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# All-Trans and 9-Cis Retinoic Acid Induction of CRABP II Transcription Is Mediated by RAR–RXR Heterodimers Bound to DR1 and DR2 Repeated Motifs

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## Summary

**Two cooperating retinoic acid response elements (RAREs) in the cellular retinoic acid-binding protein II (CRABP II) gene mediate differential transcriptional transactivation by retinoic acid receptors (RARs) and retinoid X receptors (RXRs) in P19 embryonal carcinoma cells. RARE1 and RARE2 are direct repeats (DR) of two motifs separated by 2 bp (DR2) and 1 bp (DR1), respectively, and bind RAR–RXR heterodimers more efficiently than homodimers. Using all-trans and 9-cis RA, which differentially activate RARs and RXRs, and RAR and RXR dominant-negative mutants, RAR–RXR heterodimers bound to RARE1 and RARE2 are shown to be responsible for CRABP II promoter transactivation, arguing against a unique DR spacing specifying recognition by RARs. Within heterodimers, RAR and RXR independently and differentially transactivate, depending on the specific RARE. Consistent with these results, 9-cis RA increases CRABP II mRNA levels more efficiently than all-trans RA. In contrast, all-trans and 9-cis RA have identical effects on induction of RAR $\beta$ 2 transcripts.**

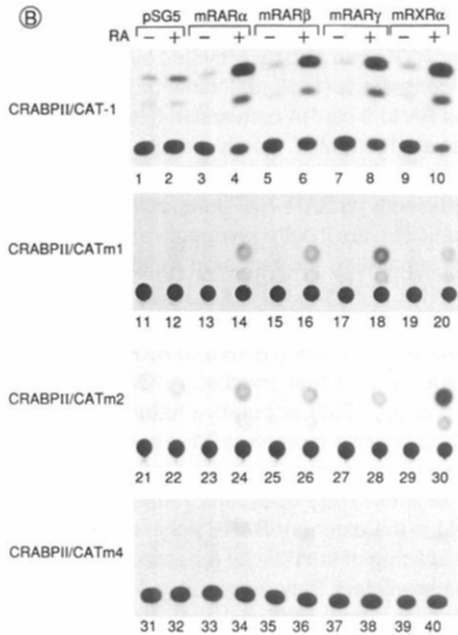
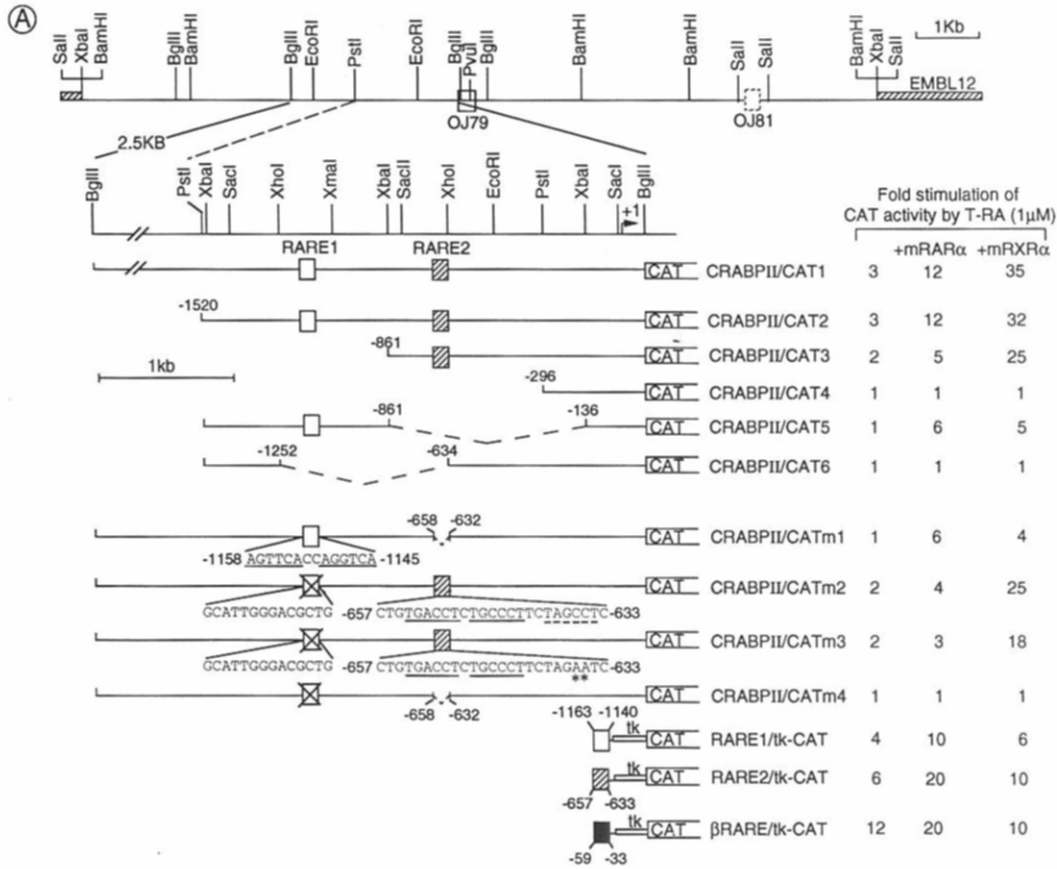
## Introduction

Retinoids appear to play a major role in embryogenesis, differentiation, and homeostasis (for reviews see Lotan, 1980; Sherman, 1986; Brockes, 1989, 1990; Saurat, 1990; Summerbell and Maden, 1990; Tabin, 1991; Mendelsohn et al., 1992). Three classes of proteins bind retinoids with high affinity: nuclear receptors that belong to the superfamily of steroid/thyroid receptors (for reviews see Evans, 1988; Green and Chambon, 1988; Beato, 1989), the cellular retinoic acid-binding proteins I (CRABP I) and II (CRABP II), and the cellular retinol-binding proteins I (CRBP I) and II (CRBP II) (for review see Bailey and Siu, 1988; Stoner and Gudas, 1989; Blomhoff et al., 1991; Chytil and Stump, 1991; Giguère et al., 1990; Maden, 1991). The three types of retinoic acid receptors (RARs), RAR $\alpha$ ,  $\beta$ , and  $\gamma$  (for review see Chambon et al., 1991; de Luca, 1991; Ruberte et al., 1991a; Kastner et al., 1992; Leroy et al., 1992), and

the three types of retinoid X receptors (RXRs), RXR $\alpha$ ,  $\beta$ , and  $\gamma$  (Hamada et al., 1989; Mangelsdorf et al., 1990, 1992; Rowe et al., 1991; Blumberg et al., 1992; Leid et al., 1992), are ligand-inducible trans regulators that modulate the transcription of target genes by interacting with cis-acting DNA retinoic acid response elements (RAREs). At the present time, there are only a limited number of RAREs that have been fully identified by mutagenesis in the regulatory region of "natural" target genes whose expression has been established to be controlled by retinoic acid (RA) at the transcriptional level. These natural RAREs appear to consist of a direct repeat (DR) of two core motifs (5'-PuG[G/T]TCA or a closely related sequence) separated by either 5 bp (DR5) in the case of human and mouse RAR $\alpha$ 2 (mRAR $\alpha$ 2) (Leroy et al., 1991a) and RAR $\beta$ 2 (mRAR $\beta$ 2) genes (de Thé et al., 1990; Sucov et al., 1990), 2 bp (DR2) in the case of the mouse CRBPI gene (Smith et al., 1991), or 4 bp (DR4) and 13 bp in the case of the mouse laminin B1 gene (Vasios et al., 1989, 1991). In addition, studies performed with a putative natural RXRE (Mangelsdorf et al., 1991) and synthetic RAREs (Kliwer et al., 1992a; Mader et al., 1992) have shown that RXRs preferentially bound to repeated core motifs separated by 1 bp (DR1).

Cotransfection experiments using expression vectors for either natural or chimeric RARs and RXRs and synthetic responsive promoters have revealed that RARs are efficiently activated by either all-trans RA or 9-cis RA at a ligand concentration of 50 nM, whereas RXRs are efficiently activated only by 9-cis RA at this ligand concentration. However, RXRs can also be activated by all-trans RA at higher concentrations (1–10  $\mu$ M), most probably owing to an all-trans RA to 9-cis RA conversion (Heyman et al., 1992; Levin et al., 1992; M. T. Bocquel et al., unpublished data; and see below). Interestingly, RARs and RXRs bind much more efficiently as RAR–RXR heterodimers to natural and synthetic RAREs in vitro irrespective of the length of the spacer separating the repeated motifs (Yu et al., 1991; Kliwer et al., 1992b; Leid et al., 1992; Zhang et al., 1992; Marks et al., 1992; Bugge et al., 1992). Moreover, transfection experiments using RAR and RXR expression vectors together with either synthetic (Kliwer et al., 1992b; Zhang et al., 1992) or putative natural (Marks et al., 1992) RA responsive promoters have suggested that RAR–RXR heterodimers may be functionally active.

A number of interesting questions remain to be addressed. What is the extent of RARE polymorphism with regard to the spacing of the directly repeated motifs? Are RAR–RXR heterodimers truly responsible for mediating the retinoid-induced transcriptional response of natural target genes in vivo, irrespective of this spacing? Can the two members of RAR–RXR heterodimeric complexes bound to a natural RARE in vivo be activated independently, and what are the respective roles of all-trans RA and 9-cis RA in RAR–RXR-mediated transactivation? How does the spacing of the directly repeated motifs affect transactivation by the two heterodimer partners?



**Figure 1. mCRABP II Promoter Activity and Identification of Two RAREs**

(A) Schematic representation of the CRABP II gene and of the reporter CAT constructs with the corresponding fold stimulation of CAT activity. The limits of the first and the last exons are represented by boxes, each of them localized by hybridization with the OJ79 and OJ81 probes, respectively (see Experimental Procedures), and relevant restriction sites are indicated. Coordinates are given with respect to the start site (+1) (see Figure 2). The open and hatched boxes correspond to the indicated sequences and the closed box to the sequence 5'-GGTAG-GGTTCCACCGAAAGTTCACCTCG-3' of the RARβ2 gene RARE. The broken underlining in CRABP II-CATm1 (nucleotides -635 to -639) denotes a related degenerate motif, into which two point mutations were introduced (indicated by asterisks) to generate CRABP II-CATm3. The fold stimulation of CAT activity was calculated taking as 1 the activity in the absence of all-trans RA (T-RA) and cotransfected receptor. CRABP II-CAT constructs were transfected in P19 EC cells, whereas the TK-CAT (tk-CAT) constructs were transfected in HeLa cells to avoid the RA induction of the TK-CAT reporter itself that occurs in P19 cells (results in the two cell types were qualitatively similar). The fold stimulations correspond to average values of several independent experiments (± 20%).

(B) CAT activity of CRABP II promoter constructs. P19 EC cells were transfected with 10 μg of the indicated reporter vector and 500 ng of either mRARα1, β2, γ1, mRXRα, or parental expression vector (pSG5). All-trans RA (1 μM) was added as indicated 16 hr later, cells were harvested 24 hr later, and CAT assays were performed.

-1521 PstI XbaI  
 CTGCAGTGATCAAAGCTCTAGACCCACGTGCCTCCTCTCCAGAAGCCTATGCCTCCTACGCGCCCGCC  
 -1451 CTCAAAATAGCATTCCGCTTAAAAATTTAGAGTCTCTGAGCTCAGGCCGACTTGTCCCACTACCTTCTGA  
 -1381 GTGTTGGTGGTGGGTTGGGGTGGGGAGAGCGCGATCCAGAGGCCCTTCTGAGAGGCCTTCTCTCC  
 -1311 CTGATCCTCTCAAGTTTCCACACCCCAATTATTCCGGATTGCCTCTATGAAGACCTGACTCGAGTGACAC  
 -1241 TGCTGGCCTCTGAGTGAGGTTAATCCTTCCAGGCCCTGTCTCAGCTCAGTTCCTCCCAACCAAGC  
 (CRABP II/RARE1)  
 -1171 CTGCCAAGTCCCGAGTTTCCAGGTCAGGGCTGCCTGTTCACCTTGGCCACCTAGATGAGAAGGGCC  
 -1101 AGGCAGGACCTTGTGGATCCTGAGGGACTACGGGGATAGCCCGGGAGACTGAGGGCGAATGCCAA  
 -1031 AGAAAGGGCAACACTTGTAGCTCCAGCACAGACACAGTGTGTTTCTGGCTTTGGGTACCCCGTGCCT  
 -961 CCGAGGCCCTCCTGTCTTCTGTCTGTCTTCCACTAGGGTTTACCCACGTAATAATCAG  
 -891 CCTCGGAGAGGCTGATTCACCGCACTTCAGCTCTAGATAGGGAGCTCAGACCCACCCCTCCACAC  
 -821 AGTCAGCCTTCCCAACCCCGGAGGCACAGCAAGCCCGGGTGGGGTGGGGTGGTGGGGGGCCT  
 -751 TGGGCTTTTCCCTCCTTGTCCGAGGGGGCCAGGGAAGCGTAGCCACCGAGAAAGCGCCTTAAT  
 (CRABP II/RARE2) XhoI  
 -681 AAAAGAGTCGTCAAGTGTGGCTGTGACCTTGCCTTCTAGCCTCGAGCGGGGCTCCTGTCTAA  
 -611 CCCTTCAATGTCCTCCAGGCTTGGAGGAAATGAATGAGTGCCTTGTGCTCACTTACGGCGTCCCA  
 AP-1  
 -541 GGCACTACTTGAACCCAGAGCTGAGTCAAGGGTGGAGGACAGAGCACCTCCCTGTCACCCAAAAGA  
 EcoRI  
 -471 ACGCTTGAATTCGAGCTGTTTCATTGAGTCCATTTTCAGCTGCCGGTTCGCCGGGGGGGGGGGGGAC  
 -401 AATCTTGCCTAGTGGGGTCTCCGGATGCCTGGAGCTGAGCTGTCCAGAGCACCAAATCTGGTAGAGAA  
 PstI  
 -331 CTA TAGGAACTGGTAAACGGGACCTGTGTTCCGCTTGCAGGCCGAGAGCATCCGTGACCCAGGCG  
 -261 AGAGCCGTGCCCTGTTCTTCCAGAGGCACAAACCCGTTTCTTGCCTTCTGACGCTTCTCGGTCCGCTA  
 NF-1 XbaI  
 -191 CTGATGCGGAGAAAGGTATCTTGGCTCTTGGAGAACCCTGGACCCACCCATTTCTAGAAACAGGAGG  
 I II  
 -121 GATTCGGAAAAGGATGGGGTGAAGTGGTCACAGAGCAGGGCCCCAGTGGTGTGGAAGCGGGGGGGG  
 III IV  
 -51 GCGGGGGCCTCATGCACCACTGGGTTATAAAAGCCTAGAGCTTGGCGCCAGCGGCTGTGCAATTGTC  
 SacI +1  
 +19 CTCCACGCGGCTTGGTCCCAAGGATCTGTCTGCAAAAGGAGACAGCAAAGTATCTTTAGCCTA  
 BglII  
 +89 AAGGACTCAGCGTCCAGTGTCTAGTTGAAGATCTAAAGAGAAAGCCACCTTGTGCCACT ATG CCT

Figure 2. Sequence of the mCRABP II Promoter Region

The sequence of the 1640 bp PstI-BglII 5' flanking fragment of the mCRABP II gene is shown, with +1 corresponding to the predominant transcription start site. Relevant restriction sites are indicated. Underlined are (5'→3') the two RAREs (CRABP II-RARE1 and CRABP II-RARE2); putative AP-2-, AP-1-, and nuclear factor 1 (NF-1)-binding sites; and four potential Sp1-binding sites (labeled from I to IV). The TATA box is boxed. The sequence of the oligonucleotide used for primer extension is underlined.

The expression of the CRABP genes has been reported to be stimulated by RA (Wei et al., 1989; Giguère et al., 1990). In particular, it has been shown that the level of CRABP II messenger RNA (mRNA) is increased in F9 teratocarcinoma cells by treatment with RA  $10^{-7}$  M (Giguère et al., 1990), in human skin by topical application of RA (Siegenthaler and Saurat, 1990; Aström et al., 1991), and in lesional hyperplastic psoriatic skin (Didierjean et al., 1991; Elder et al., 1992). In the present study, we have isolated and functionally dissected the promoter region of the mouse CRABP II (mCRABP II) gene in an attempt to answer the above questions. We have characterized two cooperating response elements of DR2 and DR1 type that can independently mediate differential activation by RAR and RXR in transfected P19 embryonal carcinoma (EC) cells. We show that RAR and RXR bind cooperatively to these DR2 and DR1 elements in vitro, and we demonstrate that RAR-RXR heterodimers bound to these elements actually mediate RA induction of the CRABP II promoter in P19 EC cells. We also provide evidence for independent transactivation by the two members of the heterodimeric complex, as well as evidence against the view that the length of the spacer between the repeated motifs provides

a code that specifies recognition of the response elements by RARs and RXRs in vivo. Finally, consistent with transfection experiment results, we show that 9-cis RA is more efficient than all-trans RA at increasing the level of CRABP II mRNA in P19 cells.

## Results

### Putative Promoter Elements in the mCRABP II Gene 5' Flanking Region

A single recombinant  $\lambda$ EMBL12 phage containing an insert of ~ 12 kb was isolated by screening a mouse genomic library with a full-length mCRABP II cDNA probe (Giguère et al., 1990; see Experimental Procedures). This recombinant hybridizes with two synthetic oligonucleotides (OJ79 and OJ81; Figure 1A and Figure 2) corresponding, respectively, to the 5' and 3' untranslated region of the cDNA and therefore contains the complete coding sequence of the mCRABP II gene. Further analysis of this phage by a combination of restriction mapping using a unique PvuII site present in the mCRABP II cDNA and Southern blotting with OJ79 and OJ81 probes identified a BglII fragment of 2.5 kb extending into the 5' flanking region of the gene (Figure

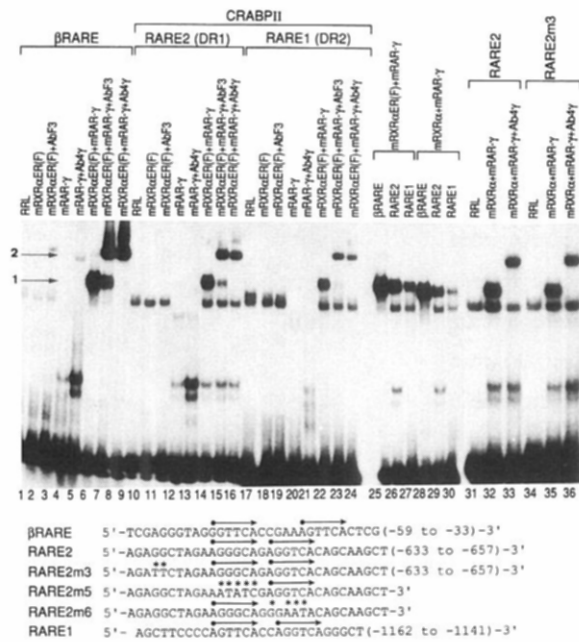


Figure 3. Binding of Heterodimers of mRAR $\gamma$  and mRXR $\alpha$  to  $\beta$ RARE, CRABP II-RARE2, CRABP II-RARE1, and Mutated CRABP II-RARE2. Gel retardation assays are shown in which mRXR $\alpha$ ER(F) (~10 fmol translated in vitro), mRAR $\gamma$  (~10 fmol translated in vitro), and mRXR $\alpha$  (~10 fmol translated in vitro) were incubated as indicated with <sup>32</sup>P-5' end-labeled oligonucleotides (~20 fmol at ~50,000 cpm) corresponding to  $\beta$ RARE, CRABP II-RARE2, CRABP II-RARE1, and mutated CRABP II-RARE2 (RARE2m3, RARE2m5, RARE2m6). (The sequence of the upper strand of each probe is given.) No binding of any combination of receptors was observed in the case of RARE2m5 and RARE2m6 (data not shown). Lanes 31-36 correspond to a different experiment than do lanes 1-30. Supershifted complexes were observed in the presence of specific antibodies, AbF3 for mRXR $\alpha$ ER(F) (lanes 7, 15, and 23) and Ab4 $\gamma$  for mRAR $\gamma$  (lanes 8, 16, 24, 33, and 36). Arrow 1 indicates the position of specific retarded RARE-receptor complexes, and arrow 2 indicates the position of antibody supershifted complexes. RRL, control assays with unprogrammed rabbit reticulocyte lysate (lanes 1, 17, and 31).

1A). The sequence of a 1650 bp PstI-BglII fragment 5' to the ATG initiator codon was determined (Figure 2). As expected, its 3' extremity overlaps with the 5' untranslated region of the cloned mCRABP II cDNA sequence (Giguère et al., 1990).

Primer extension using an oligonucleotide complementary to nucleotides +64 to +103 (underlined in Figure 2) was performed on poly(A)<sup>+</sup> RNA prepared from P19 EC cells treated with 1  $\mu$ M all-trans RA for 24 hr. A major extension product was detected whose 3' end mapped at the position indicated at +1 in Figure 2 (data not shown). This mapping is in agreement with the presence of a canonical TATA box (Corden et al., 1980), 26 bp upstream of the mRNA start site. The mCRABP II 5' flanking region contains several overlapping putative binding sites for the transcription factor Sp1 between nucleotides -41 to -62 (5'-GGCGGG-3', underlined and labeled as I to IV in Figure 2; Briggs et al., 1986), a sequence related to the nuclear factor 1 binding motif (5'-TGGN<sub>2</sub>CCA-3', from nucleotides -152 to -141; Chodosh et al., 1988; Santoro et al., 1988),

a perfect AP-1 binding site (5'-TGAGTCA-3', from nucleotides -520 to -514; Angel et al., 1987), and an AP-2 binding site (CCCCAGGCA, from nucleotides -546 to -538; Imagawa et al., 1987; Mitchell et al., 1987). No sequence homology was found between this promoter sequence and those of mCRABP I (Wei et al., 1990, 1991), mouse CRBP I (Smith et al., 1991), and rat or mouse CRBP II (Demmer et al., 1987; H. Nakshatri et al., unpublished data).

### The mCRABP II Promoter Contains Two Functional DR1 and DR2 RAREs That Bind RAR-RXR Heterodimers In Vitro Much More Efficiently Than RAR or RXR Homodimers

Various lengths of the putative mCRABP II promoter region were, with or without internal deletions, ligated to the chloramphenicol acetyltransferase (CAT) gene (see Figure 1A). These constructs were tested for CAT expression and all-trans RA (1  $\mu$ M) inducibility, following transfections into P19 EC cells together with 500 ng of expression vectors coding either for mRAR $\alpha$ 1,  $\beta$ 2, or  $\gamma$ 1 or for mouse RXR (mRXR)  $\alpha$ ,  $\beta$ , or  $\gamma$ . In the presence of the parental expression vector pSG5, the all-trans RA inducibility of CRABP II-CAT1 in P19 cells was 2- to 3-fold (see Figures 1A and 1B). When either mRAR $\alpha$ ,  $\beta$ , or  $\gamma$  expression vectors were cotransfected with this reporter, a 10- to 14-fold increase in CAT activity was observed in the presence of all-trans RA, whereas a 30- to 40-fold induction was seen with either mRXR $\alpha$  or mRXR $\gamma$  and a 15- to 20-fold induction was seen with mRXR $\beta$  (see Figures 1A and 1B; data not shown). Progressive 5' to 3' deletions resulted in a two step decrease of all-trans RA inducibility. The first decrease corresponded to deletion of the -1520 to -861 sequence (compare CRABP II-CAT2 and CRABP II-CAT3; see Figure 1A), and the second decrease occurred when the -861 to -296 sequence was deleted (compare CRABP II-CAT3 and CRABP II-CAT4). The construct containing an internal deletion between -861 and -136 in CRABP II-CAT2 was still inducible (6-fold for mRAR $\alpha$  and 5-fold for mRXR $\alpha$ ), but no induction was seen when the -1252 to -634 sequence was deleted. Thus, the CRABP II promoter appears to contain two all-trans RAREs, one located between positions -1252 and -861 and the other located between positions -861 and -634. Interestingly, in P19 cells these two elements clearly exhibit a different reactivity toward the RAR and RXR families.

Two DNA sequences that show homology with the RAREs previously described in the literature (see Introduction) were identified and named RARE1 and RARE2 (boxed in Figure 2). RARE1 is a DR of two core motifs separated by 2 bp (DR2; see Introduction; 5'-AGTTCACCGGTC-3', nucleotides -1158 to -1145), and RARE2 is a DR of two core motifs separated by 1 bp (DR1; 5'-TGACCTcTGCCCT-3', nucleotides -654 to -642; 5'-AGGGCAGAGGTC-3', on the complementary strand). We mutated RARE1 in CRABP II-CAT1, thereby creating CRABP II-CATm2, and deleted bases -633 to -657 in RARE2 of CRABP II-CAT1, creating CRABP II-CATm1 (see Figure 1A). The deletion of bases -633 to -657 eliminates the repeated motifs of RARE2 and a related degenerate motif 5'-TAGCC-3' (nucleotides -635 to -639). The latter motif

was mutated in CRABP II-CATm2 to 5'-TAGAA-3', yielding CRABP II-CATm3, which exhibited the same all-trans RA pattern of inducibility as CRABP II-CATm2 (see Figure 1A; data not shown). CRABP II-CATm4, in which both RAREs were destroyed (see Figure 1A), was not inducible by all-trans RA, whereas CRABP II-CATm1 and CRABP II-CATm2 showed the same pattern of inducibility as CRABP II-CAT5 and CRABP II-CAT3, respectively. These results strongly suggest that RARE1 and RARE2 are responsible for all-trans RA response of the CRABP II promoter (see Figures 1A and 1B; data not shown).

RARE1 and RARE2 were ligated in their natural orientation in pBLCAT2<sup>+</sup> upstream of the herpes simplex virus thymidine kinase (TK) promoter to investigate whether they could confer all-trans RA inducibility on a heterologous promoter (RARE1-TK-CAT and RARE2-TK-CAT in Figure 1A). The all-trans RA inducibility of these reporter genes was investigated in HeLa cells cotransfected with pSG5 or 500 ng of expression vectors coding for either mRAR $\alpha$ 1,  $\beta$ 2,  $\gamma$ 1, or mRXR $\alpha$ . Similar transfections were performed with the  $\beta$ RARE-TK-CAT reporter containing the RARE present in the human and mouse RAR $\beta$ 2 genes ( $\beta$ RARE; see Introduction). When cotransfected with the control parental expression vector pSG5, RARE1-TK-CAT and RARE2-TK-CAT were 4- to 6-fold all-trans RA induced by HeLa cell endogenous receptors, whereas  $\beta$ RARE-TK-CAT was 12-fold induced. The magnitude of the RARE1-TK-CAT and RARE2-TK-CAT induction was increased to 10- to 20-fold upon cotransfection with mRAR $\alpha$ 1,  $\beta$ 2, or  $\gamma$ 1 and 6- to 10-fold with mRXRs (see Figure 1A; data not shown). Thus, RARE1 and RARE2 can confer all-trans RA inducibility on a heterologous promoter, but their differential activation by RAR and RXR appears to be lost in the context of the TK promoter.

The ability of isolated RAR and RXR to bind to CRABP II response elements *in vitro* was investigated by a gel shift-retardation assay using labeled oligonucleotide probes corresponding to  $\beta$ RARE, RARE2, RARE1, RARE2m3, RARE2m5, RARE2m6 (Figure 3), *in vitro* translated mRAR $\gamma$  (similar results were obtained with mRAR $\alpha$  or  $\beta$ ; data not shown), and an antigen-tagged fusion protein, mRXR $\alpha$ ER(F), that contains the F region of the human estrogen receptor (ER) fused to the carboxy-terminal amino acid of mRXR $\alpha$ . This fusion protein contains the epitope for and can be used in supershift assays with an anti-ER(F) antibody, and it has been shown to have the same binding properties as mRXR $\alpha$  (Leid et al., 1992). At the concentrations of mRAR $\gamma$  used (~10 fmol of receptor per assay), no detectable complex was formed with any of the response elements in the absence of mRXR $\alpha$ ER(F) (Figure 3, lanes 4, 12, and 20) or mRXR $\alpha$  (data not shown). Likewise, in the absence of mRAR $\gamma$ , mRXR $\alpha$ ER(F) was unable to form any detectable specific complex with the above elements (Figure 3, lanes 2, 10, and 18). However, consistent with recent reports (Yu et al., 1991; Leid et al., 1992), a strong retarded complex was observed for  $\beta$ RARE (Figure 3, lane 6), RARE2 (Figure 3, lanes 14 and 32), RARE1 (Figure 3, lane 22), and RARE2m3 (Figure 3, lane 35) in the presence of both mRAR $\gamma$  and mRXR $\alpha$ ER(F). In each case, the complex could be supershifted

by either anti-mRAR $\gamma$  (Ab4 $\gamma$ ; Figure 3, lanes 8, 16, 24, 33, and 36) or anti-ER(F) antibody (AbF3; Figure 3, lanes 7, 15, and 23) and migrated at the same position for the four probes (Figure 3, lanes 25-30, 32, and 35). In the case of RARE2m5 and RARE2m6, where each core motif of RARE2 was separately mutated (Figure 3), no RAR-RXR complex could be detected (data not shown). Thus, RAR and RXR bind cooperatively on RARE2 and RARE1, and RAR-RXR heterodimers recognize RARE2 and RARE2m3 (where the degenerate motif 5'-TAGCC-3' has been mutated; Figure 3) with the same efficiency.

To determine the relative affinity of the RAR-RXR heterodimers for CRABP II RAREs, competition experiments were performed using RARE1, RARE2, and  $\beta$ RARE as labeled probes or "cold" competitors and *in vitro* translated mRAR $\gamma$  and mRXR $\alpha$ . The relative affinity of RAR-RXR heterodimers for  $\beta$ RARE was approximately 6 times greater than for RARE2 and 10 times greater than for RARE1, something confirmed by competition with cold RARE1 and RARE2 (data not shown).

#### **All-Trans RA- and 9-Cis RA-Induced Transcription from the CRABP II Gene Promoter Is Mediated by RARE1- and RARE2-Bound RAR-RXR Heterodimers**

To investigate whether the results of receptor binding *in vitro* could be correlated with transcriptional activation by heterodimers *in vivo*, we cotransfected mRAR $\alpha$  and mRXR $\alpha$  expression vectors with CRABP II-CATm1 (which contains only the DR2 RARE1) and CRABP II-CATm2 (which contains only the DR1 RARE2; see Figure 1A) under ligand conditions that enabled us to differentiate between RXR and RAR activation. Results from our laboratory (M. T. Bocquel et al., unpublished data) have shown that exposure of cells to 50 nM all-trans RA for 15 hr results in RAR but not RXR activation, whereas under the same conditions 50 nM 9-cis RA induces transcriptional activation through both RAR and RXR (see Figure 6).

P19 cells were transfected with either CRABP II-CATm1 or CRABP II-CATm2 and expression vectors encoding either mRAR $\alpha$  or mRXR $\alpha$  or both. The cells were treated 24 hr later with either all-trans RA (50 nM), 9-cis RA (50 nM), both ligands together, or vehicle (ethanol) for an additional 12-15 hr (Figure 4 and CAT assays not shown). In the presence of mRAR $\alpha$  alone, CRABP II-CATm1 expression was stimulated 4- to 6-fold by either all-trans RA or 9-cis RA (Figure 4, lanes 1-4), whereas a lesser stimulation was observed for CRABP II-CATm2 (Figure 4, lanes 13-16). In the presence of mRXR $\alpha$ , CRABP II-CATm2 was induced 15-fold by 9-cis RA (but not by all-trans RA; Figure 4, lanes 17-20), while under the same conditions CRABP II-CATm1 was stimulated only 4-fold (Figure 4, lanes 5-8). However, when RAR $\alpha$  and RXR $\alpha$  were cotransfected, a clear increase in all-trans RA and 9-cis RA inducibility was detected for both CRABP II-CATm1 and CRABP II-CATm2 (Figure 4, lanes 9-12 and 21-24, respectively). This increase was particularly striking in the case of CRABP II-CATm2, for which there was a marked induction by all-trans RA that was not observed with either of the two receptors alone (compare lanes 14, 18, and 22 in Figure 4). These data strongly suggest that RAR-RXR heterodimers

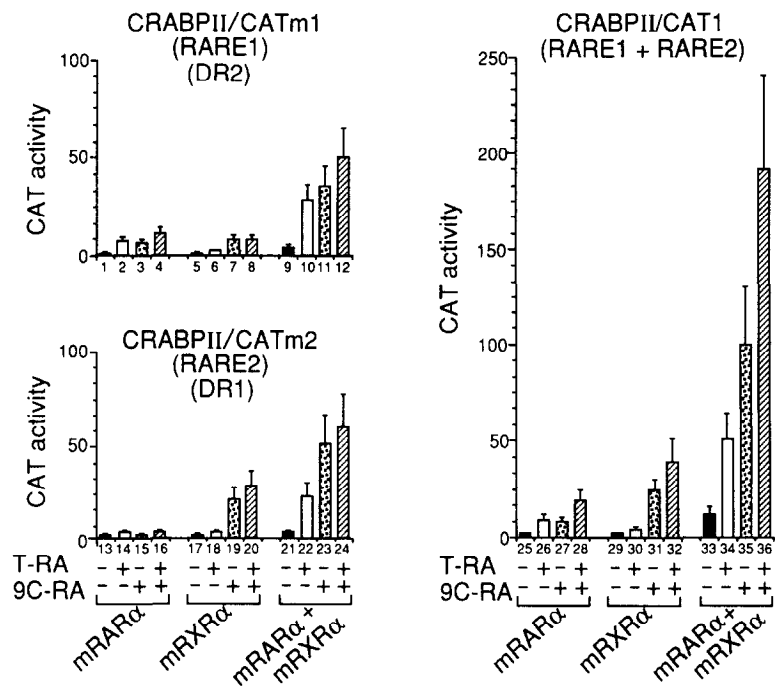


Figure 4. Cotransfection of mRAR $\alpha$  and mRXR $\alpha$  Enhance All-Trans RA- and 9-Cis RA-Induced Response of CRABP II-CATm1, CRABP II-CATm2, and CRABP II-CAT1

P19 EC cells were transfected with 10  $\mu$ g of reporter plasmids CRABP II-CATm1, CRABP II-CATm2, or CRABP II-CAT1 and 500 ng of mRAR $\alpha$  or mRXR $\alpha$  or both (as indicated). The transcriptional response of these reporter genes after a 12–15 hr treatment (as indicated) with either all-trans RA (T-RA) (50 nM), 9-cis RA (9C-RA) (50 nM), or both ligands was determined by measurement of the CAT activity that is given in arbitrary units.

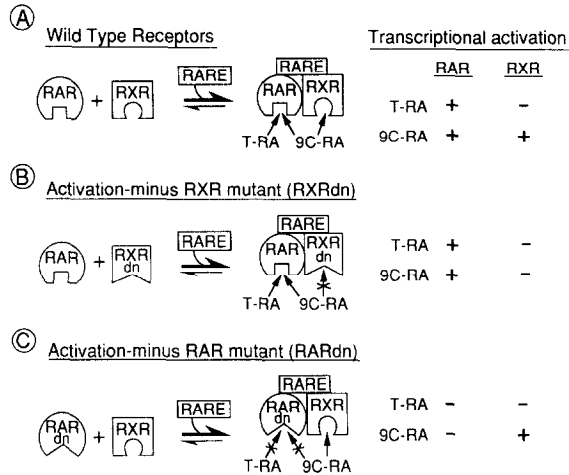
bind to and activate transcription *in vivo* from either the DR2 RARE1 or the DR1 RARE2 and also that each partner within the heterodimeric complex is able to activate transcription independently.

To support this hypothesis further, we made use of C-terminally truncated mRXR $\alpha$  and mRAR $\alpha$  mutants that have lost their ligand-dependent transcription activation function but still heterodimerize and bind RARE1 and RARE2 *in vitro* as efficiently as their wild-type counterparts (M. S. et al., unpublished data). These mutants abolished ligand-dependent transactivation by their wild-type counterparts when transfected in a 10-fold excess, most likely by competition in a molar ratio, and are therefore referred to as dominant-negative (dn) receptors hereafter. Assuming that RARE1 and RARE2 mediate transactivation by RAR–RXR heterodimers, it was therefore expected that in the presence of 50 nM all-trans RA and/or 9-cis RA, RAR–RXRdn heterodimers would stimulate transcription with the same efficiency as RAR–RXR heterodimers in the presence of only 50 nM all-trans RA. On the other hand, activation by RXR–RARdn in the presence of 50 nM 9-cis RA would only correspond to the additional activation that is seen when wild-type RAR–RXR heterodimers are induced by 9-cis RA instead of by all-trans RA (see Figure 5 for a schematic representation).

The above prediction was first tested in the case of CRABP II-CATm1, which contains the DR2 RARE1 (Figure 6A). When compared with transfection of mRAR $\alpha$  alone (Figure 6A, lanes 1–3), a cotransfection of 100 ng of mRAR $\alpha$  and 100 ng of mRXRdn in P19 cells resulted in an increased all-trans RA-dependent induction that was similar to that observed with cotransfection of wild-type mRAR $\alpha$  and mRXR $\alpha$  (compare lanes 10–12 with lanes 7–9 in Figure 6A; note that only 100 ng of RAR and RXR

expression vectors was used in the experiments shown in Figure 6, instead of 500 ng as in Figure 4). Moreover, cotransfection of mRXR $\alpha$  and mRARdn resulted in a 9-cis RA-specific activation (Figure 6A, lanes 13–15), which corresponded to the difference in the activations achieved with cotransfected wild-type mRAR $\alpha$  and mRXR $\alpha$  in the presence of 9-cis RA or all-trans RA (Figure 6A, lanes 7–9). Cotransfection of a large excess of either mRXRdn (Figure 6A, lanes 16–18) or mRARdn (Figure 6A, lanes 19–21), together with mRXR $\alpha$  and mRAR $\alpha$ , had the effect expected for transactivation by RAR–RXR heterodimers (see Figure 5). Within the heterodimers, mRAR $\alpha$  was more efficient than mRXR $\alpha$  at stimulating transcription from the DR2 RARE1 in CRABP II-CATm1.

Similar transfections were performed in P19 cells with CRABP II-CATm2 that contains the DR1 RARE2 (Figure 6B). Cotransfection of mRAR $\alpha$  with mRXRdn increased markedly all-trans RA- and 9-cis RA-dependent mRAR $\alpha$  activity (Figure 6B, lanes 11 and 12) to the same extent as cotransfection of mRAR $\alpha$  and wild-type mRXR $\alpha$  (Figure 6B, lanes 7 and 8). The cotransfection of mRXR $\alpha$  with mRARdn resulted in a 9-cis RA-dependent mRXR $\alpha$  activity (Figure 6B, lanes 13–15), which corresponded to the difference between the all-trans RA- and 9-cis RA-dependent activities when mRAR $\alpha$  and mRXR $\alpha$  were cotransfected (Figure 6B, lanes 7–9). However, the 9-cis RA-dependent activity of mRXR $\alpha$ –mRARdn heterodimers was very similar to that observed when mRXR $\alpha$  alone was transfected (compare lanes 13–15 with lanes 4–6 in Figure 6B), which suggests that mRXR $\alpha$  may act by binding RARE2 either as a heterodimer with mRAR $\alpha$  or as a homodimer. It is noteworthy that, in contrast with observations of the DR2 RARE1 (CRABP II-CATm1; Figure 6A, lanes 7–9), the RXR component of the heterodimer was as effi-



**Figure 5. Schematic Representation of All-Trans RA- and 9-Cis RA-Dependent Transcriptional Activation by Wild-Type and Mutant RAR-RXR Heterodimers**

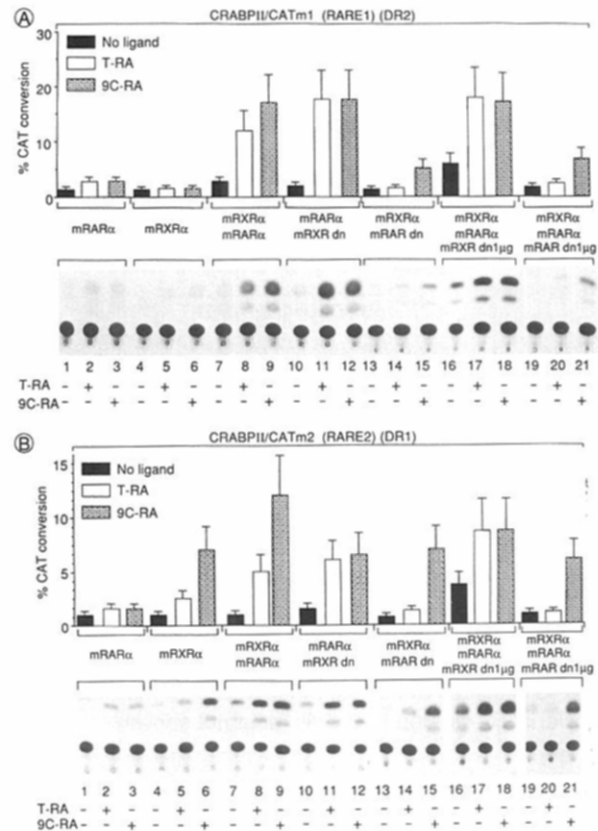
RARdn and RXRdn correspond to dominant-negative mutants of RAR and RXR that can bind to RAREs but have lost their ligand-dependent activation function. Arrows pointing from either all-trans RA (T-RA) or 9-cis RA (9C-RA) indicate that the ligand, at 50 nM concentration, is able to activate the corresponding receptor in P19 cells, whereas crossed-out arrows indicate that the receptor cannot be activated.

cient as its RAR partner at activating transcription through the DR1 RARE2 (compare all-trans RA- and 9-cis RA-dependent activities in lanes 7-9 and 16-21 of Figures 6A and 6B). Note also that cotransfection of a large excess of mRXRdn resulted in a 2- to 3-fold RA-independent activation of transcription (especially in the case of RARE2; see Figures 6A and 6B, lanes 16), which suggests that a ligand-independent transcriptional activation function may be present in mRXR $\alpha$ .

We conclude from all of the above results that RAR and RXR bind as heterodimers to either the DR2 RARE1 or DR1 RARE2, from which they can activate transcription independently. To investigate whether heterodimers bound to RARE1 and RARE2 could synergize within the CRABP II promoter, CRABP II-CAT1 was cotransfected with mRAR $\alpha$ , mRXR $\alpha$ , or the two receptors together, and the resulting all-trans RA- and/or 9-cis RA-dependent transcriptional activation was compared with that achieved with either CRABP II-CATm1 (containing RARE1 only) or CRABP II-CATm2 (containing RARE2 only) (see Figure 4). No strong increase above the activation expected from additivity was observed (~2-fold in the presence of both all-trans RA and 9-cis RA), indicating that the heterodimers bound to RARE1 and RARE2 are only weakly synergizing within the CRABP II promoter under these P19 cell transfection conditions.

**mCRABP II mRNA Levels Are Increased in P19 Cells by Either All-Trans RA or 9-Cis RA, but 9-Cis RA Is More Efficient Than All-Trans RA**

To determine whether the above transfection observations could be correlated with *in vivo* effects of all-trans RA and 9-cis RA on the activity of P19 cell endogenous RARs and



**Figure 6. Cotransfected RARdn and RXRdn Stimulate Activation of Transcription Mediated by RAR and RXR Wild Type on CRABP II-CATm1 (RARE1) and CRABP II-CATm2 (RARE2)**

P19 EC cells were cotransfected as indicated with 10  $\mu$ g of CRABP II-CATm1 (A) or 10  $\mu$ g of CRABP II-CATm2 (B) and mRAR $\alpha$  (100 ng), mRXR $\alpha$  (100 ng), mRARdn (100 ng or 1  $\mu$ g as indicated), and mRXRdn (100 ng or 1  $\mu$ g as indicated). Cells were treated with either all-trans RA (T-RA) (50 nM) or 9-cis RA (9C-RA) (50 nM) for 12-15 hr, and CAT activity was determined. Representative CAT assays and graphs corresponding to average values from several independent experiments are shown.

RXRs, mCRABP II expression was analyzed by Northern blotting of RNAs prepared from all-trans RA- and/or 9-cis RA-treated cells, using a  $^{32}$ P-labeled mCRABP II cDNA probe (Figure 7). After a 15 hr all-trans RA or 9-cis RA treatment (50 nM), mCRABP II transcripts were increased ~20-fold by all-trans RA, ~60-fold by 9-cis RA, and ~300-fold when the two ligands were added together (Figure 7). Under these suboptimal all-trans RA and 9-cis RA concentrations, some variability was observed in the extent of these increases, particularly in the further stimulation brought about by the addition of both ligands, but 9-cis RA was consistently 3- to 8-fold more efficient than all-trans RA in repeated experiments. In marked contrast, mRAR $\beta$ 2 mRNA levels were identically increased in the same cells by either all-trans RA or 9-cis RA treatment, and no significant increase was observed when the two ligands were added together. At least part of the increase in RAR $\beta$ 2 and CRABP II mRNAs was due to increased rates of transcrip-



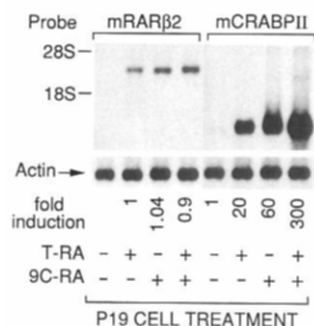


Figure 7. Inducibility of mRARβ2 and mCRABP II mRNA in P19 EC Cells Treated with All-Trans RA and/or 9-Cis RA

Shown are autoradiograms of Northern blots of poly(A)<sup>+</sup> RNA (2 μg per lane) obtained from P19 EC cells grown in the presence of all-trans RA (T-RA) (50 nM), 9-cis RA (9C-RA) (50 nM), or both ligands (50 nM) for 15 hr and hybridized with randomly primed <sup>32</sup>P-labeled cDNA probes specific for mRARβ2 and mCRABP II. Actin mRNA control is also shown, and the fold inductions are indicated. Note that in the case of mRARβ2 where no signal could be detected in the absence of RA treatment, the induction achieved with all-trans RA treatment alone was taken as 1.

tion as indicated by nuclear run-on assays performed with nuclei of RA-treated P19 cells (data not shown).

## Discussion

### The Recognition of DR Elements by RAR–RXR Heterodimers Is Not Specified by a Unique Spacing of the Repeated Motifs

Our present data clearly establish that RARE1 and RARE2 are located in the 5' flanking region of the mCRABP II gene. Both RAREs correspond to the direct repetition of a sequence identical or closely related to the core motif 5'-PuG(G/T)TCA-3', which is found in a number of natural RA-responsive promoters (see Introduction). In RARE1 these two motifs are separated by 2 bp, whereas they are spaced by 1 bp in RARE2. In this respect, RARE1 is similar to the CRBPI RARE (Smith et al., 1991), whereas RARE2 is related to the rat CRBPII putative RARE, in which the spacings between several core motifs are 1 bp (Mangelsdorf et al., 1991), as well as to response elements that have been found in the 5' flanking regions of a number of other putative RA-responsive genes (Hamada et al., 1989; Lucas et al., 1991; Rottman et al., 1991). Both RARE1 and RARE2 are different from the RAREs found in other characterized natural RA target genes, i.e., the human and mouse RARβ2 and RAREα2 genes (and possibly the human ADH3 gene; Duyster et al., 1991) and the mouse laminin B1 gene, in which the directly repeated core motifs are spaced by 5 bp and 4 bp, respectively (see Introduction).

The results of binding and transactivation studies, mainly performed with synthetic response elements and chimeric RA-responsive promoters, have suggested that the spacing between the core motifs may correspond to a discriminating recognition code for various members of the nuclear receptor superfamily, in which directly repeated motifs separated by 5 bp (DR5), 4 bp (DR4), 3

bp (DR3), and 1 bp (DR1) correspond to binding sites for RARs, thyroid hormone receptors, vitamin D<sub>3</sub> receptors, and RXRs, respectively (Umehono et al., 1991; Kliewer et al., 1992a; see also Näär et al., 1991). In addition, it has been reported that a DR1 element binds RXR–COUP-TF (ear3) heterodimers and that this heterodimeric binding results in the suppression of transactivation by RXR (Kadowaki, 1992; Kliewer et al., 1992a). However, additional systematic binding and transactivation studies have indicated that RARs can bind to and activate transcription from DR5 and DR2 elements and, with a lesser efficiency, from DR4 and DR3 elements (Mader et al., 1992). These latter observations, together with those made with RAREs of promoters of natural target genes, including the DR2 element of the mouse CRBPI gene (Smith et al., 1991) and the present CRABP II elements, indicate that if a recognition code exists, it must be highly degenerate. In fact, recent *in vitro* studies have shown that under the limiting receptor concentrations that are likely to exist *in vivo*, RAR–RXR heterodimers bind much more efficiently to the various DR1 to DR5 elements than to the corresponding homodimers (Yu et al., 1991; Kliewer et al., 1992b; Leid et al., 1992; Zhang et al., 1992; B. D., M. S., P. L., M. L., and P. C., unpublished data). Since DR elements are asymmetrical, it is possible that the above degeneracy reflects at least in part the nonequivalence of the two core motifs of a given DR for the binding of RAR–RXR heterodimers.

### Differential and Independent Transactivation by RAR and RXR Members of Heterodimers Bound to Different DR Elements

Our present cotransfection studies with CRABP II promoter constructs containing either RARE1 or RARE2 and wild-type and dominant-negative RAR and RXR demonstrate unambiguously that RAR–RXR heterodimers can bind to and transactivate from a single natural DR2 or DR1 RARE more efficiently than either of the corresponding homodimers, thus establishing that RAR–RXR heterodimers can control a bona fide RA-responsive promoter *in vivo* via DR2 and DR1 elements. Moreover, each member of the heterodimeric complex appears to be able to activate transcription independently. Interestingly, the respective contribution of the two partners of RAR–RXR heterodimers to transcriptional activation appears to be dependent on the nature of the RARE. The contribution of the RAR transactivation function(s) is clearly preponderant in the case of the DR2 RARE1, whereas the RAR and RXR transactivation functions appear to contribute equally to stimulation of transcription in the case of the DR1 RARE2. These variations in the transactivation properties may reflect modifications in the ligand-binding domain and/or the activation domain of RAR and RXR members of heterodimeric complexes bound to different RARE types. RAR and RXR dimerization domains are indeed localized within the same E region that also contains the ligand-binding domain and an activation function (see Leid et al., 1992; Nagpal et al., 1992). In this respect, we note that RAR was found to be almost exclusively responsible for activation of transcription when similar cotransfection experiments were performed in P19 cells with RXRdn mutants and the RARβ2

promoter that contains a DR5 element (M. S. et al., unpublished data). That the contribution of RAR and RXR to transcriptional activation may also be promoter context dependent is suggested by the results obtained with RARE1-TK-CAT and RARE2-TK-CAT (Figure 1A). This may possibly reflect differences in synergism between RAR and RXR and additional promoter-specific transactivators.

It is noteworthy that RXR transfected on its own could bring about a several-fold stimulation of transcription mediated by RARE2 (DR1) but not by RARE1 (DR2) (Figures 4 and 6). This observation suggests that, while RAR-RXR heterodimers bind more efficiently than either RXR or RAR homodimers to RARE2 and RARE1 (no RXR homodimer binding to either RARE1 or RARE2 was seen in vitro at low RXR concentrations; Figure 3), RXR homodimers may nevertheless bind to RARE2 at high RXR concentrations in vivo. Alternatively, RXR may be able to bind to the DR1 RARE2 (but not to the DR2 RARE1) as a heterodimer with an yet unidentified partner, which, however, is unlikely to be COUP-TF, since RXR appears to be inactive in RXR-COUP-TF heterodimers (Kliwer et al., 1992b; B. D., unpublished data).

#### **Differential Response of the CRABP II and RAR $\beta$ 2 Genes to RAR and RXR in P19 Cells and Combinatorial Effects in the RA Signaling Pathway**

The present dissection of the CRABP II promoter response to RA provides interesting insights into the question of how the RA signal can generate pleiotropic effects during embryogenesis and in homeostasis. Our data clearly suggest that the response of a given promoter is potentially dependent on the DR characteristics of the RARE, the relative RAR and RXR concentrations, and the intracellular levels of all-trans RA and 9-cis RA. In addition, studies from our laboratory (Nagpal et al., 1992) have shown that each of the three RARs and of the three RXRs possesses one transcription activation function and one transcription modulating function that are located in the C-terminal E region and the N-terminal A/B regions, respectively, and that exhibit different promoter context specificities. Diversity in RA responses could obviously result from specific combinatorial assortments of all of the above parameters.

That our transfection results do not chiefly correspond to artifacts generated by the production of high levels of RAR and/or RXR in transfected P19 cells is demonstrated by the characteristics of activated expression of the endogenous CRABP II gene when P19 cells are exposed to either all-trans RA or 9-cis RA under conditions (ligand concentrations of 50 nM) where all-trans RA induces only RAR activity and 9-cis RA induces both RAR and RXR activity (Figure 6). CRABP II gene expression was consistently induced to a higher level by 9-cis RA than by all-trans RA (Figure 7). In marked contrast, in the same cells the expression of the endogenous RAR $\beta$ 2 gene was similarly activated by either all-trans RA or 9-cis RA, thus providing clear-cut evidence that both RAR and RXR activation functions are implicated in activation of expression of the CRABP II gene, whereas only the RAR transactivation function(s) is involved in the induction of the RAR $\beta$ 2 pro-

moter. This differential response of CRABP II and RAR $\beta$ 2 promoters represents a clear example of generation of RA response diversity due to combinatorial effects, as discussed above. However, we did not find any major differences in the ability of the different RAR and RXR types ( $\alpha$ ,  $\beta$ , or  $\gamma$ ) to transactivate the CRABP II promoter in transfected P19 cells. Such differences may be masked by the nonphysiological high concentration of receptors that is generated in transfected cells. Gene disruption studies are in progress in both P19 EC cells and in the mouse to characterize the RAR and RXR types that may possibly be specifically responsible for activation of CRABP II gene expression.

The function of CRABP II during embryogenesis and in the adult is unknown, although the distribution of its transcripts is clearly distinct from that of CRABP I (Maden et al., 1988, 1989; Dollé et al., 1989, 1990; Maden, 1991; Ruberte et al., 1991b, 1992; Tabin, 1991). It has been speculated that, even though they may not be directly involved in mediating the RA signal (Breitman et al., 1981; Douer and Koeffler, 1982; Libby and Bertran, 1982), these RA-binding proteins could finely tune the actual concentration of free RA in the cell (Robertson, 1987; Maden et al., 1988; Smith et al., 1989; Balling, 1991; Boylan and Gudas, 1991; Ruberte et al., 1991b). That these proteins play an important function in the RA signaling pathway is indicated by the lethal effect of the disruption of the CRABP I gene early in mouse embryogenesis (T. Lufkin et al., unpublished data). In this respect, the observation that CRABP II gene expression is induced by both all-trans RA and 9-cis RA suggests that CRABP II may play an important role in the cellular interpretation of the RA signal. 9-Cis RA has indeed been found in a number of tissues (Heyman et al., 1992). It is presently unknown whether CRABP I and/or CRABP II binds 9-cis RA. In any event, an induction of CRABP II expression by all-trans RA would probably result both in a decrease of free all-trans RA and in a reduction in the production of 9-cis RA that appears to be derived from all-trans RA (Heyman et al., 1992; Levin et al., 1992). We note in this respect that the CRABP II mRNA levels are markedly increased in P19 cells at 50 nM all-trans RA, which corresponds to the total concentration of all-trans RA in the chicken limb (Smith et al., 1989; Tabin, 1991).

#### **Experimental Procedures**

##### **Isolation of mCRABP II cDNA and 5' Flanking Genomic DNA Clones**

mCRABP II cDNA (Giguère et al., 1990) was obtained by polymerase chain reaction amplification using oligonucleotides QJ79 (5'-GGAGACAGCAAAGTATCTTTA-3', nucleotides 63-83 in mCRABP II cDNA; Figure 2) and QJ81 (5'-AAATCACACAGACTACA-3', nucleotides 818-802 in Giguère et al., 1990) and total RNA of mouse 9.5-day-old embryos. A mouse genomic  $\lambda$ EMBL12 library was screened with a randomly primed probe corresponding to the first 130 nucleotides of mCRABP II cDNA. A recombinant  $\lambda$ EMBL12 phage containing an ~12 kb insert was isolated, mapped, and characterized (Figure 1A), and a 1.6 kb PstI-BglII fragment was subcloned into pBluescript (Stratagene) (mCRABP II/1 clone) and sequenced using the dideoxy chain termination method (Figure 2).

##### **Primer Extension Mapping of the mCRABP II Transcriptional Start Site**

The transcriptional start site was mapped by primer extension using

a synthetic oligonucleotide complementary to nucleotides +103 to +64 in the CRABP II gene sequence (underlined in Figure 2). The <sup>32</sup>P-5' end-labeled oligonucleotide was annealed at 30°C with 10 µg of poly(A) RNA prepared from all-trans RA treated (10<sup>-6</sup> M) and untreated P19 EC cells and extended using avian myeloblastosis virus reverse transcriptase. Primer extension products, along with DNA sequencing reactions on CRABP II/1 using the above oligonucleotide as a primer, were electrophoresed on 6% urea-polyacrylamide gels and autoradiographed (data not shown).

#### Construction of Promoter-CAT Reporter Genes

A DNA fragment extending in the 5' direction from the BglII site (+118) to the BglII site located at approximately -2.5 kb was subcloned into the BglII site of the CAT reporter plasmid pBLCAT3<sup>+</sup> (Luckow and Schütz, 1987), giving CRABP II-CAT1 (Figure 1A). To produce CRABP II-CAT2, 3, and 4 (Figure 1A), CRABP II-CAT1 was partially digested with PstI and XbaI and religated on itself. CRABP II-CAT5 and CRABP II-CAT6 were produced by digestion of CRABP II-CAT2 with XbaI and XhoI, respectively, and religation. The CRABP II-CATm1, m2, and m3 mutants were obtained by double polymerase chain reaction amplification reactions as previously described (Ho et al., 1989). The oligonucleotides used were as follows: for CRABP II-CATm1, 5'-GGATTGCCTGACTCGAGTGACACTGTCTGG-3', 5'-GGCAGCCCCGCGATTGGGACGCTGGGACTT-3', 5'-CCCTCAGTCTTCCCGGGGCTATCCCCGTAG-3', and 5'-AAGTCCCCAGCGTCCCAATGCGGGGCTGCC-3', generating a XmaI-XhoI fragment containing the mutated bases; for CRABP II-CATm2, 5'-CAGCAAGCCGCGGGGTGG-3', 5'-GCCTGCAGGGCGAACACAGGT-3', 5'-GAGCC-CCCGGCTCGACAATCACTTGACACG-3', and 5'-CGTGTCAGTGA-TTGTCGAGCCGGGGGCTC-3', generating a mutated SacI-PstI fragment. These two fragments were then independently inserted into CRABP II-CAT1. For CRABP II-CATm3, we used the same first two oligonucleotides as for CRABP II-CATm2 and 5'-CCCCGGCTCGAT-TCTAGAAGGGCAG-3' and 5'-CTGCCCTTCTAGAATCGAGCCGGGGG-3'; the generated PstI-SacI fragment was inserted into CRABP II-CATm2. CRABP II-CATm4 was generated by insertion of the two mutated fragments XhoI-XmaI and SacI-PstI into CRABP II-CAT1. RARE1-TK-CAT, RARE2-TK-CAT, and βRARE-TK-CAT were constructed by inserting CRABP II RARE1, RARE2, or mRARβ2 RARE (βRARE) into the HindIII-XbaI sites of pBLCAT2<sup>+</sup> (Luckow and Schütz, 1987), as indicated in Figure 1A.

#### Cell Transfection and CAT Assay

P19 EC and HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 5% charcoal-treated fetal calf serum. Cells were transfected 4-6 hr after plating using the calcium phosphate procedure as described previously (Kumar et al., 1986) with 10 µg of the CAT reporter plasmids (as described in the figure legends), along with vectors expressing mRARα1, β2, or γ1 (Zelent et al., 1989) or mRXRα, β, or γ (Leid et al., 1992), mRARαdn or mRXRαdn (M. S. et al., unpublished data) or the parental expression vector pSG5 (Green et al., 1988), and 2 µg of β-galactosidase expression vector pCH110 (Pharmacia), which was used as an internal control to normalize for variations in transfection efficiency. The total amount of DNA in each transfection was standardized to 15 µg using carrier DNA (Bluescript). The medium was changed after 15-20 hr, and the appropriate ligands (all-trans RA or 9-cis RA) dissolved in ethanol were added to a final concentration of 1 µM or 50 nM (as described in the figure legends). After an additional period of either 12 or 24 hr, the cells were harvested and cellular extracts were prepared as described previously (Petkovitch et al., 1987). Cell extracts containing 50 U of β-galactosidase activity were used for CAT assays. The CAT activity was quantified by scintillation counting (see the figure legends).

#### Gel Retardation Assays

Gel shift-retardation experiments, including antibody supershift assays, were as previously described (Nicholson et al., 1990; Smith et al., 1991). The anti-mRARγ antibody has been described (Rochette-Egly et al., 1991), and the anti-human ER region F antibody (AbF3) was a gift from D. Metzger. Receptors were synthesized by transcription-translation *in vitro* using reticulocyte rabbit lysate in the presence and absence of [<sup>35</sup>S]methionine. Radiolabeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis, and the data were used to normalize

the amount of unlabeled receptor (translated in parallel) added to gel retardation assays. The amount of receptor used per assay was approximately 10 fmol.

#### RNA Isolation and Northern Blotting

Poly(A)<sup>+</sup> RNA from P19 EC cells treated for 15 hr with 50 nM all-trans RA, 50 nM 9-cis RA, or both ligands (50 nM each) was isolated as described (Leroy et al., 1991b). RNA samples were then separated on 2.2 M formaldehyde, 1% agarose gel, transferred to nitrocellulose membrane in 20 × SSC, fixed by 2 hr baking at 80°C, and hybridized by using randomly primed <sup>32</sup>P-labeled probes corresponding to the complete cDNA of mCRABP II or to a cDNA probe specific for mRARβ2 (Zelent et al., 1991) in 5 × Denhardt's solution, 5 × SSPE, 0.1% SDS, 100 µg/ml salmon sperm DNA at 65°C. The membranes were then washed three times with 2 × SSC, 0.1% SDS at 60°C. Autoradiography was from 1 to 3 days at -80°C with an intensifying screen.

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**Note Added In Proof**

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