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A retinoic acid response element is present in the mouse cellular retinol binding protein I (mCRBPI) promoter

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Genomic and cDNA sequences for the mouse cellular retinol binding protein I (mCRBPI) are presented. A specific *cis*-acting element responsible for retinoic acid (RA) inducibility of the mCRBPI promoter was identified and characterized. Deletion mapping of a CRBPI promoter–chloramphenicol acetyltransferase reporter gene construct localized this element to a 259 bp restriction fragment located ~1 kb upstream from the transcription start-site. A sequence closely resembling the previously characterized RA response element (RARE) of the RA receptor β2 (RAR-β2) promoter, and consisting of a direct repeat of the motif 5'-GGTCA-3' separated by three nucleotides, was found within this restriction fragment. Mutation of these 5'-GGTCA-3' motifs to GGAGC and GGGGC abolished RA-inducible transcription whereas a mutation to a direct repeat of the GTTCA motif found in the RARE of the RAR-β2 promoter resulted in enhanced inducibility. Oligonucleotides containing the direct repeat of the GGTCA motif were able to confer RA-dependent transcriptional enhancement to the herpes simplex thymidine kinase promoter, as well as to bind directly all three retinoic acid receptors (RARs) α, β and γ, as determined by gel retardation/shift assays. The control of CRBPI gene transcription by RA–RAR complexes interacting with the RARE characterized here may correspond to a feedback mechanism important in regulating retinoid metabolism and action.

Key words: mouse CRBPI cDNA/retinoic acid receptors/ RAR/RARE/transcriptional control

Introduction

Several families of serum, cytoplasmic and nuclear proteins are involved in the metabolism and biological actions of retinoids (for review, see Blomhoff *et al.*, 1990). Some of these proteins mediate direct effects of retinoids on gene expression, while others are involved in their transport, storage and metabolism. Nuclear receptors for retinoic acid (RA) function as ligand-inducible *trans*-acting transcriptional factors, and appear to control RA-dependent gene expression by interaction with *cis*-acting DNA elements known as RA response elements (RAREs). Three evolutionarily conserved retinoic acid receptors, RAR-α, β and γ, all of which have homology to the larger family of steroid/thyroid hormone nuclear receptors (Evans, 1988; Green and Chambon, 1988; Beato, 1989), have been identified (Giguère *et al.*, 1987; Petkovich *et al.*, 1987; Benbrook *et al.*, 1988; Brand *et al.*, 1988; Krust *et al.*, 1989; Zelent *et al.*, 1989), as well as several isoforms of each RAR subtype (Kastner *et al.*, 1990; Leroy *et al.*, 1991; Zelent *et al.*, 1991). An additional subfamily of nuclear receptors (the RXRs), which may possibly respond to retinoic acid and/or to other uncharacterized retinoids, has been recently described (Mangelsdorf *et al.*, 1990). In addition to these RARs and RXRs, cytoplasmic binding proteins have been characterized for several retinoids, including RA (CRABP) and retinol (CRBP), and again several subtypes encoded by different genes are known to exist for these proteins (Chytil and Ong, 1984; Sundelin *et al.*, 1985; Li *et al.*, 1986; Demmer *et al.*, 1987; Levin *et al.*, 1988; Nilsson *et al.*, 1988; Giguère *et al.*, 1990; reviewed in Blomhoff *et al.*, 1990 and Chytil and Haq, 1990). The multiplicity of cellular retinoic acid (CRABP) and retinol (CRBP) binding proteins suggests that the metabolism and action of retinoids are complex and tightly regulated, and is consistent with the view that retinoids, particularly RA, play an important role in vertebrate development (for reviews see Brockes, 1989, 1990; Slack, 1987a,b; Eichelé, 1989; Summerbell and Maden, 1990). This view is further supported by the specific spatio-temporal patterns of expression observed for these retinoid binding proteins and RA receptors during mouse and chick embryonic development and in adult tissues (Dolle *et al.*, 1989, 1990; Perez-Castro *et al.*, 1989; Ruberte *et al.*, 1990, 1991; Maden *et al.*, 1989, 1990; Vaessen *et al.*, 1990; Dencker *et al.*, 1990; and refs therein).

In view of the complexity of the retinoid binding proteins and RARs, cross-regulation of the expression and/or activity of these proteins might be expected. In fact, it has been shown that the levels of both RAR-α2 (Leroy *et al.*, 1991), RAR-β1, β2 and β3 isoforms (Zelent *et al.*, 1991), CRABP I and II (Wei *et al.*, 1989; Giguère *et al.*, 1990), and CRBPI (Wei *et al.*, 1989) mRNAs are increased when embryonal carcinoma (EC) cells are treated with retinoic acid. Furthermore, a RARE exhibiting the properties of a transcriptional enhancer element has been characterized in the promoter region controlling the transcription of the human and mouse RAR-β2 isoform (de Thé *et al.*, 1990; Sucov *et al.*, 1990; Zelent *et al.*, 1991). We have now characterized the promoter region of the mouse CRBPI (mCRBPI) gene and we report here that it contains a related RARE.

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Results

Cloning of mouse CRBPI cDNA and 5′ genomic DNA sequences

The nucleotide sequence of a mCRBPI cDNA clone obtained as described in Materials and methods, and the deduced amino acid sequence (135 residues; mol. wt 15 700) is shown in Figure 1A. Two overlapping *Bss*HII sites were identified in the cDNA at nucleotides 73 and 75, and served as landmarks for characterizing genomic clones.

A single recombinant λEMBL3 phage was isolated from the screening of a mouse genomic library with a synthetic oligonucleotide (NL54) corresponding to the 5′ region of the cDNA, and was determined to have an insert of ~15 kb (Materials and methods). Based on the conserved intron/exon structure of human CRBPI (Nilsson *et al.*, 1988) and rat CRBPII (Demmer *et al.*, 1987), it was assumed that both the sequence corresponding to oligonucleotide NL54, and the *Bss*HII sites mentioned above were located within the first exon of the mCRBPI gene transcript, with the *Bss*HII sites lying 3′ to the sequence corresponding to NL54. Further analysis of this phage by a combination of restriction mapping and Southern blotting using NL54 as a probe, identified a *Bss*HII−*Bam*HI fragment extending ~2 kb in the 5′ direction of the *Bss*HII sites (data not shown). The sequence of this fragment, which was a likely candidate to contain the transcriptional start-site and *cis*-acting transcriptional regulatory elements for the mCRBPI gene, is presented in Figure 1B. As expected, its 3′ extremity overlaps the 5′ extremity of the cDNA sequence.

Mapping of the start-site and putative promoter elements in the mCRBPI 5′-flanking region

Primer extension was performed to map the mCRBPI start-site using an oligonucleotide complementary to nucleotides +132 to +170 in the genomic DNA and poly(A) RNA prepared from P19 cells cultured in the absence and presence of RA (Figure 2, and Materials and methods). Using RNA from RA-treated P19 cells (but not from untreated cells), a major extended fragment was obtained, whose 5′ end mapped at the position indicated as +1 in Figures 1B and 2. This start-site location was confirmed by S1 nuclease mapping using a ^{32}P-5′-labelled oligonucleotide (positions −49 to +41 of the genomic DNA) as a probe and the same RNA preparations (data not shown). As in the case of human CRBPI gene (Nilsson *et al.*, 1988), and unlike the rat CRBPII gene (Demmer *et al.*, 1987), no sequence resembling the canonical TATA box was present upstream of the start-site. Also as was the case for the human CRBPI gene, several copies of the core of Sp1 factor binding sites (5′-CCGCCC-3′; Jones and Tjian, 1985; Kadonaga *et al.*, 1987) were found clustered in the 5′ region of the mCRBPI gene between nucleotides −162 and −108 (labelled I−V in Figure 1B). An additional putative Sp1 binding site was identified 3′ to the transcription start-site (VI in Figure 1B). Sequences similar to the nuclear factor-1 (NF-1) binding motif (5′-TGGN$_7$CCA-3′, nucleotides −788 to −776 and −183 to −171) (Jones *et al.*, 1987), and Krox-20 or 24 binding motif (5′-CGCCCCCGC-3′, nucleotides −166 to −158; Lemaire *et al.*, 1990) were also found. Further upstream, a sequence shared by the rat CRBPII and the mCRBPI genes and consisting of the repeating dinucleotide dGdT (23 times for rat CRBPII and 26 times for mCRBPI) was observed (Figure 1B, nucleotides −1410 to −1359).

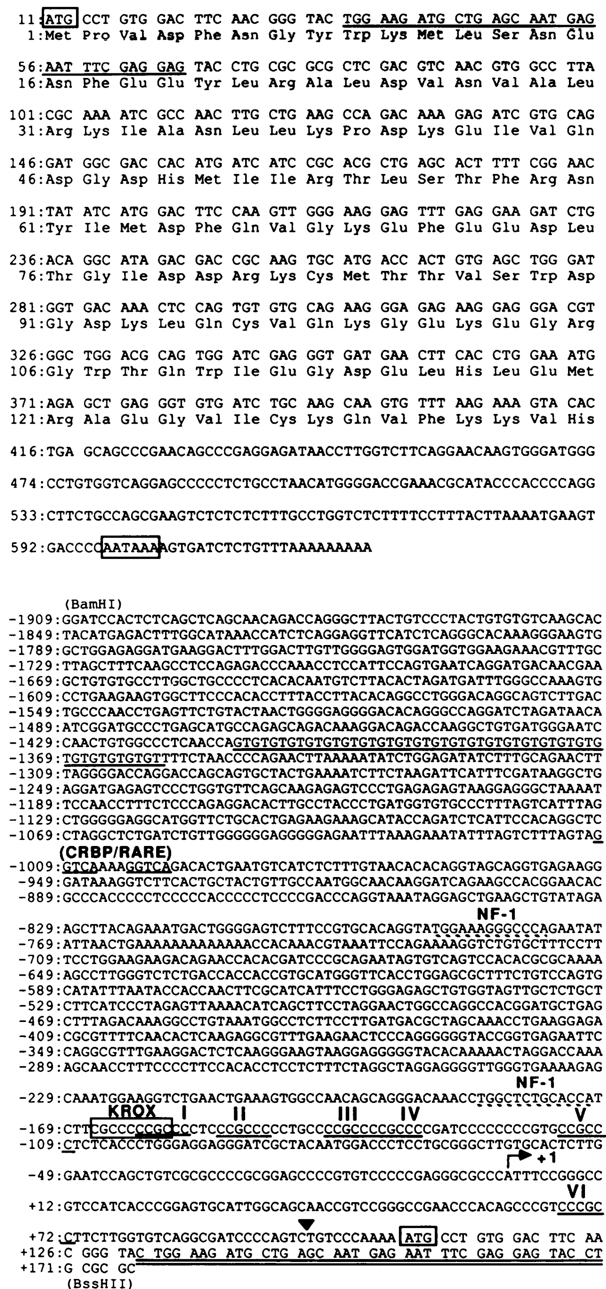

Fig. 1. (**A**) Sequence of the mouse CRBPI cDNA and predicted amino acid sequence. Underlined sequence represents the sequence of the oligonucleotide used for screening. The initiator ATG and poly(A) addition signal are boxed. (**B**) Sequence of the mouse CRBPI promoter region. The nucleotide sequence of a 2085 bp *Bam*HI−*Bss*HII fragment of the mouse CRBPI gene is shown. Nucleotides are numbered such that +1 corresponds to the predominant transcription start-site. Underlined are (5′−3′): (1) a conserved stretch of repeating dG-dT dinucleotides; (2) the retinoic acid response element (CRBP/RARE), (3) six potential Sp1 binding sites (labelled I−VI). Broken underlines represent putative NF-1 binding motifs. The putative Krox 20 or 24 (EGR-1) binding site is boxed. The sequence of the primer used for primer extension is indicated by a double line. Inverted triangle represents the first nucleotide of the cDNA.

Finally, and most importantly, a sequence closely resembling the RARE first identified in the human RAR-β gene (5′-GTTCACCGAAAGTTCA-3′, de Thé *et al.*, 1990), but consisting of a direct repeat of the motif GGTCA (instead of GTTCA) with three intervening nucleotides, was observed

in the 5′-flanking region of the mCRBPI gene (Figure 1B, nucleotides −1010 to −998).

Characterization of an in vivo functional RARE in the mCRBPI promoter distal region

Constructs containing various lengths of the putative mCRBPI promoter sequences ligated to the CAT gene were made and tested for CAT expression and RA inducibility, following transfection in P19 embryonal carcinoma cells together with a vector expressing human RAR-α1 (hRAR-α0). Representative CAT assays for each of the CRBPI−CAT constructs are displayed in Figure 3. CRBPI/CAT1 showed a low level of CAT activity in the absence of RA, and was induced (4.1 ± 0.7 fold; $n = 6$, i.e. six independent transfection experiments) upon the addition of RA (10^{-6} M). CRBPI/CAT2 was also RA inducible (3.2 ± 0.7 fold induction; $n = 5$), while the deletion of an additional 259 bp (CRBPI/CAT3) almost eliminated RA inducibility (1.4 ± 0.2 fold induction; $n = 6$). A further deletion leaving only 7 bp upstream of the mCRBPI start-site (CRBPI/CAT4) eliminated both promoter activity and RA inducibility (1.0 ± 0.1 fold induction; $n = 6$). The parental promoterless CAT expression vector pBLCAT8 + polyL showed no RA inducibility.

Since the greatest loss of RA inducibility resulted from a −1089 to −830 deletion, the corresponding 259 bp *Bgl*II−*Hin*dIII fragment was inserted into a plasmid containing the CAT gene driven by the herpes simplex virus (HSV) thymidine kinase promoter (pBLCAT8+) to yield the construct CRBPI/tk-CAT5. Note that the orientation of the inserted *Hin*dIII−*Bgl*II fragment in this construct was opposite to that in the genomic DNA (Materials and methods). Transfection of P19 cells with 10 μg of this construct resulted in a ~9-fold induction with RA when the cells were co-transfected with 10 μg of hRAR-α0 (Figure 4, lanes 7 and 8), while in the absence of the latter, a slightly lower level of induction due to P19 cell-endogenous RARs (Leroy *et al.*, 1991; Zelent *et al.*, 1991) was observed [~7-fold, lanes 9 and 10; the weak increase in CAT activity seen with 10 μg of parental vector pBLCAT8$^+$ in the presence of RA and hRAR-α0 (lanes 3 and 4) was not reproducibly observed].

As noted above, the *Bgl*II−*Hin*dIII fragment contains a putative RARE possibly consisting of a direct repeat of the motif 5′-GGTCA-3′. The possible function of this putative RARE in the RA inducibility of CRBPI/CAT1 (Figure 3) was investigated by generating in this construct the mutations shown in Figure 5A. Both of the two GGTCA motifs I and II were mutated in CRBPI-ml, whereas only one of these motifs was mutated in CRBPI-m2 and -m3. In CRBPI-m4 the mutation resulted in a direct repeat of the motif 5′-GTTCA-3′ which is present in the human and mouse

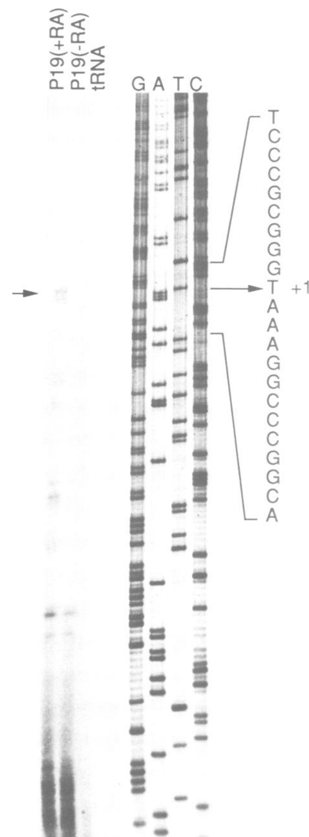

Fig. 2. Mapping of the CRBPI start-site by primer extension analysis. Products of primer extension using either 10 μg of poly(A) RNA from retinoic acid-exposed (P19+) and untreated (P19−) P19 cells, or tRNA (tRNA), were resolved on a 6% urea−polyacrylamide gel and autoradiographed. Lanes G, A, T and C contained sequencing reaction products of CRBP25 with the primer used for primer extension. The longest primer extension product is indicated by an arrow. The sequence shown on the right corresponds to the non-coding strand.

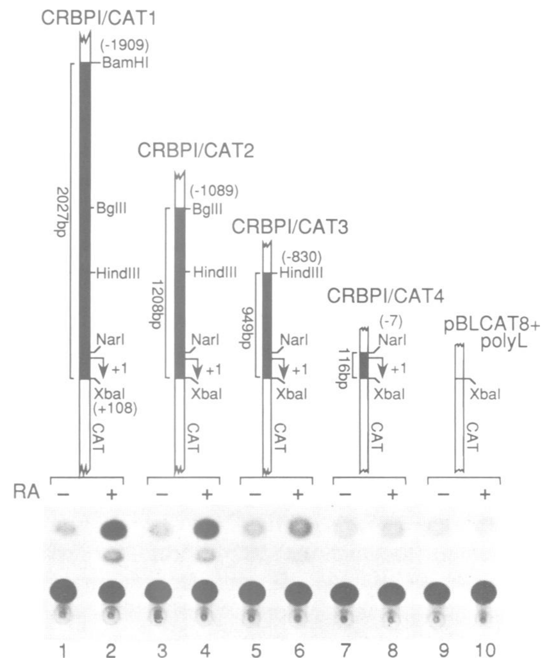

Fig. 3. Mouse CRBPI promoter activity. Plasmids (10 μg) carrying various lengths of the mouse CRBPI promoter directing the expression of the CAT gene were transiently transfected into P19 cells, along with 0.5 μg of the human RAR-α1 expression vector hRAR-α0. 10^{-6} M retinoic acid (RA) was added 16 h later (as indicated) and 24 h later cells were harvested and extracts were prepared. Transfection efficiency was standardized by co-transfection of 2 μg of the β-galactosidase expression vector pCH110. Shown in the figure is a representative assay of CAT activity following transfection with the promoter constructs depicted above. The length of the mCRBPI promoter fragment is indicated as well as relevant restriction sites (see Materials and methods). Arrow +1 indicates the relative position of the transcriptional start-site.

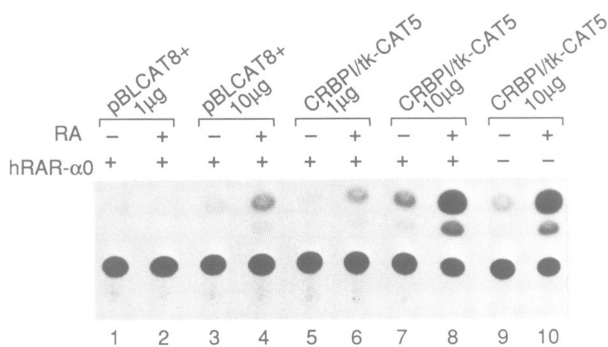

Fig. 4. Localization of a retinoic acid-induced enhancer activity to nucleotides −1089 to −830 of the mouse CRBPI promoter. A 259 bp *Bgl*II−*Hin*dIII fragment of the mCRBPI promoter (nucleotides −1089 to −830) was ligated 5′ to the thymidine kinase (tk) promoter of the CAT gene expression vector pBLCAT8+. This plasmid (CRBPI/tk-CAT5) or pBLCAT8+ containing no insert (as a control), was transiently transfected (1 or 10 µg) into P19 cells with or without the hRAR-α0 expression vector (0.5 µg). All cells were also co-transfected with 2 µg of the β-galactosidase expression vector pCH110. The figure shows the results of an assay for CAT enzyme activity in extracts from cells either untreated (−) or treated (+) with 10^{-6} M retinoic acid for 24 h.

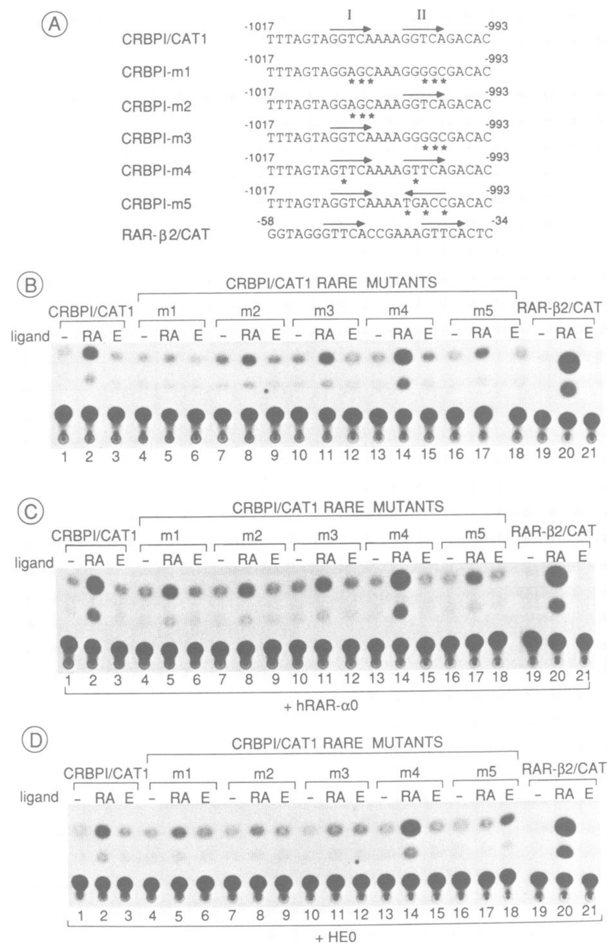

Fig. 5. Mutational analysis of mCRBPI RA response element (RARE). (**A**) Mutated sequences of the mCRBPI RARE in CRBPI/CAT1 ml to m5 are indicated by asterisk. The different constructs were transfected (10 µg) into P19 cells along with either the parental expression vector pSG5 (**B**) or the hRAR-α1 expression vector hRAR-α0 (**C**) or the human oestrogen receptor expression vector HEO (**D**), as indicated. Transfected cells were treated, also as indicated, with either no ligand (−), retinoic acid (RA) or oestradiol (E) (both at 10^{-6} M) for 24 h. The figure shows the relative CAT enzyme activity of cellular extracts (after standardization for β-galactosidase activity from the co-transfected β-galactosidase expression vector pCH110).

RAR-β2 RARE (de Thé *et al.*, 1990; Sucov *et al.*, 1990; Zelent *et al.*, 1991), whereas the mutation present in CRBPI-m5 created an inverted repeat similar to that found in oestrogen responsive elements (ERE, see Green and Chambon, 1988; Beato, 1989 for refs). Figure 5B, C and D show the activity of CRBPI/CAT1 and of the five mutants in P19 cells, when cotransfected with either the empty expression vector pSG5 as a control (Green *et al.*, 1988a), the hRAR-α0 vector expressing hRAR-α1 (Petkovich *et al.*, 1987; Leroy *et al.*, 1991) or the HEO vector expressing the human oestrogen receptor (Kumar *et al.*, 1987), respectively. When the empty pSG5 expression vector was co-transfected (i.e. when RA inducibility was mediated by P19 cell-endogenous RARs) (Figure 5B), mutation of either of the two GGTCA motifs (m2 and m3) resulted in a ~3-fold decrease of RA inducibility (average of three independent experiments), and mutation of both of them (ml) resulted in a further decrease. Mutant m4, in which both GGTCA motifs have been converted to GTTCA motifs, exhibited higher RA inducibility than CRBPI/CAT1, indicating that a directly repeated GTTCA motif is a more efficient RARE [compare also with RAR-β2/CAT (a gift from C.Mendelsohn), in which the CRBPI promoter region was replaced by the mouse RAR-β2 promoter region from +110 to approximately −3700, see Zelent *et al.*, 1991]. In contrast, very little induction was observed with mutant m5 which contains an ERE-like inverted repeat of the motif 5′-GGTCA-3′. Similar results were obtained when the hRAR-α0 expression vector was co-transfected, except that the RA inducibility of CRBPI/CAT1 and CRBPI-m4 was higher. When CRBPI/CAT1 and its mutants were co-transfected with a vector expressing hER (Figure 5D), little stimulation of CAT activity was observed in the presence of RA or oestradiol for mutants m1, m2 and m3. In contrast, expression of mutant m4 was increased in the presence of RA (presumably through endogenous receptors), but not in the presence of oestradiol, whereas the reverse situation was observed with mutant m5 which contains an ERE-like palindromic element.

To demonstrate that it can confer RA inducibility on a heterologous promoter, the CRBPI element containing the directly repeated GGTCA motifs (CRBPI RARE, nucleotides −1016 to −989 of the sequence presented in Figure 1B) was ligated (as a single copy, see Materials and methods) upstream of the tk promoter into the *Bam*HI site of pBLCAT8+ (CRBPI-RARE/tk-CAT). A control oligonucleotide in which the two GGTCA motifs were mutated as in CRBPI-ml was similarly constructed (CRBPI-RAREmut/tk-CAT, see Materials and methods and Figure 5A). These constructs were tested in transfection assays in P19 cells for both RA and oestradiol inducibility, with or without co-transfected hRAR-α0 or HEO expression vectors (Figure 6). Transfections were similarly performed with either RARβ-RARE/tk-CAT and ERE/tk-CAT constructs. RARβ-RARE/tk-CAT and ERE/tk-CAT contain the RARE

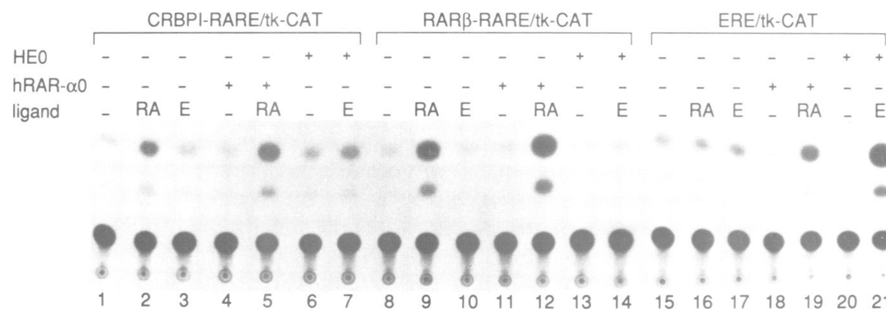

Fig. 6. Activity of a synthetic mCRBPI retinoic acid response element. Oligonucleotides containing either the mouse CRBPI RARE (oligonucleotide CRBPI-RARE, see Materials and methods), the human RAR-β2 RARE (RARβ-RARE) or an oestrogen response element (ERE) were ligated upstream of the tk promoter into CAT expression vector pBLCAT8+. CAT expression constructs (10 μg) were transiently transfected into P19 cells either with or without co-transfected hRAR-α1 (hRAR-α0) or human oestrogen receptor (HEO), as indicated. Transfected cells were treated, also as indicated, with either no ligand (−), retinoic acid (RA) or oestradiol (E) (both at 10^{-6} M) for 24 h. The figure shows the relative CAT enzyme activity of cellular extracts after standardization for β-galactosidase activity from the co-transfected β-galactosidase expression vector pCH110.

present in the human RAR-β gene (de Thé *et al.*, 1990; a gift from S.Mader) and a consensus palindromic ERE (Green *et al.*, 1988b), respectively, inserted upstream of the tk promoter of pBLCAT8+ (Materials and methods). When hRAR-α0 was co-transfected, CAT activity was induced 5- and 22-fold by RA in cells transfected with the CRBPI-RARE/tk-CAT (lanes 1−7) and the RARβ-RARE/tk-CAT (lanes 8−14) constructs, respectively. In control experiments, RA inducibility was virtually eliminated by mutation within the CRBPI RARE (CRBPI-RARE/tk-CAT, data not shown). In contrast, CAT activity was not induced by oestradiol in cells transfected with either of these two constructs, irrespective of hER co-expression. However, CAT activity was induced by both oestradiol and RA in cells co-transfected with the cognate receptors and the ERE/tk-CAT reporter (lanes 15−21).

Binding of RAR-α, β and γ to the CRBPI RARE in vitro

The ability of RARs to bind the CRBPI RARE *in vitro* was investigated using a gel shift/retardation assay and nuclear extracts prepared from vaccinia virus-infected HeLa cells overproducing hRARs (see Nicholson *et al.*, 1990; Vasios *et al.*, 1991). Specific retarded complexes (arrow 1 in Figure 7) were observed upon electrophoretic separation of incubation mixtures of a ^{32}P-end labelled CRBPI RARE oligonucleotide and extracts containing either one of the three hRAR subtypes (α, β or γ). These complexes were not observed with the corresponding mutated RARE oligonucleotide (CRBPI-RAREmut), nor with extracts from cells transfected with wild-type vaccinia virus (wt) (Figure 7). The specificity of these complexes was investigated further using a supershift assay in which an antibody preparation against each RAR subtype was added following the initial incubation of the oligonucleotide with the cell extract. In all three cases (i.e. with extracts overexpressing hRAR-α, hRAR-β and hRAR-γ) a further retarded complex was observed, indicating a specific binding of the RARs to the CRBPI RARE (arrow 2). In fact, the antibody may stabilize the RAR−CRBPI RARE interaction as suggested by the presence of a stronger signal in the case of hRAR-α. No specific complexes were observed using the mutated oligonucleotide (CRBP-RAREmut), with the exception of a faint complex which was seen using the RAR-γ-containing

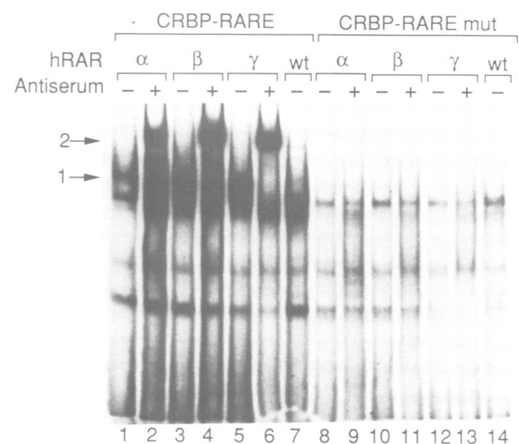

Fig. 7. Gel retardation assay. ^{32}P-end-labelled oligonucleotides containing either the normal (CRBP-RARE) or mutated (CRBP-RAREmut) mCRBPI RARE were incubated with cellular extract from vaccinia virus-infected HeLa cells overexpressing either hRAR-α1, −β2 or −γ1 [or cells infected with wild-type (wt) vaccina virus]. Where indicated, the respective specific anti-RAR α, β or γ antisera were subsequently added. Incubation mixtures were resolved on 5% polyacrylamide gels and autoradiographed (Nicholson *et al.*, 1990). Arrows indicate complexes corresponding to specific CRBPI-RARE−RAR interaction in the absence (arrow 1) and in the presence (arrow 2) of RAR antibodies.

cell extract and the anti-RAR-γ antibody preparation (Figure 7, lane 13).

Discussion

We report here the sequence of mouse CRBPI cDNA and that of the first 1909 bases of the 5′-flanking region of the corresponding genomic DNA. The cDNA-deduced amino acid sequence of mouse CRBPI (Figure 1A) is almost identical to those of human (Colantuoni *et al.*, 1985; Wei *et al.*, 1987; five amino acid changes) and rat (Sundelin *et al.*, 1985; Ile in place of Thr at position −126) CRBPIs. The sequence of the first 670 bases located upstream of the ATG initiator codon in the human CRBPI gene has been previously presented (Nilsson *et al.*, 1988). This human sequence can be readily aligned with its mouse counterpart up to nucleotide −99 (Figure 1B), whereas a lower degree of conservation

is observed further upstream. Both human and mouse CRBPI promoters lack a TATA box and contain multiple GC boxes which are putative binding sites for the transcription factor Sp1 (Kadonaga et al., 1987). The mouse CRBPI transcription start-site was mapped to an A within a sequence related to a consensus sequence, 5'-PyPyCAPyPyPyPyPy-3', found in a number of genes transcribed by RNA polymerase class II (B) (Corden et al., 1980). Furthermore, a weak similarity was also observed with the 'initiator' sequence described recently for TATA box-less promoters (Smale and Baltimore, 1989; Smale et al., 1990). Note that the human CRBPI putative start-site has been located to the base corresponding to position −9 in the mouse sequence (Nilsson et al., 1988). There is no putative NF-1 binding site in the human sequence, corresponding to those found in the 5'-flanking region of the mouse CRBPI (Figure 1B). On the other hand, seven out of nine bases of the mouse putative Krox-24 (or Krox-20 binding motif, see Lemaire et al., 1990) are conserved in the human CRBPI promoter region. A similar motif was also found in the promoter region of human (Brand et al., 1990) and mouse (Leroy et al., 1991) RAR-α genes which are also TATA box-less and exhibit GC box features found in a number of housekeeping genes. Interestingly, Krox-24, which is also known as Egr-1, is induced upon exposure of P19 cells to retinoic acid (Sukhatme et al., 1988). Thus the residual RA induction of CRBPI promoter activity observed with CRBPI/CAT3 (Figure 3) and CRBPI/CAT mutants (Figure 5) may possibly be due to such a Krox-24 induction. Alternatively it may reflect the presence of an additional weak RA response element located downstream of position −830 in the CRBPI 5'-flanking sequence.

Previous studies have shown that CRBPI mRNA levels are increased by RA treatment of whole animals (Haq and Chytil, 1988) or cultured cells (Eskild et al., 1988; Wei et al., 1989). Furthermore Wei et al. (1989) have conclusively shown that this induction corresponds to a cycloheximide-resistant primary transcriptional event. Our present results demonstrate that this direct response to RA can be mediated by the binding of RAR to a sequence which exhibits all of the properties expected for an enhancer-like RA responsive element (RARE). This RARE corresponds to a direct repeat of the motif 5'-GGTCA-3' with three intervening bases. The integrity of these two motifs is important for efficient RA induction of both the CRBPI promoter and a heterologous promoter *in vivo* (Figures 5 and 6), and all three RARs (α, β and γ) bind specifically an oligonucleotide containing these motifs *in vitro* (Figure 7).

The RARE of the mouse CRBPI gene, 5'GGTCAAA-AGGTCA-3', is very similar to the RARE which has been previously characterized in both the human (de Thé et al., 1990) and mouse (Sucov et al., 1990; Zelent et al., 1991) RAR-β2 promoter region which is composed of a direct repeat of the motif 5'-GTTCA-3'' (instead of 5'-GGTCA-3') with six intervening bases (5'-GTTCACCGAAAGTTCA-3'). It is noteworthy that the RARE of the laminin B1 gene is also composed of three directly repeated motifs, one 5'-TGACC-3' motif and two related degenerate motifs 5'-TAACC-3' and 5'-TCACC-3' (5'-GGTCA-3', 5'-GG-TTA-3' and 5'- GGTGA-3' on the opposite strand) (Vasios et al., 1989, 1991). Therefore, natural RAREs appear to be composed of the direct repetition of the motif 5'-G$^{G}_{T}$TCA-3' and/or of degenerate variations of this motif, with no strict spacing requirement between the repeated motifs, although integral multiples of a DNA helix turn may result in more efficient response elements (Sucov et al., 1990; Vasios et al., 1991).

The magnitude of the RA induction of the mouse CRBPI promoter−CAT reporter gene was lower than that observed with a similar reporter gene constructed with the mouse RAR-β2 promoter region (Figure 5), and was closer to that seen with the laminin B1 promoter region (Vasios et al., 1989). In the case of the laminin B1 promoter, it was shown that the reduced RA induction was due at least in part to the presence in the RARE of the two degenerate motifs mentioned above (Vasios et al., 1991). In the present case, the lower level of induction appears to be mainly due to the presence of a GGTCA motif direct repeat in the CRBPI RARE, which is less efficient than the GTTCA motif direct repeat present in the RAR-β2 RARE. Mutations which within the CRBPI RARE converted the GGTCA motifs to GTTCA motifs, resulted indeed in an increase in the magnitude of the RA induction (Figure 5, CRBPI-m4). In addition, the lower RA inducibility of the CRBPI promoter, when compared with the RAR-β2 promoter, may also be related to the location of the CRBPI RARE with respect to the CRBPI transcriptional start-site, which is at a greater distance than that of the RAR-β2 RARE in the case of the RAR-β2 promoter.

It has been previously reported that RARs can also activate transcription through palindromic elements composed of inverse repeats of the 5'-GGTCA-3' motif, such as those present in oestrogen (ERE, in which the repeats are spaced by 3 bp) and thyroid hormone (TRE, in which there is no strict spacer length requirement between 0 and 6 bp, see Umesono et al., 1988; de Verneuil and Metzger, 1990) response elements. Our present results confirm that a RAR can weakly transactivate the thymidine kinase (tk) promoter through a palindromic ERE, albeit with a lower efficiency than through a RARE (Figure 6). Similarly, a RAR can only weakly transactivate the CRBPI promoter region in which the RARE has been changed into an ERE (CRBPI-m5 in Figure 5).

Responsive elements of all steroid hormone receptors are composed of palindromic inverted repeats of pentameric motifs related to either 5'-GAACA-3' (for GR, PR, AR and MR) or 5'-GGTCA-3' (for the ER), with a strict requirement for a 3 bp spacer between the two arms of the palindromes (see Green and Chambon, 1988; Beato, 1989 for refs). It is well documented that steroid hormone receptors bind to their cognate responsive element as dimers, and it is likely that RARs bind also as dimers (for reviews and refs see Green and Chambon, 1988; Beato, 1989; Forman and Samuels, 1990a,b). Clearly, the dimers of steroid hormone receptors (SHRs) and those of RARs must be organized differently, since SHRs can transactivate only through pentameric motifs organized as palindromic elements exhibiting a 2-fold symmetry, whereas RARs can transactivate through similar motifs organized as either inverse or direct repeats, even though they act more efficiently through direct repeats. We note in this respect that the thyroid hormone receptors (TRs) can also transactivate through either inverse or direct repeats of similar pentameric motifs (see Glass and Holloway, 1990 for a review).

The physiological role of CRBPI is unknown. It is, however, interesting to note that it has been implicated in

the formation of retinyl esters which represent a storage form of retinol (for reviews see Blomhoff *et al.*, 1990; Chytil and Haq, 1990). A RA induction of CRBPI gene expression mediated by RAR may thus participate in a negative feedback regulation of RA synthesis from retinol. Site-directed mutagenesis of the mouse CRBPI gene in the whole mouse through homologous recombination with the CRBPI genomic sequences cloned in the present study is required to elucidate the function of CRBPI in the highly pleiotropic effect of retinoids during vertebrate morphogenesis and differentiation.

Materials and methods

Isolation of mouse CRBPI cDNA and 5′-flanking genomic DNA clones

Approximately 10^6 recombinant phages from an oligo(dT)-primed mouse testis λgt10 cDNA library (a gift from D.Duboule and B.Galliot) were screened using standard procedures with the ^{32}P-end-labelled degenerate oligonucleotide probe, 5′-TGGAAGATGC$^{GG}_{TC}$AGCA$^{G}_{A}$CGAGAATTTCG-AGGAG-3′, corresponding to the rat CRBPI sequences from amino acids 9−19 (Sundelin *et al.*, 1985; the segment of the mouse CRBPI cDNA sequence corresponding to this oligonucleotide probe is underlined in Figure 1A). Two clones were isolated, phage DNA was prepared, and the corresponding *Eco*RI DNA fragments were subcloned in pTZ19R vector for sequencing which was performed on both strands using single-stranded DNA and the dideoxy chain-termination procedure. Although independently isolated, the two inserts were identical, suggesting that the two clones correspond to sister molecules.

A mouse genomic λEMBL3 library (a gift from L.Stubbs) was screened with the ^{32}P-5′-end-labelled oligonucleotide (NL54), corresponding to the first 80 nucleotides of the mCRBPI cDNA (Figure 1A). A recombinant λEMBL3 phage containing a ~15 kb insert was isolated and a ~2 kb *Bss*HII−*Bam*HI fragment was subcloned into the *Eco*RV and *Bam*HI sites of the vector pBluescript (Stratagene) by making the *Bss*HII site blunt with Klenow DNA polymerase. This subclone (clone CRBP25) was sequenced using the dideoxy chain-termination method (Figure 1B).

Primer extension mapping of the mCRBPI transcriptional start-site

The transcriptional start-site was mapped by primer extension using the synthetic oligonucleotide 5′-AGGTACTCCTCGAAATTCTCATTGCTC-AGCATCTTCCAG-3′, complementary to nucleotides +132 to +170 in the genomic sequence (Figure 1B). The ^{32}P-5′-end-labelled oligonucleotide was annealed at 30°C with 10 μg of poly(A) RNA prepared from RA-treated and untreated P19 embryonal carcinoma (EC) cells and extended using AMV reverse transcriptase, as described previously (McKnight and Kingsbury, 1982). Primer extension products, along with DNA sequencing reactions of CRBP25 using the above oligonucleotide as a primer, were electrophoresed on 6% urea−polyacrylamide gels and autoradiographed (Figure 2).

Construction of reporter genes for CRBPI promoter activity

Single-stranded DNA was prepared from clone CRBP25 and an *Xba*I site was introduced by oligonucleotide-directed site-specific mutagenesis immediately 5′ of the initiator ATG (Figure 1B, position +108), yielding clone CRBP26. Various DNA fragments extending in the 5′ direction from this *Xba*I site up to the *Bam*HI site were subcloned into the *Xba*I and *Hin*dIII sites of the chloramphenicol acetyltransferase (CAT) reporter plasmid pBLCAT8+polyL [this plasmid was constructed from pBLCAT8+ (Klein-Hitpass *et al.*, 1988) by digestion with *Hin*dIII and *Bgl*II to remove the thymidine kinase (tk) promoter sequences]. To produce CRBPI/CAT1, 2 and 4 (Figure 3), CRBP26 was digested first with *Bam*HI, *Bgl*II and *Nar*I, respectively, and then treated with Klenow DNA polymerase and deoxynucleoside triphosphates to produce blunt ended DNA. After digestion with *Xba*I, the corresponding genomic fragments were ligated into the *Hin*dIII and *Xba*I sites of pBLCAT8+polyL in which the *Hin*dIII site had been made blunt as above. CRBPI/CAT3 was made by directly ligating the *Hin*dIII−*Xba*I fragment of CRBP26 into the *Xba*I and *Hin*dIII sites of pBLCAT8+polyL.

The CRBPI/CAT1 RARE mutants CRBPI-ml, m2, m3, m4 and m5 were obtained as follows. pUC-19 CRBPI was constructed by cloning the *Bam*HI−*Xba*I fragment of CRBP26 into the *Bam*HI−*Xba*I sites of a modified pUC-19 (in which the *Alw*NI and *Ssp*I sites were destroyed). The RARE-containing 32 bp long *Alw*NI−*Ssp*I fragment (positions nt −1025 to −994 in Figure 1B) was then replaced in pUC-19 CRBPI with synthetic oligonucleotides containing RARE sequences mutated as indicated in Figure 5A. The mutated *Bam*HI−*Xba*I fragments were then excised from pUC-19 CRBPI and inserted into pBLCAT8+polyL. The mutant oligonucleotides were: 5′-ATTTAGTCTTTAGTAGGAGCAAAGGGGCGACA-3′ for CRBPI-m1, 5′-ATTTAGTCTTTAGTAGGAGCAAAGGTCAGACA-3′ for CRBPI-m2, 5′-ATTTAGTCTTTAGTAGGTCAAAAGGGGCGA-CA-3′ for CRBPI-m3, 5′-ATTTAGTCTTTAGTAGTTCAAAAGTTCAG-ACA-3′ for CRBPI-m4, and 5′-ATTTAGTCTTTAGTAGGTCAAAATG ACCGACA-3′ for CRBPI-m5.

CRBPI-RARE/tk-CAT, CRBPI-RAREmut/tk-CAT and CRBPI/tk-CAT5 were obtained as follows. The synthetic oligonucleotides CRBP-RARE (5′-TTAGTAGGTCAAAAGGTCAGACACTGAA-3′) and CRBP-RAREmut (5′-TTAGTAGGAGCAAAGGGGCGACACTGAA-3′), and the 259 bp *Bgl*II−*Hin*dIII fragment of CRBP25, were cloned (as single copies) into either the *Bam*HI site (for the two above oligonucleotides) or into the *Hin*dIII and *Bam*HI sites of pBLCAT8+, to yield CRBPI-RARE/tk-CAT, CRBPImut-RARE/tk-CAT and CRBPI/tk-CAT5, respectively.

Cell transfection and CAT assay

P19 embryonal carcinoma (EC) cells were cultured in Dulbecco's medium containing 10% charcoal-treated fetal calf serum. Cells were transfected 8−10 h after plating by the calcium phosphate procedure as described previously (Kumar *et al.*, 1986) with 1−10 μg of the CAT reporter plasmids (as described in figure legends), along with either vectors expressing the human retinoic acid receptor α1 (hRAR-α0, Petkovich *et al.*, 1987; Brand *et al.*, 1988; Krust *et al.*, 1989) or the human oestrogen receptor (HEO, Green *et al.*, 1986; Kumar *et al.*, 1987), or the parental expression vector pSG5 (Green *et al.* 1988a), and 2 μg of the β-galactosidase expression vector pCH110 (Pharmacia) 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). The medium was changed after 15−20 h and the appropriate ligands [retinoic acid (RA) or oestradiol (E)] dissolved in ethanol were added to a final concentration of 10^{-6} M. After an additional 20−24 h the cells were harvested and cellular extracts were prepared as described previously (Petkovich *et al.*, 1987). Cell extracts containing 10−20 units of β-galactosidase activity were used for CAT assay. The CAT activity was quantified by liquid scintillation counting.

Gel retardation assay

Preparation and incubation of nuclear extracts from vaccinia virus-infected HeLa cells expressing hRARs with ^{32}P-labelled oligonucleotide probes (in the presence of 150 mM KC1) and gel retardation assays were as described previously (Nicholson *et al.*, 1990; Vasios *et al.*, 1991). Antibody preparations against hRAR-α, β and γ used in 'supershift' assays were as described (Vasios *et al.*, 1991).

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