Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 9585–9589, December 1988
Cell Biology

Sustained expression of the human protooncogene *MYCN* rescues rat embryo cells from senescence

(neuroblastoma/tumor progression/intercellular communication)

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Contributed by J. Michael Bishop, September 16, 1988

ABSTRACT **Amplification of the human gene *MYCN* may play a role in the malignant progression of human neuroblastomas. In pursuit of this possibility, previous studies have shown that the abundant expression of *MYCN* in cultured cells can elicit several aspects of the transformed phenotype. We now extend those findings by demonstrating that rat embryo cells transfected with *MYCN* can proliferate for at least 200 generations. Isolation of established cells was dependent on high expression of *MYCN* and on biological selection to eliminate untransfected cells. The established cells were not tumorigenic in syngeneic rats or athymic mice, failed to grow in soft agar, and required relatively high concentrations of serum for proliferation in culture. Our results show that enhanced expression of *MYCN* can rescue normal cells from senescence, add to the credentials of *MYCN* as an authentic protooncogene, and identify an additional biological activity that can be used in the characterization of *MYCN*.**

MYCN was discovered during surveys of human neuroblastoma cells containing karyotypic manifestations of amplified cellular DNA (1, 2). The gene is amplified most commonly in human neuroblastomas, but also in some specimens from small-cell lung cancers (3), retinoblastomas (4) and astrocytomas (5). These findings prompted the suggestion that enhanced expression of *MYCN* might contribute to malignant progression of certain neuroectodermal tumors (6). The most consistent picture has emerged for neuroblastoma, in which amplification of *MYCN* is associated with roughly 50% of advanced-stage tumors (7) and indicates poor prognosis (8). This correlation between amplification of *MYCN* and tumor stage and the observed high expression of *MYCN* in poorly differentiated cells (6, 9–13) suggest that enhanced expression of *MYCN* consequent to amplification contributes to tumor progression.

Identification of *MYCN* was facilitated by its resemblance to the protooncogene *MYC* (1, 2, 14, 15). Both *MYCN* and *MYC* comprise three exons and two intervening sequences (14, 15) and encode nuclear phosphoproteins of similar size that bind to DNA *in vitro* (16, 17). These similarities raised the possibility that *MYCN* may be an authentic protooncogene, capable of contributing to the transformation of cells and to tumorigenesis.

The oncogenic potential of *MYCN* has been addressed experimentally in cultured cells by using vectors that drive the expression of *MYCN* with a promoter and enhancer from the *U3* region of the long terminal repeat (LTR) of Moloney murine leukemia virus (Mo-MuLV). The results showed that augmented expression of *MYCN* induces tumorigenicity in an established line of rat fibroblasts (Rat-1 cells) (18) and can assist a mutant allele of the protooncogene *HRAS* in the transformation of rat embryo cells (19, 20). We now extend these characterizations by showing that the solitary action of *MYCN* can establish the sustained proliferation of early-passage rat embryo cells. Our findings reinforce the image of *MYCN* as a multipotent oncogene and sustain the view that anomalous expression of the gene might contribute to tumorigenesis.

MATERIALS AND METHODS

Molecular Clones. We used three vectors containing alleles of *MYCN*: pMP-34.1^{K}, containing *MYCN* isolated from the Kelly line of human neuroblastoma cells; pMP-34.1^{N}, containing *MYCN* from the leukocytes of a normal individual; and pMP-34.1^{K}Xho, containing a mutant allele of *MYCN* with a termination codon in the first coding exon (19). In these vectors, transcription is driven by the *U3* region in the LTR of Mo-MuLV (see Fig. 1 and ref. 19). The *MYC* expression vector pHSR-1(LTR) contains the *U3* and *R* regions of the Mo-MuLV LTR linked to a normal allele of *MYC* (see Fig. 1 and refs. 21 and 22). Molecular clone pCV108 contains the bacterial neomycin-resistance gene fused to a Mo-MuLV LTR (gift from Chris Lau, University of California, San Francisco).

Transfection and Selection of Cells in Culture. Primary cultures of rat embryo cells were prepared from 12- to 14-day-old Fisher rat embryos. After 2–4 days of growth in Dulbecco's modified Eagle's (DME) medium supplemented with 10% (vol/vol) fetal calf serum and antibiotics, the cells were seeded at a density of 1×10^6 per 100-mm dish. Transfections were performed with 75 μg of carrier DNA from salmon sperm plus 15 μg of circular *MYCN* or *MYC* vector DNA and 1 μg of pCV108 DNA for two dishes, each containing about 4×10^6 cells at the time of transfection. Approximately 24 hr after transfection, the cells were passaged at a ratio of 1:3. Selection was done in medium containing 400 μg of crude G 418 (GIBCO) per ml.

Assay for Colony Formation in Soft Agarose. For analyses of anchorage-independent growth, 5×10^4 cells were suspended in 5 ml of 0.35% low-melting-point agarose (Sea Plaque) and seeded into 60-mm culture dishes containing a 0.7% agarose base. All agarose suspensions were made in DME medium containing 10% fetal calf serum. Plates were inspected by microscopy at regular intervals for 3 weeks.

Assay for Tumorigenicity. Analyses for tumorigenicity were done by subcutaneously injecting 5×10^6 cells in 0.5 ml of DME medium into 28-day-old syngeneic Fisher rats and into 3-week-old athymic mice. Animals were monitored at regular intervals over a period of 8 weeks for the appearance of tumors.

Isolation of DNA and RNA. Cells were lysed in 4 M guanidinium isothiocyanate/25 mM sodium citrate/1% sarkcosyl, pH 7.0. The lysates were layered onto a cushion of 5.7

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Abbreviations: LTR, long terminal repeat; Mo-MuLV, Moloney murine leukemia virus.

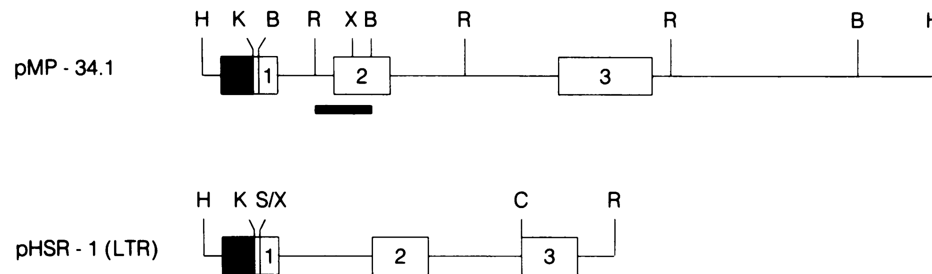

FIG. 1. Expression vectors for *MYCN* and *MYC*. The vectors illustrated here have been described in detail (19, 22). Boxed numbers 1, 2, and 3 refer to exons 1–3. The solid block 5' of exon 1 indicates the *U3* region of the Mo-MuLV LTR. The solid line under exon 2 of pMP-34.1 indicates the molecular probe pNB-1 used for analyses of *MYCN*. Restriction enzyme sites: H, *Hin*dIII; K, *Kpn* I; B, *Bam*HI; X, *Xho* I.

M $CsCl_2$/0.1 M EDTA, pH 7.0, and centrifuged in an SW 41.1 rotor (Beckmann) at 38,000 rpm for 15 hr. DNA was recovered from the viscous interphase, dialyzed against 100 mM NaCl/1 mM EDTA, pH 7.0, treated with proteinase K, extracted with phenol, and precipitated with ethanol. Polyadenylylated RNA was isolated from total RNA by chromatography through oligo(dT)-cellulose.

Analysis of DNA and RNA. DNA was cut with the appropriate restriction endonuclease, fractionated on an agarose gel, transferred to nitrocellulose paper, and hybridized with the ^{32}P-labeled insert of the *MYCN* clone pNB-1 (1). RNA was fractionated on agarose gels in the presence of 2.2 M formaldehyde and further analyzed as for DNA.

RESULTS

Establishment of Rat Embryo Cells in Culture by *MYCN*. In an attempt to establish long-term cultures of early-passage rat embryo cells, we transfected the cells with the *MYCN* expression vector pMP-34.1^{K} (Fig. 1). The cells were then passaged at a ratio of 1:3 twice a week and regularly inspected for more than 40 generations. We could not recover any cells that showed signs of proliferation after this period of time.

Reasoning that normal cells in the cultures might inhibit the proliferation of transfected cells expressing *MYCN* (23), we then used an alternative strategy. Cells were cotransfected with the *MYCN* expression vector and with the plasmid pCV108 conferring G418 resistance. Approximately 48 hr later, G418 (400 μg/ml) was added to the culture medium, and the cells were then maintained in the selection medium. Two weeks after transfection, colonies of G418-resistant cells were clearly visible. Cells from seven colonies were picked from the plate and transferred to microtiter plates. All seven foci could be established in long-term culture for more than 200 generations without indications of senescence. The doubling time of the established cells was comparable to that of an established line of rat cells (Rat-1).

To test whether establishment resulted from expression of *MYCN*, we introduced into rat embryo cells a combination of pCV108 and an *MYCN* vector containing a frameshift mutation within the second exon of *MYCN*. None of the selected clones could be established in long-term culture. These results showed that the establishment of rat embryo cells depended on expression of *MYCN* protein.

For comparison, we also transfected rat embryo cells with the *MYC* expression vector pHSR-1(LTR). After cotransfection with a selectable marker and subsequent culture in selection medium, we again obtained colonies of cells. Ten of these were isolated; all 10 proliferated into long-term cultures.

The cells established in culture by *MYCN* or *MYC* displayed a fusiform morphology (Fig. 2*W*), differing from the less refractile, flat morphology of cells into which mutant *MYCN* (Fig. 2*M*) or only plasmid pCV108 (Fig. 2*C*) had been introduced. We did not detect any morphological differences among the cells of the seven foci isolated after *MYCN* transfection or between the cells established by *MYCN* and *MYC*.

Established Cells Contain and Express Ectopic *MYCN*. To analyze *MYCN* sequences in the transfected cells, DNAs

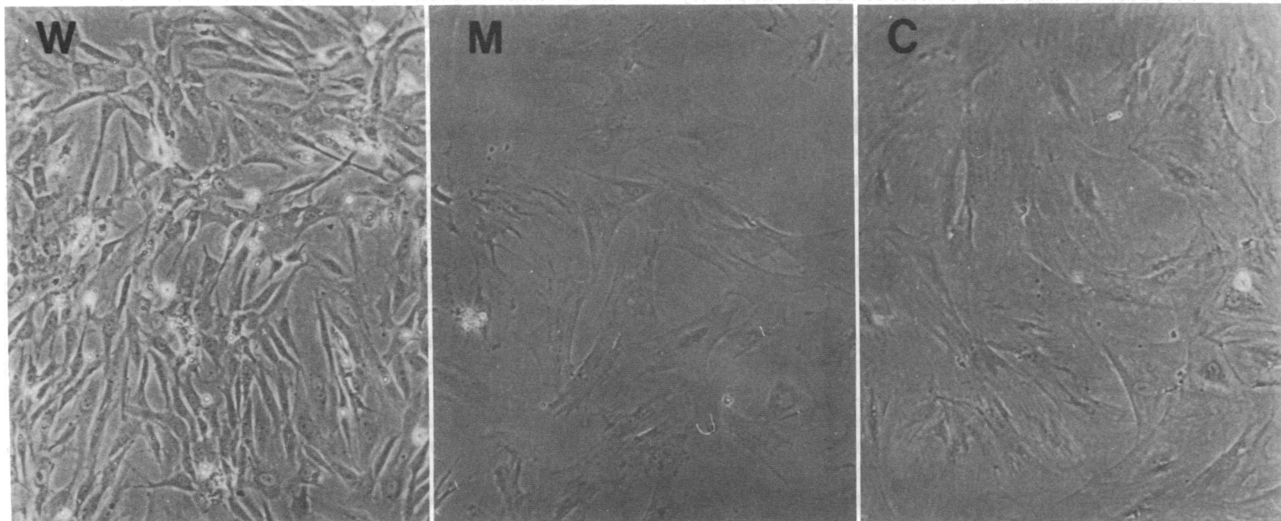

FIG. 2. Establishment of rat embryo cells in culture by *MYCN*. Rat embryo cells were transfected and selected with G418, and populations of surviving colonies were propagated for approximately 50 passages before examination by photomicroscopy. Transfections were with pCV108 alone (*C*), with pCV108 and the vector pMP-34.1^{K} carrying wild-type *MYCN* (*W*), or with pCV108 and the vector pMP-34.1^{K}Xho carrying inactive *MYCN* (*M*).

from all seven clones established after *MYCN* transfection were cut with the restriction endonuclease *Eco*RI and analyzed by electrophoresis in agarose. For the molecular probe, we used the *Eco*RI–*Bam*HI insert of plasmid pNb-1 (1), which detects a 2.0-kilobase-pair (kbp) *Eco*RI fragment in human DNA and an approximately 12-kbp *Eco*RI fragment in rat DNA (Fig. 3, lanes H and R, respectively). Inspection of the autoradiograph indicated that clones 1–7 carried from 5 to 50 copies of human *MYCN* sequences in addition to the endogenous *MYCN* of the rat. Clone 3 apparently carried the lowest number of copies, and clone 6, the highest number. For comparison, human neuroblastoma line Kelly (Fig. 3, lane N) contained approximately 120 copies of *MYCN* (1, 6). The additional faint bands in DNA of clones 1–7 presumably represented rearrangements of exogenous *MYCN* in the transfected cells. The additional strong band at *ca.* 6 kbp was most likely due to the vector sequences detected by plasmid DNA present within the molecular probe.

Analysis of polyadenylylated RNA detected (*i*) the anticipated 2.8-kb *MYCN* mRNA in the Kelly line of neuroblastoma cells that carry *ca.* 120 copies of *MYCN* (Fig. 4, lane N), (*ii*) the comparable RNA in the seven clones of cells established after transfection with *MYCN* (Fig. 4, lanes 1–7), and (*iii*) no *MYCN* RNA in normal rat embryo cells (Fig. 4, lane R). The abundance of *MYCN* mRNA in the cell lines was not inevitably proportional to the copy number of *MYCN*. For example, lines 5 and 7 carried roughly similar copy numbers of the *MYCN* gene, but the amount of *MYCN* mRNA was at least 20-fold higher in line 7 than in line 5. The molecular basis for these variations is not known.

We conclude that the establishment of rat embryo cells resulted from the abundant expression of *MYCN*. Although we did not analyze the DNA and RNA of cells established after transfection with *MYC*, we assume that the establishment resulted from augmented *MYC* expression, as in previous reports (23, 24).

Properties of Cells Established by *MYCN*. We noticed that cells established by *MYCN* and grown for prolonged periods of time without change of medium showed clear signs of senescence. In contrast, Rat-1 cells simply stopped multiplying. This observation suggested to us that the cells established by *MYCN* in culture depend on a continuous supply of exogenous growth factors for survival.

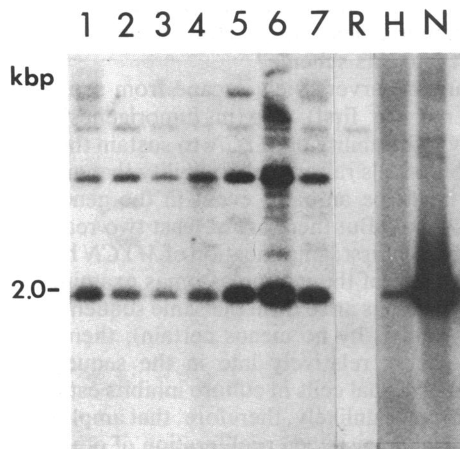

FIG. 3. Detection of transfected *MYCN* in cell lines. DNA was extracted, cleaved with *Eco*RI, and analyzed as described. Intensity of bands at 2.0 kbp reflects the copy number of human *MYCN*: a single copy in the haploid genome of human fibroblasts and *ca.* 120 copies in the Kelly line of human neuroblastoma cells (1). Lanes: 1–7, individual clones; R, untransfected rat embryo cells; H, human skin fibroblasts; N, neuroblastoma line Kelly.

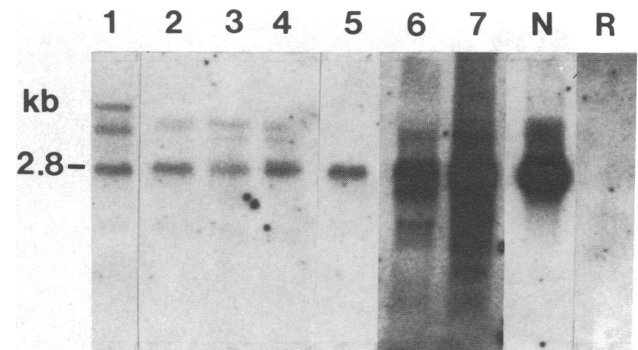

FIG. 4. Abundance of *MYCN* RNA in established rat embryo cells. RNA was isolated and analyzed in an agarose gel (20 μg per lane) as described. Lanes: 1–7, individual cell clones corresponding to the similarly numbered samples in Fig. 4; N, neuroblastoma line Kelly; R, normal rat embryo cells.

To define in more detail to what degree the rat embryo cells established in culture were dependent on serum for proliferation, we grew cells in the presence of different concentrations of fetal calf serum. Approximately 5×10^6 cells from all seven clones were seeded into 100-mm plates in 10% fetal calf serum. Approximately 15 hr later, the medium on replica plates was replaced with fresh medium containing 10%, 5%, 2%, or 0.5% fetal calf serum. Plates were inspected after 72 hr. There was no obvious difference in morphology of cells grown in 10%, 5%, or 2% fetal calf serum, although the population density at 2% was clearly lower than at 5% or 10% (Fig. 5). In contrast, cells grown in 0.5% fetal calf serum showed signs of senescence and obviously lacked mitotic activity. Comparable results were obtained when cells established in culture by *MYC* were tested.

In our previous studies, introduction of the *MYCN* expression vector pMP-34.1^{K} or pMP-34.1^{N} into rat embryo cells together with the mutationally activated *HRAS* from human bladder carcinoma cell line EJ had yielded two types of focus-forming cells (19). Type I cells had round morphology, were highly refractile, grew in soft agar, and were tumorigenic in isogeneic rats. In contrast, type II cells resembled in their morphology those cells established in culture by *MYCN* or *MYC* and were not tumorigenic. Therefore, we tested whether type II cells and the cells established by *MYCN* and *MYC* have comparable serum requirements. When we grew type II cells in 0.5% fetal calf serum, no sign of senescence or loss of mitotic activity was detectable for at least 72 hr (data not shown). These results show that cells established by *MYCN* or *MYC* are highly dependent upon exogenously supplied growth factors, whereas the focus-forming cells obtained after cotransfection with *HRAS* and *MYCN* are only partially dependent and, thus, may stimulate their own growth by autocrine mechanisms.

Rat embryo cells established by *MYCN* failed to grow in soft agar or form tumors in experimental animals (data not shown). By contrast, cells transformed by a combination of *MYCN* and *HRAS*(EJ) displayed both properties. We conclude that the cells established by *MYCN* lacked other properties of the transformed phenotype.

DISCUSSION

Establishment of Rat Embryo Cells in Culture. We have shown that the abundant expression of *MYCN* can sustain the long-term proliferation of rat embryo cells. Alleles of *MYCN* from both normal cells and the amplified DNA of tumor cells gave identical results. These findings conform to previous indications that genetic damage to the coding domain of *MYCN* is not required for transformation of cells by the gene

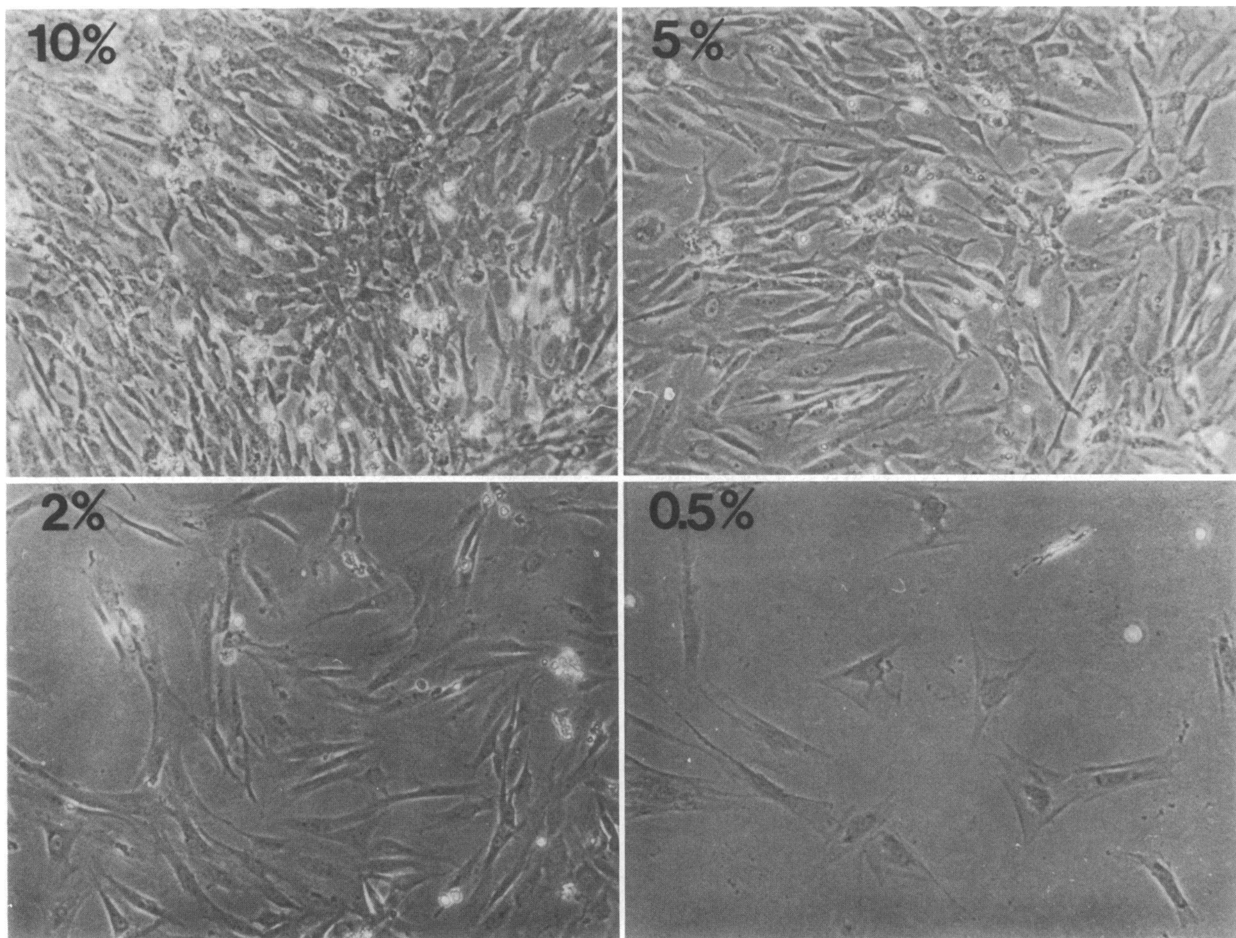

FIG. 5. Serum dependence of rat embryo cells established in culture by *MYCN*. Approximately 5×10^6 cells established by transfection with *MYCN* were seeded into 100-mm plates in medium containing 10% fetal calf serum. After 15 hr, the medium on replica plates was changed to contain 10%, 5%, 2%, or 0.5% of fetal calf serum. Photomicrographs were prepared 72 hr later.

(18–20), and they sustain the view that the abnormally high expression of an otherwise normal allele of *MYCN* contributes to the neoplastic phenotype of the neuroblastomas in which the gene is amplified.

Rat embryo cells can be established in culture by a variety of protooncogenes and oncogenes, including *MYC* (23, 24), v-*myc* (23–25), polyomavirus *tsa* encoding the large tumor antigen (26), *TP53* encoding tumor protein p53 (27), and adenovirus *E1a* (24). In the work reported here, all clones isolated after transfection with either *MYCN* or *MYC* proliferated into long-term cultures. We conclude that these two genes do not differ appreciably in their ability to sustain the proliferation of embryonic cells—a conclusion that conforms to the extensive resemblance between the proteins encoded by *MYC* and *MYCN* (16, 17). A similarity of physiological effects among *MYC* genes is also suggested by the fact that gene amplification in small-cell lung cancers can involve *MYCN*, *MYC*, or *MYCL* (a third member of the *MYC* gene family), as if the three genes might serve as surrogates for one another in tumorigenesis (for a review, see ref. 28). In other settings, however, members of the *MYC* gene family may not be physiologically equivalent. For example, only *MYCN* has been found amplified in neuroblastomas (1, 6, 7).

Establishment of rat embryo cells by *MYCN* appears not to be a fully penetrant property because proliferation of cells transfected with *MYCN* is inhibited by proximity to normal cells. The molecular basis for this behavior is unclear, but it is possible that communication with normal cells through gap junctions suppresses the proliferation of cells bearing ectopic *MYCN* (29–31). Our findings echo a previous report that transformation of rat embryo cells by alleles of either *HRAS* or *MYC* requires selection to eliminate normal cells from the culture (23).

Amplification of *MYCN* as a Step in Tumorigenesis. Malignant tumors characteristically arise from a multiplicity of events within the emerging cancer cell. Prominent among these events are various forms of genetic damage, which may contribute to both the initiation and the continued progression of tumorigenesis (32). How might amplification of *MYCN* fit into this scheme?

(*i*). Many observers view escape from senescence as an early (if not the first) step in tumorigenesis. From this perspective, the ability of *MYCN* to sustain the proliferation of rat embryo cells raises the possibility that amplification of the gene might be an early event in the genesis of human neuroblastomas. But there are at least two reasons to doubt this possibility. First, amplification of *MYCN* has been found in less than half of the neuroblastomas examined to date. If all neuroblastomas arise from the same sequence of events (a possibility that is by no means certain), then amplification probably occurs relatively late in the sequence. Second, proximity to normal cells in culture inhibits establishment by *MYCN*. It seems unlikely, therefore, that amplification of the gene could elicit sustained proliferation of otherwise normal cells in the tissues of an organism.

(*ii*). Amplification of *MYCN* has been found only in the more aggressive variants of human neuroblastoma (7), where it connotes a dire prognosis (8). These findings prompted the hypothesis that anomalous expression of *MYCN* is a late event in the genesis of neuroblastomas, contributing to malignant progression of the tumors. The ability of *MYCN* to

convert established rodent cells to a tumorigenic phenotype conforms to (but by no means proves) this hypothesis (18).

We conclude that it is presently impossible to assign *MYCN* a specific role in tumorigenesis. But the results presented here enhance the credentials of *MYCN* as an authentic protooncogene, provide an additional biological parameter for use in the characterization of *MYCN* and suggest that *MYCN* may be useful for the establishment of cell lines from various embryonic lineages.

This study was supported by grants from the National Institutes of Health (CA12705 and CA44338 to J.M.B.) and the Deutsche Forschungsgemeinschaft (to M.S.) and by funds from the G. W. Hooper Research Foundation and the German Cancer Research Center. We thank L. Vogel and I. Ulbrich for assistance with the manuscript.

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