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# Dimerization interfaces formed between the DNA binding domains determine the cooperative binding of RXR/RAR and RXR/TR heterodimers to DR5 and DR4 elements

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**We have previously reported that the binding site repertoires of heterodimers formed between retinoid X receptor (RXR) and either retinoic acid receptor (RAR) or thyroid hormone receptor (TR) bound to response elements consisting of directly repeated PuG(G/T)TCA motifs spaced by 1–5 bp [direct repeat (DR) elements 1–5] are highly similar to those of their corresponding DNA binding domains (DBDs). We have now mapped the dimerization surfaces located in the DBDs of RXR, RAR and TR, which are responsible for cooperative interaction on DR4 (RXR and TR) and DR5 (RXR and RAR). The D-box of the C-terminal CII finger of RXR provides one of the surfaces which is specifically required for the formation of the heterodimerization interfaces on both DR4 and DR5. Heterodimerization with the RXR DBD on DR5 specifically requires the tip of the RAR CI finger as the complementary surface, while a 7 amino acid sequence encompassing the 'prefinger region', but not the TR CI finger, is specifically required for efficient dimerization of TR and RXR DBDs on DR4. Importantly, DBD swapping experiments demonstrate not only that the binding site repertoires of the full-length receptors are dictated by those of their DBDs, but also that the formation of distinct dimerization interfaces between the DBDs are the critical determinants for cooperative DNA binding of these receptors to specific DRs.**

*Key words:* DNA binding specificity/DR4 and DR5 response elements/retinoic acid receptors/thyroid hormone receptor

## Introduction

Selective recognition of cognate *cis*-acting DNA elements by *trans*-acting regulatory proteins is crucial in the cascade of events that ultimately leads to the realization of specific genetic programs, which in the case of the nuclear receptor superfamily control multiple aspects of development, cell growth and differentiation, and homeostasis (Evans, 1988; Green and Chambon, 1988; Beato, 1989; de Luca, 1991; Leid *et al.*, 1992a; Mendelsohn *et al.*, 1992; Chambon, 1993, and references therein). Thus, the mechanisms that define the DNA response element repertoires of nuclear receptors are of major importance in the signal transduction pathways initiated by steroid and thyroid hormones, retinoids and vitamin D3. In this respect, most of the structure–function information available at the present time was obtained from studies of steroid receptors which specifically bind as homodimers to response elements mostly composed of palindromic (or invertedly repeated) arrangements of two PuGG(T/A)CA binding motifs separated by a 3 bp spacer [called inverted repeat (IR)3]. Selective response element recognition is due to a short sequence (the P-box or 'recognition helix', see Figure 1) which is located at the C-terminal base of the N-terminal CI finger of the DNA binding domain (DBD) and interacts directly with the binding motif, and to a weak dimerization function which encompasses the N-terminal base of the CII finger (D-box, see Figure 1) of the DBD (Green *et al.*, 1988; Kumar and Chambon, 1988; Danielsen *et al.*, 1989; Mader *et al.*, 1989; Umesono and Evans, 1989; Luisi *et al.*, 1991; for reviews see Gronemeyer, 1991 and Laudet *et al.*, 1992). The study of chimeric receptors, as well as NMR and crystal structure analyses, demonstrated that this D-box is responsible for the selection of the spacing distance between the two halves of the palindrome (Umesono and Evans, 1989; Härd *et al.*, 1990; Schwabe *et al.*, 1990, 1993a,b; Dahlman-Wright *et al.*, 1991, 1993; Freedman and Towers, 1991; Luisi *et al.*, 1991; Mader *et al.*, 1993a). In addition to the weak dimerization function present in the DBD, the presence of a strong homodimerization function has been demonstrated in the ligand binding domains (LBDs) of steroid receptors (Kumar and Chambon, 1988; Tsai *et al.*, 1988; Eriksson and Wrange, 1990; Fawell *et al.*, 1990).

In contrast to steroid receptors, the receptors for retinoids (RARs and RXRs), thyroid hormone (TRs) and vitamin D3 (VDR) bind to, and activate transcription from DNA elements consisting of both direct repeats (DRs) and IRs of the motif 5′-PuGGTCA [for reviews see Leid *et al.* (1992a) and Stunnenberg (1993)]. Retinoic acid receptors and VDR can also interact efficiently with PuGTTCA and PuGGTGA directly repeated motifs, respectively, and the existence of natural retinoic acid or vitamin D response elements composed of everted repeats has been reported (Carlberg *et al.*, 1993; Mader *et al.*, 1993c; Tini *et al.*, 1993). Moreover, RARs, RXRs, VDR and TRs can bind to the various classes of response elements as homodimers, but heterodimerization between RXR and either RAR, TR or VDR (or certain orphan receptors) increases the efficiency of DNA binding *in vitro* and transcriptional activity in transfected cells *in vivo* (Yu *et al.*, 1991; Berrodin *et al.*, 1992; Bugge *et al.*, 1992; Durand *et al.*, 1992; Hallenbeck *et al.*, 1992; Kliewer *et al.*, 1992a,b,c; Leid *et al.*, 1992b; Marks *et al.*, 1992; Zhang *et al.*, 1992a,b; Bardot *et al.*, 1993; Heery *et al.*, 1993; Mader *et al.*, 1993b; Nakshatri and Chambon, 1994). Furthermore, it has been shown that both the RAR and RXR partners can contribute to the transcriptional activity of RXR/RAR heterodimers in animal cells and yeast (Durand *et al.*, 1992; Heery *et al.*, 1993; Nagpal *et al.*, 1993).

Based on transient transfection experiments, a 'DR-spacing rule' or response element 'code' has been proposed, stating that specific spacing and/or orientation of the repeated PuG(G/T)TCA motif defines the cognate response elements of RXR, RAR, VDR or TR (Näär *et al.*, 1991; Umesono *et al.*, 1991; Kliewer *et al.*, 1992a). For example, the so-called '3-4-5 rule' predicts that promoters containing DR3, DR4 and DR5 (i.e. DRs with 3, 4 and 5 bp spacers respectively) are selectively activated by VDR, TR and RAR, respectively (Umesono *et al.*, 1991). However, a significant degeneracy of the above 'rule' was rapidly recognized (Smith *et al.*, 1991; Durand *et al.*, 1992; Mader *et al.*, 1993b). A systematic study of the binding patterns of RXR, RAR and TR expressed in *Escherichia coli* to series of DRs revealed different binding site repertoires for their homo- and heterodimers, with some overlap between the '3-4-5 rule' and the observed binding site repertoire of the various heterodimers (Mader *et al.*, 1993b). The repertoire for RAR and TR homodimers was found to be highly degenerate, allowing binding of these receptors to DRs with spacings of $>1$ and $>2$ bp [DR($n>1$) and DR($n>2$)], respectively. Heterodimerization altered the response element repertoires of RAR and TR, as RXR/RAR interacted preferentially with DR2 and DR5, while RXR/TR greatly preferred DR4 (Mader *et al.*, 1993b). Interestingly, the pattern of cooperative bindings of the DBDs closely followed the binding site repertoires of the corresponding full-length receptors, i.e. the RXR DBD on its own bound homo-cooperatively to DR1, while it bound heterocooperatively to DR2 and DR5 in the presence of the RAR DBD, and to DR4 in the presence of the TR DBD. No homocooperative binding was observed for the RAR and TR DBDs, and steric hindrance apparently precluded their binding to elements spaced by fewer than two (RAR) or three (TR) base pairs. Evidence was also provided that the observed DNA binding cooperativities were mediated by protein–protein interactions, rather than by alteration of DNA structure. Taken all together, the Mader *et al.* (1993b) study led to the conclusions that (i) the DBDs largely determine the DR response element repertoire of RAR, RXR and TR homo- and heterodimers due to the presence of homo- (RXR) and heterodimerization (RXR/RAR, RXR/TR) surfaces in their respective DBDs, and (ii) the major dimerization function which is present in the LBD (region E) (Leid *et al.*, 1992b; Au-Fliegner *et al.*, 1993; Nagpal *et al.*, 1993; and references therein) has little effect on the selectivity of DR recognition.

To investigate further the molecular mechanisms which underlie the recognition of their cognate response elements

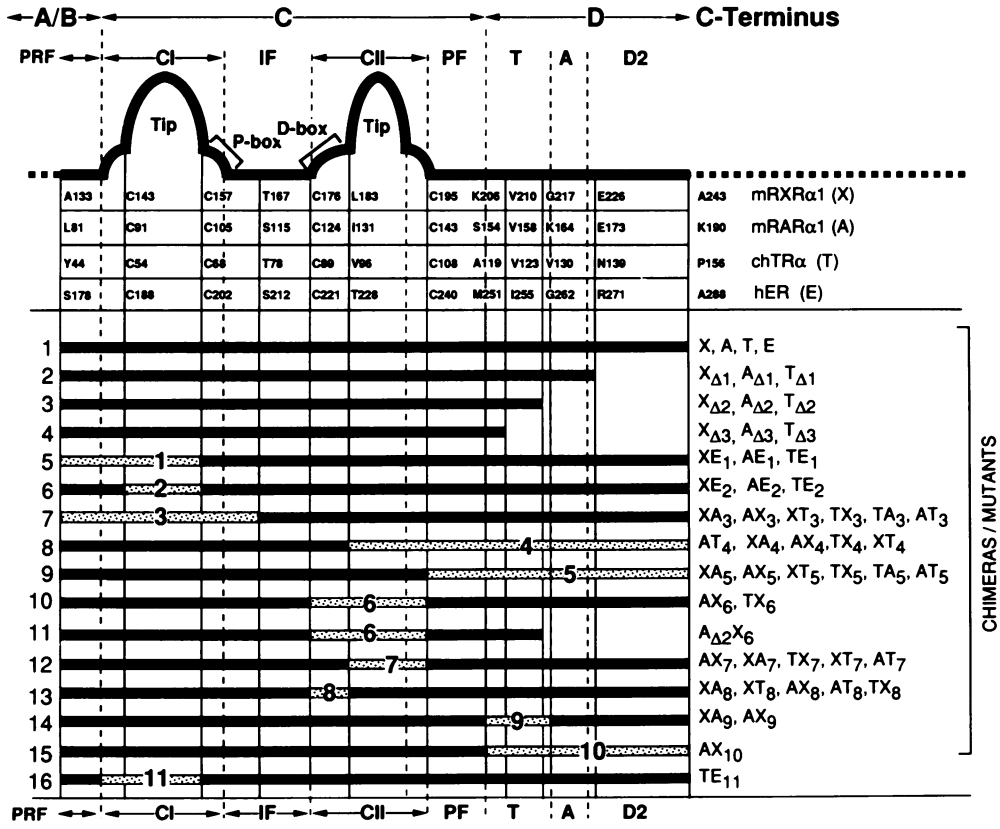



**Fig. 1.** Illustration of the mutant/chimeric DBDs used in this study. The C-terminal border of the 'prefinger region' (PRF), N-terminal CI finger, interfinger region (IF), C-terminal CII finger, post-finger region (PF), and the T- and A-boxes (see text) are indicated at the top and the bottom of the figure by dashed vertical lines. Amino acid (one letter code) positions in the wild-type receptors are given on the right side of plain vertical lines which indicate the borders of swapped segments or C-terminal truncations. Note that the amino acids indicated at the PF/T border correspond to the first amino acids of cassettes 9 and 10, and that those indicated at the T/A border correspond to the last amino acids of cassettes 9, as well as to the first amino acids which were deleted in the truncated DBDs $X_{\Delta 2}$, $A_{\Delta 2}$ or $T_{\Delta 2}$. The C-terminal truncations Δ1, Δ2 and Δ3 remove D2, the A-box and two-thirds of the T-box, respectively. The swapped segments are indicated in the chimeras by dotted boxes, the recipient DBDs by black boxes. The nomenclature of the various chimeras is as described in the 'Experimental design' section of the Results; the recipient DBD is represented by X for RXR, A for RAR, T for TR, or E for ER followed by a letter with a subscript indicating the origin and identity, respectively, of the swapped segment. For a definition of the A/B, C and D regions of nuclear receptors, and of the P- and D-box, see Introduction, Leid *et al.* (1992a) and Gronemeyer (1991).

by RAR, RXR and TR, we have now mapped the dimerization surfaces which, within the RXR, RAR and TR DBDs, are responsible for homo- and heterocooperative interactions. Two types of dimerization interfaces have been characterized, which always involve the C-terminal CII finger region (or parts of it) of a RXR monomer as one of the two dimerization surfaces. In one type of dimerization interface the second surface is provided by the N-terminal CI finger region of either RAR (RXR/RAR heterodimerization on DR5) or TR (RXR/TR heterodimerization on DR4). Furthermore, our results indicate that distinct parts of the CI finger region of RAR and TR interact with the RXR CII finger to accommodate the rotationally different location of TR and RAR monomers in DR4 and DR5 complexes. In the accompanying paper (Zechel *et al.*, 1994), we characterize a different type of dimerization interface in which the second surface is provided by the T-box region of either a second RXR (homodimers on DR1 elements) or RAR monomer (RXR/RAR heterodimers on DR2 elements).

## Results

### *Experimental design*

Figure 1 schematically represents the structure of the mutant and chimeric DBDs used to identify the dimerization surfaces which are present in the DBDs of RAR, RXR and TR and are specifically required for the formation of homodimeric RXR and heterodimeric RXR/TR and RXR/RAR complexes with the DNA elements that constitute the DNA binding site repertoire of the corresponding wild-type receptors. We use hereafter the term 'dimeric complexes' to indicate cooperative binding of the various DBDs to their cognate response elements. The term 'type 2' complex was used for non-cooperative co-binding of two DBDs or when the cooperative nature of DNA binding was ambiguous. Note that in the figures monomeric complexes are indicated by '1', and all complexes resulting from the binding of two DBD monomers by '2', irrespective of whether binding occurred cooperatively or not.

To map the DBD surfaces responsible for heterocooperative interaction between the RXR DBD (X in Figure 1) and either the RAR DBD (A in Figure 1) on DR5 or the TR DBD (T in Figure 1) on DR4, we used the wild-type RXR DBD [amino acids Ala133 to Glu242 of mRXR$\alpha$1; Leid *et al.*, 1992b], C-terminally truncated DBDs or chimeric DBDs in which specific regions of RXR were replaced by the corresponding sequences of TR, RAR or estrogen receptor (ER, E in Figure 1). The corresponding surfaces present in the DBD of RAR (Leu81 to Glu189 of mRAR$\alpha$1; Zelent *et al.*, 1989) or TR (Tyr44 to Arg155 of chicken TR$\alpha$; Sap *et al.*, 1986) were similarly mapped. To support the conclusion that a certain region of the DBDs of RXR, RAR or TR was specifically required for cooperative binding with the RXR DBD on a given DR element, we introduced the putative dimerization interface into a heterologous background; for example the RAR and the RXR heterodimerization surfaces for binding to DR5 were introduced into the TR DBD, thus yielding two chimeric TR DBDs which gained the ability to dimerize with each other on DR5. For the same purpose, chimeric DBDs that contain both dimerization surfaces of two partners, e.g. those of both the TR and RXR DBDs for binding to DR4, were also constructed, and their ability to homodimerize was assessed.

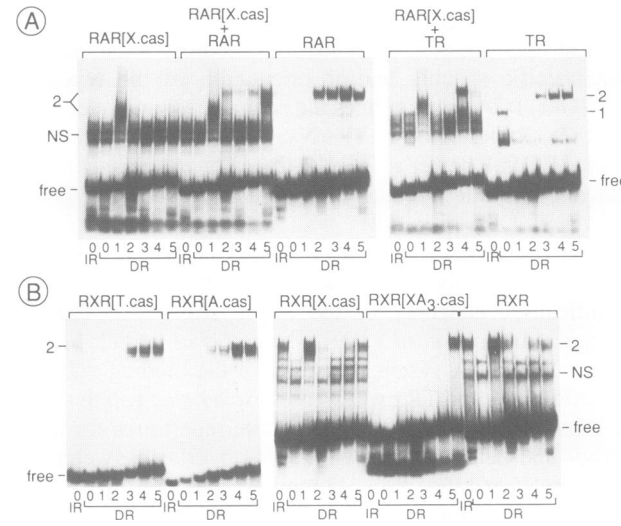



**Fig. 2.** The binding site repertoire of RXR, RAR and TR is dictated by their DBDs. Electrophoretic mobility shift assays (see Materials and methods) were carried out using bacterially expressed full-length receptors, as indicated at the top of each panel. Purified preparations of wild-type RXR and RAR, and crude extracts of the chimeric receptors and TR were used. Complexes formed by one receptor molecule and IR or DR elements are indicated by '1', those originating from homo- or heterodimer binding by '2'. The number of spacer nucleotides in the various DRs is given below each lane. For each receptor the amount of expressed wild-type or chimeric protein was determined by SDS–PAGE followed by silver staining and Western blotting, and the binding efficiencies of the various mutants were compared by EMSA using serial dilutions. Note that homodimeric RAR[X.cas] DR1 complexes are unstable and thus did not generate a discrete signal. Note also that complexes formed between DR1 and RXR[$XA_3$.cas] are less stable than the corresponding DR5 complexes and are not visible on this particular autoradiograph. NS, nonspecific complexes.

The relevance of the dimerization interfaces formed between the DBDs for the binding site selectivity of the full-length receptors was investigated by replacing the wild-type DBD by some of the chimeric DBDs in the corresponding receptors.

A given chimeric DBD was designated according to the nature of the original wild-type receptor (X, A or T) which is used as a prefix, and to the nature of the substituting region of another receptor which is used as a suffix; e.g. $TX_6$ corresponds to a chimeric TR DBD in which region 6 (Figure 1) of the RXR DBD has replaced the corresponding region of the TR DBD. The terminology used for chimeric full-length receptors is based on the same principle and indicates the identity of the swapped DBD cassette in brackets: e.g. RXR[$XA_3$.cas] (Figure 2B) corresponds to a chimeric RXR in which the wild-type RXR DBD (X in Figure 1) has been replaced by the chimeric DBD $XA_3$.

Whenever possible, a truncated DBD (designated by $\Delta$ in Figure 1) was used as one of the two dimerizing partners in electrophoretic mobility shift assays (EMSAs), since heterodimeric complexes containing a truncated DBD usually migrate with mobilities distinct from those resulting from the non-cooperative co-binding of two identical DBD monomers, thus facilitating the characterization of heterocooperative DNA binding for a given pair of DBDs. Note that some of the mutant or chimeric constructs bound only poorly or not at all to DNA as monomers; these mutants will not be discussed with the exception of some special cases where dimeric cooperative DNA binding still occurred.

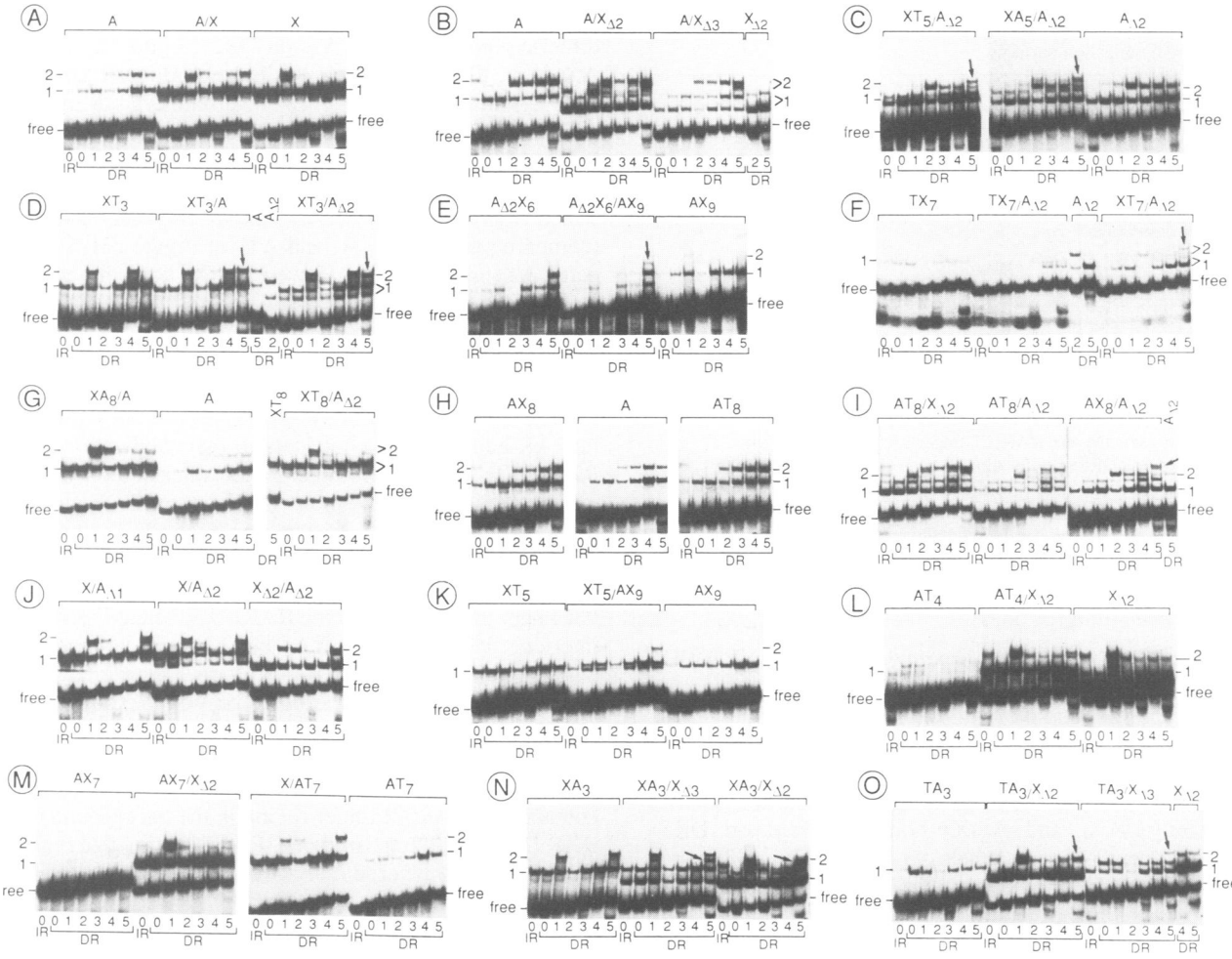


**Fig. 3.** The D-box of the RXR and the CI finger of the RAR DBDs are specifically required for the formation of a heterodimerization interface on DR5 elements. EMSAs were carried out with the truncated and chimeric RXR and RAR DBDs indicated at the top of each panel (compare Figure 1). Assay conditions were as described in Materials and methods; the amounts of DBDs chosen for each assay were based on the protein concentrations determined by SDS–PAGE and were defined as those dilutions of the DBD preparations which generated roughly equal amounts of monomeric complexes. The mutants $A_{\Delta 2}X_6$ (panel E; the corresponding non-truncated mutant $AX_6$ is shown in Figure 4G) and $XA_3$ (panel N) efficiently homodimerized on DR5. Note the double bands formed by $AX_8$ and the DR5 element (panel H). For the significance of the arrows, see text. The variability in the formation of type '1' complexes in (panel E), especially with DR2, is due to slight differences in the labeling efficiency of the IR0 and the DR0 to DR5 probes in this particular experiment.

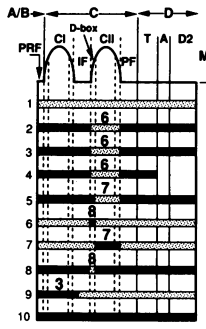
### *C-terminal sequences of RAR, RXR and TR DBDs are differentially involved in the binding of DBD monomers to the PuGGTCA motif*

The study of RAR, RXR and TR DBDs in which the C-termini were deleted to various extents (Δ1, Δ2 and Δ3 mutants in Figure 1) revealed that these regions contribute differently to the DNA binding efficiencies of the corresponding DBD monomers. For example, deletion of the D2 region strongly reduced the binding of the TR DBD (but not of the RAR or RXR DBDs) as monomers to the PuGGTCA motif, indicating that residues of the TR D2 region are involved in DNA binding (data not shown). Further deletion of residues which correspond to the so-called A-box of the orphan receptor NGFI-B/NUR77 (Wilson *et al.*, 1992) abolished the binding of the TR DBD, but did not reduce the binding of RXR or RAR DBD monomers to the PuGGTCA motif (panel $X_{\Delta 2}$ in Figure 4M, panel $A_{\Delta 2}$ in Figure 3C, $T_{\Delta 1}$ not shown). Additional truncation of a region which has been referred to as the T-box of RXRβ (H-2RIIBP; see Wilson *et al.*, 1992), resulted in a 5-fold reduced binding of the RXR DBD monomer to the PuGGTCA motif (panel $X_{\Delta 3}$ in Figure 4B), while binding of the corresponding RAR DBD ($A_{\Delta 3}$) was abolished (data not shown). Thus, the T-box region appears to contribute differently to the binding efficiencies of RXR and RAR DBDs. We conclude from these results that the C-terminus of the DBD which is necessary for efficient monomer binding to the PuGGTCA motif encompasses the T-box of RXR and RAR, but no further C-terminal sequences. In contrast, the TR DBD requires additional sequences, including the A-box and residues located in the D2 region.

### *The D-box of RXR and the N-terminal RAR CI finger are specifically required for the binding of RXR/RAR DBD heterodimers to DR5 elements*

In order to identify the sequence in the RXR DBD which forms a dimerization interface with the RAR DBD on DR5, we created various RAR/RXR and TR/RXR chimeric DBDs (Figure 1). The key mutants which allowed us to define the DR5-specific dimerization surface of the RXR DBD are depicted in Table I. That $AX_6$ and $A_{\Delta 2}X_6$ heterodimerized on DR5 with either the RAR DBD, $A_{\Delta 2}$ or $AX_9$ indicates that the RXR CII finger contains this surface (Table I, lines 2 and 4; Figure 3E, panel $A_{\Delta 2}X_6/AX_9$, and data not shown;

**Table I.** Summary of the results defining the DR5-specific RXR/RAR DBD heterodimerization surfaces




| DBDs/ Mutants | Fingers present in the mutants: CII RXR | Fingers present in the mutants: CI RAR | Homo-dimerization on DR5 | Heterodimerization on DR5 with: RAR | Heterodimerization on DR5 with: RXR | Fig. |
|---|---|---|---|---|---|---|
| X | + | - | - | + | - | 3A |
| $AX_6$ | + | + | + | + | + | 4G |
| $TX_6$ | + | - | - | + | - | 4F |
| $A_{\Delta2}X_6$ | + | + | + | + | + | 3E |
| $AX_7$ | minus D-box | + | - | - | + | 3M |
| $XA_8$ | minus D-box | - | - | - | - | 3G |
| $XT_7$ | D-box only | - | - | + | - | 3F |
| $AX_8$ | D-box only | + | (+/-) | + | + | 3H;3I |
| $XA_3$ | + | + | + | + | + | 3N |
| A | - | + | - | - | + | 3A;3B |

The RXR D-box and the RAR CI finger are required for the formation of the DR5-specific dimerization interface. For symbols, as well as for the nomenclature of the various mutant and chimeric DBDs see legend to Figure 1. Dotted boxes represent RXR, black boxes RAR or TR sequences. The zinc-complexing cysteine pairs in CI and CII fingers are indicated by dashed lines. (+/−) indicates that homocooperative binding of $AX_8$ on DR5 is less efficient than that of $AX_6$, $A_{\Delta2}X_6$ or $XA_3$.

note that $AX_9$ which heterocooperated with the RXR DBD for DR5 binding as efficiently as the RAR DBD, barely forms 'type 2' complexes when assayed alone, thus facilitating the detection of heterodimeric complexes). As expected, $TX_6$ also formed dimeric complexes with either the RAR DBD, $A_{\Delta2}$ or $AX_9$ on DR5 (Table I, line 3). Importantly, $AX_6$ and $A_{\Delta2}X_6$ homodimerized on DR5 (Table I, lines 2 and 4; panels $A_{\Delta2}X_6$, in Figure 3E and $AX_6$ in Figure 4G), indicating that each of these chimeric DBDs contains in the same molecule all the RXR and RAR sequences specifically required for the formation of a heterodimerization interface on DR5.

We next investigated whether the D-box of the RXR CII finger could be specifically involved in the dimerization of the RXR and RAR DBDs on DR5, and replaced it with the RAR and TR D-box in $AX_6$ and $TX_6$, respectively, thus generating $AX_7$ and $TX_7$ (Figure 1, line 12; Table I, line 5). Unlike $AX_6$ and $TX_6$, neither $AX_7$ nor $TX_7$ homodimerized or heterodimerized with RAR DBDs [panel $AX_7$ in Figure 3M; panel $TX_7/A_{\Delta2}$ in Figure 3F, and data not shown; this is not due to a loss of their ability to bind DNA, since $AX_7$ formed dimeric complexes with $X_{\Delta2}$ on DR5 (Figure 3M; Table I, line 5)]. That the RXR D-box is indeed required was confirmed by the inability of $XA_8$ or $XT_8$ (Figure 1, line 13; Table I, line 6) to form heterodimeric complexes with either the RAR DBD or $A_{\Delta2}$ on DR5 (panels $XA_8$/A and $XT_8/A_{\Delta2}$ in Figure 3G; note that the migrations and signal intensities of the complexes observed with DR5 correspond to the superimposition of the patterns exhibited by the individual DBDs). To determine whether sequences of the RXR CII finger were required in addition to the D-box, we constructed $XA_7$ and $XT_7$ (Figure 1, line 12; Table I, line 7). Notably, the replacement of the tip of the RXR CII finger with that of either RAR or TR still allowed the corresponding $XA_7$ and $XT_7$ chimeras to dimerize with $A_{\Delta2}$ on DR5 (Table I, line 7; arrow in panel $XT_7/A_{\Delta2}$ in Figure 3F, and data not shown). Thus, the RXR D-box appears to provide the RXR-specific surface indispensable for heterodimerization with RAR on DR5. This conclusion was further supported by the results obtained with $AX_8$, which unlike $AT_8$ or the RAR DBD (Figure 1, lines 13 and 1; Table I, lines 8 and 10) formed dimeric complexes with $A_{\Delta2}$ on DR5 (Figure 3I; in panel $AX_8/A_{\Delta2}$, the arrow points to the heterodimeric complex; note the absence of $AT_8/A_{\Delta2}$ heterodimers). Moreover, $AX_8$ (Table I, line 8) appears to contain both RXR and RAR dimerization surfaces, since it bound with higher relative efficiency to DR5 than to DR2, DR3 and DR4, which was not observed for the RAR DBD and $AT_8$ (compare panels $AX_8$, A, and $AT_8$ in Figure 3H; see also panel $AX_8$ in Figure 4J). However, $AX_8$ homodimerization and $AX_8/A_{\Delta2}$ heterodimerization on DR5 were clearly less efficient than homo- and heterocooperative interactions of $AX_6$ and $A_{\Delta2}X_6$ which, in addition to the D-box, contain also the tip of the RXR CII finger. Thus additional residues located in the tip of the RXR CII finger and non-conserved in RAR and TR may contribute to the stabilization of the RAR/RXR DBD heterodimerization interface on DR5.

That $AX_6$ and $A_{\Delta2}X_6$ homodimerize on DR5 elements (see above) indicated already that both RXR and RAR dimerization surfaces are present in these chimeras (Table I, lines 2 and 4), thus excluding a contribution of the RAR CII finger to the DR5-specific RAR/RXR heterodimerization interface. However, both $XA_3$ and $TA_3$, which contain the CI finger of RAR, but not $AX_3$ (Figure 1, line 7; Table III, lines 4 and 1) formed heterodimeric complexes with $X_{\Delta2}$ and $X_{\Delta3}$ on DR5 (arrows in Figure 3N and O, and data not shown; the heterodimeric complexes migrate more quickly than the homodimeric ones). Moreover, $XA_3$, which contains the RAR CI finger region in the background of an RXR DBD homodimerized on DR5, indicating that both the RAR and RXR heterodimerization surfaces are present in this chimera (Table I, line 9; panel $XA_3$ in Figure 3N). Thus, the CI finger of RAR appears to be the only RAR DBD region which is specifically required for the formation of a dimerization interface with the RXR CII finger/D-box region on DR5. Results obtained with a number of additional mutants which are not mentioned here supported the above conclusions. These data are contained in an extended version of the manuscript which is available upon request.

### *The D-box of RXR and the TR CI finger region are specifically required for the formation of the heterodimerization interface which determines selective binding of RXR/TR DBDs to DR4 elements*

The results obtained with the key mutants which allowed a definition of the dimerization interface formed between the DBDs of TR and RXR are summarized in Table II (additional data which support our conclusions are contained in the figures and described in the extended version of this manuscript). Mutant $XT_3$ was highly informative, since it homodimerized on DR4 (Table II, line 2), which suggested that it contains the DR4-specific heterodimerization surfaces of both RXR and TR DBDs (i.e. the CI finger region of TR; $XT_3$ in Figure 4I), while the reciprocal construct ($TX_3$) did not (Figure 4F). $TX_6$ also bound homocooperatively to DR4, and formed heterodimeric complexes with the RXR or TR DBDs (Table II, line 3; Figure 4F, panel $TX_6$, and data not shown). That the RXR CII finger contains the dimerization surface involved in the cooperative interaction with the TR DBD was further supported by the results obtained with $AX_6$, which cooperated with the TR DBD for binding to DR4 (Table II, line 4; Figure 4G).

The RXR D-box was necessary for heterocooperative

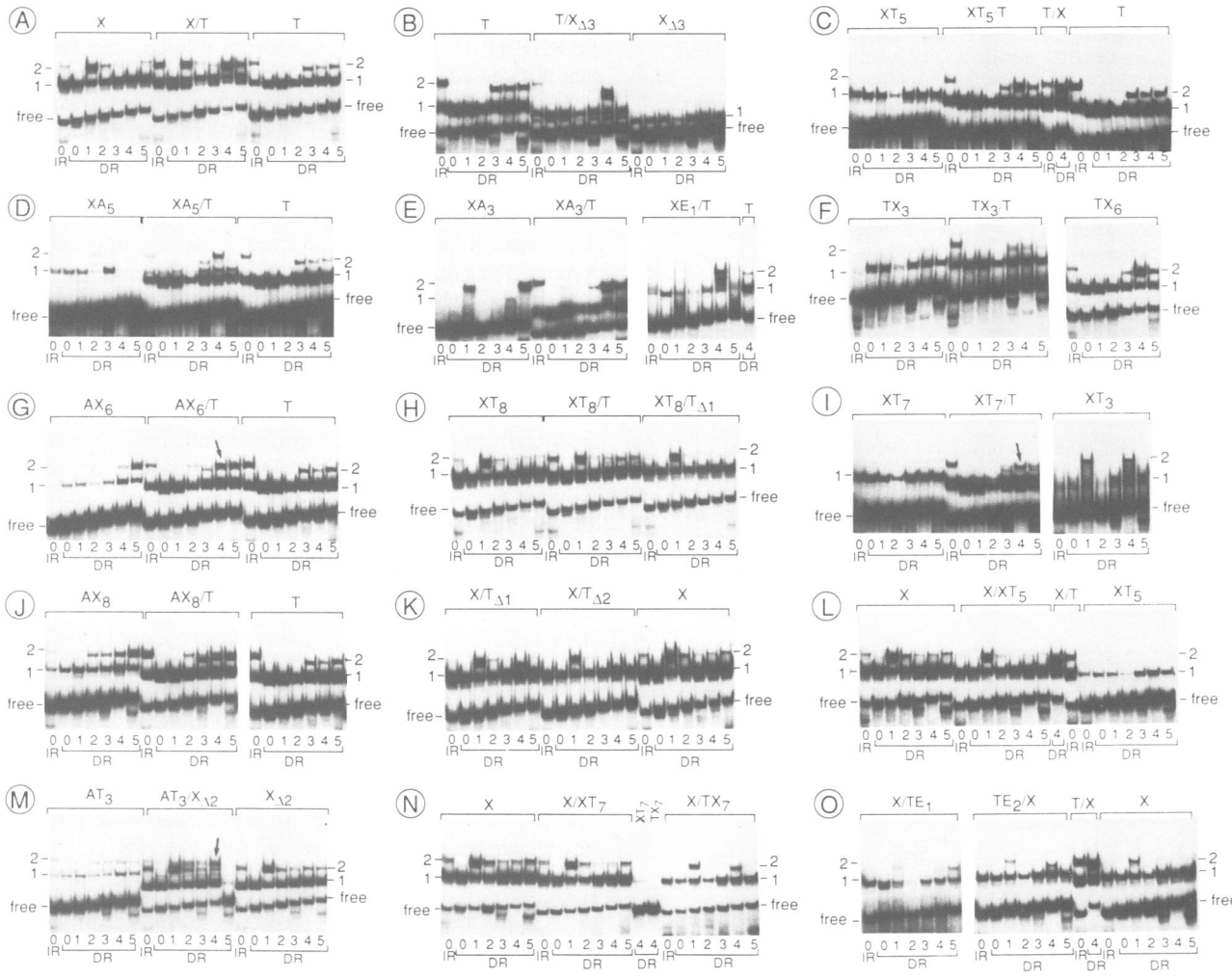


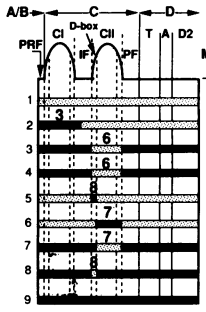
**Fig. 4.** The RXR D-box and the TR CI finger region are specifically required for DBD heterodimerization on DR4 elements. Electrophoretic mobility shift assays defining the RXR (panels **A**–**J**) and TR DBD (panels **K**–**O**) heterodimerization surfaces were performed using crude lysates of bacterially expressed mutant/chimeric DBDs as indicated at the top of each panel (for nomenclature and structure of the respective DBDs, see Figure 1). Note that non-cooperatively formed TR DBD type '2' complexes migrate more quickly than the respective RXR DBD complexes (see for example in panel A), thus facilitating the detection of dimeric complexes with a mobilty intermediate of those formed by either DBD alone. In panel B, to visualize in one gel the cooperative DR4 binding of the $T/X_{\Delta 3}$ DBDs and the non-cooperative binding of the TR DBD, respectively, 5-fold more TR DBD was used in panel T than in panel $T/X_{\Delta 3}$. In all other cases, the amounts of receptor DBDs used in each assay were calibrated as described in the legend to Figure 3. The variability in the formation of $XA_5$ and $XA_3$ type '1' complexes (panels D and E), as well as the weak homodimerization of $XA_3$ on DR1 in the panel $XA_3/T$ (panel E) are due to the low efficiency of DNA binding and the instability of the respective complexes. Note that the mutants $TX_6$ (panel F) and $XT_3$ (panel I) bound homocooperatively to DR4 elements. Arrows point to the heterodimeric complexes. In panel M ($AT_3/X_{\Delta 2}$), lane DR5 should not be considered, due to a pipetting error.

binding of RXR and TR DBDs. No cooperative binding was seen on DR4 with either T or $T_{\Delta 1}$, when the RXR D-box was replaced with that of TR ($XT_8$, Table II, line 5; see panels $XT_8/T$ and $XT_8/T_{\Delta 1}$ in Figure 4H; compare with panels X/T and $X/T_{\Delta 1}$ in Figure 4A and K), while all chimeras containing the RXR D-box, such as $XT_7$ and $XA_7$ (Figure 1, line 12; Table II, line 6) bound heterocooperatively with the TR DBD to DR4 (arrow in Figure 4I, and data not shown). Moreover, introduction of the TR D-box in $TX_6$ resulted in a chimera ($TX_7$; Table II, line 7) that unlike $TX_6$ could not homodimerize on DR4 (compare panels $TX_6$ in Figure 4F and $TX_7$ in Figure 3F). All of these observations excluded a major role of the tip of the RXR CII finger in the formation of the specific RXR/TR DBD dimerization interface, and indeed $AX_8$ (Table II, line 8) formed dimeric complexes with the TR DBD on DR4, with a signal intensity stronger than that of $AX_8$ and TR DBD on their own (Figure 4J). Thus, as it is the case for RXR/RAR cooperative binding to DR5, the RXR D-box is the only RXR DBD region which is absolutely required for the formation of a DR4-specific dimerization interface with the CI finger region of TR.

### *Distinct parts of the RAR and TR CI finger regions are involved in the formation of the dimerization surfaces which interact with the RXR D-box*

The above results indicate that the N-terminal regions of both RAR and TR DBDs provide the surfaces which are specifically required for the formation of the dimerization interfaces which specify the distinct response element preferences of RXR/RAR (DR5) and RXR/TR (DR4) DBD heterodimers. To define further which 'prefinger' and CI finger sequences provide these surfaces, we replaced these TR and RAR sequences by the corresponding sequences of ER. Unexpectedly, $AE_1$, $AE_2$ and $TE_1$, all heterocooperated with either the RXR DBD or $X_{\Delta 2}$ for binding

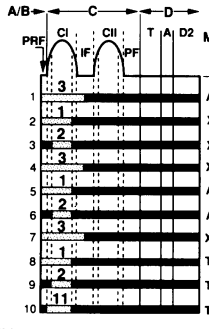
**Table II.** Summary of the results defining the sequences which are required to form the DR4-specific heterodimerization surfaces in RXR and TR DBDs




| | DBDs/ Mutants | Fingers present in the mutants: CII RXR | Fingers present in the mutants: CI TR | Homo-dimerization on DR4 | Heterodimerization on DR4 with: TR | Heterodimerization on DR4 with: RXR | Fig. |
|---|---|---|---|---|---|---|---|
| 1 | X | + | - | - | + | - | 4A |
| 2 | $XT_3$ | + | + | + | + | + | 4I |
| 3 | $TX_6$ | + | + | + | + | + | 4F |
| 4 | $AX_6$ | + | - | - | + | - | 4G |
| 5 | $XT_8$ | minus D-box | - | - | - | - | 4H |
| 6 | $XT_7$ | D-box only | - | - | + | - | 4I;4N |
| 7 | $TX_7$ | minus D-box | + | - | - | + | 3F;4N |
| 8 | $AX_8$ | D-box only | - | - | (+/-) | - | 4J |
| 9 | T | - | + | - | - | + | 4A |

Chimeras $XT_8$, $XT_7$ and $AX_8$ reveal that the RXR D-box is the only RXR CII region specifically required for the formation of the DR4-specific dimerization interface (for details see text). Symbols and nomenclature are as in Figure 1. Dotted boxes represent RXR, black boxes TR or RAR sequences. (+/−) indicates that heterocooperative DR4 bindings of the pairs $AX_8$/T and $AX_8/T_{\Delta 1}$ on DR4 are less efficient than, for example, those of the pairs X/T and $AX_6$/T.

**Table III.** Summary of results indicating that the tip of the RAR CI and the N-terminal portion of the TR DBD ['prefinger region' (PRF)] are specifically required to determine heterocooperative binding with RXR DBDs on DR5 and DR4, respectively




| | Mutants | Homo-dimerization on: DR4 | Homo-dimerization on: DR5 | Heterodimerization with X, $X_{\Delta 2}$ or $X_{\Delta 3}$ on: DR4 | Heterodimerization with X, $X_{\Delta 2}$ or $X_{\Delta 3}$ on: DR5 | Fig. |
|---|---|---|---|---|---|---|
| 1 | $AX_3,TX_3$ | - | - | - | - | 4F |
| 2 | $XE_1$ | - | + | - | unstable | n.s. |
| 3 | $XE_2$ | - | + | - | unstable | n.s. |
| 4 | $XA_3,TA_3$ | - | $XA_3^+$ $TA_3^-$ | - | + | 3N;3O |
| 5 | $AE_1$ | - | - | - | + | n.s. |
| 6 | $AE_2$ | - | - | - | + | n.s. |
| 7 | $XT_3,AT_3$ | $XT_3^+$ $AT_3^-$ | - | + | $XT_3^-$ $AT_3$ weak | 4I;4M |
| 8 | $TE_1$ | - | - | weak | + | 4O |
| 9 | $TE_2$ | - | - | + | - | 4O |
| 10 | $TE_{11}$ | - | - | + | - | n.s. |

Dotted boxes represent the CI regions swapped into a recipient DBD (either A, T or X) indicated by black boxes. Nomenclature and symbols are as in Figure 1. The chimera $TE_1$/X formed some dimeric complexes on DR4, albeit much less efficiently than on DR5, and reciprocally, the chimera $AT_3$ formed some weak complexes on DR5. n.s., data not shown.

to DR5 (Table III, lines 5, 6 and 8; panel $TE_1$/X in Figure 4O). These observations suggest that residues which are conserved in the tip of the CI finger of RAR and ER could be similarly involved in the heterodimerization interface that specifies cooperative binding with the RXR DBD on DR5.

$TE_1$ heterocooperated much less efficiently with the RXR DBD or $X_{\Delta 2}$ for binding to DR4 than $XT_3$ and $AT_3$ (Table III, lines 8 and 7; Figure 4O, and data not shown; DR5 binding by $TE_1$/X was much stronger than DR4 binding, as verified by dose−response curves). However, when the N-terminus of the TR DBD [amino acids Tyr44 to Cys54, which correspond to the 'prefinger region' and the first four amino acids of the CI finger; see Figure 1] was introduced into $TE_1$, the resulting chimera $TE_2$ heterodimerized efficiently and selectively with the RXR DBD or $X_{\Delta 2}$ on DR4 (Table III, line 9; Figure 4O). Moreover, $TE_{11}$ (Figure 1, line 16) which contains only the 'prefinger' of the TR linked to the ER CI finger heterocooperated with the RXR DBD for binding to DR4 (Table III, line 10, and data not shown; note that the 'prefinger region' sequences of TR and ER are completely divergent). In contrast, the replacement of the 'prefinger' and first four amino acids of the ER CI finger in $AE_1$ and $XE_1$ by those of RAR and RXR, respectively (generating $AE_2$ and $XE_2$), did not modify their binding properties (Table III, compare lines 8 and 9 with lines 5 and 6 and lines 2 and 3). Importantly, none of the chimeras $XE_1$, $XE_2$, $AE_1$, $AE_2$, $AX_3$, $TX_3$, $XA_3$ or $TA_3$ which contain a CI finger region other than that of TR did heterocooperate with either the RXR DBD or $X_{\Delta 2}$ on DR4 (Table III, and data not shown).

We conclude from these results that amino acids which are conserved in the tips of the RAR and ER CI fingers are specifically required for the formation of a surface which can interact with the RXR D-box surface to specify cooperative binding on DR5. In contrast, the TR 'prefinger region', but not the TR CI finger, is specifically required for the formation of a dimerization interface with the RXR D-box, which determines cooperative binding to DR4. Interestingly, $TE_2$ and $TE_{11}$, despite the presence of the tip of the ER CI finger, did not bind cooperatively with the RXR DBD on DR5 (Figure 4O, and data not shown), suggesting that the very N-terminal part of the TR DBD prevents the formation of a dimerization interface on DR5 (compare $TE_1$ with $TE_2$ and $TE_{11}$ in Table III).

### *The binding site repertoires of RXR, RAR and TR homo- and heterodimers are dictated by the specificity of the dimeric interactions between their DBDs*

The binding site repertoires of homo- and heterodimers of full-length RXR, RAR and TR appear to be largely identical to those of their isolated DBDs (Mader *et al.*, 1993b). To confirm that the DBDs dictate the DR binding site repertoire of the full-length receptors, DBD swappings were perfomed. Despite a poor solubility and reduced stability of its DNA complexes, RAR[X.cas] (RXR DBD swapped into RAR) formed homodimeric complexes selectively with DR1 (Figure 2A). No DR1 binding was seen with either the control RAR[A.cas], RAR[T.cas] or wild-type RAR (Figure 2A, and data not shown). Conversely, introducing the RAR or TR DBDs into RXR resulted in chimeras (RXR[A.cas] and RXR[T.cas]), which had lost RXR's ability to homodimerize on DR1, even though they bound efficiently to the PuGGTCA motif (Figure 2B). Interestingly, RXR[A.cas] and RXR[T.cas] bound selectively as homodimers to the same DRs as the corresponding RAR and TR DBDs, since they formed complexes with DR2 to DR5 and DR3 to DR5, respectively (Figure 2B; compare with panel A in Figure 3A and panels T in Figure 4A and B).

Heterocooperative interactions between the DBDs of RAR and RXR also appeared largely to determine the binding site repertoire of the corresponding full-length heterodimers, since RAR[X.cas] preferentially heterodimerized with RAR on DR5, and less efficiently on DR2 elements (Figure 2A, compare the binding pattern of RAR[X.cas]/RAR heterodimers with that of RAR). Similarly, RAR[T.cas] heterodimerized with RXR on DR4 (data not shown), and RAR chimeras containing the RXR DBD (RAR[X.cas]) efficiently formed heterodimeric complexes of intermediate mobility with DR4 when mixed with TR (Figure 2A). Importantly,

the RAR and RXR chimeras (RXR[$XA_3$.cas] and RAR[$XA_3$.cas]), which harbor a chimeric DBD containing both DR5-specific dimerization surfaces in a heterologous receptor environment, preferentially homodimerized on DR5 (Figure 2B and data not shown) as did the corresponding isolated DBD (see above, $XA_3$, Table I, line 9). Moreover, the chimeric RXR[$XA_3$.cas] and RAR[$XA_3$.cas] full-length receptors which contain both RXR homodimerization surfaces (see Zechel *et al.*, 1994), also homodimerized on DR1 (data not shown).

All of these results confirm the previous conclusion (Mader *et al.*, 1993b) that homo- and heterocooperative interactions between the RXR, RAR and TR DBDs essentially dictate the DR binding site repertoire of the corresponding full-length receptors, while DBD-mediated steric hindrance excludes the binding of RAR and TR homodimers to DR($n<2$) and DR($n<3$), respectively.

## Discussion

### *The D region contributes differentially to the DBDs of RAR, RXR and TR monomers and excludes binding to closely spaced DR elements*

We have shown here that, in addition to the two zinc fingers, the DBDs of RAR, RXR and TR encompass sequences located in the D regions of these receptors. Our results, which support earlier data of Lee *et al.* (1993) concerning the RXR DBD, demonstrate that the T-box regions, but no further C-terminal sequences, are required for efficient DNA binding of both RXR and RAR DBD monomers. Interestingly, the solution structures of GR, ER, RAR and RXR DBDs (Härd *et al.*, 1990; Schwabe *et al.*, 1990; Katahira *et al.*, 1992; Lee *et al.*, 1993) and the crystal structure of the GR and ER DBDs (Luisi *et al.*, 1991; Schwabe *et al.*, 1993a,b), predict that their D regions all project towards the 5′ end of the PuGGTCA motif, thus suggesting that the T-boxes of RXR and RAR could establish DNA contacts across the minor groove with the phosphate backbone located 5′ to the PuGGTCA motif. In support of this view, a mutation of three basic residues of the RXR T-box decreased the efficiency of DNA binding of the RXR DBD to DR1, but not its homocooperative binding (Lee *et al.*, 1993). Furthermore, methylation interference experiments performed with RXR/RAR heterodimers and the DR5 RARE of the RARβ2 gene suggested receptor interactions with the spacer residues located upstream of the 3′-located PuGTTCA motif (Kurokawa *et al.*, 1993). Since in RXR/RAR heterodimeric complexes, RAR appears to be bound to this 3′ motif (see Zechel *et al.*, 1994), the RAR T-box may indeed establish contacts with residues in the DR spacer.

Additional sequences in the D region are necessary for efficient binding of the TR DBD monomer to the PuGGTCA motif. Our deletion analyses of the TR DBD indicate that the region corresponding to the so-called A-box of NGFI-B (Wilson *et al.*, 1992) is absolutely required for DNA binding of the TR DBD monomer. In addition, deletion of the D2 region already resulted in a strong decrease in the efficiency of TR DBD binding. The TR A-box may play a function similar to that of the NGFI-B A-box, which was originally identified as the sequence specifically required for the recognition of the two most 5′-located adenylic residues of the motif 5′-AAAGGTCA-3′ (Wilson *et al.*, 1992). Such a possibility is in agreement with structural models of nuclear receptor DBDs (see above) which predict that the A-box projects towards sequences located 5′ of the PuGGTCA motifs, and is further supported by experiments showing that non-conservative amino acid exchanges in the TR A-box reduced or even abolished binding of full-length TR homodimers or RXR/TR heterodimers to DRs and IRs (Kurokawa *et al.*, 1993). Furthermore, a direct selection of randomized oligonucleotides by RXR/TR heterodimers revealed a preference for certain nucleotides located in the minor groove upstream of the 3′-located PuGGTCA motif of DR4 elements (Kurokawa *et al.*, 1993). Since TR is bound to the 3′-motif in DR4 RXR/TR heterodimeric complexes (see Zechel *et al.*, 1994), interactions between the spacer residues and the T- and A-boxes, as well as the D2 region, may enhance the specificity of DR4 element recognition by RXR/TR heterodimers. As RAR also occupies the 3′ position in heterodimeric RXR/RAR complexes formed on DR2 and DR5 (see Zechel *et al.*, 1994), the RAR T-box may also preferentially interact with certain nucleotides in the spacers of these DRs. In agreement with this possibility, we have previously reported that mutations in the spacer region could significantly affect DNA binding of RAR to DR2 elements (Mader *et al.*, 1993c).

The conclusion that the requirement of progressively longer sequences C-terminal to the CII fingers of RXR, RAR and TR most probably corresponds to interactions with DNA spacer sequences located upstream of the DR 3′-motif, offers a likely explanation for the distinct abilities of the various homo- and heterodimers to bind to DRs with closely spaced motifs, and supports our previous suggestion that steric hindrance may account for these differences (Mader *et al.*, 1993b). The necessary interaction of the A-box and D2 region of a TR monomer, bound to a DR 3′-motif with the spacer residues, would only allow the formation of TR homo- and heterodimeric complexes on DR($n>2$) elements. That RAR binding to the 3′ motif of a DR element requires interaction of the T-box (but not of the A-box and D2 region) with spacer residues, would only allow the formation of RAR homo- and heterodimeric complexes on DR($n>1$) elements. Similarly, the binding of RXR homodimers to DR1 could be explained by a less stringent requirement of the RXR T-box for DNA binding, since in contrast to the RAR case monomeric RXR DBD complexes can still be formed with mutants lacking most of the T-box. These proposals are supported by the observations that chimeric DBDs harbouring the C-terminal regions of the RAR DBD ($AX_6$, $XA_5$ and $TA_5$) did not bind as dimers to DR($n<2$), while those containing the C-terminal regions of the TR DBD ($TX_6$, $XT_5$ and $AT_5$) did not bind as dimers to DR($n<3$). Thus, together with the formation of specific dimerization interfaces, steric hindrance may play an important role in the specification of response element recognition, restricting the interaction of complexes containing RAR or TR to DR($n>1$) and DR($n>2$) elements, respectively. In this respect, it will be interesting to investigate the orientation and dimerization interface of RXR/RAR heterodimeric complexes bound to DR1 elements (Durand *et al.*, 1992), since, as discussed above, RAR should not be able to readily occupy the 3′-motif, whereas in the reverse orientation steric hindrance should not prevent the formation of DR1 RAR/RXR complexes [for further discussion of this point, see Zechel *et al.* (1994)].

***The CI finger regions of the RAR and TR DBDs interact differently with the RXR D-box to form the dimerization interfaces which specify binding to DR5 and DR4 elements, respectively***

We have mapped here the regions of the DBDs which are specifically required for the formation of the dimerization interfaces which are responsible for the heterocooperative binding of the DBDs of RXR and RAR to DR5, and RXR and TR to DR4. Our results are in agreement with the recent data of Perlmann *et al.* (1993) who showed that a region encompassing the CII finger of RXR forms a dimerization interface with the CI finger region of RAR on DR5, and with the CI finger region of TR on DR4. However, these authors did not provide an explanation for the distinct DR preferences of the RXR/RAR and RXR/TR heterodimers. We demonstrate here that it is in fact the D-box component of the RXR CII finger, which is specifically required for the formation of the heterodimerization interface between the RXR DBD and either the RAR (DR5) or TR (DR4) DBD. This conclusion is fully supported by results obtained with all loss-of-function ($XA_8$ and $XT_8$; Tables I and II), maintenance-of-function ($XT_7$, $XA_7$; Tables I and II, and data not shown) and gain-of-function ($AX_8$; Tables I and II) mutants. On the other hand, the tip of the RXR CII finger does not appear to be specifically required, since RXR DBD chimeras containing the tip of a heterologous CII finger formed heterodimers with RAR and TR ($XT_7$; Tables I and II). However, the tip of RXR CII finger may help in stabilizing the D-box structure, since DBD chimeras containing only the D-box in a heterologous background ($AX_8$, Tables I and II) bound less efficiently to DNA than those containing the entire RXR CII finger.

Introducing the RAR CI finger and the 'prefinger' N-terminal region in place of the corresponding regions of the DBDs of RXR or TR demonstrated that these RAR regions contain all of the residues specifically required to create the RAR heterodimerization surface for binding to DR5 (see Table III, $XA_3$ and $TA_3$). Importantly, we found that the tip of the CI finger of the ER can substitute for that of RAR, since both $AE_1$ and $AE_2$ heterodimerized as efficiently as the RAR DBD with the RXR DBD on DR5 (Table III). Taken together with the results obtained with the chimeras $XE_1$ and $XE_2$ (Table III), these observations indicate that amino acids located in the tip of the CI fingers, which are common to RAR and ER, must be essential for the formation of the dimerization surface which interacts with the RXR D-box on DR5, since the 'prefinger region' completely diverges between RAR, ER and RXR. We note in this respect that the amino acid sequence SGYHYGV is entirely conserved in the tip of the CI fingers of RAR and ER, but has significantly diverged in the TR. Perlmann *et al.* (1993) concluded from their data that the heterodimerization surfaces of both RAR and TR DBDs encompass the same region of the CI fingers ('DR-box'). Our data indicate clearly that the dimerization surfaces of the DBDs of RAR and TR which interact with the RXR D-box on DR5 and DR4 must be different, since the replacement of TR CI finger with the ER CI finger in $TE_1$ abolished nearly completely the formation of heterodimeric complexes on DR4, whereas heterodimeric complexes were formed on DR5 (Table III, and see above). Moreover, $TE_{11}$ which contains the 'prefinger region' of TR DBD, but is otherwise identical to $TE_1$, heterodimerized with the RXR DBD on DR4 (Table III), indicating that this very N-terminal region of the TR DBD contains the residues specifically required for the formation of the TR dimerization surface. Interestingly, $TE_2$ and $TE_{11}$ did not heterodimerize with the RXR DBD on DR5, suggesting that this N-terminal region of the TR DBD may preclude the formation of a heterodimer on DR5 (note that the corresponding chimera, $AE_2$, did form such DR5 complexes). Thus, the 'prefinger region' of the TR DBD, but not the CI finger, appears to determine DR4 binding specifically by two mechanisms, i.e. the formation of a DR4-specific dimerization surface and the prevention of the formation of a DR5-specific dimerization surface. That different heterodimerization surfaces of RAR and TR interact with the RXR D-box on DR5 and DR4 should in fact not be surprising, since the stereolocations of the heterodimeric partners are likely to be different on DR4 and DR5 elements.

***The dimerization interfaces formed between the DBDs of RXR and either RAR or TR determine the DR binding site repertoire of the corresponding full-length heterodimers***

We have previously reported that the DR binding site repertoire of RAR, RXR and TR homo- and heterodimers are very similar to those of the corresponding isolated DBDs, suggesting that the major dimerization domain which is present in the LBDs (region E, see Introduction for references) does not play a critical role in specific DR recognition (Mader *et al.*, 1993b). In keeping with these results, Perlmann *et al.* (1993) reported that the replacement of the RAR LBD with that of TR created a chimera that still transactivated through DR5 elements, albeit in a T3-dependent fashion. Here, we unequivocally demonstrate by DBD swapping experiments (Figure 2) that it is the identity of the DBD which determines the binding site selectivity of full-length receptor homo- and heterodimers. Moreover, we show that this selectivity is dictated by the nature of the dimerization surfaces which are present in the DBDs, irrespective of their environment in chimeric receptors.

Thus, the stronger dimerization function which is present in the E region (LBD) does not appear to be critically involved in the determination of the specific DR binding repertoire of RXR/RAR and RXR/TR heterodimers. Rather, the free energy provided by the formation of region E dimerization interfaces could be used to stabilize the binding of monomeric partners on any DR, irrespective of whether their DBDs bind cooperatively or not on this DR, whereas the additional free energy resulting from the formation of the DBD dimerization interfaces on certain DRs is apparently required for the specificity of the DR binding repertoire (see also the Discussion section in Zechel *et al.*, 1994). In fact, this additional free energy is reflected by the higher stability (Mader *et al.*, 1993b) of full-length RXR/RAR heterodimers bound to DR5 and DR2 on which the DBDs specifically heterodimerize (Zechel *et al.*, 1994).

## Materials and methods

***Plasmids***

Sequences encoding the wild-type and the truncated receptor DBDs (encompassing the amino acids indicated in Figure 1) were amplified by PCR using the pSG5-mRXR$\alpha$1 (Leid *et al.*, 1992b), pSG5-mRAR$\alpha$1 [kind

gift from H.Nakshatri (Nagpal *et al.*, 1993); mRARα1 (Zelent *et al.*, 1989)], pET3a-chTRα [Mader *et al.*, 1993b,c; chTRα (Sap *et al.*, 1986)], and the hER mutant HE0 (Kumar and Chambon, 1988) as templates. PCR products were digested with *Bam*HI, purified by polyacrylamide gel electrophoresis, and inserted into the *Bam*HI site of pET3a (obtained from F.W.Studier). The insertions of the plasmids pRAR(L81-R114)RXR(T167-E238) and pRAR(L81-M153)RXR(K206-E238) (kind gifts of S.Mader; unpublished data) were C-terminally extended by sequences coding for amino acid residues 239–242 of RXRα1 (Leid *et al.*, 1992b) with PCR primers containing *Bam*HI cloning sites, thus generating $XA_3$ and $AX_{10}$. The construction of all other plasmids encoding chimeric DBDs was performed by PCR-based mutagenesis and followed a strategy described by Perrin and Gilliland (1990). Due to their construction the DBDs contain at their N-termini the additional sequences MASMTGGQQMGRGS. Truncated DBDs harbor the additional C-terminal residues SGSGC. To enable the swapping of cassettes encoding RXR, RAR and TR wild-type and chimeric DBDs into full-length receptors, *Xho*I sites were introduced immediately downstream of the 5′ and upstream of the 3′ *Bam*HI site. This changed residues Leu81, Pro82, Arg83 and Ile188 of the RAR into Pro, Arg, Gly and Leu respectively, residues Ser134 and Phe135 in the RXR into Arg and Val respectively, and residues Tyr44, Leu45, His154 and Arg155 in the TR into Ser, Arg, Leu and Glu respectively. Plasmids containing ER-derived sequences ($TE_1$, $TE_2$, $TE_{11}$, $XE_1$, $XE_2$, $AE_1$ and $AE_2$) contain the original 5′ sequences of either TR, RXR or RAR, since no 5′ *Xho*I site was introduced. To verify that none of the above sequence modifications interferes with DNA binding or cooperativity of the RXR, RAR and TR DBDs, plasmids were constructed which code for (i) wild-type DBDs with the original N- and C-terminal amino acids, (ii) truncated DBDs without the C-terminal SGSGC, and (iii) wild-type DBDs which contain only a N-terminal methionine in addition to the amino acids indicated in Figure 1. The latter plasmids were constructed by inserting PCR-amplified and *Pvu*II/*Bam*HI-digested DNA fragments into pET3a, which had been linearized with *Nde*I, blunted with Klenow polymerase and then digested with *Bam*HI.

The chimeric full-length receptors were constructed by inserting PCR-generated and *Xho*I-digested wild-type or chimeric DBDs into the vectors RXR[AB-DE] and RAR[AB-DEF]. To obtain RXR[AB-DE] the sequences coding for amino acids 1–133[AB] and 242–466[DE] of mRXRα1 (Leid *et al.*, 1992b) were amplified in two separate PCRs, digested with *Bam*HI/*Xho*I or *Bgl*II/*Xho*I and cloned into the *Bam*HI site of an epitope-tagged pET3a (kind gift of L.Shemshedini). The recombination of the fragments resulted in the introduction of a *Xho*I site at the border between region B and D, and an out-of-frame shift of the sequences 3′ of the *Xho*I site. To generate RAR[AB-DEF] (amino acids 1–81[AB] and 189–462[DEF] of mRARα1; Zelent *et al.*, 1989), *Bam*HI/*Xho*I-digested PCR products were assembled following the same strategy. In-frame insertion of DBD cassettes into the *Xho*I sites regenerated the open reading frame in the C-terminal regions, and thus facilitated the detection of sense insertions by Western blots with monoclonal antibodies directed against the F region of RAR (anti-RARα, Ab9α; Gaub *et al.*, 1992) or the C-terminal part of the E region of RXR (anti-RXRα, AbRX6G; M.-P.Gaub, Y.Lutz, C.Rochette-Egly, unpublished results).

The sequences of the various oligonucleotides used for PCR amplification and PCR-based mutagenesis are available upon request. All constructions were verified by sequencing.

### *Expression of proteins*

The *E.coli* strain BL21(DE3)pLysS (Novagen) was transformed with the plasmids described above or pET3a-chTRα, pET3a-RARα or pET3a-RXRα (Mader *et al.*, 1993b). Cells were grown at 37°C in the presence of 100 μg/ml ampicillin and 30 μg/ml chloramphenicol to an $OD_{600}$ of 0.6–0.8. Expression of proteins was induced by 0.5 mM IPTG at 28°C for 2–3 h. For receptor DBDs, chimeric full-length receptors and TRα, 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, 100 μM DTT, 10 μM $ZnCl_2$, 10% glycerol] supplemented with protease inhibitors, and the extracts were cleared by centrifugation for 30 min at 12 000 *g*. Dilutions of the crude lysates were electrophoresed in 12% SDS–polyacrylamide gels, and the concentrations of the expressed proteins were estimated by comparison with marker proteins. The purification of full-length mRARα and mRXRα will be described elsewhere.

### *Electrophoretic mobility shift assay*

Amounts of full-length receptors or DBDs used in each assay were calibrated as described in the legend of Figures 2 and 3, respectively. 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), as well as 150 mM KCl for full-length receptors and 100 mM KCl for receptor DBDs. The protein–DNA complexes were resolved through 6% polyacrylamide gels (0.5 × TBE buffer; prerun for 2 h) at 10 V/cm and room temperature for full-length receptors and at 13 V/cm and 4°C for the DBDs. Occasionally, for EMSA with RAR[X.cas], RAR[A.cas] and RAR[T.cas], 100 mM KCl were used and electrophoresis was performed at 4°C. Protein–DNA complex formation was quantified by phosphoimaging with a Fuji BAS 2000. The IR0 and all DR elements contained the core motif PuGGTCA. For details concerning the sequences of IR0 and DR1–DR5 see Mader *et al.* (1993b).

Note that some DBDs, for example, TR DBDs containing the RXR D-box ($TX_8$, Figure 1, line 13), chimeric DBDs in which the region located C-terminal to the D-box was swapped ($XA_4$, $XT_4$, $AX_4$ and $TX_4$; Figure 1, line 8), or the mutant $AX_{10}$ (Figure 1, line 15), had all lost the ability to bind to DNA as either monomeric or dimeric complexes (data not shown), and were not considered in this analysis.

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