Plasticity of Tetramer Formation by Retinoid X Receptors
AN ALTERNATIVE PARADIGM FOR DNA RECOGNITION

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Retinoid X receptors (RXRs) are transcription factors that traditionally have been thought to bind DNA as protein dimers. Recently, however, it has been recognized that RXRs can also bind to DNA as protein tetramers. Receptor tetramers form cooperatively on response elements containing suitably reiterated half-sites, and play an important role in determining the specificity of DNA recognition by different nuclear receptors. We report here that RXR tetramers exhibit significant functional plasticity, and form on response elements possessing diverse half-site orientations and spacings. This ability of RXRs to form tetramers and related oligomers appears to contribute to the synergistic transcriptional activation observed when multiple, spatially separated response elements are introduced into a single promoter. Oligomerization may therefore be a common paradigm for DNA recognition and combinatorial regulation by several different classes of transcription factors.

Nuclear hormone receptors are hormone-regulated transcription factors, and include the steroid receptors, vitamin D3 receptors, thyroid hormone receptors, and two distinct categories of retinoid receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (reviewed in Refs. 1–7). RXRs in particular play important roles in vertebrate development and differentiation, both autonomously in response to 9-cis-retinoic acid and combinatorially in conjunction with other nuclear hormone receptors (8–14). Three different genetic loci (denoted α, β, and γ) encode RXRs (8–14).

RXRs, in common with other nuclear hormone receptors, function by binding to specific DNA sequences (denoted hormone response elements, HREs) and regulating the transcription of adjacent target genes in response to hormone (1–7). The nuclear hormone receptors share a common structural organization comprising a DNA-binding domain (including a central zinc-finger domain), a hormone-binding domain, and a variety of motifs involved in interactions with the transcriptional machinery (Fig. 1 and Refs. 1–7). Most nuclear hormone receptors can bind to DNA as protein dimers, with each receptor molecule recognizing a “half-site,” a conserved 6–8-base DNA sequence (15–18). HREs have therefore been traditionally viewed as composed of two half-sites. Both the sequence of the individual half-sites and their spacing and orientation contribute to the specificity of DNA recognition (15–25). The protein-protein interfaces involved in receptor dimer formation have been mapped to both zinc-finger and hormone-binding domains (15–18, 24–26).

It is now clear, however, that receptor dimers are not the only paradigm for DNA recognition, and that certain members of the nuclear receptor family can also bind to DNA as protein complexes larger than dimer (27). These high order oligomers include receptor trimers, tetramers, and pentamers, and bind with high cooperativity to response elements containing suitably reiterated half-sites (27). RXR tetramers have also been observed in solution (28–30). Oligomer formation by RXRs permits recognition of DNA sequences that are not recognized by receptor dimers, and contributes to isoforn-specific promoter utilization (27).

We wished to investigate the nature of these newly elucidated receptor oligomers. Here, we report that RXR tetramer formation is relatively plastic in character, and that tetramers form on response elements possessing a wide variety of half-site orientations and spacings. In fact, receptor oligomers can form cooperatively on spatially separated response elements and may contribute to the synergistic gene activation observed when multiple response elements are introduced into a single promoter. Intriguingly, the half-site spacing and orientation required for tetramer formation are non-equivalent at different positions in the response element, suggesting a corresponding anisotropism in the RXR tetramer. High order oligomer formation may be a common means of generating DNA specificity and combinatorial regulation for a variety of transcription factors.

EXPERIMENTAL PROCEDURES

Proteins and Oligonucleotide Probes—The wild-type, ΔN terminus, and the chimeric mouse RXR proteins were obtained as nuclear extracts from S99 cells infected with the appropriate recombinant baculovirus (27). The construction of the ΔN terminus RXXβ, ΔN terminus RXXγ, and the γβ RXR chimera was described previously (27). The TR-RXR chimera represents a fusion of codons 1–159 of the avian thyroid hormone receptor a-1 sequence to codons 228–464 of mouse RXRγ. The ΔNAC RXRγ, representing codons 139–228, was isolated as glutathione S-transferase fusion protein from Escherichia coli bearing a suitable pGEX plasmid vector (31). No significant functional differences were detected when the same receptor was isolated from the two different expression systems (27). Oligonucleotides were obtained as complementary, single-stranded DNAs (Operon Incorporated) and were annealed to create double-stranded DNAs with 4-base overhangs. For use as probes in electrophoretic mobility shift experiments, the overhangs were filled in with radiolabeled nucleotides and Klenow fragment of DNA polymerase I (31, 32). For use as response elements, the double-stranded DNAs were introduced into the SalI site of pD33-CAT (31). An adjacent, cryptic half-site present in the pD33-CAT vector itself (AG-GTCG) was removed from these constructs by PstI and HindIII cleavage, converting the overhanging ends to blunt with T4 DNA polymerase, and religation.

Electrophoretic Mobility Shift Assays—Receptor preparations (estimated as 20–30 ng of receptor/μl) were incubated at 25 °C for 20–25
**RESULTS**

**RXRs and γ Bind Cooperatively to Reiterated Response Elements as Protein Tetramers**—Both RXRα and γ can efficiently bind to reiterated elements containing four half-sites oriented as direct repeats (Fig. 2A). These protein-DNA complexes migrate at a position characteristic of 4-fold occupancy (4R), and form with extremely high cooperativity and no evidence of prior dimer formation (27). We have therefore defined these RXRα and RXRγ complexes as protein tetramers (27). In contrast, RXRβ primarily forms dimers (2R) and neither binds these reiterated elements as tetramers in vitro nor efficiently mediates gene activation through them in vivo (Fig. 2A and Ref. 27).

It should be noted that at high protein concentrations, RXRβ can occupy all four half-sites on a reiterated element by the non-cooperative binding of two independent RXRβ dimers (Fig. 2A); this is, however, in clear contrast to the highly cooperative binding of tetramers observed with RXRα and γ (Fig. 2A).

The various complexes observed in Fig. 2A are mediated by the corresponding RXR isoforms, as demonstrated by the ability of RXR-directed antisera to supershift the complex, and by the absence of complex formation by equivalent protein preparations isolated from uninfected Sf9 cells, or from cells infected with radiolabeled oligonucleotide probe (40,000–60,000 cpm, 20–60 ng of DNA) in 15 μl of binding buffer (10 mM Tris-Cl, pH 7.5, 3% glycerol, 13.3 μg/μl bovine serum albumin, 66.7 mM KCl, 2 mM MgCl₂, and 133 μg/ml poly(deoxyinosine-deoxycytosine)). The resulting DNA-protein complexes were then resolved by denaturing electrophoresis through a 5% polyacrylamide, 0.13% bisacrylamide gel (either at 25 °C and 200 V, or 4 °C and 320 V) and were visualized by autoradiography. The position of protein-DNA complexes representing 2-fold occupancy (2R) or 4-fold occupancy (4R) of the response element by receptor are indicated on the right, along with the location of free probe. The identity of the RXR-DNA complexes were confirmed by supershift experiments, comparing the mobilities of the complexes formed in the absence of specific antisera (panel B, left two lanes) to those of the complexes formed in the presence of anti-RXR antisera (panel B, right two lanes). Combinatorial experiments were also performed to confirm our assignment of stoichiometry to these complexes, utilizing an N-terminal deletion (ΔNγ) of RXRγ, full-length RXRγ (γ), or mixtures of the two (panels C and D). DNA elements containing two (panel C) or four (panel D) half-sites were employed, using the same form of electrophoretic analysis as in panel A. The proposed combinatorial products, expressed as mixtures of full-length (R) and deleted (r) RXR molecules, are indicated on the right of panels C and D. Small quantities of an RXRγ degradation product could be detected as a minor band flanking the major complex in the right lane of panel C. The free probe was electrophoresed off the gel in panel D to improve the resolution of the different complexes.

**FIG. 1.** The RXR constructs and response elements used in these studies. A schematic representation of RXRγ is shown (panel A) with the locations of the receptor domains thought to be involved in DNA recognition, hormone binding, receptor-receptor dimerization, and transcription (Tx) regulation indicated above. The locations of the N- and C-terminal deletions, and the junctions used to create the RXR-γ/β chimera and the TR-RXR chimera, are shown below. The core sequences of the different response elements employed in these studies are also presented (panel B); flanking sequences employed to standardize the length of the oligonucleotides and to permit cloning and radiolabeling are not shown. Arrows indicate the locations and orientation of the AGGTCA half-sites, which are numbered as 1, 2, 3, and 4 for convenience. The locations at which 2-, 5-, or 10-base spacers were inserted, as described under “Results,” are indicated above the top sequence.

**FIG. 2.** Tetramer formation by RXRα and RXRγ on reiterated DNA elements. The ability of wild-type RXRα, β, or γ to bind to DNA was tested (panel A), using a reiterated response element composed of four half-sites oriented as direct repeats with a 1-base spacer element (top, see Fig. 1B). Increasing amounts of receptor (estimated at 0, 1, 3, or 5 ng, respectively) were added to a fixed amount of radiolabeled response element, and the resulting complexes were resolved by electrophoretic shift assay and autoradiography. The position of protein-DNA complexes representing 2-fold occupancy (2R) or 4-fold occupancy (4R) of the response element by receptor are indicated on the right, along with the location of free probe. The identity of the RXR-DNA complexes were confirmed by supershift experiments, comparing the mobilities of the complexes formed in the absence of specific antisera (panel B, left two lanes) to those of the complexes formed in the presence of anti-RXR antisera (panel B, right two lanes). Combinatorial experiments were also performed to confirm our assignment of stoichiometry to these complexes, utilizing an N-terminal deletion (ΔNγ) of RXRγ, full-length RXRγ (γ), or mixtures of the two (panels C and D). DNA elements containing two (panel C) or four (panel D) half-sites were employed, using the same form of electrophoretic analysis as in panel A. The proposed combinatorial products, expressed as mixtures of full-length (R) and deleted (r) RXR molecules, are indicated on the right of panels C and D. Small quantities of an RXRγ degradation product could be detected as a minor band flanking the major complex in the right lane of panel C. The free probe was electrophoresed off the gel in panel D to improve the resolution of the different complexes.
fected by non-recombinant baculovirus (Fig. 2, A and B). 2 Furthermore, analogous complexes were formed by purified glutathione S-transferase-RXRγ fusion proteins isolated from E. coli, suggesting that RXR is likely the only protein necessary for complex formation (27).

Our assignment of stoichiometry to the various complexes in Fig. 2A was based initially on titration experiments with elements containing differing numbers of half-sites; a stepwise decrease in the mobility of the complex occurred corresponding to the number of half-sites in the element (27). A more absolute assignment of stoichiometry was obtained by a combinatorial experiment. We compared the migration pattern of DNA complexes generated by an N-terminal deletion of RXRγ (which produces a rapidly migrating DNA-protein complex) with that of the full-length RXRγ (which produces a slower migrating complex) (Fig. 2, C and D). Mixing these two different receptor derivatives on a two-half site element produced a single intermediate complex, the combinatorial result predicted if this element is indeed bound by receptor dimers (i.e. RR, Rr, rr). In contrast, mixing these same two receptor derivatives with our four half-site element produced three intermediate complexes (Fig. 2D) precisely the number predicted for combinatorial mixtures of a receptor tetramer (i.e. RRRR, RRrr, RRRrr, rrrr). We therefore conclude that both RXRα and RXRγ complexes observed on DNA elements containing four half-sites do indeed represent 4-fold occupancy by receptor tetramers.

The RXR Hormone-binding Domain Is Necessary, but Not Sufficient, to Confer Tetramer Formation—What RXR domains are involved in tetramer formation? We have previously shown that the N terminus of RXR confers the differing oligomerization properties of the α, β, and γ isoforms (27). Exchanging the N termini of the RXR β and γ isoforms exchanges the oligomerization phenotype (Fig. 3A). However, the N terminus plays a negative role in oligomerization, not a positive one; deletion of the N terminus does not affect oligomer formation by RXRγ, and actually enhances oligomerization by RXRβ (ΔN-β and ΔN-γ; Fig. 3A). Thus, the actual oligomerization interface(s) must lie outside the N-terminal domain. Indeed, deletion of both the N terminus and the C-terminal hormone-binding domain disrupted cooperative tetramer formation without abolishing dimerization (ΔNΔCγ; Fig. 3A). We conclude that the zinc-finger domain is sufficient for dimer formation, but that additional, C-terminal sequences are necessary for tetramer formation.

We next tested if the oligomerization properties associated with the C terminus of RXR were transferable to another nuclear receptor. Thyroid hormone receptor-α-1 (TRα-1) binds DNA as a dimer, but does not appear to form tetramers on 4-fold reiterated TR response elements (Refs. 1–7, and data not shown). We therefore replaced the C-terminal hormone-binding domain disrupted cooperative tetramer formation without abolishing dimerization by exchanging the N- and C-terminal deletion of RXRγ (ΔN-γ), and a combined N- and C-terminal deletion of RXRγ (ΔNΔC-γ). 0, 2, 4, 6, or 10 ng of receptor (approximately) were used for each lane. Also tested (panel B) were chimeric proteins representing fusions of the DNA-binding domain of TR to the hormone-binding domain of RXR. Two different chimeras were tested, either containing (left panel) or lacking (right panel) the first 40 amino acids in the TR N terminus. Approximately 1, 2, 4, or 8 ng of the TR-RXR chimera, and 0.75, 1.5, 3, or 6 ng of the ΔN-TR-RXR chimera were added to a fixed amount of radiolabeled response element. The response element in all cases consisted of four half-sites oriented as direct repeats and spaced by 4 bases. The identi
ty of the different receptor-DNA complexes were determined by comparison with oligonucleotide probes containing differing numbers of half-sites (data not shown).

Molecules in the Tetramer Are Non-equivalent—Nuclear hormone receptor dimers can recognize a variety of half-site orientations and spacings (15–17, 23–25). RXR homodimers, for example, bind efficiently to direct, convergent, and divergent repeats of half-sites spaced by 1, 0, or 2 bases, respectively. To dissect the topological constraints operative on the RXR tetramer, we first explored the effects of varying the spacing of the half-sites within the 4-fold reiterated element. Introduction of a 2- or 10-base spacer at the center of the element (i.e. between half-sites 2 and 3) had little or no detectable effect on tetramer formation by RXRγ (Fig. 4A), indicating that the oligomer could readily accommodate changes in spacing at this location. Formation of tetramers by RXRγ was slightly destabilized, though
not abolished, by introduction of 5 bases at the same location (Fig. 4A) presumably reflecting the non-integral rotation about the DNA imposed by this spacer. None of these changes in spacing conferred tetramer binding on the RXRβ isoform (Fig. 4A).

In contrast to its relative lack of effect at the center of the element, the 10-base insertion abolished tetramer formation by RXRγ when introduced between half-sites 1 and 2, or between half-sites 3 and 4 (Fig. 4B). Instead of tetramers, RXRγ bound to these elements relatively weakly, and as a broad complex migrating in a position characteristic of 3-fold occupancy (denoted 3R; Fig. 4B). We attribute this broadening or smearing phenomenon to an instability of this trimeric complex during the electrophoretic separation, a feature that was observed with several other non-optimal elements. Apparently the 10-base insertion at these flanking sites interferes with recruitment of a fourth receptor molecule to the DNA, resulting in formation of a receptor trimer. We conclude that the receptor N terminus of the RXRβ isoform prevents tetramer formation on the consensus reiterated element composed exclusively of direct repeats (Fig. 2A). If this interference with tetramer formation is due to a steric hindrance mediated by the RXRβ N terminus, it might be partially relieved on DNA elements with a different half-site topology. Indeed, although tetramers of RXRβ also failed to form on most of the elements tested (e.g. Fig. 4A), reiterated elements with a divergent orientation between half-sites 2 and 3 bound two molecules of RXRβ with detectable cooperativity, i.e. with some degree of tetramer formation (compare the binding of RXRβ to the \( \langle\rangle\langle\rangle\) or \( \langle\rangle\langle\rangle\langle\rangle\langle\rangle\) orientations versus the \( \langle\rangle\langle\rangle\langle\rangle\langle\rangle\rangle\langle\rangle\) orientation; Fig. 6). Nonetheless, the extent of tetramer formation by RXRβ on these divergent elements was signifi-

**FIG. 4.** The effects of varying the half-site spacing on tetramer formation by RXRβ and RXRγ. A series of reiterated response elements was created containing four-half-sites, all oriented as direct repeats as in Fig. 2, but differing in the spacing between half-sites. The elements are presented schematically above the relevant panels, with each individual half-site depicted as an arrow. The ability of wild-type RXRβ and γ to bind these elements was examined by electrophoretic shift assay as in Fig. 2. The effect of introducing different length spacers between half-sites 2 and 3 (panel A) and of introducing the same length spacer, but between different half-sites (panel B), were tested. Approximately 1, 3, or 6 ng of receptor were used in panel A, and 0, 2, 4, 6, or 8 ng of receptor in panel B. Lanes labeled “Dimer” and “Tetramer” represent the complexes resulting when saturating amounts of RXRβ or γ were bound to elements containing two or four half-sites, respectively, and were used as markers.

**FIG. 5.** The effects of varying the half-site orientation on tetramer formation by RXRγ. The same form of experiment was performed as in Fig. 4, but using response elements bearing different orientations of half-sites, as indicated by the arrows above each panel. The bottom panels represent the results for response elements comprising only three half-sites (8 indicates the presence of an 8-base spacer between the second and last half-site; see Fig. 1B). Approximately 2, 4, 6, or 8 ng of receptor was used per lane. Marker lanes are as described in Fig. 4.
were inactive in the transient transfection assays, nor did Oligomers—It was intriguing that insertion of a 10-base spacer...was performed, but using the RXRβ isofrom. Marker lanes are as described in Fig. 4.

RXR Tetramer Formation In Vitro Correlated with Reporter Gene Activation in Vivo—We tested the ability of the various response elements to mediate transcriptional activation in transient transfections (Fig. 7). Generally, the reiterated elements that permitted tetramer formation by RXRγ in vitro also conferred efficient RXRγ-mediated gene activation in transient transfection assay, whereas elements with half-site spacings or orientations that disrupted tetramer formation failed to activate reporter gene expression. This correlation was observed over a range of receptor DNA concentrations (data not shown). Similarly, the tetramer-deficient RXRβ isoform exhibited a much lower activity on all of the reiterated elements tested (Fig. 7), despite RXRβ and γ possessing near equal transcriptional activities on elements composed of only two half-sites (Ref. 27 and data not shown) and despite the use of identical expression vectors and transfection conditions. One dramatic exception to this general correlation was noted, however; our element possessing an inversion of the third half-site (Fig. 7A) conferred extremely strong transcriptional activation by RXRγ, despite failing to allow efficient tetramer formation in vitro (Fig. 7B).

Hormone has been reported to enhance dimer formation by RXRs on two-half-site elements (13). We therefore asked if the presence of hormone could influence RXR tetramer formation, perhaps accounting for the apparent discrepancy between our DNA binding studies (performed in the absence of hormone) and the transfection studies (measured in its presence). Indeed, inclusion of 9-cis-retinoic acid in the DNA binding assay significantly enhanced RXRγ tetramer formation on several of the reiterated elements tested. This effect was particularly strong for the prototype direct repeat element (Fig. 5A) and for the element containing an inverted third half-site (Fig. 5B), accounting at least partly for the strong activity of the latter element in transient transfections. Notably, hormone did not enhance RXRγ tetramer formation on the elements that were inactive in the transient transfection assays, nor did hormone confer tetramer binding by RXRβ on any of the elements tested (data not shown). Under these conditions, inclusion of 9-cis-retinoic acid had only modest effects on dimer formation by RXRγ, as determined on elements containing only two half-sites (Fig. 5A and data not shown).

Multiple, Spatially Distinct HREs May Function in an Analogous Manner as Reiterated Elements by Recruiting Receptor Oligomers—It was intriguing that insertion of a 10-base spacer in the center of a reiterated element was fully compatible with tetramer formation and with reporter gene activation. This modified element is, in essence, two dimeric response elements (>) separated by one turn of the DNA. It is known that multiple copies of a dimeric response element, when introduced into a promoter, can stimulate reporter gene expression to levels much greater than that seen with one copy (e.g. 33–36). To test if this synergy on separated dimeric response elements is related to the ability of receptors to form high order oligomers, we compared activation by RXRβ versus RXRγ on reporter genes containing multiple dimeric response elements (each separated by 18 bases). Indeed, increasing the number of dimeric response elements resulted in a synergistic increase in reporter gene activation by RXRγ, but had only a much weaker effect on RXRβ (Fig. 9, compare B to A). Thus the ability of a receptor to form oligomers on a single reiterated half-site element closely parallels the ability of the same receptor to mount a synergistic response on multiple, "separate" dimeric elements. This suggests that formation of high order receptor-DNA complexes may underlie both phenomena.

**DISCUSSION**

**RXR Tetramers Form on Reiterated Response Elements with a Broad Topological Plasticity**—The specificity of nuclear hormone receptors for their cognate response elements has traditionally been believed to operate at two levels: (a) recognition of the nucleotide sequence of the individual half-sites by each receptor monomer, mediated by contacts between the receptor...
Fig. 8. The effects of hormone on tetramer formation. The same electrophoretic mobility shift protocol as in Figs. 2, 4, and 5 was repeated, but in the presence (+) or absence (−) of 1 μM 9-cis-retinoic acid in the binding reaction. Reiterated direct repeat elements bearing two or four half-sites (panel A) and an element bearing an inverted half-site 3 (panel B) were tested. Approximately 1, 2, 3, or 4 ng of receptor were used for each lane in panel A, and 1 or 2 ng of receptor for each lane in panel B. Marker lanes are as described in Fig. 4.

Fig. 9. Effect of multiple copies of response elements on RXR-mediated reporter gene regulation. A dimeric response element (composed of two copies of an AGGTCA half-site) was introduced into the pD33-CAT reporter as either one, two, three, or four copies. The reporter (1 μg) was introduced into SL-2 cells together with 1 μg of RXRβ (panel A) or RXRγ (panel B) expression vector, the cells were incubated in the absence (hatched bars) or presence (filled bars) of 9-cis-retinoic acid and harvested, and the chloramphenicol acetyltransferase activity was determined relative to a M 9-cis RA standard, as detailed under “Experimental Procedures.” CAT activity in the absence of hormone was near zero and is not readily visible at this scale.

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zinc-finger domain and bases in the DNA, and (b) recognition of the spacing and orientation of the half-sites in an element, determined by the nature of the protein-protein interface in the receptor dimer (17–25). More recently, however, we have suggested that there is a third level of DNA recognition, conferred by the ability of certain nuclear receptors to bind cooperatively as high order oligomers to response elements containing highly reiterated half-sites (27). The ultimate affinity of a receptor for a given response element appears to be the combined consequence of all three components, and the destabilizing effects of a non-optimal half-site sequence can be counteracted if the half-sites are highly reiterated (27).

Many nuclear hormone receptors display a wide diversity in their ability to recognize dimeric response elements containing a variety of half-site orientations and spacings (1–7, 18–25). In common with these dimers, the RXR tetramer also exhibits a broad plasticity in its ability to accommodate a variety of half-site orientations and spacings in the reiterated elements examined here. Not all orientations were acceptable between all half-sites (see below), but a surprising mixture of direct, convergent, and divergent orientations could be efficiently bound by receptor tetrarmers in vitro and mediate reporter gene activation in vivo.

Tetrarmers of RXR have also been observed in solution (28–30). Although probably reflecting a similar phenomena, it is unclear if these free tetrarmers are the immediate precursors of the DNA-bound complexes we observe here. The tetrarmers in solution are destabilized by hormone (28–30), in contrast to the hormone-mediated enhancement of tetramer formation we observe on DNA. In addition, for the RXR tetrarmers in solution to be precursors to DNA-associated tetrarmers, either they must pre-exist as a mixed population of differing monomer orientations or they must rearrange on DNA binding so as to allow recognition of a wide range of different half-site orientations and spacings.

At Least Two Distinct Protein-Protein Interfaces Are Involved in Receptor Oligomerization—Dimers of nuclear hormone receptors are stabilized by protein-protein interfaces that map to both zinc-finger and hormone-binding domains (Fig. 10, A and Refs. 18–26). The protein-protein interface in the zinc-finger domain is thought to dictate recognition of half-site spacing and orientation; small changes in the spacing or orientation of half-sites can destabilize dimer binding by preventing formation of the proper receptor-receptor contacts in this zinc-finger domain (15, 17, 19, 20, 24, 25). In contrast, the dimerization interface in the hormone-binding domain exhibits significant
functional plasticity, and is able to stabilize receptor dimer formation on response elements displaying a variety of half-site orientations and spacings (see Refs. 24–26, and references therein). This contrast between the tight topological constraints imposed by the zinc-finger dimerization domain, compared with the more permissive nature of the hormone-binding domain interface, implies either the presence of a flexible “swivel” between the zinc-finger and hormone-binding domain, or the existence of multiple, alternative dimerization interfaces that are differentially invoked with the different half-site orientations (24–26).

In light of this work on dimers, it is notable that the topological requirements for tetramer formation are non-equivalent at the different half-sites in the reiterated elements. Most striking, the spacer between half-sites 2 and 3 can vary significantly in length without effect on RXR tetramer formation, whereas similar changes in the spacing between half-sites 1 and 2, or between half-sites 3 and 4, strongly impair tetramer formation. These requirements suggest that the interactions between RXRs at half-sites 1 and 2 (or between 3 and 4) may be mediated through the zinc-finger domain interfaces previously described for two-half-site elements. In contrast, the ability of half-sites 2 and 3 to accommodate a variety of spacers is consistent with the properties observed for the hormone-binding domain interface.

Thus the RXR tetramer may be thought of as a “dimer of dimers,” with one dimer bound to half-sites 1 and 2, and a second, interacting dimer cooperatively bound to half-sites 3 and 4. Two possible conceptualizations, both consistent with the data presented here, are shown in Fig. 10. In the first model (Fig. 10B), the interface holding the two dimers together is the same hormone-binding domain interface as that previously described for receptor dimers, but now oriented away from the zinc-finger domain interface. In the second model (Fig. 10C), two receptor dimers are held together by a novel interface, also located within the hormone-binding domain, but now distinct from the domain previously characterized in stabilization of receptor dimers. In either model, additional determinants mapping outside of the hormone-binding domain would function in certain contexts to restrict tetramer formation (as observed for the N-terminal deletions and the TR-RXR chimera).

The Ability of Receptors to Bind Cooperatively to Reiterated Half-sites May Contribute to the Synergistic Transcriptional Activation Observed with Multiple “Dimeric” Response Elements—Naturally occurring, hormone-responsive promoters often possess multiple copies of the relevant response element (e.g., Refs. 33–37). These multiple response elements appear to act synergistically to enhance the magnitude of the hormone response (33–39). We demonstrate here a correlation between the ability of different RXRs to bind to a single, tetramer response element, and the ability of these same receptors to synergistically activate transcription from multiple, dimeric response elements. We suggest that multiple, dimeric response elements are functionally equivalent to a single reiterated element containing a large central insertion. Thus, the synergistic effects observed with multiple HREs may reflect, in part, a cooperative binding of high order receptor oligomers to these repeated elements (36).

Recognition of Reiterated Elements May Extend to Other Members of the Nuclear Hormone Receptor Family, and Might Provide a Potential Nexus for Combinatorial Transcriptional Regulation—It is notable that many naturally occurring response elements comprise three or more half-sites (e.g., Refs. 38–40). It is tempting to suggest that these multiple half-sites are recognized by either homo- or hetero-oligomeric forms of receptors. There are several attractive consequences of such a hypothesis. (a) The same naturally occurring elements often contain non-optimal half-site sequences that, in dimeric elements, are destabilizing for receptor binding. In the reiterated elements, the destabilizing effects of the non-optimal half-sites would be compensated by the multiple DNA-protein contacts provided by the cooperative binding of a receptor oligomer. (b) Reiterated sites also provide a potent nexus for combinatorial regulation, perhaps recruiting mixed oligomers of different receptors. The constituents of the receptor oligomer might vary from cell to cell, or from response element to response element, permitting the transcriptional response to be precisely tailored in a cell-type and promoter-specific manner.

It is intriguing that the STAT family of transcription factors also bind cooperatively to reiterated DNA sites, a phenomenon that plays an important role in DNA recognition by, and functional interactions between different STAT family members (41). We suggest that oligomer formation, as observed here for RXR, may be a common means of generating DNA specificity and combinatorial regulation for a variety of transcription factors.

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REFERENCES
1. Carlson-Jurica, M. A., Schrader, W. T., and O’Malley, B. W. (1990) Endocrinol. Rev. 11, 210–220
2. Glass, C. K., and Holloway, J. M. (1990) Biochim. Biophys. Acta 1032, 157–176
3. Lazar, M. A. (1993) Endocrinol. Rev. 14, 184–193
4. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 833–840
5. Mangelsdorf, D. J., and Evans, R. M. (1995) Cell 83, 841–850
6. Beato, M., Herrlich, P., and Schutz, G. (1995) Cell 83, 851–858
7. Kastner, P. M., and Chambon, P. (1995) Cell 83, 859–870
8. Hamada, K., Gleason, S. L., Levi, B. Z., Hirschfeld, S., Appella, E., and Ozato, K. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8289–8294
9. Mangelsdorf, D. J., Ong, E. S., Dyck, J. A., and Evans, R. M. (1991) Nature 345, 322–329
10. Rowe, A., Eager, N. S., and Brickell, P. M. (1991) Development 111, 771–778
11. Levin, A. A., Sturzenbecker, L. J., Kazmier, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Klatzeisen, C., Rosenbergber, M., Lovey, A., and Gripp, J. F. (1992) Nature 355, 359–361
12. Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stern, R. B., Eichele, G., Evans, R. M., and Thaller, C. (1992) Cell 68, 397–406
13. Zhang, X.-K., Lehmann, J., Hoffmann, B., Dawson, M. I., Cameron, J., Graupner, G., Hermann, T., Tran, P., and Pfahl, M. (1992) Nature 358, 587–591
14. Mangelsdorf, D. J., Borgmeyer, U., Heyman, R. A., Zhou, J. Y., Ong, E. S., Oro, A. E., Kakiuchika, A., and Evans, R. N. (1992) Genes Dev. 6, 329–344
15. Naar, A. M., Boutin, J.-M., Lipkin, S. M., Yu, V. C., Holloway, J. M., Glass, K. C., and Rosenfeld, M. G. (1991) Cell 65, 1267–1280
16. Umesono, K., Murakami, K. K., Thompson, C. C., and Evans, R. M. (1991) Cell 65, 1255–1266
17. Forman, B. M., Casanova, J., Raaka, B. M., Gyysdaiel, J., and Samuells, H. H. (1992) Mol. Endocrinol. 6, 429–442
18. Forman, B. M., and Samuells, H. H. (1991) Nat. Neurol. 2, 587–594
19. Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. E., Yamamoto, K. R., and Sigler, P. B. (1991) Nature 352, 497–505
20. Schwabe, J. W. R., Chapman, L., Finch, J. T., Rhodes, D. (1993) Cell 75, 576–578
21. Rastinejad, F. M., Perlmann, T. P., Evans, R. M., and Sigler, P. B. (1995) Nature 375, 203–211
22. Ikeda, M., Rhee, M., and Chin, W. W. (1994) Nature 375, 1628–1638
23. Mader, S., Lery, P., Chen, J. Y., and Chambon, P. (1993) J. Biol. Chem. 268, 591–600
24. Perlmann, T., Rangarajan, P. N., Umesono, K., and Evans, R. M. (1993) Genes Dev. 7, 1411–1422
25. Kurokawa, R., Yu, V. C., Naar, A., Kyakumoto, S., Han, Z., Silverman, S., Rosenfeld, M. G., and Glass, K. C. (1993) Genes Dev. 7, 1423–1435
26. Perlmann, T., Umesono, K., Rangarajan, P. N., Forman, B. M., and Evans, R. M. (1996) Mol. Endocrinol. 10, 958–966
27. Chen, H., and Privalsky, M. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 422–426
28. Kersten, S., Kelleher, D., Chambon, P., Gronemeyer, H., and Noy, N. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8645–8649
29. Kersten, S., Fan, L., and Noy, N. (1998) Biochemistry 34, 14263–14269
30. Kersten, S., Fan, L., Chambon, P., Gronemeyer, H., and Noy, N. (1995) Biochemistry 34, 13717–13721
31. Chen, H.-W., and Privalsky, M. L. (1993) Mol. Cell. Biol. 13, 5970–5980
32. Chen, H.-W., Smith-McBride, Z., Lewis, S., Sharif, M., and Privalsky, M. L.
33. Seiler-Tuyns, A., Walker, P., Martinez, E., Merillat, A.-M., Givel, F., and Wahli, W. (1986) *Nucleic Acids Res.* **14**, 8755–8770
34. Jantzen, H.-M., Strahle, U., Gloss, B., Stewart, P., Schmid, W., Boshart, M., Miksicek, R., and Schutz, G. (1987) *Cell* **49**, 29–38
35. Klein-Hitpas, L., Kaling, M., and Ryffel, G. U. (1988) *J. Mol. Biol.* **201**, 537–544
36. Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. (1989) *Cell* **57**, 443–448
37. Norman, M. P., Lavin, T. N., Baxter, J. D., and West, B. L. (1989) *J. Biol. Chem.* **264**, 12063–12073
38. Liu, H., C., and Towle, H. C. (1994) *Mol. Endocrinol*. **8**, 1021–1037
39. Mangelsdorf, D. J., Umesono, K., Kliewer, S. A., Borgmeyer, U., Ong, E. S., and Evans, R. M. (1991) *Cell* **66**, 555–561
40. Brent, G. A., Haarney, J. W., Chen, Y., Warne, R. L., Moore, D. D., and Larsen, P. R. (1989) *Mol. Endocrinol*. **3**, 1996–2005
41. Xu, X. A., Sun, Y. L., and Hoey, T. (1996) *Science* **273**, 794–797
