Nucleic Acids Research, 1995, Vol. 23, No. 5 811–818

DNA bending by thyroid hormone receptor: influence of half-site spacing and RXR

Korina Shulemovich, Dwight D. Dimaculangan, Deborah Katz and Mitchell A. Lazar*

Division of Endocrinology, Diabetes and Metabolism, Departments of Medicine and Genetics, University of Pennsylvania School of Medicine, 611 CRB, 415 Curie Boulevard, Philadelphia, PA 19104-6149, USA

Received November 4, 1994; Revised and Accepted January 17, 1995

ABSTRACT

Transcriptional activation by thyroid hormone (T3) requires interactions between the T3 receptor (TR) and T3 response elements (TREs) composed of two copies of sequences related to AGGTCA. Direct repeats of this sequence are a functional TRE when spaced by 4 but not by 5 bp (DR4 versus DR5). TR bound as monomers, homodimers and heterodimers with retinoid X receptor (RXR) to both DR4 and DR5, with an ~10-fold greater affinity for DR4 due to reduced dissociation of the protein–DNA complex. We explored DNA bending as an additional variable which could influence the transcriptional outcome of the TR–TRE interaction. Circular permutation indicated a large distortion of the DNA following TR binding, but phasing analysis strongly suggested that this was due only in small part to DNA bending. Phasing analysis indicated that both TR/RXR and TR homodimer induced bends of ~10° in DR4, but caused little bending of DR5. Moreover, the TR homo- and heterodimers bent DR4 in opposite directions. These results indicate that in addition to regulating the affinity and spacing requirement for DNA binding by TR, the TR dimer partner may also modulate transcription by influencing the direction of the bending induced by TR binding to DNA, although this effect may be subtle, due to the modest degree of bending.

INTRODUCTION

Thyroid hormone (T3) plays a major role in vertebrate development and metabolism (1). It acts via thyroid hormone receptors (TRs), nuclear proteins which regulate gene transcription by binding to specific DNA sequences known as T3 response elements (TREs) (reviewed in 2). TREs contain repeats of the sequence AGGTCA, which is also recognized by related receptors, such as retinoic acid receptor (RAR) and vitamin D receptor (VDR) (3–5). Specificity of hormone action is at least in part dictated by a '1–2–3–4–5 rule', which states that for AGGTCA half-sites arranged as direct repeats (DRs), a 4 bp spacer (DR4) defines a TRE, while the same half-sites with a 1 bp spacer are an element responsive to RXR or peroxisome proliferator-activated receptor, a 3 bp spacer defines a VDR response element and a 2 or 5 bp spacer defines an RAR response element (RARE) (6–8).

TRs bind to TREs as monomers, homodimers or heterodimers with retinoid X receptors (RXRs) (8–14) The importance of TR/RXR heterodimers is widely acknowledged, largely on the basis of the ability of TR and RXR to interact in solution and the clearly cooperative nature of their TRE binding. The TR/RXR heterodimer preferentially binds to DR4, due to an interaction between the first zinc finger of TR and the second zinc finger of RXR which is favored by this spacing (15,16). The role of the TR homodimer is less well agreed upon, because of the weak interaction between TRs in solution, as well as a lack of cooperative binding to many TREs (6,10,11,17,18). However, TR homodimer binding is cooperative in some instances (16,19–21). Furthermore, T3 destabilizes the binding of TR homodimers to a subset of TREs, which strongly suggests that the homodimer is a facilitated interaction between two TR monomers (22–25).

In transient transfection assays of transcription DR4 was a strong TRE, whereas DR5 was inactive. TR homo- and heterodimers bound to both DR4 and DR5 *in vitro*, although TR/RXR heterodimers bound DR5, but with about 10-fold lower affinity than DR4. We therefore wondered whether factors other than DNA binding affinity might contribute to TRE specificity. One potential factor is DNA bending, which is recognized as an important regulator of procaryotic gene transcription (26–30). A variable degree of DNA bending is also associated with DNA binding by eucaryotic transcription factors, such as Jun and Fos (31,32), myc and max (33,34) and C/EBP (35). In the cases of Jun/Fos and myc/max, bending in opposite directions by homo- and heterodimeric complexes has been postulated to be of functional significance. In contrast, homo- and heterodimeric forms of C/EBP family members bend DNA in the same direction. Among members of the steroid/thyroid receptor superfamily, estrogen receptor (36), RAR (37) and TR (37–39) have been found to bend DNA. In the case of TR, the effects of its binding site and dimer partner upon DNA bending have not been systematically investigated.

To address this issue, we compared the DNA bending of DR4 and DR5 by TR homo- and heterodimers. Circular permutation analysis, which has been widely used to detect DNA bending by transcription factors, including steroid and thyroid hormone

* To whom correspondence should be addressed

receptors (36,38,39), suggested that both DR4 and DR5 were greatly bent by TR homo- and heterodimers. Phasing analysis confirmed that TR bends DNA, but indicated that circular permutation analysis greatly overestimated the magnitude of DNA bending by TR, probably because it is influenced by non-vectorial parameters (40). Using phasing analysis, maximal bending was observed when the TR/RXR heterodimer was bound to DR4. Thus there was a positive correlation between protein–DNA complex stability, the magnitude of protein-induced DNA bending and the ability of the binding site to function as a TRE. Moreover, phasing analysis revealed a qualitative difference between TR homo- and heterodimer binding, in that TR/TR and RXR/TR bent DR4 in opposite directions. These results indicate that circular permutation analysis overestimates the magnitude of DNA binding and suggest that sequence and dimer partner dependence of DNA bending may play a role in TR-mediated gene regulation.

MATERIALS AND METHODS

Transient transfection transcription assays

These assays were performed essentially as previously described (41). Briefly, a TRα1-expression vector was co-transfected into JEG-3 cells with either pUTKAT-DR4 or pUTKAT-DR5, which are chloramphenicol acetyl transferase (CAT) reporter genes driven by a thymidine kinase (TK) promoter and either DR4 (gatcagcAGGTCAtagcAGGTCAg) or DR5 (gatccAGGTCAccaggAGGTCAg) respectively, cloned into the *Bam*HI site of the pUTKAT3 plasmid (42), kindly provided by Dr R. Koenig (University of Michigan). The effect of 10 nM T3 on CAT expression was determined after normalization for β-galactosidase expression from the co-transfected pCH110 plasmid.

Production of recombinant TRα1 and RXRβ

Rat TRα1 was synthesized in *Escherichia coli* fused to 17 amino acids containing a FLAG antibody recognition site at its N-terminus, as described elsewhere (41,43). Mouse RXRβ (44) was synthesized in *E.coli* fused at its N-terminal A domain to the first 36 amino acids of β-galactosidase, beginning at amino acid 70 of the full-length RXRβ (14), so that its molecular weight (51 kDa) was ~4 kDa less than that predicted for the full-length endogenous protein (55 kDa). Soluble protein preparations were made by resuspending pellets from 500 ml bacteria grown to OD 0.3–0.5 in 5 ml 50 mM Tris, pH 8, 50 mM NaCl, 1 mM EDTA, 0.2 mM PMSF. Bacteria were treated with lysozyme (2 mg/ml final concentration) for 30 min at 0°C and then incubated for another 20 min after addition of 50 μl of a solution containing 0.5% sodium deoxycholate, 10% Triton X-100. This suspension was sonicated at 0°C, then centrifuged to remove insoluble material. Both proteins were estimated to be ~1–5% of total protein by Coomassie blue staining and were shown to be the appropriate size by Western analysis with monoclonal antibodies to TRα1 (43) and RXRβ (12). The RXRβ antibody was kindly provided by Dr K.Ozato (NIH).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed on 7.5% non-denaturing polyacrylamide gels as described elsewhere (11,18). Gels were run at 6–12 mV/cm at 4°C unless specified otherwise. For competitive dissociation experiments, equal amounts (30 000 c.p.m.) of a radiolabeled 164 bp fragment containing DR4 were incubated with TRα1 (1–5 μg of TRα1-containing bacterial extract) in the presence or absence of RXRβ (1–5 μg of RXRβ-containing bacterial extract) under previously described conditions (18) in the absence or presence of 0.01, 0.05, 0.1, 1.0 or 5.0 mg of unlabeled DR4 or DR5 double-stranded oligonucleotide and resolved on 5% acrylamide gel at room temperature. For off-rate determination experiments, equimolar (20 fmol) amounts of either DR4 or DR5 radiolabeled *Xho*I fragments were incubated for 30 min with TRα1 in the presence or absence of RXRβ at room temperature, after which the respective unlabeled fragment was added in 500-fold molar excess and incubation continued for 0, 5, 15, 30 or 60 min.

Plasmids used for gel shift analysis

For comparison of TR binding to variably spaced direct repeats (Fig. 2), the following DNA sequences (as used by Umesono *et al.*; 6) were subcloned into the *Bam*HI site of pBS, then cut out with *Xba*I and *Hin*dIII.

DR0: gatcagcttcAGGTCAAGGTCAgagagctg
DR1: gatccagcttcAGGTCAcAGGTCAgagagctg
DR3: gatccagcttcAGGTCAagAGGTCAgagagctg
DR4: gatccagcttcAGGTCAcaggAGGTCAgagagctg
DR5: gatccagcttcAGGTCAccaggAGGTCAgagagctg
TREp: gatcctcAGGTCATGACCTga

The affinity, stoichiometry and contact points of TR binding to this version of TREp has been previously reported (18). For circular permutation and phasing analysis, DRs were subcloned into bending vector pBend2, a gift of Dr S.Adhya (45). Plasmid XDR4 was constructed by cloning ctagggagcatAGGTCAcgggAGGTCAgtgttcggt into the *Xba*I site of pBend2, producing a mutated 5′ *Xba*I site. Plasmid SDR5 was obtained by the blunt-end cloning of cagcttcAGGTCAccaggAGGTCAgagag into the *Sal*I site of pBend2.

Plasmids used in phasing analysis were derived from XDR4 and SDR5 by subcloning sequences ctagatg(IB)gcatatgcgtggcatcagatctt, ctagatgcat(IB)caagcatatgtgtagatctt, ctagatgcatg(IB)ctaggtcatatgagatctt, ctagatgcatacg(IB)gcatatgcccagatctt, ctagatgcatacgtg(IB)gtcatatgagatctt, ctagatgcatacgtggt(IB)catatgagatctt and ctagatgcatacgggtcat(IB)gatgagatctt, (where IB, or the intrinsic bend, is $A_6GC_3A_6GC_3A_6$) into the *Xba*I site. All constructs were verified by direct sequencing. The series of intrinsic bend-containing vectors (JT-2–JT-11) was a gift of Dr A. Landy (46).

Circular permutation analysis

TRE (DR4 or DR5)-containing vectors were digested with a series of restriction enzymes producing equal size fragments containing the TRE at variable positions with respect to the fragment center. *Eco*RI–*Sal*I, *Nhe*I, *Xho*I, *Eco*RV, *Stu*I, *Ssp*I and *Bam*HI digests were used for XDR4. For SDR5, *Mlu*I was substituted for *Eco*RI–*Sal*I because the *Sal*I site was destroyed during subcloning. The fragments were incubated with TRα1 in the absence or presence of RXRβ as described previously (11,18) and EMSA was performed alongside the intrinsically bent standards. The fragment mobilities were measured from the bottom of the well to the center of the band and normalized to the free probe migration. Relative mobilities were normalized such that the mean of the least and greatest mobility equaled unity.

Bending angles were determined by plotting the normalized relative mobilities against the fragment center–binding site center distance (in bp) and by using the KaleidaGraph program to fit a cosine function curve to the data and determine the maximum and minimum mobilities (μ_{max} and μ_{min} respectively) (31,32,40,47). The equation $\cos(k \times \alpha/2) = \mu_{max}/\mu_{min}$ was then used to calculate the bending angle. The correction factor k is dependent upon the acrylamide concentration and was calculated from bent DNA standards to be ~1.1. Another method consists of using a standard curve generated by plotting μ_{min}:μ_{max} ratios of DNA fragments containing intrinsic bends of known magnitude (46), then extrapolating unknown bends from μ_{min}:μ_{max} ratios of each complex formed on DR4 and DR5. The overall agreement between the two methods was good, although values obtained by the best fit of the cosine function were about 10° greater than those obtained by the standard curve fitting method (data not shown), consistent with a previously reported comparison of the two methods (46).

Phasing analysis

Intrinsic bend-containing *Eco*RI–*Hin*dIII fragments of the phased plasmids were incubated with TRα1 in the presence or absence of RXRβ and resolved on EMSA. An uninterrupted six adenine sequence is bent at an angle of 18–21° (31,32,46); therefore the intrinsic bend in the fragments containing three such sequences in phase with one another (see above and Fig. 5A) was assumed to be 54°. Complex mobilities were measured on autoradiographs exposed so as to allow accurate assessment of the band centers and were defined as the distance from the top of the gel to the center of the band. In each experiment, the relative complex mobilities were calculated such that the mean of all mobilities equaled unity. The results were plotted either directly or after normalization to the free probe mobilities. The normalization to free probe migration assumes that the lack of uniform migration of the free probes is a 'background' upon which the migration of complexes containing a TR-induced bend is superimposed and is standard practice (31–35,37). The results were plotted as the function of the distance between the centers of the intrinsic bend and TRE. Cosine curves were fitted to the mobility plot using KaleidaGraph software and the difference between the slowest and the fastest mobilities (A_{PH} or the phasing amplitude) was calculated. The true bending angle α was then found by using the formula $\tan(k \times \alpha/2) = (A_{PH}/2)/[\tan(k \times IB/2)]$ (40,47). The bends were considered to be vectorial, i.e. two bends in the same direction spaced such that the distance between their centers equals n helical turns will add arithmetically and produce greatest mobility retardation. When the center-to-center distance equals $n + \frac{1}{2}$ helical turns, the total angle will equal the arithmetic difference between the two angles and the DNA–protein complex will migrate faster.

RESULTS

DR4 is a strong TRE while DR5 is inactive as a TRE

The ability of TRα1 to stimulate transcription from reporter genes containing either DR4 or DR5 upstream from the TK promoter was assessed in transient transfection experiments. In the presence of T3, TRα1 induced CAT expression 8–10-fold when the TRE was DR4, while no significant activation was observed from DR5 (Fig. 1). Furthermore, no statistically significant induction from DR5 was observed even when 10 times the amount of TR was transfected into the cells (data not shown). These results were highly reproducible and agree with observations made by others (48,49).

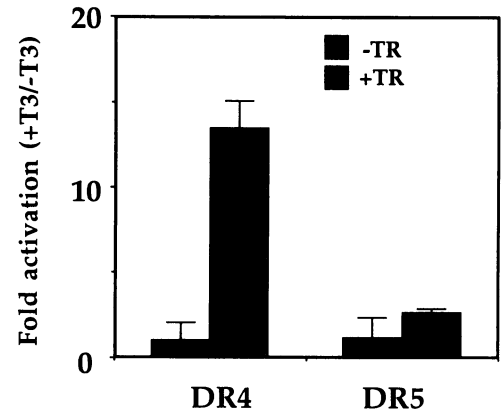

Figure 1. DR4 is a more effective TRE than DR5. JEG-3 cells were transfected with TRα1 and DR4–TK–CAT or DR5–TK–CAT in the presence or absence of T3. Results are shown as mean fold activation ± SEM (n = 3).

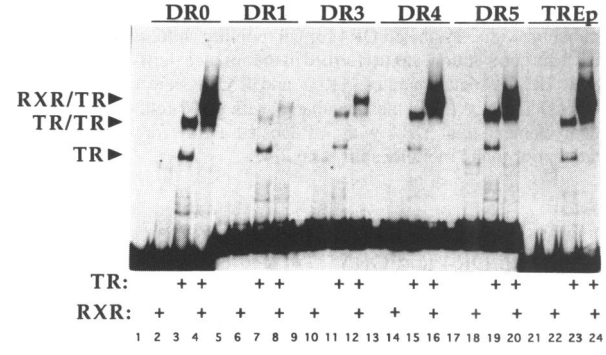

Figure 2. TR homodimer and TR/RXR heterodimer binding to direct repeats. Gel mobility shift assays of TRα1, RXRβ or both binding to direct repeats and TREp. Migration of the TR monomer (TR), homodimer (TR/TR) and heterodimer (TR/RXR) are indicated.

TR monomers, homodimers and heterodimers bind to a variety of direct repeats

We observed binding of bacterially produced TRα1 to both DR4 and DR5 (Fig. 2, compare lanes 15–16 with lanes 19–20). In the absence of RXR, TRα1 bound as monomer and homodimer, as reported by numerous groups (18,21,23,24,50). Prior methylation interference analysis has established that the monomer contacts a single AGGTCA half-site and the homodimer contacts both half-sites (18,51). In the presence of RXR, the major DNA binding form was the TR/RXR heterodimer. Under standard gel shift conditions (20 min binding reaction), the binding was similar to both elements. Figure 2 shows that when binding to other direct repeats and to an inverted repeat with no spacer (TREp) were compared, TR monomers bound similarly to all elements studied and TR homo- and heterodimer binding to DR1 and DR3 (lanes 5–12) was greatly reduced compared with DR4. However, the degree of TR homo- and heterodimer binding to

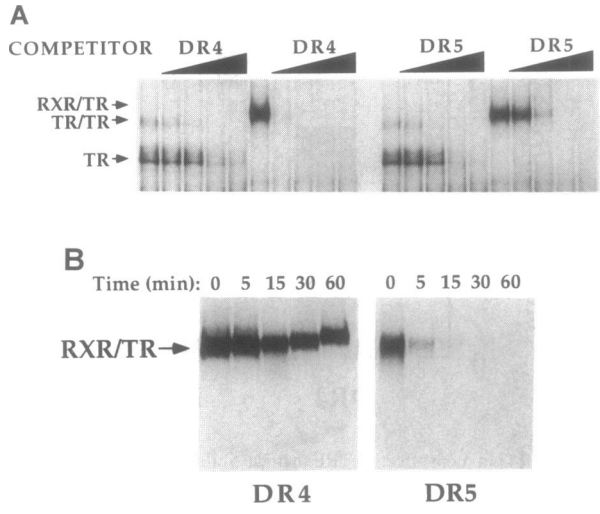

Figure 3. Increased affinity of TR/RXR for DR4. (**A**) Competition. TR was incubated with increasing amounts of unlabeled DR4 (left) or DR5 (right) prior to incubation with ^{32}P-labeled DR4 for gel mobility shift assay. For both DR4 and DR5 the competition was performed in the absence (left) or presence (right) of RXR. (**B**) Dissociation rates. TRα1 and RXRβ were co-incubated with ^{32}P-labeled DR4 or DR5, then incubated with ~500-fold excess unlabeled identical element (e.g. DR4 with ^{32}P-labeled DR4) for varying times as indicated prior to gel mobility shift assay.

DR0 (lanes 1–4) and TREp (lanes 21–24) was comparable with that seen with DR4 and DR5.

TR/RXR heterodimers have a higher affinity for DR4 than DR5

In contrast to the results shown in Figure 2, other investigators have reported selective binding of the TR/RXR heterodimer to DR4 (6). To better assess the relative binding of homo- and heterodimers to DR4 and DR5, we performed DNA competition experiments to estimate the affinity and off-rates of the various TR complexes. Figure 3A shows that cold DR4 competed 5–10-fold better than cold DR5 for TR/RXR binding to DR4, suggesting that the affinity of TR/RXR was similarly higher for DR4. In contrast, DR4 and DR5 competed equally for TR homodimer, as well as monomer, binding to DR4, indicating that these forms of TR have similar affinities for the two binding sites. Figure 3B shows that TR/RXR heterodimer dissociated from DR4 very slowly at 4°C ($T_{1/2}$ 30–60 min) and dissociated considerably faster from DR5 ($T_{1/2}$ < 5 min). In contrast, TR monomer and homodimer did not show selectively stable binding to DR4 (not shown). Thus, the increased affinity of TR/RXR for DR4 was explained predominantly by enhanced stability of the heterodimer complex; relative binding observed in gel shift experiments such as those shown in Figure 2 did not reflect this difference, possibly because the length of the binding reaction was short relative to the half-life of the TR–DNA complex.

Circular permutation analysis of DNA binding by TR

We initially employed circular permutation analysis to assess DNA bending, using probes containing DR4 and DR5 at various positions relative to the center of the pBend2 polylinker (Fig. 4A). Figure 4B and D shows representative results of circular permutation analysis of DR4- and DR5-containing fragments. The identities of the complexes (i.e. TR homodimers, TR/RXR heterodimers and TR monomers) were known from our previous studies (18). The relative mobilities of monomer, homodimer and heterodimer complexes in multiple experiments were fitted to a cosine function curve and plotted as a function of the distance between the center of the TRE and the center of the fragment (Fig. 4C and E). In all cases, the nadir of the cosine curves was near the point at which the center of the TRE was the center of the fragment (zero point), suggesting that the geometric center of the binding site was also the center of the bend, although this estimate is limited by the number of data points, as well as the non-vectorial parameters which affect the analysis (see below).

The apparent bending angles were calculated from the amplitude of the cosine curve, as described in Materials and Methods. All of the bending angles calculated from the circular permutation analysis were rather large, between 60° and 86°. As summarized in Table 1, these calculations suggested that the TR homo- and heterodimer both bent DR4 nearly 80°. DR5 was calculated to be bent to a similar extent, while monomers were calculated to induce smaller bends than homo- and heterodimers (data not shown), although the mobility anomaly of monomer–DNA complexes was magnified because they migrated farther into the gel than the dimeric complexes (for example in Fig. 4B and D). These results must be interpreted with caution, because DNA bending angles estimated by circular permutation analysis are the sum of true bending and the migration anomaly produced by tertiary structure of the protein–DNA complex; indeed, the circular permutation method has proven to be a great overestimate in the case of a number of transcription factors (35,47). Therefore, phasing analysis was used to detect true vectorial DNA bending, as well as to determine its direction.

Table 1. DNA bending angles induced by TR binding to DR4 as estimated by circular permutation and phasing analyses

	Circular permutation analysis	Phasing analysis
TR/TR	81 ± 6	10 ± 5
TR/RXR	81 ± 3	11 ± 2

Results shown are angles in degrees and are expressed as mean ± SD for circular permutation (shown as the mean results of 3–5 experiments; see Fig. 4 legend) and phasing analysis (shown as the mean results of 8–12 experiments; see Fig. 6 legend).

TR homodimers and TR/RXR heterodimers bend DNA in opposite directions

In addition to providing a more accurate assessment of the magnitude of DNA bending, phasing analysis reveals the direction of the bend relative to that of the intrinsic bend included in the DNA probes. The effect of the protein producing a branch-like configuration by binding perpendicularly to DNA is eliminated, because the probes now differ by the intrinsic bend position (Fig. 5A), not by the response element position (40). Therefore, we studied the binding of TR, in the absence and presence of RXR, to probes containing DR4 or DR5 at variable distances from an intrinsic bend. A representative experiment is shown in Figure 5B. Variations of free probe mobilities were noted, consistent with the finding of other investigators using the pBend2 polylinker (31–35,37).

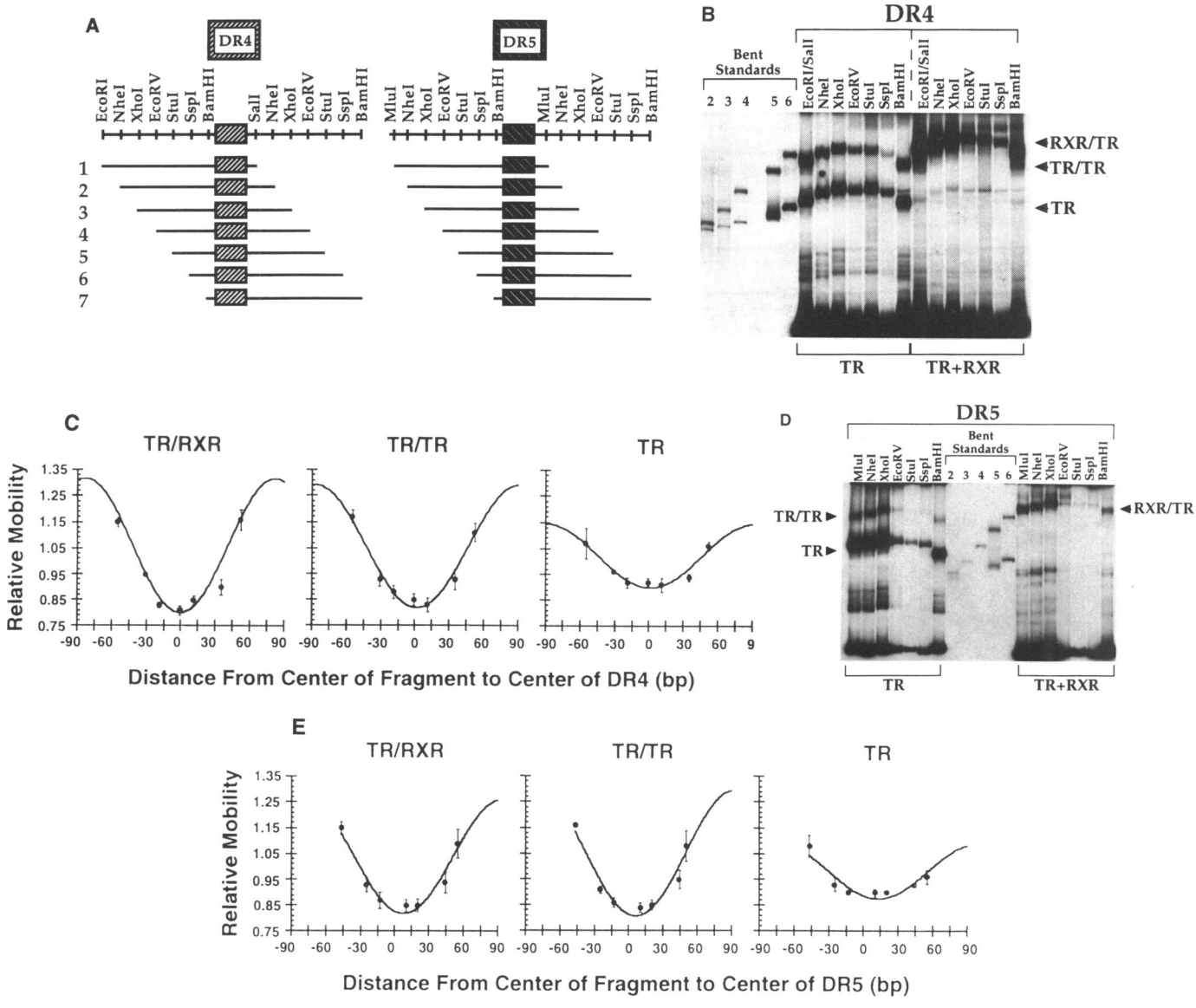

Figure 4. Circular permutation analysis of TR binding to DR4 and DR5. (**A**) Probes used containing DR4 and DR5 generated by serial restriction digests of the pBend2 polylinker resulting in variable TRE position with respect to the fragment center (see Materials and Methods). (**B**) TR binding to DR4. Standards with multiple intrinsic bends are shown at left, with the number of A tracts indicated above the lanes. Restriction enzymes above each lane refer to the sites in the polylinker as shown in (A) and are identical for TR alone (center) and TR + RXR (right). (**C**) Best fit of cosine function. Shown are the results of multiple experiments (mean ± SEM), including the one shown in (B) ($n = 3$ for TR/RXR and TR/TR, $n = 4$ for TR monomer). (**D**) TR binding to DR5. Standards with multiple intrinsic bends are shown in the center, with the number of bends indicated above the lanes. Restriction enzymes above each lane refer to the sites in the polylinker as shown in (A) and are identical for TR alone (left) and TR + RXR (right). (**E**) Best fit of cosine function. Shown are the results of multiple experiments (mean ± SD, $n = 5$), including the data shown in (D).

The mobility pattern of TR–DR4 complexes varied considerably between the TR homodimer and TR/RXR heterodimer, as shown in Figure 5B. Visual inspection of Figure 5B shows that the TR homodimer complexes had decreased variation in mobility relative to that of the unbound probes. In contrast, the TR/RXR heterodimer complexes displayed increased mobility variation compared with the free probes (after comparably short migration, data not shown). Similar results were obtained in multiple separate experiments, shown graphically in Figure 6A, in which relative mobilities were plotted as a cosine function of the distance between the centers of the TRE and the intrinsic bend, as described in Materials and Methods. This analysis revealed that TR homodimer binding had an opposite effect on the amplitude of the cosine curve than did binding of the TR/RXR heterodimer.

The same data were also plotted after normalization to the mobilities of the phased DR4 probes, a correction which is widely used by workers in this field (31–35,37). Remarkably, the curves were nearly 180° out of phase, suggesting that TR homo- and heterodimers bent DR4 in opposite directions. TR/RXR binding to DR4 induced bending toward the minor groove, whereas TR homodimer binding induced a bend toward the major groove, assuming that the center of the TR-induced bend is the center of DR4. If this assumption were incorrect, the absolute directions of the bend might differ (see Discussion), although the conclusion that the homodimer and heterodimer bend DR4 in opposite

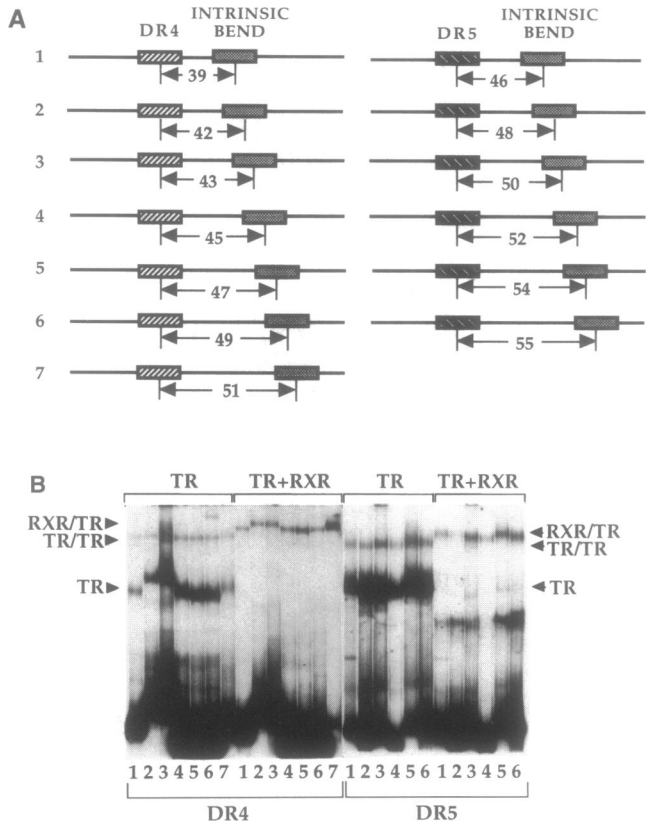

Figure 5. Phasing analysis of TR binding to DR4 and DR5. (**A**) Probes containing DR4 and DR5 variably spaced (in bp) relative to an intrinsic bend (see Materials and Methods). (**B**) A representative gel mobility shift experiment is shown. TR or TR + RXR were incubated with ^{32}P-labeled probes containing DR4 (left) or DR5 (right) phased relative to an intrinsic bend cloned into the pBend2 polylinker. Migration of the TR monomer (TR), homodimer (TR/TR) and heterodimer (TR/RXR) are shown.

directions would be unaffected. The relative mobilities of TR monomer complexes with the phased DR4 probes did not differ significantly from the relative mobilities of the free probes, suggesting very little induced bending (not shown).

TR heterodimers bend DR4 but not DR5

DR4 bending angles calculated from the amplitude of the cosine curves as described in Materials and Methods are summarized in Table 1. TR/RXR heterodimers and TR homodimers were each found to bend DR4 by ~10°. These angles are much smaller than those estimated from circular permutation analysis. This is not unprecedented, as similar overestimates of bending by circular permutation analysis have been reported for bZip transcription factors (35,47). Quantitative analysis of DR5 bending in multiple experiments using phased probes as in Figure 5B was also performed (Fig. 6C and D). In contrast to DR4, where TR binding altered the relative mobilities of the probes, binding of TR/RXR and TR homodimer caused little change in the migration pattern of the DR5-containing probes and the cosine curve of the protein–DNA complexes was essentially superimposable with the free probe migration curve (Fig. 6C). Thus TR binding to DR5 as homodimer and TR/RXR heterodimer resulted in little bending of DR5, as manifest by the small amplitude of the best fit cosine curve shown in Figure 6D. Indeed, the 'best fit' of the DR5 binding data to a cosine curve was rather poor as compared with that of the DR4 data, confirming the likelihood that the TR-induced bending of DR5 was trivial. The presence of ligand (100 nM T3, 1 mM 9-*cis*-retinoic acid or both) did not significantly alter the results on both DR4 and DR5 (data not shown), which is in agreement with one previous study of a DR4-related sequence (37).

DISCUSSION

The failure of some investigators to observe any binding of TR homodimer to DR4 or TR/RXR heterodimer to DR5 is most likely a reflection of assay sensitivity to dissociation of the protein–DNA complexes. In our experiments, where TR homodimer binding was measurable, the correlation between transcriptional activity of the TR and binding of TR/RXR, but not the TR homodimer, suggests that the TR/RXR heterodimer is the predominant transcriptional activator on DR4 in the transiently transfected JEG-3 cells used in our experiments, which contain endogenous RXR, mainly RXRα (52,53). However, the ability of the TR/RXR heterodimer to bind to DR5, albeit with lower affinity, suggested that TR-induced transcriptional activation may be regulated by additional characteristics of the TR–TRE interaction, such as DNA bending.

Circular permutation analysis suggested that the TR bends DNA. However, phasing analysis revealed that much of the migration anomaly observed in the circular permutation analysis may be attributable to factors other than vectorial bending (54). Since the circular permutation analysis was apparently greatly influenced by factors other than DNA bending, the estimates of bend centers using this analysis are likely to be inaccurate. The sensitivity of the circular permutation assay to parameters other than DNA bending has been observed for other proteins and has been postulated to reflect the binding of proteins nearly perpendicularly to DNA, creating a triangular structure whose shape varies depending on the response element position with respect to the center (40,47). Indeed, non-vectorial changes in protein conformation induced by DNA binding have been reported for other transcription factors, such as GCN4 (55) and C/EBP (35). Nevertheless, the changes detected by circular permutation may relate to TR function. For example, the results could reflect allosteric changes in the conformation of TR and/or RXR proteins induced by DNA binding, much as ligand binding is known to induce changes in TR conformation (56–58) which regulate interaction with basal transcription factors such as TFIIB (59). Indeed, it has recently been shown that DNA binding allosterically regulates the affinity of RXR for its ligand (60).

Phasing analysis revealed that TR/RXR induced significant bending of DR4, but not DR5. The 11° angle calculated from the phasing analysis of TR heterodimer binding to DR4 is similar to that previously reported in a study by Lu *et al.*, which did not directly compare TR homo- and heterodimer bending of DR4 and DR5 (37). In that study, as in the present case, the direction of the bend was apparent only after normalization to the mobilities of the unbound, phased probes. It should be noted that our observation that TR/RXR bends DR4 toward the minor groove contrasts with the results of Lu *et al.*, who concluded that TR/RXR bent the malic enzyme TRE toward the major groove (37). However, the direction of the bend is determined relative to that of the intrinsic bend and depends upon an accurate estimation

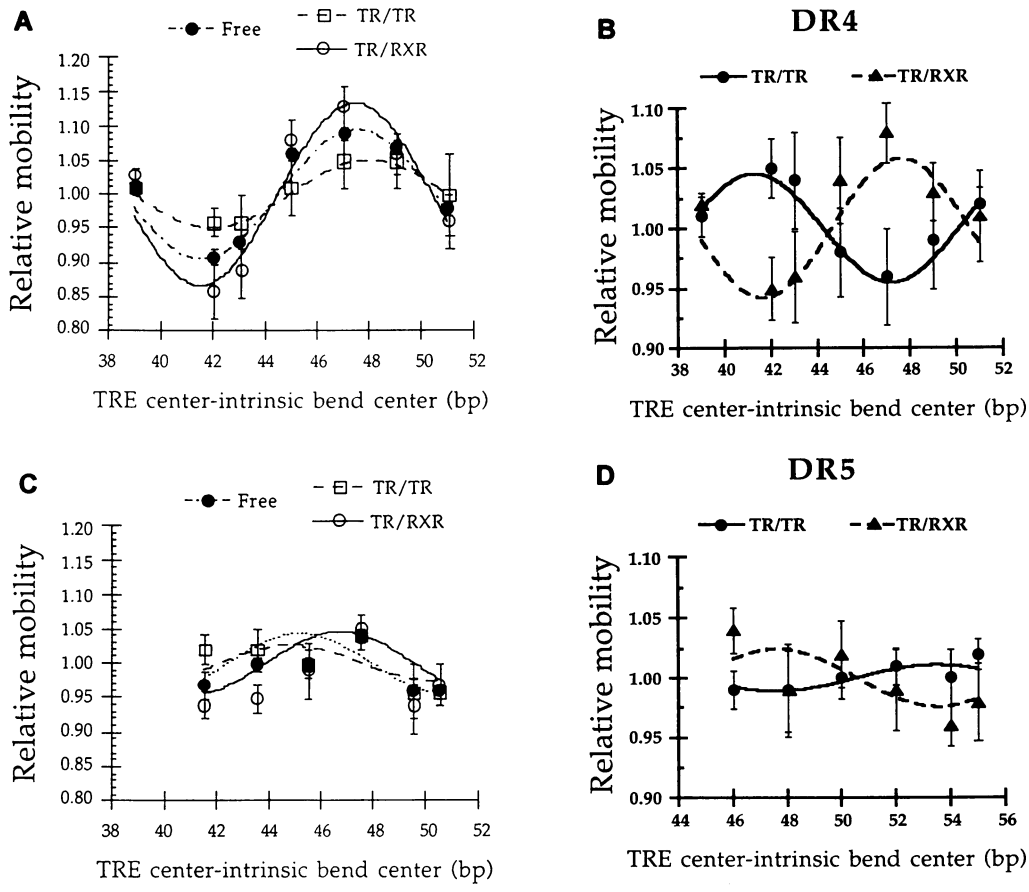

Figure 6. Direction and magnitude of TR bending of DR4 and DR5. (**A**) DR4, uncorrected for the mobilities of the free probes, which are also plotted. (**B**) DR4, corrected for free probe mobilities. (**C**) DR5, uncorrected for free probe mobilities, which are also plotted. (**D**) DR5, corrected for free probe mobilities. Relative mobilites are plotted against the distance from the center of the intrinsic bend to the geometric center of the binding site. The results of multiple experiments similar to that shown in Figure 5 were combined (mean ± SEM) and complex mobilities are plotted as the cosine function of the distance of the center of DR4 or DR5 from the known intrinsic bend. For TR/RXR, n = 12 (DR4) or 8 (DR5); for TR/TR, n = 10 (DR4) or 8 (DR5).

of the bend center. It is therefore likely that the bend centers of either the complex malic enzyme TRE or the idealized DR4 used in this experiment were not at the geometric centers of the fragments, as estimated by the circular permutation analysis, which is sensitive to factors other than DNA bending.

Although the composition of the 4 or 5 bp spacer has been shown not to influence T3 responsiveness of DRs (6), while this work was in progress the addition of the dinucleotide sequence TA 5′ to the hexameric half-site was shown to create a higher affinity binding site for the TR monomer (61). However, none of the constructs used in the present studies contained the TAAGGTCA octamer and thus the differences observed between DR4 and DR5 are not related to this additional important consideration. Of note, a variety of naturally occurring DR4 TREs, including those in the genes encoding myosin heavy chain, malic enzyme and rat growth hormone, as well as that in the Moloney leukemia virus long terminal repeat, do not contain the TA flank (6).

The induced DNA bending and the stability of the TR–RXR complex on DR4 may be interdependent, since protein-induced DNA bending may reduce the free energy of the protein–DNA complex (54,62). Thus the weak interactions between the TR and RXR sub-domains that are required for spacer length recognition (15,17) may be stabilized by bending of DR4. Such interactions may be less favored by the single additional base pair in DR5, which increases the distance between the two half-sites by one base, as well as altering their orientation by 36°. In addition to potentially stabilizing the TR/RXR–TRE complex, DNA bending may serve to reduce or increase the activation energy required for the interaction of proteins bound to distant sites on the DNA with each other and/or the polymerase complex, thereby enhancing or repressing transcription respectively (40). TR, for example, must communicate with components of the general transcription machinery, including TFIIB (59,63).

Phasing analysis also showed that the TR/RXR heterodimer and the TR homodimer bend DR4 in opposite directions. Although similar phenomena have been observed for Jun homodimers and Jun/Fos heterodimers (32), as well as myc and max (33,34), not all homo- and heterodimeric forms of transcription factors bend DNA in different directions. For example, the C/EBP-related proteins LIP and CRP3 bend DNA as homodimers in the same direction as Ig-EBP/ATF4 heterodimers (35). While the mechanism whereby TR homodimers and TR/RXR induce bends in opposite directions is unknown, such differences may play a role in transcriptional regulation (40). For example, bending in one direction may serve to facilitate interactions among DNA-bound factors and, conversely, bending in the opposite direction may prevent them from interacting; this

concept is supported by the dependence of transcriptional synergy between response elements upon their relative positions on a helical turn (64,65). Our observation that the TR homodimer and TR/RXR heterodimer bend DR4 in opposite directions would be consistent with different transcriptional roles for the TR homo- and heterodimers, which has been previously suggested by the instability of the TR homodimer on some TREs in the presence of T3 (24,25) and by different transcriptional properties of mutant TRs in which the homo- and heterodimerization capabilities have been separated (66,67). However, the modest magnitude of the bending may reduce its physiological significance.

ACKNOWLEDGEMENTS

We thank S. Adhya for the bending vector (pBend2), A. Landy for the series of intrinsic bend-containing plasmids, R. Koenig for reporter plasmids, T. Berrodin for the bacterial protein preparation, J. Perkel for oligonucleotide subcloning and T. Kerppola for valuable discussions and suggestions. This work was supported by NIH grant DK43806 (MAL). KS was supported by NIH postdoctoral fellowship grant DK08841.

REFERENCES

1 Oppenheimer,J.H. and Samuels,H.H. (eds) (1983) *Molecular Basis of Thyroid Hormone Action*. Academic Press, New York, NY.
2 Lazar,M.A. (1993) *Endocrine Rev.*, **14**, 184–193.
3 Evans,R.M. (1988) *Science*, **240**, 889–895.
4 Green,S. and Chambon,P. (1988) *Trends Genet.*, **4**, 309–315.
5 Carson-Jurica,M.A., Schrader,W.T. and O'Malley,B.W. (1990) *Endocrine Rev.*, **11**, 201–220.
6 Umesono,K., Murakami,K.K., Thompson,C.C. and Evans,R.M. (1991) *Cell*, **65**, 1255–1266.
7 Naar,A.M., Boutin,J.-M., Lipkin,S.M., Yu,V.C., Holloway,J.M., Glass,C.K. and Rosenfeld,M.G. (1991) *Cell*, **65**, 1267–1279.
8 Kliewer,S.A., Umesono,K., Mangelsdorf,D.J. and Evans,R.M. (1992) *Nature*, **355**, 446–449.
9 Zhang,X.-k., Tran,P.B.-V. and Pfahl,M. (1991) *Mol. Endocrinol.*, **5**, 1909–1920.
10 Zhang,X.-k., Hoffmann,B., Tran,P.B.-V., Graupner,G. and Pfahl,M. (1992) *Nature*, **355**, 441–446.
11 Berrodin,T.J., Marks,M.S., Ozato,K., Linney,E. and Lazar,M.A. (1992) *Mol. Endocrinol.*, **6**, 1468–1478.
12 Marks,M.S., Levi,B.-Z., Segars,J.H., Driggers,P.H., Hirschfeld,S., Nagata,T., Appella,E. and Ozato,K. (1992) *Mol. Endocrinol.*, **6**, 219–230.
13 Leid,M., Kastner,P., Lyons,R., Nakshatri,H., Saunders,M., Zacharewski,T., Chen,J., Staub,A., Garnier,J.-M., Mader,S. and Chambon,P. (1992) *Cell*, **68**, 377–395.
14 Yu,V. C., Delsert,C., Anderson,B., Holloway,J.M., Devary,O.V., Naar,A.M., Kim,S.Y., Boutin,J.-M., Glass,C.K. and Rosenfeld,M.G. (1991) *Cell*, **67**, 1251–1266.
15 Perlmann,T., Rangarajan,P.N., Umesono,K. and Evans,R.M. (1993) *Genes Dev.*, **7**, 1411–1422.
16 Kurokawa,R., Yu,V.C., Naar,A., Kyakumoto,S., Han,Z., Silverman,S., Rosenfeld,M.G. and Glass,C.K. (1993) *Genes Dev.*, **7**, 1423–1435.
17 Zechel,C., Shen,X. Q., Chambon,P. and Gronemeyer,H. (1994) *EMBO J.*, **13**, 1414–1424.
18 Lazar,M.A., Berrodin,T.J. and Harding,H.P. (1991) *Mol. Cell. Biol.*, **11**, 5005–5015.
19 Nelson,C.C., Hendy,S.C. and Faris,J.S. (1994) *Mol. Endocrinol.*, **8**, 829–840.
20 Williams,G.R., Harney,J.W., Forman,B.M., Samuels,H.H. and Brent,G.A. (1991) *J. Biol. Chem.*, **266**, 19636–19644.
21 Forman,B.M., Casanova,J., Raaka,B.M., Ghysdael,J. and Samuels,H.H. (1992) *Mol. Endocrinol.*, **6**, 429–442.
22 Miyamoto,T., Suzuki,S. and DeGroot,L.J. (1993) *Mol. Endocrinol.*, **7**, 224–231.
23 Ribeiro,R.C.J., Kushner,P.J., Apriletti,J.W., West,B.L. and Baxter,J.D. (1992) *Mol. Endocrinol.*, **6**, 1142–1152.
24 Yen,P.M., Darling,D.S., Carter,R.L., Forgione,M., Umeda,P.K. and Chin,W.W. (1992) *J. Biol. Chem.*, **267**, 3565–3568.
25 Andersson,M.L., Nordstrom,K., Demczuk,S., Harbers,M. and Vennstrom,B. (1992) *Nucleic Acids Res.*, **20**, 4803–4810.
26 Bracco,L., Kotlarz,D., Kolb,A., Diekmann,S. and Buc,H. (1989) *EMBO J.*, **8**, 4289–4296.
27 Claverie-Martin,F. and Magasanik,B. (1992) *J. Mol. Biol.*, **227**, 996–1008.
28 McAllister,C.F. and Achberger,C. (1989) *J. Biol. Chem.*, **264**, 10451–10456.
29 Perez-Martin,J. and Espinosa,M. (1993) *Science*, **260**, 805–807.
30 Zinkel,S. and Crothers,D.M. (1991) *J. Mol. Biol.*, **219**, 201–215.
31 Kerppola,T.K. and Curran,T. (1991) *Science*, **254**, 1210–1214.
32 Kerppola,T.K. and Curran,T. (1991) *Cell*, **66**, 317–326.
33 Fisher,D.E., Parent,L.A. and Sharp,P.A. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 11779–11783.
34 Wechsler,D.S. and Dang,C.V. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 7635–7639.
35 Avitahl,N. and Calame,K. (1994) *J. Biol. Chem.*, **269**, 23553–23562.
36 Nardulli,A.M., Greene,G.L. and Shapiro,D.J. (1993) *Mol. Endocrinol.*, **7**, 331–340.
37 Lu,X.P., Eberhardt,N.L. and Pfahl,M. (1993) *Mol. Cell. Biol.*, **13**, 6509–6513.
38 King,I.N., Soyza,T.d., Catanzaro,D.F. and Lavin,T.N. (1993) *J. Biol. Chem.*, **268**, 495–501.
39 Leidig,F., Shepard,A.R., Zhang,W., Stelter,A., Cattini,P.A., Baxter,J.D. and Eberhardt,N.L. (1992) *J. Biol. Chem.*, **267**, 913–921.
40 Kerppola,T.K. and Curran,T. (1993) *Nucleic Acids Mol. Biol.*, **7**, 70–105.
41 Katz,D. and Lazar,M.A. (1993) *J. Biol. Chem.*, **268**, 20904–20910.
42 Prost,E. and Moore,D.D. (1986) *Gene*, **45**, 107–111.
43 Katz,D., Berrodin,T.J. and Lazar,M.A. (1992) *Mol. Endocrinol.*, **6**, 805–814.
44 Hamada,K., Gleason,S.L., Levi,B.-Z., Hirschfeld,S., Appella,E. and Ozato,K. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8289–8293.
45 Kim,J., Zwieb,C., Wu,C. and Adhya,S. (1989) *Gene*, **85**, 15–23.
46 Thompson,J.F. and Landy,A. (1988) *Nucleic Acids Res.*, **16**, 9687–9705.
47 Kerppola,T.K. and Curran,T. (1993) *Mol. Cell. Biol.*, **13**, 5479–5489.
48 Glass,C.K., Lipkin,S.M., Devary,O.V. and Rosenfeld,M.G. (1989) *Cell*, **59**, 697–708.
49 Umesono,K., Giguere,V., Glass,C.K., Rosenfeld,M.G. and Evans,R.M. (1988) *Nature*, **336**, 262–265.
50 Brent,G.A., Williams,G.R., Harney,J.W., Forman,B.M., Samuels,H.H., Moore,D.D. and Larsen,P.R. (1992) *Mol. Endocrinol.*, **6**, 502–514.
51 Ikeda,M., Rhee,M. and Chin,W.W. (1994) *Endocrinology*, **135**, 1628–1638.
52 Davis,K.D., Berrodin,T.J., Stelmach,J.E., Winkler,J.D. and Lazar,M.A. (1994) *Mol. Cell. Biol.*, **14**, 7105–7110.
53 Rosen,E.D., O'Donnell,A.L. and Koenig,R.J. (1992) *J. Biol. Chem.*, **267**, 22010–22013.
54 Kahn,J.D., Yun,E. and Crothers,D.M. (1994) *Nature*, **368**, 163–166.
55 Gartenberg,M.R., Ampe,C., Steitz,T.A. and Crothers,D.M. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 6034–6038.
56 Bhat,M.K., Parkison,C., McPhie,P., Liang,C.M. and Cheng,S.Y. (1993) *Biochem. Biophys. Res. Commun.*, **195**, 385–392.
57 Leng,X., Tsai,S.Y., O'Malley,B.W. and Tsai,M.J. (1993) *J. Steroid Biochem. Mol. Biol.*, **46**, 643–661.
58 Toney,J.H., Wu,L., Summerfield,A.E., Sanyal,G., Forman,B.M., Zhu,J. and Samuels,H.H. (1993) *Biochemistry*, **32**, 2–6.
59 Baniahmad,A., Ha,I., Reinberg,D., Tsai,S., Tsai,M.J. and O'Malley,B.W. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 8832–8836.
60 Kurokawa,R., DiRenzo,J., Boehm,M., Sugarman,J., Gloss,B., Rosengeld,M.G., Heyman,R.A. and Glass,C.K. (1994) *Nature*, **371**, 528–531.
61 Katz,R.W. and Koenig,R.J. (1994) *J. Biol. Chem.*, **269**, 9500–9505.
62 Paolella,D.C., Palmer,C. and Schepartz,A. (1994) *Science*, **264**, 1130–1133.
63 Fondell,J.D., Roy,A.L. and Roeder,R.G. (1993) *Genes Dev.*, **7**, 1400–1410.
64 Schule,R., Muller,H., Otsuka-Murakami,H. and Renkawitz,R. (1988) *Nature*, **332**, 87–90.
65 Voz,M.L., Peers,B., Wiedig,M.J., Jacquemin,P., Belayew,A. and Martial,J.A. (1992) *Mol. Cell. Biol.*, **12**, 3991–3997.
66 Hao,E., Menke,J.B., Smith,A.M., Jones,C., Geffner,M.E., Hershman,J.M., Wuerth,J.-P., Samuels,H.H., Ways,D.K. and Usala,S.J. (1994) *Mol. Endocrinol.*, **8**, 841–851.
67 Au-Fliegner,M., Helmer,E., Casanova,J., Raaka,B.M. and Samuels,H.H. (1993) *Mol. Cell. Biol.*, **13**, 5725–5737.