Expression Patterns of Retinoblastoma and E2F Family Proteins during Corneal Development

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Purpose. To determine the expression patterns of the retinoblastoma protein and the E2F transcription factor families in limbal and corneal epithelia and in corneal keratocytes in situ during corneal development and differentiation.

Methods. Retinoblastoma protein (pRb) and its family members p107 and p130; E2F-1, -2, and -4, members of the E2F family of transcription factors; and Ki67, a marker of actively cycling cells, were localized by indirect immunofluorescence microscopy, in corneas of neonatal, juvenile, and adult rats. Presence of mRNA for pRb, p107, p130, and E2F types 1 to 5 in adult corneal epithelium was determined by reverse transcription–polymerase chain reaction.

Results. mRNA for all members of pRb and E2F families was present in adult corneal epithelium. The greatest number of Ki67-positive corneal and limbal epithelial cells were present at days 13 to 19, and Ki67-positive stromal keratocytes at day 2. pRb and E2F-2 were localized to all cells in neonatal, juvenile, and adult corneas. With age, p130 localization became more intense and nuclear in stromal keratocytes and suprabasal cells of corneal and limbal epithelia; p107, initially nuclear in limbal and corneal epithelia, became increasingly cytoplasmic in corneal epithelium. E2F-1 was initially nuclear in keratocytes and diminished after day 10. E2F-1 was localized in the basal cell layer of limbal and corneal epithelia after day 10. E2F-4 was always nuclear in limbal epithelium and cytoplasmic in corneal epithelium.

Conclusions. Expression patterns of pRb and E2F family proteins vary with corneal cell differentiation, but are most apparent with p130 and p107. Nuclear localization of p130 appears to correlate with terminal differentiation in epithelium and entrance into a quiescent state by keratocytes. In contrast, p107 is nuclear in the undifferentiated limbal basal cells and is cytoplasmic in the remainder of the corneal epithelial cells. (*Invest Ophthalmol Vis Sci.* 2000;41:1054–1062)

In the present study, the cornea was used to examine alterations in proteins associated with regulation of cellular proliferation and differentiation.[1] The developing rodent cornea provides an opportunity to examine these alterations in both epithelium and stromal keratocytes.[2–5] At birth, rat corneal and limbal epithelia consist of one or two layers of cells. At 10 days of age, the number of cell layers increases to two or three, and by 14 days, just after eyelid opening, four to five layers of cells are observed.[6] In the adult, stromal keratocytes are thought to be quiescent; however, 5% to 10% of the stromal keratocytes appear to proliferate during corneal development.[7,8] Proliferation is maximal at 1 to 3 days of age and declines until the time of eyelid opening (12–14 days). This suggests that during development the keratocytes either become quiescent or exit the cell cycle.[8–10]

One of the key regulatory proteins of the cell cycle is the retinoblastoma gene product (pRb). The product of the retinoblastoma gene is a ubiquitously expressed, 105-kDa nuclear phosphoprotein.[11,12] The Rb family consists of three members, pRb, p107, and p130.[13,14] In their hypophosphorylated state, pRb, p107, and p130 complex with members of the E2F family of transcription factors. After their phosphorylation, the complex with E2F is disrupted, allowing free E2F to activate the transcription of genes necessary for DNA synthesis. Binding of DNA and transcription occurs only when E2F is complexed with the DP family of transcription factors[11–31] The pRb and E2F families also appear to regulate entrance and exit from the cell cycle and have been associated with muscle and neuronal differentiation.[25,32–34]

Recent reports suggest that translocation of cell cycle–associated proteins from the cytoplasm to the nucleus may be another method of cell cycle regulation. It has been generally assumed that cell cycle–associated proteins, which function at least in part by binding DNA, would be present primarily in the nucleus. However, cyclin B1,[35,36] CDK 4,[37] E2F-1, and E2F-4[38] have all been demonstrated to be translocated between the cytoplasm and the nucleus in a cell cycle–dependent manner. This would allow them to have maximal affect in the nucleus when they are most needed to regulate cell cycle entrance, progression, and exit. It is unclear whether these proteins also have a functional role when they are localized in the cytoplasm.

Although there have been numerous studies of pRb and E2F function in cell lines and cultured cells, very few investi-

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gators have examined these proteins in vivo. In the present study, we used the developing cornea to correlate the expression and localization of the pRb and E2F families in cells at different states of differentiation.

Materials and Methods

Animals

Sprague–Dawley rats were used, and all procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were killed with an overdose of pentobarbital sodium.

Indirect Immunofluorescence Microscopy

Neonatal (1–14 days of age), juvenile (15 days to 6 weeks of age), and adult (3–4 months of age) rats were killed. Eyes with or without eyelids were enucleated, placed in optimal cutting temperature compound (Tissue Tek II; Laboratory Tek, Naperville, IL) and frozen on dry ice. Six-micrometer cryostat sections were placed on gelatin-coated slides and air dried overnight at 37°C. Slides were fixed in 100% methanol for 10 minutes at −20°C, washed with phosphate-buffered saline (PBS) for 10 minutes, and then blocked in 1% bovine serum albumin solution in PBS for 10 minutes. Antibody against pRb was obtained from Boehringer Mannheim (Indianapolis, IN), antibodies against p107 (C-18), p130 (C-20), E2F-1 (KH95), E2F-2 (L-20), and E2F-4 (C-20) were obtained from Santa Cruz Biotech (Santa Cruz, CA), and anti-Ki67 (MM1) from Novacastra (Newcastle-upon-Tyne, UK). The following dilutions were used for primary antibodies: 2 μg/ml pRb, E2F-2, E2F-4, and Ki67; 1 μg/ml p107 and p130; and 4 μg/ml E2F-1. Slides were incubated for 1 hour in a moist chamber at room temperature for all antibodies except pRb and E2F-1, which were incubated overnight at 4°C. Slides were rinsed with PBS and then blocked with 1% bovine serum albumin for 10 minutes. Fluorescein isothiocyanate–conjugated affinity-purified donkey anti-rabbit IgG (for p107, p130, E2F-2, and E2F-4) and anti-mouse IgG (for pRb, Ki67, and E2F-1; Jackson ImmunoResearch, West Grove, PA) were applied at a dilution of 1:50 and incubated for 1 hour at room temperature. Slides were rinsed in PBS, and coverslips were mounted with Vectashield (Vector, Burlingame, CA). Sections were viewed and photographed with a microscope (Axiophot; Carl Zeiss, Thornwood, NY). Negative controls consisted of secondary antibody alone, irrelevant monoclonal antibodies, or primary antibody preadsorbed with its respective antigen. Sections from at least four rats were examined for each antibody and time point. Antibody binding was examined in the limbal and corneal epithelia and in stromal keratocytes. In addition, staining patterns were examined to determine whether localization was cytoplasmic, nuclear, or both. Relative percentages of Ki67 binding were determined by comparing Ki67-labeled cells with total cells by means of by propidium iodide counterstaining. Cells were counted in the basal cell layers of limbal and corneal epithelia and in the corneal stroma. Ki67-labeled keratocytes were not quantitated in the limbal stroma because this area contains a mixture of cell types.

Table 1. Oligonucleotide Primer Sequences

Primer	Oligonucleotide Sequence	Annealing Temperature	Fragment Size
G3PDH	5′-ACCACAGTCCATGCCATCAC-3′ 5′-TCCACCACCCTGTTGCTGTA-3′	52°C	452 bp
E2F-1	5′-ACGCTATGAAACCTCACTAAA-3′ 5′-AGGACATTGGTGATGTCATA-3′	48°C	143 bp
E2F-2	5′-AAGAAGTTCATTTACCTCCTGA-3′ 5′-AATCACTGTCTGCTCCTTAAA-3′	50°C	386 bp
E2F-3	5′-CTGTACCCTGGACCTCAAACT-3′ 5′-AGGGGAGGCAGTAAGTTCACA-3′	54°C	450 bp
E2F-4	5′-CAAAGAGCTGTCAGAAATCTTT-3′ 5′-TTGTAGATATAATCGTGGTCTCC-3′	50°C	137 bp
E2F-5	5′-GAATTGAAGGAAAGAGAACTTG-3′ 5′-AAACCACTGGCTTAGATGAA-3′	50°C	300 bp
p107	5′-AATAGCAAAGCCGTGAACAAG-3′ 5′-GGTATAGTGTTGGCAGAAAGT-3′	50°C	438 bp
p130	5′-CCAGCCAGACGAGAATGTTAG-3′ 5′-TCCAAGTCCTCCAGCATCAGT-3′	54°C	550 bp
pRb	5′-TCTACCTCCCTTTCCCTGTTT-3′ 5′-AGTCATTTTTGTGGGTGTTGG-3′	50°C	549 bp

Isolation of Total RNA and Performance of Reverse Transcription–Polymerase Chain Reaction

Corneal epithelium was harvested, from a central 4-mm area of 12 adult rat eyes. Tissue was immediately frozen in liquid nitrogen, and total RNA was isolated using TRIzol Reagent (Gibco–Life Technologies, Grand Island, NY). Before further use, the total RNA was treated with DNase I, amplification grade (1 U DNase/1 μg total RNA; Life Technologies).

Reverse transcription–polymerase chain reaction (RT-PCR) was performed as previously described[39] using specific primers (Table 1). The primer sets for E2F-1, -2, -4, and -5 are published.[40] The primer sets for pRb, p107, p130, and E2F-3 were devised using commercial software (Oligo Primer Analysis; National Biosciences, Plymouth, MN) that selects primers based on minimal hairpin formation, minimal duplex formation, and guanine cytosine (GC) composition. Primer sets for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were purchased from Clontech (Palo Alto, CA). Samples were denatured for 1.5 minutes at 94°C, followed by 25 (G3PDH), 30 (p107, p130, pRb), or 35 (E2F-1, -2, -3, -4, and -5) PCR cycles of denaturation for 1.5 minutes at 94°C, annealing for 1 minute (Table 1), and extension for 1 minute at 72°C. The final elongation step was performed at 72°C for 7 minutes. Twenty microliters of the PCR product was then resolved on 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. The gel was photographed (Digital Science DC40 camera; Eastman Kodak, Rochester, NY). G3PDH primers were used as a positive control of cDNA quality. Samples with no cDNA were also amplified and served as negative controls. Specificity of the PCR products was checked by cycle sequencing at the Tufts DNA Sequencing Facility (Boston, MA).

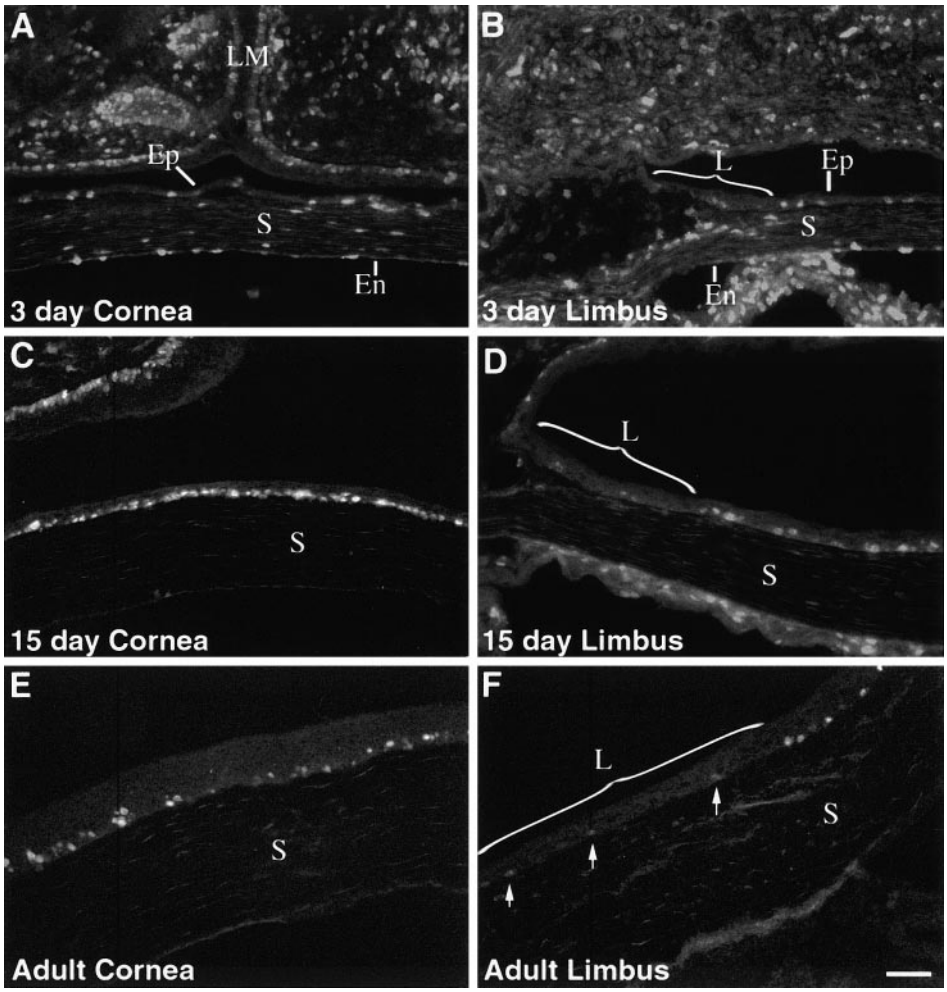

FIGURE 1. Immunolocalization of Ki67 in central cornea (**A**, **C**, and **E**) and limbus (**B**, **D**, and **F**) of 3-day-old (**A**, **B**), 15-day-old (**C**, **D**), and adult (**E**, **F**) rats. Ki67 was detected in the basal cell layer of limbal and corneal epithelia at all ages. Maximal localization of Ki67 in corneal epithelium correlated with the onset of stratification (**C**). The intensity of fluorescence was less in the limbal cells (**F**, *arrows*). LM, eyelid margin; Ep, corneal epithelium; S, stroma; En, endothelium; L, limbus. Bar, 50 μm.

RESULTS

Ki67, a marker of actively cycling cells,[41,42] was used to demonstrate the relative proliferative activity of limbal and corneal epithelial cells and central corneal keratocytes. As seen in Figure 1, Ki67 is localized in the basal layer of corneal and limbal epithelium. The number of labeled epithelial cells peaks at days 14 to 15 (Figs. 1C, 1D; Fig. 2) and then declines until it reaches adult levels (Figs. 1E, 1F; Fig. 2). Although the relative number of labeled cells varied with age, the corneal epithelium consistently had a higher percentage of labeled cells than did the limbal epithelium (Fig. 2). No Ki67 was detected in either limbal or corneal suprabasal cells. These data are consistent with previous reports that the limbal epithelium contains a mixture of slow-cycling stem cells and more rapidly cycling transient amplifying cells, that the central corneal epithelium contains a rapidly proliferating basal cell layer, and that the suprabasal cells have exited the cell cycle and become terminally differentiated.[4,5,31] Ki67 was also localized in stromal keratocytes in the developing cornea, with the maximal number of labeled cells in rats 1 to 3 days of age (Fig. 1A; Fig. 2). The number of labeled keratocytes decreased dramatically with age (Fig. 2), and by 15 days, less than 0.5% of the cells were labeled (Fig. 1C; Fig. 2). These localization patterns suggest that keratocytes are proliferative in the rat cornea at birth, then become quiescent or exit the cell cycle.

RT-PCR was used to assay for mRNA for members of the pRb and E2F families in adult corneal epithelium. The data shown in Figure 3 indicate that mRNA for all members of both families was expressed. No bands were detected in samples with no cDNA (data not shown). Based on these results, indirect immunofluorescence was then used to localize pRb, p107, p130, and E2F-1, -2, and -4. E2F-3 and -5 were not examined, because no antibody could be obtained that elicited reproducible results on rat cornea.

As seen in Figures 4, 5, and 6, members of the retinoblastoma family, pRb, p130, and p107 each had a distinct pattern of localization. pRb was present in the nuclei of all epithelial cells regardless of proliferative or differentiative state (Fig. 4). Binding intensity appeared to be greater in basal cells than in suprabasal cells. The pattern of pRb localization was the same during development as in the adult. No differences were observed between limbal and corneal epithelium. pRb was also present in the nuclei of all keratocytes, regardless of whether the cells were in a quiescent or proliferative state.

In the developing rat, p130 was initially present at low levels in the cornea (Fig. 5A) and limbus (Fig. 5E). As the epithelium began to stratify (days 7–9), the level of anti-p130 binding appeared to increase (Fig. 5B). Until day 7, p130 exhibited a weak cytoplasmic localization but became increasingly nuclear after days 9 to 11. At 15 days, nuclear localization

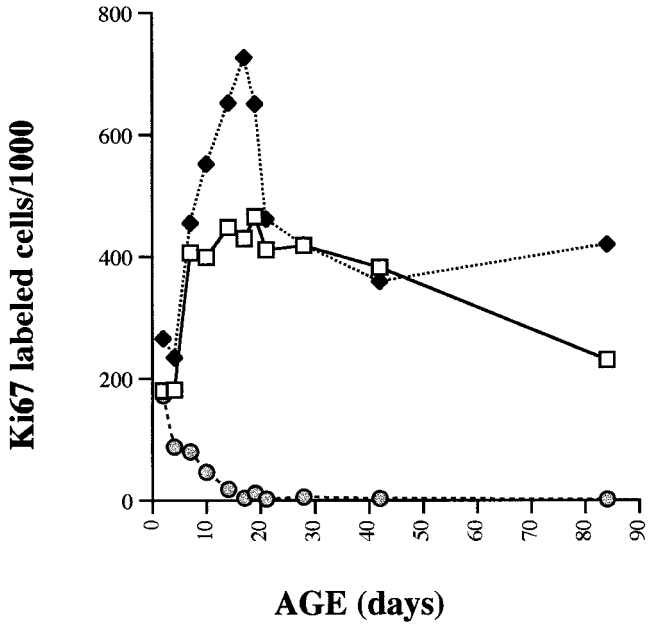

FIGURE 2. Quantitation of Ki67-labeled cells during development in (♦) corneal epithelium, (□) limbal epithelium, and (●) corneal keratocytes. Data are expressed as the number of Ki67-labeled cells per 1000 propidium iodide–labeled cells. At least four sections from four corneas were examined per time point. At least 1000 limbal epithelial basal cells, 5000 corneal epithelial basal cells, and 5000 corneal keratocytes were examined at each time point.

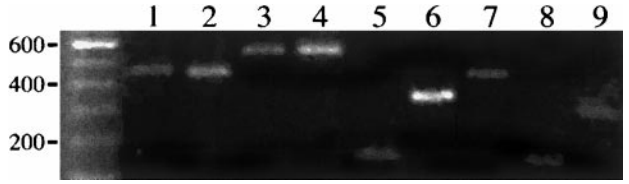

FIGURE 3. RT-PCR analysis of RNA isolated from adult rat corneal epithelium. The PCR products were visualized on agarose gels stained with ethidium bromide: *lane 1*, G3PDH; *lane 2*, p107; *lane 3*, p130; *lane 4*, pRb; *lane 5*, E2F-1; *lane 6*, E2F-2; *lane 7*, E2F-3; *lane 8*, E2F-4; and *lane 9*, E2F-5. Standard markers (in bp) are shown on the *left*.

of p130 in suprabasal cells could be clearly seen (Fig. 5C). In adult corneal epithelium, cytoplasmic p130 localization was seen in all cell layers; however, nuclear localization was present only in the suprabasal cell layers (Fig. 5D1). In the adult, limbal epithelium exhibited nuclear localization of p130 in the suprabasal cells and in some basal cells, with diffuse cytoplasmic localization in all layers (Fig. 5F). The staining intensity of anti-p130 also was altered in keratocytes during development. Anti-p130 binding was initially present at low levels (Figs. 5A, 5E) and became increasingly intense after days 9 to 11 (Figs. 5B, 5C). In the adult cornea, p130 exhibited a staining pattern similar to that of propidium iodide (data not shown), indicating a nuclear localization in the keratocytes (Figs. 5D1, 5F).

The localization pattern of p107 was also altered during development and differentiation (Fig. 6). From birth until day 7, p107 was primarily localized in the nuclei of basal cells in both corneal and limbal epithelia (Figs. 6A, 6B). Diffuse cytoplasmic localization was also present. This pattern remained relatively unchanged in the limbal epithelium during development; nuclear binding was seen even in adults (Figs. 5B, 5D, 5F). However, in corneal epithelium, the localization became increasingly cytoplasmic as the epithelium stratified (Figs. 5C, 5E). In contrast to pRb and p130, nuclear localization was observed only in basal cells. Also, the staining intensity of anti-p107 in the corneal keratocytes appeared to diminish with age (Figs. 5A, 5C, 5E). No fluorescence was observed in the central cornea in sections when p130 or p107 antibodies were preadsorbed with blocking peptides (Figs. 4, 5, 6). Low level binding was observed in the limbal vasculature of both the

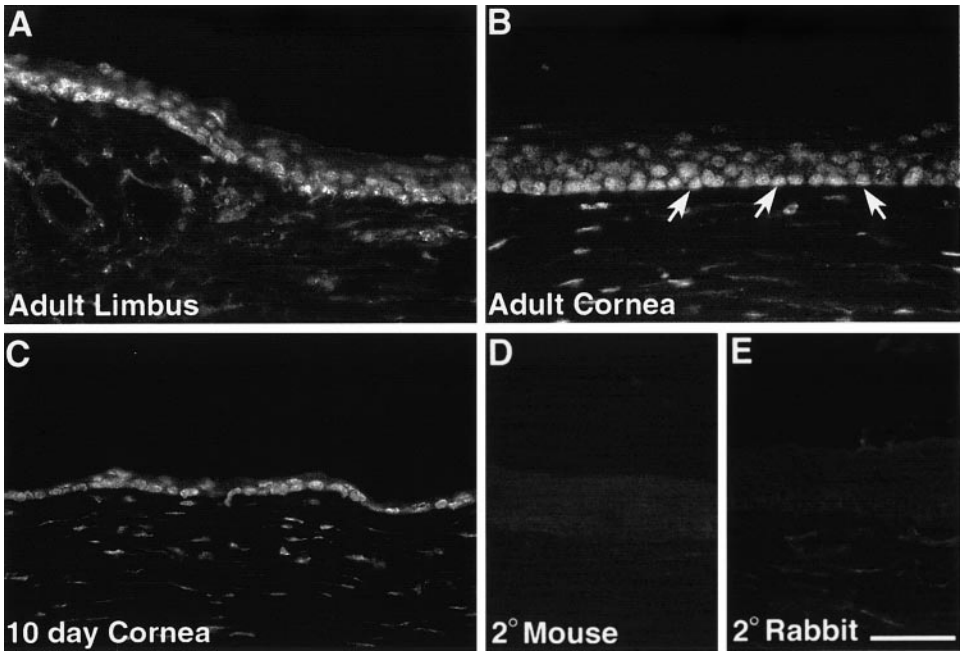

FIGURE 4. Immunolocalization of pRb in limbus (**A**) and central cornea (**B**, **C**) of adult (**A**, **B**) and 10-day-old (**C**) rats. Localization was most intense in the basal cell nuclei (*arrows*). Secondary antibody control specimens for mouse (**D**) and rabbit (**E**) antibodies are shown in adult central cornea. Secondary antibody control samples were examined for all experiments. (**D**) Representative results for Figures 1, 4, and 7; (**E**) representative results for Figures 5, 6, and 8. Bar, 50 μm.

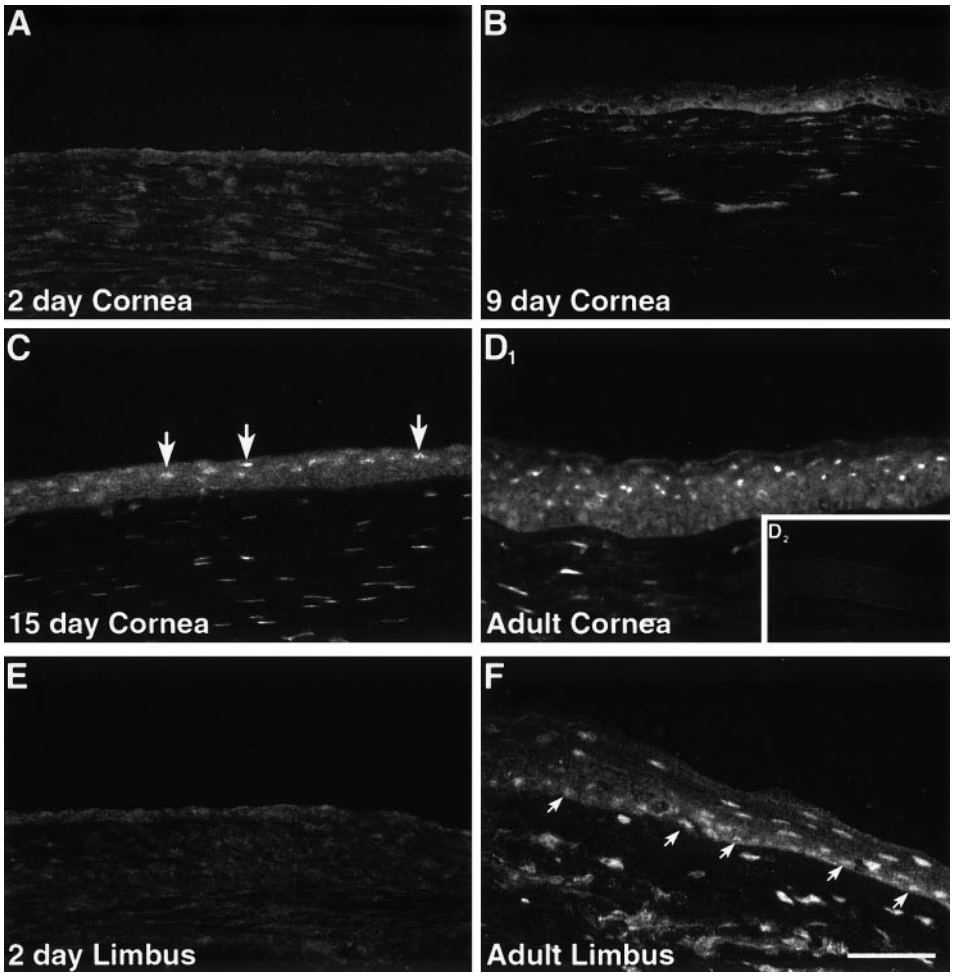

FIGURE 5. Immunolocalization of p130 in central cornea (**A** through **D**) and limbus (**E**, **F**) of 2-day-old (**A**, **E**), 9-day-old (**B**), 15-day-old (**C**), and adult (**D**, **F**) rats. Preadsorption control specimen from adult rats is shown in *inset* ($\mathbf{D_2}$). Note that at 15 days, nuclear localization of p130 was present in the suprabasal cells (*arrows*) and was absent in the basal cell layer. Compare this with Figure 1C, in which the majority of basal cells appear to be actively cycling. Also note that in contrast to its cytoplasmic localization in central cornea, p130 nuclear localization was present in some of the limbal basal cells (*small arrows*). Bar, 50 μm.

preadsorption (data not shown) and secondary antibody controls (see Fig. $8D_2$).

The binding patterns of E2F-1, -2, and -4 are demonstrated in Figures 7, and 8. In developing rats (1–10 days), E2F-1 was present at low, diffuse levels in both limbal and corneal epithelia (Figs. 7A, 7B). After 10 days, E2F-1 was present at highest levels in the basal cells of the corneal and limbal epithelium, showing an intense nuclear and diffuse cytoplasmic localization (Figs. 7C, 7D). In both limbal and corneal epithelia, the number of basal cells with nuclear localization decreased after day 17 (data not shown). E2F-1 was localized in the keratocytes of both limbus and central cornea at relatively high levels until day 10 (Figs. 7A, 7B), decreasing rapidly after day 12 in central cornea (Fig. 7C). This pattern was maintained from 17 days to adulthood (data not shown). Staining intensity also decreased in the limbal keratocytes but not to as great an extent as observed in central cornea (Fig. 7D). Anti-E2F-2 exhibited nuclear binding in epithelial cells and keratocytes, with a maximal intensity in the basal cell layer of both limbal and corneal epithelia (Figs. 8A, 8B). Some cytoplasmic localization was observed in all cell layers. No changes were detected during development (data not shown). In the adult cornea, the localization pattern of E2F-4 most closely resembled that of p107, with cytoplasmic binding in central corneal epithelium, occasional nuclear binding in limbal epithelium, and low-level diffuse localization in the keratocytes (Figs. 8C, 8D). No changes were detected during development (data not shown).

The localization patterns of the pRb and E2F families are summarized in Figures 9, 10, and 11.

DISCUSSION

We have examined the expression and localization of the pRb family of proteins and the E2F family of transcription factors in corneal cells in situ. These proteins are involved in regulation of the cell cycle, quiescence, and differentiation. The cornea provides an excellent model for this type of study, in that it contains actively cycling cells (corneal epithelial basal cells), slow-cycling cells (a subset of the limbal epithelial basal cells), terminally differentiated cells (limbal and corneal suprabasal cells), and quiescent cells (corneal keratocytes). These varying proliferative states were confirmed by the localization of Ki67.[41,42]

The major finding of the present study is that members of the pRb and E2F families exhibit differential localization, depending primarily on the differentiative state of the corneal cells. Findings of particular note include (1) pRb and E2F-2 are present in cells regardless of whether they are actively cycling; however, the intensity of antibody binding diminishes in the terminally differentiated epithelial cells; (2) p130 expression appears to be highest in terminally differentiated and quiescent cells; (3) p107 and E2F-4 are nuclear in the relatively undiffer-

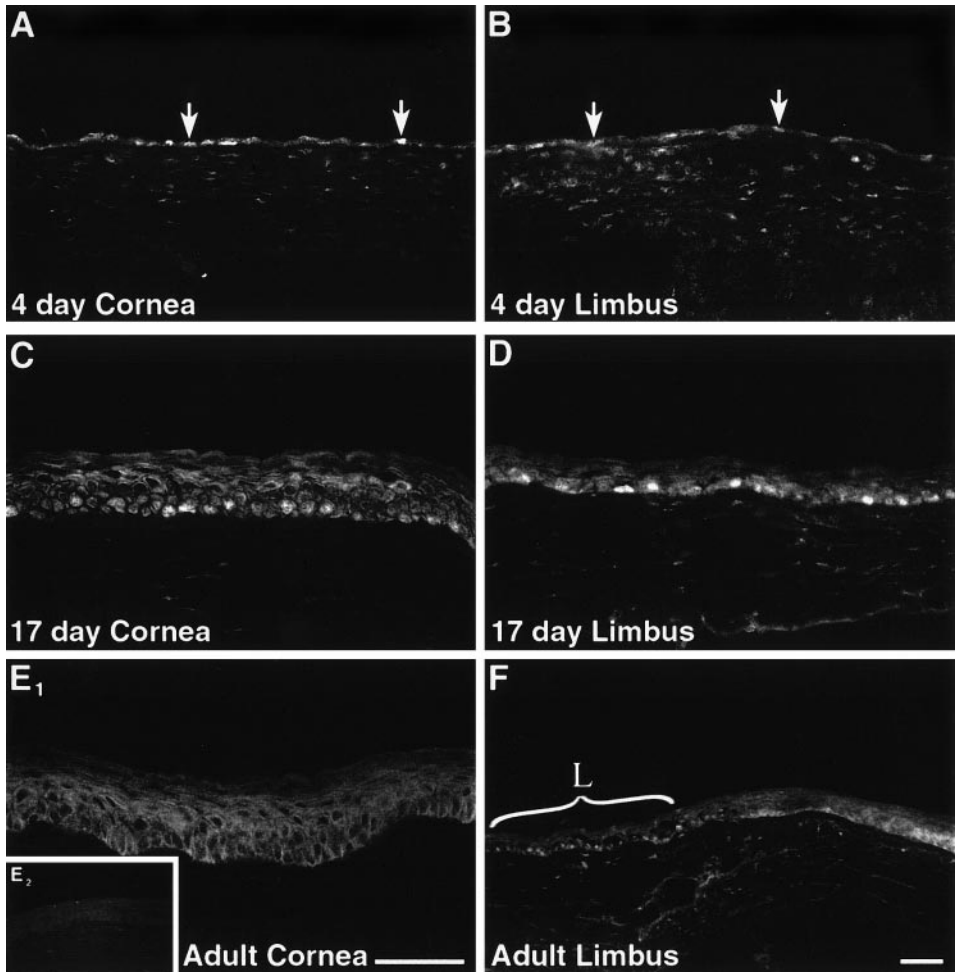

FIGURE 6. Immunolocalization of p107 in central cornea (**A**, **C**, **E**) and limbus (**B**, **D**, **F**) of 4-day-old (**A**, **B**), 17-day-old (**C**, **D**), and adult (**E**, **F**) rats. The transition from nuclear localization in the limbal epithelium to cytoplasmic localization in the corneal epithelium can be observed in (**F**). Preadsorption control is shown in *inset* (E_2). (**A** through **E**) same magnification; (**F**) lower magnification to allow better observation of the limbal–corneal transition. *Arrows*: nuclear localization in 4-day-old cornea and limbus. L, limbus. Bars, 50 μm.

entiated limbal basal cells but primarily cytoplasmic in corneal epithelial cells; and (4) E2F-1 is concentrated in cells with highest proliferative capacity.

In this study, pRb and E2F-2 were observed to be present in the nuclei of corneal cells in developing and adult rats. In both limbal and corneal epithelia, pRb and E2F-2 were present

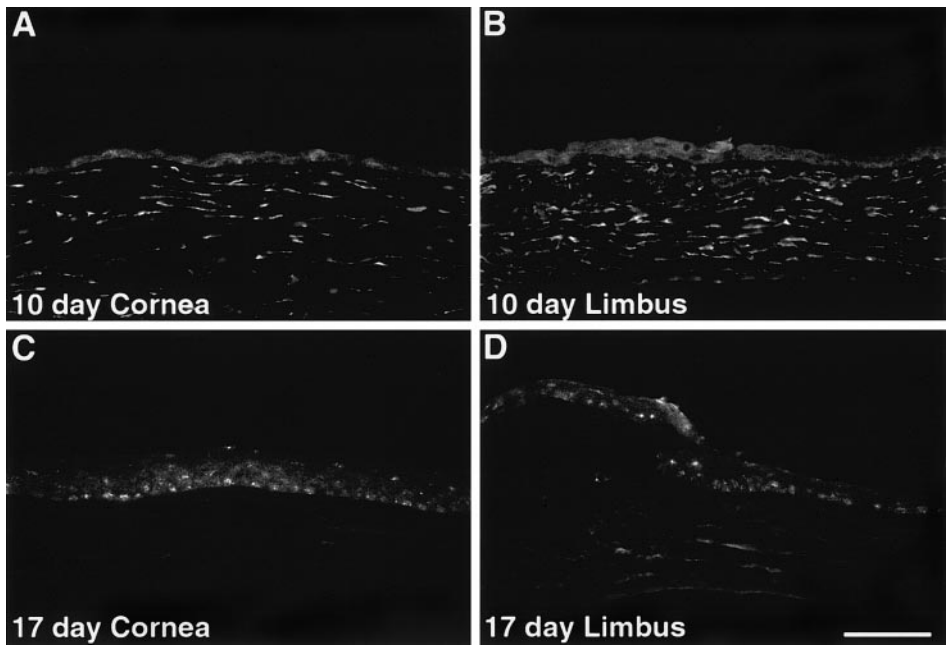

FIGURE 7. Immunolocalization of E2F-1 in central cornea (**A**, **C**) and limbus (**B**, **D**) of 10-day-old (**A**, **B**) and 17-day-old (**C**, **D**) rats. Bar, 50 μm.

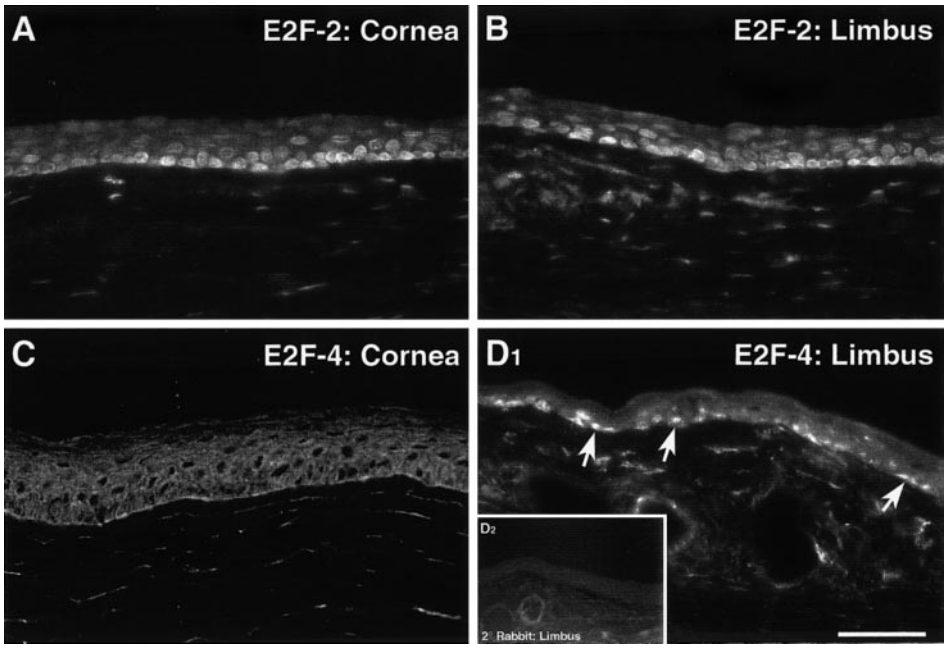

FIGURE 8. Immunolocalization of E2F-2 in central cornea (**A**) and limbus (**B**) and of E2F-4 in central cornea (**C**) and limbus ($\mathbf{D_1}$) of adult rats. ($\mathbf{D_2}$) Representative secondary antibody control in limbus; note low-level binding to vessels. *Arrows*: nuclear localization of E2F-4 in limbus. Bar, 50 μm.

at highest levels in the basal layer. This localization is consistent with the need for pRb in the regulation of cell proliferation in actively cycling cells and suggests that the need for high levels of nuclear pRb and E2F-2 diminishes during terminal differentiation of the epithelial cells. In contrast to the apparent change in levels of pRb and E2F-2 during terminal differentiation of the epithelial cells, there was no obvious change in expression patterns as corneal keratocytes became quiescent. This suggests that cells resting in G0 maintain pRb and E2F-2 so that they may reenter the cell cycle. Another possibility is that these proteins may be present in Rb-E2F family complexes that bind DNA and suppress expression of genes necessary for DNA synthesis, thus preventing cell proliferation.[43]

Expression of p130 in situ possesses characteristics similar to cells in culture, where terminally differentiated and quiescent cells express the highest levels of p130.[14,32,33,44] This is apparent in the developing rat, in which p130 was initially localized at low levels in developing corneal epithelium and then became nuclear in the suprabasal cells as the epithelium stratified and differentiated (Fig. 5). A similar change was observed in keratocytes, where nuclear localization of p130

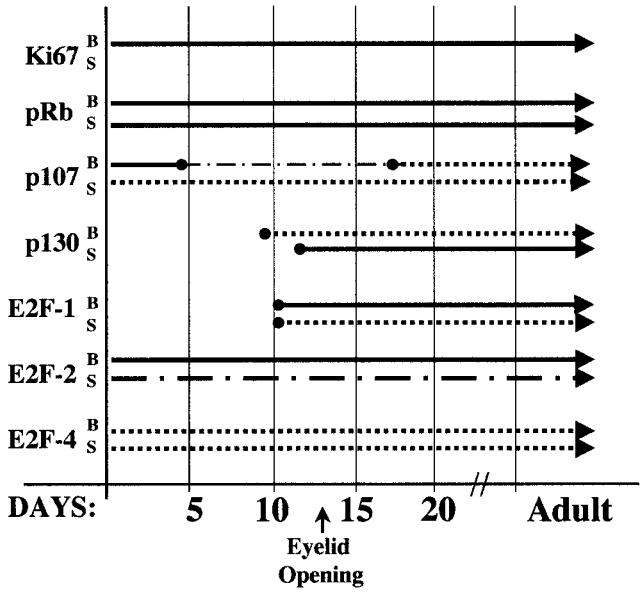

FIGURE 9. Summary of expression patterns of Ki67 and pRb and E2F families in corneal epithelium. Nuclear localization is indicated by *solid lines*, cytoplasmic localization by *dashed lines*, and both nuclear and cytoplasmic localization by *dashed–dotted lines*. (●) Change in localization. B, basal cells; S, suprabasal cells.

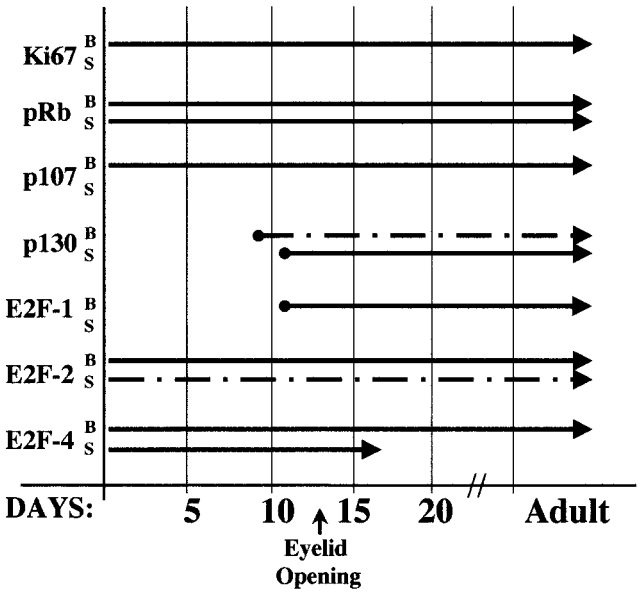

FIGURE 10. Summary of expression patterns of Ki67 and pRb and E2F families in limbal epithelium. Nuclear localization is indicated by *solid lines* and both nuclear and cytoplasmic localization by *dashed–dotted lines*. (●) Change in localization. B, basal cells; S, suprabasal cells.

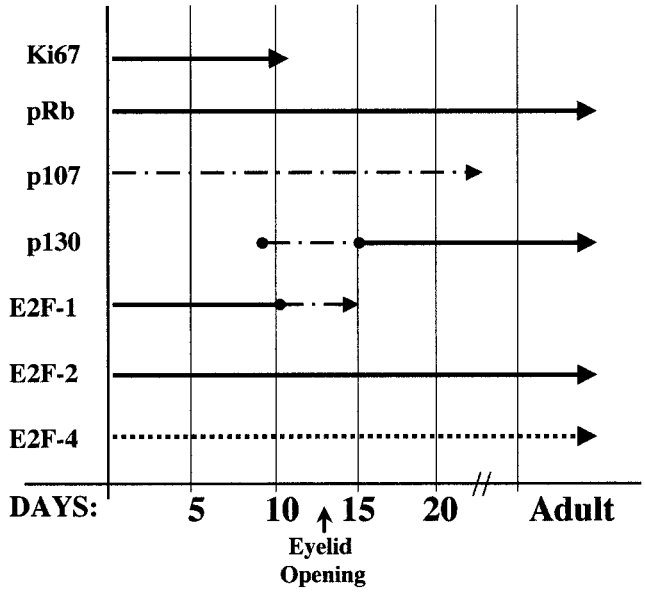

FIGURE 11. Summary of expression patterns of Ki67 and pRb and E2F families in corneal keratocytes. Nuclear localization is indicated by *solid lines*, cytoplasmic localization by *dashed lines*, and both nuclear and cytoplasmic localization by *dashed–dotted lines*. (●) Change in localization.

increased with age (Fig. 5), correlating with the decrease in keratocyte proliferation. It may play a role in actively repressing keratocytes from entering the cell cycle and in terminal differentiation of the epithelial cells. This hypothesis is supported by Paramio et al.,[45] who found that p130 is restricted to epidermal cells committed to differentiation. It is further supported by the recent report of Dagnino et al.,[46] who found, by in situ hybridization, that expression of E2F-5 (p130's most common complex partner[21,27]) is upregulated in the nonproliferative, terminally differentiated cells in both epidermal and intestinal epithelia.

One unexpected finding in our study was the cytoplasmic localization of p107 in the corneal epithelium of adult rats. Obvious nuclear localization of p107 in adult corneal epithelium was extremely rare (less than 1% of the basal cells). This is in stark contrast to the localization of Ki67, which was present in 42% of corneal epithelial basal cells. These data suggest that p107 may not play a major role in homeostatic cell proliferation of the corneal epithelium. One possible explanation for the cytoplasmic localization of p107 is that the protein is constitutively expressed in developing and adult ocular epithelia, and that its activity is regulated by entry into the nucleus.[38] This would remove the necessity for a layer of gene regulation to specifically turn off p107 transcription. The meaning of the high number of limbal cells with nuclear p107 is also unclear. We have previously shown that limbal basal cells are arrested in G1.[2] Because p107 levels in serum-stimulated cells in culture are upregulated late in G1, the present findings are consistent with the arresting of limbal basal cells after the initiation of p107 expression but before entry into the S phase. Alternatively, p107 may be repressing the transcription of a protein necessary for limbal basal cells to differentiate.

It has been suggested that p107 and p130 may be redundant or may compensate for each other.[14,44,47,48] This concept is primarily based on the findings that $p107^{-/-}$[47] and $p130^{-/-}$[48] mice are viable, with no obvious phenotype. However, mice without both proteins die shortly after birth.[48] By comparison, $pRb^{-/-}$ mice die on embryonic day 13.5.[49–51] No corneal phenotypes have been reported for any of the knockout mice, but it is not clear whether the cornea has been extensively examined. The localization patterns of p107 and p130 demonstrated in this study suggest that the proteins play different roles in the regulation of corneal cell differentiation. For example, p130 is localized in the nucleus of the terminally differentiated wing cells; whereas, p107 has a cytoplasmic localization. This suggests that p130 may play a more prominent role in terminal differentiation than does p107. We are currently examining $p107^{-/-}$ and $p130^{-/-}$ mice to determine whether p107 or p130 localization is altered to compensate for the absence of the other.

One of the intriguing and potentially important observations in this study is the differential expression of several members of the pRb and E2F families in limbal and corneal epithelia. This was most obvious in the expression of p107 and E2F-4, which were nuclear in a subpopulation of the basal cells in the limbal epithelium but cytoplasmic in the adult corneal epithelium. A differential localization was shown by p130, in that a subpopulation of the limbal basal cells exhibited nuclear localization, whereas few if any basal cells in the corneal epithelium had nuclear localization. These results may help in understanding the dynamics of stem cell proliferation in the cornea. Unfortunately, all the antibodies used for these proteins are rabbit polyclonal antibodies, and we have not been able to perform colocalization experiments.

The findings in the present study demonstrate that the members of the pRb and E2F families are differentially expressed in corneal and limbal epithelia and in corneal keratocytes. These results suggest that the level of protein expression and the pattern of localization are important mechanisms of regulation in corneal cell proliferation and differentiation and represent an initiation of experiments to understand the role of the pRb and E2F families. Obviously, however, because phosphorylation of these proteins is also a major regulatory event, changes in phosphorylation relative to cell cycle progression must be examined. In addition, the alteration of pRb–E2F family complexes should be examined. These experiments represent the next stage of our investigations.

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