Synergistic Activation of the Prolactin Promoter by Vitamin D Receptor and GHF-1: Role of the Coactivators, CREB-Binding Protein and Steroid Hormone Receptor Coactivator-1 (SRC-1)

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PRL gene expression is dependent on the presence of the pituitary-specific transcription factor GHF-1/Pit-1, which is transcribed in a highly restricted manner in cells of the anterior pituitary. In pituitary GH3 cells, vitamin D increases the levels of PRL transcripts and stimulates the PRL promoter. We have analyzed the role of GHF-1 and of the vitamin D receptor (VDR) to confer vitamin D responsiveness to the PRL promoter. For this purpose we have used nonpituitary HeLa cells, which do not express GHF-1. We found that VDR activates the PRL promoter both in a ligand-dependent and -independent manner through a sequence located between positions -45/-27 in the proximal 5'-flanking region. This sequence also confers VDR and vitamin D responsiveness to a heterologous promoter. In the context of the PRL gene, VDR requires the presence of GHF-1 to activate the promoter. Truncation of the last 12 C-terminal amino acids of VDR, which contain the ligand-dependent activation function (AF2), abolishes regulation by vitamin D, suggesting that binding of coactivators to this region mediates ligand-dependent stimulation of the PRL promoter by the receptor. Indeed, expression of the coactivators, steroid hormone receptor coactivator-1 (SRC-1) and CREB-binding protein (CBP), significantly enhances the stimulatory effect of vitamin D mediated by the wild-type VDR but not by the AF2 mutant receptor. Furthermore, CBP also increases the activation of the PRL promoter by GHF-1 and the ligand-independent activation by both wild-type and mutant VDR. (Molecular Endocrinology 13: 1141–1154, 1999)

INTRODUCTION

Tissue-specific and developmental expression of eukaryotic genes is typically governed by combinations of cell type-specific and ubiquitous transcription factors (1). GHF-1/Pit-1 is a pituitary-specific POU-Homeobox transcription factor that regulates GH, PRL, and TSHβ gene expression through binding to specific promoter sequences (2–4). The 5’-flanking region of the PRL gene contains several GHF-1 binding sites clustered in two domains: a distal enhancer (−1800 to −1500 bp), and a proximal promoter (−422 to +33 bp) both containing four GHF-1 binding sites (5). Multiple hormones, growth factors, and oncogenes act in conjunction with GHF-1 to regulate the expression of the PRL gene. The DNA elements as well as the transcription factors responsible for the regulation by some of these stimuli have been identified. Thus, different ligands of tyrosine kinase receptors, as well as the src, ras, and raf oncogenes, activate PRL gene expression, and the downstream effector for these stimuli appear to be the ubiquitous Ets factors, which bind to the proximal PRL promoter (6, 7). The proximal promoter region also contains a basal transcription element (BTE) as well as sequences conferring cAMP responsiveness [cAMP-response element (CRE)] to the PRL gene. An estrogen response element (ERE) located in the distal enhancer binds estrogen receptors and confers estradiol responsiveness to the PRL gene (8). Other ligands of nuclear receptors have also been described to regulate PRL gene transcription both positively and negatively (9).

1,25-Dihydroxyvitamin D₃ [1,25-(OH)₂D₃] (vitamin D), the active form of vitamin D₃, exerts its biological activities through binding to a specific receptor [vitamin D receptor (VDR)], a member of the nuclear hormone receptor superfamily, which also includes retinoid, thyroid hormone, and steroid hormone receptors.
Effects of CBP (but not SRC-1) increases the constitutive receptor. However, both coactivators have differential diated by the wild-type VDR, but not by the mutant deed, expression of the coactivator SRC-1 and CBP the AF2 region exhibits full constitutive activity, but requires the presence of GHF-1. A VDR mutant lacking proximal promoter region, and that this activation re- in independent manner through a VDRE located in the promoter both in a ligand-dependent and a ligand-
tem (HeLa cells). We found that VDR activates the PRL type and mutant receptors in a heterologous cell sys-
tween GHF-1 and wild-
express endogenous PRL or the pituitary-specific fac-
tor GHF-1 (18). To characterize the interaction be-
tween GHF-1 and VDR on PRL gene activation by vitamin D, the construct –3000PRLCAT was used in tran-
sient transfection assays. Since transcriptional effect of different nuclear receptors can be interfered by AP-1 factors, this construct lacks the AP-1 binding motif present in the plasmid backbone (19). As illustrated in Fig. 1B, vitamin D caused an approximately 3-fold increase in chloramphenicol acetyltransferase (CAT) activity, a value similar to that found for the increase in endogenous PRL transcripts. In contrast with the results obtained in GH3 cells, we were unable to find stimulation of the PRL gene by vitamin D in GH4C1 cells, another rat pituitary cell line (data not shown).

Vitamin D Activates the PRL Promoter in Nonpituitary HeLa Cells

Consistent with the physiological role for vitamin D in the regulation of PRL gene expression in rat pituitary cells (17), we decided to analyze the role of VDR on PRL gene activation by vitamin D, as well as the participation of GHF-1 in this response. For this purpose we used the nonpituitary HeLa cell line, which is de-
 ved from a human cervical carcinoma and does not express endogenous PRL or the pituitary-specific factor GHF-1 (18). To characterize the interaction be-
tween GHF-1 and VDR on PRL gene activation by vitamin D, the reporter plasmid –3000PRLCAT was cotransfected with VDR in the presence or absence of a GHF-1 expression vector. As shown in Fig. 2, basal CAT activity was very low in HeLa cells in the absence of GHF-1 and was not affected by vitamin D. Furthermore, this activity was not modified by expression of VDR alone, either in the presence or in the absence of the ligand. However, after expression of GHF-1, which by itself had little stimulatory effect, a strong synergistic response was observed and vitamin D caused a marked promoter stimulation. As can also be ob-
erved in Fig. 2, unliganded VDR was able to cooper-

RESULTS

Vitamin D Increases PRL Transcripts and Stimulates PRL Promoter Activity in Pituitary GH3 Cells

As shown in Fig. 1A, incubation of GH3 cells with 100 nM vitamin D for 48 h caused a significant increase of PRL mRNA levels. Quantification of Northern blots from three independent experiments showed that this concentration of vitamin D increased PRL transcripts in GH3 cells by 3.3 ± 0.2 fold. To study whether sequences contained within the 5’-flanking region of the PRL gene mediate induction by vitamin D in these cells, the construct –3000PRLCAT was used in tran-
sient transfection assays. Since transcriptional effect of different nuclear receptors can be interfered by AP-1 factors, this construct lacks the AP-1 binding motif present in the plasmid backbone (19). As illustrated in Fig. 1B, vitamin D caused an approximately 3-fold increase in chloramphenicol acetyltransferase (CAT) activity, a value similar to that found for the increase in endogenous PRL transcripts. In contrast with the results obtained in GH3 cells, we were unable to find stimulation of the PRL gene by vitamin D in GH4C1 cells, another rat pituitary cell line (data not shown).
ate with GHF-1 to cause a significant increase of CAT activity, although this constitutive activity was markedly enhanced upon incubation with the ligand. In contrast, vitamin D did not activate the PRL construct in the absence of transfected VDR, even when GHF-1 was present, showing that endogenous VDR levels are not sufficient to stimulate the PRL promoter in HeLa cells.

The same PRL promoter fragment (−3000 to +74) containing the AP-1 site in the plasmid backbone showed a stronger response to GHF-1, but the responses to VDR and vitamin D₃ were not affected (data not shown).

5′-Flanking Sequences in the Proximal PRL Promoter Confer Vitamin D Responsiveness

A series of 5′-deletion constructs were used to determine the gene elements responsible for PRL promoter stimulation by vitamin D. All constructs contained the first 74 bp of the coding PRL region and lacked the AP-1 binding site. In contrast with the results obtained with the −3000PRLCAT plasmid, CAT levels were essentially undetectable in GH3 cells transfected with shorter promoter fragments and, therefore, further mapping could not be carried out in these cells (Fig. 3B). In contrast, CAT activity was detectable in HeLa cells transfected with the different deletions. Figure 3A shows that the −21597, −2425, and −2176 constructs exhibit similar ligand-dependent activation upon cotransfection with expression vectors for VDR and GHF-1. As with the −3000PRLCAT plasmid, a weaker ligand-independent activation was also observed. Deletion to −101 bp significantly reduced vitamin D response, although constitutive activation was still observed, and both responses were totally lost in the construct extending only to −76. This gradual loss of response suggests either the existence of more than only one VDRE (some of which could be located between −176 and −101), or that the VDRE cooperates with other promoter sequences to confer full vitamin D responsiveness.

Mapping of the Vitamin D Response Element

To identify the possible vitamin D₃ response element(s) in the proximal promoter of the PRL gene, gel

Fig. 1. Vitamin D Activates PRL Gene Expression in Pituitary GH3 Cells

A, Northern blot analysis were carried out with 20 μg of total RNA from duplicate cultures of control cells and cells treated for 48 h with 100 nm vitamin D. The blot was hybridized with a labeled cDNA probe for rat PRL. The lower panel shows the ribosomal 18S RNA. B, GH3 cells were transfected with 5 μg of the PRL promoter construct −3000 PRLCAT, and CAT activity was determined after 48 h in untreated cultures and in cultures treated with 100 nm vitamin D. The data show the mean ± SD values obtained in a representative experiment performed with triplicate cultures.

Fig. 2. GHF-1 Cooperates with VDR to Stimulate the PRL Promoter in HeLa Cells in a Vitamin D-Dependent and -Independent Manner

The plasmid −3000 PRLCAT (5 μg) was cotransfected with expression vectors for GHF-1 (0.4 μg) and/or VDR (2.5 μg). After 48 h, CAT activity was determined in untreated cultures and in cultures treated with 100 nm vitamin D (black bars). The data show the mean ± sd values obtained in a representative experiment performed with triplicate cultures.

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mobility shift experiments were conducted with a labeled promoter fragment spanning from −2176 to +174 (which confers a full CAT response to vitamin D), a −2101 to +174 fragment (which shows a partial response), and a −270 to +174 promoter fragment (which is unresponsive to vitamin D in the transfection assays). The region from −176 to −101 was also used in the assays. As depicted in Fig. 4A, the promoter region between −176 and −101 contains two GHF-1 binding sites, one of them included in the composite Ets-GHF-1 binding site, which is required to mediate PRL promoter activation by oncogenic ras [Ras-responsive element (RRE)] (20). These elements could participate in the response to vitamin D3. Truncation to −2101 deletes the RRE (−165/−150). The −76 to +74 fragment, which is unresponsive to vitamin D, has lost an additional Ets binding site, the CRE (−101/−92), and the BTE (−98/−85). Since VDR can function as a homodimer or as a VDR/RXR heterodimer (21), we analyzed the formation of these complexes on the proximal promoter regions. As shown in Fig. 4B (left panel), the fragment from −176 to +74 formed a weak complex with VDR (lane 3). RXR alone also bound weakly to this fragment (lane 2), but caused the formation of VDR/RXR heterodimers that bind strongly to the promoter (lane 4), indicating a preference for heterodimeric binding to the response element. This heterodimeric complex was removed by antibodies against both VDR (lane 5) and RXR (lane 6). Identical results were obtained when the promoter region from −101 to +74 was used in the mobility shift assays (data not shown), demonstrating that the VDRE was contained within this fragment. In contrast, as shown in lanes 7–10, the region comprised between −176 and −101 did not bind the receptors although GHF-1 binding to this fragment was readily observed (not illustrated). These results suggested that the VDRE could be located between −101 and −76 bp. However, as shown in lanes 11 and 12, although the −76 to +74 region is not sufficient to confer vitamin D responsiveness to the PRL promoter in transient transfection studies, these sequences still contain the VDRE. Again, VDR binding was significantly enhanced in the presence of its heterodimeric partner RXR, showing the preference for binding of VDR/RXR heterodimers to this fragment (lane 12). These results indicate that although the VDRE is located in this promoter region, other upstream sequences are required to confer responsiveness to vitamin D3. To further map the VDRE, the −276 to −174 region was digested with Pst-1 to generate two new fragments: −276 to −210 and −29 to +74 (Fig. 4A). Each region was end labeled with 32P and used in mobility shift experiments with VDR and RXR. The formation of VDR/RXR complexes was observed with the −276 to −210 fragment (lane 14), but not with the −9 to +74 fragment (lane 16), demonstrating that the VDRE is located between −76 and −10 bp in the promoter.

Since VDR actions on the PRL promoter depended on the presence of GHF-1 and both transcription factors produced a synergistic effect, we explored the possibility that binding of GHF-1 and VDR/RXR could be cooperative. Figure 4B (right panel, lanes 1–10) shows binding of VDR and VDR/RXR alone or in combination with GHF-1 to the labeled −76 to +74 fragment. This promoter region contains one GHF-1 bind-
ing site and the VDRE. The addition of GHF-1 to the VDR or VDR/RXR binding reactions neither facilitated the binding of the receptors nor resulted in the formation of new complexes different from those formed in reactions containing each protein individually. Thus, both factors can bind independently to the promoter and do not show cooperative binding. The preference for the formation of VDR/RXR complexes, rather than VDR homodimers (21), suggests that the VDRE in the PRL promoter could consist of a direct repeat (DR) motif with a spacer of three nucleotides. This was confirmed in competition reactions with an unlabeled consensus DR-3 oligonucleotide. Binding of the VDR/RXR complex was competed specifically by this oligonucleotide (lanes 6 and 9), but not by a sequence containing a GHF-1 binding site (lane 10). The latter effectively competed binding of the GHF-1 complex (lanes 3 and 10), which in turn was not affected by the DR-3 oligonucleotide (lane 9). As also shown in Fig. 4B, binding of VDR/RXR to the PRL promoter fragment occurs irrespectively of the presence of vitamin D (lanes 5 and 8).

Since the data shown in Fig. 4B indicate that the VDRE must be contained between the nucleotides −76 and −10 in the PRL promoter, we screened this promoter region for consensus VDR-binding sites. This sequence was compared with 18 known natural VDREs (reviewed in Ref. 11). As illustrated in Fig. 5A, three overlapping putative VDREs in the −45 to −14 region were found. Oligonucleotides designed to contain all of them (A), the two most 5′-motifs (B), or the most 3′-motifs (C) were assayed in a mobility shift experiment. The A and B oligonucleotides bound VDR homodimers with a similar low affinity to the A or B oligonucleotides (lane 11), but binding of VDR/RXR heterodimers was almost undetectable (lane 12). These results suggest that the B sequence, which contains the DR-3 motifs, is the one responsible for PRL gene response to vitamin D.

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**Fig. 4.** Binding of VDR/RXR Heterodimers to the Region between Positions −76 and −10 of the PRL Promoter

A, Schematic representation of the proximal PRL promoter from nucleotides −176 to +74, showing the position of the TATA box, and the binding sites for GHF-1, Ets factors, the BTE, and the overlapping cAMP response element (CRE). 32P-labeled promoter fragments (−176/+74, −176/+101, and −76/+74) were obtained by PCR. The depicted Pst-1 restriction site was used to generate the labeled fragments, −76/−10 and −9/+74. B, In the left panels, gel mobility shift assays were performed with 150 ng of VDR and/or RXR lacking the N-terminal A/B domain (∆A/B-RXR) as fusion proteins with GST. The receptors were incubated with the 32P-labeled proximal promoter regions indicated. For supershift assays, 1 μl of the specific antibodies against VDR or RXR (αVDR and αRXR, respectively) was used in the binding reactions as indicated. The mobility of the receptor-containing complexes is indicated. ns represents a nonspecific band present in the labeled −176/+74 fragment. In the right panel the same amount of receptors was incubated with the −76/+74 promoter fragment in the presence and absence of recombinant GHF-1 (GHF-1p). As indicated, 1 μM vitamin D (Vit D) or a 50-fold excess of unlabeled oligonucleotides conforming a consensus VDRE [DR3(oli)], or the GHF-1 binding site [GHF-1(oli)], were included in the reactions.
The VDRE of the PRL Gene Is Functional

To test the functionality of the VDR-binding sites of the PRL promoter, the A and B sequences were fused to an heterologous thymidine kinase (TK) promoter driving the CAT reporter gene (A-PRLTKCAT and B-PRLTKCAT). These constructs, as well as the parental TKCAT construct, were transfected into HeLa cells together with the expression vector for VDR. The effect of vitamin D on a TKCAT reporter gene containing a consensus DR3 response element as a positive control was also analyzed. As shown in Fig. 6A, the plasmid TKCAT, which does not contain a VDRE, was unresponsive to vitamin D either in the presence or absence of VDR. On the other hand, when VDR was expressed, vitamin D induced A-PRLTKCAT and B-PRLTKCAT activity by approximately 6-fold and 5-fold, respectively. A similar induction by vitamin D (4.4-fold) was found in cells transfected with the TKCAT plasmid containing the consensus DR3 VDRE. As also shown in Fig. 6A, unliganded VDR constitutively activated the A-PRLTKCAT and B-PRLTKCAT construct, but the unoccupied receptor did not stimulate the DR3 TKCAT plasmid. These results indicate that the B sequence must be the major element used by VDR for both the ligand-dependent and -independent activation of the PRL gene.

To further test the functionality of the VDRE, this element was mutated in the context of the −176PRL-CAT plasmid. Two different mutations in the B sequence, one affecting the 5′-hemisite (−176 m1PRLCAT) and the other affecting both hemisites of the VDRE (−176 m2PRLCAT), were transfected into HeLa cells. Figure 6B shows that these mutations significantly impaired the response of the PRL promoter to vitamin D. Additionally, ligand-independent stimulation by VDR was reduced by the mutations. In contrast, the activity of these mutants was strongly stimulated by incubation with 10 μM forskolin (data not shown), showing that this element does not contribute to responses elicited by other signaling pathways.

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**Fig. 5.** Mapping of the VDRE

A, Schematic representation of the −76 to −10 fragment showing the most proximal GHF-1 binding site and the TATA Box. B, Synthetic oligonucleotides corresponding to the region between positions −45 to −14 designated as A, B, and C, showing the putatives DR3-type VDREs (black arrows). C, Gel mobility shift assay performed with GST-VDR and GST-RXR and the 32P-labeled A, B, and C sequences as indicated.

**Fig. 6.** The VDRE of the PRL Promoter Is Functional

A, The A and B sequences of the PRL promoter, as well as a consensus DR3 element, were fused to a TKCAT construct to give the A-PRLTKCAT, B-PRLTKCAT, and DR3-TKCAT constructs, respectively. HeLa cells were transfected with 5 μg of these constructs or the same amount of the parental TKCAT construct. The cells were cotransfected with 2.5 μg of VDR expression vector, and CAT activity was determined after 48 h of incubation in the presence or absence of 100 nm vitamin D (vit. D). B, HeLa cells were transfected with 5 μg of −176PRLCAT or the mutated constructs −176 m1PRLCAT and −176 m2PRLCAT. These plasmids were cotransfected with expression vectors for GHF-1 (0.4 μg) and/or VDR (2.5 μg). After 48 h, CAT activity was determined in untreated cultures and in cultures treated with 100 nm vitamin D. Schematic representations of the constructs are shown at the top. The hemisites in the VDRE are indicated by arrows, and mutations are shown as X.
VDR Activates the PRL Gene through VDR/RXR Heterodimers

Our results indicate that cotransfection of RXR was not needed for the PRL response to vitamin D. Since HeLa cells contain RXR, the endogenous receptor levels appear to be sufficient to mediate the observed response to vitamin D. To analyze whether the activation of the PRL promoter by vitamin D could be enhanced by overexpression of RXR, the −176PRLCAT construct was transfected into Hela cells in combination with the expression vectors for VDR and/or RXR in the presence of the GHF-1 vector (Fig. 7A). The responsiveness of the PRL promoter to vitamin D was only observed in the presence of VDR. Again, unliganded VDR activated the promoter, and this effect was increased by vitamin D. Overexpression of RXR did not affect basal PRL promoter activity, but potentiated both the constitutive activity of VDR and the promoter activation by vitamin D. Similar results were obtained in pituitary cells. Figure 7B shows that expression of RXR in GH3 cells did not alter basal activity, but enhanced the response to the vitamin. Thus, it is most likely that VDR activates the PRL promoter by heterodimerizing with RXR. These results are in agreement with the stimulation by vitamin D through a VDRE characterized as a DR-3 motif, since it has been proposed that VDR/RXR heterodimers mediate transactivation of DR-3-containing promoters (21, 22).

Transcriptional Activation by Wild-Type and C-Terminally Truncated VDR Mutants

To evaluate the region of VDR responsible for the ligand-dependent and ligand-independent activation, we used two truncated mutants of VDR. In the ΔABC mutant, the first 111 amino acids in the N terminus have been eliminated. This mutant lacks both the constitutive activation function AF1, and the DNA-binding domain. The ΔAF2-VDR mutant lacks the last 12 amino acids in the C terminus. Thus, this mutant does not contain the transcriptional ligand-dependent activation domain AF2. AF-2 activity appears to be due to interaction with coactivators in a ligand-dependent manner. Thus, the coactivator SRC-1 has been shown to interact with the AF-2 region of VDR (23, 24). We compared in vitro interaction of VDR and ΔAF2-VDR with other coactivators [CBP and ACTR (coactivator of thyroid hormone and retinoic acid receptors)] in glutathione-S-transferase (GST) pull-down assays. As shown in Fig. 8A in the presence of vitamin D, a significant portion of the input of 35S-labeled VDR was specifically retained by GST-ACTR immobilized in glutathione-agarose beads, while no significant binding was observed either in the absence of vitamin D or by GST alone. Deletion of the 12 C-terminal amino acids abolish binding to the coactivator, and GST-ACTR was not retained significantly by the ΔAF2-VDR mutant in the presence of vitamin D. Similar results were obtained with GST-CBP. Incubation with vitamin D increased binding of GST-CBP to the wild-type receptor, while binding to the ΔAF2-VDR mutant was unaffected. No interaction of the GST-CBP with 35S-luciferase used as a negative control was detected.

Figure 8B shows the effect of cotransfection of wild-type or mutant VDR expression plasmids on the induction of −176PRLCAT by vitamin D. Although the ΔAF2-VDR binds vitamin D with a normal affinity (25), this mutant exhibited no vitamin D-dependent activation in the presence of GHF-1. This result indicates that the last 12 amino acids in the C terminus are absolutely necessary for transcriptional activation in a ligand-dependent manner and suggests that coactivators that bind to the AF2 domain play an important role in this response. In contrast, the ΔAF2-VDR mutant was able to activate the PRL promoter in a ligand-independent manner with at least the same potency as the wild-type receptor. In contrast, the ΔABC-VDR mutant displayed neither constitutive transcriptional activity nor vitamin D-dependent transcriptional activation. This shows that the LBD is not sufficient for activation and suggests that binding to the VDRE is required.

Role of the Coactivators SRC-1 and CBP on the Activity of the PRL Promoter

As numerous reports have emphasized the requirement of coactivator factors to promote full activity of the nuclear receptors in the presence of their ligands,
it was of interest to analyze their role in the activation of the PRL gene by VDR in the absence and presence of vitamin D. For this purpose, HeLa cells were transfected with the PRL promoter construct 2300PRL-CAT, and the expression vectors for VDR, GHF-1, and the nuclear receptor coactivators, SRC-1 and CBP (which bind to the core AF2 region of nuclear receptors). Figure 9A shows the functional effects of these factors on PRL gene stimulation. In the absence of GHF-1, neither protein activated the PRL promoter (data not shown). In the presence of GHF-1, SRC-1 neither enhanced the promoter response to this pituitary factor nor the constitutive activity of VDR, but drastically potentiated the response to vitamin D. Thus, SRC-1 serves as a good coactivator for the stimulation of the PRL promoter by VDR in a ligand-dependent manner. The activating effect of SRC-1 on the PRL promoter was confirmed in pituitary GH3 cells transfected with 2300PRLCAT. As shown in Fig. 9B, overexpression of SRC-1 enhanced significantly the response to vitamin D without increasing basal promoter activity. Unlike SRC-1, CBP significantly enhanced the response to GHF-1 in HeLa cells independently of the presence of VDR. Furthermore, CBP potentiated not only the ligand-dependent, but also the ligand-independent, activation mediated by VDR (Fig. 9A). Finally, the combination of these coactivators slightly increased the effect produced by each protein alone. These results suggest that whereas SRC-1 acts specifically as a nuclear receptor coactivator and in a vitamin D-dependent manner, CBP exerts a broader role in the regulation of the PRL gene.

Since coactivators markedly enhanced the vitamin D-dependent activity of the wild-type VDR, it was also of interest to analyze their role in the activity of the VDR mutants. For this purpose, the same transfection assays described above in HeLa cells with wild-type VDR, SRC-1, and CBP were also performed with the VDR mutants (Fig. 10). Confirming the results described above, expression of SRC-1 was unable to activate the promoter construct in the absence of receptors, whereas CBP increased by approximately 6-fold the levels found in cells expressing GHF-1. The
The ΔABC-VDR mutant did not activate the PRL promoter either constitutively or in a ligand-dependent manner in the presence of coactivators. The ΔAF2-VDR mutant did not confer vitamin D responsiveness to the promoter even in the presence of coactivators. This is not surprising, since as described before (24, 25) and shown in Fig. 8A, the AF2 region of VDR was required for interaction with coactivators. However, the receptor lacking the AF2 region displayed a normal ligand-independent transcriptional activity and, although this mutant is not supposed to interact with CBP, expression of this coactivator substantially increased the constitutive activity of ΔAF2-VDR. This unexpected finding was specific for CBP, since SRC-1 did not significantly modify ligand-independent induction by the ΔAF2-VDR mutant.

Physical Interaction of VDR with GHF-1

We have previously reported that several nuclear receptors can interact with GHF-1 (26). Since the stimulation of the PRL promoter by VDR requires the presence of GHF-1, we asked whether a direct protein-protein interaction between both factors could be involved in this functional cooperation. To address this question, a GST-VDR fusion protein was immobilized on glutathione-sepharose beads and used in binding assays with in vitro translated GHF-1, labeled with [35S]methionine. To characterize the role of vitamin D in the interaction between these proteins, the binding reactions were also performed in the presence of this ligand. As shown in Fig. 11, GHF-1 interacted with VDR, and this association was independent of the presence of vitamin D. To map the GHF-1 domain responsible for this interaction, different deletion mutants of the protein were also used in pull-down assays with GST-VDR. Deletion of the homeodomain, but not of other regions of the protein, abolished the ability of GHF-1 to interact with VDR. This result indicates that the DNA-binding domain of GHF-1 is involved in binding to VDR.

DISCUSSION

It has been previously found that somatolactotroph tumor cells respond to vitamin D with a highly selective increase in PRL synthesis, raising the level of PRL mRNA without affecting the level of GH mRNA (17). We have confirmed that vitamin D increases PRL transcripts in GH3 cells and have demonstrated that vitamin D enhances PRL promoter activity in transient transfection assays. Since pituitary-specific transcription of the GH and PRL genes involves synergistic interactions between GHF-1 and other promoter-binding factors, including nuclear receptors, we decided to analyze the role of GHF-1 in the regulation of PRL gene expression by the VDR. In this report we show that in...
mediated by a sequence that binds VDR/RXR het-
constitutively and in a vitamin D-dependent manner
HeLa cells, VDR induces PRL gene activation both
constitutively and in a vitamin D-dependent manner
only after the expression of GHF-1.
Our data indicate that the response to vitamin D is
mediated by a sequence that binds VDR/RXR het-
erodimers and is located in the 5'-flanking region of
the PRL gene between the nucleotides −45 and −27.
We have identified two putative overlapping VDREs
(starting either in nucleotide −42 or −41). The hexam-
eric core of both elements shows a high homology
with the consensus VDRE sequence RRKNSA (r = A or
G; K = G or T; S = C or G). As in most VDREs identified
in natural vitamin D-responsive gene promoters, the
hemisites in the PRL promoter are arranged as direct
repeats spaced by three nucleotides. As expected for
a DR3, the PRL VDRE bound VDR/RXR heterodimers
with high affinity and showed low affinity for VDR
homodimers that have been described to bind prefer-
entially to DR6-type VDREs (21, 22). It has been re-
ported that vitamin D can increase binding of VDR/
RXR to some VDREs (27) but not to others (22), and
our data show that binding to the PRL VDRE was not
affected by vitamin D. An unusual feature of this VDRE
is its close vicinity to the TATA box of the PRL gene.
Our results suggest that binding of receptor het-
erodimers to these sequences does not compete with
binding of TATA box-binding protein (TBP) to its rec-
ognition sequence, rather a synergistic action of the
receptors with the basal transcriptional machinery
could be facilitated by the close location of the VDRE.
The PRL VDRE fused to a heterologous promoter
conferred responsiveness to vitamin D, and mutation
of this element in the PRL promoter strikingly reduced
the response to vitamin D, demonstrating that this
element is functional. Most interestingly, expression of
VD also causes a constitutive activation of the PRL
promoter, and the heterologous promoter that con-
tains the PRL VDRE was also activated upon expres-
sion of VDR. In contrast, the idealized DR3 sequence
used as a positive control only exhibits ligand-depen-
dent activation. This result suggests that the activa-
tion by the unoccupied receptor is a specific feature of
the PRL VDRE. It has been shown that other nuclear re-
ceptors are able to activate transcription constitu-
tively, and that this activation is cell and promoter
dependent (28, 29). The molecular mechanisms un-
derlying the transcriptional effects of the unliganded
receptors are not well understood. Either there is an
interaction between VDR and cellular factors that may
be necessary for hormone-independent activity or,
alternatively, the receptors may inhibit the activity of
putative inhibitory factors.
The response of successive promoter truncations to
liganded or unliganded VDR shows that a PRL pro-
moter construct extending to −76 bp does not re-
spond to the actions of this receptor although it con-
tains the VDRE. The lack of the BTE and the
overlapping CRE located between −101 and −85,
which are necessary for the activity of the PRL pro-
moter (30, 31), could be involved in the unresponsive-
ness of this promoter fragment to vitamin D. In fact,
this construct which contains one GHF-1-binding site,
does not show activation by GHF-1. However, the
presence of the BTE in the PRL promoter is not suffi-
cient to confer full responsiveness to vitamin D, since
the −101PRLCAT construct contains this element and
only exhibits a partial response to vitamin D. Thus,
other sequences located upstream of −101 bp appear
to contribute to the activation of the PRL promoter by
vitamin D. One of these sequences could be the RRE,
a composite element for binding of GHF-1 and Ets
elements (20). A direct protein-to-protein interaction be-
tween both factors has been recently described (18).
In most promoters the hormone response elements
are found clustered around binding sites for other
transcription factors, and the requirement for addi-
tional nonreceptor factors is consistent with the find-
ing that VDR acts with other transcription factors to
enhance gene expression synergistically (32). Se-
quences located between the nucleotides −176 and
−1597 did not further increase activation by vitamin D,
suggesting that factors binding to this region are not
involved in this response. However, the plasmid ex-
tending to −3000 bp showed in most experiments a
somewhat stronger regulation by vitamin D. This could
be due either to the presence of additional upstream
VDREs or to functional cooperation of the downstream
VDRE with other factors that bind to the upstream
sequences, as, for instance, the GHF-1-binding sites
identified in the distal enhancer. It is interesting that in
pituitary GH3 cells the activity of reporter plasmids
that do not contain the distal enhancer was extremely
low, precluding a detailed analysis of the response
factor in these cells.
The critical role played by GHF-1 in the stimulatory
action of vitamin D on PRL gene expression is indi-
cated by the finding that in nonpituitary cells VDR
requires the presence of GHF-1 for the activation of
this promoter. A synergistic activation of the PRL pro-
moter by GHF-1 and other nuclear receptors has been
observed previously. Thus, the estrogen receptor is
unable to activate expression of the PRL gene unless
GHF-1 is expressed (33), and as we have recently
observed, peroxisome proliferator-activated recep-
tor-α activates the PRL promoter, and this activation is
only observed upon expression of GHF-1 (34). There
are several models to explain transcriptional syner-
gism. Synergism could reflect cooperative binding be-
tween adjacent bound factors. For instance, binding
of GHF-1 could facilitate the binding of VDR/RXR to
the promoter, resulting in greater occupancy of the
cis-acting elements, thus promoting transcription. The
fact that the most proximal GHF-1-binding site is very
close to the VDRE in the promoter could suggest the
presence of a GHF-1/VDR composite element. This
type of composite element has been described in the
distal enhancer of the GHF-1 gene for GHF-1 and the
retinoic acid receptor (RAR) and is characterized by
the cooperative binding of RAR and GHF-1. However,
we do not observe cooperative binding of GHF-1 and
VDR/RXR heterodimers even in the presence of vitamin D, and our gel mobility shift assays show that the receptors and the GHF-1 bind to the promoter independently. Therefore, we favor another model for synergism involving stabilizing interactions of receptors and transcription factors such as GHF-1 or Ets with multiple target sites (some of them presumably basal transcription factors as well as coactivator proteins). The important functional role of coactivators in the regulation of PRL gene expression by VDR is demonstrated by the dramatic increase in vitamin D-dependent transactivation of the PRL promoter by VDR in the presence of SRC-1 and CBP. Both proteins bind the AF2 domain of the nuclear receptors in a ligand-dependent manner (12, 24, 25, 35), and our results show that this region is indeed involved in the recruitment of coactivators and essential for the response to vitamin D, which was lost in a VDR mutant that lacks the AF2 region. In contrast, this truncated receptor displayed a constitutive activation of the PRL promoter as strong as that of the wild-type receptor, showing that the ligand-independent activation by VDR does not require binding of coactivators to the AF2 domain. The finding that the AF2 mutant showed ligand-independent activity also dismisses the possibility that the constitutive action of VDR could be secondary to activation by residual intracellular levels of vitamin D.

Although SRC-1 and CBP had a similar effect on the vitamin D-dependent activation, a differential effect on the vitamin D-independent action of VDR was also observed. Thus, whereas VDR constitutive activity was essentially unaffected by SRC-1, CBP significantly increased the effect of unoccupied VDR. Furthermore, the vitamin D-independent activation elicited by the AF2 VDR mutant was also potentiated by CBP. The latter effect was totally unexpected, since this mutant lacks the CBP-interacting region (12). This suggests that CBP might exert this action indirectly by binding to other factor/s that, in turn, associate with VDR. The best candidate for this interaction is again GHF-1. CBP significantly potentiates the stimulatory effect of GHF-1 on the promoter. On the other hand, we have recently observed a direct protein-to-protein interaction between GHF-1 and CBP (34). This interaction maps to the POU domain of GHF-1 (36) and to the region of CBP contained between amino acids 1-1099 and 1679-1858, the same domains of interaction with other transcription factors including Ets (37).

We have also previously reported a direct association of GHF-1 with several nuclear receptors (26). This finding, as well as the important role of GHF-1 in the activation of the PRL gene by VDR, suggested that this receptor could also interact with GHF-1. Our results demonstrate that indeed this association exists, and that VDR interacts in a ligand-independent manner with the homeodomain (the DNA-binding domain) of GHF-1. Thus, although the interaction might recruit VDR and GHF-1 to the promoter, binding of VDR/GHF-1 heterodimers to the promoter was not observed.

As a whole, our data suggest that the simultaneous binding of VDR/RXR and GHF-1 to their close cognate sites in the promoter, as well as the direct interactions with other transcription factors, coactivators, and components of the basal transcription machinery, could facilitate promoter occupancy and govern transcriptional activation of the PRL promoter synergistically.

**MATERIALS and METHODS**

**RNA Extraction and Hybridization**

GH3 cells were cultured in RPMI medium containing 15% horse serum and 2.5% FCS. For the experiments the cells were incubated for 24 h in a medium containing a hormone-stripped serum by treatment with resin AG1×8 and activated charcoal. The cells were then treated for 48 h with 100 nm vitamin D. Total RNA was extracted from the cell cultures with guanidine thiocyanate. The RNA was run in 1% formaldehyde-agarose gels and transferred to nylon-nitrocellulose membranes (Nytran, Schleicher & Schuell, Dassel, Germany) for Northern blot analysis. The RNA was stained with 0.02% methylene blue. The blots were hybridized with a cDNA probe for rat PRL labeled by nick translation. Hybridizations were at 42°C with 50% formamide, and the more stringent wash was at 68°C with 50% formamide, 2× SSC, 0.1% SDS.

**Plasmids**

The constructs −3000PRLCAT and −176PRLCAT were generated from plasmids containing the 5′-flanking region of the rat PRL promoter (from −3000 to +74, and from −176 to +74 bp, respectively) in a pBL-CAT2 vector in which the thymidine kinase promoter had been deleted (38). Since the pBLCAT vector contains an AP-1 like sequence at +34/+39, which could mask some promoter responses (19), a 301-bp fragment containing this element was deleted by digestion with Asf1 and Nari. The constructs were then blunt-ended and religated. −1597PRLCAT was obtained by removing a Xbal/Nsi fragment of 1403 bp from −3000PRLCAT and then blunt-ended and religated. The −423PRLCAT plasmid was obtained by digestion of −3000PRLCAT with HindIII and religation. −101 and −76PRLCAT have been previously described (7). The mutated constructs, −176 m1PRLCAT and −176 m2PRLCAT were obtained by PCR. In the first PCR the sense oligonucleotides 5′-GACTGAGTGCAGAATTTA-3′ (for M1), and 5′-GACTGAGTGCGCATGGATATAA-3′ (for M2) containing the desired nucleotide changes, as well as the antisense oligonucleotide 5′-GACTGAGTGCAGAATTTA-3′ were used, to generate mutated fragments. After an elongation phase, a second PCR was performed using these fragments. The sense oligonucleotide for this reaction was 5′-ccacag1ttTGGC-CACTATGGTCTTCTC-3′ containing a HindIII site and as antisense 5′-GACTGAGTGCGCATG-3′. The mutated sequences were cut with HindIII and Xhol and subcloned in pBLCAT3. The mutations were confirmed by sequencing. The A oligonucleotide, 5′-gagcAGAGGAGGTCCGAAAGGTGAATTCAACTG-3′, which contains the sequences −45 to −14 of the rat PRL promoter, was cloned into HindIII/BamHI sites of pBL-CAT2 (without the AP-1 like sequence) upstream of the TK promoter to construct A-PRLTKCAT. The B oligonucleotide containing the promoter sequences from −45 to −27, 5′-agctGAGGTGCTCGAAAGGTGAATTCAACTG-3′, was also cloned into the HindIII site of pBL-CAT 2 upstream of the TK promoter and then blunt ended and religated to produce B-PRLTKCAT. The plasmid DR3-TKCAT, which contains the DR3 consensus oligonucleotide 5′-agctGAGGTGCTCGAAGGTGAATTCAACTG-3′, has
been previously described (39). The expression vectors for rat GHF-1 (40), human RXRa (41), human VDR (42), human CBP (43), and human SRC-1 (44) have been described previously. The expression vector for the ΔAF2-VDR mutant was constructed by PCR using the human VDR expression plasmid as a template and the oligonucleotides 5′-GGAGCAGGACGCATATT-3′ and 5′-CCGGATCCCTAGGATGAGCTTCCTGAG-3′ to generate a 872-bp fragment. This fragment was digested with BstXI and BamHI and cloned into the pSGS expression vector. This receptor lacks the last 12 C-terminal amino acids, which contain the AF2 region. For the ΔABC-VDR mutant, the oligonucleotides 5′-GAATTTCATGAGGAGGAGGAGGCTTG-3′ and 5′-CCGGATCTCCAGGAGATCG-3′ were used to generate a 972-bp fragment and were then digested with EcoRI and BamHI and subcloned into pSGS. This construct generates a truncated receptor lacking 149 N-terminal amino acids that include the A/B region and the DNA-binding domain (C region). pGST-VDR, which expresses a fusion protein between glutathione S-transferase (GST) and VDR, was obtained by PCR using the pSGS-VDR plasmid as a template and the oligonucleotides 5′-CCGGATCATGAGGAGGAGGCTTG-3′ and 5′-GAATTTCATGAGGAGGAGGCTTG-3′ to generate the VDR cDNA (1031 bp). This fragment was then subcloned into BamHI/EcoRI sites of the pGEX-2T plasmid. pGST-ΔA/BRXR plasmid (lacking the A/B domain), was also constructed by PCR using the pSGS-hRXR and the oligonucleotides 5′-GAATTTCATGAGGAGGAGGCTTG-3′ and 5′-GCTCTACATGACTTGGTGGCGG-3′ to generate a 1078-bp fragment that was subcloned into EcoRI/HindIII sites of the pGEX-2T plasmid. The constructs GST-ACTR and GST-CBP, which express the cDNAs coding for the amino acids 621–821 and 1–1099 of ACTR and CBP, respectively, have been previously described (45, 46). These fragments contain the nuclear receptor-interacting sequences of both proteins.

Cell Culture and Transfections

HeLa cells were cultured in DMEM containing 10% FCS and were transfected by calcium phosphate with the reporter CAT constructs. GH3 cells were shifted to DMEM and transfected with calcium phosphate with a glycerol shock. In cotransfection experiments the reporter plasmids were transfected with calcium phosphate with a glycerol shock. In cotransfection experiments the reporter plasmids were transfected with calcium phosphate with a glycerol shock. For the GHF-1 binding reactions the GST-fusion proteins were expressed in the

Protein Preparations

GHF-1 and its truncated forms cloned in Bluescript SK- (36), were used for in vitro transcription and translation following the manufacturer's recommendations of the TNT T7 coupled transcription/translation System (Promega Corp., Madison, WI). The reactions were translated in the presence of 40 μCi of [35S]methionine (Amerham Pharmacia Biotech, Arlington Heights, IL). Five microliters of the reaction product were resolved in 10% SDS-PAGE. The gel was dried and autoradiographed overnight. Recombinant purified GHF-1 was a generous gift from Dr. Castrillo. The GST-fusion proteins, VDR, SABXR, CBP, and ACTR, were expressed in the bacterial strain BL21 (DE3). They were grown at 37 C in 2× YT [Tryptone, 16 g/liter yeast extract; NaCl, 5 g/liter (pH 7)] until the absorbance reached 0.6. Then the induction was performed at 30 C for 2 h with 0.4 mM isopropyl β-D-thiogalactopyranoside and were purified following the recommendations of Pharmacia Biotech, (Piscataway, NJ).

Mobility Shift Assays

Gel retardation assays were performed with the recombinant GST-fusion proteins. Oligonucleotides corresponding to the A, B, and C fragments of the PRL promoter were used as probes. A and B oligonucleotides have been described above, and C oligonucleotide, 5′-agctgaaggaaggatatataaagtctagtcg-3′, contains the −36 to −14 promoter sequence. For the binding reaction, the proteins were incubated on ice for 15 min in a buffer [20 mM Tris HCl (pH 7.5), 75 mM KCl, 1 mM dithiothreitol, 5 μg/ml BSA, 13% glycerol] containing 3 μg/poly(dI-dC) and then for 15–20 min at room temperature with approximately 50,000 cpm of labeled double-stranded oligonucleotide end labeled with [32P]UTP, using Klenow fragment as kinase. In addition, the labeled fragment −176 to +74 was obtained by PCR using the oligonucleotides 5′-cccaagcctTGCCACATGTCTCT-3′ and 5′-AACAGCCAAGGTGACCCG-3′ as primers. The region −176 to +101 was obtained with the antisense oligonucleotide 5′-CAATCATCTATTCCGTAT-3′. For the PCRs the first oligonucleotide was previously end labeled with [32P]ATP using T4-polynucleotide kinase. Similarly, the −101 to +74 and −76 to +74 fragments were made by PCR with the 5′ oligonucleotide 5′-ATACGGAAATAGTTGAG-3′ and 5′-GAAGAGGTGCTGTAG-3′, respectively (end labeled previously with [32P]ATP). For competition experiments an excess of unlabeled double-stranded oligonucleotides were added to the binding reactions: as a DR-3 type we used 5′-agctgATCCAGAAGGTCAcg-3′ and for the GHF-1 binding site we used 5′-CCAGCATGAATAGTTGAG-3′. For supershift experiments, specific antibodies against VDR (αVDR) and RXR (αRXR) were added to the binding reactions before the addition of the labeled fragment. Finally, DNA-protein complexes were resolved on 6% polyacrylamide gels in 0.5× TBE buffer. The gels were then dried and autoradiographed at −70 C.

Protein-Protein Interactions

Pull-down assays were performed with 5 μl of in vitro translated L-[35S]methionine-labeled GHF-1, VDR, or the same amount of their truncated forms. These proteins were incubated with the fusion proteins GST-VDR, GST-ACTR, or GST-CBP or with the same amount of GST as a control, immobilized in glutathione-sepharose beads as previously described (34, 35). Where indicated, vitamin D was included in the binding reaction. The bound proteins were analyzed by SDS-PAGE and autoradiography.

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