Suppression of the Human Parathyroid Hormone Promoter by Vitamin D Involves Displacement of NF-Y Binding to the Vitamin D Response Element

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An earlier report in the literature indicated the vitamin D response element (VDRE) in the human parathyroid hormone (hPTH) promoter could be specifically bound by an unidentified transcription factor in addition to the vitamin D receptor (VDR) complex. We confirmed that OK and HeLa cell nuclear extracts formed a specific complex with the hPTH VDRE that was insensitive to competition with other VDRE sequences. However, this factor could be competed for by a consensus NF-Y DNA-binding site, and an anti-NF-Y antibody was able to supershift the bound band. Mutational analysis indicated that the NF-Y-binding site partially overlapped the 3′ portion of the VDRE. Transfection studies using an hPTH promoter construct in Drosophila SL2 cells demonstrated strong synergistic transactivation by NF-Y interactions with both the VDRE site and a previously described distal NF-Y-binding site. Finally, mobility shift studies indicated that the VDR heterodimer competed with NF-Y for binding to the VDRE sequence, and NF-Y-stimulated activity of the hPTH promoter could be suppressed in a hormone-dependent manner when the VDR heterodimer complex was co-expressed in SL2 cells. In summary, these findings establish the presence of a proximal NF-Y-binding site in the hPTH promoter and highlight the potential for synergism between distal and proximal NF-Y DNA elements to strongly enhance transcription. Furthermore, findings suggest that the repressive effects of vitamin D on hPTH gene transcription may involve displacement of NF-Y binding to the proximal site by the VDR heterodimer, which subsequently attenuates synergistic transactivation.

Despite the clear importance of parathyroid hormone (PTH) in the maintenance of calcium homeostasis, little is known about the activating and repressing factors that control the transcription of this peptide hormone. A conserved cyclic AMP response element in the promoters of the human, bovine, and more recently murine PTH genes has been described previously (1–3). Repressor DNA elements for the VDR have been identified in the human, bovine, rat, and chicken PTH promoters (4–7), although an unknown transcription factor was reported to also bind to the human VDRE (8). Negative calcium response elements have been identified in the hPTH promoter and appear to involve interactions with apurinic/apyrimidinic endonuclease/redox factor 1 (Ape1/Ref1) (9, 10). In addition, a handful of proteins have recently been shown to be important in various stages of parathyroid gland (PTG) development (11–13). Glial cells missing 2 (Gcm2) is a transcription factor in which expression is largely restricted to parathyroid tissue (14), and mice lacking the Gcm2 fail to develop parathyroid glands (15). A homozygous mutation in the human homolog gial cells missing B (GCMB) was identified as the likely cause of a case of isolated familial hypoparathyroidism (16).

We initiated studies of the PTH gene by conducting a DNA sequence comparison of different mammalian promoters to identify conserved regions that might harbor transcription factors involved in regulating promoter activity. In this analysis a highly conserved Sp1 DNA enhancer element was detected in conjunction with high levels of expression of Sp3 and Sp1 in bovine PTG cells (17). More recently we reported finding an NF-Y-binding site unique to the hPTH promoter that partially overlapped the aforementioned Sp1 element and functioned as a potent enhancer of PTH gene transcription (18). Results of the present study demonstrate that a second, proximal NF-Y-binding site is present in the hPTH promoter and partially overlaps with the repressor VDRE. Furthermore, NF-Y binding to both distal and proximal binding sites in the hPTH promotor results in synergistic enhancement of gene activity. The data suggest that one mechanism to account for repression of the hPTH promoter by vitamin D is through displacement of NF-Y binding to the proximal DNA element.

EXPERIMENTAL PROCEDURES

General—All enzymes were purchased from New England BioLabs (Beverly, MA) unless otherwise specified. Protease inhibitor mixture (Complete, Mini) was purchased from Roche Applied Science. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). The sequences (top strand) of the DNA elements used in electrophoretic mobility shift assays (EMSA) were hPTH VDRE, TCAGAT-CTGCTTTGAACCTATAGTTGAGA; human osteocalcin (hOC) VDRE, human VDR; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
bele DNA fragment was gel-purified prior to use in binding reactions, with 100 mM NaOH, 0.1 mM EDTA in 10 mM phosphate buffer at precipitation. Cleavage of the modified DNA was accomplished by treating Acrylamide sections corresponding to bound and free DNA were extracted by initial denaturation at 94 °C for 3 min and then for 30 cycles as follows: 94 °C for 30 s, 56 °C for 15 s, and 72 °C for 45 s. Following isolation of the two separate PCR products, aliquots of each were mixed, and PCR was performed again using the hPTHPromF and hPTHPromR primers under conditions described above to generate the distal NF-Y promoter fragment. The VDR promoter was isolated, digested with BamHI/XhoI, and ligated into the same sites of the luciferase reporter. Preparation of the proximal NF-Y element was similarly prepared and involved the two mutant primers HP-F-NFY-prox-mut (5'-GTCCTGCTTGAACCTAGGAGATC-3') and HP-R-NFY-prox- mutant (5'-GCCTCAAGGAAGATAGTCGTTACGCGGAG-3') (mutations are italicized and underlined). Simultaneous mutation of both NF-Y-binding sites was accomplished by using the NF-Y-distinct promoter construct as the template for PCR reactions using the NF-Y-prox-mut primers as outlined above. All mutant promoters were subject to manual sequencing analysis to verify sequence identity.

Electrophoretic Mobility Shift Assay—The double-stranded oligonucleotide probe for the hPTH VDRE possessed overhanging BglII/XhoI ends and was radiolabeled using Klenow fragment exonuclease 5' to [α-32P]dATP (3000 Ci/mmol) (PerkinElmer Life Sciences). The radiola beled DNA fragment was gel-purified prior to use in binding reactions, which were assembled as described previously (20). Briefly, indicated amounts of nuclear extracts were added to a binding solution (20 μl final volume) (buffer components were 120 mM KCl, 20 mM Tris, pH 7.5, 1.5 mM EDTA, 2 mM dithiothreitol, 10% glycerol, and 1 × protease inhibitor mixture) for 20 min followed by cell disruption with a Teflon Dounce homogenizer. Following a 30-min spin at 100,000 × g, the supernatants were removed, and the nuclear pellets were resuspended in 1 volume of cold high salt buffer (same as above with 400 mM KCl) and incubated on ice for 30 min with occasional gentle mixing. Samples were then spun at 100,000 × g for 20 min, and the supernatant fractions were collected into individual tubes, snap-frozen, and stored at −70 °C prior to use. Extracts of recombinant human VDR (hVDR) and human RXRα (hRXRα) were prepared as described previously (20).

Interference Footprinting—The ethylation interference footprint experiments were performed as described previously (20). Briefly, the hPTH VDRE was subcloned into the BamHI/SalI sites of pTZ19R (Fermentas, Hanover, MD) and excised with the combination of HindIII/EcoRI for the footprinting protocol. Single, 32P-end-labeled DNA probes in 50 μM sodium cacodylate buffer, pH 8.0, were treated with ethylen- tresurosate-saturated ethanol for 20 min at 55 °C. After precipitation with sodium acetate/ethanol and reprecipitation (3 ×), the pellets were washed with cold ethanol, dried, and resuspended in 5 μl of polyacrylamide gel electrophoresis buffer. Gels were transferred and dried, and autoradiography was performed.

Transient Transfection—Drosophila SL2 cells were maintained in Drosophila SL2 medium supplemented with 10% fetal bovine serum at 27 °C. Cells were distributed in 24-well plates on the day before transfection and transfected in triplicate with the indicated hPTH promoter luciferase reporter construct (100 ng) p97β-galactosidase expression vector (50 ng), the indicated pPac expression vectors, and carrier plasmid DNA made up to 500 ng of DNA/well. SL2 cells were transfected using Cellfectin (4 μl/well) (Invitrogen) for 3 h in medium lacking serum followed by supplementation to 7% serum. Where indicated, vitamin D was added to the serum-containing medium. Cultured HeLa and OK cells were similarly prepared and involved the two mutant primers as outlined above. The VDR promoter was isolated, digested with BamHI/XhoI, and ligated into the same sites of the luciferase reporter. Preparation of the proximal NF-Y element was similarly prepared and involved the two mutant primers HP-F-NFY-prox- mut (5'-GTCCTGCTTGAACCTAGGAGATC-3') and HP-R-NFY-prox-mut (5'-GCCTCAAGGAAGATAGTCGTTACGCGGAG-3') (mutations are italicized and underlined). Simultaneous mutation of both NF-Y-binding sites was accomplished by using the NF-Y-distinct promoter construct as the template for PCR reactions using the NF-Y-prox-mut primers as outlined above. All mutant promoters were subject to manual sequencing analysis to verify sequence identity.

Previous work indicated that a transcription factor unrelated to the VDR could also specifically bind to the hPTH VDRE (8). This factor was reportedly present in a variety of cell lines and appeared to bind to the 3' half of the VDRE probe. To begin an analysis of this binding factor, an hPTH VDRE oligonucleotide probe was synthesized (Fig. 1A) that contained the previously described half-site sequence (4) but omitted much of the distal
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Figure 1. EMSA of HeLa and OK cell nuclear extracts binding to hPTH VDRE probe. A, the oligonucleotide sequence used in the binding studies is shown. VDRE half-site sequence is bold underlined. B, binding of HeLa cell nuclear extract (3 μl) to hPTH VDRE is shown. Lanes: 1, control binding; 2, excess hPTH VDRE added; 3, excess hOC VDRE; 4, excess avian PTH VDRE; 5, excess consensus Sp1 element; 6, excess consensus NF-Y element. C, binding of OK cell nuclear extract (3 μl) to hPTH VDRE is shown. Lanes 1–6 are as in B. D, the sensitivity of bound complex from OK nuclear extract to anti-NF-Y B subunit antiserum and normal rabbit serum is shown. B, bound complex; F, free probe; SS, supershifted complex.

To localize the DNA-binding interaction, an ethylation interference experiment was performed using the recombinant heterodimer and hPTH VDRE fragment. As seen in Fig. 2C, strong interference was observed over the top strand sequence 5'-TGA, whereas on the lower strand the interference was limited to the region 5'-GGTT (Fig. 2D). Thus, the footprinting data support the previous observation that the repressor VDRE is localized to the half-site sequence 4'-TGAACC (top strand),
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Table 1

Summary of mutant competition analysis with VDR heterodimer or NF-Y complexes binding to hPTH wild-type probe

| Name                      | DNA sequence | Competes for VDR/RXR | Competes for NF-Y |
|---------------------------|--------------|----------------------|------------------|
| hPTH wild type            | CTGGTTGAACTCTATAGTGAGA | Yes                  | Yes              |
| Mutant 1                  | CTGGTTGAACTCTATAGTGAGA | No                   | No               |
| Mutant 2                  | CTGGTTGAACTCTATAGTGAGA | Yes                  | No               |
| Mutant 3                  | CTGGTTGAACTCTATAGTGAGA | Yes                  | Yes              |
| Mutant 4                  | CTGGTTGAACTCTATAGTGAGA | No                   | Yes              |

although additional weak contacts to other nucleotides outside of this sequence cannot be ruled out.

A series of mutant oligonucleotides were then used as unlabelled DNA competitors in binding experiments to compare and contrast the location of the VDR heterodimer and NF-Y-binding sites in the hPTH VDRE (Fig. 3). In Table I, Mutant 1, which disrupted the 3'-end of the VDRE, prevented competition for both VDR heterodimer and NF-Y binding, indicating that both factors were sensitive to changes in this part of the sequence. Mutations farther toward the 3'-end of the sequence (Mutant 2) prevented competition for NF-Y binding but resulted in a sequence that could now compete for VDR heterodimer binding. Mutant 3 at the 5'-end of the sequence was equally adept at displacing either VDR heterodimer or NF-Y proteins from binding, suggesting that neither complex was sensitive to this region of DNA. Finally, Mutant 4 within the VDRE could not compete for the VDR heterodimer complex but was still capable of displacing NF-Y. Thus, the different competitors localized the binding site for the NF-Y complex to the imperfect CAAT-like sequence, 5'-ACCTATAG, partially overlapping and extending 3' from the VDRE. Meanwhile, the VDR heterodimer was sensitive to changes in the previously recognized half-site VDRE (4), which was consistent with the footprinting data (see Fig. 2).

The above experiments suggested that competition between the VDR and NF-Y complexes might be occurring for binding to the hPTH VDRE. To assess this possibility, mobility shift assays were pursued using OK cell nuclear extracts containing the NF-Y complex together with increasing amounts of added recombinant heterodimer proteins. As seen in Fig. 4, control binding with the OK cell nuclear extract produced the expected strong NF-Y complex with the hPTH VDRE. When increasing amounts of recombinant VDR/RXRα extracts were added, two distinct sets of complexes were observed, NF-Y and the VDR heterodimer. As more of the heterodimer was added to the binding reactions, there was a corresponding increase in the observed bound VDRE complex together with a concomitant decrease in the amount of bound NF-Y. There was no evidence of higher order structures that would be suggestive of simultaneous binding to the same DNA probe by both factors. Thus, the data are consistent with competitive binding by these factors for their respective overlapping binding sites contained within this DNA element.

Previous data from our laboratory (18) had identified an NF-Y-binding site, hereafter referred to as NF-Ydist, which is unique to the hPTH promoter and which partially overlaps the conserved Sp1 DNA element found in a variety of mammalian PTH genes. Strong activation of the hPTH promoter by NF-Y expression was observed in that study, which raised the possibility that this might result from interactions between NF-Y complexes bound to the two sites separated by ~30 bp. To explore this issue, NF-Y activation of the wild-type hPTH promoter (hPTHpluc, see Ref. 18) was compared with analogous mutant promoter constructs that selectively inactivated binding to either NF-Ydist or the newly identified proximal NF-Y-binding site NF-Yprox. A third mutant promoter reporter that simultaneously inactivated binding to both distal and proximal NF-Y elements was also constructed. The transient transfections were carried out in Drosophila SL2 cells that lack endogenous expression of mammalian transcription factors (24) and thus provide a null background for assessing NF-Y interactions with various promoter elements (18, 25–28). As seen in Fig. 5, inclusion of expression vectors for the NF-Y complex resulted in a 38-fold induction of wild-type hPTH promoter activity. When either of the two NF-Y-binding sites was individually mutated, there was an order of magnitude decrease (to <4-fold) in the capacity of NF-Y to stimulate the reporter gene. Furthermore, no enhancement in activity was observed for the promoter containing mutations in both NF-Y-binding sites in response to co-transfection of the NF-Y complex. Thus, the data are consistent with interactions between NF-Y complexes occupying the two DNA elements to drive strong, synergistic transactivation of the hPTH promoter, whereas occupancy of either site alone produced a much more muted enhancement of gene activity.

Based on the ability of the VDR heterodimer complex to compete with NF-Y for binding to the VDRE sequence (Fig. 4), the next set of experiments sought to evaluate whether the VDR complex could suppress NF-Y-stimulated transcriptional activity in SL2 cells. To test this possibility, vectors for expression of hVDR and hRXRα in Drosophila SL2 cells were prepared and simultaneously co-transfected in increasing amounts into cells expressing a constant amount of NF-Y complex and the hPTHpluc reporter. As seen in Fig. 6A, a 1:1 ratio of transfected NF-Y complex and VDR heterodimer expression vectors resulted in a 69% decline in reporter activity in response to hormone. The repression grew to 86% at a 1:3 ratio (NF-Y/VDR heterodimer), which was maintained at the highest (1:10) amount tested. To confirm that this effect was dependent on expression of the VDR heterodimer, individual receptor vectors were analyzed in cells again transfected with the NF-Y complex and hPTHpluc. As seen in Fig. 6B, expression of either hVDR or hRXRα alone had minimal effect on NF-Y-
stimulated gene activity from the hPTH promoter. However, when both VDR and RXRs were simultaneously expressed in SL2 cells, hormone treatment again caused a ~80% decrease in reporter activity.

**DISCUSSION**

The present data affirm an earlier study (8) of an unidentified transcription factor binding to the hPTH VDRE. The identification of this factor as NF-Y is consistent with the previous report describing the presence of the factor in a variety of mammalian cell lines, and there is substantial overlap in the binding sites identified in the former and present studies. However, the possibility cannot be excluded that the factor noted in the previous report is distinct from the NF-Y complex. For example, mutation of the first two nucleotides of the core binding site identified in the earlier report, 5'-TGAACCTAT, appeared to have a significant impact on DNA-binding by the unknown factor but had no effect on NF-Y binding in the mutant oligonucleotide analysis of the present study (see Table I). Thus, although the data presented here clearly support the existence of an imperfect NF-Y-binding site, 5'-ACCTATAG, which partially overlaps with the repressor VDRE in the hPTH promoter, it remains to be seen whether NF-Y is the previously noted unidentified transcription factor.

We identified NF-Y_dist as unique to the hPTH promoter in an earlier report (18), and the present study now extends those results to establish the existence of a second NF-Y-binding site ~30 bp downstream from the former DNA element. A number of mammalian promoters exhibit multiple NF-Y-binding sites, and many of these genes appear to be regulators of the cell cycle (29, 30). It is noteworthy that strong transcriptional synergism with the wild-type hPTH promoter was observed for NF-Y expressed in SL2 cells relative to mutant promoters, inactivating either one or the other NF-Y-binding site (Fig. 4). However, in the earlier report we observed that co-transfection of a dominant negative isoform of the A subunit of NF-Y had a more modest effect on basal hPTH promoter activity in OK cells (18). Thus, the synergism observed in the SL2 cells may be an anomaly of the insect line, or conversely, NF-Y activity is restricted in some manner in OK cells, perhaps involving p300 (30), despite the ready presence of an NF-Y-binding complex in nuclear extracts (Fig. 1). Nevertheless, the presence of NF-Y in PTGs (18), together with the current SL2 cell data, highlights the potential for synergism between NF-Y complexes bound to the two DNA elements to strongly enhance transcription of the hPTH promoter.

Binding by the VDR to the hPTH VDRE (Fig. 2) was limited to the previously identified DNA half-site element (4), although binding also required heterodimerization with RXR (22). This is reconciled with a three-dimensional arrangement of heterodimer proteins and DNA is not known at this time. It is well established that the VDR heterodimer functions to acti-
NF-Y Interactions with the hPTH VDRE

Fig. 7. Transcription factor interactions with the hPTH promoter. A, a schematic is shown of the hPTH promoter with identified DNA elements indicated. The sequence corresponding to the VDRE (overhead double arrow) and NF-Y sites (underline double arrow) is shown. CRE, cAMP response element. B, binding by NF-Y complexes to the distal and proximal elements results in strong, synergistic transactivation. Binding by the VDR heterodimer to the VDRE displaces the proximal NF-Y complex and disrupts the synergism between the two NF-Y molecules. ++++++, synergistic transactivation; +, weak transactivation.

NF-Y molecules.

The distal and proximal elements results in strong, synergistic transactivation. Binding by the VDR heterodimer to the VDRE displaces the proximal NF-Y complex and disrupts the synergism between the two NF-Y molecules. ++++++, synergistic transactivation; +, weak transactivation.

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Fig. 7A outlines identified transcription factor binding sites in the region from −160 to −60 of the hPTH promoter. The VDRE/NF-Y-binding sites are highlighted and clearly indicate the overlapping nature of the two DNA elements. A potential mechanism to at least partially account for the repressive effects of vitamin D on hPTH gene transcription is also shown (Fig. 7B). NF-Y binding to both distal and proximal elements would result in strong enhancement of hPTH promoter activity. Displacement of NF-Y from the proximal element by the liganded VDR heterodimer complex can be envisioned disrupting synergistic transactivation by NF-Y and strongly attenuating promoter activity. Although the model provides a basis for future studies, there are still many unanswered questions surrounding the role of NF-Y and the hPTH promoter. For example, does NF-Y contribute to basal activity in the PTG, or are there other factors, such as hypocalcemia, that selectively enhance NF-Y interactions with the promoter? NF-Y is known to synergize with Sp1 to enhance gene transcription (27, 35, 36), suggesting that the proximal NF-Y-binding site in the hPTH promoter may also be capable of interacting with Sp1 proteins bound to the upstream Sp1 DNA element. Finally, renal insufficiency is often accompanied by the development of secondary hyperparathyroidism, which has been associated with decreased vitamin D activity in the PTG (37–42). Our data imply that unopposed NF-Y transactivation of the human promoter may be contributing to this condition; therefore drugs that attenuate NF-Y transcriptional activity may be candidates for use in treating this disorder (43, 44). Additional studies are essential to assess these and other possible roles of NF-Y in regulating hPTH promoter activity.

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