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Enhanced expression of the human gene N-*myc* consequent to amplification of DNA may contribute to malignant progression of neuroblastoma

(oncogenes/human tumors)

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ABSTRACT **Previous studies had revealed that DNA with partial similarity to the *myc* oncogene (N-*myc*) is frequently amplified in human neuroblastoma cell lines and neuroblastoma tumors. We show here for one patient that N-*myc* amplification is confined to the neuroblastoma tumor and is not present in normal tissue. N-*myc* mRNA ≈4.0 kilobases in size is detectable in neuroblastoma cell lines and tumors and in a retinoblastoma cell line. By contrast, appreciable amounts of this RNA were not present in a number of cell lines derived from other human tumors and in fibroblasts from a normal individual and from a neuroblastoma patient. Low levels of N-*myc* RNA were found in human and murine neuroblastoma cell lines lacking amplification of this gene, up to 80-fold greater levels in all cell lines carrying amplified N-*myc*. *In situ* hybridization to sections of neuroblastoma tumors revealed high expression of N-*myc* predominantly in undifferentiated neuroblasts. We hypothesize that amplification and consequent elevated expression of N-*myc* may be related to malignant progression.**

Cells from human neuroblastomas frequently display either double minute chromosomes (DMs) or homogeneously staining regions within macrochromosomes (HSRs) (1–4). Both karyotypic abnormalities are cytogenetic manifestations of amplified DNA (5). We (6) and others (7) have shown that the amplified DNA of human neuroblastomas includes a sequence with partial similarity to the cellular oncogene c-*myc* and tentatively denoted N-*myc*. Amplification of N-*myc* has been observed in numerous cell lines established from human neuroblastomas (14 of 18 lines examined to date), in tumors resected from patients prior to chemotherapy (6–9), and in occasional lines of retinoblastoma cells (ref. 9; unpublished data). The unamplified version of N-*myc* in normal cells is located at chromosomal position 2p23-24 (10), whereas the amplified gene can be translocated to any of several other chromosomes (10).

Amplification of DNA is generally accompanied by enhanced expression of genes contained within the amplified domain. This principle is exemplified both by genes responsible for resistance of cells to metabolic inhibitors (5) and by cellular oncogenes (11–17). We therefore asked whether the amplification of N-*myc* in human neuroblastomas leads to enhanced expression of the gene and how the abundance of expression might be distributed among the heterogeneous population of cells in newly resected tumors. Our findings suggest that elevated expression of N-*myc* may be a distinguishing (but not inevitable) feature of neuroblastomas that could be accounted for by gene amplification and that even normal expression of the gene may be restricted to cells of the neuroblast lineage. High expression of N-*myc* was found in only a minority of cells within the mass of a tumor examined by hybridization *in situ*. We hypothesize that amplification and elevated expression of N-*myc* may help to engender progression to higher malignancy.

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MATERIALS AND METHODS

Cell Lines and Tumors. Neuroblastoma cell lines CHP-126, NMB, and Kelly were obtained from F. Gilbert. Retinoblastoma line Y79 was also from F. Gilbert; the other retinoblastoma lines were from D. Char. Other cell lines were obtained from American Type Culture Collection. Fibroblasts from a neuroblastoma patient (AG 2202) were obtained from the Aging Cell Repository, National Institute on Aging, Institute for Medical Research. Neuroblastoma tumors from patients at University of California, San Francisco, were obtained with the help of Michael Harrison, Victor Levin, and Susan Fong. AR is a tumor from a 13-month-old female patient; JT is a tumor from a 2-year-old male patient. Both tumors were resected before chemotherapy or therapeutic irradiation.

Analysis of RNA and DNA. Our procedures have been described in detail elsewhere (11). We routinely analyzed 5 μg of polyadenylylated RNA and 10 μg of DNA per lane on 1% agarose gels. For detection of N-*myc*, we used the cloned 2.0-kilobase-pair (kbp) *Eco*RI fragment (Nb-6) containing the sequence related to the *myc* oncogene (6). For molecular hybridization, we employed conditions of high stringency (50% formamide/0.45 M NaCl/0.045 M sodium citrate, pH 7, 42°C) under which there is no cross-hybridization between c-*myc* and N-*myc* (6). For dot blot analyses of N-*myc* transcripts, polyadenylylated RNA was dissolved in 14.8% formaldehyde/1.8 M NaCl/0.18 M sodium citrate, pH 7, and spotted onto nitrocellulose filters, which then were baked and hybridized with ^{32}P-labeled Nb-6 probe.

Detection of N-*myc* Expression in Tumor Sections. Frozen tumor material was cut into sections, fixed with ethanol/acetic acid (3:1), and treated with proteinase K. For control, some sections were also treated with pancreatic RNase (100 μg/ml for 1 hr). Nb-6 fragment, labeled with ^{125}I by using random primers and reverse transcriptase, was then hybridized essentially as described elsewhere (18). Sections were washed for 1 hr in 0.3 M NaCl/0.03 M sodium citrate, pH 7, at 56°C and then washed extensively in 50% formamide/0.5 M NaCl/10 mM Tris·HCl, pH 7.5/1 mM EDTA, dried, and covered with photographic emulsion Kodak NBT2. Exposure time was 4 days.

Abbreviations: kb, kilobase(s); kbp, kilobase pair(s); HSR, homogeneously staining chromosomal region; DM, double minute.

RESULTS

Amplification of N-*myc* Is Restricted to Tumor Cells and Is Not Found in Normal Tissue of a Patient. Amplification of N-*myc* has so far been sought only in cell lines established from human neuroblastomas and in tumors resected prior to chemotherapy (6, 7, 9). We therefore obtained additional specimens of tumor (patient JT) and adjacent normal renal tissue. The tumor displayed *ca.* 40-fold amplification of N-*myc*, whereas the gene was not detectably amplified in the normal tissue (Fig. 1). We conclude that amplification of N-*myc* is a somatic rather than a germinal event and may be restricted to tumor cells.

Enhanced Expression of N-*myc* Accompanies Gene Amplification. We assessed the expression of N-*myc* by analyzing polyadenylylated RNAs extracted from tumors and cell lines. Multiple RNAs apparently derived from N-*myc* were found, but only the smallest of these [4.0 kilobases (kb)] appeared in cytoplasmic fractions (Figs. 2 and 3). We therefore presume that the larger RNAs are nuclear precursors to the mature mRNA for N-*myc*.

Neuroblastoma cell lines that displayed amplification of N-*myc* (Kelly, NMB, and IMR-32) contained relatively abundant quantities of the N-*myc* RNAs (Fig. 3*A*). By contrast, cell lines derived from human (SK-N-SH) and mouse (Neuro-2a) neuroblastomas without evident amplification of N-*myc* contained small amounts of the RNAs (recently we have found that the mouse genome contains N-*myc* that is readily detected with probes for the human gene; ref. 10). Using the RNA of SK-N-SH as a standard, we estimated that the expression of N-*myc* is enhanced by *ca.* 60- to 80-fold in the Kelly, NMB, and IMR-32 lines, 50- to 60-fold in line CHP-126, and ≈30-fold in line CHP-134 (Table 1). The relative amounts of N-*myc* RNA in the cell lines were not always in exact accord with the extent of N-*myc* amplification. The tumor from AR also contained N-*myc* RNAs (Fig. 3*A*), but the electropherogram suggested partial degradation of the RNA. A similar picture was obtained for the neuroblastoma of patient JT (not shown). To obtain a more reliable measure of the amounts of N-*myc* RNA in tumors AR and JT, we used "dot-blot" analysis and RNAs from the SK-N-SH and Kelly cell lines as standards (Fig. 3*B* and Table 1). The results indicated that the expression of N-*myc* in the tumors was elevated 20- to 30-fold over the amounts found in SK-N-SH cells.

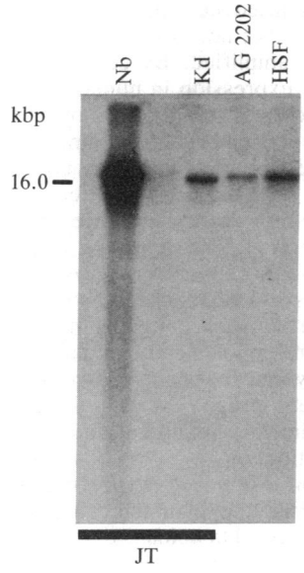

FIG. 1. Amplification of N-*myc* is a somatic mutation. Approximately 10 μg of DNA from neuroblastoma (Nb) and kidney (Kd) of patient JT and from skin fibroblasts of a neuroblastoma patient (AG 2202) and of a normal individual (HSF) was digested with the restriction endonuclease *Hin*dIII and analyzed by Southern blotting and hybridization with ^{32}P-labeled Nb-6 probe.

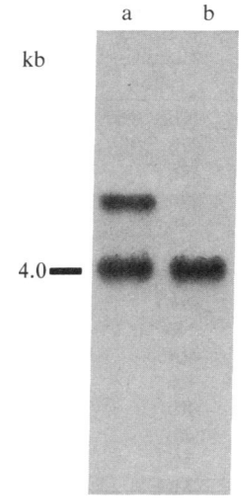

FIG. 2. Sizes of N-*myc* RNA in neuroblastoma line Kelly. Lanes: a, RNA from whole cells, and b, RNA from the cytoplasm. Polyadenylylated RNA was subjected to agarose gel electrophoresis in 2.2 M formaldehyde. N-*myc* RNA was detected by hybridization with labeled Nb-6 probe.

Specificity of N-*myc* Expression. We surveyed cell lines from a variety of human tumors for expression of N-*myc*, including retinoblastomas (McA, JD, Ng, LM, and Y79), melanomas (SK-MEL-3, SK-MEL-31, HT-144, and RPMI-7951), a colonic carcinoma (COLO 320), a bladder carcinoma (EJ), chronic myelogenous leukemia (K-562), and acute promyelocytic leukemia (HL-60). In addition, we analyzed fibroblasts of a normal individual and of a patient carrying neuroblastoma (it is not known, however, whether N-*myc* was amplified in this neuroblastoma). We found evidence of N-*myc* expression in only one instance other than neuroblastoma cells—the retinoblastoma line Y79, which we and others have shown to harbor amplified N-*myc* (ref. 7; unpublished data). Representative data are illustrated in Fig. 4. The RNA from COLO 320 provides a useful contrast, since it demonstrates the enhanced expression of c-*myc* that accompanies amplification of c-*myc* (Fig. 4, lane b, of COLO

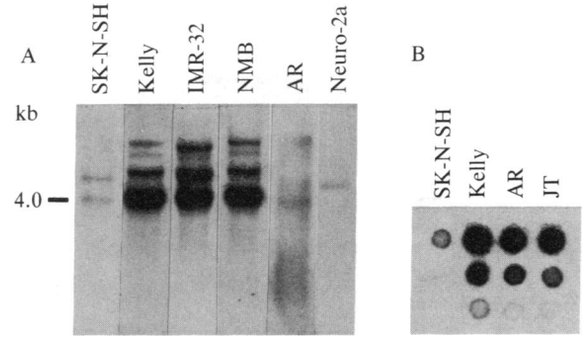

FIG. 3. Level of N-*myc* expression in neuroblastoma cells. (*A*) RNA transfer blotting analysis. SK-N-SH, Kelly, NMB, and IMR-32 are human neuroblastoma cell lines, AR is a human neuroblastoma tumor, and Neuro-2a is a murine neuroblastoma cell line. Each lane contains 5 μg of polyadenylylated RNA. (*B*) Dot blot analysis. Polyadenylylated RNA was serially diluted and spotted onto nitrocellulose filters. The filters were then baked and hybridized with ^{32}P-labeled Nb-6 probe. Spots for Kelly, AR, and JT contain, from top to bottom, 5, 0.5, and 0.05 μg of RNA and for SK-N-SH, 10, 1.0, and 0.10 μg of RNA.

Table 1. Expression and amplification of N-*myc*

Sample	Classification	N-*myc* Amplification*	N-*myc* Expression†
SK-N-SH	Nb	1	1
Kelly	Nb	100–120	60–80
NMB	Nb	100–120	60–80
IMR-32	Nb	15–20	60–80
CHP-126	Nb	100–120	50–60
CHP-134	Nb	20–25	30
AR	NbT	80–100	20–30
JT	NbT	40	20–30
Neuro-2a (mouse)	Nb	1	1
McA	Rb	1	–
JD	Rb	1	–
Ng	Rb	1	–
Y79	Rb	25	60–80
SK-MEL-3	Mel	1	–
SK-MEL-31	Mel	1	–
HT-144	Mel	1	–
RPMI-7951	Mel	1	–
COLO 320	Colon C	1	–
EJ	BlC	1	–
K-562	CML	1	–
HL-60	APL	1	–
Human skin fibroblasts		1	–

Nb, neuroblastoma cell line; NbT, neuroblastoma tumor; Rb, retinoblastoma cell line; Mel, melanoma; Colon C, colonic carcinoma; BlC, bladder carcinoma; CML, chronic myelogenous leukemia; APL, acute promyelocytic leukemia.

*Most of the data on neuroblastoma are from ref. 6. A value of 1 means a single copy per haploid genome.

†Expression was determined by RNA transfer blotting analysis, and the intensity of the signal was compared to that obtained for line SK-N-SH (value 1). Expression in AR and JT was also determined by dot-blot analysis. –, no N-*myc* RNA was detected in gels containing 20 μg of polyadenylylated RNA.

320) and that can be detected only by hybridization with a probe for c-*myc*.

It therefore appears that expression of N-*myc* may be restricted to certain neuroectodermal cells. In this view, expression of N-*myc* would be constitutive at relatively low levels in certain undifferentiated neurogenic cells but enhanced by gene amplification in neuroblastomas and apparently at least occasional retinoblastoma cell lines.

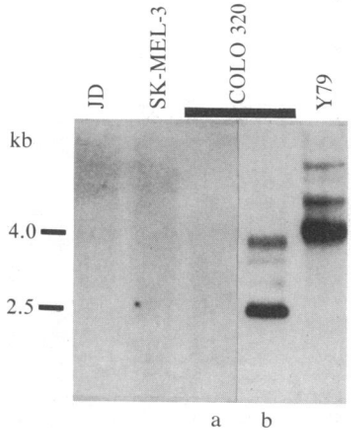

FIG. 4. Specificity of enhanced expression of N-*myc*. JD and Y79 are retinoblastoma cell lines, SK-MEL-3 is a melanoma cell line, and COLO 320 is a colonic carcinoma cell line. Lane a of COLO 320 shows the signal obtained with N-*myc* probe, and lane b shows that with c-*myc* probe. Lane b of COLO 320 and lane Y79 were run on the same gel and the hybridization solution contained an equal number of cpm of labeled N-*myc* and c-*myc* probe.

Enhanced Expression of N-*myc* Does Not Appear to Be Present in All Cells Within Neuroblastomas. Neuroblastomas are often composed of heterogeneous populations of cells, ranging from poorly differentiated neuroblasts to cells showing features of neuronal differentiation (19). The tumors from AR and JT displayed this characteristic heterogeneity (Fig. 5*A*) consisting predominantly of small, undifferentiated neuroblasts with hyperchromatic nuclei with an admixture of larger, more differentiated cells with enlarged nuclei and prominent nucleoli. We therefore used hybridization *in situ* to ask whether a similar heterogeneity might be apparent in the expression of N-*myc*. Three populations of cells were found: those marked by very dense accumulation of silver grains, indicative of relatively intense N-*myc* expression (Fig. 5*B*, arrowheads); those with evidence of intermediate expression of N-*myc*; and cells in which expression of N-*myc* was not apparent. Intense or intermediate N-*myc* expression was seen in many but not all of the small neuroblastic cells; the larger differentiated cells seemed to have fewer grains, although a statistical analysis of grain counts was not performed. The distribution of N-*myc* expression was similar in both the tumors (AR and JT) we examined. When sections pretreated with RNase were analyzed, no autoradiographic signal above background was detected (not shown).

To demonstrate that the heterogeneity of N-*myc* expression could not be attributed to an analytical artifact, we also analyzed cells from the Kelly line of neuroblastoma (Fig. 5*C*). Enhanced expression of N-*myc* was apparent in virtually all of the cells. In contrast, no expression was detected in control sections of normal kidney from the area surrounding the neuroblastoma (Fig. 5*D*) or from a Wilm tumor (not shown). We conclude that the biological heterogeneity of cells within neuroblastomas is mirrored by the distribution of expression of N-*myc* and that enhanced expression of the gene may be restricted to a limited population of tumor cells.

DISCUSSION

N-*myc* May Be Expressed Preferentially in Neuroectodermal Cells. We detected expression of N-*myc* in three related settings: mouse and human neuroblastoma cells in which the gene was not amplified; cells of human neuroblastomas bearing amplified N-*myc*; and a line of retinoblastoma cells in which N-*myc* is amplified. By contrast, we found no evidence of N-*myc* expression in human epidermal fibroblasts and in lines of cells from a variety of human malignancies representing diverse embryological lineages. It therefore appears that expression of N-*myc* may normally be limited to cells originating from the neuroectoderm and that amplification of the gene may merely enhance the usual constitutive levels of expression. Whatever its origins, the apparent specificity of N-*myc* expression may eventually prove useful for the diagnosis and evaluation of neuroblastoma in clinical contexts. In particular, it is important to distinguish neuroblastoma from morphologically similar tumors (e.g., Ewing sarcoma, embryonal rhabdomyosarcoma) of different derivations, different prognosis, and different recommended therapeutic regimens. Amplification of N-*myc* may assist in making this distinction.

Mechanism of Enhanced Expression. Amplification of DNA could suffice to explain the enhanced expression of N-*myc* in human neuroblastomas, but two observations raise the possibility that other mechanisms may also pertain. First, the relative abundance of N-*myc* RNA in tumor cells did not correlate with the extent of amplification. For example, N-*myc* was amplified 15- to 20-fold in IMR-32 cells and 100- to 120-fold in Kelly cells, yet both lines displayed *ca.* 60- to 80-fold elevation of N-*myc* expression (Table 1). By contrast, other forms of tumors have displayed a rough cor-

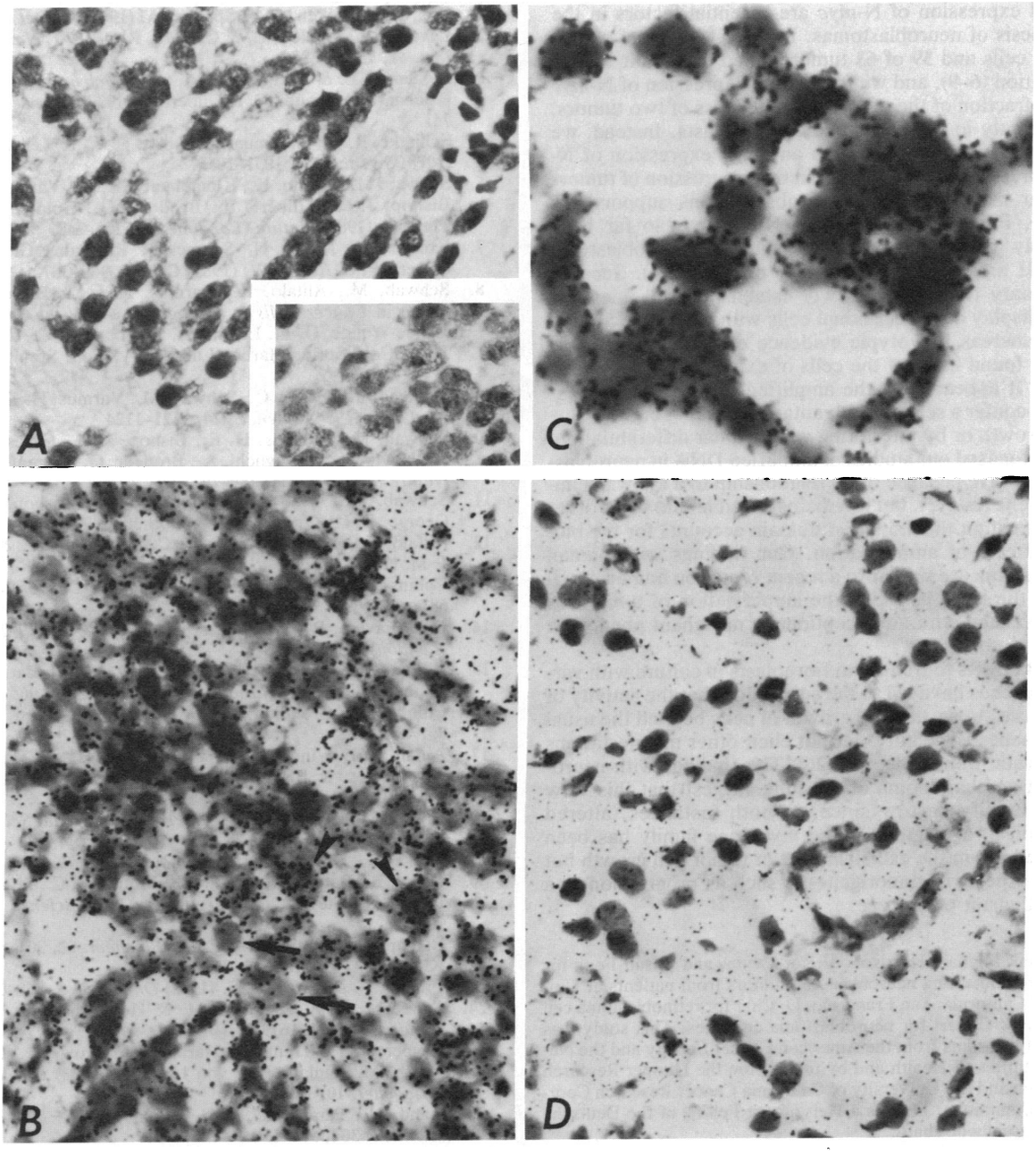

FIG. 5. Analysis of N-*myc* expression in neuroblastoma cells by *in situ* hybridization. (*A*) Human neuroblastoma, consisting predominantly of neuroblastic cells with centrally arranged neural processes forming characteristic Homer–Wright rosettes. In some areas, differentiation towards ganglion cells occurs, as indicated by nuclear and cellular enlargement with prominence of nucleoli (*Inset*; same magnification). (Hematoxylin/eosin; ×560.) (*B*) Human neuroblastoma. *In situ* hybridization with ^{125}I-labeled N-*myc* probe. Note dense grain accumulation over only a few cells (arrowheads), while others are devoid of detectable autoradiographic signal (arrows). (Autoradiograph with hematoxylin/eosin counterstain; ×560.) (*C*) Human neuroblastoma tissue culture line Kelly. *In situ* hybridization with ^{125}I-labeled N-*myc* probe reveals marked concentration of grains over the cultured neuroblastoma cells. (Autoradiograph with hematoxylin/eosin counterstain; ×1500.) (*D*) Normal human kidney, same patient as in *A* and *B*. *In situ* hybridization with ^{125}I-labeled N-*myc* probe. Sections shown in *B* and *D* were mounted on the same slide to provide identical conditions. There are individual, nonspecific grains within and between two renal tubules. The clustering of grains over the cells, as seen in neuroblastoma, is absent. (Autoradiograph with hematoxylin/eosin counterstain; ×560.)

respondence between the extent to which cellular oncogenes are amplified and the enhanced expression of these genes (11–14, 17). Second, in some of the neuroblastoma lines and tumors, we have encountered novel restriction endonuclease sites (unpublished data). Although the origin of these novel sites is not known, their presence may signal structural abnormalities that could account for the enhanced expression of N-*myc*. For example, we and others have found that amplified domains of DNA are frequently translocated from one chromosome to another (6, 7, 10), and at least in one case (the colonic carcinoma cell line COLO 320) this process is accompanied by alteration of the structure of a cellular oncogene (c-*myc*; unpublished data). Recent studies on c-*myc* have raised the possibility that translocations of DNA can activate or enhance the expression of otherwise quiescent genes (20–22).

Does N-*myc* Have a Role in the Genesis of Neuroblastomas? We have found in one patient from which tumor and normal tissue were available that N-*myc* is amplified in the neuroblastoma but not in normal kidney. This shows that amplification of N-*myc* is a somatic rather than a germinal mutation. It is unclear, however, whether the amplification and

enhanced expression of N-*myc* are essential factors in the early genesis of neuroblastomas. Four of 18 lines of neuroblastoma cells and 39 of 63 tumors failed to exhibit N-*myc* amplification (6–9), and we found high expression of N-*myc* in only a fraction of the cells within the mass of two tumors, predominantly in undifferentiated neuroblasts. Instead, we suggest that when amplification and high expression of N-*myc* occur, they may be a factor in the progression of tumors to greater malignancy. Several observations support this postulate. First, amplification of N-*myc* has so far been found only in the more advanced stages of neuroblastoma (stages III and IV) that have poor prognosis (9). Second, both primary tumors and newly established cell lines frequently display only occasional cells with DMs or HSRs (2, 23–25), whereas karyotypic evidence of amplified DNA is generally found in all of the cells of extensively propagated lines (4). It appears that the amplified DNA containing N-*myc* may confer a selective advantage on cells—e.g., by sustaining growth or by interfering with cellular differentiation. We have focused our studies of amplified DNA in neuroblastoma on N-*myc* because of its relationship to a known cellular oncogene (c-*myc*), but it remains possible that some other sequence within the amplified domain accounts for the biological effects of amplification. Our findings with human neuroblastoma are similar to a recent report on human carcinoma of the lung, in which the amplification of c-*myc* has been correlated with an exceptionally malignant phenotype (15).

Recent efforts to transform fibroblasts in culture with various oncogenes have led to the suggestion that the activity of c-*myc* serves to sustain the growth of cells beyond the usual point of senescence but does not elicit other manifestations of neoplastic transformation (26). The findings with neuroblastoma and carcinoma of the lung suggest that this view may be unduly narrow since, in both instances, altered expression of a member of the *myc* gene family has been implicated not in the establishment of tumor cell growth but in other aspects of tumorigenesis, such as progression to a more malignant phenotype.

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