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H-2RIIBP (RXRβ) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes

Michael S.Marks, Paul L.Hallenbeck[1], Toshi Nagata, James H.Segars, Ettore Appella[2], Vera M.Nikodem[1] and Keiko Ozato[3]

Laboratory of Molecular Growth Regulation, National Institute of Child Health and Human Development, [1]Genetics and Biochemistry Branch, National Institute of Diabetes and Digestive and Kidney Diseases and [2]Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

[3]Corresponding author

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H-2RIIBP (RXRβ) is a member of the nuclear hormone receptor superfamily that activates transcription of MHC class I genes in response to retinoic acid (RA). Using chemical cross-linking, co-immunoprecipitation, gel mobility shift and streptavidin-biotin DNA precipitation assays, we show that H-2RIIBP formed heterodimers with thyroid hormone (T3) and RA receptors (T3Rα and RARα). H-2RIIBP heterodimer formation required a conserved sub-domain of its C-terminal region, occurred independently of target DNA and was much more efficient than either T3Rα/RARα heterodimer or H-2RIIBP homodimer formation. Heterodimers displayed enhanced binding to target DNA elements and contacted DNA in a manner distinct from that of homodimers. A functional role for heterodimers *in vivo* was demonstrated by synergistic enhancement of MHC class I transcription following co-transfection of H-2RIIBP with T3Rα or RARα. We provide biochemical evidence that H-2RIIBP formed heterodimers with several naturally occurring nuclear proteins. The results suggest that H-2RIIBP, by virtue of its ability to heterodimerize, enhances combinatorial diversity and versatility in gene regulation mediated by nuclear hormone receptors.

Key words: dimerization/gene regulation/hormone receptors/retinoic acid/thyroid hormone

Introduction

Eukaryotic gene transcription is controlled largely by interactions of *cis*-acting DNA elements with sequence-specific DNA binding proteins. Both DNA binding and the subsequent functional activity of transcription factors are influenced by protein-protein interactions (Ptashne, 1988; Lewin, 1990). One type of interaction common to many DNA binding proteins is dimerization. Dimer formation is often a prerequisite for DNA binding and subsequent activity (Jones, 1990). The specificity of transcriptional regulation in many systems depends upon heterodimerization between different members of a family of transcription factors, such as the leucine zipper (Nakabeppu *et al.*, 1988; Landschultz *et al.*, 1989; Hai and Curran, 1991), helix-loop-helix (Murre *et al.*, 1989a,b), POU domain (Treacy *et al.*, 1991) and c-*rel* families (Ballard *et al.*, 1990; Logeat *et al.*, 1991; Nolan *et al.*, 1991).

One family of DNA binding proteins for which dimerization may be important is the nuclear hormone receptor superfamily (Evans, 1988; Beato, 1989). Members of this superfamily have a characteristic tripartite modular structure, consisting of (i) a highly conserved central DNA binding domain (DBD) characterized by 'C_4C_5 zinc fingers', (ii) a less conserved C-terminal domain that mediates binding of steroids, retinoids, thyroid hormone or other ligands and (iii) a poorly conserved N-terminal domain that has been implicated in activation or repression of target gene expression. Some members of the superfamily have been shown to form homodimers (Kumar and Chambon, 1988; Tsai *et al.*, 1988; DeMarzo *et al.*, 1991; Lazar *et al.*, 1991). Amino acid sequences that are essential for homodimer formation have been identified within both the DBD and the ligand binding domain (LBD) of the glucocorticoid and estrogen receptors (Kumar and Chambon, 1988; Eriksson and Wrange, 1990; Fawell *et al.*, 1990; Luisi *et al.*, 1991). Disruption of these sequences abolishes not only dimerization, but also transcriptional regulatory activity (Kumar and Chambon, 1988; Fawell *et al.*, 1990).

Several laboratories have provided evidence that heterodimers can be formed between members of a subgroup of the nuclear hormone receptor superfamily. Thyroid hormone (T3) receptors (T3Rs) and retinoic acid (RA) receptors (RARs), both members of this subgroup, have been shown to form heterodimers upon interaction with some DNA response elements, resulting in dual responsiveness to RA and T3 (Glass *et al.*, 1989; Forman *et al.*, 1989; Lucas *et al.*, 1991). These receptors and the vitamin D receptor also interact with other as yet undefined accessory factors, resulting in heteromeric complexes that appear to bind more avidly to target DNA elements (Murray and Towle, 1989; Glass *et al.*, 1989, 1990; Burnside *et al.*, 1990; Hudson *et al.*, 1990; Lazar and Berrodin, 1990; Liao *et al.*, 1990; Darling *et al.*, 1991; Yang *et al.*, 1991). Heterodimerization of these receptors depends upon structural features within the LBD, the disruption of which may diminish or eliminate DNA binding *in vitro* and/or transcriptional activity *in vivo* (Forman and Samuels, 1990; Darling *et al.*, 1991; O'Donnell *et al.*, 1991; Spanjaard *et al.*, 1991; Selmi and Samuels, 1991; Hudson *et al.*, 1990). These findings imply that heterodimers elicit functions that are distinct from those elicited by T3R or RAR alone.

H-2RIIBP, otherwise known as RXRβ (R.M.Evans, personal communication), is a member of the T3R/RAR subgroup of the superfamily that binds to the region II (RII) enhancer element of major histocompatibility complex (MHC) class I genes (Hamada *et al.*, 1989). The products of MHC class I genes play an important role in immune responses. The RII enhancer is part of the well described class I regulatory complex, which is highly conserved among

murine MHC class I genes and controls their expression (Kimura *et al.*, 1986; Shirayoshi *et al.*, 1987; Baldwin and Sharp, 1987). H-2RIIBP acts as an RA-responsive activator of MHC class I gene transcription in a human embryonal cell line and may be involved in the developmental control of MHC class I gene expression (Nagata *et al.*, 1992). H-2RIIBP has >85% homology in the DBD and LBD with RXRα, implicated in an RAR-independent retinoid-mediated gene regulatory pathway (Mangelsdorf *et al.*, 1990, 1991; Rottman *et al.*, 1991).

In an effort to elucidate the mechanism of its action, we analyzed the ability of H-2RIIBP to form dimers with itself and with other nuclear factors. We show here that H-2RIIBP homodimers are stabilized in the presence of specific target DNA, and that the protein binds to DNA only in dimeric form. Furthermore, we show that H-2RIIBP forms heterodimers with T3Rα and RARα that are more stable than either H-2RIIBP homodimers or T3Rα/RARα heterodimers. Heterodimerization of H-2RIIBP with these receptors leads to (i) enhanced DNA binding *in vitro*, (ii) altered DNA recognition patterns and (iii) increased transcriptional activity *in vivo*, analogous to the effects of the previously described RAR/T3R accessory factors. Our results suggest that heterodimerization with H-2RIIBP represents a combinatorial mechanism of gene regulation by which orphan receptors may affect the function of many nuclear hormone receptors.

Table I. Oligonucleotide probes used in this study[a]

	−207	−183
RII	5′-GCCAGGCGGTGAGGTCAGGGGTGGGGAA-3′	
	3′ - TCCGCCACTCCAGTCCCCACCCCTTCGG-5′	
	−207	−183
mut-RII	5′-GCCAGGCGGTGATTTCAGGGGTGGGGAA-3′	
	3′ - TCCGCCACTAAAGTCCCCACCCCTTCGG-5′	
	−175	−160
RI	5′ - GCTGGGGATTCCCCAT-3′	
	3′ - CGACCCCTAAGGGGTA-5′	
	−2514	−2490
TAT-GRE	5′-GCCTCTGCTGTACAGGATGTTCTAGCTA-3′	
	3′ - AGACGACATGTCCTACAAGATCGATCGG-5′	
TRE_P	5′-GCCAGATTCAGGTCATGACCTGAGGAGA-3′	
	3′ - TCTAAGTCCAGTACTGGACTCCTCTCGG-5′	
	−287	−255
TRE-ME	5′-GCCAGGACGTTGGGGTTAGGGGAGGACAGTGGACGA-3′	
	3′ - TCCTGCAACCCCAATCCCCTCCTGTCACCTGCTCGG-5′	
	−336	−297
ERE-B1	5′-GCCCGAGTCACTGTGACCCAACCCAAGTTATCATGACCTCTTA-3′	
	3′ - GCTCAGTGACACTGGGTTGGGTTCAATAGTACTGGAGAATCGG-5′	

[a]RII: region II of murine MHC class I genes (H-2L^d, Shirayoshi *et al.*, 1987; core sequence underlined is identical in H-2K^{b,k,d,w28}, D^{b,k,d,p} and Q10^p). mut-RII: the M1 mutant of RII (Shirayoshi *et al.*, 1987), with mutations marked in bold. RI: region I of murine MHC class I genes (Shirayoshi *et al.*, 1987; Kimura *et al.*, 1986; Baldwin and Sharp, 1987). TAT-GRE: glucocorticoid response element of the tyrosine aminotransferase gene (Jantzen *et al.*, 1987). TRE_p: artificial palindromic thyroid hormone/retinoic acid responsive element (Umesono *et al.*, 1988; Glass *et al.*, 1988). TRE-ME: thyroid response element from the rat malic enzyme gene (Petty *et al.*, 1990). ERE-B1: estrogen response element from the *Xenopus* vitellogenin B1 gene (Martinez *et al.*, 1987).

Results

Baculovirus recombinant H-2RIIBP binds to the MHC class I region II (RII) enhancer as a homodimer

Dimerization of H-2RIIBP in the absence and presence of target DNA was investigated by a series of chemical cross-linking experiments. Nuclear extracts from baculovirus-infected insect cells expressing H-2RIIBP (bH-2RIIBP) were incubated with oligonucleotides corresponding either to intact RII or to a mutated RII (mut-RII; see Table I) that does not bind H-2RIIBP (Hamada *et al.*, 1989; Marks *et al.*, 1992). Proteins were then cross-linked by adding increasing concentrations of disuccinimidyl suberate (DSS). Cross-linked products were examined by Western blotting using an anti-peptide antibody specific for bH-2RIIBP (Marks *et al.*, 1992). In the absence of DSS, bH-2RIIBP migrated in SDS−PAGE with M_r 52 000, as expected for a monomer (Figure 1A, lanes 1 and 5; Marks *et al.*, 1992). In the presence of DSS, several high M_r bands appeared in addition to the band at 52 kDa, including a prominent band of M_r 110 000. The 110 kDa complex was much more intense in the presence of intact RII than mut-RII, suggesting that the high M_r bH-2RIIBP complexes formed more efficiently or were stabilized in the presence of target DNA. To determine whether this complex in fact bound to target DNA, cross-linked products were fractionated by streptavidin−biotin-coupled DNA precipitation. Extracts containing bH-2RIIBP were mixed with biotinylated wild type RII or mut-RII oligonucleotides, reacted with DSS and precipitated with streptavidin−agarose. Streptavidin-bound and unbound products were analyzed by Western blotting (Figure 1B). As expected, biotinylated mut-RII failed to precipitate an appreciable amount of bH-2RIIBP (right, lanes 1−4). Conversely, biotinylated-RII bound and precipitated a significant amount of bH-2RIIBP, all of which was found in the 110 kDa complex in the presence of DSS (left, lanes 1−4). These results indicate that bH-2RIIBP binds to RII as a multimeric complex consistent in size with a dimer. On the other hand, most of the bH-2RIIBP that did not bind to RII and that remained in the supernatant migrated as a 52 kDa monomer, even after treatment with the highest concentration of DSS (Figure 1B, lanes 5−8). Thus, bH-2RIIBP exists primarily as a monomer in solution under these conditions, with only a small fraction present in higher M_r complexes. Sizing of bH-2RIIBP in the absence of DNA by analytical gel filtration (not shown) supports this conclusion.

It was unlikely that the 110 kDa bH-2RIIBP complex contained endogenous components of insect cell extracts since (i) no insect cell proteins were found to co-immunoprecipitate with bH-2RIIBP (Marks *et al.*, 1992) and (ii) extracts from cells infected with the wild type (WT) baculovirus failed to enhance DNA binding complex formation (Marks *et al.*, 1992 and Figure 4). Nonetheless, in order to prove that the 110 kDa complex was a homodimer, additional cross-linking experiments were performed in which we analyzed the formation of dimers between two forms of H-2RIIBP. Radiolabeled *in vitro* translated H-2RIIBP ([^{35}S]H-2RIIBP) was incubated in the absence or presence of oligonucleotides corresponding to

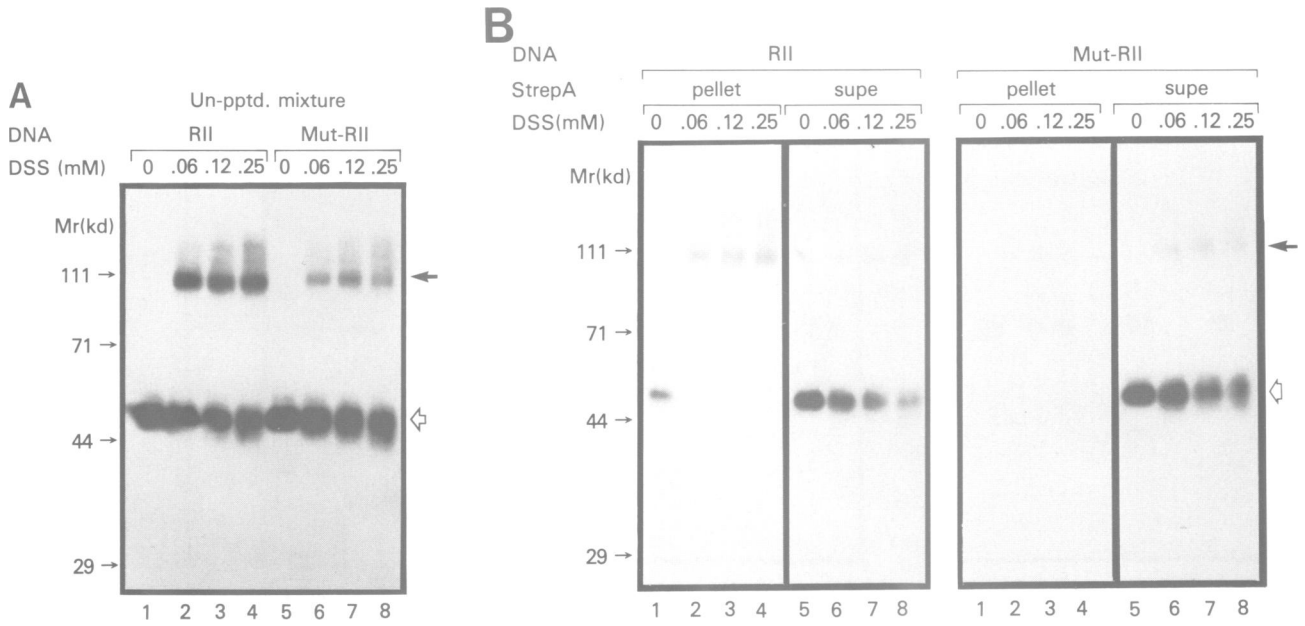


Fig. 1. H-2RIIBP binds to RII DNA as a high M_r complex. Insect cell extracts containing bH-2RIIBP were incubated with biotinylated RII or mut-RII oligonucleotides and treated with the indicated amount of DSS. **A.** Products were analyzed directly by SDS−PAGE and Western blotting with affinity purified anti-H-2RIIBP peptide antibody. **B.** Prior to SDS−PAGE, products were separated into DNA-bound (pellet) and unbound (supe) fractions by precipitation with streptavidin−agarose (StrepA). Empty arrowhead indicates the position of monomeric bH-2RIIBP; filled arrow indicates the position of the major DSS-dependent high M_r complex.

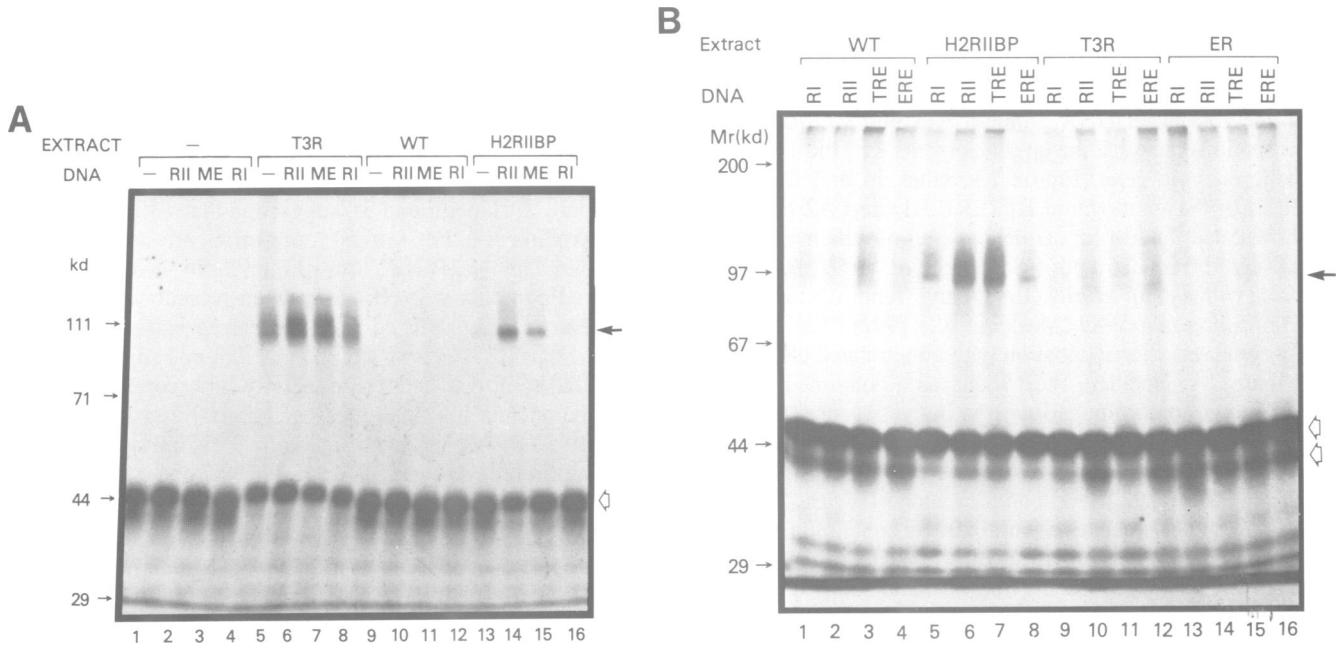


Fig. 2. H-2RIIBP forms both homodimers with itself and heterodimers with T3Rα. **A.** *In vitro* translated [^{35}S]H-2RIIBP (~1 fmol) was incubated without extracts (lanes 1−4) or with insect cell extracts (1 μg) containing WT viral proteins (lanes 9−12), bH-2RIIBP (lanes 13−16; ~2−5 pmol) or bT3R (lanes 5−8; ~1−2 pmol) in the absence (−) or presence of the indicated oligonucleotides (10 pmol). Mixtures were treated with 0.25 mM DSS and analyzed by SDS−PAGE. Oligonucleotides include the malic enzyme TRE (ME), MHC class I region I (RI) or RII (Table I). Empty arrowhead, [^{35}S]H-2RIIBP monomer; arrow, dimeric species. **B.** *In vitro* translated [^{35}S]T3Rα (~1 fmol) was incubated with insect cell extracts (1 μg) containing bH-2RIIBP (lanes 5−8), bT3Rα (lanes 9−12), or WT viral proteins alone (lanes 1−4) or with added ER (lanes 13−16) in the presence of the indicated oligonucleotides (10 pmol). Mixtures were treated with 0.25 mM DSS and analyzed by SDS−PAGE. TRE, the 16 bp core of TRE_p (see Table I); ERE, vitellogenin B1 gene ERE. Empty arrowheads, [^{35}S]T3Rα monomer (with and without internal cross-links); arrow, dimeric species.

various DNA elements and insect cell extracts containing WT viral proteins or bH-2RIIBP. The mixtures were treated with DSS and products were analyzed by SDS−PAGE. [^{35}S]H-2RIIBP migrated as a monomer with M_r 45 000 in the absence of DSS (not shown); the translation initiation site for [^{35}S]H-2RIIBP correponds to methionine 37 of the bH-2RIIBP product, accounting for its faster migration. When mixed with bH-2RIIBP and treated with DSS, a

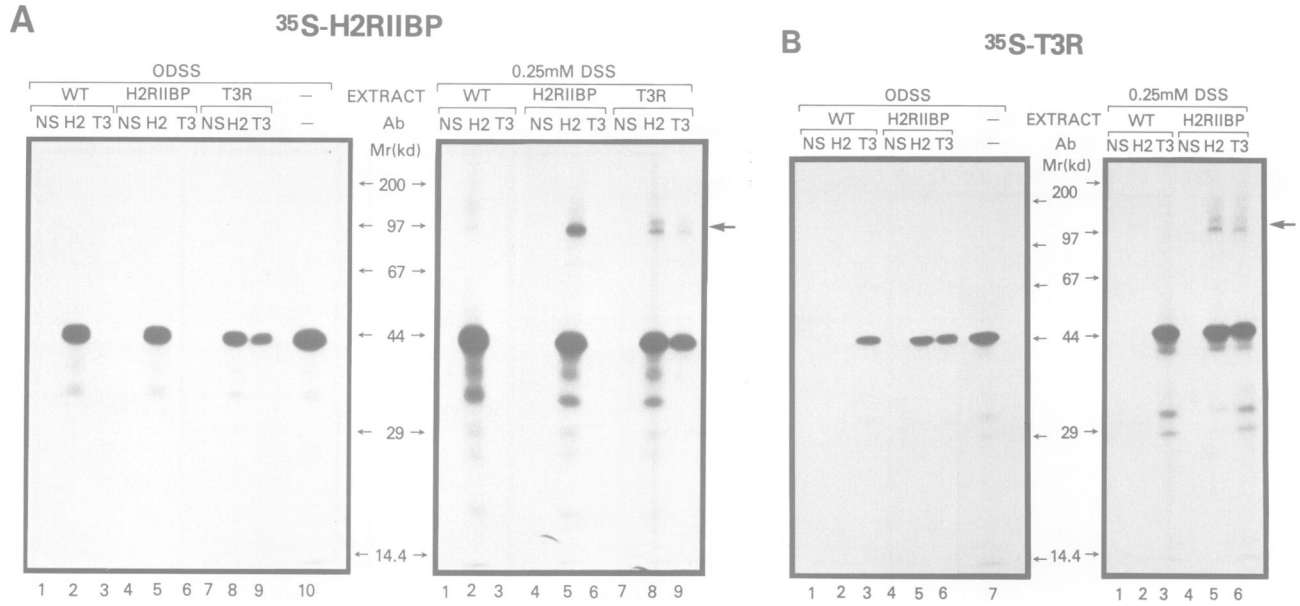


Fig. 3. H-2RIIBP/T3Rα heterodimers are stable in the absence of cross-linking. [^{35}S]H-2RIIBP (**A**) or [^{35}S]T3Rα (**B**) was incubated with 10 pmol RII oligonucleotide and insect cell extracts (125 ng) containing WT viral proteins (lanes 1–3), bH-2RIIBP (lanes 4–6), or bT3Rα (B, lanes 7–9). Mixtures were either untreated (left; 0 DSS) or treated with 0.25 mM DSS (right), and then immunoprecipitated with either control serum (N2; lanes 1, 4 and 7) or antibodies to H-2RIIBP (H2; lanes 2, 5 and 8) or to T3Rα (T3; lanes 3, 6 and 9). Eluates were analyzed by SDS–PAGE. Exposures for DSS-treated lanes were twice as long as those for untreated lanes in order to emphasize the cross-linked bands. A sample of the *in vitro* translated material was analyzed without precipitation (–; A, lane 10; B, lane 7). Arrow, dimeric species in DSS-treated samples.

significant fraction of [^{35}S]H-2RIIBP migrated as a complex with M_r 97 000 (Figure 2A, lanes 14 and 15), consistent with a dimer of bH-2RIIBP and [^{35}S]H-2RIIBP. The appearance of this complex was dependent on the presence of specific target DNA, since it was observed only when incubations included the target elements RII, the TRE of the malic enzyme gene (Figure 2A, lanes 14 and 15), or the EREs of the vitellogenin B1 (ERE-B1) and A2 genes (not shown; see Table I for sequences), all of which contain the conserved GGTCA motif (Martinez *et al.*, 1991; Näär *et al.*, 1991; Umesono *et al.*, 1991) and bind to bH-2RIIBP (Hamada *et al.*, 1989; Marks *et al.*, 1992). The band was not observed after incubation with an unrelated element, RI (Figure 2A, lane 16), most likely as a combined result of inefficient homodimer formation in the absence of specific DNA and less efficient cross-linking in the presence of reticulocyte lysate components (see below). [^{35}S]H-2RIIBP did not form a high M_r complex alone (Figure 2A, lanes 1–4) or upon incubation with extracts containing WT viral proteins (lanes 9–12), irrespective of whether RII or other DNAs were added during the preincubation. This result suggests that homodimer formation requires a higher concentration of H-2RIIBP (>10 nM in experiments not shown) than that corresponding to the *in vitro* translated product alone (10–50 pM).

H-2RIIBP forms heterodimers with T3Rα

Since H-2RIIBP can form homodimers and since other members of the T3R/RAR subgroup of nuclear hormone receptors have been shown to form heterodimers (Forman *et al.*, 1989; Glass *et al.*, 1989; Lucas *et al.*, 1991), we tested whether H-2RIIBP could also form heterodimers with members of this subgroup. As shown in Figure 2A (lanes 5–8), [^{35}S]H-2RIIBP was incorporated into a high M_r cross-linked complex when preincubated with insect cell extracts containing baculovirus recombinant rat T3Rα (bT3Rα). The average M_r of this complex was 96 000, consistent with a heterodimer of the 46 kDa bT3Rα and the 45 kDa [^{35}S]H-2RIIBP. Bands with higher M_r also appeared, and may have contained endogenous components of the extracts in addition to T3Rα and H-2RIIBP. In contrast to the H-2RIIBP homodimer, the high M_r heterodimer band was observed even in the absence of added target DNA (Figure 2A lanes 5 and 8), suggesting that T3Rα/H-2RIIBP heterodimers either formed more efficiently or were more stable than H-2RIIBP homodimers. No high M_r cross-linked complex was observed when recombinant estrogen receptor (ER) produced from a baculovirus vector (Brown and Sharp, 1990) was added (not shown), suggesting that H-2RIIBP forms heterodimers only with certain members of the nuclear hormone receptor superfamily. As shown in Figure 2B, *in vitro* translated [^{35}S]T3Rα was also incorporated into an intense 96–97 kDa cross-linked complex in the presence of bH-2RIIBP (lanes 5–8), but not the ER (lanes 13–16) or WT viral proteins (lanes 1–4). It should be noted that other factors, present either in reticulocyte lysates or insect cell extracts, could contribute to the formation or stabilization of the heterodimeric complex, and may be included in the cross-linked complexes with M_r >97 000. These results confirm that H-2RIIBP efficiently forms heterodimers with T3Rα that are stable in the absence of target DNA.

In the experiments above, it was possible that cross-linking stabilized an otherwise transient interaction between T3Rα and H-2RIIBP. We therefore tested whether formation of T3Rα/H-2RIIBP heterodimers can be detected in the absence of cross-linking reagents by immunoprecipitation. [^{35}S]T3Rα or [^{35}S]H-2RIIBP was pre-incubated with nuclear extracts containing WT or recombinant proteins in the presence of RII. The mixtures were then immunoprecipitated with control rabbit serum or antibodies specific for T3Rα or H-2RIIBP. Anti-T3R antibody

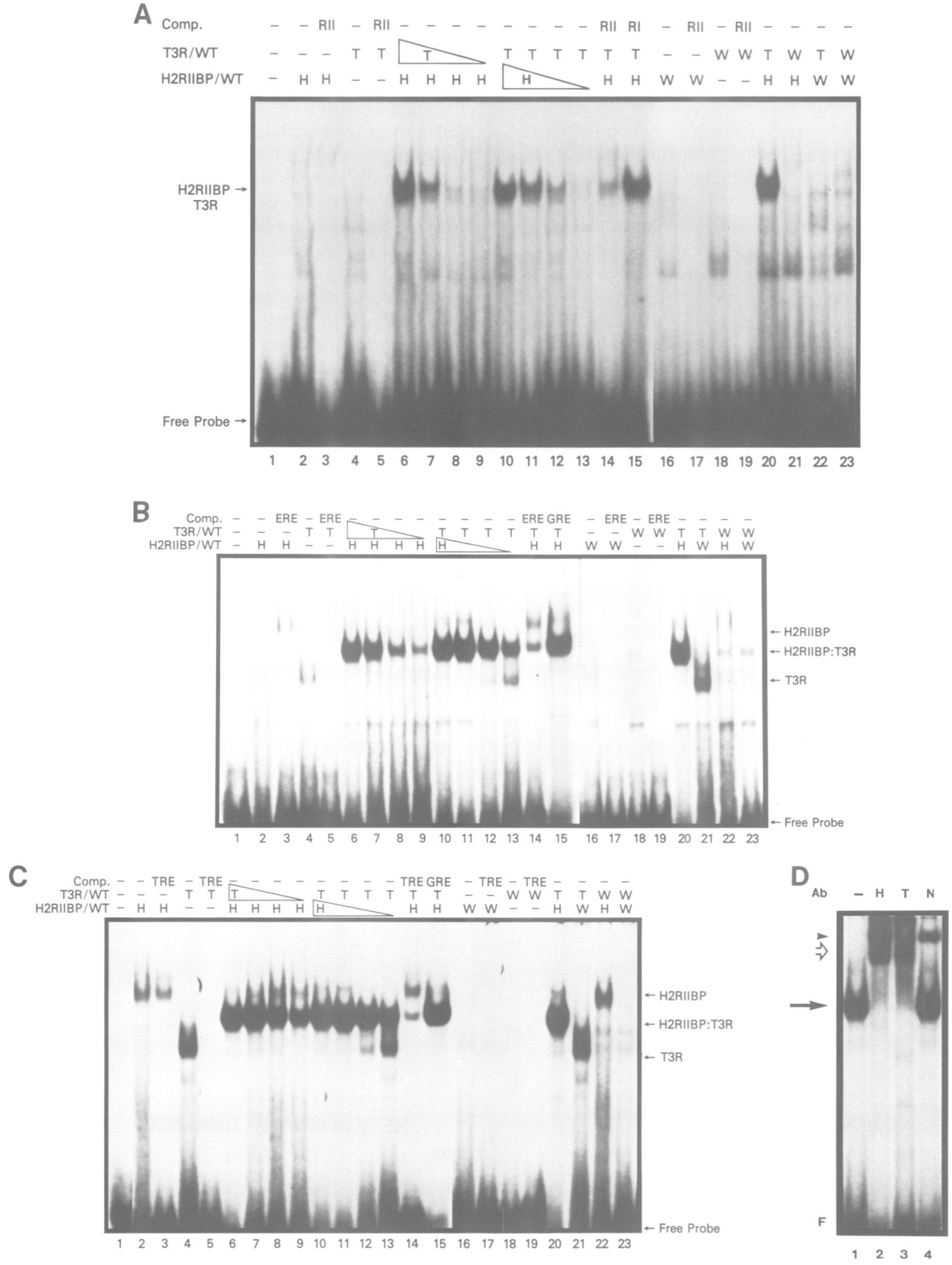


Fig. 4. H-2RIIBP and T3Rα synergistically bind to DNA response elements. **A**, **B**, and **C.** Insect cell extracts containing bH-2RIIBP (H), bT3Rα (T), WT viral proteins (W) or combinations thereof were incubated with ^{32}P-labeled probes in the absence or presence of excess unlabeled oligonucleotide, and were analyzed by gel mobility shift assay (Materials and methods). The probes and specific competitors (Comp.; lanes 3, 5, 14, 17 and 19) used were A, RII; B, ERE-B1; and C, TRE_p (Table I). Non-specific competitors (lanes 15) were region I (RI; A) and the glucocorticoid response element of the tyrosine aminotransferase gene (GRE; B and C). All lanes contained 5 μg of the indicated extract, except for lanes 1 (no extract added), lanes 7–9 (2, 0.5 and 0.2 μg of T3Rα, respectively) and lanes 11–13 (2, 0.5 and 0.2 μg of H-2RIIBP, respectively). Positions of free probe and the hetero- and putative homodimeric species of H-2RIIBP and T3Rα are indicated. **D.** Effect of antibodies on gel mobility shift. Insect cell extracts containing bT3Rα and bH-2RIIBP were incubated with ^{32}P-labeled TRE_p probe, and then either phosphate buffered saline (–, lane 1), control rabbit serum (N, lane 4) or antibodies specific for H-2RIIBP (H, lane 2) or T3Rα (T, lane 3) were added and incubation was continued for 1 h before analysis by PAGE. Filled arrow, bH-2RIIBP/bT3Rα heterodimer; empty arrow, super-shifted band; arrowhead, non-specific band produced by sera; F, free probe. As controls (not shown), anti-H-2RIIBP antisera blocked formation of a retarded band with bH-2RIIBP alone, but not bT3Rα; similarly, anti-T3Rα sera blocked formation of a retarded band with bT3Rα alone, but not bH-2RIIBP.

precipitated [^{35}S]H-2RIIBP only when the reaction mixtures contained bT3Rα (Figure 3A, left, 'T3' lanes). Similarly, anti-H-2RIIBP antibody precipitated [^{35}S]T3Rα only in the presence of bH-2RIIBP (Figure 3B, left, 'H2' lanes). Control serum failed to precipitate either receptor under any conditions (Figures 3A and B, lanes NS). When DSS was added to reaction mixtures prior to immunoprecipitation, cross-linked complexes of ~97 kDa (and higher) were also immunoprecipitated (Figure 3A and B, right). Cross-linked materials represented only a fraction of the precipitated materials, presumably due to incomplete cross-linking. These results demonstrate that H-2RIIBP forms stable heterodimers with T3Rα in solution.

Heterodimerization of H-2RIIBP with T3Rα enhances DNA binding and qualitatively alters contact sites

Binding of T3Rs to TREs is enhanced by unidentified nuclear accessory factors (Murray and Towle, 1989; Burnside *et al.*, 1990; Lazar and Berrodin, 1990; Darling *et al.*, 1991). To determine whether heterodimerization enhances DNA binding by H-2RIIBP and T3Rα, gel mobility shift assays were performed using RII, ERE-B1, and a palindromic TRE (TRE_p) as probes. All of these elements (Table I) have been previously shown to bind to bH-2RIIBP under certain conditions (Marks *et al.*, 1992). As shown in Figure 4A, either bH-2RIIBP (lane 2) or bT3Rα (lane 4) alone failed to alter the migration of a RII probe under these conditions. However, mixing of the two extracts (lanes 6, 10 and 20) resulted in the formation of an intense retarded band. This band was reduced in intensity with decreasing amounts of either extract (lanes 6–13) and was not formed by addition of equivalent amounts of WT extract (lanes 21–23), demonstrating dependence on both bT3Rα and bH-2RIIBP. The band represented a specific RII–protein complex, since it was competed by excess unlabeled RII (lane 14) but not by an unrelated oligomer (RI, lane 15). Similar results were obtained using radiolabeled ERE-B1 (Figure 4B) and TRE_p (Figure 4C) as probes, although with these probes some binding was observed using either extract alone (lanes 2 and 4). The band formed by the mixture of bH-2RIIBP and bT3Rα with these probes migrated to a unique position in between the bands formed with either protein alone,

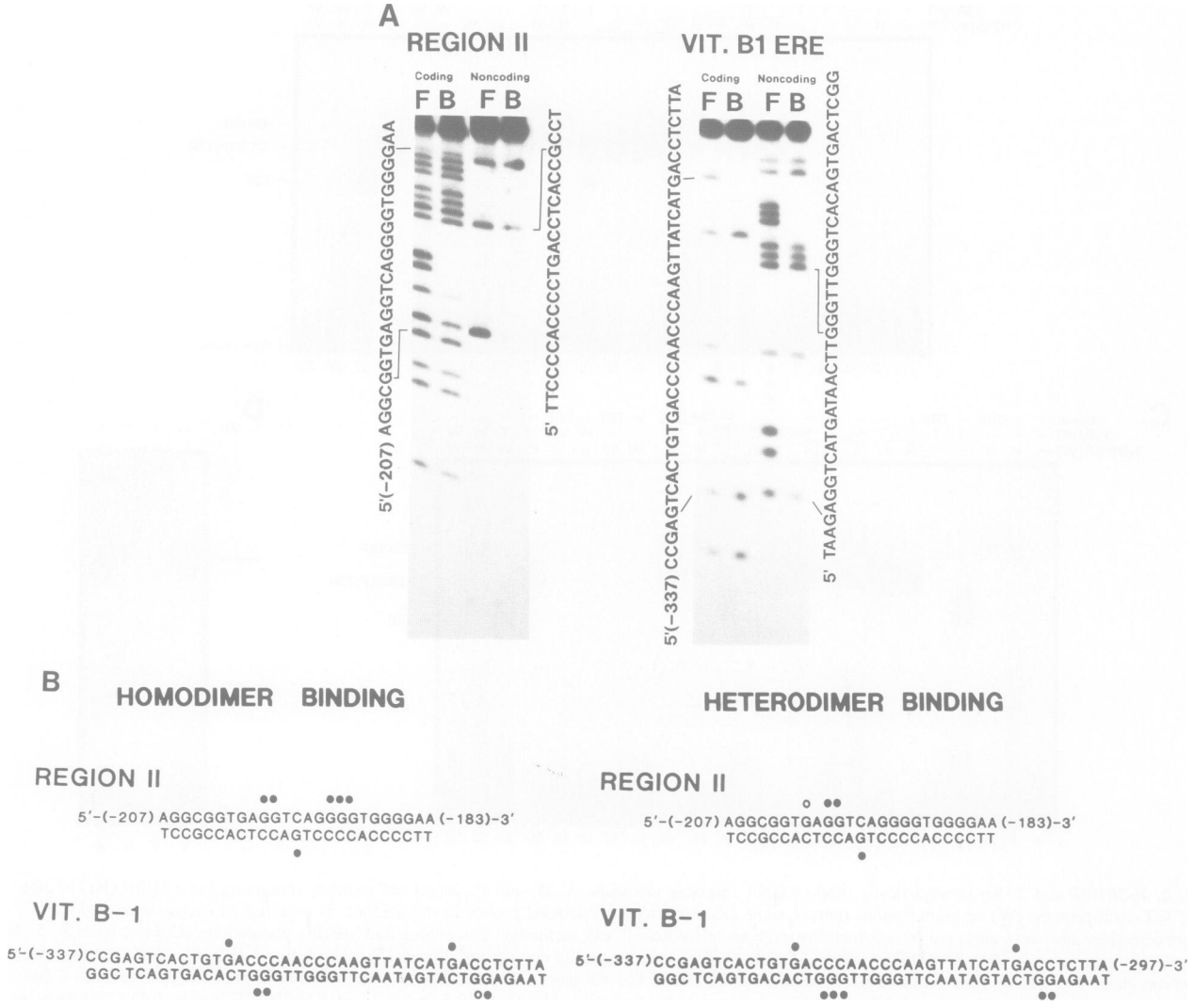


Fig. 5. H-2RIIBP homodimer and H-2RIIBP/T3Rα heterodimer contact target DNA elements differently. **A.** Results of methylation interference with the H-2RIIBP/T3Rα heterodimer binding to RII and ERE-B1. F, the pattern obtained with unbound (free) probe; B, the pattern obtained with probe bound to the heterodimer. Interference of protein–DNA interactions by methylation of G residues results in missing bands in lanes B. **B.** Summary of the interference patterns shown above for the heterodimer and comparison with that obtained for the H-2RIIBP homodimer (Marks *et al.*, 1992). G residues for which methylation abolishes binding are indicated by black circles; partial interference is indicated by open circles.

providing evidence that the novel band represented a H-2RIIBP/T3R heterodimer. Furthermore, the migration of this band was altered by an antibody specific either for T3Rα or for H-2RIIBP, but not by a control serum (Figure 4D); the band observed with bH-2RIIBP alone was not shifted by anti-T3R, and the band observed with bT3Rα alone was not shifted by anti-H-2RIIBP (not shown). These results demonstrate that a H-2RIIBP/T3Rα heterodimer binds more avidly than either homodimer to three target DNA elements.

The heterodimer bound more avidly to ERE-B1 and TRE_p than to RII (Figure 4, compare panel A with B and C). This may be a result of dual recognition of these elements: ERE-B1 and TRE_p are bound by either bH-2RIIBP or bT3Rα alone, but RII is bound only by bH-2RIIBP, albeit weakly (Marks *et al.*, 1992). Binding of the ERE-B1 probe (and to some extent, TRE_p) by H-2RIIBP alone was enhanced by the addition of excess unlabeled specific oligonucleotide (Figure 4B and C, lanes 2 and 3). This may reflect a low affinity interaction between H-2RIIBP and the DNA elements, such that an increased concentration of DNA shifts the equilibrium toward enhanced levels of the DNA–protein complex. Alternatively or in addition, this may reflect a slow 'on-rate' for DNA binding by the H-2RIIBP homodimer, which would eliminate the effect of pre-incubation with unlabeled oligonucleotide and provide an equal opportunity for both labeled probe and unlabeled competitor to bind. It should be noted that in some experiments (e.g. Figure 4B, lane 21), binding of either bH-2RIIBP or bT3Rα alone was enhanced by the control insect cell extract. It is possible that an endogenous H-2RIIBP-like protein, such as the highly homologous CF-1/XR2C (Henrich *et al.*, 1990; Oro *et al.*, 1990; Shea *et al.*, 1990), is present in sufficient amounts in the extracts to produce such an enhancing effect.

In order to determine whether the enhanced DNA binding by heterodimers reflected qualitative changes in DNA binding, methylation interference assays were performed with RII and ERE-B1. The results (Figure 5A) are summarized and compared in Figure 5B with the interference patterns observed for the H-2RIIBP homodimer (Marks *et al.*, 1992). Heterodimers showed interference by fewer G residues in RII than did H-2RIIBP homodimers; methylation of six central G residues interfered with binding by the homodimer, whereas methylation of only four residues, centering on the GGTCA motif, interfered with binding by the heterodimer. Similar results have been obtained for a thyroid hormone response element (data not shown). Although less striking, differences were also observed for the homodimer and heterodimer interference patterns with ERE-B1 (VIT.B1 ERE, Figure 5); in this case, binding by the heterodimer was blocked by methylation of an additional G residue relative to the homodimer. These results show that heterodimerization of H-2RIIBP and T3Rα not only quantitatively enhances target DNA binding, but also leads to qualitatively different protein–DNA interactions.

Localization of the H-2RIIBP dimerization domain

The previously described interactions of T3Rs and RARs with each other and with accessory factors have been shown to be mediated by a subdomain of the LBD (Forman *et al.*, 1989; Glass *et al.*, 1989, 1990; Graupner *et al.*, 1989; Darling *et al.*, 1991; O'Donnell *et al.*, 1991; Spanjaard *et al.*, 1991). Forman and Samuels (1990) and Forman *et al.* (1989) identified within this domain a series of repeated 'heptad' sequences containing hydrophobic amino acids with predicted spacings, proposed to form a leucine zipper-like structure. Alignment with the LBDs of RARs, T3Rs and other members of the subfamily shows that H-2RIIBP possesses a similar pattern of repeats (Figure 6). We therefore tested whether this region of H-2RIIBP was responsible for heterodimerization with T3Rα.

A series of C-terminally or N-terminally truncated [^{35}S]H-2RIIBP polypeptides (Figure 7A; see also Figure 6) were synthesized from deletion constructs by *in vitro* transcription and translation. Dimerization of truncated [^{35}S]H-2RIIBP with bH-2RIIBP or bT3Rα was first analyzed by chemical cross-linking experiments (Figure 7B)

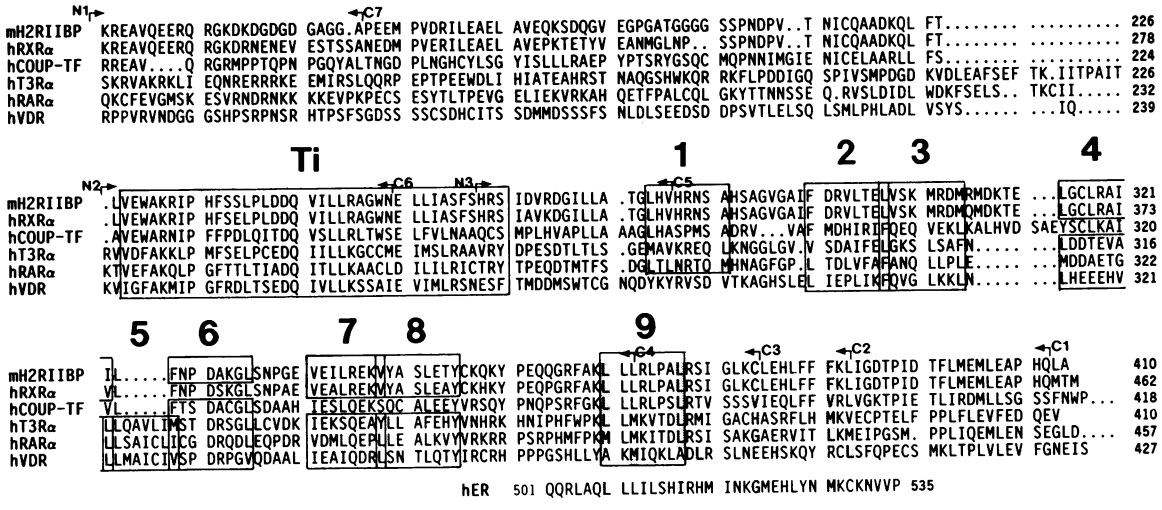
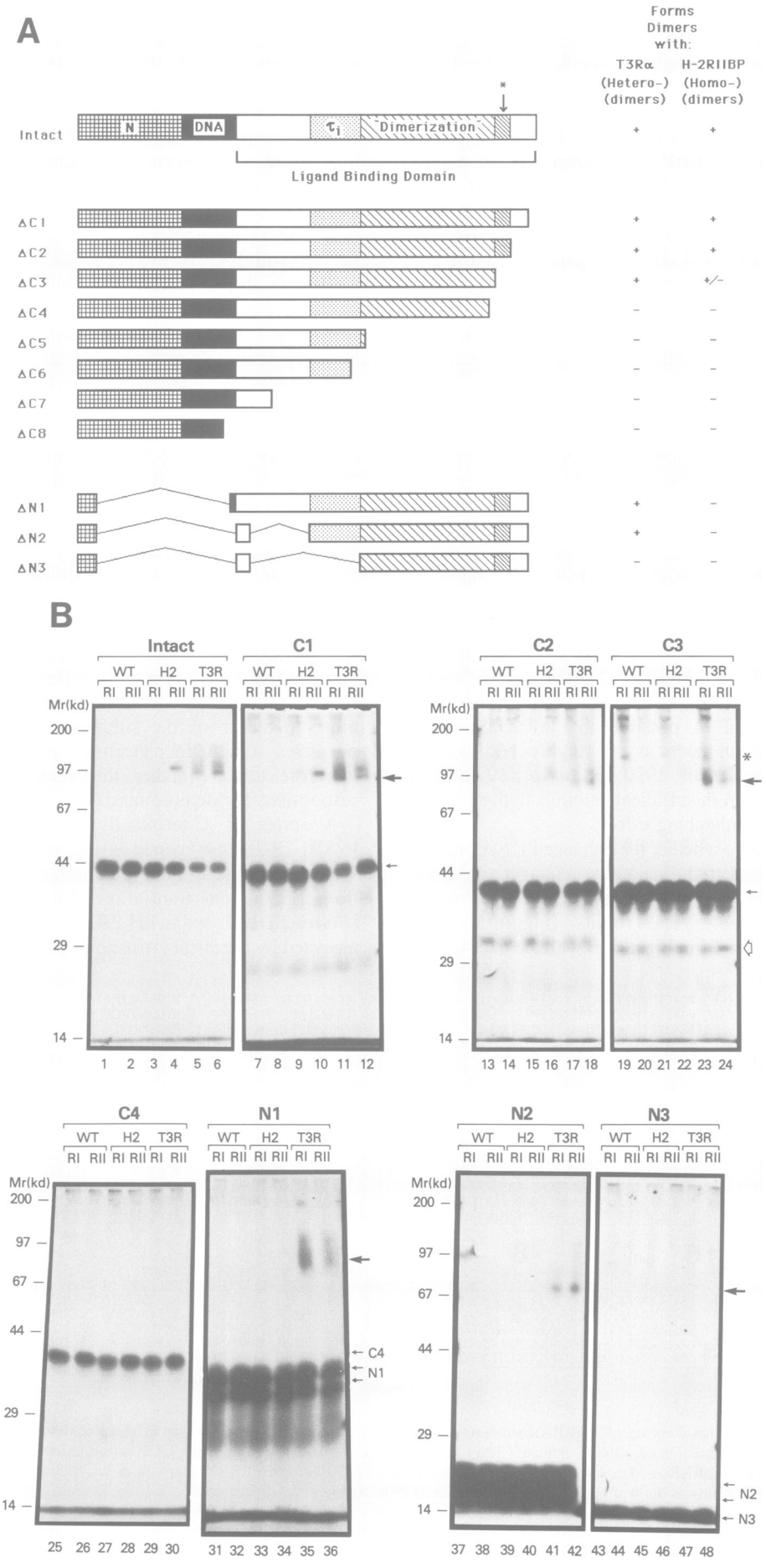


Fig. 6. Alignment of C-terminal domains of T3R/RAR subfamily members. Sequences of human vitamin D receptor (hVDR; Baker *et al.*, 1988), human RARα (hRARα; Petkovich *et al.*, 1987), human T3Rα1 (hT3Rα; Nakai *et al.*, 1988), human COUP transcription factor (hCOUP-TF; Wang *et al.*, 1989), human RXRα (hRXRα; Mangelsdorf *et al.*, 1990) and mouse H-2RIIBP (mH2RIIBP; Hamada *et al.*, 1989) are aligned with respect to the Ti and dimerization domains as defined by Forman and Samuels (1990). Regions that fit the criteria for the Ti domain and the 'heptad' repeats are enclosed by boxes. A region of the human estrogen receptor (hER; Green *et al.*, 1986), shown by Fawell *et al* (1990) to contain a homodimerization signal, is also aligned with the other sequences, with conserved amino acids indicated by shading. The beginning positions of N-terminal H-2RIIBP deletions and the ending positions of C-terminal deletions (see Figure 7A) are indicated with arrows.


A
Forms Dimers with:
T3Rα (Hetero-) (dimers)
H-2RIIBP (Homo-) (dimers)
Intact
N
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ΔC1
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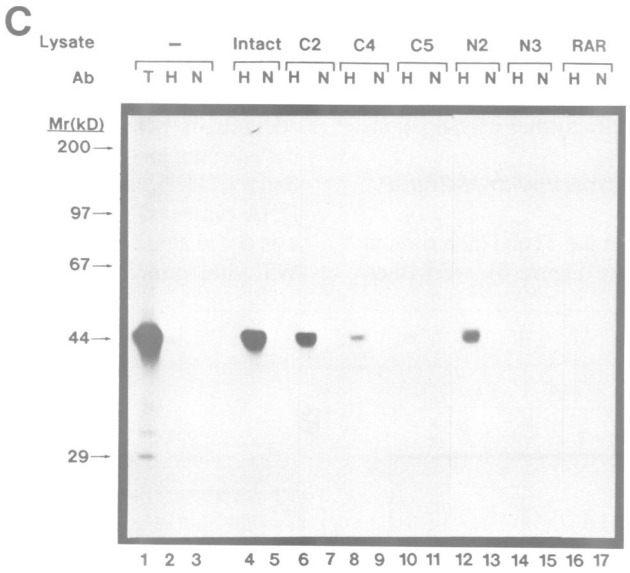


Fig. 7. Dimerization is mediated by the H-2RIIBP C-terminal domain. **A.** Schematic diagram of the putative domain organization of H-2RIIBP (Evans, 1988; Forman and Samuels, 1990) and the translated products of deletion constructs. Deleted proteins were constructed as described in Materials and methods. *, a region with homology to a domain of the ER necessary for homodimerization (Fawell *et al.*, 1990). Also shown is a summary of the results shown in panels B and C; + and − indicate formation of dimers (or lack thereof) with T3R or intact H-2RIIBP; ± indicates weak dimerization. Constructs represent deletion of the indicated number of amino acids, as shown in Figure 6: C1, 3; C2, 21; C3, 31 (disrupts ER-homodimerization domain); C4, 42 (disrupts 9th heptad); C5, 131 (deletes heptads); C6, 156 (disrupts Ti domain); C7, 238 (deletes Ti domain); C8, 275 (disrupts DBD); N1, 141 N-terminal amino acids; N2, 59 additional amino acids (deletes up to Ti domain); N3, 36 additional amino acids (deletes Ti domain). All constructs except C8 retain a 20 amino acid stretch following the DBD, containing a putative nuclear translocation signal (Evans, 1988). **B.** *In vitro* translated intact [^{35}S]H-2RIIBP or truncated polypeptides C1−C8 and N1−N3 were incubated with 10 pmol of RI (odd lanes) or RII (even lanes) oligonucleotide and insect cell extracts (1 μg) containing WT viral proteins, bH-2RIIBP (H2) or bT3Rα (T3R), as indicated (only C-terminal truncations C1−C4 are shown). The mixtures were treated with 0.25 mM DSS and analyzed by SDS−PAGE. Samples not treated with DSS were run in parallel (not shown). Small arrow, monomeric species; large arrow, dimeric species; *, putative trimeric species. **C.** Buffer- (−, lanes 1−3) or reticulocyte lysate-containing *in vitro* translated unlabeled control protein (RAR), intact H-2RIIBP or the indicated truncated polypeptides was pre-incubated with [^{35}S]T3Rα in the absence of oligonucleotide and then immunoprecipitated with antibodies (Ab) recognizing T3R (T) or H-2RIIBP (H), or with normal rabbit serum (N). Precipitated materials were analyzed by SDS−PAGE.

as in Figure 2. Intact [^{35}S]H-2RIIBP and [^{35}S]truncated polypeptides C1, C2 and C3 (deletions of 3, 21 and 31 amino acids from the C-terminus, respectively) all formed novel cross-linked complexes in the presence of bH-2RIIBP (Figure 7B). Consistent with results shown in Figure 2A, these complexes were observed only after incubation with RII DNA and had mobilities expected for a dimer of [^{35}S]H-2RIIBP and bH-2RIIBP. ^{35}S-labeled C3 shows diminished but not ablated dimerization with bH-2RIIBP, suggesting a possible role for amino acids 415−425 in homodimer stabilization. Thus, removal of the C-terminal 31 amino acids of H-2RIIBP did not eliminate homodimer formation. However, disruption of the C-terminal 'heptad' repeat (C4) or further deletion of the C-terminus (C5−C8; not shown) completely abolished homodimer formation. The lack of complex formation by N1−N3, all of which lack the DBD, is consistent with the requirement of DNA binding for stable homodimer formation (see Figures 1 and 2).

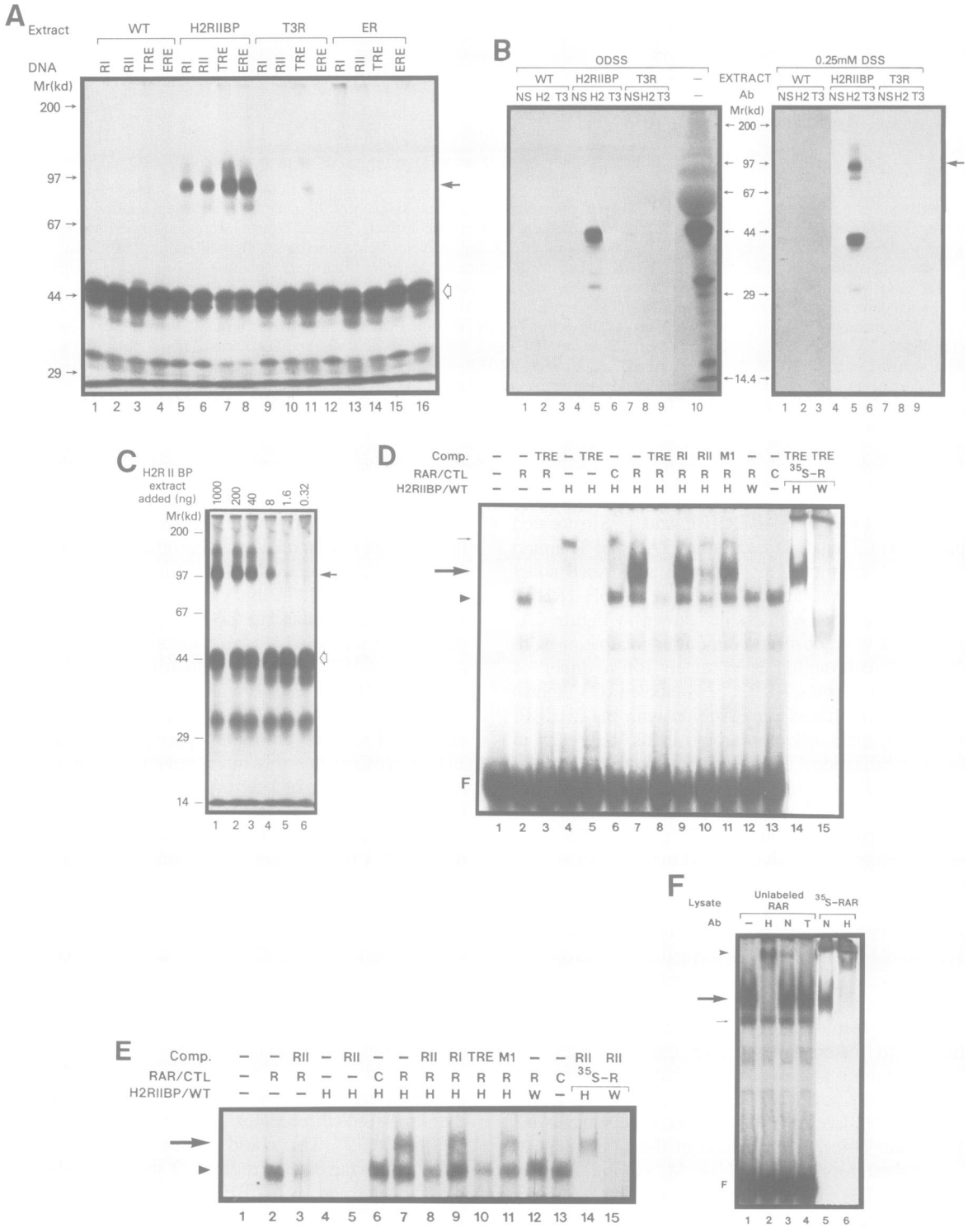
Similarly to bH-2RIIBP, bT3Rα formed cross-linked high M_r complexes with ^{35}S-labeled C-terminal deletion mutants C1−C3, but not with C4−C8 (Figure 7B and data not shown). These results suggest that the formation of H-2RIIBP/T3Rα heterodimers, like that of H-2RIIBP homodimers, requires at least an intact ninth 'heptad' repeat. Unlike bH-2RIIBP, however, bT3Rα formed cross-linked complexes with ^{35}S-labeled N-terminal deletion mutants N1 and N2. Further N-terminal deletion of the Ti domain, as in N3, abolished formation of high M_r complexes with bT3Rα (Figure 7B). Only minimal reduction of dimer formation was observed with C3, suggesting that amino acids 415−425 are less important for stabilization of heterodimers than for that of homodimers. The results of the cross-linking assay were consistent with those of a co-immunoprecipitation assay (Figure 7C). In this experiment, the ability of anti-H-2RIIBP antibodies to co-precipitate [^{35}S]T3R in the presence of unlabeled *in vitro* translated deletion mutants was assessed. [^{35}S]T3R was efficiently co-precipitated with intact H-2RIIBP, less so with mutant C2 and only poorly with mutant C4 (lanes 4−9); further C-terminal truncations eliminated specific co-immunoprecipitation (lanes 10 and 11), suggesting that this more sensitive assay could detect minimal retention of heterodimerization properties within mutant C4. This result was consistent with co-immunoprecipitation of ^{35}S-labeled deletion mutants and unlabeled T3R with anti-T3R antibodies (not shown). In addition, [^{35}S]T3R was co-precipitated with N-terminal deletion mutant N2, but not with N3 (Figure 7C, lanes 12−15). Taken together, these results indicate that the minimal region of H-2RIIBP required for heterodimerization with T3Rα consists of the Ti and dimerization domains defined by Forman and Samuels (1990). Furthermore, homodimer formation also requires at least the DBD and stabilization by amino acids 415−425.

It should be noted that all truncated [^{35}S]H-2RIIBP, except perhaps C1, consistently produced lower levels of the cross-linked complex with bT3Rα than did intact [^{35}S]H-2RIIBP. This could be due to a loss of stabilizing effects from the deleted regions. It should also be noted that several truncated H-2RIIBP polypeptides, e.g. C3 and C7, formed DSS-dependent bands with M_r higher than that

expected for dimers (asterisk in Figure 7B, lanes 19, 23 and data not shown). The migration of these bands was consistent with a trimer of each chain, suggesting that a fraction of H-2RIIBP in solution might exist in the form of homotrimers. These observations are currently under further investigation.

RARα forms more stable heterodimers with H-2RIIBP than with T3Rα

Considering the structural similarity in the Ti and dimerization domains of T3Rs and RARs (e.g. see Figure 6), we wished to determine whether RARs could also form heterodimers with H-2RIIBP. Cross-linking and co-immunoprecipitation experiments were performed as described in Figures 2 and 3 using *in vitro* translated [^{35}S]RARα. As seen in Figure 8A, addition of bH-2RIIBP to [^{35}S]RARα produced a cross-linked complex of 100 kDa (lanes 5–8), both in the presence (lane 7, TRE_p) and absence (lane 5, RI) of known specific DNA elements. No complex was observed when [^{35}S]RARα was added alone (not shown) or mixed with extracts containing WT viral proteins (lanes 1–4) or recombinant ER (lanes



13–16). Consistent with these results, [^{35}S]RARα was co-immunoprecipitated with bH-2RIIBP by anti-H-2RIIBP antibody, but not by control sera or anti-T3R antibody (Figure 8B, lanes 4–6). No immunoprecipitation of [^{35}S]RARα was detected with WT extracts. A [^{35}S]RARα–bH-2RIIBP complex was also observed in the absence of DNA by both cross-linking (see below) and co-immunoprecipitation assays (not shown). These results confirm that RARα forms a stable heterodimer with H-2RIIBP in solution independently of the presence of target DNA.

With bT3Rα, [^{35}S]RARα formed a ~100 kDa cross-linked complex only in the presence of TRE$_p$ (Figure 8A, lanes 9–12). The intensity of this complex was far less than that seen with bH-2RIIBP (lanes 5–8). Furthermore, anti-T3R antibody failed to co-immunoprecipitate [^{35}S]RARα in the presence of bT3Rα (Figure 8B, lanes 9). These results suggest that T3Rα and RARα did not heterodimerize to an appreciable level. This lack of detectable heterodimerization was not due to lower levels of available bT3Rα in extracts relative to bH-2RIIBP (Hallenbeck,P.L., Marks,M.S., Lippoldt,R.E., Ozato,K. and Nikodem,V., submitted). Furthermore, [^{35}S]RARα was incorporated into an observable cross-linked heterodimeric complex even when bH-2RIIBP was diluted >600-fold (Figure 8C, bH-2RIIBP concentration estimated to be 0.1 nM; in experiments not shown, similar concentrations of bT3Rα formed heterodimeric complexes with [^{35}S]H-2RIIBP). We conclude that both RARα and T3Rα form more stable heterodimers with H-2RIIBP than they do with each other.

We next tested whether RARα, like T3Rα (Figure 4A), could synergize with H-2RIIBP in binding to TRE$_p$ and RII. As shown in Figure 8D, reticulocyte lysate containing a control *in vitro* translated protein produced a shift in the migration of the TRE$_p$ probe (lane 13); lysate containing RARα alone produced no additional shifted band (lane 2). However, addition of bH-2RIIBP to RARα (lane 7) but not control lysates (lane 6) resulted in the appearance of a new band. This band was not observed upon addition of an extract containing WT baculovirus proteins (lane 12). The band was competed by excess unlabeled TRE$_p$ or RII (lanes 8 and 10) but not by mut-RII (lane 11) or the unrelated RI oligonucleotide (lane 9). Furthermore, the retarded probe comigrated with [^{35}S]RARα that had been pre-incubated with bH-2RIIBP and excess unlabeled TRE$_p$ and analyzed on the same gel (lanes 14,15). Similar results were obtained using RII as a probe (Figure 8E), although bands produced by endogenous factors present in the reticulocyte lysate were more prominent than the heterodimer-specific band (not shown). The RARα/H-2RIIBP specific band was eliminated by addition of an antibody specific for H-2RIIBP, but not by a T3R-specific antibody or by a control serum (Figure 8F, lanes 1–4), clearly demonstrating that b-H2RIIBP was included in the retarded band. The anti-H-2RIIBP antibody also eliminated the band observed with [^{35}S]RARα with bH-2RIIBP and unlabeled probe (Figure 8F, lanes 5 and 6). These results show that H-2RIIBP synergizes with RARα, as it does with T3Rα, in binding to DNA recognition elements.

H-2RIIBP functionally synergizes with T3Rα and RARα to activate transcription of the MHC class I promoter in vivo

In order to assess the potential functional significance of heterodimerization *in vivo*, the promoter activity of a MHC class I gene was examined in a co-transfection assay using a mammalian expression vector for H-2RIIBP combined with an expression vector for either T3Rα or RARα. The reporter for promoter activity consisted of the chloramphenicol acetyltransferase (CAT) gene fused to a 1.4 kb upstream region of the MHC class I gene, H-2L^d (Miyazaki *et al.*, 1986). H-2RIIBP has been shown to activate transcription of this reporter through RII in an RA-dependent manner after cotransfection into a human embryonal carcinoma cell line, N-Tera2 (Nagata *et al.*, 1992). The results of transfection experiments are summarized in Figure 9. In agreement with previous results, co-transfection of N-Tera2 cells with the CAT reporter and H-2RIIBP alone resulted in a 3- to 4-fold RA-dependent enhancement of CAT activity (Figure 9A). Co-transfection of the reporter with RARα or T3Rα without H-2RIIBP also resulted in a 3- to 5-fold enhancement of CAT activity that was dependent on RA or T3, respectively (Figure 9A). However, when H-2RIIBP was co-transfected together with either RARα or T3Rα, CAT activity was increased by 15- to 30-fold over background (Figure 9A). This enhancement was dependent only on the ligand of the co-transfected receptor; i.e. the enhancement produced by the combination of RARα and H-2RIIBP required RA, whereas the enhancement generated by the combination of T3Rα and H-2RIIBP required T3. It is noteworthy that RA had no effect on CAT activity when H-2RIIBP and T3Rα

Fig. 8. H-2RIIBP forms heterodimers with RARα. **A** and **B**. *In vitro* translated [^{35}S]RARα was incubated with 10 pmol of the indicated oligonucleotide (A) or RII (B) and insect cell extracts (A, 1 μg; B, 125 ng) containing bH-2RIIBP (A, lanes 5–8; B, lanes 4–6), bT3Rα (A, lanes 9–12; B, lanes 7–9) or WT viral proteins alone (A, lanes 1–4; B, lanes 1–3) or with added partially purified ER (A, lanes 13–16). Mixtures were either untreated (B, left; 0 DSS) or treated with 0.25 mM DSS (A, B right). In panel A, samples were analyzed directly by SDS–PAGE: empty arrowhead, monomer; arrow, dimer. In panel B, samples were immunoprecipitated with control serum (NS; lanes 1, 4 and 7), anti-H-2RIIBP antibody (H2; lanes 2, 5 and 8) or anti-T3Rα antibody (T3; lanes 3, 6 and 9) prior to SDS–PAGE. Lane 10 contains a sample of [^{35}S]RARα without precipitation. Arrow, dimer in DSS-treated samples. **C**. [^{35}S]RARα was incubated with the indicated amount of bH-2RIIBP extract in the absence of oligonucleotides and treated with 0.25 mM DSS before SDS–PAGE. Open arrowhead, monomer; arrow, dimer. **D** and **E**. Gel mobility shift analysis with RARα. Buffer (lanes 1, 4 and 5) or reticulocyte lysate containing *in vitro* translated unlabeled control protein (ICSBP; C, lanes 6 and 13) or RARα (R, lanes 2, 3, 7–12) or [^{35}S]RARα (^{35}S-R, lanes 14–15) was incubated with buffer (–, lanes 1–3, 13) or insect cell extract containing wild type viral proteins (W, lanes 12 and 15) or bH-2RIIBP (H, lanes 4–11, 14) and ^{32}P-labeled (lanes 1–13) or unlabeled (lanes 14 and 15) oligonucleotide probes TRE$_p$ (D) or RII (E). Binding was analyzed by gel mobility shift assay. Unlabeled specific oligonucleotides (Comp.) TRE$_p$ (D, lanes 3, 5 and 8; E, lane 10), RII (D, lane 10; E, lanes 3, 5 and 8), or non-specific oligonucleotides RI (lane 9) or Mut-RII (M1, lane 11) were added as competitors. Arrowhead, migration of unrelated reticulocyte lysate factor; small arrow, migration of putative bH-2RIIBP homodimer; large arrow, migration of bH-2RIIBP/RARα heterodimer; F, free probe. Due to multiple non-specific reticulocyte lysate bands with the RII probe, only the relevant portion of the autoradiogram is shown in panel E. **F.** Effect of antibodies on gel mobility shift. *In vitro* translated unlabeled (lanes 1–4) or [^{35}S]RARα (lanes 5 and 6) was incubated with bH-2RIIBP and ^{32}P-labeled (lanes 1–4) or unlabeled (lanes 5 and 6) TRE$_p$. After incubation for 1 h, control rabbit serum (N, lanes 3, 5) or antibody (Ab) specific for H-2RIIBP (H, lanes 2, 6) or T3Rα (T, lane 4) were added, and after an additional hour, products were analyzed by PAGE. Large arrow, bH-2RIIBP/RARα heterodimer; small arrow, non-specific reticulocyte lysate band; arrowhead, non-specific band produced by antibody alone (not shown); F, free probe.

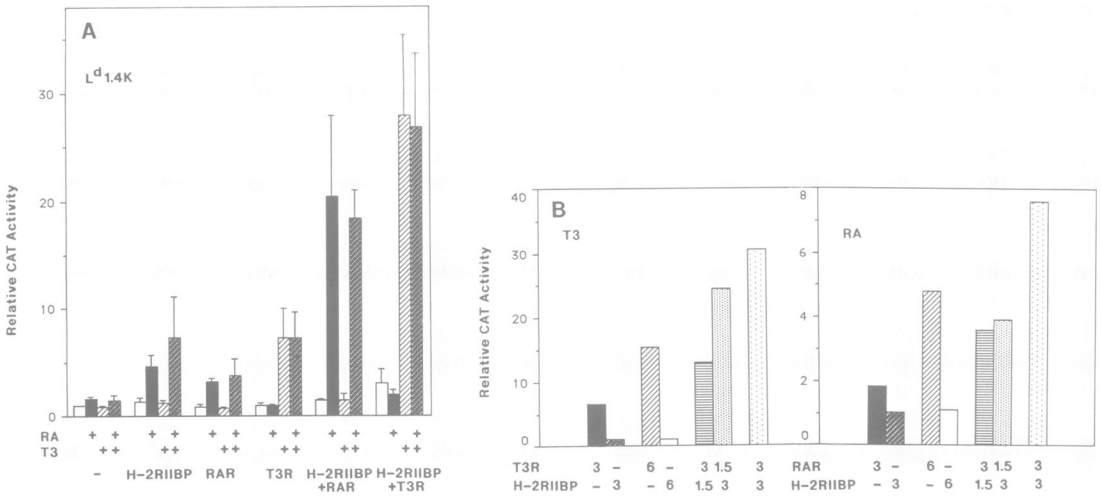


Fig. 9. H-2RIIBP synergizes with either T3Rα or RARα in activating transcription from the MHC class I promoter. **A.** Ligand specificity. N-Tera2 cells were transfected with the pL^{d}1.4K CAT reporter plasmid (2.5 μg) and expression plasmids for the indicated receptors (5 μg), and cultured with or without RA (10^{-5} M) or T3 (10^{-7} M) for 36 h. Normalized CAT activities are expressed relative to the activity produced by pL^{d}1.4K co-transfected with the control pRSV without RA or T3 treatment (value defined as 1). Values represent means of three independent determinations ± standard error. **B.** Titration of H-2RIIBP, T3Rα and RARα. The indicated amount (μg) of expression plasmids for H-2RIIBP and T3Rα (left) or RARα (right) were cotransfected with 2 μg of pL^{d}1.4K CAT as above, and cells were cultured in the presence of T3 or RA, respectively. Activities are expressed relative to the activity produced upon co-transfection of 3 μg of H-2RIIBP alone for each condition. Values represent the average of duplicate experiments.

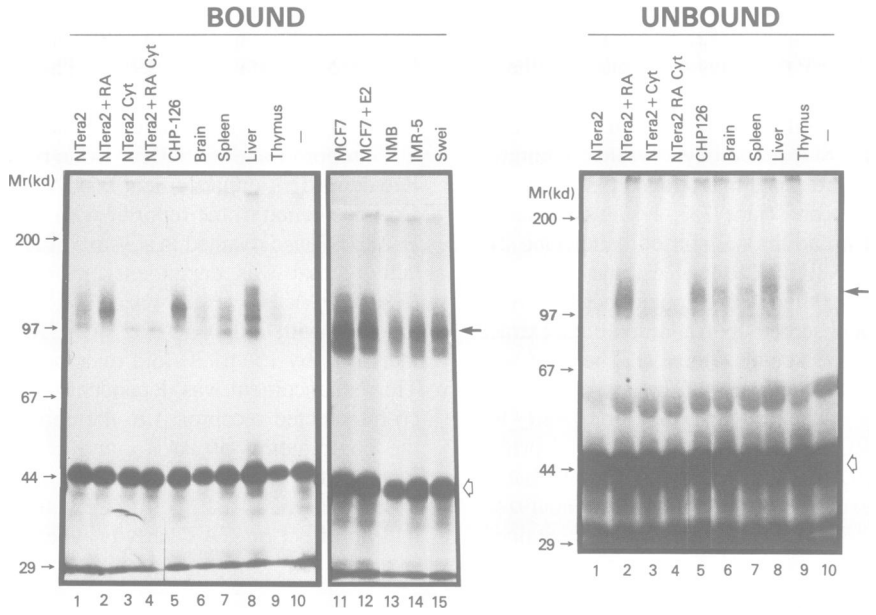


Fig. 10. H-2RIIBP forms RII binding heterodimers with proteins present in mammalian nuclear extracts. [^{35}S]H-2RIIBP was incubated with 10 pmol biotinylated RII oligonucleotide and 15 μg of nuclear or cytoplasmic (Cyt) extracts from the indicated cell lines or tissues. Mixtures were treated with 0.25 mM DSS and then fractionated by precipitation with streptavidin-agarose. Precipitated pellets (Bound) and supernatants (Unbound) were analyzed by SDS–PAGE. N-Tera2, an RA-inducible human embryonal carcinoma cell line (Andrews, 1984); lanes 2 and 4 represent extracts from N-Tera2 cells treated for 4 days with RA (5×10^{-5} M). CHP-126, NMB and IMR-5, human neuroblastoma cell lines; Swei, human Epstein–Barr virus transformed B lymphoblastoid cell line; MCF-7, human breast carcinoma cell line; MCF-7+E2, MCF-7 treated for 3 days with estradiol (10^{-7} M); brain, spleen, liver, and thymus, from female Balb/c mice; –, no extract added. Open arrowhead, [^{35}S]H-2RIIBP monomer; arrow, putative dimeric species. Monomeric [^{35}S]H-2RIIBP was precipitated with a non-specific biotinylated oligonucleotide, but dimeric species were not (not shown).

were co-transfected. Similarly, T3 had no effect when H-2RIIBP and RARα were co-transfected (Figure 9A). Functional synergism with H-2RIIBP was also observed in titration experiments (Figure 9B). The CAT activity for co-transfected receptors was clearly greater than the activities of either protein alone, although a greater level of synergistic enhancement was observed with T3Rα than with RARα (Figure 9B). These results show that H-2RIIBP enhances the ligand-dependent transactivation of MHC class I genes by either T3Rα or RARα, and suggest that H-2RIIBP heterodimers are functionally active (see Discussion). Furthermore, the lack of an observable effect on CAT activity by RA when H-2RIIBP and T3Rα were co-transfected suggests that either the RA-dependent

transactivation function in H-2RIIBP is masked upon interaction with T3Rα, or that the effect of RA on the activity of H-2RIIBP by itself may not be due to direct binding of RA or its derivative (see below).

H-2RIIBP forms heterodimers with endogenous proteins present in mammalian nuclear extracts

If heterodimerization of H-2RIIBP is physiologically relevant, H-2RIIBP would be expected to bind to target DNA in association with nuclear proteins expressed in mammalian cells. This hypothesis was tested by examining (i) whether [^{35}S]H-2RIIBP could form cross-linked complexes with a component in nuclear extracts from mammalian cells and (ii) whether these complexes could bind to RII. Extracts from a variety of human cell lines and adult mouse tissues were mixed with [^{35}S]H-2RIIBP and biotinylated RII, and analyzed by chemical cross-linking coupled with streptavidin−agarose precipitation (Figure 10, 'Bound'). Little to no RII binding high M_r cross-linked complex was observed with [^{35}S]H-2RIIBP when cell extracts were not added (lane 10). However, incubation with nuclear extracts from all cell lines and tissues examined resulted in RII binding cross-linked complexes with varying mobilities of M_r 97−122 000. Many of these complexes were also present in the fraction that did not associate with RII DNA (Figure 10, 'Unbound') and in similar samples incubated with a control oligomer (not shown), suggesting that they are formed in solution in the absence of target DNA and that some may not bind to RII. Incubations with mouse liver nuclear extracts produced the most diverse pattern of bands (lane 8), with M_r ranging from 92 000 to 135 000, suggesting the presence of several distinct factors capable of complexing with [^{35}S]H-2RIIBP and subsequently binding to RII. Cytoplasmic fractions from several cell lines generated no specific high M_r cross-linked complexes (Figure 10, lanes 3 and 4 and data not shown). In addition, no high M_r complexes were precipitated by streptavidin−agarose after incubation with a control biotinylated oligomer (RI; not shown). It is notable that extracts from RA-treated N-Tera2 cells generated a novel 107 kDa cross-linked complex that was not generated with untreated cell extracts (compare lanes 1 and 2 in Figure 10). This result raises the possibility that RA induction of a new heterodimer partner accounts for the RA dependence of H-2RIIBP transactivation of MHC class I promoters seen in N-Tera2 cells (Figure 9A; Nagata *et al.*, 1992).

Discussion

Heterodimerization is a general mechanism by which gene expression can be regulated by a small number of transcription factors during development and in response to multiple cellular signalling pathways. We show here that H-2RIIBP may be capable of conferring combinatorial diversity on the control of gene expression by nuclear hormone receptors in response to retinoic acid, thyroid hormone and perhaps other ligands.

Homodimerization of H-2RIIBP

We show that H-2RIIBP exists free in solution primarily as a monomer, with low levels of dimeric and perhaps trimeric species (Figures 1, 2A and 7B). Levels of the dimeric complex were higher in the presence of all target DNA elements examined, including MHC class I region II, malic enzyme gene and artificial palindromic TREs, and the vitellogenin A2 and B1 gene EREs (Figures 1A and 2A, and results not shown); furthermore, only dimers bound to DNA (Figure 1B). *In vitro* translated [^{35}S]H-2RIIBP formed dimeric complexes only when mixed with excess bH-2RIIBP (Figures 2A and 7), demonstrating that the complexes were homodimers and that their formation required high concentrations of monomers (>10 nM; not shown). These data and the results of gel shift analysis (Figure 4) establish a strong correlation between H-2RIIBP homodimer formation and target DNA binding. This is consistent with reports for other nuclear hormone receptors, such as glucocorticoid, estrogen and progesterone receptors, that bind to their respective hormone response elements as dimers (Kumar and Chambon, 1988; Tsai *et al.*, 1988; DeMarzo *et al.*, 1991). Similarly, oligomerization of T3Rs is required for and induced by binding to some TREs (Holloway *et al.*, 1990; Williams *et al.*, 1991), although T3R monomers can bind to others (Holloway *et al.*, 1990; Lazar *et al.*, 1991; Selmi and Samuels, 1991).

Heterodimerization of H-2RIIBP with T3Rα and RARα

We show that H-2RIIBP forms a stable heterodimer with either T3Rα or RARα (Figures 2, 3 and 8). Heterodimerization of H-2RIIBP with these receptors was not dependent on target DNA (Figures 2 and 8) and was detected with a ~100-fold lower concentration of H-2RIIBP than that required to detect homodimerization (Figure 8C and data not shown). Furthermore, heterodimers bound to DNA elements much more avidly than did putative homodimers (Figures 4 and 8D, E; Hallenbeck *et al.*, submitted). While it is possible that H-2RIIBP homodimerization could be enhanced by ligand binding (Kumar and Chambon, 1988) or by post-translational modification, these results suggest that H-2RIIBP heterodimers are more likely to occur in the natural cellular environment and to be of physiological relevance than are homodimers.

Several investigators (Forman *et al.*, 1989; Glass *et al.*, 1989; Lucas *et al.*, 1991) have demonstrated heterodimer formation between T3Rα and RARα when bound to target DNA. Our experiments indicate that heterodimers of either receptor with H-2RIIBP differ qualitatively from the T3Rα/RARα heterodimer. An association between T3Rα and RARα could not be detected by chemical cross-linking or co-immunoprecipitation assays under our experimental conditions, in which their association with H-2RIIBP was readily apparent (Figures 2, 3 and 8). An association between T3Rα and RARα was observed only in the presence of specific target DNA (Figure 8A and data not shown), much like the H-2RIIBP homodimer. To our knowledge, a direct RARα−T3Rα association in the absence of DNA has yet to be demonstrated. Thus, heterodimerization with H-2RIIBP most likely represents a higher affinity interaction than T3Rα/RARα heterodimerization. The T3Rα/RARα heterodimer may be qualitatively more similar to T3Rα homodimers (Lazar *et al.*, 1991) than to H-2RIIBP-containing heterodimers.

Both T3Rs and RARs have been shown to interact with multiple distinct nuclear proteins, or accessory factors, present in a variety of cell types (Murray and Towle, 1989; Burnside *et al.*, 1990; Glass *et al.*, 1990; Lazar and Berrodin, 1990; Darling *et al.*, 1991; Yang *et al.*, 1991). The accessory factors enhance T3R and RAR binding to

DNA, and may be necessary for full transcriptional activity *in vivo* (Hudson *et al.*, 1990; Darling *et al.*, 1991; O'Donnell *et al.*, 1991; Selmi and Samuels, 1991; Spanjaard *et al.*, 1991). Our data indicate that H-2RIIBP may be one such accessory factor. H-2RIIBP shares a number of characteristics with some of these factors, such as its intrinsic affinity for DNA (Darling *et al.*, 1991) and molecular size (Lazar *et al.*, 1991), but differs in some respects from all of the well described accessory factors (Beebe *et al.*, 1991; Darling *et al.*, 1991; Lazar *et al.*, 1991).

Dimerization of RARs and T3Rs both with each other and with accessory factors has been shown to be dependent on two broad sub-regions of the LBD, the Ti and dimerization domains (Forman *et al.*, 1989; Darling *et al.*, 1991; Spanjaard *et al.*, 1991). The latter consists of up to nine specifically spaced 'heptads' with characteristic hydrophobic residues at positions 1, 5 and 8. Alignment of the LBDs of H-2RIIBP, RARα, T3Rα and several other T3R/RAR subgroup members in Figure 6 shows that all members of this subgroup are structurally similar in both Ti and dimerization domains. We show here that disruption of either of these two domains in H-2RIIBP inhibits its ability to form heterodimers with T3Rα (Figure 7). Thus, the physical requirements for the formation of H-2RIIBP/T3Rα dimers closely parallel those observed for the formation of other heterodimers within the T3R/RAR subgroup, and correlate with previously described functional effects of mutations in RARs and T3Rs (Glass *et al.*, 1989; Graupner *et al.*, 1989; Hudson *et al.*, 1990; O'Donnell *et al.*, 1991; Selmi and Samuels, 1991; Spanjaard *et al.*, 1991).

Our data show that heterodimerization of H-2RIIBP with T3Rα results not only in quantitatively enhanced DNA binding (Figure 4), but also qualitative changes in the protein–DNA contacts, as evidenced by methylation interference data (Figure 5). The enhanced binding could well be a result of an altered protein–DNA interaction reflected by the different patterns of methylation interference. In this regard, it is striking that H-2RIIBP/T3Rα heterodimer binding was blocked by methylation of fewer G residues in RII DNA than homodimer binding, in agreement with methylation interference data for a T3Rα/liver factor heterodimer on TRE_p (Lazar *et al.*, 1991). The extended interference pattern for the homodimer may reflect a requirement for contact with more nucleotides to stabilize lower affinity interactions. Heterodimerization could alter the conformation of each partner such that the DBD is re-oriented to reveal a binding site with higher affinity than that present in homodimers. On the other hand, the enhanced DNA binding by the heterodimer may reflect only an apparent increase in affinity for DNA that is more directly related to the increased concentration of dimers relative to monomers. As illustrated in Figure 11, one may presume that (i) a monomer is favored when H-2RIIBP is present alone, (ii) dimers are favored when H-2RIIBP is present together with heterodimer partners, (iii) the DNA element binds only to pre-formed dimeric species, and thus (iv) more DNA is bound when heterodimers can be formed, resulting in apparently higher affinity. Most likely, enhanced DNA binding by the heterodimer reflects features of all of the above models.

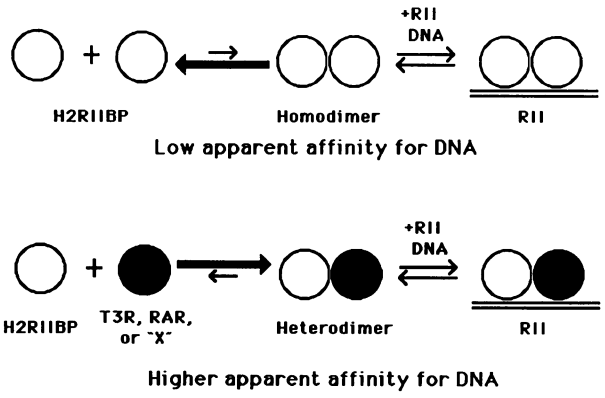


Fig. 11. Model for enhanced DNA binding by H-2RIIBP heterodimers relative to homodimers. Dynamic equilibria favor H-2RIIBP monomers over homodimers, but also favor H-2RIIBP heterodimers over monomers. RII DNA is shown to have high affinity for any dimeric species. DNA-bound complexes are more abundant for the heterodimer than the homodimer because the former is present at a higher initial concentration than the latter. 'X' indicates unknown proteins present in mammalian nuclear extracts.

Functional significance of H-2RIIBP heterodimers in vivo

We have previously shown that H-2RIIBP can mediate RA-dependent transcriptional activation of the MHC class I promoter *in vivo* (Nagata *et al.*, 1992). We show here for the first time that RARα and T3Rα also evoke mild ligand-dependent transcriptional enhancement from this promoter (Figure 9A), demonstrating that not only RA but also T3 can exert control of MHC class I gene expression. These transactivation events may require interactions of the transfected receptors with limiting amounts of endogenous H-2RIIBP present in N-Tera2 cells. Furthermore, we show that co-transfection of H-2RIIBP with RARα and more dramatically with T3Rα synergistically activates transcription from the MHC class I promoter (Figures 9A and B). Thus, H-2RIIBP potentiates not only DNA binding but also transcriptional activity of T3Rα and RARα. In agreement with these data, we have observed that H-2RIIBP potentiates T3-dependent transcriptional enhancement mediated by T3Rα through the malic enzyme TRE (Hallenbeck *et al.*, submitted). Our results are also consistent with the correlation observed among T3Rα and RARα deletion mutants between transcriptional activity and ability to bind accessory factors (Selmi and Samuels, 1991; O'Donnell *et al.*, 1991; Glass *et al.*, 1990).

The synergism of MHC class I transactivation observed by H-2RIIBP with T3Rα or with RARα is probably due to the action of heterodimers rather than to the independent action of two receptors. This is most strongly supported by the characteristic unilateral ligand dependence of synergistic enhancement. With T3Rα and H-2RIIBP, transcription was dependent only on T3, whereas with RARα and H-2RIIBP, it was dependent only on RA. Thus, in the presence of these other receptors, H-2RIIBP itself showed no apparent ligand dependence. Such unilateral ligand specificity is not readily explained by independent actions of each partner, and stands in contrast to the dependence of RARα/T3Rα synergism on ligands for both receptors (Glass *et al.*, 1989; Forman *et al.*, 1989; Hudson *et al.*, 1990). Similar RA independence may be expected for some actions of RXRα, which is highly homologous to H-2RIIBP (Mangelsdorf *et al.*, 1990). Nur77 and COUP transcription factor, two other 'orphan receptors' in the RAR/T3R subfamily, have also been shown to enhance transcriptional activity in an apparently ligand-independent manner (Power *et al.*, 1991; Davis *et al.*, 1991).

The fact that H-2RIIBP synergism is RA independent in the presence of T3Rα suggests either that the retinoid-dependent transactivation domain in H-2RIIBP is masked upon interaction with T3Rα or that a mechanism other than an RA/H-2RIIBP ligand−receptor interaction may be responsible for the previously described RA-dependent H-2RIIBP activity (Nagata *et al.*, 1992; Figure 9A). This RA dependence could reflect the ligand requirement of a heterodimer partner, such as an RAR. On the other hand, H-2RIIBP association with a factor that is expressed only after RA treatment of N-Tera2 cells (Figure 10) raises the possibility that the role of RA is to induce the expression of a novel heterodimer partner. This hypothesis is currently under examination.

Conclusions

The ability of H-2RIIBP to form heterodimers with RARα or T3Rα *in vitro*, its apparent ligand-independent synergistic transcriptional activity and its ability to associate with a variety of nuclear proteins suggest that H-2RIIBP may have pleiotropic effects on a variety of hormone-responsive genes. H-2RIIBP is likely to be capable of forming heterodimers with a variety of receptors in addition to RARα and T3Rα, resulting in more diverse patterns of DNA binding specificity and hormone responsiveness. In addition, as suggested by the changes in protein−DNA contacts upon heterodimerization (Figure 5), heterodimers are likely to have altered specificity for different hormone response elements. We speculate that the primary function of H-2RIIBP is to heterodimerize with other receptors and to modulate their activity, and thus to act as an accessory factor rather than (or in addition to) as a classical ligand receptor. Other 'orphan receptors' of the nuclear hormone receptor superfamily could conceivably have similar roles, perhaps corresponding to previously described accessory factors. Each accessory factor may be specific for dimerization with particular receptors. Thus, the availability of H-2RIIBP and other potential heterodimer partners within the cell provides a combinatorial mechanism by which increased diversity and versatility is imparted on the function of nuclear hormone receptors and thereby on hormone responses. Similar combinatorial mechanisms appear to operate widely among transcription factors, including members of the leucine zipper and the c-*rel* oncogene family (Nakabeppu *et al.*, 1988; Landschultz *et al.*, 1989; Ballard *et al.*, 1990; Hai and Curran, 1991; Logeat *et al.*, 1991; Nolan *et al.*, 1991). The data shown here provide a framework upon which the roles of other orphan receptors can be scrutinized.

During revision of this manuscript, Yu *et al.* (1991) reported observations similar to those presented here, describing dimer formation and synergistic binding of RXRβ (H-2RIIBP) with RARα, T3Rα, and the vitamin D receptor on hormone response elements, and transcriptional activation of chimeric reporters. Their data and conclusions are fully consistent with those presented here.

Materials and methods

Recombinant murine bH-2RIIBP, rat bT3Rα and human ER expressed in baculovirus vectors

Construction of a recombinant baculovirus harboring a full length mouse H-2RIIBP cDNA and preparation of nuclear extracts containing bH-2RIIBP by the modified Dignam method have been described elsewhere (Marks *et al.*, 1992). Affinity purified rabbit antibody directed to a synthetic peptide corresponding to a 22 amino acid sequence of the D domain of H-2RIIBP has also been described (Marks *et al.*, 1992). A recombinant human T3Rα in a baculovirus vector and specific rabbit antibody will be described elsewhere (Hallenbeck *et al.*, submitted). Concentrations of bH-2RIIBP or bT3Rα in the extracts were estimated as described (Hallenbeck *et al.*, submitted). Partially purified cytoplasmic extracts from Sf9 cells infected with the recombinant ER baculovirus (Brown and Sharp, 1990) were kindly given by Dr Myles Brown (Dana Farber Cancer Research Center, Boston, MA).

Plasmids

cDNA clones for mouse H-2RIIBP in pBS(+) (Hamada *et al.*, 1989), human RARα in pTZ19R (Petkovich *et al.*, 1987) and rat T3Rα1 in pSP72 (Petty *et al.*, 1990) have been described. The following C-terminal deletion mutants of H-2RIIBP were generated by *in vitro* transcription/translation of the parent plasmid that had been digested with the indicated restriction endonucleases (Boehringer Mannheim): C1, *Nhe*I; C4, *Mae*II; C5, *Sno*I; C6, *Sac*I; C7, *Mst*II; C8, *Sca*I. The remaining deletion mutants, C2, C3, N1, N2 and N3, were generated by *in vitro* transcription/translation of H-2RIIBP deletion plasmids constructed using the polymerase chain reaction (PCR). Oligonucleotide primers containing nested restriction enzyme sites were used to generate H-2RIIBP inserts containing the desired deletions and *Nco*I sites on both ends. These inserts were subcloned into the *Nco*I site of the parental plasmid from which the original *Nco*I insert had been removed. To confirm the accuracy of deletion, all deletion constructs were sequenced by the dideoxy method using a T7 polymerase kit (Pharmacia). All constructs had the expected deletions with no other changes, except for N1 which had a substitution of Thr for Met16. This alteration, however, did not affect the aim of the experiments.

In vitro translation

H-2RIIBP cDNA in pBS(+) and the deletion mutants C2, C3, N1, N2 and N3 were linearized with *Eco*RI and then transcribed *in vitro* into capped mRNA using the mCAP kit (Stratagene) with T3 polymerase; for other deletion mutants, H-2RIIBP cDNA was linearized with the enzymes described above; *Eco*RI and T7 polymerase were used for RARα; and *Sal*I and T7 polymerase for T3Rα. Purified mRNA was translated *in vitro* using rabbit reticulocyte lysates (Promega) according to Halazonetis *et al.* (1988) in the presence of [^{35}S]methionine (New England Nuclear). For H-2RIIBP deletion mutants C5, C6, C7 and C8, [^{35}S]cysteine was substituted for [^{35}S]methionine. In some experiments, free label was removed from the translated product by spun column gel filtration chromatography on Sephadex G-25 (5′/3′) into buffer B (20 mM HEPES pH 7.9, 50 mM KCl, 1 mM EDTA, 10% glycerol). In some experiments, mRNA was translated in the absence of [^{35}S]methionine and in the presence of 20−30 μM unlabeled methionine.

Chemical cross-linking

Recombinant proteins present in insect cell nuclear extracts (1.6 ng−1 μg protein) and/or obtained from *in vitro* translation reactions (20−50 000 c.p.m.; ~0.5−3 fmol receptor) were combined in a total of 50 μl of buffer A (20 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 0.05% Triton X-100) in the absence or presence of 0.05−0.2 μM double stranded oligonucleotides. Reactions were incubated at 4°C for 60 min and then 1 μl of DSS (Pierce Chemical Co.), freshly dissolved in dimethyl sulfoxide (Sigma), was added at various concentrations. Dimethyl sulfoxide alone was added as a control. After an additional 30 min at 4°C, 1 μl of 1 M NH_4Cl was added to quench unreacted DSS and incubation was continued for an additional 5 min. Unless otherwise stated, products were analyzed directly by SDS−PAGE. Oligonucleotides were not cross-linked under these conditions, since migration of ^{32}P-labeled probes in SDS−PAGE did not change following addition of DSS.

Streptavidin−biotin-coupled DNA precipitation assay

Oligonucleotides analogous to those indicated in Table I were synthesized with a biotin group on the 5′ nucleotide as described (Cocuzza, 1989). Deprotected oligonucleotides were purified by reversed phase HPLC using a C_{18} column (DuPont) with a gradient of 5−30% acetonitrile in 0.1 M triethylammonium acetate pH 7.0, and purity was assessed by mass spectrometry. For analysis of homodimerization, nuclear extracts (100 ng protein) containing bH-2RIIBP were added to 0.1 μM biotinylated oligonucleotide in 50 μl buffer A, and subjected to chemical cross-linking with DSS as described above. For analysis of heterodimerization with mammalian cell extracts, 1 μl [^{35}S]H-2RIIBP was combined with 15 μg nuclear or cytoplasmic extract and 0.1 μM biotinylated oligonucleotide in 50 μl buffer A, and then treated with DSS. After quenching excess DSS, bovine serum albumin (BSA; 0.05%) and poly(dI−dC):poly(dI−dC) (50 μg/ml; Pharmacia) were added and samples were incubated with 5−10 μl pre-washed streptavidin−agarose beads (Sigma) for 2 h at 4°C. Beads were

pelleted and washed four times with buffer A containing 0.05% (w/v) BSA. Bound materials were eluted into SDS−PAGE sample buffer at 100°C for 10 min. Bound and unbound materials were separated by SDS−PAGE on 8% polyacrylamide gels. For analysis of homodimerization, gels were transferred to reinforced nitrocellulose (Schleicher & Schuell) and analyzed by Western blotting using the antibodies specific for H-2RIIBP.

SDS−PAGE, fluorography, and Western blotting

SDS−PAGE in 8, 10.5 or 12.5% polyacrylamide gels was performed as described (Laemmli, 1970). For fluorography, gels were fixed for at least 30 min, rinsed for 15 min with H_2O and impregnated for 30 min with Enlightning (New England Nuclear) prior to drying and exposure to film. Western blotting was performed as described (Marks *et al.*, 1992; Towbin *et al.*, 1979).

Immunoprecipitation

For interactions with intact H-2RIIBP, nuclear extracts containing WT baculoviral proteins, bH-2RIIBP or bT3Rα (125 ng protein) were incubated with ^{35}S-labeled *in vitro* translated proteins in 100 μl buffer A containing 0.05 μM RII oligonucleotide and 0.5% BSA for ~60 min at 4°C; half of the reaction was cross-linked with 0.25 mM DSS. The reaction mixtures were incubated with 2 μl of antibodies specific for H-2RIIBP or T3Rα or normal rabbit serum for 16 h at 4°C. For interactions with deletion mutants of H-2RIIBP, 5 μl of unlabeled *in vitro* translated H-2RIIBP construct (previously determined by Western blotting to contain similar molar amounts of protein) were incubated with ^{35}S-labeled *in vitro* translated T3Rα without added oligonucleotides. Antibodies were added as above. Samples were further incubated with pre-washed protein A−agarose beads (15 μl; Boehringer Mannheim) for an additional 2 h. Beads were washed three times in buffer A containing 0.1% (w/v) BSA followed by another wash in buffer A without BSA. Bound materials were eluted in SDS−PAGE sample buffer and analyzed by SDS−PAGE on 8% polyacrylamide gels.

Gel mobility shift analysis

The gel mobility shift assay with bH-2RIIBP and bT3R was performed essentially as described by Hamada *et al.* (1989). Briefly, insect cell extracts containing WT viral or recombinant proteins (5 μg protein/ extract) were incubated with ^{32}P-labeled probes (~$50-100\times10^3$ c.p.m., 0.02−0.1 pmol oligonucleotides) in a 20 μl reaction containing 20 mM HEPES pH 7.9, 75−80 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.2 mg/ml poly(dI−dC):poly(dI−dC) for 45 min at 4°C. Unlabeled competitor oligonucleotides (40 pmol) were added where indicated. Reactions were fractionated on 4% polyacrylamide gels in 1×TBE buffer. For bH-2RIIBP/RARα interaction and antibody perturbation assays, a more sensitive gel mobility shift procedure was utilized, as described (Hallenbeck *et al.*, submitted). Insect cell extracts containing wild type or recombinant proteins (0.2 μg protein/extract) were incubated in some cases with reticulocyte lysates programmed with mRNA encoding RARα or a control protein (ICSBP; Driggers *et al.*, 1990) and unlabeled competitor oligonucleotides (5 pmol) where indicated in a 20 μl reaction containing 22.5 mM HEPES pH 7.9, 2.6 mM $MgCl_2$, 11.3% glycerol, 50 mM KCl, 0.125 mM EDTA, 1.25 mM sodium pyrophosphate, 0.25 mM sodium orthovanadate, 6.25 mM sodium fluoride, 0.5 mM DTT and 0.05−0.1 μg/ml poly(dI−dC):poly(dI−dC). In some experiments, [^{35}S]RARα was substituted for unlabeled lysate, and no radiolabeled probe was added. Probe (50 000 c.p.m.; 20 fmol) was added after 10 min on ice and incubation was continued for 2 h at 4°C before electrophoresis on 5% polyacrylamide gels in 0.5×TBE buffer at 4°C. To test the effect of antibodies, 2 μl of phosphate buffered saline, non-specific rabbit serum, or antibodies specific for H-2RIIBP or T3Rα were added to the reaction mixture after 1 h of incubation.

Methylation interference

RII or ERE-B1 oligonucleotides ^{32}P-labeled on one strand only were treated with dimethyl sulfate for 2 min at room temperature. Following ethanol precipitation, DNA ($1-2\times10^6$ c.p.m.) was incubated with insect cell extracts (3−5 μg protein) containing bH-2RIIBP and bT3R as described above for the gel shift assay. Following resolution by non-denaturing PAGE, unbound and shifted bands were eluted into 2−3 ml of 10 mM Tris, 1 mM EDTA and precipitated by addition of hexadecyl trimethylammonium bromide (CTAB; 0.1 mM) and ammonium sulfate (15 mM) on ice for 15 min. Pellets were dissolved in TE and extracted with phenol, re-precipitated with ethanol and cleaved with 10% piperidine. Cleavage products were resolved in a 12% acrylamide/urea gel.

Transfection and CAT assay

A mammalian expression vector for H-2RIIBP (pRSV-H-2RIIBP), a control RSV vector and a CAT reporter construct (pLd1.4K) that has 1.4 kb of the upstream region of the murine H-2L^d gene have been described (Nagata *et al.*, 1992). These DNAs were co-transfected into human embryonal carcinoma N-Tera2 cells by the calcium phosphate precipitation method and analyzed for CAT activity as described (Nagata *et al.*, 1992). The expression vector for T3Rα was pRSV-rTRα (Petty *et al.*, 1990). The expression vector for RARα, generously supplied by Dr E.Linney (Duke University, Durham, NC), contained an insert from the human RARα cDNA (Giguere *et al.*, 1987) cloned downstream of the human β-actin promoter (Gunning *et al.*, 1987) as described (Espeseth *et al*, 1989). CAT activity was normalized for transfection efficiency based on the activity of pCH110 (Pharmacia), coding for β-galactosidase. Values represent the average of two or three experiments, as indicated, ± standard error.

Nuclear and cytoplasmic extracts from mammalian cells

Mammalian cells were cultured in Dulbecco's Modified Eagle's Medium (Quality Biologicals) supplemented with glutamine, gentamicin and 10% fetal bovine serum (Gibco). For N-Tera2 and MCF-7 cells, the serum was depleted of steroids as described (Samuels *et al.*, 1979). Nuclear extracts from cultured cells were prepared according to Dignam *et al.* (1983) with a modification as described (Marks *et al.*, 1992). Cytoplasmic extracts were prepared from supernatants of cell extracts above and were centrifuged over a 1.7 M sucrose cushion. Extracts were dialyzed against buffer B prior to cross-linking experiments.

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References

Andrews,P.W. (1984) *Dev. Biol.*, **103**, 285−293.
Baker,A.R., McDonnell,D.P., Hughes,M., Crisp,T.M., Mangelsdorf,D.J., Haussler,M.R., Pike,W.J., Shine,J. and O'Malley,B. (1986) *Proc. Natl. Acad. Sci. USA*, **85**, 3294−3298.
Baldwin,A.S.,Jr and Sharp,P.A. (1987) *Mol. Cell. Biol.*, **7**, 305−313.
Ballard,D.W., Walker,W.H., Doerre,S., Sista,P., Molitor,J.A., Dixon,E.P., Peffer,N.J., Hannink,M. and Greene,W.C. (1990) *Cell*, **63**, 803−814.
Beato,M. (1989) *Cell*, **56**, 335−344.
Beebe,J.S., Darling,D.S. and Chin,W.W. (1991) *Mol. Endocrinol.*, **5**, 85−93.
Brown,M. and Sharp,P.A. (1990) *J. Biol. Chem.*, **265**, 11238−11243.
Burnside,J., Darling,D.S. and Chin,W.W. (1990) *J. Biol. Chem.*, **265**, 2500−2504.
Cocuzza,A.J. (1989) *Tetrahedron Lett.*, **30**, 6287−6290.
Darling,D.S., Beebe,J.S., Burnside,J., Winslow,E.R. and Chin,W.W. (1991) *Mol. Endocrinol.*, **5**, 73−84.
Davis,I.J., Hazel,T.G. and Lau,L.F. (1991) *Mol. Endocrinol.*, **5**, 854−859.
DeMarzo,A.M., Beck,C.A., Oñate,S.A. and Edwards,D.P. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 72−76.
Dignam,J.D., Lebowitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475−1489.
Driggers,P.H., Ennist,D.L., Gleason,S.L., Mak,W.-H., Marks,M.S., Levi,B.-Z., Flanagan,J.R., Appella,E. and Ozato,K. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 3743−3747.
Eriksson,P. and Wrange,O. (1990) *J. Biol. Chem.*, **265**, 3535−3542.
Espeseth,A.S., Murphy,S.P. and Linney,E. (1989) *Genes Dev.*, **3**, 1647−1656.
Evans,R.M. (1988) *Science*, **240**, 889−895.
Fawell,S.E., Lees,J.A., White,R. and Parker,M.G. (1990) *Cell*, **60**, 953−962.
Forman,B.M. and Samuels,H.H. (1990) *Mol. Endocrinol.*, **4**, 1293−1301.
Forman,B.M., Yang,C., Au,M., Casanova,J., Ghysdael,J. and Samuels,H.H. (1989) *Mol. Endocrinol.*, **3**, 1610−1626.
Giguere,V., Ong,E.S., Segui,P. and Evans,R.M. (1987) *Nature*, **330**, 624−629.
Glass,C.K., Holloway,J.M., Devary,O.V. and Rosenfeld,M.G. (1988) *Cell*, **54**, 313−323.
Glass,C.K., Lipkin,S.M., Devary,O.V. and Rosenfeld,M.G. (1989) *Cell*, **59**, 697−708.

Glass,C.K., Devary,O.V. and Rosenfeld,M.G. (1990) *Cell*, **63**, 729–738.
Graupner,G., Wills,K.N., Tzukerman,M., Zhang,X.-K. and Pfahl,M. (1989) *Nature*, **340**, 653–656.
Green,S., Walter,P., Kumar,V., Krust,A., Bornert,J.-M., Argos, P. and Chambon,P. (1986) *Nature*, **320**, 134–139.
Gunning,P., Leavitt,J., Muscat,G., Ng,S.-Y. and Kedes,L. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4831–4835.
Hai,T. and Curran,T. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3720–3724.
Halazonetis,T., Georgopoulos,K., Greenberg,M. and Leder,P. (1988) *Cell*, **55**, 917–924.
Hamada,K, Gleason,S.L., Levi,B.-Z., Hirschfeld,S., Appella,E. and Ozato,K. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8269–8293.
Henrich,V.C., Sliter,T.J., Lubahn,D.B., Macintyre,A. and Gilbert,L.I. (1990) *Nucleic Acids Res.*, **18**, 4143–4148.
Holloway,J.M., Glass,C.K., Adler,S., Nelson,C.A. and Rosenfeld,M.G. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8160–8164.
Hudson,L.G., Santon,J.B., Glass,C.K. and Gill,G.N. (1990) *Cell*, **62**, 1165–1175.
Jantzen,H.M., Strahle,U., Gloss,B., Stewart,F., Schmit,W., Boshart,M., Miksicek,P. and Schutz,G. (1987) *Cell*, **49**, 29–38.
Jones,N. (1990) *Cell*, **61**, 9–11.
Kimura,A., Israël,A., LeBail,O. and Kourilsky,P. (1986) *Cell*, **44**, 261–272.
Kumar,V. and Chambon,P. (1988) *Cell*, **55**, 145–156.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Landschultz,W.H., Johnson,P.F. and McKnight,S.L. (1989) *Science*, **243**, 1681–1688.
Lazar,M.A. and Berrodin,T.J. (1990) *Mol. Endocrinol.*, **4**, 1627–1635.
Lazar,M.A., Berrodin,T.J. and Harding,H.P. (1991) *Mol. Cell. Biol.*, **11**, 5005–5015.
Lewin,B. (1990) *Cell*, **61**, 1161–1164.
Liao,J., Ozono,K., Sone,T., McDonnell,D.P. and Pike,J.W. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9751–9755.
Logeat,F., Israël,N., Ten,R., Blank,V., LeBail,O., Kourilsky,P. and Israël,A. (1991) *EMBO J.*, **10**, 1827–1832.
Lucas,P.C., Forman,B.M., Samuels,H.H. and Granner,D.K. (1991) *Mol. Cell. Biol.*, **11**, 5164–5170.
Luisi,B.F., Xu,W.X., Otwinowski,Z., Freedman,L.P., Yamamoto,K.R. and Sigler,P.B. (1991) *Nature*, **352**, 497–505.
Mangelsdorf,D.J., Ong,E.S., Dyck,J.A. and Evans,R.M. (1990) *Nature*, **345**, 224–229.
Mangelsdorf,D.J., Umesono,K., Kliewer,S.A., Borgmeyer,U., Ong,E.S. and Evans,R.M. (1991) *Cell*, **66**, 555–561.
Marks,M.S., Levi,B.-Z., Segars,J.S., Driggers,P.H., Hirschfeld, S., Nagata,T., Appella,E. and Ozato,K. (1992) *Mol. Endocrinol.*, **6**, in press.
Martinez,E., Givel,F. and Wahli,W. (1987) *EMBO J.*, **6**, 3719–3727.
Martinez,E., Givel,F. and Wahli,W. (1991) *EMBO J.*, **10**, 263–268.
Miyazaki,J.-I., Appella,E. and Ozato,K. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9537–9541.
Murray,M.B. and Towle,H.C. (1989) *Mol. Endocrinol.*, **3**, 1434–1442.
Murre,C., McCaw,P.S. and Baltimore,D. (1989a) *Cell*, **56**, 777–783.
Murre,C., McCaw,P.S., Vaessin,H., Caudy,M., Jan,L.Y., Jan,Y.N., Cabrera,C.V., Buskin,J.N., Hauschka,S.D., Lassar,A.B., Weintraub,H. and Baltimore,D. (1989b) *Cell*, **58**, 537–544.
Näär,A.M., Boutin,J.-M., Lipkin,S.M., Yu,V.C., Holloway,J.M., Glass,C.K. and Rosenfeld,M.G. (1991) *Cell*, **65**, 1267–1279.
Nagata,T., Segars,J.H., Levi,B.-Z. and Ozato,K. (1992) *Proc. Natl. Acad. Sci. USA*, **88**, in press.
Nakabeppu,Y., Ryder,K. and Nathans,D. (1988) *Cell*, **55**, 907–915.
Nakai,A., Sakurai,A., Bell,G.I. and DeGroot,L.J. (1988) *Mol. Endocrinol.*, **2**, 1087–1092.
Nolan,G.P., Ghosh,S., Liou,H.-C., Tempst,P. and Baltimore,D. (1991) *Cell*, **64**, 961–969.
O'Donnell,A.L., Rosen,E.D., Darling,D.S. and Koenig,R.J. (1991) *Mol. Endocrinol.*, **5**, 94–99.
Oro,A.E., McKeown,M. and Evans,R.M. (1990) *Nature*, **347**, 298–301.
Petkovich,M., Brand,N.J., Krust,A. and Chambon,P. (1987) *Nature*, **330**, 444–450.
Petty,K.J., Desvergne,B., Mitsuhashi,T. and Nikodem,V.M. (1990) *J. Biol. Chem.*, **265**, 7395–7400.
Power,R.F., Lydon,J.P., Conneely,O.M. and O'Malley,B.W. (1991) *Science*, **252**, 1546–1548.
Ptashne,M. (1988) *Nature*, **335**, 683–689.
Rottman,J.N., Widom,R.L., Nadal-Ginard,B., Mahdavi,V. and Karathanasis,S.K. (1991) *Mol. Cell. Biol.*, **11**, 3814–3820.
Samuels,H.H., Stanley,F. and Casanova,J. (1979) *Endocrinology*, **105**, 80–85.
Selmi,S. and Samuels,H.H. (1991) *J. Biol. Chem.*, **266**, 11589–11593.
Shea,M.J., King,D.J., Conboy,M.J., Mariani,B.D. and Kafatos,F.C. (1990) *Genes Dev.*, **4**, 1128–1140.
Shirayoshi,Y., Miyazaki,J.-I., Burke,P.A., Hamada,K., Appella,E. and Ozato,K. (1987) *Mol. Cell. Biol.*, **7**, 4542–4548.
Spanjaard,R.A., Darling,D.S. and Chin,W.W. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 8587–8591.
Towbin,H., Stachelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
Treacy,M.N., He,X. and Rosenfeld,M.G. (1991) *Nature*, **350**, 577–584.
Tsai,S.Y., Carlstedt-Duke,J., Weigel,N.L., Dahlman,K., Gustafsson,J.A., Tsai,M.-J. and O'Malley,B.W. (1988) *Cell*, **55**, 361–369.
Umesono,K., Giguere,V., Glass,C.K., Rosenfeld,M.G. and Evans,R.M. (1988) *Nature*, **336**, 262–265.
Umesono,K., Murakami,K.K., Thompson,C.C. and Evans,R.M. (1991) *Cell*, **65**, 1255–1266.
Wang,L.-H., Tsai,S.Y., Cook,R.G., Beattie,W.G., Tsai,M.-J. and O'Malley,B.W. (1989) *Nature*, **340**, 163–166.
Williams,G.R., Harney,J.W., Forman,B.M., Samuels,H.H. and Brent,G.A. (1991) *J. Biol. Chem.*, **266**, 19636–19644.
Yang,N., Schüle,R., Mangelsdorf,D.J. and Evans,R.M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3559–3563.
Yu,V.C., Delsert,C., Andersen,B., Holloway,J.M., Devary,O.V., Näär,A.M., Kim,S.Y., Boutin,J.-M., Glass,C.K. and Rosenfeld,M.G. (1991) *Cell*, **67**, 1251–1266.

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Three other groups have recently described findings similar to ours [Leid *et al.* (1992) *Cell*, **68**, 377–395, Zhang *et al.* (1992) *Nature*, **355**, 441–446 and Kliewer *et al.* (1992) *Nature*, **355**, 446–449]. It has now been shown that 9-*cis*-retinoic acid is a ligand for H-2RIIBP [Levin *et al.* (1992) *Nature*, **355**, 359–361 and Heyman *et al.* (1992) *Cell*, **68**, 397–406].