Expression of Neurotransmitter Receptors and myc Protooncogenes in Subclones of a Human Neuroblastoma Cell Line

W. Sadée, V. C. Yu, M. L. Richards, P. N. Preis, M. R. Schwab, F. M. Brodsky, and J. L. Biedler

School of Pharmacy [W.S., V.C.Y., M.L.R., F.M.B.] and Brain Tumor Research Center [P.N.P.], University of California, San Francisco, California 94143; Institute f. Pathologie, Deutsches Krebsforschungszentrum, D-6900 Heidelberg, Federal Republic of Germany [M.R.S.]; and Laboratory of Cellular and Biochemical Genetics, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [J.L.B.]

ABSTRACT

Phenotypic variability of the human neuroblastoma cell line SK-N-SH was studied with the use of three subclones that interconvert at a slower rate than the parent cell line, i.e., a neuroblast-type subclone (SH-SY5Y), a nonneuronal, strongly substrate adherent subclone (SH-EP), and an intermediate type subclone (SH-IN). Rhodamine-phalloidin staining of actin fibers revealed differences in the cytoskeleton morphology of the three subclones, while the clathrin subunit proteins (heavy and light chains), components of coated vesicles, were invariant. Dramatic differences were observed for the expression of neurotransmitter systems, i.e., the μ and δ opioid receptor, the muscarinic cholinergic receptor and its effect on phosphatidylinositol turnover, and the uptake transporter for catecholamines. While these systems were strongly expressed in the parent line and the neuroblast-like clones SH-SY5Y and SH-IN, they were absent or barely detectable in the nonneuronal EP clone. Furthermore, the protooncogenes N- and c-myc were only expressed in the neuroblast containing lines, consistent with their growth characteristics of fully transformed cells. The strong c-myc expression in the absence of c- or N-myc amplification in SK-N-SH, adds a new form of high protooncogene activity in neuroblastoma cell lines. The remarkable differences of neurotransmitter systems and myc expression among the various phenotypes of human neuroblastoma cells should be considered in the therapy of neuroblastoma.

INTRODUCTION

Neuroblastoma cells are thought to represent primitive sympathetic blasts derived from the neural crest which gives rise to multiple cell lineages with neuronal, neurilemmal, or melanocytic phenotypes (1, 2). In accordance with an early stage of differentiation, neuroblastoma cells in tissue culture have been shown to undergo transdifferentiation between a neuroblast form and a nonneuronal phenotype. An example of such phenotypic interconversion has been documented with the human neuroblastoma cell line SK-N-SH, which was established from bone marrow metastases of a 4-year-old girl (3, 4). Cultures of the SK-N-SH line contain at least two morphologically distinct cell types. One is neuroblastic (N-cells) with a small cell body that may have neurite-like cell processes. These weakly substrate-adherent cells form dense aggregates with time in culture. The other variant cell type has a flat shape with strong substrate adhesiveness (S-cells) which yields monolayer cultures. These strongly adherent cells interconvert at a very slow rate. Subclones include SH-SY5Y (neuroblast, N), SH-IN (intermediate), and SH-EP (substrate adherent, S).

In addition to morphological differences among the S- and N-cells, there are coordinate biochemical changes. Thus, tyrosine hydroxylase and dopamine-β-hydroxylase are expressed only in neuroblastic subclones (N-cells) (2); furthermore, tyrosinase, a marker of melanocytes, is expressed only in S subclones (1). Protein analysis by two-dimensional gel electrophoresis revealed marked differences in the overall pattern of protein expression among N- and S-cells of SK-N-SH (5). One purpose of the present study is to extend these observations on coordinate biochemical changes among the neuroblastoma subclones. Neuronal proteins under study are the μ and δ opioid receptors which are expressed in the parent SK-N-SH line in a ratio of 5:1 (6). We have also shown that the SK-N-SH cells express a highly active uptake transporter for norepinephrine and dopamine (7). Finally, we have noticed that acetylcholine profoundly affects the turnover of phosphatidylinositol in SK-N-SH cells (8), and the presence of abundant muscarinic cholinergic receptors was readily demonstrable with the use of [3H]quinuclidinyl benzilate (this report).

The expression of clathrin, the structural protein of coated pits and coated vesicles, was also tested in the N- and S-cells of SK-N-SH (9, 10). It was of interest to determine which of the two tissue-specific forms of the clathrin LC5 is expressed by these cells, as differences were observed between LC5 expressed in normal brain and peripheral tissue (11), and whether clathrin expression is affected by phenotypic interconversions. The regulation of the proliferation and transdifferentiation of neuroblastoma cells remains poorly understood, although protooncogenes and oncogenes, e.g., the ras and src gene families, may play an important role (12, 13) with N-ras being expressed in SK-N-SH (14). The nuclear oncogene N-myc (15-16) was shown to be amplified and/or overexpressed in almost all established human neuroblastoma cell lines, and amplification in tumor tissue samples correlated with the invasiveness and clinical progression of the neuroblastoma (15). While SK-N-SH cells fail to carry amplified N-myc (16), they do express some N-myc mRNA (17). Expression of the related c-myc gene is less restricted among cell types than that of N-myc (18). We, therefore, also tested for the presence of c- and N-myc derived mRNA in N- and S-cells of SK-N-SH. The results reveal profound differences among the N and S type neuroblastoma cells.

MATERIALS AND METHODS

Materials. General chemical reagents were obtained from Sigma Chemical Co. The sources of the following materials are as indicated parenthetically: diprenorphine HCl (National Institute on Drug Abuse). Briefer list of specific materials as indicated in the text.

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2 To whom requests for reprints should be addressed.

3 Present address: Medical Biology Institute, 11077 N. Torrey Pines Rd., La Jolla, CA 92037.

4 Recipient of a Max Kade Fellowship, Austria.

5 The abbreviations used are: LC5, light chain subunit; QNB, quinuclidinyl benzilate; DAGO, [6-Ala6, MePhe7, Gly-oN-fen]enkephalin; DADL, [6-Ala6, 6-Leu7]-enkephalin; p-LC5, peripheral LC5; b-LC5, brain LC5; STV, Saline A, trypsin (0.05%); PBS, phosphate buffered saline.

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quinnucilindyl benzilate (Research Biochemicals Inc., Wayland, MA); morphicinect (Peninsula Lab, Palo Alto, CA); rhodamine-labeled phalloidin (Molecular Probes, Inc., Eugene, OR); peroxidase-conjugated goat anti-mouse immunoglobulin (Bio-Rad, Richmond, CA); molecular weight markers (Pharmacia, Piscataway, NJ).

Cell Cultures. Three subclones of the parent neuroblastoma cell line SK-N-SH, derived from a metastatic tumor in the bone marrow of a 4-year-old girl (3), were used in this study: (a) a thtice-cloned neuroblast-type (N-cell) clone, SH-SY5Y, attaches poorly to substrates, elaborates short neuritic processes, and grows slowly to high cell densities with the formation of focal aggregates (pseudoganglia); (b) an intermediate type clonal line (SH-IN) with some neuronal characteristics, that grows more rapidly and to an intermediate saturation density; (c) a clonal line lacking neuronal characteristics (SH-EP) which consists of large, flat cells that are strongly substrate adherent (S-cells) and grow to form a contiguous monolayer culture at a much lower saturation density. These clones were previously established and characterized by one of us (J. L. B.) (2, 3-5, 19).

The cells were grown at 37°C on plastic surfaces in RPMI 1640 medium supplemented with 10% fetal calf serum with 100 µg streptomycin/ml and 100 IU penicillin/ml. Unless otherwise stated, confluent SH-SY5Y and SH-IN cell cultures were harvested with 0.04% EDTA in Ca2+Mg2+-free phosphate buffered saline and mechanical shaking. The more strongly substrate adherent SH-EP cells were detached by shaking at room temperature in STV medium for 2-4 min. Control experiments established that the STV treatment did not affect the measurement of the neurotransmitter systems investigated in this study. The preparation of crude membrane homogenates followed the procedure outlined earlier (6).

Opiate Receptors. The assay of µ and δ opiate receptors on SK-N-SH cells has been previously described (6). [3H]DAGO (1 nm, 60 Ci/mmol; Amersham Corp.), [3H]DAL (1 nm, 50 Ci/mmol; Amersham), the latter in the absence or presence of 10−5 M morphicinect to block µ sites, and [3H]diprenorphine (0.3 nm, 41 Ci/mmol; Amersham) were incubated with crude cell membrane homogenates (50 mM Tris buffer, pH 7.4) at 37°C for 1 h. The bound radioactivity was determined by filtering the homogenate mixture through Whatman GF/B glass fiber filters (6). Tracer binding in the presence of 10−4 M diprenorphine served as the value for nonspecific binding.

Muscarinic Cholinergic Receptors. The receptor assay was derived from a previously developed method (20). Neuroblastoma cell homogenates were prepared as follows. Cells were detached by agitation in either STV or EDTA, resuspended in RPMI 1640 medium, centrifuged at 200 × g for 10 min, suspended in medium [120 mM NaCl; 4.7 mM KCl; 2.2 mM CaCl2; 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-1.2 mM MgSO4-1.2 mM KH2PO4-10 mM glucose, pH adjusted to 7.4 with 5 N NaOH], and homogenized for 20 s (Brinkman Polytron). One-mi aliquots (~80-120 µg protein) were incubated for 60 min at 20°C in 1.5 ml polypropylene tubes containing [3H]-(−)QNB (0.25-0.4 nm; 30 Ci/mmol; Amersham Corp.) and competing ligand. [3H]-(−)QNB labeling reached a plateau at 60 min at which time bound [3H] activity was determined by the filtration assay described for opioid tracers. The pellet was washed three times with 1 ml medium [120 mM NaCl; 4.7 mM KCl; 2.2 mM CaCl2; 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-1.2 mM MgSO4-1.2 mM KH2PO4-10 mM glucose, pH adjusted to 7.4 with 5 N NaOH] (0°C), and radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 10−4 M atropine or quinuclidinyl benzilate. Binding curves of QNB were fitted to a one-site model to obtain KD and Bmax values as described earlier (6).

Measurement of Phosphatidyl Inositol Turnover. The SK-N-SH subclones were grown in 150-cm2 culture flasks. At 80% confluency, the medium was removed and 30 ml of fresh medium containing 1 µCi/ml of ['H]inositol (14.2 Ci/mmol; Amersham) were added. Cells were harvested after 3 days and washed twice with serum-free medium. The pellet was resuspended in 0.5 ml serum-free RPMI 1640 medium to yield a protein concentration of 0.6 mg/sample, and carbachol (1 mM) was added after a 15-min preincubation period with 10 mM LiCl at 37°C. ['H]Inositol monophosphate was determined as previously described (8).

[3H]Catecholamine Uptake. The accumulation of [2,5,6-3H]dopamine (10 nm, 12 Ci/mmol; Amersham) into SK-N-SH and its subclones via noradrenergic uptake sites (uptakeoton) was measured in 17-mm culture wells as described (7). [3H]Dopamine rather than [3H]norepinephrine was used because of its 3- to 5-fold greater uptake efficiency (7). Data were normalized to protein content (±0.2 mg/sample). Nonspecific binding was defined in the presence of 10−4 M desipramine, representing −10% or less of total cell associated radioactivity. It was subtracted from all uptake data.

Detection of Clathrin Heavy Chain and LC3. Cell cultures were solubilized in 0.5% Nonidet P-40, and lysates were analyzed by electrophoresis, transferred to nitrocellulose, and reacted with monoclonal antibodies (for details, see Refs. 9-11). Antibodies used were X22 (anti-heavy chain), X16 (anti-LC3), and a control antibody (anti-lgh5b) which reacts with mouse immunoglobulin (10). Antibody binding was detected by peroxidase-conjugated antimouse immunoglobulin. Control samples included purified bovine brain clathrin, BL3 (bovine lymphoid cell line) lystate, and IM9 (human B-cell line) lystate. BL3 cells were a gift from G. Theilen, U. C. Davis.

Actin Staining with Rhodamine-labeled Phalloidin. Rhodamine phalloidin staining was performed as described by Wulf et al. (21). Briefly, cells were grown on glass coverslips, rinsed twice in PBS, fixed for 10 min in 3.7% formaldehyde in PBS, and dehydrated in absolute acetone at −20°C for 4 min. The fixed and air dried cells were stained for 20 min with 10 µl rhodamine phalloidin (1 µg/ml), washed three times in PBS, and mounted for viewing. An IMT 2 Olympus fluorescence microscope, equipped with a ×100 fluorescence objective, provided fluorescence images of cells.

Expression of N- and c-myc. The level of N- and c-myc was determined by an RNase protection assay. In short, total RNA was isolated by the quanidinium-isoctathionate procedure (22). A homogeneously labeled RNA complementary to the coding strand of a portion of the first coding exon of N-myc (EcoRI to BamHI) and c-myc (PstI to PstI) (23) was synthesized in the presence of [α-32P]UTP using a transcription vector (pGEM-4; Promega Biotech). Full-size RNA was recovered from a 6% polyacrylamide-urea gel. Of this probe approximately 106 cpm were hybridized to 10 μg of total cellular RNA in 40 mM piperazine-N, N'-bis(2-ethanesulfonic acid), pH 6.4-6.0 mM NaCl-1 mM EDTA-80% formamide at 45°C for 15 h. The exact amount of RNA was determined by measurement of UV absorption at 260 nm. Single stranded RNA was digested with 40 µg RNase A and 2 µg RNase T1/ml at 37°C for 30 min. Following extraction with phenol and precipitation with ethanol, the samples were taken up in 7 μl urea-5 mM Tris-borate buffer, pH 8.3-1.0 mM EDTA, and electrophoresed through a 6% polyacrylamide-urea gel under standard conditions (for details, see recommendations of Promega-Biotech for use of Riboprobe Kits). The gel was then autoradiographed.

RESULTS

Actin Staining with Rhodamine-Phalloidin. Distinct morphological characteristics of the three subclones SH-SY5Y, SH-IN, and SH-EP of the human neuroblastoma line SK-N-SH were demonstrated by actin staining patterns, obtained with rhodamine phalloidin (Fig. 1). The neuroblastic SH-SY5Y cells (Fig. 1A) have small cell bodies with neurite-like cytoplasmic processes (2) and form dense moundng aggregates. A few actin cables are stretching across the cell clusters, but more fluorescence activity is found on the inner surface of the cytoplasmic membrane. In addition to the cell bodies the cytoplasmic processes are made clearly visible by rhodamine-phalloidin. By contrast, the substrate adherent SH-EP cells (Fig. 1B) show a fibroblast-like actin staining pattern with a network of well organized large and fine cables. Moreover, single cells are interconnected by bridges of thin actin containing structures. Cells of the intermediate type clone, SH-IN, do not exhibit a uniform morphological or actin-staining pattern. At least three different cell types and/or growing patterns can be distinguished. Some cells are similar to the S-cell type, others to the...
N-cells, and a third type can be distinguished which exhibits a diffuse staining pattern, with fluorescence activity mainly at the inner surface of the cytoplasmic membrane and only rare transcellular cables. However, many fine intercellular connections and cytoplasmic processes are well delineated by the fluorescent dye (Fig. 1C).

The parent cell lines, SK-N-SH, expressed similar cell types; however, the relative distribution of the various cell types was highly variable and dependent upon the incubation conditions. Incubation at low initial cell density and over prolonged times tended to increase the fraction of neuroblast-like cells. Because of this variability, data presented in Tables 1–4 and Figs. 1–3 are limited to the subclones. If grown under conditions that maximize neuronal character, all results would be similar to those obtained with the SH-SY5Y and SH-IN subclones.

Expression of Clathrin Subunits. The presence of clathrin in the SK-N-SH clones was confirmed by immunoprecipitation from biosynthetically labeled cell lysates using a monoclonal antibody specific for the clathrin heavy chain (X22). There was no apparent major difference in the expression of the heavy chain or the coprecipitated light chain subunit among the three subclones of SK-N-SH (data not shown). The form of the clathrin LC<sub>α</sub> subunits present was analyzed with the use of a monoclonal antibody (X16) which reacts with the LC<sub>α</sub> subunits present in either brain or peripheral tissue clathrin. Cell lysate was prepared from each clone, separated electrophoretically, and after transfer to nitrocellulose, reacted with the X16 antibody. Antibody binding detected by peroxidase staining is shown in Fig. 2. As controls, bovine brain clathrin, and extracts of a bovine lymphoid cell line (BL3) and a human B-cell line (IM9) were also analyzed. Of the two variants of the light chain, p- and b-LC<sub>α</sub> (11), only p-LC<sub>α</sub> was expressed in all three

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Fig. 1. SK-N-SH subclones stained with rhodamine-labeled phalloidin to elaborate the actin fibers of the cytoskeleton. A, SH-SY5Y neuroblast cells (N-cells); B, substrate-adherent SH-EP cells (S-cells); C, intermediate SH-IN cells. This clone also contains some N- and S-like cells (not shown). × 1000.
NEUROBLASTOMA PHENOTYPIC MODULATION

Fig. 2. Clathrin LC, detected in neuroblastoma subclones. Monoclonal antibody, X16, specifically directed against LC, was reacted with a nitrocellulose blot of cell lysates separated by electrophoresis. Samples tested were A, molecular weight markers; B, 2 μg bovine brain clathrin; C, BL3 (bovine lymphoid cell line) lysate; D, IM49 (human B-cell line) lysate; E, SH-SY5Y lysate; F, SH-EP lysate. Amido black staining indicates the total protein on the blots. The control antibody [control immunoglobulin (Ig)] indicates background signal. The migration distances of brain (B) and peripheral (F) clathrin LC, are shown along with the positions of the molecular weight markers. The parent cell line, SK-N-SH yielded clathrin bands identical to those of its subclones.

Table 1  Expression of μ and δ opioid receptors and of muscarinic cholinergic receptors in subclones of SK-N-SH

<table>
<thead>
<tr>
<th>Subclone</th>
<th>[3H]DAGO (μ sites)</th>
<th>[3H]DADL (μ + δ sites)</th>
<th>[3H]DADL (1 nM + 10-3 M morphiceptin (δ sites))</th>
<th>[3H]diprenorphine (μ + δ sites)</th>
<th>[3H]QNB</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH-SY5Y</td>
<td>8,670 ± 380*</td>
<td>7,620 ± 110*</td>
<td>2,720 ± 810*</td>
<td>Not determined</td>
<td></td>
</tr>
<tr>
<td>SH-IN</td>
<td>9,280 ± 300</td>
<td>5,470 ± 36</td>
<td>2,340 ± 60</td>
<td>6,690 ± 180</td>
<td></td>
</tr>
<tr>
<td>SH-EP</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Not determined</td>
<td>428 ± 50</td>
<td></td>
</tr>
</tbody>
</table>
* Mean ± range; n = 2.
* Mean ± SD; n = 3.
ND, not detectable above background (~400 dpm).

Table 2  Stimulation of the accumulation of [3H]inositol monophosphate by carbachol in SK-N-SH subclones (in the presence of Li\(^+\), over 40 min)

<table>
<thead>
<tr>
<th>Subclone</th>
<th>[3H]inositol monophosphate (dpm/sample) (n = 2; mean ± range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>SH-SY5Y 2590 ± 360</td>
</tr>
<tr>
<td></td>
<td>9330 ± 50</td>
</tr>
</tbody>
</table>
* Mean ± SD; independent experiment; n = 3. Further experiments with SH-EP and SH-SY5Y repeatedly gave similar results.

Table 3  Expression of catechol uptake, system in SK-N-SH subclones

<table>
<thead>
<tr>
<th>Subclone</th>
<th>[3H]Dopamine uptake (dpm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH-SY5Y</td>
<td>132,300 ± 13,600*</td>
</tr>
<tr>
<td>SH-IN</td>
<td>153,400 ± 64,721</td>
</tr>
<tr>
<td>SH-EP</td>
<td>ND</td>
</tr>
</tbody>
</table>
* Mean ± SD, from two independent experiments; n = 8.
ND, not detectable above background (nonspecific uptake).

SK-N-SH subclones. While the quantity of p-LCa precipitated was somewhat variable between several experiments, no systematic differences in the amounts of the clathrin chains were noticeable. Further differentiation of the neuroblast cells (SH-SY5Y) with nerve growth factor (50 ng/ml) and retinoic acid (10 μM), which caused long neurite extensions after 4-6 days (24, 25), had no effect on the form of the clathrin LC, expressed (data not shown).

Expression of Neurotransmitter Systems. The three SK-N-SH subclones were tested for the presence of μ and δ opioid receptors and muscarinic cholinergic receptors (Table 1), for carbachol effects on phosphatidylinositol turnover (Table 2), and for the expression of the uptake system for catecholamine transport (Table 3). In each case, data for SK-N-SH grown under optimal conditions for neuronal characteristics were similar to those obtained with SH-SY5Y and SH-IN. The tracers used for the opioid receptors are either selective for μ sites only ([3H]DAGO), for μ and δ sites ([3H]DADL), or for μ, δ, and ε sites ([3H]diprenorphine). In the presence of 10-3 M morphiceptin, [3H]DADL labels only δ sites in the SK-N-SH cells (6). Whereas μ and δ tracer binding was indistinguishable between the neuroblast (SY-5Y) and intermediate (SH-IN) cells, [3H]diprenorphine binding was barely detectable above background in the substrate adherent SH-EP cells. In the latter cells, only [3H]diprenorphine was used because of its high affinity for all sites.

A similar expression pattern, i.e., positive for SH-SY5Y and SH-IN and negative for SH-EP, was found for the muscarinic cholinergic receptors (Table 1) and the carbachol-mediated stimulation of [3H]inositol monophosphate release (Table 2) (as well as inositol di- and triphosphate release (data not shown)). [3H]QNB labeling of the SH-IN cells was consistently higher than that of the SH-SY5Y and SK-N-SH cells, reflected also by a greater stimulation of inositol phosphate release in the SH-IN cells. Titration of the [3H]QNB binding sites on SH-SY5Y cells with unlabelled QNB confirmed the high affinity of QNB expected for muscarinic cholinergic receptors (K_D = 0.16 nM) and revealed a receptor density of 0.46 pmol/mg protein (Fig. 3). By contrast, only a small stimulation of [3H]inositol phosphate release was observed for SH-EP cells in two experiments. It was too small for quantitative evaluation and much smaller than for the other two SK-N-SH clones and the parent cell line.

Finally, the uptake of [3H]dopamine into the SH-SY5Y and SH-IN clones was much greater than that into the SH-EP clone (Table 3). In the former two clones, desipramine (10^{-6} M) greatly reduced the uptake of [3H]dopamine which is consistent
Table 4 Phenotypic differences among SK-N-SH subclones

<table>
<thead>
<tr>
<th>Subclone</th>
<th>Phenotype</th>
<th>Opioid receptors (μ and δ)</th>
<th>Muscarinic</th>
<th>Phosphatidyl inositol turnover</th>
<th>Catecholamine uptake, uptake1</th>
<th>N-myc</th>
<th>c-myc</th>
<th>Actin pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH-SY5Y</td>
<td>Neuroblast</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>Microfilaments</td>
</tr>
<tr>
<td>SH-EP</td>
<td>Nonneuronal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Poorly organized</td>
</tr>
<tr>
<td>SH-IN</td>
<td>Intermediate</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>Well organized fibrilike</td>
</tr>
</tbody>
</table>

with the uptake1 system previously described for SK-N-SH in our laboratory (7).

When SK-N-SH cells were grown under conditions that maximize nonneuronal cells (high initial density, short incubation periods), opioid receptors and the uptake1 transporter were barely detectable.

Expression of c- and N-myc Genes. The RNase protection assay of c- and N-myc messenger RNA expression in SK-N-SH (optimized culture for neuronal characteristics) and its three clones is presented in Fig. 4. Both messages were present in the SH-SY5Y and SH-IN clone while they were either barely or not at all detectable in the nonneuronal SH-EP cells. The magnitude of N-myc expression has the rank order SK-N-SH > SH-SY5Y > SH-IN > SH-EP (not detectable), while that of c-myc is SK-N-SH = SH-IN > SH-SY5Y > SH-EP. The reference neuroblastoma cell line NLF is amplified with respect to N-myc gene copy number (16) and expresses abundant N-myc mRNA but fails to express c-myc.

**DISCUSSION**

The three subclones of SK-N-SH (SH-SY5Y, SH-IN, and SH-EP) are suitable for the study of phenotypic interconversion within the neural crest lineage, because they are relatively stable and interconvert at a very slow rate (2, 5). The biochemical and anatomical differences among the three SK-N-SH subclones are summarized in Table 4. Phalloidin-mediated staining of actin fiber highlights the profound morphological changes that occur among the two extreme phenotypes, S- and N-cells (Fig. 1). However, the intermediate clone, SH-IN, did contain cells of at least three different phenotypes. All three clones invariantly express clathrin heavy chain and LC3 subunits, which hence represent constitutive proteins (Fig. 2). Expression of the peripheral, and not the brain, clathrin light chain (p-LC3) is consistent with the peripheral origin of this neuroblastoma line and agrees with earlier observations on another neuroblastoma and a retinoblastoma cell line (11).

A striking difference was observed with the expression of neurotransmitter systems among the SK-N-SH clones. The μ and δ opioid receptors, the muscarinic cholinergic receptor, and the uptake1 system were all expressed to similar degrees in the neuroblast-like SH-SY5Y and SH-IN clones, while they are either absent or at the detection threshold in the nonneuronal SH-EP cells. The expression of μ and δ opioid receptors in SH-SY5Y cells was recently confirmed (26). Together with the same result for catecholamine synthesizing enzymes (2), it appears that these neurotransmitter systems are under coordinate control that parallels observed morphological changes between the nonneuronal and neuronal cells.

Neuroblastomas generally carry amplified N-myc or less frequently c-myc, or they strongly express N-myc mRNA (15-18, 27). However, SK-N-SH was shown to have neither amplified N- nor c-myc (16, 28) nor to express high levels of N-myc (17,
3. Biedler, J. L., Nelson, L., and Spengler, B. A. Morphology and growth,
tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous
structure of continuous human neuroblastoma lines SK-N-SH, SK-N-BE2, (and 
5. Biedler, J. L., Rosen, G. E., and Brodeur, G. M. Amplification of N-myc in human neuroblastoma cell lines as 
multifunctional promoters and nerve growth factor on neurite outgrowth in cultured human 
sequence in human neuroblastoma cell lines with high c-myc expression resembles neuroepitheliomas.
Analysis of messenger RNA extracted from the three SK-N-MC clones further reveals profound changes in the level of myc 
expression with changing phenotype. Very little c-myc and no 
N-myc mRNA is detectable in the nonneuronal SH-EP clone with the use of a highly sensitive riboprobe. The c-myc message 
is highly expressed and is strongest in the SH-IN cells (similar to 
the parent SK-N-SH), and somewhat less in the SH-SYSY5 clone. Thus, c-myc expression in these clones closely parallels 
their growth characteristics. The SH-EP cells grow to slow saturation densities suggestive of a low degree of malignant 
transformation, whereas the SH-IN and SH-SYSY5 (N-cells) grow to much higher saturation densities (2). Further, the 
SH-SYSY5 clone with the lesser c-myc expression shows a considerably slower doubling time (40–60 h) than the IN clone (20– 
30 h) with the higher c-myc expression. The results of assays of tumor growth in nude mice, obtained in a parallel study, 
also correlate with c-myc expression; SH-IN is moderately tumorigenic, SH-SYSY5 weakly tumorigenic, and SH-EP, non-
tumorigenic.
On the other hand, N-myc expression in the SK-N-SH clones does not parallel growth characteristics (N-myc mRNA: SK-N-
SH > SH-SYSY5 > SH-IN > SH-EP (not detectable)). It is possible that N- or c-myc, or both, are not directly related to 
neuronal differentiation in the SK-N-SH cell system, as further differentiation of neuroblast cells by retinoic acid depresses the 
N-myc message (27), and N-myc is also expressed in a few nonneuronal cells such as small cell lung cancer that may 
alternatively amplify c-myc (18, 30). Rather, it appears more likely that N- and c-myc play complementary roles in cellular 
differentiation of neuroblastoma cells by retinoic acid depresses the 
ergrowth characteristics (N-myc mRNA: SK-N-SH, and somewhat less in the SH-SYSY5 clone. Thus, c-myc expression in 
these clones closely parallels their growth characteristics. The SH-EP cells grow to slow saturation densities suggestive of a low degree 
of malignant transformation, whereas the SH-IN and SH-SYSY5 (N-cells) grow to much higher saturation densities (2). Further, 
the SH-SYSY5 clone with the lesser c-myc expression shows a considerably slower doubling time (40–60 h) than the IN clone (20–30 h) with the higher c-myc expression. The results of assays of tumor growth in nude mice, obtained in a parallel study, also 
correlate with c-myc expression; SH-IN is moderately tumorigenic, SH-SYSY5 weakly tumorigenic, and SH-EP, non-tumorigenic.
On the other hand, N-myc expression in the SK-N-SH clones does not parallel growth characteristics (N-myc mRNA: SK-N-SH > SH-SYSY5 > SH-IN > SH-EP (not detectable)). It is possible that N- or c-myc, or both, are not directly related to neuronal differentiation in the SK-N-SH cell system, as further differentiation of neuroblast cells by retinoic acid depresses the N-myc message (27), and N-myc is also expressed in a few nonneuronal cells such as small cell lung cancer that may alternatively amplify c-myc (18, 30). Rather, it appears more likely that N- and c-myc play complementary roles in cellular growth and transformation, with c-myc predominating in the SK-N-SH lineage.

The demonstrated phenotypic variability is likely to be of clinical significance in the treatment of neuroblastoma which takes a prominent place among solid tumors of childhood. However, the status of phenotype expression in vivo is less well defined. Buck et al. (31) proposed that cells of different degree of maturation exist in vivo, while the neuroblasts grown in vitro consist of more immature cells. Accordingly, these authors observed a greater uptake of the diagnostic or therapeutic agent, 
123Iodobenzylguanidine (by the uptake, mechanism) in vivo than in vitro (31). One needs to understand better the factors that regulated neuroblastoma differentiation to improve on the rather discouraging therapeutic success against neuroblastoma.

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