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Genetics

A dominant-negative mutant of Max that inhibits sequence-specific DNA binding by Myc proteins

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ABSTRACT **Myc proteins are basic helix–loop–helix/leucine-zipper proteins that bind to specific DNA sequences. *In vivo*, Myc proteins have been found associated with Max, another basic helix–loop–helix/leucine-zipper protein. However, it is not known to what extent the dimerization of Myc with Max is required for the manifestation of the Myc-induced phenotype. To investigate this, we constructed a dominant-negative mutant of Max, named dMax, that inhibits sequence-specific DNA binding of Myc proteins. Using a rat neuroblastoma model system, we show that dMax reverts N-Myc-induced changes in cellular gene expression. A control mutant of dMax that contains a proline residue in the leucine-zipper region was unable to bind to N-Myc and did not revert the N-Myc-induced changes in cellular gene expression. These data support the hypothesis that N-Myc affects neuroblastoma gene expression through the formation of a DNA-binding heterodimeric complex with Max *in vivo*.**

myc genes encode short-lived nuclear proteins whose deregulated expression contributes to the malignant phenotype of a variety of human cancers (1–4). Myc proteins can bind to specific DNA sequences and activate transcription (1, 3, 4). A number of conserved regions are shared by the c-, L-, and N-Myc proteins. Three of these domains, localized in the N termini of Myc proteins, are required for their transcription-activating ability (5, 6). A C-terminal conserved motif consists of a basic region followed by two amphipathic helices connected by a variable-size spacer (basic helix–loop–helix, bHLH). An additional helical motif, the leucine zipper (Zip), is found immediately adjacent to the second amphipathic helix. The bHLH-Zip is thought to be a bipartite structure where the basic region is a DNA-binding element and the HLH-Zip is a dimerization surface (7–12).

Myc proteins do not form homodimers efficiently but preferentially heterodimerize *in vitro* and *in vivo* with another bHLH-Zip protein named Max (13–16). Three isoforms of Max, all encoded by the same gene, have been described. The longest form of Max (Max/p22) has a predicted size of 160 amino acids, whereas the two shorter forms lack either 9 amino acids in the N-terminal part upstream of the basic region (Max/p21, ref. 14) or 62 amino acids in the C-terminal part (ΔMax, ref. 17). The three Max variants all carry the bHLH-Zip motif and retain the ability to bind to a CACRTG sequence motif (where R is G or A) when present in homodimeric or heterodimeric complexes with Myc proteins (12, 14, 18, 19).

Recently, it has been shown that c-Myc, N-Myc, and L-Myc proteins associate with Max *in vivo* (15, 20, 21). However, it is not known to what extent the Myc–Max complexes contribute to the biological properties of Myc proteins. The identification of several naturally occurring HLH proteins that lack a basic DNA-binding domain suggested a strategy to address this issue. These HLH proteins (for example, the mammalian proteins Id1, Id2, and HLH462 and the *Drosophila* Emc) can dimerize with certain other bHLH proteins, but the heterodimers fail to bind to DNA (22–26). HLH proteins without a basic domain are, therefore, negative regulators of other bHLH proteins (27, 28). Based on these findings, we designed a mutant form of Max, dubbed dMax, in which the basic DNA-binding domain is deleted but the HLH-Zip motifs are preserved. We show here that dMax acts as a transdominant negative suppressor of DNA binding by Myc proteins through formation of a non-DNA-binding heterodimeric Myc–dMax complex and that dMax reverts N-Myc-induced changes in neuroblastoma gene expression.

MATERIALS AND METHODS

PCR Cloning and Plasmid Constructions. The d*max* gene was constructed by PCR, using the human Max/p22 cDNA as a template and the two following primers for the reaction: 5′ primer, 5′-CATGGGATCCACCATGGTGAACGTCCTGGACCACATCAAAGACAGCTT-3′; 3′ primer, 5′-CATGGAATTCTTAGCTAGCGTAATCTGGAACATCGTATGGGTATCCTCCGCTGGCCTCCATCCGGAGCTT-3′. The PCR product was digested with *Bam*HI and *Eco*RI and cloned into pSelect (Promega). The identity of the cloned PCR product was verified by DNA sequence analysis. dmaxPLZ was constructed using the Altered Sites *in vitro* mutagenesis system (Promega) and the oligonucleotide 5′-ATTCTGCCGCTTGGGGTCGTCAATATC-3′, which introduces a mutation in codon 57 of d*max*, replacing the leucine with a proline. The dmax and dmaxPLZ cDNAs were cloned into the mammalian expression vector pJ4Ω (29).

Antibodies. Two anti-N-Myc antibodies were used: the mouse monoclonal antibody AB-1 (Oncogene Sciences, Mineola, NY) and the rabbit polyclonal B1 (a gift from Gerard Evan, Imperial Cancer Research Fund, London). To detect Myc proteins, we also used the rabbit polyclonal pan-Myc (Cambridge Research Biochemicals, Valley Stream, NY) and the rabbit polyclonal 527 serum (a gift from Steven Hahn, Fred Hutchinson Cancer Research Center, Seattle). The Max antibody is a rabbit polyclonal serum that was raised against the 124 C-terminal amino acids of Max. The mouse monoclonal 12CA5 has been described (30).

Cell Culture and DNA Transfection. Culture conditions of B104 cells, the generation of N-*myc* transfectants and transfection conditions have been described (31, 32). For activation of protein kinase C, cells were incubated with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) for 45 min at 37°C. After this the cells were washed in phosphate-buffered saline (PBS) and whole cell protein extracts were subsequently prepared as described below.

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Abbreviations: bHLH, basic helix–loop–helix; Zip, leucine zipper; MHC, major histocompatibility complex; PMA, phorbol 12-myristate 13-acetate; EMSA, electrophoretic mobility shift assay.

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***In Vitro* Transcription and Translation.** c-Myc and N-Myc proteins were generated by sequential *in vitro* transcription and translation of the respective cDNAs. dmax and dmax-PLZ RNAs were cotranslated with either c-myc or N-myc RNA generated as described above.

Cell Labeling and Immunoprecipitation. For sequential immunoprecipitations, 2×10^7 cells were labeled in medium supplemented with L-[^{35}S]methionine (Tran^{35}S-label, ICN; 400 μCi/ml; 1 Ci = 37 GBq) and 2% (vol/vol) fetal calf serum for 1 h at 37°C. After this, cells were lysed in lysis buffer (PBS supplemented with 1% Nonidet P-40, 1 mM dithiothreitol, 1 mM EDTA, and protease inhibitors), sonicated on ice, and centrifuged at 10,000 × *g* to remove cellular debris. The lysate was incubated with an anti-Myc serum (a mixture of B1, AB-1, 527, and polyclonal anti-pan-Myc antibodies) and the precipitate was collected with protein A-Sepharose beads. The immune complexes were dissociated by incubation for 5 min at room temperature in 30 μl of Laemmli buffer [50 mM Tris·HCl, pH 8.0/2% (wt/vol) SDS/20% (vol/vol) glycerol]. After this, the immune complexes were diluted with 1 ml of RIPA buffer (150 mM NaCl/1% Nonidet P-40/0.5% deoxycholate/0.1% SDS/50 mM Tris·HCl, pH 8.0). The proteins that were released from the immunoprecipitate were subsequently divided in three equal parts and incubated with the Myc antibody mixture, 12CA5 monoclonal antibody, or normal mouse serum. After incubation, immune complexes were collected by binding to protein A-Sepharose beads and resolved on a SDS/12% polyacrylamide gel.

Electrophoresis-Mobility-Shift Assays (EMSAs). Preparation of whole cell protein extracts and conditions for EMSAs have been described (32). As a competitor, either 3 μg of poly(dI-dC)·poly(dI-dC) (for the NF-κB experiment) or 25 ng of sheared salmon sperm DNA (for the Myc–Max DNA binding experiment) was used. The following oligonucleotides were used: NF-κB, 5′-TGGGGATTCCCCA-3′; mutant oligonucleotide, 5′-TGCGGATTCCCGA-3′; CM1 oligonucleotide, 5′-CCCCCACCACGTGGTGCCTGA-3′.

Flow Cytometry Analysis. Cell surface expression of major histocompatibility complex (MHC) class I antigens was determined using the mouse monoclonal antibody anti-rat MHC class I molecules OX18 (Serotec) essentially as described (31) and quantitated with a FACScan (Becton Dickinson).

RESULTS

A Mutant of Max That Inhibits DNA Binding by the N-Myc Protein. A vector that directs the synthesis of dMax, a protein that lacks the 37 N-terminal amino acids of Max/p22, was constructed (Fig. 1). As a result of this, the basic region of the bHLH-Zip motif and the putative caseine kinase II phosphorylation site were deleted in dMax. To allow for the correct folding of the HLH domain in the dMax protein, 4 amino acids (VNVL) were inserted directly N-terminal of the HLH motif in dMax. These 4 amino acids are also present at the corresponding position in the Id protein, and in the Id protein the presence of these 4 amino acids just upstream of the HLH domain is known to allow correct folding of the HLH domain. As a control, we constructed a mutant of dMax, named dMaxPLZ, in which a proline replaced the first leucine of the dMax Zip. Since proline is a helix breaker, we expected that the dMaxPLZ protein would be unable to dimerize with Myc proteins. To facilitate the detection of dMax protein in transfected cells, both mutant Max constructs contained a C-terminal extension of 10 amino acids that is recognized by the monoclonal antibody 12CA5 (30).

To test whether the mutant Max proteins behaved as predicted, [^{35}S]methionine-labeled dMax and dMaxPLZ proteins were generated by sequential *in vitro* transcription and translation of the respective cDNAs. These proteins were then tested for their ability to bind *in vitro*-translated c-Myc protein. Immunoprecipitations were carried out using nonionic detergent conditions, known to preserve the Myc–Max association (15). Fig. 2 shows that a serum directed against c-Myc coprecipitates dMax but not dMaxPLZ. Furthermore, the 12CA5 antibody coprecipitates c-Myc from a mixture of c-Myc and dMax, but not from a mixture of c-Myc and dMaxPLZ. These data indicate that c-Myc forms heterodimers with dMax *in vitro* but fails to dimerize with dMaxPLZ.

We then tested the DNA-binding ability of N-Myc–dMax heterodimers. Analysis of the *in vitro*-translated N-Myc, Max, and dMax proteins by SDS/PAGE confirmed that they were correctly translated (data not shown). Fig. 3 shows that a mixture of *in vitro*-translated N-Myc and Max proteins binds to an oligonucleotide that specifies the consensus Myc–Max DNA-binding sequence in an EMSA (33). In contrast, the N-Myc–dMax heterodimer failed to bind the CACGTG motif. Antibodies against the N-Myc and Max proteins interfered with the formation of the specific DNA-

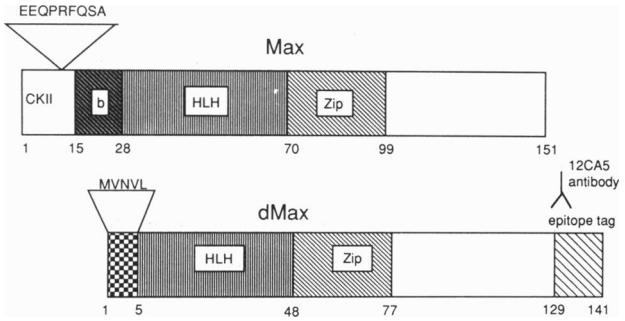

FIG. 1. Schematic representation of Max and dMax proteins. The casein kinase II site (CKII), the basic region (b), the HLH, and the Zip are indicated. The 9-amino acid insertion that differs between Max/p22 and Max/p21, the 5 N-terminal amino acids specific to dMax, and the C-terminal epitope tag are also indicated (see text for further details).

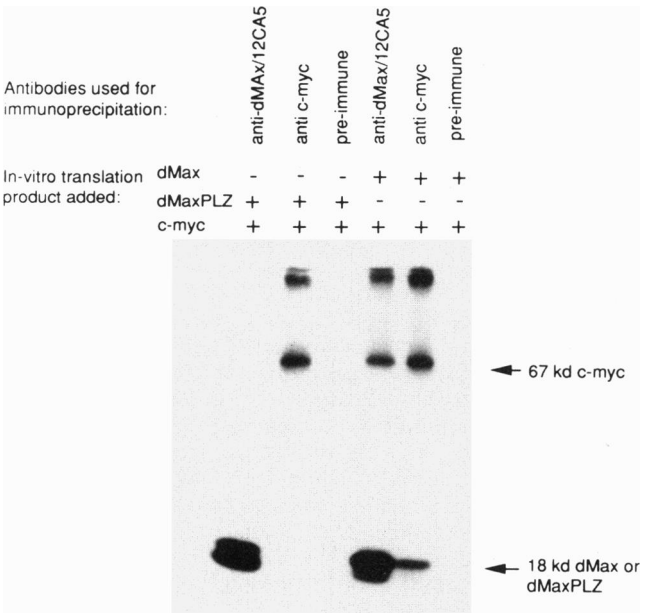

FIG. 2. Dimerization properties of mutant Max proteins. dmax or dmaxPLZ RNAs were cotranslated in a reticulocyte lysate with c-myc RNA, and the resulting ^{35}S-labeled proteins were immunoprecipitated under low-stringency detergent conditions that are known to preserve the Myc–Max heterodimer. kd, kDa.

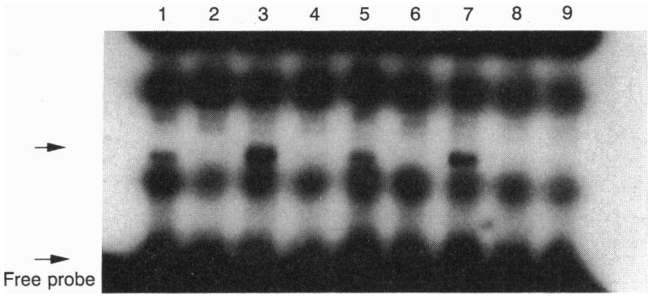

FIG. 3. EMSA of Myc–Max complexes. EMSA of the N-Myc–Max and N-Myc–dMax complexes. The labeled CM1 oligonucleotide that specifies the Myc–Max DNA-binding site was incubated with ^{35}S-labeled *in vitro*-translated proteins or with combinations of these, as indicated. Supershift experiments where performed by incubating *in vitro*-translated proteins with antibodies specific to Max and N-Myc or a nonimmune serum. The top band observed in each lane represents endogenous upstream stimulatory factor activity present in the reticulocyte lysate. The upper arrow indicates the specific N-Myc–Max or Max–Max nucleoprotein complexes. Lanes: 1, Max; 2, dMax; 3, N-Myc plus Max; 4, N-Myc plus dMax; 5, Max plus preimmune serum; 6, Max plus anti-Max; 7, N-Myc, Max, plus preimmune serum; 8, N-Myc, Max, plus anti-N-Myc; 9, N-Myc, Max, plus anti-Max.

binding complex, indicating that both N-Myc and Max were present in the complex.

Transfection of N-Myc-Expressing Rat Neuroblastoma Cells with the d*max* Gene. We have shown previously that transfection of the N-*myc* gene in the rat neuroblastoma cell line B104 leads to a drastic reduction in the expression of MHC class I antigens and of the δ isoform of protein kinase C (31, 32). Since these changes in cellular gene expression were caused by expression of N-Myc, the rat neuroblastoma cells provided a useful model system to study the effects of dMax on the N-Myc-induced phenotype. Analysis by ribonuclease protection assay showed that both the mRNAs coding for Max/p22 and Max/p21 were expressed at equivalent levels in B104 cells and in B104 N-*myc*C7 transfectants (data not shown).

B104 N-*myc*C7 cells were transfected with the selectable marker pSV2gpt and an expression vector that directed the synthesis of either dMax or dMaxPLZ. Clonal cell lines of mycophenolic acid-resistant cells were established and RNA derived from these transfectants was tested for the expression of the d*max* gene by Northern blot analysis.

Fig. 4*A* shows that several stable lines of B104 N-*myc*C7 cells were derived that express significantly more mutant dmax mRNA (0.9 kb in size) as compared to endogenous 2.0- and 1.7-kb max transcripts. Similarly, several lines were obtained that express equally high levels of dmaxPLZ mRNA (data not shown). All transfectants were subsequently tested for the expression of the N-*myc* gene. Only transfectants that had retained high levels of N-myc mRNA were used in the following experiments. To measure expression of the mutant Max proteins, transfectants were labeled with [^{35}S]methionine and immunoprecipitated with the monoclonal antibody 12CA5. Fig. 4*B* shows an 18- to 19-kDa protein is recognized by the 12CA5 antibody and not by a control antibody. Immunostaining of d*max*-transfected cells with the 12CA5 antibody showed that the dMax protein was predominantly nuclear (data not shown).

To determine whether dMax was associated with N-Myc *in vivo*, nonionic detergent lysates were prepared from [^{35}S]methionine-labeled d*max*-transfected cells and immunoprecipitated with an N-Myc antibody. To determine whether dMax was coprecipitated with N-Myc, associated proteins were released from the anti-N-Myc immunoprecipitate by incubation in a SDS-containing buffer and released proteins were

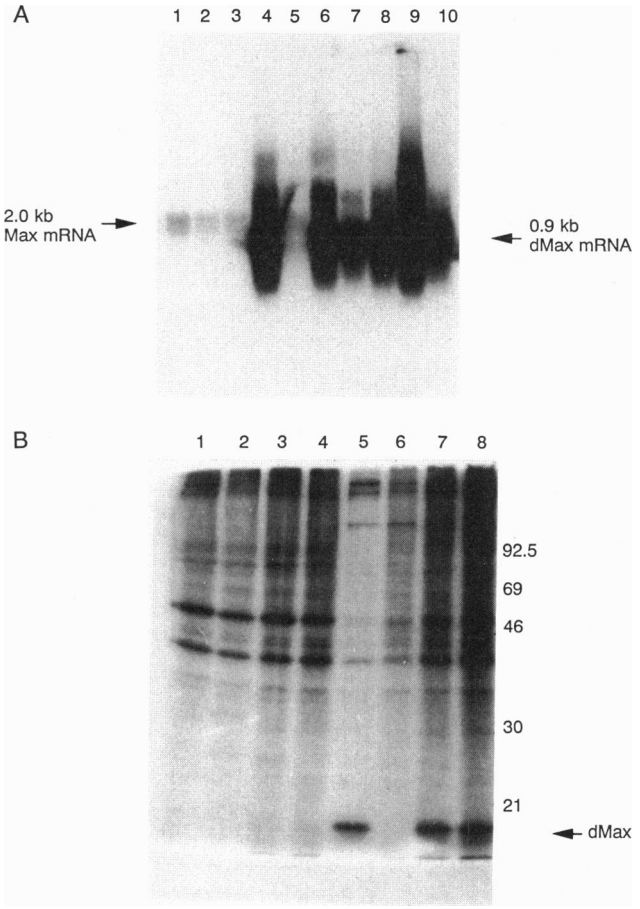

FIG. 4. Expression of Max in transfected cells. (*A*) Total cellular RNA from seven d*max*-transfected B104 N-*myc*C7 cell lines (B104 N-*myc*C7 dMax1, -3, -4, -5, -7, -9, and -12; lanes 4–10, respectively) and from parental B104 N-*myc*C7 cells (lane 1), another B104 N-*myc*-transfected cell line (B104 N-*myc*C6; lane 2), and the B104 neuroblastoma cells (lane 3) was electrophoresed through a 1% formaldehyde/agarose gel, transferred to nylon membrane, and probed for the expression of max mRNA. The dmax probe used detects both the endogenous 1.7- and 2.0-kb mRNAs that encode Max/p21 and Max/p22 and the 0.9-kb transfected dmax mRNA. (*B*) Lysates from [^{35}S]methionine-labeled cells were immunoprecipitated with either a nonimmune mouse serum (lanes 1–4) or with the 12CA5 monoclonal antibody (lanes 5–8). Lysates from four d*max*-transfected B104 N-*myc*C7 cell lines were used. Lanes: 1 and 5, dMax1; 2 and 6, dMax3; 3 and 7, dMax4; 4 and 8, dMax6. The positions of the molecular mass markers are indicated in kDa.

reimmunoprecipitated with either a polyclonal antiserum against Max or the 12CA5 antibody that recognizes only dMax. The results of this, shown in Fig. 5, indicate that both the 12CA5 monoclonal antibody and a polyclonal antiserum against the 124 C-terminal amino acids of Max could reprecipitate dMax protein from an anti-N-Myc immunoprecipitate. We conclude from this that dMax is associated with N-Myc in the transfected cells. The 12CA5 dMax antibody and the polyclonal anti-Max serum precipitated approximately equal amounts of Max protein from the N-Myc immunoprecipitate (Fig. 5). Since the polyclonal rabbit anti-Max serum precipitates Max/p22, Max/p21, and dMax, this suggests that most of the Max protein bound to N-Myc in the transfectants is dMax.

dMax Expression Reverts the N-Myc-Induced Changes in Cellular Gene Expression. Transfection of N-*myc* in B104 neuroblastoma cells reduces expression of MHC class I antigens by 10- to 15-fold (31). To assess the effect of dMax on MHC class I gene expression in the rat neuroblastoma

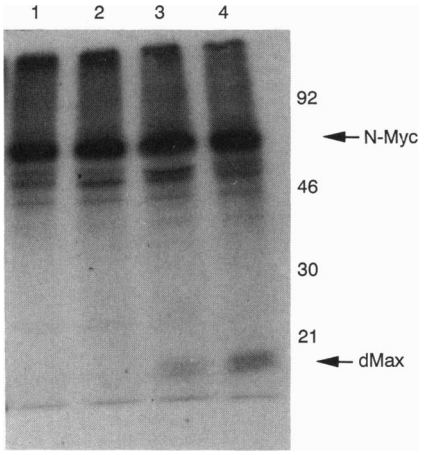

FIG. 5. Sequential immunoprecipitation of N-Myc-associated proteins. [^{35}S]Methionine-labeled lysate of B104 N-*myc* dMax4 cells was immunoprecipitated under low-stringency conditions, known to not disrupt the Myc–Max heterodimers. Immunoprecipitated N-Myc protein complexes were subsequently dissociated and reprecipitated with a preimmune serum (lane 1), an anti-N-Myc serum (lane 2), the 12CA5 monoclonal antibody (lane 3), or the polyclonal Max antiserum (lane 4). In all lanes, N-Myc protein is present in the reprecipitation because the SDS treatment used to dissociate N-Myc–Max complexes did not completely dissociate the complex between N-Myc protein and the anti-N-Myc antibody.

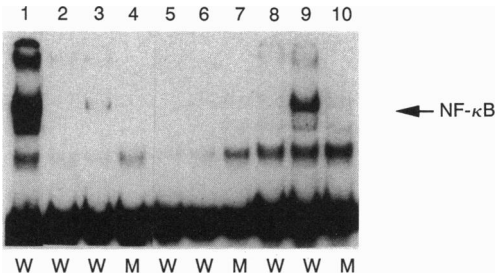

FIG. 7. Activation of NF-κB in d*max*-transfected cells. EMSA is shown of d*max*-transfected neuroblastoma cells before and after PMA treatment. Whole cell protein extracts were prepared both before (lanes 2, 5, and 8) and after (lanes 1, 3, 4, 6, 7, 9, and 10) PMA treatment. Protein extracts were incubated with either a ^{32}P-labeled oligonucleotide that specifies the NF-κB motif (lanes W) or a mutant oligonucleotide that does not bind NF-κB (lanes M). The position of the NF-κB complex is indicated by an arrow. Lanes: 1, B104; 2–4, dMax1; 5–7, dMax3; 8–10, dMax4.

cells, we measured by cell surface immunofluorescence and flow cytometry expression of MHC class I antigens in d*max* transfectants. Fig. 6 shows that B104 N-*myc* cells that express dMax at high levels express MHC class I antigens at the same levels as parental B104 neuroblastoma cells (clones dMax1 and dMax4). Importantly, MHC class 1 expression was only marginally effected in d*max*PLZ transfectants (Fig. 6 *Upper*).

N-Myc expression in neuroblastoma cells also disrupts protein kinase C-mediated signal transduction (32). As a result, the transcription factor NF-κB cannot be activated upon phorbol ester treatment of these cells. To test inducibility of NF-κB by phorbol ester in d*max*-transfected cells, protein extracts were prepared from PMA-treated d*max*-transfected cells. These extracts were then used in an EMSA with a ^{32}P-labeled oligonucleotide that specifies an NF-κB binding motif. Fig. 7 shows that transfection of d*max* restores inducibility of NF-κB by phorbol ester. However, the level of NF-κB binding to the DNA probe was lower in the PMA-treated dMax1 and dMax4 cell extracts than in the PMA-stimulated B104 cell extract. Consistent with earlier results, phorbol ester treatment of the B104/N-*myc*C7-transfected cells that expressed the *gpt*- resistance marker only failed to activate NF-κB (Fig. 7, lanes 5–7; ref. 32). We conclude that expression of dMax in B104 N-*myc* cells reverts to a significant extent the N-Myc-induced changes in cellular gene expression.

DISCUSSION

The Myc family of oncoproteins are widely believed to exert their effect on cellular physiology by acting as sequence-specific DNA binding proteins. Two distinct functional motifs are present in Myc proteins: a C-terminal bHLH-Zip motif, required to form a specific DNA-binding complex with Max, and an N-terminal transactivation domain (1, 3, 4). Dimerization of Myc with Max is observed not only *in vitro* but also *in vivo* (15, 20, 21). However, the biological significance of N-Myc–Max heterodimer formation has not been addressed in detail. We report here the construction of a mutant form of Max, named dMax, that has retained the

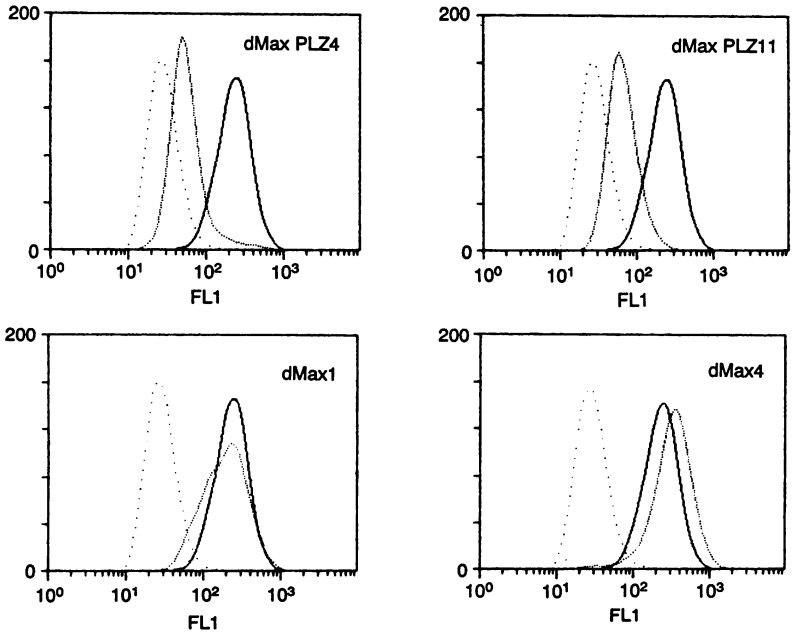

FIG. 6. Cell surface MHC class I antigen expression of d*max*-transfected cells. Cell surface staining of MHC class I antigens was performed by indirect immunofluorescence using the mouse monoclonal antibody OX18 and fluoresceinated sheep anti-mouse IgG second antibody. Immunofluorescence was quantitated by flow cytometry. Three histograms representing the fluorescent staining pattern of three cell types were overlaid in each panel. In each panel, the fluorescence is shown of both B104 and B104 N-*myc*C7 cells and one of the d*max*- or d*max*PLZ-transfected cell lines. B104, heavy solid line; B104 N-*myc*, widely spaced dotted line; dMax, closely spaced dotted line.

ability to form a heterodimer with N-Myc *in vitro* and *in vivo*. However, the N-Myc–dMax heterodimer was unable to bind DNA. Our data thus indicate that the dMax protein has the properties of a transdominant negative mutant of N-Myc, since it prevents N-Myc function by sequestering N-Myc in a non-DNA-binding N-Myc–dMax complex.

We have shown previously that overexpression of N-Myc in the rat neuroblastoma cell line B104 leads to down-modulation of the MHC class I antigens and protein kinase C type δ (31, 32). Since Myc proteins contain a transactivation domain, one would expect that suppression of gene expression by N-Myc would be an indirect effect of Myc proteins. Our present data indicate that expression of the mutant dMax protein in neuroblastoma reverts the N-Myc-induced suppression of both MHC class I gene expression and of protein kinase C. A mutant of dMax, named dMaxPLZ, was constructed that failed to form a heterodimer with N-Myc *in vitro*. Expression of dMaxPLZ in B104 N-*myc* cells hardly affected the expression of MHC class I antigens. Our data support the hypothesis that dMax acts as a transdominant negative mutant of N-Myc through dimerization with N-Myc and subsequent inhibition of N-Myc DNA binding. These data further strengthen the notion that Myc proteins bring about changes in cellular gene expression by acting as transcription factors that bind to specific DNA target sites.

Overexpression of N-Myc in the B104 rat neuroblastoma cell line does not significantly alter *in vitro* growth rate but does lead to an increase in *in vivo* growth potential (31). To investigate whether N-Myc requires dimerization with Max to enhance *in vivo* growth rate, we injected d*max*-transfected B104 N-*myc* cells subcutaneously into nude mice. Our preliminary data indicate that two independently derived d*max*-transfected cells were almost as tumorigenic as the B104 N-*myc* cells from which they were derived. This finding was surprising since dMax did have a profound effect on cellular gene expression in these cells. One possible explanation could be that dMax was not expressed at a sufficiently high level to compete out the binding of all endogenous wild-type Max protein to N-Myc. This explanation seems unlikely, however, because in sequential immunoprecipitation experiments most of the Max protein bound to N-Myc appeared to be dMax (Fig. 5). Alternatively, it cannot be ruled out at present that Myc proteins can contribute to tumorigenicity without having to bind to DNA. We have recently shown that N-Myc can form a specific complex with the product of the retinoblastoma gene pRB (34). N-Myc protein that is in complex with dMax could thus still contribute to tumorigenicity by binding to pRB.

The dominant negative mutant dMax protein could provide a useful tool to address this question regarding the biology of Myc proteins. Thus, dMax could be used to study the contribution of N-Myc to the pathogenesis of human neuroblastoma. In these tumors, both N-*myc* gene amplification and deletion of DNA at chromosome 1p3.6 have been observed (35). Introduction of d*max* in human neuroblastoma cell lines that have amplified N-*myc* in addition to other defined cytogenetic abnormalities should make it possible to dissect the contribution of N-*myc* gene amplification and genetic alterations to the malignant behavior of neuroblastoma tumor cells.

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