prepared from cell-free supernatants and stored at −70°C.
15. There was no evidence that the intrinsic infectivity of mutant virus stocks obtained from transfection experiments was different from that of wild-type virus. The median tissue culture infectious dose ($TCID_{50}$) determined by terminal dilution in MT-2 cells (*13*) was $10^{6.45}$/ml for HXB2-D and $10^{6.7}$/ml for HIVRTMC for stocks from a transfection experiment and $10^{6.95}$/ml and $10^{6.7}$/ml for HXB2-D and HIVRTMC, respectively, for stocks from a separate experiment. Furthermore, the titer of both viruses in HT4-6C cells (in plaque-forming units per milliliter) was also similar (5.2×10^4/ml for HXB2-D and 1.8×10^5/ml for HIVRTMC).
16. B. D. Chesebro and K. Wehrly, *J. Virol.* **62**, 3779 (1988).
17. B. A. Larder and S. D. Kemp, unpublished observations.
18. M. S. Smith, E. L. Hahn, J. S. Pagano, *J. Virol.* **61**, 3769 (1988).
19. G. Darby, B. A. Larder, M. M. Inglis, *J. Gen. Virol.* **67**, 753 (1986); B. A. Larder, S. D. Kemp, G. Darby, *EMBO J.* **6**, 169 (1987); P. Collins *et al.*, *J. Gen. Virol.* **70**, 375 (1989).
20. M. S. Johnson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7648 (1986).
21. B. A. Larder, D. J. M. Purifoy, K. L. Powell, G. Darby, *Nature* **327**, 716 (1987).
22. RT cloned from zidovudine-sensitive isolate A012B and resistant isolate A012D was expressed in *E. coli* (Table 1) and purified as described [(7); M. Tisdale *et al.*, *J. Virol.* **62**, 3662 (1988)]. For K_m (TTP) and K_i (AZT-TP) determination, RT assays were performed as described (*7*) by using [^{3}H]TTP and poly(rA) · oligo(dT)$_{18}$ as primer template. RT from isolate A012B gave a K_m (TTP) value of 4.5 μ*M* and K_i (AZT-TP) value of 0.01 μ*M*; RT from A012D gave a K_m (TTP) of 13 μ*M* and K_i (AZT-TP) of 0.03 μ*M*.
23. Nucleotide sequencing reactions were carried out with single-stranded M13mp18 DNA and "universal" sequencing primer as described (*8*) except dideoxy-TTP was replaced by AZT-TP. Reactions contained 1 μ*M* TTP, 40 μ*M* each of dATP, dGTP, and dCTP, and either 0.01 μ*M*, 0.1 μ*M*, or 1 μ*M* AZT-TP. Reaction products were separated by buffer gradient polyacrylamide gel electrophoresis [M. D. Biggin, T. J. Gibson, G. F. Hong, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3963 (1983)]. Both enzymes (from sensitive and resistant isolates) showed equal ability to incorporate AZT-TP into DNA template over this 100-fold range of AZT-TP.
24. M. Guyader *et al.*, *Nature* **326**, 662 (1987); L. Chakrabarti *et al.*, *ibid.* **328**, 543 (1987).
25. B. D. Preston, B. J. Poiesz, L. A. Loeb, *Science* **242**, 1168 (1988); J. D. Roberts, K. Bebenek, T. A. Kunkel, *ibid.*, p. 1171; M. S. Saag *et al.*, *Nature* **334**, 440 (1988); A. G. Fisher *et al.*, *ibid.*, p. 444.
26. We thank G. Darby and D. Richman for support and encouragement during this study, which was initiated in the laboratory of D. Richman and carried out in the Molecular Sciences Department, Wellcome, United Kingdom. We also thank P. Kellam and P. Bartlett for technical support. RT purification of A012 isolates was performed by C. Bradley and A. Emmerson. AZT-TP was supplied by W. Miller, HT4-6C cells by B. Chesebro, HXB2-D by L. Ratner, and the *E. coli* strain 5KCPo1A^{is}F′ by P. Oliver.

24 July 1989; accepted 29 September 1989

1,25-Dihydroxyvitamin D–Responsive Element and Glucocorticoid Repression in the Osteocalcin Gene

NIGEL A. MORRISON,* JOHN SHINE, JEAN-CHARLES FRAGONAS, VERONIK VERKEST, M. LYNNE MCMENEMY, JOHN A. EISMAN

The active hormonal form of vitamin D_3, 1,25-dihydroxyvitamin D_3[1,25(OH), which regulates cellular replication and function in many tissues and has a role in bone and calcium homeostasis, acts through a hormone receptor homologous with other steroid and thyroid hormone receptors. A $1,25(OH)_2D_3$-responsive element (VDRE), which is within the promoter for osteocalcin [a bone protein induced by $1,25(OH)_2D_3$] is unresponsive to other steroid hormones, can function in a heterologous promoter, and contains a doubly palindromic DNA sequence (TTGGTGACTCACCGGGTGAAC; −513 to −493 bp), with nucleotide sequence homology to other hormone responsive elements. The potent glucocorticoid repression of $1,25(OH)_2D_3$ induction and of basal activity of this promoter acts through a region between −196 and +34 bp, distinct from the VDRE.

THE IN VIVO EFFECTS OF STEROID and steroid-like hormones are mediated by specific receptors, which belong to a superfamily of trans-acting "zinc finger" proteins. After binding of the specific ligand, these receptor proteins influence gene expression via association with specific DNA elements known as hormone-responsive elements (HREs) (*1, 2*). Specific HREs are known for estrogen-, thyroid hormone–, progesterone-, and glucocorticoid-responsive genes (*1*). The estrogen-responsive element (ERE) is similar to, but distinct from, the glucocorticoid-responsive element (GRE) and the thyroid hormone–responsive element (T3RE) (*3–5*). The progesterone- and glucocorticoid-responsive elements are so similar as to be functionally identical (*1, 6*). $1,25(OH)_2D_3$ affects cellular replication and function in many cells and tissues through a typical steroidal hormone receptor (*7*) and is also thought to exert its effects through a vitamin D–responsive element (VDRE). It is not clear whether opposing effects on gene expression by different steroidal hormones are elicited through the same or different HREs.

Osteocalcin is the major noncollagenous bone protein, and its expression is stimulated by $1,25(OH)_2D_3$ and inhibited by glucocorticoids (*8*). We have therefore used promoter expression constructs of the human osteocalcin gene to investigate the influence of $1,25(OH)_2D_3$ and glucocorticoids on the activity of the osteocalcin promoter. We developed transient and stable transfection systems in a rat osteoblastic sarcoma cell line, ROS 17/2.8, which has an osteoblast (bone-forming cell) phenotype, expresses osteocalcin, and mineralizes in vitro (*9*). The promoter construct pOSCAT1 was made by exchanging the thymidine kinase (TK) promoter (*10*) of pTKCAT (*4*) for the −344- to +34-bp region of the osteocalcin gene (Fig. 1A), such that the osteocalcin promoter drives the bacterial chloramphenicol acetyl transferase (CAT) gene (Fig. 1B) (*11*). In ROS 17/2.8 cells, the promoter activity of pOSCAT1 was comparable to that of pTKCAT under the same conditions, that is, 30 times greater than background level as measured with a nonchromatographic CAT assay (*12*). This result demonstrated that the −344-bp region of the promoter was capable of directing significant CAT activity in the transfection system. However, pOSCAT1-derived CAT activity was not induced by $1,25(OH)_2D_3$, estrogen, synthetic progestin [Org 2058 (Organon International)], or thyroid hormones, but was repressed by 20 to 40% by $10^{-6}M$ dexamethasone. Dexamethasone had no effect on CAT activity derived from pTKCAT under the same conditions, indicating that the repression of pOSCAT1 by glucocorticoids is specific to the osteocalcin promoter and not an attribute of the parent plasmid.

In contrast to the lack of induction of pOSCAT1 by $1,25(OH)_2D_3$, the longer construct pOSCAT2, which contains an extra 1.0 kilobase pairs (kb) of further upstream sequences (Fig. 1B), showed a marked induction when transfected cells were treated with $1,25(OH)_2D_3$ at $10^{-7}M$. This induction was fivefold (5.0 ± 0.8) in the ROS 17/2.8 cells and up to 40-fold in the SAOS-2 human osteosarcoma cell line (*13*). Recently other workers, who have suggested that an equivalent 600- to 1000-bp region in the rat osteocalcin gene pro-

Garvan Institute of Medical Research, St. Vincents Hospital, Sydney, NSW 2010, Australia.

*To whom correspondence should be addressed.

moter contains the VDRE, have found similar levels of induction with $1,25(OH)_2D_3$ (*14, 15*). In order to evaluate the specificity and sensitivity of the regulation of this promoter, we have assessed the dose responses and actions of analogs and metabolites of both $1,25(OH)_2D_3$ and glucocorticoids. The induction of pOSCAT2 by $1,25(OH)_2D_3$ was dose-dependent (Fig. 2A), being half maximal at $10^{-10}M$ and saturated at $10^{-8}M$ $1,25(OH)_2D_3$, consistent with the dissociation constant ($5 \times 10^{-11}M$) of the vitamin D receptor for this ligand in ROS 17/2.8 cells. Different metabolites of vitamin D_3 (Fig. 2A) induced pOSCAT2-derived CAT activity to a degree consistent with their known affinities for the vitamin D receptor (*16*), suggesting that the induction is receptor mediated. Basal promoter activity of pOSCAT2 was about eight times higher than that of pOSCAT1, after normalization for transfection efficiency by co-transfection with a β-galactosidase expression vector (pCH110; Pharmacia). Thus the 1.0-kb fragment inserted into pOSCAT1 in the construction of pOSCAT2 contains sequences that boost basal promoter activity as well as confer $1,25(OH)_2D_3$ responsiveness. Dexamethasone treatment of pOSCAT2-transfected cell resulted in the same repression (20 to 40%) of CAT activity as seen for pOSCAT1; repression was also seen to a lesser extent with other glucocorticoids. Other hormones involved in calcium and bone homeostasis, including parathyroid hormone, calcitonin, human growth hormone, and thyroid hormone [for which receptors have recently been described in ROS cells (*17*)] had no effect. Estrogen, for which receptors have been described in bone cells (*18*) and which affects osteocalcin levels in vivo (*19*), did not affect CAT activity. The synthetic progestin also was without effect on this promoter. Interestingly, the large construct pOSCAT2 also responds to retinoic acid and retinol, but not through the VDRE. pOSCAT2 includes a region of the promoter (from −834 to −775 bp), which affects both basal promoter activity and the magnitude of induction to $1,25(OH)_2D_3$ and retinoic acid. This region contains the "GAGA" DNA motif AGGAGAA, suggested by Yoon *et al.* (*14*) to control $1,25(OH)_2D_3$ responsiveness of the rat osteocalcin gene on the basis of comparisons with other responsive genes. However, constructs such as pOSCAT4 (Fig. 1) containing this region, but not the VDRE, are not induced by $1,25(OH)_2D_3$; therefore this region cannot contain another specific VDRE.

Serum osteocalcin levels are reduced in patients treated with glucocorticoids, so we examined the effect of dexamethasone on osteocalcin promoter activity. Although this glucocorticoid had only a modest repressive effect (20 to 40%) on the basal activity of the osteocalcin promoter constructs, pOSCAT1 and pOSCAT2 (Fig. 2, B and C), it almost completely antagonized the induction of pOSCAT2 by $1,25(OH)_2D_3$ (Fig. 2B). Dexamethasone at $10^{-6}M$ consistently reduced induction by $1,25(OH)_2D_3$ ($10^{-9}M$) to values between 1.8 and 0.5 times that of the control. This repression was dose-dependent (Fig. 2B), being half maximal at about $10^{-9}M$ [consistent with the $1.4 \times 10^{-9}M$ dissociation constant of the glucocorticoid receptor (*20*)], and saturated at $10^{-6}M$, a concentration within the pharmacological range of corticosteroid therapy. $1,25(OH)_2D_3$ could partially reverse the repression of pOSCAT2 by dexamethasone at 10^{-9} and $10^{-8}M$ (Fig. 2B). However, even high concentrations of $1,25(OH)_2D_3$ could not reverse the repression by dexamethasone at $10^{-7}M$, suggesting a noncompetitive interaction. These results suggest that the low circulating serum osteocalcin levels associated with glucocorticoid excess could be explained by a direct influence of the hormone on the osteocalcin gene promoter, resulting in a profound antagonism of the normal function of active vitamin D_3 metabolites to induce the gene. Since pOSCAT1 is repressed by dexamethasone and pTKCAT is not, these data suggest that sequences in the −344- to +34-bp region of the osteocalcin promoter are involved in glucocorticoid repression. Dexamethasone treatment of cells transfected with pOSCAT4, which contains sequences up to −196 bp of the proximal part of the promoter (Fig. 1), resulted in similar repres-

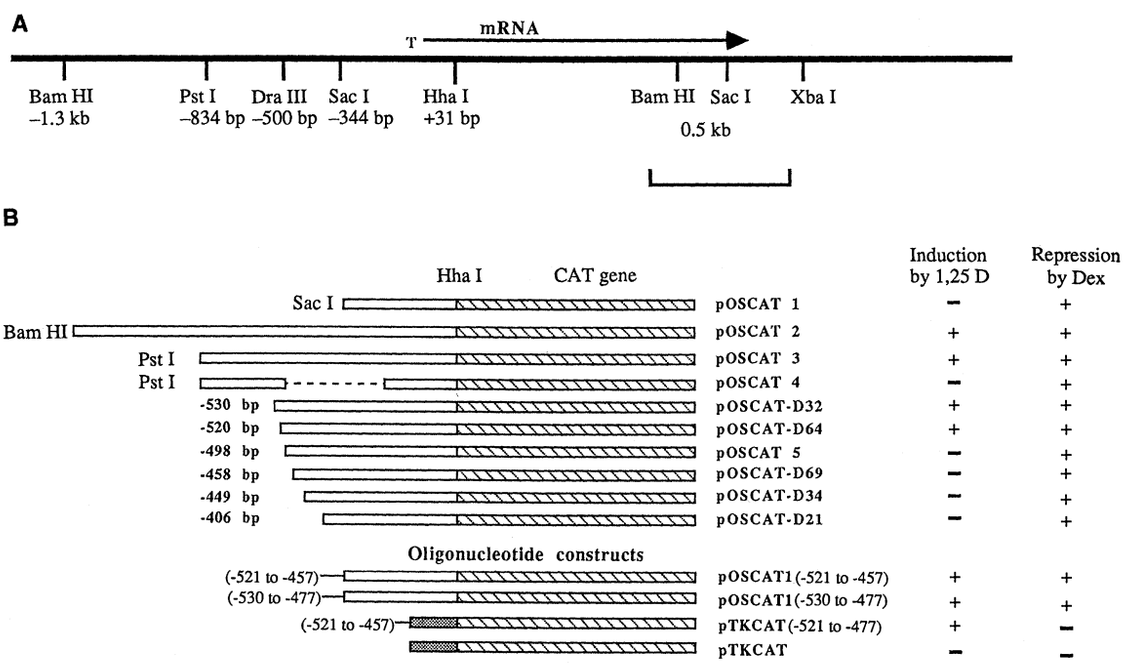

Fig. 1. Construction of an osteocalcin promoter CAT expression vectors and mapping of HREs. (**A**) Restriction map of the human osteocalcin gene isolated from a library of leukocyte DNA cloned in EMBL3 bacteriophage vector (Clontech) showing TATA (T) box, direction of mRNA synthesis (arrow), and relevant restriction sites. Numbers refer to the positions of restriction sites used to make expression constructs. Labeled overlapping oligonucleotide pairs matching the osteocalcin coding region were used to isolate clones that corresponded to the restriction map of Celeste *et al.* (*24*). (**B**) Expression vector constructs of the osteocalcin gene promoter. Osteocalcin-CAT expression vectors were made by replacing the TK promoter of the plasmid pTKCAT with the osteocalcin promoter (*25*). Relevant restriction sites used to make the constructs are indicated, as are the end points of constructs derived from unidirectional deletion. The dotted line in pOSCAT4 indicates an internal deletion. Oligonucleotide constructs containing the VDRE are shown, and their responses to $1,25(OH)_2D_3$ (1,25 D) and dexamethasone (Dex) are indicated as (+) responsive or (−) unresponsive.

sion to that seen with pOSCAT1. These data define sequences controlling the glucocorticoid repression of both basal activity and the $1,25(OH)_2D_3$ mediated up regulation to the region from −196 bp to the transcriptional start site. Computer searches of consensus GREs reveal a sequence similar to a GRE overlapping the TATA box; it is possible that glucocorticoids could modulate gene transcription via this region.

In order to evaluate the mechanism of induction by $1,25(OH)_2D_3$ and the repression by glucocorticoids, we identified the specific sequence comprising the VDRE by deletion and reconstruction analyses. Since the smaller construct, pOSCAT3 (Fig. 1), maintained responsiveness to $1,25(OH)_2D_3$, a series of nested deletions with end points between the Pst I and Sac I sites (Fig. 3) were transfected into ROS 17/2.8 cells and tested for $1,25(OH)_2D_3$ induction and dexamethasone repression of induction. When 59 bp were deleted in from the Pst I site (Fig. 3, deletion 9 at −775 bp), the basal promoter activity (measured by pCH110 co-transfection) was halved and the magnitude of $1,25(OH)_2D_3$ induction dropped by about one-third. From subsequent deletions, the specific VDRE was defined as lying between deletion 64 (−520 bp), which maintained induction, and the first deletion (69, −458 bp), which lost $1,25(OH)_2D_3$ responsiveness (Fig. 3). Two further constructs were produced from pOSCAT3 by digesting in both directions from a Dra III site (−504 to −496 bp) close to the center of this sequence. Inducibility by $1,25(OH)_2D_3$ was lost in both of these constructs that contained sequences upstream (pOSCAT4, −501 to −196 bp deleted) or downstream (pOSCAT5, −834 to −498 bp deleted) from the Dra III site (Fig. 1). The pOSCAT5 (−498 bp) end point is 22 bp downstream from the end point of the last deletion that preserves inducibility (deletion 64, −512 bp), indicating a 22-bp "essential" region that must include or be part of the VDRE. The entire 62-bp sequence between deletions 64 and 69 was synthesized and inserted as a single copy at a Hind III site upstream of pOSCAT1, which does not respond to $1,25(OH)_2D_3$. The subsequent construct [pOSCAT1 (−521 to −457 bp)] gave fivefold more induction (5.0 ± 0.1) over basal when transfected ROS 17/2.8 cells were treated with $1,25(OH)_2D_3$. A shorter oligonucleotide construct pOSCAT1 [−530 to −477 bp] gave similar results (Fig. 1), localizing the VDRE to a 44-bp region. The 62-bp sequence (−521 to −457 bp) was inserted at a Hind III site upstream of the TK promoter in the $1,25(OH)_2D_3$–unresponsive pTKCAT, which has no function in bone or calcium homeostasis, to produce pTKCAT (−521 to −457 bp). The CAT activity encoded by this construct was induced by $1,25(OH)_2D_3$ both in ROS 17/2.8 cells (2.0 ± 0.1 fold) and in the $1,25(OH)_2D_3$-responsive human breast cancer cell line MCF-7 (3.8 ± 0.3 fold) (Fig. 2C). In both ROS 17/2.8 and MCF-7 cells, this construct was unresponsive to estrogen, progestin, thyroid hormone (T3), or retinoic acid (all at $10^{-6}M$). Thus a single copy of the VDRE derived from the osteocalcin promoter clearly imparts $1,25(OH)_2D_3$ responsiveness in gene constructs and cell lines with no relation to any bone-derived source, and responds only to vitamin D metabolites.

Importantly, the same pTKCAT-derived construct pTKCAT (−521 to −457 bp) transfected into ROS 17/2.8 or MCF-7 cells was unresponsive to dexamethasone at either basal levels or after induction by $1,25(OH)_2D_3$ (Fig. 2C). By contrast, this

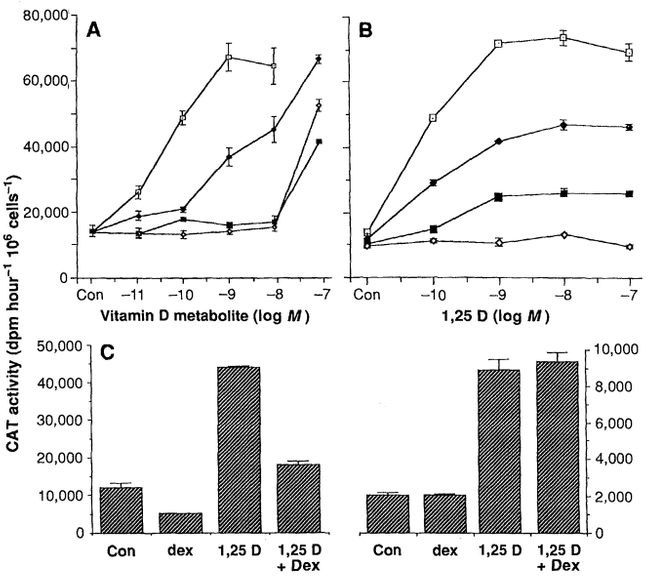

Fig. 2. Regulation by vitamin D_3 metabolites and dexamethasone (Dex) of the osteocalcin promoter expression vector transfected into rat osteoblastic sarcoma cells (*26*). (**A**) Dose response of induction of CAT activity from pOSCAT2 when transfected cells were treated with $1,25(OH)_2D_3$ (□), 1,24, 25-trihydroxyvitamin D_3 (◆), 25-hydroxyvitamin D_3 (◇), and 24,25-dihydroxyvitamin D_3 (■). Con, control. (**B**) Dexamethasone repression of $1,25(OH)_2D_3$-mediated induction of the human osteocalcin promoter. pOSCAT2-transfected ROS 17/2.8 cells were treated simultaneously for 24 hours with $1,25(OH)_2D_3$ and dexamethasone. 50% effective dose, about $10^{-9}M$. Dexamethasone concentrations: □, none; ◆, $10^{-9}M$; ■, $10^{-8}M$; ◇, $10^{-6}M$. (**C**) Effects of dexamethasone ($10^{-6}M$) on basal and $1,25(OH)_2D_3$-induced ($10^{-8}M$) CAT activity of pOSCAT1 (−521 to −457 bp) transfected into ROS 17/2.8 (left) and pTKCAT (−521 to −457 bp) transfected into MCF 7 cells (right). Both basal- and $1,25(OH)_2D_3$-induced activity of pOSCAT1 (−521 to −457 bp) were repressed by dexamethasone, whereas neither basal nor $1,25(OH)_2D_3$-induced activity of pTKCAT (−521 to −457 bp) was affected by dexamethasone.

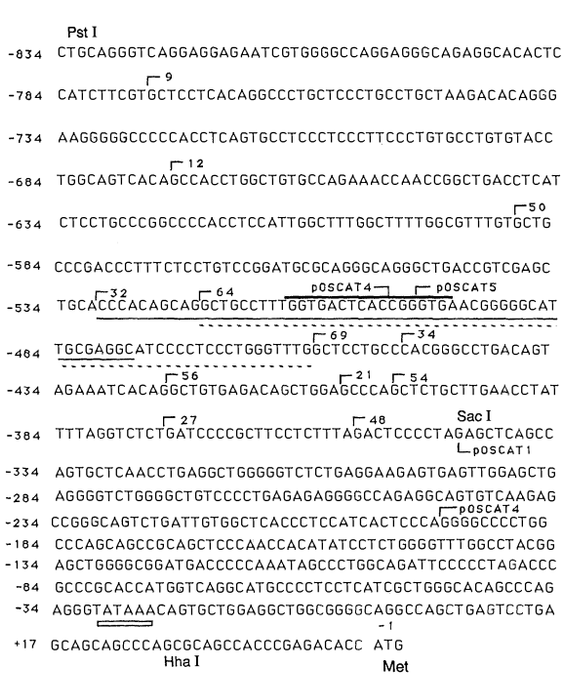

Fig. 3. Localization of HRE by sequence and deletion analysis. DNA sequence of the osteocalcin promoter from the Pst I site at the end of the promoter fragment in pOSCAT3, numbered from the putative transcription start site. Hha I, Sac I, and Pst I sites, which define pOSCAT1 and pOSCAT3, are shown. Palindromic sequences of the VDRE are overlined. The TATA box is indicated by the elongated open box. End points of deletions are indicated above the sequence. The sequences −521 to −457 and −530 to −477 are indicated by dashed and solid underlines, respectively.

same sequence inserted into pOSCAT1 [pOSCAT1 (−521 to −457 bp)] maintained dexamethasone inhibition of basal activity and $1,25(OH)_2D_3$ induction (Fig. 2C). Thus the glucocorticoid response is not mediated via the VDRE sequence but, as suggested from the glucocorticoid repression of the pOSCAT series above, is mediated through a sequence element in the more proximal section of the osteocalcin gene promoter (that is, the −196 to start-site region).

Steroid HREs identified to date are characterized by the presence of palindromic structures with the two halves of a palindrome of 5 or 6 bp separated by an intervening three-bp gap (ERE, PRE, and GRE) or by more than three bp, as in the T3RE (*5*). Since pOSCAT2 does not respond to estrogen, thyroid hormone, progestin, or glucocorticoid, the VDRE is specific for vitamin D. However, as the DNA binding domains of steroid receptors and their corresponding HREs exhibit substantial homology, we examined the VDRE for similarity with other HREs. The 44-bp region that includes the VDRE contains a complex palindromic sequence TTGGTGACTCACCGGGTGAAC, starting at −511 bp in the osteocalcin promoter, with similarity to both the consensus ERE sequence GGTCANNNTGACC (*1*) and a synthetic T3RE sequence GGTCANTGACC (*21*). This palindromic structure has strong homology to other steroid HREs, differing by nucleotide spacing between arms of the palindrome (1 versus 3 nt) or in the fourth nucleotide in the arm of the palindrome (G for C), or both. The elements that mediate the specificity of this responsive element are unknown. The equally spaced repeats of the GGTGA motif creates a double palindrome, 13 bp of which is in the 22 bp "essential" region identified by the loss of inducibility between deletion 64 and pOSCAT5. pOSCAT4 (−501 to −196 bp), which contains only 10 bp of the complex palindrome, is not inducible. No other palindromes exist in this DNA region. These data suggest that the palindrome, together with limited flanking sequences (10 bp upstream and 18 bp downstream), is sufficient for conferring $1,25(OH)_2D_3$ inducibility as a single copy. Kerner *et al.* (*22*) have also reported a VDRE in the human osteocalcin gene. The shorter sequence they identified comprises the 3′ 15 bp of the VDRE reported here and required multiple copies for restoration of full $1,25(OH)_2D_3$ inducibility in their system. Interestingly, the double palindrome contains a consensus AP1 binding site TGACTCA (*23*), suggesting possible interaction with other transcriptional activators.

The sequence of VDRE reflects the distinct roles this hormone has in cellular replication and function, as well as in bone and calcium metabolism. Thus, the VDRE palindrome is homologous to other HREs but does not respond to glucocorticoids, estrogen, progestin, retinoic acid, or thyroid hormone. Since one of the major modes of action of glucocorticoids is the repression of certain physiological responses, the mechanism of glucocorticoid repression of the osteocalcin promoter is of general significance to the actions of steroid hormones. The potential glucocorticoid receptor binding site overlapping the TATA box may represent a novel mechanism of steroidal hormone regulation of gene expression.

REFERENCES AND NOTES

1. K. R. A. Yamamoto, *Rev. Genet.* **19**, 209 (1985).
2. S. Mader, V. Kumar, H. de Verneuil, P. Chambon, *Nature* **338**, 271 (1988).
3. R. M. Evans, *Science* **240**, 889 (1988).
4. G. Klock, U. Strahle, U. Schutz, *Nature* **329**, 734 (1987).
5. C. K. Glass *et al.*, *ibid.*, p. 738.
6. A. C. B. Cato, D. Henderson, H. Ponta, *EMBO J.* **6**, 363 (1987); J. Ham, A. Thomson, M. Needham, P. Webb, M. Parker, *Nucleic Acid Res.* **16**, 5263 (1988).
7. J. A. Eisman, T. J. Martin, I. MacIntyre, J. M. Moseley, *Lancet* **ii**, 1335 (1979); D. M. Findlay *et al.*, *Cancer Res.* **40**, 4764 (1980); H. C. Freake *et al.*, *Biochem. Biophys. Res. Commun.* **101**, 1131 (1981); R. J. Frampton, S. A. Omond, J. A. Eisman, *Cancer Res.* **43**, 4443 (1983); Z. Bar-Shavit *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5907 (1983); J. M. Lemire, J. S. Adams, R. Sakai, S. C. Jordon, *J. Clin. Invest.* **74**, 657 (1984); C. D. Tsoukas, D. M. Provvedini, S. C. Manolagas, *Science* **224**, 1438 (1984); K. Colston, M. J. Colston, D. Feldman, *Endocrinology* **108**, 1083 (1981); P. H. Reitsma *et al.*, *Nature* **306**, 492 (1983); Y. Honma *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 201 (1983); J. A. Eisman *et al.*, *J. Cell. Physiol.* **138**, 611 (1989); W. F. Rigby, R. J. Noelle, K. Krause, M. W. Fanger, *J. Immunol.* **135**, 2279 (1985); G. P. Studzinski, A. K. Bhandal, Z. S. Brelvi, *Cancer Res.* **45**, 3898 (1985); A. R. Baker *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3294 (1988).
8. P. A. Price, A. S. Otsuka, J. W. Poser, J. Kristaponis, N. Raman, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1447 (1976); P. A. Price and S. K. Nishimoto, *J. Clin. Invest.* **66**, 878 (1980); I. R. Reid *et al.*, *J. Clin. Endocrinol. Metab.* **62**, 379 (1986); P. Price and S. A. Baukol, *Biochem. Biophys. Res. Comm.* **99**, 928 (1981); P. Price and S. A. Baukol, *J. Biol. Chem.* **255**, 11660 (1980).
9. G. A. Rodan and S. B. Rodan, in *Advances in Bone and Mineral Research, Annual II*, W. A. Peck, Ed. (Excerpta Medica, London, 1984), p. 244.
10. S. L. McKnight and R. Kingsbury, *Science* **217**, 316 (1982).
11. C. M. Gorman, L. F. Moffat, B. H. Howard, *Mol. Cell. Biol.* **2**, 1044 (1982).
12. M. J. Sleigh, *Anal. Biochem.* **156**, 251 (1986).
13. S. B. Rodan *et al.*, *Cancer Res.* **47**, 4961 (1987).
14. K. Yoon, S. J. C. Rutledge, R. F. Buenaga, G. A. Rodan, *Biochemistry* **27**, 8521 (1988).
15. M. B. Demay, D. A. Roth, H. M. Kronenberg, *J. Biol. Chem.* **264**, 2279 (1989); J. Lian *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1143 (1989).
16. J. A. Eisman and H. F. DeLuca, *Steroids* **30**, 245 (1977).
17. K. Sato, D. C. Han, Y. Fujii, T. Tsushima, K. Shizume, *Endocrinology* **120**, 1873 (1987).
18. E. F. Eriksen *et al.*, *Science* **241**, 84 (1988); B. S. Komm *et al.*, *ibid.*, p. 81.
19. P. J. Kelly, N. A. Pocock, P. N. Sambrook, J. A. Eisman, *J. Clin. Endocrinol. Metab.*, in press.
20. M. R. Haussler, S. C. Manolagas, L. J. Deftos, *Biochem. Biophys. Res. Comm.* **94**, 373 (1980).
21. C. K. Glass, J. M. Holloway, O. V. Devary, M. G. Rosenfeld, *Cell* **54**, 313 (1988).
22. S. A. Kerner, R. A. Scott, J. W. Pike, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4455 (1989).
23. M. Neuberg, M. Schuermann, J. B. Hunter, R. Muller, *Nature* **338**, 589 (1989).
24. A. J. Celeste *et al.*, *EMBO J.* **5**, 1885 (1986).
25. The osteocalcin CAT expression vector was made by replacing the TK promoter of the plasmid pTKCAT with the osteocalcin promoter by means of Hha I site 30 bp downstream of the putative transcription initiation site. A 1.3-kb Sac I fragment, containing most of the osteocalcin gene and 392 bp upstream of the start codon, was cloned into pBluescript (Stratagene) and from this a Pst I to Hha I fragment was ligated into pTKCAT, previously digested with Pst I and Bgl II to excise the TK promoter. The Hha I and Bgl II sites were made compatible with an oligonucleotide CCGATC. The subsequent plasmid, pOSCAT1 (verified by restriction, hybridization, and sequence analysis), has no TK promoter sequences and contains the osteocalcin promoter including the TATA and CAT boxes and the osteocalcin mRNA capping site, with the first downstream initiation codon being that of the CAT gene. pOSCAT2 was made by ligating a 1-kb Bam HI to Sac I fragment from the 2.3-kb Bam HI fragment of the genomic clone into pOSCAT1. pOSCAT3 was made by deleting 0.5 kb from the Pst I site in the polylinker of pOSCAT2 to a Pst I site in the promoter fragment. pOSCAT5 was made by cleaving pOSCAT3 with Dra III and Pst I, "blunt ending" with Klenow fragment, and ligating the blunt ends. pOSCAT4 was made by exonuclease III treatment of Dra III–Sau I–cleaved pOSCAT3 before blunt ending and religation. Unidirectional deletions were made after filling in Hind III–digested pOSCAT2 with thiodeoxyadenosine triphosphate and deoxythymidine triphosphate to protect the site from exonuclease III. This DNA was cleaved with Bam HI and, at intervals after addition of exonuclease III, treated with mung bean nuclease, religated, and transformed. Plasmid DNA prepared by alkali lysis and CsCl gradients was sequenced directly.
26. Transfections were done by calcium phosphate coprecipitation [M. Wigler *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1373 (1979)] in ROS 17/2.8 cells cultured in Hams F12 medium (Flow Laboratories) with 5% fetal calf serum (FCS). Two hours before transfection the medium was changed to Dulbecco's modified Eagle's medium with 25 m*M* Hepes, *p*H 7.2, and 2% dextran charcoal–treated FCS. Six hours after adding the DNA precipitate (50 μg in 2 ml), the cells were shocked for 1 min with 15% glycerol and then treated with 0.5 m*M* sodium butyrate (glycerol shock increased transfection efficiency 2.5-fold and butyrate 10-fold). After 16 hours the transfected cells were trypsinized, replated at 2×10^4 cells/cm^2 and, after plating, treated for 24 hours. The cells were harvested by trypsinization, washed in normal saline, and resuspended in 0.25*M* tris-HCl, pH 7.8, before lysis by three freeze-thaw cycles in liquid N_2. The brief trypsinization does not reduce CAT activity and improves reproducibility with strongly adherent cells such as ROS 17/2.8. The sensitive nonchromatographic CAT assay of Sleigh (*12*) was linear up to 0.5 unit of CAT activity (Pharmacia) with SEMs between 1% and 8%. CAT activity is expressed as (mean ± SEM) disintegrations per minute per hour per 10^6 cells of ^{14}C-acetyl groups transferred to unlabeled chloramphenicol.

27. Supported by a Centre grant to the Garvan Institute from the National Health and Medical Research Council and a grant from the New South Wales State Cancer Council. We thank R. Vedam for her contributions, M. Sleigh for providing pTKCAT and advice on extraction CAT assays, J. Faraco for help in isolating the osteocalcin genomic clone, G. Petersen for providing thiodeoxyadenosine triphosphate, and S. Rodan for providing RDS 17/2.8 cells.

8 June 1989; accepted 25 September 1989