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Biochemistry

Mouse retinoic acid receptor α2 isoform is transcribed from a promoter that contains a retinoic acid response element

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ABSTRACT **We have characterized the promoter of the mouse retinoic acid receptor α2 (mRAR-α2) isoform. This promoter contains a retinoic acid response element (RARE) that closely resembles the RARE that is present in the RAR-β2 promoter. Moreover, RAR-α2 and RAR-β2 proximal promoter sequences are similar to each other and generate transcripts whose respective start sites are located at similar positions. The RAR-α2 RARE consists of two directly repeated 5′-GTTCA-3′ motifs to which all three RARs (α, β, and γ) bind *in vitro*.**

The highly pleiotropic effects of retinoic acid (RA), a major developmental signaling molecule, are mediated by the products of three nuclear receptor genes (RAR-α, -β, and -γ), which interact with cis-acting RA response elements (RAREs) present in the promoter of RA target genes, thereby modulating their transcription (refs. 1 and 2 and references therein). Each subtype gene encodes several isoforms that are generated by alternative splicing of primary transcripts initiated from either one of two promoters. A previous report (3) showed that the mRNA level of RAR-α2, one of the two major RAR-α isoforms, is markedly increased in murine F9 and P19 embryonal carcinoma (EC) cells upon RA treatment. We have now characterized the promoter that generates this isoform and demonstrate here that it contains a RARE.†

MATERIALS AND METHODS

Mapping of the Mouse mRAR-α2 (mRAR-α2) Transcription Start Site. A 1.4-kilobase (kb) *Eco*RI–*Xho* I fragment derived from phage λG3 mRAR-α (3) was subcloned into the pBluescript SKII(+) vector (Stratagene) to yield the recombinant pEX, of which the 1.3-kb *Xba* I–*Xho* I segment was sequenced (Fig. 1*A*). The location of the mRAR-α2 start site was determined as described (4) by primer extension using the 5′-end-labeled antisense primer OY113 (Fig. 1*A*) and total RNA isolated from RA-treated (1 μM) P19 EC cells (Fig. 1*B*).

Reporter Genes for mRAR-α2 Promoter Activity. The *Xba* I–*Xho* I mRAR-α genomic DNA fragment was inserted in the *Xba* I and *Xho* I sites of the chloramphenicol acetyltransferase (CAT) reporter plasmid pBLCAT3 (6). The pBLCAT3 polylinker *Pst* I and *Sph* I sites were eliminated by digestion with *Hin*dIII and *Xba* I, followed by blunt-end ligation, which restored an *Xba* I site and yielded RAR-α2/CAT1 containing unique *Pst* I and *Sph* I sites (Fig. 2*A*). RAR-α2/CAT1 was digested with *Pst* I and *Xho* I, and the DNA ends were filled-in, yielding RAR-α2/CAT2 after ligation. RAR-α2/CAT3 and RAR-α2/CAT4 were obtained by inserting the 549-bp *Pst* I–*Xho* I fragment and the 859-bp *Sph* I–*Xho* I fragment of pEX into the corresponding sites of pBLCAT3 (Fig. 2*A*). RAR-α2m1/CAT1 was engineered from RAR-α2/CAT1 by introducing six transversions into the α2 RARE through PCR-assisted site-specific mutagenesis with four primers. The sequence of the two complementary "central" mutant primers corresponded to that of the α2 RARE m1 oligonucleotide (Fig. 3*A*). The two "external" primers were as follows: sense 5′ primer, 5′-GGACACCCAGCATGCTG-CATTCGTG-3′; antisense 3′ primer, 5′-ATCCCCTGCAG-GAAGATGCAGCGGC-3′ (nucleotides −258 to −234 and nucleotides +48 to +72, respectively; see Fig. 1*A*; the unique *Sph* I and *Pst* I restriction sites are underlined). PCR reactions were as described (7) and the final PCR product was digested with *Sph* I and *Pst* I. This mutated *Sph* I–*Pst* I cassette (Fig. 1*A*, −245 to +66) was exchanged with the corresponding *Sph* I–*Pst* I fragment of RAR-α2/CAT1 to yield RAR-α2m1/CAT1. α2RARE/tk-CAT, α2RAREm1/tk-CAT, α2RAREm2/tk-CAT, and α2RAREm3/tk-CAT were constructed by inserting the synthetic oligonucleotides α2 RARE, α2 RARE m1, α2 RARE m2, and α2 RARE m3 between the *Hin*dIII and *Xba* I restriction sites of pBLCAT8+ (8). Other materials and methods were as described previously (9, 10).

RESULTS

Isolation of the mRAR-α2 5′ Flanking Region and Mapping of the Start Site of mRAR-α2 Transcripts. The 18-kb genomic DNA insert of clone λG3 mRAR-α contains a single *Xho* I restriction site, which is also present in the 5′ UTR of the mRAR-α2 cDNA isoform (3). The sequence of a 1323-bp *Xba* I–*Xho* I fragment that hybridized to mRAR-α2-specific cDNA sequences was determined (Fig. 1*A*). As expected, its 3′ extremity overlaps with the 5′ UTR of the cloned mRAR-α2 cDNA sequence. The more-upstream genomic sequences contain recognition sites for rare-cutting restriction enzymes generally associated with CpG islands in mammalian genomes (ref. 11 and references therein), which suggests that this region contains the RAR-α2 start site.

The 5′ end of exon E3, which encodes the A region and the entire 5′ UTR of mRAR-α2 (3), was determined by PCR analysis of mRAR-α2 transcripts and primer extension. PCR was carried out with cDNA generated by reverse transcription [using the mRAR-α2 5′-UTR-specific antisense primer 5′-AAGTTGTGCAGGTTGGAGGA-3′ (3)] from poly(A)$^+$ RNA of RA-treated P19 EC cells and one of five oligonucleotide primers (Fig. 1*A*, arrows 1–5) together with a common 3′ primer (arrow 6). In all five cases, a specific PCR product of the expected size was detected (data not shown), indicating that the 5′ border of exon E3 was located upstream from

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Abbreviations: UTR, untranslated region; RA, retinoic acid; RAR, RA receptor; mRAR, mouse RAR; hRAR, human RAR; RARE, RA response element; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; EC, embryonal carcinoma.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M80673).

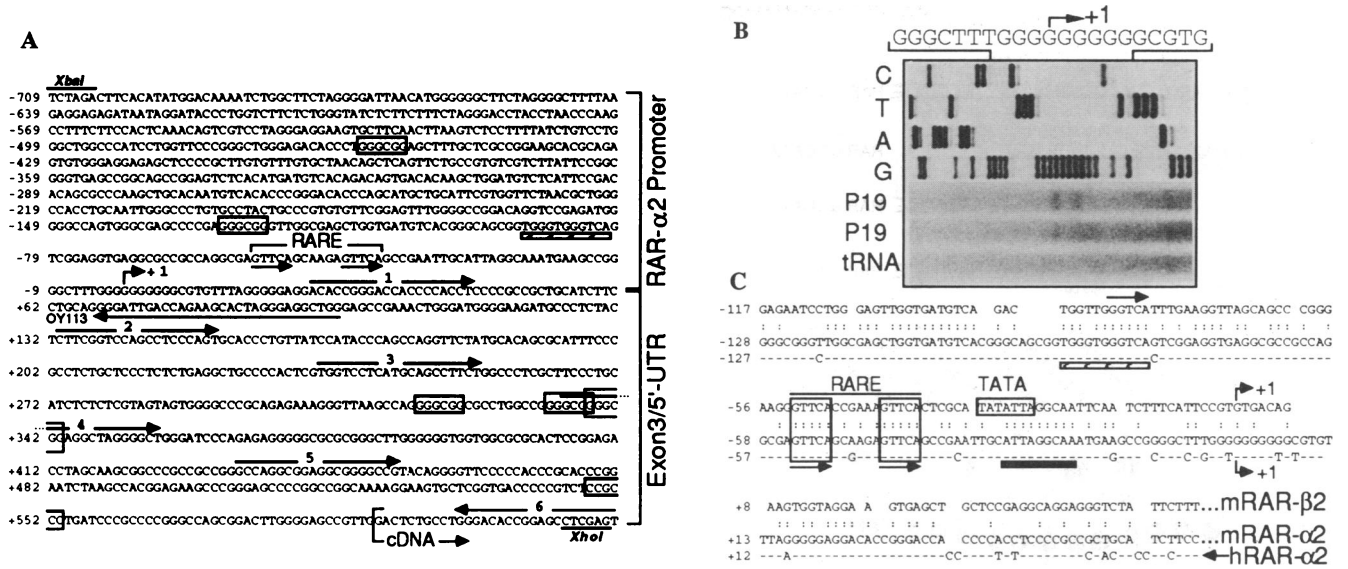

FIG. 1. Mapping of the mRAR-α2 start site. (*A*) Sequence of the mRAR-α2 promoter. The sequence of a 1323-base-pair (bp) *Xba* I–*Xho* I DNA fragment of the mRAR-α2 gene was determined on both strands by the standard chain-termination procedure (4). Position +1 corresponds to the mRNA start site (bent arrow). The 5′ boundary of the published mRAR-α2 cDNA (3) is indicated by a bracket at nucleotide 592. Six possible binding sites for factor Sp1 are boxed. The α2 RARE is bracketed and the 5′-GTTCA-3′ motifs are indicated by two arrows. The sequence 5′-TGGGTGGGTCA-3′ (see text) is underlined with a striped bar. The sequence and orientation of the oligonucleotide (OY113) used for primer extension are indicated by an arrow (nucleotides +68 to +97). Arrows 1–6 indicate the positions of primers used for PCR analysis of mRAR-α2 transcripts (see text). UTR, untranslated region. (*B*) Mapping of the mRAR-α2 start site by primer extension. The sequence presented on the top corresponds to the noncoding strand and the broken arrow indicates position +1. Lanes G, A, T, and C contained sequencing "ladders" primed with the antisense oligonucleotide 5′-CCAGCCTCCCTAGTGCT-3′ and electrophoresed through (left to right as shown here) urea/8% polyacrylamide gel alongside primer extension products obtained with either total RNA from RA-treated P19 EC cells (P19, two different RNA preparations) of yeast tRNA (tRNA). (*C*) Alignment of the sequences of mRAR-α2, human RAR-α2 (hRAR-α2), and mRAR-β2 promoters and 5′ UTRs. Gaps have been introduced for optimal alignment; colons indicate identical nucleotides. The nucleotides in the hRAR-α2 DNA sequence (unpublished results) that differ from the corresponding mRAR-α2 sequences are indicated. RARE directly repeated motifs are boxed. A putative TATA box in the mRAR-β2 promoter (5) is indicated, and the sequence 5′-ATTAGGCAA-3′, shared by both the RAR-α2 and RAR-β2 promoters, is underlined with a black bar. Other symbols are as in *A*.

the most 5′ PCR primer 1 (Fig. 1*A*), and that no intron was interrupting this 5′ UTR. Primer extension was then performed to map the 5′ end of exon E3 (Fig. 1*B*), generating a specific extended fragment whose 5′ end mapped at the position indicated as +1 in Fig. 1.

mRAR-α2 and mRAR-β2 Proximal Promoter Sequences Are Similar. The mRAR-α2 5′ region contains several putative binding sites for the transcription factor Sp1 (boxed in Fig. 1*A*), located in either the 5′ UTR or the proximal promoter sequences. No obvious TATA- or CCAAT-box sequences were noticed. However, a sequence closely resembling the RARE previously characterized in the mouse and human RAR-β2 promoters (12, 13) lies upstream of the cap site (Fig. 1 *A* and *C*, nucleotides −54 to −39). This putative RARE consists of a direct repeat of 5′-GTTCA-3′ with a 6-nucleotide spacer whose sequence differs from that of the β2 RARE. Interestingly, this presumptive RARE is located at a position (with respect to the cap site) that is similar to that of β2 RARE.

A comparison between the mRAR-α2 and mRAR-β2 (5) proximal promoter sequences reveals that the two sequences are closely related (Fig. 1*C*). The sequence 5′-ATTAGGCAA-3′ located 10 bp downstream of the putative α2 RARE is identical to a sequence located at an equivalent position in the mRAR-β2 promoter (black bar in Fig. 1*C*), where it partially overlaps the RAR-β2 TATA box (5, 12, 13). This observation, and the fact that RAR-α2 and RAR-β2 start sites are located at the same position within their respective genomic DNA sequences (13), suggests that this sequence, which is conserved in the hRAR-α2 promoter (see Fig. 1*C*), corresponds to a RAR-α2 degenerate TATA box. In fact, a mutation converting this 5′-ATTAGGCAA-3′ motif to 5′-CGACGGCAA-3′ resulted in an ≈70% reduction in the activity of the mRAR-α2 promoter (data not shown). The similarity between mRAR-α2 and -β2 5′ flanking sequences extends further upstream from the RARE, which suggests that additional upstream regulatory elements may be common to both promoters. No mRAR-β2 5′ flanking sequences located further upstream have been reported (5), but further 5′ human RAR-β2 DNA sequences have been published (14), exhibiting no significant similarities with the mRAR-α2 sequences located upstream from position −128.

The mRAR-α2 Promoter Is Active in Cultured Cells and Contains a Functional RARE. Various lengths of the putative mRAR-α2 promoter sequences were ligated to the CAT gene (Fig. 2*A*) and tested for RA inducibility in P19 EC cells. Fig. 2*B* shows the results of a typical experiment in which CAT reporter plasmids were transfected into P19 EC cells, which were subsequently treated with 1 μM RA or vehicle (ethanol). The promoterless vector pBLCAT3 was inactive (Fig. 2*B*, lanes 1 and 2); in contrast, RAR-α2/CAT1 showed a low basal level of CAT activity in the absence of RA and was induced ≈20-fold in the presence of 1 μM RA (lanes 3–6), to a level similar to that achieved with a RAR-β2 promoter CAT reporter (RAR-β2/CAT; see ref. 15). RAR-α2/CAT3 activity (lanes 11 and 12) was much lower and close to that of pBLCAT3, whereas RAR-α2/CAT2 (lanes 9 and 10) and RAR-α2/CAT4 (lanes 13 and 14) activities were similar to that of RAR-α2/CAT1 (lanes 3 and 4), showing that the sequences required for RAR-α2 promoter basal activity and those conferring RA responsiveness are contained within the 245-bp segment located upstream from the start site (*Sph* I site at −245 in Fig. 2*A*).

No RAR expression vectors were cotransfected in these experiments, indicating that RA induction was mediated by P19 cell-endogenous RARs (3, 5, 16). That RXRs (17), which

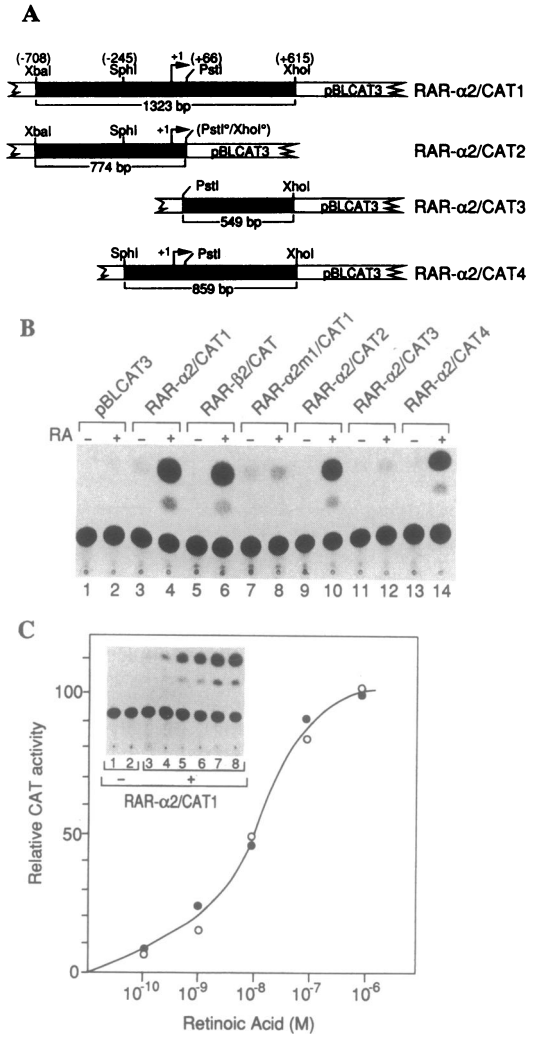

FIG. 2. RA inducibility of the mRAR-α2 promoter. (*A*) Schematic representation of the reporter constructs. mRAR-α2 gene sequences (sizes in base pairs) and the parental vector pBLCAT3 are represented as black boxes and broken white segments. Relevant restriction sites are indicated together with their positions with respect to the start site (+1). (*B*) Representative assay of CAT activity. Transfections into P19 EC cells were carried out with the constructs (5 μg) depicted in *A* (lanes 3 and 4 and 9–14), as well as with the promoterless pBLCAT3 (5 μg) (lanes 1 and 2). A similar construct containing the mRAR-β2 promoter (RAR-β2/CAT; lanes 5 and 6) and RAR-α2m1/CAT1, which carries a mutated RARE (lanes 7 and 8; see α2 RARE m1 sequence in Fig. 3*A*), were similarly assayed. Transfections were done in duplicate in the presence of 10^{-6} M RA (+, even lane numbers) or vehicle (ethanol) (−, odd lane numbers). (*C*) RA dose–response for RAR-α2/CAT1 activity. RAR-α2/CAT1-transfected P19 EC cells were treated with either vehicle (ethanol) or RA (10^{-10} to 10^{-6} M) as indicated. The relative CAT activity (expressed as percent maximal induction in treated versus untreated cells, average of two independent experiments, solid and open circles) is plotted against RA concentration. (*Inset*) A typical assay in which P19 EC cells were transfected with 5 μg of plasmid RAR-α2/CAT1 (+) and then treated with 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M RA (lanes 4–8, respectively) or untreated (lane 3). A control experiment (−) was carried out with the parental plasmid pBLCAT3 (5 μg), in the presence (lane 2) or absence (lane 1) of 10^{-6} M RA.

are also present in P19 cells (P. Kastner and P.C., unpublished observation), could be involved in this induction is unlikely, since a response was seen with as little as 0.1 nM RA (Fig. 2*C*), a concentration at which RXRs do not respond (17). The same dose–response curve as that shown in Fig. 2*C* was obtained with mRAR-β2/CAT (data not shown).

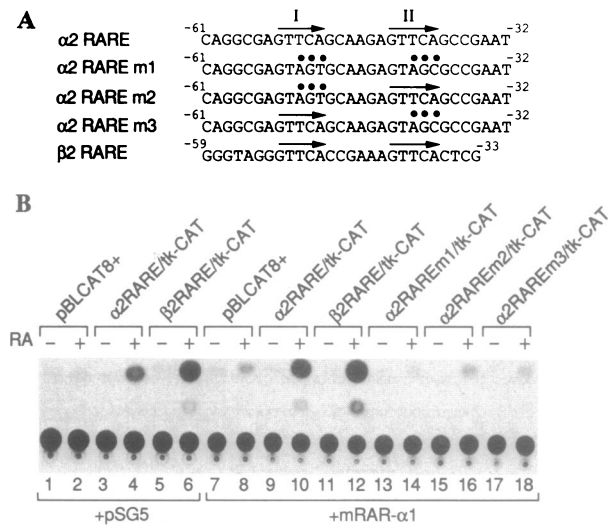

FIG. 3. Activity of a synthetic α2 RARE on the thymidine kinase (tk) promoter. (*A*) Oligonucleotides containing either the wild-type α2 RARE, or a mutated sequence (α2 RARE m1, m2, and m3) of the α2 RARE, or the β2 RARE (β2 RARE) were ligated into the CAT expression vector pBLCAT8+. Solid circles indicate mutated bases in either one of the directly repeated motifs I or II. All other symbols are as in Fig. 1. (*B*) The constructs depicted in *A* were transfected (5 μg) into P19 EC cells along with 250 ng of either the parental expression vector pSG5 (lanes 1–6) or the mRAR-α1 expression vector (lanes 7–18), as indicated. Transfected cells were exposed either to no ligand (−, odd lane numbers) or to 1 μM RA (+, even lane numbers) for 24 hr.

To prove that the above putative RARE (Fig. 1 *A* and *C*) was responsible for RA induction, the activity of the mutant RAR-α2m1/CAT1 (*Materials and Methods*) was tested in transfection experiments (Fig. 2*B*, lanes 7 and 8). Whereas its basal activity (i.e., in the absence of RA) was unchanged (compare lanes 3 and 7), its RA inducibility was virtually abolished (compare lanes 4 and 8).

The function of the α2 RARE was further investigated by inserting the directly repeated 5′-GTTCA-3′ motifs (Fig. 3*A*) upstream of the herpes simplex virus tk promoter, yielding α2RARE/tk-CAT. The plasmids α2RAREm1/tk-CAT, α2RAREm2/tk-CAT, and α2RAREm3/tk-CAT were similarly constructed using the α2 RARE mutants m1–m3 (Fig. 3*A*). These reporter genes were transfected into P19 cells either alone or together with either the parental expression vector pSG5, as a control (18), or pSG5, expressing either mRAR-α1, -β2, or -γ1 (3, 5, 16, and 19). β2RARE/tk-CAT, which contains the human β2 RARE (13) inserted upstream of the tk promoter of pBLCAT8+, was used for comparison. α2RARE/tk-CAT was RA-inducible, albeit to a lesser extent than β2RARE/tk-CAT (about one-third as efficiently, as determined by radioactivity counting; Fig. 3*B*, lanes 1–6), suggesting that the α2 RARE may have a lower affinity than the β2 RARE for RARs (see below). The magnitude of the induction was increased upon cotransfection with mRAR-α1, -β2, or -γ1 expression vectors (Fig. 3*B*, lanes 7–12, and data not shown). In contrast, no RA induction was observed with the α2 RARE mutants m1–m3 (Fig. 3*B*, lanes 13–18).

Binding of RAR-α, -β, and -γ to the mRAR-α2 RARE *in Vitro*. Binding of RARs to α2 RARE was investigated *in vitro*, using a gel retardation/shift DNA-binding assay and nuclear extracts prepared from COS-1 monkey cells transiently transfected with vectors expressing either hRAR-α1, -β2, or -γ1. Specific retarded complexes (arrow 1 in Fig. 4*A*) were observed when a ^{32}P-end-labeled α2 RARE probe was incubated with COS-1 extracts containing any one of the three hRARs. These complexes were not observed with the mutant

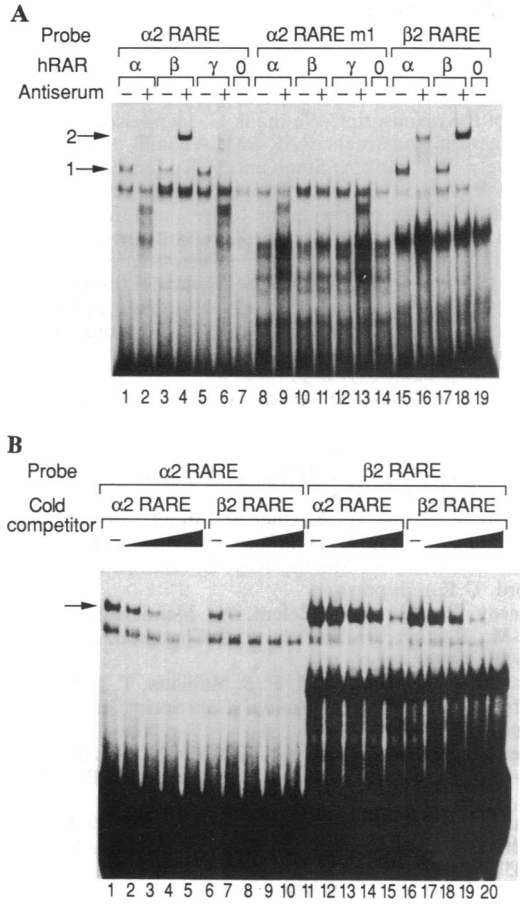

FIG. 4. Binding of RARs to the α2 RARE *in vitro*. (*A*) Specific binding of hRAR-α1, -β2, and -γ1 to α2 RARE. ^{32}P-end-labeled oligonucleotides (probe, 10 fmol, 50,000 cpm) corresponding to the wild-type α2 RARE (lanes 1–7), the mutated α2 RARE m1 (lanes 8–14), or the wild-type β2 RARE (lanes 15–19) were incubated with 4 μg of total protein extract from COS-1 cells transfected with one of the three hRAR subtypes (α1, β2, or γ1; see ref. 9) or pSG5 (0), as indicated. Arrows 1 and 2 point to complexes corresponding to specific RARE/RAR interactions in the absence (−) or presence (+) of RAR antibodies specific for either RAR-α, -β, or -γ (antiserum), respectively (see ref. 9). (*B*) Lower affinity of RAR for α2 RARE than for β2 RARE. Extracts of COS-1 cells transfected with hRAR-α1 were incubated with ^{32}P-end-labeled probes corresponding to either the α2 or the β2 RARE, as indicated, and increasing amounts of nonradioactive ("cold") competitor RARE. Incubations corresponding to lanes 1, 6, 11, and 16 contained no competitors (−); competitors were added as follows: 10 fmol for lanes 2, 7, 12, and 17; 30 fmol for lanes 3, 8, 13, and 18; 100 fmol for lanes 4, 9, 14, and 19; 300 fmol for lanes 5, 10, 15, and 20.

α2 RARE m1, or with extracts prepared from pSG5 (18)-transfected cells (Fig. 4*A*, lanes 1–14). The specificity of these complexes was further demonstrated by using a "supershift" assay (10) in which antibodies specific for each RAR subtype were added. In each case a supershifted complex was observed, indicating specific binding of hRARs to α2 RARE (arrow 2). Specific complexes were seen with a control ^{32}P-end-labeled β2 RARE oligonucleotide, as expected (lanes 15–19).

RAR Binding to α2 RARE Is Weaker Than That to β2 RARE. The observation that the magnitude of RA inducibility of α2RARE/tk-CAT is less than that of β2RARE/tk-CAT (see above) suggested that RARs may bind to α2 RARE with a lower affinity than to β2 RARE. Competition experiments were performed with α2 RARE and β2 RARE oligonucleotides (used either as radiolabeled probes or as nonradioactive competitors) and cell extracts prepared from COS-1 cells transfected with hRAR-α1. Although both α2 RARE and β2 RARE probes had similar specific activities, a smaller amount of specific complex was formed with the α2 RARE than with the β2 RARE oligonucleotide (arrow in Fig. 4*B*, compare lanes 1 or 6 with lanes 11 or 16). Moreover, a nonradioactive α2 RARE oligonucleotide was about one-third as efficient as the corresponding β2 RARE oligonucleotide at competing with either labeled α2 RARE or β2 RARE probe for binding to hRAR-α1 (Fig. 4*B*, compare lanes 1–5 with lanes 6–10, and lanes 11–15 with lanes 16–20). Competition experiments conducted with extracts prepared from COS-1 cells transfected with vectors expressing either hRAR-β2 or hRAR-γ1 yielded similar results (not shown).

DISCUSSION

A previous paper (3) reported the existence of two major mouse RAR-α isoforms, mRAR-α1 and -α2, and observed that the level of mRAR-α2 transcripts, but not that of mRAR-α1, was increased upon RA treatment of P19 and F9 EC cells (3). Moreover, mRAR-α1 is ubiquitously expressed, whereas mRAR-α2 distribution is more restricted (3). It was also found that the human and mouse RAR-α1 promoters resemble those of "housekeeping" genes (3, 20). We show here that the RA-induced accumulation of mRAR-α2 transcript is mediated through an alternative RAR-α promoter that contains a RARE.

The present sequencing data extend those published previously (3) and demonstrate that all of the 5′ UTR and region A2 coding sequences are encoded in a single exon (exon E3; see Fig. 1*A* and ref. 3). The mRAR-α2 5′ UTR (904 bp) is G+C-rich (>70%) and can form stable hairpin structures (ref. 3 and data not shown). Moreover, it contains a 702-base-long open reading frame (ORF) preceding the main ORF, which suggests that this 5′ UTR may play a role in the control of translation efficiency of mRAR-α2 mRNA and/or in its stability (ref. 3 and references therein). However, this 5′ ORF is not conserved in the hRAR-α2 5′ UTR (our unpublished results).

There is no consensus TATA box 30 bp upstream from the mRAR-α2 (and hRAR-α2) start site, but an A+T-rich sequence is present at this position. It may correspond to a degenerate TATA box, in view of its conservation in the hRAR-α2 promoter and in the highly similar RAR-β2 promoter (see below and Fig. 1*C*). There are several putative binding sites for the Sp1 factor (21) in the mRAR-α2 5′ region (Fig. 1*A*). Whether they contribute to the basal activity of the RAR-α2 promoter remains to be investigated.

The most interesting feature of the mRAR-α2 proximal promoter sequence is the presence of a RARE consisting of the directly repeated motif, 5′-GTTCA-3′, with 6 intervening bases. The role of these motifs in mediating the RA response is demonstrated by the detrimental effect of their mutation on RA inducibility of reporter genes containing either the natural mRAR-α2 promoter (Fig. 2*B*) or the heterologous tk promoter (Fig. 3). Furthermore, our *in vitro* studies demonstrate that all three RARs bind specifically to the α2 RARE (Fig. 4*A*). The mouse α2 RARE, whose sequence and location are conserved in the hRAR-α2 5′ flanking region, is strikingly similar to that of the human (13) and mouse (12) β2 RAREs, in both sequence and location (Fig. 1*C*). However, the β2 RARE is ≈3-fold more efficient than its α2 counterpart for both RA inducibility *in vivo* (Fig. 3*B*) and RAR binding *in vitro* (Fig. 4*B*), which further supports the conclusion that these RAREs mediate RA-dependent transcriptional induction.

The above results indicate that the spacer sequence located between the GTTCA repeated motifs and/or the bases flanking these motifs must modulate RARE "strength." We have changed either the intervening or the flanking bases of α2

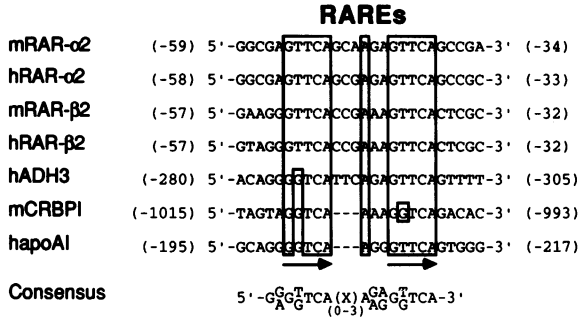

FIG. 5. Sequence similarities between RAREs. The nucleotide sequences of RAREs present in the promoter region of several genes are aligned: mouse and human RAR-α2, the mouse (5, 12) and human (13) RAR-β2, human alcohol dehydrogenase 3 (hADH3; ref. 22), mouse cellular retinol-binding protein I (mCRBPI; ref. 10), and human apolipoprotein AI (hapoA1; ref. 23). Conserved nucleotides are boxed, and directly repeated 5'-G$\frac{T}{G}$TCA-3' motifs are indicated by two arrows. A consensus RARE sequence is derived (see text).

RARE to those of β2 RARE and found that both of them contribute equally to the above 3-fold difference (P.L. and P.C., unpublished results). We note that other natural RAREs contain a direct repetition of either the GTTCA motif or the related motif GGTCA (Fig. 5; see refs. 9 and 10), whereas the length and the bases of the spacer sequence as well as the flanking bases are not conserved. Nevertheless, a consensus sequence can be derived, 5'-G$\frac{G}{A}$G$\frac{T}{G}$TCAX$_{(0-3)}$-A$\frac{GA}{AG}$G$\frac{T}{G}$TCA-3' (where X is A, C, T, or G). Further studies are required to define precisely the contribution of each base of this consensus sequence to RARE strength. An additional GGTCA motif located further upstream of the RARE is present in both the RAR-α2 and RAR-β2 promoters (see Fig. 1 *A* and *C*). Whether this single motif cooperates with the RARE to mediate RA inducibility is unknown. Interestingly, this motif is contained within the sequence 5'-TGGG-TGGGTCA-3', which is conserved in the mRAR-β2 promoter (see Fig. 1) and is also present in the proximal region of the ovalbumin gene promoter, where it has been shown to be a weak estrogen response element (24) and also to mediate the activity of the transcription factor AP-1 (25).

Previous reports (1–3, 5) have stressed similarities in the overall structural organization of the three RAR genes, as well as similarities between the amino acid sequences of the N-terminal A regions of the isoforms transcribed from the upstream P1 promoters (α1, β1/β3, and γ1), on the one hand, and of those transcribed from the downstream P2 promoters (α2, β2, and γ2) on the other (1, 2). Our present results support the idea that the RAR-α2 and RAR-β2 transcription units were derived by duplication of a common ancestor. However, the RAR-γ2 isoform is not RA-inducible (16), which suggests that it originated from a duplication event that preceded the RAR-α/β duplication. Thus the primordial RAR gene may have had two promoters, a RA-noninducible upstream promoter (P1) and a RA-inducible downstream promoter (P2), and the inducibility of the RAR-γ2 P2 promoter would have been lost. In any event, the question arises as to which RAR subtypes and isoforms can mediate the RA induction of the α2 promoter. All three RARs can bind to the mRAR-α2 RARE *in vitro* and transactivate α2RARE/tk-CAT reporter genes in cultured cells. It remains to be seen whether these RAR properties hold true under "natural" conditions within the context of the RAR-α2 promoter, and in cells and tissues where the expression of the RAR-α2 isoform is controlled by RA during development and in adult vertebrates.

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