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Genetics

N-*myc* amplification in multiple homogeneously staining regions in two human neuroblastomas

(neurotoxin-responsive Na^+ channels/gene amplification)

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ABSTRACT **Molecular characterization of two human neuroblastoma cell lines has revealed that both contain multiple homogeneously staining regions (HSRs), each representing a chromosome site of N-*myc* amplification. The newly established cell line CHP-382/JK had two cytogenetically distinct populations with several identical chromosomal abnormalities, indicating a common progenitor cell. Each population had one HSR, one on chromosome 5 at q31-34 and the other on chromosome 2 at q31-32. Chromosomal *in situ* hybridization with the N-*myc* probe pNb-1 demonstrated that both HSRs contained amplified copies of N-*myc*. Southern blot analysis confirmed amplification of N-*myc* sequences in genomic DNA of CHP-382/JK. Chromosomal features of CHP-382/JK shared with other neuroblastoma cell lines were the deletion of 1p and the presence of extra 17q material. In addition, the cells were highly reactive to monoclonal antibody PI 153/3 used to identify human neuroblastoma. CHP-382/JK cells were further characterized as neuronal cells by the expression of neurotoxin-responsive Na^+ channels. Another neuroblastoma cell line, CHP-134, contained a single cell population with three HSRs, one in the short arm of each chromosome 7 and one in the long arm of a chromosome 6. All three HSRs contained amplified copies of N-*myc* as shown by *in situ* hybridization with the N-*myc* probe pNb-1. One of the 7p HSRs was acquired during culture of CHP-134 cells, whereas the 2q HSR of CHP-382/JK was lost. Such findings highlight the continued process of N-*myc* amplification and transposition *in vitro*. To our knowledge, amplification of N-*myc* in multiple HSRs has not been documented previously in neuroblastoma cell lines.**

Cellular proto-oncogenes comprise a group of DNA sequences whose abnormal expression or function may be related to tumorigenesis (1). Recently, fragments of genomic DNA have been independently isolated from two human neuroblastoma cell lines (2, 3) and called N-*myc* because of a partial homology to c-*myc* and v-*myc* (2, 4). However, unlike c-*myc*, amplification of N-*myc* has been described, thus far only in tumors of neural origin, neuroblastoma (5) and retinoblastoma (6).

Amplified cellular DNA sequences are frequently manifested as homogeneously staining regions (HSRs) or double minutes (DMs) (7). These karyotypic abnormalities have been described in neuroblastoma tumors (8) and cell lines (7, 9) and have been shown by *in situ* hybridization to be the site of the amplified copies of the N-*myc* gene (2, 10).

Karyotypic examination of human neuroblastoma tumors and cell lines has occasionally demonstrated cells with more than one HSR-bearing chromosome. One such cell line, NGP, with 120- to 140-fold amplification of N-*myc*, has HSRs at 4p16 and 12q13. *In situ* hybridization studies showed N-*myc* amplified only on the 4p HSR, whereas the 12q HSR showed no N-*myc* amplification (2). Therefore, extra copies of N-*myc* could account for only one of the HSRs in the NGP cell line and no other cell lines with multiple HSRs have been examined for N-*myc* localization.

Here we describe cytogenetic and molecular studies of two neuroblastoma cell lines, each with multiple HSRs. We show that all detectable HSRs in both cell lines represent chromosomal sites of N-*myc* amplification.

MATERIALS AND METHODS

Establishment and Growth of Cell Lines. Neuroblastoma CHP-382 was derived from an abdominal tumor taken at autopsy from a patient with stage IV disease as classified by Evans *et al.* (11). The tumor was dispersed, placed in culture medium, and refed three times per week with RPMI 1640 medium containing 10% fetal calf serum in an atmosphere of 8% CO_2 in air at 37°C. Two distinct cell types grew: small round unattached cells and attached cells. The unattached cells were centrifuged from the used medium and added back to the CHP-382 cultures for the initial 3 weeks, at which time they were removed and placed into separate flasks. These unattached cells, CHP-382/JK, gradually adhered to the flasks and were passaged twice. An aliquot was frozen in liquid N_2. The remaining CHP-382/JK cells were passaged one additional time and examined for karyotype and cytotoxicity with monoclonal antibody PI 153/3 (12). Concurrently, clones 1, 2, and 4 were derived from CHP-382 by dilution and plating in microtiter wells.

CHP-134 was established (13) and maintained as described (14). All cultures were free of mycoplasma as detected by the method of Becker and Levine (15).

Chromosome Preparation. For karyotyping and *in situ* hybridization, the cells were treated with colchicine for 15–30 min (16). After hypotonic treatment and fixation, air-dried slide preparations were made for Giemsa banding by using standard techniques (8).

Southern Blot Analysis. DNA samples from CHP-382/JK, CHP-134, and peripheral blood leukocytes from two normal males were digested with *Eco*RI and subjected to electrophoresis in a horizontal agarose gel (0.8%) in 89 mM Tris with 89 mM boric acid in 2 mM EDTA (pH 7.8). *Hin*dIII-digested λ phage DNA was included on each gel as a reference. Gels were stained with ethidium bromide, photographed, and transferred to nitrocellulose sheets (17).

Preparation of Labeled DNA Probes and DNA Hybridization. The N-*myc* probe (pNb-1), a 1.0-kilobase (kb) *Eco*RI–*Bam*HI genomic DNA fragment in pBR322, was kindly provided by J. M. Bishop. The *Eco*RI–*Bam*HI DNA frag-

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Abbreviations: HSR, homogenously staining region; DM, double minute; kb, kilobase(s).

ment was isolated from the whole plasmid (18) and labeled with [^{32}P]dCTP (New England Nuclear) by nick-translation with enzymes from Bethesda Research Laboratories. DNA on nitrocellulose sheets was hybridized to the denatured ^{32}P-labeled probe DNA for 13 hr at 42°C in a hybridization solution containing 50% formamide and then washed six times in decreasing dilutions of 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.

***In Situ* Hybridization of N-*myc*.** Chromosomal *in situ* hybridization studies were performed as described (19). N-*myc* probe pNb-1 was ^{3}H-labeled by nick-translation to a specific activity of 4×10^{7} cpm/μg of DNA (20) and separated from free labeled nucleotides by Sephadex G-50 chromatography. Salmon sperm carrier DNA (1000-fold excess) was added. After ethanol precipitation, the DNA was resuspended in 50% formamide in 0.3 M sodium chloride/0.03 M sodium citrate with 10% dextran sulfate (pH 7.0). Denatured probe DNA (70 ng/ml) was added to the slides and hybridized for 18 hr at 37°C. Slides were washed at 39°C in 0.3 M sodium chloride/0.03 M sodium citrate, coated with liquid nuclear track emulsion (Kodak NTB-2), and developed in Kodak Dektol after 4–10 days. Chromosomes were stained by using a modified Wright/Giemsa procedure (21).

Neurotoxin-Responsive Efflux of ^{86}Rb. The assay for the efflux of ^{86}Rb was performed as described (14, 22). ^{86}Rb efflux was stimulated by veratridine (100 μM) and *Leiurus quinquestriatus* scorpion venom (5 μg/ml) and inhibited by 1 μM tetrodotoxin as characteristic of active Na^{+} channels of electrically excitable membranes (23, 24).

Reaction with Monoclonal Antibody PI 153/3. Monoclonal antibody PI 153/3 was generated against human neuroblastoma cells (12). The antibody reacts with all neuroblastoma cell lines and tumors thus far examined (25) and certain B-cell leukemias (26, 27). A cytotoxic assay (28) in the presence of complement was used to measure the antigenicity of CHP-382 and CHP-134. The assay measures the release of creatine phosphokinase from the cells (28).

RESULTS

Presence of Two or More HSRs Each with Amplified N-*myc*. Karyotype analysis of cells derived from the newly established neuroblastoma cell line CHP-382/JK revealed that 46% of the cell population contained a HSR in the long arm of chromosome 2 inserted at q31-33 and had normal number 5 chromosomes (Fig. 1*a*). The remaining cell population contained a large HSR in the long arm of chromosome 5 inserted at q31-34 and had normal number 2 chromosomes (Fig. 1*b*). No cell had both HSRs. Cells cloned from the original CHP-382 culture, clones 1, 2, and 4, showed only the presence of the HSR in the long arm of chromosome 5.

Although two cytogenetically distinct populations were isolated from the same tumor, both populations had a mode of 46 chromosomes and several identical cytogenetic abnormalities indicating a common progenitor cell. These abnormalities included a deleted chromosome 1 (p22 → pter) and a $9p^{+}$ chromosome that appeared to contain extra 17q material 17q11 → qter (Fig. 1). The population with the HSR in 2q had an additional abnormality t(4;11)(q28;q23) (Fig. 1*a*).

The chromosome of CHP-382/JK and clone 4 cells were examined by the *in situ* hybridization technique with an N-*myc* probe, pNb-1 (2). Greater than 70% of the chromosomally located grains were found, frequently in clusters, over the 2q HSR and 5q HSR (Fig. 2). The HSR-bearing chromosomes were easily identified by their overall length and morphology. Such extensive labeling, occurring only at the sites of the HSRs, indicated the presence of multiple copies of the N-*myc* sequence within both of these chromosomal regions.

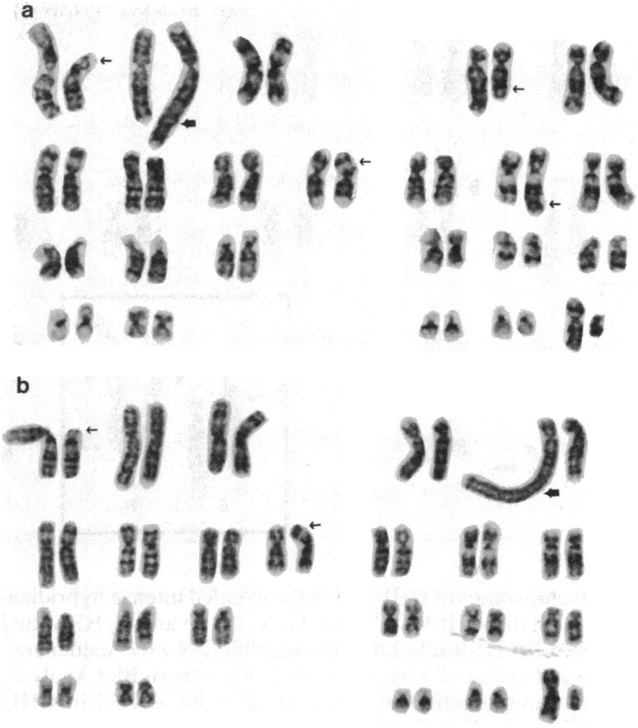

FIG. 1. Representative Giemsa-banded karyotypes from the two populations of CHP-382/JK cells. Abnormal chromosomes are marked with arrows and HSRs by heavy arrows. (*a*) 46,XY,$1p^{-}$, $2q^{+}$,$9p^{+}$,t(4;11)(q28;q23). Of note are the normal chromosomes 5 and the HSR inserted at a 2q31-33. The extra material on 9p was derived from 17(q11 → qter). (*b*) 46,XY,$1p^{-}$,$5q^{+}$,$9p^{+}$. Of note are the normal chromosomes 2, 4, and 11 and the HSR inserted at 5q31-34. Identical $1p^{-}$ and $9p^{+}$ markers are seen in both cell populations.

To determine if multiple HSRs within a single neuroblastoma cell contained amplified N-*myc* sequences, another neuroblastoma cell line, CHP-134, was examined. These cells, when originally studied, were a pseudodiploid with one HSR in the short arm of chromosome 7 and another in the long arm of chromosome 6 (7). Other chromosomal abnormalities included a $6p^{+}$, $7q^{-}$, $1p^{+}$, and $19q^{+}$ (Fig. 3). Recent analysis of the cell line showed the same two HSRs and the 1, 6, and 19 markers. In addition, the cells had acquired a third HSR in the short arm of the $7q^{-}$ chromosome (Fig. 3 *Inset*). *In situ* hybridization of the N-*myc* probe pNb-1

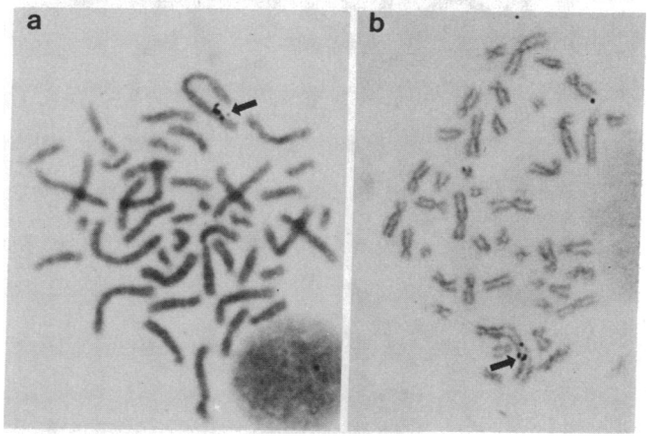

FIG. 2. Representative autoradiographs from *in situ* hybridization of ^{3}H-labeled N-*myc* probe (pNb-1) to metaphase chromosome preparations from CHP-382/JK cells. Arrows indicate clusters of grains on the relevant HSRs: 2q31-33 (*a*); 5q31-34 (*b*).

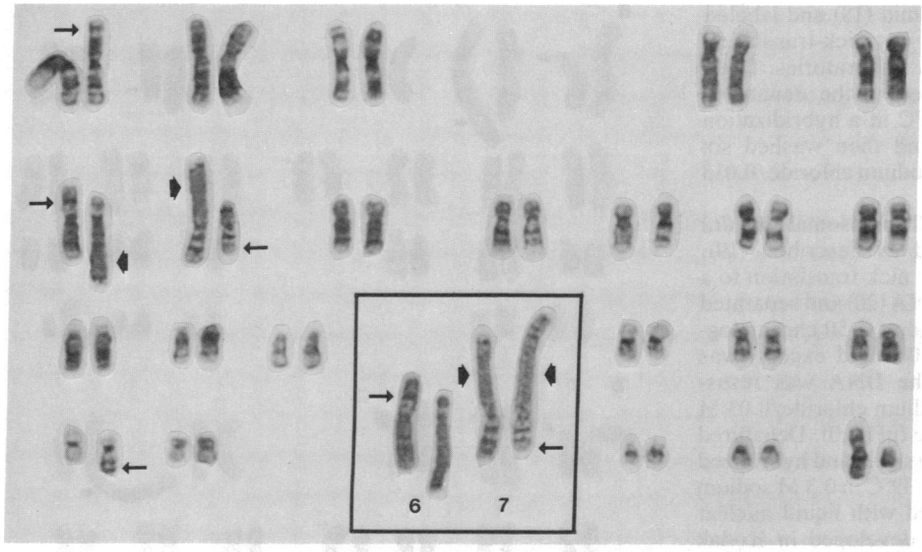

FIG. 3. Representative Giemsa-banded karyotype of early passage CHP-134 cells (29). Abnormal chromosomes are marked with arrows and HSRs by heavy arrows. 46,XY,1p$^+$,6p$^+$,6q$^+$,7p$^+$, 7q$^-$,19q$^+$. Of note are the abnormal chromosomes 6 and 7. (*Inset*) Chromosomes 6 and 7 from a cell at latter passage, showing the new HSR in the short arm of the 7q$^-$ chromosome.

to chromosomes of CHP-134 cells revealed intense hybridization to all three HSRs (Fig. 4). Thus, the 7p and 6q HRSs and the new 7p HSR all contained amplified N-*myc* sequences.

Amplification of N-*myc* Detected by Southern Blot Analysis. N-*myc* amplification was estimated to be 40-fold in CHP-382/JK cells (Fig. 5). This was determined by the Southern blot analysis of N-*myc* hybridization to 15 μg of normal DNAs and to multiple dilutions of CHP-382/JK DNA. Amplification of N-*myc* in genomic DNA from CHP-382/JK confirmed the observations obtained by *in situ* hybridization studies.

Transitory Nature of One HSR. CHP-382/JK cells (passage 3) were removed from liquid N_2 storage, grown for four passages, and then karyotyped. After this brief time in culture, cell line CHP-382/JK had no cells containing the HSR on chromosome 2 but had cells with the HSR on chromosome 5 similar to the clones derived from this tumor. *In situ* hybridization showed that N-*myc* was amplified on chromosome 5 but, as expected, no cells had detectable amplification of N-*myc* on chromosome 2.

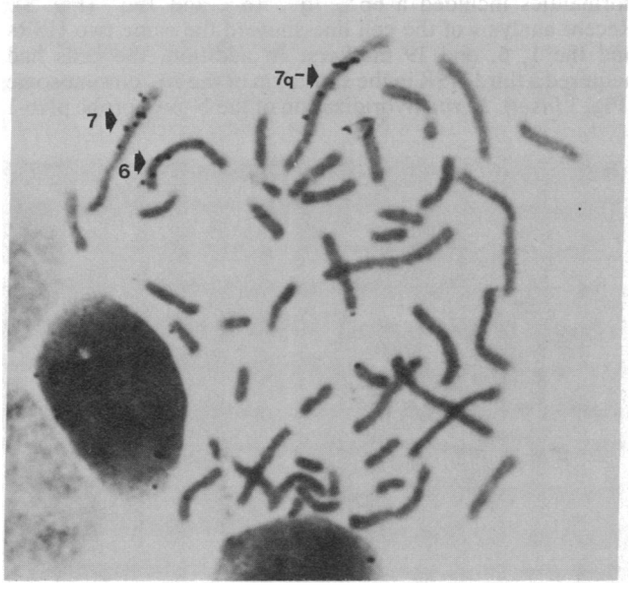

FIG. 4. Representative autoradiograph from *in situ* hybridization of ^{3}H-labeled N-*myc* probe (pNb-1) to a metaphase chromosome preparation from CHP-134 cells. Arrows indicate multiple grains on the three HSRs in chromosomes 7, 6, and 7q$^-$.

To determine whether cell line CHP-382/JK retained both populations after reconstitution from liquid N_2 storage, the cells were reconstituted and grown for 2 weeks without further passage. The presence of both populations, cells with a HSR on the long arm of chromosomes 2 and those with a HSR on chromosome 5, was confirmed (Fig. 1). Both HSRs had amplified N-*myc* (Fig. 2).

Active Na$^+$ Channels in CHP-382. To assure that the newly established cell line CHP-382/JK had neuronal properties, the neurotoxin-sensitive Na$^+$ channels were examined. The efflux of ^{86}Rb through the Na$^+$ channel, stimulated by veratridine and scorpion venom and inhibited by tetrodotoxin, has been reported for human neuroblastoma cell line CHP-134 (14). CHP-382/JK cells had active Na$^+$ channels (Fig. 6) responding to stimulation by veratridine and scorpion venom with an efflux rate constant similar to CHP-134 (14). Tetrodotoxin inhibited the stimulated ^{86}Rb efflux.

Human neuroblastoma cell lines can be induced to differentiate morphologically in the presence of retinoic acid (30). CHP-382/JK cells responded to retinoic acid by the formation of small processes. Furthermore, the cells responded to retinoic acid with increased channel activity after two treatments (Fig. 6) and this stimulated efflux was also inhibited by tetrodotoxin. Thus, this new cell line exhibited properties characteristic of neuronal cells. The expression of neuronal phenotype appeared not to be related to the karyotype of the tumor cells. Since the CHP-382/JK cells retained these properties even in the absence of the chromosome 2 HSR with amplified N-*myc*.

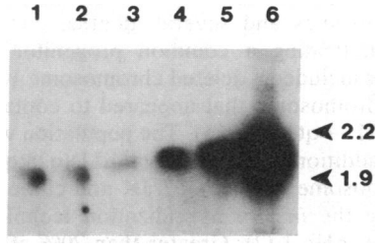

FIG. 5. Amplification of the N-*myc* gene in the neuroblastoma cell line CHP-382/JK. Genomic DNAs were digested to completion with *Eco*RI, transferred to nitrocellulose, and hybridized with ^{32}P-labeled N-*myc* probe pNb-1. Lanes 1 and 2 contained 15 μg of DNA from peripheral blood leukocytes. Lanes 3, 4, 5, and 6 contained 0.15 μg, 0.3 μg, 1.5 μg, and 15 μg of DNA, respectively, from CHP-382/JK cells. Numbers to the right indicate size of DNA in kb. Exposure time was 1 day.

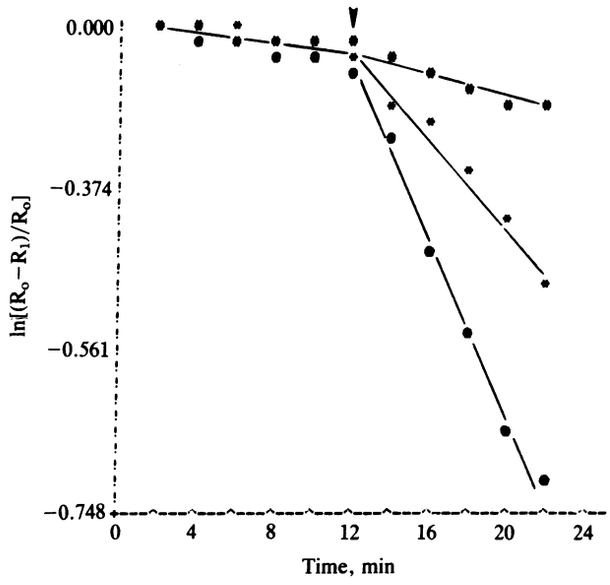

FIG. 6. Computer plot of the neurotoxin-responsive efflux of ^{86}Rb in CHP-382/JK. After growth for 5 days the cells were assayed by incubating with ^{86}Rb. The passive efflux was measured as described (14) for 12 min. At that time 100 μM veratridine and scorpion venom (5 μg/ml) were added (arrowhead) to stimulate the efflux through the Na^+ channel. Aliquots (R_1) were removed at 2-min intervals up to 22 min. The remaining ^{86}Rb was extracted from the cells with 10% trichloroacetic acid and the radioactivity was measured and used to calculate the original amount of ^{86}Rb (R_o) taken up by the cells. The efflux was plotted as the negative number (22). Addition of 1 μM tetrodotoxin (#) abolished the veratridine/scorpion venom-stimulated efflux of ^{86}Rb and did not affect the nonstimulated efflux. CHP-382/JK were grown for 4 days in medium in the presence (●) or absence (*) of 1 μM retinoic acid with two additions.

Reactivity with Monoclonal Antibody. Monoclonal antibody PI 153/3, which was generated against human neuroblastoma cells (12), was cytotoxic to CHP-382/JK cells and clone 4 in the presence of complement, as measured by the release of creatine phosphokinase from the cells (28). Cytotoxicity was evident in the early passage cells as well as those reconstituted from liquid N_2 storage (Table 1). CHP-382/JK cells were found to be highly reactive to antibody PI 153/3, thus providing further evidence that they expressed and maintained the phenotype of neuroblastoma.

The cell number and viability of CHP-382/JK and clone 4 cells were not notably different before or after storage at −70°C. However, the cell number of CHP-382/JK cells was less than either clone 4 or CHP-134 cells after 7 days in culture. The activity of creatine phosphokinase, an enzyme elevated in neuronal cells, was high for all of the cell lines.

DISCUSSION

We have established and characterized a human neuroblastoma cell line, CHP-382/JK, from a patient with stage IV disease. Forty-fold genomic amplification of the 2.0-kb *Eco*RI fragment of N-*myc* (2) was demonstrated in these cells by Southern blotting analysis (Fig. 5). The amplified N-*myc* sequences were shown to be located in two distinct HSRs by chromosomal *in situ* hybridization (Fig. 2). These two HSRs were distributed within two cell populations, no cell having both HSRs. In another neuroblastoma cell line, CHP-134, the location of amplified N-*myc* sequences was also determined with the *in situ* hybridization technique. In contrast to CHP-382/JK cells, CHP-134 cells contained three HSRs within a single cell population and N-*myc* amplification was demonstrated in all three HSRs (Fig. 4). Thus, each of the multiple HSRs in both of these cell lines (CHP-134 and CHP-382) contained amplified copies of N-*myc*. These findings are in contrast to a previous report that only one of two HSRs in the neuroblastoma cell line NGP had extra N-*myc* sequences (2). The amplification of N-*myc* has been shown to be associated with stage IV neuroblastoma (5). This property may also correlate with the ability of neuroblastoma cells to proliferate *in vitro* as well as *in vivo* since established cell lines are usually obtained only from stage IV tumors.

The two HSRs of CHP-382/JK and the three of CHP-134 occupied four distinct autosomal locations, 2q23, 5q31-34, 7p11, and 6q. In addition, in other neuroblastoma cell lines there is direct evidence for N-*myc* amplification in HSRs at other autosomal sites (10). Thus, there appears to be no preferred chromosomal site for N-*myc* integration and amplification. To date, there is no direct evidence to suggest amplification with HSR formation at 2p23-24, which is the chromosomal site of the cellular N-*myc* gene (2, 3). Amplification and transposition of the N-*myc* gene and some as yet undefined flanking sequences may occur by means of a DM intermediate. DMs as an alternative form of amplification to HSRs have been described in the neuroblastoma line CHP-126 (7) and the acute promyelocytic cell line with amplified c-*myc*, HL-60 (31). However, despite karyotypic evolution, including an additional HSR in CHP-134 and the disappear-

Table 1. Characterization of CHP-382 and CHP-134 cells

Cell line	Passage number*	Cytotoxicity, %†	CPK activity, nmol/min per cell × 10^{-4}‡	Cell number per 75-cm^2 flask × 10^7	Cell viability, %§
CHP-382					
JK	4	90	1.1	0.75	78
	13¶	89	3.0	0.65	82
Clone 4	4	56	5.4	3.8	92
	12¶	72	1.9	2.8	78
CHP-134					
	8‖	92	2.8	5.2	79
	24‖	73	5.2	10.0	93

*Passage from original culture.
†Determined by a quantitative biochemical assay (28). Cytotoxicity was measured by the activity of creatine phosphokinase (CPK) following lysis of the neuroblastoma cells by monoclonal antibody PI 153/3 (12) in the presence of guinea pig complement.
‡Assayed as described (28).
§Cells were counted in a hemocytometer and the lack of viability was measured by the uptake of trypan blue.
¶Reconstituted from frozen stock at passage 4.
‖Reconstituted from frozen stock.

ance or loss of the 2q HSR from CHP-382/JK, DMs have never been observed in either of these cell lines.

The cytogenetic studies of CHP-382 showed two distinct populations of cells. Both of these populations have several identical cytogenetic abnormalities indicating that they arose from a common progenitor cell. The common abnormalities include a del(1)(p22 → pter) and a $9p^+$ chromosome that appeared to contained an additional copy of the long arm of chromosome 17(q11 → qter). Thus, in addition to the HSRs, this newly established cell line had several cytogenetic features common to other neuroblastoma cell lines: the deletion of 1p and the presence of extra 17q material (8). CHP-382/JK also expressed other properties of neuronal cells, such as neurotoxin-responsive Na^+ channels (Fig. 6) and reactivity with monoclonal antibody PI 153/3 (Table 1). Therefore, this neuroblastoma cell line CHP-382/JK can be added to the limited number of such lines available for study.

The difference in the response of CHP-382/JK and clone 4 cells to the monoclonal antibody (Table 1) leads to the suggestion that the antigen, which is a membrane glycoprotein (28), may not be a direct product of the N-*myc* oncogene. Recently, however, the products of two different oncogenes, v-*erbB* (32, 33) and v-*fms* (34), have been shown to be membrane glycoproteins and the *erbB* product has been reported to be similar to the epidermal growth factor receptor (35). It is interesting to note that human neuroblastoma and retinoblastoma cell lines contain membrane glycoproteins that have a phenotype suggestive of increased expression of β-*N*-acetylglucosaminide $\alpha 1 \rightarrow 3$ fucosyl transferase (36, 37). The cell line CHP-382 and its clones also contained glycoproteins with these unusual fucosyl residues (unpublished observations). The relationship between the apparent enhanced activity of this specific fucosyl transferase and the amplification of N-*myc* remains to be elucidated.

The unique finding that cell lines established from two different neuroblastomas contained multiple HSRs, each with amplified copies of N-*myc*, may contribute further to defining the molecular etiology of this tumor. The acquisition and deletion of HSRs containing amplified N-*myc* in these multiple HSR-bearing cell lines further highlights the continued processes of N-*myc* amplification and transposition. This process may provide neuroblastoma with a selective advantage permitting continued proliferation both *in vivo* and *in vitro*.

Cell lines CHP-382 and CHP-134 were obtained from tumors provided by The Children's Hospital Cancer Center in December 1983 and April 1973, respectively. The assistance of Ms. Jean Kershaw in isolating CHP-382 is gratefully acknowledged. We thank Dr. J. Michael Bishop (University of California, San Francisco) for N-*myc* probe pNb-1. The technical assistance of Beatrice Sellinger is acknowledged. This research was supported by National Institutes of Health Grants CA 14489, CA 37853, and HD 08356 (M.C.G.) and GM 32592 (B.S.E.).

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