Contrasting patterns of *myc* and N-*myc* expression during gastrulation of the mouse embryo

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myc and N-*myc* are related genes whose similar protein products may be used for different purposes in vertebrate organisms. We have explored this possibility by using hybridization in situ to examine the expression of *myc* and N-*myc* during gastrulation of mouse embryos. Throughout gastrulation, *myc* RNA was most abundant in extraembryonic cells; by contrast, N-*myc* RNA was found at highest levels in the expanding primitive streak and other portions of the embryonic mesoderm. Differentiation of mesoderm to epithelioid cells was accompanied by diminished expression of N-*myc*. Expression of *myc* was not an inevitable correlate of cellular proliferation. Instead, the gene appeared to be regulated in concert with changes that affect a diversity of cellular properties, including proliferation, invasiveness, and differentiation.

[Key Words: Hybridization in situ; proto-oncogene; *myc*; N-*myc*]

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The proto-oncogenes *myc* and N-*myc* apparently arose by gene duplication. They share similar topographies (Kohl et al. 1986; Stanton et al. 1986), and their protein products have been highly conserved. In contrast, the nucleotide sequences that presumably govern transcription from the two genes differ almost entirely. It seems possible, therefore, that *myc* and N-*myc* have evolved to provide a similar biochemical function in different vertebrate cells and tissues. One strategy by which to explore this possibility is to examine the times and places at which the two genes are expressed in developing and adult organisms.

Previous work has demonstrated that the expression of *myc* is relatively widespread among different tissues (Jakobovits et al. 1985; Zimmerman et al. 1986), whereas the expression of N-*myc* appears to be more specialized. These findings were obtained primarily by examining whole embryos or tissues and offered no resolution at the cellular level. Hybridization in situ offers such resolution. In previous work, this procedure was used to examine the localized expression of either *myc* or N-*myc* at disparate times during the development of mouse and human embryos (Pfeifer-Ohlsson et al. 1985; Mugrauer et al. 1988). No coherent pattern for the expression of these genes has emerged. Now we report the use of hybridization in situ to perform a systematic study of how the expression of *myc* and N-*myc* contrasts during gastrulation of the mouse embryo. Our results

show that *myc* and N-*myc* are expressed in distinctive patterns throughout gastrulation and raise several provocative issues regarding the function of the two genes during embryogenesis. Following submission of this manuscript, Schmid et al. (1989) reported studies on the expression of *myc* in older mouse embryos. Their findings are generally in accord with ours.

Results

We analyzed the expression of *myc* and N-*myc* by hybridizing radioactively labeled DNA fragments to alternating serial sections of the gastrulating mouse embryo and its surrounding maternal implantation site. The specificity of hybridization was documented in two ways. First, we showed that a probe for α -fetoprotein hybridized under our conditions only to appropriate sites throughout the period of embryogenesis studied here. An example is shown in Figure 1, A and B, that illustrates hybridization of the probe specifically to the yolk sac of an 8.5-day embryo. Second, probes made with plasmid DNAs (pBR322 and pGem4) gave only a low level of diffuse hybridization that constituted the background for our analyses (Fig. 1C,D). We obtained the same results for prefixed, paraffin-embedded sections as we did for frozen sections. To avoid cross-reactions with more than one *myc* gene, we confirmed our findings with probes representing regions that are not conserved among the *myc* genes (see Methods).

Although our technique did not measure the number of copies of RNA in cells, we were able to assess the relative amount of RNA in different sections by using

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Figure 1. Control hybridizations. Bright-field (A) and dark-field (B) photomicrographs of a section from the same 8.5-day embryo shown in Fig. 5 hybridized with a probe for α -fetoprotein. [ys] Yolk sac. Magnification, 500 $\times$. (C) Bright-field and (D) dark-field micrographs of a transverse section from the 6.5-day embryo shown in Fig. 2 and hybridized with pGem4. The level in the egg cylinder from which this section was taken is indicated in the diagram in Fig. 2, as the horizontal line labeled T1. [pe] Primitive ectoderm. Magnification, 900 $\times$.

probes of similar specific activities, hybridizing these in saturating amounts under constant conditions, and developing all slides in a set after identical exposure times. Because cellular DNA was not denatured during the procedure, hybridization was confined to RNA in the sections. This presumption was confirmed by the failure of probes to hybridize with cells that contain numerous copies of endoreduplicated DNA (see below).

As a convenience, we describe our results in terms of gene 'expression.' However, we recognize that we have assessed only the relative levels of steady-state RNA, and that expression of the genes also may be regulated during translation. Following conventional practice, we use the term 'conceptus' to denote the entire product of the fertilized egg, and 'extraembryonic' to denote structures that are derived from the conceptus, but which ultimately contribute to formation of structures outside the embryo proper, such as the placenta. Tissues designated as 'embryonic' contribute to formation of the embryo proper.

Expression of *myc* at 6.5 days of gestation

By 6.5 days of gestation, the embryo is well-established in the maternal implantation site (Snell and Stevens 1966). Gastrulation is signaled by the appearance of the primitive streak in the midline of the embryo (Fig. 2, schematic). We examined 10 embryos at this stage for expression of *myc* and *N-myc*.

In midsagittal sections through the implantation site, we observed regionalized expression of *myc* in the maternal deciduum: The tissue that immediately surrounds the conceptus (and which is believed to be undergoing differentiation; Finn and Martin 1967) showed little or no *myc* expression, whereas the more peripheral (and still mitotic) decidual cells exhibited significant levels of *myc* RNA (Fig. 2A,B). In contrast, levels of *N-myc* RNA were invariant and relatively low throughout the deciduum (Fig. 2C,D).

RNA transcribed from *myc* was readily detectable throughout the conceptus including most of the surrounding trophoblast cells, at levels as high as any we encountered at any point in gastrulation (Fig. 2E,F). We did not detect high levels of *myc* expression in most primary giant cells, which have endoreduplicated their DNA up to 500 times (Zybina 1970; Barlow and Sherman 1972; Zybina and Grisichenko 1972; Varmuza et al. 1988), though an occasional primary giant cell expressed *myc* at high levels (Fig. 2E,F; and data compiled from complete serial sections, not shown). Because *myc* hybridization signals were not detected at high levels in most giant cells, we can be certain that there was no hybridization to chromosomal DNA in our procedure.

In contrast to the results for *myc*, *N-myc* generally was observed at low levels throughout the 6.5-day conceptus but was clearly detectable in some embryos (Fig. 2G,H). In those embryos the level of *N-myc* RNA in cells of the trophoblast appeared reduced. As it was of particular interest to determine whether *N-myc* levels were elevated in the emerging primitive streak (see below), we examined the pattern of expression for *N-myc* in cross sections where the primitive streak is more readily identified. In Figure 2, the areas marked T2 and T3 corroborate within one embryo, in cross section, the patterns of expression for *myc* and *N-myc* described above. *N-myc* expression was not elevated in the emerging primitive streak (Fig. 2, T2).

Comparison of *myc* and *N-myc* expression at 7.5 days of gestation

In the maternal decidual tissue of 7.5-day embryos, we found that expression of *myc* and *N-myc* was essentially the same as in the earlier stage (the strong signal observed for *N-myc* in the maternal blood may be caused by spurious hybridization, as probes for pBR322 and α -fetoprotein often produced hybridization signals of similar intensity in this region; data not shown).

In five embryos examined at 7.5 days of gestation, we observed a striking difference between the expression of *myc* and *N-myc* (Fig. 3). In the conceptus, levels of *myc* were highest in the extraembryonic tissues (ectopla-

Figure 2. *myc* and *N-myc* RNA in sections of 6.5-day embryos with primitive streak. (A,C,E,G) Bright-field and (B,D,F,H) dark-field photomicrographs of parasagittal sections of embryos at 6.5 days of gestation with the maternal implantation site. A,B,E,F were hybridized with the *myc* probe; C,D,G,H were hybridized with the *N-myc* probe. (A–D) Magnification, 125 $\times$; (E–H) magnification, 500 $\times$. The photomicrographs to the left of the schematic diagram show transverse sections through a single 6.5-day embryo, at levels T3 and T2 (magnification, 650 $\times$). Section T3 was hybridized with *myc*; section T2 was hybridized with *N-myc*. The schematic drawing of the 6.5-day mouse embryo was adapted from Snell and Stevens (1966) (and with permission from The Jackson Laboratory). The rose color indicates the extraembryonic structures and the yellow color indicates embryonic structures. T1 is the transverse control section shown in Fig. 1, C and D. (de) Deciduum; (em) embryo; (gc and arrowhead) giant cell; (pe) primitive ectoderm; (ps) primitive streak; (te) trophoblast.

Figure 3. *myc* and *N-myc* RNA in sagittal sections of a 7.5-day embryo. Three serial sections (A–C, 8 μ m apart) of a 7.5-day embryo hybridized with a *myc* probe (A,B) or a *N-myc* probe (C). (A,B,C) Magnification, 200 $\times$. The black box in A delineates the region in photographs B and C that are shown at higher magnification in D and E, respectively. (D,E) Magnification, 675 $\times$. [al] Allantois; [de] deciduum; [ec] ectoplacental cone; [ee] extraembryonic ectoderm; [mb] maternal blood; [pe] primitive ectoderm; [ps] primitive streak; [sgc] secondary giant cell. Arrowheads point to presumed primary giant cells.

cental cone, extraembryonic ectoderm, and allantois) and the secondary giant cells emerging from the ectoplacental cone and occasionally in presumed primary giant cells (Fig. 3B,D). All other structures in the 7.5-day conceptus exhibited lower and roughly equivalent levels of *myc* RNA. We note, in particular, that the levels of *myc* RNA were low and invariant throughout the primitive ectoderm, despite the presence of a 'proliferative zone' that contains cells with high mitotic indices (Snow 1977) and might be expected to express *myc* at high levels. Although this zone cannot be located without examination of the mitotic index in the embryonic ectoderm, we assume its presence in the serial sections examined here.

In contrast, *N-myc* expression was relatively low in the extraembryonic tissues and embryonic ectoderm, but was abundant in the primitive streak and mesoderm (Fig. 3C,E). Expression of *N-myc* was relatively low in the extraembryonic allantois, even though this tissue derives from the primitive mesoderm in which expression of *N-myc* is abundant.

To analyze more closely the difference between the primitive ectoderm and mesoderm in expression of *N-myc*, we examined an uninterrupted series of cross sections from a single late 7.5-day embryo (Fig. 4); two points emerged. First, the highest *N-myc* expression was found in the mesoderm and in occasional giant cells (Fig. 4). Little or no expression was observed in the head process (Fig. 4, T1) or in the extraembryonic structures above the amnion of the egg cylinder (data not shown). Second, there was a localized reduction of *N-myc* expression in the mesoderm. Approximately 8 μ m above the distal tip of the egg cylinder shown in Figure 4, a narrow band ($\sim$ 80 μ m in width and extending entirely around the egg cylinder) was found in which *N-myc* was not detectable (Fig. 4, T2). Paradoxically, this zone of mesoderm is the only region in the conceptus where giant cells and trophoblast showed appreciable expression of *N-myc*. The mesodermal cells throughout the egg cylinder were morphologically indistinguishable, but the differences that they displayed in expression of *N-myc* raise the possibility that the cells in dif-

Figure 4. *N-myc* RNA in transverse sections through a 7.5-day embryo. The photomicrographs to the left of the schematic diagram are transverse serial sections through a 7.5-day embryo at the levels T1-T3 shown in the diagram. As in Fig. 2, the rose color indicates extraembryonic structures and the yellow color indicates embryonic structures. T1 is $\sim 8 \mu\text{m}$ above the tip of the egg cylinder. T2 is $\sim 48 \mu\text{m}$ above T1 and T3 is $80 \mu\text{m}$ above T2. Note: In section T3, the amnion has been captured even though this is not indicated in the accompanying diagram. Magnifications [T2, T3] $475\times$; [T1] $975\times$. (am) amnion; (gc) giant cell; (hp) head process; (me) mesoderm; (pe) primitive ectoderm; (te) trophoblast; (ve) visceral endoderm.

ferent zones of the mesoderm are earmarked for different developmental fates (see Discussion).

Comparison of myc and N-myc expression at 8.5 days of gestation

By 8.5 days (4–5 somites), the maternal implantation site has probably matured fully. Expression of *myc* in this tissue no longer varied from one region to another (Fig. 5A,B). [We attribute the apparent lack of expression of *myc* in the deciduum below the embryo in this section to a coincident decrease in the density of decidual cells, readily observed at higher magnification]. We examined six embryos at the 4- to 5-somite stage and found uniform low expression of *myc* RNA throughout the 4-somite conceptus, except in chorion and giant cells (Fig. 5B,D) and allantois (data not shown).

In contrast, abundant *N-myc* RNA was regionalized in the embryonic tissues at the 4-somite stage (Fig. 5C,E,F). In particular, the neuroectoderm and the undifferentiated presomitic and cranial mesoderm of 8.5-day embryos displayed abundant expression of *N-myc*, whereas *N-myc* RNA was less abundant in the heart and somites

—both derived from the primitive mesoderm. Like the head process at 7.5 days (see above), it appears that the differentiation of primitive mesoderm is accompanied by diminished expression of *N-myc*. [The image of somites illustrated in Fig. 5C,E,F is most representative of what we have observed, but some planes of section have revealed detectable expression of *N-myc* in individual somites. We have been unable to attribute this expression to distinctive features within the somites.]

Discussion

Expression of myc and N-myc during mouse gastrulation

The proto-oncogenes *myc* and *N-myc* encode very similar proteins, but the nucleotide sequences that presumably control transcription from the two genes differ almost entirely (Kohl et al. 1986; Stanton et al. 1986). It seemed likely, therefore, that the two genes would display distinctive patterns of expression. The results reported here conform to this view (Fig. 6): RNA transcribed from *myc* was consistently abundant in ex-

Figure 5. *myc* and *N-myc* RNA in sagittal sections through an 8.5-day embryo. Serial sections of an 8.5-day embryo hybridized with a *myc* probe (A,B,D) or an *N-myc* probe (C,E,F). [D and E are 8 μ m apart.] Magnification, [A,B] 100 $\times$ or [C,D,E] 750 $\times$. The region delineated by a box in C is shown at higher magnification in E (2750 $\times$). The schematic diagram of a sagittal section through the 8.5-day embryo is from Hogan et al. (1985 reprinted with permission). (ch) Chorion; (cm) cranial mesoderm; (de) deciduum; (gc) giant cell; (ht) heart; (ne) neuroectoderm; (psm) presomitic mesoderm; (so) somites.

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Figure 6. (See facing page for legend.)

traembryonic tissues, whereas *N-myc* RNA was most abundant in the mesoderm of the conceptus. However, expression of the two genes was not mutually exclusive. For example, the primitive embryonic ectoderm displayed *myc* and *N-myc* RNA from early until midgastrulation, and most embryonic tissues displayed some expression of the two genes by 8.5 days of embryogenesis. The eventual explication of how these overlapping but distinctive patterns of expression are achieved may provide insight into the genetic program for embryogenesis.

Cellular proliferation and the expression of myc

Expression of *myc* is widely viewed as an important correlate of cellular proliferation (Cole 1986). The work reported here allows this view to be examined in a fresh context—the developing embryo and its placental precursors. The image that emerges is relatively complex. At 6.5 days of gestation, *myc* RNA is abundant throughout the conceptus. By 7.5 days of gestation, however, *myc* appears to be selectively down-regulated in the most highly proliferative tissue of the embryo, the primitive ectoderm (Snow 1977), but is expressed abundantly in placental precursors and secondary giant cells (the latter of which are invasive but not proliferating). *myc* expression also diminishes as the stromal cells of the implantation site differentiate into nonproliferative decidual cells. These findings do not conform uniformly to the view that expression of *myc* is a necessary correlate of cellular proliferation; instead, they encourage two other suggestions. First, the activity of *myc* may contribute to the invasiveness rather than, or in addition to, the proliferation of precursor placental cells. Second, the diminution of *myc* expression in decidual cells may represent not a manifestation of mitotic arrest but of cellular differentiation, much as described previously for cells in culture (Cole 1986).

Expression of N-myc and the differentiation of mesoderm

Several changes in the expression of *N-myc* occur in the embryonic mesoderm as the tissue forms and then differentiates. At the inception of mesoderm formation in the emerging primitive streak at ~6.5 days, expression of *N-myc* is negligible. However, expression becomes pronounced by 7.5 days in the undifferentiated mesoderm derived from the primitive streak and persists in the same undifferentiated tissue until at least 8.5 days of embryogenesis. As the mesoderm of the primitive streak differentiates to engender epithelioid cells of the head process, somites, and nascent heart, expression of *N-myc* diminishes. These findings raise the possibility that the activity of *N-myc* may be involved in the main-

tenance of primitive mesoderm and that it must be silenced before the mesoderm can differentiate.

In one providentially sectioned embryo, we found the 7.5-day egg cylinder girdled by an 80- μ m band of undifferentiated mesoderm within which the levels of *N-myc* were characteristically low. If these findings can be substantiated by further work, they will represent the first indication that the primitive mesoderm of the egg cylinder is in some way subspecialized. Perhaps the cells within the band have a fate distinct from those of the remaining mesoderm, or perhaps they are more advanced along the same developmental pathway.

Proto-oncogenes and embryological development

It is generally supposed that many, if not all, proto-oncogenes contribute to cellular differentiation (Bishop 1985). Perhaps the best evidence for this supposition to date has come from the both deliberate and serendipitous genetic analysis of proto-oncogenes in *Drosophila melanogaster* (Henkemeyer et al. 1987; Shilo 1987). By contrast, the evidence in mammals remains circumstantial. The data presented here add to the evidence and encourage the view that *myc* and *N-myc* may play specific and distinctive roles in murine embryogenesis. Further exploration of that view will require the use of more decisive procedures, now emerging for mammalian organisms (Frohman and Martin 1989).

Methods

Preparation of embryos

Random-bred, pregnant Swiss Webster female mice (*Mus musculus*) were purchased from Simonsen Laboratories (Gilroy, California). Embryos of the desired stage generally were removed from the maternal implantation sites by dissecting away the outer uterine muscle layers from the underlying stromal layer of the implantation sites. The sites bearing 6.5-day and 7.5-day embryos were rinsed in phosphate-buffered saline, blotted on lens paper, oriented within a capsule containing liquid embedding compound (OCT, Tissue-Tek), and frozen in a hexane-dry-ice bath. Implantation sites bearing embryos of 8.5 days contained an excess of yolk sac fluid which, upon freezing, caused formation of ice crystals and subsequent tearing of the tissue during sectioning. To eliminate this problem, implantation sites from 8.5 days were subjected to serial increasing concentrations of dimethyl sulfoxide (8, 17, 33, and 50%) in 0.1–0.15 M sodium phosphate (pH 7.3) for 10 min each, 30% sucrose for 30–60 min, and then soaked in liquid OCT for 1 hr (technique courtesy of M. Mayes, University of California, San Francisco). The sites were then frozen as described above.

Fixation and hybridization in situ

Implantation sites were sectioned serially at a thickness of 6–8 μ m at -16 to -20°C and stored at that temperature until fixed (~ 1 –3 hr). Sections were fixed on chrome alum-subbed slides at

Figure 6. Contrasting distribution of *myc* and *N-myc* RNA during gastrulation. This diagram is intended as a locator for identifying regions in which a particular combination of levels of *myc* and *N-myc* RNA are found. Expression levels were characterized as either detectable but not high (*myc* and *N-myc*), highest (abundant *myc* and abundant *N-myc*), or not detectable (no designation).

room temperature for 5 min in 4% glutaraldehyde in 0.15 M sodium phosphate (pH 7.3). The sections were rinsed in buffer containing glycine (1 mg/ml), rinsed in buffer alone, and dehydrated in graded ethanols (30–100%) for 2 min each at room temperature. Slides were stored under ethanol vapor for 1–14 days at -20°C .

Slides were hybridized with ^{32}P -labeled nick-translated probes (see below) of specific activity $1\text{--}3 \times 10^8$ cpm/ μg at a concentration of 0.05 ng/ μg in hybridization buffer (see below) for 1–3 days at $37\text{--}42^{\circ}\text{C}$. The DNA fragments were predigested with DNase I to yield an average length of 75–150 bp. Hybridization buffer consisted of 50% deionized formamide, 50 mM sodium phosphate (pH 7.0), 600 mM NaCl, 5 mM disodium EDTA, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 1 mg/ml *Escherichia coli* tRNA, 1 mg/ml sonicated salmon sperm DNA, and 20 mM dithiothreitol. After hybridization, slides were rinsed in $4 \times \text{SSC}$ (0.6 M NaCl, 0.06 M sodium citrate), soaked in $1 \times \text{SSC}$ at room temperature for 1 hr, and in $1 \times \text{SSC}$ at 37°C for 1 hr. The slides were then dehydrated twice at room temperature in 70% ethanol/ammonium acetate (20 mg/ml), 5 min each, and 95% ethanol/ammonium acetate for 5 min, and coated with emulsion (Kodak NTB-3) diluted 1 : 1 with 0.6 M ammonium acetate. Exposure times varied from 2 to 4 weeks at 4°C , but all the slides in a particular set were developed after the same period of time. Slides were soaked at $16\text{--}20^{\circ}\text{C}$ for 3 min in developer (Kodak D-19), rinsed in water for 10 sec, fixed for 3 min (Kodak Fixer), and equilibrated to room temperature in distilled water. Developed slides were then stained in hematoxylin/eosin and mounted permanently with Permount (Fisher) for analysis under the microscope (Zeiss Axiophot).

Paraffin-embedded sections were made according to the technique of Wilkinson et al. (1986). Details on this technique were communicated by A. McMahon. Hybridization to paraffin sections as well as the posthybridization procedures were as described above.

Probes

The mouse *myc* probe used was a 1.7-kb *Hind*III cDNA fragment bearing ~415 bp of exon 1, all of exon 2, and ~550 bp of exon 3 (pMcm54; Stanton et al. 1983) and was a gift from Lawrence Stanton (University of California–San Francisco, California). We also determined that exon 1 (400-bp *Bam*HI–*Xho*I fragment), which is divergent among the members of the *myc* family, gave the same pattern of hybridization as exon 3 (370 bp *Pvu*II–*Xho*I fragment) of the *myc* gene, where homology to N-*myc* has been found (both fragments were gifts from Stephanie Wright). The mouse N-*myc* probe was a generous gift from Fred Alt and Kathleen Zimmerman (Columbia University, New York) and was a 500-bp *Eco*RI fragment of the 3'-untranslated end bearing no homology to *myc* (pM2; DePinho et al. 1986; Zimmerman et al. 1986). Both probes were hybridized to mouse DNA in Southern analyses and to mRNA in Northern analyses and no cross-hybridization of either gene to the other was detected under conditions of stringent hybridization (this study; see also, Zimmerman et al. 1986; L. Stanton, pers. comm.). A 1-kb *Hind*III cDNA fragment of α -fetoprotein (pBR322-AFPI; Tilghman et al. 1979), a generous gift from Shirley Tilghman (Princeton, New Jersey), was used in all experiments as a control for specificity of hybridization: the probe hybridized only to visceral endoderm and yolk sac in the embryos. pBR322 and pGem4 vectors were used in the hybridizations to control for spurious hybridization signals due to nonspecific binding to tissues. All probes were digested with DNase I prior to nick-translation and sized to the range of 75–150 bp on a polyacrylamide gel.

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