Lack of Correlation between N-myc and MAX Expression in Neuroblastoma Tumors and in Cell Lines: Implication for N-myc-MAX Complex Formation

Giuseppe Raschella,1 Antonio Romeo, Anna Negroni, Sabina Pucci, Carlo Dominici, Manuel A. Castello, Paola Bevilacqua, Armando Felsani, and Bruno Calabretta2

ENEA (Ente Nuove Tecnologie Energia e Ambiente) CRE Casaccia, Division of Molecular Biology, Via Anguillarese, 303, 00060 S. Maria di Galeria, Rome, Italy (G. R., A. R., A. N., S. P.); Department of Pediatrics, University of Rome "La Sapienza," 00161 Rome, Italy (C. D., M. A. C.); Istituto Regina Elena per lo Studio e la Cura dei Tumori, CRS, 00158 Rome, Italy (P. B., A. F.); Istituto Tecnologie Biomediche, CNR, 00151 Rome, Italy (A. F.); and Department of Microbiology, and Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 (B. C.)

ABSTRACT

Detectable levels of MAX messenger RNA were found in a set of human neuroblastoma tumors and established cell lines. MAX mRNA levels were independent of tumor stage and N-myc genomic amplification. By contrast, N-myc mRNA transcripts were detectable only in tumors with amplification of N-myc gene and in cell lines. Analysis by reverse transcriptase polymerase chain reaction and hybridization to specific oligodeoxynucleotide probes revealed approximately equal amounts of two MAX transcripts in all cases analyzed. Immunoprecipitations with a specific antibody to MAX detected two proteins of Mr 21,000 and 22,000 in a neuroblastoma cell line with antisense oligodeoxynucleotides resulted in inhibition of N-myc expression, reorganization of the cytoskeleton to a neuronal differentiated type, and decrease in the proliferation rate. In addition, N-myc antisense transcripts restricted the differential potential of primitive neuroectodermal cell lines (6). However, despite the wealth of data associating N-myc expression with differentiation (5, 7) and proliferative (8) events, the function of N-myc remains elusive. Like c-myc, N-myc localizes to the nucleus (9) and has been shown to bind to DNA in vitro (10). Recently, a protein was identified that is able to dimerize with c-myc, N-myc and L-myc. This protein, termed MAX in humans (10) and myn in mice (11), contains a helix-loop-helix and a leucine zipper domain which are essential for dimerization (10). At the 5' of the helix-loop-helix, a basic region which is common to both MAX and myc genes allows the dimer to bind to DNA. Binding of MAX to myc proteins is highly specific; in fact, MAX fails to form a dimer with other proteins containing a helix-loop-helix or a leucine zipper domain or both (10). The myc-MAX complex has a higher affinity for the myc consensus core sequence CAGCTG than does either MAX or myc alone (10). In mice, methylation of the recognition site inhibits DNA binding (11), suggesting that DNA methylation might regulate the myc-MAX complex in vivo. c-myc-MAX and N-myc-MAX association in vivo was demonstrated in human Burkitt’s lymphoma (12) and in neuroblastoma cells (13), respectively. However, the questions of how MAX expression is regulated in the cell and whether there is a correlation with the regulation of its partner genes remain unanswered.

In this study, we compared the steady-state levels of MAX and N-myc mRNAs in a set of human neuroblastomas and cell lines. In addition, we detected comparable levels of MAX proteins in neuroblastoma lines and assessed the ability of whole cell extracts from two cell lines with opposite levels of N-myc expression to interact differentially with a myc binding consensus sequence. Our findings indicate that MAX expression is independent of N-myc, suggesting that the absence of simultaneous regulation of N-myc and MAX expression affects heterodimer formation in neuroblastoma cells.

INTRODUCTION

Neuroblastoma is the most frequent noncranial solid tumor of childhood with an occurrence of 10 per million children yearly (1). Deletion of the short arm of chromosome 1 (2) and the amplification and consequent overexpression of the N-myc oncogene (3) are common genetic features of this neoplasia. Clinical studies have demonstrated a correlation between N-myc amplification, advanced tumor stage, and poor outcome (3). Recently, we described an association between the expression of N-myc and that of the proliferation-associated genes histone H3, c-myc and p53 (4). Treatment of a neuroblastoma cell line with antisense oligodeoxynucleotides resulted in inhibition of N-myc expression, reorganization of the cytoskeleton to a neuronal differentiated type, and decrease in the proliferation rate (5). In addition, N-myc antisense transcripts restricted the differentiative potential of primitive neuroectodermal cell lines (6). However, despite the wealth of data associating N-myc expression with differentiation (5, 7) and proliferative (8) events, the function of N-myc remains elusive. Like c-myc, N-myc localizes to the nucleus (9) and has been shown to bind to DNA in vitro (10). Recently, a protein was identified that is able to dimerize with c-myc, N-myc and L-myc. This protein, termed MAX in humans (10) and myn in mice (11), contains a helix-loop-helix and a leucine zipper domain which are essential for dimerization (10). At the 5' of the helix-loop-helix, a basic region which is common to both MAX and myc genes allows the dimer to bind to DNA. Binding of MAX to myc proteins is highly specific; in

1 To whom requests for reprints should be addressed, at the Department of Microbiology, Thomas Jefferson University, 10th and Locust Streets, Philadelphia, PA 19107 (B. C.), or at the ENEA CRE Casaccia, Division of Molecular Biology, Via Anguillarese, 303, 00060 S. Maria di Galeria, Rome, Italy (G. R.).

2 The abbreviations used are: RT, reverse transcriptase; PCR, polymerase chain reaction; SSC, standard saline-citrate (1 X SSC is 0.15 M NaCl-O.015 M sodium citrate); SDS, sodium dodecyl sulfate; cDNA, complementary DNA.
LACK OF CORRELATION BETWEEN N-myc AND MAX EXPRESSION

RESULTS

Construction of a MAX-specific Probe by RT-PCR. A 5'-primer spanning the region from nucleotides -12 to 12 and a 3'-primer complementary to the sequence from nucleotides 442 to 465 of the MAX cDNA sequence reported by Blackwood and Eisenman (10) were synthesized. Total RNA from neuroblastoma cell line IMR32 was reverse-transcribed using the 3' MAX primer, and the resulting cDNA was used as template for PCR. The PCR product was purified by chromatography through a G-50 column. Probe DNA (10^-7 pmol) was incubated in a final volume of 20 

<table>
<thead>
<tr>
<th>No.</th>
<th>Stage</th>
<th>N-myc copy no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IV</td>
<td>63</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>&lt;3</td>
</tr>
<tr>
<td>3</td>
<td>IV (Metastasis)</td>
<td>&lt;3</td>
</tr>
<tr>
<td>4</td>
<td>IV</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>I</td>
<td>&lt;3</td>
</tr>
<tr>
<td>6</td>
<td>IV</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>IV (Metastasis of no. 6)</td>
<td>29</td>
</tr>
<tr>
<td>8</td>
<td>IV-S</td>
<td>&lt;3</td>
</tr>
<tr>
<td>9</td>
<td>II</td>
<td>&lt;3</td>
</tr>
<tr>
<td>10</td>
<td>IV-S</td>
<td>&lt;3</td>
</tr>
<tr>
<td>11</td>
<td>II</td>
<td>&lt;3</td>
</tr>
<tr>
<td>12</td>
<td>III</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>

Table 1 N-myc amplification and clinical stage of the neuroblastomas analyzed

Fig. 1. Northern blot analysis of neuroblastomas RNAs. A, hybridization of 10 mg of total RNA to the MAX-specific probe (exposure time, 18 h). B, hybridization of the same filter to the N-myc-specific probe (exposure time, 18 h) after removal of the previous probe. C, ethidium bromide staining of the gel. Arrows, position of the 28S and 18S ribosomal RNAs. Numbers 1 to 12 correspond to the neuroblastomas listed in Table 1.

transcripts in only the three primary tumors at stage IV and the metastasis (Fig. 1B, Lanes 1, 4, 6, and 7) with amplified N-myc gene and in the N-myc amplified cell line IMR32 (Fig. 1B). Hybridization of the same RNAs to a human c-myc probe did not reveal any detectable signal (not shown).

Detection of Two MAX Transcripts in Neuroblastoma. To determine whether the heterogeneous MAX product detected by RT-PCR in IMR32 cells reflected the presence of more than one transcript, RT-PCR was carried out with the tumor RNAs from the Northern analysis with the primers used to construct the MAX-specific probe. RT-PCR products were transferred to a nylon membrane in duplicate and hybridized with two MAX probes: MAX-3 which is identical to 20 of the 27 nucleotides inserted 5' of the basic region in several cDNAs of Manca cells mRNA (10) and able to
Fig. 2. Detection of MAX transcripts and proteins by RT-PCR and immunoprecipitation analyses. A, RT-PCR products were hybridized to probe MAX-3 after transfer to a nylon membrane (exposure time, 1 h). B, hybridization to probe MAX-4 (exposure time, 1 h). C, ethidium bromide staining of a 6% polyacrylamide gel of the RT-PCR products. Numbers 1 to 12 are as in Fig. 1. Lymph and Fibroblasts are RNA from normal resting lymphocytes and normal proliferating fibroblasts respectively. Arrows in B, close doublet revealed by probe MAX-4. In D, MAX proteins were immunoprecipitated from [35S]methionine-labeled IMR32, LAN-5, and SK-N-SH cells. As control, LAN-5 whole cell lysate was treated with preimmune serum (Lane C). Left, the position of p21–22 MAX doublet. Right, number indicates the position of a 19-kDa molecular mass marker.

Two MAX Proteins are Expressed in Neuroblastoma Cells. To investigate if MAX protein(s) expression correlates with the presence of the MAX mRNAs in neuroblastomas, immunoprecipitations were carried out using a specific antibody to MAX on whole-cell lysates prepared from [35S]methionine-labeled neuroblastoma cell lines LAN-5 which has a 50-fold N-myc amplification (22) and expresses N-myc mRNA high levels (5), IMR32 and SK-N-SH. A closely spaced doublet banding at Mr 21,000–22,000 was detected in all samples (Fig. 2D) in agreement with the reported molecular weight for MAX proteins (12). Immunoprecipitation of the cell extract from LAN-5 cell line with preimmune serum did not detect any band in the Mr 21,000–22,000 region (Fig. 2D).
genomic DNA was run on an agarose gel and transferred to a nylon membrane. A, mas for which no RNA was available for Northern and RT-PCR analyses. N-myc-specific probe (exposure time, 72 h). Samples 1, 2, 6 and 7 correspond to hybridization to the MAX-specific probe (exposure time, 72 h). B, hybridization to the consensus sequence, we carried out electrophoresis mobility shift assay experiments using whole cell extracts from LAN-5 (high level of N-myc expression) and SK-N-SH (no detectable expression of N-myc). Whole cell extracts were prepared in high salt conditions to increase the solubility of myc proteins. Different band shift patterns were obtained using LAN-5 and SK-N-SH extracts (Fig. 3A). LAN-5 shifts showed an upper band (Fig. 3A, arrow) which was not present in SK-N-SH. Although also the ubiquitous transcription factor binds to the same DNA sequence recognized by myc proteins, it seems likely that the upper band seen in LAN-5 extracts derives from the binding of N-myc-MAX heterodimers to the oligonucleotide probe which does not occur in SK-N-SH extracts due to the lack of N-myc expression. To determine whether the upper band seen in Fig. 3A depended on N-myc expression in LAN-5 cells, total extracts from LAN-5 and SK-N-SH cells were preincubated with a monoclonal antibody to N-myc before electrophoresis mobility shift assay. Preincubation with the N-myc antibody reduced the density of the upper band seen with LAN-5 cell extracts (Fig. 3B, Lanes 2 and 3), whereas the pattern seen with the SK-N-SH extracts was not modified by the treatment. In control experiments, preincubation with an unrelated monoclonal antibody did not affect the intensity of the upper band seen with LAN-5 cell extracts (Fig. 3B, Lane 4).

Southern Blot Analysis of Neuroblastoma Genomic DNA. Hybridization of EcoRI-digested genomic DNA from five primary neuroblastomas and a metastasis of one of them to the MAX-specific probe revealed several bands ranging from more than 9 kilobases to less than 1 kilobase, a pattern that was invariant in all samples (Fig. 4A). Analysis of gene dosage, after normalization of the amount of DNA for each sample, revealed no genomic amplification of the MAX gene (not shown), suggesting that the transcription of MAX does not depend on amplification of the gene. Hybridization of the same filter to N-myc-specific probe, demonstrated amplification of N-myc in two tumors at stage IV and in one metastasis (Fig. 4B, Lanes 1, 6, and 7).

DISCUSSION

The association in vivo of N-myc oncoprotein with MAX phosphoprotein has been described in human neuroblastoma (13). This result suggests a role of the N-myc-MAX dimer in this neoplasia. Using a probe obtained by RT-PCR, we have detected MAX mRNA in 10 primary neuroblastomas, two metastases, and two cell lines, at comparable levels in all samples and independent of tumor stage or N-myc genomic amplification. Anti-MAX antibody immunoprecipitated two MAX proteins in all neuroblastoma cell lines tested. Thus, it seems unlikely that MAX expression is controlled in neuroblastoma by a postranscriptional mechanism preventing translation of MAX mRNAs. By contrast, N-myc mRNA was present only in samples with amplified N-myc gene, suggesting that the latter is a limiting factor in the formation of the N-myc-MAX dimer in neuroblastoma. Unlike N-myc, the MAX levels of transcripts do not appear to depend on genomic amplification.

The detection of MAX transcripts in neuroblastomas that do not express N-myc raises the question of the function of MAX gene. It has been demonstrated in humans and mice that MAX can form an homodimer (10—12) the role of which in the cell might differ from that of the heterodimer. MAX protein(s) lacks the transactivation domain (22), yet MAX-MAX homodimers bind the same CACGTG sequence recognized by myc genes (10—12). Expression of MAX in excess over myc genes can shift the equilibrium in favor of homodimers, which in turn compete out the transactivating myc-MAX heterodimers (12), keeping the controlled genes in a state of transcriptional repression (23).

In neuroblastoma, the amplification and overexpression of N-myc is a marker of tumor aggressiveness (3). We can speculate that in tumors where only MAX expression is detectable, those genes under the transcriptional control of N-myc are silenced by MAX homodimers. The lack of expression of these genes could somehow decrease the tumor aggressiveness. The identification of neuroblastomas without N-myc amplification and overexpression, yet highly aggressive, might be explained with the activation of different pathways independent from those associated with N-myc-MAX expression. Alternatively, in tumors that do not express N-myc, the MAX protein could be in an inactive form. In light of the presence of a consensus signal for casein kinase II 3' of the leucine zipper of MAX (10) it is possible that the activity of MAX protein is related to the phosphorylation state.

All of the neuroblastoma samples analyzed revealed the presence of two MAX transcripts, one of which derives from an in-frame insertion of 27 nucleotides in a region 5' to the basic region. Since mutations outside the basic region helix-loop-helix-leucine zipper domain do not alter the ability of MAX to dimerize and bind to DNA (10, 11), it is conceivable that the presence of two MAX forms able to dimerize with N-myc serves to broaden the range of the gene activities controlled by the N-myc-MAX complex.

ACKNOWLEDGMENTS

We thank Dr. Edward B. Ziff for the gift of the anti-MAX antiserum.

REFERENCES

LACK OF CORRELATION BETWEEN N-myc AND MAX EXPRESSION


Lack of Correlation between N-myc and MAX Expression in Neuroblastoma Tumors and in Cell Lines: Implication for N-myc-MAX Complex Formation

Giuseppe Raschella, Antonino Romeo, Anna Negroni, et al.