Cellular retinoic acid-binding protein messenger RNA: levels in rat tissues and localization in rat testis

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Abstract Studies were conducted to explore the tissue- and cell-specific regulation of cellular retinoic acid-binding protein (CRABP) expression in the rat. Two studies were carried out. The first explored the regulation of CRABP mRNA levels in selected rat tissues by dietary retinoid status, and the relationship between CRABP mRNA and protein levels in different tissues. The second examined the cellular localization of CRABP expression in the testis. In order to conduct these experiments, a cDNA encoding CRABP was isolated and characterized. The DNA sequence of the coding region had 96% identity with that of the mouse CRABP cDNA and encodes a protein identical to mouse and bovine CRABP. CRABP mRNA and protein levels were quantified in five tissues from normal, retinoid-deficient, and retinol-repleted rats. Tissue CRABP and CRABP mRNA levels were highly correlated ($P < 0.01$) indicating that intertissue variability of CRABP levels mainly results from regulation of CRABP mRNA levels. Neither CRABP protein nor mRNA levels were affected by retinol deficiency, in marked contrast with results previously demonstrated with cellular retinol-binding protein (CRBP) (*J. Lipid Res.* 1990. **31:** 821–829). ^{35}S-labeled CRABP cRNA probes were used to localize CRABP mRNA within the testis of adult rats by in situ hybridization. CRABP mRNA was localized selectively in the periphery of the seminiferous tubules, primarily in type A spermatogonia. The localization of CRABP mRNA differs from that of CRABP protein, which is known to be enriched in maturing and more mature germinal cells. This difference suggests that CRABP in germ cells may be highly stable, remaining in the maturing germ cells without degradation long after CRABP mRNA levels have declined to very low levels. The specific localization of CRABP mRNA and protein presumably reflects the biological roles of retinoic acid in the development and/or later function of germinal cells.—**Rajan, N., G. L. Kidd, D. A. Talmage, W. S. Blaner, A. Suhara, and D. S. Goodman.** Cellular retinoic acid-binding protein messenger RNA: levels in rat tissues and localization in rat testis. *J. Lipid Res.* 1991. **32:** 1195–1204.

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In vertebrates, retinoids (vitamin A) represent an essential class of nutrient, required for the maintenance of differentiated epithelial structures, vision, reproductive fitness, and health (1). The most abundant retinoid found in animal tissues and in the diet is retinol, often present and stored as retinyl esters (1, 2). Recently, however, a great deal of scientific interest has been focused on the carboxylic acid analog of retinol, retinoic acid. Retinoic acid is a highly active and naturally occurring retinoid, that has multiple effects on cell growth and differentiation (3–6), and that appears to be an endogenous morphogen in embryogenesis (7, 8). The effects of retinoic acid on gene expression are thought to be mediated by a family of nuclear receptors that function as ligand-dependent transcription factors (9–13).

Within cells, retinoids in the cytoplasm are normally found associated with specific intracellular retinoid-binding proteins (14). One of these proteins, cellular retinol-binding protein (CRBP) specifically binds retinol and does not bind retinoic acid; another related protein, cellular retinoic acid-binding protein (CRABP), specifically binds retinoic acid and does not bind retinol. These binding proteins are thought to be involved in the cellular uptake and in the intracellular transport and metabolism of their retinoid ligands. They may also play a role in the functional expression of their retinoid ligands. For example, CRABP may be involved in regulating the transport of retinoic acid to the nuclear retinoic acid receptors which in turn mediate retinoid effects on gene expression.

CRABP is a 136 amino acid protein, with a single high affinity binding site for retinoic acid, which belongs to a family of small lipid binding proteins that includes CRBP,

Abbreviations: CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; PBS, phosphate-buffered saline; RBP, retinol-binding protein; SSC, saline, sodium citrate (150 mM NaCl, 15 mM Na citrate); TCA, trichloroacetic acid.

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liver and intestinal fatty acid-binding proteins, and myelin P2 (14, 15). CRABP has been detected and quantified in all rat tissues examined (21 in males and 18 in females) (16, 17). The levels of CRABP vary widely from tissue to tissue, and are minimally influenced by retinoid nutritional status or by sex (16, 17). The regulatory factors that maintain these tissue specific levels are not known. CRABP is not absolutely required for retinoic acid responsiveness; thus, cell lines, for example the premyelocytic leukemia HL60 cell line, have been reported to lack CRABP but still respond to retinoic acid (18).

With the exception of skin, in the rat the highest adult tissue levels of CRABP were found in the reproductive tract (16, 17). In the rat testis, CRABP has been localized primarily in cells in the adluminal compartment, by both immunohistochemistry (19) and radioimmunoassay (20) studies. In contrast, CRBP in the testis is localized primarily in Sertoli cells (19–21). We have previously pointed out (22) that the high concentrations of CRABP found in germinal cells suggest that retinoic acid is important in the development and/or later function of these cells. Thus, more information about the expression of CRABP and its regulation, particularly in the testis, might provide insights into the biological function of this protein.

In the present study we have isolated a cDNA for rat CRABP and used it as a hybridization probe to address questions regarding the regulation of CRABP mRNA expression in male rats. We now report detailed data on the levels of CRABP mRNA in various rat tissues, and demonstrate that the tissue levels of CRABP mRNA and protein are highly correlated. The tissue levels of CRABP mRNA were not altered in response to changes in nutritional retinol status, in contrast to the changes previously observed in CRBP mRNA levels (23). These two related retinoid binding proteins are clearly under quite distinct regulation. And lastly, we report that CRABP mRNA is localized primarily to type A spermatogonia within the testis.

EXPERIMENTAL PROCEDURES

Animals, diets, and tissue processing

The diet study reported here represents the extension of the study recently reported (23) on the effects of changes in nutritional retinol status on the tissue distribution and levels of CRBP mRNA. Portions of the same tissue RNA samples used in the previous study of CRBP mRNA were used for the present study of CRABP mRNA. The design of this diet study and the methods used to prepare tissue extracts and to isolate total tissue RNA are described in detail in the previous report (23). In brief, weanling male Sprague-Dawley rats were housed individually in a virus- and pathogen-free environment. The rats were divided into two diet groups: control and retinoid-deficient. After 10–11 weeks on the diets, and when the serum retinol levels of the retinoid-deficient rats were <3 μg/ml, four pairs of rats from each diet group were killed and their tissues were used for the study. One rat from each pair was used for CRABP mRNA measurements (the same rats were previously (23) used for CRBP mRNA assays), and the other for CRABP protein measurements (the same rats used for CRBP assays). Some retinoid-deficient rats were repleted orally with 150 μg retinol and killed 2, 4, 6, or 8 h thereafter. Retinoid status was assessed by measuring serum and liver retinol and RBP levels.

Isolation of a rat CRABP cDNA

A rat CRABP cDNA clone was isolated from a cDNA library prepared in the vector lambda gt10, with cDNA synthesized from 14 day, embryonic spinal cord poly A+ RNA. The unamplified library was screened using a bovine CRABP cDNA (24) probe labeled by nick translation (25). Two positive plaques were isolated from 250,000 that were screened. The larger of the two inserts (680 bp) was subcloned into pGem2 and sequenced by the dideoxy-chain termination method using T7 DNA polymerase (26). The entire sequence was determined by sequencing overlapping deletions generated with exonuclease III (27).

Northern blot analysis

Total RNA was isolated from rat tissues, resolved by agarose gel electrophoresis in the presence of formaldehyde, and transferred to nylon membranes. CRABP mRNA was identified by hybridization with the ^{32}P-labeled cDNA insert. Blots were washed in 0.2 × SSC/0.1% SDS at 65°C and exposed to XAR-5 film at −80°C using an intensifying screen. Southern blot analysis was performed in a similar manner after resolution of restriction enzyme digestions of rat liver DNA on non-denaturing agarose gels.

Measurement of CRABP mRNA levels

Tissue total RNA samples were stored at −70°C for several months, until used for CRABP mRNA assay. In order to verify that CRABP mRNA levels had not changed during storage, selected samples were assayed for CRBP mRNA as described previously (23). The values for CRBP mRNA so obtained agreed closely with those that had been obtained several months earlier, at the time the original study of CRBP (23) was carried out. Since CRBP mRNA was stable on storage, we assume the CRABP mRNA was comparably stable.

CRABP mRNA levels were determined using an RNase protection assay similar to the one recently reported for CRBP mRNA (23). A ^{32}P-labeled CRABP antisense

(hybridizing) RNA probe was synthesized from the CRABP cDNA clone (28). Accurately measured amounts of total RNA were hybridized to the cRNA probe for 16 h at 55°C. Non-hybridized RNA and probe were removed by digestion with RNases A and T1, and the amount of CRABP mRNA was quantified by scintillation counting after TCA precipitation of the protected hybrids (23). All RNA samples from a given tissue (across all diet groups) were assayed in a single assay. A single preparation of seminal vesicle RNA (pooled from several rats) was used as a reference standard in each assay. The absolute level of CRABP mRNA in this reference standard was determined by comparing the amount of ^{32}P-labeled CRABP cRNA probe protected by a given amount of standard seminal vesicle total RNA, with the amount of the same labeled probe protected by a known amount of sense strand CRABP RNA in a single RNase protection assay. As in our previous study (23), tissue CRABP mRNA levels are expressed in terms of pg CRABP mRNA per μg total RNA. The values have not been corrected to reflect the differences between probe size (680 nt) and mRNA size, since the latter is not precisely known.

Measurement of CRABP levels

CRABP protein levels were measured by a radioimmunoassay as described previously (16). These assays were carried out at the time of the original study (23), concurrent with the radioimmunoassays for CRBP.

In situ hybridization

CRABP mRNA was localized in sections of rat testis by in situ hybridization using methods essentially identical to those previously described for the localization of CRBP mRNA in testis and epididymis (22). Briefly, testes were obtained from adult (250–300 g) rats that had been anesthetized with isoflurane and perfused through the systemic circulation with 4% paraformaldehyde/0.5% glutaraldehyde in PBS. The testes were fixed further in the same solution at 4°C overnight, transferred to 15% sucrose in PBS for 24 h at 4°C, and then embedded, frozen, sectioned, mounted, and stored at −70°C as previously described (22). ^{35}S-Labeled CRABP cRNA probes were synthesized in vitro and used for in situ hybridization. The procedures used for preparation of the cRNA probes and for hybridization of tissue sections were identical to those described elsewhere (22, 29).

Other methods

The seminiferous tubules in testis sections were classified as being in one or another of the stages of the cycle of the seminiferous epithelium, using the classification of Roosen-Runge and Giesel (30), as described previously from this laboratory (21, 22). The statistical methods used included linear regression analysis to examine correlations between CRABP mRNA and protein levels, and *t*-test comparisons of CRABP mRNA and protein levels for given tissues between diet groups (31).

RESULTS

Molecular cloning of rat CRABP cDNA

A rat embryonic spinal cord cDNA library was generated and screened with a ^{32}P-labeled bovine CRABP cDNA probe (24). Two positive plaques contained inserts of nearly identical size. The larger, 680 nucleotide insert was sequenced and found to contain a single open reading frame of 411 nucleotides starting with an in-frame ATG at nucleotide 53. This ATG codon was preceded by the sequence CCACC, which corresponds well with the proposed translation initiation signal of Kozak (32). The nucleotide sequence of the cDNA clone and the inferred amino acid sequence are shown in **Fig. 1.** The total CRABP coding region encodes a protein of 136 amino acids excluding the initiating methionine. The cDNA also contains 52 bp of 5' and 217 bp of 3' non-coding sequences. Its 3' end falls one nucleotide short of the polyadenylation signal found in mouse and bovine CRABP cDNA (6). The region encoding CRABP is highly conserved between rat and mouse (Fig. 1), showing 96% nucleotide sequence identity and 100% amino acid sequence identity. The amino acid sequences of rat, mouse, and bovine CRABP are therefore all identical (6, 24, 33, 34).

A single CRABP mRNA was detected by Northern blotting of total RNA from rat embryonic spinal cord, seminal vesicles, and testes (**Fig. 2**). The testis CRABP mRNA was reproducibly slightly larger than the approximately 1.0 kb CRABP transcript seen in seminal vesicle and spinal cord RNA. These different sized mRNAs did not appear to be products of distinct genes. Seven separate restriction digests (with different enzymes) of rat liver DNA were examined by Southern analysis; in most cases only a single high molecular weight hybridizing band was seen (data not shown). These results are consistent with those reported by Wei et al. (24). CRABP mRNA was not detected in total RNA from liver or stomach under these high stringency conditions (blots were washed in 0.2 × SSPE at 65°C or approximately at Tm = −15); however, additional CRABP related mRNAs of about 0.7 kb (seminal vesicle), 1.6 kb (testis), 1.9 kb (all tissues), 2.2 kb (testis and stomach), and 4 kb (stomach) were seen when blots were probed and washed under reduced stringency (at approximately Tm = −40).

Retinoid nutritional status of the rats

The retinoid status of the animals in the diet study was assessed by measuring the retinol and RBP levels in

Fig. 1. Nucleotide sequence of the rat CRABP cDNA and the deduced amino acid sequence of rat CRABP. Positions at which the mouse CRABP cDNA sequence (34) differ from that of the rat are indicated above the rat cDNA sequence. In the mouse cDNA sequence, nucleotide 370 (indicated by T*) was found to be a T by Wei et al. (6) and a C by Stoner and Gudas (34). Gaps inserted into the mouse cDNA sequence to allow alignment with the rat cDNA sequence are indicated by an X. Gaps inserted into the rat cDNA sequence to allow alignment with the mouse cDNA are indicated by a dash. The putative translation initiation consensus sequence is underlined.

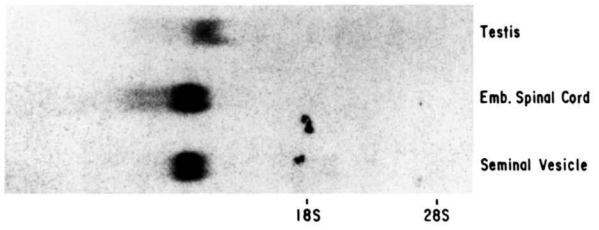

Fig. 2. Northern blot of RNA from rat tissues. Poly A$^+$ RNA from testis (30 μg), embryonic spinal cord (2.5 μg), and seminal vesicle (10 μg) were separated electrophoretically, transferred to a Nytran membrane and hybridized to a ^{32}P-labeled rat CRABP cDNA probe. Washed filters were exposed to X-ray film. CRABP mRNA is seen as a single band of about 1.0 kb in each sample. The positions of the 28s and 18s ribosomal RNAs are indicated. The intense black marks adjacent to the 18s position are exposure artifacts.

serum and liver. These values have been reported elsewhere (23). The control diet contained 2.4 mg retinol per kg of diet and maintained the rats in normal vitamin A status. The rats on the retinoid-deficient diet had very low levels of retinol in serum and liver (mean values of 1.9 μg/dl and 0.4 μg/g, respectively), and low serum (mean 12.2 μg/ml) and high liver (mean 89.0 μg/g) levels of RBP.

CRABP mRNA and protein levels and their correlation

CRABP mRNA and protein levels were measured in five different tissues from each rat: seminal vesicle, skin, testis, distal epididymis, and kidney. The levels of CRABP mRNA found in these five tissues, in each of the three diet groups, are shown in **Table 1.** The mean level of CRABP mRNA ranged from 0.27 pg/μg total RNA in the seminal vesicle to 0.01 pg/μg total RNA in the kidney.

CRABP protein levels for the same five tissues from rats in all diet groups are shown in **Table 2.** The observed CRABP levels were in good agreement with those reported in earlier studies from this laboratory (16, 17). As expected, the highest levels of CRABP were found in the seminal vesicles and the lowest in the kidney. Within each diet group, and in the study population as a whole, CRABP mRNA and protein levels across different tissues were highly correlated ($P < 0.01$); the results obtained in the control group are shown in **Fig. 3.**

Effects of nutritional retinoid status

Nutritional retinol status did not significantly affect the CRABP mRNA levels in any of the tissues examined. Thus, the CRABP mRNA levels observed in the control, the retinoid-deficient, and the retinol-repleted groups of rats were quite similar (Table 1). Similarly, nutritional retinol status did not affect any of the tissue CRABP protein levels (Table 2).

Localization of CRABP mRNA in testis

The localization of CRABP mRNA in the testis was examined by in situ hybridization, using rat CRABP antisense (hybridizing) and sense (non-hybridizing) RNA probes. Strong positive hybridization was seen in all of the approximately 500 seminiferous tubules examined, with very little to no hybridization observed in the interstitial tissue (**Fig. 4**). Within each tubule, specific hybridization was localized, usually in a punctate pattern, in the periphery of the tubule (Fig. 4A). Only a nonspecific background level of silver grains was observed when testis sections were hybridized with the sense CRABP RNA probe (Fig. 4B). At higher magnification, it was seen that the clusters of silver grains (representing localization of CRABP mRNA) were predominantly (90% or more) in type A spermatogonia and to a lesser extent (at most 10%) in type B spermatogonia (Fig. 4C and 4D). Significant hybridization was not seen in more mature germ cells, or in Sertoli, peritubular, or Leydig cells (Fig. 4C and 4D).

The seminiferous epithelium undergoes cyclical changes in morphology, number and degree of maturation of germ cells, and expression of specific proteins such as CRBP (21, 22). The intensity of CRABP hybridization observed around the periphery of a given tubule was related to its stage in the cycle of the seminiferous epithelium. Tubules with the fewest number of clusters, giving a very distinctly punctate pattern, occurred most frequently in stages 8 and 1 (30). Between stages 1 and 6 there was an increase in the number of clusters of hybridization (silver grains) ringing the periphery of the tubule. Tubules in stages 6 and 7 tended to show almost continuous patterns of hybridization around their periphery. This observed varia-

TABLE 1. Tissue CRABP mRNA levels in control, retinoid-deficient, and retinol-repleted rats

Tissue	Control	Retinoid-Deficient	Retinol-Repleted
	CRABP mRNA/total RNA (pg/10^2μg)		
Seminal vesicle	27.0 ± 6.3 (3)	20.2 ± 3.0 (4)	21.3 ± 10.3 (5)
Skin	17.3 ± 0.8 (3)	17.1 ± 7.9 (3)	15.8 ± 2.8 (4)
Testis	6.8 ± 1.0 (4)	6.4 ± 0.4 (4)	6.3 ± 1.3 (5)
Epididymis, distal	2.6 ± 0.1 (3)	1.9 ± 0.3 (3)	1.9 ± 0.5 (5)
Kidney	1.0 ± 0.5 (3)	1.2 ± 0.7 (3)	

The mean (± SD) tissue levels of CRABP mRNA were determined for the number of rats given in parentheses. Within the retinol-repleted group, the CRABP mRNA levels for 2, 4, 6, and 18 h repleted animals are reported; one animal for 2, 6, and 18 h, and two or three animals for 4 h post-retinol repletion were used.

TABLE 2. Tissue CRABP levels in control, retinoid-deficient, and retinol-repleted rats

Tissue	Control	Retinoid-Deficient	Retinol-Repleted
	μg CRABP/g wet tissue		
Seminal vesicle	32.6 ± 8.5	30.6 ± 5.3	33.8 ± 3.1
Skin	14.5 ± 5.0	17.1 ± 1.1	17.5 ± 2.2
Testis	11.6 ± 1.7	11.5 ± 1.2	12.3 ± 1.4
Epididymis, distal	6.0 ± 1.3	7.7 ± 2.9	7.8 ± 2.0
Kidney	2.0 ± 0.4	1.7 ± 0.2	2.0 ± 0.2

The mean (± SD) tissue levels of CRABP were determined from four rats for the control and retinoid-deficient groups. Within the retinol-repleted group mean CRABP levels are reported. Three rats were used for the 4 h retinol-repleted group, and one rat each for the 2, 6, and 18 h repleted group.

tion in hybridization signal pattern presumably reflects changes in the number of spermatogonia A and B associated with the different stages of the spermatogenic cycle (30, 35, 36).[2]

DISCUSSION

The goal of the present study was to explore the regulation of CRABP gene expression and metabolism in adult rats. To achieve this goal, we isolated and characterized a rat CRABP cDNA and used it to study CRABP gene expression in a variety of tissues of rats on normal diets and during retinoid deprivation and repletion, as well as to localize CRABP mRNA within the testis. The present results provide new and quantitative information about: *a*) the levels of CRABP mRNA in various rat tissues; *b*) the relationship between the levels of CRABP mRNA and protein in different rat tissues; *c*) the effects of changes in nutritional retinol status on the tissue levels of CRABP mRNA; and *d*) the cellular localization of CRABP mRNA in the testis.

The 680 nucleotide CRABP cDNA reported here included the entire coding sequence in addition to substantial non-coding sequences. This cDNA encodes a protein identical to mouse and bovine CRABP. This striking conservation of CRABP primary structure across species suggests that the structure of this relatively small protein is very tightly adapted to its biological functions.

Under the hybridization conditions used in all parts of this study, testis, seminal vesicle, and embryonic spinal cord RNA each contained a single species of CRABP mRNA, with the testis CRABP mRNA reproducibly larger than that of the other two tissues. Eskild et al. (37) very recently reported the presence of three CRABP mRNAs of 1.0 kb, 1.8 kb, and 1.9 kb in rat testis RNA. The larger transcripts predominated and were found in both pachytene and round spermatids. These larger transcripts might correspond to the bands that we have observed in a variety of adult tissues when Northern blots were probed at reduced stringency. They were not seen when hybridizations were carried out under high stringency conditions that matched those used in the present studies in either the RNase protection assays or the in situ hybridizations; the larger transcripts were therefore not measured in either of the assays reported here. Since Southern blotting failed to provide evidence of multiple closely related CRABP genes, it seems likely that the size difference seen here for CRABP mRNA in testis as compared to other tissues is due to separate transcription initiation or termination sites, or to differential post-transcriptional processing. Spermatogenic stage-specific changes in mRNA polyadenylation have been reported for the protamine 2 mRNA, with changes in polyadenylation correlated with changes in mRNA translation as spermatids mature (38). Overall, our observations suggest that the larger species of mRNA seen by Eskild et al. (37) do not represent CRABP mRNA.

The nutritional study presented here is an extension of a recent report from our laboratories describing the levels of CRBP mRNA in normal and retinoid-deficient rats (23). Retinoid deficiency substantially reduced CRBP mRNA levels in some rat tissues, including testis, lung, small intestine, and spleen (23). In an earlier and more extensive radioimmunoassay study, retinoid-deficient rats showed lower CRBP protein levels in virtually every one

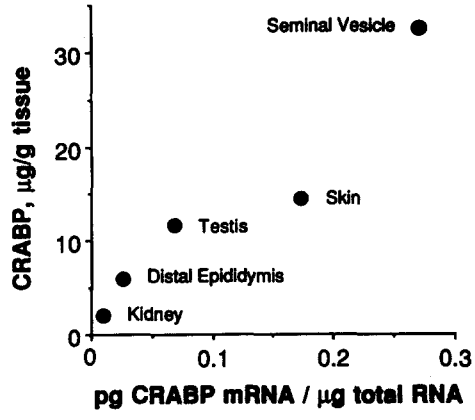

Fig. 3. Relationship between mean tissue CRABP mRNA and protein levels in control rats. The data from the control groups in Tables 1 and 2 were plotted as shown. Regression analysis showed a highly significant correlation between these sets of data ($r = 0.95$ with $P < 0.01$).

[2]Among germ cells, spermatogonia A represent the immature stem cells, from which the more mature germ cells (spermatogonia B, spermatocytes, spermatids) develop. Spermatogonia A and B and the maturing germ cells are identified by morphological characteristics (36). The stages of the cycle of the seminiferous epithelium represent different combinations of germ cell status, including spermatogonia, spermatocytes, and spermatids, that occur in the tubule during the process of spermatogenesis (30, 35, 36).

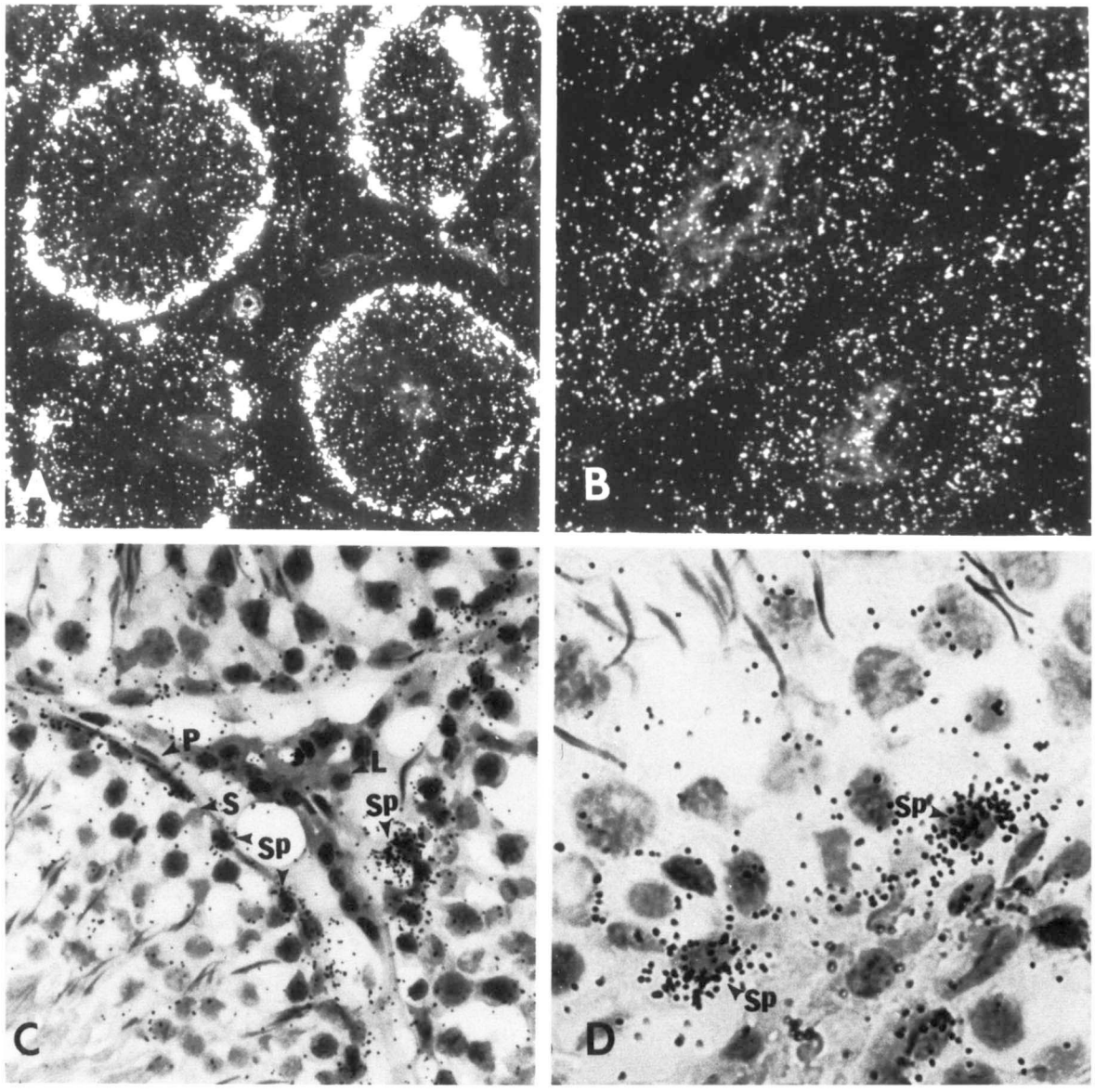

Fig. 4. Localization of CRABP mRNA in rat testis. A: Dark field micrograph of a testis section hybridized with a ^{35}S-labeled CRABP cRNA probe (×300). CRABP mRNA is selectively localized in the periphery of the seminiferous tubules. Note that the silver grains occur in a punctate pattern in some tubules. B: Dark field micrograph of a testis section hybridized with a ^{35}S-labeled CRABP sense RNA probe, indicating the background level of silver grains which represent nonspecific hybridization. Note the absence of the peripheral, punctate pattern seen in panel A. C: Bright field micrograph of a testis section demonstrating the specific localization of CRABP mRNA over spermatogonia A and the absence of hybridization over Sertoli (S), peritubular (P), or Leydig (L) cells (×1200). D: Light field micrograph of a testis section (×2000). CRABP mRNA is seen as clusters of black grains mainly above spermatogonia A (labeled Sp). All sections were exposed to Kodak NTB-2 nuclear emulsion for 6 weeks, developed, and counterstained with hematoxylin-eosin. Hybridized probe is visible as bright grains in A and as dark grains in B and C.

of 21 tissues examined, as compared to control rats (16). Within both control and retinoid-deficient rats, the tissue levels of CRBP mRNA and of CRBP protein were found to be highly correlated (23). By 2–18 h after oral repletion with retinol, the CRBP mRNA levels in the lung, testis, spleen, and small intestine had all returned to control values (23). On the basis of these and other (6) observations, we have suggested (6, 23) that in some tissues and cells, retinoids may act to directly influence CRBP gene expression.

In distinct contrast to CRBP, retinoid deficiency had no effect on the tissue levels of CRABP mRNA or protein. These observations complement and extend the previous radioimmunoassay studies from our laboratory (16, 17) that showed that CRABP levels in 20 of 21 rat tissues were not influenced by retinoid nutritional status. In the earlier study (16), only the skin CRABP level showed a slight but statistically significant decrease in retinoid-deficient rats. This effect was not seen in the present study, either at the protein or the mRNA level. In the earlier study (16) the

rats on the retinoid-deficient diet were probably clinically more retinoid-deficient at the time of killing than the deficient rats in the present study, and this may have accounted for this single difference. Within both the control and the retinoid-deficient groups, the tissue levels of CRABP mRNA and protein were found to be highly correlated. It thus appears that inter-tissue variability of CRABP levels results from regulation and variation of CRABP mRNA levels. The data suggest that tissue levels of CRABP are controlled mainly through control of CRABP synthesis, via factors that regulate CRABP mRNA levels.

The distinct and different regulation of CRBP and CRABP expression by retinoids has also been demonstrated in P19 embryonal carcinoma cells (6). In these cells, retinoic acid induced CRBP mRNA expression within 3 h independently of protein synthesis, whereas CRABP mRNA expression was induced at a considerably later time (12–24 h), and required protein synthesis. It was concluded that CRABP induction in P19 cells may be an indirect response and therefore a later event in retinoic acid-induced cell differentiation. These in vitro data support the present and earlier (16, 17) in vivo studies that indicate that, unlike CRBP, CRABP gene expression and metabolism appear to be minimally influenced by retinoid nutritional status and by the availability of retinoid ligands. Thus, the mechanisms that regulate CRABP and CRBP expression appear to be different, and the factors that regulate tissue-specific expression of CRABP remain to be determined.

The localization and levels of CRABP in different testicular cells have been studied by immunohistochemistry (19) and by radioimmunoassay of separated and cultured cell preparations (20, 39). Porter et al. (19) found positive antibody staining for CRABP in late spermatocytes and spermatids, but no staining in spermatogonia, early spermatocytes, or Sertoli cells. Blaner, Galdieri, and Goodman (20) also found that isolated pachytene spermatocytes and spermatids were highly enriched in CRABP. However, their data with Sertoli cell preparations free of germ cells, compared to preparations containing germ cells, suggested that spermatogonia and primary spermatocytes also are relatively enriched in CRABP (20). Low but definite levels of CRABP have also been found by radioimmunoassay in isolated and cultured Sertoli cells (20, 39). Since CRABP mRNA was not detected by hybridization in Sertoli cells in the present study, the results suggest that the CRABP protein detected in these cells resulted from translation of low abundance mRNA. In contrast to CRABP, both CRBP protein and mRNA are known to be localized mainly in Sertoli cells (19–22).

The present finding that CRABP mRNA is localized mainly in spermatogonia, particularly in spermatogonia A, is surprising and of interest. This finding demonstrates that, whereas CRABP protein is highly enriched in maturing and more mature germinal cells, CRABP mRNA is mainly localized in the immature stem cells. The lack of visible hybridization of CRABP cRNA probes to later germinal cells indicates that, although tissue levels of CRABP mRNA and protein correlated well, within specific cell types in a given organ or tissue, this correlation need not hold. The absence of detectable CRABP mRNA in the more mature germ cells that are enriched in CRABP suggests that CRABP synthesis might be regulated translationally, with translation and accumulation of CRABP coupled to destabilization and degradation of its mRNA. Moreover, the data suggest that CRABP in germ cells may be highly stable, remaining in the maturing germ cell without degradation, long after CRABP mRNA levels have declined to values below the limits of detection of in situ hybridization.

The high levels of CRABP found in male germ cells presumably reflect an important biological need and role for retinoic acid in the development and differentiation of these cells, and/or in the later function of the mature sperm. Retinoids in developing germ cells in the testis probably arise via transfer from Sertoli cells (see reference 22 for a more extended discussion of this point and of the role of Sertoli cells in spermatogenesis). It is not known whether Sertoli cells transfer retinol to germ cells, followed by its conversion to retinoic acid in the germ cells, or whether retinol is converted to retinoic acid in Sertoli cells, followed by retinoic acid transfer to the developing germ cells. Recent studies (40, 41) have demonstrated that vitamin A deficiency in rats leads to the developmental arrest of type A spermatogonia. These observations suggest that retinoids are required for the maturation of spermatogonia into primary spermatocytes. It is striking that CRABP gene expression is most pronounced and regulated in these same immature germ cells, perhaps playing a role in the retinoid-regulated maturation of these cells. Although the function of CRABP is currently not known, it is also conceivable that accumulated holo-CRABP in male germ cells might deliver retinoic acid to the fertilized ovum, thus serving as a source for this morphogen, adding to whatever endogenous retinoids might be in the ovum prior to the establishment of retinol transport across the placenta.

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