

Identification of Host Proteins which Interact with Vaccinia Virus Encoded E3L.

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by

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Abstract.

IFN induces an antiviral state in infected cells by modulating cellular transcription. A number of proteins are upregulated including PKR, a serine/threonine protein kinase that is activated by viral dsRNA. Once activated this protein phosphorylates eIF2- α , a component of the translation initiation complex, which results in the inhibition of protein synthesis.

The E3L protein is one of a number of proteins encoded by Vaccinia Virus (VV) which antagonise the IFN cellular response. E3L is a dsRNA binding protein which sequesters dsRNA that would ordinarily activate PKR. Therefore, within a VV infected system, protein translation is maintained. Both PKR and E3L are members of a family of proteins of diverse function which interact with dsRNA via a dsRNA binding motif (dsRBM). The amino acid sequence of these dsRBMs are highly conserved and probably form a conserved structural motif.

One of the aims of my PhD project was to determine the structure of a dsRBM by NMR spectroscopy. Studies involved the over-expression of the dsRBM region of PKR, Met1-170 and three constructs of E3L, Met1-190, Met38-190 and Met94-190, using an *E. coli* based expression system. The proteins were expressed and purified to homogeneity, however, structural analysis of these constructs was not possible as the maximum concentration of the proteins was insufficient for NMR analysis. The self association of E3L Met94-190 was also investigated. The protein was found to form dimers at a concentration up to 3.4mg/ml as determined by analytical ultra centrifugation and gel filtration, however, crosslinking analysis indicated the protein was able to form multimers.

A yeast-two-hybrid screen was undertaken to identify cellular proteins that interact with E3L. Three proteins were identified, PIC-1, a nuclear protein, H3.3 a histone protein and L23a, a ribosomal protein. All three of the proteins were found to interact with the dsRBM of E3L. Attempts to demonstrate biochemical interactions between E3L and PIC-1 and H3.3 were unsuccessful.

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Abbreviations.

APS	ammonium persulphate
CHAPS	N,N-dimethyl-N-(3 sulphopropyl)-3-[[3 α 5 β ,7 α ,12 α)-3,7,12-trihydroxy-24-oxocholan-24-YL]-amino]-1-propanaminium inner salt, or (2-[(3-cholamidopropyl)dimethylamminio]-1propane sulphonate)
DEPC	diethylpyrocarbonate
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamino tetraacetic acid
HEPES	N-[2-hydroxyethyl]piperazine
GHCl	Guanidine HCl
HRP	Horse radish peroxidase
IFN	Interferon
IPTG	Isopropyl β -D-thiogalactopyranoside
kb	kilobases
kDa	kilodaltons
MOPS	3-[N-morpholino]propanesulfonic acid
NBT	nitroblue tetrazolium
NMR	Nuclear Magnetic Resonance
magnesium acetate	$C_4H_6O_4Mg \cdot 4H_2O$
MW	Molecular weight
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulphonylfluoride

RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
VV	Vaccinia Virus
X-gal	5-bromo-4-chloro-indoyl- β -D-galactopyranoside
X-phos	5-bromo-4-chloro-indoyl - phosphate

1. Introduction.

The human body is constantly exposed to a variety of infectious agents such as bacteria, fungi, parasites and viruses. The immune system is composed of a number of mechanisms which work collectively to eliminate such agents from the host. Interferon (IFN) expression mediates the initial antiviral response. The IFNs induce a number of proteins, which together eliminate viral infection by inhibiting the growth of the infected cell and as such prevent viral replication.

1.1 Interferons.

Interferons were first discovered in 1957 by Isaacs and Lindenmann. They found that an agent was secreted by chicken chorioallantoic membranes when incubated with heat inactivated influenza virus. When this agent was transferred into cultured membrane fragments infected with competent influenza virus, it was found that this agent interfered with viral replication.

IFNs have been the subject of intense investigation over the last thirty years and are now known to constitute a large multigene family of regulatory proteins. IFNs mediate a wide variety of different biological functions including inhibition of cell growth, effects on cellular and humoral immune responses, and the establishment of the antiviral state.

The interferon gene family is classified into two groups, Type I and Type II IFN. They are produced by different cells, have alternate receptors and inducers and exert distinct biological activities. Both exhibit antiviral activities and are required to work in co-operation against certain viruses (Muller, 1994).

1.1.1 Type I IFNs.

The major functions of type I interferons are the regulation of cell growth and differentiation, the induction of MHC class I antigens and the antiviral state. Type I IFNs, IFN α and IFN β are secreted by almost all cell types except embryonal carcinoma cells (Harada, 1990). The most potent inducer of these IFNs is dsRNA produced during viral replication, although other stimuli such as cytokines and infection by protozoa, mycoplasma or bacteria, are also capable of induction.

Type I IFNs are believed to have evolved from a single ancestral gene and lack introns. The genes form a cluster on the short arm of chromosome 9 in humans, and chromosome 4 in mice. There are 18 non allelic IFN α genes of which 4 are pseudogenes, 6 non allelic IFN ω genes of which 5 are pseudogenes and a single IFN β gene (Sen and Lengyll,

1992). Recently another type I IFN, IFN τ was discovered in large quantities in the trophoblastic epithelium of the pre-implanted embryo of domestic ruminants. An essential role for IFN in embryogenesis has since been ruled out, as the disruption of the type I receptor in mice did not affect development of the embryo (Vilcek and Sen, 1996).

1.1.2 Type II IFNs.

The major functions of type II IFN, or IFN γ , is the induction of MHC class I and II antigens, macrophage activation and the induction of the antiviral state (Vilcek and Sen, 1996). IFN γ production is induced by specific antigens to which the organism has already been sensitised, but non specific T-cell activators can also induce production (Jaramillo *et al.*, 1995). The IFN has been found in all mammalian species examined so far, and is produced by T lymphocytes and Natural Killer (NK) cells.

The single IFN γ gene contains three introns and is located on chromosome 12. Sequence similarity between IFN β and IFN γ has been noted although the significance of this is not yet understood (Sen and Lengyel, 1992).

1.1.3 Interferon Biosynthesis.

The induction of IFN biosynthesis is principally as a result of viral infection, although other factors can also induce production.

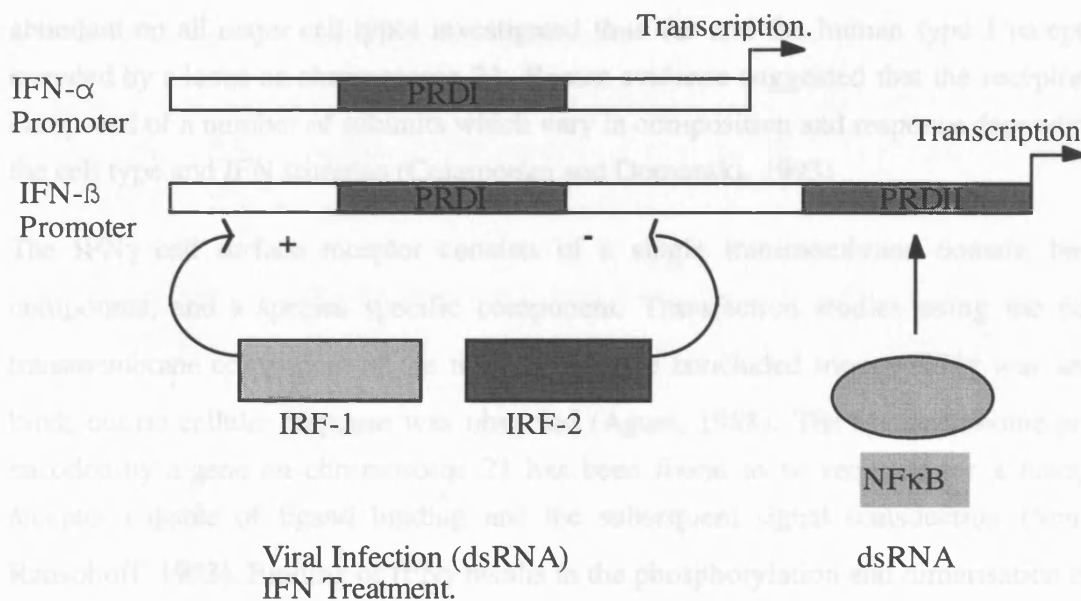
Type I.

Type I IFN induction is transient and is controlled primarily at the level of transcription although destabilising sequences present in the 3' untranslated region of the mRNA lead to a short half life (Caput, 1986). Transcriptional induction is achieved by the activation of specific transcription factors which bind to common regulatory regions of the IFN genes known as interferon response elements (IREs) (Figure 1.1). Superinduction of the IFN mRNAs is evident upon inhibition of protein synthesis, presumably as a result of the downregulation of a repressor. Induction is also enhanced by pre-treatment of the cells with low levels of IFN (Sen and Ransohoff, 1993).

Figure 1.1. Factors involved in Type I IFN biosynthesis (Jaramillo *et al.*, 1995).

Within the 5' untranslated region of the IFN β gene is a negative regulatory domain (NRD), which provides stable repression prior to induction, and four positive regulatory domains (PRDI-IV). The PRDI and II are highlighted below as these are crucial to the activation of IFN type I biosynthesis. PRDI binds to a number of *trans*-acting factors including interferon regulatory factors IRF-1 and IRF-2. The two factors are structurally similar although IRF-1 acts as a transcriptional activator and IRF-2 as a transcriptional repressor. The two proteins are induced by both IFN and virus infection, and are believed to compete for the same binding site of PRDI, the result being a transient induction of IFN β (Jaramillo *et al.*, 1995). PRDII, an NF κ B like binding site, is located downstream of PRDI and an NF κ B-like factor is required to bind to PRDII for maximal activation. Activation of both NF κ B and IRF-1 are mediated by dsRNA, leading to the transcription of IFN β (Tanguchi, 1989).

The induction of the IFN α gene is distinct to IFN β , as there is no PRDII binding site. The IFN α gene regulatory element does however contain transcriptional elements composed of 'TG sequences'. These sequences are variable and can be directly induced by different stimuli including virus, IFN and IRF-1 (Macdonald *et al.*, 1990).



1.1.5 Signal Transduction.

In response to stimuli, the rate of transcription of IFN induced genes can increase 10 fold or more, and last for hours. The proteins they encode mediate various mechanisms required to achieve the antiviral state (Samuel, 1991). The transduction process occurs through the Jak-STAT family of proteins, transferring the IFN signal from the membrane

Type II

The control of IFN γ biosynthesis is complex and not yet fully understood. The increase in IFN γ production during viral infection is believed to be mediated by IL-12, a response which is also thought to be involved in bacterial and protozoan infections. T-cell activating agents also promote IFN γ synthesis, as does IFN γ itself (Vilcek and Sen, 1996). The induction of IFN γ is thought to be regulated primarily at the level of transcription, although no known regulatory sequences have been identified to date. It is believed that positive and negative regulatory elements are located in the 5' flanking region, and as yet unknown *trans*-acting factors interact with these regions (Sen and Ransohoff, 1993).

1.1.4 Interferon Receptors.

The first stage in the IFN regulated antiviral state is the induction of the IFN induced genes (ISGs), via the cell surface receptor. Type I IFN α and β compete for the same receptor(s), whereas IFN γ binds to a distinct cell surface receptor. Type I receptors are abundant on all major cell types investigated thus far and the human type I receptor is encoded by a locus on chromosome 21. Recent evidence suggested that the receptors are composed of a number of subunits which vary in composition and response depending on the cell type and IFN stimulus (Colamonici and Domanski, 1993)

The IFN γ cell surface receptor consists of a single transmembrane domain binding component, and a species specific component. Transfection studies using the human transmembrane component of the receptor in mice concluded mouse IFN γ was able to bind, but no cellular response was observed (Aguet, 1988). The transmembrane protein encoded by a gene on chromosome 21 has been found to be required for a functional receptor capable of ligand binding and the subsequent signal transduction (Sen and Ransohoff, 1993). Binding of IFN γ results in the phosphorylation and dimerisation of the receptor which is required for signal transduction via the Jak-STAT pathway.

1.1.5 Signal Transduction.

In response to stimuli, the rate of transcription of IFN induced genes can increase in minutes, and last for hours. The proteins they encode mediate various mechanisms required to achieve the antiviral state (Samuel, 1991). The transduction process occurs through the Jak-STAT family of proteins, transferring the IFN signal from the membrane

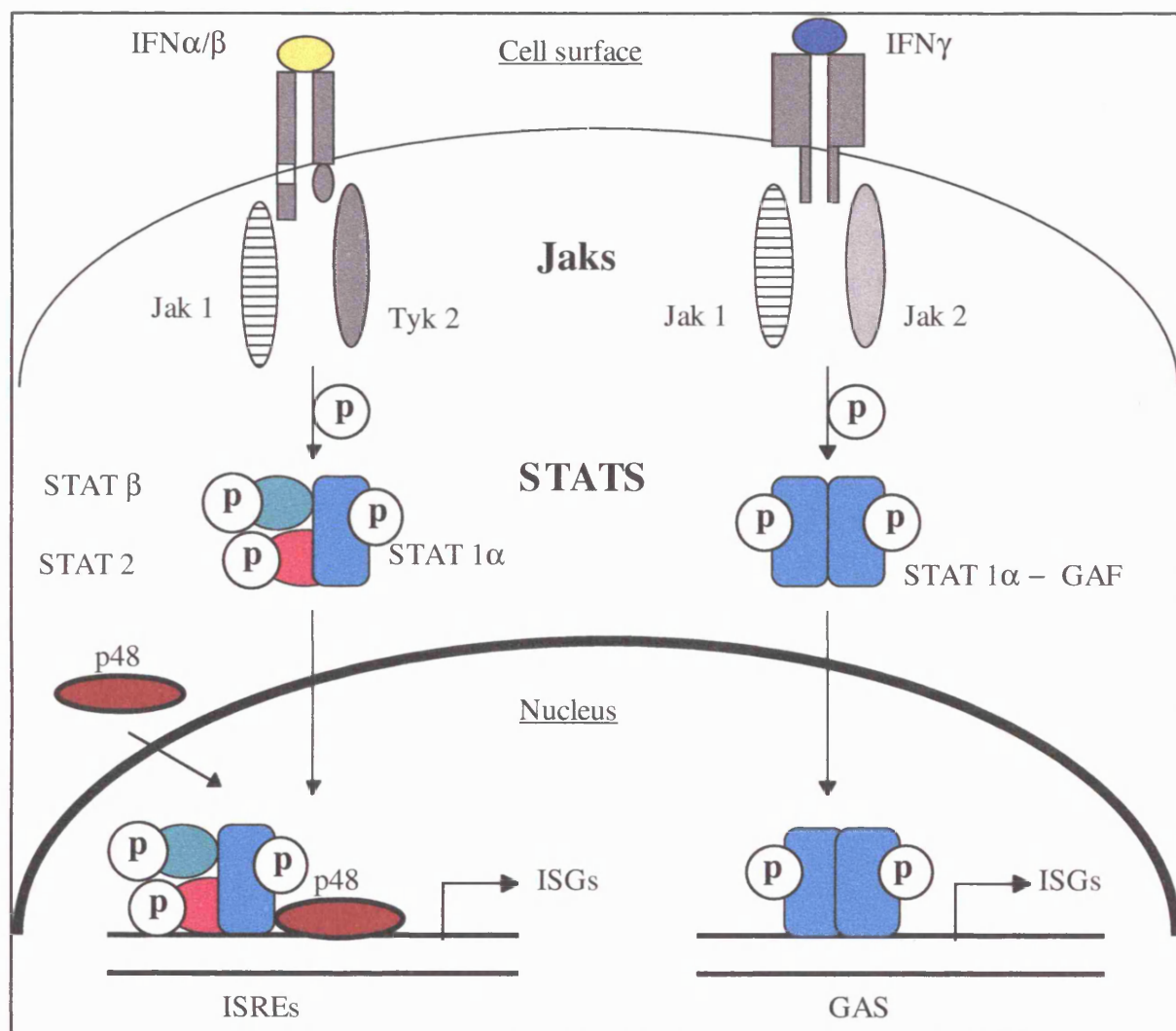
to the nucleus where cellular interferon stimulated genes (ISGs) can be induced (Figure 1.2). Jaks (Janus protein kinases) are cytoplasmic non receptor tyrosine kinases that are believed to mediate the phosphorylation events after IFN binds to the receptor. STATs (signal transducers and activators of transcription) are latent transcriptional activators that, once phosphorylated, translocate to the nucleus where they regulate gene expression (Lamb *et al.*, 1998).

Induction of ISGs can take place by alternate mechanisms. IRF-1, also known as ISGF-2, is capable of stimulating ISGs in HeLa S3 cells lacking IFN β production (Jarimillo *et al.*, 1995). It has been recognised for some time that viruses are able to activate genes directly, independently of ISGF3. This activation is thought to be mediated by dsRNA activated factors 1 and 2 (DRAF1 and DRAF2). DRAF1 binds to the classical ISRE element but only a subset of ISGs are induced as specific adenine flanking sequences are required. The RNA binding component which is found in the cytoplasm of uninfected cells, is activated in minutes following infection, whereupon it is then believed to translocate to the nucleus. The rapid initiation of this antiviral mechanism suggests it is the cells primary defence mechanism against viral attack. The exact role for DRAF 2 has yet to be elucidated (Daly and Reich, 1995).

1.1.6 Interferon Stimulated Genes.

Over 30 genes are induced by interferon, some of which are not detectable in an uninduced cell. The induction of ISGs are controlled at the level of transcription, and gene transcripts can increase 50-100 fold. Proteins induced by IFN include MHC Class I and II, which enhance antigen presentation and therefore increase immune-modulated cell lysis. Nitric oxide synthetase induction results in increased levels of nitric oxide, which has been linked with the inhibition of a number of viruses including Vaccinia Virus (Vilcek and Sen, 1996). A number of other proteins are also induced, although the functions of these proteins have yet to be elucidated (Staeheli, 1990)

Figure 1.2 Model for IFN receptor signalling (Adapted from Jaramillo, 1995). The interaction between IFN and the binding component of the specific receptor initiates the dimerisation of the receptor chains. This dimerisation leads to the activation of the Jak/Tyk 2 kinases, which *trans*-phosphorylate the receptor chains. The STAT proteins are then phosphorylated on tyrosine residues and are released from the receptor complex (Levy, 1995, Lamb *et al.*, 1998). Two complexes are then formed, IFN stimulated gene factor (ISGF3 α) if IFN α/β has bound to the surface receptors, and gamma activating factor (GAF) if IFN γ has bound. The complexes then migrate to the nucleus where GAF binds to the gamma activation sequence (GAS) and ISGF3 α combined with p48 binds to the IFN stimulated response element (ISRE) (Gaboli *et al.*, 1998, Darnell *et al.*, 1998).



1.2 Antiviral Actions of Interferon.

The response initiated by IFN allows the cell to use more than one mechanism to combat the virus. The cell is capable of obstructing viral replication at a number of stages including penetration, transcription, translation and maturation (Staeheli, 1990, and Vilcek and Sen, 1996).

Mx proteins are regulated by Type I IFN and are a family of proteins that specifically inhibit multiplication of influenza virus. These proteins are believed to stop transcription of the viral genome, however the mechanism of action remains obscure (Samuel, 1991). More recently RNA-specific adenosine deaminase (ADAR) has been detected which is induced by IFN and edits viral mRNAs and cellular pre-mRNAs. The dsRNA conformation is crucial to the enzymes editing activity due to the dsRNA binding properties of the protein. ADAR catalyses the deamination of adenosine to produce inosine leading to hypermutations in measles virus and other negative stranded RNA genomes (Lei *et al.*, 1998). A ribonuclease specific for RNA containing inosine (RNase I) has recently been discovered, which is believed to work together with ADAR in an antiviral fashion to eliminate viral RNAs (Scaddon and Smith, 1997). Two further proteins induced by interferon, dsRNA protein kinase and 2'-5' oligoadenylate synthetase, inhibit the translational machinery within an infected cell.

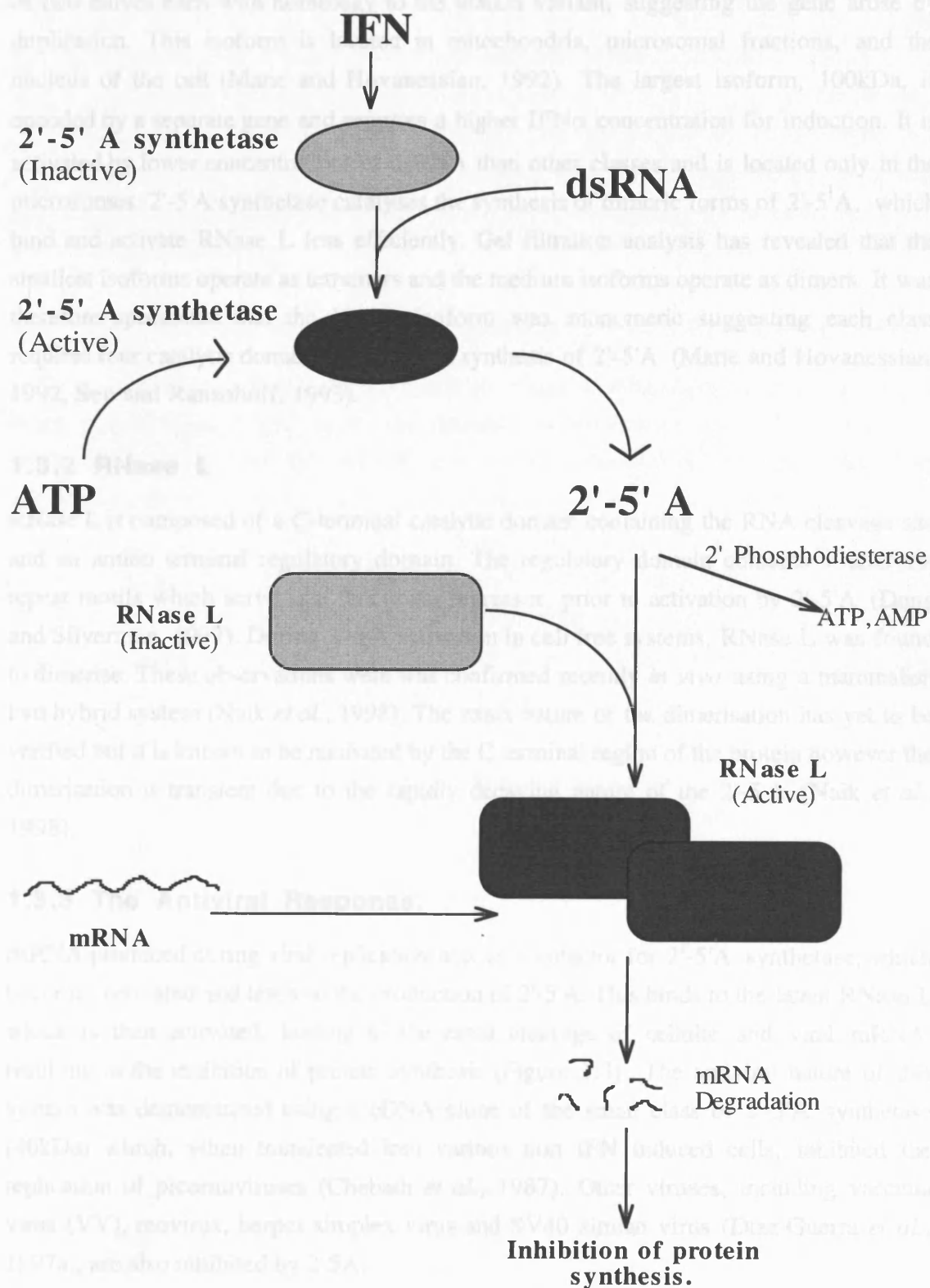
1.3 2'-5' Oligoadenylate Synthetase System

There are a number of components that are required for this system to inactivate translation (Figure 1.3). 2'-5' oligoadenylate synthetase (2'-5' A synthetase) is an IFN induced enzyme that converts ATP into 2'-5' phosphodiester linked adenosine oligomers (2'-5'-A or ppp(A2'p5)nA) of varying lengths. RNase L is a latent endoribonuclease present in the cytoplasm of cells (Salhzaha *et al.*, 1991), which when activated by 2'-5'-A cleaves single stranded RNA. A phosphodiesterase that hydrolyses 2'-5'A, limits cellular toxicity and is crucial to this system.

1.3.1 2'-5' Oligoadenylate Synthetase.

IFN (type I and II) induces 4 isoforms of 2'-5' oligoadenylate synthetase, which result from the induction of different genes with differential splicing mechanisms (Hovaniessian *et al.*, 1988). These proteins are activated by binding dsRNA, although this interaction is not well defined (Player and Torrence, 1998). The smallest proteins are 40-46kDa and are formed by alternative splicing of the same gene (Ghosh *et al.*, 1991). The differential splicing of the gene produces proteins with distinct carboxyl termini which have differing hydrophobicities. The dsRNA binding region is believed to be located within the amino

Figure 1.3. An overview of the 2'-5'A synthetase system highlighting the activation of 2'-5'A synthetase by dsRNA and the subsequent activation of RNase L.



terminal region. The second class of proteins are 69kDa and are encoded by a separate gene, which can also give rise to alternatively spliced products. The enzyme is composed of two halves each with homology to the 40kDa variant, suggesting the gene arose by duplication. This isoform is located in mitochondria, microsomal fractions, and the nucleus of the cell (Marie and Hovanessian, 1992). The largest isoform, 100kDa, is encoded by a separate gene and requires a higher IFN α concentration for induction. It is activated by lower concentrations of dsRNA than other classes and is located only in the microsomes. 2'-5'A synthetase catalyses the synthesis of dimeric forms of 2'-5'A, which bind and activate RNase L less efficiently. Gel filtration analysis has revealed that the smallest isoforms operate as tetramers and the medium isoforms operate as dimers. It was therefore speculated that the largest isoform was monomeric suggesting each class requires four catalytic domains for efficient synthesis of 2'-5'A (Marie and Hovanessian, 1992, Sen and Ransohoff, 1993).

1.3.2 RNase L

RNase L is composed of a C-terminal catalytic domain containing the RNA cleavage site and an amino terminal regulatory domain. The regulatory domain contains 9 ankryrin repeat motifs which serve as a functional repressor, prior to activation by 2'-5'A (Dong and Silverman, 1997). During 2'-5'A activation in cell free systems, RNase L was found to dimerise. These observations were confirmed recently *in vivo* using a mammalian two hybrid system (Naik *et al.*, 1998). The exact nature of the dimerisation has yet to be verified but it is known to be mediated by the C terminal region of the protein however the dimerisation is transient due to the rapidly decaying nature of the 2'-5'A (Naik *et al.*, 1998).

1.3.3 The Antiviral Response.

dsRNA produced during viral replication acts as a cofactor for 2'-5'A synthetase, which becomes activated and leads to the production of 2'-5'A. This binds to the latent RNase L which is then activated, leading to the rapid cleavage of cellular and viral mRNA, resulting in the inhibition of protein synthesis (Figure 1.3). The antiviral nature of this system was demonstrated using a cDNA clone of the small class of 2'-5'A synthetase (40kDa) which, when transfected into various non IFN induced cells, inhibited the replication of picornoviruses (Chebath *et al.*, 1987). Other viruses, including vaccinia virus (VV), reovirus, herpes simplex virus and SV40 simian virus (Diaz-Guerra *et al.*, 1997a), are also inhibited by 2-5A.

2'-5'A synthetase has also been associated with the control of cell growth. Expression of a cDNA clone of the small isoform (40kDa) of 2'-5'A synthetase resulted in a reduced rate of cell proliferation, as did the transfection of a dominant negative (C terminal

truncated) mutant of RNase L (Hassel *et al.*, 1993). Furthermore the 2'-5'A synthetase (p69) has been shown to have mRNA splicing activity, since the addition of a 2'-5'A synthetase antibody inhibited spliceosome function (Sen and Ransohoff, 1993).

The over-expression of RNase L by VV recombinants induced apoptosis in animal cells, which was further pronounced when co-expressed with 2'-5' A synthetase and highlighted a link with RNase L and apoptosis (Diaz-guerra, 1997b). Furthermore, this association was also evident in RNase L knockout mice, which were defective in both antiviral IFN action and apoptosis (Zhou *et al.*, 1998) highlighting the pivotal role of this system within the cell.

1.4 Double Stranded RNA Dependent Protein Kinase-PKR.

Following infection with VV protein synthesis inhibition was observed after 20 minutes (Metz and Esteban, 1972), work was therefore undertaken to find the cause of this inhibition. PKR (or DAI, dsI and p68 as it was also known) is now one of the most extensively studied protein kinases, due to its regulatory role in both infected and uninfected cells. Briefly, PKR is a protein kinase that is induced by IFN and activated by dsRNA produced during viral replication (Jacobs and Langland, 1996). Once activated the protein undergoes autophosphorylation (or *trans*phosphorylation by a second PKR molecule) leading to the phosphorylation of eIF2- α , a subunit of the protein synthesis initiation factor, at residue serine 51. This phosphorylation step then causes the global reduction in the initiation of protein synthesis. PKR homologues have been found in mice and rats which have 61% and 56% amino acid identity to human PKR respectively. PKR homologues in monkey and bovine cell lines have also been identified, but have not been fully characterised (Williams, 1995). Plant PKR has also been identified and is biochemically and immunologically similar to the mammalian form (Langland *et al.*, 1998).

Other protein kinases have been discovered that phosphorylate eIF2- α at serine 51, but are not regulated by dsRNA. The haemin-regulated inhibitor kinase (HRI) is a mammalian eIF2- α kinase activated under haem-deficient conditions (De Haro *et al.*, 1996). The second kinase encoded by GCN2 from *Saccharomyces cerevisiae* is activated by amino acid and purine shortages. GCN2 exerts gene specific translation of the GCN4 mRNA, required for the activation of over 30 amino acid biosynthetic genes, whilst the translation of other mRNAs are reduced. PKR and HRI can be substituted for GCN2 in yeast and activate the yeast eIF2- α kinase and subsequent translation of GCN4 however, over-expression of PKR in yeast lead to a slow growth phenotype, due to the inhibition of protein synthesis (De Haro *et al.*, 1996). An eIF2- α kinase has also been characterised from the malarial parasite *Plasmodium falciparum*, which is the first non mammalian HRI

to be identified (Mohrle *et al.*, 1997). More recently another eIF2- α kinase has been identified from a rat pancreatic islet (pancreatic eIF2- α kinase; PEK), which regulates translational control and is functional in both yeast and reticulocyte lysate models (Shi *et al.*, 1998).

The central role of PKR in virally infected cells is exemplified by the number of strategies employed by viruses to downregulate its activation and subsequent inhibition of protein synthesis. PKR has also been implicated in tumour suppression, apoptosis, cell proliferation and differentiation and signal transduction all of which will be discussed in more detail.

1.4.1 The *Pkr* Gene and Promoter Region.

The gene coding for PKR in humans, mice and rats have all been identified and characterised. The human gene is located on chromosome 2p21-22, and contains 17 exons, whilst the mouse homologue is located on chromosome 17 E2, and contains 16 exons, as an extra exon is located in the 5'UTR. The intron-exon organisation between the two are highly conserved, in fact 13 of the 15 protein exons are identical. The two dsRNA binding motifs R₁ and R₂ coding regions (discussed in section 1.4.3) are located in exon 4 and 6 respectively (Kuhlen *et al.*, 1996).

The complete sequence of both the human and mouse *Pkr* genes, and the upstream promoter regions are now known. The promoter regions of these two genes are quite distinct, but both contain motifs involved in IFN induction. Both genes contain an ISRE, crucial for mediating the IFN type I response. Upstream of the ISRE is a novel element, named a kinase conserved sequence (KCS), that is conserved in both sequence and relative position to the ISRE in the human and mouse *Pkr* gene promoter region. This sequence is believed to strengthen the type I IFN induced expression of the gene and enhance constitutive expression, producing a basal level of PKR in uninfected cells. Nuclear proteins have also been found to interact with this region, which correlates with its promoter activity, although the exact nature of the interaction has not been elucidated (Kuhlen *et al.*, 1998). A GAS site is present within the human and mouse promoters, however the human site is not as closely related to the consensus sequence as the mouse site, which could account for the poor inducibility of PKR expression by IFN type II in human cells (Thomis *et al.*, 1992). A NF-IL6 site, similar to an NF κ B site is also present on both promoters although it has not yet been demonstrated to mediate IL6 transcriptional activation by the human promoter (Kuhlen and Samuel, 1997).

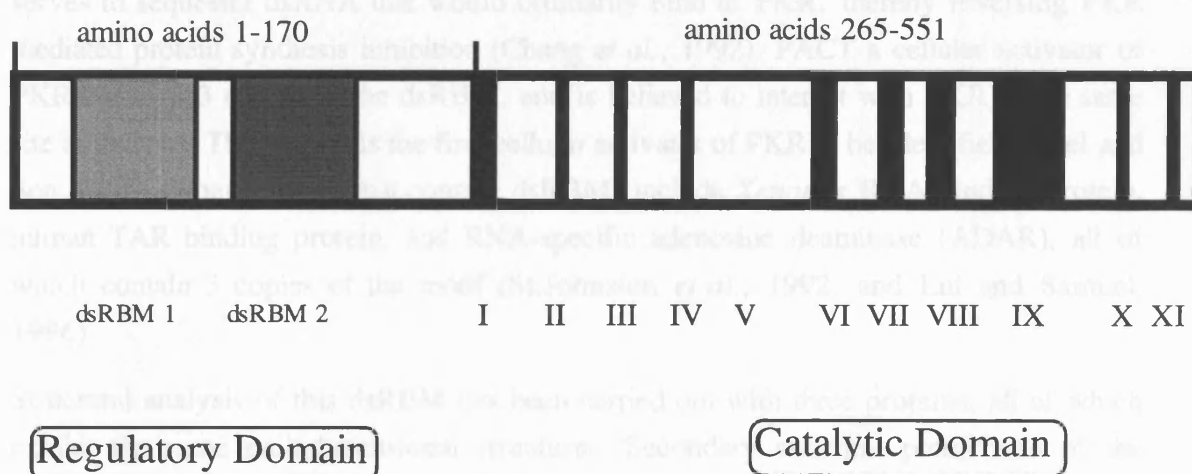
1.4.2 Localisation and Expression of the PKR.

Human PKR is present at a basal level in all cells studied. In uninfected cells PKR is found both in the nucleus and near to the ribosomes in the cytoplasm, at a ratio of 1:5 (Zhu *et al.*, 1997, Besse *et al.*, 1998). Treatment with type I IFN leads to a 5 fold increase in total PKR over 12 hours (Thomis *et al.*, 1992), however the quantity in the nucleus remains constant (Jeffrey *et al.*, 1995). The distribution of PKR within the cytoplasm is also altered by IFN treatment, as a large proportion of the protein dissociates from the ribosomes enabling PKR to bind to viral dsRNAs (Raine *et al.*, 1998).

1.4.3 Structure of the PKR protein.

PKR is a serine/threonine protein kinase composed of a regulatory and catalytic domain (Figure 1.4). The regulatory domain, which is located at the amino terminus, binds to dsRNA produced during viral replication. The catalytic kinase domain is located at the carboxyl terminus. The protein itself has a predicted MW of 62kDa, but is visualised by SDS PAGE at 68kDa due to the phosphorylation state of the protein (Meurs *et al.*, 1990).

Figure 1.4 Organisation of the PKR protein, highlighting the catalytic and dsRNA binding regions



N Terminal Region - dsRNA Binding Domain.

The cDNA clone of PKR was initially characterised from a 2.5kb mRNA which was strongly induced by IFN. When translated *in vitro* the protein was recognised by a monoclonal antibody reactive against native PKR (Meurs *et al.*, 1990). This protein has since been fully characterised and the following important domains have been identified. The dsRNA binding domain (dsRBD), located within the first 170 amino acids, consists of two tandem repeat motifs (R_1 and R_2), which are required for dsRNA binding (Green and Matthews, 1992, Patel and Sen, 1992, Barber *et al.*, 1992, McCormack *et al.*, 1992). The two dsRNA binding motifs (dsRBM) which are positioned at amino acids 11-77 and

101-169 within the human protein, are composed of a 67 amino acid consensus sequence similar to that found in other dsRNA binding proteins (St.Johnston *et al.*, 1992). R_1 is more closely related to this consensus sequence and mutations within R_1 have a more deleterious effect on dsRNA binding than equivalent mutations equivalent amino acid mutations made within R_2 . Therefore R_1 is crucial for dsRNA binding although maximal binding is attained when both dsRBMs present (100 fold more). However, the relative location of the two motifs is less important, and interchanging the location of the two motifs has no deleterious effect on dsRNA binding (McCormack *et al.*, 1994, Schemdt *et al.*, 1995, Green *et al.*, 1995).

The consensus sequence of the dsRBM is found in a number of proteins from prokaryotic and eukaryotic cells and also some of viral origin (Figure 1.5). The motif was first found in PKR and the *Drosophila* developmental protein staufer. This protein contains five copies of the motif and is required for the localisation of specific maternal mRNAs (Bycroft *et al.*, 1995). RNase III from *E. coli* which is involved in a number of mechanisms including the processing of rRNA, and cleavage of the 5' termini of cellular mRNAs, contains only 1 copy of the motif (Kharrat *et al.*, 1995). E3L encoded by VV also contains a single copy of the dsRNA binding motif. Within an infected cell, E3L serves to sequester dsRNA that would ordinarily bind to PKR, thereby reversing PKR mediated protein synthesis inhibition (Chang *et al.*, 1992). PACT a cellular activator of PKR contains 3 copies of the dsRBM, and is believed to interact with PKR at the same site as dsRNA. The protein is the first cellular activator of PKR to be identified (Patel and Sen, 1998). Other proteins that contain dsRBMs include *Xenopus* RNA binding protein, human TAR binding protein, and RNA-specific adenosine deaminase (ADAR), all of which contain 3 copies of the motif (St.Johnston *et al.*, 1992, and Lui and Samuel, 1996).

Structural analysis of this dsRBM has been carried out with three proteins, all of which exhibit the same multidimensional structures. Secondary structure predictions of the consensus sequence indicated the motif follows a α - β - β - β - α conformation. This was verified by NMR investigations of the third motif of the staufer protein (Kharrat *et al.*, 1995), the RNase III motif (Bycroft *et al.*, 1995), and more recently the two motifs of PKR (Nanduri *et al.*, 1998) (Figure 1.6).

Figure 1.5. The amino acid sequence data of the dsRBM located within various proteins is highlighted below, and the consensus sequence derived from such domains (St.Johnstons *et al.*, Ho and Shuman, 1996)

PKR
human.

MEELNTYRQKQGVVLKYOELPNSGPPHRRFTFQVIIDGREFPEGEGRSKKEAKNAAAKLAVEIL

PKR
mouse.

MDKLNKYRQMHGVAITYKELSTSGPPHRRFTFQVLIDEKEFPEAKGRSKQEARNAAAKLAVDIL

VV E3L

PVTVINEYCQITRRDWSRFIESVGPSNSPTFYACVDIDGRVFDKADGKSKRDAKNNAAKLAVDKL

Staufen
(*Drosophila*)

PISQVHEIGIKRNMTVHFKVLREEGPAHMKNFITACIVGSIVTEGEGNGKKVSKKRAAEKMLVEL

RNase
III(*E.coli*)

PKTRLQEYLQGRHLPLPYLVVQVRGEAHDQEFTIHCQVSGLSEPVVGTGSSRRKAEQAAAEQALKKL



α



β



β

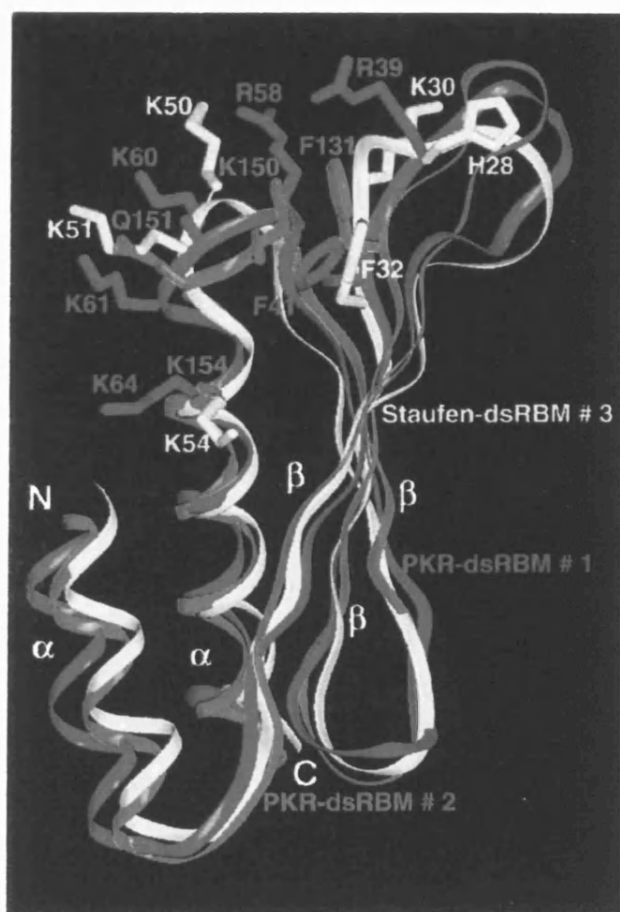


β



α

Figure 1.6 Structural resolution of PKR 1-170 highlighted the similarities between the two dsRBMs located within PKR and the third motif of the *Drosophila* Staufen protein. The picture below highlights a superimposed view of the three motifs (Nanduri *et al.* 1998).



1.4.4 Activation of PKR.

Activation by dsRNA.

PKR can be activated by a number of mechanisms within the cell, the most of extensively studied of which is dsRNA activation which will be discussed first. As already highlighted in section 1.4.3, PKR contains two dsRBMs, which interact with only dsRNA and not RNA-DNA hybrids or DNA alone. Single stranded (ss) RNA can also interact if found in an extended hairpin conformation. The interaction with dsRNA is sequence independent (Manche *et al.*, 1992), and relies on a series of 2' hydroxyl groups on both strands of the A-form of the dsRNA helix. The 2'OH groups form a number of non electrostatic interactions with the hydrophilic regions of the PKR dsRBM. The dsRBM only interacts with the minor groove of the A-form helix since the major groove is too narrow and deep for efficient binding (Bevilacqua and Cech, 1996).

The length of dsRNA required to bind and activate PKR has also been investigated. The minimum length of dsRNA required to interact is an 11bp duplex, which is the equivalent of one helical turn of the A-form dsRNA helix. Binding and activation is achieved from duplexes of 30bp up to 80bp, where maximal activation is achieved (Manche *et al.*, , 1992). Duplexes 30bp or under do not bind to PKR in a stable manner, suggesting that the region between the two motifs behaves as a hinge (Green and Matthews, 1992). It was proposed that duplexes under 30bp were unable to bind co-ordinately to both dsRBMs due to the limited range of movement of the hinge region. This would lead to an interaction with only one of the dsRBMs which is much less stable (Green and Matthews, 1992).

Further investigations found a single dsRBM of PKR interacted with a 16bp RNA duplex, however site saturation experiments indicated the dimeric form of the protein interacted with 22-24bps of dsRNA (Bevilacqua and Cech, 1996). The latter data suggested that R₁ of PKR binds to 11bp region of dsRNA, which is consistent with previous work (Manche *et al.*, 1992). It was therefore suggested that the presence of a second dsRNA binding motif compensated for the shorter RNA duplex (Bevilacqua and Cech, 1996).

More recently the crystal structure of the second dsRBM of *Xenopus laevis* complexed with dsRNA has been resolved (Ryter and Schultz, 1998). The protein interacts with 16bp of dsRNA, spanning two successive minor grooves and the major groove between the two. The protein-dsRNA complex is mediated by a number of individual interactions. The first interaction involves the N terminal of the first α helix and the first minor groove of the dsRNA. The second interaction is mediated by the loop located between the first and second β sheet, and the successive minor groove of the dsRNA. The third is

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interaction is between 7 amino acids of the N terminal of the second α helix which interacts across the major groove. The first interaction causes the dsRNA helix to deviate from the A form, thus avoiding a steric clash. These interactions lead to a widening and unwinding of the major groove, suggesting the dsRNA-dsRBM interaction is perhaps structure specific (Ryter and Schultz, 1998).

The activation of PKR as a function of dsRNA concentration gives rise to a bell shaped activation curve. The current model is based on the dimerisation of PKR, which is believed to occur concomitant with activation (Clemens and Elia, 1997). Upon dimerisation, the two PKR molecules are phosphorylated by an intermolecular effect, leading to a conformational change and the phosphorylation of eIF2- α . If however the concentration of the dsRNA is too high, the two PKR proteins interact with separate dsRNA molecules preventing the intermolecular phosphorylation and resulting activation (Kostura and Mathews, 1989). This model is consistent with evidence that high concentrations of short dsRNA molecules inhibit activation of PKR by long dsRNA by competition for the binding site. The model is also consistent with the finding that, once PKR is activated, high concentrations of dsRNA do not inhibit the phosphorylation of eIF2- α .

Dimerisation of PKR.

PKR is believed to be dimeric upon activation and subsequent phosphorylation of eIF2- α . A consensus opinion regarding the nature of the dimerisation has not yet been reached.

A model for dsRNA dependent dimerisation was proposed by Wu and Kaufman (1997), who found that dimerisation of PKR was directly attributed to the binding of dsRNA which caused a conformational change. Following dsRNA binding, both the dimerisation sites within the dsRBD, and kinase domain were exposed leading to *trans* phosphorylation between two PKR proteins. However, it was found that the dsRBD alone formed dimers in a dsRNA independent fashion. The interpretation of this somewhat confusing observation was attributed to absence of the kinase domain which revealed the dimerisation domain of the protein without a need for the dsRNA induced conformational change.

The ability of the protein to form dimers independently of dsRNA binding has been demonstrated by a number of groups (Patel *et al.*, 1995, 1996, 1998, Robertson and Mathews, 1996, Ortega *et al.*, 1996, Carpick *et al.*, 1997) (Figure 1.7). The dimerisation site has recently been located to the hydrophobic face of the first α helix in the dsRBD, and the dsRNA interaction is mediated by the charged residues on the other side of the helix. (Patel *et al.*, 1996, Patel and Sen, 1998). The PKR dimer interacts with a single

dsRNA molecule of 60bp or more, which induces a conformational change (Robertson and Mathews, 1996). The result of binding is the activation of the kinase domain and subsequent *trans* phosphorylation by the two proteins which are believed to be positioned end to end (Carpick *et al.*, 1997, Robertson and Mathews, 1996).

Recently a second dimerisation site, composed of amino acids 244-296 was identified. This region is of importance because viral directed PKR inhibitors interact with these amino acids (Gale and Katze, 1998).

Phosphorylation of PKR.

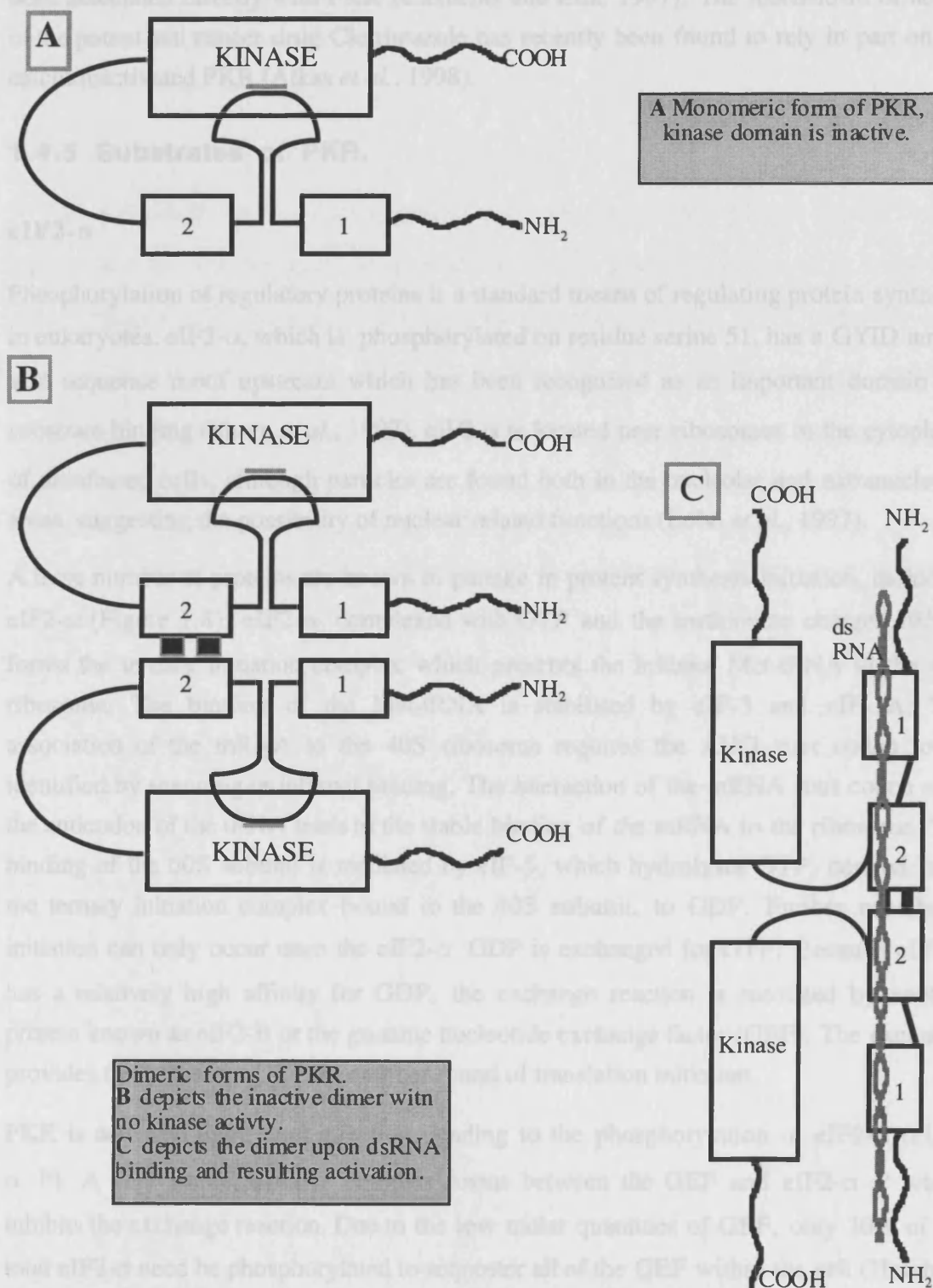
The kinase activity of the PKR protein is thought to operate in one of two ways. The first is autophosphorylation, whilst the second more likely scenario is *trans* phosphorylation between dimeric proteins (Thomis and Samuel, 1993, 1995). PKR is phosphorylated on a number of serine/threonine residues, located between the dsRBD and catalytic domain. Point mutations within this region, including threonine 258, give rise to a mutant which is less efficient at both *trans* phosphorylation of another PKR protein and the subsequent phosphorylation of eIF2- α *in vitro*. The mutated protein is also less efficient at inhibiting translation initiation *in vivo* (Taylor *et al.*, 1996).

Other Activators of PKR.

Polyanionic substances such as heparin and dextran sulphate are also able to activate PKR. The binding site for heparin is distinct from that of dsRNA, and mutants which are not activated by dsRNA or which lack the ability to dimerise may be activated by heparin (Barber *et al.*, 1995, Patel and Sen, 1998). It has therefore been suggested the binding site is located towards the C-terminal region (Patel *et al.*, 1994). Heparin oligosaccharides containing 8 sugar residues are sufficient for activation of PKR. Activated proteins are unable to *trans*- phosphorylate the catalytically inactive mutant (K295R). This suggests dimerisation is not a requirement for heparin mediated PKR activation, and the phosphorylation may be a intramolecular autophosphorylation event. This results in a less phosphorylated molecule which probably exhibits distinct functions within the cell, for example the control of cell growth via the activation of PKR in an uninfected cell (George *et al.*, 1996).

PKR is activated in cells depleted of calcium stores by a mechanism that is not fully understood. The cellular response to environmental, hormonal or nutritional stress can be reproduced by calcium ionophores such as A23187. When the cells are exposed to this agent a 2-3 fold increase in the activity of PKR is observed, which leads to an immediate

Figure 1.7 dsRNA Dependent PKR Activation. The figure highlights the monomeric and dimeric forms of PKR and activation by dsRNA. (Robertson and Mathews 1996)



inhibition of protein synthesis (Protsko *et al.*, 1995). Other cellular response pathways to stress cause the increased phosphorylation of eIF2- α , although only calcium depletion has been associated directly with PKR (Clements and Elia, 1997). The mechanism of action of the potent anti cancer drug Clotrimazole has recently been found to rely in part on the calcium activated PKR (Atkas *et al.*, 1998).

1.4.5 Substrates of PKR.

eIF2- α

Phosphorylation of regulatory proteins is a standard means of regulating protein synthesis in eukaryotes. eIF2- α , which is phosphorylated on residue serine 51, has a GYID amino acid sequence motif upstream which has been recognised as an important domain for substrate binding (Sharp *et al.*, 1997). eIF2- α is located near ribosomes in the cytoplasm of uninfected cells, although particles are found both in the nucleolar and extranucleolar areas, suggesting the possibility of nuclear related functions (Lobo *et al.*, 1997).

A large number of proteins are known to partake in protein synthesis initiation, including eIF2- α (Figure 1.8). eIF2- α , complexed with GTP and the methionine charged tRNA, forms the ternary initiation complex which presents the initiator Met-tRNA to the 40S ribosome. The binding of the Met-tRNA is stabilised by eIF-3 and eIF-1A. The association of the mRNA to the 40S ribosome requires the AUG start codon to be identified by scanning or internal binding. The interaction of the mRNA start codon with the anticodon of the tRNA leads to the stable binding of the mRNA to the ribosome. The binding of the 60S subunit is mediated by eIF-5, which hydrolyses GTP, derived from the ternary initiation complex bound to the 40S subunit, to GDP. Further rounds of initiation can only occur once the eIF2- α · GDP is exchanged for GTP. Because eIF2- α has a relatively high affinity for GDP, the exchange reaction is mediated by another protein known as eIF2-B or the guanine nucleotide exchange factor (GEF). The exchange provides the necessary GTP for another round of translation initiation.

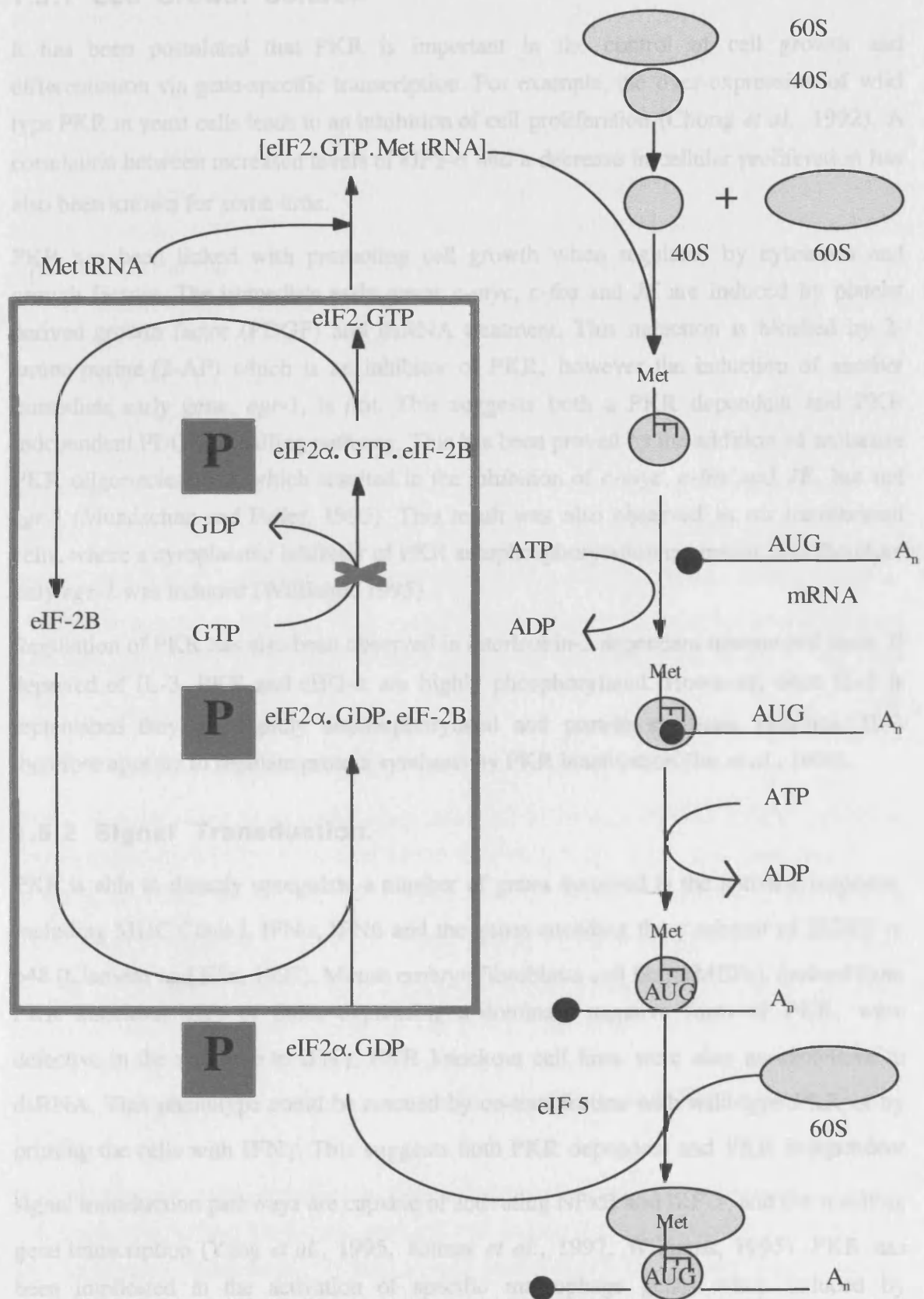
PKR is activated upon viral infection, leading to the phosphorylation of eIF2- α (eIF2- α · P). A very stable, inactive complex forms between the GEF and eIF2- α · P which inhibits the exchange reaction. Due to the low molar quantities of GEF, only 30% of the total eIF2- α need be phosphorylated to sequester all of the GEF within the cell (Hershey, 1989). The exchange of GDP to GTP is therefore prevented, and further rounds of translation initiation cannot occur (Hershey, 1993).

A 67kDa cellular protein which associates with eIF2- α has been reported to reverse the PKR mediated translational inhibition *in vivo* if over-expressed (Ray *et al.*, 1996). Another report indicated however, that this protein is required in trace amounts for protein synthesis, and over-expression did not necessarily enhance protein synthesis to any extent (Wu *et al.*, 1996).

Other Substrates for PKR.

NF κ B, a transcription factor composed of two subunits p50 and p65, is crucial for the transcription of the IFN β gene. This protein is located in the cytoplasm of an uninfected cell, complexed with the specific inhibitor I κ B. Upon activation, PKR phosphorylates I κ B, permitting the dissociation of the two proteins and also the translocation of NF κ B to the nucleus. Once in the nucleus, NF κ B controls the transcriptional regulation of a number of genes, including IRF-1, IFN β and other gene products involved in mediating the antiviral and antiproliferative response of IFN (Kumar *et al.*, 1994). Therefore, PKR is able to control the transcriptional activation of a number of IFN induced proteins through NF κ B activation and IRF-1 (Proud, 1995).

Figure 1.8 Basic translation initiation model (Hershey, 1993). P depicts phosphorylation of the eIF2- α subunit and subsequent inhibition of protein synthesis.



1.5 Role of PKR in Cell Regulation.

1.5.1 Cell Growth Control.

It has been postulated that PKR is important in the control of cell growth and differentiation via gene-specific transcription. For example, the over-expression of wild type PKR in yeast cells leads to an inhibition of cell proliferation (Chong *et al.*, 1992). A correlation between increased levels of eIF2- α and a decrease in cellular proliferation has also been known for some time.

PKR has been linked with promoting cell growth when regulated by cytokines and growth factors. The immediate early genes *c-myc*, *c-fos* and *JE* are induced by platelet derived growth factor (PDGF) and dsRNA treatment. This induction is blocked by 2-amino purine (2-AP) which is an inhibitor of PKR, however the induction of another immediate early gene, *egr-1*, is not. This suggests both a PKR dependent and PKR independent PDGF signalling pathway. This has been proved by the addition of antisense PKR oligonucleotides, which resulted in the inhibition of *c-myc*, *c-fos* and *JE*, but not *egr-1* (Mundschau and Faller, 1995). This result was also observed in *ras* transformed cells, where a cytoplasmic inhibitor of PKR autophosphorylation is present, and therefore only *egr-1* was induced (Williams, 1995).

Regulation of PKR has also been observed in interleukin-3 dependent murine cell lines. If deprived of IL-3, PKR and eIF2- α are highly phosphorylated. However, once IL-3 is replenished they are rapidly dephosphorylated and protein synthesis resumes. IL-3 therefore appears to regulate protein synthesis by PKR inactivation (Ito *et al.*, 1994).

1.5.2 Signal Transduction.

PKR is able to directly upregulate a number of genes involved in the antiviral response, including MHC Class I, IFN α , IFN β and the genes encoding the γ subunit of ISGF3 or p48 (Clemens and Elia, 1997). Mouse embryo fibroblasts cell lines (MEFs), derived from PKR knockout mice or those expressing a dominant negative form of PKR, were defective in the response to IFN γ . PKR knockout cell lines were also unresponsive to dsRNA. This phenotype could be rescued by co-transfection with wild-type PKR or by priming the cells with IFN γ . This suggests both PKR dependent and PKR independent signal transduction pathways are capable of activating NF κ B and IRF-1, and the resulting gene transcription (Yang *et al.*, 1995, Kumar *et al.*, 1997, Williams, 1995). PKR has been implicated in the activation of specific macrophage genes when induced by lipopolysaccharide or IFN α/β (Gusella *et al.*, 1995) and has also been found to modulate

the transcriptional activation of immunoglobulin κ gene (Koromilas *et al.*, 1995). More recently, activation of PKR has been associated with immunoglobulin class switching to IgE at the level of transcription via NF κ B activation. Elevated IgE levels are associated with asthma and allergies, and have been associated with viral infection for some time (Rager *et al.*, 1998).

PKR interacts with STAT1 (*see* section 1.1.5) in both human and murine cells. This interaction requires a functional dsRNA binding region of PKR, but does not lead to the phosphorylation of STAT1 *in vivo* or *in vitro*. The interaction between PKR and STAT1 leads to a decrease in STAT1 DNA binding and activation, therefore allowing PKR to control transcriptional regulation via STAT1 modulation. The exact nature of the interaction has yet to be verified, however possible mechanisms include PKR sequestering STAT1, which is normally found in combination with other STAT proteins, or PKR permitting the interaction with another protein which may then modify STAT1 DNA binding (Wong *et al.*, 1997). Cells infected with the mumps virus (Paromyxovirus) are subject to the downregulation of induction of PKR, 2-5 A synthetase and Mx proteins. This poor induction has been attributed to the degradation of the STAT1 α protein, which leads to a decrease in functional ISGF γ activation units (Yokosawa *et al.*, 1998). The interaction between PKR and STAT1 therefore may therefore inhibit the viral mediated degradation of STAT1, thereby permitting the expression of PKR and other ISGs.

1.5.3 Tumour Suppression

PKR is known to act as a growth suppressor due to the inhibition of protein synthesis. Over-expression of PKR in yeast cells leads to growth suppression, however a number of mutants, including N terminal dsRBD and a mutant eIF2- α , exhibited normal growth phenotypes in yeast (Chong *et al.*, 1992). PKR L296A, PKRdsRBD and PKR Δ 6 mutants expressed in NIH3T3 cells exhibited tumour formation when injected into nude mice (Koromilas *et al.*, 1992, Barber *et al.*, 1995). PKR is also believed to mediate IFN induced c-myc suppression. This suppression is not observed when tumourigenic inactive PKR mutants are expressed in place of wild type (Revah *et al.*, 1996, Shang *et al.*, 1998).

In direct contrast to the above results, PKR knockout mice exhibited no tumour formation and the MEFs derived from these mice did not induce tumour formation when injected into nude mice (Yang *et al.*, 1995). It was found that PKR did not control cellular

proliferation in human breast cancer, and the levels of PKR within transformed leukaemic cells did not indicate PKR acts as a tumour suppresser (Basu *et al.*, 1997).

1.5.4 Apoptosis.

Apoptosis is the programmed destruction of a cell, initiated for a number of reasons during development and homeostasis, as a defence mechanism, or as a result of ageing. Apoptosis is genetically determined, and once the cell is committed it undergoes various morphological and physiological changes including cell shrinkage, chromatin condensation, membrane blebbing and DNA degradation (Vaux and Strasser, 1996). The requirement for PKR in apoptosis is somewhat complicated due to the number of apoptotic mechanisms and signal transduction pathways with which PKR is intimately involved.

The apoptotic role of PKR was initially noted in HeLa cells infected with recombinant VV expressing WT PKR. However, expression of a catalytically inactive form of PKR was found to give a normal growth phenotype (Lee and Esteban, 1994). A functional PKR pathway has since been implicated in dsRNA, TNF α and LPS mediated apoptosis, with PKR knockout MEFs being resistant to each of the apoptotic activators (Yeung *et al.*, 1996, Der *et al.*, 1997). Recently, the upregulation of PKR by TNF α was found to enhance the downstream upregulation of the p53 tumour suppresser, which plays a pivotal role in apoptosis in a number of tumour cell lines. An NF κ B recognition sequence has been identified in the 5' region of the p53 gene which would account for the upregulation and subsequent apoptosis (Yeung *et al.*, 1998).

PKR mediated apoptosis appears to involve the downstream activation of IRF-1, which is not activated in PKR knockout cells after treatment with TNF- α or LPS. The lack of IRF-1 activation is specific, due to the ability of both TNF- α and LPS to activate NF- κ B (Williams, 1997). eIF2- α phosphorylation has also been linked to the downstream apoptotic effects of PKR. The expression of S51D, which mimics a phosphorylated serine, induced apoptosis in NIH3T3 cells, however S51A, which is unable to be phosphorylated, inhibited apoptosis (Srivastava *et al.*, 1998).

Mutant cells deficient in STAT1 are resistant to apoptotic inducers, suggesting STAT1 mediates apoptosis. An inactive form of STAT1 is believed to direct the constitutive expression of the inactive proteases required for apoptosis. The nuclear localisation may promote associations with other transcriptional factors and thus provide a mechanism by which inactive STAT1 directs transcription (Schindler, 1998). In light of these findings the interaction between STAT1 and PKR may indicate that PKR is able to modulate the transcription of specific proteases that are then activated during apoptosis.

1.5.5 Cellular Inhibitors of PKR.

P58^{IPK} (p58).

The 58kDa inhibitor of PKR was first recognised in co-infected mutant VA RNA adenovirus and influenza virus cells (Section 1.6.1). Adenovirus mutants are associated with high levels of PKR phosphorylation which decreases when co-infected with influenza virus (Lee *et al.*, 1990, Lee *et al.*, 1992). P58^{IPK} was found to inhibit the phosphorylation of PKR and resulting phosphorylation of eIF2- α (Figure 1.9). It can also downregulate PKR when over-expressed in NIH3T3 cells, leading to a transformed phenotype (Tang *et al.*, 1996). The *p58* gene is located on chromosome 13 (Korth *et al.*, 1994) and is expressed mainly in the cytoplasm of cells, which is consistent with its inhibitory role (Korth *et al.*, 1996).

P58^{IPK} is highly conserved across a number of species, and is predicted to be a 504 amino acid hydrophilic protein containing a region of similarity to eIF2- α . It is a member of the tetratricopeptide repeat (TPR) family of proteins which contain a 34 amino acid repeat sequence. P58^{IPK} contains 9 copies of this motif, which form scaffolds to mediate protein-protein interactions between itself and either PKR or the inhibitor of P58^{IPK} (I-P58^{IPK}). The carboxyl terminus of the protein has regions of homology to the DnaJ family of heat shock proteins, which is also implicated in protein-protein interactions (Lee *et al.*, 1994). The heterodimeric complex formed between PKR and P58^{IPK} requires amino acids 244-296 of PKR and is independent of the dsRBD. The eIF2- α similarity region within the TPR motif 6, and the DnaJ region of homology within P58^{IPK} are also required, as determined by deletion analysis (Polyak *et al.*, 1996, Gale *et al.*, 1996, Tang *et al.*, 1996) (Figure 1.9).

P58^{IPK} is subject to regulation by I-P58^{IPK}, forming an inactive complex within normal dividing cells. Hsp-40, a eukaryotic homologue of *E. coli* DnaJ, was also found to complex with P58^{IPK}. Therefore this may modulate the translation inhibition within a cell, suggesting a means by which influenza virus may modulate PKR activity by recruiting components of the cellular stress pathway (Melville *et al.*, 1997). More recently another modulator of P58^{IPK} has been discovered, P52^{rIPK}, which is expressed in a range of cells and shares extensive homology to Hsp-90 (Gale *et al.*, 1998).

La Antigen.

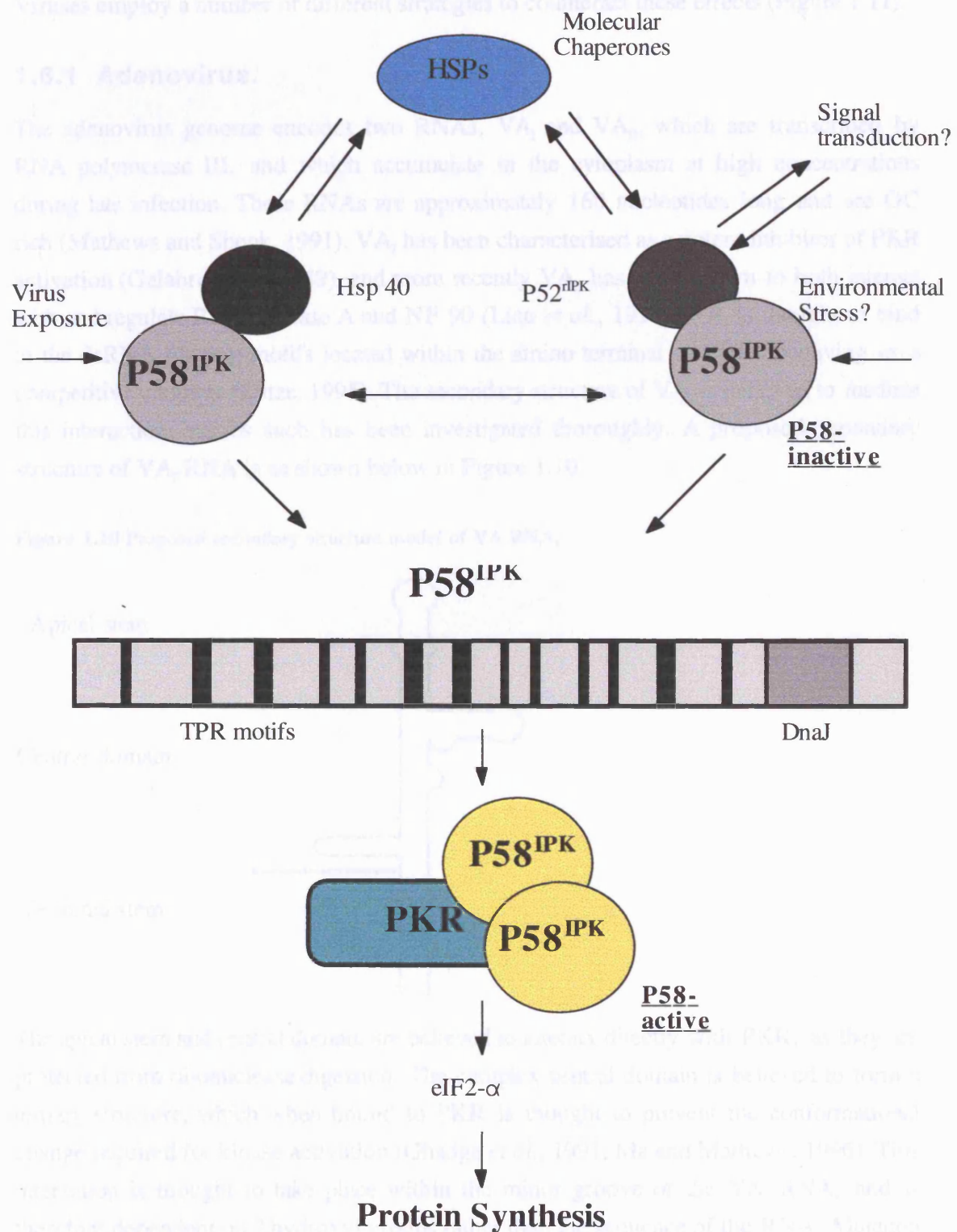
The La antigen inhibits the activation of PKR by unwinding the dsRNA. The protein converts reovirus or synthetic dsRNA into a single stranded form. This protein is found predominantly in the nucleus, but following viral attack the levels of protein increase in the cytoplasm, where it may compete with PKR (Xiao *et al.*, 1994). Characterisation of

the 47.6kDa protein found it to be an ATP-dependent helicase requiring at least one 3' overhang on the dsRNA to facilitate unwinding. A conserved motif within La proteins from a number of species shows some homology to the dsRBD of PKR, but the exact function of these motifs has yet to be found (Huhn *et al.*, 1997).

Ribosomes.

Within an uninfected cell PKR is associated with the 40S subunit of the ribosome (Zhu *et al.*, 1997). It has since been suggested that this interaction, mediated by the dsRBD of PKR, prevents activation by cellular RNA species that exhibit some secondary structure. Upon IFN treatment the level of PKR within the cytoplasm increases dramatically, and some of this associated PKR is released. This IFN induced soluble PKR is then able to bind to the viral dsRNA present in the cytoplasm (Raine *et al.*, 1998). More recently PKR has been found to interact with L18, a 60S ribosomal subunit protein that is over-expressed in colorectal cancer tissue. PKR activation by dsRNA is inhibited whilst these two proteins are associated, and results suggest over-expression of L18 may promote protein synthesis and consequently cell growth in certain cancerous tissues (Kumar *et al.*, 1999). The ability of the ribosomes to prevent the unwanted activation of PKR is not yet fully understood, although it has been suggested however that viral mechanisms to downregulate PKR (Section 1.6) may well mimic the cellular control mechanism of the ribosome.

Figure 1.9 P58^{PK} regulation of PKR (Gale *et al.*, 1998). The regulation of PKR activation by P58^{PK} and other cellular proteins resulting from a variety of environmental stimuli.



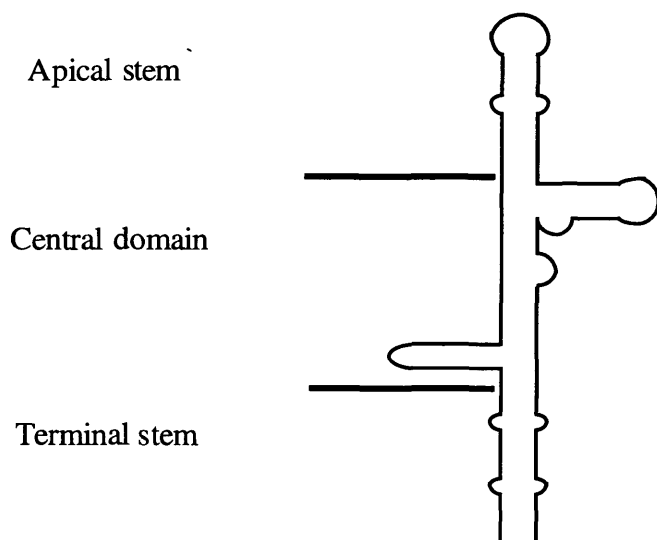
1.6 Viral Inhibitors of PKR.

The inhibitory effects of PKR upon cell activity are detrimental to viral replication. Viruses employ a number of different strategies to counteract these effects (Figure 1.11).

1.6.1 Adenovirus.

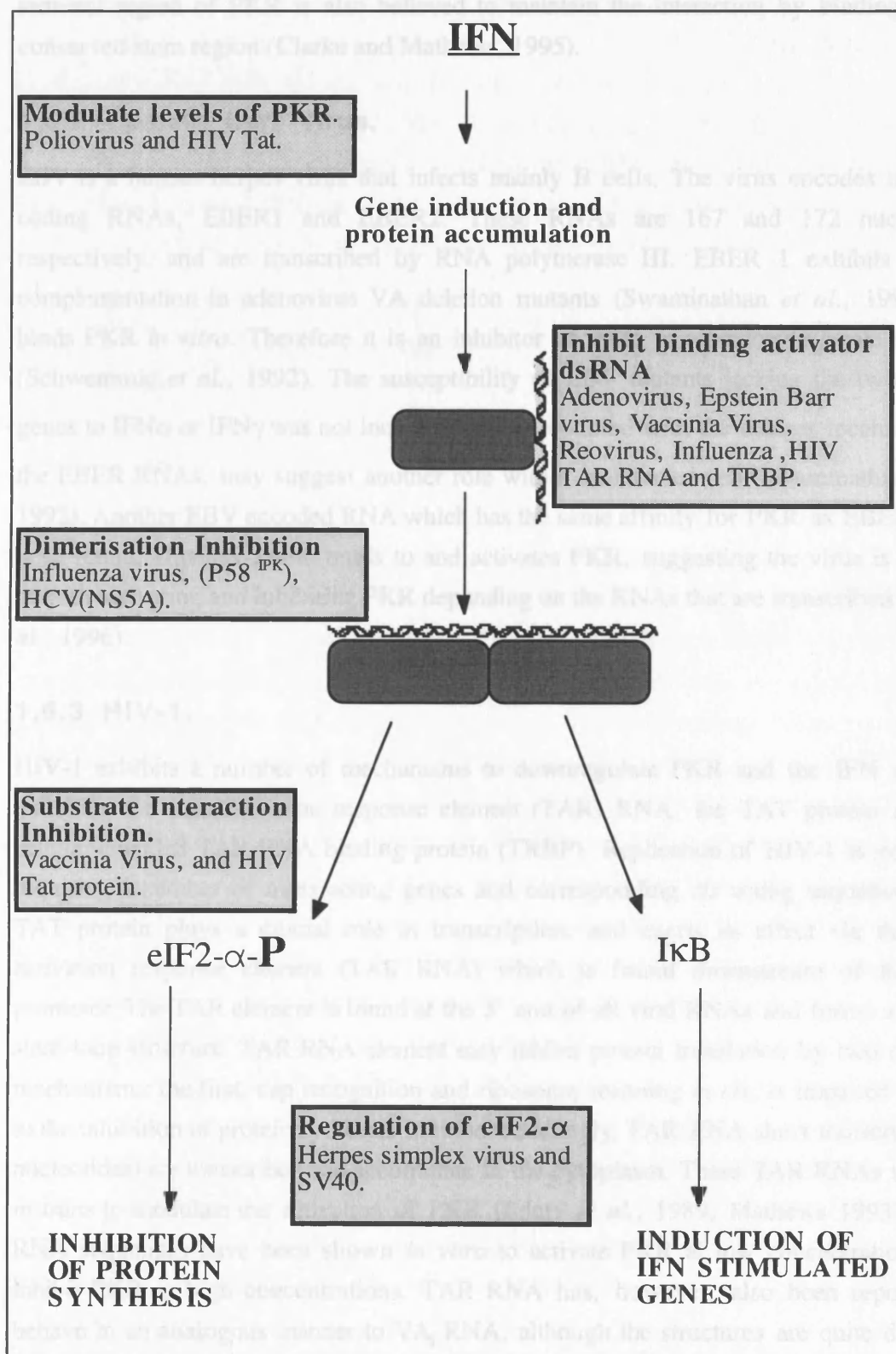
The adenovirus genome encodes two RNAs, VA_I and VA_{II}, which are transcribed by RNA polymerase III, and which accumulate in the cytoplasm at high concentrations during late infection. These RNAs are approximately 160 nucleotides long and are GC rich (Mathews and Shenk, 1991). VA_I has been characterised as a potent inhibitor of PKR activation (Galabru *et al.*, 1989), and more recently VA_{II} has been shown to both interact with and regulate RNA helicase A and NF 90 (Liao *et al.*, 1998). VA_I is thought to bind to the dsRNA binding motifs located within the amino terminal of PKR, behaving as a competitive inhibitor (Katze, 1995). The secondary structure of VA_I is believed to mediate this interaction, and as such has been investigated thoroughly. A proposed secondary structure of VA_I RNA is as shown below in Figure 1.10

Figure 1.10 Proposed secondary structure model of VA RNA_I.



The apical stem and central domain are believed to interact directly with PKR, as they are protected from ribonuclease digestion. The complex central domain is believed to form a tertiary structure, which when bound to PKR is thought to prevent the conformational change required for kinase activation (Ghadge *et al.*, 1991, Ma and Mathews, 1996). This interaction is thought to take place within the minor groove of the VA_I RNA, and is therefore dependent on 2'hydroxyl groups rather than the sequence of the RNA. Mutation analysis of the GC rich domain adjacent to the central domain causes a decrease in

Figure 1.11 PKR activation pathway and viral inhibitors. As is highlighted below a number of strategies are used by viruses to eliminate PKR activation and resulting protein synthesis inhibition.



binding affinity. The GC rich sequence is believed to mediate a slight widening of the minor groove of the RNA helix, which is disrupted if mutations are introduced. The C terminal region of PKR is also believed to maintain the interaction by binding to the conserved stem region (Clarke and Mathews, 1995).

1.6.2 Epstein Barr Virus.

EBV is a human herpes virus that infects mainly B cells. The virus encodes two non coding RNAs, EBER1 and EBER2. These RNAs are 167 and 172 nucleotides respectively, and are transcribed by RNA polymerase III. EBER 1 exhibits limited complementation in adenovirus VA deletion mutants (Swaminathan *et al.*, 1992) and binds PKR *in vitro*. Therefore it is an inhibitor of PKR in reticulocyte lysate systems (Schwemmle *et al.*, 1992). The susceptibility of EBV mutants lacking the two EBER genes to IFN α or IFN γ was not increased which, coupled with the nuclear localisation of the EBER RNAs, may suggest another role within an infected cell (Swaminathan *et al.*, 1992). Another EBV encoded RNA which has the same affinity for PKR as EBER-1 has been found. This RNA both binds to and activates PKR, suggesting the virus is capable of both activating and inhibiting PKR depending on the RNAs that are transcribed (Elia *et al.*, 1996).

1.6.3 HIV-1.

HIV-1 exhibits a number of mechanisms to downregulate PKR and the IFN system, including the transactivation response element (TAR) RNA, the TAT protein and the cellular encoded TAR RNA binding protein (TRBP). Replication of HIV-1 is complex, requiring a number of *trans* acting genes and corresponding *cis* acting sequences. The TAT protein plays a crucial role in transcription, and exerts its effect via the *trans* activation response element (TAR RNA) which is found downstream of the viral promoter. The TAR element is found at the 5' end of all viral RNAs and forms a bulged stem-loop structure. TAR RNA element may inhibit protein translation by two separate mechanisms; the first, cap recognition and ribosome scanning *in cis*, is impaired leading to the inhibition of protein synthesis initiation. Secondly, TAR RNA short transcripts (60 nucleotides) are transcribed and accumulate in the cytoplasm. These TAR RNAs then act *in trans* to modulate the activation of PKR (Edery *et al.*, 1989, Mathews 1993). TAR RNA sequences have been shown *in vitro* to activate PKR at low concentrations and inhibit PKR at high concentrations. TAR RNA has, however, also been reported to behave in an analogous manner to VA₁ RNA, although the structures are quite different (Gale and Katze 1998).

The TRBP was isolated due to its ability to bind TAR RNA (and was named accordingly). This cellular encoded protein is highly conserved across species and is expressed in all tissues examined so far. TRBP also contains two copies of the dsRBMs found in PKR, and is as efficient at binding dsRNA as PKR. It has therefore been postulated that the observed mechanism of PKR inhibition was due to the proteins ability to sequester dsRNA (Park *et al.*, 1994, Consentino *et al.*, 1995). Recently, TRBP has been found to form heterodimers with PKR at the dsRBD site in an RNA independent interaction. As such TRBP serves as a growth promoting enzyme, and therefore over-expression leads to a transformed phenotype (Benkraine *et al.*, 1997).

The TAT protein is known to regulate transcription at the level of initiation and elongation. PKR is believed to be regulated by TAT, although the mechanism has not been elucidated. Initially reports suggested that TAT over-expression and HIV-1 infection lead to a downregulation of PKR (Roy *et al.*, 1990). More recently however, it has been suggested that TAT is an inhibitor of PKR autophosphorylation as it is believed to bind directly to the kinase domain (Brand *et al.*, 1997).

1.6.4 Reovirus.

The S4 gene encodes a 41kDa protein $\sigma 3$ (365 amino acids), which is a major outer capsid component that is also capable of binding dsRNA produced during viral infection (Katze, 1992). The dsRNA molecules produced by reovirus are very potent activators of PKR, as are some reovirus mRNA species (Bischoff and Samuel 1989). Deletion analysis revealed the dsRBD is located toward the carboxyl terminus, and contains two copies of a basic motif between residues 234-240 and 291-299. An increased dsRNA binding capacity was observed by the expression of a carboxyl fragment alone, suggesting the amino terminal containing a zinc finger motif serves to inhibit the dsRNA binding in some way (Miller and Samuel 1992). More recently motif 2 (amino acids 290-299) was found to bind dsRNA while motif 1 mediated the inhibition of the amino terminal zinc finger motif (Wang *et al.*, 1996). The conserved motifs have limited homology to other dsRBMs (Section 1.4.3) resulting in a lower binding affinity. However, the large quantities of protein expressed are thought to compensate for this lower affinity. The secondary structure predictions, combined with mutational analysis resulting in the loss of dsRNA binding, indicate a similar three dimensional structure and sequence independent mechanism of binding similar to PKR (Yue and Shatkin 1996). The $\sigma 3$ protein localises to both the cytoplasm and the nucleus of the cell, suggesting the protein may exhibit an alternate function in the nucleus together with the inhibition of PKR within the cytoplasm (Yue and Shatkin, 1996).

1.6.5 Influenza Virus.

As discussed in section 1.5.5, influenza virus recruits the cellular protein P58^{IPK} which leading to an inhibition of PKR activation. Influenza encodes NS1 (non-structural protein 1), a nuclear protein that has recently been found to bind dsRNA and other RNA species including poly(A)RNA (Lu *et al.*, 1995).

1.6.6 Hepatitis C Virus (HCV)

HCV has a number of genotypes, some of which are more sensitive to IFN than others. This is believed to be due to mutations within a discrete region of the NS5A protein known as the interferon sensitivity determining region (ISDR). Due to the pivotal role of PKR in IFN mediated resistance, experiments were undertaken to investigate a link between these two proteins *in vitro*. NS5A was found to repress PKR activation via an interaction between the PKR catalytic domain (amino acids 244-296) and the ISDR together with a downstream 26 amino acid region. The catalytic region occupied by NS5A is important for PKR dimerisation, and is also important for P58^{IPK} binding. This interaction may therefore may inhibit PKR activation by similar mechanisms and also by preventing PKR dimerisation (Gale *et al.*, 1998). Mutations within the ISDR eliminated PKR inhibition *in vitro*, although other regions of NS5A may be important since mutations within the ISDR do not always lead to an IFN sensitive phenotype (Duverlie *et al.*, 1998). The downregulation of PKR by NS5A may participate in mediating malignant transformation of hepatocytes associated with HCV, and may interfere with other PKR dependent mechanisms such as apoptosis (Gale *et al.*, 1997, Gale *et al.*, 1998).

1.6.7 Other Viral Strategies to Inhibit PKR activation.

A number of other viruses have evolved mechanisms to combat PKR activation, although the detailed mechanisms by which they maintain protein translation are not known. Poliovirus infected cells are subject to a sharp decline in the level of PKR, however the small quantities of PKR remaining within the cell are highly phosphorylated due to activation by poliovirus dsRNA (Black *et al.*, 1989). The exact mechanism behind this degradation is not fully understood, although it may involve a latent heat stable cellular protease that is activated by the virus (Gale and Katze, 1998). Herpes simplex virus-1 (HSV 1) infection has been associated with a decrease in eIF2- α phosphorylation following PKR activation. Deletion of the γ_1 34.5 viral protein leads to an attenuated virus in animal systems and the premature shutdown of protein synthesis associated with high levels of eIF2- α phosphorylation. γ_1 34.5 is believed to mediate an interaction with a cellular phosphatase, identified by a yeast-two-hybrid screen, which dephosphorylates eIF2- α allowing translation to continue (He *et al.*, 1997). Simian Virus 40 infection also

inhibits the downstream activation of protein synthesis inhibition following PKR activation. The exact mechanism behind this is unresolved, but may involve the versatile large T antigen. This may either modulate the levels of eIF2-B, or mediate the phosphorylation of serine 48 on eIF2- α , instead of serine 51 which leads to protein synthesis inhibition (Swaminathan *et al.*, 1996).

1.7 Poxviridae.

VV is a member of the *poxviridae* which is composed of a large family of dsDNA viruses capable of infecting vertebrates and invertebrates, which replicate in the cytoplasm of the host cell. They are composed of a single DNA molecule of 130-300 kilobases in length with a hairpin loop at each end. The members of the *poxviridae* are subdivided into two families dependent on the host range, the *Chordopoxvirinae* infect vertebrates whilst the *Entomopoxvirinae* infect insects. The *Chordopoxvirinae* are further subdivided into genera on the basis of morphology and host range, and are genetically and antigenically related (Table 1.1).

Table 1.1 Family *Poxviridae*.

Subfamilies	Genera	Prototypal Member
<i>Chordopoxvirinae</i>	<i>Orthopoxvirus</i>	vaccinia
(infect vertebrates)	<i>Parapoxvirus</i>	orf
	<i>Avipoxvirus</i>	canarypox
	<i>Capripoxvirus</i>	sheeppox
	<i>Leporipoxvirus</i>	myxoma
	<i>Suipoxvirus</i>	swinepox
	<i>Molluscipoxvirus</i>	molluscum contagiosum
	<i>Yatapoxvirus</i>	yaba monkey tumour
<i>Entomopoxvirus</i>	<i>Entomopoxvirus A</i>	Melolontha melolontha
(infect insects)	<i>Entomopoxvirus B</i>	Amscata moorei
	<i>Entomopoxvirus C</i>	Chironimus luridus

1.7.1 Virion Structure.

Poxvirus virions are one of the largest of animal viruses (Dubochet *et al.*, 1994) and are visible by light microscopy. They exist in several forms, but the two infectious forms are IMV (intracellular mature virions) and EEV (extracellular enveloped virions). IMV remains cell associated and is a brick shaped particle wrapped in an outer membrane containing a number of surface tubules arranged in parallel rows or helices. EEV contains

a further membrane that is derived from the cisternae of the trans golgi network, this form is known as IEV (intracellular enveloped virions). This is then propelled to the plasma membrane by actin tails where it fuses with the plasma membrane. The EEV particle therefore contains proteins derived from the host cell that are absent from the IMV form (Moss, 1996).

1.7.2 Poxvirus Genome.

Several poxvirus genomes have been sequenced, including the VV Copenhagen strain (Goebel *et al.*, 1990). Within this genome a potential 263 genes were identified, with 198 major protein coding regions and 65 overlapping minor protein coding regions. The genome is very tightly packed and contains very few non-coding regions. Genes are transcribed from both strands of DNA and contain very short promoter sequences. The majority of the 'essential genes' are located in the centre of the genome with those 'non-essential genes' (in tissue culture) located at the more sequence variable ends.

1.7.3 Poxvirus Life Cycle (Figure 1.12).

Virus Entry.

The exact mechanism by which the virus attaches to the cell is still unresolved because a viral attachment protein or cell surface receptors have not been detected. The analysis of this mechanism is further complicated due to the two forms of infectious virions, IMV and EEV. The fusion with the plasma membrane of both forms occurs at neutral pH, although this occurs more rapidly with EEV (Doms *et al.*, 1990).

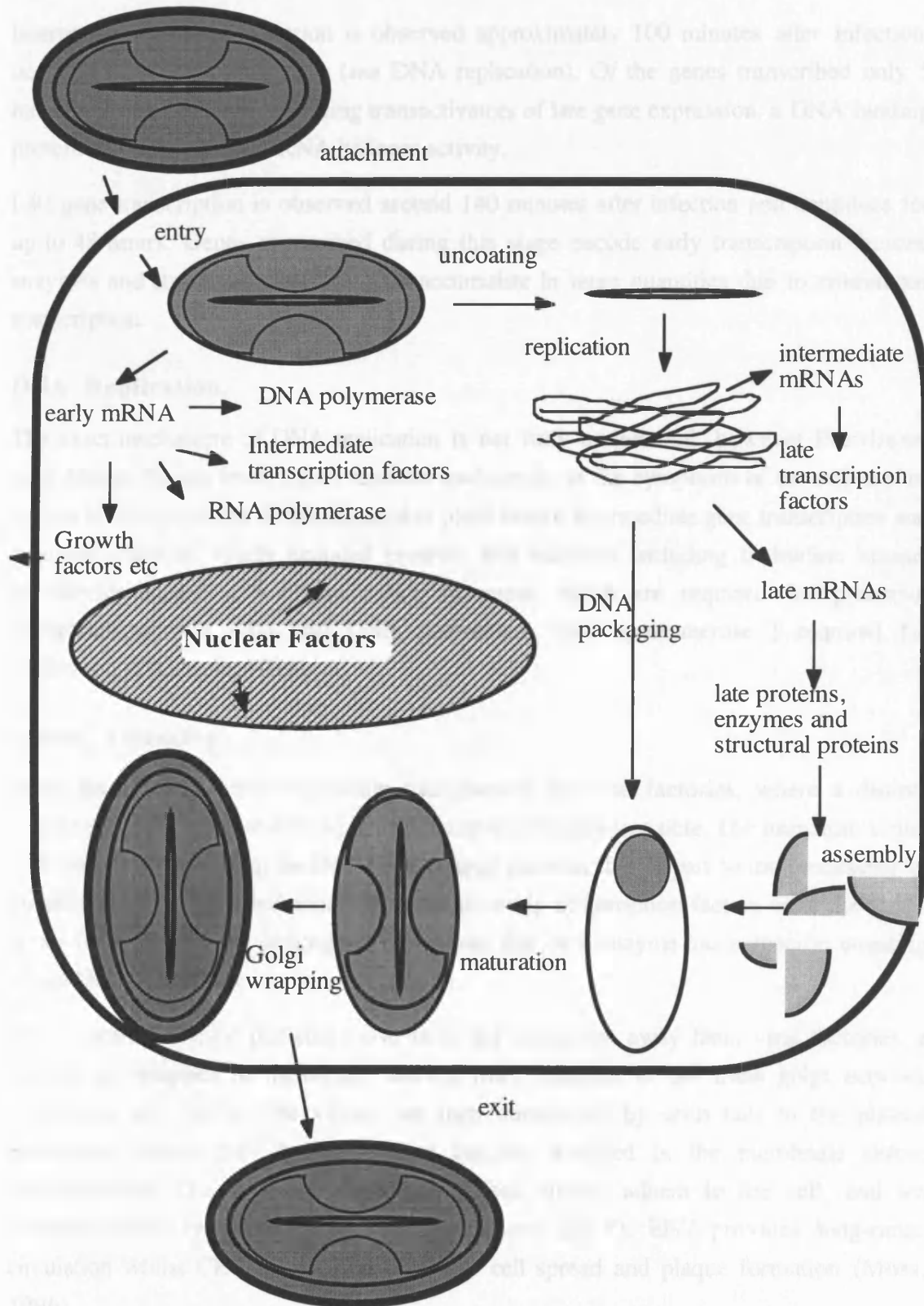
Uncoating.

The uncoating mechanism is composed of two stages, the first releases the core into the cytoplasm leading to mRNA transcription. The second is dependent on a protein either induced or encoded by the virus, as inhibitors of transcription or translation inhibit this step. A 23kDa trypsin like protein has been identified in cell extracts which may fill this role, but its action has still to be verified (Pedley and Cooper, 1987).

Transcription.

Viral transcription takes place within a stringently regulated cascade mechanism with early, intermediate and late transcription producing the required genes at the specific time during the life cycle.

Figure 1.12 Vaccinia Virus Replication Cycle, Moss (1996). The figure highlights each stage of VV replication from attachment through the exit from the cell. Each stage is discussed in more detail in the text.



Early transcription takes place immediately within the core. Early mRNA synthesis is observed within 20 minutes of infection and reaches its maximum between 1-2 hours. The products of early transcription are involved in host interactions, viral DNA replication and intermediate gene expression. (Moss, 1996).

Intermediate gene transcription is observed approximately 100 minutes after infection, occurring after DNA replication (*see* DNA replication). Of the genes transcribed only 5 have been characterised, including transactivators of late gene expression, a DNA binding protein and a protein with RNA helicase activity.

Late gene transcription is observed around 140 minutes after infection and continues for up to 48 hours. Genes transcribed during this stage encode early transcription factors, enzymes and structural proteins which accumulate in large quantities due to continuous transcription.

DNA Replication.

The exact mechanism of DNA replication is not fully understood, however Poxviruses (and African Swine fever virus) replicate exclusively in the cytoplasm of the cell at sites known as viral factories. Replication takes place before intermediate gene transcription and requires a host of virally encoded proteins and enzymes including thymidine kinase, thymidylate kinase and ribonucleotide reductase which are required for precursor synthesis, together with DNA polymerase and topoisomerase I required for replication (Traktman, 1991).

Virion Assembly.

Initial stages of poxvirus replication take place in the viral factories, where a distinct crescent shaped structure derived from cellular membranes is visible. The immature virion (IV) then matures to form the IMV brick shaped particle, due in part to the processing of the core proteins. The mechanism by which the early transcription factors enter the virion is not fully understood, although it is believed that each enzyme has a specific targeting signal (Moss, 1996).

Once mature the IMV particles move in to the cytoplasm away from viral factories, a portion are wrapped in membrane derived from cisternae of the trans golgi network (Sodiek *et al.*, 1993). The virions are then transported by actin tails to the plasma membrane, where they fuse and then become wrapped in the membrane before externalisation. The majority of the externalised virions adhere to the cell, and are therefore called cell associated enveloped virions (CEV). EEV provides long-range circulation whilst CEV is involved in cell to cell spread and plaque formation (Moss, 1996).

1.7.4 Poxviruses and the Evasion of the Immune Response.

As already discussed, poxviruses have a large genome encoding a number of genes. Recently it has become apparent that the viruses encode proteins which directly modulate a number of crucial host cell survival response mechanisms.

The viruses encode proteins which mimic a variety of host cell receptors including $\text{IFN}\alpha/\beta$, $\text{IFN}\gamma$, cytokines and chemokine receptors, dramatically limiting the hosts immune response towards the virus (For review see Smith *et al.*, 1998, Alcami *et al.*, 1998). The viruses also encode proteins which are able to modulate the cellular apoptotic response to viral infections at a number of levels (For review see McFadden and Barry, 1998).

As discussed previously (section 1.4), PKR plays a pivotal role in the IFN response of the host cell, and as such is a likely candidate to be modulated by the virus. VV has been shown to modulate PKR and the downstream processes with which it is associated, for example apoptosis. VV proteins have also been linked with 2'-5'oligoadenylate synthetase downregulation.

1.7.5 Downregulation of PKR by Vaccinia Virus.

Co infection studies of vesicular stomatitis virus (VSV) and VV found that VV was able to protect VSV from the activity of IFN by means of a factor produced early in infection (Thacore *et al.*, 1973). PKR was then found to be inhibited by extracts from VV infected cells (Rice and Kerr, 1984) which could be reversed *in vitro* by the addition of dsRNA. The factor, named SKIF (specific kinase inhibitory factor), was produced within 2 hours of infection and was dependent on protein synthesis (Whitaker-Dowling and Younger, 1984). SKIF was partially purified and its effects examined in rabbit reticulocyte extracts. It was found to prevent the dsRNA dependent inhibition of protein synthesis mediated by PKR and the phosphorylation of eIF2- α by interacting directly with the dsRNA itself (Akkaraju *et al.*, 1989). A 25kDa early protein was purified from infected cell extracts, which bound to a poly(I)(C) column, and was translated *in vitro* by a VV core (Watson *et al.*, 1991). A smaller protein of 20kDa was also recognised as SKIF but was translated from an alternative methionine.

E3L.

Sequencing of the chymotryptic fragments of the 25kDa protein encoded by VV found the protein to be encoded by ORF E3L (Goebel *et al.*, 1990), which when expressed in COS cells maintained the PKR kinase inhibition. The predicted amino acid usage (190 amino acids) revealed regions of homology to PKR at the carboxyl terminus of E3L, suggesting

the location of a dsRNA binding region that was conserved between the two proteins (Figure 1.5) (Chang *et al.*, 1992). Mutational analysis of protein confirmed the dsRNA binding region to be at the carboxyl terminus, which was sufficient to bind dsRNA and inhibit kinase activation (Chang and Jacobs, 1993).

The Role of E3L within the VV Infected Cell.

E3L expression is observed 30 minutes after infection and is detectable 3-4 hours post infection (Beattie *et al.*, 1995b). Localisation studies using confocal microscopy observed E3L in both the cytoplasm and the nucleus (Yuwen *et al.*, 1993). This function is not related to the ability to bind dsRNA, as mutational studies found expression of the dsRBM alone localised in the cytoplasm only (Chang *et al.*, 1995). The mechanism by which E3L enters the nucleus is not known, and there is no evidence of a nuclear localisation motif.

E3L deficient VV strains (VV E3L⁻ mutants) are sensitive to IFN treatment as shown by protein synthesis inhibition, and are not able to rescue VSV replication. This phenotype can be rescued by expression of the reovirus $\sigma 3$, the S4 gene product, which is another dsRNA binding protein capable of downregulating PKR (*see* section 1.6.4.) (Beattie *et al.*, 1995a). The VV E3L⁻ mutant also exhibits a restricted host range phenotype, as it was unable to replicate in HeLa, Vero or L292 cells. This restricted phenotype can be reversed by the transient expression of a number of E3L mutants, all of which maintain the capacity to bind dsRNA, suggesting the rescue of virus replication correlates with the ability to bind dsRNA (Chang *et al.*, 1995). In the case of L929 cells, the wild-type growth characteristics were restored by $\sigma 3$ (Beattie *et al.*, 1996). A similar result was observed in E3L⁻ VV infected RK-13 cells, which were susceptible to IFN treatment until complemented by E3L mutants capable of binding dsRNA (Shors *et al.*, 1997a). RNase III from *E. coli* (Section 1.4.3), another dsRNA binding protein, also reverses the restricted host range and IFN sensitive phenotypes when the full length protein is expressed transiently or the gene is inserted into the VV genome. The requirement for the full length RNase III protein and not the dsRBD alone suggests the endoribonuclease activity of the protein may also be required (Shors *et al.*, 1997b).

The Anti-Apoptotic Role of E3L.

The dsRNA induced PKR apoptotic pathway may be downregulated by proteins that sequester dsRNA and stop PKR activation. VV E3L⁻ infected HeLa cells undergo apoptosis, whereas wild-type VV infected cells do not. Complementation studies between other dsRNA binding proteins and E3L mutants revealed that the suppression of the of apoptosis induction is directly related to the dsRNA binding properties of the proteins. Further work using temperature sensitive mutants that synthesise excess dsRNA at

restrictive temperatures found the quantity of dsRNA produced within the cell has a proportional effect on apoptosis (Kibler *et al.*, 1997). E3L has also been shown to inhibit 2'-5' oligoadenylate synthetase mediated apoptosis (Rivas *et al.*, 1998).

E3L-PKR Interactions.

Recently E3L has been found to interact directly with PKR, interacting with both the dsRBD region of PKR and also an area that overlaps the substrate binding region (Sharp *et al.*, 1998, Romano *et al.*, 1995). The ability of E3L to bind to the dsRBD was not unexpected as PKR dimerisation is mediated by this region, as is the PKR-TRBP heterodimerisation (section 1.6.3). The interaction between the two is thought to be mediated by two separate motifs within E3L, the dsRBM and a PPRWF motif at amino acids 63-68 (Romano *et al.*, 1995). The amino terminal region of E3L is believed to be tethered to PKR at the site of the kinase domain, thereby preventing kinase function (Romano *et al.*, 1998). The formation of these inactive heterodimers provides an efficient mechanism for E3L to block PKR activation (Romano *et al.*, 1995, 1998). The unexpected interaction of E3L with the carboxyl terminus of PKR (amino acids 367-551) is reduced in the presence of dsRNA, as compared to the interaction with the dsRBD region which is enhanced by dsRNA. Further investigations found the dsRBD of PKR (amino acids 1-99 or 1-152) interacted with its own carboxyl terminus (367-551). The mechanism by which PKR autoregulates activation is therefore thought to be mediated by the conformation of the protein, which upon dsRNA binding is modified sufficiently to unmask the substrate binding region, resulting in the phosphorylation of eIF2- α . Due to the relatively low quantities of E3L within the cytoplasm (Yuwen *et al.*, 1993), this mechanism would appear to be an effective means of downregulating PKR (Sharp *et al.*, 1998, Romano *et al.*, 1995).

E3L Homologues.

The fowlpox virus exerts an inhibition of IFN action, however homologues of both E3L or K3L were not identified (Pollit, 1996). Orf virus is a parapoxvirus and a member of a group of viruses that cause acute cutaneous lesions that infect a variety of mammals, including humans (Haig, 1996). An ORF coding for a VV E3L like gene was identified which exhibits 31% predicted amino acid identity to E3L. Orf virus infected cells contain a lower level of phosphorylated PKR, which is also observed upon incubation with the recombinant protein derived from the E3L-like ORF. Transient expression of the recombinant protein in mammalian cells lead to the rescue of Semliki Forest Virus (SFV) from the antiviral effects of both type I and type II IFNs. The inhibition of PKR phosphorylation could not be terminated by cytosine arabinoside suggesting that the protein is an early gene product. These results suggest that the protein behaves in an analogous manner to VV E3L and sequesters the dsRNA that would ordinarily activate

PKR (Haig *et al.*, 1998). Although the orf protein shows weak homology to E3L at the dsRBM, the second motif (PPRWF) is conserved exactly. This highlights the importance of this motif and suggests that the protein may also form inactive heterodimers with PKR.

dsRNA Binding Properties of E3L.

The affinity of full length E3L and the dsRBM to dsRNA are comparable, K_D 7 to 9nM respectively, which is similar to PKR binding to VA₁ RNA (Ho and Shuman, 1996b). Characterisation of the full length protein and dsRBM expressed in *E.coli* found both protein constructs bound specifically to dsRNA (Manche *et al.*, 1992). The oligomerisation state of the full length protein was found to be dependent on the ionic strength of the buffer, as measured by glycerol gradient sedimentation, with results indicating multimer formation at the lower ionic concentrations. Crosslinking experiments undertaken in the same conditions verified these results. The oligomerisation state of the dsRBM alone, however, was not dependent the ionic strength, and the protein formed dimers in 0.2-1M NaCl. This was again verified by crosslinking studies (Ho and Shuman, 1996b). It is plausible that the ability of the protein to oligomerise is required to sequester the dsRNA that would ordinarily activate PKR. PKR contains two copies of the dsRBM and is believed to be dimeric, conversely E3L contains only one copy of the dsRBM and as such may form multimers to compete with PKR in the binding of dsRNA as well as form heterodimers with PKR itself.

K3L

Sequence analysis of the copenhagen strain of VV (Goebel *et al.*, 1990) revealed an ORF, K3L, that had 28% homology to the N-terminus of eIF2- α . This region of homology between the two proteins spans the serine 51 region, where phosphorylation by PKR takes place. The serine is not conserved in K3L and so cannot be phosphorylated. K3L is therefore believed to act as a PKR pseudosubstrate that competes against eIF2- α (Figure 1.13). K3L deleted VV mutants (VV K3L⁻) were more sensitive to IFN mediated protein synthesis inhibition, and viral replication was reduced (Beattie *et al.*, 1991).

Co-transfection studies with plasmid based K3L constructs showed K3L inhibited PKR activation by interacting with PKR at the eIF2- α binding site (Davies *et al.*, 1992). K3L modulates the level of eIF2- α phosphorylation early in infection in mouse L929 and Vero cells, and as such modulates protein synthesis inhibition. The levels of K3L protein disappear rapidly and are not detected after 3 hours postinfection, compared to E3L which is detectable 4 hours postinfection. The VV K3L⁻ mutants and E3L⁻ mutants are

below (adapted from Beattie *et al.*, 1991).

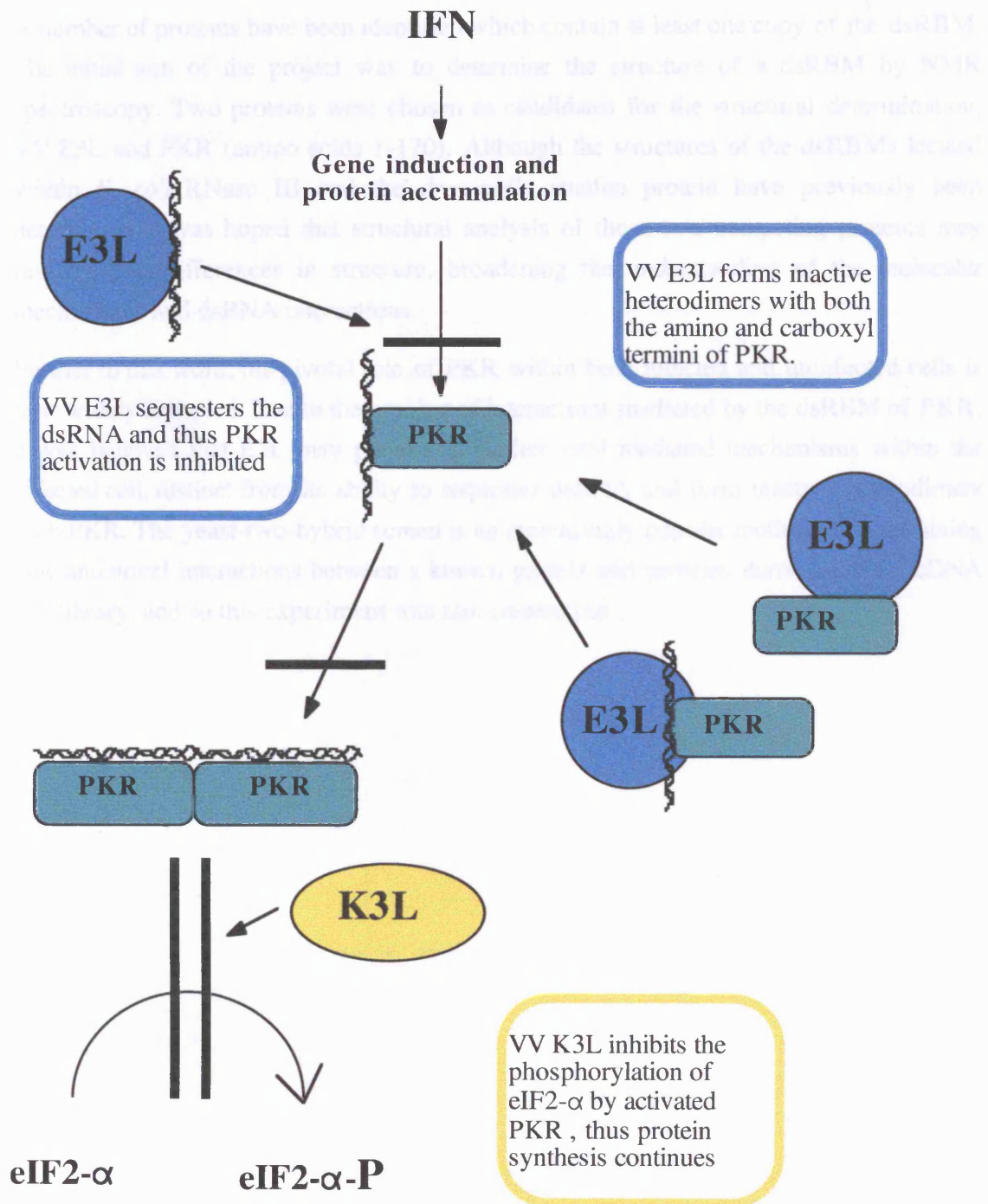
eIF2-α MPGLSCRFYOHKF**P**EVE**DV**VMVM**V**RSIAEMGAYVSLLEYNNIEGM**IL**LSEL

K3LMLAFCYSL**P**NAG**DV**IKGR**V**YE-KDYALYIYLFDPHSEA-**I**LAEVS

eIF2-α SRRRI**R**SIN KLIRI**G**RNEC**VVIRVD**KE**KGYID**LSKRRVSPEEAIKCEDK
*
K3L KMHMD**R**YVEYRDKLV**G**KTVK**V**K**VIRVD**YT**KGYID**VNYKRMCRHQ

* serine 51 residue on eIF2- α .

susceptible to IFN at 30 minutes and 2 hours post infection respectively. This implies K3L combats eIF2- α phosphorylation initially, followed by E3L which inhibits PKR activation and downstream processing. E3L is 50-100 fold more efficient than K3L (Beattie *et al.*, 1995, Davies *et al.*, 1993), (Figure 1.14).

Figure 1.14 Inhibition of PKR activation by Vaccinia Virus.

1.8 Aims of the Project.

A number of proteins have been identified which contain at least one copy of the dsRBM. The initial aim of the project was to determine the structure of a dsRBM by NMR spectroscopy. Two proteins were chosen as candidates for the structural determination, VV E3L and PKR (amino acids 1-170). Although the structures of the dsRBMs located within *E. coli* RNase III and the *drosophila* staufen protein have previously been determined, it was hoped that structural analysis of these two competing proteins may reveal subtle differences in structure, broadening the understanding of the molecular mechanisms and dsRNA interactions.

Further to this work, the pivotal role of PKR within both infected and uninfected cells is now widely accepted. Due to the number of interactions mediated by the dsRBM of PKR, it was believed that E3L may partake in further viral mediated mechanisms within the infected cell, distinct from its ability to sequester dsRNA and form inactive heterodimers with PKR. The yeast-two-hybrid screen is an increasingly popular method of determining new and novel interactions between a known protein and proteins derived from a cDNA cell library, and so this experiment was also undertaken.

2. Materials and Methods.

2.1 Strains and Vectors.

2.1.1 Bacterial Strains.

Bacterial strains used within the project are listed in Table 2.1.

Table 2.1 Highlights the strains required.

<i>E.coli</i> Strain	Source	Genotype
B834 (DE3)	Lab Stock	<i>F-ompT hsdS_B(r_B-m_B) gal dcm met</i> (DE3)
BL21 (DE3)	Lab Stock	<i>F-ompT,hsdsS_B (r_B-m_B) gal dcm</i> (DE3)
BL21(DE3) pLys S	Lab Stock	<i>F-ompT,hsdsS_B (r_B-m_B)gal dcm</i> (DE3) p lys S (cam ^R)
DH5α	Lab Stock	φ80 <i>dlacZ</i> Δ M15, <i>rec A1</i> , <i>end A1</i> , <i>gyrA</i> 96, <i>thi-1</i> , <i>hsd</i> <i>R17</i> (r _K -m _K +), <i>supE44</i> , <i>relA1</i> , <i>deoR</i> , Δ(<i>lacZYA-argF</i>) U169
JM109	Lab stock	<i>recA1</i> , <i>supE44</i> , <i>endA1</i> , <i>hsdR17</i> , <i>gyrA96</i> , <i>relA1</i> , <i>thi</i> Δ (<i>lac-proAB</i>)

2.1.2 Vectors

Plasmid vectors used within the project are listed in Table 2.2.

Table 2.2. Highlights the plasmids used.

Vector	Source	Use
pET16b	Novagen	Over-expression of histidine tagged proteins.
pUC18	Lab Stock	Electroporation transformation control.

2.1.3 Vector Constructions.

Individual constructs prepared for expression work are listed below in Table 2.3

Table 2.3. Vector constructions used.

Name	Vector Constructions	Description
pSR01	pET16b + Full length E3L with histidine tag.	E3L amino acids 1-190 cloned into the <i>Nde</i> I/ <i>Bam</i> HI site of pET16b. Primers used for PCR - oSH38 and oSH39.
pSR02	pET16b + Met 38 E3L fragment, with histidine tag.	E3L amino acids 38-190 cloned into the <i>Nde</i> I/ <i>Bam</i> HI site of pET16b. Primers used for PCR - oSH39 and oSH40.
pSR03	pET16b + Met 94 E3L fragment, with histidine tag.	E3L amino acids 94-190 cloned into the <i>Nde</i> I/ <i>Bam</i> HI site of pET16b. Primers used for PCR - oSH39 and oSR01.
pSR04	pET16b + Met 94 E3L fragment, no histidine tag.	E3L amino acids 94-190 cloned into the <i>Nco</i> I/ <i>Bam</i> HI site of pET16b. Primer used for PCR - oSH39 and oSR02.
pSR05	pET16b + PKR fragment, 1-170 with histidine tag.	PKR amino acids 1-170 cloned into the <i>Nde</i> I/ <i>Bam</i> HI site of pET16b. Primers used for PCR -oSH36 and oSH37.

2.1.4 Primers for Cloning and Sequencing.

Table 2.4 lists the primers used for PCR amplification of gene fragments for ligation into the pET16b plasmid. The restriction sites are depicted in the results section.

Table 2.4. Cloning DNA primers used within the project.

Primer	Sequence 5'to 3'
oSH36	CTAGCTCATATGGCTGGTGATCTTTCAGC
oSH37 (b/ward)	GATATTATCAGAAGAAACCTAATAGGATCCTCGATA
oSH38	CTAGCTCATATGTCTAAAATCTATATCGACGAG
oSH39 (b/ward)	TATCGAGGATCCTATTAGAATCTAATGATGACGTAACC
oSH40	CTAGCTCATATGGAGAAGCGAGAAGTTAATAA
oSR01	CTAGCTCATATGAGAGAGGATCATA
oSR02	TAGCTATCATGAGAGAGGATCATAAGT

Table 2.5 lists the primers used to verify the DNA sequence of the insert once ligated into the plasmid. The annealing sites are depicted in the results section.

Table 2.5 Sequencing DNA primers used within the project.

Primer	Sequence
oSH41 - pET16 primer	CATCATCACAGCAGCGGCC
oSH42 - E3L Primer	ACGATCTTCAACGTAGTGC
oSH43 - E3L primer	GTGCTAACCCTGTCACCG
oT7 - pET16b primer	GTAATACGACTCACT
oNB15 - PKR primer	GTAGTACTTAAATATCAA
oNB16 - PKR primer	GCAAAAAATGCCGCAGCC

2.2 Media

2.2.1 Bacterial Media

2xTY.

For 1 Litre :-

Bacto-tryptone	16g
Bacto yeast extract	10g
NaCl	5g

Luria Broth. (L.B)

For 1 Litre :-

Bacto-tryptone	10g
Bacto yeast extract	5g
NaCl	10g

Minimal Media.

For 1 Litre :-

Na ₂ HPO ₄	12g
KH ₂ PO ₄	6g
NH ₄ Cl	2g
NaCl	1g

The media was prepared excluding NH₄Cl (99.9%) and water added to 900mls. The media was then adjusted to pH 7.4, the NH₄Cl added and the media made up to 1L. To produce isotopically labelled proteins necessary for NMR analysis, ¹⁵NH₄Cl was used.

Prior to use the autoclaved media was cooled, and the following filter sterilised solutions added to 1L.

20% glucose	20mls
1M MgSO ₄	4mls
1M CaCl ₂	200μl

A solution of vitamins and trace elements was used on occasions to supplement the minimal media used to grow isotopically labelled recombinant proteins. The components used are shown below.

Solution A - Trace Elements.

FeCl ₃ ·6H ₂ O	16.2g
ZnSO ₄ ·7 H ₂ O	2.4g
CoCl ₂ ·6 H ₂ O	4.2g
Na ₂ MO ₄ ·2 H ₂ O	4.2g
H ₃ BO ₃	1.2g
MnSO ₄	3.0g
HCl	30mls
H ₂ O	570mls

Solution B - Vitamins (pH7.2)

Pantothenic Acid, calcium salt	0.4g
Folic Acid	0.4g
Nicotinamide	0.4g
Pyridoxal HCl	0.4g
Riboflavin	0.04g
Thiamine HCl	0.4g
H ₂ O	800mls

The solutions were prepared, filter sterilised and 1ml of solution A and 0.65ml of solution B was added to 1 litre of M9 minimal media.

All the above media were produced using distilled water and were autoclaved at 15psi for 20 minutes at 121°C. Agar plates were produced by the addition of 1.5% agar to the media, and poured into petri dishes whilst molten. Plates were stored at 4°C for up to 1 month. The appropriate filter sterilised antibiotics were added at the correct concentration, ampicillin (100µg/ml) to maintain selection for pET 16b and chloramphenicol (34µg/ml) when the *E.coli* strain BL21(DE3)pLys S was used to maintain the pLys S plasmid.

2.3 DNA Techniques.**2.3.1 Concentration of DNA.**

The concentration of DNA was determined by the measurement of absorbance (A) using an Ultraspec III spectrophotometer (Pharmacia). The sample was diluted in water to attain a detection range between 0.1 and 1.0, and then measured at 260nm. The nucleic acid concentrations were then calculated using the following formula,

$$\text{Concentration}(\mu\text{g} / \text{ml}) = A_{260}(A) \times \text{Dilution} \times \text{Constant}$$

Constant = 50µg ml⁻¹ A⁻¹ for double stranded DNA.

Protein contamination of plasmid DNA was assessed by comparison of the absorbance at 280nm and 260nm. An A_{260}/A_{280} ratio >1.7 was considered to be sufficiently pure for most applications. A ratio below 1.7 indicated protein contamination of the sample, and was typically re-purified by phenol extraction.

2.3.2 Ethanol Precipitation

To ethanol precipitate the DNA, 0.05 volumes of 3M Sodium acetate (pH 5.2) was mixed together with 2.5 volumes of 100% ethanol and the DNA sample itself. The DNA was precipitated by placing the sample in a dry ice/ethanol bath for 20 minutes, or incubated at -70°C for 15 minutes, or -20°C for 18 hours. The sample was then centrifuged for 15 minutes at 10,000RCF/13,000rpm in a microfuge. The ethanol was drawn off and the pellet washed in 70% ethanol to remove excess salts. The sample was then centrifuged and the ethanol drawn off once again. The pellet was left to air dry before suspension in an appropriate volume of water or TE (10mM Tris-HCl pH 8, 1mM EDTA).

2.3.3 Phenol Extraction

To remove protein contaminants, the sample was mixed with one volume of phenol:chloroform:isoamylalcohol (25:24:1). After vortexing the sample was centrifuged and the top layer removed. If a white interface was visible this procedure was repeated. An equal volume of diethyl ether was added to the aqueous DNA solution, vortexed briefly and the sample centrifuged one again. The sample was placed in a dry ice/ethanol bath for 10 minutes and the diethyl ether drawn off from the frozen lower sample. The sample was placed in a well ventilated water bath at 37°C for 15 minutes and ethanol precipitated.

2.3.4 Restriction Endonuclease Digestion.

Restriction endonucleases were purchased from Gibco-BRL and reactions carried out in the recommended reaction buffers. Digestions were performed with 1-5 μl units of enzyme per μg of DNA. Due to the high concentrations of glycerol, the volume of enzyme was less than 10% of the total reaction volume, to help prevent enzyme inhibition and star activity.

2.3.5 Agarose Gel Electrophoresis.

Electrophoresis Buffer

10xTBE.

For 1 litre:-

Tris base	108g
Boric Acid	55g
0.5M EDTA	40ml

The 10xTBE buffer was diluted down 1:10, to 1xTBE for use as a electrophoresis buffer.

Loading Buffer

Glycerol	30%
Bromophenol Blue	0.25%
Xylene cyanol FF	0.25%

Agarose Gels

Agarose (ICN) melted in 1xTBE	0.8-2%
Ethidium bromide	0.5µg/ml

Samples were mixed with loading buffer and electrophoresed at 3-4V/cm. DNA size markers, λ DNA *Hind III* digested, and ϕ X174 *Hae III* digested were used routinely. DNA was visualised under UV light and photographed.

2.3.6 DNA Extraction from Agarose

Running Buffer

1xTAE

Tris-HCl pH 7.5	40mM
Glacial acetic acid	0.57%(v/v)
EDTA (pH 8)	1mM

Agarose Gels

LMP agarose (Gibco) melted in 1xTAE	0.8-2%
Ethidium bromide	0.5µg/ml

DNA was extracted from the gel using β -Agarase I (Biolabs). The DNA sample was electrophoresed and the appropriate DNA fragment was located using UV illumination. The band was then excised using a sharp scalpel, cut into a small pieces and placed in an 1.5ml tube. The agarose was then weighed and 0.1 volumes of the manufacturers buffer (10mM bis-Tris-HCl pH 6.5, 1mM EDTA) was added. The agarose was then heated to 65°C for 10 minutes and cooled to 40°C where it was incubated for 18 hours with 1 unit of β -Agarase I per 200 μ l of 1% agarose. After incubation the salt concentration of the digested agarose was adjusted to 0.3M Sodium acetate by addition of 0.1 volumes of 3M Sodium acetate and incubated on ice for 15 minutes. The sample was then centrifuged for 15 minutes at 10,000RCF/13,000rpm in a microfuge to pellet any remaining undigested carbohydrates. The DNA was then precipitated with 2 volumes of isopropanol, mixed, chilled for 20 minutes in a dry ice/ethanol bath then centrifuged for 15 minutes at 10,000RCF/13,000rpm in a microfuge. The isopropanol was drawn off the pellet washed in 70% isopropanol air dried and the DNA pellet taken up in TE or water.

2.3.7 DNA Ligations

Typically a 3 fold molar excess of DNA insert was ligated with the vector DNA using 1 unit of T4 DNA ligase (Promega) in a 10 μ l reaction volume. The reaction was carried out in 50mM Tris-HCl, pH 7.6, 10mM MgCl₂, 1mM ATP, 1mM DTT, 5% w/v PEG 8000 at 4°C for 15-18 hours.

2.3.8 Transformation of Bacterial Cells.

CaCl₂ Heat Shock Transformations

Preparation of Competent Cells

Frozen 10% glycerol stock cells were streaked onto a 2xTY plate and incubated at 37°C for 14-18 hours so as to produce a single colony. This was then inoculated into 10mls 2xTY broth and grown at 37°C with shaking, 200rpm, for 14 - 18 hours. From this 10ml starter culture, 500mls 2xTY broth was inoculated and grown at 37°C with shaking until OD₅₉₅ 0.6-0.7. The cells were then chilled on ice for 15 minutes and transferred to pre-chilled centrifuge pots. The cells were then pelleted by centrifugation at 577RCF/2,500rpm for 5 minutes at 4°C (Sorval RC5B centrifuge, GS3 rotor). The pellet was resuspended in 250mls of pre-chilled 50mM CaCl₂, and incubated on ice for 30 minutes. Cells were pelleted once again at 577RCF/2,500rpm for 5 minutes at 4°C (sorval RC5B, ss34 rotor) and then resuspended in 50mls of pre-chilled 50mM CaCl₂ with 15% glycerol. The suspension was then aliquoted (150 μ l) and snap frozen in a dry ice/ethanol bath, and stored at -70°C for up to 6 months.

Transformation

Frozen cells were thawed slowly on ice and 10µl of the ligation mix or 1µg of plasmid DNA was added and then incubated on ice for 10 minutes. Cells were heat shocked at 42°C for 2 minutes and returned to the ice for a further 10 minutes. 200µl of 2xTY was added to the cell suspension and the cells were incubated at 37°C for 30 minutes. Various dilutions of cells were plated on 2xTY with the appropriate antibiotics and grown at 37°C for 14 -18 hours.

Electrotransformation.

Preparation of Electrocompetent Cells

A 10ml starter culture was prepared and inoculated into 1 litre of 2xTY and grown at 37°C with shaking until $OD_{550} = 0.5-0.6$. The cells were transferred to pre-chilled centrifuge pots and chilled on ice for 30 minutes. The cells were centrifuged at 1304RCF/ 3,500rpm for 15 minutes at 0°C (Sorval RC-5B centrifuge GS-3 rotor) and the pellet resuspended in 50mls of pre-chilled nanopure autoclaved water. The volume was made up to 250mls with more water and the cells pelleted again under the same conditions. The water was carefully decanted, and the cells gently resuspended in a total of 150mls of water. The cells were centrifuged once a again and the pellet resuspended in 10mls of pre-chilled sterile 10% glycerol. The suspension was centrifuged at 3055RCF/5,800rpm for 15 minutes at 0°C(ss34 rotor) and the pellet gently resuspended in 1ml of pre-chilled 10% sterile glycerol. The cells were then snap frozen in a dry ice/ethanol bath in 40µl aliquots and stored at -70°C for up to 6 months.

Electrotransformation.

An Invitrogen Electroporator II was used for this procedure and the electroporation carried out according to the manufactures instructions.

Aliquots of competent cells were thawed slowly on ice and mixed with 1µg of the positive control plasmid pUC18 or 1µl of the sample DNA of the appropriate concentration. The cells and DNA mix was transferred into a pre-chilled electroporation cuvette (0.1cm gap). The electroporator was set at capacitance 150µF, resistance 150Ω, and the power pack set to 1,500V, 25mA and 25W. The electroporator was charged prior to use and a single pulse passed through the cuvette. 1ml of 2xTY was mixed with the cells before being transferred into a sterile bijoux and incubated for 1 hour at 37°C with shaking. Cells were plated out onto 2xTY containing the appropriate antibiotics and incubated at 37°C for 14 - 18 hours.

2.3.9 Plasmid DNA Preparation from *E.coli*

Small scale preparation.

GTE

Glucose	50mM
Tris-HCl (pH 7.4)	25mM
EDTA	10mM

SDS/NaOH

SDS	1%
NaOH	0.2M

Potassium acetate/Acetic acid

Potassium acetate	3M
Acetic Acid -glacial	2M

A single colony of *E.coli* containing the plasmid was used to inoculate 10mls of 2xTY broth containing the appropriate antibiotics and grown at 37°C for 14-18 hours with shaking. 1.5mls was transferred to a 1.5ml tube and the cells pelleted by centrifugation at 10,000RCF/13,000rpm for 30 seconds in a microfuge. Cells were resuspended in 100µl of GTE to which 200µl of cell lysis SDS/NaOH solution was added. After briefly vortexing the suspension 150µl of potassium acetate/acetic acid was added, and the solution inverted several times. The cell debris was pelleted by centrifugation at 10,000RCF/13,000rpm for 30 seconds in a microfuge. The supernatant was transferred to a clean 1.5ml tube, where any remaining proteins were removed by phenol extraction. The DNA was then ethanol precipitated, washed in 70% ethanol and resuspended in TE/water.

Large Scale Preparation.

A 500ml culture was grown from a single *E.coli* colony as described earlier. The cells were then harvested by centrifugation at 2661RCF/ 5,000rpm for 10 minutes at 4°C (Sorval RC-5B centrifuge and GS-3 Rotor). The supernatant was decanted and the cells resuspended in 7.5mls GTE. 15mls of alkaline lysis solution, SDS/NaOH, was added mixed gently and incubated on ice for 10 minutes. 11mls potassium acetate/acetic acid was then added, again mixed by gentle shaking and incubated on ice for a further 10 minutes. The suspension was centrifuged at 10,000RCF/13,000rpm for 30 minutes in a microfuge at 4°C to pellet cell debris. The supernatant was decanted (28mls) and mixed with 17mls of isopropanol. After a 15 minute incubation at room temperature, the sample

was centrifuged for 20 minutes at 9,220RCF/10,000 at 4°C (ss34 rotor). The pellet was then washed in 70% ethanol, air dried and re-dissolved in 2mls of TE. 2.4g of caesium chloride, CsCl, was added to the TE and dissolved completely by incubation at 37°C for 5 minutes. The sample was then centrifuged briefly at 1,000g/2,500rpm (Sanyo, Mistral 2000i) and the supernatant loaded into the 3.9ml Beckman heat seal tubes. The volume was made up with CsCl/TE (1.2g/ml) solution and 50µl ethidium bromide (10mg/ml). The tubes were sealed and centrifuged for either 5 hours at 100,000rpm or 15-18 hours at 80,000rpm at 20°C, acceleration-deceleration 5 (Beckman TLN. 100 rotor and optima TL-ultracentrifuge).

The plasmid bands were extracted by side puncture using a 20gx1" needle and 10ml syringe. Ethidium bromide was removed by several extractions with isopropanol saturated with water and NaCl. The plasmid DNA was then diluted 3 fold with TE and ethanol precipitated centrifuged and washed with 70% ethanol. The pellet was then air dried and resuspended in TE and the concentration of the plasmid determined.

2.3.10 Oligonucleotide Purification.

Formamide Dye.

Formamide	80%
EDTA	10mM
Xylene cyanol FF	1mg/ml
Bromophenol blue	1mg/ml

Polyacrylamide Denaturing Gels

Acrylamide Stock Solution

Acrylamide	100g
Bis-acrylamide	25g
Urea	210g
Water to 450mls	

Diluent

Urea 210g
Water to 450mls

Gel Elution Buffer

Sodium acetate	0.3M
Tris-HCl (pH 8)	10mM
EDTA	0.1mM
SDS	0.5%

Oligonucleotides were synthesised by the PNACL facility at Leicester University. Before use long oligonucleotides of over 30 nucleotides, or those to be used for PCR were

purified using denaturing polyacrylamide electrophoresis. Typically the oligonucleotides were separated on a 10% denaturing gel, although smaller oligonucleotides were separated on a higher % gel. A 10% gel was prepared by mixing equal volumes of the stock solution and the diluent to a total volume of 45mls (or factors of). To this 5mls of 10xTBE was added along with 50 μ l TEMED and 50 μ l 25% APS. The solution was poured between 0.1cm x 20cm² gel plates and a comb with 4cm wells inserted. The gel was then left to polymerise at room temperature.

40 μ l TE containing approximately 75 μ g of the oligonucleotide was mixed with 40 μ l of formamide dye. The solution was then heated for 5 minutes at 95°C, to denature the oligonucleotide. The DNA was loaded into 4cm wells and electrophoresed at a constant power of 40W in 1xTBE buffer. Once completed the gel plates were removed and the gel wrapped in cling film. The oligonucleotide was visualised by UV shadowing whereby the gel was placed on top of a UV fluorescent plate and illuminated from above with a short UV light. The oligonucleotide band was then excised sliced into small pieces with a sharp sterile scalpel, and transferred to a 1.5ml eppendorf. 1ml gel elution buffer was added and the oligonucleotides eluted by incubation at 37°C for 18 hours. Gel debris was removed by centrifugation at 10,000RCF/13,000rpm for 5 minutes in a microfuge and the supernatant removed. The oligonucleotide was then ethanol precipitated and washed in 70% ethanol. The pellet was then air dried and resuspended in TE or water.

Quantitative Analysis of Oligonucleotides.

The molar concentration of the purified oligonucleotide was determined as shown below:-

$$A \quad n \times 15.4 \quad = N_1$$

$$G \quad n_1 \times 11.7 \quad = N_2$$

$$C \quad n_2 \times 7.3 \quad = N_3$$

$$T \quad n_3 \times 8.8 \quad = N_4$$

$$\Sigma N$$

$$\text{Molar concentration} = \frac{\text{OD}_{260} \times \text{dilution factor}}{\Sigma N} = \text{pmol/ul}$$

2.3.11 Sequencing DNA.

Manual Sequencing. (Sanger *et al.*, 1977)

The sequencing reaction used reagents from the Sequenase V2.0 (USB) kit. A modified version of their protocol was used for the sequencing reaction itself. 6µg of dsDNA templates were denatured using 1µl 5mM EDTA and 1µl 4M NaOH with water bringing the reaction volume to 20µl. This was incubated for 30 minutes at 37°C and the DNA recovered by ethanol precipitation in a dry ice/ethanol bath and the pellet washed with 70% ethanol and resuspended in 20µl of TE.

A primer/buffer mix was prepared using 1µl sequenase reaction buffer (40mM Tris-HCl pH 7.5, 20mM MgCl₂, 50mM NaCl), 4µl primer at a concentration of 1.25pmol/µl and 3µl water, per clone to be sequenced. 4µl aliquots of the DNA were transferred into 4 wells on a 96 well microtitre plate, to which 2µl of the primer/buffer mix were added. After mixing by brief centrifugation, the microtitre plate was wrapped in cling film and incubated at 37°C for 30 minutes. Meanwhile, a labelling mix was prepared with 6.5µl water, 0.4µl 0.1M DTT, 0.5µl α³⁵S-dATP, 0.25µl Sequenase and 0.4µl dGTP labelling mix (which was diluted 10 fold if sequencing close to the primer) for each clone to be sequenced. 2µl of the labelling mix was aliquoted into each well, mixed again by centrifugation and incubated at room temperature for 10 minutes. 2µl of the appropriate termination nucleotide mix (80µM each dNTP, 50mM NaCl and either 8µM ddATP, ddCTP, ddGTP or ddTTP), was added, mixed and incubated for 5 minutes at room temperature. Finally 4µl of stop buffer (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.005% xylene cyanol FF) was added, mixed and the reaction solution heated to 80°C for 10 minutes in a dry air oven. The reaction mix was loaded onto a 6% polyacrylamide TBE gradient gel.

Polyacrylamide Gel Electrophoresis.

40% acrylamide stock solution (40:1)

For 1 Litre

Acrylamide	380g
Bis-acrylamide	20g

If necessary the solution was deionised using 10g Doulite MB61113 mixed resin (BDH) with gentle mixing for 60 minutes. The resin was removed by gravity filtration.

10 x TBE

For 1 litre

Tris base	108g
Boric Acid	55g
0.5M EDTA	40mls

6% Top Solution

40% acrylamide stock	75mls
10 x TBE	25mls
Urea	230g
Water to	500mls

6% Bottom Solution

40% acrylamide stock solution	30mls
10 x TBE	50mls
Urea	92g
Water	50mls
Bromophenol blue	trace

Fixing Solution

Methanol	10%
Glacial acetic acid	10%

Initially PAGE gel plates (20 x 40cm) were cleaned with water and ethanol and one plate covered in silanate to allow easy separation of the plates once the gel run was finished. The two plates were placed horizontally with a 0.4mm spacer. 32mls of top solution followed by 7mls of bottom solution were mixed with 64 μ l and 14 μ l respectively of both 25% APS and TEMED. To pour the gel, 25mls of top solution followed by 7mls of the bottom solution were drawn up into a pipette. Several air bubbles were passed through the pipette to facilitate TBE gradient formation and this was introduced between the glass plates. Once complete a 36 well (3mm) comb was inserted between the gel plates and bulldog clips used to clamp the gel plates together.

Once polymerised the gel was clamped into a vertical electrophoresis apparatus with a heat diffuser metal plate. 1 x TBE running buffer was loaded into the upper and lower troughs and the comb removed. The wells were washed thoroughly with running buffer and 2-4 μ l of the sequencing reaction loaded into the appropriate wells. The DNA was electrophoresed at constant power 37W for 1-2 hours. After electrophoresis the gel was placed in fixing solution for 10 minutes, and then transferred on to 3MM Whatmann

paper. The gel was dried under vacuum at 80°C for 1 hour and exposed to autoradiography film for 14-18 hours at room temperature. Films were then developed in a Agfa-Gaevert Automatic developer.

Automated Sequencing

Automated sequencing was carried out using ABI PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit. Reaction mixes were then processed by the PNACL facility at Leicester University.

1 µg of plasmid DNA was mixed with 2 µl of 1.6 µM sequencing primer. To this 9.5 µl the terminator premix, containing dye labelled dideoxynucleotides, was added along with water to a total volume of 22 µl. The solution was mixed by pipetting and overlain with 40 µl of mineral oil in a 0.5ml 1.5ml tube. The solution was then heated to 96°C for 1 minute and cycled to 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes for 25 cycles and the temperature then held at 4°C. The cycling reactions were carried out in a Techne Cyclogene Thermal Cycler (PCR) machine. Once the reaction was complete the DNA solution was made up to 100 µl with water and mixed with 100 µl of phenol:chloroform:water (169:18:14) to extract the excess terminators. The samples were vortexed, centrifuged for 5 minutes at 10,000RCF/13,000rpm in a microfuge, and the aqueous phase removed. After ethanol precipitation the pellet was air dried and the sequence determined by PNACL. Sequence data was analysed using SeqEd, on a Macintosh computer.

2.3.12 Polymerase Chain Reaction (PCR)

The polymerase chain reaction was used to amplify specific DNA sequences. It was carried out under conditions that would minimise contamination. Reaction mixes, typically 50 µl, were overlain with 50 µl of mineral oil and cycling carried out in a Techne, Cyclogene Thermal Cycler in 0.5ml sterile tubes. Positive controls with a known PCR template were included where possible, and a negative control with no template DNA was as standard.

The reaction itself contained 0.2 µg of DNA template with 25 pmol of both forward and backward primers (Primers listed in Table 1.4). 0.3 mM of each dNTP (dGTP, dATP, dCTP, dTTP), 1 µl of DNA polymerase and 5 µl of the 10x PCR buffer supplied with the enzyme, were mixed and the volume made up to 50 µl with water. The polymerases used were :-

Taq Polymerase (Gibco)

1x Taq buffer

(20mM Tris-HCl (pH 8.4) 50mM KCl.(10mM KCl, 20mM Tris 50mM KCl)

Vent polymerase (NEB)

1 x Vent buffer

(10mM KCl, 20mM Tris-HCl (pH 8) 10mM(NH₄)₂SO₄, 2μM MgSO₂ 0.1% Triton X-100).

The *vent* polymerase was used to produce all PCR products for cloning due to the proof-reading capacity of the enzyme. *Taq* polymerase was used only as an initial control for all PCR reactions.

Cycling reactions were carried out at a variety of temperatures according to the primers and DNA to be amplified. Typically the reaction cycle parameters would be denaturation at 94°C for 30 seconds, annealing at 45-65°C for 30 seconds and extension at 72°C for 1.5minutes per kb to be amplified. Amplifications were performed for 30-35 cycles with a final cycle of 72°C for 10 minutes. PCR products were then visualised under UV light after electrophoresis of 10% of the reaction on an appropriate % agarose gel with ethidium bromide present.

2.4 Protein Techniques.

2.4.1 Protein Expression in *E.coli*

Plasmid containing the gene of interest was transformed into an appropriate *E.coli* strain and a single colony of each used to inoculate 10ml overnight starter cultures containing the necessary antibiotics. 100μl of the overnight cultures were inoculated into 10mls of 2xTY supplemented with antibiotics, and grown at 37°C, with shaking until OD₅₉₅ = 0.4-0.7. IPTG was added to a final concentration of 0.4 - 2mM and protein induction carried out between 2 and 18 hours at 37°C. 1.5ml aliquots were centrifuged and the pellet taken up in 200μl of protein loading buffer. The sample was then boiled for 5 minutes and between 2 and 10μl loaded onto the protein SDS PAGE gel (section 2.4.3).

2.4.2 Quantitative Analysis of Proteins.

An estimation of protein concentration of proteins was determined by the measurement of absorbance A₂₈₀. Due to the contamination of the sample with nucleic acids A₂₆₀ was also

measured and the following equation used to estimate the concentration of the protein in mg/ml.

$$1.55 \times A_{280} - 0.76 \times A_{260}$$

This equation was only of use if the A_{280}/A_{260} ratio was <0.6 (nucleic acid contamination w/v was less than 20%).

2.4.3 SDS Polyacrylamide Gel Electrophoresis

Resolving Gels

	12%	18%
30% Stock Acrylamide	20mls	30mls
1.5M Tris-HCl pH 8.8	12.5mls	12.5mls
10% SDS	0.5mls	0.5mls
Water	16.5mls	6.5mls
TEMED	20 μ l	25 μ l
10% APS	0.5mls	0.5mls

5% Stacking Gel.

30% stock acrylamide	4.25mls
1M Tris-HCl pH 6.8	3.13mls
10%SDS	0.25mls
Water	17mls
TEMED	25 μ l
10 % APS	0.25mls

Protein Loading Buffer

Tris-HCl pH 8	50mM
SDS	2%
β -mercaptoethanol	5%
Glycerol	20%
Bromophenol blue	0.05%

Tris - Glycine Electrophoresis Buffer

Tris-HCl	25mM
Glycine pH 8.3	250mM
SDS	1%

Stain Solution

Methanol	200mls
Glacial acetic acid	40mls
Water	200mls
Coomassie blue	1.1g
Filtered before use	

Destain Solution

Methanol	200mls
Glacial acetic acid	40mls
Water	200mls

Multiple gels were prepared simultaneously with 10 pairs of glass plates (100x100mm) with 0.4mm spacers, placed into the minigel casting apparatus (Cambridge Electrophoresis). The resolving gel was poured leaving sufficient room for the stacking gel to be poured on top. The resolving gels were overlain with water saturated isobutanol, and left to polymerise. The isobutanol was then removed, the combs inserted between the plates and the stacking gel poured. Once polymerised the gels were stored in moist conditions at 4°C.

The protein sample was mixed with an equal volume of protein loading buffer and heated to 100°C for 3-5 minutes. Following centrifugation for 5 minutes at 10,000RCF/13,000rpm in a microfuge the sample was loaded onto the gel using a Hamilton syringe and electrophoresed at 15mA. Once the dye front had reached the resolving gel the current was increased to 25mA. When the dye front had reached the bottom of the gel, the plates were removed and the gel stained for 15 - 30 minutes. The gel was then placed in destain solution to remove any excess coomassie stain.

2.4.4 Extraction of the Protein.

Sonication/Extraction Buffer

or	Tris-HCl pH 7.5	50mM
	NaHPO ₄ pH 6.5	50mM
	NaCl	100mM
	β-mercaptoethanol	1mM
	PMSF	0.5mM

where necessary,

Urea	up to 8M
Guanidine Hydrochloride	up to 6M
NaCl	up to 1M
NaBr	up to 200mM

Crude Protein Preparation.

The recombinant protein was extracted from the cytoplasm where it had accumulated after synthesis. Generally sonication was used although a french press or the addition of lysozyme was used on occasions. Because of the variable solubility of the proteins expressed, the buffer components were modified accordingly. All extraction procedures were carried out at 4°C.

Sonication

Induced cell pellets were resuspended in 5 - 10 times the volume of the cell pellet in the appropriate ice cold extraction buffer. The suspension was then cooled in an ice water bath. Sonication was carried out at 7 microns in 15 second pulses with 45 seconds between each pulse to allow the sample to cool. A coomassie dye assay (Biorad) was used to determine the required number of pulses required to ensure complete lysis of the cells. 200µl samples were removed and centrifuged at 10,000RCF/13,000rpm in a microfuge at 4°C, at several points during sonication. To ensure quantitative results a supermix of reagent and water (200µl:800µl respectively- or factors of) was prepared to which 5µl of the centrifuged sample supernatant was mixed with 1ml of supermix. After a 10 minute incubation at room temperature OD₅₉₅ was measured. A graph was then produced and once the plateau was reached and the maximum quantity of protein liberated, sonication was complete.

French Press.

The induced cell pellet was resuspended in the pre-chilled extraction buffer and then loaded into the large pre-chilled chamber. Cells were passed through the press at 12,000 lbs in⁻². This was repeated twice more to ensure maximal disruption. The sample was centrifuged for 30 minutes at 20,800RCF/15,000rpm (Sorval RC5B, ss34 rotor) to pellet insoluble proteins and cell debris. The proteins within the pellet were then extracted using the required ice cold buffer and a Dounce homogeniser. To ensure efficient extraction of the recombinant protein, the sample was homogenised very slowly to avoid frothing and denaturation of the protein. This procedure was repeated until all the recombinant protein was solubilised.

Addition of lysozyme.

Cells were resuspended in 5-10 volumes of the cell pellet in ice cold 50mM Tris-HCl pH7.5, 10% sucrose, 0.1% Triton-X 100 and 0.2mg /ml (final concentration) of lysozyme. The lysate was then centrifuged at 29,900RCF/18,000rpm (Sorval, SS34 rotor) for 45minutes. The pellet was resuspended in ice cold 50mM Tris-HCl pH8, 10% glycerol, 0.1% Triton-X 100 and 1M NaCl using a Dounce homogeniser. The centrifugation step was repeated and the extraction repeated once again. The samples were analysed by SDS PAGE and the extraction procedure repeated if necessary.

Characterisation of the Solubility of the Protein.

A simple assessment of each expressed protein provided the initial characterisation of the quantity and nature of the solubilising agents required. A small scale sonication protocol was utilised. Initially a number of 1.5ml samples of induced cell pellet were resuspended in 200µl Tris-HCl extraction buffer containing up to 8M urea. Samples were individually sonicated in extraction buffer supplemented with 1M, 2M, 4M, 6M and 8M urea then centrifuged. The soluble and insoluble pellets were then mixed with loading buffer and analysed by SDS PAGE. If this form of denaturant was not sufficient the procedure was repeated with up to 6M guanidine hydrochloride. Other chaotropic agents such as detergents, NaCl and NaBr were used where necessary.

Soluble and Insoluble Protein Processing.

Soluble protein samples were stored at 4°C ready for further rounds of purification.

Insoluble protein samples were sonicated (or passed through a french press) initially in a non-denaturing extraction buffer to remove contaminating proteins. On occasions the pellet was washed with 3 volumes 20mM-Tris HCl pH7.5, 200mM NaCl, 2mM EDTA, 1% deoxycholic acid (w/v) and 1% NP 40, to remove lipids from the sample. Following centrifugation 1,500RCF/3,500 rpm (Sorval RC5B,ss34 rotor), for 10 minutes, the pellet

was washed twice with 3 volumes of 0.5% Triton-X 100 and 1mM EDTA pH 8. The insoluble pellet was then resuspended in 5-10 volumes of extraction buffer containing the required concentration of denaturant as determined previously. This procedure was repeated when required to maximise the quantity of protein extracted.

Renaturation.

Refolding Buffer.

Tris-HCl pH7.5	50mM
NaCl	10mM
Urea	2.5M
Lysine	10mM
Reduced glutathione	0.5mM
Oxidised glutathione	0.5mM

Renaturation of denatured proteins was usually undertaken by dialysis. Dilution of the recombinant protein was also used. The sample was diluted 50 fold in refolding buffer, and incubated at 15°C for 18 hours. The sample was then dialysed against 50mM Tris-HCl pH7.5, 100mM NaCl, 0.5mM DTT, 5mM EDTA, 2.5M urea and 2% Triton-X 100, followed by dialysis into the appropriate native buffer..

2.4.5 Purification Of Proteins with Histidine Tag.

Binding Buffer

	Tris-HCl pH 8	50mM
	NaCl	100mM
where necessary	Urea	up to 8M
or	Guanidine Hydrochloride	up to 6M
	Adjust final pH to 8.0.	

Wash Buffer

	Tris-HCl pH 8	50mM
	NaCl	100mM
	Imidazole	10mM
where necessary	Urea	up to 8M
or	Guanidine Hydrochloride	up to 6M

Elution Buffer

	Tris-HCl pH 8	50mM
	NaCl	100mM
	Imidazole	100-200mM
where necessary	Urea	up to 8M
or	Guanidine Hydrochloride	up to 6M

Elution Buffer II

Small scale elution carried out with 100mM EDTA(pH 8)

For small scale purifications, an equal volume of protein (50µl) and prewashed Talon (non-nickel) metal affinity resin equilibrated in binding buffer were mixed and then agitated for 30 minutes at room temperature. The samples were then centrifuged for 1 minute at 10,000RCF/13,000rpm in a microfuge and the supernatant containing unbound proteins removed. The resin was then washed twice with 2 volumes of wash buffer and eluted with 50µl 100mM EDTA. The buffer content varied depending on the protein solubility and quantity of denaturant required.

Large scale purification of histidine tagged proteins used a column packed with Talon resin and equilibrated in the appropriate buffer followed a similar protocol. The sample was loaded onto the column, and the column then washed in several column volumes of wash buffer, again to remove any unbound proteins. Elution of the protein was achieved by progressive elution with several volumes of elution buffer containing 100mM imidazole. This was increased to 200mM imidazole if necessary. Samples were then mixed with an equal volumes of SDS loading buffer, boiled for 5 minutes and centrifuged at 10,000RCF/13,000rpm for 5 minutes in a microfuge. Fractions were then analysed by SDS PAGE. Alternatively if columns were not available binding and washing and elution steps were undertaken in a 15ml sterile tube, whereby the resin was mixed and incubated with the supernatant and then centrifuged for 5 minutes at 1,000RCF/2,500rpm (Mistral,3000i). The supernatant was then removed and the resin washed, the centrifugation repeated and the elution buffer added and the process repeated until all the histidine tagged protein had bound.

2.4.6 FXa Cleavage of Histidine Tagged Proteins.

Cleavage Buffer

	Tris-HCl	50mM
	NaCl	100mM
	CaCl ₂	1mM
where necessary	Urea	up to 0.5M

Following histidine tag purification, the protein was diluted or dialysed into cleavage buffer. FXa was added to the sample and the quantity and incubation conditions (both temperature and time) were optimised for each individual construct. The protein and cleaved histidine tag were separated by loading the sample onto a Talon metal affinity column, whereby the cleaved histidine tag was retained on the column and the authentic protein was not.

2.4.7 Double Stranded RNA Binding Assays.

Binding Buffer

HEPES pH 7.5	20mM
NaCl	300mM
magnesium acetate	5mM
DTT	1mM
PMSF	0.5mM
NP-40	0.5%
Glycerol	10%

Wash Buffer

HEPES pH 7.5	20mM
NaCl	500mM
magnesium acetate	5mM
DTT	1mM
PMSF	0.5mM
NP-40	0.5%
Glycerol	10%

Initially the Poly(rI) Poly(rC) (Pharmacia) resin slurry was centrifuged to produce a 50µl resin pellet. The resin was then equilibrated with two 0.5ml volumes of binding buffer. 50µl of the protein sample was then mixed with an equal volume of the resin and incubated at room temperature for 30 minutes with gentle agitation. The slurry was centrifuged at 10,000RCF/13,000rpm for 1 minute and the supernatant containing the

unbound fraction, carefully removed. The beads were then washed three times with two volumes of the wash buffer. The resin was then resuspended in 50µl of protein loading buffer, and heated at 100°C for 5 minutes. The crude, unbound and bound samples were then loaded onto a polyacrylamide gel and electrophoresed, stained and destained as before. DNA-Agarose (Pharmacia) was used as a negative control. The DNA agarose was equilibrated and washed in an identical fashion to the Poly(rI) Poly(rC).

2.4.8 Gel Filtration

A Superdex 75 HR 10/30 (Pharmacia) column was connected to the Pharmacia Fast Protein Liquid Chromatography (FPLC) system. Before use the column was washed in 3 column volumes of the appropriate extraction buffer (dependent on the protein sample) at a flow rate of 0.5ml/minute. Once equilibrated 500µl of the sample (or smaller volumes where possible) was loaded onto the column at a flow rate of 0.33ml/minute. Protein fractions were collected in 0.5ml aliquots and then analysed by SDS PAGE.

2.4.9 Concentration of Protein Samples.

Ammonium Sulphate Precipitation.

Ammonium sulphate was added very slowly to the sample to a final concentration of 75%w/v, with foaming kept to a minimum to avoid denaturation of the sample. The solution was then incubated at 4°C with gentle agitation for 1-4 hours. The protein precipitate was harvested by centrifugation at 4°C for 40 minutes at 20,800RCF/15,000rpm (sorval RC5B, ss34 rotor).

The pellet was then dissolved in a minimum volume of buffer (equal to the size of the pellet), and then centrifuged once again for 10 minutes at 10,000RCF/13,000rpm at 4 °C in a microfuge. Any pellet remaining at this stage was again dissolved in a minimum volume of buffer and centrifuged. This was repeated a number of times until only insoluble aggregates remained. The concentration of the soluble fractions were then determined and analysed by SDS PAGE to verify the purity of the sample.

Ultrafiltration.

Centricon 10 concentrators were used to concentrate HIS0E3LMet94-190 and used as recommended by the manufactures. Briefly, samples were loaded into the top section of the apparatus and then centrifuged at 36,900RCF/20,000rpm (Sorval RC5B centrifuge, ss34 rotor). Once the volume had decreased sufficiently, centrifugation was stopped and the concentration of the sample determined.

On occasions an amicon ultrafiltration unit was used. The membrane was incubated overnight in the extraction buffer supplemented with 1% milk to block any fortuitous interactions. The dilute sample was applied to the unit and the sample concentrated until the required concentration was achieved.

2.4.10 NMR Spectroscopy.

NMR analysis of the protein samples were undertaken in collaboration with Dr.L.Y.Lian. A concentration of at least 1mM was required for 1D NMR analysis. Initially a pure (non-isotopically labelled) sample was analysed. From this preliminary data the folded state of the protein was determined and therefore the suitability of each protein for NMR analysis decided. If the protein showed little or no aggregation, ^{15}N labelled proteins were prepared using $^{15}\text{NH}_4\text{Cl}$ (section 2.2.1). 2D NOESY analysis of the protein sample was then undertaken in a Bruker DMX 500.

2.4.11 Protein Crosslinking.

Initially a 1% glutaraldehyde solution was prepared (with water) from the 8% stock solution (Sigma). 5 μg of the native protein was mixed with glutaraldehyde to a final concentration of 0%, 0.005%, 0.01%, 0.02% and 0.05% in a total volume of 20 μl . After a 30 minute incubation, the sample was mixed with 20 μl of protein loading buffer, boiled for 5 minutes and centrifuged for 5 minutes. The results were analysed by SDS PAGE.

2.4.12 Analytical Ultracentrifugation.

Experiments were carried out on purified recombinant protein in an Optima XL-A analytical ultracentrifuge in collaboration with Dr.R Gilbert, Dept. Biochemistry, University of Leicester.

Sedimentation equilibrium studies to determine the mass of the protein, and the sedimentation coefficient were investigated.

For sedimentation equilibrium, the background absorbance was obtained at 40,000rpm. The initial mass at 15,000rpm, and 25,000rpm were obtained and the fitted mass calculated, accounting for the background absorbance. Fitted mass values were calculated using the equation :-

$$A(r) = A(r_F) \exp[HM(r^2 - r_F^2)] + E$$

Where $A(r)$ is the absorbance at radius r (cm)

$A(r_F)$ is the absorbance at the reference radius, r_F .

H is the term $(1 - \bar{v}\rho)\omega^2/2RT$

E is the baseline correction

$\bar{v}$ is the partial specific volume of the protein in ml/g(0.737)

ρ is the buffer density in g/ml

ω is the angular momentum in radians/second

R is the gas constant

T is the absolute temperature.

Data was then applied to a concentration distribution profile and the molecular weight determined.

For sedimentation coefficient analysis, the sedimentation velocity was obtained at 40krpm. The sedimentation coefficient was then calculated using the sedimentation velocity data and S value obtained from a sedimentation coefficient profile. The sedimentation coefficient data was determined using the following equation:-

$$g(s^*)_t = \left(\frac{d\{c(r,t)/c_0\}}{dt} \right) \left(\frac{\omega^2 t^2}{\ln r_m / r} \right) \left(\frac{r}{r_m} \right)^2$$

$g(s^*)_t$ is the time derivative sedimentation coefficient distribution

r is the radial position in cm

ω is angular momentum in radians/second

r_m is the radial position of the meniscus

c_0 is the loading concentration

2.4.13 N Terminal Protein Sequencing

Transfer Buffer

Tris-HCl	48mM
Glycine	39mM
Methanol	10%
SDS	0.03%

Stain

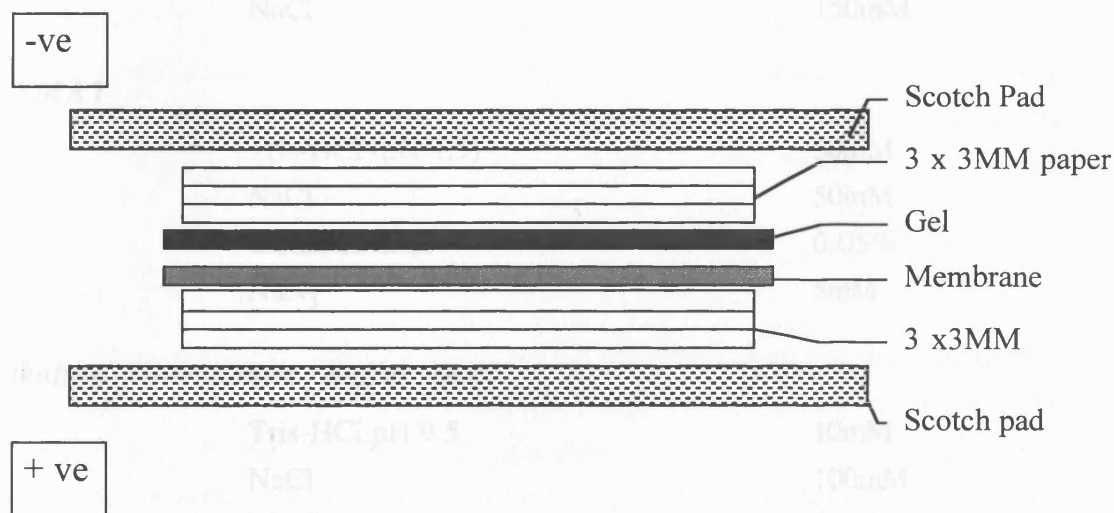
Coomassie blue	0.1%
Methanol	50%
Water	49.9%

Destain Buffer

Methanol	50%
Glacial acetic acid	10%
Water	10%

Electroblotting

The protein sample was electrophoresed on an SDS polyacrylamide gel and the equilibrated for 15 minutes in transfer buffer along with 6 pieces of 3MM blotting paper. A piece of polyvinylidene (PVDF) membrane (Biorad) was wetted in 100% methanol and then soaked in transfer buffer. The blotting cassette was constructed as indicated below. The tank was then filled with transfer buffer, and the protein transferred onto the membrane by electrophoresis at 230mA for 2 -3 hours at 4°C (Matsudaira, 1987).



Once the blotting was complete, the cassette was removed and the gel stained and destained, with the aforementioned solutions, until the protein band was clearly visible. The membrane was then washed several times in water and air dried.

Analysis

The protein band of interest was removed with a sharp scalpel and analysed by the PNACL facility at University of Leicester on 476A Protein Sequencer (Applied Biosystems). The results of the sequence were then compared to the known sequence.

2.4.14 Western Blotting.

A monoclonal antibody, 71/10 (Ribogene Inc) was reactive against PKR. Another monoclonal antibody, TW2.3 was reactive against E3L (gift of Bernard Moss).

Transfer Buffer

Tris-HCl	25mM
Glycine	250mM
Methanol	20%

Ponceau Stain

Ponceau stain	0.2%
Glacial acetic acid	7%

Alkaline Phosphatase Detection

TN

Tris-HCl pH 7.5	50mM
NaCl	150mM

TNMAT

Tris-HCl (pH 7.5)	50mM
NaCl	50mM
Skimmed milk	0.05%
NaN ₃	5mM

Alkaline Phosphatase Buffer (AP)

Tris-HCl pH 9.5	10mM
NaCl	100mM
MgCl ₂	5mM

Colour Detection Chemicals

NBT	50mg/ml dissolved in 70% DMF
X-Phos	12.5mg/ml dissolved in 50% DMF

ECL Detection Method.***PBS***

NaCl	137mM
KCl	2.7mM
Na ₂ HPO ₄	6.5mM
KH ₂ HPO ₄	1.5mM

TMT/SS

Marvel milk powder	2%(w/v)
Sheep serum	10%
Tween 20	0.5%
Dissolved in PBS	

PBS-T

Tween 20	0.05%
Dissolved in PBS	

Electroblotting

The protein was electrophoresed through SDS polyacrylamide gel and equilibrated in transfer buffer for 15 minutes along with 6 pieces of 3MM blotting paper and 1 piece of Hybond-C-super Nitrocellulose (Amersham). The blotting cassette was constructed in the same manner as with protein sequencing, with the nitrocellulose membrane replacing the PVDF membrane. The protein was then transferred by electrophoresis at 30V for 18 hours at 4°C.

Antibody Probing

The MW protein markers were visualised on the membrane with ponceau and marked on the membrane with a pencil. For detection with alkaline phosphatase detection method the membrane was blocked with TNMAT for a minimum of 15 minutes. Incubation for at least 1 hour with the primary antibody diluted in TNMAT (1:10,000 for both antibodies) followed. The membrane was then washed 3 times for 5 minutes per wash in TNMAT

and then incubated for a further 1 hour with the secondary antibody diluted 1:1000 in TNMAT (alkaline phosphatase conjugated). The membrane was washed several times in TN buffer and then briefly in AP buffer. 110µl NBT and 82.5µl X-phos were mixed with 25mls of AP buffer and poured over the membrane. When a dark pink colour developed the membrane was washed twice with water, air dried and stored in the dark.

For ECL (Amersham) detection the membrane was blocked with TMT/SS for 2 hours and then incubated with the primary antibody (diluted 1:10,000) for 1 hour. The membrane was washed 3 times for 5 minutes with PBS-T. The membrane was then incubated with the secondary antibody diluted 1:1000 in TMT/SS (HRP conjugated) for 1 hour and washed once again with PBS-T, followed by several washes with PBS alone. For detection the membrane was soaked in ECL solution number 1 for 2 minutes, then ECL solution 2 added and incubated for a further 2 minutes. The membrane was then placed in an autoradiography cassette, covered with cling film and autoradiography films exposed for various time points.

2.5 Yeast-Two-Hybrid Screen Techniques.

2.5.1 Yeast Strains.

Yeast strains of *S.cerevisiae* used within the project are listed in Table 2.6.

Table 2.6. Yeast strains available for use in the yeast-two-hybrid screen.

Strain	Source	Mating Type	Reporter Genes	Transformation Marker
Y190	Clontech	MAT a	<i>HIS3, LacZ</i>	trp 1, leu 2, cyh 2
CG-1945	Clontech	MAT a	<i>HIS3, LacZ</i>	trp 1, leu 2, cyh 2
Y187	Clontech	MAT α	<i>LacZ</i>	trp 1, leu 2

2.5.2 Bacterial Strains.

Bacterial strains used only for the yeast-two-hybrid screen are listed below in Table 2.7.

Table 2.7. Bacterial strains utilised for identification of the AD/library plasmid insert cDNA.

<i>E.coli</i> Strain	Source	Genotype
HB101	Lab stock	<i>thi</i> -1, <i>hsdS</i> 20, <i>sup</i> E44, <i>rec</i> A13, <i>ara</i> -14. <i>leu</i> B6, <i>pro</i> A2, <i>lac</i> Y1, <i>rps</i> L20 (<i>str</i> ^r), <i>xyl</i> -5, <i>mtl</i> -1.

2.5.3 Vectors.

The two vectors used throughout the yeast-two-hybrid screen are listed below in Table 2.8.

Table 2.8. Vectors used within the yeast-two-hybrid screen.

Vector	Source	Use
pAS2-1	Clontech	Used to generate fusion proteins of the bait protein with the DNA binding domain (DNA-BD) region of GAL-4 transcriptional activator. GAL -4 (1-147).
pGAD GH	Clontech	Used to generate fusion proteins from a cDNA library with activation domain (AD) region of GAL 4 transcriptional activator. GAL-4 (768-881)

2.5.4 Vector Constructions.

Individual constructs used throughout the yeast-two-hybrid screen are listed below in Table 2.9.

Table 2.9. Vector constructions used during the yeast-two-hybrid screen.

Vector Constructions	Description
pAS2-1 + Full length E3L.	Full length E3L cloned into <i>Nde I/BamHI</i> site downstream of GAL 4 DNA-BD. Selection - Trp,amp ^r
pGAD GH + HeLa cell library	cDNA library cloned into <i>EcoRI/XhoI</i> site downstream of GAL 4 AD. Selection -leu, amp ^r
pCL-1	Positive control plasmid. Encodes wild type full length Gal -4. Selection -leu, amp ^r
pVA3	Positive control plasmid used with pTD1. Encodes a DNA-BD/murine p53 fusion protein in pAS2-1. Selection -Trp. amp ^r
pTD1	Positive control plasmid used with pVA3. Encodes an AD/SV40 large T antigen fusion protein in pACT-2. Selection -leu, amp ^r
pLAM5	False positive detection plasmid. Encodes a DNA-BD/human lamin C fusion protein in pAS2-1. Selection -trp, amp ^r
pTra2 β	False positive detection plasmid. Encodes DB/Tra2 β fusion protein in pAS2-1. Selection - trp, amp ^r
pRBM	False positive detection plasmid. Encodes a BD/RBM fusion protein in pAS2-1. Selection -trp, amp ^r
pSam68	False positive detection plasmid. Encodes a BD/Sam68 fusion protein in pAS2-1. Selection - trp, amp ^r
pTstar	False positive detection plasmid. Encodes a BD/Tstar fusion protein in pAS2-1. Selection -trp, amp ^r

2.5.5 cDNA Library.

The human HeLa cDNA library (Clontech) was cloned into pGAD GH. cDNA insert size range was between 0.4 and 2.0kb (average 1.5kb). 6×10^6 independent clones within the library formed fusion proteins with the GAL-4 activation domain. The library was supplied transformed into *E.coli* DH10B strain which was then amplified before the yeast-two-hybrid screen could be carried out.

Calculation of the titre of the cDNA library.

The titre of the library was determined by dilution of the *E. coli* library stock, and the number of cfu/ml of stock calculated. 1µl of the original stock was diluted in 1ml of 2xTY broth (Diln.A = 10^{-3}). The sample was vortexed and further dilutions of 10^{-6} , and 10^{-7} prepared (Diln.B and C respectively).

1µl of the dilution A sample was mixed with 50µl of 2xTY broth and plated on 2xTY plates supplemented with ampicillin. 50µl and 100µl of dilutions B and C samples, and a further 200µl of dilution C sample were plated onto 2xTY containing ampicillin. The plates were then incubated at 37°C for 18 hours and the numbers of colonies noted. The titre in cfu/ml was then calculated.

Amplification of the cDNA library.

The quantity of library stock required to provide twice the number of independent clones required for a valid screen was calculated ($6 \times 10^6 \times 2 = 1.2 \times 10^7$).

Therefore 1.2µl of the library stock (1×10^{10} cfu/ml) was diluted into a total volume of 24mls of 2xTY media with ampicillin. This was plated onto 2xTY agar, containing ampicillin, in 100µl aliquots which after overnight incubation at 37°C, generated near confluent growth (20,000-50,000 colonies /150mm plate). The colonies were scraped into 2 litres of LB and incubated for 2-4 hours with shaking at 200rpm. The plasmid DNA was then harvested using the standard CsCl protocol described earlier in section 2.3.9., and the quantities of DNA calculated.

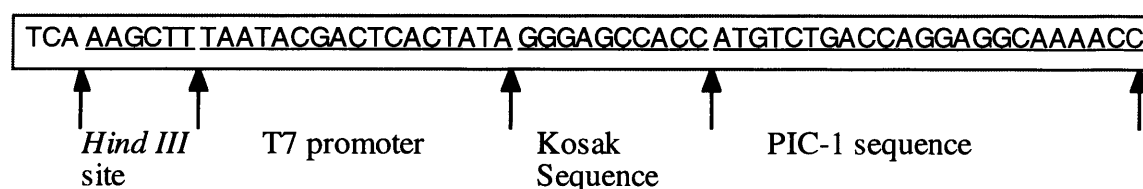
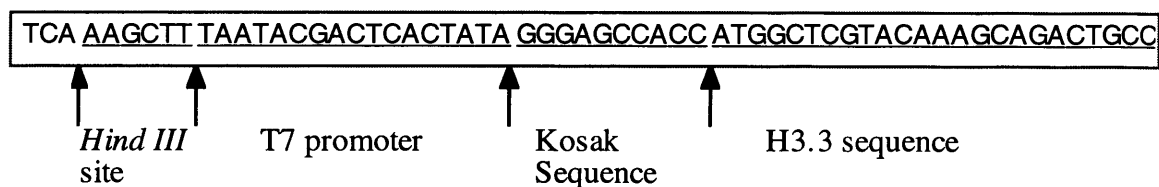
2.5.6 Primers.

The primers highlighted below in Figure 2.1 were used for PCR amplification of the cDNA library insert. The sequencing primers for the AD/library plasmid was used for some PCR amplifications as well as automated sequencing are highlighted in Table 2.10.

Table 2.10. DNA Primers used for the Sequencing of the AD/library cDNA insert.

Primer	Sequence
f/ward AD sequencing	TACCACTACAAATGGATG
o-SR03 (f/wd AD PCR)	CTATTCGATGATGAAGATACCCCAAACCC
o-SR04 (b/wd AD PCR)	GTGAACTTGCGGGGTTTTTCAGTATCTACGAT

Primers listed in Table 2.11 are those used to PCR amplify the positive interaction clones incorporating a T7 promoter region for *in vitro* transcription/ translation. A breakdown of the forward primers, highlighting the T7 promoter and Kosak sequence are shown below.

Figure 2.1.a.Forward primer (oSR05)**Figure 2.1.b.**Forward Primer (oSR07)**Table 2.11.** PCR amplification primers used for the cDNA of specific clones required for *in vitro* transcription/translation protocols

Primer	Sequence
o-F/wd PIC-1(oSR05)	TCAAAGCTTTAATACGACTCAATATAGGGAGCCACCATGTCTGAC CAGGAGGCAAAACC
o-B/wd PIC-1(oSR06)	TGAGGATCCTTATTAACTGTTGAAGTACCCCCCG
o-F/wd H3.3 (oSR07)	TCAAAGCTTTAATACGACTCACTATAGGGAGCCACCATGGCTCGT ACAAAGCAGACTGCC
o-B/wd H3.3(oSR08)	TGAGGATCCTTATTAAAGCACGTTCTCCACGTATGCG

2.5.7 Phenotype Testing.

Frozen stocks of Y190 and CG-1945 (either strain could be used for the screen) were streaked onto pre-warmed YPD plates and incubated at 30°C for 2-3 days until colonies were 2mm in diameter. From these plates, four colonies were then streaked separately onto SD media lacking leucine, tryptophan, histidine and uracil and incubated at 30°C for 5 days to verify the strains nutritional requirements. Verification of each strain was as follows in Table 2.12. A working stock plate was then streaked onto YPD and incubated at 30°C. Plates were then sealed with parafilm and kept at 4°C for up to a month.

Table 2.12. Yeast strain Verification Protocol.

Strain	SD-trp	SD-leu	SD-his	SD-ura	YPD
Y190	-	-	- ^a	+	+
CG-1945	-	-	- ^b	+	+

^a In the presence of 25mM 3-AT

^bIn the presence of 5mM 3-AT

2.5.8 Yeast Media.

YPD Media.

For 1 litre:-

Yeast extract	10g
Peptone	16g

For one litre of broth 950ml of distilled water was added, the pH adjusted to pH 5.8 with glacial acetic acid and the media autoclaved and cooled. 50mls of a 40% glucose solution was filter sterilised (0.2µm acrodisc) and added prior to use.

Synthetic Dropout (SD) Media.

For 1 litre:-

Yeast nitrogen base (Difco)	6.7g
Amino acid dropout supplement (Sigma)	
-ura	0.77g
-leu	0.69g
-trp	0.74g
-his	0.77g
-leu/-trp	0.64g
-leu/-trp/-his	0.62g

For 1 litre of broth 950mls of distilled water was added the pH adjusted to 5.8 and then autoclaved and cooled. Once again 50mls of a sterile 40% glucose solution was added to the media. Any media lacking histidine also contained 3-amino-1,2,4-triazole(3-AT) which was added as a 1M filter sterilised stock, to a final concentration of 25mM for Y190 strain and 5mM for CG-1945 strain.

2.5.9 Bacterial Media.***Minimal Media (for the selection of AD/library plasmids).***

2 x minimal salts.

For 1 litre:-

K ₂ HPO ₄	10.5g
KH ₂ PO ₄	4.5g
(NH ₄) ₂ SO ₄	1.0g
Sodium acetate	0.5g
Mg SO ₄	0.2g

SD Solution.

For 1 litre:-

-leu SD supplement	1.4g
agar (where necessary)	30g

To 1 litre with dH₂O, then pH 7 - 7.4, then autoclave.

Minimal Media Complete.

For 1L:-

2 x minimal salts	500mls
SD solution (+agar)	500mls
20% glucose (filtered)	10ml
Proline (10mg/ml-filtered)	4.06ml
Ampicillin (100mg/ml-filtered)	507.5 μ l
Thiamine -HCL(1M-filtered)	1.015ml

2.5.10 Construction of DNA-BD plasmid.

Full length E3L was excised from pET16b using restriction endonucleases, *Nde I* and *BamHI*. The insert was then ligated into *Nde I/BamHI* cut pAS2-1 and the sequence verified.

2.5.11 Transformation of Yeast Cells.**10 xTE.**

Tris-HCl	100mM
EDTA	10mM
Adjusted to pH 7.5 and autoclaved	

10 x LiAc.

1M LiAc (pH 7.5)

1 x TE/LiAc.

For 100mls :-

10 x TE	10ml
10 x LiAc	10ml
To 100ml with dH ₂ O	

1 X PEG/LiAc.

For 100mls :-

10 x TE	10mls
10 x LiAc	10mls
PEG (Mr 3550)	40g

Volume to 100mls with dH₂O, and solution filter sterilised.

Preparation of Competent Cells (Small Scale).

Once the phenotype of the yeast strain had been verified four individual colonies were inoculated into 4 x 1ml of YPD. This was vortexed briefly to disperse any clumps and then used to inoculate 4 x 50mls of prewarmed YPD. The cultures were incubated overnight at 30°C, 200rpm and harvested when OD₆₀₀ >1.5. A quantity of one of the cultures with the correct 'pink' phenotype was then transferred into 500mls of prewarmed YPD to produce OD₆₀₀ 0.2-0.3. Once again the culture was incubated at 30°C, 200rpm until OD₆₀₀ 0.8. The yeast culture was then divided into six 50ml sterile tubes and centrifuged at 1,000RCF/2500rpm (Sanyo MSE Mistral 3000i) in a swing bucket rotor for 5 minutes. Each of the pellets were washed with 50mls of sterile water, centrifuged and the pellets combined and resuspended in a total of 50mls 1xTE/LiAc. Further centrifugation permitted the final pellet to be resuspended in 1.5ml 1xTE/LiAc.

Preparation of Competent Cells (Library Scale).

Several colonies from the YPD stock plates were inoculated into 4 x 1ml of prewarmed YPD and vortexed. These were then inoculated into 4 x 150mls of prewarmed YPD and incubated overnight at 30°C, 200rpm until OD₆₀₀ >1.5. A quantity of one of the cultures with the correct 'pink' phenotype was transferred to 1 litre of prewarmed YPD to produce OD₆₀₀ 0.2-0.3 and incubated at 30°C, 200rpm. Once OD₆₀₀ 0.8 was reached the culture was divided into sterile 500ml tubes and centrifuged at 2,661RCF/5000 (Sorval RC-5B centrifuge, GS-3 rotor). The pellets were washed with sterile water, centrifuged and then combined and resuspended in a total of 200mls of 1xTE/LiAc. Further centrifugation permitted the cells to be resuspended in 8mls of 1xTE/LiAc.

Small Scale Transformation.

9mls of freshly prepared 1x PEG/LiAc was mixed with 1.5 mls of yeast competent cells. 0.1 µg of each plasmid to be transformed, along with 100µg of herring testes carrier DNA were added to 700µl of the PEG/cell mixture and vortexed for 10 seconds. Following a 30 minute incubation at 30°C with occasional agitation, DMSO was added to 10% and mixed gently by inversion. The cells were heat shocked at 42°C for 15 minutes,

with occasional agitation and then placed on ice. Cells were harvested by centrifugation at 10,000RCF/13,000rpm in a microfuge, the supernatant removed and the pellet resuspended in 0.5ml of 1 x TE.

100µl of transformed cells were incubated on the appropriate SD dropout media along with dilutions of 1:1000, 1:100, and 1:10. 100µl aliquots were also plated onto other SD dropout media to ensure the selection markers for the transformed yeast were as expected.

Before the library screen could be carried out a number of control small scale transformations were carried out to ensure E3L was not toxic to the yeast cell.

Library Scale Transformation

60mls of freshly prepared 1 x PEG/LiAc was mixed by inversion with 8mls of yeast competent cells. 0.2 - 1mg of the DNA-BD/E3L plasmid and 0.1 - 0.5mg AD/library plasmid along with 20 mg herring testes carrier DNA were divided equally and mixed with half of the cell suspension (in two separate vials). The cells were then vortexed and incubated at 30°C for 30 minutes with occasional agitation. DMSO was added to 10%, mixed by inversion and the cells heat shocked at 42°C for 15 minutes. The cells were placed on ice for 5 minutes and harvested by centrifugation at 1,000RCF/2,500rpm (Sanyo, mistral 3000i) at 20°C for 8 minutes. Cell pellets were washed twice with 40mls of sterile dH₂O and resuspended in a final volume of 10mls of 1 x TE.

200µl aliquots were plated onto SD -Trp/His/leu plates (150mm) to select for *HIS3* reporter expression (*his*⁺). 100µl of 1:1000, 1:100 and 1:10 dilutions were plated onto SD-Trp/Leu (100mm) for transformation efficiency controls and 1µl (diluted in 99µl water) plated onto SD-Leu and SD-Trp to determine the transformation efficiency of each individual plasmid. After incubation at 30°C for 3-5 days colonies were counted and the transformation efficiency calculated.

Two independent library screens were undertaken although only one gave rise to any transformants. The cause of the unsuccessful library screen was not found.

Transformation Efficiency.

As mentioned above a number of plates were prepared to ascertain the efficiency of each transformation. The calculation used is as shown below. If two plasmids were transformed simultaneously the concentration of the DNA of the limiting plasmid was used in the calculation(not the sum of the two).

$$\frac{\text{no.cfu} \times \text{total suspension volume}(\mu\text{l})}{\text{Vol.plated}(\mu\text{l}) \times \text{dilution factor} \times \text{amount of DNA used}(\mu\text{g})}$$

2.5.12 Yeast Glycerol Stocks.

Once transformed with the appropriate plasmids glycerol stocks were prepared. A single well isolated colony was taken from the plate and resuspended in 500µl of YPD or the appropriate SD media. The suspension was vortexed vigorously to disperse the cells and sterile 50% glycerol added to a final concentration of 25%. Samples were then stored at -70°C.

2.5.13 β Galactosidase Colony Lift Assay.

Z Buffer.

For 1 litre:-

Na ₂ HPO ₄ ·7H ₂ O	16.1g
NaH ₂ PO ₄ ·H ₂ O	5.5g
KCl	0.75g
MgSO ₄ ·7H ₂ O	0.246g

Adjusted to pH 7.0, autoclaved and stored at room temperature for up to a year.

X Gal Stock Solution.

X-GAL	20mg/ml
Dissolved in 70% DMF, stored at -20°C in the dark.	

Z Buffer/X-GAL Solution.

Z buffer	100mls
β-ME	0.27mls
X GAL stock soln.	1.76mls

Filter Papers

Whatman # 5 grade.

To assay for *LacZ* reporter expression of his⁺ transformants an appropriate sized filter paper was pre-soaked in the Z buffer/X GAL solution. A clean dry filter was placed over the surface of the yeast colonies and holes marked to ascertain the orientation of the filter paper. The filter paper was removed and immediately plunged (colonies facing up) into a pool of liquid nitrogen for 10-15 seconds. The filter paper was then thawed at room temperature and placed colony side up onto the pre-soaked filter paper, then incubated at 30°C from 30 minutes to 8 hours. The appearance of a blue colour indicated the colony to be a *Lac Z* activated positive interacting clone (lacZ⁺). The original plate was re-

incubated to allow re-growth of the lacZ⁺ colony. On some occasions two independent AD/library plasmids are transformed into one yeast cell. This can give rise to false positives and so to eliminate such problems the yeast colonies were re-streaked, incubated at 30°C and assayed once more to ensure only one AD/library plasmid was present in the cell. The control plasmid pCl-1 containing the full length GAL 4 gene was used as a positive control.

2.5.14 Plasmid Preparation from Yeast.

Suspension Buffer

Tris-HCl pH 8.0	10mM
EDTA	1mM
Lyticase (Sigma)	4.5units/μl
Filter sterilise and store at 4°C	

Lysis Buffer

Triton X -100	2%(v/v)
SDS	1%(v/v)
NaCl	100mM
Tris-HCl pH 8	10mM
EDTA	1mM
Filter sterilise and store at room temperature.	

Glass Beads

425-600 microns in diameter, acid washed (Sigma).

A 2cm² patch of yeast was streaked onto the appropriate SD dropout media and incubated for 3-5 days at 30°C. A portion of the patch was then transferred into a 1.5ml tube and 30μl of the suspension solution mixed thoroughly with the pellet. This was then incubated at 37°C for 30 minutes and 170μl of lysis buffer added. The suspension was then transferred to a 1.5ml tube containing 200μl (0.2g) glass beads with 200μl Phenol:chloroform:isoamyl alcohol (25:24:1). The suspension was vortexed at the highest speed for 5 minutes and centrifuged for 10 minutes at 10,000RCF/13,000rpm in the microfuge. The upper aqueous phase was removed and ethanol precipitated. The pellet was washed with 70% ethanol, air dried and resuspended in 20μl water.

Due to the small amount of plasmid DNA recovered (in comparison to the contaminating genomic DNA) the quantity was not measured by A_{260} or visualised on an agarose gel. For further analysis of the plasmid it was necessary to transform into *E.coli*.

2.5.15 Elimination of the DNA-BD/E3L Plasmid.

One of two methods were used to eliminate the DNA-BD/E3L plasmid from the yeast stock so as to allow further analysis of the AD/library cDNA insert.

Transformation of Yeast Plasmid into *E.coli*.

The first method, comprised of a yeast plasmid isolation which was then transformed into *E.coli* HB101. This strain contains a LeuB6 mutation which is complemented by the AD/library plasmid LEU2 marker, therefore once transformed into *E.coli* only the AD/library plasmid is selected for. Due to the small quantities of plasmid DNA isolated from yeast, transformation efficiencies had to be high and so bacterial cells were electroporated and to further aid the transformation, competent cells were grown at 18°C (Chuang *et al* , 1995).

Briefly each individual $his^+ /lacZ^+$ yeast colony was cultured in 3-4 mls SD -leu media for 1 day (where the DNA-BD/E3L plasmid carrying TRP1 was lost at a frequency 10-20%), followed by plasmid isolation from the yeast. 1 μ l of the plasmid solution was transformed into electrocompetent *E.coli* HB101. Cells were incubated for 1 hour at 37°C, 200rpm in 1ml of LB and then washed twice with minimal media and plated onto minimal media supplemented with thiamine, proline and ampicillin and lacking leucine, *see* section 2.5.9. Cells were grown at 37°C for 24 hours and plasmid DNA isolated.

This method however gave rise to a number of colonies which maintained the DNA-BD/E3L plasmid, and so the following protocol was used in preference.

Segregate Plasmids in Yeast by removing -trp selection.

$His^+ /lacZ^+$ colonies were cultured in 3mls SD-leu media for 1-2 days. Cultures were then diluted 1:10, 1:100 and 1:1000 and plated onto SD -leu plates for 3-5 days at 30°C. Of the resulting colonies, 24 were transferred onto orderly grids onto SD-leu and SD-trp/leu plates in duplicate. Colonies that grew on SD-leu alone (Trp auxotrophs) had presumably lost the DNA-BD/E3L plasmid, and were then assayed for *lacZ* reporter expression using a colony lift assay. All those colonies that were positive were discarded as they either contained the DNA-BD/E3L plasmid, or they contained an AD/library plasmid encoding a transcriptional activator that recognises the GAL1 promoter. Yeast colonies that did not express *lacZ* reporter expression were then cultured and the AD/library plasmid isolated.

and transformed into *E.coli* HB101 ready for further analysis or used for yeast mating procedures.

2.5.16 Yeast Mating

Once the DNA-BD plasmid containing E3L had been successfully eliminated from the yeast Y190 strain, each individual *his*⁺/*LacZ*⁺ colony was then mated with strain Y187 of the opposite mating type. Strain Y187 and Y190 were transformed with a number of specific plasmids and then 'mated' together in the combinations shown in Table 2.13.

Table 2.13. Yeast mating protocol and expected resulting phenotype.

Plasmid Strain Y187	Plasmid Strain Y190	Expected <i>Lac Z</i> phenotype	Expected <i>HIS</i> 3 phenotype	Yeast Mating ID
DNA-BD/no insert	AD/no insert	white	no growth	A
DNA-BD/E3L	AD/no insert	white	no growth	B
DNA-BD/no insert	AD/cDNA library	white	no growth	C
DNA-BD/E3L	AD/cDNA library	blue	positive growth	D
DNA-BD/lamin C	AD/cDNA library	white	no growth	E
DNA-BD/p53	AD/T-antigen	blue	positive growth	F

For each candidate AD/library plasmid to be tested, the yeast mating experiment listed in table 1.10 was prepared. The DNA-BD/p53 (murine) and AD/SV40 large T antigen were included as a positive control for the yeast mating procedure. One colony of each mating type was placed in a 1.5ml microfuge tube with 0.5mls YPD and vortexed for 10 seconds. The suspension was then incubated at 30°C with shaking, 200rpm for 18 hours. 10µl aliquots were plated onto SD -trp/leu and SD -trp/his/leu for each individual mating experiment. Cells were incubated for 3-5 days at 30°C and diploids selected. Diploids able to grow on SD dropout -trp/his/leu were then assayed for *Lac Z* reporter expression.

If the results of the assay were not as expected and *Lac Z* reporter expression was observed with the DNA-BD/lamin C and AD/library plasmid, the four plasmids shown below in 2.14 were then mated with each of the candidate AD/ library clones. The 4 cloned plasmids were kindly donated by J.Venables, Dept.of Genetics, University of Leicester

Table 2.14. Yeast mating protocol following the identification of an interaction with lamin C.

Plasmid 1 Strain Y187	Plasmid 2 Strain Y190	Expected <i>Lac Z</i> phenotype	Expected <i>His 3</i> phenotype
DNA-BD/Tra β	AD/cDNA library	white	no growth
DNA-BD/RBM	AD/cDNA library	white	no growth
DNA-BD/Sam68	AD/cDNA library	white	no growth
DNA-BD/pStar	AD/cDNA library	white	no growth

2.5.17 Analysis of Positive cDNA Library Clones.

As discussed in section 2.5.15, AD/library plasmids were transformed into *E.coli* HB101 from which plasmid DNA was isolated. *his*⁺ /*lacZ*⁺ /yeast mating⁺ clones were then analysed by PCR amplification of the cDNA insert using primers, oSR03 and oSR04 and vent polymerase. The procedure used is described in section 2.3.12 with the following cycling parameters,

94°C, 0.5 minutes

60°C, 0.5 minutes

72°C, 1 minute, for 25 cycles and then

72°C , 7 minutes for one cycle.

PCR products were visualised under UV light after electrophoresis of 10% of the reaction through an agarose gel.

After analysis of the products, either the PCR product itself or the cDNA sequence within the plasmid were sequenced using oSR03. Automated sequencing, section 2.3.11 was undertaken as manual sequencing had proven unsuccessful.

2.5.18 Protein Expression in Yeast.

Preparation of the Yeast Cultures.

A single well isolated colony transformed with DNA-BD/E3L was cultured overnight at 30°C, 200rpm in SD -trp media, along with a non-transformed colony, grown in YPD, for use as a negative control. The cultures were vortexed to disperse any clumps and then used as an inoculum for 50mls SD dropout -trp and YPD appropriately. They were then

cultured at 30°C, 200rpm until OD₆₀₀ was 0.4 - 0.6. The cultures were chilled quickly by pouring into two pre-chilled 50ml centrifuge tube per culture filled halfway with ice, prepared from sterile water. The suspension was immediately centrifuged at 1,000RCF/2,500rpm, 4°C for 8 minutes (Sanyo MSE Mistral 300I) and the supernatant containing any unmelted ice discarded. The pellets were combined and washed with 50mls cold sterile water, centrifuged once again and each pellet snap frozen in a dry ice/ethanol bath then stored at -70°C until required.

Urea/SDS Extraction Method.

Cracking Buffer Stock Solution

Urea	8M
SDS	5%(w/v)
Tris-HCl pH 6.8	40mM
EDTA	0.1mM
Bromophenol Blue	Trace

Protease Inhibitor Solution

Pepstatin A	0.1mg/ml
Benzamidine	145mM
Aprotinin	0.37mg/ml

PMSF

Stock Solution	100mM
----------------	-------

Cracking Buffer (Complete)

For 1.13ml :-

Cracking buffer stock	1ml
β-Mercaptoethanol	10μl
Protease Inhibitor Solution	70μl
PMSF	50μl

The complete cracking buffer was prepared and heated to 60°C. The OD₆₀₀ noted previously was multiplied by the volume of the culture to obtain the total number of OD₆₀₀ units (e.g. 0.6 x 55 = 33 total). This figure was then used to asses the amount of cracking buffer used for each cell pellet with 100μl of cracking buffer per 7.5 OD₆₀₀. The cell pellets were thawed by re-suspension in the cracking buffer and then transferred to a 1.5ml tube containing 80μl of glass beads per 7.5 OD₆₀₀ units of cells. The samples were heated at 70°C for 10 minutes and then vortexed for 1 minute. Cell debris was pelleted by

centrifugation at 10,000RCF/13,000rpm for 5 minutes in a microfuge at 4°C and the supernatants transferred it into a 1.5ml tube and placed on ice (1st supernatant). The remaining pellets were placed in a boiling water bath for 5 minutes and vortexed for 1 minute, they were then centrifuged once again and the supernatant collected and placed on ice (2nd supernatant). The pellet fraction and the combined supernatants were boiled briefly and stored at -70°C until required. Throughout the above procedure PMSF was added 1µl/100µl cracking buffer every 7 minutes.

TCA Extraction Method.

TCA

20% w/v TCA in water. Stored at 4°C

TCA Buffer.

Tris-HCl	20mM
Ammonium acetate	50mM
EDTA	2mM
Protease Inhibitor Solution	50µl/ml
PMSF	1mM

SDS/Glycerol Stock.

SDS	7.3%
Glycerol	29.1%(v/v)
Tris-base	83.3mM
Bromophenol Blue	Trace

Tris/EDTA Stock.

Tris -base	200mM
EDTA	20mM

TCA Laemmli Loading Buffer.

For 1ml:-

SDS/Glycerol stock.	480µl
Tris/EDTA stock	400µl
β-Mercaptoethanol	50µl
Protease Inhibitor soln.	20µl
PMSF	20µl

The cell pellets were thawed on ice and resuspended in 100µl TCA buffer per 7.5 OD₆₀₀ and the tubes placed in ice. The suspension was transferred into 1.5ml microcentrifuge tubes containing glass beads and 20% TCA (100µl glass beads and 100µl 20% TCA per 7.5 OD₆₀₀). The cells were vortexed for 4 x 1 minute with cells placed on ice for 30 seconds between each vortex. The supernatant was transferred to a fresh tube (1st supernatant). The glass beads remaining were then washed in 500µl of ice cold 1:1 mixture of 20% TCA and TCA buffer. Cells were then vortexed for a further 2 minutes with 30 seconds on ice between each minute and once again the supernatant removed and mixed with the 1st supernatant and the glass beads with unbroken cells discarded (1st sample). Proteins were pelleted by centrifugation at 10,000RCF/13,000rpm for 10 minutes in a microfuge at 4°C, the supernatant discarded (2nd sample) and the remaining pellet resuspended in TCA-Laemmli loading buffer (10µl per OD₆₀₀ unit of cells). Samples were boiled for 10 minutes and centrifuged at 10,000RCF/13,000rpm for 10 minutes at room temperature the pellet discarded (3rd sample) and supernatants transferred to a fresh tube (4th sample). The four samples were then stored at -70°C until required, although the protein of interest was believed to be present in the fourth sample only.

Protein supernatants were initially electrophoresed on SDS PAGE, and then a western blot was undertaken, using both the alkaline phosphatase detection method and ECL. Dot blots were also used for western blotting procedures in an attempt to enhance the detection of the protein. Briefly, 10µl samples of protein extracts were pipetted directly on the western blotting membrane (Hybond-C-super nitrocellulose) and the western blot protocol followed from the blocking step onward.

2.6 Biochemical Analysis of Protein-Protein Interactions

Once the yeast-two-hybrid screen was complete, *in vitro* verification of the protein-protein interactions were studied.

2.6.1 *In Vitro* Transcription /Translation.

A transcription/translation coupled reticulocyte lysate system, TNT T7 (Promega) was used as recommended by the manufacturers. The expression template was a PCR product with a T7 transcription start site as discussed in section 2.5.6.

The following components were mixed on ice and then incubated at 30°C for 90 minutes.

rabbit reticulocyte lysate	25µl
reaction buffer	2µl
T7 RNA polymerase	1µl
amino acid mix minus methionine	1µl
³⁵ S methionine (10mCi/ml)	4µl
RNasin	1µl
DNA substrate	1µg
DEPC treated water to 50µl.	

5µl of the translation mix was mixed with 15µl water and 20µl SDS loading buffer boiled for 5 minutes and the products analysed by SDS PAGE.

Detection of *in vitro* translated proteins

The quantities of protein produced were too low to be visualised by coomassie staining, and so the ³⁵S methionine labelled products were detected by fluorography. Briefly, the gel was first stained and destained as standard to visualise the MW markers. The gel was then soaked in Amplify (Amersham), a fluorographic agent that is activated by ³⁵S decay, for 30 minutes to increase the sensitivity of detection. The gel was then placed onto Whatmann 3MM paper and dried at 60°C for 1 hour. The gel was exposed to an autoradiography film at -70°C for 12-18 hours.

2.6.2 Bacterial Expression of ³⁵S labelled E3L.

E.coli BL21 expressing HIS-E3L and HIS-E3L Met94-190 were grown in minimal media (section 2.2.1) until OD₅₉₅ 0.7. IPTG was then added to a final concentration of 0.4mM. Following a 30 minute induction, cells were centrifuged, washed and resuspended in minimal media lacking MgSO₄. The MgSO₄ was replaced by an equivalent quantity of MgCl₂ and 20µCi /ml of ³⁵sodium sulphate (NEN). Following a 1 hour induction, a further 20µCi /ml of ³⁵sodium sulphate was added and the cells induced for an additional hour. Cells were then harvested mixed with SDS loading buffer, boiled for 5 minutes and centrifuged for 2 minutes at 10,000RCF/13,000rpm in the microfuge. Proteins were analysed by SDS PAGE and fluorography.

2.6.3 Immune Precipitation.

RIPA Buffer

Tris-HCl (pH 8)	50mM
NaCl	150mM
NP-40	1%
Sodium Deoxycholate	0.5%
SDS	0.1%

High Salt Buffer

NaCl	300mM
NaBr	200mM
NaHPO ₄ pH 7	50mM
NP-40	0.2%
DTT	1mM
PMSF	0.5mM

Low Salt Buffers.

	NaCl	60mM
	NaBr	40mM
or	NaCl	100mM
or	NaCl	200mM
	NaHPO ₄ pH 7	50mM
	NP-40	0.2%
	DTT	1mM
	PMSF	0.5mM

Labelled bacterial cells were resuspended in 200µl, then sonicated in both the RIPA and high salt buffer. The suspension was then centrifuged for 5 minutes at 10,000RCF/13,000rpm in a microfuge, and the supernatant drawn off. Before use the lysate was diluted 1:5 with RIPA buffer, high salt buffer or 50mM NaHPO₄, 1mM DTT, 0.2% NP-40 and 0.5mM PMSF, producing the low salt buffer. Yeast cells which contained the DNA-BD/E3L plasmid were first washed with PBS then resuspended in three cell volumes of ice cold RIPA buffer supplemented with protease inhibitors. An equal volume of glass beads was added and the suspension then vortexed for 4 x 1minute with 30 seconds on ice between each 1 minute vortex. The lysate was then centrifuged at 5,000RCF/10,500rpm for 5 minutes in a microfuge at 4°C and the supernatant drawn off.

To each 50µl bacterial / yeast lysate, 5µl monoclonal hybridoma tissue culture supernatant containing TW2.3 was added and incubated at 4°C for 1-2 hours with gentle agitation. 5µl protein A sepharose (10% slurry) equilibrated in the appropriate buffer was then added and the incubation continued for a further 2 hours. The sepharose beads were spun down for 1 minute and the washed three times in 1ml of the appropriate buffer. SDS loading buffer was added to the beads and the sample boiled for 5 minutes before resolution by SDS PAGE and visualisation by fluorography. On occasions a negative control antibody was used, gp120 reactive against the HIV-1 gp120 protein (Thiriart *et al.*, 1989).

Some of the reactions were pre-incubated (pre-cleared) with various substrates to prevent any fortuitous interactions between the protein and the sepharose. 50µl protein lysate was mixed with 5-10µl of protein A sepharose beads or 5-10µl protein A insoluble *S.aureus* suspension. These were then incubated at 4°C with agitation for 2 hours, then centrifuged and the pre-cleared lysate drawn off and mixed with fresh protein A sepharose. Blocking procedures were also undertaken to saturate non specific binding sites. 5µl (10% slurry) of protein A sepharose was incubated with BSA (2mg/ml) or 5µl of an unlabelled *E.coli* lysate (Biorad assay OD₅₉₅ 0.703) for 4 hours at 4°C with agitation. The suspension was then centrifuged at 10,000RCF/13,000rpm in a microfuge the supernatant drawn off and the radiolabelled lysate added to the blocked beads.

2.6.4 Histidine Tag Pull Down Interaction Studies.

Binding Buffer

NaCl	150mM
PMSF	0.5mM
Glycerol	1%(v/v)

with either :-

	HEPES pH7	50mM
or	MOPS pH7	50mM
or	Tris-HCl pH 7	50mM
or	NaHPO ₄ pH7	50mM
or	CHAPS pH7	50mM

Radiolabelled HIS- E3L was expressed in *E.coli* using the induction conditions discussed previously. 5 x 1ml cell pellets were resuspended in 200µl of each of the buffers listed above, in an attempt to optimise binding and protein-protein interactions, lysates were sonicated and centrifuged for 5 minutes at 10,000RCF/13,000rpm in a microfuge. BSA (50µg/µl) was then added to the lysates, in an attempt to stabilise any interactions. 100µl of the lysate was incubated with a 25µl aliquot of pre-equilibrated nickel affinity resin for

30 minutes at room temperature with agitation. After centrifugation the unbound proteins were removed and the resin washed with 2 volumes of binding buffer. The cDNA of the positive interacting clone, from the yeast-two-hybrid screen, was *in vitro* translated and 25µl of the lysate was mixed with the bound E3L and resin for a maximum of 4 hours at room temperature. The resin was washed once again in binding buffer, SDS loading buffer added to the resin, boiled and fractions separated by SDS PAGE and visualised by fluorography.

2.6.5 Poly(rI) Poly(rC) Pull Down Interaction Studies.

Binding Buffer - 1 (Madison *et al.*, 1998)

Tris-HCl pH 7.1	30mM
NaCl	30mM
MgCl ₂	2mM
DTT	1mM
Glycerol	1%(v/v)

Binding Buffer - 2 (Sierra *et al.*, 1995).

HEPES pH7	25mM
EDTA	0.1mM
KCl	40mM
DTT	1mM
Glycerol	10%(v/v)

HIS-E3L labelled lysates were expressed in *E. coli* and 100µl fractions centrifuged and resuspended in 200µl of each of the above buffers. The cells were then sonicated, centrifuged and the lysate drawn off and the volume made up to 1000µl with the appropriate buffer. The cDNA of the positive interacting clone, from the yeast-two-hybrid screen, was *in vitro* translated and 25µl of the lysate was pre-incubated with 25µl of radiolabelled HIS-E3L on ice, and at room temperature for a variety of time points. A number of 25µl aliquots of poly(rI)(rC) beads were then equilibrated in the appropriate buffer and mixed with the pre-incubated lysates from the two temperature conditions and the two individual proteins with no pre-incubation. The beads and lysates were incubated at room temperature for 30 minutes with gentle agitation. Following centrifugation the beads were washed 3 times with 2 volumes of binding buffer. The beads were then resuspended in 25µl SDS loading buffer and the proteins separated by SDS PAGE and visualised by fluorography.

2.6.6 Crosslinking of Interacting Proteins

The cDNA of the positive interacting clone, from the yeast-two-hybrid screen, was once again *in vitro* translated and incubated with HIS-E3L in the presence of 0.01% glutaraldehyde. Both labelled (^{35}S) and unlabelled HIS-E3L was expressed in *E. coli* and solubilised in 50mM NaHPO_4 , 100mM NaCl, 1mM DTT, 1% glycerol and 50mM PMSF, and ^{35}S labelled and unlabelled forms of the positive interacting clones were produced by *in vitro* transcription/translation. Glutaraldehyde was added to a final concentration of 0.01% and the sample then incubated at room temperature for 30 minutes. 25 μl of the unlabelled form of HIS-E3L was incubated with 25 μl of the labelled form of the positive interacting clone, and 25 μl of the labelled form of HIS-E3L was incubated with 25 μl of the unlabelled form of the positive interacting clone. Control reactions of each ^{35}S labelled protein alone was also undertaken. Samples were then mixed with SDS loading buffer, boiled and separated by SDS PAGE and visualised by fluorography.

2.6.7 Nuclear Extraction Procedure.

Buffer A

HEPES pH 7.9	10mM
MgCl_2	1.5mM
KCl	10mM
DTT	0.5mM

Buffer B

HEPES pH7.9	20mM
MgCl_2	1.5mM
NaCl	420mM
Glycerol	25%(v/v)
EDTA	0.2mM
PMSF	0.5mM
DTT	0.5mM

8×10^6 of C8166, a human T-lymphoblastoid cell line, were provided by B.Maddison in pellet form (fresh from culture). The cells were first incubated in 5mls of ice cold PBS for 10 minutes at 4°C and centrifuged at 10,000RCF/13,000rpm in a microfuge for 5 minutes. The cells were then resuspended in 5mls of buffer A, homogenised, then centrifuged at 10,000RCF/13,000rpm for 10 minutes. The pellet was washed in 500 μl of buffer A, centrifuged once again at 10,000RCF/13,000rpm for 20 minutes, then resuspended in 500 μl buffer B. Nuclear extracts were then stored at -70°C until required.

2.6.8 Affinity Blotting.

Native Gel Electrophoresis.

4 xTris Buffer

Tris-HCl (pH 7.5)	200mM
-------------------	-------

Resolving Gel

17.5 %Gel

For 40mls:-

30% stock acrylamide	23.32mls
4 x Tris Gel Buffer	10mls
Water	6.46mls
10% APS	0.2mls
TEMED	0.02mls

Stacking Gels

For 40mls:-

30% stock acrylamide	6.7mls
4 x Tris gel buffer	10mls
Water	23.08mls
10% APS	0.2mls
TEMED	0.02mls

Running Buffer/Blotting Buffer

Tris-HCl (pH 7.5)	50mM
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Loading Buffer

Sucrose	5%(w/v)
Dissolved in 1 x Tris running buffer	

Renaturation Buffer

NaCl	140mM
Tris-HCl (pH 7.4)	10mM
DTT	2mM
EDTA	2mM
Tween-20	0.1%(v/v)
BSA	1%

Native gels were prepared according to 2.4.3. Protein extracts from (non-radiolabelled) BL21 expressing HIS-E3L and BL21 cells alone, were electrophoresed at 22mA for 18 hours at 4°C and then blotted according to section 2.4.13 onto Hybond -C- Super Nitrocellulose for 90 minutes at 4°C. The membrane was then cut into strips and each strip was incubated in renaturing buffer for 18 hours at 4°C with agitation (Celenza and Carlson, 1991) The strips were then incubated at 4°C for 6 hours with the proteins and nuclear extracts added as shown below in Table 2.15.

Table 2.15. Affinity Blotting Procedure.

Strip Number.	Protein sample electrophoresed and blotted	Protein/nuclear extracts incubated with the strip	Use
1	<i>E. coli</i> BL21 expressing HIS-E3L	Strip underwent ECL western blot analysis	Molecular weight marker of HIS-E3L
2	<i>E. coli</i> BL21 expressing HIS-E3L	³⁵ S labelled protein derived from cDNA clone	Experimental
3	<i>E. coli</i> BL21 expressing HIS-E3L	³⁵ S labelled protein derived from cDNA clone and Nuclear Extracts.	Experimental.
4	<i>E. coli</i> BL21	³⁵ S labelled protein derived from cDNA clone	Negative control
5	<i>E. coli</i> BL21	³⁵ S labelled protein derived from cDNA clone and Nuclear Extracts.	Negative control.

The ³⁵S labelled positive interacting clone (and nuclear extracts) were removed and the membrane strips washed in renaturation buffer for 1 hour, with 3 changes of buffer. The strips were dried and visualised by autoradiography.

3. Results. Protein Expression and Purification.

3.1 Introduction.

One of the aims of my PhD project was to determine the structure of a dsRBM by NMR spectroscopy. As discussed previously dsRBMs are found in a number of proteins of diverse function however, the motifs located within them are highly conserved at the amino acid level and are believed to be structurally similar. At the time the project commenced no structural analysis of these motifs had been reported, although soon after the structural resolution of both RNase III from *E. coli* and the third motif of the *Drosophila* staufen protein were published.

For this particular project the dsRBMs located within PKR and E3L were chosen as targets for study. An *E. coli* based expression system was used to generate the recombinant proteins from the PCR amplified fragments. A PKR gene fragment, encoding amino acids 1-170, PKR Met 1-170, was chosen as it contained both dsRBMs separated by a hinge region. This fragment was particularly useful as it lacked the carboxyl terminus kinase domain which has previously been associated with toxicity with regard to over-expression. A number of E3L gene fragments were produced encoding the full length protein, E3L Met 1-190, an amino terminal deletion product, E3L Met 38-190, and finally a fragment encoding the carboxyl terminus dsRBM alone, E3L Met 94-190. Each of the target proteins were of a size suitable for NMR spectroscopy.

Recombinant expressed proteins were extracted, purified and concentrated in preparation for NMR analysis and structure determination. NMR spectroscopy was undertaken in collaboration with Dr.L-Y.Lian, NMR Centre, MSB, University of Leicester.

3.2 The Expression System.

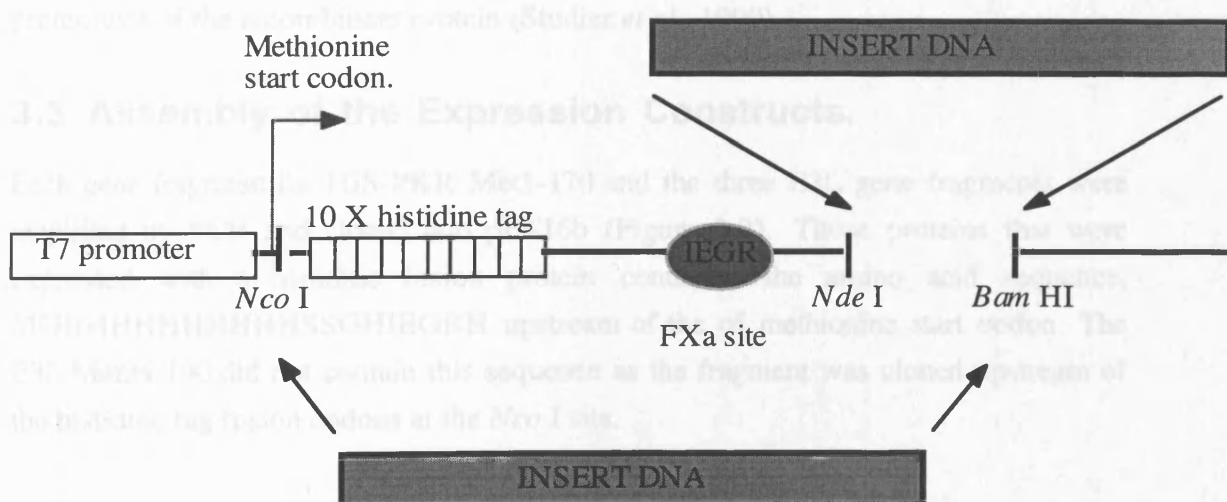
The T7 RNA polymerase system was used to produce high levels of protein expression within *E. coli*.

3.2.1 The Expression Vector.

Insert DNA is cloned downstream of the T7 promoter sequence within the pET16b expression vectors (Novagen). Between the promoter sequence and cloning site is a ribosome binding site, a methionine start codon and 10 histidine codons, which when translated form a histidine tag fusion at the amino terminus of the recombinant protein. Located between the histidine codons and the cloning region are the coding nucleotides for a FXa site, which recognises and cleaves after a IEGR amino acid sequence. This provides a simple cleavage step which removes the histidine tag from the recombinant protein. A translation termination codon is located 19 residues downstream of the cloning site, however a stop codon was incorporated at the carboxyl terminus of the inserts so that the proteins would resemble the natural molecules as closely as possible (Figure 3.1).

The plasmid can also be used for the expression of proteins without a histidine fusion. Insert DNA sequences can be cloned into the *Nco* I site, upstream of the histidine codons, thereby producing an authentic protein (Figure 3.1).

Figure 3.1 Schematic diagram representing the expression plasmid pET16b. The two separate insert DNA fragments are depicted to highlight the production of histidine tag fusion proteins (*Nde* I/*Bam* HI site) and non-histidine tag proteins (*Nco* I/*Bam* HI site).



3.2.2 Histidine Tag Purification.

As discussed in section 3.2.1 when a PCR fragment is cloned into the *Nde I/Bam HI* site, the recombinant protein is expressed as a fusion protein with a histidine tag which can be used for affinity purification. Other purification tag systems such as glutathione-S-transferase (GST) are very large, as compared to the histidine tag, which may affect the behaviour of the recombinant protein. Another significant advantage of the histidine tag system is the ability to purify proteins under denaturing conditions. Because the affinity resin does not rely on the recognition of a specific conformation adopted by the tag under native conditions, denaturing conditions can be applied which often enhance protein solubility.

Most histidine tag purification resins are nickel based, however the resin used in this project was a non-nickel affinity resin (Talon, Clontech), the composition of which is not public knowledge. Once bound to the resin, proteins were eluted using 100-200mM imidazole. On occasions EDTA was used to remove the bound protein, however this strips the resin completely and as such is only used for small scale procedures.

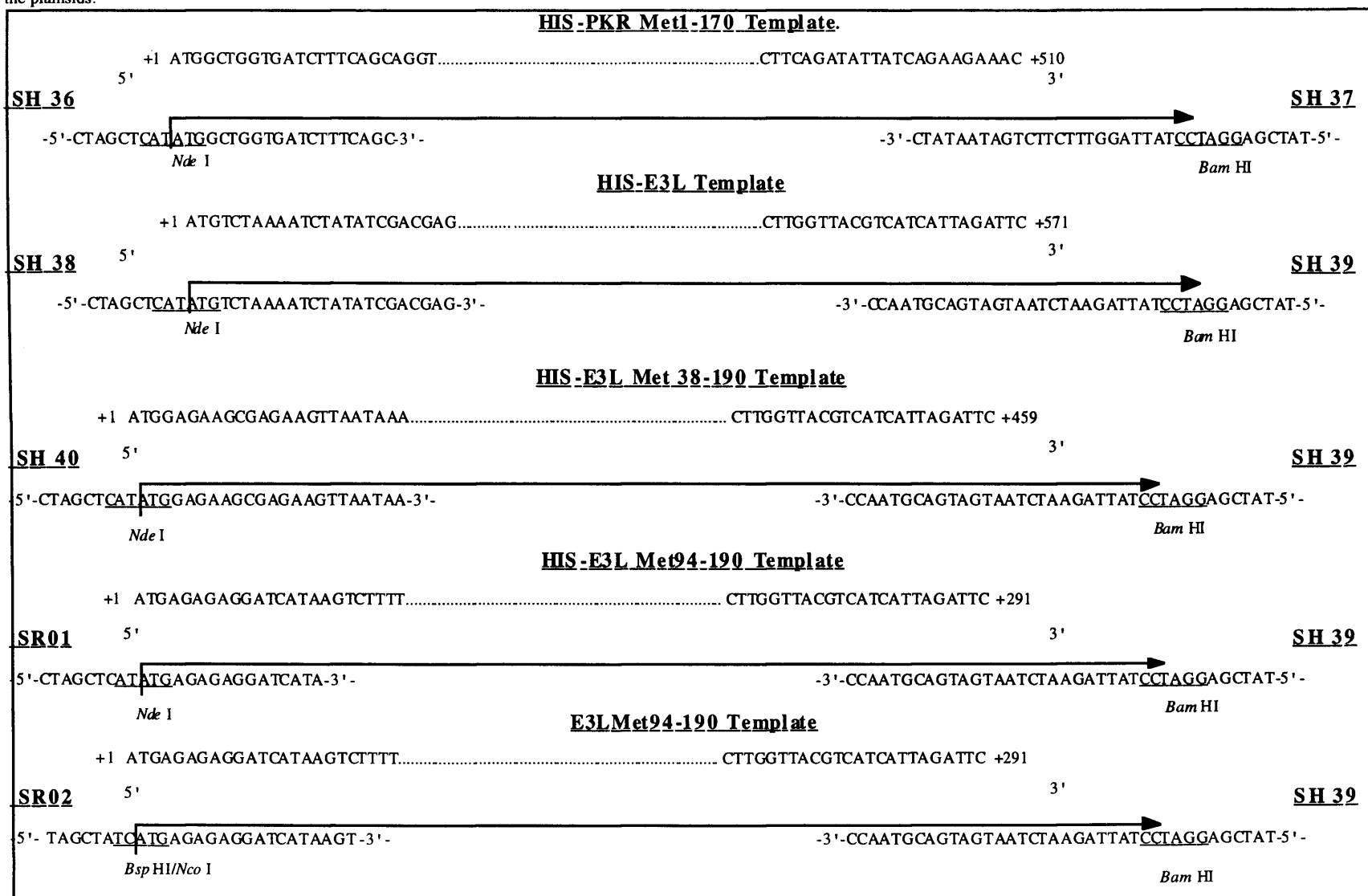
3.2.3 The Host.

The bacterial host is a lysogen of bacteriophage λ DE3. It contains an integrated copy of the phage T7 RNA polymerase gene which is under the control of the inducible *lac UV5* promoter. The addition of IPTG induces the T7 RNA polymerase resulting in transcription of the recombinant clone when growing in culture media. The BL21(DE3) and B834(DE3) strains of *E. coli* were used during expression work and are deficient in both *lon* protease and *ompT*, outer membrane protease, resulting in a reduction of proteolysis of the recombinant protein (Studier *et al.*, 1990).

3.3 Assembly of the Expression Constructs.

Each gene fragment for HIS-PKR Met1-170 and the three E3L gene fragments were amplified by PCR and cloned into pET16b (Figure 3.2). Those proteins that were expressed with a histidine fusion protein contained the amino acid sequence, MGHHHHHHHHHHSSGHIEGRH upstream of the of methionine start codon. The E3L Met94-190 did not contain this sequence as the fragment was cloned upstream of the histidine tag fusion codons at the *Nco I* site.

Figure 3.2. PCR Amplification and Cloning of Gene Products into pET16b for Protein Expression Work. The figure below highlights the primers, restriction sites and templates used to produce the plasmids.



3.3.1 Recombinant dsRBD of PKR

PCR amplification of PKR 1-170 and cloning into pET16b (pSR05), had been undertaken by B.Gale. Sequencing primers oT7, oNB15 and oNB16 were used to manually re-sequence the insert DNA. The sequence was found to be entirely correct (Figure 3.3).

3.3.2 Recombinant E3L.

Three separate E3L constructs were cloned into pET16b. We have experienced problems in this laboratory with a high error rate from non-proof reading DNA polymerases such as Taq. The PCR reactions were therefore carried out with Vent DNA polymerase, a proof reading enzyme which has 3' to 5' exonuclease activity as well as 5' to 3' polymerase activity. Incorrectly paired bases are therefore removed and the final amplified product should be an accurate copy of the original. PCR products, with restriction sites incorporated from the primers, were then digested with *Nde* I and *Bam* HI, gel purified and ligated into an *Nde* I/*Bam* HI digested pET16b plasmid. The dsRBM of E3L (start codon methionine 94) was also cloned into the *Nco* I/*Bam* HI site to produce a non histidine tagged protein. The PCR product was digested with *Bsp* I and *Bam* HI to produce the required overhang for ligation into the *Nco* I/*Bam* HI digested plasmid.

Manual sequencing of the inserts was undertaken using the sequencing primers oSH41, oSH42 and oSH43. All were found to be correct (Figure 3.4). A section of a manual sequencing autoradiogram of the full length E3L insert using oSH41 is also highlighted (Figure 3.5).

Figure 3.3. Nucleotide and amino acid usage of PKR 1-170. The annealing sites for sequencing primers, are highlighted by arrows above the corresponding nucleotide sequence.

————→ **T7 primer (upstream)**

```

ATGGCTGGTGATCTTTCAGCAGGTTTCTTCATGGAGGAACTTAATACATAC
CGTCAGAAGCAGGGAGTAGTACTTAAATATCAAGAACTGCCTAATTCAGG
ACCTCCACATGATAGGAGGTTTACATTTCAGTTATAATAGATGGAAGAGA
ATTTCAGAAAGGTGAAGGTAGATCAAAGAAGGAAGCAAAAAATGCCGCAG
CCAAATTAGCTGTTGAGATACTTAATAAGGAAAAGAAGGCAGTTAGTCCTT
TATTATTGACAACAACGAATTCTTCAGAAGGATTATCCATGGGGAATTACA
TAGGCCTTATCAATAGAATTGCCCAGAAGAAAAGACTAACTGTAAATTATG
AACAGTGTGCATCGGGGGTGCATGGGCCAGAAAGGATTTCAATTATAAATGC
AAAATGGGACAGAAAGAATATAGTATTGGTACAGGTTCTACTAACAGGA
AGCAAAACAATTGGCCGCTAACTTGCATATCTTCAGATATTATCAGAAGA
AACCTAATAG
  
```

oNB15

oNB16

Amino Acid Sequence PKR 1-170

```

MAGDLSAGFFMEELNTYRQKQGVLKYQELPNSGPPHRRFTFQVIIDGREF
PEGEGRSKKEAKNAAKLAVEILNKEKKAVSPLLLTTNSSEGLSMGNYIGLI
NRIAQKKRLTVNYESQASGVHGPGEFHYKCKMGQKEYSIGTGSTKQEAKQL
AAKLAYLQILSEETZZ
  
```

Figure 3.4. Nucleotide and Amino Acid usage of the three E3L protein constructs. The annealing sites for sequencing primers, are highlighted by arrows above the corresponding nucleotide sequence. The start codons for each of the three constructs expressed are depicted by **ATG** and **M** in the nucleotide and amino acid sequence respectively.

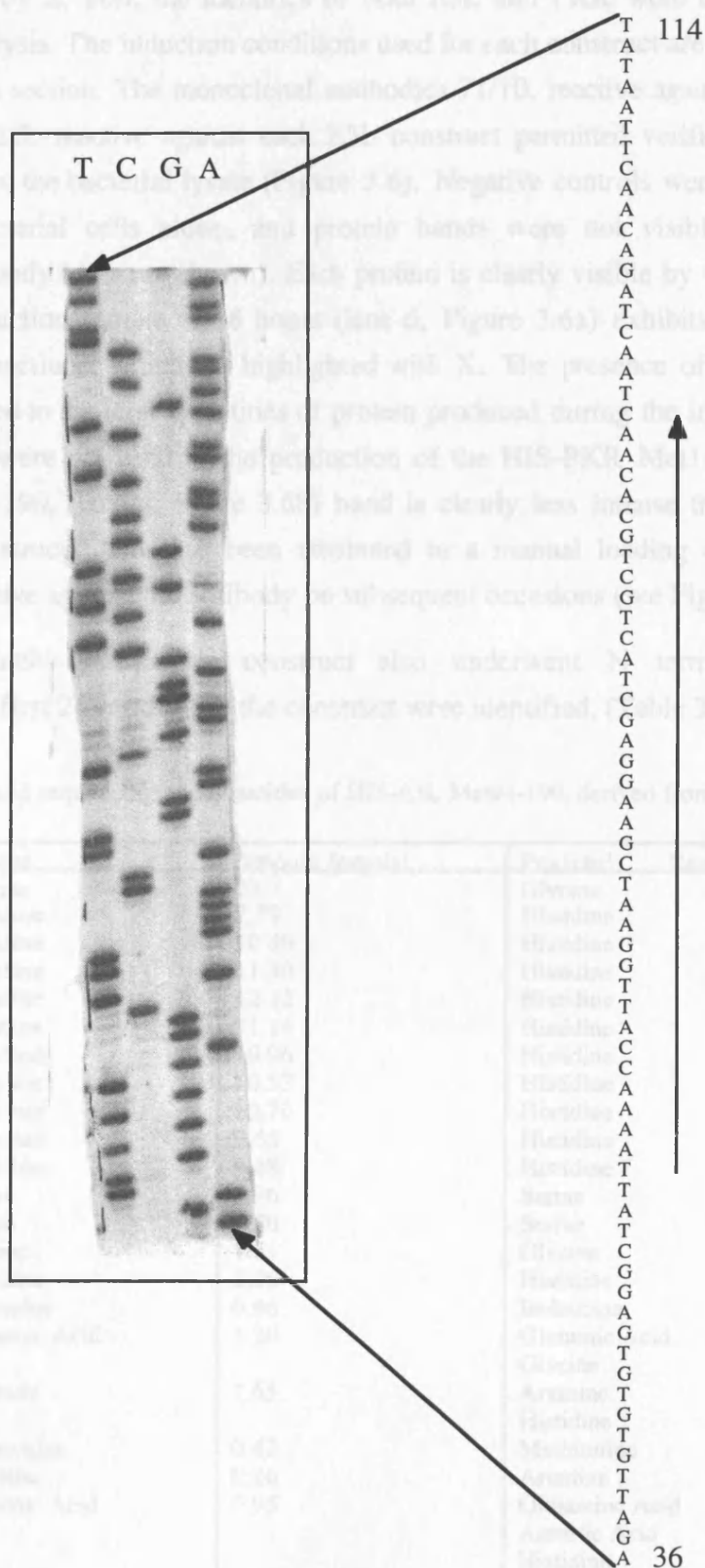
—▶ **oSH41 (upstream)**

ATGTCTAAAATCTATATCGACGAGCGTTCTAACGCAGAGATTGTGTGTGA
 GGCTATTAAAACCATTTGGAATCGAAGGAGCTACTGCTGCACAACCTAACTA
 GACAACCTTAATA**ATG**GAGAAGCGAGAAGTTAATAAAGCTCTGTACGATCTT
 —▶ **oSH42**
 CAACGTAGTGCTATGGTGTACAGCTCCGACGATATTCCTCCTCGTTGGTTT
 ATGACAACGGAGGCGGATAAGCCGGATGCTGATGCTATGGCTGACGTCAT
 AATAGATGATGTATCCCGCGAAAAATCA**ATG**AGAGAGGATCATAAGTCTT
 TTGATGATGTTATTCCGGCTAAAAAAATTATTGATTGGAAAGGTGCTAACC
 —▶ **oSH43**
 CTGTCACCGTTATTAATGAGTACTGCCAAATTACTAGGAGAGATTGGTCTT
 TTCGTATTGAATCAGTGGGGCCTAGTAACTCTCCTACATTTTATGCCTGTGT
 AGACATCGACGGAAGAGTATTCGATAAGGCAGATGGAAAATCTAAACGAG
 ATGCTAAAAATAATGCAGCTAAATTGGCAGTAGATAAACTTCTTGGTTACG
 TCATCATTAGATTCTAA

Amino Acid Sequence of E3L

MSKIYIDERSNAEIVCEAIKTIGIEGATAAQLTRQLN**M**EKREVNKALYDLQRS
 AMVYSSDDIPPRWFMTTEADKPDADAMADVIIDDVSREKS**M**REDHKSFDD
 VIPAKKIIDWKGANPVTVINEYCQITRRDWSFRIESVGPSNSPTFYACVDIDGR
 VFDKADGKSKRDAKNNAAKLAVDKLLGYVIIRFZ

Figure 3.5. Section of a manual sequencing autoradiogram for full length E3L cloned into pET16b. Nucleotides 36-114 are shown.



3.4 Protein Expression and Purification.

Once expressed by *E. coli*, the identities of both E3L and PKR were confirmed by western blot analysis. The induction conditions used for each construct are discussed in detail later in this section. The monoclonal antibodies 71/10, reactive against PKR Met 1-170, and TW2.3, reactive against each E3L construct permitted verification of the proteins present in the bacterial lysate (Figure 3.6). Negative controls were undertaken using BL21 bacterial cells alone, and protein bands were not visible with each monoclonal antibody (data not shown). Each protein is clearly visible by western blot, however the induction sample of 18 hours (lane d, Figure 3.6a) exhibits a degree of background interactions, which are highlighted with X. The presence of these bands has been attributed to the large quantities of protein produced during the induction, and such conditions were not used in the production of the HIS-PKR Met1-170 protein. The E3L Met94-190, (lane d, figure 3.6b) band is clearly less intense than the other E3L protein constructs. This has been attributed to a manual loading error, as the protein was reactive against the antibody on subsequent occasions (see Figure 3.12.5).

The HIS-E3LMet94-190 protein construct also underwent N terminal protein sequencing. The first 26 residues of the construct were identified, (Table 3.1).

Table 3.1. Amino acid sequencing and quantities of HIS-E3L Met94-190, derived from pSR03.

Cycle	Residue.	Amount (pmols).	Predicted Residues
1	Glycine	23.7	Glycine
2	Histidine	7.59	Histidine
3	Histidine	10.49	Histidine
4	Histidine	11.30	Histidine
5	Histidine	12.12	Histidine
6	Histidine	11.14	Histidine
7	Histidine	10.96	Histidine
8	Histidine	10.53	Histidine
9	Histidine	10.70	Histidine
10	Histidine	9.65	Histidine
11	Histidine	9.68	Histidine
12	Serine	3.96	Serine
13	Serine	1.50	Serine
14	Glycine	1.11	Glycine
15	Histidine	2.16	Histidine
16	Isoleucine	0.96	Isoleucine
17	Glutamic Acid.	1.20	Glutamic Acid.
18	?		Glycine
19	Arginine	1.65	Arginine
20	?		Histidine
21	Methionine	0.42	Methionine
22	Arginine	0.26	Arginine
23	Glutamic Acid	0.95	Glutamic Acid
24	?		Aspartic Acid
25	?		Histidine
26	Lysine	0.16	Lysine

Figure 3.6.A Recombinant HIS-PKR Met1-170 identification by western blot analysis

A. Western blot of induced bacterial cell pellets transformed with pSRO5, expressing HIS-PKR Met1-170. The alkaline phosphatase method was used with the monoclonal antibody 71/10. The MW markers are highlighted on the left side of the figure.

a-d = induced cell pellet expressing HIS-PKR Met1-170 after 1, 2, 4 and 18 hour inductions.

3.6 B. Recombinant E3L identification by western blot analysis.

B. Western blot of induced bacterial cell pellets transformed with pSR01-4, expressing each recombinant VV E3L protein fragment. The alkaline phosphatase method was used, with the monoclonal antibody TW2.3. The MW markers are highlighted on the left side of the figure.

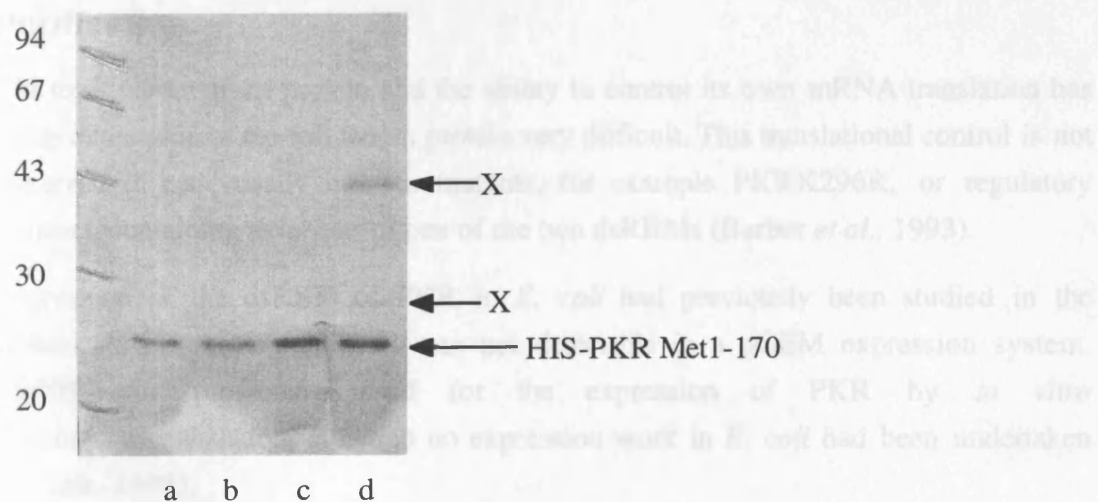
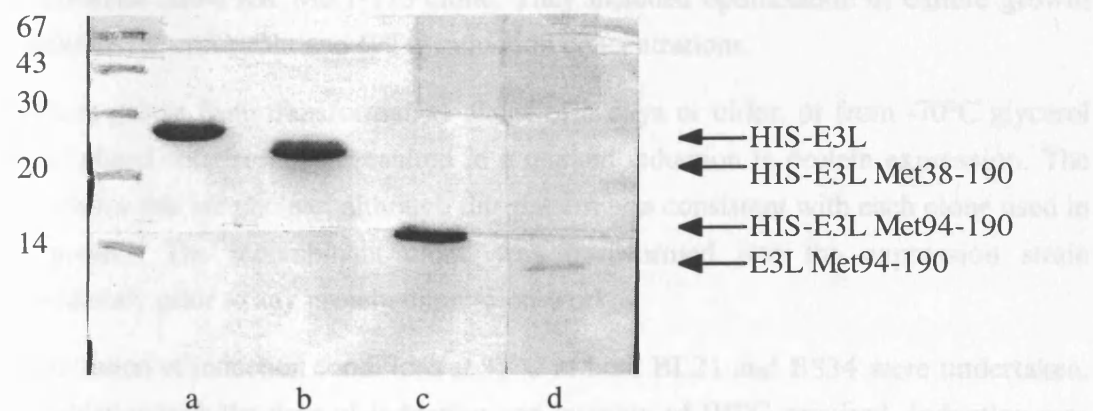
a=HIS-E3L.

b=HIS-E3L Met38-190.

c=HIS-E3L Met94-190

d=E3LMet94-190.

The protein sample loaded into Lane d required a longer exposure time and so the gel was cut to prevent the over exposure of the proteins in the other lanes .

Figure 3.6 (A) Recombinant HIS-PKR Met1-170 Expression and**Figure 3.6(B)**

3.4.1 Recombinant HIS-PKR Met1-170 Expression and Purification.

The toxic nature of the protein and the ability to control its own mRNA translation has made expression of the full length protein very difficult. This translational control is not observed in catalytically inactive mutants, for example PKRK296R, or regulatory mutants, containing deletions of one of the two dsRBMs (Barber *et al.*, 1993).

Expression of the dsRBD of PKR in *E. coli* had previously been studied in the laboratory. Expression of PKR was not detectable in a pGEM expression system. pSR05 was previously used for the expression of PKR by *in vitro* transcription/translation, although no expression work in *E. coli* had been undertaken (B.Gale, 1997).

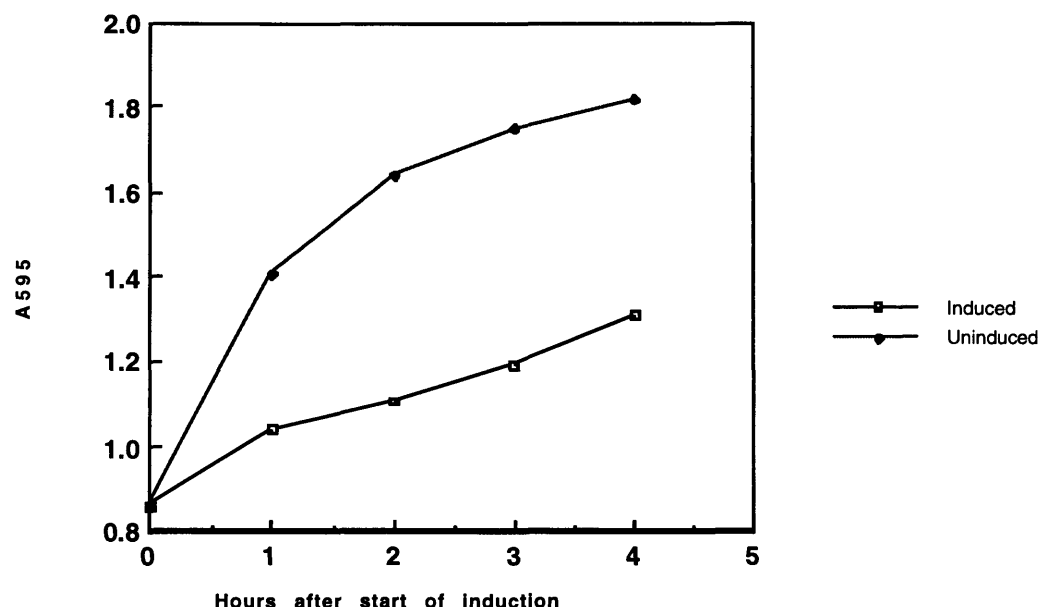
Optimising Induction Conditions of recombinant HIS-PKR Met 1-170 in *E. coli*.

A number of growth conditions were tested to maximise the expression of the recombinant HIS-PKR Met1-170 clone. They included optimisation of culture growth conditions, *E. coli* strain and IPTG induction concentrations.

Cultures grown from transformation plates of 5 days or older, or from -70°C glycerol stocks plated onto fresh agar resulted in a marked reduction in protein expression. The reasons for this are unclear, although this pattern was consistent with each clone used in the project. The recombinant clone was transformed into the expression strain immediately prior to any protein expression work.

Optimisation of induction conditions at 37°C in both BL21 and B834 were undertaken, investigating both the time of induction and quantity of IPTG required. Induction was initiated mid log phase when OD₅₉₅ reached 0.7. Western blot analysis demonstrated BL21 produced slightly enhanced levels of the recombinant protein, at a final concentration of 1mM IPTG. The recombinant protein was not visible by coomassie stain when expressed in this cell line. A decrease in growth rate was also observed upon the addition of IPTG suggesting growth inhibition by the recombinant protein (Figure 3.7.1).

Figure 3.7.1. The graph depicts the decrease in growth rate as a result of the addition of IPTG and resultant protein expression.



Due to the basal level of T7 RNA polymerase expression, this system is susceptible to leaky expression of the recombinant clone prior to the addition of IPTG. The co-expression of the natural T7 RNA polymerase inhibitor, T7 lysozyme, can reduce this basal activity. The pLys S plasmid, maintained by the presence of chloramphenicol, contains the T7 lysozyme gene in the reverse orientation to its promoter, thus producing a low level of enzyme. These levels are sufficient to permit the expression of toxic products once the cells have grown to the required cell density and the IPTG is added. A more stringent system, pLys E, places the T7 lysozyme gene in the correct orientation, producing increased levels of enzyme but reducing the expression of the recombinant protein.

pSR05 was therefore transformed into BL21pLys S cells and the level of recombinant protein expression compared. Western blot analysis of the two strains revealed an increased level of recombinant protein expression in BL21pLys S, and a reduction in leaky expression as no band was visible in the uninduced sample (Figure 3.7.2). The induction of the recombinant protein was also now visible by coomassie blue staining (Figure 3.7.3). The second band observed is believed to be lysozyme which eliminated leaky expression.

Figure 3.7 HIS-PKR Met1-170 Expression and Purification

All SDS PAGE analysis was undertaken on 12% polyacrylamide gels under reducing conditions. The MW markers are highlighted on the left side of each figure.

Figure 3.7.2. Induction Optimisation of Recombinant HIS-PKR Met1-170.

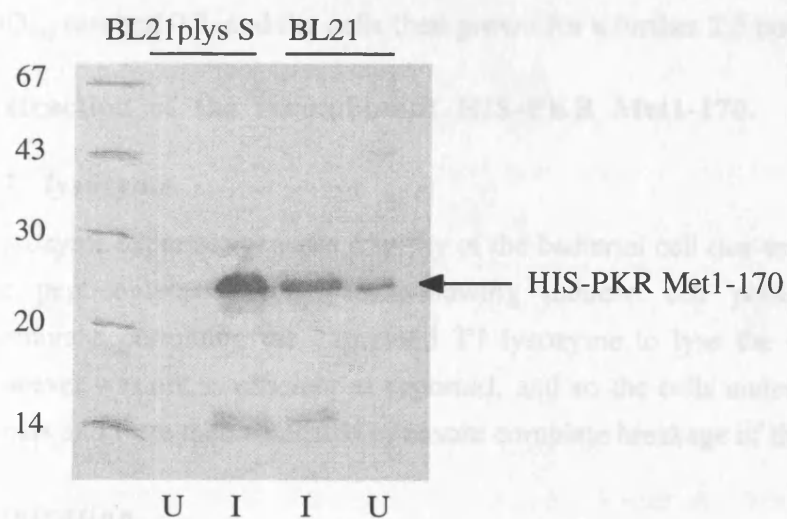
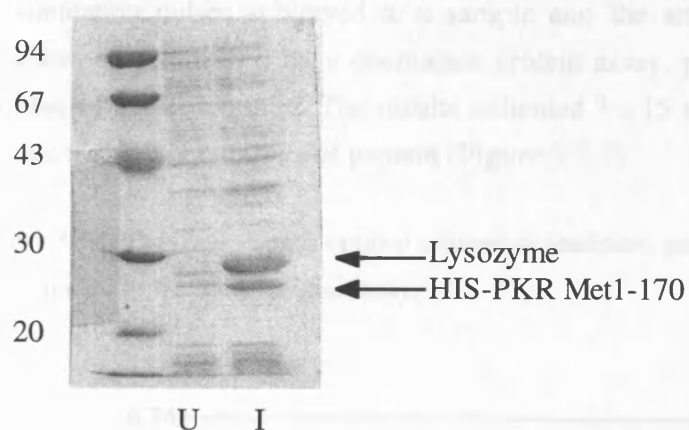
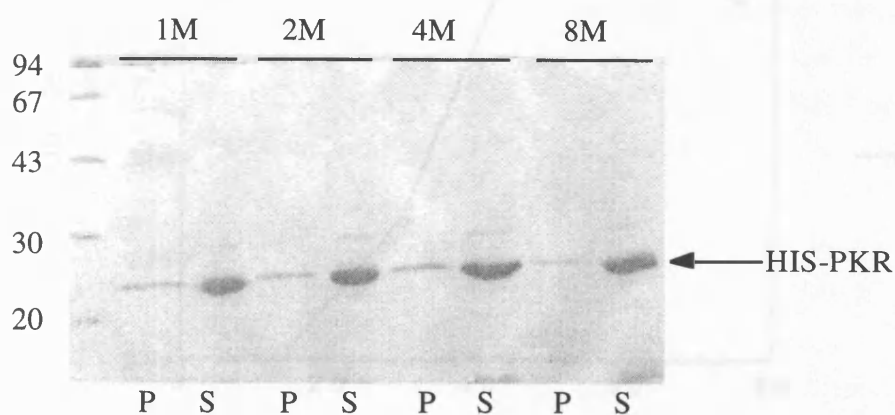
Western blot analysis of *E. coli* BL21 and BL21pLys S induction with 1mM IPTG added mid growth phase. Extracts were prepared 2 hours post IPTG induction. I-induced, U-uninduced.

Figure 3.7.3. Induction Optimisation.

Expression of HIS-PKR visible by coomassie blue stain. The protein was expressed in *E. coli* BL21 pLys S, and extracts were prepared as described above.

Figure 3.7.4. Solubility of the Recombinant Protein.

Extraction of the recombinant protein by sonication, in 50mM Tris-HCl, 100mM NaCl, 1mM β -Mercaptoethanol, and 0.5mM PMSF extraction buffer supplemented with increased concentrations of urea (1M - 8M). The concentrations of urea and the P-pellet and S-supernatant fractions are highlighted in the figure.

Figure 3.7.2.**Figure 3.7.3.****Figure 3.7.4.**

Under the induction conditions examined, optimal expression of recombinant HIS-PKR Met1-170 was observed in BL21 pLys S. 1mM IPTG was added mid log phase when OD₅₉₅ reached 0.7, and the cells then grown for a further 2.5 hours at 37°C.

Extraction of the recombinant HIS-PKR Met1-170.

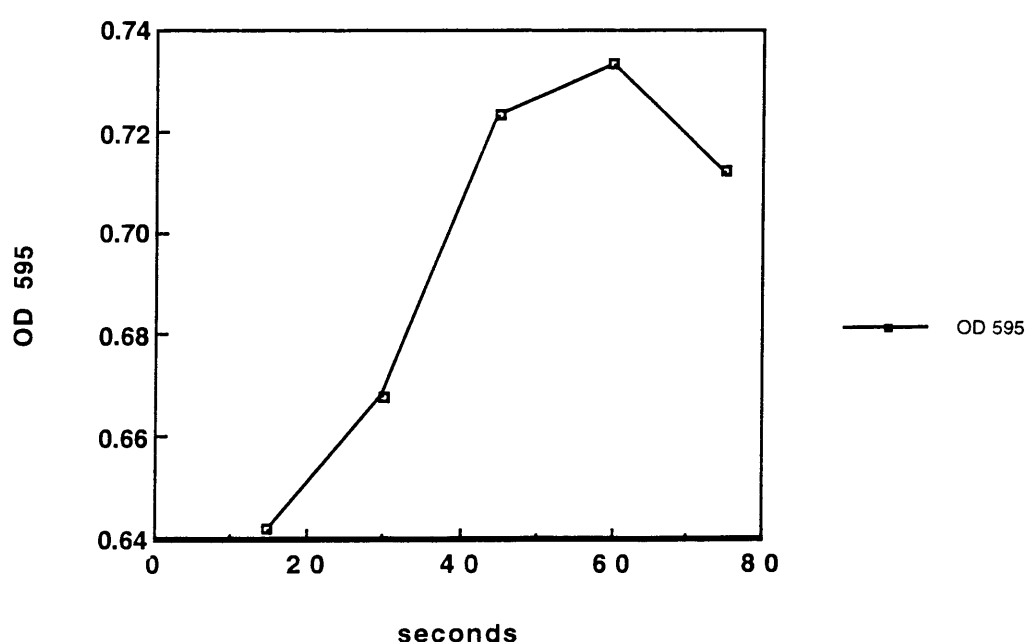
T7 lysozyme.

Lysozyme expression causes fragility of the bacterial cell due to the partial digestion of the peptidoglycan layer. Freeze/thawing induced cell pellets disrupts the inner membrane permitting the expressed T7 lysozyme to lyse the cells. This lysis event however was not as efficient as reported, and so the cells underwent three freeze/thaw cycles and were then sonicated to ensure complete breakage of the cells.

Sonication.

To ensure efficient lysis of the bacterial cells optimal sonication conditions were determined using a small scale sonication protocol. A direct comparison of the number of sonication pulses subjected to a sample and the amount of protein released into solution, as determined by a coomassie protein assay, provided data to determine the number of pulses required. The results indicated 3 x 15 second pulses were required to extract maximal quantities of protein (Figure 3.7.5).

Figure 3.7.5. The graph depicts optimal sonication conditions used to extract the expressed protein, as determined by the Biorad protein assay.



Solubility of the Recombinant Protein.

Conditions for maximal protein extraction and solubility were determined utilising small scale sonication protocols using a number of 1.5mls induced cell pellets. The soluble and insoluble fractions were then analysed by western blot analysis (Figure 3.7.4). Work had been undertaken to extract the protein in Tris-HCl, pH 7.5, extraction buffer containing no denaturant, however the protein was not solubilised in this buffer (data not shown). A significant amount of the protein was soluble in a 1M urea Tris-HCl, pH7.5, buffered extraction solution. This was a significant advantage for structural determination, as the sample was not required to undergo complex renaturation procedures.

Extraction Procedure.

The induced cell pellets were resuspended in 5-10 times the volume of the cell pellet in a Tris-HCl (pH7.5) extraction buffer, supplemented with 1M urea. The cells were then flash freeze-thawed for 3 cycles and sonicated 3 times on iced water for 15 seconds (7 microns). The extracts were then centrifuged and the supernatant drawn off in preparation for histidine tag purification. All procedures were carried out at <8°C.

Histidine Tag Purification.

The crude supernatant was mixed with pre-equilibrated Talon affinity resin, incubated for 30 minutes at room temperature, and then washed in extraction buffer supplemented with 10mM imidazole. The bound protein was eluted with extraction buffer supplemented 100mM imidazole in several 5 ml washes. The fractions were then analysed by SDS PAGE (Figure 3.7.6), and the imidazole removed by dialysis. Although a large quantity of the protein had bound to the resin, a proportion of a protein with the exact MW of HIS-PKR Met 1-170 had not bound and was present in the through wash (lane b). This suggested the conformation of the protein may have been such that the affinity tag had folded within the protein making it inaccessible to the resin. SDS PAGE analysis of the eluted protein samples verified that the majority of the cellular contaminating proteins had been removed leaving on average 5mg, in 25mls, of recombinant HIS-PKR Met1-170 from a 2L induction (Figure 3.7.6, lanes c-g).

FXa Cleavage.

PMSF was initially removed from the sample by dialysis. Small scale cleavage experiments were undertaken to ascertain the quantity of enzyme, and length and conditions of the incubation. Incubations were carried out for 18 hours at 4°C. A FXa lab stock solution, 0.86mg/ml, was incubated at quantities ranging from 0.0043 to

Figure 3.7.6. Purification of the Recombinant HIS-PKR Met1-170.

Histidine Tag Purification in 50mM Tris-HCl, 100mM NaCl, 1mM β - Mercaptoethanol, and 0.5mM PMSF supplemented with 1M urea.

a = Crude Extract.

b = Unbound Fraction (1 μ l loading).

c - g = Eluted Bound Fractions, removed with 100mM imidazole (5 μ l loading).

Figure 3.7.7. FXa Cleavage of the Histidine Tag.

0.002mg of recombinant protein was digested for 18 hours at 4°C.

a=0.43 μ g FXa and protein.

b=0.86 μ g FXa and protein.

c=1.29 μ g FXa and protein.

d=1.72 μ g FXa and protein.

e=Uncleaved Protein only.

Figure 3.7.8. Optimal FXa cleavage of the Histidine Tag.

0.002mg of recombinant protein digested for 18 hours at 4°C.

a=0.086 μ g FXa and protein.

b= 0.043 μ g FXa and protein.

c=0.02 μ g FXa and protein.

d=0.01 μ g FXa and protein.

e=0.0086 μ g FXa and protein.

f=Uncleaved -protein only.

Figure 3.7.6.

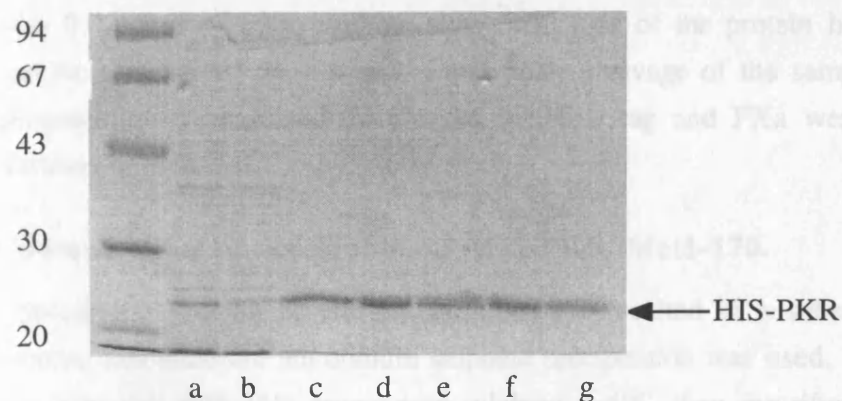


Figure 3.7.7.

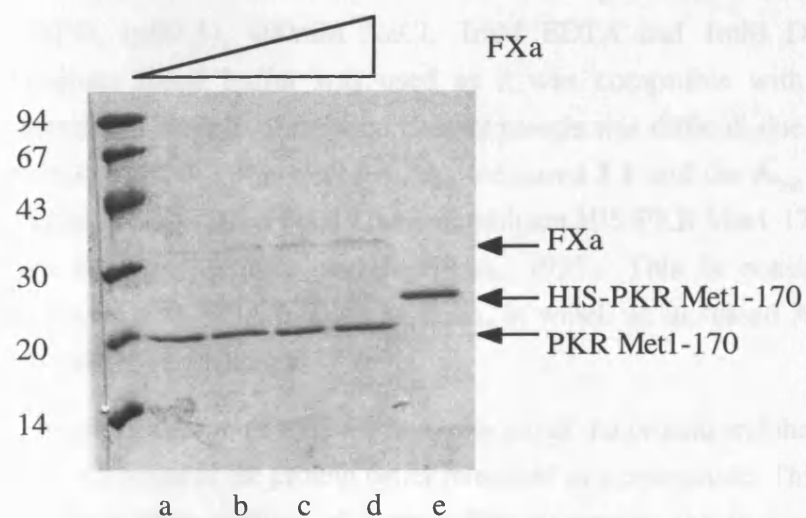
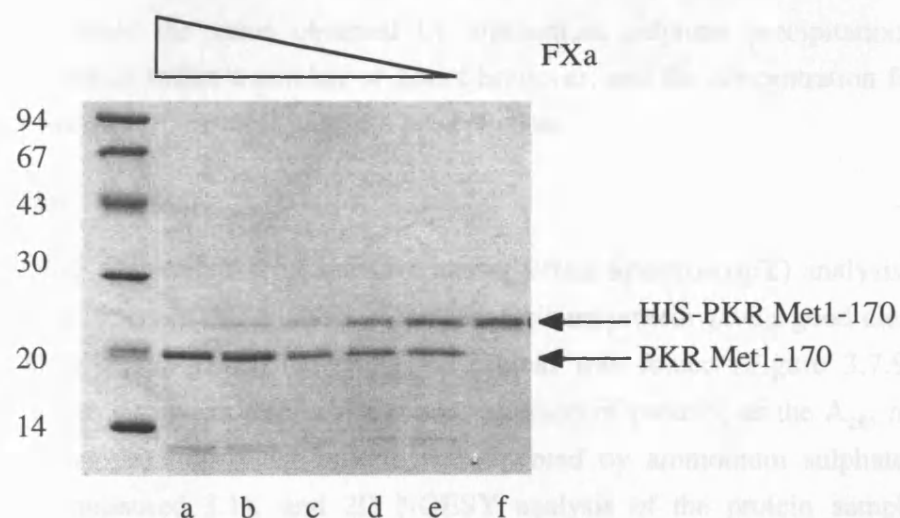


Figure 3.7.8.



1.72 μ g which were diluted in FXa cleavage buffer. The results were observed by SDS PAGE analysis (Figure 3.7.7 and 3.7.8). 0.002mg of recombinant protein was cleaved with 0.0215 μ g of FXa, without substantial loss of the protein highlighting the site specific cleavage of the enzyme. Large scale cleavage of the samples maintained the aforementioned ratios and the cleaved histidine tag and FXa were removed by gel filtration.

Concentration of Recombinant HIS-PKR Met1-170.

Concentration of the protein by ultrafiltration resulted in a substantial loss of the protein, and therefore ammonium sulphate precipitation was used. The protein sample was saturated with 75% ammonium sulphate at 4°C, then centrifuged and the protein pellet resuspended in a small volume of buffer.

The buffer used to resolubilise the recombinant protein pellet was composed of 50mM NaHPO₄ (pH7.5), 400mM NaCl, 1mM EDTA and 1mM DTT (deuterated). The phosphate based buffer was used as it was compatible with NMR spectroscopy. Quantitative analysis of the recombinant protein was difficult due to a high nucleic acid content (<19.2%), however the A₂₈₀ measured 3.1 and the A₂₆₀ measured 5.95. This was attributed to RNA bound the recombinant HIS-PKR Met1-170 which are protected from nucleases (Clarke and Mathews, 1995). This is consistent with laboratory experience with RNA binding proteins, in which an increased A₂₆₀ is not reduced by treatment with nucleases.

Following ammonium sulphate precipitation of the protein and the resolubilisation of the pellet, a portion of the protein pellet remained as a precipitate. This precipitate was then solubilised upon addition of more buffer, suggesting the first sample had reached its solubility maximum. This observation was supported by the use of a stirred cell ultrafiltration device. Following ultrafiltration the protein concentration achieved was three times the value obtained by ammonium sulphate precipitation. The protein precipitated within a number of hours however, and the concentration fell to the value obtained by ammonium sulphate precipitation.

NMR Analysis.

1D and 2D NOESY (Nuclear Overhauser Effect Spectroscopy) analysis of the protein were undertaken. 1D analysis of the recombinant protein gave a good clear spectra with well dispersed peaks, indicating the protein was folded (Figure 3.7.9). The signal however was weak due to a low concentration of protein, as the A₂₈₀ measured 1.75. The concentration of the protein was doubled by ammonium sulphate precipitation, (A₂₈₀ measured 3.1), and 2D NOESY analysis of the protein sample undertaken,

Figure 3.7.9. 1D NMR analysis of HIS-PKR Met1-170. The peaks were well distributed, indicating the protein sample was in a folded conformation and therefore suitable for further analysis. The proton chemical shifts (ppm - parts per million) arising from the chemical environments are highlighted at the bottom of the spectra.

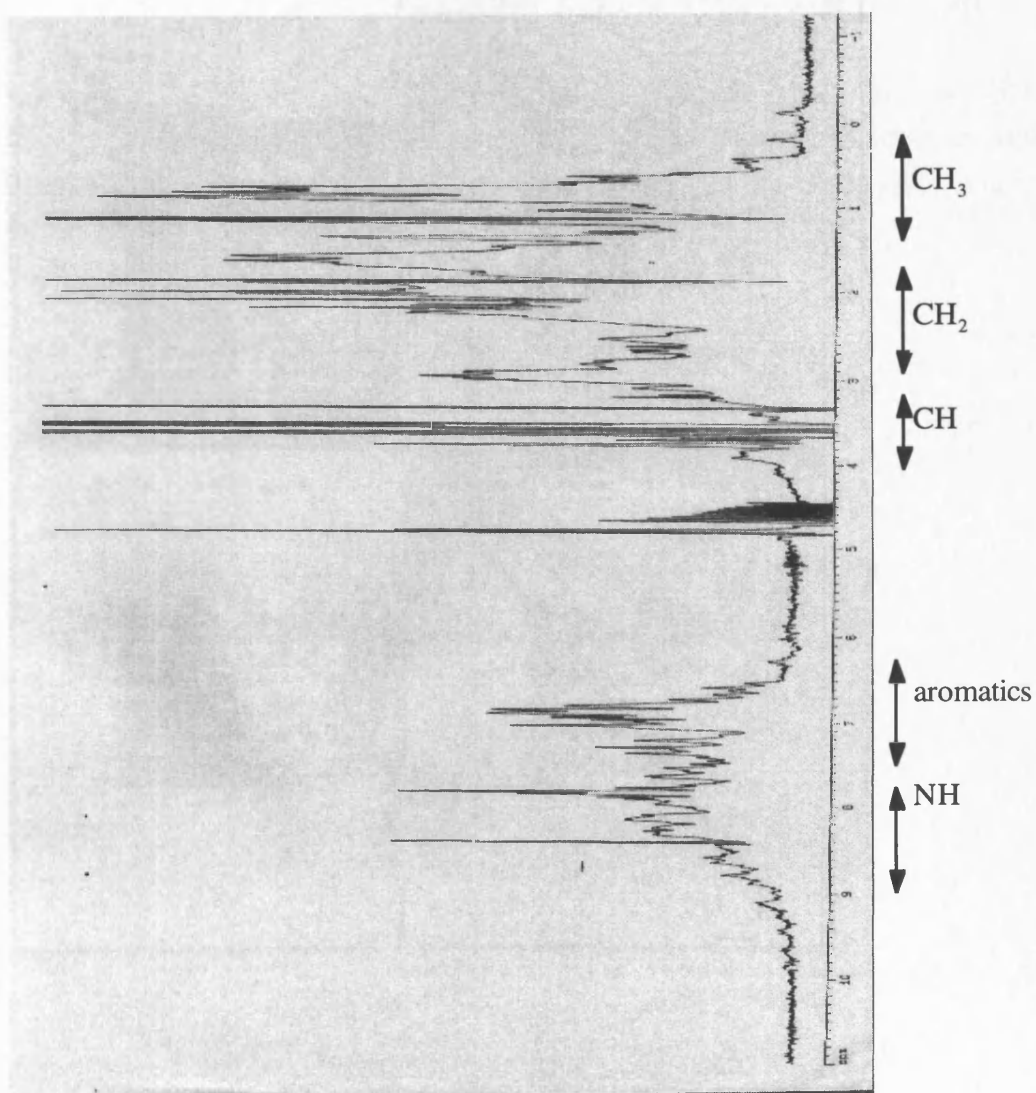
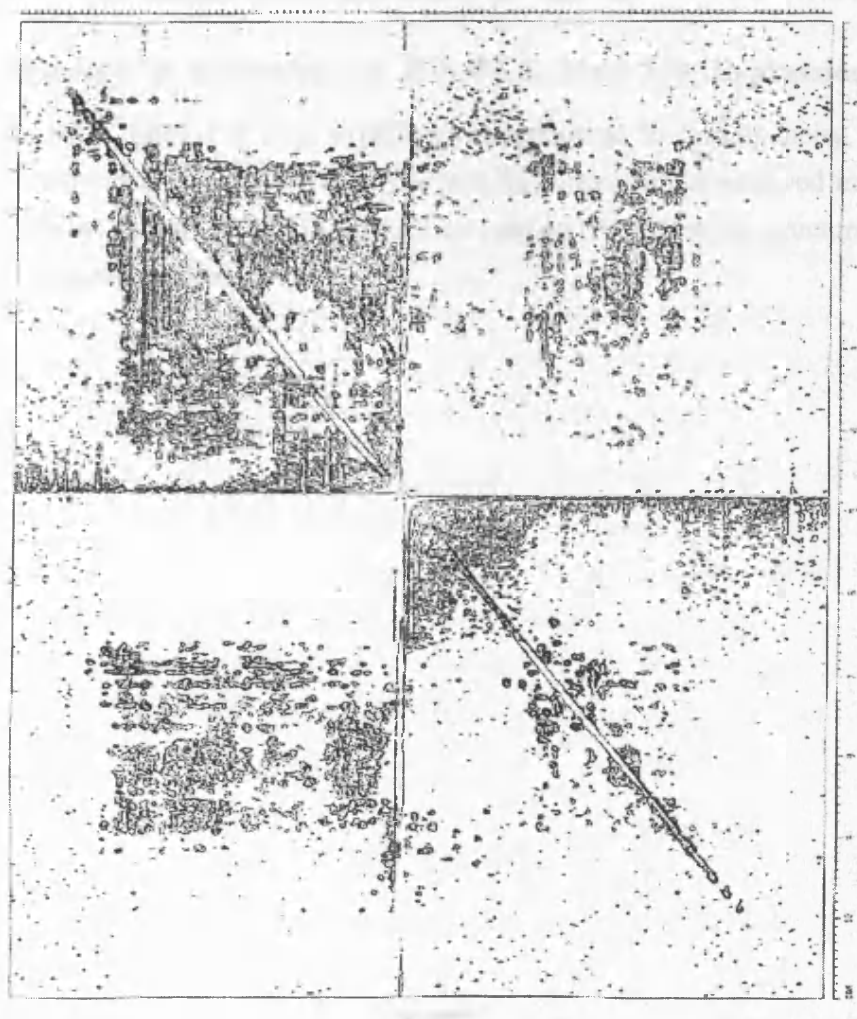


Figure 3.7.10. 2D NOESY NMR analysis of HIS-PKR Met1-170. As is clearly visible, the assignment peaks were not dispersed (when compared to figure 3.11.15) This data therefore could not be used for structural determination.



(Figure 3.7.10). The assignment peaks were not dispersed sufficiently and large “clumps” were clearly visible. For further analysis, a third dimension was required, and the isotopic labelling of the protein was required, however this was deemed futile as the maximum concentration of the sample was insufficient for NMR analysis.

Summary of Recombinant HIS-PKR Met1-170 Expression.

HIS-PKR Met1-170 was expressed and purified to homogeneity, and the apparent solubility maximum achieved. The protein concentration achieved in native conditions produced a poor 2D NOESY NMR spectra, and therefore the structural resolution of the protein was not pursued.

3.4.2 Recombinant HIS-E3L Protein Expression and Purification.

The function of E3L within an infected cell has been investigated by a number of groups, however little had been reported on the structural or biochemical analysis of the protein at the start of the project. In addition to determining the structure of the dsRBM located at the carboxyl terminus, structural analysis of the full length protein was hoped to provide insight into the role of the amino terminal region of the E3L protein, which is highly conserved between E3L and the orf virus homologue (Haig *et al.*, 1998).

Optimising Induction Conditions of recombinant HIS-E3L in *E. coli*.

A number of growth conditions were applied to maximise the expression of the recombinant HIS-E3L clone. They included optimisation of culture growth conditions, *E. coli* strain and IPTG induction concentrations.

Recombinant HIS-E3L formed the major product with a mobility of MW 27kDa within an induced cell, and was visible by coomassie blue stain (Figure 3.8.1). The growth rate of the cell was not adversely affected by the expression of recombinant HIS-E3L (data not shown) and so BL21 and B834 were used for the optimisation of induction. Transformation of pSR01 was required prior to each induction due to poor protein expression rates exhibited by transformed cells stored at -70°C, or those taken from an agar plate of 5 days or older.

Under the induction conditions examined, optimal expression of recombinant HIS-E3L was observed in *E. coli* B834. 1mM IPTG was added mid log phase when OD₅₉₅ reached 0.7, and the cells then grown for a further 2 hours at 37°C (Figure 3.8.1).

Extraction of the recombinant HIS-E3L.

Sonication.

Conditions for maximal protein extraction and solubility were determined utilising small scale sonication protocols. The soluble and insoluble fractions were then analysed by SDS PAGE (Figure 3.8.2).

The protein was solubilised in the presence of 6M urea in a Tris-HCl based extraction buffer after 3 (15 second) sonication pulses. The requirement for the chemical denaturant suggested the protein had formed highly insoluble misfolded aggregates within inclusion bodies.

Extraction Procedure.

The induced cell pellets were resuspended in 5-10 times the volume of the cell pellet in

Figure 3.8. Recombinant HIS-E3L Expression and Purification.

All SDS PAGE analysis was undertaken on 12% polyacrylamide gels under reducing conditions. The MW markers are highlighted on the left side of each figure.

Figure 3.8.1. Induction Optimisation of Recombinant HIS- E3L.

SDS PAGE analysis of *E.coli* B834 and BL21 induction with 0.5 and 1mM IPTG added respectively, mid growth phase, and the cells then grown for a number of hours at 37°C. The *E. coli* strain and number of hours of induction are highlighted on the figure.

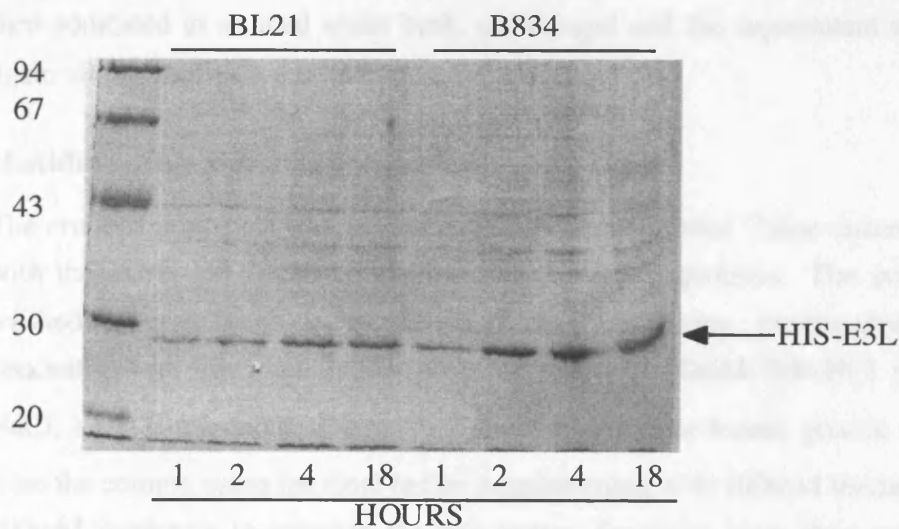
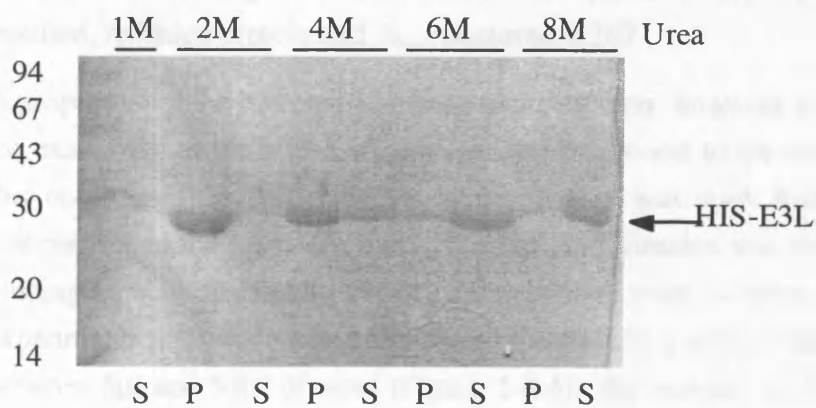
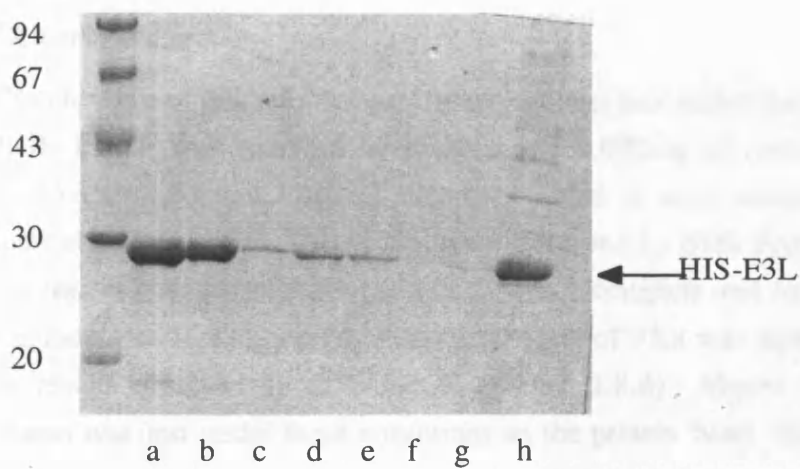
Figure 3.8.2. Solubility of the Recombinant Protein.

Extraction of the recombinant protein by sonication in extraction buffer supplemented with increased concentrations of urea. The concentrations of urea and the P-pellet and S-supernatant fractions are highlighted in the figure.

Figure 3.8.3. Purification of the Recombinant VV E3L.

Histidine Tag Purification in 50mM Tris-HCl, 100mM NaCl, 1mM β - Mercaptoethanol, and 0.5mM PMSF with 6M urea and then washed with buffer containing decreasing concentrations of urea. Protein eluted with imidazole in a standard Tris based extraction buffer, containing no urea.

- a - e= Refolded bound protein, eluted with 100mM imidazole
- f+g= Refolded bound protein, eluted with 200mM imidazole.
- h= Unbound protein, collected in initial through wash.

Figure 3.8.1.**Figure 3.8.2.****Figure 3.8.3.**

50mM Tris-HCl (pH7.5) extraction buffer, supplemented with 6M urea. Cells were then sonicated in an iced water bath, centrifuged and the supernatant drawn off. Once again all procedures were undertaken < 8 °C.

Histidine Tag Purification and Renaturation.

The crude supernatant was loaded onto a pre-equilibrated Talon column then washed with the extraction buffer to remove any unbound proteins. The column was then washed several times in extraction buffer containing progressively lower urea concentrations. The final buffer was composed of 50mM Tris-HCl pH7.5, 100mM NaCl, 1mM β -mercaptoethanol and 0.5mM PMSF. The bound protein was then eluted from the column using the final buffer supplemented with 100mM imidazole and finally 200mM imidazole to enhance the stringency. Fractions were then analysed by SDS PAGE (Figure 3.8.3). Quantitative analysis yielded 100mls crude protein extract solubilised in 6M urea. The purified protein was eluted in five 4ml fractions (lanes a-e), on which combining the first two fractions (lanes a and b), gave rise to 8mls of purified, refolded protein and A_{280} measured 0.267.

A proportion of the protein had bound to the column, however much of a protein, with the exact MW of the HIS-E3L protein, had not bound to the column at all suggesting that once again the conformation of the protein was such that the affinity tag was inaccessible to the resin. To verify that the conformation was the cause of the limited binding capacity and not a saturated Talon affinity resin, a series of small scale binding experiments were undertaken. 0.006mg of protein in a total of 50 μ l was incubated with between 5 μ l and 50 μ l of resin (Figure 3.8.4). An increase in binding efficiency was noted with the 1:1 sample, however the majority of the protein remained in the unbound fraction with all of the ratios examined. This suggests the low binding capacity is a result of the inaccessibility of the histidine tag of the recombinant HIS-E3L to the resin.

FXa cleavage.

FXa cleavage of this refolded protein proved less successful than the recombinant HIS-PKR. PMSF was removed by dialysis and 0.002mg of recombinant HIS-E3L was digested with FXa. 1.72 μ g of FXa was added to each sample, and digested for 18 hours at 4°C and 21°C. The results were observed by SDS PAGE (Figure 3.8.5). The cleavage in both temperature conditions was incomplete and so the length of digestion was increased to 48 hours. 1.72 μ g and 0.43 μ g of FXa was added to each sample, and the results observed by SDS PAGE (Figure 3.8.6). Almost all of the recombinant protein was lost under these conditions as the protein band was no longer visible by

Figure 3.8.4. Talon Affinity Resin Protein Saturation Experiment.

Aliquots of 0.006mg of recombinant protein (in a total of 50µl) solubilised in 50mM Tris-HCl, 100mM NaCl, 1mM β - Mercaptoethanol, 0.5mM PMSF and 6M urea were mixed with increasing quantities of resin. The quantities of resin used and U-unbound and B-bound fractions are highlighted on the figure.

Figure 3.8.5. FXa Cleavage of the Histidine Tag.

0.002mg of recombinant refolded protein digested for 18 hours at 4°C or 21°C.

a = Uncleaved protein.

b = 1.72µg FXa and protein digested at 4°C.

c=1.72µg FXa and protein digested at 21°C.

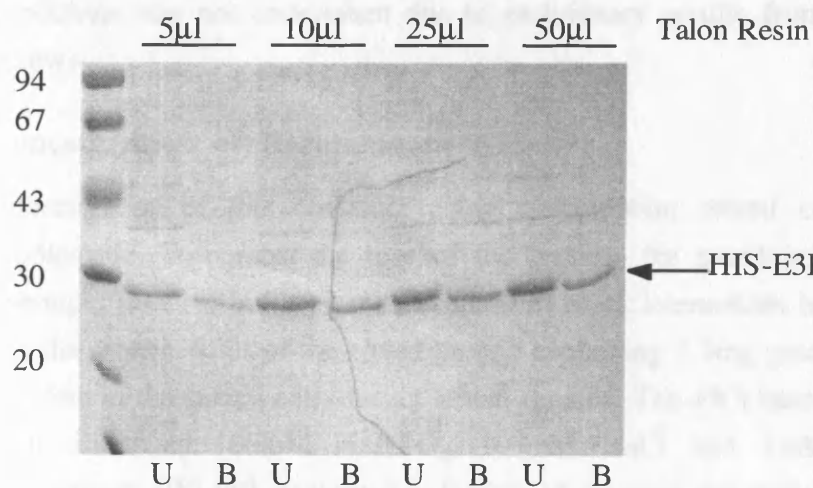
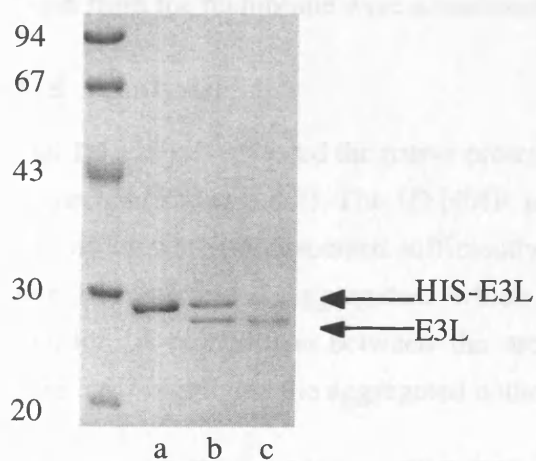
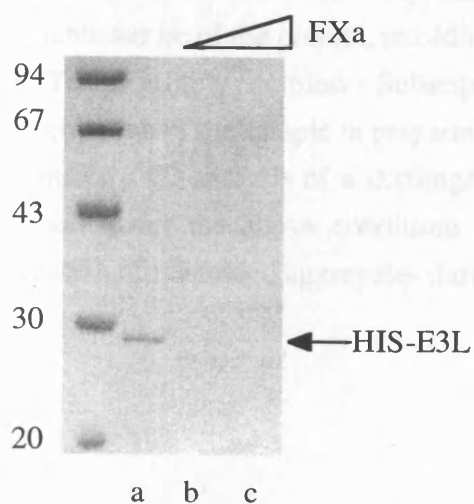
Figure 3.8.6. FXa Cleavage of the Histidine Tag.

0.002mg of refolded recombinant protein digested for 48 hours at 21°C.

a = Uncleaved protein.

b = 0.43µg FXa and protein.

c = 1.72µg FXa and protein.

Figure 3.8.4.**Figure 3.8.5.****Figure 3.8.6.**

coomassie blue staining (lanes b and c). Further work to identify optimal cleavage conditions was not undertaken due to preliminary results from 1D NMR data (see below)

Concentration of Recombinant HIS-E3L.

Concentration of this construct using ultrafiltration stirred cells (amicon) proved problematic. To combat the loss of the protein, the membrane was first incubated overnight in a 1% buffered milk solution to block interactions between the membrane and the protein. 8mls of the eluted protein containing 1.3mg protein were concentrated to 0.5ml in the stirred cell, during which time the Tris-HCl based buffer was replaced with deuterated 100mM NaHPO₄, 100mM NaCl and 1mM DTT. 0.16mg of recombinant HIS-E3L remained in 0.5mls of solution, the remainder was believed to have precipitated and then become “stuck” to the membrane. Attempts to retrieve the protein from the membrane were unsuccessful.

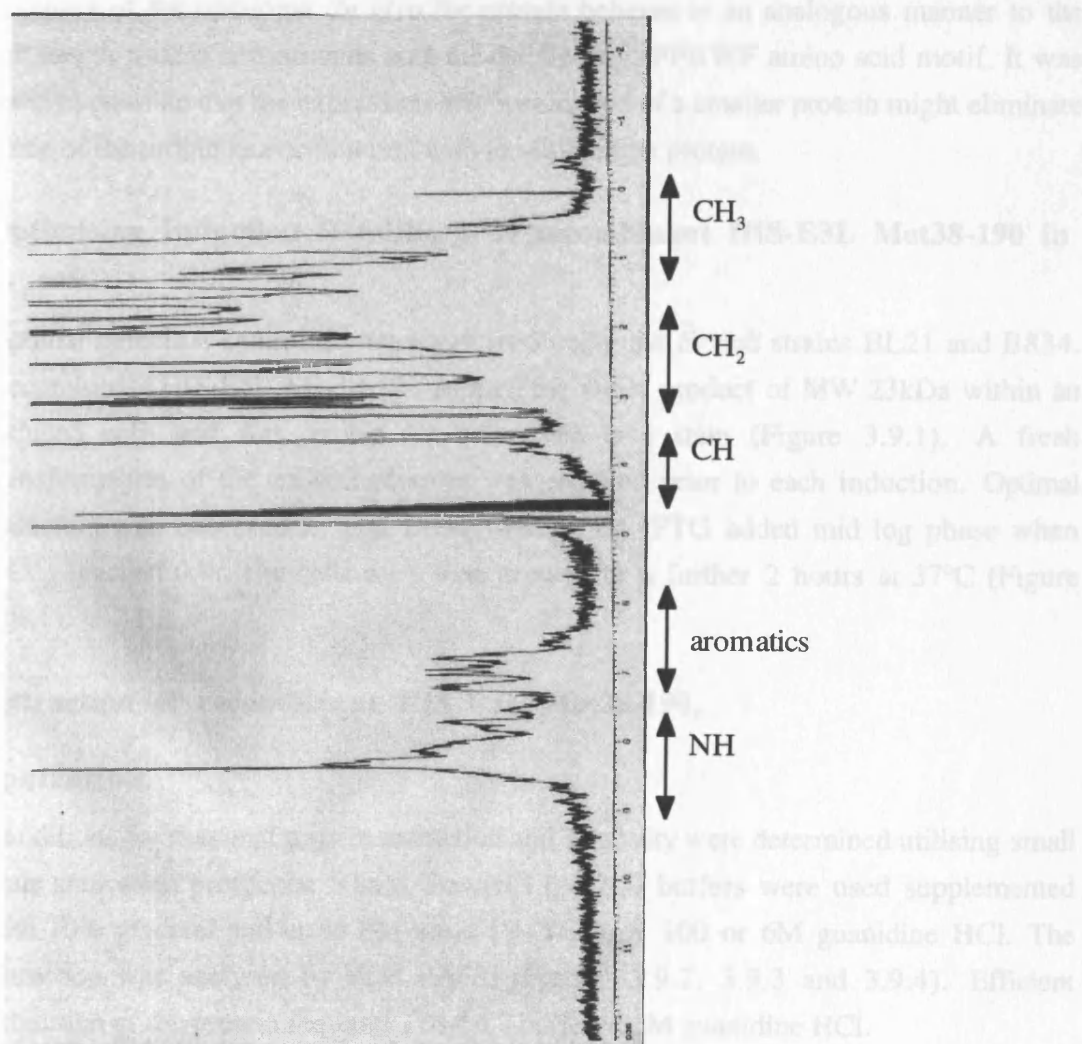
NMR Analysis.

Initial 1D analysis revealed the native protein sample (with histidine tag) had not folded as expected (Figure 3.8.7). The 1D NMR profile was composed of a number of broad peaks which were not dispersed sufficiently. This suggested the sample was mixture of native and misfolded aggregates, which was therefore of no use for structural resolution. A comparison between the aromatic region shown in Figure 3.7.9 and Figure 3.8.7 highlights the aggregated nature of this protein sample.

Summary of Recombinant HIS-E3L Expression.

Recombinant HIS-E3L was expressed and purified to homogeneity. Due to the insoluble nature of the protein, refolding of the protein was undertaken whilst bound to the Talon affinity column. Subsequent FXa cleavage of the histidine tag and concentration of the sample in preparation for NMR analysis proved to be problematic. Preliminary 1D analysis of a 0.16mg/ml sample indicated that this particular protein, purified under the above conditions was unsuitable for NMR analysis due to the formation of misfolded aggregates during the renaturation process.

Figure 3.8.7. 1D NMR analysis of HIS-E3L. The peaks are not well distributed, indicating the sample was a complex mixture of unfolded aggregates and therefore unsuitable for NMR spectroscopy. The proton chemical shifts (ppm - parts per million) arising from the chemical environments are highlighted at the bottom of the spectra.



Preparation of Lysates

Cells of the strains were disrupted using lysostaphin and homogenization of the cell debris was performed using a Dounce homogenizer (100 strokes) in 100 μ l of lysis buffer (100 mM Tris-HCl pH 7.5, 10 mM EDTA, 5 mM DTT, 10% glycerol, 0.1% Triton X-100) and centrifuged at 14,000 $\times$ g for 10 min (Ho and Brannan, 1996). The recombinant protein was precipitated by addition of 100 μ l of 100% methanol and centrifuged at 14,000 $\times$ g for 10 min. The supernatant was removed and the pellet was resuspended in 100 μ l of 100% methanol.

Protein Analysis

The protein samples were analyzed by 1D SDS-PAGE using 10% gels. The gels were stained with Coomassie Brilliant Blue G250. The protein bands were visualized using a UV transilluminator.

3.4.3 Recombinant HIS-E3L Met38-190 Protein Expression and Purification.

As discussed previously a truncated E3L, p20, protein is expressed *in vivo* due to leaky scanning of the ribosome. *In vivo* the protein behaves in an analogous manner to the full length protein and contains both the dsRBM and PPRWF amino acid motif. It was thought possible that the expression and purification of a smaller protein might eliminate some of the problems encountered with the full length protein.

Optimising Induction Conditions of recombinant HIS-E3L Met38-190 in *E. coli*

Optimal induction conditions were assessed using the *E. coli* strains BL21 and B834. Recombinant HIS-E3L Met38-190 formed the major product of MW 23kDa within an induced cell, and was visible by coomassie blue stain (Figure 3.9.1). A fresh transformation of the pSR02 plasmid was required prior to each induction. Optimal induction was observed *E. coli* B834, with 1mM IPTG added mid log phase when OD₅₉₅ reached 0.7. The cells were then grown for a further 2 hours at 37°C (Figure 3.9.1).

Extraction of recombinant HIS-E3L Met38-190.

Sonication.

Conditions for maximal protein extraction and solubility were determined utilising small scale sonication protocols. 50mM Tris-HCl (pH7.5) buffers were used supplemented with 10% glycerol and up to 8M urea, 1% Triton X 100 or 6M guanidine HCl. The extraction was analysed by SDS PAGE (Figures 3.9.2, 3.9.3 and 3.9.4). Efficient extraction of the protein required Tris-HCl buffered 6M guanidine HCl.

Addition of Lysozyme.

Extraction of the protein was attempted using lysozyme and homogenisation of the sample in 50mM Tris-HCl pH8, 1mM EDTA, 2mM DTT, 10% glycerol, 0.1% Triton-X 100 supplemented with 1M and 2M NaCl (Ho and Shuman, 1996). The recombinant protein was not soluble in either of the NaCl concentrations (data not shown).

Extraction Procedure.

The induced pellet was resuspended in 5-10 times the volume of the cell pellet in Tris-HCl pH7.5 extraction buffer. The cells were sonicated in an iced water bath,

Figure 3.9. Expression and Purification of Recombinant HIS-E3L Met38-190.

All SDS PAGE analysis was undertaken on 12% polyacrylamide gels under reducing conditions. The MW markers are highlighted on the left side of each figure.

Figure 3.9.1. Induction Optimisation of Recombinant HIS-E3L Met38-190.

SDS PAGE analysis of *E.coli* B834 induction with 0.5 and 1mM IPTG added mid growth phase, and the cells then grown for a number of hours at 37°C. The concentration of IPTG and number of hours of induction are highlighted in the figure. The uninduced sample is labelled as U.

Figure 3.9.2. Solubility of the Recombinant Protein.

Extraction of the recombinant protein by sonication in 50mM Tris-HCl, 100mM NaCl, 1mM β -Mercaptoethanol, and 0.5mM PMSF supplemented with 1% Triton X 100 or increasing concentrations of urea. The concentration of the solubilising agents and P-pellet and S-supernatant fractions are highlighted in the figure.

Figure 3.9.3. Solubility of the Recombinant Protein.

Extraction of the recombinant protein by sonication in extraction buffer supplemented with 8M urea and the extraction buffer alone, containing no solubilising agents. The P-pellet and S-supernatant fractions are highlighted in the figure.

Figure 3.9.1.

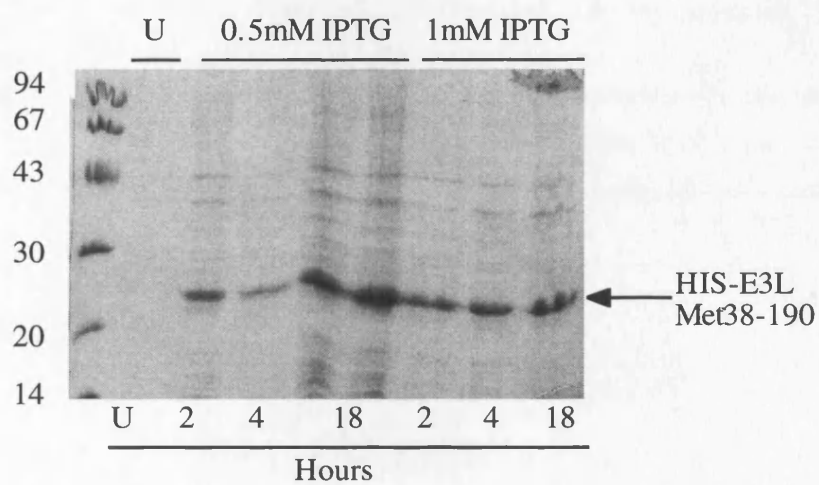


Figure 3.9.2.

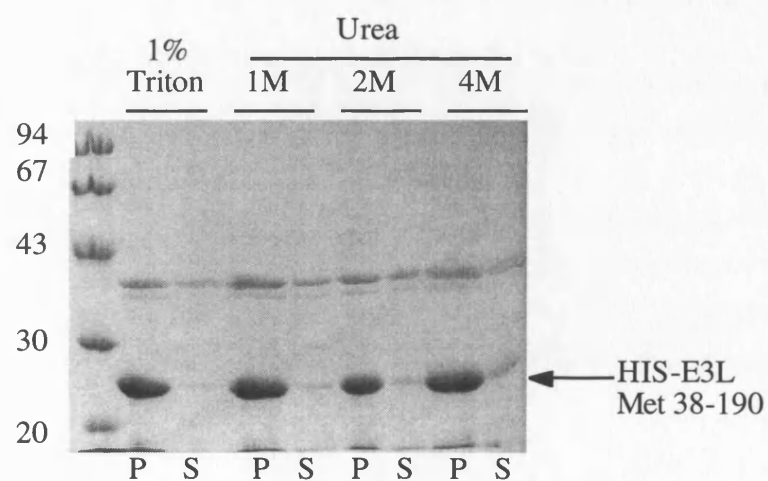


Figure 3.9.3.

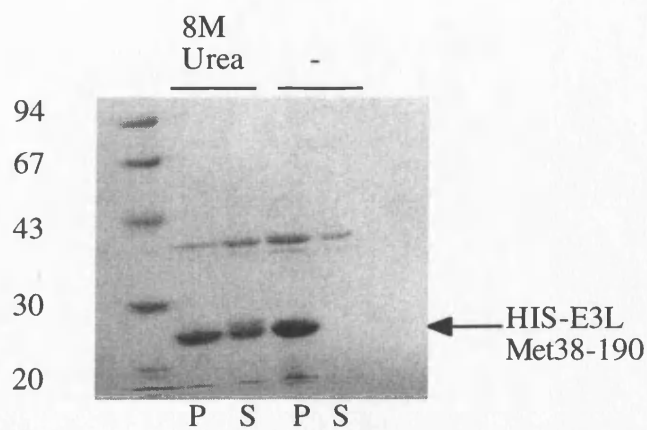


Figure 3.9.4. Solubility of the Recombinant Protein.

Extraction of the recombinant protein by sonication in 50mM Tris-HCl, 100mM NaCl, 1mM β - Mercaptoethanol, and 0.5mM PMSF supplemented with 6M guanidine HCl. The P-pellet and S-supernatant fractions are highlighted in the figure.

Figure 3.9.5. Purification of the Recombinant HIS-E3L Met38-190.

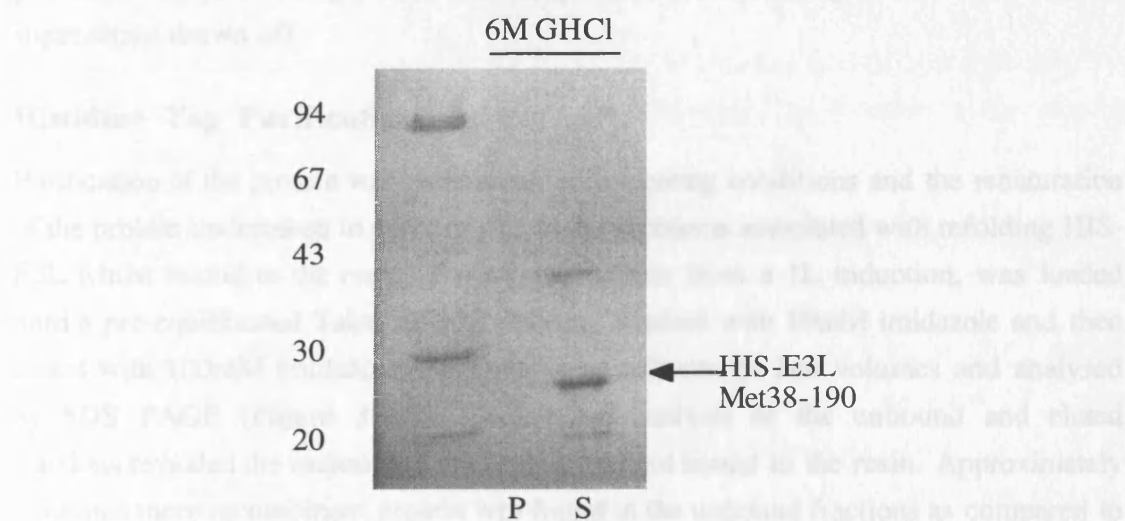
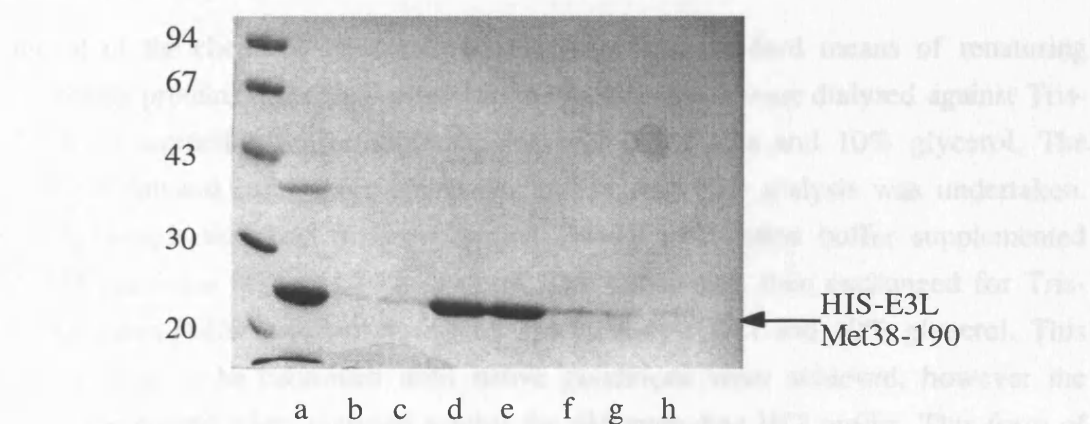
Histidine Tag Purification in 50mM Tris-HCl, 100mM NaCl, 1mM β - Mercaptoethanol, and 0.5mM PMSF supplemented with 6M guanidine HCl.

a = Unbound protein collected from initial through wash.

b + c = Column washes in buffer containing 10mM imidazole.

d + e = Initial two samples, eluted with 100mM imidazole.

f - h = Further samples, eluted with 200mM imidazole.

Figure 3.9.4.**Figure 3.9.5.**

centrifuged and the pellet resuspended in extraction buffer supplemented with 6M guanidine HCl and 10% glycerol. The sample was then centrifuged once more and the supernatant drawn off.

Histidine Tag Purification.

Purification of the protein was undertaken in denaturing conditions and the renaturation of the protein undertaken in solution due to the problems associated with refolding HIS-E3L whilst bound to the resin. The sample, 20mls from a 1L induction, was loaded onto a pre-equilibrated Talon affinity column, washed with 10mM imidazole and then eluted with 100mM imidazole. Fractions were collected in 2ml volumes and analysed by SDS PAGE (Figure 3.9.5). Quantitative analysis of the unbound and eluted fractions revealed the majority of the protein had not bound to the resin. Approximately 3.5 times more recombinant protein was found in the unbound fractions as compared to the eluted fractions.

Renaturation.

Removal of the chemical denaturant by dialysis, is a standard means of renaturing recombinant proteins. Initially the combined eluted fractions were dialysed against Tris-HCl pH7.5 extraction buffer supplemented with 0.5M urea and 10% glycerol. The protein precipitated under these conditions and so step-wise dialysis was undertaken. Here the sample was first dialysed against Tris-HCl extraction buffer supplemented with 4M guanidine HCl and 10% glycerol. The buffer was then exchanged for Tris-HCl extraction buffer supplemented with 2M guanidine HCl and 10% glycerol. This procedure was to be continued until native conditions were achieved, however the protein precipitated when dialysed against the 4M guanidine HCl buffer. This form of dialysis was repeated with increased concentrations of β -mercaptoethanol, from 1mM to 10mM, however the protein precipitated once more when dialysed against 4M guanidine HCl.

Dilution of the recombinant protein is an alternative means of renaturation. The sample was diluted 50 fold in refolding buffer, and incubated at 15°C for 18 hours. The protein started to precipitate when dialysed against 50mM Tris-HCl pH7.5, 100mM NaCl, 0.5mM DTT, 5mM EDTA, 2.5M urea and 2% Triton-X 100. When dialysed against the Tris-HCl extraction buffer alone, the protein precipitated completely.

Summary of Recombinant HIS-E3L Met38-190 Expression.

The recombinant HIS-E3L Met38-190 protein was expressed and purified to homogeneity. The protein was highly insoluble, and I was unable to remove sufficient quantities of the denaturant for any structural analysis to be undertaken.

3.4.4 Recombinant HIS-E3L Met94-190 Protein Expression and Purification.

This protein construct contains the dsRBM alone. The smaller size of HIS-E3L Met94-190 was hoped to be beneficial to both the expression and purification of the protein and the NMR structural resolution.

Optimising Induction Conditions of recombinant HIS-E3L Met94-190 in *E. coli*.

Recombinant HIS-E3L Met94-190 was expressed in BL21, with 0.4mM IPTG added when OD₅₉₅ reached 0.45. The cells were then grown for a further 4 hours at 37°C (Ho and Shuman, 1996). Recombinant HIS-E3L Met94-190 formed the major product of MW 14kDa within an induced cell, and was visible by coomassie blue stain (Figure 3.10.1). A fresh transformation of the pSR03 plasmid was required prior to each induction.

Extraction of recombinant HIS-E3L Met94-190.

Sonication and Solubility Determination.

Once again small scale sonication protocols were used to determine the solubility of the recombinant protein using Tris-HCl, pH7.5, buffers supplemented with increasing concentrations of urea. 50µl of the supernatants were immediately loaded onto 50µl Talon affinity resin, using a small scale procedure, the resin stripped using EDTA and the bound fractions analysed by SDS PAGE (Figure 3.10.2). A 500mM NaCl Tris-HCl buffered extraction was also attempted, however very little recombinant protein was solubilised (data not shown). The protein was partially soluble in 8M urea and a small quantity of recombinant protein had bound to the resin. The protein was therefore solubilised in 6M guanidine HCl so as to denature the protein more thoroughly, which was hoped would expose the histidine affinity tag.

Purification.

The cells were sonicated in a Tris-HCl extraction buffer, supplemented with 500mM NaCl. The pellet was then washed with two separate detergent solutions to remove lipids. The 0.5% Triton-X 100 wash solubilised a large proportion of the recombinant protein, however the protein itself is hydrophilic suggesting this detergent extracted sample was composed of misfolded aggregates with exposed hydrophobic residues. The remaining insoluble pellet from a 1L induction was then resuspended in 1ml of Tris-HCl extraction buffer supplemented with 6M guanidine HCl, and the results

Figure 3.10. Expression and Purification of Recombinant VV E3L dsRBM.

All SDS PAGE analysis was undertaken on 18% polyacrylamide gels under reducing conditions. The MW markers are highlighted on the left side of each figure.

Figure 3.10.1. Induction Optimisation of Recombinant VV E3L dsRBM.

SDS PAGE analysis of *E.coli* BL21 induction with 0.4mM IPTG added when the OD₅₉₅ reached 0.4, and the cells then grown for 4 hours at 37°C. The I-induced and U-uninduced samples are highlighted in the figure. Western blot analysis of the induction confirmed the expression of HIS-E3L Met94-190, Figure 3.6(B).

Figure 3.10.2. Solubility of the Recombinant Protein and Binding to Affinity Column.

Extraction of the recombinant protein by sonication in 50mM Tris-HCl, 100mM NaCl, 1mM β -Mercaptoethanol, and 0.5mM PMSF supplemented with increasing concentrations of urea, followed by histidine tag purification. The concentration of urea and the B-bound and U-unbound fractions are highlighted in the figure.

Figure 3.10.3. Purification of the Recombinant Protein.

Removal of lipids, contaminating proteins and purification of the insoluble protein.

a = Supernatant from sonication supplemented with 500mM NaCl.

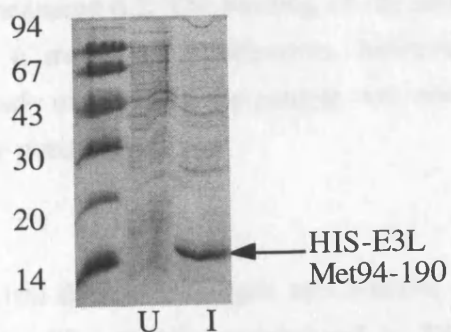
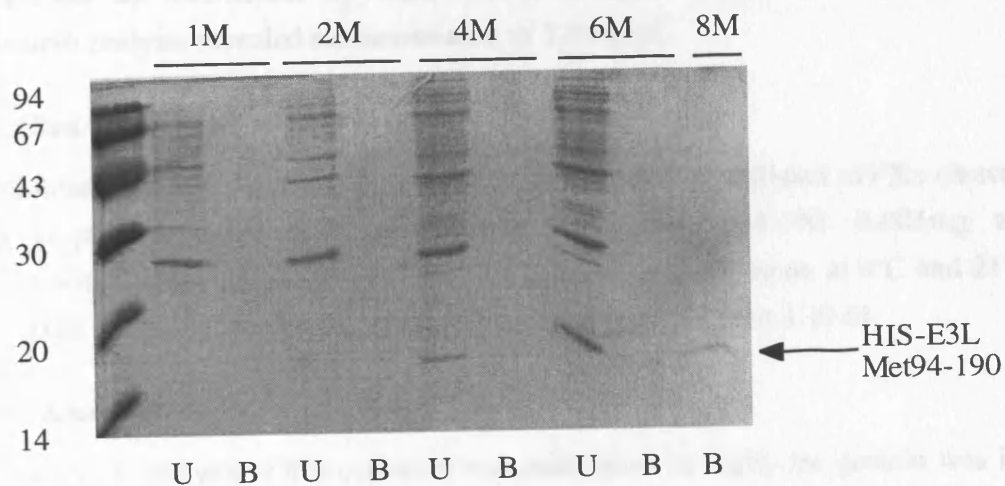
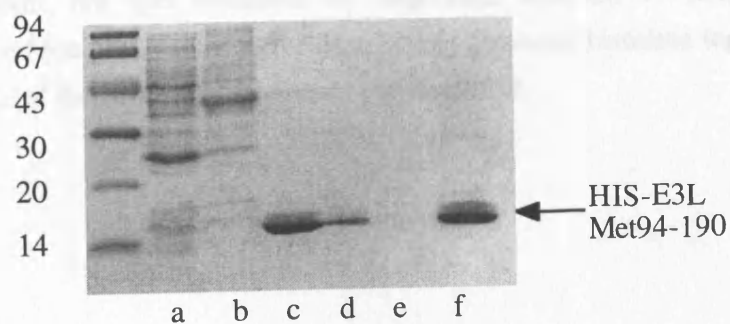
b = Deoxycholate wash supernatant.

c = Triton-X 100 wash 1 supernatant.

d = Triton-X 100 wash 2 supernatant.

e = Pellet fraction after guanidine HCl extraction.

f = Guanidine HCl extraction supernatant.

Figure 3.10.1**Figure 3.10.2.****Figure 3.10.3.**

observed by SDS PAGE (Figure 3.10.3). Quantitative analysis of the 1ml denatured sample revealed the A_{280} measured 6.2. The binding of the protein to the Talon affinity resin was inadequate as a means of purification, however due to the extensive purification procedure already undertaken, the sample was considered sufficiently pure to continue without affinity purification.

Renaturation.

Sequential dialysis of the 1ml denatured sample into a native buffer proved successful with this particular protein. The protein, solubilised in Tris-HCl pH7.5 extraction buffer supplemented with 6M guanidine HCl, was dialysed against the extraction buffer supplemented with 8M urea. Although a small decrease in concentration was evident when analysed by SDS PAGE (Figure 3.10.4), the sample was then dialysed against Tris-HCl extraction buffer supplemented with 1M urea. Further protein precipitation was evident as determined by SDS PAGE analysis (Figure 3.10.5), however quantitative analysis revealed a concentration of 1.8mg/ml.

FXa Cleavage.

The refolded protein sample was dialysed to remove PMSF and diluted in FXa cleavage buffer to produce 0.001mg of recombinant HIS-E3L Met94-190. 0.001mg was digested with 0.43µg and 0.86µg of FXa and incubated for 18 hours at 4°C and 21°C. SDS PAGE analysis revealed no cleavage had taken place (Figure 3.10.6).

NMR Analysis.

Preliminary 1D analysis of this construct was undertaken to verify the protein was in a folded conformation. The 1D spectra indicated that the protein was folded (data not shown).

Summary of Recombinant HIS-E3L Met94-190 Expression.

HIS-E3L Met94-190 was expressed and purified to homogeneity and a concentration of 1.8mg/ml was achieved. The protein was extracted using high concentrations of a chemical denaturant, but was renatured by sequential dialysis. N terminal protein sequencing verified the presence of a histidine fusion however histidine tag purification and FXa cleavage of the histidine tag proved unsuccessful.

Figure 3.10.4. Renaturation of the Recombinant Protein.

Dialysis of protein from 50mM Tris-HCl, 100mM NaCl, 1mM β - Mercaptoethanol, and 0.5mM PMSF supplemented with 6M guanidine HCl to 8M urea.

a = Soluble fraction in 6M guanidine HCl.

b = Soluble fraction in 8M urea.

Figure 3.10.5. Renaturation of the Recombinant Protein.

Dialysis of protein from 50mM Tris-HCl, 100mM NaCl, 1mM β - Mercaptoethanol, and 0.5mM PMSF supplemented with 8M urea 1M urea.

a = Soluble fraction in 8M urea

b = Soluble fraction in 1M urea.

Figure 3.10.6. FXa cleavage of the Histidine Tag.

0.001mg of recombinant protein solubilised in 50mM Tris-HCl, 100mM NaCl, 1mM β - Mercaptoethanol and 1M urea was digested at 4°C and 21°C with various quantities of FXa.

a = Uncleaved protein.

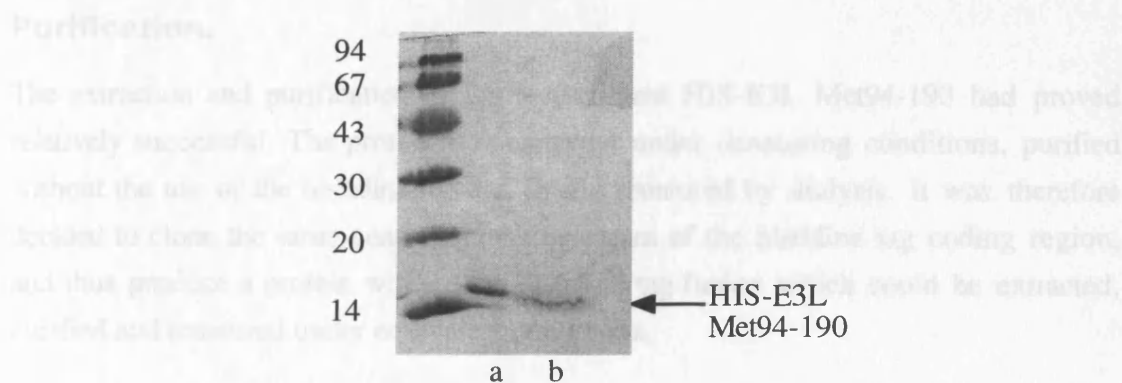
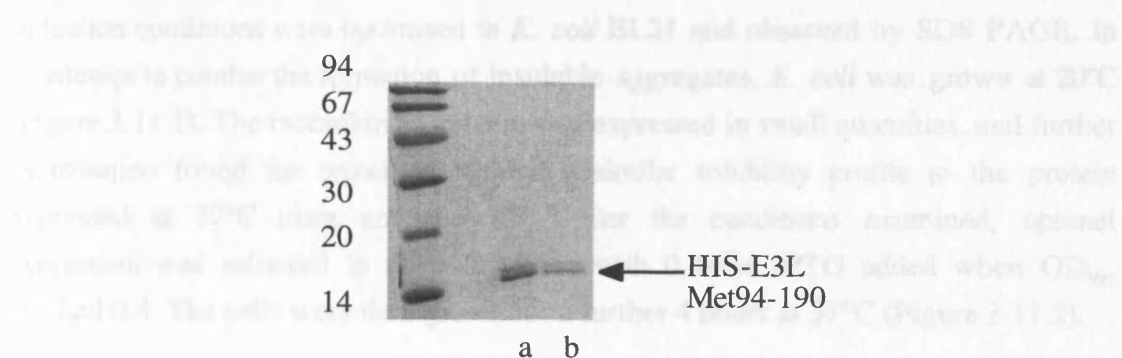
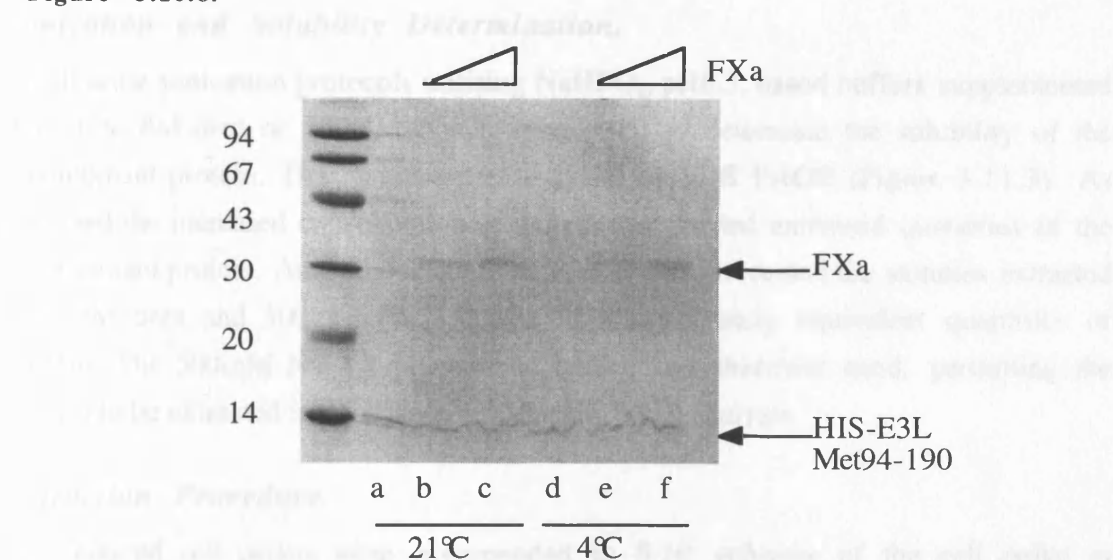
b = 0.43 μ g of FXa and protein, digested at 21°C.

c = 0.86 μ g of FXa and protein, digested at 21°C.

d = Uncleaved protein.

e = 0.43 μ g of FXa and protein, digested at 4°C.

f = 0.86 μ g of FXa and protein, digested at 4°C.

Figure 3.10.4. Recombinant E3L Met94-190 Protein Expression and Purification.**Figure 3.10.5.****Figure 3.10.6.**

3.4.5 Recombinant E3L Met94-190 Protein Expression and Purification.

The extraction and purification of the recombinant HIS-E3L Met94-190 had proved relatively successful. The protein was extracted under denaturing conditions, purified without the use of the histidine tag and finally renatured by dialysis. It was therefore decided to clone the same gene fragment upstream of the histidine tag coding region, and thus produce a protein without the histidine tag fusion which could be extracted, purified and renatured under equivalent conditions.

Optimising Induction Conditions of recombinant E3L Met94-190 in *E. coli*.

Induction conditions were optimised in *E. coli* BL21 and observed by SDS PAGE. In an attempt to combat the formation of insoluble aggregates, *E. coli* was grown at 20°C (Figure 3.11.1). The recombinant protein was expressed in small quantities, and further examination found the protein to exhibit a similar solubility profile to the protein expressed at 37°C (data not shown). Under the conditions examined, optimal expression was achieved in *E. coli* BL21, with 0.4mM IPTG added when OD₅₉₅ reached 0.4. The cells were then grown for a further 4 hours at 37°C (Figure 3.11.2).

Extraction of recombinant E3L Met94-190.

Sonication and Solubility Determination.

Small scale sonication protocols utilising NaHPO₄, pH6.5, based buffers supplemented with 1 to 8M urea or 500mM NaCl, were used to determine the solubility of the recombinant protein. The results were analysed by SDS PAGE (Figure 3.11.3). As expected the increased concentration of denaturant, yielded increased quantities of the recombinant protein. Analysis of the SDS PAGE result revealed the samples extracted with 4M urea and 500mM NaCl contained approximately equivalent quantities of protein. The 500mM NaCl supplemented buffer was therefore used, permitting the protein to be extracted in conditions suitable for NMR analysis.

Extraction Procedure.

The induced cell pellets were resuspended in 5-10 volumes of the cell pellet in NaHPO₄, pH6.5, extraction buffer. The lysate was passed through the french press, centrifuged and the pellet resuspended in 5-10 volumes of the cell pellet in NaHPO₄ extraction buffer supplemented with 500mM NaCl. The lysate was homogenised to

Figure 3.11. Expression and Purification of E3L Met94-190

All SDS PAGE analysis was undertaken on 18% polyacrylamide gels under reducing conditions. The MW markers are highlighted on the left side of each figure.

Figure 3.11.1. Induction Optimisation of Recombinant E3L Met94-190.

SDS PAGE analysis of *E.coli* BL21 induction with a number of IPTG concentrations, added when OD₅₉₅ reached 0.4. Cells were then grown for 4 and 18 hours at 37°C or 20°C. The temperatures, IPTG concentrations and number of hours of induction are highlighted in the figure.

Figure 3.11.2. Optimal Induction Conditions for Recombinant E3L Met94-190.

SDS PAGE analysis of *E. coli* BL21, induced with 0.4mM IPTG when OD 595 reached 0.4. Cells were then induced for 4 hours at 37°C. The I-induced cells and U-uninduced cells are highlighted in the figure.

Figure 3.11.3. Solubility of the Recombinant Protein.

Extraction of the recombinant protein by sonication in 50mM NaHPO₄, 1mM β - Mercaptoethanol, and 0.5mM PMSF with increasing concentrations of urea supplemented with 100mM NaCl and also 500mM NaCl. The supernatant fractions along with the I-induced sample were analysed by SDS PAGE. The concentrations of urea and NaCl are highlighted in the figure,.

Figure 3.11.1.

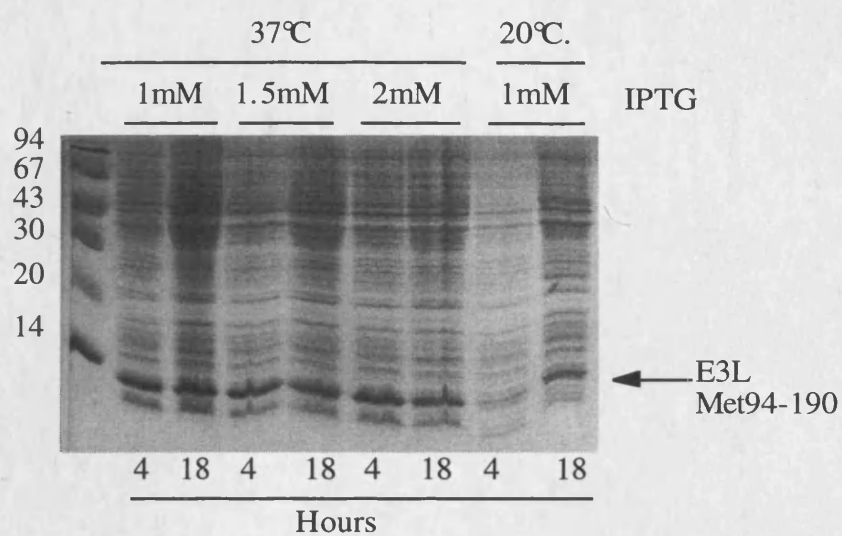


Figure 3.11.3.

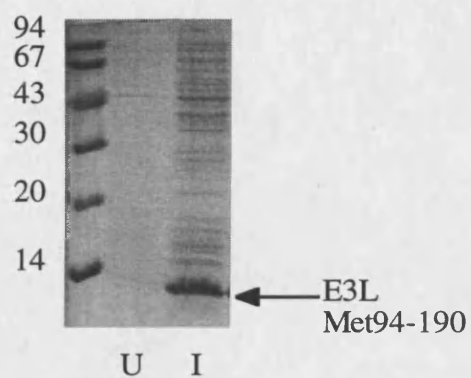
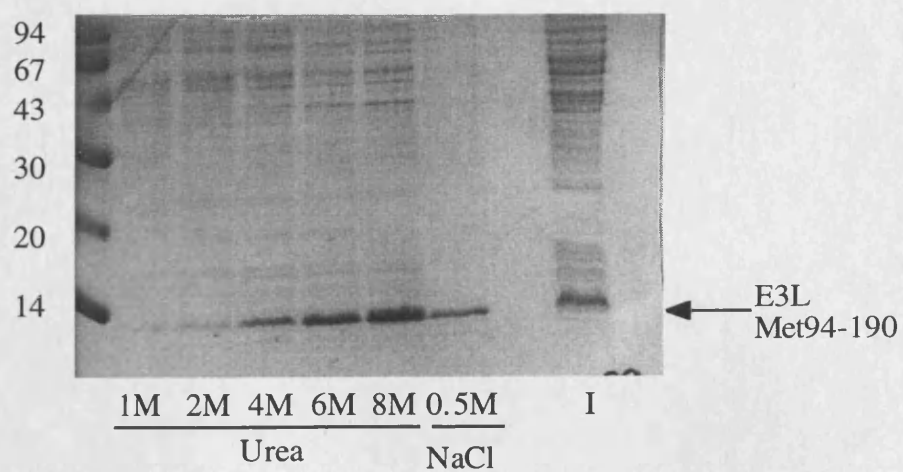


Figure 3.11.3.



ensure efficient extraction of the recombinant protein followed by centrifugation. The process was repeated until all the soluble recombinant protein had been extracted. The samples were then analysed by SDS PAGE (Figure 3.11.4).

Purification of the protein.

As there was no histidine tag on the protein other methods of protein purification were used.

Ion Exchange Chromatography.

High concentrations of NaCl are incompatible with ion exchange chromatography and so removal of the NaCl from the sample was required if this approach to protein purification was to be used. The 500mM NaCl protein sample was dialysed against a range of phosphate buffered extraction solutions containing 100-400mM NaCl. The results were observed by SDS PAGE analysis (Figure 3.11.5). The sequential decrease in NaCl concentration caused a successive increase in recombinant protein precipitation. This method of purification was therefore not utilised.

Poly(rI) (rC) purification.

The dsRNA binding capacity of the protein was exploited as a means of purification. A small scale binding protocol was undertaken to ascertain the viability of this method, and the results analysed by SDS PAGE (Figure 3.11.6). The recombinant protein did not interact with the resin quantitatively, and therefore was not a suitable means of purifying the protein.

Cibacron Blue Dyes.

A number of cibacron blue dyes which mimic NAD, were equilibrated in poly(rI) (rC) binding buffer and small scale binding protocols attempted. The results were observed by SDS PAGE (Figure 3.11.7). The recombinant protein did not bind quantitatively with any of the dyes used, and therefore the dyes were not suitable for purification.

Purification Procedure.

Because the protein was not interacting directly with the aforementioned resins, the purification procedure relied on gel filtration chromatography. During gel filtration chromatography proteins pass through a chromatographic bed, the rate at which the protein moves through the bed is dependent on the mobile phase and partitioning of the proteins between the mobile and stationary phase. Hence, larger proteins do not enter the pores of the bed and as such are eluted faster than the smaller proteins which are retained in the pores. As discussed previously the induced pellets were lysed by the

Figure 3.11.4. Extraction Procedure.

SDS PAGE analysis of the stepwise extraction of the protein in 50mM NaHPO₄, pH 6.5, 1mM β - Mercaptoethanol, and 0.5mM PMSF supplemented with 500mM NaCl.

a = Crude induction.

b -c = Supernatant from cell lysis in sodium phosphate extraction buffer.

d -g = Extraction of the recombinant protein in sodium phosphate extraction buffer supplemented with 500mM NaCl by homogenisation.

Figure 3.11.5. Removal of NaCl.

SDS PAGE analysis of extracted samples dialysed against 50mM NaHPO₄, pH6.5, 1mM β - Mercaptoethanol, and 0.5mM PMSF supplemented with decreasing NaCl concentrations. The NaCl concentrations, S-supernatant and P-pellet fractions are highlighted in the figure.

Figure 3.11.6. Purification of the Recombinant Protein.

SDS PAGE analysis of Poly (rI) (rC) binding.

a = Crude Fraction.

b = Unbound Fraction.

c = Bound Fraction.

d = Unbound Fraction.

e = Bound Fraction

f = Crude Fraction (diluted 5 fold)

g = Unbound Fraction

h = Bound Fraction.

Figure 3.11.4.

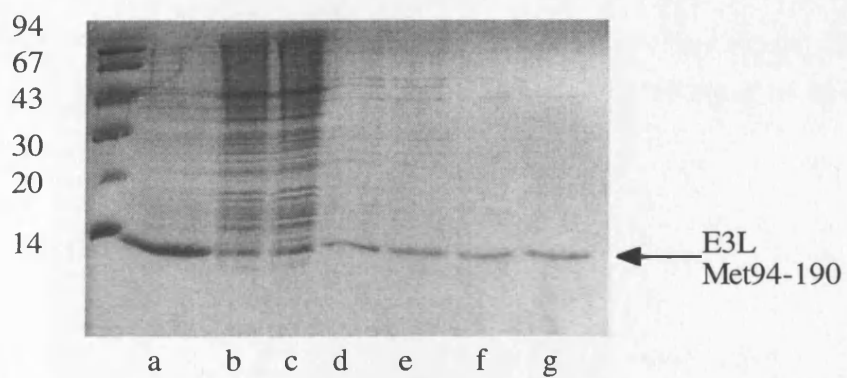


Figure 3.11.5.

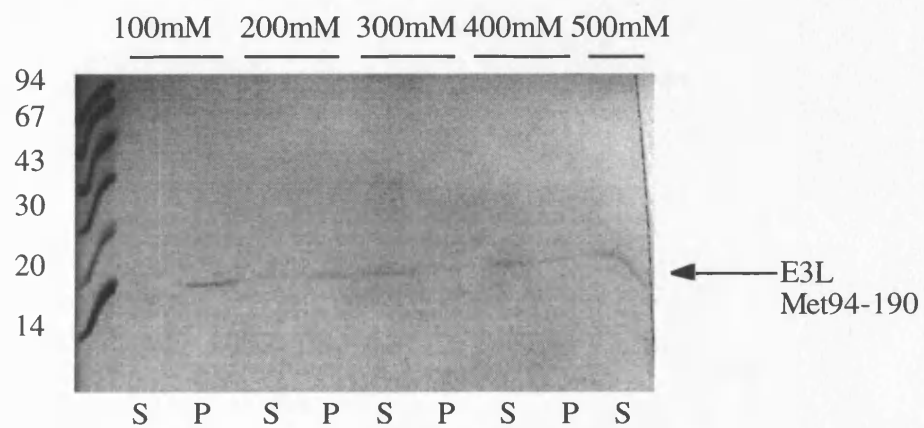


Figure 3.11.6.

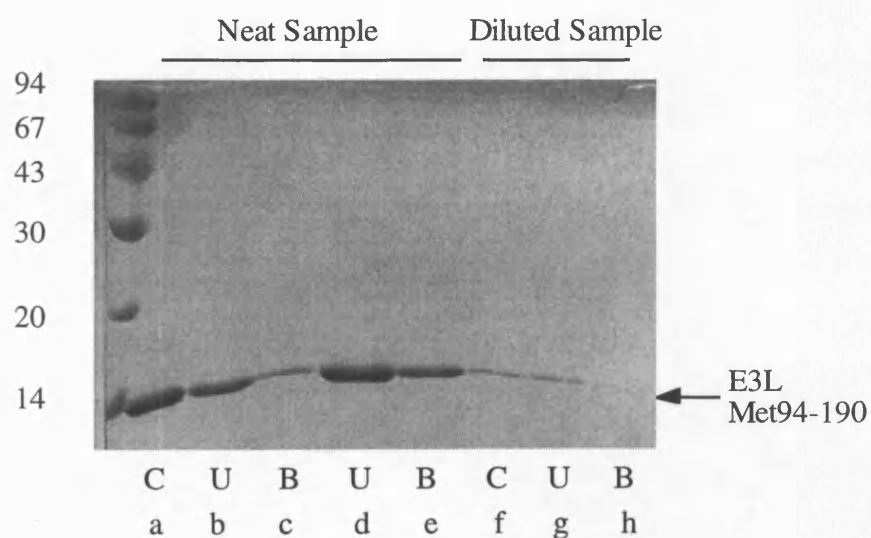


Figure 3.11.7(A and B). Purification of the Recombinant Protein.

SDS PAGE analysis of cibacron blue dye binding. The cibacron blue dye type, U-unbound, B-bound and C-crude fractions are highlighted in the figures.

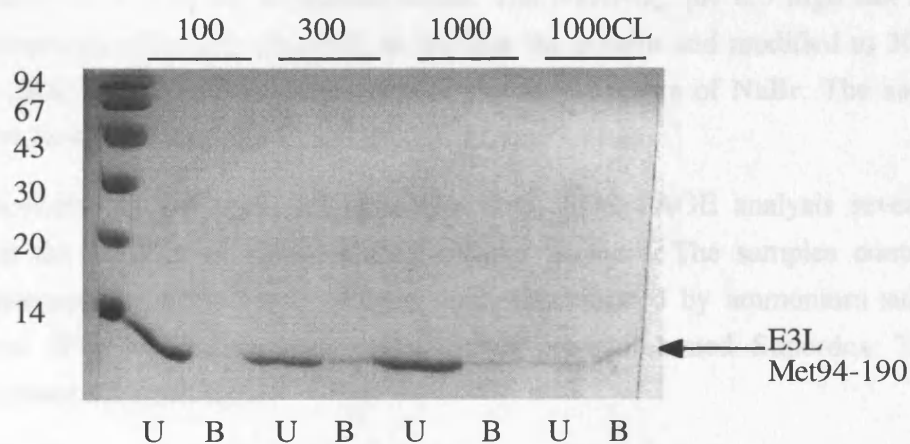
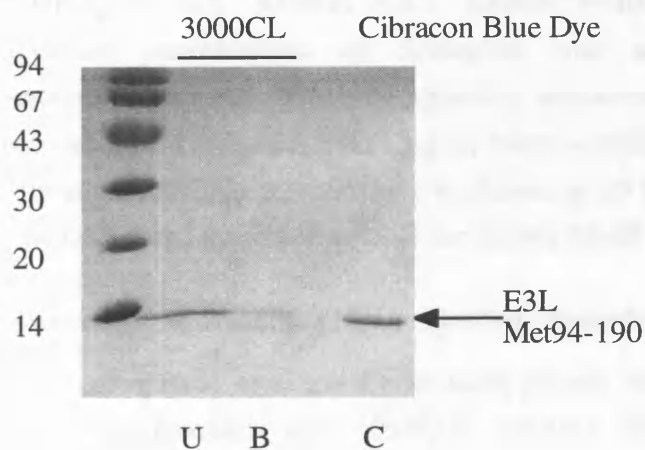
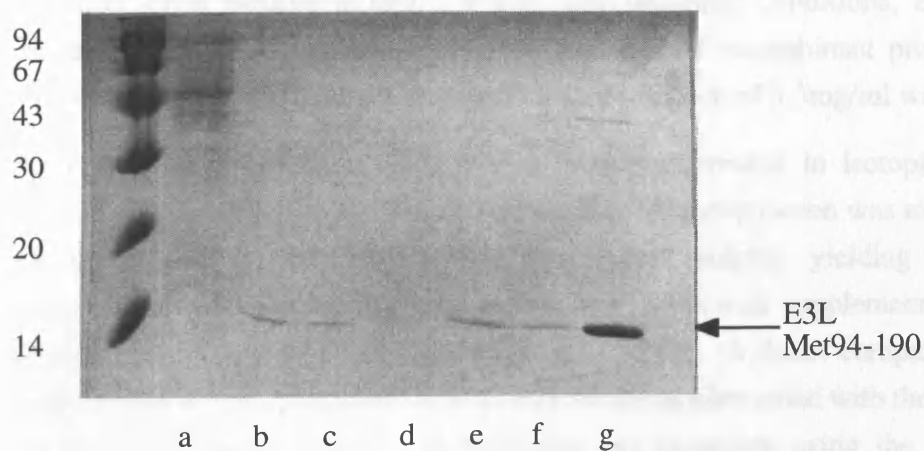
Figure 3.11.8. Purification Procedure- Extraction.

SDS PAGE analysis of the lysis of the cells and extraction of the recombinant protein.

a = Supernatant from cell lysis in sodium phosphate extraction buffer.

b - f = Extraction of the recombinant protein in 50mM NaHPO₄, pH 6.5, 1mM β - Mercaptoethanol, and 0.5mM PMSF supplemented with 300mM NaCl, 200mM NaBr and 10% glycerol by homogenisation.

g = Insoluble pellet fraction.

Figure 3.11.7.(A)**Figure 3.11.7. (B)****Figure 3.11.8.**

french press in a low salt buffer and the recombinant protein then extracted using homogenisation in a high salt extraction buffer. The NaHPO_4 , pH 6.5 high salt buffer was supplemented with 10% glycerol, to stabilise the protein and modified to 300mM NaCl and 200mM NaBr, due to the stronger chaotropic nature of NaBr. The samples extracted were then analysed by SDS PAGE (Figure 3.11.8).

Due to repetition of the high salt extraction step, SDS PAGE analysis revealed a decrease in the quantity of contaminating cellular proteins. The samples containing fewer contaminating proteins were then pooled, concentrated by ammonium sulphate precipitation (Figure 3.11.9), and applied to a pre-equilibrated Superdex 75 gel filtration column (Pharmacia).

The samples from gel filtration containing the recombinant protein, as determined by SDS PAGE analysis (Figure 3.11.10) were combined and concentrated by ammonium sulphate precipitation. The recombinant protein pellet was resuspended in 50mM NaHPO_4 , pH 6.5, 300mM NaCl, 200mM NaBr, 1mM DTT and 1% glycerol. A maximum concentration of 5.5mg/ml was achieved (Figure 3.11.11). This concentration appeared to be the solubility maximum, as once again native protein was recovered from the pellet following the initial suspension of the protein pellet following ammonium sulphate precipitation. Preliminary 1D NMR analysis indicated the protein to be folded and therefore suitable for further NMR analysis.

Expression of Isotopically Labelled Samples for NMR Analysis.

To ^{15}N isotopically label the recombinant protein, the cells were grown in M9 minimal media supplemented with $^{15}\text{NH}_4\text{Cl}$ (99.9%). Initially induction and purification conditions were reviewed using a non-labelled minimal media. Addition of IPTG was undertaken when OD_{595} reached 0.7, due to a low level of recombinant protein expression when induced at $\text{OD}_{595} = 0.4$. The induction conditions, extraction and purification procedures yielded equivalent quantities of recombinant protein as those expressed in rich 2xTY media. Once again a concentration of 5.5mg/ml was achieved.

Once optimised, recombinant E3L Met94-190 was expressed in isotopically labelled media, using $^{15}\text{NH}_4\text{Cl}$. The level of recombinant protein expression was maintained at a high level however the protein was much less soluble yielding a maximum concentration of 2.5mg/ml. To combat this, the media was supplemented with trace elements and vitamins solutions Jannsson *et al.*, 1996. A direct comparison of a ^{15}N labelled induction and extraction with the M9 media supplemented with the vitamins and trace elements was compared to an induction and extraction using the standard ^{15}N

Figure 3.11.9. Purification Procedure - Concentration.

SDS PAGE analysis of the extracted protein concentrated in preparation for gel filtration chromatography.

a-b = concentrated protein supernatant after ammonium sulphate precipitation.

c = Pooled fraction prior to concentration.

Figure 3.11.10. Purification Procedure - Gel filtration chromatography.

SDS PAGE analysis of gel filtration chromatography of the concentrated protein solubilised in 50mM NaHPO₄, pH 6.5, 1mM β - Mercaptoethanol, and 0.5mM PMSF, 300mM NaCl, 200mM NaBr, 10% glycerol.

a = Fraction eluted at 7mls.

b = Fraction eluted at 8mls.

c = Fraction eluted at 9mls.

d = Fraction eluted at 10.5mls.

e = Fraction eluted at 11mls.

f = Fraction eluted at 11.5mls

g = Fraction eluted at 12mls

h = Fraction eluted at 12.5mls.

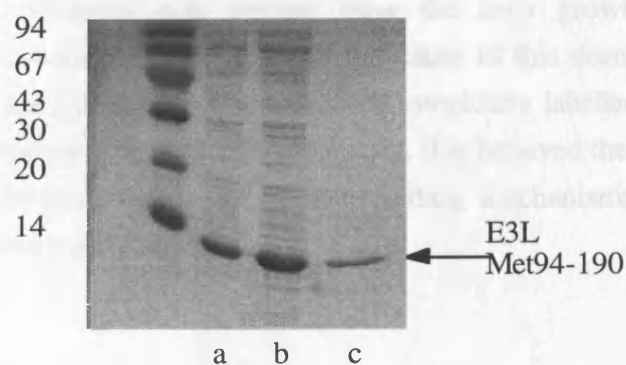
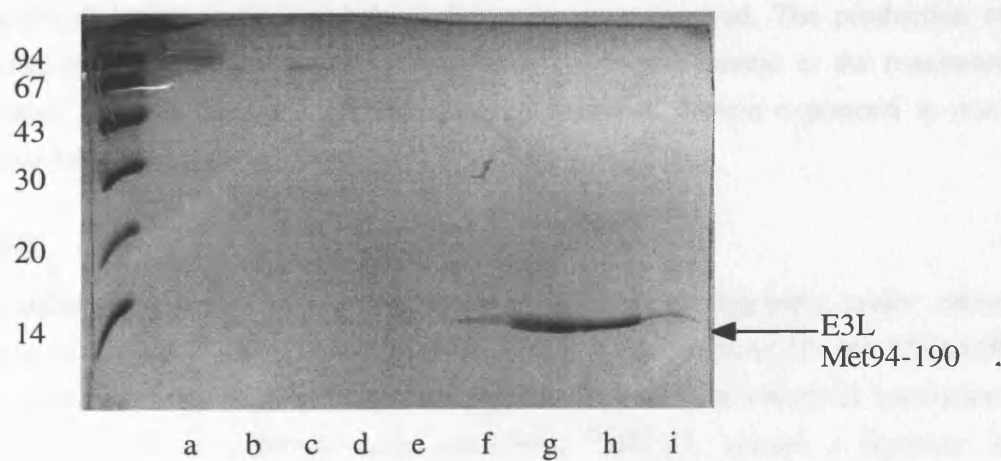
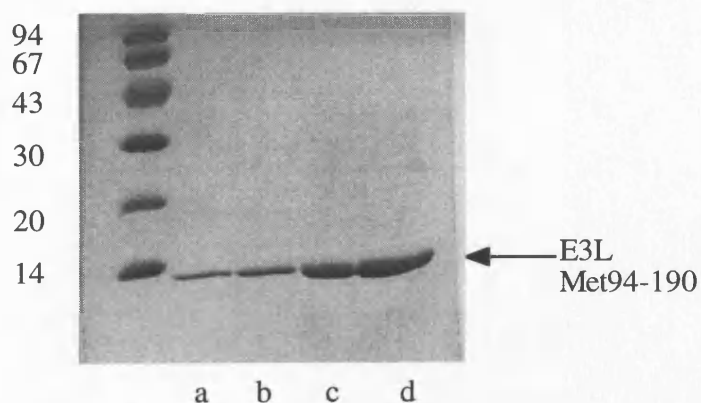
i = Fraction eluted at 13mls.

Figure 3.11.11. Purification Procedure - Concentration.

SDS PAGE analysis of the purified fractions when pooled and concentrated by ammonium sulphate precipitation and the protein then solubilised in 50mM NaHPO₄, pH 6.5, 1mM DTT, 300mM NaCl, 200mM NaBr, 1% glycerol and 0.5mM PMSF

a - b = Pooled fractions prior to concentration.

c - d = concentrated protein supernatant after ammonium sulphate precipitation.

Figure 3.11.9.**Figure 3.11.10.****Figure 3.11.11.**

labelled M9 media. When analysed by SDS PAGE (Figure 3.11.12, 3.11.13), no increase in protein solubility was evident from the cells grown in the media supplemented with the vitamins and minerals. The cause of this decrease in solubility was attributed to the ultra pure $^{15}\text{NH}_4\text{Cl}$ used in the isotopically labelled M9 media, and has been observed previously in the NMR laboratory. It is believed the incorporation of the $^{15}\text{NH}_4\text{Cl}$ must adversely effect the protein folding mechanisms within the cell leading to a less soluble protein sample.

NMR Analysis.

The results of 1D NMR analysis (Figure 3.11.14) indicated the protein was folded, and therefore suitable for further NMR analysis. 2D NOESY analysis (Figure 3.11.15) using the non isotopically labelled protein sample provided dispersed assignment peaks, however for structural resolution a third dimension was required. The production of ^{15}N labelled protein, for 3D studies, did however prove problematic as the maximum concentration achieved was lower than the concentration of protein expressed in non-isotopically labelled minimal media.

Summary.

The recombinant protein was expressed and purified to homogeneity under native conditions. A maximum concentration of 5.5mg/ml was achieved and 1D and 2D NMR analysis indicated the protein to be folded and therefore suitable for structural resolution. Growth of the cells in minimal media containing $^{15}\text{NH}_4\text{Cl}$, caused a decrease in recombinant protein solubility, which was not reversed by the addition of trace elements and vitamins. As a result of the low concentration of ^{15}N labelled protein, further NMR analysis and the structural resolution of the recombinant protein was not pursued.

Figure 3.11.12. Extraction of ^{15}N labelled recombinant protein when cells grown in the presence of additional vitamins and minerals.

SDS PAGE analysis of the stepwise extraction of the protein.

a = Crude induction.

b = Supernatant from cell lysis.

c - f = Extraction of the recombinant protein in 50mM NaHPO_4 , pH 6.5, 1mM DTT, and 0.5mM PMSF supplemented with 300mM NaCl, 200mM NaBr and 10% glycerol by homogenisation.

g = Insoluble pellet.

Figure 3.11.13. Extraction of ^{15}N labelled recombinant protein when cells grown without additional vitamins and minerals.

SDS PAGE analysis of the stepwise extraction of the protein.

a = Crude induction.

b = Supernatant from cell lysis.

c - f = Extraction of the recombinant protein in 50mM NaHPO_4 , pH 6.5, 1mM DTT, and 0.5mM PMSF supplemented with 300mM NaCl, 200mM NaBr and 10% glycerol by homogenisation.

g = Insoluble pellet.

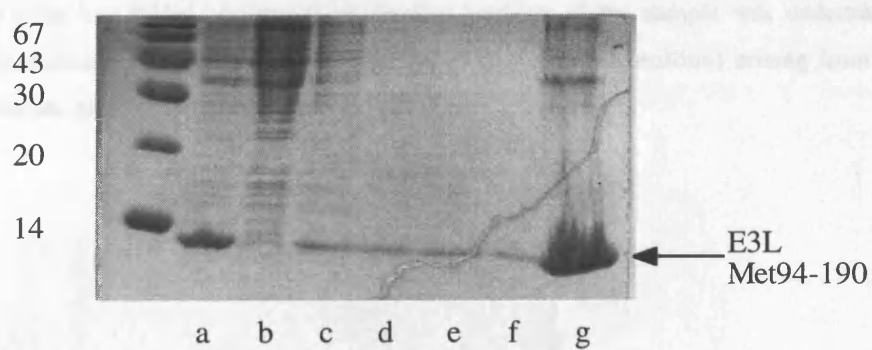
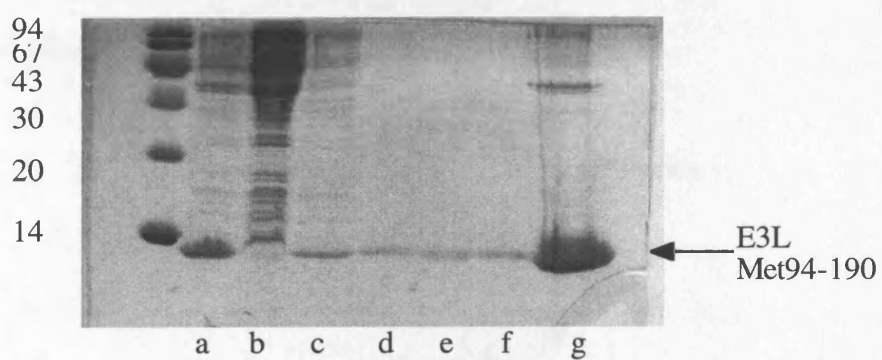
Figure 3.11.12.**Figure 3.11.13.**

Figure 3.11.14. 1D NMR analysis of E3L Met94-190. The peaks were well dispersed which indicated the protein to be in a folded conformation. Further analysis of the sample was undertaken using 2D NOESY spectroscopy. The proton chemical shifts (ppm - parts per million) arising from the chemical environments are highlighted at the bottom of the spectra.

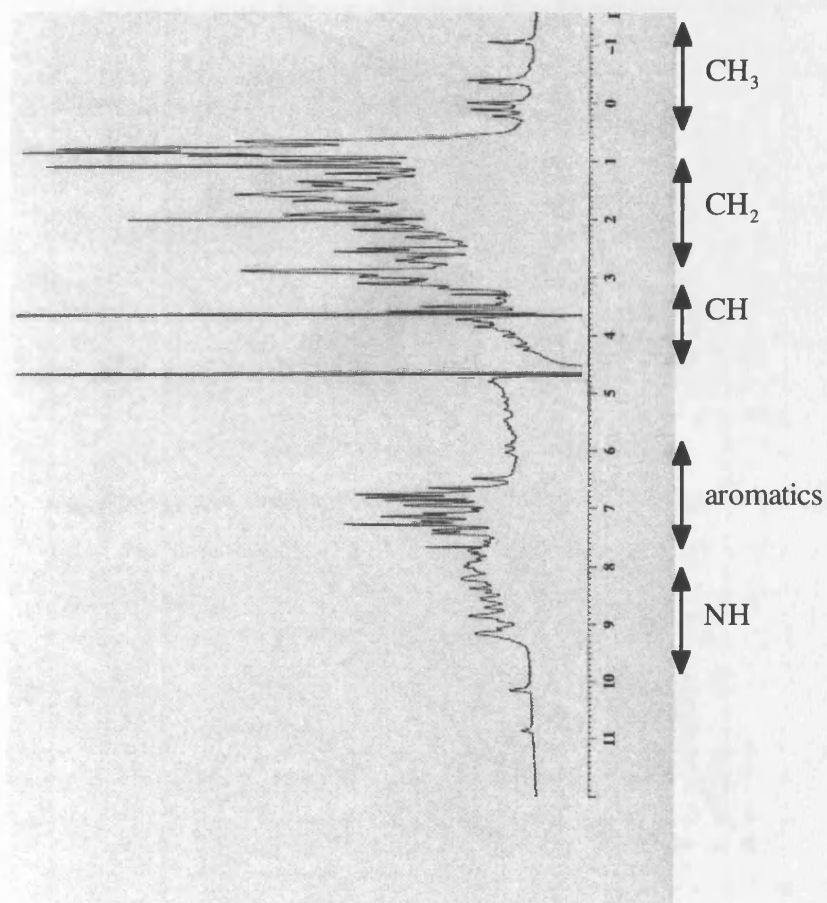
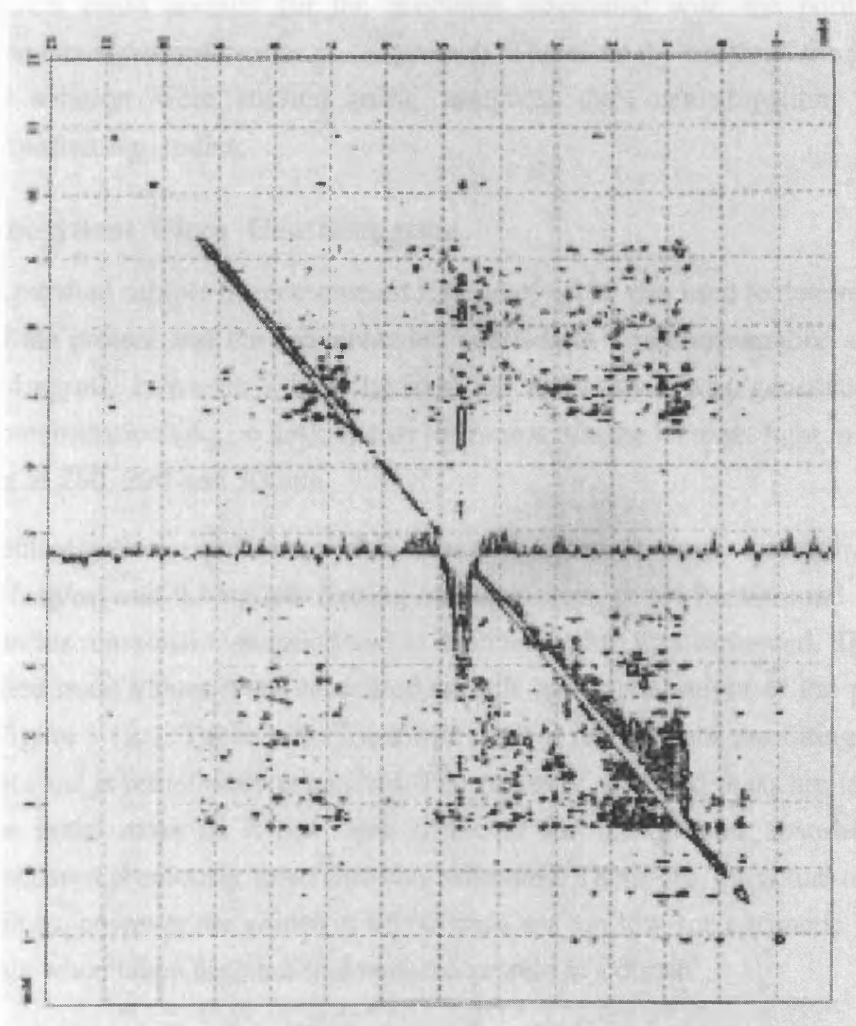


Figure 3.11.15. 2D NOESY NMR analysis of E3L Met94-190. The assignment peaks are well dispersed indicating the protein to be in a folded conformation. The signals however were insufficient for a reliable assignment of the structure and therefore a third dimension was required. The acquisition of such data from the ^{15}N labelled protein sample however did not prove succesful, due to a decrease in the final concentration.



3.4.6 Self Association of Recombinant E3L Met94-190 in solution.

PKR is known to form dimeric complexes with itself and a number of other proteins. Following incubation with glutaraldehyde, full length E3L forms multimeric complexes which could account for the problems associated with the purification and low maximum concentrations of the proteins. Therefore the nature of the protein interactions in solution were studied using analytical ultra centrifugation, gel filtration and crosslinking studies.

Analytical Ultra Centrifugation.

A purified sample of recombinant E3L Met94-190 was used to determine both the mass of the protein and the sedimentation coefficient. The concentration of the sample was 3.4mg/ml, however a high background absorbance was generated by nucleic acid contamination ($A_{260} = 2.4$), and so to combat this the incident light in the centrifuge was set at 280, 290 and 300nm.

Sedimentation equilibrium data was generated at three concentrations, 3.4mg/ml, 1.1mg/ml and 0.5mg/ml. During the acquisition of the background absorbance, some species remained in solution and as such this value was estimated. The initial mass and fitted mass values were calculated and the molecular weight of the protein determined (Figure 3.12.1, Table 3.2). The initial mass is the guess of the fitting algorithm for this data and is remarkably consistent. The values of the fitted mass are larger than those of the initial mass as it has been corrected for background absorbance, however as discussed previously this value was estimated. Therefore, the actual results are the fitted values, however the values at 25,000rpm are too low for a trimeric species and so the data when taken together indicates the protein is a dimer.

The sedimentation velocity data was also obtained at three concentrations, Table 3.2. From this, the sedimentation coefficient data revealed that the sample was composed of a single species of protein, that was a size compatible with a dimeric form of E3L Met94-190. The sedimentation coefficient of the protein was found to be 1.6S (Figure 3.12.2).

FPLC Gel Filtration Chromatography.

The analytical ultracentrifugation results are consistent with the results of the gel filtration step of the purification. The recombinant protein was consistently fractionated at 12-13mls (Figure 3.12.3) which when compared to the calibration curve for the Superdex 75 column (Figure 3.12.4) is consistent with the MW of a dimeric form of

Figure 3.12. Self Association of Recombinant VV E3L dsRBM in solution.

Figure 3.12.1. Sedimentation Equilibrium Analysis.

The lower panel is the concentration distribution profile of the protein at equilibrium at 25,000rpm, as measured at 300nm. The upper panel represents the distribution of the residuals for this fit.

Figure 3.12.2. Sedimentation Coefficient Analysis.

The lower panel represents the $g(s^*)$ obtained from the sedimentation velocity data. The upper panel represents the distribution of the residuals for this fit.

Table 3.2 Highlights the data used to determine the sedimentation equilibrium and sedimentation coefficient.

Sample	Baseline Absorbance 290nm	Baseline Absorbance 300nm	Initial mass at 15K rpm, 300nm	Fitted mass at 15K rpm, 300nm	Initial mass at 25K rpm, 300nm	Fitted mass at 25K rpm, 300nm	Sedimentation coefficient,s (S)
3.4mg/ml	0.2	0.092	23,500	32,594±379	21,334	26,952±233	1.8367± 0.0057
1.1mg/ml	0.067	0.043	22,573	32,453±1589	22,324	30,958±527	1.6550± 0.0028
0.5mg/ml	0.039	0.02	22,544	31,482±2323	19,763	25,657±1089	1.5977± 0.017

Figure 3.12.1. Sedimentation Equilibrium Profile Analysis. The centrifuge is operated until a balance is established between the sedimentation of the protein, which is under the influence of centrifugal force, and the diffusion of the protein in the opposite direction. The concentration distribution profile of the protein (lower panel) at 25,000rpm as measured at 300nm, can then be used to determine the MW of the protein (see section 2.4.12). The upper panel highlights the distribution of the residuals for this fit. The presence of a single species of protein that does not deviate from the single concentration gradient produced indicates the protein sample contains only dimeric proteins.

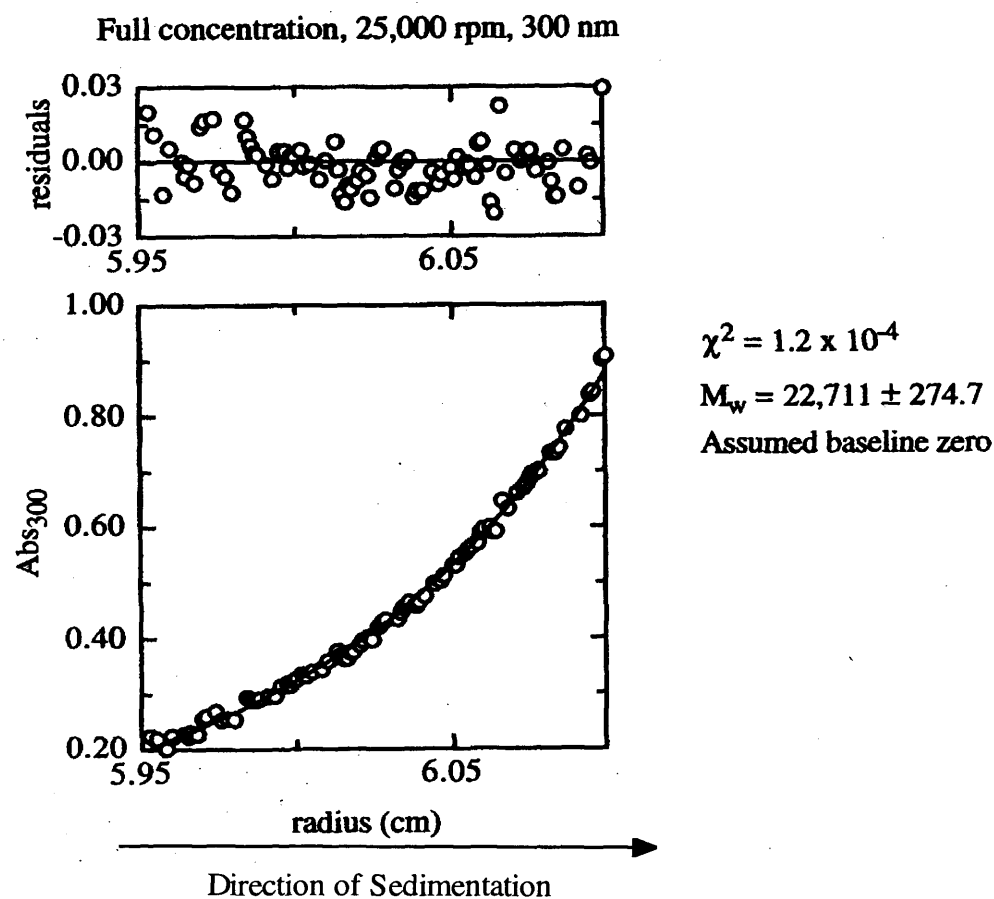


Figure 3.12.2. Sedimentation Coefficient Analysis derived from Sedimentation Velocity Data. The centrifuge is operated at high speeds which cause the randomly distributed particles to migrate outwards from the centre of rotation. A boundary is formed between the solvent that is clear from particles and the solvent which still contains sedimenting materials. The rate at which the boundary moves through the cell can then be measured and the data analysed by the $g(s^*)$ method (section 2.4.12). This gives rise to an apparent distribution of sedimentation coefficients shown by the sedimenting population from which the S value can be obtained. The upper panel highlights the residuals for this fit. Once again the sample is monodisperse indicating a single population is present.

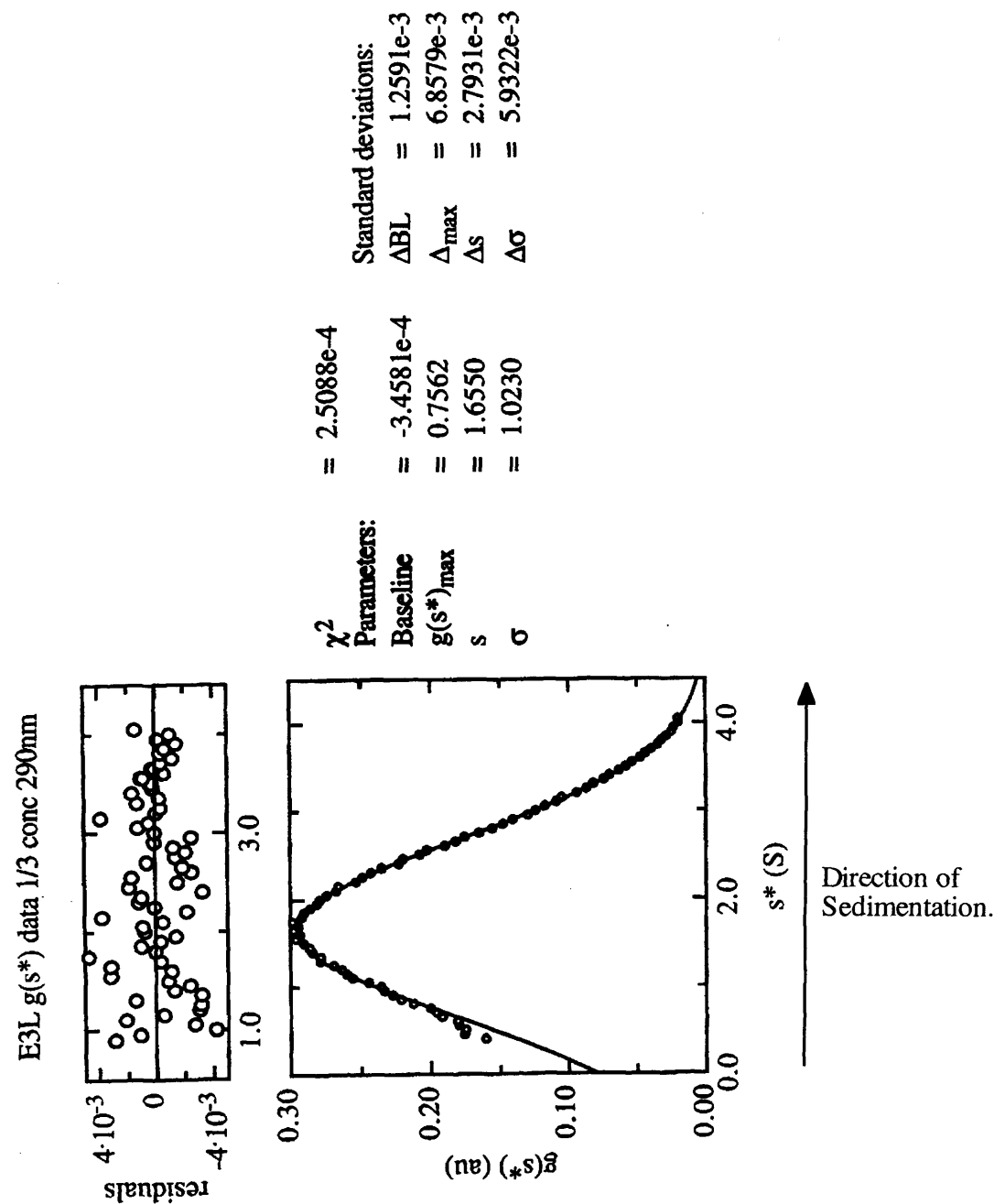


Figure 3.12.3. Elution profile of FPLC gel filtration.

Peaks depict increase in A_{280} (protein concentration) as a function of the elution volume.

Figure 3.12.4. Calibration Curve for the Superdex 75 Gel Filtration Column.

Specific proteins in 0.15M NaCl in TE, with known MW's were fractionated by gel filtration at 0.33ml/min. A calibration curve was then produced using the data obtained from the fractionation.

Figure 3.12.3.

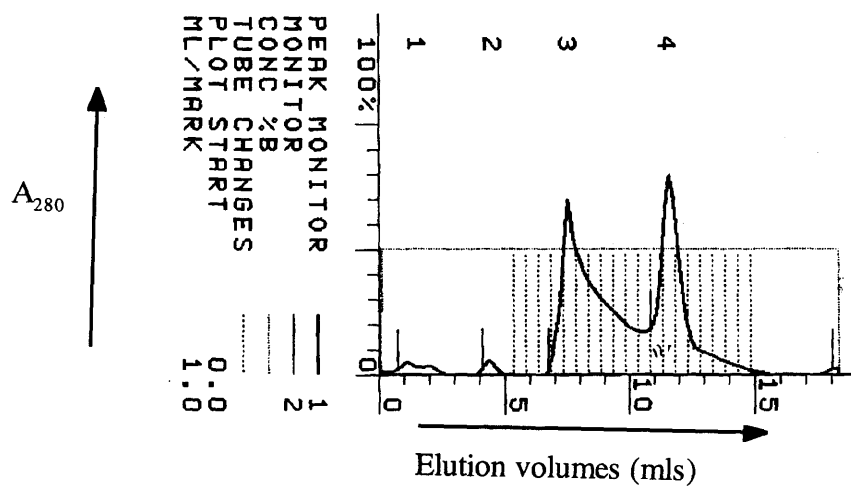
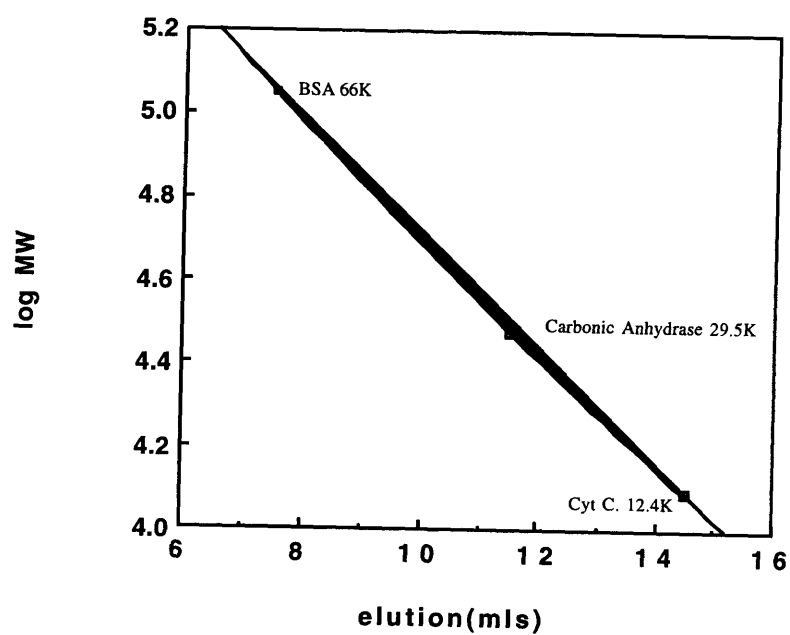


Figure 3.12.4.



the recombinant protein. The first peak on the absorbance trace depicts the fractionation of the large MW cellular contaminating proteins which are clearly visible by SDS PAGE and coomassie blue staining (Figure 3.11.10). Further to this, western blot analysis, of the gel filtration fractions confirmed the recombinant protein was present only in 12-13ml fractions verifying only a single dimeric species was present (Figure 3.12.5).

Crosslinking Studies.

A purified fraction of recombinant E3L Met94-190 was incubated with increasing concentrations of glutaraldehyde. The results were then analysed by SDS PAGE (Figure 3.12.6). The electrophoretic mobility of the recombinant protein was modified as a function of glutaraldehyde concentration. Intermolecular crosslinking was evident at 0.005% glutaraldehyde with the appearance of a ladder of species which corresponded to the formation of dimers, trimers, tetramers and multimers. These results however are in contrast to the results obtained by AUC and gel filtration.

Figure 3.12.5. Western Blot analysis of FPLC Fractions

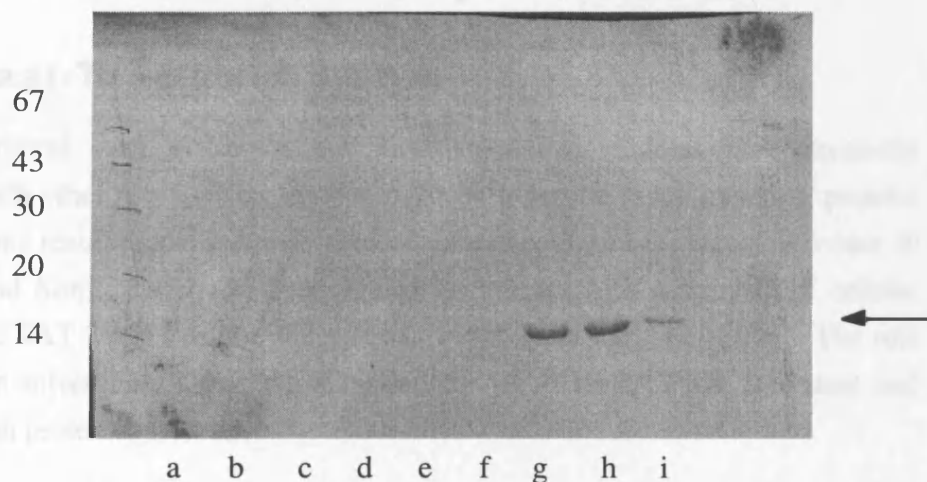
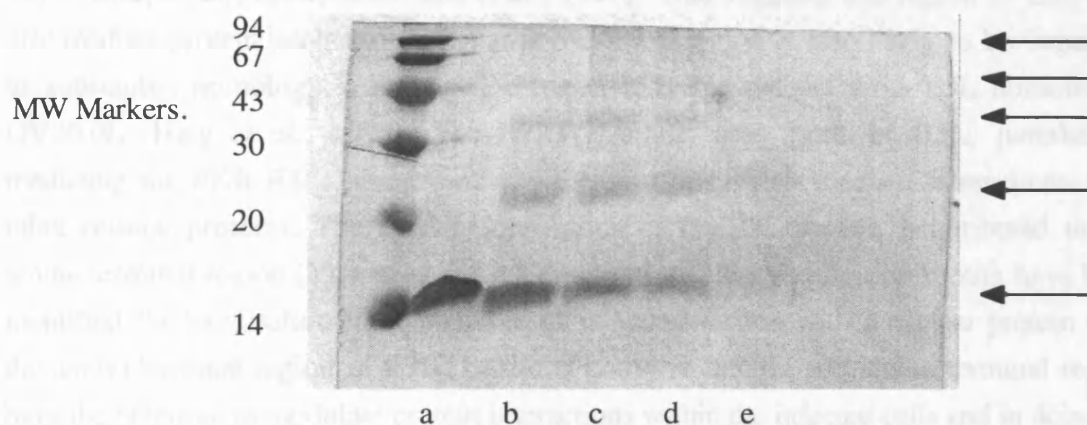
Western blot analysis using the monoclonal antibody TW3.2. The fractions used for this western are depicted by SDS PAGE analysis in Figure 3.11.10.

- a = Fraction eluted at 7mls.
- b = Fraction eluted at 8mls.
- c = Fraction eluted at 9mls.
- d = Fraction eluted at 10.5mls.
- e = Fraction eluted at 11mls.
- f = Fraction eluted at 11.5mls
- g = Fraction eluted at 12mls
- h = Fraction eluted at 12.5mls.
- i = Fraction eluted at 13mls.

Figure 3.12.6. Glutaraldehyde Crosslinking Studies.

SDS PAGE analysis of 5µg of recombinant protein solubilised in 50mM NaHPO₄, pH 6.5, 1mM DTT 300mM NaCl, 200mM NaBr and 0.5mM PMSF crosslinked with increasing final concentrations of glutaraldehyde.

- a = no glutaraldehyde.
- b = 0.005% glutaraldehyde and protein.
- c = 0.01% glutaraldehyde and protein.
- d = 0.02% glutaraldehyde and protein.
- e = 0.05% glutaraldehyde and protein.

Figure 3.12.5.**Figure 3.12.6 .**

4. Results. Yeast-Two-Hybrid Screen.

4.1 The Yeast-Two-Hybrid System

The yeast-two-hybrid system permits the identification of proteins that physically associate with each other *in vivo*. This system relies on a genetic assay in which protein-protein interactions result in the reconstitution of a functional transcriptional activator in yeast, (Fields and Song, 1989). PKR is known to interact with a number of cellular proteins such as STAT 1 and PACT (Wong *et al.*, 1997, Patel and Sen, 1998). The role of E3L within an infected cell, as determined so far, is to inhibit PKR activation and therefore maintain protein synthesis.

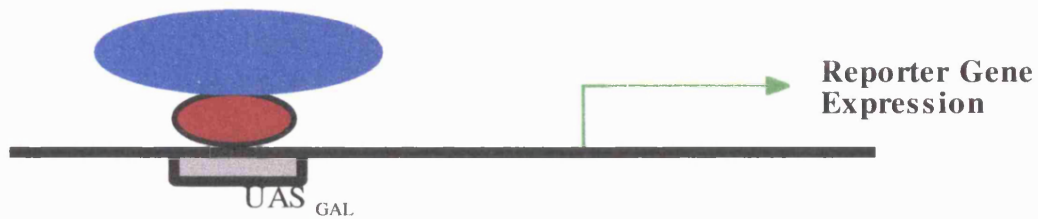
As previously discussed, sequence and predicted structure of E3L is similar to the dsRBM of PKR (Ho and Shuman, 1996). The dsRBD of PKR mediates the homodimerisation of PKR as well as interactions with STAT 1, E3L and TRBP (Wong *et al.*, 1997, Sharp *et al.*, 1998, Benkraline *et al.*, 1997). This suggests this region of E3L may also mediate protein interactions. The amino terminal region is also likely to be important as substantial homology is exhibited between E3L and the orf virus E3L homologue, OV20.0L (Haig *et al.*, 1998). The PPRWF amino acid motif of E3L, partakes in mediating the PKR-E3L interaction, and therefore may also mediate interactions with other cellular proteins. The nuclear localisation of the E3L protein is attributed to the amino terminal region (Yuwen *et al.*, 1993). As no nuclear localisation motifs have been identified, the localisation could be the result of an interaction with a nuclear protein with the amino terminal region of E3L. In short, both the dsRBD and amino terminal region have the potential to modulate protein interactions within the infected cells and in doing so may enhance VV replication. The yeast-two-hybrid screen was therefore undertaken to examine physical interactions between E3L and specific cellular proteins

4.1.1 The Basis of the Yeast-Two-Hybrid System.

Some yeast transcription factors are composed of two separable domains both of which are required for transcriptional activation. One domain, the DNA binding domain, binds to the upstream activation sequence (UAS). The second domain, the activation domain, then facilitates the assembly of the transcription complex resulting in transcription initiation. Individual expression of the two components can activate transcription provided the two are in sufficiently close proximity to form a non-covalent interaction. If the domains do not form a non-covalent interaction, the units are transcriptionally inactive. The two domains are brought into such proximity if each is expressed as fusion proteins to other individual proteins that physically interact (Figure 4.1).

Figure 4.1 Overview of the mechanics of the yeast-two-hybrid system.

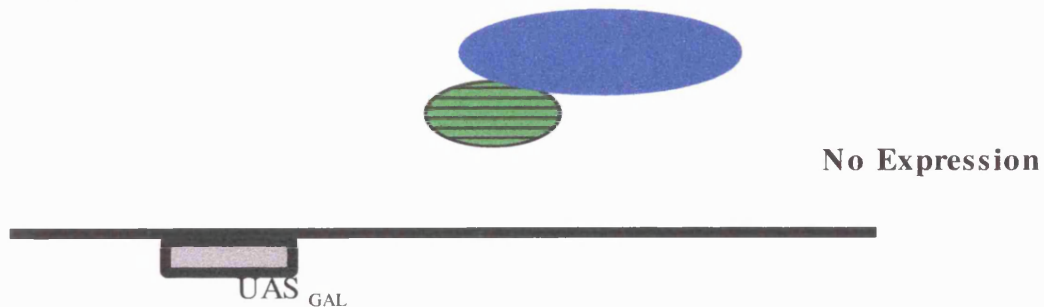
A. Native GAL 4



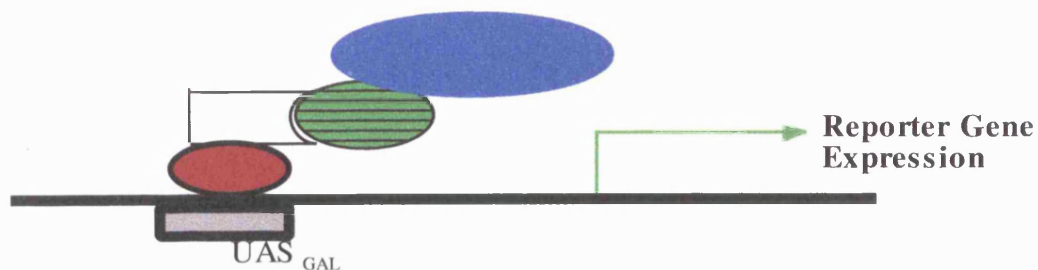
B. GAL 4 DNA BD alone and fusion protein



C. GAL 4 AD alone with fusion



D. Expression of both DNA BD and AD domain and the two fusion proteins. Due to the proteins interaction the two GAL 4 domains are in sufficient proximity to activate transcription.



Specific features of the yeast-two-hybrid system provide a simple means of assessing transcriptional activation by the use of two reporter genes located downstream of the transcription start site. The first reporter gene is a nutritional marker gene *HIS3*, and the second *Lac Z*, yields a standard colour based assay. Thus, yeast cells containing interacting proteins fused to the transcription factor can grow in the absence of histidine (His) and turn blue in the presence of a chromogenic substrate (Durfee *et al.*, 1993). Upon confirmation of the expression of the two reporter genes, false positives can be eliminated by means of a yeast genetic assay utilising the mating phenotype of yeast, which will be discussed in more detail in section 4.1.3.

4.1.2 Applications of the Yeast-two-hybrid System.

As discussed earlier the yeast-two-hybrid system provides a means of investigating protein-protein interactions *in vivo*, however the system can be utilised in a number of ways. The system can be used to verify an interaction between two proteins that are known to interact (Fields and Song 1989) and also identify novel interactions for the protein of interest (Chien *et al.*, 1991). Once the interaction has been confirmed the two proteins can be mutated or regions deleted to ascertain the exact regions required for the interaction *in vivo*. Each of these applications have been used to study PKR by Patel *et al.* (1995), who initially characterised PKR homo-dimerisation. The dimerisation region was then characterised, followed by the identification of PACT, a cellular dsRNA binding protein that interacts with PKR itself (Patel *et al.*, 1995, 1996, 1998).

4.1.3 The Yeast-Two-Hybrid Screen.

The experimental features required to facilitate the characterisation of interactions between a 'bait' protein and a protein derived from a cDNA library shall now be highlighted. A number of yeast-two-hybrid screen designs and reagents are available commercially, but perhaps the most popular is the GAL-4 yeast transcriptional activator system, which shall now be discussed (Figure 4.2).

Vectors.

Both a DNA binding domain (GAL-4 DNA-BD) expression plasmid and an activation domain (GAL-4 AD) expression plasmid were required for the screen. A number of GAL-4 DNA-BD plasmids are available commercially although those plasmids used most extensively are pAS1 and pAS2 (Bai and Elledge, 1996). A number of GAL-4 AD plasmids, containing the cDNA library clones of a variety of cell lines and sources are also available commercially. Each of the two plasmids carry nutritional markers for selection in yeast, the GAL-4 DNA-BD expressed *TRP1* and GAL-4 AD expressed *LEU2*, thus supporting growth in media lacking tryptophan and leucine respectively. The

two plasmids also contain a bacterial origin of replication and ampicillin resistance for bacterial expression and maintenance.

Strains.

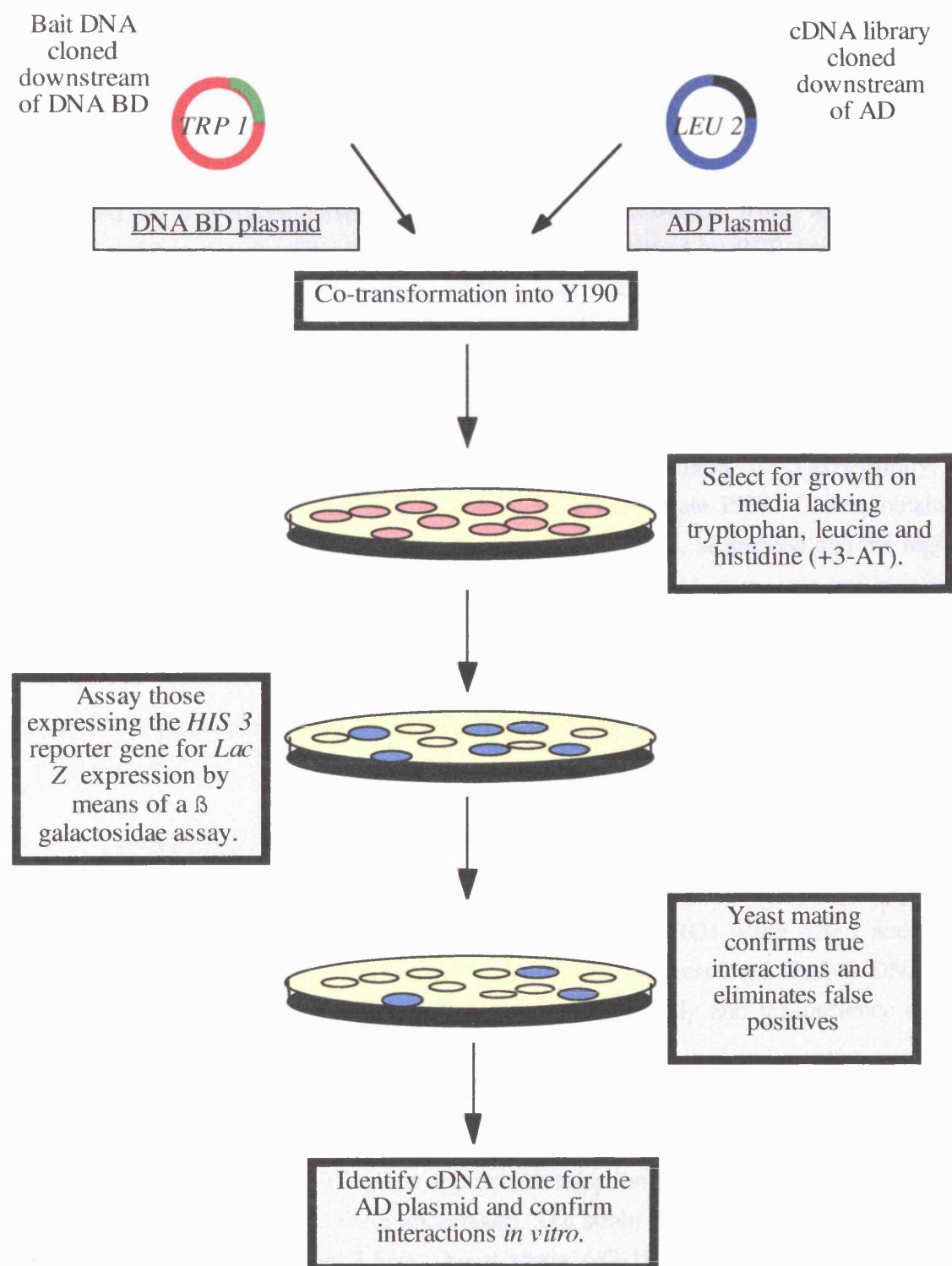
A number *S.cerevisiae* strains can be used for the screen provided the reporter genes are under the control of the same UAS to which the DNA-BD protein binds. All strains carry the *ade2-101* mutation, which confers a pink colour after several days due to the block in the adenine biosynthetic pathway. Small white colonies form at a rate of 1-2% due to spontaneous mutations that eliminate mitochondrial function, and should always be avoided (Holm *et al.*, 1993). The mating type of the yeasts should be noted as further verification of the interaction between the two proteins may utilise the ability of yeast to mate with the opposite mating type. On most occasions the yeast strain Y190 is used due to high *Lac Z* reporter activity.

The Screen.

Co-transformation of the plasmids into yeast and growth on the appropriate media, - Trp/His/Leu, provides identification of the first reporter gene, *HIS3*. 3-AT (3-amino-1,2,4 triazole) is required in the media lacking histidine as the insertion of the *HIS3* gene at the GAL UAS leads to an increased basal level of *HIS3* expression that is sufficient for growth on media lacking histidine. 3-AT at a concentration between 25 and 50mM inhibits the expression of a *HIS3* encoded enzyme leading to a reduced basal level of expression and thus only positive interactions are capable of growth on this media. The second reporter gene, *Lac Z*, encodes β galactosidase which is assayed using X-Gal. A positive result is observed if the colony turns blue in a colony lift assay (Figure 4.2).

Elimination of False Positives.

The system is prone to false positives due to the inappropriate activation of the reporter genes. The exact mechanisms are not fully understood although it may be due to non-specific interactions or interactions with other DNA binding proteins at a particular promoter. The elimination of the false positives can be achieved by analysing the ability of the library clone to activate transcription when in contact with the 'bait' protein alone and not another unrelated 'bait' protein. This process can be carried out efficiently by utilising the yeast mating assay (Harper *et al.*, 1993). The mating assay is a convenient means of bringing the two plasmids together, which are located in separate yeast strains of the opposite mating type. The opposite strains can then mate and if the two separately expressed proteins interact diploid formation is visible on media supplemented with 3-AT and lacking tryptophan, leucine and histidine. Colonies are then assayed for β -

Figure 4.2 Overview of a Yeast-two-hybrid screen.

galactosidase activity due to the reporter gene expression. By this means the AD cDNA clone can be analysed for an interaction with the bait protein and a number of other unrelated proteins in one simple experiment (Sections 2.5.15, and 2.5.16).

Identification of the cDNA Interacting Clone.

Once the interaction between the DNA-BD and the AD expressed proteins has been confirmed as a positive interaction, the plasmid is extracted from the yeast and transformed into *E.coli*. The plasmid cDNA is then amplified by PCR and the positive clones grouped according to their size and then sequenced. BLAST homology searching may then identify or give clues to an identity of the sequence.

4.2 Introduction.

A yeast-two-hybrid screen was therefore undertaken using a HeLa AD-cDNA library, as VV grows in this cell type and E3L is known to downregulate PKR. Clones obtained from the screen were confirmed as authentic interacting clones, sequenced and the region of E3L that mediated the interaction characterised further.

4.3 Preparation for the Yeast-Two-Hybrid Screen.

A number of protocols were required to be undertaken prior to the library screen to ensure a valid screen.

4.3.1 Assembly of the Expression Constructs.

The HeLa cDNA library inserts cloned individually into the GAL-4 AD plasmid (pGAD GH) was available commercially. E3L was excised from pSRO1 using *Nde* I and *Bam* HI. The fragment was then cloned into the *Nde* I/*Bam* HI restricted GAL-4 DNA-BD plasmid (pAS2-1). The plasmid was then sequenced manually and the presence of the E3L insert at the *Nde* I site verified (data not shown).

4.3.2 Yeast Control Transformations and Strain Verification.

Yeast phenotypes were verified on the appropriate selection media and a number of small scale control transformations were undertaken. The strain verification was compared to the expected results (section 2.5.7). Yeast strain CG-1945 did not grow under the expected conditions, and therefore was not used for this screen. The control transformations ensured the E3L protein did not behave as a transcriptional activator once fused to the GAL 4 DNA-BD, the two reporters, *lacZ* and *HIS3*, were expressed only when two plasmids expressing two interacting proteins were transformed, and the yeast transformation efficiency was sufficient for the library screen itself.

4.3.3 Preparation of the AD library.

The titre of the library was calculated to be 1×10^{10} cfu/ml. The required quantities of *E. coli* library stock, 1.2µl ($= 1.2 \times 10^7$) which contained twice the number of independent clones, was then grown on plates and amplified for 3 hours in 2xTY media. The plasmid DNA was extracted by a CsCl maxi prep., concentrations calculated and the DNA stored at -20°C in preparation for the screen, (section 2.5.5).

4.4 The Library Screen.

The DNA-BD/E3L plasmid and AD/library plasmids were transformed into the yeast strain Y190 simultaneously using a library scale procedure. 0.58mg of the AD/library plasmid and 2mg of the DNA-BD/E3L plasmid were used. The suspension was then plated onto 50 SD-Trp/His/Leu plates (150mm in diameter) and various dilutions prepared and plated onto SD-Trp/Leu plates (100mm) to calculate the co-transformation efficiency as discussed in section 2.5.11.

Two small scale control transformations were undertaken alongside the library screen using aliquots of the cells prepared for the library screen. The plasmids pVA3-1 and pTD1-1, expressing proteins that are known to interact, were co-transformed and the plasmid pC1-1 expressing the full length GAL 4 protein was also transformed individually. The suspensions were plated onto SD-Trp/His/Leu, SD-Trp/Leu, SD-Leu and SD-Trp plates.

Following incubation at 30°C for five days 60 colonies were visible, however after a longer incubation a further 38 colonies were observed on those SD-Trp/His/Leu plates from the library screen. The transformation efficiencies of both the library screen and the small scale transformations were calculated.

Small Scale Transformation.

Co-transformation of pVA31-1 and pTD1-1.

117 colonies visible on SD-Trp/Leu plate.

500µl total suspension volume.

100µl plated

No dilution factor.

0.1µg of limiting plasmid DNA

$$\frac{117 \times 500}{100 \times 1 \times 0.1} = 5850 = 5.85 \times 10^3 \text{ cfu } / \mu\text{g}$$

Library Scale Transformation.

Co-transformation of AD/library (pGAD GH + cDNA inserts) and DNA-BD/E3L (pAS2-1/E3L).

313 colonies visible on SD-Trp/Leu plate.

10ml total suspension volume.

100μl plated

Dilution factor 1/1000 (0.001).

580μg of limiting plasmid DNA

$$\frac{313 \times 10,000}{100 \times 0.001 \times 580} = 5.4 \times 10^4 \text{ cfu}/\mu\text{g}$$

The number of independent clones screened therefore

= co-transformation efficiency x amount of AD/library plasmid used

= $5.4 \times 10^4 \times 580 = \underline{3.13 \times 10^7 \text{ clones screened.}}$

For an efficient library screen 2-3 times the number of independent clones in the library were to be screened, 1.2×10^7 . The actual number of clones screened therefore was approximately 2.5 times the number of independent clones.

4.5 Verification of the Positive Interacting Clones.

Growth of the transformed colonies on SD-Trp/His/Leu supplemented with 3-AT exhibited the expression of the first reporter gene *HIS3* (*his*⁺). The expression of the second reporter gene, *lacZ*, was then assayed as the *HIS3* reporter is susceptible to leaky expression.

4.5.1 β Galactosidase Colony Lift Assay.

Each of the 60 colonies derived from the library screen were assayed for β galactosidase activity using the colony lift assay. Following re-incubation of the plates at 30°C, to permit re-growth of these colonies, a further 38 colonies were observed (section 4.4). The original 60 colonies were then re-assayed along with the 38 new colonies. The yeast colonies were then re-streaked, incubated at 30°C and assayed once more to ensure only one AD/library plasmid was present in the cell. Table 4.1 highlights the results of the three initial β galactosidase assays prior to the storage of the samples as glycerol stocks at -70°C.

Table 4.1. Preliminary β galactosidase assays which ensured the segregation of the AD/library plasmid within each clone exhibiting growth on -Trp/His/Leu plates.

Clone	β Gal 1	β Gal 2	β gal 3	Growth	Clone	β Gal 1	β Gal 2	β gal 3	Growth	Clone	β Gal 1	β Gal 2	β gal 3	Growth
1	-	+	+	+	34	new	-	+	+	67	new	+	+	+
2	+	+	+	+	35	new	+	+	+	68	+	+	+	+
3	+	+	+	+	36	new	+	+	+	69	+	+	+	+
4	+	+	+	+	37	new	+	+	+	70	+	+	+	+
5	+	+	+	+	38	new	+	+	+	71	+	+	+	+
6	+	+	+	+	39	new	+	+	+	72	+	+	+	+
7	+	+	+	+	40	new	+	+	+	73	+	+	+	+
8	new	-	-	+	41	new	+	+	+	74	+	+	+	+
9	+	+	+	+	42	new	+	-	+	75	+	+	+	+
10	-	-	+	+	43	new	+	+	+	76	+	+	+	+
11	+	+	+	+	44	+	+	+	+	77	+	+	+	+
12	-	-	-	-	45	+	+	+	+	78	+	+	+	+
13	+	+	+	+	46	+	+	+	+	79	+	+	+	+
14	+	+	+	+	47	+	+	+	+	80	+	+	+	+
15	+	+	+	+	48	+	-	-	+	81	+	-		+
16	+	+	+	+	49	+	+	+	+	82	+	+		+
17	+	+	+	+	50	-	+	+	+	83	+	+	+	+
18	new	+	+	+	51	+	+	+	+	84	-	+	+	+
19	new	-	+	+	52	+	+	+	+	85	+	+	+	+
20	new	+	+	+	53	new	+	+	+	86	+	+	+	+
21	new	+	+	+	54	new	+	+	+	87	new	+	+	+
22	new+	+	+		55	+	-	+	+	88	+	+		+
23	new	-	-	-	56	+	-	+	+	89	+	-	+	+
24	+	+	+	+	57	+	+	+	+	90	+	-	+	+
25	new	+	+	+	58	new	-	+	+	91	+	+	+	+
26	new	-	-	-	59	new	+	+	+	92	new	+	+	+
27	new	+	+	+	60	new	+	+	+	93	+	+	+	+
28	new	+	+	+	61	+	+	+	+	94	+	-	+	+
29	+			+	62	+	+	+	+	95	new	-	-	-
30	new	-	-	-	63	+	+	+	+	96	new	new	+	+
31	new	+	+	+	64	+	+	+	+	97	new	new	+	+
32	+	+	+	+	65	new	-	+	+	98	new	new	+	+
33	new	+	+	+	66	new	+	+	+					

4.5.2 Elimination of Duplicate Clones Derived from the Screen.

Once the clones had been assayed for expression of the second reporter gene, a number of methods were used to characterise the clones further. It was conceivable that a number of the clones were duplicates, and so work was undertaken to group analogous clones prior to verification of the interaction by yeast mating.

Preparation of the AD/library Plasmid.

For this work to be undertaken the plasmid had first to be extracted from the yeast using the yeast mini prep procedure. The crude DNA extraction yielded both plasmid and chromosomal DNA from the yeast, and therefore the quantity of the plasmid DNA could not be examined. The extracted DNA was then electroporated into *E. coli* HB101. This strain contains the LeuB6 mutation which is complemented by the LEU2 gene located on the AD/library plasmid and as such promoted the amplification of the plasmid, which was then extracted by a bacterial mini prep procedure.

The electroporation efficiency of the positive control plasmid into HB101 was between 6×10^6 and 9.8×10^7 . The plasmid DNA extracted from the transformed cells was then used for PCR and/or DNA sequencing reactions.

PCR Amplification of the insert cDNA in the AD/Library plasmid.

A number of clones were chosen at random, that exhibited the expression of both reporters. The cDNA insert region of these clones were amplified and the products electrophoresed through agarose gels. The products could then be compared and grouped according to the size of the amplification product.

The two primers required for this amplification were designed by Clontech (pSR03 and pSR04). Initially the PCR reactions were undertaken using the sequencing forward primer in place of pSR03. This primer was much shorter than pSR03 and as such annealing temperatures were decreased to 50°C. A large proportion of the reactions did not amplify a single PCR product and a number of faint bands were visible after agarose gel electrophoresis. It was therefore decided to use the forward primer designed by Clontech specifically for PCR amplification which would provide higher annealing temperatures and enhanced specificity. The primers oSR03 and oSR04 were then used for PCR cycling reactions under the conditions, described in section 2.5.17. Some single amplification products were visible, however doublets were visible in some samples, and in other amplifications no product was visible at all. Hot start PCR was used as a means to combat the poor amplification results, however no improvement was visible (data not shown). This therefore seemed an unacceptable means of grouping clones, and so alternative methods were utilised.

Sequencing of the insert cDNA in the AD/Library plasmid.

T-tract sequencing of the AD/library plasmid was then used as a means of grouping alike clones. Manual sequencing of the plasmid at the insert region using the f/ward sequencing primer was undertaken.

A number of clones were used for T-tract DNA sequence analysis, however the data was insufficient for sequence similarity analysis. This was initially believed to be due in part to the quality of the DNA derived from a mini prep., and so large scale CsCl DNA preps were undertaken. Once again the data obtained was insufficient for sequence similarity analysis (Figure 4.3).

A single clone (20) was sequenced using all 4 ddNTPs, and a section of the sequence was obtained. When a BLAST database search was undertaken, the sequence was found to be a section of E3L DNA (Figure 4.4). A comparison of the T-tract sequence of clone 20 with the T-tract sequence of clones 21, 24, 25 and 36 found each of the T-tract sequence to be identical to 20.

Outcome of Elimination of Duplicate Clones.

The cause of the variable PCR amplifications and poor DNA T-tract sequencing was attributed in part to the presence of the DNA-BD/E3L plasmid, which was not eliminated from the yeast and/or bacteria as expected. This method of identification had therefore failed and it was then decided to return to the original glycerol stocks and undertake yeast mating experiments on each of the individual 98 clones. The elimination of the DNA-BD/E3L plasmid was verified prior to undertaking the mating experiment, from which genuine interacting clones were identified. Following the mating experiment, analogous clones were grouped according to size, following PCR amplification.

4.5.3 Identification of Genuine Interacting Clones.**Elimination of the DNA-BD/E3L Plasmid.**

The glycerol stocks were streaked onto -Trp/His/Leu plates and the plasmids then segregated in yeast by removing -Trp selection, section 2.5.15. The yeast therefore contained the AD/library plasmid alone as they could no longer sustain growth on-Trp plates.

Colonies were cultured in -Leu media and then -Leu plates to remove the DNA-BD/E3L plasmid. To verify the elimination of the DNA-BD/E3L plasmid 24 colonies were streaked in duplicate onto -Leu and -Trp/Leu plates in a grid fashion. The colonies growing on the -Leu plates alone were then assayed for β -galactosidase activity. Those

Figure 4.3. Manual sequencing autoradiogram of AD/Library plasmids derived from the yeast-two-hybrid screen. A small section of AD/Library clone 34 is highlighted below. The poor quality of the sequencing reaction is clearly visible, however the cause was not fully understood. Control sequencing reactions produced clear sequence data, suggesting contamination by a second plasmid or contaminants derived from the yeast could be the cause.

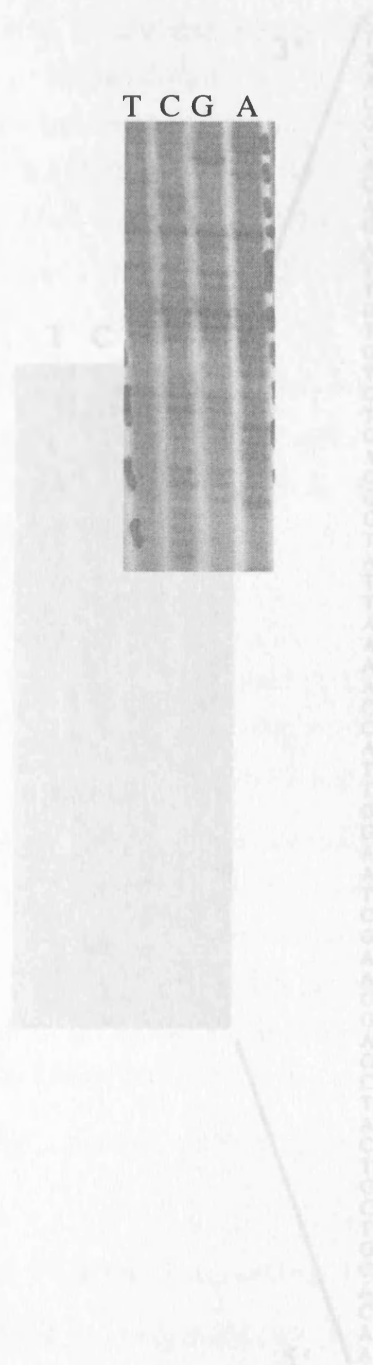
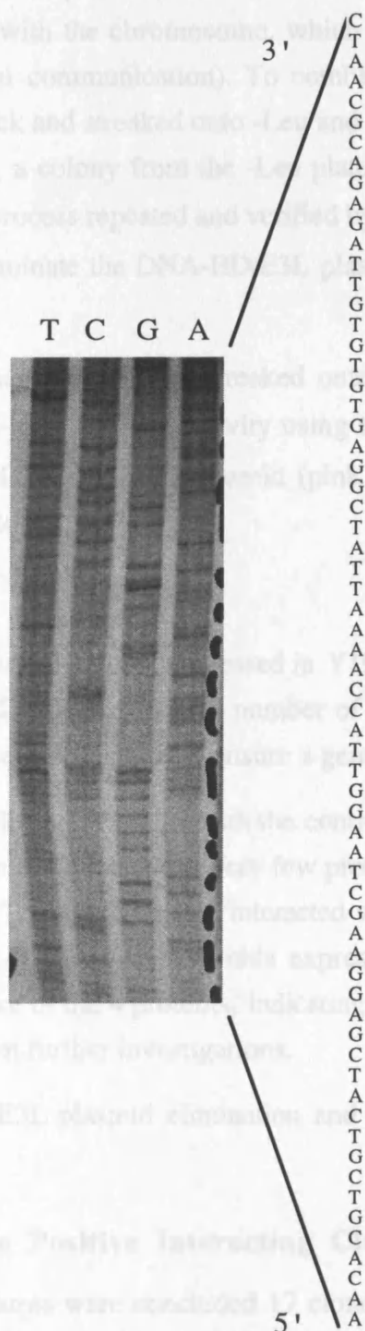


Figure 4.4. Manual sequencing autoradiogram of AD/Library plasmids derived from the yeast-two-hybrid screen. A small section of AD/Library clone 20 is highlighted below. Once again the data is of poor quality, however some sequence was derived from the autoradiogram, as depicted below. The E3L sequence obtained from this data indicates substantial contamination of the plasmid preparation by the GAL-4 BD/E3L plasmid which was believed to have been eliminated.



colonies that exhibited a positive result (blue colour) were not used for the yeast mating assay as the DNA-BD/E3L plasmid was still present, activating the reporter gene. Therefore those colonies that grew on the -Leu plate alone and exhibited a negative β galactosidase assay result were used for the yeast mating assay (Figure 4.5).

On occasions the DNA-BD/E3L plasmid was not removed by this method as it was believed to have recombined with the chromosome, which is possible after one selection event (A.Cashmore, personal communication). To combat this the clones were taken straight from the glycerol stock and streaked onto -Leu and -Trp/Leu plates. Once growth on both plates was observed, a colony from the -Leu plate was incubated in -Leu media and the plasmid elimination process repeated and verified by the β -galactosidase assay. A number of clones did not eliminate the DNA-BD/E3L plasmid, which made any further investigations impossible.

Figure 4.5 highlights two individual clones streaked onto a grid on a -Leu plate. The grids were then assayed for β -galactosidase activity using the colony lift assay. with one clone having eliminated the DNA-BD/E3L plasmid (pink colour) and the other having retained the plasmid (blue colour).

Yeast Mating.

Each individual AD/library plasmid clone, expressed in Y190 was then mated with Y187 transformed with the DNA-BD/E3L plasmid. A number of control mating reactions were undertaken simultaneously, section 2.5.16, to ensure a genuine result (Figure 4.6).

A number of the AD/library clones interacted with the control plasmid DNA-BD/lamin C. The lamin C protein is known to interact with very few proteins, and as such serves as an indicator of a false positive. Those clones that interacted with lamin C were then mated with Y187 transformed with 4 individual plasmids expressing unrelated proteins. Each clone interacted with 2 or more of the 4 proteins, indicating they were false positives and were therefore eliminated from further investigations.

The results of the DNA-BD/E3L plasmid elimination and yeast mating experiments are highlighted in Table 4.2.

Identification of Genuine Positive Interacting Clones.

Once the yeast mating procedures were concluded 17 clones remained that were genuine interacting clones, as highlighted in Table 4.2. The DNA from the Y190 strain containing only the AD/library plasmid was extracted and the DNA then transformed into the *E. coli* HB101 strain. The plasmid DNA was extracted using a RPM Spin Kit mini prep (Biolabs

Figure 4.5. Elimination of DNA-BD/E3L plasmid. Colony lift β -galactosidase assays were used to confirm the elimination of the DNA-BD/E3L plasmid. Figure 4.5a and Figure 4.5b depicts the result of the assay derived from 24 colonies derived from clone 6 and clone 10 respectively following the procedure to eliminate the DNA-BD/E3L plasmid. The 24 colonies derived from clone 10 were of no use for yeast mating experiments as the DNA-BD/E3L plasmid was present yielding a positive (blue) result in the β -galactosidase assay.

Figure 4.5a.



Figure 4.5b.



Figure 4.6. Representative results of the yeast mating procedure using the colony lift assay. The following figures depict the results of the yeast mating procedure of clone 6 as described in section 2.5.16. Figures 4.6a, 4.6b, and 4.6c, highlight the negative control mating reactions which ensured the interaction was mediated by E3L and protein derived from the HeLa cDNA library. These colonies therefore do not express the *lacZ/HIS 3* reporter genes and were only visible on -Trp/Leu plates. Figure 4.6d highlights the actual yeast mating experiment between E3L (transformed into strain Y187) and the protein derived from the library (present in strain Y190). The mating procedure therefore resulted in the expression of the *HIS 3* and *lacZ* reporter gene resulting in a blue coloured colonies that were able to grow on -Trp/His/Leu media. Figure 4.6e is a further control reaction between lamin C and the protein derived from the library. The two proteins did not interact and therefore there was no expression of the *HIS 3/LacZ* reporter genes. Figure 4.6f is the positive control reaction between p53 and the SV40 large T-antigen and Figure 4.6g is the full length GAL-4 protein used as a control for β -galactosidase expression.

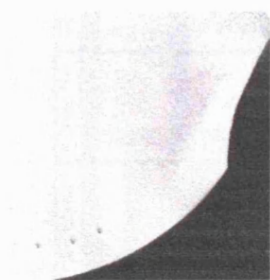
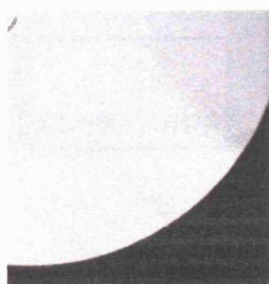
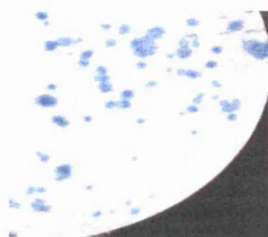
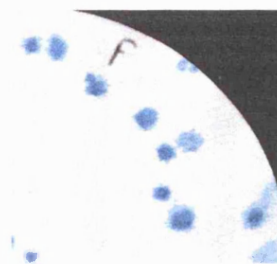
Figure 4.6a.**Figure 4.6b.****Figure 4.6c.****Figure 4.6d.****Figure 4.6e.****Figure 4.6f.****Figure 4.6g.**

Table 4.2. The table highlights the results of the screen. BD Elim. highlights those clones that did (+) or did not eliminate (-) the DNA-BD/E3L plasmid. Y.M. highlights the results of the yeast mating experiment, followed by lamin + and false + which indicates if the library derived protein interacted with the lamin C protein and 2 of the 4 other control clones respectively.

Clone No	Growth	β Gal	BD Elim	Y.M.	Lamin +	False +	Genuine	Clone No	Growth	β Gal	BD Elim	Y.M.	Lamin +	False +	Genuine
1	+	+	+	+	-	-	L23a	50	+	+	+	+	-	-	H3.3
2	+	+	+	+	+	+		51	+	+	+	+	+	+	
3	+	+	+	+	-	-	L23a	52	+	-	+	+	+	+	
4	+	+	+	+	+	+		53	+	+	+	+	+	+	
5	+	+	+	+	+	+		54	+	+	+	+	-	-	H3.3
6	+	+	+	+	-	-	PIC-1	55	+	+	+	+	+	+	
7	+	+	-	+	-	-		56	+	+	+	-	+	+	
8	+	+	+	+	-	-	L23a	57	+	+	+	+	+	+	
9	+	+	-	+	+	+		58	+	+	+	+	+	+	
10	+	+	-	+	+	+		59	+	+	+	+	+	+	
11	+	+	+	+	+	+		60	-	+	+	+	+	+	
12	+	+	+	+	+	+		61	+	+	+	+	-	-	H3.3
13	-	+	+	+	+	+		62	+	+	+	+	+	+	
14	+	+	+	+	+	+		63	+	+	-	+	+	+	
15	+	+	+	+	+	+		64	+	-	-	+	+	+	
16	-	+	-	+	+	+		65	+	+	+	+	-	-	L23a
17	+	+	-	+	+	+		66	+	+	+	+	+	+	
18	+	+	+	+	+	+		67	+	+	-	+	+	+	
19	-	+	+	+	+	+		68	-	+	-	+	+	+	
20	+	+	-	+	+	+		69	+	+	-	+	+	+	
21	+	+	+	+	+	+		70	+	+	+	+	+	+	
22	+	+	+	+	+	+		71	+	+	+	+	+	+	
23	-	+	+	+	+	+		72	+	+	+	+	+	+	
24	+	+	-	+	+	+		73	+	+	+	+	+	+	
25	+	+	+	-	+	+		74	+	+	+	+	+	+	
26	+	+	+	+	+	+		75	+	+	+	+	+	+	L23a
27	+	-	+	+	+	+		76	+	+	+	+	-	-	H3.3
28	+	+	+	+	-	-	H3.3	77	-	+	+	+	+	+	
29	+	+	+	+	-	-	L23a	78	+	+	+	+	+	+	
30	-	+	+	+	+	+		79	-	+	+	+	+	+	
31	+	+	+	+	+	+		80	+	+	+	+	+	+	
32	+	+	+	+	+	+		81	+	+	+	+	+	+	
33	+	+	+	+	+	+		82	+	+	+	+	+	+	
34	+	+	+	+	+	+		83	+	+	+	+	+	+	
35	+	+	+	+	+	+		84	+	+	+	+	+	+	
36	+	+	+	+	+	+		85	+	+	+	-	+	+	
37	+	+	+	+	+	+		86	+	+	+	+	+	+	
38	+	+	+	+	+	+		87	+	-	+	+	+	+	
39	+	+	+	+	-	-	H3.3	88	+	+	+	+	+	+	
40	+	+	+	+	+	+		89	-	+	+	+	+	+	
41	+	+	+	+	+	+		90	-	+	+	+	+	+	
42	+	-	+	+	+	+		91	+	+	+	-	+	+	
43	+	+	+	+	+	+		92	+	+	+	+	+	+	
44	-	+	+	+	+	+		93	+	+	+	+	+	+	
45	+	+	+	+	-	-	L23a	94	+	+	+	+	-	-	L23a
46	+	+	+	+	+	+		95	-	+	+	+	+	+	
47	+	+	+	+	-	-	H3.3	96	-	+	+	+	+	+	
48	+	+	+	+	-	-	L23a	97	-	+	+	+	+	+	
49	+	+	+	+	+	+		98	-	+	+	+	+	+	

101) and then amplified by PCR using the primers oSR03 and oSR04 (Figure 4.7). The results of the PCR amplification permitted the clones to be classified into one of three groups, group 1 containing 1 clone, group 2 containing 8 clones and group 3 also containing 8 clones.

A representative number of the plasmids derived from the amplification were then sequenced (automated). The resulting sequence data was then analysed using a BLAST search (<http://www.ncbi.nlm.nih.gov/>) and the identification of the clones established (Figure 4.8).

Three individual clones were identified, those being L23a, a ribosomal protein, H3.3, a histone protein and PIC-1 a nuclear protein.

Identification of the Region of E3L Necessary for the Interaction with L23a, H3 and PIC-1.

The yeast-two hybrid system provides a means of identifying the region of E3L that mediates the interaction with the proteins. A yeast mating assay was used using the full length E3L protein, and the two smaller constructs of the E3L protein Met 38-190 and Met 94-190.

Cloning of E3LMet38-190 and E3LMet94-190 into pAS2-1.

The plasmids pSR02 and pSR03 containing E3LMet38-190 and E3LMet94-190 respectively, were digested with *Nde* I and *Bam* HI, electrophoresed through an agarose gel and the E3L fragments excised and purified. The fragment was then cloned into the *Nde* I/*Bam* HI digested pAS2-1, and the insert identity verified by *Nde* I/*Bam* HI digestion (Figure 4.9). The two plasmids were then transformed into Y187 in preparation for the yeast mating procedure.

Yeast Mating.

The three DNA-BD/E3L constructs were mated with two individual AD/library plasmids containing L23a (clones 1 and 8) and H3 (clones 28 and 47) and one AD/library plasmid containing PIC-1(clone 6). Once again the control mating assays were undertaken, to ensure a valid result (Figure 4.10, Table 4.3).

The results indicated the interaction between E3L and L23a, H3 and PIC-1 were mediated by the dsRBM of E3L located between amino acids Met94-190.

Figure 4.7. PCR amplification of genuine interacting clones derived from the yeast mating protocols. The clones were then classified according to the size of the amplification product. Clone 6 was classified as group 1, clones 1, 8, 29, 45, 48, 65, 75, and 94 were classified as group 2 and finally clones 28, 39, 47, 50, 54, 61 and 76 were classified as group 3.

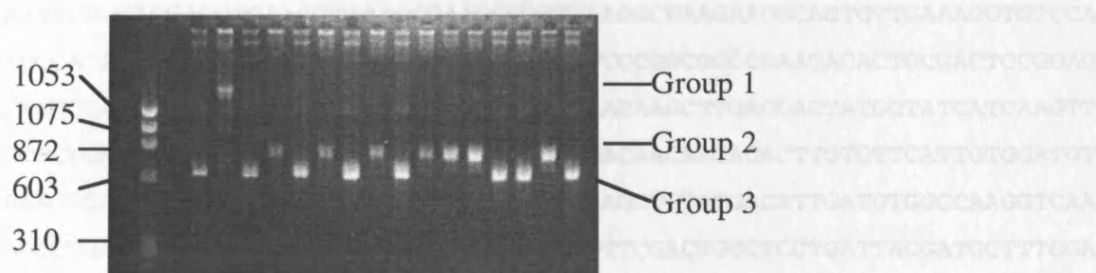


Figure 4.8. DNA sequence derived from a representative number of genuine interacting clones and identification of the protein by a BLAST a database search.

Sequence data derived from AD/library clone 3.

TAGNGCCGAAGATACNNACCAAACCCAAAAAAGAGATCCTAGAACTAGTGGATCCCCCGGGCTGCAG
 GAATTCGGCACGAGCTGAAGCCAAAGCGAAGGCTTTNAAGGCCAAGAAGGCAGTGTGAAAGGTGTCCA
 CAGCCACAAAAAGAAGAAGATCCGCACGTCACCCACCTTCCGGCGGCCGAAGACACTGCGACTCCGGAG
 ACAGCCCCAAATATCCTCGGAAGAGCGCTCCCAGGAGAAACAAGCTTGACCACTATGCTATCATCAAGTT
 TCCGCTGACCACTGAGTCTGCCATGAAGAAGATAGAAGACAACAACACACTTGTGTTTCATTGTGGATGT
 TAAAGCCAACAAGCACCAGATTAAACAGGCTGTGAAGAAGCTGTATGACATTGATGTGGCCAAGGTCAA
 CACCTTGATTTCGGCCTGATGGAGAGAAGAAGGCATATGTTTCGACTGGCTCCTGATTACGATGCTTTGGA
 TGTTGCAACAAAATTTGGGATCATCTAAACTGAGTNCAGCTGCCTAATTCTGAATATATATATATATAT
 ATCTTTTTCACCATAAAATAAAAAAANAAAACTCGAGGGGGGGCCCGGTNCCCAATTCGGCCTATAGGG
 AGTCGTATTCCATTNAACTGGCCGTCNGTTTACAACGTCTGACTGGGAAAACCCCTGATNTATGAATCGT
 AGANTCTTGAAAAACNCCCCAANGTNACTTAACTGNCATNGNGCCCCNATNTNAAATTTCTNTNTTTNT
 CNATNGTTTGCNTCTTTTATGGTACCTATACNTCCNNTAAGTTTNAACNTGGCCATGTAANCNTNGNN
 CTTTNGAATTTTTTAANTGCTTGGAATTAATGCCCANTTTTTTTTGGCCNTAAATCTTTATGAAANTTT
 TTTCCNNGGGCTTTTTTCNNAACTTTTGGNNTTTTTCCCCNGAGGTTTGNNAATCTNCANTAAAGGGG
 NCGGNTTGTTACNTTGCGCNAATTTCTAAAAGANTTANNGGTAAACNNTGGGNNAANNCTTTCC

Results of the BLAST database search.

Sequences producing significant alignments: (bits) Value

gblU43701 HSU43701	Human ribosomal protein L23a mRNA, complete ...	860	0.0
gblL13799 HUMCH13C1A	Homo sapiens (clone 01) liver expressed pr...	852	0.0
gblU02032 HSU02032	Human ribosomal protein L23a mRNA, partial cds.	833	0.0
ref NM_000984.1 RPL23A	Homo sapiens ribosomal protein L23a (RP...	829	0.0
gblAC005884 AC005884	Homo sapiens chromosome 17, clone hRPK.26...	797	0.
gblAD000091 CH19F15314	Homo sapiens DNA from chromosome 19p13.1...	777	

Alignment of the AD/library Clone sequence and Nucleotide sequence detected by the BLASTsearch

The AD/library clone nucleotide sequence is displayed on the bottom line and actual nucleotide sequence derived from the BLAST search displayed on the top line.

gb|U43701|HSU43701 Human ribosomal protein L23a mRNA, complete cds .

Length = 546

Score = 860 bits (434), Expect = 0.0

Identities = 452/456 (99%), Gaps = 2/456 (0%)

Strand = Plus / Plus

```

Query: 82  agctgaagccaaagcgaaggctttnaaggccaagaaggcagtggtgaaaggtgtccacag 141
          |||
Sbjct: 63  agctgaagccaaagcgaaggctttaaaggccaagaaggcagtggtgaaaggtgtccacag 122
Query: 142 ccacaaaaagaagaagatccgcacgtcacccaccttccggcggcgaagacactgcgact 201
          |||
Sbjct: 123 ccacaaaaagaagaagatccgcacgtcacccaccttccggcggcgaagacactgcgact 182
Query: 202 ccggagacagcccaaataatcctcggaagagcgctcccaggagaaacaagcttgaccacta 261
          |||
Sbjct: 183 ccggagacagcccaaataatcctcggaagagcgctcccaggagaaacaagcttgaccacta 242
Query: 262 tgctatcatcaagtttccgctgaccactgagctctgcatgaagaagatagaagacaacaa 321
          |||
Sbjct: 243 tgctatcatcaagtttccgctgaccactgagctctgcatgaagaagatagaagacaacaa 302
Query: 322 cacacttggtgttcattgtggatgttaaagccaacaagcaccagattaaacaggctgtgaa 381
          |||
Sbjct: 303 cacacttggtgttcattgtggatgttaaagccaacaagcaccagattaaacaggctgtgaa 362
Query: 382 gaagctgtatgacattgatgtggccaagggtcaacaccctgattcggcctgatggagagaa 441
          |||
Sbjct: 363 gaagctgtatgacattgatgtggccaagggtcaacaccctgattcggcctgatggagagaa 422
Query: 442 gaaggcatatgttcgactggctcctgattacgatgctttggatgttg-caacaaaatttg 500
          |||
Sbjct: 423 gaaggcatatgttcgactggctcctgattacgatgctttggatgttgccaacaaaa-ttg 481
Query: 501 ggatcatctaaactgagtcagctgcctaattctga 536
          |||
Sbjct: 482 ggatcatctaaactgagtcagctgcctaattctga 517

```

Analysis of the data from the DNA sequencing reaction using Seqed confirmed the match to be identical. Those nucleotides recognised as “n” could be assigned to the appropriate nucleotide as seen in the published sequence. Further analysis of the sequence derived from the AD/library plasmid and the coding region of the gene highlighted 23 nucleotides were absent from the 5' region of the coding sequence, whilst the 3' coding region was intact.

Sequence data derived from AD/library clone 6.

GAAGNTCCCCACCAAACCCANAAAAAGAGATCCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGG
CACGAGGGAAGTTACTGCAGCCGGGGGTTGTGCTGTGGGAAGGGAGAAGGATTTGTAAACCCCGGAG
CGAGGTTCTGCTTACCCGAGGCCGCTGCTGTGCGGAGACCCCGGGTGAAGCCACCGTCATCATGTCTG
ACCAGGAGGCAAAACCTTCAACTGAGGACTTGGGGGATAAGAAGGAAGGTGAATATATTAAACTCAAAG
TCATTGGACAGGATAGCAGTGAGATTCACCTCAAAGTGAAAATGACAACACATCTCAAGAACTCAAAG
AATCATACTGTCAAAGACAGGGTGTTCGAATGAATTCACCTCAGGTTTCTCTTTGAGGGTCAGAGAATTG
CTGATAATCATACTCCAAAAGAACTGGGAATGGAGGAAGAAGATGTGATTGAAGTTTATCANGAACAAA
CGGGGGGTCATTCAACAGTTTAGATATTCTTTTTATTNTTTTCTTTTCCCTCAATCCTTTTTTATTTT
TAAAAATAGTTCTTTTGTAATGTGGTGTCAAACCGGAATTGAAAACGGCACCCCTCTCTTTTGAA
ACATCTGGTAATTTGNATTCTAGTGCTCAATTATTCATTATTNGTTGGTTTCATTGGGCTGAATTTTTG
GTGGACAAGCCTTANTTCCCTTTATATTACCCTCTTCTTTTTAAAAATACCTGTGCACCANAGAGGGCA
NCTTTTTNAAGGACATTGCATTTTCANGCTTGNGGNGATAAAATTAGATCCNCCAATGCCAGGNGTTCA
TAAAGACTTTCNNANTGGCCCTGATGGTNTAGCATGGGGATACCTTTACTCCTGGACTNGNGACTTTT
NNTGGGNAGATGGAAGTTTTNCCAGANCNTACTNNGGGAAAAATGACCTTTCCTTTACTTTGANNCTCC
TTTTAAANTTGTNGGGCTTGGNCCAAAAAAAANGGGNTTCTNGTNGAACNTAATTCCCATNAAAGG
GNNCN

Results of the BLAST database search.

Sequences producing significant alignments: (bits) Value

gb|U61397|HSU61397 Human ubiquitin-homology domain protein PIC1... 837 0.0
gb|U83117|HSU83117 Human sentrin mRNA, complete cds 821 0.0
gb|U67122|HSU67122 Human ubiquitin-related protein SUMO-1 mRNA,... 771 0
ref|NM_003352.1|UBL1| Homo sapiens ubiquitin-like 1 (sentrin)... 728 0.0
gb|N32642|N32642 yx76d09.r1 Homo sapiens cDNA clone 267665 5'. ... 694 0.0
emblZ57271|HS16F6F H.sapiens CpG island DNA genomic MseI fragment. 96 2e-
17

Alignment of the AD/library Clone sequence and Nucleotide sequence detected by the BLAST search

The AD/library clone nucleotide sequence is displayed on the bottom line and actual nucleotide sequence derived from the BLAST search displayed on the top line.

gb|U61397|HSU61397 Human ubiquitin-homology domain protein PIC1 mRNA, complete cds.

```

Length = 1223
Score = 837 bits (422), Expect = 0.0
Identities = 433/437 (99%)
Strand = Plus / Plus
Query: 76 ggaagtactgcagccgggggtgtgtgtgtgggaaggagaaggatttgtaaaccccg 135
      |||
Sbjct: 21 ggaagtactgcagccggcggtgtgtgtgtgtgggaaggagaaggatttgtaaaccccg 80
Query: 136 gagcgagggttctgcttaccggagccgctgtgtgtgaggagacccccgggtgaagccaccg 195
      |||
Sbjct: 81 gagcgagggttctgcttaccggagccgctgtgtgtgaggagacccccgggtgaagccaccg 140
Query: 196 tcatcatgtctgaccaggaggcaaaaccttcaactgaggacttgggggataagaaggaag 255
      |||
Sbjct: 141 tcatcatgtctgaccaggaggcaaaaccttcaactgaggacttgggggataagaaggaag 200
Query: 256 gtgaatatattaaactcaaagtcattggacaggatagcagtgagattcacttcaaagtga 315
      |||
Sbjct: 201 gtgaatatattaaactcaaagtcattggacaggatagcagtgagattcacttcaaagtga 260
Query: 316 aaatgacaacacatctcaagaaactcaaagaatcatactgtcaaagacagggtgttccaa 375
      |||
Sbjct: 261 aaatgacaacacatctcaagaaactcaaagaatcatactgtcaaagacagggtgttccaa 320
Query: 376 tgaattcactcagggtttctctttgagggtcagagaattgctgataatcatactccaaaag 435
      |||
Sbjct: 321 tgaattcactcagggtttctctttgagggtcagagaattgctgataatcatactccaaaag 380
Query: 436 aactgggaatggaggaagaagatgtgattgaagtttatcangaacaaacggggggtcatt 495
      |||
Sbjct: 381 aactgggaatggaggaagaagatgtgattgaagtttatcangaacaaacggggggtcatt 440
Query: 496 caacagtttagatattc 512
      |||
Sbjct: 441 caacagtttagatattc 457

```

Once again analysis of the data from the DNA sequencing reaction using Seqed confirmed the match to be identical. Further analysis of the sequence derived from the AD/library plasmid and the coding region of the gene highlighted the entire coding region of the gene was present within the AD/library plasmid.

Sequence data derived from clone 47.

TCCCTTTCTACTCGACGATGAAGATACCCCCCAAACCCAAAAAAGAGATCCTAGAACTAGTGGATCCC
 CCGGGCTGCAGGAATTCGGCACGAGAAAAAATAATTTCTCTTCTTCTGTTATTGGTAGTTCTGAA
 CGTTAGATATTTTTTTTCCATGGGGTCAAAAGGTACCTAAGTATATGATTGCGAGTGAAAAATAGGGG
 ACAGAAATCAGGTATTGGCAGTTTTTCCATTTTCATTTGTGTGTGAATTTTAAATATAAATGCGGAGAC
 GTAAAGCATTAATGCAAGTTAAAATGTTTCAGTGAAAAGTTTCAGCGGTTCAACTTTATAATAATTATA
 AATAAACCTGTTAAATTTTTTCTGGACAATGCCAGCATTTGGATTTTTTTTAAAACAAGTAAATTTCTTAT
 TGATGGCAACTAAATGGTGTGTTGTAGCATTTTTATCATACAGTAGATTCCATCCATTCACTATACTTTT
 CTAAGTGAAGTTGCCTACATGCAAGTCATGTTTTTAATGTTGTCTGTCTTCTGTGCTGTTCTCTGTAAGTT
 TGCTATTAAAATACATTAAACTATAAAAAAAAAAAAAAAAAAACTCNAGGGGGGGCCCGGTCCCAATTCTG
 CCCTATAGTGAGTCGTATTACAATTCAGTGGCCCGTCGTTTTACAACGTCGTGACTGGGAAAACCTGA
 TCTATGAATCCNAGATCCTGAAAAACCCCGCAAGTTCACTTTAACTGNGCATCGTGCACCATCTCAAT
 TNCTTTCATTTATACCATCGGTTTGCCTTCTTTTATGTAAGTATACTCCNCTAAGTTTCAATCTTNGGC
 CTGNTACCCCTGATCTATAGAAATTTTTTAAAAGCCTNGAATTANNGCCCTCNTTTTTTTTGGGCCCCAAA
 TTCTTCATGAAAATNTTTTTTCNANGGCTTATTCANAACCTTTTGGAATTTTTTCCCCAAAGGTTTGCCAA
 GTTTCATAAAGGNTNTCGNTTTNCTACCTTGCCAAAAATTNN

Results of the BLAST database search.

Sequences producing significant alignments: (bits) Value

reflNM_002107.1 H3F3A	Homo sapiens H3 histone, family 3A (H3F3...	785	0.0
gb M11354 HUMHISH3B	Human H3.3 histone, class B mRNA, complete ...	785	
emblX05857 HSH33G4	Human H3.3 gene exon 4	785	0.0
emblX51897 OCH33A	Rabbit mRNA for histone H3.3A	684	0.0
gb AC002367 AC002367	Homo sapiens Xp22 PAC RPCI1-22N22		
gb M18678 MUSMH3B	Mouse histone H3.3 pseudogene (MH-921), compl 509		

Alignment of the AD/library Clone sequence and Nucleotide sequence detected by the BLAST search.

The AD/library clone sequence is displayed on the bottom line and actual nucleotide sequence of the homologous protein displayed on the top line.

ref|NM_002107.1|H3F3A| Homo sapiens H3 histone, family 3A (H3F3A) mRNA
>gil184092|gb|M11353|HUMHISH3C Human H3.3 histone class C mRNA, complete cds.

```

Length = 1305
Score = 785 bits (396), Expect = 0.0
Identities = 454/473 (95%), Gaps = 4/473 (0%)
Strand = Plus / Plus
Query: 107  tttctcttcttctctgttattggtagttctgaacgtagatannnnnnnnccatggggtca 166
          |||
Sbjct: 834  tttctcttcttctctgttattggtagttctgaacgtagatatttttttccatggggtca 893
Query: 167  aaaggtagcctaagtatatgattgtagtggaataataggggacagaaatcaggtattggc 226
          ||
Sbjct: 894  aa-ggtacctaagtatatgattgtagtggaataataggggacagaaatcaggtattggc 952
Query: 227  agtttttccattttcatttgtgtgtgaatttttaataataaatgcggagacgtaaacgatt 286
          |||
Sbjct: 953  agtttttccattttcatttgtgtgtgaatttttaataataaatgcggagacgtaaacgatt 1012
Query: 287  aatgcaagttaaaatgtttcagtgaa-aagtttcagcgggtcaactttataataattata 345
          |||
Sbjct: 1013 aatgcaagttaaaatgtttcagtgaa-aagtttcagcgggtcaactttataataattata 1072
Query: 346  aataaacctgttaaatttttctggacaatgccagcatttggannnnnnnaaaacaagtaa 405
          |||
Sbjct: 1073 aataaacctgttaaatttttctggacaatgccagcatttggatttctttaaacaagtaa 1132
Query: 406  atttcttattgatggcaactaaatggtgtttagcatttttatcatagtagattcca 465
          |||
Sbjct: 1133 atttcttattgatggcaactaaatggtgtttagcatttttatcatagtagattcca 1192
Query: 466  tccattcactatacttttctaactgagttg-cctacatgcaagt-catgtttttaatgtt 523
          |||
Sbjct: 1193 tccattcactatacttttctaactgagttgtcctacatgcaagtacatgtttttaatgtt 1252
Query: 524  gtctgtcttctgtgctgttcctgtaagtttgctattaaaatacattaaactat 576
          |||
Sbjct: 1253 gtctgtcttctgtgctgttcctgtaagtttgctattaaaatacattaaactat 1305

```

Once again analysis of the data from the DNA sequencing reaction using Seqed confirmed the match to be almost identical. Further analysis of the sequence derived from the AD/library plasmid and the coding region of the gene highlighted 50 nucleotides were absent from the 3' region of the coding sequence, whilst the 5' coding region was intact.

Figure 4.9. Restriction digest verification of cloned Met38-190 and Met94-190 E3L into pAS2-1. Following the ligation of the inserts into the plasmid, the cloned insert region size was verified by digestion with *NdeI* and *BamHI* restriction enzymes. The Met38-190 E3L and Met94-190 E3L fragments were of the correct size and are clearly visible on the 2% agarose gel, stained with ethidium bromide. The full length product visible on the gel depicts the original plasmid with the full length E3L gene cloned into the *NdeI/BamHI* site.



Figure 4.10. Identification of the region of E3L required for the interaction with L23a, H3 and PIC-1 using the three constructs full length E3L, Met38-190 E3L and Met94-190. Yeast mating procedures were undertaken as described in section 2.5.16 and Table 4.3. The three E3L constructs cloned individually into the DNA-BD plasmid and transformed into Y187 were mated with the AD/library plasmid present in Y190. The figures below depict the interaction between the AD/library plasmid derived from clone 6 (PIC-1) and the Met38-190 (Figure 4.10a) and Met94-190 E3L transformed into Y187 (Figure 4.10b) constructs. The same results were obtained with clones 28 and 47 (H3.3) and clones 1 and 8 (L23a) highlighting the interaction between E3L and the three cellular proteins were mediated by the dsRBD of E3L (amino acids 94-190).

Figure 4.10a. Met38-190 E3L.



Figure 4.10b. Met94-190 E3L.

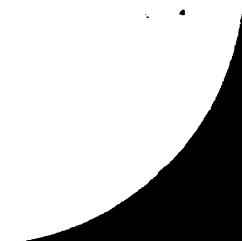


Table 4.3. The table highlights the results of the yeast mating experiment which determined the region of E3L with which each cellular protein interacts.

Plasmid 1 (Y187)	Plasmid 2 (Y190)	Growth on -Trp/His/Leu.	Lac Z expression (Blue/white)
DNA-BD/no insert	AD/no insert	-	
DNA-BD/E3L	AD/no insert	-	
DNA-BD/no insert	AD/clone no 1	-	
DNA-BD/no insert	AD/clone no 8	-	
DNA-BD/no insert	AD/clone no 28	-	
DNA-BD/no insert	AD/clone no 47	-	
DNA-BD/no insert	AD/clone no 6	-	
DNA-BD/E3L	AD/clone no 1	+	blue
DNA-BD/E3L	AD/clone no 8	+	blue
DNA-BD/E3L	AD/clone no 28	+	blue
DNA-BD/E3L	AD/clone no 47	+	blue
DNA-BD/E3L	AD/clone no 6	+	blue
DNA-BD/ E3L Met38-190	AD/clone no 1	+	blue
DNA-BD/ E3L Met38-190	AD/clone no 8	+	blue
DNA-BD/ E3L Met38-190	AD/clone no 28	+	blue
DNA-BD/ E3L Met38-190	AD/clone no 47	+	blue
DNA-BD/ E3L Met38-190	AD/clone no 6	+	blue
DNA-BD/ E3L Met94-190	AD/clone no 1	+	blue
DNA-BD/ E3L Met94-190	AD/clone no 8	+	blue
DNA-BD/ E3L Met94-190	AD/clone no 28	+	blue
DNA-BD/ E3L Met94-190	AD/clone no 47	+	blue
DNA-BD/ E3L Met94-190	AD/clone no 6	+	blue
DNA-BD/lamin C	AD/clone no 1	-	
DNA-BD/lamin C	AD/clone no 8	-	
DNA-BD/lamin C	AD/clone no 28	-	
DNA-BD/lamin C	AD/clone no 47	-	
DNA-BD/lamin C	AD/clone no 6	-	

4.5.4 E3L Protein Expression in Yeast.

Two extraction techniques, using SDS/urea and TCA, were used to obtain samples for the western blot analysis. Both extraction methods gave rise to a number of soluble and insoluble fractions (section 2.5.18) which were used to verify E3L protein expression. Two control extractions were undertaken simultaneously which included Y187 alone and Y187 transformed with DNA-BD plasmid (pAS2-1) with no insert, which are highlighted in figure 4.12.

Figure 4.11 depicts the western blot following SDS PAGE of the samples derived from Y187 transformed with DNA-BD/E3L plasmid alone. Unfortunately the DNA-BD/E3L fusion (40kDa) was not visible in any of the extractions. It was thought that the protein may have degraded during the extraction process, as a result of the large number of proteases present within yeast cells and therefore was not visible following SDS PAGE.

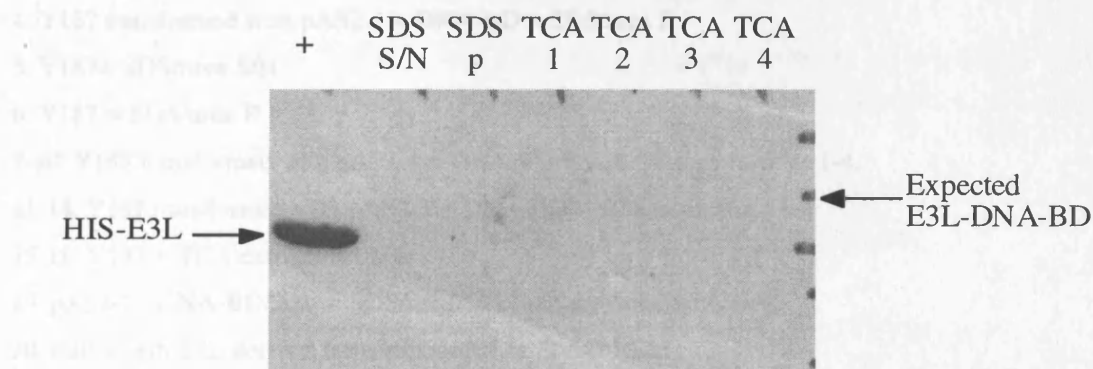
The samples were then 'dot blotted' directly onto the membrane, allowed to dry and a western blot undertaken. It was hoped this method would amplify any weak signals derived from the E3L protein. Figure 4.12 depicts a western blot/dot blot with samples from the two control extractions and Y187 transformed with DNA-BD/E3L. Unfortunately a number of the negative control extractions were western blot positive. This could have been a result of non-specific binding due to the large quantities of protein present in the extracts.

Verification of E3L protein expression by western blot was inconclusive, however electrophoresis of *Nde* I/*Bam* HI digested DNA from the clones derived from the screen, yielded a DNA fragment equivalent in size to E3L (data not shown). Also the sequencing data, discussed in section 4.5.2, highlights sequence derived from E3L, which is unlikely to have originated from the HeLa library. Further to this the results of the yeast mating indicate the two reporter genes are expressed only when the AD/library plasmid (present in strain Y190) is mated with the DNA-BD/E3L plasmid (transformed into strain Y187) and not the DNA-BD plasmid alone. Therefore the proteins detected by the yeast-two-hybrid screen, are interacting with the E3L protein in yeast.

4.6 Summary.

Three cellular proteins, L23a, H3.3 and PIC-1, were found to physically interact with E3L within a yeast -two-hybrid system. Controls to eliminate false positives were undertaken throughout the procedure and therefore the interactions were believed to be authentic. Yeast mating procedures found the dsRBM of E3L (amino acids Met94-190) to mediate the interaction with each of the three cellular proteins.

Figure 4.11. ECL western blot detection of E3L protein expression in yeast following SDS PAGE. The DNA-BD/E3L fusion was not detected by either the TCA or SDS/urea method of extraction. The positive control HIS-E3L (+) derived from bacteria was clearly visible, (30kDa) however the E3L-DNA-BD fusion protein band (41kDa) was not visible by either means of extraction. The numbers above the western blot lanes correspond to samples taken at different stages of the extraction using TCA and are referred to in section 2.5.18.

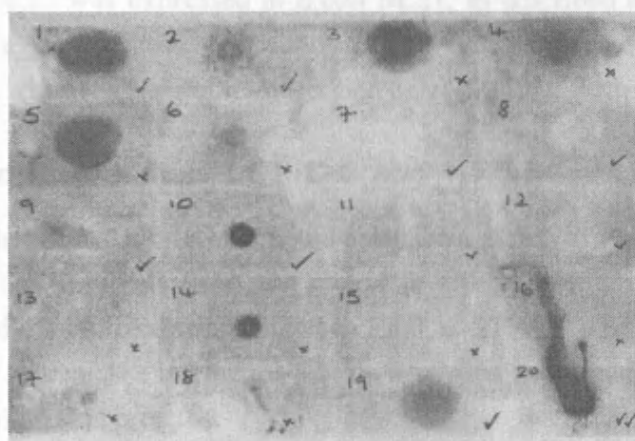


The positive or negative results expected from the blot are highlighted on the blot by a tick or cross respectively however there was no clear evidence of E3L-DNA-BD expression from either extraction method.

Figure 4.12. “Dot blot” analysis of E3L protein extraction from yeast. 10µl of each protein sample was dotted directly onto the membrane and then allowed to dry and ECL analysis undertaken. The number in the top left hand corner of each square corresponds to the sample loaded onto the membrane which are highlighted below.

1. Y187 transformed with pAS2-1 + DNA-BD/E3L = SDS/urea S/N
2. Y187 transformed with pAS2-1 + DNA-BD/E3L = SDS/urea P
3. Y187 transformed with pAS2-1 + DNA-BD = SDS/urea S/N
4. Y187 transformed with pAS2-1 + DNA-BD = SDS/urea P
5. Y187 = SDS/urea S/N
6. Y187 = SDS/urea P
- 7-10. Y187 transformed with pAS2-1 + DNA-BD/E3L = TCA extractions 1-4.
- 11-14. Y187 transformed with pAS2-1 + DNA-BD = TCA extractions 1-4
- 15-18. Y187 = TCA extractions 1-4.
19. pAS2-1+ DNA-BD/E3L = SDS/urea S/N from previous extraction.
20. Full length E3L derived from expression in *E. coli* BL21.

The positive or negative results expected from the blot are highlighted on the blot by a tick or cross respectively however there was no clear evidence of E3L-DNA-BD expression from either extraction method.



5. Results. Biochemical Analysis of Interactions.

5.1 Introduction.

Having detected 3 individual cellular proteins that interacted with E3L in a yeast-two-hybrid screen, work was undertaken to verify these interactions biochemically. The standard means of confirming such interactions include immune precipitation, affinity chromatography, crosslinking and immune fluorescence microscopy.

Expression plasmids for PIC-1 and H3.3 were obtained, however an expression plasmid for L23a was not available. Biochemical analysis was then undertaken to verify the interactions between E3L, PIC-1 and H3.3.

5.2 Protein Expression for Interaction Analysis.

5.2.1 Bacterial Expression of E3L.

Extraction of the E3L-DNA-BD fusion protein from yeast cells using urea/SDS and TCA had proved unsuccessful, section 4.5.4., as did the extraction using glass beads and RIPA buffer (data not shown). HIS-E3L was therefore expressed in *E.coli*, as this had previously been undertaken and was known to produce sufficient quantities of protein for the work described below.

³⁵S labelled HIS-E3L was expressed in *E.coli* BL21, as described in section 2.6.2. The expression of the protein was verified by SDS PAGE and fluorography. The E3L containing lysate was used for studies described below without further purification.

5.2.2 *In Vitro* Expression of PIC-1 and H3.3.

In vitro transcription/translation systems are used for a variety of purposes including the study of protein:DNA, protein:RNA and protein:protein interactions. For example, Patel and Sen (1998) detected interactions between PKR and PACT a cellular protein using a yeast-two-hybrid screen. The interaction was then verified by immune precipitation, using the two *in vitro* translated proteins.

The cDNA for PIC-1, was kindly donated by Professor P.S. Freemont, Imperial Cancer Research Fund, London. It was amplified by PCR using primers oSR05 and oSR06. The cDNA for H3.3, was kindly donated by Professor D.Wells, Stanford Medical School, Palo Alto and was amplified by PCR using primers oSR07 and oSR08. The resulting amplified DNA from each clone contained a T7 promoter site and Kosak sequence

(Kosak, 1990) (*see* section 2.5.6), to maximise protein expression during the *in vitro* transcription/translation (Figure 5.1).

As only very small quantities of protein, in the ng range, are usually produced by *in vitro* transcription/translation (Promega Applications Guide). ^{35}S -methionine was incorporated into the translation reaction allowing the products to be visualised by fluorography (Figure 5.2). Non radiolabelled proteins were produced in an analogous protocol replacing the ^{35}S methionine with unlabelled methionine. The apparent MW of each protein as determined by SDS PAGE was 22kDa for PIC-1 (Boddy *et al.*, 1996) and 23kDa for H3.3 respectively. The increased MW of the proteins when analysed by SDS PAGE when compared to the expected MWs of 11.5kDa and 15.3kDa respectively, could be the result of the phosphorylation of the protein or interference with the electrophoretic mobility by other factors such as the hydrophobicity of the protein.

The identity of PIC-1, was confirmed by immune precipitation using a polyclonal antibody reactive against PIC-1, kindly donated by P.S.Freemont (Figure 5.3). Unfortunately an antibody reactive against H3.3 was unavailable for this study. Further to this, the third lane of Figure 5.2 (-), highlights the negative control reaction where no PCR products were added to the transcription/translation lysate and the reaction yielded no protein product. Thus *in vitro* transcription/translation apparently produces labelled PIC-1 and H3.3 proteins.

5.3 Interaction Analysis.

5.3.1 Immune Precipitation.

The basis of the technique is that Protein A is known to bind to the Fc region of immunoglobulins. When it is attached to sepharose beads it can therefore be used to precipitate immune complexes. The immune precipitation was optimised using bacterial lysates expressing ^{35}S labelled HIS-E3L, and the monoclonal antibody TW2.3. The experimental procedure was undertaken as described in section 2.6.3, and the bound fractions analysed by SDS PAGE and fluorography.

The HIS-E3LMet94-190 protein was used initially as this region of the E3L protein had interacted with each of the clones within the yeast-two-hybrid screen, and was also known to adopt a folded conformation (section 3.4.4). The protein interacted with the protein A sepharose beads, non-specifically, without the addition of the monoclonal antibody TW2.3 (Figure 5.4). The quantity of protein A beads (10% slurry), used in the reaction was therefore reduced five fold to 5 μl in a 50 μl reaction, however this did not decrease the level of non-specific interactions. The salt concentration of the buffer was

Figure 5.1a. PCR amplification of PIC-1 cDNA as visualised by agarose gel (2%) electrophoresis, in preparation for *in vitro* transcription/translation protocols. The PIC-1 product is composed of 303bp which combined with the T7 promoter region and Kosak sequence increases the size of the amplification product as highlighted in section 2.5.6.

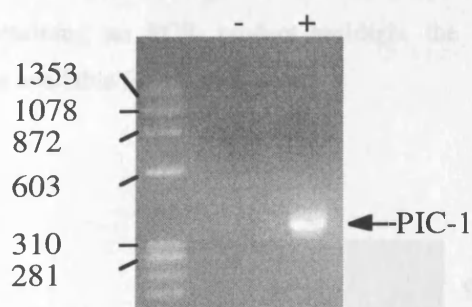


Figure 5.1b. PCR amplification of H3.3 cDNA as visualised by agarose gel (2%) electrophoresis, in preparation for *in vitro* transcription/translation protocols. The H3.3 product is composed of 408bp which combined with the T7 promoter region and Kosak sequence increases the size of the amplification product, as highlighted in section 2.5.6.

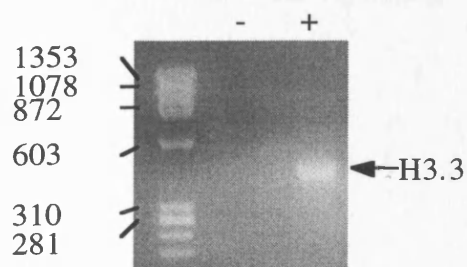


Figure 5.2. *In vitro* transcription/translation products of PIC-1 and H3.3 of amplified cDNA as determined by SDS PAGE (18%). The transcription/translation reaction was undertaken using the TNT T7 rabbit reticulocyte lysate system (Promega). The negative control containing no amplification product, and MW markers are highlighted on the autoradiogram, and H and P stand for H3.3 and PIC-1 respectively. Immune precipitation of PIC-1, figure 5.3, using a specific polyclonal antibody and the negative control reaction containing no PCR product highlight the identity of the two proteins. Unfortunately no antibody was available for H3.3.

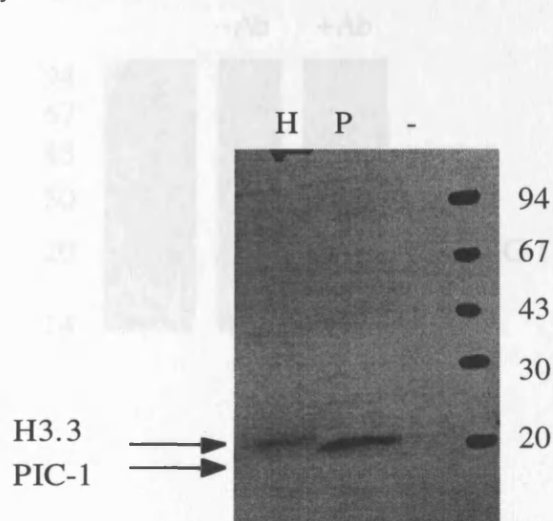


Figure 5.3. Immune precipitation analysis by SDS PAGE (18%) and fluorography of PIC-1 using a polyclonal antibody reactive against the protein. The autoradiogram below highlights the negative control reaction, which did not contain the polyclonal antibody, and positive reaction with the resulting PIC-1 protein band derived from the *in vitro* transcription/translation protocol. 25 μ l of the lysate was mixed with 5 μ l of the antibody and agitated for 2 hours at 4°C. The complex was then bound to 5 μ l of the protein A sepharose beads, incubated for 30 minutes, centrifuged and the bound fraction analysed.

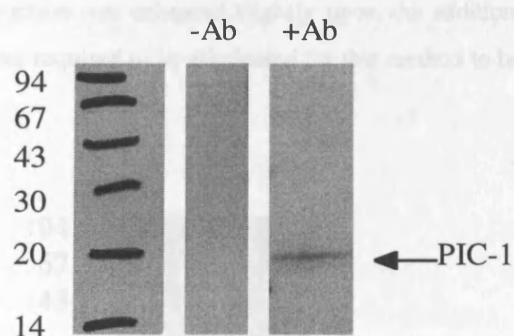
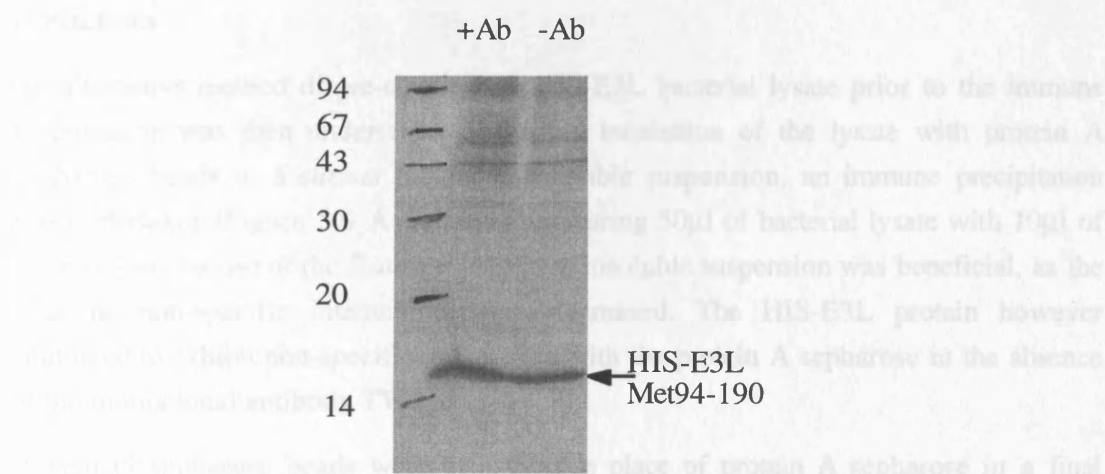


Figure 5.4. The non-specific interaction between E3L, derived from a bacterial expression system, and the protein A sepharose beads is highlighted below as analysed by SDS PAGE (18%) and fluorography. The +ve and -ve lanes refer to the addition/or not of 5µl of the monoclonal antibody TW2.3, to 50µl of HIS-E3L Met94-190 solubilised in 300mM NaCl, 200mM NaBr, 50mM NaHPO₄, pH6.5, 0.2% NP-40, 1mM DTT and 0.5mM PMSF which were then agitated for 2 hours at 4°C. The complex was then bound to 25µl of protein A resin (10% slurry) and incubated for a further 2 hours, centrifuged and the bound fraction analysed. The interaction was enhanced slightly upon the addition of the antibody, however the non-specific interactions were required to be eliminated for this method to be of use.



then reduced from 0.3/0.2M NaCl/NaBr, which had previously been used to solubilise the protein for NMR analysis, five fold to 0.06/0.04M NaCl/NaBr, and the binding analysed once again (Figure 5.5) however the non specific interactions were not eliminated.

Next the protein A sepharose beads were blocked with 2mg/ml BSA, or a non radiolabelled BL21*E.coli* lysate (which when assayed using the Biorad coomassie reagent measured 0.703). The blocking procedure is described in section 2.6.3. The blocked beads interacted with the recombinant protein in a non-specific manner once again (data not shown). The blocking procedure therefore did not eliminate the non-specific interactions.

An alternative method of pre-clearing the HIS-E3L bacterial lysate prior to the immune precipitation was then undertaken. Following incubation of the lysate with protein A sepharose beads or *S.aureus* protein A insoluble suspension, an immune precipitation was undertaken (Figure 5.6 A and B). Pre-clearing 50µl of bacterial lysate with 10µl of protein A sepharose or the *S.aureus* protein A insoluble suspension was beneficial, as the level of non-specific interactions were decreased. The HIS-E3L protein however continued to exhibit non-specific interactions with the protein A sepharose in the absence of the monoclonal antibody TW2.3.

Protein G sepharose beads were then used in place of protein A sepharose in a final attempt to eliminate the non-specific interactions. HIS-E3L, extracted in buffer containing 100 and 200mM NaCl were incubated with the two forms of sepharose and the binding compared directly both in the presence and absence of the monoclonal antibody TW2.3 (Figure 5.7). As can be seen from the unbound fractions the majority of the HIS-E3L protein bound to the protein G sepharose when buffered in 100mM NaCl. Once again however equal quantities of the protein interacted with the protein G beads in the absence of the monoclonal antibody as had previously been observed with protein A sepharose, and therefore was of no use for this particular immune precipitation.

Immune precipitation analysis could not therefore be used as the E3L protein bound to protein A and G sepharose in the absence of the antibody.

A summary of the methods used for the immune precipitation analysis is highlighted in Table 5.1.

Figure 5.5. The protein was solubilised in 300mM/200mM NaCl/NaBr (**H**igh) and 60/40mM NaCl/NaBr (**L**ow) salt buffers supplemented with 50mM NaHPO₄, pH 6.5, 0.2% NP-40, 1mM DTT and 0.5mM PMSF. 5µl of the monoclonal antibody was then added to the appropriate samples and agitated for 2 hours. The complex was then mixed with 5µl of the protein A sepharose (10% slurry) for a further 2 hours, centrifuged and the bound fraction analysed by SDS PAGE (18%) and fluorography. Once again the interaction between HIS-E3L and the protein A sepharose beads was increased upon the addition of the monoclonal antibody, however the non-specific interaction between the HIS-E3L and protein A sepharose beads was still evident under both NaCl/NaBr concentrations.

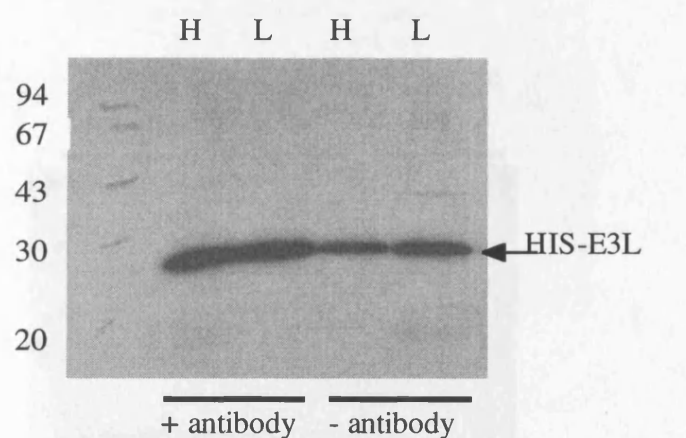


Figure 5.6. Preclearing the bacterial lysate. 50µl of the HIS-E3L bacterial lysate, solubilised in 100mM NaCl, 50mM NaHPO₄, pH 6.5, 0.2% NP-40, 1mM DTT and 0.5mM PMSF, was precleared with 5µl and 10µl of protein A sepharose beads (10% slurry) or a protein A insoluble *S.aureus* suspension. The lysate and beads/*S.aureus* were incubated for 2 hours at 4°C. Following centrifugation the bacterial lysate was mixed where necessary with 5µl of the antibody incubated for 1 hour and finally 5µl protein A sepharose beads (10% slurry) for a further 2 hours when the bound fraction was analysed. The results highlighted the pre-clearing eliminated a proportion of the non-specific interactions, however sufficient quantities of the protein bound to the beads under all conditions in the absence of the monoclonal antibody TW2.3.

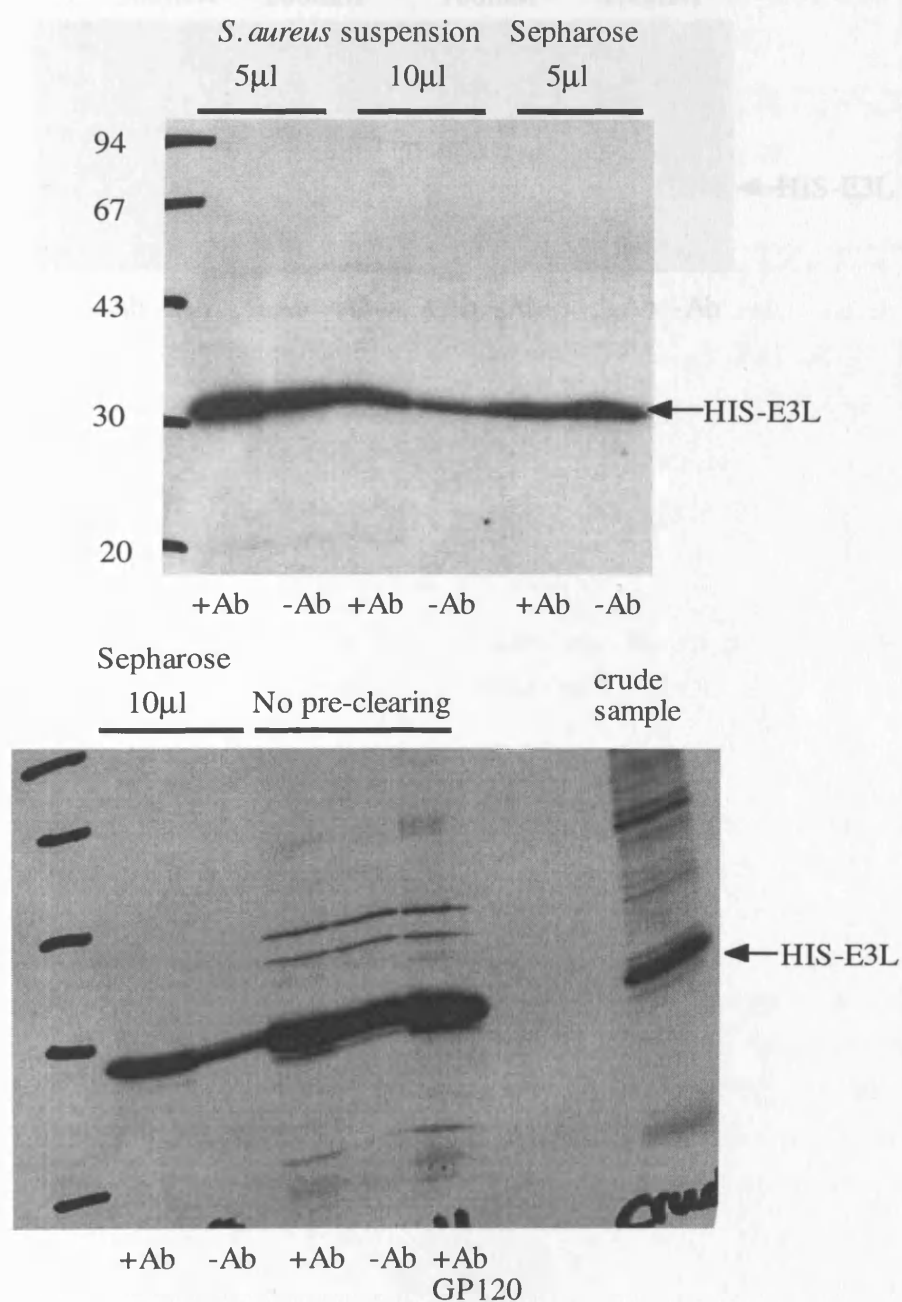
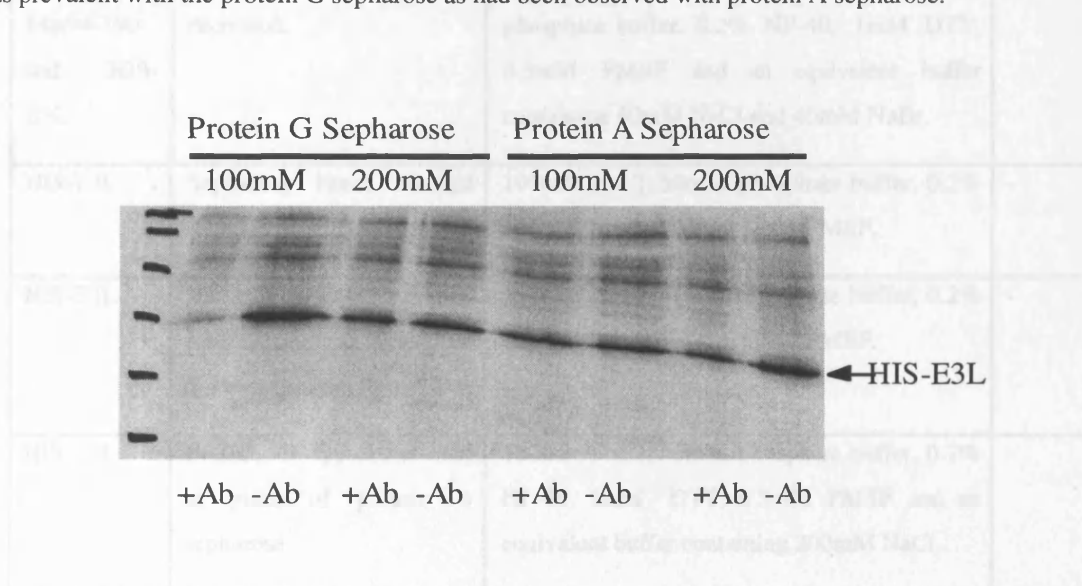


Figure 5.7. Protein G sepharose immune precipitation analysis. The bacterial lysate containing HIS-E3L, extracted 100mM and 200mM NaCl supplemented with 50mM NaHPO₄, pH 6.5, 0.2% NP-40, 1mM DTT, 0.5mM PMSF, was incubated with 5µl of the monoclonal antibody TW2.3 for 2 hours. 5µl of Protein A or G sepharose was then added where required and bound and unbound fractions analysed by SDS PAGE (18%) and fluorography. The unbound fractions highlight the non-specific interactions were as prevalent with the protein G sepharose as had been observed with protein A sepharose.



5.3.2 Affinity Chromatography

Poly (ur) (rC) Affinity Interaction Studies

The mRNA binding properties of HIS-E3L were exploited to analyse interactions with PIC-1 and H3.2, which were assumed not interact with mRNA.

PIC-1 Interaction studies

A total of 10⁸ of ³²S labelled BL21 *E. coli* expressing HIS-E3L was washed in 100µl of binding buffer 1, and the volume made up to 1mL. 25µl of both HIS-E3L and PIC-1 transcription/translation products were incubated individually on ice for 30 minutes and the binding procedure undertaken as described in section 2.4.5 (Figure 5.3). The interaction between HIS-E3L and the poly(ur)(rC) resin was specific (lane Eb), and no interaction was observed between the resin and the PIC-1 product (lane Pb). Under these conditions the two products did not interact (lane E+Pb) with the PIC-1 protein remaining in the unbound fractions (lanes Pa and E+Pa). The incubation time was therefore increased to 14 hours and the binding procedure repeated. Once again however the

Table 5.1. The table highlights the methods and conditions used to eliminate non-specific interactions between E3L and the protein A sepharose beads.

Protein	Elimination Method	Buffer	Result
HIS-E3L Met94-190 and HIS- E3L	Salt Concentration decreased.	300mM NaCl, 200mM NaBr, 50mM phosphate buffer, 0.2% NP-40, 1mM DTT, 0.5mM PMSF and an equivalent buffer containing 60mM NaCl and 40mM NaBr.	-
HIS-E3L	Sepharose beads blocked with BSA or <i>E.coli</i> lysate.	100mM NaCl, 50mM phosphate buffer, 0.2% NP-40, 1mM DTT, 0.5mM PMSF.	-
HIS-E3L	Bacterial lysate pre-cleared with sepharose beads or <i>S.aureus</i> suspension.	100mM NaCl, 50mM phosphate buffer, 0.2% NP-40, 1mM DTT, 0.5mM PMSF.	-
HIS-E3L	Protein G sepharose used in place of protein A sepharose	100mM NaCl, 50mM phosphate buffer, 0.2% NP-40, 1mM DTT, 0.5mM PMSF and an equivalent buffer containing 200mM NaCl.	-

5.3.2 Affinity Chromatography.

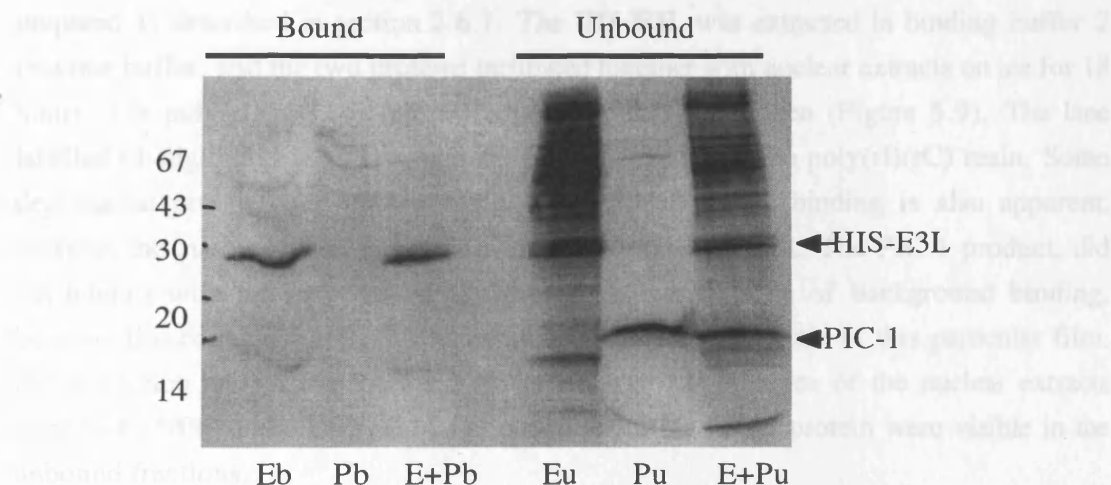
Poly (rI) (rC) Affinity Interaction Studies.

The dsRNA binding properties of HIS-E3L were exploited to analyse interactions with PIC-1 and H3.3, which were assumed not interact with dsRNA.

E3L-PIC-1 Interaction studies

A 1ml pellet of ³⁵S labelled BL21 *E.coli* expressing HIS-E3L was sonicated in 200µl of binding buffer 1, and the volume made up to 1ml. 25µl of both HIS-E3L and PIC-1 transcription/ translation product were incubated individually on ice for 30 minutes and the binding procedure undertaken as described in section 2.6.5 (Figure 5.8). The interaction between HIS-E3L and the poly(rI)(rC) resin was specific (lane Eb), and no interaction was observed between the resin and the PIC-1 product (lane Pb). Under these conditions the two proteins did not interact (lane E+Pb) with the PIC-1 protein remaining in the unbound fractions (lanes Pu and E+Pu). The incubation time was therefore increased to 18 hours and the binding procedure repeated. Once again however the

Figure 5.8. Poly(rI)(rC) “pulldown” of HIS-E3L and *in vitro* translated PIC-1. 25µl HIS-E3L (E), solubilised in Tris-HCl pH7.1, 30mM NaCl, 2mM MgCl₂ 1mM DTT and 1% Glycerol, and 25µl of PIC-1 (P), derived from the *in vitro* transcription/ translation lysate were incubated on ice for 30 minutes. 25µl of a 50% slurry of poly(rI)(rC) resin was added and agitated for 30 minutes at room temperature. Following centrifugation the bound and unbound fractions were analysed by SDS-PAGE (18%) and fluorography. Under these conditions the proteins were found not to interact.



E3L-H3.3 Interaction studies.

Evidence of an interaction between E3L and H3.3 was searched for in a similar fashion to that described above for PIC-1. 25µl of the H3.3 transcription/translation product was incubated with 25µl of HIS-E3L (extracted in binding buffer 1) for 18 hours on ice. The poly(rI)(rC) binding experiment undertaken along with the appropriate controls, no interaction was observed (data not shown). Due to the nuclear localisation of histone proteins, the experiment was then repeated using HIS-E3L extracted in nuclear buffer (binding buffer 2) however no interaction was visible (data not shown).

Finally the interaction was investigated using HIS-E3L extracted in a nuclear buffer (binding buffer 2) in the presence of nuclear extracts (Figure 5.10). Once again the interaction with HIS-E3L and the poly(rI)(rC) resin was specific (lane Eb). There was a degree of background interaction from the H3.3 transcription/translation lysate (lane Hb) however, no interaction was observed between E3L and H3.3 in the presence of nuclear extracts (lane E+H +NEb). Once again a large quantity of the H3.3 protein was visible in the unbound fraction (Lane Hu and E+H+NEu).

proteins did not interact (data not shown). Finally the HIS-E3L and PIC-1 products were incubated for 2.5 hours at 21°C. Once again no interaction between the two proteins was observed (data not shown).

The PIC-1 protein is known to interact with proteins located within nuclear dots (Boddy *et al.*, 1996) and so it was conceivable that the interaction required conditions and/or proteins found in the nucleus. Therefore nuclear extracts from a human cell line were prepared as described in section 2.6.7. The HIS-E3L was extracted in binding buffer 2 (nuclear buffer) and the two proteins incubated together with nuclear extracts on ice for 18 hours. The poly(rI)(rC) binding procedure was then undertaken (Figure 5.9). The lane labelled Eb highlights the interaction between HIS-E3L and the poly(rI)(rC) resin. Some degradation products are visible and a level of background binding is also apparent, however the major product interacting with the resin is HIS-E3L. The PIC-1 product, did not interact with the resin (lane Pb). There is some evidence of background binding, however this could in part be attributed to the longer exposure time of this particular film. No interaction was visible between the proteins in the presence of the nuclear extracts (lane E+P+NEb) and once again large quantities of the PIC-1 protein were visible in the unbound fractions.

E3L-H3.3 Interaction studies.

Evidence of an interaction between E3L and H3.3 was searched for in a similar fashion to that described above for PIC-1. 25µl of the H3.3 transcription/translation product was incubated with 25µl of HIS-E3L (extracted in binding buffer 1) for 18 hours on ice. The poly(rI)(rC) binding experiment undertaken along with the appropriate controls, no interaction was observed (data not shown). Due to the nuclear localisation of histone proteins, the experiment was then repeated using HIS-E3L extracted in nuclear buffer (binding buffer 2) however no interaction was visible (data not shown).

Finally the interaction was investigated using HIS-E3L extracted in a nuclear buffer (binding buffer 2) in the presence of nuclear extracts (Figure 5.10). Once again the interaction between HIS-E3L and the poly(rI)(rC) resin was specific (lane Eb). There was a degree of background interaction from the H3.3 transcription/translation lysate (Lane Hb), however, no interaction was observed between E3L and H3.3 in the presence of nuclear extracts (lane E+H +NEb). Once again a large quantity of the H3.3 protein was visible in the unbound fraction (Lane Hu and E+H+NEu).

Figure 5.9. Poly(rI)(rC) "pulldown" of HIS- E3L solubilised in 25mM HEPES pH7, 0.1mM EDTA, 40mM KCl, 10% glycerol and 1mM DTT, and *in vitro* translated PIC-1 supplemented with nuclear extracts. 25µl of nuclear extracts (NE), HIS-E3L (E), and PIC-1 (P), were incubated on ice for 18 hours. 25µl of a 50% slurry of poly(rI)(rC) resin was added and agitated for 30 minutes at room temperature. Following centrifugation the bound and unbound fractions were analysed by SDS-PAGE (18%) and fluorography. The two proteins were found not to interact in the presence of nuclear extracts.

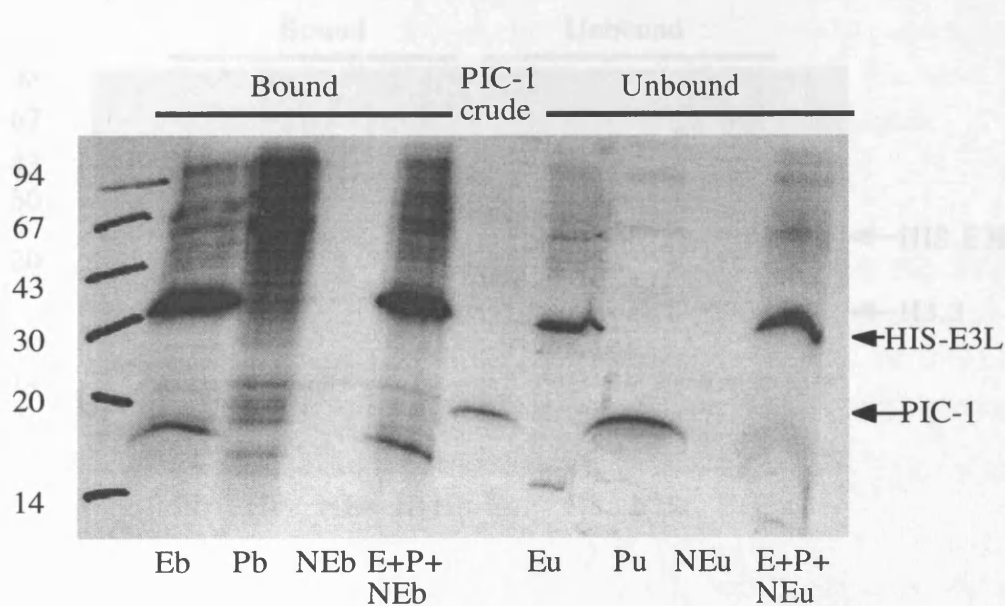
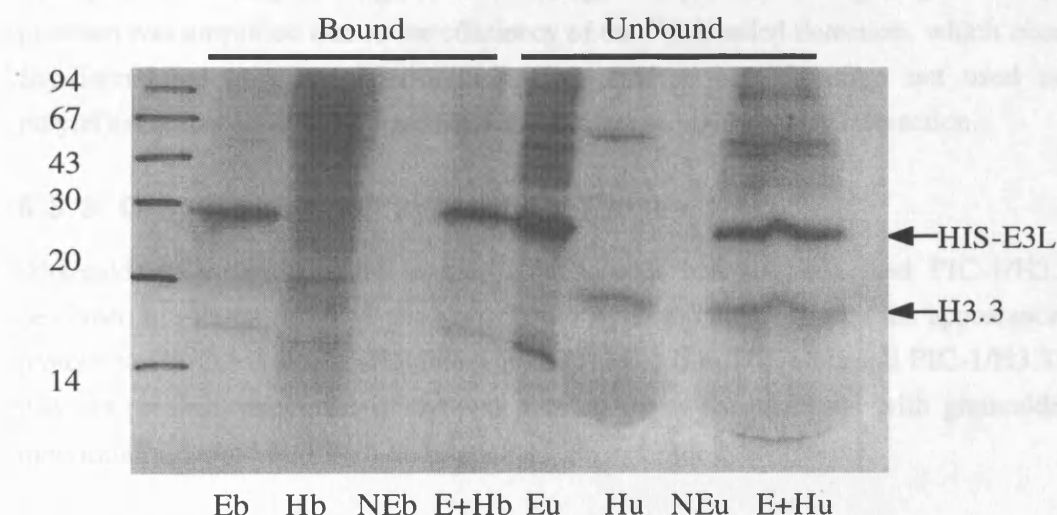


Figure 5.10 Poly(rI)(rC) “pulldown” of HIS-E3L solubilised in 25mM HEPES, pH7, 0.2mM EDTA, 40mM KCl, 1mM DTT, 10% glycerol, and *in vitro* translated H3.3. 25µl of HIS-E3L (E), and H3.3 (H), were incubated on ice for 18 hours. 25µl of a 50% slurry of poly(rI)(rC) resin was added and agitated for 30 minutes at room temperature. Following centrifugation the bound and unbound fractions were analysed by SDS-PAGE (18%) and fluorography. Under these conditions the proteins were found not to interact.



HIS-E3L solubilised in 50mM NaH₂PO₄, 400mM NaCl, 1mM DTT, 2mM MgCl₂, 1% glycerol and 0.5mM PMSF, was treated with glutaraldehyde in the presence of PIC-1. No additional protein band was visible when the HIS-E3L (³⁵S) and PIC-1, and PIC-1 (³⁵S) and HIS-E3L were crosslinked, suggesting an interaction had not taken place (data not shown). The experiment was then repeated using HIS-E3L extracted in poly(rI)(rC) buffer (Table 5.1) or nuclear buffer, in an attempt to promote the interaction between the two (Fig. 5.12). The HIS-E3L formed large multimers upon addition of glutaraldehyde (not shown) to the non-crosslinked sample (lanes E⁺ and E⁺XL respectively). The Eukaryotic protein (Euk-P⁺) had not been reported to self associate (Bayer *et al.*, 1998), and it was observed a dimeric form when mixed with glutaraldehyde (P⁺XL). Interestingly the auto-association of the HIS-E3L protein decreased slightly when incubated together with Euk-P⁺ (lane E⁺+P⁺XL). This observation cannot however be attributed to PIC-1 since a number of other proteins were present in the translation lysate. The incubation with glutaraldehyde did not highlight an interaction between E3L and PIC-1 (lane E⁺+P⁺XL and E⁺+P⁺XL).

Histidine Tag Affinity Interaction Studies.

The histidine tag purification system had previously been utilised for the production of purified HIS-E3L in preparation for NMR spectroscopy.

The histidine tag purification was undertaken in 5 buffers as discussed in section 2.6.4. An immediate problem was evident as the interaction with the histidine affinity resin was non-specific, leading to a high level of background protein binding (Figure 5.11). The problem was amplified due to the efficiency of the ^{35}S labelled detection, which obscured any interaction with the PIC-1/H3.3. This method was therefore not used as the poly(rI)(rC) interaction was specific and sufficient to highlight any interaction.

5.3.3 Crosslinking of Interacting Clones.

Glutaraldehyde was used to highlight interactions between E3L and PIC-1/H3.3 as described in section 2.6.6. Following SDS PAGE and fluorography the appearance of a protein band in the sample containing both proteins (i.e. HIS-E3L and PIC-1/H3.3) that was not present when each of the two proteins that were incubated with glutaraldehyde individually, would highlight an interaction between the two proteins.

E3L-PIC-1 Interaction studies

HIS-E3L solubilised in 50mM NaHPO_4 , 100mM NaCl, 1mM DTT, 2mM MgCl_2 , 1% glycerol and 0.5mM PMSF, was treated with glutaraldehyde in the presence of PIC-1. No additional protein band was visible when the HIS-E3L (^{35}S) and PIC-1, and PIC-1 (^{35}S) and HIS-E3L were crosslinked, suggesting an interaction had not taken place (data not shown). The experiment was then repeated using HIS-E3L extracted in poly(rI)(rC) binding buffer 2, or nuclear buffer, in an attempt to promote the interaction between the two (Figure 5.12). The HIS-E3L formed large multimers upon addition of glutaraldehyde when compared to the non-crosslinked sample (lanes E* and E*XL respectively). The PIC-1 protein (lane P*), had not been reported to self associate (Bayer *et al*, 1998), and it maintained a monomeric form when mixed with glutaraldehyde (P*XL). Interestingly the multimerisation of the HIS-E3L protein decreased slightly when incubated together with PIC-1 (lane E*+P XL). This observation cannot however be attributed to PIC-1 since a number of other proteins were present in the translation lysate. The incubation with glutaraldehyde did not highlight an interaction between E3L and PIC-1 (lanes E*+P XL and E+P* XL).

Figure 5.11. Histidine tag “pulldown” interaction analysis. 100µl of ^{35}S labelled HIS-E3L extracted in 5 individual buffers were mixed with 25µl of Talon resin for 30 minutes at room temperature and the bound fractions analysed by SDS PAGE (18%) and fluorography. Two of the samples extracted in Tris-HCl (pH7) and NaHPO_4 (pH7) supplemented with 150mM NaCl, 1% glycerol and 0.5mM PMSF are shown below. Lane E+P indicates the HIS-E3L bacterial lysate was first pre-incubated with PIC-1 and then the histidine “pulldown” experiment undertaken. Lane E highlights the “pulldown” experiment with HIS-E3L bacterial lysate bound to the resin alone. The level of non-specific interactions between the HIS-E3L bacterial lysate and the Talon resin were too great to permit this method to be used as a means of verifying interactions *in vitro*.

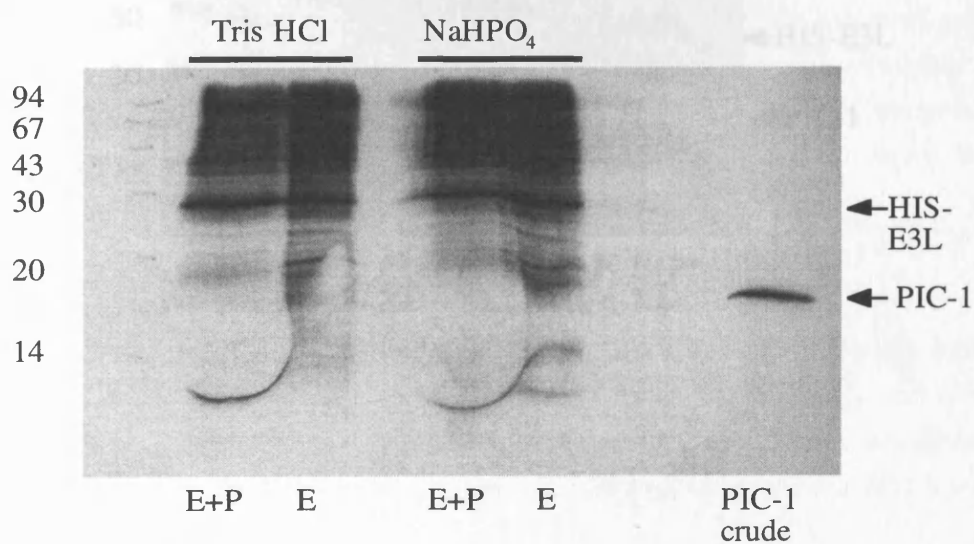
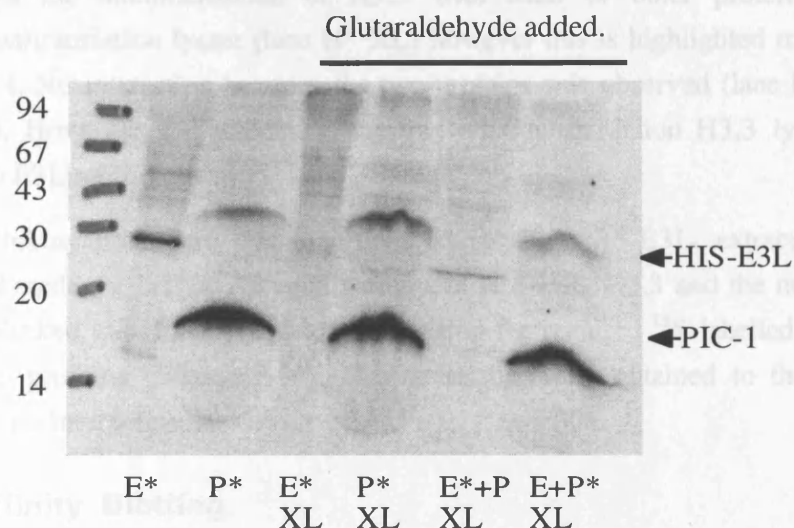


Figure 5.12. Gluteraldehyde crosslinking analysis of HIS-E3L/PIC-1 interactions. 25µl of ^{35}S labelled HIS-E3L (**E**) and 25µl of PIC-1 (**P**) were incubated together for 30 minutes at room temperature in various combinations to analyse any interactions between the two proteins. The *, highlights those proteins that were ^{35}S labelled and **XL** depicts those proteins that were mixed in the presence of 0.01% glutaraldehyde. The results were analysed by SDS-PAGE (18%) and fluorography and are shown below. No interaction between the two proteins was observed.



E3L-H3.3 Interaction studies

Evidence of an interaction between HIS-E3L, solubilised in 50mM NaHPO₄, 100mM NaCl, 1mM DTT, 2mM MgCl₂, 1% glycerol and 0.5mM PMSF, and H3.3 was investigated in a similar fashion to that described for PIC-1 (Figure 5.13). Incubation with glutaraldehyde lead to the multimerisation of HIS-E3L (lane E* XL). There was also evidence of the multimerisation of H3.3 with itself or other proteins within the transcription/translation lysate (lane H* XL) however this is highlighted more clearly in Figure 5.14. No interaction between the two proteins was observed (lane E*+P XL and E+P* XL). However, the addition of the transcription/translation H3.3 lysate lead to a decrease in E3L multimerisation.

The crosslinking procedure was then undertaken using HIS-E3L, extracted in nuclear buffer, and nuclear extracts. An equal volume of HIS-E3L, H3.3 and the nuclear extracts were crosslinked individually and together, using the required ³⁵S labelled combinations for the two proteins (Figure 5.14). Similar results were obtained to those described above, and no interaction was observed.

5.3.4 Affinity Blotting.

This protocol is similar to immunoblotting (western blot). The protocol utilises the interacting protein (derived from the yeast-two-hybrid screen) to identify and bind to the HIS-E3L protein blotted onto a nitrocellulose membrane, in place of an antibody. The technique has been used to identify proteins that interact with calmodulin (Phizicky and Fields, 1995).

The renaturation of the HIS-E3L protein in solution had not been successful, and so the protein was electrophoresed under native conditions. Following transfer from the gel, the membrane was incubated in renaturation buffer, to ensure the protein was in a native conformation. The strip used for western blot analysis served as a molecular weight marker for the HIS-E3L protein. The PIC-1³⁵S labelled *in vitro* transcription/translation product and nuclear extracts were then incubated with the appropriate strips and the results obtained. The ³⁵S labelled cellular protein did not interact with the HIS-E3L protein, as there was no radiolabelled protein band visible in the region where the HIS-E3L protein was located (data not shown).

Figure 5.13. Glutaraldehyde crosslinking analysis of HIS-E3L/H3.3 interactions. ^{35}S labelled HIS-E3L (**E**) and H3.3 (**H**) were incubated together in various combinations to analyse any interactions between the two proteins. The *, highlights those proteins that were ^{35}S labelled and **XL** depicts those proteins that were mixed in the presence of 0.01% glutaraldehyde. The results were analysed by SDS-PAGE (18%) and fluorography and are shown below. No interaction between the two proteins was observed.

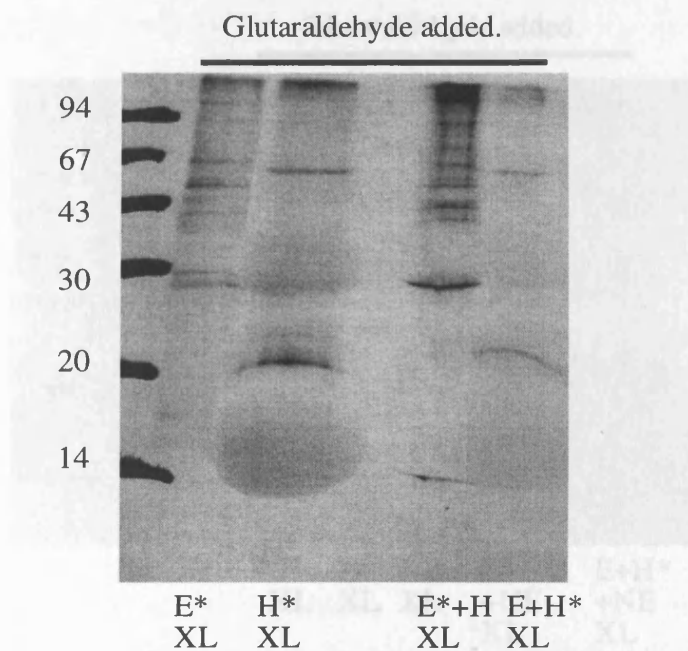
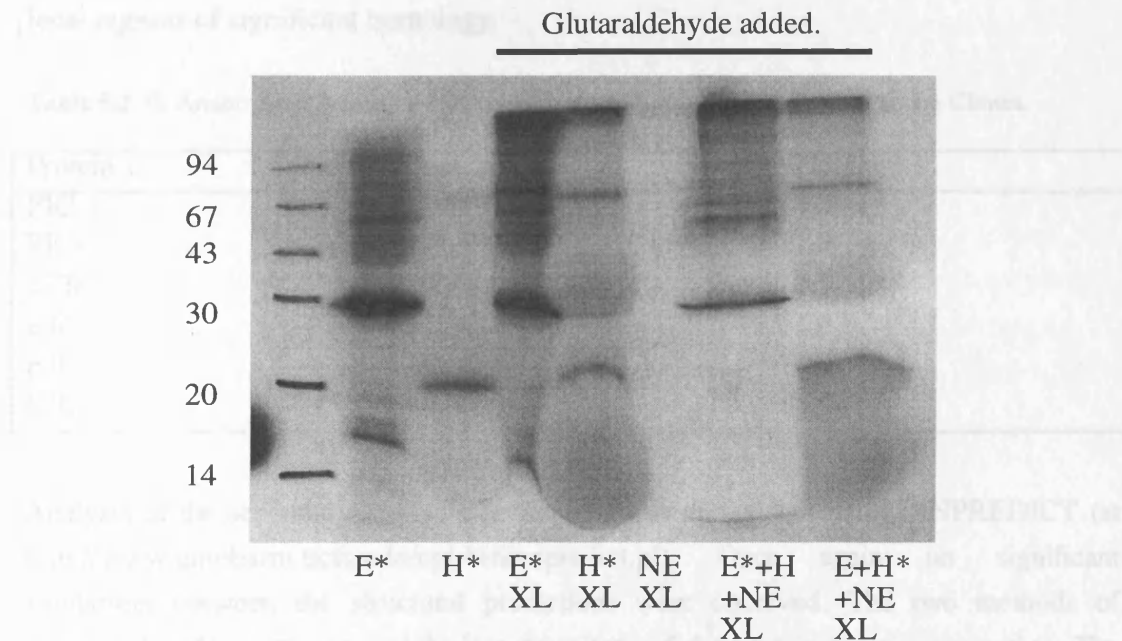


Figure 5.14. Glutaraldehyde crosslinking analysis of HIS-E3L/H3.3 interactions supplemented with nuclear extracts. ^{35}S labelled HIS-E3L (**E**) and H3.3 (**H**) were incubated together with nuclear extracts (**NE**) in various combinations to analyse any interactions between the two proteins. The *, highlights those proteins that were ^{35}S labelled and **XL** depicts those proteins that were mixed in the presence of 0.01% glutaraldehyde. The results were analysed by SDS-PAGE (18%) and fluorography and are shown below. No interaction between the two proteins was observed in the presence of the nuclear extracts.



5.3.5 Alignment Analysis and Secondary Structure Prediction of E3L and PIC-1, H3.3 and L23a.

Analysis of the secondary structure and amino acid sequence might give an insight into the nature of the protein-protein interactions. Alignment of the amino acids which is designed to determine the best overlapping sequence alignment was undertaken using Gene Stream (at <http://www2.igh.cnrs.fr/bin/align-guess.cgi>). The results found no significant similarities between PIC-1, L23a or H3.3 and E3L, Table 5.2 along with no local regions of significant homology.

Table 5.2. % Amino Acid Sequence Identity between E3L and the Cellular Interacting Clones.

Protein 1	Protein 2.	% identity.
PIC-1	L23a	14.7
PIC-1	H3.3	17.9
L23a	H3.3	22.5
E3L	PIC-1	11.8
E3L	H3.3	13.7
E3L	L23a	16.4

Analysis of the secondary structure predictions was carried out using NNPREPDICT (at <http://www.cmpharm.ucfs.edu/cgi-bin/nnpredict.pl>). Once again no significant similarities between the structural predictions were observed. The two methods of analysis therefore gave no insight into the nature of the protein-protein interaction. The entire structural prediction analysis and alignment data is found in Appendix 1.

6. Discussion.

The dsRBM of PKR and E3L interact with dsRNA. These motifs are common and are found in a number of proteins of diverse function (St.Johnston *et al.*, 1992). The E3L protein is important in Vaccinia Virus infection as it is known to downregulate PKR by two distinct mechanisms. The ability of the E3L protein to sequester the dsRNA and prevent PKR activation has been accepted for some years (Beattie *et al.*, 1991, Chang *et al.*, 1995, Shors *et al.*, 1997). More recently the E3L protein was found to form heterodimers with PKR preventing PKR activation by inhibiting the formation of PKR homodimers (Sharp *et al.*, 1998).

The results presented here include studies on the expression of regions of the two proteins in *E. coli*, the methods of induction and purification utilised and resulting solubility profile of each individual protein. Preliminary NMR analysis and hydrodynamic profiles were also undertaken where possible. The yeast-two-hybrid screen detected interactions between E3L and three cellular proteins PIC-1, histone H3 and the ribosomal protein L23a, the possible role of such interactions shall also be discussed in this section.

6.1 Protein Expression and Purification.

Eukaryotic proteins have been expressed in *E. coli* for a number of years, due to the low cost and high level of recombinant protein expression. The efficient expression of soluble eukaryotic proteins is not guaranteed due to a number of features including gene sequence, the mRNA stability and translational efficiency, the folding of the protein and also the degradation of the protein following translation (Makrides, 1996). A number of prokaryotic expression systems, expressed in *E. coli* strains deficient in specific proteases, are now available which serve to enhance recombinant protein expression. The pET16b plasmid was used throughout this project for the expression of the recombinant proteins. This system can produce histidine tag fusion proteins which facilitate efficient purification of proteins from the *E. coli* cellular protein pool, and the histidine fusion can also prevent protease digestion.

The dsRBM is present in a number of proteins (St.Johnston *et al.*, 1992), however two proteins containing the dsRBM were chosen for analysis in this project. The PKR dsRBD region was examined in an attempt to enhance the understanding of the dsRNA-dsRBM interaction mediated by the two dsRBMs, and the function of the hinge region. The amino terminal region of E3L, has recently been found to participate in the heterodimerisation with PKR (Sharp *et al.*, 1998). It was hoped the structural resolution of this region could provide insight into other mechanisms utilised within the infected cell, other than

sequestering dsRNA. The expression of the smaller E3L constructs were used to examine the structure of the dsRBM alone, which could then be compared to other dsRBM structures.

Each of the protein constructs were expressed and purified to homogeneity, however the highest concentration achieved for each protein was insufficient for NMR analysis. The expression and purification procedures used shall now be discussed, including any alternative procedures that could have been undertaken.

6.1.1 HIS-PKR Met1-170.

The protein was extracted and purified under near native conditions, to prevent problems associated with precipitation during the renaturation process.

1D NMR analysis demonstrated the protein was folded, however the concentration maximum of the protein was insufficient for structural resolution as is highlighted by the 2D NOESY analysis. The poor dispersal of signals indicated a third dimension was required to further dissipate the signals, this required the costly ^{15}N labelling of the protein. The maximum concentration of the protein extracted under these conditions however, was insufficient for NMR analysis and an obvious way forward for the project was not evident

The structure of the dsRBD of PKR has since been resolved (Nanduri *et al.*, 1998 a,b). The published extraction procedure initially denatured the protein in 6M guanidine hydrochloride. The protein sample was then purified by histidine tag affinity chromatography, and the purified recombinant protein renatured by dialysis producing a 0.9mM PKRdsRBD solution. During this project the denaturation of HIS-PKRMet1-170 was considered unnecessary, as the expressed protein was soluble in 1M urea, and was hoped to provide a homogeneous sample suitable for structural resolution. 1D NMR analysis confirmed the extracted sample was in a folded conformation, however the maximum concentration achieved was considerably lower than the published concentration. Denaturation followed by renaturation, provided a folded protein in a conformation which was consistent with the structure of other dsRBDs (Kharrat *et al.*, 1995, Bycroft *et al.*, 1995, Ryter *et al.*, 1998). The protein extracted under native conditions used in this project, may have exhibited a slight variation in conformation which then promoted precipitation of the protein at the lower concentration.

6.1.2 HIS-E3L.

The expression, purification and renaturation of this recombinant protein yielded a homogeneous protein sample as determined by SDS PAGE. 1D NMR analysis highlighted a lack of dispersal of the signals, suggesting the protein had not re-folded as

anticipated. The 1D NMR analysis indicated the sample was a complex mixture of misfolded and aggregated proteins, which exhibited a similar profile to a multi-globular protein.

Removal of the denaturant by dialysis, or dilution are standard means of renaturing proteins extracted from inclusion bodies. Aggregation of the protein during this procedure is common (Forciniti, 1994), and so the protein was refolded whilst bound to the affinity column. The resin was hoped to serve as a scaffold and also eliminate problems associated with the accessibility of the histidine fusion. Unfortunately the renaturation was incomplete leading to the formation of misfolded aggregates. The multimerisation properties of the protein may have contributed to the aggregated nature of the sample, as the decrease in concentration of the chaotropic agent, urea, may have increased the multimerisation of the protein, which was evident when the concentration of NaCl was decreased from 1M to 100mM (Shuman and Ho, 1996). The structural resolution of the dsRDBs of both PKR and *Xenopus Laevis* (Nanduri *et al.*, 1998 Ryter *et al.*, 1998), utilised proteins expressed in *E. coli* which were denatured in guanidine HCl and then renatured by dialysis. On a molar basis, guanidine-HCl is 2.5 times more powerful than urea as a chaotropic agent (Coligan *et al.*, 1995), and therefore the extraction of the protein in 6M urea may not have denatured the protein as efficiently as guanidine HCl, and the subsequent renaturation was therefore less efficient.

6.1.3 HIS-E3L_{Met38-190}.

This protein was solubilised in 6M guanidine HCl, however removal of the denaturant proved problematic. Due to the aggregation of the full length E3L whilst bound the affinity resin, renaturation was attempted in solution. Both dialysis and dilution of the sample were unsuccessful as large quantities of the protein precipitated soon after the removal of the denaturant. Multimerisation studies were not undertaken with this protein, and therefore the ability of this protein to self associate was not known. The renaturation data, together with the problems associated with the full length E3L protein indicated that this construct was not suitable for NMR structural resolution, when expressed under those conditions used.

6.1.4 HIS-E3L_{Met94-190}.

The purification and subsequent renaturation of the protein by dialysis proved successful, yielding a folded renatured protein with a concentration of 1.8mg/ml. FXa cleavage of the renatured protein was unsuccessful, suggesting the histidine tag had folded within the protein, thereby occluding the FXa cleavage site. The occlusion of the FXa site had occurred previously with the HIS-E3L protein, however the aggregation observed with that construct was not evident with HIS-E3L_{Met94-190}. It was concluded that the smaller

size of the protein contributed to the efficiency of the renaturation (Wilkinson and Harrison, 1991).

6.1.5 E3LMet94-190.

Of all the proteins expressed this construct proved to be the most successful. The protein was solubilised in 0.3M NaCl/0.2M NaBr, purified to homogeneity and concentrated by ammonium sulphate precipitation producing a maximum concentration of 5.5mg/ml (0.5mM). Preliminary 1D and 2D NMR spectroscopy indicated the protein was not aggregated and was in a folded conformation and as such suitable for preliminary NMR spectroscopy. The expression of ^{15}N labelled E3LMet94-190, required for 3D NMR analysis resulted in a decreased solubility maximum which was deemed to low for analysis by the NMR collaborators. Although this has been observed previously in the NMR laboratory, the exact cause of this apparent loss of solubility is unclear. The incorporation of the ^{15}N labelled NH_4Cl by the protein expression system may have been less efficient leading to the general downregulation of protein translation. Thus those proteins required for the efficient processing of the recombinant protein may have been downregulated leading to a high concentration of folding intermediate proteins, which are more likely to form insoluble inclusion bodies. It has also been speculated that the ultra pure $^{15}\text{NH}_4\text{Cl}$ lacks any minerals or trace elements present in standard NH_4Cl . It is thought that these elements may enhance the solubility of the recombinant protein in some way, and therefore without them the recombinant protein solubility is reduced.

6.1.6 Inclusion Body Formation.

A number of the proteins expressed have been extracted under denaturing conditions as a result of inclusion body formation. Inclusion body formation is associated with the high level of expression of a protein, which results in a concentration not ordinarily found in nature. A number of strategies can be applied to minimise the formation of insoluble proteins such as the growth of *E. coli* at a lower temperature which slows the production of the recombinant protein, or the expression of molecular chaperones which serve to promote efficient protein folding (Makrides, 1996). The expression of other fusion proteins such as thioredoxin, and glutathione-S-transferase (GST) promote the formation of soluble recombinant proteins by generating a less reducing environment in the cytoplasm, and thus favouring the formation of disulphide bonds (Hanrig and Makrides, 1998).

The solubility of recombinant proteins when expressed in *E. coli* can be estimated by the method devised by Wilkinson and Harrison (1991) (http://ftp.sunet.se/ftp/pub/Science/Molecular_Biology/mac/). The prediction analyses parameters involving the folding of the protein, such as disulphide bond formation and number of turn forming amino acids

(turns are the most difficult structures in protein formation and the number of turn forming amino acids gives an indication of the complexity of the folded protein). The hydrophobicity, charge and size are also taken into account as they affect the overall solubility of the protein. Table 5.1. highlights the predictions and actual solubilities.

Table 5.1 The table highlights the solubility prediction for each protein and the actual solubility as determined during the project. The value $CV-CV'$ is the canonical variable, CV , which is a composite parameter of the individual parameters, minus the discriminate canonical variable, CV' . If this value is high the solubility (negative value) or insolubility (positive value) of the protein can be predicted with a high degree of confidence. Low values indicate the protein is borderline and could be soluble or insoluble

Protein Construct.	% Insolubility	$CV-CV'$	Form of Extraction	Final Result.
HIS-PKR1-170	86%	1.81	1M urea.	Soluble 1M urea
HIS-E3L	53%	-0.15	6M urea	Aggregated 1M urea
HIS-E3LMet38-190	49%	0.00	6M guanidine HCl	Insoluble
HIS-E3LMet94-190	79%	1.33	6M guanidine HCl	Soluble 1M urea
E3LMet94-190	82%	1.42	0.3M NaCl/0.2M NaBr.	Soluble 0.3M NaCl/ 0.2M NaBr.

The solubility prediction data was not in line with the solubility profiles obtained within the project. The $CV-CV'$ values for both HIS-E3L and HIS-E3LMet38-190 were near to zero, making the prediction of solubility difficult. The two proteins were found to be highly insoluble, and renaturation of the proteins was unsuccessful due to aggregation and precipitation respectively. Conversely, E3LMet94-190 was predicted to be highly insoluble, but was extracted in 0.3M NaCl/0.2M NaBr and NMR analysis indicated the protein to be in a folded state. The predictive algorithm was therefore of little value for this project.

During this project the expression of proteins at reduced temperatures may have been productive, however previous experience in the laboratory had found proteins expressed under such conditions exhibited similar solubility profiles. The E3L Met94-190 was expressed at 20°C, and the solubility was found to be similar to those expressed at 37°C, data not shown. This, combined with a reduced yield demonstrated that this temperature was not of use for protein production. The use of alternative fusion proteins and molecular chaperone expression was not considered due to time constraints within the project.

6.1.7 Self Association of E3L_{Met94-190}.

E3L_{Met94-190} was found to self associate in solution, as determined by gel filtration analysis, ultra centrifugation, and crosslinking studies. Analytical ultracentrifugation and gel filtration analysis suggested that the protein was dimeric in solution. The analytical ultracentrifugation was undertaken at three concentrations, 3.4mg/ml, 1.1mg/ml and 0.4mg/ml to ascertain if oligomerisation was a function of protein concentration. The concentration of the protein did not modify the dimerisation properties, however it could be assumed that the concentration of full length E3L within the infected cell would be significantly lower than 0.4mg/ml. Gel filtration analysis indicated the MW of the protein to be approximately 20kDa, which is consistent with dimer formation. The rate of protein elution from a gel filtration column is influenced by the MW of the protein. Other factors such as the shape of the protein can increase or slow the elution rate from the column, which can lead to a misrepresentation of the MW of the sample. In this case however, the results were considered conclusive as they were consistent with the findings of the analytical ultra centrifugation.

The results of the crosslinking analysis yielded the formation of a number of increased MW protein bands, which were consistent with dimers, trimers, tetramers and multimers. Another study examined the self association of both the full length E3L and a smaller construct of 90 amino acids E3L₁₀₀₋₁₉₀ (Ho and Shuman, 1996). The full length protein was found to self associate, and form higher order oligomers as the ionic strength was reduced from 1M to 200mM NaCl. The smaller E3L₁₀₀₋₁₉₀ however formed dimers at all ionic strengths investigated, which is in contrast to the results obtained with E3L_{Met94-190} which was undertaken in 0.3MNaCl/0.2MNaBr. From this crosslinking data it could be suggested that a region important for oligomerisation is located within amino acids 94-100.

6.1.8 Further work.

Clearly a way forward for those proteins which had a maximum concentration that was deemed insufficient for NMR analysis, could be denaturation in 6M guanidine HCl as was undertaken for the dsRBD of PKR (Nanduri *et al.*, 1998). This method produced a concentration of 0.9mM which was far in excess of the values obtained when the both HIS-PKR Met1-170 and E3L Met94-190 were extracted under native conditions. However whilst this is still a worthy objective, several other dsRBM structures have now been determined which has undoubtedly eliminated the requirement for another dsRBM alone, although not perhaps the whole E3L protein.

6.2 Yeast-Two Hybrid Screen.

The yeast-two-hybrid screen was undertaken to ascertain which, if any cellular human proteins interacted with the E3L protein. The library scale transformation screened twice the number of independent cDNA clones recommended by the manufacturers, and therefore was deemed to be a fair examination of E3L and cellular protein interactions.

6.2.1 The Yeast-Two-Hybrid Screen.

The β galactosidase assay profile from those clones derived from the glycerol stock, Table 4.2, differed somewhat from the original findings, Table 4.1. The exact reason for this was unclear, one or both of the plasmids may have been eliminated from the cell during the culture growth period of the yeast resulting in a negative result. Those colonies which were originally deemed negative and then found to be positive may have not been segregated thoroughly before glycerol stock preparation. The colonies could have contained multiple GAL-4 AD/library plasmids which obscured the β galactosidase positive result, however growth of the colony in culture may have permitted the elimination of the excess plasmids leading to the positive result. Alternatively cross contamination could have taken place although this is unlikely. Those yeast colonies derived from the glycerol stock, that were $his^+/LacZ^+$ were then used to examine the interaction between E3L and cellular proteins.

Identification of duplicate interacting clones were problematic, as the analysis by PCR amplification was unsuccessful. A number of samples contained multiple amplification products, whilst others lacked amplified products completely. The primer design and cycling conditions used were those as recommended by the manufacturers, and as such were optimal for such analysis. Hot start PCR was also used however this did not affect the outcome of the amplification. The T-tract manual sequencing analysis was also unsuccessful as very few of the samples produced any legible sequence. The crude preparation of yeast DNA contained both chromosomal DNA and plasmid DNA which was then transformed into *E. coli* HB101. Although growth in the HB101 strain selected for the GAL-4 AD/library plasmid alone, the GAL-4 BD/E3L plasmid was maintained throughout as is evident from the manual sequencing data where a VV E3L gene sequence was detected. The low annealing temperature (37°C), used during manual sequencing reactions, could have induced the primer to anneal to the wrong template, however the primer should have also annealed to the correct GAL-4 AD/library template. The VV E3L sequence data derived from the experiment may therefore indicate that this plasmid was present in greater quantities. The contamination of the sample by the GAL-4BD/E3L and other contaminants derived from the yeast could also account for the poor results obtained by PCR amplification.

Due to the problems associated with grouping alike clones, all *his⁺/Lac Z⁺* clones were examined to ascertain which were genuine interacting clones using the yeast mating assay (Harper *et al.*, 1993). 10% of the clones did not eliminate the GAL-4 BD/E3L plasmid following selective growth in -*leu* media. Recombination of the yeast plasmids with the chromosome is not uncommon and is particularly prevalent with an 2 μ ori (A.Cashmore, personal communication), these clones therefore were of no use for yeast mating assays. A number of the GAL-4 AD/library plasmids, expressed in strain Y190, interacted with the mating control protein Lamin C. This protein interacts with very few other proteins and as such serves to highlight a false positive. On very rare occasions however authentic interactions between the GAL-4 AD/library protein and lamin C can occur and so the yeast mating was repeated with 4 distinct proteins cloned into GAL-4 BD plasmid. The proteins used Traß, Sam68, pStar and a human testes protein RNA recognition motif (RRM) were not necessarily an ideal choice for use as controls as they exhibit RNA binding characteristics. The RNA interaction however is distinct to that of E3L however other proteins would have been better suited to this purpose.

6.2.2 Identification Of the Region of E3L required for Interactions.

Following elimination of the false positives, sequence analysis, and the identification of the interacting clones, the region of E3L required for the interactions were analysed. It was hoped that the amino terminal region was required the interactions with PIC-1 and H3 as this could account for the nuclear localisation of the full length protein and only cytoplasmic localisation of the dsRBM (Yuwen *et al.*, 1993). This was not the case however as the three proteins interacted with the dsRBD of E3L (amino acids 94-190). As discussed previously the dsRBD of PKR mediates a number of interactions, and so the ability of this region to mediate such interactions was not unlikely.

6.2.3 E3L Protein Expression in Yeast.

The extraction of proteins from yeast cells is particularly challenging due to the high level of endogenous yeast protease expression and also the durable yeast cell wall. Protein extraction from yeast therefore is highly variable and the method of extraction difficult to predict. Yeast cells were grown to mid-log phase to prevent proteolysis by endogenous proteases produced during late log phase and were extracted with a cocktail of protease inhibitors present. The E3L protein extracted using both the SDS/urea and TCA methods was not detected by SDS PAGE and western blot analysis. It was hoped the dot blot technique would enhance the stringency of the western blot detection, however due to the high concentrations of protein present and resulting non-specific interactions, a number of the control samples gave rise to a positive result. Extraction of the protein for immune precipitation work was also undertaken using RIPA buffer and glass beads (section

2.6.3). Western blot analysis of the proteins bound to the protein A sepharose beads did not show E3L expression, data not shown.

The GAL-4 BD/E3L plasmid was known to be present within the yeast cells due to the nutritional selection of the yeast following transformation. The E3L insert was known to be present in the plasmid due to sequence and restriction digestion analysis, data not shown. This coupled with the results of the yeast mating experiment whereby a positive result was observed only when the GAL-4 AD/library plasmid was mated with the GAL-4BD/E3L plasmid and not the GAL-4/BD plasmid alone, further verifies this.

PKR expression in yeast has been detected previously however the constructs were mutants unable to exhibit the downregulating properties of the full length catalytically active protein (Chong *et al.*, 1992, Romano *et al.*, 1995). An inducible promoter, the GAL-1-CYC1 hybrid, induced when grown on galactose as the sole carbon source, was used for the expression of such constructs. The ADH1 promoter, used in the pAS2-1 plasmid, is known to be a strong promoter and has been reported to induce high levels of protein expression (Harper *et al.*, 1993). However in this case it was perceived that the level of protein expression within the yeast cells was insufficient for E3L protein detection by western blot as the protein was not detected in the soluble and insoluble fractions collected, although expression levels were sufficient to permit the yeast-two-hybrid system to work efficiently.

6.2.4 *In vitro* Interaction Analysis.

The use of *in vitro* interaction techniques are an independent demonstration of interactions required for confidence in yeast-two-hybrid results. There are a number of *in vitro* protein-protein interaction methods which can be utilised on such occasions which include immune precipitation, crosslinking, affinity chromatography and affinity blotting (Phizicky and Fields, 1995). Interaction analysis between the proteins was undertaken in native conditions using E3L expressed in *E. coli* and *in vitro* translated H3 and PIC-1. Crosslinking and Poly(rI)(rC) methods were also undertaken in the presence of nuclear extracts to provide other proteins that may be required for the interaction to take place. Each of the methods listed above were used to verify the results of the yeast-two-hybrid screen, however no interaction *in vitro* was observed.

The immune precipitation method utilised the MAb reactive against E3L, TW2.3. The antibody was previously used for western blot analysis, confocal microscopy and immune precipitation by the group who had engineered the protein (Yuwen *et al.*, 1993). Unfortunately non-specific interactions between the E3L, expressed in *E. coli* BL21, and protein A and G sepharose could not be eliminated, which resulted in this method being unsuitable. Each of the blocking and pre-clearing procedures reduced the interaction,

however the non-specific interaction was not eliminated entirely. The observed non specific interaction of proteins expressed in *E. coli* with the sepharose beads has occurred previously in the laboratory (Ben Madison, personal communication), and as yet no satisfactory resolution to this problem has been found.

Two forms of affinity chromatography were used to verify the interaction. The histidine tag affinity interaction studies were not pursued as the increased sensitivity associated with the ^{35}S labelled bacterial lysate highlighted a number of non-specific interactions. The poly(rI)(rC) resin interacted specifically with E3L and was therefore used to verify the interactions. No interaction was observed in each of the three buffers, also the addition of the nuclear extracts did not initiate an interaction between H3 and /or PIC-1 with E3L.

The results of the crosslinking analysis was similar to that of the poly(rI)(rC) affinity chromatography. The two proteins H3 and PIC-1 failed to interact with E3L both in the absence and presence of nuclear extracts. The results of the affinity blot were also inconclusive. ECL western blot analysis of the E3L protein alone detected a band at the top of the gel, which highlighted the slow movement of proteins through the native gel after 18 hours. There was no evidence of an interaction between E3L and PIC-1 as no ^{35}S labelled band was located in the appropriate lanes. The two proteins were believed to be in a native conformation, following the extensive renaturation of the blotted E3L protein, and therefore should permit the interaction between the two.

A number of reasons can account for the apparent lack of interaction *in vitro*, including the modification state of the protein. The H3.3 protein is susceptible to a number of post transitional modifications, and the PIC-1 protein can complex with a number of proteins within the cell. Although both proteins were produced in a rabbit reticulocyte transcription /translation system the correct physiological modification state required for the interaction as found within a cellular environment may not have been achieved . The physiological concentrations of the proteins is also of importance, as a weak interaction *in vivo* would require high concentrations to observe an interaction *in vitro*. The E3L protein used for interaction studies was expressed in *E. coli* BL21 which could exhibit an altered conformation resulting in no visible interaction. An ideal environment therefore to observe such interactions would be in a VV infected mammalian cell, using confocal microscopy. This was attempted however the experiment was inconclusive due to insufficient time and lack of control antibodies.

A number of other studies have undertaken the yeast-two-hybrid screen then verified the results *in vitro*. For example, Sharp *et al.* (1998) and Romano *et al.* (1998) detected the interaction between E3L and PKR. The interaction was verified *in vitro* using GST fusion binding assays. Both groups used a radiolabelled *in vitro* translated protein coupled with

a GST fusion protein expressed in *E. coli* as was attempted during this investigation. Immune precipitation experiments were also undertaken by Patel and Sen (1998), when verifying the interaction between PKR and PACT. The interaction between E3L and the two proteins therefore was not demonstrated *in vitro*. This may suggest the interactions were mediated by one or a number of other complexed proteins found within the cell, or alternatively the conditions required for the interaction were not achieved during the experiments.

It could have been assumed at the outset that one protein that could interact with E3L would be PKR. However, the failure to observe this interaction is not surprising as the expression of PKR within yeast cells would lead to the downregulation of protein expression, therefore any colonies expressing the PKR protein that interacted with E3L would not have grown. It could also have been assumed that proteins which have been found to interact with PKR would also interact with E3L, due to the relatedness of the two proteins. One explanation as to why E3L did not interact with P58^{IP} or TRBP for example, is that such an association could lead to the inhibition of protein synthesis mediated by PKR by way of the formation of active homodimers. The interaction between E3L and PACT, a proposed cellular activator of PKR, on the other hand could have eliminated the enhanced activation of PKR observed by Patel and Sen (1998).

6.2.5 Interaction Proposals.

The possible role of the Histone H3.3 Protein and VV E3L Interaction.

Histone proteins have been studied extensively due to the important role in DNA packaging. Two of each of the four histone proteins, H2a, H2b, H3 and H4 form the core which is wrapped in 146bp of DNA, forming a nucleosome (Hammiche and Richard-Foy, 1998). The H1 histone protein associates with the linker DNA between the nucleosomes, and is thought to help mediate the folding of the DNA into condensed higher order chromatin (Kaludov *et al.*, 1997). Histone genes are expressed both in a replication dependent and independent manner. The replication variants, H2a, H2b, H3 and H4 are expressed at high levels during S phase and therefore are closely linked to DNA replication. The replication independent histones, such as H3.3, are synthesised throughout the cell cycle and are the predominant histones in non dividing cell that replace the normal counterparts in nucleosomes (Thatcher *et al.*, 1994).

Histone proteins are subject to post-translational modifications including phosphorylation, acetylation and ubiquitination which are believed to be involved in transcriptional activation (van Holde *et al.*, 1992). Conformational changes within the nucleosome are noted once the histone proteins are acetylated as the association between the protein and the DNA is weakened. A link between transcriptionally active chromatin

and acetylation of the histone proteins at the conserved lysine residues on the amino terminal tails has been noted (Wade *et al.*, 1997), however a number of examples have been discovered which indicate the acetylation patterns are much more complex than originally thought (Struhl, 1998). Phosphorylation of histone proteins, particularly H1, is required for chromatin condensation prior to mitosis. Calf thymus H2A been used on numerous occasions as an exogenous substrate as a measurement of PKR phosphorylation (Gunnery and Mathews, 1998). Ubiquitination of the H2B histone protein has been linked with ongoing transcription as this modification is inhibited in the presence of transcriptional inhibitors (van Holde *et al.*, 1992). More recently the H3 protein was found to be ubiquitinated in rat spermatids however the exact purpose of this has yet to be determined (Chen *et al.*, 1998).

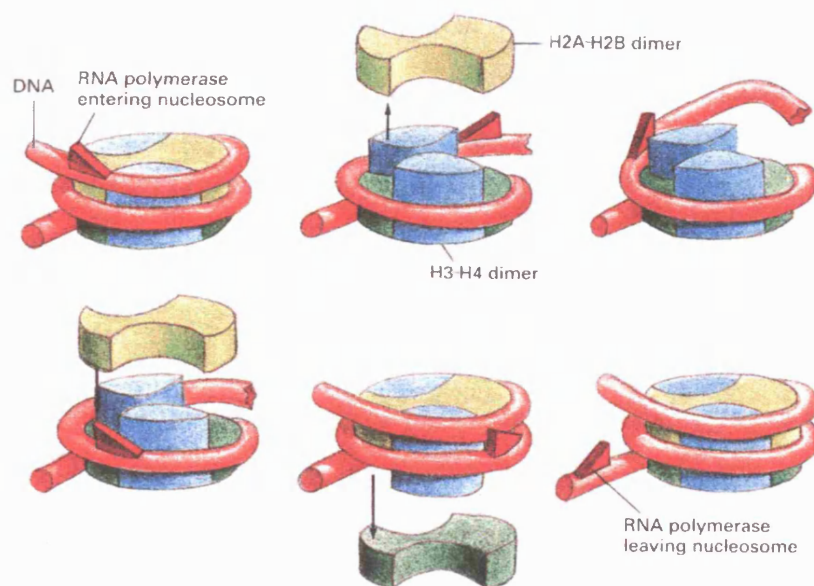
The mechanism by which the histones are displaced from the nucleosome is still not yet fully understood however a model for this event has been suggested (van Holde *et al.*, 1992) (Figure 6.1).

As the RNA polymerase tracks along the DNA transient positive supercoils are formed ahead of the transcription complex, which are believed to cause the nucleosomes to split with the displacement of the H2A/H2b dimer. The positively supercoiled DNA remains bound to the (H3/H4)₂ tetramer as the tetramer is able to switch conformation which is mediated by the H3-H3 interface (Hammiche and Richard-Foy, 1998). Following RNA transcription negative supercoiling is evident, which is believed to facilitate nucleosome restructuring.

Other viruses have been reported to interact with histone proteins. Ramsperger and Stahl (1995) suggested the SV40 T antigen interacted with both H3 and H1 which facilitated the nucleosome disruption required for efficient DNA replication. The E1 protein of HPV was also found to interact with H1, facilitating the disruption of the nucleosome (Swindle and Engler, 1998).

The interaction between E3L and H3.3 therefore may promote or inhibit the transcription of specific genes. The interaction may prevent the post transcriptional modifications of conformational change associated with active gene transcription resulting in the downregulation of proteins such as those involved in the host immune response. Parekh and Maniatis (1999) found the H3 and H4 histones located at the IFN β promoter were specifically acetylated during viral infection. The interaction of E3L with H3 therefore could prevent this and the resulting induction of IFN- β transcription. Alternatively the interaction may promote nucleosome dissociation and therefore promote gene transcription producing host cell proteins to be utilised by the virus.

Figure 6.1 As is visible from the diagram the first H2A/H2B dimer is initially displaced from the nucleosome as the RNA polymerase approaches. The DNA is then pulled away from the (H3/H4)₂ tetramer which binds to the DNA once transcribed. The first H2A/H2B dimer is then recaptured, whilst the second H2A/H2B dimer is displaced by the RNA polymerase. The nucleosome then reforms. (Alberts *et al.*, 1994).



The possible role of the L23a Ribosomal Protein and VV E3L Interaction

Ribosomal proteins are very basic with few areas of negative potential, which permit the required interaction with RNA (Ramakrishnan and White 1998). The proteins are classified into one of two groups depending on which subunit they reside, L (large) or S (small). The mammalian L23a protein therefore resides in the large subunit and is believed to be involved in the initiation of the assembly of the large ribosomal subunits due to its relatedness to the *S.Cerevisiae* L25 protein and *E. coli* L23 protein which carry out this function (Suzuki and Wool, 1993). Koyama *et al.* (1999) found the *Drosophila* L23a protein interacted with PARP (poly ADP-ribose polymerase), a nuclear protein involved in DNA repair, and concluded the protein may also play a role in DNA binding.

PKR has been reported to associate primarily with the 40S, and also the 60S ribosome (Zhu *et al.*, 1997, Raine *et al.*, 1998, Romano *et al.*, 1998). The interaction is mediated by the dsRBD and rRNA, and is believed to permit the efficient phosphorylation of eIF2- α by localising PKR to the translational machinery. The E3L dsRBM was found to displace the PKR protein from the 40S ribosome, more efficiently than the full length protein, which has been attributed to the decreased oligomerisation capabilities of the dsRBM alone (Romano *et al.*, 1998).

The L23a protein has been reported to be downregulated at the post transcriptional level following treatment of cells with IFN α/β and γ , but not when cells are subjected to serum starvation (Jiang *et al.*, 1997). The *de novo* production of antisense RNA transcripts, which were observed in tumour cells, are thought to mediate the degradation of the paired sense and antisense dsRNA by an enzyme such as RNase L activated by the 2'-5' A system. This hypothesis has yet to be verified (Jiang *et al.*, 1997).

The interaction between E3L and L23a may prevent the binding of PKR to the 60S subunit or obscure the PKR/40S interaction, thereby preventing the required localisation of PKR for the efficient phosphorylation of eIF2- α . Alternatively, the viral infection can cause stimulatory effects on cell growth by means of the Vaccinia Virus growth factor, VGF (Moss, 1996) and the interaction may enhance the translational efficiency of the ribosome.

The possible role of the PIC-1 and VV E3L Interaction.

Within the nucleus of the cell are located a number of nuclear dots (NDs) or promyelocytic leukaemia (PML) oncogenic domains (PODs) found predominantly in dividing cells (Sternsdorf *et al.*, 1997). Normal cells contain 10-20 NDs that are approximately 0.3-1 μ m in diameter and are associated with the nuclear matrix (Lamond and Earnshaw, 1998). The NDs are composed of a number of proteins three of which include PML, SP100 and ND5P2. Each of the proteins are believed to be upregulated by IFN (Muller *et al.*, 1998).

APL (acute promyelocytic leukaemia) is a haemopoietic malignancy characterised by the block in differentiation of cells at the promyelocytic stage. A translocation between the PML gene on chromosome 15 and the retinoic acid receptor α on chromosome 17, resulting in a RAR α -PML fusion protein is evident in almost all cases. The fusion of the RAR α protein to PML disrupts the nuclear dot localisation of PML leading to a micropunctate pattern within the nucleus. The effect of the translocation can be reversed by the addition of *trans* retinoic acid or arsenic trioxide (As₂O₃), which lead to the dissociation of the two proteins and the formation of the structured NDs with PML located within them. The APL blast then undergoes differentiation leading to complete remission in patients suggesting the NDs play a role in the regulation of cell growth (Koken *et al.*, 1994).

PIC-1 (PML interacting clone 1) was initially identified in a yeast-two-hybrid screen due to the strong interaction with PML (Boddy *et al.*, 1996) and is also known as SUMO-1, small ubiquitin-related modifier (Mahajan, 1997), sentrin (Okura *et al.*, 1996) and GMP1, Gap modifying protein1 (Matunis *et al.*, 1996). The protein is a member of the

ubiquitin-like protein family characterised by a ubiquitin homology domain, and exhibits 18% amino acid identity to ubiquitin itself. Structural resolution of the protein highlighted a number of structural similarities to the ubiquitin family (Bayer *et al.*, 1998). The covalent modification mediated by PIC-1 however does not target the proteins for degradation by the 26S proteasome. A number of PIC-1 protein homologues have been identified which contain the ubiquitin homology domain. The SMT3 protein, found in budding yeast and human SMT3A/B show the highest homology. In yeast the protein is a suppresser of mutations in the MIF2 centromeric protein and therefore is important in the regulation of cell cycle control (Sterndorf *et al.*, 1997).

The PIC-1 protein has been found to interact with a number of proteins including the TNF receptor and the death domain of Fas/Apo-1 by way of the yeast-two-hybrid screen. Overexpression of the PIC-1 protein prevented anti Fas/Apo-1 and TNF induced cell death (Okura, 1996). Although PIC-1 interacts with a number of proteins, covalent modification has been noted only in the case of PML (Sterndorf *et al.*, 1997, Muller *et al.*, 1998, Kamitani *et al.*, 1998), and the Ran GTPase activating protein RanGap1 (Matunis *et al.*, 1998, Mahajan *et al.*, 1998). The PML-PIC-1 conjugate is located only in the NDs whereas the free form of PML is dispersed through the nucleoplasm. Treatment of APL patients with retinoic acid results in the formation of the PIC-1-PML conjugates in the NDs. This indicates the modification modulates the intracellular localisation. RanGTPase, essential for bidirectional protein transport into the nucleus, requires an interaction with RanGap1, a GTPase activating enzyme required for the efficient hydrolysis of GTP. The RanGap1 protein must therefore be in close proximity to the nuclear pore complex to be of use. The localisation of the protein at the nuclear envelope has recently been associated with the modification by PIC-1 (Mahajan *et al.*, 1998, Matunis *et al.*, 1998). The localisation of each of the proteins would appear to be determined by the modification induced by PIC-1 indicating such proteins may play an important role in the structure and function of the NDs.

The upregulation of the ND associated proteins by IFN suggest viral infection may modulate ND composition. Herpes simplex virus 1 (HSV1), cytomegalovirus (CMV), and adenovirus have been observed to disrupt the nuclear dots which are close to the preferred location for adenovirus and HSV-1 initial DNA replication (Sterndorf *et al.*, 1997, Everett *et al.*, 1997). The HSV-1 infection is believed to mediate the degradation of the modified PML fractions by association with an early viral protein, Vmw110. Vmw110 interacts with both PML and HAUSP, a ubiquitin dependent protease, which localises to NDs during infection (Everett *et al.*, 1997, 1998). Adenovirus infection leads to structuring of the spherical nuclear dots into fibrous like structures early in infection. The cellular proteins within the ND are then either actively transported to viral factories by viral proteins, or diffuse to that region (Doucas *et al.*, 1995).

Due to the complex role of the NDs within the cell, it is difficult to speculate the role of the PIC-1/E3L interaction. The interaction could impair or enhance the modification of PML and the other unknown cellular proteins which have been reported to be modified (Muller *et al.*, 1998). If the DNA replication is deregulated by obstructing the modification of PML by PIC-1, as is the case with APL, this will enhance cellular DNA synthesis which would lead to the production of a number of cellular factors required for efficient transcription. This therefore could be of use to those viruses which replicate in the nucleus, such as adenovirus and HSV-1. DNA replication within a poxvirus infected cell is undertaken in the cytoplasm however, the nucleus of the cell is required to be present suggesting such modifications may be required. Other proteins with which PIC-1 associates such as RanGAP1, and the TNF receptors may also be affected by the association of E3L. The overexpression of PIC-1 inhibits anti Fas and TNF induced cell death, and could be enhanced by the interaction with the E3L protein. The interaction between E3L and the RanGap1 protein could lead to the downregulation of the protein transport into and out of the nucleus due to the inability to form the nuclear pore complex. Presumably the proteins to be transported into the nucleus can either be utilised by the virus or else debilitate the cellular transcription machinery making the cell less viable.

6.2.6 Further Work.

The clear way forward would be to verify those interactions derived from the yeast-two-hybrid screen *in vitro*. Confocal microscopy is now used (almost) as standard to confirm protein-protein interactions, and could provide any additional proteins or environmental conditions which would permit the interactions to take place. Following *in vitro* confirmation, the purpose of the interactions could be investigated which would provide a further insight into of the events within the VV infected cell.

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8. Appendix 1.

8.1.1 Secondary structure predictions.

E3L.

Secondary structure prediction (H = helix, E = strand, - = no prediction):

```

---EEE-----HHHHHHH--EEEHH-HHHHHHHHHHHHHHHHHHHHHHHHHHH--EEE
-----HHHEEE-----H-----EE--
---EEE--HHHH-----EEEE-----EEEE-----E-----HH
HHHHHHHHHHHH--HEEEE-

```

H3.3.

Secondary structure prediction (H = helix, E = strand, - = no prediction):

```

---H-H-----HHHHHHHH-----HHHHHHHHH-
-HHHHHH-----HHHHHHHHHH--HHHHHHHHHHHHHHHHHHHHEE-----HHHHH
-----HHHHH-----

```

L23a.

Secondary structure prediction (H = helix, E = strand, - = no prediction):

```

-----HHHHHHHHHHHHHHHHHHHH-----
-----EEEE-----HHHHHHHH-----EEEEH-----HHHHHHHH
-H--HHH-----E-----HHEE-----HHHHH-----

```

PIC-1.

Secondary structure prediction (H = helix, E = strand, - = no prediction):

```

-----HHHEEEEE-----HHHEEH-HHHHH-----
---HHHHHHHH-----HH-HHHHH-----

```

8.1.2 Alignment Analysis.

L23a + H3.3

>_ L23a 156 aa vs.

>_ H3.3 136 aa

22.5% identity; Global alignment score: 6

```

                10         20         30         40         50         60
277282 MAPKAKKEAPAPPKAEAKAKALKAKKAVLKGVHSHKKKKIRTSPTRRPKTLRLRRQPKY
      :: . . . . :      . . . : : : : . . . :      :      . :      : . . . . . :
_      MA-RTKQTARKSTGGKAPRKQL-ATKAARKSAPSTGGVK--KPHRYRPGTVLREIRRY
                10         20         30         40         50

                70         80         90        100        110
277282 PRKSAPRRNKLDHYAIIKFPLTTESAMKKIEDNNTLVFIVDVKANKHQIK--QAVKKLYD
      ...      . : : . . . . . : . . . : . : : . . . : : : : . . . :
_      QKST-----ELLIRKLPF--QRLVREIAQD----FKTDLRFQSAAIGALQEASEAYL
                60         70         80         90        100

                120        130        140        150
277282 IDVAKVNTLIRPDGEKKAYVRLAPDYDALDVANKI-GI-I
      . . . . . :      . . . : . : : : . . . : . : :
_      VGLFEDTNLCAIHAKR---VTIMPK-D-IQLARRIRGERA
                110        120        130

```

L23a + PIC-1.

>_ L23a 156 aa vs.

>_ PIC-1101 aa

14.7% identity; Global alignment score: -76

```

          10      20      30      40      50      60
471374 MAPKAKKEAPAPPKAEAKAKALKAKKAVLKGVSHKKKKIRTSPTRRPKTLRLRRQPKY
      . . . : . . : . . : . . . . . : . .
_      MSDQEAK--PSTEDLGDKKEGEYIKLVIGQDSSEIHFVKMTTHLKKLKESYCQRQG-V
          10      20      30      40      50

          70      80      90      100     110     120
471374 PRKSAPRRNKLDHYAIIKFPLTTESAMKKIEDNNTLVFIVDVKANKHQIKQAVKKLYDID
      : . . : . . : . . : . . : . . : . . :
_      PMNS-----LRFLFEG----QRIADN-----HTPKEL--GMEEED
          60              70              80

          130     140     150
471374 VAKVNTLIRPDGEKKAYVRLAPDYDALDVANKIGII
      : . . : . . . . .
_      VIEV-----YQEQTGGHST-----V
          90              100

```

H3.3 + PIC-1

>_ H3.3 136 aa vs.

>_ PIC-1101 aa

17.9% identity; Global alignment score: -45

```

          10          20          30          40          50          60
304992 MARTKQTARKSTGGKAPRKQLATKAARKSAPSTGGVKKPHRYRPGTVALREIRRYQKSTE
      :  .  :  .  ::      .  :      :  ::  .  :      .  :  :      :  ::
_      M--SDQEAKPST-----EDL-----GDKKEGEY----IKLKVIG--QDSSE
          10          20          30

          70          80          90          100         110         120
304992 LLIRKLPFQRLVREIAQDFKTDLRFQSAAIGALQEASEAYLVGLFEDTNLCAIHAKRVTI
      .  .  :  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
_      IHF-KVKMTTHLKKLKESY---CQRQGVPMNSLRF-----LFEGQRIADNHTPKELG
          40          50          60          70          80

          130
304992 MPKD--IQLARRIRGERA--
      :  .  .  :  .  .  .  :  .  .
_      MEEEDVIEVYQEQTGGHSTV
          90          100

```

E3L + L23a

>_ L23a 156 aa vs.

>_ E3L 271 aa

16.4% identity; Global alignment score: -172

```

          10          20                      30          40
957519 MAPKAKKEAPAPPKAEAKAKALKA-----KKAVLKGVHSHKKKKIRT
      :  .:      .::  .:..      :. : :....  . .
_      M---SKIIYIDERSNAEIVCEAIKTIGIEGATAAQLTRQLNMEKREVNKALYDLQRSAMVY
          10          20          30          40          50

                      50
957519 S-----PTF-----RRP-----KTL-----RLRRQPK
      :  :  .      .:      :..      .: : .
_      SSDDIPPRWFMTEADKPDADAMMSKIIYIDERSNAEIVCEAIKTIGIEGATAAQLTRQLN
      60          70          80          90          100          110

      60          70          80          90          100
957519 YPRKSAPRR-NKLDHYAII-----KFPLTTESAMKKIEDNNTLVFIVDVKANK----
      . . . .      :.. :..      .. ::: : :  :  . ::: :.. :.
_      MEKREVNKALYDLQRSAMVYSSDDIPPRWFMTE-ADKPDADAMADVIIDVSREKSMRE
      120          130          140          150          160          170

      110          120          130          140
957519 -HQIKQAV---KKLYDIDVAKVNTLIR-----PDGEKKAYVRLAPD-
      :.  . :  :.. :  :.  :::      :..      :.  . :
_      DHKSFDDVIPAKKIIDWKGANPVTVINEYQCITRRDWSFRIESVGPSNSPTFYACVDIDG
      180          190          200          210          220          230

          150
957519 --YDALDVANK-----IGII-----
      .:  :  .:      .: .
_      RVFDKADGKSKRDAKNNAAKLAVDKLLGYVIIRFZ
      240          250          260          270

```

E3L + PIC-1

>_ PIC1 101 aa vs.

>_ E3L 271 aa

11.8% identity; Global alignment score: -271

```

                                                    10
304992 MS----DQEAKP-----STE
      ::      :....
_      MSKIYIDERSNAEIVCEAIKTIGIEGATAAQLTRQLNMEKREVNKALYDLQRSAMVYSSD
          10          20          30          40          50          60

                20                      30
304992 DL-----GDKKEGE-----YIK-----LKVIGQD-----
      :.      .:: ...      ::      .:::: .
_      DIPPRWFMTEADKPDADAMMSKIYIDERSNAEIVCEAIKTIGIEGATAAQLTRQLNMEK
          70          80          90          100          110          120

                        40                      50
304992 -----SSEIHFVKVMTTHL-----KKLKES---
      :...: .  :...      :...:
_      REVNKALYDLQRSAMVYSSDDIPPRWFMTEADKPDADAMADVIIDVSREKSMREDHKS
          130          140          150          160          170          180

                                60          70
304992 -----YCQ--RQGV-----PMNSLRFL----FEGORIA
      :...: .  :...: :  :...:
_      FDDVIPAKKIIDWKGANPVTVINEYQCITRRDWSFRIESVGPSNSPTFYACVDIDG-RVF
          190          200          210          220          230

                80          90          100
304992 DNHTPKELGMEEEDVIEVYQEQTGGHSTV---
      :.  :  .... ..  :.  .
_      DKADGKSKRDAKNNAAKLAVDKLLGYVIIRFZ
          240          250          260          270

```

E3L + H3.3

>_ H3.3 136 aa vs.

>_ E3L 271 aa

13.7% identity; Global alignment score: -216

```

                                10
151611 MARTKQTAR-----KSTG-----GKA-----
      ...      :      :. :      ...
_      MSKIYIDERSNAEIVCEAIKTIGIEGATAAQLTRQLNMEKREVNKALYDLQRSAMVYSSD
              10          20          30          40          50          60

              20          30          40          50          60
151611 ---PRKQLATKAARKSAPST-GGVKKPHRYRPGTV--ALREIRRYQKSTELLIRKLPFQ-
      :: .::: . .: . . . . :      : :.. :      .. : :.. ..
_      DIPPRWFMTTEADKPDADAMMSKIYIDERSNAEIVCEAIKTIGIEGATAAQLTRQLNMEK
              70          80          90          100          110          120

              70          80          90          100          110
151611 RLVREIAQDF-KTDLRFQSAAG-----ALQEASEAYLVGLFEDTNLC-----
      : : .      :. . . . .: :      : . .:: . .::..
_      REVNKALYDLQRSAMVYSSDDIPPRWFMTTEADKPDADAMADVIIDVSREKSMREDHKS
              130          140          150          160          170          180

                                120          130
151611 ---AIHAKR-----VTIMPKDIQLARR-----IRG---E
      .: :..      :... . :...:      : : .
_      FDDVIPAKKIIDWKGANPVTVINEYQCITRRDWSFRIESVGPSNSPTFYACVDIDGRVFD
              190          200          210          220          230          240

151611 RA-----
      ..
_      KADGKSKRDAKNNAAKLAVDKLLGYVIIRFZ
              250          260          270

```