Journal of Cellular Biochemistry 73:400–407 (1999)

Characterization of an Enhancer Required for 1,25-dihydroxyvitamin D_3-Dependent Transactivation of the Rat Osteocalcin Gene

W. Bruce Sneddon and Marie B. Demay*

Endocrine Unit, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114

Abstract The sequences in the rat osteocalcin gene that lie 3′ to the vitamin D response element (VDRE) contain a GGTTTGG motif (-420 to -414) that is essential for transcriptional activation of osteocalcin-CAT (OC-CAT) fusion genes by $1,25(OH)_2D_3$. A second copy of this motif, present on the antisense strand is unable to compete for nuclear protein binding to the VDRE-associated motif, suggesting that the core element extends beyond the GGTTTGG motif. In order to examine the base requirements for both function and nuclear protein interactions with the VDRE-associated GGTTTGG enhancer motif, deletion and substitution of flanking sequences was performed in the context of both the native osteocalcin promoter and a heterologous viral promoter. These data demonstrate that the base requirements for protein-DNA interactions and transactivation are located between -430 and -414. The position of the element with respect to the VDRE is flexible and insertion of additional copies either 5′ or 3′ to the VDRE further enhances transactivation, both in the context of the native osteocalcin promoter and a heterologous viral promoter. These data demonstrate that VDR-dependent transactivation of the rat osteocalcin gene requires not only the VDRE (-456 to -442) but also sequences between -430 and -414. The protein(s) that interacts with these sequences is capable of enhancing transcription in both a position and orientation-independent fashion. J. Cell. Biochem. 73:400–407, 1999.

Key words: rat osteocalcin gene; VDRE

Studies aimed at characterizing the $1,25(OH)_2D_3$-responsive region of the rat osteocalcin gene led to the identification of an element 3′ to the VDRE which is absolutely required for ligand-dependent transactivation [Sneddon et al., 1997a]. Mutation of the bases between -420 and -414 (GGTTTGG) of the rat osteocalcin gene abrogated the $1,25(OH)_2D_3$-responsiveness of osteocalcin-CAT fusion genes in transient gene expression assays. The GGTTTGG sequence that is critical for the $1,25(OH)_2D_3$-responsiveness of the rat osteocalcin gene is also present in the human osteocalcin gene [Morrison et al., 1989] and enhances ligand-dependent transactivation mediated by the human osteocalcin VDRE [Sneddon et al., 1997a], demonstrating that these functionally important bases are evolutionarily conserved. An accessory element adjacent to VDRE-1 of the rat 25-hydroxyvitamin D_3 24-hydroxylase gene has been identified which also enhances $1,25(OH)_2D_3$-responsiveness [Ohyama et al., 1996]. This element bears no sequence similarity to the element identified in the rat osteocalcin gene and appears to contain VDRE-like half-sites which do not bind the VDR or the RXR. Although it is not known whether this latter element can augment transactivation from response elements other than a VDRE, the osteocalcin gene element has been shown to enhance transactivation mediated by a cAMP response element (CRE) and an estrogen response element (ERE) [Sneddon et al., 1997a].

Studies of the protein/DNA interactions of this GGTTTGG motif reveal that binding of the VDR/RXR heterodimer to the adjacent VDRE is not required for nuclear protein interactions with this element [Sneddon et al., 1997a], and that these sequences do not affect basal transcription. It is, therefore, likely that the VDR/

Grant sponsor: NIH; Grant number: DK 36597.

W. Bruce Sneddon's current address is Department of Pharmacology, University of Pittsburgh School of Medicine.

*Correspondence to: Marie Demay, MD, Endocrine Unit Wellman 501, Massachusetts General Hospital, Boston, MA 02114.

Received 6 November 1998; Accepted 22 December 1998

RXR heterodimer bound to the VDRE works in concert with this downstream element and the basal transcription complex to activate transcription. A second GGTTTGG motif, present on the antisense strand between -347 and -353 of the rat osteocalcin gene, also contributes to transcriptional activation by $1,25(OH)_2D_3$ [Sneddon et al., 1997b]. These sequences do not interact with the same nuclear protein(s) as the VDRE-associated GGTTTGG motif. This suggests that although the GGTTTGG motif is integral to the VDRE-associated motif, flanking sequences are also critical for enhancer function. We, therefore, examined the precise sequence and positional requirements for enhancer function of the VDRE-associated GGTTTGG motif. These studies demonstrate that the bases from -430 to -421 are required for full enhancer function, and that the position and orientation of the enhancer relative to the VDRE is flexible.

MATERIALS AND METHODS

Engineering of Osteocalcin-CAT Fusion Genes

For experiments using a heterologous promoter, oligonucleotides were inserted into the *Bam*HI site of puTKAT3 [Prost and Moore, 1986] (a kind gift of Dr. David D. Moore). Orientation and copy number of the oligonucleotides were determined by DNA sequencing.

For experiments using the native rat osteocalcin promoter, the plasmid OC-CAT, which contains 522 bp upstream from the transcriptional start site of the rat osteocalcin gene fused to the CAT reporter gene, was employed [Demay et al., 1992b]. Mutations in regions of interest were introduced by site-directed mutagenesis using the U.S.E. Mutagenesis kit (Pharmacia, Piscataway, NJ). Two oligonucleotide primers were employed in this procedure. A oligonucleotide (50-mer), which eliminated the *Xba*I site in the polylinker of pUC18 (CGAC**TC**TAGA to CGAC**ga**TAGA), was used as a selection primer. The mutation of interest was introduced into a second primer. The mismatched bases were located in the central portion of an oligonucleotide of 50–60 bases. Additional copies of $-D14D_3$ were inserted into OC-CAT using a *Bam*HI restriction site introduced by site-directed mutagenesis, changing C to A at position -413. This mutation had no effect on the $1,25(OH)_2D_3$-responsiveness of the native OC-CAT fusion gene (data not shown). The sequences from -522 to -306 (*Sac*I site) were sequenced to confirm the introduction of these mutations, the orientation of the inserted sequences, and to exclude the presence of other undesired mutations. This region was then substituted for the identical non-mutated region in the wild-type parent plasmid (OC-CAT).

Cell Culture and Transfections

ROS 17/2.8 cells were maintained in Ham's F12 medium with L-glutamine (Gibco, Grand Island, NY) supplemented with 10% (vol/vol) fetal bovine serum, penicillin, and streptomycin. From 24 h prior to transfection until harvesting, cells were cultured in medium containing charcoal-stripped fetal bovine serum. Transfections were performed using the calcium phosphate method as previously described [Demay et al., 1989]. The cells were treated with 10^{-8} M 1,25(OH)2D3 immediately after transfection and again the following day. The cells were fed and stimulated 40 h post-transfection and harvested 24 h later. CAT activity was assessed as previously described [Demay et al., 1992a]. All test plasmids were co-transfected with a control plasmid containing the luciferase gene under the control of the Rous sarcoma virus promoter. Luciferase activity was measured using a standard protocol [Ausubel et al., 1992]. The presence of $1,25(OH)_2D_3$ did not affect the level of luciferase activity. CAT activity was assessed by densitometric scanning of thin layer chromatography plate autoradiograms and each replicate was normalized for luminescence units. Relative CAT activity, presented as fold stimulation, reflects the ratio of corrected CAT activity in the presence or absence of $1,25(OH)_2D_3$.

DNA Sequencing

All DNA sequencing was carried out by the dideoxynucleotide chain termination method after subcloning into M13 vectors [Sanger et al., 1977].

Gel Retardation Assays

Oligonucleotides were labeled by filling in recessed ends with the large fragment of DNA polymerase I and [α^{32}P]-dATP. The oligonucleotide used as a probe for these studies was the sequence 3′ to the rat osteocalcin VDRE between bases -434 and -410 (-D14D3) with the addition of GATC overhanging bases. The sequence of the sense strand, and that of competi-

TABLE I. Sequence of Double-Stranded Oligonucleotides (With Added GATC Overhangs) That Were Employed as Probes or Competitors in Gel Retardation Assays[a]

$14D_3$	−458	CT**GGGTGA**ATG**AGGACA**TTACTGACCGCTCCTTCCTGGGGTTTGGCTCC	−410
$13D_3$		CT**GGGTGA**ATG**AGGACA**TTACTGACCGCTCCTTCCTGGGGT	
$dm14D_3$		CT**GGGTGA**ATG**AGGACA**TTACTGACC TTCCTGGGGTTTGGCTCC	
$dmL14D_3$		CT**GGGTGA**ATG**AGGACA**TTACTGACCatgaaTTCCTGGGGTTTGGCTCC	
$-D14D_3$		CCGCTCCTTCCTGGGGTTTGGCTCC	
−Dsdm6		CCatcCCTTCCTGGGGTTTGGCTCC	
GT2 (reverse complement)		−337 ATCTAAAATCGGTTTGGGACAAACTG	−362

[a]$14D_3$, $13D_3$, $dm14D_3$, and $dmL14D_3$ were also expressed upstream of the HSV-tk promoter (Fig. 2). The position of the sequences in the rat osteocalcin gene with respect to the transcriptional start site is shown. Note that the antisense strand of GT-2 is shown. Mutant sequences are presented in lower case type. The VDRE is in boldface type. The GGTTTGG motif is underlined.

tor oligonucleotides, is shown in Table I. The antisense strand of the GT-2 oligonucleotide is shown to emphasize the GGTTTGG motif and flanking bases. Gel retardation assay buffer composition and nuclear extract preparation were described previously [Demay et al., 1990]. ROS 17/2.8 nuclear extracts were pre-incubated with poly(dI-dC) - poly(dI-dC) at a concentration of 0.5 μg/μg of extract protein, with or without 10 or 100 ng of unlabeled competitor as indicated, for 15 min at 22°C. Subsequently, 1 ng of probe was added for an additional 15 min. The mixture was brought to 10% (vol/vol) glycerol and electrophoresed on a 4% nondenaturing polyacrylamide gel.

RESULTS

It has previously been demonstrated that the bases between -420 and -414 (GGTTTGG) of the rat osteocalcin gene are essential for $1,25(OH)_2D_3$-responsiveness of osteocalcin-CAT fusion genes containing the native osteocalcin promoter. A search for other copies of this motif revealed a second GGTTTGG motif on the antisense strand of the rat osteocalcin gene between -347 and -353 (Table I). Gel retardation assays were performed to address whether this more proximal motif was able to interact with the same nuclear proteins as the VDRE-associated motif. As shown in Figure 1, the more proximal motif (GT-2) does not compete for the specific nuclear protein-DNA complexes generated by the VDRE-associated motif (-D14D3). These data suggest that although the GGTTTGG motif is critical for the enhancer function and the nuclear-protein-DNA interactions of the VDRE-associated motif, other sequences in this oligonucleotide are integral to the core enhancer element. We, therefore, undertook mutagenesis studies, in the context of both a heterologous viral promoter and the native osteocalcin promoter, to define the sequences that comprise the core element.

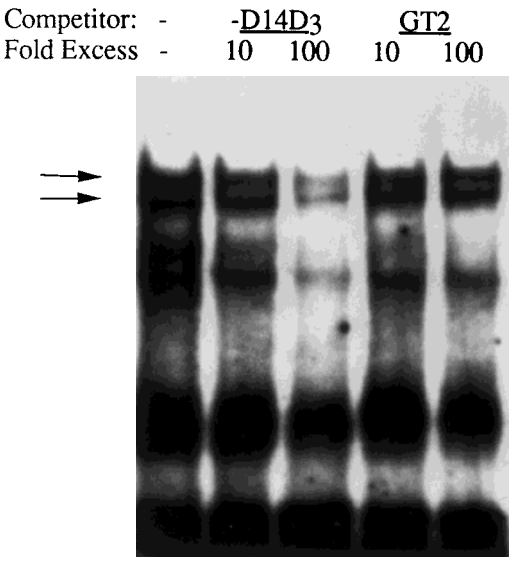

Fig. 1. The sequences required for nuclear protein-DNA interactions of the VDRE-associated enhancer extend beyond the GGTTTGG motif. -D14D3 was employed as a radiolabeled probe. Competitor oligonucleotides (10- and 100-fold molar excess) were preincubated with nuclear extracts prior to probe addition, as outlined in Materials and Methods. The specific nuclear protein-DNA interactions are indicated by arrows.

The DNA sequences 5′ to the VDRE-associated GGTTTGG (-420 to -414), were altered to determine whether the spacing between this motif and the VDRE, was critical for enhancer function. Deletion mutagenesis was carried out in the context of 14D3-tkCAT (Table I, Fig. 2). Placing the GGTTTGG motif five bases closer to the VDRE (deletion of bases -432 to -428) reduced the $1,25(OH)_2D_3$-responsiveness from 5.3- fold (with 14D3-tkCAT) to 1.6-fold (Fig. 2: dm-14D3). In order to distinguish whether the observed reduction in $1,25(OH)_2D_3$-responsive-

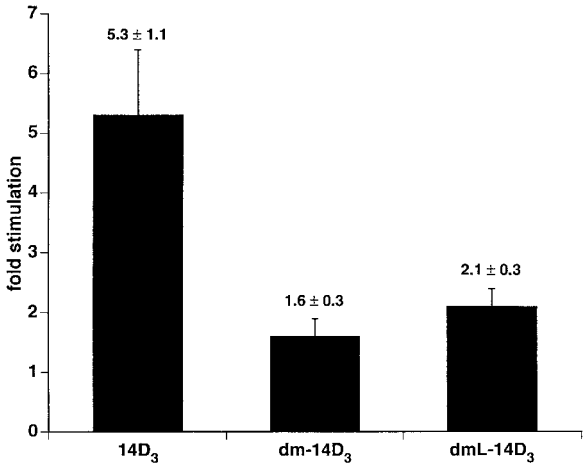

Fig. 2. The sequences between -432 and -428 of the rat osteocalcin gene are required for VDRE-mediated of transactivation using a heterologous viral promoter. One copy of the oligonucleotides 14D3, dm14D3, and dmL14D3 (Table II) were inserted in the forward orientation upstream of the HSV-tk promoter in puTKAT3. Their ability to induce CAT activity in response to 10 nM $1,25(OH)_2D_3$ was assessed after transient transfection into ROS 17/2.8 cells. Relative stimulation of CAT activity represents the mean ± S.E.M. of three independent experiments using at least two different preparations of each plasmid. Each transfection was performed in triplicate and normalized for transfection efficiency with RSV-luc cotransfection.

ness was due to the altered spacing of the GGTTTGG element with respect to the VDRE in dm-14D3-tkCAT or to the deletion of critical bases, these same bases were mutated by substitution mutagenesis (GCTCC to ATGAA; Fig. 2: dmL-14D3). These substitution mutations also reduced the $1,25(OH)_2D_3$-responsiveness to two-fold, suggesting that these bases were, in fact, an integral part of the enhancer element and that impaired transactivation was a result of base changes rather than spacing. These data demonstrate that the VDRE-associated element extends 5′ to the GGTTTGG core motif and may explain the inability of the more proximal GGTTTGG enhancer sequences (GT2) to compete for the nuclear proteins which bind to these sequences.

To confirm that the bases 5′ to the GGTTTGG motif are important in the context of the native osteocalcin promoter, OC-CAT fusion genes were engineered by site-directed mutagenesis to delete bases -422 and -421 (Table II, sdm1). This eliminated $1,25(OH)_2D_3$-responsiveness (Fig. 3), confirming that these bases are an integral part of the enhancer element. To examine if spacing was critical, the GGTTTGG motif was displaced 3′, increasing the distance between the VDRE and downstream regulatory element by 2 bases (Table II, sdm2). This spacing mutation had no effect on the $1,25(OH)_2D_3$-responsiveness of the native osteocalcin promoter (Fig. 3: sdm2-OC-CAT).

A series of mutations between -434 and -423 (Table II) were, therefore, introduced into an OC-CAT fusion gene containing the native rat osteocalcin promoter in order to define the 5′ extent of this element. Mutation of the bases between -431 and -423 of the rat osteocalcin gene (CTCCTTCCT changed to AGAAGGAAG; sdm3, Table II) eliminated $1,25(OH)_2D_3$-responsiveness in ROS 17/2.8 cells transfected with this OC-CAT fusion gene (Fig. 3: sdm3-OC-CAT), indicating that this region contains bases required for $1,25(OH)_2D_3$-responsiveness. In order to more closely delineate sequences involved in $1,25(OH)_2D_3$-mediated transactivation in this region, the bases between -431 and -427, and between -426 and -423 were mutated separately. Mutation of bases -431 to -427 (CTCCT to AGAAG) or bases -426 to -423 (TCCT to GAAG; sdm 4 and 5, Table II) also significantly reduced the response to $1,25(OH)_2D_3$ (Fig. 3: sdm4-OC-CAT , sdm5-OC-CAT). This demonstrates that residues critical for $1,25(OH)_2D_3$-responsiveness include the region encompassing -431 to -427. However, mutagenesis of bases -434 to -430 (Table II, sdm6; Fig. 3: sdm6-OC-CAT) had little effect on $1,25(OH)_2D_3$-responsiveness. These data suggest that the 5′ extent of the element required for $1,25(OH)_2D_3$-responsiveness of the rat osteocalcin gene is at, or near base -430.

To assess whether the sequences required for transactivation by the VDRE-associated enhancer were also required for binding nuclear proteins, the protein/DNA interactions of this region were examined by performing gel retardation analyses using nuclear extracts from ROS 17/2.8 cells. A double stranded oligonucleotide, $-D14D_3$ ($14D_3$ lacking the VDRE), representing the rat osteocalcin sequence between -434 and -410, was employed as a radiolabeled probe (Table I). The oligonucleotides $dm14D_3$ and $dmL14D_3$, which consist of $14D_3$ with a deletion or substitution respectively (Table I) of the sequences between -432 to -428, were employed as competitors (Fig. 4). These two mutations, which abolished the enhancer activity (Fig. 2), also failed to compete for the two shifted bands, indicating that sequences in the region

TABLE II. Site-Directed Mutants of OC-CAT Fusion Genes[a]

	−458	−410
Wild-type	CT**GGGTGA**ATG**AGGACA**TTACTGACCGCTCCTTCCTGGGGTTTGGCTCC	
sdm1	CT**GGGTGA**ATG**AGGACA**TTACTGACCGCTCCTTCCTGGttTggGGaTCC	
sdm2	CT**GGGTGA**ATG**AGGACA**TTACTGACCGCTCCTTCCTaGGtggtttggCC	
sdm3	CT**GGGTGA**ATG**AGGACA**TTACTGACCGagaaggaagGGGGTTTGGaTCC	
sdm4	CT**GGGTGA**ATG**AGGACA**TTACTGACCGagaagTCCTGGGGTTTGGaTCC	
sdm5	CT**GGGTGA**ATG**AGGACA**TTACTGACCGCTCCTgaagGGGGTTTGGaTCC	
sdm6	CT**GGGTGA**ATG**AGGACA**TTACTGAgCatcCCTTCCTGGGGTTTGGaTCC	

[a]The mutant sequences are in lower case type. The GGTTTGG motif is underlined. The VDRE is indicated by bold type face. The C to A conversion of base −413 in sdm 1, 3, 4, 5, 6 created a *Bam*HI restriction site, which was used to screen for introduction of the mutation during site-directed mutagenesis. It had no effect on the $1,25(OH)_2D_3$-responsiveness of OC-CAT fusion genes on its own (data not shown).

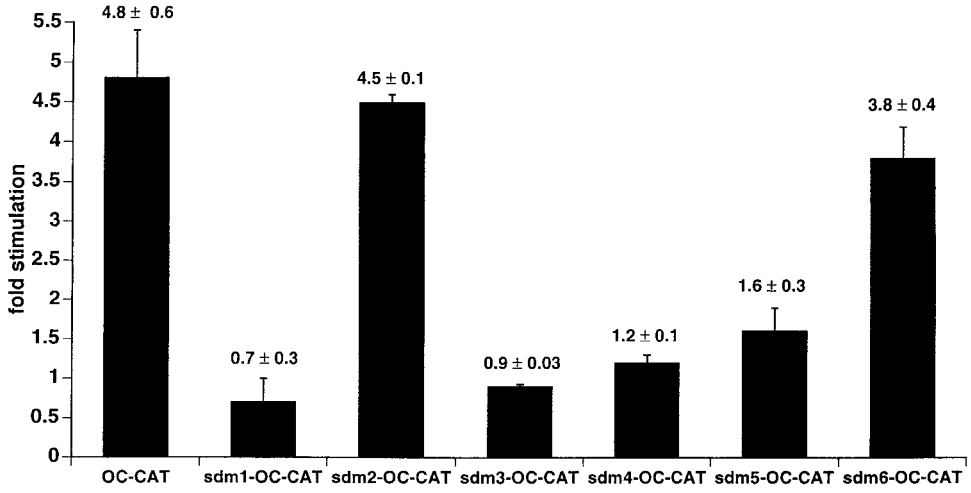

Fig. 3. Sequences required for VDRE-mediated transactivation using the native osteocalcin promoter. ROS 17/2.8 cells were transiently transfected with mutant and wild-type (OC-CAT) fusion genes and their ability to induce CAT activity in response to 10 nM $1,25(OH)_2D_3$ was assessed. The mutations are indicated in Table II. Relative stimulation of CAT activity represents the mean ± S.E.M. of three independent experiments using at least two different preparations of each plasmid. Each transfection was performed in triplicate and normalized for transfection efficiency with RSV-luc cotransfection.

between -432 and -428 of the rat osteocalcin gene were also critical for specific protein/DNA interactions with the -D14D_3 probe. Mutation of the bases between -434 and -430, (sdm6, Table II) had only a modest effect on the $1,25(OH)_2D_3$-responsiveness of the native OC-CAT fusion gene (Fig. 3) and also permitted full competition for the two bands which bind -D14D_3 (Fig. 4, -Dsdm6) indicating that the 5′ end of the sequences required for DNA-protein interactions corresponds to the 5′ boundary defined by functional studies using the native osteocalcin promoter.

To address whether this enhancer would retain function when placed upstream to the VDRE, a single copy of the sequences between -434 to -410 (-D14D_3) was subcloned upstream to the VDRE in 13D3-tkCAT (Table I, -458 to -418 of the rat osteocalcin gene) [Sneddon et al., 1997a]. While four copies of these sequences, in the absence of a VDRE, do not confer $1,25(OH)_2D_3$-responsiveness to a heterologous promoter (Fig. 5: 4X(-D14D_3)^F), insertion of one copy of -D14D_3 in the forward orientation, 5′ to the VDRE in 13D_3-tkCAT, increases $1,25(OH)_2D_3$-responsiveness two-fold (Fig. 5, (-D14D_3)^F-13D_3). Similarly, insertion of four copies of -D14D_3 in the reverse orientation 5′ to the VDRE in 13D_3-tkCAT also increases $1,25(OH)_2D_3$-responsiveness two-fold (Fig. 5, 4X(-D14D_3)^R-13D_3). Insertion of four copies of -D14D_3 in the forward orientation 5′ to the VDRE in 13D_3-tkCAT increases $1,25(OH)_2D_3$-responsiveness 2.5-fold (4X(-D14D_3)^F-13D_3). Of note, four copies of

-D14D$_3$ do not alter basal CAT activity (data not shown).

DISCUSSION

The bases between -420 and -414 of the rat osteocalcin gene are essential for transactivation by 1,25(OH)$_2$D$_3$ [Sneddon et al., 1997a]. These DNA sequences increase 1,25(OH)$_2$D$_3$-responsiveness both in the context of the native rat osteocalcin promoter and the heterologous HSV-tk promoter; therefore, their position with respect to the transcription pre-initiation complex is not crucial. The experiments described herein address the orientation and positional requirements of the GGTTTGG motif with respect to the VDRE and identify bases 5′ to this motif as being integral to this element.

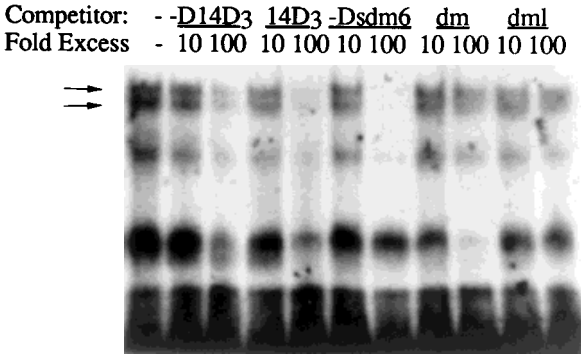

Fig. 4. The sequences required for nuclear protein-DNA interactions of the VDRE-associated enhancer include 10 bp 5′ to the GGTTTGG motif. -D14D3 was employed as a probe (Table I) in gel retardation assays. Competitor oligonucleotides (10- and 100-fold molar excess) were preincubated with ROS 17/2.8 cell nuclear extracts prior to probe addition, as outlined in Materials and Methods. The two specific protein complexes associated with the VDRE-associated GGTTTGG motif are indicated by the arrows.

The precise location of the GGTTTGG motif downstream from the VDRE is not critical for its function. This motif is also able to function upstream of the VDRE in an orientation-independent fashion. Both the functional and protein/DNA binding data define the 5′ extent of the VDRE-associated element to be at, or near base -430. These sequences are highly conserved in the human osteocalcin gene and also enhance the 1,25(OH)$_2$D$_3$-responsiveness of the human osteocalcin VDRE, demonstrating the evolutionary conservation of this functionally important motif [Sneddon et al., 1997a].

In the region between -430 and -414, the enhancer may consist of two discrete elements functioning together with the VDRE to promote transactivation of the rat osteocalcin gene. When two bases are inserted 5′ to -420 in the context of the native osteocalcin promoter, effectively shifting the GGTTTGG motif two bases away from the VDRE, 1,25(OH)$_2$D$_3$-responsive-

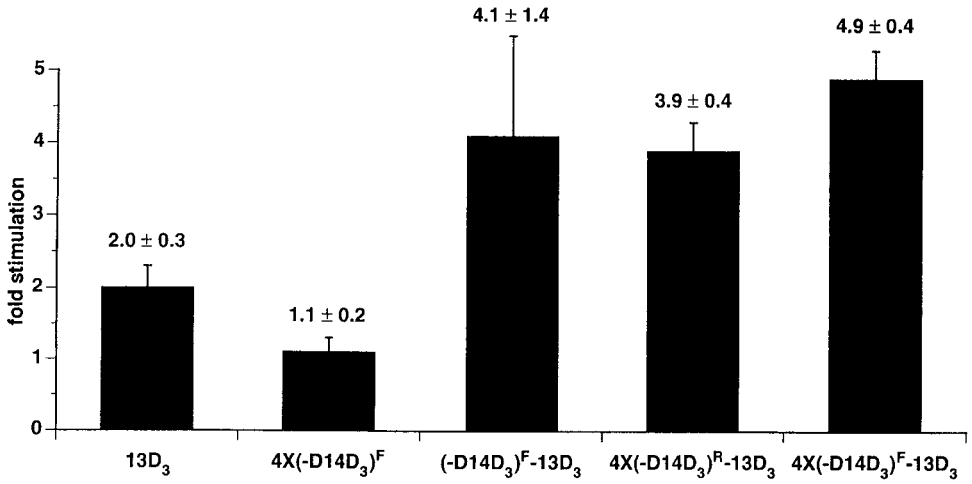

Fig. 5. The VDRE-associated GGTTTGG motif enhances 1,25(OH)$_2$D$_3$-responsiveness in a position and orientation-independent fashion. Four copies of -D14D$_3$ were inserted upstream of the HSV-tk promoter in pUTKAT3 to yield 4X(-D14D$_3$)^F. One copy of -D14D$_3$ was inserted upstream of the VDRE in 13D$_3$-tkCAT in the forward orientation to yield (-D14D$_3$)^F-13D$_3$. Four copies of -D14D$_3$ were inserted upstream of the VDRE in 13D$_3$-tkCAT in the reverse orientation to yield 4X(-D14D$_3$) R-13D$_3$. Four copies of -D14D$_3$ were inserted upstream of the VDRE in 13D$_3$-tkCAT in the forward orientation to yield 4X(-D14D$_3$) F-13D$_3$. Their ability to induce CAT activity in response to 10 nM 1,25(OH)$_2$D$_3$ was assessed after transient transfection into ROS 17/2.8 cells. Relative stimulation of CAT activity represents the mean ± S.E.M. of three independent experiments using at least two different preparations of each plasmid. Each transfection was performed in triplicate and normalized for transfection efficiency with RSV-luc cotransfection.

ness of the osteocalcin-CAT fusion gene is maintained. This suggests that the GGTTTGG motif between -420 and -414 may form a discrete element or half-site, separate from the functionally important upstream sequences between -430 and -421. The presence of the downstream antisense GGTTTGG with different flanking sequences also suggests that each GGTTTGG motif may represent one half-site of a binding motif for different transcription factor complexes.

The GGTTTGG sequence present from -420 to -414 has not been identified as a transcription factor binding site, nor have the bases between -429 and -421. The TTCC motif (reverse complement GGAA) between positions -427 and -424 is suggestive of a binding site for the Ets family of transcription factors [Woods et al., 1992; Nye et al., 1992]. Oligonucleotides representing consensus binding sites for Ets-1, Ets-2, Elk-1, and PU.1, however, fail to compete for the protein-DNA complexes which bind to the rat osteocalcin sequences between -429 and -414 (data not shown). This does not, however, rule out involvement of a novel member of the Ets family in the $1,25(OH)_2D_3$-responsiveness of the rat osteocalcin gene.

As more information becomes available, the complexity of steroid-activated transcription is becoming ever more apparent. Adjacent response elements for hepatic nuclear factor 3 are required for optimal glucocorticoid responsiveness of the phosphoenolpyruvate carboxykinase and the insulin-like growth factor binding protein genes [O'Brien et al., 1995]. Similarly, a novel accessory factor binding site adjacent to the glucocorticoid response element is required for glucocorticoid regulation of the gamma-fibrinogen gene [Woodward et al., 1997]. Thus, transcription factors binding to distinct sites interact with occupied steroid response elements to promote ligand-mediated transcriptional activation. Accessory factors, including TRIP1 [Lee et al., 1995], TIF1 [LeDouarin et al., 1995], RIP140, RIP160 [Cavailles et al., 1995; Halachmi et al., 1994], and SRC-1 [Oñate et al., 1995], contribute to transcriptional activation mediated by nuclear hormone receptors [Halachmi et al., 1994; Cavailles et al., 1994; Kurokawa et al., 1995; Suen and Chin, 1995; Cavailles et al., 1995; Oñate et al., 1995; Maurer and Notides, 1987]. The cAMP response element protein binding protein (CBP) functions as an integrator for transcriptional activation by nuclear receptors through contacts with both the ligand binding domain of the receptor and the coactivator protein p160 [Kamei et al., 1996]. CBP also has been shown to acetylate chromatin through its histone acetyltransferase activity, thereby destabilizing nucleosomes and promoting activated chromatin formation [Bannister and Kouzarides, 1996; Ogryzko et al., 1996]. The potential candidate transcription factors of the VDRE-associated GGTTTGG motif are not, however, restricted to those shown to interact with the nuclear receptor superfamily. The position and orientation independence of this novel enhancer, and its ability to modulate responses to different transcription factors suggests that it may function to coordinate transcriptional activation by several different response elements in a single gene.

REFERENCES

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. 1992. Current protocols in molecular biology. New York, NY: Greene Publishing Associates and Wiley-Interscience.

Bannister AJ, Kouzarides T. 1996. The CBP co-activator is a histone acetyltransferase. Nature 84:641–643.

Cavailles V, Dauvois S, Danielian PS, Parker MG. 1994. Interaction of proteins with transcriptionally active estrogen receptors. Proc Natl Acad Sci USA 91:10009–10013.

Cavailles V, Dauvois S, L'Horset F, Lopez G, Hoare S, Kushner PJ, Parker MG. 1995. Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. EMBO J 14:3741–3751.

Demay MB, Roth DA, Kronenberg HM. 1989. Regions of the rat osteocalcin gene which mediate the effect of 1,25-dihydroxyvitamin D_3 on gene transcription. J Biol Chem 264:2279–2282.

Demay MB, Gerardi JM, DeLuca HF, Kronenberg HM. 1990. DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin D_3 receptor and confer responsiveness to 1,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA 87:369–373.

Demay MB, Kiernan MS, DeLuca HF, Kronenberg HM. 1992a. Sequences in the human parathyroid hormone gene that bind the 1,25-dihydroxyvitamin D_3 receptor and mediate transcriptional repression in response to 1,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA 89: 8097–8101.

Demay MB, Kiernan MS, DeLuca HF, Kronenberg HM. 1992b. Characterization of 1,25-dihydroxyvitamin D_3 receptor interactions with target sequences in the rat osteocalcin gene. Mol Endocr 6:557–562.

Halachmi S, Marden E, Martin G, MacKay H, Abbondanza C, Brown M. 1994. Estrogen receptor-associated proteins-possible mediators of hormone-induced transcription. Science 264:1455–1458.

Kamei Y, Xu L, Heinzel T, Torchia J, Kurokawa R, Gloss B, Lin SC, Heyman RA, Rose DW, Glass CK, Rosenfeld MG. 1996. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell 85:403–414.

Kurokawa R, Soderstrom M, Horlein A, Halachmi S, Brown M, Rosenfeld MG, Glass CK. 1995. Polarity-specific activities of retinoic acid receptors determined by a co-repressor. Nature 377:451–454.

LeDouarin B, Zechel C, Garnier JM, Lutz Y, Tora L, Pierrat B, Heery D, Gronemeyer H, Chambon P, Losson R. 1995. The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. EMBO J 14:2020–2033.

Lee JW, Ryan F, Swaffield JC, Johnston SA, Moore DD. 1995. Interaction of thyroid hormone receptor with a conserved transcriptional mediator. Nature 374:91–94.

Maurer RA, Notides AC. 1987. Identification of an estrogen-responsive element from the 5′ flanking region of the rat prolactin gene. Mol Cell Biol 7:4247–4254.

Morrison NA, Shine J, Fragonas J-C, Verkest V, McMenemy ML, Eisman JA. 1989. 1,25-Dihydroxyvitamin D-responsive element and glucocorticoid repression in the osteocalcin gene. Science 246:1158–1161.

Nye JA, Petersen J, Gunther CV, Jonsen MD, Graves BJ. 1992. Interaction of murine Ets-1 with GGA-binding sites establishes the ETS domain as a new DNA-binding motif. Genes Dev 6:975–990.

O'Brien RM, Noisin EL, Suwanichkul A, Yamasaki T, Lucas PC, Wang J, Powell DR, Granner DK. 1995. Hepatic nuclear factor 3- and hormone-regulated expression of the phosphenolpyruvate carboxykinase and insulin-like growth factor-binding protein 1 genes. Mol Cell Biol 15:1747–1758.

Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 87:953–959.

Ohyama Y, Ozono K, Uchida M, Yoshimura M, Shinki T, Suda T, Yamamoto O. 1996. Functional assessment of two vitamin D-responsive elements in the rat 25-hydroxyvitamin D_3 24-hydroxylase gene. J Biol Chem 271: 30381–30385.

Oñate SA, Tsai SY, Tsai M-J, O'Malley BW. 1995. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270:1354–1357.

Prost E, Moore DD. 1986. CAT vectors for the analysis of eukaryotic promoters and enhancers. Gene 45:107–111.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467.

Sneddon WB, Bogado CE, Kiernan MS, Demay MB. 1997a. DNA sequences downstram from the vitamin D response element of the rat osteocalcin gene are required for ligand-dependent transactivation. Mol Endocrin 11:210–217.

Sneddon WB, Demay MB. 1997b. Definition of an element required for 1,25-dihydroxyvitamin D-Dependent Transactivation of the rat osteocalcin gene. J. Bone Miner Res 1997, 12, S1:F204.

Suen C, Chin WW. 1995. A potential transcriptional adaptor(s) may be required in thyroid hormone-stimulated gene transcription in vitro. Endocrinology 136:2776–2783.

Woods DB, Ghysdeal J, Owen MJ. 1992. Identification of nucleotide preferences in DNA sequences recognised specifically by c-Ets-1 protein. Nucleic Acids Res 20:699–704.

Woodward RN, Li M, Holland LJ. 1997. Novel accessory factor-binding site required for glucocorticoid regulation of the gamma-fibrinogen gene from *Xenopus laevis*. Mol Endocrin 11:563–576.