Analysis of the Effects of CRABP I Expression on the RA-Induced Transcription Mediated by Retinoid Receptors†

Pratap Venepally, Lakshmi G. Reddy, and Brahma P. Sani*

Kettering-Meyer Laboratories, Southern Research Institute, Birmingham, Alabama 35255

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ABSTRACT: The involvement of cellular retinoic acid-binding protein I (CRABP I) in the RA signaling was investigated by examining its effects on the interaction of retinoid receptors (RARs and RXRs) with RA-response elements (RAREs) as well as on the RA-induced transcription mediated by retinoid receptors. Analysis of the expression of mouse CRABP I from a cDNA expression plasmid in COS-1 cells revealed that this protein was about 5-fold more abundant in cytosol than in nuclei. The identity and the localization of CRABP I in the cytoplasm as well as the nuclei were also confirmed by the immunoperoxidase staining of the transfected COS-1 cells with CRABP I-specific antibody. When the nuclear extract containing a 10-fold molar excess of CRABP I was incubated with RAR α extract in the presence of [^{3}H]RA and resolved on an FPLC size-exclusion column, a 20% decrease in the bound radioactivity in the RAR α fraction was accompanied by a proportional increase in the CRABP I fraction. In contrast, the addition of CRABP I did not significantly affect the interaction of RAR α or RAR α−RXR α heterodimers with RAREs. Moreover, the coexpression of CRABP I in CV-1 cells did not markedly inhibit or enhance the transcription activated by RARs and RAR α−RXR α heterodimers under RA concentrations ranging from 10^{-10} to 10^{-6} M. These results demonstrate that CRABP I, while it might be important for RA homeostasis, is not directly involved in the retinoid receptor-mediated RA-signaling pathway.

The intricate molecular signaling pathway by which *all-trans*-retinoic acid (RA)[1] exerts its effects on cellular differentiation, growth, and development during embryogenesis as well as in the adult animal involves members of at least two gene superfamilies. One of these encodes a class of nuclear receptors belonging to the steroid/thyroid hormone superfamily called RARs (retinoic acid receptors) and RXRs (retinoid X receptors) (Mangelsdorf et al., 1994). These are DNA-binding proteins which upon activation by specific retinoid ligands induce gene transcription by interacting with distinct promoter sequences in the target genes. The second family of genes codes for a group of low molecular weight serum and cellular proteins which bind various retinoids and are involved in the transport and metabolism of the latter. In this class, two cellular RA-binding proteins, CRABP I and CRABP II, which exhibit a high degree of amino acid sequence conservation but are immunologically distinct, possess different binding affinities for RA and show distinct patterns of expression during the various stages of development (Maden et al., 1988; Perez-Castro et al., 1989; Dollé et al., 1990).

The genes and cDNAs for CRABP I and II from various species have been identified and characterized (Shubeita et al., 1987; Nilsson et al., 1988; Stoner & Gudas, 1989; Giguere et al., 1990; Wei et al., 1990; Åström et al., 1991, 1992; MacGregor et al., 1992; Eller et al., 1992). Although the exact physiological role of CRABPs remains as yet unclear, the evidence obtained so far suggests their possible involvement in the transport of RA to the nucleus (Takase et al., 1986; Bloomhoff et al., 1990) and regulation of the levels of intracellular RA. CRABPs are presumed to achieve the latter by either sequestering RA in the cytoplasm, thereby reducing its availability for nuclear receptors (Boylan & Gudas, 1991), or converting RA into more polar, oxidized metabolites (Fiorella & Napoli, 1991; Boylan & Gudas, 1992), some of which may have their own important biological activities.

There has been no evidence so far that CRABPs function as DNA-binding transcriptional factors. However, considerable evidence exists that these proteins are partly localized, in addition to their predominant cytoplasmic occurrence, in the nuclei of some tissues and cells (Sani, 1977; Sani & Donovan, 1979; Banerjee & Sani, 1980; Barkai & Sherman, 1987). In addition, evidence has also been presented for the interaction between CRABP holoprotein and the nuclei from mammary carcinoma and testicular cells (Mehta et al., 1984; Cope et al., 1984; Takase et al., 1986). In light of these observations, it was important to examine if CRABPs participated in a direct interaction with the RA-binding RARs both in the presence and in the absence of their cognate DNA-binding sequences and, also, if the expression of CRABPs affected the RA-induced transcription in an RAR subtype-specific manner. In this report we show (1) that a part of CRABP I expressed in COS-1 cells is localized in the nuclei, (2) that CRABP I does not directly interact with RAR α even though it competes partially with the latter for RA, (3) that nuclear extracts containing CRABP I do not significantly affect the binding of RAR α and RAR α−RXR α heterodimers to RA-response DNA sequences β-RARE and TREpal, and (4) that the expression of CRABP I does not markedly inhibit or enhance the transcription activated by RAR homodimers and RAR α−RXR α heterodimers in CV-1 cells.

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* To whom correspondence should be addressed. Phone: 205-581-2225. Fax: 205-581-2877.

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[1] Abbreviations: RA, *all-trans*-retinoic acid; CRABP I, cellular retinoic acid-binding protein I; PBS, phosphate-buffered saline; RAR, retinoic acid receptor; RXR, retinoid X receptor; FPLC, fast protein liquid chromatography; TREpal, palindromic thyroid hormone response element; βRARE, RA-response element from human RAR β gene.

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MATERIALS AND METHODS

Materials. *all-trans*-[11,12-^{3}H]RA (40−60 Ci/mmol) and unlabeled *all-trans*-RA were purchased from Dupont/NEN and Sigma Chemical Co., respectively. 9-*cis*-Retinoic acid was a gift from Hoffman LaRoche (Nutley, NJ). DME medium, fetal bovine serum (FBS), and antibiotics used for cell culture were purchased from Atlanta biologicals, Atlanta, GA. CV-1 monkey kidney fibroblast and COS-1 cells were obtained from American Type Culture Collection, Rockville, MD. Expression plasmid for mouse CRABP I (pSG5-CRABP I) was provided by Dr. P. Chambon (Faculté de Médecine, Strasbourg, France). Plasmids expressing human RAR α (pSG5-hRAR α) and human RXR α (pSG5-hRXR α) were obtained from Dr. Arthur Levin (Hoffman LaRoche, Nutley, NJ).

Expression of CRABP I and RAR α in COS-1 Cells. COS-1 cells were maintained in DME medium supplemented with 10% fetal bovine serum. Cells [(3−5) × 10^7] were transfected by electroporation with either pSG5-CRABP I or pSG5-RAR α expression plasmid carrying cDNA for mouse CRABP I and human RAR α, respectively (Levin et al., 1992). Cytosol and nucleosol from the transfected cells were prepared (Nervi et al., 1989), after the nuclei were found to be homogeneously purified under microscopic examination and used in the binding assays.

Assay for [^{3}H]RA Binding of Cytosol and Nucleosol by Anion-Exchange and Size-Exclusion FPLC Chromatography. Approximately 100 μg of cytosol and nucleosol from COS-1 cells transfected with expression vectors for either CRABP I or RAR α (see above) was incubated with 5 nM [^{3}H]RA in a 200 μL reaction volume. After incubation at 4 °C overnight, the unbound RA was removed by addition of 50 μL of dextran-coated charcoal suspension (0.25% activated charcoal, 0.025% dextran, MW 70,800, 5 mM Tris-HCl, pH 8.0) to samples on ice for 10 min, followed by a 5 min centrifugation at 13 000 rpm (Bailey & Siu, 1990). Two hundred microliters of the supernatant, filtered through a 0.2 μm filter, was injected onto either Mono Q (Pharmacia) anion-exchange or Superose-12 (Pharmacia) gel-filtration FPLC chromatography columns. The Mono Q column was equilibrated with 5 mM Tris-HCl, pH 8.0 (Bonelli & De Luca, 1985; Sanquer & Gilchrest, 1994). After the sample was applied, the column was eluted with buffers A (5 mM Tris-HCl, pH 8.0) and B (0.3 M NaCl and 5 mM Tris-HCl, pH 8.0) utilizing the gradient profile described by Bonelli and De Luca (1985) at a flow rate of 1.5 mL/min. For fractionation on the Superose-12 size-exclusion column, the samples (200 μL) were eluted from the column in buffer C (30 mM sodium phosphate, pH 7.5, and 150 mM NaCl) at a flow rate of 1 mL/min. Bovine serum albumin (MW 68 000), ovalbumin (MW 44 500), and cytochrome *c* (MW 12 400) were used as external molecular size markers. The bound RA in the eluted fractions was quantitated using a scintillation counter. To determine nonspecific binding in the samples, a 200-fold molar excess of nonradioactive RA was used as a competitor during the incubation with [^{3}H]RA.

In Vivo Analysis of [^{3}H]RA Binding in COS-1 Cells. On day 1, 2 × 10^7 cells, suspended in phosphate-buffered saline (PBS), were electroporated in the presence of 20 μg of pSG5-CRABP I plasmid DNA. Following electroporation, the cells were suspended in 50 mL of DME medium containing 10% FBS and plated into two 150 cm^2 cell culture dishes. Medium was changed on day 3, and on the fourth day [^{3}H]-RA was added to the cells in serum-free DME medium at a final concentration of 0.5 nM. After 2 h in the CO_2 incubator at 37 °C, the cells were washed in PBS and harvested by trypsinization for subsequent preparation of cytosol and nucleosol (Nervi et al., 1989). Two hundred microliters of cytosol and nucleosol was dialyzed against the appropriate buffer overnight at 4 °C and analyzed on Mono Q and Superose-12 FPLC columns as described elsewhere.

Immunostaining of CRABP I in COS-1 Cells. COS-1 cells (2 × 10^4) that had been transfected with pSG5-CRABP I plasmid by electroporation (Levin et al., 1992) were grown for 72 h on superfrost glass slides (Fisher Scientific) in DME medium supplemented with 10% FBS. After a brief wash in PBS, the cells were fixed in a solution of 50% acetone/50% methanol and immunostained with the ABC peroxidase reagent system (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's recommended procedure. Following incubation with monoclonal (mouse) anti-CRABP I antibody (Affinity Bioreagents, Inc., Boulder, CO), the cells were sequentially treated with biotinylated secondary anti-mouse IgG and the preformed avidin−biotinylated horseradish peroxidase complex (ABC). Subsequently, the cells were incubated with the peroxidase substrate (diaminobenzidine) and counterstained in hematoxylin, and the target protein in them was visualized by light microscopy.

Gel Retardation Analysis. The synthetic double-stranded oligonucleotides TREpal, containing one copy of thyroid hormone response element (Glass et al., 1989), and human βRARE (de Thé et al., 1990) were labeled by polynucleotide kinase ([γ-^{32}P]dATP; 4500 Ci/mmol, ICN) and by DNA polymerase I Klenow fragment ([α-^{32}P]dATP; 3000 Ci/mmol, ICN), respectively. Five to ten micrograms of nuclear extract from COS-1 cells expressing either RAR α or CRABP I was mixed separately or together with binding buffer (10 mM Hepes, pH 7.9, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, and 10% glycerol), 1 μg of poly(dIdC), and 10^{-5} M RA or 9-*cis*-RA in a volume of 19 μL. After 30 min at room temperature, the mixture was incubated with 1 μL of ^{32}P-labeled probe (10 000 cpm) for a further period of 10 min. For competition assay, 10 pmol of unlabeled competitor oligonucleotide was used along with the labeled probe. In the case of heterodimer binding analysis, equal amounts of RAR α and RXR α were used with or without CRABP I. The DNA−protein complexes were resolved by electrophoresis on a 5% polyacrylamide−2.5% glycerol gel in 0.5 × TBE buffer (Sambrook et al., 1989) and visualized by autoradiography.

Transient Transfection Assays. CV-1 monkey kidney fibroblast cells were grown in DME medium supplemented with 10% FBS. Transfection of these cells with DNA was performed essentially as described by Pfahl et al. (1990), Husmann et al. (1991), and Alam et al. (1995) with some modifications. Twenty four hours before the transfection, cells were plated at 0.5 × 10^5 per well in a 24-well cell culture plate. Four hours before the addition of DNA−calcium phosphate precipitates, the cells were fed with DME medium supplemented with charcoal-stripped FBS. The DNA used in the transfections consisted of 200 ng of reporter plasmid TRE_2-tk-CAT (Zhang et al., 1991) and 300 ng of β-galactosidase plasmid pCH110 in addition to the expression plasmid for one of the following: CRABP I, RAR α, RAR β, RAR γ, and RXR α. In experiments where the effect of CRABP I on the RAR and RXR receptor-mediated transcriptional activation was examined, 5−400 ng of CRABP I and 50 ng of either RAR α or RXR α receptor plasmids

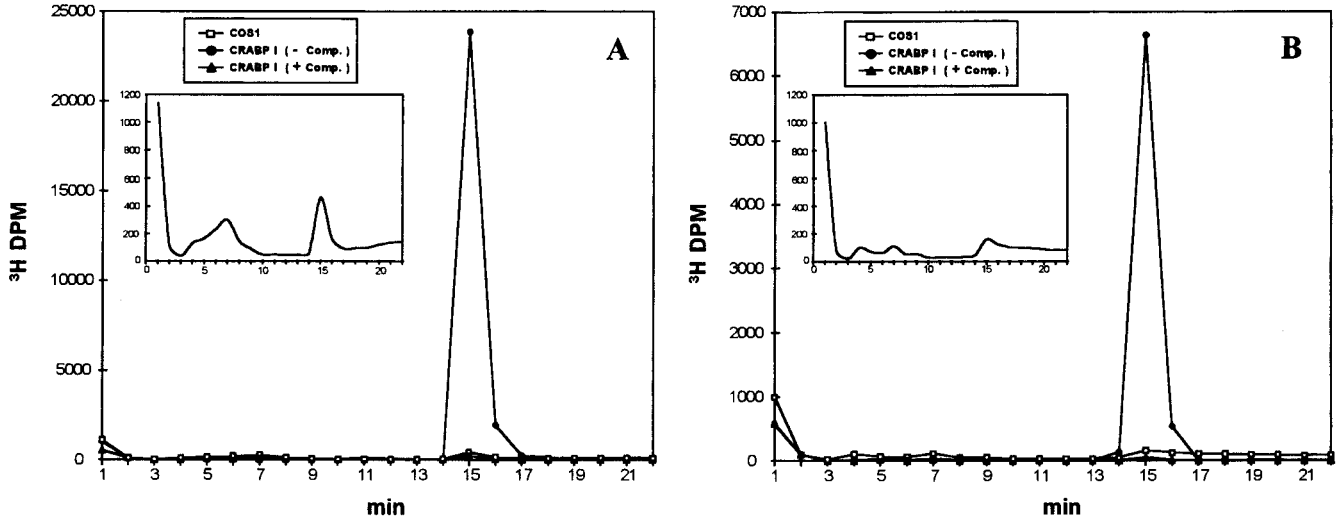

FIGURE 1: FPLC ion-exchange chromatographic analysis of COS-1 cytosolic and nuclear extracts for the expression of CRABP I. One hundred micrograms of cytosolic (A) and nuclear (B) extracts from COS-1 cells that had been either mock-transfected (COS-1) or transfected with pSG5-CRABP I, an expression plasmid for mouse CRABP I (CRABP I), was mixed with 5 nM [^{3}H]RA alone (−Comp.) or together with a 200-fold excess of unlabeled RA (+Comp.). After an overnight incubation at 4 °C, the samples (200 μL) were treated with charcoal to remove unbound RA and analyzed on a Mono Q anion-exchange FPLC column as described under Materials and Methods. The insets in (A) and (B) show an enlarged view of the chromatographic profiles for the mock cytosolic and nuclear extracts, respectively.

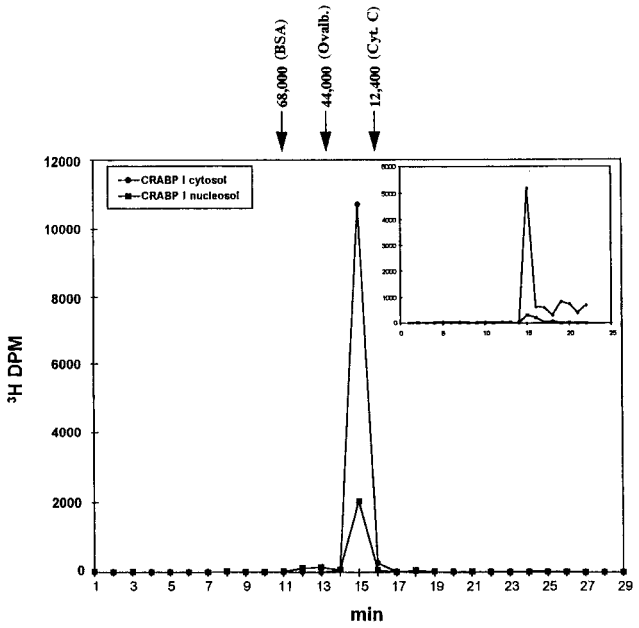

FIGURE 2: Analysis of the expression of CRABP I labeled *in vivo* with [^{3}H]RA. Cytosol (closed circles) and nucleosol (closed squares) from 2×10^7 COS-1 cells that had been transfected with CRABP I expression plasmid, and labeled *in vivo* with 5 nM [^{3}H]RA, were analyzed on a Superose-12 size-exclusion or a Mono Q anion-exchange (inset) FPLC column (Pharmacia). The elution profile for external protein molecular weight markers bovine serum albumin (BSA, M_r 68 000), ovalbumin (Ovalb., M_r 44 000), and cytochrome *c* (Cyt. C, M_r 12 400) is shown at the top of the graph.

were added together. When RAR α−RXR α heterodimer activities were investigated in the context of CRABP I coexpression, 50 ng of DNA of each of the expression plasmids for RAR α and RXR α was used with or without 50 ng of expression plasmid for CRABP I along with the other plasmids mentioned above. In all cases, enough Bluescript plasmid was added to make the final quantity of DNA in each transfection sample equal to 1000 ng. After an overnight incubation in the presence of DNA precipitates, cells were washed once with PBS and grown for 24 h in the medium containing 10% charcoal-stripped FBS and various concentrations of *all-trans* RA or 9-*cis*-RA. At the end of the incubation, cells were washed with PBS and lysed for 10 min at room temperature in 0.25 mL of lysis buffer (0.5% Triton X-100 in 100 mM Tris-HCl, pH 7.8). The resultant cell extracts were assayed for β-galactosidase activity and CAT activity as described by Alam et al. (1995). The CAT activity of individual samples was normalized for transfection efficiency by the corresponding β-galactosidase activity.

For the analysis of expression of CRABP I in CV-1 cells, various amounts of pSG5-CRABP I were transfected along with the β-galactosidase plasmid. The [^{3}H]RA-binding CRABP I activity in these cell extracts was fractionated on the Mono Q column and normalized using the β-galactosidase enzyme activity observed in the same lysates.

RESULTS

Expression of CRABP I in COS-1 Cells. In order to analyze the possible interaction between CRABP I and the retinoid receptors involved in the retinoid signaling pathway at the protein−protein and DNA−protein levels, sufficient quantities of these proteins were isolated from COS-1 cells transiently transfected with pSG5-derived eukaryotic expression plasmids (Levin et al., 1992; Allenby et al., 1993). Figure 1 shows the FPLC anion-exchange chromatographic analysis of the [^{3}H]RA binding of cytosolic (Figure 1A) and nuclear (Figure 1B) extracts prepared from COS-1 cells expressing mouse CRABP I. The extracts were loaded onto a Mono Q column equilibrated with 5 mM Tris-HCl, pH 8.0, and eluted using a gradient of 0−300 mM NaCl in 5 mM Tris-HCl, pH 8.0 (Bonelli & De Luca, 1985; Sanquer & Gilchrest, 1994). Under these conditions, cytosolic and nuclear extracts from the untransfected (mock) COS-1 cells showed a small amount of RA-binding species in the fractions eluted at 4, 7, and 15 min (see insets, Figure 1A,B). However, when the cDNA for CRABP I was expressed in these cells, only the RA-binding protein that eluted with a retention time of 15 min showed a dramatic increase in both cytosolic and nuclear occurrence (Figure 1A,B). Moreover, as seen in the figure, this protein is about 3.5-fold more abundant in the cytosol than in the nucleosol. The peak is diminished to the background levels in both cytosolic and nuclear extracts when a 200-fold excess of unlabeled RA

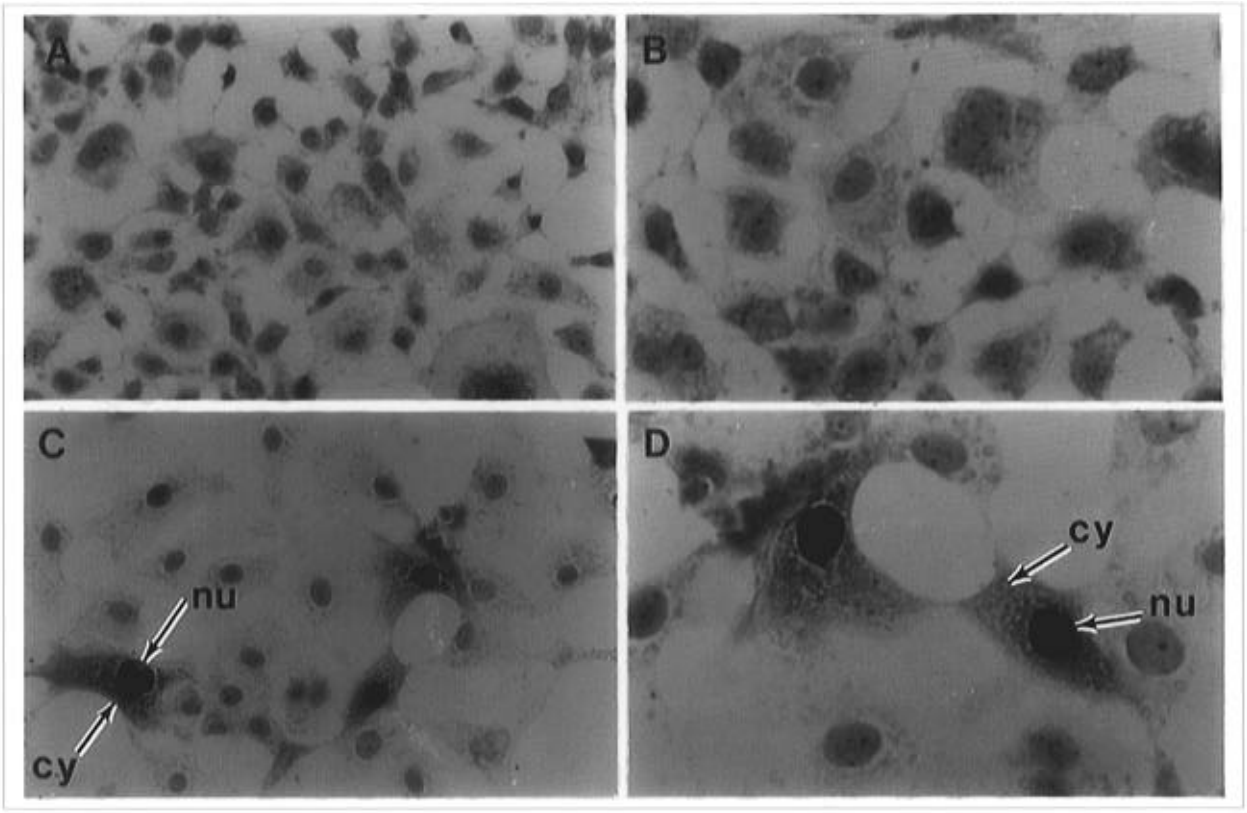

FIGURE 3: Immunocytochemical analysis of CRABP I localization in COS-1 cells. Untransfected (A and B) and transiently transfected COS-1 cells (C and D) expressing CRABP I were subjected to immunoperoxidase staining following a sequential incubation with mouse anti-CRABP I antibody, biotinylated secondary anti-mouse IgG, and a preformed Avidin−biotinylated horseradish peroxidase complex as described under Materials and Methods. The nuclei (nu) and the cytoplasm (cy) of the overexpressing cells (shown with arrows in panels C and D) are markedly brown, indicating the distribution of CRABP I at both sites. In untransfected cells CRABP I is found almost exclusively in the cytoplasm (A and B). The cells in panels A and C are magnified 200×, while those in panels B and D are magnified 400×.

was included during the incubation with [^{3}H]RA, thus confirming the specificity of the ligand interaction (Figure 1A,B).

The identity and the occurrence of CRABP I in cytosol as well as nucleosol was also examined by labeling the COS-1 cells *in vivo* with [^{3}H]RA after they had been transfected with the expression vector for CRABP I. The cytosol and nucleosol from these cells were then assayed by both size-exclusion and anion-exchange chromatographic analysis (Figure 2). As seen previously when extracts were labeled *in vitro* (Figure 1), both cytosol and nuclear extracts from *in vivo* labeled cells expressing CRABP I revealed a prominent RA-bound species that eluted at 15 min when analyzed on a Mono Q column (see inset, Figure 2). When the same extracts were resolved on a Superose-12 gel-filtration column to verify the size of the peak protein, a radioactive peak corresponding to an expected molecular weight of about 15 000 was observed in both cytosol and nucleosol of *in vivo* labeled COS-1 cells (Figure 2), suggesting that a protein consistent with the size of CRABP I is expressed in these cells. Again, in general agreement with the observation made in the case of extracts labeled *in vitro* with [^{3}H]RA (Figure 1), the cells labeled *in vivo* showed that the expressed CRABP I is about 5-fold more abundant in cytosol than in nucleosol (Figure 2).

To make sure that the CRABP I activity observed in the nuclear fractions was not due to the contaminating cytosol and that the protein was indeed present in the nuclei of the transfected cells, we have also examined the intracellular localization of the expressed CRABP I by immunoperoxidase staining of the COS-1 cells with the CRABP I-specific antibody. As seen in Figure 3A,B, the untransfected cells themselves contain a small amount of CRABP I, as evidenced in the predominant distribution of immunostained (brown) grains in the cytoplasm, which is consistent with the RA-binding activity observed in the cytosolic and nuclear fractions of untransfected COS-1 cells (insets, Figure 1). However, when the cells transfected with the CRABP I expression plasmid were examined, elevated levels of CRABP I were present in the nucleus in addition to the cytoplasm (Figure 3C,D), again in agreement with the results seen in Figures 1 and 2. Together, these observations clearly demonstrate the occurrence of functionally active CRABP I in the nucleus as well as in the cytoplasm of the transfected COS-1 cells.

Analysis of the Interaction between CRABP I and RAR α. Since a part of the CRABP I expressed in COS-1 cells was located in the nuclei, we investigated to see if nuclear CRABP I had any effect on the binding of RA to RAR α and whether there was a direct protein−protein interaction between CRABP I and RAR α that resulted in a larger complex than either protein when analyzed on a size-exclusion chromatographic column. As seen in Figure 4, when nuclear extracts from COS-1 cells expressing either CRABP I or RAR α were separately incubated with [^{3}H]-RA and analyzed on a Superose-12 column, radioactive peaks corresponding to molecular sizes of approximately 15 000 and 50 000, respectively, were observed (Figure 4A,B). When a 10-fold molar excess of CRABP I was coincubated with RAR α in the presence of [^{3}H]RA (Figure 4C), a 20% increase in the bound radioactivity observed in the fractions

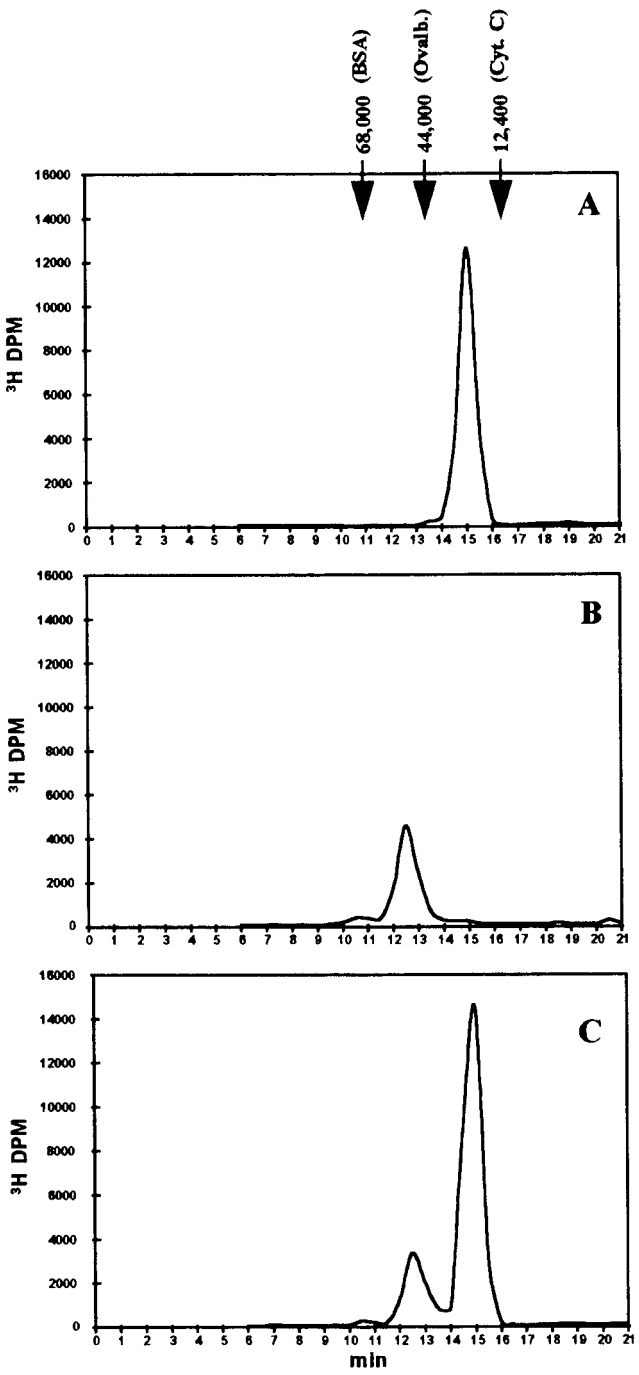

FIGURE 4: Chromatographic analysis of the interaction between CRABP I and RAR α. Samples with [^{3}H]RA (5 nM) and COS-1 nuclear extracts containing either CRABP I (A) or RAR α (B) or both CRABP I and RAR α (C) were incubated overnight at 4 °C and analyzed on a Superose-12 size-exclusion FPLC column (Pharmacia). The position of elution for external protein size markers bovine serum albumin (BSA, M_r 68 000), ovalbumin (Ovalb., M_r 44 000), and cytochrome *c* (Cyt. C, M_r 12 400) is shown at the top of the graph.

comigrating with the 15 000 molecular weight marker was accompanied by a proportional decrease in the fractions containing the 50 000 MW protein. This suggests that, at the concentration of labeled RA used in these assays (5 nM), CRABP I seems to compete partially with RAR α for the ligand. The coincubation of these proteins did not result in the appearance of additional peaks of high molecular weight, indicating that either these proteins do not directly interact with each other or a stable complex formed by them cannot be detected under the conditions used in our assays.

Gel-Shift Analysis of the Effect of CRABP I on the Binding of RAR α and RXR α to TREpal and βRARE DNA Sequence Elements. We have also examined the effects of CRABP I on the interaction of RAR α and RXR α with their DNA-binding elements. Two double-stranded oligonucleotide sequences, TREpal and βRARE, which contain the palindromic thyroid hormone response sequence (Glass et al., 1989) and an RA-response sequence from the human RAR β gene (de Thé et al., 1990), respectively, and have been shown to bind homo- and heterodimers of RAR α and RXR α with different affinities (Zhang et al., 1992), were incubated with COS-1 nuclear extracts containing the retinoid receptor RAR α or RXR α or both and analyzed by gel retardation assay.

In the presence of nuclear extracts from untransfected COS-1 cells (mock) both TREpal (Figure 5A) and βRARE (Figure 5B) probes produced a DNA−protein complex (shown by *). When the nuclear extracts from COS-1 cells expressing RAR α were incubated with the same probe fragments, an intense, heavier complex (shown by arrow, Figure 4A,B) appeared in addition to the background complex observed with the mock extracts. A similarly retarded DNA−protein complex showing a slightly stronger affinity than the band seen with RAR α extract alone was also observed when COS-1 extracts containing RAR α and RXR α were coincubated with TREpal and βRARE probes (Figure 5A,B).

The interactions of RAR α, with or without RXR α, with TREpal and βRARE probes were also examined in the presence of COS-1 nuclear extracts that contained CRABP I. As seen in Figure 5A, the coincubation of CRABP I had a slight inhibitory effect on the RAR α−TREp complex. However, this inhibitory effect was not reproducible in other experiments (data not shown). Also, the coincubation of CRABP I did not inhibit either the interaction of the RAR α−RXR α heterodimer with the TREp sequence (Figure 5A) or the complexes formed by the βRARE DNA with RAR α alone or both RAR α and RXR α (Figure 5B). Importantly, TREpal and βRARE DNA did not form additional complex-(es) when CRABP I was added to these sequences either directly or along with the RAR α and RXR α receptors. These results demonstrate that CRABP I does not participate in DNA−protein interactions with TREpal and βRARE promoter elements either directly or in association with RAR α and RXR α. All the complexes formed by TREpal and βRARE probes in the presence of COS-1 nuclear extracts appear to be specific since only an excess of unlabeled probe oligonucleotide, but not the consensus binding site for transcription factor AP1, competed for their formation (Figure 5A,B).

Analysis of the Effect of CRABP I on the Transcription Mediated by Retinoid Receptors. The effects of CRABP I on the transcription mediated by various retinoid receptors were examined by transient transfection analysis of reporter gene expression in CV-1 cells. Initially, the RA-induced transcription activated by RAR α was analyzed in the presence of increasing amounts (5−400 ng) of CRABP I expression plasmid (Figure 6A). CRABP I by itself did not support a significant level of RA-induced transcription above the background expression seen from the TRE_2-tk-CAT reporter gene at any of the concentrations. When expressed together with RAR α, CRABP I inhibited the RAR α-mediated RA-induced transcription slightly (20−30%), and such an effect did not vary with the amount of CRABP I

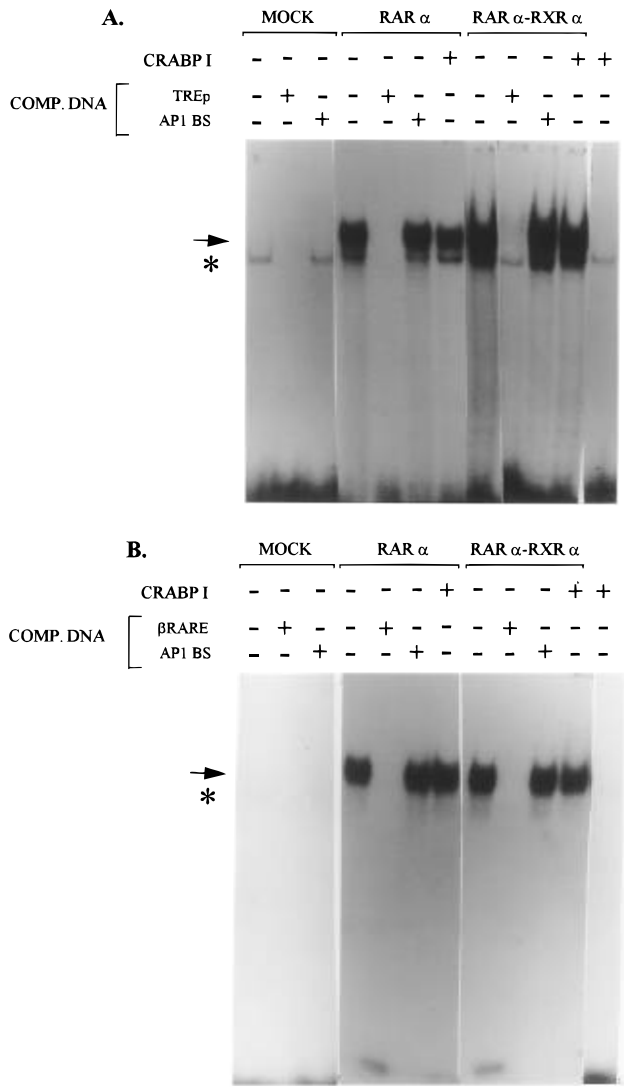

FIGURE 5: Gel mobility shift analysis of the effect of CRABP I on the interaction of RAR α and RXR α with RA-response elements. ^{32}P-Labeled double-stranded oligonucleotides representing RA-response elements TREpal (A) and βRARE (B) were incubated with nuclear extract (15 μg) from untransfected COS-1 cells (MOCK) or nuclear extract (5 μg) from transfected cells that expressed RAR α or a mixture of extracts containing both RAR α and RXR α (5 μg each). In some samples, 5 μg of CRABP I extract was added to the probes either by itself or in addition to RAR α or both RAR α and RXR α. The amount of protein in each sample was adjusted to 15 μg by addition of the mock extract. For competition assays, 10 pmol (100-fold molar excess) of competitor oligonucleotide DNA (COMP. DNA) was added along with the probe. DNA−protein complexes were analyzed by electrophoresis on a native 4% polyacrylamide gel. TREp = TREpal, the palindromic thyroid hormone response sequence (Glass et al., 89); βRARE = RA-response sequence from human RAR β gene (de Thé et al., 90); AP1BS = consensus DNA-binding sequence for AP1 transcription factor.

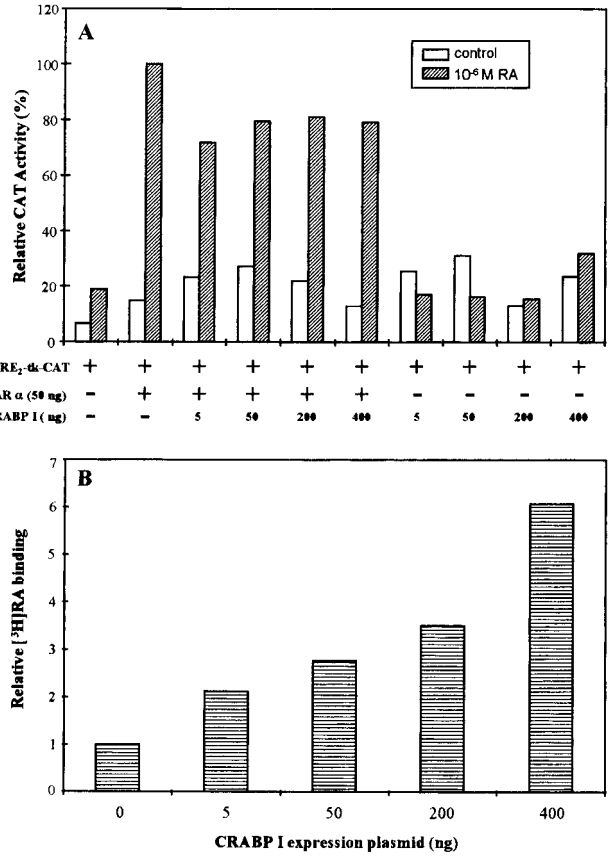

FIGURE 6: Analysis of the expression and effects of various amounts of CRABP I on the transcription mediated by RAR α in CV-1 cells. (A) Transcriptional Analysis. CV-1 cells (5×10^4) were transfected with 200 ng of TRE_2-tk-CAT reporter plasmid and expression plasmid for RAR α (50 ng) or CRABP I (5−400 ng) or both as indicated under the graph. After an overnight incubation in the presence of DNA precipitates, the cells were treated with either DMSO (control) or 10^{-6} M RA for a period of 24 h and the CAT activity in the cell extracts was assayed as described under Materials and Methods. The CAT activity is presented relative to that obtained from the cells transfected with RAR α and treated with 10^{-6} M RA. (B) FPLC chromatographic analysis of the expression of CRABP I in CV-1 cells. The [^{3}H]RA binding activity in extracts from CV-1 cells transfected with various amounts of the expression plasmid for CRABP I (5−400 ng) and the internal control β-galactosidase plasmid pCH110 was analyzed on a Mono Q anion-exchange column and normalized to the β-galactosidase activity. The values are expressed relative to the background activity in the mock (0 ng) extract.

expression plasmid used in the cotransfections. The level of basal transcription observed in the cells did not change significantly when CRABP I was expressed either singly or together with RAR α (Figure 6A). To verify that the lack of a more pronounced effect by CRABP I was not due to the low expression of the latter in the cells, extracts obtained from CV-1 cells transfected with varying amounts of CRABP I expression plasmid were assayed for the [^{3}H]RA-binding activity using anion-exchange (Mono Q) chromatography. As seen in Figure 6B, the level of CRABP I expressed in the cells increased in response to the increasing amount of the plasmid used in the transfections. It is also clear from these data that although the highest level of CRABP I expression was observed when 400 ng of the plasmid was used, even with 5 ng the amount of CRABP I was about 2−2.5-fold higher compared to the basal levels. Moreover, the amount of CRABP I obtained with 5 ng as well as at higher plasmid levels (i.e., 50, 200, and 400 ng) affected the RA-induced transcription mediated by RAR α to the same degree (Figure 6A), suggesting that, even at a 2.5-fold excess, the effect of CRABP I on the RAR α-mediated transcription reaches the saturation level.

The possibility that CRABP I might affect the RA-induced transcription mediated by various RAR subtypes differently was examined by using equal amounts of expression plasmids for CRABP I and one of the RAR subtypes α, β, and γ in cotransfection experiments. As seen in Figure 7, CRABP I by itself did not support any significant level of RA-induced transcription from the TRE_2-tk-CAT reporter gene. In contrast, each of the RAR subtypes, α, β, and γ,

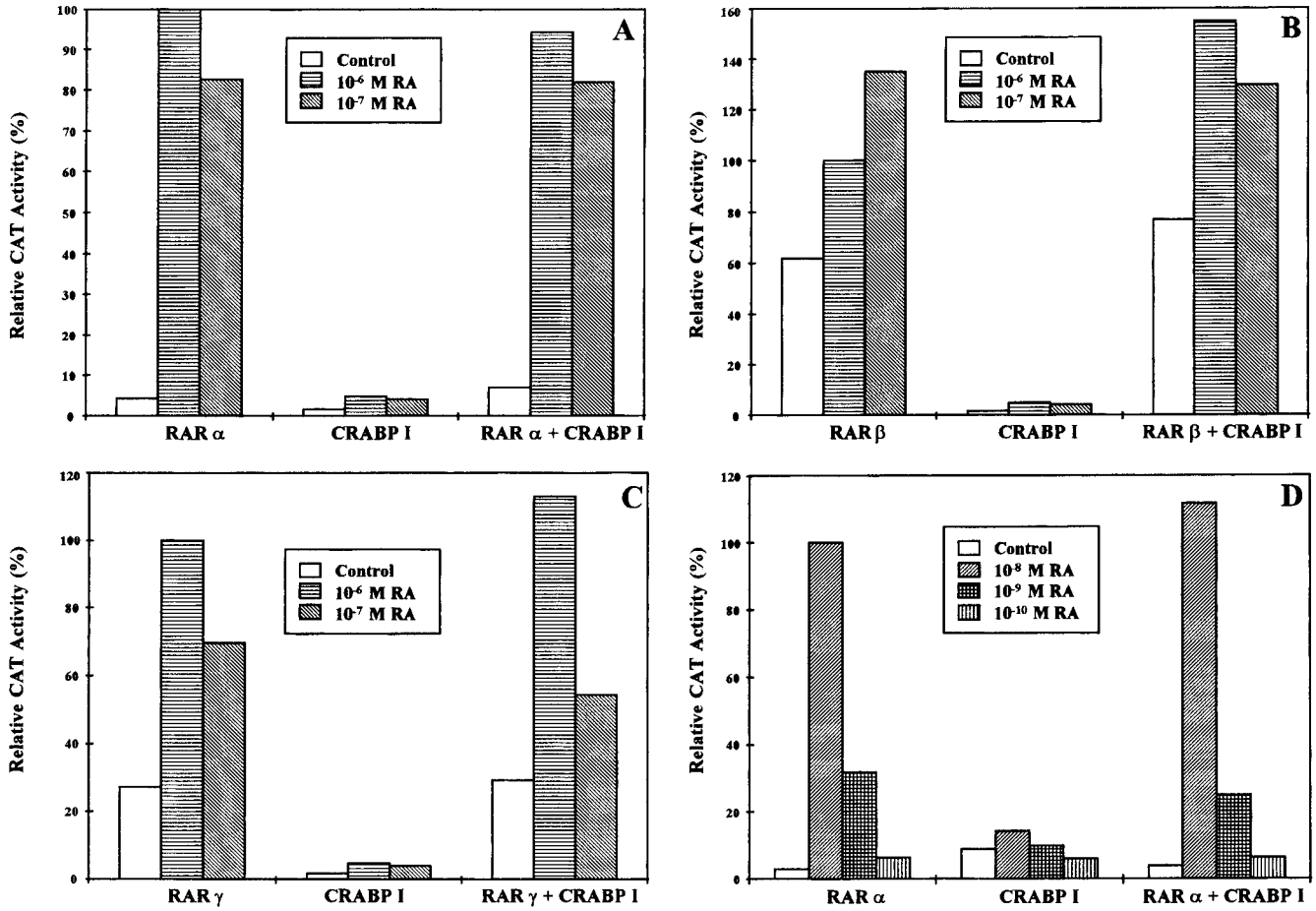

FIGURE 7: Analysis of the effect of CRABP I expression on the RA-induced transcription mediated by retinoid receptors. CV-1 cells were transfected with the reporter plasmid TRE_2-tk-CAT (200 ng) and 50 ng of expression plasmid for one of the subtypes of RA receptor [(RAR α (A), RAR β (B), and RAR γ (C)] or CRABP I or both (50 ng each). The CAT activity in the transfected cells, treated with RA (10^{-6} to 10^{-7} M) or DMSO (control) was determined as described elsewhere and normalized for transfection efficiency by the corresponding β-galactosidase activity expressed from the internal control plasmid pCH110. (D) Same as in (A) except that the cells were exposed to lower RA concentrations ranging from 10^{-8} to 10^{-10} M.

when expressed in CV-1 cells, supported a significant enhancement or in the transcription at 10^{-6} and 10^{-7} M concentrations of RA over the controls (Figure 7A−C). When CRABP I was expressed along with RAR α or RAR γ, no significant change was observed in either the basal (control) or the RA-induced transcription (Figure 7A,C). However, in the presence of CRABP I, a 50% increase in the RAR β-mediated transcription was observed at 10^{-6} M RA although the fold induction over the basal activity was still not significantly different from that seen with RAR β alone (Figure 7B). Moreover, at 10^{-7} M RA, the level of induced transcription was similar in CV-1 cells transfected with either RAR β alone or both RAR β and CRABP I (Figure 7B), suggesting that the coexpression of CRABP I did not significantly affect the RA-induced transcription mediated by RAR β.

To see if CRABP I was more effective in influencing the RA-induced transcription at lower concentrations of the ligand, the RAR α-activated transcription was also analyzed in cells that were treated with 10^{-10} to 10^{-8} M RA. As seen from Figure 7D, the coexpression of CRABP I did not have any effect on the transcriptional activity mediated by RAR α at any of the RA concentrations.

Analysis of the Effect of CRABP I on the Transcription Mediated by RAR α−RXR α Heterodimers. Since RA is known to be a potent activator of RAR α−RXR α heterodimers and many RAREs bind the heterodimers more avidly than the homodimers of either RAR α or RXR α (Figure 5; Zhang et al., 1992), we have examined the effects of the expression of CRABP I on the heterodimer-mediated transcription induced by RA and 9-*cis*-RA. CRABP I by itself did not support any significant level of transcription induced by either RA or 9-*cis*-RA from the TRE_2-tk-CAT reporter gene (Figure 8A). Coexpression of RAR α and RXR α, on the other hand, resulted in an enhanced level of induced transcription with both RA and 9-*cis*-RA at concentrations ranging from 10^{-9} to 10^{-6} M. However, when CRABP I was expressed along with RAR α and RXR α in the cells, no significant differences were observed in the induced transcription mediated by RAR α−RXR α heterodimers at any of the retinoid concentrations (Figure 8A). Similarly, when βRARE, which showed strong binding to RAR α as well as the RAR α−RXR α heterodimers (Figure 5B) but not to the RXR α (data not shown), was used as the inducible promoter in the CAT reporter plasmid, the coexpression of CRABP I did not have any effect on the retinoid-induced transcription mediated by either RAR α alone or the RAR α−RXR α heterodimer (Figure 8B). Moreover, in these cells, βRARECAT showed a very high level of induced activity in the presence of 10^{-7} M RA or 9-*cis*-RA even when the retinoid receptors were not expressed. RXR α and CRABP I, when expressed singly or together, did not activate transcription beyond the background level of transcription observed from βRARECAT.

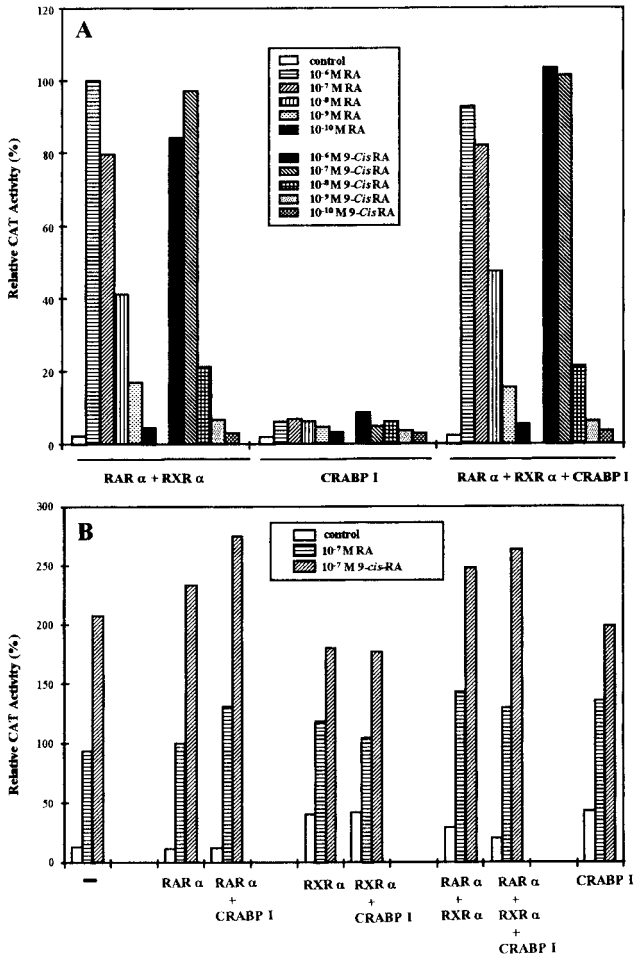

FIGURE 8: Analysis of the effect of CRABP I expression on the transcription mediated by RAR α−RXR α heterodimers. (A) Expression plasmids (50 ng each) for RAR α and RXR α were used with or without the CRABP I plasmid (50 ng) in addition to the TRE_2-tk-CAT (200 ng) reporter DNA in the transfection of CV-1 cells. The CAT activity was determined after the cells had been exposed to various concentrations (10^{-6} to 10^{-10} M) of either RA or 9-*cis*-RA for 24 h**.** (B) The CAT activity, expressed in CV-1 cells from the *β*RARECAT (200 ng) reporter plasmid, was analyzed after the expression plasmids for RAR α and RXR α were transfected either together or separately with or without the CRABP I plasmid. A 10^{-7} M concentration of either RA or 9-*cis*-RA was used to treat the transfected cells. In all cases the CAT activity was normalized to the *β*-galactosidase activity as described under Materials and Methods and in the legend for Figure 7.

DISCUSSION

Ever since the identification of CRABP as an RA-binding protein, several studies have been directed at understanding its role in the RA metabolism as well as its effects on the RA-induced gene expression. In continuation of those efforts, we have examined the possible involvement CRABP I in the RA-induced transcription, mediated by nuclear RA receptors, at the level of protein−protein and protein−DNA interactions as well as the reporter gene expression. The results presented herein provide evidence that CRABP I, partly localized in the nuclei, does not directly participate in the RA-induced transcription activated by RAR α and RXR α through their interaction with RA-response elements. This is reflected in the apparent lack of any significant effect by CRABP I on the binding of RAR α and RXR α to RAREs in gel retardation analysis (Figure 5), even though, in the absence of response elements, CRABP I competed partially with RAR α for RA (Figure 4). Moreover, overexpression of CRABP I in CV-1 cells did not result in a significant reduction or enhancement in the RA-induced transcription mediated by various RARs as well as RAR α−RXR α heterodimers under ligand concentrations ranging from 10^{-10} to 10^{-6} M (Figures 7 and 8).

These results obtained from CV-1 cells, suggesting no role for CRABP I in RA signaling, are different from those observed previously with F9 teratocarcinoma cells (Boylan & Gudas, 1991). In the latter study, stably transfected F9 cells which expressed elevated levels of CRABP I as well as wild-type F9 cells that had been transiently transfected with CRABP I expression vector exhibited reduced RAR α-activated transcription in the presence of low concentrations of RA. This suggested that CRABP I might play an important role in regulating the amount of RA available for RAR-activated transcription in the nucleus. It is possible that the reasons for the differences observed between these two studies might lie both in the cell-type and differentiation-specific variations and in the amount of exogenous CRABP I expressed in these cells. Since both CRABP I and RARs show similar binding affinity for RA (Ong & Chytil, 1978; Ishikawa et al.,1990; Yang et al., 1991), it is likely that the inhibitory effects of CRABP I on the RAR-mediated transcription would be obvious only when limited RA and excess CRABP I exist inside the cell. In this regard, it is important to note that the F9 cells, which differentiate in response to RA (Strickland et al., 1980), might be more sensitive than CV-1 cells to the intracellular levels of the retinoid. Moreover, in F9 cells, as well as in an *in vitro* assay using rat testes microsomes, it has been shown that CRABP I enhances the conversion of RA into a more polar retinoid, 4-oxo-RA (Fiorella & Napoli, 1991; Boylan & Gudas, 1992). It is possible that this increased conversion of RA into 4-oxo-RA by CRABP I has a more deleterious effect on the differentiation-specific transcription in F9 cells since RA is a better activator of cell growth and differentiation than 4-oxo-RA (Williams et al., 1987; Pijnappel et al., 1993). In contrast, the transformation of RA into 4-oxo-RA by CRABP I might have little effect on the RAR-mediated transcription in CV-1 cells as the transactivation potential of the latter retinoid is shown to be similar to that of RA in CV-1 as well as COS-1 cells (unpublished results; Pijnappel et al., 1993).

Studies conducted both *in vitro* and *in vivo* provide evidence for the involvement of CRABP I in the control of the intracellular level of available RA through sequestration and metabolism of the latter (Fiorella & Napoli, 1991; Boylan & Gudas, 1992; Fiorella & Napoli, 1994). It is suggested that such a role on the part of CRABP I, coupled with its high expression, might be an important factor in determining the susceptibility of certain tissues of developing mouse embryo to the teratogenic effects of excess RA (Dencker et al., 1990; Vaessen et al., 1990; Maden et al., 1992; Gustafson et al., 1993; Lyn & Giguère, 1994; Leonard et al., 1995). However, the recently obtained evidence that mutant mice lacking both CRABP I and CRABP II are indistinguishable from wild-type mice in their physiology and response to the exposure to excess RA indicates that CRABPs are not essential components of the RA-signaling pathway (Gorry et al., 1994; Lampron et al., 1995). Support for such a conclusion also comes from the evidence that some cell lines lacking CRABP I still retain the ability to support the RA-induced cell differentiation (Jetten et al., 1987) and that there is a lack of complete correlation between the ability of the retinoids to bind CRABP and their biological activity

(Tamura et al., 1990; Willhite et al., 1992; Asselineau et al., 1992; Keidel et al., 1993; Sass et al., 1995). Yet, the evolutionary conservation of CRABPs among various species and their ability to bind RA suggest an important role for these proteins in the regulation of the intracellular concentration of this retinoid. It is possible that a more significant role for these proteins might lie in their ability to sequester RA at times of its excess availability and release it when the animal is under vitamin A stress.

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