Contrasting patterns of c-myc and N-myc expression in proliferating, quiescent, and differentiating cells of the embryonic chicken lens

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Summary

The present study uses the polymerase chain reaction and in situ hybridization to examine c-myc and N-myc mRNA in the embryonic chicken lens at 6, 10, 14 and 19 days of development and compares the pattern of expression obtained with the developmental pattern of cell proliferation and differentiation. In the central epithelium, c-myc mRNA levels were proportional to the percentage of proliferating cells throughout development. N-myc mRNA expression in this region was relatively low and showed no correlation with cell proliferation. The ratio of N-myc to c-myc mRNA increased markedly with the onset of epithelial cell elongation and terminal fiber cell differentiation, although both c-myc and N-myc mRNAs continued to be expressed in postmitotic, elongating cells of the equatorial epithelium and in terminally differentiating lens fiber cells. Thus, increased expression of N-myc, a gene whose protein product may compete with c-myc protein for dimerization partners, accompanies the dissociation of c-myc expression and cell proliferation during terminal differentiation of lens fiber cells.

Key words: proto-oncogenes, c-myc, N-myc, chick, lens, mRNA.

Introduction

c-myc and N-myc are nuclear, DNA-binding proteins thought to play a role in cell proliferation and differentiation (reviewed by Alt et al., 1986; Boettinger, 1989). Although their exact functions are not yet known, their ability to bind DNA in a sequence-specific manner (Blackwell et al., 1990) and their structural similarity to known transcription factors (Murre et al., 1989a) suggests that they may be transcription factors. Both proteins contain leucine zipper regions similar to those in the fos- and jun-families of transcription factors (Landschultz et al., 1988), as well as the helix-loop-helix motif found in MyoD, immunoglobulin kappa enhancer binding proteins, and products of the Drosophila genes, achaete scute and daughterless (Murre et al., 1989a). Since these structural motifs have been shown to participate in protein-protein interactions (Murre et al., 1989b; O’Shea et al., 1989), their presence in c-myc and N-myc (as well as other myc family proteins, (Alt et al., 1986)) implies that these proteins have the capacity to form homodimers and heterodimers. Homodimers or higher order oligomers of c-myc have been shown to bind selectively to the sequence CACGTG (Blackwell et al., 1990). In addition, specific proteins have been identified which form heterodimers with both c-myc and N-myc altering the efficiency with which c-myc binds to the CACGTG site (Blackwood and Eisenman, 1991; Predergast et al., 1991).

Evidence that c-myc and N-myc may be developmentally important regulators of cell proliferation and differentiation has come from studies of their expression in embryonic tissues and cultured cell lines. In vitro studies indicate that c-myc expression is associated with proliferation and is usually down-regulated during differentiation (reviewed by Cole, 1986). Nonetheless, there is no apparent correlation between cell proliferation and c-myc expression in developing embryos (Pfeifer-Olszyn et al., 1985; Zimmerman et al., 1986; Downs et al., 1989; Schmid et al., 1989), and elevated c-myc expression is seen during differentiation of certain cell types (reviewed by Zelenka, 1990). One possible explanation for the lack of a clear correlation between expression of c-myc and N-myc, on the one hand, and either proliferation or differentiation, on the other hand, may be that c-myc protein has multiple functions stemming from its ability to form a variety of dimeric or oligomeric complexes. Furthermore, since N-myc and c-myc proteins may compete with each other for the same dimerization partners (Blackwood and Eisenman, 1991), the ratio of N-myc to c-myc expression may shift the population of proteins available to dimerize with c-myc. Thus, the relative concentrations of these two proto-oncogene products in developing tissues may be more significant than the absolute concentration of either. The present study investigates the developmental pattern of both c-myc and N-myc expression in the developing chicken lens to determine their relationship to each other during growth and differentiation.

The pattern of growth and differentiation in the lens
mRNAs and proteins have very short half-lives (Hann and Eisenman, 1984; Dani et al., 1984; Slamon et al., 1986; Ramsay et al., 1986; Sawai et al., 1990).

Materials and methods

Microdissection of lenses and isolation of RNA
Lenses from 6-, 10-, 14- and 19-day-old embryos of white Leghorn chickens (Truslow Farms, Chestertown, MD) were removed and cleaned of any adhering pigmented iris. The lens capsule/epithelium was separated from the fiber mass by microdissection with sharpened jeweler’s forceps. Each epithelium was further dissected into a central region, as described by Persons and Modak (1970) and Ramsay et al., 1986; Sawai et al., 1990).

Preparation of oligonucleotides
A 397 nt region of chicken c-myc mRNA (1018/1414) spanning the exon2/exon3 junction (Watson et al., 1983) and a 195 nt region of chicken N-myc mRNA (1024/1218) (Sawai et al., 1990) were chosen for reverse transcription and amplification by the polymerase chain reaction (PCR) (Mullis and Faloona, 1987; Saiki et al., 1988) (Fig. 2). The RNA secondary structure prediction program, FOLD (Zuker and Stiegler, 1981), was used to ensure that the chosen regions were free of stable secondary structures which might interfere with reverse transcription (Pallansch et al., 1990). Oligonucleotides (20-mers) used for PCR were chosen to allow discrimination between chicken c-myc (Watson et al., 1983) and N-myc (Sawai et al., 1990) mRNAs (Fig. 2). These and other oligonucleotides used in this study were synthesized on an Applied Biosystems 380B DNA synthesizer (Foster City, CA).

Reverse transcription
Reverse transcription was carried out as described in Pallansch et al. (1990) with the following modifications: oligonucleotide concentration was reduced to 1 µM, reaction volume was reduced to 12.5 µl, incubation at 37°C was reduced to 10 minutes, and the samples were heated to 68°C following reverse transcription. Molony murine leukemia virus reverse transcriptase was obtained from Stratagene (LaJolla, CA), and was used at a concentration of 15 units per reaction.

Polymerase chain reaction
PCR amplification of the cDNA synthesized by reverse transcription of 50 ng of total cytoplasmic RNA was performed as described by Pallansch et al. (1990) in a total reaction volume of 50µl. Amplification was carried out for 30 cycles in a Perkin Elmer/Cetus thermal cycler programmed for 1.5 minutes at 94°C; 2 minutes at 42°C; 3 minutes at 72°C. The final cycle was followed by a 10 minute extension step at 72°C. DNA internal standards (described below) were included in the PCR reaction at a concentration of 3 fg per reaction (c-myc) and 1 fg per reaction (N-myc). Taq DNA polymerase was obtained from Perkin-Elmer Cetus Corp. (Norwalk, CT). 32P-labeled PCR products were separated on 8% polyacrylamide/urea gels in parallel with 32P-labeled molecular weight markers (MspI digest of pBR322 (New England Biolabs, Beverly, MA) or a 123 bp DNA ladder (BRL, Bethesda, MD)). Electrophoresis was carried out at 1750 V for 90 minutes; the gels were dried and
Hybridization of PCR products to internal oligonucleotide probes

Oligonucleotides containing the reverse complement of sequences from c-myc (1242/1261) and N-myc (1102/1121) mRNAs were end-labeled with [γ-32P]ATP (sp. act. >7000 Ci/mmol, ICN, Irvine, CA) using T4 polynucleotide kinase (Pharmacia, Piscataway, NJ) as described by Sambrook et al. (1989). Approximately 5 μg of unlabeled c-myc and N-myc PCR products were electrophoresed on a 2% agarose gel at 50 V for 2-3 hours and subsequently transferred to GeneScreen Plus nylon membranes (Dupont, Boston, MA) by capillary blotting. Hybridization with 32P-labeled oligonucleotides was performed according to standard protocols (Sambrook et al., 1989) at 57°C (c-myc) or 59°C (N-myc). After appropriate washes, the membranes were autoradiographed on Kodak XAR film overnight at ~80°C.

Thymidine labeling of nuclei and quantitation of DNA by ELISA

Pulse-labeling of embryonic lenses with [3H]thymidine was performed as described by Nath et al. (1987). Epithelia were removed and the epithelial cells lysed with 1% sodium dodecyl sulfate in 0.01 M Tris, pH 7.4. [3H]thymidine-labeled DNA was purified by extraction with CHCl3:phenol:isoamyl alcohol 50:50:1 and ethanol precipitation (Sambrook et al., 1989). Duplicate aliquots of DNA were removed for determination of tritium incorporation by scintillation counting, and triplicate determinations of DNA content were made on the remainder, using the ELISA assay of Richardson and McAvoy (1989).

In situ hybridization

Lenses to be used for in situ hybridization were oriented in OCT compound (Lab Tek) and quick frozen in a dry ice/hexane bath. Sections (8 μm) were taken from the center of the lens, parallel to the lens axis (anterior to posterior pole) on a Reichert-Jung 2800E microtome, collected onto silane-treated slides and rapidly refrozen on a block of dry ice. Sections were fixed for 5 minutes at 4°C in 4% glutaraldehyde (Polysciences, Inc., Warrington, PA) and 20% ethylene glycol (Tousimis, Inc., Rockville, MD) in 0.1 M sodium phosphate buffer at pH 7.4 and rinsed in 2× saline citrate solution (SSC, 0.16 M NaCl, 0.016 M sodium citrate) as previously described (Thomas et al., 1990). Pretreatment, hybridization and post-treatment were carried out as described in the Oncor in situ procedure (Oncor, Gaithersburg, MD). Briefly, fixed tissues were pretreated with acetic anhydride, then glycinated, before dehy- dration and overnight hybridization at 52°C in a humidified chamber. 35S-labeled sense and antisense riboprobes were used at a concentration of 5×105 cts/minute/μg in 0.33 M NaCl, 0.016 M sodium citrate) as previously described (Thomas et al., 1990). Post-treatment, hybridization and post-treatment were carried out as described in the Oncor in situ procedure (Oncor, Gaithersburg, MD). Following hybridization, tissues were washed for 10 minutes in 50% formamide/2× SSC at 52°C, rinsed in 2× SSC, digested with RNAase, and again washed for 10 minutes in 50% formamide/2× SSC and rinsed in 2× SSC. Tissue sections were dehydrated in a graded alcohol series and air dried. Slides were dipped in a 1:1.5 dilution of emulsion (Ilford G-5) and exposed for 2-5 days at room temperature. Emulsion was developed in Dektol developer (Kodak, Rochester, NY), fixed and counterstained in hematoxylin and eosin. Coverslips were mounted with Permount. Micrographs were taken with a Nikon Coolpix 990 digital camera and Nikon IF-2000 microscope fitted with both bright-field and dark-field optics.

For preparation of RNA riboprobes, EcoR1 and BamH1 restriction sites were incorporated into the upstream and downstream PCR oligonucleotides, respectively, and the resulting PCR products were directionally cloned into pBlueScript(KS+) (Stratagene, LaJolla, CA) following EcoR1 and BamH1 digestion. The identity of the
cloned products was confirmed by sequencing using the dideoxy method of chain termination (Sambrook et al., 1989). 35S-labeled sense and antisense riboprobes were synthesized from the T3 and T7 promoters, respectively, using 35S-UTP (New England Nuclear, sp.act. >1000 Ci/m mole). Riboprobes were purified over a Nick column (Pharmacia) before dilution in Oncor RNA hybridization buffer.

**Results**

**Specificity of the PCR assay**

Since c-myc and N-myc mRNAs share regions of sequence homology (Fig. 2), it was necessary to confirm the specificity of the PCR reactions used to measure these proto-oncogene

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**Fig. 3.** Specificity of the PCR assays for c-myc and N-myc mRNAs. (A) Lane 1: Autoradiogram of the c-myc PCR product generated from embryonic chicken lens RNA (397 bp) and from the c-myc DNA internal standard (324 bp). Lane 2: DNA size markers (123 bp ladder). (B) Lane 1: Autoradiogram of the N-myc PCR product generated from embryonic chicken lens RNA (195 bp) and from the N-myc internal standard (120 bp). Lane 2: DNA size markers (123 bp ladder). (C) Autoradiogram showing hybridization of 32P-labelled oligonucleotide probes specific for c-myc (1242/1261 of the c-myc mRNA; shown on left) and N-myc (1102/1121 of the N-myc mRNA; shown on right) to both the c-myc and N-myc PCR products.

**Fig. 4.** (A) Autoradiogram of c-myc PCR product (397 bp) obtained from 50 ng of total RNA from 6-, 10-, 14- and 19-day-old embryonic chicken central lens epithelia with 3 fg c-myc DNA internal standard (324 bp). (B) Bar graph of relative concentrations of c-myc mRNA at 6-, 10-, 14- and 19-days of development in the central epithelium (C), equatorial epithelium (E), and fiber mass (F). Each RT/PCR assay was repeated at least 5 times using 4 independently isolated RNA preparations. Error bars represent the s.e.m. of these replicate determinations. The superimposed line graphs represent the percentage of [3H]thymidine-labeled nuclei in each region (data from Persons and Modak, 1970)
mRNAs in the developing lens. Reverse transcription of embryonic chicken lens RNA followed by PCR using oligonucleotide primers specific for chicken c-myc sequences in the presence of the c-myc internal standard generated products of the expected sizes (397 bp for c-myc; 324 bp for the c-myc internal standard) (Fig. 3A). Similarly, oligonucleotide primers specific for chicken N-myc sequences generated the expected 195 bp N-myc product and a 120 bp N-myc internal standard product (Fig. 3B). Sequencing of the subcloned c-myc and N-myc products confirmed that each was derived from the expected region of the corresponding mRNA. Oligonucleotides complementary to sequences within the amplified regions of c-myc and N-myc mRNA hybridized specifically to their respective PCR products and showed no evidence of minor products derived from the other mRNA (Fig. 3C). Thus, we conclude that the c-myc PCR product is derived exclusively from c-myc mRNA, the N-myc product exclusively from N-myc mRNA.

Developmental changes in c-myc mRNA expression

A representative autoradiogram of the PCR products generated using RNA from the central lens epithelium from chicken embryos at 6, 10, 14 and 19 days of development is shown in Fig. 4A. The compiled data from several such experiments are presented as a bar graph in Fig. 4B, along with the results obtained for the equatorial lens epithelium and the lens fibers. Superimposed on the bar graphs are line graphs indicating the labeling index in these regions of the lens as a function of developmental age (data taken from Persons and Modak (1970)). The level of c-myc mRNA in the central region of the lens epithelium was proportional to the percentage of labeled nuclei in this region throughout development (Fig. 4B). In the equatorial region, however, the levels of c-myc mRNA expression were relatively constant throughout development despite a decrease in the percentage of proliferating cells (Fig. 4B). This suggested that c-myc might continue to be expressed in cells that are in the early stages of cytodifferentiation. Significant levels of c-myc mRNA were also found in the differentiated lens fiber cells (Fig. 4B).

Developmental changes in N-myc mRNA expression

The pattern of expression of N-myc mRNA in the developing lens was distinctly different from that of c-myc. At each age examined N-myc mRNA was predominantly associated with the equatorial region of the lens (Fig. 5A,B). The levels of N-myc mRNA showed no correlation with the changes in cell proliferation that accompany development in either the central or equatorial epithelium. As with c-myc, N-myc expression seemed to continue in the differentiated fiber cells.

Expression in the annular pad

To determine the extent to which c-myc and N-myc mRNAs were expressed in the differentiating cells of the annular pad at the lens equator, lenses from 14-day-old embryos were dissected as shown in Fig. 1B. This method of dissection separates almost all the proliferating cells of the lens epithelium into a single fraction, which we have called the "inner"
The remainder of the epithelium represents the annular pad, an almost pure population of cells in the early stages of cytodifferentiation. To test whether the dissection was successful, epithelia were labeled with $[^3H]$thymidine prior to dissection, and the percentage of labeled DNA recovered in each tissue fraction was determined (Fig. 6). Approximately 93% of the labeled DNA was associated with the "inner" epithelium (average of 5 determinations). Levels of

Fig. 7. In situ hybridization of riboprobes specific for N-myc (A,C,E) and c-myc (B,D,F) mRNAs in sections of 14-day-old embryonic chicken lens, photographed with bright-field (A,B) and dark-field (C-F) illumination. Location of postmitotic annular pad cells is indicated by three arrows; region occupied by nuclei of newly formed fiber cells is indicated by two arrows. A-D at same magnification; scale bar = 100 µm. E,F at same magnification; scale bar = 100 µm.
c-myc and N-myc mRNA were then measured in similarly dissected tissues. 50% of the total c-myc mRNA of the epithelium and over 80% of the N-myc mRNA were found in the annular pad, indicating that both proto-oncogenes are actively expressed in cells that are in the early stages of cytodiagenesis (Fig. 6).

In situ hybridization

To determine whether c-myc and N-myc mRNAs are expressed in the same population of annular pad cells, in situ hybridization was performed using 35S-labeled riboprobe derived from the cloned PCR products (Fig. 7A-F). Hybridization of N-myc mRNA to the antisense N-myc riboprobe was observed in cells throughout the annular pad and in the perinuclear region of the fiber cells (Fig. 7C,E). Hybridization to the sense riboprobe in control sections was not above background (not shown). Hybridization of c-myc mRNA to the c-myc antisense riboprobe was also seen throughout the annular pad region, although the intensity of the signal was consistently lower than for N-myc using comparably labeled probes with identical exposure times (Fig. 7D,F). Again, control sections hybridized to the c-myc sense riboprobe showed no hybridization above background (not shown). In the fiber cells, hybridization to the c-myc antisense riboprobe was not above background, although c-myc mRNA was detected in these cells by the more sensitive PCR assay. These results indicate that both c-myc and N-myc mRNAs are expressed in the same population of annular pad cells of the 14-day embryonic chick lens, in a region that contains only postmitotic cells (Persons and Modak, 1970).

Discussion

The developing lens provides an opportunity to observe a single population of proliferating epithelial cells as it undergoes two types of cell cycle arrest: one leading to quiescence in the central epithelium, and one leading to terminal differentiation at the lens equator. The present results indicate that c-myc mRNA expression is distinctly different in these two instances. In the central region of the lens epithelium, the level of c-myc mRNA expression is proportional to the percentage of proliferating cells throughout development. This implies that expression of c-myc mRNA is associated predominantly with proliferating lens epithelial cells and ceases or is greatly diminished as these cells enter quiescence. The decreased expression of c-myc mRNA is a specific effect, since expression of c-fos and c-jun mRNAs increases in the central lens epithelium as cells become quiescent (Rinaudo and Zelenka, 1992). The finding that c-myc mRNA decreases as lens cells cease dividing in vivo is consistent with studies showing that c-myc mRNA is absent or at very low levels in quiescent mouse lens epithelial cells in culture (Rao and Church, 1989), as in many other quiescent cell types in vitro (reviewed by Cole, 1986). In contrast, we observed no correlation between c-myc expression and cell proliferation in the equatorial epithelium of the developing lens, where the nonproliferating cell population consists of differentiating, rather than quiescent, cells. This suggested that c-myc mRNA might be expressed in postmitotic lens epithelial cells in the early stages of differentiation in vivo, as was previously found in vitro (Nath et al., 1987). This was confirmed by direct PCR analysis of c-myc mRNA in the isolated annular pad cells by in situ hybridization. Continued expression of c-myc mRNA during differentiation has also been observed in a limited number of other cell types, including PC12 cells (Greenberg et al., 1985), chronic lymphocytic leukemia cells (Larsson et al., 1987), and postmitotic cerebellar neurons (Ruppert et al., 1986).

The present data further indicate that expression of N-myc mRNA is strongly correlated with the initial stages of lens fiber cell formation. The highest concentrations of N-myc mRNA were found in the annular pad cells at all developmental stages, although lower levels of N-myc mRNA may also be present in the proliferating or quiescent cells of the central epithelium (this study; Sawai et al., 1990; Hirning et al., 1991). This pattern of expression implies that N-myc mRNA levels increase as proliferating lens epithelial cells withdraw from the cell cycle to differentiate. Thus, although c-myc expression continues during the early stages of lens fiber cell differentiation, its expression relative to N-myc decreases, because of the increased N-myc expression. This shift in the relative levels of c-myc and N-myc expression may alter the array of proteins available to complex with c-myc, and contribute to uncoupling the usual association between c-myc expression and cell proliferation.

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References


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