted the importance of the sequences immediately flanking the LXXLL motif in determining the NR-binding specificity of the coactivator. For example, a chimeric peptide, containing GRIP1 LXM3 in the context of the flanking amino acid sequences of GRIP1 LXM2, competed as effectively as a peptide comprising GRIP1 LXM2 and its flanking amino acid sequence, for interaction with TRβ (Darimont *et al.*, 1998). This suggested that the flanking amino acid sequences are contributing to the NR-binding specificity. Furthermore, using the microinjection assays described above, McInerney and co-workers (1998), found that the 8 N-terminal flanking amino acids are not essential, while the 8 C-terminal flanking amino acids of SRC1 LXM2 are required for SRC-1 mediated coactivation of RAR, TR and ER. In particular they show that residues at positions +12 and +13 (where the first conserved leucine of the LXM is numbered +1) are essential for SRC1 rescue of ER activity and residues at positons +6, +7, +11 and +13 are essential for the rescue of RAR activity.

With a view to unravel this receptor-specificity code, Chang and co-workers (1999), employed phage display technology to screen a large combinatorial LXM-containing peptide library for NR interactions. They identified three different classes of LXXLL containing peptides that are able to interact with NRs in a ligand-dependent manner. Class I: typically have a conserved serine at the –2 position and a basic residue at the –1 position (S(+)LXXLL). Class II: have a proline at the –2 position and a hydrophobic residue at the -1 position (PΦLXXLL). Class III: have a conserved serine or threonine at the –2 position and a hydrophobic leucine or isoleucine at the –1 position ((S/T)ΦLXXLL). These different classes of LXXLL-containing peptides were found to exhibit different NR-binding properties reinforcing the view that the

amino acid sequences flanking the LXXLL motif are key determinants in defining NR-binding specificity.

1.9 The Metazoan Mediator

1.9.1 TRAP/SMCC/Mediator complex

The human thyroid hormone receptor-associated protein (TRAP) complex was originally isolated using affinity purification of an epitope-tagged TRα from HeLa cells grown in the presence of T_3 (Fondell *et al.*, 1996). Since its discovery in 1996, several TRAP-related complexes have been isolated including an SRB/MED-containing cofactor complex (SMCC) (Gu *et al.*, 1999 and Ito *et al.*, 1999), a vitamin D receptor (VDR)-interacting complex (DRIP) (Rachez *et al.*, 1998 and 1999), a SREBP-interacting complex (ARC) (Naar *et al.*, 1999), an E1A-interacting complex (human mediator) (Boyer *et al.*, 1999), the USA (upstream stimulatory activity)-derived PC2 (Malik *et al.*, 2000) and CRSP (cofactor required for Sp1) complexes (Ryu *et al.*, 1999) and the NAT (negative regulator of activated transcription) (Sun *et al.*, 1998) and mouse mediator complexes (Jiang *et al.*, 1998). A comparison of the subunit compositions of these TRAP-related complexes has revealed that, in essence, they represent either the same or a very similar cellular identity. Further, these complexes share a small subset of homologous subunits (SRB7, SRB10, SRB11, MED6, MED7, MED10 and RGR1) with the multifunctional yeast Mediator complex (Myer *et al.*, 1998 and Malik *et al.*, 2000). The yeast Mediator is a multisubunit complex that interacts reversibly with RNA polymerase II, forming a holoenzyme, and serves as both a coactivator and corepressor in yeast. It is comprised of a group of SRB proteins (suppressor of RNA polymerase B), regulatory proteins such as SIN4, GAL11, RGR1 and a group of biochemically identified MED (Mediator) proteins (Kim *et al.*, 1994). Hence the TRAP/SMCC/DRIP/ARC/CRSP/NAT/mediator complex represents the mammalian mediator and is referred to hereafter as the TRAP/SMCC/Mediator complex.

TRAP/SMCC/Mediator is a 1.5-2.0 MDa complex composed of more than 25 distinct polypeptides including homologues of yeast Mediator components SRB7, SRB10, SRB11, MED7, MED6, MED10 and the yeast regulatory factor SOH1 (suppressor of hpr1), as well as subunits unique to the mammalian mediator e.g. TRAPs, and smaller polypeptides (Malik *et al.*, 2000). These subunits range in size from 12 to 240 kDa. Additionally, although the yeast and mammalian mediators are only distantly related on the basis of these homologous subunits, ultrastructural analyses have revealed a remarkable structural similarity between the two

complexes (Asturias *et al.,* 1999 and Dotson *et al.,* 2000), perhaps suggesting an evolutionary conserved role in transcriptional activation.

In reconstituted *in vitro* transcription assays, utilising naked (chromatin-free) DNA templates, the TRAP complex was observed to enhance the transcriptional activity of liganded TR (Fondell *et al.,* 1996). Similarly, the DRIP complex has been shown to enhance the activity of VDR on naked DNA templates in cell-free ligand-dependent transcription assays (Rachez *et al.,* 1998). Since the most purified TRAP/SMCC/Mediator complex is devoid of HAT activity (Rachez *et al.,* 1999) it would appear that the TRAP/SMCC/Mediator complex plays a novel role in the coactivation of NR activity, distinct from the HAT activities associated with other classes of coactivators (such as SRC1, CBP/p300 and p/CAF). However rather intriguingly, Rachez and co-workers (1999) were able to demonstrate that the purified DRIP complex can enhance VDR transcriptional activity on a chromatinised template *in vitro,* despite its lack of HAT activity, perhaps suggesting a potential unidentified chromatin remodelling function.

1.9.2 The multi-step model of TRAP/SMCC/Mediator function

The ability of TRAP/SMCC/Mediator to enhance NR transcriptional activity, despite its lack of chromatin modifying and remodelling activity, raised questions concerning its exact role in transcriptional activation. However based on its homologous subunits shared with the yeast Mediator, which has been shown to interact with RNA polymerase II (Myer *et al.,* 1998) and its original isolation due to its ligand-dependent association with the TRα, a role as a bridging factor between promoter-bound NRs and the basal transcription machinery has been proposed. Thus the TRAP/SMCC/Mediator complex could mediate NR-enhanced recruitment of RNA polymerase II and other basal transcription factors to the promoter and therefore enhance or stabilise PIC formation. A multi-step (sequential) model has been proposed for the involvement of the TRAP/SMCC/Mediator complex in transcriptional coactivation of NRs in relation to other NR-associated coactivators (Figure 1.14). The proposed model is as follows: (1) ligand-independent recruitment of class II NR-heterodimers to their cognate HREs within the promoter regions of the target gene, together with corepressor complexes, resulting in histone deacetylation and thus repression of transcription, (2) ligand induced dissociation of the corepressor complex and concomitant association of coactivator proteins possessing intrinsic HAT activity or the ability to recruit HAT-containing coactivators, (3) HAT-mediated acetylation of nucleosomal histones, (4) exchange of the HAT-coactivator complexes with

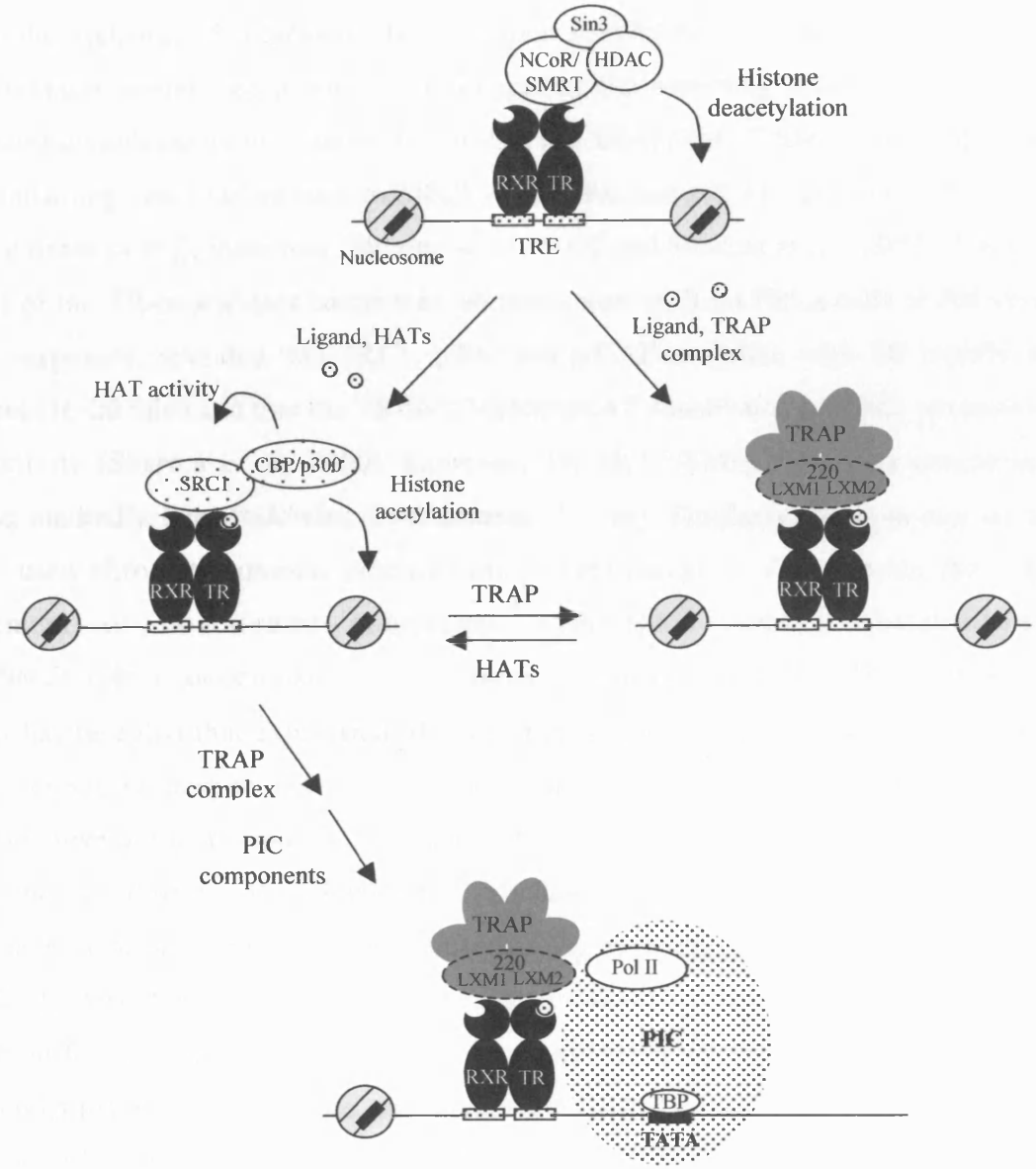


Figure 1.14 A multi-step model has been proposed for the recruitment of distinct coactivator complexes to the HRE-bound NR heterodimer. In the absence of ligand corepressor complexes are recruited to the NR heterodimer. In the presence of ligand corepressor complexes are exchanged for HAT-containing coactivator complexes and subsequently the nucleosomal histones become acetylated. Next the HAT-containing coactivator complexes are exchanged for the TRAP complex, which is thought to act as a bridging factor between the liganded NR and RNA polymerase II. NR-TRAP complexes are thought to be non-functional prior to nucleosomal histone modification. Adapted from Ito *et al.*, 2001.

TRAP/SMCC/Mediator and (5) concomitant or subsequent recruitment of RNA polymerase II and basal transcription factors to form the PIC (Figure 1.14). This sequential model therefore predicts the exchange of cofactors rather than their simultaneous binding to NRs. In support of this sequential model, recent studies investigating the assembly kinetics of different TR-coactivator complexes *in vivo*, have shown that the TRAP/SMCC/Mediator complex and the HAT-containing coactivators such as SRC1, CBP/p300 and p/CAF, are recruited to the TR at different times post T_3 induction (Sharma *et al.*, 2000 and Sharma *et al.*, 2002). For example, analysis of the TR-coactivator complexes immuno-purified from HeLa cells at different times after T_3 exposure, revealed that SRC1, p300 and p/CAF associate with TR rapidly after T_3 treatment (10-20 min) and that the TR-SRC1/p300/pCAF coactivator complex possesses potent HAT activity (Sharma *et al.*, 2000). However, TR-TRAP/SMCC/Mediator complexes were detected markedly later following T_3 treatment (1-3 hr). Similarly, Sharma and co-workers (2002), used chromatin-immunoprecipitation (ChIP) assays to demonstrate the sequential recruitment of coactivator complexes with HAT activity followed by the TRAP/SMCC/Mediator complex, to T_3-responsive promoter bound-TRα *in vivo*. Northern blot analysis has revealed that expression of T_3-responsive genes does not occur until 1-18 hours after T_3 exposure despite the formation of functional TR-SRC1/p300/pCAF coactivator complexes several minutes post T_3 treatment. This is consistent with a multi-step model, where perhaps the recruitment of the TRAP/SMCC/Mediator complex is a rate-limiting step. However promoters may possess multiple HREs, thus allowing the coexistence of NR-SRC1/CBP/pCAF and NR-TRAP/SMCC/Mediator complexes. Presumably under these conditions, the TRAP/SMCC/Mediator containing complexes would be non-functional due to the absence of nucleosomal histone modifications and therefore NR-HAT-containing complex assembly and action might be a regulatory prerequisite for NR-TRAP/SMCC/Mediator complex assembly. The next question that is raised therefore is 'what triggers the exchange of the HAT-containing coactivator complex for the TRAP/SMCC/Mediator complex?' In a recent report CBP/p300 was observed to acetylate ACTR, which resulted in dissociation of ACTR from promoter-bound ER (Chen *et al.*, 1999a). Hence, by CBP/p300 acetylation of SRC1 coactivators, the association of SRC1/CBP/pCAF coactivator complexes with promoter-bound NRs could be disrupted, thus permitting the subsequent association of NRs with the TRAP/SMCC/Mediator complex.

1.9.3 TRAP/SMCC/Mediator subunits

In order to fully characterise the TRAP/SMCC/Mediator complex, several groups have focused their attention on single TRAP/SMCC/Mediator subunits (Figure 1.15). The following is a summary of some of this research, excluding that which is specific to TRAP220, which will be discussed in more detail in section 1.9.4.

TRAP150 (also designated ARC130/DRIP130/CRSP130/Sur-2) is a distant homologue of the *C. elegans* Mediator subunit Sur-2 (suppressor of ras) and has been shown to interact with the adenoviral oncoprotein E1A (Boyer *et al.*, 1999). TRAP150 has been shown to be the subunit of the TRAP/SMCC/Mediator complex which the ETS-family transcription factor, Elk1, targets. Since Elk1 is activated by a mitogen-stimulated Ras-MAP kinase pathway, this implicates TRAP/SMCC/Mediator, through its TRAP150 subunit, as an endpoint integrator of the Ras-MAP kinase signal-transduction cascade.

TRAP100 (also designated ARC100/DRIP100) is unique to higher organisms, with no homologues in *C. elegans* or yeast. Transient over-expression of TRAP100 in mammalian cells has only a modest effect on NR activity (Rachez *et al.*, 1998 and Zhang *et al.*, 1999) and further, TRAP100 shows no interaction with NRs *in vitro* (Yuan *et al.*, 1998). However, genetic studies of the TRAP/SMCC/Mediator complex have shown that ablation of the TRAP100 subunit is embryonic lethal (Ito *et al.*, 2002b). *Trap100*$^{-/-}$ embryos exhibit severe developmental abnormalities including cardiovascular defects and poor, or abnormal, development of the central nervous system (CNS), which together eventually leads to embryonic death. Primary embryonic fibroblasts (MEFs) derived from *Trap100*$^{-/-}$ embryos exhibit severely attenuated basal and activator-dependent transcription, suggesting an important role for the TRAP100 subunit in transcription. Additionally, the mutant TRAP/SMCC/Mediator complex found in *Trap100*$^{-/-}$ cells was shown to lack the TRAP150 and TRAP95 subunits. This suggests the existence of a TRAP100/TRAP150/TRAP95 sub-module that is dependent on TRAP100 for its assembly into the TRAP/SMCC/Mediator complex.

TRAP80 (also designated DRIP77/ARC77/CRSP77) has been shown to interact with the activation domains of the tumour suppressor p53 and the herpes virus activator VP16 (Ito *et al.*, 1999). Hence TRAP80 could mediate the function of p53 and VP16, thus implicating the TRAP/SMCC/Mediator complex in p53 and VP16 signalling pathways.

The TRAP230 (also designated DRIP240/ARC240) protein encoding gene contains a long CAG trinucleotide repeat (Ito *et al.*, 1999) which translates to give a glutamine-rich C-terminal

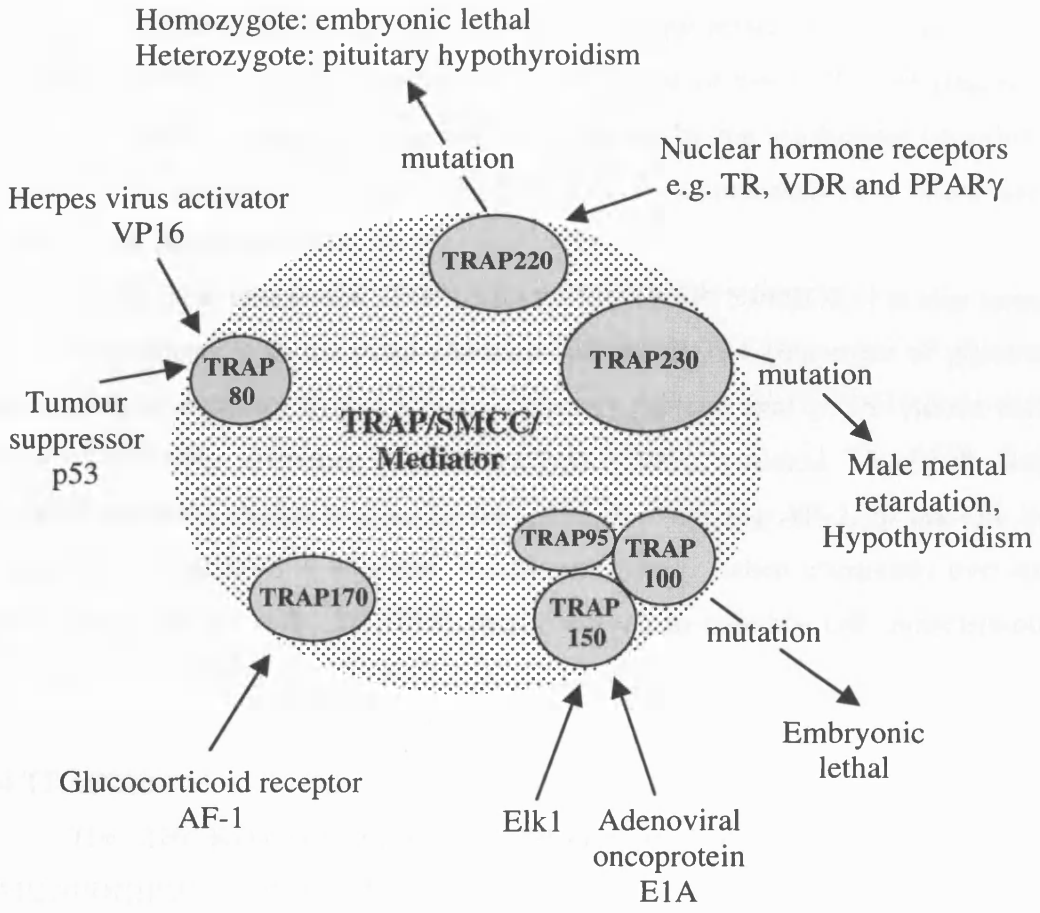


Figure 1.15 The TRAP/SMCC/Mediator complex is able to interact with numerous activators via its various subunits. Some of these subunit-activator interactions are shown, together with phenotypes resulting from mutation of the TRAP220, TRAP230 and TRAP100 subunits. Other TRAP/SMCC/Mediator subunits have been omitted for clarity.

domain. Such CAG repeats have long been associated with many hereditary diseases, most notably with neuropsychiatric disorders. A mutation in the CAG region of the TRAP230 gene has been linked with a significant susceptibility to mental retardation in males (Philibert *et al.*, 1998). Additionally, a mutation within the CAG repeat of the TRAP230 gene has also been linked with hypothyroidism, a condition characterised by the inadequate secretion of thyroid hormones. This supports the idea that TRAP230 is a component of a coactivator complex involved in TR function in humans.

TRAP170 (also designated DRIP150/ARC150/CRSP150/hRGR1) is also termed hRGR1 due to its homology with the yeast Mediator subunit RGR1 (repressor of glucose-regulated genes). Using a yeast two-hybrid screen to identify proteins that could interact with the AF-1 domain of the GR, Hittleman and co-workers (1999), isolated TRAP170. Subsequently TRAP170 has been shown to bind to the AF-1, but not the AF-2, of the GR in a ligand-independent manner both *in vivo* and *in vitro* and further, when transiently over-expressed in mammalian and yeast cells, TRAP170 was observed to enhance GR transcriptional activity (Hittleman *et al.*, 1999).

1.9.4 TRAP220

The 220 KDa subunit of the TRAP/SMCC/Mediator complex is designated TRAP220/DRIP205/PBP (PPARγ-Binding Protein) but will be referred to as TRAP220 hereafter. TRAP220 is a unique subunit of the TRAP/SMCC/Mediator complex in that it displays ligand-dependent interactions with NRs (Figure 1.15) and has been proposed to act as the anchorage subunit that holds the entire TRAP/SMCC/Mediator complex on the NR AF-2 surface in response to ligand. Consistent with the functional relevance of these ligand-dependent interactions with NRs, transient over-expression of TRAP220 in mammalian cells was observed to modestly enhance TRα-, VDR- and PPARγ-mediated transcriptional activity (Yuan *et al.*, 1998, Rachez *et al.*, 2000 and Zhu *et al.*, 1997).

TRAP220 possesses two closely spaced LXXLL motifs (LXM1 amino acids 604 to 608, and LXM2 amino acids 645 to 649), located centrally within the protein and constituting the NID of TRAP220. Apart from this NID, TRAP220 does not appear to possess any other known functional domains nor does it have any close homology with other classes of coactivators. Reports show that TRAP220 is able to bind to RXRα and its heterodimeric partners TRα, TRβ, VDR, RARα, PPARα and PPARγ, in a ligand-dependent manner (Zhu *et al.*, 1997, Yuan *et al.*,

1998 and Treuter *et al.*, 1999). However, there is some ambiguity in the literature concerning TRAP220 ligand-dependent interactions with the ERα. There are reports of ligand-dependent binding of full-length TRAP220 with ERα (Chang *et al.*, 1999, Zhu *et al.*, 1999, Burakov *et al.*, 2000 and Burakov *et al.*, 2002) but conversely there are also reports of no or very weak binding (Yuan *et al.*, 1998, Kobayashi *et al.*, 2000 and Warnmark *et al.*, 2001). Hence, the role of TRAP220 in ERα signalling remains ambiguous.

Similar to the LXMs of the SRC1 family of coactivators, the two LXMs of TRAP220 are preferentially utilised by different NRs (Yuan *et al.*, 1998 and Ren *et al.*, 2000). Initially Yuan and co-workers (1998) demonstrated, using *in vitro* binding assays (GST-pulldowns), that a fragment of TRAP220 encompassing LXM2 bound to full-length TRα with a much stronger affinity than a fragment encompassing LXM1. Moreover, using *in vitro* interaction assays, Ren and co-workers (2000) demonstrated that TRα, VDR and PPARα preferentially bind to TRAP220 LXM2 whereas RXRα preferentially binds LXM1. Taken together, a stoichiometric model for TRAP220-NR interactions was proposed. In this model one molecule of TRAP220 interacts simultaneously with both NRs of the NR-heterodimer, with LXM1 binding RXR and LXM2 binding the heterodimeric partner of RXR.

In addition to these biochemical studies that identified TRAP220 as the mediator of the ligand-dependent interactions of the TRAP/SMCC/Mediator complex with NRs, genetic studies involving the disruption of the *Trap220* gene in mice, have also been employed to investigate the physiological role of TRAP220 (Zhu *et al.*, 2000 and Ito *et al.*, 2000). Results indicate that a TRAP220 knockout in mice is embryonic lethal. *Trap220*$^{-/-}$ embryos showed retarded growth and exhibited severe heart and neurological developmental defects which eventually resulted in their death (Ito *et al.*, 2000). Further, MEFs derived from *Trap220*$^{-/-}$ mice exhibited a significant reduction in T_3/TR-dependent transcription that could be rescued by ectopic expression of TRAP220. This is consistent with the biochemical studies that show TRAP220 binds TR in a ligand-dependent manner and strongly implicates TRAP220 as a genuine coactivator for the TR. However, a small but significant level of T_3/TR-dependent transcription can be seen in *Trap220*$^{-/-}$ MEFs, suggesting the existence of an alternative T_3/TR-signalling pathway independent of TRAP220 and perhaps involving other cofactors or indeed other subunits of the TRAP/SMCC/Mediator complex. RAR- and RXR-dependent transcription was unaffected in *Trap220*$^{-/-}$ MEFs, despite reports showing both RAR and RXR are targets for TRAP220 (Zhu *et al.*, 1997 and Yuan *et al.*, 1998). This implies that TRAP220 is dispensable for retinoid

signalling in MEFs. Moreover, the transcriptional activity of p53 and VP16 in *Trap220*$^{-/-}$ derived MEFs does not show any attenuation, consistent with the proposed role of TRAP80 in p53 and VP16 signalling (Ito *et al.*, 1999).

The condition known as hypothyroidism is characterised by the inadequate secretion of thyroid hormones. Normally the pituitary gland produces thyroid-stimulating hormone (TSH) which, as its name suggests, stimulates the thyroid gland to produce thyroid hormones. However, high levels of thyroid hormone repress the production of TSH in the pituitary and thus the production of thyroid hormones is reduced. Hence the production of thyroid hormones is subject to a strict negative feedback mechanism (Figure 1.16). The ability of high levels of thyroid hormones to repress transcription of the gene encoding TSH is due to the presence of an nTRE in the promoter of the *Tsh* gene. Thus liganded TR bound to the nTRE will repress transcription and conversely TR bound to the nTRE in the absence of ligand will allow transcription of the *Tsh* gene. As well as exhibiting retarded growth, *Trap220*$^{-/+}$ mice also exhibited lowered serum levels of thyroid hormone, characteristic of hypothyroidism. Northern blot analysis revealed that *Trap220*$^{-/+}$ mice have a lowered TSH mRNA level in the pituitary and this was proposed to be the cause of the hypothyroidism. However, the exact mechanism accounting for the lowered TSH levels and hence hypothyroidism in *Trap220*$^{-/+}$ mice is unknown and the subject of much speculation. One theory posits TRAP220 as a positive cofactor for unliganded TR, whilst another suggests TRAP/SMCC/Mediator, in a manner requiring TRAP220, could act as a general coactivator for other activators required for the upregulation of the *Tsh* gene at low levels of thyroid hormone. However, whilst further research is required to unravel this mechanism, it is clear that TRAP220 plays a crucial role in the normal transcriptional control of TSH in the pituitary.

Through its various subunits, TRAP/SMCC/Mediator is able to interact, probably simultaneously, with many activators including NRs, p53, VP16, NF-κB, Sp1, Elk1, SREBP and E1A. This suggests a model for the synergistic function of multiple activators bound to a single promoter, where the TRAP/SMCC/Mediator complex functions as an end-stage integrator of signals from a diverse array of activation pathways.

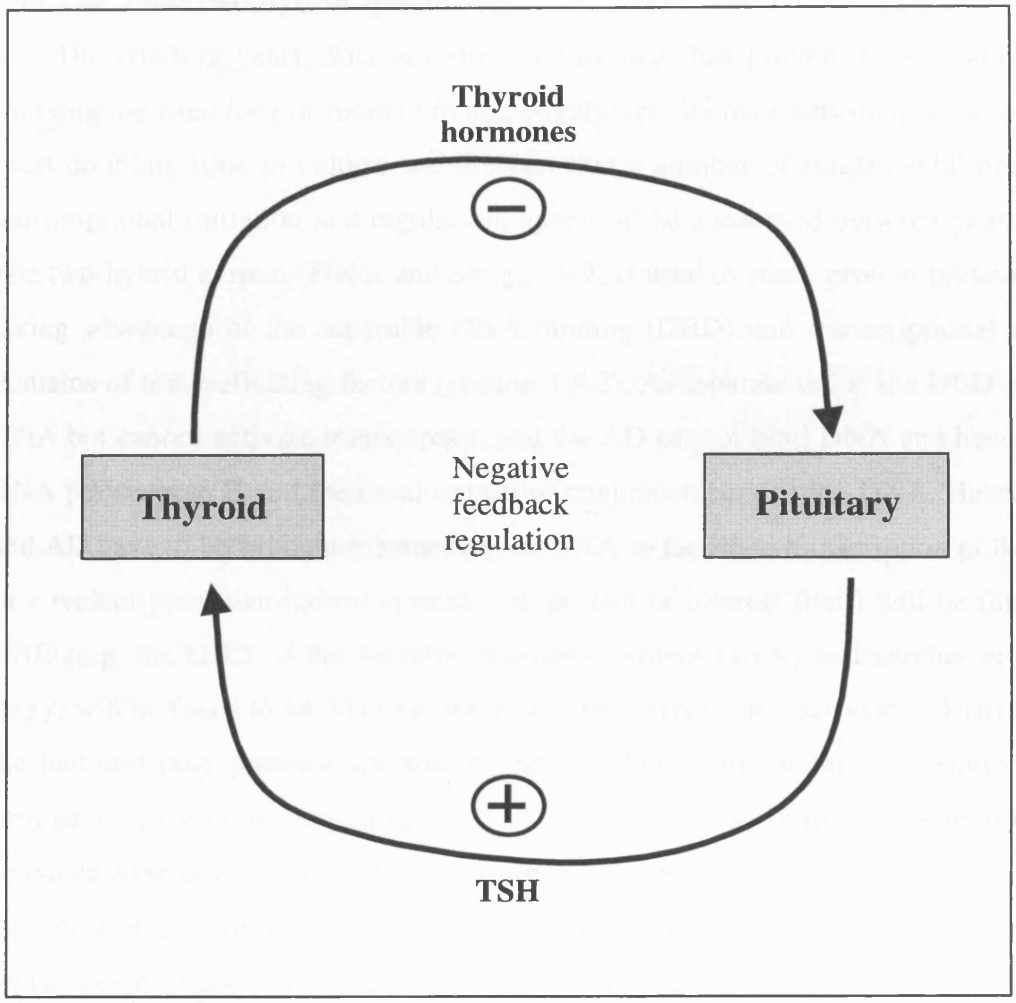


Figure 1.16 Thyroid hormone production is under the control of a negative feedback mechanism. The pituitary gland produces and secretes TSH, which binds to TSH-receptors present on the surface of thyroid cells and stimulates the production of thyroid hormones. High levels of thyroid hormones in the serum and pituitary result in the repression of transcription of the gene encoding TSH and thus the production of thyroid hormones is also lowered. Repression of transcription of the *Tsh* gene is relieved as levels of thyroid hormones fall and TSH is produced. Thus the negative feedback loop cycles.

1.10 The Yeast two-hybrid system

The budding yeast, *Saccharomyces cerevisiae,* has proved to be a powerful model for studying the functions of more complex eukaryotes. Its main advantages include its relatively short doubling time in culture and the fact that a number of fundamental processes, such as transcriptional initiation and regulation, appear to be conserved between yeast and mammals. The two-hybrid system (Fields and Song, 1989) is used to study protein-protein interactions by taking advantage of the separable DNA binding (DBD) and transcriptional activation (AD) domains of transactivating factors (section 1.4.2). As separate units, the DBD can interact with DNA but cannot activate transcription and the AD cannot bind DNA and hence cannot recruit RNA polymerase II and the basal transcriptional machinery to the DNA. Hence both the DBD and AD have to be brought together on the DNA to facilitate transcription of the reporter gene. In a typical yeast two-hybrid system, one protein of interest (bait) will be fused to a specific DBD (e.g. the DBD of the bacterial repressor protein LexA) and another protein of interest (prey) will be fused to an AD (e.g. the AD of the herpes virus activator VP16) (Figure 1.17). If the bait and prey proteins are able to interact then a functional transcription factor will be formed, consisting of both an AD and DBD. Hence transcription of the reporter gene will be activated. One potential drawback in using this system to study the interactions of transcription factors and coactivators is that such proteins may possess ADs capable of activating transcription of the reporter gene. Hence all bait and prey fusion proteins must be tested for intrinsic transcriptional activity. A simplified diagram of a typical yeast two-hybrid system can be seen in figure 1.17.

1.11 NR-mediated transactivation reporter systems

In this study the effect of transiently over-expressed coactivator proteins, on the transcriptional activity of ecoptic NRs has been assessed, by employing NR-mediated transactivation reporter systems. Such reporter systems involve transiently transfecting mammalian cells with a β-galactosidase reporter (as an internal control) and an HRE-linked firefly luciferase reporter, together with expression vectors for the NR and coactivator protein of interest (Sheppard *et al.,* 2001). Thus ectopically expressed NR should bind to its cognate HRE within the luciferase reporter and in the presence of its cognate ligand, mediate transcription of the luciferase reporter gene. Additional over-expression of a coactivator protein

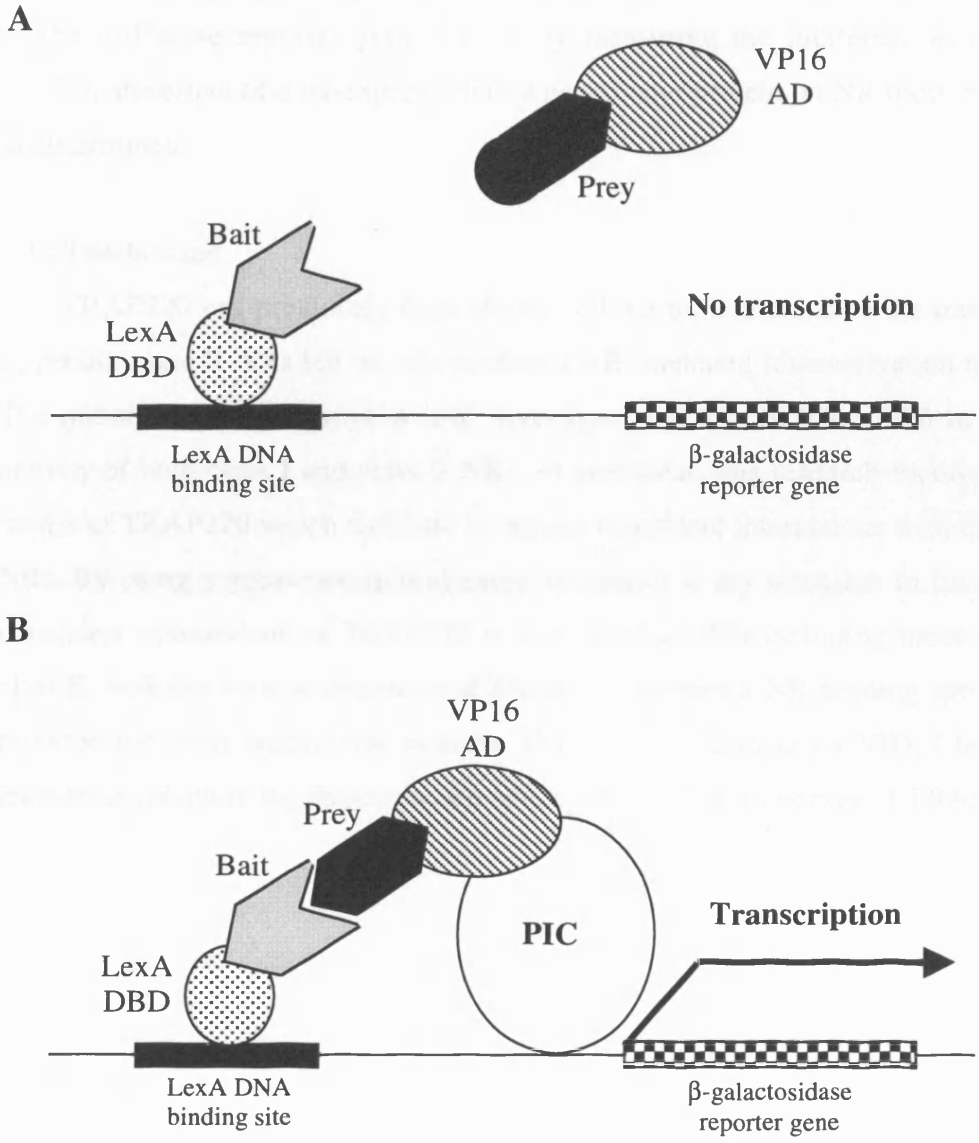


Figure 1.17 The yeast two-hybrid system. (A) The bait and prey proteins are unable to interact and hence transcription of the reporter gene cannot be activated. (B) The prey and bait proteins interact, thus RNA polymerase and the basal transcription machinery can be recruited to the DNA and hence transcription of the reporter gene is activated. Adapted from D. Heery lecture material.

could then potentially interact with the HRE-bound NR and enhance NR-mediated transcription of the luciferase reporter gene. Hence by measuring the luciferase activity of whole cell extracts, the effect of over-expression of a coactivator protein on NR transcriptional activity can be determined.

1.12 Thesis aims

TRAP220 has previously been shown to bind to, and enhance the transcriptional activity of certain class II NRs but its role in class I NR-mediated transactivation remains ambiguous. The initial aim of this work was to investigate the role of TRAP220 in the transcriptional activity of both class I and class II NRs. In particular, this research focuses upon the LXXLL motifs of TRAP220 which facilitate its ligand-dependent interactions with the AF-2 domains of NRs. By using a yeast two-hybrid assay system, it is my intention to investigate the ligand-dependent interactions of TRAP220 with a panel of NRs including those of both class I and class II, with the view to determine if TRAP220 displays a NR-binding specificity, as has been reported for other coactivator proteins. Further, by mutating its NID, I hope to identify key residues involved in the determination of the NR-binding properties of TRAP220.

CHAPTER 2
MATERIALS AND METHODS

2.1 Sources of materials

2.1.1 General suppliers

All general laboratory chemicals were of analytical grade and supplied by Fisher Chemicals or Sigma Chemical Company Ltd. unless otherwise stated. Phosphate buffered saline (PBS) was prepared using PBS tablets (1 tablet/100 ml distilled water) supplied by OXOID Ltd. Double deionised water, purified by passage through an Elgastat Option 2 water purification system (ELGA) was used to make all solutions. The pH of solutions was measured using a Corning pH meter 240. Where appropriate, sterilisation was achieved by either filtration through a 0.22 micron filter or by autoclaving for 15 minutes at 121°C and 25 lb/in^2 pressure.

2.1.2 Bacterial reagents

Tryptone and yeast extract for bacterial growth medium were purchased from OXOID Ltd. *E. coli* strain DH5α (ϕ80d*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K-, m_K+), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZY-*arg*F)U169) (Hanahan, 1983) was used for all molecular cloning applications and was purchased from Stratagene.

2.1.3 Yeast reagents

The yeast strains used were *Saccharomyces cerevisiae* W303-1B (HMLα, MATα, HMRα, *his3-11, 15 trp1-1, ade2-1, can1-100, leu2-3, 11 ura3*) and L40 (*trp1, leu2, his3, ade2,* LYS2::$(lexAop)_{4x}$-HIS3, URA3::$(LexAop)_{8x}$-*LacZ*), which were gifts from R. Rothstein and S. Hollenberg respectively. Complete Supplement Mixture minus X (CSM-X, where X represents any amino acid) was used in yeast selective minimal media and agar (for composition and preparation of yeast selective media/agar see section 2.9) and was purchased from BIO 101 Inc. Herring sperm DNA was obtained from Promega.

2.1.4 Nuclear hormone receptor ligands

All ligands were obtained from Sigma Chemical Company Ltd. with the exceptions of mibolerone, which was supplied by Steraloids Inc., promegestone (R5020), supplied by ICN and Rosiglitazone, which was a kind gift from GlaxoSmithKline Pharmaceuticals Ltd.

2.1.5 Molecular biology reagents

All enzymes and their respective buffers were purchased from Roche, ABgene or Invitrogen Life Technologies. dATP, dCTP, dGTP and dTTP were purchased from ABgene. 1kb Plus DNA ladder was purchased from Invitrogen Life Technologies. QIAquick gel extraction kit (50), QIAprep mini and maxi DNA prep kits were obtained from QIAGEN Ltd. Oligonucleotides for sequencing and PCR applications were purchased from the Protein and Nucleic Acid Sequencing laboratory (PNACL), Leicester University.

2.1.6 Protein chemistry, western blotting and immunofluorescence reagents

30% (w/v) acrylamide mix was obtained from National Diagnostics. 10x Tris-glycine-SDS running buffer and Precision Protein Standards were purchased from Bio-Rad. Nitrocellulose transfer membrane was obtained from Schleicher and Schuell and FUJI medical x-ray film was used. Enhanced Chemiluminescence system (ECL Plus™), Amplify™, Glutathione Sepharose® 4B beads and [^{35}S] labelled methionine were purchased from Amersham Pharmacia Biotech. Complete Protease Inhibitor Cocktail tablets were obtained from Roche. All primary antibodies, Horseradish peroxidase (HRP) conjugated secondary antibodies, FITC- and TRITC-conjugated secondary antibodies and lactacystin proteasome inhibitor were obtained from Autogen Bioclear UK Ltd. or Sigma, with the exception of the anti-F (ER) region primary antibody which was a gift from P. Chambon. Secondary antibodies used in immunofluorescence experiments were all purchased from Sigma. Isopropyl-β-D-Thiogalactopyranoside (IPTG) was purchased from Melford Laboratories Ltd. and DTT was purchased from USB. The TNT® coupled reticulocyte lysate system and recombinant RNasin® ribonuclease inhibitor used for *in vitro* transcription/translation reactions were purchased from Promega.

2.1.7 Tissue culture and transient transfection reagents

HeLa and COS-1 cells were purchased from the European Collection of Cell Cultures (ECACC). All tissue culture reagents were purchased from Invitrogen Life Technologies and plasticware was obtained from Helena Biosciences. CalPhos™ mammalian transfection kit was purchased from Clontech Laboratories Inc. and FuGENE™ 6 transfection reagent was purchased from Roche.

2.1.8 Assay reagents

Luciferase assay reagent and lysis buffer were purchased from Promega. β-galactosidase Galacto-Light Plus™ assay kit was purchased from TROPIX Inc. Protein assay reagent was purchased from Bio-Rad Laboratories and bovine serum albumin (BSA) protein standard was purchased from First Link (UK) Ltd.

2.2 Propagation, purification and manipulation of plasmid DNA

2.2.1 Preparation of competent *E.coli*

The *E.coli* strain DH5α was used for the propagation of plasmid DNA. DH5α cells were streaked, from a glycerol stock stored at –70°C, onto Luria Bertani (LB) agar plates and incubated at 37°C overnight. A single colony was picked and used to inoculate 10 ml LB medium which was then grown overnight in an orbital shaker at 37°C, 225 rpm. This starter culture was then diluted 1/100 and cultured at 37°C until the optical density (OD) at 600 nm was 0.5-0.6. To pellet the cells, the culture was centrifuged in pre-chilled sterile tubes (a 200 ml culture was centrifuged in 50 ml aliquots) for 5 min at 4,000 *g*, 4°C. The supernatant was discarded and the cell pellet resuspended in 15 ml ice-cold TFBI (see section 2.7). Cell suspensions were pooled into 30 ml aliquots, incubated on ice for 10 min and then centrifuged as before. The supernatant was again discarded and the cell pellet resuspended in 4 ml ice-cold TFBII (see section 2.7). The cells were quickly aliquoted into pre-chilled 1.5 ml microfuge tubes, flash frozen in dry ice/IMS and stored at -70°C, where they remained viable for several months.

2.2.2 Transformation of competent *E.coli*

50 µl aliquots of competent cells prepared, as described in section 2.2.1, were used for each transformation. In the case of previously constructed plasmids, 1 ng of DNA was used to ensure a good yield of successfully transformed cells. When transforming ligation mixtures, 5-10 µl of the ligation mix was used (see section 2.2.14). Cells and DNA were mixed in a pre-chilled 1.5 ml microfuge tube and incubated on ice for 15 min. The transformation mixture was then heat shocked by incubation at 42°C for 90 sec, followed by incubation on ice for 2 min. 950 µl LB media was then added to the heat shocked cells and they were incubated for 1 hr at 37°C. The cells were then centrifuged at 3,000 *g* for 2 min and 900 µl supernatant was discarded. The remaining 100 µl supernatant was used to resuspend the cell pellet and this cell

suspension was spread on LB agar plates containing 100 μg/ml ampicillin. These were then dried and incubated at 37°C overnight.

The transformation efficiency of the competent cells could be determined by transforming with a known amount (small, e.g. 10 pg pSG5) of plasmid DNA and then calculating:

Number of colonies obtained x dilution factor x 1/amount of DNA used (μg)

If the transformation efficiency of the competent cells was $1x10^{-6}$ - $1x10^{-7}$, and if no bacterial colonies were obtained in a no DNA control then the competent cells were deemed suitable for use.

2.2.3 Long term storage of bacterial cultures

Bacterial cultures were maintained for long periods of time by transferring 0.8 ml of a turbid bacterial culture to a cryogenic storage tube. 0.2 ml sterile glycerol was then added to the culture, the contents were vortexed, flash frozen (IMS/dry ice) and stored at -70°C.

2.2.4 Small scale plasmid DNA preparation

QIAGEN QIAprep mini prep kits were used for the small scale preparation of plasmid DNA. This DNA was primarily necessary to screen for plasmids containing the correct DNA inserts. 5 ml LB, containing 100 μg/ml ampicillin, in a sterile universal was inoculated with a single bacterial colony and grown overnight in an orbital shaker at 37°C, 225 rpm. 1.5 ml was pelleted by centrifugation in a microfuge tube at 4,000 *g* for 2 min. The remaining bacterial culture was stored at 4°C for later use. The pelleted cells were then resuspended, subjected to alkaline lysis and neutralized. The plasmid DNA was absorbed onto a silica gel membrane, washed and eluted. This technique was carried out according to manufacturers instructions and details can be found in the QIAGEN QIAprep miniprep handbook.

2.2.5 Large scale plasmid DNA preparation

In order to obtain larger quantities (up to 500 μg) of high quality plasmid DNA suitable for transient transfections and *in vitro* transcription/translation experiments etc. the QIAGEN-tip 500 plasmid maxiprep kit was used according to manufacturers instructions. A single colony, containing the plasmid of interest, was picked and used to inoculate 5 ml LB containing

100 µg/ml ampicillin. This was grown in an orbital shaker at 37°C, 225 rpm for 8 hr prior to being diluted 1/100 and grown for a further 16 hr. Isolation of the plasmid DNA was achieved by alkaline lysis of the cells, followed by immobilization of the plasmid DNA on QIAGEN anion-exchange resin, elution and precipitation in isopropanol. DNA precipitates were resuspended in 400 µl sterile H_20 and re-precipitated in 1 ml absolute ethanol, prior to being washed in 70% (v/v) absolute ethanol, dried under vacuum to remove residual ethanol and resuspended in the appropriate volume of sterile H_2O. Details of this technique and compositions of solutions used can be found in the QIAGEN maxiprep handbook.

2.2.6 Spectrophotometric quantification of DNA

All DNA was quantified and qualified by spectrophotometry, restriction digest (section 2.2.10) or by comparison of various dilutions of DNA samples with molecular weight markers of known concentration following separation by agarose gel electrophoresis (section 2.2.8). For spectrophotometric analysis, absorbance readings at 260 nm and 280 nm wavelengths were taken using a Unicam PU8600 UV/VIS spectrophotometer. At 260 nm an optical density of one is equivalent to 50 µg/ml of double stranded DNA. Pure DNA has an A_{260}/A_{280} absorbance ratio of greater or equal to 1.7. A_{260}/A_{280} absorbance ratio's lower than 1.7 usually indicate the DNA sample is contaminated with protein.

The concentration of resuspended double stranded DNA or single stranded DNA were calculated according to the following equations;

For double stranded DNA:

$$A_{260\,nm} \text{ x dilution factor x } 50 = \mu g/ml$$

For single stranded DNA:

$$A_{260\,nm} \text{ x dilution factor x } 38 = \mu g/ml$$

2.2.7 Phenol/chloroform extraction and ethanol precipitation of DNA

Phenol/chloroform extraction, followed by ethanol precipitation was preformed on all DNA samples with a low A_{260nm}/A_{280nm} absorbance ratio (i.e. lower than 1.7). An equal volume of phenol was added to the DNA solution to precipitate the protein contaminant and the mixture was vortexed for 20 sec, followed by centrifugation at 13,000 *g* for 2 min. The top aqueous layer was transferred to a clean microfuge tube to which an equal volume of

chloroform was added to remove residual phenol, vortexed for 20 sec and centrifuged at 13,000 *g* for 2 min. The top aqueous layer was transferred to a clean microfuge tube to which 0.1 volumes of 3 M sodium acetate (pH 5.2) and 3 volumes absolute ethanol were added to precipitate the DNA, vortexed for 10 sec prior to being incubated at –70°C for 15 min (or –20°C overnight). The sample was then thawed on ice and centrifuged at 13,000 *g* for 10 min and the supernantent discarded. The pellet was washed in 70% (v/v) absolute ethanol, centrifuged and the supernatant discarded as before. The resulting pellet was dried under vacuum to remove residual ethanol and resuspended in an appropriate volume of sterile H_2O.

2.2.8 Agarose gel electrophoresis

DNA was size fractionated on neutral agarose gels of between 0.8 and 2% as appropriate to the size of the fragments being resolved. Gels containing 0.5 µg/ml ethidium bromide were made and run in 1x TBE buffer as described in standard protocols (Sambrook *et al.*, 1989). DNA samples were loaded in a 1x gel loading buffer. Following electrophoresis gels were visualised by transillumination with ultra violet light and photographed. The size (kb) and amount (ng) of DNA fragments was determined by comparison with a reference DNA molecular weight standard kb ladder.

2.2.9 Purification of DNA fragments from agarose gel slices

DNA fragments were purified from agarose gel slices using a QIAquick gel extraction kit according to manufacturers instructions. The DNA was bound to the QIAquick membrane, washed and eluted in sterile H_2O.

2.2.10 Restriction digest of DNA

Plasmid DNA was digested with restriction endonucleases in order to generate compatible ends for cloning and to verify newly created plasmids. The reaction conditions were set up according to manufacturers instructions. Typically, digests were set up in a 30 µl reaction volume consisting of:

DNA	x µl
restriction endonuclease (10 units/µl)*	2 µl
10x manufacturers buffer	3 µl
sterile H_2O	x µl, to total volume of 30 µl

Digests were normally carried out at 37°C for 3-4 hr unless advised otherwise in the manufacturers instructions.

* When two or more enzymes were used simultaneously the total reaction volume was increased proportionally to prevent the glycerol concentration exceeding 10%, which can be problematical when using certain restriction endonucleases.

2.2.11 Removal of 5' terminal phosphate groups from cleaved plasmid DNA

In order to reduce the efficiency with which plasmid DNA cleaved by a single restriction endonuclease would re-ligate itself without any insert DNA, the 5' terminal phosphate groups were removed by treatment with calf intestinal alkaline phosphatase (CIAP). Restriction digests were set up and incubated as in section 2.2.10 and then 5 µl manufacturers 10x CIAP buffer and 2 µl CIAP (1 unit/µl) were added to the digestion mixture and the final volume made up to 50 µl with sterile H_2O. This mixture was incubated at 37°C for 1 hr prior to incubation at 65°C for 10 min to inactivate the CIAP.

2.2.12 Addition of 5' terminal phosphate groups to oligonucleotides

Short oligonucleotides (27-39mers) needed to generate short double stranded DNA inserts, first had to have a phosphate group added to their 5' terminus to make them compatible with cut, CIAP treated vector. The following polynucleotide kinase reaction was set up:

Oligonucleotide (10 ng/µl)	5 µl
5x T4 DNA ligation buffer*	10 µl
polynucleotide kinase	2 µl
sterile H_2O	33 µl

* T4 DNA ligation buffer was used as a source of ATP, which acts as the phosphate group donor in this reaction.

Kinase reactions were incubated at 37°C for 30 min. Kinased oligonucleotides were then ethanol precipitated (section 2.2.7) and resuspended in 10 µl sterile H_2O.

2.2.13 Annealing short complementary oligonucleotides

Kinased oligonucleotide complementary pairs from section 2.2.12 were annealed to create an oligonucleotide cassette that was subsequently used in ligation reactions. Equal quantities of oligonucleotide pairs were mixed and incubated at 100°C for 2 min, followed by 80°C for 2 min and then allowed to cool to room temperature. Annealed DNA could be stored at –20°C or used in ligation reactions (section 2.2.14).

2.2.14 Ligation of DNA fragments

Recombinant plasmids were created by annealing cut fragments using T4 DNA ligase. All restriction digests in this study resulted in the production of compatible cohesive ends. Relative quantities of cut vector and digested inserts were determined by agarose gel electrophoresis (section 2.2.8) and then typically cohesive end ligation reactions were set up so the vector:insert ratio was 1:5. Ligations were set up as follows and incubated at room temperature for 2 hr:

Vector	x µl
Insert	x µl
5x T4 DNA ligation buffer	2 µl
T4 DNA ligase (5 units/µl)	1 µl
Sterile H_2O	x µl to a total volume of 10 µl

Vector only controls were set up as above except without the addition of insert DNA.

2.2.15 Polymerase Chain Reaction (PCR)

PCR was used to generate DNA fragments needed for the construction of recombinant plasmids and also to screen newly generated recombinant plasmids for the correct DNA inserts. For the synthesis of DNA to be used for cloning, a DNA polymerase enzyme with proofreading (3' to 5' exonuclease) activity (Elongase® and manufacturers associated buffers) was used in the following typical reaction mixture:

10x reaction buffer A*	10 µl
10x reaction buffer B**	10 µl
dNTP mixture (2 mM)	10 µl
forward PCR primer (100 µM)	1 µl
reverse PCR primer (100 µM)	1 µl
DNA template (10 ng/µl)	2 µl
Elongase polymerase enzyme	1 µl
Sterile H_2O	65 µl

* 10x reaction buffer A was 600 mM Tris-SO_4, (pH 9.1), 180 mM $(NH_4)_2SO_4$, 10 mM $MgSO_4$. ** 10x reaction buffer B was 600mM Tris-SO_4, (pH 9.1), 180 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$. Buffer A and B could be differentially combined to optimize Mg^{2+} concentration.

The PCR mixture was subject to a pre-amplification denaturation step of 94°C for 2 min and then typical thermal cycling parameters were as follows: denaturation, 94°C for 1 min, annealing, 50-65°C for 1 min and extension, 68°C for 1 min per kb of target DNA, for a total of 30-35 cycles.

A DNA polymerase enzyme lacking 3' to 5' exonuclease activity (Thermoprime plus DNA polymerase and manufacturers associated buffers) was used to screen for plasmids containing the desired DNA insert. A typical PCR reaction mixture was as follows:

10x manufacturers reaction buffer IV	5 µl
dNTP mixture (2 mM)	5 µl
forward PCR primer (100 µM)	0.2 µl
reverse PCR primer (100 µM)	0.2 µl
bacterial culture*	5 µl
$MgCl_2$ (25 mM)	3 µl
Thermoprime plus DNA polymerase (5 units/µl)	0.2 µl
Sterile H_2O	31.4 µl

*Bacteria transformed with the plasmid of interest were grown in LB culture and when turbid this was used directly in the PCR.

The PCR mixture was subject to a pre-amplification denaturation step of 94°C for 3 min and then thermal cycling parameters were as follows: denaturation, 94°C for 30 sec, annealing, 50-65°C for 30 sec and extension, 74°C for 1 min per kb of target DNA, for a total of 35 cycles.

2.2.16 Synthesis of oligonucleotides

Oligonucleotides were synthesized on an Applied Biosystems model 394 machine (Protein and Nucleic Acid Sequencing Laboratory, Leicester University) at a 0.2 μM scale.

2.2.17 Oligonucleotides

Details of Oligonucleotides employed are given in table A.1 (see appendix).

2.3 Cell culture

2.3.1 Maintenance of cell lines

HeLa and COS-1 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing phenol red and supplemented with 10% (v/v) heat inactivated (56°C for 1 hr) fetal calf serum (FCS) and 1% (v/v) 0.2 M L-glutamine. Cells were grown in 60 mm tissue culture (TC) dishes at 37°C in a humidified atmosphere containing 5% CO_2. Cells were passaged at 100% confluency for a maximum of 50 passages at which point the cells were discarded. To passage cells the DMEM was aspirated and the cell layer washed twice with sterile PBS. 1 ml trypsin/EDTA was added to the cell layer and incubated at room temperature for 1 min prior to being aspirated and the cells returned to the 37°C, 5% CO_2 incubator for 2 min. Cells were then resuspended in the appropriate volume of DMEM and transferred to fresh 60 mm TC dishes (for maintenance) or 6-well plates (for transient transfections).

2.3.2 Transient transfection

Transient transfections were performed either using the calcium phosphate co-precipitation method or the non-liposomal FuGENE™ 6 transfection reagent. All volumes and quantities described below are based on transient transfection of cells seeded in 6 well plates. For transient transfections of cells seeded in 60 mm dishes, volumes and quantities were adjusted accordingly. In co-transfection experiments where multiple plasmid DNAs were used to transfect cells, μg quantities of DNA were kept constant between individual wells.

The CalPhos™ mammalian transfection kit was used for transient transfections by the calcium phosphate co-precipitation method according to manufacturers instructions. 24 hr prior to transfection, HeLa or COS-1 cells were seeded at a density of 1.3 x 10^5 cells/well in phenol red-free DMEM supplemented with 5% 2x dextran-charcoal stripped FCS. FCS was 2x dextran-charcoal stripped to remove steroid hormones which could interfere with steroid hormone-dependent protein-protein interactions. The calcium-phosphate-DNA precipitate was prepared as follows: 15.5 µl 2 M $CaCl_2$ and the appropriate plasmid DNA (table 2.1) were mixed and made up to a total volume of 125 µl with sterile H_2O. This DNA solution was added dropwise to 125 µl 2x HEPES-buffered saline (HBS) and mixed thoroughly. This mixture was allowed to precipitate at room temperature for 20 min and then added to the cells.

Cells to be transfected using FuGENE™ 6 transfection reagent were seeded at a density of 1 x 10^6 cells/well in phenol red-free DMEM supplemented with 5% 2x dextran-charcoal stripped FCS, 24 hr prior to transfection. The FuGENE-DNA complex was prepared as follows: an appropriate volume of FuGENE™ 6 reagent was diluted with serum-free DMEM to a final volume of 100 µl, so the FuGENE™ 6 reagent (µl) to DNA (µg) ratio would be 3:2. The DMEM/FuGENE solution was equilibrated at room temperature for 5 min. In a separate 1.5 ml microfuge tube the appropriate plasmid DNA solution was prepared to a final volume of 0.5-50 µl. The DMEM/FuGENE solution was then added dropwise to the DNA solution, mixed by flicking the tube and incubated at room temperature for 15 min. This FuGENE-DNA complex was then added to the cells.

In this study three different nuclear hormone receptor (NR)-mediated transcription systems were used, including a TRβ, ERα and Gal4-RXRα system, to analyse the influence of TRAP220, TRAP170 and SRC1e on NR-mediated transcription. Typically each well received a β-galactosidase reporter and a ligand responsive (T_3, E_2 or 9c-RA) luciferase reporter and then additionally a NR-expression vector and optionally a TRAP220, TRAP170 or SRC1e expression vector. Table 2.1 outlines details of plasmid DNA used.

The addition of the proteosome inhibitor, Lactacystin, at a final concentration of 5 µM, 8 hr after transfection of the cells was employed when cell-free extracts were to be prepared for western blotting. For transfected cells to be used in luciferase and β-galactosidase assays, at 16 hr post incubation with the DNA complex (calcium phosphate-DNA or FuGENE-DNA complex) the media was aspirated, replaced with fresh phenol red-free DMEM and incubated at 37°C, 5% CO_2 for a further 8 hr. The appropriate ligand (10^{-7} M 3,3',5-Triiodo-$_L$-Thyronine

(T_3) for the TRβ-mediated system, 10^{-8} M 17β-estradiol (E_2) for the ERα-mediated system, 10^{-7} M 9-*cis* retinoic acid (9c-RA) for the Gal4-RXRα-mediated system) or vehicle was added to the cells in fresh phenol red-free DMEM and further incubated at 37°C, 5% CO_2 overnight. Cells were harvested the next day (section 2.2.3).

Plasmid DNA	*Quantity DNA used	Description	Source/reference
pJ7-LacZ	0.5 μg/well	β-galactosidase expression vector used as internal control to normalize luciferase data.	E. Kalkhoven, Imperial Cancer Research Fund, London, UK.
p3ERE-TATA-LUC	1.0 μg/well	An estrogen responsive luciferase reporter. Contains 3 copies of the ER DNA response element upstream of a TATA-box, which is linked to the coding sequence of firefly luciferase.	E. Kalkhoven, Imperial Cancer Research Fund, London, UK. Legler *et al.*,(1999)
pMAL-TKLUC	2.0 μg/well	A T_3 responsive luciferase reporter which contains a single copy of a direct repeat TRE, (taken from the malic enzyme gene) upstream of the thymidine kinase promoter and firefly luciferase cDNA.	Collingwood *et al.*, 1994
pPAL-TKLUC	2.0 μg/well	A T_3 responsive luciferase reporter which contains 2 copies of a palindromic TRE, upstream of the thymidine kinase promoter and firefly luciferase cDNA.	Collingwood *et al.*, 1994
pF2-TKLUC	2.0 μg/well	A T_3 responsive luciferase reporter which contains a single copy of an everted repeat TRE (a chicken lysozyme silencer element TRE(F2)), upstream of the thymidine kinase promoter and firefly luciferase cDNA.	Collingwood *et al.*, 1994
pGAL4-EI6ΔLUC	0.6 μg/well	Luciferase reporter containing a Gal4 binding site upstream of firefly luciferase cDNA.	P.T. van der Saag, Institute for Developmental Biology, Utrecht, The Netherlands.

Plasmid DNA	*Quantity DNA used	Description	Source/reference
pMT-MOR	0.1 μg/well	Mouse estrogen receptor expression vector.	M. G. Parker, Imperial Cancer Research Fund, London, UK. Lahooti *et al.*, (1994).
hTRβ-RSV	0.2 μg/well	Human thyroid receptor β expression vector containing Rous sarcoma virus (RSV) enhancer and promoter.	Collingwood *et al.*, 1997
pGal4-RXRα	0.1 μg/well	The C-domain and domains DEF (LBD) of RXRα fused in frame with the DBD of Gal4.	P.T. van der Saag, Netherlands Institute for Developmental Biology, 3584 CT Utrecht, The Netherlands.
pSG5-RXRα	0.2 μg/well	Full-length human RXRα expression vector.	M. G. Parker, Imperial Cancer Research Fund, London, UK.
pSG5	variable	Eukaryotic expression vector.	Green *et al.*, 1988
pSG5(PT)	variable	Modified pSG5 eukaryotic expression vector. Contains modified MCS.	D. Heery lab records - Ref.8802.
pSG5(PT)-HA-TRAP220	As indicated	Full-length TRAP220 (1582 amino acids), with an N-terminal HA tag, was sub-cloned into pSG5(PT) using XmaI and NotI restriction sites.	TRAP220 cDNA provided by R. G. Roeder, The Rockefeller University, New York, USA.
pSG5-SRC1e	As indicated	Full-length SRC1e expression vector.	Kalkhoven *et al.*, 1998
pSG5-HA-GRIP1	As indicated	Full-length GRIP1, with N-terminal HA tag, expression vector.	M. R. Stallcup, University of Southern California, USA. Ding *et al.*, 1998

Plasmid DNA	*Quantity DNA used	Description	Source/reference
pFLAG(s)-7-TRAP170	As indicated	Full-length TRAP170, with N-terminal FLAG tag, expression vector.	R. G. Roeder, The Rockefeller University, New York, USA.
pCMV-p300	As indicated	Full-length p300, with C-terminal HA tag, expression vector.	E. Kalkhoven, Imperial Cancer Research Fund, London, UK.

Table 2.1 Plasmid DNA used in transient transfection experiments

* μg quantity of plasmid DNA for a single well of a 6-well plate. DNA quantity is not shown if it was varied between experiments and will be indicated elsewhere.

2.3.3 Preparation of cell-free extracts for western blotting

Cells were harvested by aspirating the DMEM and scraping the cell layer into 1 ml sterile PBS which was transferred to a sterile 1.5 ml microfuge tube. Centrifugation at 5,000 *g* for 5 min at room temperature was used to pellet the cells before discarding the supernatant. The pelleted cells were resuspended and lysed on ice in 150 µl RIPA (lysis) buffer containing protease inhibitors. Lysis was allowed to proceed for 30 min with periodic vortexing to aid efficient lysis and then the cell debris was pelleted by centrifugation at 2,000 *g*, 4°C for 2 min. The cell-free extract (supernatant) was transferred to a fresh sterile 1.5 ml microfuge tube. Cell-free extracts and cell debris pellets were stored at –20°C.

2.3.4 Preparation of cell-free extracts for luciferase and β-galactosidase assays

Cell-free extracts to be assayed for luciferase and β-galactosidase activity were prepared by essentially the same method as those to be used in western blotting but with a few alterations as outlined here. Cells were harvested by scraping the cell layer into 500 µl PBS and transferring to a fresh 1.5 ml microfuge tube. Centrifugation was at 3,000 *g* for 5 min at room temperature to pellet the cells. The pelleted cells were resuspended and lysed on ice in 100 µl lysis buffer (supplied by Promega at 5x stock). Lysis was aided by vortexing and cells were incubated at room temperature for 15 min (if cell extracts were to be used immediately in luciferase and β-galactosidase assays) or frozen at –20°C for a maximum of 1 week. Cell debris was pelleted by centrifugation at 13,000 *g* for 10 min.

2.3.5 Luciferase assay

The amount of luciferase produced due to the nuclear hormone receptor (NR)-mediated transcriptional activation of luciferase reporter constructs was quantified using the firefly luciferase assay system according to manufacturers instructions. 5 µl cell-free extract was mixed with 50 µl luciferase assay reagent at room temperature in a luminometer tube and transferred to a Optocomp I luminometer (MGM Instruments), where the light emitted over a period of 10 sec was measured. Luciferase readings of triplicate samples were measured, averaged and normalized relative to β-galactosidase activity as determined by the β-galactosidase assay outlined in section 2.3.6.

2.3.6 β-galactosidase assay

β-galactosidase enzyme was produced in cells due to the expression of the transiently transfected β-galactosidase expression vector. Therefore measurement of β-galactosidase activity in cell-free extracts was used to indirectly monitor transient transfection efficiency and normalise luciferase measurements (section 2.3.5). β-galactosidase was detected using the Galacto-Light Plus™ assay system according to manufacturers instructions. Galacton-plus substrate was diluted 1:100 with reaction buffer diluent to make reaction buffer. 5 µl cell extract was mixed with 50 µl reaction buffer in a luminometer tube and incubated at room temperature for 1 hr. 75 µl light emission accelerator was added and the light emitted over a period of 10 sec was measured. The amount of light produced by the reaction should be proportional to the amount of β-galactosidase in the sample.

2.4 Protein chemistry

2.4.1 SDS-polyacrylamide gel electrophoresis (PAGE)

Cell extracts were analysed by one-dimensional polyacrylamide gel electrophoresis using either a mini-gel system (Protean II, Bio-Rad) or large gel system (Bio-Rad), according to the method described by Laemmli, 1970. Cell extracts (mammalian and yeast) and *in vitro* translated proteins were resuspended in the appropriate volume of SDS-loading buffer and boiled for 5 min, prior to being resolved on 8-12% SDS-polyacrylamide gels (table 2.2), depending on the sizes of the proteins to be resolved.

	Resolving Gel		**Stacking Gel**
%	**8%**	**12%**	**5%**
dH_2O	4.6 ml	3.3 ml	6.8 ml
30% acrylamide	2.7 ml	4.0 ml	1.7 ml
1.5 M Tris (pH 8.8)	2.5 ml	2.5 ml	1.25 ml (pH 6.8)
10% SDS	100 µl	100 µl	100 µl
10% APS	100 µl	100 µl	100 µl
TEMED	6 µl	4 µl	10 µl

Table 2.2 Preparation of polyacrylamide gels

2.4.2 Western blotting

Protein samples were separated by SDS-PAGE and transferred from the polyacrylamide gel to nitrocellulose membrane by electrophoresis using a wet transfer apparatus (Bio-Rad). Membranes were incubated in Ponceau S stain for 5 min and then washed in sterile H_2O to monitor protein transfer. Ponceau S stained membranes could be photocopied or photographed. The following incubation steps were performed at room temperature on a rocking platform. Membranes were incubated in blocking buffer for 1 hr, to block non-specific antibody binding sites, prior to being incubated with primary antibody for 2 hr. Primary antibodies were prepared in blocking buffer containing 0.05% (v/v) Tween 20, at dilutions indicated in table 2.3. Membranes were washed in PBS containing 0.05% Tween 20 (6 x 5 min) and incubated in appropriate secondary antibody for 1 hr. Horseradish peroxidase (HRP) conjugated secondary antibodies were prepared in blocking buffer containing 0.1% Tween 20, at dilutions specified in table 2.3. Membranes were then washed as above. Proteins were visualized using ECL according to manufacturers instructions. After protein detection, membranes could be stripped by incubation in stripping buffer (see section 2.8) for 15 min at 50°C with occasional agitation, washed as above and then re-probed with antibodies or stored at 4°C wrapped in moistened saran wrap.

Primary antibody	Dilution	Secondary antibody	Dilution
HA (F-7) Mouse monoclonal (200 ug/ml)	1:500	Anti-mouse IgG-HRP (400 ug/ml)	1:2000
TRAP220 (C-19) Goat polyclonal (200 ug/ml)	1:500	Anti-goat IgG-HRP (400 ug/ml)	1:2000
TRAP220 (M-255) Rabbit polyclonal (200 ug/ml)	1:500	Anti-rabbit IgG-HRP (400 ug/ml)	1:2000
FLAG (M2) Mouse monoclonal (200 ug/ml)	1:500	Anti-mouse IgG-HRP (400 ug/ml)	1:2000
VP16 (1-21) Mouse monoclonal (200 ug/ml)	1:500	Anti-mouse IgG-HRP (400 ug/ml)	1:2000
LexA (2-12) Mouse monoclonal (200 ug/ml)	1:500	Anti-mouse IgG-HRP (400 ug/ml)	1:2000
F-tag (ERα) Mouse monoclonal	1:1000	Anti-mouse IgG-HRP (400 ug/ml)	1:2000

Table 2.3 Antibody conditions for western blotting

2.4.3 Indirect Immunofluorescence

For this method a coverslip, which cells could adhere to and grow on, was placed in each well of a 6 well TC plate, depending on the number of conditions required and then cells were seeded at 1-3 x 10^5 cells/well. Cells were cultured and transfected with appropriate plasmid DNA as described in section 2.3.2. The following steps were performed at room temperature with the coverslip undisturbed in the well. The cells were gently washed twice with sterile PBS, taking care not to disrupt the cell layer on the coverslip, and fixed by incubation in

1.5 ml 4% (w/v) paraformaldehyde for 10 min. Cells were washed in PBS (3 x 5 min) to remove excess paraformaldehyde which could interfere with fluorescence and permeabilised by incubation with 1.5 ml 0.2% (v/v) Triton X-100 (in PBS) for 10 min, followed by washing in PBS as above. To block non-specific binding sites, cells were incubated in 2 ml antibody dilution buffer (ADB-see section 2.8) for 30 min. ADB was removed and the cells probed with primary antibody for 1 hr. Primary antibodies were prepared in ADB at dilutions specified in table 2.4 to a final volume of 50 µl, which was carefully pipetted onto the coverslip and the lid of the 6 well plate was replaced to prevent drying out of the cell layer. After 1 hr cells were washed as above and incubated with FITC- or TRITC-conjugated secondary antibodies for 45 min. Secondary antibodies were prepared in ADB at dilutions specified in table 2.4, to a final volume of 100 µl. Control cells were incubated in secondary antibody only, to determine background fluorescence. Cells were again washed as above to remove unbound secondary antibody and incubated in 1 ml Hoechst stain 33258 (1 µg/ml final concentration) for 5 min. After washing in PBS (3 x 5min) each coverslip was carefully removed from its well, drained of excess solution by dabbing one edge of the coverslip on a tissue and placed cell side down onto 10 µl mounting media (section 2.8) spotted onto a microscope slide. The coverslips were secured on the microscope slides by applying a thin layer of clear nail varnish around the edge of the coverslip and allowed to dry. Slides were stored at 4°C, protected from light. Fluorescent cells were visualised using a Axioskop 2 fluorescent microscope.

Primary antibody	**Dilution**	***Secondary antibody**	**Dilution**
HA (F-7) Mouse monoclonal (200 ug/ml)	1:50	Anti-mouse IgG FITC	1:100
TRAP220 (M-255) Rabbit polyclonal (200 ug/ml)	1:50	Anti-rabbit IgG TRITC	1:100

Table 2.4 Antibody conditions for indirect immunofluorescence. *Secondary antibody concentration was 3.0-6.0 mg/ml according to manufacturer.

2.4.4 Eukaryotic *in vitro* transcription/translation

The TNT® coupled reticulocyte lysate system was used to produce [^{35}S] labelled proteins *in vitro* according to manufacturers instructions. A typical reaction mixture was set up as follows:

Rabbit reticulocyte lysate	25 µl
Manufacturers reaction buffer	2 µl
T7 RNA polymerase	1 µl
1 mM amino acid mixture minus methionine	1 µl
[^{35}S] methionine (10 mCi/ml)	2 µl
RNasin® ribonuclease inhibitor (40 units/µl)	1 µl
DNA template (0.5 µg/µl)	2 µl
Nuclease-free H_2O	x µl, to a total of 50 µl

This reaction mixture was incubated at 30°C for 90 min and then could be stored at –70°C for a maximum of 2 months.

2.4.5 Visualisation of proteins resolved by SDS-PAGE

For visualisation of radiolabelled proteins resolved by SDS-PAGE, the polyacrylamide gel was first soaked in fix solution for 15 min. The low pH of the fix solution precipitates the proteins in the gel so they cannot diffuse out. The gel was then incubated in Amplify™ solution for 30 min to enhance the intensity of the autographic image. The gel was then dried under vacuum and exposed to film overnight at –70°C.

For visualisation of non-radiolabelled proteins resolved by SDS-PAGE, the polyacrylamide gel was soaked in Coomassie Blue stain solution for 30 min, followed by incubation in destain solution until the protein bands were clearly visible on the gel. Gels were soaked in 1% glycerol for 30 min before being dried between 2 layers of cellophane.

2.4.6 IPTG-inducible expression of glutathione S-transferase (GST)-fusion proteins

Vectors encoding GST-fusion proteins were introduced into *E.coli* DH5α by transformation (section 2.2.2). The *E.coli* growing in liquid culture could then be induced to produce GST-fusion protein by the addition of IPTG, since the expression of the GST-fusion

protein is under the control of an IPTG-inducible *tac* promoter. First transformants expressing GST-fusion proteins had to be identified. To do this, small scale inductions were performed as follows. A small number of transformants were grown in 3 ml LB media (supplemented with 100 μg/ml ampicillin) aliquots in an orbital shaker at 37°C, 225 rpm for 2 hr. The culture was then split into 2x 1 ml aliquots in 1.5 ml microfuge tubes and the remaining culture was stored at 4°C. To one of the 1 ml aliquots ('induced') IPTG was added to a final concentration of 0.5 mM and the appropriate volume of sterile LB media was added to the other 1 ml aliquot ('uninduced'). The cultures were grown for a further 2 hr at 30°C. The cells were harvested by centrifugation at 13,000 *g* for 15 min. Cell pellets were lysed by boiling in SDS-PAGE loading buffer for 5 min and then total cell lysates were screened for IPTG-induced expression of the expected GST-fusion protein by SDS-PAGE (section 2.4.1).

Suitable transformants, giving good IPTG-induced expression of the desired GST-fusion protein were then grown on a large scale (e.g. 50 ml cultures). All large scale induction cultures were screened for expression of the desired GST-fusion protein by SDS-PAGE and pelleted cells could be frozen and stored at –70°C.

2.4.7 Purification of glutathione S-transferase fusion proteins

GST-fusion proteins were purified from bacterial lysates by affinity chromatography using glutathione sepharose 4B beads. First the glutathione sepharose beads were prepared as follows. 100 μl glutathione sepharose beads were mixed with 100 μl NETN buffer/0.5% (w/v) skimmed milk powder, in a 1.5 ml microfuge tube. The beads were pelleted by centrifugation at 2,000 *g*, room temperature for 2 min and the supernatant discarded. The pelleted beads were resuspended in 125 μl NETN buffer/0.5% (w/v) skimmed milk powder, centrifuged as above and the supernatant discarded. The beads were washed in this way 3 times and on the third wash the supernatant was not removed but used to re-make the slurry by pipetting.

Cell pellets of the IPTG-induced bacterial cultures expressing GST-fusion proteins (section 2.4.6), were resuspended in 5 ml ice cold NETN buffer, supplemented with 10 mM DTT and lysozyme at 5 mg/litre of culture from which the cells were pelleted (e.g. 50 ml culture, add 250 μg lysozyme). Cell suspensions were kept on ice and the cells were lysed by sonication at 12 μ amplitude for 1 min using a Soniprep 150 (MSE) sonicator. The cell debris was pelleted by centrifugation at 6,000 *g*, 4°C for 15 min.

The cleared bacterial supernatant was mixed with 125 µl of the slurry (sepharose beads prepared as above) in a 15 ml polypropylene tube and placed on a rotating wheel at 4°C for 1 hr. The glutathione sepharose beads with bound GST-fusion protein (referred to as GST-beads hereafter) were pelleted by centrifugation at 2,000 *g*, 4°C for 2 min and the supernatant discarded. GST-beads were washed with 5 ml cold NETN buffer 3 times and then resuspended in 1 ml cold NETN buffer. GST-beads could be stored at 4°C.

2.4.8 Glutathione S-transferase (GST)-pulldown assay

In vitro protein-protein interactions were assessed using GST-pulldown assays. In this study GST-nuclear hormone receptor fusion proteins were tested for interaction with *in vitro* translated proteins. A typical GST-pulldown reaction mixture contained 20-200 µl GST-beads (section 2.4.7) and 1-5 µl [^{35}S]-labelled protein (section 2.4.4) in 1 ml NETN buffer. Receptor cognate ligands (E_2, T_3 and 9c-RA) were added at a final concentration of 10^{-6} M where appropriate and the reaction mixtures were incubated at 4°C, protected from light, overnight on a rotating wheel. The next day the GST-beads were washed 3 times in 500 µl cold NETN buffer and then dried under vacuum. Analysis of *in vitro* translated [^{35}S]-labelled proteins 'pulled-down' (i.e. bound to the GST-fusion protein) was performed by SDS-PAGE (section 2.4.1) followed by visualisation of the radiolabelled protein in the polyacrylamide gel (section 2.4.5).

2.4.9 Protein assay

The protein concentration of samples was measured using the Bio-Rad protein assay reagent according to manufacturers instructions. Typically 2 µl of the sample was mixed with 200 µl protein assay reagent and 800 µl dH_2O in a cuvette, incubated at room temperature for 15-20 min and then its absorbance at 595 nm (A_{595nm}) was measured. Protein concentration was calculated using A_{595nm} values of diluted BSA standards of known concentrations.

2.5 Yeast methods

2.5.1 Yeast plasmids

Two different DNA binding domain (DBD) vectors were employed in this study: BTM116mod (Figure 2.1A) and pBL1mod (Figure 2.1B). pBTM116mod is a derivative of pBTM116 (Vojtek *et al.*, 1993), expresses the DBD of the bacterial repressor protein LexA and carries a TRP1 selection marker. pBL1mod is a derivative of pBL1 (Le Douarin *et al.*, 1995),

expresses the DBD of the estrogen receptor and carries a HIS3 selection marker. Additionally, pBL1mod encodes the sequence for the F domain of the ERα in frame with the ER DBD and hence ER-DBD fusion proteins expressed from pBL1mod can be immunologically detected by use of a monoclonal antibody raised against the F domain of the ERα. Fusion proteins containing these DBDs were generated by subcloning appropriate cDNAs in frame into the multiple cloning site.

The acidic-activation domain (AAD) vector used in this study was pASV3mod (Figure 2.2). pASV3mod is a derivative of pASV3 (Le Douarin *et al.*, 1995), contains the AAD from the herpes virus protein VP16, a nuclear localisation signal and carries the LEU2 selective marker. Fusion proteins containing this AAD were generated by subcloning appropriate cDNAs in frame into the multiple cloning site.

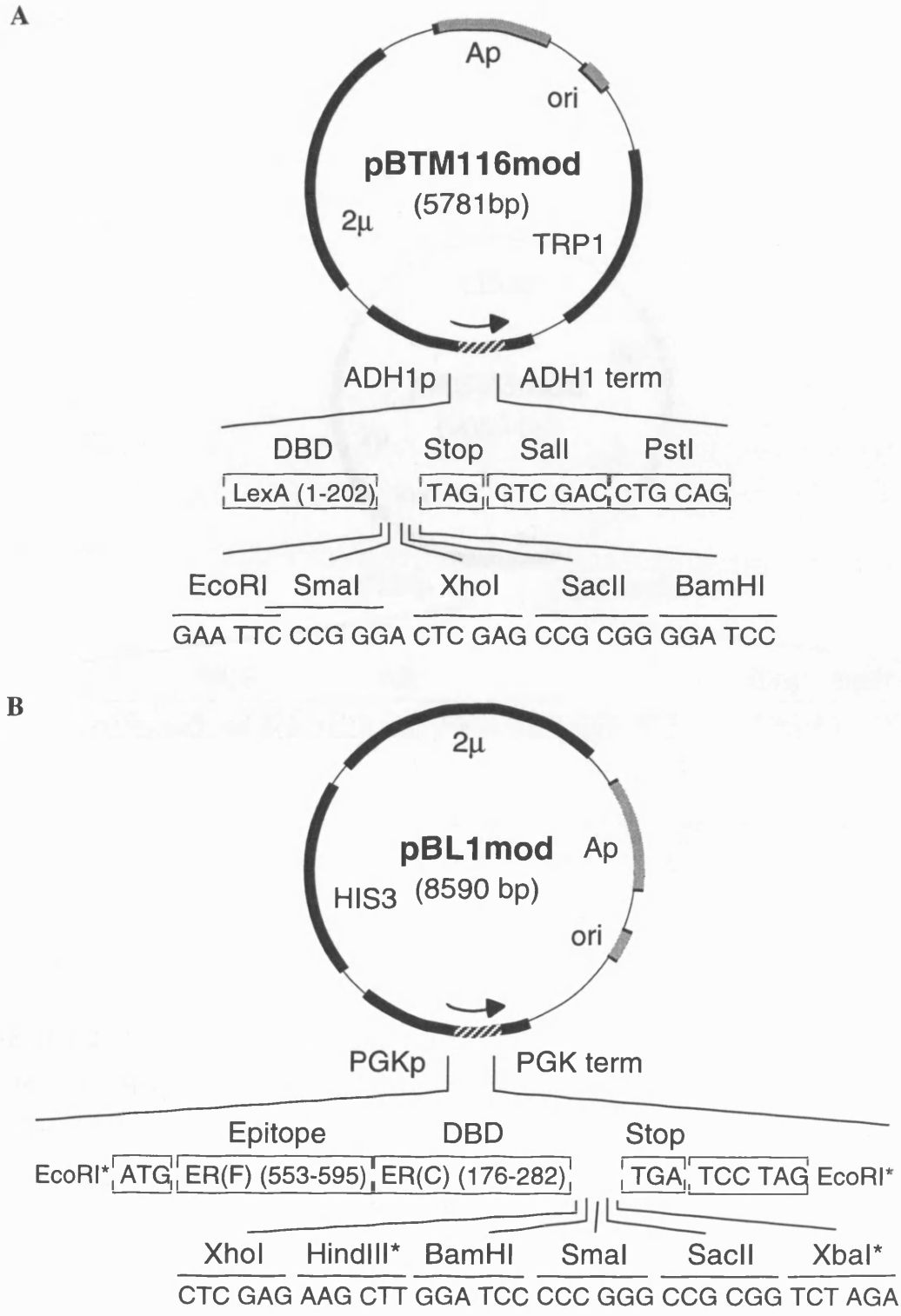


Figure 2.1 DBD-fusion protein vectors, (A) pBTM116mod and (B) pBL1mod. In both cases the DBD-fusion protein is expressed from a strong constitutive promoter, namely ADH1 (alcohol dehydrogenase 1) and PGK (phosphoglycerate kinase), for pBTM116mod and pBL1mod respectively. ori; origin of replication (in *E.*coli), Ap; ampicillin resistance (in *E.*coli), TRP1; tryptophan selective marker, HIS3; histidine selective marker, 2μ; origin of replication (in yeast). Taken from Le Douarin *et al.*, 2001.

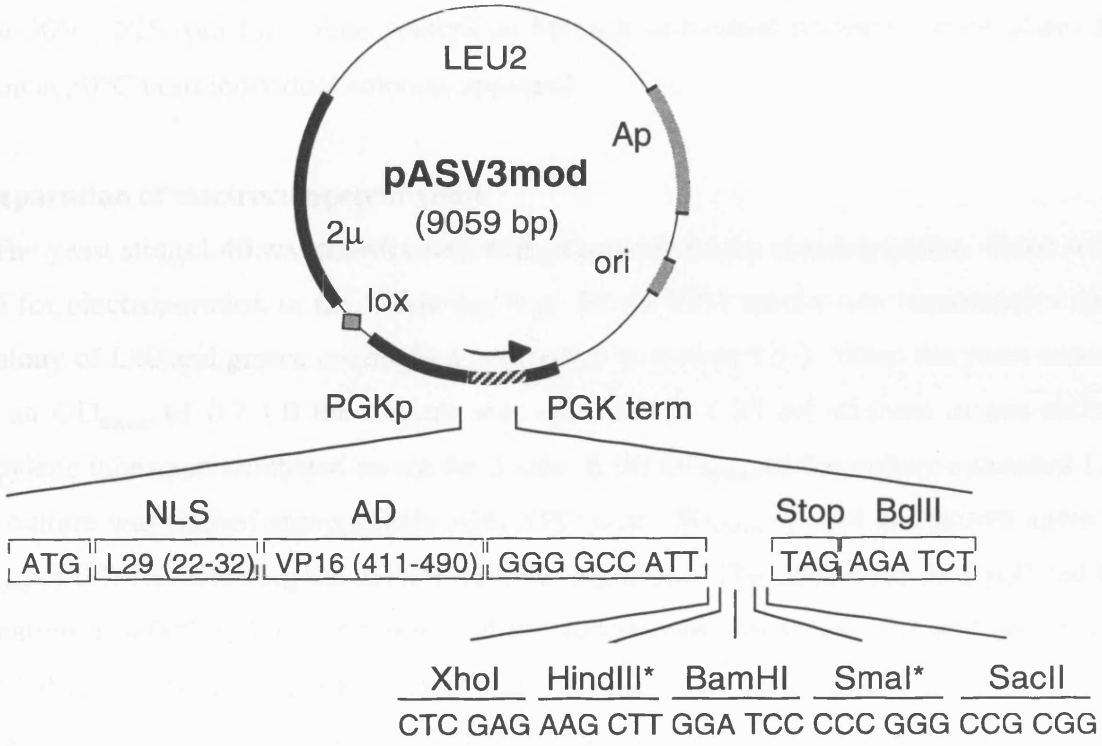


Figure 2.2 The AAD-fusion protein vector, pASV3mod. The VP16-fusion protein is expressed from the PGK (phosphoglycerate kinase) promoter. ori; origin of replication (in *E.*coli), Ap; ampicillin resistance (in *E.*coli), LEU2; leucine selective marker, 2μ; origin of replication (in yeast). Taken from Le Douarin *et al.*, 2001.

2.5.2 Growth of yeast

A variety of media was required for the maintenance and selection of yeast reporter strains used in the yeast two-hybrid system: YPG media for growing cultures and defined minimal media (CSM-X) for maintaining selection of plasmids. Media was solidified by addition of 2% bacteriological agar. Yeast was grown in culture by incubation in an orbital shaker at 30°C, 225 rpm for a time prescribed by each individual protocol, or on plates by incubation at 30°C until individual colonies appeared.

2.5.3 Preparation of electrocompetent yeast

The yeast strain L40 was transformed with plasmid DNA by electroporation. Yeast were prepared for electroporation in the following way. 50 ml YPG media was inoculated with a single colony of L40 and grown overnight as described in section 2.5.2. When the yeast culture reached an OD_{600nm} of 0.7-1.0 the culture was split into 2 x 25 ml aliquots in pre-chilled polypropylene tubes and incubated on ice for 5 min. If the OD_{600nm} of the culture exceeded 1.0, then the culture was diluted appropriately with YPG to an OD_{600nm} of ~0.4 and grown again to an OD_{600nm} of 0.7-1.0, allowing the cells to re-enter log phase. The cells were then pelleted by centrifugation at 4,000 *g*, 4°C for 5 min and the supernatant discarded. The cell pellet was resuspended in 50 ml ice cold sterile H_2O and centrifuged at 4,000 *g*, 4°C for 5 min. The supernatant was discarded and the cell pellet resuspended in 25 ml ice cold electroporation buffer (see section 2.9), followed by centrifugation at 4,000 *g*, 4°C for 5 min. This step was repeated using 10 ml ice cold electroporation buffer and each pellet (from 2 x 25 ml cultures) was resuspended in 500 µl electroporation buffer. These electrocompetent cell suspensions were combined in a pre-chilled 1.5 ml microfuge tube and placed on ice.

2.5.4 Transformation of yeast by electroporation

50 µl electrocompetent yeast cells (section 2.5.3) were mixed with 2 µg plasmid DNA in a pre-chilled 1.5 ml microfuge tube and incubated on ice for 2 min. The yeast/DNA mixture was transferred to a sterile electroporation cuvette and subjected to a short high-voltage pulse, using a Bio-Rad Gene Pulser® II, set at 450 V, 250 µF and 400 ohms. The pulse length was approximately 74 msec. The cells were rescued by addition of 1 ml sterile, ice cold H_2O and transferred to a sterile 1.5 ml microfuge tube on ice. Cells were then pelleted by centrifugation at 5,000 g, 4°C for 5 min and 900 µl supernatant was discarded. The remaining 100 µl

supernatant was used to gently resuspend the cell pellet by pipetting. Transformed yeast were spread on the appropriate solidified selective minimal media depending on with which growth selective plasmids they had been transformed. Yeast were incubated at 30°C until individual colonies appeared (usually 3-4 days). Multiple plasmid DNA's were introduced sequentially into yeast, allowing the yeast to grow on selective minimal media (3-4 days) between each successive electroporation and ensuring a high transformation efficiency.

2.5.5 Lithium acetate transformation of yeast

A modified version of the high efficiency lithium acetate method of yeast transformation developed by Schiestl *et al.*, (1993) was used to transform the yeast strain W303-1B. 10 ml YPG was inoculated with a single colony of W303-1B and grown overnight as described in section 2.5.2. The next day the culture was diluted to an OD_{600nm} of 0.1 in a total volume of 50 ml YPG and grown to an OD_{600nm} of 0.5 (~4 hr). The cells were harvested by centrifugation at 3,000 *g*, room temperature, for 5 min, the supernatant discarded and the pelleted cells resuspended in room temperature, sterile H_2O. The cells were centrifuged as above, the supernatant removed and the pellet resuspended in 250 µl 100 mM lithium acetate transformation buffer.

A sample of carrier DNA (Herring sperm DNA, 10 µg/µl) was prepared by incubation at 95°C for 5 min and then quickly chilled on ice. 50 µl transformation-ready cells were mixed with 1 µg plasmid DNA and 50 µg carrier DNA in a sterile 1.5 ml microfuge tube. To this transformation mixture, 300 µl freshly prepared, sterile, 40% (w/v) PEG 4000 was added, mixed vigorously (not vortexed) and incubated in an orbital shaker at 30°C, 225 rpm for 30 min. 40 µl DMSO was added to each transformation mixture and mixed vigorously, before being heat shocked at 42°C for 10 min. Cells were pelleted by centrifugation at 4,000 *g* for 1 min, the supernatant aspirated and the cells resuspended in 100 µl 1x TE buffer (pH 8.0). Transformed cell suspensions were spread on the appropriate solidified selective minimal media, depending on the growth selective plasmid with which they had been transformed and incubated at 30°C until individual colonies appeared (3-4 days). Multiple plasmid DNA's were introduced sequentially into yeast, allowing the yeast to grow on selective minimal media between each successive chemical transformation and ensuring a high transformation efficiency.

2.5.6 Preparation of yeast cell-free extracts

Yeast cell-free extracts were prepared for use in either western blotting or liquid β-galactosidase and protein assays. For western blotting, cell-free extracts were prepared using breaking buffer (section 2.9). For β-galactosidase and protein assays, cell-free extracts were prepared using LacZ buffer (section 2.9).

Yeast derived from a single colony, transformed with the plasmid DNA of interest, was grown in 15 ml appropriate selective minimal media overnight in an orbital shaker at 30°C, 225 rpm. When ligand-dependant protein-protein interactions were being tested, appropriate NR cognate ligand (17β-estradiol (E_2) for ER, promegestone (R5020) for PR, 9-*cis*-retinoic acid (9c-RA) for RXR, all-*trans*-retinoic acid (AT-RA) for RAR, 3,3',5-Triiodo-$_L$-Thyronine (T_3) for TR, Rosiglitazone for PPARγ and mibolerone for AR) was added to a final concentration of 10^{-6} M. Minus ligand control cultures were supplemented with an equal volume of vehicle. Cultures were shielded from light since 9c-RA isomerises to AT-RA on exposure to light. The next day the cultures were centrifuged at 4,000 *g*, 4°C for 5 min and the supernatant discarded. Cell pellets were resuspended in either 1 ml ice cold H_2O (for use in western blotting) or 1 ml ice cold LacZ buffer (for use in β-galactosidase and protein assays) and transferred to pre-chilled 1.5 ml microfuge tubes on ice. Cell suspensions were then centrifuged as above, the supernatant discarded and the cell pellet resuspended in either 150 µl breaking buffer (for use in western blotting) or 150 µl LacZ buffer (for use in β-galactosidase and protein assays). Approximately the same volume (~200 µl) of glass beads were added to the cell suspension and this was incubated on ice for 5 min. The cell suspension/glass bead mixture was then vortexed vigorously for 2 min to break open the yeast cells, before being centrifuged at 13,000 *g*, room temperature for 10 min to pellet the cell debris and glass beads. The cell-free extract was transferred to a pre-chilled 1.5 ml microfuge tube on ice.

2.5.7 Quantitative β-galactosidase liquid assay

β-galactosidase activity of the yeast cell-free extracts was measured using a colorimetric assay. 500 µl LacZ buffer was mixed with 20 µl cell extract (or 20 µl LacZ buffer to the blank), as prepared in section 2.5.6, in a plastic cuvette at room temperature. To initiate the reaction 100 µl 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was added at room temperature and timing was begun. The reaction was incubated at room temperature until a pale yellow colour developed, at which point the reaction was stopped by addition of 200 µl 1 M disodium

carbonate and the timing of the reaction was recorded. If no colour change was observed, samples were incubated at room temperature for 30 min before addition of 200 µl 1 M disodium carbonate. The OD_{420nm} of the samples were measured against a blank and the β-galactosidase activity was normalized to protein concentration of the sample, as determined by a protein assay described in section 2.4.9.

Specific β-galactosidase activity of the extracts was calculated according to the following equation:

$$\text{Units of } \beta\text{-galactosidase activity (nmol/mg/min)} = OD_{420nm} / 0.0045 \times \text{Volume} \times \text{Protein} \times \text{Time}$$

where 0.0045 is a constant to convert OD_{420nm} to nmol of substrate used, Protein is the protein concentration of the yeast extract in mg/ml, Volume is the extract volume in ml and Time is the time of reaction in min.

2.5.8 Microscopic visualisation of yeast cells

Yeast cells were grown overnight in liquid media, where necessary with or without 10^{-6} M NR cognate ligand, as described in section 2.5.2. The next day 20 µl cell culture was spotted onto a microscope slide and covered with a coverslip. Cells were visualised using a Axioskop 2 microscope. Slides prepared in this way were visualised the same day.

2.6 Composition of solutions used for molecular biology and tissue culture

Agarose gel loading buffer (6x)

0.25% (w/v) bromophenol blue dye, 30% (v/v) glycerol

Tris-borate-EDTA (TBE)

40 mM Tris base, 40 mM boric acid, 1 mM EDTA (pH 8.0)

Tris-EDTA (TE) buffer (1x)

10 mM Tris/HCl (pH 8.0), 1 mM EDTA

RIPA buffer

20 mM Tris (pH 8.0), 150 mM sodium chloride, 0.1% (w/v) SDS, 1% (v/v) NP40, 1 mM EDTA. 1 complete protease inhibitor cocktail tablet was added to 50 ml RIPA buffer.

2.7 Composition of solutions and media used for bacterial methods

Luria-Bertoni (LB) media

1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl (pH 7.0 NaOH). Solidified where required by the addition of 2% (w/v) bacteriological agar.

TFBI (transformation buffer I)

30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM magnesium chloride, 15% (v/v) glycerol (pH 5.8 with 0.2 M glacial acetic acid and filter sterilise)

TFBII (transformation buffer II)

10 mM 3-(N-morpholim)-propane sulphonic acid (MOPS), 75 mM calcium chloride, 10 mM rubidium chloride, 15% (v/v) glycerol (pH 6.5 with 1 M potassium hydroxide and filter sterilise)

2.8 Composition of solutions used for protein chemistry

Tris-glycine-SDS PAGE running buffer (10x)

250 mM mM Tris base, 2 M glycine, 35 mM SDS

Tris-glycine-SDS transfer buffer (standard)

39 mM glycine, 48 mM Tris base, 0.037% (w/v) SDS, 20% (v/v) methanol (pH 8.3)

Tris-glycine-SDS transfer buffer (for high molecular weight protein transfer)

380 mM glycine, 48 mM Tris base, 0.037% (w/v) SDS, 20% (v/v) methanol

SDS-PAGE loading buffer (4x)

62.5 mM Tris (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 20 mM β-mercaptoethanol, 0.05% (w/v) bromophenol blue

Coomassie® Blue staining solution

10% (v/v) acetic acid, 40% (v/v) methanol, 0.25% (w/v) Coomassie® Blue R-250

Destaining solution

10% (v/v) acetic acid, 40% (v/v) methanol

Fix solution

10% (v/v) acetic acid, 10% (v/v) methanol

NETN buffer

20 mM Tris (pH 8.0), 100 mM sodium chloride, 1 mM EDTA, 0.5% (v/v) NP40. 1 complete protease inhibitor cocktail tablet was added to 50 ml NETN buffer.

Blocking buffer (for western blotting)

3% (w/v) skimmed milk powder in PBS

Stripping buffer

100 mM β-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris/HCl (pH 6.7)

Mounting media

90% (v/v) sterile glycerol, 3% (w/v) n-propyl-gallate in PBS

Antibody dilution buffer (ADB)

3% (w/v) BSA in PBS

2.9 Composition of solutions and media used for yeast methods

Yeast-peptone-glucose (YPG) media

1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose. Solidified where required by the addition of 2% (w/v) bacteriological agar.

Yeast selective media

x g/l of Complete supplement mixture (CSM), where x was defined by the manufacturer depending on the particular drop-out media being used e.g. CSM-trp, 0.67% (w/v) yeast nitrogen base (without amino acids), 2% (w/v) glucose. 20 μg/ml adenine was included when the W303-1B strain was being propagated. Solidified where required by the addition of 2.2% (w/v) bacteriological agar and 1.25 mM NaOH.

Yeast electroporation buffer

10 mM Tris/HCl (pH 7.5), 1 mM magnesium chloride, 270 mM sucrose, (pH 7.5)

Lithium acetate transformation buffer

100 mM lithium acetate in 1x TE buffer

Breaking buffer

50 mM Tris (pH 8.0), 400 mM KCl. 1 complete protease inhibitor cocktail tablet was added to 50 ml breaking buffer.

LacZ buffer (for yeast assay)

60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, filter sterilised.

CHAPTER 3
RESULTS

Assessing the effects of the 220 kDa and 170 kDa subunits of the TRAP complex on Nuclear hormone Receptor-mediated transcriptional activity

3.1 Introduction

Optimal nuclear hormone receptor function requires various interacting coactivator proteins. The best characterised coactivators include the SRC1 family (i.e. SRC1/NCoA-1, TIF2/GRIP1/NCoA-2 and ACTR/RAC3/AIB1/pCIP/TRAM1/NCoA-3), CBP/p300 and pCAF (reviewed in Glass *et al.*, 2000) (see section 1.7). These coactivator proteins are assembled on NRs and function, at least in part, through intrinsic histone acetyltransferase (HAT)-mediated nucleosome modification. In an attempt to identify novel coactivator proteins, J. D. Fondell and co-workers created a HeLa-derived cell line which constitutively expressed FLAG-tagged hTRα (Fondell *et al.*, 1996). Immunopurified hTRα, in the presence of T_3, was found to be associated with a distinct set of nuclear proteins. This set of proteins was subsequently referred to as the human TR-associated protein (TRAP) complex. Significantly, TRAP-related complexes were later isolated as a vitamin D receptor (VDR)-interacting complex (DRIP) (Rachez *et al.*, 1998 and Rachez *et al.*, 1999), a SRB/MED-containing cofactor complex (SMCC) (Gu *et al.*, 1999), an E1A-interacting complex (human mediator) (Boyer *et al.*, 1999), a SREBP-interacting complex (ARC) (Naar *et al.*, 1999), the USA-derived PC2 (Malik *et al.*, 2000) and CRSP complexes (Ryu *et al.*, 1999), and the NAT (Sun *et al.*, 1998) and mouse mediator complexes (Jiang *et al.*, 1998). As well as these complexes being related to each other, they are also related to the multifunctional Mediator complex found in yeast, through a small subset of homologous subunits.

Several groups have used similar *in vitro* transcription assays, comprising a cell-free system reconstituted with purified general transcription factors and naked DNA templates bearing nuclear hormone receptor response elements (HRE), to demonstrate that TRAP/SMCC/Mediator is able to greatly enhance the function of TRα and VDR (Fondell *et al.*, 1996, Rachez *et al.*, 1998 and Ito *et al.*, 1999). This ability to enhance TRα and VDR activity led to the proposal that the TRAP complex functions as a coactivator for these NRs. Whether the TRAP complex additionally could enhance the activity of other NRs and hence be classed as a global coactivator for NRs, is yet to be determined. However a lack of any detectable HAT activity by the TRAP complex suggested its mode of coactivation was not by nucleosomal

remodelling, as had already been observed for the coactivators, CBP/p300, pCAF and the p160s.

Of particular interest to this study is the 220 kDa subunit of the TRAP complex, termed TRAP220. As previously discussed in chapter one (section 1.9.4), TRAP220 possesses two closely spaced LXXLL motifs and has been shown to bind in a ligand-dependent manner to the TRα/β, PPARα/γ, RARα, RXRα and VDR (Yuan *et al.*, 1998, Rachez *et al.*, 1999 and Treuter *et al.*, 1999). This has implicated TRAP220 as a crucial component of the TRAP complex in terms of its *in vitro* coactivation function for the TRα and VDR. Indeed it has been postulated that TRAP220 serves as an anchorage subunit, allowing the entire TRAP complex to be recruited to the AF-2 of the NR.

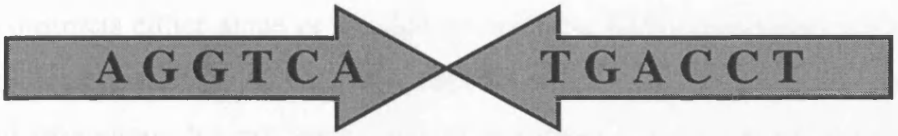
In this section, I assessed the *in vivo* function of TRAP220 and determined its involvement in NR-mediated transcription. Thus addressing the question of whether the TRAP complex can function as a global coactivator for NRs or whether it restricts its function to class II NRs?

3.2 Selection of a suitable Thyroid hormone receptor Response Element (TRE)

In order to assess the modulation of TRβ-mediated transcription by TRAP220 *in vivo*, a T_3-responsive, TRβ-mediated transactivation reporter system was established. As described in section 1.11, COS-1 and HeLa cells were transiently co-transfected with a TRAP220 expression vector, together with a TRβ expression vector and a TRE-linked firefly luciferase reporter to assess the *in vivo* coactivation function of exogenously expressed TRAP220. Additionally cells were transfected with a β-galactosidase reporter to act as an internal transfection control to normalise luciferase activity. Firstly, selection of a suitable TRE-linked luciferase reporter construct was necessary. Exogenously expressed TRβ should be able to mediate transcription from the selected luciferase reporter and hence exogenously expressed coactivator proteins could be shown to modulate this TRβ-mediated transcriptional activity. Three different TRE-linked luciferase reporters were tested, namely PAL-TKLUC, MAL-TKLUC and F2-TKLUC (Collingwood *et al.*, 1994) (Figure 3.1). These luciferase reporters each contain a different TRE configuration upstream of the viral thymindine kinase promoter and firefly luciferase gene. PAL-TKLUC contains two copies of a palindromic TRE (i.e. the TRE sequence exhibits dyad symmetry, where the same sequence is found in the 5' to 3' direction on each strand) derived from the rat growth hormone (GH) gene, MAL-TKLUC

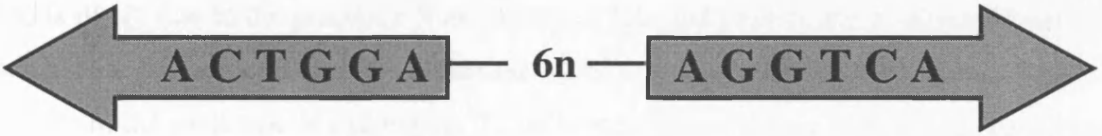
A

PAL-TKLUC



B

F2-TKLUC



C

MAL-TKLUC (DR4)

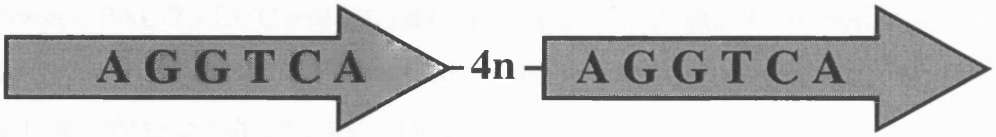


Figure 3.1 TRE configurations of the luciferase reporter constructs

(A) PAL-TKLUC contains two copies of this palindromic sequence

(B) F2-TKLUC contains a single copy of this everted repeat

(C) MAL-TKLUC contains a single copy of this direct repeat (DR4)

contains single copy of a natural direct repeat TRE, (AGGTCA-4n-AGGTCA), taken from the malic enzyme gene and F2-TKLUC contains the F2 response element from the chicken lysozyme gene, which consists of two hexameric half sites arranged as an everted repeat separated by six base pairs (ACTGGA-6bp-AGGTCA).

Cells were transiently transfected with the above TRE-linked luciferase reporter constructs either alone or in addition with the TRβ expression vector, hTRβ-RSV. Luciferase reporter activity in extracts from cells transfected with a luciferase reporter only, in the absence of exogenous ligand, were used to represent basal level of transcription of the luciferase reporter gene and were assigned the value 1. All other luciferase values were expressed relative to this. As shown in figure 3.2, the luciferase reporter activity of cells transfected only with luciferase reporters was slightly increased in the presence of exogenous T_3, as compared to the basal level of transcription. This was the case for all the TRE-linked luciferase reporters tested and is likely due to the presence of endogenous TRs and coactivator proteins which can mediate and enhance transcription of the luciferase reporter gene in the presence of exogenous T_3.

In the presence of exogenous T_3, cells transfected with a TRE-linked luciferase reporter and TRβ expression vector were expected to show enhanced luciferase reporter activity as compared to cells transfected with a luciferase reporter alone, due to the binding of the exogenously expressed TRβ to the TRE of the luciferase reporter and hence permitting TRβ-mediated transactivation. However this was not the case for the TRE-linked luciferase reporters, PAL-TKLUC and F2-TKLUC (Figure 3.2 A and B). In both cases luciferase reporter activity was not appreciably affected by the presence of exogenous TRβ. This could suggest that under these conditions, the configuration of the TREs within these luciferase reporters were not favourable for exogenous TRβ binding or TRβ/RXR heterodimers bound to these TREs were of the wrong orientation to permit productive endogenous coactivator binding. In the case of MAL-TKLUC however, the luciferase reporter activity was enhanced 14-fold above the level of basal transcription in a T_3-dependant manner, upon expression of exogenous TRβ. Therefore under these conditions, MAL-TKLUC proved to be the most suitable TRE-linked luciferase reporter to study TRβ-mediated transactivation and hence assess the modulation of TRβ-mediated transactivation by coactivator proteins.

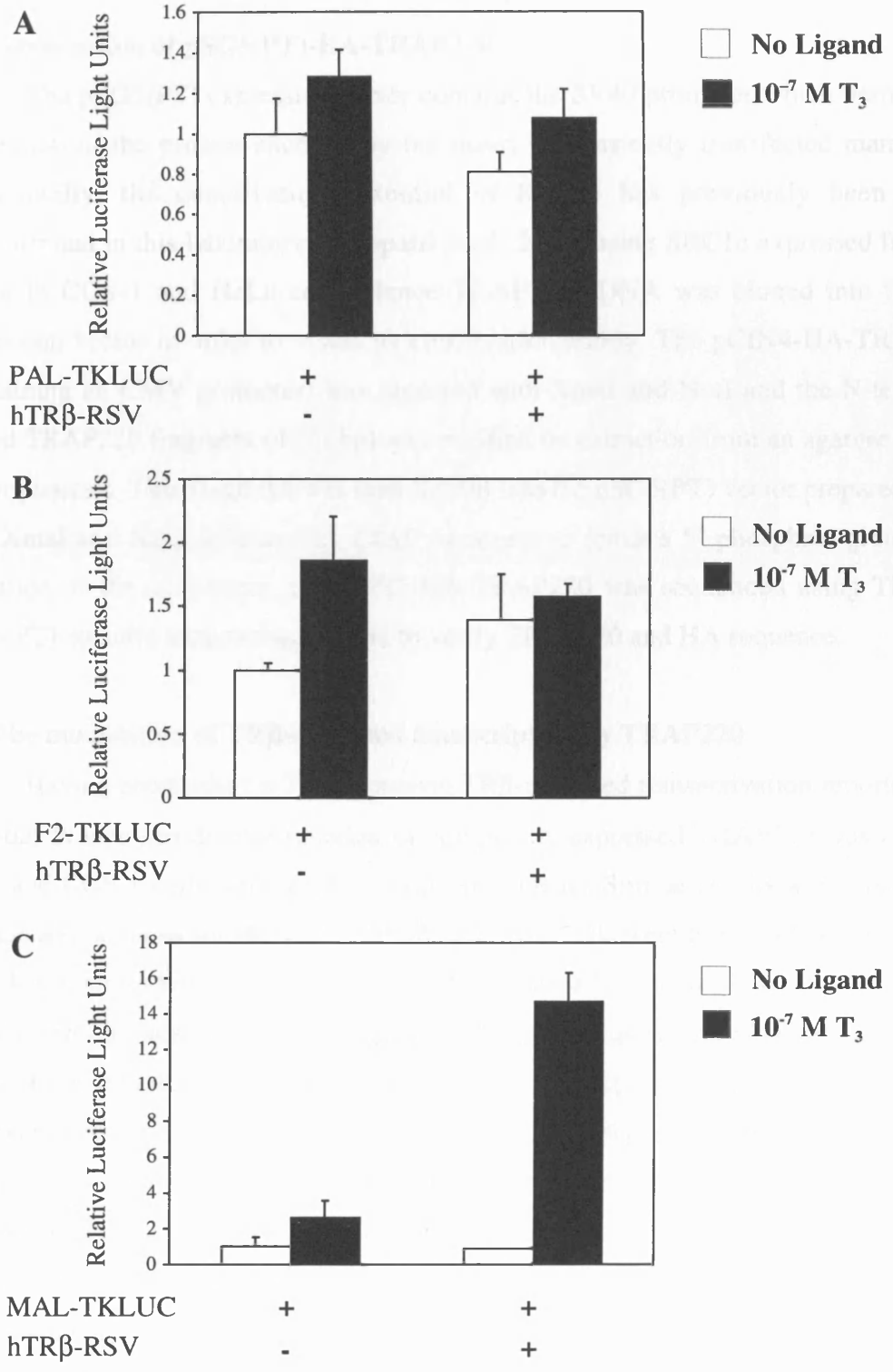


Figure 3.2 Testing TRβ-mediated transcriptional activity from three different TRE-linked luciferase reporter constructs. HeLa cells were transiently transfected, using the calcium-phosphate co-precipitation method, with either (A) PAL-TKLUC, (B) F2-TKLUC or (C) MAL-TKLUC and then additionally with or without hTRβ-RSV, in the presence or absence of 10^{-7} M T_3. Luciferase activity was measured and normalised to the β-galactosidase internal transfection control. Basal level transcriptional activity was set at 1 and all other values expressed relative to this.

3.3 Construction of pSG5(PT)-HA-TRAP220

The pSG5(PT) expression vector contains the SV40 promoter which permits high level expression of the protein encoded by the insert in transiently transfected mammalian cells. Additionally, the coactivation potential of SRC1e has previously been successfully demonstrated in this laboratory (Sheppard *et al.*, 2001) using SRC1e expressed from the pSG5 vector in COS-1 and HeLa cells. Hence TRAP220 cDNA was cloned into the pSG5(PT) expression vector in order to assess its coactivation ability. The pCIN4-HA-TRAP220 vector (containing an CMV promoter) was digested with XmaI and NotI and the N-terminally HA-tagged TRAP220 fragment (4773 bp) was purified by extraction from an agarose gel following electrophoresis. This fragment was then ligated into the pSG5(PT) vector prepared by digestion with XmaI and NotI, followed by CIAP treatment to remove 5' phosphate groups to prevent religation of the cut vector. pSG5(PT)-HA-TRAP220 was sequenced using TRAP220- and pSG5(PT)-specific sequencing primers to verify TRAP220 and HA sequence.

3.4 The modulation of TRβ-mediated transcription by TRAP220

Having established a T_3-responsive, TRβ-mediated transactivation reporter system, the potential *in vivo* coactivator function of ectopically expressed TRAP220 was assessed. Both HeLa and COS-1 cells were used in these experiments. Similar results were obtained for each cell line and so those for HeLa only are shown here. Cells were transiently co-transfected using the calcium phosphate co-precipitation method, with a β-galactosidase reporter and the MAL-TKLUC reporter alone to determine the basal level of transcription from the luciferase reporter under these conditions. Co-transfection with the TRβ expression vector resulted in an approximately 2-fold increase in transcription of the luciferase reporter gene above basal level, in a T_3-dependent manner (Figure 3.3). This is a lower level of enhancement than that seen previously (Figure 3.2) but is substantial enough for comparisons to be made within these experiments. Perhaps the increased quantity of total DNA used to transfect the cells in these experiments affected the transfection efficiency and resulted in this lowered level of TRβ-mediated transcription observed here (Figure 3.3).

The well characterised coactivator, SRC1e, (Onate *et al.*, 1995) was used in these experiments for comparative purposes and to act as a positive control for coactivator function. Here co-transfection with 1 μg of SRC1e expression vector resulted in a 1.6-fold enhancement

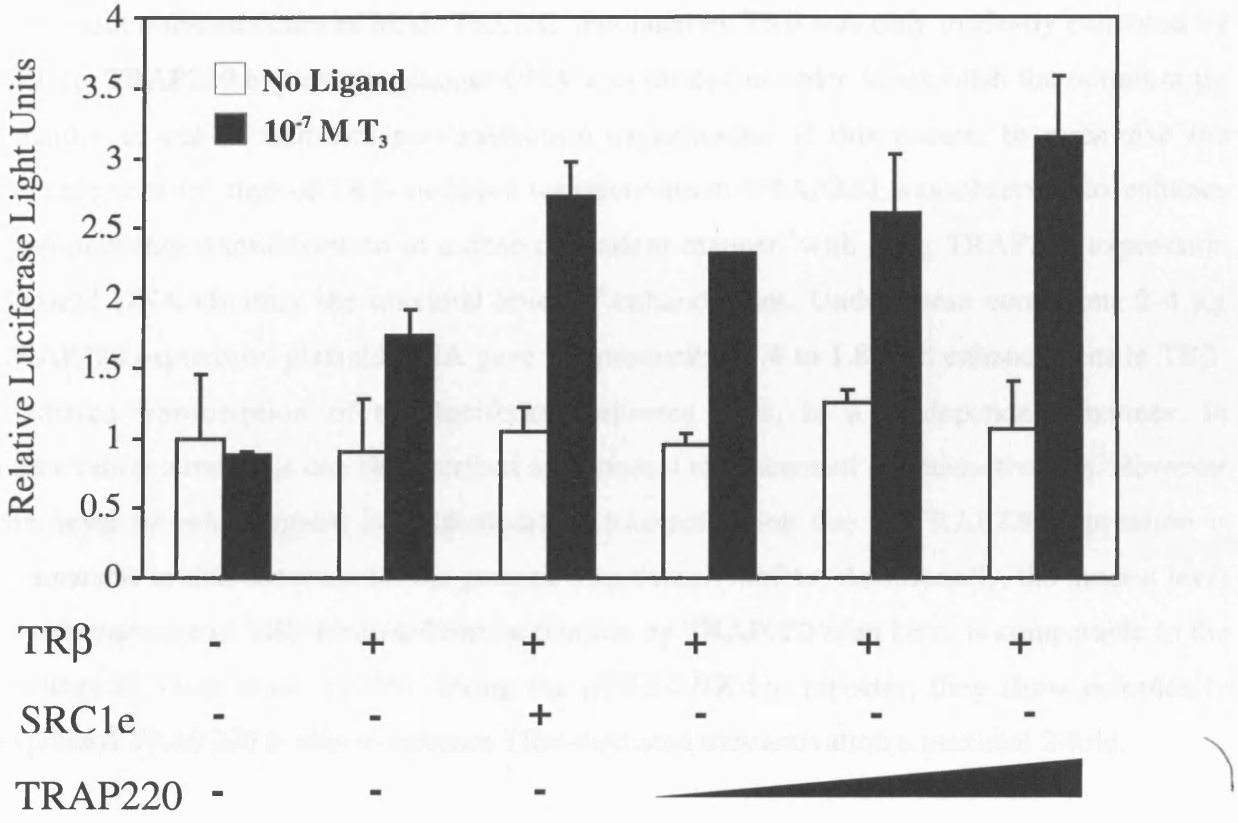


Figure 3.3 TRAP220 is able to enhance the TRβ-mediated transcriptional activity from MAL-TKLUC reporter. HeLa cells were transiently transfected, using the calcium-phosphate co-precipitation method, with 2 μg pMAL-TKLUC and 200 ng hTRβ-RSV, as indicated. Cells were additionally transfected with 1 μg pSG5-SRC1e or increasing amounts of pSG5(PT)-HA-TRAP220 (2 μg, 3 μg and 4 μg), as indicated. Luciferase activity was measured and normalised to the β-galactosidase transfection control. Basal level transcriptional activity of the luciferase reporter was set at 1 and all other values are expressed relative to this. The values shown represent the average of triplicate samples and the error bars indicate standard deviation.

in TRβ-mediated transcription of the luciferase reporter gene in a T_3-dependent manner. This is comparable to the findings of Collingwood *et al.*, (1997), who using JEG3 cells, showed T_3-dependent transactivation of MAL-TKLUC, mediated by TRβ was only modestly enhanced by SRC1e. TRAP220 expression plasmid DNA was titrated in order to establish the optimum μg quantity to use in transient co-transfection experiments of this nature, to maximise the enhancement (if any) of TRβ-mediated transactivation. TRAP220 was observed to enhance TRβ-mediated transactivation in a dose-dependent manner, with 4 μg TRAP220 expression plasmid DNA eliciting the maximal level of enhancement. Under these conditions 2-4 μg TRAP220 expression plasmid DNA gave a reproducible 1.4 to 1.8-fold enhancement in TRβ-mediated transcription of the luciferase reporter gene, in a T_3-dependent manner. In coactivation terms, this can be described as a modest enhancement of transactivation. However this level of enhancement in TRβ-mediated transactivation due to TRAP220 expression is comparable to that observed for the genuine coactivator, SRC1e. Additionally, the modest level of enhancement in TRβ-mediated transactivation by TRAP220 seen here, is comparable to the findings of Yuan *et al.*, (1998). Using the pTRE-LBK-luc reporter, they show ectopically expressed TRAP220 is able to enhance TRα-mediated transactivation a maximal 2-fold.

3.5 Assessing the effects of using an alternative transient transfection method

All transient transfection experiments described so far in this study (Figures 3.2 and 3.3), were performed using the calcium-phosphate co-precipitation method, as outlined in section 2.3.2. This transfection method involves the formation of a calcium-phosphate-DNA complex which is able to adhere to the cell membrane and enter the cell by endocytosis. The calcium-phosphate co-precipitation method is user-friendly and generally permits high levels of transfection efficiency. However this transfection method is not suitable for all cell lines and plasmid DNAs. Hence to determine if the observed low levels of enhancement in TRβ-mediated transcription of the luciferase reporter gene by both SRC1e and TRAP220, were due to an unsuitable transfection method for this DNA, an alternative method was tested.

The FuGENE™ 6 transfection reagent, described by the manufacturers as a blend of lipids (non-liposomal) and additional compounds in ethanol, was tested. This reagent is often used when transfection of certain cell lines with particular DNA has proven difficult. HeLa cells were transiently transfected using FuGENE™ 6 transfection reagent with the same plasmid DNAs, in the same quantities, as used in the TRβ-mediated transactivation system

described previously (section 3.3) when using the calcium-phosphate co-precipitation method. As shown in figure 3.4, the basal level of transcription of the MAL-TKLUC reporter was set at 1 and all other values were expressed relative to this. Co-transfection with TRβ expression vector resulted in a 3-fold increase in transcriptional activity of the luciferase reporter gene in a T_3-dependent manner, as compared to the basal level of transcription. This T_3-induced, TRβ-mediated enhancement in transcription is only slightly higher than that observed using the calcium-phosphate method (2-fold for calcium-phosphate method). Co-expression of TRAP220 resulted in a 1.9-fold enhancement in TRβ-mediated transactivation in a T_3-dependent manner. Again this level of enhancement is similar to that observed when the calcium-phosphate method was used (Figure 3.3). Hence for the cell lines (HeLa and COS-1) and plasmid DNAs used in this study there was no advantage gained by using the costly FuGENE™ 6 transfection reagent, since the cheaper, user-friendly calcium-phosphate method achieved the same results.

3.6 Assessing the effect of exogenous RXRα on TRAP220 activity

The TR has been shown to be able to bind TREs as a monomer or homodimer (Williams *et al.*, 1991). However, characteristic of a class II NR, TR binds DNA as a heterodimer with its heterodimeric partner, the retinoid-X receptor (RXR). Indeed heterodimerisation with RXR is a prerequisite to the formation of a functional transcription factor and presumably the interaction with NR coactivator proteins. In the TRβ-mediated transactivation assays described in this study thus far, cells were transiently transfected with an expression vector for TRβ and it was presumed that this exogenously expressed TRβ would heterodimerise with endogenous RXR, thus forming a functional transcription factor. However it should be considered that RXR could be a limiting factor in these assays and hence the maximal TRβ-mediated transcriptional activity and additionally coactivator-mediated enhancement in transcriptional activity may not have been observed. To investigate the effects of exogenously expressed RXR in these TRβ-mediated transactivation assays, cells were additionally transfected with an expression vector for RXRα, as indicated (Figure 3.5).

As shown in figure 3.5, expression of TRβ resulted in a 3-fold increase in transcriptional activity from the TRE-linked luciferase reporter (MAL-TKLUC) in a T_3 dependent manner as compared to basal level. This is consistent with levels previously observed in this study (Figures 3.3 and 3.4). Not unexpectedly, the cognate ligand of RXRα, 9-*cis*-retinoic acid (9c-RA), was unable to induce transactivation of the TRE-linked luciferase

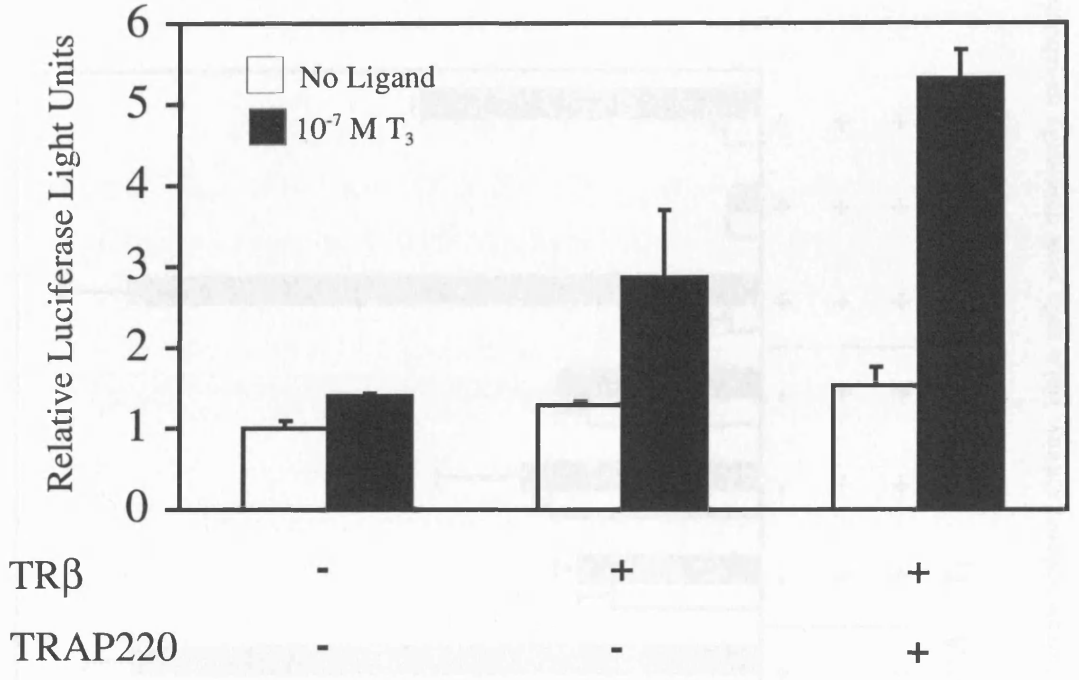


Figure 3.4 Using the FuGENE 6 transfection reagent yields similar results as the calcium-phosphate co-precipitation method of transient transfection. HeLa cells were transiently transfected with 500 ng pJ7-LacZ, 2 μg pMAL-TKLUC, 200 ng hTRβ-RSV and 4 μg pSG5(PT)-HA-TRAP220, as indicated. Luciferase activity was measured and normalised to the β-galactosidase transfection control. Basal level transcriptional activity of the luciferase reporter was set at 1 and all other values are expressed relative to this. The values shown represent the average of triplicate samples and the error bars indicate standard deviation.

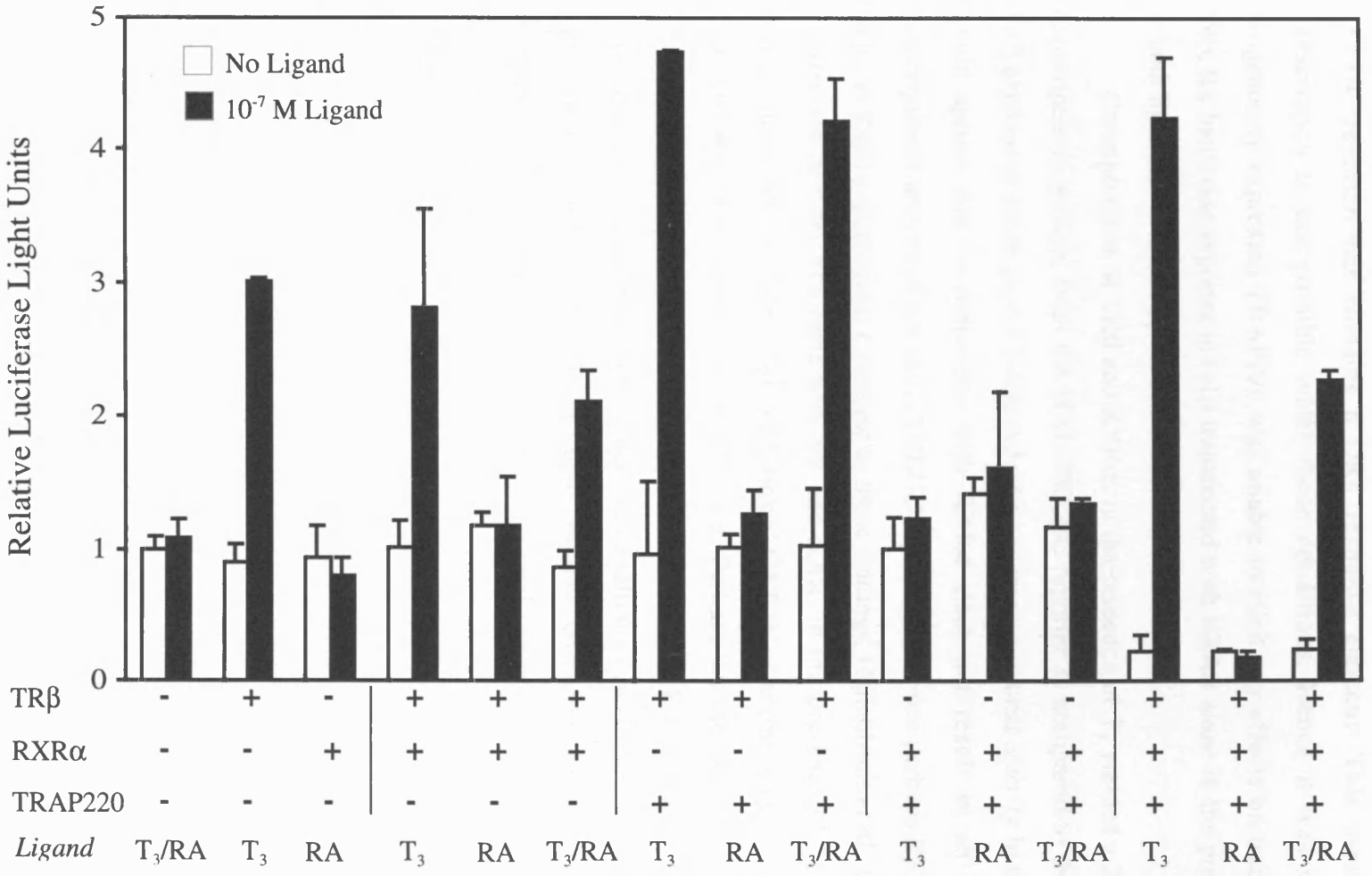


Figure 3.5 Exogenously expressed RXRα has no apparent effect on levels of TRβ-mediated transcriptional activity. HeLa cells were transiently transfected with 500 ng pJ7-LacZ, 1 ug pMAL-TKLUC and then 200 ng hTRβ-RSV, 200 ng pSG5-hRXRα and 3 ug pSG5(PT)-HA-TRAP220, as indicated. 10^{-7} M T_3 and 10^{-7} M 9c-RA (abbreviated here to RA) were used as indicated. Luciferase activity was measured and normalised to the β-galactosidase transfection control. Basal level transcriptional activity of the luciferase reporter was set at 1 and all other values are expressed relative to this. The values shown represent the average of triplicate samples and the error bars indicate standard deviation.

reporter gene in cells transfected with RXR α alone, since RXR α cannot bind to the DNA response element of MAL-TKLUC. Perhaps over-expression of RXRα led to the formation of RXRα homodimers, which bind to direct repeats of the consensus sequence AGGTCA spaced by one nucleotide (DR1). RXRα homodimers are therefore incompatible with the MAL-TKLUC reporter that contains a DR4 response element. This means potentiation of transcription is not possible under these conditions. Hence it was not surprising that exogenously expressed TRAP220 was unable to elicit any effects on transcriptional activity from the luciferase reporter in cells transfected with RXRα alone in the presence of T_3, 9c-RA or both ligands.

Co-expression of TRβ and RXRα, in the presence of T_3 yielded a 2.8-fold induction in transcriptional activity from the MAL-TKLUC reporter as compared to the basal level. Since TRβ expression alone gave a 3-fold induction in transcriptional activity in the presence of T_3, it would appear that co-expression with RXRα does not result in an increased level of transcriptional activity of the MAL-TKLUC reporter and hence perhaps RXRα is not a limiting factor in these experiments. Converse to these findings, Hallenbeck *et al.*, (1993) observed co-transfection of NIH 3T3 cells with TR and RXR, in the presence of T_3, gave an increased transcriptional activity from a ME-TRE-linked CAT reporter (malic enzyme-TRE) as compared to activity seen with transfection of TR alone. Perhaps the use of different cell lines accounts for this difference in results. The endogenous levels of RXRα in HeLa cells may be sufficient to satisfy TRβ heterodimerisation under the conditions described in this study, whereas RXR may be a limiting factor in NIH 3T3 cells. Further, co-expression of TRβ and RXRα, in the presence of 9c-RA, did not permit transcriptional activity from the MAL-TKLUC reporter above the basal level. This was not surprising since there are reports showing that the ligand-induced transcriptional activity of RXR is suppressed when it is complexed with its heterodimeric partners. In RXR/TR and RXR/RAR heterodimers, the binding of 9c-RA to RXR is blocked by its heterodimeric partner (Forman *et al.*, 1995 and Kurokawa *et al.*, 1994) and indeed RXR is referred to as a silent partner in heterodimers in which it is unresponsive to its ligand. In this situation, transactivation mediated by RXR/TR heterodimers is induced preferentially or exclusively by TR ligand. The ability of TR to block the binding of RXR ligands is thought to result from an allosteric conformational change in the RXR LBD that is induced upon heterodimerisation between the LBDs of TR and RAR. However there is an

exception to the rule, as in the case of PPAR/RXR heterodimers, RXR is an active partner. Here both receptors are independently responsive to their ligands and are synergistically activated in the presence of both ligands (Kliewer *et al.*, 1992).

Co-transfection of TRβ and RXRα, in the presence of both T_3 and 9c-RA, resulted in a 2-fold increase in transcriptional activity from the MAL-TKLUC reporter compared to basal level. This is lower than that seen for cells co-transfected with RXRα and TRβ in the presence of T_3 alone and suggests 9c-RA is having a negative effect on TRβ-mediated transactivation. This 9c-RA-dependent reduction in TRβ-mediated transactivation is indicative of 9c-RA-induced homodimerisation of RXRα, which could possibly lead to the sequestering of RXRα from the transcriptionally active RXRα/TRβ heterodimers. Similar observations have been reported by Forman *et al.*, (1995).

Additionally cells were co-transfected with expression vectors for TRβ and TRAP220 in the presence of T_3 alone, 9c-RA alone or both ligands, for comparisons with cells transfected with RXRα and TRAP220 expression vectors. As shown in figure 3.5 TRAP220 was able to enhance TRβ-mediated transcription of the luciferase reporter gene 1.6-fold in the presence of T_3 alone and a comparable 1.4-fold in the presence of both T_3 and 9c-RA. This suggests co-addition of 9c-RA in the absence of over-expressed RXRα, has no apparent effect on TRβ-mediated transactivation. However cells expressing exogenous TRβ and TRAP220 displayed no transcriptional activity from the luciferase reporter above basal level in the presence of 9c-RA alone. This is because TRβ cannot bind 9c-RA, as discussed previously.

Finally cells were co-transfected with TRβ, RXRα and TRAP220 expression vectors in the presence of T_3, 9c-RA or both ligands. Since co-expresion of TRβ and RXRα, in the presence of T_3 did not result in increased transcriptional activity from the luciferase reporter as compared to cells transfected with TRβ alone, it was not surprising that TRAP220 achieved a similar level of enhancement in TRβ-mediated transactivation in cells expressing exogenous TRβ (1.6-fold) and cells expressing exogenous TRβ and RXRα (1.4-fold). This again suggests RXRα is not a limiting factor in these experiments. However what was surprising was the effect of co-addition of 9c-RA and T_3 on the transcriptional activity from the MAL-TKLUC in cells transfected with RXRα, TRβ and TRAP220 expression vectors. In this case there was no observed enhancement in transcriptional activity by TRAP220 as compared to cells transfected with RXRα and TRβ in the absence of TRAP220. The reason for this apparent 9c-RA-dependent inhibition of TRAP220 activity is unclear and since this was not one of the primary

aims of my research, the phenomenon of 9c-RA-inhibition of TRAP220 enhanced TRβ-mediated activity was not investigated further.

3.7 TRAP220 is unable to enhance ERα-mediated transactivation

Thus far I have demonstrated that TRAP220 is able to modestly enhance TRβ-mediated transcriptional activity in a T_3-dependent manner. This is not surprising given that the TRAP complex was isolated due to its T_3-dependent association with the TR (Fondell *et al.*, 1996). However there is a degree of ambiguity in the literature regarding TRAP220 interactions with the ERα. Indeed there are reports of ligand-dependent binding of full-length TRAP220 with ERα (Chang *et al.*, 1999, Zhu *et al.*, 1999, Burakov *et al.*, 2000 and Burakov *et al.*, 2002) but conversely there are also reports of no or very weak binding (Yuan *et al.*, 1998, Kobayashi *et al.*, 2000 and Warnmark *et al.*, 2001). Therefore it was of interest to investigate the effects of TRAP220 expression on ERα-mediated transcription.

In order to do this an ERα-mediated transactivation reporter system previously established in this laboratory (Dr H. Sheppard) was utilised. HeLa cells were transiently transfected, using the calcium-phosphate co-precipitation method, with a β-galactosidase reporter (as an internal transfection control), an ERE-linked firefly luciferase reporter (p3ERE-TATA-LUC) together with an ERα expression vector. Additionally cells were transfected with either an SRC1e or TRAP220 expression vector as indicated, to assess the effects of SRC1e and TRAP220 on ERα-mediated transcription of the ERE-linked luciferase reporter gene. As with the TRβ-mediated transactivation reporter system, the genuine coactivator SRC1e was included for comparative reasons. As shown in figure 3.6, basal level of transcription was represented by cells transfected in the absence of E_2 with β-galactosidase and ERE-linked luciferase reporters only. Basal level of transcription was set at 1 and all other values were expressed relative to this. Upon expression of ERα, the transcriptional activity from the luciferase reporter was increased 85-fold over the basal level in an E_2-dependent manner. This is a very good ERα-mediated induction in transcription of the luciferase reporter gene and is comparable to inductions seen previously for this system (Dr H. Sheppard, personal communication). SRC1e expression resulted in a 6-fold, E_2-dependent, enhancement in ERα-mediated transcription of the luciferase reporter gene. This is in agreement with the findings of Kalkhoven *et al.*, (1998) and Sheppard *et al.* (2001), who observed SRC1e is able to enhance ERα-mediated transcription of an ERE-linked luciferase reporter gene 5 to 8-fold in an E_2-dependent manner.

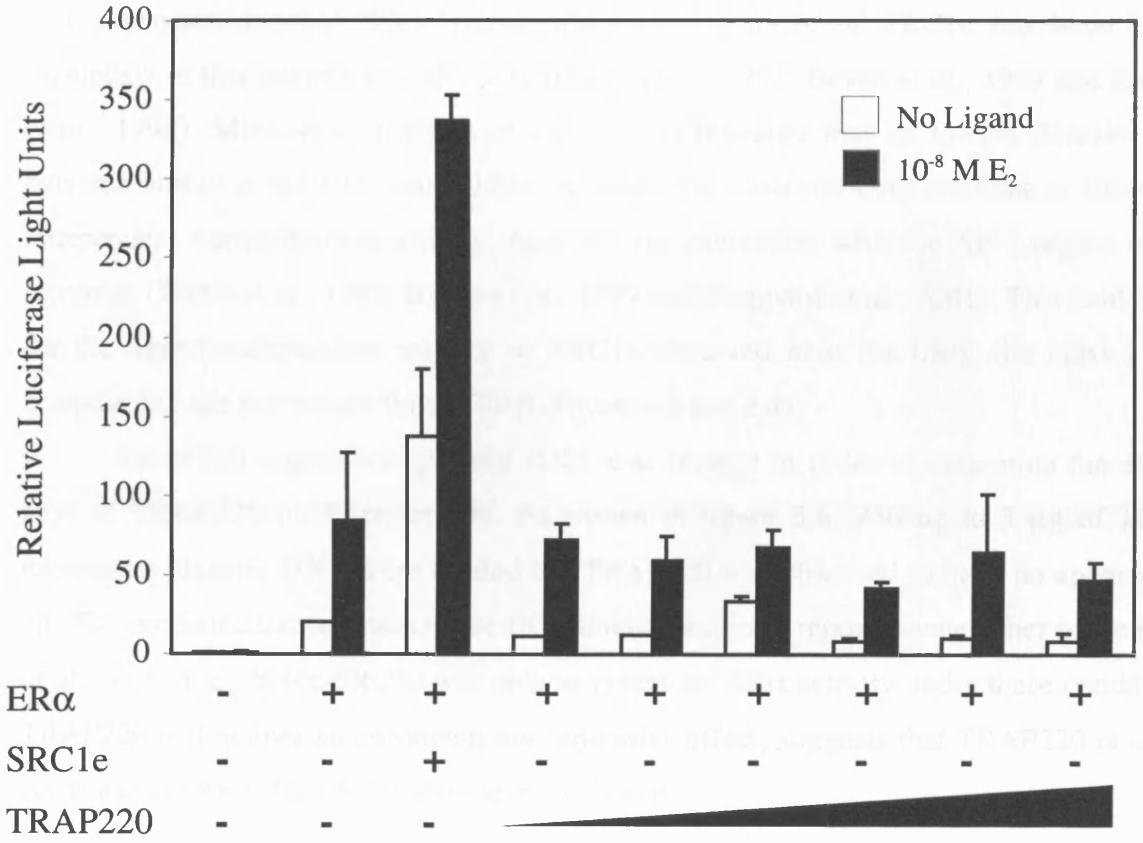


Figure 3.6 Exogenously expressed TRAP220 is unable to enhance ERα-mediated transcriptional activity. HeLa cells were transiently transfected with 500 ng pJ7-LacZ and 1 μg p3ERE-TATA-LUC. Cells were additionally transfected with 100 ng pMT-MOR, 500 ng pSG5-SRC1e or increasing amounts of pSG5(PT)-HA-TRAP220 (250 ng, 500 ng, 750 ng, 1 μg, 2 μg and 3 μg), as indicated. Luciferase activity was measured and normalised to the β-galactosidase transfection control. Basal level transcriptional activity of the luciferase reporter was set at 1 and all other values are expressed relative to this. The values shown represent the average of triplicate samples and the error bars indicate standard deviation.

Additionally SRC1e was shown here to enhance ER α-mediated transactivation 5.6-fold in an E_2-independent manner. This ligand-independent activity of SRC1e has been observed previously in this laboratory and others (Heery *et al.*, 1997, Bevan *et al.*, 1999 and Kalkhoven *et al.*, 1998). Mutational analysis of SRC1e has revealed that its Q-rich domain (located between amino acids 1053 and 1123) mediates the observed enhancement in ERα ligand-independent transcriptional activity, possibly via interaction with the AF-1 region of class I receptors (Webb *et al.*, 1998, Bevan *et al.*, 1999 and Sheppard *et al.*, 2001). This could account for the ligand-independent activity of SRC1e observed here for ERα, the class I nuclear receptor, but not previously for the TRβ (Figure 3.3 and 3.4).

TRAP220 expression plasmid DNA was titrated in order to determine the effects (if any) of TRAP220 on ERα activity. As shown in figure 3.6, 250 ng to 3 μg of TRAP220 expression plasmid DNA were titrated but TRAP220 was observed to have no apparent effect on ERα-mediated transcription of the ERE-linked luciferase reporter gene either in the presence or absence of E_2. Since SRC1e was able to potentiate ERα activity under these conditions but TRAP220 had neither an enhancing nor repressive effect, suggests that TRAP220 is unable to act as a coactivator for ERα under these conditions.

3.8 Exogenously expressed TRAP220 does not interfere with SRC1e potentiation of ERα activity

Under the same conditions, I have shown here that SRC1e, but not TRAP220, is able to potentiate ERα-mediated transcriptional activity. The reason for this observed differential usage of coactivator by ERα is unclear but I suggest it may be related to specific coactivator-nuclear receptor interactions. It is well documented that SRC1e is able to interact with the ERα and indeed potentiate ERα activity (Halachmi *et al.*, 1994, Onate *et al.*, 1995, Kalkhoven *et al.*, 1998 and Margeat *et al.*, 2001), however as discussed previously there are conflicting reports regarding TRAP220 interactions with ERα. No interaction or a very weak interaction between ERα and TRAP220 *in vivo*, could result in no potentiation in ERα transcriptional activity in the ERα-mediated transactivation assays described in this study.

Using the ERα-mediated transactivation system described above, I was able to indirectly assess TRAP220 binding to ERα. As shown in figure 3.7, SRC1e was able to enhance ERα-mediated transcription of the luciferase reporter gene 5.5-fold in an E_2-dependent

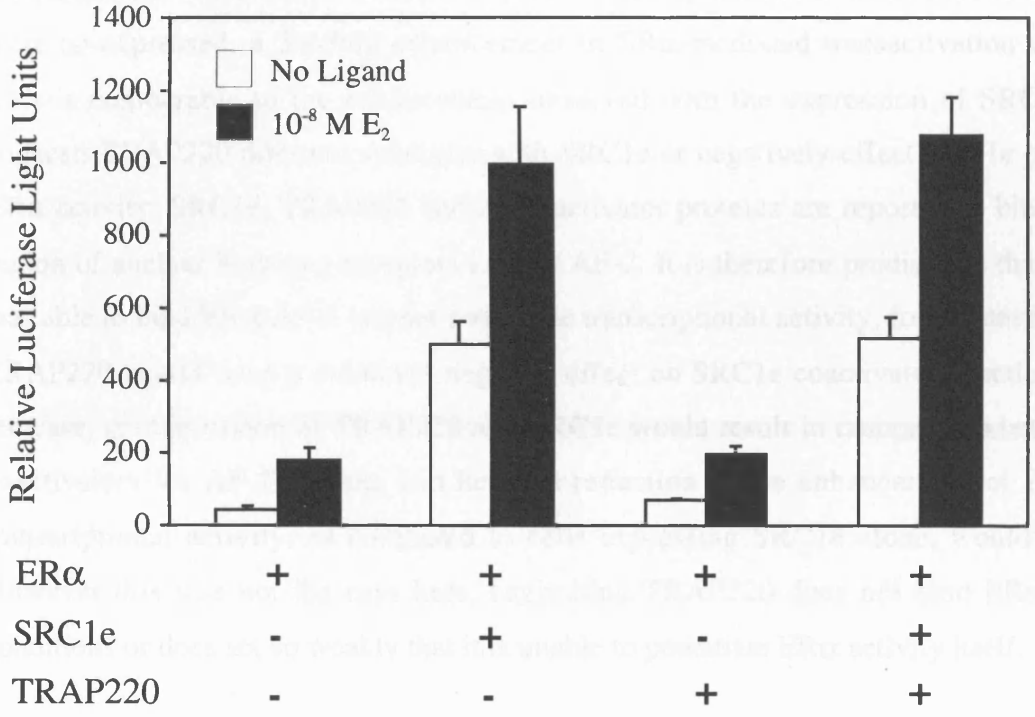


Figure 3.7 TRAP220 does not affect SRC1e activity. HeLa cells were transiently transfected with 500 ng pJ7-LacZ, 1 μg p3ERE-TATA-LUC and 100 ng pMT-MOR. Additionally cells were transfected with 500 ng pSG5-SRC1e and 500 ng pSG5(PT)-HA-TRAP220, as indicated. Luciferase activity was measured and normalised to the β-galactosidase transfection control. Basal level transcriptional activity of the luciferase reporter was set at 1 and all other values are expressed relative to this. The values shown represent the average of triplicate samples and the error bars indicate standard deviation.

manner and 2.7-fold in an E_2-independent manner. This is consistent with previous results (Figure 3.6). Also, as seen previously, TRAP220 had no apparent effect on ERα-mediated transactivation either in the absence or presence of E_2. However when SRC1e and TRAP220 were co-expressed, a 5.9-fold enhancement in ERα-mediated transactivation was observed. This is comparable to the enhancement observed with the expression of SRC1e alone and suggests TRAP220 does not synergise with SRC1e or negatively effect SRC1e potentiation of ERα activity. SRC1e, TRAP220 and all coactivator proteins are reported to bind to the same region of nuclear hormone receptors i.e. the AF-2. It is therefore predictable that if TRAP220 was able to bind ERα AF-2 but not potentiate transcriptional activity, for whatever reason, then TRAP220 would have a dominant negative effect on SRC1e coactivator function. If this was the case, co-expression of TRAP220 and SRC1e would result in competition between the two coactivators for AF-2 binding and hence a reduction in the enhancement of ERα-mediated transcriptional activity, as compared to cells expressing SRC1e alone, would be observed. However this was not the case here, suggesting TRAP220 does not bind ERα under these conditions or does so, so weakly that it is unable to potentiate ERα activity itself.

3.9 Detection of exogenously expressed TRAP220 protein

The TRβ- and ERα-mediated transactivation assays used in this study rely on the successful transient transfection of mammalian cells with various reporter constructs, as well as nuclear receptor and coactivator expression vectors. In order to examine the effects of TRAP220 on TRβ- and ERα-mediated transactivation, it is vital not only that the cells take up the TRAP220 expression plasmid DNA but that they also express full-length TRAP220 protein from the expression vector. Since TRAP220 was observed to modestly enhance TRβ-mediated transactivation and have no effect on ERα-mediated transactivation, whereas SRC1e was able to greatly enhance the transcriptional activity of ERα under the same conditions, it was necessary to assess exogenous TRAP220 expression within the transiently transfected cells. If for some reason TRAP220 protein was not being expressed, then this could account for TRAP220s observed inability to enhance ERα-mediated transactivation.

HeLa cells were transiently transfected with expression vectors for either HA-TRAP220, HA-p300 or HA-GRIP1. Each of these proteins can be distinguished from their endogenous counterparts due to their N-terminal (for TRAP220 and GRIP1) or C-terminal (for

p300) HA-tag and additionally can potentially be detected using a monoclonal anti-HA primary antibody. HA-p300 and HA-GRIP1 were used in these experiments as HA-tagged protein controls for the anti-HA primary antibody. Whole cell lysates were prepared from the transfected cells and the proteins separated using one-dimensional SDS-PAGE. Proteins were then transferred from the polyacrylamide gel to a nitrocellulose membrane which was subsequently probed with an anti-HA primary antibody. As shown in figure 3.8, the anti-HA antibody was used to successfully detect HA-p300 from cells transfected with HA-p300 expression vector (Figure 3.8, lane 2). HA-p300 appears as the major band in lane 2, corresponding to approximately 300 kDa. A series of weaker bands possibly represent HA-tagged degradation products, ranging in size from 90 to 240 kDa approximately. A non-specific band corresponding to a protein of 75 kDa was visible in all lanes, including lane 1 which corresponds to the cell lysate derived from mock transfected cells. This implies the 75 kDa band is non-specific and is not an HA-tagged breakdown product of any of the proteins being examined here. The anti-HA antibody was also able to detect HA-GRIP1 protein from cells transiently transfected with HA-GRIP1 expression vector. HA-GRIP1 appears as the major band in lane 3 (Figure 3.8), corresponding to approximately 160 kDa. Degradation products of HA-GRIP1 are also visible at approximately 110 and 130 kDa, respectively. However HA-TRAP220 was not detected in cell lysates derived from cells transfected with HA-TRAP220 expression vector (Figure 3.8, lane 4). Cells were transfected with equal quantities of HA-p300, HA-GRIP1 or HA-TRAP220 expression plasmid DNA under the same conditions and yet HA-TRAP220 could not be detected but HA-p300 and HA-GRIP1 could. Additionally HA-GRIP1 and HA-TRAP220 are both expressed from vectors containing SV40 promoters, suggesting different promoter strength is not the reason for this failure to detect HA-TRAP220. There are several possible explanations why HA-TRAP220 protein could not be detected. The HA tag at the N-terminus of TRAP220 may have been masked or occluded in some way, perhaps due to the folding of the TRAP220 protein, hence rendering it unrecognisable to the HA-specific antibody. The membrane from which the chemiluminescent image in figure 3.8 was derived was stripped and re-probed with commercially available TRAP220 specific polyclonal antibodies. These antibodies would be expected to detect endogenous TRAP220 as well as the exogenously expressed HA-TRAP220 protein and hence it was predicted that a specific band representing TRAP220 would be more intense from cells transfected with HA-TRAP220 as compared to mock transfected cells or cells transfected with HA-p300 and HA-GRIP1

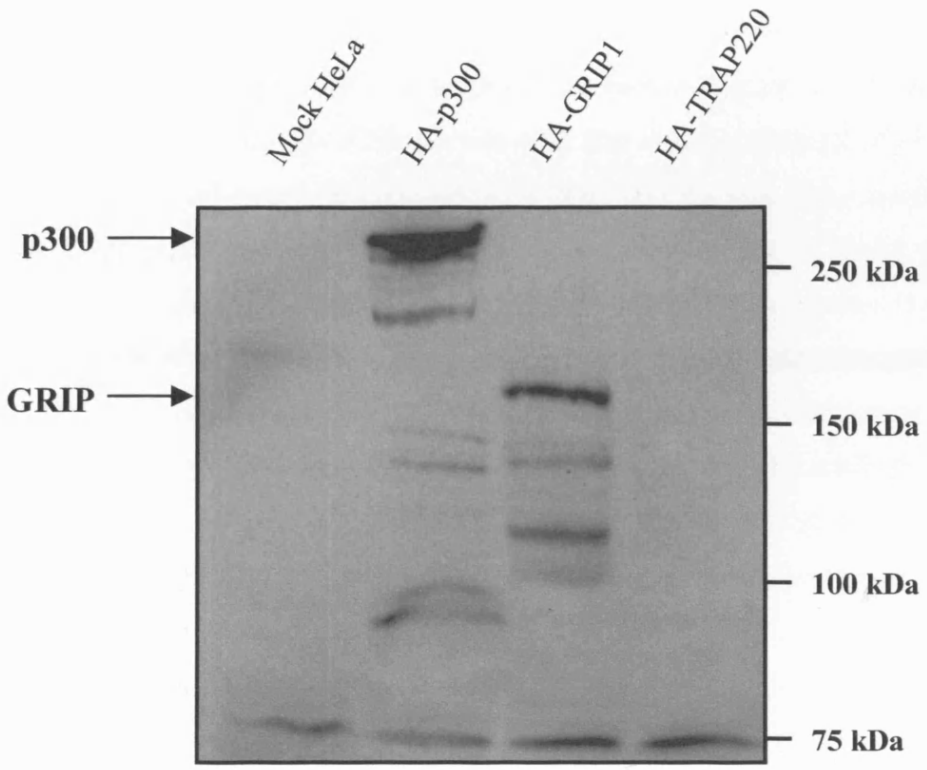


Figure 3.8 Detection of exogenously expressed HA-tagged proteins using western blotting. HeLa cells were transiently transfected with pCMV-p300, pSG5-HA-GRIP1 or pSG5(PT)-HA-TRAP220. Control cells were mock transfected. Whole cell lysates were prepared 24 hours post transfection and the proteins separated on 8% SDS-polyacrylamide gels, prior to being transferred to a nitrocellulose membrane and probed with an anti-HA primary antibody.

expression vectors. However the TRAP220 specific antibodies proved to be highly unspecific in western blotting under the conditions used here, yielding high background and failing to distinguish cells over-expressing HA-TRAP220 protein from those having endogenous levels of TRAP220 (data not shown).

In vitro transcription/translation reactions verified that full-length TRAP220 protein can be produced from the pSG5(PT)-HA-TRAP220 vector (Figure 3.10A) and therefore it was assumed that full-length TRAP220 protein was also being generated *in vivo* from this vector. Failure to detect full-length HA-TRAP220 protein could result from instability of the protein due to proteolytic enzymes. TRAP220 protein could be lost at many stages between the transient transfection of the cells to immunodetection on the membranes. HA-TRAP220 protein could be more susceptible to proteolysis within the cells than HA-p300 and HA-GRIP1. Perhaps exogenously expressed HA-TRAP220 is degraded prior to the preparation of the whole cell lysates, hence giving a negative result when using the anti-HA antibody in western blotting. To investigate this possibility, the proteasome inhibitor lactacystin, was used. Lactacystin is a *Streptomyces* metabolite that acts as an irreversible proteasome inhibitor (Fenteany *et al.*, 1995). It targets the 20 S proteasome which is an essential component of the ubiquitin-proteasome pathway for intracellular protein degradation. Lactacystin was added to the cells 8 hours post transfection to inhibit proteasome activity. Whole cell lysates were then prepared, the proteins separated by one-dimensional SDS-PAGE and transferred to a nitrocellulose membrane where they were probed with an anti-HA antibody. As shown in figure 3.9, exogenously expressed HA-GRIP1 protein was detected as a 160 kDa band in cell lysates derived from cells transiently transfected with HA-GRIP1 expression vector both in the absence and presence of lactacystin (Figure 3.9, lanes 2 and 5, respectively). However in the presence of lactacystin the band representing HA-GRIP1 (Figure 3.9, lane 5) was considerably more intense than the band representing HA-GRIP1 in the absence of lactacystin (Figure 3.9, lane 2). This demonstrates that there is more HA-GRIP1 protein present in the whole cell lysates when lactacystin was used and suggests that its addition has reduced protein degradation within the transfected cells. This data is in agreement with a recent study in which lactacystin was observed to induce an increase in the total fluorescence intensity of GFP-GRIP1 expressing cells compared to GFP-GRIP1 expressing cells in the absence of lactacystin (Baumann *et al.*, 2001). Surprisingly however HA-TRAP220 was still not detected using the anti-HA antibody

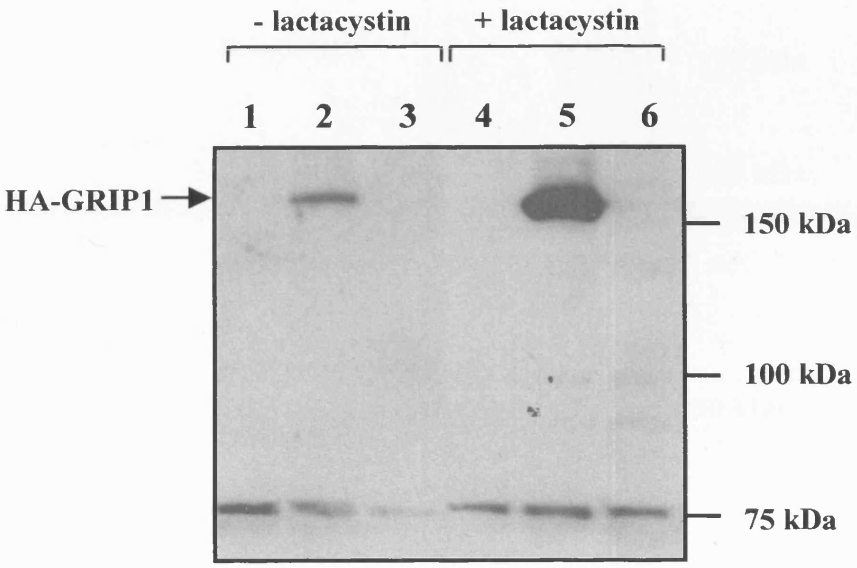


Figure 3.9 Assessing the effect of a proteasome inhibitor on exogenous protein levels. Whole cell lysates derived from mock transfected cells (lanes 1 and 4) or cells transfected with pSG5-HA-GRIP1 (lanes 2 and 5) or pSG5(PT)-HA-TRAP220 (lanes 3 and 6), in the presence or absence of 5 μM lactacystin, were subject to SDS-PAGE and the proteins transferred to nitrocellulose membrane, prior to being probed with an anti-HA antibody.

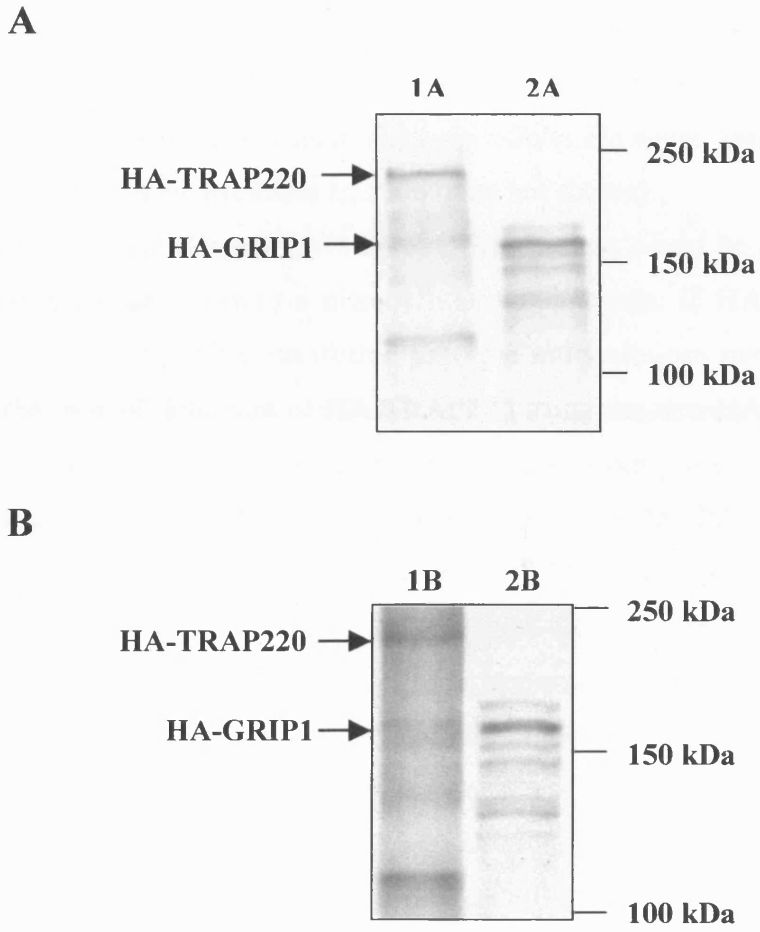


Figure 3.10 Electrophoretic transfer of proteins from a polyacrylamide gel to a nitrocellulose membrane. (A) *In vitro* translated [^{35}S]-labelled HA-TRAP220 (lane 1A) and [^{35}S]-labelled HA-GRIP1 (lane 2A) were separated by SDS-PAGE and the radiolabelled proteins visualised by autoradiography. (B) *In vitro* translated [^{35}S]-labelled HA-TRAP220 (lane 1B) and [^{35}S]-labelled HA-GRIP1 (lane 2B) proteins were transferred from a polyacrylamide gel to a nitrocellulose membrane and visualised by autoradiography.

even in the presence of lactacystin (Figure 3.9, lane 6). This suggests that cellular protein degradation is not the reason why exogenously expressed HA-TRAP220 cannot be detected using the anti-HA antibody in western blotting. To verify that the HA-TRAP220 protein was not retained within the insoluble fraction of the whole cell lysate, the cell debris was prepared for SDS-PAGE by boiling in SDS-PAGE loading buffer, prior to being subjected to vigorous vortexing to solubilise the pellet as much as possible. However anti-HA antibody failed to detect HA-TRAP220 in the insoluble fraction (data not shown).

Next I investigated whether HA-TRAP220 protein could be successfully transferred from a polyacrylamide gel onto a nitrocellulose membrane. If HA-TRAP220 protein, for whatever reason, could not be transferred onto the nitrocellulose membrane then this would account for the lack of detection of HA-TRAP220 using the anti-HA antibody. [^{35}S]-labelled HA-TRAP220 and HA-GRIP1 proteins were generated from pSG5 expression vectors, using the *in vitro* transcription/translation system and subjected to SDS-PAGE. The autoradiogram in figure 3.10A shows that an [^{35}S]-labelled protein of 220 kDa and an [^{35}S]-labelled protein of 160 kDa, corresponding to HA-TRAP220 and HA-GRIP1 respectively, were successfully generated. However there is also a weaker band approximating to 120 kDa visible in lane 1A (Figure 3.10A). This is most likely a breakdown product of TRAP220, produced due to the action of contaminating proteases in the *in vitro* transcription/translation reaction, or alternatively the result of the use of differential initiation codons by the T7 RNA polymerase. A band corresponding to 160 kDa is present in lane 2 and represents HA-GRIP1. Weaker bands representing proteins of smaller molecular weights (140-150 kDa) are present in lane 2A. [^{35}S]-labelled HA-TRAP220 and HA-GRIP1 were transferred from a polyacrylamide gel to a nitrocellulose membrane by electroblotting. The autoradiographic image of the membrane, shown here in figure 3.10B, clearly demonstrates that both HA-TRAP220 and HA-GRIP1 protein can be transferred from a polyacrylamide gel to a nitrocellulose membrane under the conditions described. Membrane-bound [^{35}S]-labelled HA-TRAP220 is represented by a band approximating to 220 kDa in lane 1B and membrane-bound [^{35}S]-labelled HA-GRIP1 is represented by a band approximating to 160 kDa in lane 2B of figure 3.10B. The membrane was additionally probed with anti-HA antibody but neither HA-TRAP220 or HA-GRIP1 were detected under these conditions (data not shown). In the case of HA-GRIP1, which has previously been shown to be detected using the anti-HA antibody (Figures 3.8 and 3.9), this is

most likely due to insufficient HA-GRIP1 protein on the membrane as a low amount of protein is generated by *in vitro* transcription/translation systems.

3.10 Immunofluorescence imaging of transiently expressed proteins

Indirect immunofluoresence can be used to visualise transiently over-expressed proteins within intact cells. This method does not involve the preparation of whole cell lysates and so eliminates the potential to lose cellular proteins at the various stages involved in the cell lysate preparation process. Therefore indirect immunofluorescence was used in this study as an alternative method for detecting transiently over-expressed HA-TRAP220 protein.

Both HeLa and COS-1 cells were transiently transfected with the expression vector for HA-TRAP220, pSG5(PT)-HA-TRAP220. For comparative reasons and in keeping with previous analyses in this study, cells were also transiently transfected with expression vectors for HA-p300 and HA-GRIP1. 48 hours post transfection, cells were fixed and probed with the appropriate antibodies. Background fluorescence was monitored by visualisation of transfected cells incubated with either a FITC-conjugated secondary antibody or TRITC-conjugated secondary antibody alone and was observed to be negligible in all cases (data not shown). As shown in figure 3.11, COS-1 (Figure 3.11A and B) and HeLa (Figure 3.11C and D) cells were transiently transfected with an expression vector for HA-TRAP220 and subsequently probed with an anti-HA primary antibody and the appropriate FITC-conjugated secondary antibody. The immunofluorescent data shown clearly demonstrates the expression of HA-TRAP220 in intact cells. In both HeLa and COS-1 cells, transiently expressed HA-TRAP220 protein was observed to be localised exclusively in the nucleus giving a diffuse staining pattern, but excluded from the nucleoli. This was not surprising given TRAP220s proposed role in RNA polymerase II transcription (RNA polymerase I transcription occurs in the nucleoli). Treatment of COS-1 cells with T_3 (10^{-7} M) did not influence the distribution of transfected HA-TRAP220 under these conditions (Figure 3.11F). Transiently transfected COS-1 cells, probed with anti-HA primary antibody (Figure 3.11A), were additionally probed with an anti-TRAP220 polyclonal antibody and a TRITC-conjugated secondary antibody. As shown in figure 3.11E, the anti-TRAP220 primary antibody also successfully recognised TRAP220 protein, displaying a similar pattern to that obtained with the anti-HA antibody.

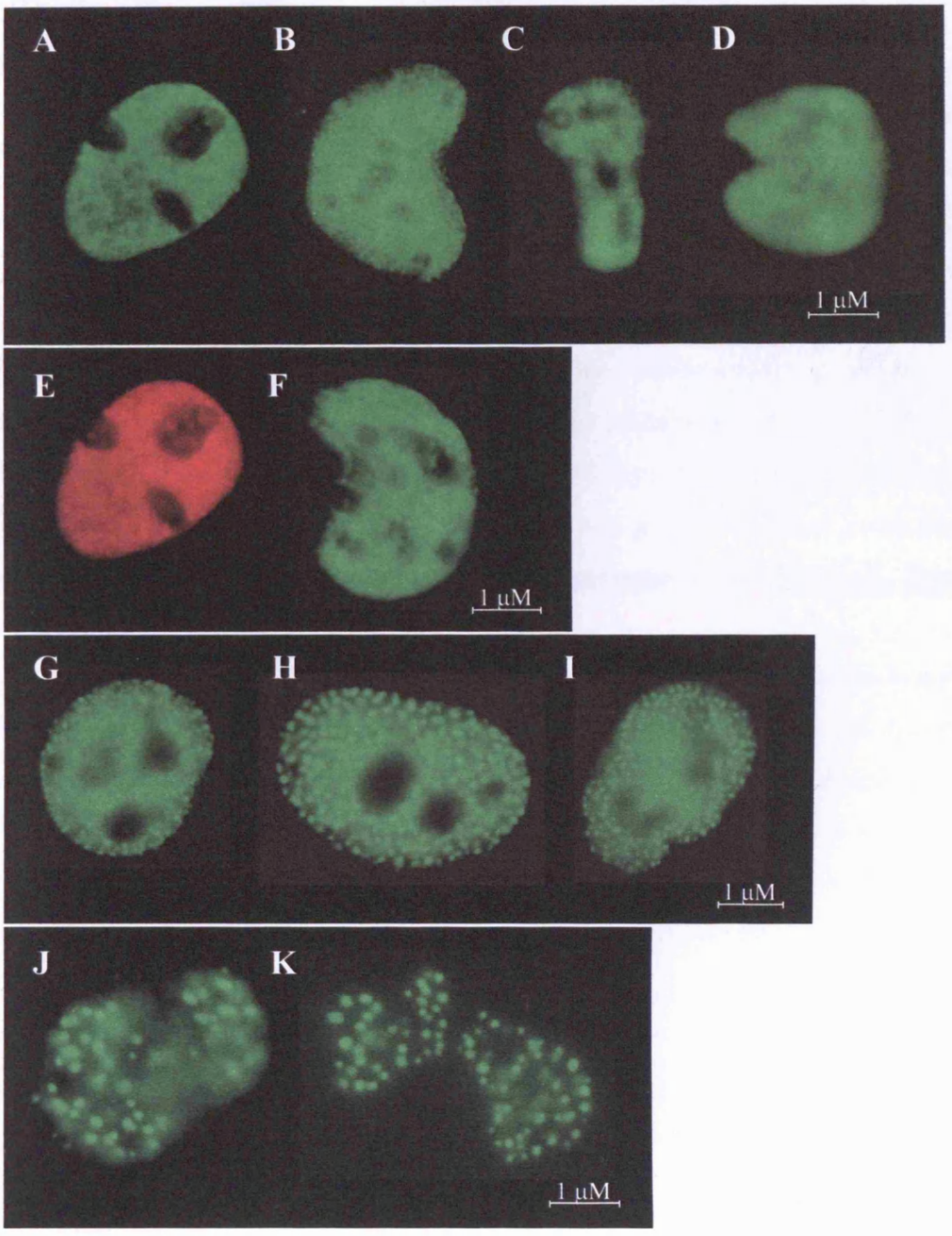


Figure 3.11 Indirect immunofluorescence was used to visualise exogenously expressed proteins. All images are at 100x magnification. COS-1 (A and B) and HeLa (C and D) cells were transiently transfected with pSG5(PT)-HA-TRAP220 and probed with anti-HA 1° antibody and FITC-conjugated 2° antibody. (E) As for A, except an anti-TRAP220 1° antibody and TRITC-conjugated 2° antibody were used. (F) COS-1 cells were transiently transfected with pSG5(PT)-HA-TRAP220, in the presence of 10^{-7} M T_3 and probed with anti-HA 1° antibody and FITC-conjugated 2° antibody. COS-1 (G and H) and HeLa (I) cells were transiently transfected with pCMV-p300 and probed with anti-HA 1° antibody and FITC-conjugated 2° antibody. COS-1 (J) and HeLa (K) cells were transiently transfected with pSG5-HA-GRIP1 and probed with anti-HA 1° antibody and FITC-conjugated 2° antibody.

HA-p300 was also observed to be localised exclusively in the nucleus, but not the nucleoli (Figure 3.11G-I). However in contrast to the distribution pattern observed for HA-TRAP220, HA-p300 gave a diffuse staining pattern overlaid with numerous brighter foci. Foci or speckles, representing p300 localisation within cells, have been reported previously (von Mikecz *et al.*, 2000) and have been suggested to function as temporary storage sites or recycling centres for multiple factors required for mRNA biogenesis (Spector *et al.*, 1993). Typically, mammalian cell nuclei contain 20-40 speckles. Additionally promyelocytic leukaemia (PML)-containing bodies appear as discrete punctate regions when using indirect immunofluorescence. It is not clear in this case whether the foci observed due to HA-p300 transient expression are PML-containing bodies, storage sites/recycling centres or a mixture of the two. Indeed these foci could simply be due to over expression of HA-p300 (K. Kindle, personal communication).

Similarly to HA-TRAP220 and HA-p300, HA-GRIP1 was observed to be localised exclusively in the nucleus, with exclusion from the nucleoli (Figure 3.11 J and K). As with HA-p300, the distribution pattern of HA-GRIP1 was distinct from that of HA-TRAP220. In agreement with recent studies (Baumann *et al.*, 2001), HA-GRIP1 gave punctate foci with a small amount of diffuse staining. The foci in this case appeared to be brighter and in fewer numbers than those observed when HA-p300 was transiently expressed (Figures 3.11G-I). Again the exact nature of these foci is unclear.

In conclusion, the use of indirect immunofluorescence has successfully demonstrated that transiently transfected cells do express HA-TRAP220 protein, as well as HA-p300 and HA-GRIP1. An anti-HA primary antibody was able to recognise the HA tag of these transiently expressed proteins and highlighted their subcellular location.

3.11 The modulation of nuclear receptor-mediated transcriptional activity by TRAP170

Although the main focus of this study will remain on the 220 kDa subunit of the TRAP complex (TRAP220), a recent report suggesting DRIP150 (also named TRAP170) acts as a coactivator in glucocorticoid receptor (GR)-mediated transcriptional activity (Hittelman *et al.*, 1999), prompted an investigation into the effect of TRAP170 on ERα- and TRβ-mediated transactivation, for comparison with the effects observed for TRAP220 (Figures 3.3-3.7).

The T_3-responsive, TRβ-mediated transactivation reporter system established in section 3.2 was utilised to assess the effect of TRAP170 expression on TRβ-mediated transactivation. HeLa cells were transiently transfected with a β-galactosidase reporter and MAL-TKLUC reporter, to determine the basal level of transcription from the MAL-TKLUC reporter under these conditions. The basal level of transcription was set at 1 and all other values were expressed relative to this. Cells were additionally transfected with a TRβ expression vector and a TRAP170 expression vector, as indicated in figure 3.12. In a T_3-dependent manner, expression of TRβ resulted in a 5-fold increase in transcriptional activity from the luciferase reporter, above the basal level. Co-expression of TRAP170 resulted in a 2.5-fold enhancement in TRβ-mediated transcription of the luciferase reporter gene in a T_3-dependent manner. This level of enhancement in TRβ-mediated transactivation is comparable to that observed for TRAP220 (Figures 3.3, 3.4 and 3.5).

The ERα-mediated transactivation reporter system utilised in section 3.7 was again used to assess the effect of TRAP170 expression on ERα-mediated transcriptional activity. The basal level of transcription was determined by transfecting cells with a β-galactosidase reporter and the ERE-linked luciferase reporter, (p3ERE-TATA-LUC). Again basal level was set at 1 and all other values were expressed relative to this. Cells were additionally transfected with an ERα expression vector and a TRAP170 expression vector. Expression of ERα resulted in a 100-fold increase in transcriptional activity from the ERE-linked luciferase reporter in an E_2-dependent manner (Figure 3.13). This is comparable with levels seen previously in this study (Figure 3.6). Co-transfection with TRAP170 resulted in a 2.6-fold enhancement in ERα-mediated transactivation in an E_2-dependent manner.

Previously in this study, TRAP220 was shown to be unable to enhance ERα-mediated transactivation (Figure 3.6) but here TRAP170 has been shown to modestly enhance ERα-mediated transactivation (Figure 3.13). Taken together this suggests that the TRAP complex could be involved in ERα-mediated transcriptional activity but perhaps its involvement is mediated through the TRAP170 subunit. Hittelman *et al.*, (1999), demonstrated under the same conditions that DRIP150/TRAP170 was able to enhance GR-mediated transactivation, but DRIP205/TRAP220 had little effect. By showing that DRIP150/TRAP170 binds to the AF-1 of the GR but not the AF-2, they suggest that DRIP150/TRAP170 mediates GR transcriptional activity in an AF-1 dependent manner. To test this hypothesis for TRAP170, a system able to measure the effects of exogenously expressed proteins on the transcriptional activity of a

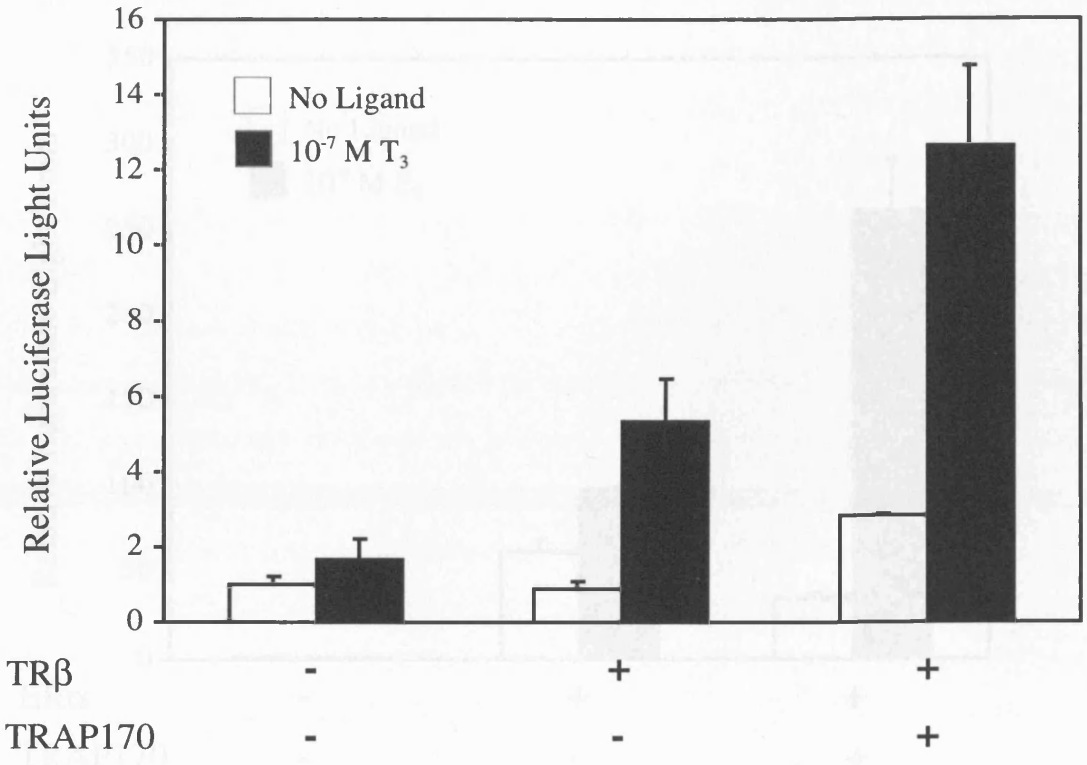


Figure 3.12 TRAP170 is able to enhance the TRβ-mediated transcriptional activity from a TRE-linked luciferase reporter. HeLa cells were transiently transfected with 500 ng pJ7-LacZ and 2 μg pMAL-TKLUC, together with 200 ng hTRβ-RSV and 3 μg pFLAG(s)-7-TRAP170, as indicated. Luciferase activity was measured and normalised to the β-galactosidase transfection control. Basal level of transcriptional activity of the luciferase reporter was set at 1 and all other values are expressed relative to this. The values shown represent the average of triplicate samples and the error bars indicate standard deviation.

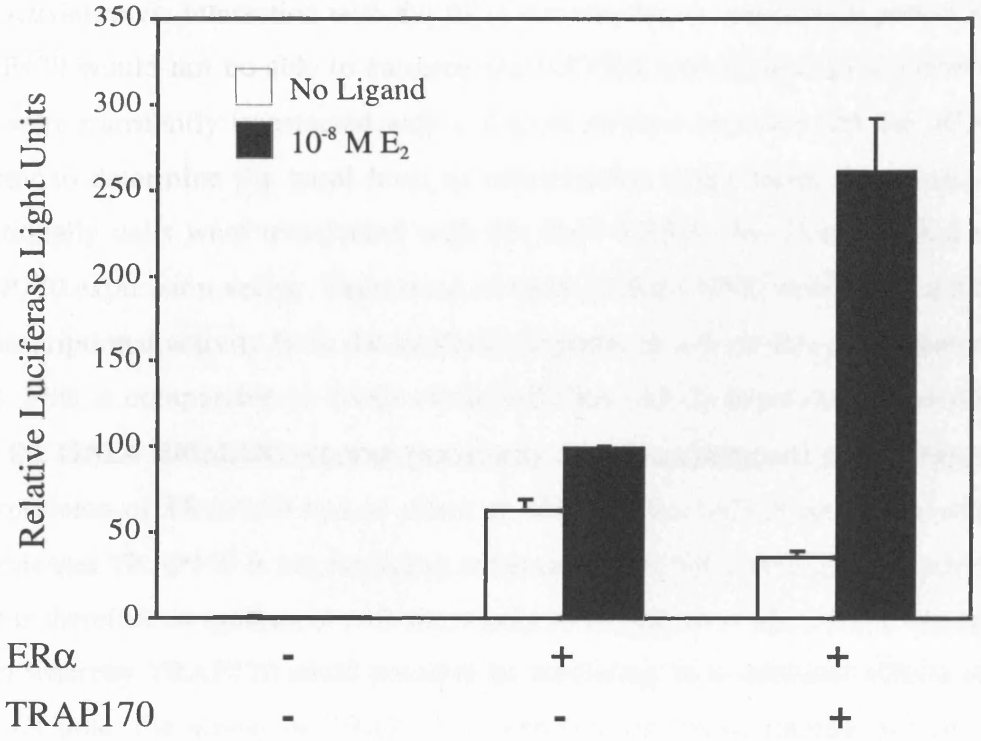


Figure 3.13 TRAP170 is able to enhance the ERα-mediated transcriptional activity from an ERE-linked luciferase reporter. HeLa cells were transiently transfected with 500 ng pJ7-LacZ and 1 μg p3ERE-TATA-LUC, together with 100 ng pMT-MOR and 3 μg pFLAG(s)-7-TRAP170, as indicated. Luciferase activity was measured and normalised to the β-galactosidase transfection control. Basal level of transcriptional activity of the luciferase reporter was set at 1 and all other values are expressed relative to this. The values shown represent the average of triplicate samples and the error bars indicate standard deviation.

luciferase reporter gene mediated by a Gal4-RXR α (AF-2) construct was utilised. The Gal4-RXRα (AF-2) construct is composed of the DNA binding domain (DBD) of the yeast transcription factor Gal4, fused to the AF-2 of RXRα. If TRAP170 enhances NR-mediated transactivation via interaction with the AF-1 domain then it would be expected in this case that TRAP170 would not be able to enhance Gal4-RXRα (AF-2)-mediated transactivation. HeLa cells were transiently transfected with a β-galactosidase reporter and the pGAL4-EI6ΔLUC reporter to determine the basal level of transcription under these conditions (Figure 3.14). Additionally cells were transfected with the Gal4-RXRα (AF-2) expression vector and the TRAP170 expression vector. Expression of Gal4-RXRα (AF-2) resulted in a 3.8-fold increase in transcriptional activity from the luciferase reporter in a 9-*cis*-RA-dependent manner (Figure 3.14). This is comparable to levels of Gal4-RXRα (AF-2) dependent transcriptional activity from the GAL4-EI6ΔLUC reporter previously reported (Sheppard *et al.*, 2001). As predicted co-expression of TRAP170 had no effect on Gal4-RXRα (AF-2) transcriptional activity. This suggests that TRAP170 is not mediating enhancement of NR transcriptional activity via the AF-2 and is therefore in agreement with the results of Hittelman *et al.*, (1999). My results support a model whereby TRAP170 could possibly be mediating its coactivator effects via the AF-1 of NRs. Of note, the ability of TRAP170 to enhance the transcriptional activity of full-length RXRα was not tested here and therefore it should be considered that perhaps TRAP170 is unable to enhance even full-length RXRα.

3.12 Discussion

3.12.1 The nature of the TRE influences TRβ transcriptional activity.

In establishing an *in vivo* TRβ-mediated transactivation reporter system, I have also demonstrated the importance of the nature of the TRE in regulating transcriptional activation by the TRβ. In this study TRβ activity was tested using three different TREs, each possessing a different configuration of the hexameric nucleotide motif of the consensus sequence AGGTCA, found in physiological TREs. Analyses of the promoter regions of T_3-inducible genes indicates that the consensus hexameric half-sites are arranged as direct repeats, everted repeats or a palindromic sequence. In this study TREs containing each of the above mentioned configurations were tested. In all of the TRE-linked luciferase reporters used, namely PAL-TKLUC, F2-TKLUC and MAL-TKLUC, the TRE were derived from the following promoters of the T_3-inducible genes; the rat growth hormone (rGH) gene, the chicken lysozyme gene and

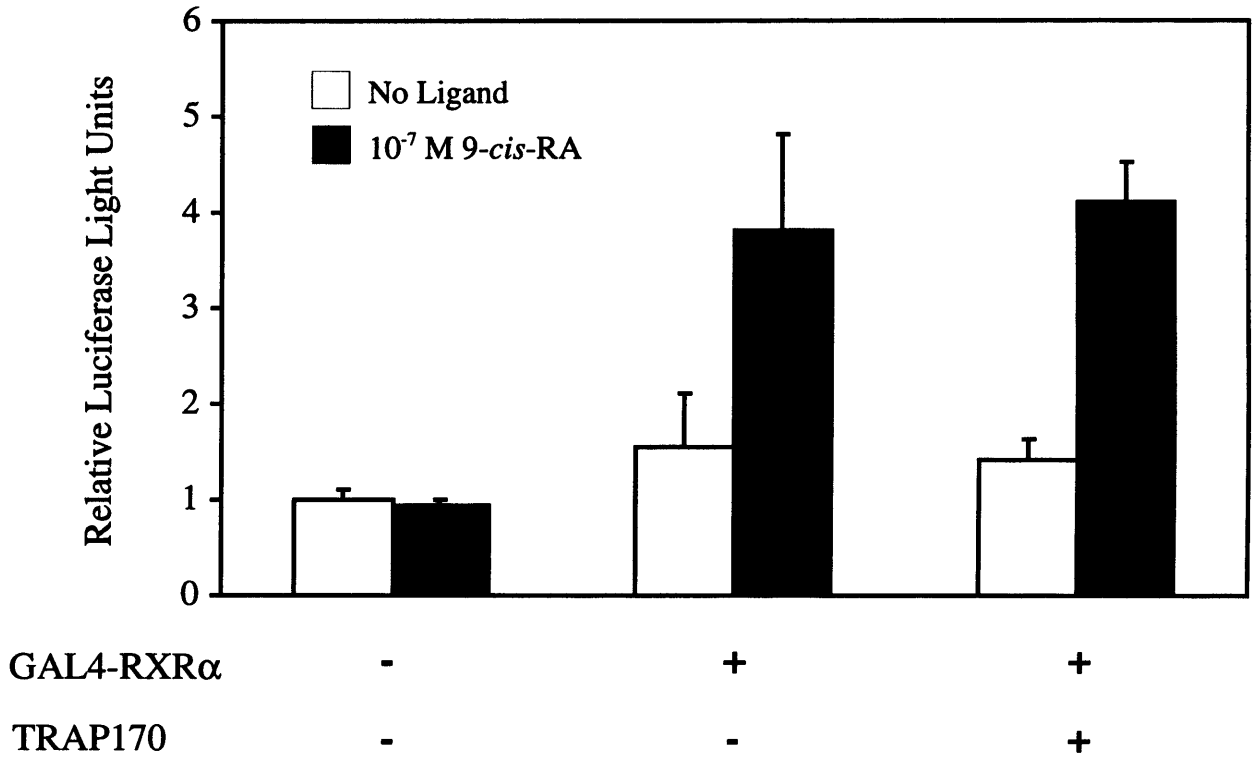


Figure 3.14 TRAP170 is unable to enhance the transcriptional activity of GAL4-RXRα (AF-2). HeLa cells were transiently transfected with 500 ng pJ7-LacZ and 600 ng pGAL4-EI6ΔLUC, together with 100 ng pGAL4-RXRα and 3 μg pFLAG(s)-7-TRAP170, as indicated. Luciferase activity was measured and normalised to the β-galactosidase transfection control. Basal level of transcriptional activity of the luciferase reporter was set at 1 and all other values are expressed relative to this. The values shown represent the average of triplicate samples and the error bars indicate standard deviation.

the malic enzyme gene, respectively. Given the natural derivation of each of the TREs tested, it could be presumed that TRβ activity would be observed from each of the TRE-linked luciferase reporters. However under the conditions used here this was not the case and the differences in configuration have been shown to impact on TRβ-mediated transcription of the luciferase gene. Results from this study show that TRβ activity from PAL-TKLUC and F2-TKLUC was negligible compared to the strong TRβ activity observed from the direct repeat (DR4) TRE (MAL-TKLUC) (Figure 3.2). In this case, the precise mechanism underlying the preferential usage of the variant TREs by TRβ is unclear but it is known that both structural orientation and the spacing between adjacent hexameric half-sites can influence nuclear receptor binding and transcriptional activity (Naar *et al.*, 1991, Umesono *et al.*, 1991, Forman *et al.*, 1992, Hallenbeck *et al.*, 1993 and Hall *et al.*, 2002a). Insertion of a single nucleotide into the half-site will result in a 3.4 angstrom extension and impose a 36° positive rotation of the double helix. Conversely, deletion of a single nucleotide would result in a 3.4 angstrom contraction of the half-site and a negative 36° rotation of the double helix. Clearly such alterations could have an effect on nuclear receptor binding. Indeed the "3-4-5" rule for nucleotide spacing between hexameric half-sites was composed by Umesono *et al.*, (1991), on discovering that the nucleotide spacing between a directly repeated AGGTCA half-site determined nuclear receptor selectivity, e.g. the direct repeat AGGTCA separated by 3 nucleotides (DR-3) is a vitamin D response element (VDRE); by 4 nucleotides (DR-4) is a TRE; and by 5 nucleotides is a retinoic acid receptor response element (RARE). Further, the orientation of the hexameric half-sites can influence nuclear receptor binding and transcriptional activity. Binding of the TRβ to the TRE can induce conformational changes within the nuclear receptor structure and binding to different TRE configurations could induce subtle different changes in conformation of the receptor. The dimerisation status of the receptor (i.e. homodimer or heterodimer with RXR) and additionally perhaps its coactivator binding preferences could be affected. Collingwood *et al.*, (1994) used gel mobility shift assays to demonstrate that homodimer formation (TR/TR) occurs most readily on everted repeats and palindromic configured response elements but were unable to demonstrate any significant homodimer formation on directly repeated response elements. Perhaps under the conditions used in this study, the conformation adopted by the MAL-TKLUC-bound TRβ induced heterodimerisation with RXR and this permitted binding to the available endogenous coactivator proteins and hence elicited a transcriptional response. Alternatively, the conformation adopted by the F2-TKLUC- and PAL-TKLUC-bound TRβ

may have induced homodimer formation which may not be the preferred dimerisation status to permit binding and enhancement of transcription by the available endogenous coactivator proteins. However in testing these three varying TRE-linked luciferase reporters, I have found a suitable reporter to demonstrate TRβ-mediated transactivation and hence investigate the effects of TRAP220 on TRβ-mediated transcriptional activity.

3.12.2 TRAP220 enhances the transcriptional activity of the class II NR, TRβ

Results from this study show that ectopically expressed TRAP220 is able to modestly enhance TRβ activity in a T_3-dependent manner (Figure 3.3). Use of alternative transient transfection methods, two different cell lines and co-expressing TRβ and RXRα all yielded similar results, suggesting that a modest level of enhancement in TRβ-mediated transactivation is the best that can be achieved in experiments of this nature. Similar modest levels of enhancement in TRα-mediated transcriptional activity by TRAP220 have also been observed previously (Yuan *et al.*, 1998). TRAP220 was able to enhance TRα activity from a TRE-linked luciferase reporter containing two copies of the DR4 TRE 2-fold, in a T_3-dependent manner. It is worth noting that the observed level of T_3-dependent TRAP220 induced enhancement in TR-mediated transactivation could have been increased, or indeed decreased, if a differently configured TRE had been utilised in these experiments. Recent reports have shown that the nature of the estrogen response element (ERE) influences the conformation of the ER coactivator binding pocket and hence the nature of the ERE is partly responsible for determining coactivator selectivity (Hall *et al.*, 2002b). Conformational changes induced by TR binding to differently configured TREs could therefore also influence coactivator binding. However in this study, TRβ was found to give optimal activity from the MAL-TKLUC reporter, containing a single copy of the DR4 TRE, as opposed to the everted and inverted TRE containing luciferase reporters (F2-TKLUC and PAL-TKLUC, respectively) and hence MAL-TKLUC was the most suitable candidate TRE-linked luciferase reporter to demonstrate TRAP220 function under these conditions. Additionally, TRAP220/PBP has been observed to only modestly enhance the transcriptional activity of two other class II nuclear receptors, PPARγ and VDR (Zhu *et al.*, 1997 and Ren *et al.*, 2000). Further, in a report showing that TRAP220/PBP has a modest effect on PPARγ-mediated transactivation, comparable to the levels observed by Zhu *et al.*, (1997), it was shown that phosphorylation of TRAP220/PBP greatly enhances its coactivator function (Misra *et al.*, 2002). By overexpressing RafBXB, an

activated upstream kinase of the MAPK signalling transduction cascade, they were able to induce TRAP220/PBP phosphorylation and this increased TRAP220/PBP coactivator function. Clearly therefore, the cellular environment is also a determinant in coactivator function. In summary, I suggest that this modest level of enhancement in class II nuclear receptor mediated transactivation is typical of TRAP220 in experiments of this nature.

A personal communication with Dr Robert Roeder (The Rockefeller University, New York), indicated that ectopic TRAP220 maybe inefficiently incorporated into the endogenous multisubunit TRAP complex. Further this research suggested that an undisclosed sequence of the TRAP220 protein had to be deleted before it could be effectively incorporated into the TRAP complex. Previous published evidence shows that while T_3 is necessary for the formation of the TR/TRAP complex, it was not required for the formation of the multisubunit TRAP complex and indeed TRAP exists as a preformed complex in the absence of ligand (Yuan *et al.*, 1998). Taken together I suggest that perhaps the ectopically expressed TRAP220 protein in this study and in the studies of other groups, may not be efficiently incorporated into the TRAP complex or incorporated in a manner that is not optimal to permit transactivation, and hence the full coactivator potential of TRAP220 may not be shown here. Despite this possible limitation, the data in this study and that of other groups (Yuan *et al.*, 1998), clearly demonstrates that ectopically expressed TRAP220 is having a modest positive effect on TRβ-mediated transcription and therefore can be deemed a genuine coactivator for TRβ.

3.12.3 TRAP220 is unable to enhance the transcriptional activity of the class I NR, ERα

The p160 coactivator SRC1e, exhibits promiscuous activity, acting as a coactivator for a number of nuclear hormone receptors, including PR, GR, ER, TR, RXR (Onate *et al.*, 1995), AR (Bevan *et al.*, 1999), PPARγ (Zhu *et al.*, 1996) and the hepatocyte nuclear factor 4 (HNF-4) (Wang *et al.*, 1998). Data from this study shows SRC1e is able to act as a coactivator for both TRβ (a class II NR) and ERα (a class I NR), reinforcing its role as a genuine coactivator for nuclear hormone receptors. However somewhat surprisingly, I show here that whilst TRAP220 was able to function as a coactivator for the class II nuclear hormone receptor TRβ (Figures 3.3, 3.4 and 3.5), it was unable to enhance the activity of ERα (Figures 3.6 and 3.7). Further, when TRAP220 and SRC1e were co-expressed, the level of enhancement in ERα-mediated transactivation observed was comparable to the level seen when SRC1e was expressed alone. This suggests that TRAP220 is having no apparent effect on ERα-mediated transactivation.

Again this could be due to inefficient incorporation into the TRAP complex. However this could also be the case for over-expression of TRAP220 in the TRβ-mediated transactivation system but a modest TRAP220-induced enhancement in TRβ-mediated transactivation was seen here and so I suggest this is not the reason for TRAP220s apparent lack of coactivator function for the ERα. Since coactivator proteins function via a direct interaction between their nuclear receptor interaction domains (NID) and the coactivator binding pocket of nuclear hormone receptors, the apparent inability of TRAP220 to enhance ERα/SRC1e activity, suggests TRAP220 does not interfere with SRC1e binding to ERα. If TRAP220 was able to bind to the ERα but for whatever reason could exert no coactivator function (Figure 3.15A), then co-expression with SRC1e could result in competition for binding to the ERα and hence attenuation in the level of SRC1e-induced enhancement of ERα-mediated transactivation (Figure 3.15E). However this was not the case here, leading me to hypothesize that perhaps TRAP220 is unable to bind the ERα (Figure 3.15B and 3.15D), or its binding to the ERα is very weak as compared to other coactivator proteins and hence it cannot enhance ERα-mediated transactivation. This hypothesis is supported by evidence in the literature showing TRAP220 is unable to bind to the ERα or does so very weakly (Rachez *et al.*, 1998, Yuan *et al.*, 1998, Kobayashi *et al.*, 2000 and Warnmark *et al.*, 2001). However there is also evidence in the literature opposing this hypothesis and detailing TRAP220 binding to the ERα (Chang *et al.*, 1999, Zhu *et al.*, 1999, Burakov *et al.*, 2000 and Burakov *et al.*, 2002). This conflicting evidence suggests there is still some ambiguity remaining concerning TRAP220/ERα interactions.

3.12.4 Subcellular localisation of TRAP220

Transient over-expression of proteins in HeLa and COS-1 cells was verified using both western blotting and indirect immunofluorescence. Whilst western blotting was used successfully to confirm the expression of transiently over-expressed GRIP1 and p300, it was unable to confirm expression of transiently over-expressed TRAP220, using either an anti-HA or an anti-TRAP220 antibody. Detailed analysis and optimisation of the transient transfection conditions, cell lysate preparation and western blotting protocols led to the conclusion that perhaps the binding of HA-TRAP220 protein to the nitrocellulose membrane induced a conformational change in which the HA-tag was unavailable for antibody recognition. This could explain why HA-TRAP220 could not be detected by western blotting. Alternatively,

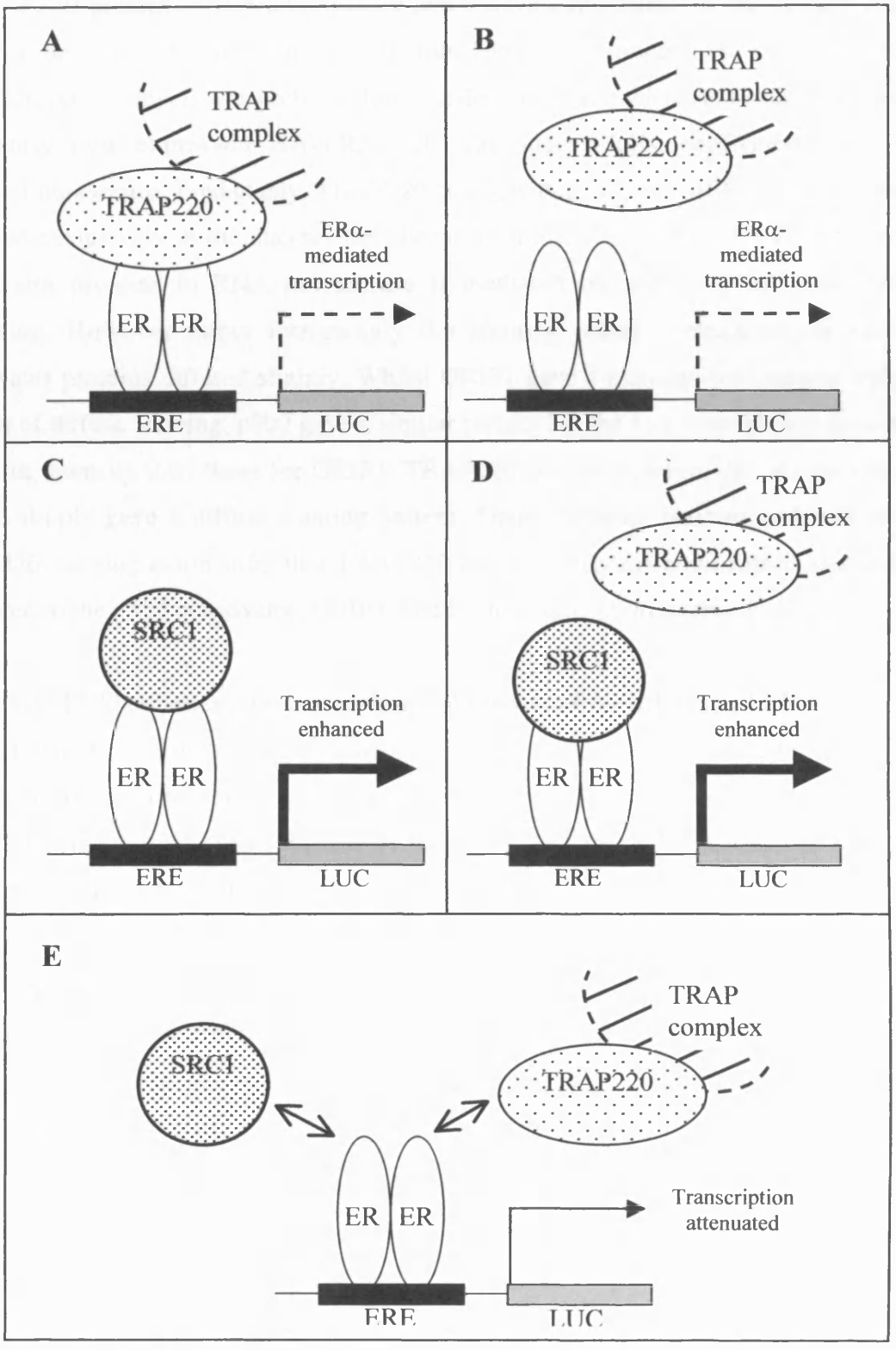


Figure 3.15 Simplified diagram depicting TRAP220 involvement in ERα activity. Other proteins involved in transcription have been omitted for clarity. (A) TRAP220 binds ERα homodimer but is unable to enhance transcription, (B) TRAP220 is unable to bind ERα homodimer, (C) SRC1e enhances transcription, (D) TRAP220 does not interfere with SRC1e enhancement in ERα-mediated transcription, (E) SRC1e activity is attenuated by TRAP220.

failure to detect HA-TRAP220 protein in cell extracts may have been due to a low abundance of TRAP220 protein, which additionally could have contributed to the modest coactivator activity observed for TRAP220 in transient transfection experiments. The primary cause of low TRAP220 protein levels was probably low transfection efficiencies. However the expression of transiently over-expressed HA-TRAP220 was successfully confirmed using indirect immunofluorescence. Predictably, TRAP220, along with p300 and GRIP1, were all found to be localised exclusively in the nucleus but absent from the nucleoli of cells. Given their role as coactivator proteins in RNA polymerase II mediated transcription this was perhaps not surprising. However rather intriguingly the staining pattern visualised for each of the coactivator proteins differed slightly. Whilst GRIP1 gave a punctate foci pattern with a small amount of diffuse staining, p300 gave a similar pattern but the foci were greater in number and fainter in intensity than those for GRIP1. TRAP220 protein however did not stain as foci but instead simply gave a diffuse staining pattern. These differing patterns and lack of foci for TRAP220 staining could infer that TRAP220 has a slightly different role in transcription as compared to the p160 coactivator, GRIP1, and the histone acetyltransferase, p300.

3.12.5 TRAP170 enhances the transcriptional activity of both TRβ and ERα

In addition to investigating the effects of TRAP220 on NR-mediated transcription, this study also assessed the effects of the 170 kDa subunit of the TRAP complex, TRAP170. TRAP170, also termed DRIP150, CRSP150 and hRGR1 (hereafter referred to as TRAP170) was observed to enhance both ERα and TRβ-mediated transcriptional activity. The level of enhancement in TRβ-mediated transcription was comparable to that observed for TRAP220 activity, however TRAP170 was also observed to modestly enhance ERα activity. This was surprising given the previous evidence that TRAP220 cannot enhance ERα activity under the same conditions but suggests that indeed the TRAP complex may be involved in ERα activity and perhaps TRAP170 serves as the TRAP complex anchor to the ERα rather than the TRAP220 subunit. Additionally studies utilising the glucocorticoid receptor (GR), a class I NR, showed that TRAP170 was able to enhance GR-mediated transcriptional activity and bind to the AF-1 of the GR in a ligand-independent manner. However TRAP170 was shown to be unable to bind to the AF-2 of GR, either in the absence or presence of ligand (Hittelman *et al.*, 1999). Evidence of this nature suggests that TRAP170 is functioning as an AF-1 dependent coactivator. In support of this theory, TRAP170 was unable to enhance the transcriptional

activity of Gal4-RXRα (AF-2), presumably due to the lack of the RXRα AF-1 domain. However, neither full-length RXRα nor RXRα-AF-1 were tested in these experiments. Hence it can only be speculated that TRAP170 functions via the AF-1 of RXRα. A recent report has shown that SRC1 is able to interact with the AF-1 of the AR (Bevan *et al.,* 1999) via a polyglutamine (Q-rich) domain. Sequence analysis of TRAP170 however has not revealed such a polyglutamine domain and suggests a unique mode of interaction for the TRAP170 subunit with the TRβ and ERα.

NRs contain two activation domains, namely AF-1 and AF-2, located in the N-terminus and C-terminus, respectively. AF-1 is a weak constitutive activation function that is silent in the full-length NR until it is relieved from its repression by ligand binding to the LBD. AF-2 constitutes a stronger ligand-dependent activation function. Studies using mutant NRs, in which either the A/B region (AF-1 containing) or the LBD (AF-2 containing) were deleted, have shown that the A/B region (AF-1) can only weakly stimulate transcription, at 1-5% of the efficiency of the wild type NR (Kumar *et al.,* 1987), but the LBD (AF-2) strongly stimulates transcription to levels of 60-80% of the wild type NR (Tora *et al.,* 1989). Additionally, the AF-1 and AF-2 containing domains, expressed as separate polypeptides, have been shown to functionally interact in a ligand-dependent manner to reconstitute the full transcriptional activity of the NR (Kraus *et al.,* 1995). This suggests that the activities of the AF-1 and AF-2 synergise to facilitate the optimal transcriptional activity of the NR. It is possible that cellular proteins, distinct from the basal transcription factors, may play a role in mediating the AF-1 /AF-2 synergism. Evidence that the AFs of NRs can have a squelching effect (transcriptional interference) on the activity of other NRs (Tasset *et al.,* 1990 and Barettino *et al.,* 1994) suggested the existence of a common mediator or group of mediator molecules. Subsequent studies revealed the identity of these mediator molecules as coactivator proteins. For example, the interaction of the N- (AF-1) and C-terminal (AF-2) regions of the ER, when expressed as separate polypeptides, has been shown to be facilitated by the coactivator SRC1 (McInerney *et al.,* 1996). Further studies have shown that cooperativity between the AF-1 and AF-2 of several NRs, including the PPARγ (Gelman *et al.,* 1999), ERα (Benecke *et al.,* 2000 and Kobayashi *et al.,* 2000), ERβ (Tremblay *et al.,* 1999), AR (He *et al.,* 1999) and the RARα1 (Bommer *et al.,* 2002) are mediated through coactivators interacting simultaneously with the two AFs. Results from this study and that of Hittleman and co-workers (1999), suggest that the TRAP complex is able to functionally link the AF-1 and AF-2 domains of the TRβ, ERα and GR, via interaction

of the TRAP170 subunit with the AF-1 and interaction of the TRAP220 subunit with the AF-2. The inability of TRAP170 to potentiate the transcriptional activity of the TRβ and ERα in the absence of ligand, despite being predicted to function via the constitute AF-1, is in agreement with the model whereby AF-1 activity is silent in the full-length NR until it is released from repression by the LBD due to ligand binding.

3.12.6 Summary

In summary, TRAP220 has been demonstrated to be a genuine coactivator for the TRβ but shown not to exhibit any coactivator function with regard to the ERα. The precise reason for this receptor selectivity exhibited here by TRAP220 is unclear but could be due to selective NR binding by TRAP220. The nature of TRAP220 selective interactions with different NRs will be addressed in chapters 4 and 5 of this study. Additionally TRAP170 has been shown here to act as a coactivator for both ERα and TRβ. However whilst TRAP220 exerts its coactivator functions via interaction with the C-terminal AF-2 containing domain of the NR, TRAP170 is proposed to exert its coactivator function via interaction with the N-terminal AF-1 containing domain of NRs. This presents a clearer picture of how the TRAP complex can associate with and activate the transcriptional activity of numerous NRs.

CHAPTER 4
RESULTS

The Nuclear Receptor binding properties of TRAP220

4.1 Introduction

The TRAP220 subunit of the TRAP/SMCC/Mediator complex has been shown to interact, in a ligand-dependent manner, with RXRα and several class II NRs including, TRα/β, PPARα/γ, RARα and VDR (Zhu *et al.,* 1997, Yuan *et al.,* 1998 and Treuter *et al.,* 1999). Moreover, TRAP220 has been shown to enhance TRα-, VDR- and PPARγ-mediated transcription when transiently over-expressed in mammalian cells (Yuan *et al.,* 1998, Rachez *et al.,* 2000 and Zhu *et al.,* 1997). Consistent with this, results shown previously in this study (Chapter 3; Figures 3.3, 3.4 and 3.5) demonstrate the ability of transiently over-expressed TRAP220 to modestly enhance TRβ-mediated transcriptional activity. Further, TRAP220 haploinsufficient mice (*Trap220*$^{-/+}$) exhibit mutant phenotypes associated with defects in thyroid hormone signalling (e.g. hypothyroidism) (Ito *et al.,* 2000). Similarly, PPARγ-mediated transcription is attenuated in *Trap220*$^{-/-}$ and *Trap220*$^{-/+}$ MEFs (Zhu *et al.,* 2000). Thus, the combined data from these biochemical and genetic studies implicate the TRAP complex, in a manner that requires TRAP220, in the signalling pathways of the class II NRs TR, VDR and PPAR.

Genetic studies, utilising TRAP220 knockout mice, have failed to identify any mutant phenotypes associated with defects in signalling pathways of the class I NRs such as ER. Further, there is a degree of ambiguity in the literature regarding TRAP220 ligand-dependent interactions with the ERα. Some reports detail strong TRAP220-ERα interactions (Chang *et al.,* 1999, Zhu *et al.,* 1999, Burakov *et al.,* 2000 and Burakov *et al.,* 2002), whereas others report no or very weak TRAP220-ERα interactions (Yuan *et al.,* 1998, Kobayashi *et al.,* 2000 and Warnmark *et al.,* 2001). Thus, if the TRAP/SMCC/Mediator complex is involved in the coactivation of class I NRs, it may do so via a subunit distinct from TRAP220. Consistent with this possibility, transiently over-expressed TRAP220 was shown to have no effect on ERα-mediated transactivation (Chapter 3; Figures 3.6 and 3.7). Moreover, under the same conditions, SRC1 was able to strongly enhance ERα-mediated transactivation (Chapter 3; Figures 3.6 and 3.7) and this enhancement was unaffected by the presence of ectopic TRAP220 (Chapter 3; Figure 3.7). It was thus proposed that the inability of TRAP220 to have an effect on

ERα-mediated transactivation may be due to an inability to bind to the ERα or perhaps it exhibits weak binding that is insufficient to support transcriptional activation.

Therefore in this section of the study I investigated the NR-binding properties of TRAP220 and established whether it displays a NR-binding selectivity that could account for its observed class II specific coactivator functions.

4.2 Interaction of the TRAP220 NID with NRs

4.2.1 Construction of LexA-TRAP220 NID expression vectors

Two different LexA-TRAP220 NID expression vectors were generated for this study (Figure 4.1A). They differed in the length of the TRAP220 sequence which was taken to represent TRAP220s NID. TRAP220 amino acids 503-667 were used to generate a short form of the NID and amino acids 335-667 were used to generate a long form of the NID. These two over-lapping sequences were designed to include the two closely spaced LXXLL motifs (LXM1 and LXM2) of TRAP220. Use of the protein secondary structure prediction computer program ('PredictProtein') (Rost and Sander, 1993) ensured that the boundaries of the (503-667) and (335-667) fragments did not reside in the middle of a potentially important section of secondary structure e.g. α-helix. Double-stranded DNA fragments encoding NID (503-667) and NID (335-667) were generated using PCR and included a 5' *Ksp* I restriction site and a 3' *Bam*H I restriction site. Purified PCR fragments were ligated into the LexA-DBD expression vector, BTM116mod, which had been previously prepared by *Ksp* I/*Bam*H I digestion followed by CIAP treatment. BTM116mod-TRAP220(335-667) and BTM116mod-TRAP220(503-667) were verified by sequencing.

4.2.2 Characterisation of the LexA-TRAP220 NID fusion proteins

Before the LexA-TRAP220 NID fusion proteins could be used to assess TRAP220 interactions with NRs, they first had to be tested for their suitability for use in the yeast two-hybrid experiments used in this study. If, when tethered to DNA, these TRAP220 NID proteins were capable of activating transcription of the β-galactosidase reporter gene in the absence of an ectopic VP16-fusion protein, then they would be unsuitable for use in these experiments. This was because any further activation of transcription of the β-galactosidase reporter gene due to interaction between the LexA-fusion protein and the VP16-NR-LBD fusion protein, could be masked by the transcriptional activity of the LexA-fusion protein alone. However, if in

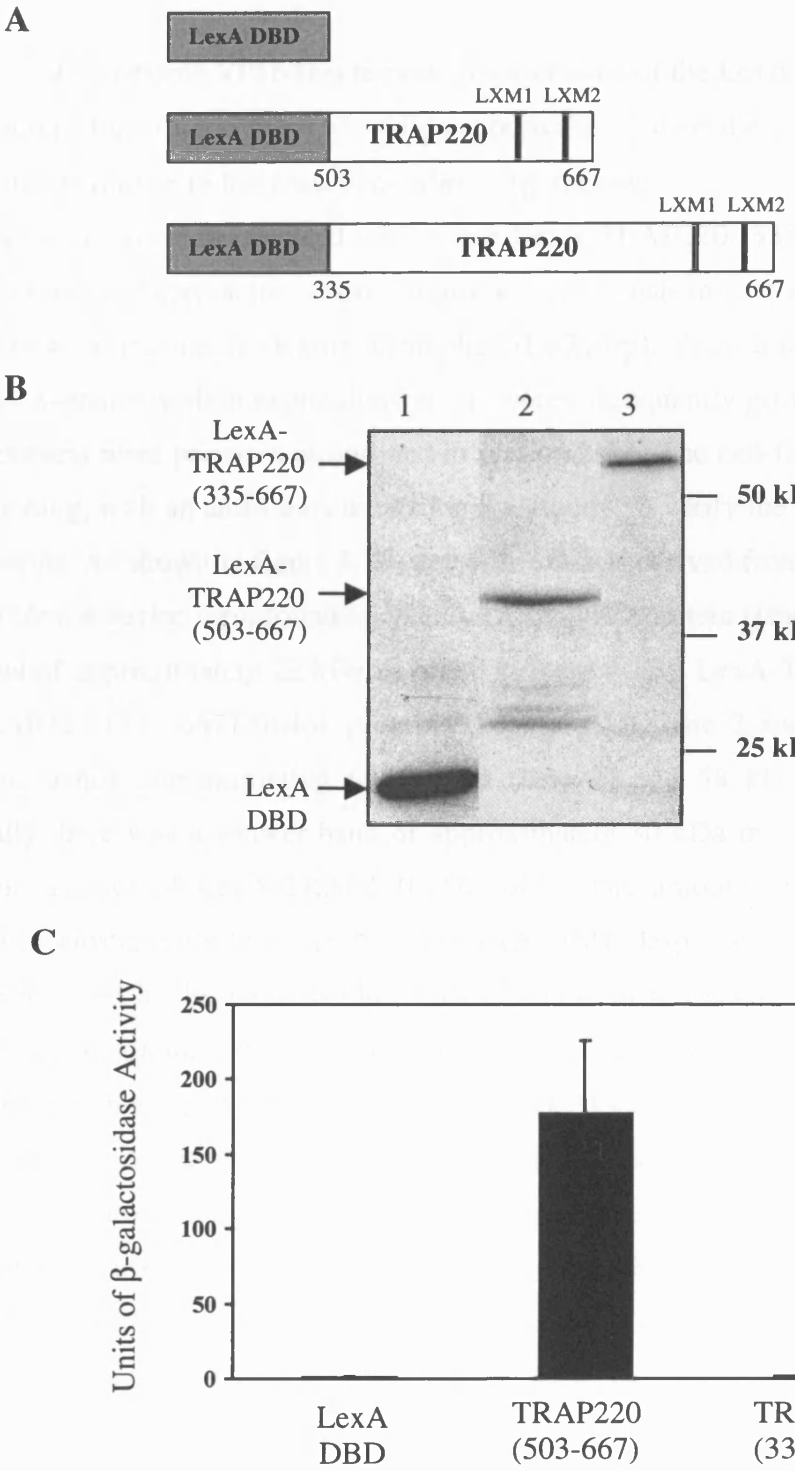


Figure 4.1 Assessing the suitability of the LexA-TRAP220 NID constructs for yeast two-hybrid assays. (A) Schematic representation of LexA-TRAP220 NID fusion proteins. Bars represent LXM1 and LXM2, as indicated. (B) Western blot showing the expression of LexA-DBD (lane 1), LexA-TRAP220 (503-667) (lane 2) and LexA-TRAP220 (335-667) (lane 3), in L40 yeast, (C) yeast two-hybrid β-galactosidase assay showing the activity of the LexA-DBD fusion proteins in the absence of an ectopic activation domain.

the absence of an ectopic VP16-fusion protein, expression of the LexA-fusion protein did not induce transcription of the β-galactosidase reporter gene then the LexA-fusion protein was deemed suitable for use in the yeast two-hybrid experiments.

L40 yeast were transformed with either LexA-TRAP220 (335-667), LexA-TRAP220 (503-667) or LexA expression vectors (Figure 4.1A). Transformants were selected by growth on complete selective media lacking tryptophan (CSM-trp). Yeast transformants, carrying the desired LexA-fusion protein expression vector, were subsequently grown in CSM-trp and then cell-free extracts were prepared as outlined in section 2.5.6. The cell-free extracts were used in western blotting, with an anti-LexA monoclonal antibody, to verify the expression of the LexA-fusion proteins. As shown in figure 4.1B, cell-free extracts derived from yeast transformed with the BTM116mod vector were found to contain LexA-DBD protein (lane 1), as evident from the strong band of approximately 22 kDa in lane 1 (Figure 4.1B). LexA-TRAP220 (503-667) and LexA-TRAP220 (335-667) fusion proteins (Figure 4.1B, lane 2 and 3, respectively) were detected as bands approximating to 40 kDa (lane 2) and 58 kDa (lane3), respectively. Additionally there was a weaker band of approximately 30 kDa in lane 2, which could be a breakdown product of LexA-TRAP220 (503-667). The amount of LexA fusion proteins detected was substantially less than that for LexA-DBD, despite equal quantities of total cell protein being used in the western blot. This observation has been noted previously (Dr D. Heery, personal communication) and suggests the yeast can more readily synthesize the smaller LexA-DBD protein as opposed to the larger LexA-TRAP220 NID fusion proteins.

Yeast cell-free extracts were tested for β-galactosidase activity to determine if any of the LexA-fusion proteins were capable of activating transcription in the absence of an ectopic protein bearing a transcriptional activation domain. As shown in figure 4.1C, expression of either the LexA-DBD protein or the LexA-TRAP220 (335-667) fusion protein did not result in transcription of the β-galactosidase reporter gene. However expression of the LexA-TRAP220 (503-667) fusion protein resulted in a high level of reporter activity (177 units of β-galactosidase activity). This was unexpected given that TRAP220 (503-667) is simply a shorter form of TRAP220 (335-667), which did not activate reporter activity when expressed alone. Further, this was not due to increased expression of this protein relative to the others, as evidenced from the western blot data in figure 4.1B.

Proteins possessing intrinsic transcription activation domains (AD), are often rich in acidic, proline or glutamine residues and are observed to activate transcription when tethered to DNA. In this instance, amino acids 503-667 of TRAP220 have been fused to the DBD of LexA and hence can bind to the LexA binding sites in the promoter region of the yeast β-galactosidase reporter gene. LexA-TRAP220 (503-667) was seen here to activate β-galactosidase reporter activity which suggests it may possess an AD. However it is more likely that in fusing TRAP220 (503-667) to the LexA DBD, an AD has been created fortuitously. Perhaps the TRAP220 (503-667) protein folds in such a way as to expose or create a surface to which yeast proteins possessing activation domains or proteins of the basal transcriptional machinery can bind and hence transcriptional activity was observed. The extended TRAP220 NID (335-667) possibly does not expose or create such a surface and hence transcription is not activated. Alternatively, the TRAP220 NID (335-667) could possess an AD and a repression domain, that is not present in the (503-667) NID. Therefore using amino acids 503-667 of TRAP220 to represent its NID would not be suitable for the yeast two-hybrid experiments employed in this study and so the longer form of TRAP220 NID (335-667) has been used to represent the TRAP220 NID in all subsequent experiments. TRAP220 (335-667) will be referred to as TRAP220 NID hereafter.

4.2.3 The TRAP220 NID interacts differentially with TRβ and ERα

With a suitable LexA-TRAP220 NID fusion protein selected it was possible to assess the interaction of the TRAP220 NID with NRs using the yeast two-hybrid system described in section 1.10. Yeast expressing the LexA-TRAP220 NID fusion protein were additionally transformed with either an expression vector for VP16-ERα-LBD or VP16-TRβ. Double-transformants, expressing both LexA-TRAP220 NID and a VP16-NR fusion protein, were selected and propagated in CSM lacking tryptophan and leucine and cell-free extracts were prepared as outlined in section 2.5.6. In a control experiment, individual yeast transformants co-expressing LexA-DBD protein (minus the TRAP220 NID fusion) and each of the aforementioned VP16-NR fusion proteins, showed no transcriptional activity from the β-galactosidase reporter gene (data not shown). This confirmed that any transcriptional activity observed in subsequent experiments incorporating the LexA-TRAP220 NID fusion protein must be as a result of interaction between the TRAP220 NID and the NR in question.

Expression of the VP16-NR fusion proteins was verified by western blotting using an anti-VP16 monoclonal antibody. Figure 4.2A shows an anti-VP16 western blot of cell-free extracts derived from L40 yeast co-transformed with LexA-TRAP220 NID and VP16-TRβ (lane 1) or LexA-TRAP220 NID and VP16-ERα-LBD (lane 2). VP16-TRβ is evident as a strong band of approximately 59 kDa (Figure 4.2A, lane 1) and VP16-ERα-LBD is represented by an equally strong band approximating to 43 kDa (lane 2).

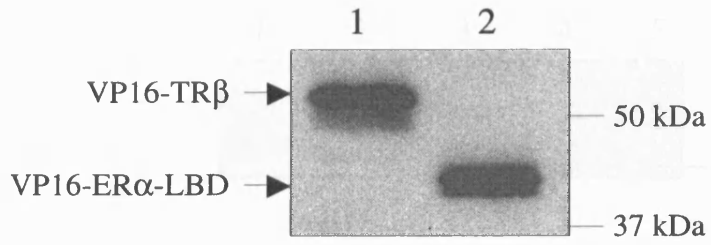
As shown in figure 4.2B, co-expression of VP16-TRβ and LexA-TRAP220 NID resulted in a strong ligand-dependent activation of the β-galactosidase reporter (109 units of β-galactosidase activity) and negligible activity in the absence of ligand. However co-expression of VP16-ERα-LBD and LexA-TRAP220 NID resulted in no ligand-independent activity and a relatively low ligand-dependent activation of the β-galactosidase reporter (16 units of β-galactosidase activity) as compared to VP16-TRβ and LexA-TRAP220 NID co-expression. This 7-fold lower reporter activation was obtained despite ectopic proteins being expressed at comparable levels and suggests a difference in interaction between the ERα and the TRβ with the NID of TRAP220. This data suggests that whilst TRAP220 is able to bind the ERα in a ligand-dependent manner, the interaction is weak as compared to its interaction with the TRβ. Further this data is in support of the hypothesis proposed in chapter 3 of this study, which postulated that the apparent lack of coactivator function exhibited by TRAP220 for ERα could be due to very weak or no interaction between TRAP220 and the ERα.

4.2.4 TRAP220 exhibits class-specific NR binding

The yeast two-hybrid data shown in figure 4.2B demonstrates that binding of the TRAP220 NID to the TRβ induces stronger transcriptional activity from the β-galactosidase reporter gene than when the TRAP220 NID binds to the ERα. This indicates that the interaction between TRAP220 and the TRβ may be stronger than the interaction between TRAP220 and the ERα. Significantly, TRβ is a class II NR and ERα is a class I NR, suggesting that perhaps TRAP220 is exhibiting NR-class binding specificity. To test this supposition, the interaction of the TRAP220 NID with a panel of NRs, including those from both class I and class II, were tested using the yeast two-hybrid assay system.

Yeast were sequentially transformed first with the LexA-TRAP220 NID expression vector and then with an expression vector for either VP16-AR-LBD, VP16-PR-LBD, VP16-RARα-LBD, VP16-RXRα-LBD or VP16-PPARγ-LBD. As shown in figure 4.3A, expression

A



B

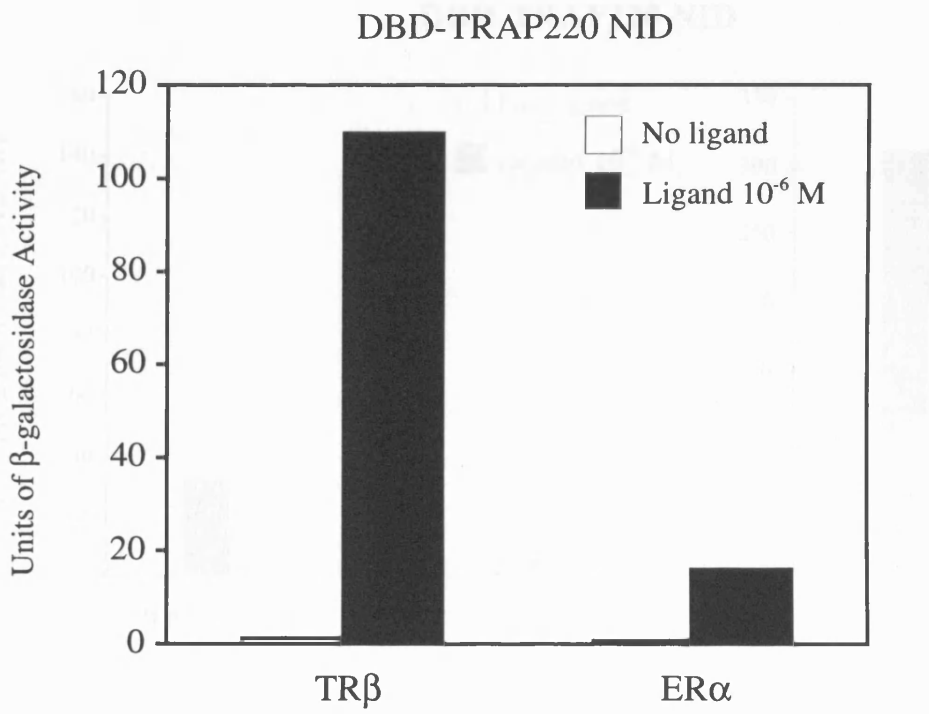


Figure 4.2 Assessing the interaction of TRAP220 NID with TRβ and ERα. (A) Western blot analysis, using an anti-VP16 antibody, shows the expression of VP16-TRβ (lane 1) and VP16-ERα-LBD (lane 2) in transformed L40 yeast cells, (B) Yeast two-hybrid β-galactosidase assay using cell-free extracts derived from yeast co-expressing LexA-TRAP220 NID and either VP16-TRβ or VP16-ERα-LBD. Reporter activity of cell-free extracts is expressed as units of β-galactosidase activity. The cognate ligands for the TRβ and ERα were T_3 and E_2, respectively.

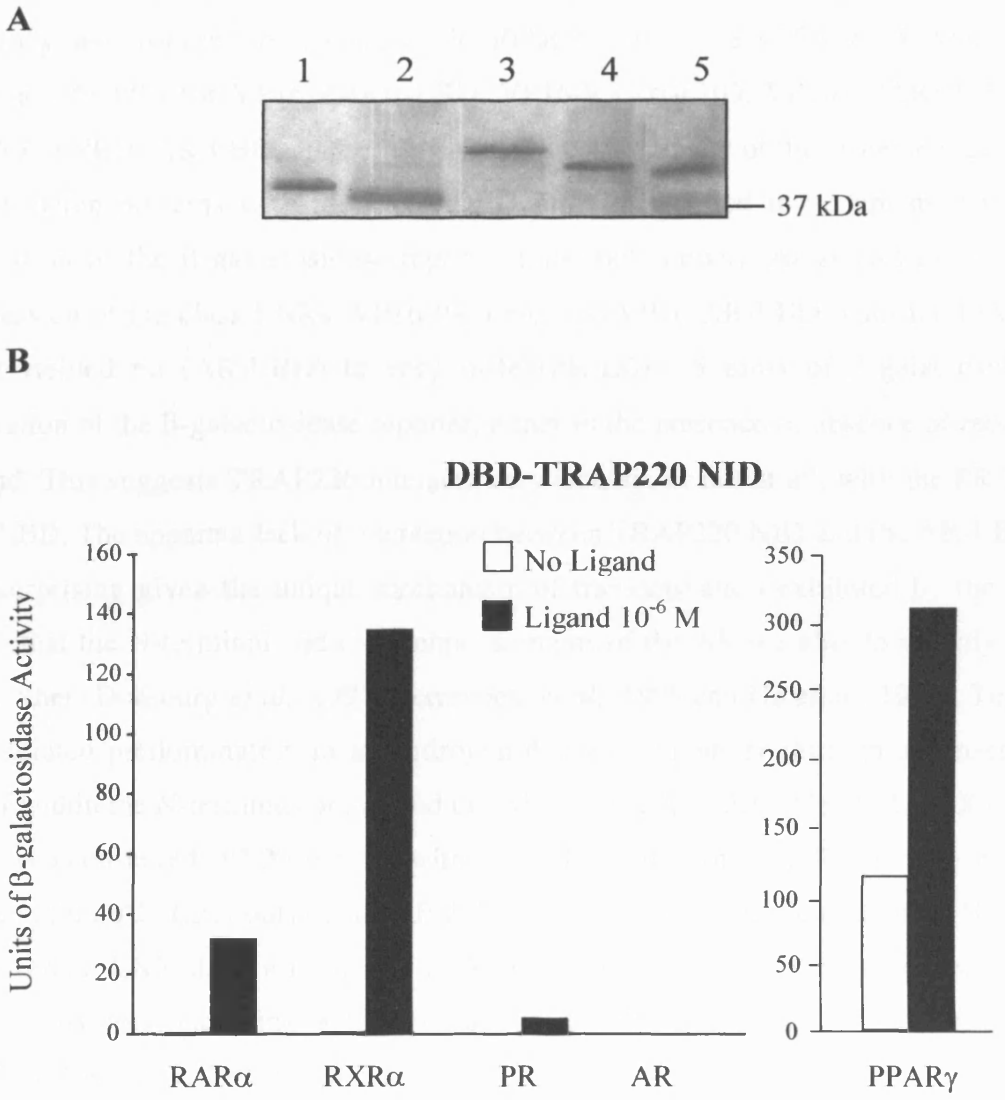


Figure 4.3 (A) Western blot analysis, using an anti-VP16 antibody, to detect VP16-NR fusion proteins in cell-free extracts derived from yeast co-transformed with the LexA-TRAP220 NID expression vector and either an expression vector for VP16-RARα-LBD (lane 1), VP16-RXRα-LBD (lane 2), VP16-PPARγ-LBD (lane 3), VP16-PR-LBD (lane 4) or VP16-AR-LBD (lane 5). (B) Yeast two-hybrid β-galactosidase assay showing TRAP220 NID interactions with the LBDs of RARα, RXRα, PR, AR and PPARγ. The receptor cognate ligands were AT-RA, 9c-RA, R5020, mibolerone and rosiglitazone for RARα, RXRα, PR, AR and PPARγ, respectively.

of the VP16-NR-LBD fusion proteins was verified by western blotting using an anti-VP16 antibody and observed to be comparable. Relative sizes were 37.5 kDa, 35 kDa, 42 kDa, 41.5 kDa and 41 kDa for VP16-RARα-LBD, VP16-RXRα-LBD, VP16-PPARγ-LBD, VP16-PR-LBD and VP16-AR-LBD, respectively. Co-expression of any of the aforementioned VP16-NR-LBD fusion proteins with the LexA-DBD protein, resulted in no apparent transcriptional activation of the β-galactosidase reporter (data not shown). As shown in figure 4.3B, co-expression of the class I NRs, VP16-PR-LBD and VP16-AR-LBD, with the LexA-TRAP220 NID yielded no (AR-LBD) to very little (PR-LBD; 5 units of β-galactosidase activity) activation of the β-galactosidase reporter, either in the presence or absence of receptor cognate ligand. This suggests TRAP220 interacts very weakly, or not at all, with the PR-LBD and the AR-LBD. The apparent lack of interaction between TRAP220 NID and the AR-LBD is perhaps not surprising given the unique mechanism of transactivation exhibited by the AR. Reports show that the N-terminal and C-terminal domains of the AR are able to directly interact with each other (Doesburg *et al.*, 1997, Berrevoets *et al.*, 1998 and He *et al.*, 1999). This interaction is mediated predominately in an androgen-dependent manner through a conserved FXXLF motif within the N-terminus of AR and the AF-2 of the AR-LBD (He *et al.*, 2000). Additionally there is a conserved WXXLF motif within the N-terminus of the AR which is able to bind to a region of the AR-LBD outside the AF-2, in an androgen-independent manner (He *et al.*, 2000). This variant LXXLL motif, namely FXXLF, has subsequently been identified within known AR coactivators, including ARA54 (Kang *et al.*, 1999), ARA55 (Fujimoto *et al.*, 1999) and ARA70 (Yeh *et al.*, 1996), and has been shown to mediate the interaction of these coactivators with the AR AF-2 in an androgen-dependent manner (He *et al.*, 2002). Further the AR AF-2 has been observed to preferentially interact with FXXFL-containing coregulators as opposed to the SRC1 family of LXXLL-containing coregulators (He *et al.*, 1999). Despite this, LXXLL-containing coregulators such as SRC1, TIF2 and RIP140 have been observed to bind the AF-2 of the AR in a ligand-dependent manner (Bevan *et al.*, 1999 and Heery *et al.*, 2001).

Figure 4.3B also shows that co-expression of VP16-RARα-LBD and LexA-TRAP220 NID resulted in a modest ligand-dependent activation of the β-galactosidase reporter (32 units of β-galactosidase activity). Additionally co-expression of VP16-RXRα-LBD and LexA-TRAP220 NID resulted in a strong ligand-dependent activation of the β-galactosidase reporter (134 units of β-galactosidase activity). However co-expression of LexA-TRAP220 NID and

VP16-PPARγ-LBD yielded the greatest activation of the yeast reporter gene, giving 121 units of β-galactosidase activity in the absence of ligand and 302 units of β-galactosidase activity in the presence of ligand. This ligand-independent activity observed here for the PPARγ-LBD, was not seen for any of the other NRs tested and could be attributed to the nature of the cognate ligands for PPARγ. It has been reported that PPARγ can be transcriptionally activated by a variety of xenobiotic and natural compounds, including fatty acids, eicosanoids and synthetic antidiabetic thiazolidinedione drugs (Lambe *et al.*, 1996 and Krey *et al.*, 1997). Hence it is highly likely that the natural ligands of PPARγ will be present within the yeast cells prior to the addition of the synthetic PPARγ ligand rosiglitazone. This could result in the apparent ligand-independent interaction observed between the TRAP220 NID and PPARγ. Addition of rosiglitazone resulted in an enhancement in transcriptional activity from the β-galactosidase reporter as compared to activity in its absence, suggesting that the natural ligands of PPARγ were not at saturating levels prior to the addition of rosiglitazone and rosigltazone has indeed induced a genuine ligand-dependent interaction between PPARγ and the TRAP220 NID.

This data suggests that the NID of TRAP220 is exhibiting a NR class specificity. It appears to preferentially interact with the class II NRs, showing a strong interaction with TRβ, RXRα and PPARγ and a modest interaction with RARα. TRAP220 NID showed a very weak interaction with the class I NRs, PR and ER, and was observed to exhibit no apparent interaction with the AR.

4.2.5 Differential usage of TRAP220 LXM1 and LXM2

Thus far, the data in this study has demonstrated that the NID of TRAP220 was able to bind, to varying degrees, to a range of NRs. It was not clear if these interactions were facilitated through both LXMs of TRAP220 or whether each receptor had a preference for one of the motifs. Reports suggest that NRs do have a preference for LXMs in multiple LXM containing coactivators. For example, Darimont *et al.*, (1998) show TRβ has a preference for LXM2 of GRIP1, whereas the GR prefers LXM3 of GRIP1. Similarily, Leers *et al.*, (1998), demonstrate that a mutation in LXM2 of TIF2 is most deleterious to interactions with PPARγ while mutations in LXM1 of TIF2 has the greatest effect in TIF2/RXR binding. Further Kalkhoven *et al.*, (1998), demonstrate that ER preferentially utilises LXM2 of SRC1 and mutation of this LXM significantly reduces *in vitro* binding of SRC1 to ER and *in vivo* function of SRC1 in ER-mediated transactivation assays.

In order to investigate the contribution of each of the LXMs of the TRAP220 NID for binding to different NRs, two mutant LexA-TRAP220 NID fusion proteins were generated. As shown in figure 4.4A, either LXM1 or LXM2 (but not both) of the TRAP220 NID were mutated by replacing the conserved leucine residues at positions +4 and +5 with alanine residues to give TRAP220 NID mut1 and TRAP220 NID mut2, respectively. These TRAP220 mutant NID constructs were verified by sequencing and then used to transform L40 yeast. The expression of the correctly sized LexA-TRAP220 NID mut1 and mut2 fusion proteins was verified by western blotting using an anti-LexA antibody (Figure 4.4B). As shown in figure 4.4B, the LexA-TRAP220 NID mutant proteins were expressed at comparable levels and each was represented by a band approximating to 58 kDa, as calculated. Additionally, the LexA-TRAP220 NID mutants were tested for any intrinsic transcriptional activity, which would render them unsuitable for use in the yeast two-hybrid assays. Cell-free extracts were prepared from yeast expressing either LexA-lamin, LexA-VP16(AAD), LexA-TRAP220 NID mut1 or LexA-TRAP220 NID mut2 only (i.e. minus a VP16-NR fusion protein), and tested for β-galactosidase activity. LexA-lamin and LexA-VP16 were employed as a negative and positive control, respectively, in these experiments. LexA-VP16 consists of the DBD of LexA fused to the acidic activation domain (AAD) of VP16. Hence the VP16(AAD) can be recruited to the promoter region of the β-galactosidase reporter gene by way of its fusion with the LexA-DBD which will bind to the LexA binding sites within the promoter of the β-galactosidase reporter gene. In such close proximity to the promoter region, the VP16(AAD) should be able to activate transcription from the β-galactosidase reporter gene. LexA-lamin consists of the DBD of LexA fused to amino acids 66-230 of the human lamin C protein and has been shown previously not to exhibit intrinsic transcription activating capabilities in yeast when expressed either alone or together with VP16-AAD (Shih *et al.*, 1996).

As shown in figure 4.4C, expression of the LexA-VP16 fusion protein greatly promoted transcriptional activity from the β-galactosidase reporter (78 units of β-galactosidase activity), whereas LexA-lamin was, as expected, unable to promote any transcriptional activity. Comparable to the LexA-lamin negative control, expression of either LexA-TRAP220 NID mut1 or LexA-TRAP220 NID mut2 resulted in negligible transcriptional activity from the β-galactosidase reporter. Therefore as for the wildtype LexA-TRAP220 NID, the two TRAP220 NID mutant proteins (mut1 and mut2) were suitable for use in the yeast two-hybrid experiments

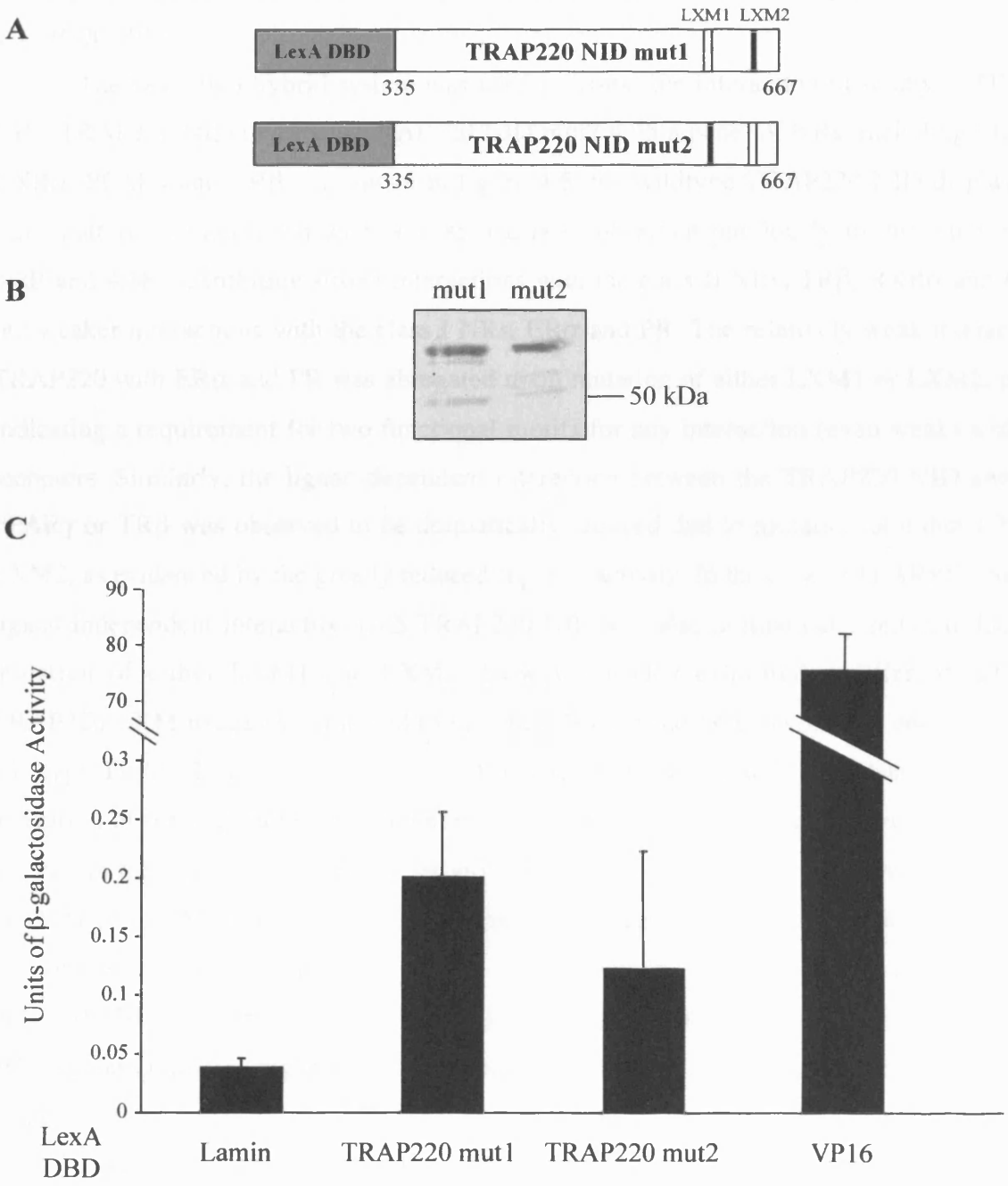


Figure 4.4 (A) Schematic representation of TRAP220 NID mutants 1 and 2. Black bars represent wt LXXLL motif, open bars represent mutant LXXAA motif. (B) Western blot, using a anti-LexA antibody, showing the expression of LexA-TRAP220 NID mut1 and mut2 in yeast cell-free extracts, (C) Yeast two-hybrid β-galactosidase assay showing the activity of LexA-Lamin, LexA-TRAP220 NID mut1, LexA-TRAP220 NID mut2 and LexA-VP16(AAD) in the absence of an ectopic activation domain.

and these amino acid substitutions do not affect the weak intrinsic activity of the LexA-TRAP220 NID.

The yeast two-hybrid system was used to assess the interaction of wildtype TRAP220 NID, TRAP220 NID mut1 and TRAP220 NID mut2 with a panel of NRs, including PR, ERα, RXRα, PPARγ and TRβ. As shown in figure 4.5, the wildtype TRAP220 NID displayed the same pattern of interaction with NRs as had been observed previously in this study (Figure 4.2B and 4.3B), exhibiting strong interactions with the class II NRs, TRβ, RXRα and PPARγ but weaker interactions with the class I NRs, ERα and PR. The relatively weak interaction of TRAP220 with ERα and PR was abrogated upon mutation of either LXM1 or LXM2, perhaps indicating a requirement for two functional motifs for any interaction (even weak) with these receptors. Similarly, the ligand-dependent interaction between the TRAP220 NID and either PPARγ or TRβ was observed to be dramatically reduced due to mutation of either LXM1 or LXM2, as evidenced by the greatly reduced reporter activity. In the case of PPARγ the apparent ligand-independent interaction with TRAP220 NID was also dramatically reduced due to the mutation of either LXM1 and LXM2. However RXRα exhibited a different pattern of TRAP220 LXM usage as compared to the other NRs tested here. Typically, co-expression of wildtype TRAP220 NID and RXRα-LBD resulted in strong ligand-dependent transcriptional activation of the β-galactosidase reporter (115 units of β-galactosidase activity). However in contrast to the reduction in reporter activity observed for PR, ERα, PPARγ and TRβ, mutation of TRAP220 LXM2 resulted in a small increase in β-galactosidase activity. The significance, if any, of this small increase in activity due to LXM2 mutation is unknown. Conversely, mutation of TRAP220 LXM1 resulted in a dramatic decrease in transcriptional activity from the β-galactosidase reporter, suggesting RXRα requires TRAP220 LXM1 to be functional in order to permit interaction.

Taken together these data suggest that the NRs, PR, ERα, TRβ and PPARγ all require TRAP220 to have two functional LXMs for effective interactions. However the strong interaction between RXRα and TRAP220 appears to be primarily dependent on the presence of a functional LXM1, as mutation of LXM2 was observed to have little effect on reporter activity.

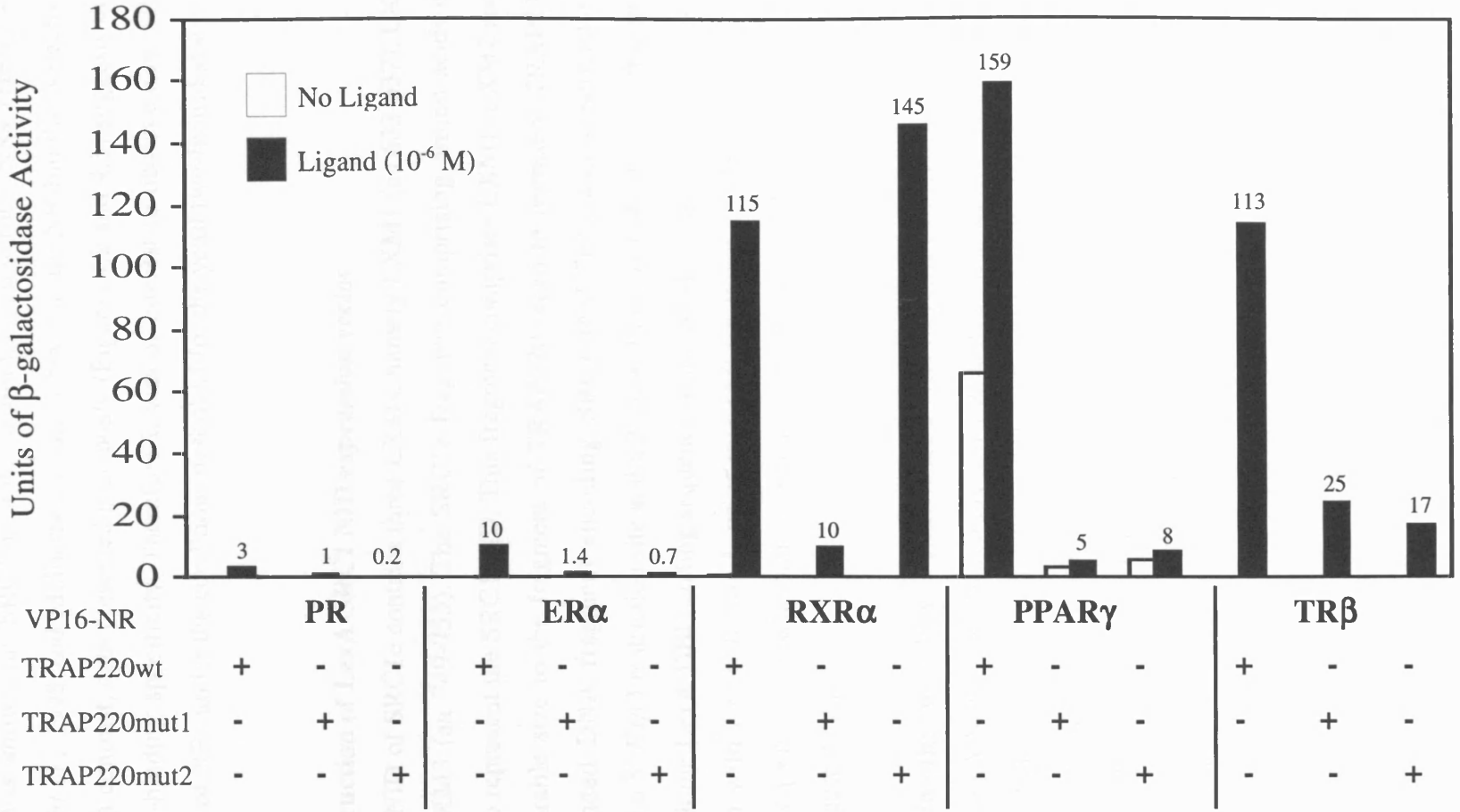


Figure 4.5 Assessing the contribution of TRAP220 LXM1 and LXM2 in NR interactions. Yeast β-galactosidase reporter assay testing the interactions between LexA-TRAP220 NID -wt, -mut1 and -mut2 fusions proteins with VP16-PR-LBD, -ERα-LBD, -RXRα-LBD, -PPARγ-LBD and -TRβ fusion proteins. Receptor cognate ligands were R5020, E_2, 9c-RA, rosiglitazone and T_3 for PR, ERα, RXRα, PPARγ and TRβ, respectively. Numbers represent respective units of β-galactosidase activity in the presence of ligand.

4.3 SRC1 NID interactions with NRs

Reports show that SRC1 is able to bind to numerous NRs (Heery *et al.*, 1997, Kalkhoven *et al.*, 1998 and McInerney *et al.*, 1998) but no NR-binding selectivity has been described. In contrast, data presented previously (Figures 4.2 and 4.3) suggests that TRAP220 exhibits NR-binding specificity. Therefore it was of interest to observe how SRC1 interacts with a panel of NRs under the conditions described for the yeast two-hybrid system used in this study.

4.3.1 Construction of LexA-SRC1 NID expression vector

The NID of SRC1e contains three LXMs, namely LXM1 (aa 633-637), LXM2 (aa 690-694) and LXM3 (aa 749-753). The SRC1e fragment comprising amino acids 431-761 was taken here to represent the SRC1 NID. This fragment comprises LXM1, LXM2 and LXM3 and is of comparable size to the fragment of TRAP220 taken to represent TRAP220 NID. The double-stranded DNA fragment encoding SRC1 (431-761) was generated by PCR and incorporated a 5' *Ksp* I restriction site and a 3' *Bam*H I restriction site. This fragment was fused in frame with the LexA-DBD coding sequence in the BTM116mod vector, previously prepared by digestion with *Ksp* I/ *Bam*H I, prior to CIAP treatment. BTM116mod-SRC1 NID was verified by sequencing. A schematic representation of the LexA-SRC1 NID fusion protein can be seen in figure 4.6A.

4.3.2 Assessing the suitability of LexA-SRC1 NID in yeast two-hybrid assays

The LexA-SRC1 NID fusion protein was tested for intrinsic transcriptional activity in the yeast two-hybrid system. L40 yeast were transformed with the LexA-SRC1 NID expression vector and transformants were selected by growth on CSM lacking tryptophan. Verification of expression of the LexA-SRC1 NID fusion protein in L40 yeast was performed by western blotting using an anti-LexA antibody. As shown in figure 4.6B, yeast transformants expressed LexA-SRC1 NID as a fusion protein approximating to 58 kDa. In addition, the western blot shown in figure 4.6B also shows that yeast clones transformed with either the expression vector for LexA-TRAP220 NID or LexA-SRC1 NID, expressed these fusion proteins at comparable levels. Further, the calculated molecular weight (mw) of both LexA-TRAP220 NID and LexA-SRC1 NID is 58 kDa. The slight difference in mobility observed between LexA-SRC1 NID and

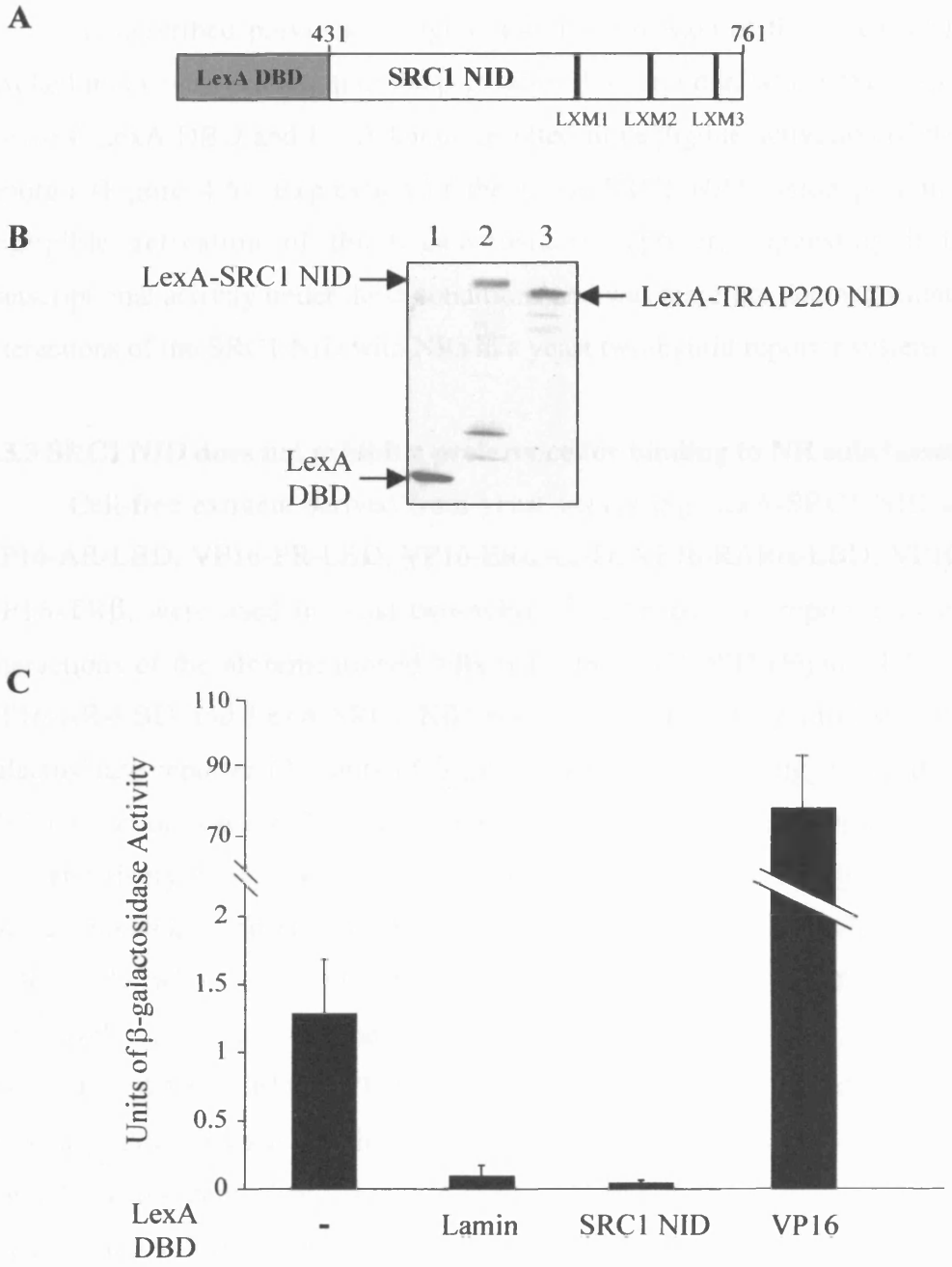


Figure 4.6 (A) Schematic representation of the LexA-SRC1 NID fusion protein. Black bars represent the 3 LXXLL motifs of SRC1. (B) Western blot, using an anti-LexA antibody, showing the expression of LexA-DBD (lane 1), LexA-SRC1 NID (lane 2) and LexA-TRAP220 NID (lane 3) in transformed L40 yeast. (C) Yeast two-hybrid β-galactosidase assay showing the activity of LexA-DBD, LexA-Lamin, LexA-SRC1 NID and LexA-VP16 fusion proteins in the absence of an ectopic activation domain.

LexA-TRAP220 NID was most likely due to the different amino acid compositions of the two proteins.

As described previously (Figure 4.4C), expression of the LexA-VP16 fusion protein resulted in a strong activation of the β-galactosidase reporter, whilst expression of the negative controls, LexA-DBD and LexA-lamin, resulted in negligible activation of the β-galactosidase reporter (Figure 4.6). Expression of the LexA-SRC1 NID fusion protein also resulted in negligible activation of the β-galactosidase reporter, suggesting it has no intrinsic transcriptional activity under these conditions and was therefore deemed suitable for testing the interactions of the SRC1 NID with NRs in a yeast two-hybrid reporter system.

4.3.3 SRC1 NID does not exhibit a preference for binding to NR subclasses

Cell-free extracts derived from yeast expressing LexA-SRC1 NID and one of either VP16-AR-LBD, VP16-PR-LBD, VP16-ERα-LBD, VP16-RARα-LBD, VP16-RXRα-LBD or VP16-TRβ, were used in yeast two-hybrid β-galactosidase reporter assays to assess the interactions of the aforementioned NRs with the SRC1 NID (Figure 4.7). Co-expression of VP16-AR-LBD and LexA-SRC1 NID resulted in a relatively modest activation of the β-galactosidase reporter (25 units of β-galactosidase activity), suggesting the SRC1 NID/AR-LBD interaction is weak. This is in agreement with earlier studies (Ding *et al.*, 1998, He *et al.*, 1999 and Heery *et al.*, 2001) and is probably due to the preference of the AR for binding to FXXLF motifs as opposed to LXXLL motifs. Co-expression of either VP16-PR-LBD or VP16-RXRα-LBD, with LexA-SRC1 NID resulted in 81 and 95 units of β-galactosidase activity, respectively, in a ligand-dependent manner (Figure 4.7). Co-expression of either VP16-ERα-LBD, VP16-RARα-LBD or VP16-TRβ with LexA-SRC1 NID resulted in 160, 156 and 136 units of β-galactosidase activity respectively, in a ligand-dependent manner. Additionally, a marked ligand-independent interaction was observed between the retinoid receptors, RARα and RXRα, with the SRC1 NID (50 and 28 units of β-galactosidase activity, respectively) and to a lesser extent between TRβ and the SRC1 NID (6 units of β-galactosidase activity). The significance of this ligand-independent interaction with retinoid receptors is unclear but has been reported previously in yeast (Heery *et al.*, 1993 and Heery *et al.*, 1994).

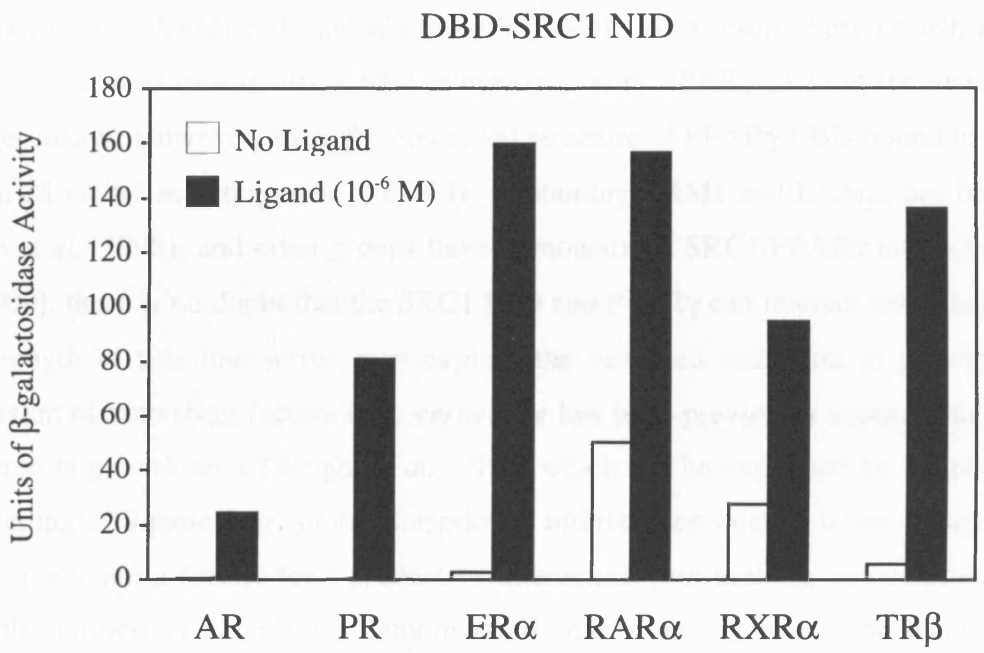


Figure 4.7 Assessing the interaction of the SRC1 NID with a panel of NRs. Yeast two-hybrid β-galactosidase assay using cell-free extracts derived from yeast transformed with LexA-SRC1 NID expression vector and an expression vector for either VP16-AR-LBD, VP16-PR-LBD, VP16-ERα-LBD, VP16-RARα-LBD, VP16-RXRα-LBD or VP16-TRβ. The receptor cognate ligands were mibolerone, R5020, E_2, AT-RA, 9c-RA and T_3 for AR, PR, ERα, RARα, RXRα and TRβ, respectively.

4.4 Transcriptional interference in the yeast two-hybrid system

It was observed that yeast co-transformed with LexA-SRC1 NID and VP16-PPARγ-LBD expression vectors, grew very slowly compared to yeast co-transformed with expression vectors for LexA-SRC1 NID and any other VP16-NR. As a result, these growth arrested yeast could not be used to test SRC1 NID interactions with PPARγ-LBD in the yeast two-hybrid reporter assays. However, since the co-crystal structure of PPARγ-LBD bound to rosiglitazone and an 88 amino acid fragment of SRC1e, containing LXM1 and LXM2, has been described (Nolte *et al.,* 1998), and other groups have demonstrated SRC1/PPARγ interactions (Yang *et al.,* 2000), there is no doubt that the SRC1 NID and PPARγ can interact and it is probable that the strength of this interaction may explain the observed reduction in growth rate. Over-expression of activating factors in *S. cerevisiae* has been previously reported to cause severe reduction in growth rates (Wright *et al.,* 1991) which can be explained by the phenomenon of 'squelching'. 'Squelching' or transcriptional interference occurs when competition exists between activating factors for a productive interaction with limiting transcription factors. For example, Gilbert *et al.,* (1993) demonstrated squelching by over-expressing a potent E_2-inducible activator (a chimera of the ER in which the A/B region had been replaced by the AAD of VP16) in yeast. This led to the inhibition of an endogenous yeast reporter and gradually caused complete cell growth arrest. It is therefore possible that PPARγ-LBD and SRC1-NID are able to interact in a ligand-dependent manner under the conditions of this yeast two-hybrid system, but that the recruitment of the AAD-NR to the LexA promoter is so efficient that it is interfering with normal yeast cell transcriptional processes i.e. titrating transcriptional intermediary factors (TIFs) and the RNA polymerase II machinery away from normal and essential yeast cell transcription. Hence the yeast cells exhibit a reduction in normal cell growth.

In order examine the possible squelching effects induced by SRC1-NID and PPARγ-LBD co-expression, L40 yeast cells expressing LexA-SRC1 NID and VP16-PPARγ-LBD or LexA-SRC1 NID and VP16-ERα-LBD were grown in culture overnight in the absence or presence of 10^{-6} M receptor cognate ligand. Aliquots of yeast cell culture were then mounted onto microscope slides and the yeast cell morphology visualised (Figure 4.8). A clear difference in the appearance of the yeast cells expressing PPARγ-LBD as opposed to the ERα-LBD was immediately apparent. The majority of yeast cells expressing LexA-SRC1 NID and VP16-ERα-

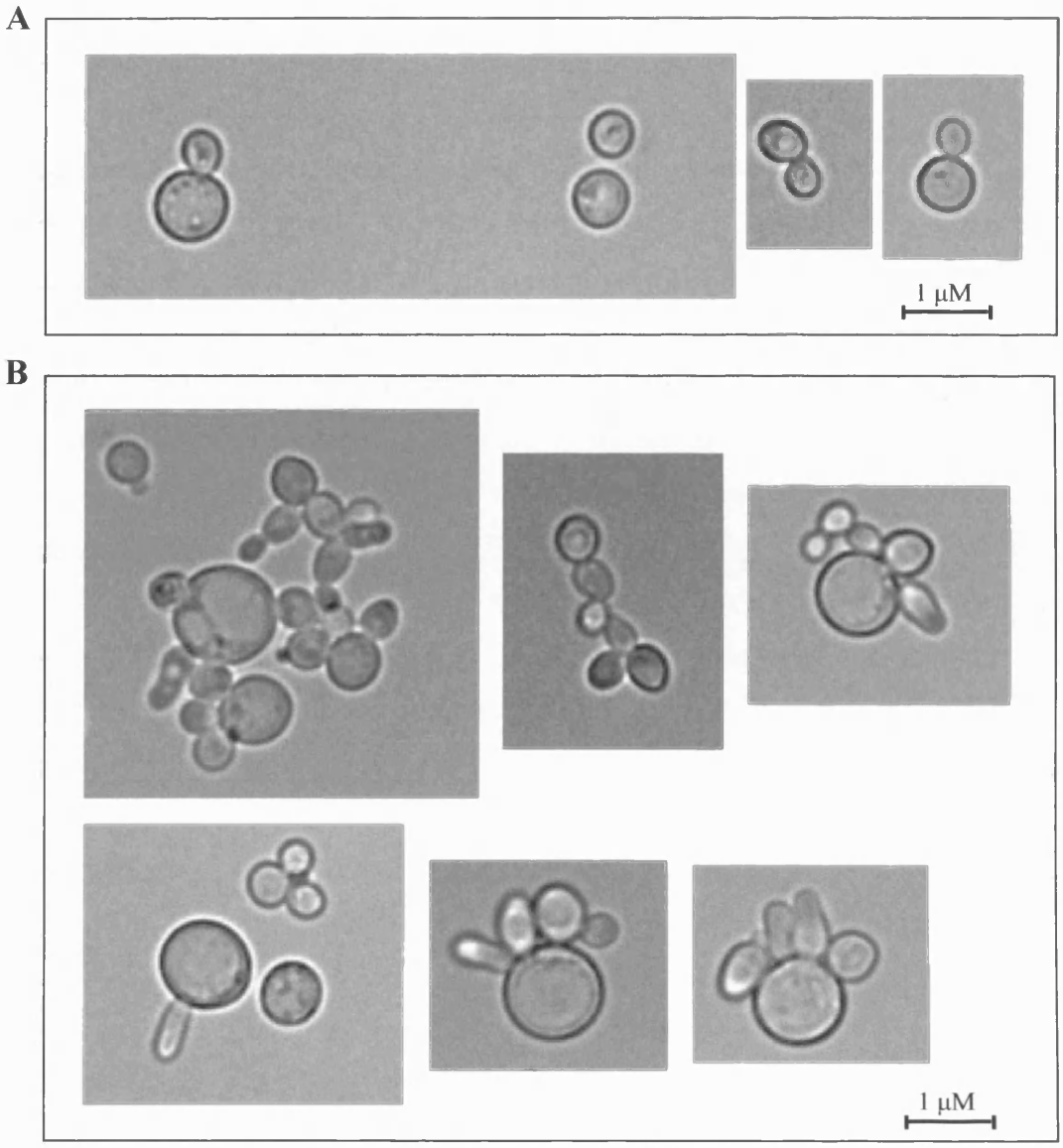


Figure 4.8. Light microscopy was used to visualise L40 yeast cells growing in liquid culture. All images are at 40x magnification. (A) Yeast cells expressing LexA-SRC1 NID and VP16-ERα-LBD, in the presence of 10^{-6} M E_2. (B) Yeast cells expressing LexA-SRC1 NID and VP16-PPARγ- LBD, in the presence of 10^{-6} M rosiglitazone.

LBD appeared rounded or oval and were frequently observed to have a single budding daughter cell which is characteristic of normal yeast cell growth (Figure 4.8A). No difference in the appearance of the yeast cells was observed whether they were grown in the presence or absence of E_2, suggesting E_2-induced squelching is not occurring. However yeast expressing LexA-SRC1 NID and VP16-PPARγ-LBD exhibited several abnormalities in their appearance (Figure 4.8B). A large degree of clumping of the yeast cells was observed, probably due to ineffective separation of the daughter and parental cells. Additionally, yeast cell nuclei were observed to be enlarged, daughter cells were elongated and multiple daughter cells remained attached to the parental cells. The same abnormalities were observed in the presence and absence of rosiglitazone, suggesting that ligand-induced squelching is evident whether the PPARγ ligand is endogenous to yeast or ectopic rosigliazone. Similar morphological abnormalities have recently been reported for the yeast strain W303-1B, when over-expressing NR and coactivator proteins (Sheppard *et al.*, paper in press). Therefore, I conclude that these severe growth abnormalities could be a result of squelching and suggest that the SRC1 NID may interact strongly with PPARγ-LBD, thereby generating a potent activator of transcription.

4.5 The interactions of TRAP220 core LXXLL motifs with NRs

It has been observed previously that polypeptide sequences of 8-10 amino acids in length encompassing the signature motif LXXLL, are sufficient to bind liganded NR LBDs and this has been referred to the as the core LXXLL motif (Heery *et al.*, 1997 and Heery *et al.*, 2001). The NID of TRAP220 used in this study contains two core LXXLL motifs (LXM1 and LXM2) and has been shown to exhibit NR class binding specificity (Figure 4.2 and 4.3). However the NID of SRC1, containing three core LXXLL motifs (LXM1, LXM2 and LXM3) was observed not to exhibit this specificity (Figure 4.7). To explore the nature of the specificity exhibited by the TRAP220 NID in more detail, the LXXLL core motifs derived from TRAP220 and SRC1 were assessed for their ability to bind NRs.

4.5.1 Characterisation of the ER-DBD-core LXXLL motif fusion proteins

ER-DBD fusion protein expression vectors were generated encoding sequences corresponding to amino acids –1 to +8 of TRAP220 LXM1 and LXM2, and SRC1 LXM2, fused in frame with the ER-DBD (Figure 4.9A). Expression vectors for ER-DBD-TRAP220

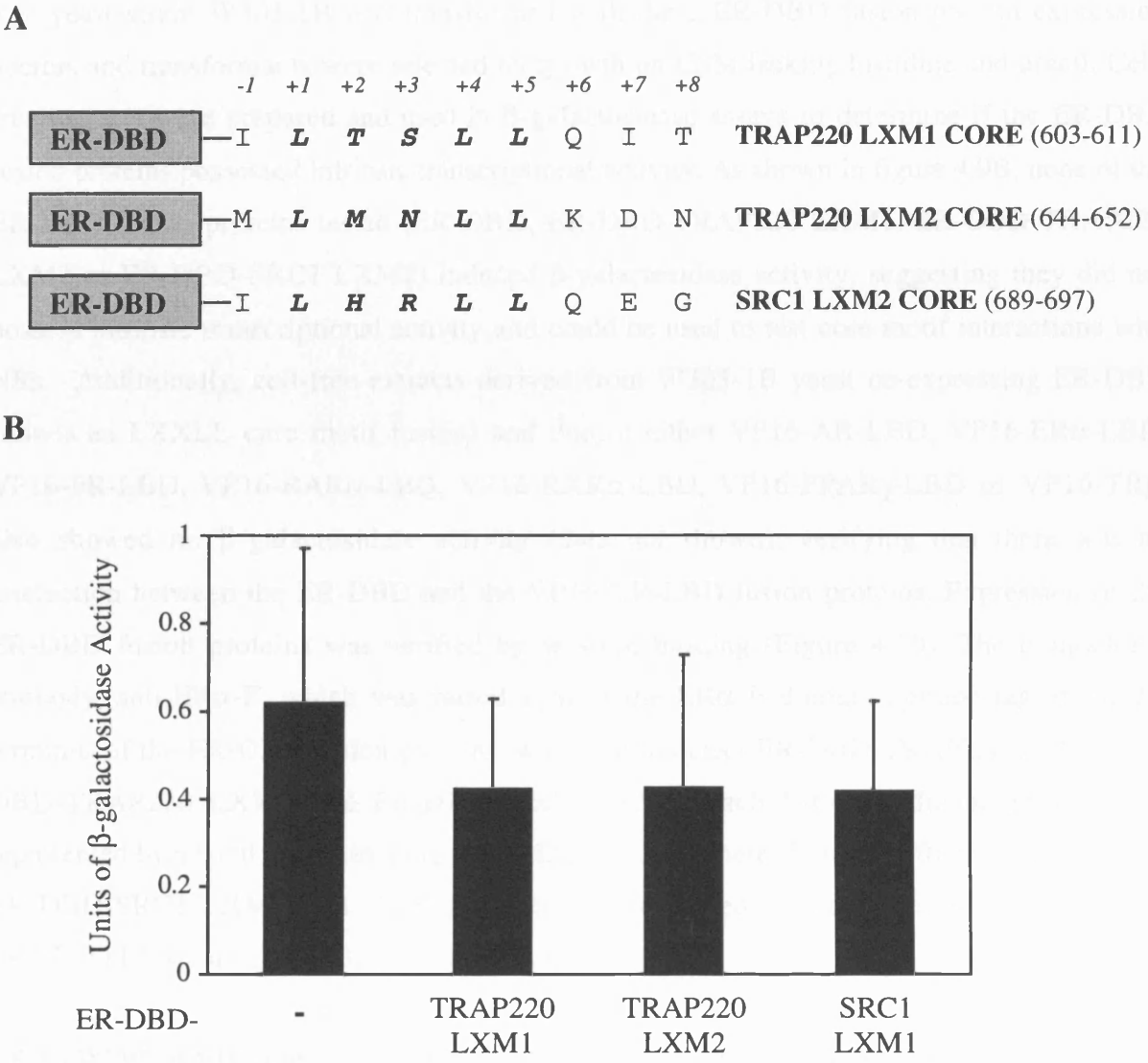


Figure 4.9. Testing ER-DBD fusion proteins for intrinsic transcriptional activity. (A) Schematic representation of the ER-DBD fusion proteins. (B) Yeast two-hybrid β-galactosidase assay using cell-free extracts derived from yeast expressing ER-DBD, ER-DBD-TRAP220 LXM1 CORE, ER-DBD-TRAP220 LXM2 CORE and ER-DBD-SRC1 LXM2 CORE fusion proteins, as indicated.

LXM1, ER-DBD-TRAP220 LXM2 and ER-DBD-SRC1 LXM2 were verified by sequencing. The yeast strain W303-1B was transformed with these ER-DBD fusion protein expression vectors and transformants were selected by growth on CSM lacking histidine and uracil. Cell-free extracts were prepared and used in β-galactosidase assays to determine if the ER-DBD fusion proteins possessed intrinsic transcriptional activity. As shown in figure 4.9B, none of the ER-DBD fusion proteins tested (ER-DBD, ER-DBD-TRAP220 LXM1, ER-DBD-TRAP220 LXM2 or ER-DBD-SRC1 LXM2) induced β-galactosidase activity, suggesting they did not possess intrinsic transcriptional activity and could be used to test core motif interactions with NRs. Additionally, cell-free extracts derived from W303-1B yeast co-expressing ER-DBD (minus an LXXLL core motif fusion) and one of either VP16-AR-LBD, VP16-ERα-LBD, VP16-PR-LBD, VP16-RARα-LBD, VP16-RXRα-LBD, VP16-PPARγ-LBD or VP16-TRβ, also showed no β-galactosidase activity (data not shown), verifying that there was no interaction between the ER-DBD and the VP16-NR-LBD fusion proteins. Expression of the ER-DBD fusion proteins was verified by western blotting (Figure 4.10). The monoclonal antibody, anti-ERα-F, which was raised against the ERα F domain epitope tag at the N-terminus of the ER-DBD fusion proteins, was used to detect ER-DBD-TRAP220 LXM1, ER-DBD-TRAP220 LXM2 and ER-DBD-SRC1 LXM2. Each ER-DBD fusion protein was represented by a band approximating to 16 kDa. As shown here (Figure 4.10), the expression of ER-DBD-SRC1 LXM2 was slightly reduced as compared to the expression of ER-DBD-TRAP220 LXM1 and ER-DBD-TRAP220 LXM2.

4.5.2 TRAP220 NID and core LXXLL motifs display different NR-binding properties

To assess the activity of ER-DBD-core motif proteins in yeast two-hybrid assays, the yeast strain W303-1B, containing p3ERE-LacZ as the β-galactosidase reporter, was used. As shown in figure 4.11A, TRAP220 LXM1 was able to bind to all of the NRs tested in a ligand-dependent manner. Notably, the β-galactosidase activity measured due to the co-expression of ER-DBD-TRAP220 LXM1 and any of the VP16-NR-LBDs tested was similar, with the exception of the VP16-RXRα-LBD. Co-expression of ER-DBD-TRAP220 LXM1 and VP16-RXRα-LBD resulted in activation of the β-galactosidase reporter which was 2-3 fold greater than that resulting from co-expression of ER-DBD-TRAP220 LXM1 with any of the other VP16-NR-LBD fusion proteins tested. This difference possibly reflects the preference of RXRα

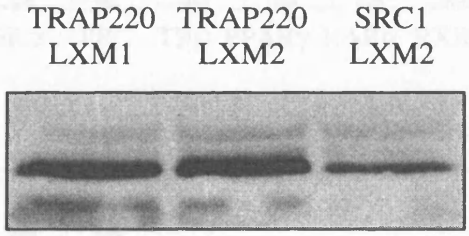


Figure 4.10 Over-expressed ER-DBD fusion proteins can be detected in the yeast strain W303-1B. A monoclonal antibody raised against the ERα F domain epitope tag at the N-terminus of the ER-DBD fusion proteins was used in western blotting to detect ER-DBD-TRAP220 LXM1 CORE, ER-DBD-TRAP220 LXM2 CORE and ER-DBD-SRC1 LXM2 CORE fusion proteins, as indicated.

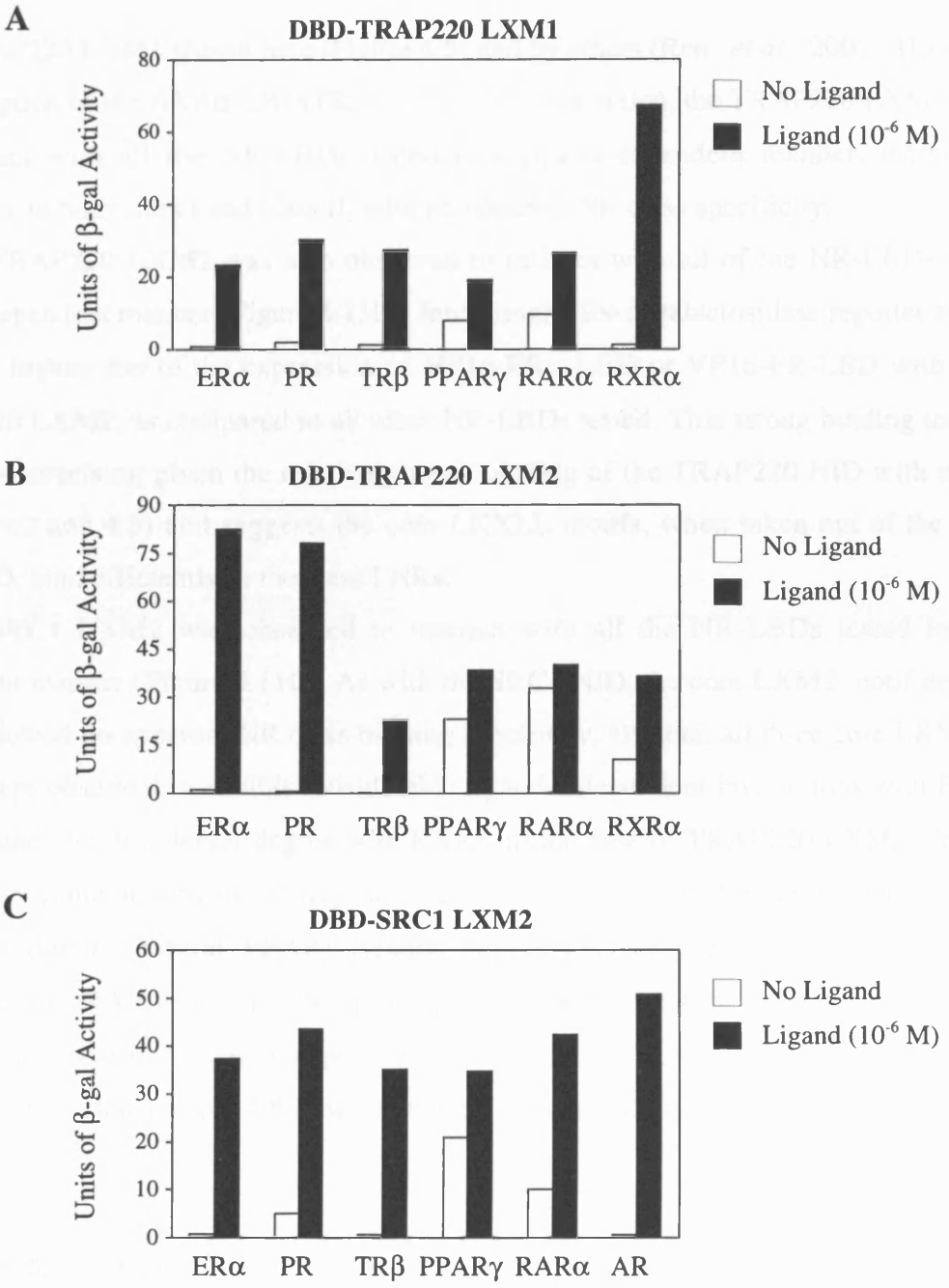


Figure 4.11 Assessing the NR-binding specificities of LXXLL core motifs derived from TRAP220 and SRC1e. Yeast β-galactosidase reporter assays using cell-free extracts derived from yeast transformed with an expression vector for (A) ER-DBD-TRAP220 LXM1 CORE, (B) ER-DBD-TRAP220 LXM2 CORE, (C) ER-DBD-SRC1 LXM2 CORE, and an expression vector for a VP16-NR-LBD, as indicated.

for TRAP220 LXM1 shown here (Figure 4.5) and by others (Ren *et al.*, 2000). However, with the exception of the RXRα-LBD/TRAP220 LXM1 interaction, the TRAP220 LXM1 was found to interact with all the NR-LBDs tested in a ligand-dependent manner, including those belonging to both class I and class II, with no apparent NR class specificity.

TRAP220 LXM2 was also observed to interact with all of the NR-LBDs tested in a ligand-dependent manner (Figure 4.11B). Intriguingly, the β-galactosidase reporter activity was 2-4 fold higher due to the expression of VP16-ERα-LBD or VP16-PR-LBD with ER-DBD-TRAP220 LXM2, as compared to all other NR-LBDs tested. This strong binding to the class I NRs was surprising given the relatively weak binding of the TRAP220 NID with class I NRs (Figure 4.2 and 4.3) and suggests the core LXXLL motifs, when taken out of the context of their NID, bind efficiently to the class I NRs.

SRC1 LXM2 was observed to interact with all the NR-LBDs tested in a ligand-dependent manner (Figure 4.11C). As with the SRC1 NID, the core LXM2 motif derived from SRC1 showed no apparent NR class-binding specificity. Of note, all three core LXXLL motifs tested were observed to exhibit considerable ligand-independent interactions with PPARγ and RARα (and also to a lesser degree with RXRα in the case of TRAP220 LXM2). The ligand-independent interactions of PPARγ have been seen previously (Figure 4. 3 and 4.5) and are probably due to natural PPARγ ligands endogenous to yeast. The ligand-independent interactions of RARα have also been seen previously (Figure 4.7). Similar ligand-independent interactions between retinoid receptors and the third LXXLL motif derived from SRC1e have been reported (Heery *et al.*, 2001) when using a similar yeast two-hybrid system as that used in this study.

4.6 Flanking amino acid sequence contributes to NR-binding specificity

The NID of TRAP220 was observed to preferentially bind to the class II NRs, PPARγ, RXRα and TRβ, as opposed to the class I NRs, ERα and PR (Figure 4.2 and 4.3). However as shown in figure 4.11, this NR-binding specificity was not shared by the TRAP220 LXXLL core motifs (LXM1 and LXM2) when they were taken out of the context of the NID. This suggests that sequences outside the core LXXLL motifs are contributing to the observed NR-binding specificity. To investigate the influence of the flanking amino acid sequence, the interaction of an extended TRAP220 LXM1 core motif with NR-LBDs was examined.

4.6.1 Construction of the ER-DBD-TRAP220 LXM1-extended expression vector

An oligonucleotide cassette encoding amino acids 600 to 612 of TRAP220 was generated and ligated with the vector pBL1mod, which was previously prepared by digestion with *Ksp* I and *Bam*H I followed by CIAP treatment. The resulting ER-DBD-TRAP220 LXM1-extended (ext) fusion protein expression vector was verified by sequencing. The TRAP220 LXM1 extended motif comprised 4 amino acids N-terminal to LXM1 (-4 to -1) and four amino acids C-terminal to LXM1 (+6 to +9), overall giving a thirteen amino acid peptide fused to the ER-DBD (Figure 4.12A).

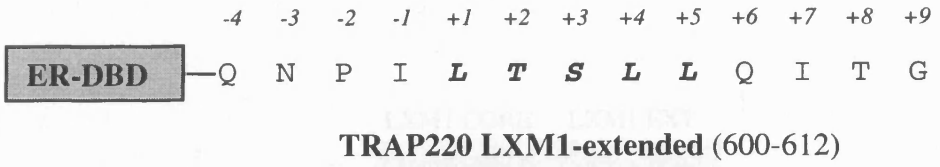
4.6.2 Characterisation of the ER-DBD-TRAP220 LXM1-ext fusion protein

W303-1B yeast were transformed with the expression vector for ER-DBD-TRAP220 LXM1-ext and transformants were selected by growth on CSM lacking histidine and uracil. Cell-free extracts derived from W303-1B yeast over-expressing only ER-DBD or ER-DBD-TRAP220 LXM1-ext were used in β-galactosidase assays to confirm that, as for ER-DBD expression, the expression of ER-DBD TRAP220 LXM1-ext resulted in negligible activation of the β-galactosidase reporter (Figure 4.12B), suggesting that this fusion protein does not possess intrinsic transcriptional activity. Co-expression of ER-DBD-TRAP220 LXM1-ext and VP16-AAD also resulted in no activation of the β-galactosidase reporter (data not shown). Therefore the fusion protein, ER-DBD-TRAP220 LXM1-ext, was suitable for use in the yeast two-hybrid experiments described in this study. Expression of the ER-DBD-TRAP220 LXM1-ext fusion protein in cell-free extracts was confirmed by western blotting using monoclonal anti-ERα-F antibody. As shown in figure 4.13B, both ER-DBD-TRAP220 LXM1-core and ER-DBD-TRAP220 LXM1-ext fusion proteins were detected and represented by bands of comparable intensity approximating to 15 and 16 kDa, respectively.

4.6.3 Differential NR-binding of the core and extended LXM1 derived from TRAP220

Interestingly, in the presence of VP16-TRβ, the ER-DBD-TRAP220 LXM1-ext fusion protein was able to activate the β-galactosidase reporter 3.5 fold above the level observed for the ER-DBD-TRAP220 LXM1 core fusion protein, in a ligand-dependent manner (Figure 4.13C). This suggests that the additional amino acids flanking the core motif contained within the LXM1 extended motif fusion protein may be stabilising or enhancing the interaction with

A



TRAP220 LXM1-extended (600-612)

B

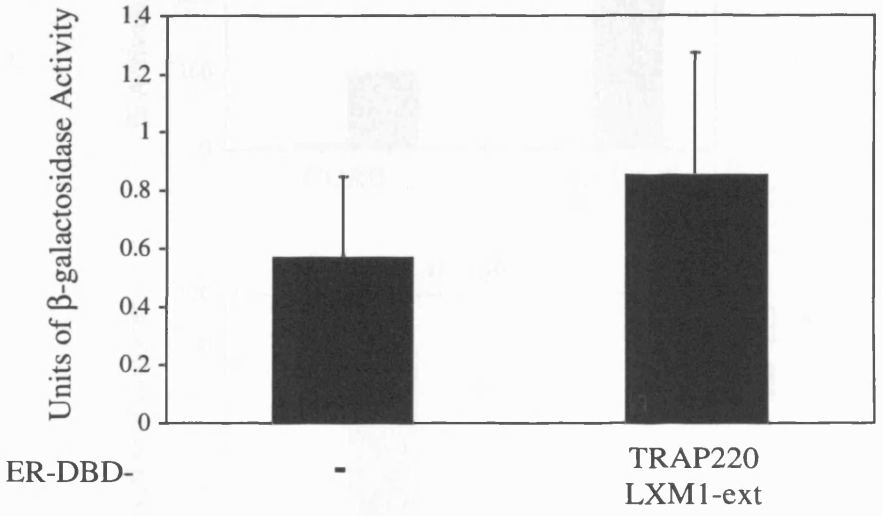


Figure 4.12 Testing the ER-DBD-TRAP220 LXM1-extended fusion protein for intrinsic transcriptional activity. (A) Schematic representation of the ER-DBD fusion protein comprising thirteen amino acids of TRAP220 LXM1 (-4 to +9). (B) Yeast two-hybrid β-galactosidase assay using cell-free extracts derived from yeast expressing ER-DBD or ER-DBD-TRAP220 LXM1-extended fusion proteins, as indicated.

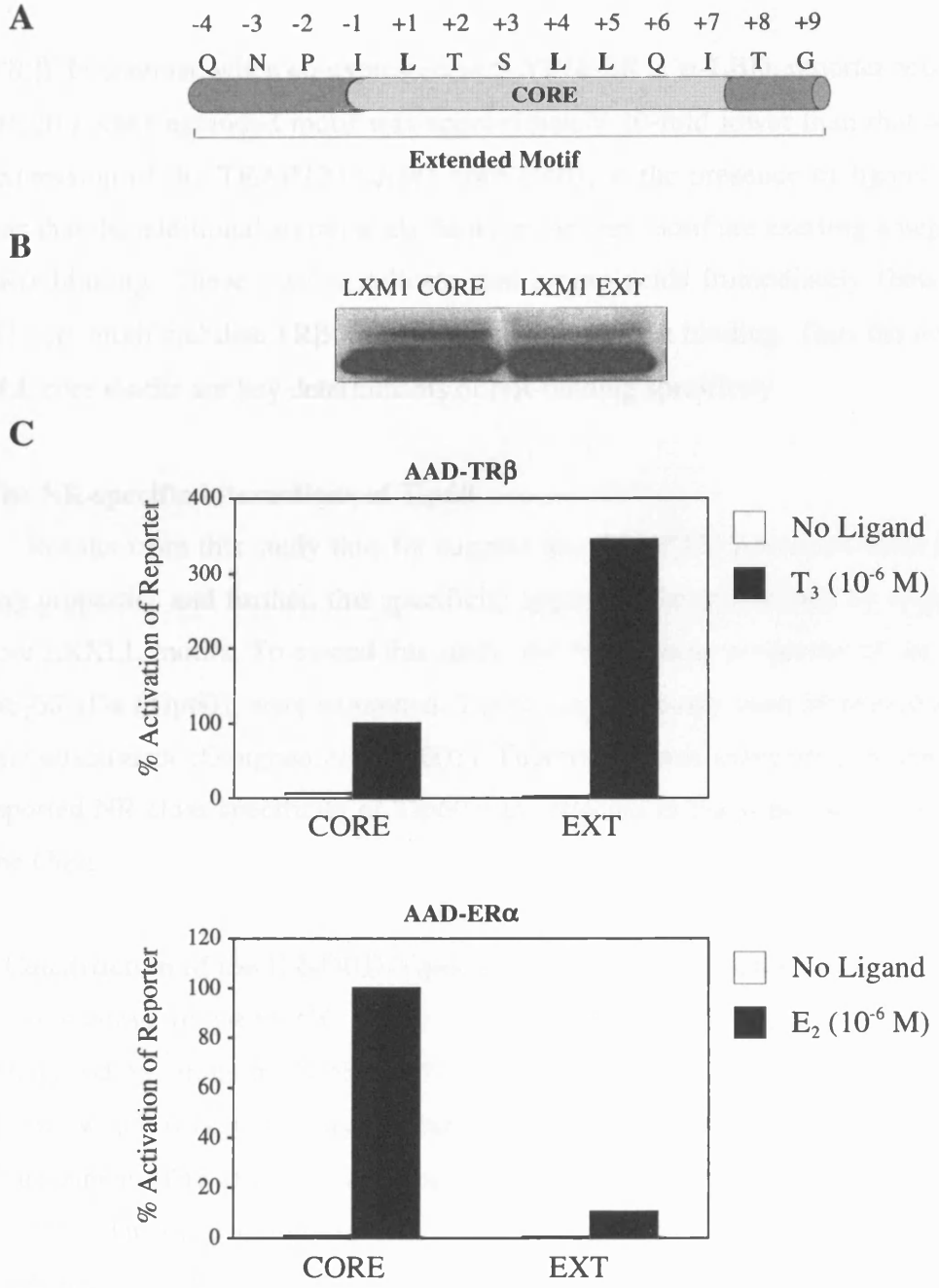


Figure 4.13 Amino acid sequence flanking the core LXXLL motifs contributes to NR-binding specificity. (A) Schematic representation of TRAP220 extended and core LXXLL motif sequences. (B) Western blot, using the anti-ERα-F antibody, was used to detect ER-DBD-TRAP220 LXM1 CORE and ER-DBD-TRAP220 LXM1-extended in W303-1B yeast. (C) Yeast two-hybrid experiments showing the interaction of TRAP220 LXM1 core (CORE) and extended (EXT) motifs with TRβ and the ERα LBD.

the TR β. In contrast, when co-expressed with VP16-ER α-LBD, reporter activation by the TRAP220 LXM1 extended motif was approximately 10-fold lower than that achieved due to the expression of the TRAP220 LXM1 core motif, in the presence of ligand. In this case it appears that the additional amino acids flanking the core motif are exerting a negative influence on ERα binding. These results indicate that amino acids immediately flanking TRAP220 LXM1 core motif stabilise TRβ interaction but reduce ERα binding. Thus the residues flanking LXXLL core motifs are key determinants of NR-binding specificity.

4.7 The NR-specific interactions of Tip60

Results from this study thus far suggest that TRAP220 possesses class II specific NR-binding properties and further, this specificity appears to be determined by sequences flanking the core LXXLL motifs. To extend this study, the NR-binding properties of the Tat-interactive protein-60 kDa (Tip60), were examined. Tip60 has previously been identified as a class I NR specific coactivator (Gaughan *et al.*, 2001). Therefore it was interesting to determine whether the reported NR class specificity of Tip60 was reflected in the yeast two-hybrid assay system decribed here.

4.7.1 Construction of the ER-DBD-Tip60 LXM expression vector

In contrast to TRAP220, Tip60 contains only one LXXLL motif. An oligonucleotide cassette encoding amino acids 485 to 497 of Tip60 was generated and ligated with the vector pBL1mod, which was previously prepared by digestion with *Ksp* I and *Bam*H I followed by CIAP treatment. The resultant expression vector encoded a thirteen amino acid sequence derived from Tip60, (encompassing the LXXLL motif flanked by four amino acids on the C- and N-terminals, respectively) fused in frame with the ER-DBD (Figure 4.14A). The ER-DBD-Tip60 LXM expression vector was verified by sequencing.

4.7.2 Characterisation of the ER-DBD-Tip60 LXM fusion protein

W303-1B yeast were transformed with the expression vector for ER-DBD-Tip60 LXM fusion protein and transformants were selected by growth on CSM lacking histidine and uracil. Cell-free extracts were prepared as outlined in section 2.5.6 and used in western blotting to verify the expression of the ER-DBD-Tip60 LXM fusion protein. As shown in figure 4.14B,

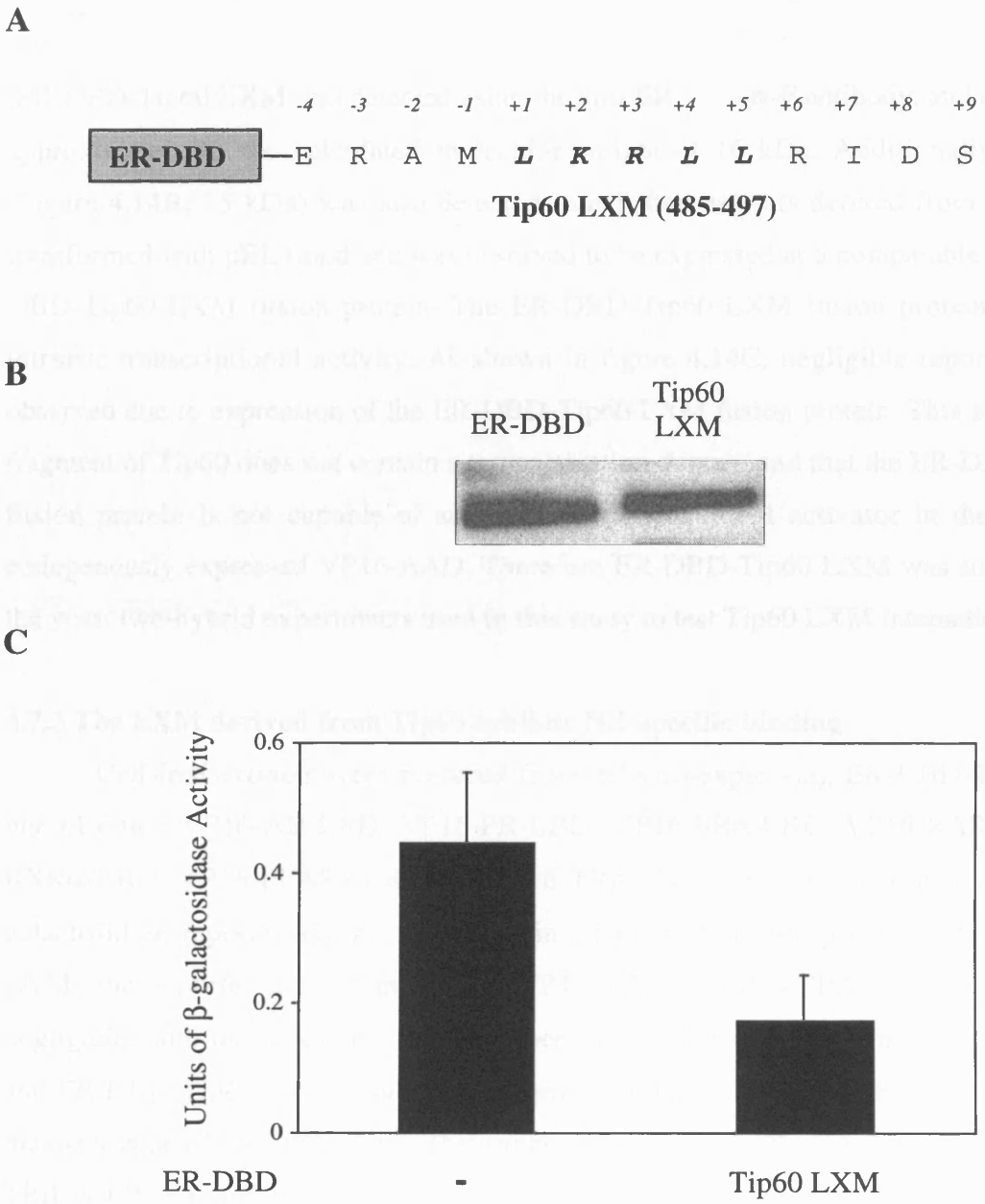


Figure 4.14 Characterisation of the ER-DBD-Tip60 LXM fusion protein. (A) Schematic representation of the ER-DBD-Tip60 LXM fusion protein. (B) Western blotting, using the anti-ERα-F antibody, shows expression of ER-DBD and ER-DBD-Tip60 LXM fusion protein in yeast cell-free extracts. (C) Yeast two-hybrid β-galactosidase assay using cell-free extracts derived from cells expressing ER-DBD and ER-DBD-Tip60 LXM fusion protein, respectively.

ER-DBD-Tip60 LXM was detected using the anti-ER α-F antibody, and corresponded approximately to the calculated molecular weight of 16 kDa. Additionally, the ER-DBD (Figure 4.14B; 15 kDa) was also detected in cell-free extracts derived from W303-1B yeast transformed with pBL1mod and was observed to be expressed at a comparable level to the ER-DBD-Tip60 LXM fusion protein. The ER-DBD-Tip60 LXM fusion protein was tested for intrinsic transcriptional activity. As shown in figure 4.14C, negligible reporter activity was observed due to expression of the ER-DBD-Tip60 LXM fusion protein. This suggests that this fragment of Tip60 does not contain a transactivation domain and that the ER-DBD-Tip60 LXM fusion protein is not capable of acting as a transcriptional activator in the absence of an endogenously expressed VP16-AAD. Therefore ER-DBD-Tip60 LXM was suitable for use in the yeast two-hybrid experiments used in this study to test Tip60 LXM interactions with NRs.

4.7.3 The LXM derived from Tip60 exhibits NR-specific binding

Cell-free extracts were prepared from cells co-expressing ER-DBD-Tip60 LXM and one of either VP16-AR-LBD, VP16-PR-LBD, VP16-ERα-LBD, VP16-RARα-LBD, VP16-RXRα-LBD, VP16-PPARγ-LBD or VP16-TRβ. These cell-free extracts were used in β-galactosidase reporter assays. As shown in figure 4.15, in the presence of ER-DBD-Tip60 LXM, the reporter activation due to VP16-TRβ or VP16-PPARγ-LBD expression was negligible, both in the absence and presence of ligand. Co-expression of VP16-PPARγ-LBD and ER-DBD-Tip60 LXM resulted in reporter activity barely above the level observed due to the expression of ER-DBD alone. This suggests there is no interaction between Tip60 LXM and TRβ or PPARγ, and is in agreement with a previous study who were able to show, using a mammalian two-hybrid system, that full-length Tip60 did not bind to the TR (Gaughan *et al.*, 2001). In the presence of ligand, the reporter activity due to the co-expression of ER-DBD-Tip60 LXM and VP16-RARα-LBD was also weak (7 units of β-galactosidase activity), whilst co-expression of ER-DBD-Tip60 LXM and VP16-RXRα-LBD resulted in a marginally higher level of reporter activity (20 units of β-galactosidase activity). The class I NRs, AR and PR, were also observed to exhibit relatively weak interactions with the Tip60 LXM (15 and 10 units of β-galactosidase activity, respectively), in a ligand-dependent manner. However, co-expression of VP16-ERα-LBD and ER-DBD-Tip60 LXM resulted in strong ligand-dependent

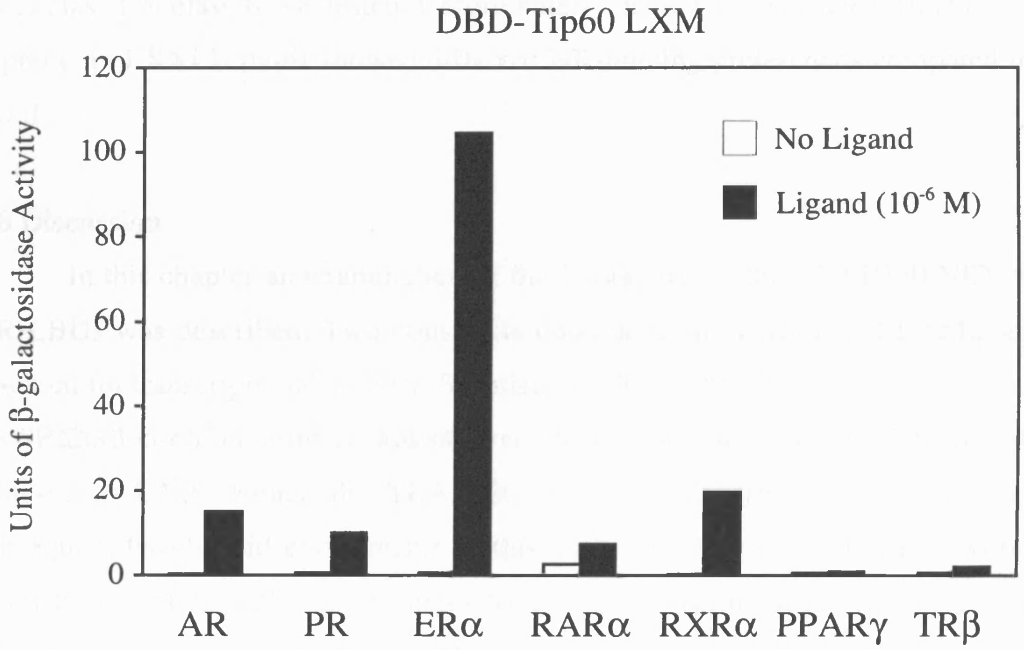


Figure 4.15 Assessing the interaction of the LXM derived from Tip60 with a panel of NR-LBDs. Yeast two-hybrid β-galactosidase assay using cell-free extracts derived from yeast transformed with ER-DBD-Tip60 LXM expression vector and then an expression vector for either VP16-AR-LBD, VP16-PR-LBD, VP16-ERα-LBD, VP16-RARα-LBD, VP16-RXRα-LBD, VP16-PPARγ -LBD or VP16-TRβ. The receptor cognate ligands were mibolerone, R5020, E_2, AT-RA, 9c-RA, rosiglitazone and T_3 for AR, PR, ERα, RARα, RXRα, PPARγ and TRβ, respectively.

transcriptional activity from the β-galactosidase reporter, suggesting a strong ligand-dependent interaction between Tip60 LXM and the ERα-LBD.

These results suggest the lone LXM derived from Tip60 exhibits a NR-binding specificity, preferentially binding to the ERα under the conditions used here as opposed to any other class I or class II NR tested. Unfortunately, there was insufficient time to test whether the Tip60 core LXXLL motif showed different NR-binding preferences compared to its extended motif.

4.8 Discussion

In this chapter an examination of the interaction of the TRAP220 NID with a panel of NR-LBDs was described. Two constructs containing the LXM1 and LXM2 sequences were assessed for transcriptional activity. Surprisingly the TRAP220 (503-667) construct, but not the TRAP220(335-667) construct, was observed to activate transcription of the reporter gene when tethered to DNA. Hence the TRAP220 (503-667) construct was deemed unsuitable for subsequent two-hybrid experiments in this study. Reports show that is possible to generate novel transcription activating proteins by attaching peptide sequences to a DBD (Ma *et al.*, 1987 and Lu *et al.*, 2000). Indeed Ma *et al.*, (1987), demonstrated that an array of peptides encoded by random fragments of the *E. coli* genome, when fused to the GAL4-DBD, could activate transcription up to 30% as effectively as the intact GAL4 protein. It is therefore likely that by fusing the TRAP220 (503-667) sequence to the LexA-DBD, an artificial yeast activator protein has been generated.

Interestingly, Treuter *et al.*, (1999), were able to demonstrate that by fusing amino acids 425-973 of TRAP220 to the DBD of GAL4, transcriptional activation of reporter genes could be achieved in both yeast and mammalian cells. This led to the proposal that TRAP220 contains intrinsic transcriptional activity. TRAP220 amino acids 503-667 (this study) and 425-973 (Treuter *et al.*, 1999) have both been shown to support transcriptional activation in yeast whereas TRAP220 amino acids 335-667 (this study) was shown to exhibit no transcriptional activity. Thus, by extending the TRAP220 sequence included in the DBD-fusion protein at the N-terminus, the transcriptional activity was lost. Perhaps therefore it should be considered that the additional N-terminal TRAP220 sequence (335-502) could contain a transcriptional repression domain. Whilst this proposal is unlikely, it cannot be discounted.

Several NR coactivators have been found to display preferences for NR subclasses. For example Tip60, was reported to preferentially bind to class I NRs whilst showing little affinity for the class II NRs, TR, VDR and RXR (Gaughan *et al.*, 2001). This led to the proposal that Tip60 is a class I specific coactivator. Using a yeast two-hybrid system, a thirteen amino acid peptide derived from Tip60 and encompassing the LXXLL motif, was observed to preferentially bind to the class I NR, ERα, but showed no, or very weak, binding to all of the other class I and class II NRs tested. Perhaps amino acids outside this thirteen amino acid stretch are additionally required to permit Tip60 to bind other class I and II NRs. In contrast to the NR class I specificity exhibited by Tip60, results from this study suggest that TRAP220 is a NR class II specific coactivator. The NID of TRAP220 was observed to preferentially bind to the class II NRs, TRβ, RXRα-LBD, PPARγ-LBD and to a lesser extent RARα-LBD, whilst showing weak binding to the class I NRs, PR-LBD and ERα-LBD, and no binding to the AR-LBD. This data supports the hypothesis proposed in chapter 3 of this study. TRAP220 was observed to be unable to enhance ERα-mediated transactivation or interfere with SRC1 enhancement of ERα-mediated transactivation, prompting the supposition that the inability of TRAP220 to enhance ERα activity in transient transfection experiments, was due to weak or no interaction with the ERα. However, with the exception of the AR-LBD, TRAP220 was observed to bind to all of the NRs tested but displayed a clear class II preference.

The NID of TRAP220 contains two LXXLL motifs and the data from these yeast two-hybrid experiments show that it is capable of binding to a panel of NRs. However it was not clear whether these interactions were mediated through a single LXM or if both LXMs were required. Therefore, in order to test TRAP220 LXM usage by different NRs, two mutant TRAP220 NIDs were generated. The two conserved leucine residues at positions +4 and +5 were replaced by alanines in either LXM1 or LXM2, thereby abolishing the signature NR-binding LXXLL motif. Results indicated that mutation of either motif abolished the weak binding to the class I NRs, ERα and PR and dramatically reduced the binding to the class II NRs, PPARγ and TRβ. This suggested the observed NR interaction of the TRAP220 NID was mediated by two functional LXXLL motifs. Presumably over-expression of the NR-LBDs in yeast could lead to homodimerisation. Class II NRs preferentially heterodimerise with RXR but are capable of homodimerisation (Williams *et al.*, 1991, Zhang *et al.*, 1992b, Yen *et al.*, 1992 and Heery *et al.*, 1994). Under the conditions in yeast, which lack endogenous NRs, the over-expressed class II NR-LBDs would only be able to homodimerise. Hence the necessity for two

functional LXXLL motifs suggests one molecule of TRAP220 binds to a NR homodimer, with each LXXLL motif interfacing with one of the AF-2 surfaces of the LBD-homodimer. This is consistent with the co-crystal structures of agonist-bound LBD homodimers with peptides derived from SRC1 and GRIP1 NIDs, which show that both LBDs in the NR dimer are occupied by an LXXLL core α-helix (Shiau *et al.,* 1998, Nolte *et al.,* 1998, Darimont *et al.,* 1998 and Xu *et al.,* 2001). However RXRα-LBD was observed to require only a functional LXM1 to facilitate strong binding. Perhaps the RXRα/LXM1 interaction is strong enough to overcome the loss of the second LXM and sufficient to stabilise the RXRα-LBD-homodimer/TRAP220-NID complex. In a recent report, *in vitro* binding experiments have shown that RXRα preferentially binds TRAP220 LXM1, whilst TR, VDR and PPAR preferentially bind TRAP220 LXM2 (Ren *et al.,* 2000). Taken together, the necessity for two functional LXXLL motifs and the preference of RXRα for TRAP220 LXM1, supports the stoichiometric model proposed for TRAP220/NR-dimer complex formation. With RXR positioned on the 5' half-site of the nuclear hormone receptor response element (HRE) and its heterodimeric partner occupying the 3' half-site, one molecule of TRAP220 will bind to the heterodimer so that LXM1 interfaces with the RXR-AF-2 and LXM2 interfaces with the AF-2 of the heterodimeric partner NR, thus forming an activated transcription complex. The stoichiometry therefore of NR-dimer complexes with cofactors containing a single LXXLL motif e.g. Tip60, must differ from the stoichiometric model proposed above and is yet to be determined.

For comparative purposes the binding of the NID of SRC1 to a panel of NRs was also tested. In contrast to TRAP220, which showed a clear NR class preference, the NID of SRC1 was observed to bind to both NR classes with no apparent selectivity. The NID of SRC1 contains three LXXLL motifs, compared to the two LXXLL motifs of TRAP220 and perhaps this allows SRC1 an advantage over TRAP220 for NR-binding promiscuity. It has been shown that NRs do display differential usage of the LXMs within p160 coactivators (Darimont *et al.,* 1998, Leers *et al.,* 1998 and Kalkhoven *et al.,* 1998). Hence by containing three LXMs, p160s increase their chance of binding to different NRs by utilising different combinations of LXM pairs to facilitate binding to the AF-2 surfaces of the NR dimers. Results from this study imply that two functional LXXLL motifs of TRAP220 are optimally required to facilitate binding to NR-dimers. However since TRAP220 only has two LXMs, differential usage of LXM pairs of TRAP220 by NRs is not an option.

In order to investigate the NR-binding specificity of TRAP220 in more detail, the NR-binding properties of its two LXXLL core motifs were examined in turn. Remarkably TRAP220 LXM1 and LXM2 core motifs did not exhibit the same NR-binding specificity as that observed for the TRAP220 NID, and they were able to bind to all the NRs tested in a ligand-dependent manner. Therefore it would appear that whilst the core LXXLL motifs are critical for NR/cofactor binding, they are not solely responsible for imparting NR-binding specificity. Indeed previous studies utilising members of the p160 family of coactivators have shown that differential usage of the multiple LXMs of p160s is influenced by the amino acid sequence flanking the core LXXLL motif (Darimont *et al.*, 1998, McInerney *et al.*, 1998, Mak *et al.*, 1999 and Needham *et al.*, 2000). In particular the use of chimeric peptides has highlighted the importance of the core LXXLL motif flanking sequence in determining NR-binding specificity. For example, the LXM3 of GRIP1 was shown to have a lower affinity for TRβ-LBD than GRIP1 LXM2, but by replacing the flanking sequence of GRIP1 LXM3 with the flanking sequence derived from GRIP1 LXM2, a chimeric peptide exhibiting strong TRβ-LBD binding was generated (Darimont *et al.*, 1998). Similarly, the use of chimeric peptides has highlighted the importance of the core LXXLL amino acid flanking sequence in determining binding specificity to the class I NRs, ER and GR (Needham *et al.*, 2000).

Since LXM1 and LXM2 derived from TRAP220 did not exhibit the NR-binding specificity shown for the TRAP220 NID, it was proposed that this specificity could have been introduced due to the presence of the core LXXLL motif flanking amino acid sequences contained within the NID but which were absent from the core LXXLL motifs taken to test NR interactions. An extended version of the TRAP220 LXM1 core motif, incorporating additional wildtype flanking amino acid sequence, was generated and its NR-binding properties assessed. Strikingly, this addition of flanking amino acid sequence to the core LXM1 was enough to alter its NR-binding properties. The extended TRAP220 LXM1 sequence showed increased interaction (3.5 fold) with TRβ, but a 10-fold reduced interaction with the ERα-LBD. Thus the extended thirteen amino acid LXM1 sequence displays selective NR-binding properties similar to the TRAP220 NID. This suggests, as for the p160 family of coactivators, that the amino acid sequence flanking the core LXXLL motifs are a key determinant with respect to NR-binding specificity.

The thirteen amino acid peptide derived from Tip60 and incorporating its LXXLL motif was found to bind strongly to the ERα-LBD. However the NID of TRAP220 exhibits weak

interaction with the ERα-LBD and by extending the TRAP220 LXM1 core motif to a thirteen amino acid peptide, NR-binding specificity was introduced and reduced binding to the ERα-LBD was observed. This implies that the flanking amino acid sequences of Tip60 and TRAP220 LXM1 impose opposite NR-binding preferences with respect to the ERα-LBD. Sequence analysis of Tip60 and TRAP220 LXM1 extended sequences did not reveal any striking differences that could account for their opposing NR-binding preferences. Indeed the overall properties of the amino acids occupying specific positions in the core LXM and flanking sequences were similar. For example, in addition to the conserved leucine residues at positions +1, +4 and +5, both Tip60 LXM and TRAP220 LXM1 contain hydrophobic residues at positions –1, -2 and +7, basic polar residues at positions –3 and +6 and acidic polar residues at position +8. The differences between the two peptides, with respect to the nature of the residues, arise at position –4 where Tip60 has a acidic polar residue and TRAP220 LXM1 has a basic polar residue, position +9 where Tip60 has a acidic polar residue and TRAP220 LXM1 has a hydrophobic residue, and the core 'XX' position (+2 and +3) where Tip60 has two basic polar residues and TRAP220 LXM1 has two acidic polar residues. Rather interestingly, SRC1 LXM2 also possesses two basic polar residues at positions +2 and +3 and was observed to bind strongly with the ERα. Therefore perhaps ERα preferentially requires basic polar residues at these positions to facilitate optimal binding. The nature of the core LXXLL motif flanking amino acid sequence, with regard to NR-binding, will be discussed further in chapter 5.

In conclusion, the NID of TRAP220 was shown to interact with a panel of NR-LBDs and this interaction was dependant on, and mediated by two functional LXXLL motifs. RXRα-LBD was the exception in these experiments, requiring only functional TRAP220 LXM1 for interaction. Further, the NR-binding of the TRAP220 NID was NR-class specific, with preferential binding to class II NRs over class I NRs observed. However the core LXXLL motifs derived from TRAP220 did not display the same NR-binding selectivity as the TRAP220 NID and were able to bind to both class I and II NRs. By using an extended TRAP220 LXM1 peptide it was noted that this NR-binding specificity could be introduced by the presence of flanking amino acid sequence to TRAP220 LXM1. Hence the amino acid sequences flanking the core LXXLL motif have been implicated in the NR-binding specificity of TRAP220.

CHAPTER 5
RESULTS

Sequence Determinants of the Nuclear Receptor-binding Specificity of TRAP220

5.1 Introduction

The SRC1 family (p160s) of coactivators are able to form ligand-dependent interactions with a wide range of NRs, apparently exhibiting no NR-class binding selectivity. However other coactivators do display NR-binding selectivity. For example, Tip60 has been categorised as a class I specific coactivator due to its preferential binding to class I NRs (Gaughan *et al.*, 2001). In agreement with this, yeast two-hybrid data described previously shows that the extended LXXLL motif derived from Tip60 preferentially binds the class I NR, ERα (Chapter 4; Figure 4.15). In contrast, NRIF3 specifically binds RXR and TR (Li *et al.*, 2001), perhaps suggesting a class II NR-binding specificity. Thus, the existence of coactivators specific to certain NRs or NR subclasses, adds another level of complexity, and indeed regulation, to NR signalling.

Previously in this study, the NID of TRAP220 was shown to exhibit strong ligand-dependent interactions with TRβ, PPARγ and RXRα (Chapter 4; Figures 4.2 and 4.3). However, TRAP220 NID binding to the class I NRs, PR and ERα, was relatively weak and no binding was observed with the AR (Chapter 4; Figures 4.2 and 4.3). Thus TRAP220 appears to exhibit a NR-class binding specificity, preferring to bind class II NRs as opposed to class I. Intriguingly, the core LXXLL motifs derived from TRAP220 did not display the same NR-class binding specificity as the NID (Chapter 4; Figure 4.11). However, a class II-specific binding preference was re-introduced by extending the core LXXLL motif to encompass amino acids –4 to +9 (Chapter 4; Figure 4.13). Thus it was proposed that the amino acid sequences immediately flanking the core LXXLL motifs of TRAP220 could be contributing to the NR-binding specificity observed for the NID of TRAP220.

Described in this section of the study, is an examination of the core LXMs of TRAP220 and the amino acid sequences immediately adjacent to them, performed with the intention of identifying key residues involved in determining the NR-binding selectivity of the TRAP220 NID.

5.2. Identifying the key residues involved in determining the NR-binding selectivity of TRAP220 LXM1

5.2.1 Mutagenesis of LXM1 within the TRAP220 NID

To investigate the contribution to NR-binding specificity of the amino acids within the extended LXXLL motifs, a series of TRAP220 NID mutants were generated. All of the mutations (with the exception of the 'spacer' mutant - see below) involved an exchange of amino acids within the extended TRAP220 LXM1 sequence for the corresponding amino acids of the extended SRC1 LXM2 sequence (Figure 5.1). The LXM2 of SRC1 was chosen as this sequence shows strong interactions with both class I and class II NRs (Heery *et al.*, 2001). Both SRC1 LXM2 and the LXM of Tip60 possess basic polar residues at the +2 and +3 positions, and have been reported to strongly bind the class I NR, ERα, whilst TRAP220 LXM1 possesses acidic polar residues at these positions and the extended TRAP220 LXM1 motif showed weak binding to the ERα (Figure 4.13). This, together with a recent report demonstrating that the amino acid occupying the +2 position has a strong impact on the ability of core LXXLL motifs to bind to different NR-LBDs (Heery *et al.*, 2001), led us to speculate that perhaps the amino acids at positions +2 and +3 contributed to NR-binding specificity. Hence TRAP220 NID mutant E, which incorporated an exchange of amino acids at the +2 and +3 positions of LXM1, for those in the corresponding positions in SRC1 LXM2, was generated (Figure 5.1). TRAP220 mutant F incorporated an exchange of amino acids at positions -4, -3, -2 (N-terminal flank), +2, +3 ('XX' position), +7, +8 and +9 (C-terminal flank) of LXM1 with those in corresponding positions of SRC1 LXM2 (Figure 5.1). TRAP220 mutant G incorporated a single amino acid exchange at the –2 position (P602K) (Figure 5.1), since this position has been suggested to define subclasses of coactivators (Chang *et al.*, 1999).

The spacing between LXXLL core motifs in the SRC1 family is highly conserved, with 51 amino acids separating LXM1 from LXM2 and LXM2 from LXM3, of SRC1 (Heery *et al.*, 1997). By comparison, the spacing between LXM1 and LXM2 of TRAP220 is 36 amino acids. Both LBDs of the NR-dimer are occupied by a single LXXLL α-helix (Shiau *et al.*, 1998, Nolte *et al.*, 1998 and Darimont *et al.*, 1998) and hence the spacing between adjacent LMXs of coactivator proteins could influence specific NR-coactivator interactions. Indeed previous studies have shown that reducing the spacing between GRIP1 or TRAP220 motifs can negatively influence NR-binding (McInerney *et al.*, 1998 and Ren *et al.*, 2000). This suggests there may be an optimal spacing requirement for specific NR interactions. To examine whether

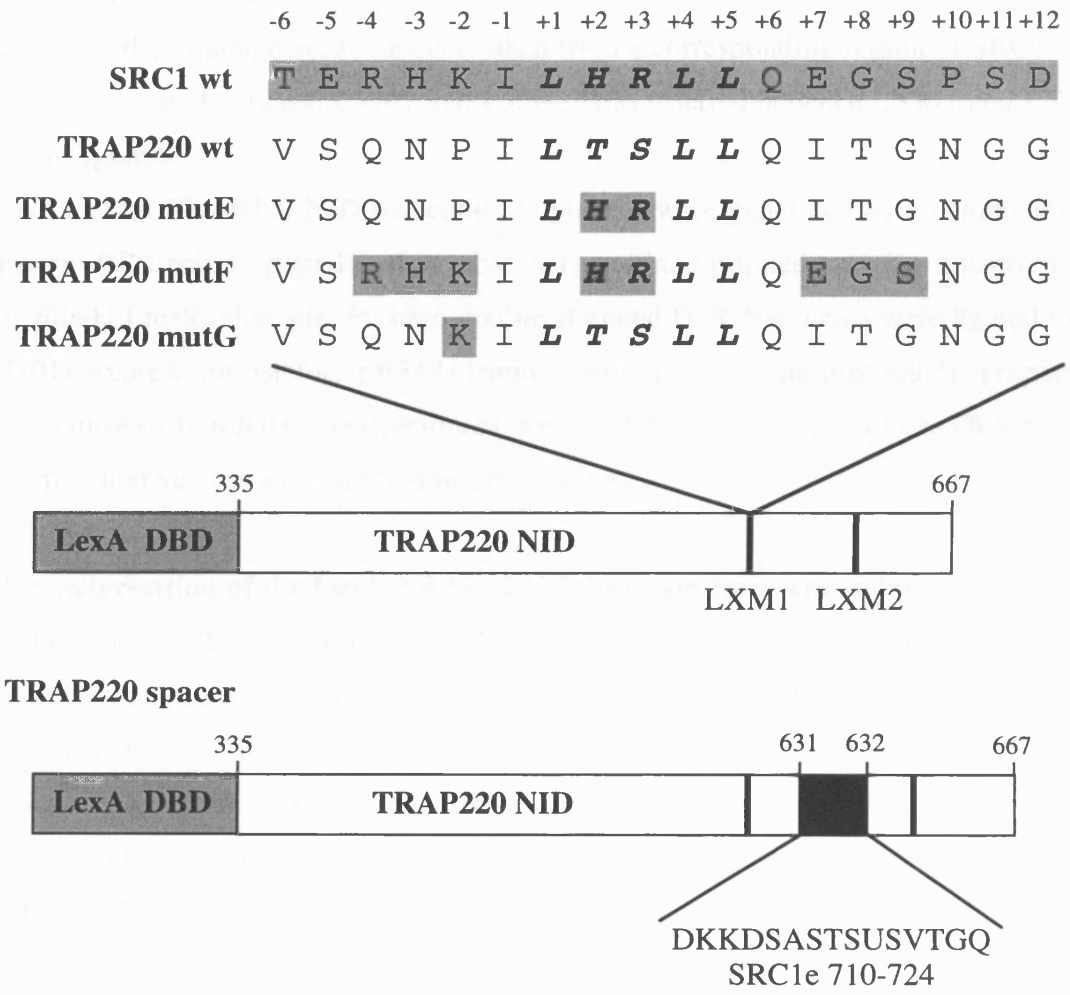


Figure 5.1 Schematic representation of the LexA-TRAP220 NID mutants. Wild type TRAP220 LXM1 (not shaded) and SRC1 LXM2 (shaded) sequences are shown and amino acid exchanges between TRAP220 and SRC1 sequences are highlighted. The additional SRC1-derived sequence used to generate the TRAP220 spacer mutant is indicated.

the spacing between LXM1 and LXM2 is a determinant of the TRAP220 preference for NR classes, the TRAP220 NID 'spacer' mutant was generated in which the spacing between LXM1 and LXM2 core motifs was increased to 51 amino acids, as in SRC1 family members. To achieve this, a fifteen amino acid sequence taken from a corresponding region of SRC1 (amino acids 710-724, located between LXM2 and LXM3) was inserted between LXM1 and LXM2 of TRAP220 (Figure 5.1).

All mutant TRAP220 NID nucleotide sequences were generated by recombinant PCR, using mutant PCR primer pairs listed in table A.1, and incorporated a 5' *Ksp* I restriction site and a 3' *Bam*H I restriction site. Purified double stranded PCR fragments were ligated into the LexA-DBD expression vector, pBTM116mod, which had been previously prepared by digestion with *Ksp* I/ *Bam*H I and treatment with CIAP. The resultant LexA-TRAP220 NID mutant expression vectors were verified by sequencing.

5.2.2 Characterisation of the LexA-TRAP220 NID mutant fusion proteins

The mutant LexA-TRAP220 NID fusion proteins were tested for intrinsic transcriptional activity before they could be used to assess mutant TRAP220 NID interactions with NRs. This ensured that the mutations incorporated within the NID of TRAP220 had not unintentionally generated an artificial transcriptional activation domain. L40 yeast were transformed with an expression vector for either LexA-lamin, LexA-VP16, LexA-TRAP220 mutant E, F, G or spacer and transformants were selected by growth on CSM lacking tryptophan. Cell-free extracts were prepared and used in β-galactosidase reporter assays. As shown in figure 5.2A, cell-free extracts derived from yeast expressing LexA-VP16 showed high levels of β-galactosidase activity (71 units of β-galactosidase activity). However, cell-free extracts derived from yeast expressing Lex-lamin or any of the LexA-TRAP220 NID mutants (E, F, G and spacer) showed negligible β-galactosidase activity. This confirmed that none of the LexA-TRAP220 NID mutant fusion proteins were able to activate transcription from the yeast β-galactosidase reporter gene in the absence of an ectopic VP16-AAD.

Expression of the LexA-TRAP220 NID wildtype and mutant fusion proteins was assessed by western blotting using an anti-LexA antibody. As shown in figure 5.2B, the LexA-TRAP220 NID wildtype and mutant fusion proteins were all represented, at comparable levels,

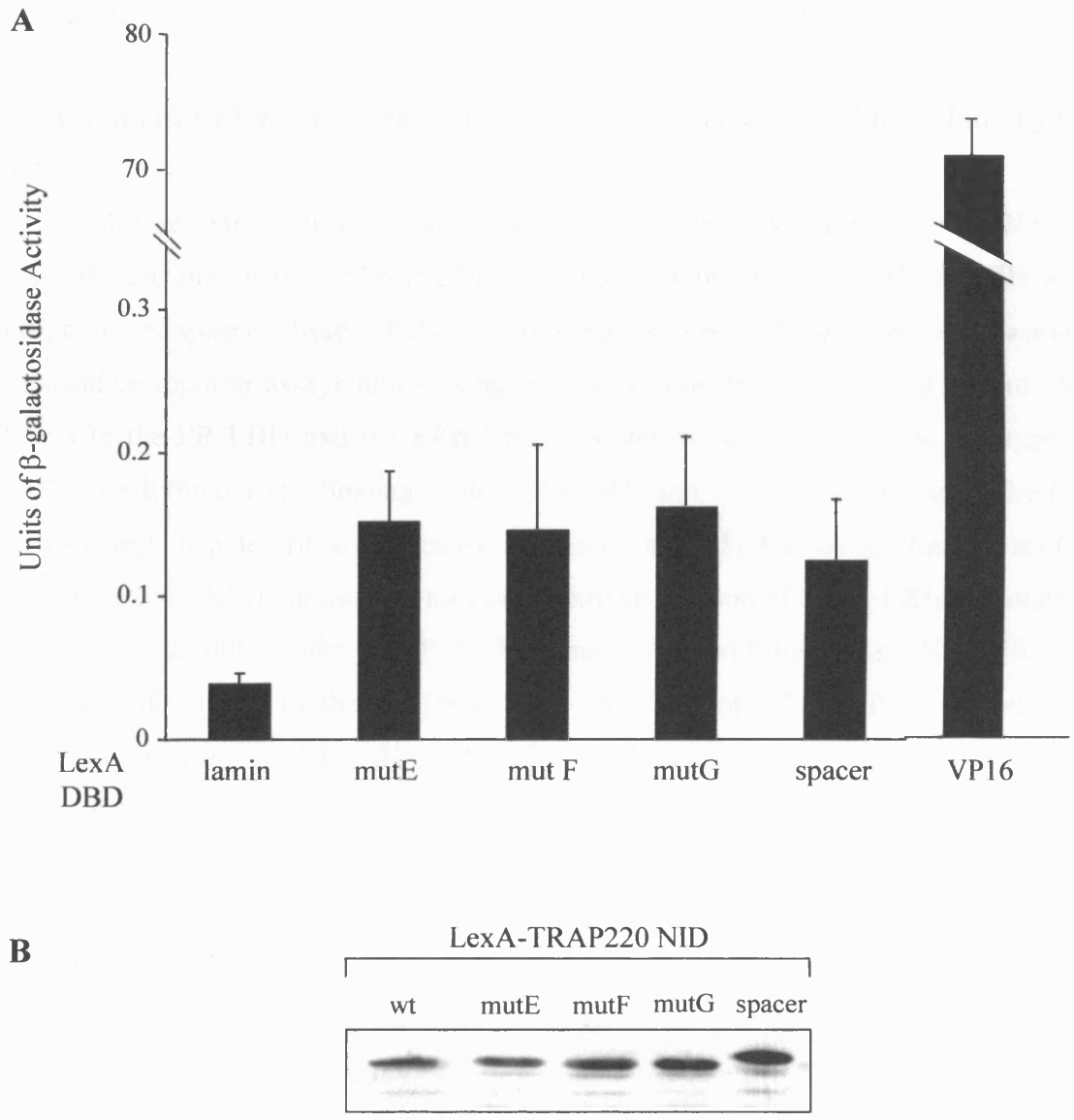


Figure 5.2 (A) Yeast two-hybrid β-galactosidase assay testing the transcriptional activity of LexA-lamin, LexA-VP16 and the LexA-TRAP220 NID mutants E, F, G and spacer, as indicated, in the absence of an ectopic activation domain. (B) Western blot, using an anti-LexA antibody, showing the expression of LexA-TRAP220 NID wt and the LexA-TRAP220 NID mutants E, F, G and spacer.

by bands approximating to 58 kDa. The LexA-TRAP220 NID spacer mutant displayed slightly lower mobility, approximating to 60 kDa, due to its additional amino acid sequence.

5.2.3 Mutations within the extended TRAP220 LXM1 sequence influence binding to class I NRs

Cell-free extracts were prepared from L40 yeast expressing VP16-PR-LBD or VP16-ERα-LBD and one of the following LexA-fusion proteins; LexA-TRAP220 NID wt, mutE, mutF, mutG or spacer. These cell-free extracts were subsequently used in yeast two-hybrid β-galactosidase reporter assays to assess the interactions of the wildtype and mutant TRAP220 NIDs with the PR-LBD and the ERα-LBD. As shown in figure 5.3, the wildtype NID of TRAP220 exhibited weak binding to the ERα-LBD and very weak binding to the PR-LBD, consistent with data described previously (Figure 4.2 and 4.3). Exchange of amino acids +2 and +3 of TRAP220 LXM1 for those in the corresponding position of SRC1 LXM2 (mutant E), had little effect (< 2-fold) on the TRAP220 NID interaction with the class I NRs, PR and ERα. Similarly, replacement of the proline at the –2 position of TRAP220 LXM1 with a lysine residue, as found in SRC1 LXM2 (mutant G), had little effect on TRAP220 NID interactions with the PR and the ERα. However, TRAP220 NID mutant F, in which eight amino acids at positions -4, -3, -2, +2, +3, +7, +8 and +9, had been exchanged for those in corresponding positions in SRC1 LXM2, exhibited a strongly enhanced ligand-dependent binding to the class I NRs, PR and ERα (4-fold and 10-fold, respectively). Therefore by exchanging the amino acids immediately flanking the TRAP220 core LXM1, for those derived from the corresponding positions of the high affinity class I NR-binding SRC1 LXM2 motif, the NR-binding properties of the TRAP220 NID have been changed. This further implicates the flanking amino acid sequences in determining NR-binding specificity. Increasing the spacing between TRAP220 LXM1 and LXM2 to 51 amino acids, was observed to have little effect on TRAP220 NID interactions with both the PR-LBD and the ERα-LBD, suggesting that increased spacing is not sufficient to allow strong interaction with class I NRs (Figure 5.3).

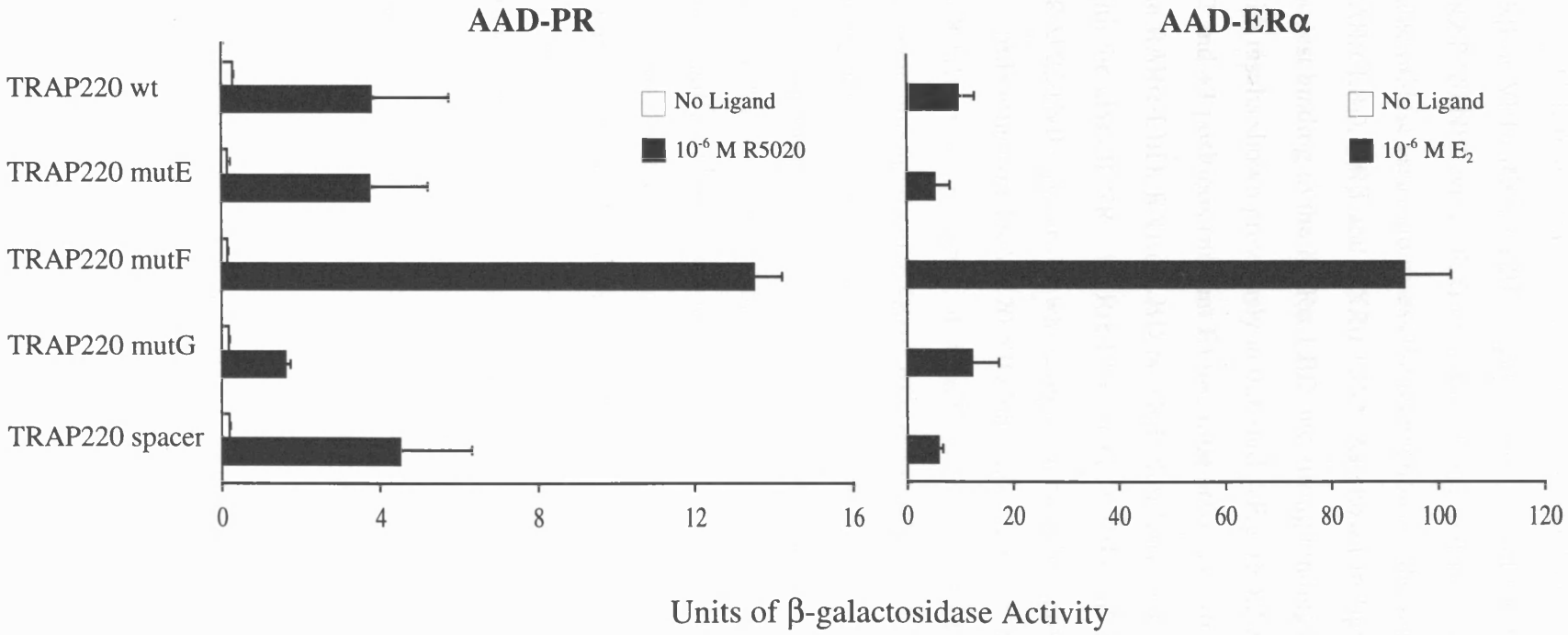


Figure 5.3 Assessing the NR-binding properties of the mutant TRAP220 NIDs with class I NRs. Yeast were transformed with either an expression vector for VP16-PR-LBD or VP16-ERα-LBD, together with an expression vector for one of the following LexA-fusion proteins; LexA-TRAP220 NID wt, LexA-TRAP220 NID mutE, LexA-TRAP220 NID mutF, LexA-TRAP220 NID mutG or LexA-TRAP220 NID spacer, as indicated. The β-galactosidase activity of cell-free extracts are shown.

5.2.4 The effects of mutations within the extended TRAP220 LXM1 sequence on class II NR interactions

Cell-free extracts were prepared from L40 yeast expressing VP16-RARα-LBD, VP16-TRβ or VP16-RXRα-LBD, together with one of the following LexA-fusion proteins; LexA-TRAP220 NID wt, E, F, G or spacer. These cell-free extracts were used in yeast two-hybrid β-galactosidase assays to assess the interactions of the wildtype and mutant TRAP220 NIDs with RARα-LBD, TRβ and RXRα-LBD. As shown in figure 5.4, the NID of TRAP220 showed modest binding to the RARα-LBD and strong binding to the TRβ and RXRα-LBD, consistent with results shown previously in this study (Figure 4.2 and 4.3). Exchange of amino acids at the +2 and +3 positions (mutant E) had little effect (< 2-fold) on TRAP220 NID interactions with the RARα-LBD, RXRα-LBD or TRβ. Similarly mutant F displayed comparable interactions with the class II NRs, RARα-LBD, RXRα-LBD and TRβ as those exhibited by the wildtype TRAP220 NID. However, whilst replacement of the proline at position –2 for lysine (mutant G) had little effect on TRAP220 NID binding to the TRβ, the ability of mutant G to bind the LBD of RARα in the presence of ligand was reduced ~2-fold. The ligand-dependent interaction between mutant G and RXRα-LBD was slightly reduced (~2-fold) compared to wild type and the ligand-independent binding to RXRα-LBD was increased. Perhaps therefore, the amino acid occupying the –2 position plays some role in RXRα-recruitment, although results here suggest that the mere exchange of a single amino acid at the –2 position is not sufficient to significantly affect the NR-binding properties of the TRAP220 NID.

As was the case for the class I NRs, PR and ERα, the insertion of a fifteen amino acid sequence between LXM1 and LXM2 of TRAP220 to increase the spacing to 51 amino acids, did not adversely effect the TRAP220 NID interactions with the class II NRs, TRβ or RXRα-LBD (Figure 5.4). However, somewhat surprisingly the reporter activity due to the interaction of the TRAP220 NID spacer mutant with the RARα-LBD was slightly reduced in a ligand-dependent manner, as compared to the wildtype TRAP220 NID. This suggests that correct spacing maybe more critical for interactions with the RARα-LBD.

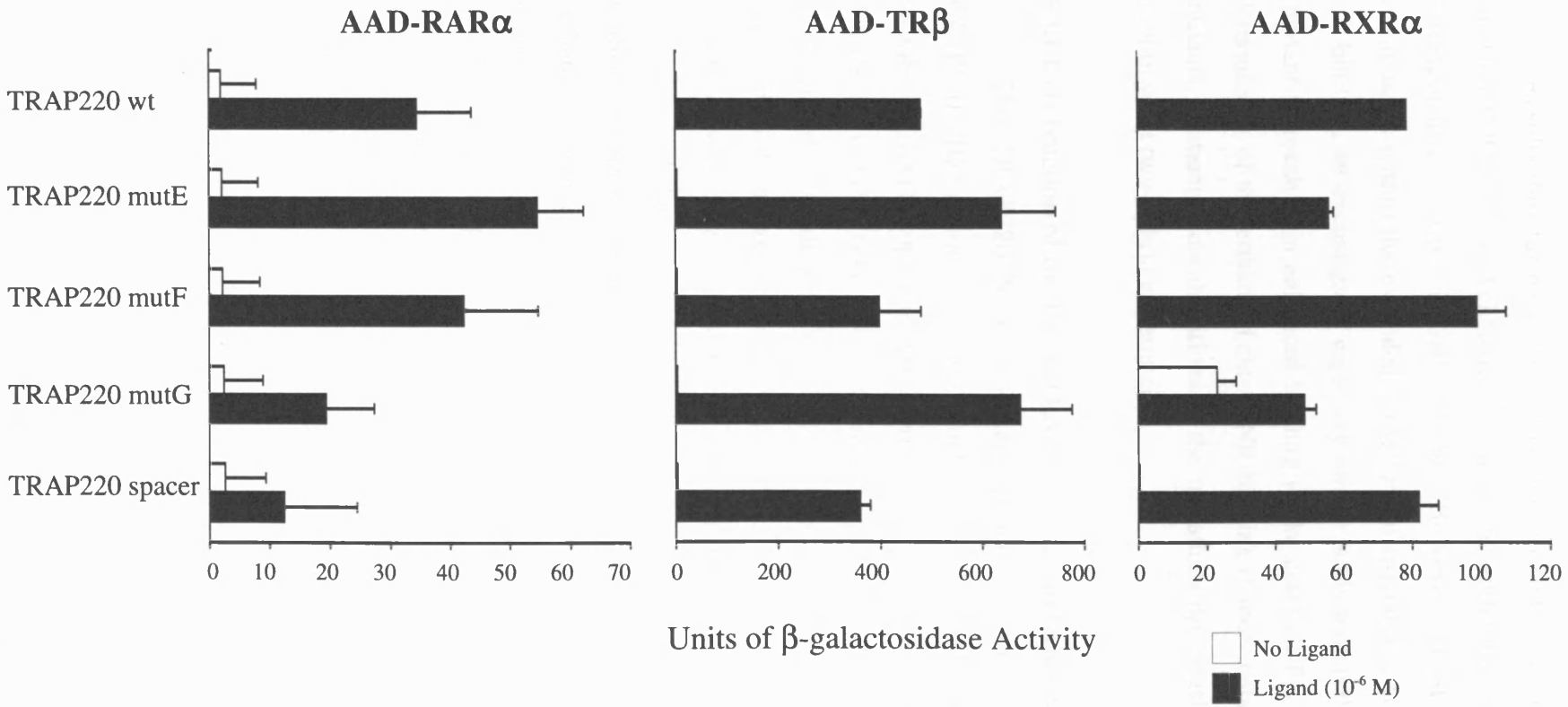


Figure 5.4 Assessing the NR-binding properties of the mutant TRAP220 NIDs with class II NRs. Yeast were transformed with either an expression vector for VP16-RARα-LBD, VP16-TRβ or VP16-RXRα-LBD together with an expression vector for one of the following LexA-fusion proteins; LexA-TRAP220 NID wt, LexA-TRAP220 NID mutE, LexA-TRAP220 NID mutF, LexA-TRAP220 NID mutG or LexA-TRAP220 NID spacer, as indicated. The β-galactosidase activity of cell-free extracts are shown and the ligands were, AT-RA, T_3 and 9c-RA for RARα, TRβ and RXRα, respectively.

5.3 Combinatorial effects of the mutations within the extended LXM1 on NR-binding specificity of the TRAP220 NID

Results thus far have shown that it is possible to change the NR-binding properties of the TRAP220 NID by exchanging amino acids within the extended LXM1 motif for those in corresponding positions in SRC1 LXM2. However, whilst exchange of single, or pairs of, amino acids within the extended LXM1 motif (mutants E and G) did not dramatically affect NR-binding, an exchange of eight key amino acids within the extended LXM1 of TRAP220 (mutant F) resulted in enhanced binding to the class I NRs. Therefore to investigate the key determinants of this enhanced class I NR-binding in more detail, a further set of TRAP220 NID mutants, containing combinations of the mutations previously examined, were generated and used in yeast two-hybrid experiments.

5.3.1 Construction of the LexA-TRAP220 NID combinatorial mutants

The TRAP220 NID combinatorial mutants were based on the mutations of the TRAP220 NID mutants E, F and G, and involved an exchange of amino acids in the extended TRAP220 LXM1 sequence with amino acids in corresponding positions in SRC1 LXM2 (Figure 5.5). To assess the contribution of the +2 and +3 amino acids in the observed enhancement in binding of TRAP220 NID mutant F with class I NRs, a mutant similar to mutant F, with the exception that amino acids +2 and +3 remained the same as in wildtype TRAP220, was generated (mutant A). Additionally, to assess the contribution of the N-terminal and C-terminal flanking amino acid sequences to the core TRAP220 LXM1, in defining NR-binding specificity, mutants B and mutant C were generated. In addition to amino acid exchanges at the +2 and +3 positions, mutant B also incorporated amino acid exchanges at positions +7, +8 and +9, to assess the importance of the C-terminal flanking amino acid sequence. Conversely, mutant C was generated to assess the importance of the N-terminal amino acid sequence, by incorporating exchanges at positions -4, -3 and –2, in addition to the +2 and +3 position amino acid exchanges. Finally, a TRAP220 NID mutant was generated which could further examine the importance of the N-terminal flanking amino acid sequence (mutant D). This mutant incorporated a proline instead of a lysine residue at the –2 position, in addition to an exchange at the +2 and+3 positions. All of the TRAP220 NID combinatorial mutants are depicted in figure 5.5. Expression vectors for the LexA-TRAP220 NID mutants A-

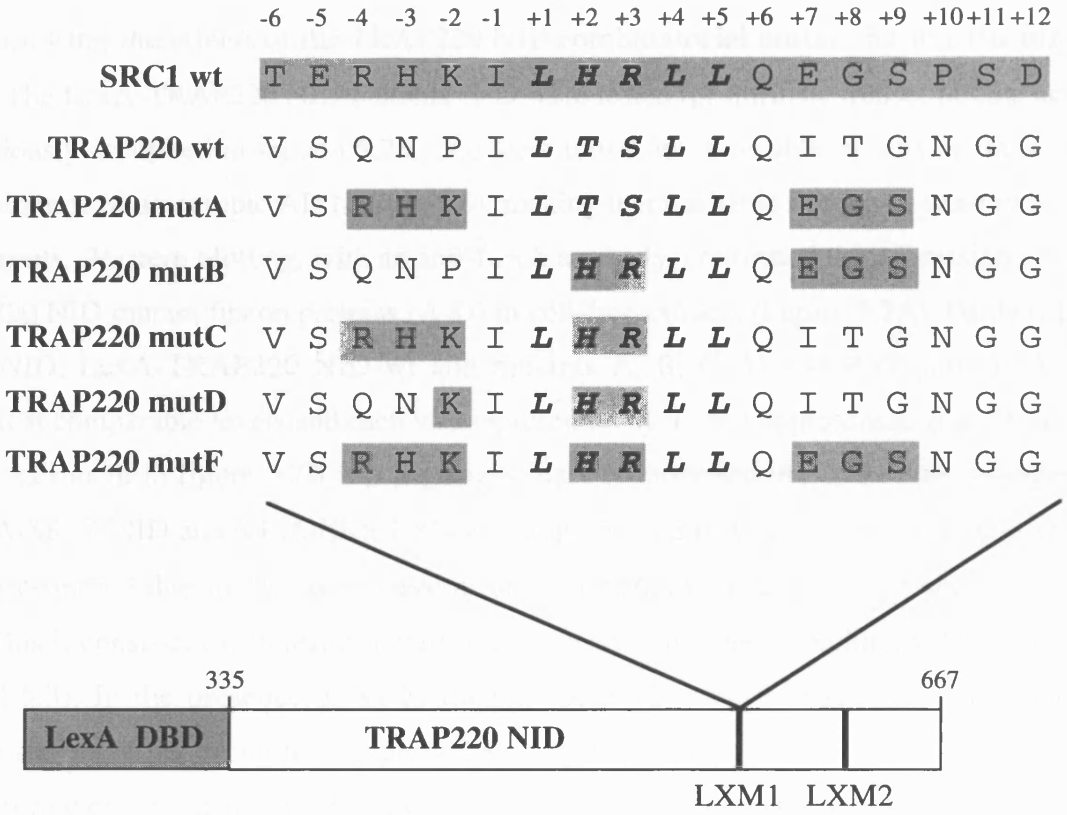


Figure 5.5 Schematic representation of the LexA-TRAP220 NID combinatorial mutants. Amino acid exchanges between TRAP220 (not shaded) and SRC1 (shaded) sequences are highlighted. The TRAP220 NID mutF sequence has been included for comparative purposes.

D were constructed as previously described for the LexA-TRAP220 mutants E-G (section 5.2.1) and verified by sequencing.

5.3.2 Assessing the effects of the TRAP220 NID combinatorial mutations on ERα-binding

The LexA-TRAP220 NID mutants A-D were tested for intrinsic transcriptional activity, as previously described in section 5.2.2, and were found not to be able to activate transcription in the absence of an ectopic AD (Figure 5.6), making them suitable for use in yeast two-hybrid experiments. Western blotting, with an anti-LexA antibody, confirmed the expression of LexA-TRAP220 NID mutant fusion proteins (A-D) in cell-free extracts (Figure 5.7A). Further, LexA-SRC1 NID, LexA-TRAP220 NID wt and mutants A, B, C, D and F (Figure 5.7A) were detected at comparable levels and each was represented by a band approximating to 58 kDa.

As shown in figure 5.7B, the β-galactosidase reporter activity due to the co-expression of LexA-SRC1 NID and VP16-ERα-LBD was high in contrast to the very low β-galactosidase activity observed due to the co-expression of the LexA-TRAP220 NID wt and VP16-ERα-LBD. This is consistent with results obtained previously under these conditions (Figure 4.2, 4.5, 4.7 and 5.3). In the presence of VP16-ERα-LBD, the LexA-TRAP220 NID mutant F was observed to promote strong transcriptional activity from the β-galactosidase reporter, which was a 19-fold enhancement over the level observed due to the expression of the wildtype LexA-TRAP220 NID. All of the combinatorial TRAP220 NID mutants (A-D) were observed to exhibit increased ERα-binding as compared to the wildtype NID. However, none were as efficient at binding the ERα-LBD as mutant F, which contained the entire SRC1 LXM2 extended motif. Mutant A displayed an 8-fold enhanced reporter activation due to binding the ERα-LBD, over the wildtype NID. This suggests that the amino acids at the +2 and +3 positions may be important in the context of the extended LXXLL motif, although alone (mutant E) they have only a minimal effect on NR-binding. Similarly, enhanced ERα-LBD interaction was observed for mutant C (6-fold), suggesting that the N-terminal flanking sequences (-4, -3 and -2) in combination with the +2 and +3 amino acids, have an important influence on TRAP220 NID NR-binding specificity. Moreover, a similar increase was observed for mutant D, which has a single amino acid exchange at the –2 position (P to K) in combination with the +2 and +3 amino acid exchange. This was in contrast to the results obtained for mutant G, which contained the P to K change only and mutant E, which had the +2

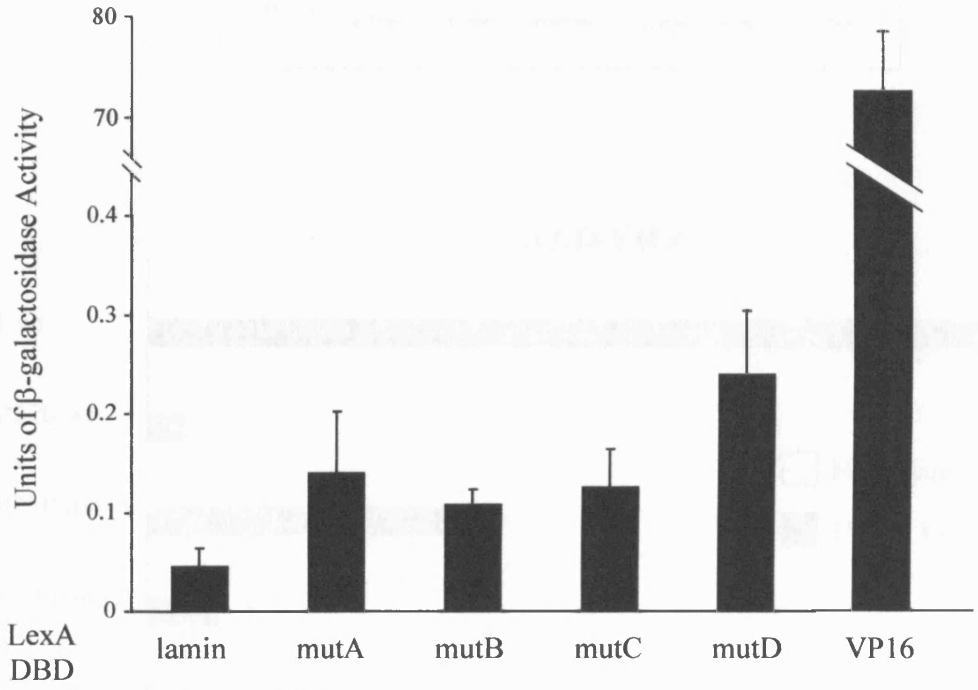


Figure 5.6 Testing the LexA-TRAP220 NID mutant fusion proteins for intrinsic transcriptional activity. L40 yeast were transformed with an expression vector for either LexA-lamin, LexA-TRAP220 NID mutA, LexA-TRAP220 NID mutB, LexA-TRAP220 NID mutC, LexA-TRAP220 NID mutD, or LexA-VP16, as indicated. Cell-free extracts were used in β-galactosidase reporter assays.

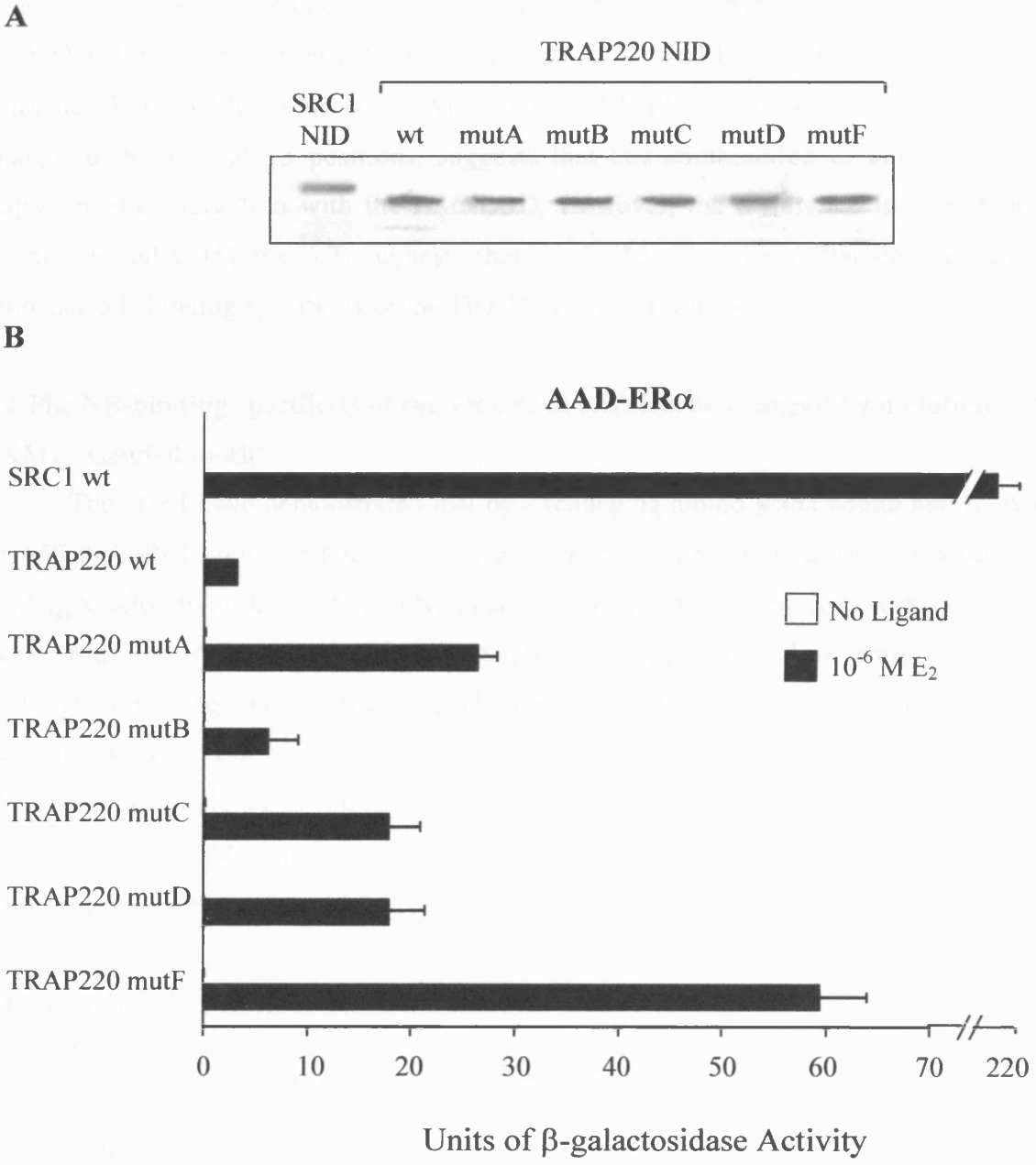


Figure 5.7 Assessing the NR-binding properties of the mutant TRAP220 NIDs with the ERα-LBD. (A) Western blot, using an anti-LexA antibody, showing the expression of LexA-SRC1 NID, LexA-TRAP220 NID wt and the mutant LexA-TRAP220 NID fusion proteins (A-D, F), in cell-free extracts. (B) Yeast two-hybrid β-galactosidase assay testing the interactions of the SRC1 NID, wt TRAP220 NID and the mutant TRAP220 NIDs with the ERα-LBD.

and +3 change only, neither of which showed a strong increase in binding to the ER α-LBD (Figure 5.3). Thus in combination, these mutations influence NR-binding specificity of TRAP220 LXM1. The relatively weak interaction of the ERα-LBD with mutant B, which contained SRC1 LXM2 sequence at the C-terminal flank (+7, +8 and +9), coupled with the change at the +2 and +3 positions, suggests that this combination of amino acids is less important for interaction with the ERα-LBD. However, the difference in ERα binding of mutants F and C (Figure 5.7) suggests that the LXM1 C-terminal flanking sequence does influence NR-binding specificity of the TRAP220 NID in some way.

5.4 The NR-binding specificity of the TRAP220 NID can be changed by mutation of the LXM2 extended motif

Thus far I have demonstrated that by exchanging amino acids within and flanking the TRAP220 LXM1 core motif for those of corresponding positions in SRC1 LXM2, the NR-binding specificity of the TRAP220 NID can be changed, and enhanced binding to class I NRs can be achieved. A TRAP220 NID LXM1 mutant, which contained the entire thirteen amino acid sequence of the SRC1 LXM2 extended motif (mutant F), gave the greatest enhancement in ERα binding over the wildtype NID, thereby suggesting that perhaps this sequence at the LXM1 position confers ERα binding. Therefore it was of interest to determine if this effect was restricted to the LXM1 position or whether this thirteen amino acid sequence could also permit ERα binding in the context of TRAP220 LXM2.

5.4.1 Construction of the LexA-TRAP220 NID LXM2 mutant (H)

To assess the effects of replacing the extended LXM2 sequence of TRAP220 (thirteen amino acids) with that of SRC1 LXM2, LexA-TRAP220 NID mutant H was generated (Figure 5.8). TRAP220 NID mutant H, incorporated amino acid exchanges at positions –4, -2, -1, +2, +3, +6, +7, +8 and +9, for those in corresponding positions in SRC1 LXM2, which in effect replaced the extended LXM2 of TRAP220 with that of SRC1 LXM2. The LexA-TRAP220 NID mutant H was generated as described previously in section 5.2.1 by recombinant PCR using mutant primer pairs listed in table A.1, and verified by sequencing.

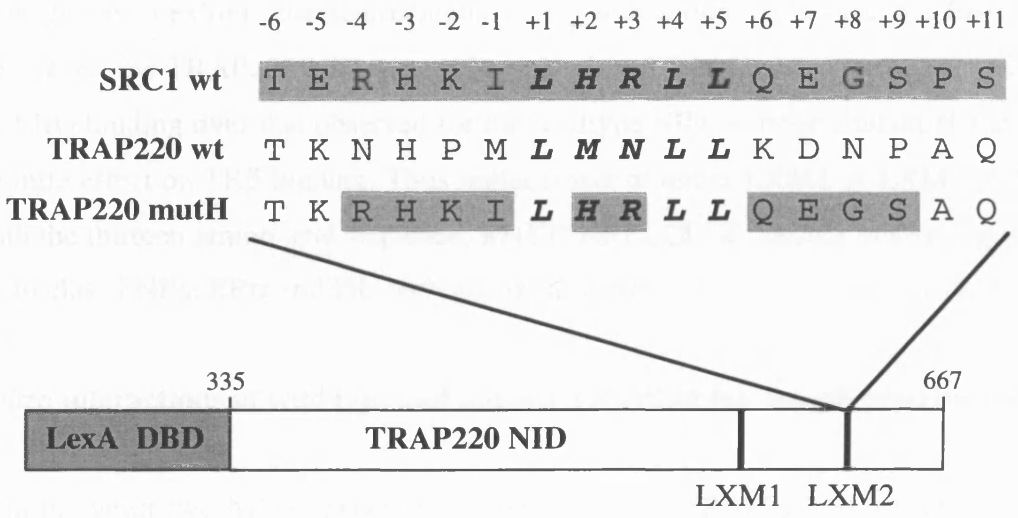


Figure 5.8 Schematic presentation of the LexA-TRAP220 NID mutant H fusion protein, which possesses a mutation within the LXM2. Wild type TRAP220 LXM2 and SRC1 LXM2 sequences are shown. SRC1-derived sequences are highlighted by shaded boxes.

5.4.2 The thirteen amino acid extended SRC1 LXM2 sequence confers ERα-binding

LexA-TRAP220 NID mutant H was found not to possess intrinsic transcriptional activity (Figure 5.9) and therefore its NR-binding properties could be tested in yeast two-hybrid experiments. As shown in figure 5.10, the wildtype TRAP220 NID exhibited typically weak binding to the ERα-LBD but strong binding to the TRβ. Under the same conditions, the SRC1 NID was shown to exhibit characteristically strong interactions with both the ERα-LBD and TRβ. However, the TRAP220 NID mutant H yielded an 8-fold increase in reporter activity due to ERα-LBD binding over that observed for the wildtype NID. Further, mutant H was observed to have little effect on TRβ binding. Thus replacement of either LXM1 or LXM2 of TRAP220 NID with the thirteen amino acid sequence, RHKILHRLLQEGS, results in a strong increase in binding to class I NRs, ERα and PR, without significantly affecting binding to class II NRs.

5.5 *In vitro* interactions of wild type and mutant TRAP220 full-length proteins with NR-LBDs

In the yeast two-hybrid experiments described, the TRAP220 NID LXM1 mutant that most effectively bound to the ERα-LBD contained the entire thirteen amino acid sequence of the extended SRC1 LXM2 motif in place of the corresponding wildtype TRAP220 LXM1 sequence. The same mutation was incorporated into a full-length TRAP220 protein in order to establish whether this mutation was sufficient to facilitate binding to the ERα-LBD *in vitro*.

5.5.1 Construction of a full-length TRAP220 LXM1 mutant F expression vector

pBTM116mod-TRAP220 NID mutant F was cleaved at its unique restriction sites, *Kpn*I and *Xho*I, and the 63bp fragment encoding amino acids 559-621 of TRAP220 and including the mutation (F) of the extended LXM1 motif was purified. pSG5(PT)-HA-TRAP220 (expressing wild type full-length TRAP220 protein) was also digested with *Kpn* I and *Xho* I, and the corresponding 63bp fragment of wild type TRAP220 sequence discarded. The remaining pSG5(PT)-HA-TRAP220 *Kpn* I/*Xho* I cut vector was treated with CIAP and ligated with the 63bp mutant F TRAP220 fragment. The resultant pSG5(PT)-HA-TRAP220 LXM1 mutant F vector was verified by sequencing and additionally, transcribed and translated *in vitro*, in the presence of [^{35}S]-labelled methionine, to verify expression of full-length TRAP220 protein (Figure 5.11, lane 3). As shown in figure 5.11, [^{35}S]-labelled protein, corresponding to

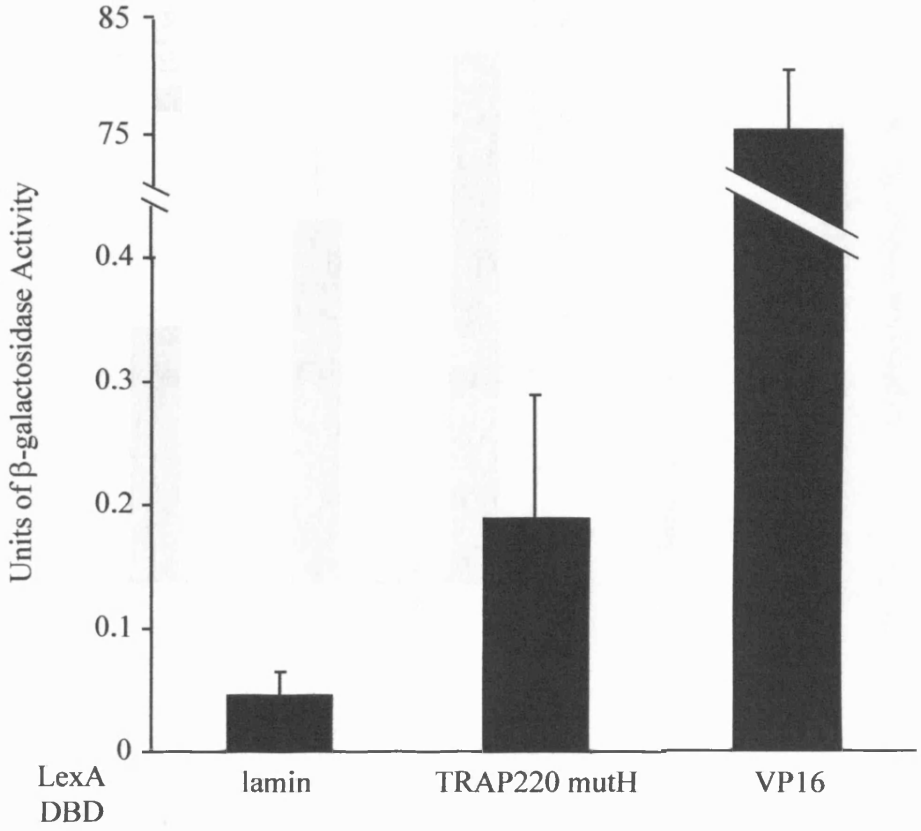


Figure 5.9 Testing the LexA-TRAP220 NID mutant H fusion protein for intrinsic transcriptional activity. L40 yeast were transformed with an expression vector for either LexA-lamin, LexA-TRAP220 NID mutH, or LexA-VP16, as indicated. Cell-free extracts were used in β-galactosidase reporter assays.

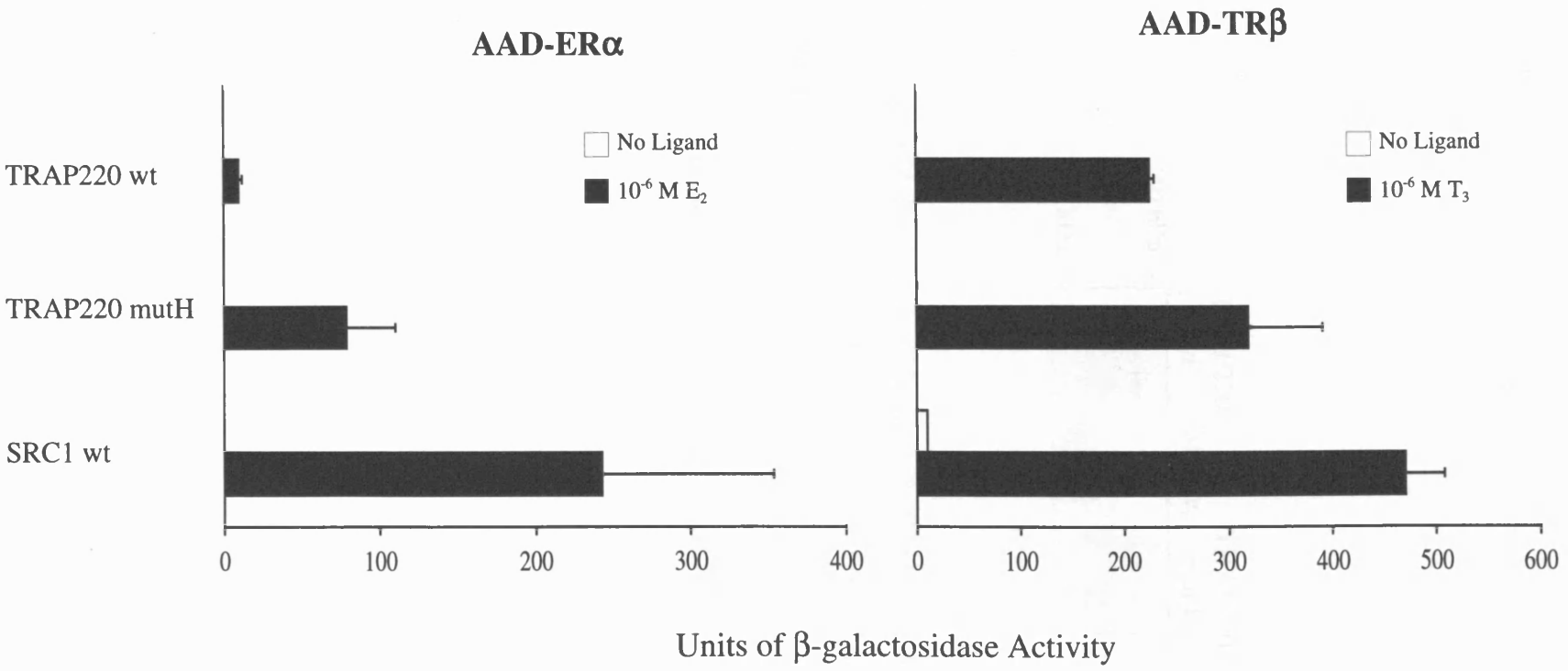


Figure 5.10 Assessing the NR-binding properties of the TRAP220 NID mutant H with ERα-LBD and TRβ. Yeast were transformed with either an expression vector for VP16-ERα-LBD or VP16-TRβ, together with an expression vector for one of the following LexA-fusion proteins; LexA-TRAP220 NID wt, LexA-TRAP220 NID mutH, or LexA-SRC1 NID, as indicated. The β-galactosidase activity of cell-free extracts are shown.

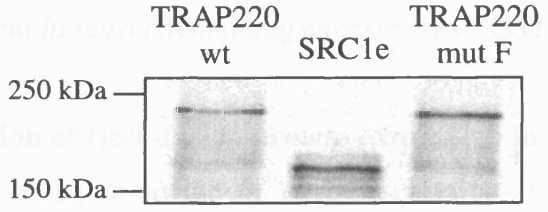


Figure 5.11 Generation of full-length TRAP220 LXM1 mutant F protein *in vitro*. [^{35}S]-labelled full-length wild type TRAP220 (lane 1), [^{35}S]-labelled full-length wild type SRC1e (lane 2) and [^{35}S]-labelled full-length mutant TRAP220 (lane 3) proteins were generated *in vitro*, separated by SDS-PAGE and visualised by autoradiography.

approximately 220 kDa was generated when using pSG5(PT)-HA-TRAP220 LXM1 mutant F vector in an *in vitro* transcription/translation experiment. Additionally, [^{35}S]-labelled full-length wild type TRAP220 protein was generated from the pSG5(PT)-HA-TRAP220 expression vector, and was represented by a band approximating to 220 kDa (Figure 5.11, lane 1), which ran at approximately the same position as the band representing the *in vitro* translated mutant TRAP220 protein. Together with the sequencing data, this confirmed that full-length mutant TRAP220 protein was being expressed from the pSG5(PT)-HA-TRAP220 LXM1 mutant F vector, *in vitro.* [^{35}S]-labelled full-length SRC1e protein was also generated from the pSG5-SRC1e expression vector for comparative purposes (Figure 5.11, lane 2), since SRC1e was used in subsequent *in vitro* NR-binding experiments ('GST-pulldowns').

5.5.2 Optimisation of GST-fusion protein expression in *E. coli.*

In order to examine the *in vitro* interactions of full-length mutant and wild type TRAP220 proteins with the LBDs of NRs, the Glutathione-S-Transferase (GST) gene fusion system or 'GST-pulldown' system was employed. This involved producing GST-NR-LBD fusion proteins which were subsequently immobilised on Glutathione-sepharose beads ('GST-beads') and incubated with [^{35}S]-labelled proteins. Initially production of the GST-fusion proteins in *E. coli* was optimised. *E. coli* strain DH5α was transformed with an expression vector for either GST protein alone, GST-ERα-LBD, GST-RXRα-LBD or GST-TRβ-LBD. Transformants were grown on a small scale to determine which clones were capable of producing either GST, GST ERα-LBD, GST-RXRα-LBD or GST-TRβ-LBD fusion proteins upon IPTG induction. The GST-fusion protein expression vector, pGEX, contains an IPTG-inducible *tac* promoter which controls the expression of the GST-fusion protein. It also contains an internal *lacI^q* gene. The *lacI^q* gene encodes a repressor protein that can bind to the operator region of the *tac* promoter thus preventing expression of the GST-fusion protein until induction by IPTG. Occasionally, basal or 'leaky' expression of the GST-fusion protein can occur under non-induced conditions, making it difficult to isolate clones containing the insert in the proper orientation. 'Leaky' expression occurs from a *lac* promoter located between the 3' end of the *lacI^q* gene and the *tac* promoter. Therefore small scale inductions are essential to identify IPTG-inducible GST-fusion protein expressing clones.

As shown in figure 5.12, clones transformed with an expression vector for either GST alone, GST ERα-LBD, GST-RXRα-LBD or GST-TRβ-LBD were grown in the absence or presence of IPTG, the cells harvested and subjected to SDS-PAGE. Each of the clones selected was shown to express either GST alone, GST ERα-LBD, GST-RXRα-LBD or GST-TRβ-LBD fusion proteins only upon IPTG induction and no GST-fusion protein expression was observed in the uninduced cultures. GST protein was represented by a band approximating to 26 kDa, and GST-ERα-LBD, GST-RXRα-LBD and GST-TRβ-LBD fusion proteins were expressed as 58 kDa, 52 kDa and 58 kDa proteins, respectively. These clones were used in large scale inductions to generate sufficient GST-fusion proteins to produce 'GST-beads' for use in *in vitro* binding experiments.

As described in sections 2.4.6 and 2.4.7, IPTG-induced DH5α, expressing either GST alone or a GST-NR-LBD fusion protein, were harvested and the cells lysed by sonication. The cell lysates were incubated with Glutathione-sepharose beads. SDS-PAGE analysis of samples collected during the preparation of the bacterial lysates revealed that the majority of the GST-fusion proteins were located in the post-sonicate pellet and were therefore insoluble. High level expression of foreign fusion proteins in *E. coli* frequently results in the formation of an insoluble product or aggregate, which is often referred to as an inclusion body. However it is possible to increase the solubility of the GST-fusion proteins by changing the growth parameters. Hence the bacterial growth conditions were optimised in order to maximise the production of soluble GST-fusion proteins. As well as adding lysozyme to aid the lysis of the bacterial cells, three different growth conditions were tested as follows:

1) Cultures were grown to an OD_{600nm} of 0.9 prior to IPTG induction at 30°C for 2 hours.
2) As for condition 1 except the time allowed for IPTG-induction was reduced to 1 hour and the temperature was reduced to 28°C. Lowering the growth temperature and inducing for a shorter period of time have both been found to increase the production of soluble non-degraded fusion proteins.
3) As for condition 2 except DTT, at a final concentration of 10 mM, was added to the NETN buffer at all stages. DTT is a reducing agent able to prevent the formation of disulphide bonds which may contribute to the production of insoluble GST-fusion proteins, and it has also been shown to increase the binding of some GST-fusion proteins to Glutathione-sepharose beads (Frangioni *et al.*, 1993).

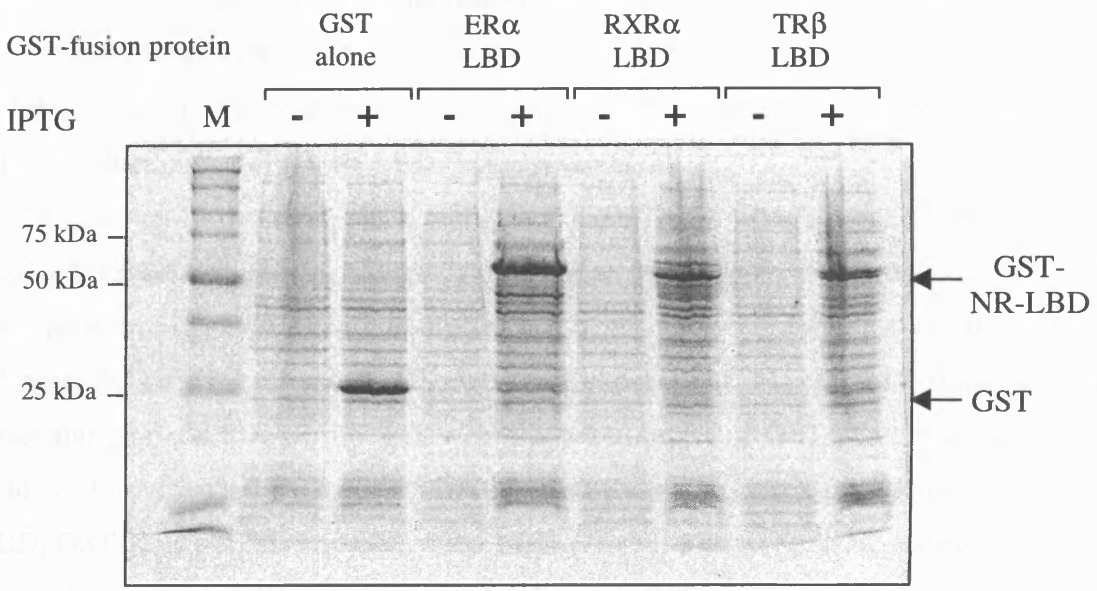


Figure 5.12 IPTG-inducible production of GST-fusion proteins in DH5α. Coomassie Blue stained SDS-polyacrylamide gel showing the IPTG-induced expression of GST protein and the GST-ERα-LBD, GST-RXR-LBD and GST-TR-LBD fusion proteins. Cultures of DH5α, transformed with either an expression vector for GST alone, GST-ERα-LBD, GST-RXRα-LBD or GST-TRβ-LBD, were grown in the absence ('uninduced'; -) or presence ('induced': +) of 0.5 mM IPTG and subsequently a sample of each was subjected to SDS-PAGE. 'M' represents the protein standards marker lane.

As shown in figure 5.13, soluble GST-ERα-LBD, GST-RXRα-LBD and GST-TRβ-LBD fusion proteins were all generated in sufficient quantities to facilitate the production of ERα-LBD-, RXRα-LBD- and TRβ-LBD-bound glutathione-sepharose beads. However there was a slight increase in the quantity of soluble GST-fusion protein produced with each successive growth condition used, with growth condition (1) yielding the least soluble GST-fusion protein available for GST-bead generation and growth conditions (2) and (3) yielding marginally more soluble GST-fusion proteins (1 < 2 < 3). Hence growth condition (3) was utilised for all subsequent productions of GST-NR-LBD fusion proteins.

Prior to GST-pulldown analysis, a sample of each GST-NR-LBD bead preparation was analysed by SDS-PAGE. This permitted an estimation of the relative amounts of GST-fusion proteins present in each GST-bead preparation and hence allowed normalisation of the volume of GST-beads to be used in each GST-pulldown experiment. Figure 5.14 shows a typical Coomassie blue stained SDS-PAGE gel, where equal volumes of GST-bead preparations have been loaded. As evidenced by the variation in intensity of the bands representing GST, GST ERα-LBD, GST-RXRα-LBD and GST-TRβ-LBD (26, 58, 52 and 58 kDa, respectively), there are not equal amounts of GST-fusion proteins in each GST-bead preparation and hence the volume of each GST-bead preparation was normalised prior to use in GST-pulldown experiments. Additionally, a series of bands representing lower molecular weight proteins were present in the GST-ERα-LBD, GST-RXRα-LBD and GST-TRβ-LBD bead preparations. Despite use of protease inhibitors, these are likely GST-fusion protein degradation products. However non-degraded GST-NR-LBD fusion proteins remained the dominant species and hence these GST-bead preparations, when normalised, were suitable for use in GST-pulldown experiments.

5.5.3 Wild type and mutant TRAP220 proteins display different *in vitro* NR-binding specificities

Normalised amounts of GST, GST-ERα-LBD, GST-RXRα-LBD and GST-TRβ-LBD, immobilised on Glutathione-sepharose beads, were incubated with equal amounts of *in vitro* translated, radiolabelled full-length SRC1e and TRAP220 wild type proteins or a TRAP220 LXM1 mutant F protein. As shown in figure 5.15, the GST control was unable to bind to SRC1e, wild type TRAP220 or mutant TRAP220, either in the absence or presence of E_2, 9c-

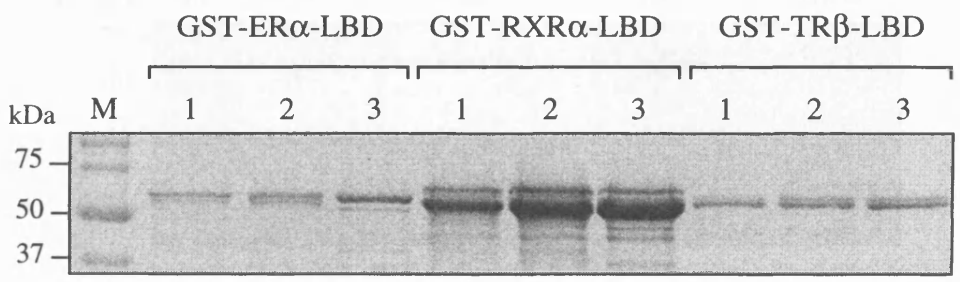


Figure 5.13 Optimisation of the bacterial growth conditions was required to maximise the production of soluble GST-fusion proteins. IPTG-induced bacterial cultures were grown under three different conditions (1, 2 and 3) as outlined in section 5.5.2, the cells were broken open by sonication and the lysates collected. Lysates were then incubated with Glutathione-sepharose beads to generate GST-beads, subjected to SDS-PAGE and the gel stained with Coomassie Blue. 'M' represents the protein standards marker lane.

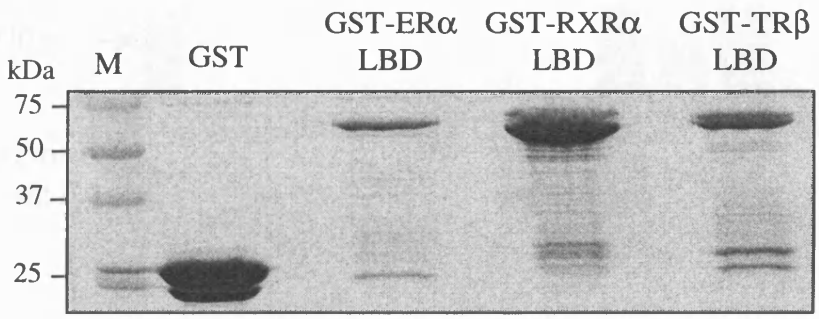


Figure 5.14 Normalisation of the 'GST-beads' was required prior to use in *in vitro* binding experiments. IPTG-induced bacterial cultures were grown under optimised growth conditions, the cells harvested and lysed, and the lysates collected. Lysates were incubated with equal quantities of Glutathione-sepharose beads to generate GST-beads. An equal sample of each GST-bead preparation was subjected to SDS-PAGE and the gel stained with Coomassie Blue. 'M' represents the protein standards marker lane.

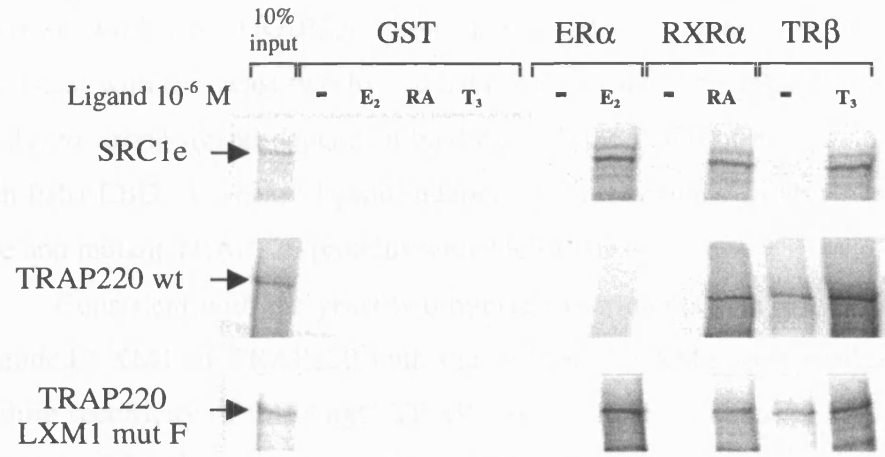


Figure 5.15 Interaction of wild type and mutant TRAP220 proteins with NR-LBDs *in vitro*. Normalised amounts of GST, GST-ERα-LBD, GST-RXRα-LBD and GST-TRβ-LBD proteins were immobilised on Glutathione-sepharose beads and incubated with equal amounts [^{35}S]-labelled full-length TRAP220 wild type (wt), TRAP220 LXM1 mutant F, or SRC1e wildtype protein (as control), in the presence or absence of 10^{-6} M cognate ligand, as indicated (9c-RA is abbreviated here to RA). Bound proteins were analysed by SDS-PAGE and autoradiography. One tenth of the total [^{35}S]-labelled protein used in the pull-down is shown for comparison (10% input).

RA or T_3. Predictably, SRC1e was shown to bind to the LBDs of ERα, RXRα and TRβ in a receptor cognate ligand-dependent manner. Under the same conditions, wild type TRAP220 was observed to bind to the LBDs of RXRα and TRβ in a ligand-dependent manner. In contrast, wild type TRAP220 showed little, if any, detectable binding to the ERα-LBD, consistent with the yeast two-hybrid data. Remarkably, the TRAP220 LXM1 mutant F protein displayed strong ligand-dependent binding with the LBDs of RXRα and TRβ, and additionally with ERα-LBD. A weaker ligand-independent interaction was also observed for both the wild type and mutant TRAP220 proteins with TRβ-LBD.

Consistent with the yeast two-hybrid experiments, exchange of the thirteen amino acid extended LXM1 of TRAP220 with that of SRC1 LXM2, was sufficient to change the NR-binding specificity of full-length TRAP220, allowing it to bind to the class I NR, ERα, whilst having no deleterious effects on class II NR-binding.

5.6 Assessing the coactivator function of mutant TRAP220 protein *in vivo*.

Yeast two-hybrid and *in vitro* binding experiments, have both shown that wild type TRAP220 exhibits very little interaction with the ERα, whilst a mutant form of TRAP220 (mutant F) exhibits strong binding with the ERα. It was therefore postulated that the newly acquired ERα-binding ability of the TRAP220 mutant might enable it to potentiate ERα transcriptional activity *in vivo*. To test this theory, a full-length TRAP220 mutant F protein was utilised in the ERα-mediated transactivation system previously described (section 3.7). HeLa cells were transiently transfected with a β-galactosidase reporter (as an internal transfection control), an ERE-linked firefly luciferase reporter (p3ERE-TATA-LUC) and an ERα expression vector. Additionally cells were transfected with either an SRC1e, wild type TRAP220 or mutant TRAP220 expression vector as indicated (Figure 5.16). SRC1e was included for comparative reasons. As shown in figure 5.16, basal level of transcription was represented by cells transfected in the absence of E_2, with β-galactosidase and ERE-linked luciferase reporters only. Basal level of transcription was set at 1 and all other values were expressed relative to this. Upon expression of ERα, the transcriptional activity from the luciferase reporter was increased 67-fold over the basal level in an E_2-dependent manner, consistent with levels seen previously (Figure 5.16). SRC1e expression resulted in a 10-fold, E_2-dependent, enhancement in ERα-mediated transcription of the luciferase reporter gene and

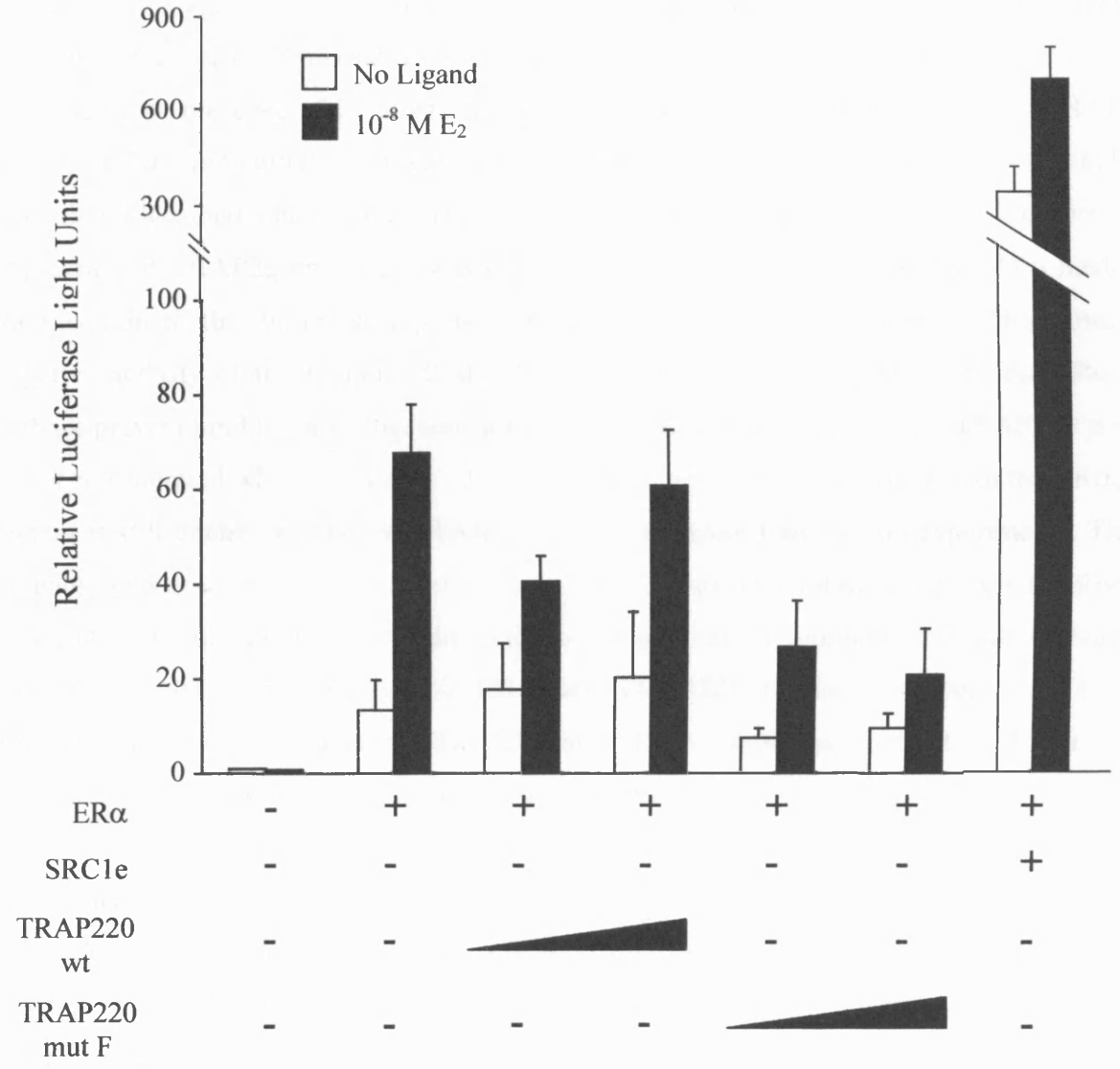


Figure 5.16 Both mutant and wild type TRAP220 are unable to enhance ERα-mediated transcriptional activity. HeLa cells were transiently transfected with 500 ng pJ7-LacZ and 1 μg p3ERE-TATA-LUC. Cells were additionally transfected with 100 ng pMT-MOR, 500 ng pSG5-SRC1e or increasing amounts of pSG5(PT)-HA-TRAP220 wt (500 ng and 1 μg), or pSG5(PT)-HA-TRAP220 LXM1 mutant F (500 ng and 1 μg), as indicated. Luciferase activity was measured and normalised to the β-galactosidase transfection control. Basal level transcriptional activity of the luciferase reporter was set at 1 and all other values are expressed relative to this. The values shown represent the average of triplicate samples and the error bars indicate standard deviation.

also a 5-fold enhancement in an E $_2$-independent manner, as seen and discussed previously (Figure 3.6). Typically, expression of wild type TRAP220 protein was observed to have no apparent effect on ERα-mediated transcription of the ERE-linked luciferase reporter in either the absence or presence of E_2, suggesting little or no interaction between wild type TRAP220 and the ERα. Intriguingly, expression of the TRAP220 mutant F protein was not able to potentiate ERα-mediated transactivation in either the absence or presence of E_2. Moreover, the expression of TRAP220 mutant F was observed to modestly repress (~3-fold) ERα-mediated transcription of the luciferase reporter gene in a ligand-dependent manner. This dominant negative activity of the mutant TRAP220 suggests it is able to interact with the ERα and perhaps prevent binding of endogenous coactivators. Thus, although a mutant TRAP220 protein has been generated which is capable of strong ligand-dependent interactions with the ERα, this mutant is still unable to potentiate ERα activity in transient transfection experiments. This is probably due to its inability to be assimilated into endogenous TRAP complexes (R. Roeder, personal communication). This additionally could explain the anomalous results obtained in transfected cells expressing ectopic ERα and TRAP220 proteins compared to *in vitro* transcription experiments using purified ERα and TRAP complexes (Kang *et al.*, 2002), where the TRAP complex is shown to enhance ERα activity.

5.7 Discussion

Yeast two-hybrid experiments revealed that the NID of TRAP220 exhibits a NR-binding specificity, preferentially binding to class II NRs as opposed to class I NRs. By contrast, the NID of SRC1 was observed to bind to both class I and class II NRs with no apparent selectivity. The core LXXLL motifs derived from TRAP220 (LXM1 and LXM2) did not display the NR-binding specificity exhibited by the NID and were found to bind to both class I and class II NRs. Intriguingly, the NR-binding specificity was re-established by extending the core LXM1 sequence at the N- and C-terminal flanks. Thus it was proposed that the NR-binding specificity of the TRAP220 NID is influenced by the amino acid sequences flanking the core LXXLL motif.

In order to determine which residues in the extended TRAP220 LXM1 sequence are important for defining TRAP220s NR-binding specificity, a panel of TRAP220 NID mutants (A-H) were used in yeast two-hybrid experiments. Initially, it was confirmed that the extended

LXM1 sequence is a major determinant of TRAP220 NR-binding specificity, by exchanging the entire LXM1 for the corresponding sequence of SRC1 LXM2 (mutant F). This resulted in a strongly enhanced interaction of the TRAP220 NID with the ERα and PR, but had little or no effect on binding to class II NRs. A similar result was recently reported in which replacement of TRAP220 LXM1 (-5 to +9) with the corresponding sequence from TIF2 LXM2, enhanced binding to the ERα in contrast to wild type TRAP220 (Warnmark *et al.*, 2001). This effect is not restricted to LXM1, as data from this study also shows that exchange of the LXM2 extended motif of TRAP220 with that of SRC1 LXM2 (mutant H) results in enhanced binding to the ERα-LBD, without affecting binding to the TRβ.

Detailed mutational analysis of the TRAP220 extended LXM1 sequence was undertaken to further identify key residues important in defining TRAP220s NR-binding specificity. A previous study used phage display technologies to identify subclasses of LXXLL sequences that show differential interactions with the ERα (Chang *et al.*, 1999). Both LXMs of TRAP220 fall into the subclass typified by having a conserved proline residue at the –2 position and exhibiting relatively weak interactions with the ERα. The unique structure of proline means that its presence in α-helices creates a distortion or 'kink'. Perhaps therefore the coactivator binding pocket of class I NRs, such as PR and ERα, cannot optimally accommodate the distorted α-helices created from the extended LXXLL motifs of TRAP220. By replacing the proline residue at the –2 position, the local helical structure of the LXXLL motifs might be altered to enhance class I NR-binding. In contrast to the LXMs of TRAP220, LXM2 of PGC-1, which has been shown to be the major contributor of PGC-1 interactions with the LBDs of several NRs including TRβ1, RXRα, PPARα, GR and ERα (Knutti *et al.*, 2000, Tcherepanova *et al.*, 2000, Vega *et al.*, 2000, Delerive *et al.*, 2002, Wu *et al.*, 2002), belongs to the third subclass of LXXLL sequences typified by having a conserved serine (or threonine) residue at the –2 position. Recent studies have shown that PGC-1 displays a unique mode of interaction with the LBDs of TRβ and PPARγ, whereby the presence of a serine residue at the –2 position of LXM2 appears to negate the need for a functional charge clamp (Wu *et al.*, 2002 and Wu *et al.*, 2003). Mutant forms of TRβ and PPARγ, in which the conserved glutamic acid residue in helix 12 (forming one half of the charge clamp responsible for recognition of the coactivator LXXLL motif) had been mutated, thus abolishing the charge clamp, were still able to recognise and bind the LXM2 of PGC-1 due to the presence of the serine residue at the –2 position. Computer modelling revealed that the conserved serine residue of PGC-1 LXM2 is in close

proximity to Glu-471 (the conserved glutamic acid residue in helix 12 forming one half of the charge clamp) of PPARγ, thus placing it in an ideal position for charge clamp recognition. This suggests that the nature of the amino acid occupying the –2 position of extended LXM sequences is important with regard to NR-binding. In p160s, three lysine residues flanking the LXM1 motif, including a lysine at the –2 position, have been shown to be targets for acetylation by CBP/p300, and this event assists the dissociation of NR/p160 complexes (Chen *et al.,* 1999a). This lysine residue at the –2 position potentially makes electrostatic contact with Glu-380 in helix 5 of the ERα LBD (Chang *et al.,* 1999), thus stabilising and enhancing the ERα/p160 interaction. Therefore a basic lysine residue at the –2 position may be optimally required for ERα-binding. However exchange of the proline residue at the –2 position in TRAP220 LXM1 (mutant G) for a lysine residue, did not significantly change the NR-binding properties of the TRAP220 NID and this mutant retained low affinity binding to class I NRs. Similarly, substitution of proline for alanine at the –2 position of TRAP220 LXM2 had little effect on binding with the TRα, VDR or PPARγ (Ren *et al.,* 2000).

Due to the α-helical nature of the LXXLL motif, the non-conserved +2 and +3 positioned amino acids are predicted to be solvent exposed and hence not in direct contact with the AF-2 surface of the NR-LBD (Heery *et al.,* 1997, Nolte *et al.,* 1998 and Shiau *et al.,* 1998). Amino acids occupying these positions would therefore not be expected to contribute to the NR-binding specificity exhibited by some NR cofactors. Despite this fact, several studies have highlighted the importance of the +2 and +3 amino acids in NR/cofactor interactions. Mutation of the +2 and +3 amino acids of the variant FXXLL motif of NSD1 to alanines (ST-AA) abolished binding to both class I and class II NRs (Huang *et al.,* 1998). Similarly, a +3 mutation in TIF2 LXM3 has been reported to reduce its interaction with the ERα (Warnmark *et al.,* 2002). Use of GRIP1 chimeric peptides, in which the flanking amino acid sequence of the low affinity GR-binding LXM2 motif was exchanged for that of the high affinity GR-binding LXM3 motif, revealed that the chimeric GRIP1 LXM2 motif still could not bind to the GR as strongly as the wild type LXM3 (Darimont *et al.,* 1998). This suggests the non-conserved +2 and +3 positioned amino acids could be contributing to NR-binding specificity. In contrast, replacement of the +2 and +3 amino acids of GRIP1 LXM2 for alanines was found to have no significant effect on TRβ-binding (Darimont *et al.,* 1998). Results of the yeast two-hybrid experiments in this study show that exchange of the +2 and +3 amino acids of TRAP220

LXM1, for positively charged equivalents of SRC1 LXM2 (mutant E) did not by itself enhance binding of the TRAP220 NID to class I NRs, or indeed alter its interactions with class II NRs.

TRAP220 NID mutants A-D were designed to assess the effects of combinatorial changes in the extended TRAP220 LXM1 sequence on NR-binding specificity. All of these mutants displayed enhanced interaction with the ERα as compared to the wild type NID and highlighted the relative importance of each component of the extended LXXLL motif in determining NR-binding specificity. For example, ERα binding to mutant F was greater than to mutant A, indicating that the +2 and +3 positions are important for optimal binding but an exchange of these amino acids alone is not sufficient to change the NR-binding specificity of the TRAP220 NID. Similarly, exchange of the +2 and +3 amino acids resulted in increased ERα binding only when combined with an exchange of the N-terminal (-4, -3 and –2), or to a lesser extent the C-terminal (+7, +8 and +9) flanking sequences (compare mutant E with mutants B and C). This suggests that the N-terminal flanking sequence is more important than the C-terminal flanking amino acid sequence in determining NR-binding specificity. Further, the similarity of ERα interaction with mutants C and D suggests that the –2 position plays a key role in the interaction of the ERα with the N-terminal flanking sequence of TRAP220 LXM1, possibly via the Glu-380 residue in helix 5 of the ERα (Chen *et al.,* 1999a). Taken together, these results indicate that the exchange of the entire extended LXM1 sequence of TRAP220 for the corresponding sequence of SRC1 LXM2 is required for optimal enhancement in binding to the ERα and that residues within the core motif and flanking sequence cooperate to determine NR-binding specificity.

In addition to investigating the key residues of the extended LXM1 sequence which may be contributing towards the NR-binding specificity exhibited by TRAP220, the spacing between the LXMs of TRAP220 was also investigated to assess if it too has any influence over NR-binding specificity. Other studies have shown that a reduction in the spacing between LXXLL motifs in p160s (McInerney *et al.,* 1998) or TRAP220 (Ren *et al.,* 2000) has adverse effects on NR interactions. This suggests specific spacing between adjacent LXMs is a steric prerequisite for binding NR dimers and that a minimal sequence length is required to generate a folded or flexible domain which can permit docking of both LXM1 and LXM2 with both AF-2 surfaces on NR dimers. The spacing between adjacent LXMs of p160s is highly conserved (51 amino acids), even across species, and differs to that found between the LXMs of TRAP220 (36 amino acids). To investigate whether this reduced spacing between the LXMs of TRAP220, as

compared to p160s, was contributing to its NR-binding specificity and in particular, its weak binding to class I NRs, a mutant TRAP220 NID in which the spacing between LXM1 and LXM2 was increased to 51 amino acids was generated and used in yeast two-hybrid experiments. This 'spacer' mutant showed no enhanced interaction with class I NRs, nor was its binding to class II NRs adversely affected. Thus, while a minimal spacer sequence may be required to allow contact with both AF-2 surfaces, the exact spacing does not appear to be critical. Moreover, the absence of any effect of the spacer mutation on binding to class II NRs, is consistent with the hypothesis that rather than folding into a rigid domain, the NID may be a flexible or largely unstructured sequence, accommodating interactions with the AF-2 surfaces of each member of the NR dimer.

In support of the yeast two-hybrid data, *in vitro* binding studies were also used to demonstrate that the NR-binding specificity of TRAP220 can be changed by replacement of its extended LXM1 sequence for that of SRC1 LXM2. An *in vitro* translated full-length mutant TRAP220 protein was found to bind to the LBDs of TRβ, RXRα and ERα, whereas a full-length wild type TRAP220 protein bound the LBDs of TRβ and RXRα but showed very little, if any, binding to the ERα-LBD.

It was demonstrated previously that over-expressed wild type TRAP220 protein had no effect on ERα activity *in vivo*, and nor did it interfere with SRC1-mediated enhancement in ERα activity (chapter 3; Figures 3.6 and 3.7). This suggested that wild type TRAP220 is unable to bind to the ERα. Yeast two-hybrid and *in vitro* binding experiments confirmed that wild type TRAP220 exhibited relatively weak ligand-dependent binding with the ERα (chapter 4, Figure 4.2 and chapter 5, Figure 5.15). It was therefore predicted that a mutant TRAP220 protein, capable of strong interactions with the ERα-LBD, might potentiate ERα transcriptional activity. Surprisingly, over-expressed mutant TRAP220 protein was observed not to enhance ERα activity in transient transfection experiments, suggesting that enhancing TRAP220 binding to the ERα is not sufficient to allow it to potentiate ERα transcriptional activity under these conditions. Conversely, a mutation of the –3 position amino acid of the LXM of the Thyroid Receptor Binding Protein (TRBP) (S884Y), which was observed to enhance the binding of TRBP to the ERβ, was also observed to increase the coactivator potential of TRBP for the ERβ (Ko *et al.*, 2002). Thus in this case, increased binding to a given NR correlates with increased coactivation of that NR. However, in contrast to TRBP, TRAP220 functions as part of a multi-subunit complex, where it makes as yet undefined contacts with other subunits within

the complex. Inefficient assembly of the ectopically expressed mutant TRAP220 proteins into the functional TRAP complexes might therefore explain why in the case of mutant TRAP220, strong ERα binding does not correlate with the ability to potentiate ERα transcriptional activity. Indeed the expression of mutant TRAP220 protein was found to modestly repress ligand-dependent ERα activity, suggesting the mutant TRAP220 protein can bind the ERα and is perhaps competing with endogenous coactivators for ERα binding.

In summary, the NR-binding specificity of TRAP220 can be changed by replacement of its extended LXM1 or LXM2 sequences with the corresponding sequences derived from the SRC1 LXM2. Mutational analysis revealed that the entire 13 amino acid extended sequence of SRC1 LXM2 in the context of TRAP220 LXM1 or LXM2 is needed to facilitate strong binding of TRAP220 to class I NRs. Amino acids within the core motif and flanking sequences cooperate to bring about this change in specificity. In contrast to the intriguing effects on NR-binding specificity achieved here by mutation of the core motif and flanking amino acid sequences, an increase in the spacing between TRAP220 LXXLL motifs, to resemble that found in p160s, had little effect on NR-binding.

CHAPTER 6
DISCUSSION

6.1 TRAP220 enhances the transcriptional activity of TRβ but not ERα

Analyses of the TRAP/SMCC/Mediator complex has revealed that its TRAP220 subunit is unique, displaying ligand-dependent interactions with the AF-2 of NRs. Consistent with the functional relevance of these NR-interactions, ectopic expression of TRAP220 has been shown to induce a modest enhancement in TRα-, VDR- and PPARγ-mediated transcription in transiently transfected cells (Yuan *et al.*, 1998, Rachez *et al.*, 2000 and Zhu *et al.*, 1997). In agreement with this, exogenously expressed TRAP220 was shown here to induce a moderate enhancement of TRβ activation of a DR4-driven reporter gene (Chapter 3). The relatively weak coactivator activity of TRAP220 in these transient transfection experiments, and those of other groups, might be due to the inefficient assembly of exogenous TRAP220 proteins into functional TRAP/SMCC/Mediator complexes. In contrast, transiently over-expressed TRAP220 failed to have any significant affect on ERα-mediated transcription of a 3ERE-TATA-LUC reporter gene, nor did it interfere with the SRC1e-induced enhancement of ERα-mediated transactivation. Thus, the inability of TRAP220 to have a dominant negative affect on ERα activity raised questions concerning its NR-binding properties. Does TRAP220 form a functional interaction with the ERα (or other class I NRs) or is it selective for the class II NRs with which it has previously been shown to bind and coactivate?

6.2 The NR-binding specificity of TRAP220

Yeast two-hybrid and GST pull-down experiments were used in this study to compare the interactions of TRAP220 proteins with class I and class II NRs. Consistent with reports detailing weak ERα/TRAP220 interactions (Yuan *et al.*, 1998, Kobayashi *et al.*, 2000 and Warnmark *et al.*, 2001), both the NID of TRAP220 and full-length TRAP220 proteins displayed weak ligand-induced interactions with the ERα. Moreover, whilst the NID of SRC1 showed no apparent preference for binding to a panel of NRs, including those from both class I and class II, the NID of TRAP220 bound preferentially to TRβ, RXRα, PPARγ and to a lesser extent RARα, with binding to ERα, PR and AR observed to be dramatically reduced. Hence it would appear that TRAP220 displays a NR-binding specificity, preferentially binding to class II as opposed to class I NRs. Other NR-coactivators have also been observed to display NR-binding specificities, suggesting the existence of distinct coactivator classes that are specific for certain NRs or NR subclasses.

6.3 Amino acid sequences flanking LXXLL motifs influence NR-binding selectivity

To understand the molecular basis of the differential NR-binding properties displayed by TRAP220, a detailed analysis of TRAP220/NR-binding was undertaken. The NR-binding properties of the core LXXLL motifs of TRAP220 (LXM1 and LXM2) were assessed in yeast two-hybrid experiments. Remarkably, this revealed that TRAP220 core LXXLL motifs do not display the same NR-binding selectivity as the NID of TRAP220. Indeed TRAP220 LXM1 and LXM2 core motifs displayed strong interactions with all NR LBDs tested. Previous studies have shown that the NR-binding specificity of other coactivators is determined in part by LXXLL core motifs (Heery *et al.*, 1997, McInerney *et al.*, 1998, Ding *et al.*, 1998 and Heery *et al.*, 2001) but also involves sequences immediately flanking the core motif (McInerney *et al.*, 1998 and Needham *et al.*, 2000). In agreement with this, an extended TRAP220 LXM1 sequence containing amino acids at positions –4 to +9, showed increased interaction with TRβ (3.5 fold) but a 10-fold reduced interaction with ERα LBD. Thus the extended 13 amino acid LXM1 sequence displays selective NR-binding properties similar to the TRAP220 NID or full-length protein, suggesting that amino acid sequences flanking the core LXXLL motif are contributing to the NR-binding selectivity exhibited by TRAP220.

6.4 Sequence determinants governing the NR-binding specificity of TRAP220

To date the co-crystal structure of the TRAP220 NID in complex with an agonist-bound NR LBD has not been reported and details of the TRAP220/NR LBD interface are poorly understood. However this study clearly demonstrates that TRAP220 exhibits a NR-binding selectivity that is partly determined by amino acid sequences immediately flanking the LXM1 core motif, suggesting that these amino acids may contact the NR LBD. Moreover, I show that it is possible to change the NR-binding properties of TRAP220 by exchanging amino acids at positions –4 to +9 of TRAP220 LXM1 (mutant F) and LXM2 (mutant H) for those in the corresponding positions in the SRC1 LXM2 sequence. These mutant TRAP220 proteins (mutants F and H) showed increased binding to the class I NR, ERα (and also to PR in the case of mutant F) but binding to class II NRs was relatively unaffected. This confirmed that the extended LXM1 and LXM2 sequences of TRAP220 (-4 to +9) are major determinants of TRAP220 NR-binding specificity. Further detailed analysis revealed that exchange of the entire TRAP220 LXM1 extended sequence for the SRC1 LXM2 extended sequence was optimally required for enhanced binding to ERα and residues within the core motif and flanking sequence

cooperate to determine NR-binding specificity. For example, exchange of amino acids at the +2 and +3 positions, or at the –2 position of the TRAP220 LXM1 sequence for those in the same positions in SRC1 LXM2, were not sufficient to change the NR-binding selectivity of the TRAP220 NID. However combinations of amino acid exchanges, for example –2 coupled with a +2 and +3 exchange, or –4, -3, -2 coupled with a +2 and +3 exchange, were observed to enhance ERα/TRAP220 interactions but not to the same extent achieved when the entire TRAP220 extended LXM1 sequence was exchanged for the SRC1 LXM2 extended sequence. Thus key residues within the TRAP220 NID have been identified which contribute to the NR-binding specificity of TRAP220.

6.5 How the spacing between adjacent LXXLL motifs influences NR-binding

Crystallographic data has shown that each NR LBD of a NR-dimer is occupied by a single LXXLL α-helix (Nolte *et al.*, 1998, Darimont *et al.*, 1998 and Shiau *et al.*, 1998). Hence if a single molecule of TRAP220 interacts with a NR-dimer, via its two LXXLL motifs, then the spacing between LXM1 and LXM2 core motifs could impose NR-binding specificity where different NRs require different spacing between adjacent LXMs to facilitate binding. Previous studies have shown that a reduction in the spacing between LXXLL motifs in p160s (McInerney *et al.*, 1998) or TRAP220 (Ren *et al.*, 2000) adversely affects NR interactions. This work demonstrated that increasing the spacing between LXM1 and LXM2 of TRAP220, so that it resembles the spacing found in p160s, has no significant affect on class I or class II NR interactions. Thus whilst there appears to be a minimal spacing requirement necessary to facilitate TRAP220/NR interactions, the spacing itself does not appear to impose NR-binding specificity.

6.6 A unique coactivator binding site

Adding a new twist to the complexity of NR-coactivator interactions, a recent report detailing the crystal structure of the farnesoid X receptor (FXR) revealed the existence of a second unique coactivator binding site (Mi *et al.*, 2003). An agonist-bound FXR-LBD monomer was crystallised in complex with two separate LXXLL peptide motifs. The first LXXLL peptide motif was observed to bind in the traditional coactivator binding pocket (see section 1.8.1), whereas the second LXXLL peptide motif bound directly adjacent to the first peptide on helix 3. The second LXXLL peptide motif interacted with both the FXR and the first

peptide through hydrophobic contacts but lacked the electrostatic charge clamp that is characteristic of the traditional coactivator binding pocket. In the normal cellular environment FXR heterodimerises with RXR. It would therefore be interesting to observe if the second coactivator binding site of FXR is prefered to the coactivator binding site of the RXR LBD, or whether coactivator proteins containing three or more functional LXXLL motifs utilise all three coactivator binding sites of the RXR-FXR heterodimer. To date little is known about this unique coactivator binding site of the FXR and the mechanisms by which it is utilised by coactivator proteins. Perhaps it enables FXR to interact with a wider range of coactivators by providing an alternative binding surface for those coactivators which normally exhibit very weak or no interaction with the traditional coactivator binding pocket of FXR. Alternatively, cooperative binding may be employed whereby binding of an LXXLL peptide motif to the first binding site permits binding of an LXXLL peptide motif to the second binding site, thus perhaps stabilising the coactivator-FXR interaction. Whether the second coactivator binding site additionally imposes coactivator-specific binding properties has yet to be investigated.

6.7 The TRAP/SMCC/Mediator complex, its TRAP220 subunit and ERα-mediated transcriptional activity

Recently, chromatin immuno-precipitation (ChIP) experiments have been used to demonstrate that TRAP proteins are recruited to NR-regulated promoters, including ERα- and AR-responsive promoters (Shang *et al.*, 2000, Wang *et al.*, 2002 and Burakov *et al.*, 2002), and another recent report has shown that the TRAP/SMCC/Mediator complex can be purified from HeLa cell nuclear extracts using GST-ERα LBD in the presence of ligand (Kang *et al.*, 2002). Additionally, microinjection of HeLa cell nuclei with anti-TRAP220 antibodies was observed to down-regulate ERα activation of a reporter gene (Llopis *et al.*, 2000). Moreover, GST-ERα LBD failed to retain any TRAP/SMCC/Mediator components in *Trap220*$^{-/-}$ MEF nuclear extracts (Kang *et al.*, 2002), suggesting recruitment of the TRAP/SMCC/Mediator complex to the ERα LBD requires the TRAP220 subunit. Taken together this data implies that the TRAP/SMCC/Mediator complex, in a manner that optimally requires TRAP220, does function in class I NR-mediated transcriptional activity. This would appear to contradict both evidence presented here, and that of others groups (Rachez *et al.*, 1998, Yuan *et al.*, 1998, Kobayashi *et al.*, 2000 and Warnmark *et al.*, 2001), detailing the relatively weak interactions of TRAP220 with class I NRs. However it should be considered that whilst the TRAP220 subunit may

facilitate binding, albeit weak, with the AF-2 of class I NRs, other TRAP/SMCC/Mediator subunits may be optimally required to facilitate binding of the TRAP complex to NRs. Indeed the TRAP170 subunit has been shown to bind to the GR AF-1 domain (Hittleman *et al.*, 1999) and rather interestingly the TRAP230 subunit possesses a glutamine (Q)-rich domain. Such Q-rich domains within SRC1 have previously been shown to facilitate ligand-independent interactions with the AF-1 domains of class I NRs (Bevan *et al.*, 1999 and Heery *et al.*, 2001). Further studies will be required to determine TRAP230s involvement, if any, in ligand-independent binding to NRs. Hence weak TRAP220/NR AF-2 interactions may be stabilised and/or complimented by interactions of other TRAP subunits with the NR, meaning that TRAP220 functions optimally only in conjunction with other subunits of the TRAP/SMCC/Mediator complex.

6.8 Future directions

As a consequence of this study a greater understanding of the complexity of NR-coactivator interactions has been gained. Knowledge of coactivator LXXLL motif sequence determinants that govern specific NR interactions could perhaps be used to predict the NR-class specificity, if any, of novel coactivator proteins. Additionally, this knowledge could be used to design peptides, or other agents, to interfere with specific NR-coactivator interactions. These peptides could be used therapeutically to attenuate the activity of specific NRs or alternatively as an experimental tool. As an extension to this study, and to further explore the molecular mechanism underlying the NR-specific interactions of TRAP220, mutagenesis of the NR LBDs could be explored with the view to identify NR LBD residues that are important for TRAP220 binding. Taken together, the key residues of the TRAP220 NID that contribute to NR-binding specificity and the key residues of the NR LBDs involved in TRAP220 interactions, could assist in developing a model of the TRAP220/NR LBD interface. Additionally, based on the data from this study and the knowledge of existing crystal structures of NR LBDs in complex with LXXLL peptides or coactivator fragments, advanced computer modelling programs could be used to further define the TRAP220/NR LBD interface. Ultimately however, structural studies involving TRAP220 extended LXXLL peptides, or preferably the stably folded NID, in combination with biochemical studies should provide a clearer insight into the selective interactions of TRAP220 with different NRs.

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APPENDIX

Primer name	Sequence 5'-3'
TRAP220-KpnI-F	GGTACCACTACACCAACCAACACC
TRAP220-XhoI-R	CTCGAGCCAATGGTAGACCCCCCG
TRAP220-335F	AAAACCGCGGACTTATGCACCCCTGTAT
TRAP220-503F	AAAACCGCGGTCCATCCCTGTGACGATG
TRAP220-667R	AAAAGGATCCCTATTCTAAAGGGCTGCTTCC
SRC1-431F	AAAACCGCGGCATAGTAATTCTAGCAAC
SRC1-761R	AAAAGGATCCCTATCTTAAATCTTTCTCATC
TRAP220Mut1-F	CCAATTCTTACCAGTGCAGCACAAATCACAGGGAACGGG
TRAP220Mut1-R	CCCGTTCCCTGTGATTTGTGCTGCACTGGTAAGAATTGG
TRAP220Mut2-F	CCGATGCTCATGAACGCAGCAAAAGATAATCCTGCCCAG
TRAP220Mut2-R	CTGGCGAGGATTATCTTTTGCTGCGTTCATGAGCATCGG
TRAP220MutA-F	CGGCATAAAATTCTAACCAGTCTCTTACAGGAGGGTAGCAACGGGGGGTCTACCATTGGC
TRAP220MutA-R	GCTACCCTCCTGTAAGAGACTGGTTAGAATTTTATGCCGAGACACCTTGCTGAAGTC
TRAP220MutB-F	CAGAACCCAATTCTACACCGGCTCTTACAGGAGGGTAGCAACGGGGGGTCTACCATTGGC
TRAP220MutB-R	GCTACCCTCCTGTAAGAGCCGGTGTAGAATTGGGTTCTGAGACACCTTGCTGAAGTC
TRAP220MutC-F	CGGCATAAAATTCTACACCGGCTCTTACAAATCACAGGGAACGGGGGGTCTACCATTGGC
TRAP220MutC-R	CCCTGTGATTTGTAAGAGCCGGTGTAGAATTTTATGCCGAGACACCTTGCTGAAGTC
TRAP220MutD-F	AAGGTGTCTCAGAACAAAATTCTTCACCGGTTGTTGCAAATC
TRAP220MutD-R	GATTTGCAACAACCGGTGAAGAATTTTGTTCTGAGACACCTT
TRAP220MutE-F	GTGTCTCAGAACCCAATTCTTCACCGGTTGTTGCAAATCACAGGGAACGGG

Primer name	Sequence 5'-3'
TRAP220MutE-R	CCCGTTCCCTGTGATTTGCAACAACCGGTGAAGAATTGGGTTCTGAGACAC
TRAP220MutF-F	CGGCATAAAATTCTACACCGGCTCTTACAGGAGGGTAGCAACGGGGGGTCTACCATTGGC
TRAP220MutF-R	GCTACCCTCCTGTAAGAGCCGGTGTAGAATTTTATGCCGAGACACCTTGCTGAAGTC
TRAP220MutG-F	AAGGTGTCTCAGAACAAAATTCTTACCAGTTTGTTGCAAATC
TRAP220MutG-R	GATTTGCAACAAACTGGTAAGAATTTTGTTCTGAGACACCTT
TRAP220MutH-F	CGGCATAAAATTCTACACCGGCTCTTACAGGAGGGTAGCGCCCAGGATTTCTCAACCCTT
TRAP220MutH-R	GCTACCCTCCTGTAAGAGCCGGTGTAGAATTTTATGCCGCTTGGTGTTGCCGGCCAT
TRAP220lxm1core-F	GGGGCATTCTTACCAGTTTGTTGCAAATCACATAG
TRAP220lxm1core-R	GATCCTATGTGATTTGCAACAAACTGGTAAGAATGCCCCGC
TRAP220lxm2core-F	GGGGCATGCTCATGAACCTTCTCAAAGATAATTAG
TRAP220lxm2core-R	GATCCTAATTATCTTTGAGAAGGTTCATGAGCATGCCCCGC
TRAP220spacer-F	GATAAAAAGGACAGTGCATCTACTTCTGTGTCAGTGACTGGACAGGTCTCTTCGATGGCCG GC
TRAP220spacer-R	CTGTCCAGTCACTGACACAGAAGTAGATGCACTGTCCTTTTTATCAGGTGGCGGCGTGTGA TG
TRAP220ext-F	GGGGCCAGAACCCAATTCTTCCCAGTTTGTTGCAAATCACAGGGTAG
TRAP220ext-R	GATCCTACCCTGTGATTTGCAACAAACTGGTAAGAATTGGGTTCTGGCCCCGC
TRAP220-232-F	CTGGATGACAAGACTGCATCT
TRAP220-529-F	GCAGAGACAGTTGAAGACATG
TRAP220-709-F	CAGAGGGAGCTATTTTCAATG

Primer name	Sequence 5'-3'
TRAP220-1369-F	GGGAGTTCAGTGGATTCTTCT
TRAP220-1531-F	GAGAGTTGGTCCAAATCACC
TRAP220-36-R	TTGACGCACAAGCTTAAT
TRAP220-91-R	GGTTTCACTCCAGGGTCT
TRβ-1-F	AAAAGGATCCATGACAGAAAATGGCCTT
TRβ-456-R	AAAACCGCGGCTAATCCTCGAACACTTCCAG
TRβ-181-F	CTGGCCAAGAGGAAGCTGATA
PPARγ-173-F	AAAAGGATCCATGTCTCATAATGCCATC
PPARγ-475-R	AAAACCGCGGCTAGTACAAGTCCTTGTAGA
pSG5(PT)-411-R	AGTTTGGACAAACCACAACTAGAATGC
pSG5(PT)-F	CCTACAGCTCCTGGGCAAC
BTMF	GCAGAGCTTCACCATTGAAG
BTMR	TCTTTAAGCGGGCCTTAA
PD199	CTGGCAATTCCTTACCTTCC
JRL26	ATGGCCGACTTCGAGTTT

Table A.1. Oligonucleotides used in molecular biology applications

PUBLICATION

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An Extended LXXLL Motif Sequence Determines the Nuclear Receptor Binding Specificity of TRAP220*

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The interaction of coactivators with the ligand-binding domain of nuclear receptors (NRs) is mediated by amphipathic α-helices containing the signature motif LXXLL. TRAP220 contains two LXXLL motifs (LXM1 and LXM2) that are required for its interaction with NRs. Here we show that the nuclear receptor interaction domain (NID) of TRAP220 interacts weakly with Class I NRs. In contrast, SRC1 NID binds strongly to both Class I and Class II NRs. Interaction assays using nine amino acid LXXLL core motifs derived from SRC1 and TRAP220 revealed no discriminatory NR binding preferences. However, an extended LXM1 sequence containing amino acids −4 to +9, (where the first conserved leucine is +1) showed selective binding to thyroid hormone receptor and reduced binding to estrogen receptor. Replacement of either TRAP220 LXXLL motif with the corresponding 13 amino acids of SRC1 LXM2 strongly enhanced the interaction of the TRAP220 NID with the estrogen receptor. Mutational analysis revealed combinatorial effects of the LXM1 core and flanking sequences in the determination of the NR binding specificity of the TRAP220 NID. In contrast, a mutation that increased the spacing between TRAP220 LXM1 and LXM2 had little effect on the binding properties of this domain. Thus, a 13-amino acid sequence comprising an extended LXXLL motif acts as the key determinant of the NR binding specificity of TRAP220. Finally, we show that the NR binding specificity of full-length TRAP220 can be altered by swapping extended LXM sequences.

The nuclear hormone receptors (NRs)[1] are a family of structurally related, ligand-regulated transcription factors that exert both positive and negative control of gene expression in metazoans (1). The NRs are subdivided into classes depending on their DNA binding and dimerization properties. Class I comprises the steroid hormone receptors, including the estrogen (ER), androgen (AR), and progesterone (PR) receptors, which function as homodimers. The largest group (Class II) function as heterodimers with the 9-*cis* retinoic acid receptor (RXR), and includes receptors for retinoic acid (RAR), vitamin D (VDR), thyroid hormone (TR), and peroxisome proliferators (PPARs). Binding of cognate ligand induces a conformational change in the ligand binding domain (LBD) of NRs, which influences their function with respect to subcellular localization, dimerization, cofactor binding, and transcriptional activity (2).

A wide range of NR cofactors have been identified which perform distinct functions at target promoters, including chromatin modification and remodeling and recruitment of the RNA polymerase II holoenzyme (3). A number of cofactors have been shown to bind the LBD directly via short amphipathic α-helices containing the LXXLL sequence (4, 5). Structural studies have demonstrated that a hydrophobic channel (AF2) is exposed on the surface of the LBD as a consequence of ligand binding (6–11). This channel accommodates the LXXLL α-helix, which is held in place by hydrophobic interactions and a charged clamp involving two amino acids (lysine and glutamate) that are conserved throughout the NR family (12, 13). The minimal sequence that can bind the AF2 surface (the LXXLL core motif) is contained within 8 amino acids (−1 to +7) (14).

Different cofactors have been shown to have variable numbers of functional LXXLL motifs. The p160 coactivators SRC1, TIF2/GRIP1, and ACTR/AIB1/pCIP have homologous NR interaction domains (NID) containing three LXXLL motifs (4, 5, 15). These motifs are highly conserved both in sequence and spacing, and it has been shown that at least two (preferably adjacent) motifs are required for high affinity binding of SRC1 to Class I receptors (4, 5, 15, 16). Thus, the presence of multiple LXXLL motifs in coactivators facilitates cooperative binding to the AF2 surfaces of NR dimers. The mammalian mediator complex TRAP/DRIP/SMCC/ARC/CRSP contains a single subunit capable of binding to NR LBDs (17, 18). This protein, termed TRAP220, DRIP205, or PBP, contains two LXXLL motifs within the NID that are required for ligand-dependent binding to NRs (19–21). Other cofactors including TIP60 (22), TIF1α (23), PGC1 (24), *Fushi tarazu* (25), and ASC-2/RAP250/TRBP/PRIP/NRC/AIB3 (26), contain a single functional LXXLL motif, although the stoichiometry of binding of these proteins to NR dimers has yet to be determined. The transcriptional repressor RIP140 contains nine functional LXXLL motifs (4), and a similar number have been identified in the ERα-binding protein PELP1 (27).

Several cofactors have been found to display preferences for NR subclasses. For example, TIP60 has been reported to bind the Class I receptors, but displayed little interaction with VDR, TR, or RXR (22). The p160s interact with a wide range of NRs, although individual LXXLL motifs derived from these proteins display differential binding to NRs (14, 38). In addition, we have shown that LXXLL sequences derived from CBP and RIP140 show selectivity that is at least partly determined by the LXXLL core sequences (14). Similarly, LXXLL motifs (or

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[1] The abbreviations used are: NRs, nuclear hormone receptors; LBD, ligand binding domain; NID, nuclear receptor interaction domain; DBD, DNA binding domain; ER, estrogen receptor; E_2, estradiol; HA, hemagglutinin; GST, glutathione *S*-transferase; VDR, vitamin D receptor; PPAR, peroxisome proliferator-activated receptor; RXR, 9-*cis* retinoic acid receptor; RAR, retinoic acid receptor; T_3, 3,3′,5-triiodo-L-thyronine; TR, thyroid receptor; TRAP, thyroid hormone receptor-associated protein.

variants such as LXXIL and FXXLL) from other cofactors, such as PERC (31), NRIF3 (32), and NSD1 (33) have been reported to display selectivity in their interactions with NRs.

TRAP220 was isolated on the basis of its interaction with Class II NRs such as TR, VDR, and PPARs, and it has been shown that ablation of either of its two LXXLL motifs had differential effects on binding to NRs. RXR binding was disrupted by mutation of LXM1, whereas TR, RAR, and VDR showed a preference for binding the distal motif, LXM2 (20, 28). TRAP220 has also been reported to interact with ERα, although this appears to be relatively weak (18, 19, 29, 30). Recent reports have demonstrated that TRAP220 interacts more strongly with ERβ (30, 50). In addition, data from other studies have suggested that sequences flanking the core motif also influence NR binding specificity (30, 34–39).

In this study we assessed the interaction of TRAP220 NID, LXXLL peptides, and full-length proteins with a panel of NR LBDs. We show that TRAP220 displays stronger interactions with certain Class II receptors in comparison to steroid receptors. We describe mutational analyses and mapping experiments that shed light on the molecular basis of the NR binding specificity of TRAP220.

MATERIALS AND METHODS

Plasmid Constructions—The following plasmids used in transient transfection experiments have been described previously: pSG5-SRC1e (16) and p3ERE-TATA-LUC (40). The reporter plasmid pMAL-TK-LUC (41) and hTRβ-RSV (42) were gifts from K. Chatterjee. The expression construct pCIN4-TRAP220 was a gift from R. Roeder. A cDNA-encoding full-length TRAP220 with an N-terminal HA tag was subcloned into the modified vector pSG5(PT) using unique *Xma*I and *Not*I sites.

For yeast two-hybrid interaction assays, the p3ERE-lacZ reporter (46), and vectors pBL1 and modified pASV3 (43) expressing the human ERα DNA binding domain (DBD) and the VP16 acidic activation domain (AAD), respectively, were gifts from R. Losson and P. Chambon. The constructs AAD-AR-LBD-(625–919), AAD-PR-LBD-(633–933) (44), AAD-ERα-LBD-(282–595) (4), AAD-RARα-LBD-(200–462), and AAD-RXRα-LBD-(230–467) (14) have been described previously. The constructs AAD-PPARγ-LBD-(173–475) and AAD-TRβ-(1–456) were generated by cloning PCR fragments into the modified pASV3. The ER-DBD-LXXLL core motif fusion proteins including TRAP220 LXM1-(603–611) and TRAP220 LXM2-(644–652) and the TRAP220 LXM1 extended (600–612), were generated by ligation of phosphorylated, annealed oligonucleotide pairs into the pBL1 vector. ER-DBD-SRC1 LXM2 (formerly referred to as DBD-SRC1 motif 2) has been described previously (4). TRAP220 NID-(335–667) and SRC1 NID-(431–761) were produced by PCR and cloned into a modified pBTM116 vector generating LexA-TRAP220 NID and LexA-SRC1 NID, respectively. The LexA-TRAP220 NID-(335–667) mutants (A-H, spacer, mut1 and mut2) were generated using recombinant PCR techniques. All constructs generated by PCR were sequenced. The expression of fusion proteins in yeast was monitored by Western blotting using antibodies recognizing VP16, LexA (Autogen Bioclear), or the ERα F domain epitope tag at the N terminus of the ER-DBD fusion proteins, as described previously (43).

For *in vitro* glutathione *S*-transferase (GST) pull-down assays, the control GST construct was a modified version of pGEX-2TK vector (Amersham Biosciences). The constructs GST-TRβ-LBD and GST-RXRα-LBD were gifts from K. Chatterjee and E. Kalkhoven, respectively. GST-ERα-LBD has been described previously (4). pSG5(PT)-TRAP220 LXM1 mutant F was generated by replacing a *Xho*I/*Kpn*I fragment of pSG5(PT)-TRAP220 with the corresponding fragment from pBTM116-TRAP220 NID mutant F.

Cell Culture and Transient Transfections—Maintenance of HeLa cells and transient transfection protocols were as described (45). The transfected DNA included pJ7-lacZ internal control plasmid (500 ng/well), p3ERE-TATA-LUC (1 μg), or pMAL-TK-LUC (2 μg) luciferase reporter plasmids, with either pMT-MOR (100 ng) or pRSV-TRβ (200 ng) expression vectors and varying amounts of pSG5-SRC1e or pSG5-TRAP220, as indicated. Empty pSG5 expression vector was used to standardize the amount of transfected DNA. The ligands used were 10^{-8} M 17β-estradiol (E_2 (for ERα)) and 10^{-7} M 3,3′,5-triiodo-L-thyronine (T_3) (for TRβ).

Yeast Two-hybrid Interaction Assays—Saccharomyces cerevisiae W303–1b (HMLα MATα HMRα *his3–11, 15 trp1–1 ade2–1 can1–100 leu2–3, 11 ura3*) was transformed sequentially with p3ERE-LacZ reporter plasmid, ER-DBD fusion protein expression plasmids and AAD-NR-LBD expression plasmids using the lithium acetate chemical transformation method (43). L40 (*trp1 leu2 his3 ade2* LYS2::$(lexAop)_{4\times}$-HIS3 URA3::$(LexAop)_{8\times}$-*LacZ*) was transformed sequentially with LexA-fusion protein expression vectors and AAD-NR-LBD expression vectors using electroporation (43). Transformants containing the desired plasmids were selected on appropriate media and grown to late log phase in 15 ml of selective medium (yeast nitrogen base containing 2% w/v glucose and appropriate supplements) in the presence of 10^{-6} M receptor cognate ligand (E_2, T_3, promegestone (R5020), 9-*cis*-retinoic acid (9c-RA), all-*trans*-retinoic acid (AT-RA), rosiglitazone, mibolerone) or an equivalent amount of vehicle. Preparation of cell-free extracts was by the glass bead method and β-galactosidase assay of the extracts were performed as described (43, 46). Reporter β-galactosidase activities in the presence or absence of ligand were determined for two transformants for each condition, in replicated experiments, as stated. Ligands were purchased from Sigma with the exception of rosiglitazone, which was a generous gift from GlaxoSmithKline.

GST Pull-down Assays—Recombinant cDNAs in pSG5 or pSG5(PT) expression vectors were transcribed and translated *in vitro* in the presence of [^{35}S]methionine in reticulocyte lysate (Promega) according to the manufacturer's instructions. GST fusion proteins were expressed in *Escherichia coli* DH5α using isopropyl-β-D-thiogalactopyranoside induction, purified on glutathione-Sepharose beads (Amersham Biosciences), and normalized amounts were incubated with ^{35}S-radiolabeled protein in NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 10 mM dithiothreitol) containing complete protease inhibitors (Roche Molecular Biochemicals) in the presence or absence of 10^{-6} M cognate ligand (E_2, 9c-RA or T_3), as described previously (16). Samples were washed three times, and bound proteins were separated on SDS-polyacrylamide gels. Radiolabeled proteins were visualized by autoradiography.

RESULTS

TRAP220 Enhances the Transcriptional Activity of TRβ but Not ERα—Previous studies have shown that ectopic expression of the mammalian mediator complex protein TRAP220/DRIP205/PBP modestly enhances TRα/β, VDR, and PPARγ-mediated transcription in a ligand-dependent manner (19–21). We assessed the ability of TRAP220 to stimulate TRβ-mediated activation of the T_3-responsive reporter gene, MAL-TK-LUC. This reporter contains a single thyroid response element (TRE) consisting of a direct repeat spaced by four nucleotides (DR4) within a thymidine kinase promoter and driving a firefly luciferase gene (41). As shown in Fig. 1*A*, increasing amounts of transiently transfected TRAP220 expression vector enhanced TRβ-mediated transcription of the MAL-TK-LUC reporter ~2-fold in the presence of ligand. This is comparable to previously observed levels of TRAP220 enhancement of TRα-driven expression of T_3-responsive reporters (19). Under similar conditions, the p160 nuclear receptor coactivator, SRC1e, also modestly enhanced TRβ activation of MAL-TK-LUC (Fig. 1*A*). Co-expression with RXRα did not significantly increase the levels of reporter activity achieved in the presence or absence of coactivators (data not shown).

We also assessed the ability of TRAP220 to enhance the activity of the Class I receptor ERα. As shown in Fig. 1*B*, TRAP220 did not enhance ERα-mediated transcription of the 3ERE-TATA-LUC reporter gene (40), under similar transfection conditions to those used for TRβ. In contrast, co-transfection of SRC1e strongly enhanced ERα-mediated transcription both in the presence and absence of ligand, as reported previously (4, 16, 44, 45). The expression and nuclear localization of both TRAP220 and p160 coactivators in transiently transfected cells was confirmed by indirect immunofluorescence using anti-HA antibodies (data not shown).

TRAP220 NID Exhibits Nuclear Receptor Binding Specificity—The ability of TRAP220 to enhance transcription by TRβ but not ERα suggested that TRAP220 exhibits NR binding preferences. We therefore decided to compare the ability of SRC1 and TRAP220 NIDs to bind to a panel of NR LBDs in

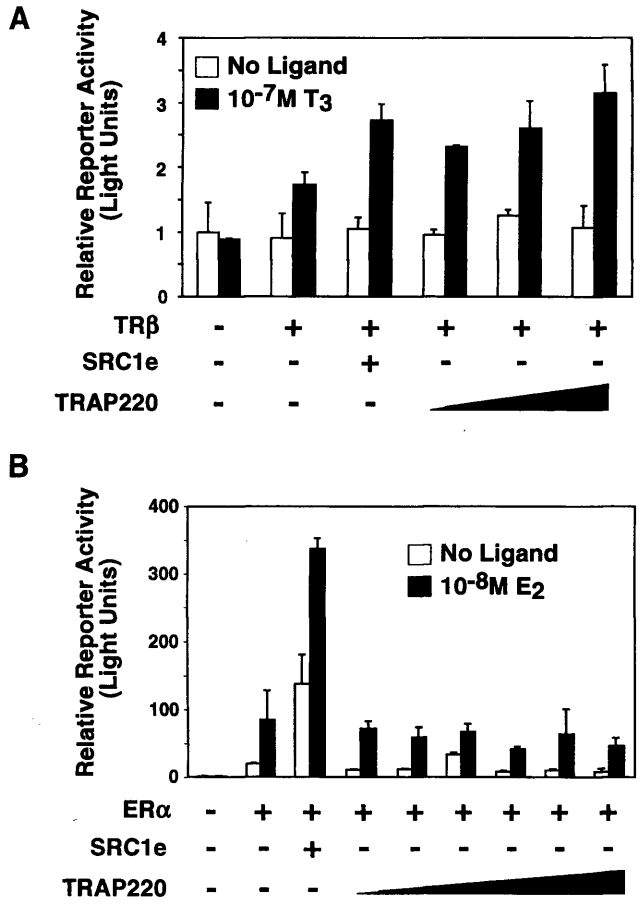

FIG. 1. **Effects of ectopically expressed TRAP220 on ligand-induced reporter activation by TRβ and ERα.** *A*, HeLa cells were transiently transfected as described under "Materials and Methods" with pMAL-TK-LUC reporter plasmid (2 μg) and expression vectors for TRβ (200 ng), SRC1e (500 ng), or TRAP220 (2, 3, and 4 μg) as indicated. Luciferase activity was measured 24 h post addition of ligand (10^{-7} M T_3, *black bars*) or vehicle (*white bars*), and the data were normalized to a β-galactosidase internal control. Reporter activity was expressed relative to that obtained for reporter in the absence of ligand (set at 1). The data represent the mean of triplicate samples, and *error bars* are shown to indicate S.D. Similar results were obtained in replicate experiments. *B*, reporter assays as in *A* except that HeLa cells were transiently co-transfected with the p3ERE-TATA-LUC reporter (1 μg), together with expression vectors for ERα (100 ng), SRC1e (500 ng), or TRAP220 (0.25, 0.5, 0.75, 1, 2, and 3 μg) as indicated. In these experiments the ligand was E_2 (10^{-8} M).

yeast two-hybrid experiments. Fusion proteins (shown schematically in Fig. 2), consisting of the LexA-DNA binding domain (DBD) fused to amino acids 431–761 of SRC1 encompassing LXM1, LXM2, and LXM3 (SRC1 NID; Fig. 2*A*), or amino acids 335–667 of TRAP220, encompassing LXM1 and LXM2 (TRAP220 NID; Fig. 2*B*), were assessed for binding to AAD-NR-LBD fusion proteins. Western blots using an antibody recognizing LexA confirmed that these constructs were expressed to similar levels in L40 (see Fig. 6*B*), and neither LexA-SRC1 NID nor LexA-TRAP220 NID was able to activate transcription of the reporter either alone or in the presence of VP16-AAD (data not shown). As shown in Fig. 2*A*, strong ligand-dependent reporter activation was observed between SRC1 NID and the LBDs of the PR, ERα, RARα, RXRα, and full-length TRβ, (ranging from 80–160 units of reporter activity) and also a weaker but significant interaction with the AR LBD (25 units reporter activity). We also observed a significant binding to retinoid receptors in the absence of ligand (50 and 30 units of reporter activity for RARα and RXRα, respectively). The TRAP220 NID fusion protein also displayed strong ligand-dependent reporter activation when co-expressed with RXRα LBD, TRβ, and PPARγ LBD (140, 110 and 300 units of reporter activity respectively, Fig. 2*B*), and an intermediate level of reporter activation when co-expressed with RARα-LBD (40 units of reporter activity). Although no ligand-independent interactions were observed between TRAP220 NID and retinoid or thyroid receptors, the reporter activity indicated a strong interaction with PPARγ-LBD in the absence of exogenous ligand (120 units of reporter activity), consistent with our previous observation that yeast cells may contain endogenous ligands for PPARγ.[2] In contrast to SRC1, the TRAP220 NID showed a greatly reduced ability to interact with Class I receptors, showing relatively weak ligand-dependent reporter activities when co-expressed with ERα LBD (20 units), PR LBD (3 units), and no detectable interaction with AR LBD. Thus, our results indicate that while the SRC1 NID interacts strongly with both steroid and Class II NRs, the TRAP220 NID shows a marked preference for binding to the Class II NRs such as TRβ, RXRα, and PPARγ.

To determine whether both LXXLL motifs of TRAP220 are required for binding the panel of LBDs used in this study, we generated LexA-TRAP220 NID constructs in which either LXM1 or LXM2 was mutated, by replacing the leucines +4 and +5 with alanines. As shown in Fig. 2*C*, interaction of the TRAP220 NID with TRβ and PPARγ LBD was dependent on the presence of two functional LXXLL motifs, as mutation of either motif resulted in a significant reduction in reporter activation. Similarly, the weak interaction with ERα LBD was abrogated by mutation of either LXM1 or LXM2, indicating a requirement for both motifs. In contrast, interaction of TRAP220 NID with RXRα LBD was severely reduced by mutation of LXM1 but largely unaffected by mutation of LXM2. This indicates that the RXRα LBD preferentially interacts with LXM1, consistent with a previous study that showed selective binding of RXRα LBD (albeit weak) to TRAP220 LXM1 in GST pull-down assays (28).

The NID and Core LXXLL Motifs of TRAP220 Exhibit Distinct NR Binding Specificities—It has been shown previously that 8–10 amino acid sequences encompassing the signature motif LXXLL are sufficient to bind to liganded NR LBDs, and this has been referred to as the core LXXLL motif (4, 14). To explore the nature of the specificity exhibited by the TRAP220 NID in more detail, we assessed the ability of LXXLL core motifs derived from SRC1 and TRAP220 to bind NRs. We generated ER-DBD fusion proteins containing sequences corresponding to amino acids −1 to +8 of TRAP220 LXM1 and LXM2, and SRC1 LXM2 (Fig. 3*A*) and confirmed their expression in W303-1b cells by Western blots (Fig. 3*B*). As shown in Fig. 3*C*, TRAP220 LXM1 core motif was able to bind all NR LBDs tested in a ligand-inducible manner. Notably, similar levels of reporter activation were achieved due to interactions with Class I and Class II NRs, with the exception of the RXRα LBD, for which the reporter activity was consistently 2–3-fold higher. This potentially reflects the preference of RXRα for TRAP220 LXM1 shown here (Fig. 2*C*) and in other studies (28). Similarly, the TRAP220 LXM2 core motif was also able to bind all NR LBDs tested in a ligand-dependent manner. Binding of TRAP220 LXM2 to the Class I receptors ERα and PR was 2–4-fold greater than binding to AR and the Class II receptors TRβ, PPARγ, RARα, and RXRα. In contrast to TRAP220 LXM1, LXM2 did not exhibit any enhanced ligand-induced interaction with RXRα. As expected and shown for comparison, the SRC1 LXM2 core motif induced high levels of ligand-de-

[2] V. H. Coulthard and D. Heery, unpublished results.

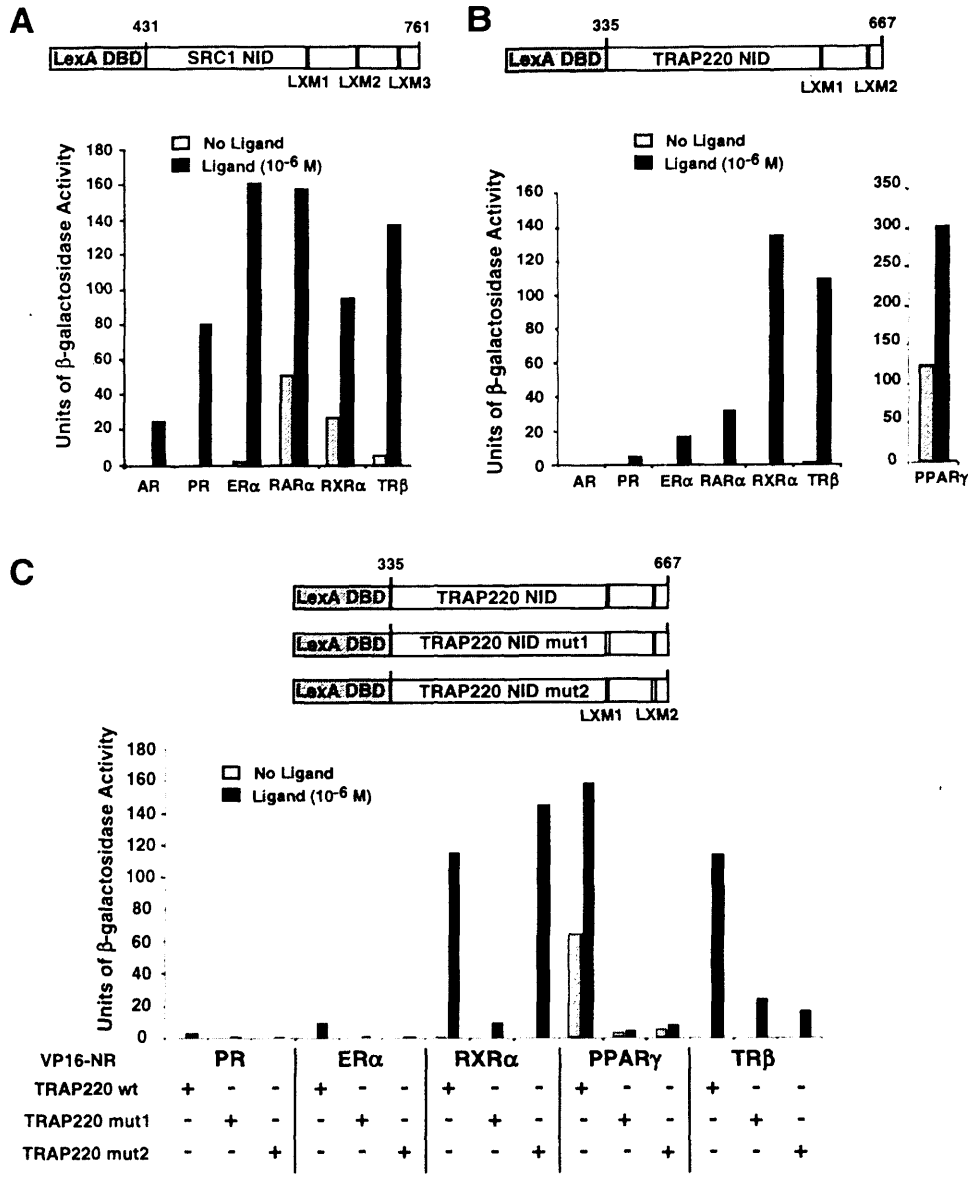

FIG. 2. **Interaction of SRC1 and TRAP220 NIDs with NR subclasses.** *A*, yeast two-hybrid interactions of the SRC1 NID with NR LBDs. Schematic representation of the LexA-SRC1 NID bait protein in which the LexA-DBD is fused in-frame with amino acids 431–761 of human SRC1. The LXM1, LXM2, and LXM3 sequences are indicated. Prey vectors consisted of the acidic activation domain of VP16 (AAD) fused in-frame with full-length TRβ, or the LBDs of AR, PR, ERα, RARα, RXRα, and PPARγ. Transformants of the yeast reporter strain L40 co-expressing bait and prey proteins were cultured overnight in the presence or absence of 10^{-6} M cognate ligand; T_3, mibolerone, R5020, E_2, AT-RA, 9C-RA, or rosiglitazone. Reporter activity in cell-free extracts is expressed as units of β-galactosidase activity. *Shaded bars* and *black bars* represent reporter activity in the absence or presence of cognate ligand, respectively. A representative experiment is shown, and similar results were obtained in replicate experiments. *B*, yeast two-hybrid interactions of the TRAP220 NID with NR LBDs. The LexA-TRAP220 NID fusion protein is shown schematically, and consisted of LexA-DBD fused to amino acids 335–667 of TRAP220. The LXM1 and LXM2 sequences are indicated. Reporter assays to assess interactions with VP16-NR proteins were performed as in *A*. *C*, yeast two-hybrid interactions of wild-type and mutant TRAP220 NID constructs with VP16-NR-LBDs. LexA-TRAP220 NID wild-type and mutant constructs are shown schematically. Mut 1 contains the mutation LL-607/8-AA, which inactivates the LXM1 motif, whereas Mut 2 contains the mutation LL-648/9-AA resulting in loss of LXM2 binding to NRs. Reporter activation was determined as in *A*.

pendent reporter activity with all NR LBDs tested (Fig. 3*D*). The three core motifs displayed considerable ligand-independent interaction with PPARγ and RARα LBDs (and to a lesser degree with RXRα LBD in the case of TRAP220 LXM2). Taken together, our results show that while the TRAP220 and SRC1 NIDs appear to have quite distinct NR binding preferences, LXXLL core motifs derived from these domains show little selectivity for NRs. This suggests that LXXLL core motifs are only partial determinants of the specificity of coactivator interactions with NRs, and that other sequences in the NID contribute to specificity.

To investigate the potential influence of amino acids flanking TRAP220 core LXXLL motifs in determining NR binding specificity, we generated an additional ER-DBD fusion protein containing the sequence −4 to +9 of TRAP220 LXM1 (*extended*, Fig. 4*A*). As shown in Fig. 4*B*, in the presence of AAD-TRβ, the TRAP220-extended motif was able to activate the reporter 3.5-fold above the level observed for the TRAP220 LXM1 core motif, in a ligand-dependent manner, suggesting that flanking sequences stabilize the interaction with TRβ. In contrast, when co-expressed with ERα LBD the reporter activation by TRAP220 extended motif was ~10-fold lower than that achieved by the TRAP220 LXM1 core motif, in the presence of ligand. These results indicate that amino acids immediately flanking TRAP220 LXM1 core motif stabilize TRβ interaction and reduce ERα binding, demonstrating that residues flanking LXXLL core motifs are key determinants of NR binding specificity.

The NR Binding Specificity of TRAP220 NID Can Be Altered by Exchange of Extended LXXLL Motifs—The crystal structures of NR LBDs in complex with LXXLL peptides (12, 34) or a polypeptide containing part of the SRC1 NID (13) revealed that the conserved leucine residues make intimate contacts with a hydrophobic groove on the LBD surface, whereas amino acids +2, +3, +6, and +7 are solvent-exposed. Sequences flanking the core motif were undefined in these structures (12,13), thus it remains unclear as to how the LBD makes contacts with NID sequence outside the core motif. However, there is evidence that sequences immediately flanking core LXXLL motifs play a role in determining NR binding specificity (14, 28, 30, 34–39). For example, a number of LXMs contain a cluster of positively charged residues at positions −4, −3, and −2, that are potentially involved in contacting the conserved glutamic acid in helix 12 of the LBD, which has been proposed to be part of a charged clamp (13). To investigate the contribution of amino acids within extended LXXLL motifs to NR binding specificity, we made a series of TRAP220 NID mutants (*A–H*; Figs. 5*A* and 6*A*). All of the mutations involve an ex-

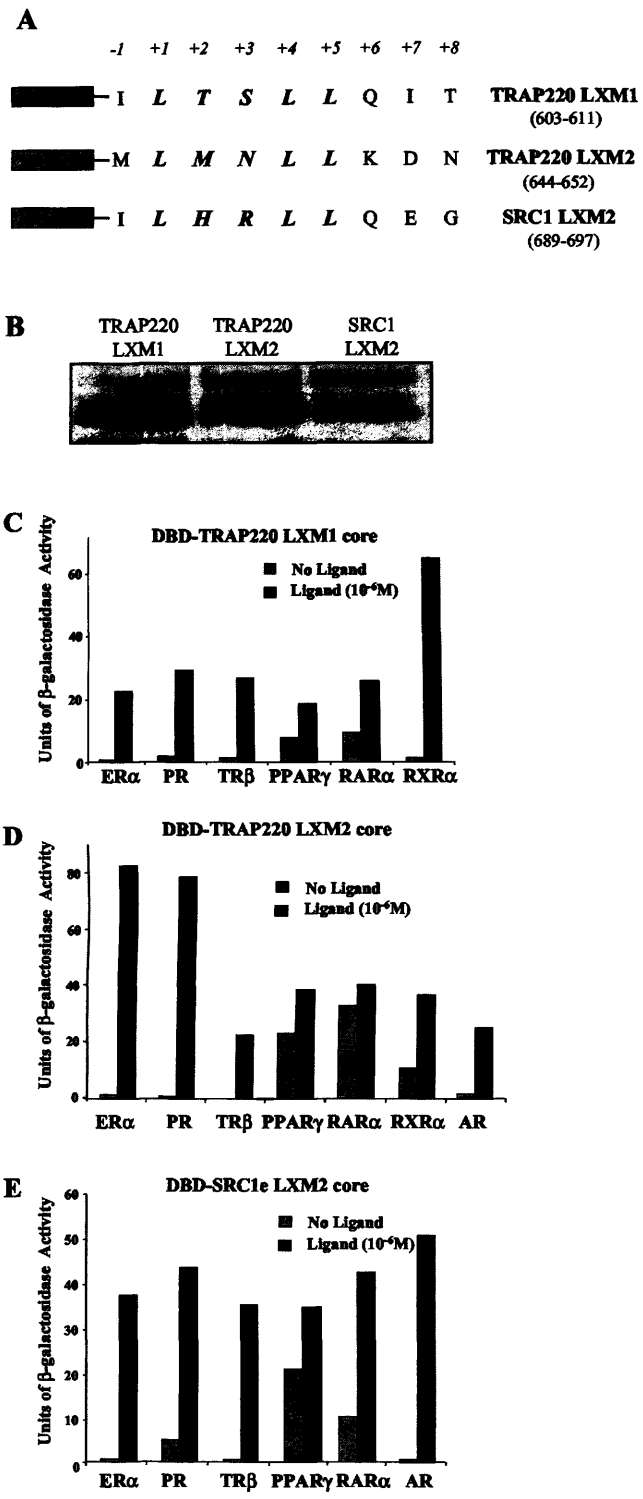

FIG. 3. **Interaction of TRAP220 and SRC1 LXXLL core motifs with NRs.** *A*, schematic representation of LXXLL core sequences from SRC1 or TRAP220 fused in-frame with the ERα DBD, including TRAP220 LXM1 (amino acids 603–611), TRAP220 LXM2 (amino acids 644–652), and SRC1 LXM2 (amino acids 689–697). *B*, Western blot detection of the DBD fusion proteins in equivalent amounts of cell free extracts from yeast transformants, using the antibody recognizing the ERα F domain epitope. *C–E*, yeast two hybrid experiments assessing interaction of DBD-TRAP220 LXM1, DBD-TRAP220 LXM2 or DBD-SRC1 LXM2 with VP16-AAD-NR fusion proteins. Reporter activation assays were carried out on cell-free extracts of W303-1b clones carrying the 3ERE-lacZ reporter and co-expressing bait and prey fusion proteins, as described in the legend to Fig. 2. *Shaded* and *black bars* represent reporter activity in the absence or presence of cognate ligand, respectively. A representative experiment is shown, and similar results were obtained in triplicate experiments.

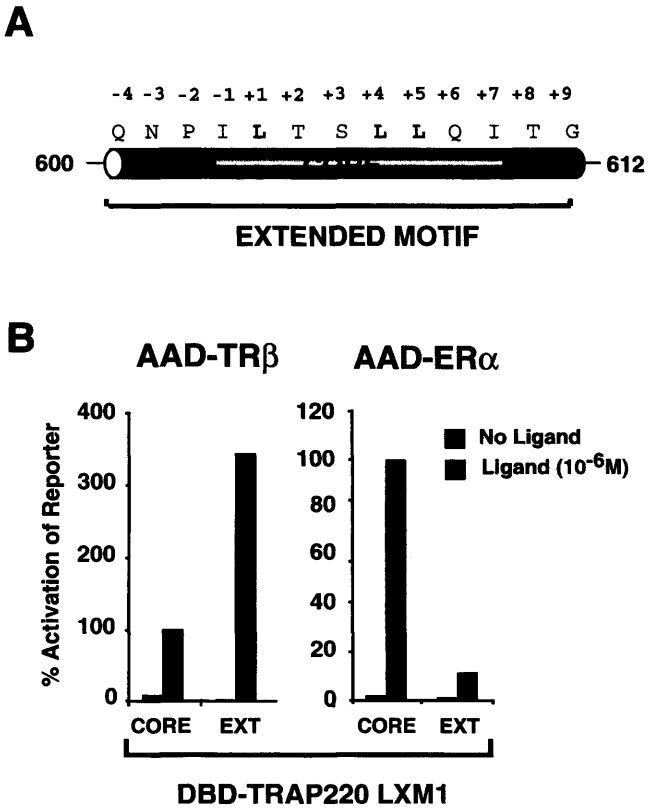

FIG. 4. **The extended TRAP220 LXM1 core motif exhibits selective NR binding.** Yeast two-hybrid experiments as in the legend to Fig. 3 showing the interaction of TRAP220 LXM1 core and extended motifs with TRβ and the ERα LBD. Reporter activities were determined as described in the legend to Fig. 2. *Shaded* and *black bars* represent reporter activity in the absence and presence of 10^{-6} M receptor cognate ligand, respectively.

change of amino acids in the extended TRAP220 LXM1 sequence for the corresponding amino acids of SRC1 LXM2. We chose SRC1 LXM2 as this sequence shows strong interactions with both Class I and Class II NRs (14). Western blotting confirmed that wild type and mutant proteins were expressed at similar levels in the yeast reporter strain (Fig. 5*B*). Exchange of amino acids +2 and +3 of TRAP220 LXM1 for those of SRC1 LXM2 (mutant E) had little effect (<2-fold) on TRAP220 NID interaction with the Class I receptors PR and ERα (Fig. 5*C*) or the Class II receptors RARα, TRβ and RXRα (Fig. 5*D*). Similarly, the replacement of a proline at position −2 that has been suggested to define subclasses of coactivators (37) with a lysine residue as found in SRC1 LXM2 (mutant G), had little effect on TRAP220 NID interactions with the Class I receptors, PR and ERα (Fig. 5*C*) or the Class II receptor TRβ (Fig. 5*D*). However the ability of mutant G to bind RARα and RXRα LBDs in the presence of ligand was slightly reduced (~2-fold), and curiously we observed an increase in ligand-independent interaction with RXRα LBD (Fig. 5*D*). We also generated a mutant in which the entire TRAP220 extended LXM1 sequence (−4 to +9) was replaced with the corresponding SRC1 LXM2 sequence (mutant F), incorporating a total of eight amino acid exchanges at positions −4, −3, −2, +2, +3, +7, +8, and +9. Remarkably, this mutant displayed a strongly enhanced binding to Class I receptors PR and ERα (4- and 10-fold respectively, Fig. 5*C*). Importantly, the interaction of TRAP220 mutant F with the Class II receptors RARα, TRβ, and RXRα was unaffected and was similar to that displayed by wild-type TRAP220 (Fig. 5*D*). Taken together, our results indicate that replacement of the 13 amino acids incorporating

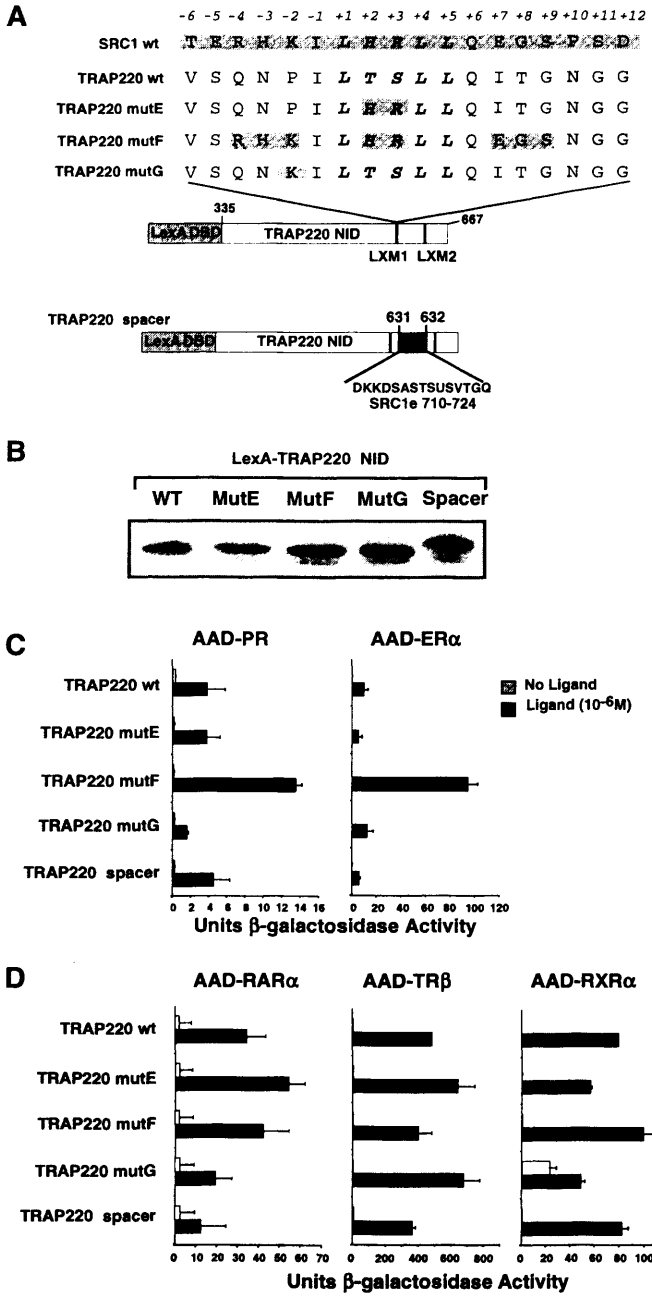

FIG. 5. **Effects of mutations altering LXM sequence and spacing on binding of the TRAP220 NID to NRs.** *A*, schematic representation of the LexA-TRAP220 NID mutants. Amino acid exchanges between TRAP220 and SRC1 sequences are highlighted by the *shaded boxes*. The additional SRC1-derived sequence used to generate the TRAP220 spacer mutant is indicated. *B*, Western blot showing the expression of the LexA-TRAP220 NID fusion proteins in cell-free extracts used in reporter assays. The antibody used was a mouse monoclonal antibody recognizing the LexA DBD. *C*, yeast two-hybrid experiments, performed as in Fig. 2, showing the interaction of LexA-TRAP220 NID wt and mutant proteins with AAD-PR and AAA-ERα fusion proteins, in the absence (*white bars*) or presence (*black bars*) of cognate ligand (R5020 and E_2, respectively). *D*, yeast two-hybrid experiment as in *C* using AAD-RARα, AAD-TRβ, and AAD-RXRα constructs with or without AT-RA, T_3, and 9C-RA, respectively. The data represent the mean reporter activity of two transformants, and *error bars* indicate S.D.

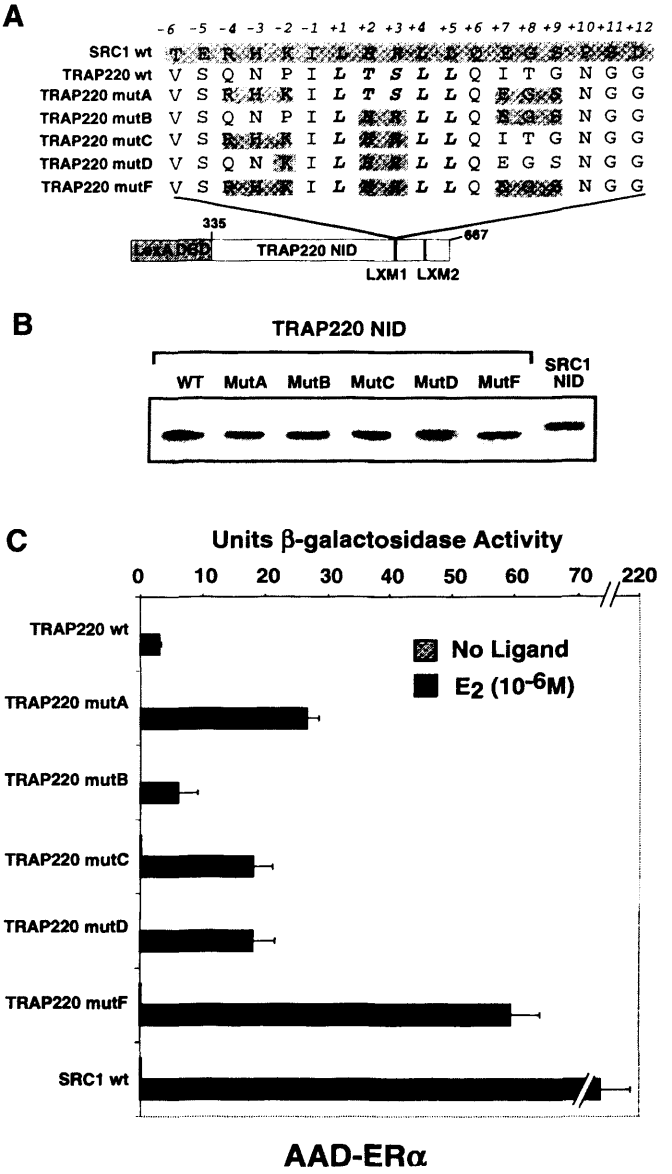

FIG. 6. **Combinatorial effects of mutations in the extended LXM1 on the binding of the TRAP220 NID to ERα.** *A*, schematic representation of LexA-TRAP220 NID mutants. The *shaded boxes* highlight amino acids from SRC1 LXM2 used to replace the corresponding amino acids in TRAP220 LXM1. *B*, Western blot as in Fig. 5 showing the expression levels of the LexA-DBD fusion proteins. *C*, yeast two-hybrid experiment showing interaction of LexA-SRC1 NID and LexA-TRAP220 NID wt and mutant proteins with AAD-ERα as described in the legend to Fig. 2.

TRAP220 LXM1 with the corresponding SRC1 LXM2 sequence is sufficient to change the NR binding properties of the TRAP220 NID. In contrast, amino acid exchange at position −2 or +2 and +3 is not in itself sufficient to permit strong interactions with Class I receptors.

The spacing between LXM core motifs in the p160 family is highly conserved, with 51 amino acids between LXM1 and LXM2, and between LXM2 and LXM3, of SRC1 (4). By comparison, the spacing between LXM1 and LXM2 of TRAP220 is 36 amino acids. Previous studies have shown that reducing the spacing between the GRIP1 or TRAP220 motifs can negatively influence NR binding properties (28, 35), suggesting there may be an optimal spacing requirement for specific NR interactions. To examine whether the spacing between LXM1 and LXM2 is a determinant of the TRAP220 preference for NR classes, we generated the mutant (LexA-TRAP220 NID spacer) in which the spacing between TRAP220 LXM1 and LXM2 core motifs was increased to 51 amino acids, as in p160 proteins. To achieve this, a 15 amino acid sequence taken from a corresponding region of SRC1 (amino acids 710–724, located between LXM2 and LXM3) was inserted between LXM1 and LXM2 of TRAP220 (Fig. 5*A*, *spacer*). As shown in Fig. 5*C*, this mutation did not significantly alter the binding of TRAP220

NID to PR or ERα, suggesting that increased spacing between the TRAP220 LXXLL motifs is not sufficient to allow a strong interaction with Class I receptors. Moreover, this sequence insertion did not adversely affect the interaction of the NID with TRβ or RXRα (Fig. 5*D*), although reporter activation due to interaction with AAD-RARα was slightly reduced (Fig 5*D*). Thus, the difference in spacing between LXMs in TRAP220 and SRC1 does not appear to be a critical determinant of the distinct NR binding preferences of these proteins.

Combinatorial Effects of Mutations in LXM1 on the NR Binding Specificity of the TRAP220 NID—Having determined that exchange of the 13 amino acids comprising LXM1 can alter the NR binding specificity of the TRAP220 NID (mutant F), we used an expanded panel of TRAP220 NID mutants (*A–D*) to investigate the specificity determinants in more detail (Fig. 6*A*). Yeast two hybrid experiments were carried out to assess the ability of these mutants to interact with the ERα LBD. Western blots confirmed that the wild-type and mutant proteins were expressed at similar levels in yeast (Fig. 6*B*). Mutant A is similar to mutant F with the exception that amino acids +2, +3, are wild type. Mutant B contains SRC1 sequence at positions +2, +3, +7, +8, and +9 and thus assesses the contribution of N-terminal flanking sequence. Mutant C contains SRC1 sequence at positions −4, −3, −2, +2, and +3, to assess the contribution of the C-terminal flanking sequence. Finally, mutant D contains SRC1 sequence at positions −2, +2, and +3. As shown in Fig. 6*C*, all these TRAP220 NID mutants showed increased ERα binding compared with wild type NID. However, none were as efficient at binding ERα-LBD as mutant F, which contained the entire SRC1 LXM2 extended motif. Mutant A displayed 8-fold enhanced reporter activation due to binding ERα-LBD, compared with a 19-fold enhancement seen for mutant F under similar conditions (Fig. 5*B*). This suggests that the amino acids at positions +2 and +3 are important in the context of extended LXXLL motif, although alone (mutant E) they have only a minimal effect on NR binding (Fig. 5C). Similarly, enhanced ERα-LBD interaction was observed for mutant C (6-fold) (Fig. 6*C*), suggesting that the N-terminal flanking sequences (−4, −3, −2) in combination with the +2, +3 amino acids, have an important influence on TRAP220 NID NR binding specificity. Moreover, a similar increase was observed for mutant D (7-fold), which has a single amino acid exchange at the −2 position (Pro to Lys) in combination with the +2 and +3 amino acid exchange. This is in contrast to the results obtained for mutant G, which contained the Pro to Lys change only, and mutant E, which has the +2, +3 change only, neither of which showed a strong increase in binding to ERα (Fig. 5*C*). Thus, in combination these mutations influence the NR binding specificity of TRAP220 LXM1. The relatively weak interaction of AAD-ERα with mutant B, which contained SRC1 LXM2 sequence at the C-terminal flank (+7, +8, +9), coupled with the change at the +2 and +3 position suggests that this combination of amino acids is less important for interaction with ERα. However, the difference in ERα binding of mutants F and C (Fig. 6*C*) suggests that the LXM1 C-terminal flanking sequence does influence NR binding specificity of the TRAP220 NID.

To examine whether mutation of the distal LXXLL motif would also alter TRAP220 NR binding specificity, we generated mutant H, which replaces the LXM2 sequence −4 to +9 with the corresponding SRC1 LXM2 sequence (Fig. 7*A*). As shown in Fig. 7*B*, this mutation resulted in a 8-fold increase in reporter activity due to ERα binding, but had little effect on the ability of the TRAP220 NID to bind TRβ. Thus replacement of either LXM1 or LXM2 in the TRAP220 NID with the 13 amino acid sequence, RHKILHRLLQEGS, results in a strong increase in

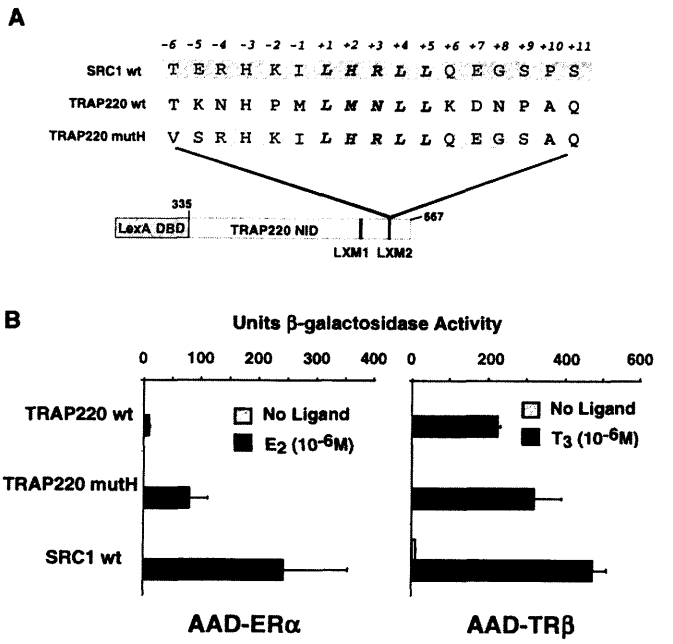

FIG. 7. **Mutation of the extended LXM2 sequence, and its effect on the interaction of TRAP220 NID with ERα.** *A*, schematic representation of highlighting sequence exchanges in LexA-TRAP220 mutant H. *B*, yeast two-hybrid experiment showing the binding of LexA-SRC1 NID and LexA-TRAP220 NID wt and mutant H proteins to AAD-ERα and AAD-TRβ as in Fig. 2.

binding to the Class I receptors ERα and PR, without affecting binding to Class II NRs. In contrast, altering spacing between LXM1 and LXM2 to that seen in SRC1, did not alter NR binding. Thus, we conclude the extended LXXLL motif is the principle NR binding specificity determinant of TRAP220.

Altering the NR Binding Specificity of Full-length TRAP220—To support the conclusions from our yeast two-hybrid data, we introduced mutations similar to that in mutant F into the LXM1 motif of full-length TRAP220 protein, and assessed its ability to bind NRs *in vitro* (Fig. 8). Bacterially expressed GST, GST-ERα-LBD, GST-TRβ-LBD, and GST-RXRα-LBD fusion proteins were tested for binding to *in vitro* translated, radiolabeled full-length SRC1e and TRAP220 wild-type proteins, or a TRAP220 LXM1 mutant F protein. Equal amounts of GST fusion proteins and radiolabeled proteins were used in each experiment. As expected, SRC1e showed strong ligand-dependent binding to ERα, TRβ, and RXRα LBDs. Under the same conditions, TRAP220 wild-type protein showed strong ligand-dependent or ligand-stimulated binding to RXRα and TRβ, respectively. In contrast, TRAP220 wild-type protein showed little, if any, detectable binding to ERα LBD, consistent with our yeast two-hybrid experiments. Remarkably, the TRAP220 LXM1 mutant F displayed strong interactions with TRβ, RXRα LBDs, and also the ERα LBD in the presence of ligand. Thus, swapping of a single extended LXM sequence is sufficient to change of specificity of the TRAP220 coactivator, allowing it to bind the Class I receptor ERα.

DISCUSSION

TRAP220/DRIP205/PBP was identified as a consequence of its strong ligand-dependent binding to Class II NRs such as TRα/β, VDR, and PPARγ. In contrast, several groups have shown that the interaction of TRAP220 with ERα is comparatively weak (18, 19, 30, 51). In this study we compared the interactions of TRAP220 proteins with Class I and Class II NRs using yeast two-hybrid and GST pull-down experiments. Consistent with this, our results show that TRAP220 NID (Figs. 2,

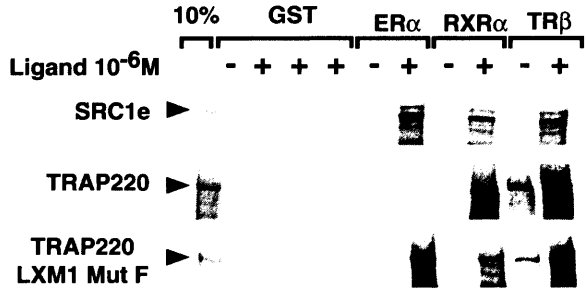

FIG. 8. **Interaction of wild type and mutant TRAP220 proteins with GST-NRs *in vitro*.** Normalized amounts of GST, GST-ERα-LBD, GST-RXRα-LBD, and GST-TRβ-LBD proteins were immobilized on glutathione-Sepharose beads and incubated with ^{35}S-labeled full-length TRAP220 wild-type (*wt*), TRAP220 LXM1 mutant F, or SRC1e wild-type protein (as control), in the presence or absence of 10^{-6} M cognate ligand as indicated (E_2, 9C-RA, or T_3, respectively). Bound proteins were analyzed by SDS-PAGE and autoradiography, as described under "Materials and Methods." One-tenth of the total ^{35}S-labeled protein used in the pull-down is shown for comparison (10% input).

5, 6, and 7) or full-length TRAP220 proteins (Fig. 8) displayed only weak ligand-induced interactions with ERα (or other Class I NRs) in yeast two-hybrid and GST pull-down assays. While the SRC1 NID showed no apparent preference for binding to the panel of NRs used here (Fig. 2*A*), TRAP220 NID bound preferentially to TRβ, RXRα, PPARγ, and to a lesser extent RARα, whereas its binding to ERα, AR, and PR was dramatically reduced (Fig. 2*B*). To understand the molecular basis of these differential interactions, we undertook an indepth analysis of TRAP220/NR binding.

The presence of multiple LXXLL motifs in p160s and other coactivators is thought to facilitate co-operative binding to NRs and may be important for selective interactions through differential usage of LXXLL motifs (15, 16, 35, 52). Similarly, mutagenesis of TRAP220/DRIP205 LXXLL motifs revealed preferential interaction of RXRα LBD with LXM1, whereas LXM2 showed preferential binding to the LBDs of the heterodimeric partners of RXR such as TRα, VDR, and PPARα (28). Our yeast two-hybrid experiments indicate that mutation of the conserved leucines in either LXM1 or LXM2 results in greatly reduced binding to Class II receptors (Fig. 2*C*), suggesting that NR LBD dimers contact both TRAP220 motifs. This is consistent with the crystal structures of agonist-bound LBD homodimers or RXRα LBD heterodimers in combination with short LXXLL peptides, or a partial SRC1 NID, which show that both LBDs in the NR dimer are occupied with a LXXLL core α-helix (12, 13, 34, 53). An exception in our experiments was the RXRα LBD, which required only a functional LXM1 for strong binding (Fig. 2*C*).

To investigate whether the LXXLL motifs from TRAP220 display the same NR specificity as demonstrated by its NID, we examined their NR binding properties. Remarkably, TRAP220 LXM1 and LXM2 core motifs displayed strong interactions with all NR LBDs tested (Fig. 3, *C* and *D*), in contrast to the NID (Fig. 2*B*). Previous studies have shown that the NR binding specificity of other coactivators is determined in part by LXXLL core motifs (4, 14, 35, 52) but also involves sequences immediately flanking the core motif (35, 38). As shown in Fig. 4, an extended LXM1 sequence, containing additional amino acids on the N- and C-terminal flanks, showed increased interaction (3.5-fold) with TRβ, but a 10-fold reduced interaction with ERα LBD. Thus, the extended 13 amino acid LXM1 sequence displays selective NR binding properties similar to the TRAP220 NID (Fig. 2*B*), or full-length protein (Fig. 8).

To determine which residues in the extended LXM1 are important for its NR subclass selectivity, we used a panel of TRAP220 NID mutants (A–H). Initially, we confirmed that the extended LXM1 sequence is a major determinant of TRAP220 NR binding specificity, by exchange of the LXM1 for the corresponding sequence of SRC1 LXM2 (Mutant F, Fig. 5*A*). This resulted in strongly enhanced interaction of the TRAP220 NID with ERα and PR (Fig. 5*C*) but had little or no effect on binding to Class II NRs (Fig. 5*D*). A similar result was recently reported in which replacement of TRAP220 LXM1 (residues −5 to +9) with the corresponding sequence from TIF2 LXM2, enhanced binding to ERα, in contrast to wild-type TRAP220 (30). However, this effect is not restricted to LXM1, as our data show that exchange of the extended TRAP220 LXM2 sequence for SRC1 LXM2, (Mutant H) also results in enhanced binding to ERα LBD, without affecting binding to TRβ (Fig. 7*B*).

Other studies have shown that a reduction in the spacing between LXXLL motifs in p160s (35) or TRAP220 (28) has adverse affects on NID/NR interactions. This suggest that a minimal sequence length is required to generate a folded or flexible domain which can permit docking of both LXM1 and LXM2 with both AF2 surfaces on NR dimers. However, we noted that the spacing between p160 LXMs (51 amino acids) is highly conserved, even across species, and differs from that found between TRAP220 LXMs (36 amino acids). To investigate whether this differential spacing is a feature of the NR selectivity of TRAP220, we generated the mutant designated spacer in which the distance between LXM1 and LXM2 in the TRAP220 NID is increased to 51 amino acids, using a sequence derived from the SRC1 LXM2/LXM3 spacer region (Fig. 5*A*). However, the spacer mutant showed no enhanced interaction with steroid receptors (Fig. 5*C*), nor was binding to Class II NRs adversely affected (Fig. 5*D*). Thus, while a minimal spacer sequence may be required to allow contact with both AF2 surfaces, the exact spacing does not appear to be critical. Moreover, the absence of any effect of the spacer mutation on binding to Class II NRs is consistent with the hypothesis that rather than folding into a rigid domain, the NID may be a flexible or largely unstructured sequence, accommodating interactions with AF2 surfaces on different NR dimers.

Other TRAP220 NID mutants were used to examine the importance of different residues within the extended LXM1 sequence for NR binding specificity. A previous study used phage display to identify subclasses of LXXLL sequences that show differential interactions with ERα LBD (37). The LXMs of TRAP220 fall into the subclass typified by having a conserved proline residue at the −2 position, which shows relatively weak interactions with ERα. In p160s, three lysine residues flanking the LXM1 motif, including a lysine at the −2 position, have been shown to be targets for acetylation by CBP/p300, and this event assists the dissociation of NR/p160 complexes (47). This lysine residue at the −2 position potentially makes electrostatic contact with Glu-380 in helix 5 of the ERα LBD (47). Our data show that exchange of the proline at −2 in LXM1 for lysine (Mutant G) did not significantly alter the binding properties of the TRAP220 NID (Fig. 5, *C* and *D*). Similarly, substitution of proline for alanine at position −2 of LXM2 had little effect on TRAP220 binding to TRα, VDR, or PPARα (28). Thus, mutation of this residue in LXM1 is not sufficient on its own to enhance TRAP220 binding to steroid receptors.

Several studies have highlighted the importance of the +2 and +3 amino acids in NR/cofactor interactions. Mutation of the +2 and +3 amino acids of the variant FXXLL motif of NSD1 to alanines (Ser-Thr to Ala-Ala) abolished binding to both Class I and Class II NRs (33). Similarly, a +3 mutation in TIF2 LXM3 has been reported to reduce its interaction with ERα (30). However, in our experiments, exchange of the +2 and +3 amino acids of TRAP220 LXM1 for the positively charged equivalents in SRC1 LXM2 (Mutant E) did not by itself result

in a strong increase in binding of TRAP220 NID to Class I NRs (Fig. 5*C*), or alter interaction with Class II NRs (Fig. 5*D*).

Mutants A–D (Fig. 6*A*) were used in NR binding assays to allow us to investigate the effect of combinatorial changes in the extended LXM1 sequence. All of these mutants displayed enhanced interaction with ERα compared with wild-type NID (Fig. 6*C*). For example, ERα binding to Mutant F was greater than to Mutant A, indicating that the +2, +3 positions are important for optimal binding. Similarly, exchange of residues +2, +3 resulted in increased ERα binding only when combined with exchange of N-terminal (−4, −3, −2), or to a lesser extent the C-terminal (+7, +8, +9) flanking sequences (compare Mutant E in Fig. 5*C* with Mutants B and C in Fig. 6*C*). The similar extent of ERα interaction with Mutants C and D also suggests that the −2 position plays a key role in the interaction of ERα with the N-terminal flank, possibly via the Glu-380 residue in helix 5 of ERα (47). Taken together, our results indicate that exchange of the entire extended LXM1 motif for the SRC1 LXM2 sequence is required for optimally enhanced binding to ERα, and that residues within the core motif and flanking sequence cooperate to determine NR binding specificity.

The L*XX*LL core motif forms a two-turn α-helix that is clamped in position on the LBD surface by electrostatic interactions with conserved lysine and glutamate residues (13). Unfortunately, the crystal structures available to date offer little structural insight into how flanking sequences in extended L*XX*LL motifs might determine receptor specific contacts with LBDs. A recent study has revealed the existence of a second charged clamp in the GR LBD that appears to anchor the third L*XX*LL motif of TIF2 (LXM3). This involves interactions with the +2 and +6 amino acids, further highlighting the importance of the +2 position amino acid and C-terminal flank in coactivator interactions with Class I NRs (58). Further structural studies involving extended L*XX*LL peptides, and preferably stably folded NIDs will be required to visualize selective interactions of different NRs with TRAP220 and other cofactors such as TIP60, NRIF3, and ASC-2.

Purified TRAP/DRIP/mediator complexes have been shown to enhance the activity of NRs in cell-free or purified *in vitro* transcription assays (17, 50, 54). In comparison, only very modest enhancement of TRα/β, VDR, and PPARγ-mediated transcription has been observed due to ectopic expression of the NR binding subunit TRAP220/DRIP205/PBP in transiently transfected cells (17, 19–21). Consistent with this, we observed that exogenously expressed TRAP220 induced a very moderate enhancement of TRβ activation of a DR4-driven reporter gene (Fig. 1*A*). The relatively weak coactivator activity of TRAP220 in transfection experiments might be due to inefficient assembly of exogenous TRAP220 proteins into functional mediator complexes. By contrast, TRAP220 failed to enhance ERα-mediated transcription of the 3ERE-TATA-LUC reporter gene (Fig 1*B*). Nonetheless, chromatin immunoprecipitation experiments have suggested that TRAP/DRIP proteins are recruited to NR-regulated promoters, including ERα- and AR-responsive promoters (51, 55, 56), and microinjection of HeLa cell nuclei with anti-TRAP220/PBP antibodies down-regulated ERα activation of a reporter gene (57). In addition, a recent report has shown that the TRAP complex can be purified from HeLa cell nuclear extracts using GST-ERα LBD in the presence of agonist ligand (50). In contrast to reports suggesting that TRAP170 interacts with steroid receptors, GST-ERα AF1 did not retain TRAP complex in HeLa cell nuclear extracts (50). Thus, recruitment of TRAP complexes to the promoters of estrogen-regulated genes may involve additional interactions with other components of this multiprotein complex.

In conclusion, we have demonstrated that TRAP220 exhibits preferential binding to Class II NRs and only weak interactions with steroid receptors. This binding specificity is determined by an extended L*XX*LL motifs of 13 amino acids in length, which when exchanged is sufficient to alter the specificity of full-length TRAP220 protein. It will be of interest to undertake mutagenesis of NR LBDs to further explore the molecular mechanism of selective interactions between NRs and their cofactors.

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