Isoform-specific amino-terminal domains dictate DNA-binding properties of RORα, a novel family of orphan hormone nuclear receptors

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Three isoforms of a novel member of the steroid hormone nuclear receptor superfamily related to the retinoic acid receptors have been identified. The three isoforms, referred to as RORα1, RORα2, and RORα3, share common DNA- and putative ligand-binding domains but are characterized by distinct amino-terminal domains generated by alternative RNA processing. An exon encoding a functionally important subregion of the amino-terminal domain of the RORα2 isoform resides on the opposite strand of a cytochrome *c*-processed pseudogene. Binding site selection using in vitro-synthesized proteins reveals that the RORα1 and RORα2 isoforms bind DNA as monomers to hormone response elements composed of a 6-bp AT-rich sequence preceding a half-site core motif PuGGTCA (RORE). However, RORα1 and RORα2 display different binding specificities: RORα1 binds to and constitutively activates transcription from a large subset of ROREs, whereas RORα2 recognizes ROREs with strict specificity and displays weaker transcriptional activity. The differential DNA-binding activity of each isoform maps to their respective amino-terminal domains. Whereas truncation of the amino-terminal domain diminishes the ability of RORα1 to bind DNA, a similar deletion relaxes RORα2-binding specificity to that displayed by RORα1. Remarkably, transfer of the entire amino-terminal region of RORα1 or amino-terminal deletion of RORα2 confers RORE-binding specificities to heterologous receptors. These results demonstrate that the amino-terminal domain and the zinc finger region work in concert to confer high affinity and specific DNA-binding properties to the ROR isoforms and suggest a novel strategy to control DNA-binding activity of nuclear receptors.

[*Key Words*: DNA-binding protein; processed pseudogene; transcription; alternative RNA splicing; retinoic acid receptor]

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Nuclear receptors constitute a rapidly expanding class of ligand-activated transcription factors that directly transduce hormonal signals to the nucleus (Evans 1988). This superfamily of regulatory proteins includes receptors for steroids, retinoids, and thyroid hormones, as well as a large number of closely related gene products, referred to as orphan nuclear receptors, for which no ligand have been found (for references, see Laudet et al. 1992). Nuclear receptors share a common modular structure composed of four major domains that have originally been defined by amino acid sequence conservation and function (Giguère et al. 1986; Krust et al. 1986). The central DNA-binding domain is the most conserved among nuclear receptors and is composed of two zinc finger motifs that serve as interfaces in both DNA–protein and protein–protein interactions (Freedman 1992). The ligand-binding domain, located at the carboxy-terminal end of nuclear receptors, shows moderate conservation and performs a number of functions that include ligand binding, transcriptional activation and repression, nuclear translocation, and dimerization (Truss and Beato 1993). In contrast, both the amino-terminal domain and the hinge region separating the DNA- and ligand-binding domains are poorly conserved between receptors and their functions remain to be fully delineated. The amino-terminal region of a number of receptors has been shown to contain a *trans*-activation domain that in some instances may specify target gene activation (Tora et al. 1988; Nagpal et al. 1992). The mechanism(s) by which the amino-terminal domain specifies target gene activation is

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not understood but it has been suggested that distinct amino-terminal domains possess differential ability to interact with cell- and target gene-specific transcription factors (Meyer et al. 1989; Tasset et al. 1990).

Nuclear receptors control the activity of primary target genes by binding to short DNA sequences known as hormone response elements (HREs). These DNA-binding proteins can be grouped in four general categories according to the types of HREs they recognize and physical interactions displayed between receptor monomers. The first group includes steroid hormone receptors such as the glucocorticoid and estrogen receptors that bind DNA as homodimers and recognized HREs configured as inverted repeats of the consensus half-sites AGAACA or AGGTCA spaced by 3 bp (Klock et al. 1987; Martinez et al. 1987). The second group is composed, among others, of the thyroid hormone (T_3R), vitamin D3 (VD_3R) and retinoic acid (RAR) receptors that bind DNA as heterodimers with the retinoic X receptor (RXR) (Yu et al. 1991; Bugge et al. 1992; Kliewer et al. 1992; Leid et al. 1992b; Marks et al. 1992; Zhang et al. 1992) and recognize HREs configured as direct or everted repeat of the core half-site motif PuGGTCA separated by spacers of defined length (Näär et al. 1991; Umesono et al. 1991; Tini et al. 1993). The third group comprises receptors such as RXR and COUP-TF that display the ability to bind DNA as homodimers to direct repeat HREs (Mangelsdorf et al. 1991; Tran et al. 1992). The fourth and most recently defined group includes a number of orphan nuclear receptors, apparently binding as monomers, that interact with HREs configured as a single half-site preceded by a short AT-rich sequence (Lavorgna et al. 1991; Wilson et al. 1991; Tsukiyama et al. 1992; Harding and Lazar 1993; Wilson et al. 1993).

The diversity in HRE configuration and their interactions with receptor monomer, homodimer, and heterodimer suggest that nuclear receptors must employ a vast repertoire of molecular mechanisms to achieve high DNA-binding specificity and affinity. DNA-binding specificity of nuclear receptors is dictated primarily by the two zinc finger motifs through subdomains referred to as the P-box, which specify half-site sequence recognition (Danielsen et al. 1989; Mader et al. 1989; Umesono and Evans 1989), and the D- and DR-boxes, which dictate proper half-site spacing (Perlmann et al. 1993). Amino acids carboxy-terminal of the zinc finger region have also been implicated in monomeric, homodimeric, and heterodimeric high affinity DNA-binding and/or sequence recognition by RXR, T_3R, and the orphan nuclear receptors NGF1-B and FTZ-F1 (Ueda et al. 1992; Wilson et al. 1992; Kurokawa et al. 1993; Lee et al. 1993; Wilson et al. 1993; Predki et al. 1994). Finally, a dimerization interface localized in the ligand-binding domain is required for high affinity binding by nuclear receptor homo- and heterodimers (Fawell et al. 1990; Yu et al. 1991; Leid et al. 1992b).

In this paper, we report the cloning and functional characterization of RORα (RAR-related orphan receptor), a gene encoding a novel subfamily of orphan nuclear receptors that bind as monomers to closely related HREs composed of a single half-site core motif PuGGTCA preceded by a 6-bp AT-rich sequence. Apparent differential promoter usage and alternative splicing of the RORα transcription unit generate three isoforms, referred to as RORα1, RORα2, and RORα3, that are distinct in their amino-terminal region but that are otherwise identical in their presumptive DNA- and ligand-binding domains. A striking feature of these orphan receptors is that their respective amino-terminal domains influence DNA-binding specificity of each isoform.

Results

Cloning of RORα1, RORα2, and RORα3

ROR was isolated as part of a screen to identify RAR- and RXR-related genes that might play a direct or even an indirect role in vitamin A physiology. The DNA-binding domain of the human RARα was used as a probe to screen recombinant DNA libraries to search for unrecognized nuclear receptors related to the RARs. A partial cDNA clone (λrB5) was first isolated from a total rat brain cDNA library, and nucleotide sequence analysis revealed a novel polypeptide that contains the characteristic zinc finger structure of nuclear receptor DNA-binding domain (data not shown). The insert of λrB5 was then used to screen under high-stringency conditions human retina and testis cDNA libraries, and several positive clones were isolated and characterized. We determined the complete nucleotide sequence of one cDNA (λhT19) as well as the 5′ and 3′ ends of several independent cDNAs (Fig. 1). We identified three classes of cDNA containing long open reading frames of 1569, 1668, and 1644 nucleotides that are referred herein as RORα1, RORα2, and RORα3, respectively. RORα2 (λhT3) and RORα3 (λhT19) share a common 5′ end that encodes the first 45 amino acid residues of their open reading frames, after which they diverge for the next 168 and 134 nucleotides, respectively. The 5′ end of the RORα1 (λhR5) clone is completely distinct from the 5′ ends of RORα2 and RORα3 and encodes the first 66 amino acid residues of this open reading frame. Restriction endonuclease mapping and sequence analyses indicate that RORα1, RORα2, and RORα3 are colinear from the exon encoding the first zinc finger of the putative DNA-binding domain to the 3′ end of each clone. Each presumptive initiator methionine codon is preceded by an upstream in-frame terminator codon, and the open reading frames are predicted to encode proteins of 523, 556, and 548 amino acid residues, respectively. The size of each protein has been verified by in vitro translation of RNA derived from these cDNA clones and found to correspond to the predicted molecular weight (data not shown). After the terminator codon is a short 192-nucleotide 3′-untranslated region (UTR) with a consensus polyadenylation signal (AATAAA) found 18 nucleotides upstream of a polyadenylated tract.

The three distinct but related RORα1, RORα2, and RORα3 polypeptides, diagramed in Figure 2C, contain characteristic DNA- and ligand-binding domains of nu-

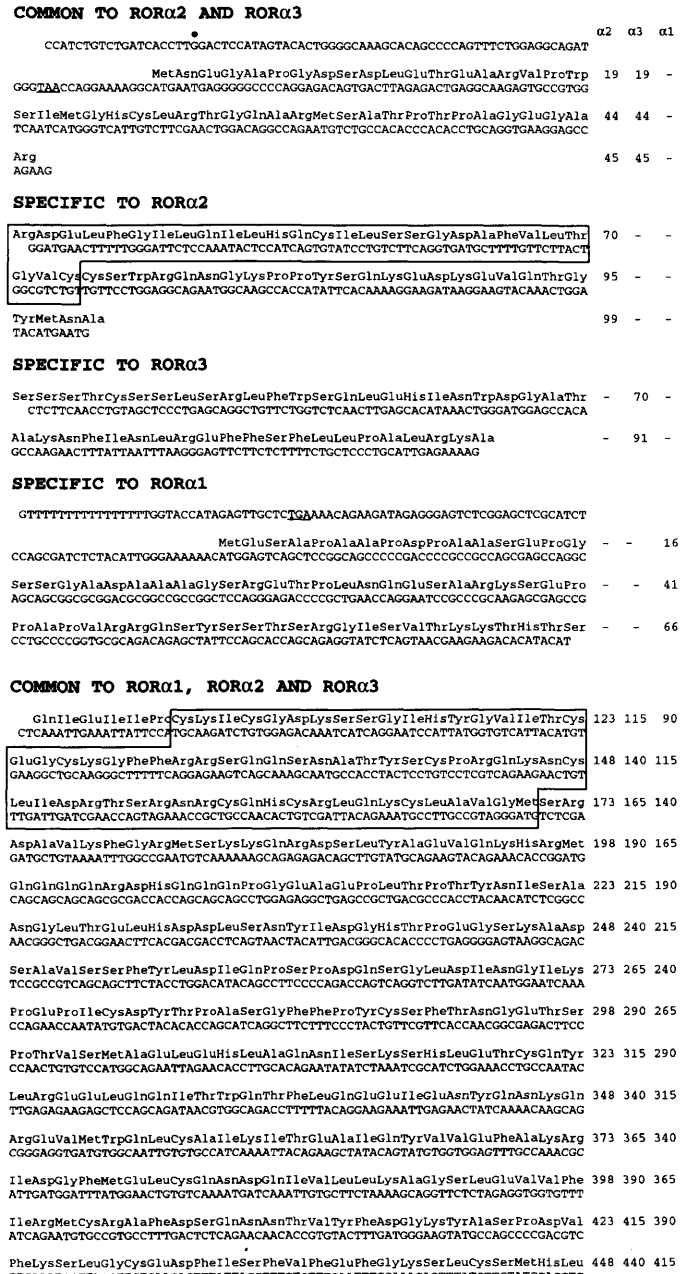

Figure 1. Nucleotide sequence of RORα cDNA and deduced amino acid sequences of RORα proteins. The DNA sequence encoding the three proteins is divided into RORα2 and RORα3 common and specific amino-terminal domains, an RORα1 5′-specific amino-terminal domain, and a region common to all three RORα isoforms. The amino-terminal sequences specific to RORα1, RORα2, RORα3 are derived from clones λhR5, λhT3, and λhT19, respectively. The carboxy-terminal sequence common to the three isoforms is derived from clone λhT3. The boxed amino acids in the region specific to RORα2 represent the exon encoded on the opposite strand of the cytochrome *c*-processed pseudogene (see Fig. 2). The boxed amino acids in the region common to all three isoforms represents the zinc finger region that is part of the DNA-binding domain. Upstream in-frame stop codons present in the 5′ UTR region of the three cDNA clones and a potential polyadenylation signal are underlined; (●) The 5′ end of the λhT19 cDNA insert encoding RORα3. The complete RORα1, RORα2, and RORα3 cDNA sequences have been submitted to GenBank under accession numbers U04897, U04898, and U04899, respectively.

clear receptors. Sequence alignment of RORα1 with a number of human nuclear receptors showed a high degree of identity with the DNA-binding domain of RARα (67%) and the ligand-binding domain of Rev–Erbα (30%) (Fig. 2A). However, the highest level of identity was observed in the presumptive DNA-binding domain (77%) of the *Drosophila* orphan receptor DHR3 (Koelle et al. 1992). Surprisingly, the similarity between RORα and DHR3 is higher within short amino and carboxy regions immediately adjacent to the zinc finger region (Fig. 2B).

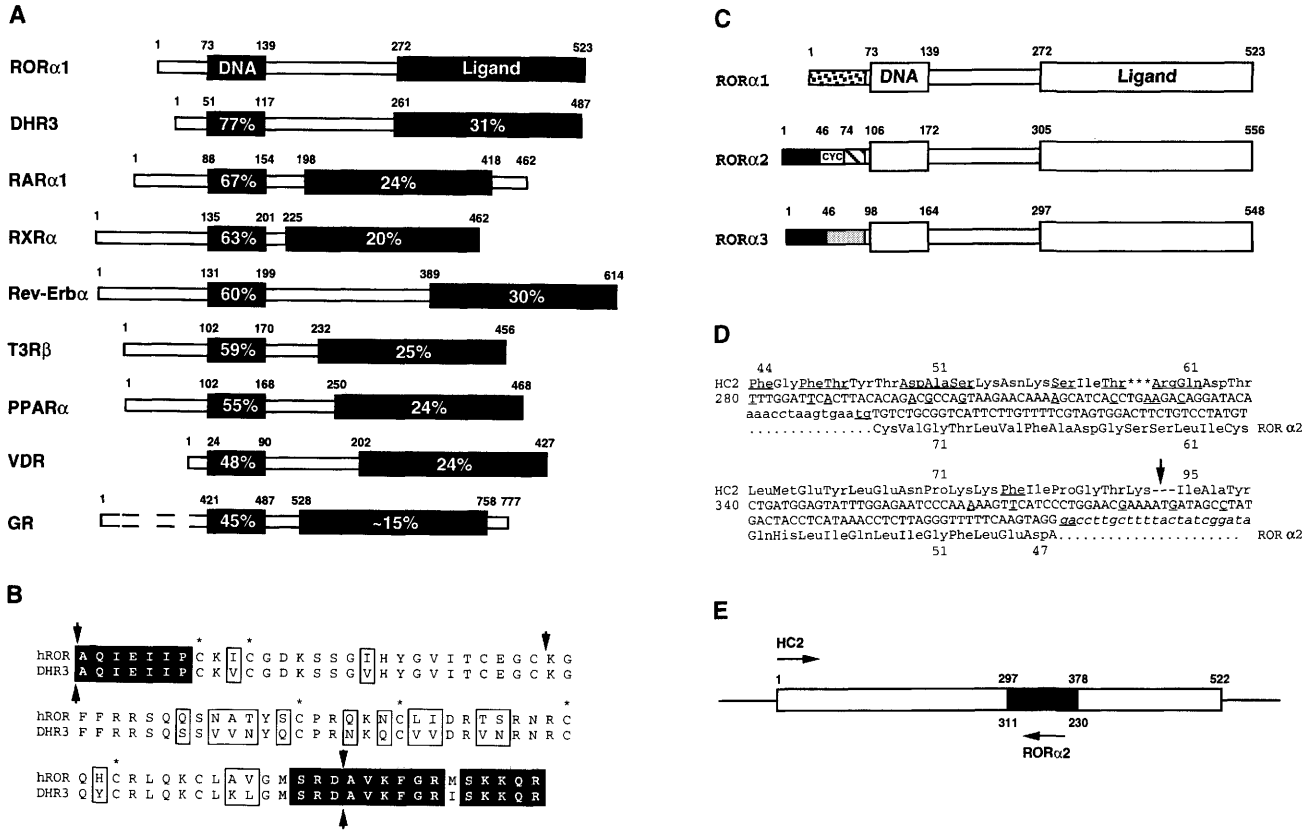

Figure 2. RORα1, RORα2, and RORα3 are members of the steroid hormone nuclear receptor superfamily and arise from alternative RNA processing. (*A*) Schematic amino acid comparisons between human RORα1 and various members of the steroid hormone nuclear receptor family. With the exception of DHR3, all sequences are for human receptors. Amino acid sequences have been aligned schematically according to the functional domain structure of nuclear receptors. The percentage of amino acid identity of each receptor with RORα1 in the putative DNA- and ligand-binding domains is indicated inside each domain. (DHR3) *Drosophila* hormone receptor 3 (Koelle et al. 1992); (RARα1) retinoic acid receptor (Giguère et al. 1987); (RXRα) retinoid X receptor (Mangelsdorf et al. 1990); [(Rev–ErbAα (ear1)], orphan receptor encoded on the reverse strand of the c-erbAα gene (Miyajima et al. 1989); (T_3Rβ) thyroid hormone receptor (Weinberger et al. 1986); (PPARα) peroxisome proliferator-activated receptor (Issemann and Green 1990); (VDR) vitamin D3 receptor (Baker et al. 1988); (GR) glucocorticoid receptor (Hollenberg et al. 1985). (*B*) Comparison of the amino acid sequence surrounding the DNA-binding domain of RORα with DHR3. (□) Dissimilar residues; (■) two regions of high similarity immediately adjacent to the two zinc finger motifs. Intron–exon boundaries are indicated by arrows. The asterisks (*) indicate conserved cysteine residues in the DNA-binding domain. (*C*) Schematic representation of the gene products RORα1, RORα2, and RORα3. The amino-terminal region common to RORα2 and RORα3 is represented by a solid rectangle. The specific exon to RORα2 that is encoded on the opposite strand of the cytochrome *c*-processed pseudogene HC2 is represented by the rectangle marked with the abbreviation CYC. Two regions of the amino-terminal domain specific to RORα2 and RORα3 are represented by hatched and shaded boxes, respectively. The RORα1 amino-terminal domain is shown as a dotted boxed. Open boxes represent region common to the three RORα isoforms. The amino acid position of each domain boundary is shown for each isoform. (*D*) Analysis of the genomic sequence surrounding the RORα2 amino-terminal exon encoded within the human cytochrome *c*-processed pseudogene. The nucleotide and deduced amino acid sequences of the cytochrome *c*-processed pseudogene are on the sense strand (Evans and Scarpula 1988) and those of the RORα2 amino-terminal exon correspond to the antisense strand. The numbered amino acid sequence of the human somatic cytochrome *c* gene is shown above the nucleotide sequence, with difference between the somatic cytochrome *c* gene and the processed HC2 pseudogene indicated by underlines. Numbers below the amino acid sequence on the antisense strand denote position within the RORα2 protein. Consensus AG and GT splice donor and acceptor sites are underlined on the antisense strand. The arrow denotes the position of a 42-bp deletion in the HC2 pseudogene. (*E*) Schematic representation of the overlapping genomic organization of the cytochrome *c* pseudogene and RORα transcription unit. The HC2 pseudogene is shown as an open box, and the RORα2 exon as a black box. Arrows indicate the direction of transcription.

In addition, RORα and DHR3 share similar intron–exon boundaries (represented by arrows in Fig. 2B) delineating the amino and carboxy ends of the zinc fingers region, although the DHR3 gene has lost the intron separating the two exons encoding each zinc finger of RORα. Further amino acid sequence comparisons of RORα1, RORα2, and RORα3 show distinct amino-terminal domains with no similarity with other nuclear receptors, including DHR3. However, a search of the nucleotide sequence data base (GenBank release 77.0) revealed an

82-nucleotide region of the RORα2 amino-terminal domain with complete identity with the previously characterized human cytochrome *c* pseudogene HC2 (Evans and Scarpula 1988). As shown in Figure 2D, the nucleotide sequence coding for amino acid residues 46–73 of the amino-terminal domain of RORα2 corresponds to the opposite DNA strand of the HC2 cytochrome *c* pseudogene and is flanked by consensus AG and GT intron splice acceptor and donor dinucleotides, respectively. The 3′ end of the intron also contains a characteristic polypyrimidine tract, a feature associated with splice acceptor sites. It thus appears that the RORα transcription unit uses at least two different promoters and five alternatively spliced exons to generate three novel members of the nuclear receptor superfamily, one of which created by the random integration of a cytochrome *c* processed pseudogene (Fig. 2E).

RORα1 binds to an asymmetric HRE composed of an AT-rich region upstream of a single core motif half-site, PuGGTCA

The high degree of similarity between the DNA-binding domain of ROR and a subset of nuclear receptors led us to believe that the RORα-binding site (RORE) might contain one or more core half-site, PuGGTCA. We therefore tested whether RORα1 could bind to a series of well-characterized natural and synthetic hormone response elements configured as direct, inverted, and everted repeats of the core half-site PuGGTCA. Because a number of nuclear receptors bind DNA with high affinity only in the form of heterodimers with the coregulator RXR, we performed the DNA-binding reaction in the presence or absence of RXRβ. Of nine different HREs tested, significant binding was observed with the DR-2 CRBP-I RARE, synthetic TREpal, and the γF-HRE (Fig. 3). The coregulator RXRβ has no effect on RORα1 binding to DNA, although it is essential for RAR binding to these elements (Fig. 3, lanes 6,13,20). Because the three HREs bound by RORα1 share no common configuration of the core half-sites, we decided to perform DNA-binding site selection using a polymerase chain reaction (PCR)-based strategy to better define the DNA-binding properties of RORα. Binding of RORα1 to known HREs allowed us to use TREpal as a marker to localized bound DNA after electrophoresis. Bound oligonucleotides were excised from the region of the gel comigrating with TREpal, amplified by PCR, and subjected to EMSA. After the fifth round of selection, the selected material was subcloned into the vector pSK$^+$ and inserts, from 30 independent clones were subjected to sequence analysis. Of these, 25 inserts contained a single PuGGTCA while the remaining 5 inserts did not display any form of consensus sequence among themselves or with the first 25 inserts. As shown in Table 1, a single and invariant core half-site motif PuGGTCA preceded by the AT-rich consensus sequence (A/G/T)(T/A)(A/T)(T/A)C(A/T) was observed. Comparison of the nucleotide sequences surrounding the half-site motifs AGGTCA present in the DR-2 CRBP-I RARE, TREpal, and the γF–HRE oligonucleotides used in our studies reveals that an AT-rich motif is located upstream of one of the two core half-site motifs PuGGTCA of each these HREs.

The amino-terminal domain influences DNA-binding activity

We then investigated whether the three RORα isoforms had distinct abilities to activate transcription from the γF–HRE. The cDNAs encoding RORα1, RORα2, and RORα3 were inserted in the mammalian expression vector pCMX (Umesono et al. 1991), and the resulting plasmids were cotransfected in P19 embryonal carcinoma cells with a luciferase reporter construct driven by the thymidine kinase (TK) promoter linked to three copies of the γF–HRE (Tini et al. 1993). While cotransfection of RORα1 led to a 25-fold stimulation in luciferase activity, both RORα2 and RORα3 failed to enhance significantly enzymatic activity (Fig. 4A). Although this observation can be explained by a lack of transcriptional activity of the RORα2 and RORα3 amino-terminal domains in P19 cells, the localization of a strong *trans*-activation domain in the common carboxy-terminal region of ROR isoforms (J. Torchia and V. Giguère, unpubl.) and the lack of transcriptional activity of the RORα2 and RORα3 isoforms in a number of cell lines (data not shown) led us

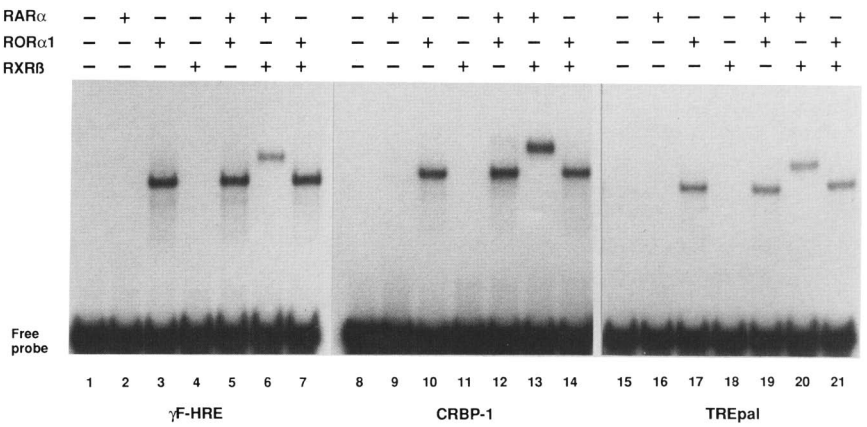

Figure 3. RORα1 recognizes diverse HREs in absence of the coregulator RXR. Radiolabeled γF–HRE, CRBP-I RARE, and TREpal were incubated with reticulocyte lysate programed with RORα1, RARα1, or RXRβ alone or in combination. Probes were also incubated with unprogrammed lysate as a control (lane *1,8,15*).

Table 1. *Consensus sequences binding to RORα1*

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S1-      GGGAAGTCAAGTGGTAAATTTAGGTCATAT
S2-   CTTGCAATCCAATACTACAAATCGAGGTCA
S3-                        GAATGTAGGTCATTCATCATGATAACCCCT
S4-                     CTTGATACAGGGTCATCACTATTCGGTTCA
S5-                  GATGAAGATTTAGAGGTCATTTAGCCTGCG
S6-                  CTCCCAAAGATCAAGGTCACGGTGATCGAG
S7-     TATGAATAGGTTATGTAATTCAAGGTCAACG
S8-                        GTTATACAGGTCAAAGGTATGCCATGCACC
S9-         CAATTCATCCATAAGGAGTAGGTCACTAAG
S10-        GTCGTTTATACGTTAATTGGGTCATTGCAA
S11-                       ATAACTGGGTCACACGACACTGCGTTACTC
S12-                    ACCATCTTAGAGGTCATTCGTTACCCACGT
S13-                  ATGATATTTAGAGGTCATCGGGGTTACTAA
S14-                   CACCCATACAAGGTCATCGTCGGTTAACTG
S15-                       GTATCCGGGTCAATGCGAGGAGAGGGTGTC
S16-          CGAAAAGACTTCAAATAAGGTCAAAAGGTC
S17-        GATACAAGGGTCGTACAAAGGTCAGTATCT
S18-        AAATAGGTCGCGGCATGAAGGTCAAGTTAC
S19-       ATACAGGCGCAACGTATCTAGGTCACCGGG
S20-     CTCATTTCGTATTGCATCTAAAGGTCACTG
S21-           AAGGGCTAGACAACAAGGTCAAGTGTACCT
S22-                 GCCATGGTCAAGGTCAGCTGTTACCCCCTG
S23-     GTCATCTAACTCTAATTTTGAAGGTCATTC
S24-                    AATAAACGAGGTCAATGACTTGAAATGCA
S25-            CTTTACACACGAAACTAGGTCATTGTCCCC
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Consensus[a]

	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	+1
G	7	3	2	0	4	5	4	25	25	0	0	0	2
A	10	9	15	9	6	11	21	0	0	0	0	25	7
T	6	10	7	15	3	7	0	0	0	25	0	0	10
C	2	3	1	1	12	2	0	0	0	0	25	0	6
	(C)	**T**	**A**	**T**	C	**A**	**A**	G	G	T	C	A	(G)
		A	T	**A**		**T**	**G**						

[a]The numbering system is relative to the AGGTCA core motif. Nucleotides in parentheses indicates any nucleotide but that one.

to investigate whether the failure of these two proteins to activate transcription could be correlated with lack of DNA-binding activity. As shown in Figure 4, B and C, although in vitro-translated RORα1 strongly bound both the γF–HRE and consensus REREα1, RORα2 and RORα3 failed to bind both HREs with high affinity, although extremely weak binding could be detected with longer exposure. Thus, the observation that RORα2 and RORα3 failed to bind to the natural γF–HRE and the consensus REREα1 with high affinity shows that a region distinct from the central zinc finger DNA-binding domain of nuclear receptors appears to influence DNA-binding properties of the various ROR isoforms.

To assess the potential role of the amino-terminal domain in DNA binding by the RORα isoforms, deletion mutants of both RORα1 and RORα2 were constructed (Fig. 5A) and in vitro-translated ROR mutants were assayed for their ability to bind to the consensus REREα1. Deletion of the amino-terminal domain of RORα1 (RORα1ΔN23-71) considerably reduces its ability to bind the REREα1 consensus site (Fig. 5B, lane 3), indicating that the amino-terminal domain influences ROR DNA-binding properties. In contrast, deletion of most of the amino-terminal domain of RORα2 (RORα2ΔN26-103) results in a marked increase in binding to REREα1 (Fig. 5B, lane 5). Selective deletion of the RORα2 amino-terminal domain using mutants RORα2ΔN3-45, RORα2-ΔN46-103, and RORα2ΔN71-103 demonstrates that the apparent DNA-binding inhibitory function localizes to amino acid residues 46–71 (Fig. 5B, lanes 6–8, respectively). These results show that while the amino-terminal region of the RORα2 appears to exert an inhibitory influence on DNA binding, the amino-terminal domain of RORα1 isoform is necessary for full DNA-binding activity. Therefore, the distinct amino-terminal domains of each ROR isoform appear to exert both positive and negative influences on RORα DNA-binding function. To evaluate the activity of the amino-terminal RORα1 and

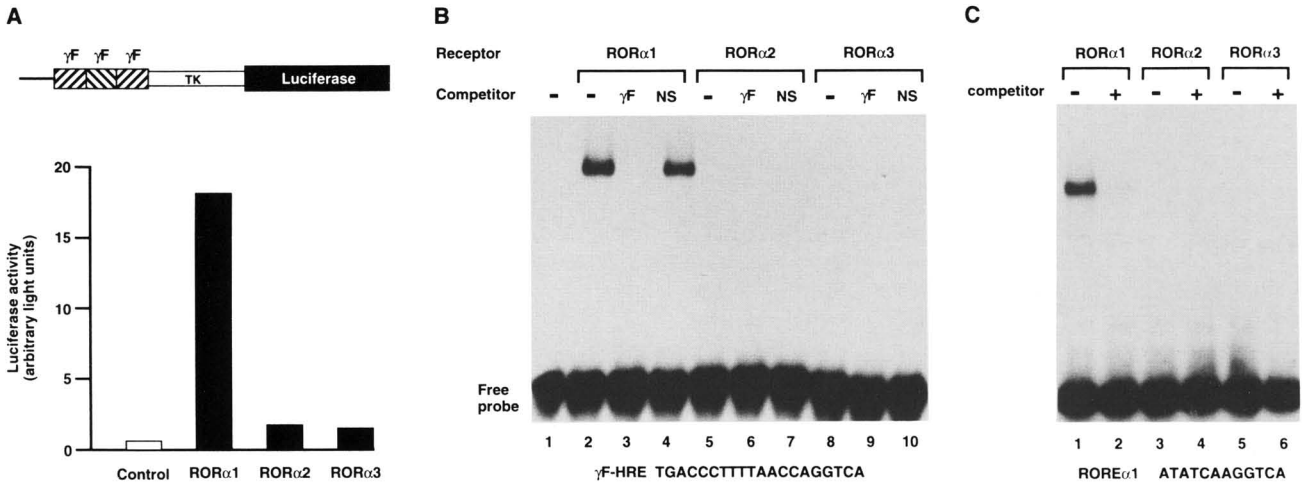

Figure 4. RORα isoform-specific DNA-binding and *trans*-activation. (*A*) Comparison of RORα1, RORα2, and RORα3 in a cotransfection assay. P19 cells were transfected with 2 μg of γF–HRE$_3$TKLUC reporter, and 250 μg of pCMX (control), pCMXRORα1, pCMXRORα2, or pCMXRORα3 expression vectors and harvested 36 hr later. (*B*) Interaction of RORα1, RORα2, and RORα3 with γF–HRE in vitro. Approximately 0.1 ng of radiolabeled γF–HRE was incubated with reticulocyte lysate programed with RORα1, RORα2, or RORα3 mRNA. Probe was also incubated with unprogrammed lysate as a control (lane *1*). Cold γF–HRE (lanes *3,6,9*) and a nonspecific competitor (NS) (lanes *4,7,10*) were used at 100 molar excess. (*C*) Interaction of RORα1, RORα2, and RORα3 with REREα1 in vitro. Experimental conditions were as described above.

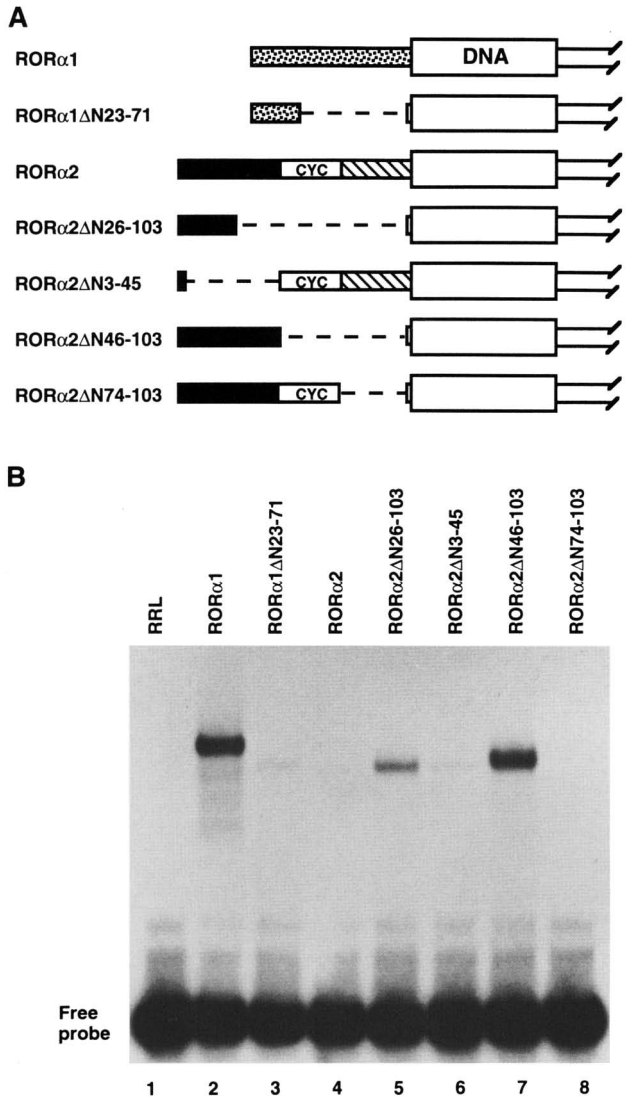

Figure 5. The amino-terminal domain influences DNA-binding activity. (*A*) Schematic representation of RORα1 and RORα2 mutants used for the DNA-binding analysis. (*B*) EMSA analysis of in vitro translated RORα1 and RORα2 mutants using ROREα1 probe. (Lane *1*) Unprogrammed lysate; (lanes *2–8*) programmed lysates as indicated above each lane.

RORα2 truncated mutants in vivo, expression vectors were cotransfected in P19 cells together with the γF–HRE_3TKLUC reporter gene. Whereas the RORα1Δ23-71 showed reduced transcriptional activity as compared with RORα1 (~35%), RORα2ΔN46-103 was able to stimulate luciferase activity by fourfold, or ~25% of the activity displayed by RORα1 (data not shown). RORα2, as shown previously in Figure 4, was inactive in this assay. These data indicate a correlation between the ability of ROR isoforms and amino-terminal mutants derived from them to recognize the γF–HRE and activate transcription from this element.

Table 2. *Consensus sequences binding to RORα2*

S1	CTCGGAGCGTCTAAATTTAATTGGGTCATCC
S2	CTACATATGGCCAGATTATTTAGGTCAGCTG
S3	CTGGATTAATCTAGGTCATGGCAAGGTTGGG
S4	CTAATTACCGGATTCGAATGTGGGTCAGTCC
S5	CCATATATAAGTAGGTCAACCAATTCTCGA
S6	CCAATAATATGTAGGTCAGGGAGTGGTTAG
S7	CCACTTGGAGACTACGTAATTAGGTCATCGA
S8	AAACCCTAAATAGGTCAGTGGGTCAGCTAGC
S9	TAAACCCCTGACCCATAGTATCTAGGTCAAG
S10	CTAAAAAAAAGTAGGTCACGAGCGGCTGG
S11	CGAGTACTCTTGTCAAATGTAGGTCACGAG
S12	GGGGGATTTGACAAAAAAGTAGGTCATGACC
S13	CGAAATTTAGGTCAAAGGTTATTTAACTAG
S14	CCATATAATGGGATCTCAGAATTGGGTCAA
S15	CTAGTTTTTATCTGGGTCAAGGGGGGCTAA
S16	GCTCCTTCAGTCAGGATTTAATTAGGTCAG
S17	GCCAGTAGACGACATTAATTAGGTCAGTAGT
S18	CTAGATAAATATAGGTCAGACTGGGTAGTA
S19	AATACTGTGAGTAGTTAATTAGGTCACCAG
S20	GGATCTAGGTCACTAGGTCAAATCAGACTA
S21	GGAGAGGTGTTTGGGTATCTAGGTCAGTAG
S22	CCACATATAAGTAGGTCAACCAATTCTCGA
S23	GGGTCCCCGGAAACTGGGTCAGTCGGTCCT
S24	ATTTATGTAGGTCAAGCAGCACTAATTGAG
S25	CTAGTTATCGGTTGAACAGAAGTGGGTCAA
S26	CATGAATTACAGAAAAAAGTAGGTCACCTA
S27	CTGGAAAACTAGGTCAAGGCTATGGGCAAG
S28	CATGAATCAAAATGTGGGTCAATTGTAGAC
S29	CTGGAATCGCTTAGTAAAGTAGGTCAGTGG
S30	CTAGATAAAATTAGGTCAAACGGCTGGCCC
S31	CCTACGAAAGTAGGTCACTCAGATGTCAGA
S32	CTAGTTTAGAACTAGGTCAATCCACCCTAG
S33	GACTATTTAAATCCGGATAACTGGGTCAAC
S34	CTAATTATGGGAATCAAACTAGGTCACGTT
S35	CTAGTTAATATCTAGGTCAACCGCGTCGGG
S36	GCTTAACTAGGTCATCGCAGTGGGTAAGG

Consensus[a]

	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	+1
G	7	4	0	0	13	0	8	36	36	0	0	0	10
A	17	15	36	22	2	0	28	0	0	0	0	36	14
T	10	17	0	14	9	36	0	0	0	36	0	0	5
C	2	0	0	0	12	0	0	0	0	0	36	0	7
	A T	**T** A	A	**A** T	**G** C	T	**A** G	G	G	T	C	A	**A** G

[a]The numbering system is relative to the AGGTCA core motif.

RORα1 and RORα2 recognize closely related but distinct sets of HREs

The finding of a cryptic DNA-binding activity that is activated by selective deletion of RORα2 amino-terminal domain, coupled with the observation that the

Figure 6. DNA-binding specificity of RORα isoforms. EMSA analysis of in vitro-translated RORα1 (*A*) and RORα2 (*B*) using ROREα2 probe and mutant ROREα2 oligonucleotides as competitors. Only the 6-bp AT-rich sequence upstream of the AGGTCA motif is shown for each mutant competitor at the top of each group. The base that is substituted for a G residue is underlined. Cold competitors were used at 5-, 25- and 100-fold molar excess, as indicated below the sequence of the competitor. The bar graphs below the autoradiograph indicate percent of total binding for each lane as determined by phosphorimaging.

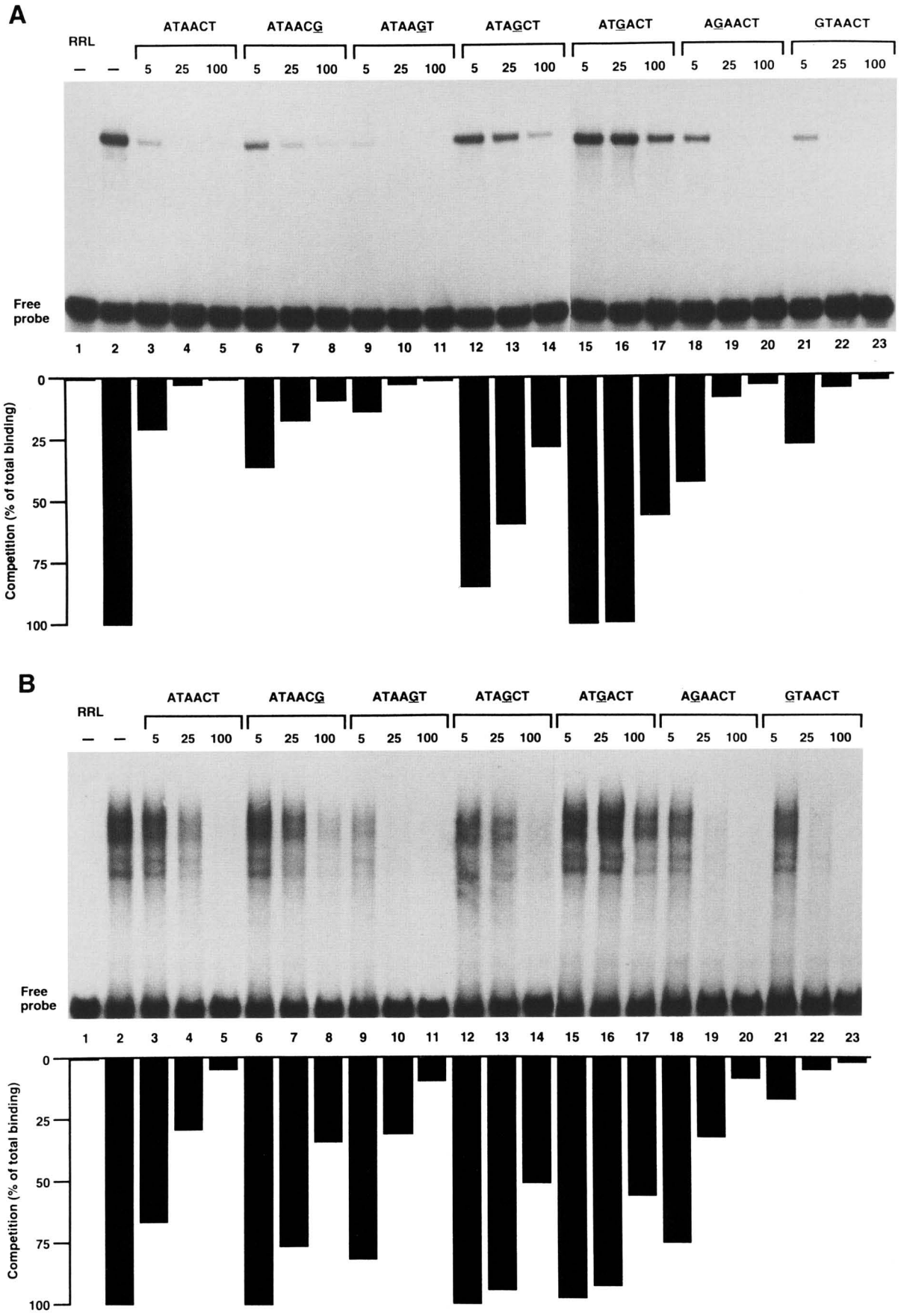

Figure 6. *(See facing page for legend.)*

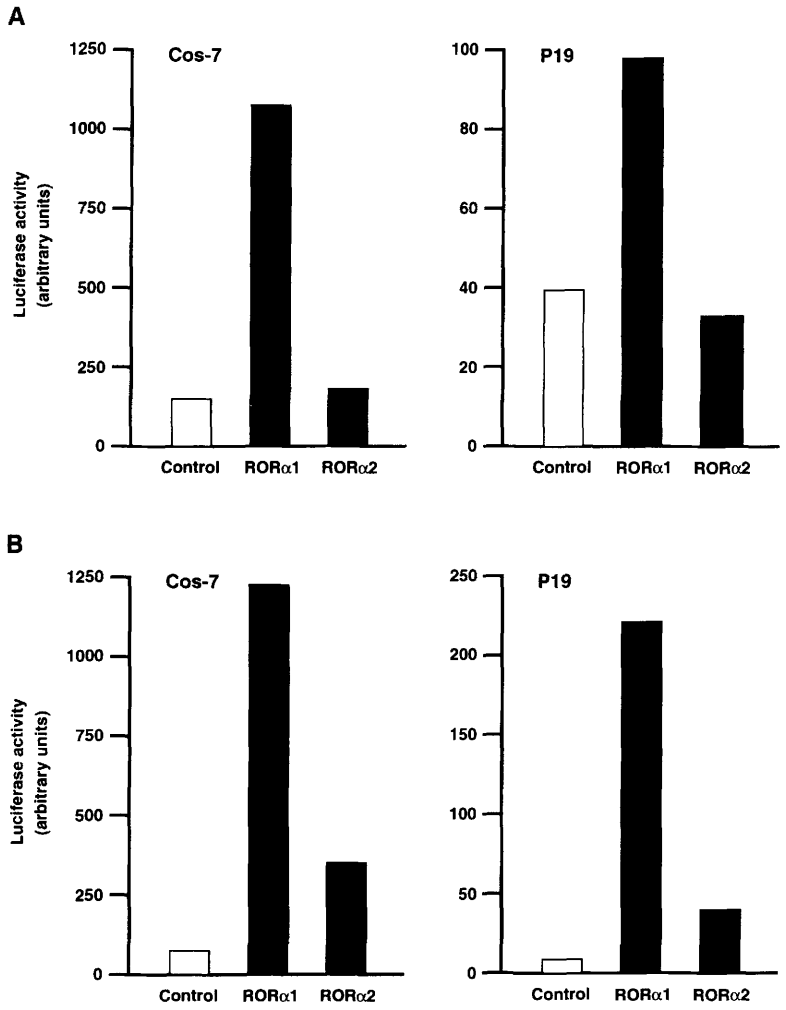

Figure 7. *Trans*-activation of the ROREα1$_3$TK-LUC (*A*) and ROREα2$_3$TKLUC (*B*) reporter genes by RORα1 and RORα2 in P19 and Cos-7 cells. Cells were transfected with 2 μg of ROREα1$_3$-TKLUC or ROREα2$_3$TKLUC reporter each containing three copies of the respective binding site upstream of the thymidine kinase promoter, and 500 ng of pCMX (control), pCMXRORα1, or pCMXRORα2 expression vectors and harvested 36 hr later.

RORα1 amino-terminal domain also plays a crucial role in ROR DNA-binding activity, led us to explore the possibility that the RORα2 isoform might recognize a distinct sets of HREs. We therefore repeated the DNA-binding site selection with in vitro-translated full-length RORα2. Data obtained from sequence analysis of 48 inserts isolated from the slower migrating complex is displayed in Table 2. As observed previously with RORα1, 36 of the 48 inserts contained a single PuGGTCA preceded by a 6-nucleotide AT-rich sequence. However, unlike the consensus ROREα1, two nucleotides located in the AT-rich region are absolutely invariant in the ROREα2: a T at position −1 and an A at position −4. We then investigated RORα1- and RORα2-binding preferences within the AT-rich upstream sequence by performing a competition analysis with mutant oligonucleotides in which the 6 bases upstream of the PuGGTCA half-site (ATAACT) were individually changed to a G. The ability of mutant ROREα2 to compete with labeled consensus ROREα2 for binding to RORα1 and RORα2 was determined by EMSA. Figure 6A shows that mutant oligonucleotides with a G at position −1, −3, and −4 fail to fully compete for binding to RORα1 even at a 100-fold molar excess. However, bases at position −3 and −4 in the AT-rich region are more important than the T at position −1 for binding to RORα1 (Fig. 6A, cf. lane 7 with lanes 13 and 16). Figure 6B shows the same competition analysis for binding to RORα2. As observed with binding to RORα1, bases at position −1, −3, and −4 appear to be the most important for binding to RORα2. However, as predicted by the binding site selection experiments, the T at position −1 is more important for binding to RORα2 than to RORα1. Quantitation using phosphorimaging technology shows that the mutant oligonucleotide at position −1 compete for binding to RORα1 by ~65% at fivefold molar excess while no competition is observed for binding to RORα2 (Fig. 6A,B, lane 6). At 25-fold molar excess, mutant oligonucleotides at position −1 compete for binding to RORα1 and RORα2 by ~80% and ~20%, respectively (Fig. 6A,B, lane 7). Positions −3 and −4 appear to be equally important for binding to RORα1 versus RORα2 (cf. lanes 14 and 17 between Fig. 6A,B).

We also examined the ability of RORα1 and RORα2 to activate transcription from luciferase reporter constructs driven by the TK promoter linked to three copies of

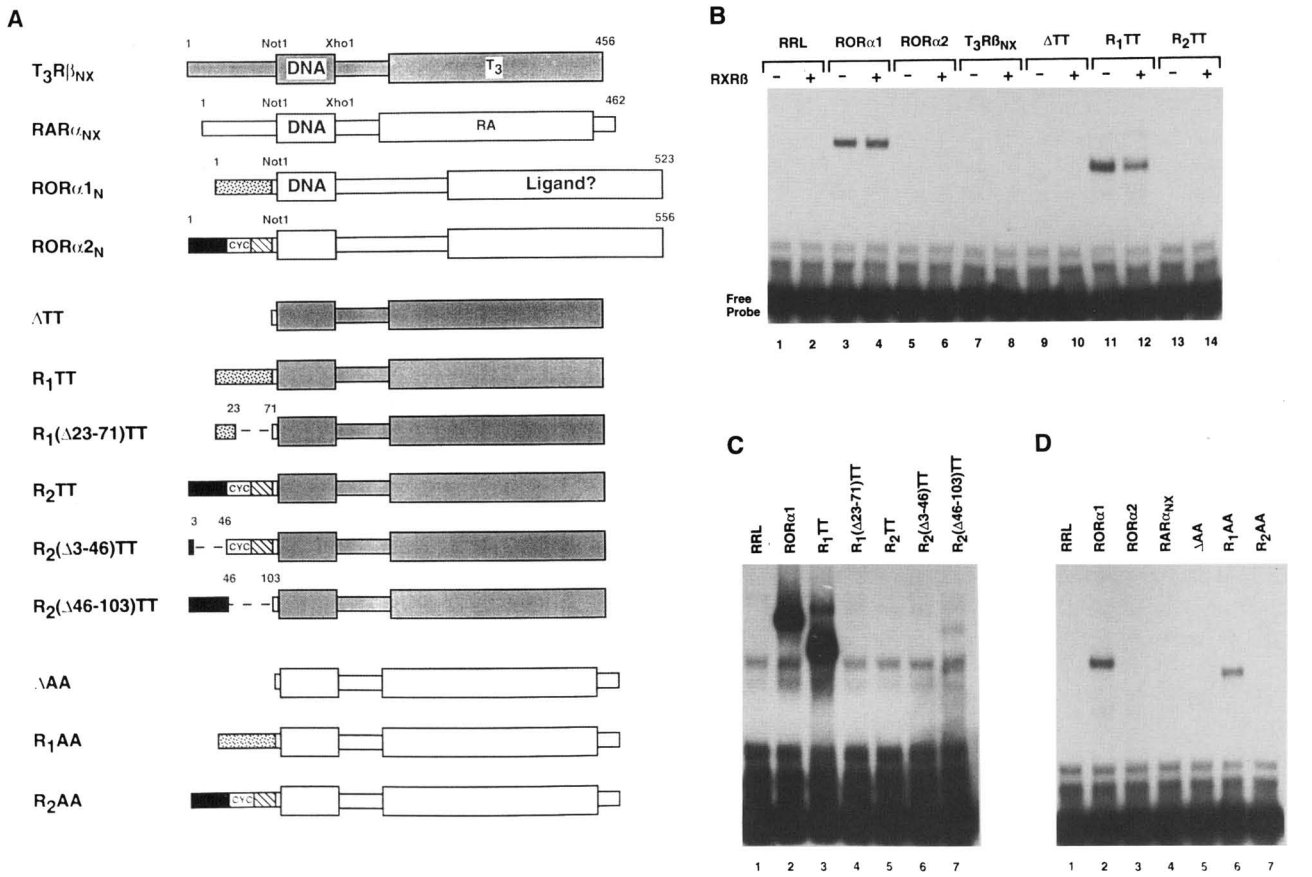

Figure 8. The RORα1 and RORα2 amino-terminal domains impose DNA-binding specificity to T_3Rβ and RARα. (*A*) Schematic representation of the chimeric receptors used in this study. The numbers above the boxes indicate amino acids. Chimeric receptors are named by letters referring to the origin of the domain; for example, R_1TT has the amino-terminal domain of RORα1 and the DNA- and ligand-binding domains of the T_3Rβ. (*B*) RORα–T3Rβ chimeric receptors ability to bind ROREα1 in the presence or absence of RXRβ. Lysates were programed as indicated at the top of each lane. (*C*) Amino-terminal deletion chimeric receptors ability to bind ROREα1. (Lane *1*) Unprogramed lysate; (lane *2*) lysate programed with RORα1; (lane *3–7*) programed lysates as indicated at the top of each lane. The arrows indicate specific retarded R_2(Δ46–103)TT complexes. The asterisk indicates a nonspecific band present in reticulocyte lysate. (*D*) RORα–RARα chimeric receptors ability to bind ROREα1. Lysates programed as indicated at the top of each lane.

ROREα1 or ROREα2 in two distinct cell types, P19 and COS-7 cells. As shown in Figure 7A, cotransfection of the expression vector pCMXRORα1, together with the ROREα1$_3$TKLUC, leads to 6- and 2.5-fold induction of luciferase activity in COS-7 and P19 cells, respectively. However, no induction can be observed when the expression vector pCMXRORα2 is introduced in COS-7 or P19 cells. In contrast, pCMXRORα2 leads to a fourfold induction in luciferase activity when cotransfected with ROREα2$_3$TKLUC both in COS-7 and P19 cells (Fig. 7B). RORα1 activates transcription from the ROREα2$_3$TKLUC construct by 7- and 21-fold in COS-7 and P19 cells, respectively. These data demonstrate that the ability of each receptor isoform to *trans*-activate gene expression correlate well with their ability to bind distinct HREs and that RORα2 can function as a constitutive transcriptional activator. Differences in transcriptional ability between RORα1 and RORα2 also indicate that this activity is modulated by the amino-terminal domain.

The RORα1 and RORα2 amino-terminal domains impose DNA-binding specificity to heterologous nuclear receptors

If the amino-terminal region plays a direct role in dictating DNA-binding properties of ROR isoforms, it might be possible to replace the amino-terminal domain of a related nuclear receptor with the amino-terminal domain of RORα1 and RORα2 to produce hybrid receptors with a new DNA-binding specificity. To test this possibility, the amino-terminal domain of the human thyroid hormone receptor β [from $T_3R\beta_{NX}$, also referred to as TTT in Thompson and Evans (1989)] was substituted with various regions of the amino-terminal domains of RORα1 and RORα2 (Fig. 8A). The DNA-binding activities of the hybrid receptors were then tested using ROREα1 as a probe. $T_3R\beta_{NX}$ or a mutant lacking its amino-terminal domain (ΔTT), alone or in presence of RXRβ, does not recognize ROREα1 as a binding site (Fig.

8B, lanes 7–10). R_1TT, a hybrid receptor that possesses the amino-terminal domain of RORα1 and the DNA- and ligand-binding domains of T3Rβ, binds ROREα1 apparently as a monomer (Fig. 8B, lane 11). When RXRβ is added to the reaction, binding is greatly reduced indicating that the RXR/R_1TT heterodimer (Fig. 8B, lane 12), formed through interactions between intact RXR and T_3Rβ DNA- and ligand-binding domains dimerization interfaces (Kurokawa et al. 1993), does not bind with high affinity to the ROREα1. Formation of functional RXR/R_1TT heterodimers were observed when either TREpal or γF–HRE was used as a probe (data not shown). As control, a hybrid receptor containing only a portion of the RORα1 amino-terminal domain (ΔN23–71) was also tested. Although weak DNA-binding activity can be detected with RORα1ΔN23-71, the R_1(ΔN23-71)TT hybrid receptor does not bind ROREα1 (Fig. 8C, lane 4). Similarly, we were unable to transfer novel DNA-binding specificity to the T_3Rβ using the entire RORα2 amino-terminal domain or the ΔN3-46 derivative (Fig. 8C, lanes 5,6). However, the hybrid receptor R_2(ΔN46-103)TT that does not contain the inhibitory function characterized previously in the amino-terminal domain of the native RORα2 weakly recognizes ROREα1 (Fig. 8C, lane 7). We then tested whether this observation is limited to the T_3Rβ or that the RORα1 amino-terminal domain could also impart novel DNA-binding specificities to a nuclear receptor not known to bind DNA as a monomer such as the RAR. We therefore engineered a series of hybrid receptors in which the amino-terminal region of RORα1 and RORα2 was substituted for the amino-terminal domain of RARα (Fig. 8A). As shown in Figure 8D, the hybrid receptor R_1AA that possesses the amino-terminal domain of RORα1 and the DNA- and ligand-binding domains of RARα, binds ROREα1 as a monomer (Fig. 8D, lane 6) as observed previously with the hybrid receptor R_1TT. It should be noted that none of these synthetic hybrid receptors show transcriptional activity when cotransfected with the ROREα1_3TKLUC reporter gene in P19 or Cos-7 cells (data not shown). We attribute this lack of activity to the possible formation of unproductive heterodimers between the hybrid receptors and endogenous RXR.

Discussion

In this paper, we describe the cloning and functional characterization of a novel gene family referred to as RORα, so named because of its close relationship with the RAR gene products and because it falls into the category of "orphan receptors," nuclear receptors for which no ligand has been identified [e.g., ERR1 and ERR2 (Giguère et al. 1988), ear1 and Rev–ErbAα (Lazar et al. 1989; Miyajima et al. 1989), COUP-TF (Wang et al. 1989), and HNF-4 (Sladek et al. 1990)]. The RORα gene generates at least three different isoforms that have common DNA- and ligand-binding domains but are distinguished by discrete amino-terminal domains. We demonstrate that two of the RORα gene products bind as monomers to closely related but clearly distinct HREs configured as a single core half-site motif PuGGTCA preceded by a short AT-rich sequence. However, the most remarkable feature of RORα is that the distinct DNA-binding properties observed for each isoform are dictated by their specific amino-terminal domains and that these properties can be transferred to heterologous receptors. These results demonstrate that the amino-terminal domain and the zinc finger region work in concert to confer high affinity and specific DNA-binding properties to the RORα isoforms and suggest a novel strategy to control DNA-binding activity of nuclear receptors.

A novel family of orphan nuclear receptor with an unusual gene organization

Comparison of the domain structure and predicted amino acid sequence of RORα with that of other members of the nuclear receptor superfamily shows greatest similarity with the *Drosophila* DHR3 orphan receptor (Fig. 1). Numerous vertebrate nuclear receptor genes have *Drosophila* homologs such as COUP-TF and SVP (Mlodzik et al. 1990), RXR and USP (Oro et al. 1990), ELP and FTZ-F1 (Tsukiyama et al. 1992), and possibly Rev–ErbAα and E75A (Segraves and Hogness 1990). In the case of RXR and USP, the function of these two proteins as coregulators in nuclear receptor-based hormone response systems has been conserved during evolution (Yao et al. 1992; Koelle et al. 1993; Thomas et al. 1993). Among vertebrate receptors, RORα is related most closely to RAR and RXR in their respective DNA-binding domains, whereas the ligand-binding domain shares a higher degree of similarity with Rev–ErbAα (ear1). However, the genomic organization of the ROR gene is most reminiscent to that of the three RAR genes in which each transcription unit generates multiple isoforms by alternative splicing and promoter usage of a single gene (Leid et al. 1992a). In addition, alternative splicing of the RORα transcription unit leads to the inclusion of one exon, which resides on the opposite strand of a cytochrome *c*-processed pseudogene [Fig. 2 and Evans and Scarpula (1988)]. Retroposons have been shown in the past to generate transposable elements, pseudogenes, and functional gene families and influence the expression of nearby genes (Weiner et al. 1986; Samuelson et al. 1988). In the instance described here, the fortuitous presence of splicing signals combined with the introduction of point mutations within the processed pseudogene generated a functional exon that confers novel DNA-binding properties to a transcription factor (see below). Thus, transformation of a processed pseudogene into a functional exon represents a novel role of reverse transcription in shaping the human genome and its gene products.

RORα belongs to the class of monomeric nuclear receptors

Although RORα is related most closely to RAR in its zinc finger region and genomic organization, its DNA-binding properties match most closely those of the or-

phan receptor Rev–ErbAα. As observed previously with Rev–ErbAα (Harding and Lazar 1993), RORα1 and RORα2 isoforms constitutively activate transcription and bind DNA as monomers (no intermediate sized band resulted from EMSA analysis of truncated RORα1 mutants; data not shown) to HREs configured as a single core motif half-site PuGGTCA preceded by a 6-bp AT-rich sequence [(A/T)(A/T)A(A/T)NT(A/G)GGTCA] (Tables 1 and 2). The RORα- and Rev-ErbAα-binding sites are practically indistinguishable, and the two receptor systems should be expected to control overlapping gene networks. RORα1 can bind to TREp and direct repeat HREs providing that an AT-rich sequence precedes one of the two PuGGTCA half-site core motifs. These results suggest that a subset of natural HREs containing PuGGTCA half-site core motifs and the proper 5′ upstream AT-rich sequence could serve as dual response elements. Interestingly, we show that the γF–HRE, an enhancer element that confers retinoic acid responsiveness to the γF–crystallin promoter (Tini et al. 1993), acts as a strong HRE for RORα1 (Fig. 4). It will be of interest to investigate the possible interactions between RAR and RORα in the control of γF–crystallin gene expression.

Two other orphan nuclear receptors, NGF1-B and FTZ-F1, apparently bind DNA as monomers to HREs closely related to the ROR and Rev–ErbAα-binding sites (Wilson et al. 1991; Ueda et al. 1992). These observations contrast with the model of the molecular mechanism of action of nuclear receptors in which these proteins bind DNA as homo- or heterodimers to HREs composed of inverted, everted, or direct repeats of a core half-site motif (Kumar and Chambon 1988; Tsai et al. 1989; Yu et al. 1991; Kliewer et al. 1992; Leid et al. 1992b; Kurokawa et al. 1993; Perlmann et al. 1993; Predki et al. 1994). Taken together, these results suggest that ROR, Rev–ErbAα, NGF1-B, and FTZ-F1 form a distinct subfamily of monomeric nuclear receptors that recognize asymmetric HREs containing a single core motif half-site PuGGTCA preceded by a short 2- to 5-bp AT-rich sequence. Monomeric nuclear receptors characterized to date have putative homologs in *Drosophila* or *Caenorhabditis elegans*. From an evolutionary perspective, it is tempting to suggest that RORα, Rev–ErbAα, NGF1-B, and FTZ-F1 and their homologs may represent prototypes of the ancestral nuclear receptor. The most recent sequence comparison (Laudet et al. 1992) and functional analyses (e.g., Wilson et al. 1993) of nuclear receptors suggest that the family probably evolved from a monomeric zinc finger protein able to recognize a single PuGGTCA motif. As new receptors were generated during the course of evolution, more complex and specific HREs could be created by elongation of the binding sites 5′ upstream of the primordial PuGGTCA motif. This hypothesis implies that novel DNA-binding determinants, other than the zinc finger region, would play a role in specific DNA binding by monomeric nuclear receptors.

Amino-terminal domains dictate ROR isoforms DNA-binding properties

We have demonstrated that two RORα isoforms differ in their ability to recognize closely related HREs as defined by a PCR-based unbiased selection of target binding sites (Tables 1 and 2). The RORα1 isoform binds to the consensus site [(A/G/T)(T/A)(A/T)(T/A)C(A/T)AGGTCA] while, in sharp contrast, the RORα2 isoform is able to efficiently bind only the more stringent consensus [(A/T)(T/A)A(A/T)(C/G/T)TAGGTCA], in which nucleotides at position −1 and −4 in relation to the AGGTCA motif are invariant. The RORα3 isoform does not recognize either site with high affinity. Mutational analysis of the RORα2 amino-terminal domain shows that deletion of amino acids 46–103, but not amino acids 71–103 or 3–45 relaxes the DNA-binding specificity of RORα2 to that displayed by RORα1 (Fig. 5). Therefore, the more stringent DNA-binding specificity displayed by RORα2 appears to be imposed upon by amino acids 46–74, a region corresponding to the exon encoded on the opposite strand of the cytochrome *c*-processed pseudogene. Considered on their own, these results would lead us to conclude that a region of the amino-terminal domain of RORα2 exerts an inhibitory function on DNA binding while the amino-terminal domain of RORα1 would play a neutral role. However, the most dramatic result reported in this study is the ability to transfer the DNA-binding properties of RORα1 and RORα2 to the T_3R and RAR by exchanging their respective amino-terminal domains (Fig. 8). Chimeric receptors R_1TT, R_2TT, R_1AA, R_2AA and amino-terminal deletion mutants linked to the $T_3Rβ$ possess the DNA-binding properties of the corresponding wild-type and mutant RORα isoforms. These observations suggest that the amino-terminal region of the RORα isoforms can work in concert with an heterologous zinc finger region capable of recognizing the PuGGTCA half-site motif to confer the ability to the DNA-binding domain to bind monomeric HREs with high affinity and specificity. Taken together, these results provide evidence of a complex domain organization and function of the amino-terminal region of RORα1 and RORα2 and show a direct role for the amino-terminal domain in modulating DNA binding. Chen et al. (1993) have recently demonstrated that differences in DNA sequence specificity between c-*erbA* (T_3R) and the v-*erbA* oncogene are also determined in part by amino acids that localized to the amino-terminal domain. In that case, amino-terminal determinants are involved in the discrimination of a single base pair at position 4 of the half-site core motif AGGTCA in HREs composed of repeated half-site motifs. Whether DNA-binding specificity imposed by the c-*erbA* amino-terminal region can be transferred to an heterologous receptor remains to be investigated.

We were surprised to find that determinants conferring site-specific DNA binding to RORα isoforms are located within the amino-terminal domain. Several mechanisms for imposing DNA-binding specificities via the amino-terminal domain can be envisioned. The amino-terminal domains of RORα1 and RORα2 could alter the tertiary structure of the zinc fingers and adjacent carboxy-terminal regions, which are common to both isoform, so that contacts between the central DNA-

binding domain and the 5′ AT-rich sequence are nonequivalent for each isoform. On the other hand, the amino-terminal domains could make nonspecific contact with DNA sequence surrounding the binding site that would result in a change in the tertiary conformation of the HRE so that the DNA-binding domain would recognize distinct sequences upstream of the half-site. Finally, the amino-terminal region could function as a separate DNA-binding domain recognizing the A/T-rich sequence upstream of the PuGGTCA motif. Although this model is supported by the fact that DNA-binding specificity can be transferred by exchange of the amino-terminal region between heterologous receptors, it should be noted that no significant level of amino acid sequence homology can be detected among RORα1, RORα2, and Rev–ErbAα (which bind a closely related HRE) in their respective amino-terminal domain. In addition, recognition of the AT-rich sequence 5′ upstream of the PuGGTCA motif has been shown to involve amino acids carboxy-terminal to the second zinc finger (Ueda et al. 1992; Wilson et al. 1992). This region is highly conserved between RORα and DHR3 (Fig. 2B), an observation that suggests an important functional role for these residues. One aim of future studies will be to determine the nature of the putative protein–DNA and intramolecular interactions for each isoform and the exact amino acid involved in each type of interactions.

Materials and methods

Molecular cloning and analysis of cDNA and genomic clones

The partial cDNA clone λrB5 was isolated from a λgt11 adult rat brain cDNA library using a hybridization probe derived from the cDNA encoding the human RARα (Giguère et al. 1987) and a hybridization mixture contained 35% formamide as described previously (Giguère et al. 1988). The clone λhR5 was isolated from a human retina λgt11 cDNA library (gift of J. Nathans, Johns Hopkins University, Baltimore, MD) using the insert from λrB5 as a probe under the same hybridization conditions. The clones λhT3 and λhT19 were isolated from a human testis λgt11 cDNA library (Clonetech) using the insert from λhR5 as probe. For this screening, the hybridization mixture was modified to 50% formamide. The *Eco*RI inserts derived from the three λ phages were subcloned in pBluescript KS+ (Stratagene) to generate pSKhR5 (RORα1), pSKhT3 (RORα2), and pSKhT19 (RORα3). DNA sequencing was performed as described by Giguère et al. (1990). Genomic clones containing the exons encoding the DNA-binding domain were obtained using a RORα1 cDNA fragment as probe to screen a human genomic phage library. Exon-bearing fragments were identified by hybridization with RORα1 cDNA probes. The genomic sequence encoding each of the two zinc finger region were determined on one strand and compared with the RORα1 cDNA sequence to identify the exon boundaries.

Plasmid construction

The expression vectors pCMXRORα1, pCMXRORα2, and pCMXRORα3 were constructed as followed. Plasmid pSKhT3 was cut with *Bst*EII (nucleotide position 73, see Fig. 1) and the ends repaired with the Klenow fragment of DNA polymerase I. *Kpn*I linkers were added to these ends by standard procedures, and the plasmid was subsequently cut with *Bam*HI at a site located in the polylinker of pSK+. The resulting *Kpn*I–*Bam*HI fragment was then introduced into the *Kpn*I–*Bam*HI sites of the expression vector pCMX (Umesono et al. 1991) to generate pCMXhRORα2. To create pCMXhRORα3, a *Kpn*I linker was added to pSKhT19 at the common *Bst*EII site (nucleotide position 62), and the plasmid was cut with *Bgl*II (nucleotide position 374). The resulting *Kpn*I–*Bgl*II DNA fragment was then exchanged with the corresponding fragment in pCMXhRORα2. Plasmid pCMXhRORα1 was generated by cutting pSKhR5 with *Kpn*I (nucleotide position 18) and *Bgl*II, and the resulting *Kpn*I–*Bgl*II DNA fragment was then introduced in the *Kpn*I–*Bgl*II sites of pCMXhRORα2. These manipulations created expression vectors with specific amino-terminal domains but identical 3′ sequences.

Mutant RORα1Δ23-71 was generated by partial digestion with *Xmn*I to linearized pCMXhRORα1, followed by complete digestion with *Not*I and repair with Klenow. *Sal*I linkers (8-mer) were added, and the plasmid was religated. Mutant RORα1Δ23-71 carries three additional amino acids, Gly-Arg-Pro, at the deletion junction. Mutant RORα2Δ26-103 was generated by cutting pSKhT3 with *Bst*BI (nucleotide position 167), repaired by Klenow and recut with *Kpn*I. The resulting *Kpn*I–blunt fragment encoding amino acids 1–26 common to RORα2 and RORα3 was then introduced into pCMXhRORα1Δ23-71 from which the amino-terminal region was removed by digestion with *Sal*I, followed by repair with Klenow and digest with *Kpn*I. The *Sal*I site is recreated during ligation, which results in mutant RORα2Δ26-103 carrying three additional amino acids, Arg-Arg-Pro, at the deletion junction.

To create mutant RORα2Δ3-45, we used a pair of oligonucleotide primers, one containing the sense strand encoding amino acids 46–51 with a 5′ tail containing a *Kpn*I site and the sequence encoding the first 2 amino acids of RORα2 (5′-CCAGGGTACCATGAATAGGGATGAACTTTTTGGG-3′), and the other containing the antisense sequence encoding amino acids 99–104 with a 5′ tail containing a *Sal*I site complementary to mutant RORα2Δ26-103 (5′-GGATCCGTCGACCAATAATTTCAATTTGAGC-3′), for the PCR using pSKhT3 as template. The amplified fragment was digested with *Kpn*I and *Sal*I and then reintroduced into the *Kpn*I and *Sal*I sites of pCMXRORα2Δ26-103. To generate mutant RORα2Δ46-103 and RORα2Δ74-103, we used the T7 promoter primer 23-mer (New England Biolab) and oligonucleotides containing the antisense sequences encoding amino acids 39–45 and 67–73, respectively, with a 5′ tail containing a *Sal*I site complementary to mutant RORα2Δ26-103 (5′-GGATCCGTCGACGGGCTCCTTCACCTGCAGG-3′ and 5′-GGATCCGTCGACAGACGCCAGTAAGAACAAA-3′), for the PCR using pCMXhRORα2 as template. The amplified fragments were cloned back into the pCMX vector as described above. The cloning procedure led to the addition of 2 amino acids, Arg-Pro, at the deletion junction of each mutant.

The construction of $T_3R\beta_{NX}$ has been described (Thompson and Evans 1989). To construct pCMX$T_3R\beta_{NX}$, a *Kpn*I–*Bam*HI fragment containing $T_3R\beta_{NX}$ was subcloned into the *Kpn*I–*Bam*HI sites of pCMX. The $T_3R\beta$ mutant ΔTT lacking the amino-terminal region was constructed by introduction of a synthetic oligonucleotide duplex (5′-GTACCACCATGGGGC-3′) containing a consensus methionine initiator codon in place of the amino-terminal-coding *Asp*718–*Not*I amino-terminal fragment of $T_3R\beta_{NX}$. Chimeric receptors R_1TT and R_2TT were constructed by exchanging *Kpn*I–*Not*I fragments gene rated by PCR with the *Kpn*I–*Not*I fragment encoding the amino-terminal domain of $T_3R\beta_{NX}$. The PCR fragments were generated using the T7 promoter primer and oligonucleotides modifying the

sequence encoding amino acids 101–103 (in reference to RORα2) to a *Not*I site in RORα1 (5′-CCCGAATTCGCGGCCGCTGAGATGTATGTGTCTTC-3′) and RORα2 (5′-CCCGAATTCGCGGCCGCTGAGCATTCATGTATCCA-3′), respectively. The creation of the *Not*I site resulted in the mutation of amino acids 101–103 from Ile-Glu-Ile to Arg-Pro-Leu. Manipulations of the *Kpn*I–*Not*I fragment of RORα1 were carried out by partial digestion with *Not*I due to the presence of an endogenous *Not*I site within the amino-terminal domain of RORα1. Amino-terminal mutant derivatives pCMXR$_1$(ΔN23-71)TT,pCMXR$_2$(ΔN3-46)TT, and pCMXR$_2$(ΔN46-103)TT were constructed by first adding a *Sal*I linker at the *Not*I site of pCMXR$_2$TT to create pCMXR$_2$TT$_S$, and then by exchanging the *Kpn*I–*Sal*I fragments among pCMXRORα1Δ23-71, pCMXRORα2Δ3-46, and pCMXRORα2Δ46-103 and pCMXR$_2$TT$_S$.

The construction of RARα$_{NX}$ and pCMXRARα$_{NX}$ have been described (Giguère et al. 1987; Predki et al. 1994). The RARα mutant ΔAA lacking the amino-terminal domain was constructed by introducing the carboxy-terminal-coding *Not*I–*Nhe*I fragment of RARα$_{NX}$ in place of the corresponding T$_3$Rβ fragment in ΔTT. Hybrid receptors R$_1$AA and R$_2$AA were constructed by introducing the *Kpn*I–*Not*I amino-terminal-coding fragments of RORα1 and RORα2 in the *Kpn*I—*Not*I sites of ΔAA.

Plasmid TKLUC and γF–HRE$_3$TKLUC have been described (Tini et al. 1993). ROREα1 (5′-TCGACTCGTATATCAAGGTCATGCTG-3′) and ROREα2 (5′-TCGACTCGTATAACTAGGTCAAGCGCTG-3′) oligonucleotides were cloned into the *Sal*I–*Bam*HI sites of the polylinker in three copies arranged in the sense, antisense, and sense orientation to create the reporter gene ROREα1$_3$TKLUC and ROREα2$_3$TKLUC, respectively. All constructs described above were confirmed by sequencing and RORα proteins were analyzed by PAGE using [^{35}S]methionine in the in vitro translation reaction.

In vitro synthesis of ROR proteins and EMSA

pCMX-based plasmids containing various RORα isoforms and mutants, T$_3$Rβ$_{NX}$, RARα$_{NX}$, and plasmid pSKmRXRβ (Mangelsdorf et al. 1992) containing the mouse RXRβ were linearized with *Bam*HI and *Acc*I, respectively. Capped RORα, RARα$_{NX}$ and T$_3$Rβ$_{NX}$ mRNAs were synthesized in vitro using T7 polymerase, whereas RXRβ mRNA was synthesized with T3 RNA polymerase. These mRNAs were used to synthesize RORα and RXRβ protein in vitro using rabbit reticulocyte lysates (Promega). Probes for EMSA were radiolabeled by end-filling with Klenow. Approximately 0.1 ng of probe was used in each reaction with a total of 5 μl of programmed reticulocyte lysate in a buffer containing 10 mM Tris-HCl (pH 8.0), 40 mM KCl, 6% glycerol, 1 mM DTT, and 0.05% NP-40 in a final volume of 20 μl. To prevent single-stranded binding, 10 ng of a nonspecific oligonucleotide was included in the binding reaction. As a control, probes were also incubated with the same amount of unprogrammed lysate. Competitors and probes were added prior to the addition of lysate. The following oligonucleotides and their compliments were used as probes or competitors where indicated: γF–HRE, 5′-TCGACAGTGACCCTTTTAACCAGGTCAGTGAG-3′; CRBP-I, 5′-AGCTTTAGTAGGTCAAAAGGTCAGACACG-3′; TREpal, 5′-AGCTTATCTCCTCAGGTCATGACCTGAATCTTACA-3′; ROREα1, 5′-TCGACTCGTATATCAAGGTCATGCTG-3′; ROREα2, 5′-TCGACTCGTATAACTAGGTCAAGCGCTG-3′; ROREα2, single base pair substitution mutants were based on ROREα2 in which a G residue replaces a base in the 5′-AT-rich region as indicated in Figure 6A.

PCR-assisted DNA-binding site selection from random oligonucleotides

To select for the binding sites of RORα1 and RORα2, we synthesized by PCR a mixture of 70-base oligonucleotides using as template the random oligomer 5′-CGCGGATCCTGCAGCTCGAGN$_{30}$GTCGACAAGCTTCTAGAGCA-3′ and the forward and reverse primers 5′-CGCGGATCCTGCAGCTCGAG-3′ and 5′-TGCTCTAGAAGCTTGTCGAC-3′, respectively (gifts from A.T. Look and T. Inaba, St. Jude Children Research Hospital, Memphis, TN). Prior to the amplification reaction, the forward primer was end-labeled with polynucleotide kinase and [γ-^{32}P]ATP. The amplification reaction was carried out using 20 pmoles of random oligomer, 100 pmoles of ^{32}P-labeled forward primer and 100 pmoles of reverse primer for three cycles, with each cycle consisting of 1 min at 94°C, 2 min at 52°C and 3 min at 72°C. Double-stranded mixed oligomer, as well as labeled TREpal probe as a marker, were incubated with in vitro-synthesized RORα1 or RORα2 protein in the binding buffer for 10 min, and the complexes were separated by electrophoresis through a 4% polyacrylamide gel in 0.5× TBE. A band migrating at the same position of a band containing radioactivity in the lane loaded with RORα1 or RORα2 protein and ^{32}P-labeled TREpal was excised and eluted in the elution buffer (0.5 M NH$_4$ acetate, 1 mM EDTA at pH 8.0). Bound DNA was recovered by ethanol precipitation and amplified by PCR using 100 pmoles of ^{32}P-labeled forward primer and 100 pmoles of reverse primer for 12 cycles using the conditions described above. The selection procedure was repeated four times for RORα1 and six times for RORα2. The products were then digested with *Xho*I and *Sal*I and cloned into Bluescript KS+, and white colonies were picked and subjected to sequence analysis.

Cell culture and transfection assays

P19 and Cos-7 cells were maintained in α-minimal essential medium (MEM) containing 7% fetal calf serum. These cells were transfected by a calcium phosphate coprecipitation technique with 2 μg of TK promoter-based luciferase reporter plasmids, 1 μg of RSV-βgal, 500 ng of appropriate expression vector, and 7 μg of pUC18 as described previously (Giguère et al. 1986). β-Galactosidase and luciferase assays were carried out as described elsewhere (Giguère et al. 1990).

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