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Contrasting roles for c-Myc and L-Myc in the regulation of cellular growth and differentiation *in vivo*

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Although *myc* family genes are differentially expressed during development, their expression frequently overlaps, suggesting that they may serve both distinct and common biological functions. In addition, alterations in their expression occur at major developmental transitions in many cell lineages. For example, during mouse lens maturation, the growth arrest and differentiation of epithelial cells into lens fiber cells is associated with a decrease in L- and c-*myc* expression and a reciprocal rise in N-*myc* levels. To determine whether the down-regulation of L- and c-*myc* are required for mitotic arrest and/or completion of differentiation and whether these genes have distinct or similar activities in the same cell type, we have studied the consequences of forced L- and c-*myc* expression in the lens fiber cell compartment using the αA-crystallin promoter in transgenic mice (αA/L-*myc* and αA/c-*myc* mice). With respect to morphological and molecular differentiation, αA/L-*myc* lenses were characterized by a severely disorganized lens fiber cell compartment and a significant decrease in the expression of a late-stage differentiation marker (MIP26); in contrast, differentiation appeared to be unaffected in αA/c-*myc* mice. Furthermore, an analysis of proliferation indicated that while αA/L-*myc* fiber cells withdrew properly from the cell cycle, inappropriate cell cycle progression occurred in the lens fiber cell compartment of αA/c-*myc* mice. These observations indicate that continued late-stage expression of L-*myc* affected differentiation processes directly, rather than indirectly through deregulated growth control, whereas constitutive c-*myc* expression inhibited proliferative arrest, but did not appear to disturb differentiation. As a direct corollary, our data indicate that L-Myc and c-Myc are involved in distinct physiological processes in the same cell type.

Key words: differentiation/lens/*myc*/proliferation/transgenic mice

Introduction

Current evidence supports the view that Myc oncoproteins (c-, N- and L-Myc) function in part as sequence-specific transcription factors involved in the regulation of cellular growth, differentiation and programmed cell death (apoptosis) (for reviews, see DePinho *et al.*, 1991; Evan and Littlewood, 1993). These highly related nuclear phosphoproteins share many biochemical properties, including the capacity to bind the same consensus DNA recognition sequence (Blackwell *et al.*, 1990; Halazonetis and Kandil, 1991; Kerkhoff *et al.*, 1991; Prendergast and Ziff, 1991; Alex *et al.*, 1992; Ma *et al.*, 1993) and to transactivate gene expression (Kato *et al.*, 1990; Barrett *et al.*, 1992) through interaction with another basic region helix–loop–helix leucine zipper (bHLH/LZ) protein, Max (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991; Wenzel *et al.*, 1991; Barrett *et al.*, 1992; Blackwood *et al.*, 1992; Kato *et al.*, 1992; Mukherjee *et al.*, 1992). On the biological level, members of the Myc family are related in so far as their deregulated expression can effect the malignant transformation of cells in culture and in transgenic mice (reviewed in Morgenbesser and DePinho, 1994), apparently through a common genetic pathway or shared genetic elements, including interaction with Max and possibly regulation of similar gene targets (Mukherjee *et al.*, 1992; Lahoz *et al.*, 1994).

Although members of the Myc family are structurally and functionally similar, their conservation as three distinct genes from fish to man (Schreiber-Agus *et al.*, 1993), their dispersed chromosomal positions (Crews *et al.*, 1982; Dalla Favera *et al.*, 1982; Neel *et al.*, 1982; Taub *et al.*, 1982; Kohl *et al.*, 1983; Schwab *et al.*, 1983; Nau *et al.*, 1985; Campbell *et al.*, 1989) and marked differences in their transactivation potentials (Barrett *et al.*, 1992), oncogenic activities (DePinho *et al.*, 1987; Birrer *et al.*, 1988; Moroy *et al.*, 1990) and developmental patterns of gene expression (for examples, see Jakobovits *et al.*, 1985; Zimmerman *et al.*, 1986; Mugrauer *et al.*, 1988; Downs *et al.*, 1989; Schmid *et al.*, 1989; Hirning *et al.*, 1991; Mugrauer and Ekblom, 1991; Yamada *et al.*, 1992; K.A. Mahon and R.A.DePinho, unpublished results) suggest that they may perform distinct physiological functions. Evidence for complementary, but not fully redundant, activities among the Myc family comes from the phenotypic analysis of c-Myc- and N-Myc-deficient mice. Although inactivation of c-*myc* or N-*myc* results in embryonic lethality by mid-gestation (Charron *et al.*, 1992; Stanton,B.R. *et al.*, 1992; Davis *et al.*, 1993; Sawai *et al.*, 1993), establishing that each is required for normal development, the survival of these mutant embryos to such relatively late stages of embryogenesis suggests that *myc* family genes may have overlapping functions at earlier, but not later, stages of development. It remains to be determined whether the lack of complementation between c- and N-*myc* beyond early gestation reflects differential cell type-specific functions or inadequate overlap in their spatial patterns of expression during mid-gestation.

Extensive analysis of *myc* family gene expression in evolutionarily distant vertebrates has demonstrated that the three genes display distinctive patterns of temporal and spatial expression during development and that marked changes in their steady-state expression often occur as cells progress from an actively proliferating state to a terminally differentiated one (for review, see DePinho *et al.*, 1991). These findings have fueled speculation that members of the Myc family influence processes that guide cells through their growth and maturation. During ontogeny, c-*myc* transcripts are found in a wide variety of developing tissues, whereas abundant N- and L-*myc* expression is limited to fewer tissues. In the mid-gestational mouse, enhanced c-*myc* expression correlates well with active proliferation and its down-regulation accompanies mitotic arrest and onset of differentiation (Schmid *et al.*, 1989). Furthermore, in most cell culture and transgenic mouse experiments, forced c-*myc* expression prevents withdrawal from the cell cycle and inhibits differentiation, indicating that down-regulation of c-*myc* is required for mitotic arrest and terminal differentiation in these cell lineages (for examples, see Coppola and Cole, 1986; Dmitrovsky *et al.*, 1986; Leder *et al.*, 1986; Prochownik and Kukowska, 1986; Schoenenberger *et al.*, 1988; Sandgren *et al.*, 1989; Trudel *et al.*, 1991; Cartier *et al.*, 1992). In contrast to c-*myc*, high levels of N- and L-*myc* mRNA have been detected in both proliferating tissues and some post-mitotic, but actively differentiating, cell types (Grady *et al.*, 1987; Mugrauer *et al.*, 1988; K.A.Mahon and R.A.DePinho, unpublished data). Their expression in post-mitotic cells has led to the view that N- and L-*myc* may be involved more directly in processes of differentiation rather than proliferation.

In an effort to understand the biological roles of members of the Myc family *in vivo*, we have exploited both the transgenic mouse technology and the experimental attributes of the developing mouse lens. The embryonic lens provided an ideal system for the study of Myc function, because of the differential patterns of expression of the three *myc* family genes therein; c- and L-*myc* transcripts are abundant in mitotically active, undifferentiated cells of the lens placode and c-*myc* transcripts are also found in proliferating lens epithelial cells, but as these cells enter growth arrest and begin to mature into elongated lens fiber cells, c- and L-*myc* are down-regulated and N-*myc* is up-regulated (Yamada *et al.*, 1992; see also below). This decrease in c- and L-*myc* expression coincident with mitotic arrest and onset of differentiation, coupled with the availability of lens-specific promoters capable of driving transgene expression across this developmental transition, afforded the opportunity to compare the physiological consequences of late-stage c- or L-*myc* expression on the control of proliferation and differentiation in the same cell type.

Results

Production and gross analysis of αA/L- and αA/c-myc transgenic mice

As shown in Figure 1, mouse L- and c-*myc* DNA fragments capable of encoding functional L- and c-Myc oncoproteins (Legouy *et al.*, 1987; Mukherjee *et al.*, 1992) were placed under the control of the αA-crystallin promoter that has been shown previously to direct high level transgene expression specifically to the lens fiber region (Chepelinsky *et al.*, 1985; Overbeek *et al.*, 1985; Mahon *et al.*, 1987; Nakamura *et al.*, 1989). Fourteen αA/L-*myc* and eight αA/c-*myc* transgenic founder mice were generated, with transgene DNA copy numbers ranging from 1 to 50.

Transgenic founders and their offspring were examined for gross lens defects by slit lamp examination, in which a narrow slit of intense light is focused upon the lens to highlight opacifications (cataracts). In all αA/L- and αA/c-*myc* transgenic founders and their transgenic progeny, bilateral cataracts ranging in severity from mild to dense were detectable by slit lamp as soon as the mice opened their eyes (data not shown); non-transgenic lenses were clear. These cataracts were predominantly central (nuclear region) in location, with occasional opacifications located more posteriorly, near the retina (posterior subcapsular region). Since the nuclear region of the adult lens corresponds anatomically to the portion of the lens formed during embryonic life, cataracts found in this region are typically associated with inherited abnormalities that impact upon embryonic lens growth and development (Sorsby, 1972). Lastly, although the αA/L-*myc* eyes and lenses were normal in size, three of the αA/c-*myc* lines that were afflicted with moderate or dense cataracts also exhibited mild microphthalmia and microphakia (data not shown).

A detailed histological and molecular characterization was conducted on selected αA/L- and αA/c-*myc* lines possessing mild (L-*myc* line 10 and c-*myc* line 32), moderate (L-*myc* line 1 and c-*myc* line 30) or very dense cataracts (L-*myc* line 3 and c-*myc* line 33). The purpose of these studies, outlined below, was to determine and compare the impact of deregulated L-Myc versus c-Myc on lens fiber cell proliferation, differentiation and apoptotic cell death.

Myc and max expression in αA/L- and αA/c-myc lenses

Transgene and endogenous *myc* family gene expression was analyzed by Northern and Western blot analysis and by RNA *in situ* hybridization assays. In Northern and Western blot studies, significantly higher levels of the mature 3.6/3.8 kb L-*myc* transcripts and their corresponding 60 kDa protein were detected in αA/L-*myc* transgenic lenses compared with the levels observed in age-matched, non-transgenic controls (Figures 2A and 8A); the level of transgene expression correlated strongly with the severity of lens opacification. In addition to the mature 3.6/3.8 L-*myc* transcripts, 1.6/1.8 kb L-*myc* transcripts that are generated from alternative usage of polyadenylation signals in the second intron (Kaye *et al.*, 1988; R.A. DePinho and F.W.Alt, unpublished observations) were present in the higher expressing transgenic lines (lines 3 and 1) and detected, on longer exposure, in the lower expressing transgenic (line 10) and control mice (data not shown). The physiological significance of these smaller L-*myc* transcripts, which encode the transactivation domain (TAD) (Barrett *et al.*, 1992) but do not encode structures essential for nuclear localization, DNA binding and protein oligomerization, has not yet been determined (see below). Transcripts larger than 3.8 kb represent unprocessed

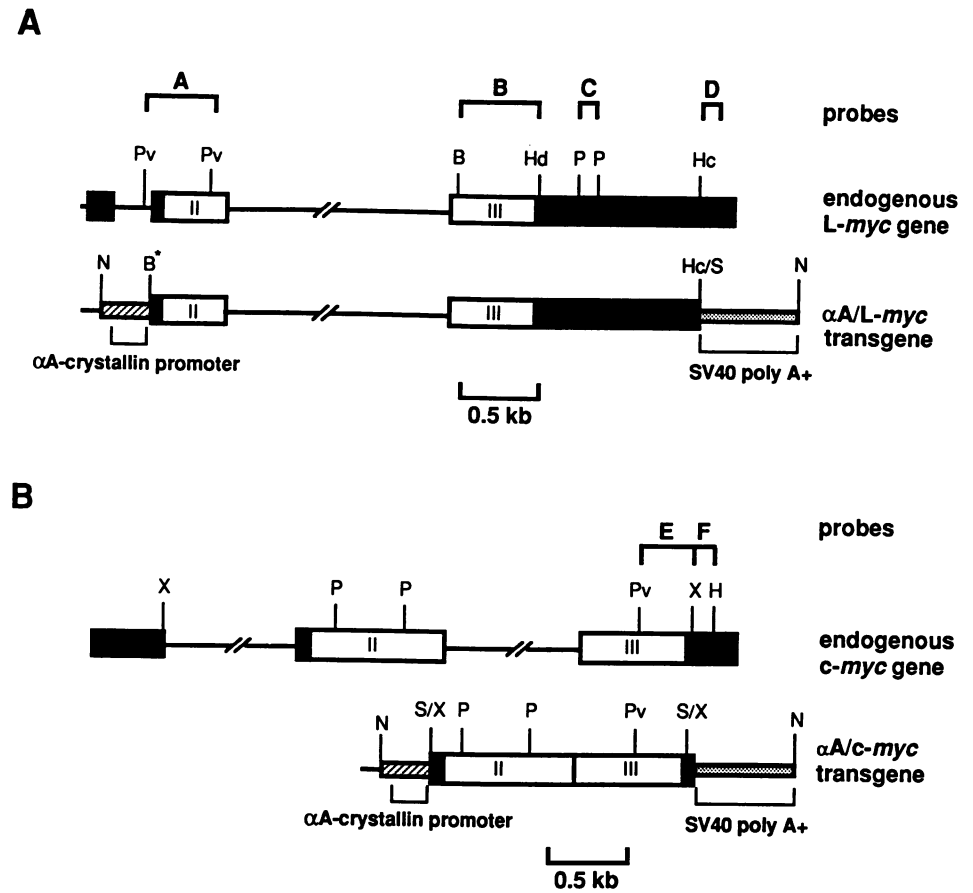

Fig. 1. Partial restriction maps of the mouse L- and c-*myc* genes and the αA/L-*myc* and αA/c-*myc* transgenes. (**A**) This panel depicts the endogenous murine L-*myc* gene and the αA/L-*myc* transgene, which contains exons 2 and 3 of the mouse L-*myc* gene flanked at the 5′-end by the mouse αA-crystallin promoter (−364 to +45) and at the 3′-end by the SV40 polyadenylation signal sequence, both derived from the plasmid CPV1. In order to insure high level expression of the transgene, exon 1 sequences that possess negative regulatory elements were removed (Xu *et al.*, 1991). The 3′-most 180 bp of the L-*myc* gene were removed for the creation of a probe unique to the endogenous gene. (**B**) This panel depicts the endogenous mouse c-*myc* gene and the αA/c-*myc* transgene, which contains a small portion of the 5′-end of exon 1, all of exon 2 and most of exon 3 derived from the mouse c-*myc* cDNA flanked at the 5′-end by the mouse αA-crystallin promoter and at the 3′-end by the SV40 polyadenylation signal sequence. In these diagrams, *myc* introns are shown as solid lines and *myc* exons are shown as boxed regions; the translated regions are white and the untranslated regions are black. The αA-crystallin promoter and SV40 polyadenylation sequences are represented by cross-hatched and stippled boxes respectively. Probes used for Northern blotting (probes A, B and E) and *in situ* hybridization (probes C and D) analyses are indicated. Probes were derived by use of the restriction sites noted, whereas probe D was generated by PCR. B, *Bam*HI; Hc, *Hinc*II; Hd, *Hin*dIII; N, *Not*I; P, *Pst*I; Pv, *Pvu*II; S, *Sma*I; X, *Xho*I. * Indicates that site was derived from a synthetic linker.

mRNAs, as determined by intronic probes (data not shown). A similarly elevated level of c-*myc* expression and its correlation with cataract severity was shown for the αA/c-*myc* transgenic lenses (Figure 2C). Finally, in contrast to tumor cell lines wherein deregulation of one *myc* family gene has been associated with repression of other *myc* family members (Nisen *et al.*, 1986; Moroy *et al.*, 1990; Ma *et al.*, 1991), the over-expression of L- or c-*myc* transgenes did not significantly affect the level of endogenous N-*myc* expression in lens fiber cells (Figure 2B and C). Similarly, levels of the Myc binding partner, *max* were within the normal range of expression in all αA/L- and αA/c-*myc* lines tested (Figure 2B and C).

The regional distribution of endogenous L-*myc* and αA/L-*myc* transgene transcripts was determined by RNA *in situ* hybridization assays. In the normal lens, transcripts encoded by the endogenous L-*myc* gene were detected in the mitotically active, undifferentiated cells of the lens placode (Figure 3H, p = placode; see also Figure 1A for probe D). With progression through development to E14.5, a significant decrease in the endogenous L-*myc* transcript levels was observed in transgenic and non-transgenic lenses (Figure 3E and F, only the transgenic lens is shown here). In particular, in the anterior epithelial cells, L-*myc* transcripts were below the limit of detection by RNA *in situ* hybridization, but L-Myc protein was detected, albeit at low levels, by immunohistochemical methods (data not shown). In contrast, an L-*myc* probe capable of hybridizing to both transgene and endogenous transcripts (Figure 1, probe C) detected abundant L-*myc* expression in the lens fiber cells in transgenic lenses, thus demonstrating that high level αA/L-*myc* transgene expression was properly directed to the lens fiber cell compartment (Figure 3C and D). N-*myc* transcripts localized to the lens equatorial region, as observed previously (Yamada *et al.*, 1992), and the level of N-*myc* expression was comparable in both transgenic (Figure 3G) and non-transgenic lenses (data not shown). In the αA/c-*myc* lenses, RNA *in situ* hybridization studies confirmed that the transgene was expressed at high levels in the lens fiber cells of the equatorial region (data not shown, but see Materials and methods for details on probes).

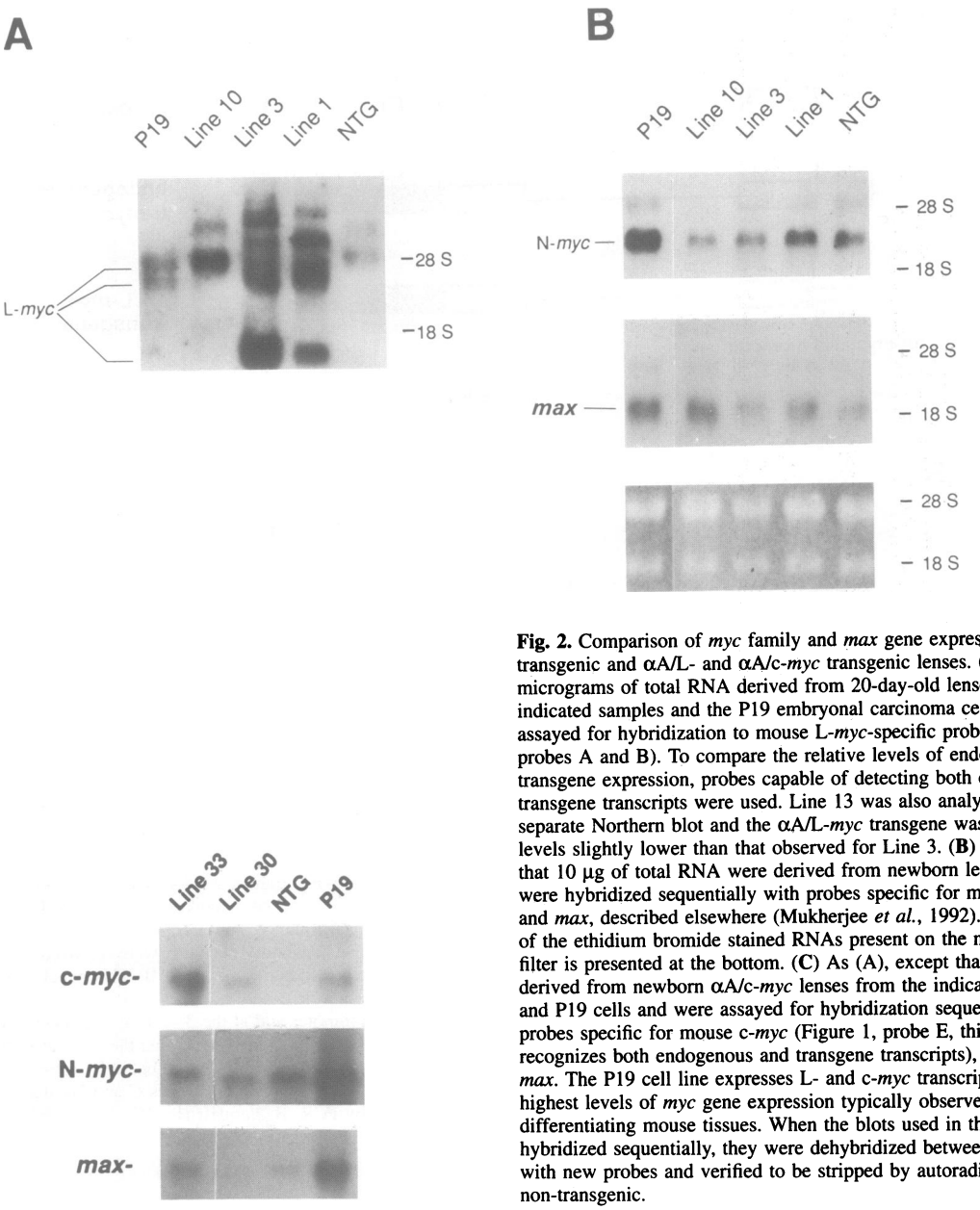

Fig. 2. Comparison of *myc* family and *max* gene expression in non-transgenic and αA/L- and αA/c-*myc* transgenic lenses. (**A**) Five micrograms of total RNA derived from 20-day-old lenses from the indicated samples and the P19 embryonal carcinoma cell line were assayed for hybridization to mouse L-*myc*-specific probes (Figure 1, probes A and B). To compare the relative levels of endogenous and transgene expression, probes capable of detecting both endogenous and transgene transcripts were used. Line 13 was also analyzed on a separate Northern blot and the αA/L-*myc* transgene was expressed at levels slightly lower than that observed for Line 3. (**B**) As (A), except that 10 μg of total RNA were derived from newborn lenses and filters were hybridized sequentially with probes specific for mouse N-*myc* and *max*, described elsewhere (Mukherjee *et al.*, 1992). A photograph of the ethidium bromide stained RNAs present on the nitrocellulose filter is presented at the bottom. (**C**) As (A), except that RNAs were derived from newborn αA/c-*myc* lenses from the indicated samples and P19 cells and were assayed for hybridization sequentially to probes specific for mouse c-*myc* (Figure 1, probe E, this probe recognizes both endogenous and transgene transcripts), N-*myc* and *max*. The P19 cell line expresses L- and c-*myc* transcripts at the highest levels of *myc* gene expression typically observed in actively differentiating mouse tissues. When the blots used in this study were hybridized sequentially, they were dehybridized between incubations with new probes and verified to be stripped by autoradiography. NTG, non-transgenic.

Distinct morphological consequences of deregulated L- and c-myc in the adult lens

An important experimental attribute of the lens as a developmental genetic system is the maintenance of its cells throughout life; as such it provides a permanent record of its development. Consequently, the anatomical location of morphological defects in the adult lens can often provide clues about the inciting pathophysiological processes and the developmental stage of its actions. A histological analysis performed on the αA/L- and αA/c-*myc* adult lenses showed that cells of the anterior epithelial layer and the equatorial region (where lens epithelial cells withdraw from the cell cycle and begin to differentiate) appeared unaffected (Figure 4) in both L-*myc* and c-*myc* transgenic lenses. However, in all αA/L-*myc* lenses examined, the distinctive orderly arrangement of central lens fiber cells, arrayed along well-defined axes in the normal adult lens (Figure 4A, central fibers are arranged along a horizontal plane), was replaced by a highly disorganized pattern (Figure 4B, central fiber orientation is chaotic). Thus, late-stage αA/L-*myc* transgene expression selectively affected the most differentiated cells of the adult lens, the central lens fiber cells. In contrast, the morphology of lens fiber cells in the αA/c-*myc* lines was indistinguishable from that of age-matched, non-transgenic controls (Figure 4C and D).

A classical late-stage differentiation marker is repressed in αA/L-myc, but not αA/c-myc, lenses

Since the central portion of the adult lens forms during embryonic development, the presence of pathology (cataracts and lens fiber cell disorganization) in this region prompted further analyses. A histological survey of developmental stages extending from formation of the lens placode (E9.5) through the lens fiber cell denucleation stages (E17.5 to post-natal day 4) failed to detect any morphological abnormalities at the light microscopic level (Figure 5A–C, only E14.5 is shown). Development of

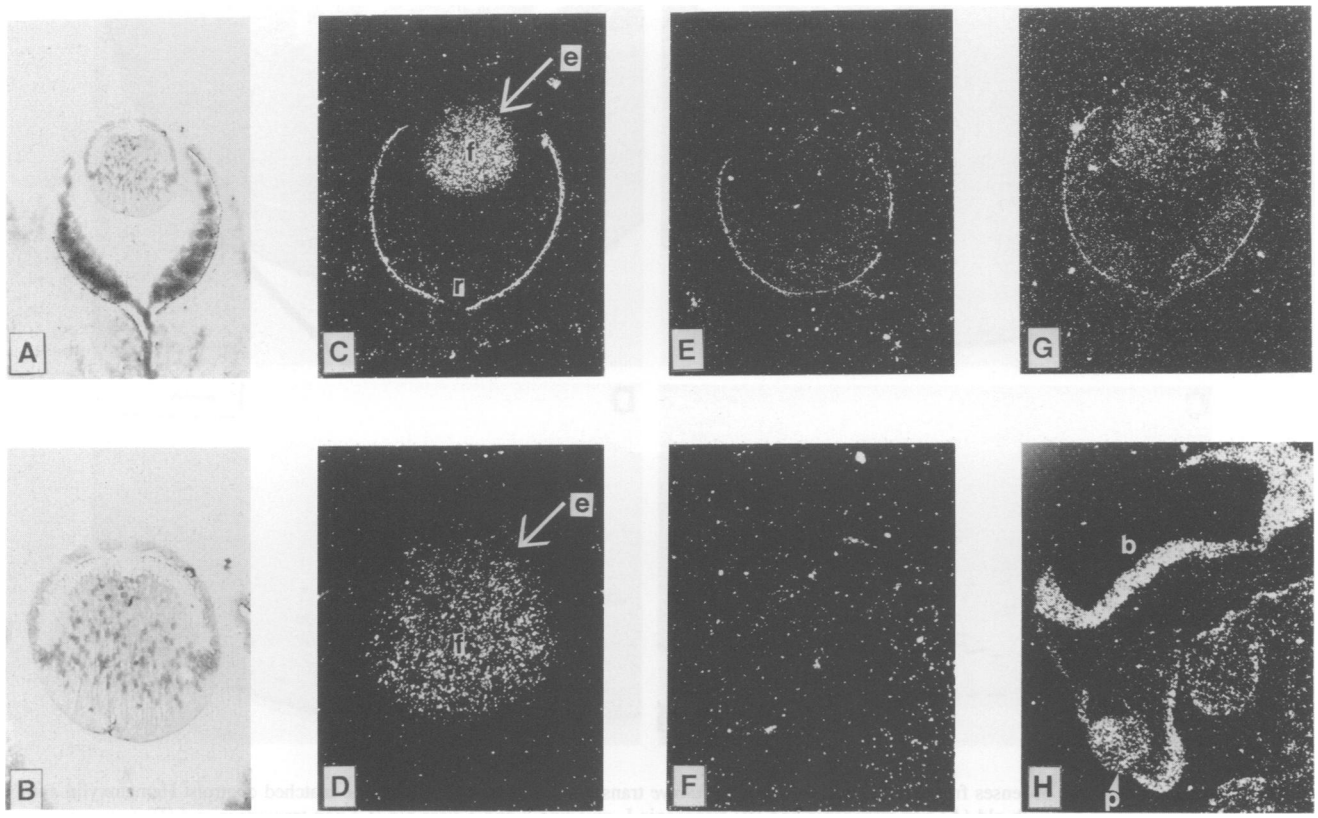

Fig. 3. *In situ* hybridization of L- and N-*myc* transcripts on coronal sections of normal and αA/L-*myc* transgenic E14.5 embryos. (**A**–**G**) Eye sections from E14.5 transgenic mice (line 1) were stained with hematoxylin and eosin (A and B) or were hybridized with ^{35}S-labeled antisense riboprobes for both endogenous and transgenic mouse L-*myc* transcripts (C and D) (probe C, Figure 1A) specific for endogenous mouse L-*myc* transcripts (E and F) (probe D, Figure 1A) and specific for mouse N-*myc* transcripts (G). The anterior epithelial layer is facing the top of each figure. Photomicrographs were shot at 125× (A, C, E and G) or 250× magnification (B, D and F). (**H**) Section through the lens placode from an E9.5 non-transgenic lens were hybridized with a ^{35}S-labeled anti-sense L-*myc* riboprobe (Figure 1, probe C). Control hybridizations with sense transcripts to L-*myc* gave no staining above background (data not shown). The white ring in C, E and G reflects the pigmented epithelium of the retina. b, brain; e, anterior epithelial layer; f, lens fiber cells; p, lens placode; r, sensory retina.

the lens was also assessed at the molecular level through an analysis of the expression of several stage-specific differentiation markers, including the three classes of crystallins, α, β and γ (reviewed in McAvoy, 1980; Piatigorsky, 1981), and MIP26, the 26 kDa major intrinsic polypeptide of the plasma membrane of lens fiber cells. The α- and β-crystallins represent early-stage markers, with α-crystallin expressed earliest and distributed throughout the epithelial and fiber cell compartments (Zwann, 1983) and β-crystallin expressed initially in equatorial cells and in lens fiber cells subsequently (McAvoy, 1980; Piatigorsky, 1981). Expression of the γ-crystallins (McAvoy, 1980; Piatigorsky, 1981) and MIP26 (Broekhuyse *et al.*, 1976; Vermorken *et al.*, 1977) is restricted to the central lens fiber cells.

Both indirect immunofluorescence and Western blot studies of embryonic and post-natal αA/L-*myc* and αA/c-*myc* lenses revealed a normal amount, onset and distribution of α- and β-crystallin expression (Figures 5D–F and 6A; only α-crystallin is shown), indicating that expression of early-stage differentiation markers was unaffected. A slight decrease in the level of γ-crystallin immunofluorescent staining was apparent in only some of the highest expressing αA/L-*myc*, but not αA/c-*myc*, transgenic lines (data not shown). Strikingly, in contrast to the α- and β-crystallins, the levels and pattern of MIP26 expression were consistently reduced and blotchy throughout the lens fiber cell compartment in the αA/L-*myc* transgenic lenses (Figure 5H), while a normal staining pattern for MIP26 was detected in the αA/c-*myc* lenses (Figure 5I). To quantitate more accurately the relative impact of transgene expression on early- versus late-stage differentiation markers, protein lysates derived from single αA/L-*myc* or αA/c-*myc* E16.5 lenses were immunoblotted and assayed for αA-crystallin and MIP26 expression by Western blot analysis. For these experiments, lens protein lysates were separated into water-soluble, cytoplasmic, crystallin-containing and water-insoluble membrane fractions, the latter containing MIP26 and membrane-associated α-crystallins (Takemoto *et al.*, 1988). An analysis of both cytoplasmic and membrane proteins derived from multiple samples at this stage indicated that α-crystallin isoforms were highly expressed in all lenses tested (Figure 6A, only the membrane fraction is shown). In striking contrast to the level of α-crystallin, a profound reduction in MIP26 was observed in all E16.5 αA/L-*myc* lens samples (Figure 6A, αA/L-*myc*). Densitometric quantitation of the reduction in MIP26 relative to α-crystallin (Figure 6B) demonstrated a clear reciprocal relationship, with the highest levels of αA/L-*myc* transgene

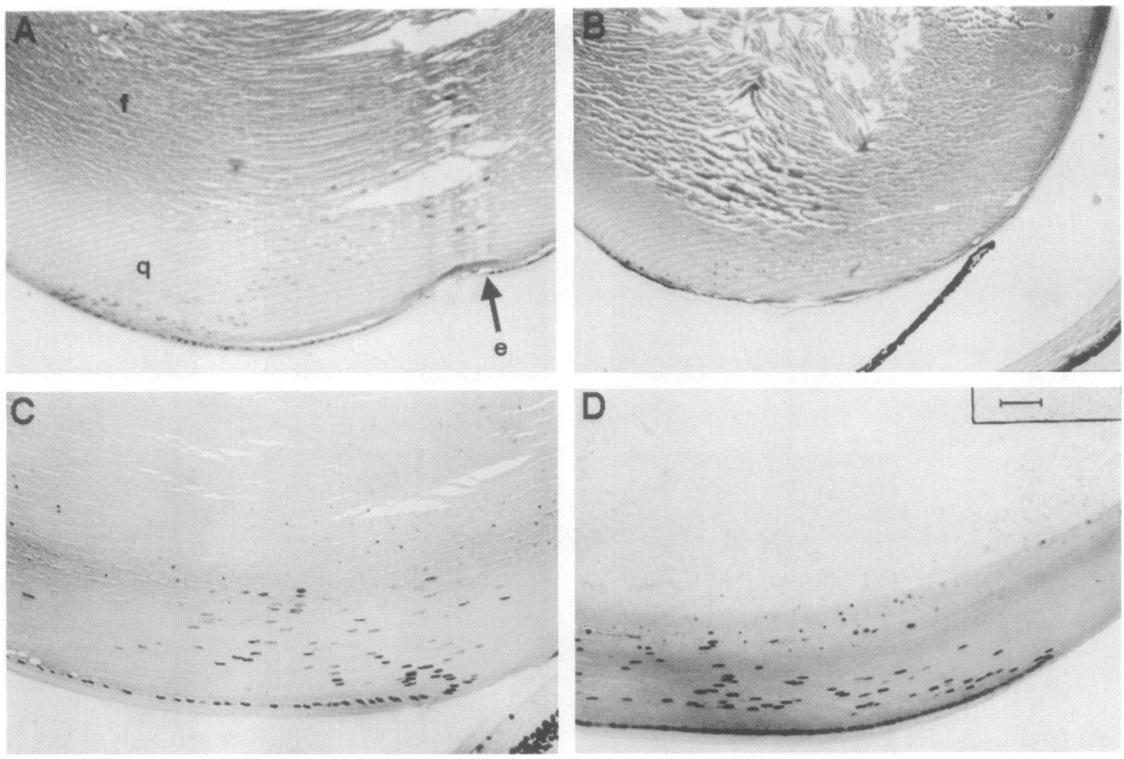

Fig. 4. Histological analysis of lenses from adult αA/L-*myc* and αA/c-*myc* transgenic and non-transgenic, age-matched controls. Hematoxylin and eosin stained sections of 1-month-old (**A**) non-transgenic and (**B**) transgenic L-*myc* line 3 and 1-year-old (**C**) non-transgenic and (**D**) transgenic c-*myc* Line 33 lenses. e, anterior epithelial cells; q, equatorial region; f, lens fiber cells. Although only the highest expressing lines are shown, the results were similar in the other lines examined. The disorganized pattern in the center of the αA/L-*myc* lenses was observed consistently in all specimens examined and was not a consequence of the fragility of lenses at this developmental time period.

expression correlating with the lowest levels of MIP26. Similar analyses confirmed that MIP26 expression was unaffected in embryonic αA/c-*myc* lenses (Figure 6A, αA/c-*myc*).

Late-stage expression of c-myc, but not L-myc, is associated with entry into S phase in the lens fiber cell compartment

In previous transgenic- and cell culture-based experiments conducted in other cell types, constitutive expression of c-*myc* has generally resulted in both deregulated cell growth and impaired expression of late-stage differentiation markers (reviewed in Morgenbesser and DePinho, 1994). The inability of these c-*myc*-expressing cells to withdraw from the cell cycle made it unclear whether c-*myc* expression disrupted normal differentiation directly or indirectly, through an inability of these cells to undergo mitotic arrest. Therefore, we were particularly interested in determining whether L- and c-Myc play a role in lens growth control and whether the impaired differentiation brought about by forced L-*myc* expression was associated with inappropriate progression through the cell cycle in the lens fiber cell compartment. *In situ* bromodeoxyuridine (BrdU) incorporation was utilized as a highly sensitive assay to evaluate cellular proliferation in the αA/L- and c-*myc* lenses. BrdU, a thymidine analog, is incorporated into newly synthesized DNA during S phase and can be detected by anti-BrdU monoclonal antibodies (Gratzner, 1982).

In non-transgenic embryos, nuclear-associated BrdU-containing DNA staining was found in many cells of the anterior epithelial layer and was not detected in the lens fiber region (Figure 7A and B); the absence of staining in some epithelial cells can be attributed to both the brief duration of the BrdU pulse and asynchrony in the cell cycle. In addition, other tissues with high proliferative activity exhibited a similar pattern of staining (Figure 7A, retina) and staining was not detected when the anti-BrdU antibody was omitted (Figure 7C), indicating that the stained regions specifically contained BrdU-containing DNA and represented mitotically active cells. When αA/L-*myc* transgenic lenses were assayed, the pattern of BrdU-containing DNA staining was indistinguishable from that of age-matched, non-transgenic lenses (Figure 7D); BrdU reactivity was detected in the epithelial layer only and was absent in the lens fiber cells, indicating that αA/L-*myc*-induced developmental abnormalities are a direct consequence of aberrant differentiative, rather than proliferative, processes. In contrast to the lack of nuclear staining in non-transgenic and αA/L-*myc* transgenic lens fiber cells, nuclear-associated staining was detected in several lens fiber cell nuclei of the equatorial region of all αA/c-*myc* transgenic embryos tested (Figure 7E–H), correlating well with the pattern of αA/c-*myc* transgene expression in this region. Because only some αA/c-*myc* equatorial lens fiber cells incorporated BrdU during the short pulse, the cell cycle profile was also assayed for expression of proliferating cell nuclear antigen (PCNA) in these embryonic lenses. PCNA expression has been shown to be restricted to the G_1 and S phases of the cell cycle (Woods *et al.*, 1991). Histochemical detection of PCNA in lens fiber cells clearly confirmed that progression

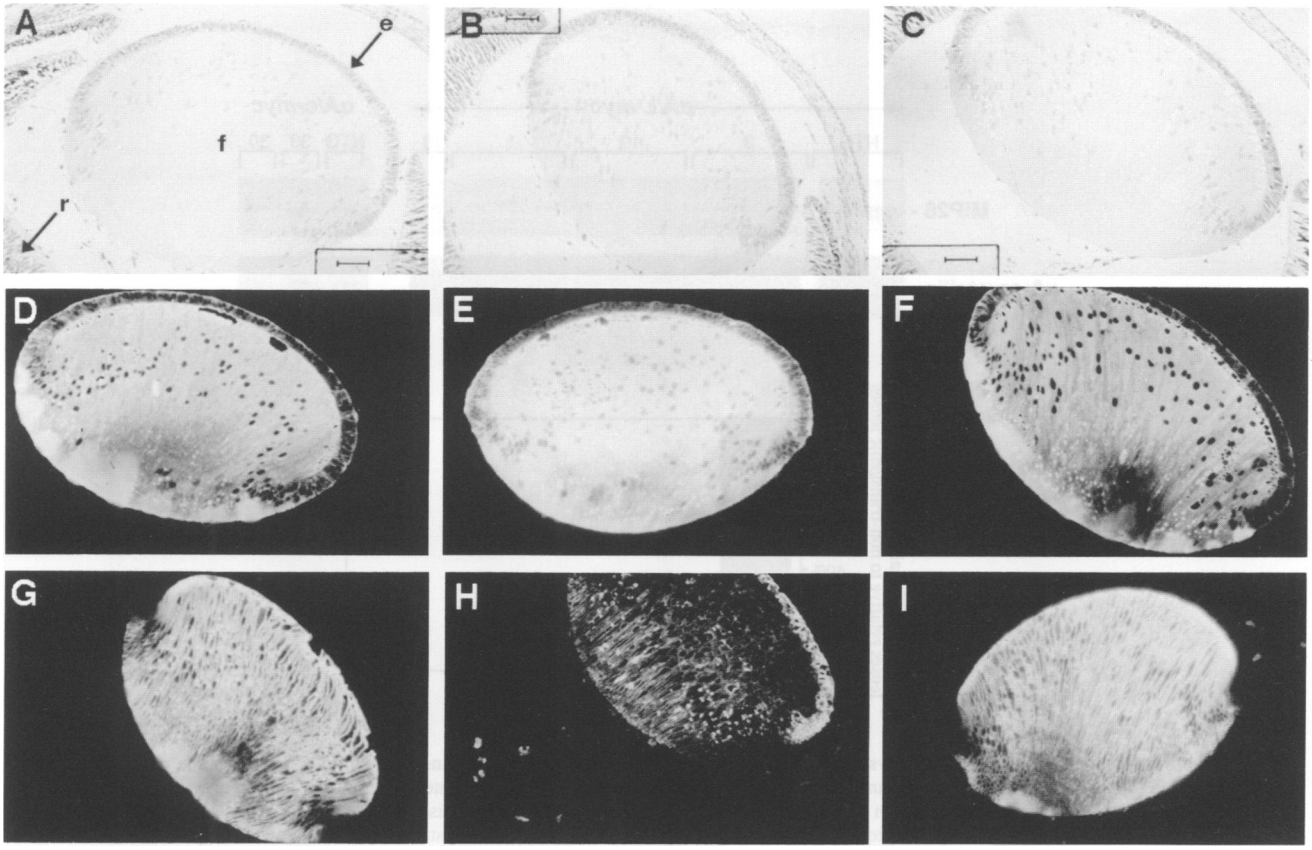

Fig. 5. Comparative morphological development and expression of stage-specific markers in embryonic lenses of αA/L- and αA/c-*myc* transgenic and non-transgenic, age-matched controls. (**A**−**C**) E14.5 embryonic eye sections from non-transgenic (A), αA/L-*myc* line 3 transgenic (B) and αA/c-*myc* line 33 transgenic (C) mice were stained with hematoxylin and eosin. (**D**−**I**) E14.5 embryonic eye sections from non-transgenic (D and G), line 3 transgenic (E and H) and line 33 transgenic (F and I) mice were assayed by indirect immunofluorescence with polyclonal antisera to α-crystallins (D−F) and MIP26 (G−I). All photographs were taken at 62.5× magnification and the scale bar indicates a distance of 80 μm. In control immunofluorescent experiments, specificity of these antibodies was demonstrated by incubation with the pre-immune sera as the primary antibody, which did not contribute to increased staining over the background levels observed in other tissues (data not shown). In all panels, the anterior epithelial layer is positioned facing the upper right corner. Sections stained with the same antibody were photographed with equivalent exposure times. Although only the highest expressing lines are shown, the results were similar in the lower expressing trangenic lines.

through the cell cycle was indeed occurring in this cell population and that this process was not restricted to just a few of these cells (data not shown). Thus, forced c-*myc* expression in lens fiber cells is associated with inappropriate progression through the cell cycle. Moreover, we observed abnormal morphology of the nuclei found in the equatorial region. This abnormal nuclear morphology is similar to that observed in polyploid cells and may reflect continued cycling without accompanying cell division, as evidenced by the lack of mitotic figures or tumor formation.

Deregulated L- and c-myc transgene expression is not associated with apoptotic cell death in the lens

A proposed role for high level c-*myc* expression in processes of apoptosis in normal developing tissues (Buttyan *et al.*, 1988; Riegel *et al.*, 1990; Strange *et al.*, 1992) and in transformed cells (Evan *et al.*, 1992) prompted us to examine whether abundant c- or L-*myc* transgene expression was associated with apoptosis in lens fiber cells. Such an analysis is particularly relevant under physiological circumstances, wherein cells that are normally destined to growth arrest and terminal differentiation continue to receive growth promoting instructions, such as those provided by elevated *myc* transgene expression (Evan *et al.*, 1992). In the normal developing lens, apoptosis has been shown to occur in lens epithelial cells (Silver and Hughes, 1973; Ishazaki *et al.*, 1993), but has not been observed in lens fiber cells.

On the histological level, αA/L-*myc* and αA/c-*myc* lenses did not exhibit nuclear pyknosis or fragmentation typically associated with apoptosis (Figures 4 and 5A−C). Since the histological features associated with apoptosis can progress rapidly and escape light microscopic detection (Kerr *et al.*, 1987), the highly sensitive TUNEL assay was used to detect apoptosis-associated internucleosomal DNA cleavage *in situ* through the histochemical detection of terminal deoxynucleotidyl transferase-mediated attachment of biotinylated dUTP molecules to the free 3′-OH groups in apoptotic nuclei (Gavrieli *et al.*, 1992). In the non-transgenic and transgenic embryos, nuclear staining was observed in some cells of the anterior epithelial cell layer, retina and brain (Figure 7I, K and L), a finding consistent with previous experiments documenting apoptotic cell death in these regions by other methods (Silver and Hughes, 1973; Cowan *et al.*, 1984; Young, 1984; Ishazaki *et al.*, 1993). In contrast, multiple independent assays failed to detect nuclear staining in the lens fiber cell

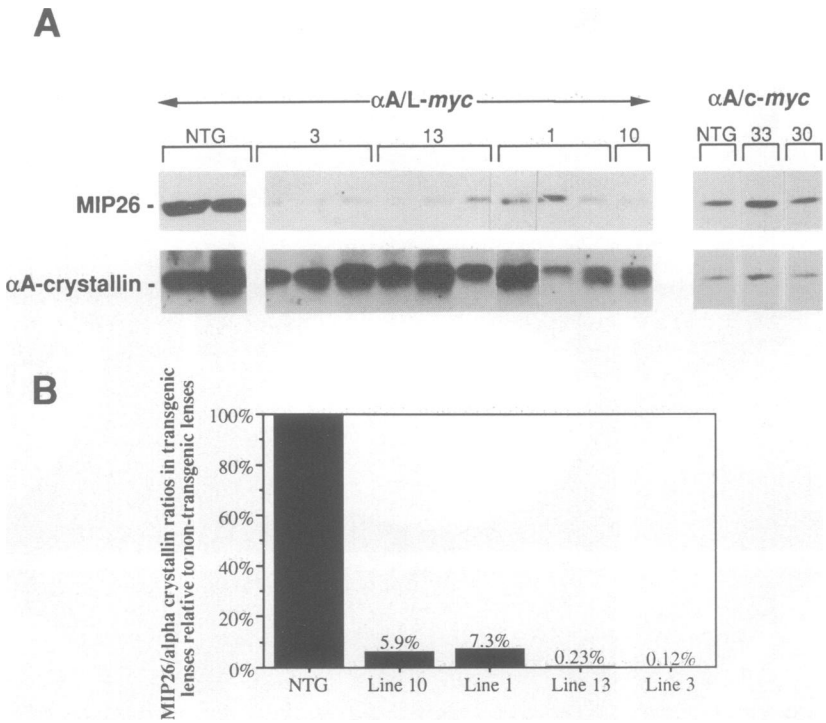

Fig. 6. Western blotting and densitometric analysis of MIP26 expression in transgenic and non-transgenic lenses. (**A**) Membrane protein lysates from equivalent numbers of lenses of the indicated samples were fractionated and the blots were incubated with an anti-MIP26 antisera, exposed and subsequently incubated with the anti-α-crystallin antisera without stripping off the anti-MIP26 antisera. (**B**) The expression levels of MIP26 bands from αA/L-*myc* transgenic lenses were quantitated by densitometry and normalized to the αA-crystallin band. For each transgenic line, the results were averaged and are shown relative to the levels in non-transgenic mice (set at 100%) from left to right in order of increasing levels of transgene expression.

compartment of αA/L-*myc*, αA/c-*myc* and non-transgenic control samples. Nuclear staining was also absent in tissues that normally undergo apoptosis when TdT and biotinylated dUTP were excluded from the assay (Figure 7J), indicating that staining represented attachment of dUTP to free 3′-OH groups in apoptotic cells. Together, these results indicate that forced expression of L- or c-*myc* alone (even with c-*myc*-induced progression through the cell cycle) is insufficient to induce apoptosis in lens fiber cells.

Forced expression of L-Myc proteins lacking a transactivation domain does not affect lens fiber cell differentiation

To begin to understand the molecular action of the full-length L-Myc protein in lens fiber cells, a construct encoding a mouse L-Myc protein lacking the TAD under the control of the αA-crystallin promoter (αA/L-*zip*) was used to generate three trangenic lines: line 1, one copy; line 2, 3–4 copies; and line 3, 15 copies. We have demonstrated previously that L-*zip* dominantly interferes with the transforming activities of wild-type *myc* family genes (Mukherjee *et al.*, 1992), indicating that L-*zip* encodes a biologically active gene product. Abundant expression of the L-Zip protein was verified by Western blotting, which demonstrated the presence of a 45 kDa band in the αA/L-*zip* lens lysate (Figure 8A, lane 4), this is consistent with the size expected for the mutant protein. This band was absent in the age-matched, non-transgenic control (Figure 8A, lane 3) and could not be detected when absorbed antisera was used (Figure 8A, lane 9), indicating that the band represents stable expression of L-Zip in transgenic lenses.

In contrast to the αA/L-*myc* mice, the αA/L-*zip* mice did not develop cataracts (Figure 8B) or exhibit abnormalities in cellular morphology (Figure 8C) or in the expression of crystallins and MIP26 (Figure 8D, MIP26; crystallin data not shown). In addition, mitotic arrest of lens fiber cells occurred normally (Figure 8E). It is unlikely that insufficient levels of the L-Zip protein contribute to the lack of a phenotype, as steady-state levels of L-Zip protein (Figure 8A, lane 4) actually exceeded those observed for the L-Myc protein in developmentally altered αA/L-*myc* lenses (Figure 8A, lane 5).

Discussion

In this study, the developing mouse lens was utilized as a model system to determine whether developmentally regulated changes in *myc* family gene expression play a role in the control of cellular proliferation and/or differentiation *in vivo* and whether Myc family proteins serve distinct or redundant biological roles within the same cell type. During mouse lens development, the down-regulation of L- and c-*myc* coincides with progression towards proliferative arrest and active differentiation of lens fiber cells (Yamada *et al.*, 1992; this study). Employing a gain-of-function transgenic approach, we demonstrated that continued expression of L- or c-*myc* across this developmental transition results in impaired differentiation or deregulated cell cycle progression respectively. The failure of transactivation-incompetent L-Myc mutant

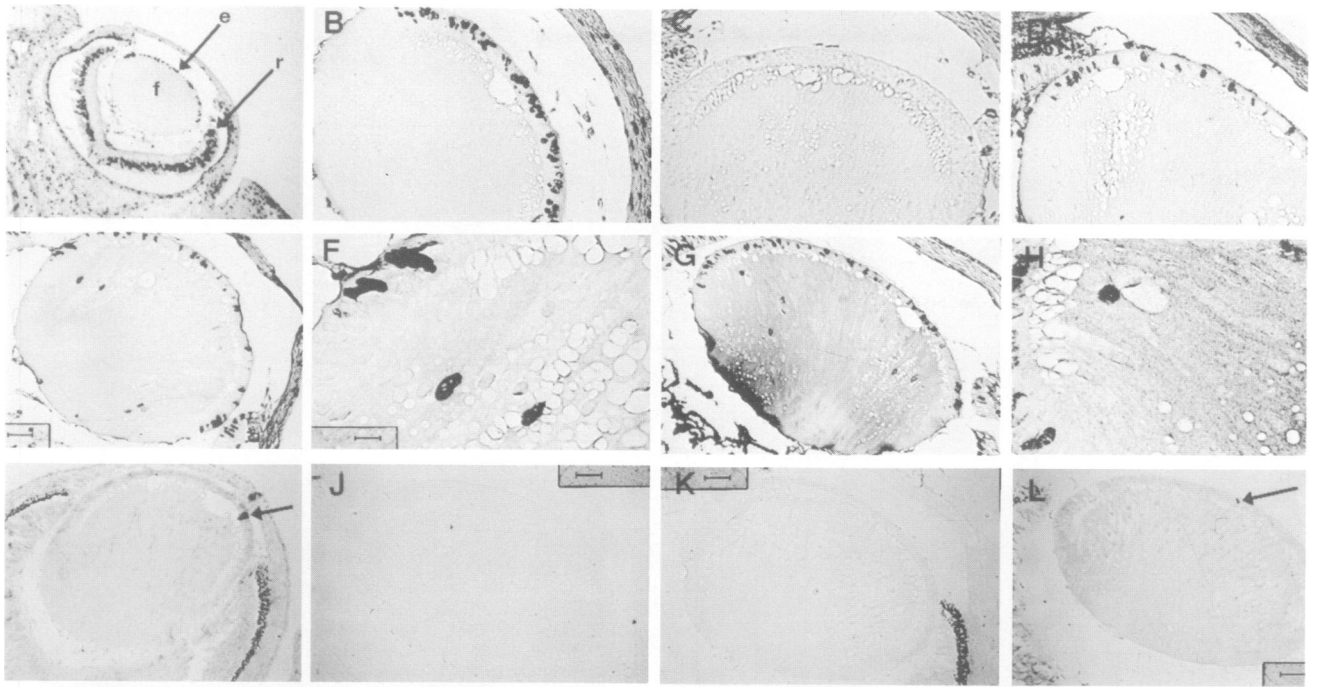

Fig. 7. Cellular proliferation and death in epithelial and lens fiber cells in E14.5 lenses of αA/L-*myc* and αA/c-*myc* transgenic and non-transgenic controls. (**A**−**H**) Sections through E14.5 eyes from BrdU-treated non-transgenic and transgenic mice were assayed by indirect immunoperoxidase methods using an anti-BrdU-containing DNA antibody. (A−C) Non-transgenic control sections were incubated with (A and B) or without the anti-BrdU-containing DNA antibody (C). (D−G) Sections from αA/L-*myc* transgenic line 3 (D), αA/c-*myc* transgenic line 32 (E and F) and line 33 (G and H) were incubated with the anti-BrdU-containing DNA antibody. BrdU-labeled DNA-containing nuclei appear brown. The slight differences in the number of stained nuclei in non-transgenic versus transgenic samples was not a consistent finding and may represent minor variations in pulse time. (**I**−**L**) E14.5 sections were assayed for the presence of DNA 3′-OH groups that are generated during apoptotic internucleosomal cleavage utilizing the TUNEL assay. Non-transgenic control (I−J), line 3 (K) and line 33 (L) sections were incubated with (I, K and L) or without (J) the TdT enzyme and biotinylated dUTP molecules. Arrows point to representative peroxidase stained nuclei exhibiting cellular proliferation (A−H) or death (I−L). In all panels, lenses are oriented with the anterior surface facing the upper right corner. The dark staining in the retina reflects the pigmented layer, rather than peroxidase activity. Magnification 25× for panels A, E and G and 62.5× for panels B−D, F and H−L. Although only the highest expressing lines are shown, the results were similar in the other lines examined. e, anterior epithelial layer; f, lens fiber cells; r, retina.

proteins to induce cataracts or molecular anomalies when introduced into the same compartment indicates that L-Myc exerts its actions in part through functions encoded within the TAD and rules out the possibility that the observed anomalies represent non-specific sequelae due to foreign protein over-production. Most significantly, these data suggest that c- and L-*myc* down-regulation are required genetic events for normal lens cell growth and differentiation and demonstrate that L- and c-Myc perform different functions in the same physiological setting *in vivo*.

Contrasting roles for L- and c-myc in the developing lens

Continued L-*myc* expression in the mature lens fiber cell compartment resulted in severe morphological disorganization of central lens fiber cells, a profound and consistent reduction in MIP26 expression, less dramatic and variable decreases in expression of the γ-crystallins and normal expression patterns for the α- and β-crystallins. Aberrant lens cell differentiation in the face of normal cell cycle control resulting from forced L-*myc* expression provides the first clear *in vivo* evidence for a direct role for L-Myc in cellular differentiation, rather than proliferation. Our findings stand in contrast to those of previous cell culture-based or transgenic experiments in other cell types in which constitutive *myc* expression that was associated with abnormal differentiation was always in the context of deregulated cellular proliferation (reviewed in Morgenbesser and DePinho, 1994). In addition, the effects of forced L-*myc* expression in the lens differ significantly from those of several viral oncoproteins, including the SV40 T-antigen and the human papillomavirus type 16 E6 and E7 proteins (Mahon *et al.*, 1987; Griep *et al.*, 1993), in which impaired lens fiber cell differentiation is accompanied invariably by inappropriate proliferation. On the other hand, our findings are reminiscent of the perturbed development but lack of deregulated growth associated with the forced expression of polyoma large T antigen in lens fiber cells (Griep *et al.*, 1989).

In contrast to L-Myc, constitutive c-*myc* expression in the lens fiber cell compartment leads to inappropriate cell cycle progression, without a block to cellular differentiation. These findings indicate that the down-regulation of c-*myc* is required for lens fiber cells to exit from the cell cycle, but is not necessary for the initiation and completion of lens cell differentiation. The capacity of cycling lens fiber cells to differentiate properly was not anticipated based on the apparent antagonistic relationship between cellular differentiation and proliferation. In normal development, many cell types do not complete their differentiation program until they have exited the cell cycle; in cancer cells, deregulated growth is associated with a block to differentiation. However, the findings of this study are in accord with the presence of elevated c-*myc* transcript levels during differentiation of cultured lens epithelial cells (Nath *et al.*, 1987) and with the actions

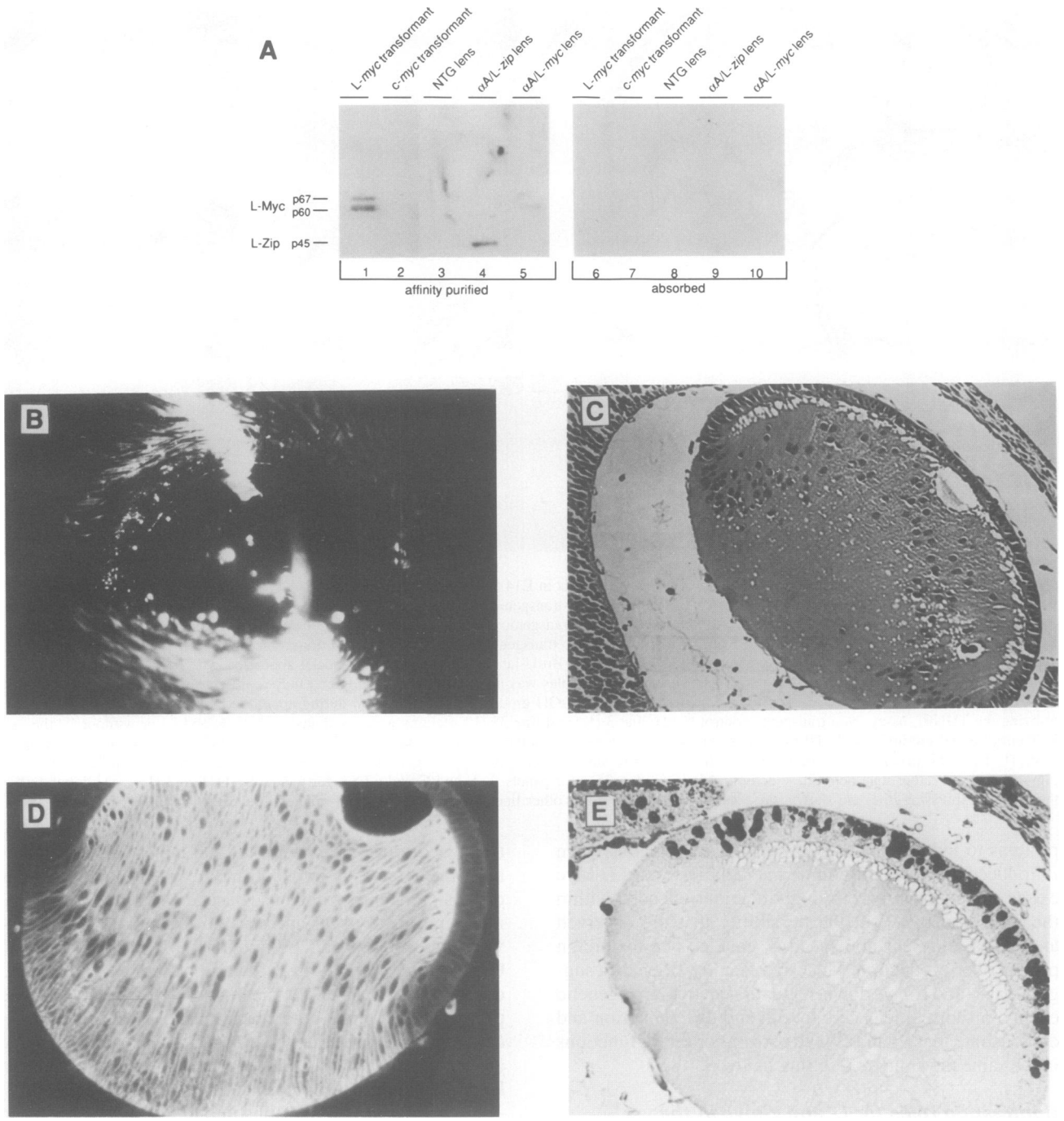

Fig. 8. Expression of the αA/L-Zip protein and phenotype of the αA/L-*zip* transgenic mice. (**A**) Fifty micrograms of protein lysates from the indicated cell lines and newborn lenses were incubated with an affinity-purified anti-mouse L-Myc antibody (lanes 1–5) or the affinity-purified antibody absorbed with the immunizing mouse L-Myc peptide (lanes 6–10). L-*myc* and c-*myc* transformants are rat embryo fibroblasts transformed by a mutant Ha-*ras* and either mouse L-*myc* or mouse c-*myc* respectively (Mukherjee *et al.*, 1992) and serve as positive and negative controls respectively for the antibody. The 60 kDa L-*myc*-encoded protein initiates from an AUG codon near the 5′-end of the second exon; the basis for the larger 67 kDa form is less clear (DeGreve *et al.*, 1988). (**B**–**E**) Phenotypic analysis of αA/L-*zip* line 3 lenses. (B) Slit lamp examination of an adult eye. (C and D) A section through an E14.5 eye was stained with (C) hematoxylin and eosin or (D) assayed by indirect immunofluorescence with an anti-MIP26 antisera. (E) An E14.5 αA/L-*zip* embryo was exposed *in utero* to BrdU and was assayed by indirect immunoperoxidase using an anti-BrdU-containing DNA antibody. (C–E) 400× magnification.

of c-*myc* in cardiac muscle development *in vivo* (Jackson *et al.*, 1990). With respect to the latter, although c-*myc* down-regulation normally accompanies mitotic arrest and differentiation of cardiac myocytes, constitutive high-level c-*myc* transgene expression in these cells led to inappropriate proliferation, but did not alter cardiac actin expression or disturb normal cardiac architecture (Jackson *et al.*, 1990). These data suggest that, in some cell types, withdrawal from the cell cycle may not be required for normal differentiation and that c-*myc*-induced proliferation may not provide a sufficient block to cellular differentiation.

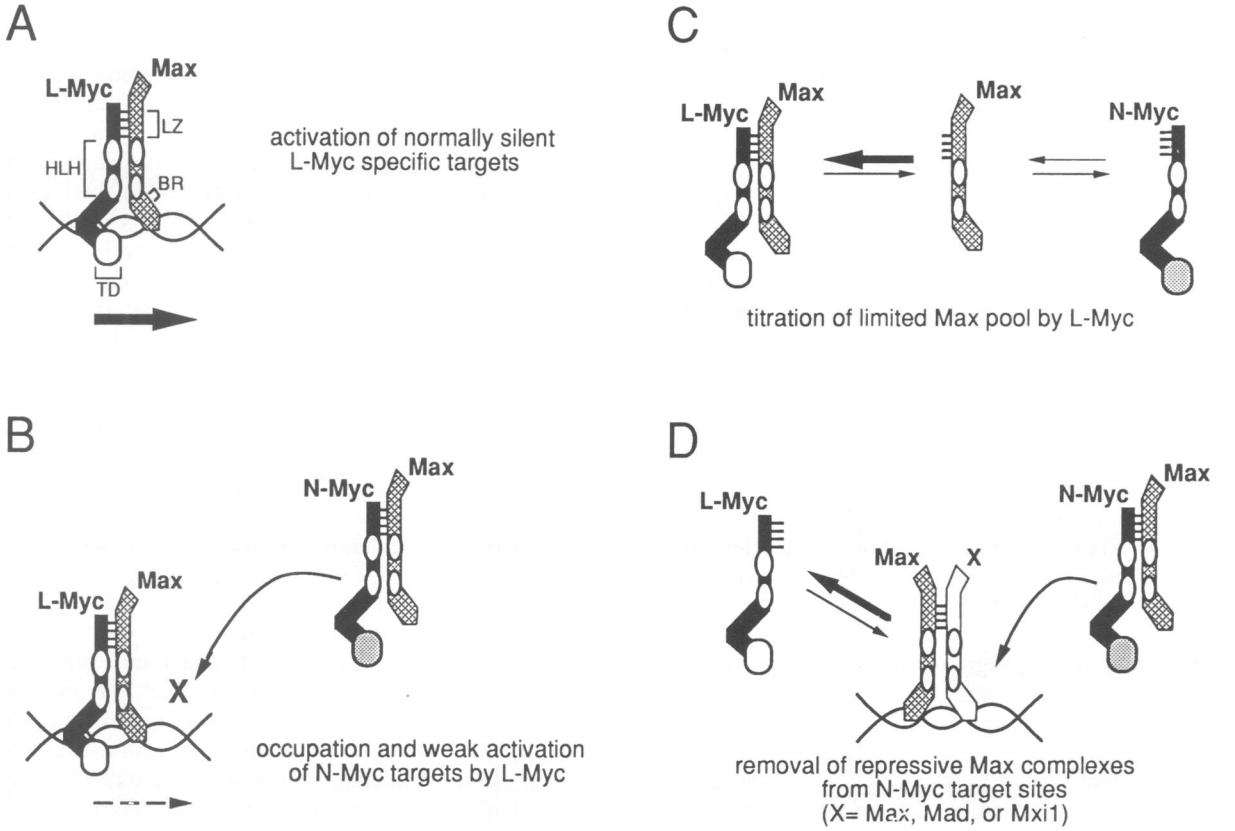

Fig. 9. Models of proposed interactions and activities of L-Myc, Max, Mad and Mxi1 proteins in αA/L-*myc* lens fiber cells. (**A**) Late-stage expression of L-Myc may result in inappropriate activation of normally silent L-Myc-specific gene targets in lens fiber cells. (**B**) N-Myc−Max and L-Myc−Max may share common differentiation-specific gene targets and the occupation of those shared sites in N-*myc*-expressing lens fiber cells by the weakly transactivating L-Myc−Max complex could attenuate the normal N-Myc−Max activation of genes involved in the differentiation of these cells. (**C**) Intracellular Max protein levels may be limiting, and the titration of this limited pool by L-Myc could result in a decrease in functional N-Myc−Max heterodimers. (**D**) Max may be abundant and Max homodimers (or Max−Mad or Max−Mxi1 heterodimers) may occupy and repress N-Myc targets. Excess L-Myc protein may sequester a portion of the Max pool and allow access to target sequences by the N-Myc−Max transactivation complex. BR, basic region; HLH, helix−loop−helix; LZ, leucine zipper; TD, transactivation domain.

The distinct phenotypes of the αA/L- and αA/c-*myc* mice indicate that L- and c-*myc* perform different roles in the lens and suggest that within the same cellular context, *myc* family members may have separable biological activities. This finding distinguishes our study from previous studies employing other cell types, wherein strikingly similar physiological consequences were brought about by over-expression of different *myc* family members, thus supporting functional redundancy among members of the *myc* family in those cell lineages. For example, L- and c-*myc* over-expression can lead to impaired immunodifferentiation and transformation of thymocytes in transgenic mice (Spanopoulou *et al.*, 1989; Moroy *et al.*, 1990) and can block the induction of erythrocyte differentiation in cultured mouse erythroleukemia cells (Coppola and Cole, 1986; Dmitrovsky *et al.*, 1986; Prochownik and Kukowska, 1986; Birrer *et al.*, 1989). In lens cells, as opposed to thymocytes or erythrocytes, L- and c-*myc* appear to influence distinct developmental pathways, perhaps through the regulation of unique gene targets and/or interaction with and modulation of a distinct set of cellular proteins.

The phenotypic differences between c- and L-*myc* are not likely to reflect differences in expression of c-*myc* versus L-*myc*, since the broad range of transgene expression levels observed in the 14 different αA/L-*myc* lines overlapped significantly with the range found in the eight independently derived αA/c-*myc* lines; despite this extensive overlap, the phenotypes induced by each transgene were consistent and non-overlapping. Lastly, although the lens is a highly specialized organ, the experimental manipulation of other critical growth and developmental regulators, e.g. Rb and p53, has established the lens as a powerful model system for the study of gene function and has allowed for extrapolation of the role of such genes in the control of growth and development in other cell types (Morgenbesser *et al.*, 1994, and references therein). On this basis, we believe that the functional insights into the Myc family provided by this study will likely extend beyond the lens cell type and could have more general relevance to other cell types.

Towards an understanding of the molecular mechanisms underlying Myc actions in the developing lens

Our initial characterization of the distinct cellular phenotypes of the αA/L- and αA/c-*myc* lenses provides an experimental framework upon which the differential molecular actions of members of the Myc family could be more clearly conceptualized (Figure 9). In particular, the known biochemical properties of each Myc family member, together with the biological results of this study,

have provided insight into the question of whether members of the Myc family function through common genetic pathways in their regulation of growth and differentiation *in vivo*. With regard to the actions of L-Myc, the weak transactivation potential of L-Myc relative to other Myc family members, (Barrett *et al.*, 1992), the ability of wild-type and dominant-negative forms of L-Myc to abrogate N-Myc transforming activity in rat embryo fibroblasts (Mukherjee *et al.*, 1992) and the failure of the mutant L-*zip* to induce a phenotype in N-*myc*-expressing lens fiber cells all argue strongly for the regulation of common growth-related gene targets by L-Myc and N-Myc in fibroblast transformation, but distinct targets in the developing lens fiber cell. The lack of phenotype with L-*zip* indicates that, in the lens fiber cell, high levels of transgene-encoded L-Myc do not interfere with endogenous N-Myc activity through the competitive occupation of shared genetic targets by a weakly transactivating L-Myc−Max complex (Figure 9B) or through the titration of a limited intracellular pool of Max leading to a decrease in the formation of active N-Myc−Max complexes (Figure 9C).

Instead, our results suggest that L-Myc functions as a positive transcriptional regulator of L-*myc*-specific gene targets or modulates the activities of key cellular proteins through interactions mediated by its TAD (Figure 9A). The requirement for the L-Myc TAD, as demonstrated by the αA/L-*zip* experiment, raised the possibility that the action of the L-*myc* transgene could also involve the shorter L-Myc protein, whose transcripts are present at high levels in αA/L-*myc* lenses. This protein, which lacks DNA and dimerization binding motifs, may sequester general transcription factors (e.g. the TATA binding protein that has been shown to interact with the c-Myc TAD; Hateboer *et al.*, 1993) or key regulatory proteins (e.g. pRb or p107; Rustig *et al.*, 1991; Kratzke *et al.*, 1992; Gu *et al.*, 1994) and thereby impact upon their biological activities. An important role for such a protein in the αA/L-*myc* phenotype appears unlikely in that transgenic mice harboring an αA-driven L-*myc* short form do not possess congenital cataracts (J.Horner and R.A.DePinho, unpublished results). Thus, the full-length L-Myc protein appears to be required for the αA/L-*myc*-induced phenotype, presumably by activating normally quiescent L-Myc-specific gene targets in post-mitotic lens fiber cells. Since L-*myc* is normally expressed in immature lens cells only, these gene targets may be involved in the maintenance of an undifferentiated state (e.g. a state prior to MIP26 expression). With the identification of candidate Myc-responsive targets involved in lens growth and differentiation, these transgenic lines will provide a useful biological system in which to clearly address this question.

The deregulation of L- or c-myc in the lens does not lead to the development of tumors

In contrast to many other cell types, over-expression of c-*myc* (or L-*myc*) was insufficient to bring about malignant transformation in the lens. The observation that the expression of other viral oncoproteins in the lens resulted in the formation of lens tumors possessing developmentally immature lens fiber cells demonstrates that the lens is susceptible to neoplastic conversion (Mahon *et al.*, 1987; Griep *et al.*, 1993). The basis for the lack of tumors in the αA/L- and αA/c-*myc* lenses may relate to stage- or tissue-specific restrictions in L- and c-*myc* oncogenic potential, to levels of L- and c-*myc* expression that are sub-threshold with respect to their transforming activity or to a requirement for the activation of specific cooperating oncogenic agents. In particular, the transforming potential of *myc* family genes may be restricted in the lens due to the presence of specific anti-oncogenic agents. These anti-oncogenic factors could include rising levels of the potent anti-Myc repressive complexes Mad−Max (Ayer *et al.*, 1993) and Mxi1−Max (Zervos *et al.*, 1993). Indirect support for this possibility derives from the observations that both Mad and Mxi1 have been shown to dramatically suppress Myc-induced transformation (Lahoz *et al.*, 1994) and that their levels of expression typically increase as many cell types progress towards terminal differentiation (Ayer and Eisenman, 1993; L.Chin and R.A.DePinho, unpublished observations). Finally, although apoptotic cell death is one mechanism through which malignant cells may be eliminated (for review, see Wyllie, 1992), apoptosis was not observed in the αA/c- or αA/L-*myc* transgenic lens fiber cells, indicating that the lack of lens neoplasia in our mice is not a consequence of tumor cell suicide.

Our study has clearly demonstrated that members of the Myc family exert distinct physiological effects in the same cell type *in vivo*. These differential activities, coupled with the experimental merits of the ocular lens, provide an ideal model system in which to determine whether these activities are executed through distinct gene targets and an expanding network of Myc interacting proteins.

Materials and methods

Production of the αA/c-myc, αA/L-myc and αA/L-zip transgenic mice

The αA-crystallin expression cassette, CPV1, contains a polylinker flanked by the murine αA-crystallin promoter (Chepelinsky *et al.*, 1985) and the SV40 splice and polyadenylation sequences (Gorman *et al.*, 1982) in a Bluescript-based vector (D.Silversides and P.A.Overbeek, unpublished results). The *myc* fragments inserted into the polylinker were as follows: for αA/c-*myc*, a 1.4 kb *Xho*I fragment of the mouse c-*myc* cDNA pmc-*myc*54 (Stanton,L.W. *et al.*, 1983; graciously provided by Dr Ken Marcu; see Figure 1B); for αA/L-*myc*, a mouse L-*myc* genomic fragment that contains the entire second exon and extends to the *Hinc*II site located in the 3′ untranslated region of the third exon (Legouy *et al.*, 1987; see Figure 1A); and for αA/L-*zip*, the transactivation domain of L-*myc* was removed as described previously (Mukherjee *et al.*, 1992). Gel-purified transgene inserts were introduced into the germline of C57Bl/6×CBA (B6/CBA) F1 mice (Jackson Laboratories) by pronuclear microinjection as described previously (Hogan *et al.*, 1986). Outbred CD1 females (Charles River) served as pseudopregnant recipients for microinjected eggs and B6/CBA mating partners were used to propagate the transgenic lines. Genomic DNA was prepared from tail tips as described previously (Hogan *et al.*, 1986) and was analyzed for the presence of the transgene by Southern blotting (Sambrook *et al.*, 1989).

RNA isolation and Northern blotting analyses

Total RNA was isolated from lenses and P19 embryonal carcinoma cells (McBurney *et al.*, 1982) by the LiCl−urea method (Auffray and Rougeon, 1980) and assayed by Northern blotting as described previously (Sambrook *et al.*, 1989). RNAs were judged to be intact and evenly loaded by ethidium bromide staining. The mouse c- and L-*myc*-specific probes are indicated in Figure 1, whereas the probes for mouse N-*myc* and *max* (*myn*) have been described elsewhere (Mukherjee *et al.*, 1992).

Histological analyses, RNA in situ hybridization and indirect immunofluorescence

Embryos were staged as 0.5 days at noon on the day the vaginal plug was found. Samples were fixed in 4% paraformaldehyde in phosphate-

buffered saline (PBS), pH 7.0, or 10% buffered formalin and processed through paraffin embedding using standard procedures. Sections measuring 3 μm in thickness were cut parallel to the optic nerve, affixed to poly-L-lysine-coated slides, dewaxed in xylene, rehydrated through an ethanol series and stained with hematoxylin and eosin. RNA *in situ* hybridization was performed as described previously (Nakamura *et al.*, 1989), except that the slides were incubated with 2 mg/ml glycine for 5 min at room temperature following proteinase K (PK) digestion and hybridization to ^{35}S-containing riboprobes was performed at a probe concentration of 5×10^4 counts/min/μl. Inserts for the mouse L-*myc* and c-*myc* riboprobes are shown in Figure 1. The N-*myc* riboprobe contains a 560 bp *Cla*I–*Pst*I fragment of the mouse N-*myc* third exon (DePinho *et al.*, 1986). For indirect immunofluorescence, paraffin-embedded sections were rehydrated, rinsed in PBS and blocked in 3% bovine serum albumin (BSA) in PBS at room temperature for 20 min. Polyclonal rabbit IgG antisera to MIP26 (Bok *et al.*, 1982; a generous gift of Dr Joseph Horwitz) and to α-, β- and γ-crystallins (graciously provided by Dr Sam Zigler) were diluted in 3% BSA/PBS, incubated on sections for 1 h at room temperature and washed in PBS. The secondary antibody, an FITC-linked anti-rabbit IgG antibody, was diluted, incubated and washed in PBS. For quantitation, limiting dilutions were performed with the primary and secondary antisera to determine the highest dilution for optimal staining.

Antibody preparation, protein isolation and Western immunoblot analyses

For Western blot analyses of crystallin and MIP26 expression, lenses were homogenized in ice-cold 0.1 M Tris, pH 7.4, the water-soluble lens proteins were separated from water-insoluble lens membrane proteins by centrifugation at 4°C for 20 min (Takemoto *et al.*, 1988) and the protein concentration was determined by the Bradford assay. The membrane pellets were resuspended in an ice-cold solution of 0.1 M Tris, pH 8.0, 7 M urea, 5 mM EDTA (Broekhuyse *et al.*, 1976). Proteins were separated by SDS–PAGE (12.5% polyacrylamide) and electroblotted to nitrocellulose; for the analysis of MIP26, the membrane samples were not boiled prior to electrophoresis as this treatment causes MIP26 aggregation (Wong *et al.*, 1978). Blots were blocked for 1 h at room temperature in PBS containing 2.5% chicken ovalbumin, 2.5% milk and 0.1% Tween-20. The primary antibody incubations were carried out for 1 h with the anti-crystallin and MIP26 antisera (see above) diluted 1:10 000 in blocking solution, followed by a donkey anti-rabbit IgG horseradish peroxidase-conjugated antibody at a 1:10 000 dilution as the secondary antibody. Peroxidase activity was detected with enhanced chemiluminescence (Amersham). Densitometric analysis was performed within the linear range of the film.

For Western blotting analysis of L-Myc and L-Zip, a peptide corresponding to the 16 C-terminal amino acid residues of mouse L-Myc, CRQQQLQKRIAYLSGY, was synthesized and purified and polyclonal rabbit antibodies were prepared and affinity-purified as described previously (Schreiber-Agus *et al.*, 1993). Western analysis was the same as for crystallins and MIP26, except that proteins were extracted from cells and lenses by homogenization in ice-cold 8 M urea and were separated by SDS–PAGE (8% polyacrylamide); the blots were blocked in PBS containing 5% milk, 5% chicken ovalbumin and 0.1% Tween 20, the primary antibody was the affinity-purified anti-L-Myc antibody at 5 μg/ml and the secondary antibody was used at a 1:15 000 dilution. Controls for non-specific binding of the affinity-purified antibody were performed by pre-incubating the antibody with its immunizing peptide linked to BSA at a ratio of 1:5 for 24 h at 4°C. Following centrifugation, the supernatant was used as the primary antibody.

Bromodeoxyuridine immunoperoxidase histochemistry and TUNEL assay

Pregnant mice were injected intraperitoneally with BrdU dissolved in PBS at a dose of 100 μg/g body wt (Miller and Nowakowski, 1988). After 1 h, the embryos were fixed in 10% buffered formalin for 2 h at room temperature. Rehydrated sections were rinsed well in PBS and incubated with 10% methanol, 3% H_2O_2 for 30 min, to inactivate endogenous peroxidases, and rinsed in PBS again. In order to facilitate binding of the mouse anti-BrdU monoclonal antibody, which recognizes BrdU in single-stranded DNA, the nuclear and DNA binding proteins were mildly digested with 200 μg/ml pepsin in 0.01 N HCl for 20 min at 37°C (Hayashi *et al.*, 1988), such that tissue damage was avoided. DNA was denatured in 2 N HCl for 45 min, neutralized in 0.1 M sodium borate, pH 8.5, for 10 min and rinsed in PBS. Sections were incubated with the anti-BrdU antibody diluted 1:20 overnight at 4°C in a humidified chamber, washed in PBS and incubated with a biotinylated anti-mouse IgG antibody followed by the avidin–biotin–peroxidase complex from a Vectastain ABC detection kit as per the manufacturer's instructions (Vector Laboratories). Staining was achieved with a 10 min incubation with diaminobenzidine/H_2O_2 as substrates for peroxidase. Slides were mounted and photographed under bright-field optics with a Nikon microscope. For detection of apoptosis *in situ*, the TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated dUTP nick end labeling] assay was performed as described previously (Gavrieli *et al.*, 1992), except that all solutions were made with DEPC-treated water and the pre-incubation and incubation PK solution contained 20 mM EDTA. For detection of incorporated biotinylated dUTP, the sections were blocked in diluted horse serum for 30 min, washed in PBS and incubated with the avidin–biotin–peroxidase complex followed by diaminobenzidine/H_2O_2 as described above.

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