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RXRβ: A Coregulator That Enhances Binding of Retinoic Acid, Thyroid Hormone, and Vitamin D Receptors to Their Cognate Response Elements

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Summary

The retinoic acid receptor (RAR) requires coregulators to bind effectively to response elements in target genes. A strategy of sequential screening of expression libraries with a retinoic acid response element and RAR identified a cDNA encoding a coregulator highly related to RXRα. This protein, termed RXRβ, forms heterodimers with RAR, preferentially increasing its DNA binding and transcriptional activity on promoters containing retinoic acid, but not thyroid hormone or vitamin D, response elements. Remarkably, RXRβ also heterodimerizes with the thyroid hormone and vitamin D receptors, increasing both DNA binding and transcriptional function on their respective response elements. RXRα also forms heterodimers with these receptors. These observations suggest that retinoid X receptors meet the criteria for biochemically characterized cellular coregulators and serve to selectively target the high affinity binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate DNA response elements.

Introduction

Retinoic acid has been implicated to play a critical role in many aspects of vertebrate development and homeostasis (reviewed in Lotan et al., 1980; Sporn and Roberts, 1983). The effects of retinoic acid are apparently mediated by specific nuclear receptor proteins that are members of the steroid and thyroid hormone (T_3) receptor superfamily of transcriptional regulators (reviewed in Yamamoto, 1985; Giguere et al., 1987; Petkovich et al., 1987; Brand et al., 1988; Benbrook et al., 1988; Zelent et al., 1989; Kastner et al., 1990; Evans, 1988; Green and Chambon, 1988; Beato, 1989; O'Malley, 1990; Glass and Rosenfeld, 1991). These receptors are characterized by a highly conserved 68 amino acid domain that forms the zinc finger structures conferring sequence-specific DNA-binding capabilities to each receptor. In addition, a less well-conserved C-terminal region of approximately 220 amino acids functions as a ligand-binding domain (reviewed in Evans, 1988; Green and Chambon, 1988) and facilitates receptor dimerization (Glass et al., 1989, 1990; Forman et al., 1989; Fawell et al., 1990).

Three genes that encode high affinity retinoic acid receptors (RARs), termed α, β, and γ, have been identified (Giguere et al., 1987; Petkovich et al., 1987; Benbrook et al., 1988; Brand et al., 1988; Krust et al., 1989; Zelent et al., 1989). The transcripts of each of these genes encode a series of nuclear receptor proteins, differing primarily in N-terminal sequence, that bind retinoic acid and activate transcription from retinoic acid–responsive promoters in response to nanomolar concentrations of retinoic acid. These observations, in conjunction with the patterns of expression of RARs during ontogeny and in the developed organism (e.g., Brand et al., 1988; Durston et al., 1989; Krust et al., 1989; Zelent et al., 1989; Yang et al., 1991), have suggested that this class of RARs mediates many of the developmental effects of retinoic acid in vivo. Recently, a distinct nuclear receptor, referred to as retinoid X receptor α (RXRα), has been identified (Mangelsdorf et al., 1990, 1991). This receptor differs considerably from the RARs within the ligand-binding domain and is incapable of high affinity binding of retinoic acid itself. However, it is reported to be capable of mediating a transcriptional response to retinoic acid, but only at much higher concentrations ($>10^{-6}$ M) (Mangelsdorf et al., 1990; Mangelsdorf et al., 1991). RXRα, in response to retinoic acid, appears to be capable of activating target genes such as the cellular retinol binding protein type II by binding to specific response elements that are distinct from those that are regulated by RARs (Mangelsdorf et al., 1991), raising the possibility of distinct pathways of retinoid actions.

The retinoic acid, thyroid hormone, and vitamin D receptors, as well as the retinoid X receptor, activate transcription from response elements containing two or more degenerate copies of the consensus motif AGGTCA (Figure 1). The orientation and spacing of the core binding motifs appear to dictate selective transcriptional effects by each of these receptors (Umesono et al., 1991; Näär et al., 1991; Mangelsdorf et al., 1991). A palindromic arrangement of core motifs, AGGTCA TGACCT (TRE-pal) (Glass et al., 1988), confers transcriptional responses to thyroid hormone receptor, RAR, and RXRα (Glass et al., 1988; Umesono et al., 1988; Näär et al., 1991; Mangelsdorf et al., 1991). In contrast, when arranged as direct repeats (AGGTCA $[N]_x$ AGGTCA), relatively specific transcriptional responses are observed that are dependent on the spacing between half-sites (Umesono et al., 1991; Näär et al., 1991; see Figure 4A). Spacings of 1, 3, 4, and 5 bp (Figure 1) confer relatively specific transcriptional responses for RXRα, vitamin D receptor, thyroid hormone receptor, and RAR, respectively, while a spacing of 2 bp can permit negative regulation by the thyroid hormone receptor (Mangelsdorf et al., 1991; Umesono et al., 1991; Näär et al., 1991). Similarly, restricted specificity can be observed with inverted core motif palindromic orientations (Baniahmad et al., 1990; Näär et al., 1991).

Analysis of the DNA-binding properties of the RAR has

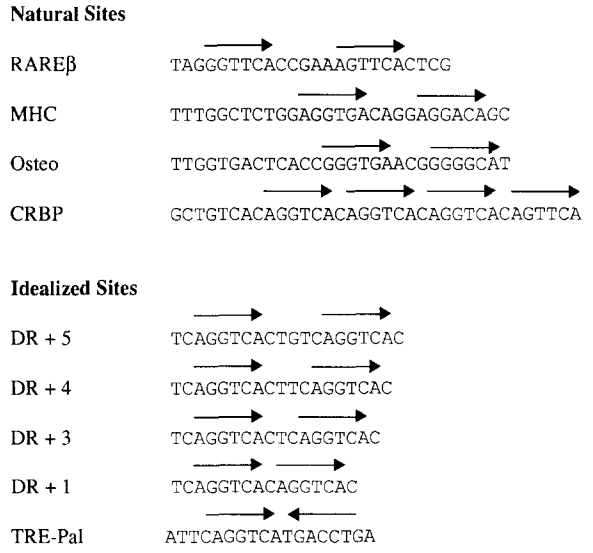


Figure 1. Nuclear Receptor DNA-Binding Elements

Natural sites: RARβ response element (RAREβ), myosin heavy chain thyroid hormone receptor response element (MHC), rat osteocalcin vitamin D receptor response element (Osteo), cytoplasmic retinol binding protein II RXRα response element (CRBP).

Idealized sites: direct repeat (DR) synthetic elements spaced by 5 bp (DR+5, retinoic acid response element), 4 bp (DR+4, thyroid hormone response element), 3 bp (DR+3, vitamin D response element), 1 bp (DR+1 RXRα response element), or the palindromic thyroid hormone response element/retinoic acid response element (TRE-Pal).

suggested that high affinity binding to response elements requires interactions with additional nuclear factors referred to as RAR coregulators (Glass et al., 1990). Biochemical analyses of these protein(s) indicated that they are widely expressed and exhibit different molecular weight profiles in different cell types (Glass et al., 1990). Coregulatory proteins have been found to interact with the RAR via conserved C-terminal sequences that function as a dimerization interface in the estrogen and thyroid hormone receptors (Glass et al., 1989; Forman et al., 1989; Fawell et al., 1990). The heteromeric interactions of RAR with specific coregulators has proved to be prototypic for a subset of nuclear receptors. Thus, binding of the thyroid hormone receptor to representative response element sequences has been reported to be significantly enhanced by addition of a protein or proteins, one of which has been termed thyroid hormone receptor auxiliary protein (Darling et al., 1989, 1990; Burnside et al., 1990; O'Donnell et al., 1991; Beebe et al., 1991; Näär et al., 1991). Purified vitamin D receptors bind to vitamin D response elements with low affinity, but exhibit high affinity binding in the presence of additional nuclear proteins (Liao et al., 1990; Ozono et al., 1990). While the identity of the coregulator proteins that interact with retinoic acid, thyroid hormone, and vitamin D receptors are not known, the potential biological actions of these coregulators make their identification and functional characterization important for an understanding of the molecular basis of retinoic acid, thyroid hormone, and vitamin D action. Furthermore, an analysis of these proteins may provide insight into the molecular mechanisms by which the orientation and spacing of identical core binding motifs can confer selective responsivity to specific nuclear receptors.

Although the thyroid hormone receptor has been found to be capable of forming heterodimers with the RARs (Forman et al., 1989; Glass et al., 1989), it failed to enhance RAR binding to sequences such as the β retinoic acid response element that confers retinoic acid responsiveness in vivo. Therefore, the DNA-binding properties of thyroid hormone–RAR heterodimers were markedly different than the DNA-binding properties of heterodimers formed with RAR coregulator activities in most cell types. To identify cDNA clones encoding proteins that exhibit the properties of biochemically defined coregulators, an expression screening strategy was devised based on the ability of coregulator proteins to bind to retinoic acid response elements and to interact with the RAR. Using this strategy, a clone encoding a fusion protein exhibiting both of these properties was identified that is a member of the retinoid X receptor subclass of nuclear receptors, referred to here as RXRβ. Subsequent analysis of the full-length RXRβ indicated that it preferentially increased RAR binding to retinoic acid response elements, compared with other classes of DNA response elements containing identical core DNA-binding motifs. RXRβ also augmented the transcriptional activity of the RAR on promoters containing these elements. Remarkably, the identical protein exerted similar specific effects on the thyroid hormone and vitamin D receptors, acting preferentially to increase binding to their respective response elements. The ability of RXRβ heterodimeric complexes to facilitate element-specific DNA binding of retinoic acid, thyroid hormone, and vitamin D receptors may account, in part, for the ability of differentially configured core binding motifs to selectively confer positive or negative transcriptional responses to specific nuclear receptors.

Results

Cloning of a cDNA Encoding an RAR Coregulator

RAR coregulators were initially found to have the properties of forming heterodimeric complexes with RARs and of increasing their binding to cognate DNA response elements (Glass et al., 1990). Coregulator proteins also exhibited intrinsic DNA-binding properties, because they could be purified on DNA affinity columns containing retinoic acid response elements. Cross-linking analyses showed that in many cell types (HeLa, F9, PC12, CV-1), the predominant coregulator proteins had molecular weights of ~55 kd (data not shown). Based on these observations, a cloning strategy was devised, as shown in Figure 2A. Initially, a cDNA library from a tissue shown to contain RAR coregulators (a rat thyroid C-cell tumor) was screened using the retinoic acid response element from the β RAR gene (see Figure 2). A total of more than 10^6 plaques were screened, and 11 phage expression fusion proteins that specifically bound the RARβ response element were identified. These fusion proteins failed to bind recognition sequences for the transcription factors Pit-1 or C/EBP (Figure 2A). The initial positive clones, once plaque purified

and shown not to be RAR fusion proteins, were then subjected to a secondary protein–protein screening based on the ability to bind to in vitro translated ^{35}S-labeled retinoic acid receptor (Figure 2B). One of the 11 fusion proteins reproducibly bound to ^{35}S-labeled RAR protein, but not to ^{35}S-labeled Pit-1 or ^{35}S-labeled C/EBP proteins. The isolation and DNA sequencing of this cDNA insert, as well as a series of additional clones obtained by nucleic acid hybridization with the original cDNA insert, revealed a cDNA with an open reading frame encoding a protein of 451 amino acids (Figure 2C). This protein, initially referred to as RCoR-1, was characterized by a 68 amino acid putative DNA-binding motif that corresponded to the known cys-cys zinc finger DNA-binding motif present in the steroid receptor gene superfamily. A data base search revealed that the predicted protein differed by only 2 amino acids from a previously reported murine nuclear receptor, referred to as H-2 RIIBP (Hamada et al., 1989), identified based on its ability to bind a specific cis-active element in MHC class I genes. This protein also exhibited remarkable homology to the RXRα protein (Mangelsdorf et al., 1990), exhibiting 95% identity in the DNA-binding domains and greater than 90% identity in the ligand-binding domain and differing only in two, highly diverged regions of 10 and 15 amino acids, as indicated by the boxed regions in Figure 2C. The N-termini were markedly diverged. Based on this sequence similarity to RXRα, this protein will be referred to as RXRβ in this manuscript.

RNA blot analysis and RNAase protection assays utilizing a specific probe from the 5′ region of the cDNA revealed that the 2.4 kb RXRβ mRNA is widely expressed, except for notably low levels of expression in liver, pancreas, and intestine (Figure 3B). Consistent with its role as a coregulator, it is coexpressed with RARα in several cell types (Figure 3A). Therefore, RXRβ transcripts are expressed in most cell types in which retinoic acid has been suggested to exert regulatory functions. RXRβ mRNA is particularly highly expressed in P19 stem cells, which have been shown to differentiate in response to retinoic acid into several cell types that include cardiac and neuronal phenotypes. RXRβ mRNA levels were increased (3-fold) in response to thyroid hormone in the GC pituitary cell line (Figure 3B).

To determine whether RXRβ actually satisfied the criteria of biochemically defined retinoic acid coregulators, bacterially expressed or in vitro translated protein was evaluated for the ability to enhance binding of the RAR to specific sites (Figure 4A), using the avidin–biotin complex DNA (ABCD) assay (Glass et al., 1988). RXRβ increased the binding of the RAR to the RARβ response element as effectively as the coregulators present in the HeLa nuclear extracts in which the coregulator activity was initially identified (Figure 4A). The coregulator effect of RXRβ was also observed on the palindromic thyroid hormone–retinoic acid response element (TRE-pal), but not on another, unrelated DNA element (Figure 4A).

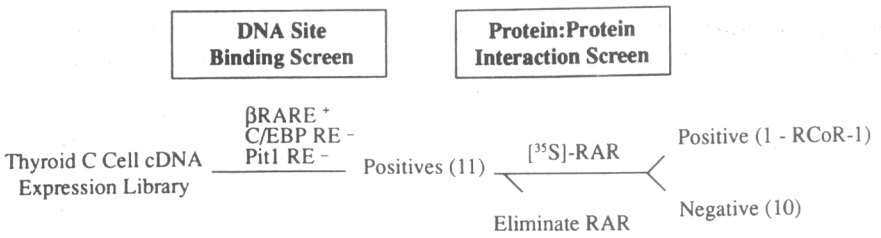
These effects were confirmed using DNA electrophoretic mobility shift analysis, employing bacterially expressed RXRβ and an RARα expressed in vitro. At low protein concentrations, RXRβ itself bound only weakly to a RARβ response element, while binding of the RAR was virtually undetectable using gel shift analysis (Figure 4B). However, the simultaneous addition of both proteins resulted in a striking increase in binding of both proteins to the RARβ response element, consistent with the marked effect of RXRβ on RAR binding in the ABCD analysis (Figure 4B). The presence of RAR in the putative heterodimeric complex on DNA was confirmed by use of an antibody directed against a peptide at the RAR C-terminus, which significantly disrupted the increased DNA-binding activity observed when RAR was added in addition to RXRβ (data not shown). Specificity of the effects of RXRβ action was suggested by its failure to enhance binding of an unrelated transcription factor Pit-1 to its recognition element. It also had little or no reproducible effects on the binding of a rat COUP-related nuclear receptor, even though these experiments were performed under conditions of RXRβ excess (Figure 4B). Failure of RXRβ to enhance binding was also observed at lower concentrations of Pit-1 or COUP-related protein (CRP), at which minimal DNA binding was observed (data not shown). Furthermore, the effects of RXRβ were examined in the case of estrogen receptor, which binds to the vitellogenin estrogen response element as a homodimer (Kumar and Chambon, 1988; Fawell et al., 1990). On both ABCD assays (data not shown) and gel shift analysis (Figure 4B), no evidence of enhanced binding of estrogen receptor or heterodimer formation with RXRβ was observed. We also find no evidence of enhancement of binding of the glucocorticoid receptor to its cognate recognition element by RXRβ (data not shown).

The binding of RXRβ–RAR heterodimers to the RARβ response element and TRE-pal DNA response element was confirmed using the bifunctional chemical cross-linking agent disuccinimidyl suberate (DSS) (Figure 4C). Identical heteromeric complexes were obtained regardless of which receptor was radiolabeled. The RXRβ and RARs were also shown to be capable of forming heterodimers in solution (data not shown). These data indicated that the RXRβ protein fulfilled the criteria for an RAR coregulator.

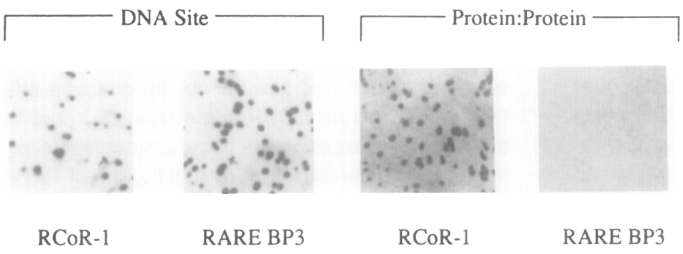
The C-Terminal Interaction Domain Is Required for RAR–RXRβ Heterodimer Formation

The protein regions involved in RAR and RXRβ interactions were determined using a series of RAR truncations. The C-terminus has been demonstrated to serve as both a ligand-binding and protein interaction domain for other steroid receptors. A truncation of 57 amino acids of C-terminal sequence in the RAR produced minimal effects on heterodimeric interactions. However, truncation of 110 C-terminal amino acids markedly impaired the RXRβ–RAR interaction (Figure 5A). A mutation in the first zinc finger that prevented RAR binding to its cognate DNA element did not decrease RXRβ–RAR interactions in solution (data not shown); however, this mutation abolished the enhanced binding of the RXRβ–RAR heterodimers, suggesting that intact DNA-binding domains of both heteromeric partners were required for effective DNA binding (Figure 5A). Furthermore, an isolated region of the RAR

A.



B.



C.

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GAATTCCGGGGGGAGGCTGGCCGGGACGGG ATG GGC GAC ACG GGG CGG GAT TCC CGA AGC CCA GAC AGC TCC TCC   75
                               Met Gly Asp Thr Gly Arg Asp Ser Arg Ser Pro Asp Ser Ser Ser   15

CCA AAT CCC CTT TCC CAG GGG ATC CCT CCC TCT TCT CCT CCT GGC CCA CCT CAC ACC CCT TCA GCA CCT CCA CCT   150
Pro Asn Pro Leu Ser Gln Gly Ile Pro Pro Ser Ser Pro Pro Gly Pro Pro His Thr Pro Ser Ala Pro Pro Pro   40

CCA ATG CCA CCC CCG CCA CTG GGC TCC CCC TTC CCA GTC ATC AGT TCT TCC ATG GGG TCT CCT GGT CTG CCC CCT   225
Pro Met Pro Pro Pro Pro Leu Gly Ser Pro Phe Pro Val Ile Ser Ser Ser Met Gly Ser Pro Gly Leu Pro Pro   65

CCG GCT CCC CCA GGA TTC TCC GGG CCT GTC AGC AGC CCT CAG ATT AAC TCC ACA GTG TCG CTC CCT GGG GGT GGG   300
Pro Ala Pro Pro Gly Phe Ser Gly Pro Val Ser Ser Pro Gln Ile Asn Ser Thr Val Ser Leu Pro Gly Gly Gly   90

TCT GGC CCC CCT GAA GAT GTG AAG CCA CCA GTC TTA GGG GTC CGG GGC CTG CAC TGT CCA CCC CCT CCA GGT GGC   375
Ser Gly Pro Pro Glu Asp Val Lys Pro Pro Val Leu Gly Val Arg Gly Leu His Cys Pro Pro Pro Pro Gly Gly   115

CCT GGG GCT GGC AAA CGA CTT TGT GCA ATC TGC GGG GAC CGA AGC TCA GGC AAG CAC TAT GGG GTT TAC AGC TGC   450
Pro Gly Ala Gly Lys Arg Leu Cys Ala Ile Cys Gly Asp Arg Ser Ser Gly Lys His Tyr Gly Val Tyr Ser Cys   140

GAG GGC TGC AAG GGT TTC TTC AAG CGC ACC ATT CGG AAG GAC CTG ACC TAC TCA TGC CGA GAT AAC AAA GAC TGC   525
Glu Gly Cys Lys Gly Phe Phe Lys Arg Thr Ile Arg Lys Asp Leu Thr Tyr Ser Cys Arg Asp Asn Lys Asp Cys   165

ACA GTG GAC AAG CGC CAG CGG AAT CGC TGT CAG TAC TGT CGA TAT CAG AAG TGC CTG GCC ACC GGC ATG AAA AGG   600
Thr Val Asp Lys Arg Gln Arg Asn Arg Cys Gln Tyr Cys Arg Tyr Gln Lys Cys Leu Ala Thr Gly Met Lys Arg   190

GAG GCG GTA CAG GAG GAG CGA CAA CGG GGG AAG GAC AAA GAC GGG GAT GGG GAT GGG GCT GGG GGA GCC CCT GAG   675
Glu Ala Val Gln Glu Glu Arg Gln Arg Gly Lys Asp Lys Asp Gly Asp Gly Asp Gly Ala Gly Gly Ala Pro Glu   215

GAG ATG CCG GTG GAC AGG ATC CTG GAG GCA GAG CTT GCT GTG GAA CAG AAG AGC GAC CAA GGC GTT GAG GGT CCC   750
Glu Met Pro Val Asp Arg Ile Leu Glu Ala Glu Leu Ala Val Glu Gln Lys Ser Asp Gln Gly Val Glu Gly Pro   240

GGG GCC ACC GGG GGT GGT GGC AGC AGC CCA AAT GAC CCA GTG ACT AAC ATC TGC CAG GCA GCT GAC AAG CAG CTG   825
Gly Ala Thr Gly Gly Gly Gly Ser Ser Pro Asn Asp Pro Val Thr Asn Ile Cys Gln Ala Ala Asp Lys Gln Leu   265

TTC ACA CTC GTG GAG TGG GCA AAG AGG ATC CCG CAC TTT TCC TCC CTA CCT CTG GAC GAC CAG GTC ATA CTG CTG   900
Phe Thr Leu Val Glu Trp Ala Lys Arg Ile Pro His Phe Ser Ser Leu Pro Leu Asp Asp Gln Val Ile Leu Leu   290

CGG GCA GGC TGG AAT GAG CTC CTC ATT GCG TCC TTC TCC CAT CGG TCC ATC GAT GTC CGA GAT GGC ATC CTC CTG   975
Arg Ala Gly Trp Asn Glu Leu Leu Ile Ala Ser Phe Ser His Arg Ser Ile Asp Val Arg Asp Gly Ile Leu Leu   315

GCC ACG GGT CTT CAT GTG CAC AGA AAC TCA GCC CAT TCT GCA GGC GTG GGA GCC ATC TTT GAT CGG GTG CTG ACA   1050
Ala Thr Gly Leu His Val His Arg Asn Ser Ala His Ser Ala Gly Val Gly Ala Ile Phe Asp Arg Val Leu Thr   340

GAG CTA GTG TCC AAA ATG CGT GAC ATG AGG ATG GAC AAG ACG GAG CTT GGC TGC CTG CGG GCA ATC ATA CTG TTT   1125
Glu Leu Val Ser Lys Met Arg Asp Met Arg Met Asp Lys Thr Glu Leu Gly Cys Leu Arg Ala Ile Ile Leu Phe   365

AAC CCA GAC GCC AAG GGG CTC TCC AAC CCT GGA GAG GTG GAG ATC CTT CGG GAG AAG GTG TAT GCC TCA CTG GAG   1200
Asn Pro Asp Ala Lys Gly Leu Ser Asn Pro Gly Glu Val Glu Ile Leu Arg Glu Lys Val Tyr Ala Ser Leu Glu   390

ACC TAC TGC AAG CAG AAG TAT CCT GAG CAG CAG GGC CGG TTT GCC AAG CTG CTA CTA CGT CTT CCT GCC CTC CGC   1275
Thr Tyr Cys Lys Gln Lys Tyr Pro Glu Gln Gln Gly Arg Phe Ala Lys Leu Leu Leu Arg Leu Pro Ala Leu Arg   415

TCC ATC GGC CTC AAG TGT CTG GAG CAC CTG TTC TTC TTC AAG CTC ATT GGC GAC ACC CCC ATT GAC ACC TTC CTC   1350
Ser Ile Gly Leu Lys Cys Leu Glu His Leu Phe Phe Phe Lys Leu Ile Gly Asp Thr Pro Ile Asp Thr Phe Leu   440

ATG GAG ATG CTT GAG GCT CCC CAC CAG CTA GCC TGA GTCCGGATGCACACGGAGGGAGAGTGTCGCTGAGGAGGACTTGAGCCGCGC   1437
Met Glu Met Leu Glu Ala Pro His Gln Leu Ala OPA  *                                          451

GGGGGCGAGAACCATGGACAGGTGCAGAGCAGGAGGGGACTTGCCCATCCTGCCAGGGGTCTGGCAACACTTAACAGGGTTTGCGTGGTCCCCAAGTCA   1536
GAGCAGAGGGACCCCAGATCCCTGTGA                                                                 1564
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C-terminus (amino acids 186–461) failed to bind to DNA in the presence of RXRβ. Chemical cross-linking analysis with DSS revealed that the RAR–RXRβ heterodimers bound to the RARβ response element could be effectively cross-linked when intact, or minimally truncated (53 amino acids), RARs were used. Effective cross-linking was not observed with further truncation of the C-terminus (Figure 5C). Truncation of the RAR N-terminus had no effect on the formation of RXRβ–RAR heterodimers. Truncation of the C-terminal 25 amino acids of RXRβ reduced heterodimer formation, while deletion of most of the C-terminal domain (to amino acid 222) abolished both RXRβ–RAR interaction and the enhanced binding of RAR to cognate DNA elements (Figure 5B). These data suggested that both the C-terminal ligand-binding domain of the RAR and the C-terminal domain of RXRβ served as dimerization interfaces for effective formation of heteromeric complexes required for high affinity binding of RAR to specific DNA elements, consistent with the properties of biochemically defined coregulator proteins.

RXRβ Selectively Enhances Binding of RAR, Vitamin D Receptor, and Thyroid Hormone Receptor to Their Specific DNA Response Elements

We next examined the sequence specificity of DNA binding of RXRβ–RAR heterodimers. Analysis of specificity was performed using a series of naturally occurring response elements (Figure 1) for retinoic acid (RARβ response element [deThé et al., 1990; Sucov et al., 1990], laminin B1 retinoic acid response element [Vasios et al., 1989]; thyroid hormone [rat growth hormone AB site; Beebe et al., 1991]; myosin heavy chain thyroid hormone response element [Izumo and Mahdavi, 1988]; and vitamin D [osteocalcin, vitamin D response element; Kerner et al., 1989; Ozono et al., 1990; Figure 1]). In addition, idealized response elements containing the core motif AGGTCA in different arrangements (Figure 1) were tested. Direct repeats of this motif spaced by 3 (DR+3), 4 (DR+4), and 5 (DR+5) bp confer relatively selective positive regulation by vitamin D, thyroid hormone, and retinoic acid, respectively (Umesono et al., 1991). The palindromic arrangement TRE-pal confers positive regulation to both thyroid hormone and retinoic acid in transient transfection assays (Glass et al., 1988; Umesono et al., 1988). RXRβ dramatically enhanced the binding of the RAR on the RARβ response element, TRE-pal, and the DR+5 synthetic response elements (Figure 6A). In contrast, under the conditions of these experiments, RXRβ–RAR heterodimers exhibited much lower binding affinities for thyroid hormone response elements (myosin heavy chain, DR+4, growth hormone AB) or vitamin D response elements (osteocalcin, DR+3). The DNA-binding properties of the RXRβ–RAR heterodimers were indistinguishable from those observed for heterodimers between RAR and partially purified coregulator proteins of the identical molecular weight present in HeLa and F9 teratocarcinoma cells.

Based on the DNA site specificity of enhanced binding effects that RXRβ conferred on the RAR, it became of considerable interest to evaluate whether RXRβ might exert similar effects on either the thyroid hormone or vitamin D receptors. The ability of RXRβ to interact with thyroid hormone receptor was evaluated using the identical series of DNA-binding sites, as shown in Figure 6C. While the thyroid hormone receptor was capable of binding to thyroid hormone response elements, including growth hormone, myosin heavy chain, and the TRE-pal response elements (Figure 6C), as well as to a synthetic DR+4 cis-active element (data not shown), the addition of RXRβ markedly enhanced its binding to each of these sites. In contrast, RXRβ enhanced to a much lesser extent the binding of the thyroid hormone receptor to a series of sites that were response elements for the other nuclear receptors including RARβ response element and the osteocalcin (vitamin D response element) (Figure 6C), as well as the laminin B1, DR+3, and DR+5 sites (data not shown). Similarly, the ability of the RXRβ to interact with the vitamin D receptor was evaluated using both a natural vitamin D response element (osteocalcin) and a synthetic vitamin D response element (DR+3). These experiments revealed marked enhancement of vitamin D receptor binding to the osteocalcin site (Figure 6D) and to the DR+3 site (data not shown). RXRβ enhanced, to a much lesser extent, the binding of vitamin D receptor to thyroid hormone response elements (DR+4, MHC, TRE-pal, growth hormone AB site) and retinoic acid response elements (RARβ response element, laminin B1, DR+5; Figure 6D and data not shown). Vitamin D receptor binding to the TRE-pal element was not positively enhanced.

Based upon the similarity of RXRβ to the RXRα family, the capacity of RXRβ to bind to an RXR response element (CRBPII or DR+1 sites) (see Mangelsdorf et al., 1991) was also evaluated. In parallel to the properties of RXRα

Figure 2. Cloning of an RAR Coregulator

(A) Expression screening strategy for a cDNA-encoding RAR coregulator. A λgt11 cDNA library prepared from a rat medullary thyroid C cell tumor was screened using a concatamerized RARβ response element as probe, as described in the Experimental Procedures. Phage-expressing fusion proteins that generate binding signals were plaque purified, evaluated for their differential ability to bind RARβ response element but not Pit-1 or C/EBP DNA-binding site probes, and screened using ^{35}S-labeled RAR prepared by in vitro translation using rabbit reticulocyte lysate. This protein–protein interaction screen yielded a single positive fusion protein, referred to as RCoR-1.

(B) Example of the two-stage RARβ response element and ^{35}S-labeled RAR binding screen. Representative signals for the RCoR-1 and retinoic acid response element BP3, 1 of the 10 plaques negative for the protein–protein interaction screen, are shown.

(C) Primary DNA sequence and deduced amino acid sequence. Based on isolation of three independent clones, one encompassing the entire coding sequence, the full sequence of RCoR-1–RXRβ was determined, with full sequence analysis of both strands obtained using the dideoxy nucleotide sequencing method (Sanger et al., 1977). The bars indicate a transactivation domain. The putative DNA-binding domain is shown in reverse lettering, while the boxed C-terminal regions delineate the areas in this domain diverged from RXRα.

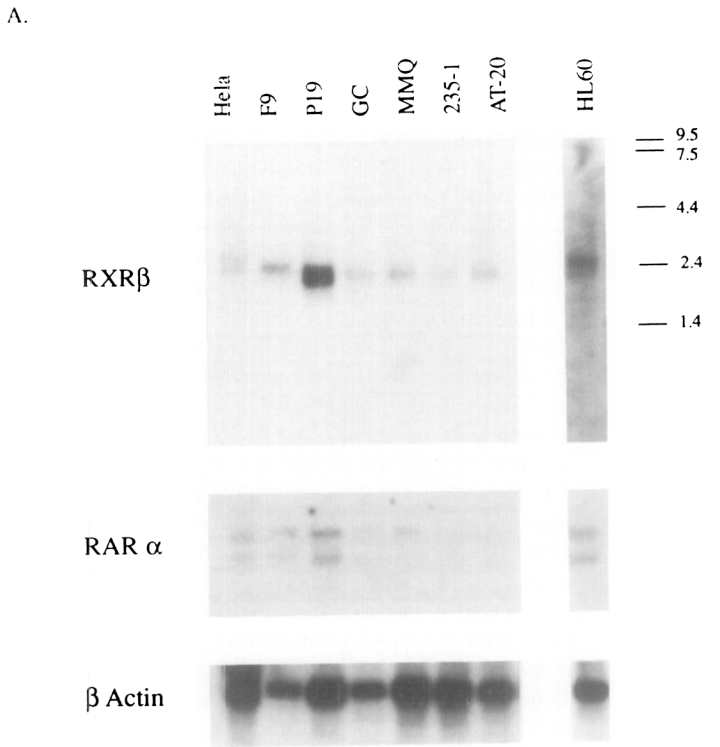


Figure 3. Expression of RXRβ Transcripts

(A) RNA blot analysis of RXRβ mRNA. Poly(A)-selected RNA (4 μg) from HeLa (human cervical) cells, F9 and P19 murine embryonic cell lines, pituitary cell lines (rat GC, MMQ, 235-1, or mouse AtT20), and human promyelocyte leukemic cells (HL60) were size fractionated under denaturing conditions and RXRβ transcripts identified as described in the Experimental Procedures (upper panel). Migration of RNA size standards is indicated (kb). The same blot was also hybridized to an RARα-specific probe (middle panel), demonstrating that both transcripts are expressed in these cell lines. Hybridization using a β-actin probe is shown (lower panel) for comparison.

(B) RNAase protection analysis of RXRβ transcript expression. RNA from a series of rat tissues (20 μg of total RNA) was used to evaluate expression of RXRβ using a 298 nucleotide runoff antisense RXRβ RNA probe that would generate a 240 nucleotide fragment if protected by hybridization. Roughly equivalent expression of RXRβ transcripts was detected in all tissues tested, except liver, intestine, and pancreas. RNA prepared from hormone-treated GC pituitary cells revealed similar expression in control (C), retinoic acid (RA, 10^{-7} M)–treated and dibutyryl cAMP (1 mM)–treated cells (data not shown), while treatment with thyroid hormone (10^{-7} M) for 36 hr increased RXRβ transcript levels ~3-fold. Assay control was provided by addition of yeast RNA. Migration of probe (P) and DNA ladder (BRL) is shown.

(Mangelsdorf et al., 1991), RXRβ was found to bind much more effectively to the CRBPII element (Figure 6A) than to other elements. However, while RAR itself bound relatively ineffectively to the CRBPII element, addition of RAR significantly enhanced RXRβ binding, implying that the RAR–RXRβ heteromeric complex bound to this site with a higher affinity than RXRβ alone. The enhanced binding of RAR by RXRβ on this site was confirmed using the ABCD assay (data not shown). In contrast to the RAR, thyroid hormone and vitamin D receptors were not capable of forming high affinity heterodimers with RXRβ on this site (Figures 6C and 6D).

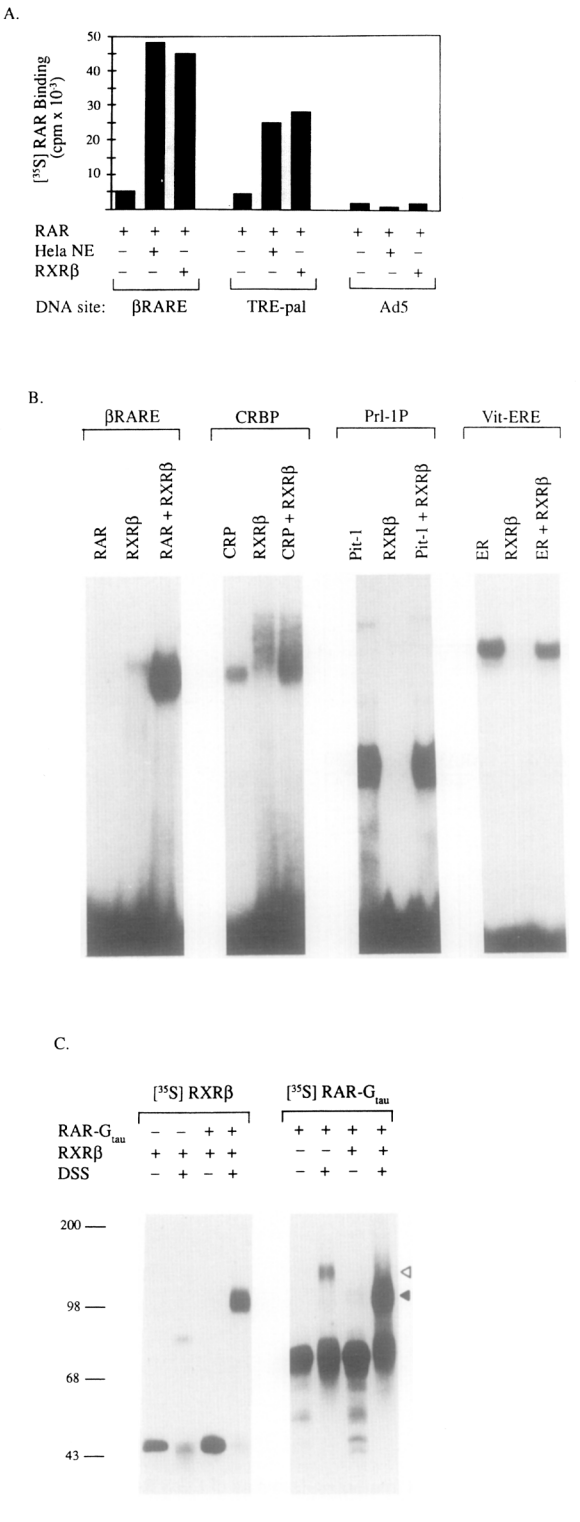


Figure 4. RXRβ Interacts Cooperatively with RARs on Retinoic Acid DNA Response Elements

(A) ABCD assay. In vitro translated ^{35}S-labeled human RARα was generated by in vitro transcription and translation. Labeled receptor (100,000 cpm) was added, in the presence or absence of 15 fmol of RXRβ also generated in translation in reticulocyte lysates, to 100 fmol biotinylated RARβ response element, TRE-pal, or control (Ad5) DNA sequences. Where indicated, 10 μg of HeLa nuclear protein (Glass et al., 1990) was added. Results are the average of duplicate determinations differing by less than 5%. Similar results were obtained in 4 experiments of comparable design.

(B) Effects of RXRβ on RAR DNA gel shift analysis. Equimolar amounts (10 fmol) of in vitro translated RAR, rat CRP, Pit-1, or human estrogen receptor were utilized. A 10-fold molar excess of bacterially expressed RXRβ was added to radiolabeled RARβ response element, CRBPII, Prl-1P, or Vit-ERE DNA sites (0.2 ng). The marked enhancement of binding with the simultaneous addition of RXRβ and RAR was observed in 12 comparable experiments. Addition of antisera against the C-terminus of RAR abolished the gel shift observed with RXRβ addition, confirming the presence of RAR in the DNA complex (data not shown), as predicted by the ABCD assay results shown in (A). In contrast, addition of RXRβ resulted in no or minimal enhancement of binding of CRP, Pit-1, or estrogen receptor over a wide range of protein concentrations including those that gave a minimal gel shift.

(C) Cross-linking analysis confirms that the RAR–RXRβ heterodimers bind to the RARβ response element. In these experiments, the RAR–G_t fusion protein (Glass et al., 1989) was utilized to permit clear distinction of crosslinked homodimers versus heterodimers. ^{35}S-labeled RXRβ or ^{35}S-labeled RAR–G_t was preincubated with equimolar amounts of unlabeled RXRβ or RAR–G_t, respectively, or with binding buffer alone. Following precipitation of protein–DNA complexes with streptavidin–agarose and extensive washing, cross-linking was performed using DSS (0.5 nM, 10 min), and the samples were analyzed by SDS–polyacrylamide gel electrophoresis. The molecular weights of RAR–G_t and RXRβ are 79 kd and 50 kd, respectively. With both receptors present, in each case cross-linked species migrated at ~130 kd, consistent with heterodimeric binding to the DNA site. Open arrows indicate migration of homodimers; closed arrows indicate migration of heterodimers. Molecular weight standards: myosin heavy chain (200 kd), phosphorylase A (98 kd), bovine serum albumin (68 kd), ovalbumin (43 kd), and DNAase I (18 kd).

RXRα Can Act as an RAR Coregulator

Further evidence for a general functional relationship between coregulators and the retinoid X receptor subclass was obtained by directly assessing the ability of RXRα to interact with the RAR. RXRα protein was shown to be capable of enhancing the binding of RARs to retinoic acid response elements, vitamin D receptors to vitamin D response elements, and thyroid hormone receptors to thyroid hormone response elements (Figure 6B). Based upon these data, the RXRα would itself appear to be capable of serving as a coregulator. We find no evidence that the RXRα and RXRβ proteins themselves form stable high affinity heterodimeric complexes (data not shown).

Effects of RXRβ on Nuclear Receptor-Induced Transcriptional Activation

Based on the selective effects of RXRβ on RAR binding, the action of RXRβ on nuclear receptor–dependent gene transcription was evaluated. In these experiments, a series of specific response elements were utilized. Naturally occurring response elements that mediate transcriptional responses to retinoic acid, thyroid hormone, and vitamin D were placed in an identical promoter context. A series of synthetic sites were also evaluated that contained identical core binding motifs (AGGTCA), but with the variable spacing (DR+1, DR+3, DR+4, DR+5; see Figure 1), that confer relatively selective responses to RXRα, vitamin D receptor, thyroid hormone receptor, and RAR, respectively. Experiments were performed in CV-1 cells, which contain low levels of endogenous RARs and no detectable thyroid hormone or vitamin D receptors. We found that

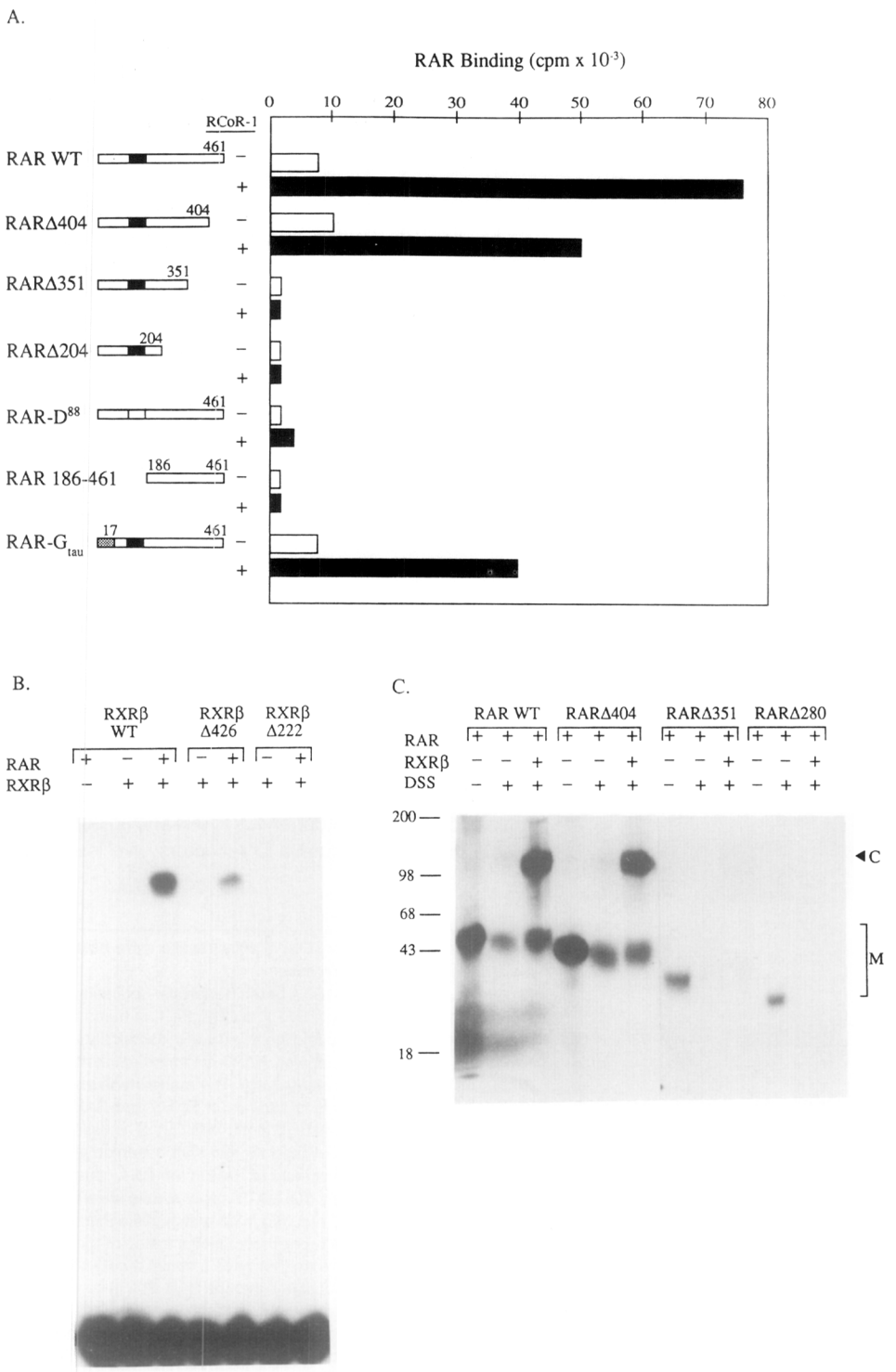


Figure 5. The Formation of RAR–RXRβ Heterodimeric Complexes Depends on the C-Terminal Interaction Domains of Each Receptor

(A) Effects of C-terminal truncation of the RAR. Serial truncations of the RAR were evaluated using the ABCD assay with 10^5 cpm of ^{35}S-labeled RARα and the RARβ response element as probe. In addition, a mutation of the first cysteine in the first zinc finger (D^{88}) that renders the RAR incapable of binding to DNA and a C-terminal interaction domain alone (RAR 186–461) failed to bind in the presence of RXRβ. The RAR–G_τ fusion protein (Glass et al., 1989), containing the glucocorticoid receptor major transactivation (τ) domain, mimicked the actions of wild-type RAR. Results are the average of duplicate determinations differing by less than 5%. Similar results were obtained in 2 additional experiments of comparable design.

(B) Effects of C-terminal truncations of RXRβ on heterodimer formation. RXRβs truncated at residues 426 or 222 were evaluated for their ability to cooperatively bind with RAR on the RARβ response element, using protein-perturbated DNA gel shift analyses. Similar results were obtained using the ABCD assay (data not shown).

(C) Cross-linking analysis of interactions between C-terminal truncation mutations of the RAR and RXRβ. Following ABCD binding as performed in (A), chemical cross-linking was performed using DSS (0.5 mM), and cross-linked products were analyzed using SDS-polyacrylamide gel electrophoresis. In this case the migration of the heterodimer is indicated by the arrow (at ~105 kd). Monomers are designated M.

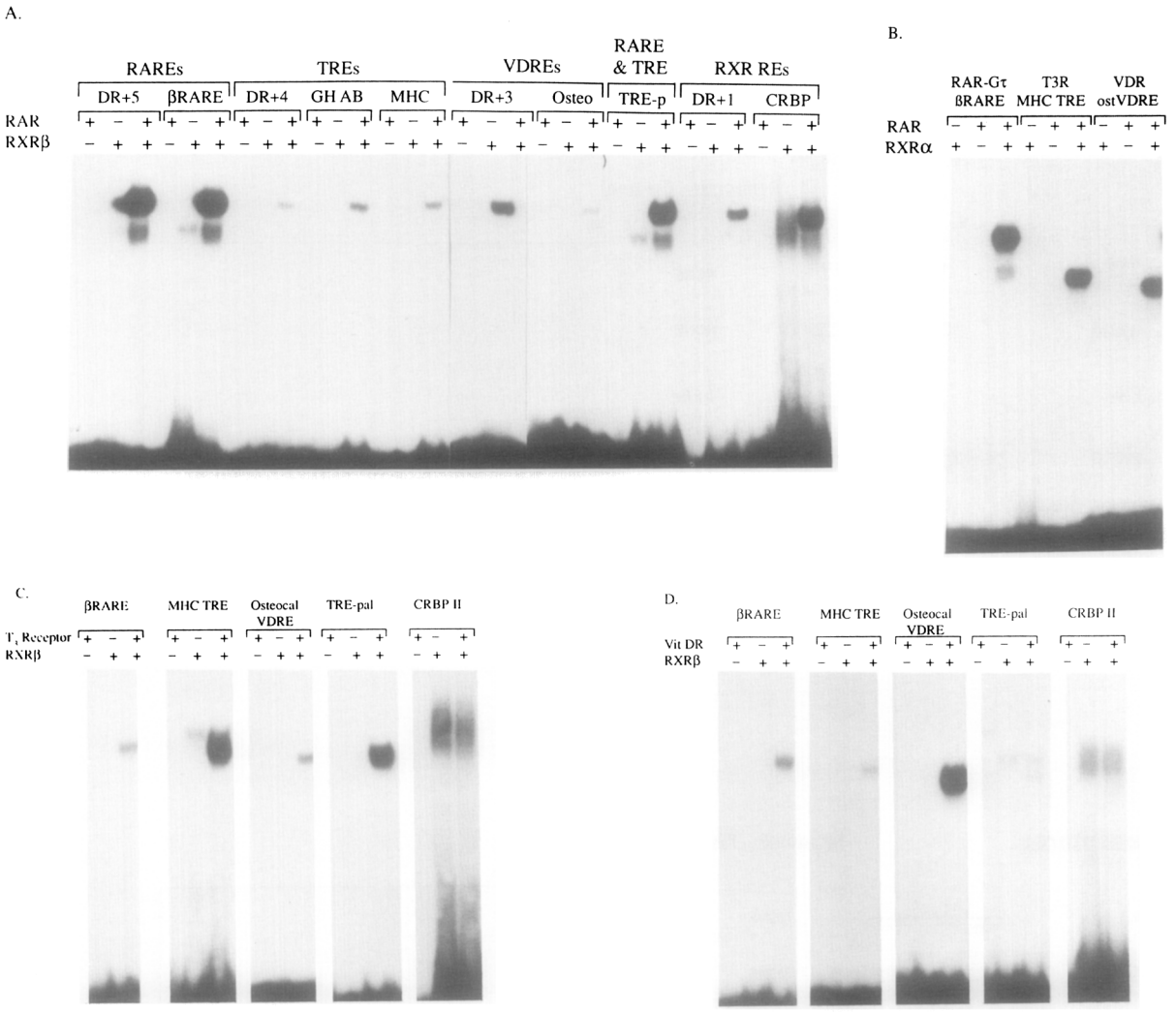


Figure 6. DNA Response Element Specificity of Coregulator Effects

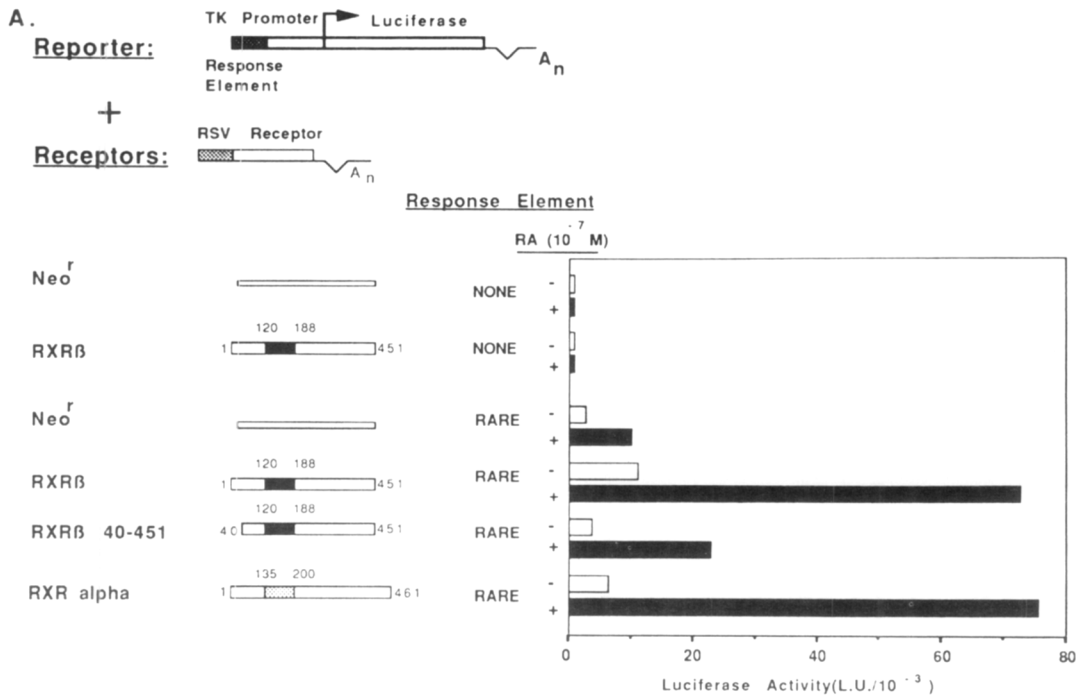
(A) Effect of RXRβ on RAR binding. A series of native and synthetic elements were evaluated for their ability to effectively bind RAR–RXRβ heterodimeric complexes, using protein-perturbated DNA gel shift analysis. Native sites used were RARβ response element and laminin B1 retinoic acid response element, myosin heavy chain, growth hormone AB thyroid hormone response elements, osteocalcin vitamin D response element, and the cytoplasmic retinol binding protein II (CRBPII) RXRα response element. Synthetic sites were direct repeats of AGGTCA core motifs spaced by 1, 3, 4, or 5 bp (DR+1, DR+3, DR+4, DR+5) (see Figure 1). All sites were added at equivalent concentrations. The same amounts of bacterially expressed RXRβ and in vitro translated RAR were used for each of the binding sites. Concentrations of in vitro translated proteins were used in which only minimal binding was observed for each receptor alone. Similar results were obtained in 6 separate experiments. Addition of anti-RAR sera abolished this RXRβ-RAR-mediated gel shift (data not shown). The probe used for DR+1 was several base pairs shorter than other probes used, and therefore, for the purposes of this figure, the position of the free probe was moved to place it at the same position of other probes in the photograph.

(B) RXRα interacts with RAR, thyroid hormone receptor, and vitamin D receptor. In vitro translated RXRα (~10 fmol) was utilized to evaluate their ability to enhance binding by equivalent concentrations of RAR, thyroid hormone receptor, or vitamin D receptor on the RARβ response element, myosin heavy chain thyroid hormone response element (MHC TRE), or the osteocalcin vitamin D response element (OstVDRE), respectively. The upper row (labeled RAR) indicates the absence (–) or presence (+) of RAR (RAR-G$_\tau$), thyroid hormone receptor (T3R), or vitamin D receptor (VDR). Similar results were obtained in 2 additional experiments of similar design.

(C–D) RXRβ enhances binding of thyroid hormone and vitamin D receptors to their cognate DNA response elements. The effects of RXRβ on binding of equivalent concentrations of thyroid hormone receptors or vitamin D receptors to a series of DNA elements were evaluated. Chemical cross-linking followed by ABCD binding assays confirmed the formation of RXRβ–thyroid hormone receptor heterodimers on the myosin heavy chain TRE site (data not shown). RXRβ preferentially enhanced binding of the thyroid hormone and vitamin D receptors to the myosin heavy chain TRE and osteocalcin VDRE (and to DR+3), respectively. Similar results were obtained in 5 additional experiments of similar design that also gave comparable data for other synthetic and natural response elements.

the addition of any of these elements to the TK promoter increased TK fusion gene expression 2.5- to 3-fold in a ligand-independent fashion.

As shown in Figure 7A, endogenous RARs were capable of activating transcription from promoters containing the RARβ response element or DR+5 sites in response to low concentrations (10^{-8} to 10^{-7} M) of retinoic acid. Expression of RXRβ resulted in an approximately 2-fold increase in



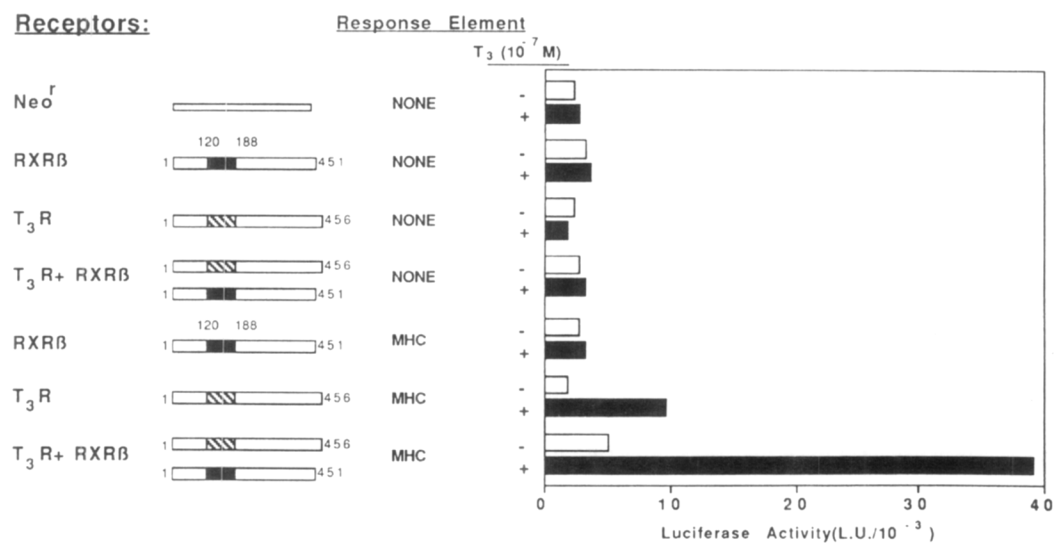


Figure 7. RXRβ Enhances Retinoic Acid and Thyroid Hormone–Dependent Transcription from Promoters Containing Retinoic Acid and Thyroid Hormone Response Elements

(A) Effects of RXRβ on retinoic acid–dependent transcription. CV-1 cells were transfected with TK luciferase reporter plasmids either lacking or containing a single copy of the RARβ response element placed 109 bp upstream of the transcriptional start site. The transcriptional response of these reporter genes to retinoic acid was determined in the presence of coexpressed RXRβ or a control protein (neo'). RXRβ 40–451 is an N-terminally deleted protein lacking amino acids 1–40.

(B) Effects of RXRβ on thyroid hormone–dependent transcription. CV-1 cells were transfected with TK reporter plasmids either lacking or containing a single copy of the myosin heavy chain thyroid hormone response element. Cells were cotransfected with expression plasmids encoding the thyroid hormone β receptor and/or RXRβ. An expression plasmid encoding a control protein (neo') was used to balance total amounts of transfected expression plasmid. In all cases, 0.5 μg of each expression plasmid and 3.0 μg of each reporter plasmid were used. Results represent the mean of duplicate determinations differing by less than 10% and are representative of more than five experiments.

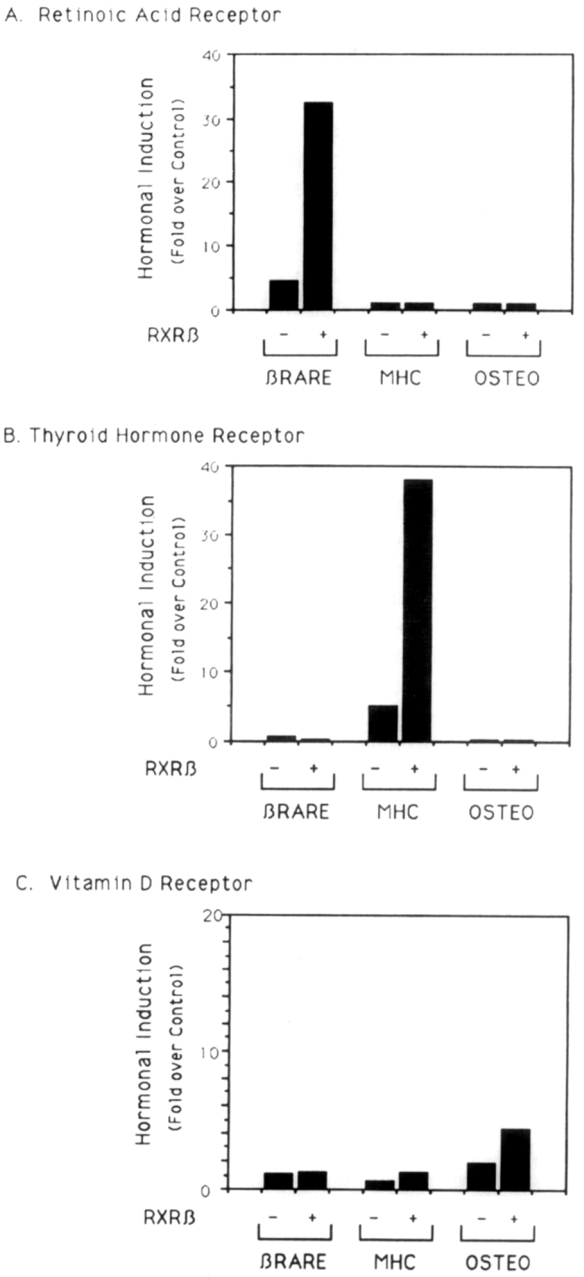


Figure 8. RXRβ Enhances the Ligand-Dependent Transcriptional Effects of RAR, Thyroid Hormone Receptor, and Vitamin D Receptor Only on Appropriate Response Elements

In these experiments, transfections were performed in CV-1 cells using both RXRβ and RARβ, thyroid hormone receptor, or vitamin D receptor expression vectors cotransfected (0.05 μg and 0.5 μg, respectively) with TK reporter plasmids (3 μg) containing one copy of the RARβ retinoic acid response element (βRARE), the myosin heavy chain thyroid hormone response element (MHC), or the rat osteocalcin vitamin D response element (OSTEO). Effects of hormonal induction (10^{-7} M retinoic acid, thyroid hormone, or vitamin D, 40 hr treatment) were in each case compared with the neomycin expression vector transfection control. Results represent the average of duplicate determinations differing by less than 5%.

(A) DNA site specificity of the RXRβ actions on retinoic acid–induced transcription. In 5 experiments performed, the presence of RXRβ increased the stimulation by retinoic acid (10^{-7} M) on the RARβ response element from 4- to 5-fold to 30- to 35-fold above control.

the basal transcription activity and a marked increase in the total retinoic acid–dependent transcriptional stimulation observed (Figure 7A).

Several lines of evidence support the idea that RXRβ enhances retinoic acid–dependent transcription by forming heterodimers with endogenous RARs. First, the effects of RXRβ were site specific, as it did not augment retinoic acid–dependent transcription from promoters containing thyroid hormone response elements (e.g., myosin heavy chain) or vitamin D response elements (e.g., osteocalcin) (Figure 8A) or a series of synthetic elements (DR+1, DR+3, DR+4) (data not shown). RXRβ did augment retinoic acid–dependent transcription on the TRE-pal element, which conferred regulation to both thyroid hormone and RARs (data not shown). Second, although RXRα has been reported to activate transcription in response to retinoic acid (Mangelsdorf et al., 1991), it is unlikely that the effects of RXRβ on promoters containing the RARβ response element result from direct activation by retinoic acid, because we found that the concentration of retinoic acid used in these experiments (10^{-7} M) was insufficient to activate it on a naturally occurring response element (CRBPII). Third, the ability of RXRβ to enhance retinoic acid–dependent transcription was dependent on C-terminal sequences that are required for cooperative binding with the RAR (data not shown). The final argument for heterodimer function is the direct in vitro evidence of enhanced binding of RAR–RXRβ heterodimers to retinoic acid response elements. The ability of RXRβ to augment transcriptional responses in these experiments probably reflected the limiting concentration of endogenous RXRβ or related molecules in the transfected cells.

The ability of RXRβ expression to modify transcriptional responses to thyroid hormone and vitamin D was similarly evaluated using transient transfection analyses. RXRβ selectively enhanced the actions of thyroid hormone receptor on the myosin heavy chain thyroid hormone response element (Figure 8B), somewhat increasing basal expression of promoters containing this site and markedly increasing transcription in the presence of thyroid hormone. RXRβ enhanced vitamin D effects, to a much lesser extent, on promoters containing the osteocalcin response element (Figure 8C). Similarly, RXRβ selectively enhanced transcriptional actions of retinoic acid, thyroid hormone, and vitamin D receptors on the DR+5, DR+4, and DR+3 elements, respectively, but in each case the effects on DR+4 and DR+5 were considerably less than those observed on native response elements (data not shown). Thus, RXRβ

(B) DNA site specificity of the RXRβ actions of thyroid hormone–induced transcription. In 5 experiments, the presence of RXRβ increased the stimulation by thyroid hormone receptors on the myosin heavy chain thyroid hormone response element fusion from 3- to 4-fold to 30- to 40-fold.

(C) DNA site effects of the RXRβ on the actions of vitamin D-induced transcription. In 4 separate experiments, vitamin D produced only 2-fold stimulation in expression of promoters containing a single copy of the rat osteocalcin VDRE. RXRβ produced a 1.8- to 2.2-fold additional increase in response to vitamin D relative to controls.

appeared to enhance basal and ligand-stimulated transcriptional responses selectively on those natural or synthetic response elements normally responsive to retinoic acid, thyroid hormone, or vitamin D, respectively.

Discussion

Identification of an RAR Coregulator

Combinatorial regulation of gene expression as a consequence of the formation of specific heterodimers has been experimentally established for several classes of transcription factors (e.g., Rauscher et al., 1988; Goutte and Johnson, 1988; Halazonetis et al., 1988; Kouzarides and Ziff, 1988; Glass et al., 1989; Murre et al., 1989a, 1989b; Vinson et al., 1989; Agre et al., 1989; Herskowitz, 1989; Schuermann et al., 1989; Turner and Tjian, 1989; Benezra et al., 1990; Treacy et al., 1991). In contrast to homodimeric binding of glucocorticoid and estrogen receptors (e.g., Tsai et al., 1988; Kumar and Chambon, 1988; Fawell et al., 1990), the initial biochemical evaluation of the RAR revealed the necessity for specific nuclear factors, referred to as coregulators, that formed heterodimers with the RAR, and permitted high affinity binding to DNA response elements (Glass et al., 1990). As described in this manuscript, we have utilized a strategy that screened for two functional properties that initially served to define RAR coregulators: binding the RARβ response element and high affinity association with the RAR to clone a coregulator, referred to as RXRβ. Application of this approach should prove useful in the isolation of other coregulator molecules within the ligand-dependent nuclear receptor gene family and for other classes of transcription factors.

RXRβ is widely expressed in many tissues on which retinoic acid exerts regulatory effects. RXRβ forms a heteromeric complex with RARs that preferentially bind, with high affinity, to those DNA sites (e.g., RARβ response element, laminin B1, TRE-pal) that physiologically serve as strong retinoic acid response elements. Thus, in contrast to the behavior of thyroid hormone receptor–RAR heterodimers, the DNA-binding properties of the RXRβ–RAR complex correspond to those of biochemically defined retinoic acid coregulators. Furthermore, the predicted molecular weight of RXRβ is consistent with those of the predominant coregulator in several cell types. RXRβ enhances the ability of RAR, but not thyroid hormone or vitamin D receptors, to stimulate promoters containing retinoic acid response elements. Together, these data indicate that RXRβ fulfills all of the criteria predicted for an RAR coregulator. Analyses of mutant RAR and RXRβ molecules indicated that the cooperative DNA-binding effects required functional DNA-binding domains and intact C-terminal interaction domains. These data are consistent with the prediction that interactions between the extended dimerization interfaces of the two receptors are required for retinoic acid coregulator actions, and even relatively small truncation in this 200 amino acid region drastically compromised RAR–RXRβ heterodimer formation. Thus, while DNA-binding domains of nuclear receptors alone appear to be capable of protein–protein interaction (Luisi et al., 1991; Yang et al., 1991), effective functional interactions between RXRβ and the RAR require the full C-terminal interaction domains of both receptors.

Retinoid X Receptors as Nuclear Receptor Coregulators

While direct binding of retinoic acid to the RXRα receptor has not been reported, the ability of promoters containing specific elements, such as the TRE-pal site, to be regulated at much higher levels of retinoic acid ($>10^{-5}$ M) led to the proposal that a metabolite of retinoic acid serves as a regulatory ligand (Mangelsdorf et al., 1991). Because we find that both RXRα and RXRβ increase the binding of RAR, thyroid hormone receptor, and vitamin D receptor to their respective response elements, the retinoid X receptor subclass may subserve functions in addition to those proposed in activation of genes related to retinoic acid metabolism, as in the cellular retinol binding protein II promoter (Mangelsdorf et al., 1991). Because the retinoid X receptor family is evolutionarily conserved in Drosophila (Oro et al., 1990), perhaps preceding the appearance of the RAR, it is tempting to speculate that a major function of the C-terminus of these proteins is to serve as a dimerization interface, in order to permit high affinity binding of additional nuclear receptors to specific DNA response elements. Interestingly, RXRβ is widely expressed at high levels, except in those few tissues in which RXRα is well expressed (Mangelsdorf et al., 1991).

The Role of the Coregulator in Mediating the DNA Site-Specific Effects of RAR, Thyroid Hormone Receptor, and Vitamin D Receptor

One of the most intriguing aspects of the biological actions of the RAR, thyroid hormone receptor, vitamin D receptor, and estrogen receptor is their ability to regulate distinct, although occasionally overlapping, patterns of gene transcription. Systematic analyses using synthetic DNA sites revealed that different spacing and orientation of core binding motifs can constitute a code that permits selective actions by RAR, thyroid hormone receptor, vitamin D receptor, and estrogen receptor (Umesono et al., 1991; Näär et al., 1991). These observations have been proposed to explain the selective responses to related nuclear receptors by many naturally occurring response elements containing a direct repeat organization of the core motif (Umesono et al., 1991). The surprising capacity of a single coregulator to form stable heterodimers with three highly related nuclear receptors and to simultaneously provide a discriminatory function in DNA site targeting suggests that information is contributed by both RXRβ and each ligand-dependent nuclear receptor in order to achieve appropriate binding site specificity. Because the RAR is often present at less than 1000–5000 molecules per cell (Nervi et al., 1989; Kurokawa et al., 1990), coregulators would serve critical functions in permitting these relatively low levels of RAR to exert selective positive transcriptional actions.

In concert with the observed DNA site-specific actions of RXRβ on nuclear receptor binding, this protein generally enhances the transcriptional actions of RAR, thyroid hormone receptor, and vitamin D receptor on their respective

DNA response elements. However, the reported inhibition of RXRα-dependent transactivation of promoters containing a CRBPII element by RAR might be explained by the failure of the RXRα–RAR heterodimers, which preferentially bind on this site, to function as transactivators in this context. It is therefore suggested that the identical RXRβ–RAR heterodimer might function as a positive transactivator on one class of DNA sites (e.g., retinoic acid response element, DR+5), but be unable to transactivate and thus serve as an inhibitor on a second class of response elements (CRBPII, DR+1). Consistent with this view, we have previously provided evidence that other nuclear receptor heterodimeric complexes can fail to activate or even inhibit transcription on a subset of DNA sites to which they are bound (Glass et al., 1988; Holloway et al., 1990; Näär et al., 1991; Lipkin et al., 1991). These observations raise the additional, intriguing possibility that expression of the RAR itself could inhibit RXR-dependent gene expression mediated by response elements of the DR+1 class (e.g., CRBPII), while simultaneously activating target genes containing response elements of the DR+5 class (e.g., RARβ response element) as a consequence of forming heterodimers with retinoid X receptors.

While the experiments reported in this manuscript provide strong evidence that members of the retinoid X receptor class can serve as coregulators of RAR, thyroid hormone receptor, and vitamin D receptor, biochemical characterization of RAR coregulators (Glass et al., 1990) and thyroid hormone receptor auxiliary proteins (Murray and Towle, 1989; Burnside et al., 1990; Darling et al., 1991; O'Donnell et al., 1991; Beebe et al., 1991) suggests that additional coregulators are expressed in cell-specific patterns. Furthermore, although RXRβ increased vitamin D receptor binding to the osteocalcin vitamin D response element, coexpression led to little increase in vitamin D receptor–dependent transcription, suggesting that an additional, unidentified coregulator may be required for full vitamin D action. Therefore, a complex combinatorial pattern of interactions between nuclear receptors and their coregulators may serve to refine the precise patterns of gene activation during mammalian organogenesis.

Experimental Procedures

Cloning and Sequencing of RCoR-1

cDNA prepared using rat medullary thyroid tumor (generous gift of Dr. B. Roos) mRNA as template was rendered double stranded, ligated to EcoRI adapters, size fractionated on a Sephacryl S-400 spin column, and cloned into λgt11 as we have previously described (Lin et al., 1987). Expression screening was performed by modification of the method of Singh et al. (1988) (Vinson et al. 1988; Ingraham et al., 1988) using a double-stranded oligonucleotide corresponding to the RARβ response element sequence (5′-GGGTTCACCGAAAGTTCAC-3′). A total of 10^6 plaques was screened by ligated, radiolabeled (>10^8 cpm/μg) RARβ response element probe, and 23 were picked for further expression screening by RARβ response element/Pit-1 and C/EBP binding sites. Eleven positive plaques were taken to plaque purity and subjected to a protein–protein interaction screen using 2 × 10^6 to 3 × 10^6 cpm/ml of ^{35}S-labeled human RARα, prepared by in vitro translation using rabbit reticulocyte lysate (Glass et al., 1989) under binding and washing conditions virtually identical to those used for the DNA site screen. The EcoRI insert of the one initial positive clone was then used to rescreen several cDNA libraries (MTC, P-19, pituitary, kidney), and three additional, overlapping clones were selected for further characterization. One of the cDNA clones, 1.6 kb in length, putatively encoded the entire open reading frame of RCoR-1/RXRβ. Both strands of the RCoR-1 cDNA inserts were sequenced entirely by enzymatic procedures using dideoxy nucleotides and a T7 polymerase (Sequenase) on double-stranded DNA template (Sanger et al., 1977), utilizing oligonucleotide primers. In addition, the secondary screen yielded a full-length clone encoding the human RXRα.

RNA Analysis

Total RNA was prepared using a guanidinium thiocyanate procedure (Ingraham et al., 1988). Oligonucleotide (dT)-selected RNA was size fractionated under denaturing conditions using formaldehyde–1% agarose gels (Lin et al., 1987). ^{32}P-labeled probes were generated using random primers (>8 × 10^8 cpm/μg) using a 290 bp fragment corresponding to N-terminal coding information of RXRβ as template. Blots were washed at 65°C, 2× SSC for 30 min. RNAase protection studies were performed using a uniformly radiolabeled 326 nucleotide probe containing 290 bp of 5′ information from the N-terminus of RXRβ and vector sequences, as previously described (Emeson et al., 1989; He et al., 1989). RNAase protection assays used total RNA (20 μg). The GC pituitary cells were either untreated (control) or treated with dibutyryl cAMP (10^{-3} M), retinoic acid (10^{-7} M), or thyroid hormone (10^{-7} M) for 48 hr prior to harvest.

Preparation of Nuclear Extracts and Partial Purification of Nuclear Proteins

Nuclear extracts from HeLa and GC cells were prepared as previously described (Glass et al., 1990) in the presence of the protease inhibitors phenylmethylsulfonyl fluoride, antipain, leupeptin, and benzamidine. Nuclear extracts were then dialyzed against buffer H (20 mM HEPES [pH 7.8], 50 mM KCl, 20% glycerol, 1 mM β-mercaptoethanol, and 0.1% Nonidet P40) and stored at −80°C until used. DNA affinity matrices consisting of the TRE-pal sequence and RARβ response element sequence were prepared and used for partial purification of coregulator activities as described (Kadonaga and Tjian, 1986; Glass et al., 1990).

Expression of Wild-Type Proteins for DNA-Binding Studies

The full-length cDNA encoding RXRβ was subcloned as a KpnI–XbaI fragment into PBKSII vector (Stratagene). This construct was used to generate RXRβ Δ426 and RXRβ Δ222 mutants by introducing a stop codon at the appropriate site. Plasmids were linearized with NotI restriction enzymes, and capped mRNA transcripts prepared using a T3 RNA polymerase system were used to program translation in a rabbit reticulocyte system in the presence or absence of [^{35}S]methionine. The specific activity of the proteins translated in the presence of [^{35}S]methionine was determined by precipitation with trichloroacetic acid and SDS–polyacrylamide gel electrophoresis. To generate bacterial RXRβ, the full-length KpnI–XbaI fragment of RXRβ was cloned in-frame into a modified T7 expression vector PHB40P (pMET, Studier and Moffat, 1986), which contained a T7 terminator codon downstream of the inserted fragment. Bacterial RXRβ was expressed in the Escherichia coli strain BL21 (DE3) as described (Studier and Moffat, 1986), using preparatory procedures as previously described (Ingraham et al., 1990). The RARα, β thyroid hormone receptor, vitamin D receptor, retinoid X receptor, estrogen receptor, and CRP were produced by in vitro transcription/translation using a rabbit reticulocyte lysate system as previously described (Glass et al., 1987). The mutant RARs used in these studies have been described previously (Glass et al., 1990).

DNA Binding and Cross-Linking Assays

DNA probes containing biotin-11-dUTP were prepared and used in DNA-binding assays as previously described (Glass et al., 1989). Sense strands of oligonucleotides used in these studies were as follows: TRE-pal (5′-aaggggatccGTAAGATTCAGGTCATGACCTGAGGAGA-3′), the myosin heavy chain thyroid hormone response element (5′-aaggggatccTTTGGCTCTGGAGGTGACAGGAGGACAGC-3′), Vit-ERE (5′-aaggggatccGTA AGA TTC AGG TCA CAG TGA CCTGAGGAGA-3′), RARβ response element (5′-aaggggatccGGGTAGGGTTCACCGAAAGTTCACTCG-3′), CRBPII (5′-aaggggatccGCTGTCACAGGTCACAGGTCACAGGTCACAGTTCA -3′), osteocalcin vitamin D response element (5′-aaggggatccTTGGTGACTCACCGGGTGAACGGGGGCATTG-3′), growth hormone AB thyroid hormone response ele-

ments (Brent et al., 1989) (5′-aaggggatccAGTGGAAAGGTAAG-ATCAGGGACGTGA-3′), laminin B1 retinoic acid response element (5′-aaggggatccCAGACAGGTTGACCCTTTTTCTAAGGGCTTA-ACC-TAGCTCACCTC-3′), and the direct repeat elements listed in Figure 1. Lowercase letters represent sequences added for ABCD assays, permitting incorporation of seven biotin residues per double-stranded oligonucleotide. Oligonucleotides were purified by nondenaturing polyacrylamide electrophoresis and quantitated by a fluorometric procedure (Labarca and Paigen, 1980).

The effects of RXRβ on the DNA-binding properties of the RXRα, thyroid hormone receptor β, and vitamin D receptor were analyzed using an ABCD assay as previously described (Glass et al., 1990), incubating in vitro translated or bacterially expressed RXRβ protein with 60,000–100,000 cpm of TCA-precipitable ^{35}S-labeled receptor (10 fmol) for 20 min at room temperature in the presence of 1 μg poly(dI-dC), and binding to biotinylated DNA probes (usually 0.5–2 nM) at room temperature for 40 min prior to washing and quantitation (Glass et al., 1988). For gel shift analysis, the same oligonucleotides used for ABCD experiments were end labeled by kinasing with [γ-^{32}P]ATP, filled with dNTPs, and purified by nondenaturing polyacrylamide gel electrophoresis. Aliquots (1–5 μl) of either in vitro translated (5–20 fmol) or bacterially expressed proteins (1 μl) were preincubated for 5 min in 20 μl of binding buffer (10 mM HEPES [pH 7.4], 50 mM KCl, 1 mM dithiothreitol, 5% glycerol) containing 1 μg of poly(dI-dC), 0.1 μg of heat-treated salmon sperm DNA, and 100 μg of bovine serum albumin, and samples were incubated for an additional 30 min at room temperature (25°C) after addition of probe (0.1–0.5 ng). One-sixth of each reaction was loaded onto a 4.5% nondenaturing 0.5 × Tris–borate–EDTA polyacrylamide gel, electrophoresed at 275 V for 90 min, and autoradiographed for 2–12 hr at −80°C.

For cross-linking experiments performed with the RAR and RXRβ bound to DNA, protein–DNA complexes were precipitated with streptavidin–agarose and washed three times with buffer H lacking β-mercaptoethanol. The protein–DNA–streptavidin–agarose complexes were washed one additional time in 20 mM KPO_4 (pH 7.4), 50 mM KCl, 20% glycerol and resuspended in a total volume of 50 μl in the same buffer. One microliter of a 25 mM solution of DSS in dimethyl sulfoxide was added, mixed thoroughly, and allowed to react at room temperature for 10 min. Control samples received 1 μl of dimethyl sulfoxide alone. Reactions were quenched by addition of 5 μl of 1 M ethanolamine. Cross-linked protein complexes were released from DNA by addition of 100 μl of SDS sample dyes and boiling for 5 min, and sample volumes containing equal amounts of total radioactivity were analyzed by SDS–polyacrylamide gel electrophoresis.

Transfection Analysis

The Rous sarcoma virus (RSV)–based expression vector RSV-RAR WT and RSV-thyroid hormone receptor β WT containing cDNA coding sequences for the human RARα and thyroid hormone receptor β have been described previously (Glass et al., 1989; Holloway et al., 1990). A KpnI–XbaI fragment corresponding to full-length rat RXRβ was placed in the polylinker of the pCMV 1 expression vector and was preceded by the cytomegalovirus promoter region and followed by the human growth hormone termination and polyadenylation signal, as previously described (Ingraham et al., 1990). RSV-VDR WT containing cDNA coding sequence for the human vitamin D receptor has been described previously (Liao et al., 1990) and was a generous gift from Dr. J. W. Pike. Reporter plasmids containing the TK promoter fused to the firefly luciferase cDNA have been described previously (Glass et al., 1989, 1990). Transfection of CV1 cells (Chen and Okayama, 1987) and analysis of luciferase activity as previously described (Holloway et al., 1990; deWet et al., 1987) were performed using 0.05 μg of CMV-RXRβ, expression plasmid, and 0.5 μg of other nuclear receptor expression plasmids, except where otherwise noted.

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