The DNA Binding Pattern of the Retinoid X Receptor Is Regulated by Ligand-dependent Modulation of Its Oligomeric State*

(Received for publication, February 3, 1997, and in revised form, March 5, 1997)

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The retinoid X receptor (RXR) regulates target gene transcription via its association with cognate DNA response elements either as a homodimer or as a heterodimer with a number of other nuclear receptors. We previously demonstrated that, in solution, RXR forms tetramers with a high affinity and that ligand binding leads to dissociation of receptor tetramers to smaller species. Here it is shown that RXR tetramers form stable complexes with direct repeats (DR-1 or DR-5) or palindromic (TRP$_{max}$) response elements. Binding of RXR tetramers to cognate DNA occurs with a significantly higher affinity as compared with dimers. Ligand binding by DNA-bound RXR tetramers results in their dissociation to DNA-bound dimers, a process that is completely reversed upon removal of the ligand. Formation of stable tetramer-DNA complexes requires binding of two oligonucleotides/tetramer. It is proposed that ligand-dependent modulation of the oligomeric state of RXR is a regulatory feature of this nuclear receptor.

The retinoid receptors, retinoid X receptors (RXRs) and retinoic acid receptors (RARs), are ligand-activated transcriptional regulators that belong to a superfamily of nuclear receptors that mediate the effects of small lipophilic ligands on gene transcription. RARs can bind and are activated by the vitamin A metabolite all-trans-retinoic acid, while both RXRs and RARs can bind and are activated by the 9-cis isomer of retinoic acid (9cRA) (1, 2) although the physiological role of this ligand is not clear at present (3, 4). Besides RXR and RAR, the hormone nuclear receptor superfamily also includes the steroid receptor, thyroid hormone receptors (TR), vitamin D receptor (VDR), and peroxisome proliferator-activated receptor as well as a number of orphan receptors (for reviews, see Refs. 5–8). These proteins modulate transcription via their association with specific nucleotide sequences, termed response elements (REs), that are located in the promoter region of target genes. Recognition sequences for thyroid and retinoid receptors consist of everted, inverted, or direct repeats of the consensus motif RG(G/T)TC

This work was supported by National Institutes of Health Grant CA68150 and United States Department of Agriculture Grant 89-065-3-601. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: RXR, retinoid X receptor; RAR, retinoic acid receptor; 9cRA, the 9-cis isomer of retinoic acid; TR, thyroid hormone receptor; VDR, vitamin D receptor; RE, response elements; EMISA, electrophoretic mobility shift assay.

EXPERIMENTAL PROCEDURES

Proteins—Recombinant RXRs lacking the N-terminal A/B domain (RXR$\Delta$A/B) was obtained by overexpression in E. coli and isolated as described previously (47, 50). Isolated protein was found to possess 0.65–0.85 mol of ligand binding sites/mol of protein. The receptor showed the expected ligand selectivity as it tightly bound 9cRA and several RXR-selective ligands but not all-trans-retinoic acid (51). The
DNA Binding Pattern of RXR Oligomers

RXR Associates with DNA Containing a Single Response Element as a Dimer and as a Tetramer—We previously showed that RXR exists in solution as a monomer, dimer, and tetramer and that tetramers become the predominant species of the oligomeric state of the two DNA complexes, with the slower moving band most consistent with a complex comprised of a DNA-bound RXR dimer, while the slower moving band most likely represents a DNA-bound tetramer. Hence, these data demonstrate that RXR can bind to DNA containing a single response element either as a dimer or as a tetramer.

**RESULTS**

**DNA Binding by RXR**

DNA binding by RXR is essential for its activity, it was of interest to explore which of the receptor oligomers can bind to cognate DNA. The DNA binding pattern of RXR was analyzed by EMSAs in the presence of a DR-1 RE (Fig. 1, lane 1), a DR-5 RE (lane 3), or a TREpal RE (lane 2). As previously reported (11, 13), RXR and TREpal displayed a lower affinity for RXR as compared with DR-1 and required higher protein concentrations for visualization of RXR-DNA complexes. Nevertheless, two distinct RXR-DNA complexes, with the slower moving band being the more pronounced complex, could be observed with all three REs. Thus, RXR appears to have two modes of association with DNA containing two half-sites of its recognition sequence. The presence of two RXR-DR-1 complexes in EMSAs carried out with receptor obtained by expression in *Escherichia coli* was described as prepared by Lupisella et al. (52).

**Oligonucleotides**—The following oligonucleotides containing the following response elements were used: DR-1, a response element containing two direct repeats separated by a single nucleotide (5'-TCGAAGGC-GGGTCAGGAGGTCACTCGTCGA-3'); DR-5, a response element composed of two direct repeats spaced by 5 nucleotides (5'-AGCTTGGG-GCAGGGGTCAGGTCAGAATT-3'); TREpal (5'-AGCTTGGGC-GCCTACAGGTCACTCAGTGAATT-3'); half-site, 5'-AGCTTGGGGCGAGGGTGTCAGAATT-3'; DR-1, 4'-TCGACGGCCAAACCG-GAATCTTAAGGCTAAGGGCTAGGGGTCAGAGGTCACTCA-ATGGTCACACCGGATAAGGAGATTTGTGCTCGA-3'. The oligonucleotides were synthesized and purified at the Cornell DNA synthesis facility. Single-stranded DNA was annealed (53), and double-stranded DNA isolated on Centrекс centrifugal filter units. Oligonucleotides were end-labeled with [32P]dCTP by filling in with Klenow fragments, and free nucleotides were removed with the Qiagen nucleotide removal kit.

**Electrophoretic Mobility Shift Assays (EMSA)**—EMSAs were carried using oligonucleotides containing a DR-1 RE (lane 1), TREpal (lane 2), DR-5 (lane 3), or a half-site (lane 4) (see “Experimental Procedures” for sequence details). 1 μl of [32P]-labeled oligonucleotide (50–200 nM) and 1 μl of 2.4 mg/ml dl-dC were mixed with the indicated amounts of RXR or 1 μl of 2.4 mg/ml dI-dC were mixed with the indicated amounts of RXR to a final concentration of 10 nM RXR, 0.8, 0.1, or 0.01 nM EDTA, 0.4 mM dithiothreitol; 100 mM KCl, and 15% glycerol. Ligands were added from a concentrated solution in ethanol or Me2SO to a final concentration of 1 μM, and mixtures were incubated for 15 min at room temperature. Protein-DNA complexes were resolved by electrophoresis on 5% polyacrylamide gels (0.5 x TBE, 2–3 h prerun at 100 V, 2 h run at 25 mA/gel), the gel was dried, and protein-DNA complexes were visualized by autoradiography. During electrophoresis, the gel was cooled with circulating water at 12°C.

**Ferguson Analyses**—Binding of RXR to DR-1 RE was analyzed by EMSAs using polycrylamide gels with various acrylamide concentrations. The mobilities of receptor-DNA complexes were analyzed by the method of Ferguson as described by Bollag and Edelstein (54). The retardation factors (R<sub>f</sub> values) of individual bands in gels of differing acrylamide concentrations were plotted versus the acrylamide concentration, and the resulting slopes were compared with those of protein standards with known molecular masses. These standards were ovalbumin (20 kDa), bovine serum albumin (67 kDa; monomer, dimer, and tetramer), transferrin (76 kDa), trypsinogen (21 kDa), and trypsin (26 kDa). The compositions of the two DNA-RXR complexes were determined by analyzing EMSAs using the method of Ferguson, a procedure that allows for determining the molecular weight of species resolved by electrophoresis under nondenaturing conditions (54). RXR/DNA mixtures were resolved on polyacrylamide gels of varying acrylamide concentrations. The R<sub>f</sub> values of the two RXR/DNA bands in gels of differing acrylamide concentrations were plotted versus the acrylamide concentration, and the resulting slopes were compared with those of protein standards with known molecular masses. Fig. 2 shows the Ferguson analysis relating the negative value of the slopes to the molecular weights of the standards. The mobilities of the two RXR-DNA complexes (arrows in Fig. 2) corresponded to molecular masses of 110 ± 14 and 181 ± 5 kDa (mean ± S.E.M., n = 3). As the molecular masses of RXRΔAB and the DR-1 oligonucleotide are 40,270 and 18,200 Da, respectively, the molecular masses of the species represented by the faster moving band is most consistent with a complex comprised of a DNA-bound RXR dimer, while the slower moving band most likely represents a DNA-bound tetramer. Hence, these data demonstrate that RXR can bind to DNA containing a single response element either as a dimer or as a tetramer.

**RXR Tetramers Associate with DR-1 RE at an Oligonucleotide:Tetramer Ratio of 2:1**—The data in Fig. 2 identified the oligomeric state of the two DNA-RXR complexes observed in COS-1 cells and insect Sf9 cells was previously noted (11, 50), but their compositions were not explored.

To examine whether the faster moving band might represent monomeric DNA binding by RXR, EMSAs were performed using an oligonucleotide containing a single half-site (Fig. 1, lane 4). No specific complex between RXR and oligonucleotides containing a half-site could be observed even at very high RXR concentrations ([RXR] = 10 μM). These data are in agreement with the reported cooperativity of DNA binding by this receptor (55) and indicate that RXR monomers have a very low affinity for the response element. Thus, the faster moving band apparent in Fig. 1 reflects a receptor-DNA complex of a higher order than monomer.

The compositions of the two DNA-RXR complexes were determined by analyzing EMSAs using the method of Ferguson, a procedure that allows for determining the molecular weight of species resolved by electrophoresis under nondenaturing conditions (54). RXR/DNA mixtures were resolved on polyacrylamide gels of varying acrylamide concentrations. The R<sub>f</sub> values of the two RXR/DNA bands in gels of differing acrylamide concentrations were plotted versus the acrylamide concentration, and the resulting slopes were compared with those of protein standards with known molecular masses. Fig. 2 shows the Ferguson analysis relating the negative value of the slopes to the molecular weights of the standards. The mobilities of the two RXR-DNA complexes (arrows in Fig. 2) corresponded to molecular masses of 110 ± 14 and 181 ± 5 kDa (mean ± S.E.M., n = 3). As the molecular masses of RXRΔAB and the DR-1 oligonucleotide are 40,270 and 18,200 Da, respectively, the molecular masses of the species represented by the faster moving band is most consistent with a complex comprised of a DNA-bound RXR dimer, while the slower moving band most likely represents a DNA-bound tetramer. Hence, these data demonstrate that RXR can bind to DNA containing a single response element either as a dimer or as a tetramer.

**RXR Tetramers Associate with DR-1 RE at an Oligonucleotide:Tetramer Ratio of 2:1**—The data in Fig. 2 identified the oligomeric state of the two DNA-RXR complexes observed in...
EMSAs. However, these data are not precise enough to ascertain whether DNA-bound RXR tetramers are associated with 1 or 2 oligonucleotides/tetramer, i.e. whether each dimer within the tetramer can separately interact with a response element or whether only two of the tetramer subunits are involved in DNA-receptor interactions.

To distinguish between these two possibilities, EMSAs were carried out using a mixture of a short (31-base pair) and an extended (93-base pair) oligonucleotide containing a DR-1 response element. It was reasoned that if RXR tetramers associate with two DR-1 REs simultaneously, a complex in which a tetramer is bound to one short and one extended oligonucleotide will form when both oligonucleotides are present. Such a complex is expected to display a mobility that is intermediate between tetramer-DNA complexes observed in the presence of each of the DR-1 REs separately. In the presence of the extended DR-1 (Fig. 3, lanes 1 and 2), a predominant band with a low mobility and a minor band of higher mobility were observed. The addition of 9cRA to mixtures of RXR with the extended oligonucleotide led to a significant decrease in the concentration of the slower moving band with a concomitant increase in the concentration of the faster moving band (Fig. 3, lane 9). In the absence of limiting DNA (Fig. 4), the dimeric complex gradually became the prominent complex, the dimeric complex then diminished until it reached a peak at a protein concentration of about 50 nM (lane 3) and then gradually diminished (Fig. 4A, lanes 4–6). Concomitantly with the diminishment of the tetrameric complex, the dimeric complex gradually became the prominent species. In contrast, in the presence of excess DR-1, both the dimeric and the tetrameric RXR-DNA complexes intensified monotonously as the protein concentration was raised (Fig. 4B).

The origin of the apparent concentration dependence of DNA binding by RXR under conditions of a limiting DNA concentration is not completely clear to us at the present time. This behavior is especially puzzling if the concentration dependence of the oligomeric state of RXR in solution is taken into account; at low protein concentrations, where the concentrations of tetramers are low (47), the tetrameric species seems to be the predominant DNA-bound RXR. On the other hand, at high protein concentrations, where tetramers are the predominant species in solution (47), RXR-DNA complexes appear to be mainly dimeric. Thus, the apparent concentration dependence of protein-DNA complexes in Fig. 4A cannot be explained based...
on the thermodynamic properties of the system, suggesting that under some conditions EMSAs do not properly report on the equilibrium situation. It is possible that kinetic effects, such as differential instabilities of complexes during electrophoresis, play a role in bringing about the observed profiles. In support of this explanation, an examination of the bottom region of the gel shows that, in contrast to what could be expected, the amount of free DNA and the region across which free DNA diffuses increased continuously as the protein concentration was raised. This behavior is indicative of gradual dissociation of complexes during electrophoresis, which became more pronounced at higher protein concentrations. The origin of the instability of protein-DNA complexes at high protein concentration is not clear. Nevertheless, these observations demonstrate that under different conditions EMSAs can result in the appearance of either the tetrameric or the dimeric complex or in the appearance of both RXR-DNA complexes. Hence, unless a range of conditions is explored, the presence of more than one species might be overlooked.

The dissociation of protein-DNA complexes during electrophoresis in Fig. 4A, lane 1, appears to be minimal. The relative distribution of tetrameric and dimeric RXR-DNA complexes in this lane may thus be used to assess the relative binding affinities of receptor dimers and tetramer for DR-1 RE. The concentration of RXR used in lane 1 was 12.5 nM, a concentration in which the fraction of RXR tetramers in solution is significantly lower than the fractions of either monomers or dimers (47). Nevertheless, the tetrameric RXR-DNA complex predominated, indicating that the binding energy driving the formation of the tetrameric RXR-DR-1 complex is significantly stronger as compared with that of the dimeric RXR-DR-1 complex.

Overall, the observations in Figs. 3 and 4 indicate that both RXR tetramers and dimers can bind to oligonucleotides containing two half-sites of their RE and that formation of a stable RXR tetramer-DNA complex requires the association of each dimer within the tetramers with a separate RE. The data also show that the affinity of RXR for tetramers is higher than the affinity toward dimers.

Ligand Binding Induces Dissociation of DNA-bound RXR Tetramers to DNA-bound RXR Dimers in a Reversible Fashion—It was previously demonstrated that RXR tetramers, the predominant species of the unliganded receptor in solution, dissociate into dimers and monomers upon binding of ligand (48, 49). The observations in this paper that RXR tetramers can bind to cognate DNA raise the question of whether DNA-bound RXR tetramers respond to their ligand in the same vein. Fig. 5 clearly demonstrates that binding of 9cRA by DNA-bound RXR leads to a significant decrease in the fraction of RXR bound as a tetramer concomitantly with an increase in the fraction of RXR bound as a dimer. The dimeric RXR-DNA complexes were enhanced upon the addition of ligand over the entire range of protein concentrations used in the experiment depicted in Fig. 4. The same effect was observed using a variety of RXR-selective ligands (data not shown), indicating that tetramer dissociation is a general response of this receptor to cognate ligands. These observations demonstrate that, similar to the behavior of the protein in solution, ligand binding by DNA-bound RXR tetramers leads to their dissociation to DNA-bound dimers.

We previously suggested that ligand-induced dissociation of RXR tetramers might serve to regulate the activity of the receptor by varying the ratio between tetrameric and dimeric species. An important feature of such a regulatory mechanism would be the ability to “switch off,” i.e., that the tetramer retains the ability to reversibly reassociate to form tetramers following removal of the ligand.

EMSA of apo-RXR with a DR-1 RE showed the expected presence of tetrameric and dimeric protein-DNA complexes (Fig. 6A, lane 1). The addition of 9cRA resulted in the dissociation of the tetrameric complex with a concomitant enhancement of the dimeric complex (Fig. 6A, lane 2). Holo-RXR was then depleted of its ligand by photobleaching. The protein was exposed to 2-min bouts of UV light for a total of 8 min, resulting in degradation of 9cRA, which could be followed by the decrease of the absorbance of the ligand (Fig. 6B). If ligand-induced tetramer dissociation is a reversible process, depletion of RXR of its ligand would result in reassociation of the protein to tetramers. Indeed, apo-RXR generated following photodegradation of 9cRA reassociated to form tetramers, which is reflected by the reappearance of tetrameric RXR-DNA complex (Fig. 6, lane 3). The similarity of the relative distribution of the two RXR-DNA complexes prior to the addition and following
the depletion of ligand (compare lanes 1 and 3) indicates that the ligand-induced dissociation of DNA-bound tetramers was fully reversed upon removal of the ligand. These data provide additional support to our hypothesis that variations in the local concentrations of the ligand for RXR may serve as an on/off switch that regulates activation/deactivation cycles of the receptor by modulating its oligomeric state.

The A/B Domain of RXRa Is Not Involved in the Self-association of the Receptor—Our studies of RXR, most importantly the characterization of the physicochemical properties of receptor oligomers (47–49), relied on usage of a truncated protein lacking the A/B domain (RXRaΔAB). An important question that has remained unanswered is whether the A/B domain might influence the self-association of the receptor or the response of protein oligomers to ligand binding. Due to technical difficulties in obtaining pure full-length receptor, the assays described below were carried out using crude extracts of E. coli expressing full-length RXRa. EMSAs of mixtures of the bacterial extract with DR-1 RE gave rise to two bands (Fig. 7, lanes 1–3). As expected, the mobilities of these bands were slower than the mobilities of the two bands observed when the truncated protein was used (data not shown). The presence of RXR within the two complexes was confirmed by supershifting using antibodies against RXR (Fig. 7, lanes 7–9). The addition of 9cRA led to a significant decrease in the population of the slower moving band with a concomitant enhancement of the faster moving band (Fig. 7, lanes 4–6). Thus, full-length RXRa, similarly to RXRaΔAB, forms both dimeric and tetrameric complexes with DR-1 and responds to its ligand by dissociation of the DNA-bound tetramers to DNA-bound dimers. These results demonstrate that deletion of the A/B domain has little effect on the characteristics of self-association of RXRa. The data also indicate that the truncated receptor RXRaΔAB is a valid model for studying the self-association of RXR and the factors that regulate this process.

DISCUSSION

Previous studies of the self-association of RXR in solution revealed that this receptor forms tetramers with a high affinity and in a cooperative fashion and that tetramers become the predominant species at concentrations in the range of 50-100 nM. It was further demonstrated that ligand binding by RXR leads to rapid dissociation of tetramers to monomers and dimers (47–49). Here it is shown that tetramers can form stable complexes with REs consisting of two direct or inverted repeats of the consensus half-site. Analyses of tetramer-DNA complexes indicated that they are formed with a significantly higher affinity as compared with dimeric DNA complexes. Tetrameric DNA complexes were found to contain two REs/tetramer and did not reveal the presence of a species consisting of a tetramer and a single RE. These observations suggest that the association of each dimer within the tetramer with a separate RE is necessary to stabilize the interaction of tetramers with cognate DNA and thus that binding of the two REs to receptor tetramers proceeds cooperatively. As free dimers associate with REs with a high affinity, the observation that association of a RE with each of the dimers within RXR tetramers depends on DNA binding by the other leads to the conclusion that dimers within RXR tetramers adopt a somewhat different conformation from that of free dimers. The observed cooperativity in association of REs with tetramers also indicates that individual subunits within tetramers communicate between themselves. This last point is in agreement with our previous observation that ligand-induced dissociation of
RXR tetramers proceed cooperatively (49), pointing at the existence of efficient communication between individual subunits of RXR tetramers.

These observations raise the question of whether RXR tetramers might be able to associate with cognate DNA in vivo. The requirement for binding of two REs for the formation of a stable tetramer-DNA complex leads one to speculate that perhaps in special promoter contexts, DNA binding by RXR tetramers allows the receptor to serve as a bridging factor between two distant regulatory elements on the chromatin. The existence of regulatory sequences far upstream from the transcriptional start site is common to many eukaryotic promoters, and it has been suggested that such distant regulatory elements could be brought close to the start site via a DNA loop mediated through protein-protein interactions (56). One example that might be relevant in this context is that of two retinoic acid REs separated by 30 nucleotides that have recently been identified in the promoter region of the retinol-binding protein gene. The two regions were found to be present on the same side of the DNA helix, and occupancy of both by retinoid receptors was shown to be necessary for transactivation. It has been proposed that protein-protein interactions between receptors bound at the two regions might play a role in regulating the transcription of this gene (57). An intriguing possibility is that DNA binding by RXR tetramers might be involved in regulating the expression of genes that contain such a complex RE within their promoter regions.

It was previously shown that four RXR molecules can cooperatively bind to the RARE of the CRBPI gene, a RE that consists of 4 or 5 half-sites separated by a single base pair (58). It is not clear, however, whether the mode by which multiple RXR subunits associate with REs containing multiple adjacent repeats is similar to the mode by which RXR tetramers associate with REs composed of only two half-sites as reported in the present work. While the tetrameric RXR-DNA complexes formed with a single RE reflect binding of DNA to preformed tetramers, the association of four RXR subunits with the CRBPI RE might simply reflect association of two separate receptor dimers with the complex RE.

The data in the present work demonstrate that, similarly to the effect of ligand on the oligomeric state of RXR in solution, binding of ligand to DNA-bound RXR tetramers induces their dissociation and leads to a concomitant increase in the population of DNA-bound receptor dimers (Fig. 5). In addition, the ligand-induced tetramer dissociation was found to be fully reversible, that is, upon removal of the ligand, the receptor reassociated and efficiently formed tetrameric RXR-DNA complexes (Fig. 6). Ligand-induced increases in the population of RXR dimers, and in DNA binding by these dimers, has been previously reported (20, 59). To account for these findings, it was suggested that ligand binding by RXR enhances the affinity of receptor monomers for each other. In conflict with this hypothesis, measurements of the equilibrium dissociation constants governing dimer formation by RXR showed that they are very similar in the absence and in the presence of ligand (48). The data in the present work, in agreement with the previous reports (20, 59), show that the population of the DNA-bound RXR dimers is indeed significantly increased upon binding of 9cRA but indicate that this response stems from ligand-induced weakening of receptor tetramers and not, as previously suggested, from changes in the affinity of receptor monomers for each other.

The observations reported here further support our postulate that binding of ligand to RXR may regulate the activity of the receptor by modulating the relative distribution of receptor monomers, dimers, and tetramers (47–49). According to this model, the bulk of apo-RXR in cells is sequestered in the form of tetramers that serve as a transcriptionally inactive reservoir for the receptor. Changes in the distribution of receptor species occur upon increases in the cellular level of 9cRA to a concentration that is sufficient for efficient binding to the receptor (see Ref. 49 for discussion of the cooperative nature of the association of 9cRA with RXR). Ligand binding leads to dissociation of receptor tetramers into dimers that can activate the homodimeric pathway of RXR and into monomers that can then interact with partner receptors and participate in heterodimeric pathways.

Similar to the present observations that unliganded RXR is bound to DR-1 as a tetramer while the liganded receptor associates with the RE as a dimer, it has been reported that unliganded VDR binds to a DR-3 RE mainly as a dimer but dissociates into monomers in the presence of 1,25(OH)2-vitamin D3 (21). It was also shown that TR binds to cognate DNA as a monomer and a dimer in the absence of ligand but binds exclusively as a monomer in the presence of T3 (19, 60, 61). These findings, taken together, suggest that modulation of the oligomeric state of hormone nuclear receptors might be a general mode of response of these proteins to their ligands. If receptor homo-oligomerization serves to sequester active subunits into an inactive reservoir as suggested above, then the question arises of why unliganded RXR self-associates to homotetramers while VDR and TR only form homodimers. An important distinction between RXR versus VDR and TR is that while RXR homodimers are transcriptionally active, homodimers of both VDR and TR are poor transcriptional activators, since these receptors seem to function predominantly via heterodimers with RXR (5, 8). It hence might be speculated that formation of homodimers by TR and VDR is sufficient to render these receptors relatively inactive. In contrast, inactivation of RXR requires sequestration of both monomers and dimers, a need that can be fulfilled by the formation of homo-oligomers of a higher order, i.e. tetramers.

In summary, the data reported here demonstrate that RXR tetramers can bind to cognate DNA containing a single response element with high affinity and that formation of RXR tetramer-DNA complexes requires a binding stoichiometry of two REs/tetramer. The data further show that ligand binding reversibly induces dissociation of DNA-bound receptor tetramers concomitantly with enhancement of the population of DNA-bound RXR dimers. Additional studies will be required to delineate the implications of this mode of DNA binding by RXR for the in vivo function of the receptor.

Acknowledgments—We thank Peter Reczek for providing bacterial extracts containing full-length RXRα and Hoffman La Roche for the gift of 9cRA.

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J. Biol. Chem. 1997, 272:12771-12777.
doi: 10.1074/jbc.272.19.12771

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