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The Orphan Receptor Rev-ErbAα Activates Transcription via a Novel Response Element

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Rev-ErbAα (Rev-Erb) is a nuclear hormone receptor-related protein encoded on the opposite strand of the α-thyroid hormone receptor (TR) gene. This unusual genomic arrangement may have a regulatory role, but the conservation of human and rodent Rev-Erb amino acid sequences suggests that the protein itself has an important function, potentially as a sequence-specific transcriptional regulator. However, despite its relationship to the TR, Rev-Erb bound poorly to TR binding sites. To determine its DNA-binding specificity in an unbiased manner, Rev-Erb was synthesized in *Escherichia coli*, purified, and used to select specific binding sites from libraries of random double-stranded DNA sequences. We found that Rev-Erb binds to a unique site consisting of a specific 5-bp A/T-rich sequence adjacent to a TR half-site. Rev-Erb contacts this entire asymmetric 11-bp sequence, which is the longest nonrepetitive element specifically recognized by a member of the thyroid/steroid hormone receptor superfamily, and mutations in either the A/T-rich or TR half-site regions abolished specific binding. The binding specificity of wild-type Rev-Erb was nearly identical to that of C- and N-terminally truncated forms. This binding was not enhanced by retinoid X receptor, TR, or other nuclear proteins, none of which formed heterodimers with Rev-Erb. Rev-Erb also appeared to bind to the selected site as a monomer. Furthermore, Rev-Erb activates transcription through this binding site even in the absence of exogenous ligand. Thus, Rev-Erb is a transcriptional activator whose properties differ dramatically from those of classical nuclear hormone receptors, including the TR encoded on the opposite strand of the same genomic locus.

Rev-Erb (also known as *ear1*) is encoded on the noncoding strand of the α-thyroid hormone receptor (TR) gene, such that its mRNA is complementary to that of the TR splice variant, TRα2 (also called c-ErbAα2) (34, 41). This unusual relationship is likely to serve an important role in the regulation of gene expression from this locus (31, 42). Rev-Erb mRNA is expressed in many cell types but is most abundant in muscle, brown fat, and brain (34). Furthermore, Rev-Erb protein is highly conserved between rats and humans (35) and is translated in vivo (18), suggesting an important function. Indeed, Rev-Erb contains a putative DNA binding domain (DBD) containing two zinc fingers of the Cys_4 type which are characteristic of steroid and thyroid hormone receptors (12, 15).

Rev-Erb is an orphan receptor (45) most similar to the TR/retinoic acid receptor (RAR) subgroup of nuclear receptors (13, 34). These receptors all contain an identical sequence in the first zinc finger of the DBD, referred to as the P box, which specifies binding to response elements containing the sequence AGGTCA (4, 10, 54), often arranged in tandem as direct or inverted repeats with variable spacing (43, 55). The unique actions of peroxisomal proliferators, vitamin D, thyroid hormone (T3), and retinoic acid are due at least in part to the ability of their receptors to differentially recognize direct repeats spaced by 1, 3, 4, and 5 bp, respectively (26, 55). Furthermore, these receptors bind to DNA with highest affinity as heterodimers with retinoid X receptors (RXRs) (8, 24, 25, 36, 39, 60, 61) and COUP-TF (5, 52), two members of the TR/RAR subgroup. Rev-Erb shares considerable sequence identity in the domains implicated in receptor homo- and heterodimerization (13, 23, 39, 44, 50).

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We considered whether Rev-Erb functions as a ligand-dependent transcriptional activator, as a constitutive transcriptional activator, or as a heterodimerization partner for other members of the TR/RAR subgroup. To address these issues, an iterative, polymerase chain reaction (PCR)-based DNA binding site selection technique (6, 59) was used to determine the ideal binding site for Rev-Erb. Remarkably, the selected Rev-Erb element (RevRE) is composed of a unique 5-bp A/T-rich sequence adjacent to a single AGG TCA half-site. Competition experiments confirmed the importance of the entire RevRE for DNA binding, and methylation and acylation interference assays demonstrated that bases in both the half-site and the A/T flank of the RevRE are directly contacted by Rev-Erb. Rev-Erb did not form functional heterodimers with TR, RXR, or other nuclear proteins and appeared to bind to the RevRE as a monomer. However, Rev-Erb had the ability to activate transcription via the RevRE site in the absence of added ligand. Therefore Rev-Erb acts via a mechanism distinct from that of better-studied nuclear receptors, including the TR which is encoded on the opposite strand of the same genomic locus. In addition, this is to our knowledge the first report demonstrating that two transcriptional activators are encoded on overlapping genes transcribed from opposite strands of the same genomic locus.

MATERIALS AND METHODS

Synthesis of Rev-Erb proteins in bacteria. Glutathione *S*-transferase (GST) fusion proteins were produced in *Escherichia coli* from pGEX-2TK (22). GST-RevT, containing amino acids 21 to 292 (numbered as in the human cDNA) of Rev-Erb, was generated by introducing via PCR a stop codon and *Stu*I and *Eco*RI restriction sites following nucle-

otide 1058 in the rat Rev-Erb cDNA (34) and subcloning the *Bam*HI-*Eco*RI fragment of the PCR product into pGEX-2TK. GST-Rev-Erb, containing all of the Rev-Erb sequences except the N-terminal 21 amino acids, was produced by subcloning the *Hin*dIII-*Nae*I fragment of the rat Rev-ErbAα cDNA into the *Hin*dIII-*Stu*I sites of GST-RevT, which removed the stop codon at amino acid 292 and substituted the entire additional coding region of the rat Rev-ErbAα cDNA, followed by its native stop codon after amino acid 614. Proteins were expressed in *E. coli* DH5α cells as previously described for production of TRα (23) and purified to 50 to 75% homogeneity by glutathione-Sepharose chromatography (Pharmacia).

Synthesis of Rev-Erb in reticulocyte lysate. Full-length Rev-Erb was synthesized in reticulocyte lysates from a *Bam*HI-to-*Nae*I fragment of the human Rev-Erb cDNA subcloned into pBluescript (pBS), using the TNT-T7 kit (Promega). Truncation products were made by runoff transcription/translation on cDNA templates restricted with *Xho*I (to synthesize Rev-199), *Eco*RV (to synthesize Rev-288), and *Eco*RI (to synthesize Rev-554).

Preparation of DNA fragments. For DNA binding site selection studies, the bottom strands of N_4AGGTCAN_{12} and N_{20} were 5′-AGACGGATCCATTGCAACCTCCCCCN_{12} TGACCTN_4GGGCCTGATCTGTAGGAATTCGGA-3′ and 5′-AGACGGATCCATTGCAACCCCCCCCCCN_{20}GGCCCT GATCTGTAGGAATTCGGA-3′, respectively. These oligonucleotides were made double stranded by using Klenow enzyme and amplified by PCR with primers A and B, which were 5′-CATCGACGCTCGTACACACTGTCCGTCTAGA TGACTCCGAATTCCTACAG-3′ and 5′-TCGTAAGCTGA CCTAGCATGCTACGCAATGCTGTAGACGGATCCATT GCA-3′, respectively.

PCR mixtures contained 10 ng of template, 200 ng (1.75 nM) each of primers A and B, 0.2 mM deoxynucleoside triphosphates, 2 U of *Taq* polymerase, and 1× PCR buffer (5 mM KCl, 10 mM Tris [pH 8.4], 5 mM $MgCl_2$, 0.1 mg of gelatin per ml) in 100 μl. Cycling conditions used to generate the initial double-stranded DNA were 4 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 3 min followed by 25 cycles of 94°C for 15 s, 62°C for 15 s, and 72°C for 15 s. In subsequent rounds of PCR, a single cycle of 94°C for 1 min, 62°C for 1 min, and 72°C for 3 min was followed by 25 cycles of 94°C for 15 s, 62°C for 15 s, and 72°C for 15 s. Labeled probes were obtained in a 50-μl reaction by using the same conditions but with a final concentration of 0.5 mM dATP, dGTP, and dTTP and 30 μCi of [^{32}P]dCTP. The reaction mixture was incubated at 95°C for 1 min, 37°C for 2 min, and 72°C for 3 min for a single cycle, and the probe was separated from free nucleotides on a Nick column (Pharmacia). PCR products were purified on 7.5% nondenaturing polyacrylamide gels.

EMSA. For the electrophoretic mobility shift assay (EMSA), the standard 30-μl binding reaction mixture contained labeled (30,000 cpm) or unlabeled (30 to 90 ng) DNA and Rev-Erb (from bacteria or reticulocyte lysate as described above) in 1× binding buffer plus 200 μg of poly(dI-dC) per ml. After incubation at room temperature for 20 min, reaction mixtures were loaded on a 7.5% polyacrylamide gel and the complexes were separated in 0.5× Tris-borate-EDTA at room temperature. Gels were dried prior to autoradiography. Bacterially expressed TRα1 and RXR (also known as H2RIIBP [16]), which have been described previously (5), and reticulocyte lysate-synthesized RXRα (38) were used in the heterodimerization studies.

Selection of binding sites. The selection method of Blackwell et al. (6, 7) was modified as follows. The double-stranded randomers (30 to 90 ng) were prepared as described above, incubated with 500 ng of purified GST-RevT, and subjected to EMSA as previously described (30). Bound DNA was excised from the portion of the gel between the free probe and the wells, eluted into buffer (0.5 M ammonium acetate, 10 mM $MgCl_2$, 0.1% sodium dodecyl sulfate, 1 mM EDTA) at 37°C for 8 to 16 h, PCR amplified and gel purified as described above, and subjected to EMSA with GST-RevT. This process was repeated six times. Fragments were further selected by affinity chromatography with GST-RevT prebound to glutathione-Sepharose (Pharmacia) at 4°C for 2 h in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–80 mM KCl–1 mM dithiothreitol–5% glycerol–25 μg of denatured herring sperm DNA per ml and then washed extensively with the same buffer. Bound protein and DNA were eluted with 50 mM reduced glutathione. DNA bound to GST-RevT was purified by phenol-chloroform extraction, PCR amplified, gel purified, and subjected to a second round of affinity chromatography. The pool selected from N_4AGGTCAN_{12} was subjected to one additional round of EMSA. Fragments were subcloned into *Xba*I-*Sph*I sites of pBS, PCR amplified with primers A and B, and subjected to EMSA with GST-RevT. Inserts in positive transformants were amplified by PCR, gel purified, labeled by a single round of PCR as described above, and then studied in the EMSA. Strongly binding inserts from 19 transformants selected from N_4AGGTCAN_{12} and 23 selected from N_{20} were sequenced by the dideoxy-chain termination method.

Methylation and acylation interference assays. The RevRE-containing insert from a selected clone (ACTCCCAGAATG TGGGTCAT) was labeled on either strand by using Klenow enzyme to fill in one 3′ overhang (*Xba*I or *Hin*dIII digested) prior to digestion with the second enzyme. The gel-purified, labeled DNA was modified by treatment with dimethyl sulfate (DMS) or diethylpyrocarbonate (DEPC) as described by Sturm et al. (51). The modified probes (300,000 cpm) were incubated with 5 μg of GST-RevT or GST-Rev-Erb in a 40-μl reaction mixture and subjected to EMSA. The protein-DNA complexes and free probe were eluted from the wet gel, and the DNA was cleaved with piperidine (3) and electrophoresed on a sequencing gel.

Cell culture, transfection, and CAT assays. The RevRE-TKCAT reporter plasmid was made by ligating four copies of the sequence GATCCAGAATGTAGGTCAGGATC (the Rev-Erb binding site is underlined) into the *Bam*HI site of pUTKAT-3 (46) in the following orientation: ←←→←. The additional nucleotides at both ends of the RevRE resulted in separation between AGGTCA half-sites of 13 bp in the ←← arrangement, 20 bp in the ←→ arrangement, and 6 bp in the →← arrangement. JEG-3 choriocarcinoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum and switched to DMEM plus 10% anion-exchange resin and charcoal-treated serum (47) 12 h prior to calcium phosphate transfection (32) in pairs or triplicate with 5 μg of RevRE-TKCAT or pUT-KAT-3, along with 5 μg of plasmid pCDM (2) unmodified or expressing rat TRα1 (33) or human Rev-Erb (35) cDNA. The fold activation of RevRE-TKCAT varied somewhat for different preparations of the Rev-Erb expression plasmid; 5 μg of β-galactosidase expression plasmid pCH110 (Pharmacia) was also included to control for transfection efficiency. After 16 h, cells were treated for 2 min with 20% dimethyl sulfoxide in serum-free medium and then washed prior to addition of fresh DMEM plus 10% stripped serum. Cell

extracts were prepared 24 h after dimethyl sulfoxide shock, normalized for protein concentration, and assayed for chloramphenical acetyltransferase (CAT) activity. Expression of β-galactosidase varied insignificantly, and Rev-Erb had no significant effect on transcriptional activity of the enhancerless reporter plasmid.

RESULTS

Rev-Erb binds to an asymmetric site composed of an A/T-rich region 5′ to a single AGGTCA half-site. Since the DBD of Rev-Erb is as similar to those of TR and RAR as the latter are to each other (amino acid identities of 49% [33 of 67] in all three, 57% between TR and Rev-Erb, 61% between RAR and Rev-Erb, and 60% between TR and RAR [34]), we initially studied the binding of Rev-Erb to an inverted repeat of the AGGTCA motif (TREp), which is bound by both TR and RAR. A GST-RevT fusion protein containing amino acids 21 to 292 was produced in bacteria for use in these studies (the highly conserved DBD is from amino acids 132 to 199). However, although TR and RAR bound TREp well (5), binding of GST-RevT to TREp was barely detectable (data not shown; see Fig. 5).

To better understand the DNA-binding properties of Rev-Erb, we devised a PCR-based strategy based on the sequence amplification and binding (SAAB) method of Blackwell et al. (6), in which the binding site is selected from a pool of random oligonucleotides by iterative binding, separation of bound from free, and PCR amplification. Hypothesizing that Rev-Erb, like TR, RAR, vitamin D_3 receptor, PPAR (peroxisomal proliferator-activated receptor), and related receptors, would recognize two AGGTCA half-sites with a specific orientation and spacing (26, 43, 55), we initially selected from a biased pool of oligomers containing four randomly generated base pairs 5′ to one AGGTCA motif followed by 12 random base pairs ($N_4AGGTCAN_{12}$). Six rounds of EMSA selection using GST-RevT were followed by two rounds of selection on GST-RevT immobilized on beads and one additional round of EMSA selection (Fig. 1A). After the ninth round of selection, there was an ~70-fold enhancement of RevT binding to the selected pool (Fig. 1B). The selected material was subcloned into pBS, and inserts from 100 individual clones were assayed for DNA binding to GST-RevT in the EMSA (data not shown). Of these, approximately 30% contained inserts which bound tightly to Rev-Erb, and 19 of these were sequenced. Surprisingly, a second AGGTCA motif did not emerge. Instead, as shown in Fig. 2A, the consensus sequence (A-[A/T]-N-T) was nearly invariant 5′ to the AGGTCA in strongly binding fragments, while no preference for specific 3′ sequences, including a second TR half-site, was ascertained.

To confirm the requirement for the AGGTCA motif in an unbiased manner and to determine whether Rev-Erb had additional sequence preference 5′ to the A/T-rich region (only four random bases were present 5′ to the AGGTCA in the initial pool of oligonucleotides), the selection was repeated with a starting pool of oligomers containing 20 random base pairs (N_{20}). Selection of binding sites was confirmed in the EMSA (Fig. 1C), and the selected pool of fragments was subcloned into pBS as described above. Sequences of 20 fragments which were determined to bind tightly to Rev-Erb are shown in Fig. 2B. This analysis proved in an unbiased manner that a single TR half-site was necessary for Rev-Erb binding, although it was noted that a G residue can substitute for the first A in the AGGTCA. Furthermore, these studies confirmed the requirement for the 5′ A-(A/T)-N-T sequence and revealed an additional preference for an A or T as the fifth base upstream from the AGGTCA. No evidence for a second AGGTCA half-site or any further upstream or downstream components of the RevRE emerged from the starting material (N_{20}).

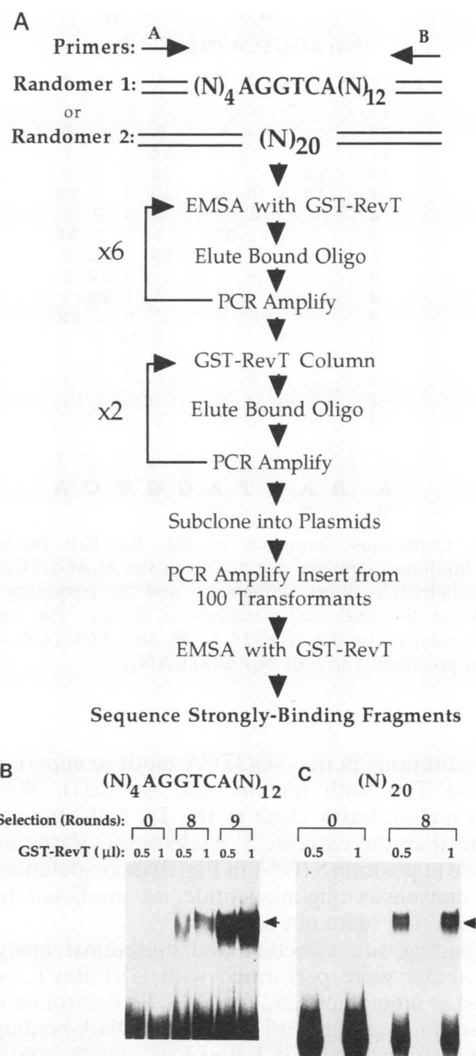

FIG. 1. Selection of a consensus Rev-Erb binding site. (A) Strategy. $N_4AGGTCAN_{12}$ and N_{20} are the two starting pools of oligonucleotides (N indicates A, C, G, or T at random) which were amplified by PCR using primers A and B (see Materials and Methods). (B and C) Enhancement of Rev-Erb DNA binding in selected pools. After the indicated number of rounds, selected oligonucleotides were ^{32}P labeled and studied in the EMSA with ~190 and 380 ng of GST-RevT.

Mutational analysis of the A/T-rich region and AGGTCA half-site. The relative importance of specific bases within the RevRE was evaluated with a series of mutant RevREs, whose ability to compete with a labeled consensus RevRE for binding to Rev-Erb was studied. The data from these experiments are summarized in Fig. 3A and shown in Fig. 3B. The middle panel of Fig. 3B shows that mutants 9-11C, 8-10C, and 7-9C failed to compete for binding to GST-RevT even at a 625-fold molar excess, confirming the importance of the A/T-rich sequence. Indeed, point mutations in this region were as effective in preventing binding as were single

	A				B			
	(N4) AGGTCA (N12)				N20			
POSITION	**A**	**C**	**G**	**T**	**A**	**C**	**G**	**T**
13	–	–	–	–	5	4	4	4
12	–	–	–	–	5	2	7	5
11	–	–	–	–	**11**	3	2	**7**
10	**15**	0	1	3	**20**	2	1	0
9	**13**	0	0	**6**	**13**	0	0	**10**
8	2	9	3	5	4	4	9	6
7	0	1	1	**17**	2	2	3	**16**
6	●	–	–	–	**13**	0	**10**	0
5	–	–	●	–	1	0	**22**	0
4	–	–	●	–	0	1	**22**	0
3	–	–	–	●	0	0	1	**22**
2	–	●	–	–	0	**23**	0	0
1	●	–	–	–	**23**	0	0	0
-1	2	7	4	5	4	7	5	7
-2	6	5	2	6	3	5	3	11
-3	6	3	6	3	5	3	6	3

11	*10*	*9*	*8*	*7*	*6*	*5*	*4*	*3*	*2*	*1*
A	**A**	**A**	**N**	**T**	**A**	**G**	**G**	**T**	**C**	**A**
T		**T**			**G**					

FIG. 2. Consensus sequence of the Rev-Erb binding site. Strongly binding fragments selected from the $N_4AGGTCAN_{12}$ and N_{20} oligonucleotides were sequenced, and the frequency of each nucleotide at the indicated positions is shown. The numbering system is relative to the AGGTCA. ●, the AGGTCA sequence present in positions 1 to 6 of $N_4AGGTCAN_{12}$.

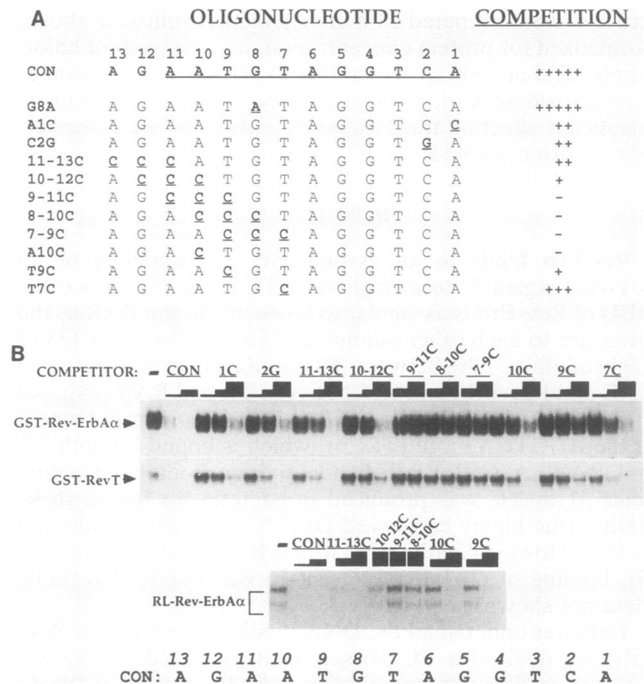

FIG. 3. DNA-binding specificity of Rev-Erb. (A) Consensus RevRE sequence and mutants used for competition. The consensus sequence determined from the binding site selection is underlined within the CON oligonucleotide. Mutants are named by the base substitution which is underlined and bold for each oligonucleotide. Competition results shown in panel B are summarized as follows: +++, the level of competition of the CON oligonucleotide with itself; ++, 50% competition between 25- and 125-fold molar excess; +, 50% competition between 125- and 625-fold molar excess; –, no competition detected at 625-fold molar excess. (B) Competition for binding to Rev-Erb. Unlabeled consensus and mutant RevREs were added in the molar excesses shown to binding reaction mixtures containing the labeled consensus RevRE and either GST-RevT (middle), GST-Rev-Erb (top), or RL-Rev-Erb (bottom). Unlabeled competitors were in 25-, 125-, and 625-fold molar excess, as indicated by the bars above the lanes.

base substitutions in the AGGTCA motif (compare mutants A10C and T9C with mutant A1C or C2G). Within the A/T-rich region, bases close to the TR half-site were more important than the extreme 5′ nucleotides. Variation of the nucleotide at position 8 (G8A in Fig. 3A) competed as well as did the consensus oligonucleotide, as predicted from the SAAB selection (data not shown).

The binding site selection and mutational analysis described above were performed with GST-RevT, which is truncated at amino acid 292 (of 614). To determine whether the C terminus of Rev-Erb influenced DNA-binding specificity, we synthesized GST-Rev-Erb, which contains the intact C terminus and lacks only the N-terminal 21 amino acids. Figure 3B (top) shows that the mutant oligonucleotides competed for binding to GST-Rev-Erb in a pattern which was virtually identical to that observed for GST-RevT. The competition analysis was also repeated with full-length, wild-type Rev-Erb synthesized in reticulocyte lysate (RL-Rev-Erb) in order to determine whether the GST moiety and absence of the N-terminal 21 amino acids influenced the DNA-binding specificity of the GST fusion proteins (Fig. 3B, bottom). Here, too, mutations in the A/T-rich region and AGGTCA motif profoundly reduced binding affinity, and RL-Rev-Erb displayed nearly the same binding preferences as did GST-RevT and GST-Rev-Erb. Thus, the DNA-binding specificity of Rev-Erb is determined entirely by amino acids 21 to 292. Note that RL-Rev-Erb formed two complexes with the consensus RevRE, consistent with the two major translation products of 70 and 55 kDa which were seen when the cDNA is translated in vitro (data not shown) (35). The smaller species could result from initiation at the second in-frame methionine residue (amino acid 107), as is used by one form of rat Rev-Erb (34), or could represent a C-terminal degradation product. In any event, no differences were observed in the DNA-binding specificities of these two complexes.

Rev-Erb contacts both the half-site and the A/T-rich flank of the selected binding site. The ability of Rev-Erb to contact bases within the A/T-rich and (A/G)GGTCA regions of the RevRE was determined using methylation (DMS) and acylation (DEPC) interference assays, in which protein-DNA interaction was prevented by modification of purines. DMS interference detected contact with G residues, while DEPC interference identified important A and G (A>G) residues. Analysis of both strands of the RevRE by these two methods revealed that modification of bases within the A/T-rich and GGGTCA sites completely interfered with binding, with the exception that acylation of the downstream A in the GGGTCA motif did not interfere with binding despite the clear preference during selection (Fig. 4). Furthermore, the interference patterns for GST-RevT and GST-Rev-Erb were indistinguishable with both methods. Thus, Rev-Erb contacts a span of 10 to 11 bp, and the DNA binding of a C-terminal truncation was again nearly identical to that of full-length Rev-Erb.

Rev-Erb binds to DNA in the absence of other proteins, most likely as a monomer. We studied the ability of Rev-Erb to form DNA-binding heterodimers with other nuclear receptors, including RXR, which heterodimerizes with TR and RAR to stabilize their interaction with DNA. Figure 5A shows that RXR and TR did not bind to the RevRE.

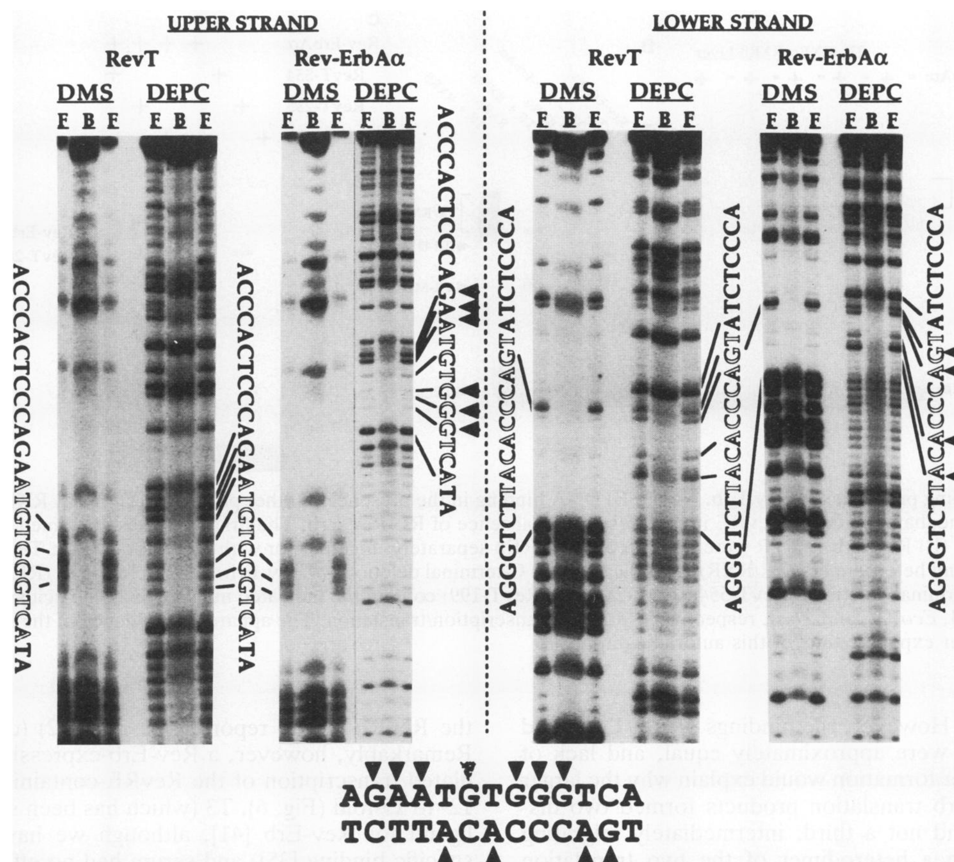

FIG. 4. DNA contact by Rev-Erb determined by methylation (DMS) and acylation (DEPC) interference analysis. Results are shown with both lower and upper strands of a selected fragment, using both GST-RevT and GST-Rev-Erb. DMS treatment modifies G residues; DEPC cleaves at A and G residues (A>G [18, 19]). Arrows point to bases whose modification interferes with binding. Lightly shaded arrow at bottom indicates weak contact of the G at position 8. Cleavage patterns of free (F) and bound (B) DNAs are shown.

Furthermore, although Rev-Erb is homologous to TR and RAR in C-terminal regions involved in heterodimerization (23, 39, 44, 50), Rev-Erb did not bind to the RevRE as a heterodimer with RXR, with TR, or with proteins in liver nuclear extract which have been shown to heterodimerize with TR and RAR. In contrast, TR bound to TREp as monomer, homodimer, and heterodimer with RXRα and -β as previously demonstrated (8, 24, 25, 36, 39, 60, 61). However, Rev-Erb did not bind to TREp as a heterodimer with TR or with RXR and bound poorly to direct repeats of the AGGTCA motif spaced 3, 4, and 5 bp apart (the preferred response elements of vitamin D receptor, TR, and RAR, respectively) unless the spacer sequences provided the A/T-rich region of the RevRE (18). Thus, despite the sequence conservation between Rev-Erb, TR, and RAR, Rev-Erb has unique DNA-binding characteristics, which suggests a distinct mode of action and potential target genes.

To better understand which regions of the Rev-Erb protein were required for DNA binding, full-length and truncated forms of the protein were synthesized in reticulocyte lysate. As discussed earlier, in vitro translation of the wild-type Rev-Erb cDNA resulted in two major protein species, and two complexes were seen in the EMSA (see also Fig. 3B and Fig. 5A and B), the more greatly retarded of which was presumably full-length Rev-Erb (Fig. 5C). Figure 5C shows that RevT-288 (truncated after amino acid 288) bound to the RevRE, which was not surprising since this protein is nearly identical to the Rev-Erb portion of GST-RevT. However, DNA binding was eliminated by truncation immediately after the second zinc finger (RevT-199), which removes amino acids distal to the P and D boxes, a region recently shown to be important for the DNA-binding specificity of two nuclear receptor superfamily members, NGFI-B (also known as *nur77* [19, 40]) and RXRβ (58). Thus, a region between 21 and 288, but not the zinc fingers alone, is necessary and sufficient for DNA binding. Removal of the last 60 amino acids from the C terminus of Rev-Erb (Rev-554) also eliminated DNA binding, suggesting that the C terminus of Rev-Erb is required to expose this domain in the full-length protein.

The truncated and full-length Rev-Erb formed complexes with very different mobilities, as expected from their molecular weights. RevT-288 and full-length Rev-Erb were mixed prior to EMSA in order to determine whether these complexes contained Rev-Erb monomers or homodimers; observation of an intermediately migrating complex would be strong evidence for dimer formation (27, 53). In contrast, even though both RevT-288 and full-length Rev-Erb contain the D box which is involved in homodimerization of other nuclear receptors (17, 20, 37), Fig. 5C shows that no intermediate complexes were observed when the translation products were mixed, suggesting that each species bound as a monomer. This experiment does not rule out the possibility that sequences deleted from RevT-288 are important for

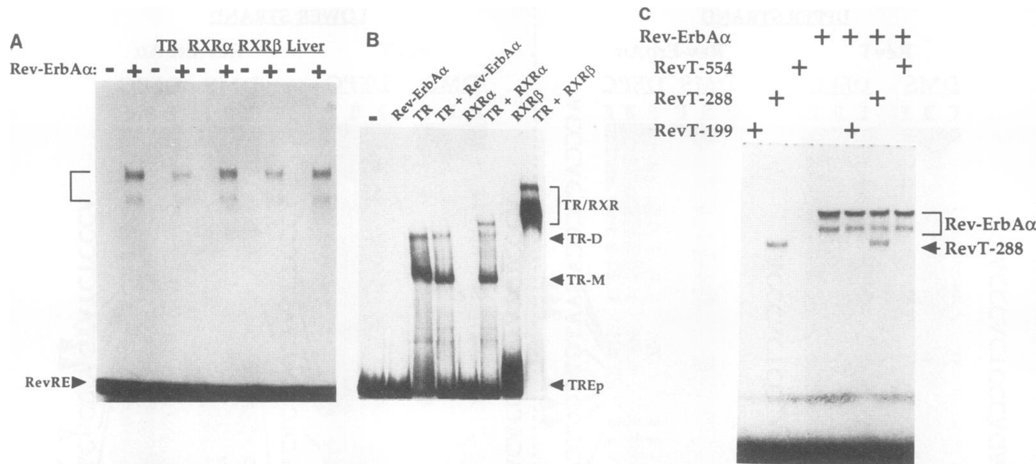

FIG. 5. DNA-binding properties of Rev-Erb. (A and B) DNA binding in the presence of other proteins. (A) TRα1, RXRα, RXRβ, or liver nuclear extract was incubated with the RevRE in the presence or absence of RL-Rev-Erb. The first two lanes contained the RevRE with or without RL-Rev-Erb. (B) Rev-Erb and TR were incubated with TREp separately, together, or with RXRα or -β. The TR monomer (TR-M), homodimer (TR-D), and heterodimers (TR/RXR) are indicated. (C) C-terminal deletions of Rev-Erb. Human Rev-Erb (RL-Rev-Erb) contains 614 amino acids. C-terminal deletions (RevT-554, RevT-288, and RevT-199) contain the indicated number of amino acids due to digestion of the cDNA with *Eco*RI, *Eco*RV, and *Xho*I, respectively, prior to transcription/translation. The apparent confluence of the signal from the free probe is due to greater exposure time of this autoradiograph.

homodimerization. However, the bindings of RevT-288 and wild-type Rev-Erb were approximately equal, and lack of Rev-Erb homodimer formation would explain why the larger and smaller Rev-Erb translation products formed two discrete complexes and not a third, intermediately migrating complex containing a heterodimer of the two translation products.

Rev-Erb activates transcription via the RevRE. To examine the ability of Rev-Erb to regulate gene transcription, four copies of the RevRE were inserted upstream to the thymidine kinase promoter driving expression of CAT. Separation between direct repeats of the AGGTCA half-sites was 13 bp, and separation between inverted repeats was 6 bp, as a result of cloning sites in the RevRE-containing insert used to generate this reporter (see Materials and Methods). TRα1 had no effect on expression from the RevRE-containing reporter (Fig. 6), although it did mediate strong, T3-dependent activation with a T3 response element substituted for the RevRE in the reporter plasmid (32) (data not shown). Remarkably, however, a Rev-Erb-expressing plasmid stimulated transcription of the RevRE-containing reporter gene 12- to 15-fold (Fig. 6). T3 (which has been suggested to be a ligand for Rev-Erb [41], although we have not observed specific binding [35]) and serum had no effect on transcriptional activation by Rev-Erb. These data indicate that Rev-Erb is a constitutive transcriptional activator or is activated by a ligand present in stripped serum or in the JEG-3 cells themselves.

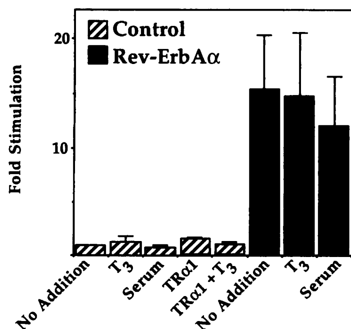

FIG. 6. Evidence that Rev-Erb activates transcription from the Rev-RE. JEG-3 cells were cotransfected with a CAT expression plasmid driven by the minimal thymidine kinase promoter, containing four copies of the RevRE upstream, along with a TRα1 or Rev-Erb expression plasmid. After transfection, cells were incubated for 24 h with DMEM plus 10% stripped calf serum, supplemented with 10 nM T3 and 10% calf serum where indicated.

DISCUSSION

We have shown that Rev-Erb is a sequence-specific DNA-binding protein and transcriptional activator. Rev-Erb and TRα are thus the first examples of sequence-specific DNA-binding proteins encoded on opposite strands of the same eukaryotic genomic locus. Rev-Erb binds specifically to an asymmetric 11-bp sequence, the longest nonrepetitive element specifically recognized by a member of the thyroid/steroid hormone receptor superfamily, and binds poorly to tandemly arranged half-sites such as TREp. Rev-Erb also binds poorly to direct repeats, unless the 5′ or spacer sequences provide the specific A/T-rich portion of the RevRE (18) (suggesting a mechanism by which a subset of hormone-responsive target genes could also respond to Rev-Erb). The DNA-binding properties of Rev-Erb have diverged from those of the TR and RAR despite the genomic relationship between Rev-Erb and the TR which previously led us to speculate that they arose by gene duplication and inversion (34). These observations are consistent with recent analyses of the primary amino acid sequences of all steroid/thyroid hormone receptor superfamily members, which suggest that Rev-Erb diverged from TRα1, TRβ, and RAR before these diverged from each other (1, 29). The findings that Rev-Erb seems to bind DNA as a monomer and does not bind DNA as a heterodimer with RXR, TR, or TR/RAR heterodimerization partners present in liver nuclear extract are also interesting in this context, given the homology of Rev-Erb in portions of the C terminus felt to be important for

homo- and heterodimerization of TR and RAR (13, 45). Our experiments have not ruled out the possibility that Rev-Erb can form heterodimers with as yet undiscovered factors or that Rev-Erb homo- or heterodimers bind to a site greater than 20 bp in length, which would not have been detected by our analyses. It is also formally possible that the bacterial Rev-Erb and reticulocyte lysate-derived Rev-Erb do not heterodimerize because of abnormal posttranslational modification of the proteins.

Rev-Erb's ability to activate transcription in the absence of exogenous ligand is unusual for steroid and thyroid hormone receptors. It is important to note that the transactivation by Rev-Erb was less than 10% of that which we routinely observe for the T3-stimulated TR (using TREp or a direct AGGTCA repeat with a 4-bp gap as the TRE), and it is possible that the activity of Rev-Erb can be enhanced by an as yet unknown ligand. Indeed, we do not know whether transcriptional regulation by Rev-Erb requires an intracellular ligand that is present in JEG-3 cells or, alternatively, reflects a truly autonomous function. Nonetheless, ligand-independent transactivation has recently been demonstrated for other orphan receptors (all with the identical P box as in Rev-Erb and TR), including COUP-TF (21), HNF-4 (49), and NGFI-B (11, 57). However, COUP-TF (28, 56) and HNF-4 (49) bind DNA as homodimers, with the COUP-TF homodimer binding tightly to TREp (5) and to variably spaced direct repeats of the AGGTCA half-site (9). Moreover, COUP-TF forms DNA-binding heterodimers with TR and RAR (5, 52) as well as RXR (24).

PCR-based, unbiased screening methods, referred to as SAAB (6, 7) or cyclic amplification and selection of targets (CASTing) (59), have been previously used to identify the DNA-binding sites of transcription factors such as c-Myc (6, 7), myogenin (59), and p53 (14). The present report describes the first successful application of a similar strategy to a member of the nuclear receptor family of transcription factors. It is interesting to compare this method with a genetic selection in *Saccharomyces cerevisiae* which was used to uncover a rat genomic DNA sequence specifically bound by the orphan receptor NGFI-B (57). Both methods are unbiased, but the yeast selection method has the advantage of identifying functional binding sites in endogenous genes, although it does not prove that the selected genomic sequences actually function as natural response elements. Also, if heterodimeric interactions can modulate DNA binding, the yeast system might yield different information than a biochemical strategy using purified proteins. However, there are also several advantages to the selection method which we have used. While it does not address the possibility of physiologically relevant heterodimerization partners, screening with purified protein in vitro ensures that the selected binding sites are recognized by Rev-Erb in the absence of other nuclear proteins, such as would be present in yeast cells. Furthermore, the PCR-based method provides a large number of independent binding sites which allow generation of a consensus sequence. In contrast, the yeast genetic selection method revealed only two NGFI-B binding sites, both contained within a single genomic fragment.

Although the NGFI-B binding site (two A residues 5′ to an AGGTCA half-site [57]) competed only weakly for binding to Rev-Erb (18), there are remarkable similarities between the DNA-binding properties of Rev-Erb and NGFI-B. Both bind to asymmetric elements containing a single TR half-site, in contrast to the direct repeat motifs recognized by other nuclear receptor homodimers and heterodimers. The DNA-binding specificity of both Rev-Erb and NGFI-B is dependent upon amino acid residues distal to the P and D boxes; the fact that there is little similarity in the actual amino acid residues in this region of the two proteins probably accounts for their different DNA-binding specificities. Additionally, in an assay similar to that employed here, NGFI-B was determined to bind DNA as a monomer (57), although its heterodimerization properties have not been studied. Furthermore, both Rev-Erb and NGFI-B can activate transcription in the absence of exogenous ligand. These similarities suggest that Rev-Erb and NGFI-B are prototypes of a subfamily of orphan receptors capable of activating transcription from complex elements composed of distinct sequences 5′ to a single TR half-site in the absence of endocrine ligand. Another likely member of this subfamily is the ecdysone-inducible E75 protein, which is involved in the metamorphosis of *Drosophila melanogaster* (48), since it is 79% identical to Rev-Erb in the DBD (with considerable similarity in the adjacent region implicated in DNA-binding specificity). Further study of the expression and properties of these and additional members of this intriguing subfamily of receptor-related transcription factors will be needed to shed light on their biological functions.

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