Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 5050–5054, August 1985
Developmental Biology

Cell-type-specific pattern of *myc* protooncogene expression in developing human embryos

(embryogenesis/*in situ* hybridization/epithelial cells/developmental control)

SUSAN PFEIFER-OHLSSON*, JAN RYDNERT†, ANTON SCOTT GOUSTIN‡, ERIK LARSSON§, CHRISTER BETSHOLTZ§, AND ROLF OHLSSON*¶

*Department of Oncology, †Department of Gynecology, ‡Unit of Applied Cell and Molecular Biology, University of Umeå, S-901 87 Umeå, Sweden; and §Department of Pathology, University of Uppsala, S-751 85 Uppsala, Sweden

Communicated by Tomas Hökfelt, April 8, 1985

ABSTRACT **The expression of viral oncogenes in cells transformed by acutely transforming retroviruses profoundly alters proliferation and differentiation in the target cell, suggesting that the cellular homologues of the viral oncogenes, the protooncogenes, have a role in normal cell proliferation and differentiation. To investigate the possible developmental role of protooncogenes in human embryogenesis, we have determined the spatial distribution of *myc* gene transcripts in early human embryos by using *in situ* hybridization of a labeled *myc* exon to thin sections. The results indicate a stage- and cell-type-specific regulation of c-*myc* gene expression in primarily epithelial cells of late first trimester embryos. Furthermore, the data suggest that the linkage between c-*myc* gene expression and cellular proliferation holds for only a restricted set of embryonic cells.**

A role for protooncogenes in the growth and differentiation of normal cells is implied by the action of viral oncogenes in retroviral transformation (1–4). Several lines of evidence support this view, including the identification of genes coding for platelet-derived growth factor and the epidermal growth factor receptor as the *sis* and *erbB* protooncogenes, respectively (5–7). The developing embryo may provide a suitable model system in which to establish the role for various cellular oncogenes in the control of normal proliferative and differentiation processes. A few studies have demonstrated a temporal course of protooncogene expression in embryonic tissues of both mouse and human (8, 9). The c-*fms* and c-*fos* oncogenes, for example, appear to display a stage- and cell-type-specific pattern of expression correlated to the progression of mouse embryogenesis (8).

The c-*myc* oncogene is apparently a key correlate of normal cell proliferation. One event common to the mitogenic activation of mouse T and B lymphocytes and mouse fibroblasts is the transcriptional activation of the c-*myc* gene in the early G_1 phase (10–13). The finding that c-*myc* gene expression in early human placenta parallels the proliferative activity of the cytotrophoblasts further strengthens the evidence linking c-*myc* gene expression to the control of cell proliferation *in vivo* (14). Moreover, evidence from the mouse leukemic cell line WEHI-3B suggests that the linkage between the proliferative state and c-*myc* gene expression may extend to myeloid cells as well (15).

Were the abundant presence of c-*myc* gene products necessary for the proliferation of all normal cells, we would predict that most or all proliferative cells of the human embryo should display significant levels of *myc* transcripts during some part of their cell cycle. We have surveyed the spatial distribution of transcriptionally active c-*myc* oncogenes in sectioned human embryos and fetuses, staged between 3 and 10 weeks after conception‖, through the application of *in situ* and blot hybridization techniques. The results presented here add support to a role for active c-*myc* genes in cellular proliferation, although this linkage to proliferation appears to be restricted to only a subset of embryonic cells.

MATERIALS AND METHODS

Tissue Samples and RNA Extraction. Embryonic and placental tissue was obtained from routine elective abortion at the Department of Gynecology, Umeå University Hospital. Gestational age was estimated by ultrasound analysis as well as patient history. Recovered patient materials were dissected without delay and frozen at −80°C for subsequent RNA extraction (16) or fixed in 4% (wt/vol) paraformaldehyde for subsequent histological analysis (14).

Hybridization Analysis. The probe used in this study was the 1.4-kilobase *Cla* I/*Eco*RI fragment excised from the plasmid pMC.41.3RC, a subclone of the third exon of the human c-*myc* oncogene (17). Electrophoresis and transfer of total embryonic RNA and subsequent medium-stringency hybridization were performed as described (14, 18). *In situ* hybridization was performed on 4-μm paraffin sections of formalin-fixed human embryos (staged 21–50 days postconception), with ^{125}I-labeled c-*myc* or pBR322 DNA probes, prepared by nick-translation using ^{125}I-labeled dCTP (Amersham) as precursor (14, 19). For autoradiography, stripping film (Kodak AR10) was used with exposure for 1–2 weeks at 4°C. After development and fixation, the underlying tissue was May–Grünwald stained to allow histological identification.

RESULTS

Analysis of c-*myc* Transcripts in Extracted Embryonic RNA. Proper assessment of c-*myc* transcript abundance in individual human embryos was limited, for ethical and practical reasons, to early stages of pregnancy (8–11 weeks gestational age). Equal amounts of RNA extracted from human placentas and embryos were analyzed for the presence of c-*myc* transcripts by blot hybridization analysis using the third exon of the human c-*myc* gene as a ^{32}P-labeled probe. As shown in Fig. 1*a*, embryos of late first trimester pregnancies display more abundant c-*myc* transcripts than the corresponding

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¶To whom reprint requests should be addressed.

‖We have found it convenient to use the term "embryo" throughout most of the paper, even though some of the staged samples are older than 9 weeks postconception, where the term "fetus" is strictly proper. We attempt to use the embryological term "weeks postconception" throughout this work, except where clinical data only allows an estimate of "gestational age" (weeks since last appearance of menstruation).

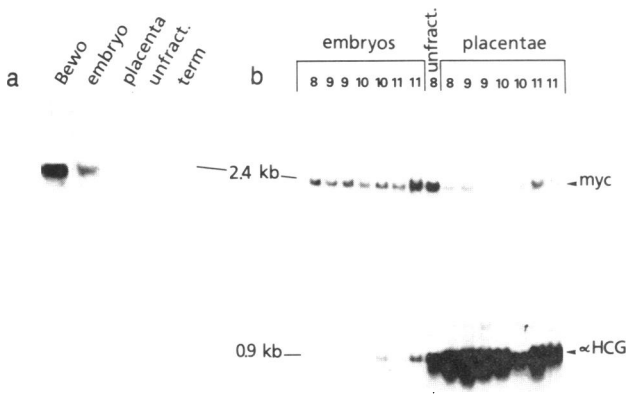

FIG. 1. Expression of the c-*myc* gene during human development. Total RNA, extracted from staged human embryos and corresponding extra-embryonic tissues, was subjected to blot analysis (18) using a ^{32}P-labeled human c-*myc* third-exon probe. (*a*) Relative *myc* transcript abundance in identical amounts of total RNA (30 μg per lane) of embryo, placenta, and unfractionated tissue from a pregnancy of 11 weeks (gestational age) as well as RNA from the choriocarcinoma cell line (BeWo) and RNA from term placenta (term). (*b*) Levels of *myc* transcripts (10 μg of total RNA per lane) in developing human embryos and the corresponding placentas. The gestational age in weeks is given above each lane. For the 8-week sample, RNA from unfractionated (unfract.) material is shown in addition to RNA from pure placenta. The presence of transcripts related to the α-subunit of human chorionic gonadotropin (α-HCG) was detected by a second hybridization using a ^{32}P-labeled cDNA clone. kb, Kilobases.

placentas of the same pregnancy. In comparison with steady-state levels of *myc* RNA of a choriocarcinoma cell line, BeWo, which are on par with those of the early placenta (14), embryonic levels are lower by a factor of 3–4, based upon total cellular RNA input (Fig. 1*a*). The relatively high levels of c-*myc* transcripts could reflect the proliferative capacity of the multipotent embryonic cells. This stage of embryogenesis is characterized by dramatic growth and tissue remodeling, even though the main organ systems have already been laid down (20). However, the *myc* transcript abundances in whole embryos do not vary extensively during the latter part of the first trimester (Fig. 1*b*). Thus, a sustained c-*myc* gene expression in late first trimester embryos contrasts with the decrease in c-*myc* gene expression in placenta during the second month of the first trimester (14). Transcripts hybridizing to cDNA for the α-subunit of human chorionic gonadotropin are most abundant in placental samples (Fig. 1*b*). These data underline the differential control of c-*myc* and chorionic gonadotropin α-subunit gene expression in the embryonic and extra-embryonic cells, respectively.

***In Situ* Localization of Transcriptionally Active *myc* Protooncogenes in Thin Sections of Whole Human Embryos.** The ^{125}I-labeled probe containing a major portion of the third exon of the human c-*myc* gene was hybridized to the tissue sections and excess probe was washed away as described (14). After autoradiography and histological staining, the occurrence of transcriptionally active c-*myc* genes can be visualized by the pattern of silver grains associated with various histological structures. The low hybridization signal detected in the 3-week embryo (a portion of which is shown in Fig. 2*a*) indicates that *myc* gene expression is low in very early human embryos. Corresponding placental tissues of the same section display a very strong *myc* hybridization signal (*cf.* figure 4 in ref. 14), ruling out possible artifacts, including the loss of probe-accessible RNA. However, older embryos (6–10 weeks postconception) display abundant *myc* RNA, primarily in epithelial cell layers (Fig. 2 *c–j*), despite declining *myc* RNA levels in the corresponding placentas (Fig. 1 and ref. 14). An adjacent section taken from the 6-week embryo was hybridized to an ^{125}I-labeled pBR322 probe as a control (Fig. 2*b*).

In older embryos/fetuses (7–8 weeks after conception), the pattern of c-*myc* gene expression appears to be quite similar to that of the 6-week embryo. In addition to parts of the connective tissue, epithelial cell layers in both skin and gut represent the major cell type showing a very active c-*myc* gene (Fig. 2 *c–e*). The expression is particularly high in the skin epithelia in the region of the mouth (Fig. 2 *h–j*), which may correlate with the dramatic remodeling and growth of the embryonic facial skin at this stage of development (20). Note that in the part of the section corresponding to early stages of embryonic tooth-bud development (Fig. 2 *h* and *i*, arrow), the relatively low *myc* gene expression in the ameloblasts as well as the enamel organ itself correlates with a more differentiated morphology, even though these cells are proliferating at this stage (20).

A magnification of this region further reveals that c-*myc* gene activity is associated with the proliferative germinal cell layers of the skin (Fig. 2*j*). Similarly, the germinal epithelium of the gut displays abundant c-*myc* transcripts (Fig. 2 *d* and *e*). The *myc* gene activity may therefore reflect the proliferative potential of these structures, where one encounters a highly proliferative basal layer (for example, the stratum germinativum of skin) underlying a less proliferative intermediate cell layer (20).

A summary of the *in situ* hybridization results is shown in Table 1. A number of tissues displaying a major epithelial component also display abundant *myc* transcripts. Embryonic cartilage, heart, and liver display generally low levels of c-*myc* gene activity, although we do find occasional cells in the liver that show abundant *myc* transcripts; these *myc*-positive cells can be found lining the endothelial tissue of blood sinuses in the liver (data not shown).

DISCUSSION

The activation of the c-*myc* gene in platelet-derived growth factor-stimulated mouse fibroblasts (10, 11, 13), Con A- or bacterial lipopolysaccaride-stimulated mouse T and B lymphocytes (10, 12), and growth factor-stimulated human cytotrophoblasts (21) occurs at a point early in the G_1 phase of the cell cycle, suggesting that the *myc* gene product is a key correlate to growth stimulation in a variety of cell types. One generalization drawn from these data is that the *myc* gene product functions as a central courier in the cell proliferation process; we would therefore expect that most cells of the

Table 1. Distribution of c-*myc* transcripts in human embryos

Tissue type	*myc* RNA steady-state levels per cell	% *myc*-positive cells in whole tissue*
Cartilage	−/+	<1
Brain	−/++	>20
Intestine	−/++++	>30
Kidney	−/++	>20
Liver	−/++	<2
Lung	−/++	>20
Skin	−/++++++	>30
Connective	−/+++	<5
Retina	−/+	<1

The data are based on *in situ* hybridization analyses of human embryos staged 7–10 weeks postconception. The fluctuations of the *myc* RNA steady-state levels per cell are estimated from the over-all distribution of silver grains over background in thin sections of whole tissues.

*Where *myc*-positive cells contain concentrations of silver grains at least 3-fold higher (+) than nonspecific background levels in histological details.

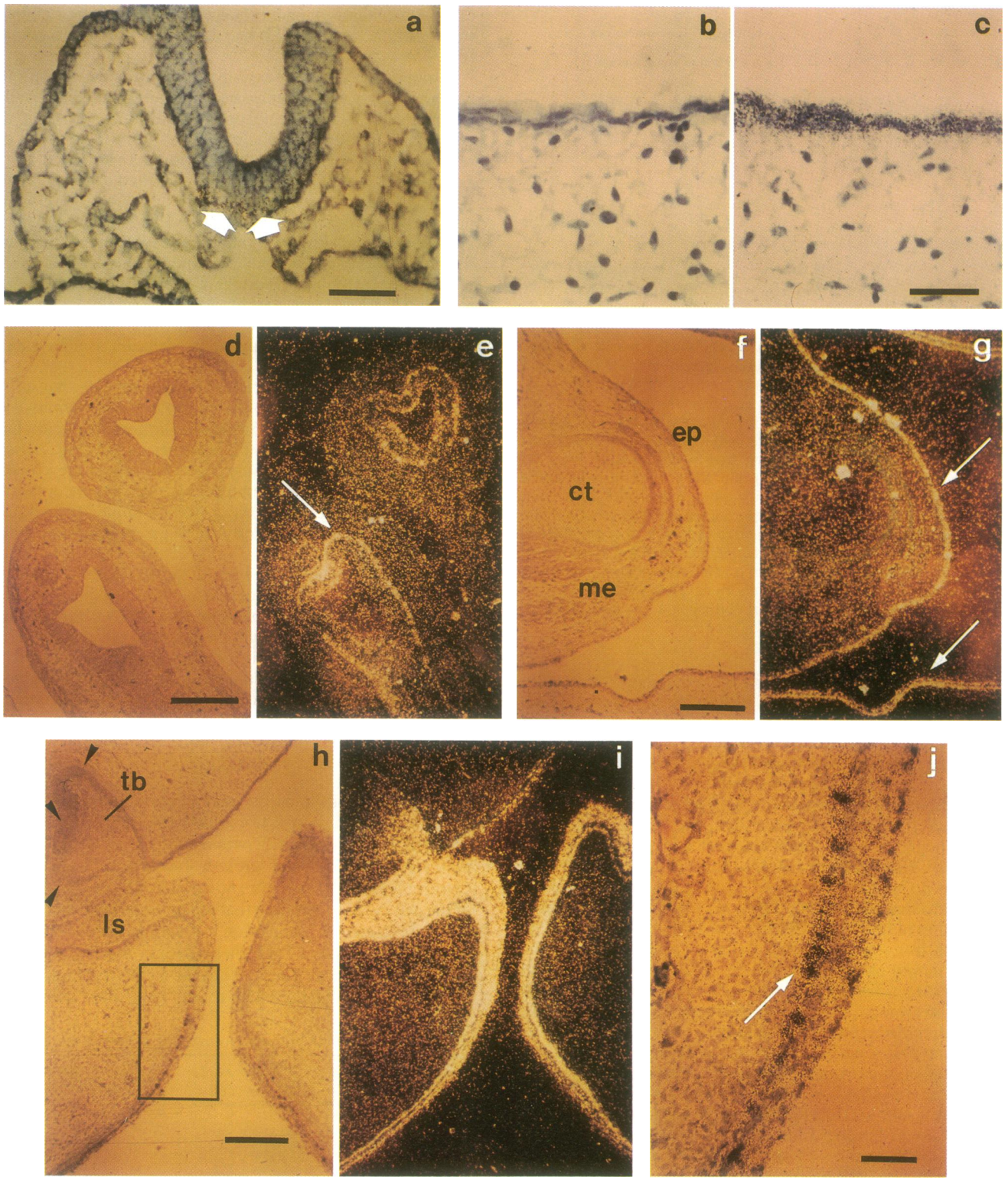

FIG. 2. Spatial distribution of c-*myc* transcripts as revealed by *in situ* hybridization of ^{125}I-labeled probe to thin sections of human embryos. All stages of embryogenesis are given as time from conception. (*a*) A 3-week embryo, showing active c-*myc* gene expression in cells at the base of the neural fold (arrows). (*b* and *c*) pBR 322 control and c-*myc* probes, respectively, hybridized to skin of a 6-week embryo. (*d* and *e*) Bright- and dark-field views of developing intestine of a 7-week embryo showing c-*myc* gene activity in both basal (arrow) and luminal epithelial layers. (*f* and *g*) Bright- and dark-field views of a 7-week embryo in the region of the anterior limb bud; c-*myc* gene activation is confined to the epithelia (ep, arrows), despite the proliferative nature of the stromal mesenchyme (me) and cartilaginous (ct) masses. (*h* and *i*) A 7-week embryo in the region of the future mouth, including a tooth bud (tb); note the absence of hybridization signal in the more interior regions of the tooth bud containing ameloblasts (arrowheads). ls, Lip sulcus. (*j*) An enlargement of the boxed region in *h* showing a magnification of a region of epithelia of the mouth region. Note the trilaminar distribution of silver-grain density, in particular in *i*, reminiscent of the trilaminar make-up of embryonic skin characteristic at this stage (20). Bars correspond to 40 μm in *a–c* and *j* and to 100 μm in *d–i*.

rapidly growing early embryo should harbor significant amounts of c-*myc* transcripts.

In the early human placenta, a tissue displaying a broad range of cellular proliferative activity, c-*myc* gene expression

is confined to the most proliferative cytotrophoblasts (14). Like the placenta, early human embryos actively express the *myc* protooncogene, yielding the canonical 2.4-kilobase transcript (Fig. 1). Because of the more polymorphic and multipotent nature of the embryo as compared to the extra-embryonic tissues, it was of considerable interest to assay individual cells for their level of *myc* transcripts. Using *in situ* hybridization, we have here shown that the *myc* gene is actively expressed in only a subset of human embryonic cells. This is surprising, since the entire embryo is, in fact, growing at a remarkable rate, the mass increasing by several orders of magnitude between the third and ninth week after conception. Despite this high proliferative activity, embryos staged 3–4 weeks following conception display low levels of active c-*myc* genes, although the corresponding extra-embryonic tissues of the very same section display very active c-*myc* genes (*cf.* figure 4 of ref. 14). A higher level of c-*myc* transcripts is apparent in embryos 5–10 weeks following conception, where the *myc* transcripts are most detectable in the proliferative germinal cells of the skin and the gut epithelia as well as parts of connective tissue. However, most other cell types do not display abundant *myc* transcripts, suggesting that embryonic cellular proliferation is not always mediated by *myc* gene products. These inferences rely on the assumption that most embryonic cells proliferate at these stages of embryogenesis, albeit at different rates. Quantitative data on the dynamics of cycling cells in the early human embryo, necessary for a direct comparison between *myc* transcription and proliferative rate, are not available. Nonetheless, it should be noted that, for example, the embryonic mesenchyme and cartilage cell masses of the limb bud and head grow rapidly at this stage but exhibit little or no *myc* gene transcripts, in contrast to the limb-bud and head epithelia (*cf.* Fig. 2 *g* and *i*).

We argue from these data that c-*myc* gene activity is not a simple marker of proliferative activity but reflects additional tissue-specific gene regulation operating during human embryogenesis. This notion suggests that the pivotal step committing these early embryonic cells to DNA synthesis (22) cannot simply be the result of the appearance of c-*myc* products. Recently, Stewart *et al.* (23) have produced several strains of transgeneic mice, carrying a transfected murine mammary tumor virus promoter-*myc* conjugate, which all appear to display a normal pattern of development (23). Although 2 of 13 different mouse strains established produced adenocarcinomas during their second or third pregnancies, the expression of the fusion *myc* gene in some (and in one case all) of the tissues examined, suggests that high-level c-*myc* gene expression is not sufficient to grossly derange the timing and pattern of cell proliferation during development. This surprising result suggests that one or more additional and complementary properties are needed to potentiate the excessively active c-*myc* gene and so derange coarse controls on cellular growth. Evidence for such a situation has recently been described for resting human B cells, in which the specific activation of the c-*myc* gene by anti-immunoglobulins only renders the activated B cells competent to respond to B-cell growth factor; hence the presence of abundant *myc* transcripts is not *per se* sufficient to promote B-cell proliferation in the absence of additional signals (24).

The discontinuous distribution of *myc*-positive cells seen in some sections (Fig. 2*j*) suggests that these cells do not express the *myc* gene in synchrony with the surrounding field of neighboring cells. We have found several examples of epithelial cell layers that display relatively sharp zones, in which one side is very active in c-*myc* gene transcription while the other side is essentially inactive (Fig. 2 *i* and *j*, as well as data not shown). A cell cycle-specific appearance of transcriptionally active c-*myc* genes, coupled to a rapid decay of *myc* mRNA, may provide an explanation of this discontinuous, apparently unsynchronous distribution of *myc*-positive cells in epithelial layers (see also ref. 14). If it is assumed that *myc* mRNA decays at the same rate in all embryonic cells, transcription of the *myc* protooncogene would appear to be under cell type-specific and developmental controls.

We have provided evidence for developmental control of the pattern of embryonic c-*myc* gene expression during the first trimester of pregnancy. Moreover, the temporal control of expression of the embryonic and extra-embryonic c-*myc* genes differ markedly during the first trimester. Such a situation could result from interactions between many different cellular events, including localized synthesis of growth factors or growth factor receptors or perhaps localized *trans*-activation of tissue-specific enhancer elements within the *myc* locus. Both explanations push back the control of c-*myc* gene expression another step. The ultimate control over these regulatory genes may result from a differentiation program internal to the cells, or from external physiological signals, or both. Moreover, since our results are incompatible with a general role for the active c-*myc* gene in all proliferative cells, we speculate that the activated cells have options in which proliferative pathway to choose. The recent findings that the adenovirus *E1a* gene (25) and the c-*myb gene* (26) can substitute for c-*myc* (27) in one step of the transformation process may signify that the c-*myb* gene or other putative cellular analogs of *E1a* (26, 28) can replace the *myc*-specific function in these *myc*-negative embryonic cells.

pMC.41.3RC, a plasmid subclone of the third exon of the human c-*myc* gene, and the plasmid cDNA clone of human chorionic gonadotropin subunit α mRNA were kindly provided by Drs. R. C. Gallo and H. C. Fiddes, respectively. This work was supported by grants from the Gustav V Jubileums Foundation of Sweden (Lions Project 305/83 and 355/84) and the Swedish Cancer Research Society (RmC, Project 1955).

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