Defective lens fiber differentiation and pancreatic tumorigenesis caused by ectopic expression of the cellular retinoic acid-binding protein I

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SUMMARY

All-*trans* retinoic acid, a metabolite of retinol, is a possible morphogen in vertebrate development. Two classes of cellular proteins, which specifically bind all-*trans* retinoic acid, are thought to mediate its action: the nuclear retinoic acid receptors (RAR α, β, γ), and the cytoplasmic binding proteins known as cellular retinoic acid-binding proteins I and II (CRABP I and II). The function of the retinoic acid receptors is to regulate gene transcription by binding to DNA in conjunction with the nuclear retinoid X receptors (RXR α, β, γ), which in turn have 9-*cis* retinoic acid as a ligand. Several lines of evidence suggest that the role of the cellular retinoic acid-binding proteins is to control the concentration of free retinoic acid reaching the nucleus in a given cell. Here, we have addressed the role of the cellular retinoic acid-binding protein I in development by ectopically expressing it in the mouse lens, under the control of the αA-crystallin promoter. We show that this ectopic expression interferes with the development of the lens and with the differentiation of the secondary lens fiber cells, causing cataract formation. These results suggest that correct regulation of intracellular retinoic acid concentration is required for normal eye development. In addition, the generated transgenic mice also present expression of the transgene in the pancreas and develop pancreatic carcinomas, suggesting that overexpression of the cellular retinoic acid-binding protein is the cause of the tumors. These results taken together provide evidence for a role of the cellular retinoic acid-binding protein in development and cell differentiation. The relevance of these findings to the possible role of the cellular retinoic acid-binding proteins in the transduction of the retinoic acid signal is discussed.

Key words: CRABP I, cataract, retinoic acid, mouse eye, lens, pancreas, tumor, fibre differentiation

INTRODUCTION

Retinoic acid plays an important role during embryonic development of vertebrates (Tickle et al., 1982; Durston et al., 1989). Vitamin A is also of prime importance for the maintenance of vision. Congenital blindness and eye malformations due to maternal vitamin A deficiency have been described in the literature (Thompson et al., 1964; Warkany and Schraffenberger, 1946). Administration of retinol or retinoic acid (RA) prior to organogenesis of the eye prevents the appearance of eye abnormalities associated with vitamin A-deficiency in rats (Dowling and Wald, 1960; Wilson et al., 1953). However, retinoic acid alone can not prevent the degeneration of the retina because it cannot be converted to retinal, which is required for the visual metabolic pathway.

Maternal hypervitaminosis A has also been shown to induce ocular abnormalities, such as anophthalmia, microphthlamia, exophthalmos and cataract (Murakami and Kameyama, 1965; Geelen, 1979). The nature of ocular abnormalities depends on the timing of hypervitaminosis A treatment. In rats, anophthalmia is induced by maternal hypervitaminosis A when the optic evagination just starts to develop. Teratogenic treatment with vitamin A, immediately after the period of optic evagination and optic cup formation, induces microphthalmia. Exophthalmos is produced at the stage when the eye has already developed, but before the orbit has reached its final shape. Congenital cataract is induced by hypervitaminosis A treatment late in the fetal life (Murakami and Kameyama, 1965; Geelen, 1979; Shenefelt, 1972). Thus, this complex teratogenic effects in embryos may suggest that retinol and retinoic acid are important in eye development.

The molecular basis for the teratogenic action of retinoic acid has not been fully elucidated. Two different types of cellular proteins are thought to mediate retinoic acid action: the nuclear receptors, which consist of the retinoic acid receptors (RARs , ,) (Petkovich et al, 1987; Giguere et al., 1987; Zelent et al., 1989), and the cytoplasmic binding proteins, known as cellular retinoic acid-binding proteins (CRABP I and II) (Ong and Chytil, 1975; Giguere et al.,

1990). The function of the RARs is to act as RA-inducible transcriptional factors, which interact with specific responsive elements of different genes (Glass et al., 1989; De The et al., 1990; Vasios et al., 1989). The RARs interact for efficient DNA binding with the retinoid X receptors (RXRs , ,) which in turn have 9 *cis*-retinoic acid as ligand (Zhang et al., 1992; Kliewer et al., 1992; Heyman et al., 1992; Leid et al., 1992). Both of the CRABPs bind retinoic acid, but the affinity of CRABP II for retinoic acid seems to be lower than that of CRABP I (Chytil and Stump, 1991). Based on the finding of two opposing anteroposterior gradients in the chicken limb, one of CRABP I and the other of retinoic acid, Maden et al. (1988) suggested that the function of the CRABPs is to modulate the action of endogenous retinoids by sequestering retinoic acid and thus preventing it from activating the RARs. Recent evidence suggests that this may be the case. Boylan and Gudas (1991) reported that overexpression of CRABP I in F9 cells reduces retinoic acid activation of transcription by the RARs, suggesting that less retinoic acid reaches the nucleus. In a parallel study, Fiorella and Napoli (1991) presented evidence that the complex retinoic acid-CRABP is a substrate in retinoic acid metabolism, therefore sequestering retinoic acid and serving as an intermediate for its efficient catabolism. In both instances, CRABP would be modulating the concentration of free retinoic acid in the nucleus, hence supporting the initial hypothesis.

CRABP I is expressed in the retina but not in the lens of normal mice (Perez-Castro et al., 1989; Dolle et al., 1990, Ruberte et al., 1992). To investigate the role of CRABP I during development, we ectopically expressed it in the mouse lens using the A-crystallin promoter. We show that, in the resulting transgenic mice, the ectopic CRABP I interferes with lens fiber differentiation, causing the formation of cataract. In addition, these mice present expression of the transgene in the pancreas and develop pancreatic carcinomas. Together these results suggest a role of CRABP I during development and cell differentiation.

MATERIALS AND METHODS

DNA construct and transgenic mice generation

The paAp(BS) plasmid, a bluescript KS(−) derivative vector containing the 412 bp *Bgl*II-*Bam*HI A-crystallin promoter (D. Silversides, personal communication) was cut at the *Hin*cII site located immediately downstream of the promoter. A 493 bp *Pvu*II-*Sty*I fragment of the bovine CRABP I cDNA (Wei et al., 1987) was ligated to the *Hin*cII site. The *Sty*I enzyme was chosen to remove the endogenous cDNA polyadenylation site. This whole construct was cut at the *Sma*I site downstream of the cDNA. A 2.5 kbp *Bgl*II-*Bam*HI fragment, containing the SV40 intervening sequence and polyadenylation site was ligated to the *Sma*I site. The whole construct was released from the plasmid as a *Not*I fragment. This DNA was microinjected into (B6CBA F_2 hybrid) fertilized eggs from which 17 F_0 transgenic mice were obtained. Seven transgenic lines were generated, out of which lines 10, 37 and 38 expressed the transgene.

RNA isolation

Total RNA from embryos, microdissected embryo parts and adult tissues were isolated as reported by Chomczynski and Sacchi (1987). The isolated RNAs purified by the above procedure were quantitated by spectrophotometric determination. To isolate RNA from the eyes, excised eyes were flash frozen in liquid nitrogen and pulverized with a Bessman Tissue pulverizer chilled with liquid nitrogen. The pulverized sample was treated in the same manner as all other tissues.

Northern blot analysis

10 µg of total RNA were separated in a 1% formaldehyde-agarose gel and blotted onto nylon. The filter was prehybridized and then hybridized to a cRNA ^{32}P-labeled probe. Prehybridization, hybridization and washing conditions were as recommended by Stratagene.

In situ hybridization

Embryos or tissues were isolated and fixed for 24 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C. Following fixation, the embryos were placed in 0.5 M sucrose in PBS at 4°C for 24 hours, and embedded in OTC (Miles Laboratories). Sections were cut (8-10 µm thickness), placed on poly-L-lysine-coated slides, and stored at −70°C. Slides bearing cryostat sections were treated exactly as in the procedure described by Perez-Castro et al. (1989). Briefly, slides were postfixed with 4% paraformaldehyde, dehydrated in a series of ethanol, acetylated in 0.1 M triethanolamine (pH 8.0) and 0.25% acetic anhydride for 10 minutes, and washed in 30 mM NaCl and 3 mM sodium citrate for 10 minutes. After dehydration in a series of ethanol, the sections were prehybridized at room temperature for 2 hours in hybridization solution (50% formamide, 0.08% bovine serum albumin, 0.6 M NaCl, 0.01 M Tris-HCl pH 7.5, Denhardt's solution, 0.12 mM EDTA, 0.1 µg of salmon sperm DNA/ml, and 0.05 µg of total yeast RNA/ml). Sections were hybridized at 45°C overnight in 20 µl of hybridization solution containing 10% dextran sulfate, 0.1% SDS, 10 mM dithiothreitol, and ^{35}S-labeled RNA at a concentration of 2.5 ng/ml. Slides were washed in 50% formamide, 0.15 M NaCl, 15 mM sodium citrate, 10 mM dithiothreitol at 50°C for 30 minutes and 75 mM NaCl, 7.5 mM sodium citrate for 30 minutes at room temperature. Remaining unhybridized probe was digested with RNase A at 20 µg/ml in RNase buffer (0.5 M NaCl, 0.1 mM Tris pH 8.0, and 1 mM EDTA for 30 minutes at room temperature). Slides were washed in RNase buffer for 10 minutes, and in 30 mM NaCl and 3 mM sodium citrate at 50°C for 2 hours, dehydrated in a series of ethanol containing 300 mM ammonium acetate and coated with a 1:1 dilution of photographic emulsion NTB-2 in water. Slides were developed after 4 days of exposure.

Preparation of riboprobes

^{35}S-labeled RNA probes were prepared as described by Perez-Castro et al. (1989).

For ^{32}P-labeled RNA probes, the procedure was the same as that for ^{35}S probes, except that 100 µCi of ^{32}P- UTP (800 Ci/mmol) were dried down and 40 µM of cold UTP was added.

RNAse protection assay

Isolated total RNA (50-75 µg) was dried and dissolved in 30 µl of hybridization buffer (80% formamide/40 mM Pipes pH 6.4/400 mM NaCl, and 1 mM EDTA) containing a 20-fold excess of probe (2-7×10^5 cts/minute), heated at 85°C for 10 minutes, and incubated at 45°C for at least 8 hours. To the mixture, 350 µl of 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 300 mM NaCl containing 40 µg/ml of RNAse A and 2 µg/ml of RNAse T1 were added, and incubated for 30 minutes at 30°C. 50 µg of proteinase K and 20 µl of 10% SDS were added to the reactions, and incubated at 37°C for 15 minutes. Each sample was phenol extracted and ethanol precipitated with tRNA as carrier. The RNA samples were then dissolved, denatured and fractionated on a 6% sequencing gel (Zinn et al., 1983).

Analysis of cataracts

For gross examination of the lens, animals were anaesthetized with Avertin and their eyes dilated with neosynaphrine (2.5%). Animals were then examined under a dissecting microscope.

Immunohistochemistry

Eyes were dissected and immediately frozen in OTC. 8 μm sections were placed on poly-L-lysine-coated slides and stored at −70°C. Sections were fixed in 1:1 acetone:chloroform and blocked with 10% goat serum and 1% bovine serum albumin (BSA) for 30 minutes, incubated with primary turkey antibody against CRABP (Kato et al., 1985) for 2 hours and washed extensively with phosphate-buffered saline (PBS). Immediately afterwards, sections were blocked once more with 10% goat serum and 1% BSA for 15 minutes, and then rinsed several times with PBS. FITC-conjugated goat antiserum to turkey immunoglobulin G (1:500) was applied for 30 minutes at room temperature. Slides were washed extensively in PBS, mounted with cover slips and immediately examined under a fluorescence microscope.

Preparation of eyes and tissues for histology

Eyes and tissues from other organs were dissected and fixed in 4% paraformaldehyde overnight at 4°C. A window of sclera was removed from the eye just behind the cornea in order to permit better penetration of fixatives to the posterior segment. Tissues were dehydrated with three changes of 95% ethanol for one hour each, followed by the same regimen with a 100% ethanol, embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin, and examined.

RESULTS

Generation of transgenic mice

To express CRABP I ectopically in the lens, a linear DNA construct consisting of the mouse A-crystallin promoter (Chepelinsky et al., 1985) and the coding sequence of the bovine CRABP I (Fig. 1A) (Wei et al., 1987) was microinjected into fertilized mouse eggs. Seven transgenic lines were produced, and their progeny analyzed for transgene expression. Three lines, 10, 37 and 38, expressing the transgene were obtained. They contained 2, 10 and 5 copies of the transgene, respectively. All the F_0 mice appeared phenotypically normal, although they eventually died of pancreatic endocrine tumors between 7 to 9 months.

Cataracts in the αA/CRABP transgenic mice

Starting with the F_1 generation, all subsequent generations of these transgenic lines developed cataracts that were characterized by a central lenticular opacity (Fig. 2A,B). Analysis indicated that cataracts were more severe in homozygous compared with heterozygous transgenic mice of line 10. However, only homozygous mice from the lines 37 and 38 had cataracts.

Expression of the CRABP I transgene

(a) Northern blot analysis

A variety of different tissues from progeny of transgenic animals were tested for expression of the transgene by northern blots using an SV40 probe. No hybridization was detected in any organ tested except the eye (Fig. 1B). This shows that the transgene transcript is expressed exclusively in the eye until 4.5 months of age at which time the transgene can also be detected in the pancretic tumors (see 'transgene expression in the pancreas').
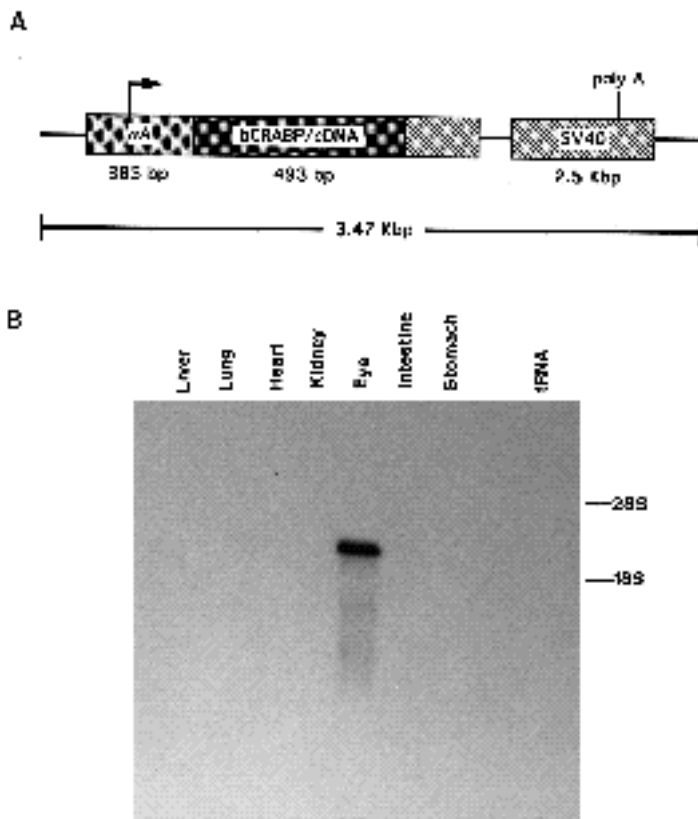

Fig. 1. (A) Diagram showing the construct used for the generation of A/CRABP transgenic mice. (B) Northern blot analysis of total RNA from a 2 month old transgenic animal from line 10, showing specific expression of the transgene in the eye. RNA from different organs was hybridized with an SV40 probe. The different organs showed the absence of transcript except for the eyes. The size of the expected transcript was 2.85 kbp. The 28S and 18S bands were used as markers.

(b) In situ hybridization

In order to detect the expression of the transgene in the lens, normal and transgenic whole eyes were analyzed by in situ hybridization. The probes used to detect the trans gene were 240 bp of SV40 and 493 bp of the CRABP I bovine cDNA.
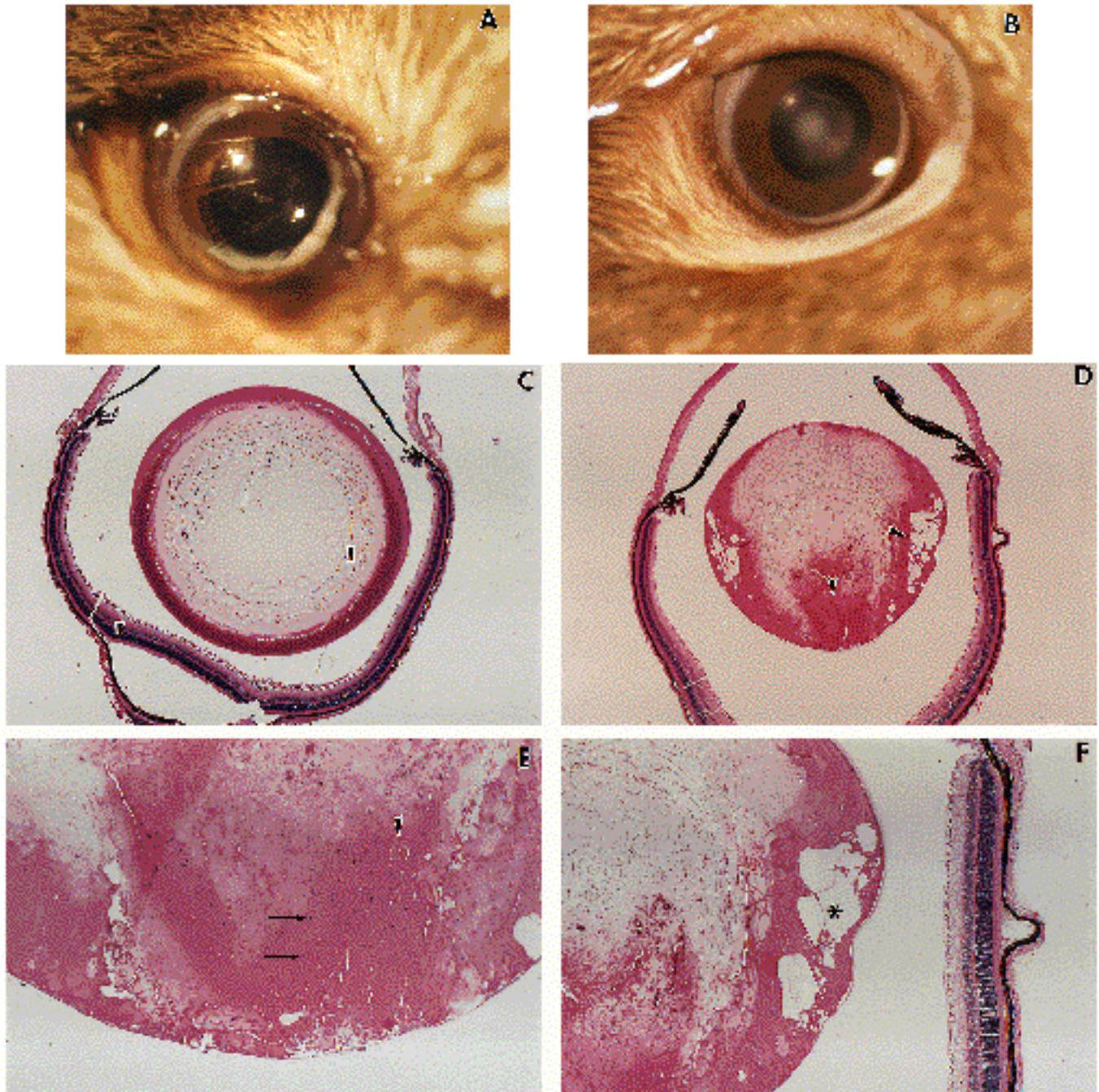

Fig. 2. Phenotype of transgenic mice containing the αA/CRABP transgene. (A) Normal eye from a non transgenic littermate with a transparent lens. (B) Eye of an αA/CRABP homozygous transgenic mouse from line α10 showing an opaque lens. (C) Section of a 5 months old normal eye showing a normal lens (l) and retina (r) (40×). (D) Section of an eye from a transgenic littermate showing a cataractous lens and a normal retina (40×). Note the flattened anterior surface of the lens. The lens nucleus contains many retained pyknotic nuclei and abundant eosinophilic material. The nuclei are accumulated in the posterior pole of the lens. The lens fibers at the lens equator are disorganized and contain many clefts. Arrowheads point to areas of the section that have been magnified in E and F. (E) Magnified view (100×) of the posterior portion of the cataractous lens. Arrowhead points to a Morgagnian globule. Small arrows show the retention of the pyknotic nuclei. (F) High magnification (100×) of the equatorial region of the cataract, which consists of many clefts (asterisk) and disorganized lens fibers.
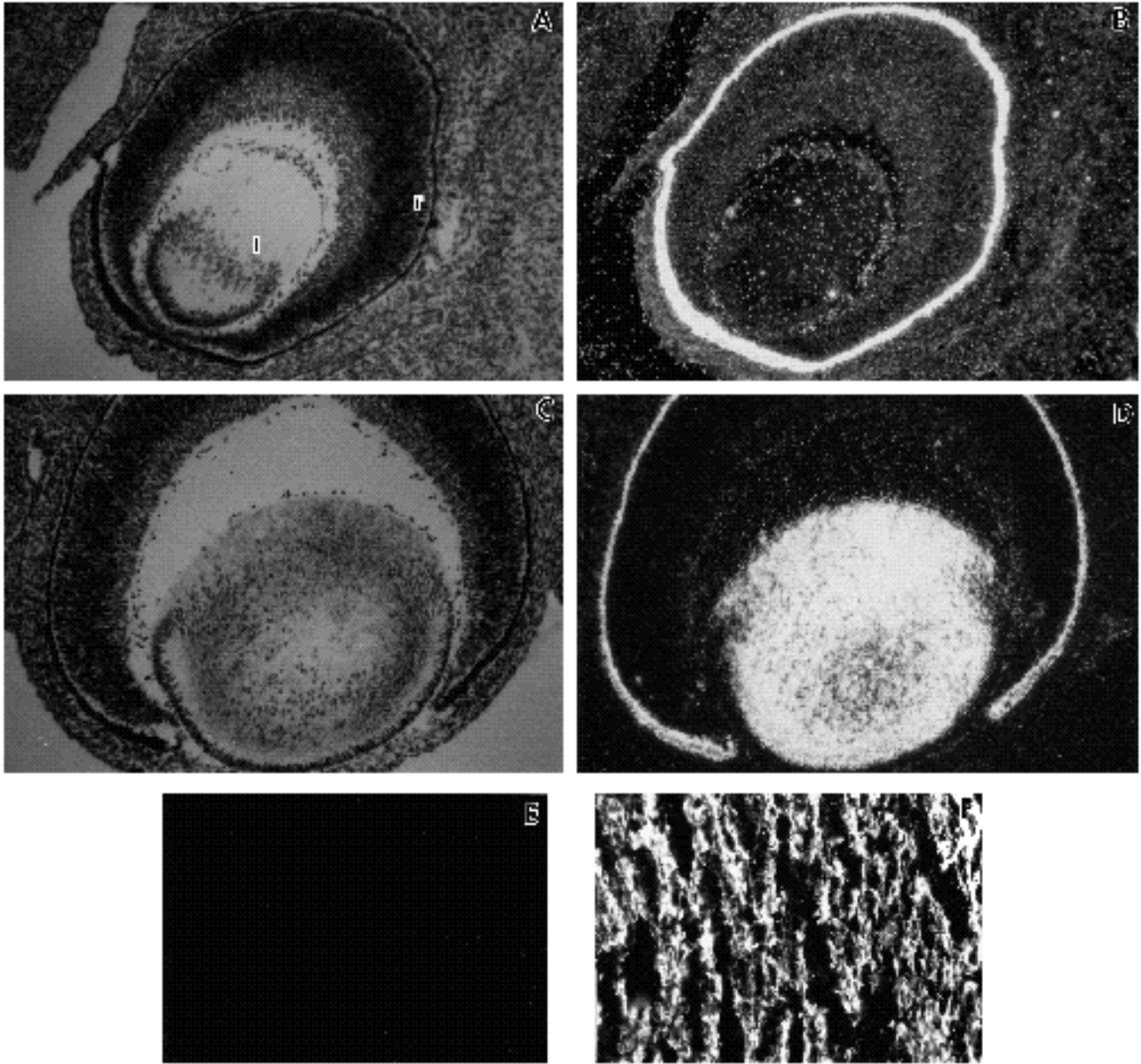
Fig. 3. Expression of the transgene in the eyes of transgenic mice from line   10. (A) Transverse section of a normal embryo at 14.5 days p.c. showing the developing lens and retina. (B) Dark-field photomicrograph of the same section probed with the SV40 antisense probe. No hybridization grains are seen in the lens, and background hybridization grains are observed in the retina. Non-specific hybridization is noted in the pigmented epithelium. (C) Transverse section of a 14.5 days p.c. transgenic embryo. (D) Dark-field photomicrograph of the same section probed with the SV40 antisense probe. Strong hybridization is detected in the lens, indicating the presence of the transgenic transcripts. Only background hybridization is also seen in the retina, as seen in the non-transgenic mouse eye shown in B. Again, non-specific hybridization is detected in the pigmented epithelium. (E) Immunostaining with antibodies to CRABP in section through an eye of a newborn non-transgenic mouse, showing only the lens cells. No staining can be detected indicating the total absence of endogenous CRABP I. (F) Immunostaining with antibodies to CRABP I of a transgenic littermate. All the lens cells are stained, showing the presence of CRABP I.

Whole-eye sections hybridized with SV40 antisense probe show very strong hybridization only in lenses of transgenic animals, indicating the expression of the transgene (Fig. 3B,D). Controls were done with the SV40 sense probe, and no hybridization grains were observed in transgenic and non-transgenic eye sections. The retina did not show hybridization above the level of background. These results are consistent with results from experiments using the bovine cDNA probe: hybridization grains are seen only in lenses of transgenic eye sections, showing the presence of CRABP I transcripts (data not shown). In contrast, hybridization with non-transgenic eye sections showed the absence of CRABP I transcripts. This indicates that the CRABP I expression in the lens is due to the transgene.
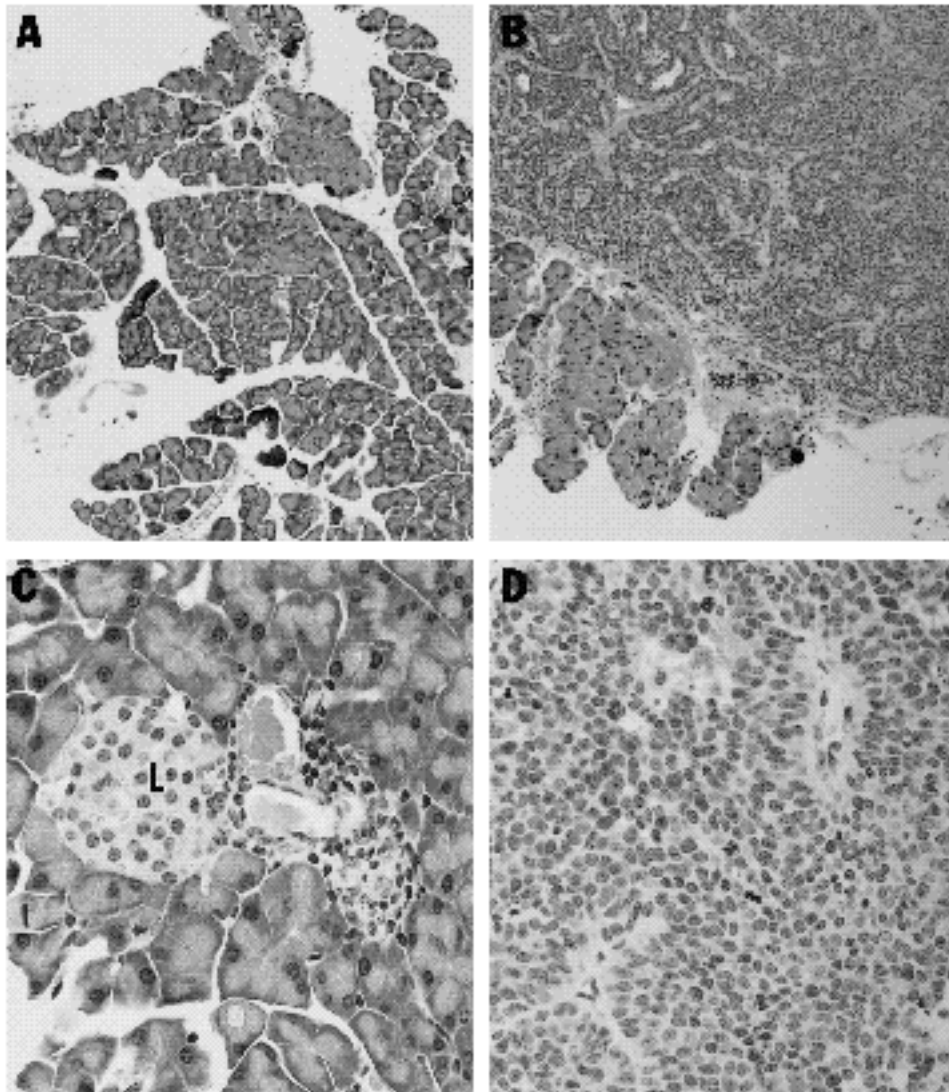
Fig. 4. Histologic characterization of the pancreatic tumors present in transgenic mice from line 38 carrying the αA/CRABP I transgene. (A) Section showing a normal pancreas of a 5 months old mouse (40×). (B) Section showing a pancreatic carcinoma in a 5 months old transgenic mouse (40×). Normal cells can be seen in the lower left side of the panel. (C) Higher magnification (100×) of section shown in A, showing a Langerhans islet (L) surrounded by acinar cells. (D) Higher magnification (100×) of section shown in B, showing the tumor cells.

Transgenic transcripts are detected in the lens of embryos as early as 12.5 days post coitum (p.c.). This is in agreement with reports showing developmental regulation of the αA-crystallin promoter (Overbeek et al., 1985).

(c) Immunohistochemistry

We ascertained further that CRABP I is expressed in the lenses of the transgenic animals by immunohistochemistry using a polyclonal antibody against CRABP I (Kato et al., 1985). As shown in Fig. 3E,F, in the non-transgenic lens, no immunostaining can be detected, indicating the total absence of endogenous CRABP I (Fig. 3E). Conversely, in the transgenic lens, all the lens cells are immunostained (Fig. 3F).

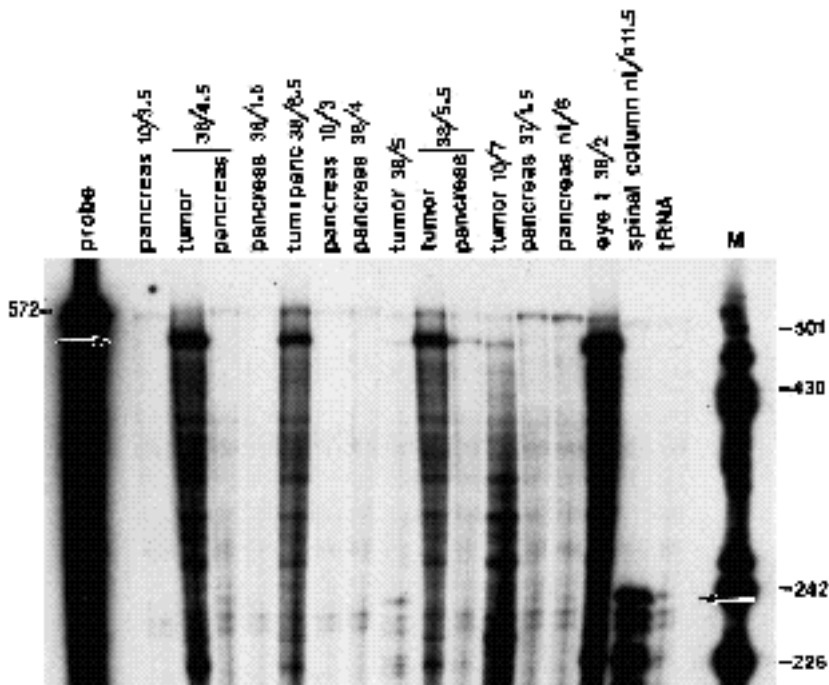## Transgene expression in the pancreas

Because animals were dying prematurely at 7-9 months, we investigated their cause of death. At autopsy, these animals had multinodular tumor of the pancreas. Systematic investigation showed that these tumors became visible at around 4.5 months of age. The tumors were characterized histolog-

ically and were found to derive from the islets of Langerhans (Fig. 4C,D).

Expression of the transgene in the tumors was analyzed. Total RNA from tumors and pancreas of the different transgenic lines at different ages was isolated. In order to detect the transgenic transcript, RNase protection analysis was performed using the bovine CRABP cDNA as a probe. A 493 bp protected fragment is expected for the transgenic CRABP transcript, and a 235 bp protected fragment for the endogenous CRABP (Fig. 5). The 493 bp protected band was detected in all the RNA tumor samples, but it was not detected either in the apparently normal pancreatic tissue samples of the same transgenic mice of 4.5 months of age, or in pancreatic tissue samples of younger transgenic mice. It was not until 5.5 months of age that the transgene transcript was detected in these apparently normal pancreatic tissues.

Histological analysis of cataractous eyes

We studied histologically the lens of animals from line 10. In the wild-type lens, a single layer of mitotically active

Fig. 5. Analysis of pancreas and tumors of mice from transgenic lines 10, 37 and 38. The lineage of the animals from which tissue samples are obtained is denoted by the number before the slash (/). The age of the animal is indicated in every lane after the slash. Total RNA isolated from the tissues was analyzed by RNase protection assay using a 572 bp RNA probe, specific for the CRABP sequences. A 493 bp protected band was expected for the transgenic transcripts and a 235 bp protected band was expected for the endogenous transcripts. The 493 bp protected band (indicated by the arrow on the left), was found in the lane that contains RNA from the eye of a transgenic animal, in the lanes corresponding to all tumors, and in the lanes that contain RNA from apparently normal pancreatic tissue of animals that presented tumors and were at least 5.5 months of age. Transgenic transcripts were not found in lanes that contain apparently normal pancreatic tissue of animals o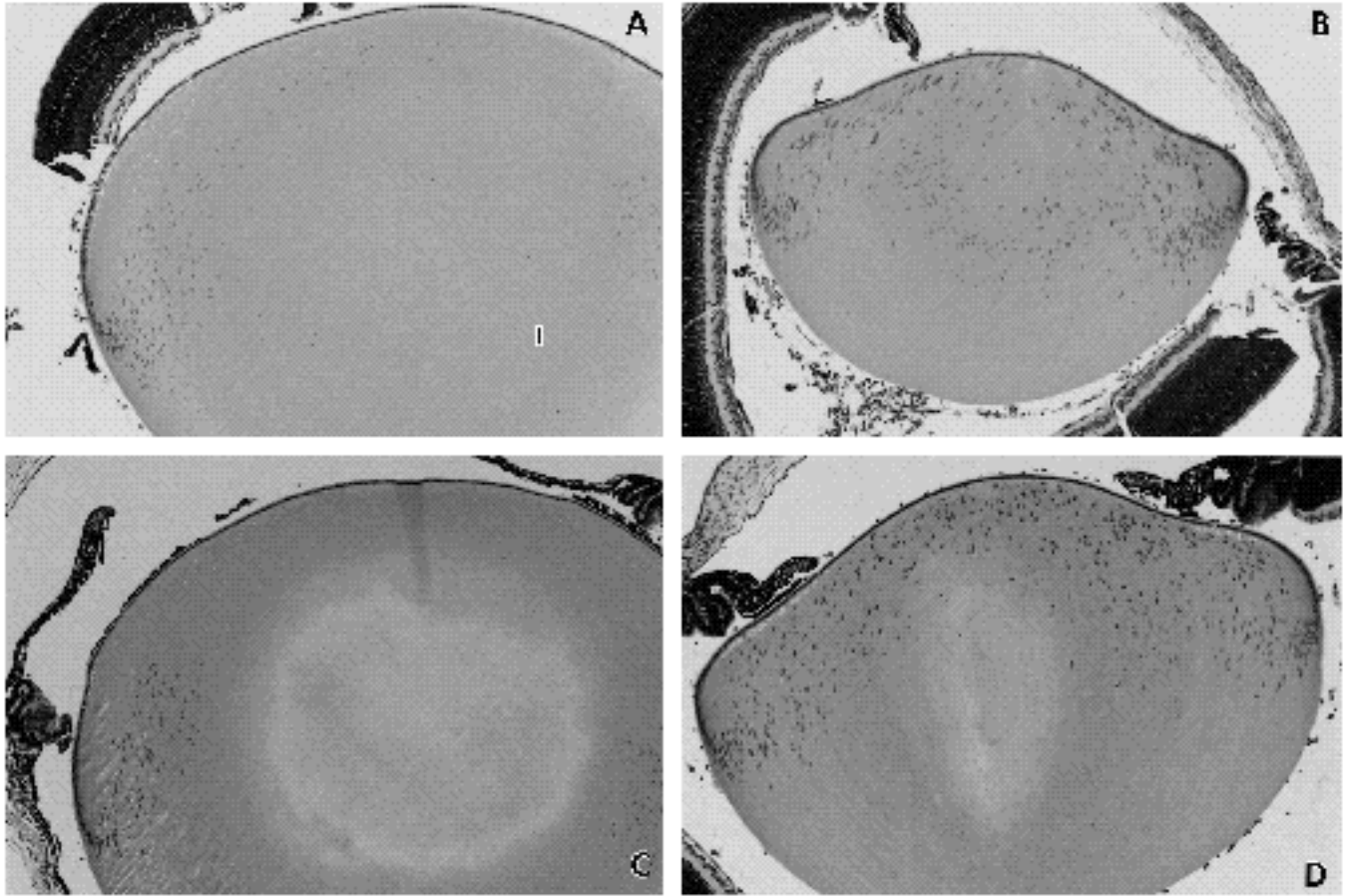f 4.5 months of age or younger. The 235 bp protected band (indicated by the arrow on the right), was found in the lane with sample from spinal column of a non-transgenic embryo of 11.5 days p.c., which is known to contain high levels of endogenous CRABP I transcripts. None of the other lanes show this protected band. The marker used is PGem2 digested with *Hpa*II, labeled with ^{32}P (M).

epithelial cells lines the anterior part of the lens. At the equator, these cells cease to divide and begin to differentiate into elongated lens fibers which ultimately lose their nuclei (Fig. 2C) (McAvoy, 1980). Interestingly, the transgenic lens is cataractous when the mice opened their eyes (day 14 after birth). We studied histologically the development of the abnormal phenotype. Accumulation of nuclei seems to become apparent between day 2 and day 3 after birth, when most of the primary and some secondary fibers in normal lenses have finished their process of denucleation. When eyes from e14, e16 and e18 day embryos, and newborns were examined, no appreciable difference from lenses of their normal littermates is found. At day 4 and day 8 after birth, lenses are characterized by the retention of the nucleus in many of the secondary lens fibers (Fig. 6B,D). The normal elongation of the lens fibers is also impaired, so that the anterior lens surface becomes flattened (Fig. 6B,D). By 4 weeks after birth, the transgenic lenses are grossly cataractous (Fig. 2D). Histologically, the lens nucleus is more eosinophilic. There are many retained nuclei in the lens cells anteriorly and posteriorly (Fig. 2E). The lens equator consists of many clefts and disorganized lens fibers (Fig. 2E,F). Morgagnian globules are noted occasionally in the cytoplasm of lens cells (Fig. 2E).

Histological comparison of heterozygous and homozygous transgenic lenses of line 10 is shown in Fig. 8. Lenses from both, heterozygous and homozygous transgenic animals show histologic abnormalities. However, lenses from heterozygous transgenic animals present a milder cataract than homozygous transgenic ones. This can be clearly distinguished by comparing the amount of tissue that presents clefts in each case (Fig. 7A,B), and by comparing the severity of the disorganization of the lens fibers at the equator (Fig. 7C,D). The alteration in shape of the heterozygous transgenic lens is not as severe as in the homozygous transgenic lens (Fig. 7A,B). This indicates that the elongation process of the homozygous transgenic lens fiber cells is more severely impaired than in the heterozygous transgenic lens. The retention of nuclei in the anterior part of the lenses seems to be similar in both cases (Fig. 7E,F), and the lens nucleus is more eosinophilic also in both cases (Fig. 7A,B). Fig. 9 shows the same histological comparison for transgenic lenses of line 37. Lenses from homozygous transgenic animals of line 37 show severe histologic abnormalities, such as the accumulation of highly eosinophilic material in the lens nucleus (Fig. 8C), disorganization of the lens fibers (Fig. 9F) and retention of pyknotic nuclei (Fig. 8I). The severity of the cataract in the lens of 37 homozygous mice is only as severe as the cataract of an heterozygous lens from line 10. The lens of heterozygous transgenic animals of line 37 is almost completely normal, with the exception of very few lens fiber cells that have retention of nuclei in the anterior part of the lens (Fig. 8H). In all cases, all other tissues from these eyes are normal.

Effects of the ectopically expressed CRABP I on the expression of the γF-crystallin gene

The role of CRABP on retinoid metabolism during development has not been elucidated. It has been postulated that CRABP may sequester free retinoic acid, thus reducing the availability of retinoic acid reaching the nucleus at a sufficient level for the differential regulation of gene transcription (Maden et al., 1988; Boylan and Gudas, 1991, Fiorella and Napoli, 1991). The effects of the transgenic CRABP I on the expression of intrinsic lens proteins such as the F-crystallin gene was examined (Maurer-Orlando et al., 1987).

Fig. 6. Progression of the abnormal phenotype during early postnatal development of homozygous transgenic mice from line 10, containing the A/CRABP transgene. (A) Section of a 4 days old normal eye, showing a normal lens (l). There is still retention of the nucleus in some of the secondary lens fibers (100×). (B) Section of an eye from a 4 days old homozygous transgenic mouse (100×), showing retention of the nucleus in many more of the secondary lens fibers. The normal elongation of the lens fibers is also impaired so that the anterior lens surface becomes flattened. (C) Section of an 8 days old normal eye, showing a normal lens (100×). The lens seems to be very clear and the lens fibers do not present any nuclei in the anterior or posterior part of the lens. (D) Section of an eye from an 8 days old homozygous transgenic mouse showing the flattened shape of the anterior part of the lens, and many retained pyknotic nuclei (100×).

We decided to look at the F-crystallin gene, because it has three half-site repeats related to the consensus sequence (A/G)GGTCA in its promoter region, two of which are responsible of conferring ligand-dependent activation by retinoic acid (Tini et al., 1993). A downregulation of the F-crystallin gene was expected in the transgenic lens if CRABP served to sequester free retinoic acid. With this purpose, a F-crystallin-specific probe in northern blot analysis was used (data not shown). Interestingly, no difference in the expression of the F-crystallin transcript from normal and transgenic lenses was detected.

DISCUSSION

Our results suggest that ectopic expression of CRABP I in the lens alters the normal differentiation of secondary lens fibers by interfering with the process of denucleation.

During normal lens development, at day 15 of embryogenesis, the posterior epithelial cells have fully occupied the lens vesicle lumen and their nuclei have migrated anteriorly. These constitute the primary lens fibers that will be forming the lens nucleus. From this point onwards, secondary lens fibers will derive from lens epithelial cells at the equator (Vrensen et al., 1991). The fact that transgenic lenses show histologic abnormalities on and after day 2 after birth indicates that the secondary lens fibers are affected. Interestingly, the ectopic expression of CRABP begins at around day 12 in embryogenesis. These facts taken together indicate that the expression and accumulation of CRABP I preceded the appearance of the phenotype in these transgenic mice. This temporal relationship further supports the hypothesis that ectopic expression of CRABP I in the lens is causally related to the formation of cataracts, and that there may be a threshold requirement for the amount of the ectopic CRABP I to induce this abnormal phenotype. These conclusions are further strengthened by the observations that in two of the lines (37, 38), cataracts are observed only in homozygous animals and that, in line 10, more severe cataracts were observed in homozygous compared with heterozygous mice. We presume in these cases that homozygosity confers a higher level of CRABP I expression than heterozygosity because of the gene dosage effect. Similar gene dosage effects have been reported for several other
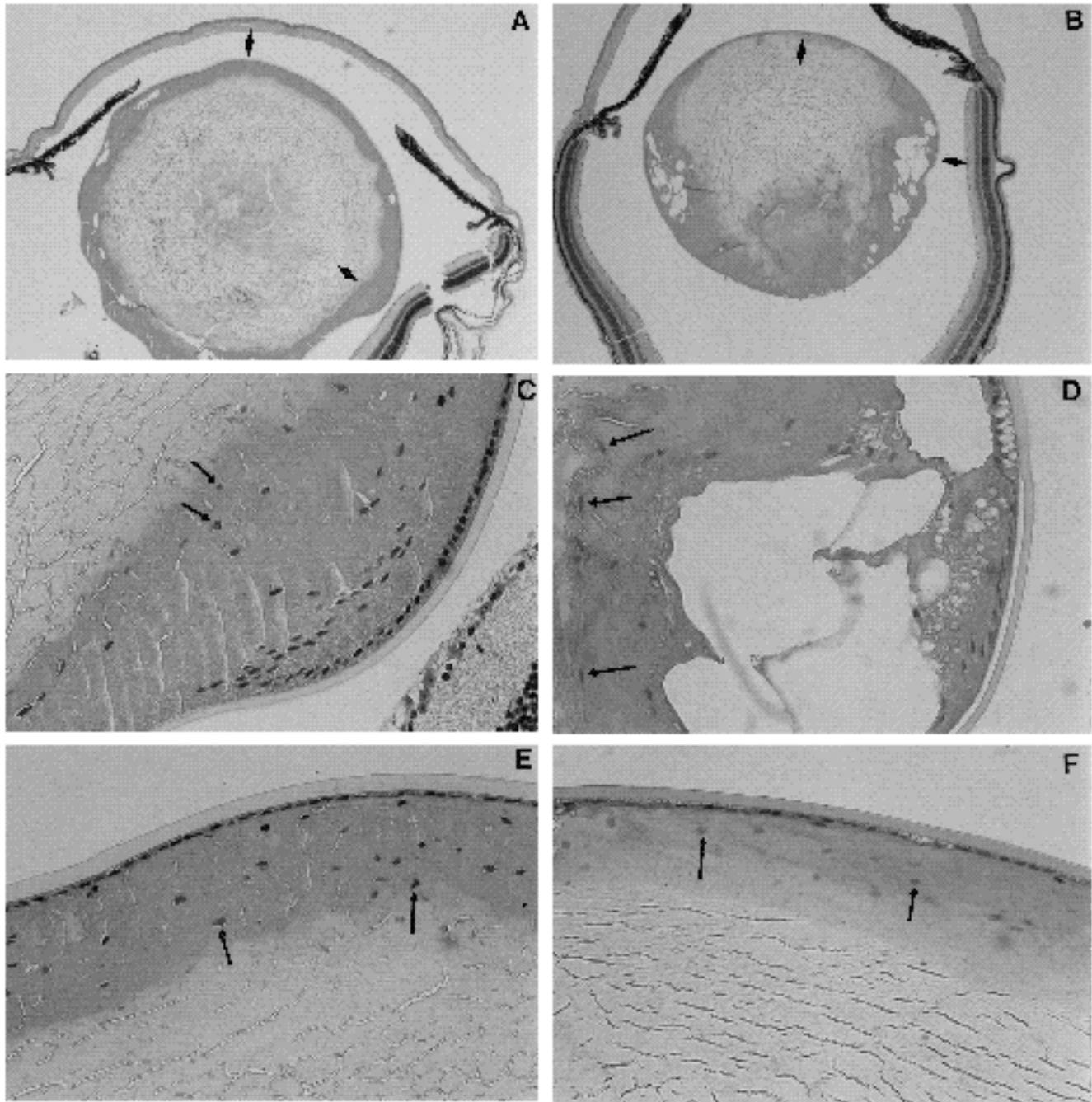
Fig. 7. Histological comparison of the cataracts present in homozygous and heterozygous transgenic mice of line 10. (A) Section of an eye from an adult 10 heterozygous mouse showing a cataractous lens (50×). Rhomboids show magnified areas in C and E. Note that clefts are present at the edges of the lens, and the alteration of the lens shape, although the severity of the cataract is milder than in the homozygous transgenic lens. The lens nucleus presents abnormal eosinophilic material. (B) Section of an eye of an adult 10 homozygous mouse showing a cataractous lens (50×). Rhomboids show magnified areas in D and F. (C) Magnified view (400×) of the equatorial region of the heterozygous transgenic lens. Arrows show the disorganization of the lens fibers. (D) Magnified view (400×) of the equatorial region of the homozygous transgenic lens. Arrows show the disorganization of the lens fibers. Note the presence of clefts. (E) High magnification (400×) of the anterior part of the heterozygous transgenic lens. Arrows show the abnormal retention of nuclei. (F) High magnification (400×) of the anterior part of the homozygous transgenic lens. Arrows show the abnormal retention of nuclei.

transgenes expressed in the lens of transgenic mice (Breitman et al., 1989; Griep et al., 1989; Capetanaki et al., 1989; Harrington et al., 1991).

We would like to emphasize that many different proteins, such as chloramphenicol transferase and -galactosidase,

have been expressed in the lens of transgenic mice without generating a phenotype (Goring et al., 1987; Wawrousek et al., 1990). Thus, the expression of a protein in the lens is not enough per se to produce an abnormal phenotype. In contrast, when an oncogene or a viral gene has been
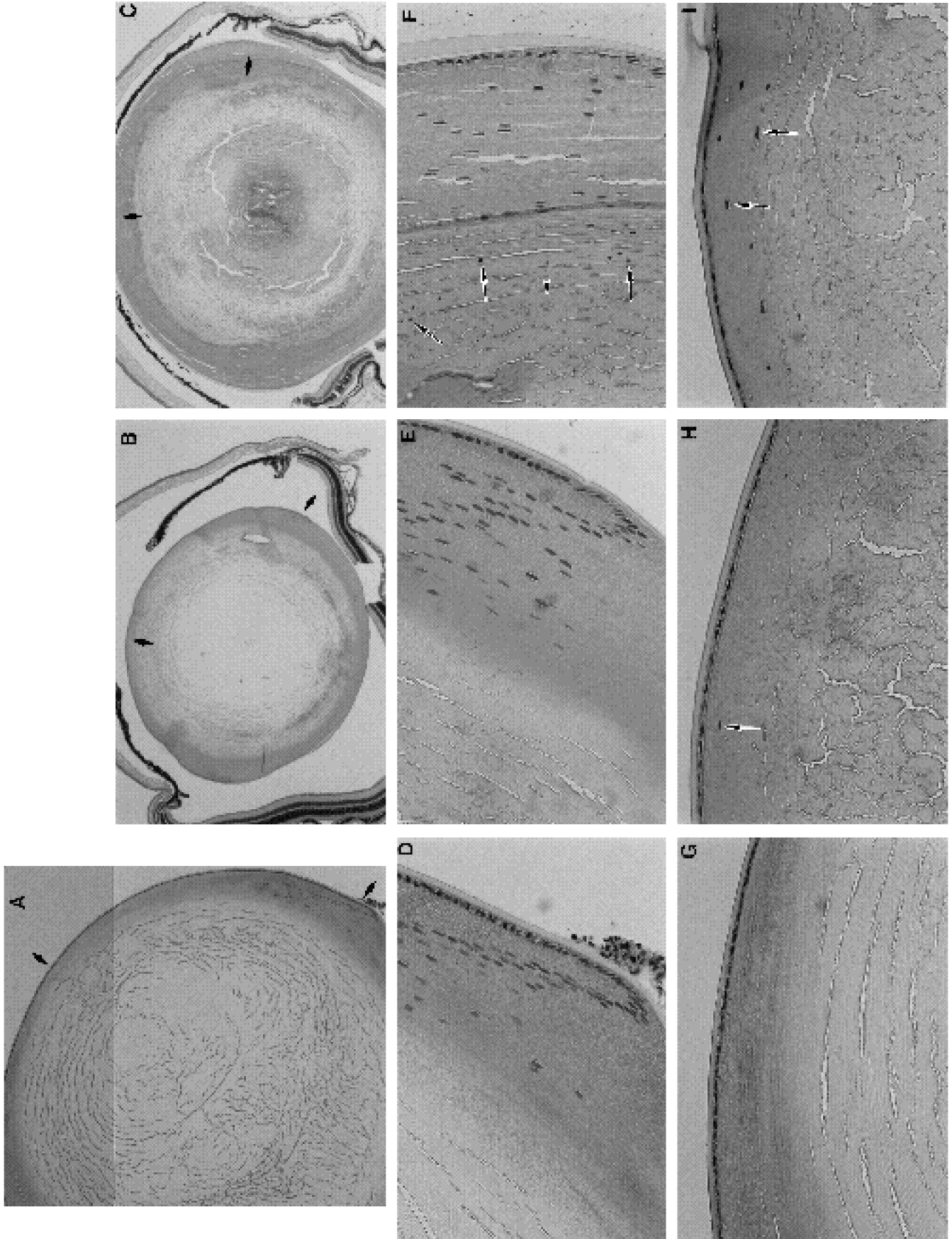
expressed in the lens, tumor formation or microphthalmia have been reported (Mahon et al., 1987; Khillan et al., 1987; Griep et al., 1989). An interesting report by Capetanaki et al. (1989) showed that overexpression of the vimentin gene in the lens of transgenic mice inhibits lens cell differentiation and leads to cataract formation. However, formation of cataract in these mice does not start until they are at least five months old.

A very interesting and unexpected result in all our transgenic lines is the expression of the transgene in the pancreas. These observations exclude the possibility of the existence of genomic positional effects on the expression of the transgene. Expression of the transgene in the pancreas was not expected, since detailed studies on the regulation of the murine A-crystallin promoter have shown lens-specific expression (Wawrousek et al., 1990). However, a recent study by Srinivasan et al. (1992) shows by PCR analysis that the A-crystallin is also expressed in non-ocular tissues, raising the possibility of pancreatic expression due to the promoter itself. Another possible explanation is that the combination of the promoter and the CRABP I cDNA may be promoting expression outside the lens. This seems likely, since other regulatory sequences could exist in the downstream region of the gene. The possibility that a coding region may serve as a transcriptional enhancer element has been previously shown for the murine histone H3 gene (Hurt et al., 1989). However, what seems peculiar about our A/CRABP I transgenic mice is the time of appearance of the transgenic transcript in the pancreas at around 5.5 months of age. This could be due to the fact that, under our assay conditions, we are not able to detect the transgenic transcript until it is very abundant. This would seem to be the case since our observations suggest that the tumors arise from the Langerhans islets which, under normal conditions, only represent 1% of the whole pancreatic mass.

Fig. 8. Comparison of histological eye sections of heterozygous and homozygous transgenic mice of line 37. (A) Section of an eye of an adult non-transgenic mouse showing a normal lens. (Compound picture of two different exposures of 100× magnification each). Rhomboids point to magnified areas in D and G. (B) Section of an eye of an adult 37 heterozygous mouse showing a lens with normal features (50×). The torn tissue in this photograph is an artifact of sectioning. Rhomboids point to magnified areas in E and H. (C) Section of an eye of an adult 37 homozygous mouse showing a cataractous lens (50×). Note that the severity of the cataract resembles the severity of the cataract of a lens from an 10 heterozygous mouse. The lens nucleus presents abnormal eosinophilic material. Rhomboids point to magnified areas in F and I. (D) Magnified view (400×) of the equatorial region, showing normal differentiating fiber cells. (E) Magnified view (400×) of the equatorial region of the transgenic heterozygous lens, showing the differentiating fiber cells with no apparent abnormalities. (F) Magnified view (400×) of the equatorial region of the transgenic homozygous lens. Arrows show the disorganized differentiating fiber cells and arrowhead points to a Morgagnian globule. (G) High magnification (400×) of the anterior part of a normal lens. (H) High magnification (400×) of the anterior part of the heterozygous transgenic lens, showing the retention of the only abnormal pyknotic nucleus in this lens. (I) High magnification (400×) of the anterior part of the homozygous transgenic lens. Arrows show the abnormal retention of pyknotic nuclei.

An interesting question, but one for which we do not have yet a conclusive explanation, is the cause of pancreatic carcinomas. Kato et al. (1985b) by radioimmunoassay experiments, reported the presence of both the cellular retinol-binding protein I (CRBP I) and CRABP I in the Langerhans islets of the rat. They suggested that the retinoids and their binding proteins may play important metabolic roles within the islet cells, and hence that they may be involved in some way in the endocrine function of the islets. If this is also the case in the mouse, then overexpression of CRABP I in the islets would lead to the deregulation of the normal homeostasis of the retinoids in mouse pancreatic islet cells. Experiments are under way to address this issue.

What is the function of CRABP I in normal development? It is likely that CRABP I is involved in regulating retinoid homeostasis in cells that are the targets for retinoid. For example, in the mouse embryo, CRABP I has been found in all of the tissues that are known to be teratogenic targets of retinoic acid (Perez-Castro et al., 1989; Maden et al., 1990; Ruberte et al., 1992). Thus, CRABP I could be controlling the actual concentration of free retinoic acid in these tissues, and when retinoic acid exceeds certain saturation levels (i.e. maternal hypervitaminosis A), then CRABP I would no longer be able to regulate the concentration of retinoic acid reaching the nucleus, and consequently teratogenesis occurs. Conversely, at a higher concentration of CRABP I, it could exert its effect by preventing retinoic acid from reaching the nucleus to a level sufficient for the differential regulation of gene transcription. This hypothesis has been supported by three different sets of experiments. First, two opposing gradients have been observed in the anteroposterior axis of the chicken limb, one of retinoic acid and another reciprocal gradient of CRABP. It was proposed that these two gradients would result in a flatter distribution of retinoic acid-CRABP complex, but in a steeper gradient of free retinoic acid available for binding to the Retinoic acid receptors (RARs) (Maden et al., 1988). Second, experiments with F9 EC cells in vitro showed that increased expression of CRABP I reduces the expression of retinoic acid induced genes (Boylan and Gudas, 1991), supporting the idea that CRABP I sequesters retinoic acid within the cell, thereby preventing it from reaching its nuclear targets. Third, Fiorella and Napoli (1991) have recently shown in an in vitro system that CRABP I modulates the concentration of free retinoic acid not only by sequestering retinoic acid but also by serving as a conduit for its efficient catabolism.

Two recent transgenic experiments in which the reporter gene *lacZ* was expressed under the control of the retinoic acid responsive element (RARE) from the RAR, confirms the existence of retinoic acid in both the lens and retina (Rossant et al., 1991; Balkan et al 1992). In our case, ectopic expression of CRABP I in the lens interferes with the normal program of differentiation from epithelial to lens fiber cells. The effect of the ectopic CRABP I on lens fiber differentiation could be explained as a change in the concentration of free retinoic acid, either because this ectopic CRABP I binds retinoic acid, or because the ectopic CRABP I increases the catabolism of retinoic acid. In both instances, the concentration of the normally available free retinoic acid would have been altered, thus affecting differential regulation of gene transcription, which is ultimately reflected in

the alteration of the program of differentiation from epithelial to lens fiber cells.

To see if we could determine the mode of action of the ectopic CRABP I in our transgenic mice, we examined the effects of the transgenic CRABP I on the expression of the intrinsic lens protein gene, F-crystallin. The F-crystallin gene has a RARE in its promoter region (Tini et al., 1993). A downregulation of the F-crystallin gene was expected in the transgenic lens if CRABP sequesters free retinoic acid. By northern blot analysis of total RNA using a F-crystallin-specific probe (Maurer-Orlando et al., 1987), we could not detect any difference in the expression levels of the F-crystallin transcripts in normal and transgenic lenses. One possible explanation for this result is that the changes in transcription rates of the F-crystallin gene are not detected by examination of steady state levels of transcripts in whole lenses because of the relatively large amounts of F-crystallin transcripts that are present in the lens. The potential effects of the transgene on the expression of other lens proteins are the subject of future studies.

We believe that transgenic mice bearing the A-crystallin/CRABP transgene provide us with the opportunity to elucidate the interactions between retinoids, nuclear receptors and retinoid-binding proteins. It will be interesting to cross some of the RAR-*lacZ* transgenic mice (Rossant et al., 1991; Balkan et al 1992) with our transgenic A-crystallin/CRABP mice and look for changes in the pattern of *lacZ* expression which might result from changes in intracellular concentrations of retinoic acid in transgenic mice.

The results of our transgenic experiments suggest that retinoic acid is involved in the development of the lens and in lens fiber differentiation. We have presented the first evidence that CRABP I interferes with normal development and cell differentiation when ectopically expressed, suggesting an important role for this protein in embryonic development, probably by regulating retinoid homeostasis in the cells where it is normally present.

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