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Vitamin D receptor 3′-untranslated region polymorphisms: lack of effect on mRNA stability

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Abstract

Allelic variation at the 3′-end of the vitamin D receptor (VDR) gene has been associated with a 3–5-fold increased risk of developing prostate cancer and with differences in bone mineralization. This genetic diversity does not alter the VDR protein structurally, but instead may be a marker(s) of other, nearby polymorphisms that influence message stability or translation. The work reported here was instigated to identify additional VDR 3′-UTR polymorphisms that may have functional significance and to then test whether these genetic variants alter message stability. Initially, four novel, frequently occurring sequence variants were identified that associated with two common haplotypes that were described previously. These common sequence variants were not found within three message-destabilizing elements that we mapped within the 3′-UTR of the vitamin D receptor mRNA. Furthermore, the two VDR 3′-UTR haplotypes conferred an identical half-life on a heterologous β-globin reporter gene, in an in vitro assay. We therefore conclude that common polymorphisms within the VDR 3′-UTR do not influence message stability.

Keywords: Vitamin D receptor; Polymorphism; Message half-life

1. Introduction

The biologically active form of vitamin D, 1α,25-dihydroxyvitamin D_3, functions through a nuclear receptor to regulate mineral homeostasis and bone metabolism, as well as cellular differentiation and proliferation [1]. Recent studies have associated restriction fragment length polymorphisms (RFLPs) in the 3′-region of the VDR gene with normal physiological variability in circulating osteocalcin and vitamin D levels and with differences in bone mineralization [2,3]. Three independent studies, by Taylor et al. [4] and Ingles et al. [5,6], associated normal genetic variation at the 3′-end of the vitamin D receptor (VDR) gene with altered risk of prostate cancer in Caucasian and African-American populations. RFLPs in intron 8 (*Bsm*I, *Apa*I) and in exon 9 (*Taq*I) are in linkage disequilibrium with a poly(A) microsatellite ($(A)_{14-17}$ = S (short), $(A)_{18-24}$ = L (long)) in the VDR 3′-untranslated region (3′-UTR) of Caucasians and define two common haplotypes, BAt,S and baT,L (where b, a, and t are cut by

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*Bsm*I, *Apa*I, and *Taq*I, respectively, while B, A, and T are not cut). Taylor's study [4] focused on a *Taq*I RFLP in exon 9 (codon 352) that did not change the protein sequence of the VDR. Men who were homozygous for the *Taq*I RFLP (genotype tt) had a one-third decreased risk of prostate cancer than did men who were either homo- or heterozygous for the T allele (genotype Tt or TT). In analogous studies, Ingles et al. [5,6] examined the poly(A) length polymorphism in the 3′-UTR and found that Caucasian men who harbored either one or two L alleles (LL or LS) had a 4–5-fold increased risk of prostate cancer than did individuals homozygous for the short allele (SS). Among African-American men, only weak linkage disequilibrium exists between the *Bsm*I and poly(A) microsatellite markers [8]. However, a haplotype (BL) was identified in this ethnic group that conferred prostate cancer risk [6]. The one common feature of the 'at risk' allele in the two ethnic groups was the long (L) microsatellite in the 3′-UTR. Allelic differences (including possibly the size difference of the microsatellite) in the 3′-UTR of the VDR may affect message translation or stability. A decreased abundance of the vitamin D receptor could then lead to reduced tissue responsiveness to 1α,25-dihydroxyvitamin D_3 and increased risk of prostate cancer.

Several recent papers have examined differences in the function of two primary polymorphic forms of VDR. Verbeek et al. [9] and Mocharla et al. [10] used the reverse transcriptase-polymerase chain reaction (RT-PCR) assay to measure the abundance of the mRNAs from two different VDR genotypes in normal lymphocytes, and in leukemia and prostate carcinoma cell lines. Mocharla et al. [10] reported no difference in the amount of mRNA transcribed from either *Bsm*I allele (B or b) in cells that were homo- and heterozygous at this locus. Conversely, Verbeek et al. [9] consistently detected about 30% fewer transcripts from the *Taq*I (t) allele than from the T allele. They observed no difference, however, in the half-life of these two polymorphic transcripts. In an independent study, Carling et al. [11] employed the ribonuclease protection assay to measure significantly lower VDR message levels in parathyroid adenomas of patients who were homozygous for the b, a, T, or baT alleles than in patients who were homozygous for the B, A, t, or BAt genotype. Finally, Gross et al. [12] did not detect any variation in message levels when examining the *Bsm*I alleles (B vs. b) in heterozygous (Bb) fibroblasts. The latter group also examined expression from *Bsm*I B and b alleles, and cellular responsiveness to 1α,25-dihydroxyvitamin D_3, in dermal fibroblasts. They noted no significant differences in protein function between alleles. The BB and bb cell lines used by this group, however, included a variety of genotypes at the *Apa*I and *Taq*I polymorphic sites downstream of *Bsm*I. Thus, it cannot be presumed that the *Bsm*I genotype is indicative of polymorphic sites further downstream in the 3′-UTR.

Concurrently with the studies referred to above, we set out to examine mRNA stability bestowed by VDR 3′-UTRs of known sequence in order to establish the mechanism whereby normal allelic variation in the VDR gene may lead to a modified physiological response that can alter prostate cancer risk. The rationale for these experiments is two-fold. First, it is well known that the 3′-UTR is a major regulator of message half-life [13]. Second, areas of linkage disequilibrium do not extend very far along the >70-kb VDR gene, in the populations in which the associations with prostate cancer risk were demonstrated. For example, a *Fok*I RFLP in the VDR translation initiation site is not in linkage disequilibrium with the 3′-polymorphisms [14,15] and even the 3′-*Bsm*I and poly(A)$_n$ variants (which are separated by only 3 kb of sequence) are not tightly linked in all ethnic groups [8]. Initially, we sequenced the VDR 3′-UTR from eight individuals in order to detect frequent polymorphisms associated with the poly(A) microsatellite haplotypes. Two common VDR alleles were then inserted into a reporter plasmid, downstream of the stable rabbit β-globin gene that was under transcriptional control of the c-*fos* promoter. This reporter plasmid has been used extensively to measure the half-life conferred by a heterologous 3′-UTR. We were thus able to measure directly whether allelic differences in the VDR 3′-UTR encoded differences in message stability. In concurrent work, we identified three redundant, non-cooperative destabilizing elements in the VDR 3′-UTR. None of these sequence elements included the common polymorphisms. Additionally, the rare sequence variants did not alter message half-life conferred by nearby destabilizing elements.

2. Materials and methods

2.1. Detection of polymorphisms in the VDR 3′-UTR

To identify polymorphisms within the vitamin D receptor 3′-UTR, DNA was obtained from the leukocytes of eight individuals. Five individuals had previously been shown by genotype analysis to be homozygous for an $(A)_{18-24}$ tract (L) and another three individuals were homozygous for a $(A)_{12-17}$ tract (S). The entire 3.1 kb of 3′-non-coding sequences were amplified by the polymerase chain reaction technique and sequenced, without prior insertion into a vector. The frequency of a novel 5-nt insertion (+AGCCC after nt 2517, see Section 3) was assessed in an additional 25 African-Americans, 25 Asians, 25 Latinas, and 67 Caucasians.

2.2. Plasmid construction

The two relatively common polymorphic forms of the VDR 3′-UTR were amplified from leukocyte DNA and then inserted immediately after the rabbit β-globin gene in the plasmid pBBB (kindly provided by Ann-Bin Shyu [16]; by the following multi-step procedure. First, the 3.1-kb of 3′-UTR DNA (from immediately after the translation stop codon to, but not including, the poly(A) addition site) was amplified by the polymerase chain reaction (PCR) technique as two overlapping fragments. The primer pairs used were F9/R6 (1547-bp product) and F2/R11 (2009-bp product) (Table 1). The two PCR products were digested at a common *Not*I restriction enzyme site, and with *Bgl*II (cuts adjacent to the primer sequences), and ligated together into the *Bgl*II site of pBBB to give pVDR-3.1(L) and pVDR-3.1(S). The integrity of all VDR sequences in pVDR-3.1(L) and pVDR-3.1(S) was confirmed by dideoxynucleotide sequencing using the ThermoSequenase kit (Amersham).

Deletions were produced by digesting pVDR-3.1(L) with unique cutting restriction enzymes or by amplifying specific DNA fragments with primer pairs (Table 1; also see Fig. 4, top). Constructs deleted of their 3′-sequences initiated at the *Sma*I site (except for one that started immediately after the translation termination signal (TGA) and which was designated with an asterisk) and were denoted by an L and a number. The number represents the last nucleotide, before the deletion site, that is included in the construct. Numbering begins after the TGA. Constructs deleted of their 5′-sequences were denoted by an R and a number that indicates the initiating nucleotide. All these latter constructs terminate at a common 3′-end point, immediately upstream of the poly(A)-addition site. Other constructs were prepared by PCR amplification of specific DNAs. All VDR sequences within the plasmid constructs were validated by dideoxynucleotide sequencing as described above.

Plasmid pGAPM was kindly provided by Greg Goodall [17]. The plasmid pTRIamp-β-globin was prepared by digesting pBBB with *Pst*I after which the end was filled in with T_4 DNA polymerase. The plasmid was then digested with *Bam*HI and the 835-bp β-globin fragment was ligated into the *Sma*I/*Bam*HI sites of the vector pTRIamp19 (Ambion).

2.3. Cell culture and transfections

Mouse NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Cells were plated to approximately 50% confluence on 60 mm dishes, grown overnight and then transfected using the liposome transfection methodology. Briefly, 3 μg of the experimental DNA (pBBB-X) was combined with 1 μg of pCD_{NEO} in 150 μl of serum-free DMEM (DMEM-s/f). This mixture was added to an equal volume of DMEM-s/f containing 10–12 μl of TransfectAMINE reagent (Life Technologies) and DNA–liposome complexes were allowed to form for 30 min at room temperature. The complexes were then diluted to 1.5 ml total with DMEM-s/f and added to a plate of NIH3T3 cells that had been washed once with DMEM-s/f. After 5 h at 37°C, 5% CO_2, the DNA–liposome mixture was removed and the cells were grown in DMEM–10% FBS and incubated as above for 2 days. Cells were then trypsinized and replated on two to four 100-mm plates in DMEM–10% FBS containing 300 μg/ml geneticin (G418-sulfate; Life Technologies). Colonies (100–300) were pooled after 12–14 days and maintained in DMEM–10% FBS plus 200 μg/ml geneticin. For analysis of mRNA

decay, cells were plated on 60-mm dishes, grown to 80–90% confluence, then washed two times with phosphate buffered saline (PBS) and serum starved in DMEM–0.5% FBS. After 24 h, the cells were stimulated with DMEM–20% FBS for 0–8 h. Two stably transfected cell lines were prepared from each construct and message half-life was measured two or three times for each cell line (four to six times total).

2.4. RNA preparation and analysis of mRNA decay

Total RNA was isolated at specific times after serum stimulation, by the Purescript RNA isolation method (Gentra Systems). Cells were washed once with PBS and then lysed with cell lysis solution. Protein and DNA were precipitated and removed and total RNA was recovered by isopropanol precipitation. Transcripts derived from transfected DNA, and cellular GAPDH, were detected by RNase protection analysis using in vitro synthesized complementary RNA probes. The pBBB, pVDR-3.1(S) and pVDR-3.1(L) transcripts (β-globin or β-globin/vitamin D receptor fusion mRNAs; Fig. 1) were detected using probes synthesized from the plasmid pTRIampβ-globin digested with *Nco*I, using T_7 RNA polymerase and 200 Ci/mmol of [α-^{32}P]UTP. The 278 nucleotide (nt) probe protected a 188-nt fragment within the β-globin coding region. As an internal, loading control, a 120-nt fragment of the mouse GAPDH mRNA was detected using a 175-nt riboprobe [17]. RNase protection assays were performed as described by the manufacturer (Ambion), using 10 μg of total RNA. Protected products were analyzed on 6% polyacrylamide–8 M urea gels which were dried prior to quantification. The amount of specific mRNA was measured by PhosphorImager analysis using Imagequant version 3.21 (Molecular Dynamics, Sunnyvale, CA) and normalized with respect to the GAPDH internal standard. To correct for the increase in GAPDH message levels during serum stimulation, the GAPDH mRNA data for each time point (approximately 50 independent values) were pooled and a regression line was fitted to this data to calculate the expected GAPDH message levels. The β-globin mRNA levels were then normalized to these expected GAPDH transcript levels.

3. Results

3.1. Polymorphisms in the 3′-UTR of the vitamin D receptor message

The entire vitamin D receptor 3′-UTR was sequenced in order to identify genetic variations that might regulate message stability. DNA was isolated from the leukocytes of eight individuals who were chosen on the basis of their genotype at the $(A)_n$ microsatellite; five individuals were homozygous 'long' (L) and the other three were homozygous 'short' (S) at this site. The entire vitamin D receptor 3′-UTR from each test subject was amplified as a series of overlapping fragments and sequenced manually, using the dideoxynucleotide sequencing protocol of Sanger et al. [18], without prior subcloning. This approach avoids erroneous identification of either amplification or cloning artifacts. The method is also sensitive enough to detect heterozygosity at individual nucleotides. We observed seven polymorphisms, four of which appeared to be common (present in six (S) or ten (L) out of sixteen total alleles sequenced) and three that were rare (see below). In this limited study, the common sequence

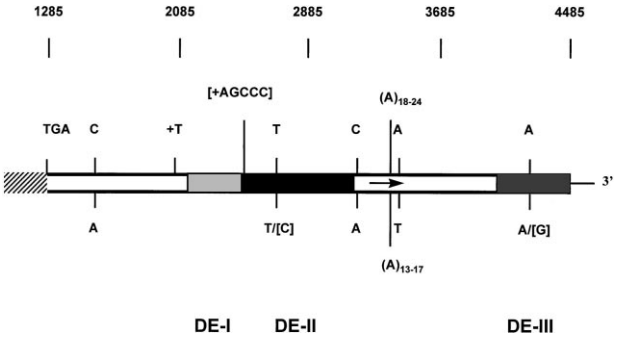

Fig. 1. Polymorphisms within the vitamin D receptor 3′-UTR. Two common haplotypes of the vitamin D receptor 3′-UTR are shown schematically as a double line with the stippled box at the left representing part of the coding region and TGA is the VDR translation termination signal. The horizontal arrow depicts a forward oriented Alu-like sequence. Sequences are numbered at the top according to Baker et al. [19]. Genetic variations are shown as those that associate most commonly with the baT,L haplotype (top) or with the BAt,S haplotype (bottom). Three polymorphisms that occur infrequently are shown in brackets. Three destabilizing elements (see later) are depicted as gray or black filled boxes and designated as DE-I, DE-II and DE-III at the bottom.

variants associated exclusively with the L or S poly-(A) tract (Fig. 1). The nucleotide location (relative to the sequence of Baker et al.; [19]), and the haplotype the frequent polymorphisms associated with, are designated as C1588A, T2074del, C3185A, A3424T (where the initial nucleotide was associated with the L allele and the latter nucleotide was associated with the S allele). Rare genetic variants were heterozygous in the eight individuals examined and included a 5-nt insertion (AGCCC2517del, found on about 7% of L alleles among Caucasians and Latinas, but not among African-Americans or Asians), and T2697C and A4107G sequence conversions (where the C and the G, respectively, were found once only in 16 alleles sequenced). The $(A)_n$ microsatellite centers around nt 3400 (Fig. 1). In addition to the poly(A) microsatellite, only one of these polymorphisms, the T2074del, corresponded with any of the VDR sequence variants previously reported to differentially influence gene expression [3].

3.2. Half-life conferred by the polymorphic vitamin D receptor 3′-UTRs

Calculation of mRNA decay rate following short-term activation of an inducible promoter (i.e. the c-*fos* promoter) is a widely used method to measure message stability (Fig. 2) [16,17]. Degradation of the β-globin gene, which is very stable when regulated by its homologous 3′-UTR ($t_{1/2} > 18$ h; see below) [20], is often used as the reporter in these constructs. Insertion of a heterologous 3′-UTR after the β-globin translation termination site signals the β-globin gene to be degraded with the kinetics characteristic of the substituted 3′-UTR. The two allelic forms of the vitamin D receptor mRNA 3′-UTR were prepared as described in Section 2 and inserted into the unique *Bgl*II restriction enzyme site in the reporter plasmid pBBB (Fig. 2). Fig. 3A illustrates the nucleotides that differ between the two constructs: the top line represents the L allele and the bottom line the S allele. Only the more frequent polymorphisms were included in the constructs. After stable integration of the chimeric molecules into mouse NIH3T3 cells, the c-*fos* promoter was silenced by 24 h growth in media containing low serum (0.5%) and then induced by serum growth factors (MEM+20% FBS). A synchronized pool of mRNA was synthesized (the c-*fos* promoter shuts off after 30–60 min [16,17]), after which message degradation was measured. Fig. 3B and C show representative autoradiograms from ribonuclease protection assays that measured the half-life ($t_{1/2}$) of pBBB and pVDR-3.1(S). The VDR message present at each time point was measured, corrected relative to the internal control (GAPDH), and graphed (Fig. 3D). The VDR message half-life was calculated from the linear descending slope. Both allelic forms of the VDR 3′-UTR conferred almost identical half-lives on the heterologous β-globin gene (pVDR-3.1(L), $t_{1/2} = 5.3 \pm 1.4$ h and pVDR-3.1(S), $t_{1/2} = 5.2 \pm 0.4$ h).

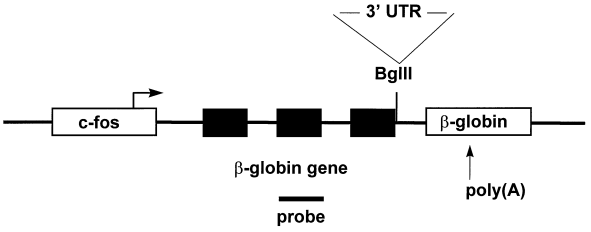

Fig. 2. Reporter vector pBBB [16]. Transcription of the rabbit β-globin gene (filled boxes) is under control of the c-*fos* promoter (open box at left). A unique *Bgl*II cloning site, for insertion of heterologous 3′-UTR sequences, is located immediately after the β-globin translation termination signal. The rabbit β-globin poly(A) addition site (open box at right) encodes efficient message termination and polyadenylation. The β-globin gene coding region probe (exon 2) utilized for ribonuclease protection assays is illustrated (thick line, bottom).

Table 1
Vitamin D receptor mRNA 3′-untranslated region primers

F9	5′-ggagatctAGGACAGCCTGTGCGGTGC-3′
R6	5′-GACAATGGGGCCAGGTGGAC-3′
F2	5′-GGGGTTCCGTGATGTAG-3′
R11	5′-ggagatctGCAAAGCTTTCTACATTGGTTGACT-3′
F7	5′-ggaagatctCGGATCACCGAGAGTAGCCGA-3′
R3	5′-ggagatctCCCACCTTGGAGTAAACGGAC-3′
F10	5′-ggagatctCGGAGTGATACAACTTCAAGTGC-3′
F13	5′-gggagatctGCCCAGGGCTGAGTAACTGATAT-3′
R12	5′-gggagatctTGAGGGGATTGACTCGTTTAGC-3′
F12	5′-ggagatctGGGGAACTCCTGAAATATCAGTTAC-3′
R13	5′-gggagatctGCCCAGGGCTGAGTAACTGATAT-3′
F11	5′-ggagatctTTGGAGTCATGGTCCGGTG-3′

R, reverse primers; F, forward primers. To facilitate localization of primers on the VDR 3′-UTR, search GenBank with the ascension number JO3258. Nucleotides depicted as small letters are *Bgl*II restriction enzyme cutting sites used to facilitate cloning into the reporter plasmid.

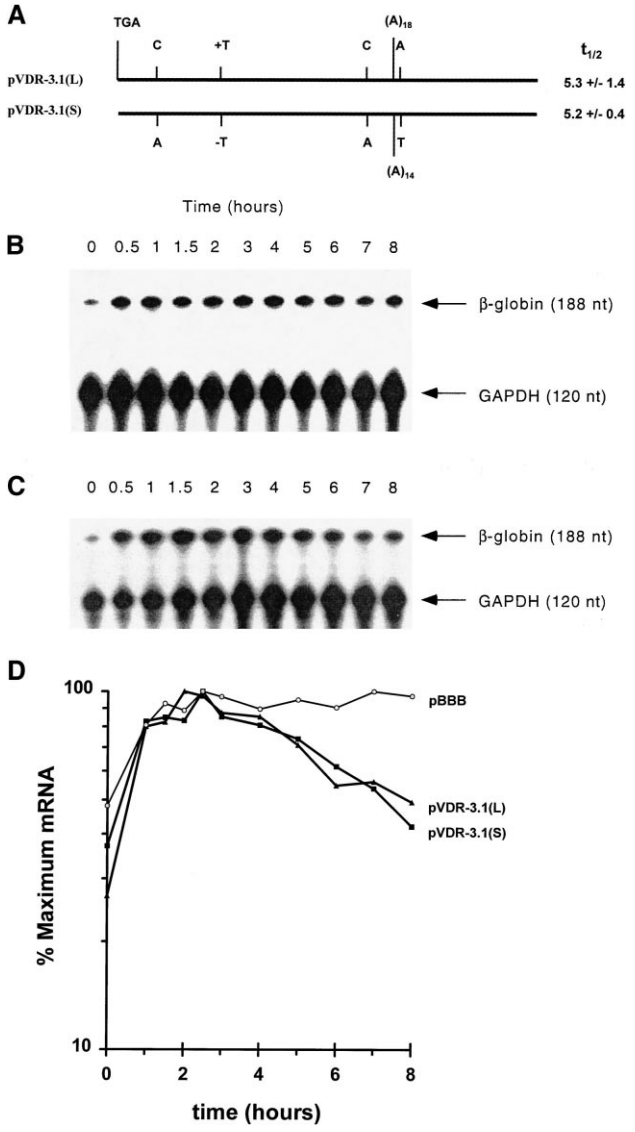

Fig. 3. (A) Half-lives conferred by the VDR 3′-UTRs. The plasmids pVDR-3.1(L) (top) or pVDR-3.1(S) (bottom) are identical except for the nucleotides indicated. These constructs were transfected into NIH3T3 cells and half lives ($=t_{1/2}$) measured as described in Section 2. All measurements were repeated 4–6 times. (B,C) Representative autoradiographs of ribonuclease protection assays for pBBB and pVDR-3.1(S), respectively. (D) The β-globin or β-globin-VDR fusion mRNA, at each time point, was measured by PhosphorImage analysis and corrected relative to the internal GAPDH loading control. Each time point was plotted as a percentage of the maximum induced level of β-globin mRNA. Message half-life was calculated from the descending slope. open circles, pBBB; filled squares, pVDR-3.1(L); filled triangles, pVDR-3.1(S).

3.3. Destabilizing elements within the vitamin D receptor 3′-UTR

To identify vitamin D receptor 3′-UTR functional elements that regulate mRNA decay rates, a series of 3′-deleted DNA fragments were prepared by digesting the pVDR-3.1(L) construct with the unique-cutting restriction enzymes *Apa*I, *Sac*I, *Kpn*I, *Sph*I, and *Bam*HI (Fig. 4, top; the translation termination signal (TGA) and a polymorphic *Alu*-like repeat sequence (arrow) are shown for reference). Half-lives were measured as described in Section 2. Surprisingly, the VDR 3′-UTR, deleted of anywhere from 400 to 1900 nt of its 3′-sequences (Fig. 4, constructs L2689, L2337, L1702, L1219), still conferred a half-life of approximately 4–5 h on the chimeric β-globin message. This was similar to the half-life of the full-length VDR 3′-UTR (pVDR-3.1(L), $t_{1/2}=5.3\pm1.4$ h). However, when another 500 nt was deleted, the VDR 3′-UTR lost its ability to destabilize the β-globin message (Fig. 4, construct *L933, $t_{1/2}>18$ h). Thus, a VDR destabilizing element (=DE-I) was located within the 500 nt delimited by the *Bam*HI and *Sph*I restriction enzyme sites. This was confirmed by the 5′- and 3′-truncated construct 841–1283, which encompassed only 442 nt of the VDR non-coding sequences between the primer pair F7 and R3 (Table 1). These sequences destabilized the β-globin message to $t_{1/2}=4.4\pm0.4$ h.

The *Alu*-like repeat element (Fig. 4, arrow) contains the poly(A)$_n$ tract that has been associated with prostate cancer risk [5,7]. This polymorphic length allele, or other sequences in this vicinity, could differentially affect stability of the VDR mRNA. Thus, to further characterize these sequences, a 1150-bp region was amplified between the primer pair F10 and R10 (Fig. 4, construct 1195–2342) and inserted into pBBB. Interestingly, these sequences destabilized the β-globin message independent of, and as efficiently as did DE-I ($t_{1/2}=4.5\pm1.1$ h). To further demarcate this second destabilizing element (DE-II), the 1150-bp DNA sequence was dissected into 710-bp (primer pair F10 and R12) and 480-bp (primer pair F13 and R10) fragments and the half-life conferred by these sequences was measured. The smaller fragment (construct 1851–2342) included all of the *Alu*-like repetitive element, but did not destabilize the β-globin message ($t_{1/2}>18$ h). However,

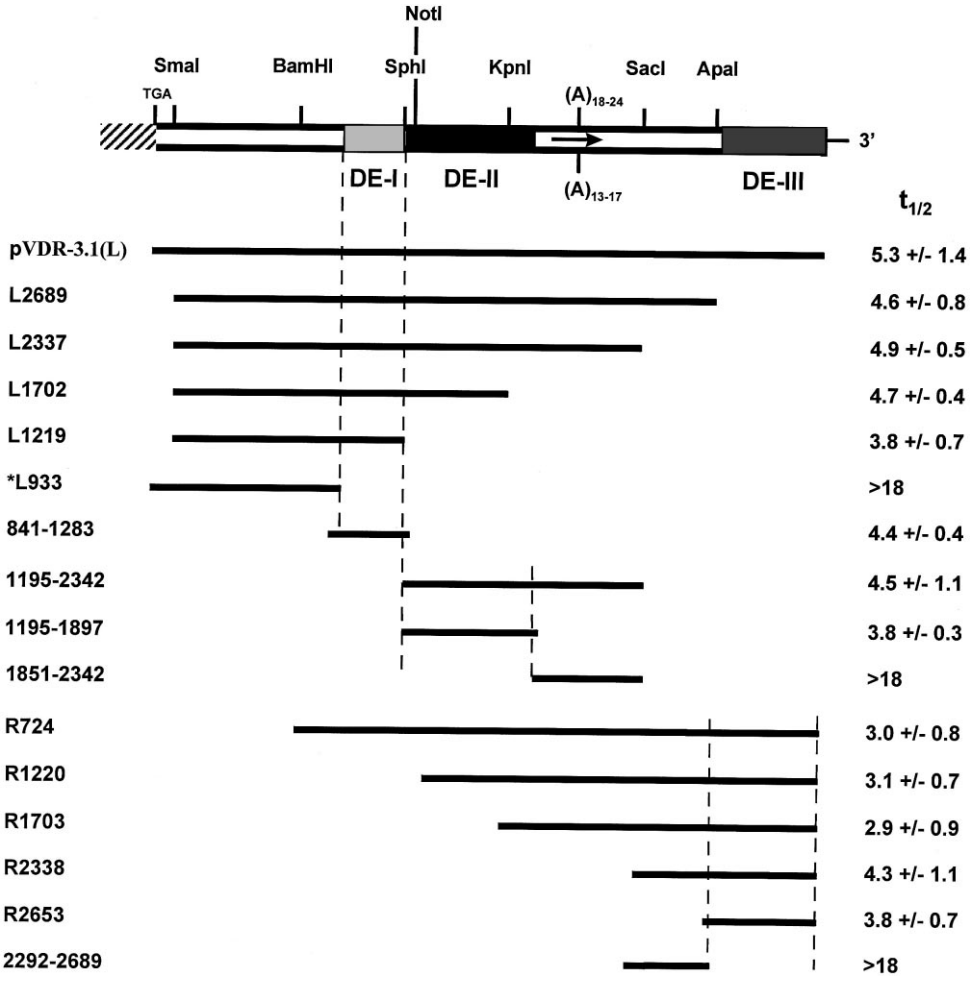

Fig. 4. Half-lives of vitamin D receptor 3′-UTR deletion constructs. At the top is a schematic diagram of the vitamin D receptor 3′-UTR depicting the translation termination signal (TGA), the variable length poly(A)$_n$ tract within the *Alu*-like repeat sequence (horizontal arrow), and unique restriction enzyme cutting sites. The three destabilizing elements (DEs) identified by deletion analysis are shown as gray or black filled boxes. The lines at the bottom represent DNA sequences that are present in the constructs. Construct names are shown at the left and half-lives ($t_{1/2}$) conferred on the heterologous reporter gene are shown at the right.

the larger fragment (construct 1195–1897) did signal β-globin mRNA destabilization ($t_{1/2} = 3.8 \pm 0.3$ h). Thus, DE-II was located adjacent to, but independent of, DE-I.

Deletions from the 5′-end of the VDR 3′-UTR were prepared by restriction enzyme digestion at the unique *Bam*HI, *Not*I, *Kpn*I or *Sac*I sites and also by PCR amplification. Interestingly, after deletion of approximately 85% of the 5′-sequences (including DE-I and DE-II), the remaining 430 nt of sequences (delimited by the primer pair F12 and R11) destabilized the β-globin message at a rate similar to that of the full-length 3′-UTR (Fig. 4, $t_{1/2} = 3.8 \pm 0.7$ h for construct R2653). The singularity of this element (DE-III), and its upstream border, were confirmed by the 5′/3′-deletion construct 2292–2689 (amplified using the primer pair F11 and R13) which included approximately 400 nt of sequences that did not direct β-globin message turnover ($t_{1/2} > 18$ h).

Interestingly, none of the common polymorphisms, including the (A)$_n$ tract, were situated within the destabilizing elements. The rare sequence variants, however, were either very near to or were within the DNA fragments containing the three destabilizing elements (summarized in Fig. 1).

3.4. Effects of individual polymorphisms on message stability

To compare whether specific sequence variations altered message stability, constructs were prepared containing the alternative polymorphic form of individual DEs (all constructs in Fig. 4 are either the L or the common allele). The function of DE-I was not significantly altered when either sequence variant was present at T2074del (construct 724–1283, $t_{1/2}$ = 4.6 ± 0.8 h (+T) vs. $t_{1/2}$ = 3.6 ± 0.6 h (del), or at AGCCC2517del (construct 841–1283, $t_{1/2}$ = 4.4 ± 0.4 h (del) vs. $t_{1/2}$ = 4.1 ± 0.7 h (+AGCCC). Likewise, DE-II (construct 1195–2342) displayed similar $t_{1/2}$ values when three sites (C3185A, $(A)_{19}3400(A)_{14}$, A3424T) were altered simultaneously ($t_{1/2}$ = 4.5 ± 1.1 h (L) vs. 3.9 ± 1.7 h (S)). DE-III (construct R2653) conferred similar half-lives when position 4107 was substituted with an A ($t_{1/2}$ = 3.8 ± 0.7 h) or a G ($t_{1/2}$ = 4.2 ± 0.5 h). We have not yet investigated whether AGCCC2517del or T2697C influence the function of DE-II.

4. Discussion

Restriction fragment length polymorphisms (*Bsm*I, *Taq*I, and *Apa*I) and a variable length $(A)_n$ microsatellite (short (S) = A_{13-17}; long (L) = A_{18-24}) in the 3′-portion of the vitamin D receptor gene have been associated with variability in prostate cancer risk and with physiological differences in bone density [2,4–6]. Additionally, 3′-UTR sequence differences in two common VDR haplotypes (baT,L and BAt,S) have been associated with a small variability (less than two-fold) in VDR gene expression [3]. The genetic differences in the latter study were detected after comparing only two VDR alleles and may not represent true polymorphisms. Here we identified seven novel sequence variants in the VDR 3′-UTR. Interestingly, only one of these, T2074del (and also the poly(A) tract described previously), corresponded with the genetic variants previously reported to differentially regulate gene expression [3]. These discrepant results are likely due either to differences in methodology, or to differences in the source of the alleles sequenced. Incorrect nucleotides can be misincorporated during PCR amplification of a DNA fragment, even when using high-fidelity enzymes such as rTth polymerase (personal observation). Detection of such incorrect nucleotides will be selected for if the PCR products are then cloned into a vector before sequencing.

Allelic variation in the 3′-UTR of a messenger RNA could affect a number of post-transcriptional processes including message stability. We chose the 'short-term promoter activation' technique to examine whether known nucleotide differences in the VDR 3′-UTR conferred different half-lives on a heterologous β-globin reporter gene. We found that the two variant 3′-non-coding regions signaled almost identical rates of message degradation (Fig. 3A–D; pVDR-3.1(L), $t_{1/2}$ = 5.3 ± 1.4 h vs. pVDR-3.1(S), $t_{1/2}$ = 5.2 ± 0.4 h). Thus, it appears that common genetic variation in the long VDR 3′-UTR does not contribute to disease states by directly altering message stability (and therefore abundance of the mRNA). This conclusion is supported by the identification of three distinct destabilizing elements within this region. These elements function in a non-additive, redundant, and sequence locality-independent fashion (i.e. the distance between the translation termination site and the DE does not alter its destabilizing abilities). Thus, in the mouse NIH3T3 cell system, it appears that all three elements would have to be altered for message half-life to be changed significantly. This is significant since none of the common polymorphisms we identified were situated within the DEs (Fig. 1) and the rare sequence variants did not affect message stability conferred by individual DEs under our experimental conditions (see Section 3). However, we suggest that the three VDR destabilizing elements may not always function contemporaneously to constitutively destabilize messenger RNA. An alternative hypothesis is that an individual DE could moderate VDR message stability, independent of the other two DEs, during differentiation, development and/or hormonal (effector) stimulation. Thus, a polymorphism within or adjacent to a DE might modulate the activity of that functionally relevant DE only in a specific tissue (e.g. normal prostate epithelial cells) or during a specific stage of growth or effector stimulation. In support of this premise, a recent example from another system has demonstrated that, under hypoxic conditions, a protein is induced that binds one of two (normally de-

stabilizing) AREs (message stability elements) within the vascular endothelial growth factor gene 3′-UTR, resulting in message stabilization [21]. Additionally, a sequence element (105 bp) in the glucose transporter (GLUT1) mRNA 3′-non-coding region mediates message destabilization but can also function as a site through which the cytokine, tumor necrosis factor-α stabilizes the message 5-fold [22].

As an alternative to regulating message stability, it is possible that the VDR 3′-UTR interacts with other, upstream sequences to regulate transcription, translation, or RNA processing. A recently identified translation initiation site RFLP (*Fok*I), which is not linked to the 3′-VDR polymorphisms, encodes two different sized proteins and has been associated with differences in bone mineralization [14,15]. It is possible that a polymorphic functional element in the VDR 3′-UTR interacts diversely with these two translation start sites, leading to differences in VDR levels and, subsequently to changes in prostate cancer risk and in bone mineralization. However, the recent work by Mocharla et al. [10] and Gross et al. [12] suggests that this may not be the case. Both groups used RT-PCR to measure transcription from two polymorphic forms of the VDR in homozygous or heterozygous cell systems (normal lymphocytes, leukemia, and prostate carcinoma cells). They noted neither allele-specific differences in message levels nor differences in protein abundance or function. One problem with these studies, however, is that the genotype upstream and downstream of the *Bsm*I and *Taq*I RFLPs is not known and this could significantly effect interpretation of the results. Conversely, Carling et al. [11] did measure allelic differences in VDR message levels in parathyroid adenomas. Thus, genetic diversity may exert tissue-specific differences on VDR transcription.

Another possible explanation for the association between genetic variation in the vitamin D receptor 3′-region and bone loss or prostate cancer risk, is the existence of an unknown, but nearby, gene in linkage disequilibrium with the VDR polymorphisms. However, even the *Bsm*I and poly(A)$_n$ variants (which are separated by only about 3 kb of sequence) are not tightly linked in all ethnic groups [8]. Therefore, additional work is necessary to define the allelic variation that alters the phenotype at this locus. Only then will we understand the role that vitamin D plays in prostate cancer risk and bone metabolism.

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