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The Nerve Growth Factor-Responsive PC12 Cell Line Does Not Express the Myc Dimerization Partner Max

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Heterodimerization of Max with the nuclear oncoprotein Myc and the differentiation-associated proteins Mad and Mxi1 enables these factors to bind E-box sites in DNA and control genes implicated in cell proliferation and differentiation. We show that in the PC12 pheochromocytoma tumor cell line, functional Max protein is not expressed because of the synthesis of a mutant *max* transcript. This transcript encodes a protein incapable of homo- or heterodimerization. Furthermore, the mutant Max protein, unlike wild-type Max, is incapable of repressing transcription from an E-box element. Synthesis of mutant *max* transcripts appears to be due to a homozygous chromosomal alteration within the *max* gene. Reintroduction of *max* into PC12 cells results in repression of E-box-dependent transcription and a reduction in growth rate, which may explain the loss of Max expression either during the growth of the pheochromocytoma or in subsequent passage of the PC12 cell line in vitro. Finally, the ability of these cells to divide, differentiate, and apoptose in the absence of Max demonstrates for the first time that these processes can occur via Max- and possibly Myc-independent mechanisms.

A significant advance in our understanding of the biochemical basis of Myc oncoprotein function resulted from the identification of the Myc dimerization partner Max (1, 3, 49). The ability of Myc to promote transformation, cellular proliferation, and apoptosis is believed to require dimerization with Max (1, 2); moreover, studies in vivo with a lymphoma cell line have shown that essentially all of the newly synthesized Myc protein is associated with Max (14). Myc and Max belong to a growing family of nuclear proteins containing the basic region (b), helix-loop-helix (HLH), and leucine zipper (LZ) motifs (bHLH-LZ), which permit dimerization and DNA binding (38, 46, 50). Binding of Max to Myc permits specific binding to the sequence CACGTG, called an E-box Myc site, and related sequences (11, 12, 36, 51). Through binding to DNA, Myc is capable of activation of transcription from a promoter containing multiple CACGTG motifs (3, 29, 35, 37, 52). Max binds to all three of the Myc oncoproteins, c-Myc, N-Myc, and L-Myc, and appears to be essential in vivo for the transforming activity of these proteins in the rat embryo fibroblast transformation assay (1, 45, 65). In addition to the Myc proteins, two other bHLH-LZ proteins have been isolated which bind to Max, Mad and Mxi1 (7, 66). These proteins are induced during differentiation of the myeloid cell lines U-937, HL-60, and ML-1 (6, 40, 66), and in the case of U-937 cells, Mad has been shown to replace c-Myc as the dimerization partner for Max during differentiation of these cells (6). Current evidence suggests that neither Myc, Mad, nor Mxi1 is capable of homodimerization and DNA binding in vivo, although these activities have been observed for Myc in vitro (20, 35, 36). Max therefore appears to occupy a pivotal position as an obligate partner for dimerization and DNA binding, dimerizing with Myc proteins in proliferating cells and dimerizing with Mad or Mxi1 in differentiating cells.

Current models of normal Myc function invoke a fundamental role for Myc as a transcription factor which regulates the cell cycle. Myc has been suggested to be necessary for the G_1-to-S (31, 33, 34) and the S-to-G_2/M (60) transitions. An alternative view regards Myc as an inhibitor of growth arrest or differentiation (19, 24, 39). More recently, Myc has been proposed as a component of an apoptotic program (4, 22, 59). It is not clear whether Myc is absolutely required for any of these processes or whether Myc simply influences these processes under certain physiological conditions or in a limited spectrum of cell types. However, Max has been shown to be required for Myc-induced transformation (1) and for cell cycle progression and apoptosis (2).

In this report, we show that functional Max protein is not expressed in the rat tumor cell line PC12 (28) because of aberrant processing of *max* transcripts. These mutant transcripts do not encode the dimerization domain and C-terminal sequences of the normal Max protein, and their translation product appears to be nonfunctional. The synthesis of these transcripts appears to be due to a homozygous chromosomal rearrangement or translocation within the *max* gene. Reintroduction of Max into PC12 cells represses transcription dependent on the E-box Myc site and has a mild growth-inhibitory effect. This suggests that loss of Max expression may have conferred a selective growth advantage on the cells either during the origin of the tumor or in subsequent cell culture in vitro. The identification of a cell line in which functional Max is not expressed challenges the model that cellular proliferation and apoptosis have an absolute requirement for Myc-Max heterodimers. Either Myc is capable of influencing these processes in a Max-independent fashion, or Myc does not have an essential role in these processes in PC12 cells.

MATERIALS AND METHODS

Cell culture, transfection, and immunoprecipitation. PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum and 5% horse serum (Hyclone). Cells were transfected by electroporation (26). For immunoprecipitation of Max, a 50-cm^2 dish was labeled overnight with 500 μCi of $^{32}PO_4$. Cells were lysed in 500 μl of 20 mM Tris (pH 7.4)–50 mM NaCl–1 mM EDTA–0.5% Nonidet P-40 (NP40)–0.5% deoxycholate–0.5% sodium dodecyl sulfate (SDS)–0.2 mM phenylmethylsulfonyl fluoride (PMSF)–0.7

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μg of pepstatin per ml–5 μg of aprotinin per ml–10 mM NaF–50 mM β-glycerolphosphate on ice for 20 min, followed by sonication twice for 15 s each at 25 W. The lysate was spun for 15 min at 15,000 × *g*. Anti-Max CT is a rabbit polyclonal antibody raised against amino acids 111 to 132 of mouse Max (LQT NYPSSDNSLYTNAKGGTIS). Anti-Max loop antibody was raised against amino acids 40 to 60 of mouse Max (KDSFHSLRDSVPSLQGEKASR). Polyclonal serum (20 μl) was added to 100 μl of lysate and left on ice for 1 h. Then 50 μl of a 50% slurry of protein A-Sepharose (Pharmacia) was added, and the lysate was rocked for 2 h. The Sepharose pellet was washed twice in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris [pH 8]), once with 2 M NaCl–10 mM Tris (pH 7.4)–1% NP40–0.5% deoxycholate, and finally washed with RIPA buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE).

Construction of MTMax and induction with zinc. *max* was subcloned into the inducible vector pMV-33, containing the zinc-inducible mouse metallothionein promoter (a gift of Riccardo Dalla-Favera, Columbia University). Stable PC12 clones containing the inducible *max* plasmid were selected in hygromycin B (100 μg/ml). Induction of *max* expression was done with 120 μM $ZnSO_4$ for 12 h. Clones were screened for expression of *max* by RNase protection or immunoprecipitation of $^{32}PO_4$-labeled cells with the anti-Max antibody as described above.

Transient transcriptional transactivation assay. The E-box reporter construct M4TKluc contains four contiguous copies of the Myc/Max binding site CACGTG inserted 15 nucleotides (nt) 5′ of the thymidine kinase (TK) minimal promoter, which is 5′ of the luciferase gene. The control plasmid TKluc contains only the TK minimal promoter 5′ of the luciferase gene. Both plasmids were constructed by replacement of the chloramphenicol acetyltransferase (CAT) gene from pM4MinCAT or pMinCAT (gifts from Leo Kretzner [37]) with the luciferase gene from pGL2 (Promega). Three micrograms of luciferase reporter was cotransfected with plasmids expressing Max, Max9, or Max^{pc12} from the cytomegalovirus (CMV) promoter together with 1 μg of the β-galactosidase expression vector CMVgal as an internal control for transfection efficiency. All transfection mixes were brought to 25 μg of total DNA by using the empty expression vector pcDNAIamp. Luciferase and β-galactosidase activities were measured 2 days posttransfection in a luminometer (Berthold) according to the supplier's instructions. For measurement of β-galactosidase activity, we used the Galacto-Light kit (Tropix).

Plasmid construction and cDNA library screen. Rat *max* was cloned by reverse transcription-PCR with oligonucleotides which hybridized to sequence flanking the mouse *max* open reading frame (ORF): 5′ primer, AGAGAAGCTTGCCGT AGGAAATGAGCGATAACG; 3′ primer, TACGTCGGATCCTTAGCTGGC CTCCATCCGGAGTTT. The PCR product was cloned into the vector pcDNA Iamp (InVitrogen) to give pCMVrMax. The shorter splice variant of *max* encoding the 151-amino-acid protein was used to generate all RNase protection probes. pSVrMax, in which *max* is under the control of the simian virus 40 (SV40) early promoter, was constructed by subcloning rat *max* from pCMVrMax into the vector pSVK-3 (Promega). For production of the Max 5′ antisense probe, pCMVrMax was digested with *Sma*I and *Eco*RI and religated to give pCMVrMax5′. For production of a rat c-*myc* antisense probe, a 190-bp *Xho*I-*Bst*YI fragment containing sequence from *myc* exon 3 was purified from pRmyc1.2 (a gift from P. A. Overbeak, Baylor College of Medicine, Houston, Tex.) and subcloned into pcDNAIamp to give prMycXho/Bst. The max^{pc12} cDNA was cloned from a PC12 cDNA library in λgt11 (Clontech). The 1.2-kb *Eco*RI insert from the λ clone was subcloned into pcDNAIamp to give $pMax^{pc12}$. For in vitro translation of max^{pc12}, the max^{pc12} ORF (nucleotides 127 to 432 in Fig. 3A) was amplified by PCR and subcloned into pcDNAIamp to give $pMax^{pc12}$ORF. Glutathione *S*-transferase (GST) fusion genes were constructed by subcloning PCR-amplified fragments from pCMVrMax, $pCMVMax^{pc12}$, or pCMVMyc (49) into the vector pGSTag (54).

RNase protection. Antisense RNA was synthesized in vitro from 1 μg of linearized plasmid by using SP6 polymerase and [^{32}P]dCTP as label (44). The *max* probe was synthesized from pCMVrMax linearized with *Hin*dIII. The *max*5′ probe was synthesized from pCMVrMax5′ linearized with *Hin*dIII. The *max*3′ probe was synthesized from pCMVrMax linearized with *Sma*I. The *myc* probe was synthesized from prMycXho/Bst linearized with *Hin*dIII. Total RNA (20 μg) from PC12 or Rat1a cells or 2 μg of mRNA from rat tissue was hybridized with 5×10^5 cpm of probe overnight, digested with RNase A and RNase T_1, and separated on a 6% denaturing polyacrylamide gel (5).

In vitro translation and GST pull-down assay. GST fusion proteins were purified from isopropyl thiogalactopyranoside (IPTG)-induced *Escherichia coli* (54). *max* or max^{pc12} was transcribed in vitro. Approximately 1 μg of RNA was translated and labeled with [^{35}S]methionine in vitro with a wheat germ extract (Promega). Labeled Max or Max^{pc12} was added to 200 ng of GST protein in Sepharose beads, incubated, and washed, and bound proteins were resolved on polyacrylamide gels (30).

Northern (RNA) and Southern blotting. Polyadenylated RNA from PC12 or Rat1a cells was purified with oligo(dT) (InVitrogen FastTrack). Northern blots were probed either with the full-length *max* ORF, with a 680-bp *Xba*I fragment from max^{pc12} containing sequence unique to this transcript (i.e., not found in the normal *max* transcript), or with an 800-bp *Eco*NI-*Eco*RI fragment from pCMVMyn (49) containing sequence from the 3′ untranslated region of the *max* mRNA. For Southern blots, genomic DNA from PC12 cells, Rat1a cells, or NEDH rat tissue was isolated, digested with restriction enzyme, and blotted onto nylon filters (58). The mouse intronic probe used was an 8-kb *Xba*-*Sma* fragment from p22Xba (40a). All probes were labeled with random hexanucleotides (Boehringer Mannheim).

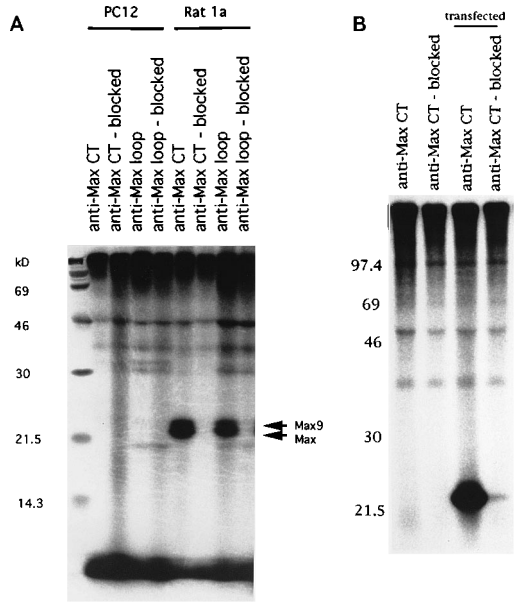

FIG. 1. Absence of normal *max* expression in PC12 cells. (A) PC12 cells or Rat1a cells were labeled overnight with $^{32}P_i$. Cell lysates were incubated with anti-Max CT or anti-Max loop antibody, immunoprecipitated, and resolved on a polyacrylamide gel. Where indicated, the antibody was blocked by incubation with peptide prior to addition to cell lysate. (B) PC12 cells were transfected with *max* expressed from the CMV promoter. The following day, cells were labeled with $^{32}P_i$ and immunoprecipitated as before.

RESULTS

Normal Max protein is not expressed in PC12 cells. Given the dependence of the activities of Myc, Mad, and Mxi1 on Max, we were interested in the possible regulation of Max at the transcriptional or post-transcriptional level. We analyzed Max in a group of cell lines, including PC12 cells, a pheochromocytoma tumor cell line which serves as a model for nerve growth factor (NGF)-induced differentiation (28). In all cell lines examined except PC12 cells, two phosphoproteins were specifically immunoprecipitated from phosphate-labeled or methionine-labeled cells with two different anti-Max antibodies (Fig. 1A and unpublished observations). These two proteins most likely correspond to Max and Max9, related Max proteins produced by alternative splicing. Max9 contains an additional nine amino acids near the N terminus (13, 49). We found no evidence for regulation of either Max protein levels or phosphorylation during the cell cycle in BALB/c-3T3 cells or during differentiation of F9 cells (not shown). However, unexpectedly, we did not detect Max protein in PC12 cells (Fig. 1A). Despite the failure to detect endogenous Max protein in PC12 cells, Max expressed from exogenous vectors transfected into PC12 is easily detected by immunoprecipitation of phosphate-labeled cells (Fig. 1B), indicating that *max* transcripts can be normally translated and Max protein can be phosphorylated in these cells.

Analysis of *max* expression by RNase protection revealed that only a fraction of the *max* ORF is expressed as mRNA in PC12 cells. Using the full-length rat *max* ORF as probe, we found complete protection of the probe in Rat1a cells (Fig. 2B, arrow 1), whereas in PC12 cells, only 150 nt of the probe was

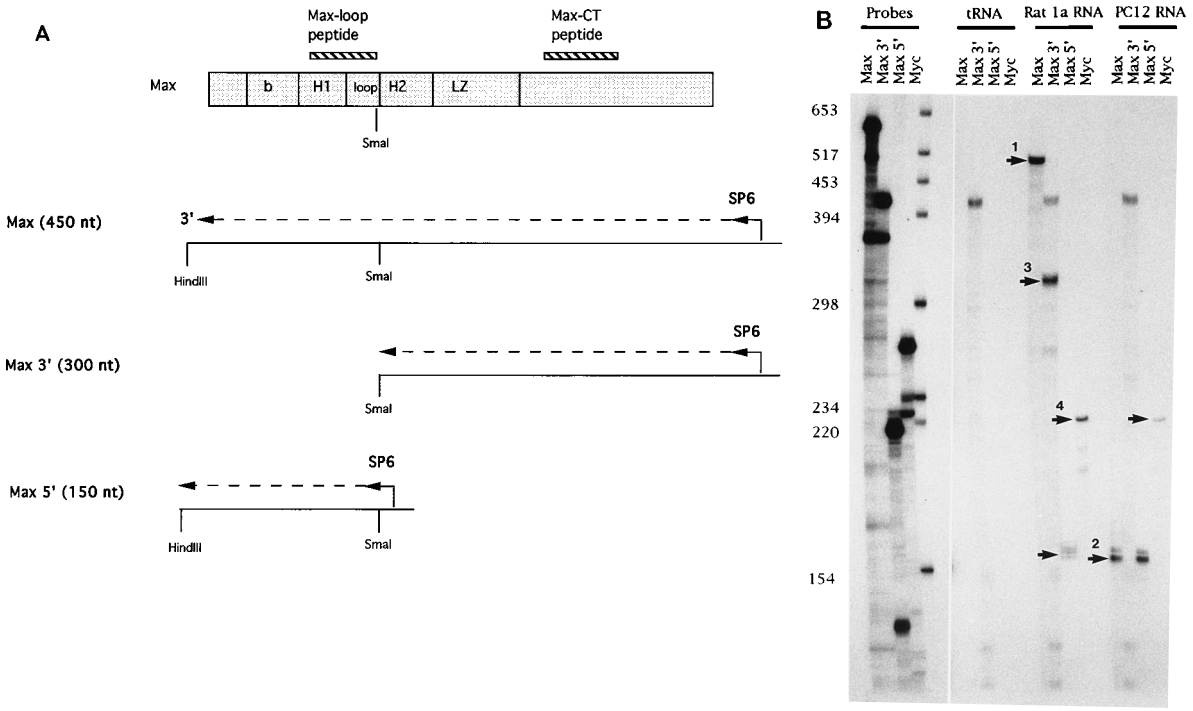

FIG. 2. Analysis of *max*-related transcripts in PC12 cells. (A) Antisense *max* probes used for RNase protection assays. (B) RNase protection assays with antisense *max* probes. The *max* probe protects a 500-nt fragment in Rat1a cells, corresponding to the full-length *max* ORF (arrow 1), whereas only 150 nt of the probe is protected in PC12 cells (arrow 2). The *max*5′ probe protects a 150-nt fragment in both Rat1a and PC12. The *max*3′ probe protects a 300-nt fragment in Rat1a (arrow 3) but gives no protection product in PC12. (Note that this probe gives an artifactual protection product seen in all lanes, including the tRNA negative control.) RNA protected by the *myc* probe is indicated by arrow 4. Numbers to the left refer to DNA size markers (in nucleotides), which run slightly faster than RNA of the corresponding size.

protected (arrow 2). We determined the region of *max* expressed in PC12 cells by using probes specific for either the first 150 nt of the *max* ORF (Fig. 2A, *max*5′) or the remaining 300 nt of the *max* ORF (Fig. 2A, *max*3′). The *max*5′ probe protected 150 nt in both Rat1a cells and PC12 cells, whereas the *max*3′ probe protected 300 nt in Rat1a cells and gave no protection product in PC12 cells (Fig. 2B). PC12 cells therefore express a *max* transcript containing approximately the first 150 nt of the normal *max* ORF. PC12 cells express *myc* mRNA at low levels (Fig. 2B, arrow 4); indeed, *myc* has previously been shown to be induced in PC12 cells by NGF, epidermal growth factor (EGF), and fibroblast growth factor (FGF) with immediate-early kinetics (27).

Cloning from a PC12 library of a novel *max* cDNA which encodes a protein unable to dimerize with Myc or Max. The RNase protection experiments described above indicate that a novel *max* transcript is expressed in PC12 cells. We therefore screened a λgt11 PC12 cDNA library with the rat *max* ORF. From 300,000 plaques, a single plaque hybridized to the probe. The nucleotide sequence of the 1.2-kb insert from this clone was found to contain an ORF which is predicted to encode a novel protein which we call Max^{pc12}, consisting of the 48 amino-terminal amino acids of rat Max fused to a 53-amino-acid sequence of unknown origin (Fig. 3). The *max*^{pc12} transcript is identical to the previously described *max* message up to nt 331 of the *max* cDNA sequence (49). This position coincides with an exon boundary of the chicken *max* gene (62). The *max*^{pc12} transcript therefore appears to result from aberrant processing or another fault in expression of exons downstream of this exon.

The predicted amino acid sequence of Max^{pc12} contains the basic region, helix 1, and loop regions of Max but does not contain helix 2, the leucine zipper, or the carboxyl terminus of Max (Fig. 3B). The crystallographic structure of the Max homodimer shows that helix 2 and the leucine zipper constitute a continuous α helix, which forms the major part of the dimerization interface (23). The absence of these structures from Max^{pc12} would be predicted to render the protein incapable of dimerization. We tested directly, by in vitro translation, the ability of the cDNA to encode Max^{pc12} and of Max^{pc12} to dimerize with Myc or Max by measuring the ability of the in vitro-translated protein to bind to various GST fusion proteins. As expected, in vitro-translated Max bound to GST-Max and GST-Myc beads, but it did not bind to GST or GST-Max^{pc12} (Fig. 4B). In vitro translation of the *max*^{pc12} cDNA yielded a product of the anticipated size for Max^{pc12}, which was recognized by the anti-Max loop antibody (Fig. 4C). However, in contrast to in vitro-translated Max, in vitro-translated Max^{pc12} failed to bind to GST-Max, GST-Myc, or GST-Max^{pc12} (Fig. 4C). These results indicate that the PC12 mRNA sequence encodes a product, Max^{pc12}, which is unable to substitute functionally for Max as a Myc dimerization partner in PC12 cells and is most likely a nonfunctional variant of Max. Indeed, we do not detect expression of Max^{pc12} by immunoprecipitation from methionine-labeled cells with the antiloop antibody (results not shown). We also tested the ability of Max^{pc12} to repress transcription from an E-box element. Figure 5 shows that wild-type Max and Max9 repress transcription approximately fourfold in PC12 cells, whereas Max^{pc12} had no effect.

Max^{pc12} transcripts are not found in a range of rat tissues. Comparison of Rat1a and PC12 RNA by Northern blot analysis with the *max* ORF as the probe revealed multiple large transcripts in PC12 cells which were not seen in Rat1a cells (Fig. 6A). Sequences unique to *max*^{pc12} are not expressed in

A

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1    CGG GCG AGT GAG TGA GTG AGT GTG TGC GTG GGG GCA CTC GGC TTG TTG TTG TCG GTG ACT TCC CCC TTC CCT CCA

76   CCC CTC CCC TCC CCG GCC GCC GCT GCA GTG GCC GCT CCC TGG GCC GTA GAA ATG AGC GAT AAC GAT GAC ATC GAG
                                                                 MET SER ASP ASN ASP ASP ILE GLU

151  GTG GAG AGC GAC GCT GAC AAA CGG GCT CAC CAT AAT GCA CTG GAA CGA AAA CGT AGG GAC CAC ATC AAA GAC AGC
9    VAL GLU SER ASP ALA ASP LYS ARG ALA HIS HIS ASN ALA LEU GLU ARG LYS ARG ARG ASP HIS ILE LYS ASP SER

226  TTT CAC AGT TTG CGG GAC TCA GTC CCA TCA CTC CAA GGA GAG AAT CTC ATC TGT CTC CTA TGT ACC CAC CAA AGG
34   PHE HIS SER LEU ARG ASP SER VAL PRO SER LEU GLN GLY GLU LYS Leu ile cys leu leu cys thr his gln arg

301  AAC AGC CAT GGC CTC CAG AGG CAT CTG CTA CAT GGA ATT ATA CCC AAG AAG AAA GAG GCT TCT ATG GAA TTT GAA
59   asn ser his gly leu gln arg his leu leu his gly ile ile pro lys lys lys glu ala ser met glu phe glu

376  GAT GTT CTA GAA GGA GAA AAT GCG GAA GAC TCA GAA TTT GGA TTC CAG GAA GAG TAA ACT TCA GGA CGG AAA ACC
84   asp val leu glu gly glu asn ala glu asp ser glu phe gly phe gln glu glu OCH

451  GCT CCC TCT TGA ATA CTA ACA ATT ATG TAT AAG GGC AAG GAT ACA CCC AGA AAA GGA ACA AAA GTC ATT ATC GTG

526  TAG GGA TAA GAT TGA CCT ACT TCA GTG ATA ACA GCT GCT AGA TGG TTA CTA ATA ATT TAT CAT AAC AGA GGG AGT

601  GGA TGA TGA ACA GGA TCA TTT CTT GAC CAG GAC GAC TTA ATA GTG CTT GCC TTT TGC TTC CTC CAT AAA ATT CCT

676  CCA CTC AGT GCT CAG GGA ATC CTG AGG AAG AAG ATC CAG AAA AAC TAA GAT CCA GAG CAG ATG AAG GGC ACC AGA

751  GCA AAG CCC TCT AAA TCA ACT AAT CAA GGC ACA CAT AAG CTC ACA GAG ACT GAA ACT GCA AGC ACA GGA CCT GCC

826  TAT GTG AGT GCA CCA GGA CCT CTG CAT ACA TGT TGT GAC TTT TAG CTG GGT GCT TTT ATG AGA CTC CTG ACT ATG

901  AGA AAG AGT GGG TCT CTG ACT TCT GTG CCT ACT CTT GGG ACT TTT CCT CCT GTT GGG TTT CTA TGT CTA ACT GCT

976  ATA TGG TAG GTT TTT TTC TTC ATC TTA TAT TTT ATA TTC CAC ATG TGG CCG TTA TCC CTT AGA AGC CTG TCC TTT

1051 TCT AAT AAG AGA CAG AAA TGG AGT CAA TCT AGA AGG GAG GAA AGA TGG AAA GGA ACT GGA AAA ATT AGA GGG AGG

1126 AAA AAC TGG AAT CAA TAT ATA TTG TAT GAA AAA AAT AAT CTA TGT TCA ATA AAA GGG GGA AAT AAT GGA GAG AGT

1201 TAT TTG GCA AGA ATT AAA AAA GAA GAT ATG ACA AAA CCG
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B

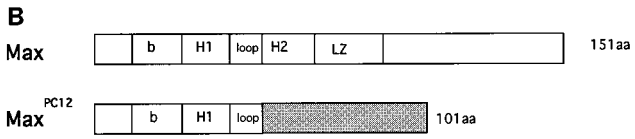

FIG. 3. Novel *max* transcript expressed in PC12. (A) *max*^{pc12} sequence. The normal Max protein sequence is shown in uppercase and the novel protein sequence is shown in lowercase letters. (B) Schematic representation of the Max protein variant encoded by *max*^{pc12}. aa, amino acids.

Rat1a, whereas multiple PC12-specific transcripts are detected (Fig. 6B). Initially, we speculated that the novel transcript cloned from PC12 cells might result from a regulated splicing event specific to certain cell types. Alternatively, the transcript might result from a mutation in the *max* gene resulting in aberrant processing of *max* RNA. If the former were the case, we might expect to find similar transcripts in RNA from normal rat tissues. We therefore screened mRNA from a series of rat tissues by RNase protection, with antisense *max*^{pc12} as a probe (Fig. 7). If normal *max* is expressed, only 150 nt are protected, corresponding to the 5′ fragment of *max* present in the *max*^{pc12} probe, as seen in Rat1a cells (Fig. 7, arrow 2). Expression of the *max*^{pc12} transcript results in a 310-nt protection product, as observed in PC12 (Fig. 7, arrow 1). *max* but not *max*^{pc12} transcripts are detected in mRNA from rat brain, heart, muscle, kidney, intestine, lung, and liver. As expected, *myc* mRNA is also detected in all of these tissues, although only at low levels in brain and heart (Fig. 7, arrow 3). Thus, *myc* and *max* but not *max*^{pc12} transcripts could be detected in the rat tissues analyzed and in NIH 3T3 and CTLL-2 cells (not

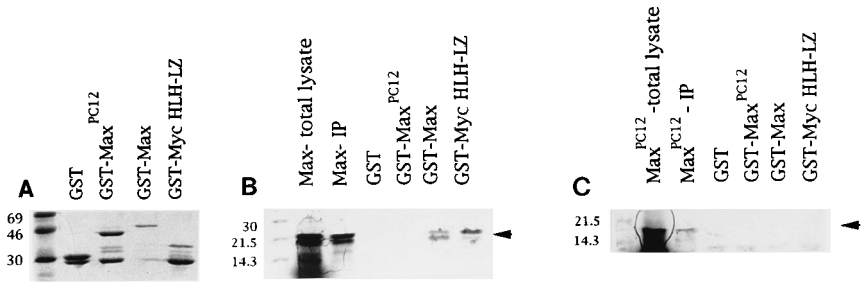

FIG. 4. Max^{pc12} fails to bind Myc, Max, or itself. (A) GST fusion proteins after purification on GST-Sepharose beads. GST-Max and GST-Max^{pc12} contain the entire Max and Max^{pc12} proteins, respectively, fused to GST. GST-MycHLH-LZ contains the C terminus of Myc, including the HLH-LZ motif, fused to GST. (B and C) Binding of in vitro-translated Max (B) and Max^{pc12} (C) to GST fusion proteins. Max and Max^{pc12} were transcribed in vitro and translated in wheat germ extracts. Proteins were precipitated with 200 ng of GST fusion protein and separated on 12% (B) or 15% (C) polyacrylamide gels. Lanes marked IP were immunoprecipitated with the anti Max-loop antibody. Arrows indicate the Max and Max^{pc12} proteins.

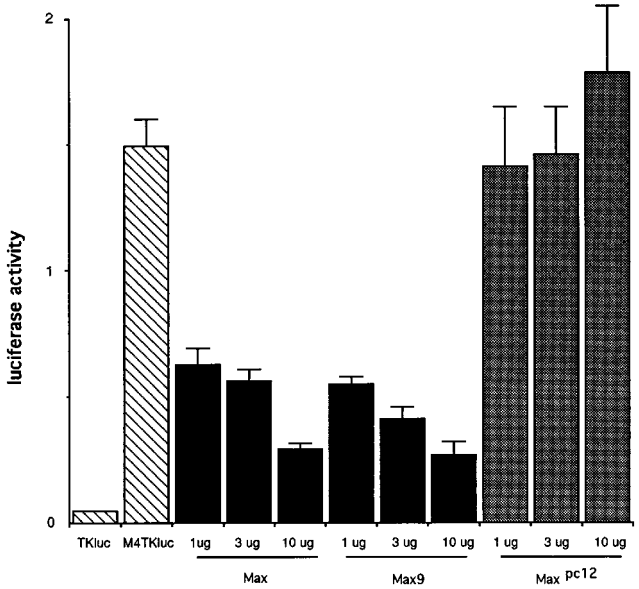

FIG. 5. Max but not Max^{pc12} can repress transcription from an E-box element in PC12 cells. PC12 cells were transiently transfected with 3 μg of a reporter plasmid containing four copies of the consensus Myc/Max binding site and the TK minimal promoter 5′ of the luciferase gene (pM4TKluc) or the TK minimal promoter alone (TKluc). Max, Max9, and Max^{pc12} expression plasmids were cotransfected as indicated. Bars indicate the standard error of the mean calculated from triplicate experiments.

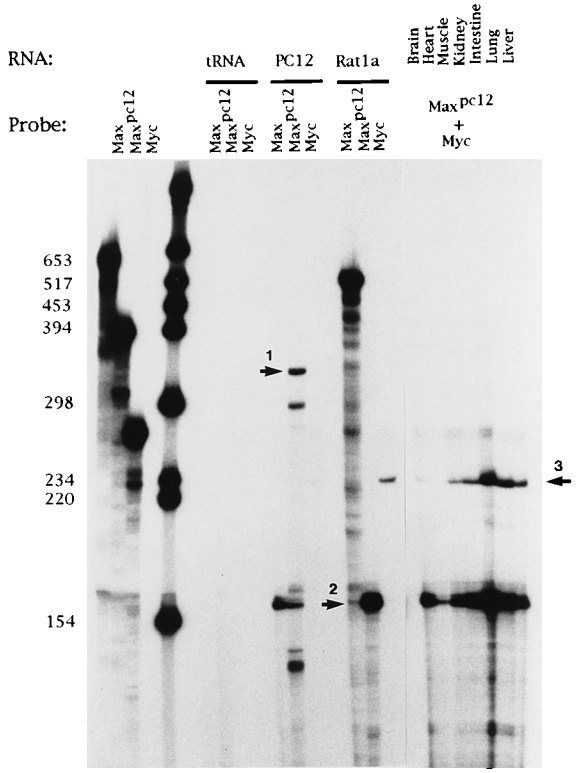

FIG. 7. RNase protection by the *max*^{pc12} transcript in PC12 and Rat1a cells and seven rat tissues. The *max*^{pc12} probe is a 310-nt fragment of *max*^{pc12} containing the *max*^{pc12} ORF (150 bp of the normal *max* transcript and 160 bp unique to the *max*^{pc12} transcript). Conditions were as for Fig. 2 except that 2 μg of mRNA was used per reaction for rat tissues.

shown). In PC12 cells, additional RNase protection products besides the *max*^{pc12} transcript are also detected, presumably corresponding to multiple aberrantly processed transcripts (Fig. 7), in agreement with the Northern analysis (Fig. 6A and B).

Aberrant processing of *max* mRNA in PC12 cells appears to be due to a chromosomal rearrangement or translocation of the *max* gene. Max was also absent from the PC12 subline U2, which fails to arrest growth in response to NGF (16), and from a morphologically transformed PC12 clone stably transfected with the E1a oncogene (15), as assayed by immunoprecipitation and RNase protection (not shown). The consistent absence of Max from different sublines of PC12 suggested a mutational origin for the aberrant processing of *max* in PC12 cells. Furthermore, the absence of any wild-type *max* transcripts indicated that both *max* alleles might be affected. Initial comparison of Southern blots of PC12 and Rat1a DNA with the *max* ORF as the probe failed to reveal mutations within the PC12 *max* exons (Fig. 8B). Since no exons 3′ of the exon encoding the basic region, helix 1, and loop regions of Max are expressed in PC12 cells (Fig. 2A and 6C), we wondered whether a rearrangement of the intron 3′ of this exon could account for the aberrant processing of downstream exons (Fig. 8A; the intron in question is designated C). Using a probe from *max* intron C, we identified a homozygous PC12 chromosomal alteration by comparing PC12 cells with Rat1a cells (Fig. 8C). To exclude the possibility that the mutation observed is a polymorphism, we also compared PC12 cells with DNA from the NEDH strain of rat, from which PC12 cells are derived (Fig. 8C). In Rat1a cells and NEDH rat cells, a 9.5-kb *Hin*dIII fragment is detected, whereas in PC12 cells, a 15-kb fragment is detected. Similarly, an 8.5-kb *Pvu*II fragment is detected in Rat1a cells and NEDH rat cells, versus a 7-kb fragment in PC12 cells. These observations demonstrate that PC12 cells are mutant with respect to both Rat1a cells and the NEDH rat, confirming that the mutation arose during the formation of the tumor or during subsequent in vitro selection of the PC12 cells.

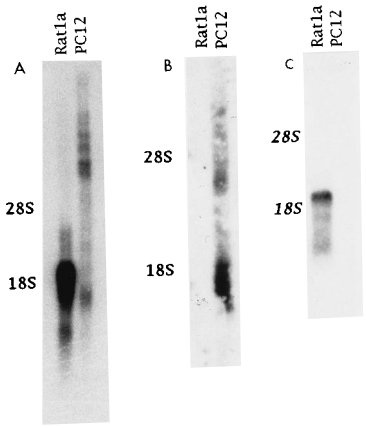

FIG. 6. Northern blots of *max* transcripts expressed in PC12 and Rat1a cell lines. (A) Rat1a or PC12 mRNA probed with *max* reveals multiple large transcripts in PC12. (B) A probe containing sequences specific to *max*^{pc12} detects transcripts in PC12 but not Rat1a. (C) A probe containing sequences from the *max* 3′ untranslated region detects transcripts only in Rat1a cells.

The observed genomic mutation may reflect either a rearrangement within the *max* gene or a chromosomal translocation in which the breakpoint falls within this intron of the *max* gene. A rearrangement within this intron of *max* is entirely consistent with the results of RNase protection, in which no

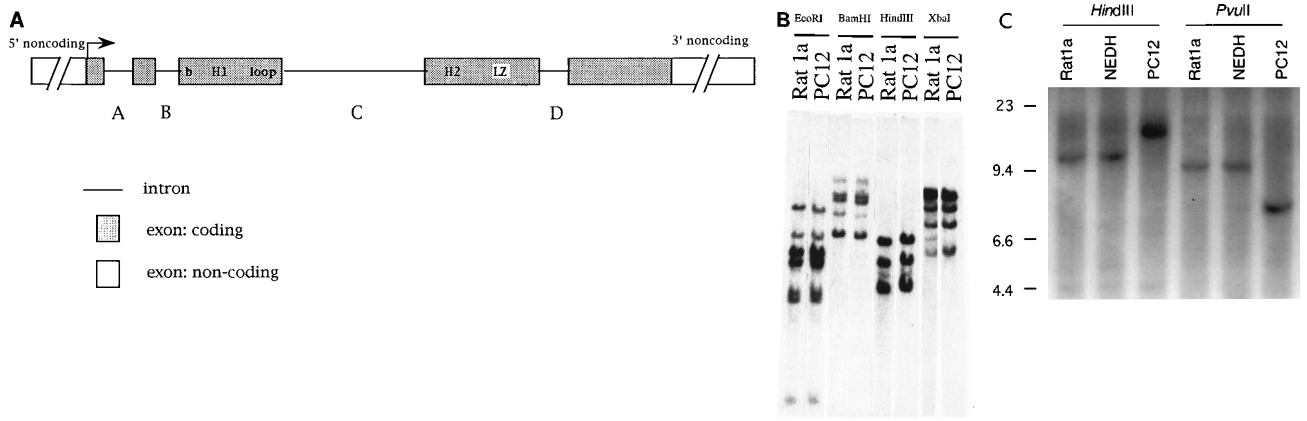

FIG. 8. Southern blots of the *max* gene in PC12 cells and Rat1a cells. (A) Schematic representation of the *max* gene intron-exon structure. (B) Rat1a and PC12 genomic DNA probed with the *max* ORF. (C) Rat1a, NEDH rat, and PC12 genomic DNA probed with a fragment of intron C.

coding exons 3′ of this intron are expressed. Furthermore, Northern blotting with the *max* 3′ untranslated region showed no expression of 3′ noncoding exons (Fig. 6C).

Max exerts a mild growth-inhibitory effect in PC12 cells. To assess the effect of reintroducing Max into PC12 cells, we cloned stable lines of PC12 cells containing *max* under the control of the Zn^{2+}-inducible mouse metallothionein promoter. These clones all express a low level of Max protein in the absence of the inducer. Max protein expression is induced in these clones approximately 10-fold by Zn^{2+}, to a level comparable with endogenous Rat1a expression of Max (Fig. 9A). Clones expressing Max were indistinguishable from control clones expressing the empty expression vector or parental PC12 cells, both in their morphology and in their ability to differentiate in response to NGF (not shown). The basal uninduced expression of Max appeared to result in a slight reduction in the rate of cellular proliferation, at least for cells at low density (Fig. 9B). Surprisingly, induction of Max resulted in no further growth retardation (results not shown). This might be explained if the basal level of Max expressed in uninduced cells was already saturating with respect to its growth-inhibitory potential.

DISCUSSION

We have shown that in the well-characterized rat tumor cell line PC12, functional Max protein is not expressed because of aberrant processing of the *max* mRNA. We base this conclusion on the following observations. (i) Immunoprecipitation with two independent anti-Max antibodies fails to detect Max protein in PC12 cells but readily detects both Max splice variants in Rat1a cells. (ii) By RNase protection, we show that only the sequence encoding the N terminus of Max is expressed in PC12, whereas 3′ exons encoding helix 2 of the helix-loop-helix motif, the leucine zipper, and the C terminus of Max are not expressed in PC12 cells. (iii) By Northern blotting, we show that sequences in the 3′ untranslated region of the normal *max* message are not expressed. Therefore, all exons 3′ of the exon encoding the basic region, helix 1, and loop of the helix-loop-helix motif fail to be expressed in PC12 cells (Fig. 8A). (iv) We have also shown by Northern blotting that PC12 cells express multiple polyadenylated transcripts of abnormally large size relative to wild-type *max* mRNA, suggesting a dysfunction in the splicing of *max* mRNA in these cells. (v) We have cloned a *max*-related cDNA from PC12 cells. In agreement with the RNase protection results, this cDNA contains only the first 150 nt of the *max* ORF fused to a novel sequence. The protein encoded by this cDNA cannot functionally substitute for Max, as determined by its inability to dimerize with Myc or Max in vitro and its inability to repress transcription from an E-box. (vi) Finally, we have identified a homozygous mutation in the PC12 *max* gene which is consistent with the aberrant processing of *max* transcripts in these cells.

The observation of homozygous mutation of *max* in PC12 cells suggests that loss of *max* expression may have been a selected event either in the development of the rat pheochromocytoma or during subsequent cell culturing in vitro. However, we find that reintroduction of Max into PC12 cells reduced the rate of cellular proliferation only slightly. We have observed a comparable weak growth-inhibitory effect of Max expression in PC12 cells assayed by the ability of Max to inhibit neomycin-resistant colony formation, an effect also observed in Rat1a cells (results not shown). Ectopic expression of Max has also been observed to retard the growth rate in murine erythroleukemia cells (18). In normal tissues, Max expression appears to be constitutive and ubiquitous (10, 14, 40, 64). Although the formation of Myc-Max heterodimers is associated with proliferation of cells, Mad-Max or Mxi1-Max heterodimers appear to be associated with differentiation of various cell types, including the myeloid cell lines U-937, HL-60, and ML-1 (6, 40, 66) and the adipocyte cell line 3T3-L1 (40b). In PC12 cells, we have observed a high level of *mxi1* expression even in the absence of NGF, but little or no expression of *mad* (40b). Mutational loss of Max expression might therefore release cells from growth inhibition due to transcriptional effects of Mxi1-Max complexes or complexes of Max with as yet uncharacterized Max dimerization partners. In agreement, Max expression in PC12 repressed E-box-dependent transcription from a test promoter, although surprisingly, cotransfection of Mxi1 did not potentiate this repression (unpublished results). Despite this repression at the transcriptional level, the observed growth-inhibitory effect of exogenously expressed Max appears to be weak and was achieved at low Max levels. Possibly the ability of PC12 to survive and proliferate in the absence of Max reflects decreased reliance by these cells on Myc-, Max-, and Mxi1-regulated pathways for control of proliferation. Such a decrease could arise from other, uncharacterized oncogenic mutations.

max has been mapped to human chromosome 14q22-24 (25, 64). This position is associated with a t(12;14)(q13-15;q23-24) chromosomal translocation in uterine leiomyomas and deletions in malignant lymphomas and B-cell chronic lymphocytic

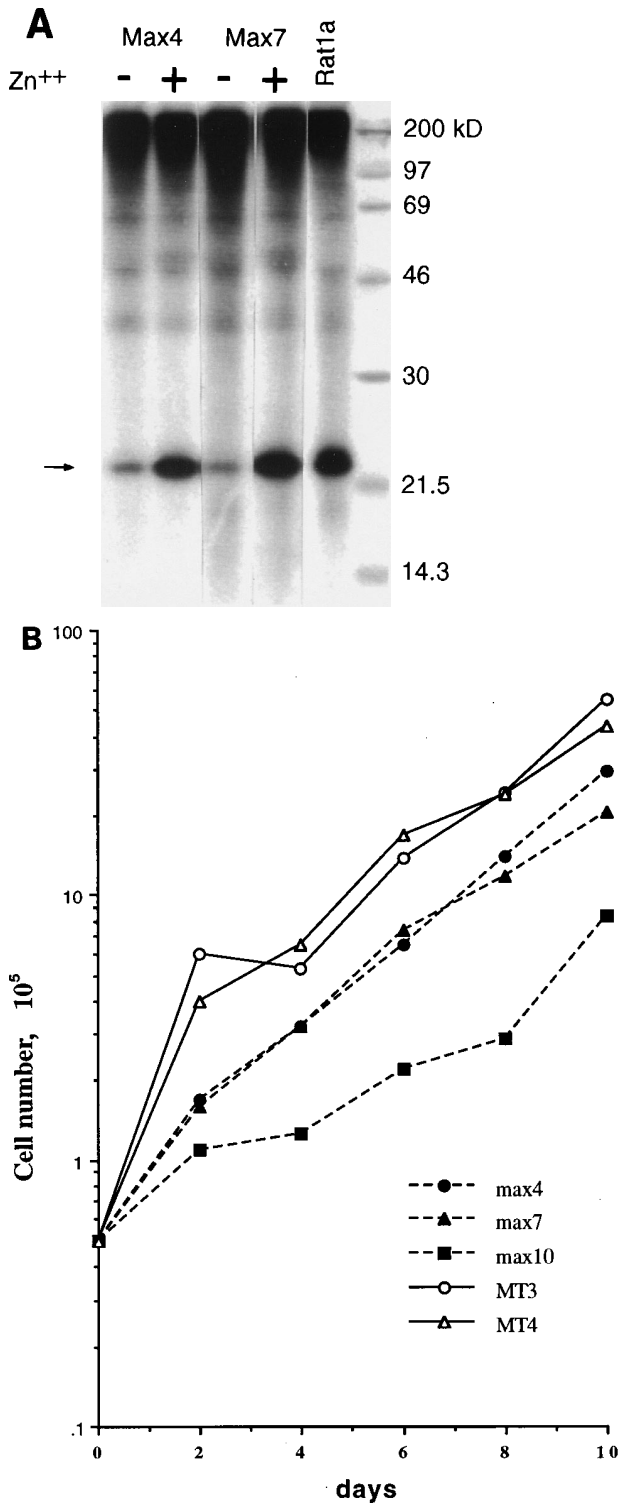

FIG. 9. Expression of Max in PC12 cells under the control of an inducible promoter. (A) Detection of Max protein expression in two PC12 clones. PC12 clones Max4 and Max7 contain the rat *max* gene under the control of the Zn^{2+}-inducible mouse metallothionein promoter. Cells were treated with Zn^{2+} for 12 h or not treated. Max protein was detected by immunoprecipitation from $^{32}PO_4$-labeled cells followed by electrophoresis on an SDS–12% polyacrylamide gel. Max immunoprecipitated from Rat1a cells is shown for comparison. (B) Growth curves for three Max-expressing PC12 clones (Max4, Max7, and Max10) and two control clones containing the empty vector (MT3 and MT4). Cell nuclei were counted every 2 days. On day 0, 0.5×10^5 cells were plated. At each time point, the cell number was determined three times for each clone.

leukemia (63). Conceivably, these mutations are specifically associated with loss of Max function. The notion that Max might function as a tumor suppresser rests on the assumption that certain tumors can grow in the absence of Myc-Max dimers. This appears to be the case for the PC12 tumor cell line. We examined 2 rat pheochromocytomas and 12 human pheochromocytomas and related paragliomas (kindly provided by A. Tischler, New England Medical Center) for mutation of the *max* gene. By probing of Southern blots of DNA from these tumors with the *max* gene, we found no evidence for mutation of *max* (unpublished observations). We conclude that mutation of *max* is unlikely to be a common genetic lesion in this class of tumor, although we cannot exclude the existence of homozygous point mutations in the *max* gene, not detected by our Southern blot analysis, which might disrupt *max* function.

An important question arising from this work is whether Myc exerts any biological effects when expressed in PC12 cells. The evidence on this point is conflicting. We observe a low level of expression of endogenous Myc mRNA in PC12 cells (Fig. 2B). Indeed, *myc* has been shown to be transcriptionaly induced in these cells by NGF, EGF, and FGF (27). Ectopic expression of Myc in PC12 cells was reported to transform cells in a manner similar to transformation by E1a (43). We have constructed PC12 clones containing c-Myc under the control of the inducible metallothionein promoter. We see no significant effect of Myc induction in these cells on cellular proliferation, as measured by fluorescence-activated cell sorting analysis (unpublished observations). Nevertheless, we cannot exclude other effects of Myc in PC12 cells not measured in our assays. Evidence has been presented for transcriptional transactivation by c-Myc from Myc/Max binding sites in PC12 cells (53). However, we do not see this effect in PC12 cells even when *max* is cotransfected (unpublished observations). These conflicting results might be due to subtle differences in the reporter and expression plasmids used. However, we have examined the effect of Myc on two related reporter constructs, using Myc under the control of either the strong CMV promoter or the weaker SV40 promoter, and have so far failed to observe transcriptional transactivation by Myc in these cells.

Myc has recently been shown to inhibit transcription from an initiator element in vitro via interaction with the initiator-binding basal transcription factor TFII-I (55) and to be capable of transcriptional repression in vivo through the initiator (41, 47). In vitro association of Myc with the transcription regulator YY-1 (61), the tumor suppressor protein Rb (57), the Rb-related protein p107 (29), and the basal transcription factor TBP (32) has also been observed. For p107 and TBP, this association was demonstrated to occur in vivo (29, 42). Myc may therefore still function in PC12 cells and execute certain of its functions via interaction with these or other proteins, although, as indicated above, the evidence for an effect of Myc in PC12 cells is conflicting.

It is currently unclear whether normal mammalian cells can divide in the absence of Myc. Knockouts of N-*myc* and c-*myc* in mice are both embryonic lethal, arguing a crucial role for Myc proteins in embryogenesis (17, 21). However, the early embryo undergoes a number of divisions in the absence of either N-Myc or c-Myc. This could be due to functional redundancy between N-Myc and c-Myc, or it may reflect the ability of cells to proliferate in the absence of Myc function but the failure of these cells to respond appropriately to developmental cues. Conceivably, an additional mutation(s) in PC12 may have released these cells from Myc dependence.

PC12 cells are the first cell line to be described which lacks Max expression and should prove a useful tool in the analysis of those Myc, Mad, and Mxi1 functions, if any, which are

independent of Max. We see a high basal level of activity for an E-box element in PC12 cells, which is significantly repressed by Max (Fig. 5). Surprisingly, neither Myc, Mad, nor Mxi1 had any effect on this element in the presence or absence of Max (unpublished observations). This result suggests that additional factors that are either absent or present at limiting levels in PC12 cells may be required for transcriptional activation by Myc-Max complexes or repression by Mad-Max and Mxi1-Max complexes. Other promoters proposed as targets for Myc activation can now be tested for dependence on Max.

The identification of a cell line in which functional Max protein is not expressed permits a refinement of current models of Myc and Max function in proliferation, differentiation, and apoptosis. Our results show that the proliferation of PC12 cells and their well-characterized ability to differentiate in response to NGF must take place without reliance on Max function. In addition, Max is dispensable for apoptosis in PC12, since PC12 cells undergo classical apoptosis under conditions of NGF deprivation and low serum levels (8, 9, 48, 56). Thus, the functions of Myc, Mad, and Mxi1 which are dependent upon interaction with Max must be dispensable in PC12 for traversal of the cell cycle, differentiation, and apoptosis.

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