

Overexpression of the Cellular Retinoic Acid Binding Protein-I (CRABP-I) Results in a Reduction in Differentiation-specific Gene Expression in F9 Teratocarcinoma Cells

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Abstract. Treatment of F9 teratocarcinoma stem cells with retinoic acid (RA) causes their irreversible differentiation into extraembryonic endoderm. To elucidate the role of the cellular retinoic acid binding protein-I (CRABP-I) in this differentiation process, we have generated several different stably transfected F9 stem cell lines expressing either elevated or reduced levels of functional CRABP-I protein. Stably transfected lines expressing elevated levels of CRABP-I exhibit an 80–90% reduction in the RA induced expression of retinoic acid receptor (RAR) β , laminin B1, and collagen type IV (α 1) mRNAs at low exogenous RA concentrations, but this reduction is eliminated at higher RA concentrations. Thus, greater expression of

CRABP-I reduces the potency of RA in this differentiation system. Moreover, transfection of a CRABP-I expression vector into F9 cells resulted in five- and threefold decreases in the activation of the laminin B1 RARE (retinoic acid response element) and the RAR β RARE, respectively, as measured from RARE/CAT expression vectors in transient transfection assays. These results support the idea that CRABP-I sequesters RA within the cell and thereby prevents RA from acting to regulate differentiation specific gene expression. Our data suggest a mechanism whereby the level of CRABP-I can regulate responsiveness to RA during development.

RETINOIC acid (RA),¹ a derivative of retinol (vitamin A), influences the proliferation and cellular differentiation of a wide variety of cell types (Sporn and Roberts, 1983). For example, RA has been shown to inhibit differentiation of keratinocytes (Fuchs and Green, 1981), and promote differentiation of human promyelocytic leukemia cells (Breitman et al., 1980), melanoma cells (Lotan and Lotan, 1981), human neuroblastoma cells (Haussler et al., 1983) and mouse teratocarcinoma stem cells (Strickland et al., 1980). In addition, retinoids can suppress or reverse the malignant phenotype both in vivo and in vitro (Sporn and Newton, 1981; and Lotan, 1980). RA exhibits striking effects on vertebrate limb development (Thaller and Eichele, 1987; Tickle et al., 1982) and limb regeneration (Maden, 1983), lending support to the idea that RA plays a pivotal role in pattern formation during development.

F9 murine teratocarcinoma stem cells resemble the pluripotent inner cell mass cells of mouse blastocysts, and thus, provide an important model system with which to study critical early events in mouse development during the time when initial cell lineage commitments are made. F9 mouse teratocarcinoma stem cells differentiate into primitive endoderm

in response to RA and into parietal endoderm, an epithelial extraembryonic cell type in the mouse blastocyst, in response to RA and dibutyl cAMP (Strickland et al., 1980). When F9 stem cells differentiate, the genes encoding laminin B1 and collagen IV (α 1) are transcriptionally activated between 24 and 48 h after exposure to RA (Hosler et al., 1989) and their mRNA levels become 50–60-fold higher by 60 h (Wang et al., 1985). In contrast, within 2 h after RA addition the *Era-1/Hox 1.6* gene is transcriptionally activated (LaRosa and Gudas, 1988a, b). (The *Era-1* gene appears to be the same gene as that named *Hox 1.6* by Baron et al., 1987.) Decreases in the expression of specific genes such as *c-myc* (Griep and DeLuca, 1986; Dony et al., 1985; Campisi et al., 1984) and *REX-1* (Hosler et al., 1989) in RA-treated F9 cells have also been reported. Thus, these classes of genes represent molecular markers of the parietal endoderm differentiation pathway.

Although the mechanism whereby RA regulates gene expression and differentiation is not completely understood, it is likely that high-affinity RA receptors and/or binding proteins are involved. Recently, four high-affinity nuclear retinoic acid receptors were identified and shown to be members of a large family of genes that encode nuclear steroid hormone receptors. These four genes, which presumably act as transcription factors, have been designated RAR α , RAR β ,

1. *Abbreviations used in this paper:* RA, retinoic acid; RACT, retinoic acid plus dibutyl cAMP and theophylline.

RAR γ , and RXR α , (Brand et al., 1988; Petkovich et al., 1987; Benbrook et al., 1988; Krust et al., 1989; deThé et al., 1989; Giguere et al., 1987; Mangelsdorf et al., 1990). RAR α and γ mRNAs are constitutively expressed in F9 stem cells whereas the RAR β mRNA level increases 20-fold after RA addition (Krust et al., 1989; Hu and Gudas, 1990).

In addition to these nuclear RA binding proteins, F9 cells express a high-affinity cytoplasmic RA-binding protein designated the cellular RA-binding protein (CRABP-I; Eriksson et al., 1986; Stoner and Gudas, 1989). Both the bovine CRABP-I cDNA (Shubeita et al., 1987) and the mouse CRABP-I cDNA (Stoner and Gudas, 1989) have been cloned and sequenced. The mouse CRABP-I gene encodes a single, low abundant mRNA species with an approximate length of 800 nucleotides. The CRABP-I mRNA level decreases two- to threefold after treatment of F9-Wt cells with RA alone; however, the CRABP-I mRNA level increases \sim 20-fold between 24 and 48 h after treatment with RA plus dibutyl cAMP (Stoner and Gudas, 1989). The mRNA encodes a 15-kD protein that exhibits a high affinity for RA ($K_d = 10$ nM, Grippo and Gudas, 1987) and does not appear to contain any DNA binding motifs. A second mouse CRABP gene, referred to as CRABP-II, has recently been cloned and its expression studied during mouse embryogenesis (Giguere et al., 1990). The CRABP-II mRNA is expressed at high levels during early mouse development and in adult mouse skin. In addition, CRABP-II mRNA levels are induced 50-fold in F9 teratocarcinoma cells treated with RA for 48 h (Giguere et al., 1990). The levels of CRABP-I and CRABP-II mRNAs have not been directly compared in F9 cells, so which of the genes is predominantly expressed is unknown.

CRABP-I mRNA expression has been detected in the developing mouse nervous system, the eye region, and the craniofacial region (Perez-Castro et al., 1989). In mouse limb buds, Dolle et al. (1989) showed that the CRABP-I mRNA was expressed along a proximo-distal gradient with maximal expression at the distal portion of the day 10 developing mouse limb. This suggests that the CRABP-I may play a role in mediating the putative effects of RA during pattern formation of the limb. It is of interest that the patterns of CRABP-I mRNA expression and those of RAR β and RAR γ were mutually exclusive at day 14.5, suggesting different functions for these proteins (Dolle et al., 1989).

As yet no function has been defined for CRABP-I. Our results reported here support the idea that CRABP-I functions either to sequester RA within the cytoplasm or to enhance the metabolism of RA to inactive derivatives, thus limiting the RA specific activation of differentiation-specific genes.

Materials and Methods

Cell Culture

Mouse F9 teratocarcinoma stem cells were maintained at 37°C in 10% CO₂ in tissue flasks with DMEM supplemented with 10% heat-inactivated calf serum (Gibco Laboratories, Grand Island, NY) and 2 mM glutamine as described previously (Wang et al., 1985). For cell growth experiments, F9 stem cells (2×10^4 cells) were plated on gelatinized multiwell tissue culture plates containing 2 ml of medium. Retinoic acid (Sigma Chemical Co., St. Louis, MO) was added 6–8 h after plating (Wang and Gudas, 1984). All RA stock solutions were made in 100% ethanol; the final [ethanol] in

DMEM was never >1%. Cells were harvested beginning the following morning (day 1) and counted in a Coulter counter (Coulter Electronics, Hialeah, FL).

Plasmids

The full-length CRABP-I cDNA (Stoner and Gudas, 1989) was cloned into the Eco RI restriction site within the expression vector pMT64AA (Glanville et al., 1982). This vector contains the mouse metallothionein I promoter and an Eco RI/Hind III polylinker followed by a poly-A addition site spanning an additional 200 bases. The anti-sense CRABP-I vector was constructed in a similar manner and contains the CRABP-I cDNA in the reverse orientation. The pSV2 neo plasmid (Southern and Berg, 1982) was used as a selection marker for the generation of stably transfected cells. The pc156 plasmid contains a partial cDNA specific for the murine laminin B1 gene within the Pst I site of pBR322 (Wang and Gudas, 1983). The pc15 contains a partial cDNA specific for the collagen type IV ($\alpha 1$) gene within the Pst I site of pBR322 (Wang and Gudas, 1983). The pEra-1-993 E1.0 plasmid contains the 3' Eco RI fragment of the Era-1/Hox 1.6 cDNA cloned into pUC-9 (LaRosa and Gudas, 1988a). The pMT-c-myc 1 plasmid contains the c-myc Sac I/Hind III fragment (Stanton et al., 1984) cloned into the Sma I/Hind III site of pMT64AA. The pAct-1 plasmid contains a partial cDNA coding for the murine β actin gene (Spiegelman et al., 1983). The pSG5-RAR α and the pSG5-RAR β plasmids contain the entire protein coding regions of the human RAR α and the human RAR β genes, respectively. The RAR α cDNA was cloned into the Eco RI site within pSG5 and the human RAR β cDNA was cloned into the Eco RI/Bam HI site within pSG5 (Petkovich et al., 1987; Brand et al., 1988). The REX-1 cDNA was cloned into the Eco RI site within pUC9 (Hosler et al., 1989). All vector inserts were isolated by the band intercept procedure before random primer labeling (Schleicher & Schuell application update #364, 1981).

Production and Initial Characterization of the Growth Properties of Stably Transfected Cell Lines

All transfections were carried out using the calcium phosphate precipitation procedure (Graham and Van der Eb, 1973). Cotransfections were performed at a 10:1 molar ratio of the gene of interest to the neomycin resistance gene in pSV2 neo, which was used as a selectable marker. Selection was carried out in medium containing 300 μ g/ml of G418 sulfate (Gibco Laboratories) for 14–21 d. Resistant foci were subsequently cloned and grown up in mass culture for screening for the stable expression of the transfected gene. Screening G418-resistant clones for the elevated or reduced expression of the CRABP-I gene was carried out by the RNA slot blot procedure as described previously (Schleicher & Schuell application update 372, 1983). Clones were grown in 100 μ M zinc chloride for 4 d, after which cytoplasmic RNA extracts were prepared for slot blotting onto nitrocellulose sheets. All hybridizations and washing procedures were carried out as described (Stoner and Gudas, 1989). Each blot was stripped and reprobed with actin to insure that equal amounts of RNA had been blotted. All probes were labeled by the random primer procedure as described previously (Feinberg and Vogelstein, 1984). The cell lines characterized in this paper are designated as follows: untransfected F9 wild-type stem cells, F9-Wt; a G418-resistant clone of pSV2 neo transfected F9 wild-type stem cells, F9-Wt-S-neo-1; two CRABP-I sense transfected F9 wild-type stem cell clones number 11 and 15, F9-Wt-S-CRABP-I-11 and F9-Wt-S-CRABP-I-15; two CRABP-I anti-sense transfected F9 wild-type stem cell clones number 18 and 27, F9-Wt-AS-CRABP-I-18 and F9-Wt-AS-CRABP-I-27.

Growth curves were performed to determine whether alterations in the level of CRABP-I mRNA influenced the proliferation of the transfected F9 lines in the presence or absence of different concentrations of RA, plus 100 μ M zinc chloride, 250 μ M dibutyl cAMP, and 500 μ M theophylline (CT; Sigma Chemical Co.). Drug treatment is abbreviated as RACT to indicate the addition of RA plus dibutyl cAMP and theophylline. Previous experiments (Dionne, C., unpublished) demonstrated that 100 μ M zinc chloride was not toxic to F9-Wt cells, and that this concentration of zinc chloride did not influence the RA-associated differentiation of F9 cells. In the absence of any drugs, the F9-Wt, F9-Wt-S-neo-1, CRABP-I sense transfected and CRABP-I anti-sense transfected lines exhibited generation times of \sim 14–16 h. In the presence of 1×10^{-6} M RA, 100 μ M zinc chloride, and CT, the generation times were also \sim 14–16 h for all of the lines (data not shown). In the presence of 1×10^{-4} M RA, 100 μ M zinc chloride, and CT, reductions in the growth rates of all of the lines were measured; no cytotoxicity was seen, as the cell numbers increased over the 4-d period in all of the lines and very few dead cells (<1%) were observed in the wells.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated by the guanidine isothiocyanate method as described previously (Chirgwin et al., 1979). RNA was isolated at 0, 12, 24, 36, 48, and 96 h after addition of retinoic acid at various concentrations as described in the figure legends; 250 μ M dibutyl cAMP, 500 μ M theophylline, and 100 μ M zinc chloride were also added to the samples. The combination of RA, dibutyl cyclic AMP, and theophylline is designated RACT or RA plus CT. Time 0 is defined as 6–8 h after trypsinization and plating of the cells. The RNA was size fractionated on 1.2% agarose/2.2 M formaldehyde gels, transferred to nitrocellulose, and hybridized with a random primer labeled probe as described previously (Stoner and Gudas, 1989). Blots were exposed to Kodak XAR-5 film at -70°C with an intensifying screen. The hybridization signals were quantitated by scanning densitometry using a Helena Laboratories (Beaumont, TX) densitometer linked to an automatic integrator. All of the Northern analyses shown in this manuscript were performed at least twice using two different RNA preparations from cells cultured from two different thawed vials of each of the cell lines.

Genomic DNA Isolation and Southern Blot Analysis

Genomic DNA was isolated from F9 stem cells and F9 clones as described (Herrmann and Frischauf, 1987). Restriction endonuclease digestions and Southern blotting were carried out as described previously (Southern, 1975). The hybridizations were performed as described previously (Stoner and Gudas, 1989).

[^3H]Retinoic Acid Binding Assay

The levels of [^3H]RA binding in cytoplasmic extracts of F9 stem cells and F9 transfectants were measured by the sucrose gradient procedure (Grippe and Gudas, 1987) or FPLC chromatography analysis (Nervi et al., 1989). Briefly, 400 μ g (F9-Wt-S-neo-1 and CRABP-I sense transfectants) or 600 μ g (CRABP-I anti-sense transfectants) of total cytoplasmic protein was incubated in a final volume of 200 μ l in the presence of 2 mM dithiothreitol, 10 mM Tris, pH 7.4, and 75 nM [^3H]RA (specific activity 53–55 Ci/mmol; New England Nuclear, Boston, MA). Each sample was incubated in the dark at 4°C for 5 h. Two controls consisted of incubating extracts with a 100-fold molar excess of unlabeled RA, or 100-fold molar excess of unlabeled retinol (Sigma Chemical Co.). Unbound [^3H]RA was removed by charcoal extraction. For sucrose gradient analysis, the samples were applied to a 4.3-ml gradient of 5–15% sucrose in PBS and spun at 220,000 g for 21 h at 4°C . After centrifugation, 280- μ l fractions were collected from the bottom of each tube and counted by liquid scintillation. For FPLC analysis, the samples were fractionated over a Superose 12 HR 10/30 size-exclusion column (Pharmacia Fine Chemicals, Piscataway, NJ) at 4°C maintaining a flow rate of 0.5 ml/min with PTG buffer (5 mM sodium phosphate, pH 7.4, 10 mM thioglycerol, and 10% glycerol) plus 0.4 M KCl as the eluent. Each 0.5-ml fraction was counted by liquid scintillation.

Transient Transfection and CAT Assay

The transient transfections and chloramphenicol acetyltransferase (CAT) assays were performed as described (Vasios et al., 1989). Briefly, F9-Wt stem cells were cotransfected by the calcium phosphate precipitation method with various combinations of the plasmids, as indicated in the figures; pSG5-RAR α (1 μ g), pMT-CRABP-I-sense (5 μ g), pMT-CRABP-I anti-sense (5 μ g), pL-477/-432 Lam TKCAT (10 μ g), pRAR β -RARE CAT (10 μ g), pRSV-CAT (10 μ g) and varying amounts of pUC9 DNA (to bring the total amount of transfected DNA to 20 μ g). pL-477/-432 LamTKCAT vector contains the laminin B1 RARE (Vasios et al., 1989). The pRAR β -RARE CAT vector contains the RAR β RA responsive element (deThé et al., 1990) inserted into the Hind III and Bam HI restriction sites of pBLCAT 2. The RSV-CAT vector contains the Rous sarcoma viral LTR (Gorman et al., 1982a) and was used as an additional control. The p β Ac lacZ (3 μ g) construct was included in all transfections to normalize for any differences in transfection efficiency. The precipitate was removed after 12 h and the cells were treated with 100 μ M zinc chloride with or without 1 μ M RA for 24 or 48 h. Cells were harvested and extracts were prepared for the CAT assay (Herbomel et al. 1984; Gorman et al., 1982b). Protein equivalent to 20 units of β -galactosidase activity was used for each CAT assay. Quantitation of the monoacetylated [^{14}C]chloramphenicol product was performed by liquid scintillation analysis.

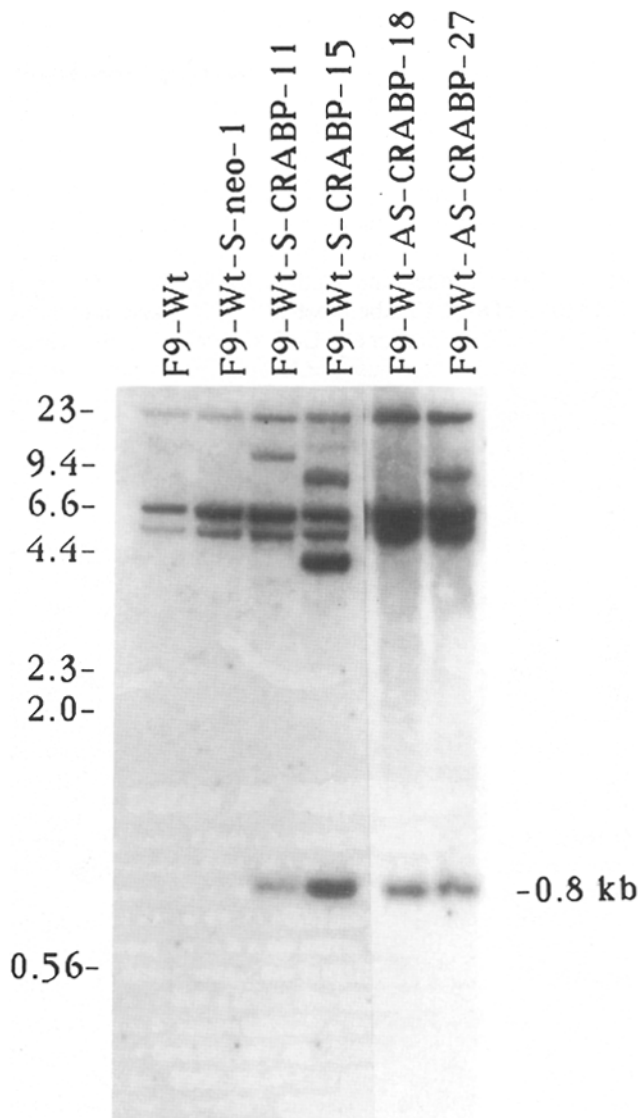


Figure 1. Southern blot analysis of CRABP-I sense and CRABP-I anti-sense F9 transfectants. Genomic DNA was isolated and digested with the restriction enzyme Eco RI. Fragmented DNA was fractionated through a 1% agarose gel and transferred to nitrocellulose. The blot was probed with random primer labeled CRABP-I cDNA (2×10^6 cpm/ml). DNA/Hind III size markers are indicated. The 0.8-kb band corresponds to the integrated CRABP-I cDNA insert. The exposure time was 36 h. This experiment was performed twice with identical results.

Results

Isolation of Stably Transfected F9 Cell Lines Containing CRABP-I Sense or Anti-sense cDNA

To determine the function of CRABP-I, we have transfected mouse F9 teratocarcinoma stem cells with a plasmid containing the mouse full-length CRABP-I cDNA in either the sense orientation or in the anti-sense orientation under the transcriptional control of the heavy metal inducible mouse metallothionein I promoter. F9 stem cells were cotransfected with either CRABP-I sense cDNA or CRABP-I anti-sense cDNA and the pSV2neo gene followed by selection in G418

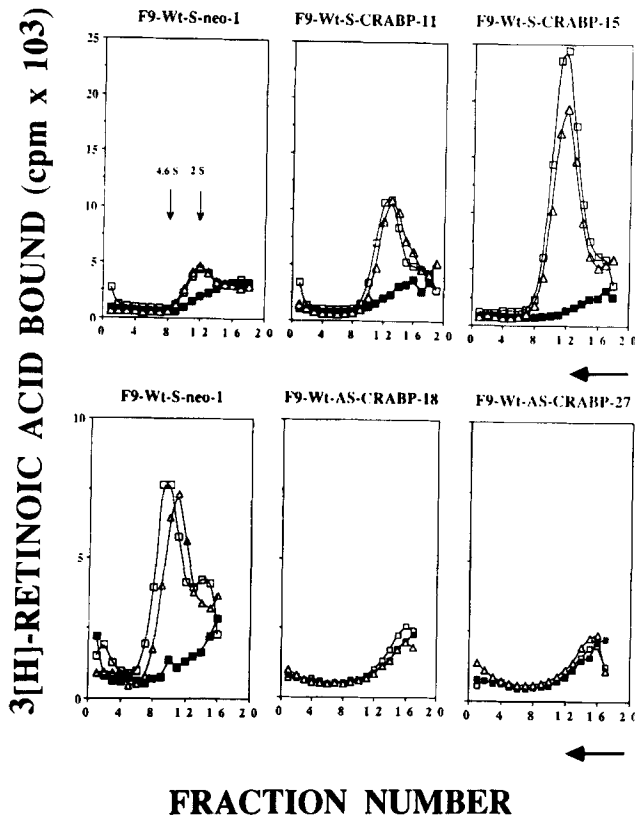


Figure 2. Sucrose gradient analysis of $[^3\text{H}]\text{RA}$ binding activity in undifferentiated F9 stem cells and CRABP-I transfectants. Cytoplasmic extracts were prepared from F9-Wt-S-neo-1 stem cells, F9-Wt-S-CRABP-I-11, F9-Wt-S-CRABP-I-15, F9-Wt-AS-CRABP-I-18, and F9-Wt-AS-CRABP-I-27 grown for 4 d in the presence of 100 μM zinc chloride in the absence of any media change. Total cytoplasmic protein was incubated with either $[^3\text{H}]\text{RA}$, a 100-fold excess of nonradioactive RA, or a 100-fold excess of nonradioactive retinol. CRABP $[^3\text{H}]\text{RA}$ complexes were assayed by the sucrose gradient procedure. Gradients were fractionated and each aliquot was counted in a scintillation counter. The results are expressed as the total number of $[^3\text{H}]\text{RA}$ counts per minute as a function of fraction number. CRABP-RA complexes possess a sedimentation coefficient of 2S. The arrow at the bottom left indicates the direction of sedimentation. Incubation conditions: (\square) $[^3\text{H}]\text{RA}$ only; (Δ) $[^3\text{H}]\text{RA}$ plus 100-fold excess nonradioactive retinol; (\blacksquare) $[^3\text{H}]\text{RA}$ plus 100-fold excess nonradioactive RA. The experiments represented in the top three panels were carried out with 400 μg total cytoplasmic protein while those in the bottom three panels were performed with 600 μg total cytoplasmic protein. The top and bottom panels have different y-axes. Each experiment was performed twice using cytoplasmic extracts from cells cultured from two different thawed vials of each of the cell lines.

sulfate. G418-resistant colonies were isolated and screened by the RNA slot blot procedure for either the elevated expression of CRABP-I mRNA in the case of the sense transfectants or for the decreased expression of CRABP-I mRNA in the case of the anti-sense transfectants. For the CRABP-I sense transfectants, we identified 3 stably transfected cell lines out of 25 that expressed a higher level of CRABP-I mRNA as compared to the F9 stem cell control. For the CRABP-I anti-sense transfectants, we identified 4 clones out of 28 that appeared to exhibit a decreased level of CRABP-I mRNA. Two CRABP-I sense-transfected lines, F9-Wt-S-

CRABP-I-11 and F9-Wt-S-CRABP-I-15, and two CRABP-I anti-sense transfected lines, F9-Wt-AS-CRABP-I-18 and F9-Wt-AS-CRABP-I-27, were chosen for further analysis.

Southern Analysis of Stably Transfected Lines

Southern blot analysis revealed the typical genomic restriction pattern for the endogenous CRABP-I gene within F9 stem cells (Stoner and Gudas, 1989) characterized by three Eco RI restriction fragments of 22, 5.6, and 4.9 kb (Fig. 1). These three Eco RI fragments were present in all of the F9 transfectants in addition to several other restriction fragments created by the random integration of the transfected CRABP-I cDNA. Most notable was a 0.8-kb Eco RI fragment which corresponded to the 0.8-kb CRABP-I cDNA contained within the transfected plasmid DNA (Fig. 1). These results indicate that each transfected line is unique and that two lines did not arise from the same transfected cell. In addition, each transfected line contains at least one exogenous CRABP-I cDNA of the correct nucleotide length.

Analysis of the CRABP-I mRNA and the Functional CRABP Protein in the Stably Transfected Lines

RNA from untreated F9-Wt stem cells and from the lines F9-

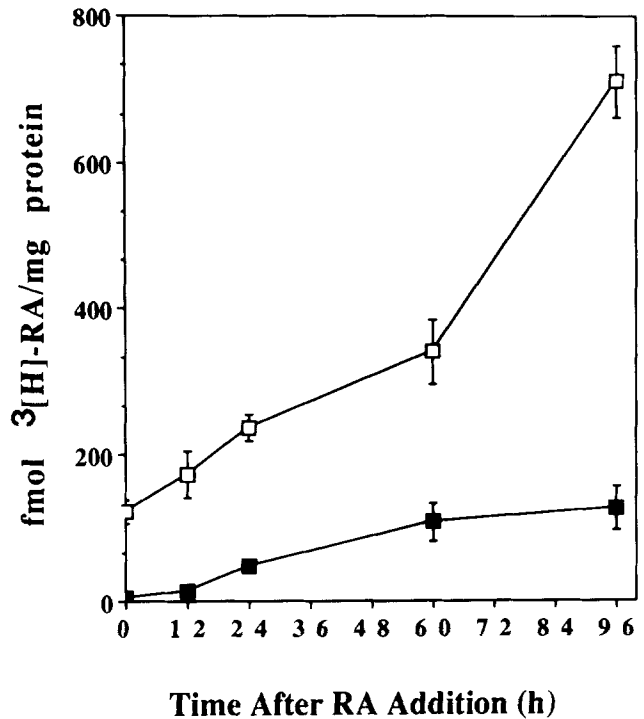


Figure 3. FPLC analysis of $[^3\text{H}]\text{RA}$ binding activity in F9-Wt-S-neo-1 and F9-Wt-S-CRABP-I-15 after the addition of RACT. Cytoplasmic extracts of F9-Wt-S-neo-1 and F9-Wt-S-CRABP-I-15 cells were prepared at the indicated times after the addition of RACT plus zinc chloride and incubated with either $[^3\text{H}]\text{RA}$ or a 100-fold excess of nonradioactive RA. CRABP $[^3\text{H}]\text{RA}$ complexes were assayed by FPLC chromatography. Each fraction was counted in a scintillation counter and the level of CRABP $[^3\text{H}]\text{RA}$ binding was determined. The results are expressed as the number of femtomoles $[^3\text{H}]\text{RA}$ bound/milligram protein as a function of time. Symbols: (\blacksquare) F9-Wt-S-neo-1; (\square) F9-Wt-S-CRABP-I-15. This experiment was performed twice.

Wt-S-CRABP-I-11 and F9-Wt-S-CRABP-I-15 was analyzed on Northern blots. The transfected CRABP-I sense mRNA could be distinguished from the endogenous CRABP-I mRNA by the fact that the transfected CRABP-I transcript was 200 nucleotides longer than the endogenous CRABP-I transcript as a result of a 200-bp polylinker-poly A addition sequence downstream of the CRABP-I cDNA. Therefore, on Northern blots the transfected CRABP-I mRNA in F9-Wt-S-CRABP-I-11 and F9-Wt-S-CRABP-I-15 was 1,000 nucleotides, whereas the endogenous CRABP-I mRNA was 800 nucleotides (data not shown).

Both sucrose gradient assays (Grippo and Gudas, 1987) and FPLC assays (Nervi et al., 1989) were used to compare the overall increase or decrease in CRABP functional protein in the specific transfected lines with the levels in untransfected F9-Wt cells and the F9-Wt-S-neo-1 line. These assays measure CRABP protein derived from both the endogenous CRABP-I gene and the transfected CRABP-I cDNA as well as the CRABP-II gene. Nuclear retinoic acid receptors are not detected in these assays, as primarily cytoplasmic protein is present in the extracts. Moreover, the nuclear RA receptor protein levels are extremely low in F9 cells; as a result, no [³H]RA binding can be detected when nuclear protein extracts of F9 cells are prepared and assayed on sucrose gradients (Gold, J., unpublished).

Cytoplasmic protein extracts were prepared from untransfected F9 stem cells and from the transfected stem cell lines after all of the lines were grown in the presence of 100 μM zinc chloride for 4 d in the absence of RACT. The control extracts, prepared from the F9-Wt-S-neo-1 line, bound 604 fmol of RA/mg protein in the sucrose gradient assay (Fig. 2). This is in agreement with previous results using untransfected F9 stem cells (Grippo and Gudas, 1987). This level

of [³H]RA binding remained the same in the presence of 100-fold excess of nonradiolabeled retinol, but the [³H]RA binding was effectively eliminated when extracts were incubated in the presence of a 100-fold excess of nonradioactive retinoic acid. F9-Wt-S-CRABP-I-11 extracts bound 1,743 fmol of RA/mg protein, a level approximately three times the level of the control, whereas extracts of the second CRABP-I sense clone, F9-Wt-S-CRABP-I-15, bound 5,150 fmol of RA/mg protein, almost 10 times the level detected in the control extracts (Fig. 2). This [³H]RA binding in both transfected lines was not competed by the presence of a 100-fold excess of nonradiolabeled retinol but was abolished by the presence of a 100-fold excess of nonradiolabeled RA. Very similar results were obtained using a Superose 12 column/FPLC assay; the F9-Wt-S-neo-1 line exhibited 561 ± 65 fmol of RA/mg protein and the F9-Wt-S-CRABP-I-15 line bound 4,967 ± 258 fmol of RA/mg protein when these lines were grown for 4 d in the presence of 100 μM zinc chloride, whereas the F9-Wt-S-neo-1 line bound 532 fmol of RA/mg protein and the F9-Wt-S-CRABP-I-15 line bound 1,004 fmol of RA/mg protein when the lines were grown for 4 d without zinc chloride. Therefore, F9 stem cells stably transfected with the CRABP-I sense cDNA under the transcriptional control of the mouse metallothionein I promoter exhibited elevated levels of functional CRABP protein.

Sucrose gradient analysis was also performed on extracts prepared from the CRABP-I anti-sense cell lines. 600 μg of total cytoplasmic protein from each line was used to detect any reduction in [³H]RA binding in the anti-sense transfected lines. The control F9-Wt-S-Neo-1 extracts bound 624 fmol of RA/mg protein (Fig. 2). In contrast, neither of the two CRABP-I anti-sense lines, F9-Wt-AS-CRABP-I-18 or F9-Wt-AS-CRABP-I-27, showed any detectable [³H]RA binding

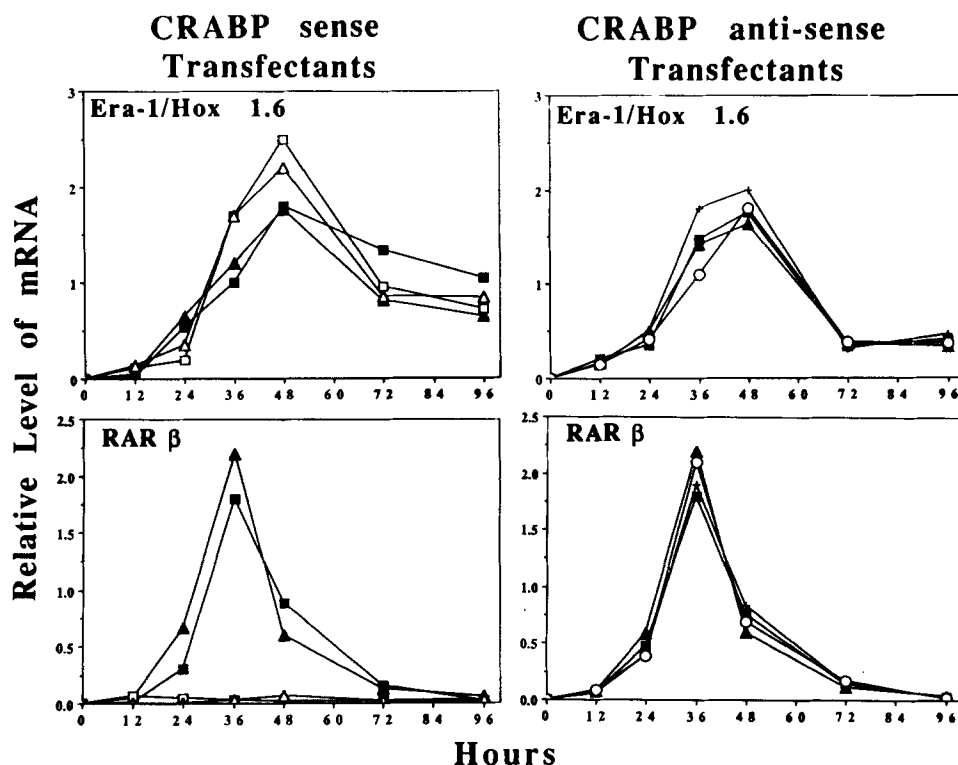


Figure 4. Northern blot analysis of early RA genes. Total cellular RNA (35 μg) was isolated at the indicated times after addition of 1×10^{-6} M RA, CT plus zinc chloride and fractionated on a 1.2% agarose/formaldehyde gel. The RNA was transferred to nitrocellulose and probed with a random primer labeled cDNA probe for either ERA-1/Hox 1.6 or RAR β (1×10^6 cpm/ml and 2×10^6 cpm/ml, respectively). The relative mRNA levels are expressed in arbitrary units determined by densitometry scanning of the Northern gels followed by normalization of the ERA-1/Hox 1.6 and RAR β mRNA level to the actin mRNA level for each sample. These results are expressed in relative RNA units. Symbols: (■) F9-Wt; (▲) F9-Wt-S-neo-1; (□) F9-Wt-S-CRABP-I-11; (△) F9-Wt-S-CRABP-I-15; (×) F9-Wt-AS-CRABP-I-18; (○) F9-Wt-AS-CRABP-I-27. This experiment was performed three times; one Northern analysis is shown.

(Fig. 2). A Superose 12 column/FPLC system was also used to measure [^3H]RA binding and more precisely determine the reduction in CRABP functional protein in the F9-Wt-AS-CRABP-I-18 and F9-Wt-AS-CRABP-I-27 lines. From this analysis, we estimate that extracts from these two lines express >20-fold less CRABP functional protein than the control F9-Wt-S-neo-1 extracts (data not shown).

A time-course experiment was then carried out to examine the levels of CRABP protein in F9 stem cells and the F9-Wt-S-CRABP-I-15 transfectant after RACT addition (Fig. 3). Cells were treated with RACT plus 100 μM zinc chloride at day 0, and cytoplasmic extracts were prepared at specific times thereafter. The CRABP protein levels in the F9-Wt-S-neo-1 line rose slowly after drug addition from 13 fmol of RA/mg protein at 12 h to 128 fmol of RA/mg protein at 96 h. In the CRABP-I sense transfectant, the CRABP protein levels increased from 173 fmol of RA/mg protein at 12 h after drug addition to 711 fmol of RA/mg protein at 96 h. Thus, the CRABP protein level was 20–25-fold higher at the time of drug addition (0 h), and 10-fold higher at 12 h after drug addition in the F9-Wt-S-CRABP-I-15 line as compared with the F9-Wt-S-neo-1 line (Fig. 3). By 96 h after addition of RACT plus zinc, the F9-Wt-S-CRABP-I-15 line exhibited a CRABP protein level that was 5–7-fold higher than that in the F9-Wt-S-neo-1 line. It is important to note that in all of the lines tested, the total number of femtomoles of RA bound/mg protein at 96 h after RACT addition in Fig. 3 differs from the total number of femtomoles of RA bound/mg protein at 96 h in the absence of RACT in Fig. 2. One possible explanation may be that the stability of the CRABP protein is reduced in the presence of RACT in all of the cell lines. However, at the time of RACT addition, the CRABP-I sense transfectant possesses much more functional CRABP protein than the “control” line F9-Wt-S-neo-1.

The Effect of Altered CRABP-I Expression on Differentiation-specific Gene Expression

To examine whether an elevated level of CRABP-I influences the differentiation process of F9 teratocarcinoma cells, the expression of three classes of genes was examined. These include primary response (cycloheximide insensitive) mRNAs induced within the first 12 h of differentiation, namely Era-1/Hox 1.6 and the nuclear RA receptor RAR β , genes that exhibit reduced expression during the first 12–18 h after RA addition such as *c-myc* gene and REX-1, and genes that exhibit increased expression between 24 and 48 h after RA treatment such as laminin B1 and collagen type IV ($\alpha 1$).

RNA was isolated from F9-Wt and F9-Wt-S-neo-1 control lines, F9-Wt-S-CRABP-I-11 and F9-Wt-S-CRABP-I-15 sense transfectants, and F9-Wt-AS-CRABP-I-18 and F9-Wt-AS-CRABP-I-27 anti-sense transfectants at 12-h intervals through 96 h after the addition of RACT ([RA] = 1×10^{-6} M) and 100 μM zinc chloride. The ERA-1/Hox 1.6 mRNA levels in the CRABP-I sense anti-sense transfectants were similar to those in identically treated F9-Wt and F9-Wt-S-neo-1 cells (Fig. 4). In marked contrast, RAR β mRNA levels were not detectably induced in response to RACT in the F9-Wt-S-CRABP-I-11 and F9-Wt-S-CRABP-I-15 transfectants (Fig. 4), whereas the RAR β mRNA levels increased 20-fold at 36 h in the F9-Wt and F9-Wt-S-neo-1, as observed previously. In the CRABP-I anti-sense transfectants, RAR β mRNA ex-

pression was not altered relative to identically treated F9-Wt and F9-Wt-S-neo-1 cells (Fig. 4).

The steady state levels of both REX-1 and *c-myc* mRNAs in F9-Wt and F9-Wt-S-neo-1 cells decreased between 0 and 12 h after RACT treatment. In the CRABP-I sense as well as in the CRABP-I anti-sense transfectants, a similar decline in REX-1 and *c-myc* mRNA levels was observed in the presence of 1×10^{-6} M RA plus CT (data not shown).

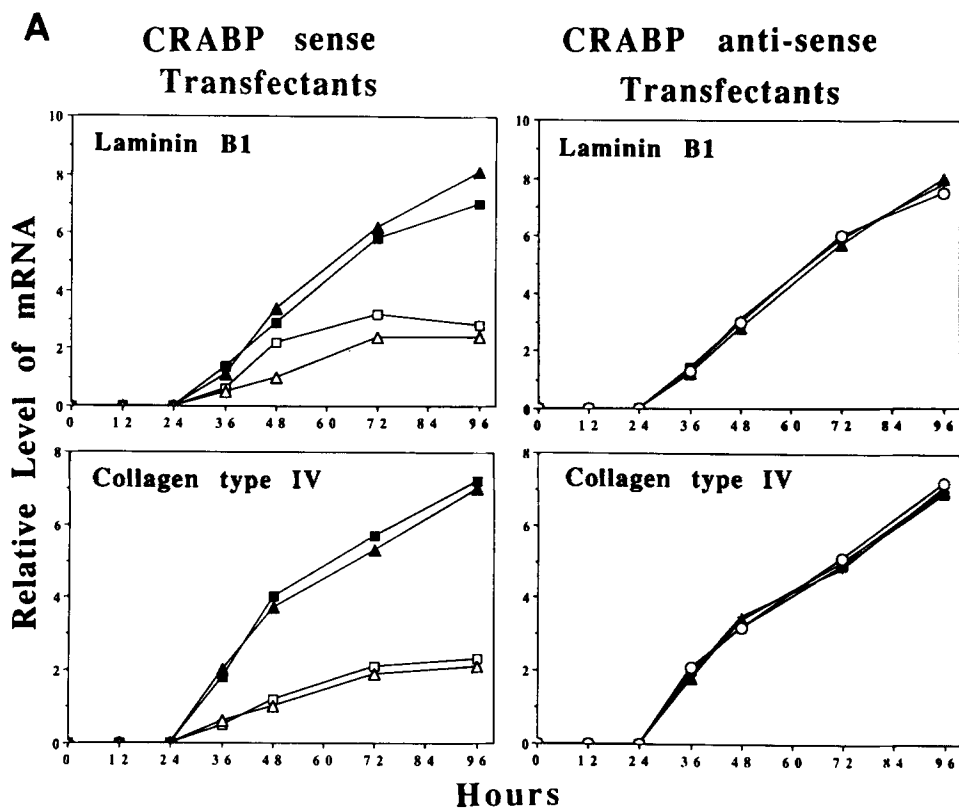
The steady-state levels of laminin B1 and collagen type IV ($\alpha 1$) mRNAs increased ~ 60 -fold in F9-Wt and F9-Wt-S-neo-1 cells by 96 h after RACT addition (Fig. 5, A and B). In contrast, the expression of laminin B1 and collagen type IV ($\alpha 1$) mRNAs in the F9-Wt-S-CRABP-I-11 and F9-Wt-S-CRABP-I-15 transfectants increased <15-fold by 96 h after RACT addition. In the CRABP-I anti-sense lines F9-Wt-AS-CRABP-I-18 and F9-Wt-AS-CRABP-I-27, laminin B1, and collagen type IV ($\alpha 1$) steady-state mRNA levels increased ~ 60 -fold in response to RACT, an increase of a magnitude similar to that in RACT treated F9-Wt and F9-Wt-S-neo-1 cells.

In summary, transfected F9-Wt cell lines that display elevated levels of CRABP-I sense transcripts and protein exhibited marked differences in their levels of expression of specific differentiation associated genes as compared to identically treated untransfected F9-Wt cells or F9-Wt-S-neo-1 cells. One rapidly RA induced gene, RAR β , was expressed at a greatly reduced level in both the F9-Wt-S-CRABP-I-11 and F9-Wt-S-CRABP-I-15 cell lines while the mRNA levels of another rapidly induced gene, Era-1/Hox 1.6, were only marginally affected by the elevation in CRABP-I. In addition, two genes expressed at later times after RACT addition, laminin B1 and collagen type IV ($\alpha 1$), were not expressed at levels as high as those observed in the RACT treated F9-Wt and F9-Wt-S-neo-1 cells. The decrease in functional CRABP-I protein in the CRABP-I anti-sense transfectants did not affect the RACT (1×10^{-6} M RA) induced expression of the differentiation-specific genes examined in these studies.

Effects of Different Concentrations of RA on the Expression of Differentiation-specific Genes in CRABP-I Sense and CRABP-I Anti-Sense Transfectants

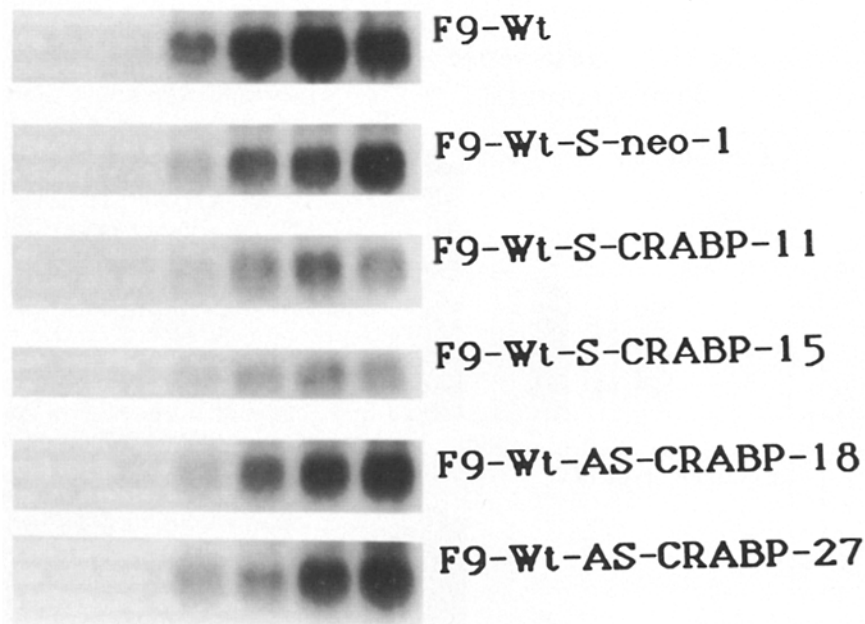
The above results support the hypothesis that CRABP-I functions, at least in part, to sequester intracellular RA. In this capacity CRABP-I may act to regulate the concentration of cytoplasmic RA and/or regulate the delivery of RA to the nuclear RA receptors. If this is indeed the case, it should be possible to reduce or eliminate the effect of overexpression of CRABP-I by increasing the concentration of RA used to initiate differentiation. To test this possibility, cells were harvested at 36 or 72 h after the addition of varying concentrations of RA (plus CT) and the expression of the differentiation-specific genes was monitored by Northern analysis.

After addition of 1×10^{-8} M RA or 1×10^{-6} M RA for 36 h, the magnitude of the RAR β mRNA induction was reduced in the two CRABP-I sense transfectants relative to the F9-Wt and F9-Wt-S-neo-1 lines (Fig. 6, A and B). However, after addition of 1×10^{-4} M RA, the levels of RAR β mRNA were similar for the four cell lines, F9-Wt, F9-Wt-S-neo-1, F9-Wt-S-CRABP-I-11, and F9-Wt-S-CRABP-I-15.



B Laminin B1

0 12 24 36 48 72 96



At all RA concentrations examined at the 36-h time point, the levels of Era-1/Hox 1.6 mRNA were similar in the F9-Wt, F9-Wt-S-neo-1, and the two CRABP-I sense transfectants F9-Wt-S-CRABP-I-11 and F9-Wt-S-CRABP-I-15, and these levels increased with increasing RA concentrations (Fig. 6 A).

The RA dose-dependent expression of Era-1/Hox 1.6 and RAR β mRNAs in the two CRABP-I anti-sense transfectants was similar to that in identically treated F9-Wt and F9-Wt-S-neo-1 control cells (Fig. 6 A).

The steady-state levels of REX-1 mRNA in the absence of

Figure 5. Northern blot analysis of late RA induced genes. (A) Northern blot analysis was carried out as described for Fig. 4. The random primer radiolabeled probes used in the hybridizations were laminin B1 and collagen IV ($\alpha 1$) cDNAs (1×10^6 cpm/ml and 1×10^6 cpm/ml, respectively). Densitometry results are expressed in relative RNA units. (A) Symbols: (■) F9-Wt; (▲) F9-Wt-S-neo-1; (□) F9-Wt-S-CRABP-I-11; (△) F9-Wt-S-CRABP-I-15; (×) F9-Wt-AS-CRABP-I-18; (○) F9-Wt-AS-CRABP-I-27. This experiment was performed three times; an autoradiogram of the lamin B1 Northern blot is also shown (B).

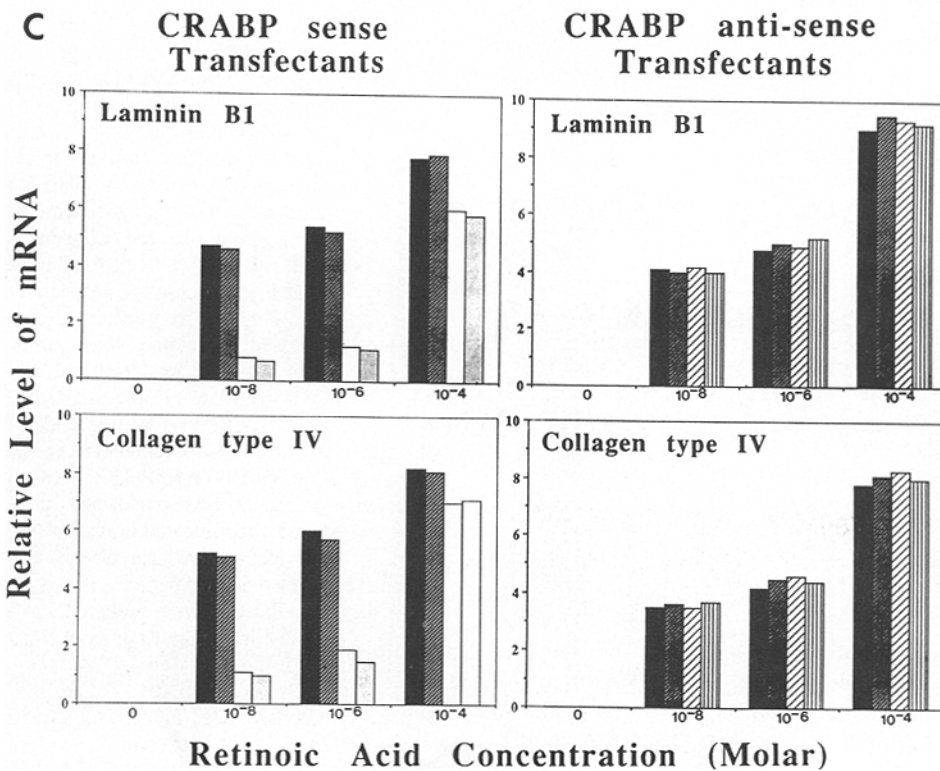
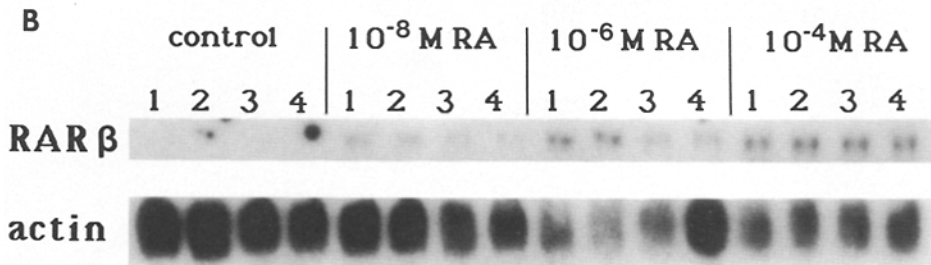
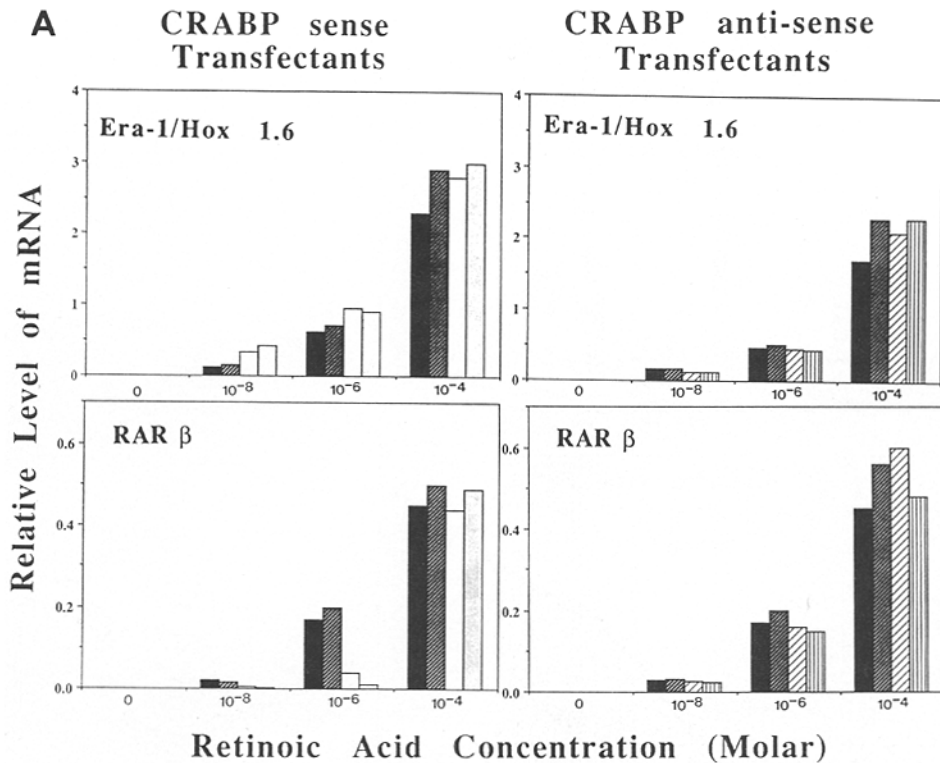


Figure 6. The expression of early and late RA induced genes at varying RA concentrations. (*A* and *B*) Total cellular RNA (35 μ g) was isolated at 36 h after the addition of 1×10^{-4} M RA, 1×10^{-6} M RA, or 1×10^{-8} M RA, plus 250 μ M dibutyl cAMP, 500 μ M theophylline, and 100 μ M zinc chloride. The Northern blot was prepared as described in the Materials and Methods. The hybridization probes were radiolabeled ERA-1/Hox 1.6 and RAR β cDNAs (1×10^6 and 2×10^6 cpm/ml, respectively). Densitometry results are expressed in relative RNA units after normalization to the actin mRNA in each sample. (*B*) An autoradiogram of some of the RNA samples hybridized to the RAR β cDNA is also shown. This blot was subsequently stripped and reprobed with actin (1×10^6 cpm/ml) to quantitate the relative levels of RNA in each lane. F9-Wt (lane 1), F9-Wt-S-neo-1 (lane 2), F9-Wt-S-CRABP-I-11 (lane 3), F9-Wt-S-CRABP-I-15 (lane 4). (*C*) Total cellular RNA (35 μ g) was isolated at 72 h after the addition of 1×10^{-4} M RA, 1×10^{-6} M RA, or 1×10^{-8} M RA, plus 250 μ M dibutyl cyclic AMP, 500 μ M theophylline, and 100 μ M zinc chloride. Northern blot analysis was performed as described in Materials and Methods. The hybridization probes were radiolabeled laminin B1 and collagen IV (α 1) cDNAs (1×10^6 cpm/ml for both). Densitometry values are expressed in relative RNA units after normalization to the actin mRNA in each sample. Symbols for *A* and *C*: (■) F9-Wt; (▨) F9-Wt-S-neo-1; (□) F9-Wt-S-CRABP-I-11; (▩) F9-Wt-S-CRABP-I-15; (⊗) F9-Wt-AS-CRABP-I-18; (▧) F9-Wt-AS-CRABP-I-27. This experiment was performed twice.

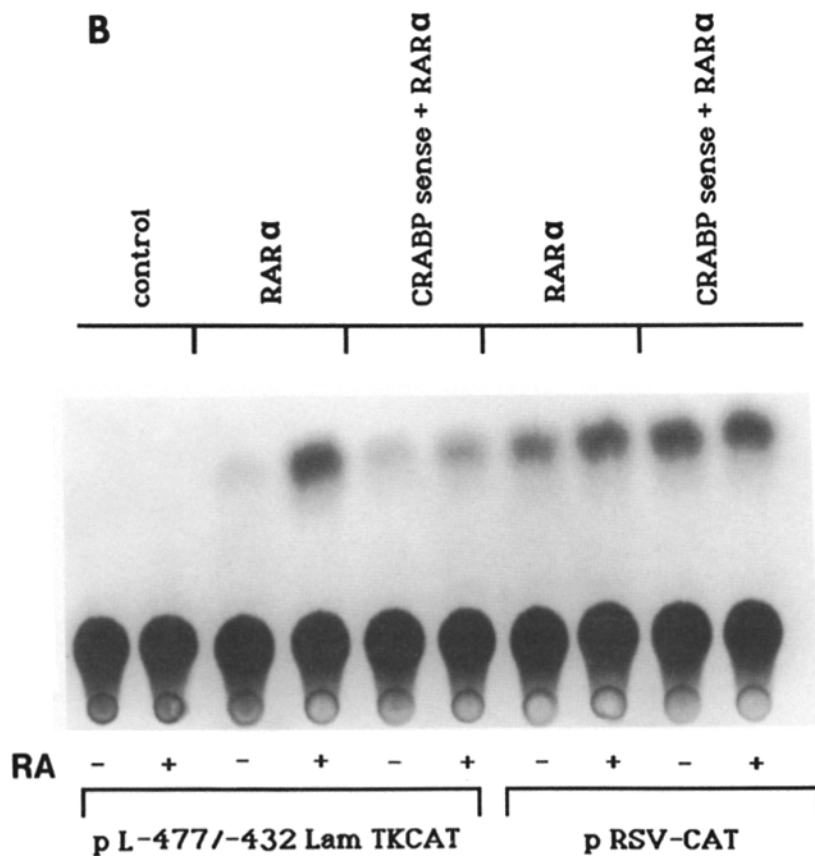
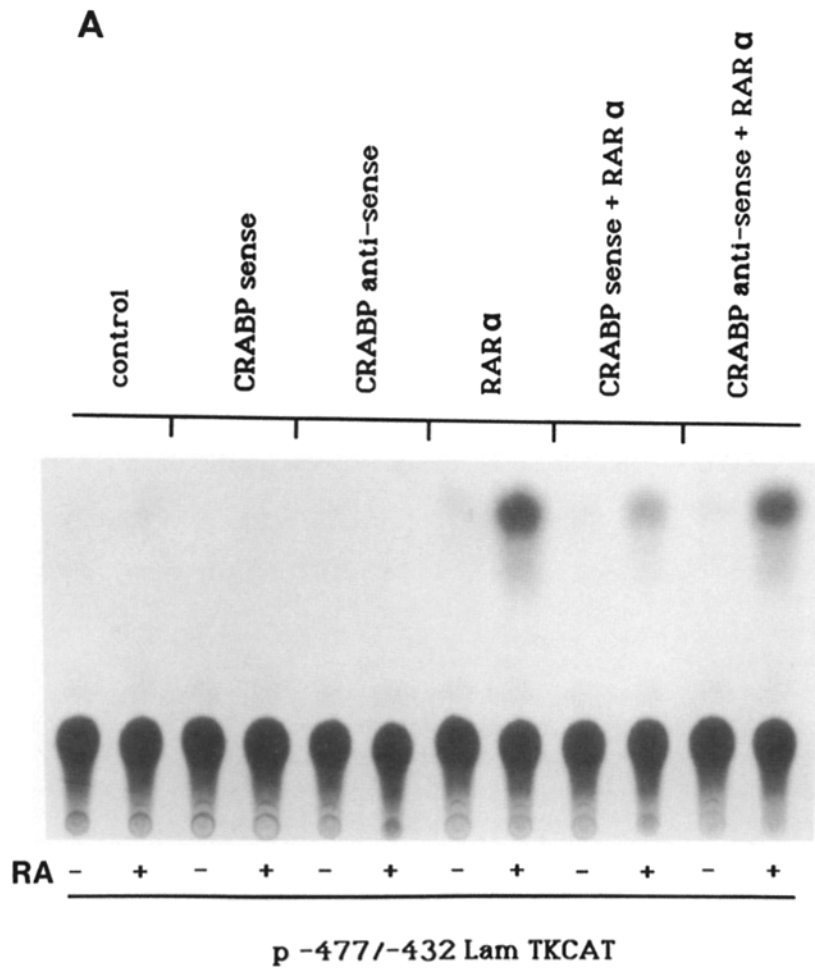


Figure 7. Effect of CRABP-I sense and CRABP-I anti-sense expression on the activation of the laminin B1 RA response element by RAR α in F9-Wt cells. (*A* and *B*) F9-Wt stem cells were cotransfected with the indicated plasmids (see Materials and Methods) and treated with 100 μ M zinc chloride with or without 1×10^{-6} M RA for 48 h. Extracts were prepared and a volume of extract containing 20 units of β -galactosidase activity was assayed for CAT activity (see Materials and Methods). This experiment was performed three times with similar results; one experiment is shown.

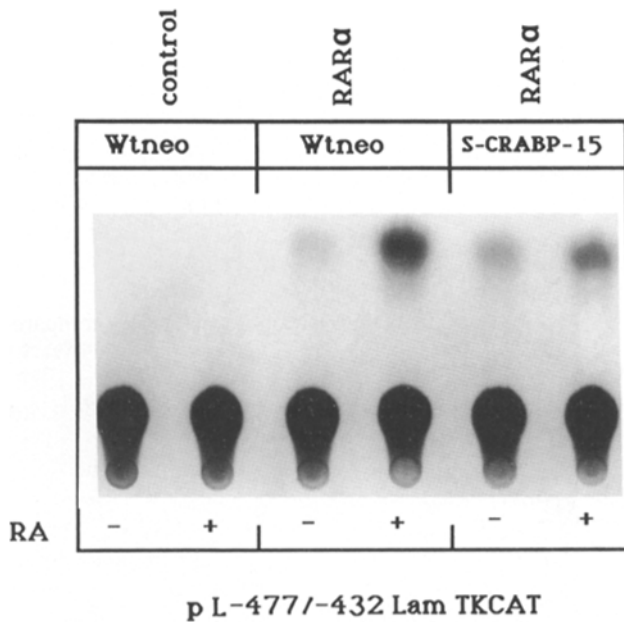


Figure 8. The activation of the laminin B1 RARE in the F9-Wt and F9-Wt-S-CRABP-I-15 lines. F9-Wt and F9-Wt-S-CRABP-I-15 stem cells were cotransfected with the indicated plasmids and treated with 100 μ M zinc chloride with or without 1×10^{-6} M RA for 48 h. Extracts were prepared and CAT assays performed as described in Materials and Methods. This experiment was performed twice with similar results; one assay is shown.

drugs were elevated by two- to threefold in the CRABP-I sense transfectants relative to the F9-Wt and the F9-Wt-S-neo-1 control lines at 72 h (data not shown). This suggests that small amounts of RA derived from the calf serum may continually reduce the REX-1 steady-state mRNA level in

the F9-Wt stem cells and that this does not occur to as great an extent in the two sense transfectants. The concentration of RA taken up by the cells from the serum is apparently not high enough to cause cell differentiation. In the presence of varying RA concentrations (plus CT), the REX-1 mRNA levels were also two- to threefold higher in the sense transfectants than in the F9-Wt control line at 72 h (data not shown). The reduction of REX-1 mRNA in response to RA in the CRABP-I anti-sense transfectants was similar to that in identically treated F9-Wt and F9-Wt-S-neo-1 cells lines at doses of RA between 10^{-8} and 10^{-4} M (data not shown). Additionally, the levels of *c-myc* mRNA declined after RACT addition to a similar extent in both F9-Wt and the CRABP sense and anti-sense transfectants (data not shown).

At both 1×10^{-8} M RA plus CT and 1×10^{-6} M RA plus CT, laminin B1 and collagen type IV (α 1) mRNA levels were significantly lower in the F9-Wt-S-CRABP-I-11 and F9-Wt-S-CRABP-I-15 lines than in the identically treated F9-Wt and F9-Wt-S-neo-1 lines. In striking contrast, at 1×10^{-4} M RA plus CT, the levels of collagen type IV (α 1) and laminin B1 mRNAs in the two CRABP-I sense transfectants were very similar to the identically treated F9-Wt and F9-Wt-S-neo-1 cells (Fig. 6 C). Thus, the inhibition of differentiation associated with CRABP-I overexpression can be reversed at high exogenous RA concentrations. The RA dose-dependent expression of laminin B1 and collagen type IV (α 1) mRNAs in the two CRABP-I anti-sense transfectants was similar to that in identically treated F9-Wt and F9-Wt-S-neo-1 control cells (Fig. 6 C).

Effect of CRABP-I Overexpression on the Activation of the Laminin B1 Gene

The next question we addressed was whether the reduction in the RA-associated laminin B1 mRNA expression in the

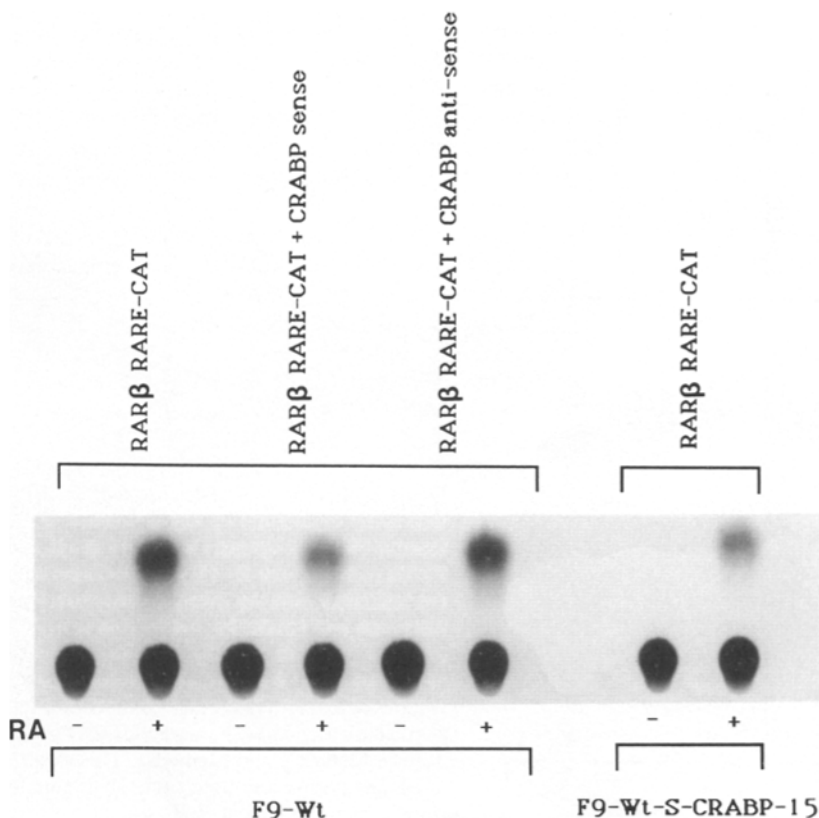


Figure 9. Effect of CRABP-I and CRABP-I anti-sense expression on the activation of the RAR β RA responsive element. F9-Wt and F9-Wt-S-CRABP-I-15 stem cells were cotransfected with the indicated plasmids and treated with 100 μ M zinc chloride with or without 1×10^{-6} M RA for 48 h. Extracts were prepared and CAT assays performed as described in Materials and Methods. This experiment was performed twice with similar results; one assay is shown.

presence of elevated CRABP-I level was due to decreased transcriptional activation of the laminin B1 gene. The pL-477/-432 LamTKCAT construct contains the laminin B1 5' flanking 46 bp RARE and has been shown to activate CAT expression in the presence of RA when cotransfected with RAR α (Vasios et al., 1989). F9-Wt cells were transfected with either the pMT-CRABP-I sense or pMT-CRABP-I anti-sense construct, pSG5-RAR α , and the pL-477/-432 LamTKCAT reporter construct, followed by treatment with 100 μ M zinc chloride with or without 1×10^{-6} M RA for 24 or 48 h. The presence of the pMT-CRABP-I sense construct resulted in a two- to threefold reduction in CAT activity at 24 h (data not shown) and a five- to sixfold reduction in CAT activity at 48 h when compared to the CAT activity in extracts from cells transfected with pSG5-RAR α and pL-477/-432 LamTKCAT without the pMT-CRABP-I vector (Fig. 7 A). Thus, transactivation of the laminin B1 RARE by RAR α is reduced in the presence of high CRABP-I levels.

Transfection of the pMT-CRABP-I anti-sense construct with pSG5-RAR α and pL-477/-432 LamTKCAT did not result in any reduction in CAT activity when compared with the CAT activity in extracts from cells transfected only with pSG5-RAR α and pL-477/-432 LamTKCAT when 1×10^{-6} M RA was used (Fig. 7 A). Thus, the reduction in LamTKCAT expression observed when the pMT-CRABP-I sense construct is cotransfected is not the result of the titration of transcription factors by the metallothionein I promoter. Transfection of the pMT-CRABP-I vector plus the pL-477/-432 LamTKCAT vector without the pSG5-RAR α resulted in no increase in CAT activity (Fig. 7 A), demonstrating that the CRABP-I protein itself does not functionally activate transcription through the laminin B1 RARE. As control for any potential nonspecific effects of CRABP-I expression in the transient transfection assays, a RA-unresponsive promoter, RSV, was employed. Transient transfection of the pMT-CRABP-I sense construct, pSG5-RAR α , and the RSV-CAT expression vector followed by treatment with or without 1×10^{-6} M RA did not result in any change in CAT activity as compared to a similar transfection without the pMT-CRABP-I construct (Fig. 7 B).

The transient transfection assays described above all involve introduction of expression vectors into the F9-Wt cells. The next series of experiments were performed to compare the activation of the laminin B1 RARE by RAR $\alpha \pm$ RA in the untransfected F9-Wt control cells with the activation of this RARE in the F9-Wt-S-CRABP-I-15 line. Both cell lines were transfected with pL-477/-432 LamTKCAT and pSG5-RAR α , and the lines were cultured for 48 h in the presence of 1×10^{-6} M RA and 100 μ M zinc chloride. Extracts were then prepared and CAT assays were performed. The CAT activity from the F9-Wt-S-CRABP-I-15 extracts was increased 1.9-fold by RA, whereas in the F9-Wt extract the CAT activity was increased 8.3-fold by RA (Fig. 8). These results are consistent with those reported in Fig. 7, and demonstrate that stable overexpression of the CRABP-I also results in decreased activation of the laminin B1 RARE.

Effect of CRABP-I Overexpression on the Activation of the RAR β RARE

A RA-responsive element (RARE) was recently found in the RAR β 5' flanking DNA (deThé et al., 1990). This RARE differs from the laminin B1 RARE in that the RAR β RARE

can be activated by RA in F9 cells without cotransfection of the RARs (Fig. 9). Experiments similar to those reported in Figs. 7 and 8 were performed, but with the RAR β RARE. In the first series of experiments, the F9-Wt stem cells were transfected with the RAR β RARE/TKCAT vector \pm the pMT-CRABP-I vector. The F9-Wt cells were then cultured with or without 1×10^{-6} M RA plus 100 μ M zinc chloride for 48 h. The introduction of CRABP-I cDNA into F9-Wt cells by transient transfection resulted in a threefold reduction in CAT activity (Fig. 9).

In a second series of experiments, designed to compare the responses of the F9-Wt cells with those of the F9-Wt-S-CRABP-I-15 line, both lines were transfected with the RAR β RARE/TKCAT construct and then cultured with or without 1×10^{-6} M RA plus 100 μ M zinc chloride 48 h. Again, 3.5-fold less CAT activity was observed in the presence of higher levels of CRABP-I when the high levels resulted from stable integration of the exogenous CRABP-I cDNA into the chromosomal DNA of the F9-Wt-S-CRABP-I-15 line (Fig. 9).

Effects of a Reduction in CRABP-I Expression on the Activation of the Laminin B1 Gene and the RAR β RARE

Overexpression of the CRABP-I protein leads to a reduction in the differentiation of F9 cells in response to exogenous RA concentrations ranging from 1×10^{-8} to 1×10^{-6} M (Figs. 4-9). At these concentrations of RA, the CRABP-I anti-sense transfected lines exhibit no detectable differences from F9-Wt in the magnitude of their expression of differentiation-specific genes (Figs. 4; 5, A and B; 6, A and C; 7 A; 9). However, as the CRABP-I anti-sense transfectants express much less CRABP-I protein than F9-Wt (Fig. 2), we reasoned that the antisense transfectants might be more responsive to RA than F9-Wt at very low external RA concentrations. Under these conditions, we predict that most or all of the intracellular RA would be sequestered by the CRABP-I in F9-Wt cells, whereas in the CRABP-I anti-sense transfectants, some RA would still be available for interaction with the nuclear RA receptors leading to the activation of differentiation specific genes.

Therefore, to compare the responses of the F9-Wt-S-neo-1 control line and the F9-Wt-AS-CRABP-I-27 line to lower external RA concentrations, both lines were transfected with pL-477/-432 LamTKCAT and pSG5-RAR α . One of the CRABP-I sense transfectants, F9-Wt-S-CRABP-I-15, was also included in these experiments for comparison. These lines were subsequently cultured for 48 h in the presence of varying exogenous RA concentrations from 1×10^{-10} M to 1×10^{-7} M and 100 μ M zinc chloride. Extracts were prepared and CAT assays were then performed. The CAT activities of the F9-Wt-AS-CRABP-I-27 extracts were 6.5- and 3.8-fold higher than those of the F9-Wt-S-neo-1 line at 1×10^{-10} M and 1×10^{-9} M RA, respectively (Fig. 10 A). At 1×10^{-8} M and 1×10^{-7} M RA, the CAT activities of the F9-Wt-S-neo-1 and F9-Wt-AS-CRABP-I-27 lines were approximately equivalent (Fig. 10 A). We conclude that the CRABP-I anti-sense transfectant is more responsive than the F9-Wt-S-neo-1 line to low external RA concentrations (1×10^{-10} , 1×10^{-9} M RA). By comparison, the CRABP-I sense transfectant exhibited less CAT activity than the F9-

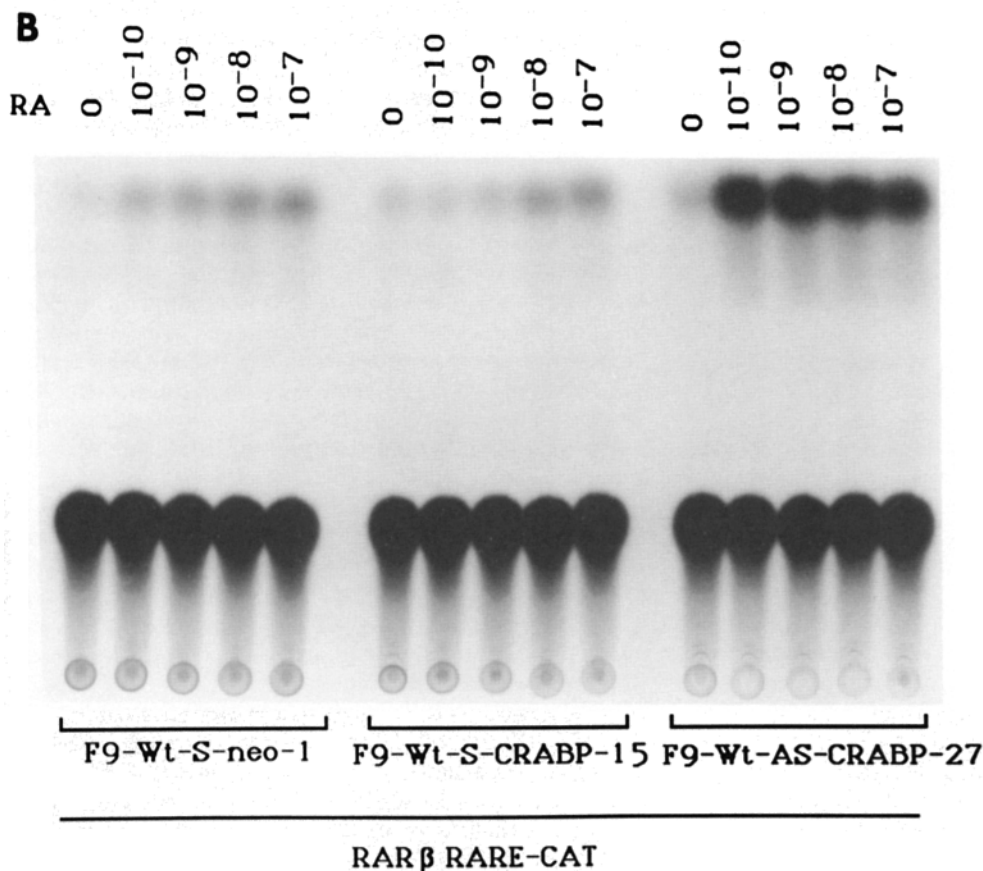
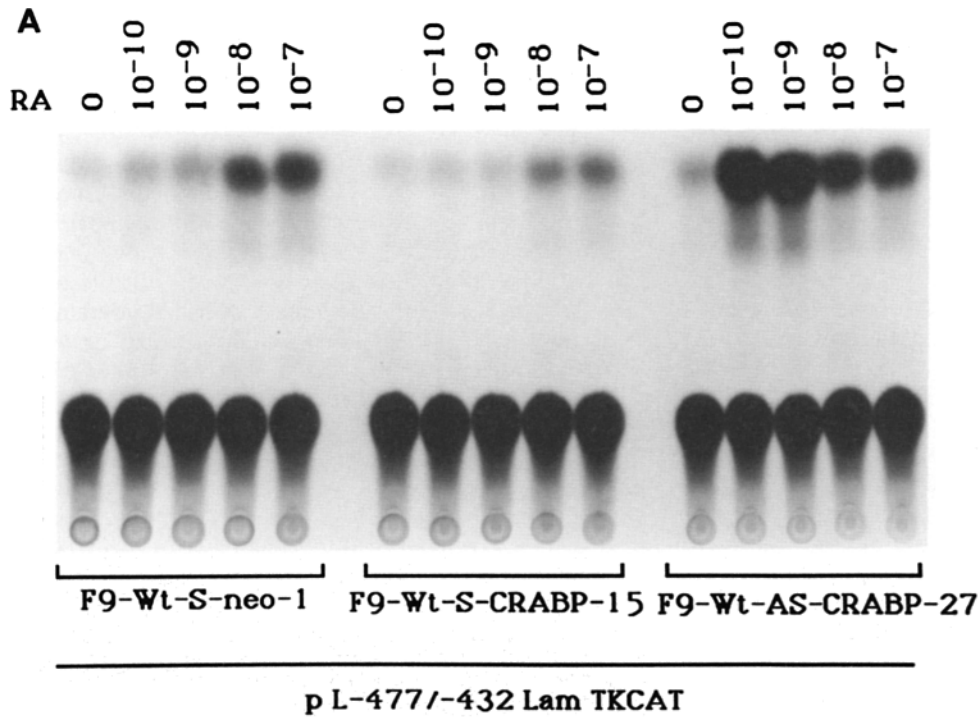


Figure 10. The activation of the laminin B1 RARE and RAR β RARE in the F9-Wt-S-neo-1, F9-Wt-S-CRABP-I-15, and F9-Wt-AS-CRABP-I-27 lines at varying RA concentrations. (A and B) F9-Wt, F9-Wt-S-CRABP-I-15, and F9-Wt-AS-CRABP-I-27 stem cells were cotransfected with the indicated plasmids and treated with 100 μ M zinc chloride with or without varying doses of RA (1×10^{-10} – 1×10^{-7} M) for 48 h. Extracts were prepared and CAT assays performed as described in Materials and Methods.

Wt-S-neo-1 line over the entire range of RA concentrations tested (Fig. 10 A).

In a second experiment, the same three cell lines were transfected with the RAR β RARE/TKCAT construct and then cultured in the presence of varying exogenous RA concentrations plus zinc chloride for 48 h. Again, 3.3- and 2.5-

fold higher CAT activities were seen in the F9-Wt-AS-CRABP-I-27 extracts than in the F9-Wt-S-neo-1 extracts at 1×10^{-10} M and 1×10^{-9} M RA, respectively (Fig. 10 B). Even at 1×10^{-8} and 1×10^{-7} M RA, the CRABP-I antisense transfectant exhibited CAT activities that were ~ 1.7 -fold greater than those of the F9-Wt-S-neo-1 line (Fig. 10 B).

In contrast, the CRABP-I sense transfectant exhibited less CAT activity than the F9-Wt-S-neo-1 line over the range of RA concentrations tested (Fig. 10 B).

In conclusion, the RA-dependent transcriptional activation of both the laminin B1 gene and the RAR β gene is inversely correlated with the level of CRABP-I protein in the cells (Figs. 7-10).

Discussion

There has been much speculation about the function of the CRABP-I protein, and about whether this protein is present in the nucleus. Some researchers have suggested that the CRABP-I facilitates RA action by transporting RA to the nucleus, whereas others have proposed that the CRABP-I sequesters RA in the cytoplasm and/or enhances RA metabolism to inactive derivatives. Such actions would decrease the number of RA molecules in the nucleus. The experiments reported here support this second hypothesis. The presence of additional functional CRABP-I protein in the F9-Wt-S-CRABP-I-11 and F9-Wt-S-CRABP-I-15 transfected lines results in lower induced levels of RAR β , laminin B1, and collagen IV (α 1) mRNAs (Figs. 4-6) and a smaller reduction in the level of REX-1 mRNA in response to RACT. Moreover, the presence of a CRABP-I expression vector reduces the CAT transcription driven by the murine laminin B1 RARE in a transient expression system (Fig. 7 and 8). Finally, the presence of a CRABP-I expression vector partially prevents the transcriptional activation of the CAT gene by the RAR β RARE in a transient expression assay (Fig. 9).

In contrast to the "early" gene RAR β , the Era-1/Hox 1.6 gene is not greatly affected by the overexpression of the CRABP-I gene (Figs. 4 and 6). Although we do not understand the reason for this difference, one possibility is that the activation of the Era-1/Hox 1.6 gene is more sensitive to RA and its metabolites or RA:RAR complexes. It will be important to fully define the RA responsive regions of these two genes in order to help explain this difference. That the Era-1/Hox 1.6 gene is appropriately expressed under conditions in which laminin B1 and collagen type IV (α 1) gene expression is inhibited (Figs. 5 and 6) suggests that Era-1/Hox 1.6 does not regulate the subsequent expression of laminin B1 or collagen type IV (α 1). Conversely, RAR β gene expression is reduced under conditions in which the expression of laminin B1 and collagen type IV (α 1) genes is reduced (Fig. 5 and 6), consistent with prior findings that RARs can transactivate the laminin B1 RARE (Vasios et al., 1989).

We had previously suggested that an appropriate level of functional CRABP-I protein was necessary, although not sufficient, for F9 teratocarcinoma stem cell differentiation in response to RA (Stoner et al., 1989; Wang et al., 1984). This suggestion was based on the isolation of a differentiation-defective F9 mutant line, RA 3-10, that exhibited less than 5% of the level of functional CRABP-I protein observed in F9-Wt cells (Wang et al., 1984). However, we have recently demonstrated that the differentiation defect in the RA 3-10 mutant is not corrected by transfection of the wild type CRABP-I cDNA (Boylan, J., and L. Gudas, unpublished). Thus, the RA 3-10 mutation may not lie within the CRABP-I gene itself. For instance, a negative regulatory protein may be overexpressed in the RA 3-10 mutant line, resulting in

both reduced expression of the CRABP-I gene and inhibition of the differentiation process.

The F9-Wt-S-CRABP-I-15 cytoplasmic extract binds 10-25-fold more RA/mg protein than the F9-Wt and F9-Wt-S-neo-I extracts (Figs. 2 and 3). If CRABP-I sequesters RA, how much would a 10-fold increase in the number of CRABP-I molecules/cell reduce the free RA internal concentration in F9 cells? The F9 differentiation model system is a complicated one, and the levels of internal RA, CRABP-I, and RAR β mRNA all change over time after the addition of RA to the culture medium. Detailed measurements of all of these parameters would be required to quantitatively demonstrate the importance of CRABP-I in modulating the levels of free RA under a variety of conditions. Nevertheless, if we assume that a higher free RA concentration within the cell results in more RA that is available for interaction with RARs in the nucleus, then an increase in the CRABP-I protein concentration within the cell should result in a decreased ability of the cell to differentiate in response to a given external RA concentration. This is generally what we observe. However, at very high external RA concentrations, most of the internal RA should be free in both the F9-Wt and F9-Wt-S-CRABP-I-15 lines. We do observe less inhibition of differentiation in the F9-Wt-S-CRABP-I-15 line compared with the F9-Wt line at the highest exogenous RA concentration tested, 1×10^{-4} M RA, consistent with this idea (Fig. 6). Conversely, at very low exogenous RA concentrations (i.e., 10^{-11} to 10^{-9} M), we predict that most or all of the intracellular RA would be bound to the CRABP-I protein in F9-Wt cells resulting in negligible or no activation of differentiation-specific genes. However, at these very low external RA concentrations the CRABP-I anti-sense transfectants should express differentiation markers to a greater extent than F9-Wt cells because the anti-sense transfectants have over 20-fold less CRABP-I protein than F9-Wt cells (Fig. 2). We do observe a markedly greater activation of the laminin B1 and RAR β genes in the CRABP-I anti-sense transfectants than in the F9-Wt cells (Fig. 10), but only at very low RA concentrations (1×10^{-10} , 1×10^{-9} M). Again, these results strongly support the general model that the response of the cell to RA varies inversely with the CRABP-I level.

CRABP-I mRNA and protein levels as well as RA levels have been assayed in sections of developing mouse and chick limbs (Maden et al., 1988, 1989; Thaller et al., 1987; Kwarta et al., 1985; Tickle et al., 1982). Dolle et al. (1989) reported that CRABP-I mRNA was expressed along a proximo-distal gradient with maximal expression in the most distal portion of the day 10 developing mouse limb. Dolle et al. (1989) also clearly showed that CRABP-I transcripts were excluded from regions of the day 14.5 developing mouse limb that express RAR β and RAR γ transcripts. This striking observation is consistent with the results reported here; CRABP-I expression may reduce a cell's ability to respond to RA, or even prevent a cell from responding to RA, depending on both the internal CRABP-I protein concentration and the external RA concentration to which the cell is exposed. Thus, in cells in the developing limb where RAR γ and RAR β are expressed and a differentiation response to RA is presumably desired, few CRABP-I transcripts are present. Conversely, in the more distal sections of the limb

bud, where CRABP-I transcripts are high, no RA-associated differentiation response may be desired.

We have provided evidence that the level of CRABP-I protein expression can profoundly influence the degree of differentiation in response to RA. We suggest that the CRABP-I protein likewise indirectly regulates gene expression in developing animals, either within the context of local RA concentration gradients or possibly via gradients of CRABP-I itself.

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