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The dimerization interfaces formed between the DNA binding domains of RXR, RAR and TR determine the binding specificity and polarity of the full-length receptors to direct repeats

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Heterodimers of retinoid X receptor (RXR) and retinoic acid receptor (RAR) bind preferentially to directly repeated elements with spacing of two (DR2) or five (DR5) base pairs, due to the specific heterocooperative interaction of their DNA binding domains (DBDs) on these elements. We have demonstrated in the accompanying paper that the heterodimeric DBD interface that is responsible for the cooperative binding to DR5 elements, specifically involves the D-box of the RXR CII finger and the tip of the RAR CI finger. We show here that a second type of dimerization interface, which specifically implicates the RAR T-box and the RXR CII finger to the exclusion of the D-box, determines the selective binding to DR2 elements. Interestingly, the same type of dimerization interface (RXR T-box and CII finger) is responsible for the cooperative binding of homodimers of the RXR DBD to DR1 elements. Based on the three-dimensional structure of the glucocorticoid receptor DBD, modeling of RXR/RAR, RXR/TR and RXR/RXR DBD cooperative interactions predicts that in all cases the DBD contributing the CII finger, i.e. that of RXR, has to be positioned 5′ to its cooperatively bound partner. This binding polarity of the DBDs is conferred upon the full-length receptors, since crosslinking experiments indicate that RXR is always 5′ to RAR in complexes between either DR5 or DR2 and RXR/RAR heterodimers. The possible significance of these observations for transactivation by retinoic acid receptors is discussed.

Key words: binding site repertoire/response elements/retinoic acid receptors/thyroid hormone receptor

Introduction

The formation of heterodimers between either the retinoic acid receptor (RAR) or thyroid hormone receptor (TR) and the retinoid X receptor (RXR) not only increases the efficiency with which they bind DNA, but also results in specific response element repertoires. While RAR homodimers bind promiscuously to direct repeats (DRs) of the motif PuG(G/T)TCA with spacings of 2, 3, 4 or 5 bp (DR2 to DR5), TR homodimers bind preferentially to DR3, DR4 and DR5, and RXR homodimers bind almost exclusively to DR1 (Zhang *et al.*, 1992; Mader *et al.*, 1993a,b). However, RXR/RAR heterodimers bind to, and activate transcription from, DR5, DR2 and DR1 elements, whereas RXR/TR heterodimers are selective for DR4 elements [for references see Leid *et al.* (1992a) and Introduction of the accompanying paper (Zechel *et al.*, 1994)]. We have recently reported that the DR element binding repertoires of both homo- and heterodimers of RAR, RXR and TR correlates very closely with those of the corresponding DNA binding domains (DBDs) (Mader *et al.*, 1993a; Zechel *et al.*, 1994). Whether this is also the case for response elements composed of inverted or everted repeats (Baniahmad *et al.*, 1990; Näär *et al.*, 1991; Mader *et al.*, 1993b; Park *et al.*, 1993; Tini *et al.*, 1993) is unknown. Moreover, taking advantage of the observation that TR has a much higher preference than RXR for binding to a PuGGTCA motif (G motif) over a PuGTTCA motif (T motif), we showed that RXR/TR DBD heterodimers bound more efficiently to DR4 in which PuGGTCA was the 3′ motif (DR4T/G) than to DR4 in which it was the 5′ motif (DR4G/T), and thus proposed that the binding of RXR/TR DBD heterodimers occurs with a given polarity (anisotropic complexes; Mader *et al.*, 1993a). In keeping with this conclusion, the corresponding full-length RXR/TR heterodimers transactivated more efficiently from reporter genes containing the DR4T/G response element (Mader *et al.*, 1993a). Similarly, exploiting the observation that RXR has a higher preference for binding to G motifs over T motifs than does RAR, we found that RXR/RAR DBD heterodimers bound preferentially to DR2G/T rather than to DR2T/G elements. Furthermore, transactivation assays performed with DR*n*T/G and DR*n*G/T-based reporter genes revealed that the corresponding full-length receptors activated more efficiently from DR2G/T and DR5G/T than from DR2T/G and DR5T/G reporters. Taken together, these results led us to predict that both in DR2 and DR5 RAR/RXR heterodimeric complexes, as well as in DR4 RXR/TR heterodimeric complexes, RXR occupies the 5′ motif of the corresponding DR element, and that this polarity is determined by the nature of the cooperative interactions between the respective DBDs (Mader *et al.*, 1993a).

In the accompanying paper (Zechel *et al.*, 1994), we report that the dimerization interface that is responsible for the heterocooperative binding of RXR and RAR DBDs to DR5 elements specifically involves the D-box (Umesono and Evans, 1989) of the C-terminal CII finger of RXR and the tip of the N-terminal CI finger of RAR. Similarly, the RXR/TR DBD heterodimerization on DR4 elements appears to be due to the formation of an interface which specifically requires the RXR D-box and the TR 'prefinger region', but not the TR CI finger. If, as predicted, RXR is always bound to the 5′-located motif of DR elements, it is unlikely, in view of the rotational shift along the DNA, that the same RXR and RAR dimerization surfaces could specify the binding of RAR/RXR heterodimers to both DR2 and DR5 elements.

The question also arises as to which RXR dimerization surfaces are involved in the specific RXR homodimer binding to DR1 elements.

We demonstrate here that a second type of dimerization interface, involving the part of the RAR region which corresponds to the T-box (Wilson *et al.*, 1992) and the CII finger region of RXR (to the exclusion of the RXR D-box), is responsible for heterocooperative binding of RXR/RAR DBDs to DR2 elements. We also show that the same type of dimerization interface is formed between two RXR DBDs homocooperatively bound to DR1 elements. Based on the three-dimensional structure of homodimers of the glucocorticoid receptor (GR) DBD (Luisi *et al.*, 1991), modeling of the dimerization interfaces of RXR/RAR DBD and RXR/TR DBD heterodimers bound to DR2 and DR5, and DR4 elements, respectively, predicts that in all cases the RXR DBD is bound to the 5′ motif of the DR element. In accordance with this, crosslinking studies demonstrate that full-length RXR/RAR and RXR/TR heterodimers are bound in the 5′-RXR/RAR-3′ and 5′-RXR/TR-3′ orientation to DR5 and DR4 elements, respectively. Thus, the binding polarity of full-length heterodimers on specific DR elements appears to be essentially dictated by their DBDs.

Results

The cooperative binding of RXR DBD homodimers to DR1 elements specifically requires the T-box of one monomer and the C-terminal CII finger (to the exclusion of the D-box) of the other monomer

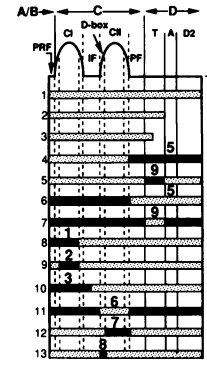
To identify the components of the dimerization surfaces which are specifically involved in the homocooperative binding of the RXR DBD to DR1 elements, we used the wild-type RXR DBD [amino acids Ala133 to Glu242 of mRXRα1 (Leid *et al.*, 1992b); denoted by X in the figures and tables], C-terminally truncated RXR DBDs, or chimeric DBDs in which specific regions of the RXR DBD had been replaced with the corresponding sequences of either TR, RAR or the estrogen receptor (ER) [indicated by T, A and E, respectively in the figures and tables; for nomenclature and schematic representations of the various constructs, see Tables I and II, and Figure 1 of Zechel *et al.* (1994)].

The binding site repertoires of the C-terminally truncated $X_{\Delta 2}$ were indistinguishable from that of the full-length RXR DBD (Table I, lines 1 and 2; Figure 1A; Figure 2, compare lanes 17–20 with lanes 1–4), indicating that the D2 and the A-box regions are not required for homocooperative DR1 binding. Further deletion of the T-box generated $X_{\Delta 3}$, which was unable to form homodimers [Table I, line 3; Figure 1B; for definition of A- and T-boxes, see Wilson *et al.* (1992)]. This was not due to an intrinsic inability of $X_{\Delta 3}$ to bind DNA, since monomeric complexes were formed with all elements ('1' in Figure 1B, right panel; Figure 2, lanes 21–24). Thus, the T-box appears to be required for the formation of the RXR dimerization interface mediating homocooperative binding to DR1. Indeed, replacing only the T-box of the RXR DBD by that of RAR abolished homocooperative DR1 binding (XA_9, line 5 in Table I, and Figure 1D and F).

To investigate whether the presence of two T-box regions would be sufficient for the formation of the RXR homodimerization interface, we constructed AX_5 and TX_5 in which sequences C-terminal to the RXR CII finger replace those of the RAR or TR DBD (Table I, line 6). No DR1 homodimeric complexes were formed by either AX_5 or TX_5, indicating that two T-box regions do not form a DR1-specific RXR dimerization interface on their own (Table I, line 6; Figure 1E). That only one T-box region is involved in the DR1 dimerization interface was supported by the heterodimerization of XA_9 and $X_{\Delta 2}$ which efficiently formed a complex of intermediate mobility on DR1 (Table I, line 5; arrow in Figure 1D). Moreover, XA_9 formed heterodimers also with AX_9, a chimera which carries only the RXR T-box (Table I, line 7; arrow in Figure 1F). Thus, the presence of one RXR T-box alone in one DBD monomer is sufficient for dimerization on DR1.

To map the second dimerization surface required for RXR homodimerization on DR1, we investigated (i) which of the various RXR DBD chimeras containing the RXR T-box homocooperatively bound to DR1, and (ii) which RAR and TR DBD chimeras harboring different RXR regions gained the ability to bind heterocooperatively to DR1 with either the RXR DBD, $X_{\Delta 2}$ or AX_9. The N-terminal CI finger of RXR did not appear to contain the second dimerization surface, since its replacement with the corresponding regions from ER, RAR or TR generated chimeras (XE_1, XE_2, XA_3 and XT_3), all of which could efficiently homodimerize on DR1 elements (Table I, lines 8, 9 and 10; Figure 1G and panel XE_2 in Figure 1H). However, swapping of the CII fingers generated the mutant TX_6 (Table I, line 11) which allowed definition of the region of the RXR DBD that contained the second dimerization surface. In contrast to the parental TR DBD, TX_6 heterodimerized with DBDs

Table I. Summary of the results defining the RXR DBD homodimerization surfaces



DBDs/ Mutants	RXR region present in the mutants: CII	RXR region present in the mutants: T-box	Homo-dimerization on DR1	Heterodimerization on DR1 with X or $X_{\Delta 2}$	Fig.
X	+	+	+	+	1A;2
$X_{\Delta 2}$	+	+	+	+	1A;2
$X_{\Delta 3}$	+	-	-	(-)	1B;2
XT_5,XA_5	+	-	-	(-)	1C;3D
XA_9	+	-	-	+	1D
AX_5,TX_5	-	+	-	+	1E
AX_9	-	+	(*)	+	1F;1C
XE_1	+	+	+	+	1G;1H
XE_2	+	+	+	+	1H
XT_3,XA_3	+	+	+	+	1G
TX_6	+	-	-	+	1I
XT_7,XA_7	D-box only	+	-	+	1J;1K
XT_8,XA_8	minus D-box	+	+	+	1L;2

The T-box and the CII finger (excluding the D-box) are specifically required for the formation of the homodimerization interface of the RXR DBDs bound to DR1 elements. T, T-box; A, A-box (for a definition, see Wilson *et al.*, 1992). For amino acid sequences encompassing the 'prefinger region' (PRF), regions C and D, the two zinc fingers (CI and CII) and region D2, see Zechel *et al.* (1994) and references therein. In the schematic representation of the chimeras the RXR sequences are indicated by dotted boxes, heterologous sequences (T for TR, A for RAR, and E for ER) by black boxes. The zinc complexing cysteine pairs of CI and CII fingers are indicated by dashed lines. The recipient DBD is represented in a one letter code, with X = RXR, A = RAR, T = TR and E = ER, followed by a letter indicating the origin of the swapped DBD segment with a subscript number identifying this segment [for further details on the nomenclature, see Zechel *et al.* (1994)]. (−) indicates that $X_{\Delta 3}$, as well as XT_5 and XA_5, failed to dimerize with RXR DBDs providing the complementary surface, possibly due to steric hindrance problems, and (*) indicates that AX_9 showed a weak, but selective DR1 binding, possibly reflecting RAR/RXR heterodimerization on DR1 (see Discussion). IF, interfinger region, PF, 'post-finger region'. 'Fig.' refers to the figures which display the corresponding EMSA data.

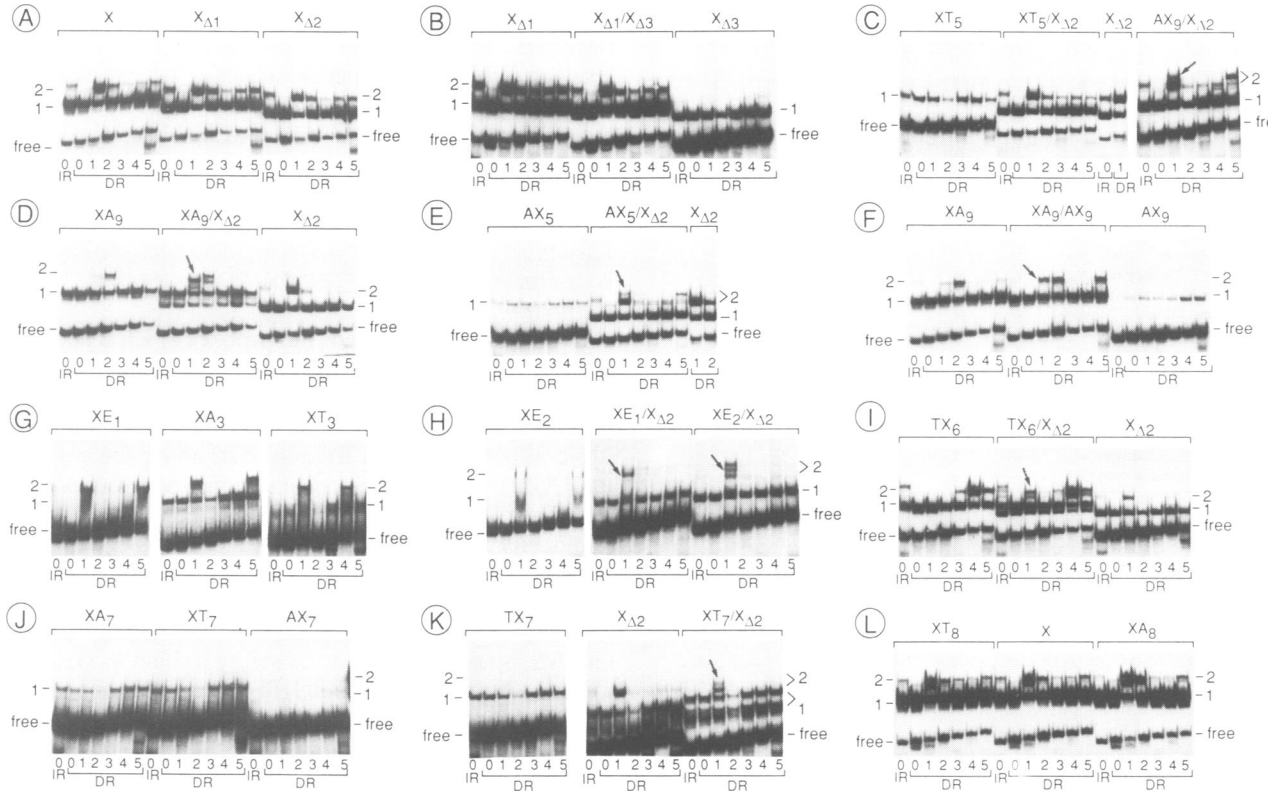


Fig. 1. RXR DBD homodimerization on DR1 elements involves the CII finger of one monomer and the T-box of the other monomer. Electrophoretic mobility shift assays were carried out using crude lysates of bacterially expressed wild-type, truncated or chimeric RXR DBDs, as indicated at the top of each panel [for the structure and nomenclature of the various mutants, see Tables I and II, and Zechel *et al.* (1994)]. Complexes originating from the binding of one DBD monomer to DRs with varying spacer lengths (1 to 5 bp; DR1 to DR5) and a non-spaced inverted repeat (IR0) of the motif PuGGTCA are indicated by '1', while complexes originating from cooperative or non-cooperative binding by '2'. The number of nucleotides separating the various DRs is given below each lane. The arrows point to dimeric complexes formed between two different RXR mutants and DR1. For each receptor DBD the amount of expressed recombinant protein was determined by SDS–PAGE, and the DNA binding efficiencies of the various mutants were compared by EMSA using serial dilutions. To compare different DBD mutants the optimal receptor concentrations (shown in the figures) were arbitrarily defined as the dilutions which generated roughly equal amounts of monomeric complexes. Note, that in cases where the probe became limiting (panel C) the monomers of a low affinity mutant (XT_5) are no longer visible when assayed together with a high affinity DBD ($X_{\Delta 2}$), most likely due to competition for the common binding site, and that unstable monomeric complexes were formed by XE_1, XA_3, XT_3 (panel G), XE_2 (panel H) and AX_7 (panel J).

containing the RXR T-box, forming dimeric complexes of an intermediate mobility with $X_{\Delta 2}$ or AX_9 (arrow in Figure 1I, and data not shown). Thus, the RXR CII finger appears to provide the second RXR dimerization surface for DR1 binding.

To map further the sequences of the RXR CII finger which specifically contribute to the second dimerization surface, we constructed XA_7 and XT_7 (replacement of the RXR CII finger residues C-terminal to the D-box; Table I, line 12). No homocooperative DR1 binding could be detected (Figure 1J), even though XT_7 could form dimeric complexes with $X_{\Delta 2}$ on DR1 (Table I, line 12; arrow in Figure 1K). Thus, replacement of the tip and C-terminal part of the RXR CII finger with the corresponding regions of TR or RAR removed or inactivated the second RXR homodimerization surface. XT_8 and XA_8, in which the RXR D-box had been replaced with those of TR or RAR, homodimerized on DR1 elements (Table I, line 13; Figure 1L). Dose–response curves comparing the homocooperative DR1 bindings of XT_8 and XA_8 (Figure 2, lanes 9–12 and 5–8, respectively) with those of the RXR DBD, $X_{\Delta 1}$ and $X_{\Delta 2}$ (Figure 2, lanes 1–4, 13–16 and 17–20), confirmed that the exchange of the RXR D-box did not reduce the cooperativity of DR1 binding. Thus, the RXR D-box does not appear to be specifically required for the formation of

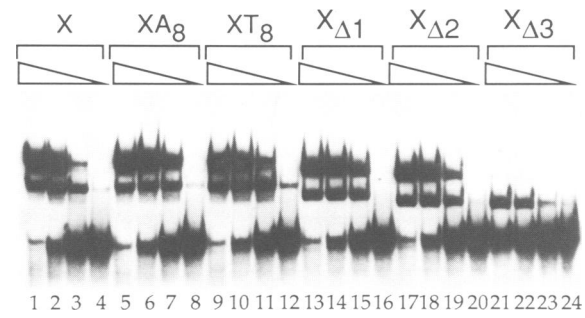
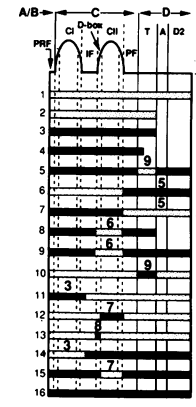


Fig. 2. The T-box and the RXR CII finger (to the exclusion of the D-box) are required for RXR DBD homocooperative DR1 binding. Dose–response experiments were carried out with decreasing amounts of bacterially expressed wild-type RXR, and truncated or chimeric RXR DBDs, as indicated at the top of each panel (for the structure of the various DBDs see Tables I and II). In crude lysates, the concentrations of the respective DBDs were calibrated by SDS–PAGE using serial dilutions. Equal amounts of the various DBDs were 5-fold diluted in serial steps. Note that the binding of the $X_{\Delta 3}$ monomers is 5-fold less efficient than that of DBDs harboring the RXR T-box, and that $X_{\Delta 3}$ cannot homodimerize.

the RXR homodimerization interface. We conclude, therefore, that the T-box of one RXR DBD monomer and residues located in the C-terminal CII finger (to the exclusion of the D-box) of a second DBD monomer are specifically

Table II. Summary of the results defining the DR2-specific heterodimerization surfaces in RXR and RAR DBDs



	DBDs/ Mutants	Region present in the mutants CII RXR	Region present in the mutants T-box RAR	Homo-dimerization on DR2	Heterodimerization on DR2 with RAR	Heterodimerization on DR2 with RXR	Fig.
1	X	+	-	-	+	-	3A;3B
2	$X_{\Delta2}$	+	-	-	+	-	3A
3	$A_{\Delta2}$	-	+	(*)	-	+	3I;3B
4	$A_{\Delta3}$	-	-	-	-	-	n.s.
5	AX_9	-	-	-	-	-	1F;1C
6	XA_5	+	+	(+/-)	(+/-)	+	3D;3I
7	AX_5,AT_5	-	-	-	-	-	1E;3H;3E
8	$A_{\Delta2}X_6$	+	+	(+/-)	(+/-)	+	3C
9	AX_6	+	+	(+/-)	(+/-)	+	3K
10	XA_9	+	+	+	+	+	1D
11	XA_3	+	-	-	+	-	3G
12	XA_7	D-box only	-	-	-	-	1J;3J
13	XA_8	minus D-box	-	-	+	-	3L
14	AX_3,AT_3	-	+	-	-	+	3F
15	AT_7	-	+	-	-	+	n.s.
16	A	-	+	-	-	+	3A

The RXR CII finger (excluding the D-box) and the RAR T-box are specifically required for the formation of the heterodimerization interface responsible for cooperative binding of RXR/RAR DBDs on DR2 elements. Dotted boxes represent RXR or TR sequences, whereas black boxes correspond to RAR sequences. For symbols and nomenclature, see Table I. (+/−) XA_5, $A_{\Delta2}X_6$ and AX_6 homodimerization or heterodimerization with RXR DBDs is weak, due to a reduced stability of the DR2 heterodimerization interface caused by (i) a reduced affinity of the mutant for DNA binding and/or (ii) the requirement for additional RXR sequences. (*) indicates that the formation of 'type 2' complexes by $A_{\Delta2}$ occurred more efficiently on DR2 than on DR3, DR4 or DR5. n.s., data not shown.

required for the formation of the homodimerization interface responsible for cooperative binding to DR1. Note that results obtained with additional mutants which are not discussed here fully support this conclusion (an extended version of this manuscript describing these data is available upon request).

The RXR CII finger (to the exclusion of the D-box) and the RAR T-box are specifically required for the formation of the dimerization interface responsible for cooperative binding of RXR/RAR DBDs on DR2 elements

Neither the D2 regions, nor the A-boxes of RXR and RAR are required for heterocooperative binding to DR2, since the RAR DBD and $X_{\Delta2}$, and the RXR DBD and $A_{\Delta2}$ efficiently formed dimeric complexes on DR2 (Table II, lines 2 and 3; Figure 3A and B) [note that, unlike the wild-type RAR DBD, $A_{\Delta2}$—which contains all the sequences required for efficient binding of an RAR DBD to the PuGGTCA motif (amino acid residues Leu81 to Lys164 of RARα1; Zelent *et al.*, 1989)—bound more efficiently to DR2 than to DR3, DR4 or DR5 (see for example panel $A_{\Delta2}$ in Figure 3I)]. In contrast, the RAR T-box was required for heterodimerization on DR2, since (i) $A_{\Delta3}$ lost the ability to bind to DNA and to heterodimerize with RXR DBDs (Table II, line 4), and (ii) an RAR DBD containing the RXR T-box (AX_9) lost the ability to heterodimerize with $X_{\Delta2}$ on DR2 (Table II, line 5; panel $AX_9/X_{\Delta2}$ in Figure 1C). Moreover, introducing

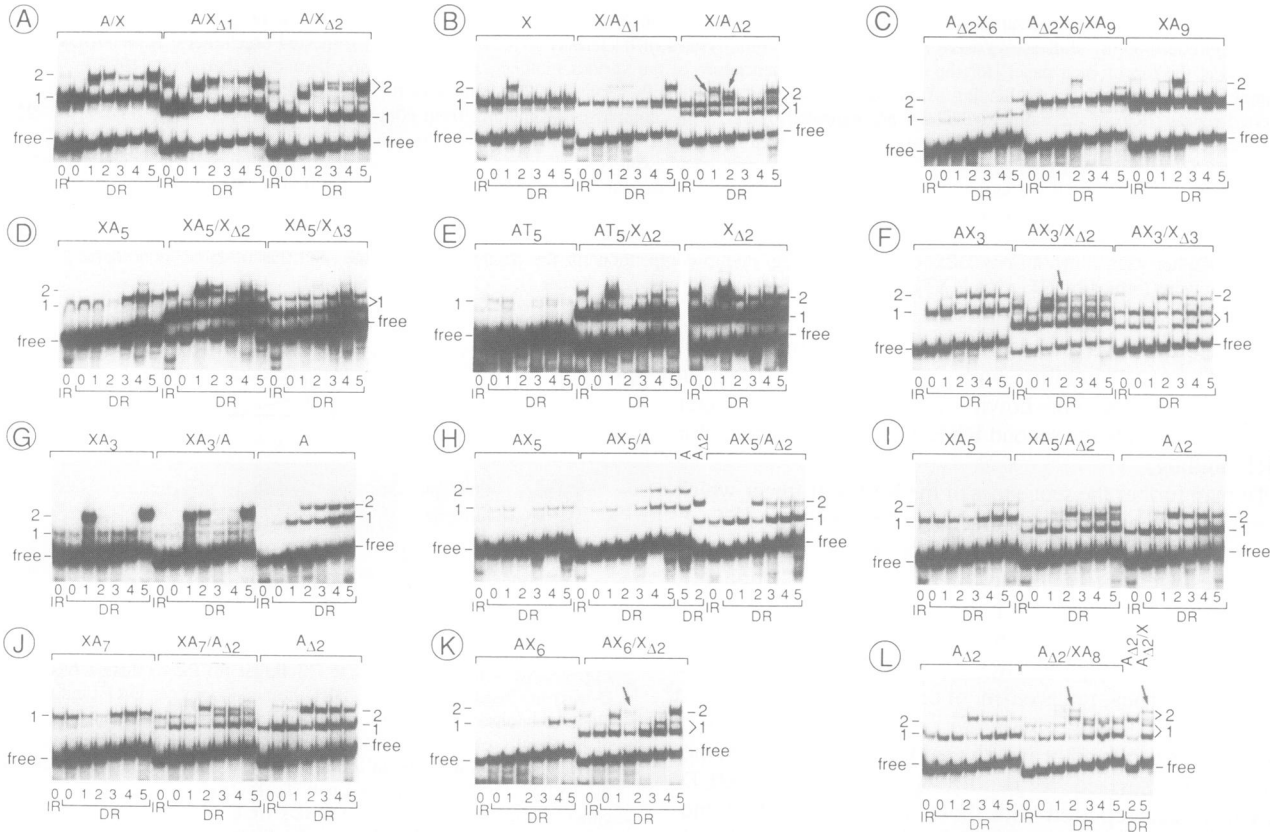


Fig. 3. RXR/RAR DBD heterodimerization on DR2 elements requires the CII RXR finger (to the exclusion of the D-box) and the RAR T-box. EMSAs were performed with crude lysates of the bacterially expressed DBDs indicated at the top of the gels (for the structure and nomenclature of the various DBDs and the protein amounts used, see Figure 1 and Tables I and II). In panel B, 5-fold less RXR DBD was used with $X/A_{\Delta1}$ than with X, to visualize the differences in cooperative binding of the RXR and RAR DBDs to DR2 and DR5 elements. The arrows in lanes DR1 and DR2 point to the appearance of a novel dimeric DR1 complex, in addition to the RXR homodimer, which migrates with the same mobility as the heterodimeric DR2 and DR5 complexes (see Discussion). Such presumable heterodimeric complexes were also efficiently formed between $AX_3/X_{\Delta2}$ and the DR1 elements in panel F. Note that $X_{\Delta3}$ formed weak if any dimeric complexes with XA_5 (panel D) or AX_3 (panel F) on DR2. Arrows in panels C, F, K and L indicate DR2- and DR5-specific heterodimerization.

the sequences located C-terminally to the RAR CII finger into RXR or TR DBDs generated chimeras (XA_5 in Table II, line 6; TA_5, data not shown) which efficiently formed dimeric complexes with $X_{\Delta 2}$ on DR2 (panel $XA_5/X_{\Delta 2}$ in Figure 3D), while the reciprocal constructs did not (AX_5 and AT_5, Table II, line 7; panels $AX_5/X_{\Delta 2}$ in Figure 1E and $AT_5/X_{\Delta 2}$ in Figure 3E). Finally, both RAR CI and CII fingers could be partially or entirely replaced with the corresponding fingers of RXR or TR (Table II, AX_3 and AT_3, line 14; $A_{\Delta 2}X_6$, line 8; AX_6, line 9; AT_7, line 15) without impairing RAR/RXR heterodimerization on DR2 (arrows in panels $AX_3/X_{\Delta 2}$ in Figure 3F, $AX_6/X_{\Delta 2}$ in Figure 3K, and data not shown). All of these results suggest that the T-box is the only component which is specifically required for the formation of the DR2-specific RAR heterodimerization surface. In accordance with this, swapping the RAR T-box into the RXR DBD generated XA_9 (Table II, line 10) which efficiently homodimerized on DR2 elements (panel XA_9 in Figures 1D and 3C).

The D2, A-box and T-box regions of the RXR DBD do not appear to contribute specifically to the DR2-specific heterodimerization interface, since $X_{\Delta 2}$ and XA_9 heterodimerized with the RAR DBD on DR2 (Table II, lines 2 and 10). Similarly, the RXR CI finger is not specifically involved in this dimerization interface, since XA_3 efficiently formed heterodimeric complexes on DR2 with the RAR DBD (Table II, line 11; panels XA_3/A in Figure 3G). In contrast, AX_5 (Table I, line 6) or TX_5 did not bind heterocooperatively to DR2 when mixed with RAR or $A_{\Delta 2}$ (panels AX_5/A and $AX_5/A_{\Delta 2}$ in Figure 3H, and data not shown; note the stronger signal on DR2 with $A_{\Delta 2}$ alone), suggesting that the RXR CII finger and the interfinger region could be required for the formation of the DR2-specific dimerization interface. In this respect, we note that XA_5, $A_{\Delta 2}X_6$ and AX_6 (Table II, lines 6, 8 and 9) did not heterodimerize as efficiently with the RAR DBD or $A_{\Delta 2}$ on DR2 as the RXR DBD or $X_{\Delta 2}$ (panel $XA_5/A_{\Delta 2}$, Figure 3I, and data not shown). Thus, the RXR CII finger is required for the formation of an RXR/RAR DR2-specific dimerization interface but, in contrast to the DR1 RXR/RXR homodimer case, adjacent C-terminal residues could also be involved. Within the RXR CII finger, the tip and C-terminal part appear to be specifically required for RXR/RAR heterodimerization on DR2 elements, as no heterodimeric DR2 complexes were formed by XA_7 and $A_{\Delta 2}$ or XT_7 and $A_{\Delta 2}$ and the DR2 element (XT_7, see Table I, line 12; XA_7, Table II, line 12; panel $XA_7/A_{\Delta 2}$ in Figure 3J). In contrast, the RXR D-box is not specifically required for RXR/RAR heterodimerization on DR2, as XA_8 and XT_8 (Tables I and II, lines 13) in which a heterologous D-box replaces that of RXR, efficiently dimerized with the RAR DBD or $A_{\Delta 2}$ on DR2 (arrow in panel $A_{\Delta 2}/XA_8$ in Figure 3L, and data not shown). Thus, heterocooperative binding of RAR and RXR DBDs to DR2 elements results from the formation of a dimerization interface which specifically requires the RAR T-box and the CII finger region of RXR to the exclusion of the D-box.

The dimerization surfaces of RAR, RXR and TR DBDs determine the polarity of the cooperative binding of the corresponding full-length receptors to DR elements

DNA elements consisting of directly repeated motifs are inherently asymmetric, implying that, unless the dimerization

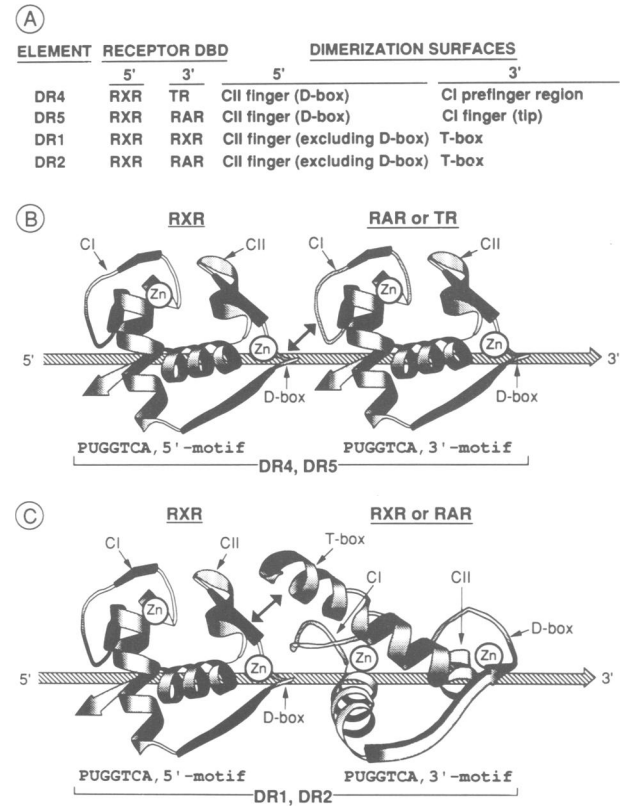


Fig. 4. Modeling of the two types of dimerization interfaces which results in homo- and heterocooperative interactions among the DBDs of RXR, RAR and TR. (**A**) Summary of the protein–protein interactions that are responsible for cooperative DNA binding to DR1 (RXR), DR2 and DR5 (RXR and RAR), and DR4 elements (RXR and TR) and of the orientations of the monomers on DNA (see text, Zechel *et al.*, 1994, and below). (**B**) Model derived from the ribbon representation of two monomers of the GR DBD bound to its cognate response element (Luisi *et al.*, 1991). The DNA representation has been omitted for the sake of clarity, and only its 5′ → 3′ polarity is indicated by a hatched arrow. In contrast to the GR or ER DBD–DNA co-crystal, in which the dimer displays a dyad axis to accommodate binding to its palindromic response element (Luisi *et al.*, 1991; Schwabe *et al.*, 1993a, b), two GR-like DBD monomers are represented here in the same orientation to reflect the binding of RAR, RXR and TR DBDs to DR5 and DR4 [see Zechel *et al.* (1994)]. The monomers are represented facing the same side of the DNA helix, assuming identical protein–DNA interactions for each monomer and a center to center distance of the PuGGTCA motifs of roughly one helical turn, as would be expected for the binding to DR4 (center to center distance of 10 bp) and DR5 (center to center distance of 11 bp) elements. Note that the overall structure of the DBDs of all nuclear receptors is believed to be very similar (Schwabe *et al.*, 1990, 1993a,b; Luisi *et al.*, 1991; Katahira *et al.*, 1992; Lee *et al.*, 1993). From this representation, it can be predicted that in the heterodimer the RXR DBD which contributes the CII finger D-box surface has to occupy the 5′ position, if an interface (double arrow) is to be formed with the CI finger region surface of either RAR or TR DBD. (**C**) Similar modeling to that in (B), except that the 3′ located monomer was rotated by ~90° against the 5′ positioned monomer to reflect their relative orientation on DR1 or DR2 elements. Note that 90° corresponds to a roughly average angle for monomers bound to DR1 and DR2 elements. In addition, the 3′ located monomer is schematically represented with a helical T-box region according to a three-dimensional NMR analysis of the structure of the RXR DBD (Lee *et al.*, 1993), even though the structure of the corresponding sequence in RAR is unknown. This model illustrates the possibility to form interfaces (double arrow) between a 5′ located CII finger surface (excluding the D-box) and a 3′ located T-box, as experimentally determined in this study, resulting in anisotropic RXR/RXR and RXR/RAR complexes on DR1 and DR2, respectively.

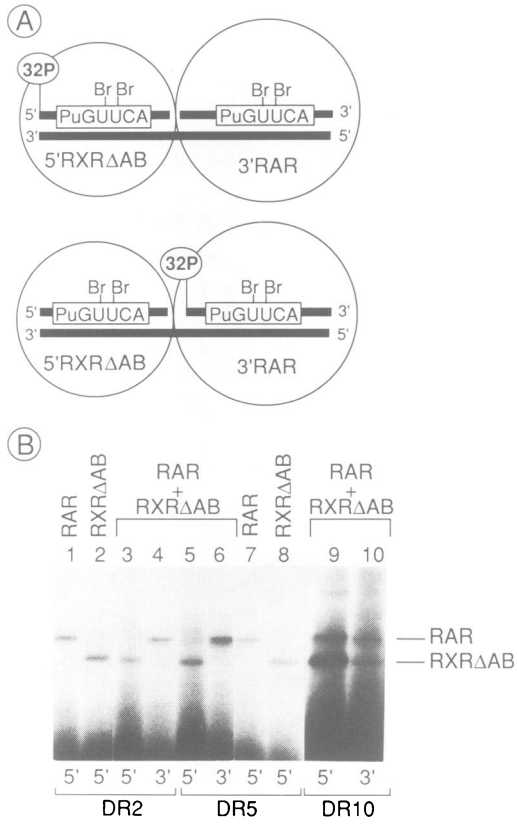


Fig. 5. RXR occupies the 5′ motif and RAR the 3′ motif in full-length receptor heterodimeric DR2 and DR5 complexes, while both motifs are similarly occupied by RAR and RXR in DR10 complexes. (**A**) The 5′ or the 3′ motif in gapped DR2, DR5 and DR10 elements was labeled with ^{32}P (for details see Results, and Materials and methods). BrdU replaced thymidine residues in PuGTTCA motifs as indicated. In protein–DNA adducts, the truncated RXR (RXRΔAB) was covalently bound to the 5′ and RAR to the 3′ motif as indicated by circles of different sizes. (**B**) Electrophoresis of protein–DNA adducts under denaturing conditions. Crude extracts or purified bacterially expressed RXRΔAB and RAR (see Materials and methods) were analyzed separately or mixed, as indicated at the top of each panel. The more rapidly migrating band corresponds to RXRΔAB–DNA adducts, and the more slowly migrating one to RAR–DNA adducts. The various elements and the ^{32}P-labeled motif are indicated below each lane.

interface is highly flexible, it is unlikely that a given RXR/RAR heterodimer can bind cooperatively to a cognate DR element in configurations corresponding to the two possible polarities (e.g. 5′-RXR/RAR-3′ and 5′-RAR/RXR-3′). In view of the rigid structure of the receptor DBDs [similar overall structures were observed for the DBDs of the GR, ER and RAR (Härd *et al.*, 1990; Schwabe *et al.*, 1990; Luisi *et al.*, 1991; Katahira *et al.*, 1992; Lee *et al.*, 1993)], it can therefore be predicted that the cooperative binding of a given heterodimer to a given DR occurs in a polar fashion [see also Leid *et al.* (1992a) and Mader *et al.* (1993a)]. Based on the three-dimensional structure of GR and ER DBD–DNA complexes (Luisi *et al.*, 1991; Schwabe *et al.*, 1993a,b), modeling of the dimerization interfaces involved in the cooperative binding of the RAR, RXR and TR DBDs predicts that the receptor DBD contributing the CII finger region (RXR) has to be bound 5′ to its partner which contributes either the T-box region (RXR and RAR on DR1 and DR2, respectively) or the CI finger region (TR and RAR on DR4 and DR5, respectively). This polar cooperative binding of the two types of DBD dimer is depicted in the models shown in Figure 4B and C, in which the ribbon representation of two GR-like or ER-like monomers is assembled on DR elements. We have neglected in these models the differences which may exist between DR4 and DR5 complexes (Figure 4B), and between DR1 and DR2 complexes (Figure 4C). The T-box of the RXR DBD is represented according to the recently described solution structure of the RXR DBD (Lee *et al.*, 1993).

We have shown in the accompanying study (Zechel *et al.*, 1994) that the same DBD regions which are required for cooperative binding of the isolated DBDs on certain DRs are also necessary for cooperative binding of full-length receptors on these elements. These results strongly suggest that the cooperative binding of full-length receptors occurs with a given polarity. To investigate whether the polarity of the cooperative binding of RXR/RAR and RXR/TR DBDs indeed results in a polar binding of the corresponding full-length receptor heterodimers on the various DR elements, we crosslinked partially purified bacterially expressed receptors to BrdU-substituted DRs which contained a gap between the two motifs, and in which only one of the oligonucleotides constituting the gapped strand was radiolabeled (Figure 5A). To facilitate the identification of the crosslinking products in denaturing SDS–PAGE, the heterodimers were assembled from receptors of different sizes (a full-length RAR and an N-terminally truncated RXRΔAB). When RXRΔAB/RAR heterodimers were crosslinked with BrdU-substituted DR2 and DR5 (Figure 5B), RXRΔAB bound covalently to the 5′ motif in both DR2 (lane 3) and DR5 (lane 5) complexes, while RAR (lanes 4 and 6) always bound to the 3′ motif [the position of the respective RXRΔAB/DNA and RAR/DNA adducts are apparent from SDS–PAGE of crosslinked homodimeric RXRΔAB (lanes 2 and 8) and RAR (lanes 1 and 7)]. Similarly, crosslinking of TR/RXR heterodimers to DR4 elements revealed that RXR bound to the 5′ motif, whereas TR bound to the 3′ motif (data not shown). Interestingly, no such binding polarity of full-length RXR/RAR heterodimers was seen with more widely spaced repeated motifs (DR10; Figure 5B, lanes 9 and 10), even though RXR/RAR heterodimers bound cooperatively to such elements (J.-Y.Chen, S.Kato, H.Gronemeyer and P.Chambon, unpublished results).

Discussion

The RXR C-terminal CII finger (to the exclusion of the D-box) and the T-box of either RXR or RAR are specifically required for the formation of the dimerization interfaces which specify cooperative binding of RXR homodimers and RXR/RAR heterodimers to DR1 and DR2, respectively.

We have identified here two distinct dimerization surfaces of the RXR DBD which are specifically required for homo-cooperative binding of RXR to DR1 elements. Moreover, we have uncovered an alternative dimerization interface between the DBDs of RAR and RXR, which is responsible for their cooperative binding to DR2 elements and is distinct from that directing their binding to DR5 elements (see Zechel *et al.*, 1994). That the RXR T-box corresponds to one of the dimerization surfaces involved in the formation of the RXR/RXR homodimerization interface on DR1, was demonstrated not only by loss-of-function experiments

(which are prone to misinterpretation), but also by gain-of-function experiments in which swapping the T-box of RXR into a heterologous DBD resulted in the selective formation of heterodimeric DR1 complexes with the RXR DBD (see $AX_9/X_{\Delta 2}$, Table I). Gain-of-function experiments also showed that the RXR CII finger provides the second RXR dimerization surface which, together with the T-box, forms the interface that is specifically required for homodimerization on DR1. In addition, our data exclude a specific role of the RXR D-box of the CII finger region in the formation of the DR1-specific dimerization interface (see XT_8 and XA_8, Table I). Our present results are in keeping with the previous suggestions of Wilson *et al.* (1992) who proposed that the RXR T-box may be involved in the binding of RXR to direct repeats, and with those of Lee *et al.* (1993) who showed that the T-box is necessary for the formation of the RXR DBD homodimerization interface. Interestingly, the results obtained by Wilson *et al.* (1992), using a series of RXR/NGFI-B chimeras, suggest that the CII finger of the NGFI-B orphan receptor can replace the RXR CII finger for the formation of a DR1-specific dimerization interface. In this respect, we note that the amino acid sequences of the tip of the CII fingers of RXR and NGFI-B are very similar to each other (much more so than to other members of the receptor family, e.g. RAR or TR), which suggests that the conserved sequence [DKR(Q/R)RNR] may in fact provide the dimerization surface which interacts with the RXR T-box in RXR homodimers.

We have shown in the accompanying paper (Zechel *et al.*, 1994) that the formation of a dimerization interface which specifically requires the tip of the RAR CI finger and the D-box of RXR determines the cooperative binding of RXR/RAR heterodimers to DR5. We have also demonstrated in the same study that the same D-box is specifically required for the formation of the interface which dictates the binding of RXR/TR heterodimers to DR4. We show here that the formation of the alternative interface which specifies the cooperative binding of RXR/RAR to DR2 specifically requires the RAR T-box and the RXR CII finger (possibly including the 'post-finger region'). However, as it is the case for the DR1-specific RXR DBD homodimerization interface, the RXR D-box does not appear to be specifically involved in the formation of the DR2-specific RXR/RAR DBD heterodimerization interface. Thus, two distinct portions of the RXR CII finger specifically contribute to the dimerization interfaces which dictate cooperative binding to closely (DR1 and DR2) and more widely (DR4 and DR5) spaced DR elements.

The dimerization interfaces in the DBDs of RXR/RAR and RXR/TR heterodimers specify the formation of anisotropic complexes with specific DR elements

Modeling of the homo- and heterodimeric protein–protein interactions of the RXR DBD, based on the three-dimensional structure of the GR and ER DBDs, indicates that the two types of asymmetric dimerization interfaces (Figure 4A) which have been defined in this and the accompanying paper (Zechel *et al.*, 1994) should result in a binding polarity of the asymmetric dimers to specific DR elements (Figure 4B and C). For example, the interaction between the RXR CII finger and the RAR T-box on DR2 elements is possible only when RXR occupies the 5′ position (Figure 4C). Similarly, the formation of interfaces between the D-box of RXR and either the TR N-terminal prefinger region or the tip of the RAR CI finger requires that RXR is bound to the 5′ motif (Figure 4B). The existence of such RXR/TR and RXR/RAR binding polarities is supported by our previous observations which suggested that cooperative binding of the corresponding DBDs required RXR to be bound to the DR 5′ motif, and also showed that transactivation by full-length heterodimers from cognate reporters was higher when RXR was bound to the DR 5′ motif (Mader *et al.*, 1993a). Furthermore, using an RXR mutant containing the P-box of GR, Perlmann *et al.* (1993) have provided evidence that RXR is bound to the 5′ motif in DR5–RXR/RAR complexes. A similar approach has been used by Kurokawa *et al.* (1993) to provide evidence for the polarity of RXR/TR heterodimer binding to DR4. However, it is difficult to rule out the possibility that in these latter studies P-box exchanges and the use of cognate chimeric response elements have created artifactual conditions. Our present crosslinking experiments prove the polarity of the binding of RXR/RAR heterodimers to DR2 and DR5 elements (RXR bound to the 5′ motif and RAR bound to the 3′ motif, Figure 5). Moreover, we have observed the same polarity (RXR bound to the 5′ motif) for RXR/TR heterodimers bound on DR4 elements (data not shown). Using a different crosslinking strategy, Kurokawa *et al.* (1993) observed a binding polarity of the RXR, but not the RAR partner of RXR/RAR heterodimers on DR5. In addition, these authors reported that an RXR chimera containing the TR A-box could be crosslinked to both the 5′ and the 3′ motif of a DR4 element. This result is not compatible with the RXR/TR dimerization interface as defined here and in the study of Perlmann *et al.* (1993), and may be related to difficulties in the interpretation of crosslinking data.

Interestingly, in contrast to the RXR/RAR heterodimeric complexes formed with DR2 and DR5 elements, no binding polarity was observed using a widely spaced DR element (DR10), even though [due to the presence of dimerization functions in their ligand binding domains (LBDs)] RXR/RAR still bound cooperatively to DR10, albeit less efficiently than to DR2 or DR5. Thus, both the specificity of the DR response element repertoire and the polarity of the receptors on DR elements appear to be determined by the asymmetry of their DBD dimerization interfaces.

Previous studies have shown that RXR/RAR heterodimers can bind to (Mangelsdorf *et al.*, 1991; Nakshatri and Chambon, 1994) and transactivate from (Durand *et al.*, 1992) a DR1 retinoic acid response element. It is difficult to envisage the possibility that the RAR partner could be bound to the 3′ motif in such DR1 complexes, because RAR binds as a dimer only to DR($n>1$), possibly due to the binding of its T-box to the spacer of DR elements (see Zechel *et al.*, 1994). In contrast, RXR can bind as a homodimer to DR1 elements. Therefore, the polarity of RXR/RAR heterodimers in DR1 complexes may possibly be opposite (RXR bound to the 3′ motif) to that in DR2 and DR5 complexes. In this respect it is interesting to note that AX_9 (Table I), which harbors the T-box of RXR in an RAR DBD background, formed inefficiently, but selectively, homodimers on DR1 (Figure 1F). In considering the model presented in Figure 4C, this mutant may bind cooperatively to DR1 through the formation of a dimerization interface between the RXR T-box of one AX_9 monomer and RAR

CII finger in the other AX_9 monomer. Assuming that a similar interface [perhaps stabilized by additional factor(s)], can be established in wild-type RAR/RXR heterodimers bound to DR1, the polarity of such a DR1 heterodimeric complex would be 5′-RAR-RXR-3′, opposite to the polarity of RXR/RAR heterodimers bound to DR2 elements. Additional heterodimeric complexes have been shown to be formed with RXR on DR1 elements. Whether heterodimerization of RXR and chicken ovalbumin upstream promoter-transcription factor (COUP-TF), and RXR and peroxisome proliferator activated receptor (PPAR), on DR1 elements (Kliewer *et al.*, 1992a,b; Bardot *et al.*, 1993; Gearing *et al.*, 1993; Issemann *et al.*, 1993; Keller *et al.*, 1993; Marcus *et al.*, 1993) occurs with the 5′-RXR-COUP-TF-3′ and 5′-RXR-PPAR-3′ polarity (CII finger of RXR interacting with the T-box of either COUP-TF or PPAR) or with the opposite polarity (CII finger of either COUP-TF or PPAR interacting with the T-box of RXR) remains to be established.

In addition to direct repeats, inverted and everted repeats have been reported to function as response elements for thyroid hormone, retinoic acid or vitamin D (Baniahmad *et al.*, 1990; Näär *et al.*, 1991; Bugge *et al.*, 1992; Carlberg *et al.*, 1993; Mader *et al.*, 1993b; Park *et al.*, 1993; Tini *et al.*, 1993). We have previously reported that bacterially expressed full-length RXR, RAR and TR bound selectively and cooperatively to IR0 elements, and that the TR DBD exhibited a weak cooperativity for IR0 binding (Mader *et al.*, 1993a). Computer models based on the structure of the GR and ER DBDs strongly suggest that the two types of DR interface discussed above (see Figure 4) cannot be formed on inverted or everted repeats. For example, binding to an IR0 element positions the two DBD monomers such that D-box/D-box (as seen for IR3 binding of GR and ER; Luisi *et al.*, 1991; Schwabe *et al.*, 1993a,b), D-box/CI finger or CII finger/T-box interfaces cannot form. As is the case for the DR elements whose spacer lengths are not compatible with the formation of the DBD dimerization interface (e.g. DR10, see above), the other dimerization functions which overlap with the LBD (Leid *et al.*, 1992b; Au-Fliegner *et al.*, 1993; Darling *et al.*, 1993; Kurokawa *et al.*, 1993; Nagpal *et al.*, 1993; Perlmann *et al.*, 1993; Rosen *et al.*, 1993, and references therein) must be responsible for cooperative binding to inverted and/or everted repeats.

Biological implications of the formation of anisotropic DNA complexes

The presence of multiple and independent dimerization surfaces in the RXR and RAR DBDs, which can form different dimerization interfaces allowing cooperative binding to a variety of specific DR elements, is an important additional parameter for building up the combinational complexity which is necessary to account for the highly pleiotropic effects of the retinoic acid signal (Leid *et al.*, 1992a; Chambon, 1993, and references therein). That the multiple homo- and heterodimers bind to their cognate response elements in a specific orientation resulting in anisotropic complexes, represents an additional factor contributing to the complexity of this signaling pathway. Indeed, the two possible orientations of a given response element (with respect to the polarity of the promoter environment), may lead to distinct transcriptional outcomes. Depending on the relative orientation of the response element within the promoter regions of different target genes, interactions of the receptor functional domains with other regulatory factors bound 5′ or 3′ of the dimeric receptor complex may be facilitated or excluded. In this respect, since synergistic effects have been observed for a composite element made up of a degenerate DR1 and a Pit-1 binding site (Rhodes *et al.*, 1993), it would be interesting to test the result of an inversion of the DR1 element on this synergism. Similarly, direct or indirect interactions with the basic transcriptional machinery may be influenced by the response element orientation. Moreover, depending on the relative orientation of the response element, the effect of receptor-induced DNA bending (King *et al.*, 1993; Lu *et al.*, 1993; our unpublished results) could be drastically different, with respect to the modulation of the interaction with other promoter-bound transacting factors and/or the basic transcriptional machinery.

Finally, it is worth pointing out that the three-dimensional conformation of the DBDs of RXR/RAR bound to DR5 and DR2 (and, possibly, DR1) elements are different (compare Figure 4B and C), which may through allosteric effects lead to distinct conformations of other receptor functional domains, such as the LBD or the activation functions (AF-1 and AF-2). Thus, the protein or ligand interactions which may occur with the various functional receptor domains may be characteristic of a given response element. Clearly, the multiple dimerization surfaces which are present in RAR and RXR and allow the polar recognition of different response elements, may be important to increase the combinatorial possibilities of interactions between these receptors and any other factor(s) which participate in the control of transcription of retinoic acid target genes.

Materials and methods

Plasmids, expression of proteins and electrophoretic mobility shift assays

The construction, structure and expression of the various DBD mutants and chimeras are described in the accompanying paper (Zechel *et al.*, 1994), and the plasmids pET3a-RARα, pET3a-RXRΔAB and pET3a-chTRα in Mader *et al.* (1993a). To obtain crude lysates of the various DBDs and the full-length RAR and TR, cells were lysed by sonication in 2% of the original culture volume in lysis buffer [50 mM Tris–HCl (pH 8.0), 100 mM KCl, 0.1 mM DTT, 10 μM $ZnCl_2$, 10% glycerol], supplemented with a cocktail of protease inhibitors. Lysates were cleared by centrifugation for 30 min at 12 000 *g*. Protein concentrations were determined by comparative SDS–PAGE. Overexpression and purification of the RXRΔAB and RAR will be described elsewhere.

Protein amounts used in each EMSA were calibrated as described in the legend of Figure 1. Proteins and 10 fmol of ^{32}P-labeled oligonucleotide probe (125 000 c.p.m.) were incubated for 15 min in a final volume of 20 μl binding buffer [10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, 0.4 mM DTT, 5% glycerol], containing 2 μg poly(dI-dC)(dI-dC) and 100 mM KCl. In some cases, for EMSAs defining the RXR DBD homodimerization interface, a final concentration of 50 mM KCl was used for the mutants XE_1, XA_3, XT_3 and XE_2. The protein–DNA complexes were resolved in 6% polyacrylamide gels (0.5 × TBE buffer; prerun for 2 h) at 13 V/cm and 4°C. Protein–DNA complex formation was quantified by phosphoimaging with a Fuji BAS 2000. The sequences of IR0 and DR1–DR5 are given in Mader *et al.* (1993a) (details are available upon request).

Crosslinking

The gapped, double-stranded oligonucleotides used for crosslinking experiments are schematically depicted in Figure 5A. BrdU residues were incorporated during oligonucleotide synthesis. The DR5 probe consisted of a 5′ half-site (5′-TCGAGGGTAG**GG(BrdU)(BrdU)CA**CCG-3′) and a 3′ half-site (5′-AA**AG(BrdU)(BrdU)CA**CTCGCACTCG-3′), which were annealed to a continuous complementary strand. Either the 5′ or the 3′ half-

site was kinased with [γ-^{32}P]ATP (see Figure 5A). For the preparation of the DR2, DR4 and DR10 probes the same strategy was applied except that the half-sites of the DR4 element contained the motif PuGG(BrdU)CA (detailed sequences are available upon request). The binding efficiency of homo- and heterodimeric RXR, RAR and TR to the gapped DR elements was indistinguishable from that of non-gapped, but otherwise identical oligonucleotides. 80 ng of bacterially expressed RXRΔAB were incubated with equal amounts of full-length RAR or TR, and 40 fmol of ^{32}P-labeled (500 000 c.p.m.) gapped DR probe in a final volume of 20 μl binding buffer [10 mM Tris−HCl (pH 8.0), 0.1 mM EDTA, 50 mM DTT, 80 mM KCl, 2 μg poly(dI-dC)(dI-dC), 5% glycerol] in micro-tissue culture plates (Falcon 3072; Becton Dickinson) for at least 15 min at room temperature. Preparations of purified RXRΔAB and RAR, as well as crude lysates of RAR and TR were used. UV crosslinking was performed at 0°C for 30 min using a wavelength of 312 nm. After addition of an equal volume of 2 × SDS sample buffer, the samples were heated at 100°C for 5 min and loaded into 4% SDS stacking gels. The DNA−protein complexes were resolved through 10% SDS−polyacrylamide gels, and visualized by autoradiography. The same protocol was used for crosslinking of homodimeric RXRΔAB, RAR and TR, except that larger amounts of proteins were used.

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