Functional Genetic Variation of the Vitamin D Receptor in Malignant Melanoma and Psoriasis

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

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July 2005

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Abstract

The vitamin D receptor (VDR) is the nuclear receptor for 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active form of vitamin D. Classically involved in the regulation of calcium and phosphate homeostasis and bone mineralisation, $1,25(OH)_2D_3$ has recently been demonstrated to suppress proliferation and promote differentiation in a number of cell types. This has led to the study of the $1,25(OH)_2D_3$ pathway in malignancies such as melanoma and hyperproliferative disorders such as psoriasis.

Genetic variation of the VDR has previously been associated with prognosis in malignant melanoma and occurrence and treatment response in psoriasis.

Here, the major promoter region of the VDR, the 1a promoter region, was screened for variation. The novel polymorphism, A-1012G, was discovered and was predicted to lie within a GATA-3 binding site, such that the site is only present in the case of the A allele. GATA-3 is a T-cell specific transcription factor involved in polarising the T-cell response to a type 2 (Th2) response and suppressing Th1 responses. It was hypothesised that the A allele would be a risk factor in malignant melanoma, due to decreased immunosurveillance but beneficial in psoriasis, due to a reduction in Th1-mediated inflammation and keratinocyte hyperproliferation.

The affect of this polymorphism was studied in 191 malignant melanoma patients, 206 psoriasis patients and 80 controls. AA genotype were associated with increased risk of both occurrence (P=0.015) and metastasis (P=0.008) in melanoma. The A allele was under-represented in psoriasis patients with no family history compared to those with a family history (P=0.01), and associated with response to the vitamin D analogue Dovonex (P=0.04).

GATA-3 was shown to bind at the A but not the G allele of A-1012G in gel-shift assays. *In vitro* studies demonstrated that T cells with AA and AG genotypes were more sensitive to $1,25(OH)_2D_3$ treatment than those with GG genotype.

Acknowledgements

I would like to thank my supervisor, Dr. Howard Pringle for his support and innumerable, and I mean that, suggestions.

Also Dr Joy Osborne and Dr Peter Hutchinson for their enormous input into this project.

Thanks to my family for their support during many years of "not getting a proper job."

And most importantly to Sarah, for putting up with me. I would never have got this far without her.

I'd also like to thank Howard, Joy, Peter and the Psoriasis Association for showing enough faith in this work that it is still ongoing.

Patient samples and data for the malignant melanoma and psoriasis studies were collected and documented by Dr Joy Osborne and Dr Peter Hutchinson.

I am grateful to Professor RJ Thakker, May professor of medicine at Oxford University, who supplied the wildtype and mutant GATA-3 expression plasmids.

Thanks to everybody in the lab for their help and friendship at work and for joining me in the pub when I'd had enough.

Table of Contents

Abstract i
Acknowledgements ii
Table of Contents iii
Table of Figures vii
Table of Figures vii
List of Tables viii
Abbreviations ix

1. General Introduction 1
1.1. Vitamin D 2
1.1.1. Formation and Metabolism 3
1.1.2. Regulation of $1,25(OH)_2D_3$ Levels 6
1.1.3. Biological Functions of Vitamin D 7
1.2. The Vitamin D Receptor 9
1.2.1. VDR: A Nuclear Hormone Receptor 10
1.2.2. Transcriptional Activation of Target Genes by the VDR 13
1.2.3. The VDR Gene 17
1.2.4. Promoter Regions 19
1.3. Genetic Variation of the VDR 20
1.3.1. Single Nucleotide Polymorphisms and PolyA Microsatellite Polymorphisms 21
1.3.2. Association Studies 22
1.3.3. Functional Effects of Polymorphisms 27
1.3.4. 5′-Splice Variants 28
1.4. Aims and Objectives 31
1.4.1. In Silico Analysis of VDR Gene Structure 31
1.4.2. Screening of the VDR Gene for Novel Variation 31
1.4.3. Association of VDR Polymorphisms with Disease 31
1.4.4. Determination of VDR Polymorphism Function 31

2. Gene Structure 32
2.1. Introduction 32
2.1.1. 5′ Splice Variation 32
2.1.2. Analysis of Gene Homology 33
2.1.3. Analysis of Promoter Homology 35
2.2. Methods 36
2.2.1. BLAST 36
2.2.2. Dotplots 37
2.2.3. PIPMaker 37
2.2.4. VISTA 38
2.2.5. TraFac 38
2.3. Results 39
2.3.1. Conservation of Exon Sequences from Mouse to Human 39
2.3.2. Conservation of Genomic Sequence from Frog and Mouse to Human 48
2.3.3. Conservation of Promoter Regions from Mouse to Human 51
2.4. Discussion 58
2.5. Conclusion 62

3. Screening for Novel Variation 63
3.1. Introduction 63
3.1.1. VDR Polymorphisms 63
3.1.2. SSCP 64
3.1.3. Prediction of Transcription Factor Binding 66
3.2. Materials and Methods 67
3.2.1. Materials 67
3.2.2. Buffers 68
3.2.3. DNA Samples 68
3.2.4. Thermal Cyclers 69
3.2.5. OligoNucleotides 69
3.2.6. PCR Amplification of the Region of Interest 70
3.2.7. SSCP Gel Preparation 70
3.2.8. Sample Preparation 70
3.2.9. Silver Staining of SSCP Gels 70
3.2.10. Sequencing 71
3.2.11. TESS analysis 71
3.3. Results 72
3.3.1. SSCP screening of Exon 2 72
3.3.2. SSCP Screening of the 1A Promoter Region 72
3.3.3. TESS Prediction of Transcription Factor Binding 77
3.4. Discussion 82
3.5. Conclusion 86

4. *VDR* Polymorphisms and Malignant Melanoma 87
4.1. Introduction 87
4.1.1. Malignant Melanoma 87
4.1.2. $1,25(OH)_2D_3$, Melanoma and the Sun 88
4.1.3. VDR Polymorphisms and Melanoma 89
4.1.4. Genotype Screening in Larger Population 90
4.2. Materials and Methods 92
4.2.1. Materials 92
4.2.2. The Melanoma Cohort 92
4.2.3. The Control Population 92
4.2.4. Oligonucleotides 93
4.2.5. RFLP 94
4.2.6. Induced RFLP 94
4.2.7. Statistics 95
4.3. Results 96
4.3.1. RFLP in Genotype Screening 96
4.3.2. Hardy-Weinberg Equilibrium 96
4.3.3. A-1012G and Melanoma Occurrence 96
4.3.4. A-1012G and Metastasis 98
Polymorphism 99
4.3.5. A-1012G and G-1520C, Fok1 and Taq1 Haplotypes 100
4.3.6. VDR genotypes and Breslow Depth 102
Breslow Thickness Group 102
4.4. Discussion 103
4.5. Conclusion 107

5. VDR Polymorphisms and Psoriasis 108
5.1. Introduction 108
5.1.1. Psoriasis: An Inflammatory Disease 108
5.1.2. 1,25$(OH)_2D_3$ and VDR in Psoriasis 109
5.2. Materials and Methods 112
5.2.1. The Psoriasis Cohort 112
5.2.2. RFLP and Induced RFLP 112
5.2.3. Statistical Analysis 112
5.3. Results 113
5.3.1. Hardy-Weinberg Equilibrium 113
5.3.2. VDR Genotype and Psoriasis Susceptibility and Severity 113
5.3.3. VDR Genotype and Response to Calcipotriol 114
5.4. Discussion 118
5.5. Conclusion 120

6. Functional Analysis of A-1012G 121
6.1. Introduction 121
6.1.1. Gel-Shift Assays 121
6.1.2. T-cell Polarisation Assays 122
6.2. Materials and Methods 123
6.2.1. Materials 123
6.2.2. Buffers 123
6.2.3. GATA-3 Plasmids 125
6.2.4. Oligonucleotides 125
6.2.5. Cell Culture 125
6.2.6. Transfections 126
6.2.7. Nuclear Extracts 127
6.2.8. Bradford Protein Assay 127
6.2.9. Preparation of Double-Stranded DNA Probes 127
6.2.10. Gel-Shift Assays 128
6.2.11. Transfer and Visualisation 128
6.2.12. T-Cell Assays 128
6.2.13. mRNA Extraction 129
6.2.14. RT-PCR 129
6.3. Results 130
6.3.1. Gel-Shift Binding Assays 130
6.3.2. T-cell Assays 133
6.4. Discussion 135
6.5. Conclusion 137

7. General Discussion 138
7.1. VDR Gene Structure 138
7.2. Genetic Variation of the VDR 140
7.3. VDR Genotype in Malignant Melanoma 142
7.4. VDR Genotype in Psoriasis 144
7.5. Molecular and Cellular Effects of A-1012G 145
7.6. Further Work 146
7.7. Conclusion 148

I. Appendix I: Bibliography 149

II. Appendix II: Association Studies 178

III. Appendix III: Exonic Alignments from BLAST 181

IV. Appendix IV: SSCP Results with no Variation 189

V. Appendix V: Genotype Screening Results 191

VI. Appendix VI: Publications Arising from this Work 202

Table of Figures

Figure 1.1	The multi-step activation of 7-dehydrocholesterol to the active form, $1,25(OH)_2D_3$	4
Figure 1.2	The Structure of the human VDR	12
Figure 1.3	A diagrammatic representation of transcriptional regulation by the VDR	15
Figure 1.4	The structure of the VDR gene as published in the three major reports of the sequence	18
Figure 1.5	The exon-intron organisation of the chromosomal VDR gene	18
Figure 1.6	The splicing pattern of the 5′ exons of the VDR gene as reported by Crofts *et al*	29
Figure 2.1	The percentage conservation of the VDR exons between mouse and human	40
Figure 2.2	Comparisons of the VDR exon sequences in human and mouse using the VISTA software	41
Figure 2.3	Dotplot comparisons of human, mouse and rat VDR genomic DNA, exons 1f-2	44
Figure 2.4	Dotplot comparisons of human, mouse and rat genomic DNA, exons 2-9	46
Figure 2.5	Percentage identity plot comparing the human and mouse VDR sequences	49
Figure 2.6	VISTA comparing the human VDR sequence to the mouse and frog sequence	50
Figure 2.7	Analysis of the 1a and 1d promoter region by TraFac	53
Figure 2.8	Analysis of the 1f promoter region by TraFac	55
Figure 2.9	Analysis of the 1c promoter region by TraFac	57
Figure 3.1	A schematic representation of single stranded conformation polymorphism analysis.	65
Figure 3.2	SSCP analysis of exon two, revealing three polymorphisms: Fok1, C59T and C156T	74
Figure 3.3	SSCP analysis of the 1A/Dp5 promoter region, showing the A-1012G polymorphism	76
Figure 3.4	TESS prediction of transcription factor binding at the G-1520C locus	79
Figure 3.5	TESS prediction of transcription factor binding at the A-1012G locus	81
Figure 3.6	A diagram representing the T-cell polarisation pathways and the involvement of VDR	85
Figure 4.1	Schematic of the induced RFLP method	91
Figure 4.2	Examples of the screening results for each polymorphism	97
Figure 4.3	Survival curve showing metastasis in A-1012G genotypes	98
Figure 4.4	Survival curve showing metastasis in A-1012G/Fok1 genotype combinations	99
Figure 4.5	(A) A-1012G genotype and (B) G-1520C/A-1012G haplotypes in controls and patients	101
Figure 4.6	(A) A-1012G genotype and (B) G-1520C/A-1012G haplotypes in melanoma patients with and without metastasis	101

Figure 5.1 The chemical structure of Calcipotriol (Dovonex) compared to that of $1,25(OH)_2D_3$ 110
Figure 5.2 The proportion of A-1012G alleles and genotypes in patients with and without a family history of psoriasis 113
Figure 5.3 The proportion of A-1012G alleles and genotypes in patients who responded to Dovonex treatment compared to patients who did not respond to treatment 115
Figure 6.1 Gel shift assays with nuclear extracts from Jurkat cell lines and Normal dermal fibroblasts 131
Figure 6.2 Gel-shift assay using GATA-3-transfected HeLa cells 132
Figure 6.3 The results of T-cell polarisation assays 134

List of Tables

Table 1.1 The number of association studies with the various *VDR* polymorphisms 23
Table 1.2 Association studies in diseases with a strong immunological element to their aetiology 24
Table 1.3 Association studies in cancer 25
Table 2.1 The accession numbers of the human exons in the NCBI database and the chromosomal positions of each VDR exon on human chromosome 12 and mouse chromosome 15 36
Table 2.2 The conservation of the exonic sequences between mouse and human as analysed by Blast 39
Table 3.1 Oligonucleotide primers used to amplify the 1a promoter region for SSCP 69
Table 4.1 The stratification of the melanoma cohort 93
Table 4.2 Oligonucleotides used in genotype analyses 93
Table 4.3 A-1012G allele and genotype frequencies in controls and malignant melanoma 96
Table 4.4 Genotype and genotype combination associations with metastasis 99
Table 4.5 G-1520C/A-1012G genotype combinations in the control and melanoma populations 100
Table 4.6 Percentage metastasis in individuals of different A-1012G genotypes and tumour thickness 102
Table 5.1: The proportion of A-1012G, Fok1 and Taq1 genotypes and A-1012G/Fok1 genotype combinations in psoriasis patients with and without a family history of disease 114
Table 5.2 The association of VDR alleles with occurrence, family history, age of onset, use of second-line treatment and response to Dovonex 116
Table 5.3 The association of VDR genotypes and genotype combinations with occurrence, family history, age of onset, use of second line treatment and Dovonex response 117
Table 6.1 Oligonucleotides used to produce gel-shift probes and RT-PCR primers 126

Abbreviations

$1,25(OH)_2D_3$	1α-,25-dihydroxy-vitamin D_3
$25(OH)D_3$	25-hydroxy-vitamin D_3
AF-2	activation function 2
AIDS	Acquired immunodeficiency Syndrome
APC	antigen presenting cell
bp	base pairs
CBP	CREB binding protein
C/EBP	CCAAT / enhancer binding protein
chIP	chromatin immunoprecipitation assay
DBP	vitamin D binding protein
DNA	deoxyribonucleic acid
DRIP	vitamin D receptor binding protein
DR-3	direct repeat separated by 3 bases
EMEM	Eagle's minimum essential medium
ER	oestrogen receptor
FCS	foetal calf serum
HAT	histone acetyltransferase
HBSS	Hank's buffered saline solution
HDAC	histone deacetylase
HR	hazard ratio
IFN-γ	interferon gamma
IL	interleukin
kb	kilobases
MAZ	myc associated zinc finger protein

MHC	major histocompatability complex
mRNA	messenger ribonucleic acid
NCoA62	nuclear co-activator
NFAT	nuclear factor of activated T-cells
NR	nuclear receptor
OR	odds ratio
PABP	polyA binding protein
PASI	psoriasis area severity index
PCR	polymerase chain reaction
PIP	percentage identity plot
PMCA	plasma membrane Ca^{2+} ATPase
PNACL	protein and nucleic acid chemistry laboratory
polyA	polyadenyl
PTH	parathyroid hormone
RAR	retinoic acid receptor
RFLP	restriction fragment length polymorphism
RNA polII	RNA polymerase II
RXR	retinoid X receptor
SKIP	ski-interacting protein
SNP	single nucleotide polymorphism
SRC	steroid receptor co-activator
SSCP	single-stranded conformational polymorphism
TBE	tris borate EDTA buffer
TEMED	N,N,N′,N′-tetramethylethylenediamine
TESS	transcription element search system
TFIIB	transcription factor IIB

TGF-β	transforming growth factor beta
Th	T-helper
TNF	tumour necrosis factor
UTR	untranslated region
UV	Ultraviolet
VDR	vitamin D receptor
VDRE	vitamin D response element
VDRR	vitamin D resistant Rickets
WINAC	WSTF-including nucleosome assembly complex
WSTF	Williams syndrome transcription factor

1. General Introduction

"A horse! A horse! My kingdom for a horse!"

Richard III. Act 5 Scene IV. William Shakespeare, 1597.

It's difficult to find a quotation about vitamin D. Even with the power of the internet, not one prominent thinker can be found who has ever touched upon the subject, or at least thought about it long enough to come up with those few pithy words with which to sum up the entire subject area and provide an easy opening for PhD theses. At least Richard was a local celebrity. And perhaps his fabled hunchback was due to weak bones caused by vitamin D deficiency, not an angle that many scholars will have considered. Tony Robinson could do a whole series trying to prove that.

Vitamin D research to date has made little impression on the public consciousness, neither ethically controversial enough or likely to produce the sort of scientific breakthrough that makes headlines. The importance of vitamin D science is not in one big effect on any one particular organ or disease but in the fact that it is important, in a significant if not necessarily vital way, in so many different organs, systems and diseases. Effects of the vitamin D system might be apparent in the bone, gut, kidney, skin, teeth or immune system, in fracture risk, diabetes, malignant melanoma or AIDS.

Thus, the genetics of the vitamin D receptor, intrinsically involved in vitamin D signalling, may have effects in a variety of cells, organs and diseases.

1.1. Vitamin D

In 1919, Sir Edward Mellanby, in two lectures at the Royal College of Surgeons, reported the existence of an "anti-rachitic factor" (Mellanby, 1919). Working with dogs with induced Rickets, he showed that the disease could be cured by the manipulation of the animals diet and concluded that "Rickets is a deficiency disease which develops in consequence of the absence of some accessory food factor or factors." The presence of anti-rachitic factor in fats and oils, but not lean meat, bread or orange juice led him to believe that this factor was not the previously discovered water-soluble B (vitamin B), or antiscorbutic factor (vitamin C) but rather had a very similar distribution to, and could in fact be, fat-soluble A (vitamin A). In 1922, McCollum *et al.* (McCollum et al., 1922) showed that fats in which vitamin A had been destroyed were still able to increase calcium deposition in experimental rats and concluded that there was therefore a substance, distinct from vitamin A, responsible for bone development. McCollum called this substance vitamin D.

In his lectures, Mellanby noted that other studies did not support the dietetic theory of Rickets. He reported that Findlay had found that dogs on a controlled diet developed Rickets when confined but not when given daily exercise while Ferguson in a study into infantile Rickets in Glasgow reported that the factors implicated most strongly in the development of Rickets were associated with confinement in overcrowded houses and not with diet. He also reported that Hess and Unger working in the United States had found that the breast-fed children of black women in New York were nearly always rachitic.

The reason for the initial difficulty in characterising vitamin D is that it is not a true vitamin. Unlike vitamins A, B and C which are obtained solely through the diet, vitamin D, although it can be obtained from dietary sources, is also produced in the skin, in a reaction catalysed by sunlight. Mellanby confined his dogs indoors and so denied them this source of vitamin D. Findlay, in exercising his dogs showed that allowing the dogs exposure to sunlight stopped the development of rickets, although he put the effect down to exercise. Ferguson's finding that confinement in overcrowded houses was the main cause of rickets was due to the reduced exposure to sunlight, and Hess and Ungers' findings that rickets was particularly prevalent among the black population in

New York was due to the increased pigmentation of black skin reducing vitamin D formation.

In his lectures, Mellanby had highlighted most of the issues that are still important, and controversial, in Vitamin D research today.

1.1.1. Formation and Metabolism

Vitamin D exists in two forms: vitamin D_2 or ergocalciferol, which is produced by plants and vitamin D_3 or cholecalciferol, produced by animals and in humans. Vitamin D can be obtained through dietary sources, such as oily fish and liver. More recently, there has been an effort to increase dietary vitamin D through fortification of foods. For example, breakfast cereals, margarines and cows milk are now vitamin D fortified (Faulkner et al., 2000; Gregory et al., 1990; Holick et al., 1992).

The major source of vitamin D is through the bioactivation of 7-dehydrocholesterol (provitamin D_3) to previtamin D_3 in the skin. In this reaction, ultraviolet (UV) light with a wavelength of 290-300nm (MacLaughlin et al., 1982) is absorbed by cutaneous stores of 7-dehydrocholesterol causing cleavage of the C^9-C^{10} bond and the formation of previtamin D_3 (Holick et al., 1977). Previtamin D_3 is thermodynamically unstable and quickly undergoes isomerization to vitamin D_3. 7-dehydrocholesterol is localised within the bilipid membrane of dermal and epidermal cells. This allows previtamin D_3 to maintain the thermodynamically unfavourable conformation that allows easy isomerization to vitamin D_3. The vitamin D binding protein (DBP), an α_1-globulin which carries vitamin D_3 in the blood, has a much higher binding affinity for vitamin D_3 compared to previtamin D_3. The DBP binds vitamin D_3 as it is formed and thus removes it from the skin into the circulation, leaving unconverted previtamin D_3 in the skin. This shifts the previtamin/vitamin equilibrium and allows isomerization to continue (Holick et al., 1981).

Once vitamin D_3 is obtained either through the diet or by the bioactivation of 7-dehydrocholesterol it requires activation to its hormonally active form, 1α-,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$). This occurs via two sequential enzyme catalysed hydroxylation reactions. The process of $1,25(OH)_2D_3$ formation is summarised in ***figure 1.1.***

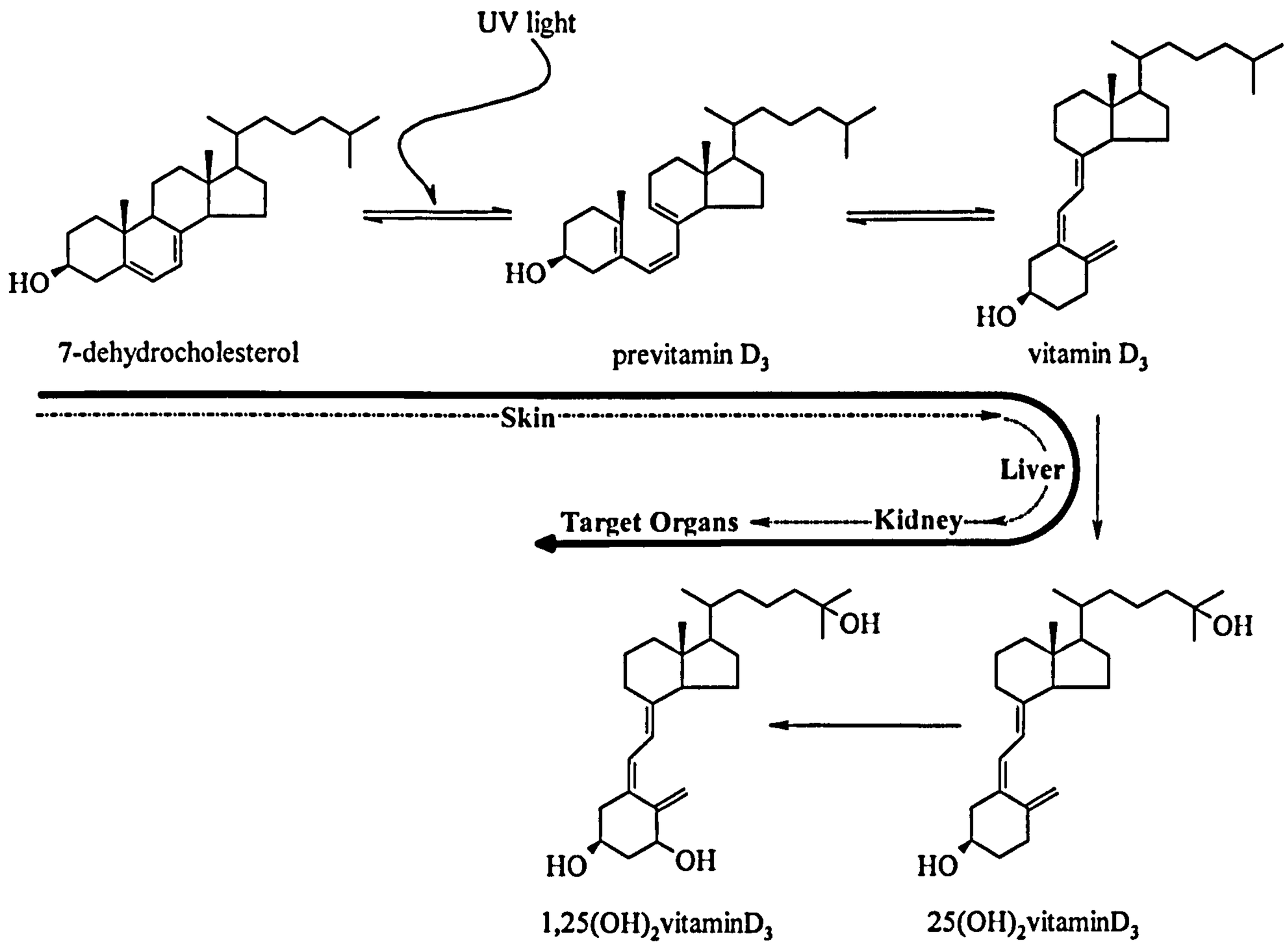

Figure 1.1: The multi-step activation of 7-dehydrocholesterol to the active form, $1,25(OH)_2D_3$. 7-dehydrocholesterol is altered to previtamin D_3 in a UV-catalysed reaction. Previtamin D_3 then isomerises to vitamin D_3. This then undergoes 25-hydroxylation in the liver followed by 1α-hydroxylation in the kidney to produce the active hormonal form, $1\alpha,25(OH)_2D_3$. Adapted from Brown *et al.* (Brown et al., 1999).

The 25-hydroxylation step takes place primarily in the liver (Ponchon et al., 1969), although other tissues have been shown to be capable of catalysing this reaction, including keratinocytes (Lehmann et al., 2001). A microsomal cytochrome P450 (CYP2C11) was shown to be able to 25-hydroxylate vitamin D_3 but was only demonstrated in rats (Hayashi et al., 1988). A mitochondrial 25-hydroxylase has been demonstrated in rat, rabbit and human. The human homologue of this protein, P450c27, coded for by the *CYP27* gene, was cloned by Guo *et al.* (Guo et al., 1993) and shown to be capable of hydroxylating vitamin D_3 at several sites, the major product being $25(OH)D_3$ while the 26- and 24-hydroxylated forms are minor products of this reaction.

The 1α-hydroxylation step occurs primarily in the proximal tubule cells of the kidney (Fraser and Kodicek, 1970), although several other tissues have been shown to have 1α-hydroxylase activity, most notably macrophages (Adams and Gacad, 1985) and keratinocytes (Bikle et al., 1986a; Bikle et al., 1986b; Lehmann et al., 2001). The 1α-hydroxylase is expressed on the mitochondrial membrane and coded for by the *P450c1α* gene. When cloned in rat kidney, the gene was shown to have 53% similarity and 44% identity with the 25-hydroxylase (Shinki et al., 1997) and so was identified as a member of the *CYP27* family and termed *CYP27B1*. The *CYP27B1* gene has been cloned from human kidney (Monkawa et al., 1997) and keratinocytes (Fu et al., 1997) and the gene has been localised to chromosome 12q13. This locus has been identified as a site of mutation in vitamin D resistant rickets (VDRR) and it has been shown that mutations of the 25-hydroxylase are the cause of VDRR type 1 (Fu et al., 1997).

$1,25(OH)_2D_3$ is broken down by oxidative metabolism and side chain cleavage. The process begins with the addition of a hydroxyl group at carbon 24, catalysed by the 24-hydroxylase enzyme. Like the 25-hydroxylase, the major site of 24-hydroxylase expression is the kidney (Omdahl et al., 2003). The 24-hydroxyl group is then further oxidised to a ketone (Makin et al., 1989). Carbon 23 is then oxidised followed by the cleavage of the side chain to give calcitroic acid, a biologically inert metabolite. Both $1,25(OH)_2D_3$ and $25(OH)D_3$ can be substrates in this side chain cleavage reaction providing a mechanism by which levels of both the active hormonal form and its precursor can be down-regulated.

1.1.2. Regulation of 1,25(OH)$_2$D$_3$ Levels

Vitamin D is involved in several feedback loops corresponding to its role in calcium and phosphate homeostasis. As 1,25(OH)$_2$D$_3$ promotes calcium and phosphate absorption, levels are increased in response to hypocalcaemia and hypophosphataemia and reduced in response to hypercalcaemia and hyperphosphataemia. Parathyroid hormone (PTH) has been shown to mediate 1,25(OH)$_2$D$_3$ regulation in response to calcium levels (Omdahl et al., 1972). Insulin and insulin-like growth factor have also been implicated in 1,25(OH)$_2$D$_3$ regulation (Gray, 1987). 1,25(OH)$_2$D$_3$ is also directly self-regulating.

25-hydroxylation is relatively uncontrolled and the level of 25(OH)D$_3$ is directly dependent on the level of vitamin D$_3$ in the circulation. The level of circulating 25(OH)D$_3$ is often used as an indication of vitamin D status. From this relationship, however, it is clear that the 25-hydroxylation reaction cannot be a major checkpoint in controlling the levels of the active hormonal form. Levels of 1,25(OH)$_2$D$_3$ therefore seem to be controlled by regulation of the 1α- and 24-hydroxlases.

Increased calcium and phosphate reduces the level of serum 1,25(OH)$_2$D$_3$ (Portale et al., 1989; Weisinger et al., 1989). Bland *et al.* (Bland et al., 1998) showed that calcium directly suppressed expression of the 1α-hydroxylase. Conversely, a reduction in calcium or phosphate levels increases 1,25(OH)$_2$D$_3$ (Hughes et al., 1975; Portale et al., 1989). Reduction in phosphate leads to induction of the 1α-hydroxylase (Shinki et al., 1997; Tanaka and Deluca, 1973) and suppression of the 24-hydroxylase (Tanaka and Deluca, 1973).

Garabedian *et al.* (Garabedian et al., 1972) showed that PTH was required to mediate calcium-regulated 1,25(OH)$_2$D$_3$ production. Calcium is detected by the calcium-sensing receptor and decreases PTH secretion and parathyroid cell growth (Brown and MacLeod, 2001). PTH appears to be important for the control of 1,25(OH)$_2$D$_3$ levels in response to calcium but not phosphate (Hughes et al., 1975). PTH has been shown to increase 1α-hydroxylase mRNA levels (Brenza et al., 1998; Murayama et al., 1998) and reduce 24-hydroxylase activity (Henry, 1981).

1,25(OH)$_2$D$_3$ is also self-regulated by negative feedback. Both the 1α- and 24-hydroxlases have been shown to be regulated at the mRNA level in response to 1,25(OH)$_2$D$_3$ (Monkawa et al., 1997; Ohyama et al., 1991) and the 24-hydroxylase gene

contains two vitamin response elements for transcriptional control by $1,25(OH)_2D_3$ and its receptor (Chen and DeLuca, 1995). Xie *et al.* (Xie et al., 2002) have shown, however, that $1,25(OH)_2D_3$ levels in keratinocytes are controlled by the induction of the 24-hydroxylase only, with no effect on the 1α-hydroxylase. This is possible due to the activity of the 24-hydroxylase. The ability of the 24-hydroxylase to metabolise both the substrate ($25(OH)D_3$) and the product ($1,25(OH)_2D_3$) of the 1α-hydroxylase allows $1,25(OH)_2D_3$ levels to be controlled without modulation of the 1α-hydroxylase.

1.1.3. Biological Functions of Vitamin D

The classical function of vitamin D is the regulation of bone mineralisation (Reichel et al., 1989). The major target tissues in this system are considered to be intestine, skeleton, parathyroid gland and kidney.

In the intestine, active calcium absorption is a three step process. $1,25(OH)_2D_3$ increases calcium absorption through the plasma membrane, through the cytoplasm and into the circulation. Calcium is absorbed into the enterocyte through calcium channels, transported through the cell bound to calbindin and released into the plasma through an ATP-driven mechanism. $1,25(OH)_2D_3$ mediates calcium influx into the enterocyte through control of the calcium channels (Roche et al., 1986), particularly the ECaC1 and ECaC2 calcium channels (Van Cromphaut et al., 2001). Calbindin expression is also under transcriptional control of $1,25(OH)_2D_3$ (Buckley and Bronner, 1980; Darwish and DeLuca, 1992; Kessler et al., 1986). Calcium is pumped from the enterocyte into the plasma via the plasma membrane Ca^{2+} ATPase (PMCA). $1,25(OH)_2D_3$ up-regulates the expression of this protein and increases the flux of calcium ions across the membrane (Hoenderop et al., 2002; Kip and Strehler, 2004).

As well as controlling the level of mineral ions available for bone formation, vitamin D may also be involved directly in bone remodelling through direct effects on osteoblasts (van Leeuwen et al., 2001) and osteoclasts (Bar-Shavit et al., 1983), the principal cells involved in bone remodelling. When calcium is readily available, $1,25(OH)_2D_3$ has been shown to induce terminal differentiation and matrix deposition in osteoblasts (Owen et al., 1991). In situations of hypocalcaemia, $1,25(OH)_2D_3$ increases osteoclast numbers (Holtrop et al., 1981) and calcium resorption (Raisz et al., 1980), through the induction of osteoclast differentiation (Bar-Shavit et al., 1983; Orcel et al., 1990).

The parathyroid is the major calcium sensing organ. PTH, calcitonin and $1,25(OH)_2D_3$ together regulate calcium homeostasis. PTH is up regulated in response to low calcium levels. PTH increases $1,25(OH)_2D_3$ which up-regulates calcium absorption. While PTH up-regulates $1,25(OH)_2D_3$ synthesis, $1,25(OH)_2D_3$ inhibits the formation and release of PTH (Cantley et al., 1985). Calcitonin also up-regulates $1,25(OH)_2D_3$ in response to low calcium levels (Rasmussen et al., 1972) and reduces resorption of calcium from bone (Friedman and Raisz, 1965).

Circulating levels of $1,25(OH)_2D_3$ are mainly controlled in the kidney. PTH increases $1,25(OH)_2D_3$ levels through induction of the 1α-hydroxylase and suppression of the 24-hydroxylase (Brenza et al., 1998; Henry, 1981). Conversely, $1,25(OH)_2D_3$ suppresses the activity of the 1α-hydroxylase and increases the activity of the 24-hydroxylase, thus reducing plasma levels of $1,25(OH)_2D_3$ (Monkawa et al., 1997; Ohyama et al., 1991).

That vitamin D may play a role outside bone mineralisation was first suggested in 1979 by Stumpf *et al.* (Stumpf et al., 1979) who used radiolabelled $1,25(OH)_2D_3$ to identify sites of $1,25(OH)_2D_3$ localisation in rats. As well as enterocytes, osteoblasts, kidney tubules and the parathyroid, $1,25(OH)_2D_3$ was also found in a number of non-classical sites including keratinocytes in the skin and a number of immune cells: promyelocytes, macrophages and T-cells.

$1,25(OH)_2D_3$ has been shown to regulate the terminal differentiation of keratinocytes and inhibit keratinocyte proliferation (Hosomi et al., 1983; Smith et al., 1986). These properties have led to $1,25(OH)_2D_3$ and its analogues being used in the treatment of hyper-proliferative skin disorders such as psoriasis (Smith et al., 1988) and scleroderma (Boelsma et al., 1995). $1,25(OH)_2D_3$ also acts on cells of the outer root sheath of the hair follicle to promote differentiation (Limat et al., 1993). $1,25(OH)_2D_3$ has been shown to have an anti-proliferative effect on melanoma cell lines (Danielsson et al., 1998) but this has not been shown in normal melanocytes.

Keratinocytes are the only location in the body where every step of the 7-dehydrocholesterol to $1,25(OH)_2D_3$ activation process have been reported within a single cell type (Bikle et al., 1986a; Lehmann et al., 2001). It has been proposed that this is a mechanism that protects against the damaging effects of UV exposure, through the induction of metallothionein, an antioxidant protein (Lee and Youn, 1998).

$1,25(OH)_2D_3$ affects several cells of the immune system. $1,25(OH)_2D_3$ was at first shown to be generally immunosuppressive, inhibiting the activity of macrophages and lymphocytes (Wientroub et al., 1989). More recently, specific actions of $1,25(OH)_2D_3$ on different cells of the immune system have been reported.

$1,25(OH)_2D_3$ inhibits the differentiation and maturation of dendritic cells, the major antigen presenting cell (Berer et al., 2000; Penna and Adorini, 2000), reducing T-cell activation (Piemonti et al., 2000). This is through the inhibition of production of IL-12 (Lemire, 1995, D'Ambrosio, 1998), a cytokine essential to T-cell maturation. $1,25(OH)_2D_3$ also acts directly on T-cells. $1,25(OH)_2D_3$ inhibits IFN-γ production by T-lymphocytes (Reichel et al., 1987) through inhibition of IFN-γ mRNA expression (Cippitelli and Santoni, 1998). Boonstra *et al.* (Boonstra et al., 2001) showed that $1,25(OH)_2D_3$ induces the polarisation of T helper (Th) cells to Th2 cells and inhibits Th1 polarisation.

$1,25(OH)_2D_3$ induces the differentiation of monocytes and macrophages from myelopoietic stem cells (Amento et al., 1984). $1,25(OH)_2D_3$ induces the fusion of macrophages to giant multinucleated cells (Bar-Shavit et al., 1983; Tanaka et al., 1984) and increases the cytotoxicity of alveolar macrophages (Abe et al., 1984). Macrophages are themselves able to produce $1,25(OH)_2D_3$ through expression of the 1α-hydroxylase (Adams and Gacad, 1985).

1.2. The Vitamin D Receptor

The genomic actions of $1,25(OH)_2D_3$ are mediated through its nuclear receptor, the vitamin D receptor (VDR). The VDR is a ligand-activated transcription factor. Binding of $1,25(OH)_2D_3$ to the receptor causes a conformational change, allowing the VDR to form a heterodimer with the retinoid X receptor (RXR) and bind to vitamin D response elements (VDREs), specific DNA sequences in the promoter region of target genes. Further co-activator or co-repressor proteins then bind to regulate target gene expression.

1.2.1. VDR: A Nuclear Hormone Receptor

The VDR is a member of the nuclear receptor superfamily, a large group of nuclear proteins thought to derive from a common ancestor (Aranda and Pascual, 2001). All receptors in this family share a common modular domain structure, with different regions corresponding to different functionality. Typically, a nuclear receptor can be divided into five functional domains: a variable NH_2-terminal region (the A/B region), a DNA-binding domain (C), a variable hinge region (D), a conserved ligand-binding domain (E) and a variable COOH-terminal domain (F). The functional domains of the VDR are shown in *figure 1.2.*

The A/B region is the most variable within the family and is thought to control receptor specificity. The A/B domain is also the target for phosphorylation which can alter the receptor's transcriptional activity (Shao and Lazar, 1999). In the VDR the A/B domain is short in comparison to other receptors (Sone et al., 1991) and has little functional activity. The A/B region of the VDR is subject to variation from polymorphism and the alternative splicing of 5′ exons.

The DNA binding domain (C) is highly conserved throughout the nuclear receptor family. The crystal structure of the VDR DNA-binding domain was initially inferred by comparison with homologous nuclear receptors, particularly the thyroid receptor (Rastinejad et al., 1995) and RXR (Lee et al., 1993) and later by direct x-ray crystallography of the VDR bound to VDREs (Shaffer and Gewirth, 2002). The region contains two zinc fingers that confer DNA-binding ability on the domain. Two α-helices form the core of the domain: the first, the recognition helix, binds the major groove of DNA and contains the first zinc finger and the "P box", the amino acid sequence that recognizes core DNA motifs. The second helix binds to the phosphate backbone, at right angles to the recognition helix, and contains the second zinc finger and the "D box", an amino acid sequence involved in dimerisation. Two further helices, the T- and A-boxes, immediately C-terminal of the zinc fingers are also important for DNA binding. The T-box also contains residues that are important in dimerisation of the VDR and RXR DNA-binding domains (Hsieh et al., 1995).

The D domain is highly variable between nuclear receptors. The domain forms a hinge region between the DNA-binding domain and the ligand-binding domain and allows the DNA binding domain to rotate. Beyond simply linking the DNA and ligand-binding

domains, residues in this region have been shown to be important in the interaction between VDR and its co-activators, specifically transcription factor IIB (TFIIB) (Blanco et al., 1995). Using deletion constructs of the VDR MacDonald *et al.* (MacDonald et al., 1995) showed that the domains at the junction of the hinge region and at the carboxy-terminal end of the ligand-binding domain were vital for this interaction.

As with the DNA-binding domain, the structure of the ligand-binding domain was first deduced by comparison with other nuclear receptors including the retinoic acid receptor γ (RARγ) (Renaud et al., 1995) and the RXR (Bourguet et al., 1995). More recently the crystal structure of the VDR ligand-binding domain has been solved (Rochel et al., 2000; Tocchini-Valentini et al., 2001). The ligand-binding domain is made up of 12 α-helices interspersed with β-sheets, which fold in such a way as to form a cavity into which the ligand binds. Tocchini-Valentini *et al.* (Tocchini-Valentini et al., 2001) showed that this domain maintains its conformation whether binding the natural ligand, $1,25(OH)_2D_3$, or the agonistic side-chain analogues which are used therapeutically. Upon ligand binding the receptor undergoes a conformational change to fold the ligand into a "mouse-trap" like pocket (Moras and Gronemeyer, 1998). This conformational change also forms the surfaces necessary for interactions with other proteins of the transcriptional machinery.

The ligand-activated VDR alone does not form a stable complex with DNA so in order to strongly bind DNA and activate transcription, VDR heterodimerises with the RXR (Carlberg et al., 1993). As well as the T-box region of the DNA-binding domain (Hsieh et al., 1995), two further regions within the ligand-binding domain (amino acids 244-263 and 317-395) mediate heterodimer formation (Jin et al., 1996; Rosen et al., 1993; Whitfield et al., 1995). The folding of the ligand-binding domain on binding $1,25(OH)_2D_3$ positions the necessary amino acids to allow RXR binding (Moras and Gronemeyer, 1998).

The final major functional domains of the VDR are involved in transactivation. The region between amino acids 244-263 is implicated in RXR heterodimerisation but Whitfield *et al.* (Whitfield et al., 1995) showed that lysine 246, within this region, is not involved in heterodimerisation but its mutation causes severe impairment of transactivation. It is likely that this residue is involved in interaction with other co-activator proteins. The second domain involved in transactivation is known as the ligand-dependent activation function (AF-2) domain and is located in the extreme

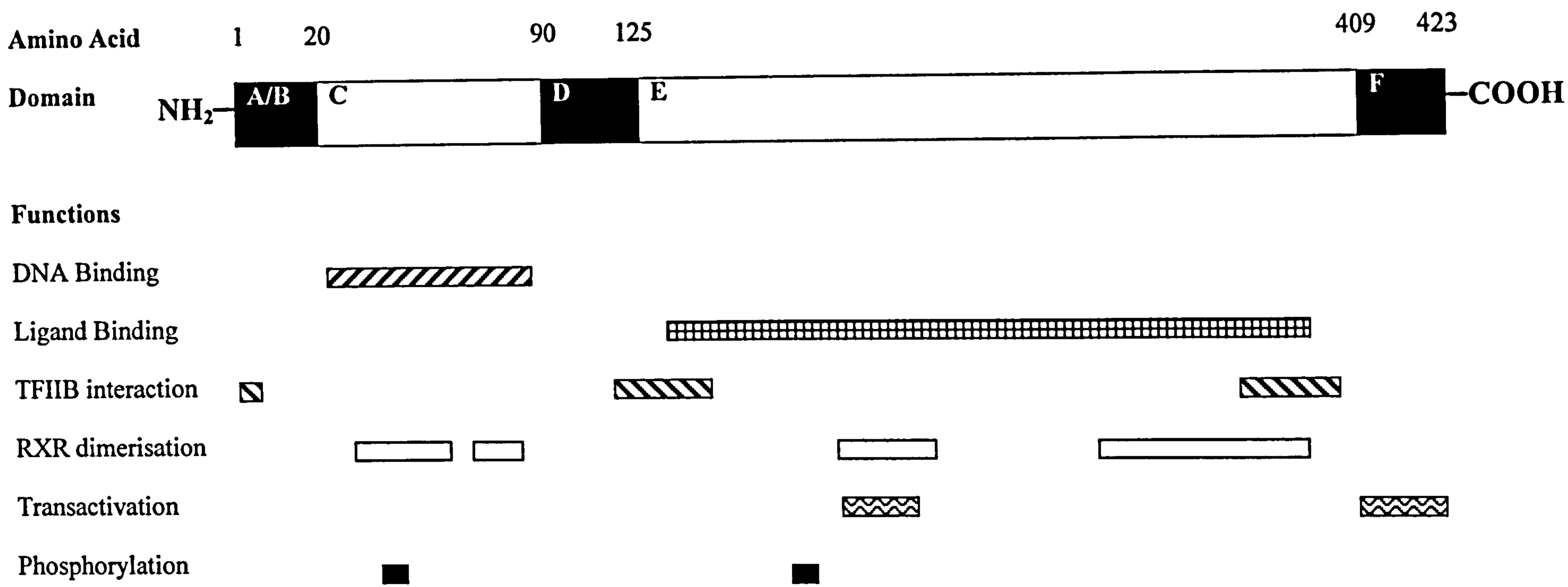

Figure 1.2: The Structure of the human VDR. The standard structure of a nuclear hormone receptor is shown, with the variable NH_2-terminal domain A/B, the DNA binding C region, the hinge D domain, the ligand binding E domain and the variable carboxy-terminal F domain. The corresponding amino acids are shown above. The regions responsible for the major functions of VDR are shown below.

carboxy-terminal F domain. Jurutka *et al.* (Jurutka et al., 1997) showed that mutation of residues in this region had no effect on DNA or ligand binding, on heterodimerisation with RXR or interaction with TFIIB but abolished transcriptional activity. The mechanism by which VDR interacts with co-factors to transcriptionally regulate target genes is further discussed in *1.2.2.*

The transcriptional activity of VDR can be regulated by phosphorylation. Phosphorylation of the VDR occurs rapidly in response to $1,25(OH)_2D_3$. The major site of phosphorylation appears to be serine 208 in the ligand binding domain (Jurutka et al., 1993) and serine 51 between the two zinc fingers of the DNA binding domain (Hsieh et al., 1991). Phosphorylation at serine 208 by casein kinase C causes a conformational change which increases the transactivation potential of VDR (Jurutka et al., 1996) while phosphorylation at serine 51 by protein kinase C appears to reduce the receptor's DNA binding ability (Hsieh et al., 1993). Therefore VDR phosphorylation appears to be part of a complex system of crosstalk between cell-signalling pathways.

1.2.2. Transcriptional Activation of Target Genes by the VDR

The VDR-RXR heterodimer acts by binding to specific DNA sequences in the promoter of $1,25(OH)_2D_3$-regulated genes, known as vitamin D response elements (VDREs). Protein complexes are then recruited to either up-regulate gene expression in the case of positive VDREs or suppress gene expression in the case of negative VDREs. Although the sequence of VDREs varies considerably, both between genes and between positive and negative VDREs, a consensus VDRE has been described as a direct repeat of two six-base half-sites separated by three nucleotides (DR-3). This is the ideal distance to bring the interacting residues of the VDR and RXR into contact without other domains becoming too close and causing steric hindrance (Rastinejad et al., 1995).

The consensus sequence of the VDRE has been described as RRKNSA (Schrader et al., 1995) or RGKTCA (Liu et al., 1996) (R = A or G, K = G or T, S = C or G, N = any base) but is being constantly updated as new $1,25(OH)_2D_3$-responsive genes are discovered.

The VDR-RXR heterodimer binds at the VDRE, in the case of positive VDREs with the VDR binding the 3′ half site and the RXR the 5′ half site. It was initially suggested that this orientation was reversed to produce a negative transcriptional effect (Haussler et al.,

1998) but it seems likely that the mechanism of up- or down-regulation is much more complex than this and is dependent on other factors such as cellular background and co-factors. The study of the co-factor proteins involved began only very recently. Understanding of the transcription process has been enhanced by the development of the chromatin immunoprecipitation (ChIP) assay (Orlando, 2000) which has allowed the study of transcription factor binding over a time-course following $1,25(OH)_2D_3$ treatment.

In order to activate gene transcription, the VDR-RXR heterodimer must first bind to the DNA at the VDRE and then recruit several distinct co-activator complexes, each responsible for a separate process necessary for gene transcription. Recent ChIP assays have revealed that VDR associates with the promoter regions of target genes independent of ligand binding (Kato et al., 2004). This interaction appears to be through a novel chromatin remodelling complex containing the Williams syndrome transcription factor (WSTF) (Lu et al., 1998), known as the WSTF-including nucleosome assembly complex (WINAC). In the case of positive VDREs, ligand binding then caused the recruitment of further histone acetylases, TRAP220 and TIF2, to the promoter in a cyclic manner. Histone acetylation loosens the chromatin structure, opening up the promoter to allow further transcriptional complexes to bind. In the case of negative VDREs, VDR and WINAC again appear to reside in the promoter in a ligand-independent manner. The co-repressor complex, NCo-R and a histone deacetylase HDAC2, are then recruited in a ligand-dependent, cyclic manner to tighten chromatin structure and repress target gene expression. The ligand-independent association of the VDR-RXR heterodimer has not been reported in other nuclear receptors, where promoter targeting appears to be induced by ligand binding (Belandia et al., 2002).

The best characterised group of co-factors are the steroid receptor co-activators (SRCs), of which three have been identified to date. The SRCs are ligand-dependent co-activators which possess the LXXLL motif, known as the nuclear receptor (NR) box, through which the SRCs bind to the AF-2 domain of VDR (Heery et al., 1997). SRC-1 and SRC-3 both possess histone acetyltransferase (HAT) activity (Chen et al., 1997; Spencer et al., 1997), suggesting a role for SRCs in the early stages of transcription. However, this HAT activity has been shown to be weak and not essential for SRC transcriptional co-activation (Liu et al., 2001), suggesting that SRCs also play an important role in protein-protein interactions with other co-factors with stronger HAT

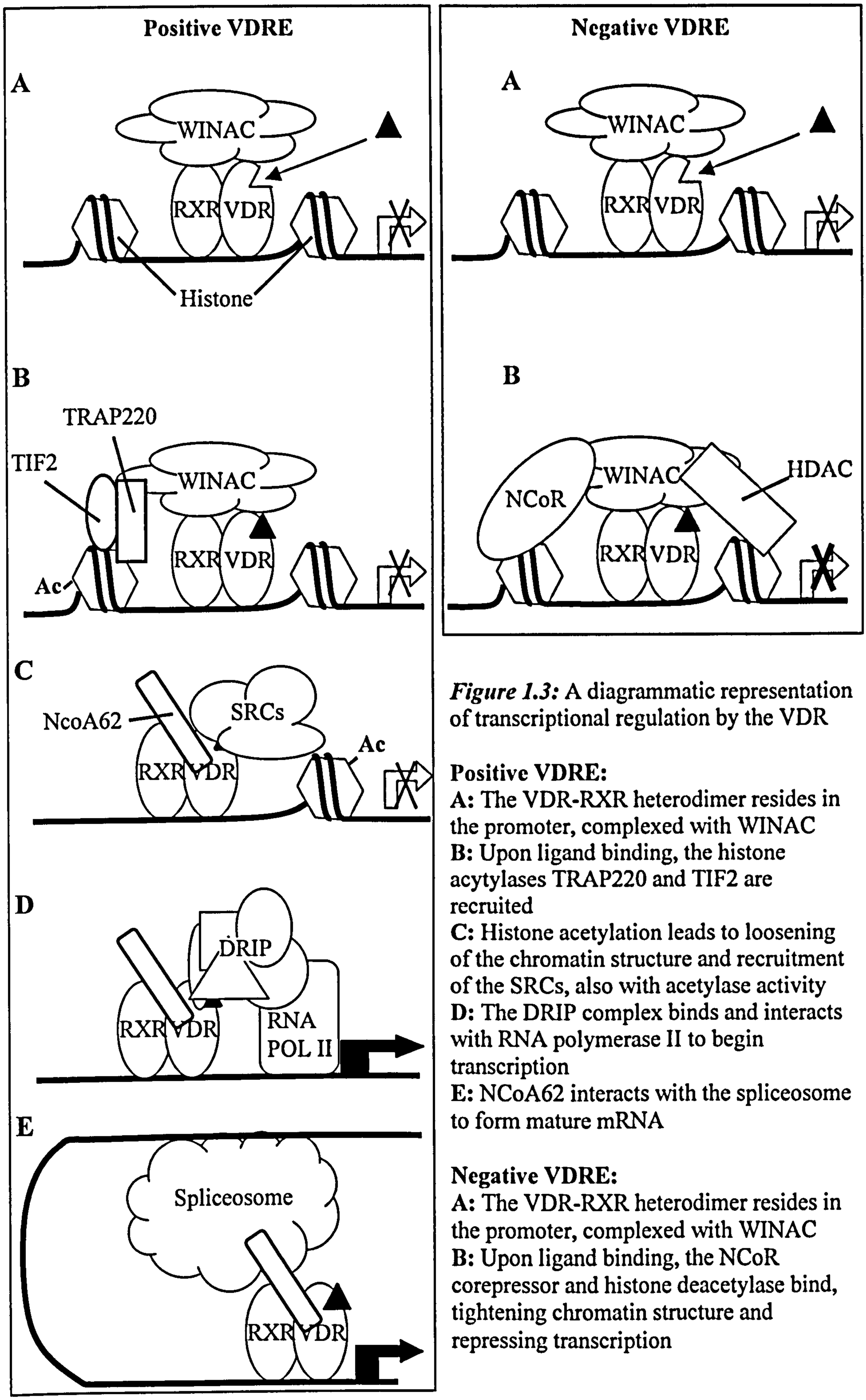

Figure 1.3: A diagrammatic representation of transcriptional regulation by the VDR

Positive VDRE:
A: The VDR-RXR heterodimer resides in the promoter, complexed with WINAC
B: Upon ligand binding, the histone acytylases TRAP220 and TIF2 are recruited
C: Histone acetylation leads to loosening of the chromatin structure and recruitment of the SRCs, also with acetylase activity
D: The DRIP complex binds and interacts with RNA polymerase II to begin transcription
E: NCoA62 interacts with the spliceosome to form mature mRNA

Negative VDRE:
A: The VDR-RXR heterodimer resides in the promoter, complexed with WINAC
B: Upon ligand binding, the NCoR corepressor and histone deacetylase bind, tightening chromatin structure and repressing transcription

activity such as p300 and CREB binding protein (CBP). The mechanism of transcriptional regulation by the VDR is summarised in *figure 1.3*.

The Vitamin D receptor interacting protein (DRIP) is a large protein complex which acts in a ligand-dependent manner (Rachez et al., 1998). DRIP has been shown to be essential for VDR-mediated transcription (Rachez et al., 1999). The mechanism of action of DRIP appears to be functionally distinct from the SRC proteins. Unlike the SRCs, DRIP does not show any HAT activity (Rachez et al., 2000) but appears to be important after the chromatin remodelling step. DRIP interacts directly with RNA polymerase II (RNA pol II), dependent on the presence of liganded VDR (Chiba et al., 2000). ChIP analysis using the oestrogen receptor (ER) showed that the SRC and DRIP complexes associated and dissociated reciprocally, with DRIP recruited to the promoter as SRC disassociated followed by SRC association as DRIP disassociated and this process continued cyclically.

The 62 kD nuclear co-activator (NcoA62) or Ski-interacting protein (SKIP) associates with VDR and mediates $1,25(OH)_2D_3$ signalling (Baudino et al., 1998). Unlike the SRC and DRIP complexes, NCoA62 does not contain the LXXLL motif and does not interact with VDR through the AF-2 domain (Baudino et al., 1998). NcoA62 binds to the VDR-RXR heterodimer preferentially to either the VDR monomer or homodimer but this interaction is only moderately increased by ligand binding (MacDonald et al., 2001; Zhang et al., 2001). Although NCoA62 does not interact directly with the SRC proteins, it does form a large ternary protein complex with the SRCs and the liganded VDR-RXR heterodimer (Zhang et al., 2001). As well as binding to the VDR-RXR/SRC complex, NCoA62 has been shown to directly interact with the spliceosome (Zhang et al., 2003a), a multi-protein complex responsible for the splicing out of introns from pre-mRNA to form mRNA (reviewed in (Smith and Valcarcel, 2000)). ChIP analysis revealed that VDR, SRC and NCoA62 occupied the promoter in a cyclic manner and NCoA62 binding was a relatively late event (Zhang et al., 2003a). This, and the ability of NCoA62 to interact with the both the VDR-RXR/SRC complex and the spliceosome suggests NCoA62 may act as a bridging mechanism between early transcription events mediated by the SRCs and the later, spliceosome-mediated mRNA processing. The protein interactions involved in transcriptional regulation by VDR are summarised in *figure 1.3*.

At least fifty genes that undergo transcriptional regulation by $1,25(OH)_2D_3$ have been reported (Hannah and Norman, 1994). These include genes that are associated with the classical actions of vitamin D: osteocalcin (Demay et al., 1990), osteopontin (Noda et al., 1990), PTH (Okazaki et al., 1988) and the 1α- and 24-hydroxylases (Takeyama et al., 1997; Zierold et al., 1995) all contain VDREs. Consistent with their roles in $1,25(OH)_2D_3$ metabolism, the 1α-hydroxylase contains a negative VDRE and the 24-hydroxylase two positive VDREs. Of interest with regard to the antiproliferative, prodifferentiation functions of $1,25(OH)_2D_3$ are the VDREs in the promoters of transforming growth factor β (TGF-β) (Wu et al., 1999) and p21 (Liu et al., 1996). P21 is a cell cycle-regulating protein which acts to arrest the cell cycle at the G_1/S checkpoint and TGF-β has been shown to affect the differentiation of human bone cells (Wu et al., 1997).

1.2.3. The VDR Gene

The cDNA sequence of the VDR was first reported in 1988 by Baker *et al.* (Baker et al., 1988) as ten exons: eight coding exons and two 5′ untranslated exons which coded for a protein 427 amino acids in length. Miyamoto *et al.* (Miyamoto et al., 1997) confirmed the sequence of the 8 coding exons but also reported additional 5′ sequence. The sequence of the first exon reported by Baker was shown to be incomplete and a further 5′ non-coding exon was found between the two 5′ exons reported by Baker. The eight coding exons were named 2-9 and the three non-coding 5′ exons 1a-1c. In 1998, Crofts *et al.* (Crofts et al., 1998) reported three further 5′ exons, exons 1d-1f, which with exon 1a-1c undergo complex alternative splicing with transcription initiating in exon 1a, 1d or 1f. The order and nomenclature of the VDR exons as reported in these three studies are shown in ***figure 1.4***.

There are two in-frame ATG translation start codons, nine bases apart, at the 5′ end of exon two, which code for proteins of 424 or 427 amino acids. There is also an in-frame ATG codon in exon 1D which, depending on exon splicing, can code for additional 23 or 50 amino acid-extended proteins.

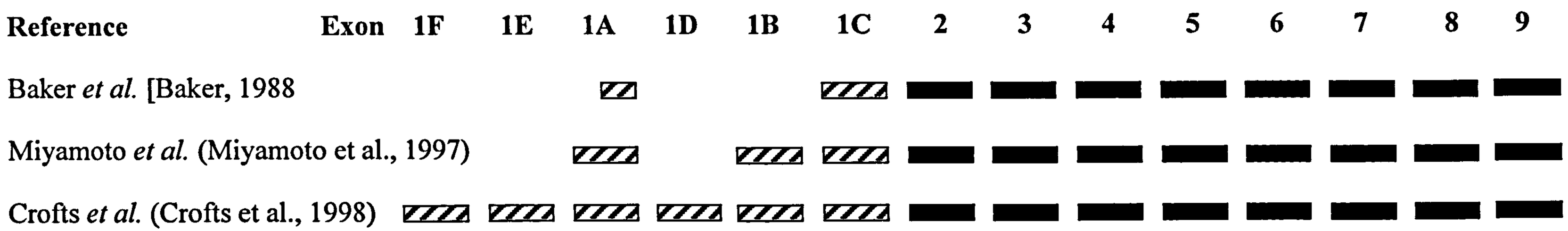

Figure 1.4: The structure of the VDR gene as published in the three major reports of the sequence.
The coding exons (solid blocks) are numbered 2-9 and the alternatively spliced 5′ exons (hashed blocks) are numbered 1A-1F.

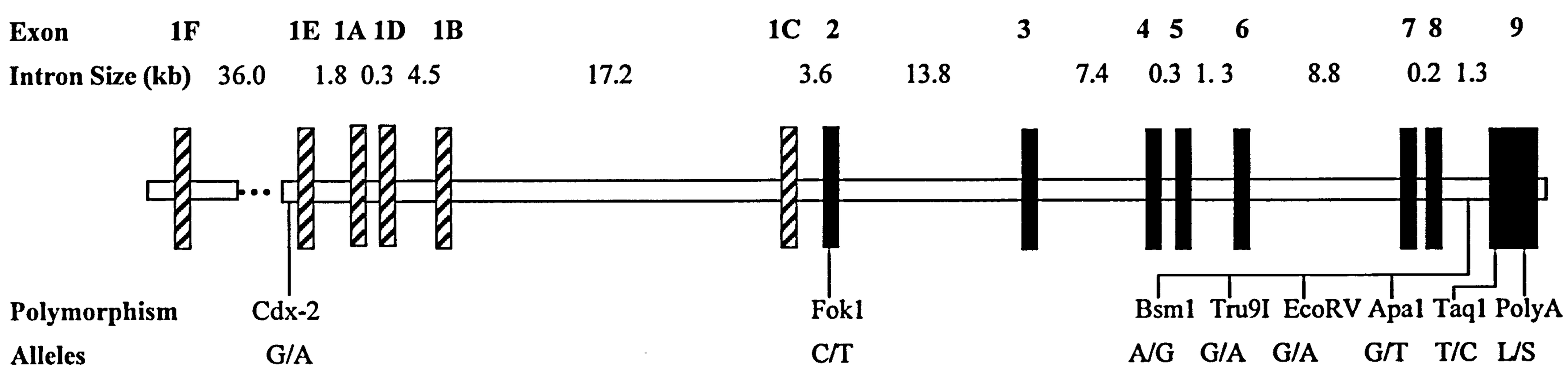

Figure 1.5: The exon-intron organisation of the chromosomal VDR gene. Approximate intron sizes are shown above each intron in kb. The positions of polymorphisms of the VDR gene are shown with their respective alleles below.

The VDR gene has been localised to chromosome 12q12-14 (Sone et al., 1990). The VDR gene is poorly reported in the NCBI database[1], with the original sequence as reported by Baker (Baker et al., 1988) (Accession number: NM 000376) still used as standard despite not including exon 1d-1f or a complete sequence for exon 1a, although these exons are reported as separate sequences (1d-1f: AF080454-AF040456 and 1a: AB002158). However, the use of the cosmid clones reported by Crofts *et al.* (Crofts et al., 1998) combined with the NCBI chromosome 12 sequence and the Celera commercial database[2] has enabled the exon-intron structure to be elucidated as shown in *figure 1.5.*

1.2.4. Promoter Regions

According to the splicing patterns reported by Crofts *et al.* (Crofts et al., 1998), transcription can begin either in exon 1a, 1d, or 1f. This means that the regions immediately upstream of each of these three exons are potential promoter regions. As can be seen in *figure 1.5*, the intron between exons 1a and 1d is only 300 base pairs (bp) long and the proximity of these exons means that the majority of the promoter region is common to both. Exon 1f, however, is more than 37 kilobases (kb) upstream and must have an entirely separate promoter region.

The promoter region 5′ of exon 1a was first characterised by Miyamoto *et al.* (Miyamoto et al., 1997). Approximately 2000bp upstream of the transcription start site was sequenced. Although the region does not contain TATA or CAAT boxes which are present in many conventional promoters the region is GC rich and contains a cluster of five Sp-1 transcription factor binding sites from nucleotides –74 to –18 relative to the exon 1a transcription start site. Sp1 is a ubiquitous transcription factor that usually binds immediately upstream of the transcription start site (Dynan and Tjian, 1983; Gidoni et al., 1984). Binding sites for other common factors including GATA-1, AP-1 and AP-2 were reported. Several constructs of this region were cloned into a luciferase vector and transfected into HeLa cells to assess their promoter activity. The 464bp proximal to exon 1a showed the highest activity while the addition of the next 122bp caused a drop in activity, suggesting that a negative regulatory region was present between nucleotides

[1] www.ncbi.nlm.nih.gov
[2] http://myscience.appliedbiosystems.com/

–586 and –464. Cloning of longer constructs up to 1935bp caused the promoter activity to rise again.

When Crofts *et al.* (Crofts et al., 1998) reported the existence of the novel 5' exons 1d-1f, they reported transcripts originating from exon 1f and 1d as well as those originating from 1a as previously reported. This suggested that VDR transcription may be dependent on multiple promoters. Crofts *et al.* also carried out transfection assays, in NIH 3T3 and COS 7 cells, to assess promoter activity. They confirmed the findings of Miyamoto *et al.* (Miyamoto et al., 1997) that the most active region was the 488bp region immediately upstream of exon 1a and found similar variation in activity of the 1a promoter as reported by Miyamoto *et al.* Little activity was found in the intron between exons 1a and 1d, suggesting that transcription from both exons is indeed controlled by a common promoter, upstream of exon 1a. Transcriptional activity was also found in the 1f promoter, although at significantly reduced levels. The 172bp proximal to exon 1f were shown to have the highest activity and, like the 1a promoter, the 1f promoter was also shown to have negative as well as positive regulatory regions.

Byrne *et al.* (Byrne et al., 2000) reported that the region immediately 5′ of exon 1c showed several characteristics of a conventional type II promoter including GC rich regions, Sp-1 and AP-2 sites and a TATA box. In transfection assays, they showed that this region possessed promoter activity and was responsive to oestrogen and retinoic acid. They also reported transcripts initiating in exon 1c in MCF7 cells. This conflicts with the findings of Crofts *et al.* (Crofts et al., 1998) who reported transcripts initiating only from exons 1a, 1d and 1f and creates the possibility that, depending on cell background, transcripts may be initiated in more exons than first reported.

1.3. Genetic Variation of the VDR

The vast majority of research into the genetic variation of the VDR has centred on four restriction fragment length polymorphisms (RFLPs) within the gene and its introns and a polyadenyl (polyA) microsatellite length polymorphism. Another potentially very significant and as yet little investigated source of variation arises from the differential splicing of the exons at the 5′ end of the gene.

1.3.1. Single Nucleotide Polymorphisms and PolyA Microsatellite Polymorphisms

Four single nucleotide polymorphisms (SNPs) that are restriction fragment length polymorphisms (RFLPs) have been extensively studied in the VDR gene. Two of these, Bsm1 (Morrison et al., 1992) and Apa1 (Faraco et al., 1989) are located in intron 8 while a Taq1 (Morrison et al., 1994) polymorphism is located in the 3'-untranslated region of exon 9. The fourth RFLP is at the 5′ end of exon 2. It is a C to T polymorphism that alters a codon three codons upstream of the wildtype start codon from an ACG codon to an alternative ATG start codon. This polymorphism was first reported as a variation on the original reported sequence (Saijo et al., 1991) and subsequently identified as a Fok1 restriction polymorphism (Arai et al., 1997). Two further restriction polymorphisms have been reported in intron 8, EcoRV (Morrison et al., 1992) and Tru9I (Ye et al., 2000) but these have been far less extensively studied. Morrison *et al.* (Morrison et al., 1994) and Durrin *et al.* (Durrin et al., 1999) also reported a number of polymorphisms in the 3' untranslated region (UTR) of exon 9. The alleles of the RFLPs follow a standard nomenclature. The allele unrestricted by the enzyme is represented by the capital initial of the enzyme and the restricted allele is represented by the lower case initial. Consequently, the Fok1 polymorphism has alleles F (unrestricted) and f (restricted), Bsm1 has alleles B and b, Apa1 A and a and Taq1 T and t. More recently a further SNP was reported in the promoter region upstream of exon 1e (Arai et al., 2001). The polymorphism was within a binding site for the Cdx-2 transcription factor and is therefore known as the Cdx-2 polymorphism.

In 1994 Morrison *et al.* (Morrison et al., 1994) sequenced the 3'-region of the VDR in individuals homozygous for the Bsm1, Apa1 and Taq1 polymorphisms. They reported a length polymorphism of a polyadenyl (polyA) sequence at the 3'end of exon 9. The polymorphic sequence was either 18 adenosines long (Long, L) or 12 adenosines (Short, S). Ingles *et al.* (Ingles et al., 1997a) have since identified twelve alleles at this locus but these can still be categorised as short (13A-17A) or long (18A-24A).

The 3′ polymorphisms, Bsm1, Apa1, Taq1 and the polyadenyl length polymorphism have been shown to be in linkage disequilibrium. Linkage disequilibrium is a measure of the non-random co-occurrence of alleles of different polymorphisms in a single haplotype (Wall and Pritchard, 2003). Practically, this means that in areas of high linkage disequilibrium, there will be a limited number of haplotypes and the genotype at

one polymorphism can be used to predict the genotype at another linked polymorphism with some accuracy. The high levels of linkage of the 3′ polymorphisms was noted in the initial reports (Morrison et al., 1994; Morrison et al., 1992), the two most common haplotypes being BAt and baT. In 1996 Uitterlinden *et al.* (Uitterlinden et al., 1996) showed that in a Caucasian population the baT haplotype represented 48% of the population and the BAt haplotype 40%, suggesting a high level of linkage. Ingles *et al.* (Ingles et al., 1997a) showed linkage between the L allele at the polyA polymorphism and the b allele at Bsm1 and between the S allele and the B allele. Combining these studies, the two dominant 3′ haplotypes are baTL and BAtS. This linkage disequilibrium pattern has been confirmed by Nejentsev *et al.* (Nejentsev et al., 2004) who showed that the coding exons are covered by one block of linkage disequilibrium, a region downstream of exon 9 is covered by another block and the 5' exons by a third block of high linkage disequilibrium. Interestingly, the Fok1 polymorphism was shown to be in an island of very low linkage disequilibrium.

1.3.2. Association Studies

When Morrison *et al.* (Morrison et al., 1992) first reported the 3′ polymorphisms they reported that genotype was linked to osteocalcin levels and in 1994 (Morrison et al., 1994) they reported that genotype could be used to predict bone mineral density. Since these original studies, over 300 association studies have been carried out on one or more of the VDR polymorphisms. Over two thirds of these study associations with the 3′ cluster of polymorphisms while nearly 100 look at associations with the Fok1 polymorphism.

The range of diseases and phenomena which have been studied in association with VDR polymorphisms are shown in ***table 1.1***, in order of the number of studies in each case.

The association of the 3′ polymorphisms with bone mineral density, bone mass and growth has been thoroughly studied, particularly in Caucasian, Japanese and Chinese populations. Associations with other phenomena linked to bone mineral density have also been reported such as risk of fracture, bone loss, periodontal disease, rickets and osteoporosis. Where only one of the polymorphisms has been studied the high level of linkage has allowed the results to be compared. Many of the results have been contradictory and differences between populations were high, particularly between

ethnic groups, males and females and pre-and post-menopausal women. Effects have also been seen in other organs and disease states related to the classical actions of vitamin D, particularly on the calcium sensing functions of the parathyroid and on the development of hyperparathyroidism. All of the studies linked with the classical actions of vitamin D are shown in ***appendix II.***

	Cdx-2		FokI		BsmI		ApaI		TaqI		polyA		Total	
Association	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Bone Mineral Density	1		26	8	3	7	13	6	11	6			81	27
Prostate Cancer	1		4	2	5	3	1	4	8	1	2	4	21	23
Type I Diabetes			5	2	5	2	3	2	2	3			16	9
Breast Cancer			1	2	5	2	4		5	3	1		16	7
Osteoporosis			1	1	7	2	3		3	1			14	4
Bone Loss			1	1	6	2		1	4				11	4
Osteocalcin levels					2		1		2				6	0
Psoriasis			1		2	3	2	1	1	1			6	5
Fracture Risk	1				2	1		1	1	1			4	3
Macrophage Phagocytosis			1		1		1		1				4	0
Colorectal Cancer			1		3	1				1			4	2
Periodontal Disease					1				2				3	0
Grave's Disease			1		1		1						3	0
Multiple Sclerosis					2		1						3	0
Type II Diabetes				1	2	1	1	1		1			3	4
Autoimmune hepatitis			2										2	0
Crohn's Disease			1					1	1				2	1
Parathyroid Function			3		9		5		3				2	0
Asthma							1						1	0
Rickets			1										1	0
Osteoarthritis									1				1	0
Height			1										1	0
Insulin-dependent Diabetes					1								1	0
PBMC Growth			1										1	0
Total	3	0	51	17	84	24	37	17	45	27	3	4	224	89

Table 1.1: The number of association studies with the various *VDR* polymorphisms where association were found (+) or not found (-). Two experiments including rarely studied polymorphisms are excluded, one found an association of the Tru9I polymorphism with diabetes, the other associated EcoRV genotype with prostate cancer.

VDR polymorphisms appear to have an effect on a significant group of diseases where inflammatory responses, immunity or auto-immunity play a role in disease aetiology. The 3′ polymorphisms have been associated with diabetes, Graves' disease, Crohn's disease, multiple sclerosis, autoimmune hepatitis, asthma and susceptibility and response to treatment in psoriasis. These studies are summarised in *table 1.2.*

Associations		No Association	
Study	**Polymorphism**	**Study**	**Polymorphism**
Asthma			
Raby et al., 2004	A		
Autoimmune Hepatitis			
Fan et al., 2003	F		
Vogel et al., 2002	F		
Crohn's Disease			
Simmons et al., 2000	T	Simmons et al., 200	F, A
Grave's Disease			
Ban and Taniyama, 2000	A, B		
Ban et al., 2000	F		
Growth Inhibition of PBMC			
Colin et al., 2000	F		
Macrophage Phagocytosis			
Selvaraj et al., 2004	F, B, A, T		
Psoriasis			
Holick et al., 1996	B	Kontula et al., 1997	B
Lee and Park, 2002	A	Mee and Cork, 1998	B
Park et al., 1999	A	Okita et al., 2002	A, B, T
Saeki et al., 2002	F, B, T		
Multiple Sclerosis			
Fukazawa et al., 1999	B		
Niino et al., 2000	A, B		
Type I Diabetes			
Audi et al., 2004	F	Fassbender et al., 2002	B, T
Fassbender et al., 2002	F	Turpeinen et al., 2003	F, A, B
Ban et al., 2001	F	Chang et al., 2000	T
Marti et al., 2004	F, B	Guja et al., 2002	A
Motohashi et al., 2003	B	Gyorffy et al., 2002	F,T
Skrabic et al., 2003	B, A, T		
Chang et al., 2000	B, A		
Gyorffy et al., 2002	B, A, Tru9I		
Guja et al., 2002	F, T		
Type II Diabetes			
Oh and Barrett-Connor, 2002	A, B	Malecki et al., 2003	F, A, B, T
Speer et al., 2001	B		
Insulin-Dependent Diabetes			
Hauache et al., 1998	B		

Table 1.2: Association studies in diseases with a strong immunological element to their aetiology. F=Fok1, B=Bsm1, A=Apa1, T=Taq1.

The association of VDR 3′ genotype with several cancers has been studied. These are summarised in *table 1.3*. In breast cancer, genotype has been linked with occurrence and metastasis. There is a growing body of evidence of an association with prostate cancer risk. An association has also been reported with colorectal cancer.

Associations		No Association	
Study	**Polymorphism**	**Study**	**Polymorphism**
Breast Cancer			
Cui et al., 2001	A, T	Curran et al., 1999	F
Curran et al., 1999	A, T	Bretherton-Watt et al., 2001	B
Bretherton-Watt et al., 2001	B	Dunning et al., 1999	T
Guy et al., 2003	B	Guy et al., 2003	F
Hou et al., 2002	B, A, T	Buyru et al., 2003	B, T
Guy et al., 2004	F, B, p(A)	Newcomb et al., 2002	T
Lundin et al., 1999	T		
Ruggiero et al., 1998	B		
Sillanpaa et al., 2004	A, T		
Colorectal Cancer			
Boyapati et al., 2003	B	Ingles et al., 2001	B
Kim et al., 2001b	B	Peters et al., 2004	T
Speer et al., 2000	B		
Ingles et al., 2001	F		
Malignant Melanoma			
(Hutchinson et al., 2000)	F,T		
Prostate Cancer			
Bodiwala et al., 2004	C, F, T	Blazer et al., 2000	T, p(A)
Cheteri et al., 2004	B	Cheteri et al., 2004	F, p(A)
Chokkalingam et al., 2001	F, B	Correa-Cerro et al., 1999	F
Correa-Cerro et al., 1999	T, p(A)	Gsur et al., 2002	T
Furuya et al., 1999	T	Habuchi et al., 2000	A, T
Habuchi et al., 2000	B	Kibel et al., 1998	T, p(A)
Hamasaki et al., 2001	T	Liu et al., 2003	B
Hamasaki et al., 2002	T	Ma et al., 1998	T
Huang et al., 2004	B, A, T	Maistro et al., 2004	A, T
Ingles et al., 1997c	p(A)	Oakley-Girvan et al., 2004	B, A, T
Ma et al., 1998	B	Suzuki et al., 2003	B, A, T
Medeiros et al., 2002	T	Tayeb et al., 2003	T
Oakley-Girvan et al., 2004	F	Watanabe et al., 1999	T, p(A)
Taylor et al., 1996	T		
Xu et al., 2003	F		

Table 1.3: Association studies in cancer. C=Cdx-2, F=Fok1, B=Bsm1, A=Apa1, T=Taq1, p(A)=polyA length polymorphism.

Despite the lack of linkage between Fok1 and the 3′ polymorphisms, the Fok1 genotype is associated with very similar biological functions. Again, the majority of the studies report an association with bone mineral density and associated phenomena. Associations are also reported with diabetes, Graves disease, autoimmune hepatitis and psoriasis. Fok1 genotype has been shown to have an effect on growth inhibition of peripheral blood mononuclear cells by $1,25(OH)_2D_3$ and on the activity of macrophages(Selvaraj et al., 2004). These effects on immune cells may suggest a mechanism by which *VDR* polymorphisms affect diseases with an inflammatory or immune aetiology.

In contrast to the 3′ polymorphisms, Fok1 shows no association with occurrence of breast cancer, suggesting that there are mechanistic differences in the way the polymorphisms exert their effects. Similarly in prostate cancer, few associations have been found between Fok1 genotypes and occurrence, even where associations were found with the 3′ polymorphisms although there is some evidence that genotype is linked to prostate cancer risk and progression. Fok1 genotype has been linked to risk of malignant melanoma and the combined genotype of Fok1 and Taq1 have been linked with tumour thickness (Hutchinson et al., 2000). More recently, Nieto *et al.* (Nieto et al., 2004) have reported an association of Fok1 genotype with the progression to AIDS from HIV infection. Strangely, though, the Ff heterozygote is reported as the risk genotype, suggesting that either the effects are through an unusual mechanism or the association is coincidental.

The Cdx-2 polymorphism has only been discovered recently and hence fewer associations have been reported. Arai *et al.* (Arai et al., 2001) reported that Cdx-2 was associated with bone mineral density in a Chinese population and as the Cdx-2 protein is a major gut transcription factor, it seems likely that the Cdx-2 polymorphism of the VDR may affect the calcium absorption pathways in the gut and therefore bone mineral density. This association could not be confirmed in a large Caucasian population although an association was found with risk of fracture (Fang et al., 2003). Cdx-2 genotype has also been linked with prostate cancer risk (Bodiwala et al., 2004).

1.3.3. Functional Effects of Polymorphisms

Although association studies have clearly shown that VDR polymorphism has an effect on many biological processes and diseases, the mechanism by which the polymorphisms exert these effects is much less clear.

As the Bsm1, Apa1, EcoRV and Tru9I polymorphisms are all in a cluster in intron 8 they cannot have any effect on protein structure. Taq1 is a synonymous polymorphism at the beginning of exon 9 so also does not affect protein structure. The proximity of the polymorphisms to each other and to the polymorphisms in the 3′ UTR and the high level of linkage in this region suggest that effects ascribed to the RFLPs may be due to their linkage to more functional polymorphisms in the UTR. The UTR of genes has been shown to affect mRNA stability and therefore the levels of protein that can be translated from a single messenger molecule (Decker and Parker, 1995). Durrin *et al.* (Durrin et al., 1999), however, although they reported the presence of destabilising regions within the VDR UTR, did not find any polymorphisms within these regions and found no association of the 3′ RFLPs with mRNA stability.

The length polymorphism of the polyA tail also has no effect on protein structure but does alter the mRNA structure. Whitfield *et al.* (Whitfield et al., 2001) showed that the L allele was more active than the S allele in transcriptional assays in transfected fibroblasts. It was suggested that this increased activity was due to increased RNA stability but the linkage results of Durrin *et al.* (Durrin et al., 1999) would dispute this. A mechanism may exist whereby the length of the polyA site may affect translation levels without altering mRNA stability. The site may form a binding site for certain polyA binding proteins (PABPs), which interact with the translation machinery at the 5′ end of the mRNA to enhance translation (Munroe and Jacobson, 1990). If the L allele bound PABPs with greater affinity than the S allele, translation could be enhanced without an effect on mRNA stability.

Fok1 is the only RFLP that structurally alters the VDR protein. By forming an alternative start codon the Fok1 variant T (f) allele codes for a 3 amino acid-extended, 427 amino acid protein, with a slightly longer N-terminal A/B domain. Several studies have shown that the shorter protein, termed the M4 protein (methionine at codon 4), coded for by the F allele has greater transcriptional activity than the longer M1 protein, coded for by the f allele (Arai et al., 1997; Colin et al., 2000; Whitfield et al., 2001).

The longer M1 protein has been shown to interact less efficiently with TFIIB and this reduced affinity for TFIIB was associated with a concurrent reduction in transcriptional activity (Jurutka et al., 2000). This provides a molecular mechanism by which the Fok1 polymorphism has a direct functional effect on VDR activity.

SNPs within promoter regions can have dramatic effects due to the nature of promoter activity. Transcription factors bind to promoter regions through short core binding sequences, often only six or ten bases in length. Alteration of a single base within these transcription factor binding elements can have a significant effect on transcription factor binding and therefore on promoter activity. The promoter polymorphism reported by Arai *et al.* (Arai et al., 2001) is a G to A polymorphism within a binding site for the intestinal transcription factor Cdx-2. In electrophoretic mobility shift assays, Cdx-2 binding was shown to be significantly reduced in the G allele and in transfection assays the transcriptional activity of the G allele was shown to be decreased to 70% of the activity of the A allele.

1.3.4. 5'-Splice Variants

Crofts *et al.* (Crofts et al., 1998) reported that there were six exons upstream of the translation start codon in exon two of the VDR gene. These 5' exons were reported to be involved in a complex pattern of splicing that could potentially produce fourteen different VDR mRNAs.

Crofts *et al.* reported that transcription could begin in exons 1f, 1a or 1d. Transcripts beginning in exon 1f could then either splice in exons 1e, 1c, both or neither before the incorporation of exon 2 with the translation start codon. Transcripts beginning in exons 1a or 1d could splice in exon 1b, 1c, both or neither before exon 2. They also reported transcripts that contained a fragment of intron 2 spliced in after exon 2 which contained a premature stop codon and would code for a severely truncated protein. The splicing patterns reported by Crofts *et al.* are shown in ***figure 1.6.***

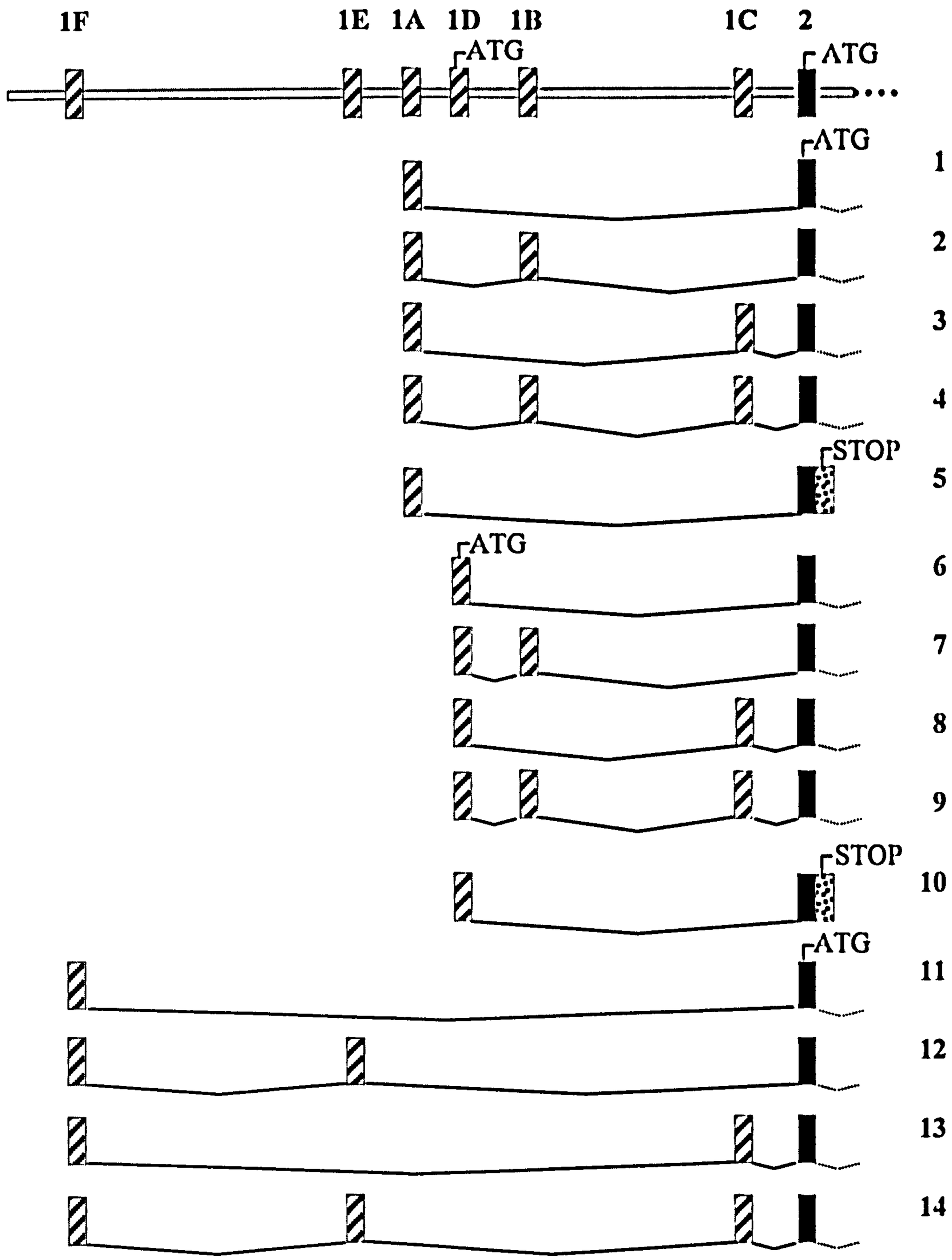

Figure 1.6: The splicing pattern of the 5′ exons of the VDR gene as reported by Crofts *et al.* (Crofts et al., 1998). All variants code for the wildtype 424 amino acid protein except for transcript 6 which codes for a 450 amino acid protein, transcript 8 which codes for a 474 amino acid and transcripts 5 and 10 which include a fragment of intron 1 containing a premature stop codon and codes for a truncated protein. Adapted from Crofts et al (Crofts et al., 1998).

Of particular importance in terms of potential functional affects are the transcripts initiating from 1d. Exon 1d contains an ATG codon and in the case of transcripts 6 and 8 this codon is in frame with the previously described coding regions of the VDR gene.

Translation from this alternative ATG site can lead to extended proteins of 450 (known as the VDRB2 protein) and 477 (VDRB1) amino acids. The VDRB1 protein has since been isolated from a variety of cell backgrounds by the same group (Sunn et al., 2001) but the VDRB2 protein was not found. Sunn *et al.* also reported that transactivation by VDRB1 was lower compared to that of wildtype VDR (VDRA). However, further experiments have shown that VDRB1 has significantly higher transactivational activity (Gardiner et al., 2004) and the results were sensitive to experimental conditions, particularly transfection efficiency and carrier vector. Apart from this variation in transactivation potential no major functional differences between VDRA and VDRB1 have been reported. Clearly, because of the extended A/B domain these isoforms could have a significantly different functionality to the 424 amino acid protein. The existence of a membrane receptor for $1,25(OH)_2D_3$ has been hypothesised but never proven and this is an area of great controversy (reviewed in (Farach-Carson and Davis, 2003)). It is possible that the extension of the A/B domain may confer membrane-binding capability and preliminary investigations by Huhtakangas *et al.* (Huhtakangas et al., 2003) suggest that a form of VDR is present in caveolae, small infoldings of the plasma membrane, in animal models. Whether this is the VDR and whether it is the wildtype or extended protein has not been confirmed. It is worth noting that in the case of these longer proteins, the Fok1 polymorphism no longer has an effect on protein length but becomes a non-synonomous polymorphism, the ACG codon in the F allele coding for threonine and the ATG of the f allele coding for methionine. The Fok1 polymorphism, therefore, has a protein-altering effect on the VDR in all transcripts, although the effect may be functionally different in 1d transcripts.

Transcripts initiating in exons 1a and 1f do not contain any additional ATG codons and so translation from these transcripts must begin at the exon 2 start codon. These transcripts will therefore code for the 424 or 427 amino acid receptor, dependent on Fok1 genotype. These transcripts therefore have no effect on protein function. However, the different mRNA sequences 5′ of the translation start site may provide binding sites for factors involved in translational control and may provide a mechanism for differential translational control of the variant transcripts. The splice variants may be

important in binding the different translational machinery present in different cell backgrounds.

1.4. Aims and Objectives

1.4.1. In Silico *Analysis of VDR Gene Structure*

The VDR gene will be analysed using a range of recently developed software. The evolutionary conservation of both the exonic and promoter region sequences will be analysed to identify the functionally important regions of the gene. This may help to establish how fundamental is the complexity of the 5' region to VDR function. It may also identify the relative importance of the various potential promoter regions.

1.4.2. Screening of the VDR Gene for Novel Variation

Having identified regions of likely importance to VDR function, these will be screened for novel genetic variation using single stranded conformational polymorphism analysis.

1.4.3. Association of VDR Polymorphisms with Disease

The affect of VDR polymorphisms on occurrence, Breslow depth and metastasis will be studied in a cohort of 194 malignant melanoma patients.

The association of VDR genotypes will also be tested with psoriasis occurrence, severity and response to Dovonex (calcipotriol) treatment.

To assess the affect on occurrence, VDR genotype will be compared in disease populations compared to 80 controls.

1.4.4. Determination of VDR Polymorphism Function

Any polymorphisms that show an association with melanoma and psoriasis will be assessed for functional affects on VDR activity at the molecular and cellular level.

2. Gene Structure

2.1. Introduction

The complex structure of the VDR gene was discussed in *chapter 1.3.4*. Alternative splicing of the six 5′ exons produces fourteen alternative mRNA molecules, two of which code for variant proteins. The functional significance of neither the alternative splicing of the 5′ exons, which produces 12 significantly different untranslated regions, nor of the two alternative proteins has been elucidated.

In this chapter, the genetic sequence of the human *VDR* is compared with those of the mouse (*Mus Musculus*) and the rat (*Rattus Norvegicus*). Analysis of the evolutionary conservation of the VDR gene will reveal the functionally important regions of the gene. The more conserved a region of a gene is, the more likely it is to be functionally important. Thus if the complex pattern of 5′ exons were conserved, particularly those coding for alternative proteins, it would suggest that these variants were necessary for the normal function of the VDR.

The promoter regions will also be analysed for conservation of transcription factor binding sites between species. This may determine which regions are under the control of particular transcriptional systems and identify the functionally important promoter regions.

2.1.1. 5′ Splice Variation

Crofts *et al.* (Crofts et al., 1998) reported a complex pattern of splice variation at the 5′ end of the VDR gene, shown in *figure 1.6*. Transcription was shown to begin in exons 1a, 1d and 1f and transcripts could include both, either or neither of exons 1e and 1c in the case of 1f transcripts and 1b and 1c in the case of 1a and 1d transcripts. Two transcripts were also detected including a fragment of intron 2 containing a premature stop codon. This variation produced fourteen mRNA molecules, two of which, beginning in exon 1d, coded for alternative, N-terminally extended, proteins. One of these proteins (VDRB1) was later identified in several cell backgrounds (Sunn et al., 2001). The function of this alternative protein has not yet been discovered, although it has been suggested that VDRB1 may be a membrane receptor for $1,25(OH)_2D_3$. Byrne

et al. (Byrne et al., 2000) also reported transcripts beginning in exon 1c, although this was only shown in MCF-7 breast cancer cells. The 10 transcripts initiating in exons 1f and 1a do not contain alternative translation start codons and so do not code for alternative proteins. They may however provide alternative binding sites for proteins of the translational machinery. One reason for this variation may be the presence of different transcriptional and translational proteins in different cells and tissues. The alternative promoters and mRNA transcripts may allow for the differential regulation expression of VDR in different tissues and for the differential expression of the VDR protein isotypes. This 5′ variation creates several sources of functional variation: the two transcripts beginning in exon 1d potentially code for two alternative proteins; the twelve further mRNA transcripts which do not affect protein structure provide a mechanism for differential interaction with the translational proteins; the three (1a, 1d and 1f (Crofts et al., 1998)) or four (1c (Byrne et al., 2000)) initial exons allow transcription to be regulated from at least three promoter regions.

2.1.2. Analysis of Gene Homology

Of approximately 3,200,000,000 base pairs of the human genome, less than five per cent are within the coding regions of genes. The completion of several genomes in recent years has allowed the comparison of the human genome with genomes at varying degrees of evolutionary distance. The NCBI database[3] now contains the genomes of nine mammals and two other vertebrates. Functional regions of the genome are likely to be conserved through evolution, with the more basic functional elements such as the coding exons of active genes being better conserved. The conservation of these sequences occurs because there is a selection pressure against any genetic change that would alter the function of the active protein.

The level of evolutionary conservation depends on the evolutionary distance between the two organisms. For instance, the frog, which diverged from humans approximately 350 million years ago, is likely to show conservation within exons but little homology in the intronic regions. The mouse, which diverged from humans only 80 million years ago is also likely to show homologous exon sequences but will also have in common

[3] http://www.ncbi.nlm.nih.gov/mapview/

functionally important non-exonic regions, such as regulatory regions within gene promoters (reviewed in (Nobrega and Pennacchio, 2004)).

The usefulness of comparative genomics has led to the development of a variety of software for this type of analysis. Shorter sequences can be analysed using the BLAST software at the NCBI (Altschul et al., 1990). This allows base-by-base comparison of two sequences. This is a useful tool for comparing exonic sequences but less practical for comparisons of an entire gene including the introns. This has led to the development of less literal, more graphic comparison software. The most basic form of this is the nucleic acid dot plot (Pustell and Kafatos, 1982), such as is available at the Molecular Toolkit.[4] The two DNA sequences form the x and y axes of the plot. A sliding window of defined size moves along each sequence. Where the sequences within the window contain fewer mismatches than the defined mismatch limit, a dot is generated at the appropriate coordinate. Thus, regions of homology appear as a diagonal line.

When comparing genomic sequences of phylogenetically relatively close organisms such as human and mouse, functional regions will often be masked by common repeat regions which are non-functional but make up a large proportion of genomic DNA. The RepeatMasker programme[5] identifies common repeat regions and masks them to allow the functional regions to be identified. The dot plot format has been expanded and integrated with the repeat masker software and improved annotation tools to create the percentage identity plot (PIP) (Schwartz et al., 2000). The PIP again compares the identity of two sequences, presenting the results as a percentage identity dot plot annotated with exon positions and the repeat regions revealed by RepeatMasker. Similarly, the VISTA software (Couronne et al., 2003) allows comparison of large genomic sequences from several species, identifying common repeat regions. Unlike Pipmaker, VISTA allows comparison of more than two organisms simultaneously and illustrates the percentage identity as a curve rather than a dot plot.

Here, the evolutionary conservation of the VDR gene will be determined by comparison of the human gene with that of the mouse, rat and frog by dot plots, percentage identity plots and using the VISTA software. The conservation of the exon sequences will be determined using BLAST.

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[4] http://arbl.cvmbs.colostate.edu/molkit/dnadot/index.html
[5] http://www.repeatmasker.org/

2.1.3. Analysis of Promoter Homology

The conservation of promoter regions is more difficult to analyse due to the short length of transcription factor binding sites. Most transcription factors bind to sequences of 6-12 base pairs, much shorter than the "window" employed in analyses such as dot plots and PIPs. Therefore only regulatory regions containing larger clusters of transcription factor binding sites, such as the cluster of Sp1 sites often found immediately upstream of a gene's transcription start site, can be identified by the basic comparative tools. In order to overcome this problem, the TraFac database (Jegga et al., 2002) combines the percentage identity plot with software such as the Transcription Element Search System (TESS)[6] that predicts transcription factor binding sites. Based on experimental data, transcription factor binding prediction software such as TESS defines a matrix for each transcription factor based on the likelihood of transcription factor binding given the presence of a particular base at each position of the binding site. The degree to which the actual sequence matches the optimal sequence for binding produces a likelihood score enabling the most likely transcription factor binding sites in a particular sequence to be predicted. The TraFac database can then combine these predictions for the comparable promoter regions of two species with a PIP to identify areas of high sequence homology within the promoter region that also contain binding sites for the same transcription factors. As transcription factors usually bind in complexes containing a number of proteins, the software can also identify sequences that bind a number of transcription factors in parallel positions in both species, thus identifying regions under common regulatory control.

Here, the promoter regions upstream of exons 1f, 1a and 1d, the initial exons in transcripts reported by Crofts *et al.* (Crofts et al., 1998), and 1c, as reported by Byrne *et al.* (Byrne et al., 2000) are analysed by the TraFac software in order to identify conserved regulatory regions and perhaps elucidate the difference between the mechanisms controlling transcription from the alternative initial exons.

[6] http://www.cbil.upenn.edu/tess

2.2. Methods

2.2.1. BLAST

In order to demonstrate the evolutionary conservation of the exons, human and mouse exonic sequences were compared using the BLAST software (Altschul et al., 1990). The human exon sequences were obtained from NCBI. The sequences of the corresponding mouse exons were predicted by the TraFac software (see *2.2.5*). Accession numbers for the human exons and chromosomal positions of the human and mouse exons are shown in *table 2.1*. In order to find the percentage conservation, the Blast 2 sequences programme[7] was used. In order that less well-conserved sequences were not excluded from analysis, reward for a match was increased to 2, penalty for a mismatch was reduced to –1, word size reduced to 7 to allow for the relatively short sequence length and the statistical significance threshold "expect" value increased to 200.

Exon	Human		Mouse
	Accession Number	Position (Chromosome 12)	Position (Chromosome 15)
1f	AF080456	46623119-46622913	
1e	AF080455	46587073-46586917	
1a	AB002158	46585081-46585005	98368316-98368245
1d	AF080454	46584709-46584614	98367936-98367843
1b	AB002159	46580007-46579887	98363539-98363426
1c	AB002160	46562824-46562744	98348496-98348421
2	AB002161	46559165-46559018	98344949-98344802
3	AB002162	46545227-46545097	98336862-98336732
4	AB002163	46537738-46537554	98329838-98329654
5	AB002164	46537299-46537179	98329431-98329326
6	AB002165	46535851-46535680	98327295-98327124
7	AB002166	46526858-46526707	98319293-98319142
8	AB002167	46526501-46526385	98318949-98318833
9	AB002168	46525055-46521588	98317675-98314470

Table 2.1: The accession numbers of the human exons in the NCBI database and the chromosomal positions of each VDR exon on human chromosome 12 and mouse chromosome 15.

[7] http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi

2.2.2. Dotplots

Dotplot comparison of the human, mouse and rat VDR genes was carried out using the Molecular Toolkit nucleic acid dot plot software (Pustell and Kafatos, 1982)[8]. The genomic sequences used for comparison were human chromosome 12, 46633119-46511591; mouse chromosome 15, 98440000-98310000; rat chromosome 7, 136760000-136644000. In all three organisms, the *VDR* gene is on the reverse strand of the DNA so the reverse complement of these sequences was used such that the output could be viewed in the appropriate orientation for the gene. In order to determine the position and conservation of the exons, the human mRNA sequence including all exons, produced by combining the sequences shown in ***table 2.1***, was plotted against the human, mouse and rat genomic DNA. For clarity, the sequence were analysed in two parts, exons 1f-2 and exons 2-9.

2.2.3. PIPMaker

The human and mouse genomic sequences used for dotplot comparison, that is human chromosome 12, 46633119-46511591 and mouse chromosome 15, 98440000-98310000, were also used to produce a percentage identity plot (PIP)[9] (Schwartz et al., 2000). The human sequence was first analysed for common human repeat regions by the RepeatMasker software[10] and repeat regions in the sequence "masked" so that regions of functional homology were not masked by homologous regions of non-functional repeats. RepeatMasker produces a mask file annotating all the repeat regions in the sequence.

The human and mouse chromosomal sequences were uploaded to PipMaker with the mask file from RepeatMasker and an exons file containing the position of the exons in the human sequence. PipMaker returned two results files, a PIP and a DotPlot.

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[8] http://arbl.cvmbs.colostate.edu/ molkit/dnadot/index.html
[9] http://pipmaker.bx.psu.edu/pipmaker/
[10] http://www.repeatmasker.org

2.2.4. VISTA

To confirm the results of the PipMaker analysis, similar analysis was carried out using the VISTA software[11] (Couronne et al., 2003). The chromosomal sequence of interest was specified as chromosome 12, 46633119-46511591. This sequence was automatically annotated for repeats and exon positions and compared to the relevant mouse and frog genomic sequences. The annotation, however, was based on the *VDR* gene structure available at the NCBI, that is not including exons 1f, 1e, 1b or a complete sequence of 1a. The positions of these exons were therefore annotated manually.

Separate VISTAs were also produced comparing the sequence of each exon in mouse and human. The human and mouse sequences of each exon were uploaded to the mVISTA programme[12] and compared using a window size of 10 in order to provide a more detailed analysis of exon homology.

2.2.5. TraFac

The *VDR* human and mouse sequences were available on the TraFac databases. Again, however, the exon positions were based on those available at the NCBI, excluding exons 1f, 1e, 1d and 1b. The sequence of the complete mRNA, including the missing exons, was submitted to Dr Bruce Aronow, Associate Professor; Co-director, Computational Medicine Center, Cincinnati Children's Hospital. This was uploaded to the TraFac database and used to predict the position of the exons in the mouse. These sequences are now available on the TraFac database[13] named VDR_Hs and VDR_Mm. These sequences were used to identify conserved regions of transcription factor binding in the promoter regions upstream of exon 1f, 1a, 1d and 1c. The regions upstream of these exons were then used to produce TraFac analyses, showing transcription factor binding sites present in the equivalent regions of both sequences (Jegga et al., 2002). To identify the areas of strongest homology and reduce the likelihood of false positives resulting from the coincidental presence of similar sequence motifs within the region, TraFac analysis was restricted to identifying binding sites in parallel positions in the compared sequences.

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[11] http://pipeline.lbl.gov/cgi-bin/gateway2
[12] http://gsd.lbl.gov/vista/mvista/submit.shtml
[13] http://trafac.cchmc.org/trafac/ index.jsp

2.3. Results

2.3.1. Conservation of Exon Sequences from Mouse to Human

The human and exon sequences were compared using Blast. The full Blast alignments are shown in ***appendix III***. The percentage of identities (matching bases) and gaps (missing bases) in each exon alignment are shown in ***table 2.2***.

Exon	Identities Identities/exon size (%)	Gaps Gaps/exon size (%)
1A	52/72 (72%)	0 (0%)
1D	74/95 (77%)	2/95 (2%)
1B	68/113 (60%)	5/113 (4%)
1C	56/77 (72%)	5/77 (6%)
2	137/148 (92%)	0 (0%)
3	120/131 (91%)	0 (0%)
4	161/185 (87%)	0 (0%
5	75/116 (64%)	15/116 (12%)
6	149/172 (86%)	0 (0%)
7	127/151 (84%)	0 (0%)
8	104/117 (88%)	0 (0%)
9	1754/3223 (54%)	334/3223 (10%)

Table 2.2: The conservation of the exonic sequences between mouse and human as analysed by Blast. Identities are shown as number of identities over the length of exon with the percentage conservation shown in parentheses. Similarly, the number of gaps are shown over exon length with the percentage in parentheses.

Exons 1f and 1e could not be identified in mouse. A genomic Blast search of the human sequences against the entire mouse genome did not find any significant areas of homology. Exons 1a and 1d showed fairly high levels of conservation, 72% and 77% respectively. 1b was less well conserved, with only 60% identities and 4% gaps. 1c was also fairly well conserved (72%) but with a relatively high number of gaps (6%). Exons 2-4 were very well conserved (92%, 91% and 87%) with no gaps in any exon. Exon 5

was less well conserved, with only 64% identities and 12% gaps. Exons 6-8 were also well conserved (86%, 84% and 88%) with no gaps while exon 9 was the least well conserved with 54% identities and 10% gaps. These results are shown graphically in *figure 2.1*.

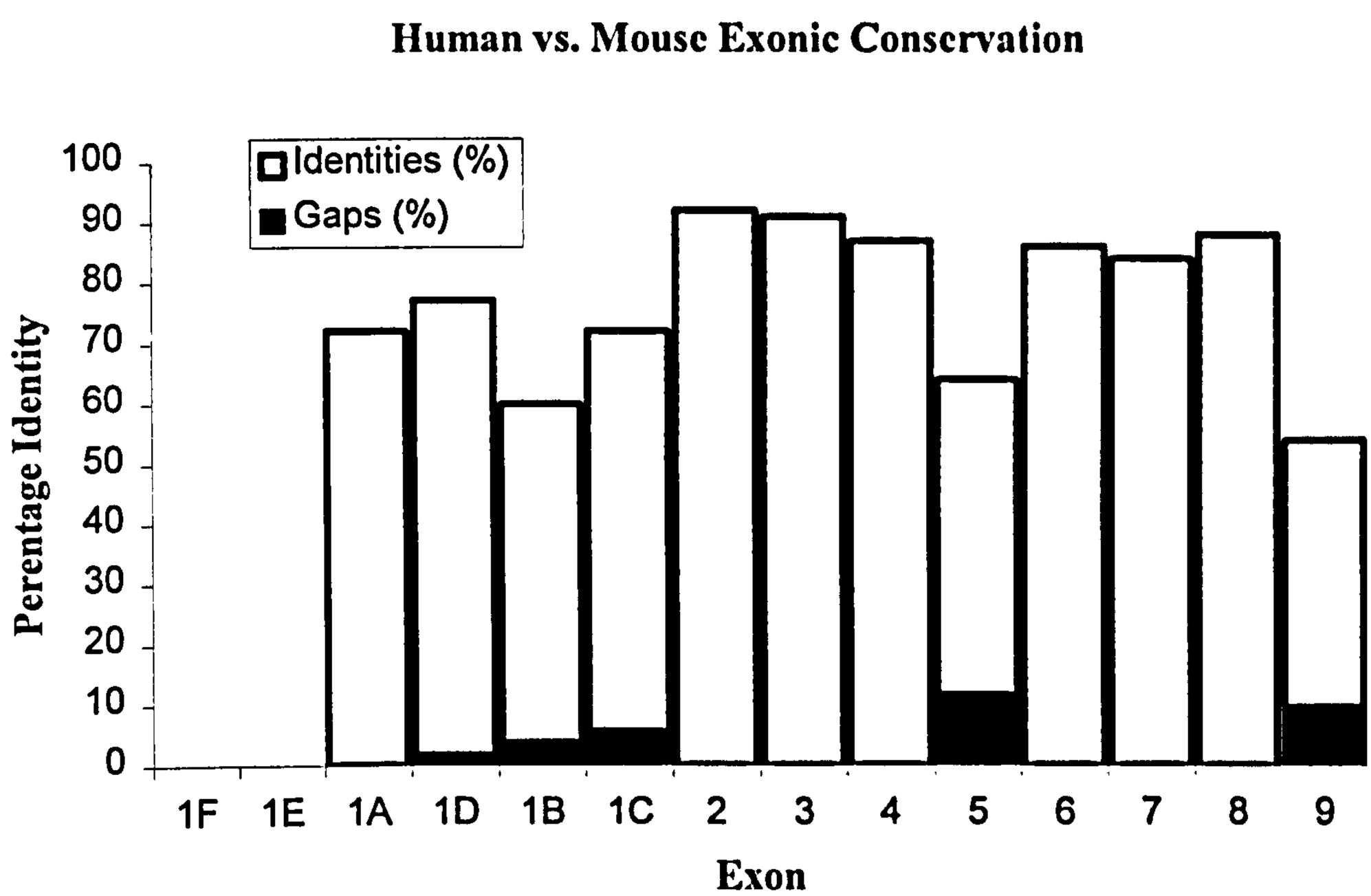
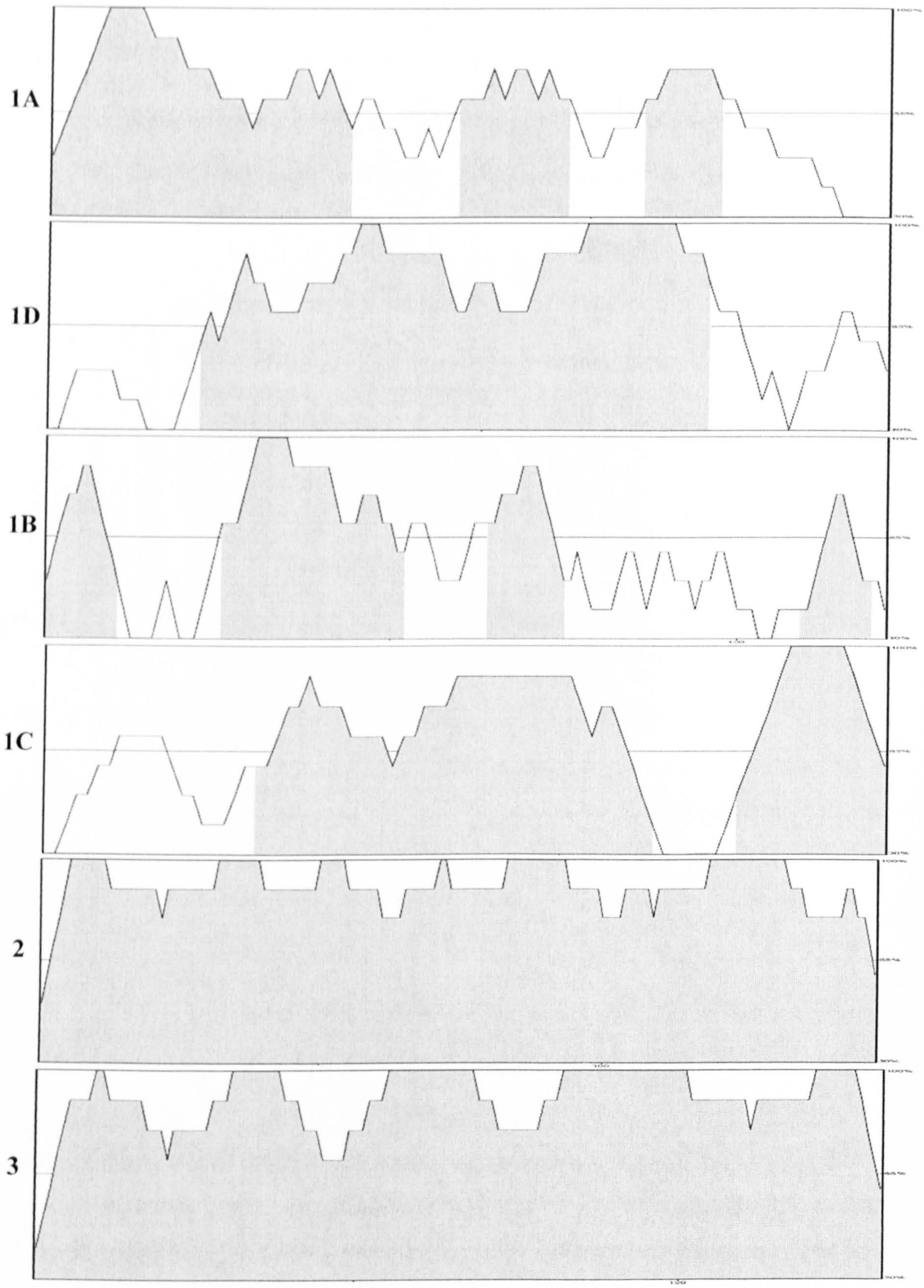


Figure 2.1: The percentage conservation of the VDR exons between mouse and human. Percentage conservation is shown as open bars and the percentage of gaps as closed bars.

To confirm these results and show the exonic conservation in more details, VISTAs were produced comparing each exon in human and mouse. These are shown in *figure 2.2*.

Exon 1a shows three blocks of conservation, a large block at the 5′ end and two further smaller blocks. Exon 1d shows a large region of homology in the centre of the exon with less homology at the extremes. Exon 1b shows less homology with four small regions of conservation with relatively large gaps in between. Exon 1c shows low homology at the 5′ end, a large central region of conservation and a highly conserved region at the 3′ end. Exons 2 and 3 show very high levels of conservation for their entire length. Exon 4 is similar but with a drop off in homology towards the 3′ end. Exon 5


1A
1D
1B
1C
2
3

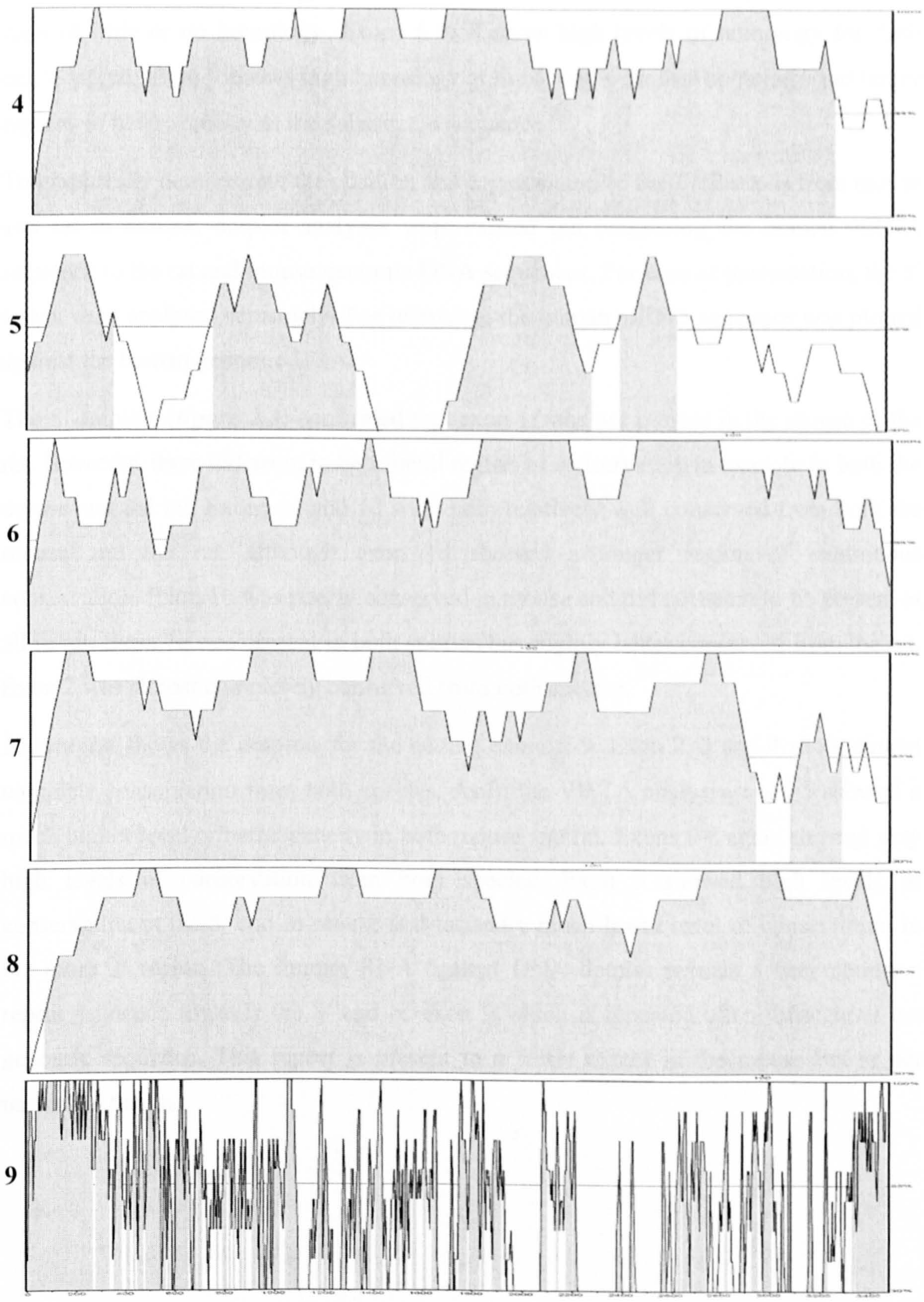


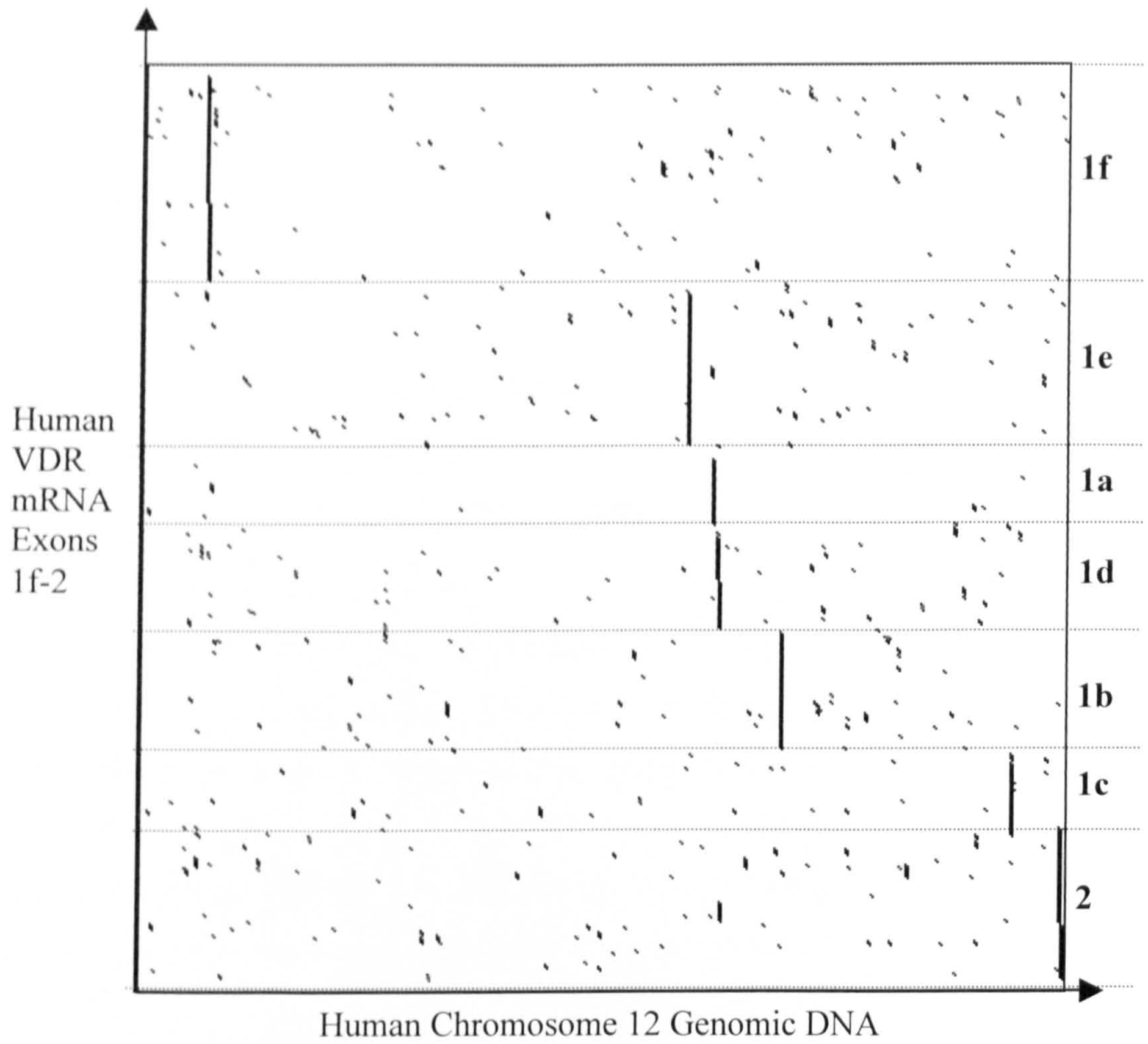
Figure 2.2: Comparisons of the VDR exon sequences in human and mouse using the VISTA software. The data is shown as a percentage identity graph, with regions with identity over 75% shown as coloured blocks.

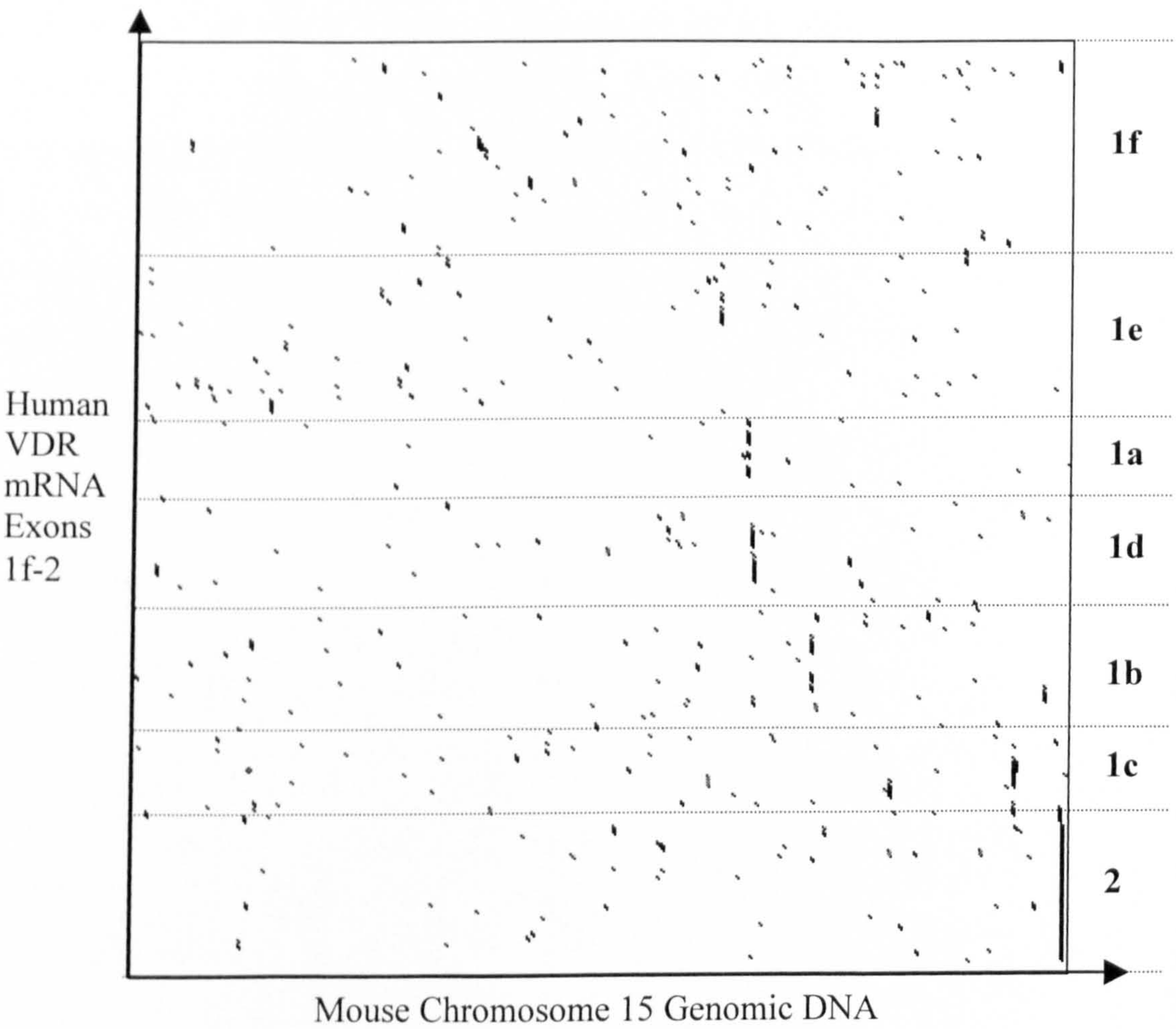
shows much lower homology, with 4 small blocks of conserved sequence and large gaps of little or no homology. Exons 6 to 8 show high levels of homology for their entire length. Exon 9 shows high homology at the 5′ end with less homology and larger regions of heterogeneity in the subsequent sequence.

To graphically demonstrate the position and conservation of the *VDR* exons from mouse and rat to human, dotplot analyses were carried out comparing the human mRNA sequence to the rat and mouse genomic DNA sequences. For ease of presentation, the 5′ exons were analysed separately. For reference, the human mRNA sequence was plotted against the human genomic DNA.

The 5′ dotplots (***figure 2.3***) confirmed that exon 1f was not present in the mouse or the rat. However, there did seem to be a small region of conservation in exon 1e in both the mouse and the rat. Exons 1a and 1d were both relatively well conserved from both the mouse and the rat, although exon 1d showed a longer region of continuous conservation. Exon 1b was poorly conserved in mouse and did not seem to be present at all in rat. Exon 1c was present in both species but slightly better conserved from the rat. Exon 2 was almost completely conserved from both species.

Figure 2.4 shows the dotplots for the coding exons 2-9. Exon 2, 3 and 4 show almost complete conservation from both species. As in the VISTA analysis, exon 5 showed a much higher level of heterogeneity in both mouse and rat. Exons 6-8 again showed very high levels of conservation from both species. Exon 9 showed high levels of conservation at the 5′ end in mouse and rat and a much lower level of conservation in the more 3′ region. The human RNA against DNA dotplot reveals a very common repeat sequence towards the 3′ end of exon 9 which is repeated often throughout the genomic sequence. This repeat is present to a lesser extent in the mouse but is not present in the rat.


Human
VDR
mRNA
Exons
1f-2
1f
1e
1a
1d
1b
1c
2
Human Chromosome 12 Genomic DNA


Human
VDR
mRNA
Exons
1f-2
1f
1e
1a
1d
1b
1c
2
Mouse Chromosome 15 Genomic DNA

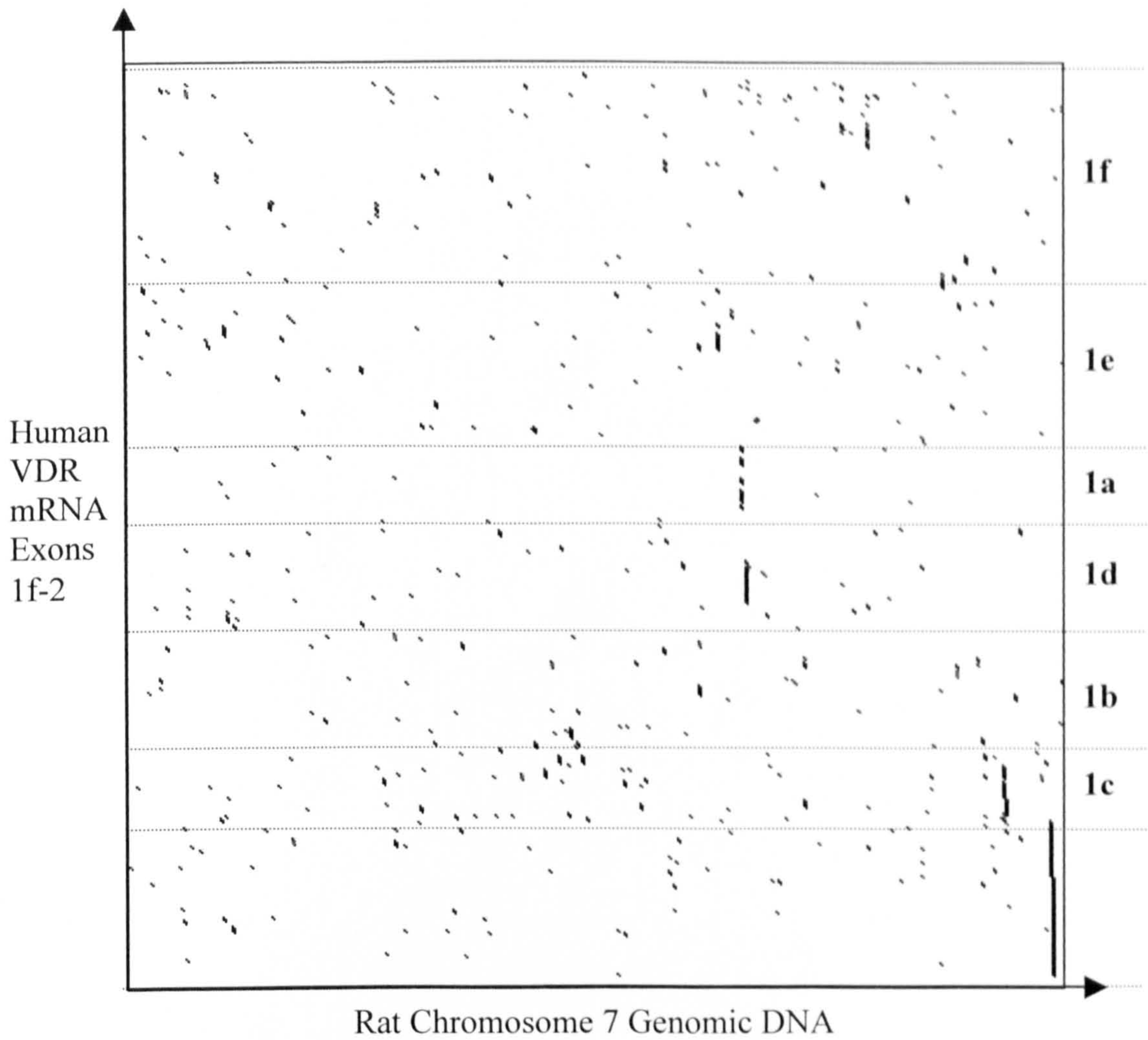
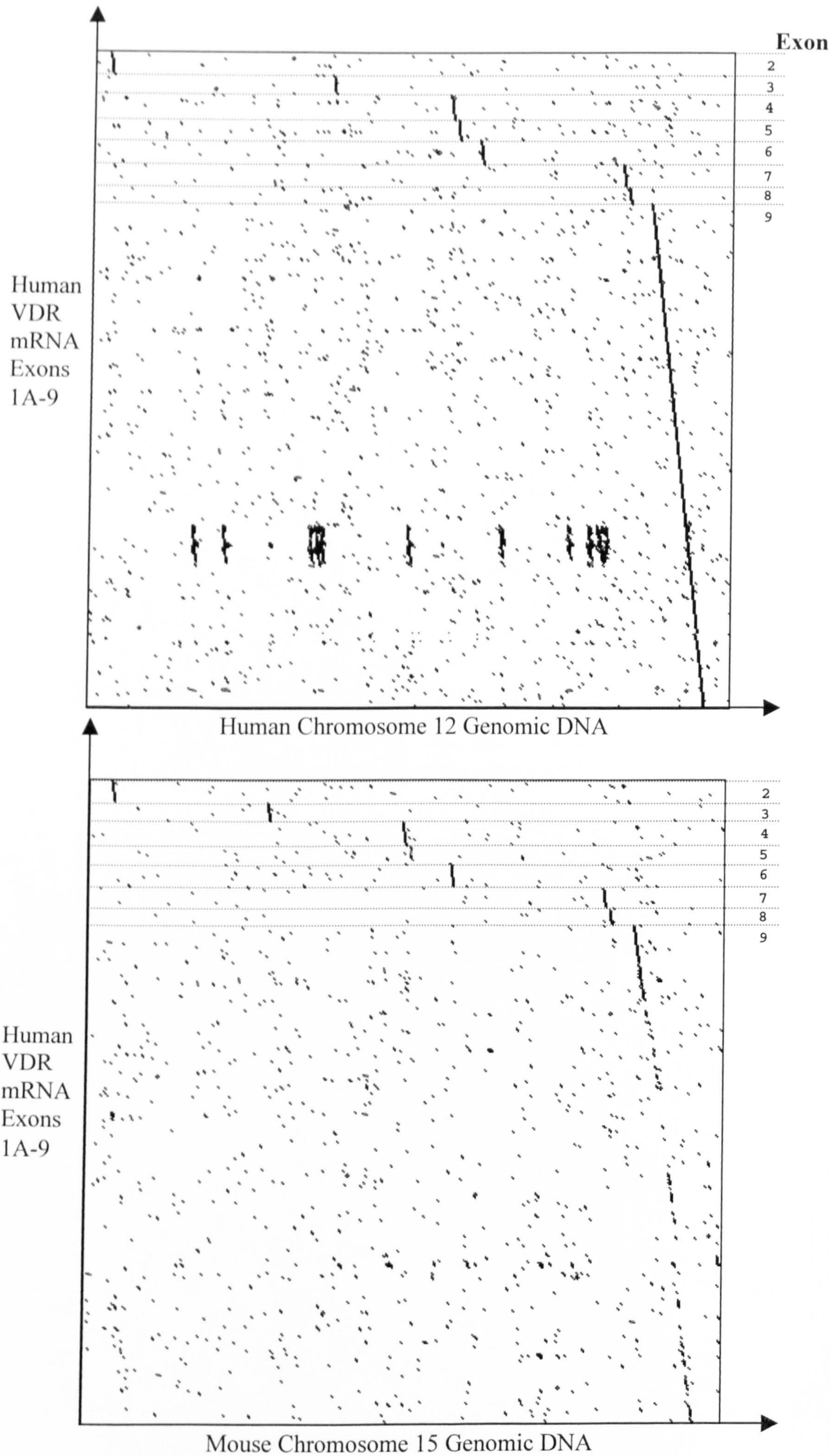


Figure 2.3: Dotplot comparisons of human, mouse and rat genomic DNA (x axes) plotted against human mRNA, exons 1f-2 (y axes). Dotplot analysis was carried out with a window size of 23 and a mismatch limit of 5.


Exon
2
3
4
5
6
7
8
9
Human
VDR
mRNA
Exons
1A-9
Human Chromosome 12 Genomic DNA
2
3
4
5
6
7
8
9
Human
VDR
mRNA
Exons
1A-9
Mouse Chromosome 15 Genomic DNA

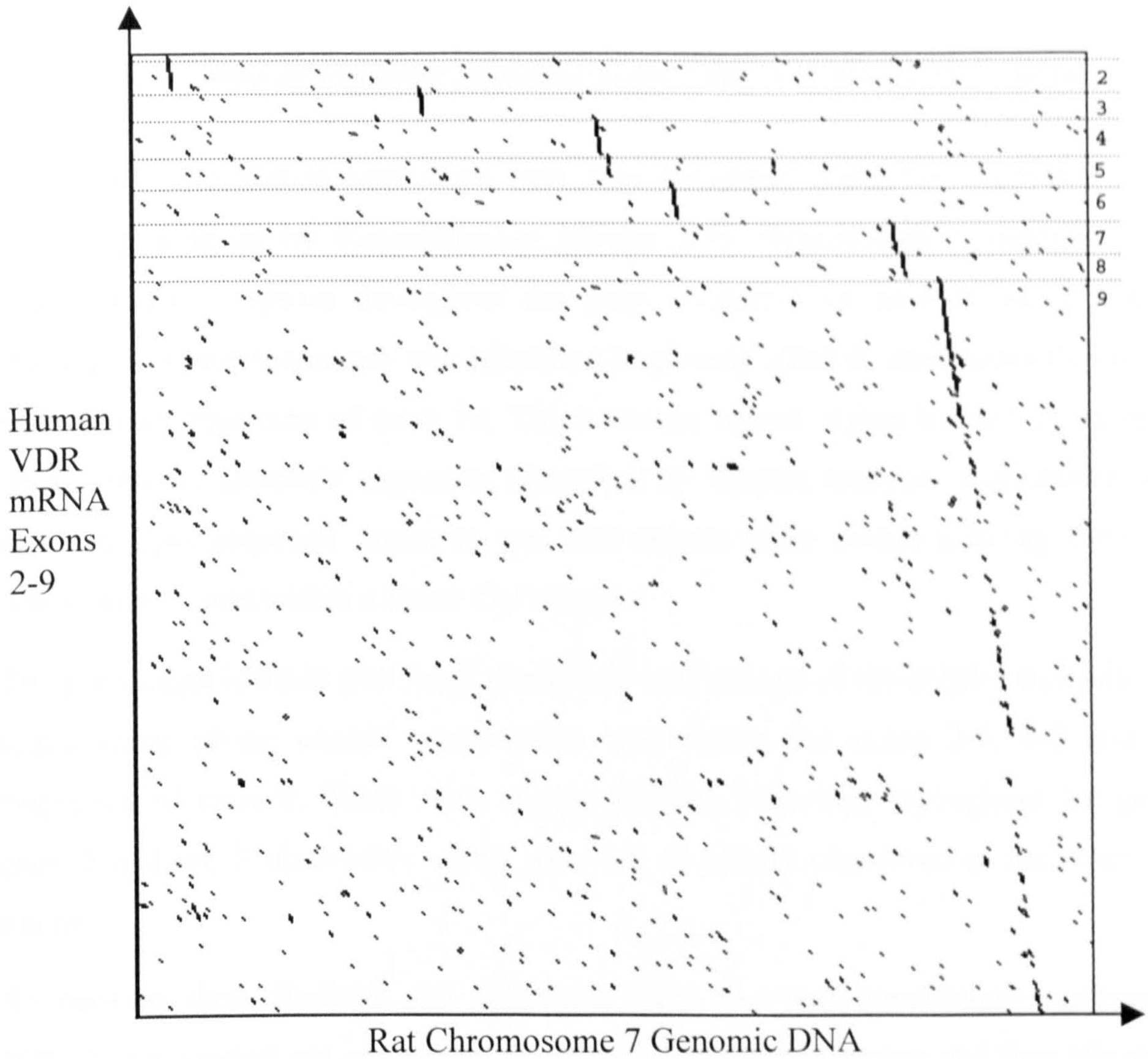


Figure 2.4: Dotplot comparisons of human, mouse and rat genomic DNA (x axes) plotted against human mRNA, exons 2-9 (y axes). Dotplot analysis was carried out with a window size of 25 and a mismatch limit of 7.

2.3.2. Conservation of Genomic Sequence from Frog and Mouse to Human

The mouse and human sequences were also compared using the PipMaker software following analysis by RepeatMasker (*figure 2.5*). RepeatMasker identified various common repeat regions throughout the gene. In particular, an 8kb region consisting entirely of repeat sequences was identified beginning 22kb downstream of exon 1f and ending 6 kb upstream of exon 1e. The common repeat region located in exon 9 and throughout the genomic sequence, identified by dotplot analysis, was shown to be a repeated CpG sequence. Exon 1a was also shown to be within a strong CpG island, while exon 1f was within a lesser CpG region.

The percentage identity plot itself confirmed the findings of the dotplot analysis: a band representing strong exonic conservation was shown for exons 2-4, 6-8 and at the beginning of exon 9. There were regions of high homology throughout the gene but exon 5 and the 5′ alternative exons were not as clearly conserved as the other coding exons.

To confirm these findings and to extend them to a non-mammalian comparison, a VISTA was carried out comparing the human sequence to mouse and frog (*figure 2.6*). VISTA also generates a map of common repeats, and this confirmed the results of the RepeatMasker analysis. The human to mouse comparison confirmed the results of PipMaker, with exons 2-4, 6-8 and the coding region of exon 9 showing very high levels of evolutionary conservation. Again, exon 5 did not appear to exist in the mouse sequence and exons 1a-1f were less well conserved than the coding exons. Again there were regions of high similarity throughout the intronic sequences, notably a cluster of conserved sequence around exons 1a, 1d and 1b and several regions of high homology upstream of exon 1f.

In contrast, the frog genomic sequence showed no significant conservation in the intronic sequences but exons 2-4 and 6-8 and the coding region of exon 9 were well conserved. The 5′ exons 1a-1f were not present in the frog and exon 5 also showed no homology.

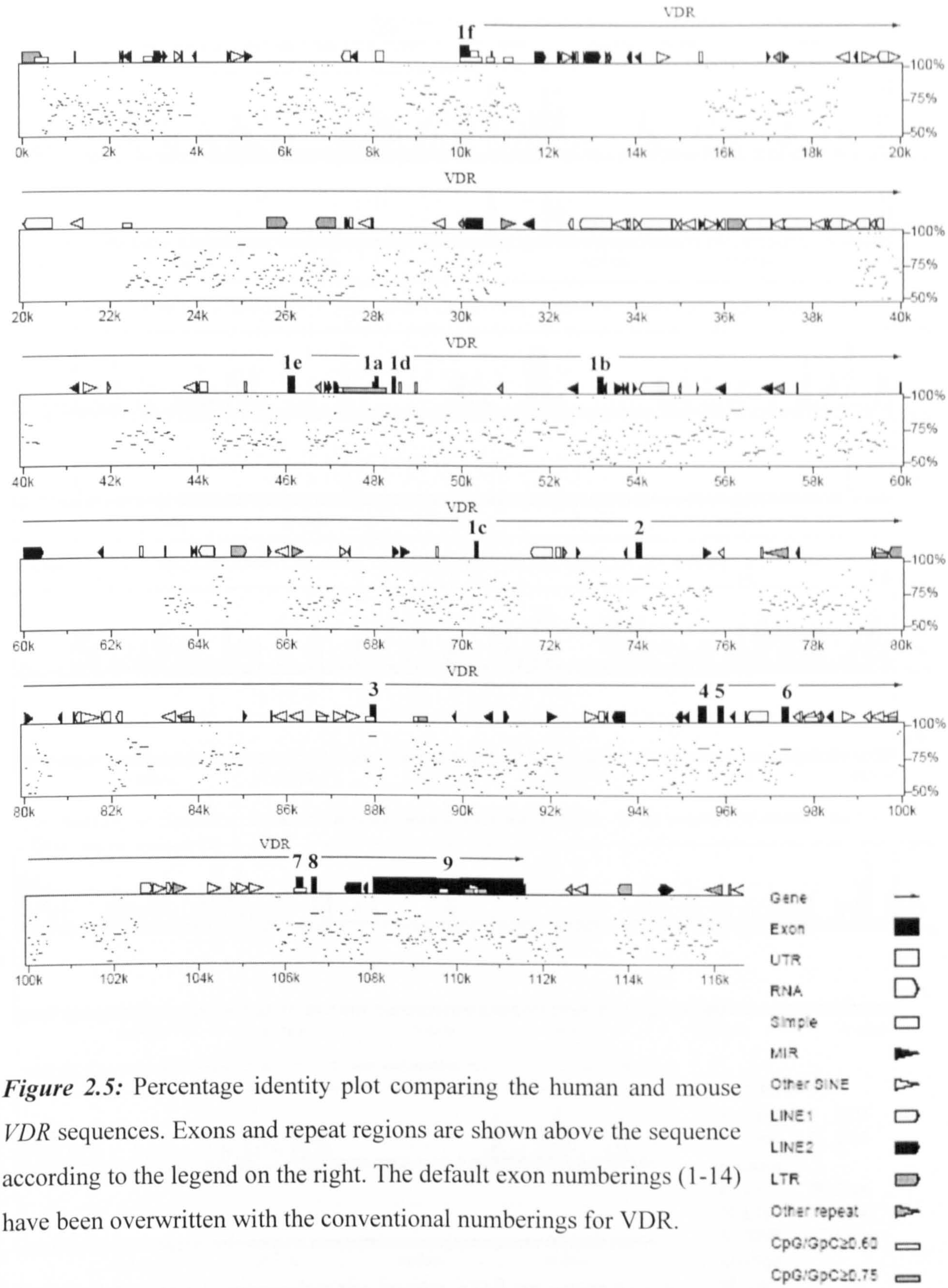


Figure 2.5: Percentage identity plot comparing the human and mouse *VDR* sequences. Exons and repeat regions are shown above the sequence according to the legend on the right. The default exon numberings (1-14) have been overwritten with the conventional numberings for VDR.

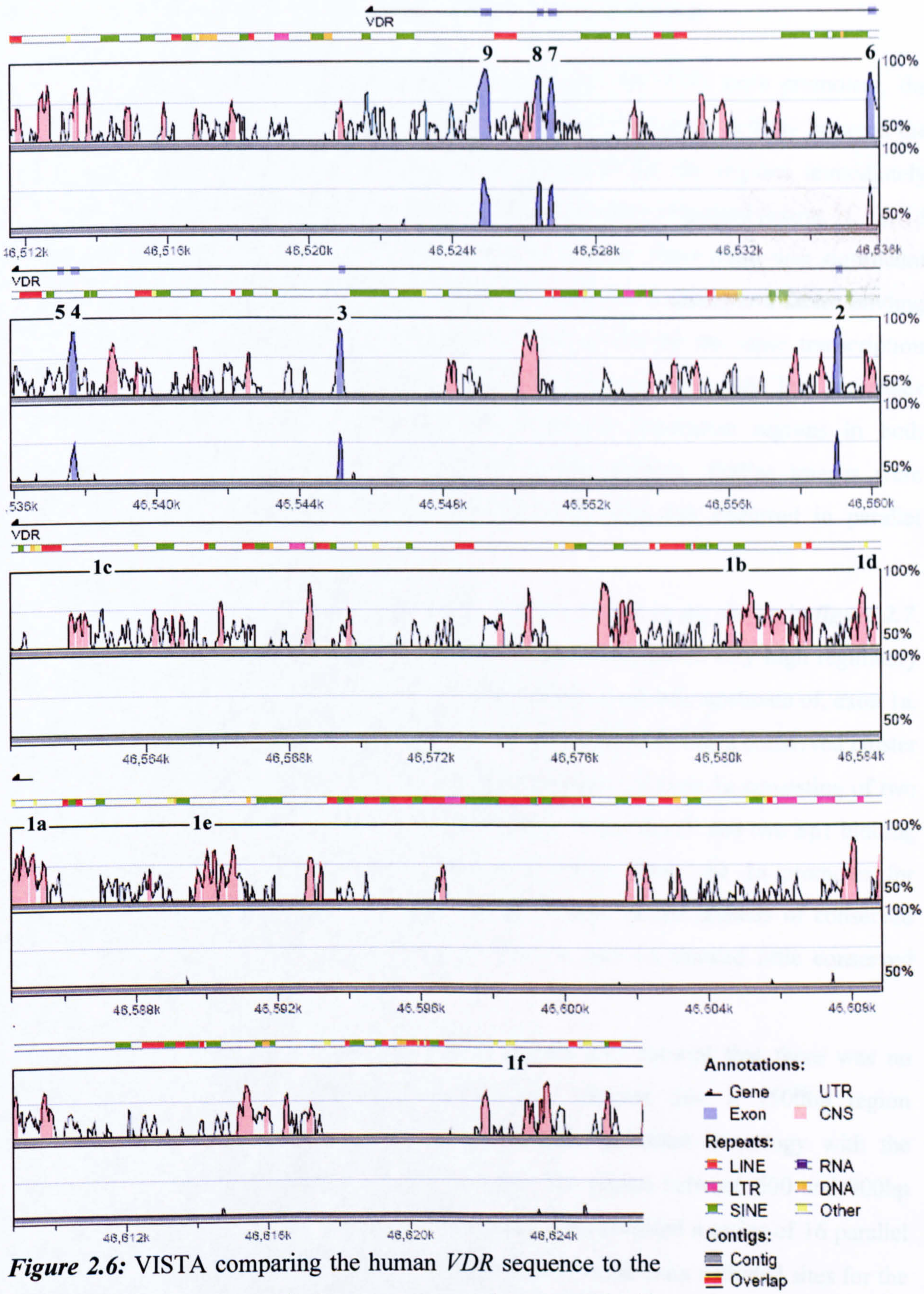


Figure 2.6: VISTA comparing the human *VDR* sequence to the mouse and frog sequence. The human sequence is annotated with the exon numbers and repeat regions. Note that due to the genes position on the reverse strand of chromosome 12, the exons are in reverse order relative to the coding sequence. Below this, percentage identity plots for the mouse and frog sequences are aligned.

2.3.3. Conservation of Promoter Regions from Mouse to Human

In order to identify functionally important regions of the VDR gene promoters, the TraFac software was used to analyse the conservation of promoter activity between the mouse and human VDR. A regulogram was produced for the regions immediately upstream of the four exons from which transcription has been reported (exons 1f, 1a, 1d and 1c). The regulograms were used to identify regions where there was significant sequence homology and a significant number of conserved transcription factor binding sites, that is, where the software predicted a binding site for the same transcription factor in both sequences. These regions were then analysed to produce TraFac images showing transcription factor binding sites present in equivalent regions in both sequences. To further improve the accuracy of the analysis, TraFac images were produced showing only transcription factor binding sites that occurred in parallel positions in the two sequences.

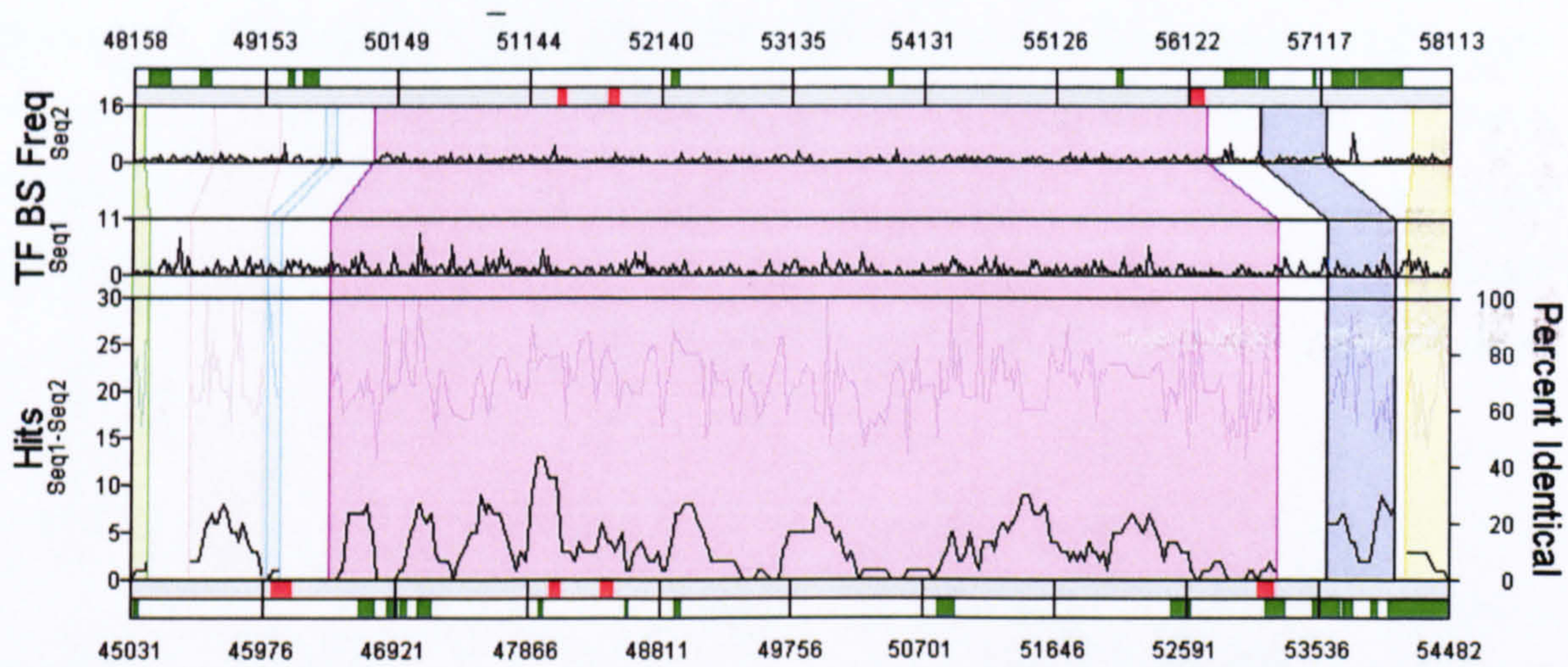
The results of the analysis of the region around exons 1a and 1d are shown in *figure 2.7*. Sequence homology was high throughout the region. A region of very high regulatory conservation (number of "hits") occurred within, and immediately upstream of, exon 1a. The TraFac analysis for this region is shown. Of particular note was a conserved cluster of transcription factor binding sites immediately upstream of exon 1a consisting of two binding sites for the general zinc finger transcription factor MAZF and two Sp1 binding sites. Three further blocks of fairly high conservation cover the 1a promoter for ~1500bp upstream of exon 1a but these did not contain similar clusters of conserved parallel binding sites. The region between exons 1a and 1d showed little conserved regulatory activity.

The regulogram for the 1f promoter region (*figure 2.8*) showed that there was no significant conservation of the exonic sequence. Despite this, a 1500bp region immediately upstream of the human exon showed significant homology with the corresponding region of the mouse chromosome. The region between 600 and 900bp upstream of exon 1f is shown in the TraFac image. This revealed a series of 16 parallel binding sites in three tight clusters. The closest cluster to the exon included sites for the general transcription factors AP1 and AP4 while the second cluster, containing 9 tightly packed transcription factor sites, seemed to contain sites for more tissue-specific transcription factors such as the nuclear factor of activated T-cells (NFAT),

progesterone receptor, Hepatic nuclear factor 4 and pre-B-cell leukaemia transcription factor 1.

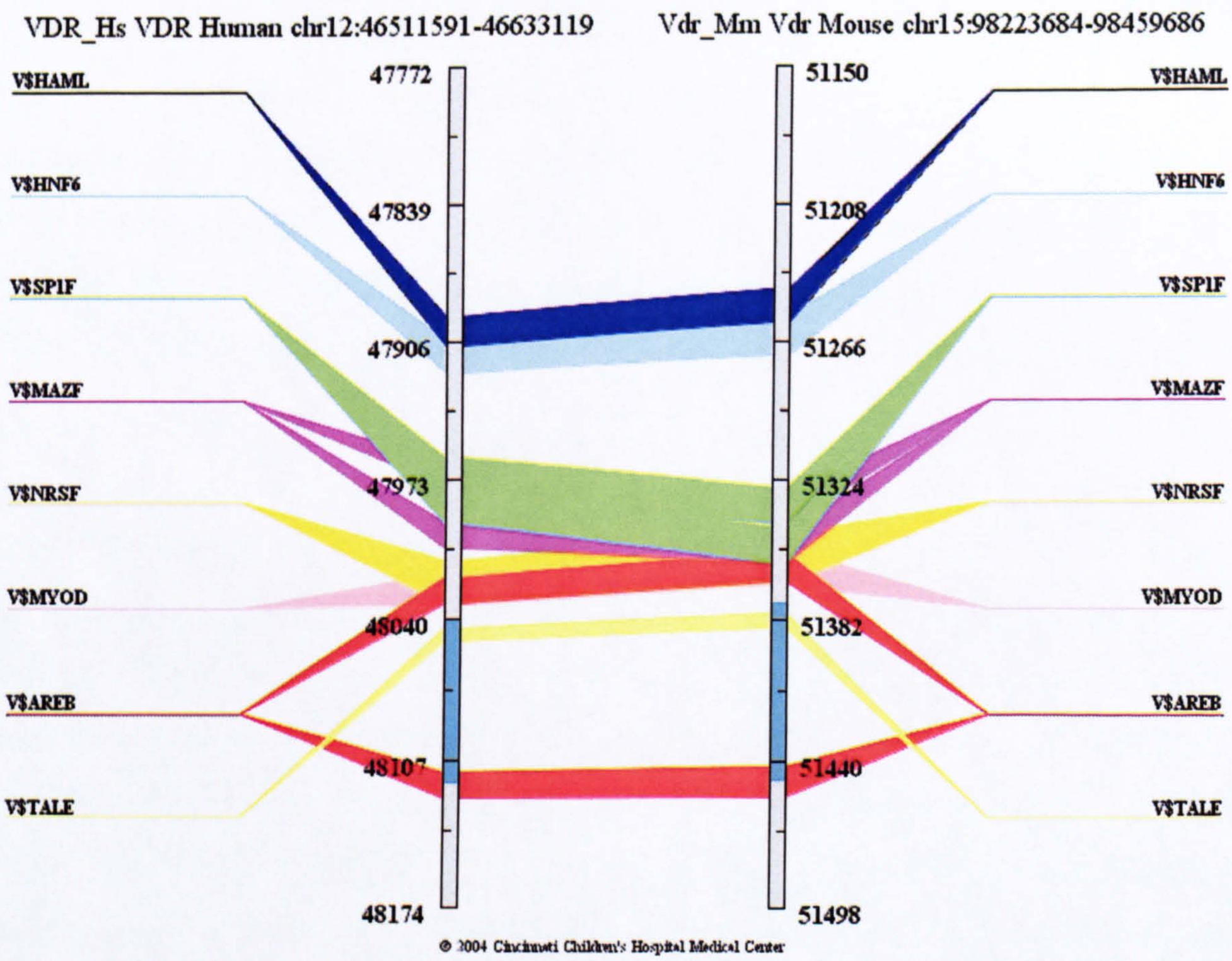
Figure 2.9 shows the regulogram for the region including exons 1c and 2. In this region, there were significant gaps in the sequence homology between the two species, notably a 1200bp break between the two exons. For 2500bp upstream of exon 1c, sequence homology was relatively high. Despite this, TraFac did not reveal any significant conservation of transcription factor binding sites. Further upstream of this region, several more significant breaks in sequence homology occurred.

Regulogram:



Exon: 1e 1a 1d 1b

TraFac Image:



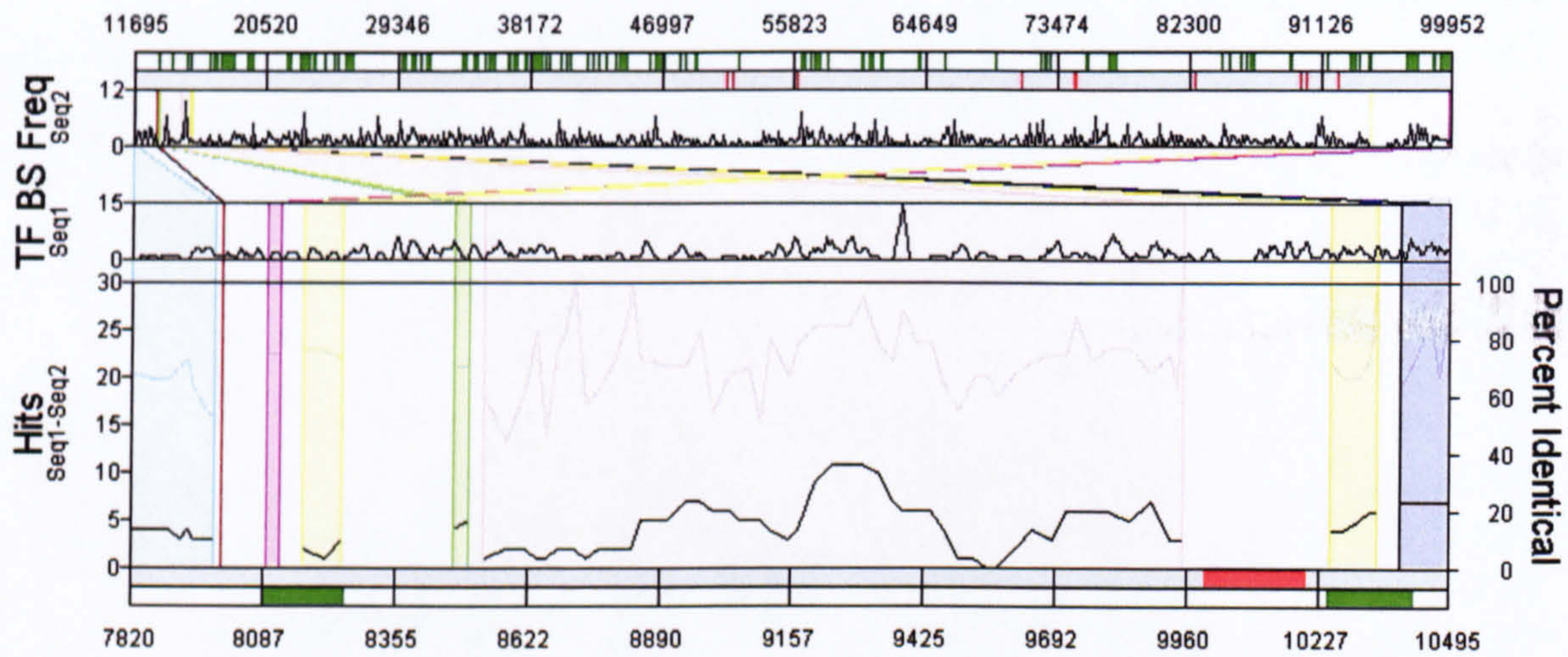
Legend:

Transcription Factor	Description
V$HAML	Runt-related transcription factor 2 / CBFA1 (core-binding factor, runt domain, alpha subunit 1)
V$HNF6	Liver enriched Cut - Homeodomain transcription factor HNF6
V$SP1F	Sp1
V$MAZF	Myc associated zinc finger protein (MAZ)
V$SP1F	Sp1
V$MAZF	MAZR
V$NRSF	neuron-restrictive silencer factor
V$MYOD	myoblast determining factor
V$AREB	AREB6 (Atp1a1 regulatory element binding factor 6)
V$TALE	TALE Homeodomain class recognizing TG motives
V$AREB	AREB6 (Atp1a1 regulatory element binding factor 6)

Figure 2.7: Analysis of the 1a and 1d promoter region by TraFac. The regulogram shows the two aligned sequences, with the mouse sequence along the top and the human sequence below. Exons are shown as red blocks and common repeat regions as green blocks. Between the two sequences are graphs showing (from top to bottom) the number of predicted transcription factor binding sites for each sequence, the percentage identity and the number of conserved transcription factor binding sites (hits) between the two sequences.

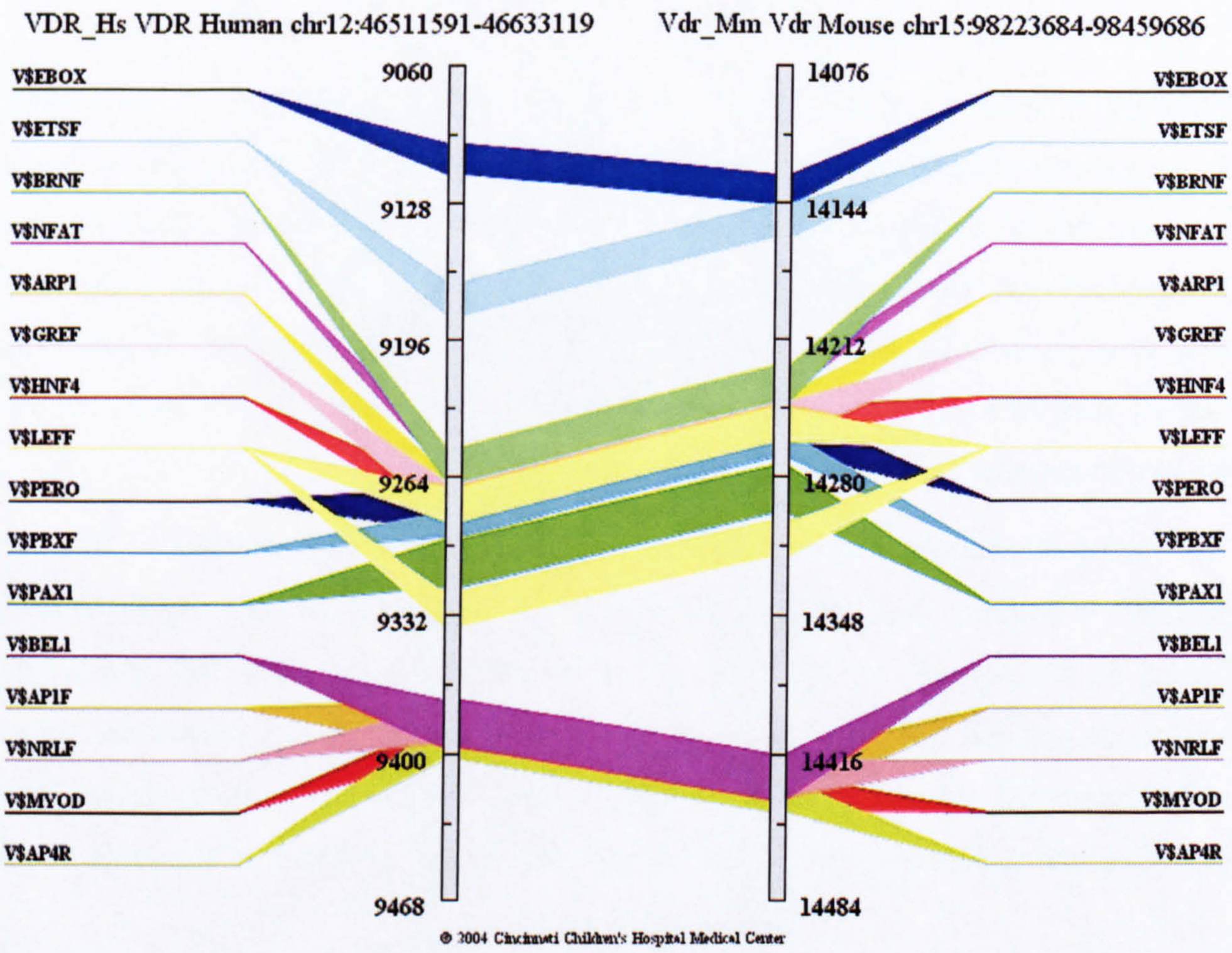
The region immediately upstream of exon 1a, showing a high number of hits, was analysed to produce the TraFac image shown. The two sequences are aligned with the human on the left and the mouse on the right. Exons are shown in dark green. Only conserved, parallel transcription factor binding sites are shown to demonstrate regions under conserved regulatory control in both species. The key to the transcription factor identities and a description of each transcription factor is shown in the table above.

Regulogram:



Exon: 1f

TraFac Image:



Legend:

Transcription Factor	Description
V$EBOX	Member of b-zip family, induced by ER damage/stress
V$ETSF	c-Ets-1(p54)
V$BRNF	POU factor Brn-2
V$NFAT	Nuclear factor of activated T-cells
V$ARP1	apolipoprotein AI regulatory protein 1
V$GREF	Progesterone receptor binding site
V$HNF4	Hepatic nuclear factor 4
V$LEFF	TCF/LEF-1, involved in the Wnt signal transduction pathway
V$PERO	PPAR/RXR heterodimers
V$PBXF	pre-B-cell leukemia transcription factor 1
V$PAX1	Pax-2
V$LEFF	TCF/LEF-1, involved in the Wnt signal transduction pathway
V$BEL1	Bel-1 similar region
V$AP1F	NF-E2 p45
V$NRLF	Neural retinal basic leucine zipper factor (bZIP)
V$AP1F	AP-1
V$MYOD	Myf5 myogenic bHLH protein
V$AP4R	activator protein 4

Figure 2.8: Analysis of the 1f promoter region by TraFac. The regulogram shows the two aligned sequences, with the mouse sequence along the top and the human sequence below. Exons are shown as red blocks and common repeat regions as green blocks. Between the two sequences are graphs showing (from top to bottom) the number of predicted transcription factor binding sites for each sequence, the percentage identity and the number of conserved transcription factor binding sites (hits) between the two sequences.

The region 600bp upstream of exon 1f, showing a high number of hits, was analysed to produce the TraFac image shown. The two sequences are aligned with the human on the left and the mouse on the right. Only conserved, parallel transcription factor binding sites are shown to demonstrate regions under conserved regulatory control in both species. The key to the transcription factor identities and a description of each transcription factor is shown in the table above.

Regulogram:

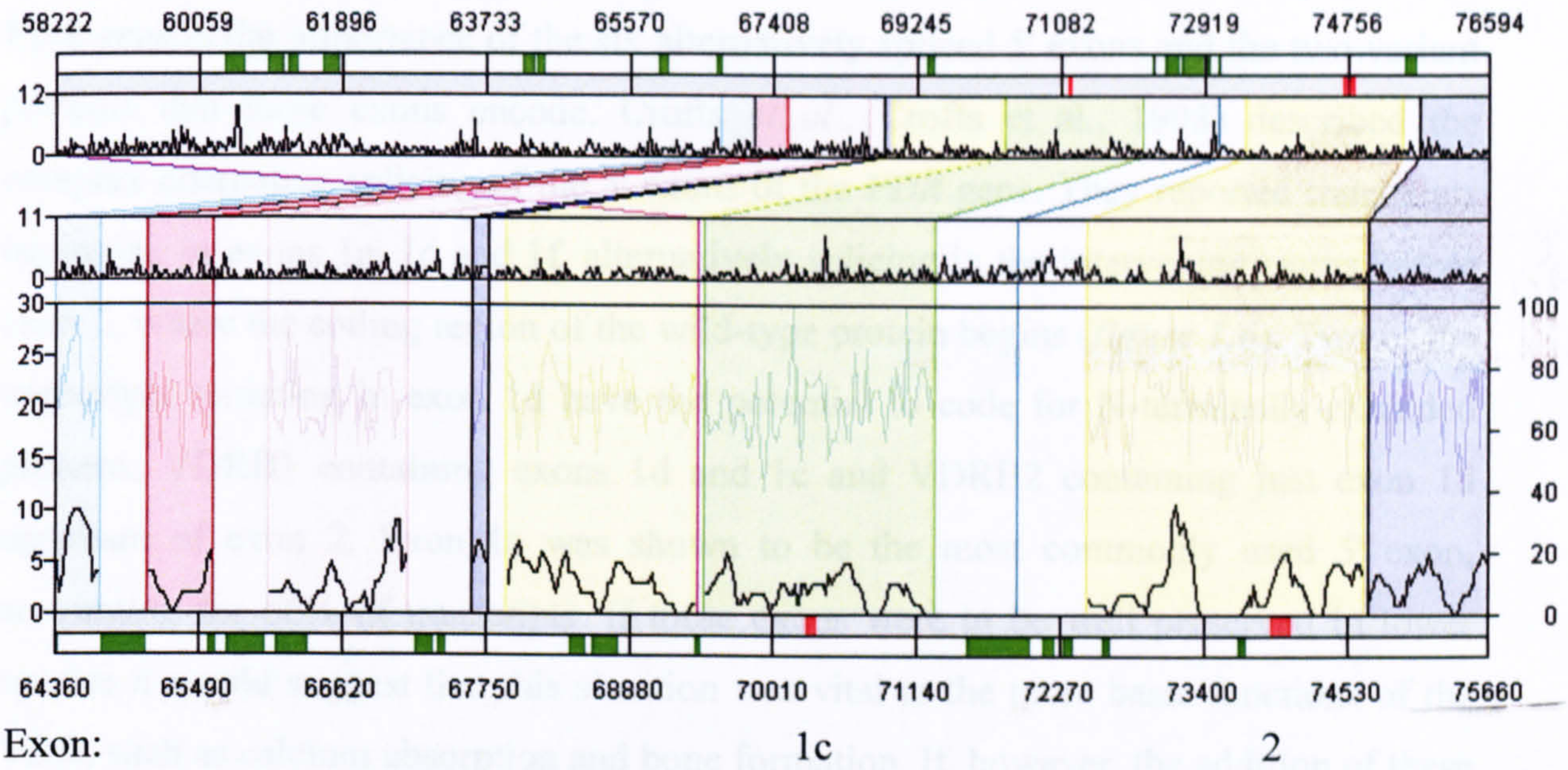


Figure 2.9: Analysis of the 1c promoter region by TraFac. The regulogram shows the two aligned sequences, with the mouse sequence along the top and the human sequence below. Exons are shown as red blocks and common repeat regions as green blocks. Between the two sequences are graphs showing (from top to bottom) the number of predicted transcription factor binding sites for each sequence, the percentage identity and the number of conserved transcription factor binding sites (hits) between the two sequences.

There were no regions of significant transcription factor binding site conservation so a TraFac analysis was not produced.

2.4. Discussion

Perhaps the most significant question to be addressed by evolutionary analysis of the *VDR* gene is the importance of the six alternatively spliced 5' exons and the two variant proteins that these exons encode. Crofts *et al.* (Crofts et al., 1998) described the complex alternative splicing of the 5' exons of the *VDR* gene. They reported transcripts beginning in exons 1a, 1d and 1f, alternatively splicing in the intervening exons before exon 2, where the coding region of the wild-type protein begins (***figure 1.6***). Two of the transcripts initiating in exon 1d have the potential to code for N-terminally-extended proteins, VDRB1 containing exons 1d and 1c and VDRB2 containing just exon 1d upstream of exon 2. Exon 1a was shown to be the most commonly used 5' exon, accounting for 66% of transcripts. If these exons were to be well preserved in lower species it would suggest that this situation was vital to the more basic functions of the VDR, such as calcium absorption and bone formation. If, however, the addition of these exons was shown to be an evolutionarily relatively recent event, it would indicate that they were involved in more complex function only present in higher organisms, such as the adaptive immune system. As well as exonic conservation, the conservation of promoter sequences and, more accurately, of groups of transcription factor binding sites can be used to identify important regulatory regions. In the case of the *VDR*, a strongly conserved promoter region upstream of a 5' exon might suggest that that particular exon is the initial exon in a major transcript.

BLAST comparison of the mouse and human genes revealed that there were no exons homologous to the human exons 1f and 1e in the mouse (***table 2.2, figure 2.1***). Exons 1a, 1d and 1c all showed over 70% conservation; exon 1b was slightly less well conserved at 60%. Despite exon 1a being reported as the initiating exon in the majority of VDR transcripts, exon 1d was slightly better conserved than exon 1a and this was confirmed by the exonic VISTAs (***figure 2.2***). Exon 1a was well conserved at the 5' end but contained two significant regions of lower homology while exon 1d contained a large central block of high conservation. This would bear out the theory that exon 1d, containing an alternative ATG translation start codon, acts as a coding exon while 1a codes for a 5' UTR. Coding regions are better conserved throughout evolution because genetic alterations to these regions are more likely to lead to impaired protein function and therefore are subject to greater negative selection pressure than changes to UTRs, which do not affect protein structure. This would also suggest that the alternative

proteins encoded by exon 1d perform an essential function such that loss of this function would incur this negative selection pressure. The genomic VISTA comparing the mouse and the frog sequence to the human (*figure 2.6*) supported the findings of the BLAST analysis in that exons 1a, 1d and 1c were relatively well conserved from the mouse while exon 1b was less homologous and exons 1e and 1f did not appear in the mouse. The frog, evolutionarily more distant to humans than the mouse, did not contain any of the 5' exons, although all of the coding exons were well conserved.

Dotplot comparison of the 5' exons of the rat and mouse gene to the human (*figure 2.3*) confirmed the results of the BLAST and VISTA analysis. Exon 1f was not present in either the mouse or the rat and only a small part of exon 1e appeared to be conserved. Exon 1b was poorly conserved in the mouse and not present in the rat. Exons 1a and 1d were well conserved in both, supporting the supposition that transcripts initiating in exon 1d are important to the basic function of the VDR while the lack of exon 1f in the mouse suggests that transcripts initiating here are involved in more complex functioning that is unique to humans. The fact that exon 1b, which is not involved in transcripts coding for alternative proteins, was evolutionarily more divergent than 1d and 1c further confirms that expression of the alternative transcripts including 1d and 1c confers a selection advantage leading to conservation of the protein-coding sequences.

Gardiner *et al.* (Gardiner et al., 2004) have carried out similar evolutionary analysis and have confirmed these findings. They reported over 75% identity of exon 1d between human and mouse and rat. They also confirmed that VDRB1 has greater transcriptional activity at ligand concentrations greater than $1x10^{-9}$ molar. An alternative, N-terminally altered, VDR has been reported in chicken (Lu et al., 1997), although this is due to use of an alternative translation start codon rather than alternative splicing. Gardiner *et al.* (Gardiner et al., 2004) reported that the transcript encoding this variant chicken VDR has greater than 50% homology with human exon 1d. This may suggest that exon 1d transcripts and proteins are not solely a mammalian phenomenon.

Promoter analysis revealed that the promoter region upstream of exon 1a contained motifs compatible with exon 1a being the major initiating exon. Immediately upstream of 1a, a cluster of conserved transcription factor binding sites includes a pair of binding sites for the Sp1 transcription factor, a pattern commonly seen in promoter regions. The pattern of two pairs of sequential sites for Sp1 and Myc-associated zinc finger protein (MAZ) is a commonly seen pattern in promoter regions (Song et al., 2001). The myc

pathway is involved in cell-cycle progression and differentiation (Marcu et al., 1992). Similarly, runt-related transcription factor 2 / CBFA is involved in differentiation, particularly in osteoblasts (Ducy et al., 1997), indeed Cbfa1 knockout results in the complete loss of bone formation. Myoblast determining factor (myoD) is essential for the differentiation of the myoblasts of muscle fibres. Homeodomain transcription factor HNF6 in involved in liver differentiation (Wilding and Gannon, 2004).

There was very little conservation of the short intron between exons 1a and 1d and this agrees with the finding of Crofts *et al.* (Crofts et al., 1998) who reported little promoter activity in this region. However, this suggests that exon 1a and exon 1d are under the control of the same promoter region and does not provide a mechanism by which transcripts from the two exons could be differentially expressed.

There is some evidence that the 1f promoter is active in embryological development. The promoter contains binding sites for LEF-1, part of the Wnt signalling pathway, which is expressed during murine embryogenesis (Oosterwegel et al., 1993). A Pax-2 binding site was also conserved. Pax-2 is an important developmental transcription factor in embryogenesis, particularly in the development of the kidney (reviewed in (Dressler, 1995)). The use of different promoters in embryogenesis and mature cell function would echo the situation found in RAR genes (Mendelsohn et al., 1994). However, apart from LEF-1 and Pax-2, the 1f promoter contains binding sites for other transcription factors not specifically involved in embryogenesis.

Although sequence comparison suggested that exon 1f did not exist in the mouse, promoter analysis did not support this view. A strongly conserved region of regulatory control was found 500bp upstream of exon 1f containing binding sites for both general transcription factors, such as the activator proteins AP-1 and AP-4, and for several tissue-specific factors. It could be argued that the conserved region found in the 1f promoter is in fact part of a promoter for a neighbouring gene, particularly given the 36kb intron between exon 1f and 1a. A neighbouring gene, COL2A1 is within approximately 3kb of exon 1f. However, COL2A1 is in the same chromosomal orientation as VDR, such that the 5′ regulatory region of COL2A1is approximately 60kb away from the observed conserved region in the 1f promoter. The presence of a 1f promoter would suggest that transcripts may occur in the mouse from an exon equivalent to the human 1f. However, as this exon forms part of the UTR and not a coding region, the sequence has diverged through evolution such that there is no

significant homology. It is therefore possible that an exon 1e also exists in the mouse that has similarly diverged in sequence, and indeed a small part of exon 1e did appear to be conserved in mouse and rat. That the sequences of these exons are so divergent does imply that 1f transcripts are functionally less important than those initiating in exons 1a and 1d, which are much more homologous in sequence.

Although Byrne *et al.* (Byrne et al., 2000) reported transcripts initiating in 1c, TraFac analysis of the 1c promoter region did not reveal any significant conservation. Although the 1c promoter contained several conserved binding sites, these sites were randomly distributed with no clusters of strongly conserved regulation. Notably, the Sp1 sites and TATA box reported by Byrne *et al.* (Byrne et al., 2000) did not appear to be conserved from the mouse, suggesting either that transcripts beginning in exon 1c are unique to humans or primates, or that exon 1c transcripts are not expressed and the promoter-like sequences are coincidental, the transcripts found by Byrne *et al.* either fragments of longer molecules or artefacts of the immortal cancer cell line (MCF-7) in which they were found. Neither Crofts *et al.* (Crofts et al., 1998) or Miyamoto *et al.* (Miyamoto et al., 1997) found transcripts beginning in 1c.

The analysis performed by the RepeatMasker programme and shown in ***figure 2.5*** supports the probability that exon 1a is the major initiating exon. Exon 1a and its promoter are shown to be within a strong CpG island. These are regions that are methylated as a means of transcriptional control and are commonly found in the promoter region of genes, proximal to the 5' exon. The position of the CpG island suggests that 1a is indeed the major 5' exon of the *VDR* gene. However, RepeatMasker also revealed a CpG island around exon 1f. This island contains a lower proportion of CpG compared to exon 1a but nonetheless, 1a and 1f are the only 5' exons where transcription is likely to be controlled by methylation. This may mean that transcripts from these exons can be turned on or off depending on cell background, or at different stages of development.

BLAST analysis revealed that all of the coding exons, that is exons 2-9, were highly conserved between mouse and human. Exons 2-4, coding for the DNA binding domain, and 6-8, encoding the ligand-binding domain, showed 84-92% conservation, highlighting the major functional domains of the protein; exon 5, which codes for the hinge region, was significantly less homologous (64%). Exon 9 showed only 54% homology over its entire length but this exon consists mainly of the 3' UTR and the

coding region at the 5' end of the exon showed much increased homology. This pattern was confirmed by VISTA, PIP and dotplot analysis and, even in the evolutionarily more distant frog, each of the coding exons apart from exon 5 showed over 75% homology. This evolutionary conservation mirrors the homology of the human nuclear hormone receptor family, where the DNA and ligand binding domains are conserved and the hinge and 5′ regions subject to a greater degree of variation (Aranda and Pascual, 2001).

2.5. Conclusion

In conclusion, *in silico* analysis of the *VDR* gene suggested a basic functional role for the alternative protein or proteins encoded by transcripts beginning in exon 1d. The strong homology of exons 1d and 1c in the mouse and rat was indicative of a coding region.Although no sequence homologous to exons 1f and 1e were found in the mouse or rat, the strong conservation of the promoter region upstream of 1f suggests that these exons may exist but with such an altered sequence as to be unrecognisable. Conservation of transcription factor binding sites upstream of 1a supports its role as the major initiating exon. CpG islands around exons 1a and 1f supported the assertion that transcripts can initiate in both exons and that they may be differentially expressed in different tissues or stages of development. No evidence was found to support the existence of transcripts from exon 1c.

The coding exons 2 to 9 were well conserved with the exception of exon 5, which codes for the hinge region. This echoes the homology of the human nuclear receptor family, and points to the DNA-binding domain and ligand-binding domain as the key functional regions of the protein.

3. Screening for Novel Variation

3.1. Introduction

3.1.1. VDR Polymorphisms

A vast number of studies have been carried out attempting to associate genotypes of the four *VDR* restriction fragment length polymorphisms (RFLPs) – Fokl, Bsml, Apal and Taql – with many varying diseases and phenomena. Many of these studies were successful in finding associations and these were summarised in ***chapter 1***. These studies have enabled the identification of organs, tissues and diseases in which the *VDR* has a functional role. However, to focus on these polymorphisms is essentially to pick, entirely at random, four out of many more polymorphisms that must exist within the *VDR* gene. It has been estimated that there are at least 15 million polymorphisms within the human genome (Botstein and Risch, 2003), that is approximately one every 200 bases. The *VDR* gene spans approximately 96.5 kb so a very approximate estimation suggests that there are around 480 SNPs within the *VDR* gene. Therefore, to concentrate on the four RFLPs may be to overlook many associations. Moreover, of the four RFLPs, only Fokl has a proven functional effect on the protein, the two alleles producing proteins differing in length by three amino acids (Arai et al., 1997). Bsml and Apal are in the final intron and as such can have no effect on protein structure (Faraco et al., 1989; Morrison et al., 1992) while Taql is a synonymous polymorphism in exon 9 (Morrison et al., 1994). These 3' polymorphisms have been shown to be in linkage disequilibrium with a length polymorphism of the polyA tail and it has been suggested that this may affect the stability of the mRNA, determining how many protein molecules can be translated from each mRNA molecule, although this has not been proven. It is therefore likely that there are many more polymorphisms of the *VDR* gene than those that have currently been studied and that these novel polymorphisms may demonstrate more functionality and consequently show stronger disease association than the RFLPs.

The exons of the *VDR* have previously been screened for variation (Brown et al., 2000) and a further two polymorphisms were found, a synonymous C to T SNP in codon 16 and a C to T substitution 8 bases downstream of the 3′ end of exon 2. Of the non-exonic sequences, the promoter regions are most likely to yield functional polymorphisms. This

is for two reasons. Firstly, promoter regions are probably the most functional non-exonic sequences in the gene, in that they control the levels of the gene product, through transcriptional regulation and can effectively turn a gene on or off. Secondly, the nature of transcriptional regulation means that single base changes can considerably alter promoter activity. Transcription factors generally bind to small sequences of DNA within the promoter region. For example, the VDR, as discussed in ***chapter 1***, binds to a six base half site. A polymorphic base would therefore constitute one sixth of a VDR binding site and the substitution of one base for another would be likely to significantly affect transcription factor binding. Given that transcriptional control tends to cascade from the promoter-bound transcription factor through many co-factors to the transcriptional machinery, an SNP within a transcription factor binding site would have the potential to significantly affect the transcription, and therefore expression, of the target gene and through this mechanism produce observable biological effects.

In an attempt to find novel functional polymorphisms of the *VDR*, the major promoter region, that upstream of exons 1a and 1d, was screened for variation.

3.1.2. SSCP

To screen for variation, single-stranded conformational polymorphism (SSCP) analysis was used (Beier, 1993). SSCP is based on the principle that the sequence of single-stranded DNA determines its secondary structure and therefore different alleles of an SNP will show different secondary structure. SSCP uses a polyacrylamide-based gel to create a matrix that separates DNA on the basis of secondary structure, or conformation, as well as size. Samples are denatured to give single stranded DNA and separated by gel electrophoresis to give a pattern of migration representing the different conformers present in the sample (***figure 3.1***). In this case, the region of interest was amplified from 36 control DNA samples and analysed by SSCP. Variation in the migration pattern between individuals would suggest variation in the DNA sequence. It is estimated that by screening 36 control individuals, over 99% of polymorphisms occurring at the level of 5% or more in the normal population would be detected. In order to validate the technique, exon 2, containing the Fok1 polymorphism was also screened. As well as the common Fok1 polymorphism, exon two has also been reported to contain a synonymous C to T polymorphism at the third base of codon 16 (Brown et al., 2000).

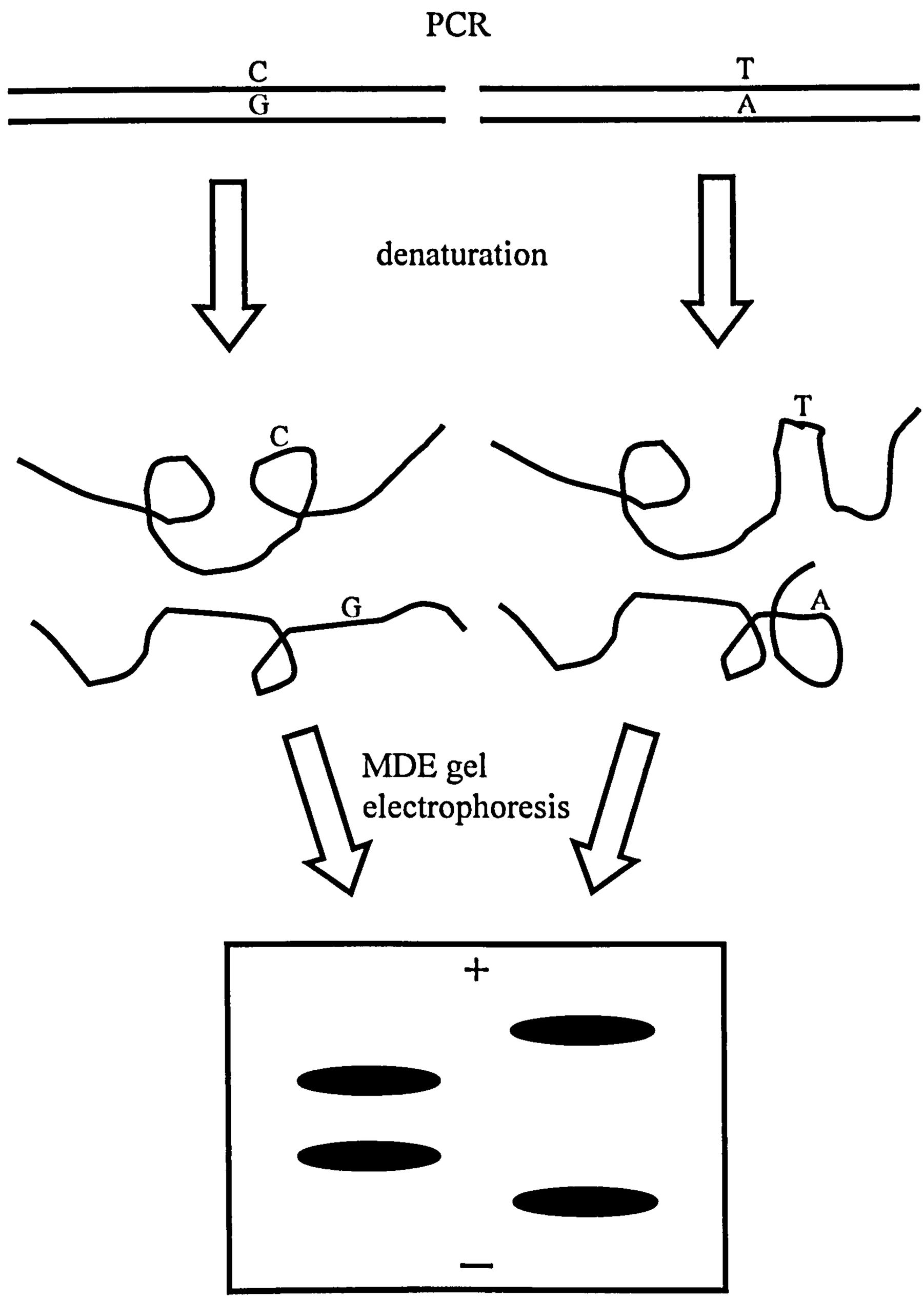


Figure 3.1: A schematic representation of single-stranded conformation polymorphism analysis. The area around the polymorphic loci is amplified by PCR. The DNA is then heated to denature it to single strands and cooled to allow it to form secondary structure. Variation at a polymorphic base will cause the variant sequences to adopt different

conformations. On subsequent MDE polyacrylamide gel electrophoresis, the bands separate according to secondary structure, such that the different alleles show different migration patterns.

3.1.3. Prediction of Transcription Factor Binding

In order to predict the probable affect of promoter polymorphisms, the sequence at each locus was analysed using the Transcription Element Search System (TESS) (Schug and Overton, 1997). This software uses matrices for each transcription factor to predict the likelihood of transcription factor binding given a particular sequence. Each allele of a polymorphism was submitted to TESS and any change in predicted transcription factor binding would suggest a potential mechanism by which that polymorphism can cause a functional affect.

3.2. Materials and Methods

3.2.1. Materials

Ammonium Sulfate	Sigma
Ammonium Persulfate	Sigma
BigDye Mix	PNACL, University of Leicester
Boric Acid	Fluka
Bromophenol blue	Sigma
Centri-sep spin columns	Princeton Separations
dNTPs	Invitrogen
EDTA	Sigma
Formamide	Sigma
Glisseal	Borer Chemie
Glycerol	Sigma
100 bp Ladder	Gibco
MDE 2x gel solution	BioWhittaker
Magnesium Chloride	Sigma
β-mercaptoethanol	BDH
N,N,N′,N′-tetramethylethylenediamine (TEMED)	Sigma
QIAquick PCR Purification Kit	Qiagen
Silver nitrate	Sigma
Sodium hydroxide	Sigma
Taq DNA polymerase	Promega
Tris	Roche
Xylene cyanol	Sigma

3.2.2. Buffers

AJ PCR Buffer (Working Concentration)

45mM Tris-HCl (pH 8.8), 11mM $(NH_4)_2SO_4$, 4.5mM $MgCl_2$, 110μg/ml bovine serum albumin, 16.7mM β-mercaptoethanol, 4.4 mM EDTA (pH 8.0), 200 mM dNTPs in water

Ethidium Bromide Solution

10 mg/ml Ethidium Bromide in water

Silver Staining

Fixer	10% IMS, 0.5% glacial acetic acid
Stain	0.1% silver nitrate
Developer	1.5% NaOH, 0.16% formaldehyde
Neutraliser	0.75% Na_2CO_3, 4% glycerol

10 x TBE Buffer

108g/L tris, 55G/L boric acid, 5mM EDTA, pH8.0 in water

SSCP Denaturing Loading Buffer

95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol, 10mMsodium hydroxide

3.2.3. DNA Samples

SSCP screening was carried out using 36 anonymised samples of genomic DNA extracted from blood. These samples comprised white Caucasian individuals who required a blood test at the Leicester Royal Infirmary. Each individual gave written informed consent.

3.2.4. Thermal Cyclers

PCR was carried out using a GeneAmp PCR System 9700, a Perkin-Elmer DNA Thermal Cycler 480 and a Progene FPROG050.

3.2.5. OligoNucleotides

The oligonucleotide primers used to amplify the 1a promoter region for SSCP are shown in ***table 3.1***.

Primer	Sequence	Amplicon Position (bp)
1A/Dp1 F	CTGATGACGGCATGTGCT	-2119 to -1755
1A/Dp1 R	CAGCCTTTGTTGGAGGAGAG	
1A/Dp2 F	CAGTGGGATGTGCAGAGAGA	-1979 to -1529
1A/Dp2 R	GCTAGCGGTGATTCTTGTGG	
1A/Dp3 F	AGATGTCAGGCCAGTCAAGC	-1676 to -1329
1A/Dp3 R	GGTATCATGGCAACTTTCTGG	
1A/Dp4 F	ATGGTCCATTCCCAAGTTCA	-1411 to -1051
1A/Dp4 R	CAGAGGGACAAGGTGAAAGG	
1A/Dp5 F	AGCAGATTTGCTGGGCTCTA	-1163 to -818
1A/Dp5 R	TGCTTCCCTTGACTGTGTGA	
1A/Dp6 F	TCCCACTGCACAGTGAGTTC	-914 to -554
1A/Dp6 R	AAGTGGAAACCGGAGTTGC	
1A/Dp7 F	GATATCGGGTGGGAGCAAT	-594 to -232
1A/Dp7 R	TGGGACAGAGTTGTCGATGA	
1A/Dp8 F	ACAGGCTGAAGCGGGTATC	-281 to +126
1A/Dp8 R	CCGAGTCCCTATCCTGAGAC	
1A/Dp9 F	GCAAGAGAGGACTGGACCTG	-94 to +334
1A/Dp9 R	GCGGAGCATTTCTCCTAAGC	
1A/Dp10 F	TCTCAGCGGTAAACTTGGCTA	+186 to +535
1A/Dp10 R	AGACCCAACTCCACCATCAC	
Exon 2 F	CTGGCACTGACTCTGGCTCT	-48 to +199*
Exon 2 R	TGCTTCTTCTCCCTCCCTTT	

Table 3.1: Oligonucleotide primers used to amplify the 1a promoter region for SSCP. Amplicon position is shown relative to the beginning of exon 1a except *relative to the beginning of exon 2.

3.2.6. PCR Amplification of the Region of Interest

Exon 2 was amplified using primer pair "exon 2." The 1a promoter region was amplified using overlapping primer pairs "1A/Dp1-1A/Dp10." PCR was carried out in 50µl reactions containing 200nM of each primer, 1 unit Taq polymerase and 1µl target DNA in AJ PCR buffer. PCR was carried out for 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds.

3.2.7. SSCP Gel Preparation

20x20cms gel plates were washed with water and 75% alcohol 5 times to remove grease. 1mm spacers were placed between the plates and glisseal used to create a seal. The plates were sealed in a gel-pouring rack and the gel solution prepared. MDE 2x gel solution was used at 0.6-0.8x final concentrations in 0.6x TBE buffer depending on the amplicon size. 10% ammonium persulfate solution was added to 5µl/ml and N,N,N',N'-tetramethylethylenediamine (TEMED) was added to a final concentration of 0.5µl/ml. The gel was poured between the plates using a syringe and 1mm combs added. The gel was then allowed to set at room temperature for 2 hours.

3.2.8. Sample Preparation

3µl PCR product was added to 9µl SSCP denaturing loading buffer and heated to 95°C for 3 minutes, chilled on ice and loaded onto the gel. The gels were run in 0.6% TBE buffer at 4°C. The gels were run at 500V for 30 minutes and then at 270-350V overnight depending on amplicon size.

3.2.9. Silver Staining of SSCP Gels

The gel was removed from the gel plates and fixed by two 3 minute washes in fixer. The gel was incubated in silver nitrate stain for 15 minutes and then rinsed twice in water. The gel was incubated in the dark in developer for up to 20 minutes. The gel was then placed in neutralising solution for 1 hour and photographed.

Bands that showed altered migration were picked from the gel with a 25G needle and re-amplified by PCR before sequencing.

3.2.10. Sequencing

PCR reactions were purified using the QIAquick PCR purification kit according to the manufacturer's instructions. 20μl BigDye sequencing reactions were set up containing 2-8.5μl PCR product, 8μl BigDye Mix, and 3.5pM of the appropriate primer. Samples were then amplified by PCR for 35 cycles with 96°C denaturation, 10 seconds, 50°C annealing, 5 seconds and 60°C extension, 4 minutes. Unincorporated DyeDeoxy terminators were removed using centri-sep spin columns and the sequence analysed by the Protein and Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester.

3.2.11. TESS analysis

The sequence including each allele of each polymorphism of interest was submitted to the Transcription Element Search System (TESS)[14] (Schug and Overton, 1997). The search was limited to strings and matrices representing human transcription factors.

[14] http://www.cbil.upenn.edu/tess/

3.3. Results

3.3.1. SSCP screening of Exon 2

In order to validate the SSCP method, exon 2, containing the Fok1 RFLP, was screened in the panel of 36 DNA samples. The results are shown in ***figure 3.2.*** As expected, varying patterns of migration were observed between samples, due to the Fok1 polymorphism. Upon sequencing, sample 2 was shown to be an example of an FF wildtype homozygote (C allele, coding for a 423 amino acid protein), sample 6 an ff homozygote (T allele, coding for a 427 amino acid protein) and sample 7 a heterozygote.

Samples 3, 5, 30 and 34 showed further variation, most easily noted by altered migration of the bottommost band. On sequencing, this was shown to be a variation on the F allele with a C to T polymorphism 8 bases downstream of the 3' exon boundary. This was designated C156T, taking the position from the beginning of exon 2. Samples 3, 5 and 30 were homozygous F for the Fok1 polymorphism and heterozygous for the C156T polymorphism while sample 34 was also homozygous F at the Fok1 polymorphism but was genotype TT at the C156T polymorphism.

A further polymorphism was shown in case 24. This was again a variation on the F allele showing a C to T polymorphism in the third base of codon 16, designated C59TT, which has no affect on protein sequence.

3.3.2. SSCP Screening of the 1A Promoter Region

The promoter region upstream of exons 1a and 1d was divided into 10 amplicons. Parts 2 and 7 failed to amplify. Parts 1, 4, 6, 8, 9, and 10 were screened and showed no variation. Part 3 showed signs of variation but bands showed poor separation and were unable to be sequenced. These results can be seen in ***appendix IV.***

Part 5 showed three different patterns of migration ***(figure 3.3)***, suggesting the presence of a single polymorphism. Upon sequencing this proved to be an A to G polymorphism 1012bp upstream of the exon 1a transcription start site. This polymorphism was designated A-1012G. Sample 2 is an example of a GG homozygote, 3 an AA homozygote and 4 a heterozygote.

Figure 3.2: SSCP analysis of exon two, revealing three polymorphisms: Fok1, C59T and C156T. The bands that were picked for reamplification and sequencing and the resulting sequencing traces at each locus are also shown. The polymorphic base is marked on the sequencing traces with an arrow.

	Fok1	C59T	C156T
Trace 1 is wildtype at all three loci:	F	C	C
Trace 2 is variant f at the Fok1 locus:	f	C	C
Trace 3 is variant T at the C156T locus:	F	C	T
Trace 4 is variant T at the C59T locus:	F	T	C

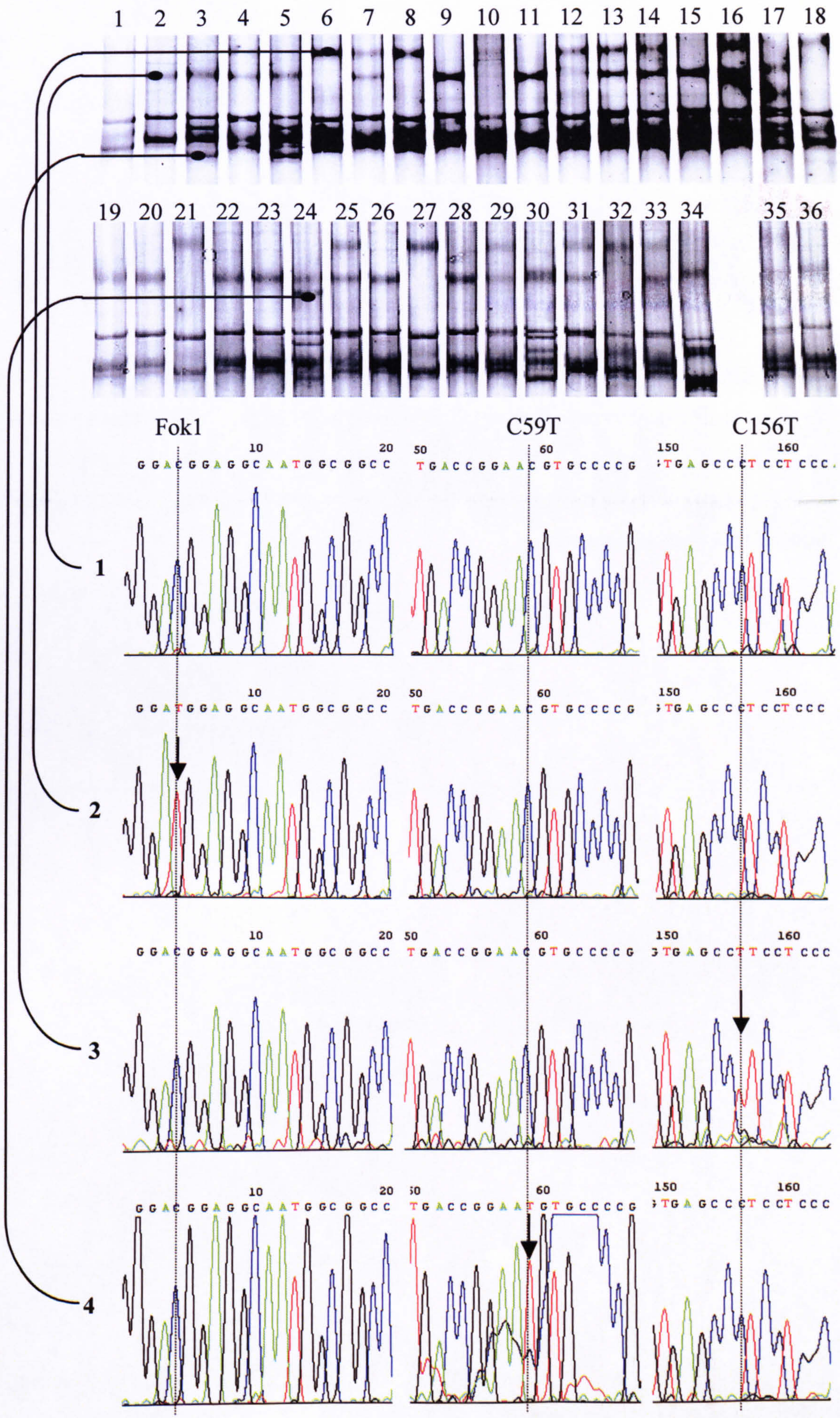
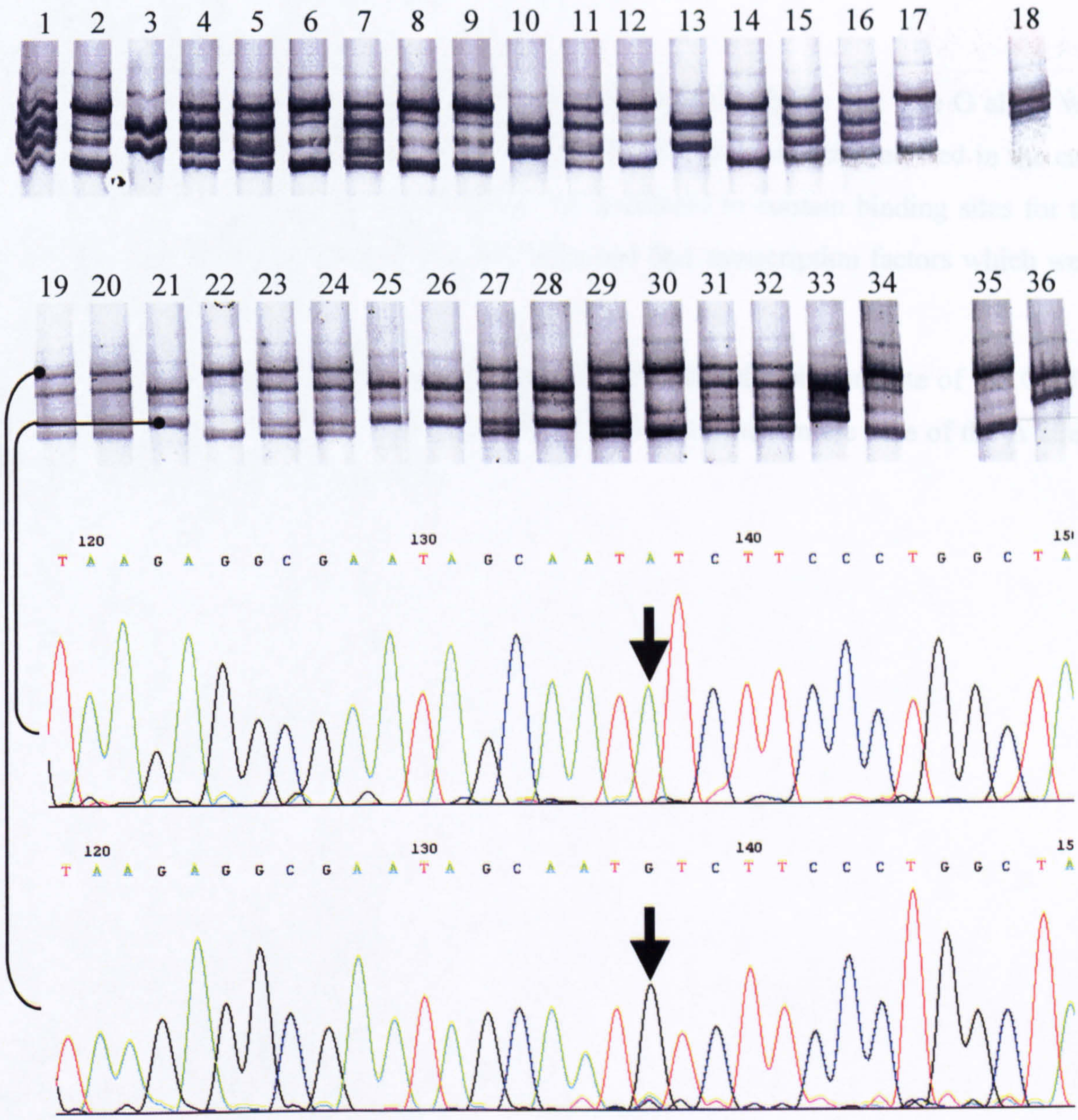

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
Fok1
C59T
C156T
1
2
3
4

Figure 3.3: SSCP analysis of the 1A/Dp5 promoter region, showing the A-1012G polymorphism. Sample two shows a GG homozygote, 3 an AA homozygote and 4 a heterozygote. The bands that were picked for reamplification and sequencing and the resulting sequencing traces are also shown. The polymorphic base is marked on the sequencing traces with an arrow.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
120 130 140
T A A G A G G C G A A T A G C A A T A T C T T C C C T G G C T A
120 130 140
T A A G A G G C G A A T A G C A A T G T C T T C C C T G G C T A

3.3.3. TESS Prediction of Transcription Factor Binding

The sequence of each allele of the A-1012G polymorphism and another recently reported common 1a promoter polymorphism (NCBI SNP database rs7139166), designated G-1520C, were analysed against the TESS database of transcription factor binding sites.

The results for the G-1520C polymorphism are shown in ***figure 3.4***. The G allele was predicted to contain a binding site for GATA-2. This site was not predicted in the case of the C allele. Conversely, the C allele was predicted to contain binding sites for the CCAAT / enhancer binding protein (C/EBP) and Sp1 transcription factors which were not found in the G allele.

The A-1012G polymorphism was predicted to be within the binding site of the GATA binding proteins GATA-1, GATA-2 and GATA-3 and Lmo2 in the case of the A allele but not of the G allele (***figure 3.5***).

Figure 3.4: TESS prediction of transcription factor binding at the G-1520C locus, with the G allele on the left and the C allele on the right. Factor denotes the name of the transcription factor that is predicted to bind, Model denotes the string or matrix used to predict the binding of this factor, "Beg" defines on which number of the sequence the predicted binding site begins (G-1520C is at base 40 of the submitted sequence), Sns denotes which strand the predicted site is on, Normal (N) or reverse (R), Length denotes the length of the predicted site and Sequence shows the sequence in the region, a black base representing a perfect match, a blue base a reasonable match and a red base a mismatch with the defining matrix. Note the predicted C/EBP site present in the C but not the G allele and the GATA-2 site in the G allele that is replaced by an Sp1 site in the C allele.

G

Factor	Model	Beg	Sns	Len	Sequence
T00113 c-Ets-2	R04343 ()	8	R	6	CTTCCC
T02256 AML1a	R05028 ()	11	R	6	CCCACA
T00996 SRY T00997	M00148 (mid_c)	19	N	7	aatcacc
T00529 MZF-1	M00083 (mid_c)	21	R	8	tcaccgct
T02256 AML1a	M00271 (mid_c)	23	R	6	accgct
T00539 NF-1	R00802 ()	31	N	6	CTTTCC
T00308 GATA-2	M00076 (mid_c)	37	N	10	cacgatgctt
T00459 C/EBPbeta T00581 T00017	M00109 (mid_c)	43	N	14	gctttgggcaaggg
T00104 C/EBP	M00116 (mid_c)	43	R	14	gctttgggcaaggg

C

Factor	Model	Beg	Sns	Len	Sequence
T00113 c-Ets-2	R04343 ()	8	R	6	CTTCCC
T02256 AML1a	R05028 ()	11	R	6	CCCACA
T00996 SRY T00997	M00148 (mid_c)	19	N	7	aatcacc
T00529 MZF-1	M00083 (mid_c)	21	R	8	tcaccgct
T02256 AML1a	M00271 (mid_c)	23	R	6	accgct
T00108 C/EBP T00104 C/EBPalpha T00105 T00107 T01388	M00159 (mid_c)	29	R	13	agctttcccacca
T00539 NF-1	R00802 ()	31	N	6	CTTTCC
T00759 Sp1	R08207 ()	35	R	6	CCCACC
T00459 C/EBPbeta T00581 T00017	M00109 (mid_c)	43	N	14	gctttgggcaaggg
T00104 C/EBP T00108 C/EBPalpha	M00116 (mid_c)	43	R	14	gctttgggcaaggg

Figure 3.5: TESS prediction of transcription factor binding at the A-1012G locus, with the G allele on the left and the C allele on the right. Factor denotes the name of the transcription factor that is predicted to bind, Model denotes the string or matrix used to predict the binding of this factor, "Beg" defines on which number of the sequence the predicted binding site begins (A-1012G is at base 52 of the submitted sequence), Sns denotes which strand the predicted site is on, Normal (N) or reverse (R), Length denotes the length of the predicted site and Sequence shows the sequence in the region, a black base representing a perfect match, a blue base a reasonable match and a red base a mismatch with the defining matrix. Note the binding sites predicted in the A allele for the GATA proteins, GATA-1, 2 and 3, and LMO2. These sites are not present in the G allele.

A

Factor	Model	Beg	Sns	Len	Sequence
_00000 DEF	I00073 (DEF)	43	R	8	AATAGCAA
T00304 GATA-1 T00305 GATA-1A T00267 T00306	M00128 (mid_c)	46	R	13	AGCAATATCTTCC
T00311 GATA-3	M00077 (mid_c)	48	R	9	CAATATCTT
T02251 Lmo2 T02250	M00278 (mid_c)	48	R	9	CAATATCTT
T00308 GATA-2	M00076 (mid_c)	48	R	10	CAATATCTTC
T00311 GATA-3	R08270 ()	50	N	6	ATATCT
T00113 c-Ets-2	R04343 ()	54	R	6	CTTCCC
_00000 LBP-1	I00191 (LBP-1)	58	N	5	CCTGG
_00000 NF-IL6	I00088 (NF-IL6)	59	R	9	CTGGCTAAA
_00000 C/EBP	I00034 (C/EBP)	59	N	10	CTGGCTAAAG

G

Factor	Model	Beg	Sns	Len	Sequence
_00000 DEF	I00073 (DEF)	43	R	8	AATAGCAA
T00113 c-Ets-2	R04343 ()	54	R	6	CTTCCC
_00000 LBP-1	I00191 (LBP-1)	58	N	5	CCTGG
_00000 NF-IL6	I00088 (NF-IL6)	59	R	9	CTGGCTAAA
_00000 C/EBP	I00034 (C/EBP)	59	N	10	CTGGCTAAAG

3.4. Discussion

The SSCP technique was validated by the analysis of exon 2, containing the FokI polymorphism. The altered migration of the two alleles was clearly observable. As well as this common polymorphism, two further, rarer, polymorphisms were observed: C59TT, and C156T. These polymorphisms had previously been reported by Brown *et al.* (Brown et al., 2000). C59TT is a synonymous base change and is therefore unlikely to have any functional affect. C156T is 8 bases downstream of the 3' exon boundary and as such can have no affect on the protein structure. However, during mRNA processing the spliceosome assembles at the exon boundary in order to remove the intronic sequences from the pre-mRNA molecule (Smith and Valcarcel, 2000). Although C156T is not within the core binding site for the spliceosome, which extends 6 bases into the intron, it may be close enough to alter pre-mRNA conformation and affect spliceosome binding efficiency. Crofts *et al.* (Crofts et al., 1998) reported that some of the 5'-variant VDR transcripts contained a fragment of intron 2 containing a premature stop codon and it is possible that C156T may alter the likelihood that this intronic sequence is spliced into the mRNA molecule. It is not clear what the function of these molecules is. They code for a severely truncated protein containing neither the DNA- or ligand-binding domains that would seem unlikely to have any useful function. It is possible, however, that the short mRNA molecules may control functional VDR protein levels by competitively binding translational proteins.

Analysis of the 1a promoter region revealed a novel A to G polymorphism 1012 bp upstream of exon 1a, designated A-1012G and probable variation in amplicon 3 which it was not possible to sequence. This failure, and the difficulty in amplifying parts 2 and 7, is probably due to the nature of the sequence in this region. The TRAFAC, VISTA and PIP analysis shown in ***chapter 2*** revealed common repeat regions were present in this part of the promoter. This makes priming of alternative targets much more likely and will reduce PCR specificity and efficiency and cause contamination of sequencing reactions with alternative template sequences.

Since this SSCP analysis was carried out, improved genomic sequence, including variation data, for this region of chromosome 12 has been submitted to the NCBI database (GI:32891816). These findings confirm the presence of A-1012G (rs4516035) and also of a G to C polymorphisms 1520 bp upstream of exon 1a (rs7139166),

designated G-1520C. This polymorphism would account for the variation observed in SSCP of part 3 of the promoter. A much rarer polymorphism is also present at 699 bp upstream of exon 1a (rs11574007). This is a poly-G sequence that in the wildtype consists of eight Gs but in the variant allele contains nine Gs. These reports were confirmed by Nejentsev *et al.* (Nejentsev et al., 2004) who sequenced the entire VDR gene and reported 245 SNPs, including all of those discussed here.

To assess the likely impact of these polymorphisms on promoter activity, each allele of G-1520C and A-1012G polymorphism was submitted to the TESS database. TESS predicted the likely binding of transcription factors at each locus, and determined whether each polymorphism would affect that binding.

At the G-1520C locus the G allele was predicted to contain a binding site for GATA-2 while the C allele was predicted to contain a binding site for the CCAAT / enhancer binding protein (C/EBP) and Sp1 transcription factors. GATA-2 is a transcription factor important in development although its affects in adults are less well described. Sp1 is a ubiquitous transcription factor that is involved in the regulation of transcription from most promoters. Wietzke *et al.* (Wietzke et al., 2005) showed that $1,25(OH)_2D_3$ regulated a promoter region upstream of exon 1c through Sp1 signalling. However, Sp1 usually binds to clusters of transcription factors in the GC-rich region immediately proximal to the initiating exon and Wietzke *et al.* showed that $1,25(OH)_2D_3$-responsiveness mapped to a response element 261bp upstream of the exon. G-1520C is comparatively distal to exon 1a and is not in a particularly GC-rich region. C/EBP is also a general transcription factor. A further C/EBP binding site is located 14 bases upstream of G-1520C. VDR has been reported to up-regulate C/EBP expression (Christakos et al., 2003) and C/EBP enhances the VDR-mediated activation of the $25(OH)D_3$ 24-hydroxylase (Dhawan et al., 2005). Dhawan *et al.* report two further putative C/EBP binding sites in the VDR promoter, one of which begins at –1490 bp relative to exon 1a, only 30 bases downstream of G-1520C. The additional binding site at G-1520C in the C allele may increase C/EBP-mediated transcriptional regulation of the VDR.

The A-1012G polymorphism was predicted to be within the binding site of GATA-1,2 or 3 and Lmo2. The inability of TESS to predict which of the GATA proteins will bind demonstrates the limitations of *in silico* analysis. All GATA binding proteins, as the name suggests, bind to the core sequence GATA or similar. Which protein actually

binds depends on the peripheral sequence, the binding of the appropriate co-factors nearby and, perhaps most importantly, cell background. Different GATA proteins are specific to different cell types and it is perhaps the availability of protein within the cell that is most likely to affect which of the GATA proteins will bind. TESS predicts that GATA-3 is the most likely of the GATA proteins to bind based on sequence.

Merika & Orkin (Merika and Orkin, 1993) demonstrated that GATA-3 was able to bind DNA constructs containing the core sequence AGATAT, as contained in the A allele (in reverse orientation) while none of sixty-three constructs shown to bind GATA-3 contained the sequence AGACAT, as found in the G allele of A-1012G. This demonstrates likely differential transcription factor binding in the two alleles of A-1012G that provides a mechanism by which A-1012G may have a functional affect. GATA-3 is a T-cell specific transcription factor that is involved in the differentiation of T-helper cells. Naïve T-cells have the potential to develop to T-helper 1 (Th1) or T-helper 2 (Th2) cells. Th1 cells are involved in the activation of macrophages and a cell-mediated immune response; Th1 development is characterised by increased interferon gamma (IFN-γ) production. Th2 cells activate B-cells and produce an antibody-mediated immune response; Th2 development is characterised by increased interleukin-4 (IL-4) and IL-5 secretion (Rengarajan et al., 2000). The two pathways are self-reinforcing, such that once a Th1 response is initiated, Th2 polarisation is repressed and *vice versa.*

$1,25(OH)_2D_3$ has been shown to up-regulate GATA-3 expression and favour Th2 polarisation in mice (Boonstra et al., 2001). This signalling can be assumed to be mediated through the VDR. If the A allele at A-1012G binds GATA-3 while the G allele does not, the levels of VDR in cells expressing GATA-3 that contain one or two copies of the A allele (AA or AG individuals) are likely to be significantly higher than in cells containing only the G allele (GG individuals). This increased level of VDR would amplify any affect of $1,25(OH)_2D_3$ signalling on T-cell polarisation. The T-cell polarisation pathways and the potential modulating affects of the VDR are shown in ***figure 3.6***. The fact that GATA sites are predicted at the G allele of G-1520C and the A allele of A-1012G would suggest that, if GATA signalling was indeed occurring through both sites, a haplotype effect would occur whereby the $G^{1520}A^{1012}$ haplotype would be significantly more active than any other haplotype.

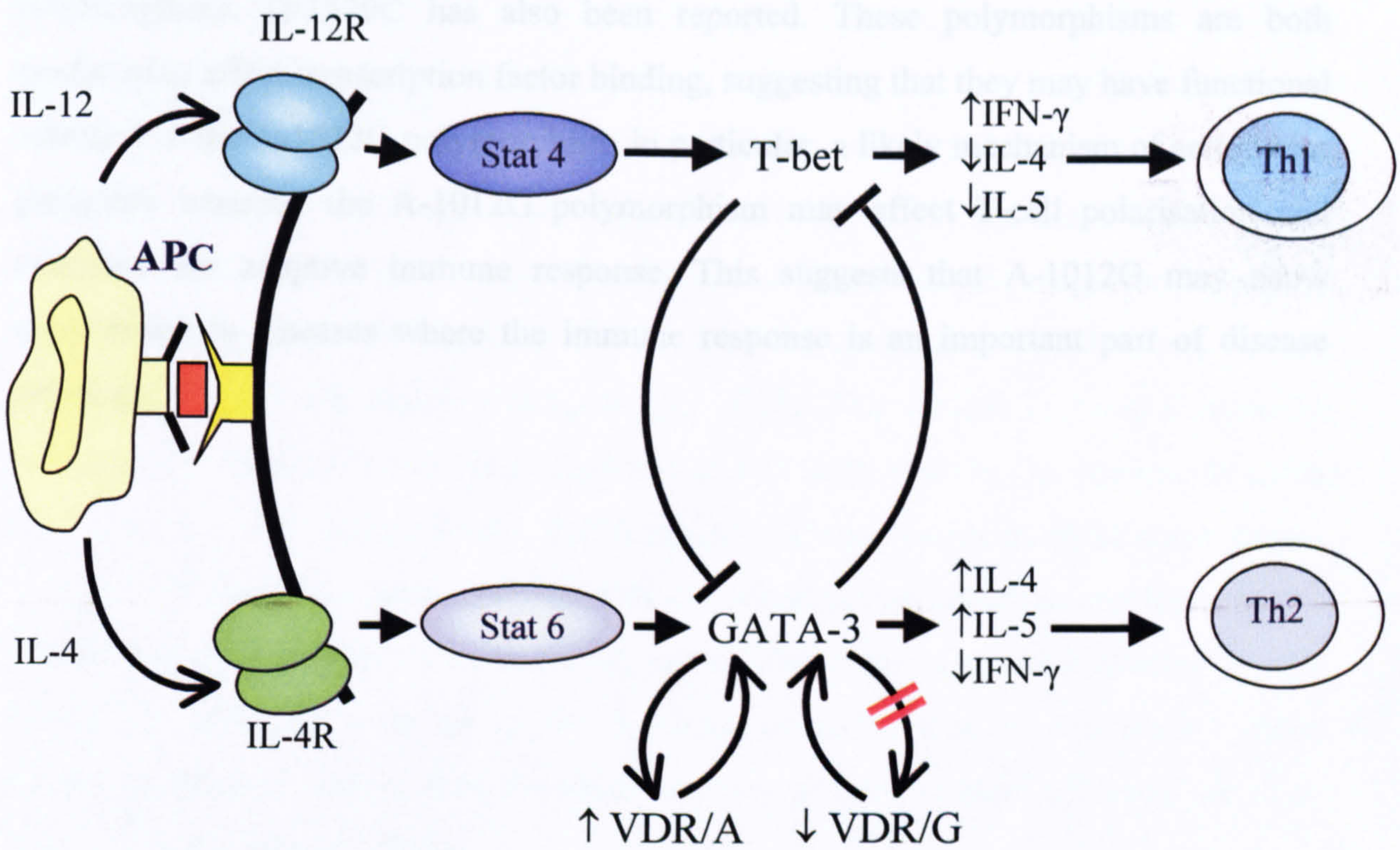


Figure 3.6: A diagram representing the T-cell polarisation pathways and the involvement of VDR. Following antigen presentation by the antigen presenting cell (APC), Th1 or Th2 responses can be initiated. The Th1 pathway involves signalling through IL-12 to the IL-12 receptor (IL-12R), then through Stat 4, and T-Bet to produce a Th1 response characterised by raised interferon gamma (IFN-γ) and decreased IL-4 and IL-5. The Th2 pathway involves signalling through IL-4, the IL-4 receptor, stat 6 and GATA-3 to elicit a Th2 response characterised by increased IL-4 and IL-5 and decreased IFN-γ. The two pathways are self-reinforcing, T-bet down-regulating GATA-3 and *vice versa.*

$1,25(OH)_2D_3$ signals through the VDR to upregulate GATA-3 expression and favour Th2 development. In the case of the A allele of A-1012G, VDR may be up regulated by GATA-3, amplifying this effect.

Adapted from Rengarajan *et al.* (Rengarajan et al., 2000).

3.5. Conclusion

A novel polymorphism, A-1012G was found in the 1a promoter region. A further polymorphism, G-1520C has also been reported. These polymorphisms are both predicted to affect transcription factor binding, suggesting that they may have functional affects. For the A-1012G polymorphism in particular, a likely mechanism of action was proposed, whereby the A-1012G polymorphism may affect T-cell polarisation and modulate the adaptive immune response. This suggests that A-1012G may show associations in diseases where the immune response is an important part of disease aetiology.

4. *VDR* Polymorphisms and Malignant Melanoma

4.1. Introduction

4.1.1. Malignant Melanoma

Malignant melanoma, although less common than basal cell or squamous carcinoma, is the most lethal form of skin cancer. The incidence in the western world has increased dramatically in the last 10 years. In Europe, there are approximately 59,400 new cases each year and 15,900 deaths (Ferlay et al., 2001). The disease is more commonly diagnosed in women (33,300 cases compared to 26,100 in men) but proportionally more fatal in men (7,600 female deaths, 8,300 men). Incidence varies north to south across Europe, with incidence particularly high in the Scandinavian countries and lower in the Mediterranean. Mortality rates, however, are similar across Europe. Incidence is still increasing, although more so in the southern countries than in the north, where incidence, although higher than the south, appears to have levelled off since the mid-nineties (de Vries et al., 2003).

Melanoma develops from the malignant transformation of melanocytes, mesenchymal cells in the basal layer of the epidermis that secrete the pigment melanin, which gives the skin its colour and protects against the potentially damaging affects of sunlight by absorbing ultraviolet radiation. The skin is divided into two distinct layers, the upper epidermis which derives from ectoderm and the lower dermis, which is of mesodermal origin. The dermis is the supporting layer of the skin and consists of fibroblasts and collagen fibres. The epidermis consists mainly of keratinocytes with interspersed melanocytes, Langerhans cells and merkel cells. Melanocytes are dendritic with long processes extending between the surrounding keratinocytes. Melanin granules are transferred along these processes to neighbouring keratinocytes.

In Caucasians, melanocytes occur at a ratio of approximately one melanocyte to every five keratinocytes. Melanocytes can also occur in clusters or naevi. These melanocytic naevi are very common, the average Caucasian has at least twenty-five of these lesions. These lesions are benign, although an excessive number is a risk factor for melanoma (Rivers, 1996). Normal naevi are symmetrical with even pigmentation but lose this symmetry and develop an irregular shape and colouration to form dysplastic naevi,

which are associated with an increased risk of malignant melanoma. Malignant melanoma may arise from existing naevi or *de novo* from normal skin. Melanoma development generally consists of a horizontal or radial growth phase where the melanoma spreads along the epidermis. This is followed by a vertical growth phase that leads to invasion through the basal layer and into the dermis, from where tumour cells can reach the circulation or lymph, leading to metastasis. Tumour thickness is measured on the Breslow scale and Breslow depth is the most important prognostic factor (MacKie et al., 1985). Patients with the thickest tumours (>3.5mm) show only a 31% five year survival rate while those with 1.5-3.49 thick tumours show a 67% survival rate and 93% of patients with tumours less than 1.5mm survive.

4.1.2. 1,25(OH)$_2$D$_3$, Melanoma and the Sun

The anti-proliferative, pro-differentiation affects of 1,25(OH)$_2$D$_3$ would suggest that it would delay the development of melanoma. Indeed, 1,25(OH)$_2$D$_3$ has been shown to decrease proliferation and increase differentiation of melanoma cells *in vitro* (Colston et al., 1981; Danielsson et al., 1998; Danielsson et al., 1999; Mason et al., 1988). Epidemiologically, low serum levels of 1,25(OH)$_2$D$_3$ have been associated with increased risk of melanoma (Cornwell et al., 1992).

However, the mode of synthesis of 1,25(OH)$_2$D$_3$ is at odds with its beneficial effects in melanoma. For most people, dietary intake of vitamin D is insufficient (Glerup et al., 2000), such that the cutaneous synthesis of 1,25(OH)$_2$D$_3$ through exposure to sunlight is essential to maintain appropriate levels. However, UV radiation is a serious risk factor for melanoma. Increased UV exposure has been associated with increased risk of melanoma (MacKie et al., 1985). It has been found that up to two thirds of melanoma cases are caused by UV exposure (Koh et al., 1996), although people who get irregular exposure, particularly if it leads to burning, such as office workers on holiday, are at greater risk than those who get regular exposure, such as those who work outdoors (Elwood, 1996; Hakansson et al., 2001).

The recent increased awareness of melanoma and the role of UV-induced damage in its aetiology has led to the increased use of stronger and stronger sun block. However, all but the weakest sun creams block 1,25(OH)$_2$D$_3$ synthesis and regular use has been associated with decreased serum levels of 1,25(OH)$_2$D$_3$ (Matsuoka et al., 1988). Some

vitamin D scientists now argue that the increased fear of the sun, along with poor nutrition, has led to vitamin D deficiency becoming a serious problem. This issue has become particularly controversial in the United States, where vitamin D deficiency is a particular problem in the large black population, due to the reduced vitamin D production as the greater levels of melanin in dark-coloured skin absorb more UV radiation.

4.1.3. VDR Polymorphisms and Melanoma

The VDR mediates the anti-proliferative, pro-differentiation effects of $1,25(OH)_2D_3$. As cancers such as melanoma are characterised by poor differentiation and uncontrolled proliferation, it would follow that any polymorphisms affecting the VDR may be associated with the development of cancer. Indeed, VDR polymorphisms have been associated with disease susceptibility and prognosis in psoriasis (Park et al., 1999), breast (Ingles et al., 1997b), colonic (Kim et al., 2001a) and prostate (Ingles et al., 1997b) cancer.

Hutchinson *et al.* (Hutchinson et al., 2000) found that the FokI and TaqI polymorphisms, particularly the genotype combination fftt were associated with susceptibility and prognosis in malignant melanoma. As described in ***chapter 1.3.3*** the F and T alleles have been associated with increased VDR activity, the F allele producing the shorter protein with greater transactivation potential and the T allele perhaps with increased mRNA stability. On this basis, the fftt genotype combination would represent the lowest level of transcription of the least active protein, which would associate fftt with decreased $1,25(OH)_2D_3$ effects, allowing increased proliferation and reduced differentiation of melanoma cells.

However, this hypothesis assumes that the FokI and TaqI polymorphisms are exerting their effects in the melanoma cells themselves. The potential binding of GATA-3 at the A-1012G polymorphisms suggests a mechanism by which *VDR* polymorphisms could affect the immune response to cancer. As described in ***chapter 3.4***, GATA-3 directs the polarisation of T-cells to Th2 cells, up-regulates the B-cell response and concurrently represses the Th1-regulated cytotoxic T-cell and macrophage response. Tatsumi *et al.* (Tatsumi et al., 2002) have shown that the Th1 response is much more effective against melanoma and renal cell carcinoma, which exhibits a similar antigen profile. Of 18

melanoma patients, 6 showed a Th1 response to melanoma antigens and all had no current evidence of disease while of 9 patients who raised a Th2 response, 7 developed metastasis. If the A allele at A-1012G were to be associated with the Th2 response and down-regulate the Th1 response, the A allele would be a risk factor in development and progression of melanoma. In this case the F and T alleles of the Fok1 and Taq1 polymorphisms, associated with increased VDR activity, would exaggerate this affect.

In this chapter, A-1012G genotype was determined in control and malignant melanoma populations in order to test the hypothesis that the A allele of A-1012G is associated with increased risk and prognosis in melanoma and that this affect is exaggerated by the more active F and T allele of the Fok1 and Taq1 polymorphisms.

4.1.4. Genotype Screening in Larger Population

In order to screen for genotype in the large control and melanoma populations, a more efficient method than SSCP was developed. Although it is theoretically possible to genotype control and patient populations by analysing the migration patterns in SSCP, the use of poly-acrylamide gels and silver staining make the technique impractical on a large scale. The RFLP technique is less labour-intensive; it requires the additional step of restriction digestion but the results are analysed by electrophoresis on agarose rather than polyacrylamide gels, which are quicker to pour and run and can be photographed directly using ethidium bromide rather than silver staining. For the G-1520C, Fok1 and Taq1 polymorphisms, RFLP analysis is straightforward, as in each case, one allele of the polymorphism falls within the natural restriction site of a restriction endonuclease (NlaIII, Fok1 and Taq1 respectively). This is not the case for the A-1012G so a method was developed to introduce a restriction site at the polymorphism during the initial PCR amplification. The forward primer was designed to bind immediately upstream of the polymorphic base. The primer contained a mismatched base that mutated the local sequence to form an EcoRV binding site in the case of the A allele (*figure 4.1*). In order to allow the mismatched primer to bind, the stringency was reduced by lowering the annealing temperature for the first three cycles of PCR.

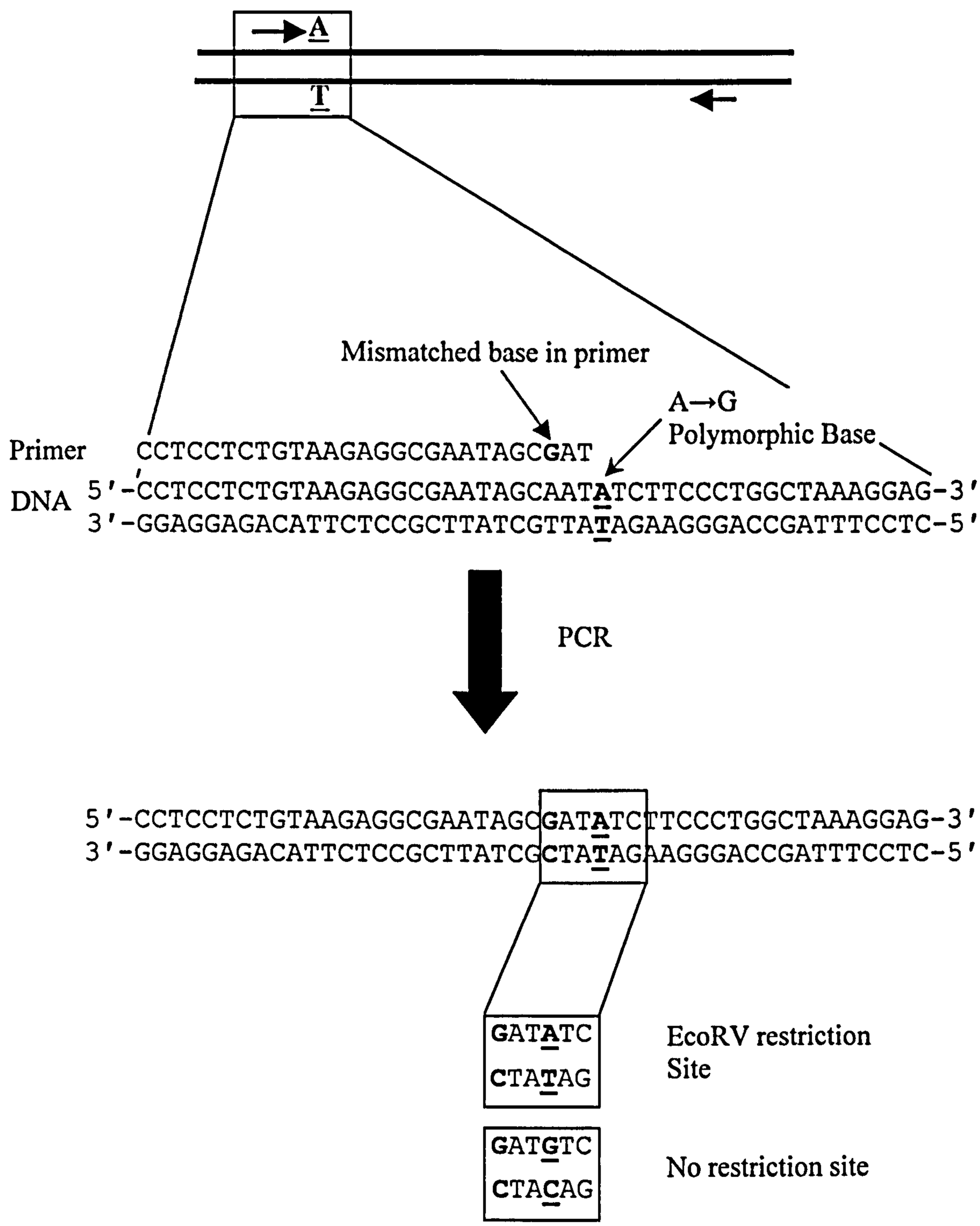


Figure 4.1: Schematic of the induced RFLP method, as used to screen for A-1012G genotype. In order to form an EcoRV restriction site in the A allele, the forward primer contained a mismatched base to mutate the sequence. The mismatched base and polymorphism are marked. The resultant sequence in the case of each polymorphism, the A allele containing the EcoRV restriction site, is shown below.

4.2. Materials and Methods

4.2.1. Materials

Fok1 restriction enzyme	New England Biolabs
Taq$^{\alpha}$1 restriction enzyme	New England Biolabs
EcoRV restriction enzyme	New England Biolabs
SeaKem agarose	Cambrex Bio Science
Nusieve agarose	Cambrex Bio Science

Further PCR reagents and buffers are shown in ***chapter 3.2.1.***

4.2.2. The Melanoma Cohort

A total of 191 patients who presented with malignant melanoma at the Dermatology Department, Leicester Royal Infirmary since 1994 were recruited to the study.

In order that the cohort was a true representation of melanoma patients for susceptibility analysis, the cohort was stratified according to Breslow depth, in proportion to the spectrum of tumours presenting in Leicestershire (Osborne and Hutchinson, 2001). This distribution and the numbers allocated to each group are shown in ***table 4.1***. There were therefore 176 patients in the melanoma cohort, 110 females, 66 males, mean age 54.3 years.

For the metastatic analysis, all patients for whom there was five-year follow-up data were included, giving a total of 171, with 18 patients who suffered metastases.

4.2.3. The Control Population

The frequency of A-1012G genotypes in the samples used for SSCP suggested that the A allele appeared at approximately 50% in a normal population. Aiming to detect a difference of 20% between controls and patients, power calculation suggested that given a test power of 0.9 at the 0.05 confidence level, with a 2-tailed test, 80 controls would be sufficient with a disease population of 176. There were 80 UK white Caucasian

controls consisting of individuals not known to have cancer. The available controls comprised 40 females and 40 males, mean age 56.3 years (5-89 years).

Breslow Depth	% Leicestershire 1994-2004 (n=738)	Numbers in Present Study n (%)
In situ	13.8%	24 (13.6%)
<0.75mm	26.6%	49 (27.8%)
0.75 – 1.49mm	21.5%	35 (19.9%)
1.5 – 3.49mm	21.0%	38 (21.6%)
≥3.5mm	17.1%	30 (17.0%)

Table 4.1: The stratification of the melanoma cohort. The cohort was stratified by Breslow depth in accordance with the proportion of malignant melanoma patients presenting in Leicestershire (1994-2004) (Osborne and Hutchinson, 2001).

4.2.4. Oligonucleotides

Table 4.2 shows the oligonucleotides that were used in genotype screening.

Primer	**Sequence**
G-1520C F	TGCAGAGAATGTCCCAAGGT
G-1520C R	GTCCTGCCAGTCTGATGGAT
A-1012G F	CCTCCTCTGTAAGAGGCGAATAGCGAT
A-1012G R	GGACAGGTGAAAAAGATGGGGTTC
Fok1 F	CTGGCACTGACTCTGGCTCT
Fok1 R	TGCTTCTTCTCCCTCCCTTT
Taq1 F	CAGAGCATGGACAGGGAGCAAG
Taq1 R	CGGCAGCGGATGTACGTCTGCAG

Table 4.2: Oligonucleotides used in genotype analyses.

4.2.5. RFLP

G-1520C, Fok1 and Taq1 genotypes were assessed by RFLP analysis. Control and patient DNA was amplified with primer pair G-1520C, Fok1 and Taq1 respectively for 35 cycles of PCR: 94°C, 30 seconds, 60°C, 30 seconds, 72°C, 30 seconds.

For G-1520C, restriction digestion was carried out using NlaIII in a 20μl reaction containing 15μl PCR product, 1x NEBuffer 4 (20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol), 100μg/ml bovine serum albumin, 2.5 units NlaIII. Digestion reactions were incubated at 37°C, 18 hours and analysed on 3% Nusieve, 1% Seakem agarose gels in 1xTBE.

For Fok1, restriction digestion was carried out in a 20μl reaction containing 15μl PCR product, 1x NEBuffer 4, 2.5 units Fok1. Reactions were incubated at 37°C, 18 hours.

For Taq1, restriction digestion was carried out in a 20μl reaction containing 15μl PCR product, 1x NEBuffer 3 (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol), 100μg/ml bovine serum albumin, 2.5 units $Taq^{\alpha}1$. Reactions were incubated at 65°C, 18 hours.

4.2.6. Induced RFLP

A-1012G genotype was assessed by induced RFLP. This was based on a method developed by Noll *et al.* (Noll et al., 2001). Control and patient DNA was amplified with primer pair A-1012G for 3 cycles of PCR: 94°C, 30 seconds, 55°C, 30 seconds, 72°C, 30 seconds, followed by 37 cycles of 94°C, 30 seconds, 65°C ,30 seconds, 72°C, 30 seconds.

Restriction digestion was carried out using EcoRV in a 20μl reaction containing 15μl PCR product, 1x NEBuffer 3 (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol), 100μg/ml bovine serum albumin, 2.5 units EcoRV. Reactions were incubated at 37°C, 18 hours.

4.2.7. Statistics

Genotype frequencies in patients and controls were analysed by contingency tables. The affect of genotype on risk of metastasis was analysed by logistic regression. Survival analysis and power calculations were carried out using the Unistat statistical package. Haplotypes were predicted by submitting the genotypes at each polymorphism to the Hap website (http://research.calit2.net/hap/). Hardy-Weinberg equilibrium was assessed by the Arlequin statistical package (Schneider et al., 2000).

4.3. Results

4.3.1. RFLP in Genotype Screening

Example results of RFLP for each polymorphism are shown in *figure 4.2*. The induced RFLP method designed to screen A-1012G genotype was verified by comparison with SSCP. For each polymorphism, RFLP analysis produced distinct, distinguishable patterns for each homozygote and heterozygote. Complete genotyping results for controls and patients can be seen in *appendix V*.

4.3.2. Hardy-Weinberg Equilibrium

All polymorphisms were in Hardy-Weinberg disequilibrium in controls and patients apart from A-1012G, which was in Hardy-Weinberg equilibrium in controls but not in patients (P=0.02).

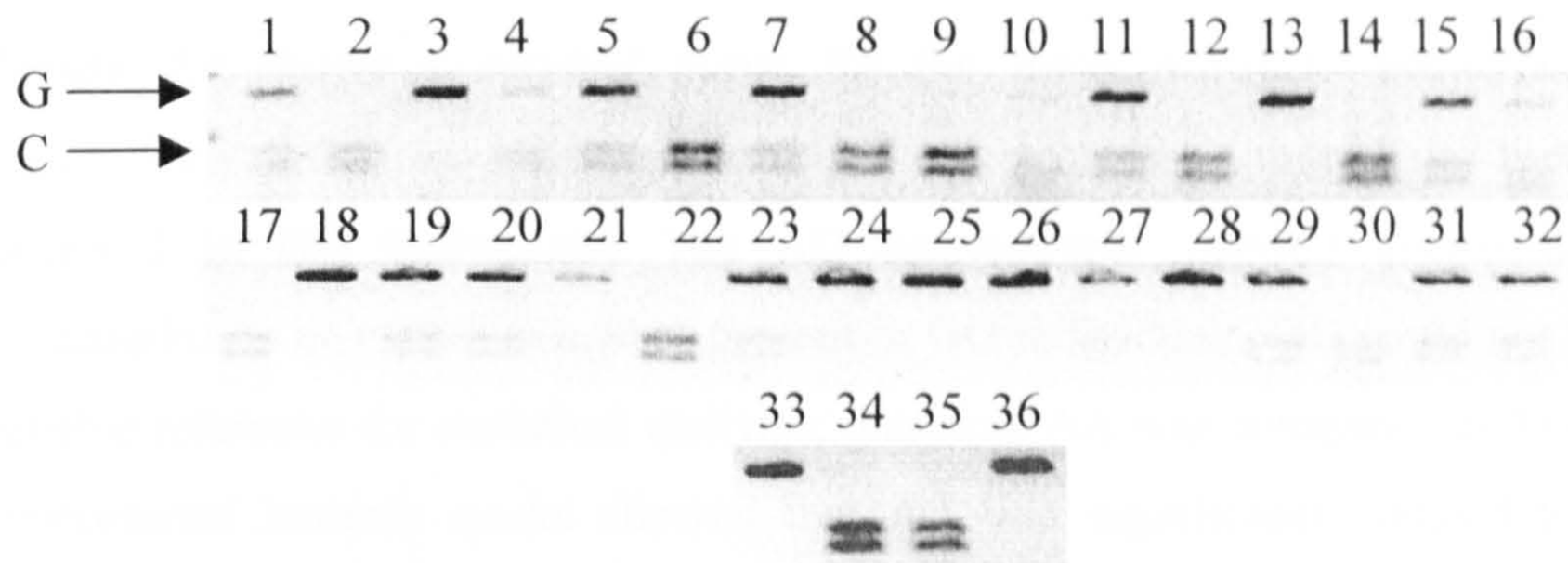
4.3.3. A-1012G and Melanoma Occurrence

A-1012G genotype was linked to melanoma occurrence. The allele and genotype frequencies in controls and patients are shown in *table 4.3*. The A allele was significantly over-represented and the G allele under-represented in melanoma patients compared to controls (P=0.03). Similarly the AA genotype was significantly over-represented and the GG genotype significantly under-represented in melanoma compared to controls (P=0.015). This is shown graphically in *figure 4.6 (A)*.

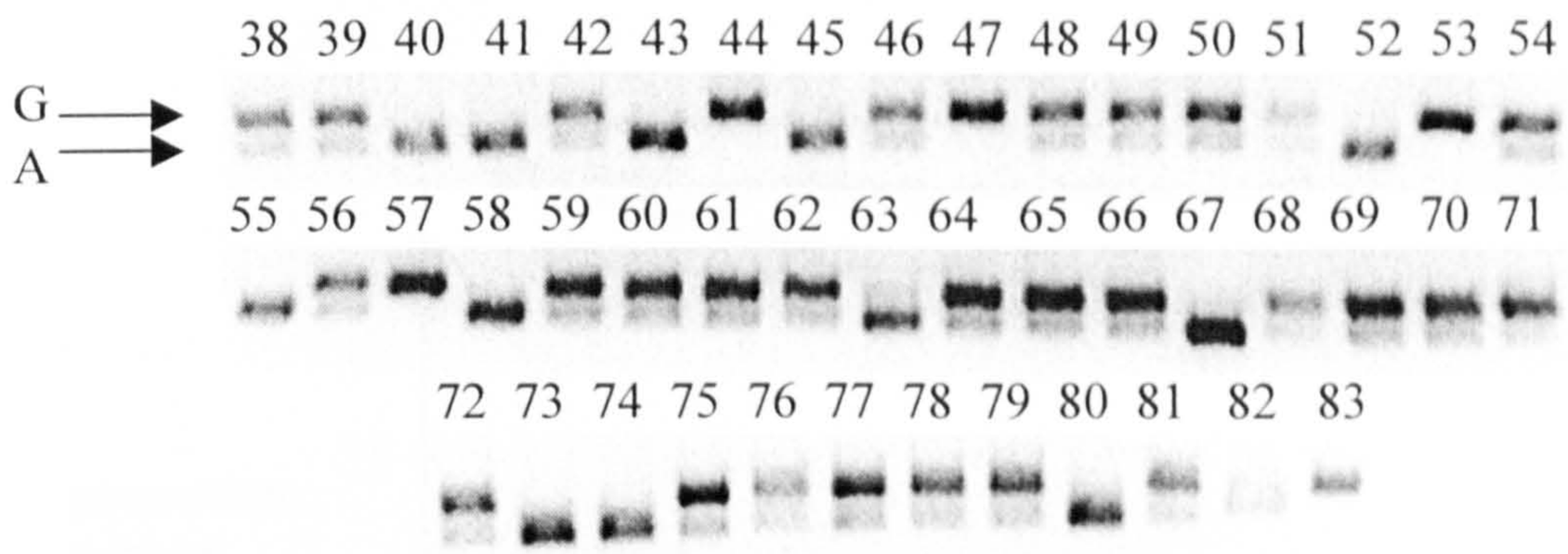
	Alleles		**Genotypes**		
	A	**G**	**AA**	**AG**	**GG**
Controls	86 (54%)	74 (46%)	22 (27%)	42 (52%)	16 (20%)
MM patients	244 (64%)	140 (36%)	66 (38%)	92 (53%)	16 (9%)
	P=0.03		P=0.015		

Table 4.3: A-1012G allele and genotype frequencies in controls and malignant melanoma. Statistical comparisons are by contingency tables with 2 degrees of freedom.

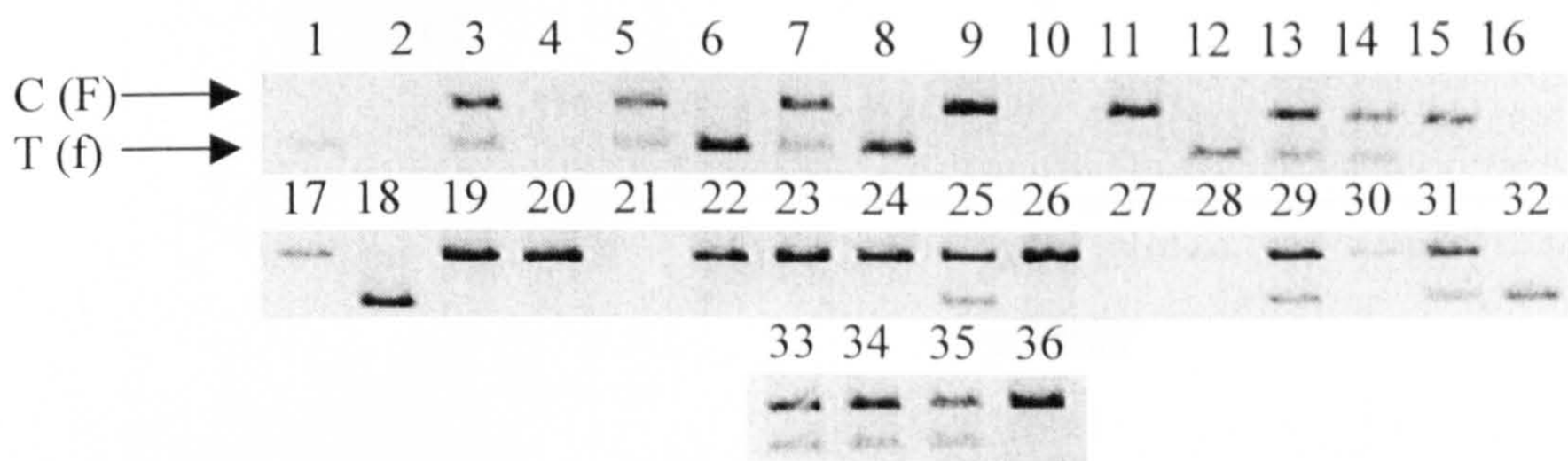
G-1520C



A-1012G



Fok1



Taq1

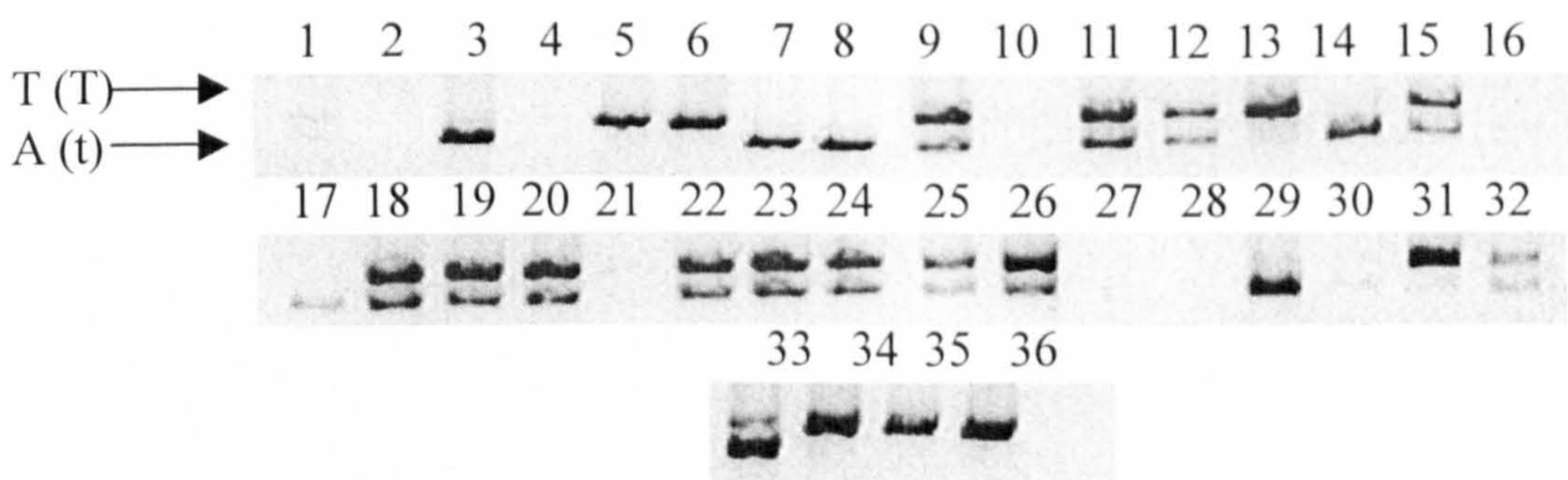


Figure 4.2: Examples of the screening results for each polymorphism. In each case the bands representing each allele are marked.

4.3.4. A-1012G and Metastasis

Figure 4.4 shows a survival curve for the three A-1012G genotypes. AA was significantly linked to metastasis and GG was protective; indeed, no metastasis was observed in GG individuals. The AG heterozygote showed intermediate risk of metastasis. As no metastases were present in GG individuals, GG could not be used as a reliable reference for statistical analysis; therefore AA was compared to AG/GG. Cox's proportional hazards model showed that AA was significantly related to metastasis (Hazard Ratio (HR), 2.9, 95% confidence interval 1.1-7.5, P=0.03).

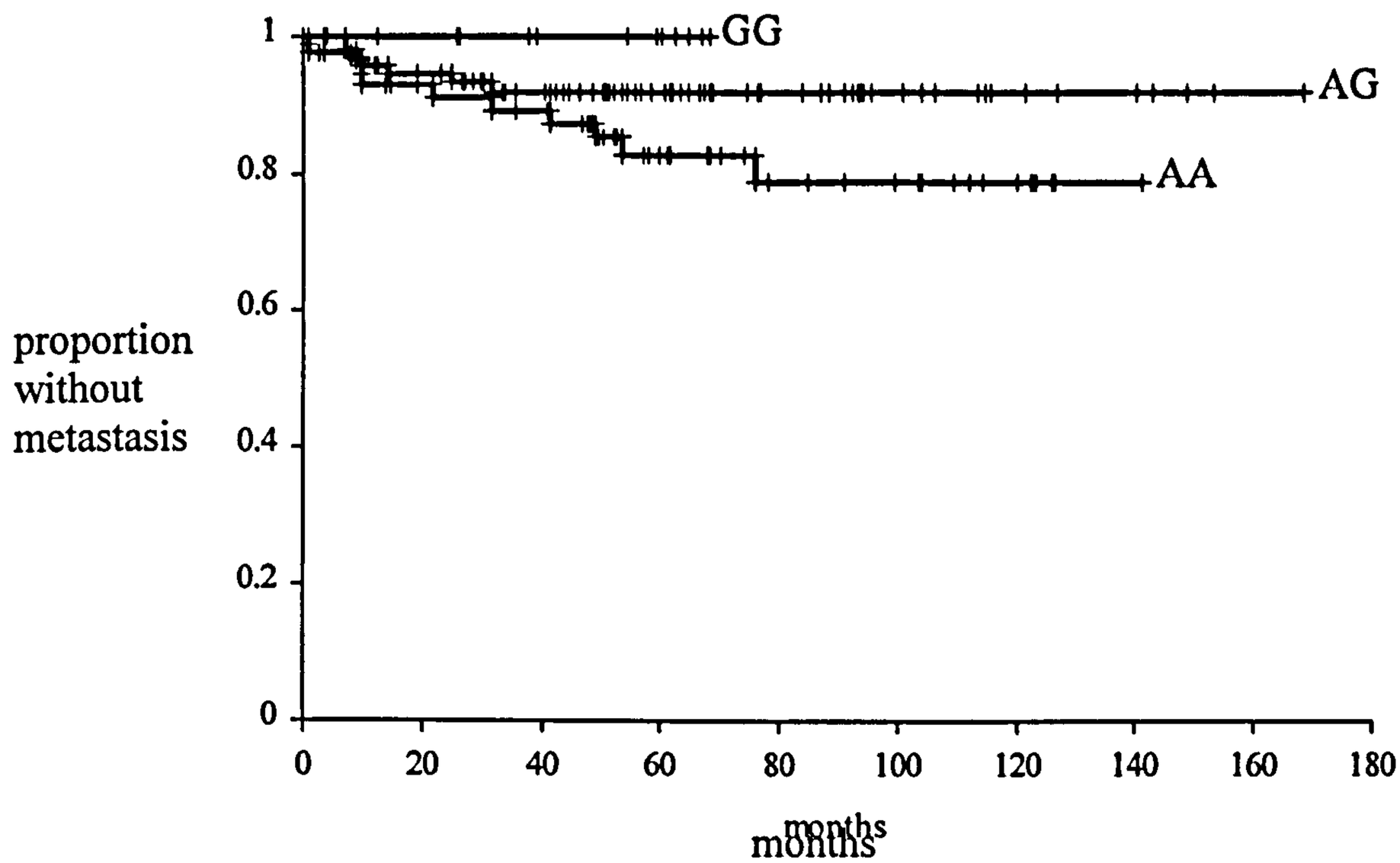


Figure 4.3: Survival curve showing metastasis in A-1012G genotypes.

Polymorphism	Genotype	Number	HR	95% CI	Significance
A-1012G	GG/AG	110	1		
	AA	59	**2.9**	1.1-7.5	*P*=0.03
Fok1	FF/Ff	140	1		
	ff	30	1.3	0.4-4.1	*P*=0.6
Taq1	TT/Tt	143	1		
	tt	26	1.5	0.5-4.6	*P*=0.5
Fok1/Taq1	other genotypes	165	1		
	fftt	4	2.2	0.3-16.3	*P*=0.5
A-1012G/Taq1	other genotypes	130	1		
	AATT	27	2.5	0.9-7.4	*P*=0.1
	AAtt	11	3.1	0.8-11.2	*P*=0.1
A-1012G/Fok1	other genotypes	130	1		
	AAFF	30	**4.3**	1.5-12.4	*P*=0.006
	AAff	9	**8.6**	2.5-29.6	*P*=0.001
	AAFF/Ff	50	1		
	AAff	9	**4.7**	1.2-18.6	*P*=0.03

Table 4.4: Genotype and genotype combination associations with metastasis. Bold numerals represent statistically significant associations

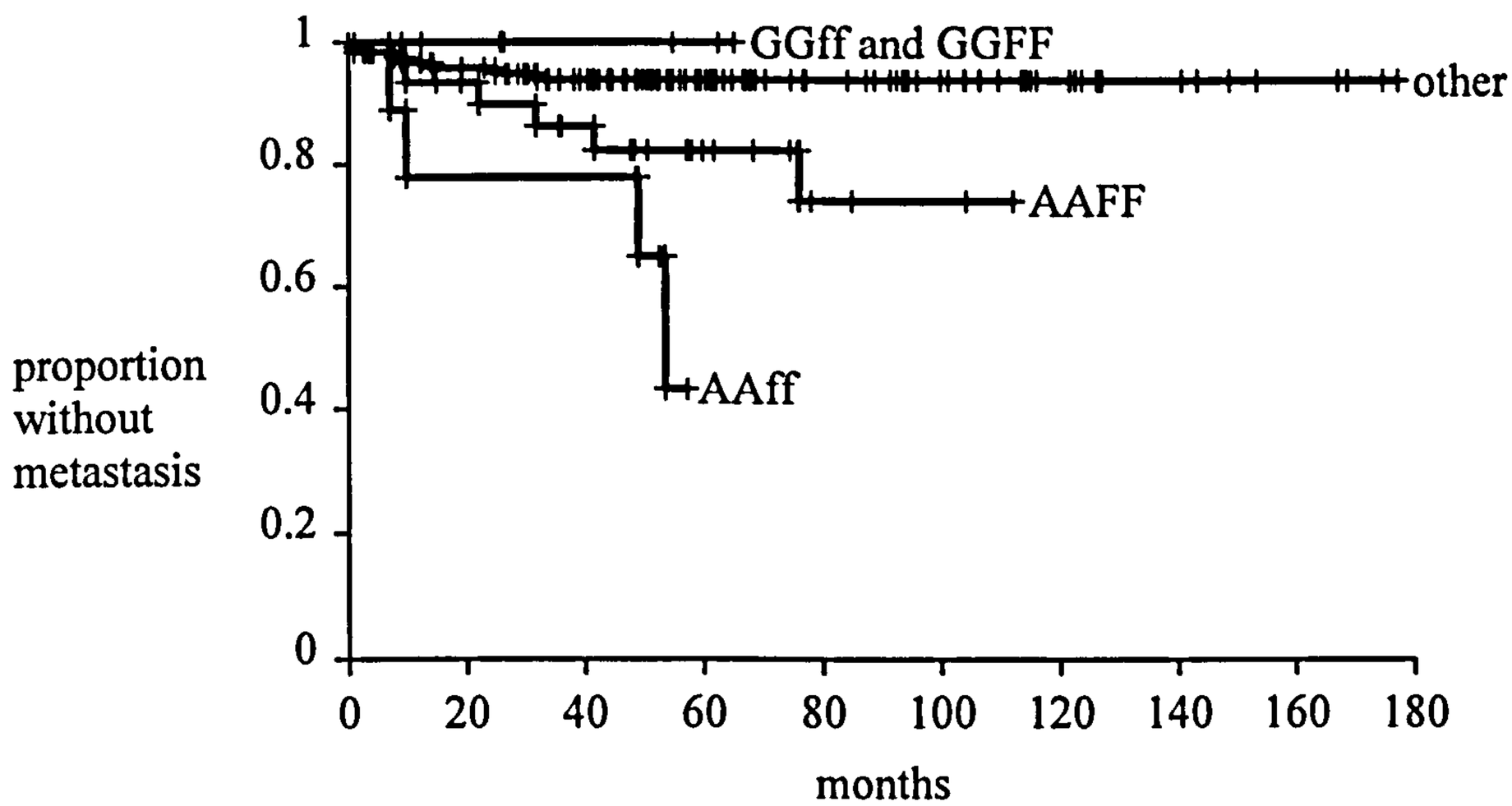


Figure 4.4: Survival curve showing metastasis in A-1012G/Fok1 genotype combinations.

4.3.5. A-1012G and G-1520C, Fok1 and Taq1 Haplotypes

There was strong linkage disequilibrium between G-1520C and A-1012G (composite disequilibrium constant $p=2.6x10^{-12}$ in controls and $1.5x10^{-10}$ in melanoma), with G at G-1520C linked with A at A-1012G. There was no significant linkage disequilibrium between any other pair of polymorphisms. G-1520C and A-1012G genoptye combinations are shown in ***table 4.5***.

Fok1 and Taq1 genotypes were not related to melanoma occurrence. Fok1 genotype alone was not significantly related to metastasis (P=0.6). In order to test whether Fok1 and Taq1 genotypes had a modifying effect on the main A-1012G association, each Fok1 and Taq1 homozygote was analysed in combination with the AA risk genotype at A-1012G. Both Fok1 homozygotes augmented the effects of A-1012G (AAFF, HR 4.3 (1.5-12.4), AAff, HR 8.6 (2.5-29.6). A survival curve for A-1012G/Fok1 genotype combinations is shown in ***figure 4.4***. There was a significant statistical interaction between A-1012G and Fok1 (P=0.025). The Taq1 polymorphism was not associated with metastasis, either singly or in combination with other polymorphisms. These results are summarised in ***table 4.4***.

G-1520C genotype was not itself linked with occurrence or metastasis (P=0.7 and 0.1). However, the G-1520C/A-1012G haplotype CA was strongly associated with melanoma occurrence (P=0.0006, ***table 4.5***). As shown in ***figure 4.5***, the increase in AC haplotype from controls to melanomas was approximately equivalent to the total increase in the AA genotype at A-1012G.

No such pattern was observed with G-1520C/A-1012G haplotypes in metastasis. As shown in ***figure 4.6***, the CA and GA haplotypes both increased in patients with metastasis, although neither increase was significant (P=0.5 and 0.06 respectively).

		Controls			Melanomas		
		G-1520C			G-1520C		
		GG	GC	CC	GG	GC	CC
A-1012G	AA	19 (25%)	1 (1%)	0 (0%)	46 (27%)	9 (5%)	10 (6%)
	AG	9 (12%)	32 (42%)	0 (0%)	22 (13%)	61 (35%)	9 (5%)
	GG	0 (0%)	0 (0%)	15 (20%)	0 (0%)	0 (0%)	16 (9%)

Table 4.5: G-1520C/A-1012G genotype combinations in the control and melanoma populations.

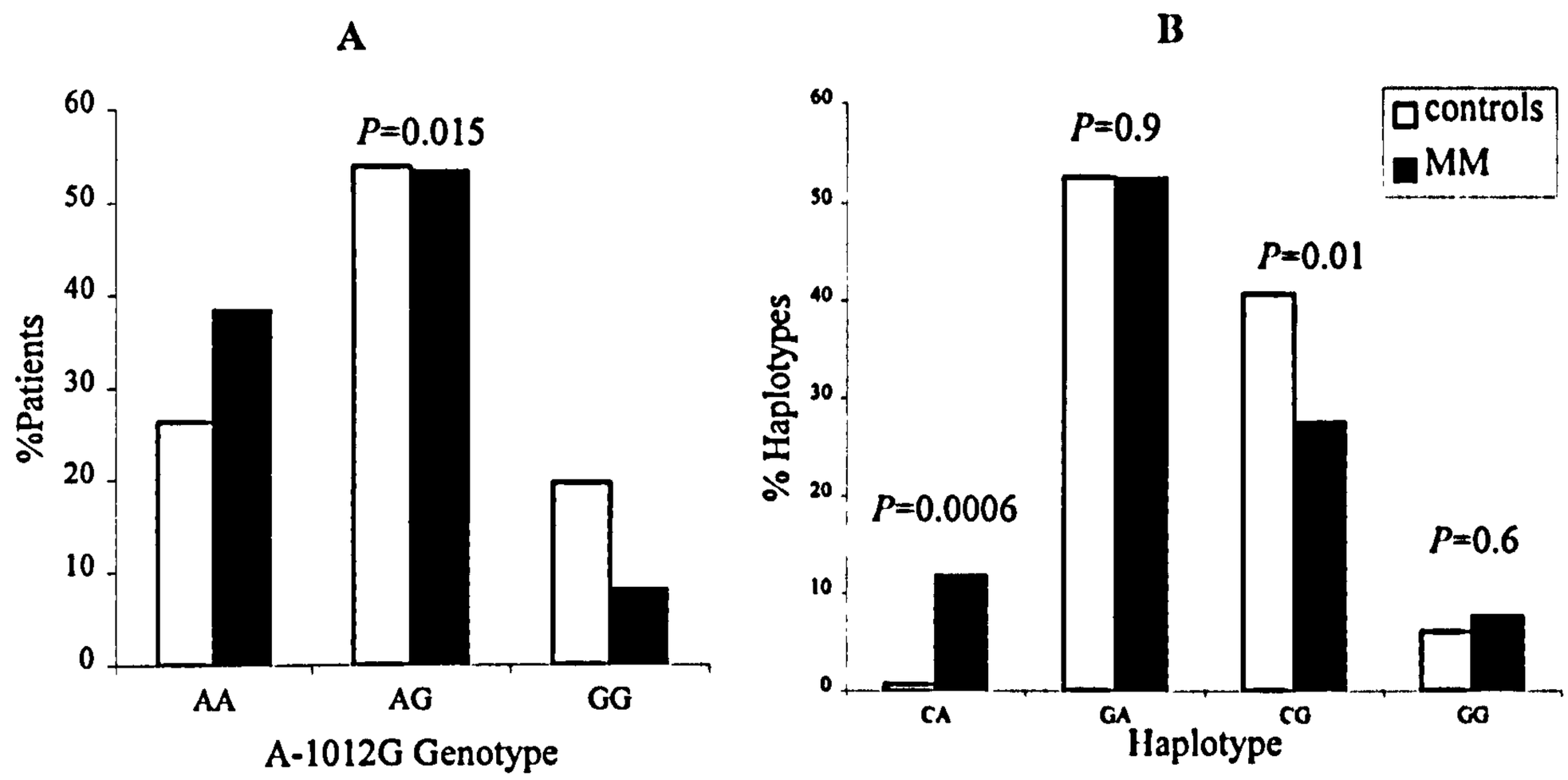


Figure 4.5: (A) A-1012G genotype and (B) G-1520C/A-1012G haplotypes in controls and patients. The increase in the CA haplotype is approximately equivalent to the increase in the AA genotype.

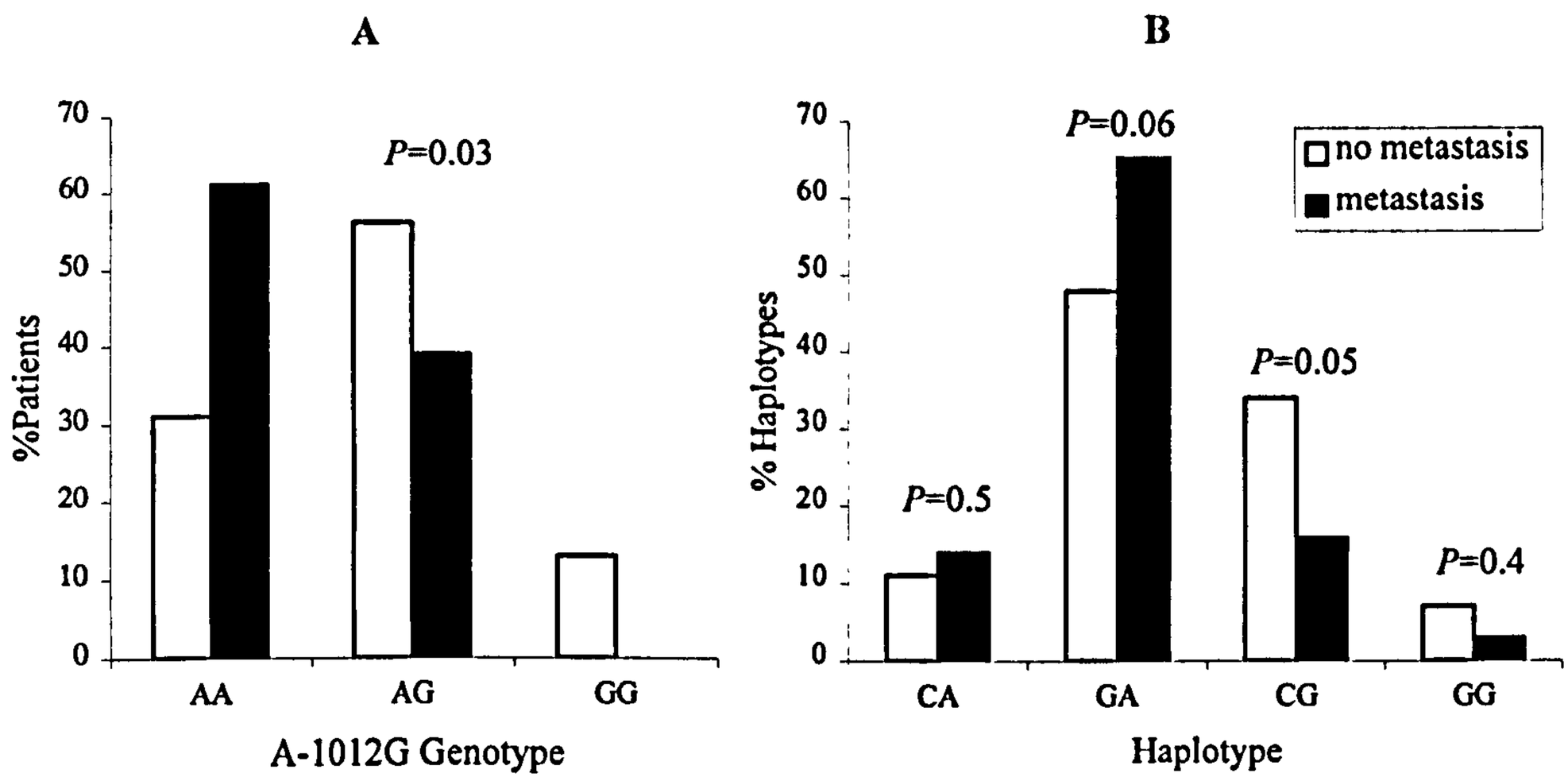


Figure 4.6: (A) A-1012G genotype and (B) G-1520C/A-1012G haplotypes in melanoma patients with and without metastasis.

4.3.6. VDR genotypes and Breslow Depth

AA genotype at A-1012G was associated with Breslow thickness. Comparing tumours of Breslow depth ≥0.75mm to those <0.75mm, AA was associated with thicker tumours, (OR 1.9 (1.0-3.6), *P*=0.04). Taq1 was associated with tumour thickness ≥1.5mm (OR 3.1 (1.2-8.3), *P*=0.02). Fok1 genotype alone was not associated with Breslow thickness but the fftt genotype combination was strongly associated with the thickest tumours (≥3.5mm) (OR 24 (3.0-225), *P*=0.005).

The frequency of metastasis when Breslow depth and A-1012G genotypes are combined is shown in ***table 4.6***. 67% of individuals with genotype AA and the thickest tumours (≥3.5mm) suffered metastases. Risk decreased with Breslow depth but, even in tumours <0.75mm, 8% of patients suffered metastases with AA genotype. In AG heterozygous individuals, metastasis occurred only in tumours ≥1.5mm, but to a lesser extent than in AA individuals. No metastasis occurred in GG individuals, even in the thickest tumours.

	Breslow Thickness Group				
	In situ	**<0.75mm**	**0.75-1.49mm**	**1.5-3.49mm**	**≥3.5mm**
GG	0% (0/3)	0% (0/5)	0% (0/3)	0% (0/4)	0% (0/2)
AG	0% (0/13)	0% (0/30)	0% (0/17)	17% (3/18)	27% (4/15)
AA	0% (0/7)	8% (1/13)	12% (2/16)	14% (2/14)	67% (6/9)

Table 4.6: Percentage metastasis in individuals of different A-1012G genotypes and tumour thickness. Numbers of patients in each group are shown in parentheses (metastasis/total patients).

4.4. Discussion

RFLP and induced-RFLP analysis proved to be suitable techniques for genotyping on a large scale. The PCR and subsequent enzyme restriction reactions were carried out on 96-well plates, allowing the use of multichannel pipettes for the mastermixes and relatively high-throughput screening. The technique was less labour-intensive than SSCP and more cost-effective than SSCP or a sequencing-based technique. There is a risk of genotyping errors with this technique, particularly through failure of the restriction reaction giving a false "uncut" or through misinterpretation of an incomplete digestion of a "cut" homozygote as a heterozygote. This risk was minimised by incubating the digestion reactions overnight with an excess of restriction enzyme. Given that the risk of an error occurring should be equal in different test populations, this sort of error should not cause a significant difference in genotype frequency between populations to be overlooked or for a false positive result to be observed. However, when smaller populations are considered, such as patients with metastasis or the rarer haplotypes, individual errors may have more of an effect. The relatively small numbers of controls and melanoma patients, particularly those with metastases, do impose some limitations on the study. When genotypes of different polymorphisms were combined, and when cases were stratified by Breslow depth as well as genotype the numbers in each group were reduced to such an extent that limited value can be placed on the results. *P* values were not corrected for multiple testing. In hypothesis-driven association studies, the application of a multiple testing correction such as the Bonferroni adjustment is more likely to lead to the discarding of genuine results (a type 2 error) than the avoidance of false positives (a type 1 error) (Perneger, 1998).

The results confirmed the hypothesis that the A allele of A-1012G was associated with occurrence and metastasis in malignant melanoma. AA genotype at A-1012G was significantly over-represented in malignant melanoma patients compared to controls. The fact that A-1012G was in Hardy-Weinberg equilibrium in controls but not in patients strengthens the case for an association of A-1012G genotype with melanoma (Gyorffy et al., 2004). The hypothesis was based on the predicted affect of the A allele of A-1012G in T-lymphocytes, amplifying GATA-3 signalling and biasing the T helper response to Th2 and reducing the Th1 response. Tatsumi *et al.* (Tatsumi et al., 2002) showed that patients with advanced melanoma were much more likely to exhibit Th2 responses to melanoma antigens than disease free patients or normal controls. Thus the

modifying affect of VDR on T-cell responses may be influencing the immune response to melanoma. The immune response may be important both in the early detection and elimination of tumour cells, thereby affecting occurrence, and in the resistance to cancer spread, thus affecting metastasis.

The genotype combinations of G-1520C and A-1012G were significant in association with melanoma occurrence, with the CA haplotype significantly linked with increase risk. This might suggest a function, unique to this one haplotype, involving signalling of two interacting transcription factors binding simultaneously on the one allele. Sp1 and C/EBP binding proteins were both predicted to bind at the C allele of G-1520C while GATA-3 was predicted to bind at the A allele of A-1012G (*chapter 3.3.3*). VDR has been reported to up-regulate C/EBP expression (Christakos et al., 2003) and C/EBP enhances the VDR-mediated activation of the 25(OH)D_3 24-hydroxylase (Dhawan et al., 2005). Dhawan *et al.* reported two further putative C/EBP binding sites in the VDR promoter, one of which begins at –1490 bp relative to exon 1a, only 30 bases downstream of G-1520C. Tong *et al.* (Tong et al., 2005) showed that GATA-3 and C/EBP form protein complexes to mediate target gene expression while Cousins *et al.* (Cousins et al., 2000) reported GATA-3 and C/EBP binding sites in the promoter of the IL-5 gene. It may be that the strength of the haplotype effect is due to its localisation in a particular cell type. C/EBP binding might be expected to enhance the signal of several transcription factors binding to the *VDR* promoter but the effect is localised to T-cells only in the case of the A allele at A-1012G, with the GATA-3 binding site present. The fact that this haplotype affect is not associated with metastasis, while A-1012G is associated with both occurrence and metastasis, suggests that A-1012G may be acting through a different mechanism, perhaps in different cell types, at different stages of tumour development.

The affects of the Fok1 polymorphism were unclear. Individually, Fok1 was not associated with either occurrence or outcome. However, in combination with A-1012G, both Fok1 homozygotes were associated with increased risk of metastasis: AAFF, HR 4.3 (1.5-12.4), P=0.006 and AAff, HR 8.6 (2.5-29.6), P=0.001. AAff was associated with significantly greater risk than AAFF, HR=4.7 (1.2-18.6), P=0.03. There was also a statistically significant interaction between A-1012G and Fok1 (P=0.025), confirming that the combined affect of A-1012G and Fok1 is more than the sum of the two individual affects. It is difficult to explain how both F and f alleles can be associated

with greater risk, particularly given the statistical interaction between Fok1 and A-1012G, which would suggest that one particular genotype at each polymorphism, taken in combination, exerts an effect via a mechanism which can only occur in the presence of both appropriate genotypes on the same allele. The F allele of the Fok1 genotype has been associated with greater VDR activity so it would be expected that this allele would amplify any affect of the A-1012G mechanism, such that the GATA-3 responsive promoter in the A allele of A-1012G activates transcription of the more active protein transcribed by the F allele of Fok1. This cannot explain how the AAff genotype combination is also associated with increased risk. One explanation may be that VDR genotypes have different affects in different cells. In T-cells, the more active alleles would be a disadvantage, as their presence would increase the Th1 to Th2 switch. However, in melanocytes and melanoma cells, VDR mediates the antiproliferative, pro-differentiation effects of $1,25(OH)_2D_3$. Here the more active alleles would be advantageous in suppressing tumour growth. A further explanation may be that stratification of the population by more than one polymorphism leads to numbers in each group being too small for accurate comparison, particularly when one of the genotypes is rare. For example, only nine individuals had the A-1012G/Fok1 genotype combination AAff (***table 4.4***).

If A-1012G acts on the immune response rather than in the cancer cells themselves, it is likely that A-1012G genotype would have a similar effect in other cancers where the immune response is similar to that in melanoma. Similar results might be expected in breast cancer, which also exhibits a similar antigen profile, and renal cell carcinoma, in which Tatsumi *et al.* (Tatsumi et al., 2002) showed a similar link between T-cell response and metastasis.

Taq1 genotypes or genotype combinations were not associated with melanoma occurrence or metastasis. However, the fftt Fok1/Taq1 genotype combination was strongly associated with deeper tumours. This would strengthen the hypothesis that the Taq1 polymorphism acts on melanoma cells while the A-1012G polymorphism acts to affect the immune response to melanoma. Fok1, as it affects VDR protein structure, would be expected to modify the effect of any *VDR* polymorphism, independent of cell background.

The observed linkage disequilibrium, with G-1520C and A-1012G strongly linked while Fok1 and Taq1 showed no linkage is confirmed by Nejenstsev *et al.* (Nejentsev et al.,

2004). They report that three blocks of linkage disequilibrium cover the VDR gene, the first downstream of exon 9, the second covering the coding region from exon 9 to exon 2 and the third covering the gene upstream of exon 2 with FokI in a small region of no linkage between the second and third blocks. Thus Taq 1, in block 2 and FokI between blocks 2 and 3 showed no linkage, while A-1012G and G-1520C, both in block 3 were in marked linkage disequilibrium with each other.

The melanoma cohort was stratified according to Breslow thickness such that the study population was representative of melanoma patients in the general population. This avoids the potential for bias in any associations that may occur if an unrealistic number of more or less severe tumours are used in comparison.

A-1012G appeared to increase risk of metastasis at least partially independently of Breslow depth, with the association of the A genotype with metastasis much stronger than that with Breslow depth. This suggests that although Breslow depth is a good prognostic indicator, it is not comprehensive, with some thin tumours metastasising while some thick tumours do not. The independence of the two measures also means that taking the two together should significantly increase the accuracy of prognostic prediction. As shown in ***table 4.5***, 67% of patients with the thickest tumours (≥3.5mm) and AA genotype developed metastases and even in patients with thinner tumours, significant levels of metastasis was seen in AA genotypes. No metastases were seen in GG individuals even with those with the thickest tumours. However, the stratification of the cohort into Breslow groups and genotypes has led to some groups comprising small numbers, particularly in the GG genotypes as GG is rare in melanoma. These small numbers mean that these results are currently of limited value. If this pattern can be confirmed in a larger cohort, A-1012G genotype combined with Breslow depth may be a useful prognostic indicator for melanoma treatment.

4.5. Conclusion

VDR genotype was associated with melanoma occurrence and prognosis, the strongest associations being with the A-1012G polymorphism. The A allele at A-1012G was shown to be a risk factor for both occurrence and metastasis, which supported the hypothesis that the A allele is associated with a down-regulation of the Th1 immune response. The G-1520C/A-1012G haplotype CA was strongly associated with occurrence, to the extent that this haplotype may account for the entire effect of A-1012G. This effect was not seen with metastasis. The FokI/TaqI genotype combination fftt was associated with deeper tumours, but not with metastasis. These findings may be related to the tissue-specific actions of the VDR. The association of A-1012G genotype with metastasis was independent of Breslow depth, and combining A-1012G genotype with Breslow depth may improve the prediction of melanoma prognosis.

5. VDR Polymorphisms and Psoriasis

5.1. Introduction

5.1.1. Psoriasis: An Inflammatory Disease

Psoriasis is a common dermatological disorder. It occurs in approximately 1-2% of the population (Nevitt and Hutchinson, 1996) and is characterised by inflammation of the dermis and hyperproliferation of keratinocytes, leading to epidermal thickening. The most common type of psoriasis, psoriasis vulgaris or chronic plaque psoriasis features scaly plaques, commonly on the scalp, lower back, elbows and knees. The severity of psoriasis varies with time and can be affected by infection (Telfer et al., 1992), cutaneous injury, alcohol and stress (Seville, 1989).

Psoriasis is considered to be a genetic disease. Concordance studies in twins have shown the disease to be strongly heritable at least in some patients (Elder et al., 1994) although no single gene accounts for all cases nor do the genetic variants linked with occurrence cause psoriasis in all individuals. A number of candidate genes have been described in patients with a family history. Nineteen loci have currently been identified: PSORS1-7 and 9 and eleven further loci (Sagoo et al., 2004), the most significant region is the PSORS1 locus on chromosome 6p21 within the major histocompatability complex (MHC) (Nair et al., 1997; Trembath et al., 1997). The high level of genetic linkage in the region has made identification of the actual genes responsible difficult. Candidate genes include *corneodesmosin* (Tazi Ahnini et al., 1999), *HCR* (Asumalahti et al., 2000), *SEEK 1* and *SPR1* (Holm et al., 2003).

T-lymphocyte responses have been implicated in the inflammatory response in psoriasis (Bata-Csorgo et al., 1995). Cutaneous T-cell infiltration is found in psoriasis and analysis of lesional T-cells has suggested that the majority of these T-cells show a Th1 phenotype (Friedrich et al., 2000; Horrocks et al., 1997). Austin *et al.* (Austin et al., 1999) showed that the majority of T-cells found in the psoriatic epidermis produced Th1 type cytokines. The ratio of IFN-γ-producing (Th1) to IL-4-producing (Th2) cells significantly increased in psoriatic compared to healthy skin. Tumour necrosis factor-α (TNF-α) expression was also shown to be significantly increased in psoriatic T-cells. TNFα has been shown to be co-expressed with IFN-γ in Th1 effector cells (Res et al., 1994) and these Th1 type cytokines appear to mediate the proliferation of psoriatic

keratinocytes. Co-culture with T-cells derived from psoriatic lesions has been shown to promote keratinocyte growth *in vitro* (Prinz et al., 1994), probably through IFN-γ (Bata-Csorgo et al., 1995).

5.1.2. 1,25(OH)$_2$D$_3$ and VDR in Psoriasis

There are several factors that suggest that the VDR may be active in psoriasis. Calcipotriol, (Dovonex®), an analogue of 1,25(OH)$_2$D$_3$ (***figure 5.1***), is commonly used to treat psoriasis (Bourke et al., 1993b; Holick, 1989; Kragballe et al., 1998). The analogue is used rather than the natural ligand to avoid calcaemic affects, which can be observed with 1,25(OH)$_2$D$_3$ treatment (Bourke et al., 1993a). Calcipotriol is one hundred times less active on calcium metabolism than 1,25(OH)$_2$D$_3$ while showing similar anti-proliferative, pro-differentiation effects (Kragballe et al., 1998). The structure of Dovonex compared to 1,25(OH)$_2$D$_3$ is shown in ***figure 5.1***. The VDR is expressed in normal and psoriatic keratinocytes (Feldman et al., 1980; Milde et al., 1991; Solvsten et al., 1997) and higher induction of VDR transcription by calcipotriol treatment is associated with better clinical response (Chen et al., 1996). As in other cell backgrounds, 1,25(OH)$_2$D$_3$ has been shown to reduce proliferation (Matsumoto et al., 1990; Smith et al., 1986) and induce the terminal differentiation (Bikle et al., 1993; Matsumoto et al., 1991; Pillai and Bikle, 1991) of keratinocytes.

Further to the direct affects on keratinocytes, 1,25(OH)$_2$D$_3$ may also be beneficial in psoriasis by suppression of the inflammatory response. Reichel *et al.* (Reichel et al., 1987) showed that 1,25(OH)$_2$D$_3$ inhibits IFN-γ production by T-lymphocytes and this would be expected to reduce the IFN-γ induced keratinocyte hyper-proliferation and associated inflammation. Mathieu *et al.* (Mathieu and Adorini, 2002) showed that 1,25(OH)$_2$D$_3$ inhibits the maturation of dendritic cells, thereby inhibiting antigen presentation by the dendritic cells to T-lymphocytes and reducing the T-cell response.

VDR polymorphisms have been linked with occurrence and response to calcipotriol in several studies. Most studies have focused on the 3' polymorphisms, which are in strong linkage disequilibrium, the abTL alleles of Apa1, Bsm1, Taq1 and the polyA length polymorphism (Ingles et al., 1997a; Uitterlinden et al., 1996) being strongly linked. As the abTL haplotype has been linked with greater VDR activity, it might be expected that the less active ABtS haplotype would be linked to psoriasis occurrence,

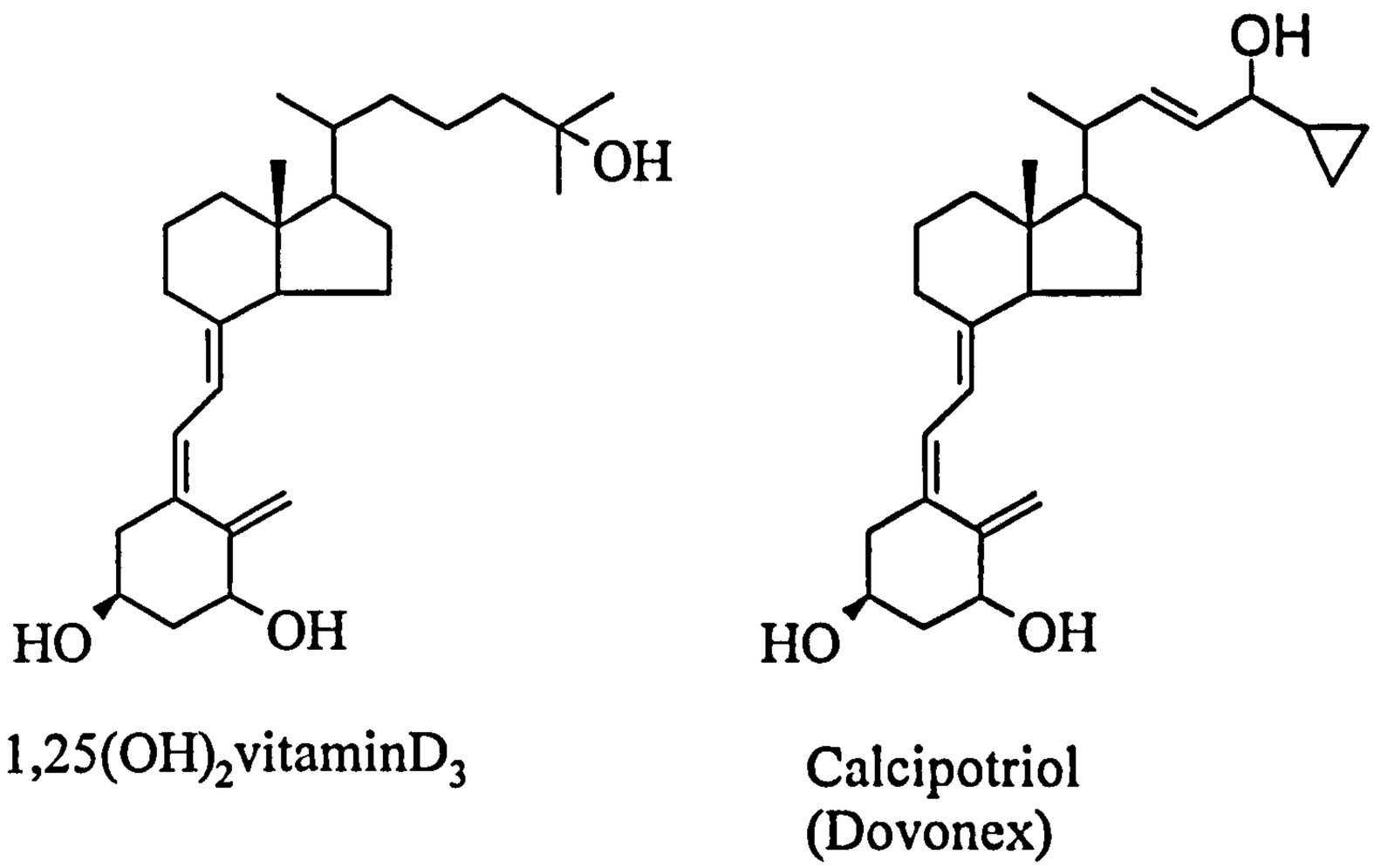


Figure 5.1: The chemical structure of Calcipotriol (Dovonex) compared to that of $1,25(OH)_2D_3$. Dovonex has a modified side chain with a 22, 23 double bond, 24-hydroxyl group and a 25-cyclopropyl group.

given the beneficial affects of $1,25(OH)_2D_3$, while the more active abTL haplotype would be linked to improved calcipotriol response. Park *et al.* (Park et al., 1999) reported an association of the A allele of Apa1 with psoriasis occurrence. This was confirmed by Lee *et al.* (Lee and Park, 2002). Saeki *et al.* (Saeki et al., 2002) reported an association of the b allele of Bsm1 and the T allele of Taq1 with occurrence. Given the known linkage of the 3' polymorphisms, abTL, the data of Lee *et al.* and Park *et al.* conflict with that of Saeki *et al.* but are in agreement with the predicted activities of the polymorphisms. Holick *et al.* (Holick et al., 1996) reported an association of the b allele of Bsm1 with calcipotriol response and Saeki *et al.* (Saeki et al., 2002) found that the F allele of Fok1 was associated with improved response. Both of these associations are in agreement with the known allelic activities. Several studies failed to find any association of *VDR* polymorphisms with any variable in psoriasis (Kontula et al., 1997; Mee and Cork, 1998; Okita et al., 2002).

While Fok1 and the 3' polymorphisms may affect the $1,25(OH)_2D_3$ actions on keratinocytes and its general immunosuppressive affects, the A-1012G polymorphism provides a mechanism whereby *VDR* polymorphisms might specifically act to alter the immune response. Contrary to the case in melanoma, down-regulation of the Th1

response, as predicted via GATA-3 signalling in the A allele of A-1012G, is beneficial in psoriasis. Therefore, the A allele of A-1012G would be expected to be negatively associated with psoriasis occurrence and positively associated with response to $1,25(OH)_2D_3$ treatment. Also contrary to the situation in melanoma, the more general affects of $1,25(OH)_2D_3$, the general inhibition of cellular proliferation and promotion of terminal differentiation, would be beneficial in both the psoriatic keratinocytes, by reducing hyperproliferation, and in the lesional T-cells, in down-regulating the inflammatory response. Therefore, any affect of the A-1012G polymorphism would be expected to be amplified by the more active alleles of the Fok1 and 3' polymorphisms.

In this chapter, VDR genotype are analysed in a psoriasis cohort to test the hypothesis that the A allele of A-1012G is protective against psoriasis occurrence and associated with less severe disease and improved response to calcipotriol treatment and that this affect is amplified in combination with the more active F and T alleles of Fok1 and Taq1.

5.2. Materials and Methods

5.2.1. The Psoriasis Cohort

White Caucasian psoriasis patients were recruited who had suffered from psoriasis for at least five years and had been treated with calcipotriol (Dovonex, Leo Pharma, Ballerup, Denmark). Patients were asked to assess their response to calcipotriol as none, some, good or very good. Most patients had experienced more than one different type of treatment and, where Dovonex had some affect, patients were asked to to compare its effectiveness with that of other treatments. Age of disease onset and the presence or absence of the disease in a sibling or parent was recorded. There were 205 psoriasis patients, comprising 86 females and 119 males, mean age 44 years. 88 patients had a positive family history in siblings or parents. A Psoriasis Area Severity Index (PASI) was compiled for each patient and the patients were also asked to estimate, compared to current involved area (A), how many times lesser an area had been involved at best (t) and how many times greater an area had been involved at worst (T) within the past few years such that a chronic basal index (A/t) and a chronic maximal index (A/T) could be recorded. As a further measure of severity, recourse to second-line treatments was also recorded. The same control cohort as that used in the melanoma cohort was used: 80 controls, 40 females, 40 males, mean age 56.3 years.

5.2.2. RFLP and Induced RFLP

DNA was extracted from patient blood samples using the DRI ChargeSwitch DNA extraction kit according to the manufacturer's instructions.

Genotypes of A-1012G, Fok1 and Taq1 were analysed by RFLP and induced RFLP as described in ***chapter 4.2.4*** and ***4.2.5***.

5.2.3. Statistical Analysis

As described in ***chapter 4.2.6*** genotype frequencies in patients and controls were analysed by contingency tables. Genotype associations were analysed by logistic regression.

5.3. Results

Complete genotyping results for patients and controls are shown in *appendix V*. The associations of VDR alleles in psoriasis are shown in *table 5.2* and the associations of VDR genotypes and genotype combinations in psoriasis are shown in *table 5.3*.

5.3.1. Hardy-Weinberg Equilibrium

There was no divergence from Hardy-Weinberg equilibrium at any of the polymorphisms studied in patients or controls.

5.3.2. VDR Genotype and Psoriasis Susceptibility and Severity

There was no significant difference between any VDR allele or genotype frequency in the controls and the psoriasis cohort. However, when the psoriasis cohort was separated into those with a family history and those without, the A allele and the AA genotype were significantly under-represented in those with no family history (P=0.01 and 0.04 respectively, *table 5.1, figure 5.2*).

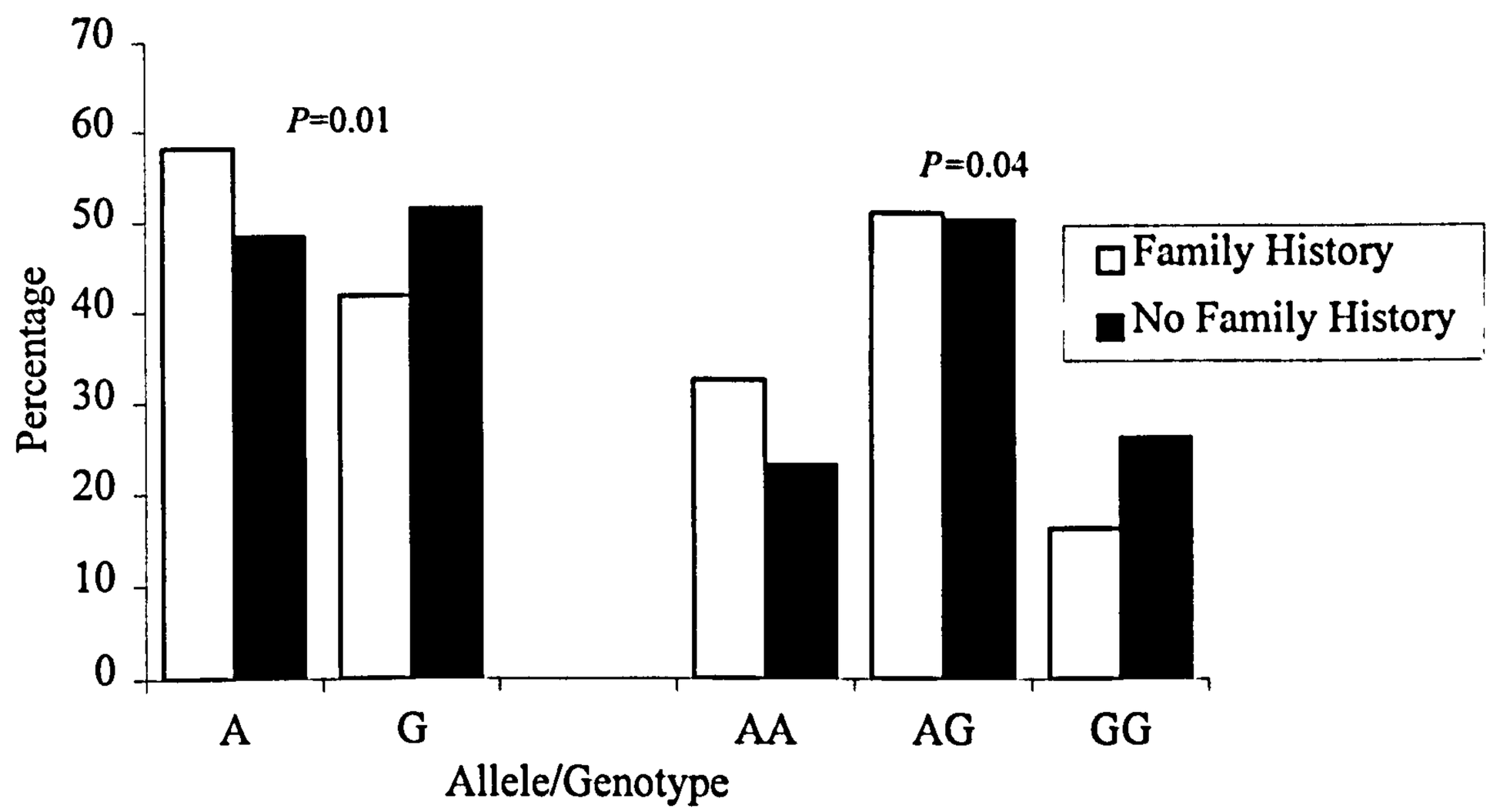


Figure 5.2: The proportion of A-1012G alleles and genotypes in patients with and without a family history of psoriasis. The A allele and AA genotype are significantly under-represented in patients with no family history.

	Family History	No Family History	
A-1012G			
AA	29 (33%)	24 (21%)	
AG	46 (52%)	60 (52%)	*P*=0.04
GG	13 (15%)	32 (28%)	
	88	**116**	
Fok 1			
FF	35 (40%)	39 (34%)	
Ff	42 (48%)	58 (50%)	*P*=0.4
Ff	11 (13%)	18 (16%)	
	88	**115**	
Taq 1			
TT	34 (39%)	47 (41%)	
Tt	44 (50%)	56 (48%)	*P*=0.8
tt	10 (11%)	13 (11%)	
	88	**116**	

	Family History	No Family History	
A-1012G/Fok1			
AAFF	14 (16%)	5 (4%)	*P*=0.005
AAFf	11 (13%)	15 (13%)	
AAff	4 (5%)	4 (3%)	
GGFF	3 (3%)	10 (9%)	
GGFf	8 (9%)	14 (12%)	
GGff	2 (2%)	7 (6%)	
AGFF	18 (20%)	24 (21%)	
AGFf	23 (26%)	29 (25%)	
AGff	5 (6%)	7 (6%)	

Table 5.1: The proportion of A-1012G, Fok1 and Taq1 genotypes and A-1012G/Fok1 genotype combinations in psoriasis patients with and without a family history of disease

The AAFF genotype combination was more significantly under-represented in patients with no family history compared to those without (*P*=0.005) and the AAFF genotype was also under-represented in patients with no family history compared to controls (OR 0.31 (0.10-0.95), *P*=0.04, ***table 5.1***). The FF genotype was not individually associated with family history (*P*=0.4). Taq1 was not associated with family history, individually (*P*=0.8) or in combination.

VDR genotype was not significantly associated with the severity of psoriasis by any of the severity measures used, although by each severity measure, AA genotype at A-1012G was weakly associated with less severe disease.

There were no significant associations of *VDR* genotype with age of onset.

5.3.3. VDR Genotype and Response to Calcipotriol

All three polymorphisms were associated with response to calcipotriol treatment. The AA genotype at A-1012G was significantly associated with response compared to GG/AG (OR 2.18 (1.0-4.71), *P*=0.04, ***figure 5.3***). The TT genotype at Taq1 was also associated with treatment response (OR 1.97 (1.04-3.76), *P*=0.03). Fok1 was not

individually significantly associated with response (P=0.2). However, the AAFF genotype combination was associated with response (OR 4.11 (0.92-18.38), P=0.03). Similarly, the AATT genotype combination (OR 5.64 (1.28-24.82), P=0.005) and the FFTT combination (OR 3.22 (1.18-8.74), P=0.01) were significantly associated with response to calcipotriol.

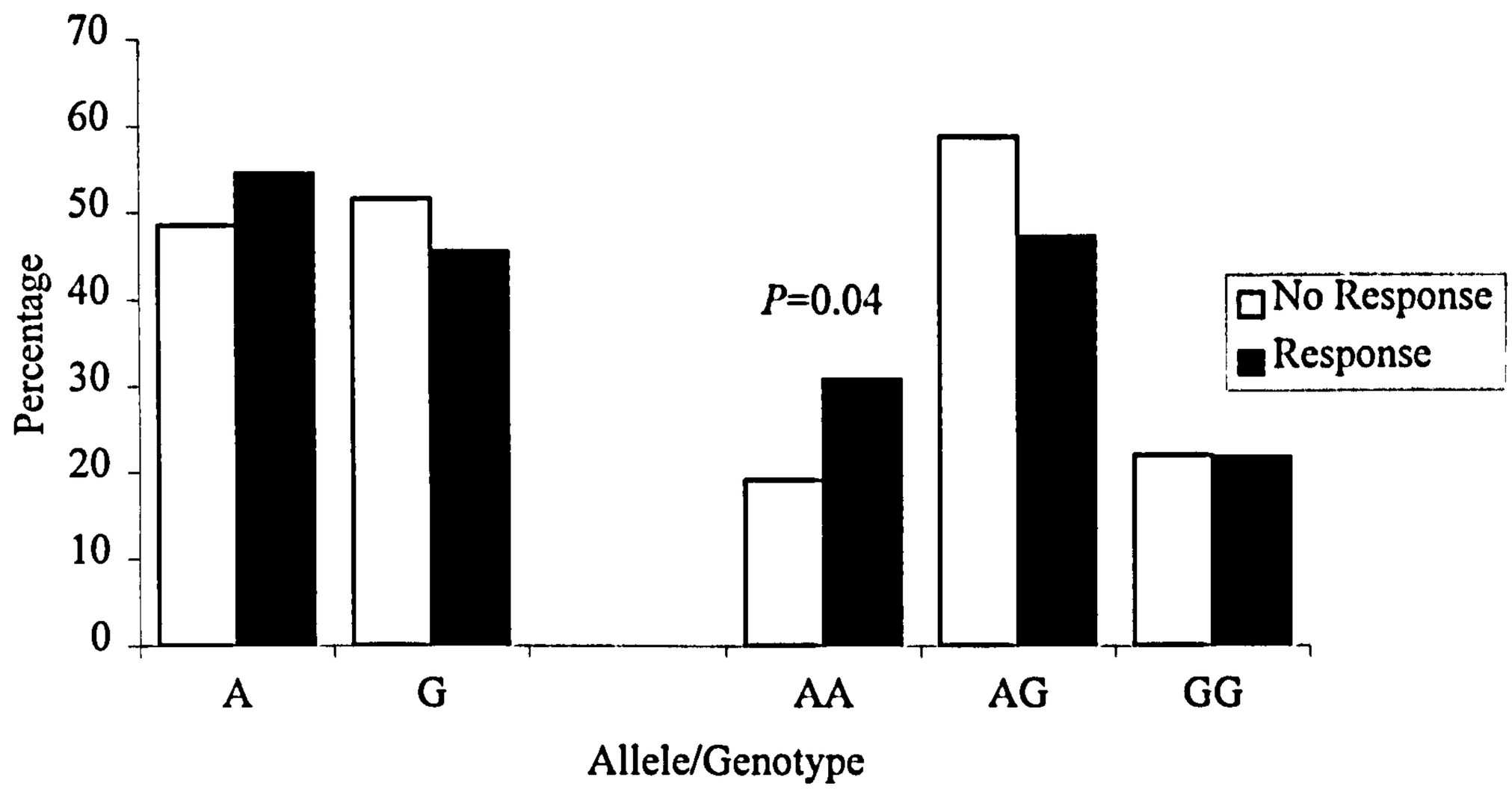


Figure 5.3: The proportion of A-1012G alleles and genotypes in patients who responded to Dovonex treatment compared to patients who did not respond to treatment. The AA genotype is significantly over-represented in patients who responded to Dovonex treatment.

	Allele	Occurrence		Family History		Age of Onset		Second-line Treatment		Calcipotriol Response	
		Psoriasis (*n*=205)	Control (*n*=80)	Negative (*n*=117)	Positive (*n*=88)	≥40 years (*n*=30)	<40 years (*n*=175)	Received (*n*=58)	Not received (*n*=147)	Response (*n*=137)	No response (*n*=61)
A-1012G											
	G	0.48	0.52	**0.53**	**0.41**	0.53	0.47	0.47	0.48	0.46	0.53
	A	0.46	0.54	**0.47**	**0.59**	0.47	0.53	0.53	0.52	0.54	0.47
		P=0.7		***P*=0.01**		*P*=0.4		*P*=0.9		*P*=0.2	
Fok1											
	f	0.39	0.36	0.41	0.37	0.32	0.41	0.42	0.38	0.39	0.42
	F	0.61	0.64	0.59	0.63	0.68	0.59	0.58	0.62	0.61	0.58
		P=0.5		*P*=0.3		*P*=0.2		*P*=0.4		*P*=0.5	
Taq1											
	t	0.36	0.44	0.35	0.36	0.37	0.35	0.39	0.34	**0.32**	**0.42**
	T	0.64	0.56	0.65	0.64	0.63	0.65	0.61	0.66	**0.68**	**0.58**
		P=0.07		*P*=0.9		*P*=0.8		*P*=0.4		***P*=0.05**	

Table 5.2: The association of *VDR* alleles with occurrence, family history, age of onset, use of second-line treatment and response to Dovonex. Bold figure represent statistically significant results.

	Genotype	Frequency of genotype in psoriatic patients	Psoriasis occurrence	No family history (n = 117) vs positive family history (n = 88)	Age onset ≥ 40 years (n = 30) vs < 40 years (n = 175)	Second-line treatment (n = 58) vs no second-line treatment (n = 147)	Calcipotriol response (n = 137) vs no calcipotriol response (n = 61)
A-1012G	GG/AG		1	1	1	1	1
	AA	0.26	0.92 (0.51–1.64) *P* = 0.8	**0.53 (0.28–0.99)** ***P* = 0.04**	0.85 (0.34–2.12) *P* = 0.7	0.77 (0.38–1.58) *P* = 0.5	**2.18 (1.00–4.71)** ***P* = 0.04**
FokI	ff/Ff		1	1	1	1	1
	FF	0.36	0.80 (0.46–1.40) *P* = 0.4	0.77 (0.43–1.36) *P* = 0.4	1.95 (0.89–4.26) *P* = 0.09	1.22 (0.65–2.29) *P* = 0.5	1.47 (0.77–2.82) *P* = 0.2
TaqI	tt/TT		1	1	1	1	1
	TT	0.40	1.49 (0.83–2.68) *P* = 0.2	1.07 (0.61–1.88) *P* = 0.8	0.87 (0.39–1.94) *P* = 0.7	1.01 (0.54–1.88) *P* = 1.0	**1.97 (1.04–3.76)** ***P* = 0.03**
A-1012G/FokI	Rest		1	1	1	1	1
	AAFF	0.09	0.70 (0.30–1.62) *P* = 0.4	**0.24 (0.08–0.69)** ***P* = 0.005**	1.10 (0.30–4.02) *P* = 0.9	1.96 (0.75–5.16) *P* = 0.2	**4.11 (0.92–18.38)** ***P* = 0.03**
A-1012G/TaqI	Rest		1	1	1	1	1
	AATT	0.12	2.26 (0.76–6.73) *P* = 0.1	0.66 (0.29–1.53) *P* = 0.3	1.55 (0.53–4.51) *P* = 0.4	0.98 (0.39–2.50) *P* = 1.0	**5.64 (1.28–24.82)** ***P* = 0.005**
FokI/TaqI	Rest		1	1	1	1	1
	FFTT	0.18	2.21 (0.89–0.51) *P* = 0.09	1.08 (0.52–2.23) *P* = 0.8	1.90 (0.77–4.69) *P* = 0.2	1.79 (0.84–3.81) *P* = 0.1	**3.22 (1.18–8.74)** ***P* = 0.01**

Table 5.3: The association of VDR genotypes and genotype combinations with occurrence, family history, age of onset, use of second line treatment and Dovonex response. In each case, odds ratio for the more active genotype is shown compared to residual genotypes.

5.4. Discussion

In general, the findings support the hypothesis that the A allele of A-1012G, in association with the more active F and T alleles of Fok1 and Taq1 would be beneficial in psoriasis. The AA genotype was under-represented in patients with no family history and the AAFF genotype combination significantly associated with occurrence in patients with no family history. All three polymorphisms were associated with response to calcipotriol treatment.

The AA genotype was significantly under-represented in patients with no family history of psoriasis compared to patients with a family history. The AAFF genotype combination showed a similar, stronger, association and AAFF was also significantly under-represented in patients without a family history compared to controls. Family history was defined for the purposes of this study as the existence of an affected parent or sibling. Psoriasis is a heritable disease, with the most significant susceptibility locus in the major histocompatability complex on chromosome 6p21, although the genes responsible for the heritability of psoriasis have not yet been defined. It seems likely that the presence of a risk genotype at 6p21 or at one of the other susceptibility loci has a stronger affect on disease occurrence than polymorphism of the *VDR* and in the case of patients with a family history of psoriasis, these effects overwhelm the affects of the A-1012G and Fok1 polymorphisms. In patients where the stronger susceptibility alleles are not present, the VDR genotypes become significant. However, the candidate susceptibility genes at 6p21, *corneodesmosin* (Tazi Ahnini et al., 1999), *HCR* (Asumalahti et al., 2000), *SEEK 1* and *SPR1* (Holm et al., 2003) are more likely to be expressed in the psoriatic keratinocytes than in the lesional T-cells, so even in patients with a family history, A-1012G acting in T-cells might be expected to modify the inflammatory response. It may be that it is the more general anti-proliferative, pro-differentiation affects of the *VDR* on the keratinocytes that are overwhelmed by the presence of the stronger susceptibility alleles, so that any allelic associations lose significance.

There was no association of age of onset with any of the polymorphisms. Early age of onset has been associated with the presence of a family history of psoriasis (Henseler and Christophers, 1985; Swanbeck et al., 1995) so it might have been expected that the A-1012G and Fok1 polymorphisms would be linked to age at onset, given their

association with family history. The association of FF with age of onset greater than 40 years approached significance (OR=1.95 (0.89-4.26), P=0.09) but no similar trend was seen for A-1012G.

There appeared to be a very weak association, that was not statistically significant, of A-1012G with less severe disease by all of the measures used to assess severity. No similar trends were seen for FokI and TaqI. This may be due to the difficulty in assessing severity of psoriasis. A PASI provides a measure of severity at a given time, but due to the cyclical nature of the disease, this may not give a good estimation of long term severity. In order to address this, the chronic basal and chronic maximal indices were used as severity measures. However, these are dependent on patient estimations of greatest and least involved area and may be less accurate because of this. Recourse to second line treatment was also used as an indicator of more severe disease but this may be confounded by other factors, such as the involvement of more sensitive areas of the skin and the patient's tolerance of the disease.

The strongest associations of the *VDR* genotypes were seen with response to calcipotriol treatment. AA and TT genotypes at the A-1012G and TaqI loci were significantly associated with response to treatment and AAFF, AATT and FFTT genotype combinations were also associated with calcipotriol response. This was to be expected, as treatment with the ligand of the VDR can be expected to accentuate any polymorphic affects on the receptor. Further, by adding the ligand in pharmacological concentrations, the variable becomes less dependent on the vagaries of endogenous ligand levels, that is serum levels of $1,25(OH)_2D_3$. Serum levels have been shown to vary considerably between individuals, dependent particularly on diet, sun exposure and skin type. The level of ligand is likely to influence the magnitude of any polymorphic affect, and therefore is likely to be a confounding factor in any association analysis of polymorphisms of the *VDR*. However, patients treated with calcipotriol are likely to have higher cutaneous levels of ligand for the VDR, reducing the confounding affect of variable endogenous levels of $1,25(OH)_2D_3$ on the association analysis. This affect may also have weakened the associations of genotype with the other psoriasis variables tested.

The analysis of calcipotriol response was based on patient-reported response to treatment. This may introduce inaccuracies, although by comparing patients with no response to those reporting any response, this variable should have been minimised.

However, the variability introduced by self-reporting would be expected to reduce the significance of the associations and it may be that a clinician-assessed study would find more significant associations.

The hypothesis was based on the predicted down-regulation of the Th1 response in the A allele of A-1012G and the amplifying affects of the more active alleles of Fok1 and Taq1. This hypothesis is supported by the results. Recent findings have suggested that the effective psoriasis treatments PUVA (Klosner et al., 2001) and fumaric acid esters (Ockenfels et al., 1998) act by switching the T-cell response from Th1 to Th2, suggesting that the hypothetical mechanism of A-1012G action is likely to be effective in psoriasis. As predicted, the more active F and T alleles of Fok1 and Taq1 amplified the effect of A-1012G. These polymorphisms are also likely to affect the actions of VDR in psoriatic keratinocytes to reduce proliferation and induce terminal differentiation. Unlike in melanoma, the more active *VDR* alleles are likely to be beneficial in psoriasis through the suppression of both the affected skin cells and the immune response. It is therefore difficult to determine which of these mechanisms is dominant. Furthermore, it is not known whether GATA-3 is expressed in psoriatic keratinocytes. GATA-3 expression has been demonstrated in the specialised keratinocytes of the hair follicle (Kobielak et al., 2003) but expression in keratinocytes of the normal skin has not been determined. If GATA-3 were expressed in psoriatic keratinocytes, A-1012G, Fok1 and Taq1 could all act in both the keratinocytes themselves and the lesional immune cells.

5.5. Conclusion

These findings supported a role for VDR in the aetiology of psoriasis. The lack of association of *VDR* genotypes with occurrence in patients with family history suggests that VDR has a modifying rather than an initiating affect on the disease and that other genetic determinant are greater risk factors for susceptibility. Unsurprisingly, response to calcipotriol treatment was strongly associated with VDR genotype. If these findings are supported by further studies, VDR genotyping may be useful in predicting response to treatment and determining the most appropriate treatment for each patient.

6. Functional Analysis of A-1012G

6.1. Introduction

A-1012G genotype has been shown to have an affect on susceptibility and prognosis in malignant melanoma and on non-familial susceptibility and calcipotriol response in psoriasis. All of these findings support the hypothesis that the A allele of A-1012G is associated with a down-regulation of the Th1 response, through GATA-3 binding at the A-1012G locus. Based on this hypothesis, the A allele would cause reduced immunosurveillance in the case of prostate cancer and malignant melanoma, leading to increased risk both of occurrence and metastasis. In psoriasis, however, the suppression of the Th1 response is beneficial, reducing the inflammatory response and suppressing the production of Th1 cytokines such as IFN-γ and TNF-α which have been shown to cause keratinocyte hyperproliferation.

Here, the mechanism of action of A-1012G is examined at the cellular and molecular level. In order to assess the polarising affect of A-1012G in T-cells, peripheral blood mononuclear cells were isolated from healthy donors of known genotype and their response to $1,25(OH)_2D_3$ treatment assessed *in vitro*. At the molecular level, gel-shift assays were used to determine the binding of transcription factors at A-1012G in the case of the A and G alleles.

6.1.1. Gel-Shift Assays

Gel-shift or electro-mobility shift assays (EMSA) are an established method for studying transcription factor binding to promoters. Double stranded DNA probes representing the promoter region of interest, in this case containing the different alleles of A-1012G, are labelled and mixed with nuclear proteins. The samples are then separated on a polyacrylamide gel. DNA probes that do not bind protein run through the gel relatively quickly while DNA probes with protein bound form a much larger complex and their progress through the gel is retarded or shifted. The labelled DNA probes are detected and any protein-DNA interaction will appear as a shifted band. Typically, gel-shift probes have been labelled with a radioisotope and the gels dried and exposed to x-ray film. Here, a non-radioactive method was developed through the

synthesis of biotin-labelled oligonucleotides which were annealed to form a double-stranded, biotin-labelled probe. Following poly-acrylamide gel electrophoresis, the samples were transferred to a nylon membrane and visualised by chemiluminescence.

6.1.2. T-cell Polarisation Assays

The hypothetical mechanism by which A-1012G exerts its effects in cancer and psoriasis involves the modification of the T-cell response from Th1 to Th2. The distinct signalling pathway and cytokine profile of the two responses enables the polarisation to be measured. As shown in ***figure 3.5***, the Th1 pathway involves signalling through stat-4, T-bet and increased IFN-γ expression while the Th2 pathway involves increased expression of stat-6, GATA-3, IL-4 and IL-5. By measuring the expression of these cytokines, the polarisation of T-cells to Th1 or Th2 can be assessed.

In this study, volunteers were genotyped and one individual of each genotype was asked to donate a blood sample. Peripheral blood mononuclear cells were isolated from the blood and cultured in the presence or absence of $1,25(OH)_2D_3$. mRNA was then extracted and gene expression assayed by RT-PCR. In this way, any polarising affect of $1,25(OH)_2D_3$ on T-cells of the different genotypes should be apparent. As only one volunteer was studied for each genotype, this experiment can only provide an indication of T-cell response in each genotype and act as a preliminary study for further work.

6.2. Materials and Methods

6.2.1. Materials

Anti-GATA-3 Antibody	Santa-Cruz Biotechnology
CD3/CD28 T-cell Expander DynaBeads	Dynal
Dulbecco's Modified Eagle Medium (DMEM)	BioWhittaker
Ficoll-Paque	Amersham Biosciences
Foetal Calf Serum (FCS)	BioWest
Hanks Buffered Saline Solution (HBSS)	BioWhittaker
Lipofectamine	Invitrogen
Lipofectamine Plus Reagent	Invitrogen
Minimum Essential Medium Eagle (EMEM)	BioWhittaker
Oligo d(T) DynaBeads	Dynal
Poly (dI-dC)	Sigma
Protein Assay (Bradford) Reagent	Bio-Rad
RPMI 1640 Cell Culture Medium	BioWhittaker
40% 39:1 Acrylamide : Bis-acrylamide	Flowgen
Tri-Reagent	Sigma

6.2.2. Buffers

Lysis Binding Buffer

100mM tris hydrogen chloride, pH 8.0, 500mM lithium chloride, 10mM EDTA, pH 8.0, 1% SDS, 5mM DTT

Wash buffer with SDS

10mM tris hydrogen chloride, pH8.0, 0.15mM EDTA, 0.1% SDS

Wash buffer without SDS

10mM tris hydrogen chloride pH 8.0, 0.15M lithium chloride, 1M EDTA

Nuclear Extract

Buffer A 10mM HEPES pH 7.8 10mM potassium chloride, 2mM magnesium chloride, 1mM DTT, 0.1mM EDTA, 1:100 dilution of protease and phosphatase inhibitor cocktails

Buffer B 10% Igepal CA630, 1:100 dilution of protease and phosphatase inhibitor cocktails

Buffer C 50mM HEPES pH 7.8 50mM potassium chloride, 300mM sodium choride, 1mM DTT, 0.1mM EDTA, 10% glycerol, 1:100 dilution of protease and phosphatase inhibitor cocktails

Gel-Shift Binding Buffer

20% Ficoll, 5mM magnesium chloride, 5mM DTT, 1:100 dilution of protease and phosphatase inhibitor cocktails

Sucrose Dye Solution

0.25% xylene cyanol, 0.25% bromophenol blue, 10% sucrose

Maleic Acid Buffer

100mM maleic acid, 150mM sodium chloride, pH8.0

Blocking Solution

1% (w/v) blocking reagent in maleic acid buffer

Wash Solution

0.3% (v/v) tween in maleic acid buffer

Solution A

Streptavidin solution A diluted 1:1000 in blocking solution

Solution B

Biotinylated alkaline phosphate solution B diluted 1:500 in blocking solution

CDP-Star Substrate Buffer

0.1M Tris HCl pH 9.5, 0.1M sodium chloride, 50mM magnesium chloride

6.2.3. GATA-3 Plasmids

Wildtype and mutant GATA-3 cloned into the pcDNA3.1 plasmid were kindly supplied by Prof. R.J. Thakker, May Professor of Medicine, University of Oxford .

6.2.4. Oligonucleotides

Oligonucleotides used for PCR and gel-shift probes are shown in ***table 6.1***.

6.2.5. Cell Culture

Jurkat cells were cultured in RPMI 1640 supplemented with 10% FCS. HeLa and Sk-mel-28 cells were cultured in MEM with 10% FCS. Human dermal fibroblasts were cultured in RPMI 1640 with 10% FCS.

6.2.6. Transfections

The day before transfection, HeLa cells were seeded in T175 flasks to give approximately 60% confluence on the day of transfection. 20μg plasmid was pre-complexed with 20μl Plus reagent in 600μl EMEM without FCS and incubated for 15 minutes at room temperature. 30μl Lipofectamine reagent was diluted in 600μl EMEM without FCS. DNA/Plus complexes were mixed with diluted Lipofectamine and incubated at room temperature for 15 minutes. Growth medium was removed from the cells, the cells were washed in HBSS and 10ml MEM without FCS was added. The plasmid DNA/Plus complexes in Lipofectamine were added to the cells, incubated for 3 hours at 37°C, 5% CO_2. The transfection medium was then removed and replaced with full growth medium. The transfected cells were cultured for 24 hours before the nuclear proteins were extracted.

Gel-Shift Probe	**Sequence**
A-1012G/A F Bt	^{Bt}GTAAGAGGCGAATAGCAATATCTTCCCTGGCTAAAGGAGG
A-1012G/A R	CCTCCTTTAGCCAGGGAAGATATTGCTATTCGCCTCTTAC
A-1012G/G F Bt	^{Bt}GTAAGAGGCGAATAGCAATGTCTTCCCTGGCTAAAGGAGG
A-1012G/G R	CCTCCTTTAGCCAGGGAAGACATTGCTATTCGCCTCTTAC
GATA-3 +VE F Bt	^{Bt}CCTTTATTAGGTGTCCTCTATCTGATTGTTAGCAATTATT
GATA-3 +VE R	AATAATTGCTAACAATCAGATAGAGGACACCTAATAAAGG
RT-PCR Primer	**Sequence**
T-BET RT F	TTGTGGACGTGGTCTTGGT
T-BET RT R	AGTCACGGCAATGAACTGG
IFN-γ F	GAGTGTGGAGACCATCAACGA
IFN-γ R	GGATTAAGTGAGACAGTCACAGG
GATA-3 F	CAATGCCTGTGGGCTCTACT
GATA-3 R	ATGCAAGTCGAAAGGGACTG
IL-4 F	GGCAGTTCTACAGCCACCAT
IL-4 R	TTCAGCTCGAACACTTTGAA
IL-5 F	TGAGGATGCTTCTGCATTTG
IL-5 R	TTTGGCCGTCAATGTATTTC
VDR F	GTTCCAGGTGGGACTGAAGA
VDR R	TCGGCTAGCTTCTGGATCA
p21 F	GACTTTGTCACCGAGACAC
p21R	TAGAAATCTGTCATGCTGG
β-actin F	TCATCACCATTGGCAATGAGCG
β-actin R	CTAGAAGCATTTGCGGTGGACG

Table 6.1: Oligonucleotides used to produce gel-shift probes and RT-PCR primers. ^{Bt}=biotin

6.2.7. Nuclear Extracts

Jurkat cells, which grow in suspension, were pelleted by centrifugation and washed in 15ml PBS. Medium was removed from adherent cell lines and the cells were detached by scraping, resuspended in 15ml PBS and pelleted by centrifugation. Cells were resuspended in 1ml PBS, transferred to 1.5ml eppendorfs and placed on ice. Cells were centrifuged for 30 seconds, 13,000 rpm at 4°C, resuspended in 400μl buffer A and incubated on ice for 15 minutes. 25μl buffer B was added and the samples mixed vigorously. The nuclei were pelleted by centrifugation, 13,000rpm, 30 seconds and resuspended in Buffer C. The samples were incubated with shaking at 4°C, 20 minutes and sonicated twice at 14-16 microns, 15 seconds, to disrupt the nuclear membrane. The samples were centrifuged, 13,000rpm, 5 minutes, 4°C and the supernatant transferred to a clean tube. Protein content was determined by the Bradford assay.

6.2.8. Bradford Protein Assay

Protein standards were prepared by dissolving bovine serum albumin in nuclear extract buffer C to give a range of concentrations between 0.1 and 10 mg/ml. Bradford reagent was diluted 1:5 in ultra-pure water. 1μl of each protein and standard, in duplicate, was added to 1ml Bradford reagent in a cuvette, and incubated for 15 minutes at room temperature. The absorbance of each sample and standard at 595nm was determined. A standard curve was constructed and used to determine nuclear extract protein concentration.

6.2.9. Preparation of Double-Stranded DNA Probes

A non-radioactive method was developed based on the method of Furia *et al.* (Furia et al., 2002). Forward and reverse strand oligos were synthesised and annealed to form double stranded probes as follows. The forward oligos were labelled with biotin at the 5' end. 25μl each of 200μM forward and reverse oligo were mixed, and heated to 95°C in a PCR machine for 15 minutes. The probes were then left in the PCR block to cool slowly for at least 2 hours. The probes were then diluted 1:100 for use in the gel-shift assays.

6.2.10. Gel-Shift Assays

10μl binding reactions were prepared containing 5μg nuclear extract, 1μg poly(dI-dC), in 1x binding buffer and incubated at room temperature for 5 minutes. 1μl probe was added and the reactions were incubated on ice for 30 minutes. For the supershift assays, 1μl antibody was added after 5 minutes. 1.5μl sucrose dye solution was added and the samples separated at 120V for 90 minutes on a 6% polyacrylamide gel in 0.5x TBE at 4°C. To improve binding specificity, the salt concentration of the binding buffer was increased for the assays with GATA-3 transfected cells. 125mM potassium chloride and 20mM HEPES was added to the binding buffer.

6.2.11. Transfer and Visualisation

Separated DNA was transferred from the gel to Biodyne B membrane at 300mA in 0.5x TBE at 4°C for 30 minutes. The membrane was then air-dried for 15 minutes, fixed by baking at 80°C for 30 minutes (later by cross-linking in a UV oven for 3 minutes) and blocked in blocking buffer for 30 minutes. The membrane was incubated in streptavidin solution A for 10 minutes and washed twice for 2 minutes in wash solution. BAP solution B was added for 10 minutes and the membrane again washed twice in wash solution. The membrane was again incubated in solution A for 10 minutes, washed twice for 2 minutes, incubated in solution B for 10 minutes and washed twice for 15 minutes. Substrate buffer was added for 5 minutes and CDP-star added at 1:250 dilution for 5 minutes. The membrane was exposed to x-ray film for 10 seconds to 1 minute and developed.

6.2.12. T-Cell Assays

Healthy volunteers, who had given written, informed consent, provided a buccal swab. DNA was extracted using the ChargeSwitch DNA extraction kit and genotyped as described in ***chapter 4.2.6***. Volunteers with AA, AG and GG genotypes were asked to give a 9ml blood sample.

The blood was diluted 1:1 with HBSS and layered on top of 9ml Ficoll-Paque. This was centrifuged at 2000rpm for 30 minutes, separating into an upper layer containing serum,

an interphase containing the mono-nuclear cells and a lower layer containing granulocytes. The upper layer was removed and the interphase transferred to a clean tube, washed in HBSS and counted. Cells were seeded at $1x10^5$ cells/ml and treated with 50nM $1,25(OH)_2D_3$ in ethanol. This is within the range used by Colin *et al.* (Colin et al., 2000) and similar to that used by Mahon *et al.* (50nM) (Mahon et al., 2003) and Boonstra *et al.* (40nM) to induce T-cell polarisation in mouse T-cells (Boonstra et al., 2001). Control cells were treated with vehicle only. T-cells were activated using 1.25µl/ml CD3/CD28 antibody bound beads, which hold CD3 and CD28 antibodies in close proximity to mimic the action of antigen presenting cells without the need for antigen (Trickett and Kwan, 2003). Cells were lysed into tri-reagent at day 0 and 7.

6.2.13. mRNA Extraction

500µl chloroform was added to 1ml cell lysis in tri-reagent, mixed and incubated at room temperature for 5 minutes. The sample was centrifuged at 13,000rpm, 4°C for 15 minutes, separating into an upper aqueous layer containing RNA, an interphase containing DNA and a lower organic layer containing protein. The aqueous layer was removed to a clean eppendorf and re-extracted by the addition of a further 500µl tri-reagent and 200µl chloroform, and centrifugation at 13,000rpm, 4°C, 10 minutes. The aqueous layer was transferred to a clean eppendorf and the RNA precipitated by the addition of 1µl mussel glycogen and 500µl isopropanol, standing at room temperature for 10 minutes and centrifuging at 13,000rpm, 4°C for 10 minutes. The pelleted RNA was washed in 70% ethanol and resuspended in 20µl of ultra-pure water and 80µl lysis binding buffer was added. MRNA was extracted from the total RNA using oligo d(T) Dynabeads. The dynabeads were reconditioned by pelleting in the magnet and twice resuspending in lysis binding buffer. 30µl reconditioned beads were added to the 100µl total RNA and allowed to bind at room temperature for 5 minutes. The beads with mRNA bound were washed twice in wash buffer with SDS and twice in wash buffer without SDS and were then resuspended in 30µl ultra-pure water.

6.2.14. RT-PCR

RT-PCR was carried out for Th1 cytokines T-Bet and IFN- and IFN-γ, Th2 cytokines GATA-3, IL-4 and IL-5, VDR and p21 and housekeeping gene β-actin using the

primers listed in *6.2.3* for 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds. Products were analysed on a 3% agarose gel. The intensity of bands was analysed using the Scion Image Version 4.02 (Beta) densitometry software.

6.3. Results

6.3.1. Gel-Shift Binding Assays

In order to assess binding at the A-1012G polymorphism, 40-mer biotin-labelled probes were generated to represent the A and G alleles. To demonstrate GATA-3 binding, a probe representing the GATA-3 binding element of the mouse IL-5 promoter was also used. Nuclear proteins were extracted from Jurkat cells, which express GATA-3, and binding compared to nuclear extracts from normal dermal fibroblasts, which do not. These results are shown in *figure 6.1*. Binding was demonstrated in Jurkat nuclear extract with both the A and G probes. However, while the G allele produced two bands, an extra band was apparent in the A allele, suggesting additional protein binding. A similar pattern to the G allele was seen with the positive control IL-5 GATA-binding probe, although this binding was weaker than that with the A and G probes.

To confirm whether the additional binding in the A allele was GATA-3, expression vectors for wildtype and mutant GATA-3 were transfected into HeLa cells which do not constitutively express GATA-3. Super-shift assays were carried out using an anti-GATA-3 monoclonal antibody. *Figure 6.2* shows the results of these assays. A triplet of bands were common to all lanes where nuclear extract was present.

The A probe with GATA-3-transfected nuclear extract showed a band that was not present in the untransfected HeLa cells or cells expressing mutant GATA-3. This band was not observed with the G probe. Furthermore, when anti-GATA-3 antibody was added to this sample, this band disappeared and a super-shifted band was observed. A super-shift did not occur in the GATA-3 transfected cells with the G probe.

A shift was also observed that occurred with all nuclear extracts but only in the G allele.

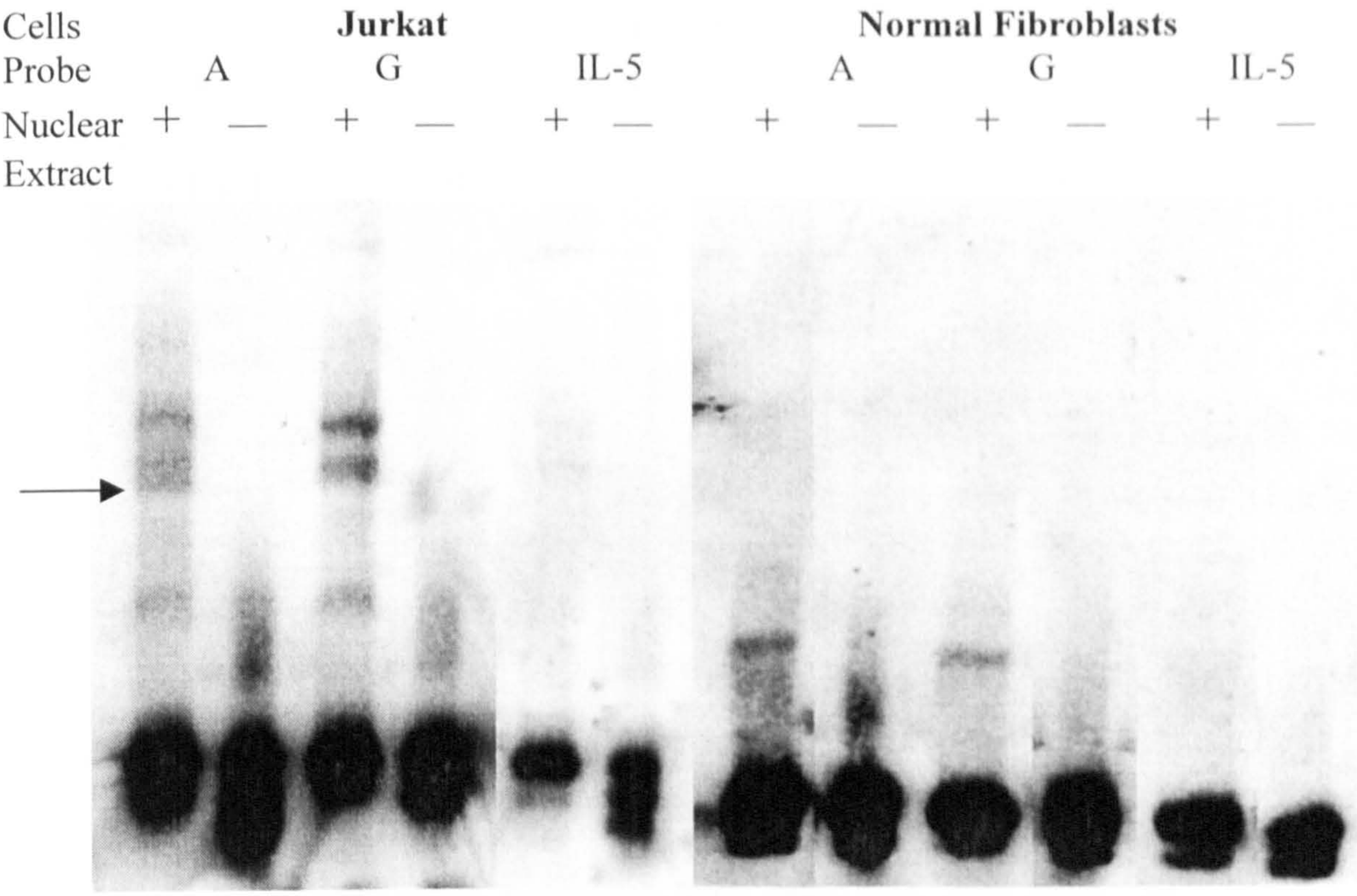


Figure 6.1: Gel-shift assays with nuclear extracts from Jurkat cell lines and normal dermal fibroblasts. Three probes were used, representing the A and G allele of A-1012G and the GATA-3 binding element of the IL-5 promoter. Binding reactions were in low salt buffer (5mM $MgCl_2$) and DNA was fixed to the membrane by baking at 80°C. The band representing differential binding in the A allele compared to the G allele is marked with an arrow.

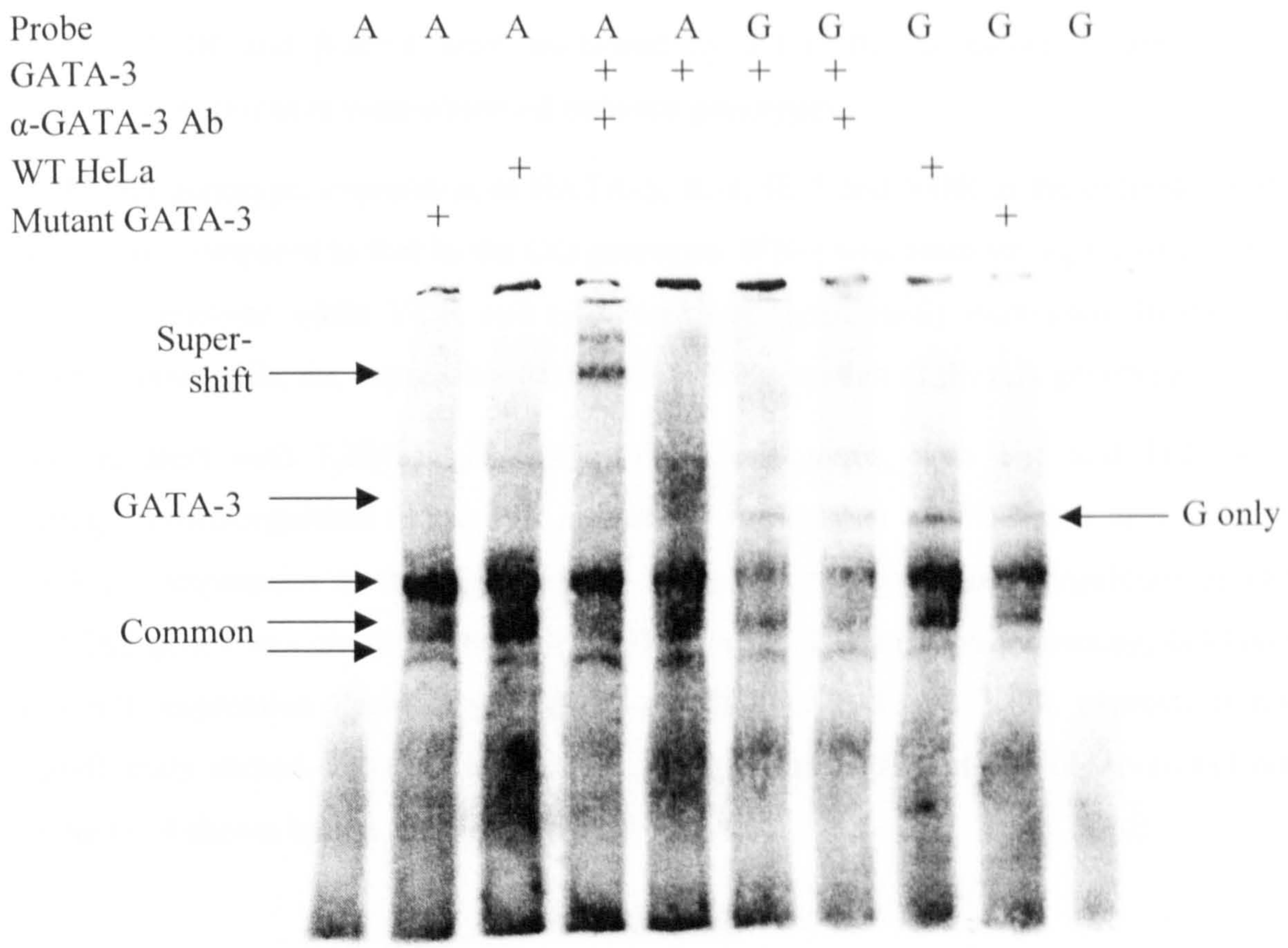


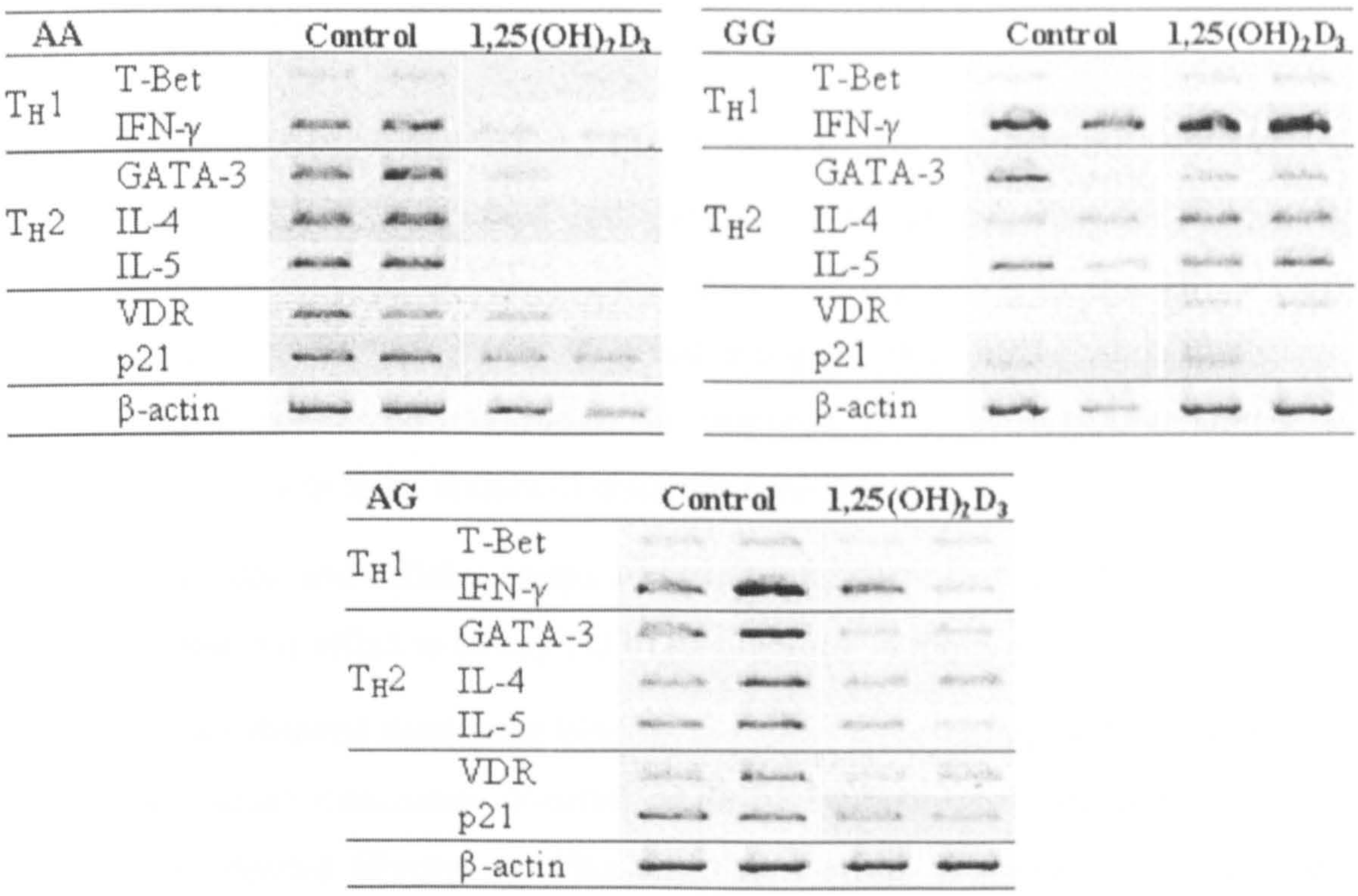
Figure 6.2: Gel-shift assay using GATA-3-transfected HeLa cells. Probes represent the A and G allele of A-1012G. Cells were transfected with expression plasmids for wildtype or mutant GATA-3 or were untransfected. Supershift assays were performed using anti-GATA-3 antibody. Reactions were in high salt buffer (5mM $MgCl_2$, 125mM KCl, 20mM HEPES) and DNA fixed to the membrane by UV crosslinking. A band found only in wildtype GATA-3-transfected cells with the A probe, representing GATA-3 binding and the supershift of this band are marked. A triplet of bands common to all lanes and a shift occurring only in the G allele are also marked.

6.3.2. T-cell Assays

T-cells from donors of each genotype, AA, AG and GG, were treated with $1,25(OH)_2D_3$, cultured for 7 days and the expression of Th1 and Th2 cytokines as well as p21, VDR and β-actin were measured by RT-PCR. As shown in ***figure 6.3***, significant differences were observed between genotypes.

In the AA genotype, expression of GATA-3, IL-4, IL-5 and VDR in the untreated cells was strong compared to that in the GG genotype. IFN-γ was more strongly expressed in the GG genotype while VDR and p21 were not significantly expressed. In the AG heterozygous cells, the expression profile was similar to that of the AA genotype.

On treatment with $1,25(OH)_2D_3$, all of the T-cell genes, both Th1 and Th2, were strongly down-regulated in the AA individual. Expression of VDR was also reduced while p21 expression remained fairly high. In the GG genotype, down-regulation of Th1 and Th2 genes was not observed, with T-Bet and IL-4 expression increasing, GATA-3 and p21 expression decreasing slightly and IFN-γ, IL-5 and VDR expression not significantly altered. In the AG genotype, some down-regulation was observed but not to the level shown by the AA genotype.



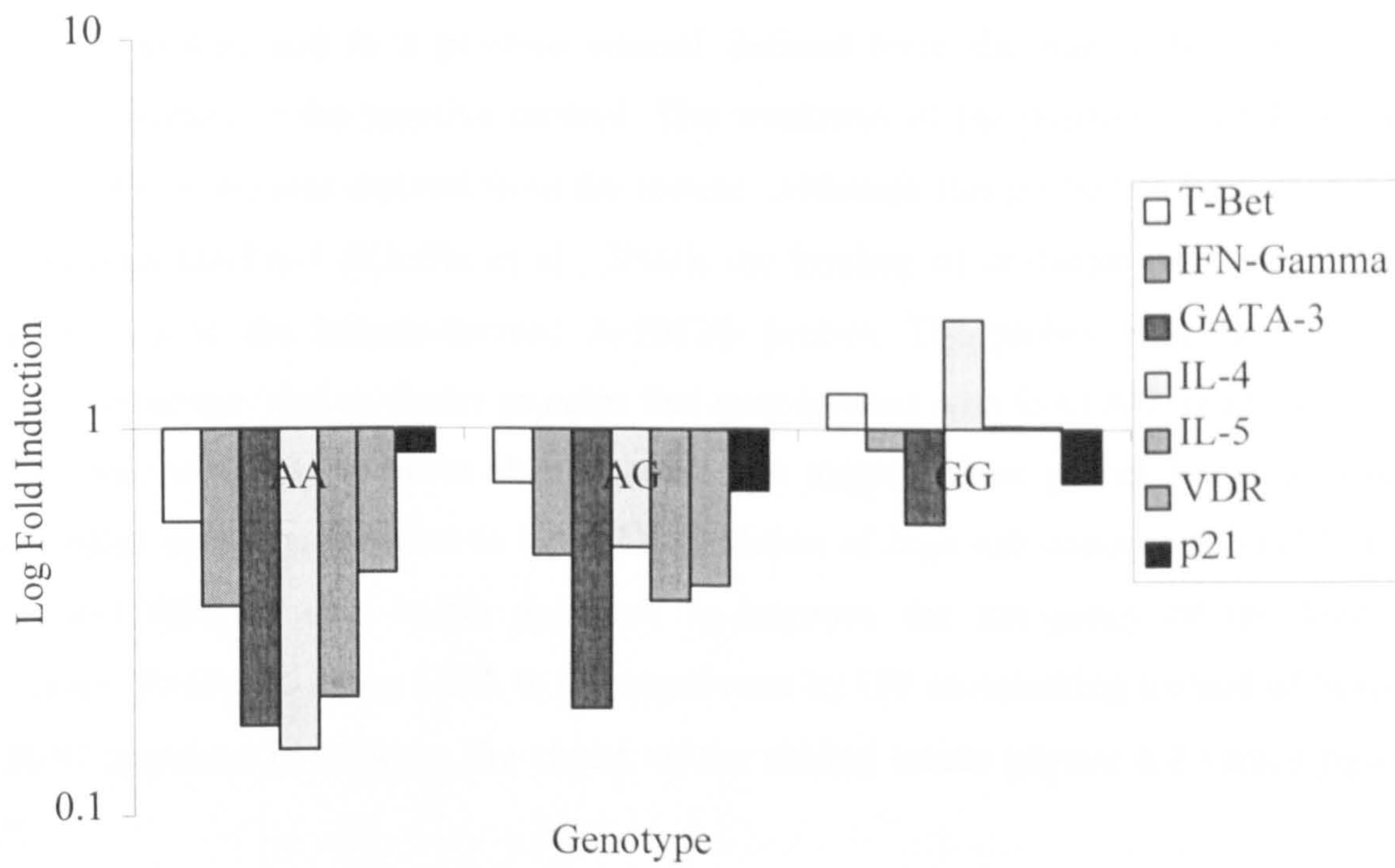


Figure 6.3: The results of T-cell polarisation assays. T-cells were treated for 7 days with 50nM $1,25(OH)_2D_3$, mRNA was extracted and RT-PCR carried out for T-cell cytokines, VDR, p21 and housekeeping gene β-actin. mRNA levels after 7 days are shown. The graph shows the fold induction of each gene by $1,25(OH)_2D_3$ treatment compared to expression in vehicle only treated cells following normalisation to β-actin expression. The results are the average of duplicate experiments.

6.4. Discussion

In this chapter, molecular and cellular assays were used to confirm the hypothesis that A-1012G genotype influences the T-cell response in malignant melanoma and psoriasis through GATA-3 binding in the A allele and down-regulation of the Th1 response. Binding at the A-1012G locus was assessed using gel-shift assays while the affect of genotype on T-cells gene expression in response to $1,25(OH)_2D_3$ treatment was analysed using T-cells from donors of different genotype.

Both the molecular and cellular assays appeared to confirm that A-1012G affects T-cell activity and that this effect is through GATA-3 binding in the A allele.

Gel-shift assays showed significant binding to probes representing both alleles in Jurkat cell nuclear extract (leukaemic T-cells) while no binding was observed with nuclear proteins from dermal fibroblasts. This suggests that the promoter region around A-1012G is active in T-cells. With Jurkat nuclear protein, two bands were common to the A and G probes and to a positive control derived from the mouse IL-5 promoter, although weaker in the positive control. The weakness of the positive control may be because the probe was derived from the mouse. Although this probe has been shown to bind human GATA-3 (Kieffer et al., 2002), the binding of co-factor proteins may be weaker than in the human-derived A-1012G probes. The probes were designed as 40mers to ensure that co-factor proteins that may interact with GATA-3 and bind to the DNA close to A-1012G were able to bind. The length of the probes may, however, allow other unrelated proteins to bind. The addition of high salt concentration (125mM KCl) and HEPES as a buffer appeared to improve the stringency of the binding reactions. Similarly, fixing DNA to the membrane by UV crosslinking instead of baking at 80°C appeared to improve the clarity of the shifted bands (*figure 6.2* versus *figure 6.1*).

An additional band was observed in the A allele compared to the G allele, suggesting that A-1012G does indeed affect transcription factor binding. It may be that the two common bands represent proteins of the GATA-3 complex while the additional band represents GATA-3 binding in the case of the A allele. It is also possible that the common bands are completely unrelated to GATA-3 and represent binding elsewhere in the probe. However, the similar migration patterns in the A-1012G probes and the IL-5

promoter positive control would suggest that the bands represent binding of the GATA-3 complex.

To confirm that the binding patterns observed did represent differential GATA-3 binding in the A and G alleles, wildtype or mutant GATA-3 was expressed in HeLa (human cervical carcinoma) cells which do not usually express GATA-3. An antibody to GATA-3 was also used to perform super-shift assays. A shift was observed that occurred only with wild-type GATA-3-transfected nuclear extract and only in the case of the A allele. When an antibody to GATA-3 was added, the specific band was super-shifted, confirming that it does represent GATA-3 binding. This binding did not occur in mutant GATA-3-transfected cells or in untransfected cells. The mutant C318R GATA-3 contains a mutation in the second zinc finger, leading to loss of the zinc ion and elimination of DNA binding (Nesbit et al., 2004). The absence of binding in the mutant protein suggests that the observed binding is specific binding through the GATA-3 DNA-binding domain.

Gel-shift assays showed binding in the case of the G allele and not the A allele was weaker in cells transfected with wildtype of mutant GATA-3 than in untransfected HeLa cells. This is probably indicative of the over-expression of GATA-3 proteins in transfected cells and the concurrent under-expression of all other cellular proteins. This, and the absence of super-shift bands with the G probe, suggests that the binding is unrelated to GATA-3. *In silico* analysis (***chapter 3.3.3***) did not predict that any protein would bind exclusively to the G allele. This binding may be related to a further mechanism by which the G allele is protective in malignant melanoma and disadvantageous in psoriasis. In particular, GG was found to be protective against metastasis in melanoma, with no metastasis occurring in any GG individual. This might suggest a separate mechanism, additional to the affect of A-1012G in T-cells, which is particularly active in melanoma cells. HeLa cells are human cancer cells so may express similar proteins to malignant melanoma cells. Further gel-shift assays using nuclear extracts from melanoma cells would help to clarify whether transcription factor binding to the G allele and not the A allele is affecting A-1012G action.

T-cell polarisation assays were used to test the hypothesis that the A allele of A-1012G was linked to Th2 polarisation, through GATA-3 signalling. The T-cell assays did indeed show significant differences in response to $1,25(OH)_2D_3$ between the A-1012G genotypes. The affects, however, appeared to be a more general suppression of T-cell

cytokine expression, rather than a polarisation towards Th2. The AA and AG individuals both showed significant suppression of gene expression while expression was unaffected or increased in GG cells. Boonstra *et al.* (Boonstra et al., 2001) showed that $1,25(OH)_2D_3$ promoted Th2 polarisation in mice. This has not been confirmed in humans and was not observed here. Instead, the basal level of VDR appeared to be much higher in AA and AG genotypes, rendering the cells much more responsive to the suppressing affects of $1,25(OH)_2D_3$. The levels of the housekeeping gene β-actin appeared to drop slightly in AA cells, suggesting that AA cells were more sensitive to the anti-proliferative affects of $1,25(OH)_2D_3$ while GG cells were unaffected by $1,25(OH)_2D_3$ treatment.

It may be, therefore, that the A allele of A-1012G is acting to increase levels of the VDR in cells that express GATA-3, leading to increased sensitivity to $1,25(OH)_2D_3$ treatment. This modified hypothesis is still compatible with the observed affects of A-1012G in melanoma and psoriasis. The differential binding of GATA-3 at A-1012G localises its affects to T-cells. In the case of the A allele, VDR expression is relatively high due to up-regulation by GATA-3. This sensitises the cells to the suppressive affects of $1,25(OH)_2D_3$, leading to reduced immunosurveillance and increased risk of melanoma. In psoriasis, the protective affect is again caused by the increased sensitivity of T-cells to $1,25(OH)_2D_3$, reducing the secretion of IFN-γ, believed to modulate the inflammatory response and keratinocyte hyper-proliferation in psoriasis (Bata-Csorgo et al., 1995).

It must be stressed that these T-cell assays were only carried out in one individual of each genotype, so no firm conclusions can be drawn. To confirm these results, a larger study would be required using several volunteers of each genotype.

6.5. Conclusion

Gel-shift assays supported the hypothesis that A-1012G was affecting GATA-3 binding to the VDR promoter. The A allele was shown to bind GATA-3 while the G allele did not. T-cell assays appeared to confirm that A-1012G genotype affected T-cell response. A-1012G did not, however, affect T-cell polarisation, instead, presence of the A allele appeared to increase VDR levels, sensitising T-cells to $1,25(OH)_2D_3$ treatment.

7. General Discussion

The expression of the VDR in such a wide array of cells and tissues means that genetic variation of the receptor can have wide-range effects. The number of diseases and phenomena that VDR polymorphisms have been associated with, from rickets, osteoporosis and fracture risk to prostate and breast cancer to diabetes and psoriasis, exemplifies this.

In the skin, the abundance of ligand for the VDR is likely to exaggerate any effects. Vitamin D is formed in the skin and keratinocytes have the ability to both 25- and 1α-hydroxylate it to produce the active hormone. It is not surprising, then, that the novel *VDR* polymorphism A-1012G is important in two very different skin disorders, malignant melanoma and psoriasis.

7.1. VDR *Gene Structure*

The structure of the *VDR* gene provides a potential mechanism whereby VDR expression can be differentially regulated and the protein have different functional effects. Six exons upstream of the wildtype translation start codon in exon 2 can be alternatively spliced and the regions upstream of at least four of these exons, 1a, 1d, 1f and 1c, have been reported to show promoter activity. To add further complexity, an alternative start codon in exon 1d can produce alternative proteins, VDRB1 and VDRB2 with significantly extended amino-terminal regions. The AF-1 region is contained within the amino-terminal region of nuclear transcription factors and affects protein interactions and cellular localisation. Gardiner *et al.* (Gardiner and Eisman, 2003) have shown that the VDRB1 localises in nuclear speckles in the absence of ligand, changing to diffuse nuclear localisation upon $1,25(OH)_2D_3$ treatment. This appears to rule out the possibility that VDRB1 is a membrane receptor for $1,25(OH)_2D_3$ and offers little explanation for the function of the alternative protein.

Apart from encoding an alternative protein, the alternative splicing of the 5' exons produces mRNA molecules with different 5' ends, which may provide binding sites for different translational proteins and allow different transcripts to be translated in different tissues. Perhaps the most important function of the 5' variation is to allow the VDR expression to be controlled by different promoters. This could provide a

mechanism for VDR to be differentially expressed in response to different transcription factors controlled by different signalling pathways.

Here, the conservation of the VDR gene sequence across species was studied to determine to what extent the 5' complexity is conserved through evolution and therefore how important it is to basic VDR function.

Comparison of the human sequence with the frog VDR highlighted the major functional domains of the VDR with only exons 2-4 and 6-9, coding for the DNA and ligand binding domains, conserved.

Comparing the human sequence with the mouse and rat *VDR*, evidence exists for the conservation of alternative exons, promoters and proteins. Comparison of the exons showed that exon 1d, containing the alternative start codon was well conserved. While exons 1f and 1e were not well conserved, the promoter region upstream of exon 1f showed a well-conserved region of transcription factor binding sites, suggesting that, although the sequence of the exon has evolved beyond recognition, transcription still occurs from an exon 1f in mouse and, furthermore, the exons are under similar transcriptional control in mouse and human. Given the non-coding nature of the exon, this change of sequence is not unlikely. The conservation of the promoter region suggests that expression of VDR from this promoter region is important to VDR activity, perhaps in a specific cell type or tissue.

The conservation of the 1a promoter region, and the type of transcription factor binding sites present, including two Sp1 sites, supports the findings of Crofts *et al.* (Crofts et al., 1998) that the majority of VDR transcripts originate in exon 1a. The fact that exon 1a is within a GC-rich CpG island, and is therefore susceptible to control by methylation also suggest that the 1a region is an important region for gene regulation. Strikingly, the 1f region was also shown to be within a CpG island, suggesting that regulation of gene expression through the 1f region is also important.

The 1c promoter region has recently been studied and shown to be hormone responsive, sensitive to regulation by $1,25(OH)_2D_3$ and oestrogen through Sp1 signalling (Wietzke et al., 2005). However, TraFac analysis did not show particularly strong conservation of the 1c promoter region. While the 1c promoter region may confer hormone responsiveness, transcripts beginning in exon 1c have only been reported in two breast cancer cell lines (Byrne et al., 2000; Wietzke and Welsh, 2003) and were not found in

the more extensive panel of cell types studied by Crofts *et al.* (Crofts et al., 1998). Although it is possible that the 1c promoter could regulate the expression of transcripts initiating in the upstream exons, it seems likely that the 1a promoter region is the major constitutive promoter while the 1c promoter has a more minor modifying affect.

There is little evidence to suggest how transcription is differentially initiated in exon 1a or 1d. The two exons are separated by only 400 bases and largely share the same promoter region, that upstream of exon 1a. The region between 1a and 1d does not show any significant promoter activity (Crofts et al., 1998) and here, the region was found to show little conservation, despite the homology of exon 1d between species. It may be that transcription is initiated in one exon or the other by the assembly of differing transcription complexes in response to the availability of transcription factors in different cell and tissue types.

Considering the coding region of the gene, the most evolutionarily varied exon is exon five, coding for the hinge region between the DNA and ligand binding domains. This highlights these two domains as the most basically functional domains of the receptor. The alteration within the hinge region may reflect the fact that while the structure of DNA and of $1,25(OH)_2D_3$ has remained constant through evolution, other proteins that interact with the receptor have evolved, such that the shape and size of the DNA and ligand binding domains have not been required to evolve while the region between the two has altered in size and shape to incorporate differing interactions between the VDR and other proteins involved in the VDR-DNA complex.

7.2. *Genetic Variation of the* VDR

Genetic variation of the *VDR* has been associated with many and varied phenomena but this research has focused almost exclusively on the four restriction fragment length polymorphisms, Fokl, Bsml, Apal and Taql and a length polymorphisms of a 3′ polyadenyl microsatellite. Of these, only Fokl has a clear functional effect, the variant producing an alternative translation start codon and a protein three amino acids longer than that encoded in the wildtype Fokl allele. Bsml, Apal and Taql have no affect on protein structure although they are in linkage disequilibrium with the polyadenyl length polymorphism which, it has been suggested, may affect mRNA stability, and therefore protein levels.

Here, the VDR gene was screened for novel variation. It was considered that single nucleotide polymorphisms within promoter regions were likely to have distinct functional affects, given the nature of transcription factor binding. Transcription factors bind to short sequences within promoter regions and single base changes within these binding sites are likely to have significant affect on binding. As discussed above, the 1a promoter region appears to be the major VDR promoter region, apparently controlling transcription from exon 1a, the major site of transcription initiation and exon 1d, containing the alternative start codon. The 1a promoter region was therefore screened for variation.

The A-1012G polymorphism was discovered and has since been confirmed in a submission to the NCBI SNP database. A further common polymorphism was reported at G-1520C.

In silico analysis suggested that both polymorphisms would affect transcription factor binding. A-1012G was predicted to bind GATA-3 in the A allele and not in the G allele. G-1520C was predicted to bind GATA-2 in the G allele and Sp1 or CCAAT enhancer binding protein (C/EBP) in the case of the G allele.

The simple polymorphic affect on GATA-3 binding at A-1012G, the clear function of GATA-3 in T-cell development (Rengarajan et al., 2000) and the reported involvement of $1,25(OH)_2D_3$ and the VDR in Th2 polarisation through GATA-3 signalling (Boonstra et al., 2001) enabled the formulation of a hypothesis for A-1012G function.

It was hypothesised that the A allele of A-1012G would allow GATA-3 binding to the VDR promoter, up-regulating VDR expression. This would increase the sensitivity of T-cells to $1,25(OH)_2D_3$, increasing the Th2 polarising affect of $1,25(OH)_2D_3$ and concurrently down-regulating Th1 response.

It was therefore predicted that A-1012G would have an affect in malignant melanoma and psoriasis, two diseases where *VDR* polymorphisms have been shown to have an affect and where the T-cell response is important in disease aetiology.

In malignant melanoma the Th1 response has been shown to be more effective in combating tumour development (Tatsumi et al., 2002). It was predicted that the A allele of A-1012G, associated with down-regulation of the Th1 response, would be associated with tumour development and metastasis.

In psoriasis, the inflammation and hyper-proliferation of keratinocytes has been shown to be caused by an overactive Th1 response (Austin et al., 1999). Furthermore, vitamin D analogues are a commonly used and successful treatment of psoriasis. It was therefore predicted that the A allele of A-1012G, associated with a reduced Th1 response would be associated with reduced occurrence, reduced severity and improved response to vitamin D analogue treatment.

7.3. VDR *Genotype in Malignant Melanoma*

The hypothesis was confirmed in malignant melanoma, with the A allele of A-1012G associated with increased risk of melanoma and with increased risk of metastasis. No metastasis was observed in melanoma patients with the GG genotype, suggesting that the G allele is strongly protective. The AG heterozygote showed a risk of melanoma and of metastasis intermediate between that of GG and AA genotypes, suggesting a co-dominant or allele dosage affect.

The FokI genotype was shown to increase the risk of metastasis in combination with the A allele at A-1012G. However, both FokI homozygous genotypes, in combination with AA at A-1012G, were associated with increased risk compared to residual genotypes, suggesting that the observed affect does not correlate to a true molecular function and may be an artefact of the relative rarity of the homozygous genotype combinations. Alternatively, it may be indicative of the different affects of the VDR in different cell types. According to the hypothesis studied here, a more active VDR would be associated with increased immunosuppression in T-cells and therefore lead to increased metastatic risk. However, in melanoma cells, a more active VDR might be associated with decreased proliferation and increased differentiation, which would be expected to slow tumour development.

The G-1520C promoter polymorphism was not associated with occurrence or metastasis. However, the G-1520C/A-1012G haplotype CA was significantly increased in melanoma compared to controls. Indeed, the increase in the AC haplotype was equivalent to, and could account for, the increase in the A allele of A-1012G. This suggests that binding of transcription factors at the two sites on the same allele has a much greater affect than the sum of the individual affects. The two transcription factors predicted to bind to the C allele of G-1520C are both relatively general transcription

factors and it would be expected that the affect of binding of a general transcription factor at G-1520C would up-regulate gene expression independently of A-1012G genotype and be additional to any affect of binding at A-1012G. However, GATA-3 is a much more cell-specific transcription factor, with its expression limited to relatively few cell types, particularly T-cells. The affect of the A allele at A-1012G might be to localise the affect of the polymorphism in T-cells, where it is then amplified by the binding of Sp1 or C/EBP at G-1520C. Thus the affect of G-1520C would only be observed in T-cells in the presence of the C allele at G-1520C with A of A-1012G on the same allele.

The AC haplotype was not increased in metastasis, suggesting that different mechanisms may be involved in tumour development and metastasis.

When the cohort was stratified according to A-1012G genotype and tumour thickness, a very high risk of metastasis was seen in individuals with AA genotype and the thicker tumours. Even in patients with thinner tumours, a significant risk of metastasis was observed with the AA genotype. No metastasis was observed in GG individuals, even in those with the thickest tumours. This finding is potentially clinically significant. Breslow depth is currently the best prognostic indicator in melanoma. If A-1012G genotype was also considered, the accuracy of prognostic prediction could be significantly improved. However, because of the rarity of metastasis in the melanoma cohort, stratification according to genotype and tumour thickness produced very small groups, reducing the strength of these findings. To confirm the pattern, it would be necessary to significantly increase the number of metastases analysed, and to measure risk the number of non-metastatic melanoma would need to be increased proportionally. The rarity of the GG genotype in melanoma caused the stratified groups in the GG genotype to be very small, including from 2-5 cases. Metastasis was observed in approximately 10% of the melanomas studies so to increase the number of metastases to 100, which, assuming an equivalent distribution to that observed in this study, would give tumour depth/genotype groups of at least 10, an overall cohort of 1000 patients would be required. While this is a large number, the genotyping of DNA extracted from paraffin-fixed metastatic tissue might make it practical to study larger numbers than are possible using DNA from patient blood samples, as was used in this study.

7.4. VDR *Genotype in Psoriasis*

In psoriasis, the hypothesis was largely confirmed. While no association was observed with A-1012G genotype in patients compared to controls, the A allele was significantly under-represented in patients without a family history of psoriasis compared to those with a family history. Psoriasis is, to a debatable degree, a genetic disease (Elder et al., 1994). It seems that the presence of a stronger psoriasis susceptibility gene is enough to overwhelm any affect of *VDR* variation while in patients without a family history *VDR* polymorphisms become more important. Although the A allele was not significantly under-represented in patients with no family history, it is likely that the association exists but is masked to a certain extent by the presence of stronger susceptibility loci.

No association was found with severity of psoriasis. The A allele was associated with reduced severity by every measure used but the associations did not approach significance. This may be due to the difficulty in assessing severity. Psoriasis is a cyclical disorder and the point at which the patient presented may not be representative of the mean severity of their disease. The maximal and basal indices of severity were introduced in an attempt to provide more objective measures but these rely on patient estimation of worst and best case severity and this may introduce too great an error into the calculation. Furthermore, the patients recruited to this study were patients presenting at dermatology clinics. This may introduce a bias towards more severe psoriasis in that patients with mild psoriasis either do not see a doctor at all or are treated by their GP and only the more severe cases are referred to the dermatologist. It may be that the cohort does not include enough mild cases of psoriasis to see an association with severity.

As predicted, the A allele at A-1012G was associated with response to calcipotriol treatment. Given the application of pharmacological levels of ligand, it is not surprising that this gives the strongest associations with *VDR* polymorphisms.

In psoriasis, the association of *VDR* haplotypes were more straightforward than in melanoma, with the F and T alleles of the Fok1 and Taq1 polymorphisms, associated with increased VDR activity, amplifying the affect of the A-1012G polymorphism. In psoriasis, unlike melanoma, the affects of VDR do not conflict in the different cell types involved. While in melanoma the suppression of T-cells is a disadvantage but the suppression of melanoma cells would be an advantage, in psoriasis, the suppression of

both the T-cells and the psoriatic keratinocytes would be an advantage, reducing both the inflammatory response and the hyper-proliferation of the keratinocytes.

7.5. Molecular and Cellular Effects of A-1012G

The gel-shift assays at A-1012G confirmed GATA-3 binding in the A allele and not in the G allele. The presence of several other shifts on the same probe suggested the region is an active promoter with several transcription factor binding sites present in the region. A band-shift that occurred in the G allele and not the A allele suggests that there may be a further mechanism of A-1012G action. This does not appear to be related to GATA-3 signalling. This may account for the apparently different mechanism of A-1012G action in melanoma occurrence and metastasis. To determine what this protein is, extraction of the band from the gel-shift gel and analysis by mass spectrometry may be useful, although it might be difficult to extract enough protein from the small amount that causes a gel-shift.

Gel-shift assays were not carried out at the G-1520C locus. These would confirm how G-1520C affects transcription factor binding and would confirm whether GATA-2 does indeed bind in the G allele while Sp1 or C/EBP bind in the A allele, as suggested by *in silico* prediction or whether the affect is simply the differential binding of one of those proteins.

T-cell assays appeared to show that A-1012G genotype affected T-cell response to $1,25(OH)_2D_3$ treatment. However, the affect seemed to be a more general suppression of gene expression rather than a polarisation to Th2 as hypothesised. VDR levels were significantly higher in AA T-cells compared to GG and this made them more susceptible to $1,25(OH)_2D_3$ treatment. This still supports the hypothesis as in both melanoma and psoriasis, the reduction of the Th1 response was predicted to cause the observed affects. It seems, therefore, that the affect of the A allele of A-1012G is to increase the levels of the VDR in cells expressing GATA-3, particularly T-cells, and to sensitise these cells to the affects of $1,25(OH)_2D_3$.

7.6. Further Work

The findings reported here are currently being investigated further, supported by a grant from The Psoriasis Association. This project has four main aims:

- To assess whether the association of A-1012G genotype with calcipotriol response can be confirmed in a larger, clinician assessed study
- To demonstrate whether calcipotriol does have a polarising affect on lesional T-cells in psoriasis
- To extend the study of genotype affect on *in vitro* T-cell response to $1,25(OH)_2D_3$ to include more significant numbers of each genotype
- To confirm the molecular mechanism of A-1012G action

The calcipotriol response study is being extended to include a greater number of patients and to introduce more controlled experimental conditions. The data presented in ***chapter 5*** were based on patient-assessed calcipotriol response and were subject to the inconsistencies of variability in treatment between patients. To improve the accuracy of the experiments, the patients will be asked to leave several test psoriatic plaques untreated for two weeks, a dermatologist will then assess the severity of their psoriasis. The test plaques will then be treated for four weeks with Dovonex and the response to this treatment again assessed by a dermatologist. This more objective assessment of treatment response is expected to increase the significance of genotypic associations.

To assess the affect of Dovonex on lesional T-cells, patients involved in the study will be asked to give biopsies of the involved skin before and after treatment. In situ hybridisation for IFN-γ, IL-4 and IL-5 will be used to determine the proportion of Th1 and Th2 cytokine expressing T-cells. The total number of T-cells will be identified by immunohistochemistry for the CD3 antigen.

The *in vitro* T-cell assays, as described in ***chapter 6***, will be repeated in further volunteers. If the effects shown here are repeated in further individuals of each genotype, the mechanism of action of A-1012G will be confirmed.

Gel-shift assays to demonstrate GATA-3 binding at A-1012G are being repeated in more depth to confirm allele-dependent GATA-3 binding. Further assays will be used to assess the affect of differential binding on promoter activity. Reporter gene assays will

be used to assess the activity of promoters containing each genotype. Additionally, GATA-3 expression plasmids will be transfected into normal dermal fibroblasts of known genotype. Fibroblasts do not usually express GATA-3 and the affect of over-expressing GATA-3 on VDR mRNA levels in the case of each allele will demonstrate the difference in GATA-3 regulation of VDR in each promoter allele.

In addition to the psoriasis project outlined above, it is planned to extend the studies in melanoma. The number of metastatic melanoma cases is being increased by using DNA extracted from archival paraffin-fixed metastatic tissue for genotyping. If the link between Bresolw depth, A-1012G genotype and prognosis can be confirmed in greater numbers, this may provide a clinically applicable prognostic indicator.

The affects of A-1012G/G-1520C haplotypes in psoriasis is being investigated, although initial results suggest that there is no similar haplotype affect as was shown in melanoma. Gel-shift assays are being carried out to assess the affect of G-1520C on transcription factor binding.

The possibility that A-1012G affects are due to linkage with another, more significant polymorphism is also being addressed. The fact that G-1520C is less significantly associated with melanoma and psoriasis than A-1012G suggests that there is not a more functional polynorphism upstream. To determine whether a more functional polymorhism exists downstream, the closest reported common polymorphism (rs7299460), downstream of exon 1d, will be analysed for association with melanoma and psoriasis.

A further possible area for future investigation arises from the likelihood that A-1012G genotype will have an effect on further diseases. Other cancers with similar antigen profiles to melanoma, such as prostate and breast cancer may show similar effects. Many diseases are classified as T-cell mediated. T-cell response is implicated in type 1 diabetes, asthma, inflammatory bowel disease, Crohn's disease and multiple sclerosis and A-1012G genotype may show affects in any of these diseases.

7.7.Conclusion

In conclusion, a novel polymorphism, A-1012G, was discovered upstream of exon 1a , in the major promoter region of the *VDR*. It was predicted that the T cell-specific transcription factor, GATA-3 would bind at this locus in the A allele but not in the G allele. The A allele of this polymorphism was associated with occurrence and metastasis in malignant melanoma and with calcipotriol response and occurrence in non-familial psoriasis. These findings were consistent with A-1012G being associated with increased sensitivity to $1,25(OH)_2D_3$ in T cells through up-regulation of the VDR by GATA-3. Gel-shift assays confirmed the binding of GATA-3 at the A allele of A-1012G and T-cell assays showed that A-1012G genotype affects T-cell response to $1,25(OH)_2D_3$.

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II. Appendix II: Association Studies

Association studies of VDR polymorphism in diseases and phenomena related to the classical actions in vitamin D. F=Fok1, B=Bsm1, A=Apa1, T=Taq1.

Associations		No Association	
Study	**Polymorphism**	**Study**	**Polymorphism**
Bone Mineral Density			
Ames et al., 1999	F	Braga et al., 22	F, B
Arai et al., 1997	F	Cheng and Tsai, 1999	F
Arai et al., 2001	C	Eccleshall et al., 1998	F
Baltzer et al., 1999	B	Efstathiadou et al., 21	F
Barger-Lux et al., 1995	B	Francis et al., 1997	T
Bell et al., 2001	A	Garnero et al., 1996	B, A, T
Bernad et al., 1999	B	Ho et al., 1999	B, T
Chen et al., 2001	B	Holmberg-Marttila et al., 2	B
Chen et al., 2002	F	Jaramillo-Rangel et al., 1999	B, A, T
Choi et al., 2000	F	Kim et al., 21c	F
Ferrara et al., 2002	F, B	Kubota et al., 21	F
Ferrari et al., 1995	B	Kurabayashi et al., 1999	F, A
Ferrari et al., 1998	F, B, A	Kurabayashi et al., 24	F
Ferrari et al., 1999	F, B	Lau et al., 1999	B, A, T
Fleet et al., 1995	B	Long et al., 24	A
Fontova Garrofe et al., 2000	B	Tsai et al., 1996	B, A, T
Garnero et al., 1995	B, A, T		
Gennari et al., 1999	F		
Gomez et al., 1999	B		
Gross et al., 1996	F		
Harris et al., 1997	F		
Hauache et al., 1998	B		
Houston et al., 1996	B		
Kanan et al., 2000	F		
Keen et al., 1997a	T		
Kiel et al., 1997	B		
Kubota et al., 2001	F		
Kurabayashi et al., 1999	T		
Kurabayashi et al., 2004	A, T		
Laaksonen et al., 2004	F		
Lau et al., 2002	F		
Lorentzon et al., 2001	F, B, A, T		
Lucotte et al., 1999	F		
Ma et al., 2001	B		
Marc et al., 2000	B		
Masi et al., 2002	F		
Matsushita et al., 2004	F, A, T		
McClure et al., 1997	B, A, T		
Morita et al., 2004	B, T		
Murakami et al., 1998	B		
Nakamura et al., 2002	F		
Obermayer-Pietsch et al., 2003	F		
Pollak et al., 2001	B		
Qin et al., 2004	A		

Associations		No Association	
Study	Polymorphism	Study	Polymorphism
Strandberg et al., 2003	F		
Taguchi et al., 2003	B		
Tofteng et al., 2002	F, B		
Uitterlinden et al., 1996	B, A, T		
van der Sluis et al., 2003	B, A, T		
Vidal et al., 2003	F, B		
Videman et al., 2001	T		
Viitanen et al., 1996	B		
Yamada et al., 2003	F		
Zajickova et al., 2002	F, B, A, T		
Zhang et al., 2003b	A		
Zhang et al., 2003c	F, A, B, T		
Bone Loss			
Gomez et al., 1999	B	Rapuri et al., 24	F, A
Guardiola et al., 1999	B	Zmuda et al., 1997	B
Karkoszka et al., 1998	B	Ho et al., 1999	B, T
Kikuchi et al., 1999	B		
Owada et al., 1996	B		
Rapuri et al., 2004	B, T		
Seko et al., 2004	F, T		
Videman et al., 2001	T		
Zmuda et al., 1997	T		
Fracture Risk			
Fang et al., 2003	C	Aerssens et al., 2	B
Houston et al., 1996	B	Ensrud et al., 1999	A, T
McClean et al., 2003	T		
Ramalho et al., 1998	B		
Osteoporosis			
Chen et al., 2002	F	Lisker et al., 23	F
Borjas-Fajardo et al., 2003	B	Sosa Henriquez et al., 1998	B
Chen et al., 2003	B	Zhang et al., 1998	B
Fountas et al., 1999	B, A, T	McClean et al., 23	T
Huang et al., 2000	B, A, T		
Lisker et al., 2003	B		
Langdahl et al., 2000	B		
Alvarez-Hernandez et al., 2003a	B, A, T		
Periodontal Disease			
de Brito Junior et al., 2004	B, T		
Hennig et al., 1999	T		
Tachi et al., 2001	T		
Rickets			
Fischer et al., 2000	F		
Osteoarthritis			
Keen et al., 1997b	T	Aerssens et al., 1998	B

Associations Study	Polymorphism	No Associations Study	Polymorphism
Height			
Ruza et al., 2003	F		
Osteocalcin Levels			
Sheehan et al., 2001	T		
Morrison et al., 1992	B, E, A		
Naves et al., 2003	B, T		
Parathyroid Function			
Fernandez et al., 1997	B		
Yokoyama et al., 2001	A		
Carling et al., 1998	B, A, T		
Sosa et al., 2000	F, B		
Menarguez et al., 1999	B		
Ferrari et al., 1999	F, B		
Yokoyama et al., 1998	B, A		
Alvarez-Hernandez et al., 2003b	F, B, A, T		
Carling et al., 1997	B, A, T		
Tagliabue et al., 1999	B		

III. Appendix III: Exonic Alignments from BLAST

Exons 1f and 1e were not identified in the mouse.

Exon 1a

Identities = 52/72 (72%)

```
Human: 1  ctgcttgtcaaaaggcggcagcggagccgtgtgcgccgggagcgcggaacagcttgtcca 60
          ||||||||||||| || || || ||||| |  || | || ||||| || | |  ||||||
Mouse: 1  ctgcttgtcaaaaagctgctgccgagccctaagcacgggaagcgccgagctgtgtgtcca 60

Query: 61 cccgccggccgg 72
          ||  | | || |
Sbjct: 61 ccgccagaccag 72
```

Exon 1d

Identities = 74/95 (77%), Gaps = 2/95 (2%)

```
Query: 1  gtttccttcttctgtcggggcgccttggcatggagtggaggaataagaaaaggagcgatt 60
          ||||| ||| |  | |||| ||| ||| | ||||||||||||| ||||||  |||| ||||
Sbjct: 1  gtttctttcctgggacggg-cgctttgacttggagtggaggagtaagaag-ggagtgatt 58

Query: 61 ggctgtcgatggtgctcagaactgctggagtggag 95
          ||||||||||||||||| ||  ||   |  ||||||
Sbjct: 59 ggctgtcgatggtgctaaggtctctggaggtggag 93
```

Exon 1b

Identities = 68/113 (60%), Gaps = 5/113 (4%)

```
Query: 1   gctcctgaacct----agcccagctggacggagaaatggactctagcctcctctgatagc 56
           |||||||| ||     |||     ||   |||||||||||||| |||||  ||||| |
Sbjct: 1   gctcctgacccccgagagctttcgtgcctggagaaatggactccagcctgttctgaga-t 59

Query: 57  ctcatgccaggccccgtgcacattgctttgcttgcctccctcaatcctcatag 109
           |||  | ||||||| ||||  | | | |    |   |  |||  |   | | |
Sbjct: 60  ctccagtcaggccctgtgctgagttcctgagctcattaactcgcttacctttg 112
```

Exon 1c

Identities = 56/77 (72%), Gaps = 5/77 (6%)

```
Query: 1  aagcctttgggtctgaagtgtctgtgagacctcacagaagagcacccctgggctccactt 64
              |||| |||  ||    |||||||| | || ||| ||||||||| |||||||
Sbjct: 5  agttcttttggttggacagatctgtgagtcttcccaggagagcacccttgggctc----- 59
```

```
Query: 65 acctgccccctgctccttcag 81
            ||  ||||||||||||||
Sbjct: 60 tgctcgcccctgctccttcag 76
```

Exon 2

Identities = 137/148 (92%)

```
Query: 1   ggatggaggcaatggcggccagcacttccctgcctgaccctggagactttgaccggaacg 60
           ||||||||||||||||| |||||||| ||||||||||||||||||| ||||||||||||||| |
Sbjct: 1   ggatggaggcaatggcagccagcacctccctgcctgaccctggtgactttgaccggaatg 60

Query: 61  tgccccggatctgtggggtgtgtggagaccgagccactggctttcacttcaatgctatga 120
           |||| |||||||||||| ||||||||||||||||||||| ||||| |||||||| |||||||
Sbjct: 61  tgcctcggatctgtggagtgtgtggagaccgagccacgggcttccacttcaacgctatga 120

Query: 121 cctgtgaaggctgcaaaggcttcttcag 148
           ||||||||||||||||| || ||||||||
Sbjct: 121 cctgtgaaggctgcaagggtttcttcag 148
```

Exon 3

Identities = 120/131 (91%)

```
Query: 1   gcgaagcatgaagcggaaggcactattcacctgccccttcaacggggactgccgcatcac 60
           ||| |||||||||||| ||||| || ||||||||||||||||| || || ||||||||||||
Sbjct: 1   gcggagcatgaagcgcaaggccctgttcacctgccccttcaatggagattgccgcatcac 60

Query: 61  caaggacaaccgacgccactgccaggcctgccggctcaaacgctgtgtggacatcggcat 120
           |||||||||||| || |||||||||||||||||||||||||||||| |||||||| |||||
Sbjct: 61  caaggacaaccggcgacactgccaggcctgccggctcaaacgctgcgtggacattggcat 120

Query: 121 gatgaaggagt 131
           ||||||||||||
Sbjct: 121 gatgaaggagt 131
```

Exon 4

Identities = 161/185 (87%)

```
Query: 1   tcattctgacagatgaggaagtgcagaggaagcgggagatgatcctgaagcggaaggagg 60
           |||| || |||||||||||| |||||| | ||||| ||||||||| ||||| |||||||||
Sbjct: 1   tcatcctcacagatgaggaggtgcagcgtaagcgagagatgatcatgaagaggaaggagg 60

Query: 61  aggaggccttgaaggacagtctgcggcccaagctgtctgaggagcagcagcgcatcattg 120
           | |||||||||||||||||||||| |||||||||||||||||||||||| |||| ||| || |
Sbjct: 61  aagaggccttgaaggacagtctgaggcccaagctgtctgaggagcaacagcacattatcg 120

Query: 121 ccatactgctggacgcccaccataagacctacgaccccacctactccgacttctgccagt 180
           |||| ||||| || |||||||| |||||||||||||||||||||  |||||||| |  | |
Sbjct: 121 ccatcctgctcgatgcccaccacaagacctacgaccccacctatgccgacttccgggact 180
```

```
Query: 181 tccgg 185
           |||||
Sbjct: 181 tccgg 185
```

Exon 5

Identities = 75/116 (64%), Gaps = 15/116 (12%)

```
Query: 1   cctccagttcgtgtgaatgatggtggagggagccatccttccaggcccaactccagacac 60
           |||||| ||||||   | |   ||  ||||||| || || | |||||          |||
Sbjct: 1   cctccaattcgtgcagacgtaagtacagggagctattctccaaggcc----------cac 50

Query: 61  actcccagcttctctggggactcctcctcctcctgctcagatcactgtatcacctc 116
           |||  ||||||||| || ||   |||||||||   ||| ||||  |  | | ||||
Sbjct: 51  act--cagcttctccggaga---ctcctcctcaaactctgatctgtacacccccctc 101
```

Exon 6

Identities = 149/172 (86%)

```
Query: 1   acatgatggactcgtccagcttctccaatctggatctgagtgaagaagattcagatgacc 60
           ||||||||||  || ||||||| ||||   ||||||||| ||||||||  || |||||||
Sbjct: 1   acatgatggaaccggccagcttttccacgatggatctgaatgaagaaggctccgatgacc 60

Query: 61  cttctgtgaccctagagctgtcccagctctccatgctgccccacctggctgacctggtca 120
           | ||||||||||| || ||||| | ||||||||||||||||||||||||||||| || ||||
Sbjct: 61  cctctgtgaccctggacctgtctccgctctccatgctgccccacctggctgatcttgtca 120

Query: 121 gttacagcatccaaaaggtcattggctttgctaagatgataccaggattcag 172
           ||||||||||||||||||||| |||||||| |||||||| || || |||||
Sbjct: 121 gttacagcatccaaaaggtcatcggctttgccaagatgatccctggcttcag 172
```

Exon 7

Identities = 127/151 (84%)

```
Query: 2   gacctcacctctgaggaccagatcgtactgctgaagtcaagtgccattgaggtcatcatg 61
           |||||||||||||| |||||||| || ||||| |||||||||||||||||||| ||||||
Sbjct: 2   gacctcacctctgatgaccagattgtcctgcttaagtcaagtgccattgaggtgatcatg 61

Query: 62  ttgcgctccaatgagtccttcaccatggacgacatgtcctggacctgtggcaaccaagac 121
           |||||||||||  |||| || ||| |||| ||||||||||||  |||||||| |||||||
Sbjct: 62  ttgcgctccaaccagtcttttaccttggatgacatgtcctgggactgtggcagccaagac 121

Query: 122 tacaagtaccgcgtcagtgacgtgaccaaag 152
           ||||| ||   | ||| ||| ||  ||| ||
Sbjct: 122 tacaaatatgacatcactgatgtctccagag 152
```

Exon 8

Identities = 104/117 (88%)

```
Query: 1    ccggacacagcctggagctgattgagcccctcatcaagttccaggtgggactgaagaagc 60
            | || |||| |||||||||||| || |||||||| ||||||||||||||| ||||||||||
Sbjct: 1    ctgggcacaccctggagctgatcgaacccctcataaagttccaggtggggctgaagaagc 60

Query: 61   tgaacttgcatgaggaggagcatgtcctgctcatggccatctgcatcgtctccccag 117
            ||||| | |||||||| || ||||| |||||||||||||||||||| ||||||||||
Sbjct: 61   tgaacctccatgaggaagaacatgtgctgctcatggccatctgcattgtctccccag 117
```

Exon 9

Identities = 1754/3223 (54%), Gaps = 334/3223 (10%)

```
Query: 1    atcgtcctggggtgcaggacgccgcgctgattgaggccatccaggaccgcctgtccaaca 60
            | || |||||||| ||||| ||   |||| |||| ||||| ||||||||||| |||||||
Sbjct: 1    accgacctggggtacaggatgctaagctggttgaagccattcaggaccgcctatccaaca 60

Query: 61   cactgcagacgtacatccgctgccgccacccgcccccgggcagccacctgctctatgcca 120
            |||||||||| |||||||||||||||||||||||||||||||||||||| |||||| ||||
Sbjct: 61   cactgcagacctacatccgctgccgccacccgcccccgggcagccaccagctctacgcca 120

Query: 121  agatgatccagaagctagccgacctgcgcagcctcaatgaggagcactccaagcagtacc 180
            |||||||||||||||| || |||||||| |||||||||||||||||||||||| |||||||
Sbjct: 121  agatgatccagaagctggctgacctgcgaagcctcaatgaggagcactccaaacagtacc 180

Query: 181  gctgcctctccttccagcctgagtgcagcatgaagctaacgccccttgtgctcgaagtgt 240
            | | |||||||||||||||| |||  ||||||||||| || ||||||||||| || ||||
Sbjct: 181  gttccctctccttccagccggagaacagcatgaagctcacacccccttgtgctagaggtgt 240

Query: 241  ttggcaatgagatctcctgactaggacagcctgtggcggtgcctgggtggggctgctcct 300
            | |||||||||||||||||||| |||   |||||  | ||||||||||| |||| ||||||
Sbjct: 241  tcggcaatgagatctcctgaccagggtggcctgcagtggtgcctgggtagggccgctcct 300

Query: 301  ccagggccacgtg-ccaggcccggggctggcggctactcagcagccctcctcaccccgtc 359
            |  |||||  ||| |||||||| |||||||  ||  | ||||||  |  |   ||||  |
Sbjct: 301  ctggggccctgtgcccaggccctgggctggttgcagcccagcagtgcctcctgcccctcc 360

Query: 360  tggggttcagcccctcctctgccacctcccctatccacccagcccattctctctcctgtc 419
            ||| ||||||| ||||||||||    ||| | ||   |  || ||| || ||||||| |
Sbjct: 361  tggagttcagctcctcctctgccgtggcccgtgtctgtctggctcatcctttctcctgcc 420

Query: 420  caacctaac---------ccctttcctgcgggct-----tttccccggtcccttgagacc 465
            || ||| ||         |||||||||  | ||     || | || |||||||||| |
Sbjct: 421  cagcctcacacctgatctccctttcctgtagactgcaggttgctcctgtcccttgagatc 480

Query: 466  tcagccatgaggagttgctgtttgtttgacaaagaaacccaagt---------------g 510
            ||||  | |||    |||||||| |||||||||||||| |||||               |
Sbjct: 481  tcagttaggagacactgctgtttatttgacaaagaaactcaagtgtgggatgggggaag 540

Query: 511  ggggcagagggcagaggctggaggc--agggccttgcccagag-atgcctccaccgctgc 567
             ||| |||||||||||||||| ||||  || ||  |||| || | |||||||| |     |
Sbjct: 541  agggtagagggcagaggctgaaggcagagagctctgcctagggaatgcctccgc-----c 595
```

```
Query: 568   ctaagtggctgctgactgatgttgaggga-acagacaggagaaatgcatccattcctcag 626
             |||| |||  ||| ||  |||  |||||  ||| ||| ||| |||  ||||||||||||
Sbjct: 596   ataaggggccactg-cttgtgtcaagggaggcaggcagaagagatgagtccattcctcag 654

Query: 627   ggacagagacacctgcacctcccccactgcaggccccgcttgtccagcgcctagtgggg 686
             ||||  ||  ||||| |||| |||| | | ||  ||   ||        |||| ||| |
Sbjct: 655   ggac--aggtacctgtacctaccccgattccaaacctacct--------gcctggtgaga 704

Query: 687   tctccctctcctgcctactcacgataaataatcggcccacagctcccaccccaccccctt 746
              ||||  | |||||||||  | | || | || |  ||||      |  ||| |     |
Sbjct: 705   actcctgcccctgcctacaaagggtacacaacctaccca-----tcatccctagtgtgtc 759

Query: 747   cagtgcccaccaacatcccattgccctggttatattctcacgggcagtagctgtggtgag 806
             | ||  || ||  |  ||   || |   ||  |||||| ||     | || |  |
Sbjct: 760   ccgtctcctcctgccacc---tgtc--tgtattattctgacccaggggag-tcagtcact 813

Query: 807   gtgggttttcttcccatcactggagcac-caggcacgaacccacctgctgaga-gaccca 864
             |||||   || | ||    ||||   || || | ||  |  || ||||  ||| |||| |
Sbjct: 814   gtggggcctccttcctctgctggtatactcatggactcattca-ctgccaagatgaccaa 872

Query: 865   aggaggaa--aaacagacaaaaacagcctcac-ag--aagaatatgacagct----gtcc 915
             |      |  | ||  || ||     |||| | ||    | |  |  || ||    |
Sbjct: 873   atactctaccacactaaccaaggagccctccctagccctgcagttcccacctttgaggtt 932

Query: 916   ctgtca--ccaagctcacagttcctcgccctgggtctaaggggttggttgaggtggaagc 973
              ||| |    |||  | ||  |||    |||  |   ||| ||    ||         | |
Sbjct: 933   ttgttatggtaagtccccaagtccagtacctctggtaaagtgg--acttcttcccccatc 990

Query: 974   cctccttccacggatccatgtagcaggactgaattgtccccag---tttgcagaaaagca 1030
             |||    ||| ||| | |   | ||  | || |  ||||||||    | | | | |
Sbjct: 991   cctagaaccagggacctagaaaccaccattggaaagtccccagggaatggaatacagagc 1050

Query: 1031  cctgccgacctcgtcctcccccctgccagtgccttacctcctgccca---ggagagccagc 1087
             ||   | |    |  |   |  |||||   |  | |       ||   ||||  ||| |
Sbjct: 1051  ccaagcaatgaggagcaggcaaagccagcctcccagcaggacaacaaatggaggtccatc 1110

Query: 1088  cctccctgtcctcctcggatcaccgagagtagccgagagc--ctgctcccccaccccctc 1145
               ||  ||| | ||  |       |   | ||||  ||||  |||   |||| || |  |
Sbjct: 1111  tatctttgt-caccaagctgaggtgtctgaagcctggagctactgggacccctcctctcc 1169

Query: 1146  cccaggg----gaga-gggtctgga-gaag--------------cag-------tgagcc 1178
              | |  |     ||| | ||| |||  |||              |||         ||||
Sbjct: 1170  tctatagctattagatgtgtcaggaccaagttgaccccaactttcagaacgtccctagcc 1229

Query: 1179  gcatcttc-tccat----ctggcaggg-------tgggatggagg--agaagaattttca 1224
                |||||  ||| |    ||| ||| |           |   |||  || ||    || |
Sbjct: 1230  ctgtcttcatcccttcccctgccagtgccttaccacctacccaggaaagcaggtccttga 1289

Query: 1225  gac-cccagcggctgagtcatgatctccctgccgcctcaatgtggttgcaaggccgct-g 1282
             | | |||||    |      |||||  ||  |      | |   | |  |   ||  ||
Sbjct: 1290  gccacccaggccttacaatttgatcaccaagagtatcccagaagttatctcagcttctcc 1349

Query: 1283  ttcacccaca---gggctaagagctagcgctgccgcaccccagagtgtgggaagggaga- 1338
              |||||| ||   ||   || || |  | |  |  |||||||         ||  |  |
Sbjct: 1350  atcacccccacttggagcaacag-tgtcacatctccaccccacttaccaataaaagctac 1408
```

```
Query: 1339 ----gcggggcagt---ctcgggtggctagtcagagagagt-gtttgggggttccgtgat 1390
                 |    ||||    | || ||||     |   | ||| |    |  |  ||   |
Sbjct: 1409 cctttccacccagtggaatgggatggcagaaaaccaacagtggcccagatggacctccaa 1468

Query: 1391 gtagg---gtaaggtg------ccttcttattctcactc-caccacccaaaagtcaaaag 1440
            ||  |    | |||||      |   | |   ||| | | |  ||   |||||  | | |
Sbjct: 1469 gtctgcttttcaggtgtaaagccagacatgagctctcccacctcagggaaaaggtagagg 1528

Query: 1441 gtgcctgt--gaggcaggggcggagtgatacaacttcaagtgcatgct--ctctgca--- 1493
            | ||  ||  ||    | ||   |||||||     || ||| | | ||  |||| ||
Sbjct: 1529 gggcaggttagaacttgtggtacagtgata-ttggtccagttcctcctgactcttcactc 1587

Query: 1494 -gccagcc--cagcccagctggtgggaagcgtctgtccgtt--tactccaaggtggggtc 1548
              ||| ||   || | |  | |    | |   | ||| | |  |   | | ||  |  ||
Sbjct: 1588 taccacccaaaagtcaatgtagacctacgaaacggtcaggtgatgggctagggacgtatc 1647

Query: 1549 tttgtgagagtgagctgtaggtgtgcgggaccggtacagaa------aggcgttcttcga 1602
            ||             ||     |||    | || || ||| |      |  | |  |   |
Sbjct: 1648 ttcaaactccatttcttcctctgtagtagcccagtccaggactttggaaccctggtctca 1707

Query: 1603 ggtggatcacagaggcttctt-cagatca------gtgcttgagtttgggga---atgcg 1652
                   || | |  |||||| | |||||      |||   | ||  |||||   ||||
Sbjct: 1708 ttcaccccaaatatacttcttgctgatcatgcgttgtgggggtgtgggggacccatgca 1767

Query: 1653 gccg-cattccct----------gagtca-------------ccag-gaatgttaaagtca 1688
            |  |   ||| ||          || |||             |||| |||||||  |   |
Sbjct: 1768 gaggaggttctcttcataagggggactcacagaaggaccttccagtgaatgtttgaccta 1827

Query: 1689 gtg---ggaacgtgact----gccccaa-------ctcctggaagctgtgtccttgcacc 1734
            | |   | | |   |||    || ||||       ||   |  ||  |   | | || ||
Sbjct: 1828 gggaatgcagccacactggaagcaccaatagagggctaaggtcagacggagcatggcccc 1887

Query: 1735 tgcatccgtag-ttccctgaaaacccagagaggaatcagacttcaca-ctgcaagagcct 1792
               | ||  || |||||||||| || ||||  ||||| | || ||| | | |  | |||
Sbjct: 1888 acgagccccagattccctgaaaatcctgagatcaatcacatttaacatcagaagaaccct 1947

Query: 1793 tggtgtccacctggccccatgtctctcagaattcttcaggtggaaaaacatctgaaagcc 1852
            | |||||||||||||| |||| || |||||   |||| | || |||||||||||| |
Sbjct: 1948 tagtgtccacctggccctatgtttcccagaa---ttcaagcgg-aaaacatctgagaatg 2003

Query: 1853 acgtt-ccttactgcagaatagcatatatatcgcttaatcttaaatttattagatatgag 1911
             | || || || ||||  |  ||||  | |  |||| || ||||| | |||  ||  |||
Sbjct: 2004 gctttcccatattgcattacggcatgcagaaggctttattttaaagtcattccatcagag 2063

Query: 1912 ttgttttcagactcagactccatttgtattatagtctaatatacagggtagcaggtacca 1971
             |||| ||||| | |||| |||  |||| | |  ||||| |   ||||| |  || ||
Sbjct: 2064 gtgttctcagaattagaccccacctgtactgtgttctaacactgagggt-ggtggcac-- 2120

Query: 1972 ctgatttggagatatttatgggggagaacttacattgtgaaacttctgtacattaatta 2031
            ||||||  |||   | | || | |            ||||||| | ||||| |||||| |
Sbjct: 2121 ctgattcagagcagtgtgtgtgtg------------tgtgaaaatactgtatattaatga 2168
Query: 2032 ttattgctgttgttattttaca-------agggtctagggagagacccttg--------- 2075
            |||||| |  ||       |||       |      |   | | ||||| |
Sbjct: 2169 ttattgttactgcacacacacacacatacaaaaaaaaaaaaaaaaaccctagagaaagacc 2228
```

```
Query: 2076 tttgattttagctgcagaacgtattggtccagcttgctcttcagtgggagaaaacacttg 2135
           ||||||||||||| |||||||  |||| ||  |||| ||  |||   ||||  ||    ||
Sbjct: 2229 tttgattttagcagcagaacacattgatctggcttactactcaacaggagggaaagtctg 2288

Query: 2136 taagttgctaaacgag-tcaatcccctcattcaggaaaactga---cagaggagggcgtg 2191
            |||||| ||| | || || |  || | ||   |  || ||||   |||||||| | |||
Sbjct: 2289 aaagttgataagctagttcgactcctttataatgagaacctgacatcagaggagagtgtg 2348

Query: 2192 actcacccaagcatatataactagctagaagt--gggccaggacaggcccggcgc----g 2245
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Sbjct: 2349 atttacctgggctcatataattagaagcatctccaggcctggagaaaatttgaacttatg 2408

Query: 2246 gtggctcacgcctgtaatcccag-cagtttgggaggtcgaggtaggtggatca---cctg 2301
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Sbjct: 2409 gtcccgggctgctttgtagtgagcctgtttccactgctctggtagctgagccaggctatg 2468

Query: 2302 agg----tcgggagttcgagaccaacc--tgaccaacatggagaaaccctgtctc----t 2351
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Sbjct: 2469 aggaaaccctggatatcagcataaacctatgtctgggactacgatccctttccctgggt 2528

Query: 2352 attaaaaata-caaaaaaaaaaaaaaaaaaaatagccgggcatggtggcgcaagcct--- 2407
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Sbjct: 2529 aataaactcagccacccatcctaaggaggcaagcacttggcctctttgagtgagacttgg 2588

Query: 2408 -----gtaatcccagc----tactcaggaggctgaggcagaagaa------ttgaaccca 2452
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Sbjct: 2589 ctttaggactctctgcatcagccttgtgtgcatcaggcactggaaagcctctgtcttccc 2648

Query: 2453 ggaggtggaggttgcagtgagctgagatcgtgccgttactctccaacctggacaacaaga 2512
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Query: 2513 gcgaaactccgtcttagaagtggac-caggacaggaccagattttggagtcatggtc--- 2568
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Sbjct: 2709 ggggtccatc-actgagaagtgcccaaagggaagtcagggagctgggaggaaaagacagc 2767

Query: 2569 ----cggtgtccttttcactacacca--tgtttgagctcag--acccccactctcattcc 2620
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Sbjct: 2768 aggaccaagtaggattccgaagccgaactgggagagaacagcatcatctatttttattct 2827

Query: 2621 ccaggtggctgacccagtccctgggggaagccctggatttcagaaagagcaa---gtctg 2677
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Sbjct: 2828 gaatgt-acaaaggcagggccttgaaagagctc-acacttccgaggcggaaatgggtacc 2885

Query: 2678 gatctgggacccttccttccttccctggcttgta-actccaccaac-ccatcagaagga 2735
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Sbjct: 2886 aaaatgagagatcagcaagatttcccaagataggagaatcctcctgctgcctgcgtggga 2945

Query: 2736 gaa-----ggaaggagactcacctctgcctcaatgtgaatcagaccctaccccaccacga 2790
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Sbjct: 2946 gaaagtaggggaggagtccaaaccaggccacca-gagatggttgagataaatgacaa--a 3002
Query: 2791 tgtggccctggcctg-ctgggctctccacctcagccttggataatgctgttgcctcatct 2849
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Sbjct: 3003 tctggtggaagtcagacatggctctaaagagcttcctaagagacttcccgagagagagac 3062
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Query: 2850 ataacatgcatttgtct-ttgtaatgtcaccaccttcccagctctccctctggccctgcc 2908
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Query: 2909 tt-cttcggggaactcctggaaatatcagttactcagccctgg 2950
           || |    |  ||     |    |  || || |  ||| || |
Sbjct: 3120 ttgccgatgataataaaagatgctcacactttcatagcgcttg 3162
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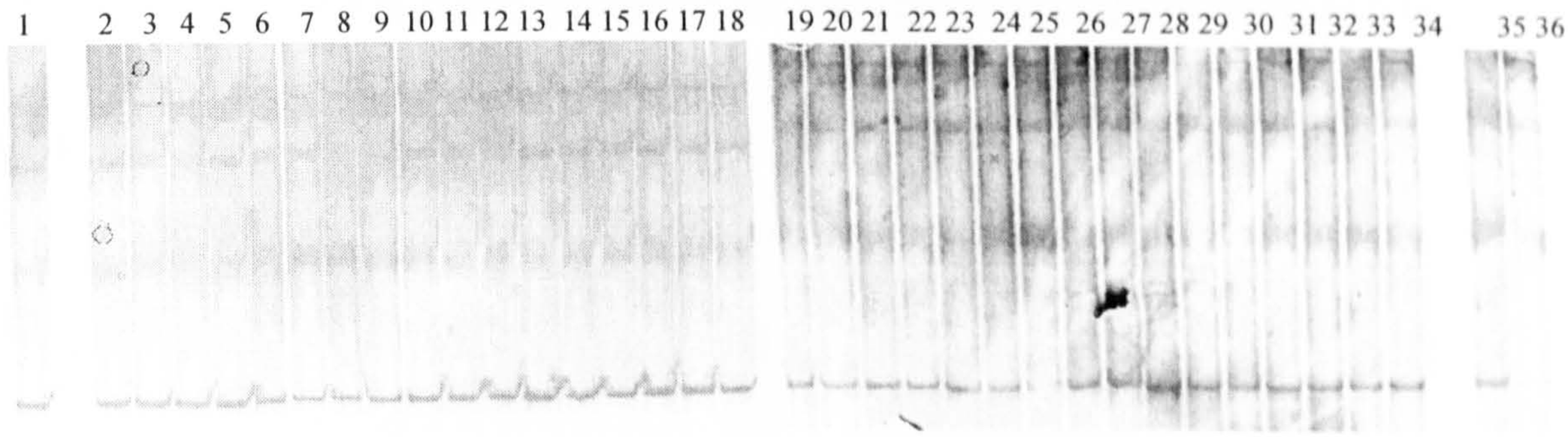
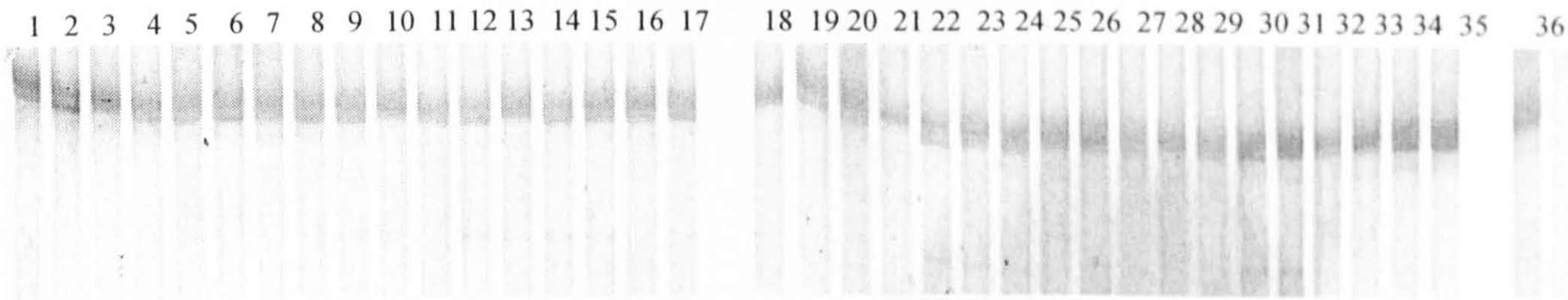
IV. Appendix IV: SSCP Results with no Variation

All amplicons were amplified in 36 normal individuals.

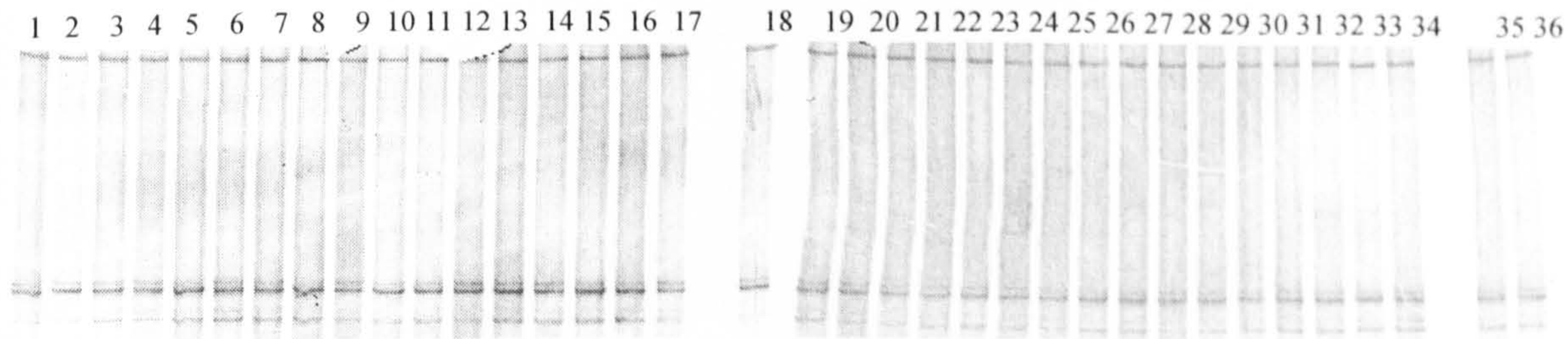
1A/Dp1



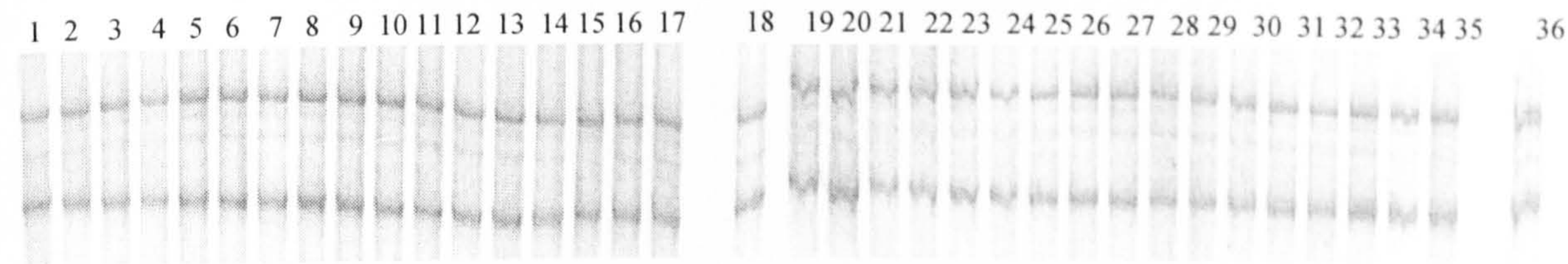
VDR 1A/Dp3



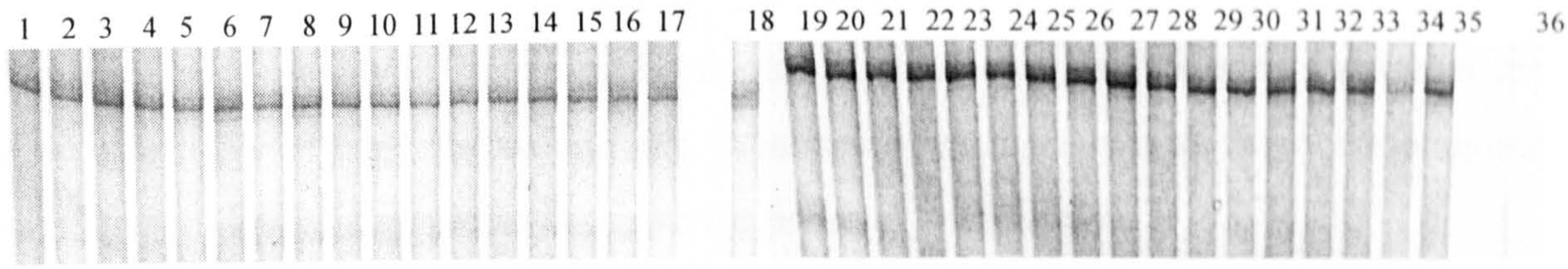
VDR 1A/Dp4



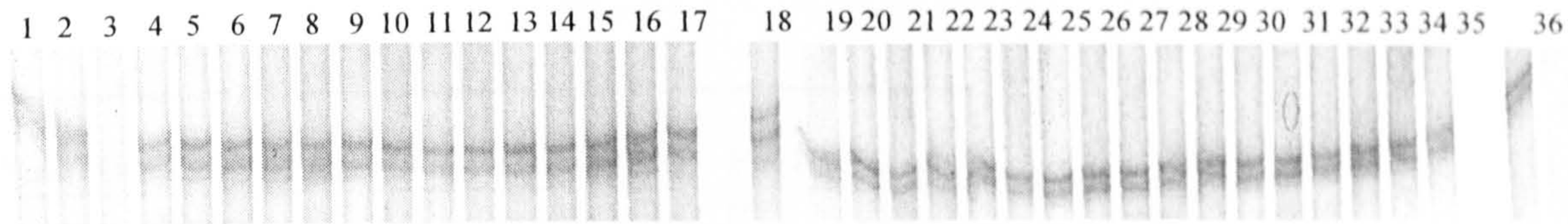
VDR 1A/Dp6



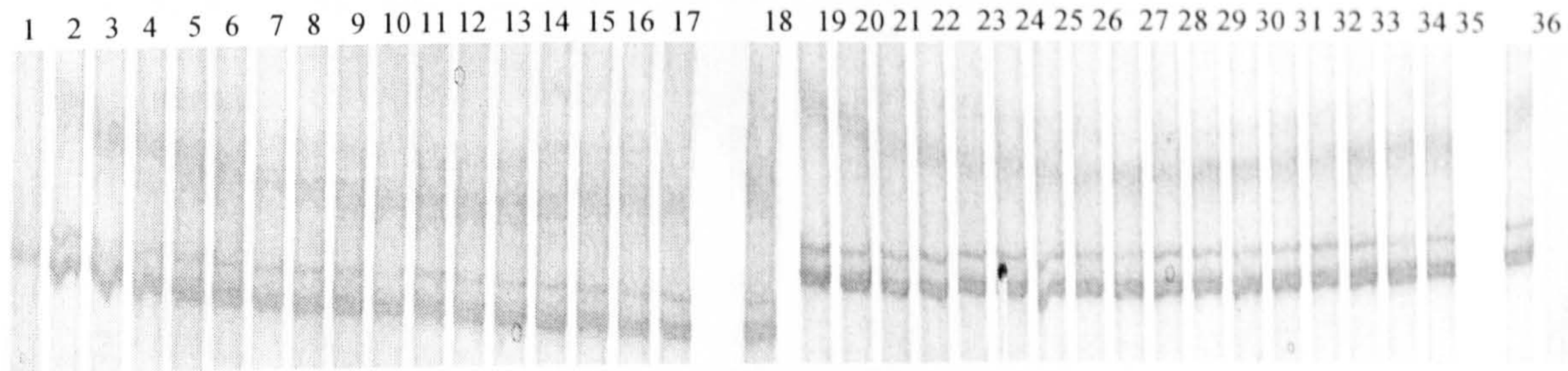
VDR 1A/Dp8



VDR 1A/Dp9



VDR 1A/Dp10



V. Appendix V: Genotype Screening Results

The results of genotype screening in controls, melanoma and psoriasis patients are shown with age at presentation and sex. Presence of metastasis is shown for melanoma patients and dovonex response is shown for psoriasis patients.

Controls

Number	Sex	Age	G-1520C	A-1012G	FokI	TaqI
1	male	65	GC	AG	ff	Tt
2	male	74	CC	GG		
3	male	74	GG	AA	Ff	tt
4	female	69	GC	AG		
5	male	53	GC	AG	Ff	TT
6	male	82	CC	GG	ff	TT
7	female	65	GC	AG	Ff	tt
8	female	68	CC	GG	ff	tt
9	male	38	CC	GG	FF	Tt
10	female	65		AA		
11	female	74	GC	AG	FF	Tt
12	male	61	CC	GG	ff	Tt
13	female	55	GG	AA	Ff	TT
14	male	47	CC	GG	Ff	tt
15	female	77	GC	AG	FF	Tt
16	male	50	GC	AG		
17	male	66	CC	GG	FF	tt
18	female	77	GG	AA	ff	Tt
19	male	51	GC	AG	FF	Tt
20	male	80	GC	AG	FF	Tt
21	male	64	GG	AA		
22	female	66	CC	GG	FF	Tt
23	female	70	GC	AG	FF	Tt
24	male	58	GG	AG	FF	Tt
25	male	56	GG	AA	Ff	Tt
26	female	56	GG	AA	FF	Tt
27	male	77	GC	AG		
28	female	76	GG	AA	FF	
29	male	76	GC	AG	Ff	tt
30	male	79	CC	GG	FF	Tt
31	male	41	GC	AG	Ff	TT
32	male	65	GC	AG	ff	Tt
33	female	79	GG	AA	Ff	Tt
34	female	79	CC	GG	Ff	TT
35	female	43	CC	GG	Ff	TT
36	female	68	GG	AA	FF	TT
37	male	75				
38	male	15	GC	AG	FF	TT

Number	Sex	Age	G-1520C	A-1012G	Fok1	Taq1
39	female	59	GC	AG	Ff	Tt
40	male	52	GG	AA	ff	Tt
41	male	54	GG	AA	FF	Tt
42	female	14	GC	AG	ff	Tt
43	female	58	GG	AA	FF	Tt
44	female	53	CC	GG	Ff	Tt
45	female	35	GG	AA	FF	TT
46	male	65	GG	AG	Ff	TT
47	female	7	CC	GG	FF	Tt
48	female	21	GG	AG	Ff	Tt
49	female	5	GG	AG	Ff	TT
50	female	18	GG	AG	FF	TT
51	female	32		GG		
52	male	20		AA	Ff	tt
53	male	43	CC	GG	Ff	TT
54	female	9	GC	AG	ff	Tt
55	female	80	GG	AA	ff	tt
56	female	75	GG	AG	FF	
57	male	20	CC	GG	FF	tt
58	male	75	GG	AA	Ff	tt
59	male	89	GG	AG	FF	Tt
60	male	85	GG	AG	Ff	Tt
61	female	58	GC	AG	FF	tt
62	female	14	GC	AG	Ff	TT
63	female	53	GC	AA		Tt
64	male	81	GC	AG	Ff	tt
65	male	61	GC	AG	Ff	TT
66	male	62	GC	AG	Ff	Tt
67	female	77	GG	AA	FF	Tt
68	female	70	GC	AG		
69	female	87	GC	AG	Ff	TT
70	female	31	GC	AG	Ff	Tt
71	female	26	GC	AG	Ff	TT
72	male	5	GC	AG	Ff	TT
73	male	69	GG	AA	FF	Tt
74	male	56	GG	AA	Ff	Tt
75	male	11	GG	AG	FF	Tt
76	male	61	GC	AG	FF	TT
77	male	75	GC	AG	FF	tt
78	female	49	GC	AG	Ff	Tt
79	female	27	CC			
80	male	46	GC	AG	Ff	TT
81	female	5	GG	AA	FF	TT
82	male	89				
83	male	31		AG		

Malignant Melanoma

Number	Sex	Age	G-1520C	A-1012G	FokI	TaqI	Metastasis
1	female	82	GC	AG	Ff	TT	
2	male	61	GC	AG	FF	Tt	
3	female	84	GG	AG	FF	Tt	
4	female	42	GG	AA	Ff	Tt	
5	male		GC	AG	Ff	Tt	
6	male	59	GC	AG	Ff	TT	
7	female		CC	AG	FF	Tt	
8	female	65	CC	AG	Ff	TT	
9	male	41	GG	AA	FF	TT	metastasis
10	female	82	CC	AG	Ff	TT	
11	male	52	CC	AG	Ff	Tt	
12	male	62	GG	AA	FF	tt	metastasis
13	male	54	GC	AG	FF	tt	metastasis
14	female	79	GG	AA	FF	TT	metastasis
15	male	60	GG	AG	FF	TT	
16	female	61	GC	AG	Ff	tt	
17	male	72	GG	AG	Ff	Tt	metastasis
18	male	76	GC	AG	Ff	Tt	metastasis
19	male	56	GC	AG	Ff	TT	metastasis
20	female		GG	AA	Ff	tt	
21	female	75	GC	AG	FF	Tt	metastasis
22	male	67	GG	AG	FF	TT	
24	male	36	GG	AG	ff	tt	
25	female	61	GC	AA	FF	TT	
26	female	92	GC	AA	FF	TT	metastasis
27	female	27	GG	AA	FF	tt	
28	male	67	GG	AA	Ff	TT	
29	female	54	GG	AA	Ff	TT	
30	female	82	GG	AA	FF	tt	metastasis
31	male		GG	AG	Ff	Tt	
32	male	57	GG	AA	ff	Tt	
33	female	40	GG	AA	FF	TT	
35	female	39	CC	AA	ff	Tt	metastasis
36	male	47	GG	AA	FF	TT	
38	male	57	CC	AG	FF	TT	metastasis
40	female	28	CC	AA	FF	Tt	
41	male	57	GG	AA	Ff	Tt	
42	female	74	GG	AG	ff	TT	
43	male		GG	AA	FF	TT	
45	female	58	GG	AA	FF	TT	metastasis
46	male	40	CC	AA	FF	tt	
47	male	52	GC	AA	ff	TT	metastasis
48	female	83	GC	AA	FF	TT	
49	male	55	CC	AA	FF	TT	
50	female	64	GG	AA	ff	Tt	
51	male	45	CC	AA	FF	Tt	
52	male		CC	AA	FF	TT	

Number	Sex	Age	G-1520C	A-1012G	Fok1	Taq1	Metastasis
53	female	77	CC	AA	Ff	TT	
54	female	45	GC	AA	Ff	TT	
55	female	46	GC	AG	Ff	Tt	
56	male	55	CC	AA	FF	TT	
57	female	77	GG	AA	ff	TT	
58	female	26	GC	AG	ff	TT	
59	female	30	GC	AG	FF	tt	
60	female	45	GC	AG	FF	Tt	
62	female	45	GC	AG	Ff	Tt	
63	female	78	GC	AA	Ff	Tt	
64	female	72	GG	AG	Ff	TT	
65	male	66	CC	AA	Ff	Tt	
66	female	36	GC	AG	FF	Tt	
67	female	53	CC	AA	Ff	TT	
68	female	59	GC	AA	FF	Tt	
69	male	41	GG	AA	FF	TT	
70	female	51	GG	AA	Ff	TT	
71	male	54	GG	AG	FF	Tt	
72	male	45	GC	AG	Ff	TT	
73	female	41	GC	AG	Ff	Tt	
74	female	41	GC	AG	Ff	TT	
75	female	48	GC	AG	Ff	TT	
76	female	68	CC	AG	Ff	Tt	
77	female	49	GC	AG	FF	Tt	
78	male	61	GC	AG	FF	Tt	
79	female	52	GC	AG	FF	TT	
80	male	56	GC	AG	FF	TT	
81	female	44	GC	AA	Ff	Tt	
82	male	24	GG	AG	Ff	TT	
83	female	54	GC	AG	Ff	Tt	
84	female	41	CC	AG	FF	Tt	
85	male	68	GG	AG	Ff	tt	
87	female	36	GC	AG	FF	Tt	
88	female	48	CC	AG	Ff	Tt	
89	female	49	GC	AG	Ff	Tt	
90	female	41	GC	AG	Ff	Tt	
92	male	66	GC	AG	FF	TT	
93	male	69	GC	AG	FF	TT	
94	female	33	GG	AA	FF	TT	
95	female	59	GC	AG	Ff	TT	
96	female	22	GC	AG	Ff	tt	
97	female	29	GG	AG	Ff	Tt	
98	female	59	GC	AG	ff	Tt	
99	female	51	GC	AG	ff	Tt	
100	female	68	GC	AG	ff	Tt	
102	female	56	GC	AG	Ff	Tt	
103	female	25	GC	AG	Ff	Tt	
104	female	43	GG	AA	Ff	Tt	
105	male	60	GC	AG	Ff	TT	
106	male		GC	AG	Ff	tt	
107	male	68	CC	GG	FF	Tt	

Number	Sex	Age	G-1520C	A-1012G	Fok1	Taq1	Metastasis
109	male	30	GG	AG	Ff	tt	
110	female	52	CC	GG	Ff	Tt	
111	female	22	GG	AA	Ff	TT	
112	male	54	CC	GG	ff	TT	
113	female	45	GC	AG	Ff	Tt	
114	male	53	GC	AG	Ff	tt	
115	male	59	GC	AG	FF	Tt	
116	female	69	GG	AA	FF	TT	
117	female	57	GG	AA	FF	Tt	
118	female	61	CC	GG	Ff	tt	
119	female	48	CC	GG	ff	TT	
120	female	61	GG	AG	FF	Tt	
121	female	29	CC	GG	ff	Tt	
122	female	49	GG	AA	Ff	TT	
123	female	86	CC	GG	FF	Tt	
124	male		GC	AG	Ff	TT	
125	male	88	GG	AA	Ff	Tt	
126	female	49	CC	GG	Ff	TT	
127	female	62	GC	AG	FF	TT	
128	male	67	GC	AG	Ff	TT	
130	male		GG	AA	FF	Tt	
131	female	60	GC	AG	FF	TT	
132	male	67	GG	AA	ff	Tt	metastasis
133	female	63	CC	AG	Ff	tt	
134	female	31	GG	AG	ff	tt	
135	female	77	GG	AA	Ff	tt	
136	female	41	GG	AG	ff	Tt	
137	female	49	GC	AG	ff	Tt	
138	female	50	GC	AG	FF	Tt	
139	female		CC	GG	ff	TT	
140	female	28	CC	GG	Ff	TT	
141	female	36	GC	AG	FF	Tt	
142	male	55	GC	AA	FF	tt	
143	female	30	GG	AA	FF	Tt	
144	male	65	GC	AG	Ff	TT	metastasis
145	male	48	GC	AG	Ff	Tt	
146	male	72	GG	AA	FF	TT	
147	male	66	GC	AG	FF	Tt	
148	female	37	GG	AA	FF	Tt	metastasis
149	male	71	GG	AA	FF	tt	
150	female	51	GG	AG	Ff	Tt	
151	female	77	CC	GG	ff	Tt	
152	male	44	GG	AA	Ff	TT	
153	male	57	CC	GG	ff	TT	
154	female	82	GG	AA	FF	Tt	
155	male	80	GG	AG	Ff	Tt	
157	female	33	GG	AA	Ff	tt	
158	female	88	CC	GG	Ff	tt	
159	female	19	GG	AG	Ff	tt	
160	male	66	GC	AG	Ff	Tt	
161	female		GG	AG	FF	TT	

Number	Sex	Age	G-1520C	A-1012G	Fok1	Taq1	Metastasis
162	male	58	GC	AG	Ff	Tt	
163	male	47	GC	AG	Ff	Tt	
165	female	45	GC	AG	FF	Tt	
166	female	33	GC	AG	FF	Tt	
167	female	57	GG	AA	ff	tt	metastasis
169	male	48	GG	AA	FF	TT	
170	female	47	GG	AG	Ff	TT	
171	male	72	GG	AA	Ff	TT	
172	female	40	CC	GG	Ff	tt	
173	male	81	GG	AA	Ff	Tt	
174	female	23	GC	AG	ff	TT	
175	male	51	GG	AA	Ff	Tt	
176	female	54	GC	AG	Ff	Tt	
177	female	71	GC	AG	FF	TT	
178	female	57	CC	GG	FF	Tt	
179	female	37	GG	AA	FF	TT	
180	female	49	GG	AA	ff	tt	
181	female	56	GC	AG	FF	Tt	
183	female	66	GG	AA	FF	tt	
185	male	42	GG	AA	ff	Tt	
186	female	46	GG	AG	FF	Tt	
188	female	56	GC	AG	Ff	Tt	
189	female	30	CC	GG	FF	TT	
193	female	47	GG	AA	FF	TT	

Psoriasis

Number	Sex	Age	G-1520C	A-1012G	FokI	TaqI	Dovonex Response
1	male	56	GC	AG	ff	Tt	response
2	female	49	GC	GG	FF	TT	response
3	female	55	GG	AG	ff	TT	no response
5	male	74	GC	AG	Ff	Tt	no response
6	male	59	GG	AA	Ff	TT	response
10	male	62	GC	AG	Ff	TT	response
15	male	23	GG	AA	Ff	Tt	response
16	male	50	GG	AA	FF	tt	response
20	male	43	GC	AG	FF	tt	response
21	female	49	GC	AG	Ff	tt	response
27	male	38	GG	AG	ff	TT	response
28	female	39	GC	AG	FF	Tt	no response
31	male	59	GC	AG	Ff	tt	no response
35	male	55	GG	AA	Ff	TT	response
40	male	36	GC	AG	Ff	tt	no response
41	male	27	GC	AG	Ff	TT	response
44	male	29	GG	AA	ff	Tt	no response
50	male	37	GC	GG	Ff	Tt	no response
53	male	52	GG	AA	FF	TT	response
54	female	44	GC	AG	Ff	Tt	response
55	female	72	GG	AA	Ff	TT	response
57	female	28	GC	AG	Ff	Tt	no response
58	female	37	GG	AA	Ff	Tt	response
66	male	64	GC	AG	FF	tt	response
68	male	67	GC	GG	ff	TT	response
70	female	70	GG	AA	FF	TT	response
71	female	-13	GC	AG	FF	Tt	response
78	male	66	GC	GG	ff	Tt	response
79	female	50	GC	GG	Ff	Tt	response
83	female	57	GC	AG	FF	TT	response
97	female	36	GG	AA	ff	TT	response
101	male	42	GC	AG	ff	Tt	response
105	female	38	GG	AA	Ff	Tt	response
108	female	37	GC	AG	FF	Tt	response
110	female	69	GG	AG	ff	tt	no response
111	female	43	GG	AA	FF	TT	response
120	male	34	CC	GG	FF	TT	response
123	male	59	GG	AG	FF	TT	response
127	male	36	GC	GG	Ff	Tt	no response
130	male	45		AG	FF	Tt	response
131	male	71	GC	GG	Ff	Tt	response
132	female	50	GC	AG	Ff	Tt	response
135	male	56	GG	AA	FF	Tt	response
139	male	54	GG	AA	FF	TT	response
140	female	63		AA	Ff	Tt	no response
141	female	74	CC	AG	ff	TT	response
143	female	66	GC	GG	ff	Tt	response

Number	Sex	Age	G-1520C	A-1012G	FokI	TaqI	Dovonex Response
144	female	29	GC	AG	Ff	TT	response
145	male	47	GC	AG	FF	TT	response
147	male	42	GG	AA	FF	Tt	response
148	male	63	GC	GG	Ff	Tt	response
149	female	78	GC	AG	Ff	Tt	response
150	female	68	GC	GG	FF	Tt	response
151	male	65		AG	Ff	Tt	response
152	male	36	GC	AG	FF	TT	response
153	male	36	GC	GG	Ff	TT	response
154	female	39	GC	AG	Ff	Tt	response
155	male	35	GG	AG	ff	Tt	response
157	male	74	GC	GG	FF	TT	response
158	male	79	GC	AG	FF	tt	no response
159	female	78	CC	GG	FF	TT	response
160	female	70	GC	GG	ff	Tt	response
161	male	37	GC	AG	Ff	TT	no response
162	male	47	GG	AG	FF	TT	response
163	female	33	GG	AG	Ff	Tt	response
165	female	40	GC	AG	FF	Tt	no response
166	male	47	GC	AG	FF	TT	response
167	female	76	CC	GG	Ff	Tt	no response
168	female	37		GG	FF	TT	response
169	female	43	GG	AA	Ff	Tt	response
170	female	47	GC	AG	FF	TT	response
172	female	25	GC	AG	FF	TT	no response
173	male	43	GC	GG	ff	Tt	response
176	female	21	GG	AA	Ff	TT	no response
177	female	27	GG	AA	ff	Tt	response
178	male	38	CC	GG	FF	Tt	no response
179	female	63	GC	AG	FF	TT	no response
180	female	39	GG	AG	FF	TT	response
181	male	46			Ff	TT	no response
182	female	19	CC	GG	FF	Tt	response
183	male	36	GG	AA	Ff	tt	response
187	male	48	CC	GG	Ff	Tt	response
188	male	19	GC	AG	Ff	TT	no response
189	male	29	GC	AA	Ff	TT	no response
190	male	53	GG	AA	FF	TT	response
191	male	46	GG	AG	FF	TT	no response
192	male	46	GG	AA	FF	Tt	response
193	male	51	GC	AG	Ff	Tt	response
194	female	36	GC	AG	Ff	tt	response
199	female	31	GC	AG	FF	TT	response
200	female	30		AA	Ff	TT	response
202	female	58	GC	AG	ff	Tt	no response
203	male	26	GG	AA	Ff	TT	no response
204	male	52	GC	AG	FF	Tt	response
205	male	60	GC	AG	Ff	tt	response
209	female	40	CC	GG	Ff	Tt	response
211	female	27	GC	AG	ff	Tt	response
212	male	38	GG	AA	Ff	TT	response

Number	Sex	Age	G-1520C	A-1012G	Fok1	Taq1	Dovonex Response
214	male	39		AG	FF	TT	response
215	female	60	GG	AA	ff	TT	response
216	male	36	GC	AG	Ff	Tt	response
218	male	29	GG	AA	FF	TT	response
219	female	69	GG	AA	Ff	Tt	no response
220	male	36	GC	GG	Ff	TT	response
222	female	75	GC	GG	Ff	Tt	
226	female	30	GC	AG	Ff	tt	no response
227	female	18	CC	GG	FF	TT	response
228	male	51	GG	AA	FF	TT	response
229	female	48	GC	GG	FF	Tt	response
230	female	80	GC	GG	ff	Tt	response
231	female	57	GG	AA	Ff	Tt	response
232	male	51	GG	AA	Ff	tt	no response
233	female	39	GG	AA	Ff	Tt	response
235	male	41	GC	GG	Ff	tt	response
236	female	61	CC	GG	Ff	Tt	no response
237	female	46	GG	AA	Ff	Tt	response
238	male	63	GG	AA	Ff	Tt	no response
239	male	21	GG	AG	FF	TT	no response
241	male	68	GG	AA	FF	TT	response
242	female	90		AG	ff	TT	response
244	female	52	GG	AG	FF	TT	no response
248	male	56	CC	GG	FF	Tt	no response
251	male	37	GC	AG	FF	TT	response
252	female	24	GG	AA	ff	Tt	response
253	female	83	GC	GG	Ff	Tt	response
254	female	80	GC	AG	Ff	Tt	no response
260	male	41	GC	AG	FF	TT	response
262	male	33	GC	AG	Ff	Tt	no response
263	female	31	GC	AG	Ff	TT	response
264	male	35	GC	AG	Ff	Tt	response
265	female	36	GC	AG	FF	TT	no response
269	male	65	GC	AG	Ff	Tt	response
272	female	44	GG	AA	Ff	TT	response
273	male	32	GC	AG	Ff	Tt	response
274	male	46	CC	GG	Ff	TT	response
275	male	22	GC	AG	FF	tt	response
280	female	36	GC	AG	Ff	Tt	no response
290	male	59	GC	AG	FF	TT	response
293	male	61	CC	GG	FF	TT	response
296	female	41	GG	AA	Ff	Tt	response
297	male	33	CC	GG	ff	TT	no response
298	female	22	CC	GG	FF	Tt	response
300	male	43	GG	AA	FF	TT	response
302	male	35	CC	GG	ff	Tt	response
303	male	25	GG	AG	ff	Tt	no response
304	male	19	GC	AG	Ff	Tt	
308	female	24	GG	AA	Ff	TT	response
311	female	24	GG	AA	FF	TT	response
315	male	34	GG	AG	Ff	Tt	response

Number	Sex	Age	G-1520C	A-1012G	Fok1	Taq1	Dovonex Response
319	female	34	GG	AA	FF	TT	response
322	female	31	GC	AG	FF	TT	response
327	male	20	GC	AG	ff	TT	response
328	male	52	GG	AA	Ff	Tt	response
329	female	51	GC	GG	ff	TT	no response
330	male	69	GC	AG	Ff	Tt	no response
331	male	43	GC	AG	FF	Tt	response
334	female	37	GG	AG	FF	tt	response
337	male	33	GC	AG	Ff	TT	no response
341	female	43	GC	AG	Ff	TT	response
347	male	58	GG	AG	Ff	tt	response
350	male	28	GC	GG	Ff	Tt	no response
353	male	41	GG	AA	Ff	TT	response
354	female	27	GC	AG	FF	Tt	no response
355	male	37	GG	AA	Ff	Tt	
357	male	41	GG	AA	FF	Tt	response
358	female	17	CC	GG	Ff	Tt	no response
360	male	69	GC	GG	Ff	TT	no response
366	male	43	GG	AG	Ff	Tt	no response
367	male	26	GC	AG	Ff	Tt	response
368	male	26	GC	AG	Ff	Tt	response
369	male	37	GG	AA	ff	TT	response
370	male	30	GC	AG	FF	Tt	response
372	male	33	GC	AG	Ff	tt	response
374	male	48	GG	AA	FF	Tt	response
375	female	43	GC	AG	FF	Tt	no response
377	male	76	GG	AA	Ff	tt	response
379	male	55	GG	AA	Ff	TT	response
380	female	38	GC	AG	Ff	Tt	no response
382	male	39	GC	GG		tt	no response
383	male	45	CC	AG	Ff	TT	response
388	male	31	GC	AG	ff	tt	response
389	female	31	GC	GG	Ff	TT	no response
390	female	46	GG	AA	FF	Tt	no response
392	female	42	GG	AG	FF	TT	response
395	male	41	GC	GG	Ff	TT	response
396	male	67	GC	AG	FF	Tt	
398	male	38	GC	AG	Ff	TT	response
405	male	29	GG	AA	ff	Tt	no response
407	male	32	GC	AG	FF	Tt	no response
408	male	29	GG	AA	Ff	Tt	no response
409	male	40	CC	GG	Ff	Tt	response
410	female	78	GG	AG	FF	TT	response
411	male	34	GC	AG	FF	Tt	
416	female	34	GG	AG	Ff	TT	no response
418	female	22	GG	AA	Ff	Tt	no response
421	female	43	GG	AG	FF	TT	no response
423	male	22		AG	Ff	tt	no response
424	female	32	GC	AG	Ff	Tt	response
426	female	47	GC	AG	Ff	tt	no response
428	male	36	GG	AG	Ff	TT	no response

Number	Sex	Age	G-1520C	A-1012G	Fok1	Taq1	Dovonex Response
430	female	44	GG	AG	Ff	Tt	no response
431	male	41	GC	AG	Ff	Tt	response
432	female	53	GG	AA	FF	Tt	response
433	male	60	GG	AA	FF	Tt	no response
436	female	21	GG		Ff	TT	no response
440	male	42	GG	AA	ff	Tt	response
441	male	20	GG	AG	FF	Tt	no response
443	male	55	GG	AG	Ff	Tt	response
446	male	51	GC	GG	Ff	TT	response
447	female	25		AA	Ff	TT	response
463	male	55	GC	AG	Ff	TT	no response
464	male	52	GC	AG	Ff	Tt	response
473	male	26		AG	FF	Tt	response
476	female	38	GC	AG	Ff	TT	response
482	male	77	GC	AG	FF	TT	response
487	male	66	CC	GG	FF	Tt	response
490	male	27	GC	AG	Ff	TT	response
492	male	40	GG	AA	Ff	TT	
493	male	36	CC	GG	Ff	Tt	no response
495	female	34	CC	GG	FF	Tt	
503	female	25	GC	AG	FF	tt	no response
506	male	37	GG	AG	FF	Tt	no response
512	male	39	CC	GG	Ff	TT	no response

VI. Appendix VI: Publications Arising from this Work

The following two abstracts are for posters presented to the 12th Workshop on Vitamin D, Maastricht, The Netherlands, July 6th-10th, 2003.

A NOVEL POLYMORPHISM IN THE 1A PROMOTER REGION OF THE VITAMIN D RECEPTOR IS ASSOCIATED WITH ALTERED SUSCEPTIBILTY AND PROGNOSIS IN MALIGNANT MELANOMA.

[1]J.A.Halsall,[2] J.E.Osborne,[2] P.E.Hutchinson,[1] L.Potter,[1] J.H.Pringle[1] Department of Pathology, University of Leicester, Leicester, UK, LE2 7LX. [2]Department of Dermatology, Leicester Royal Infirmary, Leicester, UK, LE1 5WW

$1,25(OH)_2D_3$, the hormonally active form of Vitamin D, has been shown to inhibit proliferation and promote differentiation in a number of cell types, including malignant melanoma (MM) cells. The vitamin D receptor (VDR) mediates the genomic actions of $1,25(OH)_2D_3$. Several restriction fragment length polymorphisms (RFLPs) of the VDR have been reported. Of these, the *FokI* RFLP in exon 2 and the *TaqI* RFLP in exon 9 have been associated with susceptibility and prognosis in MM. The VDR 1A promoter region was screened by single stranded conformational polymorphism (SSCP) analysis and a novel A to G polymorphism was found at position –1012bp relative to the exon 1A transcription start site. The aim of the study was to investigate the effect of this polymorphism on occurrence and outcome of MM, as assessed by metastasis. For occurrence the MM patients were stratified into Breslow depth groups corresponding to the proportions known to occur in all MMs presenting in Leicestershire. There was a total of 198 MM cases, 176 of which were stratified, and 80 controls. To screen for the polymorphism a 150bp region around the polymorphism was amplified by PCR. The forward primer was designed to introduce a restriction site adjacent to the polymorphism such that in subsequent RFLP analysis the A allele was restricted and the G allele remained uncut. AA and AG were significantly associated with the occurrence of MM (odds ratios (OR) 3.0, 95% confidence interval (CI) 1.3-7.0,p=0.01, and 2.2 (1.0-4.8), p=0.05 respectively). On univariate analysis, with Cox's proportional hazards model, AA and AG were associated with increased metastatic rate, hazard ratio (HR) 3.2 (1.3-7.6), p=0.005. On multivariate analysis, including age at presentation, sex, skin type, eye and hair colour and tumour site, results were similar, HR 3.8 (1.3-10.8), p=0.01. The A allele of this VDR 1A promoter region polymorphism is therefore associated with both increased occurrence and poorer outcome of MM.

THE EFFECTS ON PERSONAL CHARACTERISTICS WHICH ARE RISK FACTORS FOR POOR PROGNOSIS IN MALIGNANT MELANOMA OF A NOVEL POLYMORPHISM IN THE 1A PROMOTER REGION OF THE VITAMIN D RECEPTOR: INDEPENDENT ASSOCIATION WITH BLUE EYES AND FAIR SKIN.

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Patient characteristics, such as age, sex, skin type, eye and hair colour are known to be risk factors for the development of malignant melanoma (MM). However the effect of these variables on tumour outcome is less investigated, though increased age at presentation with MM, male sex, and acral situation are reported to be adverse factors. Depth of the MM within the dermis, Breslow thickness (BT), at presentation is the single most reliable estimator of prognosis for MM. The aim of the present study was to investigate the effects of all of the above variables on MM outcome, as estimated by BT and to investigate any association of these variables with a newly described A to G promoter polymorphism at -1012bp relative to the 1A transcription start site of the VDR gene. It was confirmed that age at presentation (age>50), odds ratio (OR) 4.3, 95% confidence interval (CI) 2.0-8.88, p<0.001, male gender, OR 2.0 (1.1-3.7), p=0.03, tumours on head and neck and subungual situation, OR 5.4 (1.8-15.6), p=0.002 were adverse factors. Novel findings were of increased risk in individuals with skin types 1 (fair skin) and 3 (darker Caucasian skin), OR 2.3 (1.2-4.1), p=0.008 and also hazel and green eyes and black and brown hair but not at significant levels. VDR promoter homozygote A genotype was not associated with age at presentation of MM, male sex , site or hair colour. It was associated with blue eyes compared to other eye colours, OR 2.1, (1.1-3.9), p=0.02 and skin type 1 compared to other skin types, OR 1.8 (0.9-3.4), p=0.09. On multivariate analysis with eye colour and skin type and including BT, there was a negative relationship of the interaction term and homozygote A genotype, OR 0.18 (0.04-0.76), p=0.02, and the adjusted OR for blue eyes was 3.2 (1.5-6.8) p=0.003 and skin type 1 5.0 (1.5-15.9), p=0.007. The homozygote A genotype is therefore associated with fair skin and blue eyes, an association of VDR polymorphism with hair colour and skin type that has not previously been reported.

The following abstract was selected for oral presentation at the Leicestershire Research Prize day, 11th September 2003.

A NOVEL POLYMORPHISM IN THE 1A PROMOTER REGION OF THE VITAMIN D RECEPTOR IS ASSOCIATED WITH ALTERED SUSCEPTIBILTY AND PROGNOSIS IN MALIGNANT MELANOMA.

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1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the hormonally active form of Vitamin D_3, has been shown to inhibit proliferation and promote differentiation in a number of cell types, including malignant melanoma (MM) cells. The vitamin D receptor (VDR) mediates the genomic actions of $1,25(OH)_2D_3$. Several restriction fragment length polymorphisms (RFLPs) of the VDR have been reported. Of these, the *FokI* RFLP in exon 2 and the *TaqI* RFLP in exon 9 have been associated with susceptibility and prognosis in MM. The VDR 1A promoter region was screened by single stranded conformational polymorphism (SSCP) analysis and a novel A to G polymorphism was found at position –1012bp relative to the exon 1A transcription start site. Patients and controls were then screened by PCR induction of a RFLP site adjacent to and dependent on the polymorphism followed by RFLP analysis. The aim of the study was to investigate the effect of this polymorphism on occurrence and outcome of MM, as assessed by metastasis and recurrence. For occurrence the MM patients were stratified into Breslow depth groups corresponding to the proportions known to occur in all MMs presenting in Leicestershire. There was a total of 198 MM cases, 176 of which were stratified, and 80 controls. AA and AG were significantly associated with the occurrence of MM (odds ratios (OR) 3.0, 95% confidence interval (CI) 1.3-7.0,p=0.01, and 2.2 (1.0-4.8), p=0.05 respectively). On univariate analysis, with Cox's proportional hazards model, AA and AG were associated with increased rate of metastatic or recurrence, hazard ratio (HR) 3.2 (1.3-7.6), p=0.005. On multivariate analysis, including age at presentation, sex, skin type, eye and hair colour and tumour site, results were similar, HR 3.8 (1.3-10.8), p=0.01. The A allele of this VDR 1A promoter region polymorphism is therefore associated with both increased occurrence and poorer outcome of MM. This is the strongest reported association of a VDR polymorphism with MM outcome which suggests that the promoter polymorphism may have a significant functional role in VDR gene transcription and indicates an important role for $1,25(OH)_2D_3$ and receptor genotype in MM progression.

British Journal of Cancer (2004), 1 – 6
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www.bjcancer.com

Full Paper

A novel polymorphism in the 1A promoter region of the vitamin D receptor is associated with altered susceptibilty and prognosis in malignant melanoma

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The association of Taq I and Fok I restriction fragment length polymorphisms of the *vitamin D receptor* with occurrence and outcome of malignant melanoma (MM), as predicted by tumour (Breslow) thickness, has been reported previously. We now report a novel adenine–guanine substitution −1012 bp relative to the exon 1a transcription start site (A-1012G), found following screening by single-stranded conformational polymorphism of this promoter region. There was a total of 191 MM cases, which were stratified according to conventional Breslow thickness groups, cases being randomly selected from each group to form a distribution corresponding to the known distribution of Breslow thickness in our area, and this population ($n = 176$) was compared to 80 controls. The A allele was over-represented in MM patients and, with GG as reference, odds ratio (OR) for AG was 2.5, 95% confidence interval (CI) 1.1–5.7, ($P = 0.03$) and AA 3.3, CI 1.4–8.1, ($P = 0.007$). The outcome was known in 171 of 191 patients and the A allele was related to the development of metastasis, the Kaplan–Meier estimates of the probability of metastasis at 5 years being: GG 0%; AG 9%, CI 4–16%; AA 21%, CI 12–36%; ($P = 0.008$), and to thicker Breslow thickness groups ($P = 0.04$). The effect on metastasis was independent of tumour thickness and A-1012G may have predictive potential, additional to Breslow thickness. Neither the Fok I nor Taq I variants (f and t) were significantly related to the development of metastasis, although there was a strong relationship of fftt with the thickest Breslow thickness group ($P = 0.005$). There was an interaction between the A-1012G and Fok I polymorphisms ($P = 0.025$) and the Fok I variant enhanced the effect of the A allele of the A-1012G polymorphism on metastasis, the probability of metastasis for AAff at 5 years follow-up being 57%, CI 24–92%.
British Journal of Cancer advance online publication, 6 July 2004; doi:10.1038/sj.bjc.6602006 www.bjcancer.com
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Keywords: VDR; promoter polymorphism; malignant melanoma

The polymorphisms of the *vitamin D receptor (VDR)*, reported in the literature, comprise a cluster of tightly linked polymorphisms at the 3′-end and two polymorphisms at the 5′-end of the gene. The 3′ polymorphisms are Apa 1 (Faraco *et al*, 1989) and Bsm 1 (Morrison *et al*, 1992) in intron 8, Taq 1(Morrison *et al*, 1992) in a silent site in exon 9 and a length polymorphism of a polyadenyl (polyA) microsatellite in the 3′-untranslated region (Ingles *et al*, 1997b), classified into long (L) and short (S) variants (L demonstrates linkage disequilibrium with b, a, T). The 5′ polymorphisms are Fok 1(Saijo *et al*, 1991) situated in exon 2, 10 base pairs upstream from an ATG translation start point, and a recently described polymorphism in the promoter region, situated at −3731 bp relative to the exon 1a transcription start site (Arai *et al*, 2001) within a binding element of Cdx-2, which is a caudal-related homeodomain transcription factor. The 3′ region polymorphisms do not affect VDR protein structure, while Fok 1 (C–T transition) alters an ACG codon resulting in a further upstream start codon and a three amino-acid extended protein (Saijo *et al*, 1991). Both the 3′ and Fok 1 polymorphisms have been reported to be functional in terms of VDR transactivation (Whitfield *et al*, 2000). Cdx-2 is important during the development of the intestine and in adults it has been shown to regulate VDR expression in the small intestine (Yamamoto *et al*, 1999). The expression of Cdx-2 has also been found in other tissues such as the brain and prostate. Polymorphism at the Cdx-2-binding site significantly alters the transcriptional activity of the *VDR* promoter region (Arai *et al*, 2001).

Since 1996, there have been many reports of associations of polymorphisms of the *VDR* with systemic carcinomas. The 3′ polymorphisms have been reported to be associated with the occurrence and outcome, as assessed by metastasis or presence of adverse prognostic markers, of prostatic cancer (Taylor *et al*, 1996; Ingles *et al*, 1997c; Ingles *et al*, 1998; Ma *et al*, 1998; Habuchi *et al*, 2000; Hamasaki *et al*, 2001; Medeiros *et al*, 2002), breast cancer (Curran *et al*, 1999; Lundin *et al*, 1999; Ingles *et al*, 2000; Bretherton-Watt *et al*, 2001; Cui *et al*, 2001; Schondorf *et al*, 2003) and renal cancer (Ikuyama *et al*, 2002). Fok 1 polymorphisms have been reported to be associated with the outcome of prostate cancer (Hamasaki *et al*, 2002) and occurrence of breast (Ingles *et al*, 1997a) and colon (Wong *et al*, 2003) cancers. However, other studies have failed to find an association with prostate cancer

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Received 29 December 2003; revised 27 April 2004; accepted 17 May 2004

(Jenkins *et al*, 1997; Dunning *et al*, 1999; Luscombe *et al*, 2001) or breast cancer (Correa-Cerro *et al*, 1999; Furuya *et al*, 1999; Watanabe *et al*, 1999; Blazer *et al*, 2000; Chokkalingam *et al*, 2001), but many of these (Jenkins *et al*, 1997; Correa-Cerro *et al*, 1999; Furuya *et al*, 1999; Watanabe *et al*, 1999; Blazer *et al*, 2000) were relatively limited studies of approximately 100 or less cases. The Cdx-2 polymorphism has been linked with increased risk in cancer of the prostate (Bodiwala *et al*, 2004).

We have previously reported an association with the occurrence and, particularly, the outcome of malignant melanoma (MM), as assessed by Breslow tumour thickness and polymorphisms at the Fok 1 and Taq 1 restriction sites (ttff) (Hutchinson *et al*, 2000). We now describe a new polymorphism in the promoter region upstream of the exon 1a transcription start site, A-1012G. We report investigations into the relationship of the A-1012G and A-1012G/Taq 1 and A-1012G/Fok 1 genotype combinations with the occurrence and outcome of MM, in terms of the development of metastasis and as predicted by Breslow thickness and compare the strength of the A-1012G polymorphism with the corresponding relationships of Taq 1 and Fok 1.

MATERIALS AND METHODS

Single-stranded conformational polymorphism (SSCP) analysis

All oligonucleotide primers used for PCR are shown in Table 1. A ~2500 bp region from 120 bp upstream of exon 1e to 70 bp downstream of exon 1d including exon 1a was amplified by PCR in 10 overlapping amplicons of ~350 bp (primers 1A/Dp1-10) for 35 cycles with an annealing temperature of 60°C in PCR buffer (45 mM Tris-HCl (pH 8.8), 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 110 $\mu g\,ml^{-1}$ BSA, 6.7 mM β-mercaptoethanol 4.4 μM EDTA (pH 8.0), 200 μM dNTPs). Each amplicon was screened for variation by SSCP in 36 control samples. A measure of 3 μl PCR product was added to 9 μl denaturing loading buffer (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol, 10 mM sodium hydroxide) and heated to 95°C for 3 min, chilled on ice and loaded onto a 0.6–0.8 × MDE (BioWhittaker, 50620) gel, depending on amplicon size, in 0.6% TBE buffer and run at 500 V for 30 min followed by 270–350 V overnight, depending on amplicon size. Where variation was found, variant bands were reamplified from the gel and sequenced by Big Dye sequencing on an ABI prism 377 sequencer.

The possible effects of sequence variation on promoter activity were determined by the analysis of potential transcription factor-binding sites within variable regions using the TESS database (Schug and Overton, 1997).

Genotype screening

To screen for the promoter polymorphism in patients and controls, a 150 bp region around the polymorphism was amplified using primer pair SNP1 (Table 1). PCR cycling was carried out with a 55°C annealing temperature for three cycles followed by 65°C for 37 cycles. The forward primer was adjacent to the polymorphism and mutated the sequence to introduce an *Eco*RV restriction site in the A allele but not in the G allele such that in subsequent *Eco*RV digestion (37°C, 20 h), followed by agarose-gel electrophoresis, the A allele was restricted and the G allele remained uncut. Fok 1 and Taq 1 were amplified with the appropriate primers (Hutchinson *et al*, 2000) for 35 cycles with an annealing temperature of 60°C. PCR products were then digested with Fok 1 (37°C, 20 h) or Taq 1 (65°C, 20 h). The Fok 1 F and Taq 1 T alleles were refractory to digestion, while the f and t alleles were restricted.

MM studies

Patients with a diagnosis of MM who attended the Pigmented Lesion Clinic between 1995 and 1997 were recruited. Patients with lentigo maligna melanoma were not included. It was attempted to recruit all patients, but this was not always possible in busy clinics. Documented data were age at presentation, gender, skin type based on the Fitzpatrick classification (Fitzpatrick, 1988), eye colour, hair colour at age 21 years, tumour site and Breslow group and presence of metastases on follow-up. Breslow thickness (defined as the vertical thickness of the tumour from the granular layer of the epidermis to the deepest part of the melanoma) was determined by specialist pathologists. On the basis of Breslow thickness, patients were divided into five conventional Breslow thickness groups (0–4); *in situ*, <0.75 mm, 0.75–1.49 mm, 1.5–3.49 mm and ≥3.5 mm (Mackie *et al*, 1985). For the occurrence studies, a subsample of patients were stratified according to Breslow thickness group to conform with frequencies within groups according to the reported distribution in the Leicestershire area ($n = 738$) (Osborne and Hutchinson, 2001). Controls consisted of UK Caucasian patients not known to have cancer. This study was approved by the local Ethics Committee and written informed consent was given by the patients.

Table 1 Oligonucleotide sequences used as PCR primers

Primer pair	Forward primer	Reverse primer	Amplicon position (bp)
1A/Dp1	CTGATGACGGCATGTGCT	CAGCCTTTGTTGGAGGAGAG	−2119 to −1755
1A/Dp2	CAGTGGGATGTGCAGAGAGA	GCTAGCGGTGATTCTTGTGG	−1979 to −1529
1A/Dp3	AGATGTCAGGCCAGTCAAGC	GGTATCATGGCAACTTTCTGG	−1676 to −1329
1A/Dp4	ATGGTCCATTCCCAAGTTCA	CAGAGGGACAAGGTGAAAGG	−1411 to −1051
1A/Dp5	AGCAGATTTGCTGGGCTCTA	TGCTTCCCTTGACTGTGTGA	−1163 to −818
1A/Dp6	TCCCACTGCACAGTGAGTTC	AAGTGGAAACCGGAGTTGC	−914 to −554
1A/Dp7	GATATCGGGTGGGAGCAAT	TGGGACAGAGTTGTCGATGA	−594 to −232
1A/Dp8	ACAGGCTGAAGCGGGTATC	CCGAGTCCCTATCCTGAGAC	−281 to +126
1A/Dp9	GCAAGAGAGGACTGGACCTG	GCGGAGCATTTCTCCTAAGC	−94 to +334
1A/Dp10	TCTCAGCGGTAAACTTGGCTA	AGACCCAACTCCACCATCAC	+186 to +535
SNP1	CCTCCTCTGTAAGAGGCGAATAGCGAT	GGACAGGTGAAAAAGATGGGGTTC	−1039 to −861
Fok 1	CTGGCACTGACTCTGGCTCT	TGCTTCTTCTCCCTCCCTTT	−48 to +199[a]
Taq 1	CAGAGCATGGACAGGGAGCAAG	CGGCAGCGGATGTACGTCTGCAG	−260 to +338[b]

PCR = polymerase chain reaction; bp = base pair. All amplicon positions are relative to the exon 1a start site except [a]relative to the start of exon 2 and [b]relative to the start of exon 9.

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Statistics

Occurrence and Breslow thickness data were analysed by contingency tables (Unistat Statistical Package, version 5.0, Unistat, UK) and logistic regression (Stata software package, version 7.0, Stata Corporation, Texas, USA). Metastatic data were analysed using the Kaplan–Meier product limit estimator (Unistat), with the log-rank comparison statistic, and by the Cox's proportional-hazard model (Stata). Interactions were assessed by the Cox's likelihood-ratio test (Stata).

RESULTS

Promoter screening

Single-stranded conformational polymorphism revealed variation in the 1A/Dp5 region (−1163 to −818 bp). Sequencing of the variant bands revealed an A–G polymorphism, at −1012 bp relative to the exon 1a transcription start site (Figure 1). Therefore, the sequence in the region was either: −1020 ATAGC*AATATC*TTC or −1020 ATAGCAATGTCTTC.

Analysis with the TESS database revealed a strong GATA-3 core-binding site at this locus in the A allele (given in italics above), which was not present on analysis of the G allele.

MM studies

The percentages of MM in each Breslow thickness group presenting in Leicestershire in the past 10 years are as follows: *in situ*, 13.8%; <0.75 mm, 26.6%; 0.75–1.49 mm, 21.5%; 1.5–3.49 mm, 21.0% and ⩾3.5 mm, 17.1%. A total of 191 patients were recruited. The numbers of patients randomly chosen for allocation to the respective thickness groups from the 191 patients were 24 (13.6%), 49 (27.8%), 35 (19.9%), 38 (21.6%) and 30 (17.0%). The total number of MM patients for the occurrence study was therefore 176 (mean age 54.3 years, 110 females) and there were 80 controls (mean age 56.2 years, 40 females). Age or sex did not have a statistically significant relationship with *VDR* promoter genotype in either controls or MM patients. In the metastatic outcome part of the study, there was a total of 171 of 191 patients in whom the outcome was known.

Table 2 shows allele and genotype frequencies in controls and MM patients. Genotype frequencies conformed to the Hardy–Weinberg equilibrium in both subject groups. The A allele was over-represented in the MM patients ($P=0.03$). Similarly, AA genotype was more and GG less frequent in MM patients ($P=0.03$). Correcting for age and sex and with GG as reference, odds ratio (OR) for AG was 2.5, 95% confidence interval (CI) 1.1–5.7 ($P=0.03$) and AA 3.3, CI 1.4–8.1, ($P=0.007$).

The mean time to metastasis was 31 months (range 1–171 months) ($n=19$) and mean follow-up time in patients not developing metastasis was 75 months (3–255 months) ($n=152$). Figure 2 shows Kaplan–Meier curves for metastasis-free times for

Table 2 Allele and genotype frequencies of A-1012G in MM patients and controls

	Alleles		Genotypes		
	A	G	AA	AG	GG
Controls	86 (54%)	74 (46%)	22 (27%)	42 (52%)	16 (20%)
MM patients	244 (64%)	140 (36%)	66 (38%)	92 (53%)	16 (9%)
	$P=0.03$			$P=0.03$	

MM = malignant melanoma.

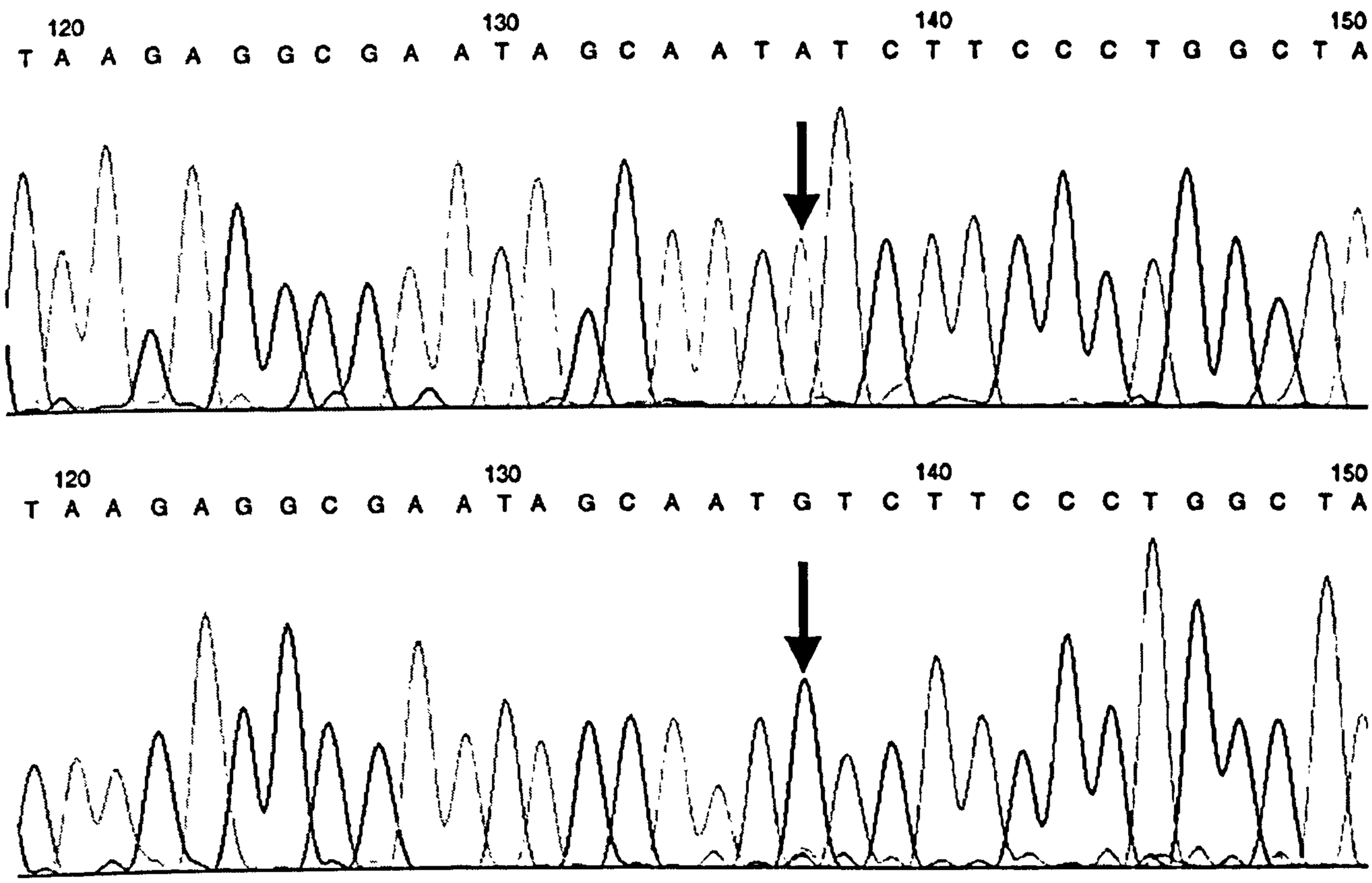

Figure 1 Sequencing traces of the region around the polymorphism, the variable base is marked by an arrow.

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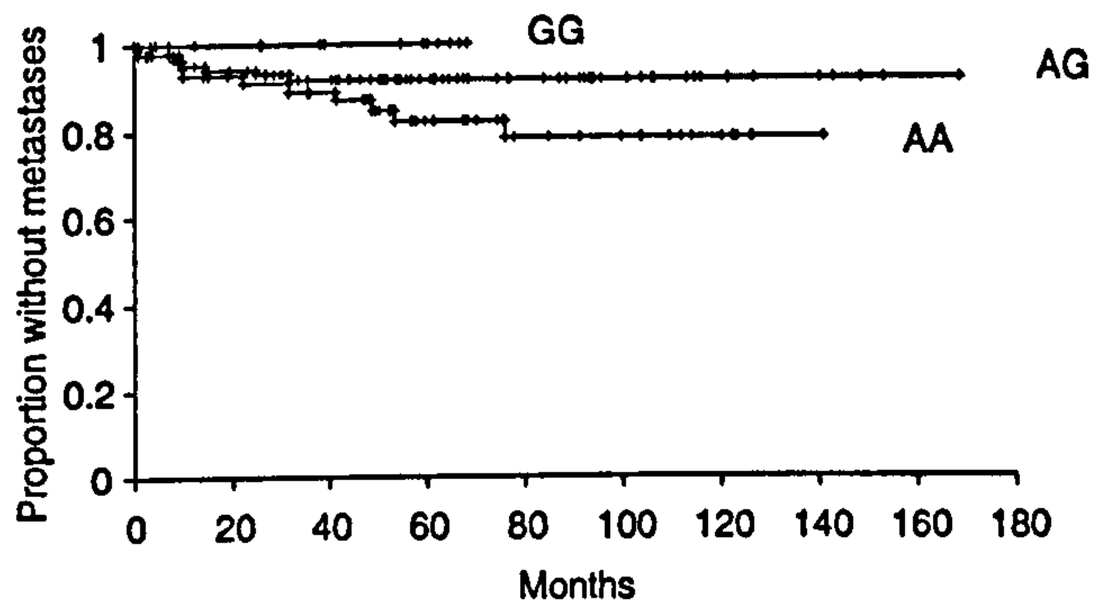

Figure 2 Kaplan–Meier estimates of the probability of metastasis for A-1012G genotypes.

Table 3 Results of Cox's proportional-hazard regression analysis (univariate) of metastatic rate on A-1012G, Fok 1 and Taq 1 genotypes, and on A-1012G genotype combinations with Fok 1 and Taq 1

Polymorphisms	Genotype	Number	HR	95% CI	Significance
A-1012G	GG/AG	110	1		
	AA	59	**2.9**	1.1–7.5	$P=0.03$
Fok	FF/Ff	140	1		
	Ff	30	1.3	0.4–4.1	$P=0.6$
Taq	TT/Tt	143	1		
	Tt	26	1.5	0.5–4.6	$P=0.5$
Fok/Taq	Other genotypes	165	1		
	ff/tt	4	2.2	0.3–16.3	$P=0.5$
A-1012G/Taq	Other genotypes	130	1		
	AATT	27	2.5	0.9–7.4	$P=0.1$
	Aatt	11	3.1	0.8–11.2	$P=0.1$
A-1012G/Fok	Other genotypes	130	1		
	AAFF	30	**4.3**	1.5–12.4	$P=0.006$
	AAff	9	**8.6**	2.5–29.6	$P=0.001$
	AAFF/Ff	50	1		
	AAff	9	**4.7**	1.2–18.6	$P=0.03$

HR = hazard ratio; CI = 95% confidence interval for hazard ratio estimate. Bold numerals represent statistically significant results.

the promoter genotypes, which differed significantly ($P=0.008$). The Kaplan–Meier estimates of the probability of metastasis at 5 years follow-up were: GG 0%; AG 9%, CI 4–16%; AA 21%, CI 12–36%. The results of Cox's proportional-hazard regression analysis are shown in Table 3. As there were no metastases with genotype GG, hazard ratios (HR) for AA and AG compared with GG were not reliable and therefore AA *vs* AG/GG are shown, confirming a significantly worse prognosis for AA. Inclusion of the covariates, age at onset, male gender, skin type, eye and hair colour and lesional site made no significant impact on this (results not shown).

Conversely, the A-1012G polymorphism was not strongly associated with Breslow thickness ($n=191$), although for Breslow thickness groups two, three and four combined *vs* groups zero and one combined (≥1.5 mm *vs* <1.5 mm depth); OR for AA *vs* other genotypes was 1.9 (CI 1.0–3.6, $P=0.04$). The relationship of promoter polymorphism and metastasis was reinvestigated correcting for Breslow thickness, when HR for AA *vs* any other genotype was 2.7, which was very similar to the uncorrected value of 2.9, suggesting that the effect of the polymorphism on metastasis was largely independent of depth of invasion. Table 4 shows the

Table 4 Proportion of patients developing metastases in relation to Breslow group of the melanoma and A-1012G genotype

	Breslow thickness group				
	In situ	<0.75 mm	0.75–1.49 mm	1.5–3.49 mm	≥3.5 mm
GG	0% (0/3)	0% (0/5)	0% (0/3)	0% (0/4)	0% (0/2)
AG	0% (0/13)	0% (0/30)	0% (0/17)	17% (3/18)	27% (4/15)
AA	0% (0/7)	8% (1/13)	12% (2/16)	14% (2/14)	67% (6/9)

() = number of patients developing metastasis/total number patients in each group.

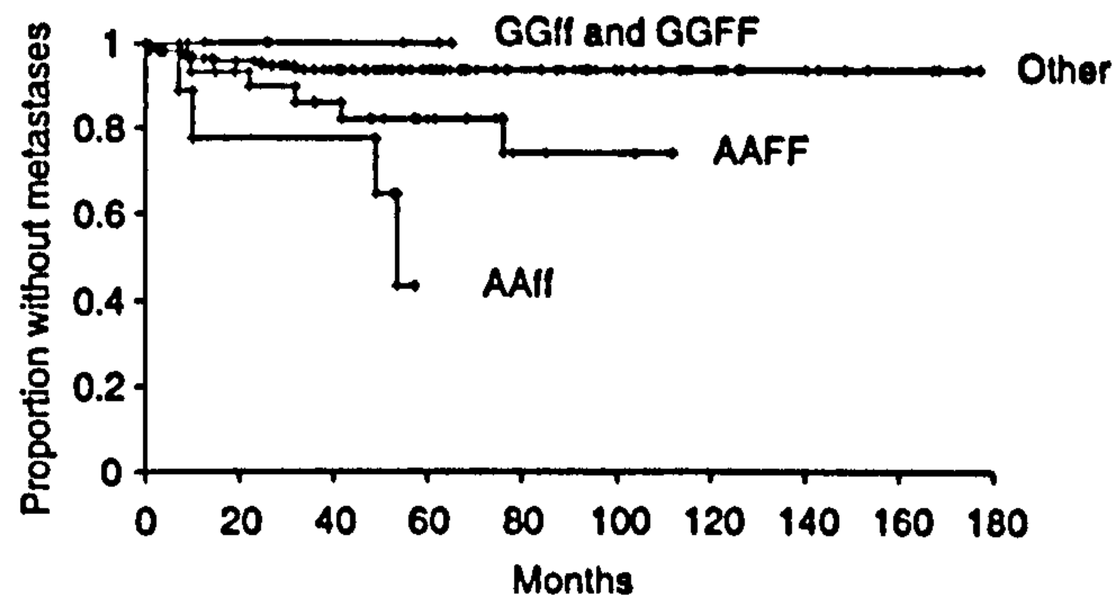

Figure 3 Kaplan–Meier estimates of the probability of metastasis for A-1012G/Fok 1 combination genotypes.

proportion of patients in each promoter genotype/Breslow thickness group who developed metastasis. The highest proportion occurred in patients who had both AA genotype and the thickest tumours (Breslow thickness ≥3.5 mm, group 4). However, some patients with thin tumours developed metastasis but only in the presence of the AA genotype.

The Taq 1 homozygote variant (tt) was associated with Breslow thickness groups three and four combined (Breslow thickness ≥1.5 mm) when corrected for age at presentation, sex, skin type eye and hair colour and site of MM (OR 3.1 CI 1.2–8.3, $P=0.02$) but the Fok 1 homozygote variant (ff) was not (OR 1.4, CI 0.6–3.5, $P=0.5$). The fftt genotype combination was strongly associated with the thickest Breslow thickness group, group 4 (OR 24, CI 3–225, $P=0.005$). Similarly, tt, ff and ttff were not significantly associated with poorer prognosis in terms of metastasis (Table 3).

There was some correlation between A-1012G with Fok 1 polymorphisms (F and A), Spearman's rank correlation 0.15 ($P=0.01$) but not with Taq 1 ($P=0.5$). There was a statistically significant interaction of the A-1012G with Fok 1 polymorphisms ($P=0.025$) but not with Taq 1. Considering A-1012G/Fok 1 and A-1012G/Taq 1 genotype combinations (Figure 3), the Kaplan–Meier estimates of the probability of metastasis at 5 years follow-up were AAff 57%, CI 24–92%; AAFF 18%, CI 8–38%; GGff 0%; GGFF 0% and all other genotypes 6%, CI 3–13%, ($P=0.001$). On Cox's proportional-hazard model regression (Table 3), there was a strong effect of AAff, which was associated with a greater metastatic rate than AAFF/Ff (HR 4.7, $P=0.03$) when corrected for the other covariates. This difference was greater than the corresponding comparison of ff *vs* FF/Ff (HR 1.3, $P=0.6$).

DISCUSSION

This newly described polymorphism of the 1a promoter, A-1012G, has been found to be related to occurrence of MM (Table 2), the A allele being over-represented in the MM population. When investigating the effect of a variable on occurrence of a

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malignancy, it is necessary to employ a sample of patients which is representative in terms of severity of the disease in general if, as in this case, the variable is also related to the outcome. Failure to do this may be a cause of some of the conflicting results in the literature. The cases were therefore stratified according to Breslow thickness, which is the best single predictor of MM outcome.

The promoter polymorphism was clearly related to outcome as assessed by the development of metastasis (Figure 2, Table 3). There was a weaker relationship with thicker Breslow thickness groups. We have previously reported a relationship of Taq 1 and Fok 1 variant gene combination (ttff) with increased Breslow thickness (Hutchinson *et al*, 2000). This was true in the present study ($P<0.005$), but the ttff genotype combination was not significantly associated with metastasis.

The relationship between the A-1012G genotype and development of metastasis was also analysed by Cox's proportional-hazard regression, correcting for Breslow group, when the promoter genotype retained significance. The *VDR* would therefore appear to be related to both Breslow thickness and development of metastasis, but the effect on metastasis is at least partially independent of tumour thickness. This finding has potential importance in the interpretation of molecular mechanism of tumour spread. Also, the inclusion of the *VDR* promoter genotype when predicting MM outcome with Breslow thickness group should potentially enhance precision (Table 4). The highest proportion of metastases occurred in patients who had both AA genotype and the thickest tumours (Breslow thickness group 4). However, some thinner tumours developed metastasis, but usually in the context of AA genotype. Studies on a larger scale are required to further investigate the predictive capacity of this polymorphism.

Neither the Fok 1 nor Taq 1 polymorphism was significantly related to the development of metastasis. However, the promoter and Fok 1 combination genotype (AAff) was greatly associated with a higher propensity to metastasis (Table 3). The Fok 1 polymorphism appears to augment the effect of the promoter polymorphism, as evidenced by a significant statistical interaction on Cox's regression and a significant difference in prognosis associated with AAff compared with AAFf/AAFF genotype combinations (Table 3), despite no similar difference between ff and Ff/FF.

The mechanism of action of this promoter polymorphism is not known. One possibility is that the polymorphism modulates docking of a transcription factor. The analysis of this region with the TESS database (Schug and Overton, 1997) demonstrates that the polymorphism is within the core sequence of a likely GATA-3-binding site in the A allele, while this binding site is not present in the G allele. Merika and Orkin, (1993) demonstrated that DNA strands containing the core sequence AGATAT (the reverse orientation of the A allele) bound human GATA-3, while zero of 63 sequences shown to bind GATA-3 contained the core sequence AGACAT (the reverse orientation of the G allele). GATA-3 is an important transcription factor directing the polarisation of naïve T cells to Th-2 cells (Rengarajan *et al*, 2000). 1α,25-dihydroxyvitamin D_3 has been shown to upregulate GATA-3 gene expression and the GATA-3 protein promotes polarisation to Th-2 (Boonstra *et al*, 2001). The present findings would suggest that the GATA-3 response element in the A allele of the *VDR* promoter may produce a positive feedback loop and amplify the GATA-3-induced polarisation. Therefore, this polymorphism may influence immune response to cancer, particularly in cancers, such as MM, which show high expression of MAGE antigens. Such tumours are susceptible to Th-1 responses (Tatsumi *et al*, 2002). The argument would be that the A allele is associated with a Th-1 to Th-2 switch, which reduces the Th-1 cytotoxic response to cancer cells. A further possibility is that A-1012G is a determinant of whether transcription is initiated in exon 1a or 1d. Exon 1d contains an alternative start codon, which can lead to the expression of a significantly N-terminally extended protein (VDRB1). This is reported to have greater transactivation potential than the short protein, translated at the conventional start codon in exon 2 (VDRA) (Gardiner and Eisman, 2003), although this has not been consistently reported (Sunn *et al*, 2001). The majority of evidence is that vitamin D and the VDR have a protective effect in cancer (Osborne and Hutchinson, 2002). Therefore, if A-1012G were determining the transcription start site, then the G allele would be expected to be associated with the VDRB1 protein and the A allele with the shorter VDRA protein, which would be further altered by the f allele of the Fok 1 polymorphism. This would be compatible with the finding of enhanced effect of AAff over AA found in the present study.

In conclusion, the novel *VDR* promoter polymorphism, A-1012G, is related to MM occurrence and outcome as predicted by Breslow thickness but more particularly with the development of metastasis. This relationship is considerably stronger than the relationships of the Taq 1 and Fok 1 polymorphisms and their genotype combinations. The Fok 1 variant enhanced the effect of the A-1012G/Fok 1 polymorphism on metastasis. The effect on metastasis is at least partially independent of tumour thickness and A-1012G may have predictive potential in addition to Breslow thickness.

ACKNOWLEDGEMENTS

We are grateful to Dr Anthony A Fryer, Honorary Senior Lecturer, Human Genomic Research Group, Institute of Science and Technology in Medicine, Keele University Medical school, University Hospital of North Staffordshire, for his helpful suggestions and corrections to the text.

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Vitamin D receptor gene polymorphisms, particularly the novel A-1012G promoter polymorphism, are associated with vitamin D_3 responsiveness and non-familial susceptibility in psoriasis

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Psoriasis is a genetically determined disease characterized by hyperproliferation and disordered maturation of the epidermis. Th1 lymphocytes are implicated in its pathogenesis. The vitamin D receptor (VDR) is a candidate modifying gene, having immunosuppressive effects and being involved in anti-proliferative and pro-differentiation pathways in keratinocytes. There is suggestive evidence that the A allele of the A-1012G polymorphism is associated with down-regulation of the Th1 response, via GATA-3. The F and T alleles of *Fok*1 and *Taq*1 have been associated with increased VDR activity. The present study aimed to test the hypothesis that the A allele of A-1012G is protective for occurrence and severity of psoriasis and enhances therapeutic response to vitamin D analogues and that these effects would be additive to those of *Fok*1 and *Taq*1. The study group comprised 206 psoriasis patients who had received topical calcipotriol treatment and 80 controls. There was no significant linkage disequilibrium between any pair of the three polymorphic sites (P=0.3–0.8). The A, F and T alleles were positively associated with calcipotriol response: AA genotype (compared to AG/GG), odds ratio (OR)=2.18 (P=0.04); TT, OR=1.97 (P=0.03); AAFF genotype combination, OR=4.11 (P=0.03); AATT, OR=5.64 (P=0.005); and FFTT, OR=3.22 (P=0.01). Comparing patients without, to patients with, a family history of psoriasis, the A allele was under represented (P=0.01) and the AAFF genotype combination even more so (compared to residual genotypes) (OR=0.24; P=0.005). AAFF was also under-represented in patients without a family history compared to controls (OR=0.31; P=0.04). There were no associations of family history with *Fok*1 and *Taq*1. There were no associations of severity of psoriasis with any polymorphism. In conclusion, the A-1012G, *Fok*1 and *Taq*1 *VDR* polymorphisms were associated with response to calcipotriol. A-1012G and *Fok*1 were associated with susceptibility to non-familial psoriasis. *Pharmacogenetics and Genomics* 15:349–355 © 2005 Lippincott Williams & Wilkins.

Pharmacogenetics and Genomics 2005, 15:349–355

Keywords: psoriasis susceptibility, psoriasis treatment, VDR, VDR polymorphisms

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Sponsorship: This work was supported by a grant from The Psoriasis Association (Registered Charity no. 257414).

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Received 20 January 2005 Accepted 24 February 2005

Introduction

Psoriasis is a common inflammatory skin disease, characterized by hyperproliferation [1,2] and disordered maturation of keratinocytes [3]. Th1 lymphocytes have been implicated in its pathogenesis [4–6]. 1,25 dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) is the ligand of the vitamin D receptor (VDR), which is a transcription factor, the receptor–hormone complex binding to hormone response elements in regulatory regions of target genes and modulating gene transcription. $1,25(OH)_2D_3$ signalling is of potential interest in psoriasis because of the anti-proliferative [7,8] and pro-differentiation effects of $1,25(OH)_2D_3$ on keratinocytes [7] and its immunosuppressive effects [9,10], which are thought to be mediated by an inhibitory effect on Th1 lymphocytes, via inhibition of maturation of dendritic cells [11]. The VDR is expressed on both normal [12,13] and psoriatic [14,15] keratinocytes. Topical vitamin D analogues are effective treatments of psoriasis [16] and treatment response has been shown to vary with keratinocyte *VDR* mRNA expression [17]. Therefore, the VDR is a candidate modifying gene of psoriasis.

A number of predominantly restriction fragment length polymorphisms (RFLP) have been reported in the *VDR* gene and include a cluster towards the 3′ end, *Bsm*1 (alleles Bb) and *Apa*1 (Aa) in intron 8, and *Taq*1 (Tt) in exon 9, and a polyadenyl microsatellite length polymorphism (LS) in the terminal untranslated region. These polymorphisms are tightly linked (abTL) [18–21]. Although these polymorphisms are silent in terms of translation, there is evidence of functionality in terms of transcriptional activity of the VDR, with the L allele being more active [22]. At the 5′ end in exon 2, another

Table 1 Results of previous studies on correlations of vitamin D receptor polymorphisms on psoriasis incidence, age of onset, severity and calcipotriol response

	Allele	Psoriasis occurrence	Family history	Age onset psoriasis	Psoriasis severity	Calcipotriol response
*Apa*1	a	NS[f,g] Negative[d,e]	NS[d]	NS[g]	NS[d,g]	NS[a,f]
*Bsm*1	b	NS[b,c,d,e,g] Positive[f]		NS[b,c,g]	NS[c,g]	NS[b,c,e,f] Positive[a]
*Taq*1	T	NS[d,g] Positive[f]		NS[g]	NS[g]	NS[f]
*Fok*1	F	NS[f]				Positive[f]

Results have been expressed in terms of the alleles reported to be more transcriptionally active. Note reported 3′ linkages are abTL [21]; NS, not significant.
[a]Holick *et al.* (1996) [33]; 48 North American patients.
[b]Kontula *et al.* (1997) [27]; 19 Finnish patients.
[c]Mee and Cork (1998) [28]; 92 British patients.
[d]Park *et al.* (1999) [29]; 104 Korean patients, 104 controls.
[e]Lee *et al.* (2002) [30]; 43 Korean patients, 104 controls.
[f]Saeki *et al.* (2002) [32]; 115 Japanese patients, 69 controls, 54 patients in calcipotriol response study.
[g]Okita *et al.* (2002) [31]; 50 patients, 86 controls.

RFLP, *Fok*1 (alleles Ff), produces a C > T substitution, resulting in a new ATG translation start codon upstream of the normal start codon and a three amino acid longer VDR protein [23], with the shorter protein being more active (F allele) [24]. Recently, we have reported a new A > G polymorphism (A-1012G) situated in the 1A promoter region of the VDR gene [25]. The A allele allows expression of a putative binding site in the *VDR* promoter for GATA-3 [25], a transcription factor directing the polarization of naïve T-cells to Th2 cells [26]. In turn $1,25(OH)_2D_3$ has been shown to up-regulate *GATA-3* gene expression. Therefore, there is an apparent amplification circuit between the *VDR* and *GATA-3*, which would result in a Th1 to Th2 switch.

There have been a number of previous studies on associations of predominantly 3′ end VDR polymorphisms with, variously, psoriasis occurrence [27–32], age of onset [27,28,31], severity [28,29,31] and response to the topical vitamin D analogue, calcipotriol [27,28,30,33], and there is a single report of Fok 1 on calcipotriol response [32] (Table 1). There were many non-significant associations [27–32]; some significant associations, although involving different polymorphisms, were apparently conflicting in view of known allele linkages (i.e. Lee *et al.* [30] and Park *et al.* [29] versus Saeki *et al.* [32]); some might be expected in terms of known activity [29,30,32,33] but some would be unexpected [32]. However, most of these studies included small numbers, which may account for a number of the conflicting results.

We hypothesized that the A allele of the A-1012G polymorphism (associated with decreased Th1 activity) might have a protective effect on the occurrence and severity of psoriasis and enhance the effect of vitamin D analogues, and that this effect might be more evident in genotype combinations with the transcriptionally more active alleles of the 3′ and 5′ polymorphisms. The present study aimed to test this hypothesis in a large group of psoriatic patients and controls, using *Taq*1 (as a representative of 3′ polymorphisms) and *Fok*1 and, furthermore, in view of the diversity of previous reports, to investigate the primary association of these polymorphisms and their genotype combinations with psoriasis parameters, with particular reference to the transcriptionally more active alleles (T and F).

Patients and methods

White Caucasian patients with chronic plaque psoriasis of at least 5 years duration, such that they were familiar with the treatment responsiveness of their psoriasis, and who had previously received topical calcipotriol (Dovonex, LEO Pharma, Ballerup, Denmark) treatment, were recruited from patients attending the Dermatology Department of the Leicester Royal Infirmary. Ethical Committee approval for the study was obtained and patients provided their written informed consent. Demographic details were obtained, including age of onset and a family history of psoriasis in parents and siblings, as well as details of all previous and current treatments. The patients were asked to grade their response to calcipotriol treatment into none, some, good or very good. A Psoriasis Area and Severity Index (PASI) was compiled for each patient. As additional measures of severity, intended to reflect more long-term severity, two further indices were devised: the chronic basal and chronic maximal indices. For the basal index, at the time of the PASI estimation, patients were asked to estimate how many times less extensive their psoriasis had been at best (t) over the past few years. Current percentage body area (A) involvement was calculated from the PASI recordings and the chronic basal index was estimated as A/t. Similarly, for the chronic maximal index, patients were asked to estimate how many times more extensive the psoriasis had been at worst (T) and the index was A/T. Blood was taken for genotyping. Control patients were patients attending the hospital who had no inflammatory skin disease or known malignancy.

Genotyping

DNA was isolated from patient blood samples using the CST Genomic DNA Purification Kit (DRI, Sittingbourne, UK). To determine A-1012G genotypes, polymerase chain reaction (PCR) was carried out using forward primer CCT CCT CTG TAA GAG GCG AAT AGC GAT and reverse GGA CAG GTG AAA AAG ATG GGG TTC in PCR buffer (45 mM Tris-HCl (pH 8.8), 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 110 μg/ml bovine serum albumin, 6.7 mM β-mercaptoethanol 4.4 mM ethylenediaminetetracatic acid (pH 8.0), 200 mM each dNTP) for three cycles with an annealing temperature of 55°C followed by 37 cycles with an annealing temperature of 37°C. The forward primer contained a mismatch adjacent to the polymorphism which mutated the sequence to form an *Eco*RV restriction site in the case of the A allele such that in subsequent digestion with *Eco*RV (New England Biolabs, Beverley, Massachusetts, USA, 37°C, 18 h) followed by agarose-gel electrophoresis the A allele was restricted and the G allele remained uncut.

To screen for *Fok*1 and *Taq*1 genotypes, 35 cycles of PCR were carried out with primers CTG GCA CTG ACT CTG GCT CT and TGC TTC TTC TCC CTC CCT TT (*Fok*1) and CAG AGC ATG GAC AGG GAG CAA G and CGG CAG CGG ATG TAC GTC TGC AG (*Taq*1). On digestion with *Fok*1 (37°C, 18 h) and *Taq*1 (65°C, 18 h), the f and t alleles were cut and the F and T alleles remained refractory to digestion.

Statistical analysis

The frequencies of the alleles of each polymorphic site were compared between patient groups using contingency tables (Unistat Statistical Package, London, UK). Analysis of genotype associations was by logistic regression (Stata software package, version 7.0, Stata Corporation, Texas, USA). First, the effects of the individual A-1012G, *Fok*1 and *Taq*1 genotypes were investigated and then the effects of genotype combinations. In line with the hypothesis to be tested by the study, the genotype combinations of primary interest were AAFF, AATT and FFTT, which were examined in regression models with all remaining genotypes as the reference category. To investigate whether any effect of these were the result of additive or synergistic associations, multivariate logistic regression was performed on the component genotypes, where statistical significance of the interaction term would indicate synergism. *P*-values have not been corrected for multiple testing due to the possibility of type 2 errors and therefore the results require verification with a further data set.

Hardy–Weinberg equilibrium and linkage disequilibrium (with a view to haplotype determination by an expectation–maximization algorithm) were evaluated by the Arlequin statistical package [34].

Results

Two hundred and five patients were recruited, comprising 86 females and 119 males with a mean age of 44 years (range 17–90 years). Mean age of onset of psoriasis was 23 years (2–76 years) and 88 patients had a positive family history in their parents and/or siblings. Mean PASI was 9.3 (0.6–43.9). There were 80 controls with a mean age of 56 years (range 5–89 years) and 39 were females.

There was no evidence of divergence from Hardy–Weinberg equilibrium at any of the polymorphic sites ($P = 0.3$–0.8), nor of linkage disequilibrium for any pair of polymorphic loci ($P = 0.2$–0.5). Haplotype estimation was therefore not performed.

Occurrence

Comparing all patients with controls, there were no significant differences in frequencies of any of the alleles (Table 2) or individual genotypes (Table 3). There were also no differences between the frequencies of genotype combinations in patients and controls (Table 3).

Table 2 Results from present study on correlations of alleles of vitamin D receptor polymorphisms on psoriasis occurrence, presence of family history of psoriasis, age of onset, severity, in terms of recourse to second line treatments, and calcipotriol response

Allele		Occurrence		Family history		Age onset		Second-line treatment		Calcipotriol response	
		Psoriasis (*n*=205)	Control (*n*=80)	Negative (*n*=117)	Positive (*n*=88)	≥ years (*n*=30)	<40 years (*n*=175)	Received (*n*=58)	Not received (*n*=147)	Response (*n*=137)	No response (*n*=61)
A-1012G											
	G	0.48	0.52	0.53	0.41	0.53	0.47	0.47	0.48	0.46	0.53
	A	0.46	0.54	0.47	0.59	0.47	0.53	0.53	0.52	0.54	0.47
		P=0.7		*P*=0.01		*P*=0.4		*P*=0.9		*P*=0.2	
*Fok*1											
	f	0.39	0.36	0.41	0.37	0.32	0.41	0.42	0.38	0.39	0.42
	F	0.61	0.64	0.59	0.63	0.68	0.59	0.58	0.62	0.61	0.58
		P=0.5		*P*=0.3		*P*=0.2		*P*=0.4		*P*=0.5	
*Taq*1											
	t	0.36	0.44	0.35	0.36	0.37	0.35	0.39	0.34	0.32	0.42
	T	0.64	0.56	0.65	0.64	0.63	0.65	0.61	0.66	0.68	0.58
		P=0.07		*P*=0.9		*P*=0.8		*P*=0.4		*P*=0.05	

n, Number of patients.

Table 3 Results from present study on correlations of genotypes and genotype combinations of vitamin D receptor polymorphisms on psoriasis occurrence, presence of family history of psoriasis, age of onset, severity, in terms of recourse to second line treatments, and calcipotriol response

	Genotype	Frequency of genotype in psoriatic patients	Psoriasis occurrence	No family history (n=117) versus positive family history (n=88)	Age onset ≥ 40 years (n=30) versus <40 years (n=175)	Second-line treatment (n=58) versus no second-line treatment (n=147)	Calcipotriol response (n=137) versus no calcipotriol response (n=61)
A-1012G	GG/AG		1	1	1	1	1
	AA	0.26	0.92 (0.51–1.64) P=0.8	0.53 (0.28–0.99) P=0.04	0.85 (0.34–2.12) P=0.7	0.77 (0.38–1.58) P=0.5	2.18 (1.00–4.71) P=0.04
*Fok*1	ff/Ff		1	1	1	1	1
	FF	0.36	0.80 (0.46–1.40) P=0.4	0.77 (0.43–1.36) P=0.4	1.95 (0.89–4.26) P=0.09	1.22 (0.65–2.29) P=0.5	1.47 (0.77–2.82) P=0.2
*Taq*1	tt/Tt		1	1	1	1	1
	TT	0.40	1.49 (0.83–2.68) P=0.2	1.07 (0.61–1.88) P=0.8	0.87 (0.39–1.94) (P=0.7)	1.01 (0.54–1.88) P=1.0	1.97 (1.04–3.76) P=0.03
A-1012G/ *Fok*1	Rest		1	1	1	1	1
	AAFF	0.09	0.70 (0.30–1.62) P=0.4	0.24 (0.08–0.69) P=0.005	1.10 (0.30–4.02) (P=0.9)	1.96 (0.75–5.16) P=0.2	4.11 (0.92–18.38) P=0.03
A-1012G/ *Taq*1	Rest		1	1	1	1	1
	AATT	0.12	2.26 (0.76–6.73) P=0.1	0.66(0.29–1.53) P=0.3	1.55 (0.53–4.51) (P=0.4)	0.98 (0.39–2.50) P=1.0	5.64 (1.28–24.82) P=0.005
*Fok*1/*Taq*1	Rest		1	1	1	1	1
	FFTT	0.18	2.21 (0.89–0.51) P=0.09	1.08 (0.52–2.23) P=0.8	1.90 (0.77–4.69) (P=0.2)	1.79 (0.84–3.81) P=0.1	3.22 (1.18–8.74) P=0.01

n, Number of patients.

Family history

When patients without a family history were compared with patients with a family history, the A allele was under-represented in the negative family history group ($P = 0.01$). (Table 2) as was the AA genotype compared to AG/GG ($P = 0.04$) (Table 3). This was more pronounced when AA was compared with GG [AA, OR = 0.33 ($P = 0.01$) and AG, OR = 0.54 ($P = 0.1$)]. Similarly the F allele of *Fok*1 and the FF genotype were under-represented in patients with no family history, but not significantly, whereas *Taq*1 appeared neutral. The AAFF combined genotype was significantly under-represented in patients with no family history of psoriasis ($P = 0.005$). When testing for synergy the interaction term just failed to reach significance ($P = 0.06$). The combined genotype AAFF was also negatively related to no family history compared to controls [OR = 0.31 (0.10–0.95) ($P = 0.04$)] but there was no significant difference between patients with a positive family history and controls [OR = 1.28 (0.59–3.16) ($P = 0.6$)] (results not shown).

Age of onset

There were no significant relationships between alleles or genotypes with age of onset (Tables 2 and 3).

Severity

AA was associated with less severe disease by all of the measures but not significantly ($P = 0.4$–0.9) (data not shown). With all other genotypes and genotype combinations, there were no significant correlations or consistent trends as illustrated by use of second-line treatments (Table 2).

Response to calcipotriol

Comparing patient groups showing response with those showing no response, the A, F and T alleles were all associated with response, but only T significantly ($P = 0.05$). (Table 2). AA, FF and TT genotypes were all positively associated with response (Table 3) with AA and TT significantly so ($P = 0.04$ and 0.03, respectively). The combined genotypes AAFF, AATT and FFTT were more strongly correlated ($P = 0.03$, 0.005 and 0.01, respectively) (Table 3). There were no significant interactions between AA and FF or TT, nor between FF and TT.

Discussion

According to the basic hypothesis tested by the study, the A, F and T alleles of the A-1012G, *Fok*1 and *Taq*1 polymorphisms, respectively, would have a protective effect on the susceptibility to psoriasis. We found no association between the VDR allele or genotype frequencies and the occurrence of psoriasis overall (Tables 2 and 3). However, the A allele was significantly under-represented in patients without a family history compared to those with a family history ($P = 0.01$), as were AA genotype and AAFF combined genotype ($P = 0.04$ and 0.005, respectively), but there was no effect of *Fok*1 or *Taq*1 alone. AAFF combined genotype was also significantly under-represented in patients without a

family history compared to controls ($P = 0.04$). Our interpretation is that patients with a family history of psoriasis will develop psoriasis irrespective of VDR genotype. Psoriasis has been shown to be strongly heritable, at least in some patients [35]. Currently, 19 candidate susceptibility loci have been identified, including PSORS1–7 and 9 [36], with the most significant region being the PSORS1 locus on chromosome 6p21 within the major histocompatability complex (MHC) [37,38]. Interestingly, candidate genes, including *corneodesmosin* [39], *HCR* [40], *SEEK 1* and *SPR1* [41] are highly expressed in keratinocytes and would appear to be exerting an effect on these cells rather than on immune cells. It appears likely that these familial risk factors are strong enough to overwhelm any effect of *VDR* polymorphisms. However, our results suggest that patients without a family history of psoriasis will be somewhat protected against the development of the disease by the A allele of the promoter polymorphism, particularly when in combination with the F allele of *Fok*1. We have chosen to use the presence of a family history rather than one of the above genetic markers, which lack complete sensitivity for familial disease (e.g. the PSORS1 site fails to account for 50% or more of psoriasis familial clustering) [42,43]. Previous studies also failed to detect a direct effect of representatives of the 3′ polymorphisms on psoriasis susceptibility [27–32]. Furthermore, other studies have variously reported a negative relationship of the a allele of *Apa*1 [29,30] (a linked to T allele of *Taq* 1) with occurrence, a positive relationship with the b allele of *Bsm*1 [32] (b linked to T) and a positive correlation of the T allele of *Taq*1 [32]. In view of the known 3′ linkages, the data on *Apa*1 apparently conflict with those on *Bsm*1 and *Taq*1, but would appear more plausible in view of the known function of the 3′ polymorphisms. There are no previous reports on an association of the *Fok*1 polymorphism and incidence.

It is known that there is a relationship between age of onset and the presence of a family history of psoriasis [44] and our results would suggest that the A and F alleles would protect against later onset psoriasis. However, we found no significant relationships between VDR genotype and age of onset. One interpretation of this is that age of onset is not a sensitive surrogate marker of familial disease. Previous studies on the 3′ polymorphisms have similarly found no significant relationship with age of onset [27,28,31]. There are no previous reports on an association of the *Fok*1 polymorphism and age of onset of psoriasis.

We found no association of any of the VDR polymorphisms with severity of psoriasis. This was also true in the literature for the 3′ polymorphisms [28,29,31]. A possible explanation is the difficulty encountered in defining the severity of psoriasis. PASI is a good estimator of severity at a particular point in time and the use of second-line treatment may give some indication of longer-term severity. We attempted to devise more objective measures of long-term severity, the basal and maximal severity indices. We used all of these measures but were unable to detect an effect.

Response to treatment with the vitamin D analogue calcipotriol showed the strongest correlation with *VDR* genotypes in the study. The hypothesis was that the A, F and T alleles would be associated with greater response. This was confirmed, although, of all the individual polymorphisms, only the T allele and AA and TT genotypes were significant ($P = 0.03$–0.05). However, all of the genotype combinations, AAFF, AATT and FFTT were significantly associated with enhanced response ($P = 0.03$–0.005). Several previous reports found no association with the 3′ polymorphisms [27,28,30,32], although Holick *et al.* [33] reported a highly significant association with the bb genotype of *Bsm*1 (b is linked to T), which is in agreement with the present results. A study investigating the *Fok*1 polymorphism and treatment response reported a positive relationship with the F allele [32], which again is in agreement with the present results.

In general, the results of the present study support predictions of the VDR allelic associations with psoriasis susceptibility, in patients without a family history but not in patients with a family history, and vitamin D analogue response but not psoriasis severity. These predictions were based on a Th1 to Th2 switching effect of the A allele of A-1012G and the reported enhanced target transcription by the VDR of the F and T alleles of *Fok*1 and *Taq*1, given the anti-proliferative, pro-differentiation and immunosuppressive effects of the VDR. Recently, it has been proposed that the major immunosuppressive mechanism occurs via inhibition of maturation of dendritic cells [11], such that antigen presentation is impaired, with down-regulation of co-stimulatory molecules, and down-regulation of interleukin (IL)-12 and up-regulation of IL-10 production [45,46]. Consequently, T lymphocyte proliferation is inhibited and the lymphocyte phenotype switched towards Th2 [11]. In addition $1,25(OH)_2D_3$ has a direct effect on lymphocytes, inhibiting IL-2 [47] and interferon-γ [48] secretion. Given that the F and T alleles of the *Fok*1 and *Taq*1 polymorphisms are associated with greater VDR-mediated transcription [22], the above VDR effects would be expected to be enhanced in these alleles.

The effect of A-1012G may be quite separate. The A allele forms a putative binding site in the *VDR* promoter for GATA-3 [25], a transcription factor directing the polarization of naïve T-cells to Th2 cells [26]. In turn

$1,25(OH)_2D_3$ has been shown to up-regulate *GATA-3* gene expression. Therefore, there is an apparent amplification circuit between *VDR* and *GATA-3*, which would result in a Th1 to Th2 switch. Again, if the A allele were in combination with alleles associated with greater VDR transcription (e.g. the F or T alleles of *Fok*1 and *Taq*1), a greater effect would be expected. Therefore the F and T alleles might have a beneficial effect in psoriasis via increase in the general anti-proliferative, pro-differentiation and immunosuppressive effects of the VDR, but also more specifically in relation to the A allele.

Of the individual polymorphisms, correlations were strongest for A-1012G. Odds ratios and significance levels were enhanced in genotype combinations (Table 3). These were found to be additive and not synergistic effects. The present study supports the role of Th1 lymphocytes in the development of disease in non-familial psoriasis and their suppression in treatment response. Interestingly, it has been proposed that a mode of action of fumaric acid esters and PUVA, which are effective treatments for psoriasis, occurs via a Th1 to Th2 switch [5,49]. Whether A-1012G affects GATA-3 binding to the *VDR* promoter, and whether this then affects T-cell polarization in response to $1,25(OH)_2D_3$ treatment, is currently under investigation.

In conclusion, the A allele of the A-1012G polymorphism was protective against the development of non-familial psoriasis and this was more evident in the AAFF genotype combination. None of the polymorphisms were associated with psoriasis severity. Calcipotriol response was enhanced in the presence of the A and T alleles, AA and TT genotypes and AAFF, AATT and FFTT genotype combinations. With the exception of severity, the results support the predictions of effects of the polymorphisms based on reported functions, particularly for A-1012G and for genotype combinations with *Fok*1 and *Taq*1.

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