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$_3$

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ABSTRACT 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$], plays an important role in the regulation of mineral homeostasis. As well as being the major steroid hormone that regulates calcium metabolism, 1,25(OH)$_2$D$_3$ suppresses transcription of the gene encoding parathyroid hormone, a peptide that plays a dominant role in regulating extracellular calcium levels. To identify DNA sequences that may mediate this transcriptional repression, nuclear extracts containing the 1,25(OH)$_2$D$_3$ receptor were examined for binding to sequences in the 5′-flanking region of the human parathyroid hormone gene. A 25-base-pair (bp) oligonucleotide containing the sequences from −125 to −101 from the start of exon 1 binds nuclear proteins recognized by monoclonal antibodies against the 1,25(OH)$_2$D$_3$ receptor. The sequences in this region contain a single copy of a motif (AGGTTCA) homologous to the motifs repeated in the up-regulatory 1,25(OH)$_2$D$_3$-response elements. When placed upstream to a heterologous viral promoter, the sequences contained in this 25-mer oligonucleotide mediate transcriptional repression in response to 1,25(OH)$_2$D$_3$ in GH4C1 cells but not in ROS 17/2.8 cells. This down-regulatory element, therefore, differs from the up-regulatory 1,25(OH)$_2$D$_3$-response elements both in sequence composition and in the requirement for particular cellular factors other than the 1,25(OH)$_2$D$_3$ receptor for repressing transcription.

Parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] both play major roles in regulating calcium homeostasis. PTH regulates the conversion of 25-hydroxyvitamin D to the active metabolite 1,25(OH)$_2$D$_3$. 1,25(OH)$_2$D$_3$, in turn, is important in controlling PTH gene transcription. Silver et al. (1), using an in vivo rat model, have demonstrated a decrease in PTH mRNA levels to <4% of basal 48 hr after i.p. injection of 1,25(OH)$_2$D$_3$. Nuclear run-off studies showed a decrease in PTH gene transcription to 10% of control. Russell et al. (2) have observed similar phenomena in dispersed bovine parathyroid cells.

To address whether the decrease in PTH gene transcription in response to 1,25(OH)$_2$D$_3$ is mediated by upstream regulatory sequences, Okazaki et al. (3) established stable GH4C1 cell lines, expressing a human PTH (hPTH) 5′-regulatory region-neomycin fusion gene. A 60% decrease in reporter-gene mRNA levels was seen in response to 1,25(OH)$_2$D$_3$. This effect was mediated by 684-base pairs (bp) of the 5′-flanking region of the hPTH-encoding gene. Absence of a cell line that expresses PTH in a 1,25(OH)$_2$D$_3$-responsive fashion has hampered subsequent identification of the bases in the PTH gene that mediate the transcriptional effects of 1,25(OH)$_2$D$_3$. Other investigations have focused on identifying sequences in the upstream regulatory region of the PTH gene that can bind the 1,25(OH)$_2$D$_3$ receptor. Farrow et al. (4) demonstrated that sequences in the bovine PTH gene, between −485 and −100 could bind a protein that comigrated with the 1,25(OH)$_2$D$_3$ receptor on Southern/blotting blots.

Transcription of the osteocalcin, osteopontin, and calbindin-D9K genes is induced by 1,25(OH)$_2$D$_3$. Furthermore, the sequences in these genes that can bind the 1,25(OH)$_2$D$_3$ receptor and mediate its effects on gene transcription have been identified (5–9). With the assay developed for studying 1,25(OH)$_2$D$_3$-receptor binding to the 1,25(OH)$_2$D$_3$-response element of rat osteocalcin gene (5), the 5′ regulatory sequences in the hPTH gene were examined for their ability to compete with the rat osteocalcin 1,25(OH)$_2$D$_3$-response element for receptor binding. A 25-base-pair (bp) oligonucleotide, which contains sequences −125 to −101 from the start of exon I of the hPTH gene (10) could compete with the rat osteocalcin 1,25(OH)$_2$D$_3$-response element for receptor binding. Gel-retardation assays showed direct binding of this hPTH oligonucleotide to proteins recognized by monoclonal antibodies against the 1,25(OH)$_2$D$_3$ receptor (11). Examination of the bases in this sequence reveals one exact copy of a sequence repeated in the 1,25(OH)$_2$D$_3$-response element of the mouse osteopontin gene (7). When placed upstream from the herpes simplex virus thymidine kinase promoter, this oligonucleotide could confer down-regulation of chloramphenicol acetyltransferase (CAT) activity in response to 1,25(OH)$_2$D$_3$ after transfection into GH4C1 cells.

MATERIALS AND METHODS

Generation of DNA for Competition Assays. The 748-bp Bgl II fragment of the hPTH gene (10) was inserted into the BamHI site of pUC18. Restriction fragments were purified on agarose gels and isolated by DEAE-membrane (Schleicher & Schuell) interception. Specific sequences of interest were amplified by the PCR, with the previously mentioned plasmid as a template. PCR products were purified by using Mermaid (Bio 101, La Jolla, CA), quantitated, and used in competition assays. Control PCR reactions were done without template and treated similarly. Synthetic oligonucleotides were synthesized on an Applied Biosystems model 380A synthesizer.

Gel-Retardation Assays. Assessment of DNA binding to the 1,25(OH)$_2$D$_3$ receptor was done as described (5). Porcine intestinal nuclear extract was used as a source of 1,25(OH)$_2$D$_3$ receptor. Monoclonal antibodies against the 1,25(OH)$_2$D$_3$ receptor (11) were used to identify specific bands that contain receptor protein. Oligonucleotide probes were labeled by filling in recessed ends with the large fragment of DNA polymerase I and [α-32P]dATP.

Abbreviations: 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; PTH, parathyroid hormone; hPTH, human parathyroid hormone; CAT, chloramphenicol acetyltransferase.

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Construction of CAT Fusion Genes. Oligonucleotides were synthesized corresponding to the sequences of interest plus bases to permit subcloning into the BamHI site of PUTKAT3 (from D. Moore, Massachusetts General Hospital). Orientation and copy number of oligonucleotides were determined by DNA sequencing.

Cell Culture and Transfections. GH4C1 cells were maintained in Dulbecco’s modified Eagle’s medium (GIBCO), supplemented with 10% (vol/vol) fetal bovine serum, penicillin, and streptomycin. Twenty-four hours before transfection, cells were fed with medium containing charcoal-stripped fetal bovine serum. Transfections were done by lipofection (GIBCO) with 20 μg of test plasmid used per 100-mm dish. Cells were treated with 10^-8 M 1,25(OH)2D3 immediately after transfection and then treated again the following day. Fresh medium with or without 10^-8 M 1,25(OH)2D3 was added 40 hr after transfection. Cells were harvested 24 hr later. ROS 17/2.8 cells were maintained and transfected as described (5). After normalization for protein content, CAT activity was assessed by TLC (12). Quantitation was done by densitometric scanning of autoradiograms [E-C densitometer, CE-C Apparatus (St. Petersburg, FL), with a Hewlett-Packard 3390A integrator].

DNA Sequencing. All DNA sequencing was done by the dideoxynucleotide chain-termination method after subcloning into M13 vectors (13).

RESULTS

The 748-bp Bgl II fragment from the hPTH gene, which included sequences capable of conferring 1,25(OH)2D3 down-regulation in stably transfected rat pituitary GH4C1 cells (3), was digested with HindIII to yield two fragments, a 5’ 122-bp fragment and a 3’ 626-bp fragment. A 50-fold molar excess of the 3’ fragment competed with the osteocalcin 1,25(OH)2D3 response element for receptor binding, whereas the same molar excess of the 5’ fragment did not compete (Fig. 1). This result suggests that sequences that compete for 1,25(OH)2D3 receptor binding are present in the 626-bp HindIII-Bgl II fragment. PCR was then used to generate shorter regions within this fragment. The resultant products were used as competitors of 1,25(OH)2D3 receptor binding. Control PCR products generated in the absence of template did not inhibit receptor binding. Analysis of receptor-binding inhibition by the PCR products (Table 1) confirmed that this region contained sequences that competed for 1,25(OH)2D3-receptor binding. The shortest sequence that inhibited receptor binding extended from -135 to -54. Sequences between -75 and +5 did not compete for binding. Overlapping oligonucleotides corresponding to the sequences from -175 to -65 were synthesized and tested for their ability to inhibit 1,25(OH)2D3 receptor binding (for results of these competition assays, see Fig. 2A). Large molar excesses of all three oligonucleotides competed for receptor binding, although similar amounts of a control oligonucleotide (AP-1) did not compete. Direct binding assays using these oligonucleotides as radioactive probes revealed numerous poorly resolved bands on gel-retardation assays. To clarify the interaction of these sequences with the 1,25(OH)2D3 receptor, shorter oligonucleotides representing their regions of overlap were synthesized and examined for their ability to inhibit 1,25(OH)2D3 receptor binding. Only one of these oligonucleotides, P15 (-125 to -101), competed for binding to the 1,25(OH)2D3 receptor (Fig. 2B).

To establish more directly that the 1,25(OH)2D3 receptor bound to P15, this oligonucleotide was used as a radioactive probe in gel-retardation assays, and the interaction of the shifted bands with monoclonal antibodies against the 1,25(OH)2D3 receptor was examined. Higher-resolution gels revealed that the lower band generated by the osteocalcin 1,25(OH)2D3 response element was, in fact, a doublet and that only the lower of these two bands was a 1,25(OH)2D3 receptor-dependent (Fig. 3, osteocalcin). Migration of the uppermost band (A), as well as the lowest band (B), was retarded by adding XVE110 (11), a monoclonal antibody against the porcine 1,25(OH)2D3 receptor (antibody 1). Addition of VIIIID6C12 and VD2F12, antibodies that interact with the DNA-binding domain of the receptor (11), decreases the intensity of these bands, presumably by competing with the DNA probe for 1,25(OH)2D3-receptor binding (antibodies 2 and 3). Addition of a nonspecific antibody of the same species had no effect on the migration of these bands (antibody 4). When P15 was used directly as a ligand for 1,25(OH)2D3 receptor binding, two bands were seen that comigrated with bands A and B of the osteocalcin 1,25(OH)2D3 response element (Fig. 3, hPTH). Like the complexes generated with the osteocalcin probe, the migration of these two bands was retarded by adding XVE110. Also, addition of VIIIID6C12 and VD2F12, the antibodies that interact with the DNA-binding domain of the receptor, decreased the intensity of these bands, whereas the nonspecific mouse immunoglobulin had no effect on their intensity or migration.

Analysis of the sequences in the P15 oligonucleotide revealed a motif identical to that repeated in the rat osteopontin 1,25(OH)2D3 response element (AGGTTCCTA) (7); however, only one copy, on the antisense strand, was present (Table 2). To address whether this motif played a role in 1,25(OH)2D3 receptor binding, mutations were made in the guanine doublet (M2-P15), as well as in two bases further upstream (M1-P15). Effects of these mutations on the ability of this sequence to compete with the osteocalcin 1,25(OH)2D3-

<table>
<thead>
<tr>
<th>PCR product</th>
<th>Binding inhibition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>-564 to +5</td>
<td>87 ± 0.9</td>
</tr>
<tr>
<td>-175 to +5</td>
<td>93 ± 4.4</td>
</tr>
<tr>
<td>-175 to -54</td>
<td>67 ± 6.2</td>
</tr>
<tr>
<td>-135 to +5</td>
<td>68 ± 4.7</td>
</tr>
<tr>
<td>-135 to -54</td>
<td>78 ± 3.2</td>
</tr>
<tr>
<td>-75 to +5</td>
<td>16 ± 2.5</td>
</tr>
</tbody>
</table>

PCR products corresponding to the sequences indicated were used to compete with the rat osteocalcin 1,25(OH)2D3-response element for binding to the 1,25(OH)2D3 receptor. The numbers indicated reflect percentage decrease in intensity of 1,25(OH)2D3-receptor-dependent bands with addition of the same molar excess of each competitor PCR product. Results represent the mean of three independent experiments, done with products from three separate PCR reactions. The experiments used 500 pg of rat osteocalcin 1,25(OH)2D3-response element as a radiolabeled probe and 1000-fold molar excess of each of the PCR products. Relative intensities of both 1,25(OH)2D3-receptor-dependent bands were equally affected. Control PCR products generated without template had no effect on the bands generated.
response element (Fig. 4A) and with P15 (Fig. 4B) were then assessed. The mutations in the guanine doublet (m2-P15) abolished the ability of this sequence to compete with both the osteocalcin 1,25(OH)₂D₃-response element (Fig. 4A) and with P15 (Fig. 4B) for receptor binding. The mutation further upstream (m1-P15) had a modest effect. In the competition assays using P15 as a probe, only the 1,25(OH)₂D₃ receptor-associated bands were inhibited by the rat osteocalcin 1,25(OH)₂D₃-response element (Fig. 4B).

The affinity of P15 for the 1,25(OH)₂D₃ receptor seems at least an order of magnitude less than that of the osteocalcin sequence 9D3 (Fig. 4A and B). This apparent decrease in affinity may reflect an inherently altered affinity of DNA for the receptor or may reflect the relative abundance and affinity of other proteins in the nuclear extract that interact with P15 and decrease its ability to compete with 9D3 for receptor binding.

To establish that binding of the 1,25(OH)₂D₃ receptor to these oligonucleotides from the hPTH gene is functionally relevant, transfection experiments were undertaken to show that these sequences mediate transcriptional responsiveness to 1,25(OH)₂D₃. Because no available cell lines express the PTH gene in a 1,25(OH)₂D₃-responsive fashion, two surrogate cell lines were chosen: the rat osteosarcoma cell line ROS 17/2.8, which expresses many 1,25(OH)₂D₃ receptors (14), and GH4C1 cells, which, in stable transfection experiments, demonstrate transcriptional repression of the hPTH gene in response to 1,25(OH)₂D₃ (3).

P15, the 25-bp oligonucleotide that directly binds the vitamin D receptor, and P12, the 54-bp oligonucleotide that contains the sequences present in P15, were placed upstream of the herpes simplex virus thymidine kinase promoter and examined for their ability to modulate CAT activity in response to 1,25(OH)₂D₃. Transfection experiments in ROS 17/2.8 cells revealed no significant 1,25(OH)₂D₃ responsiveness (Table 3). After transfection into GH4C1 cells, however, both P12 and P15 oligonucleotides could repress gene transcription in response to 1,25(OH)₂D₃. Although other regions of the hPTH gene may interact with the 1,25(OH)₂D₃ receptor, its interactions with the sequences in P15 are likely to play a significant role in transcriptional repression of the hPTH gene by 1,25(OH)₂D₃.

**DISCUSSION**

Sequences that can bind the 1,25(OH)₂D₃ receptor and mediate its up-regulatory effects on gene transcription have been identified in the rat osteocalcin (5, 8), human osteocalcin (6), mouse osteopontin (7), and rat calbindin-D9K (9) genes. The sequences in the hPTH gene that can repress gene transcription in response to 1,25(OH)₂D₃ share some homology with the sequences found in the up-regulatory elements. One notable difference, however, is that the up-regulatory elements all contain two direct repeats of a motif resembling those found in other steroid-respons eelements; only one
such motif is apparent in the hPTH sequence described. Further investigations will be necessary to determine whether the single motif (AGGTTTCA) from the hPTH gene is sufficient for specific interactions with the 1,25(OH)₂D₃ receptor. It seems unlikely that a single motif can distinguish the 1,25(OH)₂D₃ receptor from the retinoic acid and thyroid hormone receptors because the spacing between two direct repeats of a similar motif determines the binding specificity of that sequence (15). The sequences that flank the motif in P15 may, therefore, be critical in its interactions with the 1,25(OH)₂D₃ receptor. The flanking sequences, as well as the motif itself, are well preserved in both the human (10) and bovine (16) PTH genes. Allowing for insertion of a single base, followed by a single-base deletion five bases downstream, 21 out of 25 bases from the corresponding bovine sequences are identical to those in P15, and 45 out of 53 are identical to those in P12. This conservation across species gives further credence to the suggestion that the sequences flanking the motif interact with proteins important in the transcriptional regulation of the PTH gene by 1,25(OH)₂D₃.

Although the sequences described above (P12 and P15) can bind the 1,25(OH)₂D₃ receptor and mediate transcriptional responsiveness to 1,25(OH)₂D₃, these data do not preclude the possibility that other sequences in the hPTH gene interact with the receptor. Based on the PCR data (Table 1), the current investigations were focused on the more proximal regions of the gene. However, sequences between −564 and −175 or further upstream could also contain 1,25(OH)₂D₃-receptor-binding sites. Furthermore, the ability of P11 to compete with the osteocalcin 1,25(OH)₂D₃-response element for receptor binding remains unexplained. The more proximal sequences in P11 (−145 to −123) are represented in P14, which cannot compete for binding to the 1,25(OH)₂D₃ receptor (Fig. 2 B). More distal sequences in P11 (−175 to −146) may compete for binding to the 1,25(OH)₂D₃ receptor or interfere with other factors necessary for receptor binding.

The mechanism of transcriptional repression by steroid hormones is poorly understood. It is possible that steroid receptors mediate their down-regulatory effects by binding to consensus sequences that differ from those responsible for up-regulation. Binding of steroid receptors to these negative response elements may cause distinctive conformational changes, thereby repressing transcription instead of activating it. Sequences in the preopiomelanocortin gene that mediate down-regulation in response to dexamethasone, for example, bind the glucocorticoid receptor but do not contain a classic glucocorticoid-response element (17). Mutation of these sequences results in a parallel loss of receptor binding and of transcriptional repression, suggesting that they contain a "negative glucocorticoid-response element." The sequences in the hPTH gene that confer down-regulation do differ from the up-regulatory 1,25(OH)₂D₃-response elements in that only a single motif is present. If the sequences flanking this motif contain bases necessary for receptor binding, the sequences in P15 may represent a "negative 1,25(OH)₂D₃-response element." However, because down-regulation is not seen in the ROS 17/2.8 cells, cellular factors other than

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**Table 2. Sequences of hPTH oligonucleotides**

<table>
<thead>
<tr>
<th></th>
<th>−145</th>
<th>−135</th>
<th>−125</th>
<th>−115</th>
<th>−105</th>
<th>−96</th>
</tr>
</thead>
<tbody>
<tr>
<td>P12</td>
<td>CACCCGCCCAA</td>
<td>TGGTTGTTG</td>
<td>TATGTTGTCG</td>
<td>TTTGAACCTA</td>
<td>TAGTTGAGAT</td>
<td></td>
</tr>
<tr>
<td>P15</td>
<td>TATGTTGTCG</td>
<td>ATACACAGAC</td>
<td>AAACTGGGAT</td>
<td>ATCAACTCTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1-P15</td>
<td>TATGTTGTCG</td>
<td>ATACACAGAC</td>
<td>AAACTGGGAT</td>
<td>ATCAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M2-P15</td>
<td>TATGTTGTCG</td>
<td>ATACACAGAC</td>
<td>AAACTGGGAT</td>
<td>ATCAAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The motif homologous to the mouse osteopontin sequence is shown with boldface letters. Mutant bases are shown with lowercase letters.

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**Fig. 4.** Mutations in P15 affect binding to the 1,25(OH)₂D₃ receptor. (A) Using 1 ng of rat osteocalcin 1,25(OH)₂D₃-response element (9D3) as a radioactive probe, abilities of the P15 mutants M1-P15 and M2-P15 to inhibit 1,25(OH)₂D₃-receptor binding are assessed. (B) Using 1 ng of hPTH oligonucleotide P15 as a radioactive probe, abilities of the P15 mutants M1-P15 and M2-P15 to inhibit 1,25(OH)₂D₃-receptor binding are assessed.

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**Table 3. Functional assessment of hPTH oligonucleotides**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Relative stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>P12TKCAT-A</td>
<td>1.17 ± 0.04</td>
</tr>
<tr>
<td>P12TKCAT-B</td>
<td>0.38 ± 0.06</td>
</tr>
<tr>
<td>P15TKCAT-A</td>
<td>0.55 ± 0.04</td>
</tr>
<tr>
<td>P15TKCAT-B</td>
<td>0.35 ± 0.08</td>
</tr>
<tr>
<td>pUTKAT3</td>
<td>0.96 ± 0.08</td>
</tr>
</tbody>
</table>

Oligonucleotides P12 and P15 were placed upstream of the herpes simplex virus thymidine kinase promoter and tested for their ability to modulate CAT activity in response to 10⁻⁸ M 1,25(OH)₂D₃ after transfection into ROS 17/2.8 and GH4C1 cells. Each independent transfection was done in triplicate. Results represent the mean and SEM of three independent transfections. Letters after the oligonucleotide refer to orientation of the oligonucleotide (A, correct orientation; B, opposite orientation); NE, not examined. The parent plasmid (pUTKAT3) is not responsive to 1,25(OH)₂D₃ in either cell line.
the 1,25(OH)$_2$D$_3$ receptor are likely required for down-regulation of the hPTH gene. The requirement of other factors has been described for genes that are transcriptionally down-regulated by the glucocorticoid receptor. Interactions between AP-1 and the glucocorticoid receptor (18–20) determine whether the same glucocorticoid-response element mediates activation or repression: in cells containing both c-fos and c-jun, repression in response to dexamethasone is observed, whereas, in relative c-jun excess, activation is seen (18). Cell-specific transcriptional repression by steroid receptors may also be caused by interference with the binding and/or action of positive transcription factors. This interference is thought to be the mechanism by which the glucocorticoid receptor mediates transcriptional down-regulation of the gene encoding the human glycoprotein α subunit. The glucocorticoid receptor is thought to interact with a proximal region of this gene that contains a tissue-specific enhancer and two functional cAMP-response elements. The receptor has been shown to bind these DNA sequences (21); however, the inhibition of transcription may depend on interactions of the receptor with other transcription factors, rather than on its DNA binding (22).

An accessory factor is thought necessary for high-affinity interactions of the 1,25(OH)$_2$D$_3$ receptor with its up-regulatory-response elements (23–25). Recent characterization of the retinoid X receptor has demonstrated that this protein increases the affinity of the retinoid acid receptor, the thyroid receptor, and the 1,25(OH)$_2$D$_3$ receptor for their respective response elements (26, 27). However, the transcriptional consequences of the retinoid X receptor–1,25(OH)$_2$D$_3$ receptor interactions are modest (26). Other accessory factors may, therefore, be required for interactions of the 1,25(OH)$_2$D$_3$ receptor with its response element. In the context of particular DNA sequences, different accessory factors may interact with the receptor and determine the transcriptional consequences of receptor binding.

The observation of down-regulation by these hPTH sequences in GH4C1 cells raises interesting questions regarding the cell-specificity of factors required for down-regulation in response to 1,25(OH)$_2$D$_3$. This down-regulation suggests that the GH4C1 cell and the parathyroid cell may contain related cell-specific transcription factors not expressed in ROS 17/2.8 cells. The best-characterized pituitary transcription factor, Pit-1, is a member of the POU family of homeodomain proteins (28). Examining the sequences in Pit1 reveals a poorly conserved Pit-1 consensus sequence [A(T/A)(T/ A)ATNCAT] on both the sense (6/10 conserved bases) and antisense (7/10 conserved bases) strands. A POU family member, or unrelated cell-specific transcription factor may be a critical determinant of 1,25(OH)$_2$D$_3$-induced PTH gene down-regulation in the parathyroid gland. Further analysis of the binding and functional properties of mutant P15 oligonucleotides should clarify how cellular factors interact with the 1,25(OH)$_2$D$_3$ receptor to mediate down-regulation of hPTH gene transcription.

We are grateful to Dr. David D. Moore and Dr. J. Larry Jameson for helpful discussions. This work was supported by three grants from the National Institutes of Health (DK36597 and Program Project Grants DK11794 and AM-14881).