Retinyl Ester Storage Is Normal in Transgenic Mice with Enhanced Expression of Cellular Retinol-Binding Protein Type I

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ABSTRACT This report describes the production and characterization of transgenic mice with high expression of human cellular retinol-binding protein type I [hCRBP(I)]. In initial experiments, overexpression of hCRBP(I) was driven by the strong promoter SRα, but no transgenic offspring were produced. When we used the less efficient mouse metallothionein I promoter fused to the hCRBP(I) cDNA for microinjection, we obtained 12% transgenic offspring. Two of these transgenic mice (409/1 and 401/2) expressed mRNA and immunoreactive hCRBP(I) in several organs. Both lines had relatively high contents of hCRBP(I) in intestine, testis and epididymis. On the other hand, only 401/2 transgenic mice had high contents of hCRBP(I) in kidney. Effects on storage of vitamin A were studied by measuring the concentration of retinyl esters in different organs. The concentrations of retinyl esters in liver, lung and kidney did not significantly differ between transgenic and control mice, and the concentration of total retinol in plasma was within the normal range in transgenic mice. Furthermore, feeding mice a diet with high or low concentrations of vitamin A for 2 wks resulted in no marked differences in the concentrations of retinyl esters in liver, kidney, lung, intestine and testis in transgenic mice compared with control mice. Therefore, in spite of high expression of hCRBP(I) in several organs, the transgenic mice had normal storage of retinyl esters in all organs studied. The present in vivo study indicates that the CRBP(I) content alone does not control retinyl ester storage. J. Nutr. 126: 2709–2719, 1996.

INDEXING KEY WORDS:
• vitamin A • cellular retinol-binding protein I
• transgenic mice • metallothionein promoter

Within cells, retinoids are found associated with specific intracellular retinoid-binding proteins, nuclear receptors or in large lipid droplets in specialized storage cells. The intracellular binding proteins solubilize and protect their ligands within the aqueous environment and are believed to be involved in the regulation of retinoid metabolism [reviewed by Ong et al. 1994].

For retinol, two intracellular binding proteins have been characterized: cellular retinol-binding protein type I [CRBP(I)]3 and type II [CRBP(II)] [Ong et al. 1994]. These proteins belong to a family of cytosolic proteins whose members bind small hydrophobic ligands, such as retinoids and fatty acids [Sweetser et al. 1987]. In rats, CRBP(I) is present in a wide variety of organs, whereas only the small intestine contains high levels of CRBP(II) in adult rats [Crow and Ong 1985].

Recent data suggest that CRBP are important for proper intracellular metabolism of retinol. The esterification of retinol with long-chain fatty acids by the enzyme lecithin:retinol acyltransferase (LRAT) uses retinol in complex with CRBP(I) or CRBP(II) as a substrate [Herr and Ong 1992, Ong et al. 1987 and 1988]. Furthermore, the hydrolysis of retinyl esters to retinol was suggested to be stimulated by apoCRBP(I) [Boerman and Napoli 1991]. It has therefore been hypothesized that storage and hydrolysis of retinyl esters are controlled in part by the relative amounts of apoCRBP(I) and holoCRBP(I)

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3 Abbreviations used: CRBP[I], cellular retinol-binding protein type I; CRBP[II], cellular retinol-binding protein type II; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; LRAT, lecithin:retinol-acyl transferase; MT, metallothionein, PCR, polymerase chain reaction.

0022-3166/96 $3.00 © 1996 American Institute of Nutrition.
Manuscript received 8 February 1996. Initial review completed 19 March 1996. Revision accepted 1 July 1996.

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(Napoli 1994). Recently, it was demonstrated that retinyl ester synthesis was increased up to 1.8 times by coexpression of CRBP[I] or overexpression of CRBP[II] in the human intestinal Caco-2 cell line (Liosoos et al. 1995). These studies indicated that CRBP[I] and CRBP[II] contents are determinants of intracellular esterification.

To investigate in vivo the hypothesis that the contents of CRBP[I] control the metabolism of retinol, we produced transgenic mice expressing the human CRBP[I] gene under the control of the mouse metallothionein I promoter. In this report, we addressed whether overexpression of CRBP[I] controls the ability to produce and store retinyl esters in different organs of these animals.

**MATERIALS AND METHODS**

**Construction of vectors.** The PstI fragment containing the complete protein coding region of the human CRBP[I] cDNA (provided by Ulf Eriksson, Ludwig Institute for Cancer Research, Stockholm, Sweden) (Colantuoni et al. 1985) was inserted into the XhoI site of the expression vector pCDL-SRα296 (provided by Hiroyoshi Ariga, The Institute of Medical Science, The University of Tokyo, Japan) (Takebe et al. 1988) to produce the vector SRα-hCRBP[I] (Fig. 1, top).

A mouse metallothionein [mMT] promoter– regulated CRBP construct [mMT-hCRBP[I]] (Fig. 1, top) was then produced by exchanging the MscI-Csal fragment [i.e., SRα promoter] of the SRα-hCRBP vector with the promoter and transcription initiation site derived from the mouse metallothionein I gene [EcoRI-BamHI fragment of the mouse metallothionein-human growth hormone fusion plasmid, MTHGH111] (Palmiter et al. 1983).

**Production of transgenic mice.** Transgenic mice were produced according to standard procedures (Hogan et al. 1986). F2 zygotes from mated F1 [C57BL/6] × CBA/J, Hartland Olac, Blackthorn, U.K.]) hybrids were microinjected with a solution of 1 mg/L of gene construct in 10 mmol/L Tris-HCl (pH 7.4) with 0.25 mmol/L EDTA, using a Leitz micromanipulator system.

Animals were treated in accordance with the ethics rules of the Norwegian government. Collection of organs was performed after anesthesia of mice with Domicum-Hynnorm [1:1]. Organs were washed with cold PBS (Dulbecco’s PBS, BioWhittaker, Walkerville, MD) pH 7.4 and stored immediately in liquid nitrogen until use.

**Diets.** Mice consumed food and water ad libitum, and body weights were recorded routinely. Mice were fed a nonpurified diet (EWAR Sverige AB, Södertelje, Sweden) containing 4.3 mg retinyl acetate/kg. A vitamin A–enriched diet (Special Diets Services, Witham, Essex, U.K.) containing 40 mg retinyl acetate/kg and a vitamin A–deficient diet (Special Diets Services) containing 0.20 mg retinyl acetate/kg were fed to groups of mice for 2 wk before killing. Except for vitamin A, the diets were similar in composition [approximately 5 g fat, 55 g carbohydrate and 17 g protein per 100 g diet]. To induce expression of the mMT-hCRBP[I], 75 mmol/L ZnSO4 was supplemented in drinking water for 2 wk or 3 mo before killing.

**Polymerase chain reaction analysis.** Transgenic mice were detected by polymerase chain reaction (PCR) analyses as described previously (Innis and Gelbberg 1990). Total DNA was isolated from mouse tails or spleen by proteinase K-phenol extraction as described (Hogan et al. 1986).

**Cultivation and transfection of P19 cells.** P19 embryonic carcinoma cells were cultured in Dulbecco’s...
modified Eagle’s medium containing 10% fetal calf serum, penicillin [10^6 U/l], streptomycin [0.17 mmol/L], amphotericin B [2.7 μmol/L], gentamycin [0.2 g/L] and L-glutamine [2 mmol/L] (Flow Laboratories, McLean, VA). Cells were transfected 5–6 h after plating by the calcium phosphate method (Cullen 1987), with 10 μg of SRα-hCRBP[I], mMT-hCRBP[I] or control plasmid together with 2 μg of the β-galactosidase expression vector pCH110 (Pharmacia, Stockholm, Sweden) used as an internal standard. The total amount of DNA in each transfection was standardized to 20 μg using carrier DNA (pBluescript, Stratagene Cloning Systems, La Jolla, CA). The medium was changed after 20 h. After an additional 24 h (in the absence or presence of 75 μmol/L ZnCl₂ as indicated), the cells were harvested and cellular extract prepared by sonication (2 × 15 s at 50% efficiency) and centrifugation (20,000 × g for 30 min).

**Cultivation, transfection and retinol-binding assay of HL60 cells.** The HL60 cell line was purchased from American Type Culture Collection (Rockville, MD) and cultivated in Iscove medium (Flow Laboratories) with 10% fetal calf serum [Sigma Chemical, St. Louis, MO], gentamycin [0.1 g/L] and L-glutamine [2 mmol/L]. HL60 cells [10⁶] cells were transfected with 10 μg of SRα-hCRBP[I] using the DEAE-dextran (molecular weight 50,000; Pharmacia) method described by Vaheri and Pagano (1965). The DEAE-dextran (250 μg) was diluted with 1.5 mL of buffer [0.025 mol/L Tris-HCl, pH 7.5, 0.14 mol/L NaCl, 0.56 mmol/L Na₂HPO₄, 5.8 mmol/L KCl, 0.9 mmol/L CaCl₂ and 1 mmol/L MgCl₂], and the DNA was added. This solution was then added to the cells and incubated for 30 min. Control cells were treated similarly, but without DNA added to the transfection buffer. Three days after transfection, cell lysate from HL60 cells [10⁶] cells in 200 μL of 0.025 mol/L Tris-HCl, pH 7.8] was prepared by sonication (2 × 10 s at 50% efficiency) and centrifugation (20,000 × g for 30 min). This supernatant was incubated with 0.03 μmol/L [11,12-3H]retinol [2.52 TBq/mmol, TRK 646, Amersham, Buckinghamshire, U.K.] for 3 h. Free retinol was separated from protein-bound retinol by charcoal extraction. The CRBP[I]-retinol complex was separated from other proteins by gel filtration on fast protein liquid chromatography [Superose 12, Pharmacia]. The elution buffer was 0.15 mol/L NaCl and 0.05 mol/L phosphate buffer, pH 7.0; elution speed was 0.5 mL/min. Fractions [0.5 mL] were collected and radioactivity measured in a scintillation counter.

**Preparation of organ extract.** Organs were homogenized with a Dounce homogenizer in five volumes of buffer (wt/v) [0.01 mol/L sodium phosphate, pH 7.5, containing 0.14 mol/L NaCl, aprotinin [24 trypsin inhibitor units/mL], phenethylsulfonyl fluoride [PMSF, 0.5 mmol/L] and leupeptin [1 mmol/L]]. Tissue was taken from the upper half of the small intestine. Organ extracts were prepared by sonication (2 × 15 s at 50% efficiency) and centrifugation (20,000 × g for 30 min). Protein concentrations in the organ extracts were determined by the dye-binding assay of Bradford (1976), according to the protocol from Bio-Rad Laboratories [München, Germany].

**Immunoblot analysis of CRBP[I].** Proteins from cells or organ extracts were separated on a 14% polyacrylamide gel [Tris-Glycine PAGE gels, Novex, San Diego, CA], and the proteins were transferred to polyvinylidene fluoride membranes [LC 2002, Novex] by electrophotography. The membranes were incubated in PBS (pH 7.4) containing 0.1% Tween 20 overnight at room temperature. The membranes were then incubated for 2 h with a polyclonal rabbit anti-hCRBP[I] antiserum (diluted 1:200 or 1:1000). After washing in PBS with 0.1% Tween 20, the membranes were reacted with biotin-labeled anti-rabbit immunoglobulin G [H+L] from goat (Vectastain, Burlingame, CA). The membranes were washed in PBS with 0.1% Tween 20, and immunoreactive protein was visualized with avidin-biotin peroxidase complex (ABC) [Vectastain] and substrate solution (0.09% 4-chloro-1-naphthol in PBS containing 28% methanol and 0.07% H₂O₂). The production of polyclonal antiserum against recombinant human CRBP[I] will be reported elsewhere. Densitometry was performed using a Computer Densitometer model SI from Molecular Dynamics and the software program ImageQuant [Molecular Dynamics GmbH, Krefeld, Germany]. Contents of immunoreactive protein [i.e., endogenous mouse CRBP[I] plus transgene human CRBP[I] in transgenic mice were related to the mean content in control mice [100%]. Differences between transgenic and control mice were tested as unbalanced repeated measures models with compound symmetry in the covariance matrices [BMDP/Dynamic Release 7.0, 1993, BMDP Statistical Software program 5V]. P values ≤ 0.05 were considered significant.

**Preparation and analysis of mRNA.** To analyze mRNA contents of hCRBP[I], total RNA was first prepared from various organs of both transgenic and control mice by homogenization in guanidine isothiocyanate and centrifugation through cesium chloride as described [Eskild et al. 1991]. Polyadenylated mRNA was then isolated from 150 μg of total RNA using Dynabeads Oligo (dT)₂₅ [Dynal A.S., Oslo, Norway] and separated by electrophoresis in 1.5% agarose gels with 6.7% formaldehyde. The mRNA was transferred to Biotrans nylon membranes [ICN Schwarz/Mann Biotec, Cleveland, OH] [Eskild et al. 1991] and hybridized with a BamHI-SalI fragment of human CRBP cDNA [Colantuoni et al. 1985] labeled with [32P]dCTP [ICN no. 33004X] using a random-priming kit [RPN 1607, Amersham]. The filters were stripped and reprobed with a 32P-labeled cDNA for human glyceraldehyde-3-phosphate dehydrogenase [G3PDH] [Clontech Laboratories, Palo Alto, CA]. Autoradiography was performed using Amersham Hyperfilm MP.

**Determination of retinol and retinyl esters.** Total retinol [retinol plus retinyl esters], retinol and retinyl
FIGURE 2 Studies with cell cultures. Top: Immunoblot analysis of cellular retinol-binding protein I [CRBP(I)] in transfected P19 cells. Cellular extracts from transfected P19 cells (44 μg protein/sample) were separated on a 14% polyacrylamide gel, blotted to membranes and incubated with a polyclonal rabbit anti-human CRBP(I) antiserum diluted 1:200. Column 1: cells transfected with control plasmid. Column 2: cells transfected with SRα-hCRBP(I). Column 3: cells transfected with MT-hCRBP(I). Column 4: cells transfected with MT-hCRBP(I) and incubated with 75 μmol/L ZnCl₂ for 24 h. Data are presented as arbitrary values obtained after densitometric scanning of the bands visualized on the membrane. This experiment was repeated once, with similar results. Bottom: Analysis of [3H]retinol-binding in cell lysates from HL60 cells. Cell lysates were incubated with [3H]retinol and extracted with charcoal, and cell proteins were separated by fast protein liquid chromatography (Superose 12 column). The diagram illustrates the radioactive retinol bound to cell lysates from control cells (○) and cells transfected with SRα-hCRBP(I) (●). The peak between fractions 25 and 28 describes the radioactive retinol bound to the CRBP(I) protein.

FIGURE 3 Expression of mRNA for hCRBP(I) in different organs from transgenic and control mice. Two transgenic lines were designated 401/2 and 409/1. The founder mice were F₀, with subsequent generations indicated by the given number in subscript. Northern blots with mRNA isolated from different mouse organs were probed successively with [32P]-labeled cDNA for human cellular retinol-binding protein I [hCRBP(I)] and glyceraldehyde-3 phosphate dehydrogenase [G3PDH]. Above: Organs from the two founder lines, 409/1 and 402/1, and control mice. Left, next page: Organs from transgenic and non-transgenic littermate [control] from 409/1-F₂. Right, next page: Organs from transgenic and non-transgenic littermate [control] from 402/1-F₂.

Transfection of cells with SRα-hCRBP(I) and mMT-hCRBP(I). To confirm translation of protein from the

RESULTS

Transfection of cells with SRα-hCRBP(I) and mMT-hCRBP(I). To confirm translation of protein from the
gene constructs, P19 cells were transfected with either SRα-hCRBP[I] or mMT-hCRBP[I], and immunoreactive protein was determined by Western analysis and densitometry. As shown in the top portion of Figure 2, very little CRBP[I] was detected in control P19 cells, whereas cells transfected with SRα-hCRBP[I] gave a strong CRBP[I] reaction. A much weaker expression of CRBP[I] was observed in cells transfected with mMT-hCRBP[I]. A 100% increase in CRBP[I] protein was observed when such cells were incubated with 75 μmol/L ZnCl₂. These data are in accordance with the expected relative efficiencies of the different promoters used and demonstrate that immunoreactive CRBP[I] is produced from the gene constructs. In a parallel study, in which we transfected HL60 cells with the SRα-hCRBP[I] construct, it was shown by retinol-binding assay that hCRBP[I] retained its retinol-binding capacity (Fig. 2, bottom).

Production of transgenic mice. The HindIII-Thh111I fragment (1745 base pairs) of SRα-hCRBP[I] was microinjected into mouse zygotes as described in Materials and Methods. Nine or 10 microinjected zygotes were transferred into oviducts of seven pseudopregnant mice. Eleven offspring were born from these recipients, and none of the offspring were found to be transgenic by PCR analysis. The number of offspring obtained was low compared with the number of offspring obtained after control injections or injection of other gene constructs in parallel studies (6–9 offspring per mouse and about 22% transgenic mice).

Transgenic mice carrying the mMT-hCRBP(I) construct. In the next series of experiments, the EcoRI-Thh111I fragment (2805 base pairs) of mMT-hCRBP[I] was microinjected into zygotes and transferred to eight pseudopregnant mice. Three to six offspring were born per recipient (total 33 offspring), and four of these (12%) were found to be transgenic by PCR analysis. As shown in the bottom part of Figure 1, the PCR product produced from DNA preparations of mouse tails had the expected size of 373 base pairs. Two of the four transgenic founder lines were studied further. When these two mice (401/2 and 409/1) were 17 mo of age and had received zinc supplementation for 2 wk, liver, kidneys and testes were removed and subjected to Northern analysis. As shown in the top part of Figure 3, control mice (10 mo of age) expressed endogenous CRBP[I] mRNA in all organs stud-
The transgenic mice had higher CRBP[I] mRNA levels in several organs compared with the endogenous contents in control mice. Expression of the transgene varied among different organs and also between the two transgenic founder mice (401/2 and 409/1). Compared with the control mice, mouse 401/2 had greater levels of CRBP[I] mRNA in kidney and testis. No markedly higher expression was found in liver. Mouse 409/1 had greater expression of the transgene in both liver and kidneys (Fig. 3).

**Northern analysis of CRBP[I] in transgenic F2 and F3 generations.** To confirm expression of the transgene in subsequent generations from the two founder lines, we analyzed CRBP[I] mRNA contents in mice from the F3 generation of 409/1 and in the F2 generation from 401/2 in several organs. As shown in Figure 3 (left and right), a high level of CRBP[I] mRNA was observed in testis, intestine and epididymis of both transgenic lines compared with non-transgenic littermates (control mice). In addition, the 409/1-F3 transgenic mice [Fig. 3, left] had greater content of CRBP[I] mRNA in liver than did the control mice, whereas the 401/2-F3 transgenic mice [Fig. 3, right] expressed high contents of hCRBP[I] mRNA in kidney. Neither of the transgenic founder lines expressed elevated CRBP[I] mRNA contents in the lungs as compared with controls.

**Immunoblot analysis of CRBP[I] in transgenic F2 and F3 generations.** The contents of immunoreactive CRBP[I] protein [i.e., endogenous mouse CRBP[I] plus transgene human CRBP[I]] in organs from the F2 generation of 409/1 and F2 generation of 401/2 were analyzed. Figure 4 shows a typical result obtained with liver extracts from transgenic mice and non-transgenic littermates (control mice). Although the content varied among the transgenic mice from the two transgenic lines, a generally higher content of immunoreactive CRBP[I] was observed in the liver of transgenic mice compared with the control mice. In line 409/1, the mean relative content of CRBP[I] [relative contents determined by densitometry] in transgenic mice [relative to the mean contents in control mice (100%)] was 285% [18 observations, four mice], and in line 402/1 the mean relative content was 162% [14 observations, four mice].

In kidneys [Fig. 4], the content of CRBP[I] was not different from the value for controls in the 409/1-F3 transgenic mice [mean relative content of 108% [nine observations, four mice]]. The 401/2-F2 transgenic mice, however, had significantly more immunoreactive CRBP[I] compared with the controls [relative mean content 531% [seven observations, four mice]]. The contents of immunoreactive CRBP[I] in lung from transgenic mice were not different from those in controls [Fig. 4]. The mean relative CRBP[I] content in 409/1-F3 transgenic mice was 111% [12 observations, four mice], whereas the mean relative content in the 401/2-F2 transgenic mice was 104% [nine observations, four mice].

Figure 4 shows the results obtained with testes from the two founder lines. The mean content of CRBP[I] in testes from transgenic 409/1-F3 was 15 times greater than for the non-transgenic littermates [mean relative content 1530% [10 observations, two mice]]. Similarly, testes from 401/2-F3 transgenic mice showed a six times higher content of immunoreactive protein [mean relative value 5.9% [six observations, two mice]]. Significant differences in the levels of CRBP[I] (P < 0.05) between transgenic and control mice were found in testes and liver from both transgenic lines and in kidney from line 409/1. No significant differences were found for lung from both lines or for kidney of line 401/2.

To confirm that the hCRBP[I] protein retained the ability
to bind retinol, we examined the specific binding of retinol to extracts from testes by the retinol-binding assay. The retinol-binding capacity of CRBP[I] was seven times higher in transgenic mice extract compared with extracts from control mice (data not shown), indicating that hCRBP[I] expressed in mice retained its retinol-binding capacity.

Finally, we analyzed the content of immunoreactive protein in intestine. As shown in Figure 4, high contents of CRBP[I] were detected in intestine from both transgenic founder lines. In non-transgenic littermates (control mice), CRBP[I] immunoreactive protein was not detected. In both transgenic and control mice, an additional protein of higher molecular weight was recognized by the antiserum. Because CRBP[III], a homologue of CRBP[I], is slightly larger than CRBP[I] (Crow and Ong 1985) and is abundantly present in intestine (Crow and Ong 1985, Ong et al. 1994), this band probably represents cross-reactivity of the hCRBP[I] antiserum with CRBP[III].

In summary, the expression of CRBP[I] protein in liver, kidney, lung, testis and intestine is similar to that found using Northern analysis.

Zinc induction of hCRBP[I]. In the next series of experiments, the effect of zinc induction on the expression of hCRBP[I] was studied in 8-wk-old mice after 2 wk of zinc supplementation. Zinc induced the expression of CRBP[I] mRNA in the intestine and kidney of the 401/2-F3 transgenic mice compared with non-transgenic littermates (control mice) [Fig. 5]. In testes, however, high contents of CRBP[I] mRNA were observed regardless of zinc supplementation. These data were confirmed by Western analysis in both 401/2-F3 and 409/1-F1 [Fig. 6]. Higher contents of CRBP[I] were found in intestine from zinc-supplemented transgenic mice compared with transgenic mice given ordinary drinking water. No additional effect, however, was observed in testes after zinc induction.

Vitamin A concentrations in liver, kidney, lung and plasma. The vitamin A concentrations in liver, kidney, lung and plasma were first analyzed in the two founder lines (409/1-F0 and 401/2-F0). No marked effects on total retinol (i.e., retinol plus retinyl esters) were observed in transgenic mice compared with control mice, although the concentrations in lung were slightly lower in both lines of transgenic mice and the concentration of total retinol in kidney from 409/1-F0 was higher than in controls (Table 1). We examined this further by measuring the vitamin A concentrations in transgenic offspring using non-transgenic littermates as controls. No difference in the total retinol concentration was observed in liver, lung or kidney from 401/2-F2, (Table 1). When we induced the content of hCRBP[I] by zinc supplementation in the drinking water for 3 mo, the concentration of total retinol was still not different in transgenic mice compared with control mice (Table 1). The concentration and distribution of retinyl esters did not differ in transgenic mice and control mice (data not shown).

We then examined whether different amounts of vitamin A in the diet for 2 wk would affect total retinol concentrations in transgenic mice differently than in control mice. First, we compared the concentration of total retinol in liver, lung and kidney obtained in transgenic and control mice after feeding a diet with a 10 times higher concentration of vitamin A compared with a normal diet (Table 1). No difference was observed between transgenic and control mice. Furthermore, feeding the mice a diet low in vitamin A (about 1/20 the concentration of vitamin A as in control diet) resulted in similar concentrations of total retinol in both transgenic and control mice (Table 1).

Thus, despite higher levels of CRBP[I] protein in transgenic mice in liver and kidney, no effects on the concentrations of total retinol or retinyl esters were observed.

Vitamin A concentrations in intestine and testis. We found a high level of hCRBP[I] protein in the small intestine, and we examined whether this had an effect on retinyl ester storage. Retinol and retinyl ester concentrations were not different in transgenic mice compared with control mice given a normal diet and zinc supplementation in drinking water for 2 wk (Table 2). After 3 mo of zinc supplementation, no difference was found between transgenic and control mice. We also tested the intestinal concentration of retinol and retinyl esters in mice given a diet enriched with vitamin A and mice fed a vitamin A–deficient diet but did not find any significant differences between transgenic and control mice (Table 2).

In testes we found a significantly higher concentration of retinol but not retinyl ester in transgenic mice fed a normal diet and zinc for 2 wk compared with control mice (Table 3). The concentrations of retinyl esters in testes of mice fed diets with high and low vitamin A concentrations for 2 wk did not differ between transgenic and control mice (Table 3).

**DISCUSSION**

This report describes the production and characterization of transgenic mice that express human CRBP[I]. In our first attempt to produce transgenic mice, we used a strong constitutive promoter (SRa) fused to the hCRBP[I] cDNA. In transfection studies with P19 cells, this construct increased the content of CRBP[I] in the cells. However, no transgenic offspring were produced when this construct was microinjected into zygotes subsequently transferred into pseudopregnant mice. Instead, a low number of offspring were obtained compared with results after injections of other transgenes.

Several studies have shown that CRBP[I] is expressed in a very specific temporal and spatial pattern.
Transgenic
(401/2-F3)

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**FIGURE 5** Expression of mRNA for hCRBP[I] gene in different organs from transgenic mice and non-transgenic littermates (control mice) given ordinary drinking water or drinking water supplemented with 75 mmol/L ZnSO₄ for 2 wk before killing. Two transgenic lines were designated 401/2 and 409/1. The founder mice are F₀, with subsequent generations indicated by the given number in subscript. A Northern blot with mRNA isolated from different organs from 401/2-F₃ was probed successively with ³²P-labeled cDNA for hCRBP[I] [human cellular retinol-binding protein I (hCRBP[I])] and glyceraldehyde-3-phosphate dehydrogenase (G3PDH).

**FIGURE 6** Immunoblot analysis of cellular retinol-binding protein I (CRBP[I]) in testes and intestine from transgenic mice and non-transgenic littermates (control mice) given ordinary drinking water or drinking water supplemented with 75 mmol/L ZnSO₄ for 2 wk before killing. Two transgenic lines were designated 401/2 and 409/1. The founder mice are F₀, with subsequent generations indicated by the given number in subscript. Organ extracts containing 90 µg protein/well (testes) or 128 µg protein/well (intestine) were separated on a 14% polyacrylamide gel, blotted to membranes and incubated with a polyclonal rabbit anti-human CRBP[I] antiserum diluted 1:1000. The data displayed are from one typical immunoblot experiment. Organs were analyzed twice, with similar results. Tg = transgenic mouse, Ctr = control mouse.

during embryonic development in mice (Maden et al. 1989, Perez-Castro et al. 1989). Both excess and deficiency of retinol (or retinoic acid) may cause abortion as well as abnormalities during embryonic development (Kochhar 1973, Thompson et al. 1969). Thus, the low number of offspring and the absence of transgenic mice born when the strong SRα promoter was used may be due to a lethal effect of high contents of CRBP[I] on embryos.

The mouse metallothionein promoter was able to promote the expression of human CRBP[I] in a variety of mouse organs, as shown by Northern and immunoblot analyses. Two lines of transgenic mice (409/1 and 401/2) were produced with different distributions and contents of expression of hCRBP[I]. Both transgenic lines expressed relatively high contents of hCRBP[I] in intestine, testes and epididymis. On the other hand, only the 401/2 transgenic mice had high contents of hCRBP[I] in kidney.

The different pattern of hCRBP[I] mRNA expression in the two lines of transgenic mice is probably due to integration of the injected DNA at different sites and/or in different copy numbers (Palmiter and Brinster 1986). In other studies using the mouse metallothionein promoter (Hamer 1986, Palmiter et al. 1983), the expression of the gene in various organs often follows...
the pattern expected for the endogenous metallothionein gene. A main difference in the expression pattern between hCRBP[I] in the present transgenic mice and the endogenous metallothionein gene is the low expression of the transgene in the liver. However, low transgene expression in liver was also observed in other studies (Kelley et al. 1988, Stenzel-Poore et al. 1992).

The relative amounts of apoCRBP[I] and holocrbp[I] have been hypothesized to control retinol esterification and retinyl ester hydrolysis (for a review, see Napoli 1994). On the basis of these in vitro observations, we examined the effects of overexpression of CRBP[I] on the ability of the transgenic mice to store retinyl esters.

The liver is the main storage site for retinyl esters in the body, and it contains high contents of endogenous CRBP[I] (Kato et al. 1985) and LRAT (Herr and Ong 1992). Similarly, kidneys have high contents of CRBP[I] (Kato et al. 1985). In a parallel study (Astrid Nilsson, MATFORSK, Ås, Norway, personal communication), we found that cultivated liver stellate cells transfected with a SRa-CRPB[I] expression vector esterified more retinol than did control cells. In general, several in vitro studies have suggested that CRBP[I] is a main carrier of retinol as well as a regulator of retinol esterification (for a review, see Napoli 1994). In contrast to these in vitro results, our present in vivo study showed no effects of overexpression of CRBP[I] on retinyl ester storage in liver or kidney.

Schmitt and Ong (1993) showed that CRBP[I] and LRAT are found in testes and that especially the Sertoli cells have high CRBP[I] contents. Overexpression of CRBP[I] had, however, no consequence on retinyl ester storage in testes in transgenic mice.

The small intestine contains high contents of CRBP[I] but very low contents of CRBP[I] (Crow and Ong 1985). Lissoos et al. (1995) recently reported that retinyl ester synthesis is increased 1.8 times in a transfected human intestinal Caco-2 cell line with a two times greater content of CRBP[II] protein. The two transgenic lines studied in the present report expressed high contents of hCRBP[I] in the intestine. We observed, however, that concentrations of retinyl esters in the intestine from transgenic mice were not significantly different from those of control mice. In a parallel study, we found that the large increase in intestinal CRBP[I] in transgenic mice is mainly due to a large expression of hCRBP[I] in enterocytes and to some de-
It has been suggested that apoCRBP[II] regulates retinyl ester hydrolysis [Boerman and Napoli 1991]. Because increased CRBP[II] expression did not change retinol concentrations in most organs, the intracellular apoCRBP[II] contents must have been increased in several organs in our study. Our observation that retinyl ester concentrations were not changed in CRBP[II] transgenic mice therefore does not support the hypothesis that apoCRBP[II] is controlling retinyl ester hydrolysis.

It has also been suggested that CRBP[II] plays an important role in the conversion of retinol to retinoic acid [for a review, see Napoli 1994]. We are therefore currently investigating vitamin A function in these hCRBP[II] transgenic mice. Our approach is to look for signs of vitamin A deficiency or toxicity in different organs. At the same time, we plan to examine in more detail the cell-specific expressions in intestine and testis, the two organs with the highest expression of the transgene.

In conclusion, our present in vivo study demonstrated that the content of CRBP[II] did not control retinyl ester storage in vivo. The data instead suggest that factors other than CRBP[II] contents are determinants of retinyl ester storage in different organs. These factors

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**TABLE 2**

Concentrations of free retinol and retinyl esters in the small intestine from transgenic mice and control mice fed a normal diet, a vitamin A–enriched diet or a vitamin A–deficient diet

<table>
<thead>
<tr>
<th>Diet treatment</th>
<th>Retinol</th>
<th>Retinyl esters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/g intestine</td>
<td></td>
</tr>
<tr>
<td>Normal diet, zinc for 2 wk²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>401/2-F₃ (4)</td>
<td>5.0 ± 1.3</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>Control (4)</td>
<td>4.8 ± 1.5</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td>Normal diet, zinc for 3 mo³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>401/2-F₃ (4)</td>
<td>3.5 ± 1.2</td>
<td>8.6 ± 4.5</td>
</tr>
<tr>
<td>Control (3)</td>
<td>3.8 ± 1.4</td>
<td>10.4 ± 6.5</td>
</tr>
<tr>
<td>Vitamin A–enriched diet, zinc for 2 wk²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>409/1-F₃ (6)</td>
<td>950 ± 320</td>
<td>1520 ± 850</td>
</tr>
<tr>
<td>Control (3)</td>
<td>640 ± 290</td>
<td>1800 ± 920</td>
</tr>
<tr>
<td>Vitamin A–deficient diet, zinc for 2 wk²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>401/2-F₃ (5)</td>
<td>0.27 ± 0.27</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Control (6)</td>
<td>0.25 ± 0.18</td>
<td>1.7 ± 0.4</td>
</tr>
</tbody>
</table>

1 Values are means ± SD. Two transgenic lines were designated 401/2 and 409/1. The founder mice are F₀, with subsequent generations indicated by the given number in subscript. Mice were given zinc in the drinking water for 2 wk or 3 mo as indicated. The diets enriched or deficient in vitamin A were fed to the mice for 2 wk before killing. The numbers of mice analyzed are given in parentheses. Control = non-transgenic littermate.

2 Mice were 8 wk old.
3 Mice were 8 mo old.


Previous reports showed that organ contents of the cellular retinol-binding proteins are regulated by retinoid status [Blaner et al. 1986, Kato et al. 1985]. As expected, vitamin A–enriched or vitamin A–deficient diets led to mice for 2 wk altered the concentrations of retinol and retinyl esters in most organs studied in both control and transgenic mice. However, mice over-expressing hCRBP[II] had concentrations of retinyl esters similar to those in control mice.

Surprisingly, in spite of high expression of CRBP[II] in several organs, we did not observe altered concentrations in organ retinol, except for a significantly higher concentration in testes from transgenic mice fed a normal diet compared with control mice. This is in contrast to the higher retinol binding observed when HL60 cells were transfected with the same hCRBP[II] cDNA construct and to the enhanced retinol binding observed in a fraction from testes in transgenic mice compared with control mice. Thus, higher expression of CRBP[II] in vivo does not simultaneously increase the concentration of the retinol-CRBP[II] complex, which is the substrate for LRAT [Herr and Ong 1992].

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**TABLE 3**

Concentrations of free retinol and retinyl esters in testes from transgenic mice and control mice fed a normal diet, a vitamin A–enriched diet and a vitamin A–deficient diet

<table>
<thead>
<tr>
<th>Diet treatment</th>
<th>Retinol</th>
<th>Retinyl esters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/g testis</td>
<td></td>
</tr>
<tr>
<td>Normal diet, zinc for 2 wk²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>409/1-F₃ (4)</td>
<td>0.24 ± 0.03</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Control (5)</td>
<td>0.14 ± 0.08</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Normal diet, zinc for 3 mo³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>401/2-F₃ (3)</td>
<td>0.30 ± 0.05</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>Control (5)</td>
<td>0.30 ± 0.17</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Vitamin A–enriched diet, zinc for 2 wk²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>401/2-F₃ (5)</td>
<td>0.38 ± 0.15</td>
<td>4.1 ± 1.5</td>
</tr>
<tr>
<td>Control (5)</td>
<td>0.33 ± 0.10</td>
<td>3.1 ± 1.0</td>
</tr>
<tr>
<td>Vitamin A–deficient diet, zinc for 2 wk²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>409/1-F₃ + 401/2-F₂ (3)</td>
<td>0.12 ± 0.04</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>Control (3)</td>
<td>0.10 ± 0.06</td>
<td>0.9 ± 0.4</td>
</tr>
</tbody>
</table>

1 Values are means ± SD. Two transgenic lines were designated 401/2 and 409/1. The founder mice are F₀, with subsequent generations indicated by the given number in subscript. Mice were given zinc in the drinking water for 2 wk or 3 mo as indicated. The diets enriched or deficient in vitamin A were fed to the mice for 2 wk before killing. The numbers of mice analyzed are given in parentheses. * Significantly different from control (P < 0.05, Student’s t test).

2 Mice were 8 wk old.
3 Mice were 17 wk old.
could be the availability of retinol for CRBP[I] or the concentrations or activities of enzymes such as LRAT.

ACKNOWLEDGMENTS

We are grateful to Ulf Erikson [Ludwig Institute for Cancer Research, Stockholm, Sweden] for providing the human CRBP[I] cDNA, Hiroyoshi Ariga [The Institute of Medical Science, The University of Tokyo, Japan] for providing the pCDL-SRa296 vector and Ruth Paulsen [Institute of Medical Biochemistry, University of Oslo, Norway] for providing the MThGH111 vector. We thank Grethe Økern, Linda Kvam, and Kari Holte for excellent technical assistance.

LITERATURE CITED


