Isoform-specific amino-terminal domains dictate DNA-binding properties of ROR α , 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 ROR α 1, ROR α 2, and ROR α 3, 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 ROR α 2 isoform resides on the opposite strand of a cytochrome *c*-processed pseudogene. Binding site selection using in vitro-synthesized proteins reveals that the ROR α 1 and ROR α 2 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, ROR α 1 and ROR α 2 display different binding specificities: ROR α 1 binds to and constitutively activates transcription from a large subset of ROREs, whereas ROR α 2 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 ROR α 1 to bind DNA, a similar deletion relaxes ROR α 2-binding specificity to that displayed by ROR α 1. Remarkably, transfer of the entire amino-terminal region of ROR α 1 or amino-terminal deletion of ROR α 2 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.

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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 nu-

clear 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 (T₃R), vitamin D₃ (VD₃R) 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, T₃R, 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 ROR α (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 ROR α transcription unit generate three isoforms, referred to as ROR α 1, ROR α 2, and ROR α 3, 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 ROR α 1, ROR α 2, and ROR α 3

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 RAR α was used as a probe to screen recombinant DNA libraries to search for unrecognized nuclear receptors related to the RARs. A partial cDNA clone (λ rB5) 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 λ rB5 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 (λ hT19) 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 ROR α 1, ROR α 2, and ROR α 3, respectively. ROR α 2 (λ hT3) and ROR α 3 (λ hT19) 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 ROR α 1 (λ hR5) clone is completely distinct from the 5' ends of ROR α 2 and ROR α 3 and encodes the first 66 amino acid residues of this open reading frame. Restriction endonuclease mapping and sequence analyses indicate that ROR α 1, ROR α 2, and ROR α 3 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 ROR α 1, ROR α 2, and ROR α 3 polypeptides, diagramed in Figure 2C, contain characteristic DNA- and ligand-binding domains of nu-

COMMON TO ROR α 2 AND ROR α 3

CCATCTGTCTGATCACCTTGGACTCCATAGTACACTGGGGCAAGACAGCCCCAGTTTCTGGAGGAGAT 19 19 -
 MetAsnGluGlyAlaProGlyAspSerAspLeuGluThrGluAlaArgValProTrp
 GGGTAACAGGAAAGGCGAATGAGGGGGCCCCAGGAGACAGTGAATAGAGACTGAGGCAAGGTGCCCTGG
 SerIleMetGlyHisCysLeuArgThrGlyGlnAlaArgMetSerAlaThrProThrProAlaGlyGluGlyAla
 TCAATCATGGGTCTTGTCTTCAACTGGACAGGCGAGAAATGTCTGCCACACCCACACCTGCAGGTGAAGGAGCC 44 44 -
 Arg
 AGAAG 45 45 -

SPECIFIC TO ROR α 2

ArgAspGluLeuPheGlyIleLeuGlnIleLeuHisGlnCysIleLeuSerSerGlyAspAlaPheValLeuThr 70 - -
 GGATGAACCTTTTGGGATCTCCAAATACTCCATCAGTGATCTCTCTCAGGTGATGCTTTTGTCTTACT
 GlyValCysCysSerTrpArgGlnAsnGlyLysProProTyrSerGlnLysGluAspLysGluValGlnThrGly 95 - -
 GGCCTCTGTCTTCTCGAGGAGAGATGGCAAGCCACATATTCACAAAGGAAGATAAGGAAGTACAACTGGA
 TyrMetAsnAla 99 - -
 TACATGAATG

SPECIFIC TO ROR α 3

SerSerSerThrCysSerSerLeuSerArgLeuPheTrpSerGlnLeuGluHisIleAsnTrpAspGlyAlaThr - 70 -
 CTCTTCAACCTGTAGCTCCCTGAGCAGGCTGTTCTGGTCTCAACTTGAGCACATAAAGTGGATGAGGACACA
 AlaLysAsnPheIleAsnLeuArgGluPhePheSerPheLeuLeuProAlaLeuArgLysAla - 91 -
 GCCAAGAACTTTATTAATTTAAGGAGTCTTCTCTTTTCTGCTCCCTGCATTGAGAAAAG

SPECIFIC TO ROR α 1

GTTTTTTTTTTTTTTTTTGGTACCATAGACTTGCTCTGAAACAGAGATAGAGGAGTCTCGGAGCTGCATCT
 MetGluSerAlaProAlaAlaProAspProAlaAlaSerGluProGly - - 16
 CCAGCGATCTCTACATTGGGAAAAAATCGAGTCCGCGAGCCCGGACCCCGCGCAGGAGCAGGCG
 SerSerGlyAlaAspAlaAlaGlySerArgGluThrProLeuAsnGlnLeuSerAlaArgLysSerGluPro 41 - -
 AGCAGCGCGCGGAGCGCGCGCGCGCTCCAGGAGACCCCGTGAACAGGAATCCGCGCAGCAGGAGCGG
 ProAlaProValArgArgGlnSerTyrSerSerThrSerArgGlyIleSerValThrLysLysThrHisThrSer - 66 -
 CCGCCCCGGTGCAGACAGAGCTATTCCAGCAGCAGAGGATCTCAGTAACGAAGAAGACATACAT

COMMON TO ROR α 1, ROR α 2 AND ROR α 3

GlnIleGluIleIleProCysLysIleCysGlyAspLysSerSerGlyIleHisTyrGlyValIleThrCys 123 115 90
 CTCAAATTCGAAATATTTCCTGCAAGATCTGGGAGCAAAATCATCAGGAATCAATTATGGTGTCTATTACATGT
 GluGlyCysLysGlyPheArgSerGlnGlnSerAsnAlaThrTyrSerCysProArgGlnLysAsnCys 148 140 115
 GAAGCTGCAGGGCTTTTTCAGGAGACTCAGCAAGCAATGCCACTACTCTCTCTCGTCAGAAAGACTGT
 LeuIleAspArgThrSerArgAsnArgCysGlnHisCysArgLeuGlnLysCysLeuAlaValGlyMetSerArg 173 165 140
 TTGATTGATCGAACCTAGAAACCGCTGCCAAGCTGTGATTACAGAAATGCCCTTGGCGTAGGATCTCTCGA
 AspAlaValLysPheGlyArgMetSerLysLysGlnArgAspSerLeuTyrAlaGluValGlnLysHisArgMet 198 190 165
 GATGCTGTAAAAATTTGGCCGAATGTCAAAAAGCAGAGAGACGCTTGTATGCAGAGTACAGAAACCCGAGTG
 GlnGlnGlnArgAspHisGlnGlnProGlyGluAlaGluProLeuThrProThrTyrAsnIleSerAla 223 215 190
 CAGCAGCAGCAGCGGACCCAGCAGCAGCTGGAGAGCTGAGCCGCTGACGCCACCTACACATCTCGGCC
 AsnGlyLeuThrGluLeuHisAspAspLeuSerAsnTyrIleAspGlyHisThrProGluGlySerLysAlaAsp 248 240 215
 AACGGCTGACGAACTTCACGACGACCTCAGTAACATGACGGGCACACCTGAGGGGATGAGGAGCAGC
 SerAlaValSerSerPheTyrLeuAspIleGlnProSerProAspGlnSerGlyLeuAspIleAsnGlyIleLys 273 265 240
 TCGCCCTGACGAGCTTCTACTGACATACAGCTTCCCGACAGCTCAGGTCTTGATATCAATGGAATCAAA
 ProGluProIleCysAspTyrThrProAlaSerGlyPhePheProTyrCysSerPheThrAsnGlyGluThrSer 298 290 265
 CCAGAACCAATATGTGACTACACACGACATCAGGCTCTTCTCCCTACTGTTCGTTTCAACAGCGGAGACTTCC
 ProThrValSerMetAlaGluLeuGluHisLeuAlaGlnAsnIleSerLysSerHisLeuGluThrCysGlnTyr 323 315 290
 CCAACTGTGCTCCATGGCAGAAATGAGAACACTTGCACAGAAATATATCAAAATCGCATCGGAACTGCCAATAC
 LeuArgGluGluLeuGlnIleThrTrpGlnThrPheLeuGlnGluGluIleGluAsnTyrGlnAsnLysGln 348 340 315
 TTGAGAGAAGAGCTCCAGCAGATTAACGTGGCAGACCTTTTACAGGAAGAAATGAGAACTATCAAAACAGCGA
 ArgGluValMetTrpGlnLeuCysAlaIleLysIleThrGluAlaIleGlnTyrValValGluPheAlaLysArg 373 365 340
 CGGGAGGTGATGTGGCAATGTCTGCCATCAAAATACAGAGCTATACAGTATGTGGTGAAGTTTGGCAAGCG
 IleAspGlyPheMetGluLeuCysGlnAsnAspGlnIleValIleValLysAlaGlySerLeuGluValValPhe 398 390 365
 ATTGATGGATTATGAACTGTGCAAAATGATCAAAATGTGCTCTTAAAGCAGGTCTCTAGAGTGGTGT
 IleArgMetCysArgAlaPheAspSerGlnAsnAsnThrValTyrPheAspGlyLysTyrAlaSerProAspVal 423 415 390
 ATCAGAAATGTGCGCTTTGACTCTCAGAACACACCGTGTACTTTGATGGGAAGTATGCCAGCCGACGCTC
 PheLysSerLeuGlyCysGluAspPheIleSerPheValPheGluPheGlyLysSerLeuCysSerMetHisLeu 448 440 415
 TTCAAATCTTAGTGTGTGAAGCTTTATTAGCTTTGTGTTGAATTTGGAAGAGTTTATGTTCTATGACCTGT
 ThrGluAspGluIleAlaLeuPheSerAlaPheValLeuMetSerAlaAspArgSerTrpLeuGlnGluLysVal 473 465 440
 ACTGAAGATGAATTCATTATTTCTGCTATTGTACTGATGTACGAGATCGCTCATGCTGCAAGAAAGGTA
 LysIleGluLysLeuGlnGlnLysIleGlnLeuAlaLeuGlnHisValLeuGlnLysAsnHisArgGluAspGly 498 490 465
 AAAATTGAAAAACTGCAACAGAAAAATTCAGTAGCTCTTCAACAGCTCTACAGAAAGATCACCAGAAGATGGA
 IleLeuThrLysLeuIleCysLysValSerThrLeuArgAlaLeuCysGlyArgHisThrGluLysLeuMetAla 523 515 490
 ATACTAACAAAGTTAATATGCAAGGTGTCTACATTAAAGAGCTTATGTGAGCAGCATACAGAAAGCTAATGCA
 PheLysAlaIleTyrProAspIleValArgLeuHisPheProProLeuTyrLysGluLeuPheThrSerGluPhe 548 540 515
 TTTAAAGCAATATACCCAGACATTGTGCGACTTCATTTCTCCATTATACAGGAGTGTCTCACTCAGAAATT
 GluProAlaMetGlnIleAspGly* 556 548 523
 AACAACAAAAAATTAACCGAGACCTTTATATGGCCCTGCACAGACCTGGAGCGCCACACAGCTGCATCTTT
 TGTGTATCGGGTACGCGAAGGAGGGGAAACATGAAACAAATTAAGTGAACCTGTTTCTCAAAAAA

Figure 1. Nucleotide sequence of ROR α cDNA and deduced amino acid sequences of ROR α proteins. The DNA sequence encoding the three proteins is divided into ROR α 2 and ROR α 3 common and specific amino-terminal domains, an ROR α 1 5'-specific amino-terminal domain, and a region common to all three ROR α isoforms. The amino-terminal sequences specific to ROR α 1, ROR α 2, ROR α 3 are derived from clones λ hR5, λ hT3, and λ hT19, respectively. The carboxy-terminal sequence common to the three isoforms is derived from clone λ hT3. The boxed amino acids in the region specific to ROR α 2 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 λ hT19 cDNA insert encoding ROR α 3. The complete ROR α 1, ROR α 2, and ROR α 3 cDNA sequences have been submitted to GenBank under accession numbers U04897, U04898, and U04899, respectively.

clear receptors. Sequence alignment of ROR α 1 with a number of human nuclear receptors showed a high degree of identity with the DNA-binding domain of RAR α (67%) and the ligand-binding domain of Rev-Erb α (30%) (Fig. 2A). However, the highest level of identity was ob-

served in the presumptive DNA-binding domain (77%) of the *Drosophila* orphan receptor DHR3 (Koelle et al. 1992). Surprisingly, the similarity between ROR α and DHR3 is higher within short amino and carboxy regions immediately adjacent to the zinc finger region (Fig. 2B).

Amino-terminal domains dictate DNA binding of ROR α

Figure 2. ROR α 1, ROR α 2, and ROR α 3 are members of the steroid hormone nuclear receptor superfamily and arise from alternative RNA processing. (A) Schematic amino acid comparisons between human ROR α 1 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 ROR α 1 in the putative DNA- and ligand-binding domains is indicated inside each domain. (DHR3) *Drosophila* hormone receptor 3 [Koelle et al. 1992]; (RAR α 1) retinoic acid receptor [Giguère et al. 1987]; (RXR α) retinoid X receptor [Mangelsdorf et al. 1990]; [(Rev-Erb α [ear1]), orphan receptor encoded on the reverse strand of the c-erbA α gene [Miyajima et al. 1989]; (T3R β) thyroid hormone receptor [Weinberger et al. 1986]; (PPAR α) 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 ROR α 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 ROR α 1, ROR α 2, and ROR α 3. The amino-terminal region common to ROR α 2 and ROR α 3 is represented by a solid rectangle. The specific exon to ROR α 2 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 ROR α 2 and ROR α 3 are represented by hatched and shaded boxes, respectively. The ROR α 1 amino-terminal domain is shown as a dotted boxed. Open boxes represent region common to the three ROR α isoforms. The amino acid position of each domain boundary is shown for each isoform. (D) Analysis of the genomic sequence surrounding the ROR α 2 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 ROR α 2 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 ROR α 2 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 ROR α transcription unit. The HC2 pseudogene is shown as an open box, and the ROR α 2 exon as a black box. Arrows indicate the direction of transcription.

In addition, ROR α 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 ROR α . Fur-

ther amino acid sequence comparisons of ROR α 1, ROR α 2, and ROR α 3 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

Table 1. Consensus sequences binding to ROR α 1

S1-	GGGAAGTCAAGTGGTAAATTTAGGTCATAT
S2-	CTTGCAATCCAATACTACAAATCGAGGTCA
S3-	GAATGTAGGTCATTTCATGATAACCCCT
S4-	CTTGATACAGGTCATCACTATTTCGGTTCA
S5-	GATGAAGATTTAGAGGTCAATTTAGCCTGCG
S6-	CTCCCAAAGATCAAGGTCAACGGTGATCGAG
S7-	TATGAATAGGTTATGTATTTCAAGGTCAACG
S8-	GTTATACAGGTCAAAGGTATGCCATGCACC
S9-	CAATTTCATCCATAAGGAGTAGGTCACTAAG
S10-	GTCGTTTATACGTTAATTGGGTCAATTGCAA
S11-	ATAACTGGGTCAACGACACTGCGTTACTC
S12-	ACCATCTTAGAGGTCAATTCGTTACCCACGT
S13-	ATGATATTTAGAGGTCAATCGGGTTACTAA
S14-	CACCATACAAGGTCAATCGTCGGTTAACTG
S15-	GTATCCGGGTCAATCGGAGGAGGGGTGTC
S16-	CGAAAGACTTCAAATAAGGTCAAAGGTC
S17-	GATACAAGGTCGTACAAAGGTCAAGTATCT
S18-	AAATAGGTGCGCGCATGAAGGTCAAGTTAC
S19-	ATACAGGCGCAACGTATCTAGGTCAACCGG
S20-	CTCATTTTCGTATTGCATCTAAAGGTCACTG
S21-	AAGGGCTAGACAACAAGGTCAAGTGTACCT
S22-	GCCATGGTCAAGGTCAAGTGTACCCCTG
S23-	GTCATCTAACTCTAATTTTGAAGGTCAATTC
S24-	AATAAACGAGGTCAATGACTTGAAATGCA
S25-	CTTTACACACGAAACTAGGTCAATTGTCCTCC

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								

^aThe 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 ROR α 1 strongly bound both the γ F-HRE and consensus RORE α 1, ROR α 2 and ROR α 3 failed to bind both HREs with high affinity, although extremely weak binding could be detected with longer exposure. Thus, the observation that ROR α 2 and ROR α 3 failed to bind to the natural γ F-HRE and the consensus RORE α 1 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 ROR α isoforms, deletion mutants of both ROR α 1 and ROR α 2 were constructed (Fig. 5A) and in vitro-translated ROR mutants were assayed for their ability to bind to the consensus RORE α 1. Deletion of the amino-terminal domain of ROR α 1 (ROR α 1 Δ N23-71) considerably reduces its ability to bind the RORE α 1 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 ROR α 2 (ROR α 2 Δ N26-103) results in a marked increase in binding to RORE α 1 (Fig. 5B, lane 5). Selective deletion of the ROR α 2 amino-terminal domain using mutants ROR α 2 Δ N3-45, ROR α 2 Δ N46-103, and ROR α 2 Δ N71-103 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 ROR α 2 appears to exert an inhibitory influence on DNA binding, the amino-terminal domain of ROR α 1 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 ROR α DNA-binding function. To evaluate the activity of the amino-terminal ROR α 1 and

Figure 4. ROR α isoform-specific DNA-binding and *trans*-activation. (A) Comparison of ROR α 1, ROR α 2, and ROR α 3 in a cotransfection assay. P19 cells were transfected with 2 μ g of γ F-HRE₃TKLUC reporter, and 250 μ g of pCMX (control), pCMXROR α 1, pCMXROR α 2, or pCMXROR α 3 expression vectors and harvested 36 hr later. (B) Interaction of ROR α 1, ROR α 2, and ROR α 3 with γ F-HRE in vitro. Approximately 0.1 ng of radiolabeled γ F-HRE was incubated with reticulocyte lysate programmed with ROR α 1, ROR α 2, or ROR α 3 mRNA. Probe was also incubated with unprogrammed lysate as a control (lane 1). Cold γ F-HRE (lanes 3,6,9) and a nonspecific competitor (NS) (lanes 4,7,10) were used at 100 molar excess. (C) Interaction of ROR α 1, ROR α 2, and ROR α 3 with RORE α 1 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-HRE₃TKLUC 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,

Table 2. Consensus sequences binding to RORα2

S1	CTCGGAGCGTCTAAATTTAATTGGGTCAATCC
S2	CTACATATGGCCAGATTATTTAGGTCAAGCTG
S3	CTGGATTAACTAGGTCAATGGCAAGGTTGGG
S4	CTAATTACCGGATTGGAATGTGGGTCAATCC
S5	CCATATATAAGTAGGTCAACCAATCTCGA
S6	CCAATAATATGTAGGTCAAGGAGTGGTTAG
S7	CCACTTGGAGACTACGTAATTAGGTCAATCGA
S8	AAACCCTAAATAGGTCAAGTGGGTCAAGCTAGC
S9	TAAACCCCTGACCCTAGTATCTAGGTCAAG
S10	CTAAAAAAGTAGGTCAAGGCGGCTGG
S11	CGAGTACTCTGTCAAATGTAGGTCAAGGAG
S12	GGGGGATTTGACAAAAAGTAGGTCAATGACC
S13	CGAAATTTAGGTCAAGGTTATTTAACTAG
S14	CCATATAATGGGATCTCAGAATTGGGTCAA
S15	CTAGTTTATCTGGGTCAAGGGGGGCTAA
S16	GCTCCTTCAGTCAGGATTTAATTAGGTCAAG
S17	GCCAGTAGACGACATTAATTAGGTCAAGTAGT
S18	CTAGATAAATATAGGTCAAGCTGGGTAGTA
S19	AATACTGTGAGTAGTTAATTAGGTCAAGCAG
S20	GGATCTAGGTCAATAGGTCAAGTCAAGCTA
S21	GGAGAGGTGTTTGGGTATCTAGGTCAAGTAG
S22	CCACATATAAGTAGGTCAACCAATCTCGA
S23	GGGTCCCGGAAACTGGGTCAAGTGGTCCCT
S24	ATTTATGTAGGTCAAGCAGCACTAATTGAG
S25	CTAGTTATCGGTTGAACAGAAGTGGGTCAA
S26	CATGAATTACAGAAAAAGTAGGTCAACCTA
S27	CTGGAAACTAGGTCAAGGCTATGGGCAAG
S28	CATGAATCAAAATGTGGGTCAATGTAGAC
S29	CTGGAATCGCTTAGTAAAGTAGGTCAAGTGG
S30	CTAGATAAATAGGTCAACGCTGGGCC
S31	CCTACGAAAGTAGGTCAACTAGATGTCAGA
S32	CTAGTTTAGAATAGGTCAATCCACCTAG
S33	GACTATTAAATCCGATAAATGGGTCAAC
S34	CTAATTATGGGAATCAAACTAGGTCAAGCTT
S35	CTAGTTAATATCTAGGTCAACCGGCTCGGG
S36	GCTTAATAGGTCAATCGAGTGGGTAAAG

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
	<u>A</u>	<u>T</u>	<u>A</u>	<u>A</u>	<u>G</u>	<u>T</u>	<u>A</u>	<u>G</u>	<u>G</u>	<u>T</u>	<u>C</u>	<u>A</u>	<u>A</u>
	<u>T</u>	<u>A</u>		<u>T</u>	<u>C</u>		<u>G</u>						<u>G</u>

^aThe numbering system is relative to the AGGTCA core motif.

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.

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 RORα₁₃TKLUC (A) and RORα₂₃TKLUC (B) reporter genes by RORα1 and RORα2 in P19 and Cos-7 cells. Cells were transfected with 2 μg of RORα₁₃TKLUC or RORα₂₃TKLUC 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

Amino-terminal domains dictate DNA binding of ROR α

Figure 8. The ROR α 1 and ROR α 2 amino-terminal domains impose DNA-binding specificity to T₃R β and RAR α . (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, R₁TT has the amino-terminal domain of ROR α 1 and the DNA- and ligand-binding domains of the T₃R β . (B) ROR α -T₃R β chimeric receptors ability to bind RORE α 1 in the presence or absence of RXR β . Lysates were programmed as indicated at the top of each lane. (C) Amino-terminal deletion chimeric receptors ability to bind RORE α 1. (Lane 1) Unprogrammed lysate; (lane 2) lysate programmed with ROR α 1; (lane 3–7) programmed lysates as indicated at the top of each lane. The arrows indicate specific retarded R₂(Δ46–103)TT complexes. The asterisk indicates a nonspecific band present in reticulocyte lysate. (D) ROR α -RAR α chimeric receptors ability to bind RORE α 1. Lysates programmed as indicated at the top of each lane.

RORE α 1 or RORE α 2 in two distinct cell types, P19 and COS-7 cells. As shown in Figure 7A, cotransfection of the expression vector pCMXROR α 1, together with the RORE α 1₃TKLUC, 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 pCMXROR α 2 is introduced in COS-7 or P19 cells. In contrast, pCMXROR α 2 leads to a fourfold induction in luciferase activity when cotransfected with RORE α 2₃TKLUC both in COS-7 and P19 cells (Fig. 7B). ROR α 1 activates transcription from the RORE α 2₃TKLUC 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 ROR α 2 can function as a constitutive transcriptional activator. Differences in transcriptional ability between ROR α 1 and ROR α 2 also indicate that this activity is modulated by the amino-terminal domain.

The ROR α 1 and ROR α 2 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 ROR α 1 and ROR α 2 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 T₃R β _{NX}, also referred to as TTT in Thompson and Evans (1989)] was substituted with various regions of the amino-terminal domains of ROR α 1 and ROR α 2 (Fig. 8A). The DNA-binding activities of the hybrid receptors were then tested using RORE α 1 as a probe. T₃R β _{NX} or a mutant lacking its amino-terminal domain (ΔTT), alone or in presence of RXR β , does not recognize RORE α 1 as a binding site (Fig.

8B, lanes 7–10). R₁TT, a hybrid receptor that possesses the amino-terminal domain of ROR α 1 and the DNA- and ligand-binding domains of T₃R β , binds RORE α 1 apparently as a monomer (Fig. 8B, lane 11). When RXR β is added to the reaction, binding is greatly reduced indicating that the RXR/R₁TT heterodimer (Fig. 8B, lane 12), formed through interactions between intact RXR and T₃R β DNA- and ligand-binding domains dimerization interfaces (Kurokawa et al. 1993), does not bind with high affinity to the RORE α 1. Formation of functional RXR/R₁TT heterodimers were observed when either TREpal or γ F-HRE was used as a probe (data not shown). As control, a hybrid receptor containing only a portion of the ROR α 1 amino-terminal domain (Δ N23–71) was also tested. Although weak DNA-binding activity can be detected with ROR α 1 Δ N23–71, the R₁(Δ N23–71)TT hybrid receptor does not bind RORE α 1 (Fig. 8C, lane 4). Similarly, we were unable to transfer novel DNA-binding specificity to the T₃R β using the entire ROR α 2 amino-terminal domain or the Δ N3–46 derivative (Fig. 8C, lanes 5,6). However, the hybrid receptor R₂(Δ N46–103)TT that does not contain the inhibitory function characterized previously in the amino-terminal domain of the native ROR α 2 weakly recognizes RORE α 1 (Fig. 8C, lane 7). We then tested whether this observation is limited to the T₃R β or that the ROR α 1 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 ROR α 1 and ROR α 2 was substituted for the amino-terminal domain of RAR α (Fig. 8A). As shown in Figure 8D, the hybrid receptor R₁AA that possesses the amino-terminal domain of ROR α 1 and the DNA- and ligand-binding domains of RAR α , binds RORE α 1 as a monomer (Fig. 8D, lane 6) as observed previously with the hybrid receptor R₁TT. It should be noted that none of these synthetic hybrid receptors show transcriptional activity when cotransfected with the RORE α 1₃TKLUC 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 ROR α , 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-ErbA α (Lazar et al. 1989; Miyajima et al. 1989), COUP-TF (Wang et al. 1989), and HNF-4 (Sladek et al. 1990)]. The ROR α 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 ROR α 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 ROR α 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 ROR α 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 ROR α 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-ErbA α 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, ROR α 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-ErbA α (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 ROR α 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.

ROR α belongs to the class of monomeric nuclear receptors

Although ROR α 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-ErbA α . As observed previously with Rev-ErbA α (Harding and Lazar 1993), ROR α 1 and ROR α 2 isoforms constitutively activate transcription and bind DNA as monomers (no intermediate sized band resulted from EMSA analysis of truncated ROR α 1 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 ROR α - and Rev-ErbA α -binding sites are practically indistinguishable, and the two receptor systems should be expected to control overlapping gene networks. ROR α 1 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 γ F-HRE, an enhancer element that confers retinoic acid responsiveness to the γ F-crystallin promoter (Tini et al. 1993), acts as a strong HRE for ROR α 1 (Fig. 4). It will be of interest to investigate the possible interactions between RAR and ROR α in the control of γ F-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-ErbA α -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-ErbA α , 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 ROR α , Rev-ErbA α , 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 ROR α 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 ROR α 1 isoform binds to the consensus site [(A/G/T)(T/A)(A/T)(T/A)C(A/T)AGGTCA] while, in sharp contrast, the ROR α 2 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 ROR α 3 isoform does not recognize either site with high affinity. Mutational analysis of the ROR α 2 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 ROR α 2 to that displayed by ROR α 1 (Fig. 5). Therefore, the more stringent DNA-binding specificity displayed by ROR α 2 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 ROR α 2 exerts an inhibitory function on DNA binding while the amino-terminal domain of ROR α 1 would play a neutral role. However, the most dramatic result reported in this study is the ability to transfer the DNA-binding properties of ROR α 1 and ROR α 2 to the T₃R and RAR by exchanging their respective amino-terminal domains (Fig. 8). Chimeric receptors R₁TT, R₂TT, R₁AA, R₂AA and amino-terminal deletion mutants linked to the T₃R β possess the DNA-binding properties of the corresponding wild-type and mutant ROR α isoforms. These observations suggest that the amino-terminal region of the ROR α 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 ROR α 1 and ROR α 2 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* (T₃R) 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 ROR α 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 ROR α 1 and ROR α 2 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 non-equivalent 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 ROR α 1, ROR α 2, and Rev-ErbA α (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 ROR α 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 λ rB5 was isolated from a λ gt11 adult rat brain cDNA library using a hybridization probe derived from the cDNA encoding the human RAR α (Giguère et al. 1987) and a hybridization mixture contained 35% formamide as described previously (Giguère et al. 1988). The clone λ hR5 was isolated from a human retina λ gt11 cDNA library (gift of J. Nathans, Johns Hopkins University, Baltimore, MD) using the insert from λ rB5 as a probe under the same hybridization conditions. The clones λ hT3 and λ hT19 were isolated from a human testis λ gt11 cDNA library (Clontech) using the insert from λ hR5 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 (ROR α 1), pSKhT3 (ROR α 2), and pSKhT19 (ROR α 3). 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 ROR α 1 cDNA fragment as probe to screen a human genomic phage library. Exon-bearing fragments were identified by hybridization with ROR α 1 cDNA probes. The genomic sequence encoding each of the two zinc finger region were determined on one strand and compared with the ROR α 1 cDNA sequence to identify the exon boundaries.

Plasmid construction

The expression vectors pCMXROR α 1, pCMXROR α 2, and pCMXROR α 3 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 pCMXhROR α 2. To create pCMXhROR α 3, 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 pCMXhROR α 2. Plasmid pCMXhROR α 1 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 pCMXhROR α 2. These manipulations created expression vectors with specific amino-terminal domains but identical 3' sequences.

Mutant ROR α 1 Δ 23-71 was generated by partial digestion with *Xmn*I to linearized pCMXhROR α 1, followed by complete digestion with *Not*I and repair with Klenow. *Sall* linkers (8-mer) were added, and the plasmid was religated. Mutant ROR α 1 Δ 23-71 carries three additional amino acids, Gly-Arg-Pro, at the deletion junction. Mutant ROR α 2 Δ 26-103 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 ROR α 2 and ROR α 3 was then introduced into pCMXhROR α 1 Δ 23-71 from which the amino-terminal region was removed by digestion with *Sall*, followed by repair with Klenow and digest with *Kpn*I. The *Sall* site is recreated during ligation, which results in mutant ROR α 2 Δ 26-103 carrying three additional amino acids, Arg-Arg-Pro, at the deletion junction.

To create mutant ROR α 2 Δ 3-45, 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 ROR α 2 (5'-CCAGGGTACCATGAATAGGGATGAACCTTTTGGG-3'), and the other containing the antisense sequence encoding amino acids 99–104 with a 5' tail containing a *Sall* site complementary to mutant ROR α 2 Δ 26-103 (5'-GGATCCGTCGACCAATAATTTCATTGAGC-3'), for the PCR using pSKhT3 as template. The amplified fragment was digested with *Kpn*I and *Sall* and then reintroduced into the *Kpn*I and *Sall* sites of pCMXROR α 2 Δ 26-103. To generate mutant ROR α 2 Δ 46-103 and ROR α 2 Δ 74-103, 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 *Sall* site complementary to mutant ROR α 2 Δ 26-103 (5'-GGATCCGTCGACGGGCTCCTTCACCTGCAGG-3' and 5'-GGATCCGTCGACAGACGCCAGTAA-GAACAAA-3'), for the PCR using pCMXhROR α 2 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 T₃R β _{NX} has been described (Thompson and Evans 1989). To construct pCMXT₃R β _{NX}, a *Kpn*I–*Bam*HI fragment containing T₃R β _{NX} was subcloned into the *Kpn*I–*Bam*HI sites of pCMX. The T₃R β mutant Δ TT 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 Asp718–*Not*I amino-terminal fragment of T₃R β _{NX}. Chimeric receptors R₁TT and R₂TT were constructed by exchanging *Kpn*I–*Not*I fragments generated by PCR with the *Kpn*I–*Not*I fragment encoding the amino-terminal domain of T₃R β _{NX}. The PCR fragments were generated using the T7 promoter primer and oligonucleotides modifying the

sequence encoding amino acids 101–103 (in reference to ROR α 2) to a *NotI* site in ROR α 1 (5'-CCCGAATTCGCGGC-CGCTGAGATGTATGTGTCTTC-3') and ROR α 2 (5'-CCCGAATTCGCGGCCGCTGAGCATTATGTATCCA-3'), respectively. The creation of the *NotI* site resulted in the mutation of amino acids 101–103 from Ile-Glu-Ile to Arg-Pro-Leu. Manipulations of the *KpnI*–*NotI* fragment of ROR α 1 were carried out by partial digestion with *NotI* due to the presence of an endogenous *NotI* site within the amino-terminal domain of ROR α 1. Amino-terminal mutant derivatives pCMXR α 1(Δ N23-71)TT, pCMXR α 2(Δ N3-46)TT, and pCMXR α 2(Δ N46-103)TT were constructed by first adding a *SalI* linker at the *NotI* site of pCMXR α 2TT to create pCMXR α 2TT_S, and then by exchanging the *KpnI*–*SalI* fragments among pCMXROR α 1 Δ 23-71, pCMXROR α 2 Δ 3-46, and pCMXROR α 2 Δ 46-103 and pCMXR α 2TT_S.

The construction of RAR α _{NX} and pCMXRAR α _{NX} have been described (Giguère et al. 1987; Predki et al. 1994). The RAR α mutant Δ AA lacking the amino-terminal domain was constructed by introducing the carboxy-terminal-coding *NotI*–*NheI* fragment of RAR α _{NX} in place of the corresponding T β R β fragment in Δ TT. Hybrid receptors R β 1AA and R β 2AA were constructed by introducing the *KpnI*–*NotI* amino-terminal-coding fragments of ROR α 1 and ROR α 2 in the *KpnI*–*NotI* sites of Δ AA.

Plasmid TKLUC and γ F–HRE β TKLUC have been described (Tini et al. 1993). RORE α 1 (5'-TCGACTCGTATATCAAGGT-CATGCTG-3') and RORE α 2 (5'-TCGACTCGTATAACTAG-GTCAAGCGCTG-3') oligonucleotides were cloned into the *SalI*–*BamHI* sites of the polylinker in three copies arranged in the sense, antisense, and sense orientation to create the reporter gene RORE α 1 β TKLUC and RORE α 2 β TKLUC, respectively. All constructs described above were confirmed by sequencing and ROR α proteins were analyzed by PAGE using [³⁵S]methionine in the *in vitro* translation reaction.

In vitro synthesis of ROR proteins and EMSA

pCMX-based plasmids containing various ROR α isoforms and mutants, T β R β _{NX}, RAR α _{NX}, and plasmid pSKmRXR β (Mangelsdorf et al. 1992) containing the mouse RXR β were linearized with *BamHI* and *AccI*, respectively. Capped ROR α , RAR α _{NX} and T β R β _{NX} mRNAs were synthesized *in vitro* using T7 polymerase, whereas RXR β mRNA was synthesized with T3 RNA polymerase. These mRNAs were used to synthesize ROR α and RXR β 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 μ l 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 μ l. 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: γ F–HRE, 5'-TCGACAGTGACCCTTTTAAACCAGGT-CAGTGAG-3'; CRBP-I, 5'-AGCTTTAGTAGGTCAAAAGGT-CAGACACG-3'; TREpal, 5'-AGCTTATCTCCTCAGGTATGACCTGAATCTTTACA-3'; RORE α 1, 5'-TCGACTCGTATATCAAGGTCATGCTG-3'; RORE α 2, 5'-TCGACTCGTATACTAGGTCAAGCGCTG-3'; RORE α 2, single base pair substitution mutants were based on RORE α 2 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 ROR α 1 and ROR α 2, we synthesized by PCR a mixture of 70-base oligonucleotides using as template the random oligomer 5'-CGCGGATCCTGCAGCTCGAGN₃₀GTCGACAAGCTTCTAGAGCA-3' and the forward and reverse primers 5'-CGCGGATCCTGCAGCTCGAG-3' and 5'-TGCTCTAGAAGCTTGCTCGAC-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 -³²P]ATP. The amplification reaction was carried out using 20 pmoles of random oligomer, 100 pmoles of ³²P-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 ROR α 1 or ROR α 2 protein in the binding buffer for 10 min, and the complexes were separated by electrophoresis through a 4% polyacrylamide gel in 0.5 $\times$ TBE. A band migrating at the same position of a band containing radioactivity in the lane loaded with ROR α 1 or ROR α 2 protein and ³²P-labeled TREpal was excised and eluted in the elution buffer (0.5 M NH₄ acetate, 1 mM EDTA at pH 8.0). Bound DNA was recovered by ethanol precipitation and amplified by PCR using 100 pmoles of ³²P-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 ROR α 1 and six times for ROR α 2. The products were then digested with *XhoI* and *SalI* 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 μ g of TK promoter-based luciferase reporter plasmids, 1 μ g of RSV- β gal, 500 ng of appropriate expression vector, and 7 μ g 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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