Cross-Talk among RORα1 and the Rev-erb Family of Orphan Nuclear Receptors

Barry Marc Forman, Jasmine Chen, Bruce Blumberg, Steven A. Kliewer, Robert Henshaw, Estelita S. Ong, and Ronald M. Evans*

The Howard Hughes Medical Institute (E.S.O., R.M.E.)
The Salk Institute for Biological Studies (B.M.F., J.C., B.B., S.A.K., R.H)
Gene Expression Laboratory
San Diego, California 92186–5800

We have cloned Rev-erbβ, a novel isoform of the Rev-erbα orphan nuclear receptor. The DNA binding domains of Rev-erbα and β are highly related to each other and to the retinoic acid related orphan receptor (ROR)/RZR subfamily of nuclear receptors. Indeed, we find that all three receptors bind as monomers to the sequence AATGT-AGGTCA. Whereas RORα1 constitutively activates transcription through this sequence, both isoforms of Rev-erb are inactive. When coexpressed, both Rev-erb isoforms suppress the transcriptional activity of RORα1. Our data define Rev-erb and ROR/RZR as a family of related receptors with opposing activities on overlapping regulatory networks. (Molecular Endocrinology 8: 1253–1261, 1994)

INTRODUCTION

Important biological processes including development (1–3), cell-cycle control (4, 5), apoptosis (6, 7), and oncogenesis (8–10) are regulated by complex transcriptional networks. Precise control of these networks requires the organism to coordinate and integrate signals from multiple regulatory pathways. One mechanism to achieve this regulation is the establishment of cross-talk among distinct transcription factors.

Transcriptional cross-talk occurs among several classes of transcription factors, including the nuclear hormone receptors (11–13). These receptors comprise a superfamily of transcription factors that initiate nuclear responses to steroids, retinoids, vitamin D_3, and thyroid hormone (14). An increasing number of proteins that possess sequence similarity and functional homology with members of the nuclear receptor superfamily have been identified. These proteins are referred to as orphan receptors since their potential ligands have yet to be described (15). An understanding of the physiological role of orphan receptors will require a dissection of the transcriptional networks that are regulated by these proteins.

Response elements for nuclear receptors contain six-nucleotide core-binding sites flanked on the 5′-end by a one-to-six nucleotide extension (16–20). Target gene recognition is specified by the DNA binding domain (DBD), which is composed of two zinc finger motifs that fold into two helical domains and a third helix extending from the second zinc finger (21–23). Domain swap analysis identified five amino acids (P-box) at the base of the first finger as being crucial in directing receptor recognition of the six-nucleotide core-binding site (24–26). Subsequent structural analysis has revealed that the P box is located within the first helix, which forms specific contacts with the core-binding site (22, 23). Thus, the P box/first helix serves as a recognition helix that specifies and contacts the six-nucleotide core-binding site (24–26).

Sequences extending C-terminal of the DBD have been shown to form a third helix (21). This helix overlaps with the T/A box domain of the orphan receptor NGFI-B (27, 28). In the orphan receptors NGFI-B and SF1, this region allows these receptors to specifically recognize sequences 5′ to the core-binding site. Optimal binding sites for several nuclear receptors include sequences that extend 5′ to the core binding site (16–20). The precise role and boundaries of the T/A box have not been defined for most of the nuclear hormone receptors.

We have cloned and characterized Rev-erbβ, a novel isoform of the Rev-erb family of orphan receptors (29, 30). Rev-erbα is an orphan nuclear hormone receptor that is encoded on the noncoding strand of the thyroid hormone receptor α-gene. Monomers of Rev-erbα bind with high affinity to a 5′-extended core-binding site. Rev-erbα mRNA is widely expressed and induced dramatically during the course of adipocyte differentiation (31). However, little is known about the specific role of

0888-8809/94/1253-1261$03.00/0
Molecular Endocrinology

this receptor in adipogenesis or in other physiological processes.

Rev-erbβ shares sequence similarity with the DBD of Rev-erbα and with the retinoic acid-related orphan receptor (ROR)/RZR (18, 32). RORα/RZRα was first identified using highly degenerate polymerase chain reaction (PCR) primers designed to amplify nuclear receptor-related cDNAs (32). RORα/RZRα is expressed in a variety of tissues (32) although its physiological function is also unclear. Subsequent studies have revealed that there are at least three different isoforms of RORα/RZRα (18). Like Rev-erbα, RORα/RZRα monomers bind with high affinity to an extended core-binding site (18). We find that Rev-erbα, Rev-erbβ, and RORα1 bind as monomers to a common 11-nucleotide response element. RORα1 is constitutively active when bound to this response element, whereas both Rev-erb α and β are inactive. When coexpressed, both Rev-erb isoforms suppress the transcriptional activity of RORα1. Our data suggest that the transcriptional activities of RORα1 and the Rev-erb orphan receptors are integrated through an overlapping network of responsive genes.

RESULTS

Cloning of Rev-erbβ from Mouse Liver

A cDNA library from mouse liver was screened with an oligonucleotide probe spanning the loop of the first zinc finger of the *Xenopus* peroxisome-proliferator activated receptors (33). Several overlapping cDNA clones were identified, the largest of which (λML19) contained an approximately 3.6 kilobase (kb) insert. Sequence analysis revealed a polyadenylation sequence at the 3′-end and an open reading frame of at least 1629 base pairs (bp) at the 5′-end of the clone (Fig. 1). An in-frame translation stop codon cannot be found upstream of the first methionine codon in λML19 suggesting that the full-length protein may contain additional amino acids upstream of those described here.

The open reading frame of λML19 encodes a 542-amino acid protein (Fig. 1). Comparison of this sequence with the Genbank database revealed that this is a novel clone possessing sequence similarity with members of the nuclear receptor superfamily. A 67-amino acid region spanning amino acids 69–135 is highly related to the zinc finger domain of the nuclear receptor superfamily (Fig. 1 and Fig. 2A). The relationship among the zinc finger domains of Rev-erbα (29, 30), λML19 (Rev-erbβ), ROR/RZR, and other nuclear receptors is depicted in the dendogram of Fig. 2A. Note that the zinc finger regions of λML19 and Rev-erbα are more closely related to each other than to other nuclear receptors. Similarly, the putative ligand-binding domain, spanning amino acids 363–536 of λML19, exhibits closest sequence similarity with the ligand-binding domain of Rev-erbα (data not shown). λML19 and Rev-erbα possess 96% and 75% sequence identity in the DBD and putative ligand-binding domain, respectively (Fig. 2B). Thus, λML19 appears to be a novel isoform of Rev-erb, which we refer to as Rev-erbβ (also known as RVR; V. Giguere, personal communication). Unlike isoforms of the thyroid hormone and retinoid receptors, the Rev-erb isoforms are more distantly related to each other as sequence identity between the Rev-erb isoforms is only 22% in the region between the DNA and ligand-binding domains (Rev-erbβ amino acids 162–362). The amino acid sequence of Rev-erbβ is notable for a serine-rich region at its N terminus (Fig. 1).

The dendogram shown in Fig. 2A also reveals that the zinc finger regions of Rev-erb α/β subfamily are closely related to the α- and β-isoforms of ROR/RZR. Indeed, these two subfamilies are more closely related to each other than to any other subfamily. Furthermore, sequence similarity extends for 12–26 amino acids downstream of the zinc finger region (Fig. 2B). This region corresponds to the T/A box of NGFI-B, which is a crucial determinant of NGFI-B binding to nucleotides extending 5′ of the core-binding site. Although the precise borders of the T/A box have not been defined for Rev-erb or ROR/RZR, the close relationship between these two subfamilies in both this region and the zinc finger region raises the possibility that Rev-erb α/β and ROR/RZR may recognize related response elements.

Tissue Distribution of Rev-erb mRNA

To further characterize Rev-erbβ, Northern blot analysis was performed to compare the expression pattern of Rev-erbα and Rev-erbβ in a variety of mouse tissues (Fig. 3). Rev-erbα and Rev-erbβ are both widely expressed and share a similar pattern of expression. Highest levels of Rev-erb mRNA were seen in the brain, lung, liver, skeletal muscle, and kidney. Lower levels of expression were detected in the heart, and minimal expression is seen in the spleen and testis. A control probe (human β-actin) indicated that similar amounts of RNA were loaded in all lanes (data not shown). Next we compared the distribution of Rev-erb α and β with the distribution of RORα1. Northern blot analysis was performed with a probe that specifically recognizes human RORα1 but not RORα2 or RORα3. In human tissues, the RORα1 transcript is extremely large (>12 kb) and is expressed most highly in skeletal muscle and in the brain (Fig. 3C). Lower levels of expression are seen in the the heart, liver, and pancreas. Previous analyses (32) of RORα expression used a probe common to all three RORα isoforms and revealed the presence of an abundant transcript of more than 12 kb as well as several smaller transcripts. When compared with the results of Fig. 3C, it is clear that RORα1 is encoded by the large transcript (>12 kb) and is thus the most abundantly expressed isoform of RORα. Our data indicate that Rev-erb α/β mRNAs are coexpressed with RORα1/RZRα1 in the heart, brain, liver, and skeletal muscle.

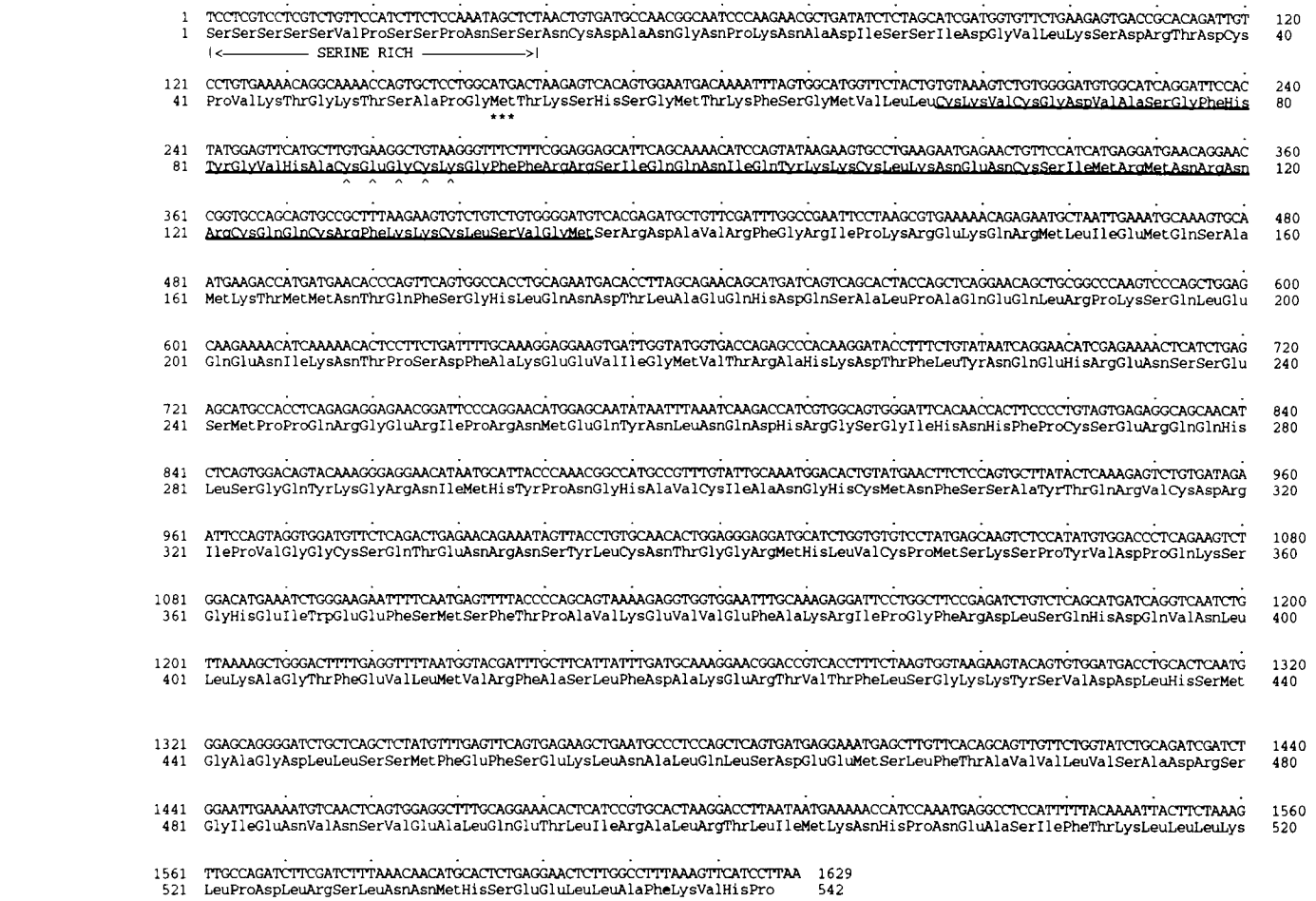

Fig. 1. Nucleotide and Predicted Amino Acid Sequence of Mouse Rev-erbβ

The sequence of mouse Rev-erbβ derived from the 5′-end of λML19 is shown here. The open reading frame is 1629 bp long and can encode a 542- amino acid protein. Since an in-frame translation stop codon cannot be identified upstream of the first methionine codon, the full-length Rev-erbβ coding sequence may contain additional sequences upstream of those shown here. The 67-amino acid DBD is *underlined*, and the P box/recognition helix is indicated by a symbol (⌒). The first methionine codon in the open reading frame is located at nucleotide 154 (*). The serine-rich region spanning amino acids 1–13 is noted. The Genbank Accession number is U09504.

Rev-erb and the Orphan Receptor RORα1 Bind to the Same Target DNA Sequence

To gain insight into the transcriptional properties of Rev-erbβ we sought to identify a DNA-binding site that serves as a functional response element. Since the zinc finger regions of the Rev-erb isoforms are 96% identical (Fig. 2B), it seems likely that both isoforms could recognize a similar six-nucleotide core-binding site. Furthermore, sequence identity continues for 24 of the 26 amino acids immediately C-terminal to the zinc finger domain (T/A box region), suggesting that both Rev-erb isoforms recognize a similar 5′-extension. It is notable that the zinc finger region and T/A box are also conserved between Rev-erb and the ROR/RZR families of orphan receptors (18, 32). Indeed, homology between these two receptor families are 65% and 75% (9 of 12) in the zinc finger region and T/A box (Fig. 2B), respectively. This prompted us to explore the possibility that Rev-erb α/β and ROR/RZR orphan receptors recognize an overlapping set of response elements.

Binding site selection and PCR amplification had previously been used to determine high-affinity binding sites for Rev-erbα (19), RORα1, and RORα2 (18). The selected binding site for Rev-erbα, ($^{A/}_{T}$)A($^{A/}_{T}$)ANT-AGGTCA, contains a five-nucleotide A/T-rich sequence followed by the six-nucleotide AGGTCA core-binding site. The optimal RORα1-binding site has recently been shown to be a highly related sequence: ($^{T/}_{A}$)($^{A/}_{T}$)($^{T/}_{A}$)N($^{A/}_{T}$)-AGGTCA (18). These optimized binding sites support high-affinity binding of their respective receptor monomers. Thus, we decided to compare binding of Rev-erbα, Rev-erbβ, and RORα1 to a common sequence, AATGT-AGGTCA (RE).

Receptor binding to the RE was analyzed using electrophoretic mobility shift analysis with ^{32}P-labeled DNA probes (Fig. 4A). *In vitro* translated Rev-erbα, Rev-erbβ, and RORα1 were all capable of binding to the RE

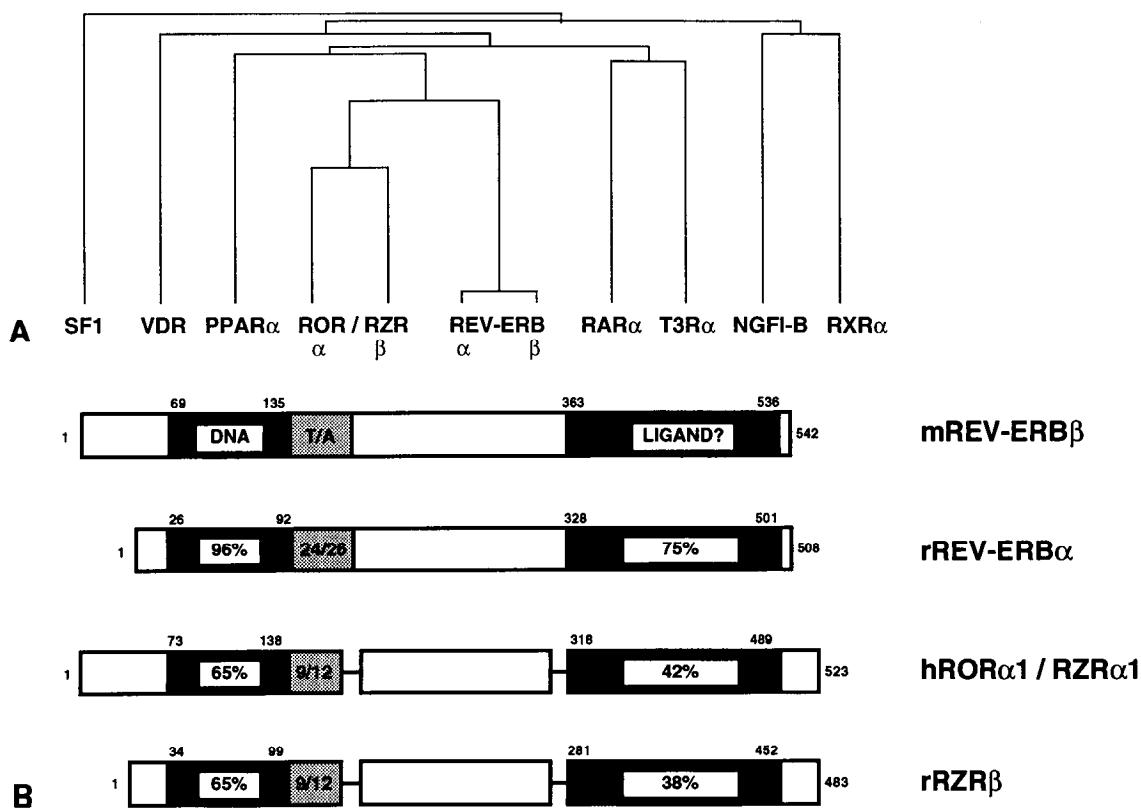

Fig. 2. Amino Acid Sequence Similarity between Rev-erbβ and Other Members of the Nuclear Receptor Superfamily

A, Relationship between the zinc finger portion of the DBD of mouse Rev-erbβ (amino acids 69–135) and other members of the receptor superfamily. Rev-erbα and Rev-erbβ comprise a subfamily that is most closely related to the ROR/RZR subfamily. Other receptors included for comparison are SF1 [mouse steroidogenic factor 1 (34)], VDR [human vitamin D receptor (51)], PPARα (mouse peroxisome proliferator-activated receptor-α (52)], RORα [human RAR-related orphan receptor (18)], rat RZRβ (Becker-Andre, 1993 Genbank accession number L14610), rat Rev-erbα (29), RARα [human retinoic acid receptor-α (53, 54)], T_3Rα [human thyroid hormone receptor α (55)], NGFI-B [rat nerve growth factor-inducible protein-B (35)], and RXRα [human retinoid X receptor-α (56)]. Dendograms were created using the PILEUP program (Genetics Computer Group, version 7.2, University of Wisconsin, Madison, WI). B, Amino acid sequence identity among mouse Rev-erbβ, rat Rev-erbα, human RORα, and rat RZRβ. The DNA binding (DNA) and putative ligand- binding domains (LIGAND?) are shown as *solid boxes*. Percent amino identity was calculated using the GAP program (Genetics Computer Group, version 7.2, University of Wisconsin) and is shown for the DNA- and ligand-binding domains. The T/A box is *stippled*. The number of identical amino acids/total amino acids in this region is indicated. *Numbers* represent the amino acid delineating each domain.

sequence (AATGT-AGGTCA). In contrast, no binding was detected using a ^{32}P-labeled binding site for NGFI-B (NBRE, AA-AGGTCA), an orphan receptor with the same P box/recognition helix as Rev-erb and ROR but a divergent T/A box region (27, 28). Mixing experiments employing combinations of all three proteins did not provide evidence for dimerization interactions among these proteins.

The relative affinities of each receptor for the RE were determined by competition analysis with an excess of unlabeled RE (Fig. 4B). Binding of Rev-erbα and RORα1 was decreased by ~50% with 3.3 nM unlabeled RE, whereas 10 nM was required for ~50% competition of Rev-erbβ. As expected, the NBRE served as a poor competitor with approximately 50% competition with 33 nmol/liter unlabeled NBRE. These data indicate that Rev-erb and ROR/RZR form a family of receptors capable of binding to similar DNA targets.

Rev-erb Blocks Transcriptional Activation by RORα1

We examined whether Rev-erbα, Rev-erbβ, or RORα1 could activate transcription through the RE sequence. CV-1 cells were transiently transfected with a reporter construct containing two copies of RE (RE-2 TK-LUC) linked to the Herpes thymidine kinase promoter (TK-LUC) (Fig. 5A). RORα1 constitutively activated the RE-2 TK-LUC reporter but failed to activate the TK promoter alone or a TK reporter containing three copies of the NBRE (NBRE-3 TK-LUC). Thus, RORα1 binds and activates transcription specifically through the RE sequence. Although both Rev-erb isoforms are capable of binding to the RE, no trans-activation was observed through the two-copy RE reporter (Fig. 5B) or a four-copy RE reporter (data not shown).

Since both Rev-erb isoforms could not activate

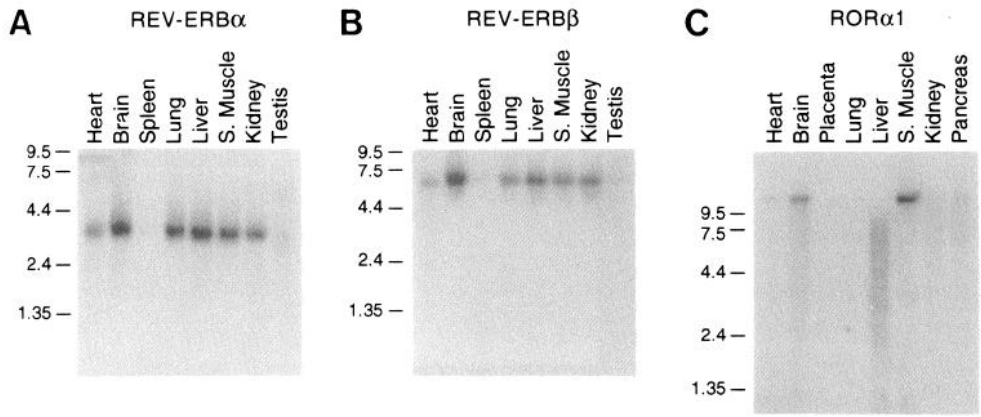

Fig. 3. Tissue Distribution of Rev-erbα, Rev-erbβ, and RORα1

A, Northern blot analysis of Rev-erbα. Immobilized Poly A$^+$ from the indicated mouse tissues was probed with a labeled fragment spanning nucleotides 891–1848 of rat Rev-erbα (3×10^9 cpm/μg). The blot was autoradiographed for 1 day. B, Northern blot analysis of Rev-erbβ. The blot was probed as in panel A with a fragment spanning nucleotides 759–1431 of mouse Rev-erbβ (1×10^9 cpm/μg) and autoradiographed for 5 days. C, Northern blot analysis of RORα1. Immobilized Poly A$^+$ from the indicated human tissues was hybridized with antisense probe-spanning nucloetides 33–298 of human RORα1 (18). The probe was obtained by assymetric PCR using Taq Polymerase in the presence of [^{32}P]dCTP. The specific activity of this probe was 1×10^9 cpm/μg of input DNA. The blot was autoradiographed for 4 days.

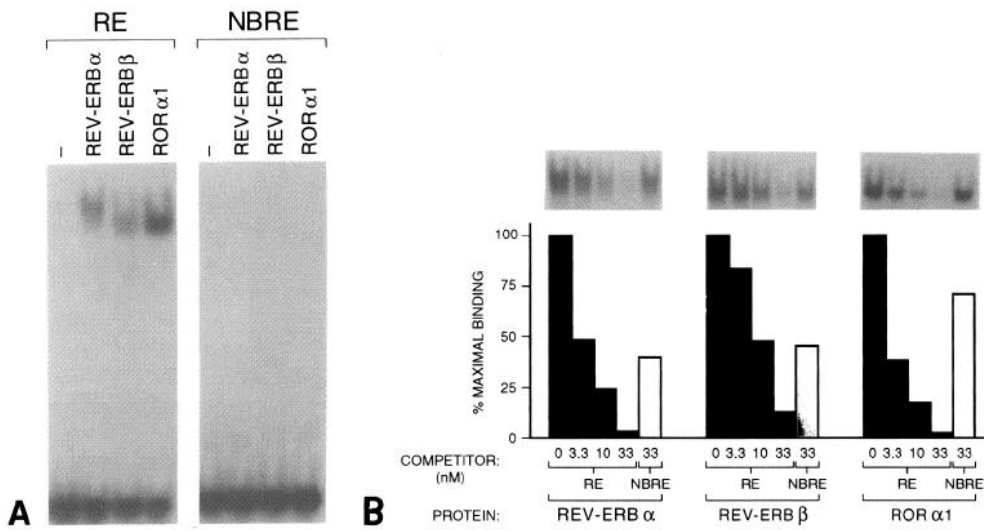

Fig. 4. DNA-Binding Properties of Rev-erbα, Rev-erbβ, and RORα1

A, Binding of Rev-erbα, Rev-erbβ, and RORα1 to the RE and NBRE sequence. *In vitro* translated proteins were incubated with ^{32}P-labeled probes and electrophoresed through a 5% nondenaturing polyacrylamide gel. The control lane contained (–) unprogrammed reticulocyte lysate. B, Relative affinity of Rev-erbα, Rev-erbβ, and RORα1 for the RE sequence. Mobility shift experiments were performed as in panel A using ^{32}P-labeled RE. Unlabeled competitor DNA was added in increasing concentrations (RE, 0–33 nM; NBRE, 33 nM) to the reaction mix. Complex formation was quantified using a Molecular Dynamics (Sunnyvale, CA) phosphorimager and plotted. The autoradiograph of each complex is shown *above the bar graph*. The amount of complex formed without competitor DNA was defined to be 100% maximal binding.

through the RORα1 response element, we asked whether Rev-erb could compete with RORα1 for activation. CV-1 cells were transfected with RE-2 TK-LUC, RORα1 expression vector, and increasing amounts of Rev-erb expression vectors (Fig. 5B). Cotransfection of RORα1 with equal amounts of either Rev-erb isoform led to significant decreases in RORα1-dependent activity. Inhibition was complete or nearly complete with a 5-fold excess of Rev-erbα and Rev-erbβ, respectively.

DISCUSSION

We describe the cloning and characterization of Rev-erbβ, a novel member of the nuclear receptor superfamily. Rev-erbβ is highly related to Rev-erbα in its DNA binding and putative ligand-binding domains. Both Rev-erb isoforms share significant sequence similarity with the DBDs of the ROR/RZR subfamily of orphan nuclear

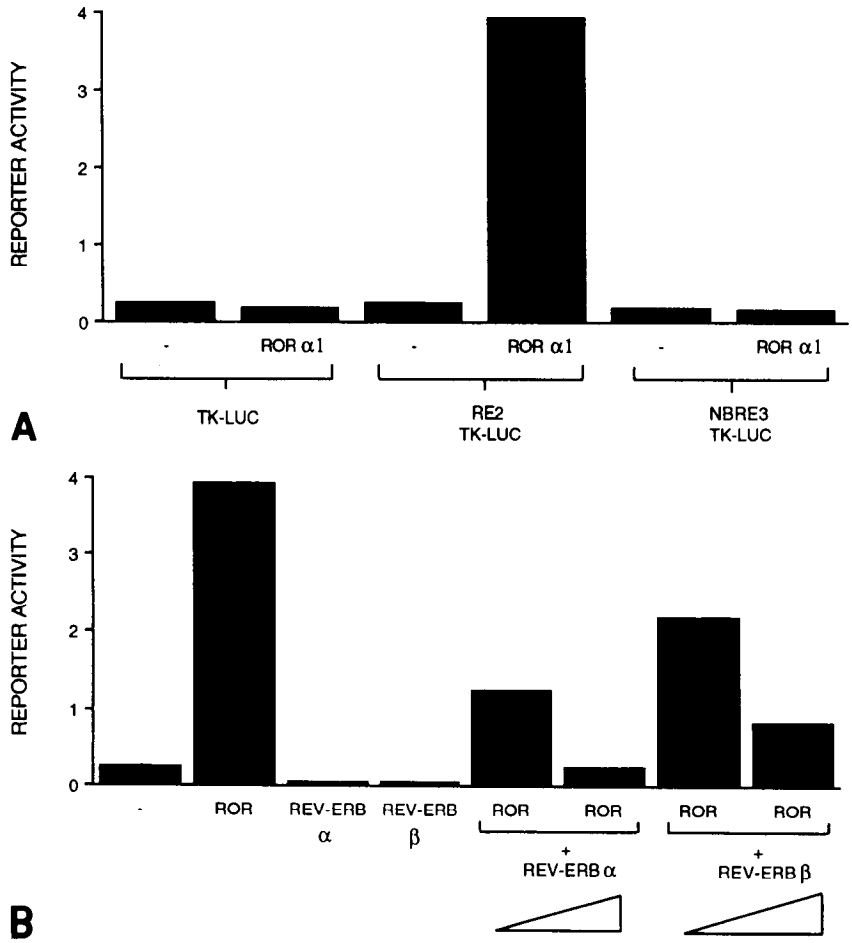

Fig. 5. Transcriptional Properties of Rev-erbα, Rev-erbβ, and RORα1 in Transfected Cells

A, RORα1 activates transcription through the RE but not NBRE sequence. CV-1 cells (7×10^3) were transfected with 30 ng of the indicated reporter construct (TK-LUC, RE2 TK-LUC, or NBRE3 TK-LUC) along with 4 ng of a control expression vector or a human RORα1 expression vector (CMX-hRORα1). CMX-βgal (50 ng) was used as an internal control to normalize for differences in transfection efficiency. The reporter activity represents luciferase activity normalized by B-galactosidase activity. B, Rev-erbα and Rev-erbβ both suppress RORα1 activity. CV-1 cells were transfected with 30 ng RE2 TK-LUC as in panel A along with 4 ng CMX-hRORα1, 20 ng CMX-rRev-erbα, or 20 ng CMX-mRev-erbβ. When ROR and Rev-erb were coexpressed, 4 ng CMX-hRORα1 were used with a 1:1 (4 ng CMX-Rev-erb) or 1:5 (20 ng CMX-Rev-erb) ratio of ROR-Rev-erb expression vectors.

receptors (18, 32). Sequence similarity is maintained in both the zinc finger domain, which recognizes the six-nucleotide core-binding site, as well as in the T/A box that determines sequence-specific binding to the 5′-extension (27, 28). Indeed, we demonstrate that both Rev-erb α/β and RORα1 bind to the same extended binding site.

RORα1 activates transcription constitutively through the RE element. It is not known whether this activity is truly constitutive or due to the presence of an endogenous agonist in CV-1 cells or cell culture media. Both isoforms of Rev-erb were inactive on this response element but were capable of suppressing RORα1 activity on this sequence. Since Rev-erb α and β bind with high affinity to this element, we conclude that Rev-erb α/β compete directly with RORα1 for activation on this element.

The mRNA expression pattern for Rev-erb α/β and RORα1 indicate that Rev-erb α and β are coexpressed with RORα1/RZRα1 in several tissues including the heart, brain, liver, and skeletal muscle. Our studies indicate that Rev-erb α/β and RORα1 comprise a subfamily of orphan receptors that can compete for overlapping networks of response elements. In order to elucidate the physiological consequences of this competition, future experiments will be required to determine the relative expression of Rev-erb α/β and RORα1 proteins in the heart, brain, liver, and skeletal muscle.

Several examples of cross-talk have been demonstrated among the nuclear hormone receptors. For example, the monomer binding orphan receptors SF-1 (34) and NGFI-B (35) bind to distinct but overlapping extended core-binding sites (28). It has been hypothesized that these orphan receptors may compete for transcriptional regulation of the steroid 21-hydroxylase

gene (36). Competitive regulatory networks have been demonstrated between other members of the receptor superfamily. For example, the viral oncogene v-erbA can suppress transcriptional activation by the thyroid hormone receptor (37–39), while the orphan receptor chicken ovalbumin upstream promoter-transcription factor can block activation by several receptors (40). In contrast, synergistic activation results from interactions between retinoid X receptor and the peroxisome proliferator-activated receptor (11, 41, 42). In the case of the glucocorticoid receptor, differential interactions with distinct members of the AP-1 family of transcription factors determine whether the hormonal response will be positive or negative (43). The opposing activities of Rev-erb α/β and RORα1 establish a new transcriptional network regulated by cross-talk among members of the nuclear receptor superfamily.

Harding and Lazar (19) have previously suggested that Rev-erbα acts as a constitutive activator of transcription. Using a similar response element, we have found that Rev-erbα is not transcriptionally active. In contrast, expression of Rev-erbα blocks transcription of the constitutively active receptor RORα1. It is not clear why Rev-erbα originally appeared constitutively active. Indeed, the same authors now support our findings that Rev-erbα is transcriptionally inactive in several cell lines (44). Like Rev-erbα, the N-terminally truncated Rev-erbβ is also transcriptionally inactive. Similarly, we have recently found that a full-length clone of Rev-erbβ (provided by V. Giguere) behaves in an indistinguishable fashion from the truncated clone described here. Although natural target genes for Rev-erb are not known, it remains possible that Rev-erb could exhibit positive transcriptional activity when acting through a native response element/promoter. Alternatively, Rev-erb isoforms may require a specific ligand or other stimulus in order to exhibit transcriptional activity.

Despite exhaustive efforts we have not been able to identify ligands or other activators of Rev-erbα, Rev-erbβ, or RORα1. No enhancement of trans-activation by Rev-erbα, Rev-erbβ, or RORα1 was seen when cells were treated with added serum, retinoids, fatty acids, vitamin E, cholesterol, bile acids, eicosanoids, dehydroepiandrosterone, pregnenalone, or phenobarbitol (B. M. Forman, J. C., T. Perlmann, and R. M. Evans, unpublished observations). It remains possible that Rev-erb α/β and RORα1 could be activated directly by a specific ligand or indirectly by posttranslational modifications resulting from activation of second messenger systems (15, 45–47). If such pathways exist, then Rev-erb would block RORα1 responses in the absence of these signals and activate RORα1-responsive genes in the presence of these signals. In either case, it is clear that signaling pathways for RORα1 and Rev-erb α/β are integrated through their overlapping network of responsive genes. A further understanding of this signaling pathway will require a detailed understanding of the biological functions of these orphan receptors.

MATERIALS AND METHODS

Isolation of Rev-erbβ cDNA

The λML19 clone of Rev-erbβ was isolated by screening an adult mouse liver λZAP cDNA library (Stratagene, La Jolla, CA) with a synthetic oligonucleotide (GGNTTYCAYTAYGGNGTNCAYGC) under conditions previously described (48). This oligonucleotide is a mixture of all possible DNA sequences encoding the amino acid sequence GFHYGVHA, a sequence present in the loop of the first zinc finger in the *Xenopus* peroxisome proliferator-activated receptor-α, β, and γ isoforms (33).

Plasmid Construction

pBluescript KS (+)-ML19 was generated from λML19 by the automatic excision process. The entire Rev-erbβ cDNA was excised by digestion with *Hin*dIII and *Bam*HI sites in the polylinker of pBluescript KS (+)-ML19 and cloned into the corresponding sites of the pCMX expression vector (49). This generated pCMX-mRev-erbβ, which drives expression of mouse Rev-erbβ under control of the T7 promoter *in vitro* and the cytomegalovirus enhancer in transfected cells. To create pCMX-rRev-erbα, the *Hin*dIII/Asp718 fragment of rat Rev-erbα was excised from pBluescript-rRev-erbα (29) and cloned into pCMX-PL1. The resulting vector was digested with *Bam*HI and *Hin*dIII, and the natural reading frame was regenerated by introduction of an oligonucleotide containing the wild type reading frame with an optimized translation start codon. Human RORα1 was cloned from clone λHR5 (18) into pCMX and kindly provided to us by Kazuhiko Umesono. RE2 TK-LUC was constructed by inserting two directly repeating copies of the RE oligonucleotide (→ →) into the *Hin*dIII site of TK-LUC. The RE oligonucleotide is as follows:

5′ AGCTTAGAATGTAGGTCAA 3′
3′ ATCTTACATCCAGTTTCGA 5′

Northern Blot Analysis

A Northern blot containing 2 μg polyA$^+$ RNA/lane from several mouse tissues was screened according to the distributor's recommendations (Clontech, Palo Alto, CA). To probe for Rev-erbα mRNA, mouse tissues were probed with a 957-bp ^{32}P-labeled fragment spanning the *Xba*I-*Eco*RI sites (nucleotides 891–1848) of rat Rev-erbα (29). This probe was selected to avoid detection of the c-erbAα2 transcript encoded by the opposite strand of a 3′-Rev-erbα exon (29). Using the same blot, Rev-erbβ mRNA was detected with a 672-bp *Bst*XI-*Pst*I fragment spanning nucleotides 762–1434 of mouse Rev-erbβ. RORα1 expression in human tissues was determined using an antisense DNA probe spanning nucleotides 33–298 of human RORα1 (18). The probe was obtained by assymetric PCR using Taq Polymerase in the presence of [^{32}P]deoxycytosine triphosphate.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays were performed with *in vitro* translated proteins in a rabbit reticulocyte lysate system (TNT, Promega, Madison, WI). Proteins were mixed with 100,000 cpm of Klenow-labeled probes in the following reaction buffer: 10 mM Tris, pH 8, 100 mM KCl, 6% glycerol, 0.05% NP-40, 1 mM dithiothreitol, and 100 ng/μl poly dI·dC. The reaction was incubated for 20 min at room temperature and then electrophoresed through a 5% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA electrophoresis buffer (TBE). The RE sequence is described above. The NBRE oligonucleotide sequence is as follows:

5′ AGCTGAGAGGTCATGCA 3′
3′ CTCTCCAGTACGTTCGA 5′

For competition studies, the reaction was performed as above with the indicated concentrations of unlabeled probes.

Transient Transfection Assay

One day before transfection 7×10^3 CV-1 cells were plated in Dulbecco's modified essential medium (DMEM) containing 10% resin charcoal- stripped (50) calf bovine serum. The next day, cells were transfected by lipofection using DOTAP according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). Transfection assays contained 30 ng reporter construct, 50 ng CMX-βgal as a control for transfection efficiency, and 4–20 ng CMX expression vector. After 2 h the liposomes were removed and the cells were treated for 36 h with phenol red-free DMEM containing 10% resin charcoal-stripped fetal bovine serum. Cells were harvested and assayed for luciferase and β-galactosidase activities. All points in each experiment represent the average of triplicates that varied by less than 10%. Each experiment was repeated three times with similar results.

Acknowledgments

The authors would like to thank Vincent Giguere and David Moore for mutual exchange of sequence information before publication. We thank Remco Spanjaard and Bill Chin for rat Rev-erbα and Kazuhiko Umesono for CMX-hRORα1; Debabrata Chakravarti, Ira Schulman, and Henry Sucov for critical review of this manuscript; and Elaine Stevens for expert secretarial assistance.

Received March 24, 1994. Revision accepted May 27, 1994. Accepted June 13, 1994.

Address requests for reprints to: Ronald Evans, Gene Expression Laboratory, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, California 92037–1099.

This work was supported by the Howard Hughes Medical Institute, the National Institute of Health (R.M.E.), Tobacco-Related Disease Research Program (B.M.F.), Jane Coffin Childs Memorial Fund for Medical Research (S.A.K.), and the American Cancer Society (B.B.).

*R.M.E is an Investigator of the Howard Hughes Medical Institute at the Salk Institute for Biological Studies.

REFERENCES

1. Shi YB, Brown DD 1993 The earliest changes in gene expression in tadpole intestine induced by thyroid hormone. J Biol Chem 268:20312–20317
2. Weintraub H, Davis R, Tapscott S, Thayer M, Krause M, Benezra R, Blackwell TK, Turner D, Rupp R, Hollenberg S, Yuan Z, Lassar A 1991 The myoD gene family: nodal point during specification of the muscle cell lineage. Science 251:761–766
3. Hurban P, Thummel CS 1993 Isolation and characterization of fifteen ecdysone-inducible Drosophila genes reveal unexpected complexities in ecdysone regulation. Mol Cell Biol 13:7101–7111
4. Hollingsworth Jr R, Chen PL, Lee WH 1993 Integration of cell cycle control with transcriptional regulation by the retinoblastoma protein. Curr Opin Cell Biol 5:194–200
5. Prives C 1993 Doing the right thing: feedback control and p53. Curr Opin Cell Biol 5:214–218
6. Fesus L 1993 Biochemical events in naturally occurring forms of cell death. FEBS Lett 328:1–5
7. King LB, Ashwell JD 1993 Signaling for death of lymphoid cells. Curr Opin Immunol 5:368–373
8. Korsmeyer SJ 1992 Chromosomal translocations in lymphoid malignancies reveal novel proto-oncogenes. Annu Rev Immunol 10:785–807
9. Gilmore TD 1991 Malignant transformation by mutant Rel proteins. Trends Genet 7:318–322
10. Sluyser M 1991 Nuclear hormone receptor variants: their role in malignancy and progression to hormone resistance in cancer. Acta Endocrinol (Copenh) 1:48–53
11. Kliewer SA, Umesono K, Noonan DJ, Heyman RA, Evans RM 1992 Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. Nature 358:771–774
12. Shemshedini L, Knauthe R, Sassone-Corsi P, Pornon A, Gronemeyer H 1991 Cell-specific inhibitory and stimulatory effects of Fos and Jun on transcription activation by nuclear receptors. EMBO J 10:3839–3849
13. Miner JN, Yamamoto KR 1992 The basic region of AP-1 specifies glucocorticoid receptor activity at a composite response element. Genes Dev 6:2491–2501
14. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889–895
15. O'Malley BW, Conneely OM 1992 Orphan receptors: in search of a unifying hypothesis for activation. Mol Endocrinol 6:1359–1361
16. Wilson TE, Fahrner TJ, Johnston M, Milbrandt J 1991 Identification of the DNA binding site for NGFI-B by genetic selection in yeast. Science 252:1296–1300
17. Kurokawa R, Yu VC, Naar A, Kyakumoto S, Han Z, Silverman S, Rosenfeld MG, Glass CK 1993 Differential orientations of the DNA-binding domain and carboxy-terminal dimerization interface regulate binding site selection by nuclear receptor heterodimers. Genes Dev 7:1423–1435
18. Giguere V, Tini M, Flock G, Ong E, Evans RM, Otulakowski G 1994 Isoform-specific amino-terminal domains dictate DNA-binding properties of RORα, a novel family of orphan nuclear hormone receptors. Genes Dev 8:538–553
19. Harding HP, Lazar MA 1993 The orphan receptor Rev-ErbA alpha activates transcription via a novel response element. Mol Cell Biol 13:3113–3121
20. Katz RW, Koenig RJ 1993 Nonbiased identification of DNA sequences that bind thyroid hormone receptor alpha 1 with high affinity. J Biol Chem 268:19392–19397
21. Lee MS, Kliewer SA, Provencal J, Wright PE, Evans RM 1993 Structure of the retinoid × receptor alpha DNA binding domain: a helix required for homodimeric DNA binding. Science 260:1117–1121
22. Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB 1991 Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352:497–505
23. Schwabe JW, Chapman L, Finch JT, Rhodes D 1993 The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell 75:567–578
24. Umesono K, Evans RM 1989 Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell 57:1139–1146
25. Danielsen M, Hinck L, Ringold GM 1989 Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. Cell 57:1131–1138
26. Mader S, Kumar V, de Verneuil H, Chambon P 1989 Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. Nature 338:271–274
27. Wilson TE, Paulsen RE, Padgett KA, Milbrandt J 1992 Participation of non-zinc finger residues in DNA binding by two nuclear orphan receptors. Science 256:107–110
28. Wilson TE, Fahrner TJ, Milbrandt J 1993 The orphan receptors NGFI-B and steroidogenic factor 1 establish

monomer binding as a third paradigm of nuclear receptor-DNA interaction. Mol Cell Biol 13:5794–5804
29. Lazar MA, Hodin RA, Darling DS, Chin WW 1989 A novel member of the thyroid/steroid hormone receptor family is encoded by the opposite strand of the rat c-erbA alpha transcriptional unit. Mol Cell Biol 9:1128–1136
30. Miyajima N, Horiuchi R, Shibuya Y, Fukushige S, Matsubara K, Toyoshima K, Yamamoto T 1989 Two erbA homologs encoding proteins with different T3 binding capacities are transcribed from opposite DNA strands of the same genetic locus. Cell 57:31–39
31. Chawla A, Lazar MA 1993 Induction of Rev-ErbA alpha, an orphan receptor encoded on the opposite strand of the alpha-thyroid hormone receptor gene, during adipocyte differentiation. J Biol Chem 268:16265–16269
32. Becker-Andre M, Andre E, DeLamarter JF 1993 Identification of nuclear receptor mRNAs by RT-PCR amplification of conserved zinc-finger motif sequences. Biochem Biophys Res Commun 194:1371–1379
33. Dreyer C, Krey G, Keller H, Givel F, Helftenbein G, Wahli W 1992 Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. Cell 68:879–887
34. Ikeda Y, Lala DS, Luo X, Kim E, Moisan MP, Parker KL 1993 Characterization of the mouse FTZ-F1 gene, which encodes a key regulator of steroid hydroxylase gene expression. Mol Endocrinol 7:852–860
35. Milbrandt J 1988 Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. Neuron 1:183–188
36. Wilson TE, Mouw AR, Weaver CA, Milbrandt J, Parker KL 1993 The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase. Mol Cell Biol 13:861–868
37. Sap J, Munoz A, Schmitt J, Stunnenberg H, Vennstrom B 1989 Repression of transcription mediated at a thyroid hormone response element by the v-erb-A oncogene product. Nature 340:242–244
38. Damm K, Thompson CC, Evans RM 1989 Protein encoded by v-erbA functions as a thyroid-hormone receptor antagonist. Nature 339:593–597
39. Hermann T, Hoffmann B, Piedrafita FJ, Zhang XK, Pfahl M 1993 V-erbA requires auxiliary proteins for dominant negative activity. Oncogene 8:55–65
40. Cooney AJ, Leng X, Tsai SY, O'Malley BW, Tsai MJ 1993 Multiple mechanisms of chicken ovalbumin upstream promoter transcription factor-dependent repression of transactivation by the vitamin D, thyroid hormone, and retinoic acid receptors. J Biol Chem 268:4152–4160
41. Issemann I, Prince RA, Tugwood JD, Green S 1993 The retinoid X receptor enhances the function of the peroxisome proliferator activated receptor. Biochimie 75:251–256
42. Keller H, Dreyer C, Medin J, Mahfoudi A, Ozato K, Wahli W 1993 Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. Proc Natl Acad Sci USA 90:2160–2164
43. Diamond MI, Miner JN, Yoshinaga SK, Yamamoto KR 1990 Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. Science 249:1266–1272
44. Dumas B, Harding HP, Choi H-S, Lehmann K, Chung M, Lazar MA, Moore DD 1994 BD73, a new orphan member of the nuclear hormone receptor superfamily closely related to Rev-Erb. Mol Endocrinol 15:996–1005
45. Power RF, Mani SK, Codina J, Conneely OM, O'Malley BW 1991 Dopaminergic and ligand-independent activation of steroid hormone receptors. Science 254:1636–1639
46. Lydon JP, Power RF, Conneely OM 1992 Differential modes of activation define orphan subclasses within the steroid/thyroid receptor superfamily. Gene Expr 2:273–283
47. Ignar-Trowbridge DM, Teng CT, Ross KA, Parker MG, Korach KS, McLachlan JA 1993 Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. Mol Endocrinol 7:992–998
48. Blumberg B, Mangelsdorf DJ, Dyck JA, Bittner DA, Evans RM, De Robertis EM 1992 Multiple retinoid-responsive receptors in a single cell: families of retinoid "X" receptors and retinoic acid receptors in the *Xenopus* egg. Proc Natl Acad Sci USA 89:2321–2325
49. Umesono K, Murakami KK, Thompson CC, Evans RM 1991 Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. Cell 65:1255–1266
50. Samuels HH, Stanley F, Casanova J 1979 Depletion of L-3,5,3′-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. Endocrinology 105:80–85
51. Baker AR, McDonnell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J, O'Malley BW 1988 Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci USA 85:3294–3298
52. Issemann I, Green S 1990 Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 347:645–650
53. Petkovich M, Brand NJ, Krust A, Chambon P 1987 A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 330:444–450
54. Giguere V, Ong ES, Segui P, Evans RM 1987 Identification of a receptor for the morphogen retinoic acid. Nature 330:624–629
55. Nakai A, Sakurai A, Bell GI, DeGroot LJ 1988 Characterization of a third human thyroid hormone receptor coexpressed with other thyroid hormone receptors in several tissues. Mol Endocrinol 2:1087–1092
56. Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM 1990 Nuclear receptor that identifies a novel retinoic acid response pathway. Nature 345:224–229