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Rat cellular retinol-binding protein: cDNA sequence and rapid retinol-dependent accumulation of mRNA

(vitamin A/lung)

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ABSTRACT **Cellular retinol-binding protein (CRBP) may be an important mediator of vitamin A action. We report here the identification of a cDNA clone corresponding to the rat CRBP gene. The cDNA is 695 nucleotides long, with an open reading frame corresponding to a protein of 134 amino acids. The deduced amino acid sequence is identical with that of rat CRBP. The nucleotide sequence shows 90.5% similarity with the human CRBP cDNA sequence. Genomic DNA analysis indicates that CRBP is present in one, or at most two, copies within the rat genome. Analysis of mRNA reveals a single species in every tissue tested and suggests that the isolated cDNA is full-length. Finally, when retinol-deficient rats are fed retinyl acetate for 4 hr, about 4-fold accumulation of CRBP-specific mRNA is observed in the lungs. This rapid effect suggests that the micronutrient retinol may directly influence the expression of its specific intracellular binding protein.**

Retinol (vitamin A alcohol) is an essential micronutrient in all vertebrate species. Animal studies indicate that lack of this vitamin in the diet results in sterility, blindness, loss of growth, and, eventually, death (1). These changes are linked to the fundamental involvement of retinol in cellular differentiation. The vitamin is required for the maintenance of most, if not all, epithelial cells (1). Retinol deficiency leads to a replacement of normal epithelia by squamous, often keratinizing, cells (1). Replenishing the animal with retinol results in the repopulation of the epithelia by normal cells (2). These effects on cellular differentiation strongly suggest that altered genomic expression is an important component of the mechanism of vitamin A action. Many vitamin A-dependent changes in the expression of specific genes have been reported (for review, see ref. 3).

The genomic-expression model of retinol action gained great impetus from the discovery of a specific intracellular protein capable of binding retinol (4). Cellular retinol-binding protein (CRBP) binds its ligand with high affinity and specificity. It has been proposed that this protein transports retinol to specific sites within the nucleus, where the principal effects of the vitamin have been localized (3, 5, 6). Evidence from animal and cell culture studies support this hypothesis, but the precise function of CRBP is not yet clearly defined (5).

Elucidation of the role of CRBP should be facilitated by an understanding of events at the molecular level. Thus, we decided to isolate full-length cDNA clones of the rat CRBP gene and to use these clones to examine the effect of retinol on the expression of its binding protein.

MATERIALS AND METHODS

Construction of cDNA Library. Total RNA was extracted from the proximal caput of the epididymides of 20 Sprague–Dawley rats by the CsCl cushion procedure (7, 8). Poly(A)$^+$ RNA was isolated as described (9). A cDNA library was constructed in plasmid pBR322 by the method of Gubler and Hoffman (10).

Oligonucleotides. Oligonucleotides for use as library probes and sequencing primers were synthesized with a Biosearch Sam-One series II DNA synthesizer employing phosphotriester chemistry. Oligonucleotides were purified as described (11). Sequences of the oligonucleotide probes were based on the sequence of the human CRBP gene (12) and the amino acid sequence of rat CRBP (13). Best-guess codon assignments were made with the codon-utilization data of Grantham *et al.* (14).

Library Screening. The pBR322 library was screened by the "colony-lift" procedure, using oligonucleotides labeled with [γ-^{32}P]ATP (7000 Ci/mmol; New England Nuclear; 1 Ci = 37 GBq) and bacteriophage T4 polynucleotide kinase (15).

Subcloning and Sequencing. The largest cDNA insert within a positive clone (263 bases) was excised from pBR322 DNA by *Pst* I digestion and subcloned into M13 as described (16), except that the UT481 strain of *Escherichia coli* was used. The positive M13 phage were plaque-purified, and single- and double-stranded DNAs were prepared. Single-stranded M13 DNA was then subjected to the Sanger dideoxy sequencing technique (17). Sequencing kits were purchased from New England Nuclear and used according to the manufacturer's instructions, except that reactions were performed at 37°C and NaCl was omitted from the reaction buffer. Clones of interest were sequenced at least three times in each orientation.

Nick-Translation. The pBR322 derivative containing the longest positive cDNA insert was subjected to nick-translation with kits purchased from New England Nuclear and used as recommended by the manufacturer.

λgt11 Library Screening. In search of a larger cDNA clone, a rat liver λgt11 library was screened as described (18). Phage producing positive signals were then plaque-purified and λgt11 DNA was isolated as follows. Phage-infected *E. coli* were grown to confluence on 150-mm dishes and eluted into SM buffer (100 mM NaCl/10 mM $MgSO_4$/50 mM Tris·HCl, pH 7.5/2% gelatin). After centrifugation at 10,000 × *g* for 15 min at 4°C, the supernatant was subjected to DNase and RNase I treatment (10 μg/ml each) for 30 min at room temperature. Phage were then pelleted by centrifugation in a Beckman Ti 45 rotor at 35,000 rpm for 3 hr and resuspended in SM buffer with 20 mM EDTA and 0.5% sodium dodecyl sulfate. Proteinase K was added to 500 ng/ml, and the solution was left at 65°C for 1 hr. The reaction was terminated by phenol extraction followed by phenol/chloroform extraction (1:1, vol/vol) and chloroform extraction. The final aqueous phase was dialyzed overnight against $T_{10}E_1$ buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA), and then nucleic acids were precipitated with ethanol. The insert was excised

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Abbreviation: CRBP, cellular retinol-binding protein.

with *Eco*RI, subcloned into M13, and subjected to sequencing as described above. Sequence analysis was aided by computer access to the National Biomedical Research Foundation data bank (Washington, DC).

Blotting Analysis of DNA and RNA. Total RNA was isolated from rat liver, lung, and testes as described above (7, 8). Fifteen micrograms of total RNA from each tissue was subjected to agarose/formaldehyde electrophoresis as described (19). Genomic DNA was prepared by a modification (A. Rinaldy, personal communication) of the CsCl cushion

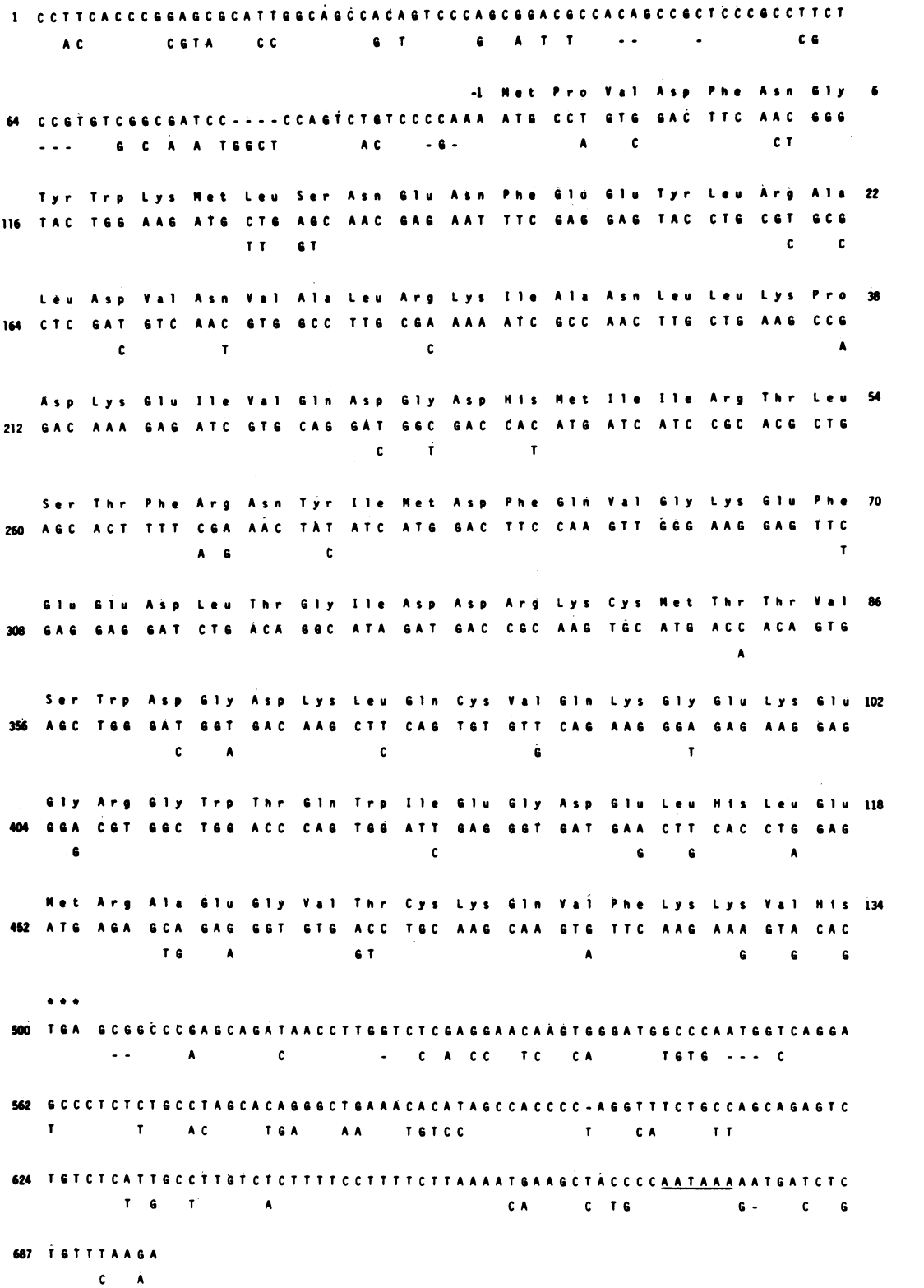

FIG. 1. Nucleotide sequence of the rat CRBP cDNA. For comparison, the differences with the human sequence are provided below the rat cDNA sequence. The deduced rat protein sequence is given above the cDNA sequence. The putative polyadenylylation signal in the 3′ untranslated region is underlined. Nucleotide positions are indicated to the left, and amino acid positions are indicated to the right. Dashes indicate gaps inserted to maximize homology.

method for RNA isolation (8). Rat liver was homogenized by hand in 4 M guanidinium thiocyanate solution and layered onto a 5.7 M CsCl cushion. After ultracentrifugation (Beckman SW41 rotor; 30,000 rpm for 24 hr), the DNA was collected as a viscous solution between the CsCl and the homogenate. This solution was extracted once with phenol/chloroform (1:1, vol/vol), extracted once with chloroform, and then ethanol-precipitated. The DNA was dissolved in $T_{10}E_1$, treated with proteinase K (50 μg/ml) in 0.1% sodium dodecyl sulfate, reextracted as above, and then reprecipitated with ethanol. This DNA (15–20 μg) was cut with various restriction endonucleases and separated in a 1% agarose gel. The gel was then processed using a modification of the Southern procedure (20, 21). Both genomic DNA and RNA were transferred to GeneScreen*Plus* (New England Nuclear) and processed as suggested by the supplier except that a Hybrid Ease hybridization chamber (Hoefer Scientific, San Francisco) was used. To prepare probes, DNA inserts were purified from agarose gels essentially as described (22). DNA and RNA blots were probed with DNA inserts subjected to the random oligonucleotide-labeling method (23). These probes consistently were labeled to a specific activity greater than 10^9 dpm/μg.

Manipulation of Retinol Status. Male Sprague–Dawley rats (Holtzmann, Madison, WI) were made retinol-deficient as described (24, 25). Vitamin A status of the animals was determined by measuring serum retinol levels fluorometrically (26). Animals were considered retinol-deficient only when their serum retinol levels were below 10 μg/100 ml. Retinol-deficient rats were given (intragastrically) 100 μg of retinyl acetate in 0.2 ml of cottonseed oil or 0.2 ml of cottonseed oil alone and were killed 4 hr thereafter. Lung RNA was isolated and analyzed as described above.

RESULTS

Isolation and Characterization of cDNA Clones. To begin the search for a cDNA clone corresponding to the rat CRBP gene, a cDNA library was constructed in the vector pBR322. Proximal caput of the epididymis was used for RNA isolation and library construction because it is the richest source of rat CRBP (27). Approximately 5×10^4 colonies were screened, and 7 positive clones were isolated. The largest insert was subcloned and subjected to sequencing as described in *Materials and Methods*. Comparison with the human CRBP gene sequence (12) and the rat CRBP amino acid sequence (13) allowed the positive identification of the clone. It contained 263 bases corresponding to the 3′ portion of the gene, including the entire 3′ untranslated region. This insert was nick-translated without isolating the fragment from the pBR322 vector sequences. This probe was used to search for full-length clones from a rat liver cDNA library constructed in the phage λgt11. Duplicate "plaque-lifts" were screened either with the probe described above or with nick-translated pBR322 without any insert. Only plaques positive with the former and not the latter were isolated and purified. After screening of approximately 4×10^5 plaques, one such plaque was identified. It was isolated, subcloned, and sequenced.

Sequence Analysis. The sequence is indicated in Fig. 1. The insert is 695 nucleotides long, including a 5′ untranslated region of 94 base pairs and a 3′ untranslated region of 199 base pairs. The −3 position relative to the ATG start codon contains an adenine, as in nearly all eukaryotic sequences (28). The initiation codon at position 95 is followed by an open reading frame of 402 nucleotides, corresponding to a protein of 134 amino acids. The 3′ untranslated region ends 10 bases beyond a potential polyadenylylation site (AATAAA); the poly(A) tail was not found.

The amino acid sequence deduced from this cDNA shows identity with the protein sequence for rat CRBP (13). Within the coding region, the nucleotide sequence shares 90.5% similarity with the human CRBP gene sequence, which indicates a very strong evolutionary conservation.

Genomic DNA Analysis. To further characterize the cDNA clone, rat genomic DNA was cut with various restriction endonucleases and subjected to Southern blot hybridization. As shown in Fig. 2, there are two to four hybridizing bands in each lane, depending on the restriction enzyme. This result suggests that the CRBP gene is present within the rat genome in not more than two copies. The existence of an extensive multigene family that includes CRBP is not supported by these data.

mRNA Analysis. To determine the number and size of mRNA species hybridizing with the cDNA clone, RNA blot analysis was performed. As shown in Fig. 3, the probe recognizes a single band of the same size in liver, lung, and testis RNA. The signal appears at approximately 700 bases, indicating that the cDNA clone is full-length or very nearly so. Relative hybridization intensities are consistent with CRBP protein levels in these tissues (29).

Effect of Retinol on Accumulation of CRBP mRNA in the Lungs. To determine whether CRBP gene expression is influenced by retinol, blot analysis was performed on total RNA isolated from the lungs of retinol-deficient rats and of retinol-deficient rats 4 hr after oral administration of retinyl acetate. Lungs were chosen because that organ is the first to show morphological signs of regeneration after administration of vitamin A to deficient rats (2). Fig. 4 shows representative data from six separate animals and demonstrates that the signal in the lung RNA increases significantly upon supplementation of deficient animals. Densitometry performed on a total of 12 lung samples (7 deficient, 5 supplemented) revealed a 4-fold increase. Thus, the amount of CRBP mRNA in the lungs appears to respond quickly to changes in the vitamin A status of the animals.

DISCUSSION

We report here the isolation and characterization of a cDNA clone corresponding to the rat CRBP gene. The amino acid sequence deduced from this clone is identical to that of rat

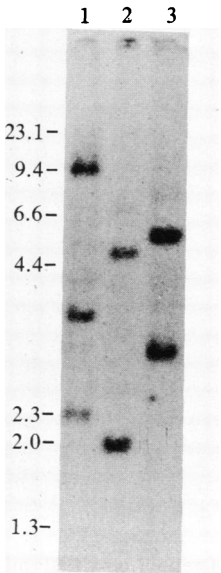

FIG. 2. Southern blot analysis of rat genomic DNA (15–20 μg per lane), using CRBP cDNA as a probe. Before electrophoresis, genomic DNA was digested with *Bam*HI (lane 1), *Hin*dIII (lane 2), or *Bgl* II (lane 3). Size markers (in kilobases) are shown at left.

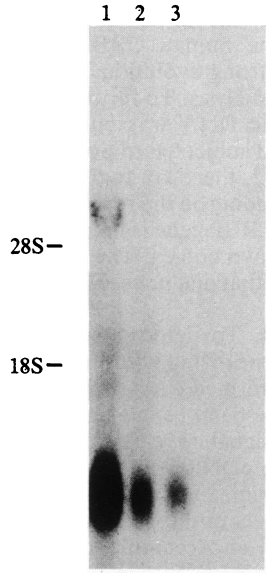

FIG. 3. Blot analysis of total RNA (15 μg per lane) from various tissues, using CRBP cDNA as a probe. Lanes: 1, liver; 2, lung; 3, testis. The positions of 18S and 28S rRNA are indicated at left.

CRBP, and the nucleotide sequence is extremely similar to that derived from the human CRBP gene. RNA blot analysis indicates the presence of a single mRNA species. The size of that mRNA suggests that the isolated cDNA is full-length or very nearly so. This cDNA should be a useful probe in studying the mechanism by which vitamin A acts.

Currently, most attempts to understand the molecular basis of retinol action focus on the effects of the vitamin on genomic expression. Many such changes in the expression of specific gene products have been reported (3). In contrast to the effects on cells in culture, genomic effects appear to be very rapid in the whole animal. It has been reported that 1 hr after retinyl acetate administration to retinol-deficient rats, *in vitro* translation patterns of testicular poly(A)$^+$ RNA reveal significant alteration (30). More recently, a doubling of

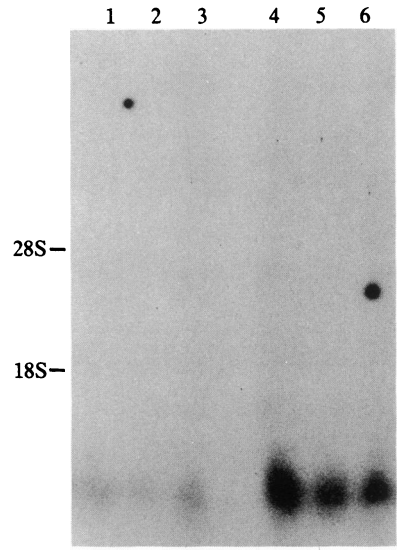

FIG. 4. Effect of retinol on CRBP mRNA in lungs. Total lung RNA (15 μg per lane) from six individual rats was subjected to blot hybridization analysis with the CRBP cDNA probe. Lanes 1–3: total lung RNA from retinol-deficient rats. Lanes 4–6: total lung RNA from retinol-deficient rats 4 hr after supplementation with retinyl acetate.

testicular chromatin template capacity was seen 4 hr after administration of retinyl acetate to deficient rats (31). Under identical conditions, we now report the rapid, *in vivo* accumulation of a specific mRNA in the lungs. The genomic-expression model, which predicts a direct interaction between retinol and genomic components, is certainly supported by the speed with which CRBP mRNA levels increase after refeeding. Whether this increase is due to enhanced transcription and whether retinol repletion produces similar effects in other tissues remain to be determined.

The interaction of retinol with the genome of the cell is believed to be mediated by CRBP. Free retinol is very hydrophobic and associates nonspecifically with lipid fractions, but the CRBP-dependent delivery of retinol to specific sites in isolated nuclei has been demonstrated (31). These sites are still present in isolated chromatin (32), where free retinol shows only a nonspecific interaction with the lipid-rich nuclear envelope. Our finding that CRBP mRNA rapidly accumulates in the lung after retinyl acetate repletion of deficient animals strengthens the hypothesis that CRBP may be an important mediator of vitamin A action. Moreover, it is of interest that retinol is capable of influencing the genomic expression of its own intracellular binding protein in a rapid manner, suggesting a direct interaction of the vitamin with the genome.

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