Identification of a Novel Retinoic Acid Response Element in the Promoter Region of the Retinol-binding Protein Gene*

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We have previously demonstrated that the retinol-binding protein (RBP) gene is induced by retinoids in hepatoma cells. In this report, we define in greater detail the region that mediates the retinoic acid response of the gene. It consists of two degenerate retinoic acid response elements, separated by 30 nucleotides that encompass a GC-rich Sp1 consensus-like sequence. We demonstrate that the entire region, as well as each element taken singly, can bind the retinoic acid receptors as homo- and heterodimers with low affinity. However, only the entire region is able to confer retinoic acid inducibility to a heterologous promoter. We also show that the correct phasing of the DNA segment is necessary to achieve full responsiveness. Site-directed mutants in each element retained partial induction after transfection, while the double mutant was no longer responsive, suggesting that the two elements act synergistically. Mutational analysis of the Sp1 binding site and cotransfection experiments revealed that Sp1 or a related protein plays an important role in the transcription of the gene. Thus, the retinoic acid induction of the RBP gene is mediated by a novel and complex responsive unit formed by two distinct elements located in a specific sequence context and the interplay of the retinoid receptors with Sp1 is required for induction.

Retinoids play a pivotal role during embryonic development and in physiological processes such as reproduction, morphogenesis, homeostasis, and disease (1, 2). These pleiotropic effects are mainly mediated by two distinct classes of receptors, the retinoic acid receptors (RAR)[1] and the retinoid X receptors (RXR) that bind the DNA as heterodimers. They belong to the superfamily of the steroid/thyroid hormone nuclear receptors and act as ligand-induced transcription factors. Both types of receptors are encoded by three different genes, α, β, and γ, each generating several isoforms by alternative splicing and promoter usage (see Giguere (3) and references therein) (4, 5). Retinoic acid (RA) and its isomer 9-*cis*-RA are the two bioactive forms of the retinoids; RARs bind both molecules with high affinity, whereas RXRs bind only 9-*cis*-RA (3, 6, 7). The ligand specificity is due to differences in the ligand binding domain located at the C terminus of the protein (4, 5).

RAR and RXR recognize specific sequences on the DNA, designated "retinoic acid response elements" (RARE), which consist of the direct repetition of two core motifs (A/GGG/TTCA) (8), found in the regulatory regions of several "natural" target genes, and in DNA segments able to trans-activate heterologous promoters (2, 3). It has been proposed that the spacing and/or orientation of the repeated core motifs represents a discriminating recognition code for some nuclear receptors. Directly repeated (DR) motifs separated by 1 (DR1), 3 (DR3), 4 (DR4), and 5 (DR5) nucleotides correspond to specific binding sites for RXR, vitamin D_3 receptor, thyroid hormone receptors, and RAR, respectively (8). More recent studies, however, have shown that such a "rule" must be highly degenerate because RAR and RXR can bind to elements with DR other than DR5 and DR1. Moreover, in several natural promoters, response elements formed by the same core sequences arranged as inverted and everted repeats with spacers of different length are still able to bind the RAR (3, 4). RXR can heterodimerize with other members of the family, broadening the repertoire of genes regulated by these factors (3, 9–12). Finally, the polarity of the RAR-RXR dimer in the binding appears to be another important element in determining the specificity of the genes to be induced in response to different stimuli and metabolic conditions (13–15).

The retinol-binding protein (RBP) is the carrier molecule for retinol or vitamin A alcohol in the bloodstream from liver storage to the tissues requiring the vitamin (16). It binds a single molecule of retinol and as holo-RBP interacts with transthyretin to form a ternary complex, the active circulating form. RBP is one of the many proteins involved in the transport and metabolism of retinoids that, being hydrophobic molecules, require specific binding proteins (1, 3, 5). Such a multiplicity coincides with the supposedly tightly controlled retinoid concentration in the cell. In many cases, this fine tuning is exerted at the level of transcription of the corresponding genes (17–20). The RBP has been shown to be regulated both *in vivo* and *in vitro* at the level of secretion by the presence of the ligand (21–24). We have demonstrated that the RBP gene is regulated at transcriptional level by RA and retinol in hepatoma cells in culture (25). We also showed that the stimulation is reproduced on a chimeric RBP-CAT gene introduced via transfection (25).

In this report, we describe the RA response of the RBP gene in greater detail and demonstrate that the induction is mediated by the direct binding of the RAR and RXR homo- and heterodimers to a novel and composite response unit. It has a bipartite structure and requires the presence of both elements to which homo- and heterodimers containing receptors cooperatively bind to achieve full induction. We also show that the

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[1] The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; RA, retinoic acid; RARE, retinoic acid response element; RBP, retinol-binding protein; DR, directly repeated; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; TRE, 12-*O*-tetradecanoylphorbol-13-acetate-responsive element; EMSA, electrophoresis mobility shift assay; bp, base pair(s).

sequence context and the correct phasing of the DNA segment where the response elements are located play an important role in this process.

Finally, we show that Sp1 or a related protein is a major activator of *RBP* and acts in concert with the retinoid receptors for the maximal induction of the gene.

MATERIALS AND METHODS

Cell Culture—HepG2, HeLa, CV-1, L, and COS 7 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% normal (Life Technologies, Inc.) or delipidized fetal calf serum (Institut J. Boy, Reims, France). *Drosophila melanogaster* Schneider SL2 cells (26) were grown at 25 °C in Schneider medium (Life Technologies, Inc.), supplemented with 10% heat-inactivated normal or delipidized fetal calf serum.

Plasmids and Cell Transfection—The RBP-CAT1, 2, 3, 4, and 5 plasmids and the positive control RARE-thymidine kinase (TK)-CAT used in transfection assays are described elsewhere (25, 27). The pSV-luciferase plasmid (25) was used as a control for the efficiency of transfection. The RBP-TK-CAT1, 2, and 3 plasmids contain DNA fragments from *RBP*, inserted upstream to the herpesvirus TK gene promoter (from −105 to +51) (28). In particular, the RBP-TK-CAT 1 and 2 carry the DNA segments, from −220 to −130 bp and from −220 to −88 bp, with respect to the RBP gene transcription start site, respectively. They were obtained after digestion of the plasmid RBP-CAT4 with *Bam*HI and *Sma*I or with *Bam*HI and *Nar*I, respectively. The RBP-TK-CAT3 plasmid contains two copies of the *Bam*HI-*Nar*I fragment, cloned in a head-to-tail arrangement. The RBP-CAT4 link10 and 12-bp constructs were generated by introducing a 10- and a 12-bp long *Hin*dIII linker, respectively, into the *Sma*I site of the RBP-CAT4 plasmid. mtA, mtB, mtA-B TK-CAT, and Sp1mt-RBP-CAT2 were constructed by site-specific mutagenesis with the polymerase chain reaction technique (29), using specific primers containing the mutated nucleotides. The following oligonucleotides were used for the polymerase chain reaction-mediated mutagenesis, as probes or competitors in electrophoresis mobility shift assays: β-RARE oligo, AAGGGTTCACCGAAAGTTCACTCGCAT; 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (TRE) oligo, CTAGTGATGAGTCAGCCGGATC; protection A oligo, CCGCTCCACT-GTGCCCGAGGCTGTCCTGGAGGTA; protection mtA oligo, CCGCTC-CACTGTGCCCGAGGCaGatCTGGAGGTA; protection B oligo, GGGC-TCCGGTGAGTCAGGGCGCGTTATGCA; protection mtB oligo, GGGC-TCCGaTGAGTCAGccCGCGTTATGCA; Sp1-RBP mt oligo, AGGTA GtCtaGaCCACAGGGACCCT.

The expression vectors pSG5-hRARα, β, and γ and hRXRα (a kind gift from Prof. P. Chambon, Strasbourg, France) were used for cotransfection experiments in HepG2, CV-1, and COS 7 cells. In *D. melanogaster* Schneider SL2 cells the expression vector pPacRARβ, containing the RARβ cDNA cloned downstream to the *D. melanogaster* actin 5C promoter (30), was transfected together with the target constructs. RSV-Sp1 and pPac-Sp1 were a kind gift from Prof. L. Lania (31).

HepG2, COS 7, and Schneider SL2 cells (5 × 105) were plated 4–5 h prior to transfection performed with the calcium phosphate coprecipitation technique (32) using 20 μg of total DNA. The precipitate was removed 12 h later, and fresh medium containing ethanol, RA, or 9-*cis*-RA (Sigma and a gift from Prof. E. Gionti) was added for a further 24 or 48 h. Cells were harvested, the extracts were prepared, and the luciferase activity was measured on an aliquot in a luminometer, to normalize for variations in transfection efficiency (25). CAT assay was performed as described elsewhere (33). The acetylated *versus* the non-acetylated [¹⁴C]chloramphenicol form was quantitated by cutting and counting the spots from thin layer chromatograms, using a liquid scintillation counter (Beckman). Alternatively, CAT enzymatic activity was determined using the CAT-enzyme-linked immunosorbent assay kit (Boehringer Mannheim). Transfections were performed three to five times, using different DNA preparations. The *bars* indicated in the figures indicate the standard deviations.

Nuclear Extract Preparation and Electrophoresis Mobility Shift Assay—Nuclear extracts were prepared according to Dignam *et al.* (34) and Lee *et al.* (35), with minor modifications. Cells were washed in phosphate-buffered saline, and the packed cell volume was resuspended in buffer A (10 mM Hepes, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol and phenylmethylsulfonyl fluoride). Cells were then lysed in a buffer containing 1% Nonidet P-40 by 10–15 strokes with an ice-cold Dounce homogenizer. The cell homogenate was centrifuged for 3 min at $12,000 \times g$; the nuclear pellet was resuspended in buffer C (20 mM Hepes, 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and dithiothreitol) and

incubated on ice for 30 min. The nuclear homogenate was pelleted in a microcentrifuge ($12,000 \times g$), and the supernatant containing the nuclear extract was dialyzed against buffer D (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and phenylmethylsulfonyl fluoride). The dialyzed extract was frozen in liquid nitrogen and stored at −70 °C. Double-stranded oligonucleotides and DNA fragments were 5′- or 3′-end-labeled with T4 polynucleotide kinase and [γ-³²P]ATP or with the Klenow fragment of DNA polymerase I and [α-³²P]dATP, respectively (36). Labeled DNA (0.05 ng) was incubated at 20 °C for 15 min with 5 μg of nuclear extracts in the presence of 2–5 μg of poly(dI-dC) in a final volume of 20 μl, containing 20 mM HEPES, 4% Ficoll, 40 mM KCl. For each competition experiment, a 100-fold molar excess of each nonradioactive double-stranded oligonucleotide was added. Antibody supershift experiments were performed with polyclonal antibodies raised to synthetic peptides derived from unique sequences of the RARβ and RXRα, respectively (Santa Cruz Biotechnology, Santa Cruz, CA). The antibodies were added to the standard electrophoresis mobility shift assay (EMSA) mixture and incubated at 4 °C overnight. The DNA-protein complexes were analyzed by electrophoresis on a 5% polyacrylamide gel in 0.25 × Tris borate-EDTA buffer at 4 °C, and the gel was dried and autoradiographed at −70 °C.

DNase I Footprint Experiments—The DNA fragment used for footprinting analysis was obtained from the RBP-CAT4 plasmid after digestion with the enzymes *Bam*HI and *Nar*I (at positions −220 and −88). This fragment was then end-labeled with [α-³²P]dATP or [α-³²P]dCTP, using the Klenow fragment of DNA polymerase I. Approximately 5×10^4 cpm of the end-labeled probe were mixed with partially purified cellular extracts from HeLa cells infected with recombinant vaccinia viruses carrying the cDNA corresponding to RARβ and RXRα (a kind gift from Dr. H. Stunnenberg, EMBL, Heidelberg, Germany) in a solution containing 25 mM HEPES, pH 7.8, 5 mM $MgCl_2$, 34 mM KCl, and 0.8 μg dipoly(dI-dC) on ice for 30 min. Samples were then digested with DNase I for 2 min on ice, and the reaction was stopped with 4 μl of 125 mM Tris-HCl, pH 7.6, 125 mM EDTA, pH 7.6, 3% SDS, and 2.25 μl of a solution containing 20% (w/v) proteinase K and 10% (w/v) yeast tRNA. The reaction mixtures were then incubated at 55 °C for 20 min, and DNA was precipitated with 0.1 volume of 3 M sodium acetate and 3 volumes of ethanol. The DNA pellet was resuspended in 4 μl of formamide loading buffer, incubated at 95 °C, and resolved on 8% acrylamide, 7 M urea sequencing gel. A G + A Maxam-Gilbert ladder (37) of the probe was performed and co-electrophoresed in adjacent lanes.

RESULTS

Identification of the Retinoic Acid Response Unit of the RBP Gene—We previously demonstrated that a chimeric RBP-CAT gene (RBPCAT1 plasmid) transfected into HepG2 cells is induced 3-fold in RA-treated *versus* untreated cells by the endogenous levels of the retinoic acid receptors (25). Moreover, co-transfections with an expression vector for RARβ determined an increase in CAT activity that was proportional to the concentration of the ligand present in the medium (25). In order to localize the sequences that mediate the RA response, RBP-CAT constructs, carrying shorter DNA fragments derived from the initial 1200-bp long segment of the RBP-CAT1 plasmid, were cotransfected into HepG2 exposed to 10^{-7} M RA or to the vehicle only with an expression vector for RARβ (25) in a ratio reporter plasmid/expression vector of 1/0.1. RBP-CAT 2, 3 and 4 plasmids, which carry 334-, 253- and 220-bp long segments, respectively, from the RBP gene transcription start site, elicited a 6-fold induction, under these conditions (Fig. 1). The RBP-CAT5 construct, which has only 130 bp from *RBP*, did not show a significant response to the treatment. Similar levels of induction were obtained in cotransfections with the RARβ or RARβ/RXRα expression plasmids, probably because of the sufficient levels of the endogenous RXRα present in these cells (7) (data not shown). No differences were observed among the various RARα, β, or γ expression vectors (data not shown). In all experiments, a RARE-TK-CAT plasmid was used as a positive control. A 20-fold-induction in RA-treated *versus* untreated cells was obtained (Fig. 2) (25).

To examine whether the RA-responsiveness could be transferred to a heterologous promoter, the DNA segment from
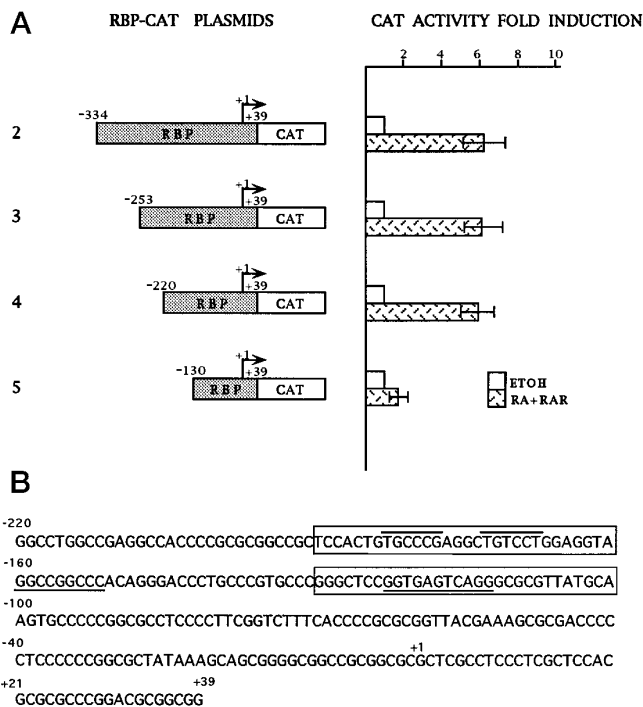
A

RBP-CAT PLASMIDS   CAT ACTIVITY FOLD INDUCTION

B

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-220
GGCCTGGCCGAGGCCACCCCGCGCGGCCGC TCCACTGTGCCCGAGGCTGTCCTGGAGGTA
-160
GGCCGGCCC ACAGGGACCCTGCCCGTGCCC GGGCTCCGGTGAGTCAGGGCGCGTTATGCA
-100
AGTGCCCCGGCGCCTCCCCTTCGGTCTTTCACCCCGCGCGGTTACGAAAGCGCGACCCC
-40
CTCCCCCCGGCGCTATAAAGCAGCGGGGCGGCCGCCGGCGCGCTCGCCTCCCTCGCTCCAC
+21              +39
GCGCGCCCGGACGCGGCGG
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FIG. 1. **Localization of the retinoic acid response unit in the RBP gene promoter.** *A*, DNA fragments of various lengths, derived from the RBP-CAT1 plasmid, were fused to the CAT gene, to generate the RBP-CAT chimeric genes 2–5. Twenty micrograms of each construct were transfected together with the expression vector for the RARβ (2 μg) and 1 μg of pSV-luciferase into HepG2 cells exposed to 10^{-7} M RA or to ethanol for 48 h. CAT activity is reported as fold induction over the basal level, taken as 1, after normalization to luciferase activity for variation in transfection efficiency. The data shown are the mean of at least five independent experiments, using different DNA preparations. The *bars* indicate the standard deviations. *B*, the sequence of the DNA segment from *RBP* that mediates the RA response. This fragment is contained in the RBP-CAT constructs 2 to 4. Regions A and B are *boxed*, putative RARE are *overlined*, and the binding sites for the transcription factors Sp1 and AP1 are *underlined*; *+1* indicates the transcription start site.

−220 to −130 was cloned in the pTK-CAT vector to generate the RBP-TK-CAT1 construct (Fig. 2, *panel A*). No RA induction was obtained in cotransfections with the receptor expression vector into HepG2 cells. To reconstitute the induction, the DNA fragment extending from −220 to −88 was isolated from the RBP-CAT4 plasmid, and one or two copies were cloned into the same vector to generate the RBP-TK-CAT2 and RBP-TK-CAT3 plasmids. Cotransfection of these plasmids with the RARβ expression vector led to a 6- and 12-fold higher CAT activity into RA-treated HepG2 cells (Fig. 2, *panel A*).

To test whether the lack of response of the RBPCAT5 plasmid could have been due to the disruption of the binding site for a transcription factor, 10- and 12-bp long DNA linkers were cloned in the unique *Sma*I site at position −130 of the RBP-CAT4 construct to generate the plasmid RBP-CAT4-link10 and RBP-CAT4-link12, respectively. With the 10-bp linker a stimulation of CAT activity similar to that obtained with the parental construct was obtained. On the contrary, after RA treatment, the 12-bp linker did not elicit any increase over the basal level (Fig. 2, *panel A*).

The RA Response of the RBP Gene Can Be Reproduced in Different Cell Types and Is Dependent on Both RARβ and RXRα Receptors—The RA induction mediated by the −220 to −88 DNA segment was then tested in different cell types to assess whether it can be reproduced in cells of nonhepatic origin. RBP-TK-CAT2 and 3 plasmids were transiently transfected into HeLa and L cells, as described above. The extent

and the time course of the stimulation were identical to those obtained in HepG2 cells (data not shown).

To determine the requirements of the different RAR and RXR and the functional role played by each in induction, experiments were carried out in simian CV-1 cells, which have very low or undetectable levels of the endogenous RAR and RXR (38). A 6-fold higher CAT activity was obtained in RA-treated (10^{-7} M) CV-1 cells cotransfected with the RBP-TK-CAT2 and the RARβ expression plasmid in the same ratio used in HepG2 cells (Fig. 2, *panel B*). An equivalent induction was obtained with the RXR expression vector transfected into cells exposed to 10^{-7} M 9-*cis*-RA; a 10–12-fold induction was, instead, produced cotransfecting half of the amount of both receptors in the presence of 10^{-7} M RA. Therefore, RAR and RXR expression plasmids together elicited an induction 2-fold higher than that produced by each homodimer, indicating that the heterodimer fully stimulates the transcription of the gene. The target plasmid was also transfected in a 20-fold lower amount together with different proportions of the expression vectors so as to maintain low DNA concentrations and to rule out an illegitimate trans-activation due to receptor overload. Under these conditions, the RBP-TK-CAT2 plasmid was induced proportionally to the amount of the transfected receptors, from a 3-fold with a ratio of 1/0.05 to 9-fold with a ratio of 1/0.5 (Fig. 2. *panel C*). The RBP-TK-CAT3 construct elicited a 14-, 10-, and 6-fold induction, respectively, with the receptors transfected in the same proportions. These results show that the retinoid receptors trans-activate the *RBP* promoter carrying plasmids proportionally and, therefore, they are present in nonsaturating amounts. Finally, a higher (15- and 25-fold) and earlier stimulation was produced after transfection of RBP-TK-CAT2 and 3 plasmids into COS 7 cells exposed to RA for 48 h. This can be attributed to the high levels of receptors synthesized from the transfected expression vectors under the control of the SV-40 enhancer-promoter region (39) (data not shown).

To test whether the RAR-containing heterodimers mediate the induction of the RBP gene, transient transfections were performed in *D. melanogaster*-derived Schneider-SL2 cells (26). Insect cells lack endogenous RAR, and RA is not physiologically active (40); therefore, any increase in CAT activity from a transfected reporter gene, in the presence of an RAR expression vector and of the ligand, is exclusively due to the transfected receptor. RBP-TK-CAT2 and 3 constructs were introduced in cells exposed to RA (10^{-6} M), along with *Drosophila*-specific expression vectors for the various RARs. Each plasmid produced a 6- and 12-fold higher CAT activity, respectively, in treated *versus* untreated cells (Fig. 3). Equivalent stimulation was observed in cells transfected with the RXRα plasmid and exposed to 10^{-6} M 9-*cis*-RA. Simultaneous expression of half of the amount of both RARβ and RXRα receptors resulted in a higher increase of CAT activity, suggesting that the RAR/RXR heterodimer is more efficient in stimulating the transcription of *RBP*. A lower stimulation was obtained by both receptors in the presence of the two ligands, RA and 9-*cis*-RA, probably due to the 9-*cis*-RA-triggered RXR homodimerization (12). No CAT activity enhancement was observed in cells transfected with the reporter construct and the expression plasmids in the absence of the ligand, clearly indicating that the effects observed are directly due to the formation of the RA-receptor complex and to its binding to the DNA.

Cis-elements and Trans-acting Factors Binding the RBP Promoter Region—EMSAs were performed to localize the sequence elements interacting with the retinoic acid receptors. The DNA segment that mediates the RA response (from −220 to −88) was used as probe and challenged with nuclear extracts from RA-treated HepG2 cells. The specific DNA-receptor complex
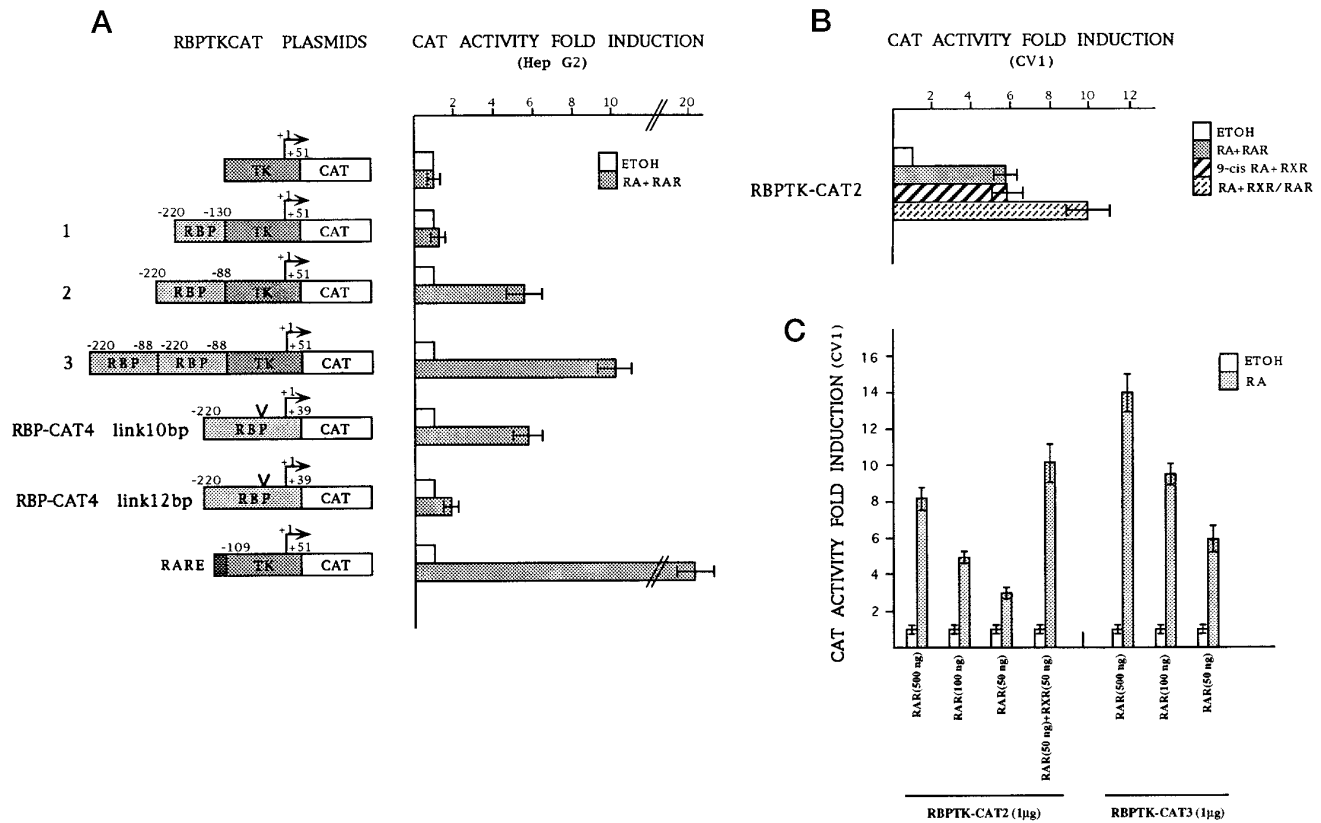
FIG. 2. **Identification of the DNA segment responsible for the RA induction of the RBP gene.** *Panel A*, schematic representations of the RBP-TK-CAT constructs carrying fragments of different lengths from *RBP* cloned in the pTK-CAT plasmid and transfected into HepG2 cells. TK-CAT and RARE-TK-CAT represent the negative and the positive control, respectively, used in these experiments. *Panel B*, the RBP-TK-CAT2 plasmid was co-transfected into CV1 cells (20 μg), with 2 μg of the expression vectors for RARβ and RXRα, or with 1 μg of each receptor in the presence of RA or 9-*cis*-RA (10^{-7} M). *Panel C*, 1 μg of RBP-TK-CAT2 and 3 plasmids was transfected into CV1 cells together with the expression vectors for RARβ, RXRα, or both, in ratios of 1/0.5, 1/0.1, and 1/0.05, respectively, in the presence of RA or 9-*cis*-RA (10^{-7} M). When both receptors were transfected, half of the amount of each receptor was used. In all cases, 1 μg of pSV-luciferase was present in the precipitate and used as internal control for transfection efficiency. CAT activity is indicated as fold induction over the basal level, taken as 1. The data shown are the average of at least five independent experiments, using different DNA preparations. The *bars* indicate the standard deviations.
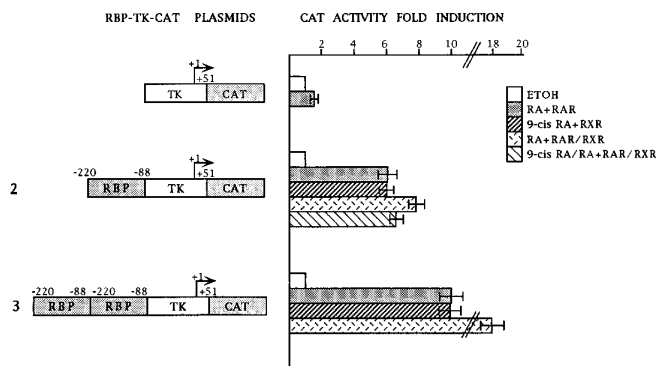

FIG. 3. **The retinoid receptors mediates the RA response of RBP in insect cells.** *D. melanogaster* Schneider SL2 cells were transfected with 10 μg of the reporter plasmids RBP-TK-CAT2 and 3, together with 1 μg of the insect expression vectors for RARβ, RXRα, or both, in the presence of 10^{-6} M RA, 9-*cis*-RA, or both, or the vehicle only. When both receptors were transfected, half of the amount of each receptor was used. CAT activity is reported as fold induction over the basal level from cells transfected with the reporter plasmid only. The *bars* indicate the standard deviations.

was, however, only barely detectable (data not shown). We decided, therefore, to use receptor-enriched extracts such as those from COS 7 cells transfected with the RARβ/RXRα expression vectors. A DNA-protein complex was obtained only with extracts from transfected COS 7 cells, the specificity of which was demonstrated by competitions with the same non-radioactive fragment (Fig. 4, *panel A, lane 3*) and a synthetic

RARE derived from the *RARβ* promoter (β-RARE) (*lanes 1, 2,* and *7*). The remaining bands could be due to the binding of a member of the Sp1 family of transcription factors and to AP1, because recognition sequences for these two factors appear in the sequence of the probe. A GC-rich sequences is, in fact, present at position -160 to -151 and a canonical TRE site at position -120 to -112 (see Fig. 1). Competition experiments with a 100-fold molar excess of unlabeled oligonucleotides, corresponding to the Sp1 site of the SV40 promoter or to the TRE site of the collagenase promoter (41, 42), showed that the Sp1 oligo completely eliminated the three major distinct bands, while the TRE oligo removed the broader band (*lanes 4* and *5*). A combination of nonradioactive oligonucleotides corresponding to Sp1, TRE, and β-RARE abolished all the major retarded bands (*lane 8*). The specificity of Sp1 or of a Sp1-related protein and of AP1 binding was confirmed in EMSA in which probes with mutated sites did not form any complex (data not shown). Finally, the intensity of Sp1 and AP1 complexes was the same in extracts from transfected and untransfected COS 7 cells, indicating that the transfection did not alter the binding capacity of general transcription factors. A β-RARE oligo used as probe in parallel experiments detected a single band only with extracts from transfected COS 7 cells (*lanes 9* and *10*).

To confirm that RAR and RXR were contained in the DNA-protein complex detected, gel supershift assays were carried out by adding anti-RARβ and anti-RXRα antibodies to the reaction mixture (Fig. 4, *panel B*). The specific complex formed by the RAR/RXR heterodimer was abolished by both antibod-

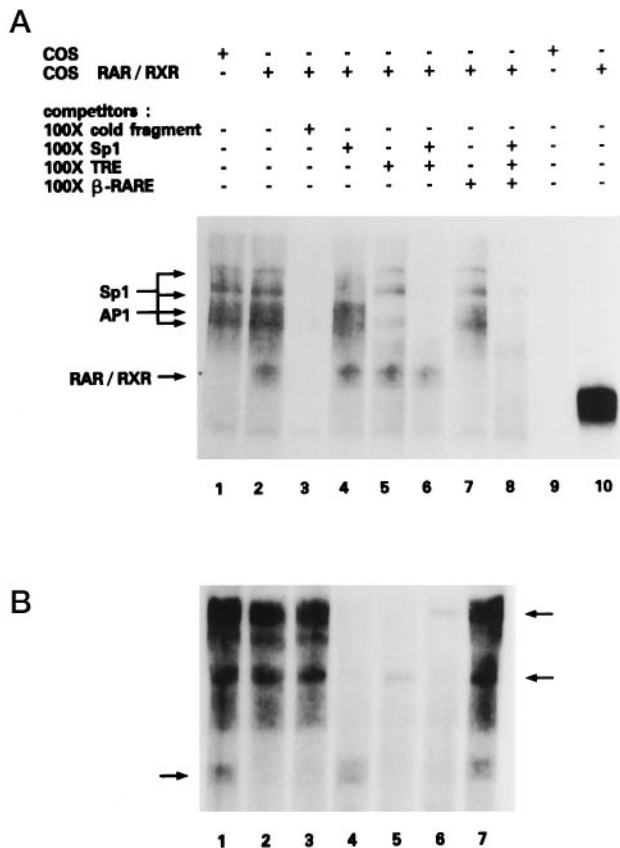FIG. 4. **Analysis of the nuclear factors that bind *RBP* sequences.** *Panel A*, electrophoresis mobility shift assays were performed using nuclear extracts from untransfected or transfected COS 7 cells (5 μg) and the DNA fragment from *RBP* that mediates the RA response (-−20 to −88) as a probe (*lanes 1–8*). The competitors, used in a 100-fold molar excess, are indicated on the *top* of *panel A*. The position of the specific retarded complexes is indicated on the left. A β-RARE oligonucleotide was used as control and challenged with the same extracts (*lanes 9* and *10*). *Panel B*, antibody gel mobility supershift experiments were carried out using the same extracts from transfected COS 7 cells and the same DNA fragment as a probe. The retarded complex containing the RARβ/RXRα heterodimer (*lane 1*) was abolished by the addition of anti-RARβ and RXRα antibodies, but no supershifted bands were detectable (*lanes 2* and *3*). Nonradioactive oligonucleotides, corresponding to Sp1 and AP1, were added to the EMSA mixture (*lane 4*). In the presence of both unlabeled oligonucleotides and of the anti-RARβ and RXRα antibodies, specific supershifted bands became apparent (*lanes 5* and *6*). A preimmune serum was added in *lane 7*. On the left is indicated the RAR/RXR-containing complex and on the right the supershifted complexes.

ies, but no supershifted bands could be detected, probably due to the comigration with DNA-protein complexes formed by other factors (*lanes 1–3*). The addition of nonradioactive oligonucleotides, corresponding to Sp1 and AP1 eliminated all the bands but the one containing the RAR/RXR heterodimer, as expected (*lane 4*). The inclusion in the reaction of the specific antibodies, together with the unlabeled oligonucleotides, abolished the retarded complex, and partial mobility supershifts appeared (*lanes 5* and *6*). A preimmune serum or an unrelated antibody did not affect the pattern observed (*lane 7*).

To better define at nucleotide level the binding of the RAR/RXR heterodimer, DNase I footprinting analysis was performed using the same DNA fragment as probe and partially purified RARβ/RXRα receptors obtained from HeLa cells infected with recombinant vaccinia viruses carrying the corresponding cDNAs. As shown in Fig. 5, two protected regions were produced on the coding strand: one, designated A, extends from nucleotide −190 to −160 and a second, designated B, from −130 to −100 with respect to the transcription start site (*lane*
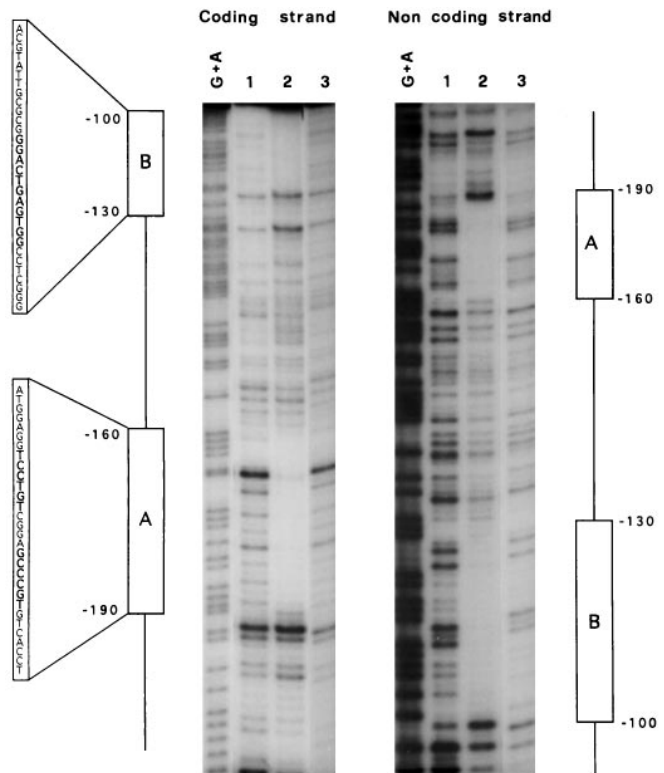

FIG. 5. **The RARβ-RXRα heterodimers bind the retinoid response unit in *RBP*.** The *Bam*HI-*Nar*I fragment from *RBP* extending from −220 to −88 was used as probe and digested with DNase I in the absence (*lane 1*) or in the presence of nuclear extracts from HeLa cells infected with recombinant vaccinia viruses carrying the cDNA for the RARβ and RXRα receptors (*lane 2*) or with an empty virus (*lane 3*). The G + A *lane* illustrates the Maxam-Gilbert ladder of the probe co-electrophoresed in adjacent lanes. The protected A and B regions are *boxed* and their positions relative to the transcription start site are indicated, along with their sequences.

2). Similar protected regions were produced on the noncoding strand (*lane 2*). No protections were obtained using extracts from HeLa cells infected with an empty virus, indicating that the two protected regions are specific (*lanes 3*).

We next asked whether the two protected regions were able to bind the RAR-containing heterodimers separately. Oligonucleotides corresponding to the A or the B protected regions were synthesized, labeled, and used as probes in EMSA. Extracts from HeLa cells infected with recombinant vaccinia virus vectors or from COS 7 cells transfected with receptor expression vectors were used as a source of proteins. In all cases, the pattern obtained was the same as the one illustrated in Fig. 6. Oligo A produced a complex that was specifically competed for by the same nonradioactive oligo (*panel A*, *probe A*, *lanes 2* and *4*) and by a β-RARE oligo (*probe A*, *lane 3*). No specific complexes were generated using extracts from COS 7 cells mock-transfected or transfected with a vector without an insert (*probe A*, *lane 1*). Oligo B, which includes the overlapping TRE site, produced two specific bands (*panel B*, *probe B*, *lane 2*), because they were competed for by the same nonradioactive oligo (*lane 5*). The faster migrating complex was competed for by a radioinert β-RARE oligo (*lane 3*); the slower migrating one, detected with extracts from both transfected and untransfected cells, was abrogated by a TRE oligo (*lanes 1* and *4*). This last oligonucleotide, used as probe and challenged with extracts from transfected COS 7 cells, formed a single complex specifically competed for by the same unlabeled oligo (*panel B*, *probe TRE*, *lanes 1* and *3*), but not by a β-RARE oligo (*lane 2*). These results, therefore, demonstrate that each protection can bind
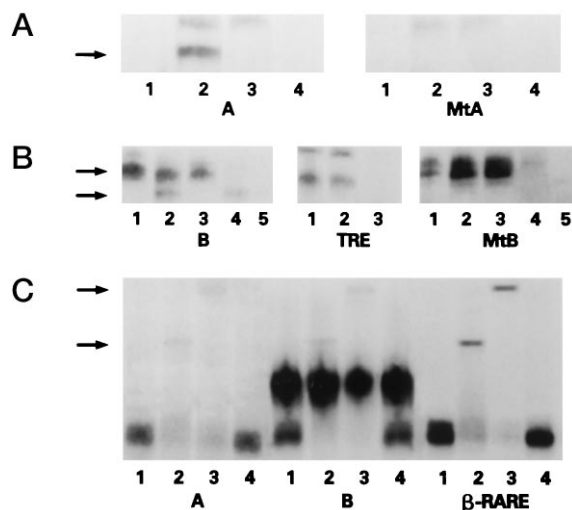
FIG. 6. **The RARβ-RXRα heterodimer binds regions A and B, separately.** Electrophoresis mobility shift assays were performed using as probes oligonucleotides corresponding to the protected region A and to its mutant A1 (*panel A*); protected region B, its mutant B, and a TRE (*panel B*). Nuclear extracts from COS 7 cells untransfected or transfected with the RARβ/RXRα receptor expression vectors were used as sources of proteins (*lanes 1* and *2* in *panel A* and *B*). Competitions were carried out with extracts from transfected cells in the presence of a 100-fold molar excess of a nonradioactive β-RARE oligo (*lanes 3* in *panels A* and *B*), the same radioinert oligo used as a probe (*lanes 4* in *panels A* and *B*) and a TRE oligo (*lanes 5* in *panel B*, *probes B* and *Mtb*, and *lane 3*, *TRE probe*). *Panel C*, antibody gel mobility supershift experiments were carried out using the same extracts from transfected COS 7 cells and oligonucleotides corresponding to regions A and B as probes. The retarded complex containing the RARβ/RXRα heterodimer (*lanes 1*) was diminished in intensity and supershifted by the addition of anti-RARβ and RXRα antibodies (*lanes 2* and *3*). A β-RARE oligonucleotide produced a specific complex that was partially supershifted by the addition of the specific antibodies (*lanes 2* and *3*). This last probe was used at a concentration of at least 10-fold lower than the A and B probes. In all *lanes 4* of *panel C*, a preimmune serum was added. The *arrows* indicate the supershifted complexes.

the RARβ/RXRα heterodimer, independently, and that the B region can also bind AP1 specifically.

Supershift experiments were performed to confirm the presence of RARβ and RXRα in the retarded complexes (Fig. 6, *panel C*). With probe A, each antibody diminished the intensity of the specific complex and produced partial supershifts (*panel C*, *probe A*, *lanes 1–3*). Also the DNA-protein complex formed by probe B was attenuated by both antibodies and supershifted complexes became apparent (*probe B*, *lanes 1–3*). A β-RARE oligonucleotide, used as a control probe and incubated with the same extracts, produced an intense retarded complex (*probe β-RARE*, *lane 1*) that was only partially supershifted when the anti-RARβ and anti-RXRα antibodies were added to the EMSA mixture (*probe β-RARE*, *lanes 2* and *3*). A preimmune serum or an unrelated antibody did not affect the intensity of the retarded complex, nor did it produce supershifts with either probe (*all lanes 4* in *panel B*).

Finally, EMSA performed in parallel with oligonucleotides corresponding to the protected regions A and B showed a 30–50-fold lower binding affinity than that of a β-RARE oligonucleotide (data not shown).

Effects of Mutations in Region A and B on the Expression of the RBP Promoter—Analysis of the A and B protected regions did not reveal any homology to known RAREs. The only elements found in the A region were an examer TGTCCT identical to the half-site of the thyroid hormone response element of the human myosin heavy chain gene (43) and to the half-site of the RARE present in the rat acyl-coenzyme A oxidase (ACO) gene (44). Spaced by 4 nucleotides, there was another examer TGC-
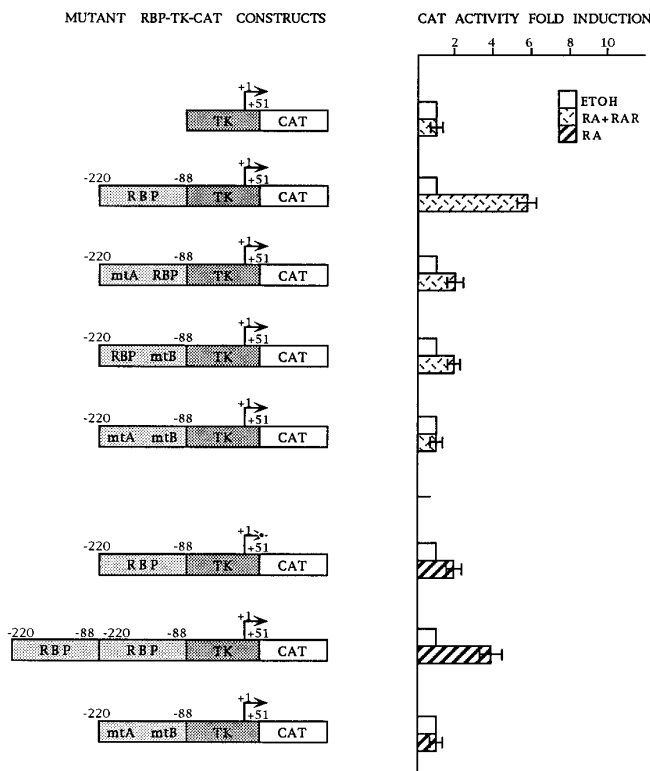

FIG. 7. **The RA response of *RBP* requires both the A and B regions.** Mutations in the A and B were produced by site-directed mutagenesis. The mutated regions were inserted in the RBP-TK-CAT2 vector, to generate mtA-RBP-TK-CAT, mtB-RBP-TK-CAT, and mtA-mtB-TK-CAT plasmids. Twenty micrograms of each plasmid were transfected along with 2 μg of the expression vectors for RARβ or RXRα and 1 μg of the pSV-luciferase into HepG2 cells exposed to RA or to the vehicle only. The same plasmids were also transfected into cells exposed to 10^{-7} M RA only, as indicated. CAT activity is reported as fold induction over the basal level, taken as 1. The data shown are the means of at least five independent experiments, using different DNA preparations. The *bars* indicate the standard deviations.

CCG, homologous to the more upstream half-site of the phosphoenol pyruvate carboxykinase gene response element (45). The B element showed homology with the RARE in the region II enhancer of major histocompatibility complex class I genes (46), with a single nucleotide deletion, that generates a TRE site that overlaps the putative RARE. On the basis of this recognition, site-directed mutagenesis was performed on the two examers of the A element and on the nucleotides neighboring the AP1 site in the B element, generating mtA1, mtA2, and mtB oligonucleotides, respectively. They were used as probes in EMSA with nuclear extracts from untransfected and transfected COS 7 cells. Neither mtA1 nor mtA2 formed specific retarded complexes (see an example in Fig. 6, *panel A*, *probe mtA1*, *lanes 1* and *2*). As expected, no competition occurred with nonradioactive oligonucleotides corresponding to β-RARE or to the probe (*lanes 3* and *4*). The mtB probe produced only the band corresponding to the AP1 complex (Fig. 6, *panel B*, *probe mtB*, *lanes 1* and *2*) that was abrogated by the addition of an unlabeled TRE and by the same oligo (*lanes 4* and *5*), but not by a radioinert β-RARE (*lane 3*).

To verify whether each protected region, taken separately, could confer RA responsiveness and to assess the functional effects of the mutations, A and B oligonucleotides and their corresponding mutants were cloned in the pTKCAT plasmid. No RA induction was observed either with the wild type or with the mutant-carrying constructs after transfection into HepG2 or CV-1 cells, in the presence of RARβ and RXRα expression vectors (data not shown). mtA and mtB were then inserted in

A

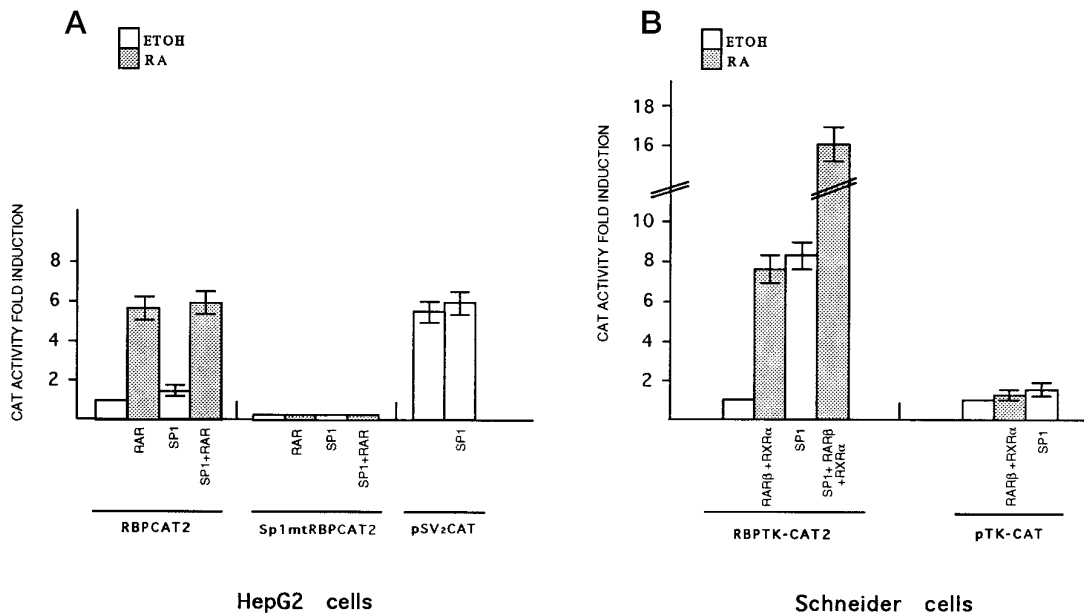HepG2 cells

B

Schneider cells

FIG. 8. **Sp1 plays an important role in the activity of *RBP*.** *Panel A*, RBP-CAT2 and Sp1mtRBPCAT2 plasmids were introduced by transfection into HepG2 cells. The reporter constructs (20 μg) were transfected together with expression vectors (2 μg) for RARβ, Sp1, or both, into cells exposed to 10^{-7} M RA or to ethanol alone. As a control, the pSV2CAT plasmid was transfected together with the Sp1 expression vector. *Panel B*, *Drosophila* Schneider SL2 cells were transfected with the RBP-TK-CAT2 (10 μg) or with the pTKCAT vector alone. Expression vectors for Sp1, RARβ/RXRα, or a combination of both, as indicated, were cotransfected into cells exposed to 10^{-6} M RA or to the vehicle only.

the RBP-TK-CAT2 construct to generate mtA-RBP-TK-CAT and mtB-RBP-TK-CAT, respectively. mtA-mtB-TK-CAT plasmid contains both mutations. The single mutant-carrying plasmids produced only 30% of the CAT activity obtained with the parental RBP-TK-CAT2 vector. The plasmid carrying both mutations lost RA stimulation completely (Fig. 7). The same reporter plasmids were also transfected into HepG2 cells exposed to 10^{-7} M RA only, without receptor expression vectors. Under these conditions, RBP-TK-CAT2 and 3 constructs were induced by 2- and 4-fold, respectively, while the double mutant was no longer responsive. Similar results were obtained in HeLa cells (data not shown). Altogether these data showed that, albeit at a low level, the endogenous receptors can trans-activate the transfected constructs and that the response is abrogated by the mutations in regions A and B.

The Retinoic Acid Receptors Interact with Other Transcription Factors—The data presented indicate that 1) Sp1 or a related protein recognizes and binds a GC-rich site located between the two regions where the retinoid receptors bind, and 2) site-directed mutagenesis of this sequence abrogates the binding. To investigate whether Sp1 plays any role in the basal or in the ligand-activated transcription of the RBP gene, the same mutation was introduced in the RBP-CAT2 construct, so generating the Sp1mt-RBP-CAT2 plasmid. Transfection of the parental plasmid with the RAR/RXR expression vectors resulted in a 6-fold induction of CAT activity, as shown above (Fig. 8, *panel A*). Overexpression of Sp1 only slightly increased the basal level of transcription of the same target plasmid and did not further enhance the induction when transfected in combination with the RARβ/RXRα expression vectors. The mutant plasmid, by contrast, showed very low CAT activity, indistinguishable from that of the vector itself and not enhanced by the presence of the Sp1 or the receptors expression plasmids. Mutation in the Sp1 site is thus sufficient to silence *RBP*. The lack of induction observed with the RBP-CAT2 construct in the presence of the Sp1 expression vector was repeatedly observed also with pSV2CAT, a typical Sp1 target promoter (Fig. 8, *panel A*) (41), suggesting that HepG2 cells contain high levels of this factor that do not allow further trans-activation in

conditions of overexpression.

To investigate further the role played by Sp1 in the basal transcription of the gene and possible interactions with the RAR, transient transfection experiments were carried out in *Drosophila*-derived Schneider SL2 cells, which are devoid of Sp1 and RAR. The RBP-TK-CAT2 produced CAT activity that was about 8-fold higher than the basal level, when cotransfected with an expression vector for Sp1, under the control of a *Drosophila*-specific promoter (Fig. 8, *panel B*). An 8-fold increase occurred with the RAR and RXR expression plasmids; overexpression of the three proteins in combination resulted in an increase of about 16-fold. The pTKCAT vector itself did not produce any increase when transfected under the same conditions.

DISCUSSION

In this article we report the identification of a novel and composite retinoic acid response unit that mediates the RA induction of the RBP gene. We have previously demonstrated that in HepG2 hepatoma cells retinoids transcriptionally activate *RBP*; that the endogenous receptors can induce a transfected RBP-CAT chimeric gene and that a higher stimulation is observed in the presence of a cotransfected RAR expression vector (25).

One of the unique features of the *RBP* responsive unit is that it consists of two distinct regions, designated A and B, and that full induction is achieved only in the presence of both elements. In fact, each region singly and separated from its own promoter is not able to confer RA inducibility. Site-directed mutants also indicate that the two regions function synergistically. DNA-protein binding assays further validate this conclusion: the binding affinity of each element is at least 50-fold lower than a canonical RARE, like the one in the *RARβ* promoter, used as a control.

Another interesting property of this responsive unit is that it can bind both RAR and RXR homodimers and RAR/RXR heterodimer; the heterodimer is, however, more efficient in stimulating the RBP gene transcription. This dual specificity could be attributed to the degenerate sequence of the response ele-

ments with respect to classical RAREs and the resulting low binding affinity of the receptors. This may also explain the 2-fold increase in CAT activity observed when both receptors are cotransfected, as compared to the results with single receptors. Moreover, it is unlikely that the induction takes place via heterodimer formation with the endogenous receptors, since both RAR and RXR receptors trans-activate the reporter construct at comparable levels when transfected alone.

A specific sequence context and a steric constraint are also necessary for stimulation, as shown by the results with the linker-carrying plasmids. The fact that only the 12-bp spacer dramatically reduces RA stimulation suggests that the helical phasing of this region of the promoter influences the extent of the response. This implies also that the homo- and heterodimers bound to these DNA sequences must be positioned on the same side of the double helix, *i.e.* at a distance of an integral number of helix turns to interact with each other, so as to participate in an active transcription complex that mediates the stimulation.

It has been shown that the relative potency and specificity of the RARE is dependent on both the configuration and nucleotide sequence of the repeats (3). In fact, the more degenerate the recognition sequence, the lower the extent and the specificity of the response. The response unit we identified follows this rule because the RA response is not as potent as that produced by the element in the *RARβ* promoter, and it is not as specific (6, 38). Preliminary data show that this DNA segment confers responsiveness to thyroid hormones also by binding the corresponding receptors (data not shown). This suggests that it may function as a composite response unit that integrates the response to multiple members of the steroid/thyroid hormone receptor superfamily (3, 44, 47–49). A similar element has been described in the promoter region of the rat oxytocin gene (47) and mediates the selective response of the gene to RA, thyroid hormones, and estrogens. A pleiotropic regulatory element has been described in the medium-chain acyl coenzyme A dehydrogenase gene promoter (50) that is positively transactivated by RA and HNF4 (the orphan receptor hepatocyte nuclear factor 4) and repressed by the chicken ovalbumin upstream promoter transcription factor. Whether different hormones or stimuli modulate the RBP gene expression through the same sequence motifs remains to be elucidated.

Interplay between Retinoic Acid Receptors and General Transcription Factors—The data presented clearly show the important role that Sp1 or a related protein plays in the basal transcription as well as in the RA induction of the RBP gene. It is well documented that Sp1 is a positive regulator of eukaryotic gene expression, acting in concert with other regulatory or constitutive factors (51). Evidence is emerging that Sp1 functionally interacts also with nuclear receptors bound to adjacent sites. This raises the possibility that an Sp1-RARE control unit, formed by two regulatory elements, may exist and differentially regulate the expression of target genes. In the case of the ApoAI and Oct3/4 genes, repression takes place, with the RARE having a dominant negative effect on the positive one exerted by Sp1 (52–54). In the case of RARγ2, there is a positive cooperation between RARs and Sp1, because Sp1 enhances the RA response of the promoter (55). The *RAR*γ2 promoter contains a functional RARE surrounded by several GC boxes, with no TATA box. The so-called "tethering" activity of Sp1 might function in this site to anchor the basal transcription complex to a promoter lacking the direct binding of transcription factor IID (55). The *RBP* promoter harbors an Sp1 binding site located between the A and B regions; it has a canonical TATA box and additional putative Sp1-binding sites around it. Should an Sp1-RARE unit exist in the RBP gene, it would include both A

and B elements in order to function. The binding of Sp1 might recruit more Sp1 molecules and possibly other factors on the promoter to direct the transcription. Moreover, it interacts with the RAR-containing homo- and heterodimers to induce fully the gene. These effects may require the transactivation domain and the tethering activity of this factor. Sp1 comprises a family of transcription factors, some of which, like Sp3, have been shown to have effects opposite to those exerted by Sp1 and Sp2 (31). Whether other members of this growing family participate to the transcription of the RBP gene or to the interaction with the retinoid receptors is not known at the moment and remains to be elucidated.

The B region of the responsive unit recognizes and binds the retinoid receptors and the general transcription factor AP1, in contrast to other canonical TRE that bind AP1 only (42). This dual specificity is probably due to the sequence context, because mutagenesis of the nucleotides neighboring the AP1 site abolishes the binding of the homo- and heterodimers, but not that of AP1. The B element is then *per se* a complex one. Preliminary evidence, in fact, indicates that the AP1 complex, by transducing signals from different stimuli, like 12-*O*-tetradecanoylphorbol-13-acetate, interferes with RA induction of *RBP*, as demonstrated for other genes (data not shown) (56, 57).

In summary, we have identified a novel response unit in the RBP gene promoter that enables modulation of the transcription in conditions of exposure to high intracellular concentrations of retinoids. The increased RBP levels allows the removal of the excess of retinoids as part of the cellular response to maintain retinoid homeostasis, the alteration of which is toxic and harmful to the cell. This complex receptor binding unit may also enable modulation of RBP gene transcription in response to a variety of metabolic and physiological signals. The pattern may differ in different cell types as a function of the receptors and of the ligand present.

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Identification of a Novel Retinoic Acid Response Element in the Promoter Region of the Retinol-binding Protein Gene

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