N-myc Proto-Oncogene Expression During Organogenesis in The Developing Mouse as Revealed by In Situ Hybridization

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Abstract. The N-*myc* proto-oncogene is expressed during embryogenesis, suggesting that it plays a role in normal development. Since the *myc*-family oncogenes have been implicated in the control of cell growth, the embryonic expression may reflect rapid proliferation known to occur in development. Alternatively, N-*myc* expression may be involved in specific differentiation stages. In many embryonic tissues, early and late differentiation events occur in different locations. By in situ hybridization of tissue sections, we now demonstrate a restricted expression of N-*myc* mRNA to a few tissues and to areas where the first differentiation stages occur. N-*myc* expression was most strongly expressed in the developing kidney, hair follicles, and in various parts of the central nervous system. In these tissues, expression was restricted to a few cell lineages. In all lineages, expression was confined to early differentiation stages, and, at onset of overt differentiation, the level of expression decreased dramatically. Several rapidly proliferating tissues showed very little, if any, N-*myc* expression. In the brain, post-mitotic but not yet differentiated cells expressed high levels of N-*myc* mRNA. Therefore, N-*myc* expression is not a simple marker for proliferation in the embryo. Rather, N-*myc* expression seems to be a feature of early differentiation stages of some cell lineages in kidney, brain, and hair follicles, regardless of the proliferative status of the cell. The results raise the possibility that N-*myc* may participate in the control of these early differentiation events.

PROTO-ONCOGENES have been shown to be expressed during embryonic development (Müller et al., 1982) suggesting that a physiological role of oncogenes is to control normal development (for reviews see Bishop, 1983; Weinberg, 1985; Müller, 1986; Adamson, 1987). It is not yet clear whether the expression of oncogenes in embryonic cells is related to proliferation, to differentiation processes, or to both. Although several lines of evidence point to a role of oncogenes in cell cycle control, they may have additional roles, unrelated to proliferation, during development.

The members of the *myc*-oncogene family encode nuclear proteins believed to be important for regulation of cell growth. Three well-defined members of the *myc*-family are known to date, c-*myc* (Sheiness and Bishop, 1979; Sheiness et al., 1980; Alt et al., 1986), N-*myc* (Kohl et al., 1983; Schwab et al., 1983), and L-*myc* (Nau et al., 1985; Legouy et al., 1987). They are found in many species and the mRNA sequences have been deduced from cDNAs of both human and mouse. The known *myc* oncogenes are all expressed at high levels in the embryo (Jacobovits et al., 1985; Zimmerman et al., 1986; Sejersen et al., 1986). Cells expressing c-*myc* during embryogenesis have been identified in human embryos and mouse embryonic cerebellum by in situ hybridization techniques (Pfeifer-Ohlsson et al., 1985; Ruppert et al., 1986). There is recent evidence, however, that N-*myc* and c-*myc* expression are separately regulated (Zimmerman et al., 1986; Sejersen et al., 1987), and it has been clearly documented that the expression of N-*myc* and L-*myc* is very restricted with respect to tissue and stage in the newborn mouse, while that of c-*myc* is more generalized. Northern blotting of total RNA revealed particularly high levels of N-*myc* expression in developing brain and kidney in newborn mice (Zimmerman et al., 1986).

For the developing brain, the areas of proliferation have been characterized previously (Miale and Sidman, 1961; Hinds, 1968) and there are also data on proliferation of some cell types in the developing kidney (Ekblom et al., 1983). In the developing kidney, proliferation and differentiation occur in the same anatomical compartments, and cells seem to proliferate both during early and late differentiation. In some areas of the developing brain, however, post-mitotic but not yet differentiated cells can be found in defined locations. In such cases, the differentiation process occurs after cessation of cell proliferation and migration to the target locations (Miale and Sidman, 1961; Angevine and Sidman, 1961). Thus, these embryonic tissues can be used to study the relationship between cell proliferation, cell differentiation, and expression of the N-*myc* oncogene. We have therefore performed in situ hybridization of sections of mouse embryos with an N-*myc* probe.

Since Northern hybridization of total RNA showed strong signals in developing brain and kidney in newborn mice and

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embryos (Zimmerman et al., 1986; this work), we expected to see expression in many cell lineages in these tissues. In situ hybridization revealed, however, that N-*myc* mRNA was remarkably restricted to a few cell lineages in the developing kidney and brain. Furthermore, expression occurred only during the earliest stages of differentiation of the cells and it was shut off when overt differentiation started. An unexpected strong signal was seen in developing hair, but also here, the expression was confined to the early differentiation stages.

Materials and Methods

Embryos and Tissues

Hybrid mouse embryos 129× NMRI were used. The day of the vaginal plug was designated as day 0. To minimize RNA degradation, tissues collected for Northern blotting of total RNA were immediately frozen on dry ice after microsurgery, and care was taken to perform the microsurgery immediately after decapitation. Frozen sections from embryonic kidneys and newborn mice were prepared as described by Holland et al. (1987) except that embryonic kidneys were fixed in 4% paraformaldehyde for 1 h only.

Isolation of RNA

For preparation of total RNA, 0.5 g of frozen tissue were homogenized in 5 ml of 4 M guanidinium thiocyanate (Fluka AG, Buchs, Switzerland; purum p.a.) using a Dounce tissue homogenizer and a tight fitting S-pistill. In the presence of high amounts of chromosomal DNA, the homogenate was squeezed through a 20-gauge needle to shear the high molecular weight DNA. The RNA was then purified by ultracentrifugation (SW41 Ti rotor; 35,000 rpm, 20°C, 16 h) through a 2.1-ml cushion of 5.7 M cesium chloride (Chirgwin et al., 1979). The supernatant was removed by suction and the RNA pellet was dissolved in sterile double-distilled H_2O. Total RNA was harvested by ethanol precipitation. The concentration of the RNA was determined by reading the absorbance at 260 nm. The purity of the RNA was tested by determining the A_{260}/A_{280} ratio and by agarose gel electrophoresis.

Preparation of Labeled Probes

For nick translation, the 2.3 kb Bgl II/Eco RI fragment of the murine genomic clone p277.3 carrying parts of the second intron and the whole third exon of the N-*myc* gene (DePinho et al., 1986) was purified from a 1% agarose gel and labeled with 30–40 μCi [α-^{32}P]dCTP (6,000 Ci/mmol; Amersham-Buchler, Braunschweig, FRG) using a nick translation kit (Bethesda Research Laboratories, Bethesda, MD). The reaction was carried out in a final volume of 50 μl according to the supplier's instructions. The reaction was stopped by addition of 40 μl TNE (10 mM Tris-Cl, 10 mM NaCl, 2 mM EDTA, pH 8.0). The labeled DNA was collected by isopropanol precipitation for 15 min at room temperature (RT)[1] in the presence of 1 μg/μl carrier tRNA. After washing with 70% ethanol the pellet was redissolved in 10 mM Tris-Cl, 1 mM EDTA, pH 8.0, and heat denatured by boiling for 5 min. The probe was chilled on ice and added to the hybridization mixture. Probes were labeled to a specific activity of 3.5×10^8–1.5×10^9 cpm/μg DNA.

To prepare single-stranded RNA probes, the same N-*myc* probe used for nick translation was subcloned into pSP65 (Promega Biotec, Madison, WI) and Bluescript vectors (Stratagene, La Jolla, CA). ^{35}S-labeled single-stranded RNA probes (8.5×10^8 dpm/μg sp act) were synthesized by a modification of the method of Melton et al. (1984). Briefly, the transcription reaction was performed at 40°C for 60 min in a final volume of 20 μl containing 40 mM Tris-Cl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 2.5 mM each of ATP, CTP, GTP, 30 U RNase inhibitor (Amersham-Buchler), 1 μg template DNA, 100 μCi [α-^{35}S]UTP (>1,000 Ci/mmol; Amersham-Buchler), and 16 U SP6 RNA polymerase (Amersham-Buchler). The salt conditions for T3 RNA polymerase (Genofit, Heidelberg, FRG) reaction were 40 mM Tris-Cl, pH 8.0, 8 mM $MgCl_2$, 6 mM spermidine, and 50 mM NaCl. After in vitro transcription, 7.5 U DNase I (FPLC pure, Pharmacia Fine Chemicals, Freiburg, FRG) together with 30 U RNase inhibitor and 20 μg *Escherichia coli* tRNA (Boehringer, Mannheim, FRG) were added to remove the template DNA. After incubation for 15 min at 37°C, the RNA was extracted once with phenol/chloroform (1:1) and once with chloroform. Unincorporated nucleotides were removed by ethanol precipitation of the labeled RNA (2 M ammonium acetate, 2 vol ethanol) for 10 min on dry ice. The RNA was then redissolved in 10 mM DTT. The antisense (−) strand (i.e., complementary to N-*myc* mRNA) was synthesized using SP6 RNA polymerase after linearizing the template DNA with Hind III. The sense (+) strand was synthesized using T3 RNA polymerase after linearizing with Eco RI, and was used for control hybridization. Probe length was reduced to an average size of 100–200 nucleotides by limited alkaline hydrolysis (Cox et al., 1984) and was checked by formaldehyde-agarose gel electrophoresis (Lehrach et al., 1977). Probes were stored in 50% formamide and 10 mM DTT at −20°C.

Northern Blotting

Equal amounts of total RNA were subjected to electrophoresis on 1% agarose gels after denaturation with glyoxal (McMaster and Carmichael, 1977). After electrophoresis, the lane with the molecular weight marker (Hind III/Eco RI-cut λ-DNA) was cut from the gel and stained with ethidium bromide (5 μg/ml). Transfer to Hybond N (Amersham-Buchler) was carried out as described by the manufacturer using 20× SSC. The RNA was crosslinked to the nylon membrane by ultraviolet irridation. Glyoxalation was reversed by baking the filters under vacuum at 80°C for 2 h. Blots were prehybridized in 50% deionized formamide, 5× SET (0.75 M NaCl, 0.15 M Tris, 10 mM EDTA, pH 8.0), 2.5× Denhardt's solution, 0.5% SDS, and 0.1 mg/ml herring sperm DNA for 1–2 h at 42°C. The heat-denatured, nick translated N-*myc* probe (2.4×10^6 cpm/ml) was added to the prehybridization mixture and hybridization was performed at 42°C for 15–20 h. Filters were washed twice in 2× SSC/0.1% SDS at 42°C for 15 min, then twice in 0.1× SSC/0.1% SDS for 15 min at 42°C. Control hybridizations with the same filters were performed under the same conditions using the nick translated 1.2-kb Pst I fragment (2.7×10^6 cpm/ml) of the murine β-actin cDNA clone pAL41 (Minty et al., 1983). For removal of the probe before subsequent rehybridization, blots were washed in boiling water for ~10 min. Northern blots were exposed to Kodak X-OMAT AR x-ray films for various lengths of time at −70°C using intensifier screens. In the liver, however, the β-actin expression decreased with advancing development, and actin expression could therefore not be used to monitor the amount of RNA in this tissue. For the different stages of liver development, we therefore stained the filter for 28 and 18 S rRNA with 0.04% methylene blue (Maniatis et al., 1982).

In Situ Hybridization of Tissue Sections with Single-stranded RNA Probes

In situ hybridization was performed according to P. Holland's protocol as described in Hogan et al. (1986). Sections (8–10 μm) were cut on a cryostat (Reichert and Jung, Nussloch, FRG) at −15°C, collected on polylysine-coated slides, rapidly dried on a hot plate at 50°C for 3 min, and air-dried for 1–2 h at RT. Sections were postfixed in 4% paraformaldehyde in PBS (20 min, RT), rinsed in PBS (three times for 5 min at RT), and dehydrated by decreasing alcohol series. After air-drying, slides were stored desiccated at −20°C. Pretreatment of sections was performed as described by Hogan et al. (1986) except that the incubation step in 2× SSC at 70°C was omitted. After incubation in 0.2 M HCl (20 min, RT) to remove basic proteins, sections were treated with self-digested pronase (0.125 mg/ml) for 10 min at RT. The pronase reaction was subsequently blocked with 0.2% glycine in PBS (30 s, RT). The slides were then rinsed in PBS, fixed in 4% paraformaldehyde, acetylated, dehydrated, and air dried. Hybridization conditions were as described by Ingham et al. (1985). Probes were used at a final concentration of ~0.24 ng/μl. Posthybridization washes including RNase A digestion were performed as described (Hogan et al., 1986). For autoradiography, the slides were dipped in emulsion (K2, Ilford Ltd., Basildon, Essex, UK), diluted 1:1 with 1% glycerol in water. After air drying, slides were exposed at 4°C for 7–11 d, developed in Kodak D19 developer (2 min at 20°C), stopped in 1% acetic acid (1 min at 20°C), fixed in 30% sodium thiosulphate (5 min at 20°C), and rinsed in distilled water. Sections were stained in toluidine blue (0.02% for 30 s), dehydrated, and mounted. Photographs were taken under bright-field and dark-field illumination.

Results

Previously performed hybridization of total RNA had shown that N-*myc* mRNA was present in the mouse embryo during

1. *Abbreviation used in this paper*: RT, room temperature.

preimplantation development, in certain teratocarcinoma cell lines, and during late organogenesis of brain and kidney (Jacobovits et al., 1985; Zimmerman et al., 1986; Sejersen et al., 1986). Only a limited amount of information was, however, available for early and late prenatal organogenesis. Differentiation is asynchronous in organs, and many rather undifferentiated cells, intermixed with terminally differentiated cells, are present during late organogenesis. It was therefore unclear whether N-*myc* expression during late organ development in newborn mice reflected N-*myc* expression by cells in early or late differentiation stages. To study this issue in more detail for the solid organs, we performed Northern blotting experiments of earlier developmental stages, and in situ hybridization of tissue sections. Embryonic day 12 was selected as a starting point since one primary interest was to study kidney development. Kidneys from 12-d embryos are composed predominantly of cells in their earliest differentiation steps. Heart, lung, and liver organogensis, however, already starts somewhat earlier in embryonic development. Brain development also starts earlier but continues postnatally (for details of mouse development, see Theiler, 1972; Jacobson, 1978; Hogan et al., 1986).

RNA Blot Analysis of N-myc Expression

We assayed total RNA of several major organs (brain, kidney, heart, lung, liver) using electrophoresis and subsequent hybridization to the N-*myc* probe. In tissues from the youngest embryos studied (12-d old), abundant expression of N-*myc* mRNA was seen in the brain and kidney. A somewhat weaker expression was seen in the lung and heart (Fig. 1), and a very weak signal could be detected in the liver from the youngest embryos (Fig. 2). In the kidney, lung, heart, and liver, expression gradually decreased with advancing embryonic development, and adult tissues showed no expression. Expression seemed to remain more constant during embryonic brain development (Fig. 1), but as also shown previously (Zimmerman et al., 1986), we found that it declined during postnatal life. In adult brain, no N-*myc* mRNA was detected with the methods used. These data suggested that N-*myc* expression indeed could be characteristic for the early stages of organogenesis, but with this method it was not possible to judge whether all cells or only a few cell types in the organs expressed N-*myc* mRNA.

In Situ Localization of N-myc mRNA in Sections

In tissues which by Northern blotting showed strong N-*myc* expression, the developing brain and kidney, it was possible by in situ hybridization of sections to identify the cells expressing N-*myc*. A remarkable, stage- and cell-lineage specific expression was noted. In addition, we found a strong expression in developing hair follicles of newborn mice. In the other tissues from newborn mice, the hybridization signal was considerably weaker, and in most tissues from newborn mice, no clear signal was obtained. The expression of N-*myc* in the mouse embryo was found to be very different from that of c-*myc* in the human embryo (see Pfeifer-Ohlsson et al., 1985).

In Situ Hybridization of N-myc in Embryonic Kidneys

In the developing kidney, at least four different cell lineages are present: the ureter epithelium, the endothelium, and two different mesenchymes. The differentiation of the ureter epithelium and the endothelium is largely an ingrowth and branching process, whereas the two mesenchymal cell popu-

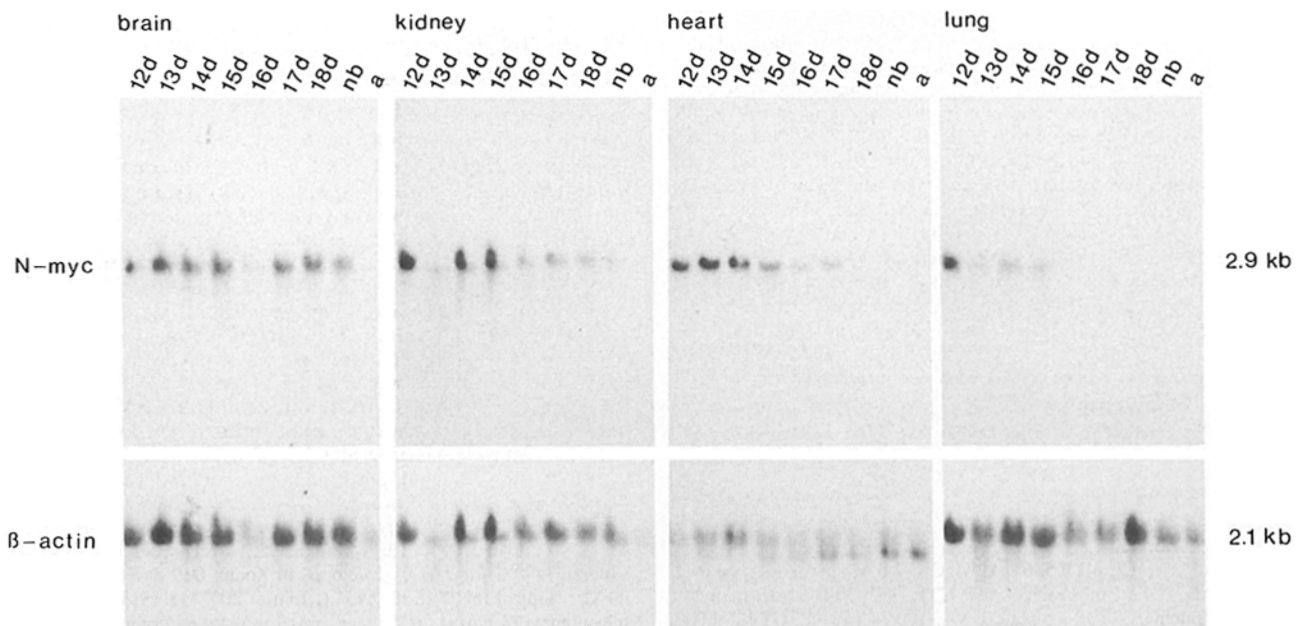

Figure 1. N-*myc* RNA expression in different tissues during murine development. Total cellular RNA (20 μg per lane) from various tissue samples isolated from days 12–18 p.c. (12–18 d), newborn (*nb*), and adult (*a*) mice was hybridized to the N-*myc* probe. To show the quality of RNA, the same filter was also hybridized to an β-actin probe. Autoradiographic exposure time was 22 h for N-*myc* (*top*) and 1.75 h for β-actin (*bottom*). Note that hybridization for actin mRNA reveals the expected shift from β-actin to α-actin during embryonic heart development.

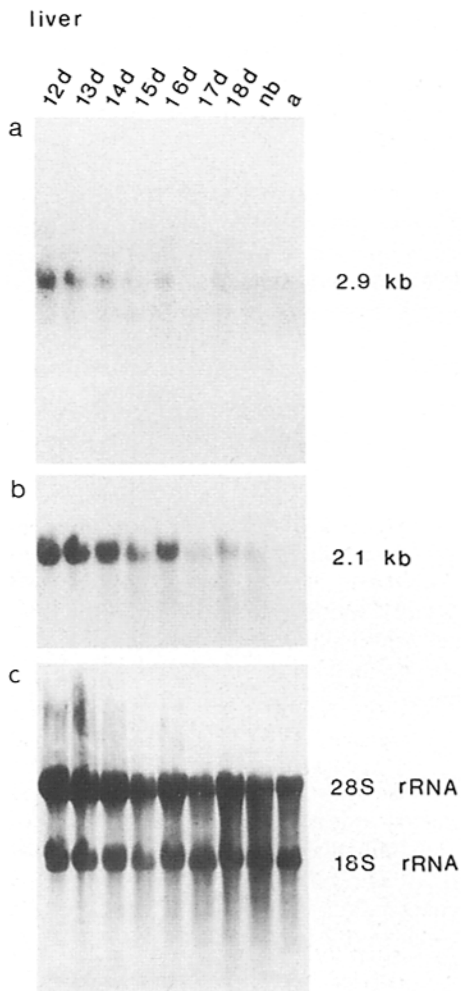

Figure 2. Northern blot analysis of N-*myc* expression in total RNA extracted from embryonic and adult mouse liver. The amount of total RNA loaded per lane was 20 μg. Total RNA was isolated from 12–18-d old embryos (*12–18 d*), newborn (*nb*), and adult (*a*) mice. (*a*) Hybridization with the N-*myc* probe. Autoradiography was for 6 d. (*b*) Control hybridization with the β-actin probe. Autoradiography was for 1.75 h. (*c*) The same filter as in *a* and *b* stained with methylene blue to visualize the 28 and 18 S rRNAs.

lations undergo dramatic morphological conversions. One part of the mesenchyme will become stroma (Aufderheide et al., 1987), and another part will convert into a new epithelium as a response to an inductive stimulus from the ureter epithelium. The early stages of differentiation of all these cell lineages occur in the cortical parts of the embryonic kidney, regardless of the age. N-*myc* expression seemed to be confined to the areas where the early differentiation steps occur, but only in one cell lineage, in the mesenchyme that converts into a new epithelium (Figs. 3 and 5). The differentiation of these mesenchymal cells in the kidney starts in the periphery of the tissues in cell clusters around the tips of the ureter epithelium (Huber, 1905; Osathanondh and Potter, 1963; Ekblom, 1981). In situ hybridization revealed that these mesenchymal cell clusters expressed N-*myc* mRNA; the N-*myc* expressing cells were located around the tips of the ureter and exclusively in the cortical parts of the tissue, regardless of the age and size of the tissue. The relative proportion of N-*myc*–positive areas was bigger in kidneys from 13-d old embryos (Fig. 3, *a* and *b*) than in those from 16-d old embryos (Fig. 3, *c* and *d*), as can be expected when expression is confined to early differentiation stages which occur only in the periphery.

The development of those mesenchymal cells which are located around the tips of the ureter is known to involve a true conversion of the mesenchyme into an epithelium. Morphologically, several stages can be distinguished during this differentiation process: condensation, comma-shape, S-shape, tubule elongation, and folding of the podocytes of the glomeruli (Fig. 4). Larger magnification of the cortical areas showed that N-*myc* mRNA was expressed exclusively during early tubule differentiation, namely in the condensates (Fig. 5, *a* and *b*) the comma- and S-shape stages (Fig. 5, *c* and *d*), but not in the subsequent stages (Fig. 5, *c–f*). Due to the continuous branching of the ureter and a proliferation of mesenchymal cells in the periphery, previously induced mesenchymal cells will be gradually displaced towards the inner parts of the kidney as development proceeds (Osathanondh and Potter, 1963). Because of this, the later differentiation events (tubule elongation and podocyte folding in the glomerulus) occur in the inner part of the developing kidneys. As can be seen both in low magnification (Fig. 3) and high magnification (Fig. 5) of 16-d–old kidneys, the inner parts, which contain the still proliferating elongating tubules and maturing glomeruli, were not expressing N-*myc* (Fig. 3, *c* and *d*; Fig. 5, *c–d*), whereas the areas which contain mesenchymal cells in their early differentiation stages around the ureter tips in the cortex were strongly positive for N-*myc* mRNA (Fig. 5, *a–d*). Hence, N-*myc* expression seems to be restricted to the precursors of one cell lineage, the mesenchymal cells which will differentiate into kidney tubules.

In Situ Hybridization of N-myc in Developing Skin

In the skin of newborn mice, N-*myc* expression could be detected in the hair follicles but it was restricted to the hair bulbs. The epidermis and the dermis were both negative for N-*myc* mRNA (Fig. 6, *a–d*). The hair bulb, the germinative zone of the hair follicle, consists of a bell-shaped mass of proliferating epithelial cells enclosing the mesenchymal dermal papilla. As soon as the cells leave this compartment, they undergo terminal differentiation. Concomitantly, the proliferation rate drastically decreases (Epstein and Maibach, 1969; Sengel, 1976). As it is known that the great majority of the hair follicles are built up during embryonic and early postnatal development (Sengel, 1976), it is not surprising that, in newborn mice, N-*myc* mRNA was present in all hair follicles. The bell-shaped mass of N-*myc* mRNA-positive cells is readily distinguishable from both the N-*myc*–negative dermal papilla and the hair shaft in sagittal sections of the developing hair (Fig. 5, *c* and *d*). Thus, in the developing skin, N-*myc* expression seems to be confined to one cell lineage, and to its earliest developmental stages.

In Situ Hybridization of N-myc in Developing Brain

Within the developing brain of newborn mice, several distinct areas with high levels of N-*myc* expression were discernible. In the cerebellum, the external granular layer showed strong N-*myc* expression (Fig. 7, *a* and *b*). This layer, the second

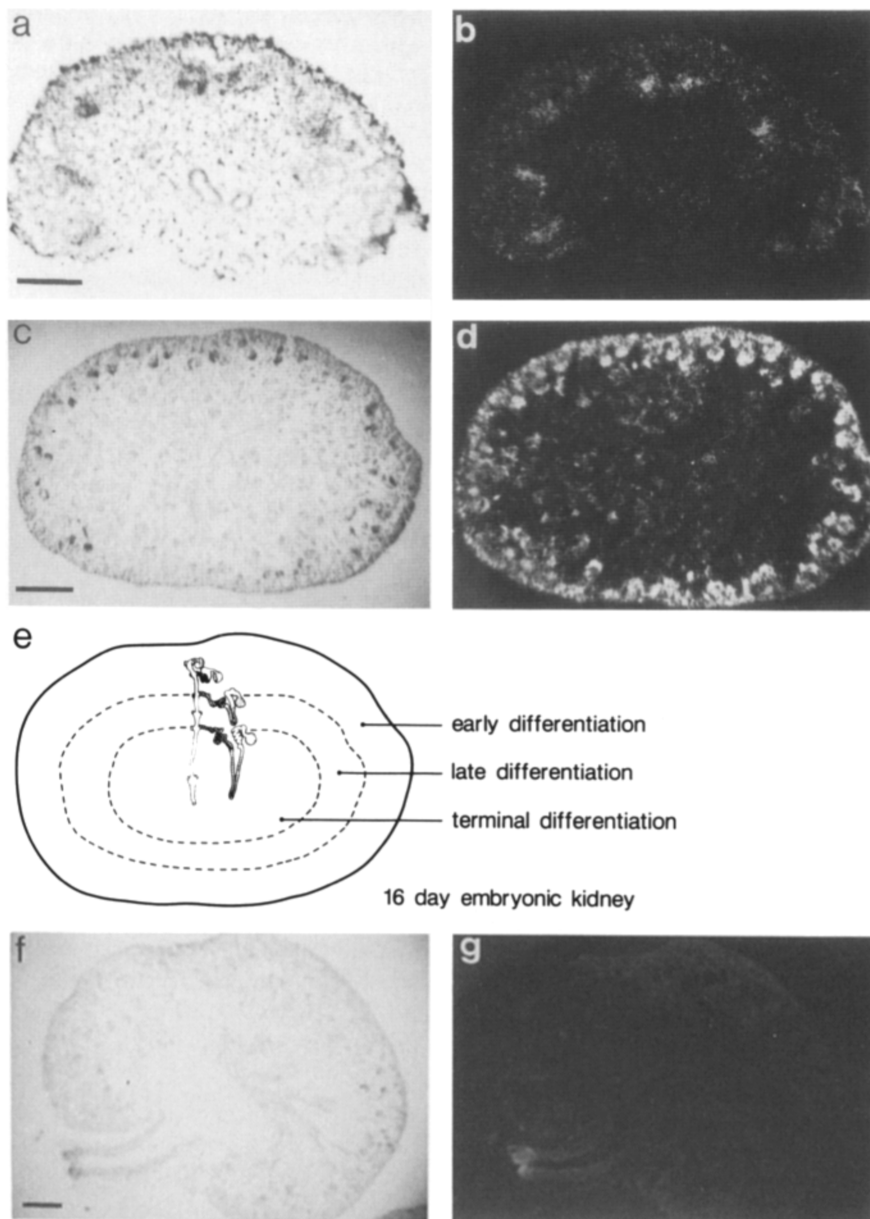

Figure 3. Expression of the proto-oncogene N-*myc* in tissue sections of the embryonic mouse kidney. Cryostat sections from frozen mouse kidneys were hybridized with antisense (−) and sense (+) single-stranded N-*myc* RNA probes. Whole 13-d mouse kidney, antisense (−) probe, bright field (*a*) and dark field (*b*) photographs. Bright field (*c*) and dark field (*d*) photography of a parasagittal section through a 16-d mouse kidney processed with the antisense (−) strand probe. Note higher density of silver grains over the nephrogenic zone compared to the inner part of the kidney. (*e*) Schematic drawing of a 16-d embryonic mouse kidney illustrating the different zones of tubule differentiation. The innermost zone also contains loose mesenchyme and the cell density is therefore lower in this region. (*f* and *g*) Autoradiograph of a midsagittal section through the same kidney as in *c* and *d* processed for in situ hybridization with the sense (+) strand probe. Bright field (*f*) and dark field (*g*) illumination. Autoradiography was for 9 d. Bars: (*a* and *b*) 100 μm; (*c*–*g*) 250 μm.

germinal layer in the cerebellum, is known to be an extremely mitotically active zone during later fetal life and the first 2 wk of postnatal murine development (Miale and Sidman, 1961). Undifferentiated neuroblasts originating from this germinal layer migrate deeper into the cerebellar cortex to their respective target layers where they terminally differentiate. One of these cortical layers, the inner granular layer, was also positive for N-*myc* although the signal intensity was much weaker. In contrast to the growth in the cerebellum, the increase in size of the neocortex and the bulbus olfactorius is due to accretion of post-mitotic neuroblasts that are generated in the respective germinal zones around the ventricles. After migration to their target layers, the neuroblasts finally differentiate into mature neurons (Miale and Sidman, 1961; Hinds, 1968; Jacobson, 1978). We found that the postmitotic but not yet differentiated cells in the neocortex (Fig. 7, *c* and *d*) and the bulbus olfactorius (Fig. 7, *e* and *f*) were expressing N-*myc*. In the bulbus olfactorius, N-*myc* expression was restricted to two of the six layers, namely to the mitral cell and the internal granular layer, which are composed of postmitotic but not terminally differentiated cells. In contrast, the three more externally located layers with differentiated cells were negative for N-*myc* (Fig. 7, *e* and *f*).

Discussion

In the embryo, overt cell differentiation is in most organs preceded by one or more distinct cell determination stages. During these stages, stem cells become progressively more determined towards certain differentiation pathways. The early determination stages can also be viewed as the first differentiation stages as opposed to terminal differentiation. In general, the determined state of a cell would not be apparent by direct inspection of any means now available but is

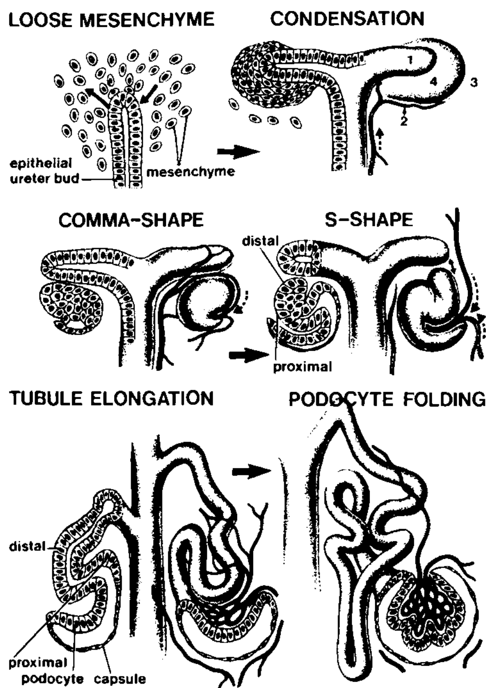

Figure 4. Scheme of kidney differentiation in vivo showing the branching of the ureter, the ingrowth of the vessels, and the conversion of the induced mesenchyme to epithelium, redrawn according to Kazimierzak (1971) and Ekblom (1984). The four different cell lineages, the ureter (*1*), the blood vessels (*2*), the uninduced mesenchyme (*3*), and the induced mesenchyme (*4*) are first seen clearly in the condensation stage. After this stage, the induced mesenchyme first condenses, develops into a comma-shaped and S-shaped structure, the tubules elongate, and simultaneously the podocytes in the glomeruli fold.

only deduced from its later behavior (Dawid and Wahli, 1979). The location of the cells which are still in these early differentiation stages has been well-defined morphologically for many organs, although very few molecular markers are available. In the present study, we show by in situ hybridization that N-*myc* expression is a feature of the earliest differentiation stages of some cell lineages in the developing brain, kidney, and hair follicles. It remains to be seen whether c-*myc* and L-*myc* expression shows a similar pattern, but Northern blotting (Zimmerman et al., 1986) suggest that each member of the *myc*-oncogene family could have a unique tissue distribution.

When sufficiently young embryonic tissues were analyzed, some N-*myc* mRNA could be detected in several other tissues as well (lung, heart, liver) and expression was, in all cases studied, strongest in the tissues from the youngest embryos. Thus, it is possible that some expression of N-*myc* could be a more general feature of early differentiation stages in the embryo. However, the cells expressing it could be assessed with certainty only in the tissues which showed exceptionally strong expression, namely in the brain, kidney, and hair follicles.

For several cell lineages, the first differentiation stages are coupled with cell proliferation, and it has been argued that the mitotic process itself is a crucial part of the differentiation of stem cells (Holzer et al., 1975; Lathja, 1979). Proliferation is a feature of early differentiation of those cells in the developing kidney, hair follicles, and cerebellum which showed a strong expression of N-*myc*. It could therefore not be distinguished whether N-*myc* expression was due to the rapid proliferation or whether it marked the early differentiation stages. In certain regions of the developing brain, however, cells stop proliferating before differentiation begins. Because of subsequent migration of the post-mitotic cells, differentiation and proliferation will occur in different locations. We could show that post-mitotic cells which were still in the early differentiation stages expressed N-*myc*. Similar findings were recently reported for human embryonic brain (Grady et al., 1987). The N-*myc* transcripts have a half-life of about 130 min (Sejersen et al., 1987), and the cells which expressed N-*myc* in the brain had been post-mitotic for more than 24 h (Hinds, 1968). Therefore, it is extremely unlikely that the N-*myc* mRNA in post-mitotic but not yet differentiated cells represents mRNA synthesized during the earlier, mitotic stages.

Many of the tissues known to continue to proliferate throughout organogenesis showed no expression of N-*myc* during late organogenesis (liver, lung). Furthermore, within those tissues showing strong expression, some rapidly proliferating cells expressed very little, if any, N-*myc*. In the developing kidney, for example, the ureter cells are known to grow rapidly, but expression was seen only in the nearby mesenchymal cells. Thus, neither N-*myc* as shown here, nor c-*myc* as previously shown (Pfeifer-Ohlsson et al., 1985; Godeau et al., 1986; Taylor et al., 1986), can be considered as a simple marker for proliferative activity during embryogenesis. The most compelling evidence that N-*myc* is associated with early cell differentiation steps rather than with cell proliferation is the fact that the post-mitotic, not yet differentiated brain cells are strongly N-*myc*-positive. In vitro cell culture experiments have also demonstrated that undifferentiated neuroblasts from neuroblastomas and pre-B cells express N-*myc*, and that decreased expression correlates with initiation of differentiation (Thiele et al., 1985; Zimmerman et al., 1986).

Taken together, current evidence suggest that N-*myc* expression in the embryo is a feature of certain stem cells which are still in the earliest differentiation stages regardless of the proliferative state of the cells. Therefore, the proto-oncogene N-*myc* can be considered as a marker for such early differentiation stages. Northern blotting experiments show that N-*myc* expression occurs also in late organogenesis (Zimmerman et al., 1986) but the in situ hybridization results establish that this is due to not yet differentiated cells which remain present in the tissues also at late organogenesis.

The identification of the cell types that express oncogenes during normal development should help us to clarify the physiological role of oncogenes. It has recently been shown for some other proto-oncogenes that they, like N-*myc*, show a remarkable tissue-specific and stage-specific expression during embryogenesis (Wilkinson et al., 1987; Shakleford and Varmus, 1987; Dony and Gruss, 1987). The expression of N-*myc* mRNA seems to be particularly interesting since it occurs during early differentiation steps and is turned off at onset of terminal differentiation. In the developing tissues which express c-*fos* mRNA, another nuclear oncogene im-

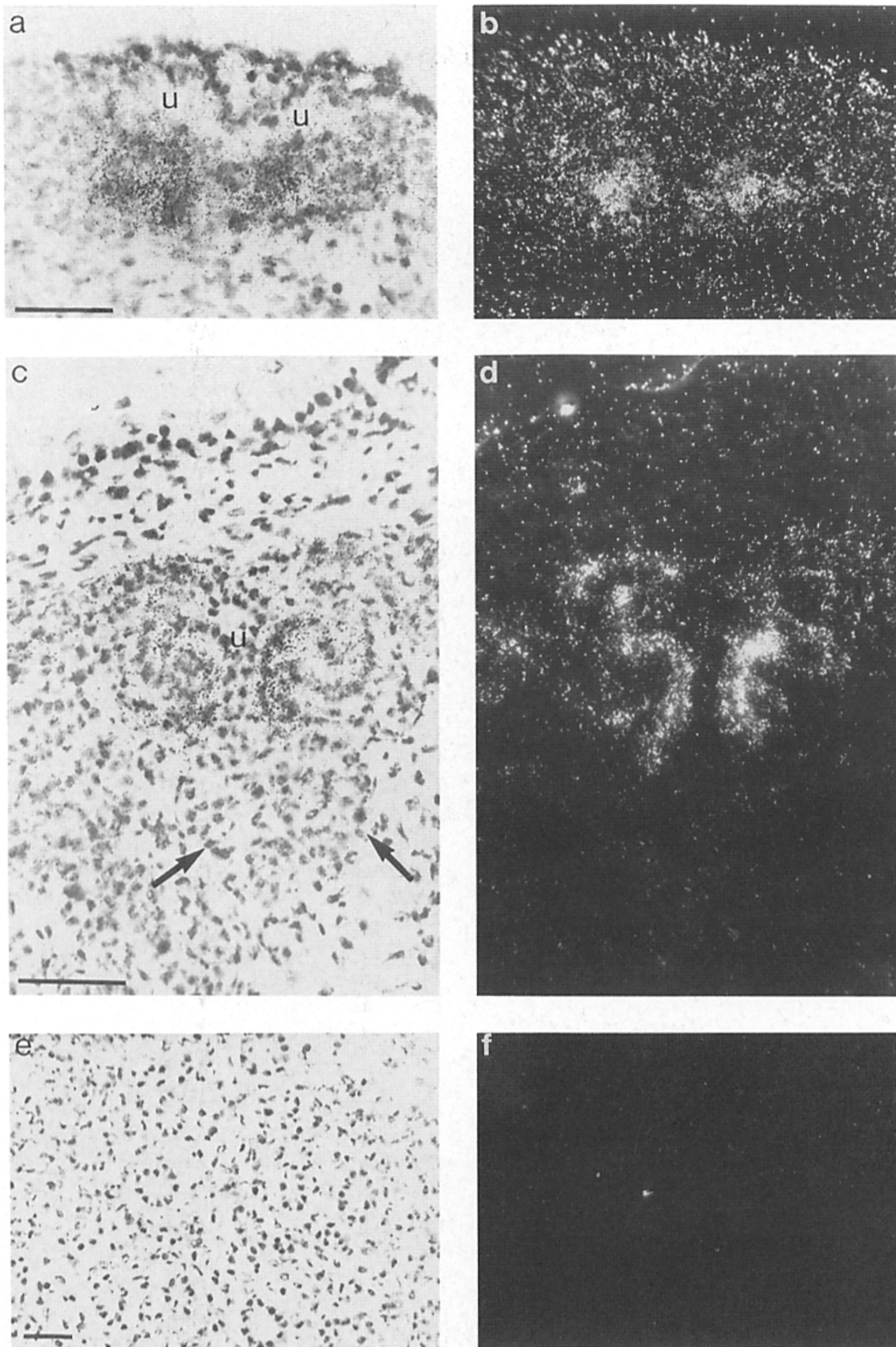

Figure 5. Analysis of N-*myc* expression with an antisense RNA-probe during the condensation of the induced mesenchyme, formation of S-shaped bodies, tubule elongation, and glomerular development in cryostat sections from mouse kidneys. Compare with scheme in Fig. 4. (*a* and *b*) Autoradiograph of the nephrogenic zone of the 13-d mouse kidney revealing the label surrounding the ureter tips. Note label only in the induced mesenchyme whereas neither the surrounding loose mesenchyme nor the ureter buds (*u*) show N-*myc* expression. Antisense (−) probe, bright field (*a*) and dark field (*b*) illumination. (*c* and *d*) Autoradiograph of a section from the cortical area reveals high density of silver grain in two S-shaped bodies found around the ureter (*u*) epithelium which shows very few grains. Note that both the renal capsule above the S-shaped tubules, and the glomerulus (*arrows*) found in the lower part of the figure show very few grains. Bright field (*c*) and dark field (*d*) illumination. (*e* and *f*) Autoradiograph of a section from the area with elongating and proliferating tubules. The density of the silver grains is low and does not differ from background levels found outside the sections. Bright field (*e*) and dark field (*f*) illumination. Autoradiography was for 11 d. For stages of nephron development, see Fig. 4. Bars, 50 μm.

plicated in growth control, the oncogene products were expressed rather late in development, and only during development of a few bones (Dony and Gruss, 1987). *Myc* and *fos* gene products have been suggested to have a direct or indirect role in regulating the expression of other genes, a proposal consistent with the fact that these oncogenes encode nuclear proteins (Slamon et al., 1984; Renz et al., 1985; Sambucetti and Curran, 1986). If the proteins were to serve as control genes in the embryo, they should be expressed during early differentiation steps rather than during late differentiation. We have here shown such a remarkable pattern for N-*myc*. The data raise the possibility that N-*myc* functions as a control gene during several early differentiation events in the developing embryo. Such functions have been directly demonstrated previously for homeobox and segmentation genes and other developmentally important control genes, which code

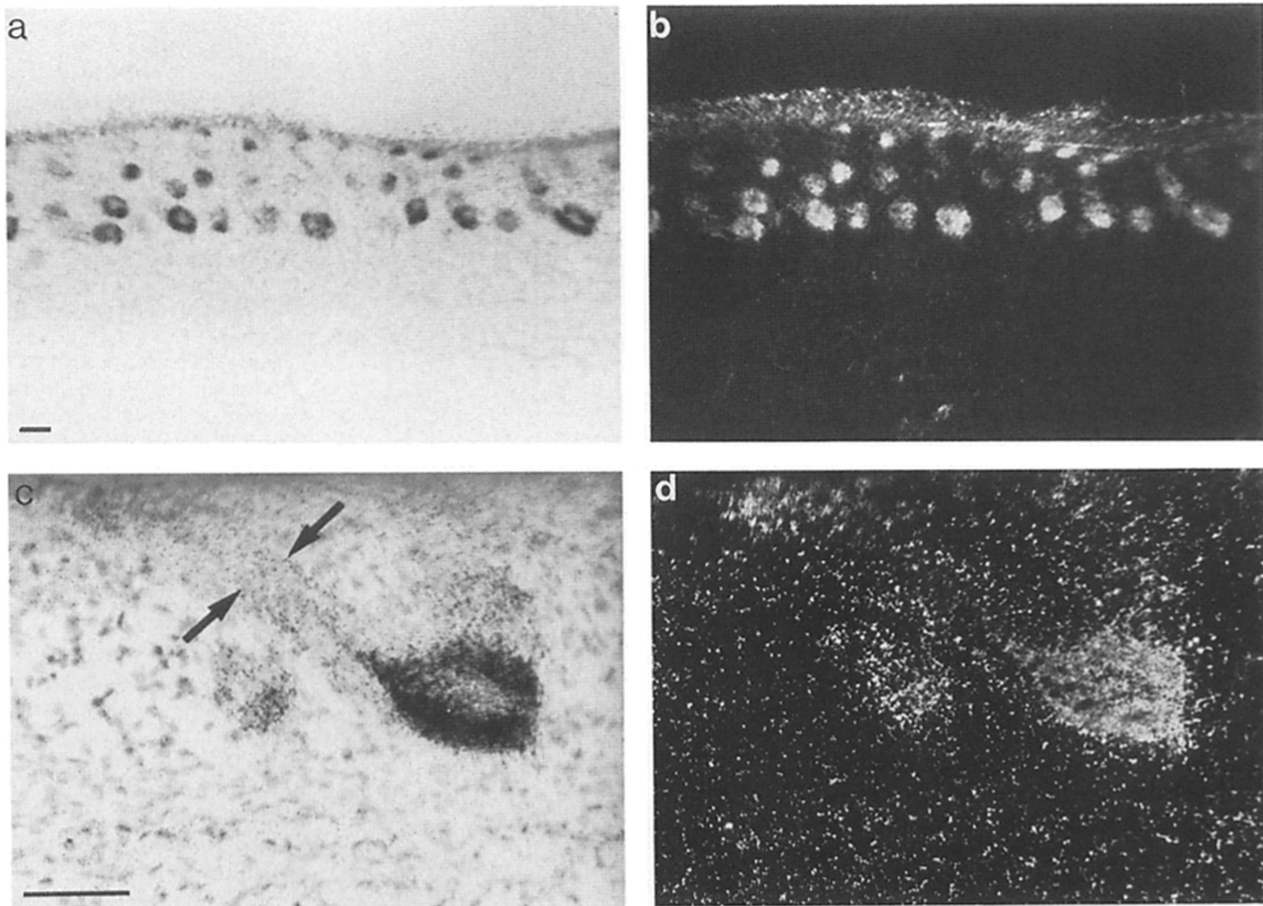

Figure 6. Localization of the N-*myc*-specific mRNA by in situ hybridization on cryostat sections of the skin region of a newborn mouse. (*a* and *b*) Autoradiograph of a section through the skin. Most of the hair follicles are cross-cut. Bright field (*a*) and dark field (*b*) illumination. (*c* and *d*) Autoradiograph of a sagittal section of a hair follicle revealing high density of silver grains in the epithelial cells of the hair bulb, but not in the hair shaft or the surrounding mesenchyme. The hair shaft is indicated by arrows. Bright field (*c*) and dark field (*d*) illumination. Autoradiography was for 9 d. Bars, 50 µm.

for nuclear proteins with DNA-binding capacity (White and Wilcox, 1984; Miller et al., 1985; Rosenberg et al., 1986). Another equally intriguing possibility is that expression of *myc* oncogenes is required to retain the cells in an undifferentiated state. It has been shown for several cell types that experimentally induced constitutive expression of c-*myc* can inhibit differentiation (Coppola and Cole, 1986; Denis et al., 1987; Lomø et al., 1987). Such an effect could be physiologically important in the embryo because it could ensure the existence of a sufficient amount of stem cells.

Because some pediatric tumors derived from nerve cells and kidney cells express high amounts of N-*myc* (Schwab et al., 1983, 1984; Nisen et al., 1986), it was important to identify the cell types that express N-*myc* during normal development. Our data may shed some light to the histogenesis of these tumors. In the Wilms' tumor, the childhood kidney cancer derived from developing kidney cells, high levels of N-*myc* expression have been reported. It is interesting that there is no amplification of the gene in Wilms' tumor as in other tumors with an elevated N-*myc* expression (Nisen et al., 1986). This could be due to a large number of determined but yet undifferentiated mesenchymal cells in Wilms' tumor. Differences in the extent of N-*myc* expression between different Wilms' tumor samples (Nisen et al., 1986) may simply be due to the varying amounts of the mesenchymal cells which fail to differentiate further. In situ hybridization studies on the expression of the N-*myc* oncogene in these tumors, brain, and skin tumors, should help to clarify these issues. We anticipate that analysis of N-*myc* mRNA in tissue sections with single-stranded antisense probes in combination with Northern blotting experiments will be useful in diagnostic histopathology.

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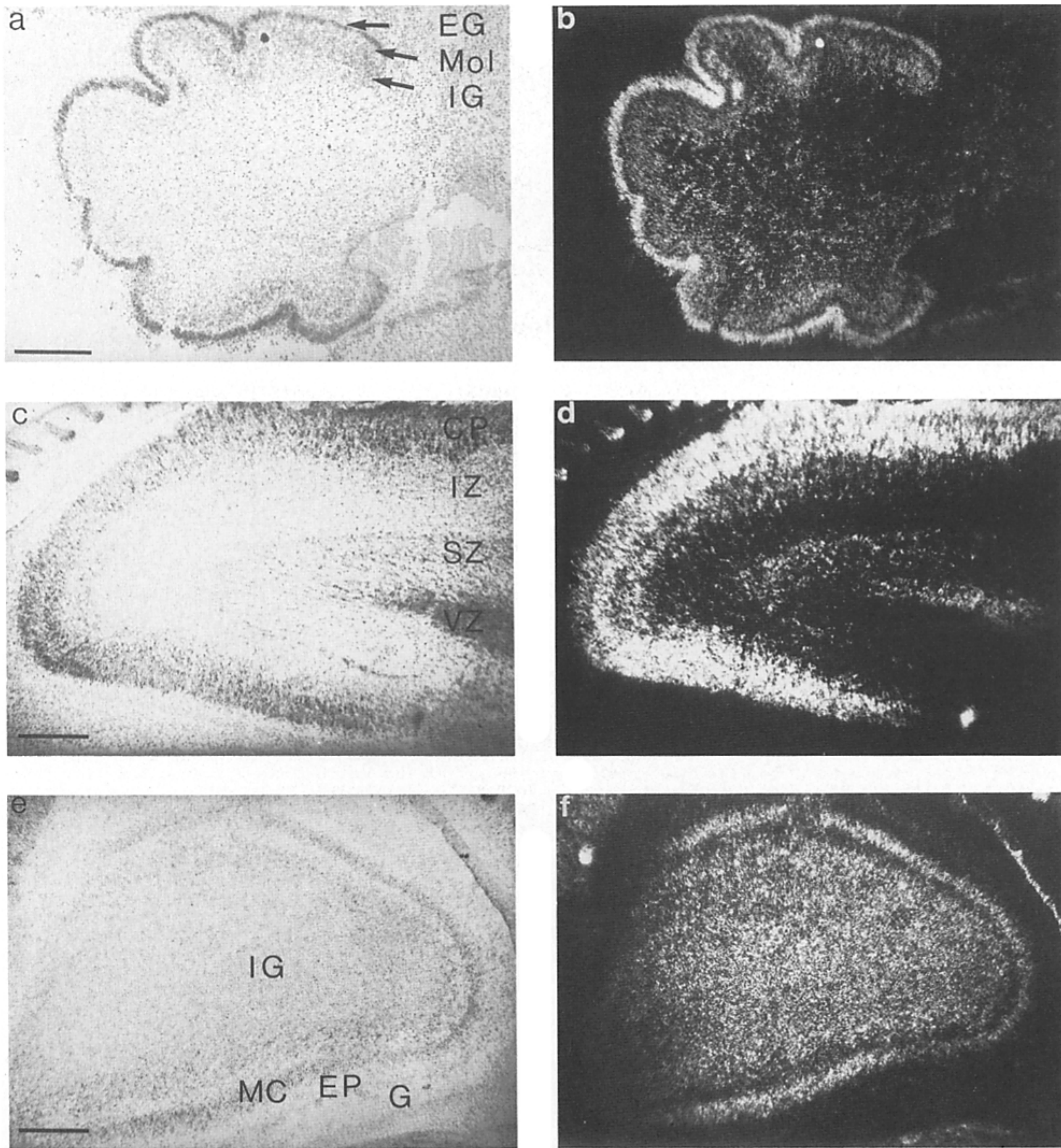

Figure 7. In situ hybridization with antisense N-*myc* RNA to sagittal sections of different areas of the brain of a newborn mouse. (*a* and *b*) N-*myc* mRNA distribution in the cerebellum of a newborn mouse. High density of silver grains are seen in the external granular layer (*EG*). The internal granular layer (*IG*) also exhibits N-*myc* expression although to a lesser degree, whereas the molecular layer (*Mol*) is devoid of silver grains. Photographed under bright field (*a*) and dark field (*b*) illumination. (*c* and *d*) Autoradiograph of a section of the neocortex of the newborn mouse. Label is evenly distributed throughout the cortical plate. The patchy label in the upper left corner, outside the brain, represents the strong signals in the hair bulbs. Photographs were taken under bright field (*c*) and dark field (*d*) illumination. Layers: (*CP*) cortical plate; (*IZ*) intermediate zone; (*SZ*) subventricular zone; (*VZ*) ventricular zone. (*e* and *f*) Localization of the N-*myc* mRNA in a section of the bulbus olfactorius. High grain density is present in the mitral cell layer and throughout the internal granular layer. Bright field (*e*) and dark field (*f*) illumination. Layers: (*IG*) internal granular layer; (*MC*) mitral cell layer; (*EP*) external plexiform layer; (*G*) glomerular layer. The innermost subventricular layer and the outermost layer of olfactory nerve fibers are not present in this section. (See Hinds, 1968; nomenclature of the different cells in the respective layers). Autoradiography was for 9 d. Bars, 200 μm.

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