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Cloning, genetic characterization, and chromosomal mapping of the mouse *VDUP1* gene

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Abstract

VDUP1 encodes a vitamin D3-inducible gene product that has been shown to be down-regulated in chemically-induced mammary tumors in rats. It has recently been reported to negatively regulate thioredoxin expression and function. We have cloned the mouse *VDUP1* gene and characterized its genomic locus. The *VDUP1* coding region spans eight exons within a total length of 2.3 kb located on mouse chromosome 3. Consensus sites for polyadenylation were identified 1.3 kb downstream of the gene, defining a long 3′ untranslated region. The minimal functional *VDUP1* promoter contains TATA and CCAAT boxes and transcription is initiated from two major start sites downstream. A direct repeat element located proximal to the TATA with homology to the USF binding site was identified as a potential regulator of *VDUP1*gene expression. Expression analysis determined that *VDUP1* mRNA was markedly induced in myeloma cells in high density cell culture, but not in sub-confluent cells arrested by serum deprivation. All samples of a panel of mouse immortalized or transformed cell lines were shown to express abundant levels of *VDUP1* mRNA.

Keywords: Thioredoxin; Vitamin D3; Gene regulation; Promoter

1. Introduction

Deregulation of cell growth control is a major effector of tumorigenesis and a target of many anti-cancer strategies. The hormonally active form of vitamin D3, 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$], and a variety of related analogs are novel putative anticancer agents with a range of actions on tumor cells including initiation of growth inhibition, cell differentiation, and apoptosis (Kubota et al., 1998; van den Bemd et al., 2000; Majewski et al., 2000). Regulation of gene expression by vitamin D3 exposure occurs primarily by transcriptional activation mediated by the vitamin D3 nuclear hormone receptor (VDR) (Lian and Stein, 1992; Chen and DeLuca, 1995; Haussler et al., 1995). Following the association of 1,25$(OH)_2D_3$ with VDR, a retinoid X receptor (RXR) is recruited to form a VDR-RXR heterodimer that binds with high affinity to regulatory regions of vitamin-D3 responsive genes, leading to enhanced or in some cases repressed gene expression. The vitamin D3 response element (VDRE) consists of guanine rich hexanucleotide direct repeats spaced by three nucleotides (Haussler et al., 1995).

By differential hybridization, Chen and DeLuca (1994) identified *VDUP1* as a gene that was strongly responsive to vitamin D3 exposure in an HL-60 model system of cellular differentiation. It was shown to encode a 46 kd protein of unknown function. Induction of *VDUP1* by vitamin D3 occurred even in the presence of the protein synthesis inhibitor cyclohexamide. Furthermore, exposure to cyclohexamide alone induced *VDUP1* several fold, suggesting that mRNA stability may be a factor in up-regulation of *VDUP1* expression. Independently, a second group determined that the *VDUP1* gene was significantly down-regulated in chemically induced rat mammary tumors (Young et al., 1996; Yang et al., 1998). Exposure of cell lines derived from these tumors to vitamin D3 resulted in elevated *VDUP1* expression and inhibition of cell growth (Yang et al., 1998).

Nishiyama et al. (1999) have recently shown by yeast two-hybrid analysis that VDUP1 specifically binds to the reduced form of the disulfide reducing protein thioredoxin

Abbreviations: VDUP1, vitamin D3 up-regulated gene 1; USF, upstream stimulation factor; TRX, thioredoxin; VDR, vitamin D3 receptor; RXR, retinoid-x-receptor; VDRE, vitamin D3 response element; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D3; BAC, bacterial artificial chromosome; pBSK-II, pBluescript-SKII; kb, kilobase; bp, base pair; RFLP, restriction fragment length polymorphism; SEAP, secreted human placental alkaline phosphatase; UTR, untranslated region; MNNU, *N*-methyl-*N*-nitrosurea; FCS, fetal calf serum; FISH, fluorescence in-situ hybridization

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(TRX) and inhibits its reducing potential. Thioredoxin is a general disulfide oxido-reductase that plays a key role in maintaining cellular redox status. Control of transcription is intimately linked to cellular redox state and over-expression of TRX has been correlated with growth promotion and cell activation (Nakamura et al., 1992, 1997; Gasdaska et al., 1995). In addition, transient expression of *VDUP1* in cells leads to reduced levels of thioredoxin protein, suggesting that it may also modulate thioredoxin expression. The biological role of VDUP1 on TRX expression and function may be to directly modulate redox regulation of cellular factors and pathways involved in cell growth, differentiation, and apoptosis. As a means to delineate the mechanisms by which *VDUP1* is regulated, we have cloned and characterized the murine *VDUP1* gene. Sequences consistent with a strong 5′ promoter region and 3′ regulatory elements were identified that may act in concert to control cellular *VDUP1* levels in response to stimuli.

2. Materials and methods

2.1. PCR cloning of mouse VDUP1 cDNA

Primers VT3F2: 5′-ATGGTGATGTTCAAGAAGAT-CAAGT-3′, and VT3R2: 5′-TCACTGCACGTTGTTGT-TGTTGTTG-3′, corresponding to the 5′ and 3′ ends of the rat *VDUP1* coding region (GenBank accession number: U30789), respectively, were generated for PCR (Bio-Synthesis Inc.). Amplification of the mouse *VDUP1* coding region was performed using the Expand kit (Boerhinger Mannheim) and a day 15 mouse embryo Quick-Clone cDNA pool (Clontech) as template. Methods were performed according to manufacturer's specifications. Amplified PCR product was gel purified and used directly as probe DNA or cloned into pBluescript-SKII (Stratagene). Automated DNA sequencing (University of Georgia) was performed to verify the integrity of the PCR product.

2.2. Cloning of the murine genomic VDUP1 gene

The coding region of the mouse *VDUP1* gene was used as probe to screen a mouse C57B6 genomic BAC library (Genome Systems Inc.). Positive clones were screened for the expected RFLP by restriction endonuclease digestion, gel electrophoresis, and Southern hybridization with the *VDUP1*-specific probe. Southern blotting and hybridization was performed as previously described (Ludwig et al., 1996). DNA probes were generated by random primer labeling (Prime-it II kit; Stratagene). For RFLP comparison to total mouse genomic DNA, genomic DNA was isolated from Sp2/0 myeloma cells using the Promega Wizard prep kit. Subcloning of the *VDUP1* gene was performed into pBluescript-SKII (Stratagene). For sequencing of the *VDUP1* gene, a 7 kb EcoRI fragment was subcloned. For sequencing of the promoter region, a 3 kb EcoRI/BamHI fragment was subcloned. Automated DNA sequencing was performed by the University of Georgia. DNA sequence analysis was performed using LaserGene software (DNA Star). Sequence analysis of the *VDUP1* promoter region utilized the TFSEARCH program at: http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH. Sequence information for the mouse *VDUP1* gene locus was entered into GenBank and given the accession number AF282825. The mouse *VDUP1* cDNA sequence was assigned the accession number AF282826.

2.3. Promoter deletion analysis of the mouse VDUP1 5′ region

Successive deletions of the mouse *VDUP1* 5′ end were amplified by PCR using Taq polymerase (Fermentas) and selected primer sets. Primers and sequence locations were V1 (REV primer) 5′- GATTGAGCCGAGTGGG-TTCA-3′; V2 (−597), 5′-AGCTCCCAAGAGGAGTCC-CC-3′; V3 (−1002), 5′-ACAGTAGCAGCACATAGGGG-3′; V4 (−1448), 5′-CTCTAGTCAGCTCCTGAGGC-3′; V5 (−452), 5′-AGCACACCGTGTCCACGCGC-3′; V6 (−403), CTGGTAAACAAGGGCCAAGT-3′; V7 (−337) 5′-AGCACTCGCGTGGAGCGCCA-3′; V8 (−281), 5′-TTGACACTCTCCTCCTC-TGG-3′. PCR products were cloned into pCR2.1 (Invitrogen) and sequenced. Promoter fragments were then cloned into a secreted alkaline phosphatase expression (SEAP) vector, pSEAP-basic (Clontech) for transient transfection analysis. Expression analysis was performed in COS-7 cells cultured in DMEM (Life Technologies) supplemented with 10% fetal calf serum (HyClone), 2 mM glutamine (Life Technologies), and Penn-Strep (Life Technologies). DNA was prepared using Qiagen MaxiPrep columns and transfected using Lipofectamine 2000 (Life Technologies) in OptiMEM (Life Technologies) according to manufacturer's specifications. SEAP activity was determined from cell culture supernatants 48 h post-transfection using the substrate p-nitrophenyl phosphate (Sigma) according to Flanagan and Leder (1990).

2.4. Primer extension mapping of 5′ transcription start sites

Primer extension was performed on total RNA prepared from Sp2/0 mouse myeloma cells using the Trizol reagent (Life Technologies) and the primer extension system from Promega. The antisense primer for extension of the *VDUP1* mRNA 5′ end was VT3F2R: 5′-ACTTGATCTTCTTGAA-CATCAC**CAT**-3′ initiating at the site of the ATG start codon (in bold). Reaction products were run on QuickPoint sequencing gels (Novex). DNA sequencing reactions were prepared (US Biochemical) using radiolabeled VT3F2R primer and run along side the primer extension reactions. Radiolabeled size markers were also run to facilitate mapping mouse *VDUP1* start sites.

2.5. Chromosomal localization of the mouse VDUP1 gene

FISH (Genome Systems Inc.) was performed on normal

mouse metaphase chromosomes using a digoxigenin dUTP labeled DNA probe generated from mouse BAC clone 7C21 containing an insert of approximately 70 kb, including mouse *VDUP1*. For verification of chromosome 3 a probe specific for the telomeric region of mouse chromosome 3 was also hybridized. Chromosome spreads were obtained from mouse embryo fibroblasts and hybridization was performed in 50% formamide, 10% dextran sulfate, and 2 × SSC. Specific hybridization signals were detected using fluoresceinated anti-digoxigenin antibody followed by counterstaining with DAPI.

2.6. Northern analysis of mouse immortalized or transformed cell lines

For determination of *VDUP1* mRNA induction by high cell density, Sp2/0-Ag14 cell cultures were seeded at 10^5 cells/ml in T-flasks containing DMEM (Life Technologies) containing 10% FCS (Hyclone) and 2 mM glutamine (Life Technologies). At either day 3, sub-confluence, or day 8, 100% confluent, the cells were harvested and total RNA prepared. Total RNA was isolated from T-flask cultures using the Trizol reagent (Life Technologies). Thirty micrograms of total RNA was run on a 1.2% agarose gel using formaldehyde/MOPs buffer as described in Sambrook et al. (1989). Northern blotting was performed using a Turboblotter apparatus (Schleicher and Schuell) according to manufacturer's instructions. Hybridization conditions were as according to methods described for ExpressHyb reagent (Clontech). *VDUP1* probe was generated by random primer labeling. Control probe for 18S rRNA (5′-CGGCATG-TATTA-GCTCTAGAATTACCACAG-3′) was P^{32} end-labeled with T4 kinase (Fermentas). For serum starvation, identical sets of cultures were seeded, as described above. On day 3, one set was harvested into Trizol reagent. The second culture was washed twice with PBS, then re-fed serum free DMEM, and cultured for an additional 2 days before harvest. Ten micrograms of total RNA from each preparation was subjected to Northern hybridization analysis. To generate the Northern blot of mouse tumor cell RNA, total RNA was prepared from confluent T-175 cultures of the cell lines NIH3T3, B16, D122-96 (Lewis lung carcinoma), P815, EL4, and 4T1. For this study, all lines were cultured in DMEM with 10% FCS, 2 mM glutamine and 10 μg of total RNA of each preparation subjected to Northern hybridization analysis. Densitiometric analysis was performed using an AlphaImager (Alpha Innotech) according manufacturer's instructions.

3. Results

3.1. Cloning and sequencing the genomic mouse VDUP1 gene

In order to clone the mouse *VDUP1* gene, a full length cDNA probe was generated by PCR, based on the sequence information of Young et al. (1996) for the rat *VDUP1* cDNA, using a mouse embryonic Day 15 cDNA pool as template. A single band of 1.2 kb was detected (not shown) and subcloned. DNA sequencing confirmed the product as the mouse version of *VDUP1*. The mouse cDNA contained a coding region of 1,188 bp, encoding 396 amino acids. Identity between the two rodent DNA sequences was determined to be greater than 94%. The *VDUP1* cDNA was then used to screen a mouse C57B6 BAC genomic library. Three independent clones were isolated and one clone digested for restriction fragment mapping analysis (Fig. 1A). Single restriction fragments from BamHI (2.1 kb), HindIII (8 kb), and EcoRI (7 kb) digestions hybridized to the *VDUP1* cDNA probe in both the BAC clone and mouse genomic DNA (not shown). The ends of the gene were then mapped by stripping the blot and subsequently reprobing with radiolabeled oligonucleotides specific for the 5′ and 3′ ends of the cDNA (not shown). A 7 kb EcoRI fragment (refer to Fig. 1A) was detected by both probes, suggesting that the coding region was contained within a sequence length of 7 kb or less. This fragment was then subcloned and the gene sequenced using forward and reverse primers that spanned the length of the coding region. Approximately 1.5 kb of sequence upstream and 1.7 kb downstream of the *VDUP1* gene was determined in addition to the coding region. Utilizing the determined cDNA sequence, a genetic map of the mouse *VDUP1* gene was then generated (Fig. 1B). The coding region spans a total of eight exons across a length of 2.3 kb. Exon sizes ranged from 50 bp (exon 8) to 249 bp (exon 1). Intron sizes ranged from 91 bp (intron 5) to 405 bp (intron 1). All intergenic splice sequences were consistent with consensus splice GT-AG rules (Jackson, 1991). Two consensus polyadenylation sites were identified more than 1.3 kb 3′ to the termination codon delineating a long 3′ UTR. The 3′ UTR was considerably thymidine rich, including several stretches of 100 bp or more possessing greater than 50% thymidine residues. The *VDUP1* 3′ UTR was, however, devoid of any consensus AUUUA elements for mRNA instability (Mitchell and Tollervey, 2000). The length and sequence context of the mouse *VDUP1* 3′ end was consistent with the 3′ UTR sequences reported for rat and human *VDUP1* cDNA (Chen and DeLuca, 1994; Young et al., 1996).

DNA sequence was determined for the immediate 5′ region of the mouse *VDUP1* gene and computer analysis performed to identify putative transcription factor binding elements. Within the 1.5 kb promoter sequence, multiple sites for myeloid-specific factors were identified (Fig. 2). In the proximal 500 bp promoter region, sequence elements for Sp1, GATA-1, two CCAAT boxes (one reverse and one forward), TATA box, and NF-κB were present. Located immediately upstream of the TATA box was a perfect octamer direct repeat, TGCACGAG spaced by three nucleotides. Within this repeat was a core CACGAG spaced by five nucleotides. This sequence is nearly homologous to the binding sequence for the upstream stimulation factor (USF)

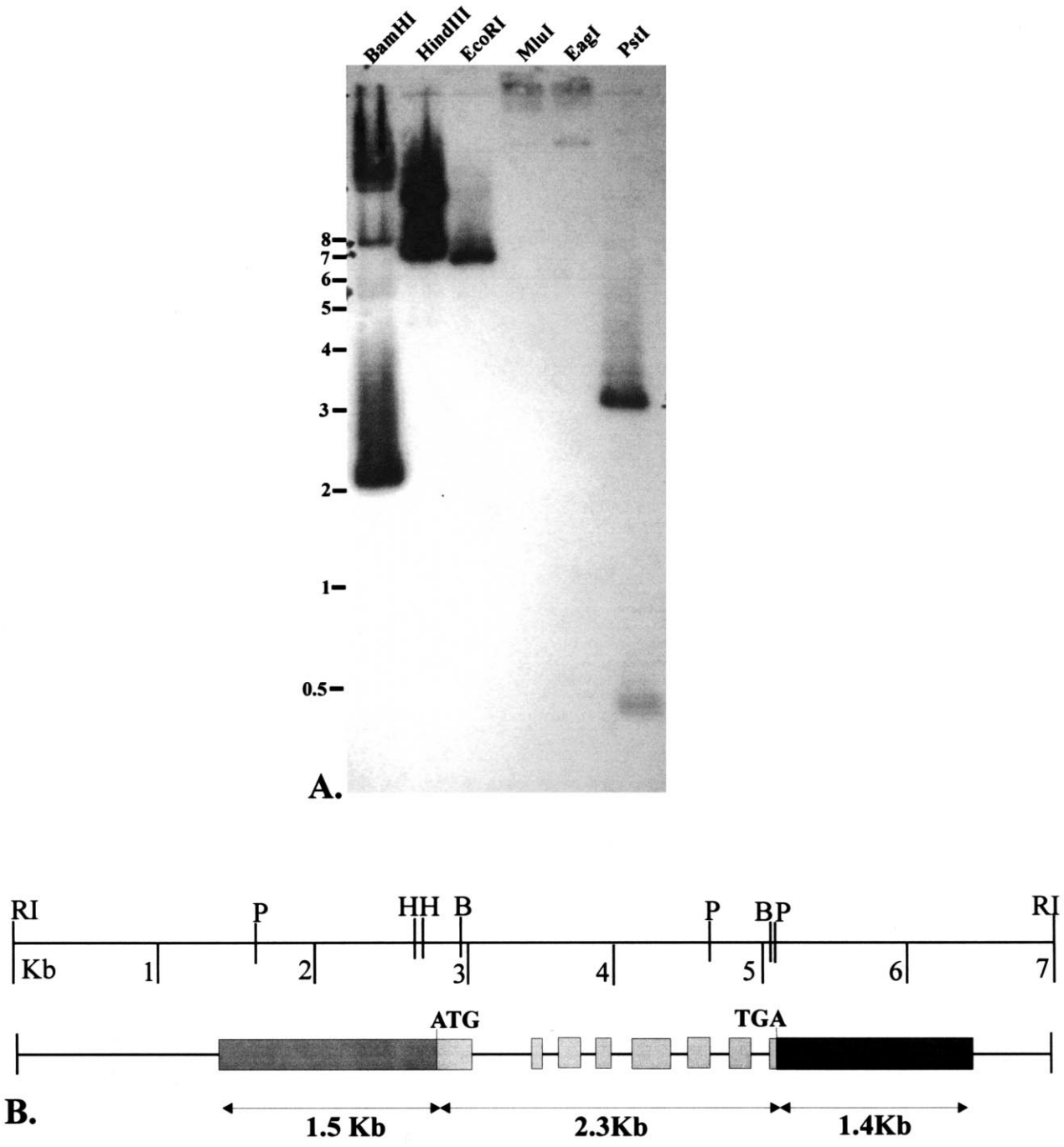


Fig. 1. (A) Southern hybridization of restriction digests of BAC clone 7C21 probed with mouse *VDUP1* cDNA. DNA size markers (Life Technologies) were extrapolated from the ethidium stained gel prior to Southern transfer. (B) Schematic restriction map of the *VDUP1* gene, based on DNA sequence and restriction profile of the locus. Start and stop codons delineating the coding region are shown. Restriction sites are: RI, EcoRI; P, PstI; H, HindIII; and B, BamHI.

and has been shown to bind USF within the HLA-B gene locus promoter (Girdlestone, 1993). Three base pair repeat spacing within this direct repeat is also compatible with the binding site for the vitamin D3 response element (VDRE). However, sequences within this repeat are considerably distinct from the VDRE repeat consensus GGGTCAxxxGGGGCA (Haussler et al., 1995). Aside from this direct repeat, sequences consistent with the VDRE binding element were not apparent within the 1.5 kb *VDUP1* promoter region.

3.2. Mapping the transcription start sites by primer extension

In order to map the 5′ end of mouse *VDUP1* mRNAs, primer extension was performed using a labeled antisense

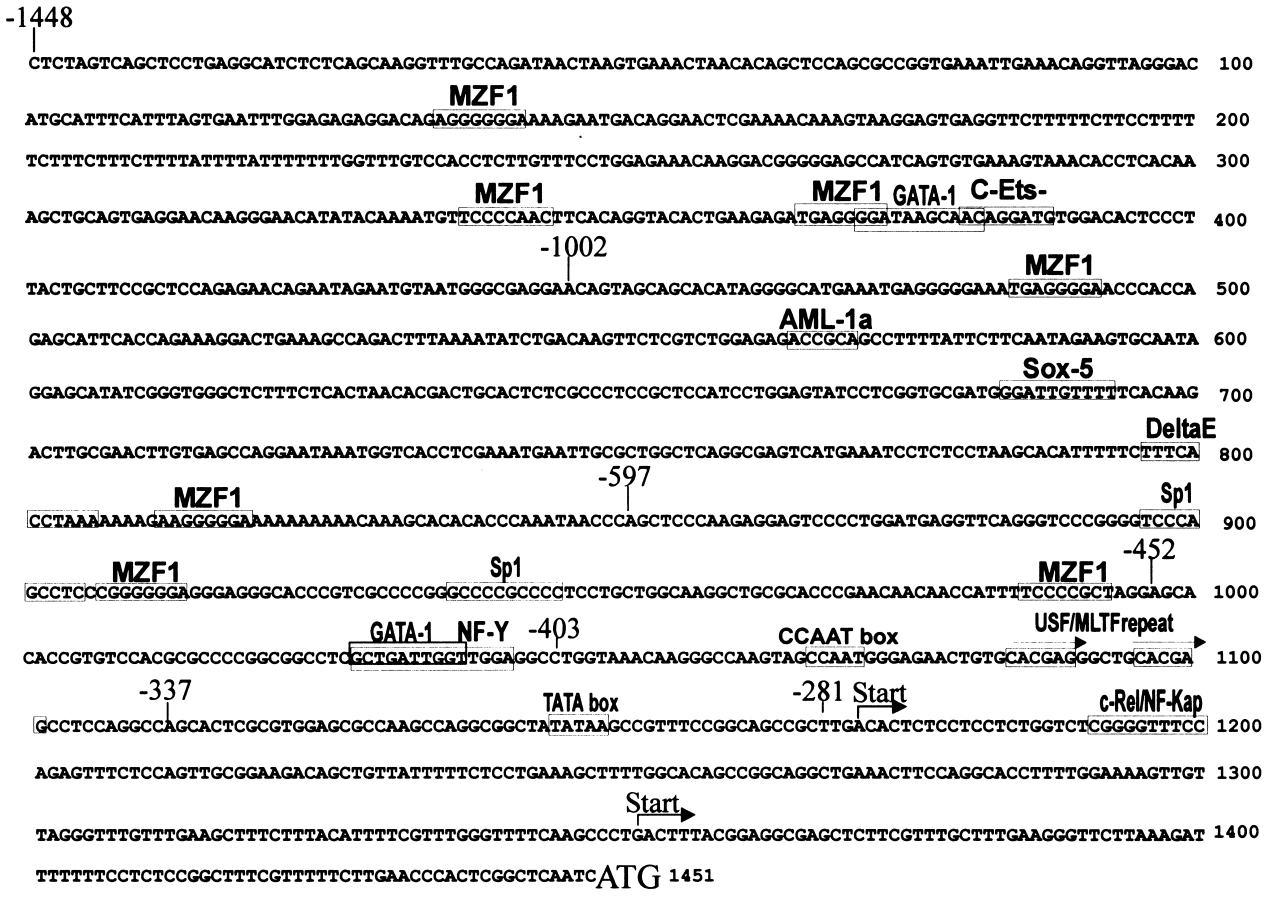
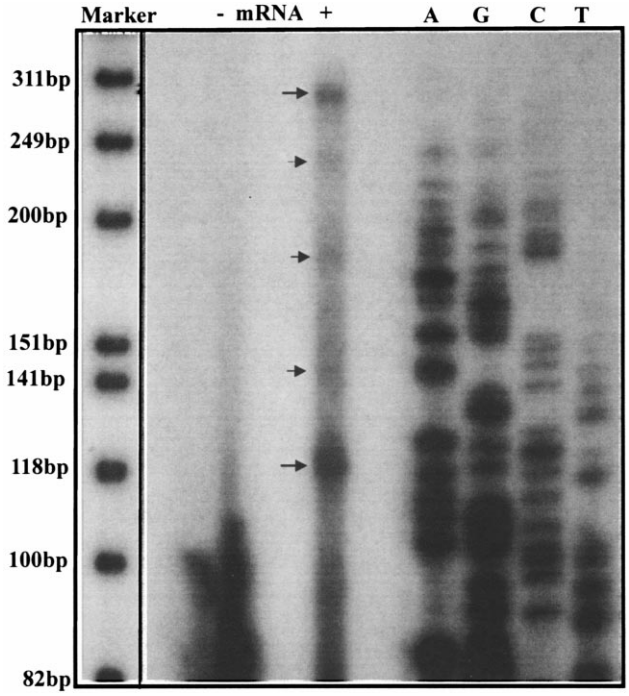


Fig. 2. DNA sequence of the *VDUP1* promoter region. Sites for putative transcription factor binding (boxed) were identified by DNA sequence homology, as described in the text. Two transcriptional start sites are shown. Sequence coordinates for *VDUP1* promoter deletion constructs are identified as bp upstream from the ATG.



oligonucleotide spanning the ATG start codon. Total RNA was prepared from mouse Sp2/0 cells and the 5′ end reverse transcribed. As shown in Fig. 3, two major start sites were identified. The first site was located approximately 20 nucleotides downstream of the TATA box. This start site generated a 5′ UTR of approximately 278 bp. The second site resulted in a smaller 96 bp 5′ UTR. Multiple weaker start sites were also identified (Fig. 3). The rat *VDUP1* 5′ UTR described by Young et al. (1996) was significantly longer, reaching over 1 kb in length. The 5′ end sequence of mouse *VDUP1* was highly homologous to the rat 5′ UTR to the 278 bp start site, but possessed little homology further

Fig. 3. Primer extension mapping of the *VDUP1* transcription start sites. DNA size markers were radiolabeled with T4 kinase and run to determine the relative size of the extension products. Control sample (- mRNA) was performed along with the extension reaction, but lacked template RNA. DNA sequence reactions were run along side the extension reaction, performed using the same radiolabeled extension primer for sequencing. A longer exposure was used to identify the sequence flanking the distal start site (not shown). Longer arrows identify major start sites, shorter arrows, minor start sites.

upstream. A 5′ UTR of 221 bp was previously reported for the human *VDUP1*gene (Chen and DeLuca, 1994), consistent with our results for the mouse *VDUP1* 5′ UTR.

3.3. Deletion analysis of the mouse VDUP1 promoter

In order to determine the minimal functional promoter unit for the mouse *VDUP1* gene, SEAP expression constructs were generated containing diminished lengths of the promoter region. Reporter gene expression was assayed by transient transfection into COS-7 cells. From a construct containing 1448 bp of *VDUP1* promoter sequences, SEAP activity was consistently 2–3 fold higher than was detected from an SV40 promoter driven SEAP control plasmid (Fig. 4). A construct containing the 1448 bp region in antisense orientation displayed no detectable

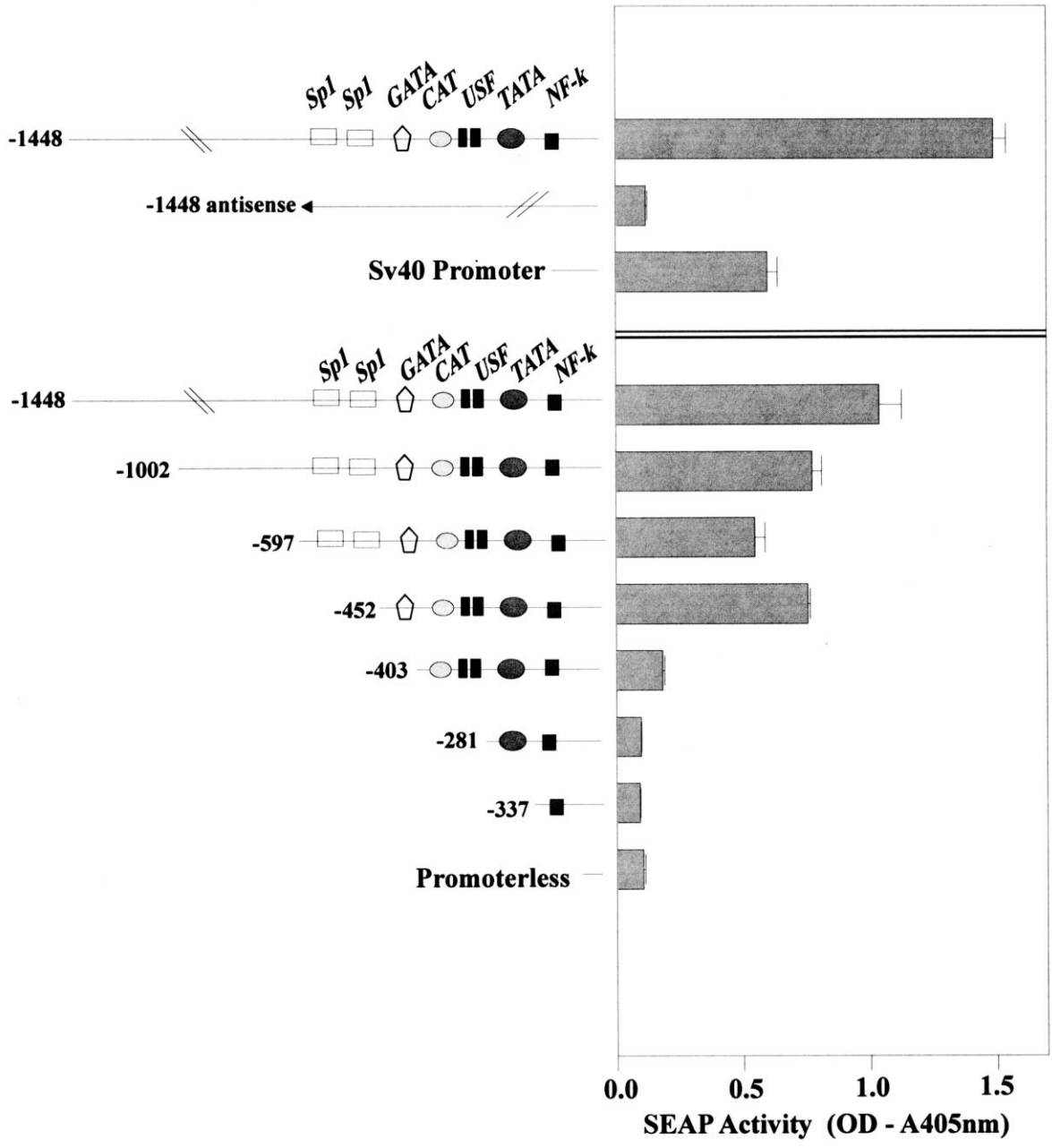


Fig. 4. SEAP enzymatic activity in culture supernatants from *VDUP1* promoter deletion constructs transfected into COS cells. Activity is shown as absorbance at 405nm. Results for each construct represents an average of three independent transfection plates. Error bars show SEM. Promoter length is shown (in bp), and putative transcription factor binding sites are identified. Upper panel shows results from one transfection in which the largest *VDUP1* promoter construct -1448 was compared to an antisense -1448 construct and to a control SV40 promoter driven SEAP. The lower panel shows the results of an independent transfection reporting SEAP activity from plasmids containing successive deletions of 5′ *VDUP1* promoter sequences. A promoterless SEAP plasmid was used as negative control for SEAP expression. Putative transcription factor binding sites are shown diagrammatically for each promoter deletion construct. All *VDUP1* constructs were sequenced prior to transfection to verify promoter integrity.

SEAP activity. Deletion of 5′ sequences between bp 1448 and 452 did not dramatically affect SEAP expression (Fig. 4). However, promoter sequence deletion to 403 bp significantly reduced SEAP expression. This region includes a perfect consensus reverse CCAAT box for NF-Y/CBF binding, which overlaps with a GATA-1 site (refer to Fig. 3). Deletion of the downstream forward CCAAT box further reduced promoter activity, and removal of the TATA element completely abolished promoter activity. Based on this information, the minimal functional promoter includes sequences at least to 452 bp upstream of the *VDUP1* coding region. Furthermore, the data support functional TATA and CCAAT boxes as required elements in basal transcription from the mouse *VDUP1* promoter.

3.4. Chromosomal mapping of the mouse VDUP1 locus

To determine the chromosomal location of the *VDUP1* gene, FISH was performed on mouse chromosome spreads. The labeled BAC probe containing the *VDUP1* gene hybridized specifically to chromosome 3 (Fig. 5). A second probe that is specific for the telomeric region of chromosome 3 was included for sub-chromosomal location mapping. Measurements of ten specifically labeled chromosomes demonstrated that the *VDUP1* gene was located at a position which was 58% of the distance from the heterochromatic–euchromatic boundary to the chromosome 3 telomere, an area that corresponds to band 3F2.2. This location is syntenic with human chromosome 1q21, a region with a high frequency of genomic instability in human tumors (Moseley and Seldin, 1989; Craig et al., 1994; Medvedev et al., 1997).

3.5. Regulated VDUP1 expression in Sp2/0 cells

High cell density induction of *VDUP1* expression in NIH3T3 cells has recently been reported (Junn et al., 2000). Using the semi-suspension myeloma cell line Sp2/0-Ag14, total RNA was prepared from sub-confluent cell cultures taken at day 3 of culture, when the cell density was 4×10^5/ml, and from confluent cultures taken at day 8 when the cell density was 2×10^6/ml. At day 8, the cells had been confluent for more than 24 h. As shown in Fig. 6A, *VDUP1* mRNA was strongly induced in the confluent cell culture. The induction was calculated to be approximately 19-fold, as determined by densitometry analysis. To determine if the effect was dependent on cell growth arrest due to culture density, sub-confluent cultures were either terminated at day 3 or cultured under serum starvation conditions for an additional 2 days. As shown in Fig. 6B, *VDUP1* levels were not induced following growth arrest due to serum starvation. To the contrary, *VDUP1* mRNA levels were reduced approximately 3-fold.

3.6. Northern analysis of mouse tumor cell lines

To investigate whether *VDUP1* expression was modu-

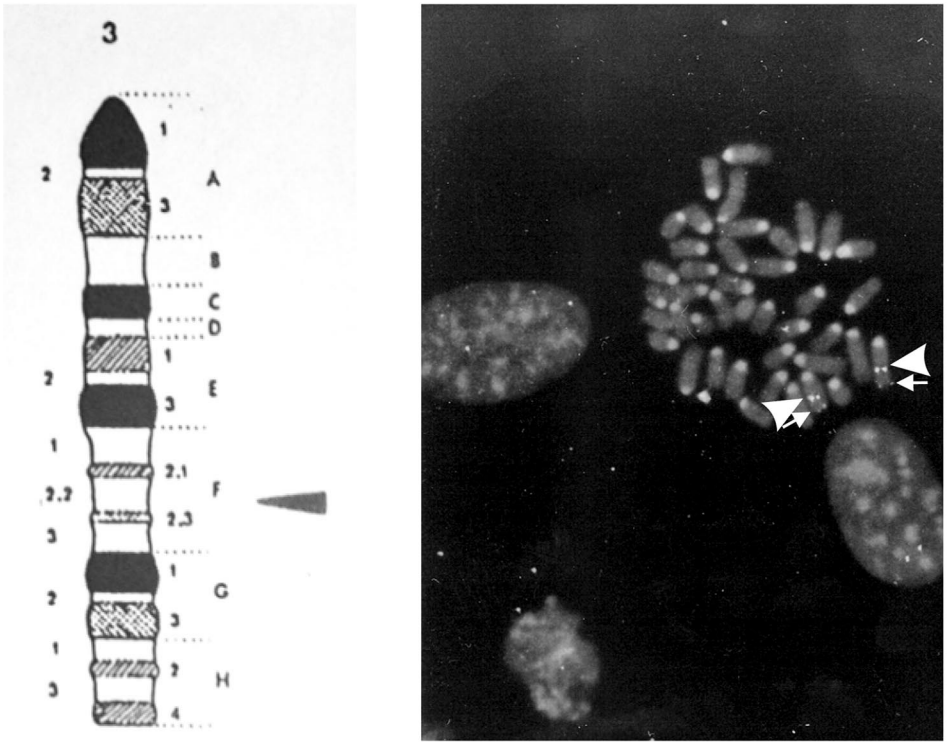


Fig. 5. Chromosomal mapping of the mouse *VDUP1* gene by FISH. Schematic of mouse chromosome 3 showing the map location of *VDUP1* at band F2.2. Large arrows on photograph designate chromosomal hybridization with *VDUP1* probe. Small arrows designate hybridization with a probe specific for the telomeric region of mouse chromosome 3 used in map location determination.

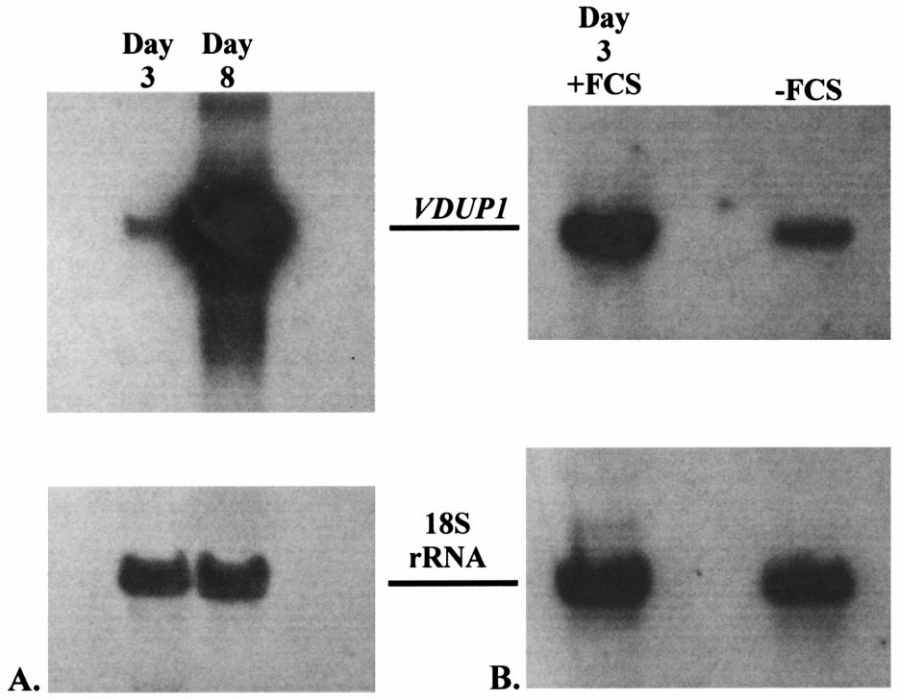


Fig. 6. Expression of *VDUP1* mRNA in Sp2/0 cells during sub-confluent and high density cell culture. (A) RNA samples were prepared from low density and high density cultures and subjected to Northern blot analysis. The *VDUP1* autoradiogram was exposed for 1 h. The blot was subsequently stripped and hybridized with the control 18S rRNA probe to normalize RNA load. (B) Sub-confluent cultures of cells were harvested at day 3 or serum starved for an additional 48 h. Both cell cultures contained approximately 3×10^5 viable cells/ml at 85–90% viability. Ten micrograms of total RNA for each sample was subjected to Northern analysis and probed for *VDUP1* and 18S rRNA expression. The *VDUP1* autoradiogram was exposed for 3 h.

lated in immortalized or tumor cells, we performed Northern analysis on cell lines of different mouse tissue origin. Total RNA from EL4 (T cell lymphoma), B16 (melanoma), D122-96 (Lewis lung carcinoma), P815 (mastocytoma), 4T1 (breast carcinoma), and the immortalized NIH3T3 fibroblast line were probed for *VDUP1* mRNA expression. All cultures were grown to confluence before preparation of total RNA. As shown in Fig. 7, all lines expressed detectable levels of *VDUP1*. By densitiometric analysis, the relative level of *VDUP1*mRNA varied between the different cell lines as much as 2-fold, with the lowest levels observed in the B16 melanoma and the highest levels detected in the Lewis lung carcinoma.

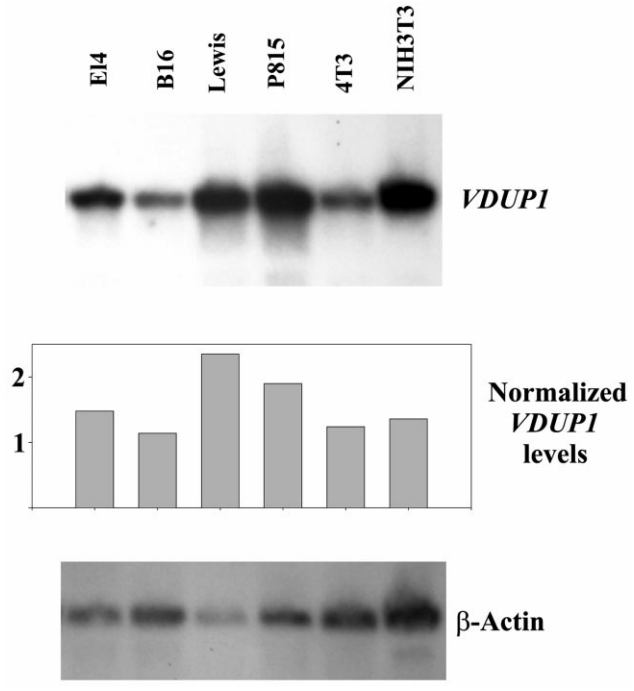


Fig. 7. *VDUP1* expression in immortalized or transformed mouse cell lines. Total RNA from EL4 T cell lymphoma, B16 melanoma, D122-96 Lewis lung carcinoma, P815 mastocytoma, 4T1 breast carcinoma, and the immortalized NIH3T3 line were subjected to Northern analysis for detection of *VDUP1* mRNA. For control, the blot was stripped and hybridized to a human β-actin probe which cross-reacts with mouse β-actin. The *VDUP1* autoradiogram was exposed for 3 h. Bands resulting from both hybridizations were quantitated by densitometry. The relative *VDUP1* mRNA level for each cell line was determined by normalizing *VDUP1* band intensity to the respective β-actin band intensity and the results depicted as a bar graph. The Y axis represent relative *VDUP1* band intensity.

4. Discussion

In this study we determined that the mouse *VDUP1* gene is contained within a 5.5 kb region on chromosome 3. The *VDUP1* promoter contains numerous *cis* regulatory elements for both general and tissue-specific transcription factors including a TATA element, CCAAT box, GATA-1

sites, and multiple MZF-1 sites. Interestingly, identification of a VDRE element for the vitamin D3 receptor was not obvious within the length of the functional *VDUP1* promoter. Chen and DeLuca (1994) originally cloned *VDUP1* as a vitamin D3 inducible gene in HL-60 cells. A prerequisite of the VDRE is a hexanucleotide repeat spaced by three base pairs. Within the *VDUP1* promoter, a perfect direct repeat was found between the TATA and CCAAT boxes (refer to Fig. 3). Given the loose consensus reported for the VDRE sequence (Haussler et al. 1995), it is possible that this region may act as a VDRE. Molecular analysis will be necessary to determine if this is indeed a VDRE binding site. Preliminary data suggests that the minimal functional promoter, containing this site, is responsive to vitamin D3 exposure (D. Ludwig, unpublished results). To the contrary, the site does, however, possess a near perfect consensus for upstream stimulation factor (USF) binding. It is possible that one or both regulatory elements may control *VDUP1* expression through this site.

Two major transcriptional start sites were identified by primer extension. One site was located approximately 20 nucleotides downstream of the TATA box, implying that it is a functional element within the *VDUP1* promoter. A second site was present further downstream, resulting in a 5′ UTR of 96 bp. Based on the combined lengths of the 278b 5′ UTR, the mouse *VDUP1* coding region, and the 1.3 kb 3′ UTR, a deduced length of 2.8 kb is generated which is consistent with the determined 2.9 kb length for human *VDUP1* mRNA (Chen and DeLuca, 1994). The minimal promoter element contained sequences in excess of 452 bp from the ATG. Full basal activity is retained within this region that contains the two CAATT boxes, TATA box, and the direct repeat element. In comparison to the SV40 promoter, the minimal *VDUP1*promoter unit expressed the SEAP reporter at levels 2–3 fold higher in transient COS transfection, implying strong basal transcriptional activity for this promoter.

Polyadenylation sequences were identified 1.3 kb downstream of the *VDUP1* translational stop codon. This long 3′ UTR was considerably thymidine rich and may impart post-transcriptional regulation of mRNA stability. Chen and DeLuca (1994) had shown that vitamin D3 induction of *VDUP1* occurred even in the presence of the protein synthesis inhibitor cyclohexamide. In addition, cyclohexamide itself was capable of inducing *VDUP1* expression in cells. No consensus AUUUA AU-rich elements (AREs) for imparting mRNA instability were noted within this 3′ UTR (Mitchell and Tollervey, 2000). Studies in which both 5′ and 3′ regulatory regions of the *VDUP1* gene are placed in the context of a reporter gene will be necessary to determine the influence of transcriptional and post-transcriptional processes on *VDUP1* expression in cells exposed to vitamin D3. Junn et al. (2000) reported that *VDUP1* was strongly inducible by exposure to a variety of stress response agents including peroxide, gamma and ultraviolet radiation, and heat shock. Furthermore, high density cell culture and exposure to TGF-β1 also were also shown to markedly elevate *VDUP1* mRNA levels. In this report, we demonstrated that *VDUP1* expression was induced 19-fold in Sp2/0 cells as a result of prolonged culture at high cell density. In addition, we showed that this effect was not a general response to growth arrest due to culture confluence, because *VDUP1* mRNA levels did not increase following growth arrest upon serum starvation. In fact, overall *VDUP1* levels were reduced under these conditions. Further characterization of the 5′ and 3′ ends of the *VDUP1* gene will enable us to determine which factors are responsible for the stress inducibility of this gene and whether this response is distinct from the response to vitamin D3 seen in HL-60 cells.

The location of *VDUP1* on mouse chromosome 3 band F2.2 is syntenic with a region of human chromosome 1q21 that is frequently mutated or lost in human cancers (Medvedev et al., 1997; Bieche et al., 1995; Keung et al., 1998). It is possible that the modulation of *VDUP1* observed in MNNU-induced rat tumors (Young et al., 1996; Yang et al., 1998) implies a tumor suppressive effect of *VDUP1* in cells. Since VDUP1 directly modulates the expression and activity of thioredoxin, which itself is correlated with cell growth promotion, down-regulation or loss of *VDUP1* may impart greater cell survival through stabilization of thioredoxin expression in tumors. We detected abundant *VDUP1* expression in all samples from a diverse set of mouse tumor cell lines, but the relative levels of *VDUP1* varied by as much as 2-fold between lines. Junn et al. (2000) have demonstrated that *VDUP1* overexpression leads to decreased cell proliferation and a concomitant increase in apoptosis. It will be interesting to determine if *VDUP1* knockout mice, if viable, are predisposed to cancer.

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