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N-*myc* Down Regulates Neural Cell Adhesion Molecule Expression in Rat Neuroblastoma

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In human neuroblastoma, amplification of the N-*myc* oncogene is correlated with increased metastatic ability. We recently showed that transfection of the rat neuroblastoma cell line B104 with an N-*myc* expression vector resulted in an increase in metastatic ability and a significant reduction in the expression of major histocompatibility complex class I antigens. We examined whether N-*myc* causes additional phenotypic changes in these cells. We showed that expression of N-*myc* leads to a dramatic reduction in the levels of neural cell adhesion molecule (NCAM) polypeptides and mRNAs. Spontaneous revertants of the high N-*myc* phenotype were found to have regained significant levels of NCAM expression, indicating that the continued expression of N-*myc* is required to maintain the low NCAM phenotype. NCAM was not reduced in B104 cells transfected with the neomycin resistance vector alone, and other neuronal markers were not specifically reduced in N-*myc*-transfected B104 cells. As NCAM functions in cell-cell adhesion, decreased NCAM expression could contribute significantly to the increased metastatic potential of N-*myc*-amplified neuroblastomas.

In human neuroblastoma, one of the most common solid tumors of childhood, amplification of the N-*myc* oncogene is correlated with increased metastatic ability (6, 10, 17, 18). The mechanism by which overexpression of the N-*myc* oncogene contributes to this increased metastatic potential is largely unknown. One of us has recently developed a model system to analyze the effects of N-*myc* by transfecting the rat neuroblastoma cell line B104 with an N-*myc* expression vector (4). The N-*myc*-transfected derivatives of this cell line showed increased in vivo growth rates and metastatic ability. These cells also had substantially diminished levels of major histocompatibility complex class I antigens at the cell surface. This finding may provide a mechanistic basis for the observation that N-*myc*-amplified human neuroblastomas have greatly reduced levels of major histocompatibility complex class I antigens (4, 11).

Here we examined whether N-*myc* causes additional phenotypic changes in B104 cells by examining the expression of the neural cell adhesion molecule (NCAM). NCAM was chosen because in avian model systems, reduced expression of NCAM polypeptides is observed after transformation of neural cells with oncogenic viruses in vitro (5, 9) and also during cell migration in embryogenesis (21).

We show here that N-*myc*-transfected rat B104 neuroblastoma cells express greatly reduced levels of NCAM polypeptides and mRNA compared with those of the parental cell line that does not express N-*myc*. As NCAM functions in cell-cell adhesion (7), decreased NCAM expression could contribute significantly to the transformed phenotype and metastatic potential of N-*myc*-amplified neuroblastomas.

MATERIALS AND METHODS

Cell culture. B104 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The derivation of N-*myc*-expressing derivatives and the isolation of revertants have been described previously (4). In short, B104 neuroblastoma cells were obtained by transfection with the human N-*myc* expression vector pmp34.1 (4). Revertant cell lines were obtained by single-cell cloning of one of the cell lines with a high N-*myc* phenotype and the screening of single-cell clones for the loss of N-*myc*.

Immunofluorescence and enzyme-linked immunosorbent assays (ELISAs). To measure cell surface NCAM expression on B104 cells and transfected derivatives, live cells were incubated with monoclonal antibody 3F4 or control myeloma medium. Our procedure for indirect immunofluorescence has been described elsewhere (22). Monoclonal antibodies 10B2.38 and 10E7.9 were developed to rat neural antigens and have been found in initial assays to show high reactivity with the surfaces of cultured rat neurons and with rat brain cells but not with four other rat organs (R. Akeson, unpublished data).

For ELISAs, cells were lifted off the plates with EDTA and plated in replicate wells of microdilution plates at 75,000 cells per well. On the following day, the abundance of each cell surface component was determined by incubating live cells with a predetermined excess of monoclonal antibody 3F4 or 3G6 and then β-galactosidase-conjugated goat anti-mouse immunoglobulin G (Bethesda Research Laboratories, Inc.). Quantification of antibody binding was performed by measuring optical density at 405 nm with a multiwell plate reader.

Western blotting (immunoblotting). Total proteins or immunoprecipitated proteins were electrophoresed through sodium dodecyl sulfate-polyacrylamide gels, transferred to Immobilon membranes (Millipore Corp.), and developed for immunoblotting with anti-NCAM and Vectastain ABC reagents (Vector Laboratories) by the recommended protocols of the manufacturer.

RNA analysis. For dot blot analysis, RNA was dried onto nitrocellulose by standard procedures (14). The filter was hybridized to 10^5 dpm/ml of NCAM probe for 18 h at 62°C, washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 62°C and twice with 0.2× SSC at room temperature, and exposed for autoradiography for 5 days at −70°C with enhancing screens. As a probe, the 500-base-pair *Pst*I-*Pst*I fragment derived from the 5′ end of the rat pR18 NCAM cDNA was used. This probe hybridizes

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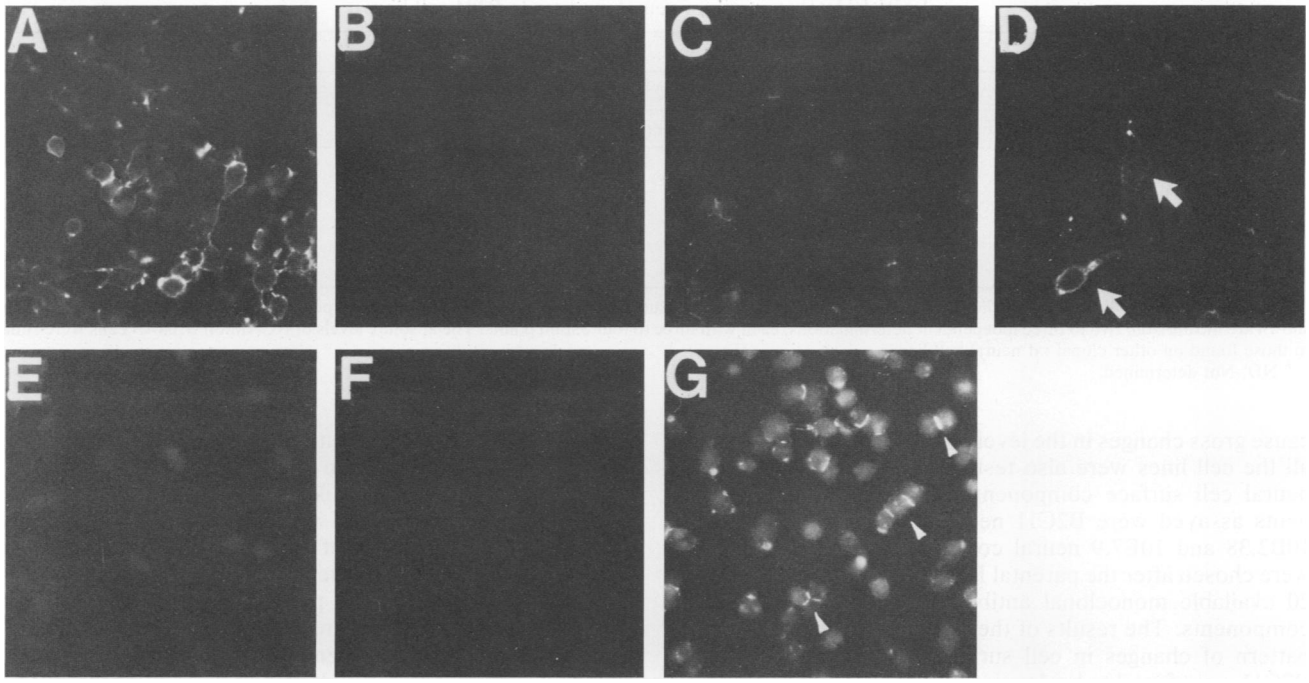

FIG. 1. Determination of NCAM levels by immunofluorescence. Live cells were incubated with anti-NCAM antibody 3F4 or control myeloma medium to determine the relative levels of cell surface NCAM by indirect immunofluorescence. Identically printed 20-s exposures of B104-wt (A), B104 N-*myc* C7 (B), B104 N-*myc* C6 (C), and B104-neo 1 (D) incubated with antibody 3F4 and of B104-wt (E) and B104 N-*myc* C7 (F) incubated with control medium are shown. Cells in panel D were somewhat sparse; two cells are indicated by arrows. Panel G is a 60-s exposure of B104 N-*myc* C6 cells. No nonspecific immunofluorescence was observed with any cells (note B104-wt [E] and B104 N-*myc* C7 cells [F]). Arrowheads in panel G indicate cell-cell contact points.

equally well with all presently known NCAM mRNAs (20). After exposure, blots were stripped and hybridized with a ubiquitously expressed actin cDNA probe. This hybridization indicated that equivalent amounts of RNA were spotted in all wells (data not shown).

For Northern (RNA) blot analysis, 5 μg of poly(A)$^+$-selected RNA was separated on a 1% agarose gel and transferred to nitrocellulose as previously described (4). The probe used was the same as the one used for dot blot analysis. The N-*myc* probe and α-tubulin probe have been described elsewhere (4).

RESULTS

Reduced NCAM expression in N-*myc*-transfected neuroblastoma. To evaluate a possible relationship between the expression of N-*myc* and NCAM in neuroblastoma cells, we assayed by immunofluorescence the levels of NCAM in B104 neuroblastoma and in two N-*myc*-transfected derivatives. Both the C6 and C7 N-*myc*-transfected derivatives of the B104 cell line had significantly reduced levels of cell surface NCAM when compared with levels in both the parental B104 cells used for transfection (B104-wt) and parental B104 cells transfected with only the neomycin resistance-encoding vector (B104-neo 1; Fig. 1, compare panels A to D photographed under identical conditions). However, the N-*myc*-transfected derivatives were not totally deficient in NCAM, as demonstrated by longer photographic exposure times (Fig. 1, panel G; note that NCAM fluorescence is particularly visible at the cell-cell contact points denoted by arrowheads). The low levels of NCAM on B104 N-*myc* C7 and C6 cells were difficult to photograph, but visual examination showed that the B104 N-*myc* C7 cells clearly expressed less NCAM than the B104 N-*myc* C6 cells. Since we have previously shown that B104 N-*myc* C7 cells express higher levels of N-*myc* than B104 N-*myc* C6 cells do, this result suggests that increased expression of N-*myc* in B104 neuroblastoma leads to a progressive loss of NCAM expression.

In order to more precisely determine the amount of cell surface NCAM remaining on the B104 N-*myc* C6 and C7 cells, quantitative ELISAs were performed with two independent monoclonal antibodies to rat NCAM. Anti-NCAM monoclonal antibody 3F4 (2) binding to the B104 N-*myc* C7 and C6 lines was 13 and 35%, respectively, of its binding to B104 parental cells. Somewhat greater reductions in NCAM polypeptide levels were found when anti-NCAM monoclonal antibody 3G6.41 (22) was used (Table 1). The results of these ELISAs are thus in agreement with the immunofluorescence assays in demonstrating a reduction in NCAM expression in B104 N-*myc* C6 cells and an even greater reduction in B104 N-*myc* C7 cells.

As controls, three distinct clonal isolates of B104 cells harboring only the neomycin resistance plasmid were tested for NCAM expression. None of these showed significant loss of NCAM with either antibody (Table 1). Furthermore, transfection of B104 cells with adenovirus 5 E1A, which induces a morphological alteration in these cells that is very similar to that induced by N-*myc*, gave cells which expressed normal levels of NCAM polypeptides (data not shown). These combined results indicate that reduced NCAM expression is tightly linked to elevated N-*myc* expression and is not due to some nonspecific effect of morphological transformation or clonal selection.

To determine if N-*myc* expression could in some way

TABLE 1. Expression of neural markers in B104 cell lines

Cell line	% Expression[a] of:				
	NCAM		10B2.38	10E7.9	B2C11
	3F4 binding	3G6.41 binding			
B104 N-*myc* C7	13	7	57	286	21
B104 N-*myc* C6	35	24	112	255	41
B104-neo 1	94	ND[b]	104	214	36
B104-neo 3	81	99	42	145	12
B104-neo 4	78	112	116	278	39

[a] B104-wt cells were defined as 100% for each component, and all other values were normalized and expressed as percentages of the B104-wt value. Values shown are means from two to three independent determinations, each with three to four datum points. The absolute levels of NCAM on B104-wt cells were similar to those found on other clonal rat neural cell lines.

[b] ND, Not determined.

cause gross changes in the levels of cell surface components, all the cell lines were also tested with antibodies for other neural cell surface components. The cell surface components assayed were B2C11 neural polypeptide (1) and the 10B2.38 and 10E7.9 neural components. These antibodies were chosen after the parental B104 cells were screened with 20 available monoclonal antibodies to neural cell surface components. The results of these assays indicate a complex pattern of changes in cell surface components. Levels of B2C11 were found to be decreased in B104 N-*myc* C6 and C7 cells. However, they were also substantially decreased in all the B104-neo control transfectants tested, suggesting that this reduction was not specifically induced by N-*myc* gene expression (Table 1). On the other hand, levels of 10E7.9 antigen were elevated in both the N-*myc* transfectants and the neomycin-resistant control lines. The levels of the 10B2.38 antigen were found to be somewhat variable among cell lines in a manner apparently unrelated to N-*myc* expression. Some of these effects could be due to normal clonal variation among cell lines or caused by effects of neomycin selection. The combined results indicate that the expression of these neural cell surface components is not consistently altered by N-*myc* expression and therefore suggest that the effects of N-*myc* on NCAM expression are specific and not a general effect on all cell surface polypeptides.

N-*myc* decreases NCAM mRNA levels. To investigate the mechanism by which N-*myc* expression leads to a reduction in cell surface NCAM polypeptides, we first examined by RNA dot blot analysis the NCAM mRNA levels in parental B104 cells and N-*myc*-transfected derivatives. As a probe, we used a fragment of the cDNA encoding rat NCAM (20). The results of this analysis (Fig. 2A) indicate that NCAM mRNA levels are reduced approximately 10-fold in the B104 N-*myc* C7 and C6 cells. The B104 N-*myc* C7 cells were found to have lower amounts of NCAM mRNA, consistent with their lower levels of NCAM polypeptide. No decrease in NCAM mRNA was observed in B104-neo-transfected cells. Thus, the quantitative decrease in NCAM polypeptide observed in ELISAs and in immunofluorescence assays is paralleled by a similar decrease in NCAM mRNA. This decrease in NCAM mRNA is sufficient to cause the observed decrease in polypeptide levels without requiring postulation of additional alterations in NCAM polypeptide metabolism. It therefore seems likely that the major alteration in NCAM metabolism caused by N-*myc* expression is at the level of mRNA abundance.

All NCAM mRNA species are reduced by N-*myc*. NCAM exists as four major polypeptide forms. Two have predicted classical transmembrane domains, one is linked to the membrane via phosphoinositide, and the fourth appears to be secreted (3, 7, 8, 20). Additional polypeptide diversity via alternative splicing has been observed in NCAM, notably in the fourth immunoglobulinlike loop (16, 19).

To determine whether N-*myc* expression has differential effects on the expression of individual NCAM mRNA and polypeptide species, Northern hybridizations to poly(A)$^+$-selected RNA from both parental B104 cells and B104 N-*myc*-transfected cells were performed. The results of this analysis indicate that parental B104 cells express a 2.9-kilobase mRNA and lower levels of a 6.7-kilobase mRNA, both of which appear to be significantly reduced in B104 N-*myc*-transfected cells (Fig. 2B). These mRNA size classes encode polypeptides of 140 and 120 kilodaltons (kDa), respectively (3, 20). Thus, the Northern blot analysis is consistent with the RNA dot blot analysis in that it shows a significant reduction in NCAM mRNA levels in N-*myc*-transfected B104 cells and extends its results to show that all forms of NCAM mRNA are reduced by N-*myc*.

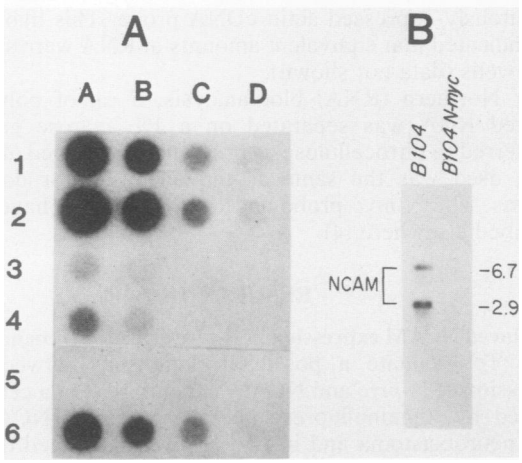

FIG. 2. Analysis of NCAM mRNA. (A) Dot blot analysis of NCAM mRNA in B104 cell lines. RNA was loaded at 5.0 (lane A), 1.5 (lane B), 0.5 (lane C), or 0.15 (lane D) μg onto a nitrocellulose filter and probed for NCAM with a rat NCAM cDNA fragment. RNA was derived from parental B104 cells (row 1), B104 cells transfected with only the neomycin resistance plasmid (row 2), and B104 cells transfected with N-*myc* (B104 N-*myc* C7 [row 3] and B104 N-*myc* C6 [row 4]). As a negative control, RNA from adult rat liver was loaded in row 5, and as a positive control, RNA derived from rat brain was loaded in row 6. (B) Northern blot analysis of NCAM mRNA in B104 cells. Five micrograms of poly(A)$^+$-selected RNA from B104-wt or B104 N-*myc* C7 cells was separated on an agarose gel, transferred to nitrocellulose, and probed for NCAM. The molecular sizes of the NCAM transcripts in kilobases are indicated.

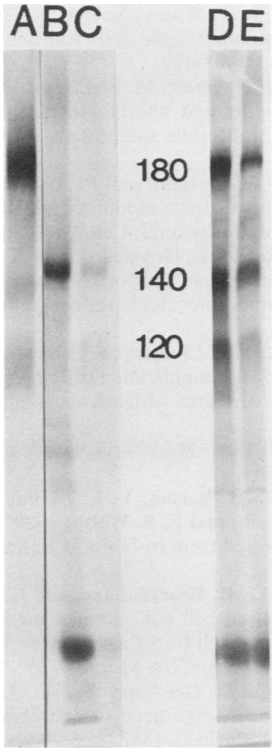

FIG. 3. Determination of NCAM polypeptide size classes. Total proteins from rat brain (lane A), clone 5.6 L cells (11) expressing recombinant 140-kDa NCAM (lane B), parental B104 cells (lane C), and immunoprecipitates of detergent-solubilized membrane proteins from parental B104 cells (lane D) and B104 N-*myc* C7 cells (lane E) made with polyclonal anti-NCAM serum were electrophoresed on a sodium dodecyl sulfate-acrylamide gel, transferred to Immobilon membrane, and processed for immunoblotting with polyclonal anti-NCAM and Vectastain ABC reagents. As the protein sample sizes were not equivalent, no quantitative comparisons should be made among any of these lanes. Molecular sizes are indicated in kilodaltons.

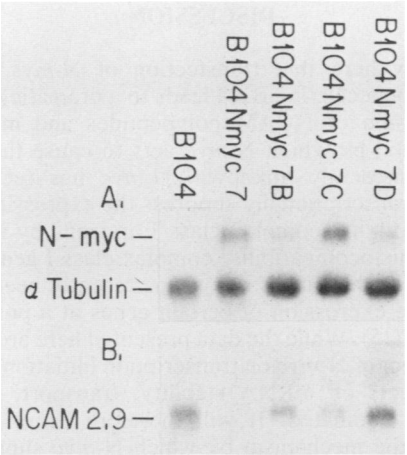

FIG. 4. NCAM mRNA levels in B104-N-*myc* revertants. Twenty micrograms of total RNA isolated from B104 cells, B104 N-*myc* C7 cells, or three independently derived single-cell clones of B104 N-*myc* C7 (B104 N-*myc* C7B, C7C, and C7D) was separated on a 1% agarose–formaldehyde gel, transferred to nitrocellulose, probed with both an α-tubulin probe and an N-*myc* probe (A), and reprobed after removal of the first signal with a rat NCAM cDNA fragment (B).

Consistent with the RNA data indicated above, when proteins extracted from parental B104 cells were immunoblotted with a polyclonal antiserum to NCAM (Fig. 3, lane C), a prominent band which migrated near the 140-kDa NCAM from rat brain cells (Fig. 3, lane A) and comigrated with 140-kDa NCAM from recombinant L cells (Fig. 3, lane B) was observed. The slight difference in mobility between NCAM isolated from brain cells and NCAM isolated from cultured cell lines has been previously observed (22) and is probably due to differences in glycosylation. A less intense band was observed at 120 kDa in the B104 cell lysate. No NCAM polypeptides could be detected with similar amounts of total protein from B104 N-*myc* C6 cells (data not shown). However, when NCAM proteins were concentrated by immunoprecipitation from parental B104 cells (Fig. 3, lane D) and also B104 N-*myc* C6 cells (Fig. 3, lane E), a major band at 140 kDa and clear bands at 120 and also 180 kDa could be observed in extracts from both cell types.

Down regulation of NCAM expression by N-*myc* is reversible. The inverse correlation between the levels of NCAM and N-*myc* in the B104 neuroblastoma cells strongly suggests that the N-*myc* gene product acts to suppress the expression of NCAM in these cells. However, it remained a formal possibility that a high level of N-*myc* expression was only tolerated in a small subpopulation of B104 cells that expressed low levels of NCAM polypeptides. If this were the case, then our procedure for obtaining clonal cell lines that expressed high levels of N-*myc* would at the same time have selected for clonal variants that were intrinsically low in their expression of NCAM. To rule out this possibility, we used previously isolated revertants of the B104 N-*myc* C7 cell line that had lost to a significant extent expression of the N-*myc* gene (4). To measure expression of NCAM in these revertant cell lines, total RNA from either B104 parental cells or B104 N-*myc* C7 cells and the revertants derived from this cell line was prepared and analyzed by Northern blotting.

To measure the level of N-*myc* expression in the revertants of the B104 N-*myc* C7 cell line, we first probed the Northern filter with an N-*myc* probe and, as an internal standard, with an α-tubulin cDNA probe. The results of this experiment (Fig. 4A) demonstrate that two subclones (C7B and C7D) of the B104 N-*myc* C7 cell line had lost to a significant extent expression of the N-*myc* gene, whereas a third subclone (C7C) had retained high levels of N-*myc* expression.

Significantly, reprobing of the filter shown in Fig. 4A with a rat NCAM cDNA fragment indicated that the two revertants of the B104 N-*myc* C7 cell line that had lost most of their N-*myc* expression had regained significant levels of the 2.9-kilobase NCAM transcript, whereas the subclone that had retained a high level of N-*myc* still manifested the low NCAM phenotype (Fig. 4B). Thus, N-*myc* transfectants that express low levels of NCAM are capable of reexpressing high levels of NCAM mRNA following the loss of N-*myc* expression. It therefore appears unlikely that the derivation of N-*myc*-transfected neuroblastoma cells depended on the selection of clonal cell lines that had a constitutive inability to express high levels of NCAM. We conclude that continued expression of N-*myc* is required to maintain the low NCAM phenotype of the N-*myc*-transfected neuroblastoma cells.

DISCUSSION

We show here that transfection of N-*myc* in the rat neuroblastoma cell line B104 leads to a dramatic decrease in the expression of NCAM polypeptides and mRNA. The mechanism(s) by which N-*myc* acts to cause the observed effects is presently unknown. N-*myc* has recently been shown to transcriptionally suppress the expression of major histocompatibility complex class I antigens by inactivating the major histocompatibility complex class I gene enhancer (12). On the other hand, c-*myc* has recently been shown to regulate the expression of certain genes at a posttranscriptional level (15). While the data presented here are consistent with an effect of N-*myc* on transcription initiation of NCAM, N-*myc* effects on mRNA stability, transport, or splicing cannot be eliminated. It will therefore be of interest to determine the mechanism by which N-*myc* suppresses the expression of the NCAM gene.

We have previously shown that expression of N-*myc* in B104 neuroblastoma cells results in the increased metastatic ability of tumor cells. In this respect, the rat neuroblastoma model system resembles human neuroblastoma, in which overexpression of N-*myc* has also been shown to be correlated with increased metastatic potential (6, 18). Evidence to suggest that reduced NCAM expression could be relevant to the induction of metastatic ability comes from the study of NCAM expression during chicken embryogenesis. In this system, it was found that premigratory neural crest cells, which are clustered adjacent to the dorsal neural tube, express readily detectable levels of NCAM polypeptide. However, as the cells leave the dorsal embryo to migrate ventrally as single cells, they do not express immunologically detectable NCAM. Upon arriving at their final anatomic location in the embryo, they reexpress NCAM and again cluster into ganglionic structures (21). Thus, migratory potential and NCAM expression are inversely related in neural crest cells. Since neural crest cells and neuroblastoma tumor cells are both derived from the neuroectodermal lineage, it is possible that alterations in cell adhesion also contribute to the migratory potential of neuroblastoma tumor cells. Thus, alterations in NCAM expression could contribute significantly to their metastatic potential. In support of this view is the recent finding that a high-metastatic subclone from a mouse melanoma cell line expressed lower levels of NCAM than the parental low-metastatic melanoma did (13). It will be of interest to investigate the levels of NCAM in human neuroblastomas with different N-*myc* expression levels and metastatic ability.

The availability of a cDNA clone of rat NCAM will allow us to reintroduce NCAM expression in B104 neuroblastoma cells that express high levels of N-*myc*. This should allow us to rigorously investigate the contribution of reduced NCAM expression to the induction of the metastatic phenotype by N-*myc* in more detail.

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