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N-*myc* Amplification Causes Down-Modulation of MHC Class I Antigen Expression in Neuroblastoma

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Summary

Amplification of the N-*myc* gene is correlated with increased metastatic ability of human neuroblastomas. We show here that overexpression of the N-*myc* gene in a rat neuroblastoma cell line following gene transfer causes down-modulation of class I histocompatibility antigen expression and increases in the in vivo growth rate and metastatic ability of these cells. N-*myc*-mediated down-modulation of MHC class I antigen expression could be reversed by treatment with interferon without affecting the steady state level of N-*myc* mRNA. No effect on MHC class I antigen expression was found when the N-*myc* gene was expressed in rat fibroblasts, indicating that some of the effects caused by N-*myc* gene amplification are cell-type-specific.

Introduction

Substantial evidence exists that implicates activated cellular oncogenes in the genesis of malignant disease. These oncogenes arise because of mutations that deregulate expression of the proto-oncogene or affect the structure of the encoded protein (Bishop, 1985; Weinberg, 1985). Once created, oncogenes are thought to confer a growth advantage, thus facilitating clonal expansion of the mutant cell.

During tumor development, sequential selection of variant cell subpopulations results in the preferential outgrowth of increasingly more malignant cells (Nowell, 1986). Oncogene activation is postulated to play a key role in triggering the clonal expansion of these subpopulations. However, the precise role of cellular oncogenes in this process of tumor progression is poorly understood.

Evidence suggesting that cellular oncogenes are involved in specific stages of tumor development comes from several sources. Study of chemically induced rat mammary carcinomas strongly implicates activation of the H-*ras* oncogene in the initiating event of tumorigenesis (Sukumar et al., 1983). Work on human neuroblastoma implicates amplification of the N-*myc* proto-oncogene in the later stages of tumor progression. These latter studies rely on diagnostic staging of the tumors. Thus, classification of a tumor as stage 1 indicates a tumor mass that is completely confined to the organ or structure in which it originated. Stages 2, 3, and 4 indicate progressive degrees of metastatic spread of tumor cells beyond the primary site (Evans et al., 1971). Analysis of tumor DNAs from patients having different stages of neuroblastoma disease has clearly shown that genomic amplification of the N-*myc* gene is more frequent in the more advanced stages of disease (Brodeur et al., 1984; Seeger et al., 1985). This suggests that the N-*myc* gene plays a crucial part in determining the degree of malignancy of neuroblastomas.

We have concentrated on this neuroblastoma model in an attempt to see whether N-*myc* amplification plays a causal role in tumor progression. To do this, we used a rat neuroblastoma cell line into which we introduced the N-*myc* gene by transfection. The traits of the transfectants were studied and found to undergo dramatic changes affecting cell antigenicity, growth rate, and metastatic ability.

Results

MHC Class I Antigen Expression in Neuroblastoma Cells

MHC class I antigens are cell-surface glycoproteins that are required for the recognition of target cells by cytotoxic T lymphocytes (Zinkernagel and Doherty, 1979). The antigens are expressed on virtually all cell types and are consequently also expressed on almost all tumor cells. Neuroblastoma and small-cell lung cancer are exceptions to this rule in that they often express very low levels of MHC class I antigens (Trowsdale et al., 1980; Lampson et al., 1983; Doyle et al., 1985). These two types of tumors are also unique in that they often show amplification of one of the genes of the *myc* gene family (Schwab et al., 1983; Kohl et al., 1983; Little et al., 1983; Nau et al., 1985, 1986). We speculated that a connection might exist between these two separate sets of observations; namely, that the low expression of MHC class I antigens in these two types of tumors could be caused by the high expression of the *myc* genes in these cells. This speculation was also inspired by earlier work that showed that expression of an adenovirus E1a oncogene could result in decrease of MHC class I antigen expression (Schrier et al., 1983).

To begin to document the relation between N-*myc* and MHC class I expression in human neuroblastomas, we extracted RNA from a number of tumor cell lines having different degrees of amplification of the N-*myc* gene. These RNAs were then analyzed by Northern blotting for the expression of both the HLA class I heavy chain gene (Figure 1A) and the N-*myc* gene (Figure 1B). The three neuroblastoma lines having amplification of the N-*myc* gene, IMR32 (25-fold amplified), LAN-5 (50-fold amplified), and NGP (120-fold amplified), all showed a dramatically reduced amount of MHC class I heavy chain transcripts when compared with two representative solid tumors of nonneuronal origin. In contrast, two of the three neuroblastoma cell lines with no N-*myc* gene amplification and no detectable N-*myc* expression (Figure 1B) showed significant amounts of MHC class I heavy chain mRNA (Figure 1A, lanes 4 and 5).

These results indicated that the low expression of MHC

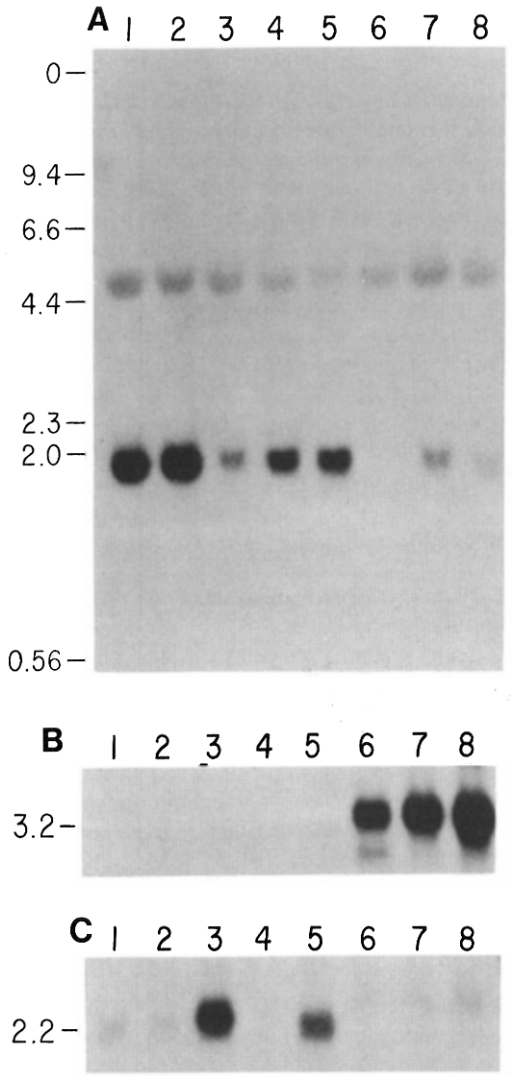

Figure 1. Northern Blot Analysis of RNA Isolated from Human Neuroblastoma Cell Lines

Twenty micrograms of total cytoplasmic RNA from the following cell lines was used: lane 1, human colon carcinoma SW480; lane 2, human kidney carcinoma ACHN; lane 3, neuroblastoma NB69; lane 4, neuroblastoma SK-N-SH; lane 5, neuroblastoma SK-N-MC; lane 6, neuroblastoma IMR32; lane 7, neuroblastoma LAN-5; lane 8, neuroblastoma NGP. The positions of the molecular weight markers are indicated. (A) Filter probed with a human MHC class I heavy chain gene probe. (B) Filter shown in (A) reprobed with a human N-*myc* probe. (C) Filter shown in (A) reprobed with a mouse c-*myc* probe.

class I heavy chain in neuroblastoma cells is indeed correlated with a high level of expression of the N-*myc* gene. One line presented an apparent exception to this. The NB69 cell line showed a low level of MHC class I mRNA but no increase in N-*myc* expression. Upon further analysis, however, these cells were found to have a high level of expression of the c-*myc* gene (Figure 1C). We thus extended the hypothesis, associating this ability to affect the expression of MHC class I antigens with both members of the *myc* gene family. In the subsequent experiments, we focused our study on the effects of the N-*myc* gene on expression of MHC class I antigens in neuroblastoma cells.

Transfection of Rat Neuroblastoma Cells with the N-*myc* Gene

The above-mentioned results indicated an inverse relationship between N-*myc* and MHC class I antigen expression. Such data could be correlative or might suggest a causal relationship. We therefore designed experiments to determine whether manipulation of N-*myc* levels could be used to modulate the expression of MHC class I antigens.

As a model system, we chose the rat neuroblastoma cell line B104 (Schubert et al., 1974). This cell line was generated by transplacental mutagenesis of pregnant rats with the chemical carcinogen ENU, causing activation of the *neu*-oncogene in these cells (Schechter et al., 1984). We specifically chose this cell line because it expresses very low amounts of c-*myc* and N-*myc* transcripts (not shown) and displays a substantial amount of MHC class I antigens at the cell surface (see below). Furthermore, we considered it advantageous that the B104 cell line was generated from a readily available inbred rat strain, BDIX, which would enable us to study the growth of transfectants in immunocompetent, syngeneic animals.

We developed derivatives of the B104 neuroblastoma cell line, each of which expressed the N-*myc* gene at a different level. To achieve this, we cotransfected the B104 cells with the plasmids pSV2neo and pmp34.1. The latter plasmid contains a genomic DNA fragment spanning the entire human N-*myc* gene and a murine leukemia virus enhancer segment in the 5′-flanking region of the gene to increase its expression (Schwab et al., 1985). Individual colonies of G418-resistant cells were picked and tested for their expression of the N-*myc* gene by Northern blot analysis. As can be seen in Figure 2A, this resulted in the generation of a series of stable transfectants having different expression levels of the N-*myc* gene.

We then determined whether the transfectants showed any modulation of the steady state level of the MHC class I heavy chain mRNA or in the transcription of the gene for β_2-microglobulin with which the 45 kd MHC class I heavy chain protein is complexed at the cell surface. For this analysis, the filter shown in Figure 2A was stripped of probe and reanalyzed with a cDNA fragment from the mouse H-2K^d gene (Figure 2B). Subsequently, the filter was reprobed yet once more with a mouse β_2-microglobulin probe (Figure 2C).

The results of these experiments clearly indicate that those cells that express a high level of the transfected N-*myc* gene exhibit decreased expression of MHC class I heavy chain mRNA. The effect of the N-*myc* gene product seems to be specific for the class I heavy chain, since no effect on the level of the β_2-microglobulin mRNA was observed in any of the transfected cell lines (Figure 2C).

We corroborated these results by measuring the levels of MHC class I antigens on the surface of the various cell lines. This was done by incubating the cells with a mouse monoclonal antibody that recognizes rat MHC class I antigens (Fukumoto et al., 1982), and then treating these cells with fluoresceinated second antibody that reacts with mouse immunoglobulins. Cell-surface fluorescence was measured using a cytofluorograph.

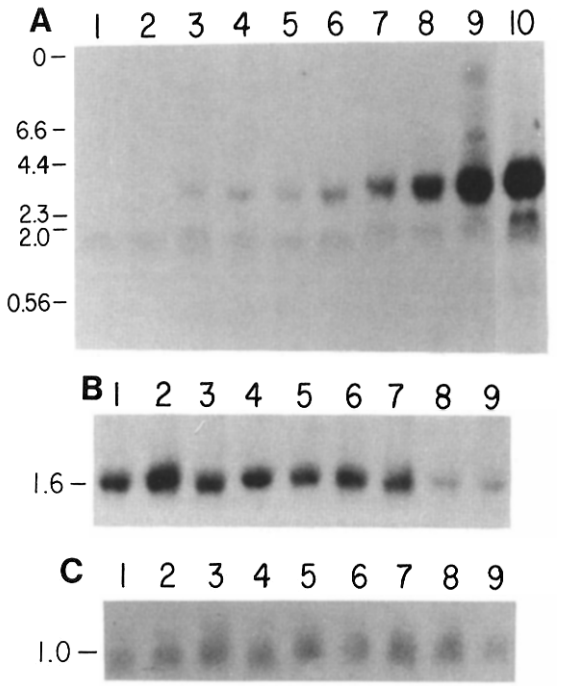

Figure 2. Northern Blot Analysis of N-*myc*-Transfected B104 Rat Neuroblastoma Cells

B104 rat neuroblastoma cells were transfected with pSV2neo and the N-*myc* clone pmp34.1 or with the pSV2neo plasmid alone. Individual colonies of G418-resistant cells were picked, and total cytoplasmic RNA was isolated. Twenty micrograms of total cytoplasmic RNA was loaded on a formaldehyde agarose gel. Lane 1, B104 neoC1; lane 2, B104 neoC2; lane 3, B104 N-*myc*C1; lane 4, B104 N-*myc*C2; lane 5, B104 N-*myc*C3; lane 6, B104 N-*myc*C4; lane 7, B104 N-*myc*C5; lane 8, B104 N-*myc*C6; lane 9, B104 N-*myc*C7; lane 10, human neuroblastoma LAN-5. (A) Filter probed with a human N-*myc* gene probe. (B) Filter shown in (A) reprobed with a mouse MHC class I heavy chain gene probe. (C) Filter shown in (A) reprobed with a mouse β_2-microglobulin probe.

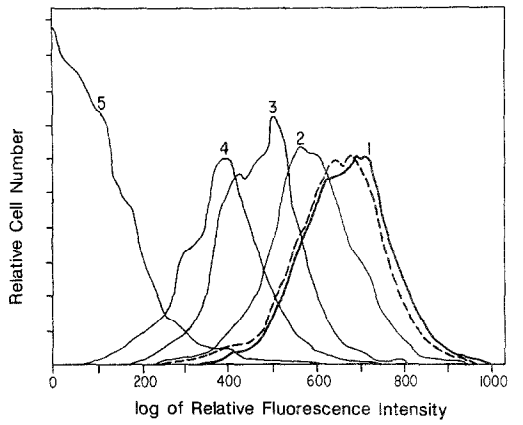

Figure 3. Cytofluorimetric Analysis of MHC Class I Antigens on N-*myc*-Transfected B104 Neuroblastoma Cells

B104 cells and N-*myc* transfected derivatives were stained with the mouse monoclonal antibody OX18, which reacts with the class I MHC antigens of the rat. After incubation with a fluoresceinated sheep anti-mouse IgG second antibody, cell-surface fluorescence was measured using a cytofluorograph. The dotted line indicates the fluorescence intensity of the B104 cells. Curve 1, B104 N-*myc*C4; curve 2, B104 N-*myc*C5; curve 3, B104 N*myc*C6; curve 4, B104 N-*myc*C7; curve 5, B104 cells stained with the fluoresceinated second antibody alone.

The results of these immunofluorescence experiments are summarized in Figure 3. As before, we observed that a low level of N-*myc* expression does not affect the expression of the MHC class I antigens. Above this threshold level, the reduction of MHC class I antigen expression is inversely proportional to the level of expression of the N-*myc* gene. We have not been able to measure N-*myc* protein levels in the transfected cells. We therefore must assume that the N-*myc* mRNA levels accurately reflect N-*myc* protein levels. We estimate that the cell-surface expression of MHC class I antigens in the line with the highest level of N-*myc* expression is reduced 10- to 15-fold. The level of N-*myc* gene expression in this particular transfectant is lower than that found in many spontaneously arising human neuroblastomas (compare lanes 9 and 10 in Figure 2A). This result suggests that the N-*myc* amplification occurring in these human tumors could have even more drastic effects on MHC class I antigen expression than those observed here.

Reversion of Phenotype

The observed inverse correlation between the levels of expression of the N-*myc* gene and that of MHC class I antigens was compatible with two hypotheses. It was possible that N-*myc* expression was only tolerated in cell variants that were intrinsically unable to express high levels of MHC class I antigens even before acquisition of the N-*myc* gene by transfection. Accordingly, our procedure for deriving clonal lines having high N-*myc* expression levels may have inadvertantly selected for cells that had preexisting lowered levels of MHC and an associated tolerance of high N-*myc* expression. Alternatively, our data may have reflected a physiological regulatory circuit in which the N-*myc* gene product is able to modulate MHC class I antigen expression.

To resolve between these alternatives, we isolated revertants of B104 cells having the high N-*myc*/low MHC class I phenotype. In order to do this, B104 N-*myc*C7 cells were plated at limiting dilution into 96 well plates. Colonies originating from single cells were grown up, and RNA was extracted for Northern blot analysis. As can be seen in Figure 4A, this resulted in the isolation of a subclone with a markedly reduced level of expression of the N-*myc* gene (compare lanes 2 and 6, Figure 4A). To measure expression of MHC class I heavy chain mRNA of this revertant, the filter shown in Figure 4A was stripped of probe and reprobed with the mouse H-2K^d cDNA probe. Figure 4B shows that this revertant expresses a 3- to 4-fold higher level of MHC class I heavy chain mRNA than the B104 N-*myc*C7 cell line. A similar conclusion was obtained when MHC class I antigen expression was measured using the immunofluorescence technique described above (data not shown).

The ability to modulate reversibly MHC class I antigen expression was also studied by treating B104 N-*myc*C7 cells with γ-interferon. This agent has been found to increase levels of MHC class I antigen expression in a variety of cell types, including those of neuronal origin (Lampson and Fisher, 1984; Wong et al., 1984).

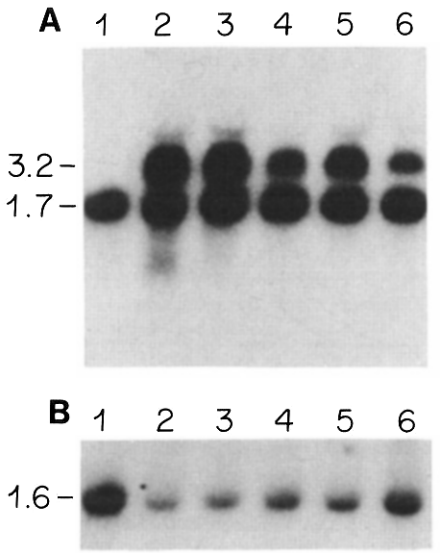

Figure 4. Northern Blot Analysis of Subclones of B104 N-*mycC7*

B104 N-*mycC7* cells were plated at limiting dilution into 96 well plates. Colonies originating from single cells were grown up and analyzed for the expression of the N-*myc* gene and the MHC class I heavy chain. Twenty micrograms of total cytoplasmic RNA derived from the following cell lines was used: lane 1, B104; lane 2, B104 N-*mycC7*; lane 3, B104 N-*mycC7* subclone A; lane 4, B104 N-*mycC7* subclone B; lane 5, B104 N-*mycC7* subclone C; lane 6, B104 N-*mycC7* subclone D. (A) Filter probed with a mixture of both the human N-*myc* probe and a rat α-tubulin cDNA. (B) Filter shown in (A) reprobed with the mouse H-2K^d cDNA probe.

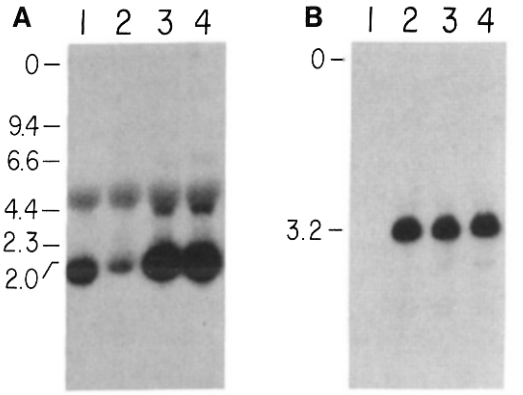

Figure 5. Interferon Treatment of B104 Neuroblastoma Cells

B104 neuroblastoma cells having a high level of N-*myc* gene expression were treated with 10^3 or 10^4 units/ml of recombinant murine γ-interferon for 18 hr. After this, total cytoplasmic RNA was isolated and analyzed by Northern blot analysis. Twenty micrograms of RNA from the following cells was loaded: lane 1, untreated B104 cells; lane 2, untreated B104 N-*mycC7* cells; lane 3, B104 N-*mycC7* cells treated with 10^3 units/ml of γ-interferon; lane 4, B104 N-*mycC7* cells treated with 10^4 units/ml of γ-interferon. (A) Filter probed with the mouse MHC class I heavy chain gene probe. (B) Filter shown in (A) reprobed with the human N-*myc* probe.

As can be seen in Figure 5A, treatment of the B104 N-*mycC7* cell line with 10^3 or 10^4 units/ml of γ-interferon for 18 hr resulted in an increase in the level of MHC class I heavy chain transcript of approximately 50-fold. A similar increase in MHC class I antigen expression was found when the untransfected B104 cells were treated with interferon (not shown).

Both these types of experiments allow the conclusion that these high N-*myc*/low MHC class I cell lines derived from cells having great plasticity in their ability to express MHC class I antigens. Conversely, it appears very unlikely that the derivation of these cell lines depended on the selection of cell clones having a constitutive inability to express a high level of MHC class I antigens.

The interferon-mediated ability to overcome repression of MHC class I antigens could be due to an inhibitory effect of interferon on N-*myc* gene expression. Indeed, others have shown that interferon strongly inhibits expression of the related c-*myc* gene (Jonak and Knight, 1984; Dron et al., 1986). However, in the present case N-*myc* expression is unaffected following interferon treatment (Figure 5B). We thus conclude that interferon restores expression of MHC class I antigens by a mechanism that circumvents the N-*myc*-mediated down-modulation of the expression of these antigens.

N-*myc* Expression, Metastasis, and Growth Rate

Clinical studies have indicated that amplification of the N-*myc* gene is more frequent in metastatic neuroblastoma than in nondisseminated tumors of this type (Brodeur et al., 1984; Seeger et al., 1985). It therefore was of interest to compare the rate of metastasis of the parental B104 cell line and the various derived transfectants. To do this, we injected 10^4 B104 cells or an equal dose of the N-*myc*-transfected derivatives in the tail vein of weanling BDIX rats. We found that 6 out of 6 animals that received the B104 N-*mycC7* cells died of widespread lung metastasis (>100 metastases per animal) within a period of 24 ± 2 days. In contrast, the 6 animals that had received an equal dose of B104 cells transfected with the pSV2neo plasmid alone showed no lung metastases at that time. This indicates that expression of the N-*myc* gene can greatly increase the rate of experimental metastasis of neuroblastoma cells as measured by the tail vein injection assay.

This metastasis assay has the disadvantage that it circumvents the events that take place during the early stages of metastatic spread of tumor cells, these including the invasion of adjacent tissue and the intravasation of tumor cells into the circulatory system. We attempted to address this limitation of the metastasis assay by injecting cells into subcutaneous sites. In so doing, we came across yet another difference between B104 cells and derived transfectants: the transfectants showed a dramatically increased growth rate when compared with the parental, untransfected cells (Figure 6A). While revealing yet another phenotypic change evoked by an overexpressed N-*myc* gene, these results precluded any meaningful comparison of metastatic rate following subcutaneous injection. Subsequent experiments explored the basis of these dramatic effects of N-*myc* gene expression on tumor growth.

The growth advantage observed for the B104 cells expressing the N-*myc* gene could be due to a decreased T cell-mediated immune response against these cells. To

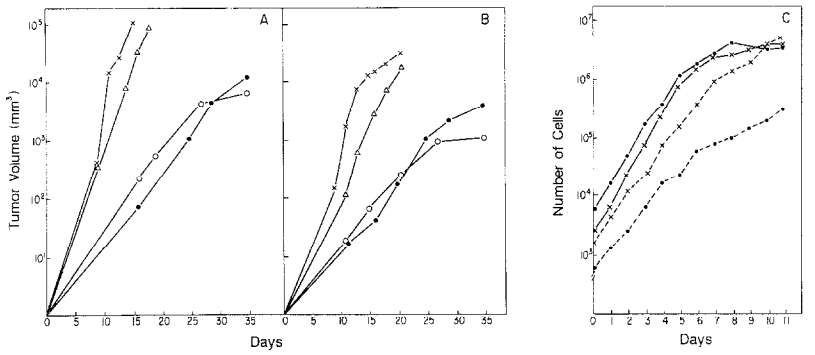

Figure 6. In Vivo and In Vitro Growth Rates of B104 Cells Having Varying Levels of N-*myc* Gene Expression

To determine in vivo growth rates, 10^6 cells were injected subcutaneously into weanling BDIX rats or 4 week old athymic nude mice. The following cell lines were used for injection: B104 N-*myc*C7 (x———x); B104 N-*myc*C6 (△———△); B104 neoC1 (●———●); B104 (○———○).

(A) Growth rates in weanling BDIX rats. (B) Growth rates in athymic nude mice. (C) Growth rates in vitro. Solid lines represent growth rates in medium containing 10% serum, and dotted lines, growth rates in medium containing 0.5% serum.

investigate this possibility, we injected the cell lines described above subcutaneously in immunodeficient nude mice. As can be seen in Figure 6B, the difference in growth rates seen previously in the immunocompetent rats was seen as well in the immunodeficient nude mice. It is therefore unlikely that differences in T cell-mediated immune surveillance account for the increased in vivo growth rate of the N-*myc* transfectants.

Of additional interest were observations that showed that the transfected and untransfected cells, while showing dramatic differences in growth in vivo, exhibited virtually no difference in growth in vitro, both when assayed in liquid medium (Figure 6c) and in semisolid medium (not shown). These observations suggest that the in vivo growth advantage of the B104 cells expressing high levels of the N-*myc* gene is caused by a greater responsiveness of these cells to some element that is present at limiting concentration in vivo. We speculate that this element is a growth factor. The small but reproducible growth advantage of the N-*myc*-transfected B104 cells in medium containing 0.5% serum is consistent with this notion (Figure 6c).

Cell-Type Specificity

To date, amplification of the N-*myc* gene has only been reported in neuroblastoma, retinoblastoma, and small-cell lung cancer (Kohl et al., 1983; Schwab et al., 1983; Lee et al., 1984; Nau et al., 1986). This finding may suggest that at least some of the effects of N-*myc* gene amplification that are advantageous for tumor growth are restricted to cells of neuroectodermal origin. We have addressed this hypothesis by measuring the expression of MHC class I antigens in derivatives of the rat fibroblast cell line Rat-1, which had acquired a high level of N-*myc* gene expression following transfection with pmp34.1 DNA (cell lines kindly provided by Michael Small).

As can be seen in Figure 7A, the level of N-*myc* gene expression in these transfected Rat-1 fibroblasts is comparable to that of the N-*myc*-transfected rat neuroblastoma cells. However, when the filter shown in Figure 7A was reprobed with a H-2K^d cDNA fragment, no reduction in the expression of MHC class I heavy chain gene was observed (Figure 7B). A similar conclusion was obtained when the cell-surface expression of MHC class I antigens of the various Rat-1 cells was measured using the immunofluorescence technique described above (data not shown). These data indicate that down-modulation of MHC class I antigen expression by N-*myc* has some cell-type specificity, as it strongly reduces MHC class I antigen expression in neuroblastoma cells but has no effect in Rat-1 fibroblasts. Whether the N-*myc*-mediated down-modulation of MHC class I antigen expression is restricted to neuronal cells only is not clear.

Discussion

We show here that a high level of expression of the N-*myc* gene in the rat neuroblastoma cell line B104 has at least three major consequences: a dramatic increase of the metastatic ability of these cells, as measured by the tail vein injection assay; a 10- to 15-fold reduction in the expression of the class I MHC antigens; and a significant increase in the in vivo growth rate, resulting in a 300-fold difference in tumor volume within 2 weeks.

The increased ability of the N-*myc*-expressing B104 cells to metastasize closely parallels observations made with human neuroblastomas, in which N-*myc* amplification has been shown to be correlated with increased metastatic ability (Brodeur et al., 1984; Seeger et al., 1985). The question arises whether the observed down-modulation of MHC class I antigens, in combination with the increased in vivo growth rate, can explain the increased metastatic ability of the N-*myc*-expressing neuroblastoma cells.

Since MHC class I antigens are required for the recognition of foreign antigen by cytotoxic T lymphocytes (Zinkernagel and Doherty, 1979), it follows that neuroblastoma cells that express the N-*myc* gene at a high level will be more resistant to T cell-mediated immune surveillance. The most compelling evidence suggesting that T cell-mediated immune responses can be effective in the prevention of metastatic spread of tumor cells was recently provided by a study on the mouse T10 sarcoma. This study showed that a highly metastatic subclone of the T10 sarcoma, which lacked expression of the H-2K alleles of the major histocompatibility complex, lost its metastatic ability upon restoration of H-2K antigen expression by gene transfer (Wallich et al., 1985). In consonance with

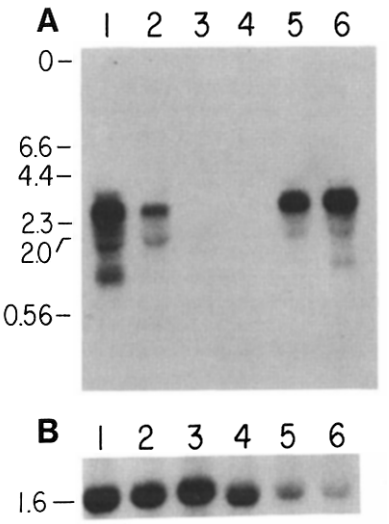

Figure 7. Northern Blot Analysis of N-*myc*-Transfected Rat-1 Cells

Twenty micrograms of total cytoplasmic RNA isolated from the following cell lines was used: lane 1, Rat-1 N-*myc* 1K-1; lane 2, Rat-1 N-*myc* 3K-1; lane 3, Rat-1; lane 4, B104; lane 5, B104 N-*myc*C6; lane 6, B104 N-*myc*C7. (A) Filter probed with a human N-*myc* probe. (B) Filter shown in (A) reprobed with the mouse MHC class I heavy chain gene probe.

this is the finding that MHC class I antigens were found to be expressed on virtually all human primary cutaneous melanomas, but only on half of the metastases derived from these tumors (Ruiter et al., 1984). We therefore feel that the N-*myc*-mediated down-modulation of MHC class I antigen expression in neuroblastoma may be an important causal factor in the progression towards a metastatic phenotype.

We have also observed that B104 neuroblastoma cells that express the N-*myc* gene at a high level show a dramatic growth advantage in immunocompetent syngeneic rats and in immunodeficient nude mice. Since successful establishment of a tumor at a secondary site depends both on the rate of killing of the spreading tumor cells and on the proliferation rate of the cells at the secondary site, it is likely that the increased growth rate of the N-*myc*-expressing cells also contributes to the increased ability of N-*myc*-amplified neuroblastomas to metastasize.

Modulation of MHC class I antigen expression was previously shown to be a property of the adenovirus type 12 E1a region (Schrier et al., 1983), which encodes gene products known to be involved in *trans*-regulation of gene expression (Berk et al., 1979; Nevins, 1981). There are a number of similarities between the *myc*- and E1a genes, including a nuclear localization of their gene products and an ability of each to cooperate with *ras* oncogenes in the transformation of primary cells (Yee et al., 1983; Land et al., 1983; Ruley, 1983; Persson and Leder, 1984). Other evidence suggests that the c-*myc* protein, like E1a, can enhance the transcription of other genes (Kingston et al., 1984). The present results represent the first demonstration of a cellular gene that is sensitive to regulation by a *myc* gene, in this case a negative regulation.

Expression of the N-*myc* gene in Rat-1 fibroblasts did not lead to a reduction of MHC class I antigen expression (Figures 7A and 7B). However, it did make these cells tumorigenic (M. Small, N. Hay, M. Schwab, and M. Bishop, unpublished). This finding indicates that some, but not all, of the phenotypic effects that are brought about by a high level of N-*myc* gene expression are cell-type-specific. Amplification of the N-*myc* gene has so far only been observed in cells of neuroectodermal origin (Kohl et al., 1983; Schwab et al., 1983; Lee et al., 1984; Nau et al., 1986). We speculate that the reason for this apparent tissue specificity of N-*myc* gene amplification is that some of the effects of N-*myc* gene amplification that are advantageous for tumor growth can only be elicited in cells of neuroectodermal origin.

It is difficult to assess at this point whether the observed regulation of MHC class I antigen expression by the N-*myc* gene product is of significance to the physiology of certain types of nonmalignant cells. In a number of in vitro model systems, however, there is a clear inverse correlation between the level of expression of a *myc* gene and the expression of MHC class I antigens. Thus, F9 teratocarcinoma cells, which undergo morphological differentiation upon addition of retinoic acid to the culture medium, lose expression of both c-*myc* and N-*myc* at the same time that they begin to express MHC class I antigens (Stern et al., 1975; Rosenthal et al., 1984; Dony et al., 1985; Jakobovits et al., 1985). Similar observations have been made in differentiating U937 leukemia cells (Yarden et al., 1984; Einat et al., 1985).

Such data indicate an inverse correlation between *myc* expression and MHC class I antigen expression and at the same time point to a relationship between expression of *myc* genes and maintenance of an undifferentiated state. Further evidence for this notion was recently provided by finding that constitutive high levels of c-*myc* expression could prevent DMSO-induced differentiation of MEL cells (Coppola and Cole, 1986), and by the finding that retinoic acid-induced differentiation of neuroblastoma cells is preceded by an early decline in the N-*myc* mRNA level (Amatruda et al., 1985; Thiele et al., 1985). Since MHC class I antigens are a marker of differentiated cells, our data suggest that overexpression of the N-*myc* gene in neuroblastoma can cause at least a partial reversion to a less differentiated state. In agreement with this view, in situ hybridization experiments have shown that high N-*myc* expression is found predominantly in the most undifferentiated cells of a tumor mass (Schwab et al., 1984).

Our data also show that the N-*myc*-mediated down-modulation of MHC class I antigen expression can be reversed by γ-interferon. This finding should make it possible experimentally to modulate MHC class I antigen expression of neuroblastoma cells in vivo and thus allow study of the role of T cell-mediated immune responses in the rejection of tumor cell metastases in more detail.

Experimental Procedures

Cell Culture

B014 cells were grown in Dulbecco's Modified Eagle's medium (DME) supplemented with 10% fetal calf serum. Transfections were per-

formed essentially as described by Van der Eb and Graham (1980). Twenty-four hours after transfection, cells were split 1:10 into medium containing 1 mg/ml of G418, and refed every third day.

To determine in vitro growth rates of cells, 5×10^3 cells were plated onto 24 well plates in 2 ml of DME medium supplemented with either 10% or 0.5% fetal calf serum. To determine growth rates, cells were harvested daily, and the number of cells per well was counted in triplicate using a Coulter counter. During growth rate experiments, cells were refed every third day.

Northern Blot Analysis

RNA isolation and Northern blot analysis were performed as described by Schrier et al. (1983). To eliminate influences of growth rate and cell cycle on the expression of the genes studied, RNA was isolated from exponentially growing cells in all cases. The probe used for detection of N-*myc* transcripts was a 500 bp XbaI–BamHI fragment isolated from pmp34.1, spanning most of exon 2 of the human N-*myc* gene (Schwab et al., 1985). c-*myc* transcripts were detected using a 900 bp XbaI–SacI fragment isolated from a genomic clone of the mouse c-*myc* gene (Land et al., 1983). This fragment spans exon 2 of the c-*myc* gene. Human MHC class I heavy chain transcripts were detected with an HLA B7 cDNA probe (Sood et al., 1981). Rat MHC class I heavy chain transcripts were detected with a fragment comprising the first 820 bp of the mouse H-2K^d cDNA (Lalanne et al., 1983), using low stringency hybridization (40% formamide at 42°C). Transcripts of the rat β_2-microglobulin gene were detected at the same low stringency conditions using a 900 bp EcoRI–HindIII fragment derived from mouse genomic DNA. This fragment spans exon 2 of the mouse β_2-microglobulin gene (Parnes and Seidman, 1982). α-tubulin transcripts were detected using an α-tubulin cDNA (Lemischka et al., 1981).

To remove probe, filters were washed twice in 50% formamide, 10 mM Tris (pH 7.4), and 1 mM EDTA at 70°C for 30 min.

Immunofluorescence

To measure cell-surface expression of MHC class I antigens on rat cells, cells were removed from the culture plates using phosphate buffered saline supplemented with 2 mM EDTA. Cells (5×10^5) were resuspended in 25 μl of a 1:100 dilution of the monoclonal antibody OX18 (obtained from Serotec, Bichester, England) in RPMI medium containing 10% calf serum. Incubation was for 30 min on ice. To remove excess antibody, cells were washed once with RPMI medium and subsequently were incubated with a 1:20 dilution of FITC-labeled sheep anti-mouse IgG serum (obtained from Cooper Biomedical, West Chester, PA) in RPMI medium. Incubation was for 30 min on ice. After this, cells were washed three times with RPMI medium and resuspended in 0.5 ml of PBS containing 2% calf serum. Fluorescence was quantitated using an Ortho Diagnostics model 2150 cytofluorograph.

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