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# Molecular cloning and analysis of functional cDNA and genomic clones encoding bovine cellular retinoic acid-binding protein

**(nucleotide sequence/cDNA expression in COS-7 cells/gene structure/tissue-specific gene expression)**

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**ABSTRACT** **A recombinant cDNA clone, pCRABP-HS1, encoding cellular retinoic acid-binding protein was isolated from a bovine adrenal cDNA library. COS-7 cells transfected with pCRABP-HS1 produced a biologically active retinoic acid-binding protein molecule of the expected molecular mass (15.5 kDa). RNA blot hybridization analysis using pCRABP-HS1 as a probe revealed a single 1050-nucleotide mRNA species in bovine adrenal, uterus, and testis, tissues that contain the highest levels of retinoic acid-binding activity. No hybridization was detected in RNA extracted from ovary, spleen, kidney, or liver, which contain relatively low levels of cellular retinoic acid-binding protein activity. Analysis of genomic clones isolated from an *Eco*RI* bovine genomic library demonstrated that the bovine cellular retinoic acid-binding protein gene is composed of four exons and three introns. Two putative promoter sequences were identified in the cloned 5′ sequence of the gene.**

Retinoic acid (RA), an endogenous metabolite of retinol (vitamin A) (1), is a potent regulator of epithelial cell proliferation and differentiation (2). In retinoid-deficient animals, stem cells fail to develop into mature epithelial sheets and frequently undergo abnormal differentiation characterized by keratinization and excessive cellular proliferation (3). RA, which cannot be reduced to retinol *in vivo* (4), is able to maintain normal proliferation and differentiation of epithelial cells, indicating that RA may be the active retinoid in these important physiological processes. Data from several *in vitro* systems in which RA is 100- to 1000-fold more active than retinol (5–7) support this hypothesis. RA has been shown to regulate the expression of genes involved in cell proliferation (8–11) and differentiation (7, 12–15). However, the molecular mechanism(s) of RA action remains an unsolved problem in retinoid biochemistry.

A specific cellular RA-binding protein (CRABP) (16) has been identified in retinoid-target tissues (17) and several retinoid-responsive cell lines (18). Rat testis CRABP is a single, acidic polypeptide chain (≈15 kDa) with a $K_d$ for RA of 4 nM (19, 20). Bovine adrenal CRABP (21) consists of 136 amino acid residues. RA-induced differentiation of F9 and PCC4aza1R embryonal carcinoma cells has been exploited to investigate the molecular mechanism of RA action and the function of CRABP. Collectively, the data indicate that the action of RA in the control of gene expression is mediated by CRABP. The ability of several retinoids (22, 23) and arotinoids (24, 25) to promote murine embryonal carcinoma cell differentiation is correlated with their affinity for CRABP. Mutants of F9 (ref. 26, J. H. Winston and A.M.M., unpublished results) and PCC4aza1R (27) embryonal carcinoma cells that exhibit normal RA uptake but lack functional CRABP fail to differentiate upon exposure to RA. Mutants that retain functional CRABP but fail to respond to RA were also isolated (28). Complementation analysis performed by fusion of the CRABP$^-$ and CRABP$^+$ differentiation-defective mutants yields a RA-responsive hybrid that contains functional CRABP. Nuclear localization of RA has been demonstrated in Y-79 retinoblastoma (29), F9 (22), and PCC4aza1R (30) cells. Preformed RA–CRABP complexes, but not free RA, were able to promote specific, saturable binding of RA to nuclear acceptor sites in isolated nuclei (refs. 17 and 31; A.M.M., unpublished data). However, CRABP itself does not bind to the chromatin acceptor sites (31). The available data on RA action in embryonal carcinoma cells have been incorporated into the following model. RA–CRABP complexes formed in the cytosol transport RA to nuclear RA acceptor sites in chromatin. RA binding to the specific acceptor sites culminates in the enhanced expression of one subset, and the decreased expression of a second subset, of RA-responsive genes. Apo-CRABP released upon nuclear RA binding may be either recycled to the cytosol to form additional RA–CRABP complexes or degraded.

To further define the function of CRABP in the molecular mechanism of RA action and to examine the regulation of CRABP expression, we have cloned and analyzed a functional cDNA and genomic clones encoding bovine CRABP.

## MATERIALS AND METHODS

**Enzymes, Radioisotopes, and Oligonucleotides.** DNA polymerase I, DNA, ligase, polynucleotide kinase, the Klenow fragment of DNA polymerase I, and restriction enzymes were obtained from commercial sources. [11,12-$^3$H]RA was provided by P. Sorter of Hoffmann–La Roche. All-*trans*-[11,12-$^3$H]RA was purified from the isomeric mixture by reverse-phase HPLC as described (32). Oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) 380A automated DNA synthesizer and purified by either gel electrophoresis or HPLC.

**Isolation of Bovine CRABP cDNA Clones.** A bovine adrenal cDNA library constructed in the pcD expression vector (33) (kindly provided by D. Russell, University of Texas Health Science Center, Dallas) was screened with two oligodeoxynucleotide probes (34, 35) deduced from the published amino acid sequence of bovine adrenal CRABP (21), to identify cDNA clones derived from CRABP mRNA. Probe A was a 17-base oligonucleotide pool with 16-fold degeneracy, corresponding to amino acids 87–92. Probe B was a 61-base oligonucleotide, corresponding to the preferred codons for amino acids 87–106.

**Isolation of Bovine Genomic CRABP Clones.** An *Eco*RI* bovine genomic library in bacteriophage λ Charon 4A (kindly provided by D. P. Quick, University of Iowa) was screened with [α-$^{32}$P]dATP- and [α-$^{32}$P]dCTP-labeled probes, prepared

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Abbreviations: RA, retinoic acid; CRABP, cellular retinoic acid-binding protein.

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by nick-translation of the *Pst* I–*Bam*HI fragments of pCRABP-HS1 (cDNA encoding the entire CRABP protein) and pCRABP-HS2 [cDNA equivalent to the 3′ region of pCRABP-HS1, from the Lys-81 codon to the poly(A) tail] as described (35, 36).

**RNA Isolation and Blot Hybridization.** Bovine tissues were obtained from a local slaughterhouse, quick-frozen in liquid $N_2$, and stored at −80°C until use. Monolayers of COS-7 cells were washed in phosphate-buffered saline, quick-frozen on liquid $N_2$, and stored at −80°C until subsequent RNA isolation. Total RNA was extracted from tissues and cells by the guanidinium thiocyanate/CsCl gradient centrifugation method (37). RNA was denatured with 1 M glyoxal/36% dimethyl sulfoxide and subjected to electrophoresis followed by blot hybridization analysis (35). Uniformly [$\alpha$-$^{32}$P]dCTP-labeled single-stranded pCRABP-HS1/M13 DNA probes ($0.5–1 \times 10^8$ cpm/$\mu$g; $2 \times 10^6$ cpm/ml) were used for hybridization (38).

**Transient Expression of Bovine CRABP cDNA in COS Cells.** COS-7 cells (39) were transfected with 5 $\mu$g of pCRABP-HS1 DNA using DEAE-dextran (40) and a chloroquine "boost" (41). Nontransfected COS-7 cells, mock-transfected COS-7 cells, and COS-7 cells transfected with a cDNA coding for 17$\alpha$-hydroxylase (42) cloned in the pcD expression vector (33) (kindly provided by M. Waterman, University of Texas Health Science Center, Dallas) served as controls.

**Determination of CRABP Levels.** Cytosolic fractions were prepared from bovine tissues and COS-7 cells as described (32, 43). Cytosolic protein was measured as described by Bradford (44). Total CRABP (apo-CRABP plus holo-CRABP) and apo-CRABP were measured by tritiated ligand binding and high-performance size-exclusion chromatographic analysis (32, 43).

## RESULTS

**Isolation and Nucleotide Sequence of Bovine CRABP cDNA Clones.** Oligonucleotide probes deduced from the published amino acid sequence of bovine adrenal CRABP (21) were used to screen $\approx 3.5 \times 10^4$ transformants of a bovine adrenal cDNA library constructed in the pcD expression vector (33). Ten positive clones that contained inserts ranging in size from 400 to 800 base pairs (bp) were isolated. Clone pCRABP-HS1 contained a cDNA insert of ≈0.8 kilobase (kb), which would be capable of coding for the entire CRABP protein (136 amino acids). The cDNA is 731 bp in length excluding the poly(G) and poly(A) tails (Fig. 1) and contains only one open reading frame of significant length (408 bp), which codes for a sequence of amino acids identical to that previously determined for bovine adrenal CRABP (21). pCRABP-HS1 also contains 91 bp of 5′ untranslated sequence and 226 bp of 3′ untranslated sequence. A putative polyadenylylation sequence (ATTAAA) is located 15 bp upstream from the poly(A) addition site.

**Expression of the Bovine CRABP cDNA.** To confirm that the bovine cDNA we have isolated and characterized encodes an active CRABP molecule, the plasmid pCRABP-HS1 was expressed transiently in COS-7 cells. Because cytosolic

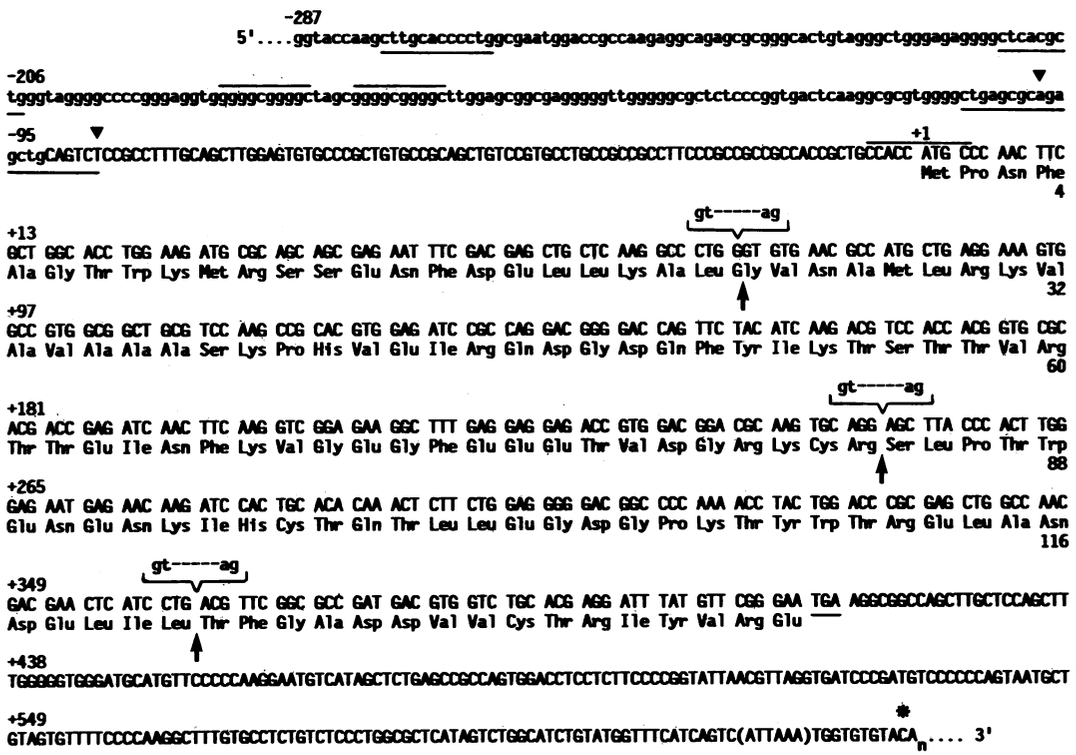


FIG. 1. Nucleotide and predicted amino acid sequence of bovine CRABP. The sequence shown is a composite of those derived from cDNA pCRABP-HS1 (uppercase) and genomic clones λ6 and λ12B (lowercase). Nucleotides are numbered above each row. Nucleotide residue +1 is the A of the initiating methionine codon (ATG), and the nucleotides on the 5′ side of this residue are indicated by negative numbers. The sequence from nucleotides −91 to +641 was determined on both strands of the cDNA clone pCRABP-HS1 and on the genomic clones λ6 and λ12B, using M13 universal primer and a series of synthetic 17-base oligonucleotides complementary to sequences in pCRABP-HS1. The deduced amino acid sequence is shown below the DNA sequence and is numbered below each row from the initiating methionine. The translational initiation sequence is overlined (uppercase) and the stop codon is underlined (uppercase). A putative polyadenylylation sequence is in parenthesis and the poly(A) addition site at nucleotide 640 is marked with an asterisk. The arrows mark the positions of the three introns in the bovine gene, and the nucleotide sequences at the exon/intron junctions are shown (lowercase) in brackets above these positions. The sequence upstream of the 5′ end of the insert in pCRABP-HS1 (−92 to −287) was derived from genomic clone λ6. Two putative Sp1 transcription factor binding sites and three potential cAMP-responsive elements are overlined and underlined, respectively. The putative cAMP-responsive element proximal to the translation initiation site contains an interrupted 12-bp palindromic sequence whose endpoints are indicated by solid triangles (▼).

CRABP can exist in either of two forms, apo-CRABP (CRABP lacking RA) or holo-CRABP (RA–CRABP), expression of pCRABP-HS1 was assayed by measuring total all-*trans*-RA binding activity (apo-CRABP plus holo-CRABP) in cytosolic preparations of COS-7 cells. Specific binding activity was calculated as the difference between all-*trans*-[$^3$H]RA binding in the presence and absence of a 100-fold molar excess of unlabeled all-*trans*-RA. No CRABP activity was detected in cytosolic fractions from nontransfected COS-7 cells, mock-transfected cells (0 and 66 hr), or COS-7 cells transfected for 66 hr with a pcD vector containing an irrelevant cDNA (17$\alpha$-hydroxylase). Significant CRABP activity was detected 24 hr after transfection with pCRABP-HS1 (Fig. 2) and increased as a function of time, reaching 40 pmol/mg of cytosolic protein by 66 hr. At all time points, a single RA-binding species was detected, which comigrated with bovine adrenal RA–CRABP complexes (≈15.8 kDA) upon high-performance size-exclusion chromatography. Unlabeled RA, but not unlabeled retinol (100-fold molar excess), competed for all-*trans*-[$^3$H]RA binding to CRABP *in vitro* (data not shown). Furthermore, RA–CRABP complex formation *in vitro* was inhibited by *p*-hydroxymercuribenzoate and recovered with excess dithiothreitol as previously described for bovine testis CRABP (43). Comparison of apo-CRABP (data not shown) and total CRABP levels at each time point demonstrated that the newly synthesized CRABP was present as the apo-protein.

**Isolation and Partial Characterization of Genomic CRABP Clones.** A bovine genomic library, constructed in the bacteriophage λ Charon 4A, was screened by hybridization, using the nick-translated inserts of clones pCRABP-HS1 and pCRABP-HS2 as probes. Ten positive clones were isolated. Restriction enzyme digestion and Southern (46) hybridization analysis indicated that these clones fall into three groups. Groups 1 (λ2, λ6, and λ7) and 2 (λ12A, λ12B, and λ21) each consist of three identical clones. Group 3 consists of four weakly hybridizing clones (λ5, λ9, λ12, and λ14) that are different from one another and from the clones in groups 1 and 2; these clones were not studied further. Representatives of groups 1 and 2 (λ6 and λ12B) were chosen for further characterization. Additional restriction enzyme mapping and Southern analysis, using the inserts of pCRABP-HS1 and pCRABP-HS2 and oligonucleotides complementary to different segments of pCRABP-HS1 as hybridization probes, showed that λ6 and λ12B contain overlapping sequences. Clone λ6 did not hybridize to pCRABP-HS2 or to oligonucleotides complementary to the 3′ region of pCRABP-HS1. Clone λ12B appeared to contain the remainder of the CRABP gene. Restriction fragments of these clones were subcloned into the bacteriophage M13 vectors mp18 and mp19 for subsequent nucleotide sequence analysis. The structure of the bovine CRABP gene and the nucleotide sequences at the exon/intron boundaries (Fig. 1) were established by DNA sequence comparison of cDNA and genomic subclones. The gene for bovine CRABP consists of four exons and three introns. The subcloned and sequenced fragment of clone λ6 is ≈1500 bp long and contains the first two exons of the CRABP gene. It begins 196 bp upstream of the 5′ end of the cDNA in pCRABP-HS1 and is colinear with pCRABP-HS1 until nucleotide position 70 (Gly-24), after which the first intron interrupts the CRABP gene coding sequence. The second exon is 179 bp from nucleotide positon 71 to nucleotide position 249 (Arg-83). The third and fourth exons were both located in genomic subclones of clone λ12B. The third exon is 114 bp long and codes for 38 amino acids (Ser-84 to Leu-121). The fourth exon is 277 bp and encodes the last 16 amino acids of CRABP (Thr-122 to Glu-137), and the whole 3′ untranslated region in pCRABP-HS1. The 5′ donor and 3′ acceptor splice sites in each of the three introns conform to the GT/AG rule and agree with the consensus sequences previously reported for exon/intron boundaries (47). The exact sizes of the introns have not been determined, but restriction enzyme digestion and Southern hybridization indicated that the CRABP gene is at least 11 kb long.

**Expression of the CRABP Gene in Bovine Tissues.** Representative bovine tissues were analyzed for CRABP-specific mRNA and CRABP activity. To minimize errors introduced by variations in the age and sex of the animal or its nutritional status, CRABP mRNA and CRABP binding activity were analyzed on duplicate samples of each tissue. With the exception of testis, tissues were obtained from female animals. Samples of total RNA were screened for CRABP-specific transcripts with $^{32}$P-labeled single-stranded cDNA probes derived from pCRABP-HS1. As illustrated in Fig. 3, increasing amounts of bovine adrenal RNA (5–20 μg) yielded a progressively stronger hybridization signal to a single mRNA species of ≈1050 bp. A single mRNA species of similar size was also detected in total RNA preparations from uterus and testis. Densitometric scanning of autoradiographs indicated that CRABP mRNA was 21- and 32-fold more abundant in adrenal tissue than in uterus and testis, respec-

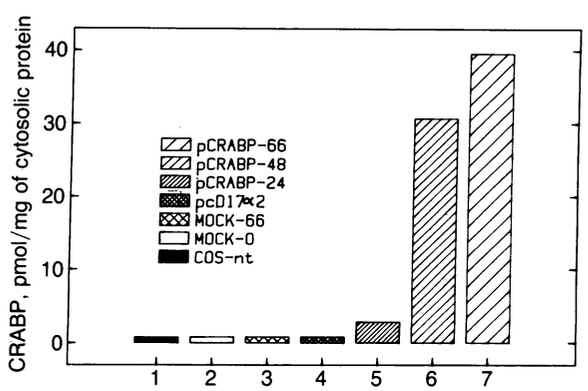


FIG. 2. Expression of pCRABP-HS1 in COS-7 cells. COS-7 cells were transfected by the DEAE-dextran protocol, as described in *Materials and Methods*, for periods ranging from 0 to 66 hr. Total (apo plus holo) CRABP activity was measured in COS-7 cell cytosolic fractions by specific [$^3$H]RA binding and HPLC analysis of [$^3$H]RA–CRABP complexes. Bar 1 (COS-nt) shows total specific [$^3$H]RA binding activity in nontransfected COS-7 cells. Bars 2 (Mock-0) and 3 (Mock-66) show total CRABP activity in mock-transfected COS-7 cells (0 and 66 hr). Bar 4 (pcD17α2) shows total CRABP activity in COS-7 cells transfected for 66 hr with a pcD vector containing a cDNA encoding 17α-hydroxylase (45). Bars 5 (pCRABP-24), 6 (pCRABP-48), and 7 (pCRABP-66) show total CRABP activity in COS-7 cells transfected with pCRABP-HS1 for 24, 48, and 66 hr.

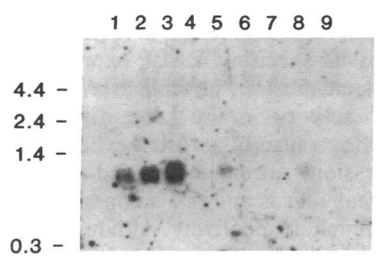


FIG. 3. Tissue-specific distribution and size of bovine CRABP mRNA. Total RNAs extracted from bovine tissues were denatured with glyoxal and dimethyl sulfoxide, fractioned by electrophoresis in a 1.5% agarose gel, and transferred to nitrocellulose. The membrane was hybridized with $^{32}$P-labeled single-stranded DNA probes derived from pCRABP-HS1 (0.5–1 × $10^8$ cpm/μg). A "ladder" of RNA (Bethesda Research Laboratories) provided size markers (given in kb at left). Lanes 1–3: 5, 10, and 20 μg of total RNA from bovine adrenal gland. Lanes 4–9: 20 μg of total RNA from bovine kidney, uterus, ovary, spleen, testis, and liver, respectively.

Table 1. Expression of biologically active CRABP in bovine tissues

| Tissue | Total CRABP activity, pmol/mg of protein |
|---|---|
| Adrenal | 3.1 |
| Uterus | 1.3 |
| Testis | 0.8 |
| Spleen | 0.4 |
| Kidney | 0.4 |
| Ovary | 0.3 |
| Liver | 0.02 |

tively. CRABP mRNA was not detected in total RNA samples (20 $\mu$g) from ovary, spleen, kidney, or liver.

The distribution of total CRABP activity in bovine tissues is given in Table 1. The highest concentration of biologically active CRABP (3 pmol/mg of cytosolic protein) was found in the adrenal gland (cortex plus medulla). Uterus and testis contained 1.3 and 0.8 pmol of CRABP activity per mg of cytosolic protein, respectively. CRABP concentrations in kidney, spleen, and ovary were approximately an order of magnitude lower than that detected in adrenal. As expected, CRABP was barely detectable in liver cytosol.

## DISCUSSION

To understand the molecular mechanism of RA action in the control of gene expression and the regulation of epithelial differentiation, and to delineate the role of CRABP in this process, we have cloned and characterized a CRABP cDNA (pCRABP-HS1) and the bovine CRABP gene. The cDNA clone pCRABP-HS1 is ≈0.8 kb long and consists of 5′ untranslated sequences, the entire coding sequence of bovine CRABP, 3′ untranslated sequences, and a poly(A) tail. The translation initiation sequence in pCRABP-HS1 (CCACCATGC) is similar to the optimal sequence for initiation by eukaryotic ribosomes (CCACCAUGG; ref. 48) except for the substitution of cytosine for guanine at position +4. The predicted amino acid sequence of the 408-bp coding region is identical to that of purified bovine adrenal CRABP (21). The identity of the pCRABP-HS1 cDNA was further verified by transient expression of the plasmid in COS-7 cells. Transfected cells produce a specific RA-binding protein of the expected size for bovine adrenal CRABP (15.5 kDa), whose RA binding activity was reversibly inhibited by *p*-hydroxymercuribenzoate (43).

Blot hybridization of total RNA from adrenal, uterus, and testis revealed a single RNA transcript with an average size of 1 kb. Under identical experimental conditions, CRABP mRNA was not detected in bovine kidney, spleen, ovary, or liver. The observed differences in CRABP mRNA levels suggest that tissue-specific CRABP expression may be regulated, in part, at the transcriptional level. Quantitation of total CRABP activity in the same tissues screened for CRABP-specific transcripts also revealed tissue-specific CRABP expression. Adrenal contained the highest CRABP activity, whereas uterus and testis contained about half and one-fourth as much, respectively. Although CRABP levels in kidney, spleen, and ovary were an order of magnitude lower than that in adrenal, CRABP activity was easily detectable by radioligand binding. Our failure to detect CRABP mRNA in kidney, ovary, and spleen might be explained by a higher sensitivity of the radioligand binding CRABP assay compared to blot hybridization analysis of total RNA. Alternatively, the CRABP activity expressed in kidney, spleen, and ovary may be encoded by a distinct gene such that CRABP mRNA in these tissues does not hybridize with the bovine adrenal cDNA probe used for blot hybridization.

The single-copy bovine CRABP gene was partially characterized by nucleotide sequence analysis of two genomic clones. Comparison of the nucleotide sequences of the cDNA and geomic subclones showed that the bovine CRABP gene contained four exons. The nucleotide sequences at the exon/intron junctions (GT/AG) agree with the consensus sequences for exon/intron boundaries (47). The structures of genes coding for three proteins related to CRABP have recently been determined: rat liver fatty acid-binding protein (49); murine aP2 (50); and rat cellular retinol-binding protein II (45). Each of these genes consists of four exons and three introns, which are located at homologous positions within the coding sequences (at amino acids 24, 84, and 119 in the numbering system used for rat cellular retinol-binding protein II) (45). The three introns of the bovine CRABP gene are located at sequences coding for amino acids 24, 83, and 121—i.e., in positions that are homologous to those occupied by introns of the three related genes.

The λ6 genomic clone contains 287 nucleotides 5′ to the translation initiation site in pCRABP-HS1. Preliminary primer-extension experiments to identify the transcription initiation site indicate that RNA transcription starts 95 nucleotides upstream of the translation initiation codon ATG. While no "TATA box" homology was discerned in the cloned 5′ sequence of the CRABP gene, this region of the gene contains several sequences that are characteristic of cis-acting elements that control expression of numerous genes (Fig. 1): two putative Sp1 transcription factor binding sites (GGGGCGGGGC; ref. 51) and three potential cAMP-responsive elements (52–54), one of which contains an interrupted 12-base sequence (5′ AGA_CTGCAGTCT 3′) similar to the 8-base palindrome 5′ TGACGTCA 3′ already identified in many genes whose expression is regulated by cAMP. It is tempting to speculate that these putative cAMP-responsive elements might direct the high level of CRABP gene expression noted in the adrenal gland.

The discovery of CRABP and cellular retinol-binding protein in cytosolic fractions of retinoid-responsive tissues led to the speculation that these retinoid-binding proteins were analogous to the steroid receptor proteins (16, 18) and that the mechanism of retinoid action was analogous to that described for steroid hormones (55, 56). However, recent findings have demonstrated that RA action cannot be equated to the steroid hormone model. CRABP shares no homology with the amino acid sequences of the highly homologous glucocorticoid (57, 58), estrogen (59, 60), and progesterone (61, 62) receptors deduced from the respective cDNA clones and thus lacks the "DNA-binding finger" domain common to these and numerous other DNA-binding proteins (63). In addition, the CRABP portion of the RA–CRABP complex does not remain associated with chromatin (31) as demonstrated for the intact steroid–receptor complexes (56). By contrast, CRABP appears to function as a specific intracellular RA-transport protein required for the delivery of RA to specific nuclear RA acceptor sites, believed to mediate RA action in cell differentiation. Using a suitable expression vector and a selectable marker such as neomycin resistance, stable cell lines expressing recombinant CRABP could be derived from CRABP$^-$, differentiation-defective embryonal carcinoma cell mutants. Restoration of RA-responsiveness upon CRABP expression would provide the ultimate demonstration that CRABP is an essential mediator of RA action in this model of RA-regulated gene expression and epithelial differentiation.

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