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Determinants of Target Gene Specificity for RORα1: Monomeric DNA Binding by an Orphan Nuclear Receptor

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The RORα isoforms are orphan members of the steroid/thyroid/retinoid receptor superfamily. Previous DNA-binding studies indicated that RORα isoforms bind to response elements consisting of a single copy of the core recognition sequence AGGTCA preceded by a 6-bp A/T-rich sequence and that the distinct amino-terminal domains of each isoform influence DNA-binding specificity. In this report, we have investigated in detail the protein determinants of target gene specificity for the RORα1 isoform and have now identified the minimal sequence both in its amino- and carboxy-terminal domains required for high-affinity DNA binding. High-resolution methylation and ethylation interference analyses and mixing of truncated proteins in a DNA-binding assay show that RORα1 presumably binds along one face of the DNA helix as a monomer. By analogy to previous studies of the orphan receptors NGFI-B and FTZ-F1, extensive mutational analysis of the RORα1 protein shows that a domain extending from the carboxy-terminal end of the second conserved zinc-binding motif is required for specific DNA recognition. However, point mutations and domain swap experiments between RORα1 and NGFI-B demonstrated that sequence-specific recognition dictated by the carboxy-terminal extension is determined by distinct subdomains in the two receptors. These results demonstrate that monomeric nuclear receptors utilize diverse mechanisms to achieve high-affinity and specific DNA binding and that RORα1 represents the prototype for a distinct subfamily of monomeric orphan nuclear receptors.

Members of the steroid/thyroid/retinoid superfamily of nuclear receptors are transcription factors that play a central role in regulating gene expression by binding to specific DNA sequences known as hormone response elements (HREs) (8). Their ability to recognize specific HREs is determined by a variety of factors: the amino acid composition of the highly conserved DNA-binding domain (DBD), the differential usage of conserved amino acid residues by distinct DBDs for base-specific contacts, and the modes of DNA binding (monomeric, homodimeric and heterodimeric) that result from differences in receptor-specific dimerization determinants (14). Studies using two-dimensional ^{1}H nuclear magnetic resonance methods showed that the core of the nuclear receptor DBDs is composed of two type II zinc-binding motifs that form a single structural unit (16, 19, 30). Furthermore, the crystal structures of both the glucocorticoid receptor and estrogen receptor DBDs bound to their cognate HREs revealed that the compact DBD structural unit is involved in both protein-DNA and protein-protein interactions (20, 29).

All nuclear receptors described to date recognize a minimal 6-bp sequence of the form AGGTCA or AGAACA referred to as a consensus half-site motif. Mutational analyses of the glucocorticoid and estrogen receptors established that three amino acids within the DBD are involved in the discrimination between the two consensus half-site motifs (6, 21, 34). This discriminatory determinant, termed the P box (34), is located at the amino-terminal end of a helix within the first of the two highly conserved zinc-binding motifs. Since all nuclear receptors recognize one of the two consensus half-site motifs, discrimination between target sites must involve mechanisms other than specific base pair contacts. Receptors that bind as homodimers, exemplified by the steroid hormone receptors, recognize two consensus half-sites arranged as inverted repeats spaced by 3 bp. Formation of stable head-to-head homodimers is dependent on discrete dimerization functions located in both the DBD and the carboxy-terminal ligand-binding domain (LBD) (5, 9, 20, 29, 34). Receptors that bind as heterodimers with the retinoid X receptor (RXR) as a partner recognize HREs composed of two consensus half-site motifs arranged as direct (23, 35), inverted or everted repeats (32). Spacing between the consensus half-sites provides discriminatory information so that RXR heterodimers with the retinoic acid, vitamin D3, and thyroid hormone receptors recognize direct repeats spaced by 2 and 5, 3, and 4 bp, respectively. As observed with homodimeric receptors, stable protein-DNA interaction and cooperative head-to-tail heterodimer formation is dependent on multiple dimerization determinants located both within the DBD and LBD (1, 18, 25, 27). Receptors that can bind DNA as monomers are able to recognize a single consensus half-site, and an increase in DNA binding affinity for the monomeric HRE is provided by an extension of the base pair contacts 5′ of a single consensus half-site motif (13, 17, 36). To date, the 5′ extensions of monomeric HREs have been found to be composed of a 1- to 6-bp-long A/T-rich sequence. Mutational analyses and domain swap experiments of the orphan receptors NGFI-B, steroidogenic factor 1 (SF-1), and FTZ-F1 showed that the 5′ extension of monomeric HREs is recognized by a distinct subdomain of the DBD abutting the second zinc-binding motif at its carboxy-terminal end (33, 37, 38).

The orphan nuclear receptor RORα is a novel member of the superfamily of steroid/thyroid/retinoid receptors (4, 13). The RORα gene generates numerous isoforms that share com-

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TABLE 1. Carboxy-terminal extension of some nuclear receptors and the 5′ A/T-rich moieties of their recognition sites

Receptor	Amino acid sequence[a]	5′ half-site	Reference
	A box		
NGFI-B	GM VKEVVRTDSLKGRRGRLPSKPKQPPDAS	AAA	37
	FTZ-F1 box		
FTZ-F1	GM KLEAVRADRMRGGRNKFGPMYKRDRALK	TCA	33
	CTE		
RORα	GM SRDAVKFGRMSKKQRDSLYAEVQKHRMQ	ATAACT	13
RVR	GM SRDAVRFGRIPKREKQRMLIEMQSAMKT	ATAACT	28
RevErbα	GM SRDAVRFGRIPKREKQRMLAEMQSAMNL	TAANT	17
	T box		
RXRα	GM KREAVQEERQRGKGRNENEVESTSSANE		19
	Helix 3		

[a] Overlines represent the following: A, a region of three amino acid residues implicated in the recognition by NGFI-B of the 5′ half-site adenine residues; FTZ-F1 box, a domain of the FTZ-F1 orphan receptor implicated in the recognition of the 5′ half site; CTE, the carboxy-terminal extension, a domain implicated in the recognition of the 5′ half-site (similarity in amino acid sequences within a subgroup of orphan nuclear receptor is indicated by boxes); and T box, a domain involved in RXR homodimer binding to the RXRE. Helix 3 (underlined) indicates a domain of RXR implicated in homodimeric binding to the RXRE.

mon DBDs and putative LBDs but are distinguished by different amino-terminal domains (13). The RORα isoforms bind to a monomeric HRE (termed RORE) composed of a 5′ 6-bp A/T-rich sequence (WWAWNT, where W represents A or T) that precedes a 3′ AGGTCA core half-site motif (13). This DNA-binding specificity is shared with two other orphan members of the nuclear receptor superfamily, Rev-ErbAα and RVR (RVR is also referred to as BD73 and Rev-Erbβ) (7, 10, 17, 28). Interestingly, the domain that extends carboxy terminal to the two zinc-binding motifs, referred to herein as the DBD carboxy-terminal extension, is highly conserved between these three proteins (Table 1).

In this study, we have examined in detail how RORα interacts with DNA. Methylation and ethylation interference analyses together with mixing experiments using truncated RORα proteins showed that RORα can bind the RORE as a monomer. We then performed an extensive mutational analysis of the domain extending from the carboxy-terminal end of the second conserved zinc-binding motif and engineered chimeric proteins between RORα and NGFI-B. This allowed us to demonstrate that RORα utilizes DNA-binding determinants distinct from those of NGFI-B and conclude that RORα represents the prototype for a novel subfamily of monomeric orphan receptors.

MATERIALS AND METHODS

Plasmid construction. The construction of plasmid pCMXRORα1 and derivative plasmids encoding mutants RΔN23-71, RΔC180-270, RΔC235*, RΔC166, and RΔC157 (translation products referred to in the text and figures as RΔN23-71 or RΔC157 to distinguish amino- and carboxy-terminal end deletions) has been described elsewhere (13, 22). Deletion mutant RΔC150 was generated by using a pair of oligonucleotide primers, one containing the antisense strand encoding amino acids 145 to 150 with a 5′ tail containing a stop codon and a *Bam*HI site (5′-GCGCGGATCCTCATTTTGACATTCGGCCAA, RΔC150) and the other containing the sense sequence (5′-GCCAACACTGTCGATTACAG; RΔC) located upstream of the *Xho*I site at nucleotide 517 of λhR5 (13), for PCR using pCMXRORα1 as the template. The amplified fragment was digested with *Xho*I and *Bam*HI and then reintroduced into the *Xho*I and *Bam*HI sites of pCMXRORα1. To generate the plasmid encoding mutant RΔC139, plasmid pCMXRORα1 was cut with *Xho*I and *Nhe*I, the ends being repaired with the Klenow fragment of DNA polymerase I before ligation. The amino-terminal deletion mutants RΔN12, ΔN25, ΔN35, ΔN45, and ΔN54 were generated by using pairs of oligonucleotide primers, one containing the sense strand encoding amino acids 12 to 17 (5′-CCAGGGTACCATGAGCGAGCCAGGCAGCAG), 25 to 30 (5′-CCAGGGTACCATGGGCTCCAGGGAGACCCC), 35 to 40 (5′-CCAGGGTACCATGTCCGCCCGCAAGAGCGA), 45 to 50 (5′-CCAGGGTACCATGGTGCGCAGACAGAGCTA), and 54 to 59 (5′-CCAGGGTACCATGAGCAGAGGTATCTCAGT), with a common 5′ tail containing a start codon and a *Kpn*I site, and the other containing the antisense sequence (5′-GGATTCCTGATGATTTGTCT; RΔN) located 3′ of the *Bgl*II site at nucleotide 351.

Plasmid pCMXNGFI-B was generated as follows. Plasmid pBS-KS-NGFI-B (the gift of J. Milbrandt, St. Louis, Mo.) containing the cDNA encoding the rat orphan receptor NGFI-B was cut with *Bst*EII, the ends were repaired with Klenow fragment, and the insert was subjected to a ligation reaction in a mixture containing *Kpn*I and *Bam*HI linkers. After digestion with *Kpn*I and *Bam*HI, the resulting *Kpn*I-*Bam*HI fragment was then introduced into the *Kpn*I-*Bam*HI sites of the expression vector pCMX (35). Proper orientation of the NGFI-B cDNA insert was confirmed by sequencing analysis. Plasmid pCMXNGFI-BΔC359X was constructed to facilitate the creation of chimeric RORα1/NGFI-B receptors. It encodes a mutant of NGFI-B receptor that is truncated at amino acid position 359 and contains an *Xho*I site at a position corresponding to the naturally occurring *Xho*I site in the RORα1 cDNA (translation product referred to as NΔC359X in the text). This plasmid was engineered as follows. A DNA fragment encoding the desired NGFI-B mutated sequence was obtained through PCR-based oligonucleotide-directed mutagenesis as described above, using two mutant primers (5′-GGCTGTGGGCATGGCTCGAGAAGTTGTCCGG and 5′-CCGGACAACTTCTCGAGCCATGCCCACAGCC) and two outside primers, one containing the antisense strand encoding amino acids 354 to 359 with a 5′ tail containing a stop codon and a *Bam*HI site (5′-GCGCGGATCCTCAGGAGGCATCTGGGGGCT) and the other containing the sense sequence (5′-CCAGCCGCTTTCCCGGGCTTG; NΔC) located 5′ of a *Sma*I site in the NGFI-B cDNA. The mutagenized and amplified DNA fragment was then excised with *Sma*I and *Bam*HI and introduced into the *Sma*I-*Bam*HI sites of pCMXNGFI-B. This cloning procedure introduced a substitution of two amino acid residues, valine 332 and lysine 333, to an alanine and a serine residue, respectively. We also constructed an NGFI-B derivative (NΔC359XS) in which valine 332 and lysine 333 were substituted for a serine and an arginine residue, respectively. These two residues are present in RORα1, and this construction thus minimizes changes in the primary amino acid sequences of the truncated and mutant receptors. Chimeric receptors RN0 and NR0 were generated by exchanging the *Xho*I-*Bam*HI fragments of plasmids pCMXRORα1ΔC166 and pCMXNGFI-BΔC359X.

Single and double point mutations (Table 2) were introduced into the RORα1 and NGFI-B DBD carboxy-terminal extensions through the generation of *Nhe*I sites by PCR-based oligonucleotide-directed mutagenesis. The following oligonucleotides were used: RΔC, NΔC, 3′CMX (5′-CCAATTATGTCACACCA), RN1S (5′-GTAGGGATGGCTAGCGATGCTGTAA), RN1A (5′-TTACAGCATCGCTAGCCATCCCTAC), RN2S (5′-ATGTCTCGAGCTAGCGTAAAATTTG), RN2A (5′-CAAATTTTACGCTAGCTCGAGAGAT), RN3S (5′-TCGAGATGCTAGCAAATTTGGCC), RN3A (5′-GGCCAAATTTGCTAGCATCTCGA), RN4S (5′-GAGATGCTGTAGCTAGCGGCCGAATGTCA), RN4A (5′-GACATTCGGCCGCTAGCTACAGCATCTC), RN5S (5′-GTAAAATTTGCTAGCATGTCAAAAAAGC), RN5A (5′-CTTTTTTGACATGCTAGCAAATTTTACAG), RN6S (5′-ATTTGGCCGAGCTAGCAAAAAGCAGAGAG), RN6A (5′-CTCTGGCTAGCTGACATTCGGCC), RN7S (5′-CCGAATGTCAGCTAGCCAGAGAGACAGC), RN7A (5′-TGTCTCTCTGGCTAGCTGACATTCGGCC), RN8S (5′-GTCAAAAAAGGCTAGCGACAGCTTGT), RN8A (5′-ACAAGCTGTCGCTAGCCTTTTTTGAC), RN9S (5′-GAGAGACAGCGCTAGCGCAGAAGTACAG), RN9A (5′-GTACTTCTGCGCTAGCGCTGTCTCTCTG), RN10S (5′-GCTTGTATGCTAGCGTACAGAAACAC), RN10A (5′-GTTTCTGTACGCTAGCATACAAGCTG), RN11S (5′-TGCAGAAGTAGCTAGCCACCGGATGCAG), RN11A (5′-CTGCATCCGGTGGCTAGCTACTTCTGCATA), NN1S (5′-AGAAGTTGTCGCTAGCGACAGCCTAAAG), NN1A (5′-CTTTAGGCTGTCGCTAGCGACAACTTCTC), NN2S (5′-GACAGACAGCGCTAGCGGGCGGCGGG), NN2A (5′-CCCGCCGCCCGCTAGC

GCTGTCTGTC), NN3S (5′-AAAGGGGCGGGCTAGCCGGCTACC), NN3A (5′-GGTAGCCGGCTAGCCCGCCCCTTT), NN4S (5′-GGGCCGGCTAGCT AGCAAACCCAAGC), NN4A (5′-GCTTGGGTTTGCTAGCTAGCCGGC CC), NN5S (5′-AAAACCCAAGGCTAGCCCAGATGCC), and NN5A (5′-G GCATCTGGGCTAGCCTTGGGTTTT). The chimeric RORα1/NGFI-B constructs (NR0 to NR5 and RN0 to RN5; Table 2) were generated by exchanging *Nhe*I-*Bam*HI fragments of the DBD carboxy-terminal extension of the RORα1 or NGFI-B sequence with the corresponding sequence of the other receptor. The nucleotide sequences of all constructs described above were confirmed by sequencing.

Methylation interference. The binding site used in this study corresponds to the sequence of the RORE oligonucleotides as previously described (13). Each oligonucleotide was uniquely end labeled with T4 polynucleotide kinase and [α-^{32}P]ATP and annealed with the complementary unlabeled oligonucleotide. Following labeling, unincorporated [α-^{32}P]ATP was removed by Sephadex G-50 chromatography. Approximately 50 fmol of oligonucleotide was partially methylated with dimethyl sulfate in the presence of 10 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia) as previously described (31). Partially methylated template was used in binding reactions as described above, and the wet gel was exposed for at least 24 h at 4°C. Bands representing bound and free fractions were excised, and DNA was recovered by electrophoretic transfer onto NA45 ion-exchange paper. Recovery of DNA was performed as described by the manufacturer (Schleicher & Schuell). DNA was cleaved by boiling in 1 M NaOH. Equal amounts (counts per minute) of DNA from bound and free fractions were analyzed on 10% sequencing gels.

Ethylation interference. A 141-bp *Hin*dIII-to-*Mlu*I fragment from pTKLuc ROREα2 that contains a single copy of the RORE upstream of the luciferase reporter gene was 5′ end labeled with [α-^{32}P]ATP and T4 polynucleotide kinase for the top strand or 3′ end labeled with [α-^{32}P]dCTP and Klenow fragment for the bottom strand. The DNA was ethylated with ethylnitrosourea essentially as described previously (39). Briefly, substrate DNA and 2 μg of denatured salmon sperm DNA were resuspended in 0.1 ml of buffer. An equal volume of ethanol saturated with ethylnitrosourea was added, and the reaction was incubated at 50°C for 1 h. The DNA was precipitated with ethanol twice. Approximately 1.5 pmol of ethylated substrate was incubated with 162 μl of R166 protein that was translated in vitro as described below. Bound and unbound DNAs were separated on a 5% polyacrylamide gel and were recovered by electroelution. The DNAs were cleaved at the sites of modification by heating at 90°C for 30 min in the presence of 0.15 M NaOH. The reaction mixtures were neutralized with HCl, and the DNAs were precipitated with ethanol. The DNAs were then analyzed on an 8% sequencing gel.

In vitro synthesis of proteins and EMSA. All of the different cDNAs encoding wild-type and mutants receptors were cloned downstream of the T7 promoter in the expression vector pCMX. The proteins were synthesized in rabbit reticulocyte lysates by using the TNT-T7 kit (Promega, Madison, Wis.) as instructed by the manufacturer. The integrity of all proteins used in this study was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using [^{35}S]methionine in the TNT-T7 protocol. Probes for the electrophoretic mobility shift assay (EMSA) were radiolabeled by end filling with Klenow fragment. Approximately 0.1 ng of probe was used in each reaction mixture with a total of 4 μl of programmed reticulocyte lysate in a buffer containing 10 mM Tris HCl (pH 8.0), 40 mM KCl, 6% glycerol, 1 mM dithiothreitol, and 0.05% Nonidet P-40 in a final volume of 24 μl. To prevent single-stranded binding, 100 ng of a nonspecific oligonucleotide was included in the binding reaction mixture. As a control, probes were also incubated with unprogrammed lysate. Binding reaction mixtures were incubated at room temperature for 30 min, and complexes were resolved on a 4% polyacrylamide gel in 0.5× TBE (1× TBE is 90 mM boric acid, 90 mM Tris, and 2 mM EDTA). Electrophoresis was carried out at 150 V for 2 h, and gels were dried and exposed to X-ray film. Bands were quantitated by PhosphorImager technology, using the software provided by the supplier (Molecular Dynamics). The following oligonucleotides and their complements were used as probes: RORE, 5′-TCGACTCGTATAACTAGGTCAAGCGCTG; NBRE, 5′-TCGACTCGTGCGAAAAGGTCAAGCGCTG; RORE M1, 5′-TC GACTCGTATAACGAGGTCAAGCGCTG; RORE M3, 5′-TCGACTCGTA TAGCTAGGTCAAGCGCTG; and RORE M4, 5′-TCGACTCGTATGACTA GGTCAAGCGCTG.

RESULTS

The orphan nuclear receptor RORα1 binds DNA as an apparent monomer. We have previously shown that the RORα1 gene product recognizes an HRE composed of a single nuclear receptor core half-site (AGGTCA) preceded by a 6-bp A/T-rich region (13). This observation suggests that RORα1 belongs to the class of nuclear receptors that bind DNA as monomers. To formally test whether RORα recognizes the RORE as a monomer, wild-type and truncated RORα1 polypeptides were mixed and subjected to EMSA (Fig. 1). We first tested translation products generated from truncated RORα1 cDNA templates lacking part of the N-terminal domain (RΔN23-71) and the hinge region (RΔC180-270), two regions of nuclear receptors that are not generally associated with a dimerization function (Fig. 1A). As shown in Fig. 1B, no intermediate-size band resulted from EMSA analysis of truncated RORα1 mutants (lanes 4 to 6). We next tested whether removal of the region that encodes the putative dimerization domain in the nuclear receptor LBDs affected DNA binding. When assayed alone, mutant RΔC235* bound DNA as well as the wild-type RORα1 (Fig. 1B, lane 9). No intermediate-size band could be observed when RΔC235* was mixed with either wild-type RORα1 (Fig. 1B, lane 11) or deletion mutant RΔN23-71 (Fig. 1B, lane 12). Furthermore, cross-linking experiments using a variety of cross-linking reagents failed to detect RORα1 dimer formation in either the presence or absence of DNA (data not shown). These data demonstrate that no apparent RORα1 homodimers are formed and that deleting putative dimerization determinants does not impair the ability of RORα1 to bind DNA. Taken together with the results of our binding-site selection experiments with intact RORα1, in which only monomer consensus sequences were recovered from a pool of 31-bp random-sequence oligonucleotides (13), these observations strongly suggest that RORα1 binds the RORE as a monomer. However, it remains possible that RORα forms heterodimeric complexes with an unidentified partner in vivo, and this possibility will require further study.

Identification of the minimal RORα1 domains required for full DNA-binding activity. We previously showed that deletion of amino acid residues 23 to 71 considerably reduced the ability of RORα1 to bind DNA (13). To determine the precise boundaries of the determinants essential for binding, we generated a series of peptides that consist of progressive amino- and carboxy-terminal deletions of RORα1 (Fig. 2A and B). As shown in Fig. 2C, deletion of the amino-terminal domain up to residue 35 (RΔN35) has no significant effect on DNA binding by RORα1 (lane 5). However, deletion of 10 additional amino acid residues (RΔN45) leads to a dramatic loss in DNA-binding activity of the in vitro-translated protein (Fig. 2C, lane 6). No further loss in DNA-binding activity is seen with mutant RΔN54 (Fig. 2C, lane 7), an observation that suggests that amino acid residues 35 to 45 play an important role in regulating the DNA-binding activity of the RORα1 isoform. As shown in previous experiments, deletion of the carboxy-terminal domain down to amino acid 166 either has no effect or increases the DNA-binding activity of RORα1. However, deletion of nine additional residues (RΔC157) considerably impairs the DNA-binding ability of the resulting peptide (Fig. 2C, lane 10, and results in Table 2 described below). Further deletion down to position 150 completely abolishes binding to the RORE (Fig. 2C, lane 11). These data clearly indicate that the zinc-binding motifs alone are insufficient to determine the DNA-binding properties of RORα1.

The minimal carboxy-terminal region of the RORα1 DBD interacts with the complete RORE and contacts the phosphodiester backbone. Recently, we have used methylation interference analysis to study the interaction of both intact and N-terminal deletions of RORα1 with their binding sites (22). These methylation interference data indicated that RORα1 contacts three guanines within the major groove of the 3′ AGGTCA element and three adenines within the minor groove of the 5′ A/T-rich half of the RORE. We concluded from these data that RORα1 is mainly oriented along one face of the DNA helix such that the zinc-binding motifs interact with the major groove of the 3′ AGGTCA element and the DBD carboxy-terminal extension interacts with the adjacent

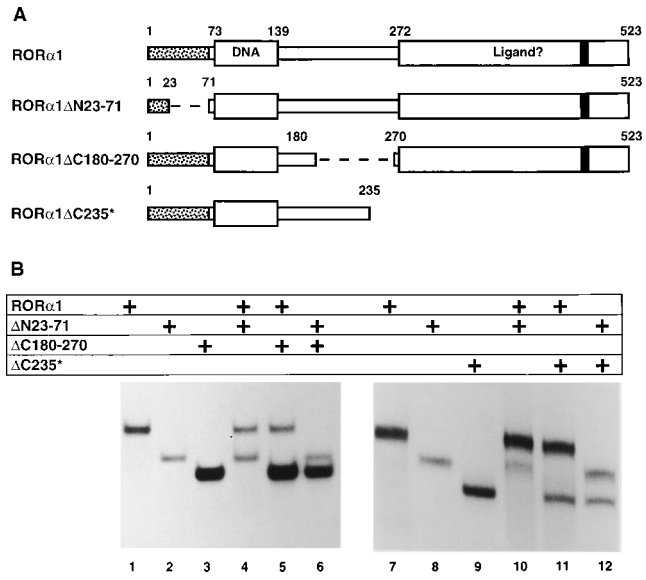


FIG. 1. Evidence that RORα1 recognizes its consensus binding site as a monomer. (A) Schematic representation of RORα1 deletion mutants used in this analysis. The black box in the LBD indicates the location of a leucine zipper-like heptad repeat involved in dimerization of a number of nuclear receptors. (B) Different combinations of lysates programmed with RORα1 and mutant derivatives were assayed by EMSA using RORE as a probe. No intermediate band is observed for any of the combinations, providing indirect evidence for monomeric binding.

minor groove of the 5′ A/T-rich half of the RORE. These studies also indicated that the contacts made by an amino-terminally deleted RORα1 mutant are shifted 5′ in the A/T-rich half of the RORE but are unaltered in the 3′ AGGTCA half. To test whether the RORα1 carboxy-terminal deletion derivative RΔC166 contains the DBD determinants necessary to make all contacts with the RORE and to test whether this peptide can contact DNA in a manner different from that of the wild-type protein, we performed methylation interference experiments with RΔC166. Figure 3A shows the results and a summary of the interference data for RΔC166. On the top strand, methylation of guanine residues at positions 2 and 3 and adenines at positions −3 and −4 strongly interfered with RΔC166 binding. Weak interference was observed at positions −6, 1, and 6. On the bottom strand, methylation of the guanine residue at position 5 and the adenine residue at position −5 also interfered with RΔC166 binding. Comparison of the interference patterns generated by RΔC166 and RORα1 (22) shows that they are similar. These results suggest that the truncated RΔC166 peptide contains all of the determinants dictating specific recognition of the RORE. This peptide and numerous derivatives were then used to complete the subsequent studies on DNA recognition by RORα1.

Many DNA-binding proteins also make specific contacts with the phosphodiester backbone. We performed ethylation interference studies (31) to determine whether phosphate contacts are also important for RORα1 binding to the RORE. On the top strand (Fig. 3B; compare lanes 2 and 3), ethylation of the phosphates 3′ to the residues at positions −2, −1, and 1 strongly interfered with RΔC166 binding. Weaker interference was observed for the phosphates 3′ to the residues at positions −5, −4, −3, and 2. On the bottom strand (Fig. 3B; compare lanes 5 and 6), binding of RΔC166 is prevented by ethylation of the phosphates 3′ to the residues at positions −6, −5, −4, 5, 6, and 7. These results are summarized at the bottom of Fig. 3B. Taken together, these results demonstrate that sequence-specific interaction of RORα does not require determinants extending beyond the DBD carboxy-terminal extension (position 166 in RORα1) and that the RORE is confined to a 12-bp region which contains a single AGGTCA motif, an observation supporting the suggestion that RORα1 binds as a monomer.

Chimeric proteins establish a role for the carboxy-terminal extension domain in recognition of the RORE 5′ A/T-rich sequence. Although the amino-terminal domain of RORα1 is required for full DNA-binding activity, we have previously demonstrated that this domain is not directly involved in base pair contacts (22). On the other hand, amino acid residues located within the DBD carboxy-terminal extension have been shown to be critical for determining DNA-binding specificity of the orphan nuclear receptors NGFI-B, SF-1, and FTZ-F1 (33, 37, 38). To first determine whether the carboxy-terminal extension of RORα1 is involved in the recognition of the 5′ A/T-rich half of the RORE, we generated chimeric proteins between RORα1 and NGFI-B (Fig. 4A). We replaced the DBD carboxy-terminal extension of RORα1 with that of NGFI-B to create the chimeric protein RN0 and also performed the reciprocal switch to generate the chimeric protein NR0. As shown in Fig. 4B, both full-length (Fig. 4B, lanes 3 to 6) and carboxy-terminally truncated mutant (Fig. 4B, lanes 7 to 10) forms of RORα1 (RΔC166) and NGFI-B (NΔC359X) bind their cognate HREs (RORE and NBRE, respectively) with high specificity. No binding of RORα1 is observed when the NBRE is used as a probe (Fig. 4B, lane 4), and no binding of NGFI-B is detected when the RORE is used as a probe (Fig. 4B, lane 5). Similarly results are obtained when the carboxy-terminally truncated peptides are used in the binding assays (Fig. 4B, lanes 8 and 9), although RΔC166 binds the NBRE with low capacity (~3% of the binding observed with the RORE; Table 2). When the chimeric peptide RN0 is used in the DNA-binding assay, a complete switch in DNA-binding specificity is observed. Although RN0 still contains the zinc-binding motifs of RORα1, RN0 no longer recognizes the RORE but now binds the NBRE with high affinity (Fig. 4B, lanes 11 and 12). The reciprocal chimeric peptide, NR0, which contains the zinc-binding motifs of NGFI-B and the carboxy-terminal extension of RORα, did not bind either of the two probes (Fig. 4B, lanes 13 and 14). Because the strategy used to generate NR0 led to an alanine-to-serine substitution at RORα1 position 140, we also constructed NR0B, which possesses an intact RORα1 DBD carboxy-terminal extension (Table 2). Nonetheless, NR0B is also inactive in the DNA-binding assay. The reason for these results is unknown, but a similar observation was made by Wilson et al. (37) in their study of the DNA-binding properties of NGFI-B and SF-1, in which a chimeric peptide containing the SF-1 zinc finger motifs and the carboxy-terminal extension of NGFI-B did not recognize the NBRE.

Mutations in the DBD carboxy-terminal extension of RORα1 affect binding to the RORE. The results presented above show a putative role for the DBD carboxy-terminal extension of RORα1 in the recognition of its binding site. However, deletions can generate changes in the overall structure of the DBD, and the observation that the chimeric peptides NR0 and NR0B did not bind the RORE called for a more detailed analysis of this domain. We therefore introduced a series of mutations into the RORα1 DBD carboxy-terminal extension and analyzed their effects on DNA binding affinity and specificity with the RORE and three RORE mutant derivatives previously shown to affect binding by wild-type RORα1 (13) (Table 2). Mutant peptides were generated via the introduction of recognition sites for the restriction enzyme

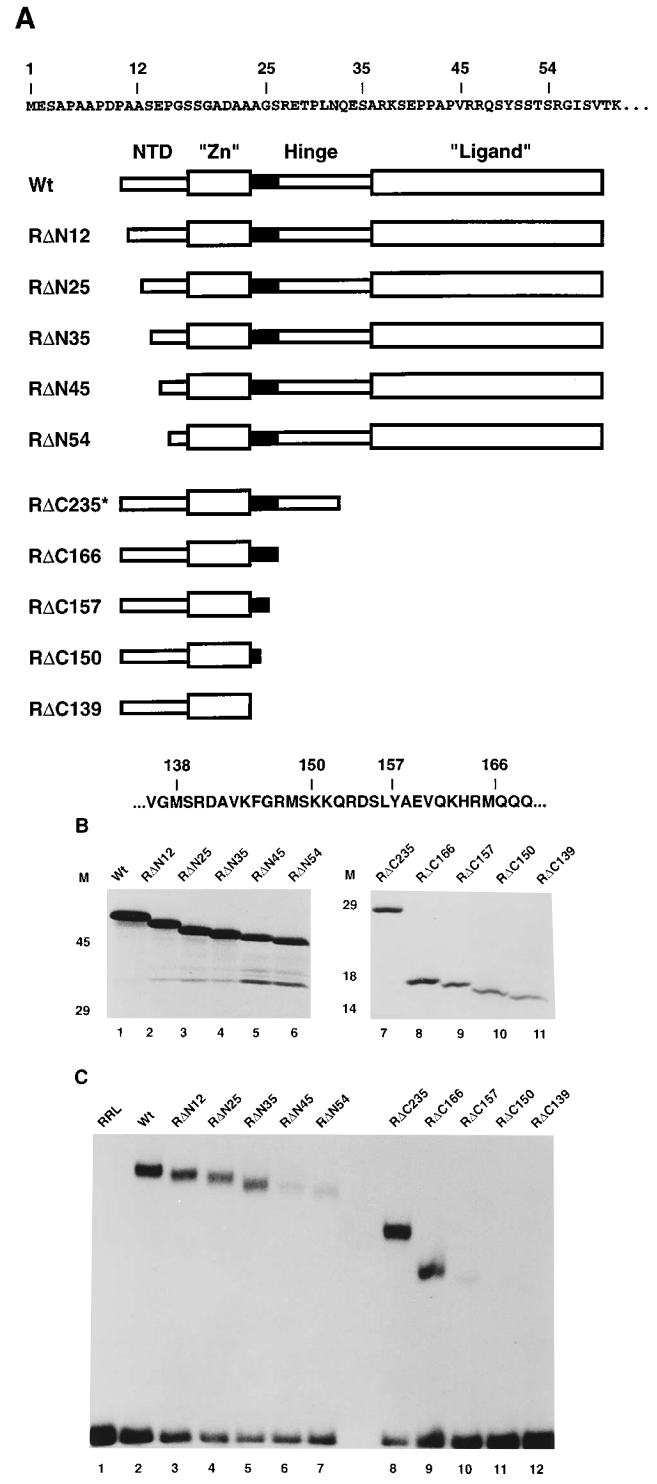
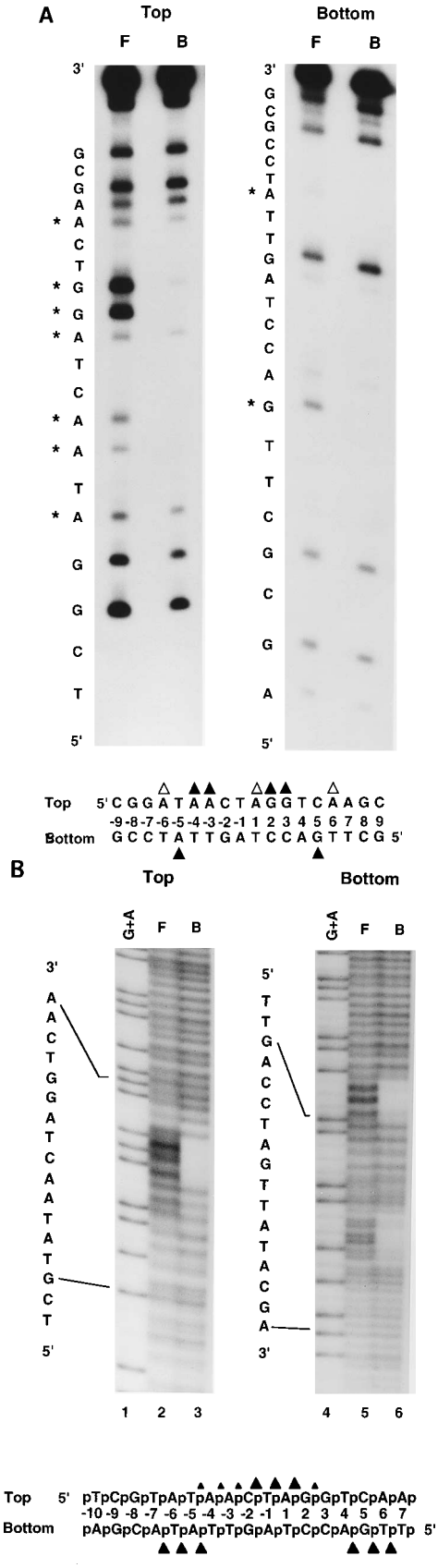


FIG. 2. Identification of the minimal RORα1 domains required for optimal DNA binding. (A) Schematic representation of the RORα1 peptides used in this study and amino acid sequences of the amino-terminal domain and DBD carboxy-terminal extension (black box). Wt, wild type. (B) Full-length (wild-type [wt]) RORα1 and amino- and carboxy-terminally truncated RORα1 peptides were synthesized by in vitro translation in the presence of [^{35}S]methionine and analyzed by SDS-PAGE on a 10% (lanes 1 to 6) or 15% (lanes 7 to 11) polyacrylamide gel followed by autoradiography and quantitation on a PhosphorImager. M, markers for proteins of known sizes (indicated in kilodaltons). (C) Equal amounts of each peptide were analyzed by EMSA for binding to the RORE. RRL, rabbit reticulocyte lysate.

*Nhe*I into the RORα1 sequence. This led to the substitution of two amino acid residues with an alanine and a serine residue. For comparison purposes and to facilitate the generation of chimeric peptides between RORα1 and NGFI-B, a similar but more limited set of mutations was also introduced into the carboxy-terminal truncated form of NGFI-B referred to as NΔC359X (Fig. 4A). As shown in Table 2, only one RORα1 DBD carboxy-terminal extension mutant (RΔC166N5) was completely unable to bind to the RORE or its mutant derivatives. This mutant peptide bears the substitution of glycine 147 and arginine 148 with the alanine and serine pair. These amino acid residues are located within a region corresponding to the previously described third helix in RXRα (Table 1 and reference 19). Mutants RΔC166N4 and RΔC166N6 with amino acid residue substitutions preceding and following the null mutation in RΔC166N5 also display significant loss in DNA-binding activity, as does mutant RΔC166N1, which contains substitutions of the first two amino acid residues of the DBD carboxy-terminal extension, including an arginine residue conserved in RORα, RVR, RevErbα, and RXRα (Table 1). Interestingly, mutant peptide RΔC166N1 displays additional DNA-binding characteristics distinct from those of RΔC166. Whereas peptide RΔC166N1 still binds the RORE relatively well (61%), binding is almost completely abolished (~1 to 2%, a 30- to 60-fold reduction in binding) when the RORE mutant binding sites M3 and M4 are used as probes. By comparison, wild-type RORα1 and its RΔC166 derivative bind site M4 with only a 5- to 10-fold-reduced ability (Table 2 and reference 13). Since the effects of the protein and binding-site mutations are additive, it is possible that the amino acids substituted in RΔC166N1 are directly involved in base pair recognition at positions other than −3 and −4, possibly at position −1. In contrast, substitution of the corresponding amino acid residues in NGFI-B (Val→Ser and Lys→Arg in mutant NΔC359XS) has no effect on binding to the NBRE (Table 2). Therefore, the combined DNA-binding activity observed with the mutant RORα1 peptides and RORE derivatives emphasizes the importance of the putative third helix in RORE recognition. Surprisingly, mutations in the region corresponding to the previously defined NGFI-B A box (37) represented by mutants RΔC166N7 and RΔC166N8 have no effect on the DNA-binding activities of these peptides. For comparison, substitution of two amino acid residues within the A box in NGFI-B (NΔ359XN3) completely abolishes the DNA-binding activity of this mutant peptide with the NBRE (Table 2). Further substitutions of amino acid residues at positions 156 (Leu→Ala) and 157 (Tyr→Ser) in mutant peptide RΔC166N9 reduces binding by a factor of 3, thus suggesting a possible involvement of this subdomain in RORE recognition. Interestingly, RΔC166N9 shows a greater reduction (4-fold) in binding to RORE mutant M1 compared with the RORE than does RΔC166 or other mutant peptides (~2-fold).

Distinct regions of the DBD carboxy-terminal extension are used by RORα1 and NGFI-B for DNA binding. The results presented above suggest that RORα1 and NGFI-B utilize distinct subdomains of the DBD carboxy-terminal extension to achieve specific recognition of their respective binding sites. To confirm these observations, we next generated a series of reciprocal and progressive chimeric peptides in which portions of the DBD carboxy-terminal extension of each receptor were substituted for one another (Table 2 and Fig. 5). As expected, the presence of the NGFI-B A box in the chimeric NGFI-B/RORα1 peptides is absolutely required for recognition of the NBRE. Chimera NR3, which possesses the NGFI-B T box but not the A box region, shows no DNA-binding activity. Progressive addition of the NGFI-B A box in chimera NR4 restores



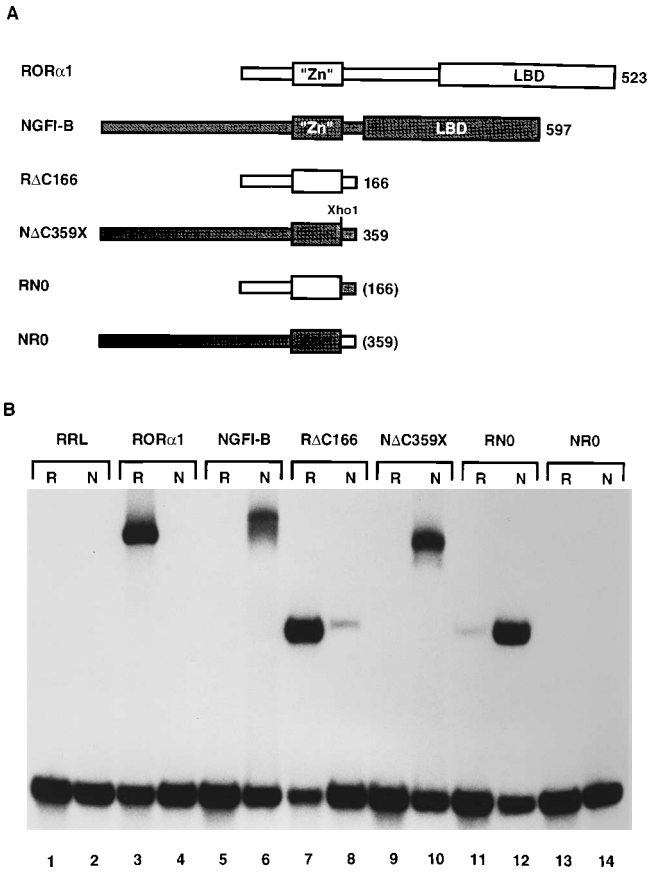


FIG. 4. The DNA-binding specificity of RORα1 resides in the DBD carboxy-terminal extension. RORα1, NGFI-B, carboxy-terminal deletion mutants RΔC166 and NΔC359X, and chimeric RORα1/NGFI-B peptides were in vitro translated, and the same amount of each protein was used for EMSA analysis with the RORE and NBRE probes. (A) The full-length, truncated, and chimeric proteins are schematically represented. "Zn" indicates the location of the zinc-binding motif. The *Xho*I site introduced in the NGFI-B sequence by in vitro mutagenesis is shown. The number at the left of each diagram represents the amino acid location at the end of each protein and therefore its length. (B) Binding of the different proteins to the RORE (R) and NBRE (N). RRL, unprogrammed rabbit reticulocyte lysate.

specific binding to the NBRE, while further addition of NGFI-B sequence to the lysine residue at position 353 leads to an almost complete recovery of DNA-binding activity of chimera NR5. In contrast, significant binding to the RORE is readily observed in chimera RN2. This peptide contains only the subregion of the DBD carboxy-terminal extension immediately adjacent to the zinc-binding motifs. Remarkably, chi-

FIG. 3. Methylation and ethylation interference with RΔC166-RORE complex formation. (A) Methylation interference. F indicates free probe, and B indicates probe bound to RΔC166. The DNA sequence is indicated at the side. Interference with RORα1ΔC166 binding is summarized at the bottom. Filled triangles indicate bases whose modification strongly interferes with RORα1ΔC166 binding; open triangles indicate bases whose methylation weakly interferes with RORα1ΔC166 binding. (B) Ethylation interference. Lanes 1 to 3, top DNA strand. Lane 1, G+A Maxam and Gilbert sequencing standard; lanes 2 and 3, unbound and bound DNAs, respectively. Lanes 4 to 6, bottom DNA strand. Lane 4, G+A Maxam and Gilbert sequencing standard; lanes 5 and 6, unbound and bound DNAs, respectively. The DNA sequence is indicated at the side. Interference with RORα1ΔC166 binding is summarized at the bottom. Large triangles indicate phosphates whose modification strongly interferes with RORα1ΔC166 binding; small triangles indicate phosphates whose ethylation weakly interferes with RORα1ΔC166 binding.

TABLE 2. DNA-binding activities of RORα and NGFI-B mutants and chimeric proteins

Name	Amino acid sequence[a]	Relative binding value[b]				
		RORE (ATAACT)	NBRE (GCGAAA)	M1 (ATAACG)	M3 (ATAGCT)	M4 (ATGACT)
RΔC166	GM SRDAVKFGRMSKKQRDSLYAEVQKHRMQ	100	3	49	24	19
RΔC157	GM SRDAVKFGRMSKKQRDSLY	42	NT[c]	8	2	3
RΔC150	GM SRDAVKFGRMS	—[d]	NT	NT	NT	NT
RΔC139	GM	—	NT	NT	NT	NT
RΔC166N1	** AS************************	61	—	12	2	1
RΔC166N2	** **AS**********************	112	7	65	26	17
RΔC166N3	** ****S**********************	107	2	58	20	13
RΔC166N4	** *****AS********************	26	—	19	4	2
RΔC166N5	** *******AS******************	—	—	—	—	—
RΔC166N6	** *********A******************	68	—	26	9	10
RΔC166N7	** ***********AS***************	122	1	48	20	23
RΔC166N8	** *************AS*************	109	2	31	13	13
RΔC166N9	** ****************AS*********	34	2	9	6	5
RΔC166N10	** ******************S*******	73	1	17	6	8
RΔC166N11	** ********************AS****	122	4	48	20	24
NΔC359	GM VKEVVRTDSLKGRRGRLPSKPKQPPDAS	NT	NT	NT	NT	NT
NΔC359XS	.. * *.....	—	100	—	—	—
NΔC359X	.. A*.....	—	80	—	—	—
NΔC359XN1	.. A*...AS.....	—	—	NT	NT	NT
NΔC359XN2	.. A*.....AS.....	—	2	NT	NT	NT
NΔC359XN3	.. A*.....AS.....	—	—	NT	NT	NT
NΔC359XN4	.. A*.....A.....	—	40	NT	NT	NT
NΔC359XN5	.. A*.....AS....	—	81	NT	NT	NT
NR0B	.. **************************	—	—	—	—	—
NR0	.. A**************************	—	—	NT	NT	NT
NR1	.. A*...AS********************	—	—	NT	NT	NT
NR2	.. A*.....A******************	—	—	NT	NT	NT
NR3	.. A*.....AS**************	—	—	NT	NT	NT
NR4	.. A*.....AS*********	—	32	NT	NT	NT
NR5	.. A*.....AS****	—	87	NT	NT	NT
RN0	** **.....	—	168	NT	NT	NT
RN1	** *****AS.....	—	—	—	—	—
RN2	** *********A*.....	13	20	14	10	6
RN3	** ************AS.....	23	8	16	12	6
RN4	** ****************AS.....	16	—	8	5	3
RN5	** ********************AS....	129	4	67	39	28

[a] All proteins were produced by an in vitro transcription-translation reaction. Asterisks indicate amino acid residues present in the RORα protein; dots indicate amino acid residues present in the NGFI-B protein. All proteins terminate immediately after the last amino acid residue shown.

[b] Expressed as a percentage of the value obtained with RΔC166 with the RORE or NΔC359X with the NBRE. Each value represents the average of at least two different experiments, each performed in duplicate.

[c] NT, not tested.

[d] —, less than 1% of the binding activity of the control.

mera RN2 shows dual binding specificity (Table 2 and Fig. 5, lanes 3 and 4). Significant binding of RN2 is observed when either the RORE or NBRE is used as a probe. It is evident, however, that chimera RN2 does not contain all determinants for proper recognition of the RORE. Peptide RN2 shows binding to the RORE reduced by a factor of 8 compared with peptide RN5 (Fig. 5 lane 5), and its binding is not affected by a mutation at position −1 in the RORE, although a mutation at position −4 further reduces binding by 50% (Table 2). Complete DNA-binding activity and specificity with the RORE and its mutant derivatives is recovered only with chimera RN5 (Fig. 5, lane 5). These results indicate that RORα1 requires two distinct subdomains of the DBD carboxy-terminal extension for proper recognition of the 5′ A/T-rich half of the RORE.

DISCUSSION

The DBD carboxy-terminal extension is a novel DNA-binding motif that characterizes monomeric DNA-binding nuclear receptors (33, 37, 38). In this study, we subjected the DBD carboxy-terminal extension of RORα1 to a detailed structure-function analysis by in vitro mutagenesis and DNA-binding assays. In addition, we used a series of NGFI-B mutants and chimeric RORα1/NGFI-B peptides and a panel of binding sites to compare the DNA-binding properties of RORα1 with those of NGFI-B. These experiments lead us to conclude that RORα1 and NGFI-B utilize distinct subdomains of the DBD carboxy-terminal extension and therefore reveal a novel strategy by which monomeric nuclear receptors recognize their cognate HREs. We suggest that RORα1 represents the pro-

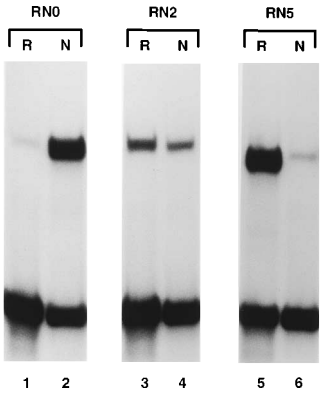


FIG. 5. Dual DNA-binding specificity by chimeric RORα1/NGFI-B peptides. Peptides were in vitro translated, and the same amount of each protein was used for EMSA analysis with the RORE (R) and NBRE (N) probes.

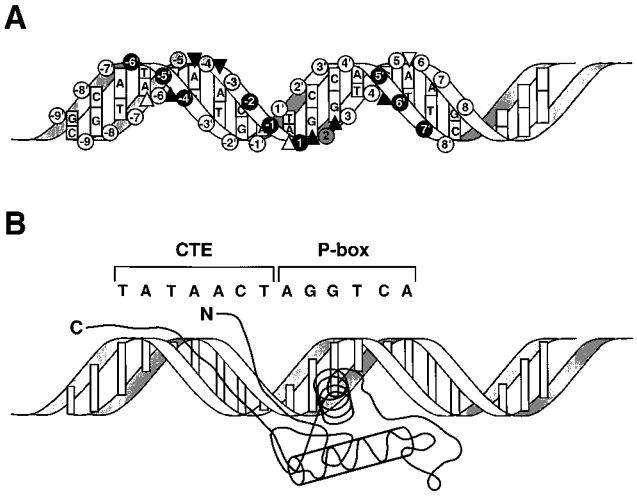


FIG. 6. RORα1 binding interference summary and model of monomeric DNA binding. (A) RORα1 ethylation and methylation interferences as shown in Fig. 3. Positions at which strong and weak G and A methylation interference occurred are indicated by closed and open triangles, respectively. Positions at which interference with modified phosphates occurred are indicated by filled (strong) and shaded (weak [these may be indirect contacts]) circles. (B) Model of RORα1 monomer-DNA complex. The RORα1 DBD is shown as being bipartite. The two zinc-binding motifs that contain the P box contact the major groove at the 3′ AGGTCA element, and the DBD carboxy-terminal extension (CTE) interacts with the adjacent minor groove at the 5′ A/T-rich element of the RORE. The zinc-binding motifs region is modeled after data presented in reference 29. The CTE is shown as a linear domain, since no structural data are available for this region of nuclear receptors. The amino-terminal domain adjacent to the zinc-binding motifs has been shown to modulate the activity of the CTE (22) and is shown here as a linear domain adjacent to the CTE.

totype for a distinct subfamily of monomeric DNA-binding nuclear receptors.

The results presented in this study allows us to refine our model of RORα-DNA interaction previously put forward by McBroom et al. (22). RORα isoforms bind DNA as monomers and preferentially recognize the consensus sequence WWAW NTAGGTCA (where W represents A or T) consisting of two distinct half-sites, a 5′ A/T-rich half (WWAWNT) and a 3′ AG GTCA half site that contains the core motif for nuclear receptors. The RORα1 DBD is bipartite, and each DBD subdomain binds to adjacent half-sites positioned along the same face of the DNA helix. The two zinc-binding motifs contact the major groove at the 3′ AGGTCA element, and the DBD carboxy-terminal extension interacts with the adjacent minor groove at the 5′ A/T-rich element of the RORE (Fig. 6). We have shown in this report that RORα1 requires two distinct subregions of the DBD carboxy-terminal extension to recognize the 5′ A/T-rich half of the RORE. These two DNA-binding determinants are adjacent to but do not include the previously defined NGFI-B A box. This model is supported by the following evidence. First, mixing of truncated and wild-type RORα1 does not generate intermediate-size bands in EMSA (Fig. 1), and RORα1 does not require RXR or nuclear extract to bind the RORE with high affinity (13). Second, the RORE contains a single nuclear receptor core half-site and is limited to 12 bp. Methylation interference studies (Fig. 3A and reference 22) showed major groove contacts at the 3′ AGGTCA half-site and adjacent minor groove contacts at the 5′ A/T-rich moieties, thus positioned on the same face of the DNA helix. Ethylation interference analysis also demonstrated that RORα1 makes a significant number of direct contacts with the phosphodiester backbone, as ethylation of about nine different phosphates caused strong interference of binding (summarized in Fig. 6A). The identified phosphate contacts are clustered in three regions. On the bottom strand, phosphate contacts are made at each end of the RORE. One group of phosphate contacts is centered near position −5, and a second group is centered one helical turn away, near position 6. The third group of phosphate contacts occurs on the top strand near the center of the RORE (near position −1). A helical projection indicates that these three clusters of phosphate contacts would all be along one face of the DNA molecule, results which are consistent with those of the methylation interference studies. Taken together, DNA-binding assays with truncated RORα1 proteins and methylation and ethylation interference analysis strongly suggest that RORα1 binds the RORE as a monomer. Third, amino acid substitutions in the DBD carboxy-terminal extension either considerably diminished or completely abolished the DNA-binding activity of RORα1 (Table 2). Mutations that affect binding are localized in both the amino- and carboxy-terminal ends of the domain. Chimeric proteins generated for this study showed that the amino-terminal region of the domain confers sequence-specific DNA binding to RORα1 (mutant RN2 in Table 2). Binding of chimeric protein RN2 is sequence specific, since introduction of a mutation at position −4 in the RORE (M4) considerably reduces its binding activity, as observed with RΔC166. Remarkably, the RN2 chimeric protein possesses dual binding specificity, as it also recognizes the NBRE (Fig. 5). The RN2 chimeric protein contains an intact NGFI-B A box, thus confirming the importance of this region for sequence-specific DNA binding for this receptor. However, addition of the corresponding A-box region in the chimeric protein RN3 and RN4 does not impart new DNA-binding activity to these proteins, although recognition of the NBRE is lost as predicted (Table 2). Complete DNA-binding activity is restored with chimeric protein RN5.

The mutational analysis presented in this report delineates the borders of the DBD carboxy-terminal extension to amino acid residues 140 to 160 of RORα1. However, prediction of precise contacts between amino acid residues of the DBD carboxy-terminal extension and base pairs in the 5′ A/T-rich half of the RORE is not possible because of the apparent complex nature of this domain in RORα1. X-ray crystallography studies of the estrogen receptor-estrogen response element complex have recently shown that such predictions, based solely on mutational analysis, can be incomplete or incorrect (29). It has also been argued that the mechanisms of monomeric DNA binding by NGFI-B and SF-1 (FTZ-F1) are basi-

cally the same (37). However, point mutations in the putative A box of FTZ-F1 do not significantly affect its interaction with DNA (33). Because FTZ-F1, NGFI-B, and RORα1 do not have a significant degree of amino acid sequence homology in the DBD carboxy-terminal extension (Table 1), it can be suggested that each one of these receptors utilizes distinct mechanisms for sequence-specific recognition of DNA. On the other hand, the DBD carboxy-terminal extensions of RORα1, Rev ErbAα, and the recently identified nuclear orphan receptor RVR (Table 1) are highly conserved, and these proteins recognize DNA in very similar manners (7, 13, 17, 28). Therefore, members of this subfamily would be predicted to utilize similar strategies for a protein monomer to recognize DNA.

In addition to the functional differences in the mechanism of specific DNA recognition between RORα1, NGFI-B, and FTZ-F1 (SF-1), the RORα1 orphan nuclear receptor has a complex functional domain organization unique so far among the members of the steroid/thyroid/retinoid receptor superfamily. Efficient binding of RORα1 to DNA requires an intact amino-terminal domain, and the presence of this domain has been shown to influence binding specificity by distinct RORα isoforms (Fig. 1 and reference 13). We have tentatively mapped a regulatory region within the RORα1 amino-terminal domain to amino acid residues 35 to 45. This short sequence contains a putative phosphorylation site for protein kinase C (Ser-35) whose covalent modification could be used to modulate the DNA-binding activity of the RORα1 isoform. Two other putative phosphorylation sites are present between amino acid residues 45 and 54, a cyclic AMP- and cyclic GMP-dependent protein kinase site (Ser-49) and a second protein kinase C site at Thr-53. Nuclear receptors are phosphoproteins (24), and it has been suggested that certain receptors can be activated through ligand-independent pathways via covalent modification of these proteins (26). It will be of interest to determine the phosphorylation status of this site under various physiological condition and/or after stimulation of signal transduction pathways involving activation of kinases and phosphatases. Furthermore, we have recently observed changes in the DNA structure induced by the binding of ROR isoforms. These changes in DNA structure require an intact hinge region that could facilitate intramolecular interactions necessary to achieve high-affinity and stable DNA binding (22).

The evolution of the nuclear receptor superfamily led to a wide variation in DNA-binding mechanisms that are united by the common utilization of the zinc-binding motifs structural unit to bind consensus core half-site motifs (14). Receptors that bind as homo- and heterodimers recognize pairs of core half-site motifs, and it is the precise orientation and spacing of these half-sites that determines binding specificity (2, 3, 11, 15, 32, 35). Binding of receptor dimers to DNA is cooperative and requires multiple independent dimerization determinants located within the DBD and LBD (9, 12, 18, 25, 27). Receptors that can bind DNA as monomers, exemplified in this study by RORα1, have developed a DNA-binding mechanism involving a bipartite DBD composed of the zinc-binding motifs and the DBD carboxy-terminal extension that is required for the recognition of extended binding sites. By analogy with the zinc-binding motifs (25, 34, 40, 41), the results presented here show that the DBD carboxy-terminal extension has a modular structure. It appears that members of different subgroups of the nuclear receptor superfamily utilize each module in the most efficient manner to achieve specific and stable DNA binding. This study led us to a greater appreciation of the role played by the DBD carboxy-terminal extension in the molecular mechanism of HRE recognition by nuclear receptors. Further elucidation of the unique DNA-binding properties of this large superfamily of transcription factors will lead to a better understanding of the crucial role played by these receptors in development, homeostasis, and diseases.

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