Regulation of Rat Cytochrome P450C24 (CYP24) Gene Expression

EVIDENCE FOR FUNCTIONAL COOPERATION OF Ras-ACTIVATED Ets TRANSCRIPTION FACTORS WITH THE VITAMIN D RECEPTOR IN 1,25-DIHYDROXYVITAMIN D3-MEDIATED INDUCTION*

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The hormone 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3 or calcitriol) is a pleiotropic secosteroid that directs many biochemical and cellular functions associated with calcium homeostasis, cellular proliferation and differentiation, and the immune response (1–4). The transcriptional regulation of target genes by 1,25-(OH)2D3 is mediated by the vitamin D receptor (VDR), a member of the ligand-dependent nuclear receptor superfamily) binds to specific control elements referred to as vitamin D response elements (VDREs) that are located in the promoter of target genes (8–9). Current evidence suggests the VDR-RXR heterodimeric complex binds to the VDRE in the unliganded state (3, 10) and is subsequently activated following ligand binding. Transactivation of the target gene is achieved through an intermediary coactivator complex that functions to link the VDR-RXR receptor complex to the RNA polymerase II holoenzyme (10–12).

The level of expression of 1,25-(OH)2D3-responsive genes in tissues is dependent on the presence of VDR and the concentration of intracellular 1,25-(OH)2D3. Steady-state levels of 1,25-(OH)2D3 in cells and the circulation are determined by a balance between 1,25-(OH)2D3 bioactivation and degradation (1). The rate-limiting step in the bioactivation pathway is catalyzed by 25-hydroxyvitamin D3 1-hydroxylase (13, 14), and the kidney is the major site of synthesis of biologically active 1,25-(OH)2D3. Metabolic inactivation of 1,25-(OH)2D3 and conversion to water-soluble products are catalyzed by the C24 oxidation pathway (15). The initial step in this pathway involves the mitochondrial cytochrome P450C24 enzyme (i.e. 25-hydroxyvitamin D3 24-hydroxylase, CYP24) (16). In the normal situation, CYP24 is expressed mainly in the kidney but can be substantially induced by 1,25-(OH)2D3 in this and many other tissues. The up-regulation of CYP24 in response to 1,25-(OH)2D3 constitutes an important negative feedback mechanism, whereby the hormone acts to regulate its ambient and cellular concentration. Hence, expression of CYP24 in kidney and other tissues serves a protective function in guarding against excessive levels of 1,25-(OH)2D3 that can be deleterious due to the attendant hypercalcemia. In addition, specific biological actions in bone have been reported for 24,25-dihydroxyvitamin D3 (24,25-(OH)2D3) (17), a metabolite whose cellular concentration is directed by regulation of CYP24 gene expression. It is clear, therefore, that regulation of CYP24 gene expression is important in the metabolism and function of metabolites from the vitamin D pathway.

Studies into the molecular mechanism by which 1,25-(OH)2D3 induces the CYP24 gene are in progress (18–21). In the rat promoter, there are two functional VDREs located at −136/−150 and −244/−258 on the antisense strand (18). To date, this dual arrangement of VDREs is unique among the 1,25-(OH)2D3-responsive genes. The two VDREs conform to the classic sequence, each consisting of two direct repeats separated by a 3-bp spacer. Gel mobility shift analysis using antibodies has established that both VDREs bind heterodimeric complexes of VDR-RXR (18). Transcriptional synergy between the two VDREs has been demonstrated and is particularly...
Evident at high levels of 1,25-(OH)\(_2\)D\(_3\) (18). Indeed, the CYP24 promoter appears to be the most responsive promoter to 1,25-(OH)\(_2\)D\(_3\) so far identified with levels of induction of 20–70-fold being observed in transient assays (21). In our previous studies on the rat CYP24 promoter, we unexpectedly observed that the proximal VDRE had greater transactivation ability than the distal VDRE, yet its affinity for the VDR-RXR complex in gel mobility shift assays was markedly lower (18). This finding raised the possibility of transcriptional cooperation between the VDR-RXR complex and nearby transcription factors (22). To investigate this possibility, we used computer analysis to identify both an Ets-binding site and a possible AP-1 site juxtaposed and downstream to the proximal VDRE. A possible gene regulatory role for an Ets-binding site in the VDRE functionality in mediating the action of 1,25-(OH)\(_2\)D\(_3\) to induce expression of the CYP24 gene.

**Experimental Procedures**

**Materials**—1,25-(OH)\(_2\)D\(_3\) was supplied by Hoffmann-La Roche. A Sequenase version 2.0 sequencing kit was purchased from U. S. Biochemical Corp. Oligonucleotides were synthesized by Bresatec (Adelaide, South Australia). The luciferase assay kit was from Promega (Madison, WI). Nickel-nitrotriacetic acid chromogaphic support and glutathione-agarose were from Qiagen GmbH (Germany).

**Promoter-Luciferase Constructs and Expression Plasmids**—Two oligonucleotide polymerase chain reaction primers engineered with KpnI sites were employed to amplify –186 bp of the CYP24 promoter sequence (encompassing the proximal VDRE at –156/–136) together with 74 bp of 5'-untranslated region using the pCYP24WT (–208)–Luc plasmid (18) as a template. The polymerase chain reaction product was cloned at the KpnI site in pG3L-Basic vector containing the firefly luciferase reporter gene as described previously (24). Mutations in promoter-luciferase constructs (see Fig. 1B) in VDRE half-sites, EBS, and glutathione-agarose were from Qiagen GmbH (Germany).

**Gel Mobility Shift Assays**—Double-stranded oligonucleotides were synthesized to contain the EBS and AP-1-like sites (CYP24-EB5A), a mutated core sequence of EBS (CYP24-mEBS), a mutated AP-1-like sequence (CYP24-mAP1), the proximal VDRE alone (CYP24-VDRE), or together with EBS as a composite oligonucleotide (VDRE + EBS). An oligonucleotide that encompassed a known Ets-1-binding site from T-cell receptor gene enhancer (TCR-Ets-1) was employed as a control (21). Each double-stranded oligonucleotide was designed with SfiI and Xbol restriction enzyme sites at the 5’ or 3’ ends as shown.

1. **Oligonucleotide CYP24-EB5A**
   - 5’-TCGACCGTCAGCTCCATCCTCTTC-3’
   - 3’-GCCGCAAGGATGTCAGAAAGAAGCT-5’

2. **Oligonucleotide CYP24-mEBS**
   - 5’-TCGACGGTTCCTCCATCCTCCTTC-3’
   - 3’-GCCGCAAGGATGTCAGAAAGAAGCT-5’

3. **Oligonucleotide CYP24-mAP1**
   - 5’-TCGACGGGCGCCCTCCTCACTCCTCCC-3’
   - 3’-GCCGCGGAGGATGTCAGAAAGAAGCT-5’

4. **Oligonucleotide CYP24-VDRE**
   - 5’-TCGACCGCGCCCTCCTCACTCCTGACCTCACCTCTCCTTC-3’
   - 3’-GCCGCGGAGGATGTCAGAAAGAAGCT-5’

5. **Oligonucleotide TCR-Ets-1**
   - 5’-TCGACGACCCACATCCTCTGGAGC-3’
   - 3’-GTGGTGTAGGAGAAGCGCT-5’

**Oligonucleotide VDRE + EBS**

Nuclear extracts were prepared from COS-1 cells (28). Each double-stranded oligonucleotide was labeled by end-filling with [\(^{32}\)P]dCTP using Klenow enzyme and purified by 12% polyacrylamide gel electrophoresis. Binding reactions for each assay contained 5 μg of nuclear protein, 1 μl of poly(dI-dC) in a final volume of 12 μl in Dignam buffer C (20 mM HEPES buffer, pH 7.6, 420 mM NaCl, 0.5 mM dithiothreitol, 5 mM MgCl\(_2\), 0.5 mM EDTA, 20% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride) and were incubated on ice for 15 min. Radiolabeled probe (200,000 cpm) was added, and samples were incubated on ice for another 30 min. For gel-shift inhibition assays, a polyclonal antibody to Ets protein DNA binding domain (PAN-Ets) was employed, and a VDR monoclonal antibody designated IgG2b (Affinity BioReagents Inc., Ne- shanic Station, NJ) was used as a control. For supershift assays, a polyclonal antibody to Ets-1 (29) and a polyclonal antibody to Erg-1/ Erg-2 (Santa Cruz Biotechnology Inc.) were employed. These antibodies were included in the binding reaction and incubated on ice for 15–30 min prior to addition of probe. Gel-shift competition assays were performed with unlabeled competitor oligonucleotide at molar excess concentra- tions (50–100-fold) by inclusion in the binding reactions. Radi- tarred DNA nuclear protein complexes were resolved on a 4% nondenaturing polyacrylamide gel using precoced low ionic strength gel running buffer (0.5× TBE) at 4 °C. The gel was dried and exposed to Kodak X-Omat AR film with an intensifying screen at –80 °C.

**Gel Mobility Shift Assays Using Purified Ets-1, VDR, and RRX**—Recombinant full-length human Ets-1 (p54Ets-1) was produced in Esch- erichia coli as a carboxyl histidine-tagged fusion protein and purified on a nickel-nitrotriacetic acid column. The tagged Ets-1 protein migrated as a single band with an apparent molecular mass of about 55 kDa in SDS-polyacrylamide gels. Recombinant VDR and RRX were produced in E. coli as N-terminal glutathione S-transferase (GST) fusion proteins. A clone pGEX-2T RRX, from Dr. George Muscat, University of Queensland, Australia, expressed GST-RRX, and a clone (pGEX-2T VDR) was created in our laboratory with human VDR cDNA cloned into pGEX2T in frame with GST sequence. The GST-VDR and GST-RRX fusion proteins were purified by affinity binding to glutathione-agarose beads.

DNA binding reactions contained recombinant Ets-1, GST-VDR, or GST-RRX with 50 ng of poly(dI-dC) in a final volume of 10 μl in binding buffer (20 mM Tris-HCl buffer, pH 8.0, 100 mM NaCl, 5 mM dithiothreitol, 1.0 mM EDTA, pH 8.0, 10% glycerol, and 100 μg/ml bovine serum albumin). Reactions were incubated at room temperature for 10 min. Radiolabeled probes (10,000 cpm) were added and samples further...
incubated at room temperature for 20 min. For supershift assays and competition binding assays, antibodies for Ets-1 and unlabeled competitor oligonucleotides, respectively, were included prior to addition of probe. Retarded complexes were resolved by 5% nondenaturing polyacrylamide gel electrophoresis. Association constants ($K_a$) for the binding of recombinant Ets-1 to CYP24-EBS and CYP24-mAP1 were determined by gel-shift assays. Increasing amounts of purified Ets-1 (0.125, 0.250, 0.500, and 1–4 μg) were incubated in a binding reaction with 1 ng of radiolabeled probe. The DNA protein complexes were resolved by 5% nondenaturing gel electrophoresis, dried, and exposed in the PhosphorImager (Molecular Dynamics). Ets-1-bound DNA complexes and unbound-probe were quantified using ImageQuant Software. The association constants, defined as the Ets-1 concentration at which 50% of the DNA probe was retarded, were determined by direct curve fitting (Sigma Plot, Jandel Scientific).

RESULTS

Identification of a Functional Ets Protein-binding Site (EBS) in the CYP24 Promoter—Previous expression studies (18) of the rat CYP24 promoter suggested that 1,25-(OH)$_2$D$_3$-mediated transactivation at the proximal VDRE (−136/−150) required the participation of a nearby transcription factor(s). An examination of sequences downstream from this VDRE revealed a likely binding site for Ets transcription factors (5'-AGAGGAT-GGA-3') at −119/−128 on the antisense strand (Fig. 1A). Members of the Ets transcription factor family bind to a common core motif (5'-GGAG/AT-3') with flanking sequence determining the specificity of binding. The site identified here with a 5'-GGAT-3' core, strongly resembles known binding sites for Ets-1 (30). Also noted was a possible AP-1-like sequence (5'-TGACTCC-3') at −132/−126, which differs from the consensus AP-1 site (5'-TGACTCA-3') by one nucleotide and overlaps the Ets-binding site (EBS).

To evaluate whether the EBS contributes to 1,25-(OH)$_2$D$_3$-dependent CYP24 promoter expression, the core EBS motif was mutated (5'-GGAT-3' to 5'-TTTT-3') in a luciferase-reporter construct that contained −298 bp of promoter sequence and both VDREs of the CYP24 promoter. The wild-type and mutated EBS promoter constructs are designated pCYP24WT(−298)-Luc and pCYP24mEBS(−298)-Luc, respectively (Fig. 1, B and C). In response to 1,25-(OH)$_2$D$_3$, the wild-type construct gave a 27.2-fold level of induction, but this was reduced to 14.2-fold when the EBS was inactivated (Fig. 2). The importance of the EBS in sustaining the 1,25-(OH)$_2$D$_3$-mediated transactivation of the CYP24 promoter constructs was explored further by cotransfection studies with an Ets-1 expression vector. Cells containing the pCYP24WT(−298)-Luc displayed more than a doubling of activity (58.2-fold) when cotransfected with Ets-1 (Fig. 2). The mutant EBS construct in the presence of Ets-1 gave the same activity (14.5-fold) as observed for this mutated construct in the absence of Ets-1 (Fig. 2). It was evident from these studies, therefore, that the EBS was critical for maximal up-regulation of the CYP24 promoter. Due to the location of the EBS in the region downstream of the proximal VDRE, subsequent studies were conducted using constructs that contained only the proximal promoter element and adjacent EBS and AP-1-like sequences.

The single VDRE construct (−186 bp of promoter sequence; pCYP24WT-Luc) expressed a 7.6-fold level of induction (Fig. 2), which is about 28% of the value obtained with the construct containing both functional VDREs (i.e. pCYP24WT(−298)-Luc). This activity for the proximal VDRE is about 2-fold higher than the contribution made by the more distal VDRE (data not shown), which reflects the cooperative synergism between the two VDREs in the rat CYP24 promoter (18). Mutation of the EBS in the proximal VDRE construct (i.e. pCYP24mEBS-Luc) resulted in a substantial loss in transactivation activity (7.6- to 4.2-fold) that was qualitatively similar to results obtained using the native two-VDRE construct (i.e. pCYP24WT(−298)-Luc) (Fig. 2). Transfected Ets-1 plasmid was ineffective in inducing transactivation of the mutated EBS construct (i.e. pCYP24mEBS-Luc, 4.3-fold induction), whereas induction was increased from 7.6- to 14.0-fold in cells containing the wild-type construct with an intact EBS (i.e. pCYP24WT-Luc) (Fig. 2).

Mutagenesis of the VDRE alone (pCYP24mVDRE-Luc, Fig.
1B) lowered induction of the wild-type construct to 1.2-fold (Fig. 2) confirming that this is the only functional VDRE present in the −186 bp promoter sequence as reported previously (24). Overexpression of Ets-1 was also investigated on the construct pCYP24mVDRE-Luc. In this situation, there was no effect on expression in the presence of hormone (Fig. 2) confirming that Ets-1 cannot transactivate in the absence of a functional VDRE. The data in Fig. 2 provide evidence for transcriptional synergy between the EBS and VDRE. For example, the 1,25-(OH)_{2}D_{3} induction of the wild-type promoter construct pCYP24WT-Luc in the presence of Ets-1 was 14.0-fold, which is greater than the sum of the individual contributions from VDRE (4.2-fold) and EBS (1.2-fold). When the constructs pCYP24WT-Luc and pCYP24mEBS-Luc were tested at a lower 1,25-(OH)_{2}D_{3} concentration (i.e., 10^{-10} M), a decrease in induction occurred for the wild-type construct (from 7.6 ± 1.1- to 2.8 ± 0.3-fold), and no induction was observed for the mEBS construct. For comparison, the induction of the −298 construct (pCYP24WT(−298)-Luc) was also examined. At this low concentration of 1,25-(OH)_{2}D_{3} induction was lowered from 27.2 ± 1.8- to 3.7 ± 0.5-fold, and mutagenesis of the EBS led to the loss of induction (1.5 ± 0.25-fold). It is clear, therefore, that the EBS is critical for the function of the proximal VDRE, especially when hormone is near physiological concentration.

Specificity of Transactivation by Ets Proteins—The role of EBS in stimulating CYP24 promoter activity was evaluated in the transactivation assay using various Ets proteins. Ets family members can be classified according to their homology to Ets-1 (31). Class I members such as Ets-1 and Ets-2 have extensive overall sequence similarity (30). Erg-1 and Fli-1, class II members, show homology to Ets-1 both in their N-terminal transactivation and C-terminal DNA binding domains, whereas class III members such as PU.1 show homology only in the DNA binding domain (30). We investigated the ability of a representative member from each of the three classes (human Ets-1, Fli-1, and PU.1) to transactivate the wild-type CYP24 promoter in COS-1 cells. Neither Fli-1 nor PU.1 further increased the 7.6-fold level of 1,25-(OH)_{2}D_{3} induction observed with the pCYP24WT-Luc construct (Fig. 3). By contrast, Ets-1 elevated 1,25-(OH)_{2}D_{3} induction of the wild-type construct to 14.0-fold (Fig. 3). The inability of Fli-1 or PU.1 to bind the EBS was not investigated.

The transactivation activity of p42Ets-1, an alternatively spliced form of Ets-1 (32), and Ets-2, both class I members of the Ets family, were also evaluated in overexpression studies. The activity of p42Ets-1 was comparable to Ets-1, where it stimulated the wild-type level of induction from 7.6- to 15.6-fold, whereas Ets-2 was less active (7.6- to 10.8-fold) (Fig. 3). It was further shown that the observed transactivation activities of p42Ets-1 and Ets-2 were completely abrogated when the EBS site in the promoter was mutated. Similar to Ets-1, neither p42Ets-1 nor Ets-2 altered basal expression of the wild-type construct or expression of the mutant VDRE construct (pCYP24mVDRE-Luc) in the presence or absence of hormone (data not shown).

EBS Binds Multiple Proteins from COS-1 Cell Nuclear Extracts—To investigate nuclear protein binding to the EBS, gel mobility shift analysis was carried out using COS-1 cell nuclear extracts and the oligonucleotide probe CYP24-EBs. Six nuclear-protein complexes (C1–C6) were consistently detected together with an inconsistently appearing band(s) between C3 and C4 (Fig. 4A). A similar profile of protein binding was observed with a control Ets-1 probe (TcRa-Ets-1) from the T-cell receptor α-gene enhancer (27), but the binding was weaker than for CYP24-EBs (Fig. 4A). Mutagenesis of the core region in CYP24-mEBS abolished the binding of all complexes except C4 and C5 (data not shown). Self-competition experiments inhibited formation of complexes C1–C3 and C6 (Fig. 4B). In contrast, competition experiments with the nonspecific probe CYP24-VDRE and mutated probe CYP24-mEBS had no effect on complexes C1–C3 and C6. However, the nonspecific and mutated probes were partially active in preventing formation of the apparent nonspecific complexes migrating between C3 and C6 (Fig. 4B). To characterize further the Ets proteins

![Graph showing fold vitamin D induction](image)
present in complexes C1–C6, gel mobility shift experiments employing various antibodies were conducted. A neutralizing polyclonal antibody (PAN-Ets) that interferes with the binding of Ets proteins to the core sequence markedly reduced the formation of complexes C1–C3 but only weakly affected C6 and had no effect on C4 or C5 complex formation (Fig. 4C). Control VDR monoclonal-neutralizing antibody was without effect (Fig. 4C). An Ets-1-supershifting polyclonal antibody (29) functioned to supershift complexes C1, C2, and C3 (data not shown).

However, migration of complexes C1–C6 was not altered by either a supershifting polyclonal antibody to the Ets proteins Erg-1/Erg-2 or an Ets-2 polyclonal supershifting antibody (data not shown). It can be concluded from the antibody gel mobility shift data that EBS binds complexes C1–C3 and C6, which are related immunologically to Ets-1. However, complexes C4 and C5 appeared to be nonspecifically bound to the CYP24-EBS probe. We have shown directly that purified Ets-1 binds to CYP24-EBS but not to CYP24-mEBS (Fig. 5). The complex was competed by self and TRcEts-1 but not with a nonspecific oligonucleotide or CYP24-mEBS. The recombinant Ets-1 complex was found to comigrate with complex C3 (data not shown, see Fig. 4 for binding complexes migration patterns).

An AP-1-like Sequence Overlaps the EBS—A possible AP-1-like site (∼132–126) with a 3-base overlap at the 3′-end of the EBS on the antisense strand (Fig. 1A) was evaluated extensively for functionality. Protein binding to this site was not observed in gel mobility shift assays with either purified c-Fos or c-Jun protein or with nuclear extracts from phorbol ester-treated cells. Furthermore, overexpression of c-Fos and c-Jun failed to alter CYP24 promoter expression (data not shown). Therefore, rather than functioning as a classical AP-1 site, the possibility was considered that the AP-1-like sequence represents extended EBS-flanking sequence. To investigate this hypothesis, both the putative AP-1-like sequence and the EBS were mutated (i.e. pCYP24mAP1-Luc and pCYP24mEBS-Luc) and compared for transactivation activity. Induction with 1,25-(OH)2D3 (7.6-fold) was lowered to 5.8-fold when the AP-1-like and EBS were mutated (data not shown). Hence, mutagenesis of the AP-1-like sequence substantially reduces transactivation by Ets-1.

To investigate further the AP-1-like sequence, transactivation experiments with Ets-1 were performed. When Ets-1 was overexpressed, induction by 1,25-(OH)2D3 of the wild-type construct was 14.0-fold, which was reduced to 8.2-fold with pCYP24mAP1-Luc (Fig. 6). By comparison, mutagenesis of the EBS core gave a 4.2-fold level of induction, and overexpressed Ets-1 was ineffective (Fig. 6). Hence, alteration of the flanking sequence substantially reduces transactivation by Ets-1. Consistent with the transactivation studies, gel mobility shift assays with CYP24-mAP1 showed reduced binding of complexes C1–C3 and C6 (data not shown). Association constants (Ka) were determined in the gel mobility shift assays for the binding of recombinant Ets-1 to CYP24-EBS and CYP24-mAP1 probes. By using the wild-type probe, the Ka value for Ets-1 was 1.2 ± 0.12 × 106 M⁻¹, which decreased by ∼25% to 0.89 ± 0.09 × 106 M⁻¹ with probe containing the mutated flanking sequence. This decrease in Ka for the CYP24-mAP1 probe supports further the proposal that the 5′-TGA-3′ sequence from the AP-1-like site is important for optimal binding of endogenous Ets proteins. Based upon the collective data, it was evident that the AP-1-like site was not functional. Therefore, subsequent studies were directed at delineating the detailed function of the EBS to facilitate the transactivation activity of 1,25-(OH)2D3 on the rat CYP24 promoter.

Ets-1 and VDR-RXR Form a Ternary Complex on DNA—Although the identity of the Ets proteins that bind to the EBS in vivo are not known, we have demonstrated that this site can respond to Ets-1 in transactivation experiments and bind recombinant Ets-1. On this basis, we investigated further the mechanism of Ets-1 action. By using gel mobility shift analysis, we showed that purified Ets-1, VDR, and RXR form a complex on an oligonucleotide (VDRE + EBS) that spans both the proximal VDRE and the EBS. When Ets-1 was incubated with the probe, a simple complex C-I was retarded (Fig. 7, lane 1), and the amount of this complex was reduced by addition of VDR and RXR with the appearance of a new slower mobility complex C-II (lane 3). Complex C-II was also generated when VDR and RXR were added to DNA prior to Ets-1 (data not shown). The complex generated by VDR and RXR alone was intermediate in size between complexes C-I and CII (Fig. 7, lane 4). Formation of the complexes C-I, C-II, and VDR-RXR was suppressed (lane 5) in the presence of excess unlabeled self-competitor oligonucleotide (VDRE + EBS), which established the specificity of these complex formations. To demonstrate that C-II results from the formation of a ternary complex between Ets-1, VDR, RXR, and the VDRE + EBS probe, compe-
Ets Proteins and Vitamin D Induction

The EBS in the CYP24 Promoter Is a Ras/Raf-responsive Element—The Ras/Raf signaling pathway plays a key role in the activation of Ets-1 through phosphorylation of threonine residue 38 (26, 33). We investigated whether a similar Ras-mediated action is involved in the transactivation of the CYP24 promoter by exogenous Ets-1. Initial tests used wild-type cEts-1 (the p54 form of chicken Ets-1) and a mutant form where threonine 38 was mutated to an alanine (i.e. T38A) (26). Overexpression of cEts-1 increased 1,25-(OH)₂D₃ induction of pCYP24WT-Luc from 7.6- to 11.6-fold (Fig. 8). When the EBS was mutated, there was no effect of overexpressed cEts-1 on induction (data not shown). By contrast, overexpression of the T38A mutant reduced the level of induction from 7.6- to 4.4-fold, a level comparable to that observed when the EBS was mutated (see Fig. 8). These results were interpreted to mean that the mutant protein competes with endogenous Ets proteins for binding to EBS but is unable to enhance transactivation. Therefore, these experiments establish threonine 38 as a critical residue for cEts-1 transactivation and imply that the Ras pathway is involved in cEts-1 function.

When Ras was overexpressed, the level of induction of the pCYP24WT-Luc construct was increased from 7.6- to 11.2-fold, and this was further increased to 16.4-fold when wild-type cEts-1 was also transfected (Fig. 8). The Ras-dependent transactivation was completely inhibited when the EBS was mutated (Fig. 8). When the T38A mutant cEts-1 was overexpressed, the Ras-mediated level of induction was lowered to 4.9-fold, which corresponded to the level of induction observed with the mutated EBS (Fig. 8). When the VDRE was mutated (i.e. pCYP24mVDRE-Luc), there was no effect of exogenous Ras on either basal expression or 1,25-(OH)₂D₃-dependent induction (Fig. 8) in keeping with the conclusion that the VDRE is critical for EBS function.

Further evidence for the involvement of endogenous Ras activity was obtained with manumycin A, an inhibitor of Ras farnesylation (35). The inhibitor was not effective at 10 μM, but when tested at 50 μM, 1,25-(OH)₂D₃-dependent induction of pCYP24WT-Luc was lowered from 7.7- to 4.1-fold, and the same result was observed with inhibitor at 100 μM. This level of expression was equivalent to that observed with mutated EBS (e.g. see Fig. 2). Additionally, there was no effect of manumycin A on induction when the EBS was inactivated. Hence, in these cells, the Ras signaling pathway appears to be entirely responsible for Ets protein activation.

Ets-1 Increases Basal Expression in Presence of a VDR Homodimer—Ets-1 was unable to alter basal expression even in the presence of overexpressed Ras. In a related manner, we have recently shown that the binding of VDR-RXR inhibits basal expression of the CYP24 promoter possibly through the action of a corepressor (24). The binding or activation of Ets-1 could be prevented by the putative corepressor. As seen in Fig.

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**Fig. 7.** Purified VDR, RXR, and Ets-1 form a ternary complex with VDRE + EBS oligonucleotide. A double-stranded oligonucleotide encompassing VDRE and the EBS site (VDRE + EBS) was labeled by end-filling with [γ⁻³²P]dCTP and incubated either with purified Ets-1 (lane 2) or VDR and RXR (lane 4), and the specific complexes are indicated by arrows. In lanes 3, 5, and 6, the probe was preincubated with purified Ets-1 for 15 min followed by addition of VDR and RXR. The specific complexes retarded (arrowed) were for Ets-1 alone (CI), VDR-RXR complex, and a unique complex (CII) resulting from the addition of VDR and RXR. CI was competed by 100-fold molar excess of self-competitor (lane 5), CYP24-VDRE alone (lane 6), and CYP24-EBS alone (lane 7). This experiment was repeated three times, and the same result was obtained.

**Fig. 8.** Ras and cEts-1 Thr-38 stimulate 1,25-(OH)₂D₃ directed induction of the CYP24 promoter. COS-1 cells were cotransfected with either pCYP24WT-Luc, pCYP24mEBS-Luc, or pCYP24mVDRE-Luc together with 10 μg of wild-type c-Ets-1 Thr-38 or mutated c-Ets-1 T38A expression clones (see Fig. 2). CYP24 promoter constructs were tested for 1,25-(OH)₂D₃-mediated transactivation in the presence or absence of transfected Ras expression clone (10 μg) (see “Experimental Procedures”). Data presented are the average of three independent experiments ± S.D.
Juxtaposed to the proximal VDRE in both the rat and human, expression in the presence of VDR

Interestingly, the proximal VDRE supports transactivation to a greater extent than does the distal VDRE. The molecular basis for this is not fully understood, but it is likely due to the measurement of basal activity. Luciferase activity of pCYP24WT-Luc is taken as 100%, and data presented are the average of three independent experiments ± S.D.

9, however, basal expression was increased when cells were transfected with a mutant form of VDR (VDRE) that strongly binds to the VDRE as a homodimer (24). Overexpression of Ets-1 (together with the mutant VDR) consistently resulted in a further increase in basal expression (Fig. 9). This result indicates that the presence of unliganded VDR-RXR on the promoter, but not VDRE, prevents the action of Ets-1. Based upon the current results and our previous work (24), we favor a model in which the putative corepressor-complex bound to unliganded VDR-RXR is responsible for EBS inactivity during basal conditions.

DISCUSSION

Previous results from our laboratory (18) and others (19, 20) have shown that the first 300 bp of rat CYP24 promoter sequence is required for the 1,25-(OH)2D3 regulation of gene expression. The hormone-initiated transactivation is mediated through two VDREs located at −136/−150 and −244/−258. Interestingly, the proximal VDRE supports transactivation to a greater extent than does the distal VDRE. The molecular basis of this observation was investigated in the current study by evaluating sites immediately adjacent to the proximal VDRE that could function to alter VDR-RXR-initiated transactivation. We subsequently identified a possible AP-1 site (−132/−126) and a consensus Ets-binding site (−128/−119) that were juxtaposed to the proximal VDRE in both the rat and human (34) CYP24 promoters. The molecular role of the two binding sites in promoting the gene-regulatory action of 1,25(OH)2D3 was the current project focus.

The presence of a functional AP-1 site in the promoter of the CYP24 gene has been suggested from various studies in which 24-hydroxylase enzyme activity was shown to be stimulated in response to treatment with the phorbol ester TPA (23, 36, 37). The role of TPA as a potent stimulator of protein kinase C (PKC) activity is well documented, and many of its actions are expressed through the Jun/Fos AP-1 system (38, 39). Also applicable to the current study is the observation that composite AP-1/Ets sites can function in a cooperative fashion to regulate gene expression (40). Consequently, the possibility was considered that the AP-1 sequence near the proximal VDRE could be involved in the action of phorbol esters to stimulate expression of the 24-hydroxylase enzyme. However, the AP-1-like sequence was found not to be a functional AP-1 site, but it was demonstrated instead to constitute a 3′-flanking sequence of the EBS on the antisense strand. Therefore, if an AP-1 site is involved in mediating the up-regulation of CYP24 gene expression, it must be located in another region of the promoter (41).

The identified Ets-binding site (EBS) was located approximately two turns of the helix downstream from the proximal VDRE. It was found to contain a 5′-GGAT-3′ core sequence that has been shown by other investigators (30) to provide specificity for the binding of Ets-1 and other closely related Ets proteins. In the corresponding position in the human CYP24 promoter, the same EBS is present although it differs by one nucleotide in the flanking region (33). The functionality of the EBS in the rat CYP24 promoter was evaluated by transient expression analysis of constructs that contained both VDREs or only the proximal VDRE. In both instances, mutagenesis of the core sequence within the EBS did not alter basal expression of the construct but lowered the level of 1,25-(OH)2D3 induction. Participation of an Ets protein in the induction mechanism was strongly supported by the finding that overexpression of either Ets-1, p42Ets-1, or Ets-2 markedly increased the transactivation response to 1,25-(OH)2D3 in which stimulation by the Ets proteins was totally dependent upon an intact EBS. For example, transactivation by Ets-1 resulted in a 14-fold induction of the proximal VDRE construct, which was reduced by 70% when the EBS was mutated. By comparison, promoter induction was not affected by overexpression of the class II Fli-1 or class III PU.1 proteins. It was concluded, therefore, that specific Ets proteins, closely related to Ets-1 and representing class I members (31), can stimulate 1,25-(OH)2D3-dependent induction in transactivation experiments. The activity of Fli-1 and PU.1 could reflect their inability to bind the EBS in the CYP24 promoter. The stimulatory action of the alternatively spliced isozyme p42Ets-1 is interesting, since this protein failed in other studies to transactivate a functional EBS in the human GM-CSF promoter. The distribution and relevance of this isozyme to CYP24 gene regulation in kidney and other tissues remains unknown.

The EBS in the CYP24 promoter bound six protein complexes in the COS-1 cell nuclear extracts as determined by gel mobility shift analysis. Formation of four of the complexes (C1–C3, C6) was inhibited in self-competition experiments. The three larger complexes (i.e. C1–C3) were supershifted with Ets-1-specific polyclonal antibody and neutralized with a PAN-Ets antibody. Based upon gel mobility shift studies with pure protein, complex C3 appeared to be an Ets-1 complex. However, the identity of this complex and the other two higher molecular weight complexes remains to be conclusively determined. The fourth and smallest Ets complex (i.e. C6) displayed lower specificity as it did not supershift, and neutralization of its binding to EBS with the PAN-Ets competitive antibody was weak. Recently, another Ets protein with similarities to Ets-1 and representing the other two higher molecular weight complexes was investigated. The fourth and smallest Ets complex (i.e. C6) displayed lower specificity as it did not supershift, and neutralization of its binding to EBS with the PAN-Ets competitive antibody was weak. Recently, another Ets protein with similarities to Ets-1 was identified in kidney and other tissues (42). Such observations emphasize the challenge in identifying a specific endogenous Ets protein(s) that stimulates CYP24 promoter induction. A similar problem has arisen in other studies where in vitro binding of multiple Ets proteins to a single functional EBS has been observed (25, 43).

Mutagenesis of the EBS and VDRE motifs in transactivation experiments provided evidence for transcriptional synergism between the two sites in response to hormone. This cooperation may reflect a direct interaction between the Ets-1 type protein and liganded VDR-RXR complex on the CYP24 promoter. Although this remains to be demonstrated, numerous other studies have established synergy between Ets-1 and transcription factors on both cellular and viral promoters (44–52), between...
Ets-1 and a partner protein (43–45, 47, 49), or an indirect interaction of Ets via the coactivator CBP (53). Ets-1 possesses internal negative regulatory domains that hinder its monomeric binding to DNA (54), and therefore, the interaction with a neighboring protein would facilitate binding to the promoter (55, 56). The lack of such an interaction would explain why the EBS is inactive when the proximal VDRE is mutated in the CYP24 promoter.

Ets proteins have been identified as key nuclear mediators of Ras/Raf action. There is recent evidence that the ability of Ets-1 to transactivate promoters is dependent upon its phosphorylation at a conserved threonine (residue 38) by the Ras/Raf pathway (26). However, phosphorylation of Ets-1 is apparently not required on some Ets-1-responsive promoters (26). Endogenous Ras was demonstrated in the current study to be important for Ets protein activation. Ras overexpression substantially enhanced the 1,25-(OH)2D3 induction of the CYP24 promoter, and the Ras response was totally dependent on an intact EBS. Furthermore, Ras overexpression potentiated transactivation by Ets-1, but Ras was ineffective with the T38A mutant Ets-1 protein in which threonine 38 was not available for phosphorylation. Interestingly, expression of the T38A mutant protein reduced the level of 1,25-(OH)2D3-induced expression to the activity observed by a CYP24 (OH)2D3. At lower hormone levels, in contrast, promoter expression would be expectedly weaker and result in lowered CYP24-mediated degradation of 1,25(OH)2D3 due to the dominant action of the proximal VDRE in which the EBS-binding proteins play a key role.

The present work represents the first report where Ets proteins have been shown to contribute to 1,25(OH)2D3-dependent promoter activity. Since several Ets proteins are coexpressed in most tissues (42, 58), it remains a challenge to determine which of these is required for activity on the CYP24 promoter. We demonstrated in COS-1 cells that signaling by Ras is essential for Ets-1 activation. However, PKC activity may also be important for CYP24 promoter expression (23, 36, 37). There are reports that 1,25(OH)2D3 stimulates PKC activity (59, 60), and recently, a plasma membrane VDR was identified that mediated PKC activation in response to 1,25(OH)2D3 (61, 62). This finding gives rise to the interesting possibility that CYP24 promoter expression in response to 1,25(OH)2D3 may be controlled at one or both extranuclear sites, involving PKC and Ras/Raf signaling. In that regard, it is evident from this study that the combined action of 1,25(OH)2D3 and mitogen-activated protein kinase pathway activity can facilitate a rapid response of the CYP24 gene to 1) tightly regulate ambient 1,25(OH)2D3 levels and, thereby, prevent elevations in the hormone and calcium that would be toxic to critical organ functions, and 2) modulate synthesis of 24-hydroxylated metabolites that express important biological functions. Whether 1,25(OH)2D3 can directly activate Ras in the CYP24 promoter system remains an interesting issue for future studies.

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