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# DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin $D_3$ receptor and confer responsiveness to 1,25-dihydroxyvitamin $D_3$

(steroid receptor/osteoblast)

MARIE B. DEMAY*, JACQUELINE M. GERARDI*, HECTOR F. DELUCA†, AND HENRY M. KRONENBERG*

*Endocrine Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114; and †Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, WI 53706

*Contributed by Hector F. DeLuca, October 13, 1989*

**ABSTRACT** **The 5′ flanking region of the rat osteocalcin gene has been shown to confer responsiveness to 1,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$] after transfection of fusion genes into ROS 17/2.8 cells. Deletion analysis has demonstrated that there are at least two domains in this 5′ flanking region that contribute to 1,25$(OH)_2D_3$ responsiveness; however, only the downstream region is able to confer 1,25$(OH)_2D_3$ responsiveness to either the native osteocalcin promoter or to a heterologous viral promoter (herpes simplex virus thymidine kinase). The proximal region responsible for 1,25$(OH)_2D_3$ induction of the rat osteocalcin gene lies 458 base pairs upstream from the transcription start site of this gene. A 25-base-pair oligonucleotide corresponding to the sequences in this region is able to confer 1,25$(OH)_2D_3$ responsiveness to the thymidine kinase promoter in an orientation-independent fashion. This sequence contains three copies of a short sequence that are homologous to "half-sites" of steroid response elements. Gel-retardation assays using porcine intestinal nuclear extract as a rich source of 1,25$(OH)_2D_3$ receptor demonstrated retardation in the migration of probes containing the sequence noted above. A monoclonal antibody directed against the 1,25$(OH)_2D_3$ receptor caused further retardation in the migration of these protein–DNA complexes. Therefore, the sequences represented in this oligonucleotide encompass the sequences necessary for binding of the 1,25$(OH)_2D_3$ receptor to DNA as well as those sequences necessary for 1,25$(OH)_2D_3$ to induce osteocalcin gene transcription.**

The rat osteocalcin gene provides an excellent model for studying the effect of 1,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$] on gene expression. Osteocalcin is a bone-specific protein (1) whose transcription is directly regulated by 1,25$(OH)_2D_3$ (2–5). The gene encoding rat osteocalcin has been cloned (2–4) and sequenced (3, 4). The availability of a cultured cell line (ROS 17/2.8) (6) that expresses the gene in a 1,25$(OH)_2D_3$-responsive fashion (7) permits the study of the regulatory elements of the rat osteocalcin gene in transient expression experiments. Furthermore, this system will facilitate future investigations aimed at examining how the 1,25$(OH)_2D_3$ receptor interacts with other tissue-specific and cellular factors to modulate gene expression.

We have described (2) two regions in the 5′ flanking region of the rat osteocalcin gene that contribute to its 1,25$(OH)_2D_3$ responsiveness. A series of deletion mutants has enabled a more precise localization of these sequences. The upstream element alone is insufficient to confer 1,25$(OH)_2D_3$ responsiveness to the native osteocalcin promoter or to a heterologous viral promoter. The more proximal element, however, contains sequences that can bind the 1,25$(OH)_2D_3$ receptor and confer 1,25$(OH)_2D_3$ responsiveness to a heterologous viral promoter.

## MATERIALS AND METHODS

**Synthesis of Chloramphenicol Acetyltransferase (CAT) Constructs.** The plasmid $POC_{2.0}CAT$ (2), which contains 1750 base pairs (bp) of the 5′ flanking region of the osteocalcin gene was linearized with *Sma* I and subjected to a series of BAL-31 (New England Biolabs) deletions from the 5′ end. The fragment containing the remaining 5′ flanking region and the CAT gene was excised with *Bam*HI, blunt-ended, and subcloned into *Hin*cII-cut alkaline phosphatase-treated pUC18. Further deletions were carried out in a similar fashion with the constructs containing 848 and 522 bp upstream from the transcription start site. Restriction fragments were blunt-ended and subcloned into *Sac* I cut blunt-ended $POC_{0.3}CAT$ (2).

For experiments using a heterologous viral promoter, oligonucleotides were inserted into the *Bam*HI site of pUTKAT3 (8) (a kind gift from David Moore, Massachusetts General Hospital, Boston). Oligonucleotides were synthesized (on an Applied Biosystems model 380A synthesizer) corresponding to sequences of interest, with the addition of nucleotides on either end that would permit subcloning into the intact *Bam*HI site of pUTKAT3.

**Cell Culture and Transfections.** ROS 17/2.8 cells were maintained in Ham's F-12 medium with L-glutamine (GIBCO) supplemented with 10% (vol/vol) fetal bovine serum, penicillin, and streptomycin. Transfections and quantitations of CAT activity were performed as described (2). Each osteocalcin–CAT fusion gene was cotransfected with a control plasmid containing the human growth hormone gene under the control of the Rous sarcoma virus promoter (9). Radioimmunoassays of the human growth hormone (Nichols, San Juan Capistrano, CA) secreted into the medium demonstrated the 1,25$(OH)_2D_3$ independence of the secretion of human growth hormone. Growth hormone measurements in the medium of 1,25$(OH)_2D_3$-stimulated and control transfections did not vary by more than 11%.

**Gel-Retardation Assays.** Restriction fragments and oligonucleotides of interest were labeled by filling-in recessed ends with the large fragment of DNA polymerase I and [$\alpha$-$^{32}$P]dATP (New England Nuclear). Porcine intestinal nuclear extracts were prepared as described (10). Nuclear extract was preincubated with 1 $\mu$M 1,25$(OH)_2D_3$ for 15 min at 22°C. Poly(dI-dC)·poly(dI-dC) (Pharmacia) was then added at a concentration of 0.27 $\mu$g/$\mu$g of extract protein in a buffer containing 155 mM KCl, 5 mM dithiothreitol, 0.75 mM

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Abbreviations: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin $D_3$; CAT, chloramphenicol acetyltransferase; HSV-tk, herpes simplex virus thymidine kinase; DR, D response.

EDTA, and 50 mM Tris·HCl (pH 7.4) for an additional 15 min. Subsequently, the probe was added (in 0.05 vol) for an additional 15 min and then 3 μg (in 0.1 vol) of either nonspecific immunoglobulin or antibody directed against the $1,25(OH)_2D_3$ receptor (11) was added to the incubation mixture. In competition experiments, the excess of unlabeled competitor DNA was added prior to the probe. The mixture was brought to 10% (vol/vol) glycerol and electrophoresed on a 4% polyacrylamide gel in 2.5% glycerol/190 mM glycine/1 mM EDTA/25 mM Tris·HCl, pH 8.5 at 7 V/cm at 4°C.

**DNA Sequencing.** All DNA sequencing was carried out by the dideoxynucleotide chain-termination method after subcloning into M13 vectors (12).

## RESULTS

Localization of the $1,25(OH)_2D_3$ response element by BAL-31 deletional analysis is depicted in Fig. 1. Numbers on the left side of each line represent the location of the 5′ end relative to the start site of transcription of the osteocalcin gene (4). Relative stimulation represents induction in response to 10 nM $1,25(OH)_2D_3$. Each determination represents the mean of three independent transfections; similar data was reproduced on at least three occasions. It is apparent that there are two regions that substantially influence responsiveness to $1,25(OH)_2D_3$. Removal of the first region between positions −1186 and −1094 dampened the response from 6.0-fold to 1.4-fold. Removal of the region between positions −1094 and −522 incrementally restored responsiveness. Deletion of the second region between positions −509 and −428 resulted in total abolition of $1,25(OH)_2D_3$ responsiveness. To further explore this region, a series of oligonucleotides corresponding to these sequences was constructed and examined for functional activity and ability to bind to the $1,25(OH)_2D_3$ receptor.

The sequences of these oligonucleotides are shown in Fig. 2 and Table 1. Data in Table 1 indicate the ability of each oligonucleotide to stimulate a heterologous promoter, herpes simplex virus thymidine kinase (HSV-tk), in response to 10 nM $1,25(OH)_2D_3$. Each arrow represents one copy of the oligonucleotide, and the direction of the arrow indicates the orientation of the oligonucleotide with respect to the promoter. As is evident from this table, a single copy of a 55-bp oligonucleotide, $1D_3$, was able to confer substantial $1,25(OH)_2D_3$ responsiveness to the heterologous HSV-tk promoter in an orientation-independent manner. Multiple copies were able to further enhance $1,25(OH)_2D_3$ stimulation. Two other oligonucleotides, $4D_3$ and $9D_3$, were able to significantly enhance transcription in response to $1,25(OH)_2D_3$. Comparison of the sequences common to these three oligonucleotides revealed the presence of three similar short motifs, two of which form an imperfect palindrome with the third. These sequences, called D-response (DR) motifs subsequently, are highlighted at the top of Fig. 2. Furthermore, M1-$1D_3$, an oligonucleotide corresponding to the sequences in $1D_3$ but including two mutations at the 5′ ends of the second and third DR motifs was nonfunctional. The same mutations in $9D_3$, represented by M1-$9D_3$, also rendered this oligonucleotide nonfunctional. Another mutant, M2-$9D_3$, involving the first two bases in the first and third DR motifs was nonfunctional as well. Oligonucleotides $2D_3$ and $8D_3$, which contain the first and part of the second DR motif, were only partially functionally active. As well, $5D_3$, which only lacks the first two bases of the first DR motif, was inactive, even in multiple copies. Therefore, both the first and the third intact DR motifs were necessary to confer $1,25(OH)_2D_3$ responsiveness to the HSV-tk promoter.

To establish that these DR motifs are binding to the $1,25(OH)_2D_3$ receptor, gel-retardation experiments were undertaken. Results of these experiments are depicted in Fig. 3. Fig. 3*A*, lane 1 shows two major bands of retarded mobility (arrows) seen with the addition of porcine intestinal nuclear extract. The migration of both these bands was further retarded by the addition of XVIE10B6A5 (11), a monoclonal antibody directed against the porcine $1,25(OH)_2D_3$ receptor (lane 2). Addition of VIIID8C12, a monoclonal antibody directed against the DNA-binding domain of the $1,25(OH)_2D_3$ receptor, caused a decrease in the intensity of these bands, presumably by competing with the DNA probe for $1,25(OH)_2D_3$ receptor binding (lane 3). The addition of non-

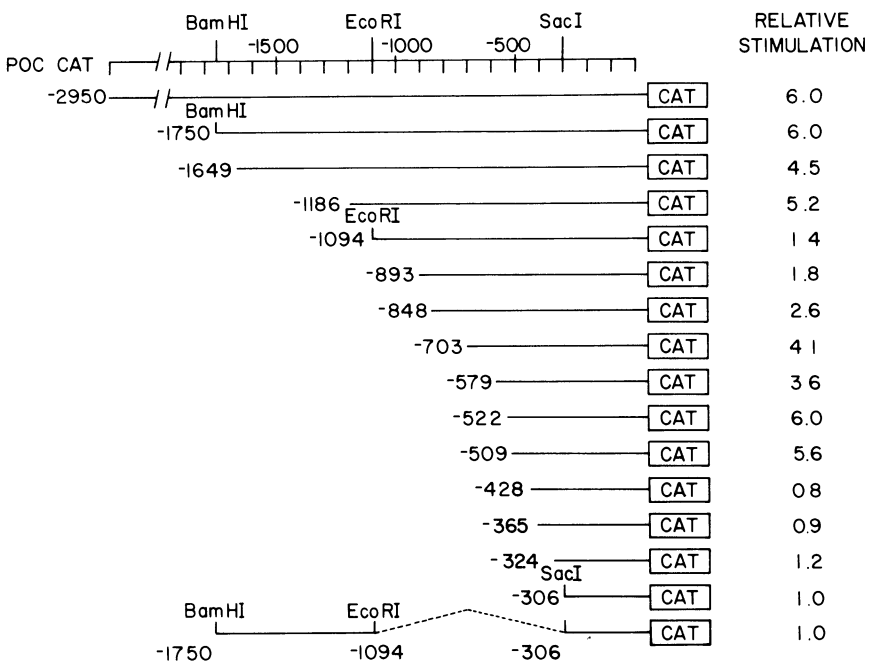


FIG. 1. Deletional analysis of the 5′ flanking region of the osteocalcin gene. A series of 5′ BAL-31 deletions of POCCAT, which contains approximately 2950 bp of the 5′ regulatory region fused to CAT is shown. Numbers on the left of each deletion represent the 5′ end of the DNA sequence relative to the transcription start site of the osteocalcin gene.

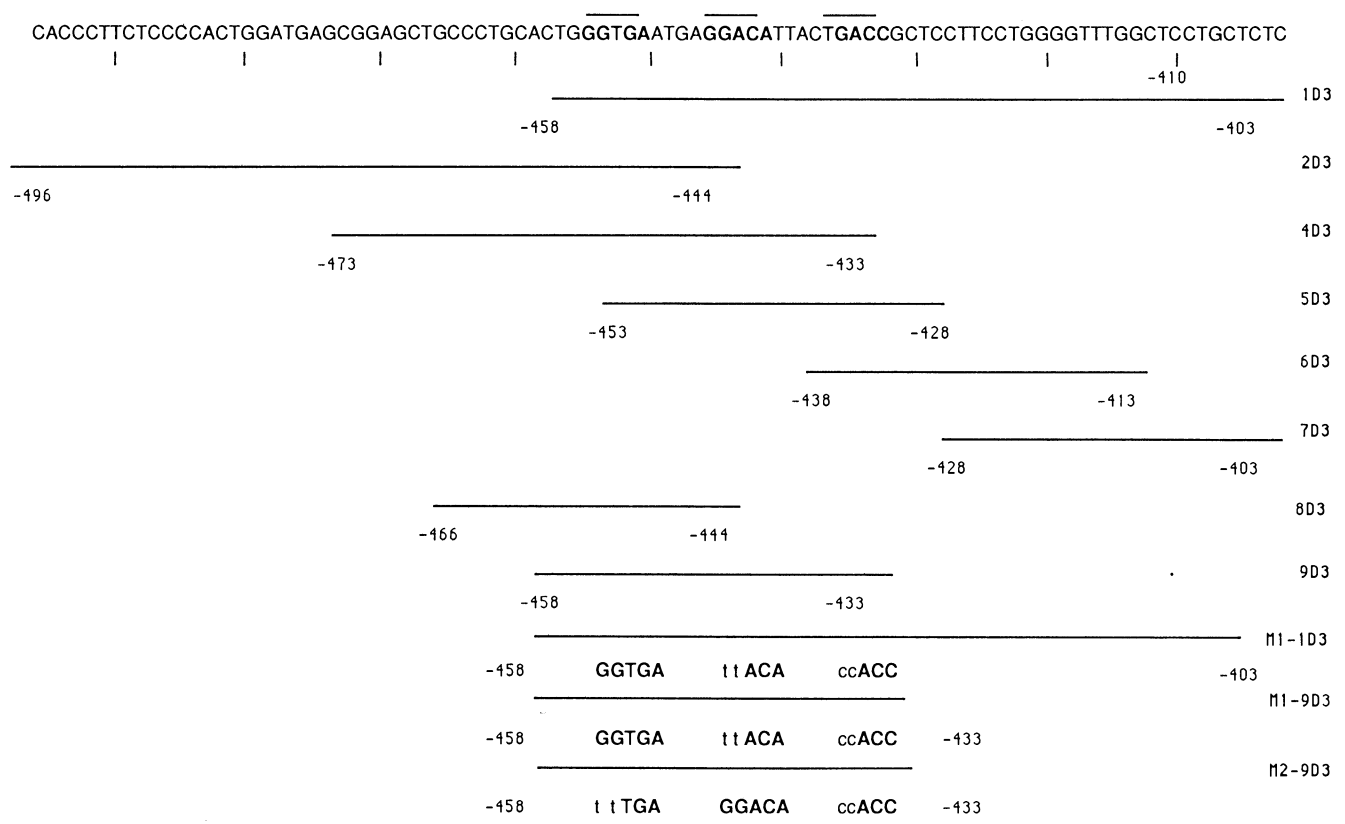


FIG. 2. Rat osteocalcin gene oligonucleotides. (*Upper*) Rat osteocalcin gene sequence from position −496 to position −403. Short lines over the sequence and boldface letters indicate the DR motifs. (*Lower*) Extents of the various oligonucleotides. M1-1D3, M1-9D3, and M2-9D3 contain mutations in the DR motifs, indicated by the use of lowercase letters.

specific mouse immunoglobulin did not have any effect on the migration of the DNA–receptor complex (lane 4). Identical results were obtained using the 25-bp oligonucleotide $9D_3$ as a probe (Fig. 3*B*). The presence of two $1,25(OH)_2D_3$ receptor-dependent bands may represent the binding of different numbers of receptors to the oligonucleotides or the binding of the $1,25(OH)_2D_3$ receptor alone (lower band) and with another protein (upper band). When $2D_3$ was used as a probe, a band of different mobility was seen that was not affected by the presence of these antibodies (Fig. 3*C*). The effects of the antibodies are, therefore, specific.

To demonstrate that the interactions of $1D_3$ and $9D_3$ with the $1,25(OH)_2D_3$ receptor are sequence-specific, competition experiments with unlabeled oligonucleotides were performed (Fig. 4). Addition of a 100-fold excess of $1D_3$ prevented

Table 1. Relative stimulation of CAT activity in response to $1,25(OH)_2D_3$

| Oligonucleotide | Sequence positions | No. copies and orientation | Relative stimulation |
|---|---|---|---|
| $1D_3$ | −458 to −403 | → | 5.2 |
| | | →→ | 7.4 |
| | | ← | 6.0 |
| | | ←← | 8.1 |
| $2D_3$ | −496 to −444 | → | 1.8 |
| $4D_3$ | −473 to −433 | ←← | 2.8 |
| $5D_3$ | −453 to −428 | →→→ | 1.3 |
| $6D_3$ | −438 to −413 | →→→← | 0.9 |
| $7D_3$ | −428 to −403 | → | 1.1 |
| $8D_3$ | −466 to −444 | →→ | 1.9 |
| $9D_3$ | −458 to −433 | → | 2.1 |
| | | ←← | 5.0 |
| M1-$1D_3$ | $1D_3$ mutant | → | 1.1 |
| | | ← | 0.9 |
| M1-$9D_3$ | $9D_3$ mutant | →←→ | 1.1 |
| M2-$9D_3$ | $9D_3$ mutant | →←→ | 0.9 |

Oligonucleotides ligated upstream to the HSV-tk promoter are indicated. Sequence positions from the 5′ flanking region of the rat osteocalcin gene represented in each oligonucleotide are indicated. The number of arrows represents the copy number of each oligonucleotide and the direction of the arrows shows the orientation of the oligonucleotide(s) relative to the promoter. Relative stimulation of CAT activity in response to 10 nM $1,25(OH)_2D_3$ is shown. See Fig. 2 for the sequences of the mutant oligonucleotides.

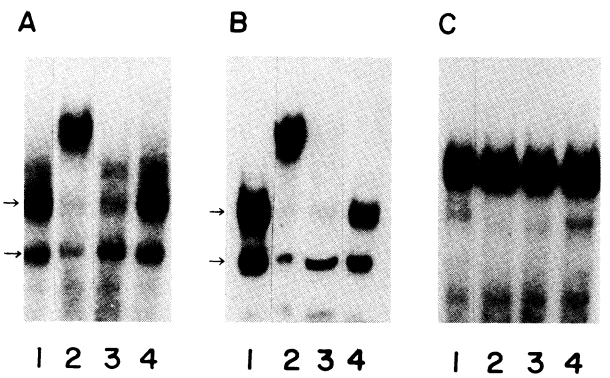


FIG. 3. Band-shift analysis using monoclonal antibodies to the $1,25(OH)_2D_3$ receptor. (*A*) Gel retardation using $1D_3$ as a probe. Nuclear extract was added to the $1D_3$ probe (lane 1). A monoclonal antibody directed against the $1,25(OH)_2D_3$ receptor (XV1E10B6A5) was added to the nuclear extract and $1D_3$ (lane 2). Antibody directed against the DNA-binding domain of the $1,25(OH)_2D_3$ receptor (VIIID8C12) was added to the extract and $1D_3$ (lane 3). Nonspecific mouse IgG was added to extract and $1D_3$ (lane 4). Arrows indicate the two major bands. (*B*) Gel retardation using $9D_3$ as a probe. Lanes 1–4 are as in *A*. (*C*) Gel retardation using $2D_3$ as a probe. Lanes 1–4 are as in *A*.

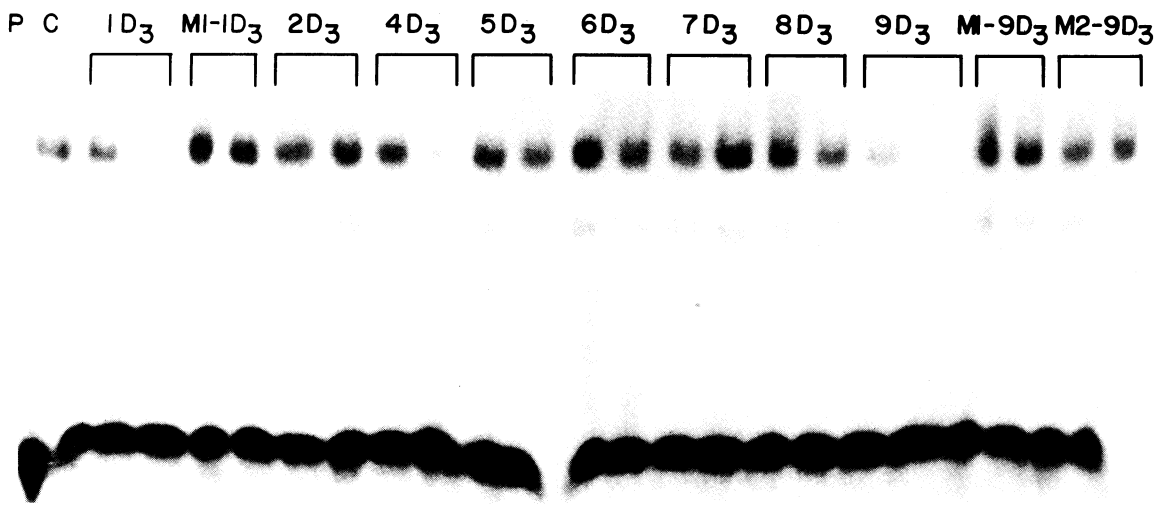


FIG. 4. Band-shift analysis using unlabeled oligonucleotides as competitors of binding to labeled $1D_3$. Lane P represents $1D_3$ probe alone. Lane C represents the control lane that contains probe and nuclear extract. The remaining lanes demonstrate the effects of addition of 10- or 100-fold excess (first and second lane of each pair, respectively) of the unlabeled oligonucleotide indicated above each pair of lanes.

binding of the $1D_3$ probe to the receptor. In contrast, M1-$1D_3$ did not interrupt binding of $1D_3$ to the $1,25(OH)_2D_3$ receptor, demonstrating that the mutations in this sequence render it nonfunctional by preventing binding of the $1,25(OH)_2D_3$ receptor. Inhibition of binding was seen as well with addition of 100-fold excess of the two functional oligonucleotides, $4D_3$ and $9D_3$. A 100-fold excess of the $9D_3$ mutants, M1-$9D_3$ and M2-$9D_3$, was also unable to block $1D_3$ binding of the $1,25(OH)_2D_3$ receptor. Oligonucleotides missing the three DR motifs, $2D_3$, $5D_3$, $6D_3$, $7D_3$, and $8D_3$, were likewise unable to prevent protein binding of $1D_3$. It should be noted, however, that when $2D_3$ was used as a radioactive probe (Fig. 3*C*), a faint lower band comigrated with the major $1,25(OH)_2D_3$ receptor-containing band in Fig. 3*A* and *B*. This faint band disappeared with the addition of monoclonal antibodies to the $1,25(OH)_2D_3$ receptor. This result suggests that $2D_3$ contains low-affinity binding sites for the $1,25(OH)_2D_3$ receptor.

As can be seen in Fig. 5, the bands generated by $1,25(OH)_2D_3$ receptor binding of $1D_3$ and $9D_3$ comigrate. In a similar fashion to $1D_3$ (lanes 1 and 2), 100-fold excess of unlabeled $9D_3$ prevented labeled $9D_3$ from binding to the $1,25(OH)_2D_3$ receptor (lanes 4 and 5). The mutants of $9D_3$, M1-$9D_3$ and M2-$9D_3$, when present in a 100-fold excess, were unable to prevent the formation of a $1,25(OH)_2D_3$ receptor–

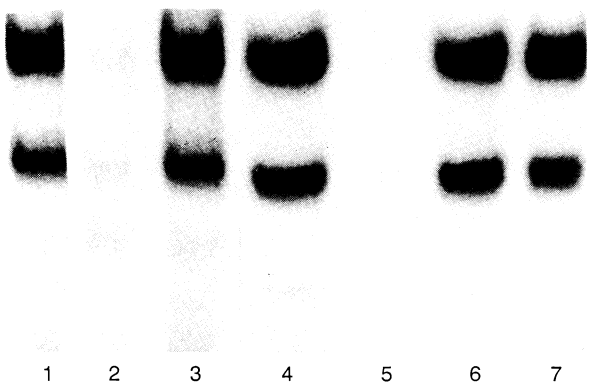


FIG. 5. Comigration of protein–DNA complexes using $1D_3$ and $9D_3$ as probes. Each lane contains pig intestinal nuclear extract with labeled $1D_3$ (lanes 1–3) or labeled $9D_3$ (lanes 4–7). Lanes: 1, no further addition; 2, 100-fold excess unlabeled $1D_3$ added; 3, 100-fold excess unlabeled M1-$1D_3$ added; 4, no further addition; 5, 100-fold excess unlabeled $9D_3$ added; 6, 100-fold excess unlabeled M1-$9D_3$ added; 7, 100-fold excess unlabeled M2-$9D_3$ added.

$9D_3$ complex (lanes 6 and 7). It is, therefore, apparent that the oligonucleotides that are able to bind to the $1,25(OH)_2D_3$ receptor, as assessed by the gel-retardation assays, are the same as those that are able to confer $1,25(OH)_2D_3$ responsiveness to a heterologous viral promoter. The three DR motifs common to these oligonucleotides, therefore, represent the proximal $1,25(OH)_2D_3$ response element of the rat osteocalcin gene.

## DISCUSSION

The proximal sequences responsible for the $1,25(OH)_2D_3$ induction of the rat osteocalcin gene lie 458 bp upstream from the transcription start site of this gene. It is of note, however, that further addition of sequences 5′ to this element result in a progressive dampening of $1,25(OH)_2D_3$ induction from 6.0-fold at position −522 down to 1.4-fold at position −1094. Addition of another 92 bp to this construct totally restores $1,25(OH)_2D_3$ responsiveness. The 656-bp fragment from position −1094 to position −1750, however, is unable to confer responsiveness to the native osteocalcin promoter (Fig. 1) or to a heterologous viral promoter (data not shown). The role of this region in osteocalcin gene transcription remains to be elucidated. It is possible that these sequences exert their effect by binding factors that inhibit the binding or subsequent effects of negative factors that interact with the sequences from position −522 to position −1094. Alternatively, this region may contain amplifiers of $1,25(OH)_2D_3$ responsiveness that can overcome the effect of the inhibitory factors simply by augmenting $1,25(OH)_2D_3$ responsiveness.

The proximal sequences, containing three DR motifs located between positions −458 and −433, confer substantial $1,25(OH)_2D_3$ responsiveness to a heterologous viral promoter. The oligonucleotide $5D_3$, which lacks only the first two bases of the first DR motif, is neither able to bind the $1,25(OH)_2D_3$ receptor nor able to restore $1,25(OH)_2D_3$ responsiveness. The oligonucleotides $2D_3$ and $8D_3$ are partially functionally active. They contain the first and part of the second DR motifs. These sequences contain the rat homologue of the $1,25(OH)_2D_3$ response element in the human osteocalcin gene (5). It is of note, however, that the proposed first half-palindrome of the human gene is poorly conserved in the rat sequence. Like the sequences in the human gene, these rat sequences are able to confer 1.8-fold $1,25(OH)_2D_3$ responsiveness. As shown in Fig. 3*C*, these sequences may

contain low-affinity binding sites for the $1,25(OH)_2D_3$ receptor.

Although $4D_3$ and $9D_3$ contain all three DR motifs and bind to the $1,25(OH)_2D_3$ receptor, they do not confer as much responsiveness as does $1D_3$. These oligonucleotides end precisely after the third DR motif and may, therefore, exclude crucial residues required for optimal activity. This hypothesis is particularly plausible since other steroid response elements contain six bases, one more than can be identified in the osteocalcin DR motifs. The exclusion of a poorly conserved sixth base from $4D_3$ and $9D_3$ may explain their relatively weak activity. Alternatively, the binding of other transcription factors to downstream sequences in $1D_3$ may permit the full transcriptional effects of $1,25(OH)_2D_3$ receptor binding.

The sequences represented in the first DR motif of the rat $1,25(OH)_2D_3$ response element (GGTGA) are found repeated in the human osteocalcin gene (GGTGAcTCACCgGGTGA) (uppercase letters represent residues included in the DR motif). The proposed $1,25(OH)_2D_3$ response element in the human osteocalcin gene (5) begins with the fourth residue in this sequence (GACTCAccggTGAACG). Mutational analysis of the proposed rat and human response elements will be required to define the precise nucleotides that confer responsiveness to $1,25(OH)_2D_3$ in each of these genes.

The probable requirement of three DR motifs for receptor binding differs from the requirement of other response elements in the steroid/thyroid receptor superfamily, except for that of the thyroid response element (TRE). It has been shown that the thyroid response element in the rat growth hormone gene encompasses three half-sites and that the presence of all three is required for maximal triiodothyronine responsiveness (13). Comparison of the base pairs represented in the rat osteocalcin $1,25(OH)_2D_3$ response element to those in other hormone response elements reveals a GGTCA motif in the estrogen response element of the vitellogenin genes (14) and a GGT(C/A)A motif in the rat growth hormone thyroid response element (13). It is evident that, allowing for a few mismatches and variation in spacing between the half-sites, there is a high degree of homology among these sequences. The spacing between these half-sites or the number of half-sites involved may play an important role in the specificity of the interaction of the steroid receptors with their hormone response elements. Extensive mutational analysis, DNase protection, and methylation interference studies will permit more precise assessment of which base pairs included in and adjacent to the DR motifs of the rat $1,25(OH)_2D_3$ response element are intimately involved in $1,25(OH)_2D_3$ receptor binding and function. Experiments using the native rat osteocalcin promoter in conjunction with the $1,25(OH)_2D_3$ response element should allow the examination of the interaction of the $1,25(OH)_2D_3$ response element with other sequences in the complex series of interactions that contribute to the regulation of osteocalcin gene expression.

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1. Price, P. (1987) in *Calcium Regulation and Bone Metabolism: Basic and Clinical Aspects*, eds. Cohn, D. V., Martin, T. J. & Meunier, P. J. (Elsevier, New York), Vol. 9, pp. 419–426.
2. Demay, M. B., Roth, D. A. & Kronenberg, H. M. (1989) *J. Biol. Chem.* **264,** 2279–2282.
3. Lian, J., Stewart, C., Puchacz, E., Mackowiak, S., Shalhoub, V., Collart, D., Zambetti, G. & Stein, G. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1143–1147.
4. Yoon, K., Rutledge, S. J., Buenaga, R. F. & Rodan, G. (1988) *Biochemistry* **27,** 8521–8525.
5. Kerner, S. A., Scott, R. A. & Pike, J. W. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4455–4459.
6. Majeska, R., Rodan, S. & Rodan, G. (1980) *Endocrinology* **107,** 1494–1503.
7. Price, P. & Baukol, S. (1980) *J. Biol. Chem.* **255,** 11660–11663.
8. Prost, E. & Moore, D. D. (1986) *Gene* **45,** 107–111.
9. Selden, R. F., Howie, K. B., Rowe, M. E., Goodman, H. M. & Moore, D. D. (1986) *Mol. Cell. Biol.* **6,** 3173–3179.
10. Dame, M. C., Pierce, E. A. & DeLuca, H. F. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 7825–7829.
11. Dame, M. C., Pierce, E. A., Prahl, J. M., Hayes, C. E. & DeLuca, H. F. (1986) *Biochemistry* **25,** 4523–4534.
12. Messing, J. (1983) *Methods Enzymol.* **101,** 20–78.
13. Brent, G. A., Harney, J. W., Chen, Y., Warne, R. L., Moore, D. D. & Larsen, P. R. (1989) *Mol. Endocrinol.* **3,** 1996–2004.
14. Klein-Hitpass, L., Schorpp, M., Wagner, U. & Ryffel, G. U. (1986) *Cell* **46,** 1053–1061.