Activated Expression of the N-*myc* Gene in Human Neuroblastomas and Related Tumors

Abstract. *In neuroblastoma lines in which the N-*myc *gene is present as a single copy, the expression of N-*myc *as messenger RNA is increased relative to that in nonneuroblastoma cell lines and tumors. The increase of expression in neuroblastomas with amplified N-*myc *genes is the result of (i) an increase in the absolute amount of expression of each N-*myc *gene and (ii) an increase in the copy number of the N-*myc *gene. A second gene—which is amplified in many of the same lines as N-*myc*—is expressed to about the same degree in most human cell lines and primary tumors regardless of origin (when normalized to gene copy number). Thus, a change in the regulation of N-*myc *expression in neuroblastomas and certain other tumors results in greatly increased expression of each N-*myc *gene copy.*

The concept that gene amplification may play a role in the genesis or progression of certain tumors is supported by the finding of amplification and increased expression of the cellular homologs of known viral oncogenes in various types of mammalian tumors and tumor lines (*1–9*). Although none of the classical oncogenes are amplified in human neuroblastomas (*10, 11*), we have previously cloned a 2.0 kb Eco RI fragment of human genomic DNA (referred to as NB-19-21) which is weakly homologous to, but distinct from, the v-*myc* (and classical c-*myc*) oncogene and which is amplified in 8 of 12 tested human neuroblastoma cell lines (*10*). Similar results have been reported by others (*11, 12*). The gene identified by the NB-19-21 sequence is referred to as N-*myc* (*10, 12*).

To further characterize the expression of the N-*myc* gene and to investigate its potential role in the biology of neuroblastomas, we have characterized the size and quantity of N-*myc*–specific RNA sequences present in various tumor types. For these analyses, RNA blotting techniques were used to assay polyadenylated [poly(A)$^+$] RNA for hybridization to the ^{32}P-labeled NB-19-21 insert (Fig. 1); the quantitative aspects of these studies were confirmed by slot blotting analyses (legend to Table 1). The 3.2-kb N-*myc*–specific RNA was present at high levels in all of the neuroblastoma cell lines in which the N-*myc* gene was amplified (Fig. 1A, lanes 2 to 7, and Table 1), and at readily detectable levels in the three neuroblastoma cell lines tested in which the gene was not amplified (Fig. 1A, lanes 8 to 10, and Table 1). The higher molecular weight bands represent precursors. However, we only detected N-*myc*–specific RNA in 4 (out of 36) nonneuroblastoma primary human tumors and tumor cell lines tested (Fig. 1A and Table 1) of which three were neuroectodermal in origin, including the Y79 retinoblastoma cell line in which the gene is amplified (*10, 13*). Even in the neuroblastoma lines in which the N-*myc* gene was not amplified, the level of N-*myc*–specific RNA was, at a minimum, 10 to 40 times greater than that of the 31 nonneuroblastoma lines in which N-*myc* RNA was not detected (Fig. 1A and Table 1). We refer to this increase in the relative level of N-*myc* expression as activation. Because the level of N-*myc* RNA per N-*myc* gene copy in amplified lines is roughly similar to that in unamplified neuroblastoma lines (Table 1), expression of most, if not all, of the amplified N-*myc* gene copies is "activated." These results indicate that N-*myc* expression shows a very restricted pattern of activation that is primarily limited to neuroblastomas and some related tumor or cell types.

To determine whether any genes in addition to N-*myc* were overexpressed or amplified in neuroblastoma lines, we have isolated a "neuroblastoma-specific" complementary DNA (cDNA) clone, pG21, by differentially screening a cDNA library prepared from poly(A)$^+$ RNA of neuroblastoma IMR-32 (legend to Fig. 1). To determine whether pG21-related sequences were amplified in various neuroblastoma lines, we screened Eco RI–digested genomic DNA from the cell lines with the ^{32}P-labeled 1.5-kb Eco RI insert of pG21; the pG21 probe hybridized to fragments of 6.6, 5.4, 4.0, 3.2, and 1.5 kb in Eco RI-digested genomic DNA from all of the lines tested (data not shown). Quantitative analyses of these blots indicated that the pG21 sequences were amplified from 75- to 500-fold in five of the neuroblastoma lines, each of which also had highly amplified N-*myc* genes (Table 1). However, the pG21-specific fragments were not amplified in three neuroblastoma lines in which N-*myc* is amplified (Table 1) or in any of the neuroblastoma or other lines in which N-*myc* is not amplified (Table 1). Like the N-*myc* gene (*10, 13*), the pG21 sequence was also extensively amplified in the Y-79 retinoblastoma line (Table 1). As we previously observed for the N-*myc* gene (*10*), we have found that pG21 is also located on the short arm of chromosome 2 (*14*). This result, in combination with those described above, suggests that the pG21 gene may be a passenger within a large N-*myc* amplification unit.

We also examined the nature and quantity of the pG21-specific RNA sequences present in the various neuroblastoma lines. The pG21 probe identified a 3.2-kb poly(A)$^+$ RNA species which comigrated with the N-*myc*–spe-

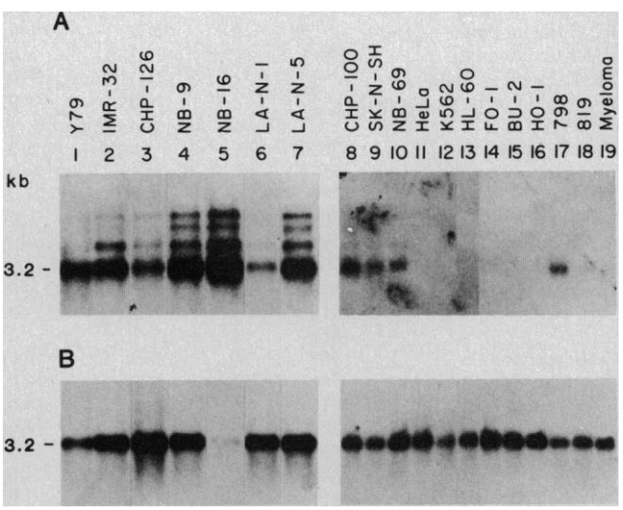

Fig. 1. Expression of N-*myc* and pG21 in human tumor cell lines. The derivation of the N-*myc*–specific probe has been described previously (*10*). The pG21 probe was isolated from an IMR-32 cDNA library by a differential screening procedure (*9*). Briefly, double-stranded cDNA prepared from IMR-32 poly(A)$^+$ RNA was cloned into the Eco RI site of lambda bacteriophage Charon 16a. Recombinant bacteriophage which hybridized to a total IMR-32 cDNA probe, but not a total HeLa cell cDNA probe, were isolated. (A) N-*myc*. Approximately 0.5 μg of poly(A)$^+$ RNA (*10*) from lines represented in lanes 1 to 7 and 10 μg from lines represented in lanes 8 to 19 were fractionated through a formaldehyde-agarose gel (1 percent) (*16*), transferred to nitrocellulose, and hybridized to approximately 5×10^6 cpm of the nick-translated 2.0-kb Eco RI insert of NB-19-21 (specific activity 3×10^8 cpm/μg) as described previously (*10*). (B) pG21. The NB-19-21 probe was removed from the filters shown in (A) by boiling in 1× standard saline citrate and the filters subsequently hybridized to approximately 5×10^6 cpm of the nick-translated 1.5-kb pG21 insert (specific activity 1×10^9 cpm/μg).

cific RNA species (Fig. 1B). However, other cross-hybridization analyses demonstrated that the N-*myc* and pG21 mRNA sequences were distinct, and we have observed that purified pG21 and N-*myc* RNA's encode distinct proteins (*15*). The pG21-specific RNA sequences were present at similar levels in all tested tumors in which the pG21 gene was present as a single copy (Fig. 1B and Table 1) and at greatly increased abundance in the five neuroblastomas and the retinoblastoma in which the gene was amplified (Fig. 1B, lanes 1 to 4 and 6 and 7, and Table 1). Thus, unlike the N-*myc* gene, expression (activation) of the pG21 gene was not restricted to a limited set of tumor types.

Table 1. Amplification and expression of N-*myc* and pG21. The measurement of N-*myc* and pG21 amplification and quantitation of DNA were as previously described (*10*). The level of N-*myc* and pG21 mRNA was quantitated by either Northern blotting (see legend to Fig. 1) or RNA slot-blotting procedures (*17*) or both procedures. Duplicate filters were hybridized with, respectively, the NB-19-21 probe, the pG21 probe, or (to control for the amount of RNA loaded), a human actin probe (pHF1, a gift from L. Kedes). The results are expressed as the amount of N-*myc* and pG21 mRNA relative to that of NB-69 (which was taken as 1). A value of <0.1 for N-*myc* RNA indicates that there was no detectable signal above background in the slot-blotting analyses; if the value was <0.05, no detectable signal was observed after assaying with the more sensitive Northern blotting analysis. ND, not determined.

Cell lines and tumors		Amplification relative to NB-69			
		N-*myc*	N-*myc* mRNA	pG21	pG21 mRNA
Neuroblastoma cell lines					
IMR-32		25	100	75	100
CHP-126		100	50	500	200
CHP-134		700	ND	1	ND
NB-19		600	ND	1	ND
LA-N-1		100	50	300	100
NB-9		300	200	100	100
NB-16		300	400	1	2
LA-N-5		50	200	200	100
CHP-100		1	2	1	1
SK-N-SH		1	1	1	0.5
NB-69		1	1	1	1
Other human tumor cell lines					
Y79 (retinoblastoma)		15	50	25	20
COLO 320 (colon carcinoma)*		1	1†	ND	ND
MC-IXC (peripheral neural epithelioma)		1	ND	1	ND
HeLa (cervical carcinoma)		1	<0.05	1	1
K562 (CML)		1	<0.05	ND	1
HL-60 (promyelocytic leukemia)		1	<0.05	1	1
T24 (bladder carcinoma)		1	<0.1	ND	ND
MCF-7 (breast carcinoma)		1	<0.1	ND	ND
FO-1 (melanoma)		ND	<0.05	ND	2
BU-2 (melanoma)		ND	<0.05	ND	2
HO-1 (melanoma)		ND	<0.05	ND	2
SK-7 (T-cell lymphoma)		ND	<0.1	ND	1
FRO 2.2 (T-cell lymphoma)		ND	<0.1	ND	1
Primary human tumors					
Breast carcinoma	753	1	<0.1	ND	0.25
	754	1	<0.1	ND	0.25
	780	1	<0.1	ND	0.25
	789	1	<0.1	ND	0.5
	792	1	<0.1	ND	0.5
	793	ND	<0.1	ND	0.5
	810	ND	<0.1	ND	1
	812	ND	<0.1	ND	1
	821	1	<0.1	ND	0.5
Colon carcinoma	785	ND	<0.1	ND	0.5
	786	ND	<0.1	ND	0.5
	815	1	<0.1	1	1
Lung carcinoma	787	ND	<0.1	ND	0.5
Stomach carcinoma	795	ND	<0.1	ND	1
	808	ND	<0.1	ND	0.5
	819*	1	0.1	ND	1
Kidney carcinoma	798	ND	1	ND	1
	803	1	<0.1	1	1
Esophageal carcinoma	801	ND	<0.1	ND	2
	816	ND	<0.1	ND	0.5
Myeloma	OSS	1	<0.05	ND	1
T-cell lymphoma	OUT	1	<0.1	ND	1
B-cell lymphoma	RM	ND	<0.1	ND	0.5

*Neuroendocrine tumors. †N-*myc* probe identified only a 5.6-kb RNA in Northern blot analysis.

N-*myc* gene amplification occurs in many but not all human neuroblastomas (*10–12*). We have now isolated a second gene which is specifically amplified in neuroblastomas and related tumors, but in a subset of those in which N-*myc* is amplified. Thus, frequent amplification of a particular gene within a certain class of tumors does not necessarily imply a role for that gene in the biology of the tumor. However, in this regard, we have now shown that the expression of individual N-*myc* genes was activated in all tested neuroblastomas including those with amplified N-*myc* genes as well as those in which the genes are unamplified. We have not been able to detect N-*myc* gene expression in other tumor tissues tested except for a retinoblastoma (also of neuroectoderm origin), two carcinomas of neuroendocrine origin, and one carcinoma of uncharacterized origin (Table 1). However, the N-*myc* gene is not activated in all tumors of neuroectoderm origin; for example, we did not find expression in three melanoma lines (Fig. 1A and Table 1). Recently, the N-*myc* gene was found to be expressed in several retinoblastomas (*13*); this result was interpreted as suggesting a special role for N-*myc* gene expression in retinoblastomas, possibly related to the retinoblastoma-specific deletion on chromosome 13 (*13*). Our studies do not support that interpretation because the level of N-*myc* gene expression (per gene copy) appears to be specifically increased in neuroblastomas (which do not have the chromosome 13 deletion), retinoblastomas, and perhaps in certain other related tumors as well. One possible explanation for the restricted pattern of N-*myc* gene expression is that N-*myc* expression may be linked to the genesis or progression of these tumors. N-*myc* activation might play a more primary role, while increased expression due to amplification may be a later event linked to tumor progression (*9, 10, 12*). A second explanation for the restricted pattern of N-*myc* gene expression is that activation of the gene may be a property of the normal cells from which these tumors derive and not be related to the neoplastic state. Examination of the expression of the N-*myc* gene in normal tissues and at different developmental stages is necessary to distinguish between these possibilities.

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JC Virus Enhancer-Promoter Active in Human Brain Cells

Abstract. *A human papovavirus, JCV, is the etiologic agent of the fatal demyelinating disease, progressive multifocal leukoencephalopathy. The JCV 98-base-pair tandem repeats, located to the late side of the viral replication origin, were shown to be a transcriptional regulatory element with enhancer-like activity in human fetal glial cells. These tandem repeats share significant homology with the 82-nucleotide rat brain–specific identifier RNA sequence.*

The JC virus (JCV), a human papovavirus, is the etiologic agent of progressive multifocal leukoencephalopathy and has been isolated from brain lesions of patients with this fatal demyelinating disease (*1*). It has also been isolated from the urine of pregnant women (*2*) and of immunocompromised patients (*3*). In contrast to the closely related viruses SV40 and BKV, JCV has an extremely restricted host range. Although human uroepithelial cells, amnion cells, and endothelial cells are able to support JCV reproduction to a limited degree, JCV grows well only in human fetal glial cells (*4–7*).

Recently, transcriptional regulatory elements called "enhancers" have been identified within the genomes of a number of viruses (*8–11*). These elements increase the level of transcription of many genes in a manner that is relatively independent of position and orientation. A second control element, the "TATA" box (T, thymine; A, adenine), also influences the level of transcription and positions the cap site of the messenger RNA (mRNA) at a distance 25 to 30 base pairs (bp) downstream. A characteristic property of enhancers is their relative host cell specificity. Some cellular enhancer elements such as those associated with the immunoglobulin heavy chain and light chain genes (*12–15*) and the insulin and chymotrypsin genes (*16*) appear to function only in specific tissues. The strong T-cell tropism of several retroviruses, including the type II human T-cell leukemia virus, is derived from information encoded within the long terminal repeats that contain their enhancer element (*17–19*). In this study, we have identified an element in the JCV genome which shows greatest enhancer-like activity in human fetal glial cells. This preferential enhancer activity is likely to be a major determinant in the restricted host range of JCV.

The region of the origin of DNA replication has recently been sequenced in the JCV genome (*20*) (Fig. 1). It contains a tandem duplication of 98 bp located in a position analogous to that of the SV40 enhancer (a 72-bp repeat) and the BKV enhancer (a 68-bp triplication). In terms of nucleotide sequence homology, SV40, BKV, and JCV are very similar except for the regions of tandem repeats. Both 98-bp repeats in JCV contain a TATA box; in contrast, the SV40 and BKV enhancer elements are located upstream of single TATA boxes. We determined

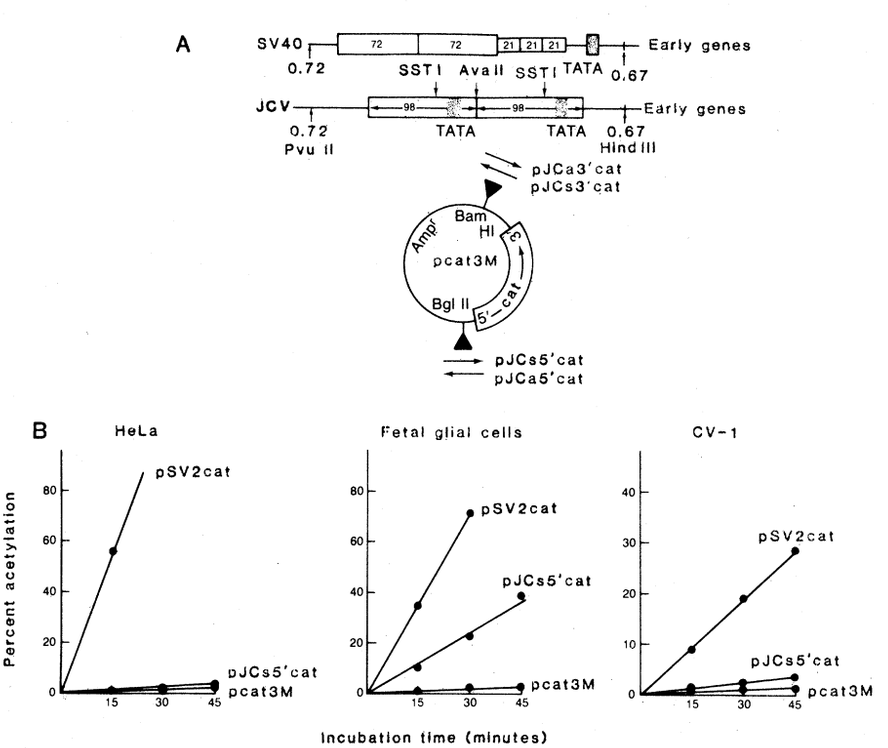

Fig. 1. (A) Construction of the JCV-cat plasmids. The 286-bp JCV fragment from 0.67 to 0.72 map units was excised with Pvu II and Hind III and ligated into the pcat3M plasmid. The pcat3M plasmid contains the bacterial CAT gene without promoter elements (*19*). The JCV fragment was placed in the Bgl II site, 5′ of the CAT gene, in both the sense (pJCs3′cat) and antisense (pJCa5′cat) orientations (relative to the JCV early transcription unit), and likewise in both the sense (pJCs3′cat) and antisense (pJCa3′cat) orientations in the Bam HI site 3′ of the CAT gene. (B) Activation of the CAT gene by JCV 98-bp repeats. Equivalent amounts (20 μg) of the CAT plasmids containing either the JCV 98-bp repeats (pJCs5′cat), the SV40 72-bp repeats and TATA box (pSV_2cat), or no promoter element (pcat3M) were transfected into the different cell types by calcium phosphate precipitation (*22*). The cells were harvested 72 hours after transfection and analyzed for CAT activity (*24*). The percent acetylation of ^{14}C-labeled chloramphenicol was determined by thin-layer chromatography and subsequent radioactive scintillation counting.