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The Retinoblastoma Protein-Binding Region of Simian Virus 40 Large T Antigen Alters Cell Cycle Regulation in Lenses of Transgenic Mice

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Regulation of the cell cycle is a critical aspect of cellular proliferation, differentiation, and transformation. In many cell types, the differentiation process is accompanied by a loss of proliferative capability, so that terminally differentiated cells become postmitotic and no longer progress through the cell cycle. In the experiments described here, the ocular lens has been used as a system to examine the role of the retinoblastoma protein (pRb) family in regulation of the cell cycle during differentiation. The ocular lens is an ideal system for such studies, since it is composed of just two cell types: epithelial cells, which are capable of proliferation, and fiber cells, which are postmitotic. In order to inactivate pRb in viable mice, genes encoding either a truncated version of simian virus 40 large T antigen or the E7 protein of human papillomavirus were expressed in a lens-specific fashion in transgenic mice. Lens fiber cells in the transgenic mice were found to incorporate bromodeoxyuridine, implying inappropriate entry into the cell cycle. Surprisingly, the lens fiber cells did not proliferate as tumor cells but instead underwent programmed cell death, resulting in lens ablation and microphthalmia. Analogous lens alterations did not occur in mice expressing a modified version of the truncated T antigen that was mutated in the binding domain for the pRb family. These experimental results indicate that the retinoblastoma protein family plays a crucial role in blocking cell cycle progression and maintaining terminal differentiation in lens fiber cells. Apoptotic cell death ensues when fiber cells are induced to remain in or reenter the cell cycle.

Regulation of the cell cycle is crucial not only for cellular proliferation but also for differentiation. Proliferating cells undergo a stringently controlled series of events that ensure that the cell becomes properly duplicated (28). This process generally consists of mitosis (M phase) followed by a gap (G_1 phase) prior to onset of DNA replication (S phase). Termination of DNA replication is followed by a second gap (G_2 phase) prior to cell division and initiation of a new cycle. Under conditions of limiting nutrients, most cell types will arrest progression through the G_1 phase of the cell cycle and enter a resting phase known as G_0 (5). Differentiating cells can also discontinue progression through the cell cycle and either enter a resting phase or, as part of their terminal differentiation, can permanently withdraw from the cell cycle so that they undergo no further proliferation. Cells with distinctive architectures, such as neurons, typically enter such a postmitotic condition during differentiation (42). The molecular basis for maintaining the postmitotic state in terminally differentiated cells has not yet been defined. Nonetheless, the maintenance of differentiated cells in a resting or postmitotic phase is critical to the survival of most multicellular organisms because loss of the block to cellular proliferation can result in tumor formation. Tumor suppressors are likely to play a critical role in maintaining terminally differentiated cells in G_0 (44). Tumor suppressor proteins have the ability to block progression through the cell cycle, while inactivation of tumor suppressor genes appears to predispose certain cell types to reentry into a proliferative state and/or loss of the capacity for terminal differentiation.

One strategy to examine the roles of tumor suppressors during differentiation is through the expression of viral transforming proteins. Simian virus 40 (SV40) large T antigen is a viral protein that interacts with tumor suppressors and is capable of transforming both primary and established cell lines (15). In transgenic mice, this protein has been found to induce tumor formation in most of the tissues in which it has been expressed (1). The transforming ability of T antigen is thought to be mediated by its interaction with the retinoblastoma (pRb) and p53 tumor suppressors (48). Both of these proteins negatively regulate cellular proliferation, and mutations in these genes are associated with cancer (22, 23, 32). It is thought that T antigen induces tumors by inactivating the growth-inhibitory activities of these tumor suppressors. A third protein that interacts with T antigen is p107, which has a domain homologous to pRb and binds to the same region of T antigen as pRb (13, 14). The role of p107 in cell cycle regulation and its relevance to tumor formation, if any, have not yet been established. Since pRb and p107 have a homologous domain and bind to the same region of T antigen, both proteins will be referred to together as the pRb family.

The ocular lens provides an excellent system for in vivo studies of cell cycle control. Normally, proliferation is confined to the epithelial cells that cover the anterior of the lens. Once the epithelial cells have been induced to begin differentiation

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into fiber cells, which occurs in the equatorial region of the lens, the cells become postmitotic (40). Lens tumors have been induced in transgenic mice by using the mouse αA-crystallin promoter (−364 to +46) to direct lens-specific expression of the SV40 early region, which encodes the large T antigen (34). In these mice, the lens fiber cells lose their phenotype of terminal differentiation and reenter the cell cycle to proliferate as tumor cells. Expression of T antigen appears to be sufficient to cause this transformation, with no secondary oncogenic events required (34). Since expression of T antigen presumably inactivates the pRb family and p53, the induction of lens tumors suggests that these tumor suppressors are involved in maintaining the postmitotic state of fiber cells.

In the current experiments, truncated versions of the SV40 early region were expressed in the lens. These experiments were initiated when one of the founder mice generated by microinjection of a full-length T antigen construct was found to have two sites of integration, one of which produced microphthalmia and lens cell death rather than lens tumors (49). The integration site associated with microphthalmia was cloned and found to contain a truncated version of the SV40 early region, which encodes a large T antigen protein that retains the binding domain for the pRb family but is missing the p53 binding domain. Additional transgenic mice have been generated to confirm that interaction of this truncated T antigen with pRb is critical to induce microphthalmia and cell death in the lens. Developmental studies have revealed that the interaction of T antigen with the pRb family induces changes in cell cycle regulation and apoptotic cell death in the normally postmitotic lens fiber cells.

MATERIALS AND METHODS

Generation of constructs and transgenic mice. The αTAg construct was described previously (34). The gr19 construct (Fig. 1A) was generated by digestion of λgr19 DNA (49) with *Eco*RI and *Pst*I releasing a 6-kb fragment for microinjection. For the VISC construct (Fig. 1A), the *Eco*RI-*Pst*I fragment from λgr19 was transferred into Bluescript(KS−), and the *supF* gene was subsequently inserted at the *Bgl*II site in the 5′ flanking mouse genomic sequences. The resulting plasmid was then digested with *Cla*I, releasing a 3.1-kb fragment for microinjection. For the αTruncTΔ268 construct (Fig. 1A), pTruncT was first created by transfer of the *Bam*HI-*Cla*I fragment from plasmid VISC into Bluescript(KS−). Next, pTruncTΔ268 was produced by replacing the *Sty*I fragment from pTruncT with the *Sty*I fragment from pSV11 (8), which contains the SV40 early region with a 268-bp deletion that removes the small t antigen splice donor. Finally, the αA-crystallin promoter was added by insertion of the *Sac*II-*Bam*HI fragment from the crystallin promoter vector (39a) into pTruncTΔ268 digested at *Sac*II-*Bam*HI. For microinjection, the resulting plasmid was digested with *Eco*RI and *Cla*I, releasing a 1.3-kb fragment. For the αTruncTΔ268-Stop construct (Fig. 1A), a PCR-based approach was used to perform site-directed mutagenesis upon pαTruncTΔ268 in order to generate a T-to-A substitution in the fourth nucleotide of the flanking mouse genomic sequences. PCRs (50-μl volumes) were performed with a mixture of 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin), 1 pg of DNA, 100 μM (each) deoxynucleoside triphosphate (dNTP), 400 nM (each) primer, and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus). First, a sense primer spanning the *Pfl*MI site (TTTAGATTCCAACCTATGGAACTGAT) and a mutant antisense primer (CTGAGTGGATTGTTTAAAGATT ATAACTG) were used to amplify a 350-bp fragment (40 cycles of 92°C for 30 s, 50°C for 3 min, and 72°C for 1 min), and this product was then combined with an antisense primer spanning the *Cla*I site (GTCGACGGTATCGATAACTTTGTT TT) to amplify a 525-bp fragment (45 cycles of 92°C for 1 min, 50°C for 3 min, and 72°C for 1 min). This 525-bp product was subsequently digested with *Pfl*MI and *Cla*I and substituted for the *Pfl*MI-*Cla*I fragment in pαTruncTΔ268. The point mutation was confirmed by dideoxynucleotide sequencing (45). The resulting plasmid was digested with *Eco*RI and *Cla*I, releasing a 1.3-kb fragment for microinjection. For the

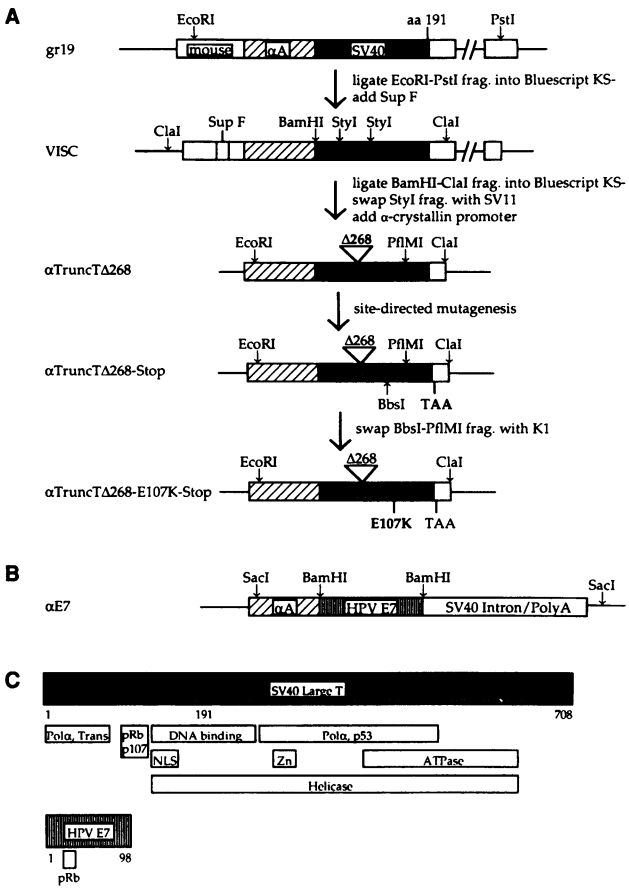

FIG. 1. Diagrams of transgenic constructs and encoded proteins. (A) Cloning strategy used to generate the truncated SV40 early region constructs. The locations of the αA-crystallin promoter, SV40 early region, mouse genomic sequences, SupF gene, and vector sequences (thin lines) are indicated. The gap represents genomic sequences not drawn to scale. Modifications made to these constructs are shown, which include deletion of a 268-bp region (nucleotides 4586 to 4854), which removes the small t antigen splice donor (Δ268); introduction of a stop codon at the 3′ end of the SV40 sequences (TAA); and substitution of glutamic acid with lysine at amino acid 107, which eliminates binding of T antigen to pRb (E107K). (B) Construct used to generate αE7 transgenic mice, indicating the locations of the αA-crystallin promoter, HPV E7 gene, SV40 small t intron and polyadenylation sequences, and vector sequences (thin lines). (C) Functional domains of SV40 large T antigen and HPV E7. The regions of large T antigen that are necessary for binding to pRb (and p107), DNA polymerase α-primase (polα), and p53 are indicated. Also shown are the areas required for transcriptional transactivation (Trans), ATPase activity, helicase activity, and the zinc finger domain (Zn) and the nuclear localization signal (NLS). This diagram is based upon information from the review by Fanning and Knippers (15). The region of HPV E7 that is required for pRb binding is also indicated (38). Frag., fragment.

αTruncTΔ268-E107K and αTruncTΔ268-E107K-Stop constructs (Fig. 1A), the *Bbs*I-*Pfl*MI fragments (nucleotides 4416 to 4558) from pαTruncTΔ268 and pαTruncTΔ268-Stop were replaced with the *Bbs*I-*Pfl*MI fragment from plasmid K1 (27), and the resulting plasmids were digested with *Eco*RI and *Cla*I, releasing 1.3-kb fragments for microinjection. The αE7 construct (Fig. 1B) was generated by insertion of the *Bam*HI fragment from pHa16E7 (4), which contains nucleotides 505 to 1176 of human papillomavirus type 16 (HPV16), into the *Bam*HI site of crystallin promoter vector, which contains the small t intron and poly(A) sequences from the SV40 early region (39a). The resulting plasmid was then digested with *Sac*I, releasing a 1.9-kb fragment for microinjection. Fragments used for microinjection were isolated by electrophoresis through 1% agarose gels in TAE buffer (40 mM Tris acetate [pH 8.5], 2 mM EDTA) and purified by Geneclean (Bio 101). Transgenic mice for all families except OVE1 were generated by pronuclear injection into one-cell-stage inbred FVB/N embryos by standard techniques (21, 51). The OVE1 founder was generated by microinjection into C3H × FVB embryos. F_1 mice were generated by matings to FVB/N partners. The OVE1 family has been maintained subsequently by brother-sister matings.

Screening of mice. Tail DNA was isolated as previously described (21). OVE1 and gr19 mice were screened by Southern hybridization with an SV40 probe as described previously (49). The VISC, αTruncTΔ268, and E107K mice were screened by PCR with an upstream primer spanning nucleotides −165 to −146 of the αA-crystallin promoter (GCATTCC AGCTGCTGACGGT) (6) and a downstream antisense primer spanning nucleotides 4407 to 4432 of SV40 (GTCCT TGGGGTCTTCTACCTTTCTC) (16). These primers amplify a fragment of 980 bp in the VISC families and a fragment of 710 bp in the Δ268 families. The αE7 families were also screened by PCR with these primers, which amplify a 1,140-bp fragment in these mice. PCR was performed in a 50-μl volume of 1× PCR buffer–1 μl of tail DNA–100 μM (each) dNTP–400 nM (each) primer–2.5 U of *Taq* polymerase (Perkin-Elmer Cetus). Reactions were run for 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, analyzed on 1% agarose gels, and then visualized by staining with ethidium bromide.

Sequencing. Double-stranded DNA sequencing was performed with dideoxynucleotides (45).

Histology. Samples for histology determination were fixed in 10% formalin, paraffin embedded, cut as 5-μm-thick sections, and stained with hematoxylin and eosin by standard techniques.

RT-PCR. For qualitative analysis, RNA was isolated from lenses of mice at approximately 3 weeks of age by using RNA Stat-60 (Tel-test B, Inc.). RNA preparations were treated with DNase I (Pharmacia) (fast-performance liquid chromatography pure) to remove contaminating genomic DNA and then were phenol extracted. Reverse transcriptase (RT) reactions were performed in a 20-μl volume of 1× PCR buffer–100 μM (each) dNTP–1 μg of total RNA–180 ng of random primers (Promega), 29 U of RNA guard (Pharmacia)–200 U of Moloney murine leukemia virus RT (Gibco BRL) at 37°C for 2 h. RT reactions were subsequently amplified by PCR with an upstream primer spanning nucleotides 10 to 34 of the αA-crystallin transcript (CCCAGAGGCTCCTGTCTGACTCA CT) (6) and a downstream primer spanning nucleotides 4407 to 4432 of SV40 (GTCCTTGGGGTCTTCTACCTTTCTC) (16). PCR was performed in a 50-μl volume of 1× PCR buffer with 4 μl of the RT reaction mixture, 100 μM (each) dNTP, 100 nM (each) primer, and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus). Reactions were run for 30 cycles at 94°C for 1 min, 62°C for 30 s, and 72°C for 1 min, analyzed on a 1.5% agarose gel, and then visualized by staining with ethidium bromide.

For quantitative analysis, RNA was isolated from embryonic day 15 (E15) lenses with RNA Stat-60 (Tel-test B, Inc.). RT reactions were run as described for the qualitative analysis. RT reactions were subsequently amplified by PCR with both the primer set described in the qualitative analysis as well as a primer set specific for the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene (30), the sense primer spanning nucleotides 118 to 137 (AGCGATGATGAACCAGGTTA), and the antisense primer spanning nucleotides 439 to 458 (GTTGAGAGATCACCTCAACC). PCR was performed in a 50-μl volume of 1× PCR buffer by using 2 μl of the RT reaction mixture, 100 μM (each) dNTP, 400 nM (each) primer, and 1 U of *Taq* polymerase (Perkin-Elmer Cetus). Reactions were run between 12 and 24 cycles (2-cycle intervals) at 94°C for 1 min, 62°C for 30 s, and 72°C for 1 min. Finally, these reactions were run on 1.5% agarose gels and analyzed by Southern hybridization (49) by using both an SV40-specific probe (nucleotides 4407 to 4706) and an HPRT-specific probe (nucleotides 118 to 458). The amount of radioactivity in each band was quantified on a blot analyzer (Betagen).

Iodination of cell extracts. Lens tissue was pooled (5 to 20 mg) and minced in 200 μl of homogenization buffer (10 mM Tris-hydrochloride [pH 7.4], 0.5 M NaCl, 1 mM β-mercaptoethanol [Bio-Rad Laboratories, Richmond, Calif.], and 1 mM EDTA, supplemented with the protease inhibitors 1 mM leupeptin [Boehringer-Mannheim Biochemicals, Indianapolis, Ind.], 1 mM phenylmethylsulfonyl fluoride [Sigma Chemical Co., St. Louis, Mo.], and 1% aprotinin [FBA Pharmaceuticals, West Haven, Conn.]). Nonidet P-40 was added to a final concentration of 1%, the sample was sonicated for 45 s, and the insoluble debris was removed by centrifugation with an Airfuge (Beckman Instruments, Fullerton, Calif. [90 min, 20 lb/in^2]). The supernatant (~200 μl) was removed and used for protein iodination (24). Two millicuries of iodine-125 (Amersham Corp., Arlington Heights, Ill.) was added, followed in succession by 20 μl of 5-mg/ml fresh chloramine T (Sigma), which was allowed to react for 1 min; 20 μl of 10-mg/ml sodium metabisulfite with mixing; and 200 μl of 10-mg/ml potassium iodide. After mixing, the lysate was dialyzed extensively against extraction buffer (50 mM Tris-hydrochloride [pH 8.0], 100 mM NaCl, 1% Nonidet P-40, 1% aprotinin) at 4°C. After dialysis, the lysate was processed for immunoprecipitation.

Immunoprecipitation and gel electrophoresis. Labeled cell extracts were immunoprecipitated as described previously (26) after being precleared with excess formalin-inactivated, heat-killed *Staphylococcus aureus* (strain Cowan I) to remove nonspecific binding proteins (36). Monoclonal antibodies directed against binding sites on the amino terminus of SV40 T antigen (PAb419, PAb430, and PAb416), against murine p53 (PAb421, 200.46), and against adenovirus E1A protein (m73) were used (36, 46). Immune complexes were collected by adsorption onto *S. aureus*, were washed extensively, and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% polyacrylamide gels as described previously (26, 36). Labeled proteins were detected by autoradiography.

BrdU immunohistochemistry. Pregnant females at 15 days postmating were injected intraperitoneally with 5-bromo-2′-deoxyuridine (BrdU) (Sigma) and 5-fluoro-2′-deoxyuridine (Sigma), an inhibitor of thymidine synthesis, in phosphate-buffered saline (PBS) (10 mM $NaPO_4$ [pH 7.5], 0.9% NaCl) at 100 μg of BrdU per g of body weight and 6.7 μg of 5-fluoro-2′-deoxyuridine per g of body weight and sacrificed 1 h later. Heads from the fetuses were obtained by necropsy, fixed in 10% formalin, paraffin embedded, and sectioned at 5 μm.

Endogenous peroxidases were blocked by incubation in 10% methanol–3% H_2O_2 in PBS for 30 min at room temperature. Protein-DNA cross-linking was disrupted by digestion with 0.02% pepsin (Sigma)–0.01 N HCl in PBS for 20 min at 37°C. Partial denaturation of DNA was achieved by incubation in 2 N HCl for 45 min at room temperature, followed by neutralization with 0.1 M sodium borate (pH 8.5) for 10 min at room temperature. Immunostaining was performed by the avidin-biotin peroxidase complex method (Vector Laboratories, kit PK-4002). Mouse anti-BrdU immunoglobulin 1 (Dako) was used as the primary antibody at 1:20 with overnight incubation at 4°C. Peroxidase was visualized with diaminobenzidine-H_2O_2 (Vector Laboratories, kit SK-4100). Sections were counterstained with hematoxylin.

Detection of apoptosis. For examination of genomic DNA, lens DNA was isolated by the tail DNA isolation procedure (21). One microgram of DNA was end labeled with 5 U of DNA polymerase I Klenow fragment (Promega), with 10 μCi of [α-^{32}P]dCTP, and analyzed by agarose gel electrophoresis as described by Rösl (43).

For in situ analysis, eyes were fixed in 10% formalin, paraffin embedded, and sectioned at 5 μm. Tissue sections on each slide were incubated with 50 U of terminal deoxynucleotidyl transferase (Pharmacia) and 1 nmol of biotin–16-dUTP (Boehringer Mannheim), as described by Gavrieli et al. (17). Biotin incorporation was detected with the avidin-biotin peroxidase complex reagent (Vector Laboratories, kit PK-4002), followed by visualization with diaminobenzidine-H_2O_2 (Vector Laboratories, kit SK-4100). Sections were counterstained with hematoxylin.

RESULTS

Microphthalmia in a transgenic family. Lens tumors can be induced in transgenic mice by using the αA-crystallin promoter (−364 to +42) to direct expression of the SV40 early region (34). The transgenic founder of family OVE1 was generated by microinjection of an αA-crystallin promoter–SV40 early region construct, and bilateral lens tumors were present in this mouse (49). Most of the transgenic F_1 offspring of the OVE1 founder mouse exhibited lens tumors similar to those of the founder. However, one mouse exhibited the unexpected phenotype of severe microphthalmia and cataracts, and transgenic offspring from this mouse showed the same phenotype (Fig. 2). Normally, the interior of the lens contains elongated fiber cells that are organized in layers (Fig. 2C and E). Histological sections of eyes of the adult mice with microphthalmia showed a small and flattened lens with disorganized, poorly differentiated fiber cells (Fig. 2D and F). Cells toward the center of the lens contained condensed and fragmented nuclei indicative of cell death. The morphology of other ocular tissues, such as cornea, iris, and retina, was not substantially affected by the lenticular changes. The transgenic retina in Fig. 2D has intact photoreceptors, while the control FVB retina exhibits loss of the photoreceptors, which is due to strain differences. The FVB mice are homozygous for the *rd* (retinal degeneration) mutation, while the OVE1 family was generated in a C3H × FVB hybrid mating, and the wild-type *rd* allele of the C3H mice is sufficient to give a normal retina.

Genomic analysis. Southern hybridizations were performed with F_1 offspring in family OVE1 (Fig. 3A). Mice with ocular tumors contain an integration site designated OVE1A, which has at least three linked copies of the transgene. Mice displaying microphthalmia contain a single copy of the transgene, and the site of integration has been named OVE1B. Since the 6.6-kb band that corresponds to the OVE1B integration site was faint in the founder mouse and was transmitted at low frequency to the offspring (1 of 26), the founder was likely mosaic for this site of integration. OVE1B homozygous mice also have a defect in hair follicle induction, which was shown to be due to insertional inactivation of the *downless* gene (49).

The phenotype of microphthalmia suggested that the early region of SV40 had been altered during integration in OVE1B. In order to characterize this alteration, the transgenic integration site (49) was subcloned, restriction mapped, and sequenced. Sequence analysis (Fig. 3B) revealed that the coding sequences had become truncated during the integration event. Rather than encoding the full 708 amino acids of the normal large T antigen, the OVE1B integration site can encode a protein consisting of the N-terminal 191 amino acids of large T antigen fused in frame to 21 amino acids encoded by the adjacent downstream mouse DNA (until a stop codon is encountered). The truncated early region can still encode a normal small t antigen.

Additional transgenic mice with microlentia. In order to confirm that the truncated early region from OVE1B can cause microphthalmia, various versions of the clone have been generated and tested (Fig. 1A). A 6.0-kb *Eco*RI-*Pst*I fragment (gr19) from the OVE1B integration site (49) and a 3.1-kb *Cla*I subclone (termed VISC) were used to generate transgenic mice. Seven gr19 and 18 VISC new founder mice were obtained (Table 1). All but two of the new transgenic mice showed cataracts, in most cases accompanied by microphthalmia and miniature lenses (termed "microlentia") analogous to the OVE1B family (Fig. 4A and B).

Various degrees of microlentia have been observed for the different families (Table 2). The least severely affected lenses are slightly smaller than normal, while the most severely affected exhibit nearly complete lens ablation, with total loss of lens fiber cells, disorganization of the epithelial cells, and a distinctive flattened architecture of the lens. Interestingly, the lens capsule remains prominent. In families in which homozygous and hemizygous mice have been compared, the homozygous mice exhibit more severe microlentia (data not shown). These variations within and between families suggest that the severity of the phenotype reflects the level of transgene expression.

Expression of truncated T antigen. RT-PCR was performed with lens RNA to assay for expression of SV40 early region transcripts. The primers were designed so that different-sized fragments would be amplified from transcripts encoding truncated large T antigen (508 bp), small t antigen (791 bp), and unspliced message (855 bp) (Fig. 5A). In the families examined, the truncated large T transcript but not the small t transcript was detected (Fig. 5B). Although the transgenic SV40 early region should be capable of producing both truncated large T antigen and full-length small t antigen through the use of alternative splice donors, the large T antigen splice site appears to be used almost exclusively in the lens. A band of approximately 650 bp was amplified in all of the VISC families (Fig. 5B), which may reflect an abnormal splicing event. This fragment does not appear to represent the normal small t antigen splice product because it does not comigrate with a control small t antigen cDNA amplified by using the same primers (data not shown). Since the 650-bp fragment hybridizes to an SV40 probe (Fig. 5F), it is not likely to be caused by inappropriate annealing of the PCR primers. The band at 855 bp could represent amplification of either unspliced message or contaminating genomic DNA. Control reaction mixtures without RT showed no amplification (Fig. 5B), indicating that this product results from amplification of

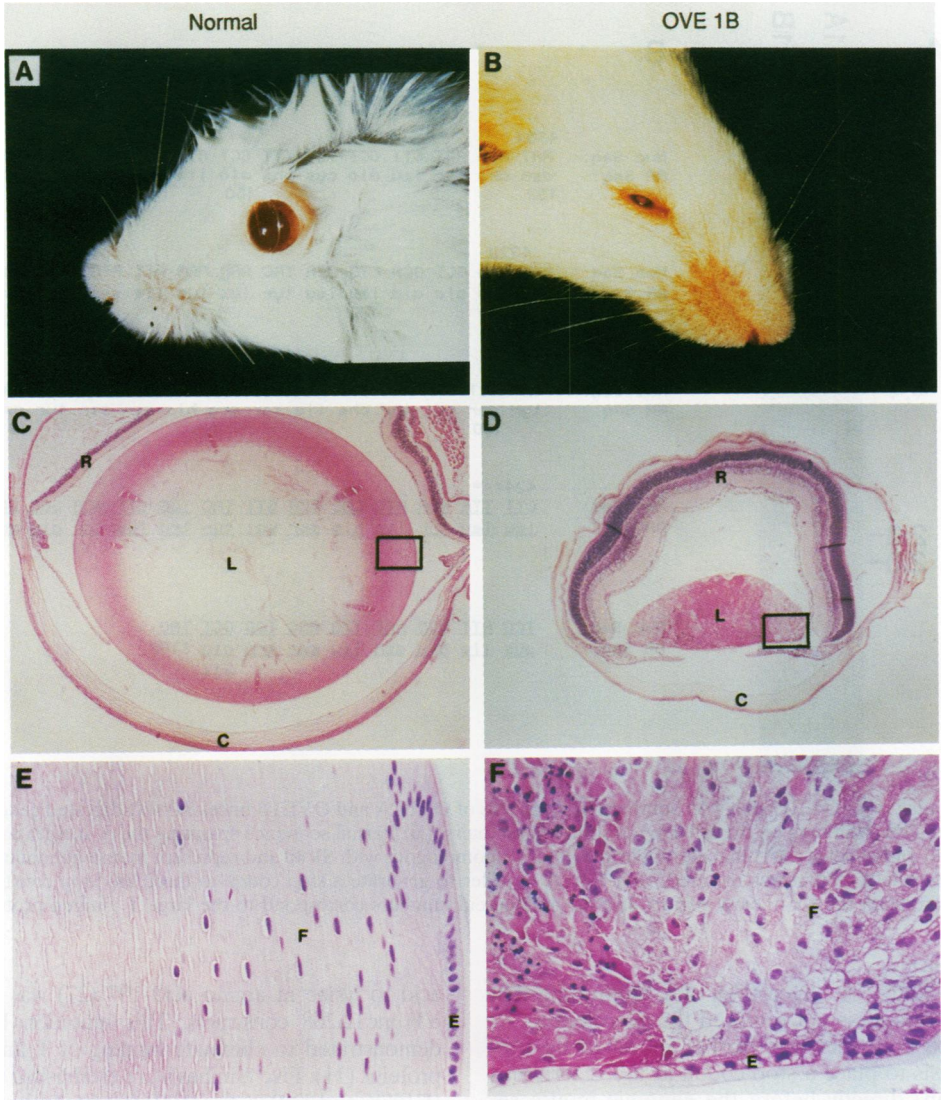

FIG. 2. Eye phenotype of OVE1B mice. Photographs of the eyes of a nontransgenic FVB mouse (A) and a homozygous OVE1B mouse (B) are shown. Eye histology from an adult nontransgenic FVB mouse (C) and an OVE1B homozygous mouse (D) is also shown. Panels E and F give higher magnifications (×400) of the boxed regions in panels C and D, respectively. OVE1B mice have small and flattened lenses with disorganized fiber cells. Cells toward the center of the lens contain condensed nuclei indicative of cell death. The difference in retinas between the nontransgenic FVB mice and OVE1B mice is not the result of transgene expression but is due to the *rd* (retinal degeneration) mutation in FVB mice. R, retina; L, lens; C, cornea; E, lens epithelial cells; F, lens fiber cells.

an unspliced transcript, which could still encode the small t antigen protein.

Lenses from OVE1B mice were analyzed for expression of the predicted truncated large T antigen protein. Because of the small amount of protein available from the microphthalmic lenses, a sensitive radioiodination protocol was used. Cellular extracts were prepared and iodinated with a chloramine T reaction mixture. The iodinated lysates were immunoprecipitated with a mixture of monoclonal antibodies directed against epitopes on the amino terminus of T antigen, and the immunoprecipitates were resolved by SDS-PAGE. An iodinated protein of about 27 kDa was detected in OVE1B lens extracts that reacted specifically with the anti-T antigen antibodies (Fig. 5G, lane 1) and not with M73 control antiserum (lane 2). This same chloramine T iodination reaction detected full-length (~94-kDa) T antigen in lysates of lens tumor (Fig. 5G, lane 3) and control SV40-transformed (29) mouse fibroblast (MKSA) cells (Fig. 5G, lane 5). Coprecipitating iodinated p53 was readily recovered from the MKSA cells but not from the lens tumor cells. The truncated T antigen in the OVE1B cells would not be able to bind p53, and no coprecipitating p53 was detected (Fig. 5G, lane 1).

Transgenic mice that produce only truncated large T antigen. The RT-PCR assays indicated that small t antigen might be poorly expressed in the transgenic lenses, suggesting that expression of truncated large T antigen is sufficient to cause microlentia. In order to confirm this prediction, we established three transgenic families with a truncated SV40 construct (αTruncTΔ268) (Fig. 1A) that is incapable of producing small t antigen because of a 268-bp deletion (nucleotides 4586 to 4854) that removes the small t antigen splice donor (8). The transgenic mice in these families exhibited a phenotype similar

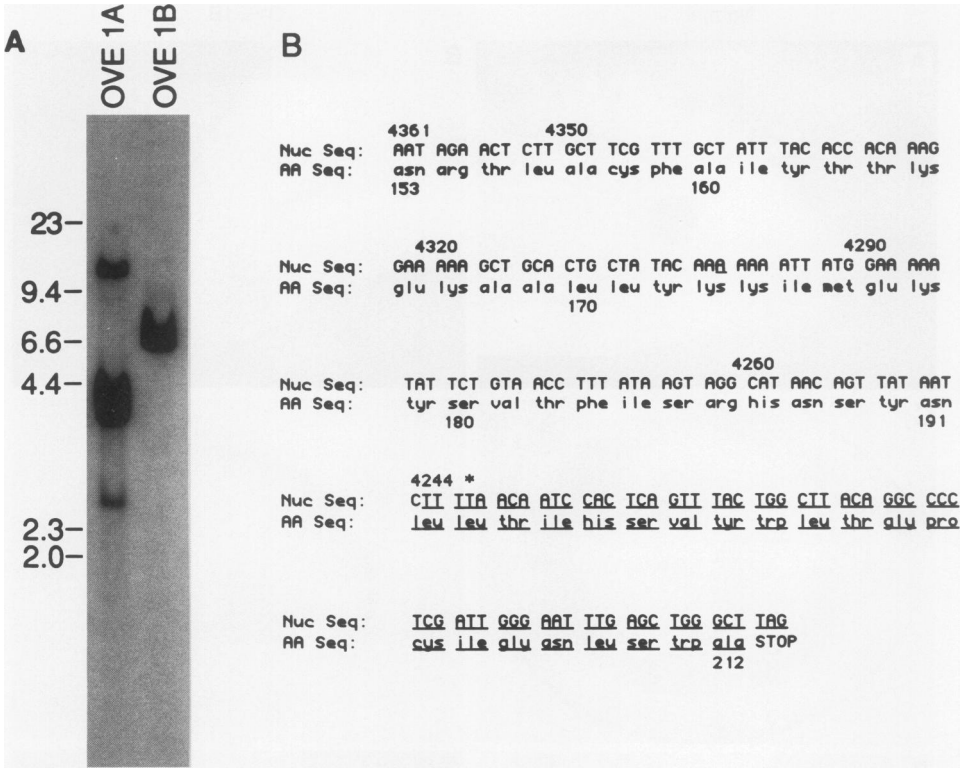

FIG. 3. Genomic analysis of OVE1 mice. (A) Southern blot analysis of OVE1A and OVE1B mice. *Bam*HI-digested genomic DNA was probed with the SV40 early region. Numbers on the left are sizes in kilobase pairs. (B) Partial sequence spanning the 3′ integration site in OVE1B. The nucleotides (Nuc.) and amino acids (AA) that are underlined are nonhomologous with SV40 and represent mouse genomic sequences (Seq.). The asterisk indicates the nucleotide that was mutated from T to A in order to generate a stop codon in the Δ268-Stop constructs (see Fig. 1). The nucleotide numbers are from the SV40 early region, and the amino acid numbers correspond to the large T antigen protein.

to that of the gr19 and VISC mice (Fig. 4C), confirming that small t antigen expression is not required for microlentia.

The TruncTΔ268 construct was further modified by site-directed mutagenesis to place a stop codon at the C terminus (αTruncTΔ268-Stop) directly before the genomic mouse sequences that encode the 21 additional amino acids (Fig. 1A). Transgenic mice in the family that was established with αTruncTΔ268-Stop exhibit microlentia, similar to the phenotype seen in mice from the other constructs (Fig. 4D). Therefore, the extra amino acids encoded by the genomic sequences are not critical for the induction of microlentia.

Truncated T antigen with a mutation in the pRb-binding region. pRb binding is one of the activities in the N-terminal region of T antigen that is important for its transformation ability (11). To determine whether binding of truncated T antigen to the pRb family is necessary for microlentia, a mutation of glutamic acid to lysine at amino acid 107 (27) was transferred into the αTruncTΔ268 constructs. This mutation has previously been demonstrated to eliminate binding of T antigen to pRb family proteins (11). Five families were established with these constructs (E107K and E107K-Stop) (Table 1). Transgenic mice in all five families have normal eyes (Fig. 4E).

TABLE 1. Summary of characteristics of transgenic founder mice

Construct	No. of founders	No. with cataracts
gr19	7	6
VISC	18	17
αTruncTΔ268	3	3
αTruncTΔ268-Stop	1	1
αTruncTΔ268-E107K	2	0
αTruncTΔ268-E107K-Stop	3	0
αE7	2	2

Transcription of the transgene in these families was confirmed by RT-PCR of lens RNA by using the same primers that were used to analyze the other families. With the Δ268 constructs, the large T transcript should amplify as a 508-bp fragment, while unspliced message should amplify a 587-bp band (Fig. 5C). In all of the families examined, the large T transcript was detected (Fig. 5D). A 587-bp band was also produced, which could represent either unspliced message or contaminating genomic DNA. Control reaction mixtures without RT showed no amplification (Fig. 5D), indicating that this product results from amplification of unspliced message.

Since the E107K mice exhibited no ocular defects, quantitative RT-PCR was performed in order to compare the level of E107K transgene expression with that of VISC. RT reaction mixtures were amplified for various numbers of cycles to determine the linear range of the assay (Fig. 5E). Amplification at 18 cycles, which falls within the linear range, was used to evaluate expression of the transgene relative to the housekeeping gene HPRT, which served as an internal control (Fig. 5F and Table 2). The level of transgenic expression in both of the E107K families examined (OVE449 and OVE450) was higher than the level of expression in one of the VISC families with severe microlentia (OVE272), indicating that the E107K mice show expression of

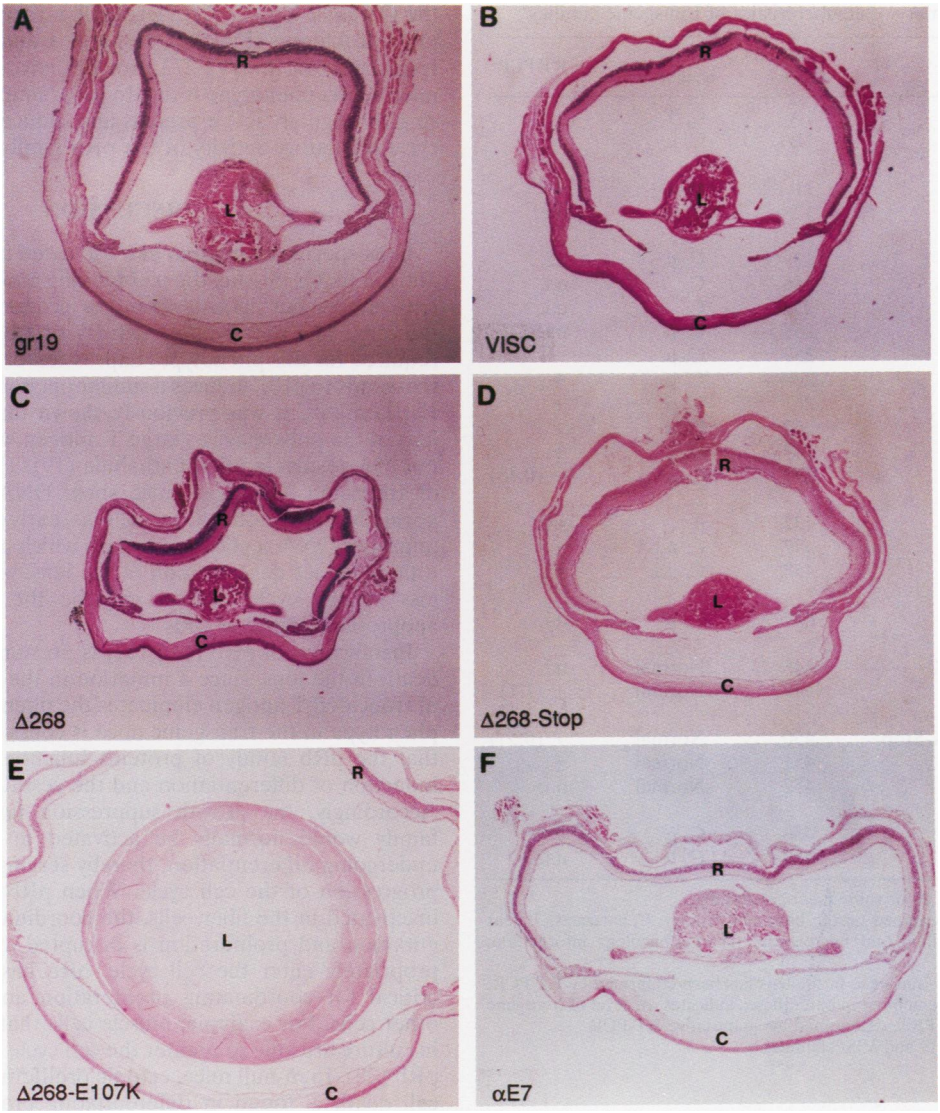

FIG. 4. Histology of representative families. Adult eye histologies of the gr19 (OVE11) homozygote (A), VISC (OVE272) heterozygote (B), αTruncTΔ268 (OVE432) heterozygote (C), αTruncTΔ268-Stop (OVE447) heterozygote (D), αTruncTΔ268-E107K (OVE449) heterozygote (E), and αE7 (OVE439) heterozygote (F) are shown. Except for the E107K family (E), the lenses all show lenticular pathology consistent with cell death. R, retina; L, lens; C, cornea.

the transgene above threshold levels. Their eyes are normal, however, which implies that binding of truncated T antigen to pRb family proteins is necessary for microlentia.

HPV E7 protein. In order to confirm that inactivation of the pRb family could induce microlentia, the gene encoding the E7 protein from HPV16 was linked to the αA-crystallin promoter for lens-specific expression in transgenic mice (Fig. 1B). The E7 protein appears to bind to and inactivate pRb in a fashion analogous to that of large T antigen (12, 38). Transgenic mice that carry the E7 construct also show microlentia (Fig. 4F), implying that inactivation of the pRb family is the critical event in the induction of lens cell death. Our attempts to generate transgenic mice that express the adenovirus E1A proteins in the lens have so far been unsuccessful.

Embryonic alterations of cell cycle. Binding of T antigen to pRb is thought to promote cell proliferation by inactivating the growth suppression ability of pRb, yet lenses expressing truncated large T antigen are smaller than normal. In order to begin to understand the process by which ablation of the lens occurs, embryonic development in a VISC family (OVE272) was studied (Fig. 6). At E15, lenses in this family appear essentially normal in size, but there is an abundance of nuclei in the interior of the lens, indicating a defect in fiber cell differentiation (Fig. 6B and D). Although this aberrant cellular morphology implies that differentiation is not proceeding normally, the cells in the interior of the lens are synthesizing β-crystallin, a fiber cell marker (37), at E15 (data not shown) indicating that lens fiber cell differentiation has been induced. Ablation of the lens is not yet apparent, indicating that degeneration occurs at subsequent stages.

To determine whether changes in cell cycle regulation accompany the changes in differentiation, DNA replication was examined by incorporation of BrdU, a thymidine analog. During normal lens development, DNA replication is confined to the epithelial cells and is prominent in the proliferative zone anterior to the equatorial region, while the fiber cells are

TABLE 2. Summary of characteristics of transgenic families[a]

Construct	Family (OVE)	Eye phenotype[b]	RT-PCR[c]
αTAg	1A	C, T	+
	1B	C, μ	+
gr19[d]	11	C, μ	+
	12	C, μ	n.i.
VISC[d]	82	C, μ	n.i.
	256	C	n.i.
	257	C	n.i.
	258	C, μ	n.i.
	259	C, μ	+
	260	C, μ	+
	261	C, μ	+
	262	C, μ	+
	263	C, μ	+
	272	C, μ	+ (0.45)
αTruncTΔ268	432	μ	n.i.
	457	C, n.i.	+
	458	C, n.i.	n.i.
αTruncTΔ268-Stop	447	C, μ	n.i.
αTruncTΔ268-E107K	448	Normal	n.i.
	449	Normal	+ (1.1)
αTruncTΔ268-E107K-Stop	450	Normal	+ (0.57)
	451	Normal	+
	452	Normal	n.i.
αE7	438	C, μ	+
	439	C, μ	n.i.

[a] Data obtained from F_1 or subsequent generations.
[b] Ocular changes determined on the basis of histology. C, cataracts; T, lens tumors; μ, microphthalmia and microlentia; n.i., no histology information; normal, no eye defects.
[c] +, expression of the transgene in the lens has been confirmed by RT-PCR. For selected families, the number in parentheses indicates the level of transgene expression relative to HPRT, determined by quantitative RT-PCR.
[d] Partial list of the gr19 and VISC families.

postmitotic and do not undergo DNA replication (Fig. 6C). In the embryonic OVE272 lens, fiber cells in the interior of the lens are found to be undergoing DNA replication (Fig. 6D). Since the αA-crystallin promoter becomes active around E12 to E13 (39), the ectopic pattern of DNA replication appears to correlate with the onset of transgene expression and precedes the degeneration of the lens. Apparently, loss of activity of the pRb family is sufficient to permit inappropriate progression of fiber cells into S phase, and this inappropriate entry into the cell cycle may initiate fiber cell death.

Apoptosis in the lens. Apoptosis, or programmed cell death, is an active process that is characterized by distinctive morphological changes, including chromatin condensation, degeneration of the nucleus, and cleavage of the DNA into nucleosome-sized fragments (3, 56). In order to examine whether lens ablation in the truncated T antigen mice occurs by apoptosis, DNA degradation in lenses from OVE272 mice was examined. By E17, lens DNA in this family was detected as mono- and oligonucleosome-sized fragments, while the DNA in nontransgenic lenses remained at a high molecular weight (Fig. 7A). DNA fragmentation was further examined in situ by end labeling of fragmented DNA in tissue sections by using terminal deoxynucleotidyl transferase and a biotinylated nucleotide. This assay detected apoptosis in many of the fiber cell nuclei of the OVE272 lenses (Fig. 7B), while no apoptosis was detected in control lenses (Fig. 7C). The appearance of apoptotic DNA fragmentation in OVE272 provides strong evidence that the microlentia phenotype is due to programmed cell death occurring through an active process and induced by loss of the cell cycle regulatory activity of the pRb family of proteins.

DISCUSSION

Lens-specific expression of a truncated form of SV40 large T antigen that is missing the p53-binding domain does not induce lens tumors but instead promotes programmed cell death in the lens. The pRb-binding activity of truncated T antigen is required for this phenotype. Expression of E7, another protein that binds to pRb, induces a similar phenotype of lens ablation. Furthermore, it was previously shown that expression in the lens of the polyomavirus large T antigen, which binds pRb but not p53, results in microphthalmia (19). The transgenic lenses of truncated T antigen mice show DNA replication in the normally postmitotic fiber cells as early as E15. Therefore, improper DNA replication in cells which are normally postmitotic precedes degeneration of the lens, which suggests that a loss of cell cycle control may be the proximal cause of apoptosis in the fiber cells.

Inactivation of pRb (or p107) is presumably critical for cell death in the lens, since a mutation in the pRb-binding region of truncated T antigen eliminates the phenotype. The fiber cell phenotype in the transgenic mice is consistent with the notion that the pRb family of proteins functions to coordinate the induction of differentiation and the cessation of proliferation. Accordingly, the growth suppression function of the pRb family would normally be activated in lens cells that are undergoing differentiation, thereby serving as a switch to stop progression of the cell cycle. When pRb family proteins are inactivated in the fiber cells, the coordination between differentiation and proliferation is disrupted and these cells inappropriately enter the cell cycle. pRb has been shown to be critical for coordinating proliferation and differentiation in other cells. For example, muscle cells that have differentiated in culture are able to reenter the cell cycle upon inactivation of pRb (20). In *rb*-null mice, ectopic proliferation coincident with cell death is found in differentiating brain cells and blood-forming tissues (7, 25, 31). Recent studies with *rb* knockout mice indicate lens defects (55), but since the mice die prenatally, microlentia and fiber cell death are not as readily apparent as in the truncated T antigen mice. The observed changes in the *rb*-null mice support the notion that inactivation of pRb, rather than p107, is the critical event that induces lens cell death in the truncated T antigen mice.

Although the pRb-binding region of truncated T antigen is critical for lens ablation, other activities in the N-terminal region may also be involved. For example, T antigen with a deletion of amino acids 17 to 27 can bind pRb but is defective for transformation (41, 57). This region has been shown to be functionally equivalent to the p300-binding activity of adenovirus E1A (57). Since the E107K mutation only affects the pRb-binding domain and results in mice with normal eyes, any additional N-terminal activities are benign by themselves. Other functions of truncated T antigen, however, could cooperate with the pRb-binding activity to induce lens cell death.

The induction of apoptosis in the truncated T antigen lenses illustrates an interesting relationship between the cell cycle and cell death. Some of the same proteins that are involved in coordinating progression through the cell cycle may also induce cell death when activated at an inappropriate time. In the lens fiber cells, the differentiation process is accompanied by the

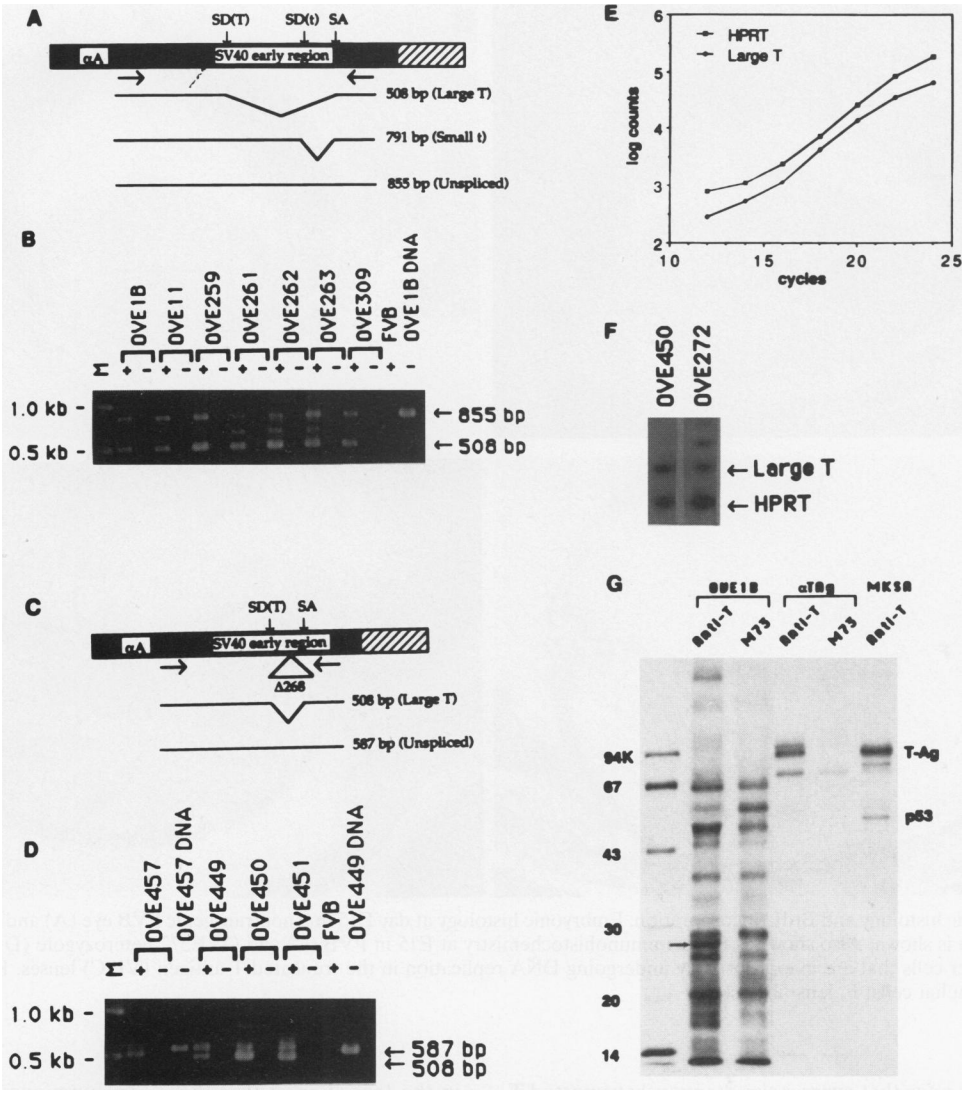

FIG. 5. Detection of transgene expression. (A) Diagram indicating the location of primers used for RT-PCR in OVE1B, gr19, and VISC mice and the predicted sizes of the amplified products. (B) Analysis of RT-PCR products by gel electrophoresis. M indicates size markers, and + and − show whether (+) or not (−) the RT reaction was performed. In the transgenic lenses, the large T antigen splice product was detected by amplification of the 508-bp fragment, while unspliced message yielded an 855-bp product. No amplification was seen in the RNA samples that were not incubated with RT. (C) Diagram indicating the location of primers used for RT-PCR in Δ268 mice and the predicted sizes of the amplified products. (D) Analysis of RT-PCR products by gel electrophoresis. As in panel B, spliced (508-bp fragment) as well as unspliced (587-bp fragment) products were detected. (E) Analysis of quantitative RT-PCR in OVE450 mice (an E107K family) illustrating the linear relationship between cycles of amplification and log of the PCR signal. PCR products were quantified by measuring radioactivity on Southern hybridizations. (F) Comparison of transgene expression in OVE450 (E107K) mice with that in OVE272 (VISC) mice by quantitative RT-PCR. After 18 cycles of amplification, PCR products were detected by Southern hybridization with SV40 and HPRT probes. The level of transgene transcription is higher in OVE450 mice relative to HPRT, than in OVE272 mice (Table 2). (G) Analysis of truncated T antigen protein in OVE1B lenses by immunoprecipitation of iodinated cellular extracts. A protein of 27 kDa, corresponding to truncated T antigen, was detected (arrow) that reacted specifically with anti-T antigen antibodies (Anti-T) but not with M73 control antiserum. Also, the anti-T antigen antibodies recognized full-length T antigen (T-Ag) (94 kDa) in lens tumors from mice expressing full-length T antigen (αTAg) and in SV40-transformed fibroblasts (MKSA).

establishment of a postmitotic state, and in the truncated T antigen lenses, entrance into the cell cycle is lethal. The notion that the cell cycle machinery can be responsible for cell death is supported by other studies. For example, expression of SV40 T antigen in the neural retina causes postmitotic photoreceptor cells to both enter the cell cycle and undergo degeneration (2). In the hormone-depleted prostate gland, quiescent cells enter the cell cycle and subsequently undergo apoptosis (9). Future studies will address which cell cycle regulatory components become activated in truncated T antigen lenses and whether any of these proteins are capable of inducing apoptosis.

Regardless of the cause of the cell death in the truncated T lenses, mice expressing full-length T antigen are able to circumvent cell death and induce proliferation of fiber cells, which leads to tumor formation. One potential explanation is that the truncated T antigen is simply a toxic protein. However, expression of truncated T antigen in tissue culture cells does not kill the cells (data not shown). In addition, when matings were done to

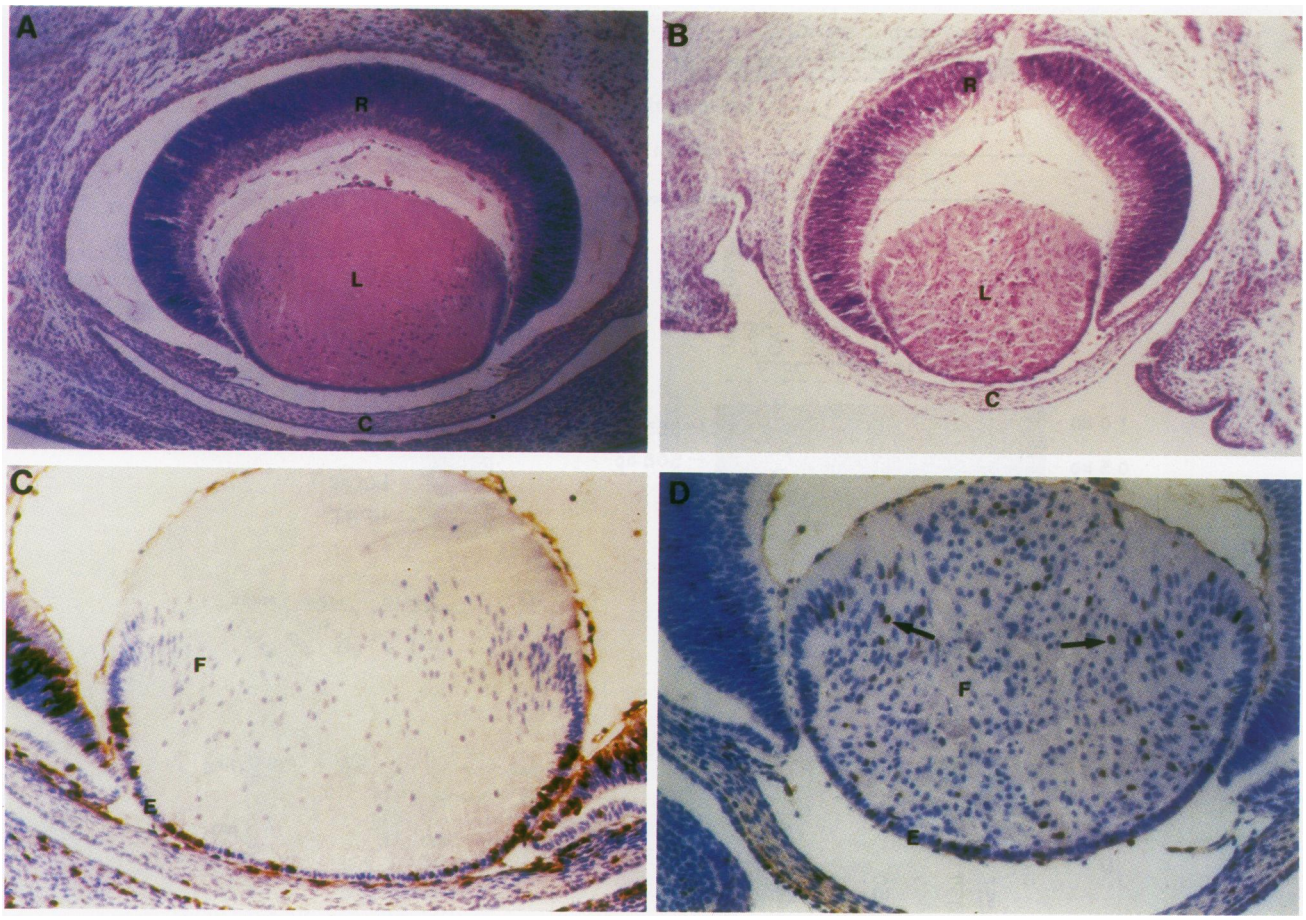

FIG. 6. Embryonic histology and BrdU incorporation. Embryonic histology at day E15 in a nontransgenic FVB eye (A) and an OVE272 (VISC) heterozygote eye (B) is shown. Also shown is BrdU immunohistochemistry at E15 in FVB (C) and OVE272 heterozygote (D) eyes. Arrows show examples of lens fiber cells that are inappropriately undergoing DNA replication in the truncated T antigen (VISC) lenses. R, retina; L, lens; C, cornea; E, lens epithelial cells; F, lens fiber cells.

generate transgenic mice that express simultaneously truncated T antigen and full-length T antigen in a lens-specific fashion, the mice developed lens tumors (data not shown). Therefore, full-length T antigen appears to contain activities that are missing in the truncated version that are required for tumor formation. Identifying these additional activities is important for understanding the molecular basis of tumor induction.

The most obvious activity of T antigen in addition to pRb binding, which could be required for lens tumorigenesis, is p53 binding. Since lens cell death occurs when the p53-binding domain is missing, the inactivation of p53 by full-length T antigen may contribute to tumor formation by suppressing programmed cell death. Studies with adenovirus E1A support this hypothesis. E1A encodes a protein that binds to pRb (54) and stimulates DNA synthesis (50), and cells expressing E1A undergo apoptosis similar to the lens cells expressing truncated T antigen (53). In E1A-induced apoptosis, p53 becomes stabilized (33), while inactivation of p53 in these cells by the E1B 55K protein or by a dominant-mutant form of p53 suppresses cell death and results in transformation (10). Experiments are in progress to determine directly whether the lens cell death induced by truncated T antigen is mediated by p53.

Although the p53-binding activity of T antigen is likely to cooperate with the pRb-binding activity to induce lens tumors, studies in which the HPV E6 and E7 proteins were expressed in the lens suggest that these two functions are not sufficient. HPV E6 interacts with p53 (47, 52), and HPV E7 binds to pRb (12, 38), but mice expressing E6 and E7 developed microphthalmia, with tumors sporadically forming after several months, suggesting that mutations had first accumulated prior to tumorigenesis (18). Therefore, the simplified hypothesis that inactivation of pRb and p53 is sufficient to cause lens tumors appears to be incomplete, and additional activities of T antigen, which presumably are not present in HPV E6 or E7, could be required for lens tumorigenesis. In addition, binding of pRb and p53 to viral proteins has been shown to be insufficient for transformation in other systems. For example, a hybrid polyomavirus-SV40 large T antigen which binds both pRb and p53 is unable to transform rat fibroblasts (35).

In our studies, pRb and related proteins presumably have been inactivated by binding to truncated T antigen. Alterations in such genes can normally occur through mutation. However, our experiments suggest that the result may not necessarily be progression toward a tumor cell phenotype. Instead, the result could be programmed cell death if the cells are induced to enter the cell cycle in a context that is incompatible with proliferation. Such a response may represent an inherent mechanism for eliminating differentiated cells with potential tumor-promoting mutations. Identification of the mechanisms that can reestablish a functional cell cycle in differentiating lens

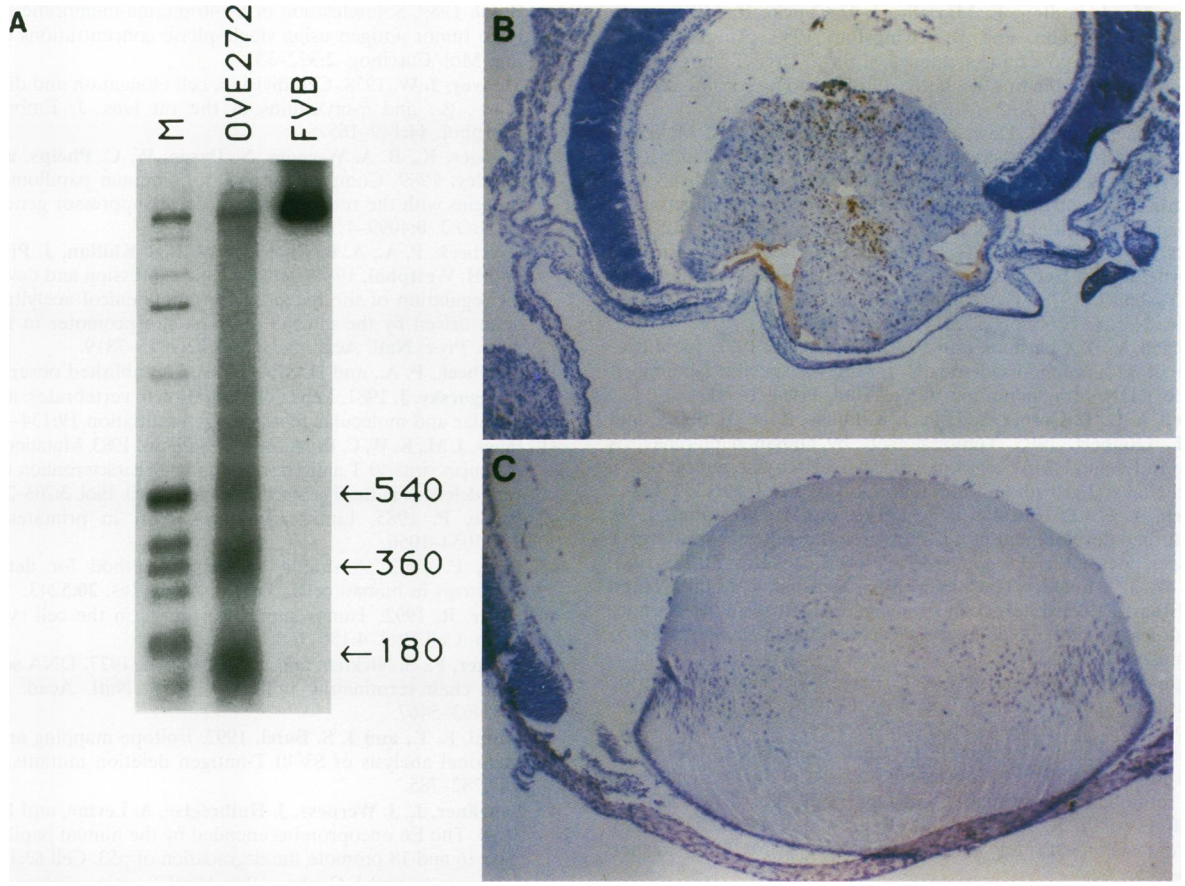

FIG. 7. Analysis of apoptotic DNA fragmentation. (A) Gel electrophoresis of lens DNA from OVE272 (VISC) heterozygote and nontransgenic FVB mice. The DNA from OVE272 mice is degraded into nucleosome-sized fragments, indicative of apoptosis. M indicates size markers. (B and C) In situ DNA fragmentation assay of OVE272 newborn (B) and nontransgenic FVB newborn (C) mouse eyes. DNA fragmentation, which is indicated by the brown staining used to detect incorporation of a biotinylated nucleotide by terminal deoxynucleotidyl transferase, is present in the fiber cell nuclei of only the transgenic lens (B).

fiber cells can provide useful insights about control points that must be altered in order to cause progression to tumorigenesis.

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ADDENDUM IN PROOF

Analogous experiments expressing the HPV E7 gene in lenses of transgenic mice have recently been reported (H. Pan and A. Griep, Genes Dev. **8**:1285–1299, 1994).

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