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The Nonconserved Hinge Region and Distinct Amino-Terminal Domains of the RORα Orphan Nuclear Receptor Isoforms Are Required for Proper DNA Bending and RORα-DNA Interactions

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RORα1 and RORα2 are two isoforms of a novel member of the steroid-thyroid-retinoid receptor superfamily and are considered orphan receptors since their cognate ligand has yet to be identified. These putative receptors have previously been shown to bind as monomers to a DNA recognition sequence composed of two distinct moieties, a 3′ nuclear receptor core half-site AGGTCA preceded by a 5′ AT-rich sequence. Recognition of this bipartite hormone response element (RORE) requires both the zinc-binding motifs and a group of amino acid residues located at the carboxy-terminal end of the DNA-binding domain (DBD) which is referred to here as the carboxy-terminal extension. In this report, we show that binding of RORα1 and RORα2 to the RORE induces a large DNA bend of ~130° which may be important for receptor function. The overall direction of the DNA bend is towards the major groove at the center of the 3′ AGGTCA half-site. The presence of the nonconserved hinge region which is located between the DBD and the putative ligand-binding domain (LBD) or RORα is required for maximal DNA bending. Deletion of a large portion of the amino-terminal domain (NTD) of the RORα protein does not alter the DNA bend angle but shifts the DNA bend center 5′ relative to the bend induced by intact RORα. Methylation interference studies using the NTD-deleted RORα1 mutant indicate that some DNA contacts in the 5′ AT-rich half of the RORE are also shifted 5′, while those in the 3′ AGGTCA half-site are unaffected. These results are consistent with a model in which the RORα NTD and the nonconserved hinge region orient the zinc-binding motifs and the carboxy-terminal extension of the RORα DBD relative to each other to achieve proper interactions with the two halves of its recognition site. Transactivation studies suggest that both protein-induced DNA bending and protein-protein interactions are important for receptor function.

The nuclear receptor superfamily encodes a diverse set of transcriptional regulators (7). This superfamily includes receptors for steroids, retinoids, and thyroid hormones as well as a large number of orphan receptors which are structurally and functionally related but whose ligands have not been identified (for references, see reference 28). The domain structures of the receptors are similar in that they each contain four structural regions (11, 26). There is an amino-terminal domain (NTD) that is not well conserved among receptors, followed by a highly conserved DNA-binding domain (DBD). The DBD is composed of two class II zinc-binding motifs which fold together to form a single structural unit (52). In some receptors, a group of amino acid residues that extends carboxy terminal to the zinc-binding motifs has also been implicated in DNA binding (31, 62, 66). These DNA-binding determinants are believed to be involved in both protein-DNA and protein-protein interactions. The DBD is separated from a moderately conserved ligand-binding domain (LBD) by a hinge region which shows little homology within the nuclear receptor superfamily. The LBD has been shown to also function in transcriptional activation and repression and dimerization (59). In contrast, the hinge region has no known function.

The nuclear hormone receptors have been shown to bind to DNA sequences containing a 6-bp element of the form AGAACA for the glucocorticoid receptor (GR) or AGGTCA for the estrogen receptor (ER), retinoid receptors, and most orphan receptors. Some receptors, such as the GR and ER, bind as homodimers to inverted repeats of these 6-bp elements (5, 24, 27, 38). Other receptors, such as the retinoic acid receptor (RAR) and thyroid hormone receptor, bind as heterodimers with the retinoid X receptor (RXR) to sites containing direct, inverted, or everted repeats of the form AGGTCA separated by different spacer lengths depending upon the nature of the receptor complex (4, 23, 32, 37, 40, 57, 63, 69, 70). Orphan nuclear receptors such as RORα, NGFI-B, SF-1, FTZ-F1, Rev-ErbAα, and RVR have been shown to bind as apparent monomers to sites containing a single 3′ AGGT CA element preceded by a 5′ AT-rich sequence (14, 18, 29, 45, 61, 65).

The DNA binding properties have been most extensively studied for those receptors which bind as homodimers. X-ray crystallographic studies of the GR DBD with GR response element (GRE) and of the ER DBD with an ER response element (ERE) indicate that the zinc-binding motifs of each receptor contact the AGAACA or AGGTCA element within the major groove (35, 52). The structures of the RAR and RXR DBDs have recently been determined in solution and were found to be similar to those of the GR and ER DBDs, suggesting that they may interact with DNA in a similar manner (25, 31). The way in which nuclear receptors that bind as monomers interact with their DNA targets is not as well understood. A number of studies indicate that the 5′ AT-rich

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sequences are constituents of the DNA-binding sites (14, 18, 65). In vitro mutagenesis and domain swap experiments have shown that for the orphan receptors NGFI-B, SF-1, and FTZ-F1, a region carboxy terminal to the zinc-binding motifs (referred to as the T- and A-boxes in NGFI-B [67] and as the FTZ-F1-box in FTZ-F1 [62]) is required for interaction with these sequences.

RORα is a novel transcription factor that belongs to the subgroup of orphan nuclear receptors (14). It has recently been demonstrated that the RORα gene generates at least three different isoforms that share common DBDs and putative LBDs but are distinguished by discrete NTDs (14). Our initial functional analysis has shown that RORα isoforms bind as monomers to a response element (RORE) composed of two moieties: a 5′ 6-bp AT-rich sequence that precedes a 3′ AGG TCA core half-site motif. A domain of RORα that extends carboxy terminal to the zinc-binding motifs contains the DNA-binding determinants necessary to recognize the 5′ AT-rich half of the RORE (12), while the zinc-binding motifs are presumed to interact with the 3′ AGGTCA half-site. Interestingly, differences in the NTDs of RORα1 and RORα2 influence DNA binding, since RORα1 binds with a more relaxed specificity than does RORα2 (14). The consensus binding sites for the two isoforms are 5′-DWWWNWAGGTCA (RORα1) and 5′-WWAWNTAGGTCA (RORα2), where D represents A, T, or G and W represents A or T. The major difference between these two sites is that RORα2 absolutely requires an A residue 4 bp 5′ of the AGGTCA element and a T residue 1 bp 5′ of the AGGTCA element for high-affinity binding. Our previous studies thus suggest a complex domain organization of RORα isoforms in which the NTD and DBD work in concert to confer the ability of these proteins to bind the monomeric hormone response elements with high affinity and specificity.

In the present study, we have extended our investigation of the functional domains of the monomeric hormone receptors and of the molecular mechanism(s) by which these receptors recognize their cognate binding sites. Numerous studies have indicated that binding of certain proteins to their recognition sequences induces DNA bending and that DNA bending may function in transcriptional activation (16, 19, 44, 47), transcriptional repression (42), DNA replication (1, 55, 64), and DNA recombination (52, 53). We have utilized circular permutation and phasing analysis to test whether binding of RORα isoforms to RORE induces DNA bending. We have found that binding of RORα1 or RORα2 to the RORE induces a large DNA bend and have determined which domains of the protein are required for DNA bending. Transactivation studies suggest that both protein-induced DNA bending and protein-protein interaction via a transactivation domain within the C terminus of RORα are involved in receptor function. We propose a model for RORα-DNA interaction in which the hinge region and the NTD of RORα isoforms are involved in orienting the class II zinc-binding motifs and the carboxy-terminal extension with respect to each other. Intramolecular interactions between multiple functional domains may therefore provide the appropriate configuration to allow strong monomer-DNA interaction by a large subset of nuclear receptors. The conformations of both the protein and the DNA within the complex may then facilitate interaction of these receptors with other components of the transcription machinery.

MATERIALS AND METHODS

DNAs. The plasmid pGVRORE contains the RORα2 RORE inserted between two direct repeats of the 375-bp *Eco*RI-to-*Bam*HI fragment of pBR322 and was used as the source of substrate for circular permutation analysis. It was constructed by inserting the complementary oligonucleotides 5′-ATAAGTAG GTCA and 5′-GATCTGACCTACTTATAGCT into pGV579 (9) that had been digested with *Sac*I and *Bgl*II. The plasmids pK10RORE, pK12RORE, pK14 RORE, pK16RORE, pK18RORE, and pK20RORE were used for phasing analysis, and each contains an RORα2 RORE and a sequence-directed DNA bend. These plasmids were constructed by replacing the 165-bp *Bam*HI-to-*Xba*I *lac* promoter DNA fragment from plasmids pK10, pK12, pK14, pK16, pK18, and pK20, which were the gift of D. Crothers (71), with the complementary oligonucleotides 5′-GATCCATAAGTAGGTCAGGATCCT and 5′-CTAGAGGAT CCTGACCTACTTATG.

The construction of plasmids pCMXRORα1, pCMXRORα2, pCMXRORα1 ΔN23-71, and pCMXRORα2ΔN46-103 has been described previously (14). To construct pCMXRORα1ΔC180-270, plasmid pSKhR5 (14) was cut with *Bpu* 1102 and *Nco*I and the ends were repaired with the Klenow fragment of DNA polymerase I. This plasmid was subsequently cut with *Kpn*I and *Bam*HI, and the resulting fragment was introduced into the *Kpn*I-*Bam*HI sites of the expression vector pCMX (63). To create pCMXRORα1ΔC235* and pCMXRORα1ΔC475*, pCMXRORα1 was cut with *Eco*RV and *Acc*I or with *Acc*I alone, respectively, and the ends were repaired with the Klenow fragment of DNA polymerase I. The cloning procedure led to addition of two amino acids, Leu and His, at the carboxy-terminal ends of each mutant which is denoted by an asterisk. Deletion mutant pCMXRORα1ΔC166 was generated by using a pair of oligonucleotide primers, one containing the antisense strand encoding amino acids 161 to 166 with a 5′ tail containing a stop codon and a *Bam*HI site (5′-GCGCGGATCCT CACTGCATCCGGTGTTTCT-3′) and the other containing the sense sequence (5′-GCCAACACTGTCGATTACAG-3′) located upstream of the *Xho*I site at nucleotide 517 of λhR5 (14), for the PCR using pCMXRORα1 as a 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 pCMX RORα1ΔC157, a similar procedure was used with a primer containing the antisense strand encoding amino acids 152 to 157 with a 5′ tail containing a stop codon and a *Bam*HI site (5′-GCGCGGATCCTCAATACAAGCTGTCTCTCT-3′) and the same sense primer as above.

In vitro synthesis of receptors and DNA binding assay. Coupled in vitro transcription and translation with T7 RNA polymerase and TNT rabbit reticulocyte lysate (Promega) was used to synthesize various RORα isoforms or deletion derivatives from pCMX- or pSK-based plasmids (according to the manufacturer's protocol). Between 2.5 and 5 μl of programmed reticulocyte lysate was used in DNA binding reactions. DNA substrates for DNA bending assays were 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. DNA binding reactions were carried out essentially as previously described (14) except that the buffer also contained 10 μg of bovine serum albumin and 6.5 μg of denatured salmon sperm DNA was substituted for the 10 ng of nonspecific oligonucleotide.

Circular permutation analysis. Circularly permuted DNA substrates containing the RORE were created by digesting pGVRORE with *Eco*RV, *Eae*I, *Bam*HI, *Eco*RI, *Hin*dIII, *Bst*NI, or *Rsa*I to release a 408-bp fragment. Each fragment was isolated and end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and then used as a substrate in a gel mobility shift assay with in vitro-translated receptor. RORα-RORE complexes were analyzed on either 5 or 8% polyacrylamide gels. The relative mobility (μ) of a protein-DNA complex was calculated as the distance migrated by the complex divided by the distance migrated by the substrate. Relative mobilities were then plotted as a function of the position of the DNA molecular end. The ratio μM/μE was calculated (from data obtained by using a 5% polyacrylamide gel), where μM and μE are the relative mobilities from substrates which contain the binding sites near the middle and the end of the DNA fragments, respectively. This ratio was used to estimate the DNA bend angle by interpolation from a standard curve of μM/μE versus bend angle which was plotted for a series of DNA fragments containing known bend angles (56).

Phasing analysis. *Pvu*II-to-*Rsa*I fragments from the plasmids pK10RORE, pK12RORE, pK14RORE, pK16RORE, pK18RORE, and pK20RORE were isolated and ^{32}P end labeled for use as binding substrates. Binding reactions were carried out as described above. A 5% polyacrylamide gel was prerun for 30 min, and the binding reactions were run at 300 V at 4°C for 12 h.

Methylation interference. The binding site used in this study corresponds to the sequences of the ROREα1 oligonucleotides as previously described (14). Each oligonucleotide was uniquely end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and annealed with the complementary unlabeled oligonucleotide. Following labeling, unincorporated [γ-^{32}P]ATP was removed by 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 (54). 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 done as described by the manufacturer (Schleicher & Schuell). DNA was cleaved by being boiled in 1 M NaOH. Equal amounts (in counts per minute) of DNA from bound and free fractions were analyzed on 12% sequencing gels.

Cell culture and transfection assays. CV-1 cells were maintained in alpha minimal essential medium containing 7% fetal calf serum. These cells were transfected by a calcium phosphate coprecipitation technique with 2 μg of RORE-thymidine kinase promoter-based luciferase reporter plasmids, 1 μg of RSVβgal, 250 ng of appropriate expression vector, and 7 μg of pUC18 as

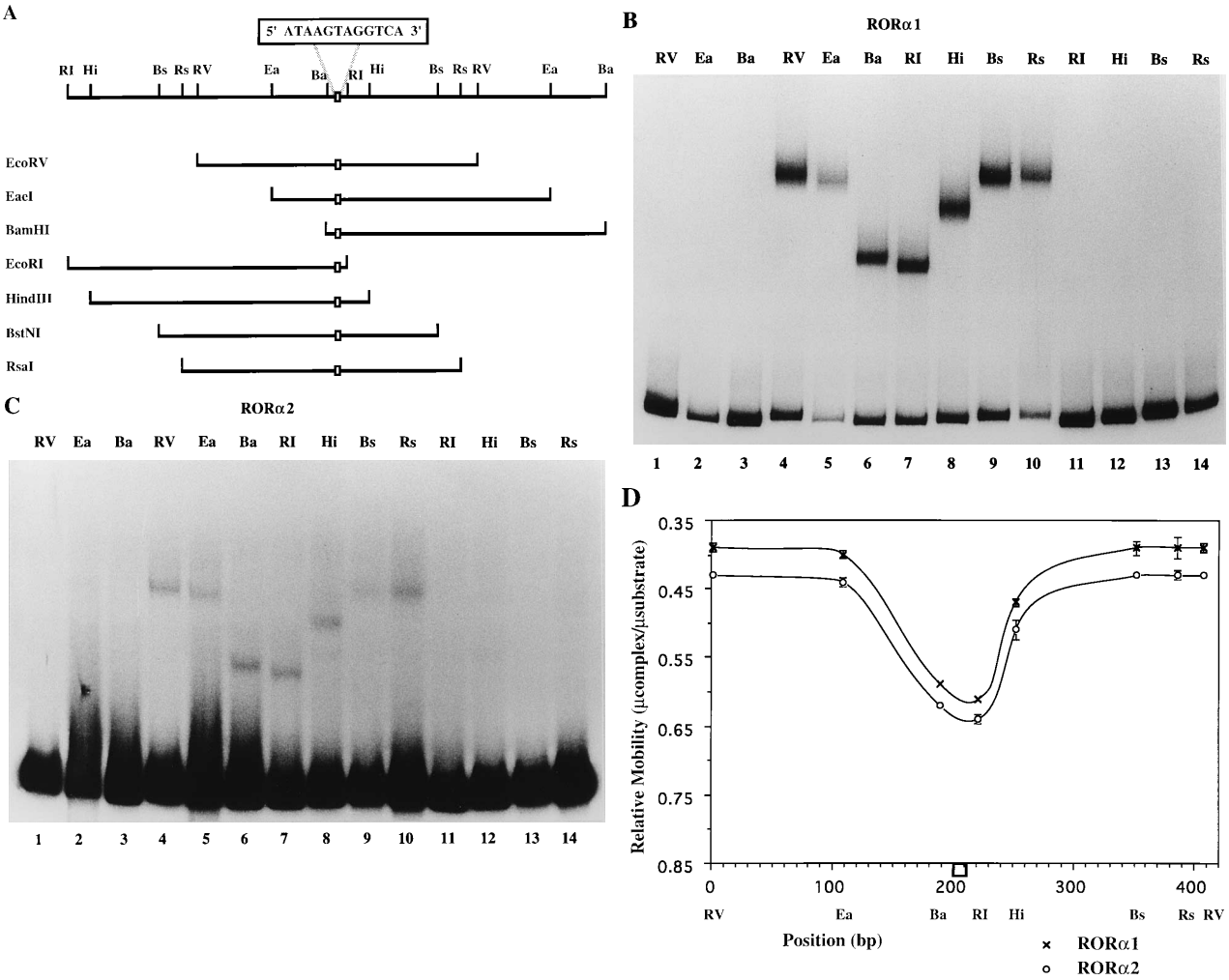


FIG. 1. Circular permutation analysis of RORα1-RORE and RORα2-RORE complexes. (A) The region of the plasmid pGVRORE that contains the RORE and the surrounding tandem duplication is diagrammed at the top. Circularly permuted substrates were generated by cleavage at restriction sites within the duplicated region as shown. Each DNA fragment has been named according to the restriction enzyme used to generate it. The RORE (box) is indicated. Restriction enzymes: RI, *Eco*RI; Hi, *Hin*dIII; Bs, *Bst*NI; Rs, *Rsa*I; RV, *Eco*RV; Ea, *Eae*I; Ba, *Bam*HI. (B) Gel mobility shift assays using the circularly permuted substrates and RORα1 on a 5% polyacrylamide gel. The corresponding substrate is indicated above each lane. Substrates were incubated with unprogrammed reticulocyte lysate (lanes 1 to 3 and 11 to 14) or with reticulocyte lysate programmed with RORα1 (lanes 4 to 10). (C) Circular permutation analysis of complexes formed with RORα2. Substrates were incubated with unprogrammed reticulocyte lysate (lanes 1 to 3 and 11 to 14) or with reticulocyte lysate programmed with RORα2 (lanes 4 to 10). (D) Relative mobilities of the complexes formed with RORα1 or RORα2 plotted as a function of the position of the DNA molecular end for each substrate. Relative mobilities were calculated as the mobility of the complex divided by the mobility of the unbound substrate. For RORα2, distances migrated by the unbound substrate were measured from a shorter exposure. Values are averages of several independent experiments, and standard deviations are indicated. The position of the RORE (box) and the position of each substrate are indicated below the graph.

described previously (14). β-Galactosidase and luciferase assays were carried out as described elsewhere (14).

RESULTS

The orphan nuclear receptors RORα1 and RORα2 induce large DNA bends. To determine whether RORα1 and RORα2 induce DNA bending at their recognition site, we have performed a circular permutation analysis (68). This assay is based on the principle that if a DNA fragment is bent near the middle, the end-to-end distance is greatly reduced. This will result in a decrease in the mobility of the protein-DNA complex compared with that of a complex containing unbent DNA. In contrast, if the DNA fragment is bent near the end, there will be little effect on the end-to-end distance and complex mobility. The circularly permuted DNA substrates used in this study were derived from the plasmid pGVRORE and are diagrammed in Fig. 1A.

As seen in Fig. 1B, there was a large decrease in the mobility of the RORα1-DNA complex when the binding site was in the center of the DNA molecule (lane 4) compared with when it was at the end of the DNA molecule (lane 7). The size of the DNA bend that was induced by RORα1 binding was estimated by comparison of the ratio of the mobilities of the most slowly and fastest-migrating complexes (μM/μE) with the bent-DNA standards of Thompson and Landy (56). In the determination of the induced bend angle, the ratio of the mobility of the complex relative to the mobility of the unbound substrate DNA was used to correct for slight differences in the migration of the substrates. The size of the DNA bend induced by RORα1 is approximately 130°. Two or three independent trials have been performed for all experiments reported. We esti-

mate the error due to measurement and interpolations from the standard curve to be ±5°.

The location of the DNA bend center can be roughly determined by plotting the relative mobilities of the complexes formed with the permutation substrates as a function of the position of the DNA molecular end (numbering according to the parent fragment) (68). As shown in Fig. 1B, the complex formed with the *Eco*RI substrate (lane 7) migrated more rapidly than the complex formed with any of the other substrates, including the *Bam*HI fragment substrate (lane 6). This indicates that the center of the DNA bend is closest to the *Eco*RI site and is thus towards the 3′ AGGTCA half of the RORE, as shown by the graph in Fig. 1D. The center of the bend cannot be determined to the exact base pair because of error in both measurement of complex mobilities and extrapolation from the plot of relative mobilities as a function of DNA position.

RORα1 and RORα2 isoforms have distinct N termini which have previously been shown to influence the binding properties of these proteins (14). We were therefore interested in determining if differences in the N termini of RORα1 and RORα2 also influence DNA bending. There was little difference between the patterns of the mobilities of the complexes obtained for RORα1 and RORα2 (compare Fig. 1B and C). The magnitude of the DNA bend induced by RORα2 may be slightly smaller, 123° ± 5°, but the bend center appears to be the same for both receptor-DNA complexes, as shown in Fig. 1D. Thus, the differences in the distinct NTDs of each RORα isoform appear to have little influence on DNA bending.

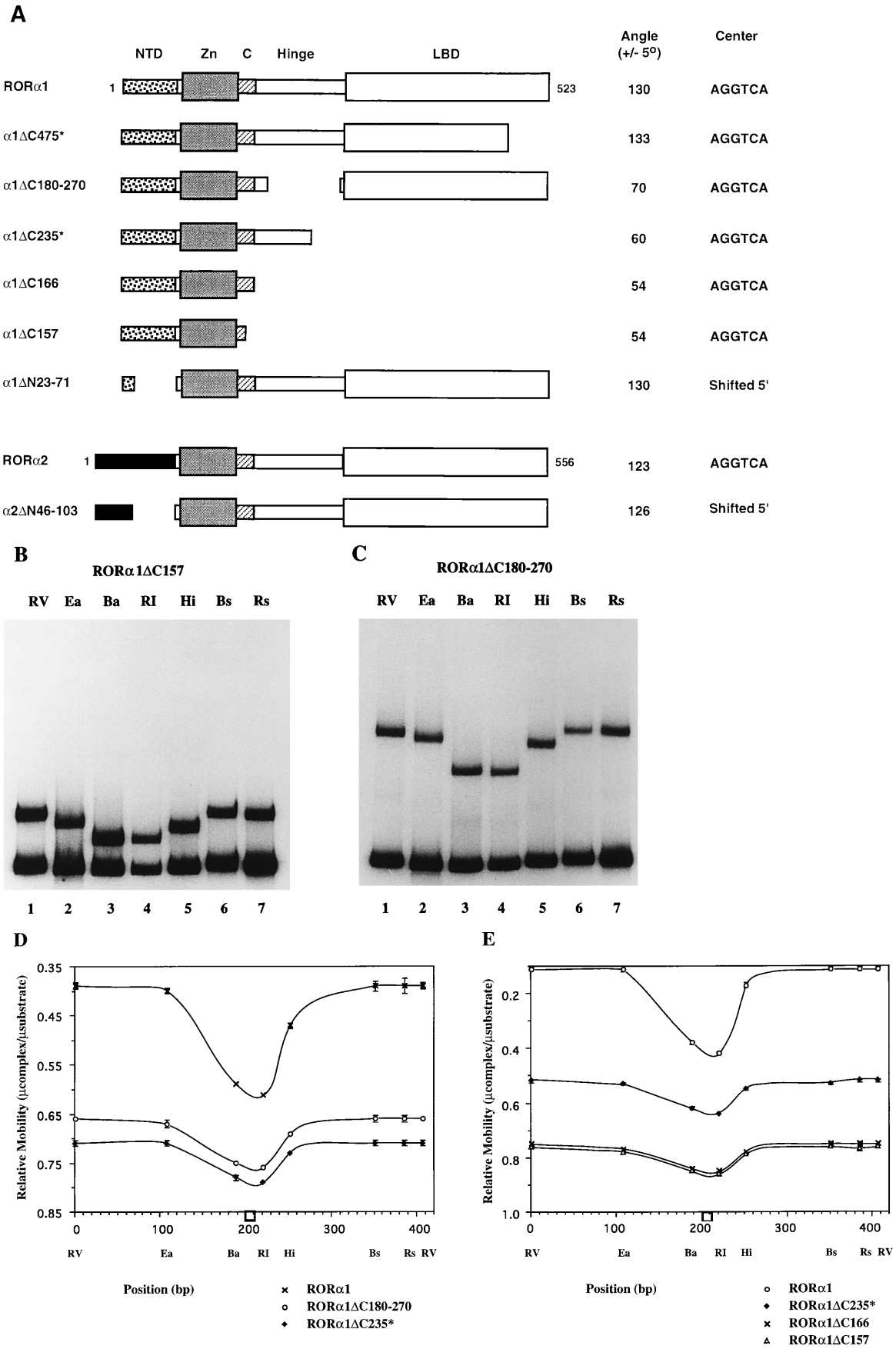
Deletion of the hinge region reduces the DNA bend angle. The DBD of RORα contains two class II zinc-binding motifs which presumably interact with the 3′ AGGTCA half of the RORE in a fashion similar to that for the ER with ERE (52). In addition, a region that extends C terminal to the zinc-binding motifs is required to interact with the 5′ AT-rich half of the RORE (12). Together, the zinc-binding motifs and the carboxy-terminal extension form a bipartite DBD that contains the information essential for DNA recognition by RORα isoforms. It is possible that full DNA bending is induced by the minimal DBD, as seen for the high-mobility-group (HMG)-binding domain (9). Alternatively, other parts of the protein in addition to the minimal DBD may be required for full DNA bending, as seen for proteins such as ABF1, RAP1, and Fos and Jun (15, 20, 21, 39). We therefore analyzed the DNA bending properties of several RORα deletion derivatives (Fig. 2A) to determine which regions of the protein are important for DNA bending. As summarized in Fig. 2A, deletion of the C-terminal 48 amino acids of RORα1 in derivative RORα1ΔC475* had little effect on either the size or the center of the DNA bend. In contrast, deletion of the residues C terminal to amino acid positions 235, 166, and 157 caused a large decrease in the μM/μE ratio, and hence the bend angle, as shown in Fig. 2B for RORα1ΔC157. Similarly, an internal deletion of residues 180 to 270 also caused a large reduction in the μM/μE ratio, as shown in Fig. 2C. The sizes of the bends induced by these derivatives are listed in Fig. 2A. Each DNA bend is reduced by at least 50% compared with that induced by intact RORα1. These results suggest that a domain of the protein which is required for maximal DNA bending appears to reside in the hinge region between the DBD and the LBD, as deletion of much of this region in derivative RORα1ΔC180-270 causes a marked reduction in the DNA bend angle. Derivative RORα1ΔC235* induced a slightly smaller bend than did RORα1ΔC180-270, suggesting that the region required for bending may extend beyond residue 270. Residues between positions 166 and 235 are likely involved in bending, as derivative RORα1ΔC166 induces a slightly smaller bend than does RORα1ΔC235*.

To determine if the DNA bend centers also changed when these residues were deleted, the relative mobilities of the complexes were plotted versus the position of the DNA molecular end. The results for studies with derivatives RORα1ΔC180-270 and RORα1ΔC235* using a 5% gel are plotted in Fig. 2D. For both of these derivatives, the bend center is towards the 3′ AGGTCA half of the binding site, as seen for the intact RORα1 protein-DNA complex. The differences in the mobilities of the complexes obtained on a 5% polyacrylamide gel were small for the RORα1ΔC157, RORα1ΔC166, and RORα1ΔC235* derivatives. We therefore used an 8% gel to obtain better resolution and therefore a better estimate of the DNA bend center. These results are plotted in Fig. 2E and show that the bend centers for each of these complexes are also towards the 3′ AGGTCA half of the RORE. Therefore, although the size of the DNA bend was reduced, the center of the DNA bend that was induced by the C-terminal deletion derivatives was similar to that for intact RORα1.

Deletion of the N terminus alters the DNA bend center. We have also studied DNA bending induced by derivatives of RORα1 and RORα2 which contain deletions within the unique NTDs of these receptors. As summarized in Fig. 2A, deletion of residues 23 to 71 of RORα1 and of residues 46 to 103 of RORα2 had little effect on the angle of the induced bend. However, as shown in Fig. 3A for RORα1, deletion of these residues caused a 5′ shift (on the top strand) in the bend center relative to that for intact RORα1, as evidenced by the comigration of the complexes formed with the *Eco*RI and *Bam*HI fragment substrates (Fig. 3A, lanes 3 and 4). This indicates that the center of the DNA bend induced by the RORα1ΔN23-71 protein is near the center of the RORE (Fig. 3B). Similar results were seen with RORα2ΔN46-103 (Fig. 3C). Thus, although differences between the N termini of RORα1 and RORα2 do not cause a drastic change in RORα-induced DNA bending, deletion of the N terminus of either receptor isoform alters the interaction of the receptor with the DNA (see below).

RORα1 and RORα2 bend the DNA towards the major groove at the center of the 3′ AGGTCA element. We have performed a phasing analysis (71) to determine the directions of the DNA bends that are induced by RORα1 and RORα2 and their derivatives. The results of the phasing analysis for RORα1 are shown in Fig. 4A. As previously observed (71), the unbound substrates show some variation in their mobilities which is believed to be due to the presence of a second, small sequence-directed bend. However, there are several differences in the patterns of mobilities for the complexes and unbound substrates, indicating that there is phasing between the RORα1-induced DNA bend and the oligo(dA) sequence-directed bend. Similar results were obtained with each deletion derivative. The results for RORα1ΔC166 are shown in Fig. 4B, in which the differences in the mobilities of the complexes are more apparent because of better resolution of the lower-molecular-weight complexes.

The mobility of each complex (or substrate) has been normalized to the average mobility of the complex (or substrate) for each experiment, and the ratio of the normalized mobility of the complex to the normalized mobility of the substrate has been plotted versus linker length in Fig. 4C to F. From the plot in Fig. 4C, it appears that for the RORα1 complexes the mobility ratio was a minimum when the linker length was 14 bp, indicating that the sequence-directed bend and the RORα1-induced bend are in-phase in this complex. Taking into consideration the standard deviations, there could be an


A
NTD
Zn
C
Hinge
LBD
Angle (+/- 5°)
Center
RORα1
1
523
130
AGGTCA
α1ΔC475*
133
AGGTCA
α1ΔC180-270
70
AGGTCA
α1ΔC235*
60
AGGTCA
α1ΔC166
54
AGGTCA
α1ΔC157
54
AGGTCA
α1ΔN23-71
130
Shifted 5'
RORα2
1
556
123
AGGTCA
α2ΔN46-103
126
Shifted 5'
B
RORα1ΔC157
RV Ea Ba RI Hi Bs Rs
1 2 3 4 5 6 7
C
RORα1ΔC180-270
RV Ea Ba RI Hi Bs Rs
1 2 3 4 5 6 7
D
Relative Mobility (μcomplex/μsubstrate)
0.35
0.45
0.55
0.65
0.75
0.85
0 100 200 300 400
RV Ea Ba RI Hi Bs Rs RV
Position (bp)
RORα1
RORα1ΔC180-270
RORα1ΔC235*
E
Relative Mobility (μcomplex/μsubstrate)
0.2
0.4
0.6
0.8
1.0
0 100 200 300 400
RV Ea Ba RI Hi Bs Rs RV
Position (bp)
RORα1
RORα1ΔC235*
RORα1ΔC166
RORα1ΔC157

error of ±1 bp in this estimate of the linker length at which the mobility ratio is a minimum. The sequence-directed bend is towards the major groove at its bend center (71). We have shown that the center of the RORα1-induced DNA bend is towards the 3′ AGGTCA half of the site. If it is assumed that the center of the RORα1-induced bend is at the middle of the 3′ AGGTCA element, then the separation of the two bend centers in the 14-bp linker substrate is 84 bp, or 8.0 helical turns (assuming 10.5 bp per turn). Since the two bends are in-phase and are separated by an integral number of turns, the RORα1-induced bend must occur in the same direction as the sequence-directed bend and is thus towards the major groove at the center of the 3′ AGGTCA element. The error in determining the direction of the DNA bend is equivalent to only ±34° of rotation for an error of ±1 bp in linker length. Since the zinc-binding motifs likely interact in the major groove of the 3′ AGGTCA element, as seen for the ER (52), the DNA appears to be bent around the RORα1 protein. Similar results were obtained for RORα1ΔN23-71, RORα2, and RORα2ΔN46-103 (Fig. 4C and D). For RORα1ΔC180-270 and RORα1ΔC235*, the mobility ratio appears to be a minimum when the linker length is between 14 and 16 bp (Fig. 4E), while for RORα1ΔC166 and RORα1ΔC157 (Fig. 4F) the mobility ratio is a minimum when the linker length is 16 bp. These results indicate that the plane of the DNA bends induced by these RORα deletion derivatives may be slightly different from that induced by intact RORα1.

N-terminal deletion of RORα1 alters protein-DNA contacts. The observation that the centers of the DNA bends induced by RORα1ΔN23-71 and RORα2ΔN46-103 were shifted relative to those produced by RORα1 and RORα2 suggests that these NTD deletion derivatives may contact DNA in a slightly different manner from the intact RORα1 and RORα2 proteins. To test this hypothesis, we have performed methylation interference experiments with RORα1 and RORα1ΔN23-71 to determine if there are any differences in the DNA contacts for these receptors (Fig. 5). Figure 5A shows the results and a summary of the interference data for RORα1. On the top strand, methylation of guanine residues at positions 2 and 3 interfered with RORα1 binding, while methylation of the adenine residue at position 1 enhanced RORα1 binding. On the bottom strand, methylation of the guanine residue at position 5 and the adenine residues at positions −7, −5, −3, 4, and 7 interfered with RORα1 binding.

The methylation interference data for RORα1ΔN23-71 are shown and summarized in Fig. 5B. On the top strand, methylation of guanines at positions 2 and 3 interfered with RORα1ΔN23-71 binding, while methylation of the adenine at position 1 enhanced binding. On the bottom strand, methylation of the guanine at position 5 and the adenines at positions −9, −7, −5, 4, and 7 interfered with binding of RORα1ΔN23-71.

The interference patterns observed for RORα1 and RORα1ΔN23-71 within the 3′ AGGTCA half of the RORE are similar; however, there are marked differences in the interference patterns observed in the 5′ AT-rich half of the RORE. RORα1 appears to contact adenine residues at positions −7, −5, and −3, while for RORα1ΔN23-71 the contact at −3 is missing and a new contact is present at −9. This indicates that for RORα1ΔN23-71, the contact positions in the 5′ AT-rich half of the RORE have been shifted upstream relative to those for RORα1, which is consistent with the observed change in the DNA bend center for the RORα1ΔN23-71–RORE complex.

DNA bending and protein-protein interactions play a role in transactivation. A large number of proteins have been shown to induce DNA bending. In the case of integration host factor (IHF), the major function of the protein appears to be to bend DNA (16, 17, 19). IHF does not appear to contact other proteins, but the IHF-induced DNA bend facilitates the interaction of other adjacent proteins. For transcription factors such as catabolite activator protein (CAP) and LEF-1, both protein-induced DNA bending and direct protein-protein interactions appear to be important for their function (2, 10, 49). To determine if there is a correlation between DNA bending and receptor function, we have assessed the abilities of various RORα1 deletion derivatives to activate transcription from the RORE. Plasmids containing the RORα1 derivatives in the expression vector pCMX were cotransfected into CV-1 cells with a luciferase reporter construct driven by the thymidine kinase promoter linked to three copies of the RORE (14). DNA binding by each RORα1 derivative was assessed, and all deletion derivatives bound DNA at a level comparable to that of intact RORα1, except that RORα1ΔC235* bound slightly better than intact RORα1, and RORα1ΔC75-140 (which has the DBD deleted) did not bind. As shown in Fig. 6, cotransfection of intact RORα1 led to an approximately 45-fold stimulation in luciferase activity compared with that of the control vector. In contrast, deletion of the DBD in derivative RORα1ΔC75-140 resulted in a total loss of transactivation. Deletion of the C-terminal 47 amino acids in RORα1ΔC475* caused a 27-fold decrease in activation compared with that of RORα1, which is due to the loss of a transactivation function (TAF) within the C terminus. The C-terminal domain is the only region of RORα1 that acts as a transcriptional activator when fused to a GAL4 DBD (58). Both RORα1ΔC235* and RORα1ΔC166 show a reduction in transactivation and induce a smaller DNA bend than does RORα1; however, it is difficult to assess the role of DNA bending, as the C-terminal TAF is also absent. The derivatives RORα1ΔC180-270 and RORα1ΔN23-71 each contain an intact C-terminal TAF but show an alteration in protein-induced DNA bending. In addition, these derivatives show a 3.5- or 3.0-fold decrease in transactivation, respectively. Thus, there appears to be some correlation between DNA bending and transcriptional activation; however, as seen for other transcription factors, protein-protein interactions (via the C-terminal TAF) are also important for transactivation.

FIG. 2. Circular permutation analysis of RORα deletion derivatives. (A) Deletion derivatives of RORα1 and RORα2 and summary of their DNA bending properties. The primary structures of intact RORα1 and RORα2 are depicted. The unique N-terminal regions (dotted and filled boxes) and the zinc-binding motifs, the carboxy-terminal extension, and the putative LBD (boxes Zn, C, and LBD, respectively) are indicated. Deletion derivatives are shown below the diagram of each intact protein. The DNA bend angles induced by binding of each protein derivative are indicated on the right. The positions of the DNA bend centers are shown (AGGTCA, complexes in which the bend center is towards this element; Shifted 5′, complexes in which the bend center is shifted 5′ relative to the 3′ AGGTCA element). (B and C) Results of circular permutation analysis on a 5% acrylamide gel of complexes formed with in vitro-translated RORα1ΔC157 and RORα1ΔC180-270, respectively. For substrate abbreviations, see Fig. 1 legend. The relative mobilities of complexes formed with RORα1 or RORα1 C-terminal deletion derivatives are plotted as a function of the DNA molecular end for each substrate as described for Fig. 1D. (D and E) Circular permutation data for the indicated receptors on 5% and 8% polyacrylamide gels, respectively.

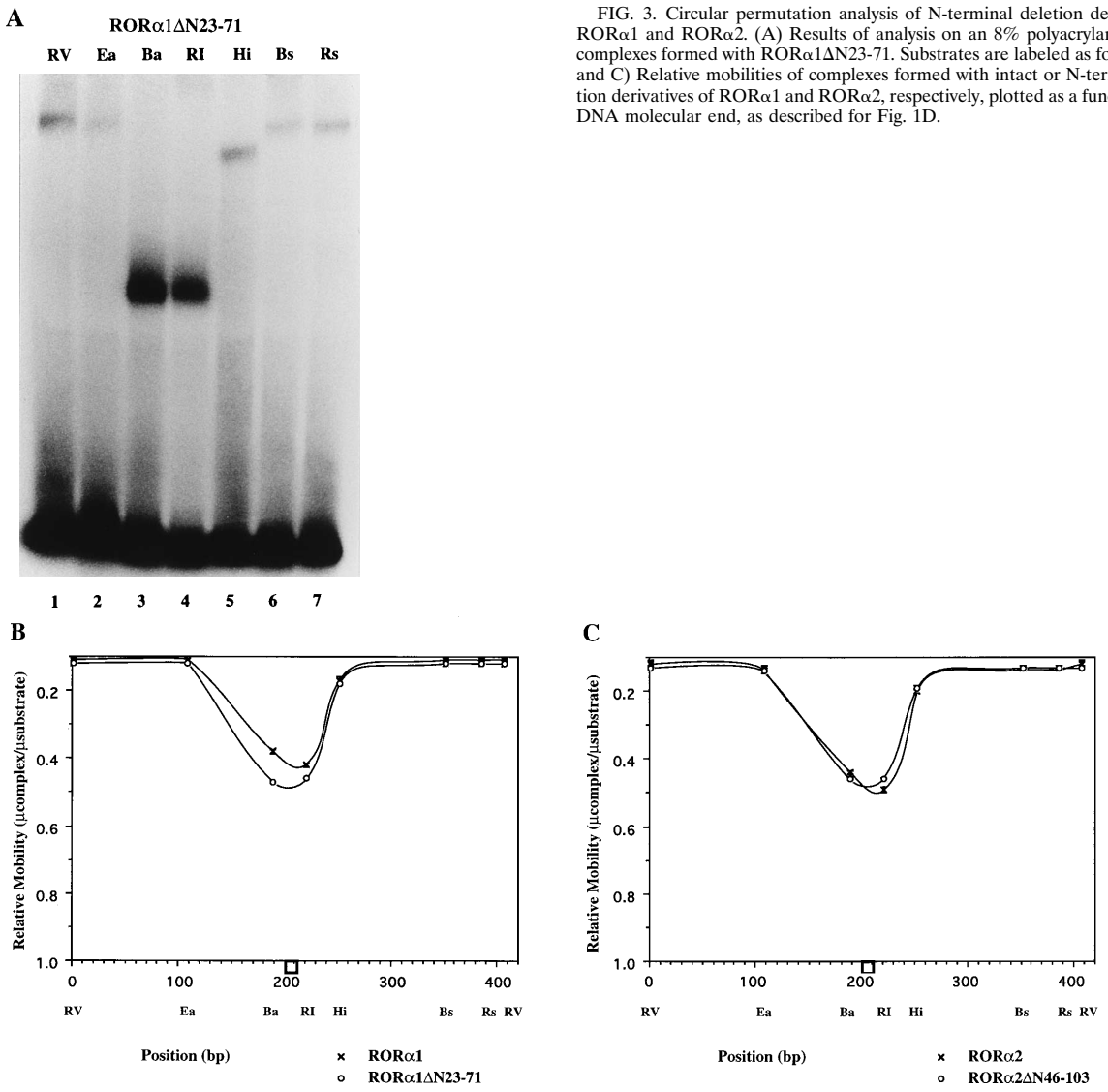


FIG. 3. Circular permutation analysis of N-terminal deletion derivatives of RORα1 and RORα2. (A) Results of analysis on an 8% polyacrylamide gel of complexes formed with RORα1ΔN23-71. Substrates are labeled as for Fig. 1. (B and C) Relative mobilities of complexes formed with intact or N-terminal deletion derivatives of RORα1 and RORα2, respectively, plotted as a function of the DNA molecular end, as described for Fig. 1D.

DISCUSSION

RORα is a novel subfamily of orphan nuclear receptor isoforms characterized by the unique ability of its distinct NTDs to modulate the DNA binding properties of each protein (14). In this report, we demonstrated by both circular permutation and phasing analysis that RORα isoforms induce large DNA bends at the RORE which may be important for receptor function. The presence of the nonconserved hinge region is required for maximal DNA bending, whereas the deletion of the NTD does not significantly change the DNA bend angle but shifts the DNA bend center 5′ relative to the bend induced by intact protein. Methylation interference studies using an NTD-deleted receptor indicate that some DNA contacts in the 5′ AT-rich moieties of the RORE are also shifted 5′. The results of these experiments allowed us to put forward a model in which the RORα NTD and nonconserved hinge region properly align the zinc-binding motifs and the carboxy-terminal extension of the DBD with respect to each other.

The DBD of RORα contains two type II zinc-binding motifs which are highly conserved among nuclear receptor proteins (8). The cocrystal structure of the ER DBD with its response element (ERE) shows that the analogous zinc-binding domain of ER contacts the DNA within the major groove at the center of the 3′ AGGTCA element (52). Presumably, the zinc-binding domain of RORα also makes major groove contacts at the 3′ AGGTCA half-site. The results of methylation interference studies with intact RORα1 are consistent with this assumption. It was seen that methylation of three guanines within the major groove (top strand positions 2 and 3 and bottom strand position 5) interfered with RORα1 binding. The analogous positions within the ERE are each contacted by the ER DBD (60).

Several minor groove contacts are made by RORα1 within the 5′ AT-rich half of the RORE (bottom strand positions −7, −5, and −3). Similar results have been observed for the monomeric binding receptor NGFI-B, in which a region C terminal to the second zinc-binding motif interacts with the minor groove of the DNA 5′ to the AGGTCA element in its binding site (65). We have recently shown that a region C terminal to the second zinc-binding motif of RORα1 is required for interaction with the minor groove of the 5′ AT-rich half of the RORE (12). The above results are consistent with a model for RORα-RORE interaction in which the RORα protein is ori-

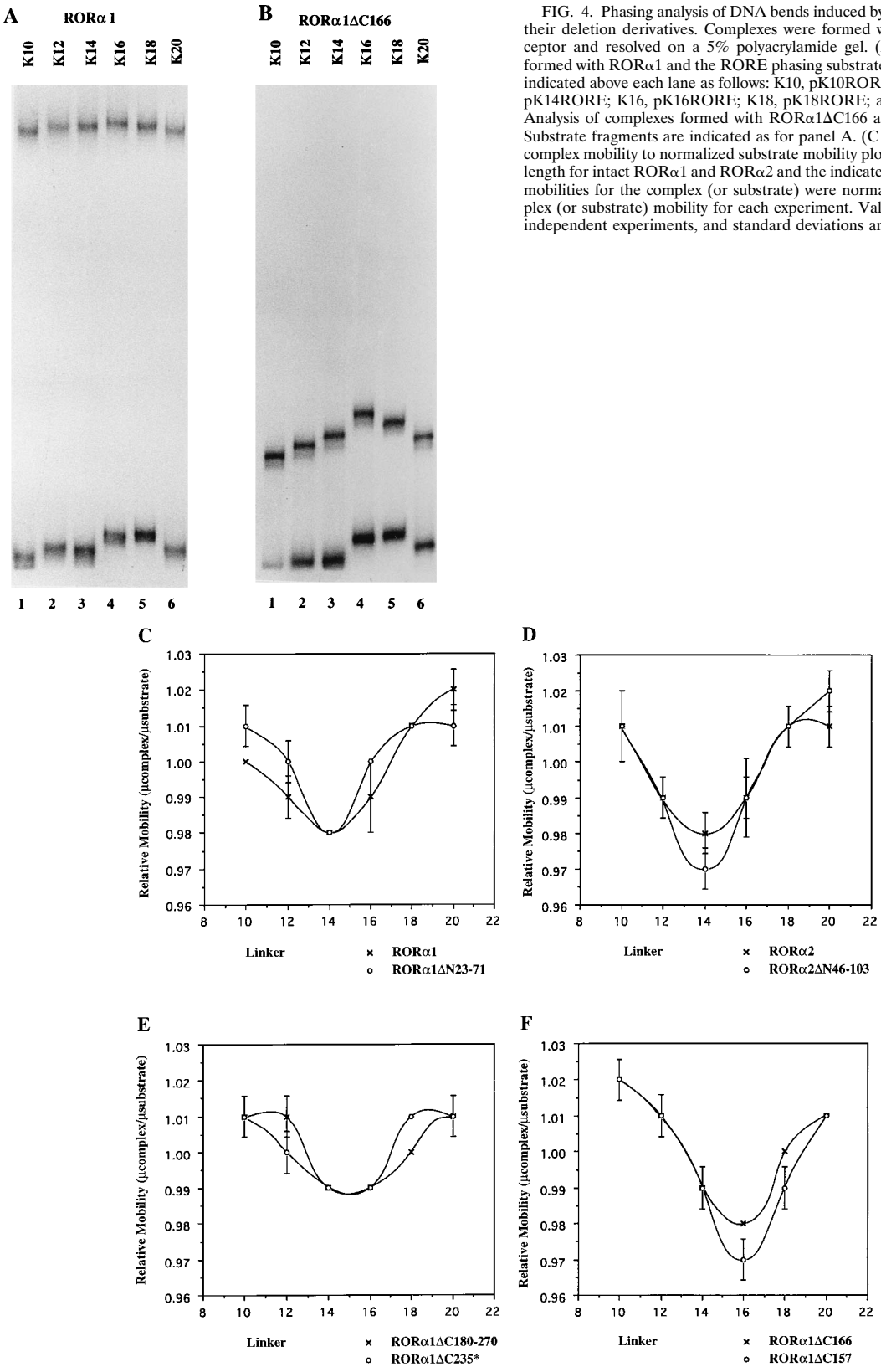


FIG. 4. Phasing analysis of DNA bends induced by RORα1 and RORα2 and their deletion derivatives. Complexes were formed with in vitro-translated receptor and resolved on a 5% polyacrylamide gel. (A) Analysis of complexes formed with RORα1 and the RORE phasing substrates. Substrate fragments are indicated above each lane as follows: K10, pK10RORE; K12, pK12RORE; K14, pK14RORE; K16, pK16RORE; K18, pK18RORE; and K20, pK20RORE. (B) Analysis of complexes formed with RORα1ΔC166 and the phasing substrates. Substrate fragments are indicated as for panel A. (C to F) Ratio of normalized complex mobility to normalized substrate mobility plotted as a function of linker length for intact RORα1 and RORα2 and the indicated deletion derivatives. The mobilities for the complex (or substrate) were normalized to the average complex (or substrate) mobility for each experiment. Values are averages for three independent experiments, and standard deviations are indicated.

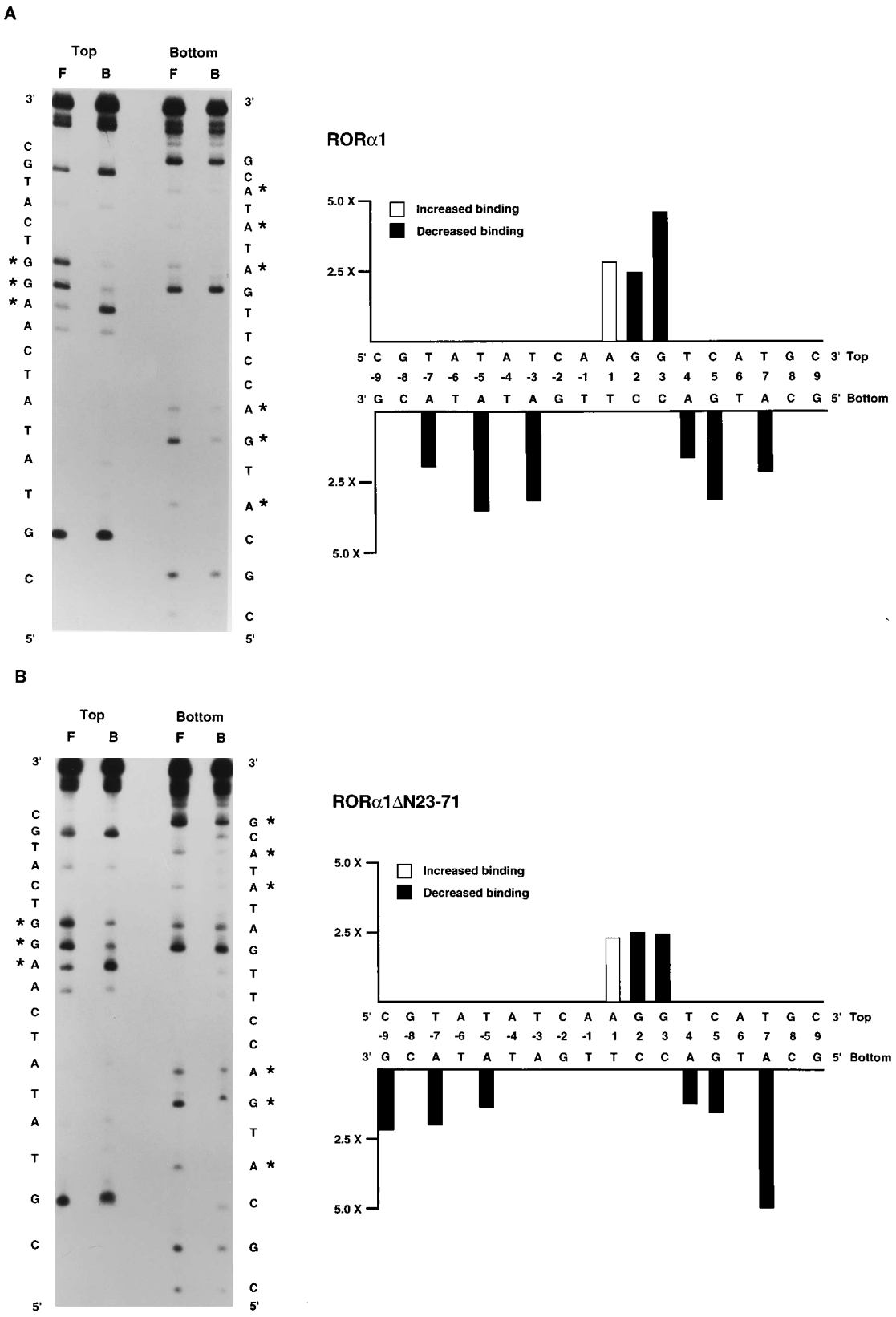


FIG. 5. Methylation interference with RORα1-RORE and RORα1ΔN23-71–RORE complex formation. (A) The interference pattern for RORα1 with the top and bottom strands of the RORE is shown on the left (F and B, free and bound DNA, respectively). The DNA sequence is indicated on the sides; asterisks indicate residues whose methylation alters DNA binding by RORα1. The interference data are summarized on the right. The fold decrease or increase in binding as determined by quantitation with a phosphorimager is indicated. (B) The interference pattern for RORα1ΔN23-71 with the top and bottom strands of the RORE is shown on the left and is labeled as for panel A. Interference data are summarized on the right.

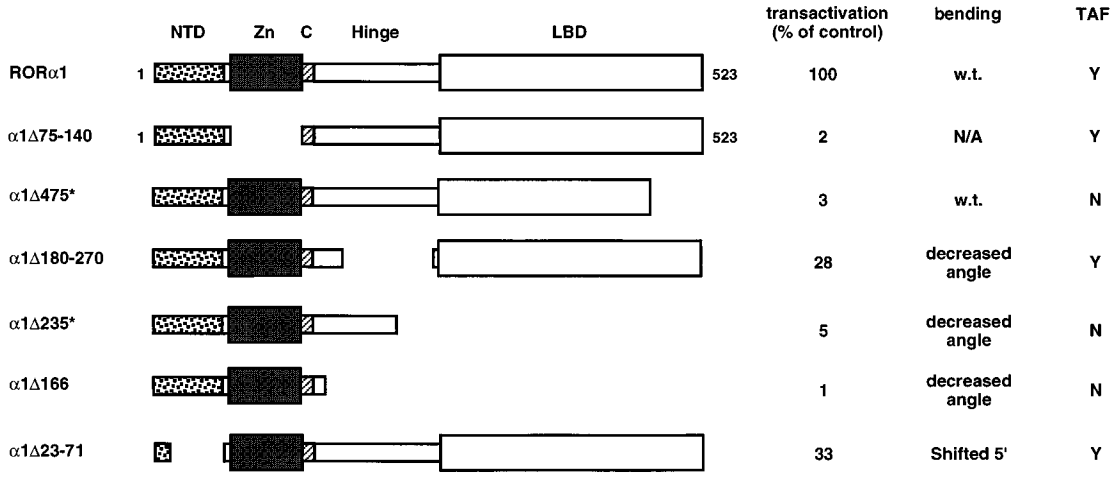


FIG. 6. Transcriptional activation by RORα1 deletion derivatives. The derivatives are illustrated and labeled as in Fig. 2. CV-1 cells were transfected with 2 μg of $RORE_3$TKLUC reporter and 250 ng of pCMX (negative control) or pCMX containing an RORα1 derivative and were harvested after 36 h. Luciferase activity is reported as a percentage of the induction obtained with intact RORα1, which is 47-fold greater than that obtained with pCMX. The DNA bending properties of each RORα1 derivative and the presence or absence of the C-terminal TAF are indicated. w.t., wild type; Y, yes; N, no; N/A, not applicable.

ented mainly along one face of the DNA helix so that the zinc-binding motifs interact with the major groove of the 3′ AGGTCA element and the carboxy-terminal extension interacts with the adjacent minor groove of the 5′ AT-rich moieties of the RORE (Fig. 7). The DNA appears to be bent around the RORα protein, since the RORα-induced DNA bend is towards the major groove at the center of the 3′ AGGTCA element.

Analysis of the DNA bending properties of several C-terminal deletion derivatives of RORα1 indicated that a region C terminal to the DBD is required for maximal DNA bending. This region appears to be localized to the nonconserved hinge region, as the derivative RORα1ΔC180-270, which has most of this region removed, shows a marked reduction in the size of the receptor-induced DNA bend. The position of the DNA bend center for each C-terminal deletion derivative was not altered, suggesting that the zinc-binding motifs and the carboxy-terminal extension contact the DNA at the same positions as the intact RORα1 protein. The above results lead us to propose a model in which the hinge region of the RORα receptor plays a structural role in orienting the class II zinc-binding motifs and the carboxy-terminal extension at the proper angle with respect to one another (Fig. 7A). When the hinge region is removed, the halves of the bipartite DBD still contact the proper DNA sequences, but they are no longer held at the proper angle with respect to each other, so the bend angle decreases and the overall plane of the bend changes slightly (Fig. 7B). To our knowledge, this is the first function ascribed to the hinge region of a nuclear hormone receptor. It will be interesting to determine if the hinge region plays a similar role in other monomeric binding receptors. Although there is no homology within the hinge regions of the nuclear receptors, it is interesting that this region appears to be longer among monomeric binding receptors, such as RORα (14), RVR (45), and RevErbAα (30), than in receptors such as RARα (13) and RXRα (36), which bind as dimers. This suggests that a large hinge region may be of functional importance

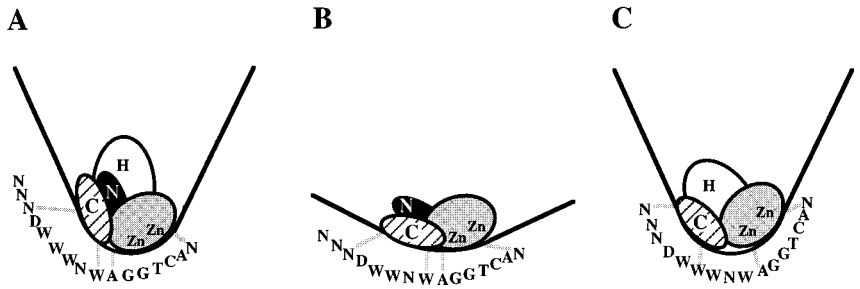


FIG. 7. Model for RORα-induced DNA bending. (A) DNA bending induced by the intact RORα receptor is diagrammed. The zinc-binding motifs (Zn) contact the 3′ AGGTCA element in the major groove, and the carboxy-terminal extension (C) contacts the AT-rich region in the adjacent minor groove. The carboxy-terminal extension and the zinc-binding motifs are held at a sharp angle relative to one another by the action of the hinge region (H) and the NTD (N). As a result, a large DNA bend (~130°) is induced by RORα. The domains of the protein are represented by the ovals. The LBD has been omitted for simplicity. DNA is represented by the black line, and the RORE sequence is shown below. Gray lines indicate the regions that are believed to be contacted by each DNA-binding element. (B) DNA bending by RORα derivatives that lack the hinge region. The carboxy-terminal extension and the zinc-binding motifs contact the same DNA elements as shown in panel A; however, the angle between the two binding elements is considerably decreased because of the absence of the hinge region. (C) DNA bending by RORα derivatives that lack the N terminus. The zinc-binding motifs still contact the 3′ AGGTCA element as in panel A. However, in the absence of the N terminus, the orientation of the zinc-binding motifs and the carboxy-terminal extension relative to each other has changed slightly. As a result, the carboxy-terminal extension no longer contacts the DNA properly so that its contacts are shifted 5′ in the AT-rich half of the RORE. Position −3 is no longer contacted, and a new contact is made at position −9. A large DNA bend is still made, but the center is shifted 5′ relative to that for the intact protein.

in monomeric binding receptors which contain two DNA-binding determinants within a single protein.

The DNA bends induced by the RORα1 and RORα2 isoforms were of similar sizes (130° and 123° ± 5°, respectively) and were each oriented towards the major groove at the center of the 3′ AGGTCA element. Thus, although differences in the NTD of the two isoforms alter the DNA binding specificities of RORα1 and RORα (14), they have little effect on the degree and direction of the induced DNA bends. We have also analyzed the DNA bending properties of N-terminal deletion derivatives of both RORα1 and RORα2. In both cases, no significant difference was seen in the induced DNA bend angles or in the plane of the DNA bend. However, the center of the DNA bend was shifted 5′ for both the RORα1ΔN23-71 and the RORα2ΔN46-103 proteins compared with the bend induced by the intact receptors. Methylation interference results indicated that the contacts made by RORα1ΔN23-71 are shifted 5′ in the AT-rich half of the RORE but are unaltered in the 3′ AGGTCA half. These results are consistent with a model in which the NTD plays a role in fine-tuning the orientation of the zinc-binding motifs and the carboxy-terminal extension with respect to one another (Fig. 7A and C). The NTD may interact with the hinge region (or directly with the carboxy-terminal extension) to properly align the halves of the bipartite DBD (Fig. 7A). When the NTD is absent, the hinge region holds the carboxy-terminal extension and the zinc-binding motifs at a slightly different angle relative to each other than in the wild-type protein. As a result, the carboxy-terminal extension no longer contacts the DNA properly, so that a base contact at position −3 is lost and a new contact is made at position −9. The end result is a complex with a large bend that is centered 5′ of the normal position. Differences in the intact NTDs of RORα1 and RORα2 may cause slight structural changes which fine-tune the interaction of the carboxy-terminal extension with the 5′ AT-rich sequences, resulting in the observed different binding specificities of the RORα isoforms (14).

Other nuclear receptors which normally bind as homodimers or heterodimers have previously been shown to bend DNA. The bend angles induced by these receptors appear to be smaller than those observed for RORα: ER, ~50° (49); ER DBD, ~34° (41); thyroid hormone receptor-RXR, ~65 to 75° (22, 34); RXR-RXR, ~92°; and RXR-RAR, 57 to 63° (34). Although no function for DNA bending by nuclear receptors has been directly demonstrated, a correlation between transcriptional activation by the thyroid hormone receptor and the degree of DNA bending induced by the receptor has been made (33). Although DNA bending may be important for transcriptional activation by hormone receptors, it is likely that protein-protein interaction with the basal transcription machinery or other transcription factors is also required for transcriptional activation. We have observed this for RORα1, in which deletion of the C terminus causes a loss of transcriptional activation which is due to the loss of an activation domain. However, there also appears to be some correlation between protein-induced DNA bending and transcriptional activation, since deletion derivatives which contain an intact C-terminal TAF but show altered DNA bending also show a decrease (~3.5-fold) in transactivation compared with that of the intact protein. These results are in keeping with those of studies of several other transcription factors which bend DNA. For example, in certain contexts, DNA bending seems to be a major function of CAP (2), yet additional evidence indicates that CAP functions by interacting with the α subunit of RNA polymerase (48). Similarly, both protein-induced DNA bending and protein-protein interactions with other transcription factors are believed to be important for the function of the transcription factor LEF-1 (10). The relative importance of the activation domain and of DNA bending for LEF-1 function appears to be similar to what we observe for RORα1.

For monomeric DNA-binding proteins such as the RORα isoforms, DNA bending may be essential to promote full contact between the bipartite DBD of the proteins and their recognition site. As has been proposed for a number of DNA-bending proteins, receptor-induced DNA bending could also be involved in the activity of these proteins by facilitating the interaction of the receptors with the basal transcription machinery. In some contexts, hormone receptors act cooperatively with other transcription factors (3, 6, 46, 51), and protein-protein interactions may be important in such cases (3, 51). It is possible that the role of receptor-induced DNA bending could be more pronounced in such contexts, as DNA bending may facilitate protein-protein interactions with the adjacent transcription factors. In the case of receptors which bind as dimers, receptor-induced DNA bending could also be required to facilitate dimerization interactions. In support of this suggestion, it has recently been demonstrated that the DNA-bending protein HMG-1 enhanced DNA binding by the progesterone receptor (43).

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REFERENCES

1. **Baur, C.-P., and R. Knippers.** 1988. Protein-induced bending of the simian virus 40 origin of replication. J. Mol. Biol. **203:**1009–1019.
2. **Bracco, L., D. Kotlarz, A. Kolb, S. Diekmann, and H. Buc.** 1989. Synthetic curved DNA sequences can act as transcriptional activators in *Escherichia coli*. EMBO J. **8:**4289–4296.
3. **Bradshaw, M. S., S. Y. Tsai, X. Leng, A. D. W. Dobson, O. M. Conneely, B. W. O'Malley, and M.-J. Tsai.** 1991. Studies on the mechanism of functional cooperativity between progesterone and estrogen receptors. J. Biol. Chem. **266:**16684–16690.
4. **Bugge, T. H., J. Pohl, O. Lonnoy, and H. G. Stunnenberg.** 1992. RXRα, a promiscuous partner of retinoic acid and thyroid hormone receptors. EMBO J. **11:**1409–1418.
5. **Drouin, J., Y. L. Sun, S. Tremblay, P. Lavender, T. J. Schmidt, A. de Léan, and M. Nemer.** 1992. Homodimer formation is rate-limiting for high affinity DNA binding by glucocorticoid receptor. Mol. Endocrinol. **6:**1299–1309.
6. **Espinás, M. L., J. Roux, J. Ghysdael, R. Pictet, and T. Grange.** 1994. Participation of Ets transcription factors in the glucocorticoid response of the rat tyrosine aminotransferase gene. Mol. Cell. Biol. **14:**4116–4126.
7. **Evans, R. M.** 1988. The steroid and thyroid hormone receptor superfamily. Science **240:**889–895.
8. **Freedman, L. P.** 1992. Anatomy of the steroid receptor zinc finger region. Endocr. Rev. **13:**129–145.
9. **Giese, K., J. Cox, and R. Grosschedl.** 1992. The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. Cell **69:**185–195.
10. **Giese, K., and R. Grosschedl.** 1993. LEF-1 contains an activation domain that stimulates transcription only in a specific context of factor-binding sites. EMBO J. **12:**4667–4676.
11. **Giguère, V., S. H. Hollenberg, M. G. Rosenfeld, and R. M. Evans.** 1986. Functional domains of the human glucocorticoid receptor. Cell **46:**645–652.
12. **Giguère, V., L. McBroom, and G. Flock.** 1994. Unpublished data.
13. **Giguère, V., S. E. Ong, P. Segui, and R. M. Evans.** 1987. Identification of a receptor for the morphogen retinoic acid. Nature (London) **330:**624–629.
14. **Giguère, V., M. Tini, G. Flock, E. S. Ong, R. M. Evans, and G. Otulakowski.** 1994. Isoform-specific amino-terminal domains dictate DNA-binding properties of RORα, a novel family of orphan nuclear receptors. Genes Dev. **8:**538–553.

15. **Gilson, E., M. Roberge, R. Giraldo, D. Rhodes, and S. M. Gasser.** 1993. Distortion of the DNA double helix by RAP1 at silencers and multiple telomeric binding sites. J. Mol. Biol. **231:**293–310.
16. **Gober, J. W., and L. Shapiro.** 1990. Integration host factor is required for the activation of developmentally regulated genes in *Caulobacter*. Genes Dev. **4:**1494–1504.
17. **Goodman, S. D., and H. A. Nash.** 1989. Functional replacement of a protein-induced bend in a DNA recombination site. Nature (London) **341:**251–254.
18. **Harding, H. P., and M. A. Lazar.** 1993. The orphan receptor Rev-ErbAα activates transcription via a novel response element. Mol. Cell. Biol. **13:** 3113–3121.
19. **Hoover, T. R., E. Santero, S. Porter, and S. Kustu.** 1990. The integration host factor stimulates interaction of RNA polymerase with NIFA, the transcriptional activator for nitrogen fixation operons. Cell **63:**11–22.
20. **Kerppola, T. K., and T. Curran.** 1991. DNA bending by Fos and Jun: the flexible hinge model. Science **254:**1210–1214.
21. **Kerppola, T. K., and T. Curran.** 1991. Fos-Jun heterodimers and Jun homodimers bend DNA in opposite orientations: implications for transcription factor cooperativity. Cell **66:**317–326.
22. **King, I. N., T. se Soyza, D. F. Catanzaro, and T. N. Lavin.** 1993. Thyroid hormone receptor-induced bending of specific sequences is modified by an accessory factor. J. Biol. Chem. **268:**495–501.
23. **Kliewer, S. A., K. Umesono, D. J. Mangelsdorf, and R. M. Evans.** 1992. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. Nature (London) **335:**446–449.
24. **Klock, G., U. Strähle, and G. Schütz.** 1987. Oestrogen and glucocorticoid response elements are closely related but distinct. Nature (London) **329:** 734–736.
25. **Knegtel, R. M. A., M. Katahira, J. G. Schilthuis, A. M. J. J. Bonvin, R. Boelens, D. Eib, P. T. van der Saag, and R. Kaptein.** 1993. The solution structure of the human retinoic acid receptor-β DNA-binding domain. J. Biol. NMR **3:**1–17.
26. **Krust, A., S. Green, P. Argos, V. Kumar, P. Walter, J.-M. Bornert, and P. Chambon.** 1986. The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. EMBO J. **5:**891–897.
27. **Kumar, V., and P. Chambon.** 1988. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell **55:**145–156.
28. **Laudet, V., C. Hänni, J. Coli, F. Catzeflis, and D. Stéhelin.** 1992. Evolution of the nuclear receptor gene superfamily. EMBO J. **11:**1003–1013.
29. **Lavorgna, G., H. Ueda, J. Clos, and C. Wu.** 1991. FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of *fushi tarazu*. Science **252:**848–851.
30. **Lazar, M. A., R. A. Hodin, D. S. Darling, and W. W. Chin.** 1989. A novel member of the thyroid/steroid hormone receptor family is encoded by the opposite strand of the rat c-erbAα transcriptional unit. Mol. Cell. Biol. **9:**1128–1136.
31. **Lee, M. S., S. A. Kliewer, J. Provençal, P. E. Wright, and R. M. Evans.** 1993. Solution structure of the RXR α DNA-binding domain: a helix required for homodimeric DNA binding. Science **260:**1117–1121.
32. **Leid, M., P. Kastner, R. Lyons, H. Nakshari, M. Saunders, T. Zacharewski, J.-Y. Chen, A. Staub, J.-M. Garnier, S. Mader, and P. Chambon.** 1992. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell **68:** 377–395.
33. **Leidig, F., A. R. Shepard, W. Zhang, A. Stelter, P. A. Cattini, J. D. Baxter, and N. L. Eberhardt.** 1992. Thyroid hormone responsiveness in human growth hormone-related genes. J. Biol. Chem. **267:**913–921.
34. **Lu, X. P., N. L. Eberhardt, and M. Pfahl.** 1993. DNA bending by retinoid X receptor-containing retinoid and thyroid hormone receptor complexes. Mol. Cell. Biol. **13:**6509–6519.
35. **Luisi, B. F., W. X. Xu, Z. Otwinowski, L. P. Freedman, K. R. Yamamoto, and P. Sigler.** 1991. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature (London) **352:**497–505.
36. **Mangelsdorf, D. J., E. S. Ong, J. A. Dyck, and R. M. Evans.** 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. Nature (London) **345:**224–229.
37. **Marks, M. S., P. L. Hallenbeck, T. Nagata, J. H. Segars, E. Appella, V. M. Nikodem, and K. Ozato.** 1992. H-2RIIBP (RXRβ) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. EMBO J. **11:**1419–1435.
38. **Martinez, E., F. Givel, and W. Wahli.** 1987. The estrogen-responsive element as an inducible enhancer: DNA sequence requirements and conversion to a glucocorticoid-responsive element. EMBO J. **6:**3719–3727.
39. **McBroom, L. D. B., and P. D. Sadowski.** 1994. DNA bending by Saccharomyces cerevisiae ABF1 and its proteolytic fragments. J. Biol. Chem. **269:** 16461–16468.
40. **Näär, A. M., J. M. Boutin, S. M. Lipkin, V. C. Yu, J. M. Holloway, C. K. Glass, and M. G. Rosenfeld.** 1991. The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. Cell **65:**1267–1279.
41. **Nardulli, A. M., and D. J. Shapiro.** 1992. Binding of the estrogen receptor DNA-binding domain to the estrogen response element induces DNA bending. Mol. Cell. Biol. **12:**2037–2042.
42. **Natesan, S., and M. Z. Gilman.** 1993. DNA bending and orientation-dependent function of YY1 in the c-fos promoter. Genes Dev. **7:**2497–2509.
43. **Oñate, S. A., P. Prendergast, J. P. Wagner, M. Nissen, R. Reeves, D. E. Pettijohn, and D. P. Edwards.** 1994. The DNA-bending protein HMG-1 enhances progesterone receptor binding to its target DNA sequences. Mol. Cell. Biol. **14:**3376–3391.
44. **Pérez-Martín, J., and M. Espinosa.** 1993. Protein-induced bending as a transcriptional switch. Science **260:**805–807.
45. **Retnakaran, R., G. Flock, and V. Giguère.** 1994. Identification of RVR, a novel orphan nuclear receptor that acts as a negative transcriptional regulator. Mol. Endocrinol. **8:**1234–1244.
46. **Rhodes, S. J., R. Chen, G. E. DiMatta, K. M. Scully, K. A. Kalla, S.-C. Lin, V. C. Yu, and M. G. Rosenfeld.** 1994. A tissue-specific enhancer confers Pit-1-dependent morphogen inducibility and autoregulation on the *pit-1* gene. Genes Dev. **7:**913–932.
47. **Rojo, F., A. Zaballos, and M. Salas.** 1990. Bend induced by the phage Φ29 transcriptional activator in the viral late promoter is required for activation. J. Mol. Biol. **211:**713–725.
48. **Russo, F. D., and T. J. Silhavy.** 1992. Alpha: the cinderella subunit of RNA polymerase. J. Biol. Chem. **267:**14515–14518.
49. **Sabbah, M., S. Le Ricousse, G. Redeuilh, and E.-E. Baulieu.** 1992. Estrogen receptor-induced bending of Xenopus vitellogenin A2 gene hormone response element. Biochem. Biophys. Res. Commun. **185:**944–952.
50. **Salvo, J. J., and N. D. F. Grindley.** 1988. The γδ resolvase bends the *res* site into a recombinogenic complex. EMBO J. **7:**3609–3616.
51. **Schüle, R., M. Muller, H. Otsuka-Murakami, and R. Renkawitz.** 1988. Cooperativity of the glucocorticoid receptor and the CACCC-box binding factor. Nature (London) **332:**8790.
52. **Schwabe, J. W. R., L. Chapman, J. T. Finch, and D. Rhodes.** 1993. The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell **75:**567–578.
53. **Schwartz, C. J. E., and P. D. Sadowski.** 1989. FLP recombinase of the 2 μm circle plasmid of Saccharomyces cerevisiae bends its DNA target. Isolation of FLP mutants defective in DNA bending. J. Mol. Biol. **205:**647–658.
54. **Siebenlist, U., and W. Gilbert.** 1980. Contacts between *Escherichia coli* RNA polymerase and an early promoter of phage T7. Proc. Natl. Acad. Sci. USA **77:**122–126.
55. **Stenzel, T. T., T. MacAllister, and D. Bastia.** 1991. Cooperativity at a distance promoted by the combined action of two replication initiator proteins and a DNA bending protein at the replication origin of pSC101. Genes Dev. **5:**1453–1463.
56. **Thompson, J. F., and A. Landy.** 1988. Empirical estimation of protein-induced DNA bending angles: applications to λ site-specific recombination complexes. Nucleic Acids Res. **16:**9687–9705.
57. **Tini, M., G. Otulakowski, M. L. Breitman, L.-T. Tsui, and V. Giguère.** 1993. An everted repeat mediates retinoic acid induction of the γF-crystallin gene: evidence of a direct role for retinoids in lens development. Genes Dev. **7:**295–307.
58. **Torchia, J., and V. Giguère.** Unpublished observations.
59. **Truss, M., and M. Beato.** 1993. Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. Endocr. Rev. **14:**459–479.
60. **Truss, M., G. Chalepakis, E. P. Slater, S. Mader, and M. Beato.** 1991. Functional interaction of hybrid response elements with wild-type and mutant steroid hormone receptors. Mol. Cell. Biol. **11:**3247–3258.
61. **Tsukiyama, T., H. Ueda, S. Hirose, and O. Niwa.** 1992. Embryonal long terminal repeat-binding protein is a murine homolog of FTZ-F1, a member of the steroid receptor superfamily. Mol. Cell. Biol. **12:**1286–1291.
62. **Ueda, H., G.-C. Sun, T. Murata, and S. Hirose.** 1992. A novel DNA-binding motif abuts the zinc fingers domain of insect nuclear hormone receptor FTZ-F1 and mouse embryonal long terminal repeat-binding protein. Mol. Cell. Biol. **12:**5667–5672.
63. **Umesono, K., K. K. Murakami, C. C. Thompson, and R. M. Evans.** 1991. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. Cell **65:**1255–1266.
64. **Williams, J. S., T. T. Eckdahl, and J. N. Anderson.** 1988. Bent DNA functions as a replication enhancer in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8:**2763–2769.
65. **Wilson, T. E., T. J. Fahrner, M. Johnson, and J. Milbrandt.** 1991. Identification of the DNA binding site for NGFI-B by genetic selection in yeast. Science **252:**1296–1300.
66. **Wilson, T. E., T. J. Fahrner, and J. Millbrandt.** 1993. The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. Mol. Cell. Biol. **13:**5794–5804.
67. **Wilson, T. E., R. E. Paulsen, K. A. Padgett, and J. Milbrandt.** 1992. Participation of non-zinc finger residues in DNA binding by two nuclear

orphan receptors. Science **256:**107–110.
68. **Wu, H.-M., and D. M. Crothers.** 1984. The locus of sequence-directed and protein-induced DNA bending. Nature (London) **308:**509–513.
69. **Yu, V. C., C. Delsert, B. Andersen, J. M. Holloway, O. V. Devary, A. M. Näär, S. Y. Kim, J. M. Boutin, C. K. Glass, and M. G. Rosenfeld.** 1991. RXRβ: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell **67:**1251–1266.
70. **Zhang, X.-K., B. Hoffmann, P. B.-V. Tran, G. Graupner, and M. Pfahl.** 1992. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. Nature (London) **335:**441–446.
71. **Zinkel, S. S., and D. M. Crothers.** 1987. DNA bend direction by phase sensitive detection. Nature (London) **328:**178–181.