Characterization of a Negative Response DNA Element in the Upstream Region of the Cellular Retinoic Acid-binding Protein-I Gene of the Mouse*

(Received for publication, September 4, 1996, and in revised form, January 9, 1997)

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A negative, regulatory DNA element from the mouse cellular retinoic acid-binding protein I gene promoter was identified. This DNA element, located approximately 1 kilobase upstream from the transcription initiation site of this gene, contained a pair of direct repeats (DRs) separated by 4 base pairs (DR4, TGAC-GTTCGGGACCT). By examining a series of reporters deleted or mutated within this DR4 region, it was concluded that the core sequence of this DR4, including both repeats and the spacer, was required for suppressive activity in the mouse embryonal carcinoma cell line P19. From gel retardation experiments, it was concluded that both repeated sequences were essential for specific protein binding, but the spacer sequence was not as critical. Specific residues required for protein binding to this DR4 were identified. In P19 cells, retinoic acid induced the binding of nuclear factors to DR4 and suppressed the activities of the reporters containing this DR4. Co-expression of retinoic acid receptor β or thyroid hormone receptor β1 (T3Rβ1) significantly inhibited the expression of this reporter in P19 cells. Gel retardation with in vitro-synthesized nuclear receptors demonstrated specific binding of this DR4 by T3Rβ1 monomers, homodimers, or heterodimers of T3Rβ1/retinoid receptor X β. A biological function of DR4 in crabp-I gene regulation in P19 cells was suggested.

Retinoic acid (RA)1 exerts pleiotropic effects in animals, and the effects are mediated through various cellular components (for review, see Refs. 1–4). The nuclear receptors for RA, including retinoic acid receptors (RARs) and retinoid receptors X (RXRs), regulate gene expression by enhancing or suppressing the transcriptional machinery via binding to DNA sequences called RA response elements (RAREs). In the cytosol, two cellular retinoic acid-binding proteins (CRABPs) exist for specific binding to RA, designated as CRABP-I and CRABP-II. It is suggested that these cytosolic receptors for RA are involved in RA metabolism, thereby controlling the amount of RA mole-

cules available to RARs/RXRs (for review, see Refs. 3 and 4). In developing embryos, both CRABPs are highly expressed. In adult animals, CRABP-I is ubiquitously expressed at low levels but is highly expressed in RA-sensitive tissues such as the eye and the testis (5–8). In contrast, CRABP-II is specific to the skin (9, 10). Studies in cultured cells showed that CRABP-II expression was directly induced by RA via an RARE located in its promoter region, whereas the regulation of CRABP-I expression involved various signaling pathways such as protein kinases (11), DNA methylation (12), and RA (13). Based upon the promoter sequence, the mouse crabp-I gene was characterized as a housekeeping gene (14). However, the upstream region of this gene contained numerous inverted repeat sequences and putative binding sites for transcription factors, indicating a complex regulatory mechanism involved in its cell type- and developmental stage-specific expression (15). The bovine crabp-I gene was also characterized (16) and seemed to have the same genomic organization and a similar promoter sequence as the mouse gene, suggesting a highly conserved regulatory mechanism for the expression of this gene among animal species.

Because the crabp-I knockout mice displayed no apparent phenotypes (17–19), the function of CRABP-I in animals remained unclear. However, it was demonstrated in transgenic mice (20) and embryonal carcinoma cells (21) that elevated CRABP-I expression was associated with abnormal cellular differentiation and RA-responsive gene expression. It was also shown in embryonic palate cells that the expression of RARβ1, transforming growth factor β, and tenasin was altered as a result of introducing anti-crabp-I oligonucleotides into the cultures (22). In addition, recent biochemical studies provided more evidence for a role of crabp-I in RA catabolism (23, 24). It is possible that an abnormally high level of crabp-I expression may disturb RA concentration, thereby affecting gene expression in specific cells at a critical time. Therefore, it is suggested that the level of crabp-I expression must be tightly controlled for certain cell types.

The study of the mouse crabp-I genomic structure revealed several interesting features within 3 kb in the upstream region of the promoter, such as a GC content of greater than 70%, 9 pairs of inverted repeats, 5 copies of GC boxes (Sp-1 sites, GGCGGG), and several potential binding sites for transcription factors, including putative hormone response elements (HREs) and consensus sequences for AP-1 and AP-2 binding (14, 15). The biological activity of this 3-kb upstream sequence was demonstrated in transgenic mouse embryos and cell cultures using an Escherichia coli β-galactosidase (lacZ) reporter (15). By examining a series of lacZ reporters deleted in various portions of the upstream sequence, the minimal promoter and cell type-specific regulatory regions were determined. From studies of transgenic mouse embryos, it was concluded that the information for developmental stage-specific regulation was
encoded within this 3-kb upstream region where both positive and negative DNA elements were located. Most notably, a DNA fragment conferring a strongly suppressive activity for this promoter was located approximately 1000–1200 bp upstream from the transcription initiation site. This 200-bp DNA segment also functioned in heterologous promoters and was able to interact specifically with proteins of nuclear extracts isolated from P19 mouse embryonal carcinoma cells in which this gene expression was regulated by RA. Within this region, a putative HRE was identified, which contained two pairs of direct repeats (DRs), each separated by 5 bp (DR5) and 4 bp (DR4), respectively. To determine if this putative response element is a functional negative response element and to better understand the regulatory mechanism for crabp-I gene expression, we conducted experiments aimed at characterizing this negative HRE in terms of its biological activities in cultured cells and its protein-binding properties. We now report the studies of detailed analysis of this negative HRE sequence demonstrated in both gel retardation and transfection experiments.

EXPERIMENTAL PROCEDURES

Techniques for Cell Cultures—P19 cells were maintained in α-minimum Eagle's medium supplemented with 2.5% fetal calf serum and 7.5% calf serum as described (15).

LacZ Reporter Constructs—The reporter deletion constructs were made by mutating the original CRABP-lacZ reporter (15) with either restriction enzyme-generated or polymerase chain reaction-generated DNA fragments to prepare the constructs. The SLA of each reporter was determined as described in the text.

FIG. 1. A strongly suppressive activity was located within a 54-bp sequence of the mouse crabp-I gene promoter region. A, the parental reporter, CRABP-lacZ, was truncated at various 5′ upstream positions, resulting in −1180, −1110, −1103, −1046, and −993 constructs, by using either restriction enzyme-generated or polymerase chain reaction-generated DNA fragments to prepare the constructs. B, the SLA of each construct was compared with the SLA of the reference construct, −993, and represented as a RLA. The RLA of the −993 reporter was arbitrarily set at 100% in each experiment.

Cell Transformation and the Quantitation of LacZ Reporter Activities—To determine the biological activities of mutants truncated at various portions of the DNA segment containing this HRE, P19 cells were plated in 24-well plates (5 × 10⁴ cells/well) and transfected with each truncated reporter DNA using the calcium phosphate precipitation method. Protein concentrations were determined using a Bio-Rad protein assay kit. The specific lacZ activity (SLA) was determined between 24 and 40 h, using orthonitrophenyl-β-D-galactopyranoside (Sigma) as substrate, as described previously (15). For oligonucleotide-generated point mutations, the reporter activity was shown as relative lacZ activity (RLA) by comparing its SLA to the SLA of the parental construct. To determine the effects of nuclear receptor expression on the putative HRE activity in P19, cells were co-transfected with the lacZ reporter and one of the nuclear receptor expression vectors. The expression vectors for the nuclear receptors were made by inserting each corresponding cDNA into a cytomegalovirus expression vector (25). The RLA of each co-transfection experiment was determined by comparing its SLA to the SLA of the control, where the RLA was arbitrarily set at 100%. For all of the assays, triplicate cultures were used in each experiment, and three to five independent experiments were conducted to obtain the means and standard errors of the mean (S.E.).

Gel Retardation Assay—Gel retardation experiments using nuclear extracts of cultured cells were as described previously (11, 15). Nuclear extracts from P19 cells were prepared using the method of Standke et al. (26). Gel retardation experiments using in vitro-translated proteins were conducted according to an established protocol (27). Each RAR and RXR cDNA was inserted into pGem2 (Promega, Madison, WI) for in vitro transcription and translation by using the TNT T7-coupled reticu-

FIG. 2. The suppressive activity of the HRE was located at a 20-bp fragment. A, within the 54-bp region, a pair of DR5 overlapped with a pair of DR4. The negative activity was determined by further deletions in the 54-bp sequence, resulting in −1046, −1027, −1020, −1012, and −993 constructs. B, the SLA of each construct was compared with the SLA of the reference construct, −993, and represented as a RLA. The RLA of the −993 reporter was arbitrarily set at 100% in each experiment.
loctye lytate system (Promega). A yeast expression vector and an antibody for TRP2 were kindly provided by Dr. H. Towle (Department of Biochemistry, University of Minnesota) (25). The protein extract was incubated with 0.4 ng of probe (5 × 10^3 cpm) in 50 μl of reaction buffer (20 mM HEPES, pH 7.6, 0.1% Nonidet P-40, 50 mM KCl, 1 mM β-mercaptoethanol, 2 μg of poly(dI-dC), and 20% glycerol) at room temperature for 30 min. The reaction mixture was then subjected to polyacrylamide gel electrophoresis as described (15). For supershift experiments, the TRP2-specific antibody was added to probe-DNA complex and incubated for another 30 min at room temperature. For competition experiments, DR4 and a palindromic thyroid hormone response element (TRE) (Ref. 29; 5'-TCGAGATCTCGAGCTACTGACCTGAGATC-3') was used.

RESULTS

A Strongly Suppressive Activity of the Mouse crabp-I Gene Upstream Region—Previous studies showed that an approximately 200-bp DNA fragment (nucleotide position -1180 to -993, according to our previous numbering system described in Ref. 15) of the mouse crabp-I gene promoter exerted a suppressive activity when it was fused to either the crabp-I minimal promoter or other heterologous promoters in P19 cells (15). To determine the exact DNA sequence required for the negative activity, serial deletion mutants were made, designated as -1180, -1110, -1103, -1046, and -993 (Fig. 1A). The SLAs of these five truncated mutants and the parental reporter were determined in P19 cells as shown in Fig. 1B. Deletions -1180, -1110, -1103, and -1046 retained the negative activity, but a further deletion to -993 position lost the suppressive activity. Therefore, the sequence between -1046 and -993 encoded the suppressive activity, which contained two overlapping DRs, one of the DR5 type (CCATGAAGGAAAAGTGA) and the other of the DR4 type (TGACCTTTGGGACCT).

To determine which of the two DRs was responsible for this negative regulatory activity, deletions truncated at various portions of this 54-bp sequence were made, designated as -1027, -1020, and -1012 as shown in Fig. 2A. The -1027 retained the complete DR5 and DR4, the -1020 was deleted in the left-side repeat of DR5, and the -1012 retained only the intact DR4. Fig. 2B shows the RLA of each deletion mutant as compared with the activity of the reference reporter, -993. It seemed that the requirement for the suppressive activity was the addition of the DR4 sequence to the reference reporter -993, as shown by the fully suppressed activity of the -1012 construct. Thus, the DNA sequence containing the suppressive activity was located between -1012 and -993, which contained the DR4 sequence.

To examine if this DNA fragment could interact with specific nuclear factors from P19, gel retardation was conducted by first using the 54-bp fragment as the probe, as shown in Fig. 3A. As expected, this fragment was specifically retarded by P19 nuclear extracts (lane 1), which could be competed out specifically by either the 54-bp fragment (lanes 2–4) or a larger fragment covering the 54-bp sequence (lanes 5–7), but not by an adjacent DNA fragment (lanes 8–10). To further define the sequence responsible for protein binding to this 54-bp region, various sequences from this 54-bp region were used as the competitors as shown in Fig. 3B. The retarded band could be competed out by the unlabeled 54-bp fragment (lanes 2 and 3), a fragment containing the DR4 sequence (lanes 8 and 9), and a RARE derived from the RARβ promoter (lanes 10 and 11); sequence reported in Ref. 25), but not by the fragments containing a half site of the DR5 (lanes 4 and 5) or the entire DR5 sequence (lanes 6 and 7). Thus, in consistence with the transfection results (Fig. 2), protein factors binding to this 54-bp fragment were specific to the DR4 site but not the DR5 site. In addition, these protein factors were able to interact with the RARE derived from the RARβ promoter. It was concluded that the DR4 of the crabp-I promoter, between -1012 and -993, functioned as a negative DNA element in P19 cells and shared some common protein factors with a typical RARE of the DR5 type.

Characterization of the DR4 Negative DNA Element—To further characterize this new negative DNA element, mutations at specific residues of this DR4 site were made by polymerase chain reaction mutagenesis, and their biological activities and protein-binding properties were determined in transfection and gel retardation experiments, respectively. The first series of mutants were mutated, three bases at a time, from the wild-type fragment (labeled with HRE) and designated as m1, m2, m3 and m4, respectively (Fig. 4A). These mutant DNA fragments were first tested for competition with the wild-type DNA fragment in gel retardation experiments as shown in Fig. 4B.
Negative Response DNA Element in the crabp-I Gene

The biological activities of trinucleotide mutants of the DR4 sequence. A, three nucleotides (underlined) were mutated in each of the mutant oligonucleotides, designated m1, m2, m3, and m4. Dashed lines indicated the wild-type sequences. B, gel retardation experiments by using end-labeled 54-bp fragment as probes. The unlabeled fragment used in each competition experiment was the 54-bp fragment (lanes 2 and 3), the m1 mutant (lanes 4 and 5), the m2 mutant (lanes 6 and 7), the m3 mutant (lanes 8 and 9), the m4 mutant (lanes 10 and 11), and the RARE of RAR, promoter (lane 12). The molecular excess of the competitor was indicated above each lane. Lane 0 showed the probes, indicated by an arrow on the left, and lane 1 showed a control reaction without competitors. An arrow on the right indicated the position of the specifically retarded band. C, the SLA of each mutant reporter was compared with the SLA of the wild-type reporter to obtain an RLA. The RLA of the wild-type reporter was arbitrarily set at 100%.

![Graph showing RLA values](image)

To determine the effects of these mutations on the biological activity of this DR4, trinucleotide mutant reporters were made by replacing the wild-type DR4 sequence in the −1020 construct with each mutant sequence and were tested in transfection experiments as shown in Fig. 4C. In consistency with the gel retardation results, the m1 mutation did not affect the suppressive activity. Interestingly, the suppressive activity was partially affected in the m2 and m4 mutants but was completely abolished in the m3 mutant. Thus, it was concluded that the 5′-flanking sequence of DR4 was not required for either the biological activity or protein binding of this DR4, whereas the DR4 core sequence, covering the regions of m2, m3, and m4, was essential for its biological activity, although mutation in the m4 sequence had no significant effects on protein binding.

To further define the specific residues required for DNA binding, point mutations (Fig. 5A) were made within the m2 and m3 regions by polymerase chain reaction, and the mutant DNA fragments were examined to determine if they could compete with the probes prepared from the wild-type DR4 fragments in gel retardation experiments. As shown in Fig. 5B, mutations in any single residue within the m2 region (lanes 4–6), a combination of any two residues (lanes 7–9), or the entire m2 region (lane 3) rendered this DNA fragment unable to compete with the wild-type DNA fragment. As expected, the wild-type DNA fragment competed efficiently (lane 2). Thus, it was concluded that all three residues of the m2 region were critical for specific protein binding to this DNA element. Using the same strategy, point and double mutations within the m3 region were made and tested to see if they could compete with the wild-type DR4 fragment as shown in Fig. 5C. Like the m3 mutant (lane 3), the m3–1 mutation (lane 4) failed to compete with the wild-type fragment, but both the m3–2 (lane 5) and m3–3 (lane 6) mutations were able to compete with the wild-type DNA sequence. Consistently, the two double mutants containing the m3–1 mutation, including m3–1.2 (lane 7) and m3–1.3 (lane 9), failed to compete, but the m3–2.3 mutant (lane 8) successfully competed with the wild-type DNA fragment. As a control, the wild-type DR4 fragment (lane 2) successfully competed in the reaction. Thus, it was concluded that the m3–1 position was the most critical residue within the m3 region for specific protein binding.

RA induction of nuclear factors binding to the negative HRE of crabp-I promoter—It was shown previously that RA affected crabp-I gene expression in P19 cells. The early effect of RA on this gene seemed to be a suppression, but the late effect was a slow accumulation of the mRNA, possibly due to the stabilization of mRNA (13). To locate the DNA sequence responsible for the effect of RA, the wild-type CRABP-lacZ reporter, the DR4 deleted reporter (−993), the DR4 wild-type reporter (−1046), and DR4 mutated reporters (m2, m3, and m4) were transfected into P19, followed by the addition of vehicle or RA (10−7 M) for 24 h (Fig. 6A). RA exerted an inhibitory effect on the wild-type reporter (CRABP-lacZ), but not the DR4 deleted (−993) or DR4 mutated reporters (m2, m3, and m4). The inhibition on RA wild-type reporter (−1046) was not clear because the reporter activity was too low. We then tested if protein factors binding to this negative HRE were induced by RA in P19 cells. As shown in Fig. 6B, the retarded bands were specifically competed by DR4 fragment (lanes 2 and 7) or RARE (lanes 5 and 10) but not by adjacent sequences (L, lanes 3 and 8; DR5, lanes 4 and 9). In addition, protein factors binding to this DR4 were strongly induced by RA (lane 6) as compared with control (lane 1). To examine if RA specifically induced nuclear factors binding to the DR4, a probe derived from the minimal promoter sequence (−153 to −104, 5′-GCC-TTAGGGCGGGAGTAGTCGGGCTCACCCCTCGTGGCC-
ACCCCGCCC-3') containing Sp-1 sites was tested in parallel experiments (lanes 14–16). Proteins binding to the minimal promoter sequence were not induced by RA (lane 16) as compared with the control (lane 15), whereas proteins binding to the DR4 sequence were induced by RA significantly (lane 13) as compared with its corresponding control (lane 12). Thus, it was concluded that protein factors binding to this negative HRE were specifically induced by RA, supporting a biological function of this negative HRE in RA-regulated crabp-I expression.

Biological Activities and DR4 Binding of T₃Rb, RARs, and RXRb—Because many genes could be induced by RA in embryonic stem cells (30) and P19 cells,² such as several RARs/RXRs, thyroid hormone receptors (31), and some homeobox genes, we then tested the effects of three RARs, RXRb, and T₃Rb on the wild-type reporter and DR4 mutants in co-transfection experiments as shown in Fig. 7A. It seemed that the expression of RXRb or T₃Rb suppressed the wild-type CRABP-lacZ reporter activity to approximately 25% and 40%, respectively. RA, suppressed this reporter approximately 40%, whereas RARb.

² L.-N. Wei, C. H. Lee, P. Filipick, and L. Chang, unpublished observations.

**FIG. 5.** Specific single-residue and double-residue mutations within the m2 and m3 regions of the DR4 site and their protein-binding properties. A, the mutated sequence in each mutant oligonucleotide was underlined on the wild-type sequence labeled with HRE. B, gel retardation experiments were conducted by using end-labeled wild-type fragments (from −1019 to −993) containing a minimal DR4 site. The unlabeled fragment used in each competition experiment was the wild type (lane 2), m2 (lane 3), m2–1 (lane 4), m2–2 (lane 5), m2–3 (lane 6), m2–1.2 (lane 7), m2–2.3 (lane 8), and m2–1.3 (lane 9), respectively. C, gel retardation experiments were conducted as described in B, with competing cold fragments prepared from the series of m3 mutations. The unlabeled fragment in each competition was the wild type (lane 2), m3 (lane 3), m3–1 (lane 4), m3–2 (lane 5), m3–3 (lane 6), m3–1.2 (lane 7), m3–2.3 (lane 8), and m3–1.3 (lane 9), respectively. Lane 0 showed the free probes, indicated by the arrow on the left, and lane 1 showed a control reaction without competitors. Each competitor was added in 100× molecular excess. The arrow on the right indicated the position of the specifically retarded band.

**FIG. 6.** RA effects on the biological activities of CRABP-lacZ reporters and nuclear proteins binding to the DR4. A, the SLA of CRABP-lacZ, the −993 reporter (the parental reporter of the DR4 mutants), the −993 reporter (DR4 deleted reporter), and DR4 mutant reporters (m2, m3, and m4) was determined in the absence (Cont) or presence (RA) of 10⁻¹⁷ M RA for 24 h, after transformation. B, RA induced nuclear factors binding to the DR4. Gel retardation experiments were conducted by using end-labeled DR4 DNA fragments (−1019 to −993) (lanes 1–13) and a minimal promoter fragment (−153 to −104; lanes 14–16) as the probes and nuclear extracts prepared from uninduced P19 cells (lanes 1–5, 12, and 15) and RA-induced P19 cells (lanes 6–10, 13, and 16). Cold competitors included wild-type DR4, “L” (from the left-side flanking sequence of this DR4, −1046 to −1020; sequence shown in Fig. 2), its adjacent DR5, and the RARE from RXRb. Lanes 0, 11, and 14 showed free probes alone, indicated with an arrow on the left. The specifically retarded bands were indicated with arrows on the right.
had no significant effects on this reporter. T₃R₁₃₁ suppressed the m3 mutant approximately 35%, and neither the RARs nor the RXRₐ had any effects on this mutant. Similar results were observed for the m2 and m4 mutants (data not shown). Thus, it was concluded that the expression of RARₐ or T₃R₁₃₁ significantly inhibited the expression of this reporter, and T₃R₁₃₁ was able to bind to this DR4 sequence as monomers, homodimers, or heterodimers of T₃R₁/RXRₐ.

The effects of RARs, RXRs, and T₃Rs on CRABP-lacZ reporter activities and their binding to the DR4 element. A, P19 cells were co-transfected with the wild-type reporter (■) or m3 mutant reporter (□), with one of the expression vectors indicated. The SLA of each transfected culture was compared with the SLA of the control expression vector and represented as a RLA. The RLA of each control reporter (□), with one of the expression vectors indicated. The SLA of each transfectant was set at 100%. B, gel retardation experiments using the DR4 segment were conducted using proteins and probes containing the DR4 (~1019 to 993), and supershift experiments using the T₃R₁₃₁ antibody. Left, the bands bound by T₃R₁ monomers and homodimers were labeled a and b, respectively, whereas the bands supershifted by the antibody were labeled c. Right, the band bound by T₃R/RXR heterodimers was labeled c, and supershifted bands were indicated with arrows. In addition to the wild-type DR4 sequence, a standard palindromic TRE was labeled c, and the wild-type DR4 sequence was labeled b. Lane 0 showed the free probe, and lane 11 showed the negative reaction using a reticulocyte lysate control.

DISCUSSION

We have identified and characterized a negative HRE located between nucleotide positions −1012 and −993 of the mouse crabp-I gene upstream region, approximately 1 kb 5’ to the transcription initiation site. A DR4 sequence (TGACCTT-TGGGGACCT) was located in this HRE, which exerted a strongly suppressive effect on the reporter activity. In addition, RA inhibited the expression of this reporter and induced specific nuclear factors binding to this HRE. Co-expression of RARₐ or T₃R₁₃₁ significantly inhibited the expression of this reporter, and T₃R₁₃₁ was able to bind to this DR4 sequence as monomers, homodimers, or heterodimers formed with RXRₐ. The entire DR4 sequence was required for the negative biological activity, but only the repeated sequences were critical for specific protein binding.

The expression of many RA-regulated genes is mediated through the nuclear receptor superfamly in either positive or negative fashion, depending upon the combination of nuclear receptors available to the cells at a specific time (for reviews, see Refs. 1, 32, and 33). We have obtained evidence that the deletion of retinoids dramatically stimulated the expression of both the endogenous crabp-I and the CRABP-lacZ reporter in transgenic mouse embryos, whereas RA suppressed the expression of this reporter in transgenic mouse embryos.3 In accordance with this observation, the current study demonstrated that RA induced nuclear factors binding to this HRE and inhibited the activities of reporters carrying this HRE in P19. In other studies, the T₃R₁ gene was shown to be induced by RA (31). In this study, it was clearly demonstrated that T₃R₁₃₁ was able to bind to this HRE and that this expression suppressed the CRABP-lacZ reporter activities. Thus, it was highly possible that RA-mediated suppression of crabp-I expression mainly involved the T₃R family. However, because many genes can be induced by RA, it remains to be determined if this DR4 can be bound by other nuclear receptor complexes.

It was demonstrated in P19 cells that the expression of the crabp-I gene was under the control of a complicated regulatory machinery involving various signaling pathways such as protein kinases (11), DNA methylases (12), and RA (13). In response to RA treatment, the endogenous crabp-I gene was first suppressed and then induced in P19 cells, which required protein synthesis (13). However, it was also reported that crabp-I gene expression was not responsive to RA in cell types such as F9 cells (9), skin cells, and fibroblasts (10). A recent report showed that crabp-I was suppressed by RA at a high concentration but was induced by RA at a low concentration in embryonic stem cells (30). The expression of this gene might respond to various cellular signals in different cell types. This was supported by the fact that the upstream DNA segments of this gene contain both strong positive (11, 15) and negative (15) activities. Thus, the expression of crabp-I was differentially regulated in various cell types under different conditions. Nevertheless, the RARs, RXRs, and T₃Rs seemed to play important roles in the regulation of crabp-I gene expression.

3 L.-N. Wei, C.-M. Lee, P. Filipick, and L. Chang, manuscript in preparation.
Previously, a model was proposed for the regulatory elements controlling crbp-I gene expression (15). It was hypothesized that both positive and negative regulatory mechanisms were needed for the control of crbp-I gene expression. An AP-1 element located further upstream of this negative HRE was shown to be involved in the elevation of CRABP-I expression in P19 cells (11). The minimal promoter activity was under the control of four Sp-1 binding sites (15). In addition, demethylation of the upstream region of this gene was closely associated with a high level of crbp-I expression in both mouse embryos and P19 cells (12). The study shown here demonstrated one additional mechanism to silence the crbp-I gene, mediated through T_{RRA} binding to this negative HRE located between the two major positive regulatory regions. Thus, the expression of this gene in a specific cell is dependent upon the DNA status and the kind of nuclear factors available to the cell. The model has been updated by the addition of new information generated from this study, as shown in Fig. 8, in which the activities of the negative HRE have been confirmed and the factors involved in this negative regulation are shown in bold.

It will be interesting to determine what are the factors binding to the sequences flanking this HRE (shown with questions marks in Fig. 8) and how these positive and negative elements interact with each other inside specific cell types where trans-acting factors are constantly affected by the signals presented to the cells.

Although mice deficient in this gene seemed normal phenotypically, several studies showed that overexpression of this gene altered cellular differentiation (20) or the expression of certain developmental genes (21). Thus, the absence of this gene was not devastating to animal survival, but overexpression of this protein affected cell differentiation in both transgenic mice and cell cultures. A tightly controlled regulatory mechanism for crbp-I gene expression, especially a mechanism to keep the gene silenced under a specific condition, could be important in certain developmental processes such as cellular differentiation. It would be interesting to examine if mutation in this promoter would cause any pathological consequences in the animals.

Acknowledgments—We thank Drs. R. Evans and V. Giguere for providing the RAR and RXR cDNAs. We thank Dr. H. Towle for providing the cDNA as well as the antibody and yeast expression vector for T_{RRA}. We also thank J. R. Wilkinson for help in supershift experiments.

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J. Biol. Chem. 1997, 272:10144-10150.
doi: 10.1074/jbc.272.15.10144

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