Functionally relevant polymorphisms in the human nuclear vitamin D receptor gene

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Abstract

The functional significance of two unlinked human vitamin D receptor (hVDR) gene polymorphisms was evaluated in twenty human fibroblast cell lines. Genotypes at both a *Fok I* restriction site (*F/f*) in exon II and a singlet (A) repeat in exon IX (*L/S*) were determined, and relative transcription activities of endogenous hVDR proteins were measured using a transfected, 1,25-dihydroxyvitamin D_3-responsive reporter gene. Observed activities ranged from 2–100-fold induction by hormone, with higher activity being displayed by the *F* and the *L* biallelic forms. Only when genotypes at both sites were considered simultaneously did statistically significant differences emerge. Moreover, the correlation between hVDR activity and genotype segregated further into clearly defined high and low activity groups with similar genotypic distributions. These results not only demonstrate functional relevance for both the *F/f* and *L/S* common polymorphisms in hVDR, but also provide novel evidence for a third genetic variable impacting receptor potency. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Vitamin D receptor; Gene polymorphisms; Pharmacogenomics

1. Introduction

The biological actions of 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] are mediated largely, if not entirely, by the vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors (Whitfield et al., 1999). This protein is found in tissues known to play a role in calcium homeostasis, and also in numerous other tissues, where it appears to regulate a variety of processes, including cell proliferation and differentiation (Haussler et al., 1998). The significance of the nuclear VDR in calcium homeostasis, as well as in certain differentiation and proliferation processes in skin and uterus, has been confirmed by gene knockout studies in mice (Li et al., 1998; Kato et al., 1999).

A simplified diagram that illustrates how nuclear hVDR mediates transcriptional activation by the 1,25(OH)$_2$D$_3$ hormone is shown in Fig. 1A. The key features of this model are: (i) liganding of nuclear VDR by 1,25(OH)$_2$D$_3$; (ii) recruitment by 1,25(OH)$_2$D$_3$-VDR of its retinoid X receptor (RXR) heteropartner that, in turn, facilitates high-affinity interaction of the dimeric complex with vitamin D responsive elements (VDREs) upstream of target genes; (iii) attraction by VDR of basal transcription factor IIB (TFIIB), the rate-limiting component of the transcription preinitiation complex; and (iv) recruitment by the heterodimer of a number of transcription coactivators, some with histone acetyl transferase (HAT) activity to modify nucleosome/chromatin organization, such as SRC-1 (Gill et al., 1998), and others like the DRIPs (Rachez et al., 1999) that target the VDR supercomplex to the TATA-box/TBP and RNA polymerase II transcription initiation machinery. The net result of this 1,25(OH)$_2$D$_3$-triggered response is the regulation of genes coding for proteins that carry out intestinal calcium absorption, bone remodeling, cell differentiation, etc. (Jurutka et al., 2001).

A modular diagram of the functional domains within the hVDR protein is presented in Fig. 1B. The details of the hVDR subdomain arrangement (see figure legend) basically follow the general pattern for the sub-

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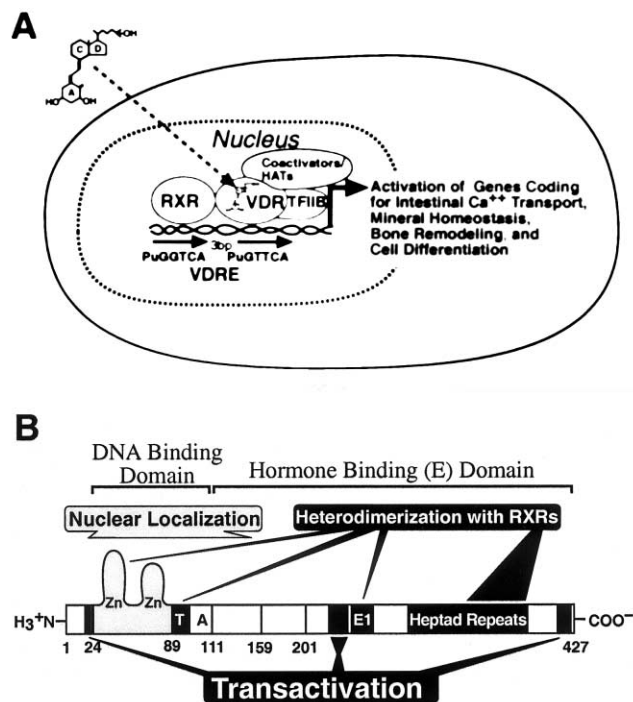*E-mail address:* haussler@u.arizona.edu (M.R. Haussler).

Fig. 1. (A) Model for transcriptional activation by $1,25(OH)_2D_3$ as mediated by a heterodimer of VDR and RXR bound to VDREs upstream of target genes in vitamin-D responsive cells. As the primary receptor, VDR is activated by $1,25(OH)_2D_3$ binding, but the RXR coreceptor apparently can remain unliganded. The receptor heterodimer associates with direct repeat-type responsive elements upstream of the target genes and the liganded heterocomplex attracts various coactivators, some with histone acetylase (HAT) activity. VDR itself recruits basal transcription factor IIB (TFIIB). Finally, the ensemble of protein-protein-DNA interactions promotes transcriptional initiation of a battery of target genes, leading to the pleiotrophic effects of the $1,25(OH)_2D_3$ hormone (Haussler et al., 1998). (B) Schematic illustration of functional domains in the VDR protein. The DNA binding domain, with two zinc finger motifs, is located near the N-terminus and also contains residues that promote nuclear localization of the receptor. The central and C-terminal region of the receptor contain subdomains that mediate ligand binding (Rochel et al., 2000). The heterodimerization (with RXR) and transactivation functions of hVDR appear to be mediated by widely separated regions of the receptor. Heterodimerization is supported by heptad repeats in the helix 7–10 region (Nakajima et al., 1994), an E1 domain (Whitfield et al., 1995), as well as residues in the T-box and first zinc finger (Hsieh et al., 1995). Transactivation regions include: (i) the extreme N-terminus, which possesses a TFIIB docking site (Jurutka et al., 2000); (ii) a centrally located domain corresponding to helix 3 in the rat TRα, hRARγ and hVDR ligand binding domain crystals (Renaud et al., 1995; Wagner et al., 1995; Kraichely et al., 1999; Rochel et al., 2000); and (iii) the extreme C-terminus, corresponding to helix 12 in the rat TRα, hRARγ and hVDR crystals (Renaud et al., 1995; Wagner et al., 1995; Jurutka et al., 1997; Rochel et al., 2000). 'A' designates the A-box, which contains important DNA-binding amino acids (Hsieh et al., 1999). The residue 159–201 segment, which is unconserved in VDRs, is encoded by a novel exon (V), not seen in other nuclear receptors for which the gene structure is known (Haussler et al., 1998).

family of nuclear receptors that heterodimerize with RXR, such as the all-*trans* retinoic acid receptors (RARs) and the thyroid hormone receptors (TRs) (Whitfield et al., 1999). For the purposes of the present communication, the most relevant regions of hVDR are the hormone-binding domain, encoded by exons VI–IX of the human gene (see also Fig. 2), the DNA binding domain/ zinc fingers, encoded by exons II–IV, and a set of discontinuous transactivation domains, including regions at the N-terminus (for TFIIB docking) (Jurutka et al., 2000), and in helices 3 and 12 (for coactivator recruitment). Since transactivation is the ultimate biochemical action of the liganded VDR and depends on all of the other capabilities of the receptor (ligand binding, nuclear localization, heterodimerization and VDRE/ DNA binding), the present study focuses on this parameter of receptor activity in order to probe for functional significance of hVDR gene polymorphisms.

The chromosomal gene for VDR has been cloned (Miyamoto et al., 1997), and several common genetic variants have been described in humans, most of which are identified by a biallelic variation in a restriction endonuclease site (Fig. 2). Genetic variation in the 3′ region of the hVDR gene is observed in specific intronic sites for *Bsm I* (Morrison et al., 1992) and *Apa I* (Faraco et al., 1989), a silent *Taq I* site in exon IX (Morrison et al., 1992), as well as in a singlet(A) repeat in the portion of exon IX encoding the 3′ UTR (Ingles et al., 1997a) (see Fig. 2, right). All of these variations near the 3′ end of the gene are in linkage disequilibrium (Morrison et al., 1992; Verbeek et al., 1997), although this linkage is weaker in some ethnic groups such as African-Americans (Ingles et al., 1997a). Interestingly, none of these polymorphisms affect the structure of the VDR protein itself, although the singlet(A) repeat in the 3′ UTR is expressed in the mature mRNA for hVDR. Singlet(A) variants are classified according to length by the number of consecutive A's in the repeat, with ≥ 17 A's scored as 'long' (L), and ≤ 15 A's considered 'short' (S).

Another polymorphic site has been found in exon II near the center of the hVDR gene (Saijo et al., 1991). This site, which is genetically unlinked to the above *Bsm/Apa/ Taq*/singlet(A) cluster, is unique among common hVDR variants described so far, in that it results in an alteration of the hVDR protein structure (Fig. 2, bottom center). Presence of the *Fok I* site (designated *f*) predicts that a 427-residue VDR protein will be produced beginning at Met-1 (M1 according to the numbering scheme of Baker et al. (1988), whereas absence of this site (denoted *F*) dictates translation from Met-4 (M4), producing a protein of 424 amino acids (Arai et al., 1997).

In an initial report (Morrison et al., 1994), allelic variation in the chromosomal gene for the vitamin D receptor was proposed to represent a major part of the genetic predisposition for low bone mineral density (BMD), and perhaps for osteoporosis and/or skeletal
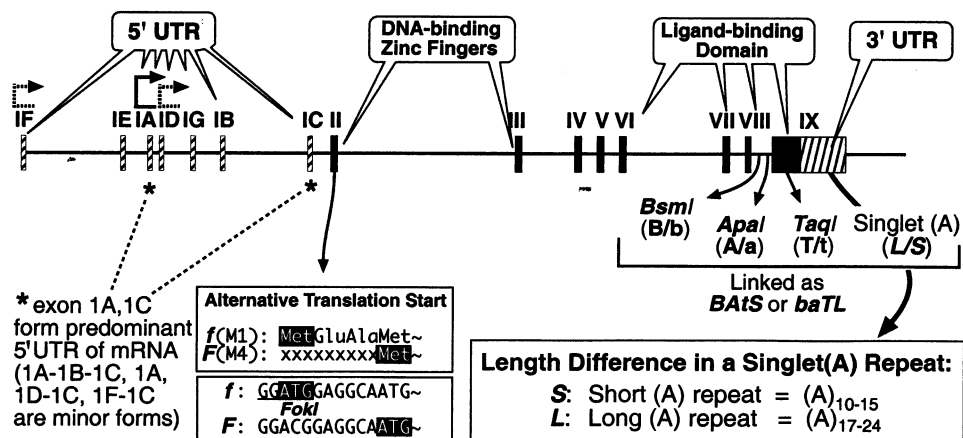
Fig. 2. The human VDR gene, with key features relevant to polymorphic variation in VDR expression and activity. The fifteen known exons are depicted at the top (Miyamoto et al., 1997; Crofts et al., 1998). The 5′ untranslated region of hVDR mRNAs is observed to be alternatively spliced in all tested human tissues (Crofts et al., 1998). The predominant hVDR mRNA in tissues tested to date contains a 5′ UTR consisting of exon IA spliced to IC; several other less abundant spliced forms have been described, implying the existence of at least three promoters for the hVDR gene, depicted by arrows above exons IF, IA and ID, respectively, (Crofts et al., 1998). Exons II and III encode the translation start site, a short N-terminal domain, and the two zinc finger motifs of the DNA binding domain (one in each exon). The overlapping ligand-binding and strong heterodimerization domains are encoded by exons VI–IX, with exon IX also containing the entire 3′ UTR. At right are shown four linked polymorphic sites in or somewhat 5′ to exon IX. The present study focuses on the singlet(A) repeat, which lies about 1 kb upstream of the polyadenylation site and exists in either a long ($L = 17–24$ A's) or a short ($S = 10–15$ A's) form (Ingles et al., 1997a). An additional site of interest to the current study is the dimorphic translational start site (Saijo et al., 1991), the two forms of which (F or f, illustrated at bottom center) are unlinked to the L/S variants (Gross et al., 1996).

fractures, although these associations have been disputed by other studies [reviewed in (Wood and Fleet, 1998)]. More recently, correlations have been reported between VDR allelic variants and risk of prostate cancer (Ingles et al., 1997b; Watanabe et al., 1999), breast cancer (Ingles et al., 1997c; Ruggiero et al., 1998; Curran et al., 1999), sporadic primary hyperparathyroidism (Correa et al., 1999), and sarcoidosis (Niimi et al., 1999). However, conflicting reports have appeared that minimize or even contradict these associations (Cheng and Tsai, 1999; Correa et al., 1999). Likewise, direct testing of hVDR alleles for activity has yielded somewhat variable results, although, when a difference is found, the b and F hVDR alleles appear to be more active than the B or f alleles (see Section 4).

One caveat in most of the above-cited studies is that correlations were sought between a single, specific polymorphism, or between the Bsm-Apa-Taq linkage group, and the physiological parameter of interest. Very few studies have attempted to control for hVDR genotype at both the Bsm/Apa/Taq/singlet(A) cluster and the Fok I site. In one example (Ferrari et al., 1998), a correlation between Fok I alleles and BMD could not be demonstrated, but 'cross-genotyping' with Bsm I alleles revealed a potentially important positive association in prepubertal girls between the ffBB hVDR genotype and low BMD (Ferrari et al., 1998).

Another caveat in the above cited studies is that a direct influence of allelic variation on VDR expression or activity was not demonstrated, leaving open the possibility that the observed correlation might be due

to linkage to another nearby site or even to a different gene. In the only two extant studies in which the potential relationship between genotype and activity of the hVDR protein was evaluated (Verbeek et al., 1997; Gross et al., 1998), no functional influence of specific alleles was observed, but again, only a single polymorphic site was examined in isolation.

In the present communication, we report an evaluation of a panel of twenty human fibroblast lines. The current protocol includes simultaneous consideration of the hVDR genotypes at both the singlet(A) and the Fok I loci, which are then correlated with activity of the endogenous VDR in the corresponding cell line. From these data, we conclude that (a) biallelic variants at the Fok I and the singlet(A) sites, in combination, affect transcriptional activation by the endogenous hVDR in the tested human fibroblasts; (b) the singlet(A) L allele is more active than the S allele; and (c) a third, unknown genetic variable appears to influence VDR activity.

2. Materials and methods

2.1. Plasmid DNAs used for transfection and in vitro transcription

The 1,25(OH)$_2$D$_3$-responsive reporter plasmid, (CT4)$_4$TKGH, contains four copies of the rat osteocalcin VDRE (Terpening et al., 1991) linked upstream of the thymidine kinase promoter-GH reporter gene

(Nichols Institute, San Juan Capistrano, CA). The hVDR expression vector, pSG5-hVDR, expressing the *F*/M4 isoform of hVDR, has been described earlier (Hsieh et al., 1991). This construct was adapted for expression of the *f*/M1 hVDR isoform by inserting the appropriate DNA codons via in vitro site-directed mutagenesis (Jurutka et al., 2000). For monitoring the efficiency of transfection, a commercial plasmid expressing β-galactosidase (CMV-βgal) was obtained from Promega Corp. (Madison, WI).

2.2. Cell lines

Cell lines DNF-BJ, DWF-CV and DWF-TW were provided courtesy of C. Bloch at the Children's Hospital, Denver, CO. Patients DWF-CV and DWF-TW are reported to have features of William's Syndrome. Other cell lines were obtained from the American Type Culture Collection, Manassas, VA, with patients Ber Lin, Be Sal and Ran Nor reported to have late-onset osteoporosis.

2.3. Transfection of cultured cells and transcriptional activation assay

Human fibroblast cell lines were cultured in DMEM:Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were transfected by electroporation (see Fig. 3, top left). Briefly, cells were collected by trypsinization, pelleted at low speed and resuspended at 5×10^6 cells per ml in 1X HeBS buffer (20 mM HEPES, pH 7.1, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose). Suspended cells (0.8 ml) were then combined with 40 μg $(CT4)_4$ TKGH reporter plasmid, 10 μg of CMV-βgal plasmid, and 450 μg carrier DNA (pTZ18U plasmid), and adjusted to a total volume of 1 ml in 1X HeBS buffer in a 0.4 cm electrode gap electroporation cuvette. Each cuvette was then subjected to electroporation in a Bio-Rad Gene Pulser II apparatus (with capacitance extender attachment) at settings of 200 V and 950 μF. After 10 min of incubation at room temperature, the electroporated cells were suspended in culture medium and then divided into six 60 mm culture dishes and incubated at 37°C for 72 h in the presence of 10^{-8} M $1,25(OH)_2D_3$ (three plates) or ethanol vehicle (also in triplicate plates). The levels of growth hormone secreted into the culture medium were then assessed by radioimmunoassay using a commercial kit (Nichols Institute) according to the manufacturer's protocol. To normalize results for the efficiency of transfection in each plate, β-galactosidase levels were assayed in cell lysates (freeze-thaw method) using reagents and instructions from a commercial kit (Promega Corp.). These steps are represented as a flow chart in Fig. 3 (left).

For the experiment depicted in Fig. 7, ROS 2/3 cells (8×10^5 cells per 60 mm dish) were transfected by calcium phosphate coprecipitation as described earlier (Jurutka et al., 2000) using 10 μg of a reporter plasmid containing 1100 bp of the natural rat osteocalcin promoter linked to the human growth hormone gene [de-
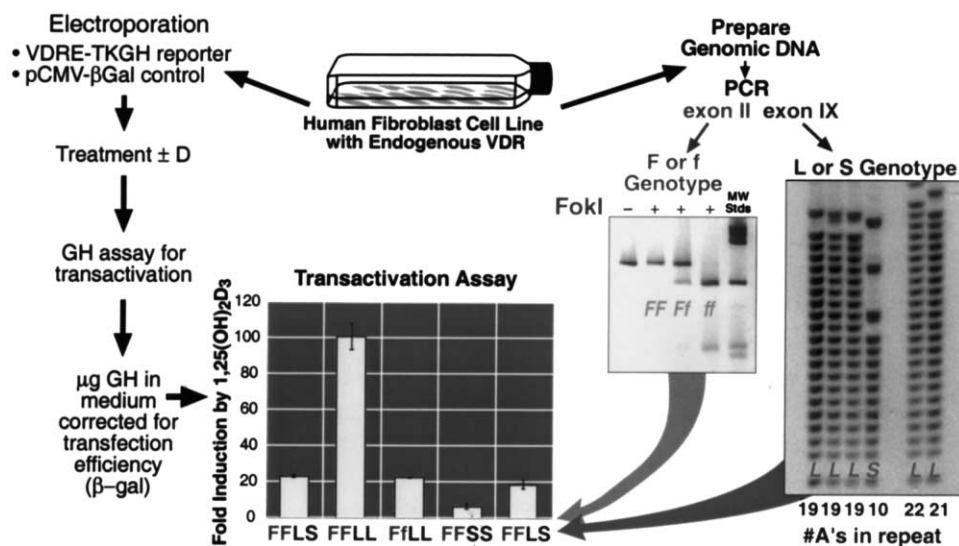

Fig. 3. Protocol for VDR genotype/phenotype analysis of human fibroblast cell lines. Cultured cells (top center) were transfected, incubated and assayed for relative transcriptional activity as depicted schematically at the left and detailed in Section 2. Results were expressed as fold-induction by $1,25(OH)_2D_3$. Cells of each line were also harvested for genomic DNA isolation and genotyping by PCR as shown at the upper right. For determination of singlet(A) repeat length, multiple PCR products from exon IX of each line were sequenced individually (see sample gel at far right). PCR products of exon II were digested with the *Fok I* restriction enzyme and resolved by electrophoresis (see sample gel at center-right). Final results for each cell line thus included the genotype at both the 3′ UTR singlet(A) site (*L/S*), and the translation initiation site (*F/f*), along with relative transactivation activity of the endogenous VDR (see sample summary graph at bottom center).

noted BGP-TKGH (Terpening et al., 1991)], along with 1.0 µg of pSG5-hVDR expressing either F/M4 or f/M1 hVDR. Sixteen hours post-transfection, the cells were washed, and refed (DMEM:Ham's F-12 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin) and treated with 10^{-8} M $1,25(OH)_2D_3$ or ethanol vehicle. After 24 h, the level of secreted growth hormone was assayed in the culture medium from each plate as described above.

2.4. Genotyping of human fibroblasts

DNA was prepared from cultured human fibroblasts (10^7 cells) using the QIAmp tissue kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. For F/f genotyping, isolated genomic DNA (500 ng) was combined with 100 ng each of primers 2a and 2b (Gross et al., 1996), along with 5 ml of 10X buffer (Perkin Elmer, Norwalk, CT) plus 1.5 mM $MgCl_2$, 2.5 mM each of dATP, dCTP, dTTP and dGTP, and 0.25 ml Taq DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). PCR conditions were: 20 cycles at 94°C for 30 s, 74°C for 30 s (with -0.1°C per cycle) and 72°C for 60 s. This was followed by 25 cycles at 94°C for 30 s, 63°C for 30 s and 72°C for 60 s. Approximately, 200 ng of unpurified PCR product was then incubated with 1 µl Fok I enzyme (New England Biolabs, Beverly, MA) and 1 µl 10X buffer in a total volume of 10 µl for 1.5 h at 37°C. The digestion mixture was electrophoresed on a 4% NuSieve (3:1) Agarose gel in TBE buffer (90 mM Tris–borate, pH8, 2 mM EDTA) to determine whether the PCR product was completely digested (indicating the ff genotype), partially digested (Ff) or completely undigested (FF).

For L/S genotyping, isolated genomic DNA (500 ng) was combined with 5′ and 3′ primers (100 ng each) (Ingles et al., 1997b) using the same PCR profile described above. PCR products at approximately 400–425 bp were resolved on 0.8% Agarose gels, excised from the gel and isolated into 20 µl of Tris–HCl, pH 8.5, using a QIAEX II extraction kit (Qiagen Inc.) according to the protocol of the manufacturer. The isolated PCR products (7.5 µl) were then cloned into the T-vector and transformed into the XL-1 Blue strain of $E.$ $coli$ using a T-vector kit (Promega Corp.). Plasmid DNA was isolated from transformed bacteria by standard methods and sequenced ('A' reaction only) using a T7 Sequenase kit (Amersham Pharmacia Biotech, Piscataway, NJ). Typical results are depicted in Fig. 3 (right).

2.5. GST coprecipitation assays

The ability of either F/M4 or f/M1 hVDRs to interact with human TFIIB was assessed as described earlier (Jurutka et al., 2000). Briefly, TFIIB-glutathione-S-transferase (GST) fusion protein was expressed from pGEX-2T-hTFIIB (Baniahmad et al., 1993) and GST alone was expressed from pGEX-4T, both in $E.$ $coli$ strain DH5α. Each protein was then coupled to glutathione Sepharose. For the GST 'pull-down' assays, pSG5-hVDR vectors expressing either F/M4 or f/M1 hVDRs were used to generate [^{35}S] methionine-labeled proteins utilizing the TNT Coupled Reticulocyte Lysate kit (Promega Corp.). The desired ^{35}S-labeled protein was then incubated with the beads in the absence or presence of $1,25(OH)_2D_3$ (10^{-6} M). Next, the unbound proteins were washed from the beads with 4×1 ml wash buffer [0.15 M KCl, 10 mM Tris–HCl, pH 7.6, 1 mM EDTA, 0.3 mM $ZnCl_2$, 1 mM dithiothreitol, 0.1% Tween-20, 1 mg/ml BSA, and the following protease inhibitors, obtained from Roche Molecular Biochemicals (Indianapolis, IN): 0.5 mg/ml Pefabloc SC, 15 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 µg/ml leupeptin]. The bound proteins were extracted from the beads into 40 µl loading buffer (2% SDS, 5% β-mercaptoethanol, 125 mM Tris–HCl, pH 6.8, and 20% glycerol), boiled for 3 min, separated by SDS-PAGE and visualized via autoradiography.

3. Results

3.1. Genotyping of human fibroblast lines

As described in Section 2 and depicted schematically in Fig. 3, genomic DNA samples were extracted from twenty human fibroblast cell lines, subjected to PCR using two independent sets of primers, and analyzed for their hVDR genotype at the polymorphic sites in exon II (F/f) and exon IX (L/S). F/f genotypes were determined by digestion of the PCR products from each line with the restriction enzyme Fok I (Gross et al., 1996). Two independent PCR reactions were performed for each line, and digestion experiments included a ff and Ff line as positive controls to monitor activity of the Fok I restriction endonuclease. A typical digestion pattern is shown at the center-right of Fig. 3, displaying the undigested PCR product (265 bp), partially digested DNA from a heterozygote, and completely digested DNA (69 and 196 bp fragments) from an ff subject. The frequencies of the F and f alleles in the present sample group were 62.5 and 37.5%, respectively. This distribution of hVDR alleles is similar to that characterized for Caucasian populations in other studies (Gross et al., 1996; Eccleshall et al., 1998; Gennari et al., 1999).

L/S genotypes were determined by sequencing of PCR products to ascertain the exact length of the singlet(A) repeat (Ingles et al., 1997b). As reported by others (Ingles et al., 1997a), we observed (Fig. 4) multiple alleles at this locus which segregate into a 'long' (L)
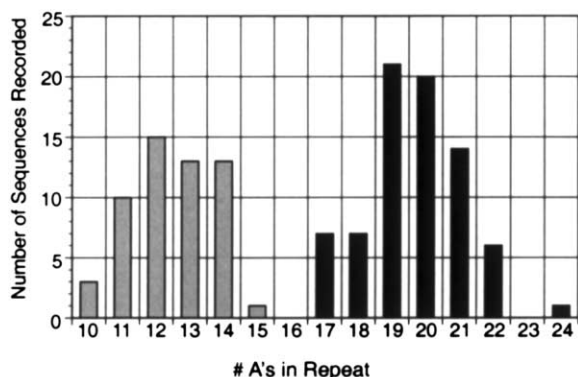
Fig. 4. Compilation of sequencing results from L/S genotyping (approximately 4–5 sequences per cell line). Variations of 1–2 A's were observed in separate sequencing reactions from the same cell line, presumably because of errors in PCR (Ingles et al., 1997a) or in the bacterial replication of these repeated sequences. Allele frequencies in the present sample of 20 cell lines were 40% S and 60% L, nearly identical to published results of 41% S for Caucasians living in the USA (Ingles et al., 1997a).

group with 17–24 A's in the repeat, and a 'short' group (S) with 10–15 A's in the repeat. The frequencies of the L and S hVDR alleles in the present panel of cell lines were 60 and 40%, respectively, similar to that earlier published for Caucasian populations (Ingles et al., 1997a).

3.2. Relative VDR transcriptional activities of human fibroblast cell lines

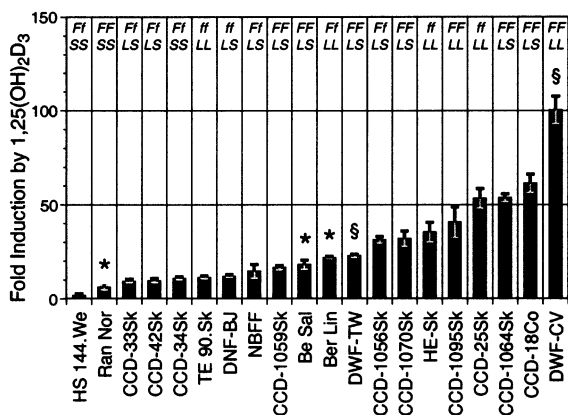Fig. 5 shows the hVDR-mediated transcription results from 20 human fibroblast cell lines after transfec-

Fig. 5. Relative transcriptional activity of endogenous VDR in 20 human fibroblast lines. Results from all cell lines (average of ≥ 6 experiments, each in triplicate ($\pm$ S.E.M.) are arranged in order of increasing fold-induction by $1,25(OH)_2D_3$. Cell lines DWF-TW and DWF-CV (§) were obtained from patients with features of William's Syndrome, and patients Ran Nor, Be Sal and Ber Lin (*) had late-onset osteoporosis. All other lines were taken from patients with no known disorders of bone or calcium metabolism. Genotypes of each line are given at the top.

tion with a $1,25(OH)_2D_3$-responsive reporter plasmid and incubation in the presence or absence of 10^{-8} M $1,25(OH)_2D_3$, expressed as fold-induction by the hormone. The assay conditions, including quantitative monitoring of transfection efficiency using a β-galactosidase vector, were designed to permit comparison of endogenous VDR activity between cell lines. The data (Fig. 5) reveal a striking spectrum of activities, ranging from only a 1.75-fold induction of the growth hormone reporter within the 72-h treatment period to a 100-fold effect of hormone, with a mean of 28 ± 24 (S.D.) fold induction. The lowest activity (1.75-fold induction by $1,25(OH)_2D_3$) was observed in a fibroblast line derived from a 5-week-old embryo. As denoted in Fig. 5, a subset of the tested fibroblast cell lines are from patients with either osteoporosis (*), in which subjects are usually normocalcemic, but can have low blood calcium, or with William's Syndrome (§), a condition frequently presenting with hypercalcemia. Interestingly, one of the William's patients (DWF-CV) contains endogenous VDR with the highest activity (100-fold induction by $1,25(OH)_2D_3$), while the other patient displays a near-average (23-fold) induction by $1,25(OH)_2D_3$. Conversely, one of the osteoporosis patients (Ran Nor) yielded a very low VDR activity in the assay (6-fold induction), while the two other osteoporotic individuals (Be Sal, Ber Lin) displayed inductions by hormone that were only slightly below average (18- and 22-fold, respectively). No other associations were noted between patient status (e.g., age, sex or medical condition) and VDR activity (see Section 4).

3.3. Correlations between either Fok I or L/S genotype and activity of endogenous VDR

Given the current interest in the Fok I and L/S hVDR polymorphisms, correlations were next sought between the genotypes of each cell line at these two loci and the relative activities of the corresponding endogenous VDRs. Accordingly, all twenty cell lines examined were grouped into ff (4 lines), Ff (7 lines) or FF (9 lines). The average fold-inductions by $1,25(OH)_2D_3$ for each group are displayed ($\pm$ S.E.M.) in Fig. 6A. Although no clear trend is evident, it is notable that the FF group displays the highest average fold-induction by the hormonal ligand. These F/f groupings were then subdivided into sets with the LS genotype (Fig. 6C, left panel) and the LL genotype (Fig. 6C, right panel), thereby controlling for the L/S genotype when evaluating the activity of endogenous F/f hVDR. Again, in both groups of data, the FF cells display the highest activity. However, none of the differences between groupings in either Fig. 6A or 6C achieve statistical significance (at the 95% confidence interval), presumably because of the small number of cell lines in each grouping or the possible existence of a novel
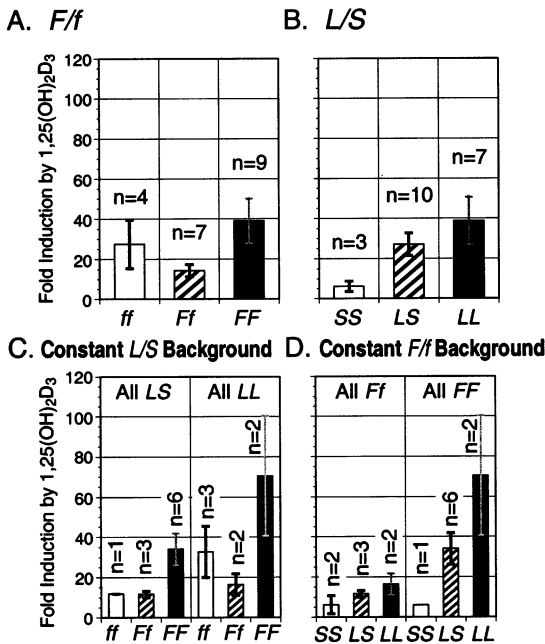
Fig. 6. Correlation of transcriptional activity with hVDR genotype. (A) All 20 cells lines are grouped by F/f genotype. Each grouping consists of the indicated number of lines, and the fold-induction values for these lines (taken from Fig. 5) are represented as the average ± S.E.M. (B) As in (A), but all 20 cell lines are grouped by L/S genotype. (C; left panel) The ten cell lines with genotype LS are grouped by F/f genotype, and the average fold-induction by $1,25(OH)_2D_3$ ± S.E.M. is shown. (C; right panel) The seven lines with an LL genotype are similarly grouped by F/f genotype and plotted. The three SS hVDR cell lines are omitted from this analysis, since they do not form a complete set of F/f groupings ($ffSS$ is missing). (D; left panel) The seven cell lines with genotype Ff are grouped by L/S genotype. (D; right panel) The nine lines with a FF genotype are similarly grouped by L/S genotype and plotted. The four ff cell lines are omitted, as they do not form a complete set of L/S groupings (again, $ffSS$ is missing).

genetic variable in hVDR activity. Nevertheless, the FF groupings show higher fold-inductions than the corresponding Ff groups, with the exception of lines with the SS hVDR genotype (see Fig. 6D), appearing to confirm an effect of the F/f polymorphism and suggesting that the F allele is more active.

When $1,25(OH)_2D_3$-stimulated transcription activities in the twenty lines are grouped by L/S genotype (Fig. 6B), a much clearer, but still not statistically significant, trend emerges, with SS having the lowest fold-induction, LL possessing the highest, and the LS genotype exhibiting intermediate activity. This trend persists when the groupings are subdivided into those with constant Ff or FF genotypic backgrounds (Fig. 6D), leading to the tentative conclusion that the L hVDR allele is more active than the S allele. The SS and ff subsets were not included in Fig. 6C and Fig. 6D, because of the low number of samples of both SS and ff homozygotes (3 and 4, respectively), as well as the complete lack of the $ffSS$ hVDR genotype in the current series of fibroblasts lines.

3.4. The F hVDR isoform is more active in transfected cells

Although the above F/f groupings did not show a rigorous association between hVDR genotype and fold-induction by $1,25(OH)_2D_3$ (Fig. 6A and C), recent results from other laboratories (Arai et al., 1997; Colin et al., 2000) have demonstrated an apparent higher activity for the F isoform of hVDR relative to the f isoform. The results presented in Fig. 6 are generally consistent with this conclusion. In addition, Fig. 7A depicts an in vitro experiment in which the F and f hVDR isoforms were expressed from a pSG5-hVDR construct and directly tested for transcriptional activity. The original pSG5-hVDR vector expresses the $F/M4$ isoform; a cDNA insert expressing the $f/M1$ was engineered into this same vector via site-directed mutagenesis [see Section 2 and (Jurutka et al., 2000)]. These two vectors were separately transfected into the VDR-deficient rat osteosarcoma line, ROS 2/3, along with the BGP-TKGH reporter plasmid. The results of this analysis (Fig. 7A) show a significant ($P < 0.001$) difference in fold-induction by $1,25(OH)_2D_3$, with the F hVDR construct displaying a greater response to $1,25(OH)_2D_3$ (4.2-fold) than the f allele construct (2.6-fold).
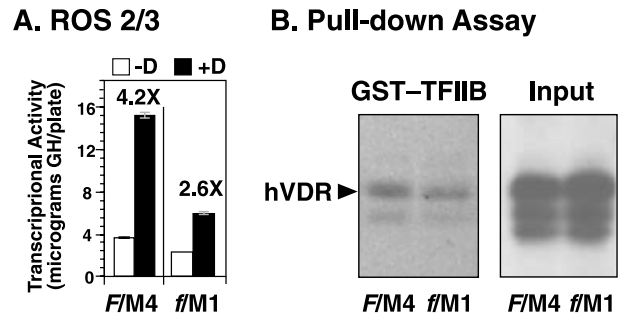

Fig. 7. Relative activities of $F/M4$ hVDR and $f/M1$ hVDR, expressed from an engineered construct. (A) An $f/M1$ hVDR cDNA, differing at the translational start site, was created from an existing $F/M4$ cDNA by site-directed mutagenesis. Both cDNAs, cloned in the vector pSG5, were used to express $F/M4$ and $f/M1$ hVDRs in VDR-deficient ROS 2/3 cells (Jurutka et al., 2000). Assays for relative transcriptional activity were performed as described above for endogenous VDR in human fibroblast cell lines, except that the BGP-TKGH reporter vector was used (see Section 2). The results shown (triplicate assays ± S.E.M.) are representative of at least three independent experiments. (B) Using the same cDNAs as in (A), ^{35}S-labeled $F/M4$ and $f/M1$ hVDRs were expressed in a coupled in vitro transcription/translation system (see Section 2) and assayed for their ability to bind a human TFIIB-glutathione S-transferase fusion protein that had been immobilized on Sepharose beads (Jurutka et al., 2000). Washed beads were denatured and subjected to electrophoresis on 5–15% SDS-PAGE gels (see Section 2), and ^{35}S-labeled protein bands were visualized by autoradiography (left panel of B). The right panel of B displays autoradiograms of ^{35}S-labeled proteins used for the pull-down assays (5% of total input).

3.5. Relative ability of F/f hVDR isoforms to interact with TFIIB, in vitro

It has earlier been reported by our group (Jurutka et al., 2000) that F hVDR interacts more efficiently with TFIIB than does the f hVDR isoform, thus providing a plausible mechanism for the greater transactivation potency of the F hVDR. Fig. 7B illustrates a typical experiment, utilizing the GST pull-down technique to compare the abilities of in vitro-synthesized, ^{35}S-labeled F/M4 and f/M1 hVDR isoreceptors to interact with an immobilized TFIIB fusion protein. Even in the face of a higher input of ^{35}S-labeled f/M1 protein, the F/M4 protein shows a reproducibly greater (approximately 2-fold) ability to interact with TFIIB, when compared with the f/M1 protein under the same conditions (Jurutka et al., 2000). Whether this difference in activity reflects that occurring under in vivo conditions is not known; however, these results provide a reasonable mechanism by which to explain the enhanced transactivation ability of the F/M4 hVDR isoform, in vitro, and are consistent with a proposed bioactivity for F hVDR that is also greater than that of f hVDR, in vivo [(Gross et al., 1996; Arai et al., 1997; Harris et al., 1997; Tao et al., 1998; Videman et al., 1998; Correa et al., 1999; Ferrari et al., 1999; Gennari et al., 1999; Kurabayashi et al., 1999; Lucotte et al., 1999; Colin et al., 2000; Sosa et al., 2000), see Section 4].

3.6. Correlation between genotype at both polymorphic loci and transactivation by endogenous VDRs

Considering the lack of genetic linkage between the $Fok\ I$ and L/S polymorphisms (Gross et al., 1996; Ferrari et al., 1998; Cheng and Tsai, 1999), plus the fact that both loci appear to affect function of the endogenous receptor, in vivo (Fig. 6), as well as evidence indicating that F hVDR is more active than f, in vitro, we attempted next to correlate the *combined* genotypes at both loci with hVDR transactivation ability. In order to condense genotypic information from both sites into a single variable, an 'allele score' was devised based on which allelic variants appear more active in the literature and in the present experiments. Since the F genotype is more active than f both in vivo and in vitro, each F hVDR allele was assigned a value of 1, while f alleles were scored as zero. Likewise, because the data in Fig. 6, panels B and D, indicate the L hVDR alleles to be more active than the S alleles, L and S alleles received scores of 1 and 0, respectively. Since the hVDR gene resides on an autosome (chromosome 12) (Szpirer et al., 1991), possible total allele scores range from 0 to 4 for both sexes. After grouping all twenty cell lines according to this formula, the average fold-induction by 1,25(OH)$_2$D$_3$ was plotted versus the allele score (Fig. 8A). A striking trend emerges from this analysis, with

each increasing increment in allele score yielding a higher average fold-induction by 1,25(OH)$_2$D$_3$. A qualitatively similar trend was seen if 1,25(OH)$_2$D$_3$-stimulated values for reporter gene production were plotted instead of fold-induction values (data not shown). Thus, the dramatic escalation of hVDR functional activity appears to correlate to the combined hVDR genotypic allele score at the F/f and L/S loci. Importantly, the difference between the two groups with allele scores of 2 and 4 achieves statistical significance by the two-tailed Student's t-test ($P = 0.035$).

Fig. 8B depicts the identical data set analyzed in A, but with each cell line plotted as an individual point. When a linear regression line is calculated for all 20 lines, the allele score shows a moderately strong, and statistically significant, positive correlation with transactivation (correlation coefficient = 0.595; $P = 0.012$). If, however, the cell lines are divided into a high group (above the $n = 20$ regression line) and low group (below the $n = 20$ line), the respective correlation coefficients are markedly improved, to 0.958 for the high group ($n = 8$, $P < 0.001$) and 0.858 for the low group ($n = 12$, $P < 0.001$). This apparent segregation of values into high and low groups argues for the existence of a new, third variable, other than the $Fok\ I$ or 3' cluster of polymorphisms containing L/S, in determining hVDR functional activity.

4. Discussion

A goal of the present study was to examine endogenous hVDR transcriptional activity in relation to hVDR genotype at unlinked polymorphic sites in both exons II and IX. The results reveal a strong correlation between genotype and VDR activity that emerges when both polymorphic sites are simultaneously considered (Fig. 8). A corollary of this conclusion is that variation at both polymorphic sites is important to hVDR functional activity, in vivo, but that considering each site separately may not reveal significant effects. Thus, the current data may explain why many attempts to correlate hVDR activity with genotype at a single locus have been unsuccessful.

The allelic distributions found in the present sample are similar to genotype frequencies in the published literature. The 62.5% frequency reported here for the F allele is comparable to the 63.5% published for Italian women (Gennari et al., 1999), the 62% for French women (Eccleshall et al., 1998) and the 61% for Mexican-American Caucasian women (Gross et al., 1996), but lower than the incidences of the F allele (68.5%) observed for Japanese women (Arai et al., 1997), or the 80.5% reported for African-American women (Harris et al., 1997). Concerning the singlet(A) L/S alleles, the frequency of the L allele in the present sample, 60%,
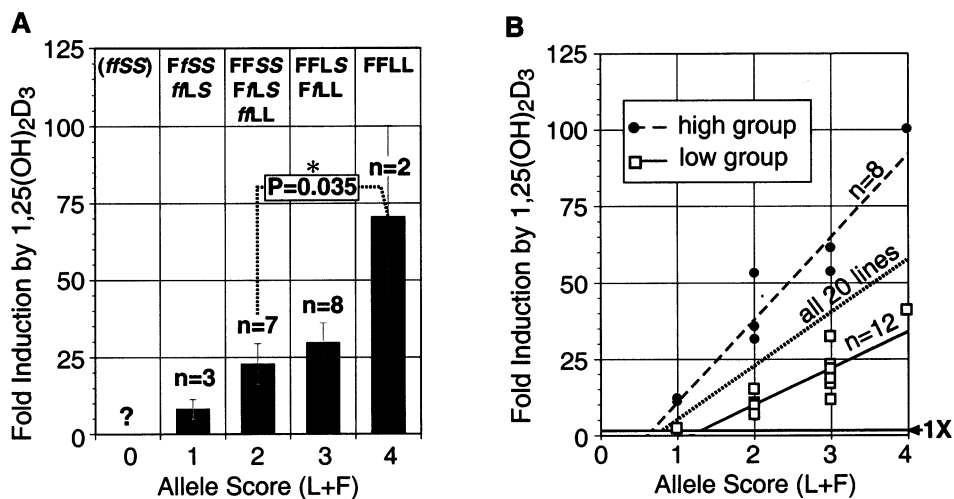
Fig. 8. Correlation of transcriptional activity with hVDR genotype at both L/S and F/f loci. (A) Results from Fig. 5 were grouped according to an 'allele score', computed as the sum of F and L alleles in each cell line. As indicated earlier, no cell lines with the $ffSS$ hVDR genotype are included in the cell lines studied; hence, no cell line with a score of zero could be evaluated. The average of the group with a score of two is significantly different from the average of the group with a score of 4 ($P = 0.035$) as assessed by the two-tailed Student's t-test. (B) The data from (A) were plotted as individual points instead of the average of each group, with each point representing a single cell line. The linear regression fit for all 20 points is represented by a dotted line. The regression line for an apparent 'high' group ($\bullet$, $n = 8$) is shown as a dashed line, while the remaining lines appear to form a 'low' group ($\square$, $n = 12$), with its regression fit illustrated as a solid line.

again resembles published results for Caucasians (59%), but is less than that noted for Hispanics (69%), African-Americans (71%), Japanese-Americans (91%), and Chinese (91%) (Ingles et al., 1997a).

The assignment of F as the more active hVDR allele is based not only on the current analysis of fibroblast lines, but also on in vitro data collected with F and f proteins expressed in transfected cells [Fig. 7 and (Arai et al., 1997; Jurutka et al., 2000)]. Another group (Colin et al., 2000) has also studied F versus f hVDR proteins, and reported a lower ED_{50} for 1,25(OH)$_2$D$_3$ with the F allele. These data indicating a more active F hVDR allele are consistent with a number of epidemiological studies which suggest that the F allele, when compared with the f allele, is associated with increased BMD (Gross et al., 1996; Arai et al., 1997; Harris et al., 1997; Tao et al., 1998; Ferrari et al., 1999; Lucotte et al., 1999), higher rates of bone turnover (Kurabayashi et al., 1999), lower risk for primary hyperparathyroidism (Correa et al., 1999; Sosa et al., 2000), lower risk for intervertebral disc degeneration (Videman et al., 1998) and lower incidence of vertebral fracture (Gennari et al., 1999). However, it should be acknowledged that not all studies have found these associations. For instance, one group (Eccleshall et al., 1998) did not observe a correlation between hVDR genotype and BMD in a large cohort of French women. Also, another group (Gross et al., 1998) was unable to correlate any hVDR-related functional parameter with F/f genotype in either cells transfected with vectors expressing F versus f hVDRs, or in a small panel of human fibroblast lines, although perhaps because of methodological limita-

tions, small differences in activity may have escaped detection. Additionally, as discussed above, the fact that only the F/f genotype was considered in the above investigations implies that the L/S genotype could have been a significant confounder in these studies.

The assignment of L hVDR as more active than S is based exclusively on observations with the present panel of fibroblast cell lines. L and S hVDR alleles do not produce different proteins, and, therefore, cannot be tested in the same fashion as F/f isoforms. As a provocative test of this assignment, we instead entertained the alternative hypothesis that S hVDR alleles are more active, and replotted the data in Fig. 8A. However, the plot of this modified allele score ($F + S$) versus transcriptional activity appeared to show an inverse correlation between hVDR allele score and bioactivity (data not shown). This exercise, plus the present data (Fig. 6 and Fig. 8), strongly support the premise that L is the more active hVDR allele. Since this polymorphism occurs in exon IX, but is expressed only in the 3' UTR of hVDR mRNA, the working hypothesis presented herein states that the L allele may produce receptor mRNA that is more stable and/or is translated more efficiently into hVDR protein than the S allele.

Pertinent to the hypothesis above, the mRNA stabilities of allelic variants in or adjacent to exon IX [i.e., considering one or more sites in the $Bsm/Taq/Apa/$singlet(A) cluster] have been evaluated in recent studies using various strategies (Morrison et al., 1994; Mocharla et al., 1997; Verbeek et al., 1997; Carling et al., 1998; Gross et al., 1998; Durrin et al., 1999).

Mirroring the epidemiological investigations, these studies have yielded conflicting results. In one set of experiments (Verbeek et al., 1997), lymphocytes heterozygous for the *Taq* polymorphism were examined. It was found that, whereas mRNA stabilities were similar for both alleles, the mRNA from the *t* allele (linked to *S*) was consistently 30% less abundant. This result was interpreted as suggesting a possible difference in transcriptional regulation between the two allelic forms studied, although no mechanism for this effect was proposed (Verbeek et al., 1997). In contrast, another study (Carling et al., 1998) examining pituitary adenomas from 42 patients showed that *B* and *t* alleles were associated with higher hVDR mRNA levels, reaching statistical significance when homozygous *BB* or *tt* lines were compared with *bb* or *TT* homozygotes. Results similar to those of Carling et al. were obtained by others (Morrison et al., 1994), using a heterologous system in which 3.2 kb of 3′ UTR from two subjects homozygous for either *BAtS* or *baTL* were linked to a luciferase reporter gene. In transfected COS-7 cells, the *BAtS* construct displayed higher luciferase activity, suggesting to these authors that either transcriptional activity of the construct itself, or mRNA stability of its transcript, were more favorably affected by attachment of the *BAtS* as opposed to the *baTL* 3′ UTR (Morrison et al., 1994). Finally, three further groups reported no significant effect of 3′ UTR allelic variants on hVDR mRNA. These studies examined *B* versus *b* mRNA abundance in blood monocytes (Mocharla et al., 1997), *B* versus *b* hVDR protein and mRNA abundance in cultured skin fibroblasts (Gross et al., 1998), and stability of globin mRNAs attached to *L* or *S* 3′ UTRs in transfected NIH3T3 cells (Durrin et al., 1999). In particular, the last study, in which methodology similar to that of Morrison et al. was used, strongly suggests that the *baTL* and *BAtS* 3′ UTRs do not confer different mRNA stabilities, at least when attached to a heterologous (rabbit β globin) mRNA (Durrin et al., 1999).

Drawing conclusions from the above investigations with respect to the *L/S* polymorphism must be done cautiously, since only two of the six studies cited above (Morrison et al., 1994; Durrin et al., 1999) actually determined *L/S* genotype in their subjects. Nevertheless, given the reasonably tight linkage between *Bsm* I and singlet(A) polymorphisms (Ingles et al., 1997a), the above discussed results, when taken together, do suggest that mRNA stability may not be a major mechanism distinguishing the activity of *L* versus *S* alleles.

The possibility remains, however, that the *L* allele in some fashion produces more VDR protein from a given unit of mRNA. While there is a paucity of data to support or refute such a conclusion, it is notable that ligand binding assays (Gross et al., 1998) seem to indicate a trend toward higher VDR abundance (expressed as N_{max}) in *bb* versus *BB* fibroblast lines, although these differences were not statistically significant. Should it be the case that *L* alleles (linked to *b*) produce more hVDR protein, what could be the mechanism for such an effect? Recent observations regarding mammalian and yeast poly(A) binding proteins (PABPs) indicate that binding of PABP to mRNA enhances translatability of mRNAs via an interaction with other proteins that interact with the 5′ end of the message (Munroe and Jacobson, 1990; Le et al., 1997). Usually, multiple PABP monomers bind to poly(A)$^+$ RNAs, with each monomer occupying approximately 27 adenylate residues (Baer and Kornberg, 1980). Intriguingly, further studies with human PABP suggest that as few as 11 consecutive A's can bind to PABP, with 25 A's giving maximum affinity (Deo et al., 1999). Thus, one could speculate that: (a) PABP may be capable of binding to the singlet(A) repeat in the hVDR gene; and (b) its ability to bind may be enhanced in long (*L*) alleles (17–24 A's) versus short (*S*) alleles (10–15 A's). Greater association of PABP with *L* alleles would then lead to more efficacious translation via a more potent interaction with translation factors such as EF-4B (Le et al., 1997).

Regardless of the potential mechanism, the endogenous VDR activities, as measured in the present experiments using a $1,25(OH)_2D_3$-responsive reporter plasmid, showed a surprising range of activities, from 1.75- to 100-fold induction by $1,25(OH)_2D_3$ It is asserted that the current results are valid when comparing cell lines within the studied group, particularly because the data were normalized for transfection efficiency as monitored by inclusion of an expression plasmid for β-galactosidase. It is possible, nonetheless, that the presence of four closely-spaced vitamin D responsive elements in the $(CT4)_4$TKGH reporter construct used could exaggerate small differences in activity that may be much more subtle under in vivo conditions, especially considering that natural promoters often possess a single, or at most, two vitamin D responsive elements (Haussler et al., 1998). Indeed, the results with the BGP-TKGH, which contains a single VDRE element, revealed more modest transactivation levels in transfected cells (see Fig. 7A) and a less dramatic difference between *F* versus *f* hVDR activity.

The absence in the current sample set of a cell line with an allele score of zero (i.e. *ffSS*) is notable because it did not allow an evaluation of the activity of this genotype. The obvious explanation for this absence is the fact that, since hVDR *ff* and *SS* homozygotes are relatively uncommon among Caucasians (19 and 15%, respectively), the combination of *ffSS* would, therefore, represent a rare genotype (estimated frequency about 3% in Caucasians). Given the observed correlation between allele score and fold-induction by $1,25(OH)_2D_3$ (Fig. 8B), it is predicted that cells with this genotype

would have very low relative transcriptional activity. A recent epidemiological study (Hutchinson et al., 2000), in which a large cohort with malignant melanoma was genotyped at the T/t and F/f loci, supports this conclusion. It was found that $fftt$ (recall that t is often linked to S) had significantly thicker tumors ($P = 0.001$). These results intimate that the $fftt$ allele combination might be associated with less active hVDR, as VDR has been reported to have antiproliferative effects that might be expected to counter the malignant phenotype (Haussler et al., 1998). Clearly, more fibroblast lines must be examined to answer these questions, and it will be interesting to determine if $ffSS$ hVDR cell lines actually possess very low hVDR activity. Should this be the case, the provocative possibility could be raised that this rare $ffSS$ hVDR genotype might be so disadvantageous with respect to calcium and bone metabolism that it has been sharply reduced in the gene pool.

The two fibroblast lines in the present panel from patients with William's Syndrome exhibited very different hVDR activities, with the endogenous VDR in the DWF-CV line displaying extremely high (100-fold) induction by $1,25(OH)_2D_3$, and the DWF-TW line displaying near-average activity (23-fold induction). The extremely high activity of the DWF-CV line (three standard deviations above the mean of 28 ± 24 fold-induction) suggests a possible association with the hypercalcemia often seen in patients with William's Syndrome. However, because the great majority of William's Syndrome cases involve a chromosomal deletion at 7q11.23 (Ewart et al., 1993), and the hVDR gene resides on chromosome 12, this syndrome would appear to be unrelated to VDR action (as seems to be the case with patient DWF-TW). However, it is conceivable that patient DWF-CV, whose fibroblasts exhibit extremely elevated VDR activity, may represent an atypical William's case that does involve increased sensitivity to $1,25(OH)_2D_3$, a mechanism that has already been speculated for isolated cases resembling Williams's Syndrome (Ghirri et al., 1999).

Concerning the three osteoporotic patients in the current sample set, two fibroblast lines showed normal hVDR activity, but a third (Ran Nor, from a 69-year-old male) displayed very low induction of the reporter gene (6.2-fold versus the average of 28-fold). The low activity of hVDR in the Ran Nor cell sample could, in theory, be related to the low bone density of osteoporosis. However, osteoporosis is a multifactorial disease and, therefore, a very large study would be required to test any relationship between its etiology and hVDR alleles.

The lowest induction of the tested reporter construct by $1,25(OH)_2D_3$ (1.75-fold) was observed in the transfected cell line HS 144.We, taken from a 5-week-old embryo. These cells represent one of two prenatal cell lines in the present panel; thus, one possible explana-

tion for the very low hVDR activity is that cells from this early gestational stage may not yet be differentiated sufficiently to express VDR at levels seen postnatally. However, the other fetal cell line, HE-SK (exact fetal age unknown), displayed a slightly above average induction of 35.4-fold. Further arguing against the above interpretation is the observation from this laboratory that, with the exception of tissues like intestine that are phenotypically responsive to vitamin D in the adult, VDR expression has actually been shown to diminish in rat and chick tissues such as muscle and liver when they mature beyond the embryonic stage (M.R. Haussler and K. Yamaoka, unpublished data). Other groups have also observed VDR expression in various embryonic and fetal tissues (Takeuchi et al., 1994; Johnson et al., 1995; Delvin et al., 1996; Johnson et al., 1996; Veenstra et al., 1998; Segura et al., 1999). Finally, given that the HS 144.We line has an allele score of 1 (genotype $FfSS$), its observed fold-induction of 1.75 resides between a predicted value of 5.3-fold stimulation by $1,25(OH)_2D_3$ using the regression line for all 19 other lines, and a prediction of negligible stimulation (i.e. close to 1.0-fold) for the 'low group' regression line (both values calculated from plots similar to that of Fig. 8B, but omitting the HS 144.We data point). Thus, the observed HS 144.We hVDR transcriptional activity is not outside the predicted range for its allele score. Regardless, the data do not rule out early developmental-stage variations in human VDR expression, a topic that deserves further study.

The presence of two distinct groupings of hVDR activity versus allele score at the F/f plus L/S loci in Fig. 8B argues in favor of the existence of another variable that influences innate hVDR activity, at least in fibroblasts. There are a number of potential variables to consider, including such parameters as age and sex of the patients from whom the cells were taken. An analysis of these variables in the current sample set reveals that, although the gender distribution between the high and low groups of Fig. 8B is similar (60 and 50% male, respectively), the average age is somewhat higher in the low group, but this difference is without statistical significance (20.9 versus 4.8 years, $P = 0.11$). Thus, based on the current data, it is contended that age and sex of the cell donors would not be satisfactory explanations for the existence of a high and a low activity group.

Instead, the hypothesis put forth in the present communication (Fig. 9) is that there exists one or more additional polymorphic variations in the hVDR gene beyond those at the F/f locus and in the 3′ cluster ($Apa/Bsm/Taq$ and L/S) that affect(s) hVDR activity. The coding exons of the hVDR gene have been studied rather extensively, and have yielded numerous point mutations causing hereditary vitamin D resistant rickets (Hawa et al., 1996; Lin et al., 1996; Whitfield et al.,

1996; Haussler et al., 1998), but no evidence has been presented for the occurrence of common polymorphic sites other than the *Fok I* site in exon II and the linked cluster of sites in the exon VIII–IX region. Yet recent investigations into the portion of the hVDR gene encoding the 5′ untranslated region (5′ UTR) have revealed a surprising complex of at least seven exons (denoted IA-IG, see Fig. 9, top left), with evidence for alternative splicing (Miyamoto et al., 1997; Crofts et al., 1998). It is possible that undiscovered common polymorphic sites may exist in this newly described complex of multiple exons at the 5′ end of the gene. Such polymorphisms could even alter VDR protein structure by introducing in-frame initiator methionine codons leading to the expression of VDRs with N-terminal extensions, as already proposed by one research group (Crofts et al., 1998).

Still another possibility is that polymorphisms in the 5′ region of the VDR gene might affect the activity of one of the three proposed hVDR promoters (Crofts et al., 1998), leading to the expression of altered quantities of VDR proteins under physiologic conditions. Here again, a precedent exists from a recent report (Arai et al., 1999 J. Bone Miner. Res. 14, S191, Abstract T084), which describes a polymorphism in a binding site for Cdx-2, a homeodomain protein related to *caudal* (position of Cdx binding site in the hVDR gene is shown in Fig. 9, top left). The importance of Cdx-2 for intestine-specific expression of hVDR was demonstrated earlier (Yamamoto et al., 1999). It is, of course, not possible to invoke this polymorphism to explain the present results in fibroblast lines, given the intestine-specific nature of Cdx-2 regulated expression (Suh et al., 1994). However, the existence of this polymorphism should certainly be considered in epidemiological studies relating VDR-mediated intestinal absorption of calcium and phosphate as they impact BMD and parathyroid gland function. Indeed, in a large cohort of Japanese women, the *A* allele at the Cdx-2 locus correlated with higher BMD in the lumbar spine, consistent with a slightly greater activity of a VDR promoter construct incorporating the Cdx-*A* type element (Arai et al., 1999, J. Bone Miner. Res. 14, S191, Abstract T084).

Rather than evoking Cdx-2 or any other known polymorphism to explain the present results, we prefer the interpretation that a novel polymorphism exists, likely located in the incompletely characterized 5′ region of the hVDR gene. A full recognition of the genetic complexity of VDR action in humans may eventually allow for accurate prediction of VDR activ-
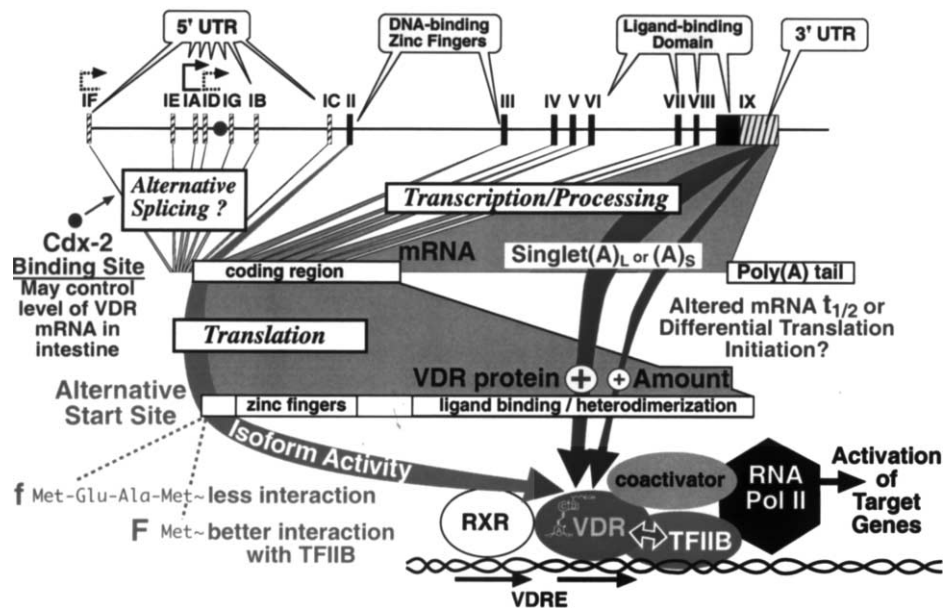

Fig. 9. Summary of common polymorphic variations in the hVDR gene that may influence VDR activity. The exon arrangement (top) is described in Fig. 2 and the associated text. The long (*L*) variant of the singlet(A) repeat in exon IX is proposed herein to be associated with increased VDR amount. A speculative mechanism for this effect is the enhanced ability of ≥ 17 consecutive As to recruit poly(A) binding protein, which would presumably stabilize VDR mRNA and/or promote its translation into hVDR protein (see text). The *F/f* polymorphism in exon II has likewise been shown to be associated with VDR activity, with the *F* isoform displaying both moderately higher transcriptional activity, as well as measurably greater interaction, in vitro, with TFIIB (Jurutka et al., 2000). Recently, a third hVDR polymorphism has been described upstream of exon IG in a binding site for the intestine-specific enhancer protein Cdx-2 (Arai et al., 1999, J. Bone Miner. Res. 14, S191, Abstract T084). This polymorphism may be significant for its effects on hVDR expression in intestinal cells (Yamamoto et al., 1999). All three of the illustrated polymorphisms are proposed to affect either the quantity of expressed VDR in human tissues (Cdx-2 and *L/S*), or the ability of VDR to interact with other proteins such as TFIIB (*F/f*, as illustrated at bottom right), in order to impact the transcription of target genes. Finally, it is suggested that other functionally relevant VDR gene polymorphisms may exist, especially in the recently-described complex of exons encoding the 5′-UTR.

ity in individual patients based on genotype, along with an enhanced ability to assess disease risk, as well as response to pharmacologic agents related to VDR action.

Acknowledgements

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