The Molecular Basis

of

Hereditary Vitamin D Resistant Rickets

Thesis of Andrew Rut for Doctor of Medicine with University of London

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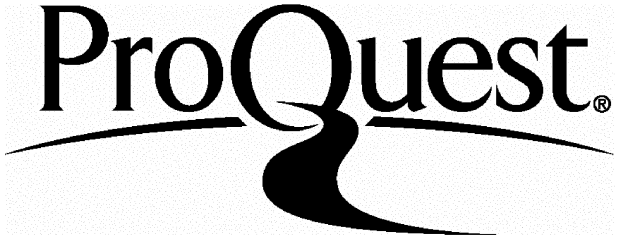

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Abstract

Hereditary vitamin D resistant rickets is an autosomal recessive disorder characterised by severe rickets, hypocalcaemia, secondary hyperparathyroidism and occasionally, the absence of body hair. It has been shown that the pathological process involves resistance of target tissues to the actions of calcitriol [$1,25(OH)_2D_3$], the hormonal form of vitamin D. Calcitriol mediates its actions through a nuclear receptor (VDR) that has been cloned and shown to be a member of the superfamily of steroid/thyroid/retinoic acid receptors that act as gene *trans*-activating factors. These receptors are organised into two discrete functional domains, one for ligand binding at the COOH-terminus and one for DNA binding at the NH_2-terminus. All nuclear hormone receptors have marked sequence conservation, particularly in the DNA binding domain. Crystallisation of this domain in the glucocorticoid receptor (GR) and has provided a structural model, enabling a stereochemical explanation of mutations in VDR that cause vitamin D resistant rickets.

Five patients with target organ resistance to the actions of $1,25(OH)_2D_3$ were studied. VDR from their skin fibroblasts were characterised. RNA was isolated, reverse transcribed, amplified, cloned and sequenced. Independent missense mutations were identified in four of the individuals, two localised to the hormone binding domain (Q149X & R271L) and two to the DNA binding domain (K42E & F44I). However, one patient with the classical phenotype had a normal cDNA sequence. The mutant receptors were expressed in VDR-deficient CV-1 cells and were found to demonstrate impaired *trans*-activation ability of an osteocalcin reporter gene linked to chloramphenicol transferase.

Finally, knowledge of the crystal structure of the DNA binding domain of GR, and its extensive homology with VDR, enabled computer-assisted modelling of the K42E & F44I mutations as well as five previously reported mutations. This provided an explanation for the defective phenotypes at the level of protein/DNA interactions.

Aim of Thesis

The primary goal of this thesis was to identify the genetic basis for vitamin D resistant rickets in five unrelated patients. A number of such patients had already been shown to have defective vitamin D receptors (VDR) [76] with mutations in the coding region of the gene [75, 150, 153, 169]. It was reasoned that any new mutations identified would provide important functional information about the receptor and how this correlated with the clinical picture of patients with end-organ resistant rickets. Expression of mutant VDR in vitro would provide confirmation of their pathological relevance. Finally, when the crystal structure of the glucocorticoid receptor DNA binding domain became available it was possible to model the effects of mutations in VDR providing an understanding of the effects of the mutations at the Angstrom level.

Chapter 1; Introduction

The important areas that will be covered in this chapter are outlined below.

- Nuclear receptors are *trans*-activating factors that regulate gene expression.

- The superfamily contains a number of important human transcription factors; ie glucocorticoid receptor, oestrogen receptor, progesterone receptor, mineralocorticoid receptor, thyroid receptor, retinoic acid receptor and retinoid X receptor.

- They are organised into two domains: the hormone binding domain and the DNA binding domain that includes a zinc finger region.

- The receptors do not operate in isolation but form complex dimers that increase the repertoire of transcriptional regulation.

- Post-translational modification in the form of phosphorylation at a number of sites in the protein and by a variety of kinases modulates the function of these receptors .

- Understanding of receptor function has been aided by the study of human mutations found in patients with end-organ resistance to the hormones.

- The crystal structure of the glucocorticoid receptor DNA binding domain has allowed modelling of the VDR and therefore an enhanced understanding of the molecular basis for vitamin D resistance.

- The ligand for VDR, vitamin D, is derived either from dietary sources or by conversion of 7-de-hydrocholesterol in the skin. After metabolism in the liver and kidney to its active form, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], vitamin D is able to bind and activate its receptor. The vitamin D receptor is a nuclear receptor that regulates transcription through interaction with response elements located in the 5' region of target genes. One such gene that has been studied extensively is osteocalcin, a bone morphometric protein the transcription of which is up-regulated by VDR. Like many proteins VDR undergo post-translational modification by phosphorylation which provides another tier of regulation to the vitamin D system.

- Vitamin D dependent rickets has been known for a long time, being first described by Daniel Whistler in his thesis of 1645. However the classic account of rickets was not published until five years later by Francis Glisson in his book *De Rachide* [110]. During the late 19th and early 20th centuries rickets appeared in epidemic proportions in the urban children of Northern Europe, North America and Northern Asia. It was believed by some to be due to lack of fresh air and sunshine; others claimed a dietary factor caused the disease. The work of Sir Edward Mellanby and Huldschinsky in 1919 showed both these notions to be correct. Mellanby demonstrated rickets to be at least in part a nutritional

disorder that could be healed with cod liver oil but hypothesised the active agent to be vitamin A [123]. McCollum tested this hypothesis by destroying vitamin A in cod liver oil by passing heated air bubbles through it and found that it was still effective in healing and preventing rickets. He named the new nutrient vitamin D [118]. In 1924 the groups of Hess and Steenbock found that irradiation of animal rations was as efficacious in curing rickets as was irradiation of the animal itself [62, 172]. The irradiation of food provided a means of largely preventing rickets as a medical problem. These observations led to the elucidation of the structures of cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) and eventually to the discovery that these compounds are further metabolised to in the body to active compounds. The discovery of metabolic activation is primarily attributable to studies conducted in the laboratories of DeLuca in the United States and Kodicek in England [37, 94]. With the chemical synthesis of vitamin D it became possible to treat and cure the majority of patients with small defined doses of the drug. Those that failed to respond provided the impetus for the identification of new diseases. Resistance to vitamin D was documented as two inherited disorders, collectively called vitamin D resistant rickets. Both conditions were characterised by classical, clinical, radiological and biochemical features of rickets, but in the face of adequate vitamin D intake and without therapeutic response to standard physiological doses of vitamin D itself. Prader et al 1961 [146] first described pseudovitamin D dependent rickets (PDDR), but it was Fraser et al in 1973 that elucidated the defect to be in renal 1α-hydroxylase [48]. It was only in 1978 that Brooks et al [18] reported rickets in association

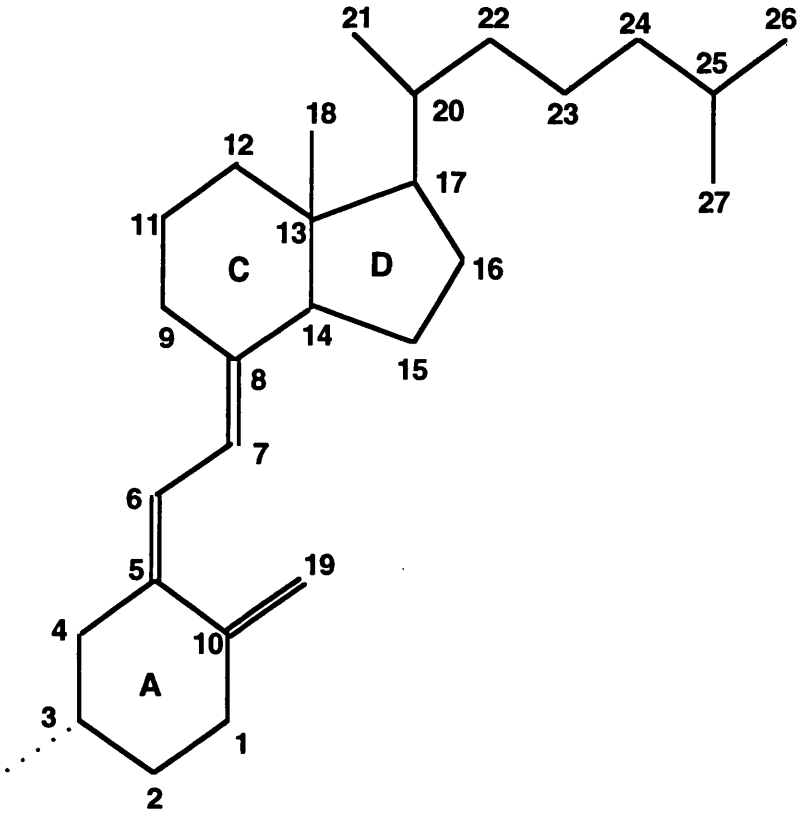
with elevated circulating levels of $1,25(OH)_2D_3$. The disorder was classified as target organ resistance to $1,25(OH)_2D_3$ and has also been referred to as hereditary vitamin D resistant rickets (HVDRR). This disease forms the basis for this thesis.

Vitamin D

Vitamin D and its analogues form a group of fat-soluble secosterols with antirachitic properties [38]. The two parent forms of vitamin D are, ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). Ergocalciferol derives its name from its immediate precursor, ergosterol, the plant sterol from which it was originally isolated. Cholecalciferol is the natural form of vitamin D and is produced by irradiation of its immediate precursor, 7-de-hydrocholesterol, a reaction that takes place in the skin [67]. Technically therefore, vitamin D is not a true hormone because it can be produced in the body. The D vitamins are related to C-21 steroids; vitamin D_2 differs from vitamin D_3 by having a double bond between the carbon positions 22 and 23 and a methyl group at C-24, (Fig. 1). Both vitamin D_2 and D_3 are metabolised along the same pathways, producing active metabolites with equivalent biological activities.

Vitamin D itself has minimal intrinsic biological activity. Sequential hydroxylation reactions, that occur first in the liver at position 25, and then in the kidney at position 1 are required for the production of the active form, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] [94]. The addition of the two hydroxyl groups enables $1,25(OH)_2D_3$ to bind with high affinity to intracellular receptors in target tissues [46].

Figure 1

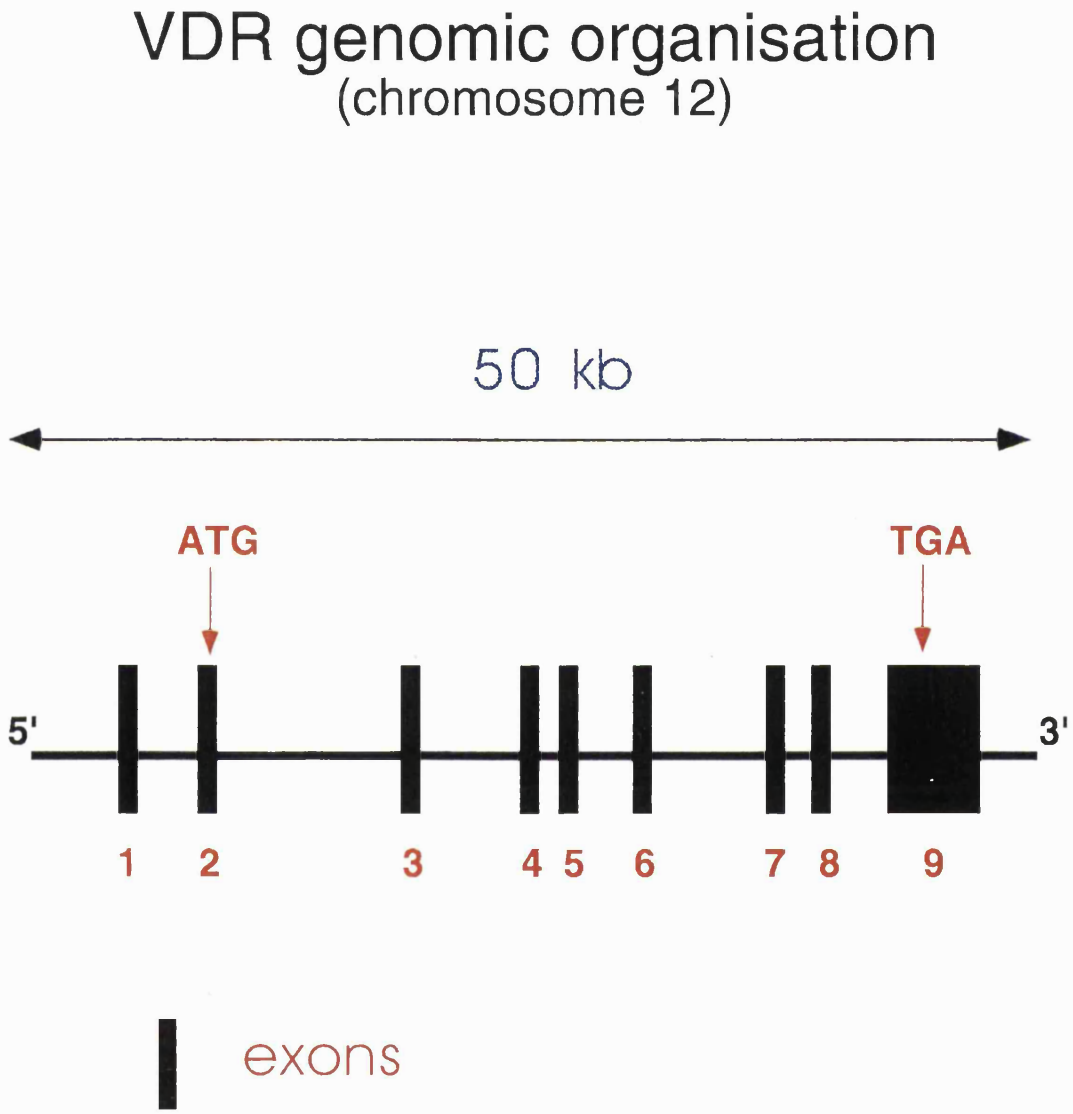


The structure of vitamin D_3 (Cholecalciferol)

1,25(OH)$_2$ vitamin D$_3$ receptors

1,25-dihydroxyvitamin D$_3$ is a eukaryotic, steroid-like hormone with an essential role in calcium and phosphorus homeostasis [38, 61]. The hormone is also known to modulate more basic cellular responses such as those involved in proliferation and differentiation, for example, of haematopoetic stem cells [1, 6] and dermal keratinocytes [70]. The mediator of 1,25(OH)$_2$D$_3$ action is the vitamin D receptor that was first isolated from chicken intestinal homogenates [142]. The purified protein was used as an antigen to generate monoclonal antibodies [140] that were used to recover vitamin D receptor cDNA from a chicken expression library [119]. The avian clones were utilised to isolate the full length, human cDNA [4] from a jejunal expression library. The nucleotide sequence consisted of 4605 bp with a 1281 bp open reading frame, 115 bp of non-coding leader sequence, and 3209 bp of 3′ untranslated sequence. The long 3′ untranslated region is characteristic of all steroid receptors and may function to stabilise mRNA and modulate translation [79]. The VDR coding region was transfected into COS-1 cells, deficient in VDR and this resulted in the production of a single receptor protein that was indistinguishable from the native receptor [4]. A eukaryotic expression system was devised to determine the function of the receptor and its characterise its domains [120]. Full length human VDR cDNA was inserted into a mammalian expression vector and transfected into VDR-deficient COS-1 cells. Following transient expression, COS-1 cells synthesised greater than 5 x 10^5 copies/cell of a ~50 kDa protein which bound 1,25(OH)$_2$D$_3$ with an affinity of 5 x 10^{-11} M,

Figure 2



A schematic representation of the genomic organisation of VDR. The nine published exons are shown; unpublished data suggests that there exists another untranslated exon 5' of what is labelled as exon 1 (Pike et al, personal communication). The coding region of VDR extends from known exons 2 to 9. Intron sequences remain unpublished [141].

bound to DNA cellulose and was phosphorylated in the presence of $1,25(OH)_2D_3$ [120]. These general properties were characteristic of wild type vitamin D receptors from a variety of tissues. The chromosomal gene was characterised by first isolating genomic clones from a human pCV-109 liver cDNA library through hybridisation screening utilising the human VDR cDNA as a probe. Clones were mapped by restriction digests, subcloned and sequenced. The organisation of the human chromosomal gene is known [141] but its sequence remains unpublished (Fig 2). Faraco et al. (1989), who identified an *Apa*I dimorphism at the VDR locus, assigned the gene to chromosome 12 by somatic cell hybrid studies [41]. By study of rat/human somatic cell hybrids, Szpirer et al. (1991) showed that the VDR gene is located on 12q in the human and chromosome 7 in the rat [174]. Labuda et al. (1991) assigned the gene to 12q12-q14 by in situ hybridisation [101]. It is noteworthy that the gene for pseudovitamin D dependent rickets due to deficiency of 25-hydroxycholecalciferol-1-hydroxylase maps to the same region. Labuda et al. (1991) used a 2-allele polymorphism revealed by a VDR cDNA probe to demonstrate linkage between it and PDDR as well as several other markers in the region [101].

The location of VDR domains involved in the principal functions of steroid and DNA binding were established through VDR cDNA deletion analysis [120]. A series of 5′ and 3′ truncations of the cDNA were created, cloned into an expression vector, and the expressed protein was evaluated specifically for DNA and ligand binding. The amino acid sequence was deduced from the original cDNA sequence published by Baker et al, (1988) and residues were

numbered accordingly [4]. Native DNA binding was localised to residues 1-114 the most prominent feature of which was the cysteine-rich Zn finger-like structures that had been shown to represent the DNA binding domain of other steroid receptors [40]. Steroid binding competence required residues 114-427. This organisation strongly suggested that the action of $1,25(OH)_2D_3$ would involve modification of gene expression through VDR. One such target gene is osteocalcin, an abundant osteoblast-specific, non-collagenous bone protein that functions in the mineralisation of bone under the direct control of $1,25(OH)_2D_3$. After the cloning of the human osteocalcin gene [26] and the demonstration that its transcription was up-regulated by $1,25(OH)_2D_3$ [135], research focused on identifying the specific promoter regions 5' of the gene. DNA fragments extending from -1339 to +10 relative to the cap site of the osteocalcin gene were isolated from genomic DNA fragment containing the human osteocalcin gene and cloned into a promoterless reporter vector. When the reporter construct was transfected into VDR-deficient ROS 24/1 cells, no basal nor $1,25(OH)_2D_3$ inducible activity was evident unless the VDR cDNA expression vector was cotransfected. 5′ deletion analysis of the osteocalcin gene promoter region, followed by site-directed mutagenesis localised a vitamin D responsive element (VDRE) to ~ 500 bp upstream of the cap site [89]. This 21 bp element (**5′-GTGACTCACCGGGTGAACGGG-3′**) bears similarity to other hormone responsive elements particularly those for oestrogen receptors (**5′-NGGTCANNNTGACCN-3′**) and thyroid hormone receptors (**5′-GGGTCATGACAG-3′** or **5′-AGGACATTAACT-3′**) providing further evidence that VDR belonged to the same family of transcription factors. The VDRE was further defined by site-

directed mutagenesis coupled to studies of *trans*-activation capability [134]. The region was shown to be complex, containing both a high affinity site recognised by VDR and a distal site that formed a consensus binding region for the AP-1 nuclear oncogene family. The binding sites did not overlap but acted synergistically The AP-1 site governed basal activity and the VDRE was inducible by vitamin D. This response element provided a direct mechanism for cross-communication between fundamentally opposing factors; those that promote cellular differentiation such as $1,25(OH)_2D_3$ [1] and those whose activities promote cell growth and division such as the proto-oncogenes c-*jun* and c-*fos*.

The vitamin D receptor and osteoporosis

The vitamin D receptor may play role in predisposing individuals to osteoporosis. In studies of twins, variation in serum osteocalcin levels was shown to have a major genetic component (Kelly et al., 1991) [88] and to be closely correlated with the genetic diversity in bone density [144]. Morrison et al. (1992) set out to ascertain whether variability in circulating osteocalcin levels may reflect allelic variation in the VDR gene, by analysing the relationship between restriction length fragment length polymorphisms (RFLPs) that define the VDR alleles and serum osteocalcin levels in a cohort of normal subjects [126]. They found that indeed RFLPs in the VDR gene predicted circulating osteocalcin levels independent of age or menopause effects. Since the osteocalcin gene and vitamin D receptor gene are encoded on different chromosomes, the interaction between these 2 genes occurs in trans. Their work demonstrated that common alleles of this *trans*-activating factor, the vitamin D receptor, were functionally different and might

contribute to physiologic variation in osteocalcin levels. Morrison et al. (1992) described preliminary analyses in monozygotic (MZ) and dizygotic (DZ) twin pairs, that indicated that the greater diversity in lumbar spine density between DZ pairs may be explained by divergence in vitamin D receptor alleles [126]. In 1994, the same researchers showed that common allelic variants in the vitamin D receptor gene might be used to predict differences in bone density, accounting for up to 75% of the total genetic effect on bone density in healthy persons [125]. The genotype associated with lower bone density was over represented in post-menopausal women with bone densities more than 2 standard deviations below values in young normal women. The molecular mechanisms by which bone density is regulated by the vitamin D receptor gene are not certain, although allelic differences in the 3-prime untranslated region may alter messenger RNA levels. Hustmyer et al. (1994), however, found no relationship between several VDR polymorphisms and bone mineral density at spine, femur, and forearm [78]. They studied 86 monozygotic and 39 dizygotic adult female twin pairs. All were white; 63 pairs were pre-menopausal, and 43 pairs were discordant for age at menopause or use of oestrogen. Therefore, much larger studies are needed to define the relevance of the vitamin D receptor to osteoporosis. At present no clear message has emerged on the relationship of VDR to osteoporosis that would result in a change in the diagnosis or therapy of this condition.

Characteristics of nuclear hormone receptors

The human vitamin D receptor is an important member of the steroid/thyroid nuclear hormone receptor superfamily of gene *trans*-activating factors [9]. Its importance to common bone diseases is only just being elucidated. A lot can be learnt from consideration of the properties of the other members of the family and so it is worth examining them in some detail here.

Like many transcriptional regulatory proteins, nuclear hormone receptors are single polypeptides that are organised into discrete functional domains. This common organisation groups these receptors into a superfamily of functionally and structurally related, hormonally regulatable transcription factors [9, 40, 189]. The superfamily includes receptors for steroid hormones such as glucocorticoid, progesterone, oestrogen, aldosterone and androgens as well as hormonal forms of vitamins A and D, thyroid hormone and peroxisomal activators. Several orphan receptors have been cloned, for which ligands are as yet unknown [131].

Upon association with a particular ligand, nuclear receptors bind to specific DNA sites located upstream of responsive genes, and up- or down-regulate the transcription of those genes, presumably by interacting with other components of the transcriptional apparatus [14]. Domains for ligand binding (located in the carboxy terminal region), DNA binding (located toward the amino terminus), nuclear localisation (within both DNA and ligand binding domains) and transcriptional modulation (localised to more variable regions of the receptors, including the N-terminus) all can confer their specific functions when linked to unrelated non-receptor proteins. In

addition, a putative dimerisation domain has been identified that overlaps part of the ligand binding domain for some receptors including VDR [128-130].

Hormone Response Elements (HREs)

The glucocorticoid response element (GRE) was first identified in DNA binding studies with the mouse mammary tumour virus promoter long-terminal repeat (MMTV LTR) [137]. Glucocorticoid inducibility was found to be mediated through a 15 base pair virtually palindromic sequence that consists of two hexameric half sites separated by three bases. A similar sequence was discovered in the controlling regions of the genes for human metallothionein IIA, chicken lysozyme, and rat tyrosine aminotransferase [9]. Based on these and other subsequently characterised GREs, a functional consensus *imperfect*, palindromic sequence was proposed: **5′-GGTACAnnnTGTTCT-3′** [9] where "n" can be any nucleotide. In nature the sequences of the half-sites of GREs may vary but the spacing of three bases is always preserved. A GRE can be constructed that is a *perfect* palindrome and that will impart glucocorticoid-inducible gene expression *in-vivo* [173]. The hormone response elements of other steroid hormone receptors also tend to palindromes with fixed intervening spacing. The oestrogen response element (ERE) sequence has been identified and is similar to the GRE: **5′-AGGTCAnnnTGACCT-3′** [92]. The palindromic nature of GREs strongly suggested that glucocorticoid receptors (GR) bind their response element as head-to-head homodimers. Crystallographic analysis of the glucocorticoid receptor DNA binding domain (GRdbd) has confirmed this. The other steroid receptors,

(progesterone, androgen and mineralocorticoid) all recognise a GRE with high affinity suggesting that they also bind to their own HREs as homodimers [24, 25]. A subgroup of non-steroid nuclear receptors that includes thyroid hormone (T_3R), retinoic acid (RAR) and vitamin D receptors (VDR) are able to recognise the same half-site element as found in an ERE (**AGGTCA**). They are able to transactivate irrespective of half-site orientation. T_3R and RAR can recognise the ERE as an inverted repeat with no half-site spacing or as a direct repeat with a spacing of four or five base pairs respectively [177, 178]. VDR can bind and activate from the same directly-repeated element if the half site spacing is three [178]. The specificity of VDR, T_3R and RAR to activate reporter genes controlled by elements that are direct repeats of ERE half-sites was termed the **"3,4,5 rule"** [178]. Binding *in-vivo* is unlikely to as regimented, because these receptors may form heterodimers with the retinoid X receptors, RXRα and RXRβ, that have 9-cis-retinoic acid as their ligand. To address this issue, Carlberg et al [22] used VDR and RXR deficient *Drosophila* SL-3 cells to study VDR/VDR and VDR/RXR mediated transcriptional responses. They identified two classes of response elements that are activated by VDR alone or by heterodimers of VDR and RXR-α. The motif **GGGTGA******GGGTGA** arranged as a direct repeat with a spacing of six nucleotides, as an *imperfect* palindrome **GGGTGATCACCT** without spacing, or as an *inverted* palindrome **TCACCC**********GGGTGA** with a spacing of twelve conferred vitamin D inducibility mediated by VDR alone. A second class of response elements , composed of directly repeated motifs (**GGTCCA***GGTCCA, AGGTCA***AGGTCA, or GGGTGA***GGGTGA**), spaced by three nucleotides is

synergistically activated by RXR and VDR but only in the presence of both ligands. Thus the nature of the response element and the ligands that are present (ie. $1,25(OH)_2D_3$ and 9-*cis*-retinoic acid) determine whether a target gene is regulated by VDR alone or by VDR and RXR.

DNA binding domain

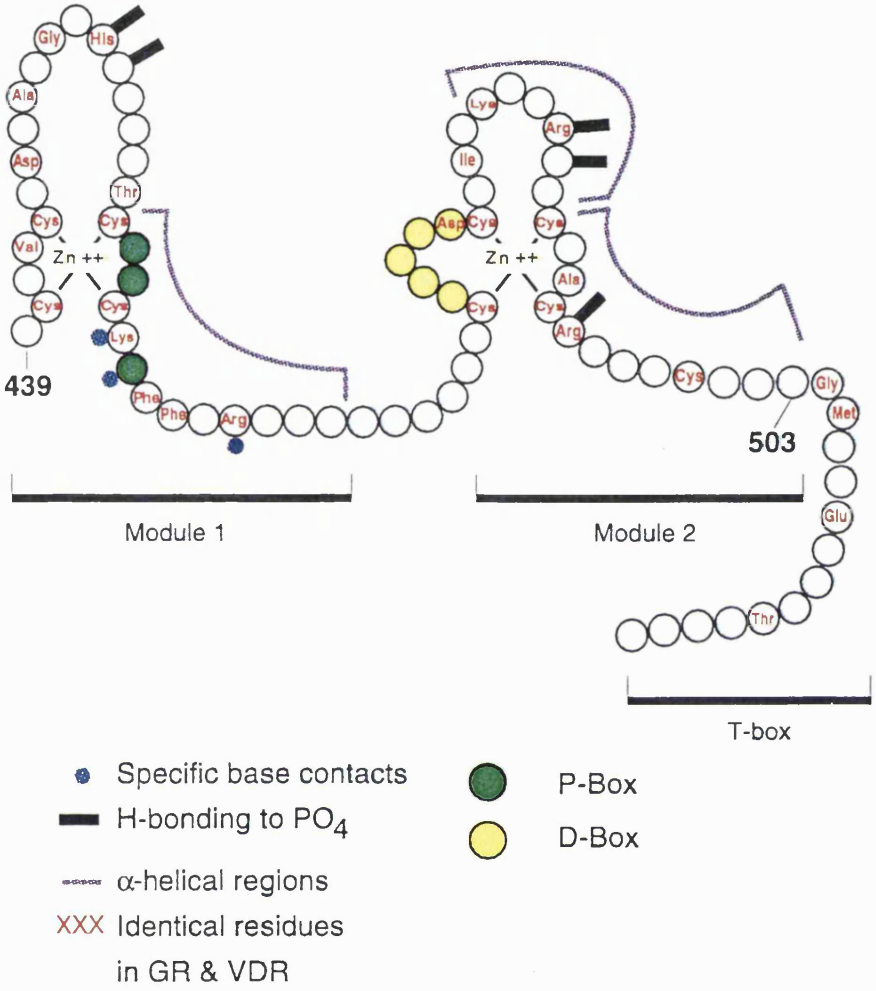
The DNA binding domain consists of 66-68 amino acids and is by far the most highly conserved region within the nuclear receptor superfamily. Several amino acids in this domain are invariant throughout the superfamily including eight cysteines that coordinate tetrahedrally two Zn^{2+} ions [49, 68] in an arrangement reminiscent of the "Zinc finger" coordination scheme originally proposed for Transcription factor IIIA (TFIIIA) of *Xenopus*. [93]. The TFIIIA motif is characterised by pairs of cysteines and histidines that tetrahedrally coordinate a zinc ion resulting in a peptide loop of twelve amino acids (consensus sequence: **Cys**-$X_{2/4}$-**Cys**-X_3-Phe-X_5-Leu-His-X_2-His)[10]. In contrast, the nuclear receptor DNA binding domain was proposed to utilise four cysteines in each of the two fingers to coordinate zinc, yielding two non-equivalent fingers, (**Cys**-X_2-**Cys**-X_{13}-**Cys**-X_2-**Cys**-X_{15}-**Cys**-X_5-**Cys**-X_9-**Cys**-X_2-**Cys**-X_4-**Cys**) [49]. Nuclear magnetic resonance spectroscopy (NMR) has indicated that the zinc fingers of GR and TFIIIA differ structurally and this has been confirmed by the crystallographic analysis of the glucocorticoid receptor DNA binding domain [107]. TFIIIA fingers act as independent, conformationally stable structural units, each contributing to DNA binding, whereas the zinc fingers of the nuclear receptors fold together as part of a

larger, unified globular domain. As a consequence the nuclear receptor fingers might be more correctly termed **zinc modules**. However, the terms modules and fingers will be used interchangeably in this thesis.

The N-terminal finger is of greatest importance in determining DNA binding specificity [55]. as demonstrated by analysis of a number of chimeric glucocorticoid receptor/oestrogen receptor(ER) derivatives. The first such chimera consisted of full length ER in which the native DNA binding domain was substituted by that of GR. Oestradiol stimulated the chimeric ER receptor to increase transcription of a reporter gene linked to a glucocorticoid response element but not an oestrogen response element. By swapping individual ER fingers for GR at the exon/intron boundary, Green et al 1988 [55] demonstrated that residues within the first Zn finger were responsible for determining target specificity, with both fingers, including a basic region immediately C-terminal to the second finger being required for DNA binding. When the results of *trans*-activating experiments from three laboratories [36, 109, 177] were combined it became apparent that three amino acids, located at the C-terminal side of the first finger played a key role in distinguishing a GRE from an ERE. A substitution of two of these amino acids in a full-length GR to the corresponding ER residues (**Gly-Ser→Glu-Gly**) switched the specificity from a GRE to an ERE but not to the level of wild-type ER as measured by induction of reporter gene activity regulated by a GRE or ERE. Studying ER and GR, Mader et al [109] demonstrated that a three amino acid alteration (**Glu-Gly-Ala→Gly-Ser-Val**) changed completely the specificity so that the chimeric ER *trans*-activated strongly from a

GRE-driven reporter but not at all from an ERE. The second finger is also required for DNA binding since deletions, amino acid insertions and point mutations into this region give rise to inactive receptors [33, 68]. In VDR as in many of the other receptors, (eg. GR) each finger is encoded for by a different exon. Umesono and Evans [177], constructed chimeric DNA binding domains of the GR and T3Rß and localised two non-contiguous "boxes" in the T3Rß DNA binding domains, one proximal (**P**) in the first finger, and one distal (**D**), in the second finger that are sufficient to impart TRE-specific recognition in the context of a GR, (Fig 3). The P-box follows the third cysteine that binds to zinc and includes the three amino acid cluster **GS**V** in GR and **EG**G** in TR. These are part of a 10 amino acid segment that is highly conserved throughout the superfamily (Fig 3). The P-box is critical for identifying the primary nucleotide sequence of the half-sites and so provides receptor specificity for the target DNA. Receptors tend to fall into subgroups which recognise the same response element core and the amino acid sequence of the P-box can be used to predict core preference. Receptors carrying the **GS**V** motif (i.e. glucocorticoid, progesterone, mineralocorticoid and androgen receptors) all recognise a GRE with high affinity. Receptors carrying the **EG**G** or **EG**A** motif (i.e. oestrogen, vitamin D, thyroid, retinoic acid and many orphan receptors) appear to bind to the ERE with high affinity. Therefore nuclear hormone receptors demonstrate high sequence homology and similar DNA response elements.

Figure 3



The glucocorticoid receptor DNA binding domain based on the published crystal structure Luisi, 1991[107].

Two Zinc-modules and the T-box are outlined as are the three helical regions. The α-helix in the first Zinc-module sits in the major groove of DNA and makes specific base contacts. It is therefore termed the recognition helix. The P-box, which is important for receptor specificity is filled in green. The D-box, which is thought to play a role in dimerisation is filled in yellow. Residues identical to those in VDR are labelled in red.

How then are they targeted to their specific genes? First the P-box motifs just described provide some specificity, second by the spacing between the half-sites and third, the sequences of the main classes of hormone response element differ principally at positions 3 and 4.

GRE	TGTTCTnnnTGTTCT	Spacing of 3 bases
ERE	TGACCTnnnTGACCT	Spacing of 3 bases
TRE	TGACCTTGACCT	No spacing

The D-box is located between cysteines 5 and 6 at the stem of the second zinc finger and is important for protein-protein interactions. It permits the correct localisation of the receptors on hormone response elements during dimerisation [177]. Compared to the structural conservation of the P-box, the D-box shows marked sequence divergence. Freedman and Towers [50] have examined the effects of exchanging the D-boxes between VDR and GR DNA binding domains. When the VDR D-box replaced those residues within the context of the GR DNA binding domain, the co-operative DNA binding normally seen was abolished. The intrinsic affinity of the fragment for its half-site remained unchanged. When the reciprocal mutation was made, where the D-box of GR was inserted within the context of the VDR DNA binding domain, the mutant protein would not dimerise on a VDRE that was a direct repeat only one that was an inverted repeat with a spacing of 3 bases. These results highlighted the importance of the D-box in orienting the receptors correctly on the response element half-sites. The crystal structure of the GR DNA binding domain in complex with DNA has thrown further light on the functional importance of zinc finger region. This will be discussed in more detail below.

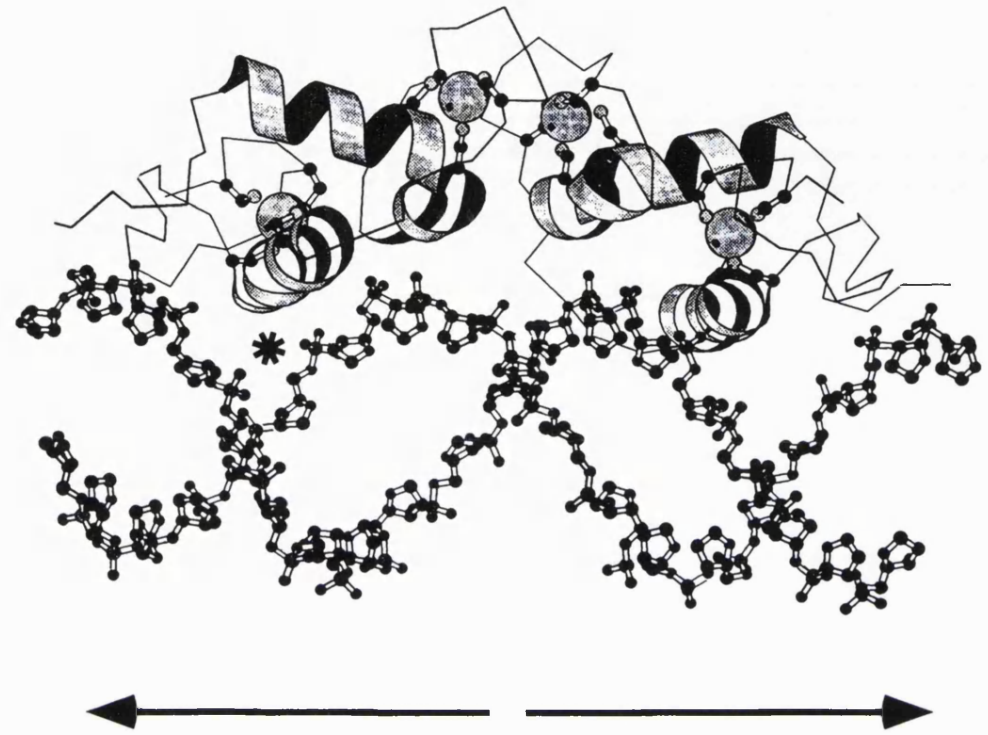
More recently another domain, C-terminal to the Zn-fingers, has been identified as important for dimerisation of nuclear receptors. Wilson et al 1992 [187] studied NGFI-B, (nur 77), an early response protein and an orphan receptor, along with RXRβ. Both these nuclear proteins bind the oestrogen response element half-site (**5′-AGGTCA-3′**). Portions of NGFI-B and RXRβ containing the Zn finger structural domain with ~20 flanking residues on either side were expressed in bacteria as fusion proteins. Bacterial sonicated extracts were used in gel electrophoretic mobility-shift assays with either CRBPII- or NBRE-containing oligonucleotides. These are the response elements for RXRβ and NGFI-B respectively. CRBPII contained five oestrogen receptor half-sites arranged as tandem repeats with a single nucleotide spacer. By making chimeric receptors, twelve residues located C-terminal to the Zn finger region of RXRβ, were found to be important for dimerisation. The domain was termed the **T**-box, Fig 3.

Because VDR also may heterodimerise with RXR, Hsieh et al [71] mutated residues in the T-box in VDR and found that binding of the receptor to VDRE was much reduced in a gel mobility shift assay. Transfection into COS-7 cells revealed reduced trans-activation of a VDRE driven reporter. In contrast mutations in the P- and D-boxes of VDR did not impair VDRE binding and demonstrated essentially normal trans-activation. Thus the determinants for DNA binding specificity in VDR and possibly thyroid and retinoic acid receptors which also bind the ERE consensus sequence (and may dimerise with RXR) are more complicated than those of steroid hormone receptors. They may be governed more by heterodimerisation and

T-box facilitated interaction than by discrete half-site recognition by the knuckle of the first Zn finger.

The three dimensional structures of the DNA binding domains of the glucocorticoid, oestrogen and retinoid X receptors have been established by nuclear magnetic resonance spectroscopy [59, 103, 159]. More recently the crystal structures of the glucocorticoid DNA binding domain (GRdbd) [107] and oestrogen receptor DNA binding [158] domain in complex with DNA have been determined. The results obtained using the different methods agree well with one another and have provided much insight into the structure and function of this region. They have confirmed many of the results obtained by mutagenesis in the GR and ER described above. I shall concentrate here on the GRdbd as this was used a template to model the effects of mutations in VDR.

Figure 4



A ribbon representation of two monomers of the glucocorticoid receptor DNA binding domain, binding to one surface of the hormone response element. Because sequence of the response element is palindromic, the monomers dimerise in a head-to-head manner.

It should be noted that:

- Only the phosphate backbone of DNA is shown for clarity.
- Zinc ions are depicted as spheres.
- The three helices in each receptor are represented as ribbons and connecting segments as lines.
- The α-recognition helix is shown sitting in the major groove of DNA as *.

The figure was prepared using MOLSCRIPT [96].

The crystal structure of the GR DNA binding domain shows that the protein assumes a compact globular fold that can be divided into two structures each with a Zn coordination centre, Fig 4. Three α-helices are present in the protein, one in the first Zn finger and two in the second. The two subdomains differ in structure and function. Residues making specific base contacts to the major groove of DNA are localised to the α-helix in the first finger. The crystal structure shows clearly that the protein dimerises upon DNA binding by forming reciprocal salt bridges and hydrogen bonds between residues that reside exclusively in the D-box of the second zinc finger. Among the important contacts are a pair of salt bridges between Arg 479 and Asp 481 of each monomer. This pair of residues correspond to Asn 60 and Asp 62 in the D-box of VDR. The strength of the dimer interface that results from these symmetrical contacts explains why GRE half-sites are always in an inverted orientation with a strict spacing of three bases.
The second zinc finger also provides phosphate contacts and a site implicated in the positive control of transcription.

Ligand binding domain

The steroid binding domain of nuclear receptors is approximately 25 kDa in size, moderately conserved and contains regions for steroid binding and ligand-dependent transcriptional activation [40, 182]. Binding of ligand results in increased affinity of the receptor for the nucleus and DNA. The conserved amino acids of this region are important for the formation of a hydrophobic pocket whereas some of the non-conserved amino acids are essential for specific ligand binding and regulation of transcription. Upon ligand binding,

steroid receptors act as dimeric transcription factors to activate or repress expression of target genes by binding to hormone response elements. Steroid receptor-mediated gene activation *in vivo* is entirely dependent upon steroid hormone. The question of how a steroid ligand modulates the gene-regulatory activity of its cognate receptor has remained unsolved. In low salt homogenates, certain steroid receptors such as glucocorticoid (GR), progesterone (PR), oestrogen (ER), and androgen (AR) receptors exist in an 8-10 S oligomeric complex in association with several non-receptor proteins such as the 90 kDa heat shock protein (hsp90) [82], hsp70 [95] and hsp56 [156]. In this high molecular weight oligomeric complex, the receptor is functionally inactive. Ligand is believed to induce dissociation of the receptor from the 8-10 S complex resulting in a smaller active 4S form that can bind its cognate response element. Receptor binding to Hsp90 is stabilised by sodium molybdate through an unknown mechanism and involves primarily the steroid binding domain of the receptor [165]. With glucocorticoid receptor hsp90 binding seems required for strong ligand binding activity, but once hormone binding has occurred, it can be sustained in the absence of hsp90 [17]. However this is not a universal relationship, since androgen receptor, oestrogen receptor and progesterone receptor do not require hsp90 for ligand binding activity. Hsp90 appears to dissociate from the receptor treated with hormone in vivo, since subsequent receptor extracts lack bound hsp90 [165]. The relevance of this work to VDR is unclear as recent evidence suggests that VDR does not bind hsp90 [74].

In addition, ligand does not appear to be essential for the binding of all nuclear receptors to DNA. Receptors for T_3, $1,25(OH)_2$ D_3 and

retinoic acid bind to DNA in the absence of ligand [16]. Hirst & Feldman, [65] have shown salt-induced activation of VDR to a DNA binding form. There have been several reports describing hormone-independent biological activities of steroid receptors *in vitro*. Hormone-free glucocorticoid receptor can bind specifically to the GRE/PRE in the long terminal repeat (LTR) of the mouse mammary tumour virus (MMTV) *in vitro* [185]. Likewise, purified progesterone receptor binds specific DNA sequences in the uteroglobin gene with similar affinity whether in hormone-bound or free state [3]. It appears that these receptors undergo structural alteration and activation by various *in vitro* manipulations such as high salt treatment.

The carboxy-terminal part of steroid hormone receptors performs functions other than just ligand binding. This domain has been shown to be important for dimerisation of the oestrogen receptor and for ligand-dependent nuclear translocation of the glucocorticoid receptor [42, 176]. The region between amino acids 241-260 in the hormone binding domain of VDR has a major role in transcriptional activation and dimerisation [74]. Previous studies have suggested the existence of two dimerisation domains in receptors, a "strong" hormone-inducible dimerisation function in the steroid binding domain and a "weak" constitutive function associated with the DNA binding domain [99]. In the oestrogen and glucocorticoid receptors, the weak zinc finger dimerisation motif mediates homodimer formation of the DNA-binding region [99, 176]. The amino acids responsible for this interaction lie in the D box as described previously. A highly conserved region has been identified within the ligand-binding domain of the mouse oestrogen receptor that is

required for both receptor dimerisation and high affinity DNA binding [42]. This domain overlaps a region essential for oestradiol binding and lies more than 250 amino acids from the DNA binding region. Binding of ligand to sequences within the steroid binding domain stabilises receptor-DNA interactions. Critical residues in the domain lie within a heptad repeat of hydrophobic residues that is conserved in a similar position in other members of the nuclear receptor family; it corresponds to residues 350-372 in VDR. Site directed mutagenesis, resulting in single amino acid substitutions in the N-terminal half of the heptad repeat prevented dimerisation. These were R507A, L511R & I518R; substitutions in the C-terminal half had no effect. Steroid binding was abolished by point mutations in the centre of the conserved region (I518R & G525R) implying that the steroid binding and dimerisation domains overlapped [42]. It is likely that if one were able to analyse the tertiary structure of VDR, the C-terminal and Zn finger dimerisation domains would be spatially juxtaposed, probably by folding of the protein.

The carboxy-terminal region participates not only in homodimer formation as in the ER and GR but also facilitates formation of heterodimers between RAR and T3R [53]. These receptors and VDR may also interact with other auxiliary nuclear proteins [21, 53, 170]. More recent evidence indicates that the auxiliary proteins are most probably the retinoid X receptor [20, 116, 190]. In VDR, the C-terminal domain is essential for complex formation with the auxiliary protein and this promotes high affinity DNA binding [128]. It is also likely that in the case of VDR, this protein is the Retinoid X-α or β receptor [22, 190]. Dimerisation with other nuclear

proteins increases the repertoire of transcriptional activation by VDR and the other receptors.

Regions required for transcriptional activation

In the oestrogen receptor two transcriptional activation functions (TAFs) have been defined using transient transfection experiments. TAF-1 in the N-terminal domain, is constitutively active while the activity of TAF-2 in the hormone binding domain requires binding of hormone [100, 182]. Danielian et al [35] identified amino acids near the C-terminal of the mouse oestrogen and glucocorticoid receptors which are essential for hormone dependent stimulation of transcription (TAF-2). This region located between residues 538 and 552 of the mouse oestrogen receptor (410-424 of VDR), is conserved in members of the nuclear receptor superfamily. Danielian used point mutagenesis of conserved hydrophobic and charged residues to demonstrate that hydrophobic residues flanking a conserved glutamine are essential for transcriptional activation. Replacement of these residues abolished transcriptional activation without significantly affecting DNA or steroid binding. These transcriptional activation functions have yet to be defined in many nuclear hormone receptors. However receptors for retinoic acid and thyroid hormone appear to lack a constitutive TAF-1 activity and this may also be the case for receptors lacking appreciable sequences N-terminal to the DNA binding domain e.g. VDR. As a consequence VDR may rely entirely on sequences in the hormone binding domain for transcriptional activation. As already mentioned, Hsieh et al have used site-directed mutagenesis to map a region between residues 241-260 in the ligand binding domain of

VDR that is important for transcriptional activation but not binding of $1,25(OH)_2D_3$ [74]. This domain is also conserved in the superfamily.

Phosphorylation

Steroid hormone receptors, including those for glucocorticoids [34] oestrogen [124], progesterone [162], and androgen [179] have been shown to exist as phosphoproteins, although the repertoire of kinases and phosphatases involved in their cellular regulation have not been fully identified. Virtually every step in steroid receptor function, from hormone binding to transcriptional modulation and receptor recycling has been postulated to be influenced by changes in receptor phosphorylation state [133].

Phosphorylation of VDR has been shown to occur in a number of species in a number of species, including mouse [143] and chicken [19] primarily on serine residues. Human VDR is an efficient substrate cAMP-dependent protein kinase, in vitro. This phosphorylation reaction is rapid and neither dependent upon, nor affected significantly by the presence of $1,25(OH)_2D_3$ [84]. Preliminary mapping experiments have revealed that the primary sites of cAMP-dependent phosphorylation, in vitro are localised between amino acids 133 and 201. Cotransfection of the murine cAMP-dependent protein kinase and human VDR into COS-7 cells not only resulted in a dramatic increase in receptor phosphorylation but also attenuated $1,25(OH)_2D_3$-dependent transcriptional activation of a reporter gene. The observations suggested a

potential role for cAMP-dependent protein kinase in the modulation of VDR-mediated gene regulation.

VDR has also been shown to be a substrate for casein kinase II (CK-II), a regulatory enzyme in the function of nuclear proteins [85]. Intact VDR produced by in vitro transcription/translation or in a baculovirus overexpression system served as efficient substrates for purified bovine CK-II and the magnitude of this phosphorylation was not affected by the addition of $1,25(OH)_2D_3$. CK-II-catalysed phosphorylation of truncated VDR suggested that the residues that can be phosphorylated may lie between Arg121 and Asp232. Site-directed mutagenesis of serine/threonine residues in this region revealed the site of phosphorylation to be at Ser208. This site of post-translational modification serves as another potential mechanism for the modulation of the activity of VDR in controlling gene transcription.

Phosphorylation may be important in ligand-independent activation of steroid receptors; Denner et al [39] noted that the transcriptional activity of chick progesterone receptors is stimulated in the absence of progesterone, by either 8-bromo-cAMP or okadaic acid (an inhibitor of phosphatases 1 and 2A); Power et al [145] reported that the neurotransmitter dopamine caused chick and human progesterone- and oestrogen-receptor mediated transcription in the *absence* of the appropriate ligand. These findings suggest that alternate pathways exist and that the signal for these pathways may be through alteration of receptor phosphorylation. The functional consequences of PK-A-mediated steroid hormone receptor phosphorylation may be distinct between the GR/PR and

VDR subfamilies, resulting in increased receptor activity in GR/PR and reduced activity in VDR. Possibly, the differential effect of phosphorylation is related to the formation of heterodimers in the VDR subfamily, while GR and PR function as homodimers.

Localisation of VDR

The subcellular localisation of hormone receptors and the mechanism of nuclear translocation upon ligand binding is still unresolved. Studies of steroid receptors in the 1960s and 1970s, principally with radioactive hormones, indicated that unstimulated receptors were located mainly in the cytoplasm. Parallel studies suggested that steroid hormone binding initiated receptor translocation to the nucleus within minutes or 0.5-4 hours after hormone exposure [180]. Subsequently, immunocytology and cell fractionation implied that even non-activated steroid receptors were predominantly intranuclear, and the evidence for receptor translocation was widely criticised as an artefact [90]. Methodological limitations may have precluded visualisation of changes in receptor distribution, particularly during the first few minutes of the receptor activation process. The development of microwave fixation of tissues accompanied by high energy immunodetection enabled Barsony et al to study VDR compartmentalisation and organisation [8]. Cultured fibroblasts were analysed from normal individuals and patients with end organ resistance to $1,25(OH)_2D_3$. Compartmentalisation of VDR in the absence of $1,25(OH)_2D_3$ was regulated by serum or oestrogen. VDR were mainly cytoplasmic in normal cells cultured without serum, but became intranuclear after addition of serum or oestrogen for at

least 18 hours. $1,25(OH)_2D_3$ initiated a rapid multistep process of reorganisation in a portion of VDR, with intranuclear accumulation occurring within 1-3 minutes. In fibroblasts from patients with vitamin D resistant rickets, VDR pattern changes were absent or severely disturbed and therefore potentially responsible for the disease. The cytoplasmic localisation of VDR and their translocation to the nucleus on hormone binding has been confirmed in HL60 human leukaemia cells [80]. The mechanism of nuclear translocation is very important to this thesis as VDR from one of the patients studied (JK) demonstrated inability to associate with nuclear material. This area will be dealt with in more detail when discussing the possible reasons for vitamin D resistance in that individual.

End-organ resistance to steroid hormones

Resistance to the actions of androgens, glucocorticoid and thyroid hormones has been well documented. Recently, the first case of oestrogen resistance was described in a young man with osteoporosis. Description of mutations in these receptors has highlighted their importance to normal biology.

Thyroid hormone resistance

Thyroid resistant syndromes are characterised by diminished clinical and biochemical manifestations of thyroid hormone action relative to the circulating hormone levels. Most patients are identified by elevated levels of T_4 and T_3 with inappropriately non-suppressed levels of TSH.

The first full report of thyroid hormone resistance appeared in 1967 and described 2 sibs of consanguineous parents who presented with deaf-mutism, delayed bone age with stippled epiphyses, goitre and high levels of protein-bound iodine [148]. The children were not iodine deficient and indeed secreted large amounts of T_4. Importantly, the secreted T_3 and T_4 were found to be the active L-isomers.

Thyroid resistance is now divided into three forms; generalised, pituitary and peripheral resistance.

Generalised Resistance (GRTH)

The majority (approximately 300 cases) belong to this category. Untreated subjects are usually clinically euthyroid at the expense of high circulating T_4 maintained by high thyroid stimulating hormone (TSH) secretion.

Pituitary Resistance

Approximately 50 cases have been described. Thyroid hormone is present in excess and results in the appropriate manifestations in the peripheral tissues but does not feed back on the pituitary.

Peripheral Resistance

One individual has been investigated and found to require high doses of T_3 to maintain normal metabolism. Because pituitary responsiveness is normal, serum TSH concentration is normal.

Molecular basis for generalised resistance to thyroid hormone

The majority of the cases arise through autosomal dominant inheritance with the remainder being sporadic. One autosomal recessive pedigree has been reported. Two genes encoding the thyroid receptors, T_3Rs (α and β), have been identified [102]. Each T_3R gene generates two proteins by alternative splicing so that there are four types of thyroid receptors. The relative expression of the two T_3R genes and the distribution of their products vary from tissue to tissue and during different stages of development. Thyroid hormone has been shown to play a key role in regulating the differential expression of the two T_3R genes and their isoforms. $T_3R\beta$ mutations have been identified in patients with generalised resistance to thyroid hormone [149]. No mutations have been found in $T_3R\alpha$ suggesting that either its products play a minor role in TSH regulation or that its mutant products are lethal. With the exception of one family with a $T_3R\beta$ deletion, all mutations have been localised to the ligand binding domain. They are clustered in two regions outside the two domains believed to be involved in dimerisation and binding to an auxiliary protein.

As generalised thyroid resistance is a dominant disorder, a key question concerns the mechanism by which the mutant alleles disrupt function. In the one family with a deletion of the $T_3R\beta$ gene only the homozygous individuals are affected; the heterozygotes are normal indicating that one copy of the gene is sufficient for normal function. It also excludes the possibility that the dominant inheritance of GRTH caused by missense mutations is due simply to a reduction in the level of normal $T_3R\beta$. It is hypothesised that the dominant inheritance of GRTH results from the mutant $T_3R\beta$ receptors interfering with the function of the

normal receptors. The most likely model for the mechanism of the dominant negative effect exerted by the mutant thyroid receptors is the formation of stable but inactive homodimers with wild type $T_3R\beta$ or heterodimers with an auxiliary protein such as RXR. These dimers compete with wild type complexes for TREs and once bound cannot be activated by T_3 as the mutations reduce receptor affinity for ligand. It is interesting that all of the ligand binding domain mutations in GRTH cluster on either side of the receptor dimerisation domain. Mutations that interfere with dimerisation of mutant receptors might not display the clinical picture of GRTH in the heterozygous state.

Androgen resistance

Androgen action is essential for the virilisation of the urogenital tract of the male embryo. The male phenotype may fail to develop due to defects in the synthesis of the principal circulating androgens (testosterone and dihydrotestosterone), or to defects in the androgen receptor itself. Androgen resistance is associated with abnormalities of male phenotypic development that range from women with complete testicular feminisation to men with hypospadias and gynaecomastia (Reifenstein syndrome) to men with infertility and/or minor degrees of undervirilisation [56]. A spectrum of defects in the androgen receptor have been defined using ligand binding assays in genital skin fibroblasts from XY men with androgen resistance. They range from absent binding to normal binding with no phenotypic correlates. Defects in the androgen receptor are much more common than mutations in other members of the steroid/thyroid receptor gene family. This

difference is likely to be due to three features of the androgen receptor:

- the gene that encodes the androgen receptor is X-linked so that hemizygous individuals express mutations
- androgen action is required for reproduction but not for life of individuals
- impairment of androgen action during embryogenesis of the male causes anatomical and/or functional abnormalities that lead to a high rate of ascertainment.

The molecular basis for the disease has been identified in many of the patients [114, 115, 122, 127]. Most of the defects are due to nucleotide changes that cause premature termination codons or amino acid substitutions within the open reading frame encoding the androgen receptor. The majority of these substitutions are localised to three regions of the androgen receptor with a dearth in the amino terminus [121]:

- the DNA binding domain
- two segments of the hormone binding domain between amino acid residues 726-772 and 829-864 .

The paucity of mutations at the amino terminus of the receptor may be due to:

- rather diffuse function of this region; a single amino acid substitution may not disrupt activity
- biased patient ascertainment; only the most severely affected patients have been investigated
- the lethal nature of mutations at the amino terminus.

The fact that one of these regions is homologous to a region of the human thyroid hormone receptor, (codons 310-347), that is a known cluster site for mutations that cause thyroid hormone resistance implies that the localisation of mutations in the AR gene is not just coincidence.

Glucocorticoid resistance

Familial glucocorticoid resistance is a hypertensive hyperandrogenic disorder characterised by increased serum cortisol concentrations in the absence of stigmata of Cushing's syndrome [2]. Plasma ACTH concentrations are also elevated, despite cortisol hypersecretion, and the hypothalamic pituitary axis is resistant to suppression by exogenous glucocorticoids. High ACTH levels participate in the pathophysiology of the disorder by stimulating adrenal secretion of salt-retaining hormones and androgens. Up to the present, the molecular basis of familial glucocorticoid resistance has been reported in three kindreds. In two kindreds there were independent missense mutations in the ligand binding domain that resulted in amino acid substitutions [77, 111]. In one kindred there was a four base-pair deletion in a splice site, leading to loss of a functioning allele [86].

Oestrogen resistance

Until recently oestrogen receptor mutations had been considered lethal. The first report of inherited oestrogen receptor mutations came from Smith et al 1994, who documented the case of a 28 year

old man with osteoporosis [166]. He presented with tall stature and progressive genu valgum and was found to have osteoporosis, unfused epiphyses and continuing linear growth in adulthood. His endocrine profile revealed elevated oestrogen concentrations, abnormal gonadotrophin secretion and no target-tissue responses to oestrogen therapy. Analysis of the oestrogen receptor gene revealed a single base-pair substitution in exon 2 generating a stop codon. These findings indicated that oestrogen receptor mutations may not be lethal and that oestrogen is important for normal male and female skeletal development.

Hereditary resistance to $1,25(OH)_2D_3$ (HVDRR)

Bearing in mind the homology of VDR to the other nuclear receptors and the fact that there are syndromes of resistance to steroid hormones and thyroid hormone, it was not surprising that vitamin D resistance was found.

Hereditary vitamin D resistant rickets (HVDRR) is an autosomal recessive disease (Mendelian Inheritance in Man #27744) that typically has its onset in early infancy with the appearance of severe rickets, hypocalcaemia, secondary hyperparathyroidism and elevated circulating levels of $1,25(OH)_2D_3$. In addition, there is sometimes total alopecia for reasons that remain unexplained [11]. Since 1978 when this disorder was first recognised [18], 34 kindreds containing 43 individuals with true resistance to

$1,25(OH)_2D_3$ have been described and characterised; for reviews [45, 47, 113].

End-organ hyposensitivity to 1,25-dihydroxyvitamin D was first reported in 1978 by the teams of Brooks and Marx [18, 117]. Subsequently, Liberman et al. (1980) [105] described a 13-year-old girl with total alopecia and rickets unresponsive to large doses of vitamin D_2. She had profound hypocalcaemia, that was unresponsive to several agents including 1,25-dihydroxycholecalciferol. Serum concentrations of 25-hydroxyvitamin D were normal but those of $1,25(OH)_2D_3$ were markedly raised. In addition, 24,25-dihydroxyvitamin D was undetectable in the serum. Administration of synthetic 24,25-dihydroxycholecalciferol was followed by normocalcaemia which persisted long after treatment was stopped. Her sister, who died at age 10 months, also had total alopecia, rickets and hypocalcaemia resistant to vitamin D_2 treatment. A defect of receptor mutation was postulated as the most likely cause of their disease. Rosen et al. (1979) [151] described 2 sisters, aged 3 and 7 years, who had onset of chronic rickets in the first year or so of life. The parents were normal second cousins. The older child lost her hair at age 16 months, and soon thereafter, bony deformities and progressive loss of teeth occurred. Vitamin D in large doses and calcium had no effect. The authors postulated absence of specific receptors for $1,25(OH)_2D_3$ in intestine and bone, or alternatively a defect in the nuclear response to $1,25(OH)_2D_3$. Although treatment with $1,25(OH)_2D_3$ had no effect, oral phosphate had significant benefit.

Work on the molecular pathogenesis of the condition was facilitated by finding that skin possessed VDR [32] and that dermal fibroblasts derived from skin biopsies could be used as a model system for the study of VDR from patients [44]. Subsequently, it was demonstrated that defects in this receptor were the likely cause of HVDRR. It was also found that $1,25(OH)_2D_3$ could induce the enzyme 25-(OH) vitamin D3-24-hydroxylase (24-hydroxylase) in multiple target tissues by a receptor-mediated process [31]. Using this bioresponse marker in cultured fibroblasts, cells from a variety of patients with HVDRR have been shown, *in-vitro*, to be resistant to the actions of $1,25(OH)_2D_3$ [27, 43, 52, 57]. Analysis has included assays of high affinity receptors by radiolabelled hormone binding, cellular uptake measurements of $1,25(OH)_2D_3$, induction of 24-hydroxylase, specific monoclonal antibody association and receptor-DNA interaction. A spectrum of molecular defects in VDR has emerged from these patients. Absent $1,25(OH)_2D_3$ binding is present in some kindred fibroblasts [23], whereas others display a quantitative deficiency of receptor e.g. Patient 4 described by Liberman et al. (1983) [104] or depressed DNA interaction e.g. Patient 3 described by Sone et al. (1990) [169]. However, all of the cell lines show an inability of $1,25(OH)_2D_3$ to activate the 24 hydroxylase enzyme.

Baker et al. (1988) [4] described the cloning and characterisation of cDNAs encoding the human vitamin D receptor. Analysis of the deduced amino acid sequence showed that the VDR protein belonged to the superfamily of trans-acting transcriptional regulatory factors, including the steroid and thyroid hormone receptors. The group showed that the single protein produced in an

expression system using the cloned cDNA was indistinguishable from the native receptor in physical properties and affinity for vitamin D [4]. Hughes et al. (1988) [75] studied 2 families: a black Haitian family with 2 affected sisters and an Arab family living in the Middle East with 2 affected brothers. In both families, the parents were consanguineous but phenotypically normal. The patients had partial alopecia. The group isolated genomic DNA and amplified the individual exons using the polymerase chain reaction (PCR) [154]. By this method, they sequenced each of the 9 exons encoding the receptor protein. The Haitian family had a glycine to aspartic acid mutation near the tip of the first zinc finger, and the Arab family had an arginine to glycine mutation at the tip of the second zinc finger. The same mutations were found in approximately 50% of clones from parents, confirming the autosomal recessive nature of the disease. The mutant residues were created in vitro by oligonucleotide directed point mutagenesis of wild type VDR cDNA and this cDNA was transfected into COS-1 cells. The protein produced was Biochemically indistinguishable from the receptor isolated from patients. The mutant cDNA was cloned into an expression vector and cotransfected with a reporter plasmid [171] into VDR deficient CV-1 cells. *Trans*-activation in mutant and wild type was measured by induction of the enzyme chloramphenicol acetyltransferase (CAT). Wild type receptors showed normal *trans*-activation whereas the mutant receptors showed no *trans*-activation. This data provided compelling evidence that HVDRR evolved in these two families as a result of inheritance of homozygous VDR alleles encoding point mutations which inactivate the $1,25(OH)_2D_3$ receptor. The single base change in the second position of each triplet codon altered a highly

conserved amino acid found at these positions in all steroid hormone receptors cloned to date. This was the first molecular identification of a disease-producing mutation in a steroid hormone receptor gene. Since then, a number of different mutations have been found as the cause of HVDRR [97, 112, 150, 152, 153, 169, 188].

Treatment of hereditary vitamin D resistance initially involved giving large doses of vitamin D and its active metabolites 1, alpha-hydroxyvitamin D_3 or 1,25-$(OH)_2$ vitamin D_3 often with little success. Balsan et al [5] and subsequently Bliziotes et al [13] demonstrated that nocturnal calcium infusions promoted normal mineralisation in these patients. The work was extended by Hochberg et al [66] who studied ten patients with the condition and gave eight of them high doses of elemental calcium (0.4-1.4 g/m2) through intracaval catheters. Clinical improvement was observed within a week and the biochemical and radiological changes returned more swiftly than with oral therapy. Therefore this group recommended the use of intravenous therapy until radiological healing of rickets was demonstrated, at which point oral therapy could be instituted.

Table 1 provides a complete list of mutations in the vitamin D receptor that have been shown to be associated with hereditary vitamin D resistant rickets prior to the publication of the work described here. Numbering of the residues is from the initiation codon at position 125 of the cDNA sequence and differs from that of some of the earlier publications [75, 150]. However it is consistent with the only initiation codon found by both Pike's group and our own [64, 97, 152, 153, 188].

Figure 5 is a schema of the vitamin D receptor, with the positions of all the mutations identified by previous groups of researchers. There are seven mutations in all, with 5 lying in the zinc finger region of the DNA binding domain. In addition, two mutations in residues 311 and 388 have been reported in abstract form [160].

Table 1

Known mutations in the vitamin D receptor

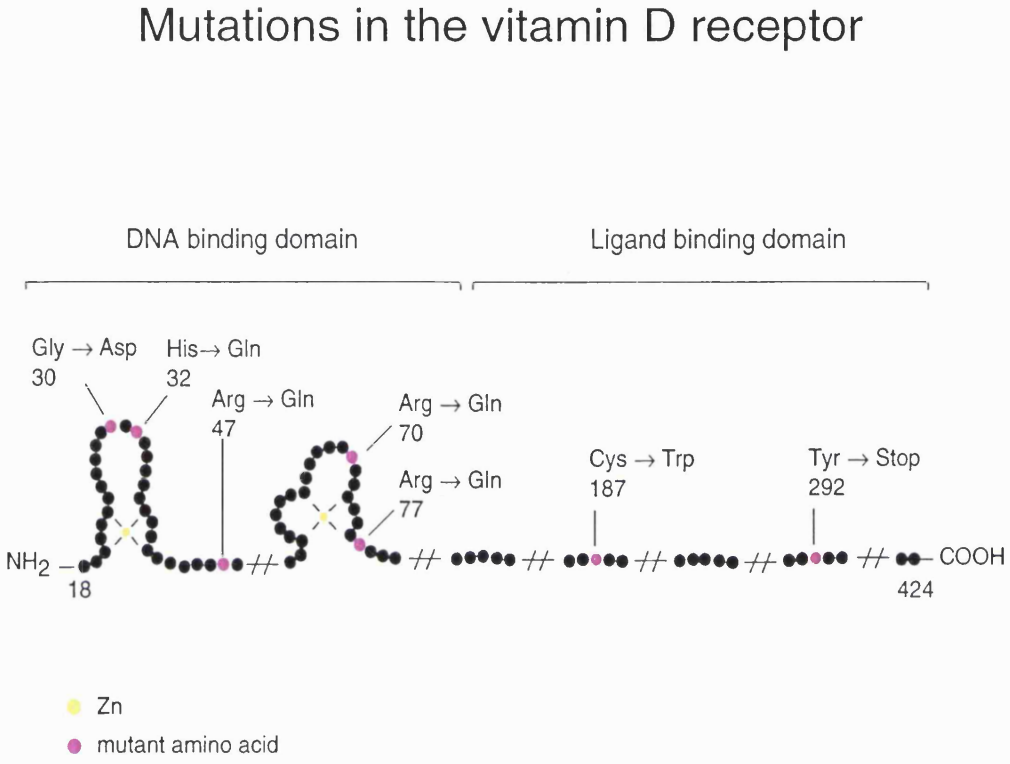
DNA binding domain

Residue	Normal	Mutant	Reference
30	Gly (GGC)	Asp(GAC)	[75]
32	His (CAC)	Gln (CAG)	[188]
47	Arg (GCA)	Gln (GAA)	[154]
70	Arg (CGA)	Gln (CAG)	[75]
77	Arg (CGG)	Gln (CAG)	[169]

Ligand binding domain

Residue	Normal	Mutant	Reference
187	Cys (TGT)	Trp (TGG)	[113]
292	Tyr (TAC)	Stop(TAA)	[150]

Figure 5



All the previosly reported and characterised mutations in the vitamin D receptor are illustrated above. Amino acids are numbered from the methionine codon at position 125 of the VDR cDNA sequence.

5 mutations lie in the DNA binding domain and 2 in the ligand binding domain.

Chapter 2; Methods

The key areas that will be covered in this chapter are outlined below.

- Vitamin D resistant rickets was documented clearly in the patients studied here.

- In all cases, patients' phenotypes were confirmed by biochemical tests and functional analysis of the vitamin D receptors .

- The molecular basis for the disorder was determined by sequencing of VDR cDNA derived from skin fibroblasts.

- Expression studies of the mutant cDNAs confirmed that the mutations detected were most likely responsible for end-organ resistance to vitamin D.

- In-vitro transcription and translation of the receptors confirmed sequence data on the sizes of the transcribed proteins.

- The DNA binding domain mutants were subjected to crystallographic modelling based on the structure of the glucocorticoid receptor DNA binding domain. This allowed structural interpretation of DNA/protein interactions.

Patients

Five patients with hereditary vitamin D resistant rickets from unrelated families were studied. Their clinical features are detailed below.

Patient CC

Lin and Uttley (1993), described the clinical and pathological features of this girl who was referred to them aged 1.3 years with delayed motor milestones and was found to be unable to weight bear [106]. She was born in the UK of non-consanguineous Indian parents who originated from Mauritius. At four months she sustained a supracondylar fracture or the right femur and in a skeletal survey assessed retrospectively demonstrated rickets. At 1.3 years she was referred to hospital There was clinical, radiological and biochemical evidence of severe rickets. Symptoms included irritability, apparent bone pain, anorexia, failure to thrive, weakness, motor and speech delay. Alopecia was striking. Biochemical changes included hypocalcaemia, 1.69 mmol/l, (normal range 2.25-2.55 mmol/l), secondary hyperparathyroidism, with parathyroid hormone at 249 ng/L, normal levels of 25-OH vitamin D_3 (4.5 ng/ml), a raised alkaline phosphatase in excess of 10,000 IU/L (normal <300 IU/L) and aminoaciduria. A jejunal biopsy specimen, sweat test and renal function measurement gave normal results. Two intramuscular injections of 300, 000 IU of cholecalciferol (25 (OH) D_3) at 1.3 and 1.4 years of age and daily oral alphacalcidol (1α(OH) D_3) between 1.4 and 2.2 years up to a maximum of 30 μg/day, failed to induce a clinical response though the plasma $1,25(OH)_2D_3$ concentration was raised at 400 pg/ml

(normal range 30-50 pg/ml). Treatment with continuous intra-atrial calcium infusions was instituted and resulted in relief of symptoms within 24 hours and biochemical resolution within 3 months and marked improvement of rickets. Infusions began at an initial dose of calcium of 200 mg/day, rising to 500 mg/day from day 7 of the treatment.

Patient TB

Simonin et al (1992) reported the clinical and pathological features of this boy who presented to them aged seven years [164]. He was the third child of Tunisian parents who were first cousins. In his first years of life he received numerous treatments for rickets but documentation was poor. At seven years of age he was noted to have advanced rickets; deformed limbs, a deformed skull with frontal bossing, dental caries, short stature and partial alopecia Serum biochemistry showed marked hypocalcaemia, 1.67 mmol/l (normal 2.1-2.6 mmol/L), elevated alkaline phosphatase 2015 mmol/L (normal 90-400), normal levels of 25-OH vitamin D_3 at 19 ng/ml (normal 19-147), elevated levels of $1,25(OH)_2D_3$, 250 pg/ml, (normal 10-65 ng/L) and secondary hyperparathyroidism. Treatment with 1 a$(OH)_2D_3$ at doses of 1-4 mg/day plus oral calcium for 3 months had no effect. This was supplemented with of vitamin D_2 at doses of 5 mg/day. There was a gradual improvement in rickets and normalisation of the biochemical parameters. The alopecia remained unchanged.

Patient FH

Kruse et al (1988) first described FH and his sister who presented to them with alopecia and rickets at 10 and 19 months of age

respectively [98]. Biochemical analysis revealed low calcium (6.0-6.5 mg/dl), low phosphate (3.4-3.8 mg/dl [normal for age: 4-6 mg/dl]) and secondary hyperparathyroidism. There was no calcaemic response to 50 mg (2 million units) of vitamin D_3/day. Both children had normal growth and showed healing of rickets by the age of 7 and 9 years. No further clinical details were available about patient FH.

Patient IS

Fraher et al (1986) described in detail the clinical and pathological features of this Kuwaiti boy who was first seen in London at the age of 3 years [47]. He was the second son of a first cousin marriage; his four siblings were normal. His birth history was unremarkable and he first came to medical attention at 7 months when he was hospitalised with a chest infection. Later in his first year, leg deformity was noted and a diagnosis of rickets made. At this point he failed to respond to physiological doses of vitamin D_2. His second year was punctuated by recurrent chest infections and anorexia with failure to thrive. He was investigated in Bombay and showed no clinical nor radiological response to three doses of 600,000 units of vitamin D_3 intramuscularly or 2.0 μg/day of $1,25(OH)_2D_3$.

On arrival to London he was a responsive child of apparently normal intellect. He had gross rickets with florid limb deformities, bowed clavicles and a dystrophic chest with typical rachitic rosary. Hair growth was normal. Biochemically, he was hypocalcaemic (1.64 mmol/l), hypophosphataemic (0.86 mmol/l) with a very high alkaline phosphatase (6700IU/l) and amino terminal parathyroid hormone (1400 pg/l). There was also generalised amino-aciduria.

Prior to treatment the serum level of $1,25(OH)_2D_3$ was 1000 pg/ml (normal adult range 20-65 pg/ml). He was treated with high doses of calcitriol (3.0-24.0 μg/day) to no avail. After 10 weeks he returned to Kuwait where he died soon after of pneumonia, aged 3 years and 3 months.

Patient J.K

Hewison et al (1994) have described this patient, who was referred to Prof J.L. H. O'Riordan via The Hospital for Sick Children [64]. She is the daughter of unrelated English parents. At the age of six weeks abnormal hair loss was observed and by sixteen months she was noted to have swollen wrists and ankles and had difficulty in walking; she was still unable to run at two years and eight months. Apart from her skeletal manifestations she was a normal, cheerful and intelligent child. Initial biochemical investigations showed the following: calcium (corrected for albumin), 2.03 mmol/L (normal, 2.25-2.55 mmol/L); phosphate, 0.78 mmol/L (normal, 1.29-1.79 mmol/L); alkaline phosphatase, 1101 U/L (normal range, <300U/L). The serum 25-hydroxyvitamin D_3 was normal, 20 nmol/ml (normal range 7.5-100 nmol/L). Skeletal x-rays revealed marked osteopenia with metaphyseal deformity typical of untreated rickets. She had been treated initially with Alfacalcidol up to 5 μg/day and because of hypophosphataemia, phosphate supplements up to 750 mg/day. There was no response within 3 months. The child was then referred by Dr J Cogswell (Poole) to The Hospital for Sick Children at the age of two years and ten months. The Alfacalcidol was stopped for one week and serum levels of $1,25(OH)_2D_3$ when first measured were 466-650 pmol/L (normal radioimmunoassay range for an adult 48-156 pmol/L) (Clemens et al, 1978 [30]). 25-

hydroxyvitamin D_3 was 87nmol/L and c-PTH, 1283 ng/L (normal, <660ng/L). Treatment with Alfacalcidol was restarted and the dose increased progressively to 12 µg/day. Phosphate supplements were stopped and replaced with calcium up to 1 gram/day. Within 8 months her biochemical profile had normalised, serum calcium, 2.30 mmol/L; phosphate, 1.16 mmol/L; alkaline phosphatase, 890 U/L, (normal range 250-1000 U/L) and radiologically the rickets had healed but alopecia persisted. Treatment was stopped after twenty months (age; four years, four months) and there has been no relapse of rickets. I first saw JK when her rickets had resolved. She is now seven and a half years old and remarkably well, except for the fact that her alopecia persists. In 1994 the results on this patient were: Serum calcium 2.52 mmol/L, albumin 50 g/L, alkaline phosphatase 491 IU/L (probably normal for her age), serum 25-hydroxy-vitamin D 176 nmol/L (normal 15-120), parathyroid hormone 19.1 pmol/L (normal 1.0-6.5), 1, 25-dihydroxyvitamin D 770 pmol/L (normal adult 25-150). So she has a normal serum calcium but a raised PTH and raised $1,25(OH)_2D_3$, results that remain in keeping with the diagnosis of end organ resistance to vitamin D.

The parents of all five patients were phenotypically normal but were not studied in detail.

Characterisation of vitamin D receptors

Initial characterisation of VDR expression and functional activity was carried out using fibroblasts cultured from skin biopsies. Tissue from patients and aged matched controls was minced and collagenase-digested before routine culture in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum (10% FCS), insulin (1iu/ml), penicillin (100u/ml), streptomycin (100 g/ml) and glutamine (2 mM). The resulting fibroblast monolayers were cultured for between 4 and 6 passages at 37°C and 5% CO_2; the functional response of these cells to treatment with $1,25(OH)_2D_3$ and their expression of VDR were determined as described below.

24-hydroxylase activity

The ability of control and patient cells to respond to treatment with $1,25(OH)_2D_3$ was assessed by the activity of the enzyme 24-hydroxylase. Flasks containing approximately 2 million cells were cultured to form a semi-confluent monolayer. 24 hours prior to assay the growth medium was supplemented with 10 nM $1,25(OH)_2D_3$ in 0.05% ethanol, which was then replaced with serum-free DMEM containing 10 nM ^{3}H-25-hydroxyvitamin D_3 (^{3}H-$25(OH)D_3$) and re-incubated for a further 3 hours at 37°C. to allow metabolism of the substrate $25(OH)D_3$. The reaction was terminated by disrupting the cell monolayer by physical agitation and the addition of methanol. Vitamin D metabolites were then extracted from the suspension as described previously [63] and separated by straight phase HPLC using a Zorbax-sil (Dupont) column (4.6 mm x 250 mm) eluted with a mixture of hexane, methanol and isopropanol (92:4:4) at 3 ml/min. Fractions were

collected at 30-second intervals and aliquots of each fraction counted for radioactivity which was plotted against the elution profile for a mixture of standard vitamin D metabolites. The production of radiolabelled 24,25$(OH)_2$ vitamin D_3 (24,25$(OH)_2D_3$) was measured as fmoles/hour/10^6cells.

Hormone binding assays

Binding of 1,25$(OH)_2D_3$ to VDR in cytosol from skin fibroblasts was determined in cells cultured to partial confluency and harvested by trypsinisation. The resulting cell pellets were washed and homogenised in TKEDM buffer consisting of Tris-HCl (10 mM; pH 7.4) 300 mM KCl, 1.5 mM EDTA, 1 mM dithiothreitol and 10 nM sodium molybdate. The homogenate was centrifuged at 100,000 g for 1 hour at 4°C. Aliquots of the cytosol (200 µl, approximately 0.5 mg protein) were incubated at 4°C for 16 hours with 0.2-3.0 nM ^{3}H-1,25$(OH)_2D_3$ in the presence or absence of a large excess of unlabelled 1,25$(OH)_2D_3$ to determine non-specific binding. Unbound hormone was removed by the addition of 50 µl dextran-coated charcoal in TKEDM buffer followed by centrifugation at 1000 g for 15 minutes. The supernatant was removed and binding determined by scintillation counting. Receptor numbers and dissociation constant (K_d) were calculated by Scatchard analysis.

Nuclear association assay

The ability of 1,25$(OH)_2D_3$-VDR complexes to associate with nuclear material was assessed by whole cell nuclear association assay, as described [63]. Briefly, cells were cultured as for cytosolic binding assays but after trypsinisation and washing the resulting pellet was

resuspended in 1 ml serum-free DMEM to a density of 10^7 cells/ml. Aliquots of these cells (100 µl) were incubated for 1 hour at 37°C with increasing doses of ^3H-1,25$(OH)_2D_3$ (0.02 - 3.0 nM) in the presence or absence of a 100-fold excess of unlabelled 1,25$(OH)_2D_3$ (to determine non-specific binding). Nuclear pellets were then isolated by washing with a Tris buffer containing 0.25 M sucrose and 1% Triton X-100 (pH 7.4) followed by centrifugation at 1000 g for 5 minutes. Pellets were dissolved and radioactivity counted. Data were analysed by Scatchard plots.

RNA isolation

RNA was isolated from skin fibroblasts for Northern hybridisation to determine the presence and abundance of mRNA, and for subsequent reverse transcription and amplification by the polymerase chain reaction [154] .

RNA was isolated using the Guanidine thiocyanate method [28]. Disposable, rubber gloves were worn throughout procedures and were changed frequently. Disposable nuclease-free plastic-ware was utilised where possible. Glass-ware was cleaned with 0.1 N NaOH, rinsed with deionised water and reserved for work with RNA. Water for solutions was treated with Diethylpyrocarbonate (DEPC) to inhibit any RNAse activity.

DEPC Water

DEPC was added to water to a final concentration of 0.1% and left to stand for at least 2 hours at 37°C. The water was then autoclaved for 20 minutes at 15 lbs/Sq in.

SOLUTION D: (500 ml) -This was made up in the original 250 g Guanidine thiocyanate container to minimise the risks of RNAse contamination (Fluka).

Guanidine Thiocyanate	250 g
DEPC H_2O	293 ml
Sodium Citrate	17.6 ml (from 0.75 M stock)
Sarcosyl	26.4 ml (from 10% stock)
(β-Mercaptoethanol	720 µl /100 ml - added just before use)

Skin fibroblasts were grown in monolayer to 50-70% confluence (i.e. Log phase) in DMEM supplemented with 10% foetal calf serum. Cells were washed twice with ice cold phosphate buffered saline (PBS). All remaining PBS was pipetted off and 2 ml of active solution D per 75 ml flask or 5 ml per 175 ml flask was added to coat the cells. The fluid was swirled for 1 minute and the lysate transferred to a 14 ml Falcon tube (2059).

The following were added sequentially vortexing vigorously between additions and for 15 seconds at the end:

0.1 x vol	2M Na Acetate pH 4.0
1.0 x vol	water saturated phenol
0.2 x vol	24:1 chloroform:Isoamyl alcohol (IAA)

The mixture was placed on ice to allow an interface to form between the two layers and spun at 10,000 g (9500 RPM on MSE 18), at 4°C for 20 minutes. The supernatant was transferred to a

fresh Falcon (2059) tube and an equal volume of Isopropanol at -20°C was added and left at -20°C Overnight. After a further spin at 10,000 g at 4°C for 20 minutes the supernatant was discarded and the remaining liquid pipetted off. The pellet was resuspended in 0.3 ml active solution D and transferred to a microfuge tube. 1 ml Isopropanol at -20°C was added and the mixture was left overnight at -20°C. RNA was sedimented at 4°C for 10 minutes and washed twice with 80% ethanol. The pellet was freeze-dried for about 5 minutes and then resuspended in 50 µl DEPC H_2O. The optical density (OD) was checked at 260 nm by adding 1 µl to 500 µl H_2O.

OD 1.0 = 40 mg/ml.

For Northern analysis 10-20 µg of RNA were aliquoted, freeze dried (30-60 mins) and the following solutions were added:

4.5 µl DEPC H_2O; the RNA pellet was mixed firmly until dissolved
2.0 µl sterile MOPS
3.5 µl formaldehyde
10 µl deionised formamide.

(To deionise formamide 1 gram of mixed bed resin was added{Biorad AG 501-X8 (D)}/10 ml of formamide and stirred for 30 mins)

Agarose gel electrophoresis and Northern transfer of RNA

1% Formaldehyde Gel

Prior to electrophoresis the gel tray, comb and electrophoresis apparatus were cleaned with 0.1 N NaOH for 30 minutes and then rinsed with deionised water. This ensured an RNAse free environment.

0.85 g agarose was added to 60 ml DEPC H_2O, dissolved by boiling and cooled to 55°C. 8.5 ml of filter sterilised 10 x MOPS was mixed with 15 ml 37% formaldehyde in a 50 ml tube and heated to 55°C. Agarose, MOPS and formaldehyde were combined in a conical flask, and 5 µl of 10 mg/ml ethidium bromide (in DEPC water) were added and the gel was poured in a fume hood. A 2 mm comb was inserted with pairs of wells taped to prevent smearing of bands with such a large quantity of RNA.

After allowing gel to set, the apparatus was filled with 1 x MOPS (non-sterile with no ethidium bromide). 2 µl of 30% glycerol/0.25% bromophenol blue loading buffer was added to samples. The samples were heated to 65 °C for 5 minutes, spun briefly and loaded on to the 1% formaldehyde gel. Samples were run at 7.5 volts/cm, constant voltage for about 3 hours, or until the bromophenol blue had migrated approximately 80% of the length of the gel. The buffer was recirculated at 950 rpm positive to negative to ensure continued buffering throughout the experiment.
At the end of the experiment, the gel was photographed on clingfilm to minimise contamination and then soaked in 10 x SSC, 2 x 20

minutes while shaking gently to remove any formaldehyde that might impair transfer of RNA to the filter.

Transfer to Genescreen Plus™ membrane

This is a nylon membrane which is positively charged on the concave side (B) to promote binding of negatively charged nucleic acids to its surface. It was chosen for its high sensitivity and ease of handling. Transfer of RNA to Genescreen was done by osmosis. Membrane has natural curl: Side A = convex

Side B = concave

- Genescreen plus (DuPont) was cut to the size of gel. Side A was marked with a pencil and the bottom right corner was cut to ensure correct orientation and labelling of bands.

- The membrane was made wet in deionised water by capillary action and then laid in 10 x SSC for 15 minutes to allow equilibration prior to blotting.

- An inverted gel tray was placed in a bath of 10 x SSC, covered with a wick of 3 mm paper and any air bubbles were rolled away with a sterile 10 ml pipette.

- The gel was slid carefully on to the wick and any air bubbles were rolled away.

- The membrane was placed carefully on to gel so that side B was in contact with the gel. If wells were still present, they were marked with a pencil on the membrane.

- The gel was surrounded with parafilm or clingfilm to prevent paper towels coming in to contact with the 10 x SSC directly.

- The membrane was covered with 6 pieces of Whatman 3 mm thickness paper (cut to size) and 2-3 inches of tissues stacked on top. The tissues were weighed down with a 500 g object. The blot was left overnight in the cold room to minimise evaporation. Tissues were changed at least twice to maximise the capillary action.

- At the end of about 16 hours the tissues and 3 mm paper were removed. The membrane was washed in 2 x SSC (to remove any residual agarose which might interfere with hybridisation). Finally the membrane was placed on 3 mm paper to air dry (face up) and then baked for 2 hours at 80°C to fix RNA on to the membrane.

RNA buffers

10x MOPS BUFFER:(200 ml)

MOPS (sodium salt)	9.25 g (0.2 M)
Sodium acetate	0.82 g (50 mM)
Na_2EDTA pH 8.0	0.4 ml (from stock)

The solution was adjusted to pH 7.0 with Glacial acetic acid (aliquoting a few drops at a time and measuring pH using pH papers).

20x SSC BUFFER:(1 litre)

NaCl	175.3 g
Sodium Citrate	88.2 g

The solution was adjusted to pH 7.0 with NaOH solution.
It was made up to 1 litre with deionised water.
If the SSC was to be used with riboprobes. 1 ml of DEPC was added and the mixture was left overnight and was sterilised by autoclaving.

Hybridisation of random prime probes to filters

Prehybridisation & Hybridisation

The method of Church and Gilbert [29] was chosen for its simplicity and good prehybridisation ability.

Prehybridisation/hybridisation buffer

1% Bovine serum albumin (BSA)	2 g of crystalline BSA
7% Sodium dodecyl sulphate (SDS)	14 g
0.5 M Na_2HPO_4/NaH_2PO_4	100 ml of 1 M stock
1 mM EDTA	0.4 ml of 0.5 M stock
Deionised water	to a final volume of 200 ml

- BSA was dissolved first by stirring vigorously at about 37°C and then SDS was added. The mixture was heated to 65°C while stirring to dissolve all SDS.

- The Genescreen plus membrane was made wet by floating it on deionised water.

- It was transferred to 2 x SSC for 5 minutes to allow equilibration prior to prehybridisation.

- Prehybridisation was performed in a lunch box at 65 °C for 15 minutes while shaking.

Labelling of cDNA probes

- 2001 bp VDR cDNA was excised from pGEM4 using *Eco*RI; this was one of the original clones obtained by Baker et al [4] (see section on cloning for details). The digestion products were run on a low melting agarose gel and extracted by the freeze and squeeze method detailed later. DNA concentration was determined by optical density at 260 nm.

- 25 ng of 2001 bp VDR cDNA was labelled for each hybridisation using the Amersham "Multiprime kit".

- To a 0.5 ml microfuge tube the following added:

cDNA to be labelled (25 ng/µl)	1 µl
Deionised water	up to 28 µl

- cDNA was denatured by boiling for 5 mins and plunged immediately on ice to prevent renaturation and the following were added:

Buffer	10 µl
Primer	5 µl
Radiolabelled ^{32}P dCTP	5 µl (10-125 pmoles)
Enzyme	2 µl

- The solution was spun briefly and incubated at 37°C for 30 mins.

- The radiolabelled probe was purified on a sephadex G-50 column and the eluent transferred to 0.5 ml microfuge tube.

Sephadex G-50 column

The radiolabelled probe was passed through a Sephadex G-50 column to remove any unincorporated nucleotides that would hybridise preferentially to RNA on the filter.

Sephadex G-50/G-80

- 10 g of Sephadex G-50/G80 were added to 250 ml of a solution of TE pH 8.0 and 0.1 M NaCl and the mixture was autoclaved at 15 lb/sq inch for 20 minutes.

- To make the column, sterile wool was inserted approximately up to 0.1 ml mark of a sterile 1 ml syringe and the remainder of the tube was filled with Sephadex G-50/80 slurry and spun for 30 seconds at 1200 rpm. The process was repeated and the tube spun for 4 mins at 1200 rpm.

- To check whether the column was ready 100 µl of TE + 0.1 M NaCl were loaded on to the G-50/80 column and spun for 4 mins at 1200 rpm and eluent volume was measured. If it was less than 100 µl the equilibration was repeated until 100 µl was obtained.

- 1 µl of the radioactive eluent was added to 5 ml of scintillation fluid and counted on the Scintillation counter. The aim was for at least 10^6 cpm/µl, ie. total of 10^8 cpm, in order to maximise the signal to noise ratio in the subsequent hybridisation.

- 25 ng of probe was denatured by boiling for 5 minutes, plunged on to ice and was spun briefly. Hybridisation was performed in a volume of 5-10 ml in a bag at 65°C overnight with agitation. Hybridisation buffer volume was kept as low as possible to promote efficient hybridisation.

Washing the Genescreen plus membrane following hybridisation

Wash buffers

Buffer A

0.5% BSA	2.5 g
5% SDS	25 g
40 mM Na_2HPO_4/NaH_2PO_4	20 ml 1 M stock
1 mM EDTA	4 ml 0.5 M stock
Deionised WATER	to 500 ml

Buffer B

1% SDS	5 g SDS
40 mM Na_2HPO_4/NaH_2PO_4	20 ml 1 M stock
1 mM EDTA	4 ml 0.5 stock
Deionised water	to 500 ml

- The membrane was removed carefully from the bag and washed under conditions of high stringency as described below:

- buffer A twice for 10 minutes at 65°C

- buffer B twice for 10 minutes at 65°C.

- Finally the membrane was washed briefly in 2 x SSC to remove any SDS and any excess SSC was allowed to drip away but membrane was kept damp to prevent permanent hybridisation.

- The membrane was placed between layers of clingfilm and autoradiographed on Kodak X-Omatic at -70°C with enhancement. The film was developed either after overnight exposure or after several days depending on intensity of signal. In the case of VDR mRNA exposure times were approximately 4 days.

Owing to the low abundance of VDR messenger RNA and the poor detection by the above method, Northern hybridisation was tried using a riboprobe to the hormone binding domain of VDR. This method was used only in detection of RNA from JK fibroblasts. It was felt that using an antisense probe to this part of the cDNA would be both more sensitive (shorter probe) and more specific (probe does not include Zn-finger domain which is conserved throughout the nuclear receptor superfamily and which might cause cross-hybridisation with other nuclear proteins). The 3′ half of the VDR cDNA coding region was amplified by PCR using primers 33a and 33b, and cloned exactly as described below. cDNA was released from the pBluescript SK$^-$ vector using *Eco*RI and *Bam*HI and was gel purified.

Manufacture of riboprobe

The Stratagene labelling kit was employed for this purpose.

Transcription reaction

The following reagents added in this order:

5X transcription buffer	5 µl

consisting of:

200 mM Tris pH8.0
40 mM $MgCl_2$
10 mM Spermidine
250 mM NaCl

Template DNA	1.5 µg
10 mM rATP	1 µl
10 mM rCTP	1 µl
10 mM rGTP	1 µl
0.75 M Dithiothreitol (DTT)	1 µl
RNAse block II	1 unit
400-800 Ci per mmole, 10 mCi/ml ^{32}P-rUTP	5 µl
T7 RNA polymerase (diluted to appropriate required concentration 10 units/µl)	10 units
DEPC water to final volume of	25 µl

The labelling mix was incubated at 37°C for 2 hours.

DNAse I added	1 µl

The mixture was further incubated at 37°C for 10 mins.

Purification:

Unincorporated nucleotides were removed by passage over Sephadex G-50 (fine) Quick Spin™ columns (Boehringer Mannheim). These were used because of their safety, speed and because they offered RNAse-free purification.

- The column was removed from its bag, inverted several times and spun at 1100 RPM for 2 minutes. This buffer was discarded.

- Keeping the column upright, the sample was applied carefully to the centre of the column bed and spun at 1100 RPM for 4 minutes.

- 1 µl of eluent was removed and incorporation assessed by scintillation counting. The aim was for 10^8 counts in total.

Prehybridisation/Hybridisation using riboprobes

60% RNA Hybridisation Mix:(20 ml)

The following were added:

freshly deionised formamide	12 ml
100 X Denhardts	1 ml
20 X SSC (DEPC treated)	5 ml
20% SDS	400 µl
1 M phosphate buffer pH 6.8 (DEPC)	
(1 M Na_2HPO_4 pH with Na_2PO_4 to give pH 6.8)	400 µl
20 mg/ml yeast RNA (tRNA)	100 µl
10 mg/ml poly-A RNA	20 µl
10 mg/ml heat denatured salmon sperm DNA	400 µl
deionised water	0.5 ml
Total	20 ml

- The membrane was prehybridised at 65°C for 2 hours in rotating cylinder after which 10 ml of Prehybridisation buffer were discarded.

- The riboprobe was added to the remainder of buffer in the cylinder. and hybridised at 65°C overnight.

Washes

The filter was washed in four separate buffers, starting with low stringency and ending at high stringency. This is thought to minimise background hybridisation.

6 washes were performed for 15 mins at 65°C;

a) 6x SSC, 0.5% SDS

b) 3x SSC, 0.5% SDS

c) 1x SSC, 0.5% SDS

3 brief, final washes were in a low salt buffer: 0.1% SSC, 0.5% SDS at 65°C.

(All wash buffers preheated to 65°C before use)

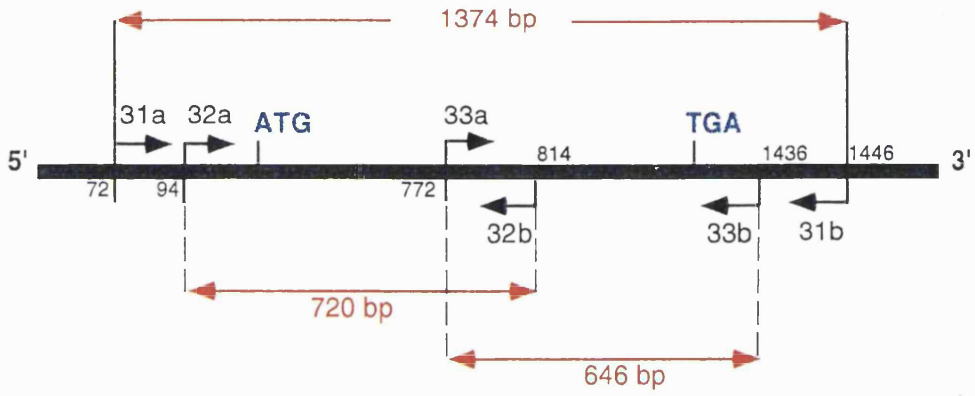
The membrane was placed between clingfilm and exposed on Kodak X-Omatic overnight with enhancement at -70°C.

First-strand cDNA synthesis and amplification of vitamin D receptor cDNA

Reverse Transcription (RT)

The organisation of VDR chromosomal gene, with eight coding exons and introns of up to 13 kb, made sequencing of genomic DNA difficult, especially as the sequences of the exon-intron boundaries remain unpublished. It was decided to accelerate the process by utilising cDNA, amplifying this by PCR and sequencing the cloned products. Total RNA was prepared from patient's skin fibroblasts as described above.[28]. All subsequent steps were carried out in **duplicate**. cDNA was synthesised from total RNA (10 µg) using primer 31b (5'-GCCCTGGAGGAGCAGCCC-3'), an 18-mer starting at position 1446 in the 3' untranslated region of the VDR cDNA sequence Fig 6. Numbering is based on the published VDR cDNA sequence [4].

Figure 6



Reverse transcription and PCR amplification of the VDR coding region. Reverse transcription was performed using primer 31b. The full-length coding region was amplified using primers 31a and 31b. Each half of the cDNA was then amplified by nested PCRs using primers 32a & 32b and 33a & 33b. Base pair numbering refers to the location of the 5'-ends of the primers with respect to published sequence, Baker et al 1988, [4].

The following components were added to a nuclease-free tube:

Primer 31b 0.1 μg/μl	2 μl
Total RNA 0.5 μg/μl	20 μl
Deionised water, to a final volume of	24 μl

The mixture was heated to 70°C for 10 minutes to denature the RNA and then plunged on ice to prevent renaturation. After a brief spun the following reagents were added from the Gibco/BRL "Superscript reverse transcriptase" kit:

5X RT-buffer	8 μl
DTT 0.1 M	4 μl
Mixed dNTP stock 10 mM each	2 μl

The tube was vortexed gently, spun and incubated at 45°C to equilibrate the contents.

Finally Superscript reverse transcriptase was added(200 units):	1 μl
Total volume	**40** μl

The reaction was incubated at 45°C for 60 minutes and stopped by refrigeration, not by addition of EDTA as this would have chelated Mg^{2+} ions in subsequent PCR amplification. Reagents were obtained from (Bethesda Research Laboratories, Gaithersgerg, MD).
5X buffer = 250 mM Tris-HCl pH 8.3 at room temperature, 375 mM KCl, 15 mM $MgCl_2$.

Amplification of the VDR coding region

The **coding region** of VDR was amplified by PCR with primers 31a (5′-GAGCACCCCTGGGCTCCA-3′) at position 72 in the 5′ untranslated region and 31b using 2 µl of the first strand mixture as template in a final volume of 100 µl (Fig 6). The PCR buffer used throughout, contained at final concentration: 50 mM KCl and 10 mM Tris/HCl pH 8.3; Perkin Elmer-Cetus, Norwalk, CT . A magnesium titration was carried out in order to optimise yield of the desired product (Fig. 7). Best amplification was obtained at 2.0 mM $MgCl_2$. The following cycling parameters were used:

1 cycle		
Denaturation	95°C	3 minutes
35 cycles		
Denaturation	95°C	1 minute
Annealing	61°C	1 minute 20 seconds
Extension	72°C	6 minutes.
1 cycle		
Extension	72°C	7 minutes

Each half of the full length coding region was further amplified using nested primers with 1 µl of the above PCR products as template for each 100 µl reaction.

For the **5′ half** these were 32a (5′-CCTGCCCCCTGCTCCTTC-3′) at position 94 to the 5′ untranslated region and 32b (5′-AGGTCAGCCAGGTGGGGC-3′) at position 814. The amplification was performed in standard buffer containing 2.5 mM $MgCl_2$. A magnesium titration was carried out to optimise yield of the desired

720 bp product. Best amplification was obtained at 2.5 mM $MgCl_2$ (Fig 7).

The following cycling parameters were used:

1 cycle

Denaturation	95°C	3 minutes

35 cycles

Denaturation	95°C	1 minute
Annealing	59°C	1 minute 20 seconds
Elongation	72°C	1 minute 40 seconds with 4 seconds extension per cycle.

1 cycle

Extension	72°C	7 minutes

For the **3′ half** primers 33a (5′-CCCAGCTCTCCATGCTGC-3′) at position 780 and 33b (5′-CCCAGGCACCGCACAGGC-3′) at position 1426 to the 3′ untranslated region were employed and the amplification was performed in standard buffer containing 1.5 mM $MgCl_2$. Excellent yields of the 646 bp product were obtained at this $MgCl_2$ concentration so no titration was performed. The following cycling parameters were used:

1 cycle

Denaturation	95°C	3 minutes

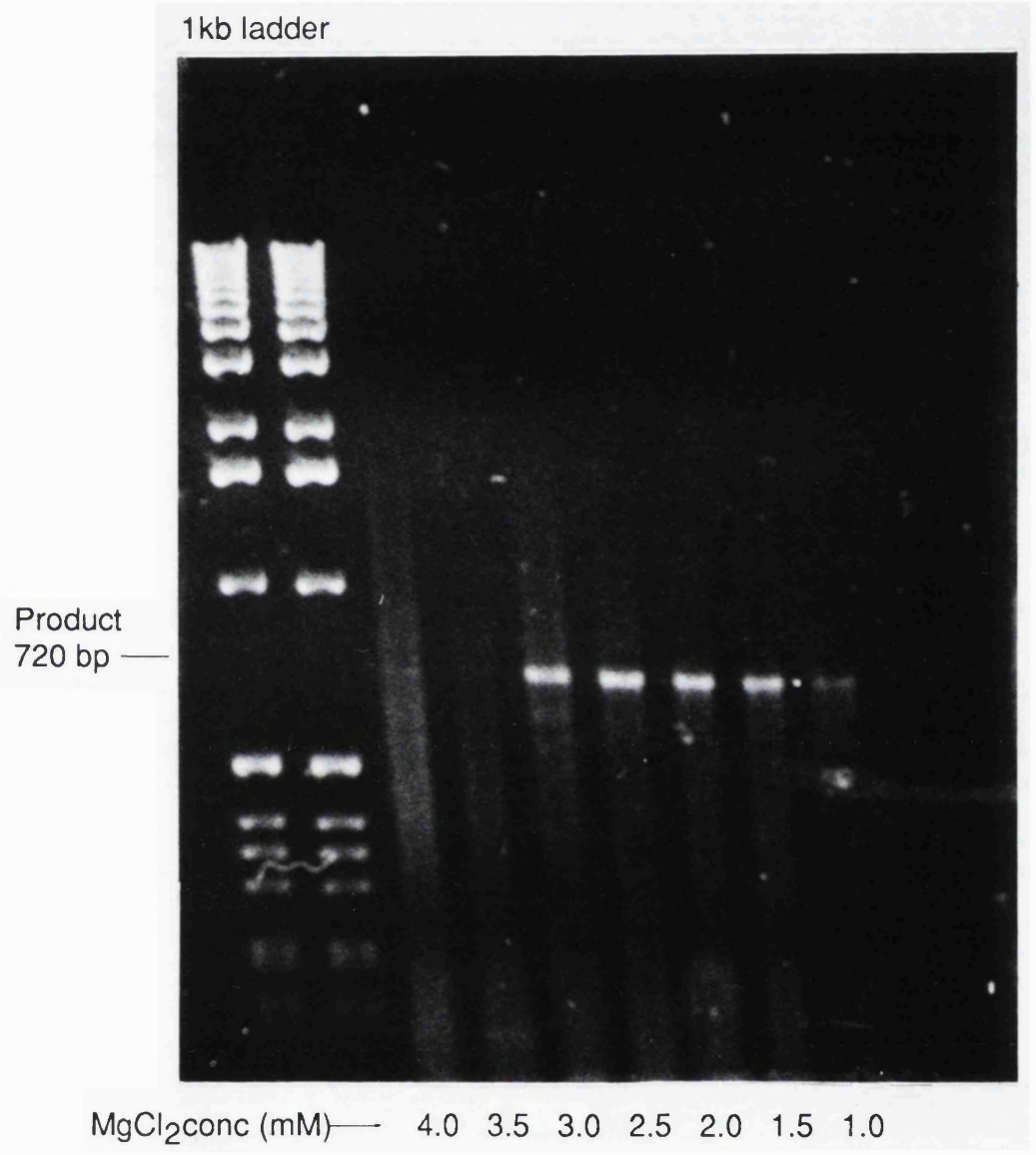
35 cycles

Denaturation	95°C	1 minute
Annealing	59°C	1 minute 20 seconds
Extension	72°C	1 minute 40 seconds.

1 cycle

Extension	72°C	7 minutes

Figure 7



Agarose gel electrophoresis of PCR products from a Mg^{2+} titration of amplification of the 5' coding region of the vitamin D receptor using cDNA from patient CC.
Primers 32a and 32b were used to amplify the region of interest.

Best amplification of 720 bp product occurred at 2.5 mM Mg^{2+}
DNA Ladder = 1 Kb

Primers were designed using the Oligo 2.0 programme (National Bioscience, Plymouth, MN). Pairs were selected for the highest and closest matching annealing temperatures. In order to achieve these aims, primers 33a and 32b overlapped by 2 bases. The primer concentration in all the above reactions was **0.16 μM**. PCR amplification was performed in a room separate from the cloning procedures and for each experiment numerous blank reactions were included. Contamination was detected during two experiments which necessitated that all reactions were repeated with fresh reagents.

Cloning and sequencing of amplified cDNA

Fill-in of PCR 3′ ends

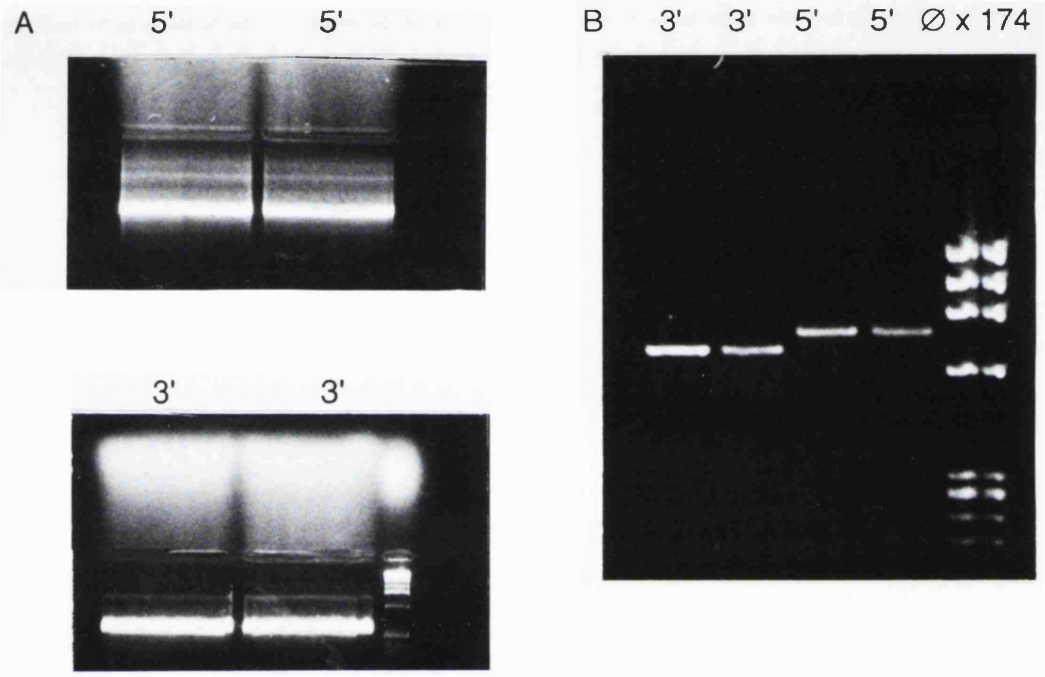
Following PCR amplification, 1 μl of Klenow fragment DNA polymerase (2 units/μl) was added to the PCR products and the reaction incubated for 30 minutes at 37°C to fill in ragged 3′ ends and prepare DNA for blunt-ended ligations. Material of interest was identified by agarose gel electrophoresis in low melting agarose and extracted by a freeze and squeeze method [147].

DNA Extraction

The band of interest was excised, its volume estimated and three volumes of Tris EDTA (TE) pH 8.0 were added. Agarose was melted by heating to 65°C for 5 minutes followed by rapid freezing on dry ice for 10 mins. The tube was thawed rapidly and tapped resulting in the agarose separating from solution. After a 15 minute spin at

13000 g the supernatant was removed and precipitated with 0.1 x volume of 3 M sodium acetate pH 5.2, and 2 x volume of absolute ethanol for 15 minutes at room temperature. DNA was pelleted by centrifugation, washed with 70% ethanol, dried briefly and dissolved in 10-15 µl of distilled water. An aliquot of each sample was run on a 1% agarose gel stained with ethidium bromide to check yield and purity (Fig 8).

Figure 8



JK PCR products using primers 32a & 32b (5') and 33a & 33b (3').

A. Products purified on a 1% LMP gel. Bands excised and purified by freeze and squeeze methods.

B. $^{1}/_{11}{}^{th}$ of product run on 1% agarose gel for quantification.

Result: Good clean amplification and purification

Cloning

Cloning procedures were performed as described [155] Reagents from (Boehringer Mannheim Corp. Indianapolis, IN) were used throughout unless stated to the contrary. The buffer set used is that tabulated below:

Table 2

	Final concentration of diluted buffer set in mmol/l (1:10 diluted set buffer)				
	A	**B**	**L**	**M**	**H**
Tris acetate	33				
Tris-HCl		10	10	10	50
Mg-acetate	10				
$MgCl_2$		5	10	10	10
K-acetate	66				
NaCl		100		50	100
Dithioerythritol (DTE)			1	1	1
Dithiothreitol (DTT)	0.5				
2-Mercaptoethanol		1			
pH at 37°C	7.9	8.0	7.5	7.5	7.5

Media and bacteriological plates

Two types of media were used.

Luria-Bertani Medium (LB)

For 1 Litre of Broth:

To 950 ml of deionised water the following were added:

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

The mixture was autoclaved for 20 minutes at 15 lb/sq. in.

Terrific Broth (TB)

For 1 Litre of Broth:

To 900 ml of deionised water, the following were added:

Bactotryptone	12 g
Bacto-yeast extract	24 g
Glycerol	4 ml

The mixture was dissolved and autoclaved for 20 minutes at 15 lb/sq in.

10X salts for TB

These were prepared separately from the medium as they are not heat stable.

KH_2PO_4	0.17 M
K_2HPO_4	0.72 M

Once dissolved the solution was filter sterilised and added to TB prior to use. Terrific broth was used for the majority of grow-ups.

SOC Medium

Bacto tryptone	2%
Bacto yeast extract	0.5%
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	20 mM

LB-Agar plates

Liquid LB was prepared as above but 15 g bacto-agar was added prior to autoclaving. The solution was cooled by stirring at room temperature. When temperature was below 50°C, ampicillin, 100 µg/ml; 5-bromo-4-chloro-3-indolyl-ß-D-galactosidase (X-gal), 80 mg/ml; isopropylthio-ß-D-galactosidase (IPTG) 20 mM, were added to give the final concentrations listed. X-Gal was prepared in dimetylformamide and IPTG in sterile distilled water. The addition of X-Gal and IPTG allowed blue/white colour selection from pBluescript II phagemids which contain the *lac* Z gene. IPTG serves as a gratuitous enhancer of *lac* Z gene expression and X-Gal as the substrate for the expressed protein. White colonies are positive because inserts interrupt the sequence of the *lac* Z gene.

MacConkey lactose indicator plates

These were used as an alternative to LB plates in some of the later experiments in an attempt to improve the colour selection between the positive and negative colonies. 94 g of MacConkey indicator medium (Difco) were added to 2 litres of distilled water and autoclaved for 20 minutes at 15 lb/sq in. When the medium had cooled below 50°C, ampicillin to a concentration of 100 µg/ml was added. MacConkey plates give pink/white selection with phagemids that contain the *lac* Z gene. White colonies are those that contain inserts.

Blunt-ended ligations

Amplified DNA was cloned into the *Sma*I site of pBluescript II SK$^-$. The vector was digested with *Sma*I at room temperature for 2 hours in buffer A and gel purified. Ligations were performed in a 10 µl volume overnight at 15°C. A 10:1 mole ratio of insert to vector was used and reactions were catalysed by T4 DNA ligase (20 Units) in a buffer containing 0.5 M Tris-HCl (pH 7.5), 70 mM $MgCl_2$, 10 mM DTT and 0.5 mM ATP. Initial transfections were into XL1-Blue E.coli but owing to problems with insert loss from these cells, later transfections were into TG1 E.coli.

Transformation of plasmid DNA into XL1-blue competent cells

Initial cloning was performed into XL1-blue strains of E.coli. 5 ng of pBluescript containing insert DNA or 0.1 ng of control plasmid pBR322 were mixed with 50 µl of freshly-thawed XL1-blue

competent cells containing 25 mM ß-mercaptoethanol in pre-chilled tubes. Cells were left on ice for 30 minutes, heat-shocked for 55 seconds at 42°C and placed on ice for 2 minutes. 0.9 ml of SOC medium pre-heated to 42°C was added to the cells and incubated at 37°C for 1 hour shaking at 225-250 rpm. To identify bacterial colonies that contained recombinant plasmids, cells were plated on LB-agar plates for blue/white colony selection. 50-200 µl of the transformation mixture was plated on to LB-agar plates containing 100 µg/ml ampicillin and X-Gal and IPTG. The plates were inverted and incubated at 37°C for 16 hours. To develop the blue colour, the plates were placed at 4°C.

Transfection of plasmid DNA into competent TG1 E.coli cells

TG1 strains of E.coli were utilised for the majority of cloning experiments owing to their robustness compared to XL1-blues. 100 µl of thawed competent TG1 cells were mixed with 1.7 µL of fresh 1:20 dilute β-mercaptoethanol and placed on ice for 10 minutes. 7-9 ng of DNA were added to the cells and left on ice for a further 30 minutes, mixing frequently by tapping the tubes. The mixture was heat-shocked for 2 minutes at 42°C and then placed on ice for 2 minutes. The mixture was warmed to room temperature. After the addition of 0.5 ml of SOC the tube was shaken for 1 hour at 37°C. 20-100 µl of the transformation mixture was spread onto LB bacteriological agar plates containing 100 µg/ml ampicillin at 37°C. The plates were incubated at 37°C for 16 hours.

Preparation of competent XL1 Blue, TG1 and DH5 alpha E.coli cells

10 µl of competent E.coli XL1 Blue, TG1 or DH5α cells were transferred into 10 ml L-broth. Cells were allowed to grow at 37°C with vigorous shaking for 16 hours. This subculture was used to inoculate 100 ml L-broth at 37°C. To monitor the growth of the culture at 37°C, optical density at 650 nm was determined every 30 minutes for 2 hours. Cells were allowed to grow until the OD reached 0.6. Cells were chilled on ice for 5 minutes and then centrifuged at 1500 g at 4 °C for 20 minutes. The supernatant was discarded and the cell pellets resuspended in ice-cold 0.1 M $MgCl_2$. Cells were respun as above and all the $MgCl_2$ was removed. The pellets were resuspended in 2 ml of ice cold $CaCl_2$, left on ice for 1 hour and refrigerated till next day. Glycerol was added to the suspension (15% of final volume), mixed and cells were aliquoted into 0.5 ml tubes which were frozen rapidly to -70°C.

Identification of positive clones

White colonies were picked from the agar plates, grown overnight in terrific broth, shaking at 37°C and plasmid DNA was extracted by alkaline lysis[191]. 1.5 ml of the overnight culture was centrifuged for 10 seconds, the supernatant was decanted and the tube vortexed to resuspend the cells completely. 300 µl of TENS was added, the tube vortexed and 150 µl of 3M sodium acetate pH 5.2 was added followed by vortexing. The cell debris was pelleted by centrifugation and the DNA in the supernatant precipitated with 0.9 ml of absolute ethanol. After centrifugation DNA was washed with 70% ethanol, dried and resuspended in 30 µl of distilled water.

TENS

10 mM Tris pH 8.0

1 mM EDTA pH8.0

0.1M NaOH

0.5% SDS

5 µl of DNA was cut to determine the presence of inserts and their orientation. For the 5′ halves *Bst*XI was used and for the 3′ halves *Nco*I plus *Pst*I were used. Figs 9 & 11 show schematically the size of digests dependent on whether the inserts were cloned in the sense or antisense orientations. Figs 10 & 12 show agarose gel electrophoresis of the digests.

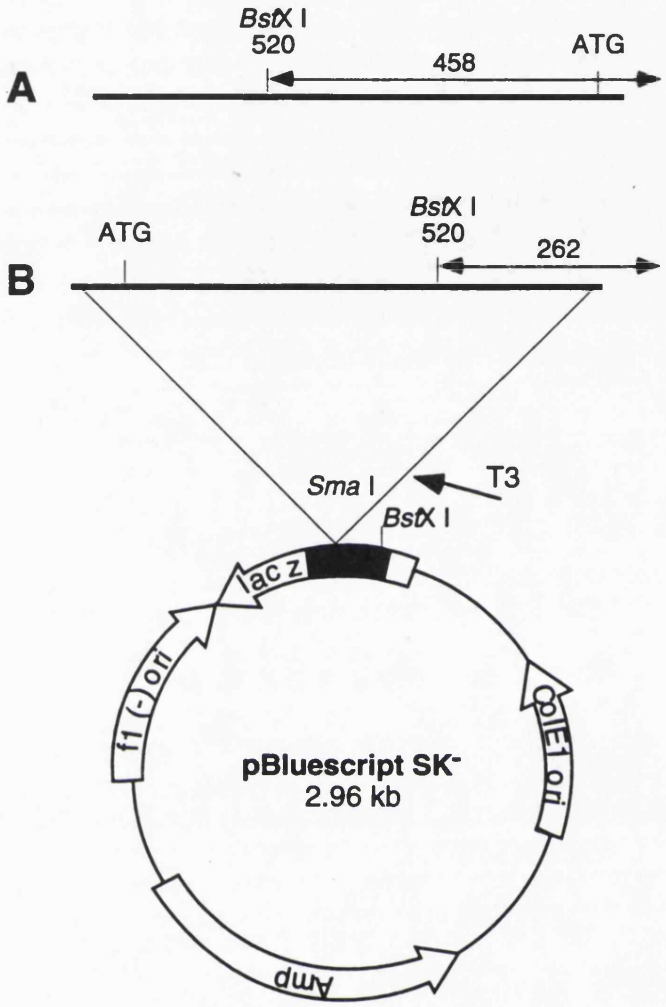
Sequencing

Positive bacterial clones were grown overnight in terrific broth. Single stranded DNA was rescued from pBluescript SK$^-$ phagemids using filamentous phage. The presence of product of appropriate size was confirmed by agarose gel electrophoresis. Sequencing was performed by the dideoxy-termination method in the Nucleic Acids Core facility at the Institute for Molecular Genetics, Baylor College of Medicine. The process was fully automated on an Applied Biosystems apparatus (Foster City CA) using M13 reverse primers tagged with fluorescent labels [167]. Sequence data was transferred to the Molecular Biology Information Resource computer network where it could be analysed using specific in-house programmes. These permitted sequence alignments of potentially mutant sequences with those of wild-type sequence. Matches could be

performed on single clones or sets of clones covering the same region. Any ambiguity in the sequence was checked with the raw, colour sequence print-outs.

All inserts were subcloned into pBluescript II KS$^-$ (in which the direction of the multiple cloning cassette is reversed with respect to pSK$^-$), to enable sequencing from the opposite orientation as only reverse primer was available for sequencing on the automated facility. Several clones from each amplification process were sequenced and the whole process was repeated starting with RT-PCR to provide unequivocal confirmation of any findings.

Figure 9

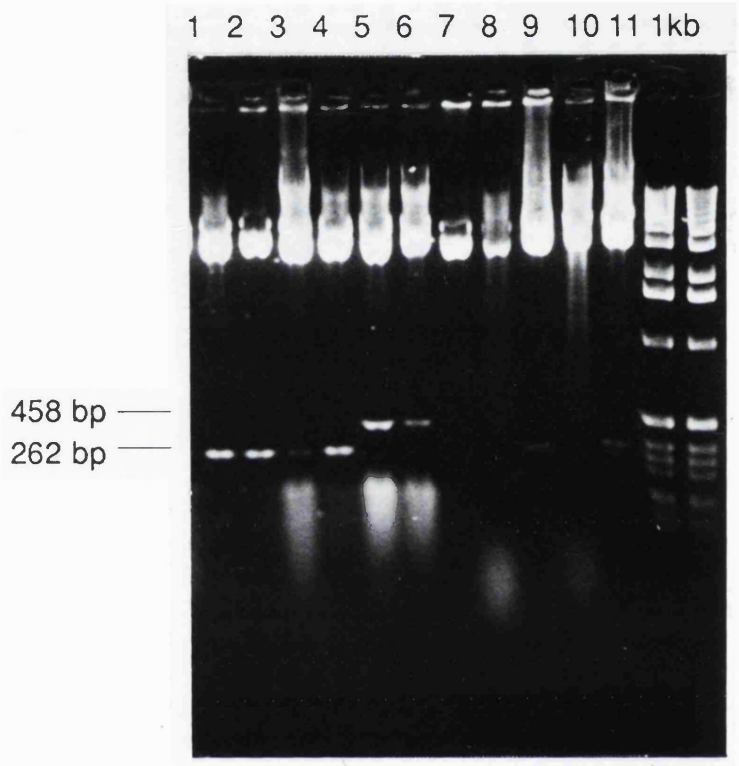


5'-half VDR cDNA cloned into the *Sma* I site of pSK$^-$. Orientation was determined by digest with *Bst*X I. Numbering of the restriction site corresponds to the published cDNA sequence.

(A) shows the insert cloned in a sense orientation with respect to the T3 and reverse primers. A fragment of 458 bp was obtained.

(B) shows the insert cloned in an antisense orientation with respect to the T3 and reverse primers. A 262 bp fragment was obtained.

Figure 10



Digests of cloned 5'-half VDR cDNA using the enzyme *Bst*XI to determine orientation of inserts.

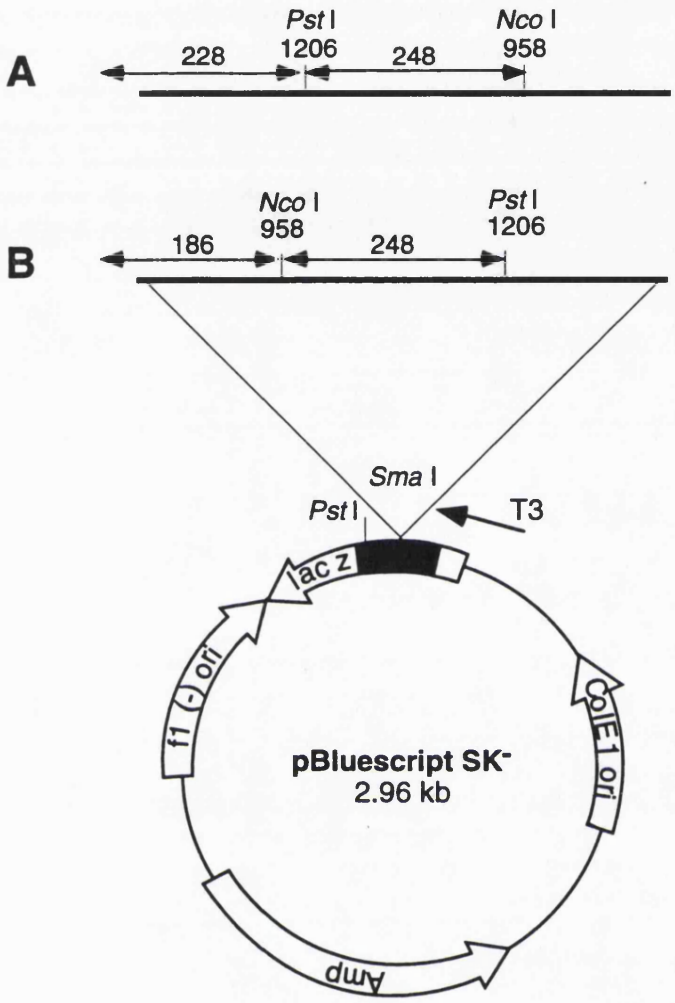
Positive clones are shown in lanes 1-6 and 9 & 11.

Those in lanes 5 and 6 are in the antisense orientation with respect to the T3 and reverse primers, while those in lanes 1,2 and 4 are in the sense orientation.

Gel = 1% agarose stained with ethidium bromide

DNA ladder = 1 kb

Figure 11

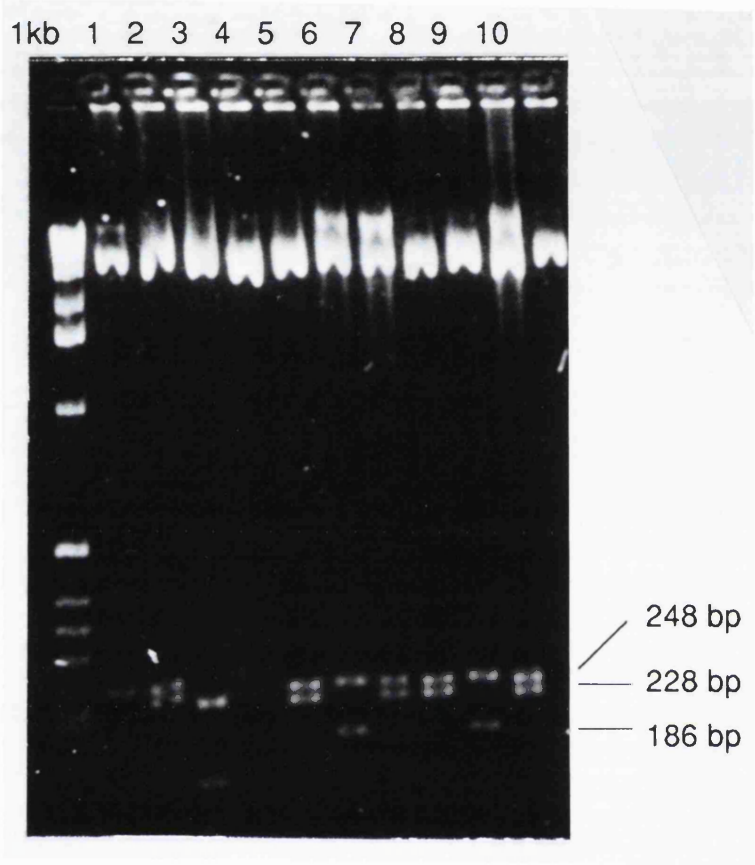


3'-half VDR cDNA cloned into the *Sma* I site of pSK$^-$. Orientation of insert was determined with *Pst* I and *Nco* I. Numbering of restriction sites corresponds to the published cDNA sequence.

(A) shows the insert in a sense orientation with respect to the T3 and reverse primers. Fragments of 228 & 248 bp were obtained following enzyme digest.

(B) shows the insert in an antisense orientation with respect to the T3 and reverse primers. Fragments of 186 & 248 bp were obtained following enzyme digest.

Figure 12



Digests of cloned 3'-half VDR cDNA using enzymes *Nco*I and *Pst*I to determine orientation of inserts. Positive clones are shown in lanes 1,2 and 5-10.

Inserts in lanes 2,5,7,8 and 10 are in the sense orientation with respect to the T3 and reverse primers.

Gel = 1% agarose stained with ethidium bromide

DNA Ladder = 1 Kb

Functional analysis of mutant receptors

The capacity of mutant receptors to stimulate transcription from vitamin D-responsive genes was examined in a cotransfection assay. Mutant VDR cDNAs were inserted into a mammalian expression vector and cotransfected into VDR-deficient CV-1 cells with a vitamin D-responsive reporter gene linked upstream of the gene for chloramphenicol acetyltransferase (CAT). CAT activity was measured following challenge with $1,25(OH)_2D_3$. The method will be detailed below.

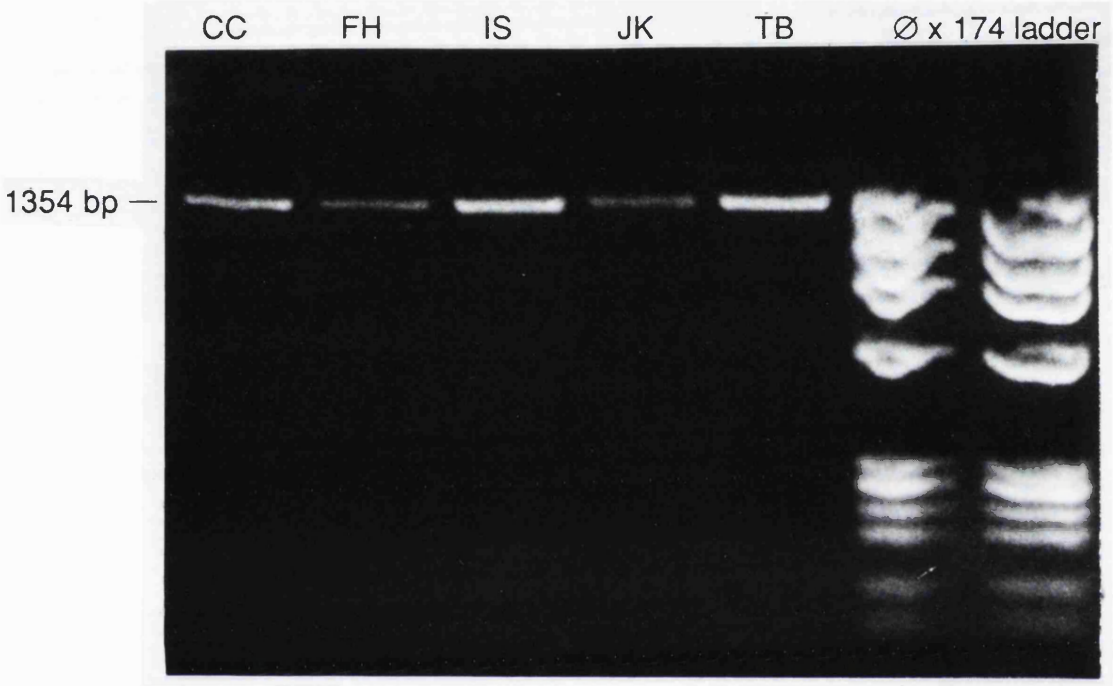
Receptor expression constructs

The mutant cDNAs were obtained by PCR amplification of reverse transcription products. The full length coding region was amplified using primers

31a (5′-GAGCACCCCTGGGCTCCA-3′) and

33b (5′-CCCAGGCACCGCACAGGC-3′) in standard buffer containing 1.5 mM $MgCl_2$ (Fig 13).

Figure 13



PCR amplification of the full-length VDR coding region, using primers 31a and 33b.

PCR products were first resolved by electrophoresis in low-melting agarose followed by purification using the freeze and squeeze method.

1/11th of the final product was examined for purity by agarose gel electrophoresis. This result is shown above.

A single 1354 bp band was demonstrated in samples from all 5 patients confirming the presence of VDR mRNA of expected size.

DNA ladder = ϕx 174 plasmid (Hae III digest)

The final volume of the reaction was 100 µl and contained 2 µl of template. The following cycling parameters were used:

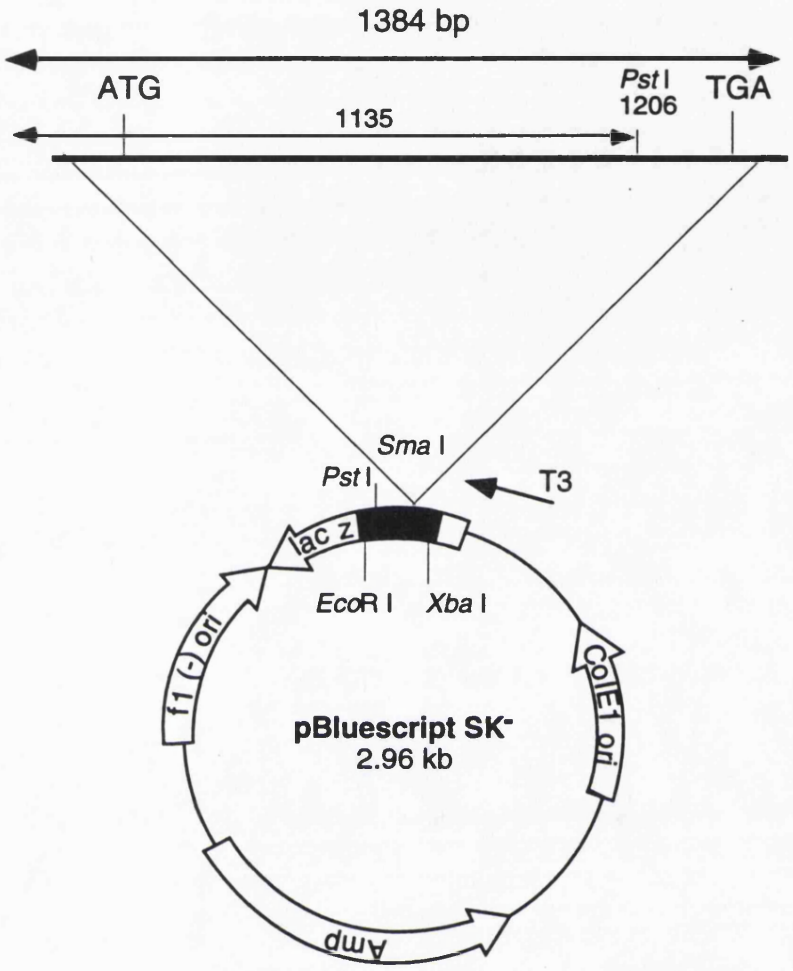
1 cycle		
Denaturation	95°C	3 minutes
35 cycles		
Denaturation	95°C	1 minute
Annealing	55°C	1 minute
Extension	72°C	1 minute
1 cycle		
Extension	72°C	7 minutes

PCR products were treated as before and cloned into a *Sma*I cut pBluescript II SK$^-$ vector. Orientation of inserts was determined by *Pst*I digest (Fig 14). Inserts were subcloned into the *Eco*RI and *Xba*I sites or the *Xba*I and *Xho*I sites of the mammalian expression vector, pSVK3 (Pharmacia LKB Biotechnology Inc. Piscataway, NJ) in the sense orientation with respect to the SV40 promoter.

Ligations in low-melting agarose

Owing to the excellent PCR yield it was decided to ligate using DNA contained in low melting agarose. The vector pSVK3 was cut with the same enzymes as the insert, gel purified and DNA extracted by the freeze and squeeze method. 1354 bp fragments were excised quickly from gel. All unstained agarose was trimmed and discarded. The gel piece was melted at 65°C for 5 minutes and then placed at 37°C. The volume was estimated (usually ~100 µl). Each ligation was performed in 0.5 ml tubes in a total volume of 5 µl at 15°C overnight.

Figure 14



Full length VDR cDNA cloned into the *Sma* I site of pSK$^-$ and cut out with *Eco*R I and *Xba* I. Orientation of the insert was determined by *Pst* I digest. This yielded a fragment of 1135 bp indicating that the insert was cloned in the antisense orientation with respect to the T3 primer.

Ligations

The tubes were kept at 37°C until all the reagents were added.

DNA in low melting agarose	3.5 µl
pSVK3 cut with appropriate enzymes	0.5 µl
10X ligase buffer	0.5 µl
T4 DNA ligase	0.5 µl
Total	5.0 µl

Transfection following ligation in low melting agarose

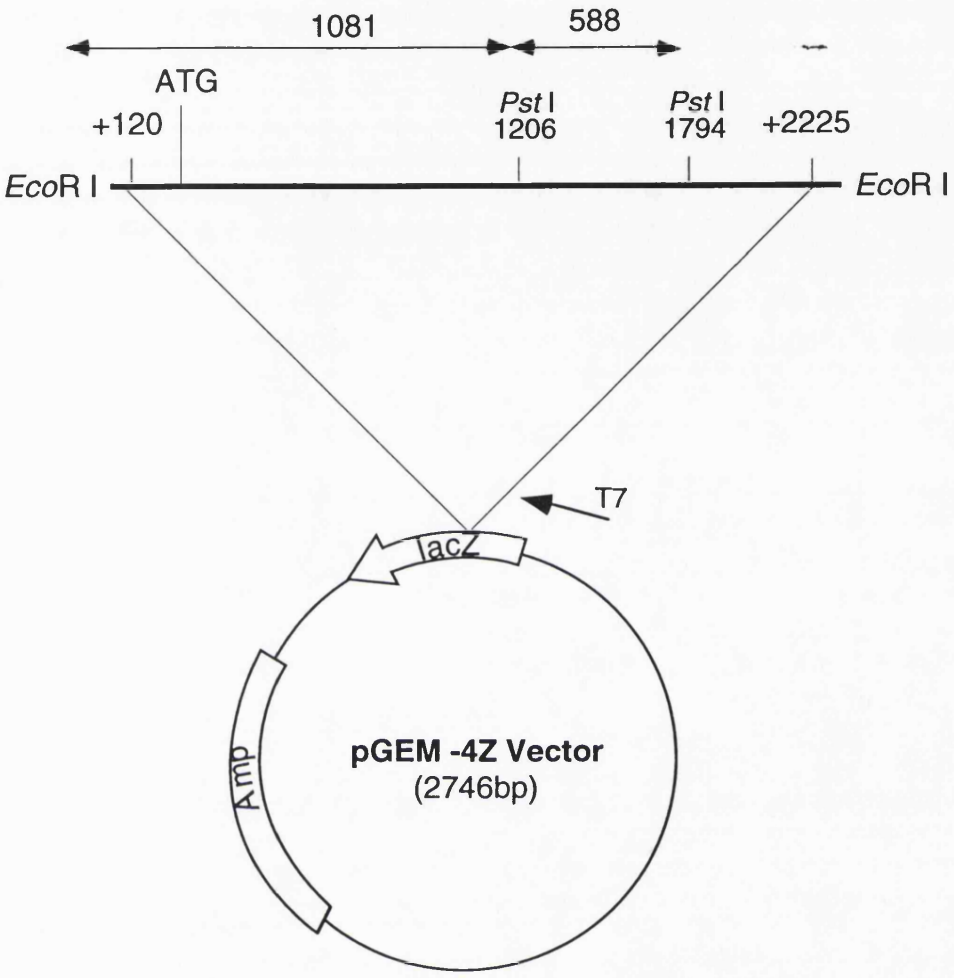
The ligations were diluted 4 fold with TE pH 8.0 to minimise the effects of agarose on ligation efficiency.

Ligation volume	5.0 µl
TE pH 8.0	15.0 µl

The mixture was heated to 65°C for 5 minutes to melt the agarose. To maintain fluidity it was placed at 37°C until an appropriate volume was extracted for transformation.

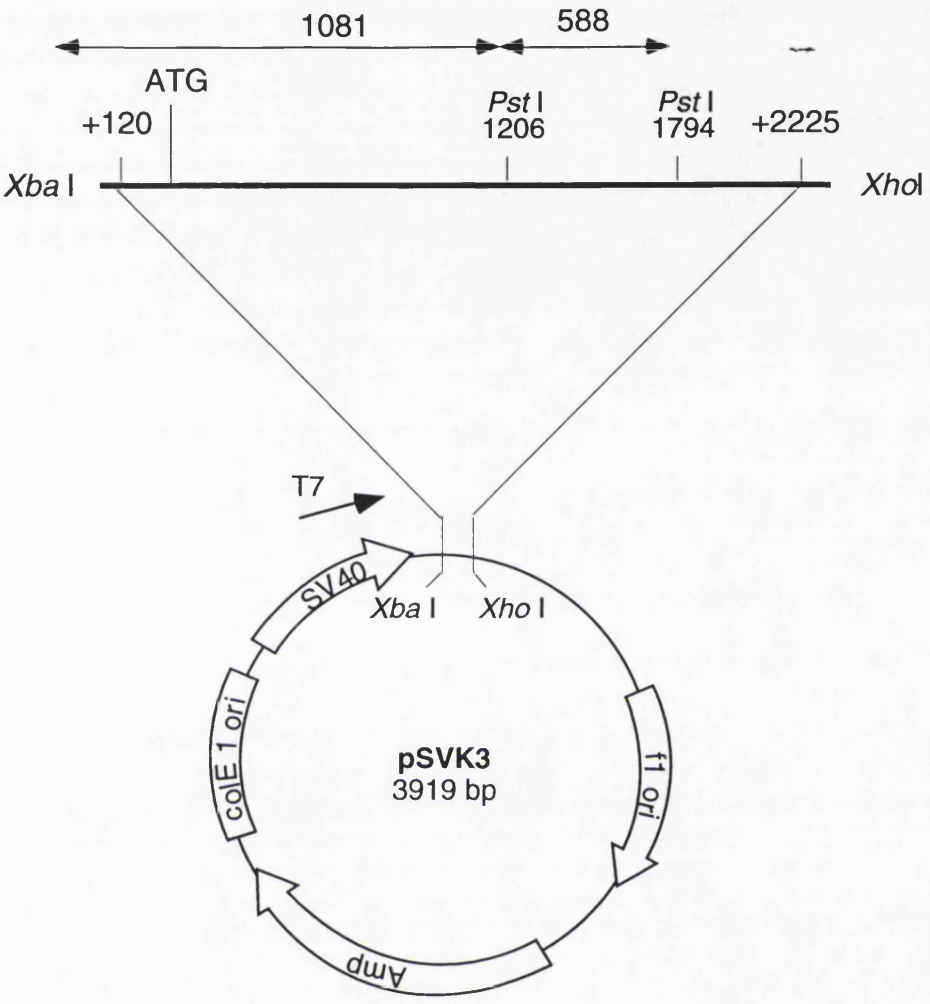
2 Kb wild-type VDR cDNA, previously cloned into *Eco*RI site of pGEM4 [4] was first subcloned into the *Eco*RI site of pKS$^-$ to expand the multiple cloning cassette and enable future directional cloning (Fig 15). Orientation was determined by *Pst*I digest. The insert was excised with *Xba*I and *Eco*RI , run on a low melting agarose gel. The band was excised melted and the DNA ligated in a sense orientation into the *Xba*I and *Xho*I sites of pSVK3 using an insert to vector ratio of 5:1, exactly as described above (Fig 16).

Figure 15



Wild type VDR cDNA cloned in an antisense orientation into the *Eco*R I site of pGEM-4Z. The construct contains the coding sequence plus approximately 700 bp of 3' untranslated region.

Figure 16



Subcloning of wild type VDR cDNA into the *Xba* I and *Xho* I sites of the expression vector pSVK3 in a sense orientation with respect to the T7 promoter. *Pst* I sites are shown to indicate how orientation was confirmed.

Competent DH5α E.coli cells were transfected with 6 ng of DNA. Transformants were plated on MacConkey lactose indicator plates and grown overnight at 37°C. The pSVK3 vector does not permit colour selection so colonies were picked, grown overnight in terrific broth and miniprepped. Positive clones were identified by digestion of plasmid DNA with *Eco*RI and *Pst*I. These clones were then grown in 50 ml of TB shaking overnight at 37°C. DNA for transfection was purified on anion exchange resin columns (Qiagen). This method was chosen for its speed and the excellent quality of DNA obtained.

Qiagen maxi preparation

The grow-up was centrifuged at 3000 g for 5 minutes and all the media was decanted. The pellet was resuspended in 10 ml of buffer P1 and 10 ml of buffer P2 was added. After gentle mixing the tube was allowed to stand for 5 minutes at room temperature. 10 ml of buffer P3 was added, mixed gently and incubated on ice for 20 minutes. The mixture was centrifuged at 15,000 g for 20 minutes at 4°C and the supernatant removed. The Qiagen-tip 500 was equilibrated with 10 ml of buffer QBT and the supernatant containing DNA was applied to the column. After 3 washes with 30 ml of buffer QC, DNA was eluted with 15 ml of buffer QF and precipitated with 0.7 volumes of isopropanol at room temperature. DNA was pelleted by centrifugation at 15,000 g for 30 minutes at 4°C, washed with 70% ethanol, air-dried and redissolved in 50 µl of distilled water. DNA concentration was estimated by optical density at 260 nM.

Qiagen Buffers

P1 50 mM Tris/HCl, 10 mM EDTA pH 8.0; 100 mg/ ml RNAse A

P2 200 mM NaOH; 1% SDS

P3 3.0 M KAc pH 5.5

QBT 750 mM NaCl; 50 mM MOPS; 15% ethanol; pH 7.0; 0.15% Triton X-100

QC 1.0 M NaCl; 50 mM MOPS; 15% ethanol; pH 7.0

QF 1.25 mM M NaCl; 50 mM Tris/HCl; 15% ethanol; pH 8.5

Reporter constructs

PCR was used to amplify 877 bp of the human osteocalcin gene promoter region starting from -833 bp 5′ and ending at +44 bp 3′, relative to the cap site of the gene (negative numbers indicating sequence extending 5′), Fig. 17. This region has been shown to contain a vitamin D response element between -510 bp and -483 bp [89, 134]. Primers were designed to incorporate restriction sites to facilitate cloning into the desired vector.

Primers R_1 (5′-CGACTGCAGGGTCAGGAGGAGAATCG-3′ with the *Pst*I site underlined) and R_2 (5′-CTGTCTAGATCTCGGGTGGCTGCGCTG-3′ with the *Xba*I site underlined) were used. The *Pst*I site is

endogenous to the osteocalcin promoter at -828 bp, whilst the *Xba*I site in primer R_2 is not native to the osteocalcin gene. With the addition of these overhangs the PCR product was 890 bp in size (Fig 18). Three bases 5′ of the restriction site were added to both primers to ensure sufficient sequence was available for future restriction enzyme cleavage.

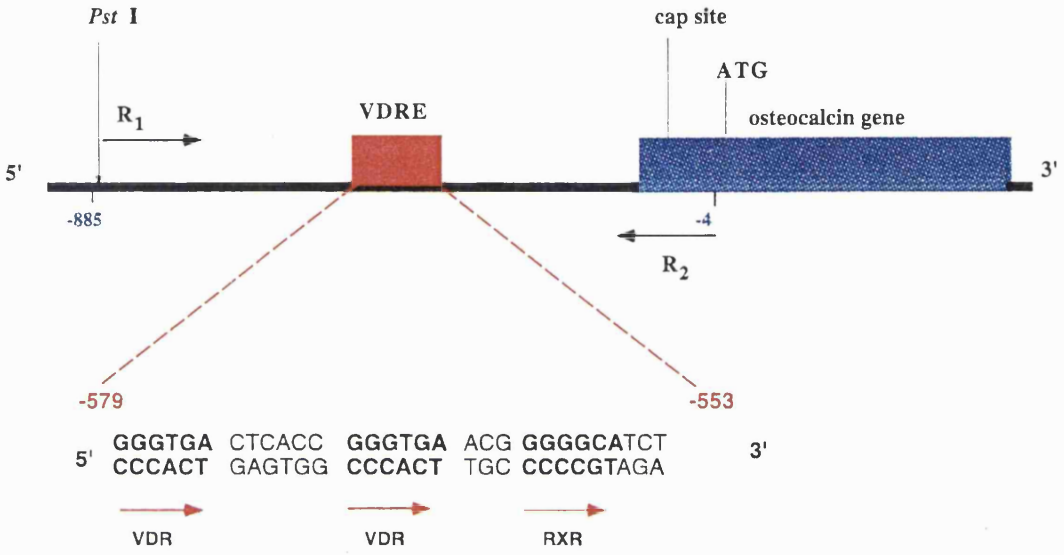
A magnesium chloride titration was performed to optimise PCR conditions. Best results were obtained in 2.0 mM $MgCl_2$.

PCR was performed in a standard buffer containing 2.0 mM $MgCl_2$ in a final volume of 100 µl containing 100 ng of genomic DNA as template (donated by Dr M. Schneider, Baylor College of Medicine). The following conditions were used:

1 cycle		
Denaturation	95°C	3 minutes
35 cycles		
Denaturation	95°C	1 minute
Annealing	58°C	1 minute 20 seconds
Extension	72°C	2 minutes
1 cycle		
Extension	72°C	7 minutes

PCR products were purified by the freeze/squeeze method. They and the chloramphenicol acetyltransferase vector, pCAT Basic (Promega Corp. Madison WI) were cut with the restriction enzymes, *Pst*I/*Xba*I in buffer H. This vector contains no promoter nor enhancer sequences.

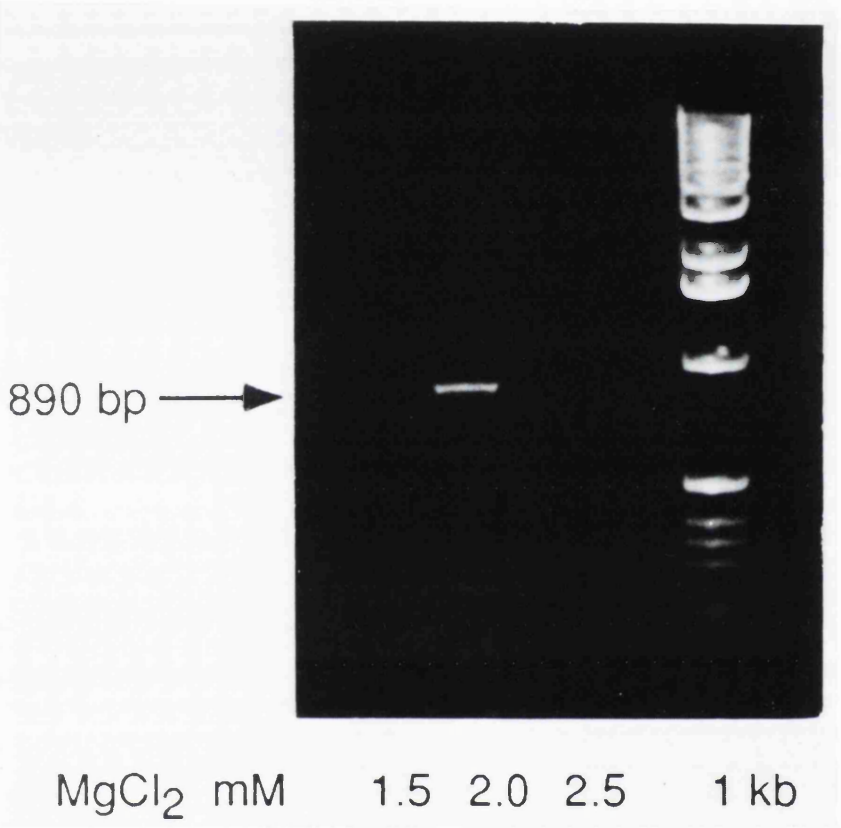
Figure 17



Amplification of the osteocalcin regulatory region with Primers R_1 & R_2

The vitamin D response element (VDRE) is shown with the directly repeated sequences highlighted. VDR is thought to bind as a homodimer to a consensus repeat with a spacing of six bases. In contrast heterodimer formation with RXR occurs to a repeat with spacing of three bases.

Figure 18



Agarose gel electrophoresis of PCR products from a Mg^{2+} titration of the osteocalcin promoter region.

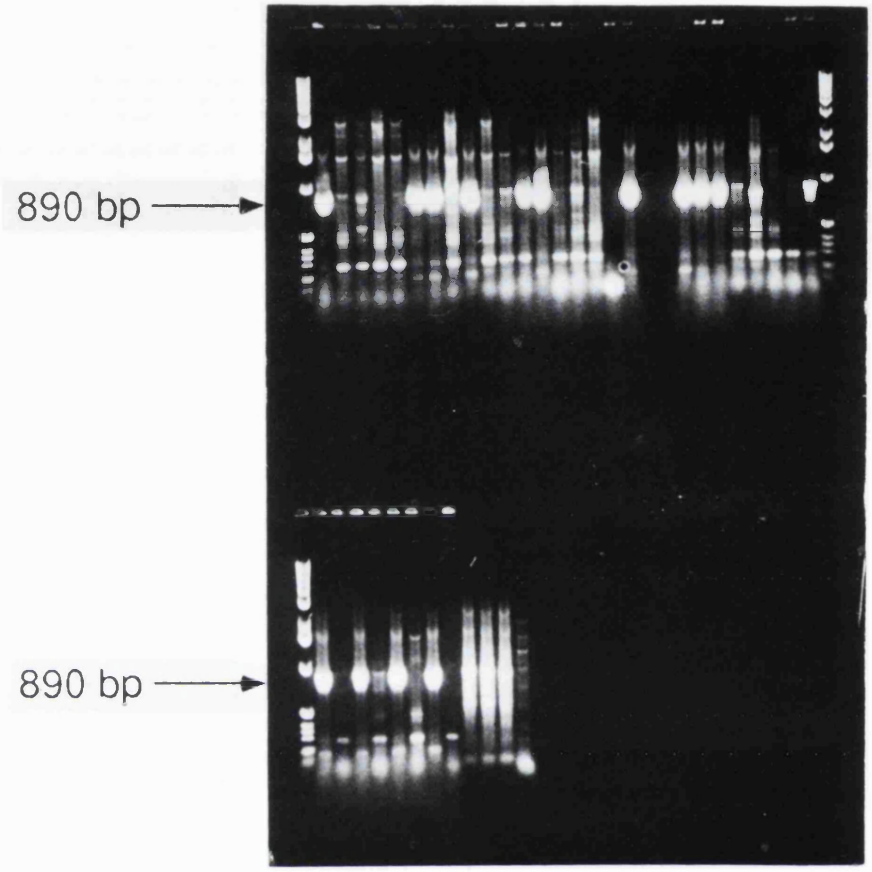
Primers R_1 and R_2 were used to amplify the region of interest.

Best amplification of 890 bp product occurred at 2.0 mM Mg^{2+}.

DNA Ladder = 1 Kb

pCAT basic was digested further with *Sma*I in buffer A to destroy the small fragment of the polylinker released by *Pst*I/*Xba*I cleavage. Ligation of the osteocalcin upstream region into the *Pst*I/*Xba*I cut pCAT basic was performed, in a volume of 10 µl, overnight at 15°C. A 10:1 mole ratio of insert to vector was used and reactions were catalysed by T4 DNA ligase (20 Units) in the presence of buffer containing 0.5 M Tris-HCl (pH 7.5), 70 mM $MgCl_2$, 10 mM DTT and 0.5 mM ATP (Boehringer Mannheim Corp). Thus the osteocalcin regulatory region was fused upstream of the chloramphenicol acetyltransferase gene. 11.7 ng of DNA was used for transformation of 100 µl of chemically competent DH5α E.coli cells. Cells were transfected as previously and plated on to MacConkey lactose indicator containing ampicillin. Positive clones were identified by PCR amplification of colonies. This method of screening was chosen as pCAT basic lacks any colour selection. 34 colonies were picked, placed in 20 µl of water and 10 µl of this mixture was transferred immediately to 1.5 ml of terrific broth containing ampicillin. 15 µl of PCR premixed PCR reagents containing buffers R_1 and R_2 were added to the 10 µl of water and amplification was performed exactly as for the osteocalcin regulatory region but with the addition of 5 minutes denaturation at 95°C prior to cycling. Fig 19 illustrates the PCR screening. One of the positive clones was grown overnight in 50 ml of terrific broth containing ampicillin. Confirmation of osteocalcin insert was obtained by miniprep and digest with *Eco*RI/*Pst*I which flank the insert in pCAT basic. DNA for transfection was purified on anion exchange resin columns (Qiagen).

Figure 19



PCR screening of colonies for inserts using primers R_1 and R_2.

Result: Many positive clones are present as demonstrated by intense product of 890 bp, which corresponds to the osteocalcin regulatory region.

Gel: 1% agarose stained with ethidium bromide

Ladder: 1 kb

Expression studies

Steroid receptor-deficient CV1 cells were grown in DMEM supplemented by 5% new-born calf serum. CV1 cells are Green Monkey kidney cells that are fibroblast-like in their morphology. 24 hours prior to transfection, cells were plated to a density of 1 x 10^6/100 mm plate. Cells were cotransfected with 5 µg of reporter plasmid and 1 µg of expression plasmid using the polybrene method [87]. Conditions for cotransfection of VDR cDNA with an osteocalcin reporter had been optimised previously in the same laboratory [134].

Polybrene method of transfection

The polybrene method [87] was chosen for CV-1 cells as it was in daily use at Baylor College, was simple and was known to work consistently with these cells.

15 ml polystyrene tubes equal in number to the plates to be transfected were set in rack. 1 ml of hepes buffered saline (HBS) was added to each 15 ml tube followed by DNA of choice. Polybrene was made up to 10 mg/ml in HBS; 5 µl was added to each 15 ml tube and left to sit for 15-30 minutes. Meanwhile plates were rinsed with Hanks medium and 10 ml of serum-free media was added to each plate. The DNA mixture was then pipetted on to the plates in a drop-wise manner and the plates were incubated for 3.5-5.0 hours at 37 °C, and an atmosphere containing 5% CO_2. The plates were rinsed with complete Hanks and the cells were shocked for 30 seconds with 5-10 ml of 25% glycerol in serum-free DMEM. Following two washes with complete Hanks, cells were cultured in

DMEM plus Nutridoma (Boehringer Mannheim Corp. Indianapolis, IN) supplemented by 10^{-7}M $1,25(OH)_2D_3$ or 0.1% ethanol (final concentration). Initial experiments were performed with new-born calf serum and subsequently stripped serum, but reporter activity was so high that no increase over baseline could be demonstrated by cotransfection with wild-type VDR cDNA. Nutridoma is a serum-substitute that contains no steroid hormones nor any growth factors that might stimulate transcription from the osteocalcin promoter region. In the case of IS, transfections were repeated three times using concentrations of $1,25(OH)_2D_3$ from 10^{-11} M to 10^{-6} M, to determine whether very high concentrations of $1,25(OH)_2D_3$ could overcome cellular resistance to the hormone.

Chloramphenicol Acetyltransferase (CAT) assays

The method of Gorman et al was used [54]. Plates were checked on the day for cell viability. Media was aspirated and cells were washed with PBS. 1 ml of TEN buffer was added to each plate and cells were scraped off the plates, transferred to a microfuge tube and pelleted at 4°C for 5 minutes. The supernatant was discarded and pellet resuspended in 0.25 M Tris pH 7.5. Cells were lysed by 3 freeze/thaw cycles in liquid nitrogen and a 37°C water bath. Debris was pelleted by centrifugation at 4°C for 5 minutes.

Protein concentration was determined by Bradford assay[15] on a microtitre plate in a Dynatech plate reader. Samples and controls were read in duplicate.

Initial CAT assays were performed with 50 µg of protein overnight, but the level of enzyme activity was found to be too high. The final experiments were carried out using 20 µg in an overnight incubation. Reactions were set-up by adding the appropriate volume of 0.25 M Tris pH 7.5 to bring the sample volume to 120 µl. 2 µl of 14C-chloramphenicol (0.2 µCi) and 40 µl of freshly made acetyl Co-A (working concentration 1.03 mM) were added, the solution vortexed and incubated overnight at 37°C. The reactions were terminated by the addition of 750 µl of ethyl acetate followed by centrifugation for 5 minutes. 600 µl of the upper phase was removed, dried and resuspended in 20 µl ethyl acetate. Samples were spotted on to thin layer chromatography plates and in tank containing 300 ml chloroform : methanol (95:5) for approximately 45 minutes or until approximately 5 cm from the top of the TLC plate. The plates were air dried and exposed overnight at room temperature with intensification on Kodak XAR5 film.

TEN

0.1M	**NaCl**	
1.0 mM	**EDTA**	**pH 8.0**
10 mM	**Tris**	**pH 8.0**

In-vitro transcription and translation

In one of the patients, in whom a stop codon was found in the steroid binding domain, it was important to confirm the presence of a truncated protein. As it was likely that such a protein would be highly unstable *in-vivo*, an *in-vitro* system was chosen.

The coupled transcription and translation rabbit reticulocyte lysate system (**TNT™** Promega) was used. Capped RNA transcripts are synthesised by T7 polymerase from DNA templates and then used to programme protein synthesis in rabbit reticulocyte lysates. ^{35}S-methionine-labelled products are separated on SDS-polyacrylamide gels.

TNT™ reagents were removed from storage at -70°C. The components of the kit were thawed and placed on ice. They were then combined as below:

TNT rabbit reticulocyte lysate	25 µl
TNT reaction buffer	2 µl
TNT T7 RNA polymerase	1 µl
Amino acid mixture	1 µl
^{35}S methionine at 10 mCi/ml	4 µl
RNasin Ribonuclease Inhibitor 40 units/µl	1 µl
DNA substrate 1 mg/ml	1 µl
nuclease-free H_2O	15 µl
Total	**50 µl**

cDNA from patients and wild-type in pSVK3 was used in the initial experiments. However wild-type and FH did not yield a detectable product. Pharmacia, the makers of pSVK3, suggested that the T7 promoter may have become corrupted during cloning. They suggested using any vector with a T7 promoter. In the case of FH, pBluescript II pKS$^-$ was used. An alternative wild-type, VDR cDNA clone was obtained from M. Haussler (Dept of Biochemistry, University of Arizona). This was known to have produced excellent

quantities of protein in their in-vitro experiments . This vector (pTZ18U; U.S. Biochemical Corp.) is shown in Fig 20.

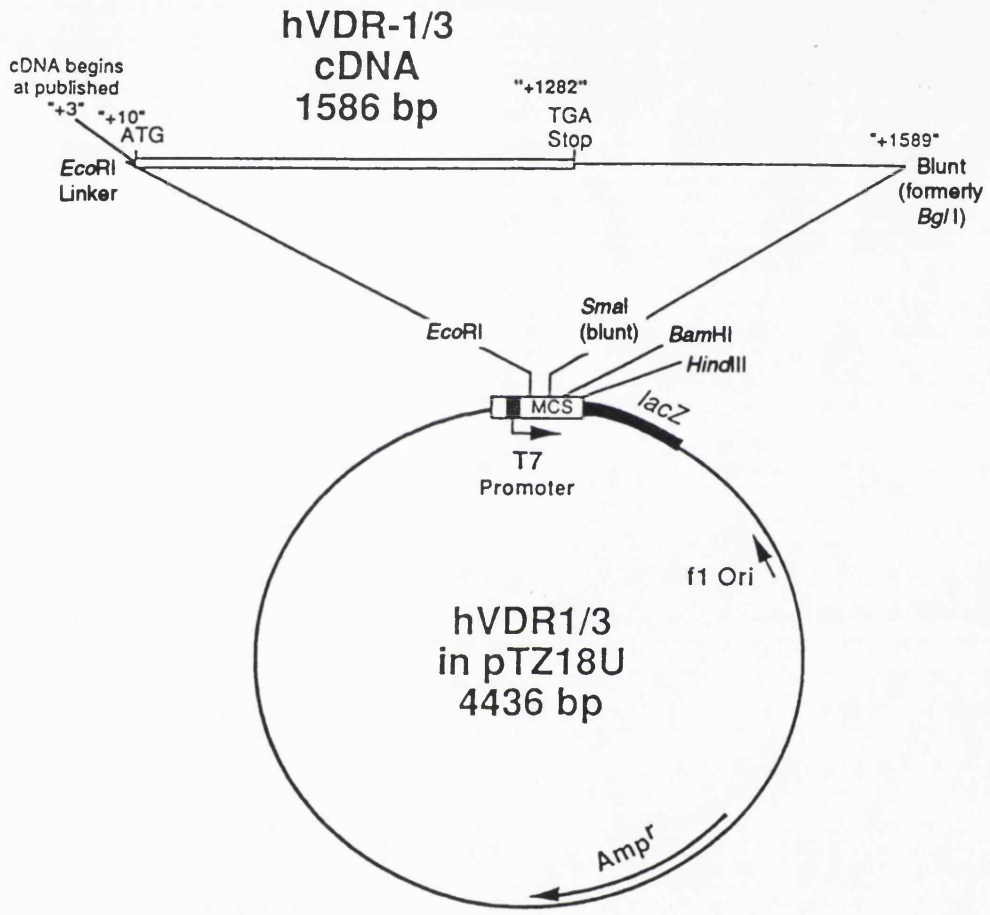
Positive control

Luciferase DNA was used as a positive control. It yields a 61 kDa protein. Reagents were combined as follows:

TNT rabbit reticulocyte lysate	25 µl
TNT reaction buffer	2 µl
TNT T7 RNA polymerase	1 µl
Amino acid mixture	1 µl
35S methionine at 10 mCi/ml	4 µl
RNasin Ribonuclease Inhibitor 40 units/µl	1 µl
Luciferase control DNA 0.5 mg/ml	2 µl
nuclease-free H_2O	14 µl
Total	**50 µl**

Reactions were incubated at 30°C for 2 hours and the products analysed by 10% SDS polyacrylamide gel electrophoresis (SDS/PAGE).

Figure 20



pTZ18U containing VDR cDNA (bases 118 to 1589) cloned in a sense orientation. The construct was used as a control in the coupled *in-vitro* transcription and translation experiments

This vector was kindly donated by Dr Mark Haussler, University of Arizona.

SDS polyacrylamide gel electrophoresis (SDS/PAGE) of proteins

Separating Gel 10%

A 10% gel was chosen as this was predicted to give the best resolution of VDR and controls. The gel was prepared by mixing the following reagents:

Acrylamide (Protogel) 30% stock	14.75 ml
1.5 M Tris pH 8.8	11.25 ml
0.5 M Tris pH 6.8	0.45 ml
20% SDS	0.225 ml
H2O	18.1 ml
Total	45 ml

The following catalysts were added just before pouring gel:

TEMED	18 µl
Ammonium Persulphate 10%	0.18 ml

Stacking Gel 3%

The following reagents mixed:

Acrylamide (Protogel) 30% stock	2 ml
0.5 M Tris pH 6.8	5 ml
SDS 20%	0.1 ml
H2O	12.69 ml

The following catalysts were added just before pouring gel:

TEMED	10 µl:
Ammonium Persulphate 10%	0.2 ml

Running Buffer (4 Litres)

The following reagents were dissolved in 4 litres of deionised water:

Glycine	115.2 g
Trizma Base	24 g
SDS (20%)	20 ml

4X SDS LOADING BUFFER:

200 mM Tris pH 6.8

8% SDS

0.4% Bromophenol blue

40% Glycerol

(400 mM Dithiothreitol (DTT) - added **just** before use)

SDS/PAGE Gel Electrophoresis

- The PAGE apparatus was washed in deionised H_2O and its glass plates were cleaned thoroughly with ethanol. The PAGE cell was filled with small layer of deionised water which facilitated the formation, and even layering of the gel.

- The PAGE cell was filled with the 10% separating gel and left to set for 30-60 minutes.

- The water layer was poured off. The stacking gel was poured carefully on to the separating gel using a Pasteur pipette. A comb was inserted and the gel left to set for 30-60 minutes.

Protein samples prepared:

The sample was mixed with the following reagents:

Sample	3 µl
4X SDS-loading buffer (1X final concentration.)	3 µl
H2O	6 µl
Total vol	12 µl

Protein molecular weight markers:

Low molecular weight rainbow marker (BRL)	25 µl
4 X SDS-loading buffer	8 µl
14C-labelled marker (Amersham)	25 µl
4 X SDS-loading buffer	8 µl

- 5 µl of sample and 32 µl of each marker were boiled for 5 mins to denature proteins. They were cooled rapidly on ice and loaded on the gel.

- The samples were electrophoresed for 5-7 hours at 100 volts and approximately 50 milliamps.

Autoradiography

The gel was removed from the plates and soaked for 30 minutes in a solution of 10% methanol/7% Acetic acid. The stacking gel was cut off and the separating gel soaked in Amplify (Amersham), a fluorographic reagent, for 30 minutes. The gel was dried at 60°C under a vacuum and put on Kodak X-Omatic overnight with enhancement.

Crystallographic modelling of VDR

Structural data on the nuclear hormone receptor DNA binding domain was available from NMR and x-ray crystallography. The 3-dimensional structures of GR [59], ER [159] and RXR [103] in complex with DNA had been resolved by NMR and that of GR by x-ray crystallography . More recently the 3D structure of ER bound to DNA has been defined [158]. The resolution of these structures was highly accurate. It was apparent from comparison of the structures that there was strong conservation in this region permitting modelling of other as yet undefined receptors eg VDR.

The co-ordinates of GR were available from the Brookhaven Protein Data Bank and these were utilised to generate computerised models of the DNA binding domain. The models were studied and manipulated using a Silicon Graphics computer and interactive computer graphics, FRODO [83]. The protein structure of GR was used as a scaffold into which the known mutations in VDR were introduced by FRODO. This programme finds the minimal energy conformation of any particular protein sequence. Some adjustments were made manually to find more reasonable stereochemical structures. The co-ordinates were saved and structures drawn using MOLSRIPT [96].

Chapter 3; Results

The key points that will be detailed in this chapter are outlined here.

- Patient phenotypes were confirmed by functional studies on receptors derived from skin fibroblasts. Of note is that VDR from all five patients failed to induce the enzyme 24-hydroxylase when challenged with $1,25(OH)_2D_3$.

- Sequencing of VDR cDNA confirmed the presence of a new start site in all patients and revealed independent missense mutations in four out of five patients.

- In one patient with definite vitamin D resistant rickets and clear lack of nuclear association by VDR, no mutation was found.

- Expression studies demonstrated that the mutations diminished or abolished the ability of the receptors to stimulate transcription of target genes.

- In one patient with a nonsense mutation, the truncated product was confirmed by in-vitro transcription and translation.

Receptor characterisation

Receptor characterisations demonstrated that the defect in all five patients with HVDRR, studied here was clearly one of VDR function.

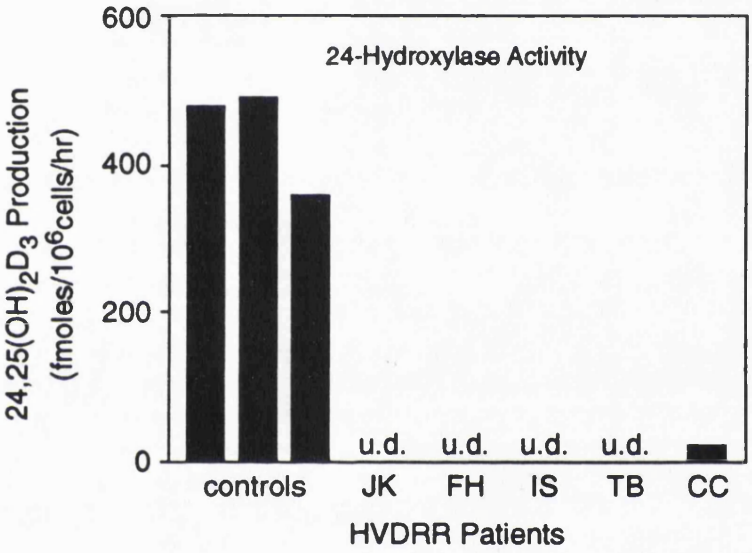
24-hydroxylase

The results in Fig 21 show the functional activity of VDR from patients and controls. It contrasts the induction of 24-hydroxylase activity in three control cell populations in response to $1,25(OH)_2D_3$, with the effectively absent response to $1,25(OH)_2D_3$ in the patients. Control fibroblasts produced 489 ± 119 fmoles/hr/10^6 cells of $24,25(OH)_2D_3$, whereas patients (FH, IS, JK & TB) showed no induction of 24-hydroxylase activity in the presence of 10^{-8} M $1,25(OH)_2D_3$ and patient CC produced only 17 fmoles/hr/10^6 cells. This indicated that fibroblasts from all patients were insensitive to $1,25(OH)_2D_3$, confirming the resistant phenotype.

Binding studies

VDR binding data was analysed on Scatchard plots. Figures 22 and 23 show results from control samples whereas Figures 24-27 show Scatchard plots from patients CC, IS, JK and TB. Maximal binding of $1,25(OH)_2D_3$ is represented as the x-axis intercept whereas the affinity of VDR for $1,25(OH)_2D_3$, K_d, is the slope of Scatchard plot. FH was studied initially by Kruse et al 1988 and Barsony et al 1989 who documented defective binding of $1,25(OH)_2D_3$ to VDR and defective nuclear uptake of VDR [7, 98].

Figure 21

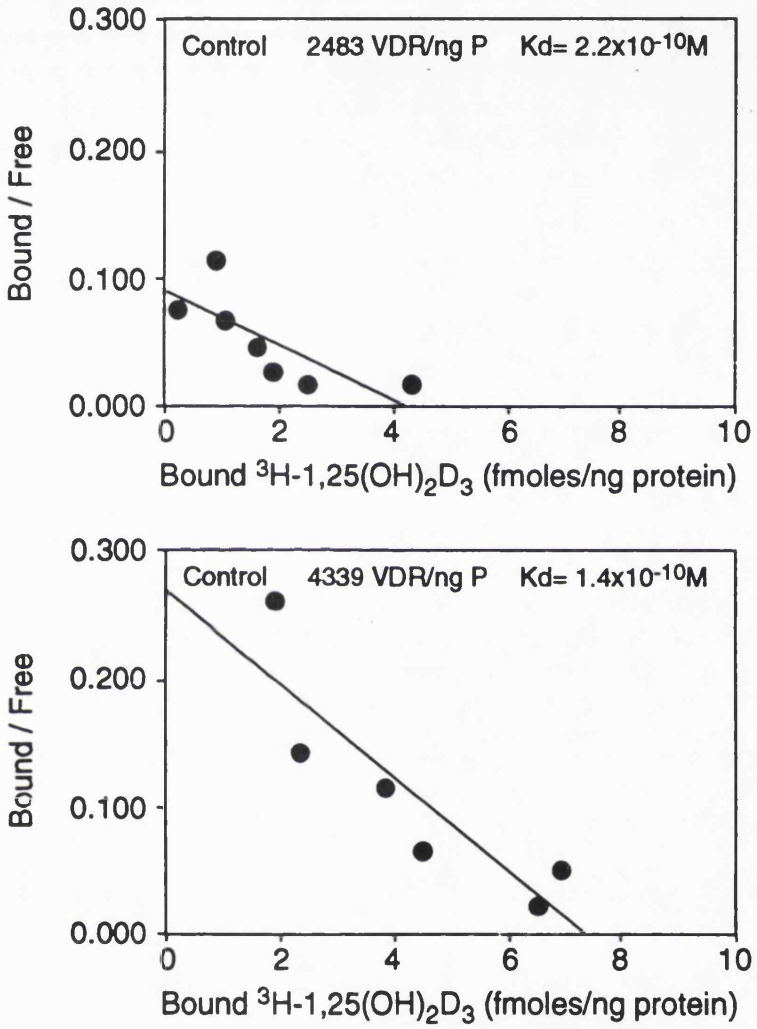


Induction of 24-hydroxylase activity in fibroblasts from patients and three controls after treatment with 10^{-8} M $1,25(OH)_2D_3$ for 24 hours.

Control cells demonstrated marked induction of 24-hydroxylase activity when challenged with $1,25(OH)_2D_3$.

There was no increase in 24-hydroxylase activity following challenge with $1,25(OH)_2D_3$ in patients JK, FH, IS and TB. Cells from patient CC demonstrated an extremely low level of activity.

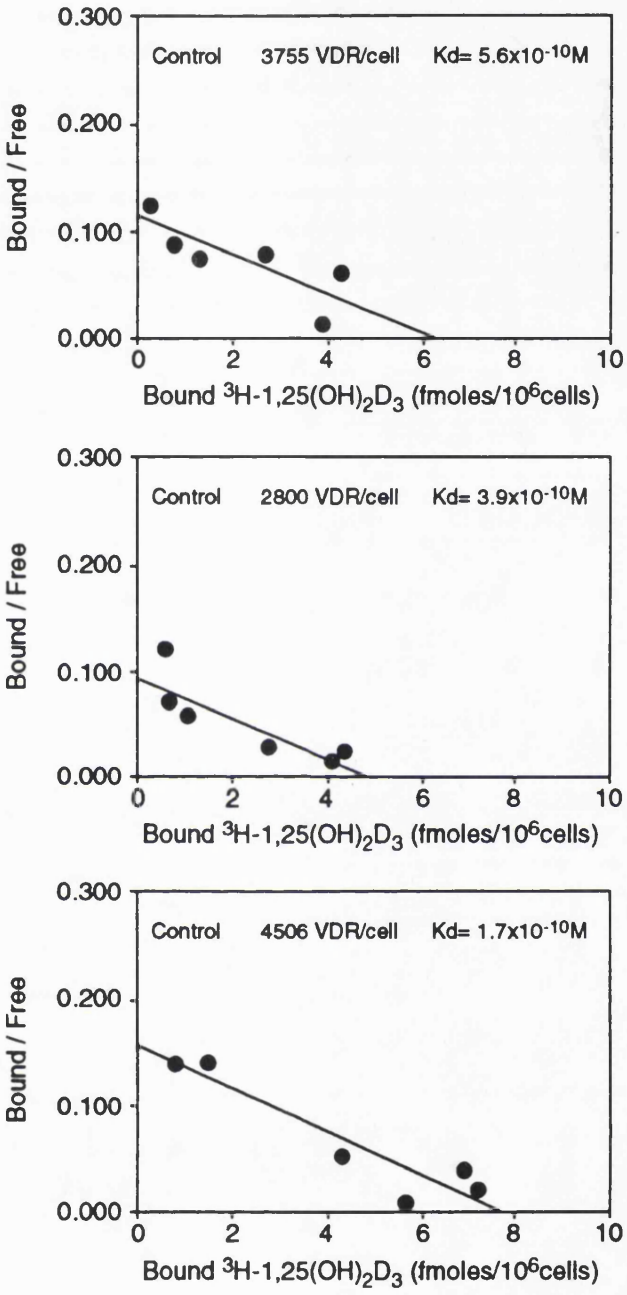
Figure 22



Scatchard plots of ^{3}H-1,25(OH)$_2$D$_3$ binding to VDR from control fibroblasts

Binding of ^{3}H-1,25(OH)$_2$D$_3$ to cytosolic VDR from skin fibroblasts, cultured from two control subjects. Results are shown as Scatchard plots in which maximal binding (fmoles/ng cytosolic protein) is represented by the intercept on the X-axis and binding affinity (K_d) by the slope of the plot.

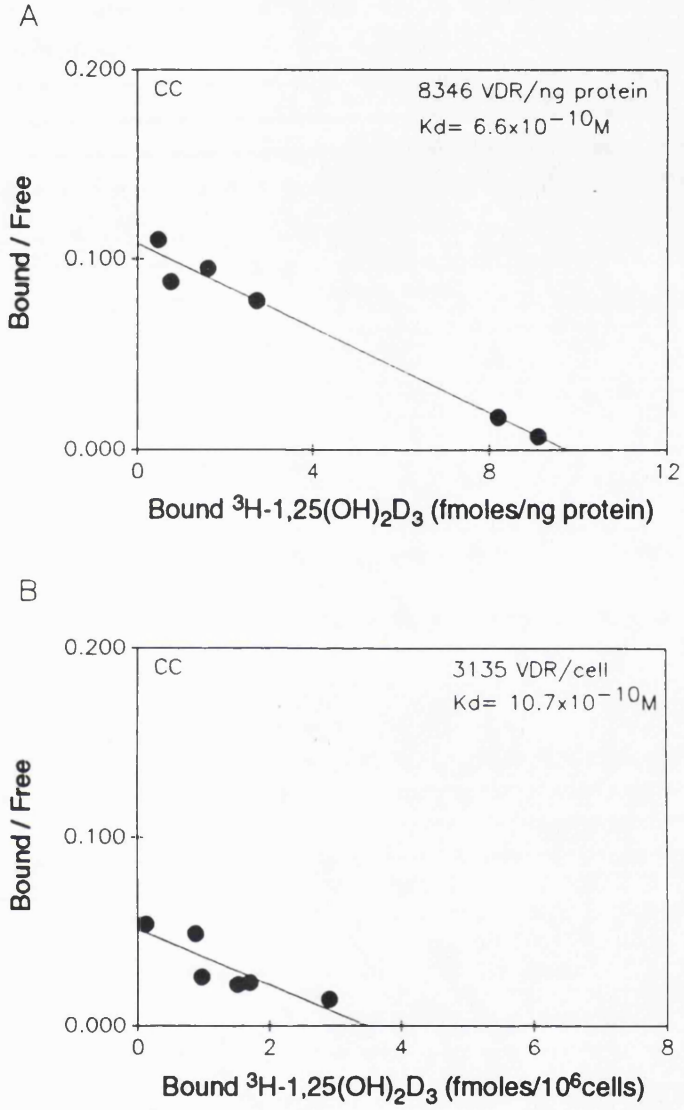
Figure 23



Scatchard plots of ^{3}H-1,25(OH)$_2$D$_3$ nuclear association in control fibroblasts

Nuclear association of ^{3}H-1,25(OH)$_2$D$_3$ in skin fibroblasts cultured from three control subjects. Results are shown as Scatchard plots in which maximal binding (fmoles/10^6 cells) is represented by the intercept on the X-axis and binding affinity (K_d) by the slope of the plot.

Figure 24

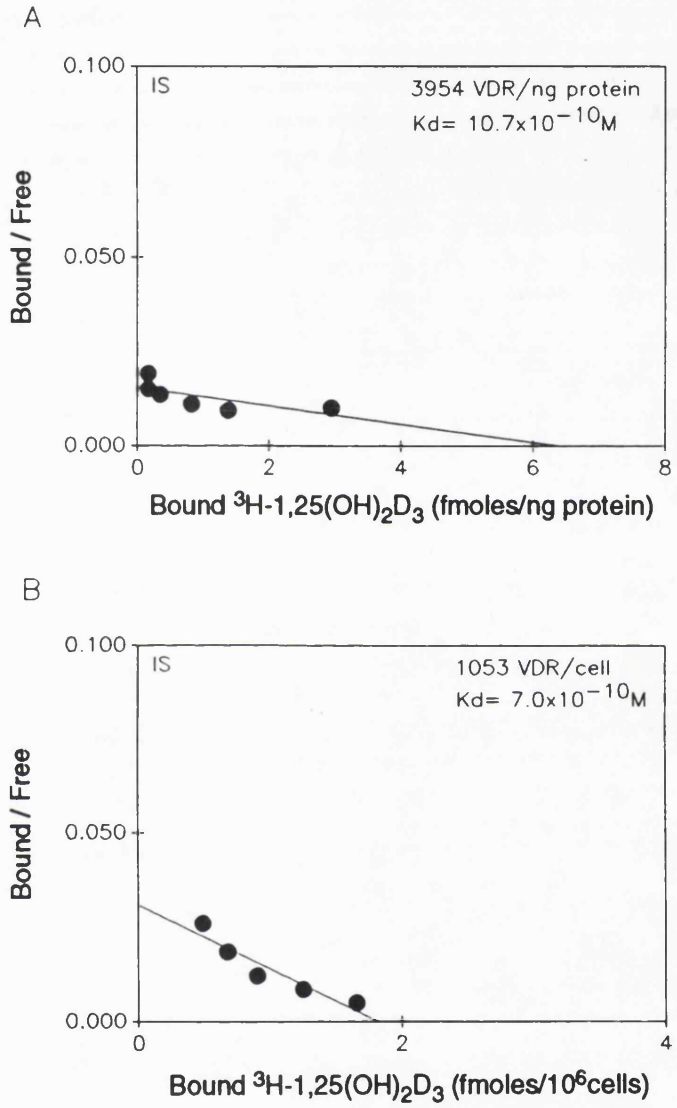


Scatchard plots of ^{3}H-1,25(OH)$_2$D$_3$ binding and nuclear association in fibroblasts from patient CC

(A) Binding of ^{3}H-1,25(OH)$_2$D$_3$ to cytosolic VDR. Maximal binding (fmoles/ng cytosolic protein) is represented by the intercept on the X-axis and binding affinity (K_d) by the slope of the plot.

(B) Nuclear association of ^{3}H-1,25(OH)$_2$D$_3$. Maximal binding (fmoles/10^6 cells) is represented by the intercept on the X-axis and binding affinity (K_d) by the slope of the plot.

Figure 25

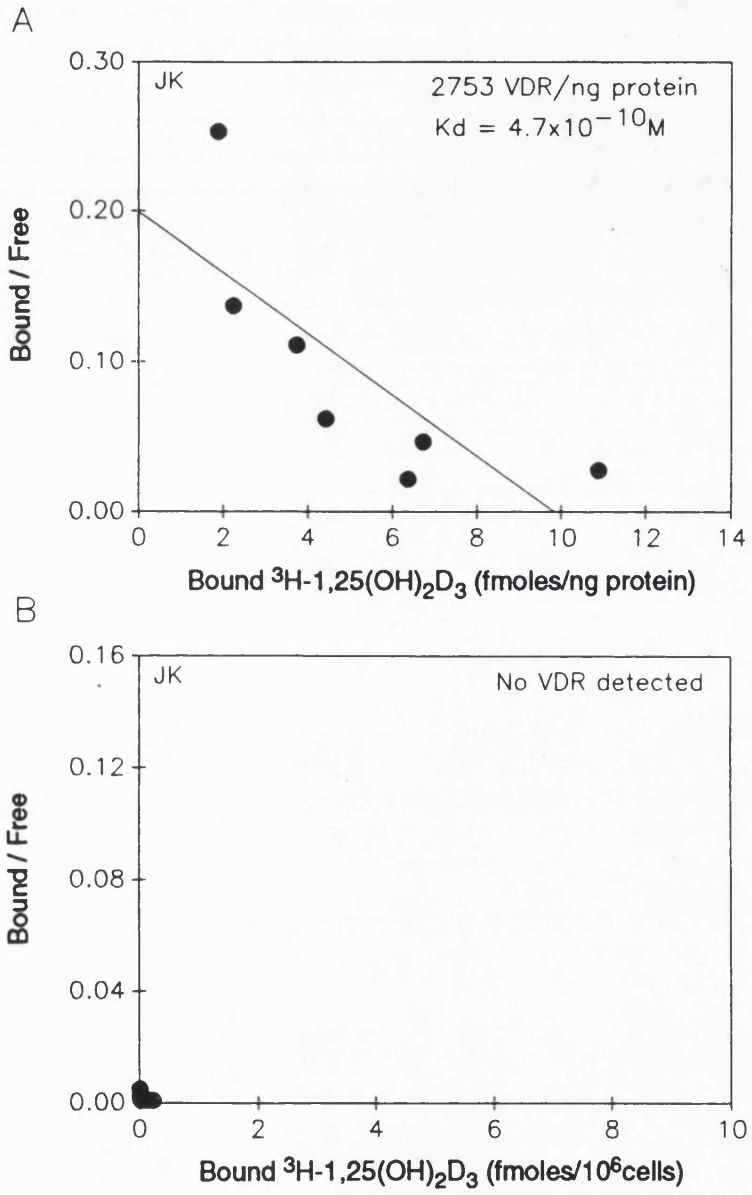


Scatchard plots of ^{3}H-1,25(OH)$_2$D$_3$ binding and nuclear association in fibroblasts from patient IS

(A) Binding of ^{3}H-1,25(OH)$_2$D$_3$ to cytosolic VDR. Maximal binding (fmoles/ng cytosolic protein) is represented by the intercept on the X-axis and binding affinity (K_d) by the slope of the plot.

(B) Nuclear association of ^{3}H-1,25(OH)$_2$D$_3$. Maximal binding (fmoles/10^6 cells) is represented by the intercept on the X-axis and binding affinity (K_d) by the slope of the plot.

Figure 26

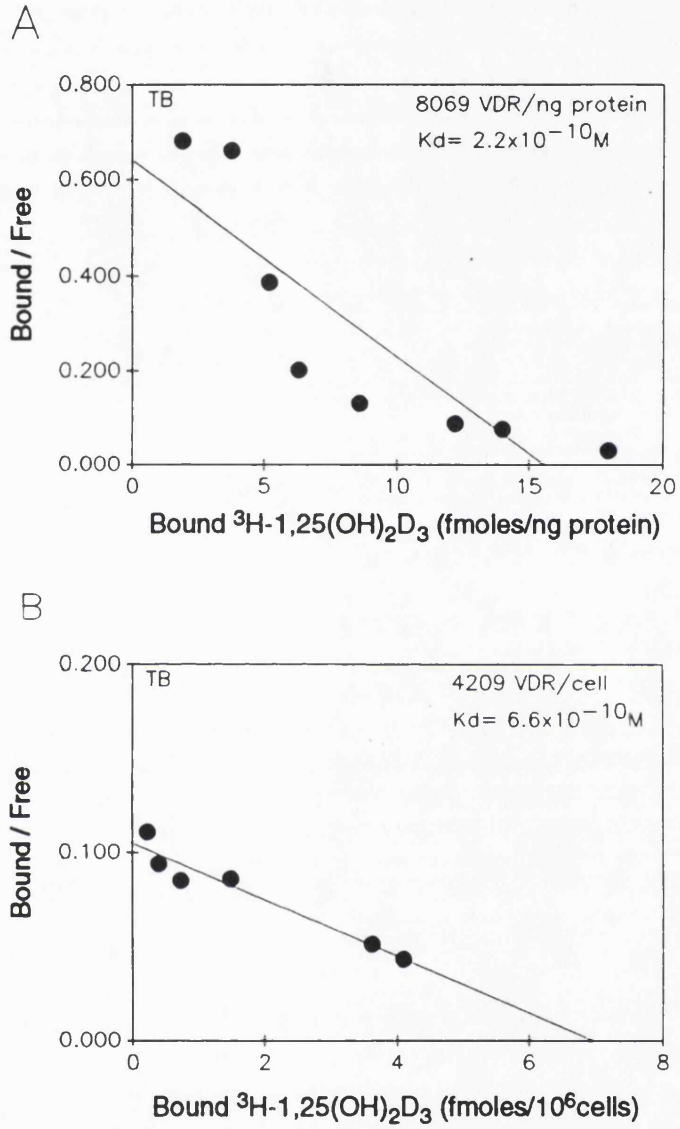


Scatchard plots of ^{3}H-1,25(OH)$_2$D$_3$ binding and nuclear association in fibroblasts from patient JK

(A) Binding of ^{3}H-1,25(OH)$_2$D$_3$ to cytosolic VDR. Maximal binding (fmoles/ng cytosolic protein) is represented by the intercept on the X-axis and binding affinity (K_d) by the slope of the plot.

(B) Nuclear association of ^{3}H-1,25(OH)$_2$D$_3$. Maximal binding (fmoles/10^6 cells) is represented by the intercept on the X-axis and binding affinity (K_d) by the slope of the plot.

Figure 27



Scatchard plots of ^{3}H-1,25(OH)$_2$D$_3$ binding and nuclear association in fibroblasts from patient TB

(A) Binding of ^{3}H-1,25(OH)$_2$D$_3$ to cytosolic VDR. Maximal binding (fmoles/ng cytosolic protein) is represented by the intercept on the X-axis and binding affinity (K_d) by the slope of the plot.

(B) Nuclear association of ^{3}H-1,25(OH)$_2$D$_3$. Maximal binding (fmoles/10^6 cells) is represented by the intercept on the X-axis and binding affinity (K_d) by the slope of the plot.

For clarity results from the binding studies are combined in the following table.

Table 3

	Cytosolic binding		Nuclear association	
Samples	**VDR/ng protein**	**K_d x10^{-10}M**	**VDR/cell**	**K_d x10^{-10}M**
Control I	**2483**	**2.2**	**3375**	**5.6**
Control II	**4339**	**1.4**	**4506**	**1.7**
Control III			**2800**	**3.9**
Mean	**3411**	**1.8**	**3560**	**3.73**
CC	8346	6.6	3135	10.7
I S	3954	10.7	1053	7.0
J K	2753	4.7	nil	nil
T B	8069	2.2	4209	6.6

FH (Listed as patient 11 by Barsony et al 1989) [7] All the available published data on patient FH is presented here.

$1,25(OH)_2D_3$ binding to receptor: Undetectable

$1,25(OH)_2D_3$ receptor uptake by nuclei: Undetectable

VDR were present in cells from patients CC, and TB as evidenced by binding of radiolabelled $1,25(OH)_2D_3$ to isolated cytosolic and nuclear protein. The numbers of VDR per ng cytosolic protein from patient CC (8346 VDR/ng protein) and patient TB (8069 VDR/ng protein) were higher than controls (3411 VDR/ng protein). The K_d values for cytosolic ligand binding in both patients were equivalent to controls. Nuclear association showed similar numbers of VDR/cell to the controls. The K_d values for nuclear association (patient CC; 10.7 10^{-10} M and patient TB; 6.6 10^{-10} M) were also comparable to those of control cells (3.7 10^{-10} M).

In fibroblasts from IS, VDR were present being detectable both by cytosolic binding assay and by nuclear association. The numbers of VDR were comparable to controls, ie. 3954/ng cytosolic protein. The K_d value for cytosolic ligand binding was approximately 10 fold higher than control (10.7 x 10^{-10} M) indicating reduced affinity for $1,25(OH)_2D_3$. K_d values for nuclear association were similar to controls (7.0 x 10^{-10} M) as were receptor numbers.

In JK fibroblasts maximal binding of $1,25(OH)_2D_3$ to VDR in cytosol was similar (2753 VDR/ng cytosolic protein) to controls (3411 VDR/ng cytosolic protein). Affinity of VDR for $1,25(OH)_2D_3$ was comparable to controls; 4.7 x10^{-10} M in J.K. cells vs. 1.8 x10^{-10} M in control cells.

JK fibroblasts however, revealed a major defect when studied by nuclear association assays. There was no detectable nuclear association of $1,25(OH)_2D_3$ (Fig 26), indicating that there were no VDR present in the nucleus [64].

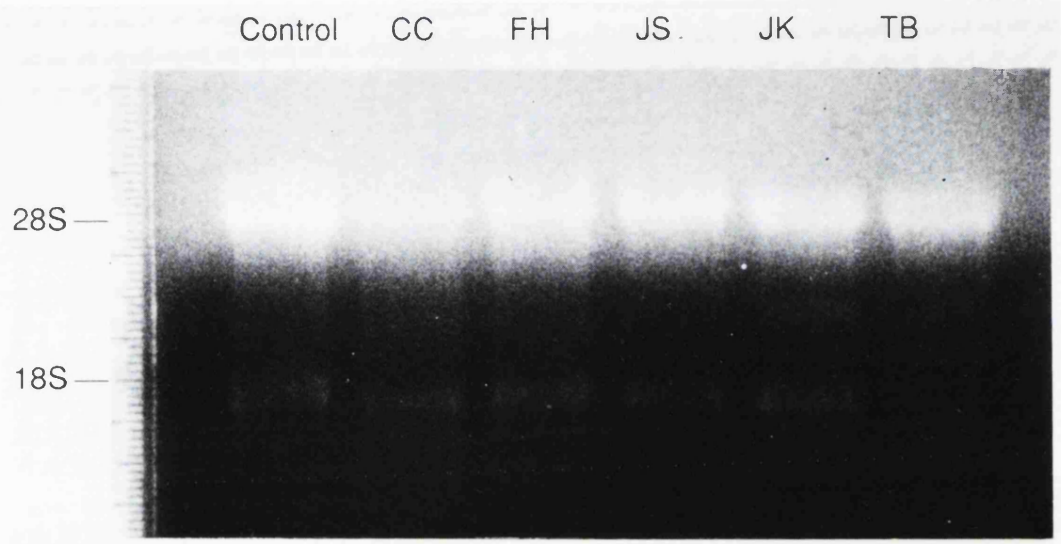
In the case of FH, binding studies were originally published by Kruse et al and Barsony et al [7, 98] and showed that in soluble extracts from whole cells, there was no saturable binding of (^{3}H) $1,25(OH)_2D_3$.

RNA isolation

Good quality RNA was purified from all patients as evidenced by 28 and 18S ribosomal bands. Loading of samples was equal as measured by ethidium bromide staining (Fig 28). Northern hybridisation confirmed the presence of 4.6 kb mRNA in the patients, but with very low abundance in FH (Fig 29). To increase sensitivity and specificity of Northern hybridisation, a riboprobe to the VDR steroid binding domain was used in Northern hybridisation against JK and control mRNA. A single band at 4.6 kb was identified, identical to that in control cells (Fig 30).

PCR analysis confirmed the presence of mRNA to the VDR coding region. Reverse transcription followed by amplification of the coding region (RT-PCR) from patients yielded a band of expected size (1353 bp) (Fig 31). Therefore although VDR mRNA may be present at low levels, it can be detected with relative ease by RT-PCR.

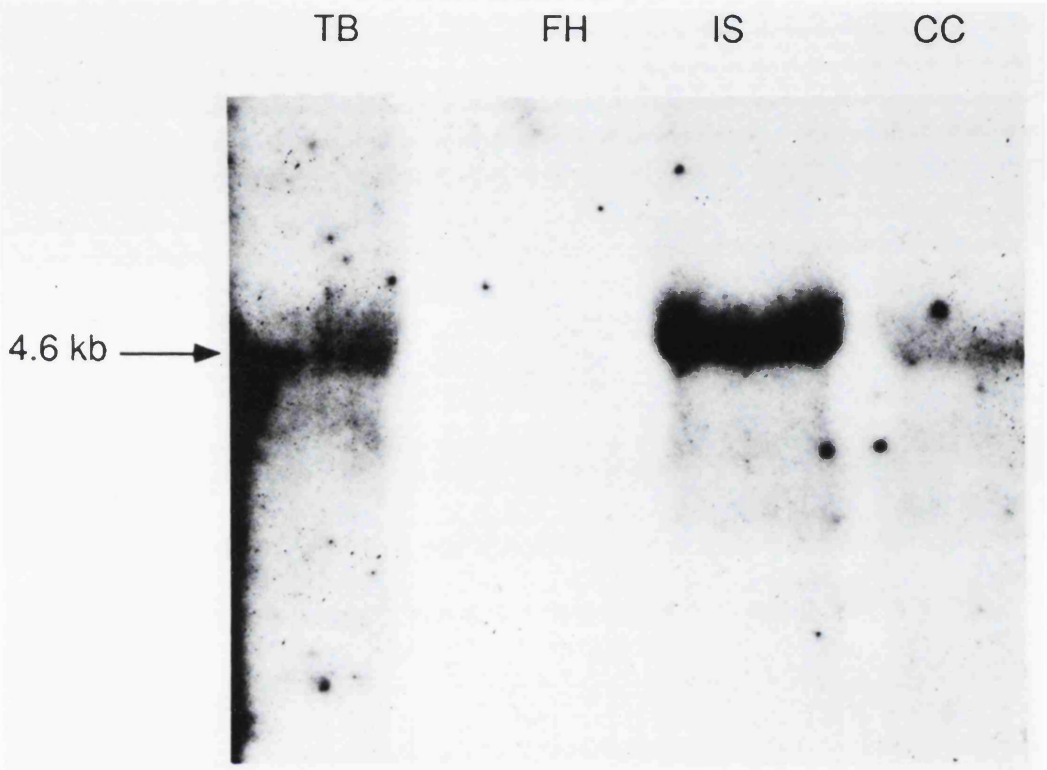
Figure 28



20µg of total RNA from control cells & patients' fibroblasts run on 1% agarose formaldehyde gel.

Ethidium bromide staining shows good quality RNA and equal loading as evidenced by the 28S and 18S ribosomal bands.

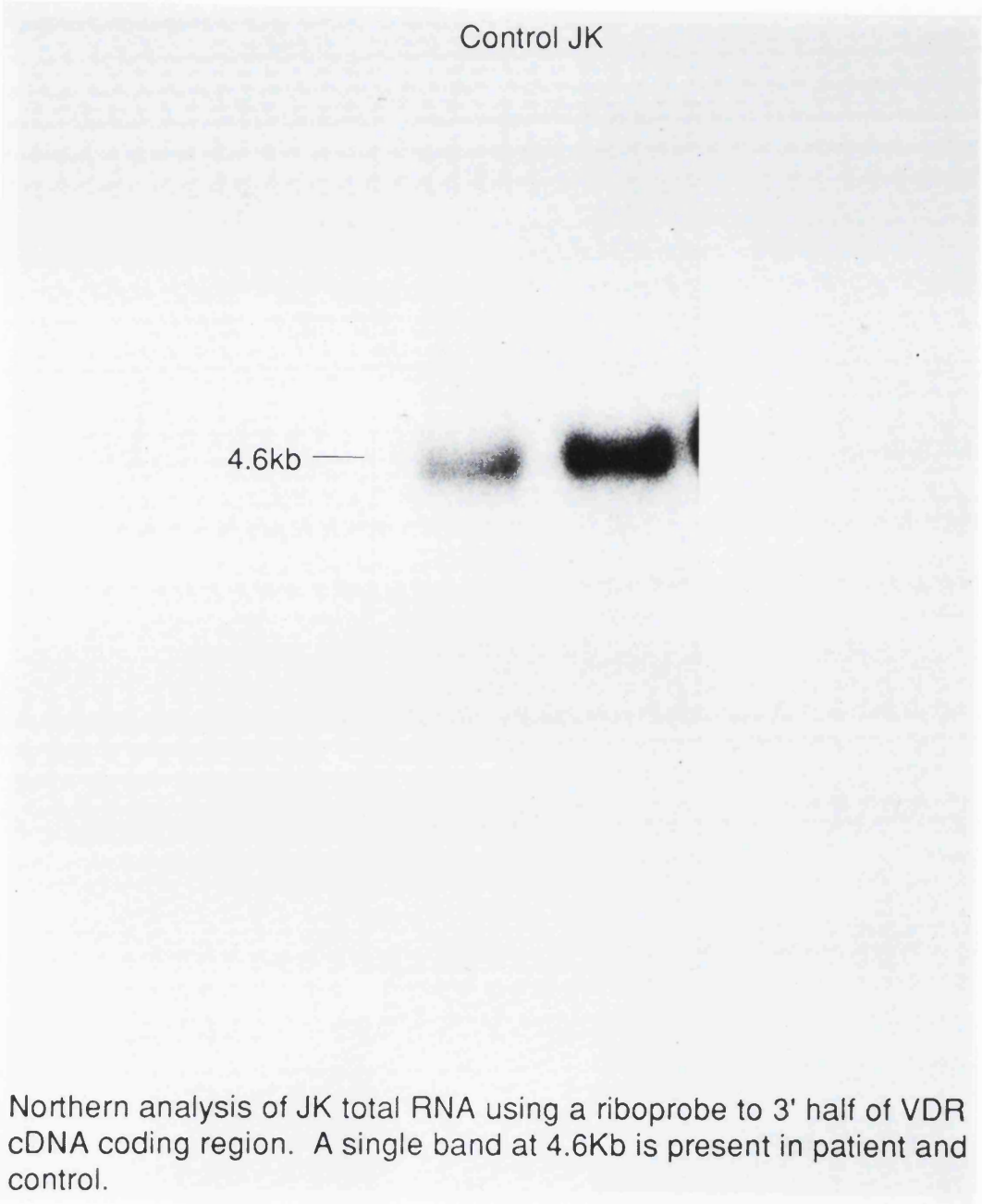
Figure 29



Northern blot of patients TB, HF, IS and CC using 2001 bp probe from +120 to 2120 bp of wild type VDR cDNA.

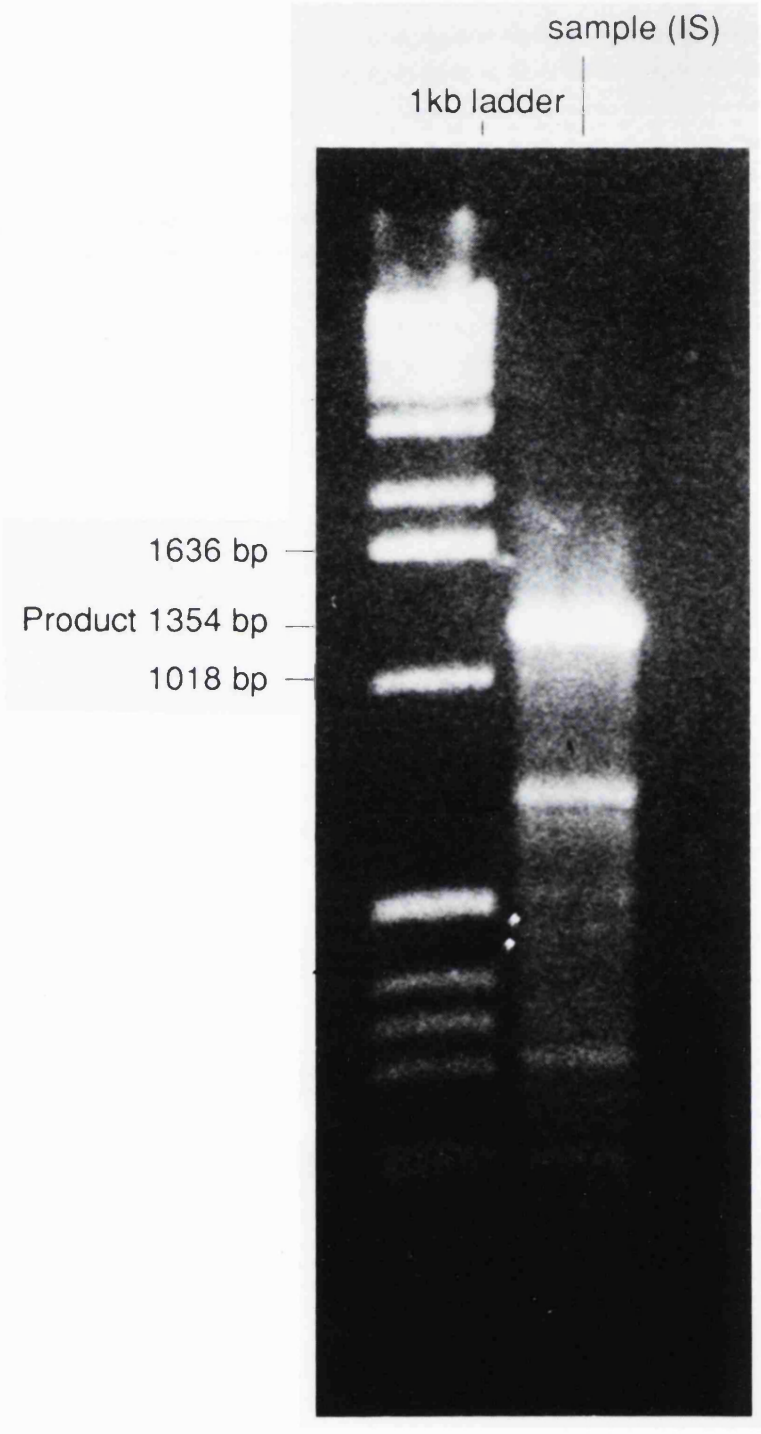
A single 4.6 kb band was present in the four patients but was of very low abundance in FH.

Figure 30



Northern analysis of JK total RNA using a riboprobe to 3' half of VDR cDNA coding region. A single band at 4.6Kb is present in patient and control.

Figure 31



Full length PCR amplification of IS cDNA coding region using primers 31a + 33b. Good yield of 1354 bp product.

Gel = 1% agarose stained with ethidium bromide

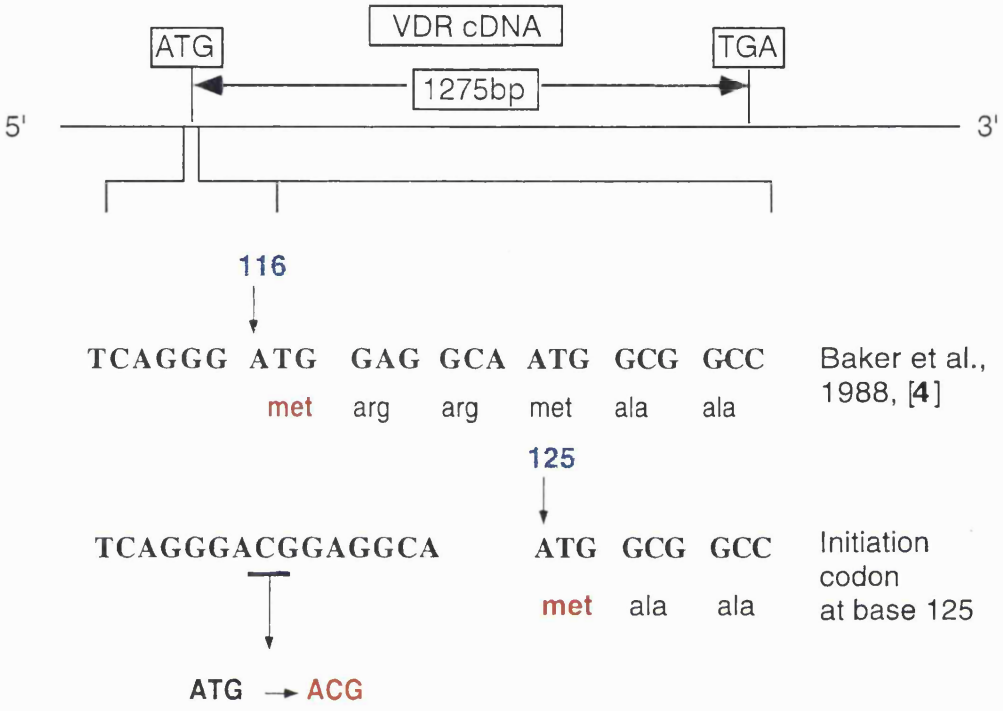
Ladder = 1kb

Sequencing

Initiation site

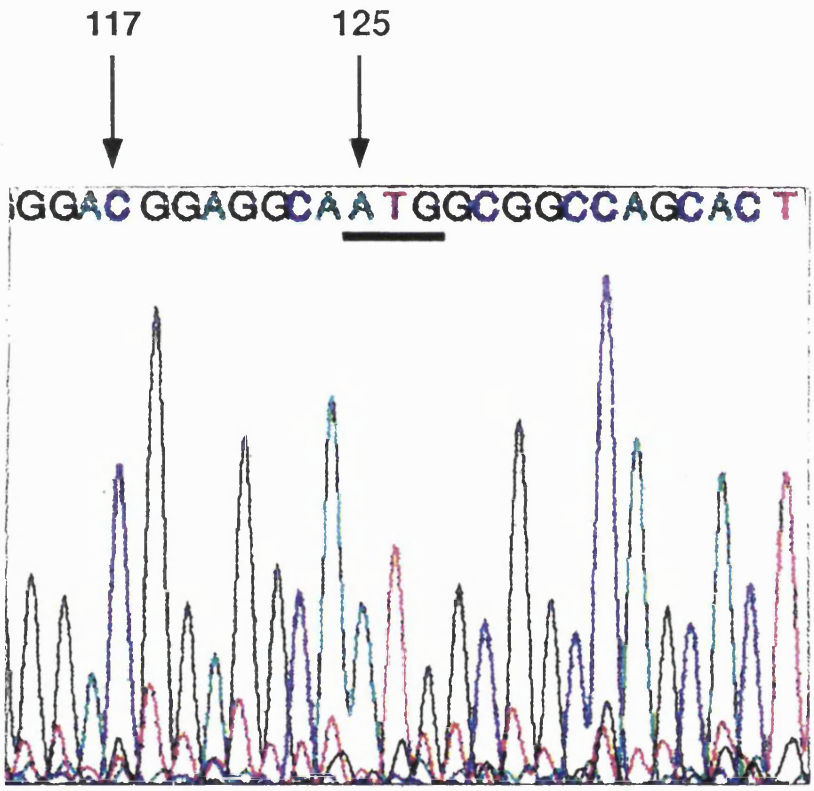
Sequencing of cDNA from all five patients demonstrated that the first in-frame ATG codon occurred at position **125** of the VDR cDNA sequence. The methionine codon at position 116 [4] was not found; instead the nucleotide sequence read ACG rather than ATG and was therefore unable to initiate translation. This is displayed schematically in Fig 32 and the sequencing data is presented in Fig 33. Other investigators have confirmed these findings in normal individuals [120, 153]. The alteration of the start site does not change the reading frame. Numbering of amino acids throughout this thesis is therefore from methionine at position **125**. Thus the numbering may differ by three amino acids, from some published data in this field.

Figure 32



The first in-frame ATG was found at position **125** of the VDR cDNA sequence as defined by Baker et al 1988 [4]. The methionine codon at position 116 was absent. This does not change the reading frame. The start site at 125 was also found in a number of normal individuals by Sajio et al 1991 [153]. Numbering of residues in this thesis is from the methionine codon at position 125.

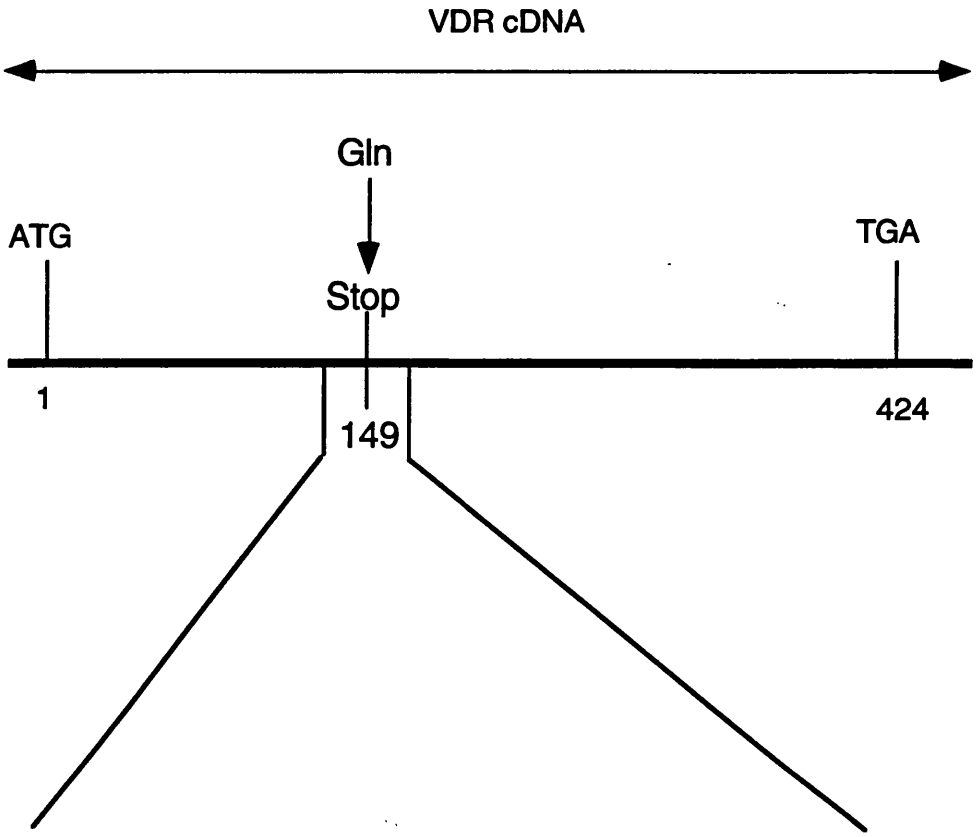
Figure 33



Nucleotide sequence around the start site (Patient TB)

The first in-frame ATG codon was found at nucleotide 125 of the VDR cDNA. A transition of T to C was found at position 117, (A**T**G→A**C**G). The change in translational start site does not alter the reading frame.

Figure 34



				*			
Wild type	GAC	TTC	TGC	**CAG**	TTC	CGG	CCT
	Asp	Phe	Cys	**Gln**	Phe	Arg	Pro
Mutant	GAC	TTC	TGC	**TAG**	TTC	CGG	CCT
	Asp	Phe	Cys	**Stop**	**********		

An illustration of the position of the mutation in VDR from patient FH with respect to wild type. cDNA from patient FH and wild type was amplified by PCR, cloned and sequenced. A mutation was found in the ligand binding domain (position 569) resulting in the formation of a stop codon.

CAG ⟶ TAG resulting in **Q149stop**

Mutations in the coding region of VDR cDNA

A different nucleotide point mutation was identified in each patient except JK. Each was confirmed by sequencing several clones derived from separate rounds of reverse transcription and PCR.

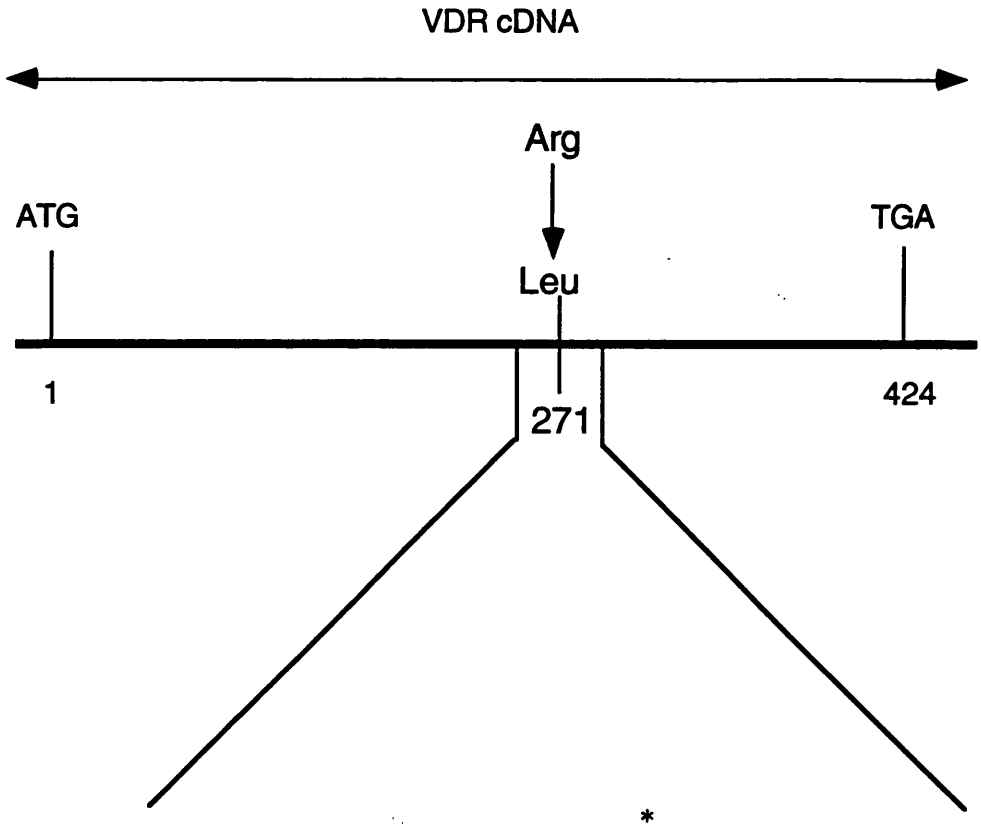
FH was found to have a C to T mutation at position 569 (**C**A G→**T**AG), replacing the normal glutamine 149 codon with a termination codon resulting in a truncated receptor; **Q149X** (Fig 34).
IS was found to have a G to T mutation at position 936, (C**G**C→C**T**C) replacing the normal arginine 271 codon with a leucine codon; **R271L.** (Fig 35)

CC was found to have a T to A mutation at position 254 (**T**T C→**A**TC) replacing the normal phenylalanine 44 codon with an isoleucine codon; **F44I.** An example of one of the sequence traces is given in (Fig 36) along with a schematic representation of the mutation (Fig 37).

TB was found to have an A to G mutation at position 248 (**A**A A→**G**AA) replacing the normal lysine42 codon with a glutamic acid codon; **K42E.** An example of one of the sequence traces is given in (Fig 38).along with a schematic representation of the mutation (Fig 37).

In JK **no** mutations were identified within the coding region of the gene.

Figure 35

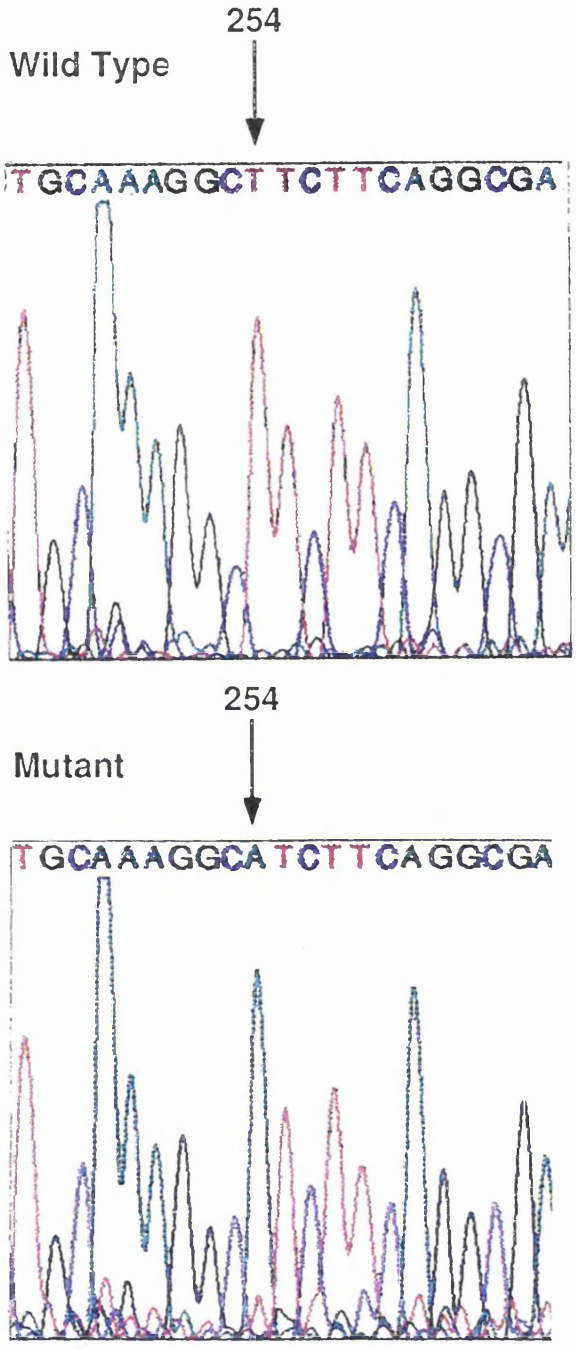


				*			
Wild type	ATC	ATG	TTG	**CGC**	TCC	AAT	GAG
	Ile	Met	Leu	**Arg**	Ser	Asn	Glu
Mutant	ATC	ATG	TTG	**CTC**	TCC	AAT	GAG
	Ile	Met	Leu	**Leu**	Ser	Asn	Glu

Illustration of the position of the mutation in VDR from patient IS with respect to wild type. cDNA from patient IS and wild type was amplified by PCR, cloned and sequenced. A missense mutation was found in the ligand binding domain (position 936) resulting in an amino acid substitution in residue 271.

CGC ⟶ CTC resulting in **R271L**

Figure 36



Nucleotide sequence around the (TTC→ATC) mutation from Wild type and patient CC

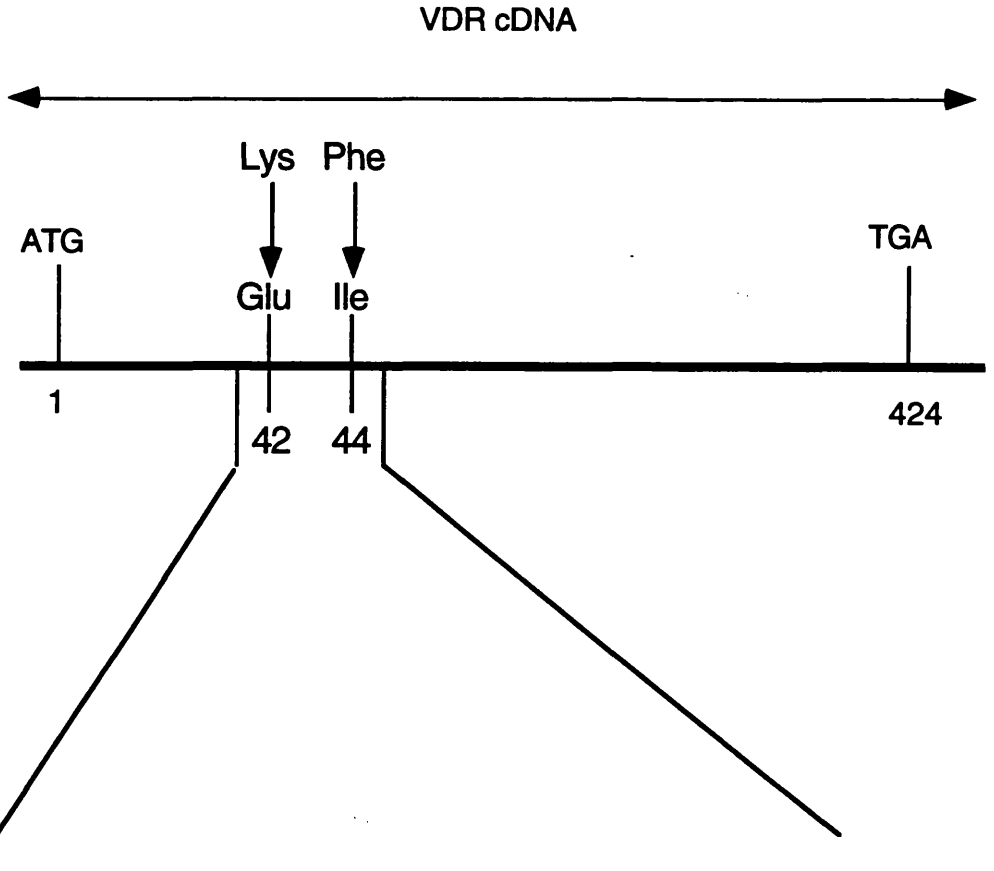
Sequencing of the sense strands is shown.

In patient CC a mutation of T→A was found at position 254 of the VDR cDNA sequence. This mutation changes the normal phenylalanine at position 44 to isoleucine.

The remainder of the VDR coding sequence was normal in all patients. To exclude Thermus aquaticus (*Taq*) polymerase and sequencing errors, all steps were performed in duplicate starting with the reverse transcription reaction and several clones of each PCR product were sequenced in both orientations.

The positions of all the mutations in the vitamin D receptor described to date are shown in Fig 39. Those in the lower part of the figure have been identified here, whereas those in the upper part of the figure have been reported by other investigators.

Figure 37



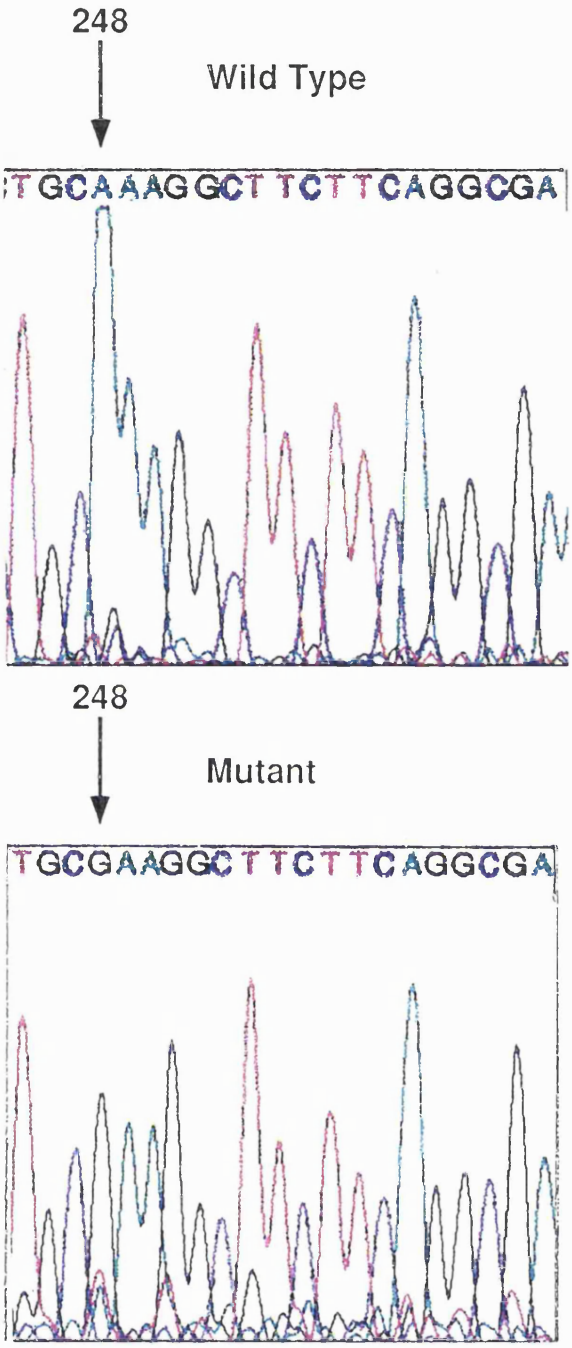
				*		*		
Wild type	GAA	GGC	TGC	**AAA**	GGC	**TTC**	TTC	AGG
	Glu	Gly	Cys	**Lys**	Gly	**Phe**	Phe	Arg
Mutant	GAA	GGC	TGC	**GAA**	GGC	**ATC**	TTC	AGG
	Glu	Gly	Cys	**Glu**	Gly	**Ile**	Phe	Arg

An illustration of the position of the mutations in the VDR DNA binding domain. cDNA from wild type and patients CC & TB was amplified by PCR, cloned and sequenced. Missense mutations at were found (positions 248 and 245), resulting in amino acid substitutions at residues 42 and 44.

Mutation AAA ⟶ GAA resulting in **K42E**; patient TB

Mutation TTC ⟶ ATC resulting in **F44I**; patient CC

Figure 38

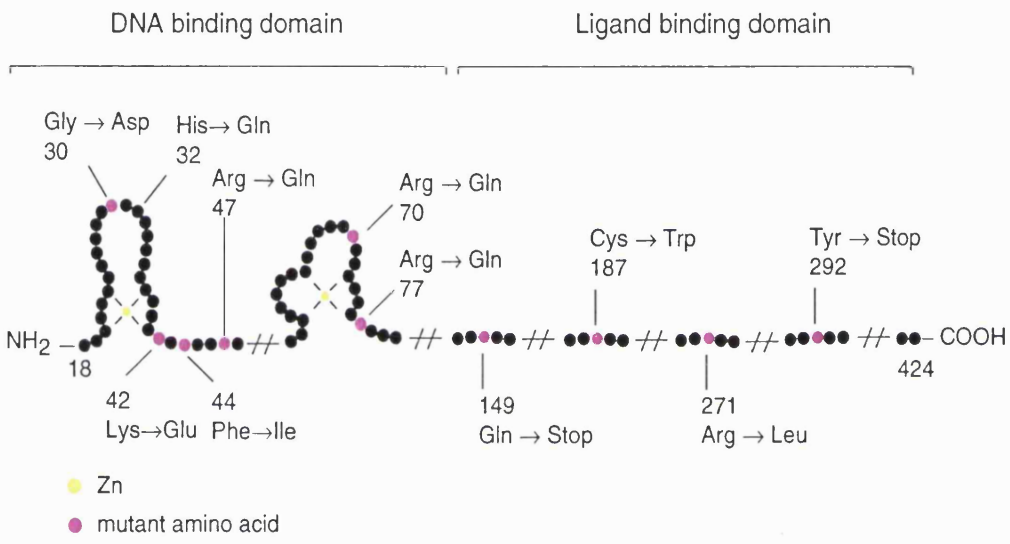


Nucleotide sequence around the (AAA→GAA) mutation from Wild type and patient TB

Sequencing of the sense strands is shown.

In patient TB a mutation of A→G was found at position 248 of the VDR cDNA sequence. This mutation changes the normal lysine at position 44 to glutamic acid.

Figure 39



All the reported and characterised mutations in the vitamin D receptor. Amino acids are numbered from the methionine codon at position 125 of the cDNA sequence.

Those mutations reported in this thesis are presented in the lower half of the figure.

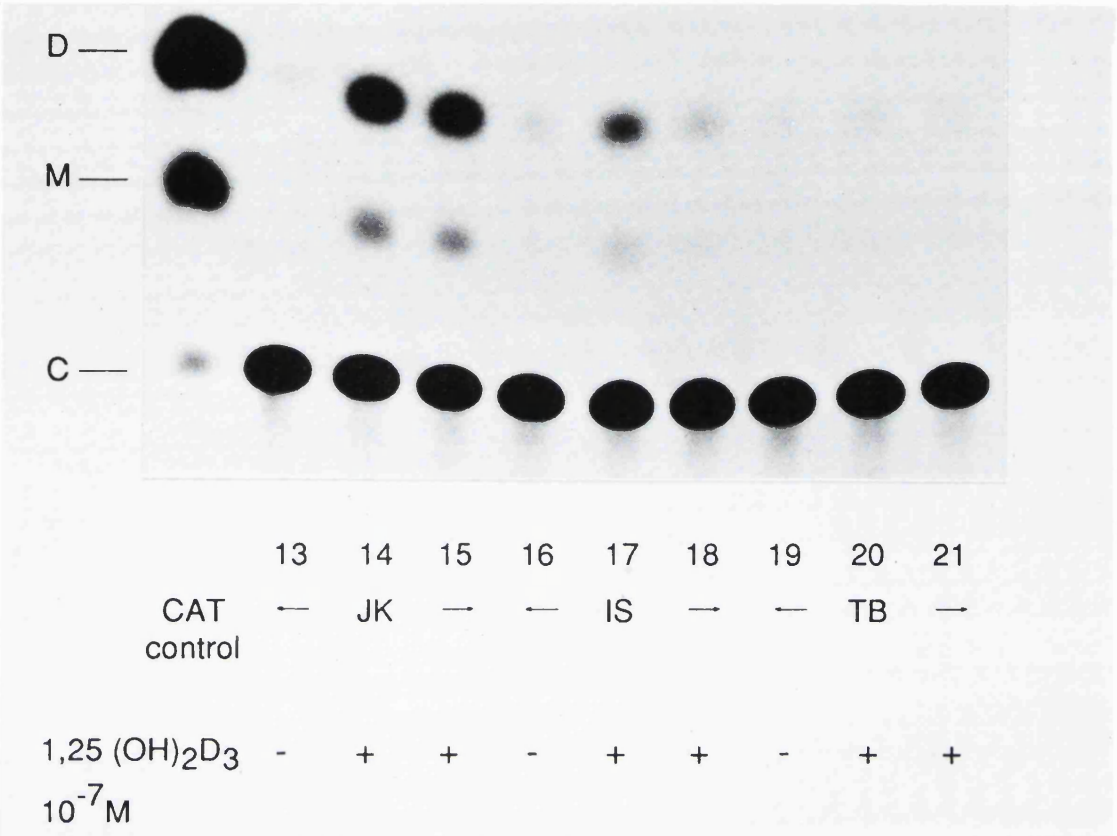
7 mutations lie in the DNA binding domain and 4 in the ligand binding domain.

Transcriptional activation by cotransfection in CV1 cells

The capacity of the mutant receptors to stimulate transcription from vitamin D-responsive genes was examined by cotransfecting cDNA from each patient with the human osteocalcin reporter plasmid, into steroid receptor-deficient CV1 cells and measuring CAT activity. Transfection with wild-type cDNA resulted in hormone-dependent induction of transcription as measured by increased CAT (Fig 40A & B),which converts chloramphenicol to its monoacetylated and diacetylated forms. The **mutant** receptors **F44I (patient CC), K42E (patient TB) & Q149X (patient FH)** were unable to induce transcription from the osteocalcin gene promoter even when incubated with high levels of $1,25(OH)_2D_3$ (10^{-7}M). However in the case of cells transfected with the **R271L (patient IS)** VDR cDNA, CAT activity could be induced by supra-physiological levels of $1,25(OH)_2D_3$. Titration with $1,25(OH)_2D_3$ at concentrations varying from 10^{-11} M to 10^{-6} M showed that CAT activity could be induced at approximately 5 x 10^{-8} M hormone, reaching maximum induction at 10^{-6} M $1,25\text{-}(OH)_2D_3$, i.e., the same as the maximum for the wild-type protein Fig 42. Consequently mutation R271L decreases the capacity of VDR to stimulate the transcription of target genes by approximately 1000 fold [97].

Transfection of VDR cDNA from patient J.K. into receptor-deficient CV-1 cells resulted in normal *trans*-activation in response to $1,25(OH)_2D_3$ as measured by conversion of chloramphenicol to its mono and diacetylated forms. (Fig 40B).

Figure 40A



Thin layer chromatograms of CAT assays from transient transfections performed with VDR cDNA from patients JK, IS and TB. The reporter plasmid contained the osteocalcin promoter fused upstream of the CAT gene.

CV 1 cells used

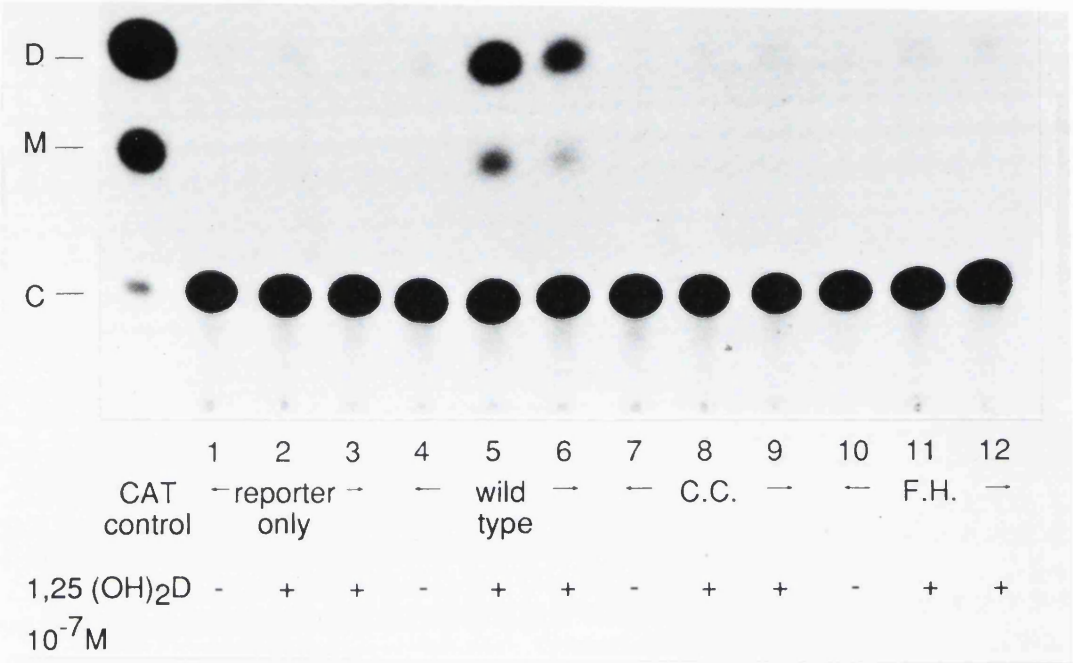
C = (^{14}C chloramphenicol)

M = Monoacetylated (^{14}C) chloramphenicol

D = Diacetylated (^{14}C) chloramphenicol

Result: Normal transactivation in cells transfected with VDR cDNA from JK, none in cells transfected with VDR cDNA from patient TB and reduced transactivation in cells transfected with VDR cDNA from IS.

Figure 40B



Thin layer chromatograms of CAT assays from transient transfections performed with cDNA from a normal control and patients CC and FH. The reporter plasmid contained the osteocalcin promoter fused upstream of the CAT gene.

CV 1 cells used

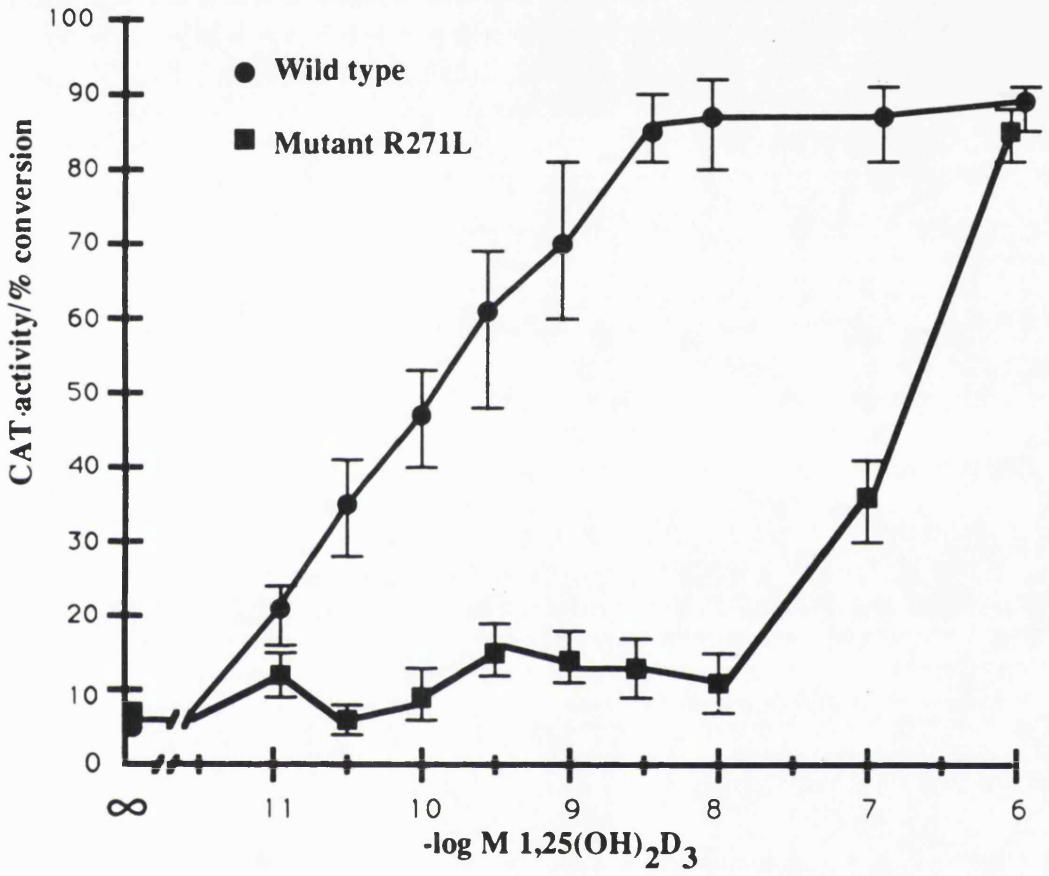
C = (^{14}C chloramphenicol)

M = Monoacetylated (^{14}C) chloramphenicol

D = Diacetylated (^{14}C) chloramphenicol

Result: Normal transactivation in cells transfected with wild type control VDR cDNA, but none in cells transfected with VDR cDNA from patients CC and FH.

Figure 41



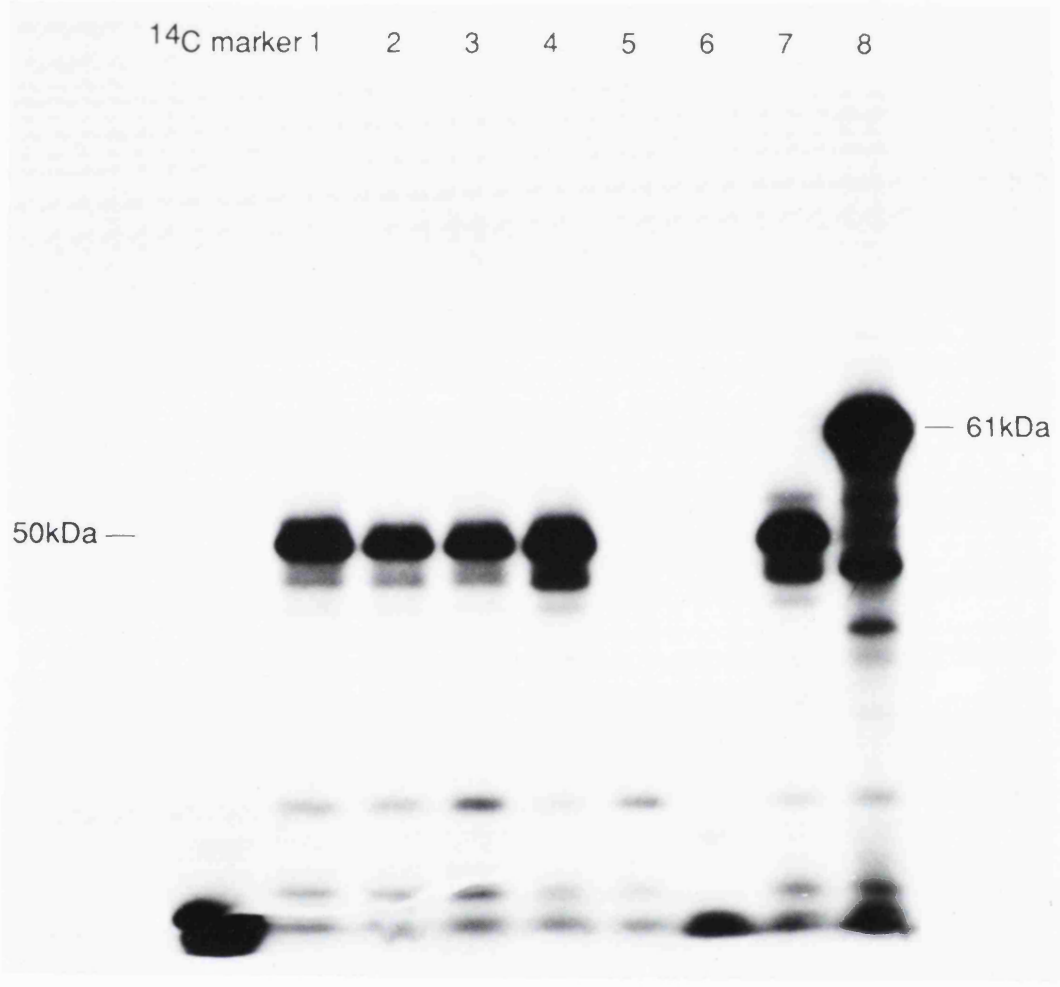
Effect of increasing concentrations of $1,25(OH)_2D_3$ on transcriptional activation by wild type cDNA and IS mutant cDNA (R271L). CV-1 cells were cotransfected with either wild type or patient IS expression plasmids (5 µg each) and the osteocalcin reporter plasmid (1 µg). Cells were treated with $1,25(OH)_2D_3$ at final concentrations ranging from 10^{-11} M to 10^{-6} M as indicated on the X-axis. Significant induction of CAT activity was observed at 10^{-7} M. CAT activity was equal to wild type at 10^{-6} M $1,25(OH)_2D_3$.

Figure modified from Kristjansson et al 1993 [97].

In-vitro transcription and translation

Mutant and wild type VDR cDNAs were transcribed and translated in vitro to confirm the presence of the translation stop codon in FH (Fig 42). Luciferase control cDNA and wild-type VDR cDNA gave appropriate translation products; 61 and ~50 kDa respectively. cDNA from Patients CC, JK, IS and TB also transcribed and translated to give a ~50 kDa protein. In contrast, FH cDNA in pSVK3 only resulted in non-specific products being identified. Using FH cDNA in pKS$^-$ (as described in methods) yielded a truncated protein of less than 30 kDa, confirming the presence of a stop codon **Q149X**.

Figure 42



10% SDS/Page of In-vitro transcription and translation of VDR cDNA from patients and wild type control.

Lane				
	1	CC	5	FH in pSVK3
	2	TB	6	FH in pKS
	3	JK	7	wild type in pTZ18U
	4	IS	8	Luciferase control

Lanes 1-4 & 7 show a 50 kDa tranlation product consistent with VDR. Lane 5 shows only non-specific translation products while lane 6 shows a truncated protein consistent with stop codon mutation Q149X.

Chapter 4; Summary

The salient points of the chapter are outlined below.

- Patient data combined with studies of VDR basic and molecular biology has allowed a much clearer understanding of the vitamin D system.

- The new mutations provided further insight into the critical importance of residues within both the DNA and ligand binding domains of VDR.

- Mutations in the DNA binding domain of VDR have only been detected in residues conserved throughout members of the nuclear receptor superfamily, suggesting that these have been essential for normal receptor function throughout evolution.

- Crystallographic modelling allowed structural interpretation of normal and mutant protein/DNA interactions at the Angstrom level.

- One patient was found to have normal VDR sequence in association with end-organ resistance to vitamin D. The pathogenesis of her disease remains to be elucidated.

- Other investigators have documented clearly that hereditary resistance to $1,25(OH)_2D_3$ is caused by mutations in VDR [75, 112, 113, 150, 153, 169, 188]. With increasing knowledge about the

structure-function relationship of the different members in the steroid/ thyroid/ retinoid receptor superfamily, it is now possible to be more specific about the effects of various mutations. This is especially true for the DNA binding domain of nuclear receptors, but increasing evidence is also accumulating regarding the function of subdomains and individual amino acids in the hormone binding domain [74, 120, 128, 150]. Discussion will be divided according to the location of base changes.

Translation initiation site

Sequencing of cDNA from all five patients demonstrated that the first in-frame ATG codon occurred at position **125** of the VDR cDNA sequence. This differs from the original data of Baker et al 1988 [4] whose published VDR cDNA sequence locates the first methionine codon (ATG) 116 bp from the cap site. This latter ATG was not identified in any of our sequencing runs. Both ATGs are in the same reading frame so could represent alternate initiation sites as occurs in thyroid receptor [183] the progesterone receptor [58] and androgen receptors [186]. Two naturally occurring hormone-binding forms of progesterone receptor have been identified. The A isoform is an N-terminally truncated version of the full-length B isoform. It is derived from the same mRNA by translation initiated at an internal methionine. Several lines of evidence suggest that the 2 isoforms serve specific functions. Tora et al 1988, demonstrated promoter and cell-specific differences in the ability of PR-A and PR-B isoforms of the chicken to activated transcription of target genes[175]. Wilson and McPhaul 1994, demonstrated 2 forms of the androgen receptor protein in human genital skin

fibroblasts [186]. The apparent molecular masses were approximately 110 kDa and 87 kDa. The 87-kDa isoform (AR-A) contained an intact C terminus but lacked the normal N terminus found in the 110-kDa isoform (AR-B). AR-A is the same size as the mutant form of AR produced in fibroblasts from an androgen-resistant individual by initiation of AR synthesis at the first internal met188 residue of AR-B, as reported by Zoppi et al 1993 [192]. The AR isoforms resembled the A and B forms of the progesterone receptor, which also are encoded by a single gene and differ by the absence or presence of an N-terminal segment. The A and B forms of the progesterone receptor differ in their ability to activate target genes and are regulated differently in various types of cells. The identification of similar forms of AR raised the possibility that the 2 isoforms also serve different functions.

In VDR it appears more likely that the true initiation codon is located at position 125. Saijo et al 1991, have confirmed the presence of one start site at position 125 in three Japanese patients with hereditary resistance to $1,25(OH)_2D_3$ and in several normal controls [153]. Based on the above findings, numbering of VDR amino acids in this thesis is from position 125.

Mutations in the hormone binding domain of the vitamin D receptor

Receptor binding data for patient **FH** was consistent with Q149X which results in deletion of the entire hormone binding domain of VDR. Binding of $1,25(OH)_2D_3$ to cytosolic extracts from FH fibroblasts was absent as was 24-hydroxylase induction verifying the phenotype. Northern analysis detected VDR mRNA of appropriate size (4.6 kb) but of very low abundance in FH (Fig 29). Confirmation that VDR mRNA was actually present in cells from this patient came from reverse transcription of VDR total RNA followed by PCR amplification using oligonucleotide primers to the 5′- and 3′-untranslated regions of the authentic receptor. Malloy et al 1990 found similarly low levels of VDR mRNA in family members from a single large kindred with vitamin D resistant rickets [112]. The molecular basis of their disease was also a stop codon but located further down stream, at position 289. Decreased androgen receptor mRNA levels have also been reported in a patient (N105) with complete testicular feminisation [115]. Sequencing revealed a mutation in the androgen receptor at amino acid 772 (Arg → Cys). Possibly, in cells from certain patients with target organ resistance to nuclear hormones, the level of mRNA transcription is depressed or mRNA turnover is increased. It is not clear that this will be the case in vitamin D resistance as recent work suggests that $1,25(OH)_2D_3$ does not up-regulate levels of VDR mRNA [157].

A truncated protein was demonstrated by in-vitro transcription and translation of FH VDR cDNA confirming the presence of the mutant stop codon in the sequence. Expression data was in keeping with

defective VDR which were unable to *trans*-activate and hence induce CAT activity. Cells cotransfected with FH cDNA and the reporter plasmid did not increase levels of CAT, as measured by conversion of ^{14}C-chloramphenicol when challenged with $1,25(OH)_2D_3$. This data established the 149 stop codon as the genetic basis of vitamin D resistance in patient FH. It is likely that this patient was studied independently by Wiese et al [184] who also confirmed the presence of the stop codon at position 149.

Patient **IS** exhibited receptor binding data consistent with the location of the mutation, namely R271L. Cytosolic binding of $1,25(OH)_2D_3$ to VDR was about 10 fold lower than controls which is in keeping with the mutation R271L and with the expression studies. However, affinity of $1,25(OH)_2D_3$ for nuclear extracts was essentially normal with reduced numbers of VDR. It is possible that the R271L mutation may be located in a region that is important for translocation of the receptor to the nucleus. Co-expression of IS VDR with the reporter construct in receptor-deficient CV-1 cells resulted in a reduced but detectable *trans*-activation response to $1,25(OH)_2D_3$. When the concentration of $1,25(OH)_2D_3$ in media was increased to 10^{-6} M, induction of CAT was equivalent to wild type (Fig 42). This implied an approximately 1000 fold reduction in VDR *trans*-activation ability as the physiological concentration of $1,25(OH)_2D_3$ in serum-free media is approximately 10^{-9} M. Sone et al 1989 [171] performed a similar experiment with receptors carrying mutations in the DNA binding domain. There was no increase in transcriptional activation even at $1,25(OH)_2D_3$ concentrations as high as 10^{-6} M.

The diminished *trans*-activation capability of the R271L mutant may comprise two components: first, ligand binding to VDR from IS is 10-fold lower than control, and second the R271L mutation may disrupt a domain of the receptor that is important for hormone-dependent transcriptional activation.

Mutations Q149X and R271L; their relevance to receptor function

These two mutations provide additional information about the ligand binding domain of VDR. This will be discussed on the background of current knowledge about this region of the receptor.

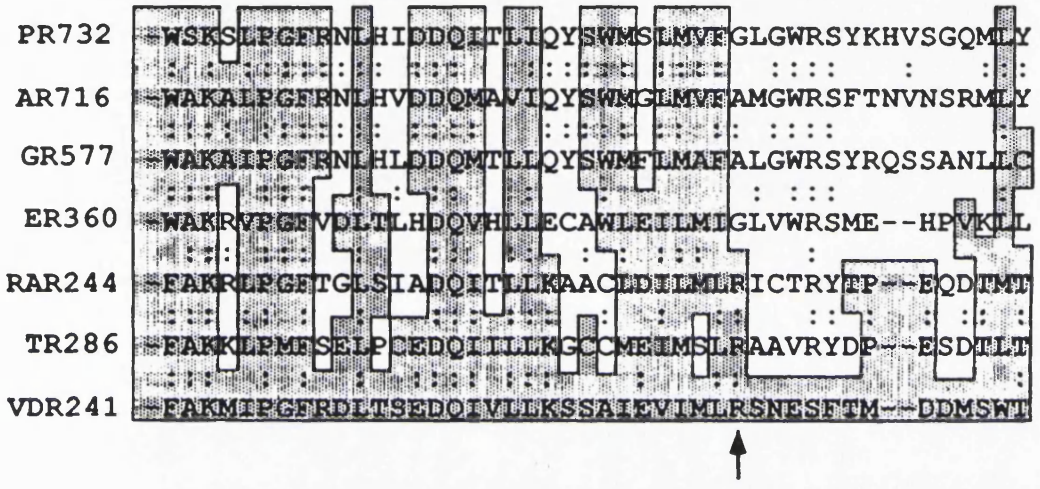
Deletion-mutant analysis has suggested that the critical point for 5′ deletions still giving high affinity wild-type binding of 1,25-$(OH)_2D_3$ lies between residues 111 and 163 [120]. The mutation at codon 149 narrows this even further by the fact that there is no detectable cytosolic binding of 1,25-$(OH)_2D_3$ [7, 98]. The C-terminal portion of the glucocorticoid receptor [132, 139], androgen receptor [81, 163], progesterone receptor [58], oestrogen receptor [100] and VDR [120] has been shown to have repressive effect on transcriptional activation in the absence of ligands. A VDR deletion mutant missing the carboxyl portion from residue 187 to the N-terminus has been shown to exhibit considerable constitutive transcriptional activity [120]. Similar data exist for the GR and the AR [81, 132, 139, 163]. This has been interpreted as evidence for transcriptional domains being present in the amino-terminal portion of the hormone binding domains. This region of the VDR [60] is rich in acidic residues that have been implicated as being important in gene activation within yeast transcription factors [69]

and steroid receptors [181]. In the mammalian cotransfection analyses reported here, no transcriptional activation by the cDNA Q149X mutant was detected. This could be explained by deletion of these transcriptional domains. Additionally, this amber mutation deletes the entire portion of the receptor that is postulated to form the actual binding pocket for 1,25-$(OH)_2D_3$ and also deletes a dimerisation domain at positions 241-260 [74]. This region is highly conserved between all the members of the superfamily and contains a leucine zipper-like motif. It is most likely involved in interaction with other nuclear proteins [74, 129, 130]. The nuclear accessory protein is most probably the Retinoid X receptor [20, 190] Point mutations within this domain do not interfere with ligand binding but affect transcriptional activation and the ability of the receptors to form heterodimers [74, 129, 130]. The R271L mutation is adjacent to this domain which by homology can be extended right up to Arg 271 (Fig. 43). The arginine residue at this site is conserved between VDR, TR and RAR so it is possible that this residue represents a critical ligand/receptor contact or it provides an important polar amino acid critical for the three-dimensional structure of this domain. From the data presented here it is not clear whether Arg271 is an essential contact residue or one that is critical for the receptor's tertiary structure. The importance of Arg271 is emphasised by the report of a mutation at the corresponding residue in AR, (A746D), causes androgen insensitivity (Reifenstein syndrome) [121].

These two novel mutations in the hormone binding domain of the VDR give further insight to the structure-function relationship of this part of the protein. There is increasing evidence that binding of $1,25(OH)_2D_3$ to VDR modifies protein-protein interactions and

transcriptional activation through modification of receptor's structure. The full understanding of the structure-function relationship of the hormone binding domains of the steroid/thyroid/retinoid receptors awaits determination of their three-dimensional structure by x-ray crystallography.

Figure 43



Conservation within a 49 amino acid sequence in the ligand binding domain of the steroid/thyroid/retinoic acid receptor superfamily. The sequence of the vitamin D receptor (VDR) along with the homologous regions of the thyroid (TR), glucocorticoid (GR), androgen (AR), progesterone receptors (PR) oestrogen (ER) and retinoic acid (RAR) receptors. Numbers refer to the first residue on the left for each receptor. Numbering of residues in VDR is from the methionine codon at position 125 of the cDNA sequence. Two dots indicate identical amino acids. Boxed and shaded areas highlight regions conserved with VDR. The arginine at position 271, that is mutated to leucine in patient IS, is indicated by the arrow.

Figure courtesy of Kristjansson et al 1993 [97].

Mutations in the DNA binding domain

The location of the two mutations in patients CC and TB ie F44I and K42E are consistent with the altered properties of the receptor found in their cultured fibroblasts. The cytosol from them contained receptors that bound $1,25(OH)_2D_3$ with a normal K_d. Translocation of the bound hormone to the nucleus, as measured in the nuclear association assays, was also of normal capacity (albeit of slightly lower affinity). Nevertheless, $1,25(OH)_2D_3$ could not induce the normal response in patients fibroblasts, as measured induction of 24-hydroxylase activity. Insensitivity to the hormone was also demonstrated in transient transfections using VDR cDNA derived from patient fibroblasts.

It might be assumed that mutations with the DNA binding domain ought to impair the ability of VDR to bind to the nucleus. However, previous investigators have demonstrated similar findings in their patients with end organ resistance to vitamin D. Patient 3, described by Liberman, [104] who has a mutation in the second zinc finger R77Z [169], had normal nuclear uptake of VDR and VDR bound $1,25(OH)_2D_3$ with standard affinity. Mutations F44I and K42E completely abolished transcriptional activation by VDR from the osteocalcin promoter confirming them as the cause of the disease. The consequences of these mutations on DNA binding will be discussed further.

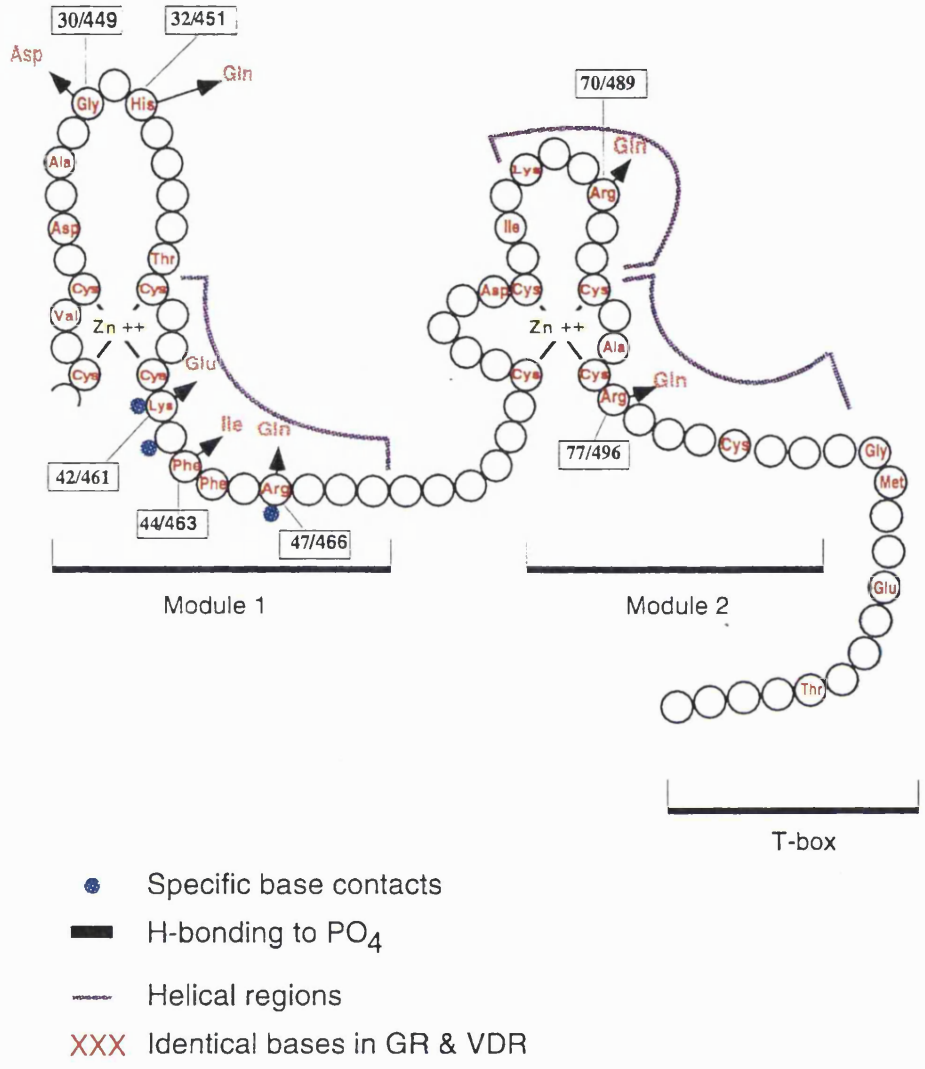
The DNA binding domain is the most highly conserved region in the nuclear receptor superfamily. 25 of the 65 residues in this zinc finger region are identical with those in the glucocorticoid receptor

(Fig 44) and 30 are identical to those in the corresponding part of the oestrogen receptor. Many of the differences in the remaining residues represent conservative changes; for example 14 of the differences between these parts of GR and VDR are conservative. It is striking that all the mutations that have been found in the DNA binding domain of VDR from patients with end-organ resistance are identical to those in the glucocorticoid (Fig 44) and oestrogen receptors. These are: glycine at residue 30 in VDR (corresponding to 449 in GR); histidine at residue 32 (451 in GR); and arginines at 47, 70 and 77 in VDR, (466, 489 and 496 in GR). These correspond with residues 16, 18, 28, 30, 33, 56 and 63 of the 84 amino acid peptide used by Schwabe et al [158]. That peptide is located between residues 179-262 of the complete human oestrogen receptor that consists of 595 amino acids.

The crystal structure of the DNA binding domains of the glucocorticoid and oestrogen receptors in complex with their respective DNA targets have established [107, 158]. Those studies provide the basis consideration of the possible effects of mutations in this region of the vitamin D receptor because of the strong homologies that exist within the superfamily of steroid hormone receptors. This modelling is undertaken with the caveat that there are differences within the receptor proteins and in the hormone response elements of DNA. Apart from the differences in the amino acid sequences, there are differences in the way the proteins bind to DNA. GR and ER bind as homodimers while VDR can bind as heterodimers with a nuclear accessory factor/RXR. Carlberg et al [22] have demonstrated that the RXR ligand, 9 *cis* -retinoic acid, and the nature of the response element determine whether VDR forms a

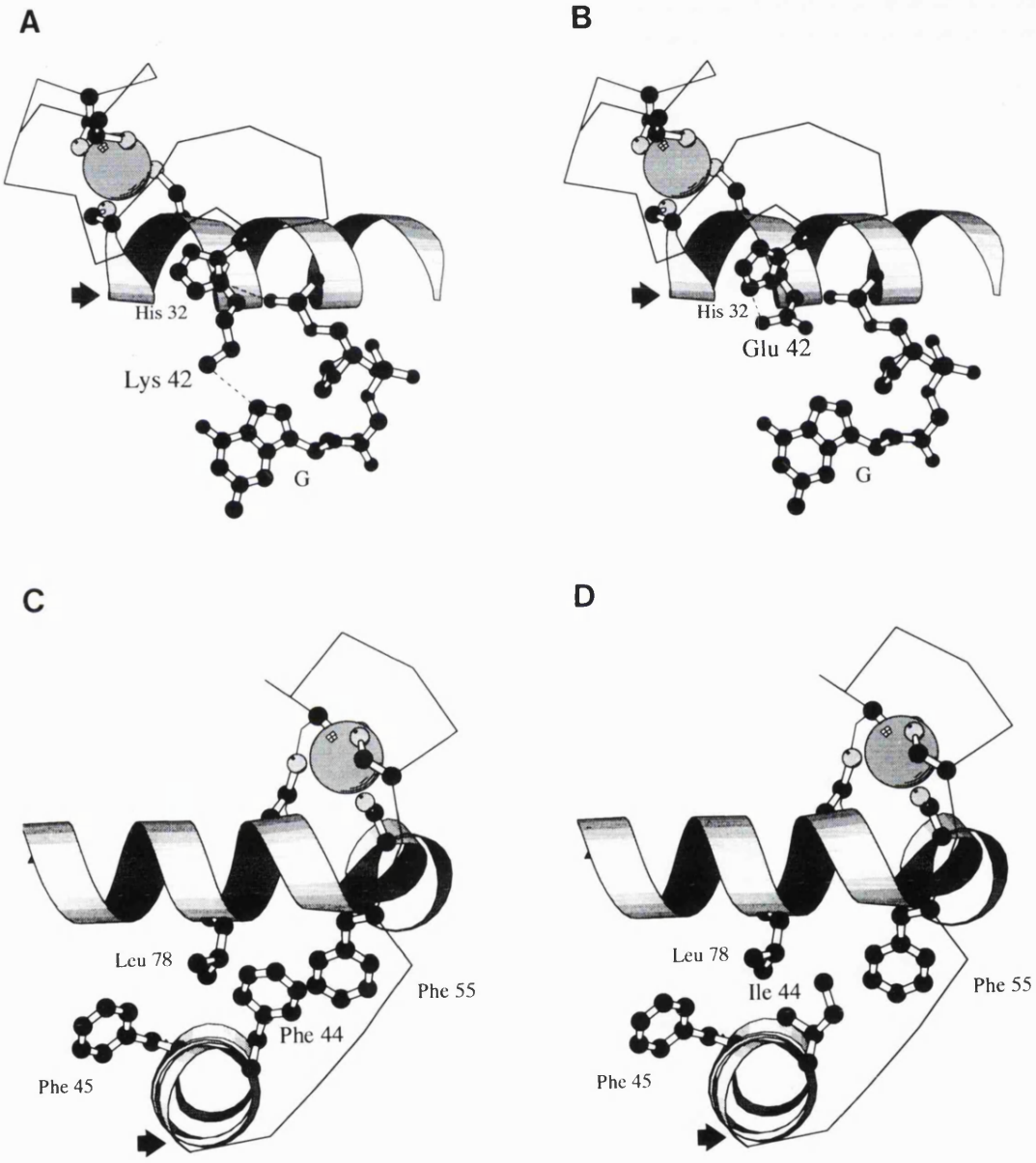
heterodimer with RXR. The spacing between the half-sites in the target genes also differs. For example GR and ER bind to palindromic sequences separated by three nucleotides [91], whilst VDR may bind to direct repeats with variable spacing [178]. However the strong homologies between the DNA binding domains of VDR, GR and ER allow the crystal structures of the last two to be used as a model for the former. This is all the more reasonable since the residues in which mutations have been found in association with rickets are identical in all three receptors. These can be conveniently considered under three headings , starting with the mutations reported here.

Figure 44



Mutations in the DNA binding domain of VDR.
The two zinc modules and the T-box are shown. Mutations in VDR are identified by arrows; boxes alongside these mutations give the residue number in VDR followed by that of the corresponding residue in GR. Labelled residues are identical in GR and VDR; those differing in the two receptors are unlabelled and are shown by open circles. Note that all mutations occur in residues that are identical in normal GR and VDR.

Figure 45



Wild type **Mutant**

Figure 45 Legend

Models of normal and mutant VDR showing the effects of substitutions at residues 42 (upper figure) and 44 (lower figure). Models were prepared from the structure of the GRdbd/DNA complex [107]. The recognition helix is indicated with an arrow. Zinc atoms are shown as spheres. Illustrations were generated using MOLSCRIPT [96].

A Lys42/DNA interactions in wild type. The axis of the recognition α-helix lies in the plane of the page and the helix is seen from the side. On the hydrophilic surface of the recognition helix, guanine is shown hydrogen-bonded (---) to Lys42. His32, in the peptide loop of the Zn finger, forms a hydrogen bond to the phosphate backbone. The interactions of Lys and His with the DNA is proposed to be conserved in the steroid/nuclear receptor superfamily.

B The effects of Lys42→Glu substitution. The view is the same as 45A. The hydrogen bond to the guanine is lost, and the bond between the phosphate backbone and His 32 could be displaced as shown.

C Phe44 interactions with the conserved hydrophobic core in wild type VDR. The packing of residues in the hydrophobic cluster is illustrated. The view is at right angles to that in Fig 45A & 45B; The α-helix that is seen from the side is the one on the carboxyl end of the second zinc finger.

D The effect of the Phe44→Ile substitution. Orientation is the same as 45C. A gap is introduced by this substitution in the hydrophobic core.

Lys42 of VDR corresponds to Lys461 of GR and by analogy would lie on the hydrophilic side of the recognition helix, where it could donate a hydrogen bond to N7 of a purine (Fig. 45A). Guanine at this position of contact is common to most hormone/nuclear receptor DNA targets including the glucocorticoid and vitamin D response elements (GRE and VDRE, respectively). VDR's His32 is conserved at the corresponding position in the superfamily and most likely would donate a hydrogen bond to the phosphate of the above guanine. Mutation of Lys42→Glu would have a number of effects (Fig. 45B). The hydrogen bond to the guanine would be lost. Furthermore, Glu42 might attract a hydrogen bond from the imidazole ring of His32, disrupting its interaction with the DNA-phosphate.

The effects of the mutation Phe44→Ile can also be explained stereochemically (Fig. 45C & D). Phe44 and Phe45 are in the VDR recognition helix but face away from the DNA surface and project into the domain's hydrophobic interior. Phe44 would pack closely against other hydrophobic residues, including Phe55 and Leu78, and buttress the Arg77 side chain to make contacts with a DNA-phosphate. The mutation Phe44→Ile (Fig. 45D) might conserve the hydrophobic nature of the core, but would not permit close packing of the well matched hydrophobic residues. Additionally, the conformation of the Ile side-chain would be highly restricted because of steric constraints imposed by the α-helix; these limitations would also result in non-optimal side chain packing. It would be expected that the mutation would affect the stability of the receptor and, through its effects on the Arg77 side chain might reduce DNA binding affinity.

The five other mutations (Fig. 44) that have been reported in the VDR DNA binding domain can be interpreted similarly. One affects Gly30 [2], another changes His32 to Gln [188] while the other three mutations affect arginines, which are mutated to glutamines at positions 47, 70 and 77 [75, 153, 169]. Gly30 might have two structural roles. Firstly, having no substituent, Gly would permit the peptide backbone to assume a special conformation orienting His32 for donation of a hydrogen bond to the phosphate backbone and Phe33 for packing against the hydrophobic interior of the domain. In addition, Gly30 would allow the peptide backbone to approach the DNA phosphates. Substitution of Gly30 by Asp would result in two adverse effects: the negative charge of the Asp carboxylate would repel the DNA-phosphates, and the Asp β-carbon would sterically prevent a close approach to DNA. The effects of the mutation His32→Gln would be critical. As already stated, His is absolutely conserved at this position in the superfamily and by virtue of its positive charge would be expected to donate a hydrogen bond to the phosphate of guanine in the VDRE; this could not be done by the mutant Gln32.

The three arginine mutations in VDR would also have important consequences. In the native protein, Arg47 could donate two hydrogen bonds to guanine in a VDRE. Substitution by Gln47 at this position introduces a side chain that is not long enough (by roughly 2Å) to reach the base; two hydrogen bonds would be lost as a consequence. Arg70 would form a salt bridge with Asp26, an interaction that appears to be conserved in the superfamily and also contact a DNA-phosphate in wild type VDR. The introduction of Gln70 could only permit one of the interactions, but not both. This

would weaken DNA binding and possibly, the stability of the protein. Arg77 would be expected to make two hydrogen bonding interactions with the phosphate backbone of the VDRE. Its aliphatic side-chain could pack against Phe44, as described above. Neither the hydrogen bonding nor the hydrophobic interactions would be conserved when the residue is substituted by Gln77. This residue has recently been found to be mutated in a case of complete androgen insensitivity, Arg614→His. This underlines the critical importance of these conserved amino acids.

The apparent tendency for deleterious mutations to occur at arginine residues in VDR may be a consequence of deamination of methyl-cytosine or cytosine at CpG in genomic DNA, resulting in mutational hotspots [12, 161]. Four of the ten known mutations in the rickets cases involve codons that harbour CpG (eg. **CGA**→**CAA** at residues 47 and 70; **CGG**→**CAG** at residue 77; **CGC**→**CTC** at residue 271). Indeed, deamination of (methyl-C)pG or CpG may explain the origin of mutations causing haemoglobinopathies [138] and many other hereditary diseases [12, 161].

The mutations in the DNA binding domain described here demonstrate that point changes in VDR can cause profound physiological effects which result in severe bone disease. By modelling with the GRdbd crystal structure, it is possible to begin to understand stereochemically the disruptive effects of these clinically significant mutations on the genomic actions of vitamin D.

End-organ resistance with no mutation

Up to now a mutation has been found in all patients with hereditary vitamin D resistant rickets in whom the nucleotide sequence has been determined, either from genomic DNA or from cDNA. In all, eleven mutations have been recognised (Fig 39): two of these are in the 1,25(OH)2D3-binding domain, seven in the DNA-binding domain, and two in the hinge region between the other two parts of the receptor. JK is the first reported patient with vitamin D resistant rickets who does not appear to have a mutation in the vitamin D receptor.

Receptor characterisation revealed absent 24-hydroxylase induction by $1,25(OH)_2D_3$, confirming the phenotype of the patient. VDR numbers in the cytosol were normal and bound ligand with normal affinity. However, no VDR were found in the nucleus so K_d was unmeasurable. VDR mRNA was present in equivalent amounts to control as determined by Northern blotting using a riboprobe to the steroid binding domain of VDR. The result was very clean indicating the usefulness of riboprobes when attempting to detect very low abundance mRNA (Fig 30). In CV-1 cells VDR cDNA generated from JK fibroblast RNA was able to induce transcription from the reporter plasmid at the same level as wild type cDNA. Thus the defect is specific to JK's cells but is distinct from the VDR which function normally in another cell system. Therefore it is important to review the available data on this patient in order to produce a working hypothesis for the molecular basis of her disorder.

Phenotypically, JK had classical end organ resistant rickets, with alopecia and raised serum 1,25(OH)2D3. She is, however, unusual in two ways. Firstly, it was possible to heal her rickets with a combination of treatment with one-alpha hydroxyvitamin D3 and oral calcium in large doses. It is difficult to say whether the 1-α hydroxyvitamin D produced the response or whether calcium achieved healing. She has remained healthy for two and a half years since treatment was stopped. It is possible that the condition might relapse at her next growth spurt, when her calcium requirements increase.

She is also unusual in that no mutation was found on sequencing her VDR cDNA. It is not likely that the methods used were inadequate since using identical techniques in parallel detected mutations in the four other patients. A possible explanation would be that the patient, whose parents were not related, was a compound heterozygote inheriting two different mutations, one from each parent and that these were missed by the techniques used. However, VDR cDNA derived from J.K. fibroblast RNA functioned normally in CV-1 cells, so she possessed at least one normal sequence. It is feasible, of course, that the patient (whose parents were normal) had a new, dominant mutation in one copy of the gene and that it was a copy of the other, normal gene that was transfected into the CV-1 cells. The chances of this are very remote as no dominant mutations have been observed to date in any patient with vitamin D resistance. It is also possible that the 5′ or 3′ untranslated regions of the vitamin D receptor are polymorphic, affecting gene expression. Against this is the fact that VDR mRNA was present in the same quantity as in control cells and VDR were

present in cytosolic extracts of fibroblasts, indicating normal mRNA and protein stability.

Consideration of the mode of action of the vitamin D receptor in other members of the superfamily of nuclear receptors, provides other possible explanations that need to be considered. Binding of hormones may be modulated by non-steroid binding proteins, which include the 90 kDa heat shock protein (hsp90). Dissociation of this protein from the glucocorticoid receptor is necessary for it to associate with DNA [156]. However, it is not clear that hsp90 is needed for the action of the vitamin D receptor. In new world monkeys, there is resistance to the action of $1,25(OH)_2D_3$ even though receptors are present and it has been suggested that there is a soluble protein that prevents intracellular binding of 1,25(OH)2D3 [51]. This is therefore not analogous to the defect described here because in this patient ligand binding of $1,25(OH)_2D_3$ was normal. Another possibility is an abnormality involving a protein that dimerises with the vitamin D receptor. VDR can form either homodimerise or form heterodimers with other proteins such as Retinoid X receptors [22, 190]. The retinoid X receptor is probably the same as the nuclear accessory protein that is important for the full transcriptional activity of VDR [168]. An abnormality in the accessory protein/retinoid X receptor could impair either dimerisation or transcriptional activation. Such an abnormality would have to be specific for the vitamin D system since the patient was otherwise normal.

Post-translational modifications of the vitamin D receptor provide another potential explanation for hormone resistance in this patient.

Phosphorylation of the receptor is known to be important and has been shown to be dependent on a number of protein kinases. Phosphorylation by kinase C-beta at serine 48 (numbered 51 in some publications) is functionally important [72]. Mutation of Ser48 to either glycine or aspartic acid which cannot be phosphorylated, abolished interaction with the VDRE as measured by gel mobility shift analysis [73]. Jurutka et al [84] have localised protein kinase A (PK-A) phosphorylation sites *in-vitro* to the VDR region between Ala133 and Ser201. However PK-A catalysed phosphorylation of VDR in transfected COS-7 cells results in a suppression of $1,25(OH)_2D_3$-stimulated VDR-dependent transcriptional activity. This suggests that in VDR, phosphorylation may play a modulatory role in hormone stimulated receptor activation. However in the case of patient JK the receptor sequence was entirely normal so that any defect in phosphorylation would have to result from altered kinase activity that was selective for the vitamin D system.

The main functional defect described here in studies of fibroblasts and transformed B-cells was a failure of receptor translocation to the nucleus, as evidenced by reduced nuclear association. Barsony et al [8] studied the compartmentalisation and subcellular organisation of VDR in normal cells and cells from patients with end organ resistance to $1,25(OH)_2D_3$. In two cell lines, 1a and 2a, derived from patients with vitamin D resistant rickets, [104] that displayed normal VDR binding to $1,25(OH)_2D_3$ and normal DNA binding to non-specific DNA but demonstrated defective $1,25(OH)_2D_3$ uptake into the nucleus, the amount of VDR by immunocytology was normal. As expected, when the patients' cells were grown in serum-free media, VDR were cytoplasmic. However

when challenged with $1,25(OH)_2D_3$, these VDR did not translocate to the nucleus. The molecular basis of the defect in patients 1a and 2a has not been published but may be analogous to that in JK. Parker et al have identified an ATP-dependent shuttle which transports the oestrogen receptor to the nucleus [136]. It is not known whether such shuttles exist for other receptors and if it is receptor-specific. If one exists for VDR, it may be defective in JK. At present, relatively little is known of the translocation process so the defect cannot be adequately explained. Further work on this patient and others with steroid hormone resistance ought to elucidate the mechanism of disease.

Conclusion

The mutations described here demonstrate that point changes in VDR can cause profound physiological effects which result in severe form of rickets. The work has furthered our understanding of VDR subdomains and provided scope for future research to unravel the basis of disease in the patient with normal VDR.

Lastly, by modelling of the protein structure of the basis of the GRdbd crystal structure, it has been possible to understand stereochemically the disruptive effects of these clinically significant mutations on the genomic actions of vitamin D.

Future work

The mutations described form an important resource as they originate from patients with a well-defined phenotype. It will be essential to discover out how the individual mutations alter binding to different vitamin D response elements and how they alter transcription of a spectrum of target genes. This will continue some of the work of Carlberg et al [22]. It is likely that some of the mutations will affect dimerisation with RXR whereas others may alter formation of homodimers.

JK, in whom no mutation was found presents the greatest challenge. Initial research could focus on identifying a transporter for VDR from cytoplasm to the nucleus. A defect in such a transporter could explain the clinical features and the receptor characteristics as described in this thesis.

Finally once the vitamin D receptor DNA binding domain is crystallised it will be very interesting to over-express the mutant proteins, from patients CC, FH, IS and TB, in an appropriate system [108]. The purified proteins could be used for x-ray crystallographic analysis and would allow confirmation of the stereochemical predictions made here.

Glossary

Amino acids

Hydrophobic side chains

Name	3 letter code	1 letter code
Alanine	Ala	A
Valine	Val	V
Isoleucine	Ile	I
Leucine	Leu	L
Methionine	Met	M
Phenylalanine	Phe	F
Tryptophan	Trp	W
Tyrosine	Tyr	Y

Polar but uncharged side chains

Serine	Ser	S
Threonine	Thr	T
Asparagine	Asn	N
Glutamine	Gln	Q

Positively charged side chains

Lysine	Lys	K
Arginine	Arg	R
Histidine	His	H

Negatively charged side chains

Name	3 letter code	1 letter code
Glutamic acid	Glu	E
Aspartic acid	Asp	D

Special amino acids

Hydrogen atom as side chain		
Glycine	Gly	G
Sulphur group as part of side chain		
Cysteine	Cys	C
Secondary amino group		
Proline	Pro	P

Abbreviations

$1\alpha(OH)\ D_3$	1-alpha hydroxy vitamin D
3′	3 prime end of a gene
5′	5 prime end of a gene
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
ACTH	Adrenocorticotrophin
AR	Androgen receptor
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C-terminal	Carboxy terminal
cAMP	Cyclic adenosine monophosphate

CAT	Chloramphenicol acetyltransferase
cDNA	Complimentary DNA
CK-II	Casein kinase II
CO_2	Carbon dioxide
cpm	Counts/minute
CRBPII	cellular retinol binding protein II
CTP	Cytidine triphosphate
D-box	Distal box (of DNA binding domain)
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTE	Dithioerythritol
DTT	Dithiothreitol
dTTP	deoxythymidine triphosphate
DZ	Dizygotic
ER	Oestrogen receptor
EDTA	ethylene diamine tetraacetic acid
ERE	Oestrogen response element
FCS	Foetal calf serum
GR	Glucocorticoid receptor
GRdbd	Glucocorticoid receptor DNA binding domain
GRE	Glucocorticoid response element
GTP	Guanosine triphosphate
HBS	Hepes-buffered saline
HCl	Hydrogen chloride

Hepes	*N*-2-hydroxyethyl piperazine *N'*-2-ethane sulphonic acid
HPLC	High performance liquid chromatography
HRE	Hormone response element
Hsp	Heat shock protein
HVDRR	Hereditary vitamin D resistant rickets
IAA	Isoamyl alcohol
IPTG	isopropyl-ß-D-thiogalactosidase
K_2HPO_4	Dipotassium hydrogen phosphate
KAc	Potassium acetate
KCl	Potassium chloride
K_d	Dissociation constant
kDa	Kilodalton
KH_2PO_4	Potassium dihydrogen phosphate
LB	Luria-Bertani Medium
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
MMTV LTR	mouse mammary tumour virus promoter long-terminal repeat
MOPS	3-(*N*-morpholino) propane sulfonic acid
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
MZ	Monozygotic
N-terminal	Amino terminal
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NBRE	NGFB-I response element

NGFI-B	nur77, an orphan nuclear receptor
NMR	Nuclear magnetic resonance
OD	Optical density
P-box	Proximal box (of the DNA binding domain)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDDR	Pseudovitamin D deficiency rickets
PK-A	cAMP-dependent protein kinase
PR	Progesterone receptor
PTH	Parathyroid hormone
RAR	Retinoic acid receptor
rATP	Riboadenosine triphosphate
rCTP	Ribocytidine triphosphate
RFLP	Restriction length fragment length polymorphism
rGTP	Riboguanosine triphosphate
RNA	Ribonucleic acid
rNTP	Ribonucleoside triphosphate
RPM	Revolutions per minute
RT	Reverse transcription
rUTP	Ribouridine triphosphate
RXRα	Retinoid X receptor alpha
RXRβ	Retinoid X receptor beta
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
T_3	Triodothyronine
T_3R	Thyroid hormone receptor
T_4	Thyroxine
TAE	Tris-acetate-EDTA buffer

TAF	Transcription activation function
Taq	*Thermus aquaticus*
TB	Terrific broth
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	(*N,N,N',N'*-) tetramethylethylenediamine
TEN	Tris-EDTA-Saline buffer
TFIIIA	Transcription factor IIIA
TKEDM	Buffer consisting of Tris-HCl (10 mM; pH 7.4) 300 mM KCl, 1.5 mM EDTA, 1 mM dithiothreitol and 10 nM sodium molybdate
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl) amino methane
TSH	Thyroid stimulating hormone
TTP	Thymidine triphosphate
UV	Ultraviolet
VDR	Vitamin D receptor
VDRE	Vitamin D response element
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactosidase
Zn^{2+}	Zinc ion

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Two Mutations in the Hormone Binding Domain of the Vitamin D Receptor Cause Tissue Resistance to 1,25 Dihydroxyvitamin D_3

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Abstract

We have identified and characterized two mutations in the hormone binding domain of the vitamin D receptor (VDR) in patients with hereditary vitamin D–resistant rickets. One patient was found to have a premature stop mutation (CAG to TAG) in the hinge region affecting amino acid 149 (Q149X) and the other demonstrated a missense mutation (CGC to CTC) resulting in the substitution of arginine 271 by leucine (R27IL) in the steroid binding domain. Eukaryotic expression analyses in CV-1 cells showed the inability of both patients' VDR to induce transcription from the osteocalcin hormone gene response element at 10^{-7} M 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$). Normal transcription levels could, however, be elicted by the missense mutated VDR (R271L) in the presence of 1,000-fold higher 1,25-$(OH)_2D_3$ concentrations than needed for the wild-type receptor. This shows that Arg 271 directly affects the affinity of the VDR for its ligand and its conversion to leucine decreases its affinity for 1,25$(OH)_2D_3$ by a factor of 1,000. Arg 271 is located immediately 3-prime to a 30 amino acid segment (VDR amino acids 241-270) that is conserved among members of the steroid/thyroid/retinoid hormone receptor superfamily. These results represent the first missense mutation identified in the hormone binding domain of VDR and further define the structure–function relationship of 1,25$(OH)_2D_3$ ligand binding to its nuclear receptor. (*J. Clin. Invest.* 1993. 92:12–16.) Key words: rickets • calcitrol • steroid • genetic • transcription-genetic

Introduction

The biological effects of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$),[1] the hormonal form of vitamin D, are mediated by its binding to a specific intracellular receptor present in target cells (1–3). The 48-kD receptor belongs to the family of steroid/thyroid/retinoid nuclear hormone receptors (4). These receptors act in *trans* by binding to specific *cis*-acting DNA sequences in the promotor regions of hormone-responsive genes, thereby modulating their transcription (5). Receptors belonging to this superfamily comprise two functional domains: a ligand binding and a DNA binding domain (4). The DNA binding domains show high evolutionary conservation in a region of 60–70 residues that form two zinc fingers essential for interaction with DNA (4, 6–10). The high amino acid homology within this region predicts a three-dimensional organization common to the individual members of the gene family. The interaction of the glucocorticoid receptor DNA binding domain with its DNA hormone response element has recently been resolved by crystallographic analysis (11), and on this basis we have analyzed the stereochemical consequences of vitamin D receptor (VDR) mutations identified in patients with tissue resistance to 1,25$(OH)_2D_3$ (12).

Structure–function relationships of the hormone binding domain are much less well understood. The potential importance of individual amino acids in this domain is inferred by their evolutionary conservation throughout the receptor superfamily (4). For example, subdomains within the carboxy-terminal half of these receptors that affect ligand binding, receptor dimerization, and transcriptional activation have been identified (9, 13–17). Further insight has been obtained from mutations intentionally created in this domain, together with reports of receptor mutations in patients with tissue resistance to the various steroid hormones (9, 13, 15, 18–26). Only one of the published mutations in the human VDR has been in the hormone binding domain (25). Here we report the identification and characterization of two mutations located in the hormone binding domain of this receptor from patients with classical features of hereditary vitamin D–resistant rickets (HVDRR).

Methods

Patients. The patients presented in early childhood with classical features of tissue resistance to 1,25$(OH)_2D_3$, including severe bowing of weight-bearing bones (rickets), and one patient had alopecia. They both had elevated parathyroid hormone and very high levels of serum 1,25$(OH)_2D_3$. The patients were unrelated but both were of Middle Eastern origin and their parents were consanguineous. Neither showed any clinical improvement by high dose 1,25$(OH)_2D_3$ therapy (up to 50 μg/d). Biochemical evaluation was consistent in both patients with HVDRR. Detailed case histories have previously been published and receptor binding data showed no detectable cytoplasmic or nuclear 1,25$(OH)_2[^3H]D_3$ association (27, 28). 25-Hydroxyvitamin-D-24-hydroxylase induction was measured in skin fibroblasts obtained by biopsies from patients and normal individuals. The conversion of 25-hydroxyvitamin-[^{3}H]D to 24,25-dihydroxyvitamin-[^{3}H]D by the 24-hydroxylase was determined as described (29) and plotted against the elution profile for a mixture of standard vitamin D metabolites. The production of radiolabeled 24,25-dihydroxyvitamin D_3 was expressed as femtomoles per hour per 10^6 cells.

This work has been reported in abstract form (1992. *Am. J. Hum. Genet.* 51:A131).

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1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; HVDRR, hereditary vitamin D–resistant rickets; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VDR, vitamin D receptor.

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RNA isolation, cDNA synthesis, and PCR amplification. Total RNA was isolated from patient skin fibroblast cultures by standard protocols (RNAzol B; Cinna/Biotex Laboratories, Houston, TX). First strand cDNA synthesis was performed with Superscript RNase H-reverse transcriptase (Bethesda Research Laboratories, Gaithersberg, MD), 10 μg total RNA, and a VDR primer specific to the early 3′ untranslated sequence (primer 31b: 5′-GCCCTGGAGGAGCAGCCC, 0.8 M final concentration; Fig. 1). The reaction was incubated at 45°C for 60 min in a total volume of 40 μl. The full-length VDR coding region was amplified by PCR (30) using primers 31a (5′-GAGCACCCCTGGGCTCCA) and 31b (0.16 μM final concentration) (Fig. 1) and 2 μl of the reverse transcription reaction as template (standard buffer conditions of Perkin Elmer-Cetus, Norwalk, CT, and 2.0 mM $MgCl_2$ with 35 cycles of 90°C for 1 min, 50°C for 1 min and 20 s, and 72°C for 6 min). To facilitate complete sequencing, each half of the coding region was amplified by a nested PCR reaction, using primers 32a (5′-CCTGCCCCTGCTCCTTC) and 32b (5′-AGGTCAGCCAGGTGGGGC) for the 5′ half and 33a (5′-CCCAGCTCTCCATGCTGC) and 33b (5′-CCCAGGCACCGCACAGGC) for the 3′ half (0.16 μM primer concentration at 2.5 and 1.5 mM $MgCl_2$, respectively, for 35 cycles at 95°C for 1 min and 20 s and 72°C for 1 min and 40 s, with 4 s extension per cycle; Fig. 1).

Cloning and sequencing of amplified DNA. The PCR products were cloned into Bluescript II pSK- plasmid (Stratagene, La Jolla, CA). Positive clones were sequenced by the dideoxy chain termination method using fluorescence labeled M13 reverse primer on an automated sequencer (Applied Biosystems, Inc., Foster City, CA). The full-length VDR coding region was subcloned in a sense orientation into the EcoR I and Xba I sites of the expression vector pSVK3 (Pharmacia, LKB Biotechnology Inc., Piscataway, NJ) (31). For exclusion of Taq polymerase and sequencing errors, all steps were performed in duplicate, starting with the reverse transcriptase reaction, and several clones of each PCR product were sequenced in both orientations.

Reporter construct. A 881-bp 5′-untranslated fragment of the human osteocalcin gene including the VDR hormone response element (32) was amplified by PCR from human genomic DNA using the following primers: 5′-CTGCAGGGTCAGGAGGAGAATCG and 5′-GTCGCGTCGGTGGGCTCT. Reaction conditions included standard Cetus buffer, 2.0 mM $MgCl_2$, and 0.2 μM primer concentration for 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min. The PCR product was cloned into the reporter plasmid pCAT Basic (Promega Corp., Madison, WI), thereby fusing the osteocalcin gene promoter/enhancer region upstream of the chloramphenicol acetyltransferase (CAT) gene.

VDR cotransfection assay in CV-1 cells. VDR-deficient CV-1 cells were grown in DME supplemented with 10% FCS and L-glutamine (2 mM). 24 h before transfection the cells were plated to a density of 1×10^6 cells/10-cm plate. Transfection was carried out by the polybrene method as described (7) using 1 μg of expression plasmid together with 5 μg of human osteocalcin reporter plasmid. After a 30-s glycerol shock, cells were cultured in DME-Nutridoma (Boehringer Mannheim Corp., Indianapolis, IN) supplemented with the desired concentrations of $1,25(OH)_2D_3$ in ethanol (final concentration 0.1%). Cells were washed with PBS and harvested by scraping 72 h later, subjected to three freeze-thaw cycles (37°C/−135°C) in 0.25 M Tris-HCl, pH 7.8, and centrifuged, and the supernatant (20 μg protein) was assayed for CAT activity (37°C overnight) (33). Products were resolved by thin layer chromatography and after autoradiography the relevant portions were excised and quantitated by liquid scintillation counting. The results are expressed as percentage CAT conversion and represent the average of three separate transfection experiments.

Results

Initial assessment of VDR bioactivity in fibroblasts of these patients was determined by $1,25(OH)_2D_3$-mediated induction of the 25-hydroxyvitamin-D-24-hydroxylase (34). Cells from both patients failed to show any significant induction of 24-hydroxylase activity when cultures were exposed to $1,25(OH)_2D_3$ at concentrations as high as 1 μM (results not shown). This contrasts with normal control fibroblasts that produced 489± 119 fmol/h per 10^6 cells of 24,25-dihydroxyvitamin D_3 at 10 nM $1,25(OH)_2D_3$ and is in agreement with the lack of response to vitamin D_3 therapy in these patients.

Mutational analysis. A different nucleotide point mutation was identified in each patient. One was found to have C → T transition at codon 149 (CAG to TAG; Fig. 2), replacing the normal glutamine codon with a termination codon and resulting in a truncated receptor missing 303 residues of the hormone binding domain. The other patient demonstrated a substitution of a G for a T in codon 271, resulting in a missense mutation (CGC to CTC), with exchange of a basic hydrophobic arginine for a nonpolar, hydrophobic leucine (Fig. 2). In both patients and in four others (unpublished results) we identified a single initiation codon (ATG) at nucleotide position 125, counting from the beginning of the published sequence (GenBank# J03258) (7). This is different from the originally reported sequence in which two putative transcription initiation sites (at 116 and 125 bp) were found (7). The former of these was absent in all of our patients, consistent with the findings of Saijo et al. (35). This could represent a polymorphism in the population resulting in two different receptor species that differ in length by 3 amino acids at the amino terminus. Our numbering of amino acids and their codons is from the single initiation site at position 125.

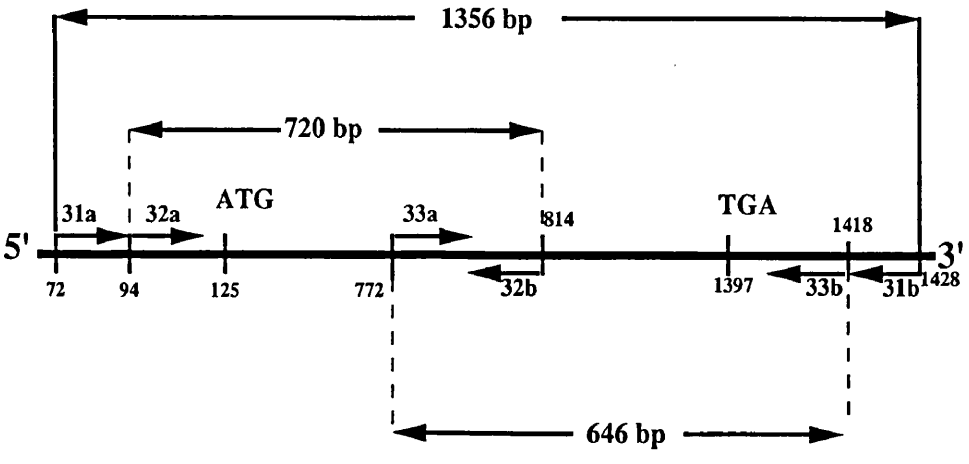

Figure 1. Reverse transcription and PCR amplification of the hVDR cDNA. cDNA was made from total RNA using primer 31b. The full-length coding region was amplified from the first strand cDNA by PCR using primers 31a and 31b. Each half of the gene was subsequently amplified by nested PCRs using primers 33a and 33b for the 3′ half and 32a and 32b for the 5′ half, giving rise to 646- and 720-bp fragments, respectively. The PCR products were then gel purified, cloned, and sequenced. Base-pair numbering refers to the location of the primer ends with respect to the cDNA sequence as originally published.

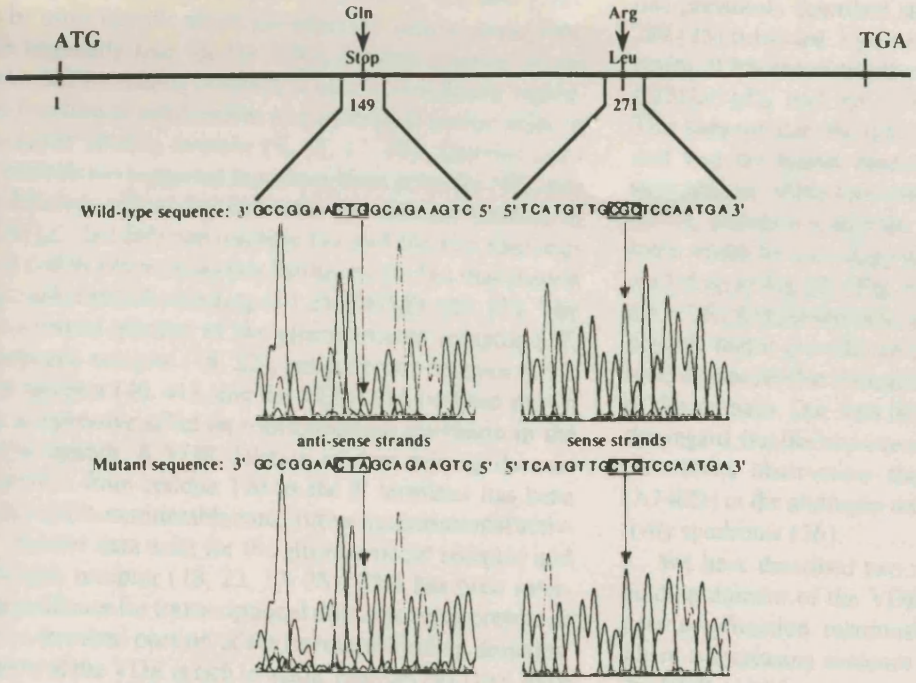

Figure 2. Sequencing data and location of mutations in two patients with HVDRR. One patient was found to have a C to T transition in codon 149 in the hinge region, changing it from CAG (glutamine) to TAG (termination codon). Shown is the actual sequence of the antisense strand from this patient with the involved codon boxed. The other patient had a substitution of a G for a T in codon 271 in the steroid binding domain, resulting in a missense mutation of CGC to CTC and an exchange of arginine for leucine. Shown is the actual sequence of the sense strand for this patient with the corresponding codons boxed.

Transcriptional activation by cotransfection in CV-1 cells. The ability of the mutant receptors to activate transcription was evaluated in receptor-deficient CV-1 cells cotransfected with VDR expression plasmid (patient or wild type) and human osteocalcin reporter plasmid. There was strong induction of CAT activity by 1 nM $1,25(OH)_2D_3$ in CV-1 cells transfected with the wild-type VDR construct but no significant induction in those transfected with receptor cDNA from the patients (Fig. 3). A titration with $1,25(OH)_2D_3$ concentration varying from 0.01 nM to 1 μM showed that CAT activity could be induced at ~ 50 nM hormone in the cells transfected with the missense mutated (R271L) VDR, reaching maximum induction at 1 μM $1,25(OH)_2D_3$, i.e., the same as the maximum for the wild-type protein (Fig. 4). Consequently, mutation R271L decreases the affinity of the VDR for the ligand by ~ 1,000-fold.

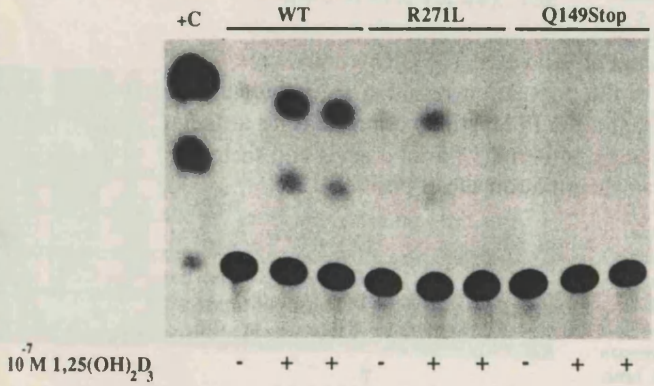

Figure 3. Comparing the transcriptional activation of the osteocalcin promotor by wild-type VDR and the two mutant VDR in the absence and presence of $1,25(OH)_2D_3$ (10^{-7} M). Wild-type VDR and mutant VDR expression vector constructs were transfected into CV-1 cells along with a human osteocalcin plasmid. Cellular extracts prepared 72 h later were examined for CAT activity. Each lane represents a separate transfection. Cells in the first lane of each set received ethanol only, while cells in the other lanes received 100 nM $1,25(OH)_2D_3$ in ethanol. Mutant VDRs show insignificant transcriptional activation of the osteocalcin promotor compared with the wild-type VDR when stimulated with $1,25(OH)_2D_3$.

Discussion

It is now well established that HVDRR is caused by mutations in the VDR (25, 35, 36). With increasing knowledge about the structure–function relationship of the different members in the

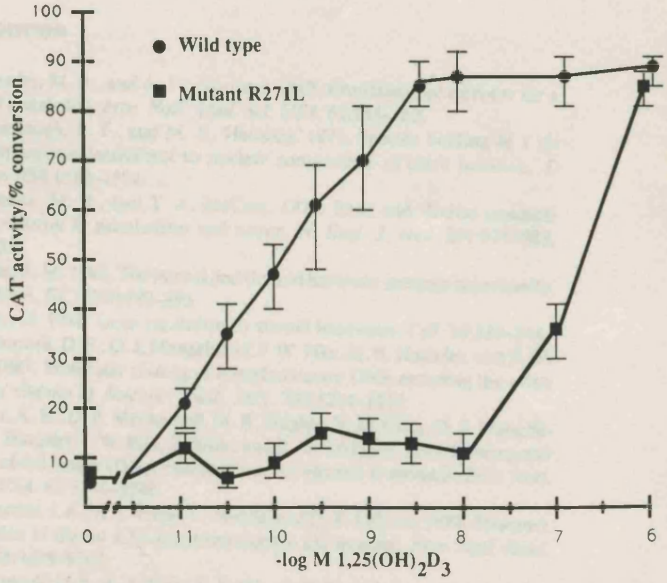

Figure 4. Effect of increasing concentrations of $1,25(OH)_2D_3$ on transcriptional activation by wild-type and missense mutated (R271L) VDR. CV-1 cells were transfected with wild-type VDR expression plasmid or the R271L mutant VDR expression plasmid (5 μg each) along with the osteocalcin reporter plasmid (1 μg). Cells were treated with ethanol only or ethanol containing the $1,25(OH)_2D_3$ needed to give the concentrations indicated. Each point represents the average of three transfections.

steroid/thyroid/retinoid receptor superfamily, it is now possible to be more specific about the effects of various mutations. This is especially true for the DNA binding domain of the receptors but increasing evidence is also accumulating regarding the function of subdomains and individual amino acids in the hormone binding domain (9, 13, 17, 25). Deletion construct analysis has suggested that the critical point for NH_2-terminal deletions still giving high affinity wild-type binding of $1,25(OH)_2D_3$ lies between residues 114 and 166 (9). Our mutation at codon 149 narrows this further by the fact that there is no detectable cytosolic binding of $1,25(OH)_2D_3$ (27, 28). The COOH-terminal portion of the glucocorticoid receptor (37, 38), androgen receptor (18, 22), progesterone receptor (39), estrogen receptor (40, 41), and the VDR (9) have been shown to have a repressive effect on transcriptional activation in the absence of ligands. A VDR deletion mutant missing the carboxyl portion from residue 190 to the 3′ terminus has been shown to exhibit considerable constitutive transcriptional activity (9). Similar data exist for the glucocorticoid receptor and the androgen receptor (18, 22, 37, 38). This has been interpreted as evidence for transcriptional domains being present in the amino-terminal portion of the hormone binding domains. This region of the VDR is rich in acidic residues (42) that have been implicated as being important in gene activation within yeast transcription factors (43) and steroid receptors (44). In the mammalian cotransfection analyses reported here, no transcriptional activation by the cDNA Q149X mutant was detected. This could be explained by deletion of these transcriptional activation domains. Additionally, a premature stop mutation at codon 149 not only deletes the entire portion of the molecule that is postulated to form the actual binding pocket for $1,25(OH)_2D_3$, but it also deletes a dimerization domain that is essential for receptor binding to the VDR hormone response element. This domain (VDR residues 241–263; Fig. 5) is highly conserved between all the members of the superfamily and is involved in interaction with nuclear proteins (13, 15, 23), possibly RXRbeta (14, 16). Point mutations within this domain do not interfere with ligand binding but affect the ability of the receptors to form heterodimers, bind with their response elements, and initiate transcription (13, 15, 23). The one previously described stop mutation at amino acid codon 789 (25) is located 3-prime to all of the above-mentioned domains. It has the same effects, with total tissue insensitivity to $1,25(OH)_2D_3$ and no specific $1,25\text{-}[^3H](OH)_2D_3$ binding. This suggests that the different domains form one functional unit and the ligand binding pocket is constructed by long stretches of three-dimensionally folded polypeptide. The R271L mutation is adjacent to the presumed dimerization domain which by homology with the other receptors can be extended up to Arg 271 (Fig. 5). This residue is conserved among the VDR, thyroid receptor, and retinoic acid receptor, and it is possible that it provides an important ligand contact or represents a polar residue critical for the three-dimensional structure of this domain. Our data do not clarify the role of Arg 271 in this regard, but the importance of this residue is emphasized by the recent observation that a mutation at this position (A746D) in the androgen receptor results in androgen insensitivity syndrome (26).

Figure 5. Conservation within a 49 amino acid sequence in the ligand binding domain of the steroid/thyroid/retinoid receptor superfamily. Shown is the sequence of the human VDR along with the homologous regions of the thyroid receptor (*TR*), retinoic acid receptor (*RAR*), estrogen receptor (*ER*), glucocorticoid receptor (*GR*), androgen receptor (*AR*), and progesterone receptor (*PR*). Numbers refer to the position of the first residue on the left for each receptor. Two dots indicate identical amino acid residues. Boxed and shaded areas show the residues conserved in VDR. Our mutation at position 271 (R271L) is indicated by the arrow.

We have described two novel mutations in the hormone binding domain of the VDR that give further insight to the structure–function relationship of this part of the protein. There is increasing evidence that binding of $1,25(OH)_2D_3$ to the VDR modifies protein–protein interaction and transcriptional activation through modification of the receptor's structure. The full understanding of the structure–function relationship of the hormone binding domains of the steroid/thyroid/retinoid receptors awaits the determination of their three-dimensional structure by x-ray crystallography.

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Tissue resistance to 1,25-dihydroxyvitamin D without a mutation of the vitamin D receptor gene

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Summary

OBJECTIVE Hereditary vitamin D resistant rickets (HVDRR) is characterized by severe rickets and is often accompanied by alopecia. Mutations in the gene encoding the vitamin D receptor have been found in this condition. In a patient with the characteristic phenotype we have investigated the functional defect and sequenced the gene to seek a mutation.

DESIGN Patient and control cell lines prepared from skin fibroblasts and peripheral blood lymphocytes were used to measure binding of $1,25(OH)_2D_3$ and to isolate vitamin D receptor mRNA. VDR cDNA was sequenced and transfected into receptor defective cells.

PATIENT A child with alopecia diagnosed as having rickets due to resistance to $1,25(OH)_2D_3$.

MEASUREMENTS Cytosolic binding and nuclear association of $1,25(OH)_2D_3$ were determined in patient and control cells, and functional response to $1,25(OH)_2D_3$ assessed by measurement of 24-hydroxylase activity. VDR mRNA was prepared, reverse transcribed, and cDNA sequenced. VDR cDNA was also transfected into VDR-deficient CV-1 cells and functional response to $1,25(OH)_2D_3$ assessed by co-transfection with a chloramphenicol acetyltransferase (CAT) reporter plasmid.

RESULTS VDR from the patient were able to bind $1,25(OH)_2D_3$ but showed no nuclear localization resulting in an absence of functional response to $1,25(OH)_2D_3$. Sequencing revealed that the VDR coding region was normal. Expression studies of the patient's VDR showed functionally normal VDR as evidenced by normal *trans*-activation in the presence of $1,25(OH)_2D_3$.

CONCLUSION These data indicate a new cause of tissue resistance to $1,25(OH)_2D_3$ which occurs in the absence of mutations in the coding region of VDR gene and which is characterized by defective nuclear localization of this receptor.

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Rickets caused by insensitivity to vitamin D can be classified as two separate and rare disorders. The first, vitamin D resistant rickets type I (VDRRI) is an autosomal recessive disorder characterized by defective 1α-hydroxylation of the substrate 25-hydroxyvitamin D_3 ($25(OH)D_3$) to the active, hormonal form, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (Scriver *et al.*, 1982; Fraser *et al.*, 1973). The second, vitamin D resistant rickets type II (VDDRII), which occurs in the face of raised circulating levels of $1,25(OH)_2D_3$, has been shown to be due to target-organ resistance to calcitriol (Brooks *et al.*, 1978; Marx *et al.*, 1978; Rosen *et al.*, 1979; Zerwekh *et al.*, 1979; Sockalosky *et al.*, 1980; Liberman *et al.*, 1980; Kodoh *et al.*, 1981; Beer *et al.*, 1981). VDRII is inherited in an autosomal recessive manner and is now more commonly called hereditary vitamin D resistant rickets (HVDRR). The salient features of HVDRR are severe rickets unresponsive to pharmacological doses of vitamin D preparations, marked hypocalcaemia, secondary hyperparathyroidism and sometimes, for reasons that remain unclear, alopecia.

There has been considerable interest in mechanisms of end-organ resistance to calcitriol, particularly in the characterization of defects in vitamin D receptors. Studies have been facilitated by the presence of the receptor in many tissues. Fibroblasts isolated from skin biopsies have been used to demonstrate abnormal binding of $1,25(OH)_2D_3$ in HVDRR patients (Feldman *et al.*, 1980; Eil *et al.*, 1981; Liberman *et al.*, 1983). It has been shown that HVDRR is a heterogeneous disease, the main sub-groups being those with defective ligand binding, those with defective nuclear localization of hormone–receptor complexes and those with defective binding of hormone–receptor complexes to DNA.

Cloning and sequencing of human VDR (Baker *et al.*, 1988) has allowed extensive analysis of the VDR gene, further clarifying defects in VDR function. In particular, several reports have shown that point mutations in the VDR gene can cause target-organ resistance to $1,25(OH)_2D_3$. A number of mutations have been described, principally in the DNA binding domain of the VDR (Hughes *et al.*, 1988; Ritchie *et al.*, 1989; Sone *et al.*, 1990; Saijo *et al.*, 1991; Rut *et al.*, 1992). In the most recent of these studies, we have outlined the specific stereochemical effects of mutations in

conserved regions of the DNA binding domain. We and others have described patients in whom resistance to $1,25(OH)_2D_3$ has originated from mutations to the steroid binding domain (Rut *et al.*, 1991; Ritchie *et al.*, 1989). Here we report a case of abnormal VDR expression in which nuclear localization of receptor–$1,25(OH)_2D_3$ complexes was absent, resulting in a decreased functional response to $1,25(OH)_2D_3$, further emphasizing the heterogeneous nature of HVDRR. Sequencing data indicated that this defect was not due to a mutation in the coding region of the VDR gene. Furthermore, we have shown that transfection of VDR cDNA from this patient into mammalian cells produced a normal *trans*activation response to $1,25(OH)_2D_3$ as measured by induction of CAT activity.

These studies suggest that some cases of HVDRR may occur in the presence of a normal coding region for VDR and the basis of this disorder would therefore appear to be abnormal VDR activity in this patient's cells. In this particular case abnormal nuclear association of $1,25(OH)_2D_3$–VDR complexes indicates a possible defect in factors associated with VDR translocation, and we discuss the possible mechanisms for this.

Materials and methods

Patient

Patient J.K. is the daughter of unrelated English parents. Her problems began at the age of 6 weeks when abnormal hair loss was observed. At 16 months she was noted to have swollen wrists and ankles and had difficulty in walking; she was still unable to run at 2 years and 8 months. Apart from her skeletal manifestations she was a normal, cheerful, intelligent child. Initial biochemical investigations showed the following: calcium (corrected for albumin), 2·03 mmol/l (normal 2·25–2·55 mmol/l); phosphate, 0·78 mmol/l (normal 1·29–1·79 mmol/l); alkaline phosphatase, 1101 U/l (normal range, < 300 U/l). Subsequent studies showed that serum 25-hydroxyvitamin D_3 was normal (20 nmol/l, normal range 7·5–100 nmol/l). Skeletal survey revealed marked osteopenia with metaphyseal deformity typical of untreated rickets. She was treated initially with Alfacalcidol up to 5 μg/day and because of hypophosphataemia, phosphate supplements up to 750 mg/day. There was no response within 3 months. The child was then referred to The Hospital for Sick Children at the age of 2 years and 10 months. The Alfacalcidol was stopped for one week and serum levels of $1,25(OH)_2D_3$ when first measured were 466–650 pmol/l (normal radioimmunoassay range for an adult 48–156 pmol/l) (Clemens *et al.*, 1978). 25-Hydroxyvitamin D_3 was 87 nmol/l and c-PTH, 1283 ng/l (normal, < 660 ng/l). Treatment with Alfacalcidol was restarted and the dose increased progressively to 12 μg/day. The phosphate supplements were stopped and replaced with calcium up to 1 g/day. Within 8 months her biochemical profile had normalized (serum calcium, 2·30 mmol/l; phosphate, 1·16 mmol/l; alkaline phosphatase, 890 U/l, normal range 250–1000 U/l) and radiologically the rickets had healed but alopecia persisted. Treatment was stopped after 20 months (age 4 years 4 months) and there has been no relapse of rickets. The patient is now 6·5 years old and well. Serum calcium (2·39 mmol/l), phosphate (1·69 mmol/l) and alkaline phosphatase (166 U/l) are normal whilst serum $1,25(OH)_2D_3$ remains slightly elevated at 115 pmol/l (normal radioreceptor assay range for adult < 100 pmol/l) (Incstar Kit; Hollis, 1986).

Cell culture

Skin fibroblasts were isolated by collagenase digestion of punch biopsies of patient and four age-matched controls. Studies were carried out on patient skin cells isolated at the age of 2 years and 9 months and functional assays were confirmed by a further skin biopsy at the age of 6 years and 6 months. Established cultures were routinely maintained in Dulbecco's Modified Eagles Medium (DMEM) (Gibco) supplemented with 10% fetal calf serum, insulin, penicillin, streptomycin and glutamine. Transformed B-lymphocytes were prepared using EDTA-treated blood isolated from J.K. and an aged-matched control. Leucocytes were isolated by centrifugation with Histopaque-1077 (Sigma) and the resulting cells resuspended in RPMI 1640 (Gibco) supplemented with 10% FCS, penicillin, streptomycin, glutamine, HEPES buffer and β-mercaptoethanol. Non-adherent cells were resuspended in RPMI containing Epstein–Barr virus (EBV) (200 μl of viral solution/10^6 cells) and after a minimum of 4 hours cells were resuspended in RPMI containing cyclosporin (20 μg/l). Transformed B-cells were then cultured in RPMI only.

Analysis of VDR

Binding of $1,25(OH)_2D_3$ to cytosolic VDR. Cells were harvested by trypsinization and centrifugation (fibroblasts) or simply centrifugation (B cells). The resulting cell pellets were resuspended in cold TKEDM buffer consisting of Tris-HCl (10 mM, pH 7·4), 300 mM KCl, 1·5 mM EDTA, 1 mM dithiothreitol and 10 mM sodium molybdate, disrupted by sonication at 4°C and then centrifuged at 100 000 *g* for 1 hour at 4°C. Aliquots of the cytosol (200 μl; approximately 0·5 mg protein) were incubated at 4°C for 16 hours with 0·2–3·0 nM ^3H-$1,25(OH)_2D_3$ (in 0·1% ethanol) in the presence or absence of a 200-fold excess of unlabelled $1,25(OH)_2D_3$ to determine non-specific binding. Unbound hormone was removed by

centrifugation with dextran-coated charcoal and bound ^{3}H-1,25(OH)$_2$D$_3$ measured by scintillation counting. Data were analysed by Scatchard plots.

Nuclear association assays. The ability of 1,25(OH)$_2$D$_3$-VDR complexes to associate with nuclei was assessed by whole cell nuclear association assay. Cells were cultured as above but pellets were resuspended in serum-free DMEM to a density of 10^7 cells/ml. Aliquots of these cells (100 μl) were incubated for 1 hour at 37°C with increasing doses of ^{3}H-1,25(OH)$_2$D$_3$ (0·02–3·0 nM in 0·1% ethanol) in the presence or absence of a 200-fold excess of unlabelled 1,25(OH)$_2$D$_3$ (to determine non-specific binding). Nuclear pellets were then isolated by washing with a Tris buffer containing 0·25 M sucrose and 1% Triton X-100 (pH 7·4). Pellets were dissolved and radioactivity counted. Data were again analysed by Scatchard plots.

24-Hydroxylase activity. The ability of control and patient cells to respond to treatment with 1,25(OH)$_2$D$_3$ was assessed by the activity of the enzyme 24-hydroxylase. Fibroblasts were cultured to semi-confluence. Twenty-four hours prior to assay DMEM was supplemented with 10^{-8}–10^{-6} M 1,25(OH)$_2$D$_3$ (in 0·1% ethanol). Single assays were carried out for each concentration of 1,25(OH)$_2$D$_3$ by replacing media with serum-free DMEM containing 10 nM ^{3}H-25-hydroxyvitamin D$_3$ (^{3}H-25(OH)D$_3$ in 0·1% ethanol) and re-incubation for a further 3 hours at 37°C. Vitamin D metabolites were then extracted from the suspension as described (Hewison *et al.*, 1989) and separated by HPLC using a Zorbax-sil column. Fractions were collected and counted for radioactivity which was plotted against the elution profile for a mixture of standard vitamin D metabolites. The production of radiolabelled 24,25(OH)$_2$vitamin D$_3$ (24,25(OH)$_2$D$_3$) was measured as fmoles/h/10^6 cells.

Analysis of VDR gene

Isolation and characterization of RNA. Total RNA was prepared from patient's skin fibroblasts by standard protocols (Chomczynski & Sacchi, 1987), resolved on a 1% agarose/formaldehyde gel and transferred by blotting to a Genescreen plus membrane. Northern hybridization was carried out at 65°C in buffer containing 60% formamide, 5 × Denhardts, 5 × SSC, 0·4% SDS, 0·02 M phosphate, 0·1 mg/l yeast tRNA, 0·01 mg/l yeast poly-A-RNA and 200 mg/l salmon sperm DNA. A 650-bp VDR riboprobe (corresponding to the steroid binding domain of VDR cDNA) was used which had been radioactively labelled using a T7 transcription kit (Stratagene) and 1 μg of template pKS-VDR cDNA. Final washes were performed at 65°C in 0·1 × SSC and 0·5% SDS.

cDNA synthesis and sequencing. All steps were carried out in duplicate. cDNA was prepared from total fibroblast RNA using a specific primer, 31b (5'GCCCTGGAGGAGCAG-CCC3'), an 18-mer starting at position 1329 in the 3' untranslated region of the VDR cDNA sequence (Baker *et al.*, 1988) and Superscript RNase H-reverse transcriptase (Bethesda Research Laboratories). The coding region was amplified by PCR with primers 31b and 31a (5'GAGCA-CCCCTGGGCTCCA3'). Each half of the full-length coding region was further amplified using nested primers. Following PCR amplification VDR cDNA was purified by electrophoresis in low melting agarose and cloned into a Sma I cut Bluescript II pSK vector. Positive clones were sequenced with M13 reverse primers with tagged fluorescent labels using an automated Applied Biosystems (ABI) system. Several clones from each amplification process were sequenced and the whole process was repeated starting with the revese transcription.

Receptor expression and transfection. VDRA expression plasmid was prepared using full length coding region cDNA cloned into a SmaI cut Bluescript II pSK-vector (as described above). The inserts were subcloned into the expression vector pSVK3 (Promega) (Mongkolsuk, 1988) in the sense orientation with respect to the T7 promoter. Wild-type VDR cDNA (2005 bp) was excised from pGEM4 (Baker *et al.*, 1988) and cloned into pSVK3. The reporter construct was made using an 881 bp fragment of the human osteocalcin regulatory region cloned directionally into a promoterless chloramphenicol acetyltransferase vector (pCAT Basic Promega), thus fusing the osteocalcin promoter upstream of the chloramphenicol acetyltransferase gene. 1 μg expression plasmid and 5 μg reporter plasmid were co-transfected into steroid receptor-deficient CV-1 cells using the polybrene method (Kawai & Nishizawa, 1984). Cells were then cultured in medium DMEM containing Nutridoma (Boehringer Mannheim) supplemented by 10^{-9}–10^{-7} M 1,25(OH)$_2$D$_3$ or vehicle (0·1% final concentration) and harvested 48 hours post-transfection. CAT activity was assessed in an overnight assay at 37°C. The products were visualized by thin layer chromatography (TLC) (Gorman *et al.*, 1982).

Results

Initial characterization of VDR expression in fibroblasts and B cells from patient J.K. indicated that VDR were present in TKEDM cytosolic extracts. Figure 1 shows representative Scatchard plots of 1,25(OH)$_2$D$_3$ binding in patient and control cytosol from both fibroblasts and B cells. In Fig. 1a

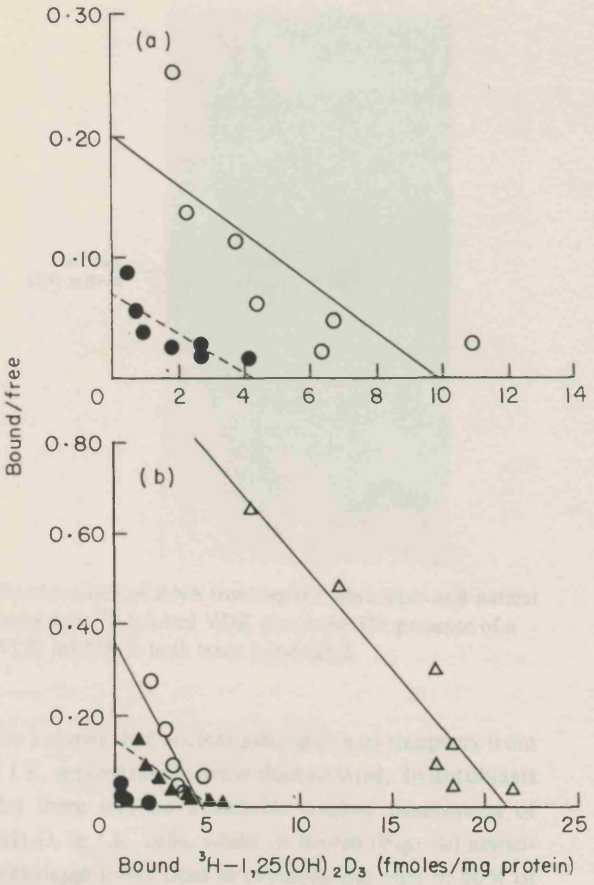

Fig. 1 Binding of ^{3}H-1,25(OH)$_2$D$_3$ to cytosolic VDR from skin fibroblasts and transformed B-cells. a, ○, Control fibroblasts were compared to ●, J.K. fibroblasts. b, ○, Control B-cells and △, B-cells treated with PMA were compared to ●, J.K. B-cells and ▲, J.K. B-cells treated with PMA. Results are shown as Scatchard plots in which maximal binding (fmoles/mg cytosolic protein) is represented by the intercept on the X-axis and binding affinity (K_d) by the slope of the plot.

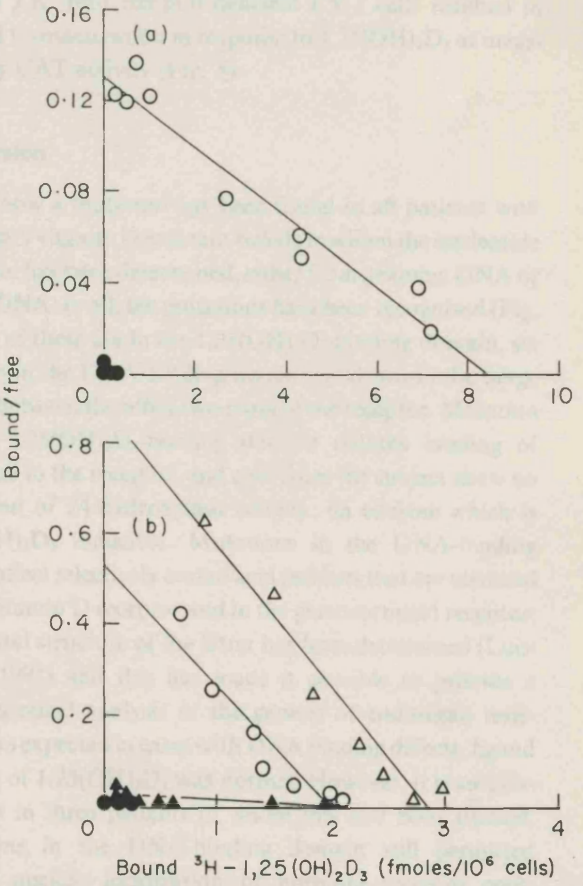

Fig. 2 Nuclear association of ^{3}H-1,25(OH)$_2$D$_3$ in skin fibroblasts and transformed B-cells. a, ○, Control fibroblasts were compared to ●, J.K. fibroblasts. b, ○, Control B-cells and ▲, B-cells treated with PMA were compared to ●, J.K. B-cells and ▲, J.K. B-cells treated with PMA. Analysis of binding was as in Fig. 2.

maximal binding of 1,25(OH)$_2$D$_3$ in fibroblasts (X-axis intercept) was lower in J.K. (2753 VDR/ng cytosolic protein) than parallel controls (6020 VDR/ng cytosolic protein), but mean values from two experiments (3254 ± 708 VDR/ng cytosolic protein) were not significantly different from pooled control values (4450 ± 1801 VDR/ng cytosolic protein, $n = 4$). The affinity of VDR for 1,25(OH)$_2$D$_3$ (K_d – slope of Scatchard plot) was $4{\cdot}7 \pm 0{\cdot}5 \times 10^{-10}$ M in J.K. cells compared to pooled control values of $3{\cdot}2 \pm 1{\cdot}4 \times 10^{-10}$ M.

Similar observations were made in transformed B-cells (Fig. 1b). J.K. cells expressed lower numbers of cytosolic VDR than did control cells. Treatment of the cells with the phorbol ester PMA (10^{-8} M) for 48 hours increased 1,25(OH)$_2$D$_3$-binding capacity in both control and J.K. cells. Binding affinity (K_d) was slightly lower in J.K. cells (3·2 and $1{\cdot}8 \times 10^{-10}$ M) than in controls (0·7 and $1{\cdot}0 \times 10^{-10}$ M).

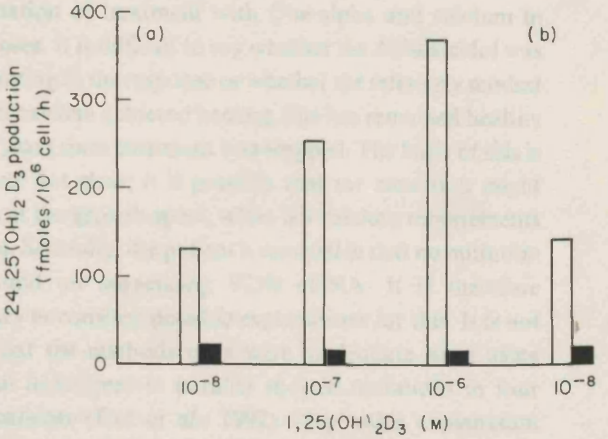

Fig. 3 Induction of 24-hydroxylase activity in fibroblasts after treatment with 1,25(OH)$_2$D$_3$ (10^{-8}–10^{-6} M) for 24 hours, $n = 1$. a, Dose-response values for control fibroblasts and J.K. fibroblasts isolated at age 2 years 9 months. b, A single dose assay carried out on fibroblasts isolated at age 6 years 6 months. □, Control; ■, J.K.

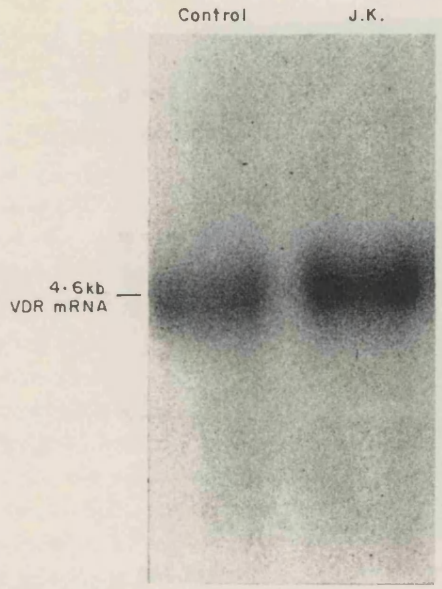

Fig. 4 Northern blot of RNA from control fibroblasts and patient J.K. probed with ^{32}P-labelled VDR riboprobe.The presence of a 4·6-kb VDR mRNA in both lanes is indicated.

Figure 2 shows that nuclear association of receptors from patient J.K. was markedly lower than controls. In fibroblasts (Fig. 2a) there was no detectable nuclear association of $1,25(OH)_2D_3$ in J.K. cells, whilst in B-cells (Fig. 2b) association was much lower than in controls, but rose to 60% of control values after PMA induction (1379 VDR/cell in J.K. *vs* 2139 VDR/cell in control). However, the K_d for VDR in PMA-treated B-cells was much higher in J.K. (59×10^{-10} M) than in the control ($0{\cdot}6 \times 10^{-10}$ M) indicating lower affinity binding.

The functional consequence of abnormal nuclear association of VDR in J.K. cells was illustrated by their inability to express 24-hydroxylase activity. Figure 3a shows that treatment of control fibroblasts with 10^{-8}–10^{-6} M $1,25(OH)_2D_3$ for 24 hours stimulated the production of $24,25(OH)_2D_3$ from $25(OH)D_3$ in a dose-dependent fashion. This response was absent in J.K. skin cells isolated at the age of 2 years and 9 months (Fig. 3a) and also when the patient was aged 6 years and 6 months (as shown by the single dose assay in Fig. 3b). Her parents had normal VDR (as measured by cytosolic binding and nuclear association) and showed normal induction of 24-hydroxylase activity.

The molecular basis of the condition was studied further in three ways. Northern blotting showed that normal length VDR mRNA (4·6 kb) was present (Fig. 4). Sequencing revealed a single initiation codon at position 125 from the CAP site. No mutations were identified within the coding region of the gene, this process being carried out in duplicate starting from RNA. Transfection of VDR cDNA from patient J.K. into receptor-deficient CV-1 cells resulted in normal transactivation in response to $1,25(OH)_2D_3$ as measured by CAT activity (Fig. 5).

Discussion

Up to now a mutation has been found in all patients with hereditary vitamin D resistant rickets in whom the nucleotide sequence has been determined, either from genomic DNA or from cDNA. In all, ten mutations have been recognized (Fig. 6): two of these are in the $1,25(OH)_2D_3$-binding domain, six of them in the DNA-binding domain, and two in the hinge region between the other two parts of the receptor. Mutation in the $1,25(OH)_2D_3$-binding domain reduces binding of hormone to the receptor, and cells from the subject show no induction of 24-hydroxylase activity, an enzyme which is $1,25(OH)_2D_3$ inducible. Mutations in the DNA-binding region affect selectively amino acid residues that are identical in the vitamin D receptor and in the glucocorticoid receptor; the crystal structure of the latter has been determined (Luisi *et al.*, 1991) and this has made it possible to provide a stereochemical analysis of the genesis of end-organ resistance. As expected in cases with DNA binding defects, ligand binding of $1,25(OH)_2D_3$ was normal. However, it is surprising that in three patients in whom this had been studied, mutations in the DNA-binding domain still permitted normal nuclear localization of hormone–receptor complexes, even though those mutations modify the specific binding to target genes. This emphasizes that nuclear uptake is dependent on other, less well defined, processes.

Phenotypically, the patient described here had classical end-organ resistant rickets, with alopecia and raised serum $1,25(OH)_2D_3$. She is, however, unusual in two ways. First, it was possible to obtain complete healing of the rickets with a combination of treatment with One-alpha and calcium in large doses. It is difficult to say whether the Alfacalcidol was contributing to the response or whether the relatively modest doses of calcium achieved healing. She has remained healthy for 2·5 years since treatment was stopped. The basis of this is of course not clear; it is possible that the condition might relapse at the growth spurt, when her calcium requirements increase. Secondly, the patient is unusual in that no mutation was found on sequencing VDR cDNA. It is therefore necessary to consider possible explanations for this. It is not likely that the methods used were inadequate since using identical techniques in parallel showed mutations in four other patients (Rut *et al.*, 1992). A possible explanation would be that the patient, whose parents were not related, was a compound heterozygote inheriting two different mutations, one from each parent, and that these were missed by the technique used. However, VDR cDNA from J.K. skin

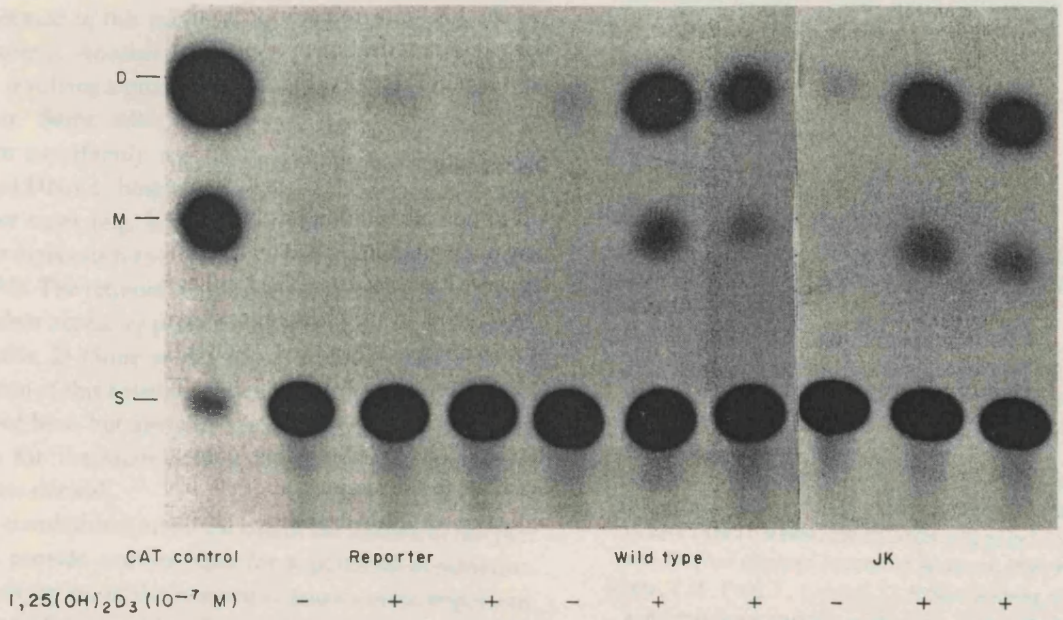

Fig. 5 CAT assays following transient transfection of VDR cDNA from patient (J.K.) and from a pGEM construct prepared from normal ('wild type') VDR cDNA into CV-1 cells. VDR cDNA was co-transfected with an osteocalcin reporter plasmid fused to the chloramphenicol acetyltransferase gene (CAT). Results show the presence of mono-(M) and di-acetylated (D) derivatives (reference standards shown as CAT control lane) after treatment of CV-1 cells with $1,25(OH)_2D_3$ (10^{-7} M). S, Substrate; Reporter, negative control lanes using cells transfected with reporter plasmid only.

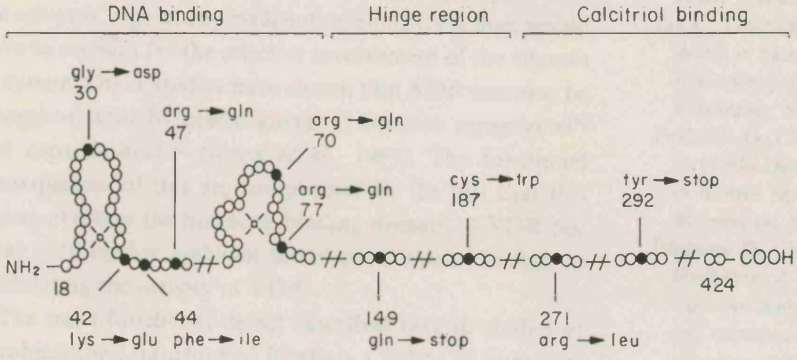

Fig. 6 Schematic representation of VDR amino acid sequence showing reported mutations (Hughes *et al.*, 1988; Ritchie *et al.*, 1989; Sone *et al.*, 1990; Saijo *et al.*, 1991; Rut *et al.*, 1992). □, Zn; ●, mutant amino acid.

fibroblasts functioned normally in CV-1 cells, so she possessed at least one normal sequence. Moreover, since end-organ resistance is inherited in an autosomal recessive fashion, the possibility of her being a compound heterozygote can be excluded. It is feasible, of course, that the patient (whose parents were normal) had a new, dominant mutation in one copy of the gene and that it was a copy of the other, normal gene that was transfected into the CV-1 cells. It is also possible that the 5′ or 3′ untranslated regions were polymorphic, affecting gene expression. Against this is the fact that VDR are present in cytosolic extracts of the patient's cells, indicating normal mRNA and protein stability.

Consideration of the mode of action of the vitamin D receptor in other members of the superfamily of steroid hormone receptors provides other possible explanations that need to be considered. Binding of hormones may be modulated by non-steroid binding proteins, which include the 90 kDa heat shock protein (hsp90). Dissociation of this protein from the glucocorticoid receptor is necessary for it to associate with DNA (Sanchez, 1985). However, it is not clear that hsp90 is needed for the action of the vitamin D receptor. In new world monkeys, there is resistance to the action of $1,25(OH)_2D_3$ even though receptors are present and it has been suggested that there is a soluble protein that prevents intracellular binding of $1,25(OH)_2D_3$ (Gacad & Adams,

1991). This is therefore not analogous to the defect described here because in this patient ligand binding of $1,25(OH)_2D_3$ was normal. Another possibility to consider is an abnormality involving a protein that dimerizes with the vitamin D receptor. Some other members of the nuclear hormone receptor superfamily, e.g. the glucocorticoid receptor, bind to target DNA as homodimers (Kumar & Chambon, 1988). In other cases (e.g. VDR), heterodimers are formed with other proteins such as retinoid Xα and β receptors (Bugge *et al.*, 1992). The retinoid Xα receptor is probably the same as the nuclear accessory protein that is important to the action of vitamin D (Sone *et al.*, 1991). An abnormality in the formation of this heterodimer could account for the findings described here, but any such abnormality would have to be specific for the vitamin D system since the patient was otherwise normal.

Post-translational modifications of the vitamin D receptor protein provide another basis for a potential explanation. Phosphorylation of the receptor is known to be important. Mutation, for example of serine[48] so that it cannot be phosphorylated, inhibits transcriptional activity experimentally (Hsieh *et al.*, 1991). In this context, it is of interest that treatment of the patient's transformed B cells with the phorbol ester PMA induced nuclear association, albeit at lower affinity than normal: this treatment would activate protein kinase C and so might stimulate phosphorylation of the receptor. Again, any explanation along these lines would have to account for the selective involvement of the vitamin D system. Other studies have shown that VDR can also be phosphorylated by casein kinase II between asparagine[160] and aspartic acid[232] (Jones *et al.*, 1991). The functional consequences of this are complicated by the fact that this region overlaps the hormone binding domain of VDR but these data further highlight the possible role of kinases in modulating the activity of VDR.

The main functional defect described here in studies of fibroblasts and transformed B-cells is a failure of translocation to the nucleus, as reflected by reduced nuclear association. It is this which has to be explained if the genesis of this patient's rickets is to be understood. At present, relatively little is known of the translocation process and so the defect cannot yet be adequately explained, but it is clear that the syndrome of end-organ resistance is heterogeneous and that we describe here a new form of the condition.

Acknowledgements

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Two mutations causing vitamin D resistant rickets: modeiling on the basis of steroid hormone receptor DNA-binding domain crystal structures

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Summary

OBJECTIVE Hereditary vitamin D resistant rickets (HVDRR) has been shown to be due to mutations in the gene encoding the vitamin D receptor (VDR). In two patients with the characteristic phenotype we have investigated the functional defect and sequenced the VDR cDNA. We report two new mutations in the DNA binding domain of the VDR gene and we have used the crystallographic structure of the glucocorticoid and oestrogen receptors (GR and ER respectively) as models to explain the stereochemical consequences of these mutations.

DESIGN Patient and control cell lines prepared from skin fibroblasts were used to measure binding of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and functional responses to this hormone. These cells were also used to isolate VDR mRNA from which cDNA was prepared and sequenced. VDR cDNA from affected and control patients was also transfected into receptor defective cells to analyse further functional responses to $1,25(OH)_2D_3$. Computer analysis of mutations in the VDR gene was carried out using the glucocorticoid and oestrogen receptors as model systems.

PATIENTS Two patients with HVDRR from unrelated families.

MEASUREMENTS Cytosolic binding and nuclear association of $1,25(OH)_2D_3$ were determined in control and affected patients, and functional response to $1,25(OH)_2D_3$ was assessed by measurement of 25-hydroxyvitamin D-24-hydroxylase activity (24-hydroxylase). VDR cDNA was sequenced and transfected into VDR-deficient CV-1 cells for further analysis of functional response to $1,25(OH)_2D_3$ following cotransfection with a chloramphenicol acetyltransferase (CAT) reporter plasmid.

RESULTS Cells from HVDRR patients I and II showed detectable numbers of VDR with normal hormone binding. However, unlike controls, the HVDRR cells did not show induction of 24-hydroxylase activity following treatment with $1,25(OH)_2D_3$. Sequencing of cDNA revealed single mutations, in patient I (Phe44 → Ile) and in patient II (Lys42 → Glu). Both these residues are conserved in the steroid/thyroid hormone receptor superfamily and stereochemical analysis has been used to deduce the importance of these amino acids and the deleterious effect of these and other mutations in the DNA-binding domain of the VDR.

CONCLUSIONS Two new mutations in the vitamin D receptor which cause hereditary vitamin D resistant rickets have been described and using molecular modelling we have been able to analyse the genesis of this inherited disease at the level of stereochemistry.

Hereditary 1,25-dihydroxy-vitamin D_3 resistant rickets (HVDRR) is most frequently an autosomal recessive disorder characterized by severe rickets, hypocalcaemia, secondary hyperparathyroidism and, occasionally, the absence of body hair (Rosen *et al.*, 1979; Liberman *et al.*, 1980). It has been shown that the pathological process involves resistance of target tissues to the actions of $1,25(OH)_2D_3$, the hormonal form of vitamin D (Feldman *et al.*, 1982; Chen *et al.*, 1984). The cloning of the receptor for $1,25(OH)_2D_3$ (VDR) (Baker *et al.*, 1988) allowed further advances in the understanding of the disease. Eleven mutations in the VDR gene have been reported and suggested as the primary cause of HVDRR (Hughes *et al.*, 1988; Ritchie *et al.*, 1989; Sone *et al.*, 1990; Saijo *et al.*, 1991; Yagi *et al.*, 1993; Kristjansson *et al.*, 1993, Wiese *et al.*, 1993). One patient with all the features of the disease was found not to have a detectable mutation in the VDR gene (Hewison *et al.*, 1993).

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The 48-kDa vitamin D receptor displays the characteristics of a steroid/thyroid nuclear hormone receptor. It is composed of two principal domains, one for hormone binding and one for DNA binding (Yamamoto, 1985; Evans, 1988; Beato, 1989; O'Malley, 1990). On binding $1,25(OH)_2D_3$ by the hormone binding domain, VDR is activated to associate with specific DNA sequence elements and modulate the transcription of downstream vitamin D-responsive genes. DNA binding and target specificity are mediated by the DNA binding domain, which includes two zinc finger-like modules (Luisi *et al.*, 1991). One of the target genes of the VDR encodes the human bone-specific protein, osteocalcin. A sequence motif positioned 500 bp upstream of the human osteocalcin gene has been identified as the hormone response element for the vitamin D receptor (Umesono & Evans, 1989; Ozono *et al.*, 1990; Kerner *et al.*, 1989). Reporter genes under the control of the human osteocalcin gene regulatory region are $1,25(OH)_2D_3$ responsive, and this system has provided a convenient means of studying the transcription activity of VDR. The biological actions of $1,25(OH)_2D_3$ can also be studied using skin fibroblasts which express vitamin D receptors (Feldman *et al.*, 1980) and exhibit 25-hydroxy-vitamin D 24-hydroxylase induction following treatment with $1,25(OH)_2D_3$ (Chandler *et al.*, 1984). This enzyme serves as a marker for evaluating functional responses to the hormone.

Here we describe two new mutations which are associated with HVDRR and map to the DNA binding domain of the VDR. We characterize the receptors functionally and describe a model to explain the stereochemical consequences of the mutations. In both the above patients as well as in three others (Kristjansson *et al.*, 1993; Hewison *et al.*, 1993), sequencing of VDR cDNA and upstream region revealed a single start codon at position 125 from the cap site. This triplet appears to be the true initiation codon, and we number the bases and residues accordingly. The remainder of the sequence indicated that in patient I there is a mutation at base 130 (TTC → ATC) resulting in an amino acid change of Phe44 → Ile44 and in patient II a mutation at base 124 (AAA → GAA) resulting in the substitution of Lys42 → Glu42. Both mutations are located in the DNA recognition helix of the DNA binding domain.

The crystal structures of the DNA binding domains of the glucocorticoid receptor (GR) and oestrogen receptor (ER) in complexes with their respective response elements have been determined and have provided details of the receptor/DNA interactions (Luisi *et al.*, 1991; Schwabe *et al.*, 1993). We have used these crystal structures as models for the interactions of the VDR with target DNA and we discuss the consequences of the above mutations as well as the previously reported mutations in the DNA binding domain of the vitamin D receptor in the light of these model systems.

Materials and methods

Patients

We studied two patients with hereditary vitamin D resistant rickets from unrelated families. The parents of both patients were phenotypically normal, but were not studied in detail. Patient I is descended from non-consanguineous Mauritian parents (Lin & Uttley, 1993). The family originate from India, but lived in Mauritius for four generations before coming to the UK. She presented at 9 months of age with failure to thrive, irritability, alopecia and rickets. Biochemical analysis showed marked hypocalcaemia (1·69 mmol/l), normal levels of 25-OH vitamin D_3 (4·5 μg/l), and markedly elevated levels of $1,25(OH)_2D_3$ (400 ng/l) with secondary hyperparathyroidism. Treatment with prolonged intra-atrial calcium infusions resulted in resolution of the symptoms and the rickets.

Patient II is Tunisian and his parents were first cousins (Simonin *et al.*, 1992; case 2). He was referred to hospital in France at the age of 6 years with rickets, partial alopecia and short stature. Serum biochemistry showed marked hypocalcaemia (1·67 mmol/l), normal levels of 25-OH vitamin D_3 (19 μg/l), elevated levels of $1,25(OH)_2D_3$ (250 μg/l) and secondary hyperparathyroidism. Treatment with $1,25(OH)_2D_3$ had no effect.

Characterization of vitamin D receptors

VDR expression and assessment of functional activity were carried out using fibroblasts cultured from skin biopsies. Cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) with L-glutamine (Gibco) supplemented with 10% fetal calf serum, insulin (1 IU/ml), penicillin (100 U/ml), streptomycin (100 g/l) at 37°C and a humidified atmosphere with 5% CO_2. The ability of control and patient cells to respond to treatment with $1,25(OH)_2D_3$ was assessed by measuring 24-hydroxylase activity as previously described (Gamblin *et al.*, 1985). $1,25(OH)_2D_3$ receptor assays were performed by described methods (Karmali *et al.*, 1989). Receptor numbers and dissociation constant (K_d) were calculated by Scatchard analysis. The ability of $1,25(OH)_2D_3$-VDR complexes to associate with nuclear material was assessed by whole cell nuclear association assay, as previously described (Hewison *et al.*, 1989). Data were again analysed by Scatchard plots.

First-strand cDNA synthesis and amplification of vitamin D receptor cDNA

Total RNA was prepared from cultured patient's skin fibroblasts by standard protocols (Chomczynski & Sacchi, 1987). All subsequent steps were carried out in duplicate. cDNA was prepared from total fibroblast RNA (10 μg) using primer 31b (5′-GCCCTGGAGGAGCAGCCC-3′) and Superscript RNase H-reverse transcriptase (Bethesda Research Laboratories) at 45°C for 60 minutes in a total reaction volume of 40 μl. The coding region was amplified by the polymerase chain reaction (PCR) (Saiki *et al.*, 1985) with primers 31b (see above) and 31a (5′-GAGCACCCCT-GGGCTCCA-3′) using 2 μl of the first strand mixture as template in a standard buffer (Perkin Elmer-Cetus) containing 2·0 mM $MgCl_2$. Thirty-five cycles of the following process were used: denaturation at 95°C for 1 minute, annealing at 61°C for 1 minute 20 seconds, extension at 72°C for 6 minutes. Each half of the full-length coding region was further amplified using nested primers. For the 5′ part these were 32a (5′-CCTGCCCCCTGCTCCTTC-3′) and 32b (5′-AGGTCAGCCAGGTGGGGC-3′). The amplification was performed in a standard buffer containing 2·5 mM $MgCl_2$ and 35 cycles of the following process: denaturation at 95°C for 1 minute, annealing at 59°C for 1 minute 20 seconds and elongation at 72°C for 1 minute 40 seconds with 4 seconds extension per cycle. For the 3′ part primers 33a (5′-CCCAGCTCTCCATGCTGC-3′) and 33b (5′-CCCAGGCACCGCACAGGC-3′) were employed and the amplification was performed in a standard buffer containing 1·5 mM $MgCl_2$ with 35 cycles of the following process: denaturation at 95°C for 1 minute, annealing at 59°C for 1 minute 20 seconds and extension at 72°C for 1 minute 40 seconds. The primer concentrations in all the above reactions was 0·16 μM. PCR amplification was performed in a room separate from the cloning procedures and for each experiment numerous blank reactions were included.

Cloning and sequencing of amplified cDNA

Following PCR amplification, the material of interest was identified by agarose gel electrophoresis in low melting agarose and extracted by a freeze and squeeze method (Qian & Wilkinson, 1991). DNA was cloned into Bluescript II pSK^-. Positive clones were sequenced with M13 reverse primers tagged with fluorescent labels, on an Applied Biosystems (ABI) apparatus. All inserts were cloned into Bluescript II pSK^- to facilitate sequencing of the whole fragment. Several clones from each amplification process were sequenced and the whole process was repeated starting with the RT-PCR.

Receptor expression vectors

The full-length coding region was amplified using primers 31a and 31b in a standard buffer containing 1·5 mM $MgCl_2$. Thirty-five cycles of the following process were used: 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute. PCR products were treated as before and cloned into a SmaI cut Bluescript II pSK^- vector. Inserts were subcloned directionally into the EcoR1 and Xba1 sites or the Xba1 and Xho1 sites of the expression vector pSVK3 (Promega) in the sense orientation with respect to the T7 promoter. Wild-type VDR cDNA, previously cloned into pGEM4 was subcloned in a sense orientation into the Xba1 and Xho1 sites of pSVK3.

Reporter constructs

PCR was used to amplify 877 bp of the human osteocalcin gene promoter region starting from −833 bp 5′ and ending at +44 bp 3′, relative to the cap site of the gene (negative numbers indicating sequence extending 5′). This region has been shown to contain a vitamin D response element between −510 bp and −483 bp (Kerner *et al.*, 1989; Ozono *et al.*, 1991). Primers were designed to incorporate restriction sites to facilitate cloning into the desired vector. Primers R_1 (5′-CGACTGCAGGGTCAGGAGGAGAATCG-3′ with the Pst1 site underlined) and R_2 (5′-CTGTCTA-GATCTCGGGTGGCTGCGCTG-3′ with the Xba1 site underlined) were used. The Pst1 site is endogenous to the osteocalcin promoter at −828 bp, whilst the Xba1 site in primer R_2 is not native to the osteocalcin gene. Thirty-five cycles of PCR were performed in a standard buffer containing 2·0 mM $MgCl_2$ under the following conditions: denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 2 minutes. The products were cloned directionally into a chloramphenicol acetyltransferase vector (pCAT Basic Promega), thus fusing the osteocalcin regulatory region upstream of the chloramphenicol acetyl-transferase gene. DNA for transfection was purified on anion exchange resin columns (Qiagen).

Transfection studies

Steroid receptor-deficient CV1 cells were grown in DMEM supplemented by 5% new-born calf serum. Twenty-four hours prior to transfection, cells were plated to a density of 1×10^6/100 mm plate. Cells were cotransfected with 5 μg of reporter plasmid and 1 μg of expression plasmid using the polybrene method (Sone *et al.*, 1989). Following a 30-s glycerol shock, cells were cultured in DMEM plus Neutrodoma (Boehringer Mannheim) supplemented by

Table 1 Characterization of ligand binding, nuclear localization and functional response of VDR in skin fibroblasts from patients and controls

	24-Hydroxylase activity (fmol/10^6 cells/h)	Cytosolic VDR (VDR/mg cytosolic protein)	Nuclear VDR (VDR/cell)
Controls	489 ± 119	3951 ± 1447 (K_d = 0·40 nM ± 0·15)	3687 ± 855 (K_d = 0·38 nM ± 0·19)
Patient I	17	8346 (K_d = 0·66 nM)	3151 (K_d = 1·07 nM)
Patient II	u.d.	8069 (K_d = 0·22 nM)	4209 (K_d = 0·66 nM)

u.d., Undetectable.

10^{-7} M $1,25(OH)_2D_3$ or 0·1% ethanol (final concentration). CAT activity was assessed as previously described (Sone *et al.*, 1989).

Molecular modelling

The coordinates of the crystal structure of the glucocorticoid receptor–DNA complex have been submitted to the Brookhaven data bank and are available from Dr Luisi. Modelling was performed with Program O. Illustrations were generated using MOLSCRIPT (Kraulis, 1991).

Results

Receptor characteristics

The results in Table 1 show both the functional activity and binding characteristics of VDR from patients and controls. It contrasts the induction of 24-hydroxylase activity in three control cell populations in response to $1,25(OH)_2D_3$, with the markedly reduced response to $1,25(OH)_2D_3$ in the two patients. The normal controls produced 489 ± 119 fmol/h/10^6 cells of $24,25(OH)_2D_3$ whereas patient II showed no induction of 24-hydroxylase activity in the presence of 10 nM $1,25(OH)_2D_3$ and patient I produced only 17 fmol h/10^6 cells. This indicated that cells from both patients were functionally insensitive to $1,25(OH)_2D_3$. VDR were present in cells from both patient I and II as evidenced by binding of radiolabelled $1,25(OH)_2D_3$ to isolated cytosolic protein. The numbers of VDR per mg cytosolic protein from patient I (8346 VDR/mg cytosolic protein) and patient II (8069 VDR/mg cytosolic protein) were somewhat higher than controls (n = 3; 3951 VDR/mg protein ± 1447). The K_d values for cytosolic ligand binding in both patients were within the error of the control value. Nuclear association showed similar numbers of VDR/cell to the controls,

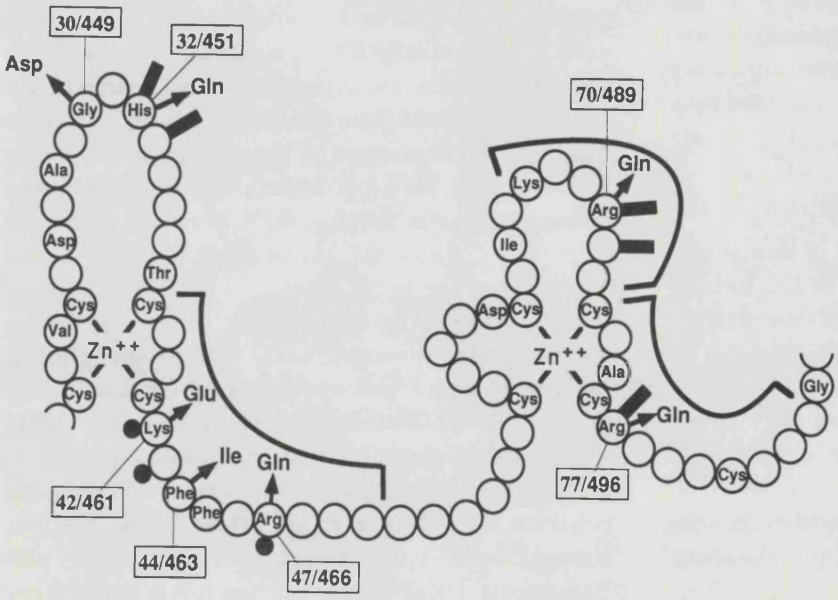

Fig. 1 Mutations in the DNA binding domain of VDR. The two zinc modules are shown. Mutations in VDR are identified by arrows; boxes alongside these mutations give the residue number in VDR followed by that of the corresponding residue in GR. Labelled residues are identical in GR and VDR; those differing in the two receptors are unlabelled and are shown by open circles. Note that all the mutations occur in residues that are identical in normal GR and VDR. Indicated symbols denote the three helical regions of GR and ER and residues that make contacts with either bases or phosphate. ●, Specific base contacts; ■, specific H-bonding to PO_4; —, helical regions.

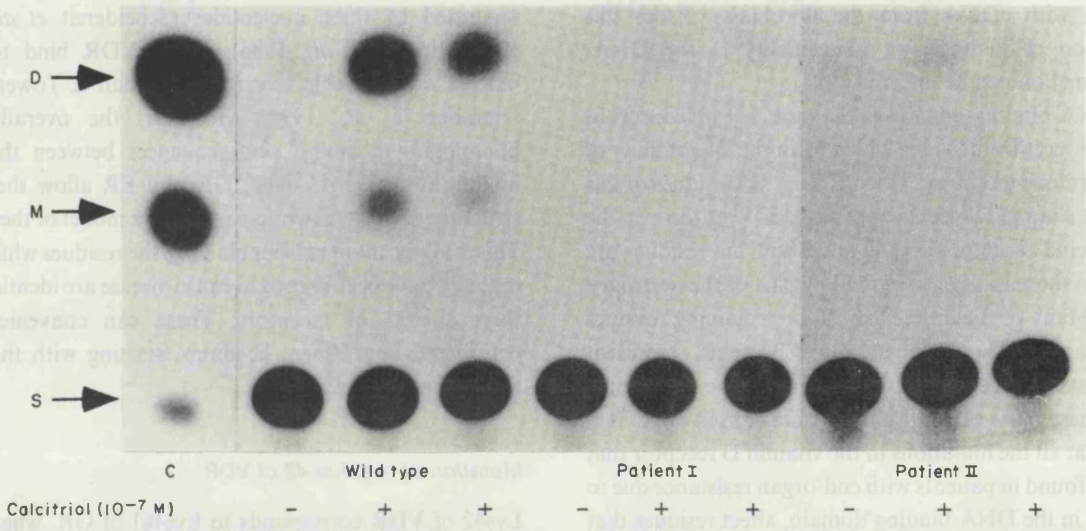

Fig. 2 Induction of CAT activity by VDR derived from patient and control cells. The CAT gene was under the control of osteocalcin gene regulatory sequences. Transcriptional activity was measured in transfected CV1 cells. Cells were treated with either 10^{-7} M $1,25(OH)_2D_3$ (calcitriol) (+) or 0·1% ethanol (−). C, CAT control; S, loaded sample of cellular extract; M, monoacetylated chloramphenicol product; D, diacetylated product.

although the K_d values for nuclear association (patient I; 1·07 nM and patient II; 0·66 nM) were slightly higher than those of control cells (0·38 ± 0·19).

Sequence analysis

Sequencing of cDNA from both patients demonstrated a single initiation codon 125 bases from the CAP site, which agreed with previous reports (McDonnell *et al.*, 1989; Saijo *et al.*, 1991; Yagi *et al.*, 1993). The first ATG (methionine) codon previously reported at position 116 (Baker *et al.*, 1988) was found to be absent in both patients (being AGC rather than ATG). Numbering of amino acids was therefore from the ATG at position 125. In patient I, sequencing revealed a point mutation (T → A) at position 130 causing the phenylalanine 44 codon (TTC) to become an isoleucine codon (ATC) (Fig. 1). In patient II, a point mutation at position 124 (A → G) was found causing the lysine 42 codon (AAA) to become a glutamic acid codon (GAA) (Fig. 1). The remainder of the sequence of the VDR coding region was normal in both patients. The findings were confirmed in clones obtained from two separate rounds of RT-PCR for each patient.

Expression of mutant VDR constructs

The capacity of the mutant receptors to stimulate transcription from vitamin D-responsive genes was examined by cotransfecting the cDNA from each patient with the human osteocalcin reporter plasmid, into steroid receptor-deficient CV1 cells and measuring chloramphenicol acetyltransferase (CAT) activity. Transfection with wild-type VDR cDNA resulted in hormone-dependent induction of transcription as measured by increased CAT (Fig. 2), which converts chloramphenicol to its monoacetylated and diacetylated forms. Neither of the mutant receptors was able to induce transcription from the osteocalcin gene promoter even when incubated with high levels of $1,25(OH)_2D_3$ (10^{-7} M).

Discussion

The two new mutations in the gene encoding the vitamin D receptor (changing Lys 42 to glutamic acid and phenylalanine 44 to isoleucine) described here add to the 11 mutations described previously. They affect the DNA binding domain of the receptor, as do five of the other mutations. The locations of these two mutations fit with the altered properties of the receptor found in the two patients, as studied in their cultured fibroblasts. The cytosol from them contained receptors that bound $1,25(OH)_2D_3$ with a normal K_d. Translocation of the bound hormone to the nucleus, as measured in the nuclear association assays, was also of normal capacity (albeit with a slightly lower affinity). Nevertheless, $1,25(OH)_2D_3$ could not induce the normal response in the patients' fibroblasts, as measured by induction of 24-hydroxylase activity. Insensitivity to the hormone was also demonstrated in CAT assays, using

constructs with cDNA from the fibroblasts. From this combination of findings, an abnormality in the DNA-binding function can be inferred.

The DNA-binding domain is the most highly conserved part of the receptor proteins which form the superfamily of steroid hormone receptors. Thus 25 out of 65 residues of this part of the vitamin D receptor are identical with those in the glucocorticoid receptor (Fig. 1) and 30 of the residues are identical to those in the corresponding part of the oestrogen receptor. Many of the differences in the remaining residues in this part of the three receptor proteins, represent conservative changes; for example 14 of the differences between these parts of VDR and GR are conservative. It is striking that all the mutations in the vitamin D receptor that have been found in patients with end-organ resistance due to mutations in the DNA-binding domain, affect residues that in normal subjects are identical to those in the glucocorticoid receptor (Fig. 1) and in the oestrogen receptor. These are, glycine at residue 30 in VDR (corresponding to 449 of GR), histidine at residue 32 (451 of GR), lysine at 42 (461 of GR), phenylalanine at 44 (463 of GR), and arginines at 47, 70 and 77 in VDR, (466, 489 and 496 in the glucocorticoid receptor). These correspond with residues 16, 18, 28, 30, 33, 56 and 63 of the 84 amino acid ER peptide used by Schwabe *et al.* (1993). That peptide corresponded to residues 179–262 of the complete human oestrogen receptor which has 595 amino acids.

The crystal structure of the DNA-binding domains of the glucocorticoid receptor and the oestrogen receptor complex to their respective DNA targets have been established (Luisi *et al.*, 1991; Schwabe *et al.*, 1993). Those studies provide a basis for consideration of the possible effects of mutations in this region of the vitamin D receptor, because of the strong homologies that exist within the superfamily of steroid hormone receptor proteins. This modelling is undertaken with the caveat that there are differences within the receptor proteins and in the hormone response elements of the DNA. Apart from differences in the amino acid sequences, there are differences in the way that the proteins bind to DNA. The glucocorticoid and oestrogen receptors bind as homodimers, while the vitamin D receptor can bind as a heterodimer with a nuclear accessory factor (NAF) (Sone *et al.*, 1991) or with the retinoid X receptor (RXR) (Yu *et al.*, 1991). Recently, Carlberg *et al.* (1993) have demonstrated that the RXR ligand and the nature of the response element determine whether a nuclear receptor is co-regulated by RXR. They have identified two types of VDRE that are activated either by a VDR homodimer (in osteocalcin), or by heterodimers of VDR and RXR (in osteopontin). The spacing between half-sites in target genes also differs; for example, GR and ER bind to palindromic sequences separated by three nucleotides (Scheidereit *et al.*, 1986; Klein-Hitpass *et al.*, 1986), whilst VDR bind to direct repeats with variable spacing (Freedman & Towers, 1991; Umesono *et al.*, 1991). However, the overall strong homologies in amino acid sequences between the DNA binding domains of VDR, GR and ER allow the crystal structure of the last two to be used as a model of the former. This is all the more reasonable since the residues which when mutated have been shown to cause disease are identical in all three species of receptors. These can conveniently be considered under three headings, starting with the mutations reported here.

Mutation at residue 42 of VDR

Lys42 of VDR corresponds to Lys461 of GR, where it lies on the hydrophilic side of the sequence recognition helix (Fig. 3a). The lysine donates a hydrogen bond to N7 and, via a water molecule, to the O6 of a guanine of the glucocorticoid response element. In the oestrogen receptor the corresponding lysine (28) also interacts with a guanine of the oestrogen response element, donating a direct hydrogen bond to the O6; additionally, the lysine donates a hydrogen bond to the side-chain of a neighbouring glutamic acid (Glu 25). This glutamic acid accepts a hydrogen bond from a C on the opposite DNA strand and from a fixed water molecule (Schwabe *et al.*, 1993). Thus, the lysine stabilizes the glutamic acid and neutralizes its negative charge. Glu25 of ER has been genetically defined as one of the residues helping to discriminate GREs and EREs by the respective receptors (Mader *et al.*, 1989). Like the ER, the VDR also has a Glu at the corresponding position 39, whereas GR has a glycine at 258. The guanine which is contacted by a lysine in both the GR and ER/DNA complexes as described above is common to most hormone response elements; as in the glucocorticoid and oestrogen response elements, a guanine may be found at the corresponding position of the vitamin D response elements. With these facts in mind, we believe that the mutation of lysine 42 → Glu in the VDR would have several effects. First, the hydrogen bond to the guanine would simply be lost. Secondly, the mutant Glu42 might attract a hydrogen bond from the imidazole of the neighbouring His 32 (see Fig. 3a) and so disrupt its own interaction with DNA phosphate. Thirdly, changing the lysine to glutamate would eliminate its stabilizing role in neutralizing Glu38 and result in a double negative charge at the N-terminus of the recognition helix.

Mutation at residue 44 of VDR

The effects of the mutation Phe44 → Ile can also be

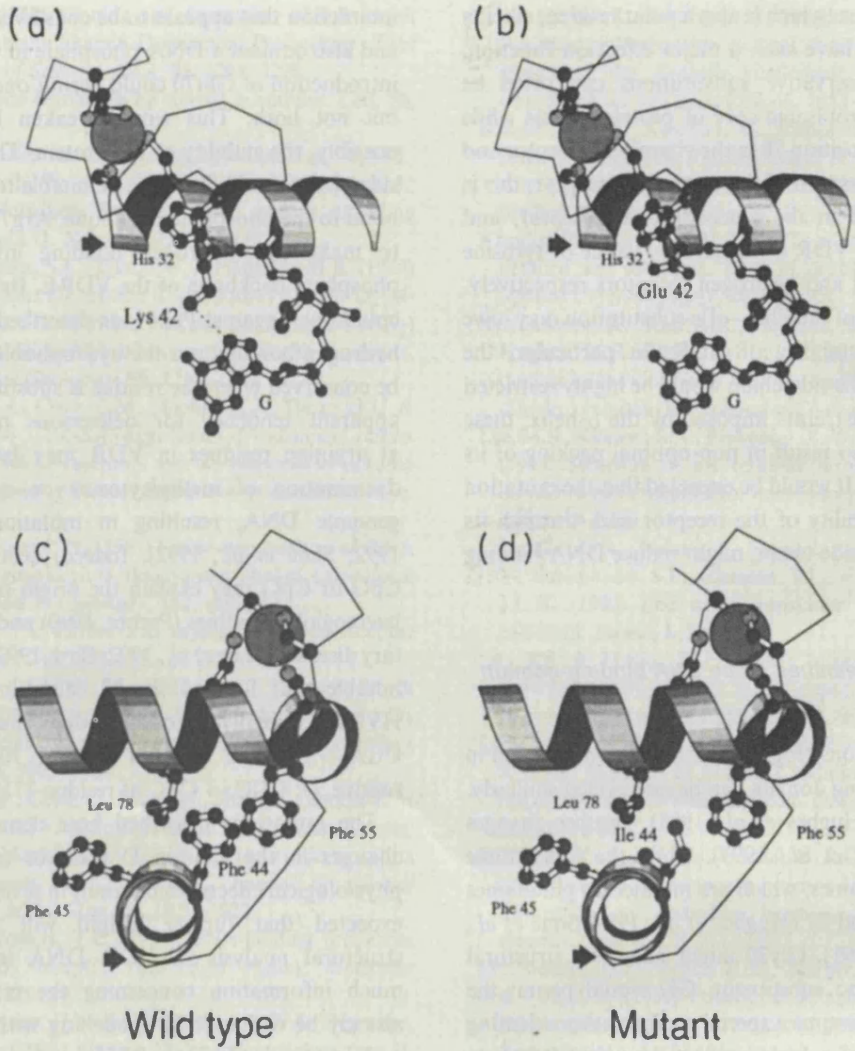

Fig. 3 Models of normal and mutant VDR showing the possible effects of substitutions at residues 42 (a and b) and 44 (c and d). Models were prepared from the structure of the GRdbd/DNA complex (Luisi *et al.*, 1991). The recognition helix is indicated with an arrow. Zinc atoms are shown as spheres. The zinc atom shown in the upper left of the illustration is the one in the amino terminal zinc module, while that in the lower half of the illustration is from the second, more carboxy terminal, zinc module. Illustrations were generated using MOLSCRIPT (Kraulis, 1991). a, Lys42/DNA interactions in wild type. The axis of the recognition α-helix lies in the plane of the page and the helix is seen from the side. On the hydrophilic surface of the recognition helix, guanine (G) is shown hydrogen-bonded (- - -) to Lys42. His32, in the peptide loop of the Zn module, forms a hydrogen bond to the phosphate backbone. The interactions of Lys and His with the DNA are proposed to be conserved in the steroid/nuclear receptor superfamily. b, The view is the same as in Fig. 3a and shows the effects of Lys42 → Glu substitution. The hydrogen bond to the guanine is lost, and the hydrogen bond from His32 could be displaced as shown. c, Shows Phe44 in the conserved hydrophobic core in wild-type VDR. The packing of residues in the hydrophobic cluster is illustrated. The view is at right angles to that in Fig. 3a and b. The α-helix that is seen from the side is the one on the carboxyl end of the second zinc module. d, Orientation is as in Fig. 3c and shows the effect of the Phe → Ile substitution. A gap is introduced by this change in the hydrophobic core.

explained stereochemically (Fig. 3c and d). By analogy with the glucocorticoid receptor–DNA and oestrogen receptor–DNA structures Phe44 and Phe45 would be in the VDR recognition helix but face away from the DNA surface and project into the domain's hydrophobic interior. Phe44 would pack closely against other hydrophobic residues, including Phe55 and Leu78, and buttress the Arg77 side-chain to make contacts with a DNA-phosphate. The mutation Phe44 → Ile (Fig. 3d) might conserve the hydrophobic nature of the core, but would not permit close packing of the well matched hydrophobic residues. It is perhaps surprising that substitution of the polar residue

Phe44 by an isoleucine (which is also a polar residue, albeit a smaller one) should have such a major effect on function. Such relatively conservative substitutions can often be tolerated in the hydrophobic core of proteins. Thus while there is a leucine at position 78 in the vitamin D receptor and the corresponding position of the oestrogen receptor, this is replaced by tyrosine in the glucocorticoid receptor, and similarly lysine 82 in VDR is replaced by valine or tyrosine in the glucocorticoid and oestrogen receptors respectively. However, the nature of the Phe → Ile substitution may have an impact on the stability of VDR. In particular, the conformation of the Ile side-chain would be highly restricted because of steric constraints imposed by the α-helix; these limitations would also result in non-optimal packing of its branched side-chain. It would be expected that the mutation would affect the stability of the receptor and, through its effects on the Arg77 side-chain, might reduce DNA binding affinity.

Mutations at other residues in the DNA binding domain of VDR

The five other mutations (Fig. 1) that have been reported in the VDR DNA binding domain can be interpreted similarly. One affects Gly30 (Hughes *et al.*, 1988), another changes His32 to Gln (Yagi *et al.*, 1993), while the other three mutations affect arginines, which are mutated to glutamines at positions 47, 70 and 77 (Hughes *et al.*, 1989; Sone *et al.*, 1990; Saijo *et al.*, 1991). Gly30 might have two structural roles. First, having no substituent, Gly would permit the peptide backbone to assume a special conformation orienting His32 for donation of a hydrogen bond to the phosphate backbone and Phe33 for packing against the hydrophobic interior of the domain. In addition, Gly30 would allow the peptide backbone to approach the DNA-phosphates. Substitution of Gly30 by Asp would result in two adverse effects: the negative charge of the Asp carboxylate would repel the DNA-phosphates, and the Asp β-carbon would sterically prevent a close approach to DNA. The effects of mutation of His32 → Gln would be critical. As already stated, His is absolutely conserved at this position in the superfamily and by virtue of its positive charge would be expected to donate a hydrogen bond to the phosphate of guanine in the VDRE; this could not be done by the mutant Gln 32.

The three arginine mutations in VDR would also have important consequences. In the native protein, Arg47 could donate two hydrogen bonds to guanine in a VDRE. Substitution by Gln47 at this position introduces a side-chain that is not long enough (by roughly 2Å) to reach the base; two hydrogen bonds would be lost as a consequence. Arg70 is predicted to form a salt bridge with Asp26, an interaction that appears to be conserved in the superfamily, and also contact a DNA-phosphate in wild type VDR. The introduction of Gln70 could permit one of the interactions, but not both. This would weaken DNA binding and, possibly, the stability of the protein. The shorter glutamine side-chain would therefore be unable to donate a hydrogen bond to the phosphate backbone. Arg77 would be expected to make two hydrogen bonding interactions with the phosphate backbone of the VDRE. Its aliphatic side-chain could pack against Phe44, as described above. Neither the hydrogen bonding nor the hydrophobic interactions would be conserved when the residue is substituted by Gln77. The apparent tendency for deleterious mutations to occur at arginine residues in VDR may be a consequence of deamination of methylcytosine or cytosine at CpG in genomic DNA, resulting in mutational hotspots (Bird, 1992; Shen *et al.*, 1992). Indeed, deamination of methyl-CpG or CpG may explain the origin of mutations causing haemoglobinopathies (Perutz, 1990) and many other hereditary diseases (Shen *et al.*, 1992; Bird, 1992). In this context it is notable that four of the 11 mutations known to cause HVDRR involve codons that harbour CpG (e.g. CGA → CAA at residues 47 and 70; CGG → CAG at residue 77; CGC → CTC at residue 271).

The mutations described here demonstrate that point changes in the vitamin D receptor can cause profound physiological effects which result in severe bone disease. It is expected that further insight will result from direct structural analysis of VDR–DNA interactions, though much information concerning the critical elements can already be deduced. By modelling with the glucocorticoid and oestrogen receptor–DNA complexes, it is possible to propose stereochemical explanations for the disruptive effects of these clinically significant mutations on the genomic actions of vitamin D. The analysis makes it possible to consider the genesis of diseases due to steroid-hormone resistance at the Ångstrom level.

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