Variant Max Protein, Derived by Alternative Splicing, Associates with c-Myc In Vivo and Inhibits Transactivation

MARCELLO ARSURA,¹ ANURADHA DESHPANDE,¹ STEPHEN R. HANN,²
AND GAIL E. SONENSHEIN^{1*}

*Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118-2394,¹ and
Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-2175²*

Received 19 May 1995/Returned for modification 7 July 1995/Accepted 20 September 1995

Max (Myc-associated factor X) is a basic helix-loop-helix/leucine zipper protein that has been shown to play a central role in the functional activity of c-Myc as a transcriptional activator. Max potentiates the binding of Myc-Max heterodimers through its basic region to its specific E-box Myc site (EMS), enabling c-Myc to transactivate effectively. In addition to the alternatively spliced exon a, several naturally occurring forms of alternatively spliced *max* mRNAs have been reported, but variant protein products from these transcripts have not been detected. Using Western blot (immunoblot) and immunoprecipitation analysis, we have identified a variant form of Max protein (16 to 17 kDa), termed dMax, in detergent nuclear extracts of murine B-lymphoma cells, normal B lymphocytes, and NIH 3T3 fibroblasts. Cloning and sequencing revealed that dMax contains a deletion spanning the basic region and helix 1 and the loop of the helix-loop-helix region, presumably as a result of alternative splicing of *max* RNA. S1 nuclease analysis confirmed the presence of the mRNA for dMax in cells. The dMax protein, prepared via in vitro transcription and translation, associated with bacterially synthesized Myc–glutathione S-transferase. Coimmunoprecipitation of dMax and c-Myc indicated their intracellular association. In vitro-synthesized dMax failed to bind EMS DNA, presumably because of the absence of the basic region. Coexpression of dMax inhibited EMS-mediated transactivation by c-Myc. Thus dMax, which can interact with c-Myc, appears to function as a dominant negative regulator, providing an additional level of regulation to the transactivation potential of c-Myc.

Max (Myc-associated factor X) was first identified as a protein belonging to the basic helix-loop-helix/leucine zipper (bHLH/LZ) family of transcription factors that can associate with c-Myc in vitro and in vivo to form heterodimeric complexes with specific DNA binding activity (9, 10). Max exists in two forms as a result of alternative splicing of an exon, termed a, which encodes a nine-amino-acid insertion in the basic region (9). Both Max products have been shown either to heterodimerize with c-Myc or to form unphosphorylated homodimers and to specifically bind to the consensus sequence CACGTG (EMS [E-box Myc site]) (1, 2, 7, 9, 14, 20, 29, 35, 36). On the other hand, Myc forms homodimers only poorly under conditions that are physiologically relevant in vivo (29). Mutational analysis of Max indicated that the helix 1, helix 2, and leucine zipper (LZ) domains are critical for EMS DNA binding of homodimers, whereas the LZ domain is critical for binding of c-Myc–Max heterodimers (36). Cotransfection of Myc and Max expression vectors indicate that Max facilitates E-box-mediated transactivation by Myc at low doses, presumably via stimulation of c-Myc binding (2, 3, 15, 23). Conversely, overexpression of Max is inhibitory, suggesting that Max homodimers, which cannot transactivate, can compete for binding. More recently, two other members of the bHLH/LZ family of transcription factors, Mad and Mxi, have been shown to dimerize with Max product, but not with c-Myc, and bind efficiently to the E-box consensus sequence (4, 5, 42).

Several other alternatively spliced forms of Max mRNAs have been reported (9, 22, 33, 40, 41). Makela and coworkers have identified an alternatively spliced Max mRNA containing

an in-frame stop codon which would yield a putative protein (termed ΔMax) truncated at the COOH terminus (33). Following in vitro transcription and translation, ΔMax was able to associate with c-Myc and bind DNA in a sequence-specific manner. However no protein product was detected in the cell lines found to express Δ*max* mRNA (33). Unlike Max, ΔMax enhanced Myc transformation activity in a Ras cotransformation assay. Subsequently, Vastrik et al. (41) identified additional alternatively spliced *max* mRNAs of 1.9, 2.4, 3.0, and 3.5 kb, in addition to the predominant wild-type (wt) 2.3-kb species. These species displayed tissue- and cell line-specific expression in Northern (RNA) blot analysis (41). The 3.5-kb mRNA encoded a protein with biological activity similar to that of ΔMax, but again the authors were unable to detect the putative protein product of this alternatively spliced *max* mRNA. Furthermore, in *Xenopus laevis*, the Max homologs XMax1 and XMax2 (resulting from alternative use of exon a) and two alternatively spliced mRNAs, termed XMax3 and XMax4, with an additional 81-base insertion in the C-terminal region of the coding sequence, termed exon b, have been identified (22, 40). While these *max* mRNAs were found to be ubiquitously expressed during early embryonic development, and both in vitro-translated XMax2 and XMax4 proteins were capable of association with Myc products, no evidence was provided for their expression in vivo. Here we have identified a variant form of Max protein, termed dMax, lacking the basic region as well as the helix 1 and loop regions, apparently as a result of alternative splicing of *max* mRNA. This nuclear dMax protein was unable to bind EMS DNA but associated with c-Myc in vitro and in vivo and repressed transactivation.

* Corresponding author. Mailing address: Department of Biochemistry, Boston University Medical School, 80 East Concord St., Boston, MA 02118-2394. Phone: (617) 638-4120. Fax: (617) 638-5339. Electronic mail address: gsonensh@med-biochem.bu.edu.

MATERIALS AND METHODS

Culture and transfection conditions. WEHI 231, A20, BALENLM 17, and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium supple-

mented as described previously (25, 31). Murine splenic B lymphocytes, prepared as described previously (17), were kindly provided by T. Rothstein (Boston University Medical School, Boston, Mass.). For transfection, WEHI 231 cells were washed once with medium and resuspended in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum at a concentration of 20×10^6 cells per ml. Cells (250 μ l) were preincubated on ice for 10 min with DNA (up to 40 μ g) and transfected by electroporation at 240 V and 960 μ F, using a Gene Pulser (Bio-Rad). Following incubation on ice for 5 min, the cell suspension was mixed with 1.75 ml of culture medium and incubated for 10 min at room temperature and then at 37°C for 24 h. For transfection of NIH 3T3 fibroblasts, cells were grown in 60-mm² tissue culture dishes to 90% confluence and transfected with a total of 35 μ g of DNA, including 5 μ g of pSV- β -gal, by the calcium phosphate precipitation technique as we have described previously (25). Cells were harvested, and the resulting extracts were normalized for β -galactosidase expression and for total protein content by using the Bio-Rad protein quantitation kit and assayed for chloramphenicol acetyltransferase (CAT) activity as described previously (27). Standard deviation was obtained by using Student's *t* test.

Eukaryotic expression and reporter vectors. The full-length dMax gene was excised with *Eco*RI from the pTA-dMax vector (prepared by cloning of the reverse transcription-PCR product as described below). The insert, containing 16 additional bp of the polylinker region of the pTA cloning vector, was subcloned in the *Eco*RI site of the eukaryotic expression vector pMT2T, containing the adenovirus major late promoter and the simian virus 40 enhancer (18). The resulting construct was called pMT-dMax. The MB2-CAT and MB2.M1-CAT reporter vectors contain the minimal herpes simplex virus 1 thymidine kinase (TK) promoter linked to the CAT reporter driven by single wt (AGGGGACACGTGGCCCGG) or mutant (AGGGGACACCTGGCCCGG) EMS-2 box oligonucleotides from the ornithine decarboxylase gene (6). Alternatively, M4Min CAT, containing four copies of the EMS driving the TK promoter-CAT reporter vector, was used (23). Expression vectors for c-Myc included pM21 (6) and pRCMV-myc (16).

Preparation of radiolabeled nuclear and whole cell extracts. For radiolabeling, cells (1×10^7 to 4×10^7) were pelleted by centrifugation and washed twice in phosphate-buffered saline. The pellet was resuspended in 1 ml of Dulbecco's modified Eagle's medium without methionine and cysteine, supplemented with 5% dialyzed calf serum, 4 mM L-glutamine, 5×10^{-8} M β -mercaptoethanol, and 500 μ Ci each of [³⁵S]methionine (1,300 Ci/mmol) and [³⁵S]cysteine (1,200 Ci/mmol) (Amersham), and incubated for 1.5 to 3 h. Nuclear salt extracts were prepared by using a modification of the method of Strauss and Varshavsky (38). The pelleted cells were swollen on ice in 1 ml of hypotonic reticulocyte standard buffer (10 mM NaCl, 3 mM MgCl₂, 10 mM Tris [pH 7.4]) for 10 min. Cells were lysed via Dounce homogenization, and nuclei were collected by centrifugation at 4,000 $\times g$ for 10 min. The nuclei were suspended in 2 packed volumes of extraction buffer (100 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.5], 350 mM NaCl, 5 mM EDTA). Proteins were extracted on ice with occasional tapping for 45 min. The nuclei were removed by centrifugation, and the supernatant, termed the salt nuclear protein extract, was adjusted to 15% glycerol and stored at -80°C until further use. The remaining proteins were reextracted from the nuclei by using 0.5 ml of radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris [pH 7.6], 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). The nuclear suspension was sheared, and the nuclear debris was removed via centrifugation in a microfuge for 30 min at full speed. This supernatant served as the RIPA or detergent nuclear protein extract and was stored at -20°C. Protease inhibitors 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 10 μ g of leupeptin per ml, which were prepared as fresh stocks, were added to buffers just prior to use.

Immunoprecipitation of c-Myc and Max from radiolabeled nuclear and cell lysates. Immunoprecipitation was performed essentially as described previously (31, 32). The anti-c-Myc and anti-Max antibodies were rabbit polyclonal antibodies generated against the whole mouse c-Myc protein fused to glutathione S-transferase (GST) and against the mouse Max protein tagged at the N terminus with a polyhistidine tract (Upstate Biotechnology Inc.), respectively. Lysates were allowed to react with the anti-Max antibody for 90 min at room temperature or the anti-Myc antibody for 90 min at 4°C. The antigen-antibody complexes were collected on protein A-Sepharose beads. Where indicated, reaction mixtures were preincubated with the cognate protein, to confirm specificity, as detailed previously (31, 32). The complexes obtained with salt or RIPA extractions were washed three times with RIPA buffer and for the anti-Max immunoprecipitates were washed once with a high-salt buffer (2 mM NaCl, 10 mM Tris [pH 7.4], 1% Nonidet P-40, 0.5% SDS). Immunocomplexes were resuspended in 50 μ l of 2 $\times$ SDS buffer (25 mM Tris [pH 6.8], 4% SDS, 20% glycerol, 0.2% bromophenol blue) containing 10% β -mercaptoethanol and boiled for 3 min. The supernatants were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE), using either a 5 to 15% gradient gel or a 12% gel, as indicated. The molecular mass markers (Rainbow Markers; Amersham) included myosin (200 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa). The gels were fixed and fluorography was performed as described previously (32).

Western blot (immunoblot) analysis of c-Myc and Max proteins from nuclear lysates. Unlabeled salt and detergent nuclear lysates (30 to 50 μ g of protein)

were subjected to SDS-PAGE on 10 or 12% gels, as indicated. For analysis of Max, two different rabbit antibodies prepared against the whole mouse Max protein tagged at the N terminus with a polyhistidine tract were used; one (described above and used at a 1:800 dilution) was purchased from Upstate Biotechnology Inc., and the other (rabbit lysate, used at a 1:100 dilution) was kindly supplied by S. Segal (National Institutes of Health). In addition, affinity chromatography-purified antibody 63-4 prepared against the whole mouse Max protein was used (1:1,000 dilution). Western blotting was performed essentially as described previously (32) except that membranes were developed by using a Dupont/NEN chemiluminescence kit.

RNA isolation, analysis, and cloning. For reverse transcription-PCR cloning, WEHI 231 cytoplasmic RNA (1 μ g) was reverse transcribed by using 5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 μ M random hexamers (Pharmacia), 1 mM each deoxynucleoside triphosphate (dNTP), 1 U of RNase inhibitor per μ l, and 2.5 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) per μ l. The reaction was carried out for 10 min at room temperature and then 45 min at 42°C. Aliquots of the reverse transcription reactions were amplified by PCR, using a set of primers specific for the murine *max* gene in the following reaction conditions: 2 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, 0.2 mM each dNTP, 2.5 U of *Taq* DNA polymerase per ml, and 5 ng of each primer per μ l. The sequences of the primers were as follows: Max 160+ (forward), 5'-ATGAGCGATAACGATGACATCGAG-3'; and Max 1020- (reverse), 5'-AGGAGAGCCTGGTAGAGCAGCGT-3'.

PCR was performed in an automated DNA Thermal Cycler (Perkin-Elmer Cetus) for 25 cycles at the annealing temperatures of 55 and 60°C. The PCR products were separated in a 1% ultrapur agarose gel (Bethesda Research Laboratories). Following transfer onto nitrocellulose filters, Southern blotting was performed for Max, using as a probe the *Eco*RI and *Xho*I insert of a full-length human Max pEPMAX vector (12), labeled by random priming (27), and hybridization conditions described above for Northern blotting. To clone the amplified DNA, an aliquot of the reaction mixture (approximately 25 ng) was subcloned into the pTA cloning vector and transfected into competent *Escherichia coli* cells (INVaF') as instructed by the manufacturer (Invitrogen). Recombinant colonies were subjected to plasmid isolation, restriction analysis, and sequencing using standard protocols (37). Two types of Max constructs were isolated: an 860-bp wt murine Max, called pTAMax, and a 752-bp dMax clone, called pTAdMax.

S1 nuclease analysis was performed essentially as described elsewhere (37). The wt murine Max, spanning from nucleotides 160 to 1020 of the published sequence (35), was amplified from plasmid pTAMax by PCR and isolated from a 1% agarose gel by using a Gene-Clean kit (American Bioanalytical). The DNA was 5' end labeled with [γ -³²P]ATP (NEN), using the T4 polynucleotide kinase enzyme (Bethesda Research Laboratories), to a specific activity of 1×10^8 to 2×10^8 dpm per μ g of DNA. The probe was resuspended at 2×10^4 dpm/ μ l in S1 nuclease hybridization buffer (80% deionized formamide, 400 mM NaCl, 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 1 mM EDTA). RNA samples (50 μ g) of *E. coli* tRNA (Sigma) and total RNA of exponentially growing WEHI 231 cells and 100 ng of the in vitro T7 RNA transcripts of the vectors pTA-Max and pTA-dMax were lyophilized, resuspended in 30 μ l of hybridization buffer containing the labeled probe, and heated to 85°C for 10 min. At the end of overnight hybridization at 58°C, 300 μ l of S1 nuclease digestion buffer (280 mM NaCl, 30 mM sodium acetate, 4.5 mM ZnSO₄, 20 μ g of sheared salmon sperm DNA per ml, 667 U of S1 nuclease [Boehringer] per ml) was added to each sample. The samples were incubated at 37°C for 45 min, and the reaction was stopped by addition of 75 μ l of termination buffer (2.5 M sodium acetate, 50 mM EDTA). After ethanol precipitation, the samples were resuspended in 30 μ l of loading buffer (80% deionized formamide, 1 $\times$ Tris-borate-EDTA (TBE), 0.02% xylene cyanol, 0.02% bromophenol blue) and loaded onto an 8 M urea-1 $\times$ TBE-6% polyacrylamide gel, and the products were visualized by autoradiography.

In vitro protein synthesis. Plasmid pTA-Max or pTA-dMax (10 μ g) was linearized with *Bam*HI, purified by ethanol precipitation, and transcribed with T7 polymerase as instructed by the supplier (Stratagene) to obtain a wt Max RNA fragment of 860 bases or a dMax RNA fragment of 752 bases. Aliquots of 100 ng of the RNAs were translated in the presence of [³⁵S]methionine (NEN) in a Flexi rabbit reticulocyte lysate system (Promega). Labeled proteins were loaded on an SDS-12% polyacrylamide gel in denaturing conditions and subjected to autoradiography.

To measure the association of in vitro-transcribed and -translated proteins with c-Myc, GST-Myc fusion protein was synthesized essentially as described by Kaelin et al. (19). The *Clal*-to-*Nsi*I fragment encoding exon III of human *c-myc* was cloned into the *Sma*I site of expression vector pGex-3X (Promega). The fusion proteins were first bound to glutathione-Sepharose beads and then reacted with the in vitro-synthesized dMax and wt Max proteins, prepared as described above. The beads were thoroughly washed with NETN (100 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.5% Nonidet P-40), and the bound fractions were eluted and analyzed by SDS-PAGE followed by fluorography.

DNA binding assay. wt Max and dMax proteins were synthesized in a rabbit reticulocyte lysate, in the presence of unlabeled methionine, as described above. Where indicated, lysates containing wt Max or dMax protein or unprogrammed reticulocyte lysate were mixed at the ratios indicated and incubated for 60 min at 30°C. A double-stranded oligonucleotide probe (5'-GCGGGACCAGCTGGTC

FIG. 1. Identification of a 16- to 17-kDa Max protein by Western blot analysis. Nuclei were isolated from the indicated cells, and proteins were extracted sequentially with salt (S) and then RIPA (R) buffer. Equal amounts of protein were subjected to SDS-PAGE analysis and Western blotting for Max. (A) Analysis of salt and RIPA extracts from WEHI 231 B-lymphoma cells (WEHI) and RIPA extracts from normal splenic B lymphocytes (B cells). Positions of the 14.3- and 21.5-kDa molecular mass markers and of Max and dMax proteins are indicated by dashes and arrows, respectively. (B) Analysis of RIPA extracts from A20 cells, BALENLM 17 cells (Bal), and NIH 3T3 fibroblasts (3T3). The bottom panel was analyzed with an affinity-purified Max antibody (see Materials and Methods).

CG-3'; the EMS is underlined) was radiolabeled with the large fragment of *E. coli* DNA polymerase (New England Biolabs) and α - 32 P-dNTPs (NEN) to a specific activity of 1×10^8 to 2×10^8 dpm/ μ g of DNA. Electrophoretic mobility shift assays were performed as follows. 32 P-labeled double-stranded oligonucleotide (25,000 cpm; approximately 2 ng) and 4 μ l of reticulocyte lysate were mixed in 70 mM NaCl–10 mM HEPES (pH 7.5)–1 mM EDTA–1 mM dithiothreitol–0.1% Triton X-100–4% (vol/vol) glycerol–5 μ g of poly(dI-dC) copolymer in a final volume of 25 μ l. This mixture was incubated at room temperature for 30 min. Competition experiments were performed with a 10-fold molar excess of unlabeled oligonucleotide added at the beginning of the incubation. For supershift experiments, antibody preparations were preincubated with the reaction mixture, in the absence of radiolabeled oligonucleotide, for 10 min at room temperature. Samples were subjected to electrophoresis at 11 V/cm in a 1 $\times$ TBE–4.5% polyacrylamide gel, dried, and subjected to autoradiography.

RESULTS

An alternative Max protein detected in nuclear extracts. To analyze the nature of Max protein expression, we first tested for optimal extraction conditions of WEHI 231 B-lymphoma cells in salt and detergent buffers. Salt conditions, commonly used for the preparation of nuclear binding factors, avoid the use of ionic detergents, which have been reported to disrupt Myc-Max interactions (10). However, we had previously shown that only 20 to 30% of the total Myc protein is extracted under salt conditions (32). Interestingly, we noted a difference in the electrophoretic mobility patterns in initial Western blots and immunoprecipitations when salt and detergent extracts were analyzed (data not shown). To examine more specifically the nature of the extracted proteins, nuclear proteins were prepared by using a sequential extraction procedure. Nuclei were first extracted under nondenaturing salt conditions and then reextracted with a detergent (RIPA) buffer. Both fractions of extracted nuclear proteins were analyzed by Western blotting (Fig. 1A). The 21- to 22-kDa wt Max proteins were observed in both the salt and detergent extracts. Surprisingly, an additional protein band was detected in the detergent extract (Fig. 1A). This lower-molecular-weight protein, which was sometimes resolved into a doublet, was also seen with a second Max antibody (data not shown). This putative Max protein had an apparent molecular size of 16 to 17 kDa and was termed dMax.

To confirm that this protein is indeed Max, immunoprecipitation of radiolabeled salt and RIPA extracts was performed. As seen in Fig. 2, proteins were detected at the positions of 21- to 22-kDa and 16- to 17-kDa proteins, with the salt and RIPA extracts, respectively, that were specifically removed upon addition of excess unlabeled Max protein, used to prepare the antiserum. These results indicate that the 21- to 22-kDa and the 16- to 17-kDa polypeptides are Max, suggesting that the 16-

to 17-kDa protein(s) represents an alternative form of Max protein. Its appearance in RIPA extracts but not in salt extracts may indicate a poor solubility in the given extraction conditions or an in vivo association with some other protein(s) that renders it salt inextractable.

Since the wt Max protein has been found to be ubiquitously expressed, we next assessed whether dMax protein could be found in normal B cells and in additional cell lines. Detergent nuclear extracts, prepared from murine splenic B lymphocytes (Fig. 1A) and from the IgM⁺ BALENLM 17 and IgG⁺ A20 B-cell lines and NIH 3T3 fibroblasts (Fig. 1B), were examined. The 16- to 17-kDa dMax protein was detected in RIPA extracts from all of these lines. To further confirm the identity of the 16- to 17-kDa band as Max, an affinity-purified Max-specific antibody was used with a duplicate of the membrane shown in Fig. 1B; a 16- to 17-kDa band was again detected (Fig. 1B, bottom panel). Thus, dMax protein is expressed in primary murine B lymphocytes, in 3T3 fibroblasts, and in several malignant B-cell lines at different stages of development.

Characterization of an alternative max mRNA. To characterize the variant form of Max, total RNA was extracted from exponentially growing WEHI 231 cells, and *max* gene expression was analyzed by cDNA cloning. The RNA was reverse transcribed, and the resulting cDNA was amplified by PCR, using specific primers in the 5' and 3' regions of the published sequence (35) (see Materials and Methods). To confirm that the amplified DNAs were Max-related forms, an aliquot of the amplified reaction mixture was subjected to Southern blot analysis. Hybridization with a radiolabeled human *max* gene probe showed two major hybridized bands of 860 and 752 bp (data not shown). Thus, an aliquot of the amplified product was subcloned into the pTA cloning vector, and a total of 20 positive clones were analyzed by restriction enzyme digestion (data not shown) and by sequencing (Fig. 3 and data not shown). Eighteen clones contained an 860-bp insert, while two of them contained an insert of 752 bp. Extensive sequence analysis of the 860-bp clone indicated 100% homology with the previously published murine *max* DNA sequence (35), showing an open reading frame encoding a 160-amino-acid polypeptide with a predicted molecular mass of 18 kDa. On the other hand, the sequence analysis of the 752-bp clone (referred to as dMax) showed 100% homology with the wt Max except for an

FIG. 2. Detection of an alternative Max protein in immunoprecipitation analysis. WEHI 231 cells were radiolabeled for 1.5 h, and nuclei were isolated. Proteins were extracted sequentially with salt and RIPA buffer and subjected to immunoprecipitation with a Max antibody in the absence (–) or presence (+) of excess unlabeled Max protein. Positions of molecular mass markers are given. Arrows indicate positions of immunoprecipitated 21- to 22-kDa and 16- to 17-kDa proteins.

FIG. 3. Structure of dMax. (A) Schematic representation of the dMax clone isolated and of the sequencing strategy. Max+ and Max- indicate the positions of the primers Max 160+ and Max 1020- used in the PCR as described in Materials and Methods. The arrows indicate the sequenced regions of the dMax clone, using the T7, SP6, and M13 (-40) contained in the parental pTA vector and using the primers Max 160+ and Max 1020-. (B) Sequence of the murine *dmax* gene. The translated amino acid sequence of the major open reading frame is shown above the nucleotide sequence. The deleted 108-bp region of the wt Max sequence is shown as an open box. The splice acceptor site (AG) is shown in boldface. The conserved exon a in human and murine species is underlined. (C) Schematic comparison of wt Max and dMax proteins. In the wt Max sequence, the black box corresponds to the basic domain, the dark gray box corresponds to the helix 1 domain, the dashed box corresponds to the loop domain, and the light gray box corresponds to the helix 2 and LZ domains. aa, amino acids.

internal deletion of 108 bp including nucleotides 64 to 171 (Fig. 3A and B). A splice acceptor site, AG, shown in boldface in Fig. 3B, was identified. The deletion leads to an open reading frame, from the same ATG of the wt Max gene, encoding a 124-amino-acid polypeptide with a predicted molecular mass of 14 kDa (Fig. 3B and C). Interestingly, the dMax clone maintained the additional exon a, identified in both human and murine Max species (9, 35). The dMax protein contains an internal deletion including amino acids 22 to 58 and therefore lacks the basic, helix 1, and loop regions and still contains the helix 2 region and the LZ domain (Fig. 3C).

To confirm that the dMax cDNA clone represents a truly expressed alternative mRNA form and not a cloning artifact, S1 nuclease analysis was carried out. An 860-bp fragment, corresponding to the amplified murine wt Max, was radiolabeled at the 5' end, hybridized to total RNA of WEHI 231 cells, and subjected to S1 nuclease digestion. As a positive

control, the same amount of probe was hybridized to the in vitro-synthesized RNA of both dMax and wt Max. Probes and S1-digested products were resolved by gel electrophoresis and autoradiography. Two major protected bands were detected with the WEHI 231 RNA: a band of the size of full-length protected probe (860 bases) and a band with an apparent molecular size of 688 bases (Fig. 4, lane 2). In contrast, no bands were detected when control tRNA was similarly analyzed, as expected (Fig. 4, lane 1). With RNA synthesized in vitro from the wt Max and dMax clones, protected bands of 860 and 688 bases, similar to those with cellular RNA, were detected. With dMax RNA, a more slowly migrating band of approximately 800 bases was also detected. Since no comigrating bands were present with cellular RNA, this band most likely results from partial digestion of the probe. The presence of the 688-base protected fragment indicates that RNA encoding dMax is present in WEHI 231 cells.

Association of dMax and c-Myc. To begin to evaluate the potential role of dMax in functional expression of c-Myc, we next monitored for in vitro and in vivo association of dMax and c-Myc. To test the ability of dMax protein to associate with c-Myc, the clones for alternatively spliced dMax, and wt Max as a control, were transcribed and translated in vitro. Translation of the RNA in a rabbit reticulocyte cell-free system yielded proteins of the expected molecular weights (data not shown), confirming expression of the cloned protein. The proteins were allowed to interact with a bacterially produced Myc-GST, or GST alone as a control, which had been prebound to glutathione-Sepharose beads. As seen in the SDS-PAGE analysis of the bound fractions, both wt Max and dMax proteins associated specifically with the Myc-GST (Fig. 5A). These results establish that the new variant dMax protein can associate with c-Myc in vitro.

To confirm the intracellular association of dMax and c-Myc, coimmunoprecipitation analysis was performed. Radiolabeled nuclear proteins were isolated by sequential extraction with salt and detergent buffers as described above. The proteins were immunoprecipitated with anti-c-Myc antibody and resolved by SDS-PAGE. Immunoprecipitated 63- to 65-kDa c-Myc proteins are seen in both extracts. In addition, a coimmunoprecipitating 16- to 17-kDa band was detected in the RIPA extract (Fig. 5B). This protein comigrates with immunoprecipitated dMax protein. Thus, on the basis of coimmunoprecipitation

FIG. 4. S1 analysis detects mRNA for the 16- to 17-kDa Max protein in WEHI 231 cells. An 860-base wt murine Max probe was used in S1 analysis with control tRNA (lane 1), total RNA from exponentially growing WEHI 231 cells (lane 2), or in vitro-expressed dMax (lane 3) or wt Max (lane 4) RNA. The probe alone is shown in lane 5. The positions of 860- and 688-base (b) DNA are indicated.

FIG. 5. dMax associates with c-Myc protein in vitro and in vivo. (A) In vitro association. Radiolabeled dMax (d) and wt Max (wt) proteins, prepared by in vitro transcription and translation, were incubated with either Myc-GST protein or GST alone prebound to glutathione-Sepharose beads. Eluted proteins were subjected to SDS-PAGE and visualized by autoradiography. Positions of bound Max and dMax proteins are indicated by arrows. (B) Intracellular association. WEHI 231 cells were labeled for 1.5 h, and nuclei were isolated. Proteins were extracted sequentially with salt (S) and detergent (R) and analyzed by immunoprecipitation with an anti-c-Myc antibody. An additional sample was similarly immunoprecipitated with an anti-Max antibody. Positions of immunoprecipitated Max and dMax are indicated by arrows.

tion analysis, dMax appears associated with c-Myc within the WEHI 231 cells.

dMax E-box DNA binding activity. To characterize the ability of dMax to bind E-box DNA, electrophoretic mobility shift analysis was performed. Unlabeled dMax and wt Max proteins, generated by in vitro transcription and translation, were tested for the ability to bind a radiolabeled double-stranded oligonucleotide containing the EMS consensus motif. Proteins within the unprogrammed rabbit reticulocyte lysate yielded one major and two minor bands upon mobility shift analysis (lane 1, Fig. 6A). These bands appeared specific, as judged by competition with excess of unlabeled probe (Fig. 6B and data not shown). Since multiple antibodies to Max or c-Myc failed to affect their migration (Fig. 6 and data not shown), these bands are likely due to binding of other members of the bHLH/LZ family of transcription factors naturally present in the reticulocyte lysate (e.g., USF and TFEB [9, 35]). Of note, an additional, faster-migrating complex was detected when the radiolabeled probe was incubated with reticulocyte lysate programmed with wt *max* RNA (lane 2, Fig. 6A). Addition of a Max antibody preparation resulted in supershift of this new band, and binding was abolished by excess unlabeled probe (Fig. 6). Thus, this specific complex appears to contain Max protein and likely represents binding of a Max protein homodimer, although the possibility of heterodimer formation involving Max and an endogenous reticulocyte protein cannot be excluded. In contrast, no additional complex was detected when in vitro-translated dMax protein was used (Fig. 6A), suggesting that dMax cannot bind DNA. (Confirmation of dMax and Max protein synthesis was achieved by using programmed lysates in the presence of [³⁵S]methionine; data not shown.) The inability of dMax to bind EMS is consistent with the absence of the basic region, which mediates DNA binding, and with the published findings of Reddy and coworkers (36), who demonstrated that point mutations within the basic region of human Max protein abrogated DNA binding to the E-box sequence.

To test whether dMax could associate with wt Max and/or affect binding to the E-box sequence, mixing experiments were performed with lysates programmed with *max* or *dmax* RNA

(Fig. 6B). Addition of lysate (4 μ l) containing wt Max protein again resulted in the appearance of a specific complex which, as judged by supershift analysis, contained Max protein. When equal volumes (2 μ l) of Max- and dMax-containing lysates were mixed, formation of the specific wt Max-containing complex was completely inhibited (Fig. 6B). This inhibition does not appear to be due simply to dilution effects, since a mixture of 3 μ l of unprogrammed and 1 μ l of wt *max*-programmed reticulocyte lysate still yielded significant levels of wt Max binding to the radiolabeled E-box-containing oligonucleotide (Fig. 6A). Since dMax is unable to bind to the E-box sequence alone, these findings suggest that the abrogation of binding of wt Max homodimers is due to interaction with dMax through the intact LZ and that this complex is not capable of binding to DNA through the intact basic domain of wt Max.

dMax is a dominant negative factor. To test whether expression of dMax is able to affect E-box-mediated transcriptional activity, cotransfection experiments were performed in WEHI 231 cells. The MB2-CAT vector (6), containing the TK promoter driven by a single copy of the second E-box consensus sequence from the ornithine decarboxylase gene, and its mutant version, MB2.M1-CAT, were used as reporter constructs. As an effector gene, the pMT-dMax vector, containing sequences for expression of dMax protein downstream of the adenovirus major late promoter, was used. Following electroporation into exponentially growing WEHI 231 cells, the activity of wt MB2 was found to be approximately fivefold higher than that of the mutant MB2.M1 (Fig. 7), suggesting that part of the promoter activity was due to binding to the E-box consensus sequence. Cotransfection of the pMT-dMax vector reduced the activity of wt MB2, in a dose-dependent fashion, to essentially that of the mutant MB2.M1 (Fig. 7). As expected, the activity of MB2.M1 was essentially unaffected by the expression of dMax. These results suggest that part of the activity was due to binding of c-Myc, which was inhibited by overex-

FIG. 6. Electrophoretic mobility shift analysis of Max and dMax binding to an EMS-containing oligonucleotide. (A) The indicated volume (microliters) of unprogrammed rabbit reticulocyte (Un) lysate or of lysates programmed with wt *max* (M) or *dmax* (d) RNA was used in mobility shift analysis with a ³²P-labeled double-stranded oligonucleotide containing an E-box element in the absence or presence of Max antibody (Ab). (B) Mobility shift analysis was performed as described above in the absence or presence of a 10-fold molar excess of unlabeled competitor oligonucleotide (C). The positions of the putative Max homodimer and supershifted complexes are indicated by an arrow and an arrowhead, respectively.

FIG. 7. Expression of dMax inhibits E-box-driven promoter activity. Exponentially growing WEHI 231 cells were electroporated, in duplicate, with 20 μ g of MB2-CAT or MB2.M1-CAT in the absence (bar 1) or presence of 1, 5, 10, and 20 μ g of pMT-dMax expression vector (bars 2, 3, 4, and 5, respectively) and enough pUC19 DNA to make up a final amount of DNA of 40 μ g. Extracts were assayed for CAT activity, and the data are presented relative to those for the MB2-CAT alone. Standard deviation was obtained by using Student's *t* test.

pression of dMax; however, the possibility that dMax acted as a nonspecific dominant negative protein on other factors involved in the regulation of E-box-containing promoters could not be excluded.

As a more direct test of the effect of dMax protein on the transactivation potential of c-Myc, cotransfection experiments were performed with NIH 3T3 cells, in which the E-box-mediated transactivating capability of the *c-myc* gene has been extensively documented (3, 15, 23, 28). The four-copy E-box-driven M4MinCAT (23) construct and its parental TK-CAT vector were used as reporters. As effectors, the pRc-CMV-myc (16) and pM21 (6) constructs, which both express full-length Myc protein, were used. NIH 3T3 cells were transfected at 90% confluence, a condition in which endogenous c-Myc expression is low, in order to maximize the transactivation of the M4MinCAT reporter plasmid by c-Myc, as recently reported by Lee and coworkers (28). When M4MinCAT vector was cotransfected in the presence of low levels of either pRc-CMV-myc (Fig. 8A) or pM21 (Fig. 8B), CAT activity was significantly enhanced, whereas the activity of TK-CAT was unaffected (data not shown). When increasing concentrations of the pMT-dMax construct were introduced in the same experimental models, the E-box-mediated transactivation by c-Myc was com-

pletely abolished (Fig. 8). These results indicate that the *dmax* gene can reduce c-Myc transcriptional activity driven by its specific E-box consensus sequence.

DISCUSSION

We have demonstrated the existence of a novel Max protein, termed dMax, encoded by an alternatively spliced form of *max* mRNA. Sequencing of dMax clones indicated the RNA contains a 108-base deletion spanning the sequences encoding the basic region and helix 1 and loop of the helix-loop-helix region but maintains the additional exon a. The expression of dMax RNA and protein in cells was confirmed by PCR and S1 analysis and by Western blotting and immunoprecipitation, respectively. Reverse transcription-PCR analysis, using an oligonucleotide spanning the break point of the alternative spliced region, further confirmed the presence of mRNA encoding dMax (data not shown). Thus, in contrast to previous reports of studies in which alternatively spliced variant forms of *max* mRNAs but not protein were observed (33, 40, 41), here an alternative *max* message has been shown to be functionally translated in vivo. Importantly, the ability of dMax to interact with c-Myc was demonstrated by using in vitro-synthesized proteins and coimmunoprecipitation of intracellularly labeled proteins. The functional interaction of dMax and c-Myc was confirmed by cotransfection analysis, which indicated that dMax can repress E-box transactivation in WEHI 231 cells and c-Myc-mediated E-box activation in NIH 3T3 cells. These findings suggest that dMax, which retains the LZ but is missing the basic region and cannot bind to DNA containing the E-box sequence, can interact with c-Myc protein but is unable to facilitate DNA binding of the heterodimer. Therefore, dMax represents a negative regulator of c-Myc transactivation.

Studies by Reddy and coworkers (36) demonstrated that Max expression constructs with deletions of the basic region failed to bind DNA. Furthermore, Cogliati et al. tested a similar construct in transfection analysis in murine erythroleukemia cells and found that Max without a basic region inhibited growth and promoted differentiation of these cells (12). There are several examples of bHLH/LZ proteins lacking a basic DNA binding domain that function as negative regulators, e.g., the Id-like proteins (39). These proteins are capable of forming dimers with other bHLH/LZ proteins, but the resultant heterodimers are unable to bind DNA. Thus, these proteins exert a dominant negative regulatory effect on transactivation activ-

FIG. 8. Expression of dMax inhibits c-Myc transactivation. (A) Ninety percent confluent NIH 3T3 cells were transfected by calcium phosphate, in duplicate, with 5 μ g of M4MinCAT reporter construct in the absence (bar 1) or presence of 100 ng of pRc-CMV-myc (bars 2 to 4) and the indicated amount of pMT-dMax expression vector (bar 3, 1 μ g; bar 4, 5 μ g) plus enough pUC19 DNA to make up a final amount of DNA of 35 μ g. Standard deviation was obtained by using Student's *t* test. (B) NIH 3T3 cells were cotransfected as described above with 5 μ g of M4MinCAT reporter construct in the absence (bar 1) or presence of 100 ng of pM21 (bars 2 to 4) and the indicated amount of pMT-dMax expression vector (bar 3, 1 μ g; bar 4, 5 μ g).

ity. The dMax protein represents a naturally occurring potential dominant negative regulator of c-Myc function.

S1 nuclease analysis indicated wt *max* RNA levels in WEHI 231 cells were greater than those for *dmax*. Consistent with this finding, higher levels of Max than of dMax nuclear protein were detected when one takes into account that Max protein was present in both salt and detergent extracts whereas dMax was extracted selectively with RIPA buffer. It is unclear why dMax is preferentially extracted in this detergent buffer, but we have found that other extraction buffers are less effective. This may explain the failure of other investigators to detect variant Max protein commensurate with observed alternatively spliced RNA species (33, 40, 41), although it should be noted that bands of molecular weight similar to that of dMax can be seen in the Max immunoprecipitates of other groups (5, 28). Coimmunoprecipitation analysis of WEHI 231 cells extracts detected only dMax in association with c-Myc protein in vivo. These observations are similar to those made by Billaud et al. (8) in B104 cells transfected with a dominant negative mutant Max construct. Sequential immunoprecipitation revealed that most of the Max protein associated with N-Myc in these cells was the mutant form. The electrophoretic mobility studies suggested that dMax can associate with Max in vitro, although alternative explanations are possible if dMax can form heterodimers with other E-box-binding proteins. The interaction of dMax with other HLH/LZ proteins, including Max binding partners, and the functional significance of c-Myc-dMax association in WEHI 231 cells, a model for tolerance in B lymphocytes, are under investigation.

The widely accepted model of how Myc functions as a transactivator suggests a central role for Max. Myc homodimerization occurs only poorly under physiological conditions; furthermore, the resulting homodimers can bind E-box DNA only weakly (29). In contrast, homodimers of hypophosphorylated Max protein bind E-box DNA but cannot transactivate it (2, 3, 11, 15, 23). Thus, it has been proposed that Myc-Max heterodimers are the functional unit. Max, which dimerizes well with c-Myc through its helix-loop-helix and LZ domains, promotes the binding to DNA, and c-Myc provides the transactivation domain (9, 10). The expression of the recently demonstrated Max binding partners Mxi1 and Mad would also affect the availability of Max, and potentially dMax, for dimerization with Myc. Mad and Mxi1 both heterodimerize with Max and bind the same E-box motif, thus serving as competitors in two ways (4, 5, 24, 42). Consistent with this hypothesis, Mad-Max heterocomplexes were found to function as transcriptional repressors (4), whereas Mxi1-Max complexes seemed to be at least transcriptionally inert when expressed in a heterologous yeast system (42). Thus, transactivation by c-Myc of specific target genes depends on the nature of each of the different DNA-binding complexes that it forms. An increase of steady-state levels of Mad and Mxi1 mRNAs during terminal differentiation of myeloid cell lines has been reported (5, 26, 42), whereas c-Myc mRNA levels dramatically decrease (13, 30). The equilibrium between Max-Max, Mad-Max, and Mxi1-Max to c-Myc-Max would ultimately control the transactivation ability of c-Myc. From the results presented here, the functionality of Myc would also be influenced by the relative levels of expression of wt Max and dMax and depend on the relative affinities of these two proteins for c-Myc. Thus, this novel variant form of Max, which is lacking the basic region required for DNA binding but which can associate with Myc, appears to provide an additional form of regulation of the transcriptional activity of c-Myc.

ACKNOWLEDGMENTS

We thank S. Segal for generously providing Myc/Max vectors and antiserum, T. Rothstein for purified B lymphocytes, and J. Cleveland, D. Dobson, and R. Eisenman for constructs.

This work was supported by grants from the NIH (CA36355 to G.E.S.; CA47399 and CA48799 to S.R.H.) and the Associazione Italiana per la Ricerca sul Cancro (M.A.).

REFERENCES

- Amati, B., S. Dalton, M. W. Brooks, T. D. Littlewood, G. I. Evans, and H. Land. 1992. Transcriptional activation by the human c-myc oncoprotein in yeast requires interaction with max. *Nature (London)* **359**:423-426.
- Amati, B., T. D. Littlewood, G. I. Evan, and H. Land. 1993. The c-myc protein induces cell cycle progression and apoptosis through dimerisation with max. *EMBO J.* **12**:5083-5087.
- Amin, C., A. J. Wagner, and N. Hay. 1993. Sequence-specific transcriptional activation by Myc and repression by Max. *Mol. Cell. Biol.* **13**:383-390.
- Ayer, D., L. Kretzner, and R. N. Eisenman. 1993. Mad: a heterodimeric partner for max that antagonises myc transcriptional activity. *Cell* **72**:211-222.
- Ayer, D. E., and R. N. Eisenman. 1993. A switch from myc:max to mad:max heterocomplexes accompanies monocyte/macrophage differentiation. *Genes Dev.* **7**:2110-2119.
- Bello-Fernandez, C., G. Packham, and J. L. Cleveland. 1993. The ornithine decarboxylase gene is a transcriptional target of c-myc. *Proc. Natl. Acad. Sci. USA* **90**:7804-7808.
- Berberich, S., N. Hyde-DeRuyscher, P. Espenshade, and M. Cole. 1992. Max encodes a sequence-specific DNA-binding protein and is not regulated by serum growth factors. *Oncogene* **7**:775-779.
- Billaud, M., K. J. Isselbacher, and R. Bernards. 1993. A dominant-negative mutant of max inhibits sequence specific DNA binding by myc proteins. *Proc. Natl. Acad. Sci. USA* **90**:2739-2743.
- Blackwood, E. M., and R. N. Eisenman. 1991. Max: A helix-loop-helix zipper protein that forms a sequence specific DNA-binding complex with myc. *Science* **251**:1211-1217.
- Blackwood, E. M., B. Luscher, and R. N. Eisenman. 1992. Myc and max associate in vivo. *Genes Dev.* **6**:71-80.
- Bousset, K., M. Henriksson, J. M. Luscher-Firzlaff, D. W. Litchfield, and B. Luscher. 1993. Identification of casein kinase II phosphorylation sites in max: effects on DNA binding kinetics of max homo- and myc/max heterodimers. *Oncogene* **8**:3211-3220.
- Cogliati, T., B. K. Dunn, M. Bar-Ner, C. M. Cultraro, and S. Segal. 1993. Transfected wild-type and mutant *max* regulate cell growth and differentiation of murine erythroleukemia cells. *Oncogene* **8**:1263-1268.
- Cole, M. D. 1986. The *myc* oncogene: its role in transformation and differentiation. *Annu. Rev. Genet.* **20**:361-384.
- Ferre-D'Amare, A. R., G. C. Prendergast, E. B. Ziff, and S. K. Burley. 1993. Recognition by max of its cognate DNA through a dimeric b/HLH/Z domain. *Nature (London)* **363**:38-45.
- Gu, W., K. Cechova, V. Tassi, and R. Dalla-Favera. 1993. Opposite regulation of gene transcription and cell proliferation by c-myc and max. *Proc. Natl. Acad. Sci. USA* **90**:2935-2939.
- Hann, S. R., M. Dixit, R. C. Sears, and L. Sealy. 1994. The alternatively initiated c-Myc proteins differentially regulate transcription through a non canonical DNA-binding site. *Genes Dev.* **8**:2441-2452.
- Huo, L., and T. L. Rothstein. 1995. Receptor-specific induction of individual AP-1 components in B lymphocytes. *J. Immunol.* **154**:3300-3309.
- Israel, D. I., and R. J. Kaufman. 1989. Highly inducible expression from vectors containing multiple GREs in CHO cells overexpressing the glucocorticoid receptor. *Nucleic Acids Res.* **17**:4589-4604.
- Kaelin, W. G., D. C. Pallas, J. A. DeCaprio, F. J. Kaye, and D. M. Livingston. 1991. Identification of cellular proteins that interact specifically with the T/E1-A binding region of the retinoblastoma gene product. *Cell* **64**:521-532.
- Kato, G. J., W. M. F. Lee, L. Chen, and C. V. Dang. 1992. Max: functional domains and interaction with c-myc. *Genes Dev.* **6**:81-92.
- Kim, K. J., C. Kanellopoulos-Langevin, R. Merwin, D. Sachs, and R. Asofsky. 1979. Establishment and characterization of Balb/c lymphoma lines with B cell properties. *J. Immunol.* **122**:549-553.
- King, M. W., E. M. Blackwood, and R. N. Eisenman. 1993. Expression of two distinct homologues of *Xenopus* Max during early development. *Cell Growth Differ.* **4**:85-92.
- Kretzner, L., E. M. Blackwood, and R. N. Eisenman. 1992. Myc and max proteins possess distinct transcriptional activities. *Nature (London)* **359**:426-429.
- Lahoz, E. G., L. Xu, N. Schreiber-Agus, and R. A. DePinho. 1994. Suppression of c-myc, but not E1a, transformation activity by max-associated proteins, Mad and Mxi1. *Proc. Natl. Acad. Sci. USA* **91**:5503-5507.
- La Rosa, F. A., J. W. Pierce, and G. E. Sonenshein. 1994. Differential regulation of the c-myc oncogene promoter by the NF- κ B Rel family of transcription factors. *Mol. Cell. Biol.* **14**:1039-1044.

26. Larsson, L. G., M. Pettersson, F. Oberg, K. Nilsson, and B. Luscher. 1994. Expression of mad, mxi 1, max and c-myc during induced differentiation of hematopoietic cells: opposite regulation of mad and c-myc. *Oncogene* **9**: 1247–1252.
27. Lawrence, R., L.-J. Chang, U. Siebenlist, P. Bressler, and G. E. Sonenshein. 1994. Vascular smooth muscle cells express a constitutive NF- κ B-like activity. *J. Biol. Chem.* **269**:28913–28918.
28. Lee, L., L. Resar, and C. Dang. 1995. Cell density and paradoxical transcriptional properties of c-Myc and Max in cultured mouse fibroblasts. *J. Clin. Invest.* **95**:900–904.
29. Littlewood, T. D., B. Amati, H. Land, and G. I. Evans. 1992. Max and c-myc/max DNA binding activities in cell extracts. *Oncogene* **7**:1783–1792.
30. Luscher, B., and R. N. Eisenman. 1990. New light on Myc and Myb. *Genes Dev.* **4**:2025–2035.
31. Maheswaran, S., H. Lee, and G. E. Sonenshein. 1994. Intracellular association of the protein product of the *c-myc* oncogene with the TATA-binding protein. *Mol. Cell. Biol.* **14**:1147–1152.
32. Maheswaran, S., J. E. McCormack, and G. E. Sonenshein. 1991. Changes in phosphorylation of myc oncogene and RB antioncogene protein products during growth arrest of the murine lymphoma WEHI 231 cell line. *Oncogene* **6**:1965–1971.
33. Makela, T. P., P. J. Koskinen, I. Vastrik, and K. Alitalo. 1992. Alternative forms of max as enhancers or suppressors of myc-ras cotransformation. *Science* **256**:373–375.
34. Pena, A., C. D. Reddy, S. Wu, N. J. Hickok, E. P. Reddy, G. Yumet, D. R. Soprano, and K. J. Soprano. 1993. Regulation of human ornithine decarboxylase expression by the c-myc · max protein complex. *J. Biol. Chem.* **268**: 27277–27285.
35. Prendergast, G. C., D. Lawe, and E. B. Ziff. 1991. Association of myn, the murine homolog of max, with c-myc stimulates methylation-sensitive DNA binding and ras cotransformation. *Cell* **65**:395–407.
36. Reddy, C. D., P. Dasgupta, P. Saikumar, H. Dudek, F. J. Rauscher III, and E. P. Reddy. 1992. Mutational analysis of max: role of basic, helix-loop-helix/leucine zipper domains in DNA binding, dimerisation and regulation of myc-mediated transcriptional activation. *Oncogene* **7**:2085–2092.
37. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. Strauss, F., and A. Varshavsky. 1984. A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome. *Cell* **33**:741–748.
39. Sun, X. H., N. G. Copeland, N. A. Jenkins, and D. Baltimore. 1991. Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. *Mol. Cell. Biol.* **11**:5603–5611.
40. Tonissen, K. F., and P. A. Krieg. 1994. Analysis of a variant max sequence expressed in *Xenopus laevis*. *Oncogene* **9**:33–38.
41. Vastrik, I., P. J. Koskinen, R. Alitalo, and T. P. Makela. 1992. Alternative mRNA forms and open reading frames of the max gene. *Oncogene* **7**:503–507.
42. Zervos, A. S., J. Gyuris, and R. Brent. 1993. Mxi1, a protein that specifically interacts with max to bind myc-max recognition sites. *Cell* **72**:223–232.