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Cooperative formation of high-order oligomers by retinoid X receptors: An unexpected mode of DNA recognition

(retinoid X response element/CRBP II element/nuclear hormone receptors)

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ABSTRACT **Nuclear hormone receptors are ligand-regulated transcription factors that function in metazoan homeostasis and differentiation. We report here that DNA recognition by at least one class of nuclear receptors, the retinoid X receptors (RXRs), can occur through an unanticipated mechanism involving the cooperative formation of protein tetramers and other high-order oligomers. Formation of these oligomeric complexes enables RXRs α and γ, but not β, to efficiently regulate transcription through response elements poorly recognized by RXR dimers. Thus, oligomer formation plays an important role in determining the specificity of DNA recognition by nuclear receptors and contributes to isoform-dependent differences in gene regulation.**

Cellular responses to retinoid, thyroid, and steroid hormones are mediated by a family of interrelated nuclear hormone receptors (reviewed in refs. 1 and 2). Members of this family function as ligand-regulated transcription factors, binding to specific sites within the DNA genome (denoted hormone response elements; HREs) and modulating the expression of adjacent target genes (1, 2). The retinoid X receptors (RXRs) in particular play important roles in vertebrate development and differentiation, both autonomously in response to the hormone 9-*cis*-retinoic acid and combinatorially in conjunction with other nuclear hormone receptors (refs. 2–10 and references therein). In common with other nuclear hormone receptors, RXRs contain a zinc-finger type II DNA binding domain linked to a C-terminal hormone-binding domain (Fig. 1*A*).

RXRs, like most other nuclear hormone receptors, have been thought to bind to DNA principally as polypeptide dimers (10, 12). Each receptor molecule binds to a "half-site," a conserved hexanucleotide DNA sequence; therefore, functional HREs are commonly composed of two half-sites (13–15). Both the sequence of the half-sites and their relative orientation and spacing determine receptor recognition; RXR homodimers bind most strongly to direct repeats of an AGGTCA half-site displayed with a 1- or 2-base spacer (10, 13–16). Nonetheless, many aspects of DNA recognition by the nuclear hormone receptors remain incompletely understood. Naturally occurring response elements frequently consist of three or more half-sites displayed in a variety of configurations (e.g., see refs. 11 and 17). The half-sites themselves can vary significantly in sequence without abolishing function, and nucleotides flanking the hexanucleotide half-site may also influence receptor recognition (e.g., see refs. 18 and 19). Many nuclear receptors are expressed in the form of multiple variants, termed isoforms; three major RXR isoforms (α, β, and γ) have been identified and are encoded by three distinct genetic loci (Fig. 1*A*; refs. 2–6). The roles of these different receptor isoforms in the regulation of different target genes remain relatively unknown.

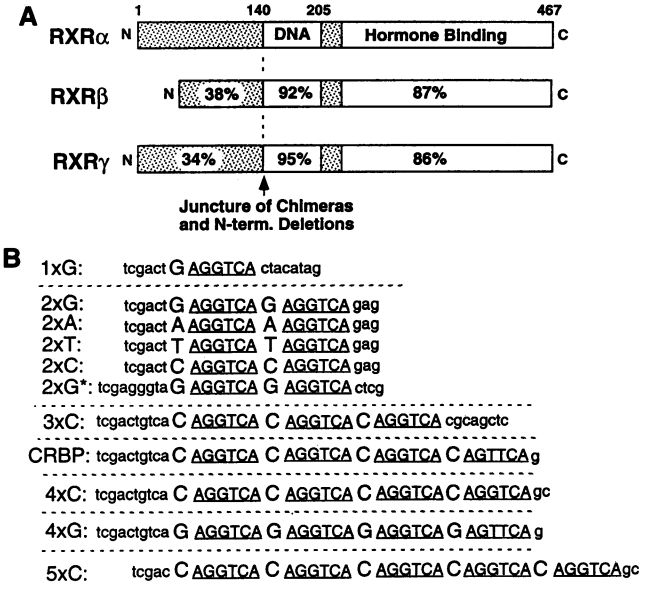

FIG. 1. RXR isoforms and response elements used in these studies. (*A*) Three major RXR isoforms are presented schematically, from N to C terminus, with the N-terminal, DNA binding, and hormone binding domains indicated. Numbers above refer to corresponding amino acid positions. Conservation of each domain among the three different murine isoforms is expressed as percentage amino acid identity. N-terminal deletions and RXRβ/γ and RXRγ/β chimeras were created at the location indicated by the arrow. (*B*) Response elements used in DNA binding and transient transfection assays are shown. Each element is designated (left) by number of half-sites present and identity of the −1 base. Sequence of each element is indicated, with the half-sites underscored and the −1 base highlighted. Flanking sequences are lowercase. CRBP indicates wild-type rat CRBP-II element (11).

We wished to better understand the ability of different receptors and their isoforms to discriminate among different response elements. We demonstrate here that a natural retinoid X response element (the cellular retinol-binding protein II element, denoted CRBP-II element; Fig. 1*B*) is actually composed of very weak half-sites, which in other contexts are not recognized by RXRs. Instead, the CRBP-II element acts as an efficient retinoid X response element due to a 4-fold reiteration of these otherwise ineffective half-sites, resulting in the cooperative recruitment of a previously uncharacterized tetrameric form of RXR to the DNA. The ability to form this tetrameric complex is RXR isoform specific. Our results suggest that, in addition to recognition of half-site sequence and spacing, nuclear hormone receptors also discriminate among different HREs through the formation of higher-order complexes on multiply reiterated half-sites.

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Abbreviations: HRE, hormone response element; RXR, retinoid X receptor.

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MATERIALS AND METHODS

Molecular Clones. Baculovirus expression constructs were prepared by introducing human RXRα, murine RXRβ, or murine RXRγ cDNAs into a pVL1393 transfer vector as *Eco*RI/*Eco*RI, *Eco*RI/*Pst* I, or *Eco*RI/*Eco*RI fragments, respectively (20), and recovering baculovirus recombinants *in vivo* by use of the BaculoGold *in vivo* recombination system (PharMingen). The ΔN-terminal RXRβ or -γ mutants were constructed by using synthetic primers (either 5′-CCGGAATTCCACCATGCGGCTCTGTGCAATCTGC-3′ for the β isoform or 5′-CCGGAATTCCACCATGCACATCTGTGCCATCTGT-3′ for the γ isoform) together with a downstream primer (complementary to the pVL1393 transfer vector) to selectively amplify the CDEF regions of these receptors by PCR (21). Chimeras exchanging the AB and CDEF domains of the β and γ RXR isoforms were created by a similar PCR procedure centered on the first cysteine in the zinc-finger domain. A glutathione *S*-transferase/RXRγ fusion construct was isolated as described (9). For transient transfection studies, the RXR molecular clones were introduced into the *Drosophila* A5C expression vector (9); the N-terminal deletion mutants and chimeras were inserted as *Eco*RI fragments derived from the pVL1393 constructs. The different HRE oligonucleotides (Fig. 1*B*) were synthesized chemically and inserted into the *Sal* I site of the pD33-CAT reporter construct (9).

Electrophoretic Mobility Shift and Transient Transfection Assays. RXR proteins were obtained by infecting Sf-9 cells with recombinant baculoviruses and preparing nuclear extracts (20). The RXRα, RXRβ, and ΔN-terminal RXRβ proteins were estimated by SDS/PAGE to be present at 20 ng/μl in the final nuclear extracts; the RXRγ and ΔN-terminal RXRγ proteins were estimated at 30 ng/μl. Electrophoretic mobility-shift assays were performed as described (9, 20). Transient transfections were performed in *Drosophila* SL-2 cells, with chloramphenicol aminotransferase activity determined relative to that of a cotransfected β-galactosidase control (9).

RESULTS

A Heptanucleotide, Not a Hexanucleotide, Is the Minimal Half-Site Sequence Efficiently Recognized by RXRs. A hexanucleotide sequence, AGGTCA, has been described as a consensus half-site for RXR receptors (10, 13–16). We observed, however, that the identity of the base 5′ of the hexanucleotide half-site had a dramatic influence on RXR binding. Direct repeats of two CAGGTCA or two TAGGTCA half-sites (denoted 2×C and 2×T, respectively; Fig. 1*B*) were relatively poorly bound by RXRα or -γ (Fig. 2). In contrast, direct repeats of two AAGGTCA or two GAGGTCA half-sites (denoted 2×A and 2×G; Fig. 1*B*) were bound with high affinity (Fig. 2). The effects of the −1 base were slightly less critical for RXRβ; binding of RXRβ to the TAGGTCA element was weak (Fig. 2, lane 8) and binding to the CAGGTCA element was weaker still (best seen in Fig. 3*A*, lane 4) but nonetheless detectably greater than that of the other two isoforms. In contrast to the −1 base, changes in more distal DNA sequences had little or no effect on RXR binding (Fig. 2, lanes 6 and 13; data not shown). We conclude that the actual half-site for RXR recognition is at least 7 nucleotides.

The CRBP-II Retinoid X Response Element Consists of Nonoptimal Half-Sites Which, Through Reiteration, Allow 4-Fold Occupancy by Receptor. Paradoxically, the rat CRBP-II promoter contains a strong RXRα and -γ-responsive element that nonetheless consists of repeats of the dysfunctional CAGGTCA half-site (11) (Fig. 1*B*). Why is this element efficiently recognized by these RXRs? The CRBP-II element is composed of three precise repeats of the CAGGTCA half-site, with an additional fourth imperfect half-site (CAGTTCA) present at the 3′ end. Perhaps the high degree of reiteration of the half-sites in the CRBP-II element compensates for their nonoptimal nature. Indeed, both RXRα and RXRγ efficiently bound to the CRBP-II element *in vitro* and generated a protein–DNA complex of slower electrophoretic mobility than that formed on a simple half-site element (Fig. 3*A*; compare lanes 3 and 9 to lanes 2 and 8). In contrast, the RXRβ isoform bound to the CRBP-II element less efficiently and with a mobility equal to that on the two half-site element (Fig. 3*A*, lanes 5 and 6). No binding of CRBP-II probe was observed in the absence of RXRs (i.e., using control extracts of baculovirus/Sf-9 cells not expressing exogenous RXRs; data not shown). The distinct mobilities of the different RXR complexes were inherent properties of the receptors and were not due to the differences in the length of the DNA probes; the 2×G and 2×G* probes, for example, differ in length, but form RXR complexes of indistinguishable mobility (Fig. 2; data not shown), and dimers of the RXRβ isoform exhibit identical mobility on both the 2×G and CRBP-II elements (Fig. 3*A*).

To determine the nature of the RXR complexes on the CRBP-II element, we used DNA elements consisting of differing numbers of half-sites (Fig. 1*B*). The RXRs did not bind to an element containing only a single half-site, indicating that these receptors cannot recognize DNA as individual protein monomers (Fig. 3*B*, lanes 1 and 8). Two GAGGTCA half-sites formed a strong complex with RXRγ with a mobility we interpret as indicative of a receptor dimer (Fig. 3*B*, lane 9). In contrast, the CRBP-II element–RXRγ complex exhibited a mobility identical to that seen with artificial elements containing four half-sites (elements denoted 4×C and 4×G; Fig. 3*B*, lanes 11–13). Abrogation of the 3′ proximal half-site in the CRBP-II sequence (leaving three half-sites intact; denoted 3×C) produced a RXRγ complex migrating between those formed by the 2×G and the native CRBP-II elements, whereas an element with five half-sites (denoted 5×C) produced a RXRγ complex with a slower mobility than that of the native CRBP-II element (Fig. 3*B*, lanes 10–14; see below). Similar results were obtained with RXRγ synthesized in bacteria and with RXRα (Fig. 4*I*; data not shown). We conclude that the

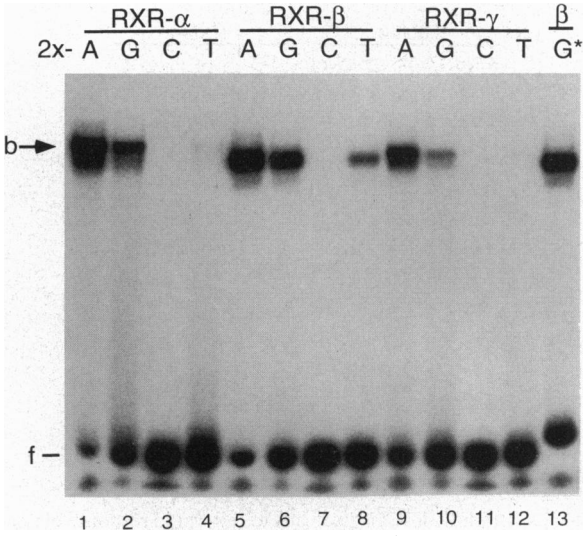

FIG. 2. Binding by different RXR isoforms to response elements bearing distinct −1 base sequences. Ability of each RXR isoform to bind to the indicated response elements was determined by electrophoretic mobility-shift assay with human RXRα, mouse RXRβ, or mouse RXRγ synthesized in a recombinant baculovirus/Sf-9 cell system. All radiolabeled DNA probes were composed of two repeats of an nAGGTCA half-site, with the −1 position indicated above (see also Fig. 1*B*). Positions of free DNA probe (f) and bound DNA–RXR complexes (b) are indicated.

native CRBP-II element contains four functional half-sites, all of which can be occupied by a RXRα or -γ protein molecule.

RXRα and -γ Bind the CRBP-II Element in a Highly Cooperative Manner As Protein Tetramers and Cooperatively Form Similar High-Order Receptor Complexes on a Variety of Reiterated Elements. Do the RXRα and RXRγ complexes on the CRBP-II element represent true protein tetramers (i.e., four interacting protein molecules), or do they simply represent independent occupancy of the four half-sites by separate receptor monomers? Arguing against the latter possibility is our observation that RXR monomers do not bind to single half-sites. More significantly, formation of a true tetrameric complex should occur cooperatively and preferentially over the formation of dimeric complexes. Indeed, even at limiting protein concentrations, both RXRα (data not shown) and RXRγ (Fig. 4*A*) preferentially and cooperatively formed tetramers on the CRBP-II element with no detectable formation of lower-order complexes. This cooperative formation of tetramers by RXRα and -γ was not unique to the native CRBP-II element but also occurred on a variety of 4-fold reiterated elements, such as the 4×C element containing four perfect CAGGTCA repeats, or the 4×G element in which the −1 position in each half-site of the CRBP-II sequence was altered to a G (Fig. 3*B*, lanes 12 and 13; Fig. 4 *B* and *C*). Identical results were observed with a purified RXRγ isolated from a glutathione *S*-transferase/*E. coli* expression system (Fig. 4*I*). In fact, oligomer formation by RXRγ was not

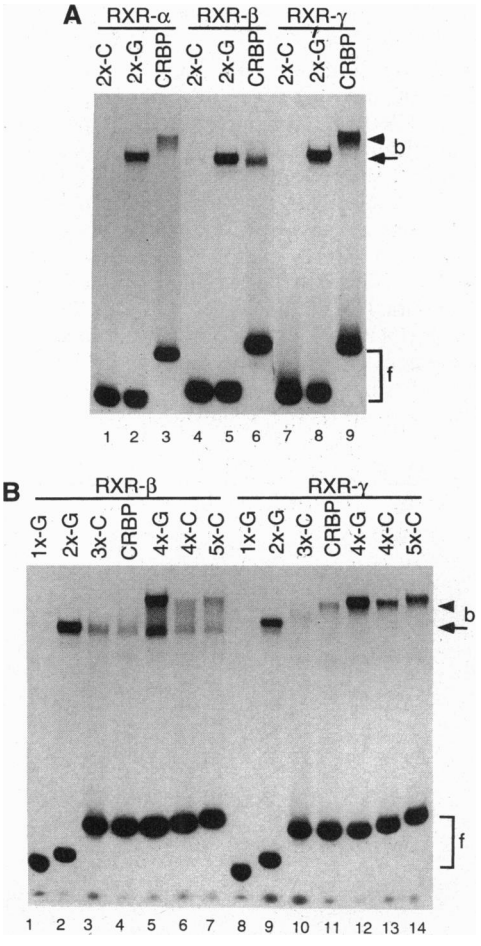

FIG. 3. Binding of RXR isoforms to reiterated response elements. (*A*) Binding of the CRBP-II element by RXR isoforms was compared by electrophoretic mobility-shift assay to that of elements possessing two half-sites. Radiolabeled DNA probes were as designated in Fig. 1*B*. Arrow (right) indicates presumptive dimeric complex; arrowhead indicates slower migrating CRBP-II complex. (*B*) Nature of the RXRγ–CRBP-II response element complex was characterized by electrophoretic mobility-shift assay, using a series of response element oligonucleotides, designated as in Fig. 1*B*. Bound (b) and free (f) DNA complexes are indicated, with arrow indicating dimeric complex and arrowhead indicating tetrameric complex.

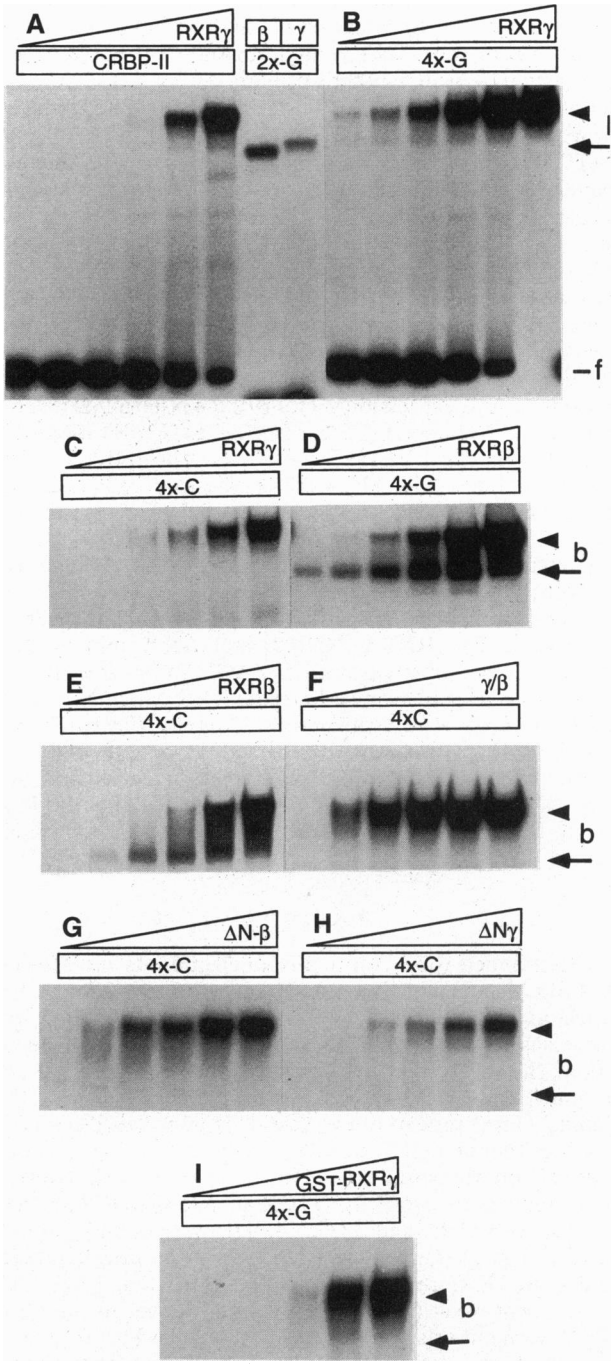

FIG. 4. Titrations of RXR isoforms on different response elements. Electrophoretic mobility-shift assay was repeated for a variety of RXR isoforms but using a range of different protein concentrations. Nature of each response element and the corresponding RXR isoform are indicated above. Murine RXRs synthesized in a baculovirus/Sf-9 system were used in *A–H*; an avian RXRγ purified from a glutathione *S*-transferase/*Escherichia coli* system was used in *I*. γ/β refers to a chimeric receptor joining the AB domain of RXRγ to the CDEF domains of RXRβ; ΔN-β and ΔNγ refer to mutant proteins bearing precise deletions of the A/B domain of RXRβ and RXRγ, respectively. For *A*, *B*, and *D*, nuclear extracts were diluted 1:60 and used at 0.35, 0.75, 1.5, 3, 6, and 12 μl per lane; for *C* and *E–H*, nuclear extracts were diluted 1:10 and used at 1.5, 3, 4.5, 6, 9, and 12 μl per lane.

restricted to tetramer formation but extended to presumptive trimers seen on a three half-site element and pentamers on a five half-site element (Fig. 3*B*, lanes 10 and 14). In common with the tetramers, these RXRγ trimers and pentamers formed cooperatively and without evidence of dimer formation, although with lower overall DNA binding affinity than that exhibited by the tetrameric forms (data not shown).

The Ability of RXRs to Cooperatively Form High-Order Complexes Is Isoform Specific and Maps to the N-Terminal Receptor Domain. In contrast to RXRγ, the RXRβ isoform bound to both the CRBP-II element and to the other reiterated elements preferentially as a dimer (Fig. 4 *D* and *E*; data not shown). Although slower-migrating complexes could be detected at high RXRβ protein concentrations, particularly on the optimized 4×C and 4×G elements (Fig. 3*B*, lanes 5–7), these RXRβ complexes were not true tetramers but instead displayed the characteristics expected of a saturation of the response element by two, noninteractive protein dimers (Fig. 4 *D* and *E*). Notably, no trimeric or pentameric complexes were detectable for the β isoform (Fig. 3*B*, lanes 3 and 7). Addition of 9-*cis*-retinoic acid modestly enhanced overall DNA binding by all three different RXR isoforms (10) but did not significantly alter the relative proportions of the different receptor–DNA complexes. Specifically, 9-*cis*-retinoic acid neither induced high-order oligomer formation by the RXRβ isoform nor interfered with the cooperative complexes formed by the α or γ isoforms (data not shown).

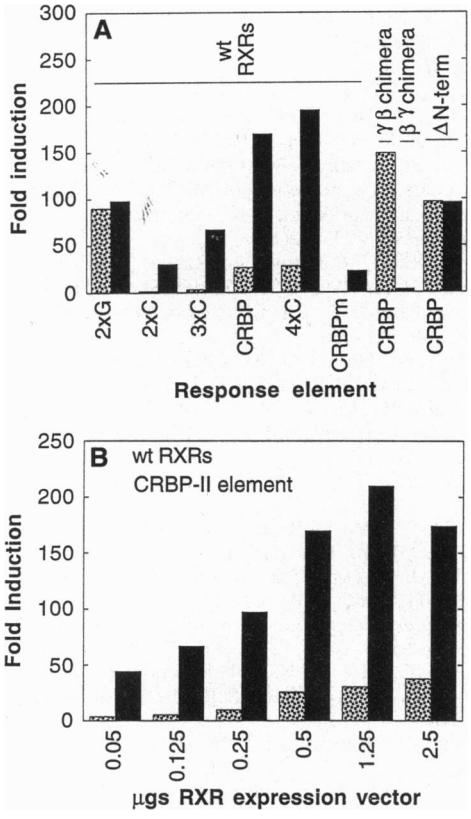

FIG. 5. Activity of different RXR and response element derivatives in transient transfections. Transient transfections were performed in *Drosophila* SL-2 cells and relative chloramphenicol aminotransferase activity was determined (expressed as fold induction in the presence versus the absence of 1 μM 9-*cis*-retinoic acid). All reporters possessed single copies of the oligonucleotides indicated except for the 2×C and 2×G reporters, which possessed two or three copies, respectively (single copy insertions of the 2× elements generated levels of reporter gene activation too low to accurately quantitate). (*A*) The indicated receptor expression constructs were introduced at 0.5 μg of DNA and reporter genes at 1 μg of DNA per plate. Stippled bars represent activity of wild-type RXRβ or RXRβ-derived mutants. Solid bars represent activity of wild-type RXRγ or RXRγ-derived mutants. CRBPm reporter contained an inactivating mutation in the second half-site of the CRBP-II element (CAGGTCA to CAGAACA). (*B*) Experiment in *A* was repeated but using a range of receptor DNA concentrations, as indicated, together with a fixed (1 μg) amount of the reporter containing the CRBP-II element. Symbols are as in *A*.

The differing abilities of the RXR isoforms to form high-order oligomers mapped to their N-terminal A/B domains. Replacement of the A/B domain of RXRβ with that of RXRγ (i.e., an RXRγ/β chimera) conferred on the chimera the ability to bind cooperatively as tetramers, even at comparatively low protein concentrations (compare Fig. 4*F* to Fig. 4*E*). Intriguingly, the A/B domain functioned not by mediating formation of these high-order complexes but by inhibiting it; deletion of the A/B domain permitted both RXRβ and -γ to form tetramers on suitably reiterated response elements (Fig. 4 *G* and *H*; data not shown). We conclude that differences in the A/B domains of the different RXR isoforms account for their different abilities to form tetramers but that the actual protein interfaces involved in this high-order oligomer formation must lie in more C-terminal regions. The protein domains involved in oligomer formation may therefore include, but must be functionally distinct from, those involved in dimer formation (12, 18, 22–24).

RXR Binding to the CRBP-II Element As a Tetramer *in Vitro* Correlates with Reporter Gene Activation *in Vivo*. RXRγ, but not RXRβ, strongly activated reporter genes linked to the CRBP-II or similarly reiterated elements, whereas RXRβ and -γ exhibited near equal activity on simple 2×G elements (Fig. 5*A*; parallel results have been reported for COS cells; see ref. 25). This isoform-specific utilization of the CRBP-II element was maintained over a range of receptor DNA concentrations (Fig. 5*B*); the weak activation by RXRβ at high receptor transfection levels presumably reflects simple saturation of the CRBP-II element with RXRβ dimers, as observed *in vitro* (e.g., Fig. 4 *D* and *E*). The ability of RXRγ to utilize the CRBP-II element was, as expected, greatly impaired by mutating an internal half-site of the CRBP-II element so as to prevent tetramer formation (CRBP-IIm; Fig. 5*A*). Exchanging the N-terminal A/B domain between β and γ isoforms exchanged the ability of the receptor to function on these highly reiterated elements (Fig. 5*A*). Deletion of the N terminus abrogated the distinction between β and γ isoforms, permitting both to activate gene expression through the CRBP-II element (Fig. 5*A*).

DISCUSSION

The ability of the CRBP-II element to function as a strong RXR response element, despite the presence of nonoptimal half-sites, is conferred by the ability of RXRα and -γ to cooperatively bind to this element as tetramers, an unusual form of receptor recognition. Although it has been previously noted that RXRs can occupy the multiple half-sites in the CRBP-II element (11), the cooperative nature of the complex, its isoform specificity, and its functional significance were not explored. Our observations strongly argue that the RXRα and -γ complex on the CRBP-II element is a true tetrameric form of receptor and is not simply two noninteractive receptor dimers. By this, we mean that the binding of four RXR molecules to the CRBP-II element is highly cooperative and interactive and occurs without the detectable formation of dimeric complexes. As such, the behavior of RXRα and -γ strongly contrasts with that of RXRβ, which does bind the CRBP-II element as dimers. Perhaps more significantly, RXRγ forms functional trimers and pentamers on elements composed of three and five half-sites. Therefore, high-order complex formation by RXRγ is not restricted to simple multiples of a dimer but must reflect protein–protein interactions able to stabilize trimeric and pentameric structures as

well. These RXR multimers may preexist in solution or, as appears more likely, may form only on interaction with an appropriate response element. If high-order RXR multimers form only on binding DNA, the initial event may be a transient binding of a RXR monomer or dimer to the DNA site; this relatively weak interaction would be subsequently stabilized by the highly cooperative and extremely rapid recruitment of additional RXR molecules to form the final multimeric complex.

Promoters can exhibit differential responses to different isoforms of the same receptor, presumably a reflection of separate physiological roles for each isoform (3, 25). This isoform-specific promoter utilization has generally been thought to be a form of combinatorial control, reflecting differences in the ability of each isoform to interact with other transcription factors bound to the same promoter (25). Our work demonstrates that isoform specificity can also operate directly at the level of DNA binding. It is particularly intriguing that the inability of RXRβ to function as a tetramer maps to its N-terminal domain; the N termini of these receptors is highly divergent among the three major isoforms (Fig. 1*A*). Furthermore, in rodents, two distinct RXRβ subspecies are expressed through alternative splicing, resulting in proteins bearing different length N termini (4, 26). The RXRβ form used here, the shorter species, is expressed at high levels in many cell lines (26). This unusual mode of expression of RXRβ may be a means by which alternative mRNA splicing can generate RXRβ subforms with different response element specificities.

The presence of more than two half-sites in one hormone response element is not unique to RXR response elements, and other classes of nuclear hormone receptors may also encode isoforms that can form high-order oligomeric complexes (e.g., see ref. 17). In this regard, it is notable that isoform-specific promoter utilization has also been reported for these other receptor classes and also maps to their N-terminal domains (e.g., see ref. 27). Therefore, we propose that response element recognition actually occurs at three interdependent levels: (*i*) recognition of the individual half-site sequence by each receptor monomer, (*ii*) recognition of the spacing and orientation of adjacent half-sites, and (*iii*) as newly elucidated here, recognition of highly reiterative half-sites by receptors able to form higher-order protein complexes. In fact, by recruiting multiple receptor molecules, and therefore multiple transcriptional activation domains, to a single promoter these high-order receptor complexes may serve as particularly strong inducers of target gene expression. Given the ability of many of these receptors to also form heterodimers (2), our observation additionally suggests a plausible mechanism of combinatorial control, by which the distinct repeats within a highly reiterated element can compete for different receptor homo- and heteromeric complexes. This may serve to integrate the inputs of different receptors, all operating at a single promoter.

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