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Genetics

# N-*myc* can cooperate with *ras* to transform normal cells in culture

**(malignant transformation/protooncogene/N-*myc* expression construct)**

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**ABSTRACT** **N-*myc*, a cellular gene bearing homology to the c-*myc* protooncogene, is frequently amplified and overexpressed in a highly restricted set of related tumors, most notably neuroblastomas and retinoblastomas. We have examined the possibility that N-*myc* may play a causal role in the genesis of these tumors by defining its ability to transform primary cells in tissue culture. Using an N-*myc* expression construct capable of producing constitutively deregulated levels of full-length murine N-*myc* mRNA, we demonstrate that a deregulated N-*myc* gene can cooperate with the activated Ha-*ras* oncogene to cause tumorigenic conversion of normal embryonic fibroblasts in a manner indistinguishable from the deregulated c-*myc* oncogene. Cell lines established from N-*myc*/*ras*-transformed foci express high levels of the N-*myc* gene, and such lines are similar to c-*myc*/*ras* transformants in their ability to grow in soft agar and cause tumors in syngeneic rats. These results illustrate that N-*myc* does encode a c-*myc*-like transforming activity and that this transforming activity is not specific for the very restricted set of tumors in which N-*myc* is normally amplified or overexpressed.**

The cellular protooncogene c-*myc* appears to be involved in the growth and division of a wide variety of normal cells (1–5). Deregulation of c-*myc* expression has been noted in a large number of different tumors (6–12), suggesting that aberrant c-*myc* expression can contribute to the development of neoplasia. Biological test systems, in which constitutively deregulated c-*myc* genes were introduced into cells in culture or into transgenic mice, have verified that altered c-*myc* expression can play a direct role in the development of malignant transformation (13–15). For example, the malignant transformation of primary rat embryo fibroblasts (REFs) requires the cooperative actions of an overexpressed c-*myc* gene and a mutant *ras* oncogene (either Ha-*ras* or N-*ras*) (13). It is thought that the two oncogenes provide distinct transforming functions, with the c-*myc* gene satisfying an "establishment" or "immortality" requirement and the *ras* gene conferring morphological transformation and loss of contact inhibition. Two viral oncogenes, adenovirus *E1A* and polyoma virus large *T*, and a single cellular gene, *p53*, have the capability to substitute for c-*myc* in a primary cell transformation assay, although the *p53* gene appears much weaker than c-*myc* in its ability to compare with *ras* (13, 16–19).

N-*myc* is a cellular gene bearing DNA sequence homology to c-*myc*. It is frequently amplified and overexpressed in a highly restricted set of related tumors, most notably neuroblastomas and retinoblastomas (20–25). Furthermore, N-*myc* gene amplification in primary neuroblastomas correlates with both tumor stage and the ability of these tumors to grow *in vitro* as established cell lines (24). Here we verify that altered N-*myc* expression can play a direct role in tumor development by demonstrating that an N-*myc* expression construct can cooperate with the activated Ha-*ras* oncogene to malignantly transform normal embryonic fibroblasts in a manner indistinguishable from the deregulated c-*myc* oncogene. This illustrates that N-*myc* encodes a c-*myc*-like transforming activity and that this transforming activity is not specific for the very restricted set of tumors in which N-*myc* is normally amplified or overexpressed.

## MATERIALS AND METHODS

**Preparation and Transfection of REFs.** Primary cultures of REFs were prepared as described elsewhere (26) from 12- to 14-day Fischer rat embryos; 3–4 days later the cells were frozen at −70°C with 10% (vol/vol) glycerol. To prepare for a transfection cells were thawed, then replated after 3–4 days at $1 \times 10^6$ cells per 10-cm plate in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. DNA was introduced into REFs by calcium phosphate precipitation as described previously (27, 28), using 25–35 μg of 3T3 carrier DNA with 2 μg of each of the appropriate plasmid DNAs per plate. The cultures were re-fed 6–9 hr after transfection and split 24 hr after the transfection into either three (experiments 1 and 2) or four (experiment 3) plates. Assuming that limited cell division occurs in the 24 hr after the transfection, each plate after this final passage contains independent transformants. This was verified by DNA analysis of isolated transformants (data not shown). The various plasmids used in the REF transfections are as follows: pT24-ras contains the mutant Ha-*ras* oncogene previously described (29); pKO-*myc*, kindly provided by Daniel Birnbaum, is a c-*myc* expression construct in which transcription of the second and third exons of c-*myc* is driven from a simian virus 40 promoter; the N-*myc* constructs are described below and in Fig. 1.

**Assay for Colony Formation in Soft Agar.** A single-cell suspension of approximately $1 \times 10^4$ cells in 5 ml of 0.28% agar was overlayed onto a 60-mm culture dish containing a 0.7% agar base. All agar suspensions were made in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Duplicate plates were prepared for each tested line. Normal REFs propagated in culture served as a nontransformed control for these studies.

**Assay for Tumorigenicity.** Cell lines propagated from transformed foci (see text) were assayed for tumorigenicity by subcutaneously injecting $1–5 \times 10^6$ cells in 0.4 ml into 7- to 10-day-old syngeneic Fischer rats. Each line tested was injected into two rats. A nontransfected control was provided by propagating normal REFs in culture until sufficient numbers of cells were attained; four rats each injected with $10^7$

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Abbreviations: REF, rat embryo fibroblast; kb, kilobase(s); LTR, long terminal repeat.

normal REFs served as controls. The rats were followed until death, or for 4 weeks in the case of the normal REF injections.

**Cloning a Genomic Murine N-*myc* Fragment.** A λ Charon 16A recombinant library was prepared from completely *Eco*RI-digested genomic DNA from a C57L murine pre-B-cell line (223-18) (ref. 30) by methods described previously (31). The genomic 7.7-kilobase (kb) *Eco*RI DNA fragment containing the murine N-*myc* gene was obtained by screening this library with a human N-*myc* probe (NB-19-21) (ref. 21). This 7.7-kb fragment was subcloned in the plasmid vector pUC9 and in the retroviral vector described below by methods previously described (31).

**Analysis of RNA and DNA.** Our procedures for RNA and DNA preparation, gel electrophoresis, and transfer to nitrocellulose membranes have been described in detail elsewhere (31, 32). For detection of oncogene RNA or DNA sequences we utilized a nick-translated *ras* probe prepared from an internal 2.9-kb *Sac* I fragment isolated from the mutant *ras* gene T24 (ref. 29), a c-*myc* probe prepared from an internal 1.4-kb *Xho* I fragment isolated from a murine c-*myc* cDNA clone (ref. 33), and a murine N-*myc* cDNA probe (unpublished data). Nick-translations and hybridization procedures were performed as previously described (31, 32).

**Manipulations of Retroviral Vectors.** Viral stocks of pRV-Nmyc1 and pRV-Nmyc2 (see below) were collected from the supernatants of NIH 3T3 murine cells in which these vectors had been introduced by DEAE-dextran cotransfection with Moloney murine leukemia virus helper DNA by the methods described by Lobel and Goff (34). The viral stocks were then used to infect 3T3 cells, from which poly(A)$^+$ RNA was isolated.

## RESULTS

**Construction of N-*myc* Expression Vector.** By screening a mouse genomic DNA library we molecularly cloned a 7.7-kb *Eco*RI fragment (designated N7.7) that contains regions strongly homologous to a human N-*myc* genomic DNA probe (NB-19-21) (ref. 21) and to a cDNA clone (pN1.8) representing the 3′ terminus of the human N-*myc* mRNA (35). Because NB-19-21 and pN1.8 contain sequences homologous to the second and third exons of c-*myc*, the N7.7 fragment encompasses the murine N-*myc* region corresponding to the known translated regions of c-*myc* (33, 36–38); mapping the locations of these c-*myc* homologous regions in N7.7 defined the transcriptional orientation of the N-*myc* gene (see legend to Fig. 1). DNA probes isolated from N7.7 identify a 2.9-kb murine N-*myc* message that is expressed in a restricted set of mouse cell lines and tissues, including neuroblastomas and developing neural tissue (unpublished data). The N7.7 DNA segment was inserted into a retroviral vector between two directly repeated Moloney murine leukemia virus long terminal repeats (LTRs) to provide the N-*myc* clone with a constitutive promoter, donor splice site, polyadenylylation signals, and enhancer sequences, any or all of which might be required for constitutive N-*myc* expression. The N7.7 fragment was inserted into this vector in both transcriptional orientations (Fig. 1*B*).

**Cooperation of N-*myc* with Mutant Ha-*ras*.** pRV-Nmyc1, which contains the N-*myc* gene in the same transcriptional orientation as the flanking LTRs, was not able to transform REFs alone but was able to cooperate with the mutant Ha-*ras* gene in a REF transformation assay to produce dense foci that overgrew the normal monolayers (Table 1). Neither pRV-Nmyc2, which contains the N-*myc* gene in the reverse transcriptional orientation with respect to the flanking LTRs, nor pN7.7, a plasmid containing only the N7.7 fragment without adjacent LTRs, was able to cooperate with *ras* to transform REFs at a detectable level in our assay (Table 1). Similarly, c-*myc* requires a cotransfected mutant *ras* gene to transform REFs, and the optimal expression of the c-*myc*/*ras* transforming activity requires that the c-*myc* gene be driven directly by a viral promoter (13). The pRV-Nmyc1/*ras* foci appeared with the same frequency and kinetics as c-*myc*/*ras* foci produced from simultaneous transfections (see Table 1, experiments 1 and 2). In both cases dense foci, which became noticeable about 9 days after the transfection, continued to expand into and eventually displaced the monolayer of normal fibroblasts. Foci from each set were picked, gradually expanded, and finally plated onto large culture dishes. Both N-*myc* and c-*myc* foci propagated at the same rate (7 of 9 N-*myc* and 6 of 9 c-*myc* foci revealed predominately transformed cells at the end of the above isolation procedure). Morphologically the N-*myc* and c-*myc* foci were quite similar macroscopically (Fig. 2*A*) and microscopically (Fig. 2 *B* and *C*). The predominant phenotypes of the propagated transformed cell lines were quite similar for N-*myc* and c-*myc* and consisted of markedly transformed cells, many of them rounded and floating (Fig. 2*D*). These lines were malignantly transformed as judged by their anchorage-independent growth in soft agar and ability to cause tumors in young syngeneic rats (Table 1). Tumors caused by either the N-*myc* or the c-*myc* transformants grew rapidly, eventually killing the host animal within 2–3 weeks.

**Analysis of DNA and RNA from REF Transformants.** Analysis of DNA prepared from the transformed REF lines confirmed the presence of multiple copies of the introduced *ras* and *myc* genes (data not shown). Total RNA prepared from the REF transformants and appropriate controls was screened for the expression of *ras*, c-*myc* and N-*myc*. All the REF transformants expressed high levels of the introduced *ras* gene as compared to the undetectable transcription of the

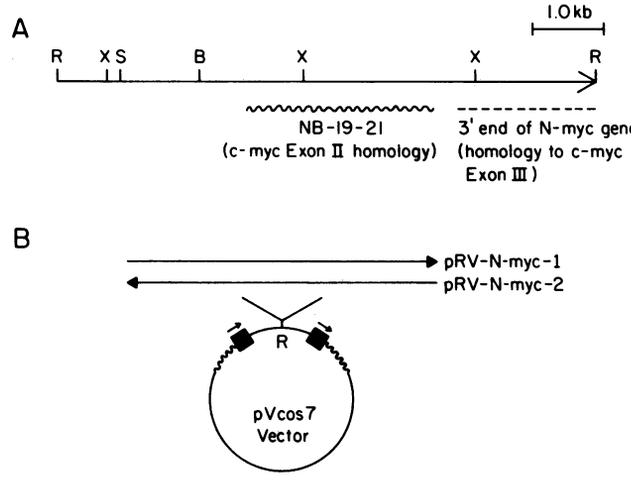


FIG. 1. (*A*) Restriction map of the murine N7.7 fragment. Restriction endonuclease sites are R, *Eco*RI; X, *Xho* I; S, *Sac* I; and B, *Bam*HI. Regions of homology to human genomic N-*myc* probe NB-19-21 and to human N-*myc* cDNA clone pN1.8 are indicated. NB-19-21 is homologous to human c-*myc* exon 2 (refs. 20 and 21), while pN1.8 represents the 3′ terminus of the human N-*myc* mRNA and contains sequences homologous to human c-*myc* exon 3 (data not shown). The region of N7.7 that cross-hybridizes to the NB-19-21 probe also is homologous to a human c-*myc* exon 2 probe (data not shown). The arrowhead indicates the direction of transcription of the N-*myc* gene deduced on the basis of the position of the c-*myc* homologous regions. (*B*) Schematic diagram of N-*myc* expression constructs. The pVcos7 vector, kindly provided by L. Lobel and S. Goff, contains two directly repeated Moloney murine leukemia virus LTRs inserted into the cosmid vector pHC79 (ref. 39); the black boxes in the diagram represent these two directly repeated LTRs. The N7.7 fragment was inserted into the unique *Eco*RI site in the vector in both directions, with the arrowheads indicating transcriptional direction of the N-*myc* gene and of the promoter in the LTRs.

Table 1. Transformation of REFs with N-*myc*, c-*myc*, and *ras* gene combinations

| Transfected DNA | Plates with transformed foci/total plates | | | Growth in soft agar, lines positive/lines tested | Tumorigenicity, rats with tumors/rats injected |
|---|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 3 | | |
| T24-ras alone | 0/6 | 0/24 | — | — | — |
| T24-ras + pKO-*myc* | 4/6 | 23/24 | — | 1/2 | 4/4 |
| T24-ras + pRV-Nmyc1 | 6/9 | 23/24 | 16/16 | 4/4 | 8/8 |
| T24-ras + pRV-Nmyc2 | 0/9 | — | 0/16 | — | — |
| T24-ras + pN7.7 | — | — | 0/16 | — | — |
| pRV-Nmyc1 alone | — | — | 0/16 | — | — |
| None | — | — | — | 0/4 | 0/4 |

The data for the first three columns are presented as the number of plates with positive foci compared to the total number of plates each particular transfection was finally divided into; due to the way the cells were passaged after transfection, each plate in the final passage has independent transformants (see *Materials and Methods*). At 2 weeks after transfection each positive plate had 1–5 foci, whereas at 4 weeks each positive plate had 5–20 foci; presumably some of these were due to metastases. Cell lines derived from c-*myc*/*ras* foci (two lines examined) or N-*myc*/*ras* foci (four lines examined) were assayed for both colony formation in soft agar and the ability to cause tumors in young syngeneic rats; each line assayed for tumorigenicity was injected into two rats. As a nontransfected control for these assays (bottom line) we used REFs propagated in culture to obtain sufficient numbers of cells. Both the colonies in soft agar and the tumors in the rats appeared and grew at approximately the same rate for either the N-*myc*/*ras* or c-*myc*/*ras* transformants. These tumors were noted in all cases within 1 week of cell injection, grew rapidly, and resulted in the death of the host animal within 2–3 weeks.

endogenous *ras* genes in untransformed REFs, the NIH 3T3 murine fibroblast cell line, and newborn mouse brain tissue (Fig. 3*A*). Normal REFs and all the lines examined expressed their endogenous c-*myc* genes, but at low and variable levels; only the lines actually produced by c-*myc*/*ras* cotransfection produced very high levels of c-*myc*, and this expression occurred from the introduced c-*myc* construct (expression of endogenous versus introduced c-*myc* genes could be distinguished by transcript size—see Fig. 3*B*). N-*myc* expression could be detected only in REF lines produced by N-*myc*/*ras* cotransfection and in developing brain (Fig. 3*C*); the lack of detectable N-*myc* expression in normal REFs is consistent with the highly tissue-specific expression of N-*myc* in normal cells (unpublished data) and in tumors (23).

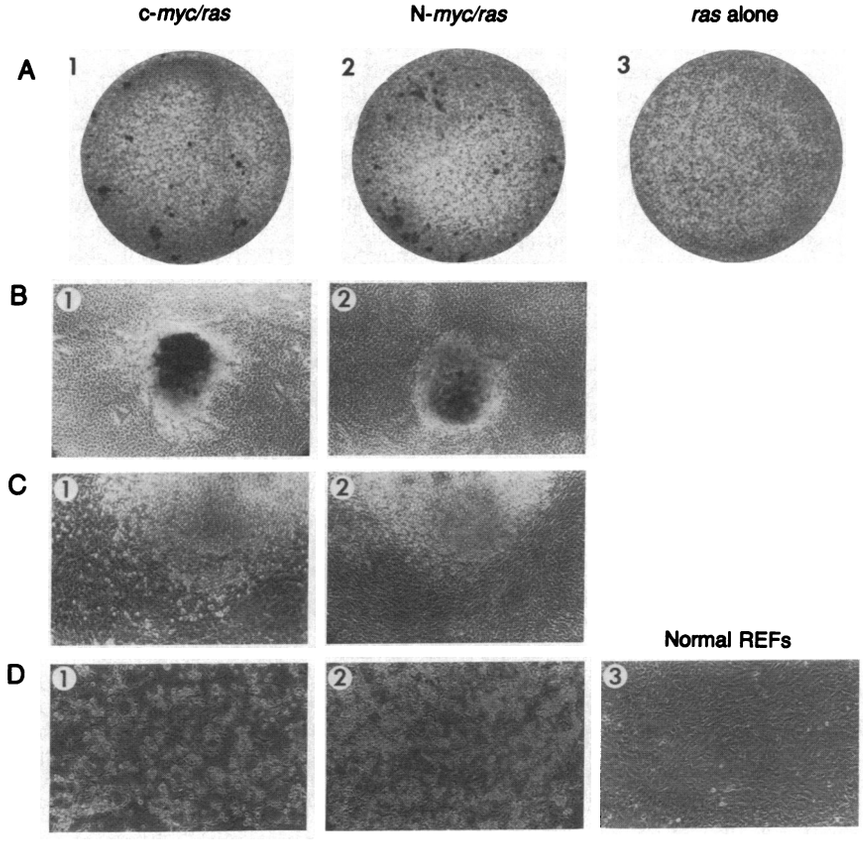


FIG. 2. Morphological comparison of c-*myc*/*ras* and N-*myc*/*ras* REF transformants. (*A*) Hemotoxylin-stained plates 24 days after transfection. (*B*) Low-power (×40) photomicrographs of two representative foci. (*C*) High-power (×100) photomicrographs showing edges of two representative foci. (*D*) High-power (×100) photomicrographs of (*1*) propagated c-*myc*/*ras* transformed REF line, (*2*) propagated N-*myc*/*ras* transformed REF line, and (*3*) normal REFs in culture.

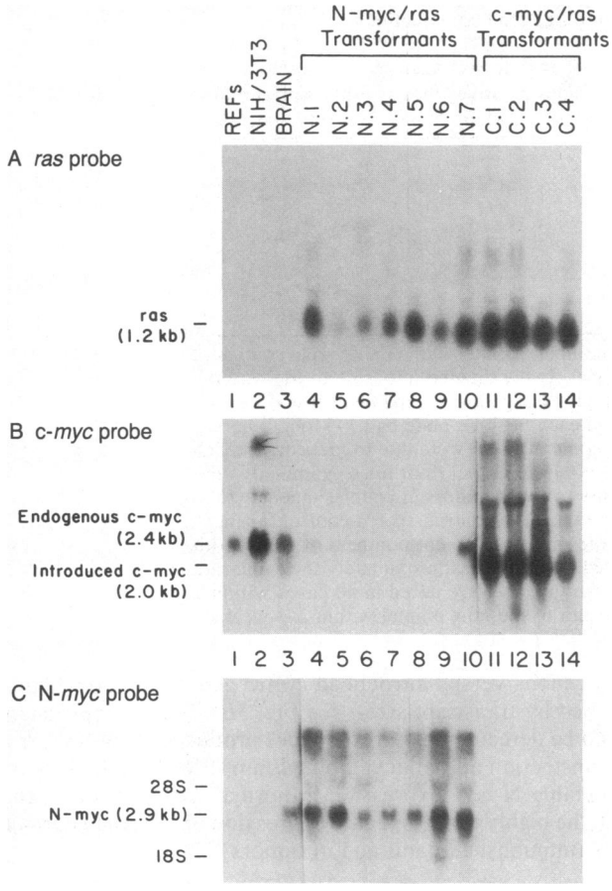


FIG. 3. Expression of introduced oncogenes in REF transformed lines. Twenty micrograms of total RNA, prepared from the indicated lines, was used in each lane. "REFs" refers to normal untransformed REFs, "BRAIN" refers to newborn mouse forebrain, N.1–N.7 refers to N-*myc*/*ras* transformants, and C.1–C.4 refers to c-*myc*/*ras* transformants. Duplicate filters were probed with the *ras* probe (*A*), the c-*myc* probe (*B*), or the N-*myc* probe (*C*), which were prepared as described in the text. The introduced c-*myc* gene is being driven from a simian virus 40 promoter linked upstream to the second exon, so RNA expressed from it is smaller than the normal endogneous c-*myc* mRNA. The levels of c-*myc* in the c-*myc*/*ras* transformants (lanes 11–14 in *B*) and N-*myc* in N-*myc*/*ras* transformants (lanes 4–10 in *C*) were very high, and thus exposures approximately 1/30th of those for the other lanes are used in this figure.

**N-*myc* Expression Vector Encodes Normal N-*myc* mRNA.** Notably, the size of the predominant N-*myc* transcript in the REF transformants is identical to that of the normal murine N-*myc* message as detected in the developing brain (compare lane 3 with lanes 4–10 in Fig. 3*C*). Furthermore, this transcript also does not hybridize with retroviral vector sequences (data not shown), and equal levels of a normal-sized N-*myc* mRNA are produced when NIH 3T3 cells are infected with viral stocks of either pRV-Nmyc1 or pRV-Nmyc2 (Fig. 4). Together, these observations suggest that the N7.7 fragment contains all the coding information necessary to produce a full-length murine N-*myc* mRNA and that the adjacent LTRs in pRV-Nmyc1 provide only an enhancer function that allows high-level expression from the normal N-*myc* promoter in inappropriate cells. The LTR enhancing function appears to be independent of its orientation with respect to N-*myc*, as is seen with other genes. Although pRV-Nmyc2 seems as effective as pRV-Nmyc1 in producing normal N-*myc* mRNA in 3T3 cells, we have shown that only pRV-Nmyc1 can efficiently cooperate with T24-ras to transform REFs. This finding raises the intriguing possibility that antisense RNA initiating in the left LTR of pRV-Nmyc2 can block translation of the normal N-*myc* message and prevent transformation. In support of this notion, we have demonstrated that N-*myc* antisense RNA is present in 3T3 cells transformed with pRV-Nmyc2 (unpublished data).

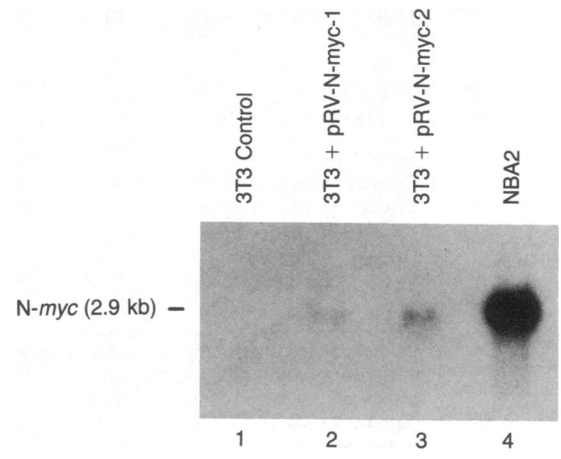


FIG. 4. pRV-Nmyc1 and pRV-Nmyc2 both produce normal-size N-*myc* mRNA. Five micrograms of poly(A)$^+$ RNA prepared from normal NIH 3T3 cells, NIH 3T3 cells infected with viral stocks of pRV-Nmyc1 or pRV-Nmyc2, or the mouse neuroblastoma NBA2 are compared for N-*myc* expression.

## DISCUSSION

**N-*myc* Encodes a Non-Tissue-Specific Transforming Function.** We have demonstrated that the transforming activity of N-*myc* is not limited to the very restricted set of tumors in which it is found to be amplified and overexpressed; N-*myc* is as potent as c-*myc* in effecting the malignant transformation of fibroblasts. Yet, *in vivo*, c-*myc* appears to play a role in the neoplastic transformation of a much wider variety of cells than N-*myc*. Recent analysis indicates that while c-*myc* is expressed in most tissues, high levels of N-*myc* expression are normally confined *in vivo* to tissues developmentally related to those that give rise to N-*myc*-expressing tumors (unpublished data). We suggest that although an oncogene's transforming activity is not necessarily tissue specific, its deregulation may preferentially occur in a cell type normally expressing this oncogene at a high level.

**N-*myc* and c-*myc* Are Members of the Same Cellular Protooncogene Family.** We have demonstrated that pRV-Nmyc1 can produce full-length N-*myc* mRNA that must encode a protein with c-*myc*-like transforming activity. Because no other assays exist for the N-*myc* protein (which has not yet been identified) or any of its activities, this expression vector may prove to be a useful tool in analyzing other functions of the N-*myc* protein. The similar transforming activities of N-*myc* and c-*myc*, together with the structural homology of the two genes, allows classification of N-*myc* as the second cellular transforming gene in the *myc* protooncogene family. N-*myc* may supply the "establishment" or "immortality" requirement previously attributed to c-*myc*. This possibility is consistent with the observation that N-*myc* gene amplification in primary neuroblastomas correlates well with the ability of these tumors to grow *in vitro* as established cell lines (24). Previously defined functional similarities between the c-*myc* and N-*myc* gene products include reduced expression in tumor lines after chemically induced cell differentiation (2, 40–42) and a correlation between gene amplification and degree of tumor malignancy (9, 24). The apparent association of *myc* gene amplification with tumor progression does not rule out a primary role for the expression of either *myc* gene in the genesis of these

tumors, as amplification may occur subsequent to (and in fact serve to potentiate) an initial event resulting in deregulated expression.

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