The Directly Repeated RG(G/T)TCA Motifs of the Rat and Mouse Cellular Retinol-binding Protein II Genes Are Promiscuous Binding Sites for RAR, RXR, HNF-4, and ARP-1 Homo- and Heterodimers*

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We show here that the element which was previously characterized as a retinoid X receptor (RXR)-specific response element (RXRE) in the rat cellular retinol-binding protein II (CRBPII) gene is not conserved in the mouse gene. However, two conserved cis-acting elements (RE2 and RE3) located in the promoter region of the mouse and rat CRBPII genes mediate trans-activation by retinoic acid receptors (RARs) and RXRs in transfected Cos-1, CV-1, and HeLa cells. The element RE3 which is the major retinoic acid (RA) response element also binds the transcription factors HNF-4 and ARP-1. HNF-4 constitutively activates the mouse CRBPII promoter, whereas ARP-1 represses the activation mediated by RARs, RXRs, and HNF-4. In contrast, RA has no effect on the activity of the mouse CRBPII promoter in the human colon carcinoma cell line CaCo-2 which constitutively expresses RARα, RARγ, RXRα, HNF-4, and ARP-1, under conditions where the activity of the RARβ2 gene promoter is readily induced by RA. Our results suggest that the CRBPII gene may not be RA-inducible in tissues expressing HNF-4 and ARP-1, and that the RA inducibility of the CRBPII gene promoter observed in transfection experiments reflects the promiscuous binding of RARs/RXRs to HNF-4 and ARP-1 response elements.

Retinoids, which are structurally related to retinol (vitamin A) and its acidic form retinoic acid (RA),[1] appear to play a major role in embryogenesis, cell growth and differentiation,

[1] The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptors; HNF-4, hepatocyte nuclear factor 4; ARP-1, apoAI regulatory protein; COUP-TF, chicken ovalbumin upstream promoter transcription factor; CRBP, cellular retinol binding protein; CRABP, cellular retinoic acid binding protein; RARE, retinoic acid response element; RXRE, retinoid X response element; tk, thymidine kinase; CAT, chloramphenicol acetyltransfer-

and homeostasis (for reviews see Refs. 1–10). The pleiotropic action of retinoids is mediated through binding to multiple receptors which belong to the family of steroid-thyroid hormone receptors, and act as ligand-inducible trans-regulators that modulate the transcription of target genes by interacting with cis-acting DNA RA response elements (RAREs). The three types of retinoic acid receptors (RARs), RARα, β, and γ, can bind and respond to all-trans (T-RA) and 9-cis (9C-RA) retinoic acids, whereas the retinoid X receptors (RXRs), RXRα, β, and γ, can bind and respond to 9C-RA, but not to T-RA (11–14). RAR and RXR bind cooperatively as heterodimers to RAREs in vitro, more strongly than the respective homodimers (15–20). These heterodimers have been shown to mediate enhanced T-RA and 9C-RA retinoid-dependent transcription from RARE-containing target promoters (see above, Refs. 14 and 21). Natural RAREs appear to consist of a direct repeat (DR) of two core motifs (5'-RG(G/T)TCA or a closely related sequence) separated by a spacer of variable length of either 5 bp (DR5; RARα2 gene (22), RARβ2 gene (23, 24), ADH3 gene (25), complement factors H gene (26), RARγ2 gene (27)), 4 bp (DR4; laminin B1 gene (28)), 2 bp (DR2; CRBPI gene (29), CRABPII gene (21)) or 1 bp (DR1; CRABPII gene (21)) (for a review, see Ref. 14).

Two classes of cellular retinoid-binding proteins, while not directly implicated in transduction of the retinoid signal, probably play important roles in retinoid metabolism and homeostasis. The cellular RA-binding proteins, CRABPI and II, bind T-RA, but not 9C-RA, with high and slightly different affinities (see Refs. 13 and 30–32 and references therein). Although the precise function of these two proteins is unknown, they may be involved in the control of the concentration of free RA within a given cell (see Refs. 7 and 33 and references therein) and in intracellular RA metabolism (see Refs. 34 and 35 and references therein). The two cellular retinol-binding proteins, CRBPI and II, may play a role in the storage and metabolism of retinol. CRBPI which is expressed in many tissues has been suggested to regulate the formation of retinyl esters, the major form of storage of retinol, whereas CRBPII, which is expressed mostly in prenatal liver, intestine, and lung just prior to birth, and in adult intestine, may be involved in the intracellular transport of retinol during intestinal absorption (32, 36–38) (see Refs. 39 and 40 for reviews).

Interestingly, the expression of the CRABPII and CRBPI genes has been previously shown to be inducible by RA,

ase; T-RA, all-*trans*-retinoic acid; 9-*cis*-RA, 9-cis-retinoic acid; PCR, polymerase chain reaction; DR, direct repeat; ER, estrogen receptor; RE, response element; bp, base pair; kb, kilobase pair.

therefore providing possible feed-back mechanisms to regulate the levels of RA (21, 29, 31, 41–43). The goal of the present study was to investigate whether the expression of the mouse CRBPII (mCRBPII) gene could also be controlled by RA, which was suggested to us by the presence of several RG(G/T)TCA related motifs in the proximal region of the rat CRBPII gene promoter (44). We show here that two putative RAREs (RE2 and RE3) are conserved in the rat and mouse CRBPII gene 5′-flanking regions. These two putative proximal RAREs (RE2 and RE3) which correspond to a DR2 and a DR1 element, respectively, are required for maximal RA induction of the CRBPII promoter upon cotransfection with RAR and RXR expression vectors, whereas the more distal response element (RE1) which was previously characterized as a RXRE in the rat CRBPII (rCRBPII) 5′-flanking sequence (45) is not conserved in the mouse gene. Interestingly, the RE3 DR1 element, which is responsible for most of the RA inducibility in transfection experiments, binds efficiently to two other members of the nuclear receptor superfamily, HNF-4 and ARP-1, both of which are expressed in liver and intestinal cells. We show that HNF-4 constitutively activates the mCRBPII promoter, whereas ARP-1 competitively represses its activation by RAR, RXR, and HNF-4. Taken together our results strongly suggest that the expression of the CRBPII gene in intestinal cells may not be controlled by RA, and that the activation of the CRBPII promoter by overexpressed RAR and RXR reflects the promiscuous binding of RA receptors to HNF-4 response elements.

MATERIALS AND METHODS

Isolation of Mouse CRBPII cDNA and 5′-Flanking Genomic DNA Clones and Mapping of the Transcription Starting Site by Primer Extension—mCRBPII cDNA was cloned by two PCR steps with reverse transcribed adult mouse intestinal poly(A) RNA. In the first step, degenerate oligonucleotides corresponding to amino acids 7–12 and a 3′-untranslated region antisense oligonucleotide 5′-GGCTTCCCCTGGGCCCATCA (nucleotides 459–478) of the rat CRBPII cDNA (46) were used to amplify the cDNA and subsequently cloned into Bluescript SK+. To obtain the sequence coding for the remaining N-terminal amino acids and 5′-UTR, an antisense oligonucleotide corresponding to amino acids 105–121 (Fig. 1A) was used to reverse transcribe intestinal mRNA, and the resulting cDNA was dG-tailed. Anchored PCR was performed with another antisense nested primer corresponding to amino acids 87–103 (Fig. 1A) as described elsewhere (47), and the PCR product was subcloned into Bluescript SK+. The unique EagI site at position 294 of the cDNA (Fig. 1A) was used to generate a full-length cDNA which was sequenced on both strands by dideoxy chain termination procedure.

A mouse genomic λEMBL3 library (a gift from L. Stubbs) was screened with the ^{32}P-5′-end-labeled oligonucleotide (OX42) corresponding to nucleotides 82–130 of the mCRBPII cDNA (*overlined* in Fig. 1A). Four recombinant λEMBL3 phages containing ~12 kb inserts were isolated and a 4.3-kb HindIII fragment was subcloned into the HindIII site of Bluescript SK+ to derive the plasmid CRBPII/SK+1. The ~2.3-kb BspHI fragment of CRBPII/SK+1 was treated with Klenow and cloned into the SmaI site of SK+ to yield the plasmid CRBPII/SK+2. Note that one of the BspHI restriction site overlaps the initiation codon of the mCRBPII cDNA (Fig. 1A).

The transcriptional starting site was mapped by primer extension using the synthetic oligonucleotide 5′-CATTACTCTCCATTTCC-CAGG-3′ complementary to nucleotides +80 to +100 in the genomic sequence (Fig. 1B). The ^{32}P-5′-end-labeled oligonucleotide was annealed at 30 °C with 3 μg of poly(A) RNA from mouse intestine and extended using avian myeloblastosis virus reverse transcriptase as described previously (48). Primer extension products, along with DNA sequencing reactions of CRBPII/SK+1 using the above oligonucleotide as a primer, were electrophoresed on 6% urea-polyacrylamide gels and autoradiographed (Fig. 1C).

Construction of Reporter Genes for mCRBPII Promoter Activity— CRBPII/SK+2 was digested with BamHI and XhoI and the fragment

containing the mCRBPII promoter sequence was ligated into BamHI-XhoI sites of pBLCAT3⁺ (49) yielding the plasmid mCRBPII/CAT1. mCRBPII/CAT2 was generated by digesting mCRBPII/CAT1 with SphI and religation. For creating mCRBPII/CAT3 and mCRBPII/CAT4 deletion mutants, nested deletions were done in CRBPII/SK+2 by first digesting with SacII and BamHI and subsequently with exonuclease III and mung bean nuclease as described previously (48). mCRBPII promoter fragments were prepared from the clones obtained with the above material which was sequentially treated with SacI, S1 nuclease, and XhoI, before ligation into the Klenow-treated BamHI site and the XhoI site of pBLCAT3⁺. Deletion end points in mCRBPII/CAT3 and mCRBPII/CAT4 were identified by sequencing. mCRBPII/CAT5 and mCRBPII/CAT10 were generated by ligating PCR-amplified fragments of CRBPII/SK+2 into BamHI-XhoI sites of pBLCAT3⁺. The oligonucleotide 5′-CCGGATCCTGT-GAACTGAGACAAAATGA-3′ plus M13 universal primer and the oligonucleotide 5′-GGGATCCCTGATGGAATGTTCTGTGTG plus M13 universal primer were used as primers for amplification of the mCRBPII fragments present in mCRBPII/CAT5 and mCRBPII/CAT10, respectively.

The reporter constructs mCRBPII/tk-CAT6, mCRBPII/tk-CAT2, and mCRBPII/tk-CAT5 were generated by ligating various lengths of mCRBPII promoter fragments into the SphI-BamHI sites of pBLCAT8⁺ (29). Promoter fragments were obtained by PCR amplification of CRBPII/SK+2 (for tk-CAT6 and CAT2) or mCRBPII/CAT4 (for tk-CAT5) using the following primers: 5′-GGGGATCCG-GACCAGGTATATAAAGATA-3′ and 5′-TTCTTGCATGCAAG-GCCTGTGAGG-3′ for mCRBPII/tk-CAT6; 5′-AAGGATCCGGT-CTGCAAGGCTCATG-3′ and TTCTTGCATGCAAGGCCTGTG-AGG-3′ for mCRBPII/tk-CAT2; 5′-GGGGATCCGGACCAGGTA-TATAAAGATA-3′ and 5′-GCTTGCATGCCTGCAGGTCG-3′ (corresponding to pBLCAT3⁺ sequence) for mCRBPII/tk-CAT5.

The point mutants mCRBPII/CAT3 mut1 to mut4 were generated by two-step PCR amplification as described by Ho *et al.* (50) using mCRBPII/CAT3 as template. The same procedure was used to generate mCRBPII/CAT3 mut5, mut6, mut7, and mut8 by using mCRBPII/CAT3 mut1, mut2, mut3, and mut7 as templates, respectively.

Expression vectors for mRARα1, mRARβ2, mRARγ1, mRXRα, mRXRβ, and mRXRγ have been described previously (17, 51). The HNF-4 expression vector was constructed by cloning HNF-4 cDNA (a kind gift from W. Zhong and J. Darnell) into the EcoRI site of pSG5 (52). The cDNA for ARP-1 was recloned from CaCo-2 cell mRNA by PCR amplification using previously published sequences as primers ((53); 5′-GGGAATTCCGGAAGTCCGGACGCAGCCC-CCATAGATATGG-3′ (nucleotides 316–346) and 5′-GGAGATCTC-CTTTTCTTTCTCTGTTTCACTCCCCCTTCTT-3′ (complementary strand, nucleotides 1599–1630)). The cDNA was cloned into the EcoRI-BglII sites of pSG5; our cloned isoform does not contain amino acids 5–48 of the published cDNA. The human estrogen receptor (ER) DNA fragment coding for amino acids 553–595 (54) was fused in-frame to the 3′-end of the HNF-4 or ARP-1 coding sequences to obtain antigen-tagged proteins.

Cell Transfection and CAT Assays—Cos-1 and CV-1 cells were cultured in Dulbecco's medium containing 5% delipidated fetal calf serum. F9, P19, and HepG2 cells were maintained in Dulbecco's medium containing 10% charcoal-treated serum. CaCo-2 cells were maintained in Dulbecco's medium with 20% charcoal-treated medium. Cells were transfected 12–16 h after plating by a calcium phosphate procedure as described previously (55) with 5 μg of reporter plasmids, 0.5 μg of expression vectors unless stated otherwise in the figure legends, and 2 μg of the β-galactosidase expression vector pCH110 (Pharmacia LKB Biotechnology Inc.) which was used as an internal control for transfection efficiency. The total amount of DNA in each transfection was standardized to 20 μg using carrier DNA (Bluescript). In all transfections, the amount of expression vector was kept constant by substituting with pSG5. The medium was changed after 15–20 h, and the appropriate ligands dissolved in ethanol were added. After an additional 20–24 h, the cells were harvested, and cell extracts were prepared as described previously (56). The CaCo-2 cell extracts were heated at 65 °C for 5 min before CAT assays. The CAT activity was quantified by liquid scintillation counting.

Gel Retardation Assay—Preparation and incubation of either *in vitro* transcribed-translated receptors and whole cell extracts with ^{32}P-5′-labeled oligonucleotide probes (in the presence of 100 mM

KCl), and gel retardations were as described previously (17, 57). Antibody preparations used in "supershift" assays were as described elsewhere (17, 28). The sequences of the oligonucleotide probes are listed in Table II.

RESULTS

Comparison of Mouse and Rat CRBPII cDNA, Transcription Starting Sites, and 5'-Flanking Genomic Sequences—The amino acid-coding sequence of the mouse CRBPII (mCRBPII) cDNA (see "Materials and Methods") and deduced amino acid sequence (134 residues, molecular mass = 18,005 daltons; see Fig. 1A) are 94.8 and 98.5% homologous to their rat (rCRBPII) counterparts, respectively (44). The

only amino acid differences correspond to valine and asparagine in rCRBPII instead of threonine and histidine at mCRBPII positions 44 and 82, respectively.

Assuming that the rat and mouse CRBPII genes have similar intron/exon organization and that sequences up to nucleotide 130 of the cDNA represent the first exon (44), we employed an oligonucleotide corresponding to nucleotides 82–130 of the cDNA (OX42, *overlined* in Fig. 1A) to probe a λEMBL3 mouse genomic library. Four clones containing DNA inserts (~12 kb) with similar restriction enzyme site patterns were obtained. Further analysis of a 4.3-kb *Hind*III fragment, which hybridized with the OX42 probe in Southern blots, identified a *Bsp*HI fragment which extended ~2.3 kb in
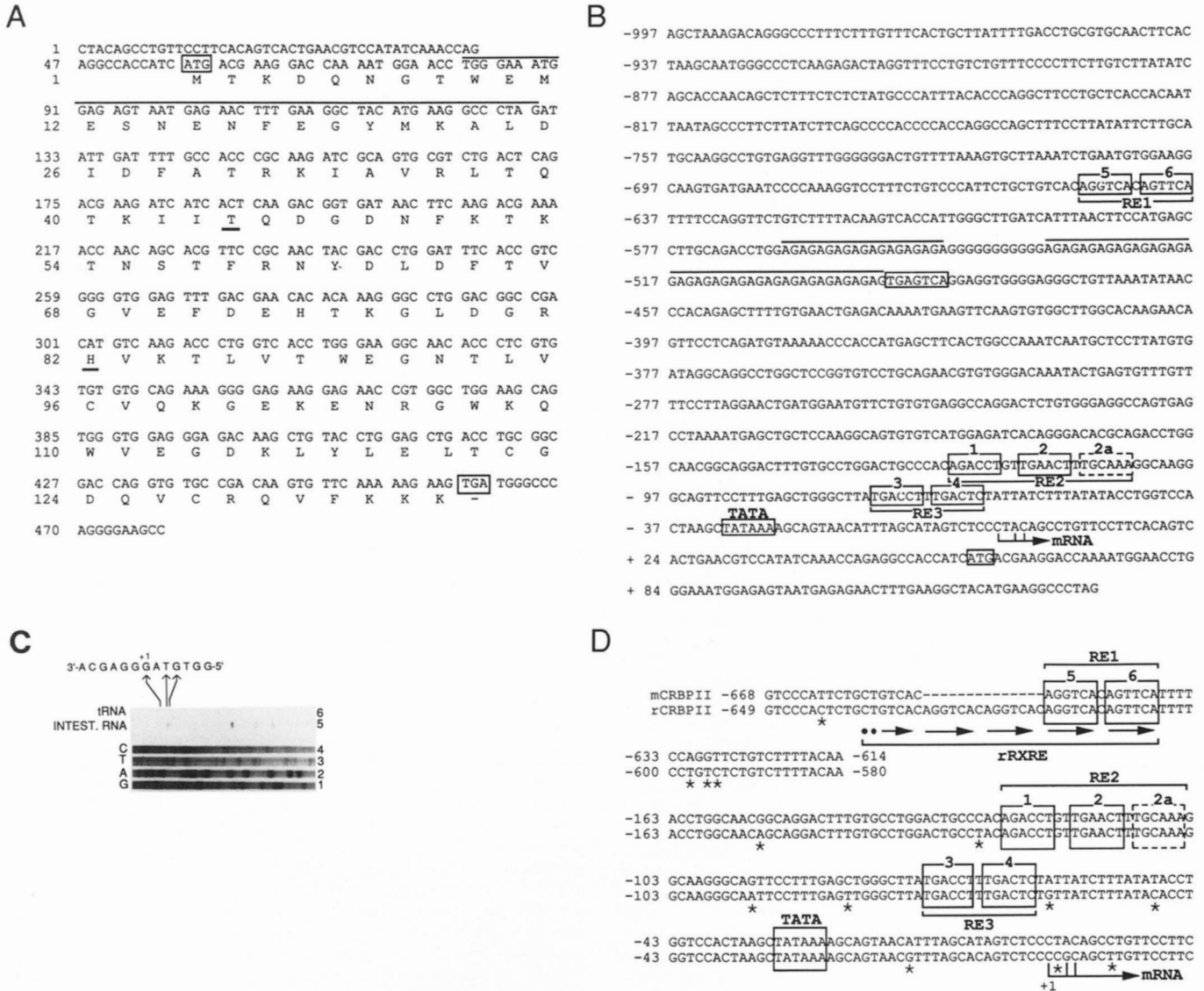
FIG. 1. *A*, sequence of the mouse CRBPII cDNA and predicted amino acid sequence (see "Materials and Methods"). The *overlined sequence* represents the sequence of the oligonucleotide used for screening the genomic library. The two amino acids that are different from those of rat CRBPII are *underlined*. Initiation and stop codons are *boxed*. *B*, sequence of the mouse CRBPII gene 5'-flanking region. Nucleotides are numbered such that +1 corresponds to the longest reverse transcribed product (see text and *panel C*). Sequences corresponding to 5'-RG(G/T)TCA or related motifs (5'-TGA(A/C)CY on the other strand) are *boxed* and *numbered 1–6*. Repeated motifs that appear in pairs are labeled as *RE1, 2,* and *3*. The TATA box, the initiation codon, and the AP-1 binding site (5'-TGAGTCA-3') are also *boxed*. Stretches of repeating AG dinucleotides are *overlined*. *C*, mapping of the CRBPII starting site by primer extension analysis. Products of primer extension using either 3 μg of poly(A) RNA from mouse intestine (*lane 5*) or tRNA (*lane 6*), were resolved on a 6% urea-polyacrylamide gel and autoradiographed. *Lanes G, A, T,* and *C* contained sequencing reaction products of CRBPII/SK+1 with the primer used for primer extension. *Arrows* represent major primer extension products. The sequence shown corresponds to the non-coding strand. *D*, alignment of the 5'-flanking sequences of mouse and rat CRBPII genes. Non-conserved bases are indicated by *asterisks*. *Arrows* indicate the repeated motifs of the rat CRBPII promoter which have been described as an rRXRE (45). Other symbols are as in *B*.

the 5' direction from the initiator codon. Approximately 1 kb of this fragment was sequenced (Fig. 1B); as expected its 3' extremity overlapped with the 5' end of the cDNA. To map the mCRBPII starting site, primer extension analysis was performed using an oligonucleotide corresponding to nucleotides +80 to +100 in the cDNA and poly(A) RNA from mouse intestine (Fig. 1C). As is the case for rCRBPII (44), several starting sites were found (Fig. 1, B and C). The 5'-most nucleotide of the longest reverse transcript, which also corresponded to the 5' end of the cDNA, was considered as position +1 of the mCRBPII gene.

A comparison between the mCRBPII and rCRBPII proximal promoter sequences reveals a high degree of similarity (Fig. 1D). Identical sequences that may be functionally important include the TATA box, and sequences corresponding to 5'-RG(G/T)TCA motifs (5'-TGA(A/C)CY, on the other strand) which have been characterized in a number of natural and synthetic response elements for nuclear receptors (see Introduction and below) (elements RE1, RE2, and RE3, motifs 1-6, in Fig. 1D). On the other hand, the CAAT boxlike sequence present at position −93 to −96 in the rCRBPII promoter (44), and the AP-1 binding site (5'-TGAGTCA-3') found at position −486 to −492 in the mCRBPII promoter (Fig. 1B) are not conserved between the two species. In addition, the mCRBPII AG repeat (*overlined* in Fig. 1B) is replaced by a TG repeat in rCRBPII. Interestingly, the sequence 5'-AGGTCACAGGTCA-3' located at position −631 to −619 in the rCRBPII promoter, which together with the RE1 element was previously characterized as corresponding to a specific RXR response element (RXRE, see Ref. 45), is not present in the mCRBPII 5'-flanking region (Fig. 1D).

Characterization of Retinoic Acid Response Elements in the 5'-Flanking Region of the Mouse CRBPII Promoter—To investigate whether response elements (REs) for retinoic acid receptors may possibly be present in the mCRBPII 5'-flanking region, the reporter plasmid mCRBPII/CAT1, which contains ~2.3 kb of 5'-flanking sequences inserted in pBLCAT3⁺ (see "Materials and Methods"), was transfected into various cell types together with vectors expressing RARα1 (also called RARα hereafter) or RXRα (see Table I for a summary of the results). Upon cotransfection with the parental expression

vector (pSG5), no significant constitutive nor T-RA- or 9C-RA-inducible mCRBPII/CAT1 expression was observed in Cos-1 cells (Fig. 2A, *lanes 22-24*; Table I). Similar results were obtained in HeLa cells, CV-1 cells, and F9 and P19 embryonal carcinoma (EC) cells (Table I, and data not shown). A weak constitutive activity (~2-3-fold above pBLCAT3⁺), which was not significantly increased by addition of T-RA or 9C-RA was observed in human colon carcinoma CaCo-2 and hepatoma HepG2 cells (Table I, and data not shown). Under similar conditions, the expression of a reporter gene containing the RA-inducible RARβ2 promoter (mRARβ2/CAT, see Ref. 29) was effectively stimulated in the same cells by endogenous RARs in the presence of either T-RA or 9C-RA (Fig. 2A, *lanes 1-3*; Table I, and data not shown).

Cotransfection of mRARα1, mRARβ2, mRXRα, and mRXRγ, but not of mRARγ1 and mRXRβ expression vectors, resulted in RA-inducible expression of mCRBPII/CAT1 in Cos-1 cells (Fig. 2A, *lanes 25-42*; the low activity seen in lane 33 was not reproducibly observed). In HeLa cells, induction of mCRBPII/CAT1 expression by mRARα1 was lower than by mRXRα, whereas in CV-1 cells only mRXRα, but not mRARα1 induced mCRBPII/CAT1 expression (Table I, and data not shown). In HepG2 and CaCo-2 cells, there was only a weak stimulation by RA when RAR or RXR were cotransfected (Table I, and data not shown). No stimulation was observed under any conditions in the F9 and P19 embryonal carcinoma cells (EC), even though the RARβ2 promoter was highly active in these cells (Table I, and data not shown). This observation was particularly surprising in view of the previous results of Mangelsdorf *et al.* (45) who reported a markedly and specific activation of the rat CRBPII promoter in F9 cells cotransfected with a RXR expression vector.

To identify the elements responsible for RA-induced stimulation of mCRBPII promoter activity, various deletion constructs were made and cotransfected into Cos-1 cells with either mRARα1 or mRXRα (Fig. 2B). mCRBPII/CAT2, which contains 760 bp upstream of the transcription starting site, showed higher basal and RA-induced activities than mCRBPII/CAT1. Deletion of the RE1 sequence (mCRBPII/CAT3) resulted in a slight increase in these activities.

TABLE I

Activity of CAT reporters for the mCRBPII promoter (mCRBPII/CAT1), the mRARβ2 promoter (mRARβ2/CAT) and the rRXRE/tk promoter (rRXRE/tk-CAT) in various cell types

Cos-1, CV-1, F9, HepG2, and CaCo-2 cells were transfected with 5 µg of CAT reporter constructs along with 0.5 µg of receptor expression vectors (or the parental vector pSG5) as indicated. The CAT activity of the reporter cotransfected with pSG5 in absence of ligands is taken as one. The basal level activity of mCRBPII/CAT1 was 2-3-fold higher than that of pBLCAT3⁺ in CaCo-2 and HepG2 cells (asterisks). Similarly, rRXRE/tk-CAT had ~3-fold higher basal level activity than pBLCAT8⁺ in CaCo-2 cells (asterisk). The results correspond to the average (±15%) of several independent transfection experiments.

| Reporter	Cell type	Relative CAT activity														
		pSG5			RARα1			RXRα			RARα1 + RXRα			rHNF-4		
		−	T-RA	9C-RA	−	T-RA	9C-RA	−	T-RA	9C-RA	−	T-RA	9C-RA	−	T-RA	9C-RA
mCRBPII/CAT1	Cos-1	1	1.5	1.5	0.2	7.5	9	2.2	13	20	1.2	13.5	23	14.5	15	20
	CV-1	1	1.5	1.5	1	1.5	1.5	2	9	14	1	6	11	44	33	31
	CaCo-2*	1	1.5	1.5	1	1.5	1.5	1	2	3	1.5	2	3	27	16	27
	HepG2*	1	1	1	0.7	1	1	0.8	2.1	2	1	1.2	1.6	10	9	7
	F9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
mRARβ2/CAT	Cos-1	1	12	10	0.5	131	146	1.5	37	40	1	105	122	2	99	94
	CaCo-2	1	11	7	1	89	77	1	38	139	2	193	306	2	29	34
	HepG2	1	34	27	0.7	48	46	2	46	48	1.8	42	33	0.9	43	35
	F9	1	231	379	4	260	257	2	313	423	3	313	345	2	230	243
rRXRE/tk-CAT	Cos-1	1	1	1	0.7	2.5	5	1.3	59	183	1.7	23	97	4	4	5
	CaCo-2*	1	0.5	0.5	0.3	0.5	0.5	0.3	3	28	0.4	3	20	1	1	1
	F9	1	4	5	0.5	5	9	1	24	16	1	35	40	8	13	15
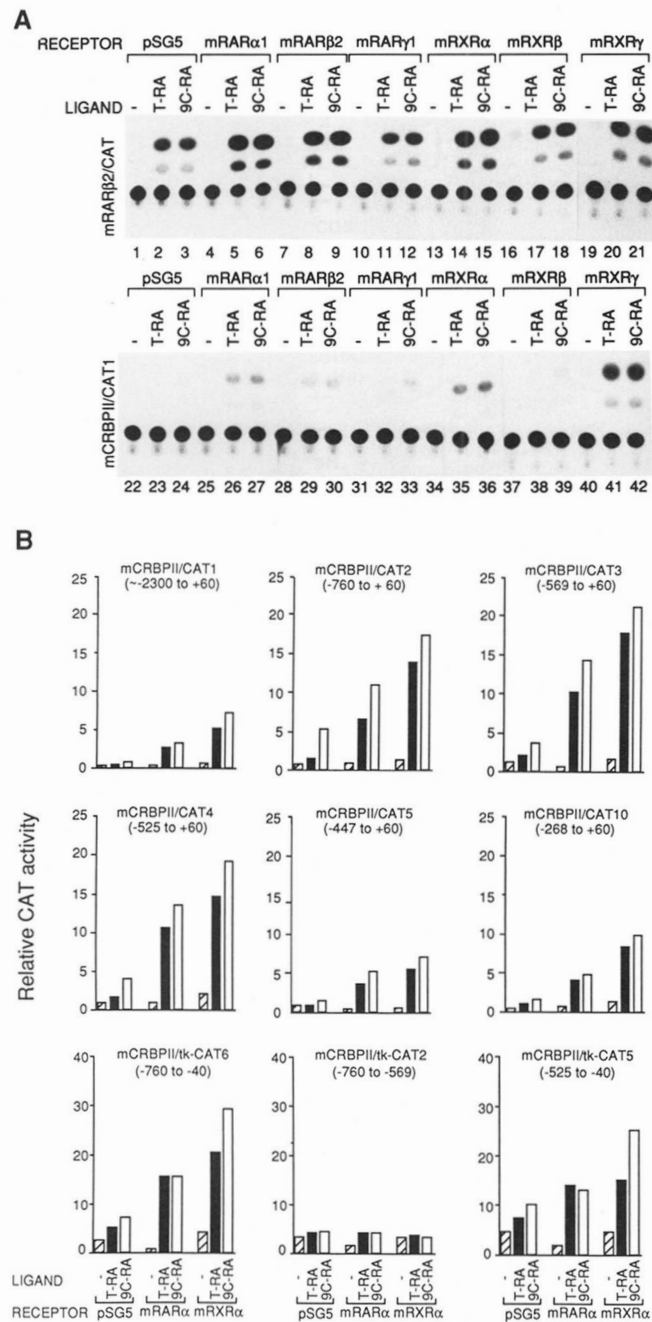
FIG. 2. **RA-inducibility of mouse CRBPII promoter activity.** *A*, 5 μg of mRARβ2/CAT and mCRBPII/CAT1 (see "Materials and Methods") were transfected with 0.5 μg of the indicated RAR or RXR expression vectors (or the parental vector pSG5) into Cos-1 cells. 10^{-6} M T-RA or 9C-RA were added 24 h later, and cells were harvested after 24 h. Transfection efficiency was standardized by cotransfection of 2 μg of the β-galactosidase expression vector pCHII0. *B*, plasmids (5 μg) carrying various lengths of the mCRBPII promoter in either pBLCAT3⁺ vector (*mCRBPII/CAT1–10*) or pBLCAT8⁺ vector (*mCRBPII/tk-CAT6, -CAT2,* and *-CAT5*) (see "Materials and Methods") were transfected into Cos-1 cells with the indicated RAR or RXR expression vector. Culture conditions and CAT assays were as in *A*. The length of mCRBPII gene 5'-flanking fragments is indicated in *parentheses*. The results (±15%) correspond to the average of several independent transfection experiments.

mCRBPII/CAT4, which lacks part of the AG repeat (*over-lined* sequence in Fig. 1*B*) was as active as mCRBPII/CAT3. Deletion of the −447 to −525 sequence resulted in parallel reduction of constitutive and RA-induced activities

(mCRBPII/CAT5). Note that this region contains an AP-1 binding motif (Fig. 1*B*). Further stepwise deletions to −268 did not alter constitutive nor RA-induced activities (Fig. 2*B*, mCRBPII/CAT10, and data not shown).

The above results indicated that a RARE(s) could be located between −268 and +60. Thus, a second series of reporter constructs were made, in which various lengths of 5'-flanking mCRBPII sequences were cloned upstream of the herpes simplex virus thymidine kinase (tk) promoter (Fig. 2*B*, and data not shown). mCRBPII/tk-CAT6, which contains RE1, RE2, and RE3 sequences, was induced by RA when cotransfected with either mRARα or mRXRα in Cos-1 cells. Constructs containing only RE1 (but not RE2 nor RE3) and the AP-1 binding site were not RA-responsive (mCRBPII/tk-CAT2, and data not shown). However, reporter constructs containing RE2 and RE3 responded to RA (mCRBPII/tk-CAT5, and data not shown). These results, together with the above deletion mutation analysis, indicated that some sequences located between positions −40 and −268 were required for RA inducibility.

The possible function of the repeated motifs present in the putative response elements RE2 and RE3 was investigated by generating mutations in mCRBPII/CAT3 (Fig. 3*A*). mCRBPII/CAT3 mut1 to mut4 contain mutations in motifs 1–4, respectively; mCRBPII/CAT3 mut5 to mut7 contain mutations in motifs 1 and 2, 2 and 3, and 3 and 4, respectively, whereas all four motifs are mutated in mCRBPII/CAT3 mut8. Mutation of motif 1 (*mut1*) had no apparent effect (Fig. 3*B*). Mutation of either motif 2 (*mut2*) or motif 3 (*mut3*) impaired both mRARα1 and mRXRα-mediated RA inducibility (note that the A → C mutation abolished both RXR homodimer and RXR·RAR heterodimer binding to the two REs, data not shown). Mutation of motif 4 also reduced RA inducibility, particularly in the case of mRXRα. Mutating motifs 1 and 2 (*mut5*) had the same effect as mutating motif 2 alone (*mut2*), whereas mutating motifs 2 and 3 (*mut6*) resulted in a strong decrease in mRXRα-mediated inducibility, and in a complete loss of induction by mRARα1. No induction was seen when motifs 3 and 4 (*mut7*) or all 4 motifs (*mut8*) were mutated. We conclude from these results that RE2, which is inactive on its own (see *mut7*), can cooperate in RA inducibility with either motif 3 (see *mut4*), motif 4 (see *mut3*) or both motifs 3 and 4 (compare mCRBPII/CAT3 and *mut5*). In addition motif 1 appears to be dispensable (see *mut1*), at least when the other motifs are intact. We note also that RE3 may be more efficient with mRXRα than with mRARα (see *mut5*), whereas the reverse situation may apply to RE2 (see *mut4*).

Binding of RARα and RXRα to mCRBPII REs in Vitro— To investigate whether the above differential effects of RE2 and RE3 *in vivo* could be correlated with differences in binding of RAR and RXRs to these REs *in vitro*, binding retardation/shift assays were carried out using RE2 and RE3 oligonucleotide probes (see Table II for their sequences) together with either *in vitro* translated hRARα1 (which is almost identical to mRARα1, see Ref. 58) or the antigen-tagged fusion protein, mRXRαER(F), which contains the F region of the human estrogen receptor (hER) fused to the C-terminal amino acid of mRXRα (called mRXRαER(F) in Ref. 17). The binding of RAR·RXR heterodimers was also tested, as they have been shown to bind much more efficiently to RAREs than either RAR or RXR homodimers (17) (see Introduction for additional references).

No detectable binding of RARα alone was observed with either RE2 or RE3 in the absence or presence of RARα antibody (Fig. 4*A*, *lanes 1* and *2*). Similarly RXRα alone did
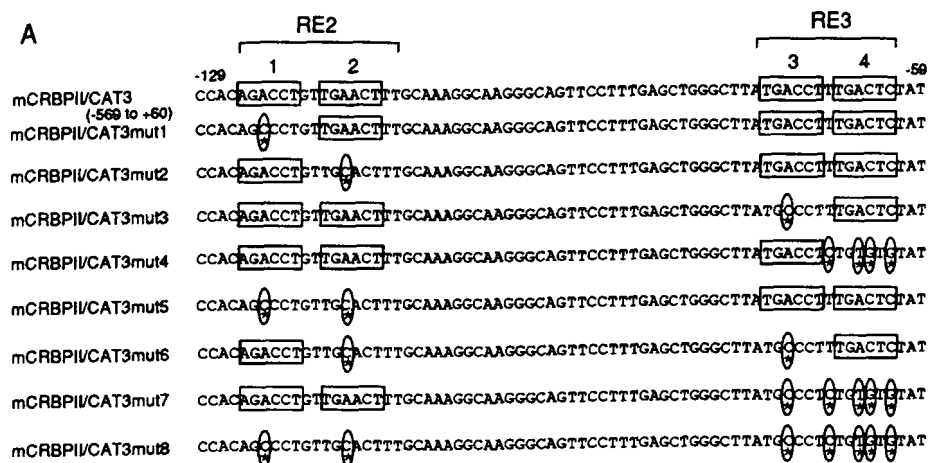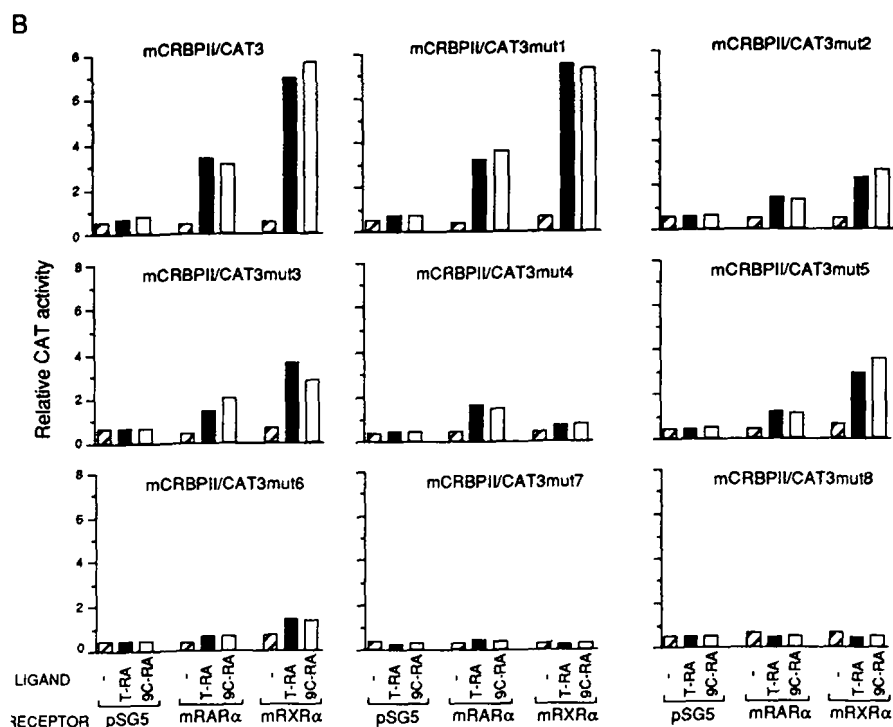
FIG. 3. **Characterization of mCRBPII RAREs by site-directed mutagenesis.** *A*, mutated sequences of mCRBPII REs in mCRBPII/CAT3 mut1 to mut8 are indicated by *asterisks* and *circles*. *B*, mutants (5 μg) were transfected into Cos-1 cells along with 0.5 μg of RAR or RXR expression vectors, as indicated. Culture conditions and CAT assays were as in Fig. 2.

not bind to either of the two probes (*lane 3*), although a supershifted complex could be detected in the RE3 case in the presence of RXR antibody (*lane 4*). In contrast, a complex was detected in the presence of both RARα and RXRα (*lane 5*), which could be supershifted by either anti-RARα (*lane 6*) or anti-RXRα (*lane 7*) antibody. Thus, RAR·RXR heterodimers bind to both RE2 and RE3 of the CRBPII gene. Note that although both the RE2 and RE3 probes were labeled to approximately the same specific activity, the intensity of the RAR·RXR complexes were different (RE3 stronger than RE2 complexes), and in both cases lower than that of the complex formed with the RARE of the RARβ2 gene (β2RARE) (Fig. 4A, and see below). Note also that, in contrast to the report of Zhang *et al.* (59), the addition of 9C-RA to the reaction did not increase by more than 2-fold the formation of complexes between RXRα and the various REs studied here and below (data not shown).

Binding of HNF-4 and ARP-1 to CRBPII RE2 and RE3 in Vitro—That the mCRBPII promoter could not be induced by RA in HepG2 and CaCo-2 cells suggested to us that other factors may interact with the CRBPII RAREs, thus prevent-

ing RAR and RXR binding. Comparison of CRBPII RE3 with the response elements of other members of the steroid hormone receptor superfamily revealed a similarity with an element present in the apolipoprotein CIII gene promoter, which binds the homodimers of orphan nuclear receptors HNF-4, ARP-1, Ear2, and Ear3 (apoCIII element in Table II) (53, 60, 61). Similarly, CRBPII RE2 along with five nucleotides extending in the 3'-direction (Fig. 1B), exhibits some similarity with both the apoCIII element and a 20-fold weaker HNF-4 binding site (HNF-4P) present in the transthyretin (TTR) gene (Table II) (60). To investigate whether these transregulators could possibly bind to the mCRBPII REs, binding assays were carried out with *in vitro* translated rat HNF-4 (rHNF-4) and human ARP-1 (hARP-1) antigen-tagged with the F region of hER (hARP-1ER(F)). We also investigated whether HNF-4 and ARP-1 could form heterodimers with each other, and with either RARs or RXRs.

HNF-4 readily formed a specific complex with RE3 (Fig. 4A, *lane 8*). Addition of RARα (*lane 9*) or RXRα (*lane 10*) did not modify the formation of the RE3·HNF-4 complex, nor generated any additional complex, suggesting that RAR

TABLE II

Nucleotide sequence of response element probes and summary of their binding to various receptors in vitro

RG(G/T) TCA motifs are indicated by arrows and degenerate nucleotides within motifs are indicated by dots. Numbers 1 to 6 over the arrows in RE1 to 3 are the same as in Figure 1B. The location of the response elements in their respective promoters is indicated. The relative binding efficiency of each receptor homodimer or heterodimer to a given response element has been simi-quantitatively scored from the results shown in Fig. 4B (± to 5+ scale in vertical columns). Note that the scores cannot be compared horizontally for the binding efficiency of the different receptors to a given response element.

GENE		RESPONSE ELEMENTS	RECEPTOR BINDING IN VITRO					
			RAR:RXR	HNF-4	ARP-1	ARP-1:RXR	(RXR)2	(RAR)2
CRBPII	RE2	5'-GATCTTTGCAAAGTTCAACAGGTCTGTGG-3' (-104 to -129)	+	±	+	±	-	-
CRBPII	RE3	5'-GATCAGAGTCAAAGGTCATAAGCCCA-3' (-61 to -82)	++	++	++++	++	++	-
CRBPII	RE1	5'-GATCCATTCTGCTGCTGTCACAGGTCA 5'-CAGTTCATTTCC-3' (-664 to -632)	±	+	+++	+	±	-
rCRBPII	rRXRE	5'-GATCCTCTGCTGTCACAGGTCAC AGGTCACAGGTCACAGTTCATTTCC-3' (-643 to -596)	++	+	+++	++	+	-
Synthetic	DR1G	5'-TCGAGGGTAGGGGTCAGAGGTCACTCG-3'	++++	+++	++++	+++	++++	-
mRARβ2	DR5T (β2RARE)	5'-TCGAGGGTAGGGTTCACCGAAAGTTCACTCG-3' (-59 to -33)	+++++	-	±	±	-	-
hapoCIII	apoCIII	5'-CGCTGGGCAAAGGTCACCTGC-3' (-67 to -87)						
mTTR	HNF4-P	5'-CCCTAGGCAAGGTTCATATGG-3' (-156 to -140)						

and RXRα do not heterodimerize with HNF-4. Like HNF-4, ARP-1 also formed a complex with RE3 (*lanes 11* and *12*). Addition of mRXRα (*lane 14*), but not mRARα (*lane 13*), along with ARP-1, resulted in an additional complex which could be supershifted with either anti-ARP-1 ER(F) or anti-RXR antibodies (*lane 15*, and data not shown; note that in all cases the binding of nuclear receptors tagged with the F region of the ER was identical to that of their wild-type counterparts). Thus ARP-1 can heterodimerize with RXRs, and the heterodimer can bind RE3. However, when both RARα and RXRα were added along with ARP-1, RAR·RXR heterodimeric and ARP-1 homodimeric complexes were predominantly formed (*lane 16*), indicating that RAR·RXR heterodimers are likely to be more stable than ARP-1·RXR heterodimers. The addition of both ARP-1 and HNF-4 to RE3 and other elements used in this study (see below) resulted in the formation of only two complexes (*lanes 17* and *18*), which migrated like those obtained with isolated ARP-1 and HNF-4, suggesting that ARP-1 and HNF-4 do not heterodimerize, in agreement with the recent results Mietus-Snyder *et al.* (62) obtained with the apoCIII element. In fact the two complexes observed here with both ARP-1 and HNF-4 and RE3 as well as other elements migrated like those obtained with the apoCIII element (data not shown).

In contrast, no complex was formed between RE2 and HNF-4 in the absence (Fig. 4A, *lane 8*) or in presence of RARα (*lane 9*) or RXRα (*lane 10*; the complex seen at the bottom of the lane is nonspecific). Similarly, ARP-1 did not bind to RE2 either on its own (*lane 11*) or together with RARα (*lane 13*) or RXRα (*lane 14*), and no specific complex could be detected when both HNF-4 and ARP-1 were added to RE2 (*lane 17*). However, weak RE2·ARP-1 and RE2· HNF-4 supershifted complexes were formed in the presence of the corresponding antibodies (*lane 12*, and data not shown).

Comparative Binding to Mouse CRBPII RE1, Rat CRBPII RXRE, and a Synthetic DR1 Element in Vitro—As mentioned above, an element containing four directly repeated AG(G/

T)TCA motifs with 1-base pair spacers was reported to be the RXR-specific response element of the rat CRBPII gene (rRXRE, see Ref. 45). However, the corresponding region in the mouse CRBPII gene contains only two of these motifs (*RE1* in Fig. 1D), which do not appear to be required for either RAR or RXR-mediated induction of the mCRBPII promoter (see Fig. 2B). Therefore, we investigated the *in vitro* binding properties of RE1 and compared them with those of rRXRE and of a synthetic element consisting of two directly repeated RGGTCA motifs separated by one bp (DR1G, see Table II). Neither rRXRE nor RE1 or DR1G bound to RARα alone (Fig. 4A, *lane 1*). Interestingly, a weak binding of mRXRα which was greatly stabilized by the cognate antibody was observed with rRXRE, but not with RE1 (compare *lanes 3* and *4* of *rRXRE* and *RE1*), whereas a RXR-specific homodimeric complex which could be supershifted by the RXR antibody was readily detected with the DR1G probe (*lanes 3* and *4*). As it was the case for RE2 and RE3, a stronger RAR· RXR heterodimeric complex was observed in all three cases, which could be supershifted by antibodies directed against either RARα or RXRα (*lanes 5-7*).

HNF-4 readily bound all three probes, albeit with different efficiencies (see also below), and this binding was not affected by the presence of either RARα or RXRα (*lanes 8-10*). ARP-1 bound very efficiently to both rRXRE, RE1, and DR1G (compare *lanes 11*). As in the case of RE3, an ARP-1·RXRα heterodimeric complex was formed with all three probes, which could be supershifted by either anti-ARP-1 or anti-RXR antibody (*lanes 14* and *15*, and data not shown). However, in the presence of RARα, RXRα, and ARP-1, the major complexes formed corresponded to a RAR·RXR heterodimeric complex and a ARP-1 homodimeric complex (*lane 16*). No HNF-4·ARP-1 heterodimeric complexes could be detected (*lanes 17*). Note that, in marked contrast with all of these results, a probe corresponding to the RARE of the RARβ2 promoter (β2RARE) was efficiently bound only by a RAR· RXR heterodimer (Fig. 4A), although weak ARP-1 homodi-
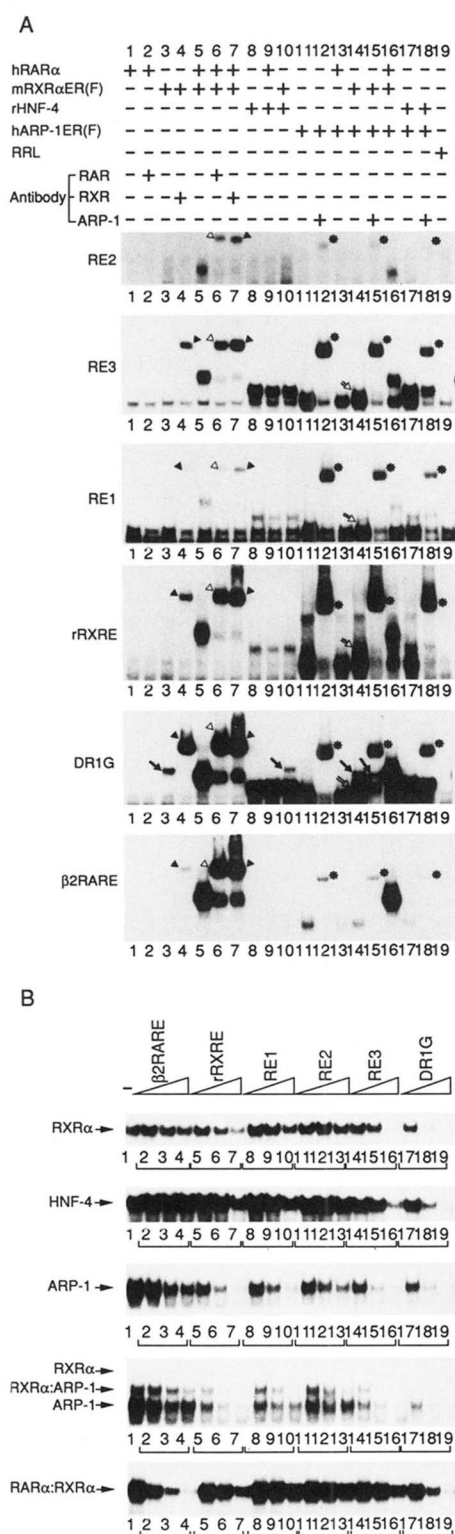
A

B

FIG. 4. **Binding in vitro of RARα, RXRα, HNF-4, and ARP-1 to various response elements.** *A,* ³²P-5′-end-labeled oligonucleotides containing mCRBPII RE1, RE2, or RE3, rCRBPII rRXRE, β2RARE of the mRARβ2 promoter or a direct repeat of RGGTCA separated by one nucleotide (see Table II for the nucleotide sequences) were incubated with *in vitro* transcribed-translated receptors, as indicated except in lanes 14 and 15 where mRXRα instead of mRXRαER(F) was added to the reactions. Where indicated, the respective antibodies were added subsequently. Incubation mixtures were resolved on 5% polyacrylamide gels and autoradiographed. *Open arrowheads, closed arrowheads,* and *asterisks* represent the retarded complexes that were supershifted by antibodies against RARα,

meric and ARP-1·:RXR heterodimeric complexes could be observed at higher receptor protein concentrations (*lanes 11* and *12, 14* and *15,* and data not shown).

Relative Efficiency of Binding of RXR, HNF-4, ARP-1, RAR·RXR and ARP-1·RXR in Vitro—The observation that the mCRBPII REs, rRXRE, and DR1G probes, although labeled to approximately the same specific activity, yielded different amounts of receptor complexes (Fig. 4*A*) indicated that the various receptors have different affinities for these REs. To further characterize these differences, gel shift competition assays were carried out, using DR1G as the labeled probe, since it gave a specific complex with all of the receptors in direct binding assays (Fig. 4*A*). Competition was also performed with the RARβ2 gene RARE (see Table II for sequences and a summary of the results).

The binding of RXR homodimer to DR1G was most efficiently inhibited by DR1G itself (*lanes 17–19,* Fig. 4*B*), whereas RE3 (*lanes 14–16*) and rRXRE (*lanes 5–7*) were less efficient at competing, although still much more efficient than RE1 (*lanes 8–10*). On the other hand β2RARE and RE2 did not compete, indicating that RXR homodimers bind very poorly, if at all, to these elements. The relative affinities of HNF-4 for the various REs were DR1G > RE3 > rRXRE ≥ RE1 > RE2 ≥ β2RARE to which HNF-4 did not appear to bind. ARP-1 homodimer, bound to the various REs with the following decreasing order of efficiency: DR1G ≥ RE3 > rRXRE ≥ RE1 > RE2 > β2RARE. Thus, HNF-4 appears to have a greater affinity for RE3 than for RE1 and rRXRE, whereas ARP-1 binds almost equally well to all three elements. RXR·ARP-1 heterodimers were most efficiently competed by DR1G. Efficient competition was also observed with rRXRE and RE3, whereas competition by RE1, and even more so by RE2 and β2RARE was weaker. RAR·RXR heterodimers bound most strongly to β2RARE (DR5T). Their relative binding efficiencies to the other REs were DR1G > rRXRE > RE3 > RE2 > RE1.

Effect of HNF-4 and ARP-1 on Constitutive and RA-induced Activity of the CRBPII Promoter—The above receptor binding results prompted us to investigate the effect of the expression of these receptors on mCRBPII promoter activity. Cos-1 cells were cotransfected with either mCRBPII/CAT2 which contains RE1, RE2, and RE3, or mCRBPII/CAT3 which contains only RE2 and RE3. Since the individual mCRBPII REs and the rRXRE exhibited different affinities for the various receptors *in vitro* (see above), tk-CAT reporter constructs containing either one of these REs were also used in parallel studies. For comparison, transfections were also performed with the mRARβ2/CAT reporter (Fig. 5, and data not shown).

mCRBPII/CAT3, which on its own has very little activity in Cos-1 cells, was stimulated ~20-fold in a ligand-independent manner when cotransfected with HNF-4 (Fig. 5, compare *lanes 1* and *4, stippled bars*). Addition of RA ligands had no

mRXRα, and ARP-1, respectively. RXR homodimers and ARP-1· RXR heterodimers are indicated by *closed arrows* and *open arrows,* respectively. *B,* ³²P-labeled DR1G (50 fmol) was incubated with rabbit reticulocyte lysates containing *in vitro* translated RXRα, HNF-4, ARP-1, RXRα, and ARP-1, or RARα and RXRα as indicated (*lane 1*). In *lanes 2–19,* non-radioactive competitor oligonucleotides corresponding to β2RARE (*lanes 2–4*), rRXRE (*lanes 5–7*), RE1 (*lanes 8–10*), RE2 (*lanes 11–13*), RE3 (*lanes 14–16*), and DR1G (*lanes 17–19*) were added. The quantity of non-radioactive competitors was 100 fmol in *lanes 2, 5, 8, 11, 14,* and *17* (2-fold excess over labeled probe), 300 fmol in *lanes 3, 6, 9, 12, 15,* and *18* (6-fold excess); 1000 fmol in *lanes 4, 7, 10, 13, 16,* and *19* (20-fold excess). Equivalent quantity of HNF-4 and ARP-1 were used in competition reaction. The sequences of the competitor oligonucleotides are shown in Table II.

effect. When RARα, RXRα, and HNF-4 were transfected, some ligand-dependent increase was observed (Fig. 5, compare *lane 4* with *8*), which disappeared when an excess of HNF-4 expression vector was transfected (*lane 9*). Similarly, the ligand-dependent activations brought about by either RARα or RXRα (*lanes 2* and *3*) were lost upon cotransfection with HNF-4 in excess (data not shown and *lane 9*). Cotransfection of ARP-1 did not affect the ligand-independent activation by HNF-4 (compare *lane 4* with *lane 7*). However, under similar conditions, ARP-1 repressed the activation of the mCRBPII promoter by either RARα (data not shown), RXRα (*lane 6*), or a combination of RARα and RXRα (*lane 10*). The ligand-independent activity of mCRBPII/CAT2, which contains RE1, was similar to that of mCRBPII/CAT3 when cotransfected with HNF-4 alone (*lane 4*). Interestingly, under the present conditions, ARP-1 which did not repress the HNF-4-mediated ligand-independent activation of mCRBPII/CAT3, repressed that of mCRBPII/CAT2 (compare *lanes 7*). However, mCRBPII/CAT3 could be repressed to the same extent as mCRBPII/CAT2, when a higher concentration of ARP-1 was used (data not shown). Thus, RE1 may be an ARP-1-negative regulatory element of the mCRBPII promoter (see below).

As in the case of the natural mCRBPII promoter constructs, RARα and RXRα stimulated RE3/tk-CAT expression, albeit to a lesser extent (Fig. 5, *lanes 2, 3*, and *5*). Similarly, HNF-4 increased the ligand-independent activity of this reporter by only 2-fold (Fig. 5, compare *lanes 1* and *4*). As with mCRBPII/CAT3, ARP-1 did not repress the stimulation generated by HNF-4 (compare *lanes 4* and *7*), whereas it reduced the ligand-dependent activation observed with cotransfected RARα and RXRα (compare *lanes 3* with *6*, and *lanes 5* with *10*). However, in contrast to the case of mCRBPII/CAT3, HNF-4 failed to block the ligand-dependent activation by cotransfected RARα and RXRα (*lanes 8* and *9*). This failure of HNF-4, as well as its inability to strongly stimulate RE3/tk-CAT (only 2-fold), suggests that HNF-4 may be less efficient at stimulating transcription from elements which contains only a single HNF-4 binding site, since genes known to be activated by HNF-4 contain either multiple HNF-4 binding sites (see Ref. 63) or binding sites for activators which can cooperate with HNF-4 (61, 62). In this respect we note that the sequence 5'-AACAGCCCTCCCCACC-3' at position −467 to −482 of mCRBPII promoter (Fig. 1B) exhibits some homology with the M element of the apoAII promoter (5'-CTCTCCCCCTCCCCACC-3') and the H element of the apoCIII promoter (5'-CTTTGCCCCTCCCTCCACC-3') which are required for efficient stimulation of these promoters by HNF-4 (61).

In contrast to RE3/tk-CAT, RE2/tk-CAT was weakly induced by RARα (*lane 2*), but not by RXRα (*lane 3*), which is in agreement with the mCRBPII/CAT mutant results shown above in Fig. 3. The ligand-independent activity of RE2/tk-CAT was weakly increased by HNF-4 expression (compare *lanes 1* and *4*), and no repressing activity was seen with cotransfected ARP-1 (*lane 7*), in agreement with the *in vitro* binding data.

The RE1 element, which in the context of the natural mouse CRBPII promoter is not required for RA response, was also tested in Cos-1 cells for its ability to confer RA inducibility to the heterologous tk promoter. RE1/tk-CAT expression was induced 3-fold by cotransfection of either RARα or RXRα (Fig. 5, compare *lanes 1, 2*, and *3*), and a 6-fold stimulation resulted from RARα and RXRα cotransfection (*lane 5*). A 2-fold ligand-independent stimulation was gener-

ated upon cotransfection with HNF-4 (*lane 4*). As with RE2/tk-CAT and RE3/tk-CAT, HNF-4 did not repress ligand-dependent activation by RARα and RXRα. However, in marked contrast to RE2 and RE3/tk-CAT, ARP-1 behaved as a repressor in all cotransfection combinations (compare *lanes 3, 4*, and *5* with *lanes 6, 7*, and *10*, respectively). The differential repressing effects of ARP-1 on these various REs may reflect differences in receptor affinities for the REs, since the binding competition data suggest that ARP-1 has similar affinities for RE1 and RE3, whereas RE1 has a lower affinity than RE3 for both HNF-4 and RAR · RXR heterodimers (Fig. 4B). These different bindings may explain why ARP-1 was more efficient at preventing the HNF-4-mediated activation of mCRBPII/CAT2, than of mCRBPII/CAT3 (see above).

rRXRE/tk-CAT, which contains the sequence 5'-AGGT-CACAGGTCA-3' in addition to the RE1 sequence (see Table II), was strongly activated by RXRα in Cos-1 cells, but not by RARα (Fig. 5, *lanes 1, 2*, and *3*). The ligand-independent activity of this reporter was 4-fold enhanced in the presence of HNF-4 (*lanes 1* and *4*, and Table I). When cotransfected with either RXRα or RARα and RXRα, an excess of HNF-4 resulted in a strong decrease in 9C-RA-dependent activation (*lane 9*, and data not shown). In Cos-1 cells, the RXRα-mediated induction was reduced by 50% when RARα was present (compare *lanes 3* and *5*). However, RARα could not further repress this RXRα-mediated stimulation in Cos-1 cells, even when cotransfected at a 10:1 ratio (data not shown). Interestingly, the magnitude of this RAR-related decrease was cell type-specific, as under similar conditions (1:1 ratio) the stimulation of rRXRE/tk-CAT expression by RXRα was drastically decreased by cotransfection of RARα in CV-1 cells (data not shown), whereas no significant decrease was observed in CaCo-2 cells, and an increase was actually observed in F9 cells (Table I, and data not shown). ARP-1 acted as a repressor of rRXRE/tk-CAT expression stimulated by RXRα (Fig. 5, *lanes 6* and *10*), but not by HNF-4 (see *lane 7*), inhibiting even more efficiently than in the case of mCRBPII/CAT2 and RE1/tk-CAT.

The ligand-independent activity of mRARβ2/CAT, whose RARE efficiently bound only RAR · RXR heterodimers *in vitro*, was not affected by HNF-4 (Fig. 5, compare *lanes 1* and *4*). Surprisingly, the ligand-dependent activity was enhanced when mRARβ2/CAT was transfected with either HNF-4 alone or in combination with RARα and RXRα (*lanes 4, 8*, and *9*). On the other hand, ARP-1 had very little effect on the RA-induced expression of RARβ2/CAT (*lanes 6* and *10*), whereas it slightly decreased the stimulation generated by HNF-4 in the presence of ligand (compare *lanes 4* and *7*). Since the β2RARE did not bind HNF-4 *in vitro* (see Fig. 4), these results suggest that the RARβ2 gene 5'-flanking sequence contains an HNF-4 (and possibly also ARP-1) binding site which has not yet been identified.

To investigate whether all of the HNF-4 stimulation of mCRBPII/CAT3 was mediated through RE2 and RE3, or through yet unidentified HNF-4 specific motifs, the effect of cotransfected HNF-4 on the activity of mCRBPII/CAT3, mCRBPII/CAT3mut5, mCRBPII/CAT3mut7, and mCRBPII/CAT3 mut8 was compared in Cos-1 cells. Mutation of RE2 (as in mCRBPII/CAT3mut5) did not specifically reduce the stimulation by HNF-4, whereas mutation of RE3, as in mCRBPII/CAT3mut7, resulted in a dramatic decrease of this stimulation (from 23-fold to 3-fold; this mutation completely abolished the binding of HNF-4 to RE3 *in vitro*, data not shown). Furthermore, mCRBPII/CAT3 mut8 which is mutated in both RE2 and RE3 was not activated by HNF-

FIG. 5. **Effect of RARα, RXRα, rHNF-4, and ARP-1 on the activity of the mCRBPII and RARβ2 gene promoters and on trans-activation mediated by mCRBPII RE1, RE2, and RE3, and rat CRBPII rRXRE.** The various CAT reporter constructs are described in "Materials and Methods." 5 μg of reporter plasmids were cotransfected into Cos-1 cells with the receptor expression vectors as indicated (0.5 μg each except in *lane 9* where rHNF-4 was 5 μg). Culture conditions were as in Fig. 2. The sequences of REs and rRXRE cloned into the *Bam*HI site of pBLCAT8+ vectors are shown in Table II. The results (±15%) correspond to average of several independent transfection experiments.
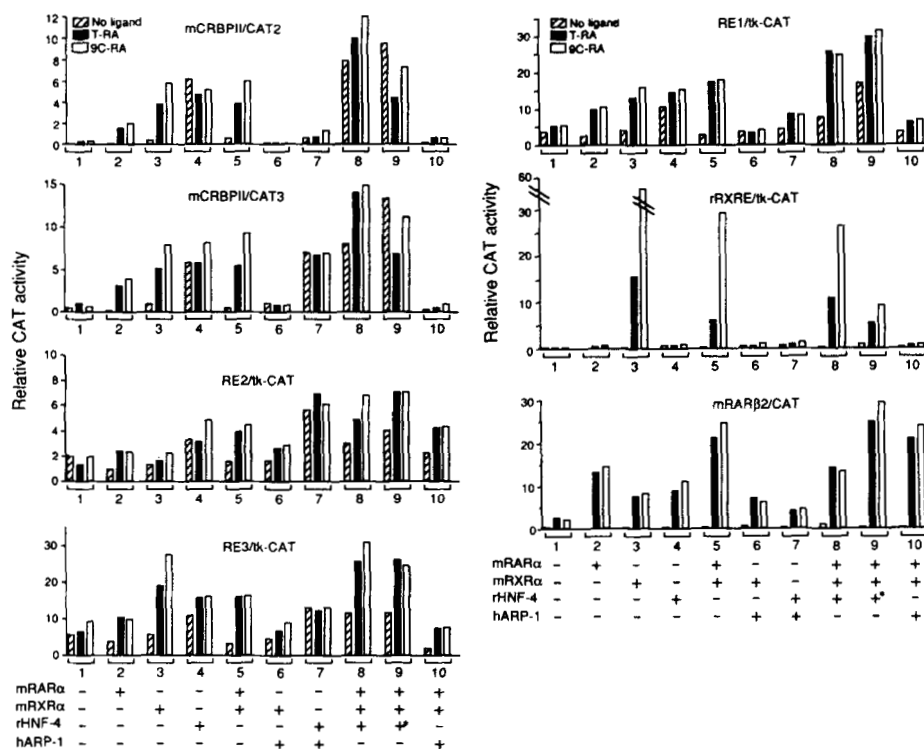
4 at all (data not shown). Thus, RE3 appears to mediate most of the stimulation generated by HNF-4. Similar results were obtained when transfections were performed in HepG2 and CaCo-2 cells in which overexpression of HNF-4 efficiently stimulated the CRBPII promoter activity (Table I, and data not shown).

DISCUSSION

Several Putative RA Response Elements Are Present in the Mouse CRBPII Promoter Which Is Cell-specifically Activated by Cotransfected RAR and RXR—We have shown here that the mCRBPII promoter responds to T-RA and 9C-RA when transfected into Cos-1, CV-1, and HeLa cells, along with vectors expressing either RARα, RXRα, or both of them (Fig. 2, Table I, and data not shown). No significant response was observed in the F9 and P19 EC cell lines, in the hepatocarcinoma cell line (HepG2) and in the colon carcinoma cell line CaCo-2, even though the mCRBPII promoter exhibited a higher basal activity in HepG2 and CaCo-2 cells, than in Cos-1, CV-1, F9, or P19 cells, and the endogenous CRBPII gene was expressed in CaCo-2 cells.[2] Under similar conditions, a well established RA-responsive promoter, the RARβ2 promoter, was readily activated in HepG2, CaCo-2, F9, or P19 cells. Thus, the cell-specific activity of the mCRBPII promoter suggests the requirement for additional cell-specific activators which may cooperate with RAR and/or RXR, or alternatively the existence of cell and promoter-specific repressors which may prevent the action of RAR and RXR.

Three putative RA response elements RE1, RE2, and RE3 have been identified in the mCRBPII promoter (Figs. 2B, 3, and 4). All three REs correspond to two directly repeated motifs, separated by either 1-bp (RE1 and RE3) or 2-bp (RE2) spacers, which are identical or related to the consensus RG(G/T)TCA motif found in the response elements of all nuclear

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[2] H. Nakshatri and P. Chambon, unpublished data.

receptors. All three REs could confer some RAR- and RXR-mediated RA inducibility to the tk promoter (Fig. 5), although RE1 and RE2 were clearly less efficient than RE3 (Fig. 5), and RE3 appeared better stimulated by RXR than by RAR (Fig. 5, see also Fig. 3, *mCRBPII/CAT3mut5 versus mCRBPII/CAT3mut4*). These transfection results are in good agreement with the data of DNA binding *in vitro*, which show that RE3 was more efficient at binding RAR·RXR heterodimers, and also RXR homodimers, than either RE1 or RE2 (Fig. 4, *A* and *B*). Note, however, that RAR·RXR heterodimers bound much more efficiently than the corresponding homodimers, consistent with previous reports (see Introduction). Thus RE3 may mediate activation by RXR homodimers only in cells which contain a large excess of RXR over RAR.

Interestingly, the effects of RE1 and RE2 were different, when tested within the mCRBPII promoter. If anything, RE1 exerted a slight inhibitory effect (Fig. 2B, compare *mCRBPII/CAT2* and *mCRBPII/CAT3*), whereas RE2, which had no effect on its own (Fig. 3, *mCRBPII/CATmut7*), clearly cooperated with RE3 (compare *mCRBPII/CAT3* and *mut5* in Fig. 3). Thus RE3 appears to be the only element which, within the CRBPII promoter, responds on its own to RAR and RXR.

The Putative Rat CRBPII RXR Response Element (RXRE) Has No Functional Equivalent in the Mouse CRBPII Promoter—Mangelsdorf *et al.* (45) concluded from a study of the rat CRBPII promoter response to RXR and RAR, that this promoter responds selectively to RXR through a unique distal element called RXRE (Fig. 1D), whose deletion results in a total loss of RA response. Thus, although the proximal region of the mCRBPII promoter which contains RE2 and RE3 is almost fully conserved in the rat CRBPII promoter (Fig. 1D), the role of these elements was not observed by these authors, most probably because they performed their experiments in F9 cells, where the mouse CRBPII promoter is not activated by RAR and RXR (Table I, see above).

Interestingly, the putative rat RXRE is not conserved in

the mCRBPII gene. In fact, mCRBPII RE1 corresponds to a truncated form of the rat RXRE from which the first two AGGTCA motifs have been deleted (Fig. 1D). Our results clearly show that the properties of mCRBPII RE1 and rRXRE are different in terms of both DNA binding *in vitro*, and RAR and RXR-mediated trans-activation in transfected cells. In contrast to RE1, and in agreement with the results of Mangelsdorf et al. (45), rRXRE very efficiently conferred RXR (but not RAR) responsiveness to a heterologous promoter in Cos-1 cells (Fig. 5, *rRXRE/tk-CAT*). Co-expression of RAR and RXR resulted in a decrease of RXR-mediated rRXRE/ tk-CAT expression in Cos-1 cells. However, instead of the drastic inhibition which could be achieved in CV-1 cells ((45) and our data not shown), a ~ 50% inhibition plateau is reached irrespective of the excess of RAR in Cos-1 cells (Fig. 5, and data not shown). Interestingly, no significant inhibition was observed when RARα was cotransfected with RXRα in CaCo-2 cells, and a stimulation was in fact seen in F9 cells when RARα and RXRα were cotransfected (Table I). Furthermore the relative effects of T-RA and 9C-RA were markedly different in Cos-1, CaCo-2, and F9 cells (Table I, and data not shown). Since rRXRE binds more efficiently RAR· RXR heterodimers than RXR homodimers (Fig. 4A), these differential cell type-related inhibitions and activations may indicate that the activation functions of the two partners of rRXRE-bound heterodimers could be mediated by cell-specific factors.

The high degree of evolutionary conservation of the retinoid signaling pathway in general, and of the CRBPII protein and proximal promoter region in particular (Fig. 1), together with the observation that RE1, the mouse counterpart of rRXRE, is not involved in mCRBPII promoter responsiveness to RA, raise the question as to whether the non-conserved rCRBPII RXRE is in fact a physiologically relevant RXR response element (see below).

The Putative CRBPII RA Response Elements, Particularly RE3, Are also Binding Sites for the Orphan Nuclear Receptors HNF-4 and ARP-1—The pattern of expression of the CRBPII gene which is mainly confined to prenatal liver and intestine, and adult intestine (46) resembles that of the apolipoprotein A, B and C genes (46, 62, 64–66). For example, the apolipoprotein CIII (apoCIII) gene is expressed predominantly in mammalian liver and intestine, and transfection studies in human hepatocarcinoma cells HepG2 and colon carcinoma cells CaCo-2 have shown that the activity of the apoCIII promoter is dependent on the binding of multiple factors to several cis-acting DNA elements (61, 62, 67, 68). Interestingly, CRBPII RE3 is very similar to one of these elements (the apoCIII C3P site, see Table II), which has been recently shown to bind several orphan members of the nuclear receptor superfamily, namely, HNF-4, ARP-1, and the ARP-1 closely related factors Ear2 and Ear3 (COUP-TF) (61, 62). The expression of HNF-4 is mostly restricted to liver, intestine, and kidney, while ARP-1, together with the closely related Ear3 (COUP-TF) is present in most tissues, and Ear2 is expressed mostly in liver (69). HNF-4 activates transcription in reporter constructs containing the C3P site, whereas ARP-1, Ear2 and Ear3 (COUP-TF) repress HNF-4 activation by competing for the same C3P site (Refs. 61 and 62 and references therein).

Our present results clearly show that both HNF-4 and ARP-1 efficiently interact with CRBPII RE3. In fact HNF-4, which binds much more efficiently *in vitro* to RE3 than to RE1, RE2, and rRXRE, acts through RE3 as a strong activator of the mCRBPII promoter (Figs. 4 and 5, and data not

shown). In all instances an excess of HNF-4 substantially decreased RA-dependent activation by RAR and/or RXR, supporting the *in vitro* data showing that these different receptors can bind to the same sites. ARP-1 binds most efficiently to RE3, but also to RE1 and rRXRE, albeit with somewhat lower efficiency (Fig. 4). Accordingly, ARP-1 represses the activation of the mCRBPII promoter and of all individual CRBPII REs, which can be generated by co-expression of either RAR and/or RXR (Fig. 5). ARP-1 was less efficient at repressing the HNF-4 induced activity, unless expressed at higher levels (Fig. 5 and data not shown), except when RE1 was present (*mCRBPII/CAT2* in Fig. 5). Thus, RE1 may in fact correspond to a negative regulatory element of the mCRBPII promoter, mediating ARP-1 repression on either RAR/RXR or HNF-4 activation. Note that the rat RXRE, which also binds ARP-1 very efficiently may play a similar role in the rat CRBPII promoter. In addition, our results indicate that ARP-1 may also act negatively on RAR/ RXR activation. This may result from competition for binding to the same element(s) (Fig. 4). Repression of RAR/RXR-mediated trans-activation by ARP-1 has been recently reported for other promoters (70–72).

As previously proposed by Ladias et al. (61) and Mietus-Snyder et al. (62) in the case of the apoCIII (in HepG2 and CaCo-2 cells), apoAII, and apoB promoters, the activity of the CRBPII promoter may primarily be modulated by the opposite effects of the activator HNF-4 and the repressor ARP-1 (or the related factors Ear2 and Ear3 (COUP-TF); note that substituting ARP-1 by COUP-TF yielded results similar to those presented here for ARP-1 (data not shown)). This possibility is supported by the results of competition experiments which have shown that the same gel shift complexes were formed between CaCo-2 or HepG2 cell extracts and either the CRBPII RE3 or the apoCIII C3P element.[2] These complexes that we could not supershift with anti-RAR or RXR antibodies have been previously shown in the case of C3P to correspond to HNF-4 and ARP-1 (COUP-TF) complexes (62). In addition, ARP-1 may also repress RAR and RXR activity by forming inactive ARP-1·RXR heterodimers, as suggested by the results of DNA binding gel shift experiments (Fig. 4). These suggestions are in agreement with the previous report of Kliewer et al. (73) who found that RXR and COUP-TF can bind as heterodimers to rRXRE *in vitro*. In this respect, we note that ARP-1·RXRα heterodimers bind more efficiently to RE3 and rRXRE than RXR homodimer (Fig. 4, and data not shown), which is in keeping with the recent report of Widom et al. (74) who observed a similar preferential binding of ARP-1·RXRα heterodimers on site A of the apolipoprotein AI (apoAI) promoter. In contrast, Tran et al. (72) found no evidence supporting COUP-TF/RXRα heterodimer binding to the rRXRE and the apoAI site A element.

Finally, it is worth noting that neither ARP-1 nor HNF-4 bind efficiently to the RARE of the RARβ2 promoter (β2RARE in Fig. 4), even though this promoter could be activated by HNF-4, and this activation (but not the RAR· RXR activation) could be repressed by ARP-1 (Fig. 5). These observations suggest that the RARβ2 promoter may contain a HNF-4/ARP-1 (Ear3/COUP-TF) response element which has not yet been identified.

Is the Expression of CRBPII Gene Controlled by Retinoic Acids in Vivo?—Although our present results and those previously reported by Mangelsdorf et al. (45) show that the rat and mouse CRBPII promoters can respond to overexpressed RXR and RAR in cotransfection experiments, for these re-

sults to be physiologically relevant, it must be demonstrated that expression of the CRBPII gene can be stimulated by RA treatment *in vivo*. No data are presently available concerning a possible RA induction of CRBPII transcripts or protein in prenatal liver or intestine, and in adult intestine, which are known to express the CRBPII protein and transcripts (37, 75, 76).[3] However, the enterocyte-like cell line CaCo-2 offers a possible alternative system to investigate the response of the CRBPII gene to the retinoic acid signal, since CaCo-2 cells constitutively express CRBPII mRNA, and at least the RARα, RARγ, and RXRα genes are also constitutively expressed in these cells.[3] Strikingly, in CaCo-2 cells, the transfected mCRBPII promoter does not respond to either endogenous or overexpressed receptors in the presence of either T-RA or 9C-RA, whereas under the same conditions efficient stimulation of the RARβ2 promoter is achieved in the same cells (Table I). In contrast, under similar transfection conditions, the CRBPII promoter is activated by overexpressed HNF-4, and RA treatment does not result in any further stimulation (Table I). Therefore, it is likely that the higher basal activity of the CRBPII promoter in HepG2 and CaCo-2 cells than in CV-1, Cos-1, F9, and P19 cells, is due to the presence of HNF-4 which is known to be expressed in liver and intestine (60) and also in CaCo-2 and HepG2 cells where it is present at a higher level than ARP-1 and Ear-3 (COUP-TF) (Ref. 62 and references therein). Note, incidentally, that HNF-4 on its own is not sufficient to activate the CRBPII promoter in F9 and P19 cells (Table I, and data not shown), even though ARP-1 is not present in these cells,[3] which suggests that EC cells lack a factor(s) necessary to mediate HNF-4 activation.

Thus, since CRBPII RE3 and RE1 response elements have a much higher affinity for HNF-4 and ARP-1 than for RAR. RXR heterodimers and RXR homodimers (Fig. 4, and data not shown), the transcription of the CRBPII gene could in fact be dependent upon the intracellular levels of HNF-4 and ARP-1, acting as positive and negative regulatory factors, respectively. Note in this respect that CRBPII mRNA was not increased in the intestine of T-RA or 9C-RA treated mice.[3] However, it cannot be excluded that RA could stimulate the CRBPII promoter in cells where HNF-4 and ARP-1 are absent or present at much lower levels than retinoic acid receptors, as it is the case in Cos-1 cells transfected with RAR and RXR expression vectors (see Fig. 2 and Table I).

In conclusion, the evidence available at the present time strongly suggests that the stimulation of the CRBPII promoter generated by overexpression of RARs and RXR in transfected cells may reflect the promiscuous binding of these receptors to HNF-4/ARP-1 response elements. Further studies will establish whether HNF-4 is in fact the major transcriptional activator of the CRBPII gene *in vivo* and whether this gene could also be induced by RA in tissues where HNF-4 and ARP-1 are lacking or present in low amounts.

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[3] H. Nakshatri and P. Chambon, unpublished results.

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