

The Expression of the Developmentally Regulated Proto-oncogene *Pax-3* Is Modulated by N-Myc*

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N-Myc is a member of the Myc family of transcription factors that have been shown to play a pivotal role in cell proliferation and differentiation. In this report, we have investigated the relationship between N-Myc and the developmental control gene *Pax-3*. Using transient transfection assays, we show that the *Pax-3* promoter is activated by both N-Myc-Max and c-Myc-Max. Moreover, we show that Myc regulation of *Pax-3* promoter activity is dependent upon a noncanonical E box site in the 5' promoter region of *Pax-3*. In addition, we show that ectopic expression of both N-Myc and c-Myc leads to increased expression of *Pax-3* mRNA. Furthermore, we show that *Pax-3* mRNA expression is cell cycle-regulated and that the 5' promoter region of *Pax-3* (bp –1578 to +56) can direct cell cycle-dependent gene expression with kinetics similar to that of the endogenous transcript. Site-directed mutagenesis of the E box site within the *Pax-3* promoter significantly altered the pattern of expression through the cell cycle. These results suggest that the Myc family of transcription factors may modulate *Pax-3* expression *in vivo*.

N-Myc is a member of the Myc family of transcription factors (c-Myc, N-Myc, L-Myc, B-Myc, and S-Myc) that are characterized by a basic DNA binding domain and dimerization domain composed of a helix-loop-helix and leucine zipper. Members of this family have been shown to play a pivotal role in cell proliferation and terminal differentiation. The forced expression of c-Myc promotes progression into S phase and inhibits differentiation and entry into a quiescent state (1). Furthermore, the deregulation of *myc* gene expression has been implicated in the pathogenesis of several tumor types. The human *NMYC* gene is frequently amplified in neuroblastoma, a childhood cancer of neural crest origin (2, 3). *NMYC* amplification in this cancer is associated with rapid tumor progression, advanced stages, and poor prognosis.

N-*myc* shares many of the properties of c-*myc*, although unlike c-*myc*, whose expression appears to be ubiquitous, N-*myc* is primarily expressed during early embryogenesis (4). In mice, N-*myc* expression is highest around E9.5, where expression is observed in early neural crest lineages, limb buds, and developing central nervous system (5). The expression of N-*myc* then declines in these tissues upon the onset of differentiation. The importance of N-*myc* in embryogenesis is demonstrated by the finding that homozygous N-*myc* null mice die

around embryonic day 11.5 with abnormalities in the limb buds and in the central and peripheral nervous systems (6). Most notably, the N-*myc*-deficient embryos showed a great reduction in the number of mature neurons, especially those derived from the neural crest such as sensory and sympathetic neurons. These defects occurred despite compensatory c-Myc increases (7), suggesting a unique role for N-Myc in development.

To function, N-Myc, like c-Myc, must heterodimerize with Max proteins. Max proteins also contain a basic DNA binding domain and a helix loop helix and leucine zipper dimerization motif (8). This heterodimerization is required for sequence specific DNA binding as well as for biological function. Myc-Max heterodimers recognize the core sequence CA(C/T)GTG, termed the E box Myc sequence. In both yeast and mammalian cells, Myc-Max complexes are capable of activating reporter gene constructs containing concatamerized E box Myc sites (9, 10). In addition, Max proteins can also heterodimerize with Mad proteins that negatively regulate cell growth. Max-Mad complexes bind to the same E box motif as the Myc-Max complexes, but in contrast to Myc-Max complexes, they repress transcription (11). Mad family members repress transcription through their association with the Sin3 proteins, which in turn recruit histone deacetylases to the complex (12). The central member of the Myc/Max/Mad network is Max, which is very stable. In contrast, the expressions of both Myc and Mad proteins are highly regulated. The *myc* genes are actively transcribed in dividing cells, but little expression can be detected in quiescent or differentiated cells. By comparison, the Mad genes are usually expressed in resting or differentiated cells with little expression in dividing cells (13). A number of target genes for c-Myc have been identified. Many of the target genes that are up-regulated by c-Myc are either rate-limiting enzymes involved in the biosynthesis and metabolic production of polyamines and pyrimidines, such as ornithine decarboxylase (14) and *cad* (15), or involved in cell cycle control such as *cdc25a* (16), *ISGF3γ* (17), and *p53* (18).

Pax-3 is a member of a family of evolutionarily conserved transcription factors (19) that have been shown to play a critical role in early embryogenesis. Disruption of the *Pax-3* gene has been shown to lead to a range of developmental abnormalities including neural tube defects, a lack of limb musculature, and deficiencies in neural crest-derived cell types (20–24). *Pax-3*, like N-*myc*, is first expressed during early embryogenesis within neural crest lineages, limb buds, and the developing nervous system. The expression of *Pax-3* in these tissues is restricted to mitotically active progenitor cells and is rapidly down-regulated upon differentiation (25, 26). Given the overlapping patterns of expression of N-*myc* and *Pax-3*, we have examined the relationship between N-Myc and *Pax-3*, and in this report we present evidence that *Pax-3* is a direct transcriptional target of N-Myc and c-Myc.

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EXPERIMENTAL PROCEDURES

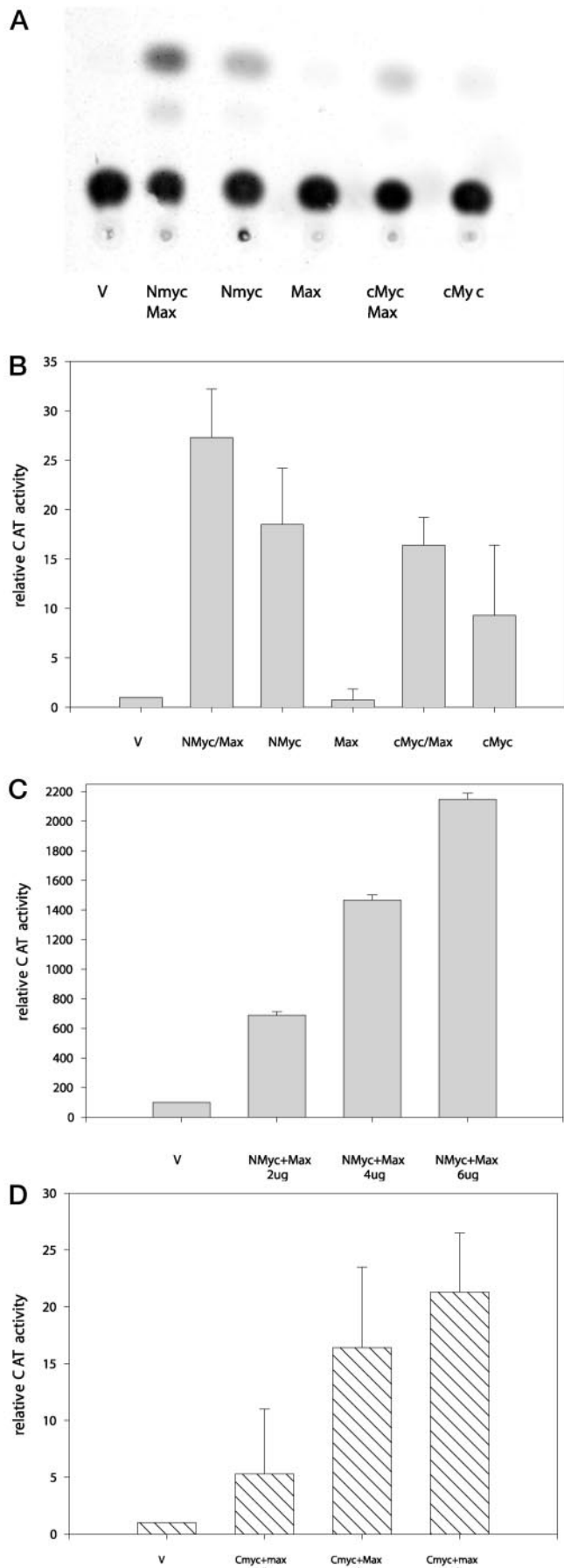


FIG. 1. Regulation of Pax-3 promoter activity by N-Myc and c-Myc. Shown are a representative CAT assay (A) and graph (B) of

Cell Culture—ND7, NIH3T3, and COS-7 cells were grown in full growth medium containing Dulbecco's modified Eagle's medium (DMEM),¹ 10% bovine calf serum, and 2 mM glutamine. Neuroblastoma cell lines (IMR-32, Kelly, SK-N-SH, C1300, SHSY-5Y, SK-N-AS, SK-N-BE, and SK-N-DZ) were grown in DMEM, 10% bovine calf serum, 2 mM glutamine, and 1% nonessential amino acids.

Transfections—To prepared stable cell lines expressing N-Myc, ND7 cells (5×10^5) were plated out on 58-cm² dishes and transfected with an N-Myc expression vector (pMiwNmyc; 2 μ g) together with 2 μ g of pcDNA3.1, which carries the neomycin resistance marker. Stable transfectants were selected by supplementing the medium 48 h after transfection with 800 μ g/ml G418. Independent clones were isolated after 1 week, when individual foci of cells were evident. These were then grown up and maintained in DMEM plus 10% serum containing 800 μ g/ml G418. Cell lines expressing c-MycER were generated by transfecting ND7 cells with pBpuro c-MycER (27) and selecting for puromycin (5 μ g/ml)-resistant clones. The retroviral vector pBpuro c-MycER comprises the c-myc coding sequences fused in frame to a modified ligand binding domain of the estrogen receptor. The fusion protein is activated by the addition of the synthetic ligand 4-hydroxytamoxifen (OHT) but is refractory to 17 β -estradiol. ND7 cells expressing c-MycER were passaged in DMEM plus 10% bovine calf serum and 1% charcoal-treated bovine calf serum for 48 h prior to Myc induction. To activate c-MycER, 100 nM OHT was added to the culture media for 4 h. This results in the translocation of the c-MycER fusion protein from the cytoplasm to the nucleus (data not shown). In transient transfection assays, NIH3T3, ND7, and COS-7 (5×10^5) cells were plated out on 58-cm² dishes and transfected as previously described (28). The transfection efficiency was normalized by co-transfecting cells with pCMV- β -gal. β -Galactosidase assays were performed according to the method of Gorman (29). Quantitation of the CAT assay was achieved using a STORM Phosphor-Imager and then using an image quantifier program to calculate percentage conversions of the [¹⁴C]chloramphenicol to its acetylated products. For cell cycle analysis, transfected ND7 cells were placed in serum-free media for 24 h. Following serum starvation, the cells were either harvested or refed with media containing serum and incubated for the indicated time periods. Luciferase assays were performed using the Promega luciferase assay system.

Western Blot Analysis—Nuclear extracts were made as described by Dignam *et al.* (30), and the protein concentration of each sample was determined using the Bio-Rad DC protein assay kit. Samples (20 μ g of nuclear protein) were separated by SDS-PAGE (7.5% resolving gel) using a Bio-Rad minigel apparatus. Proteins were transferred to a Hybond-P membrane and blocked overnight at 4 °C with 5% dried milk powder, 0.02% Tween 20 in PBS. Membranes were incubated at room temperature for 1 h at room temperature in 5% dried milk powder in phosphate-buffered saline and a 1:2000 dilution of N-Myc or c-Myc antisera and washed in phosphate-buffered saline plus 0.2% Tween 20. Membranes were then incubated in phosphate-buffered saline plus 0.2% Tween 20 containing 5% dried milk powder with a 1:10,000 dilution of goat anti-rabbit antibody coupled to horseradish peroxidase for 1 h. Immunoreactive bands were then visualized using Ultrasignal (Pierce), and the intensities of the bands were quantitated using densitometry. All membranes were subsequently checked to ensure equal loading of samples by staining the membrane with Amido Black.

Antibodies—Anti-c-Myc, -Max, -Mad, and -MyoD antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-N-Myc antibodies were from Calbiochem.

RT-PCR—Total cellular RNA was extracted using Trizol (Invitro-

¹ The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; OHT, 4-hydroxytamoxifen; RT, reverse transcriptase; EMSA, electrophoretic mobility shift assay.

NIH3T3 cells co-transfected with the Pax-3 promoter (Pax3CAT; 2 μ g) and either the null expression vector pMISV (V, 4 μ g), N-Myc and Max expression vectors (4 μ g each), N-Myc expression vector (4 μ g), Max expression vector (4 μ g), c-Myc and Max (4 μ g each), or c-Myc expression vector (4 μ g). C and D, increasing the concentration of N-Myc-Max or c-Myc-Max further enhanced Pax-3 promoter activity. The Pax-3 promoter construct (Pax3CAT; 2 μ g) was co-transfected with either increasing concentrations of N-Myc-Max (2, 4, and 6 μ g) (C) or c-Myc-Max (2, 4, and 6 μ g) (D) into NIH3T3 cells. The graph shows the mean of four independent experiments \pm S.E. (all values are normalized for pCMV- β -gal activity).

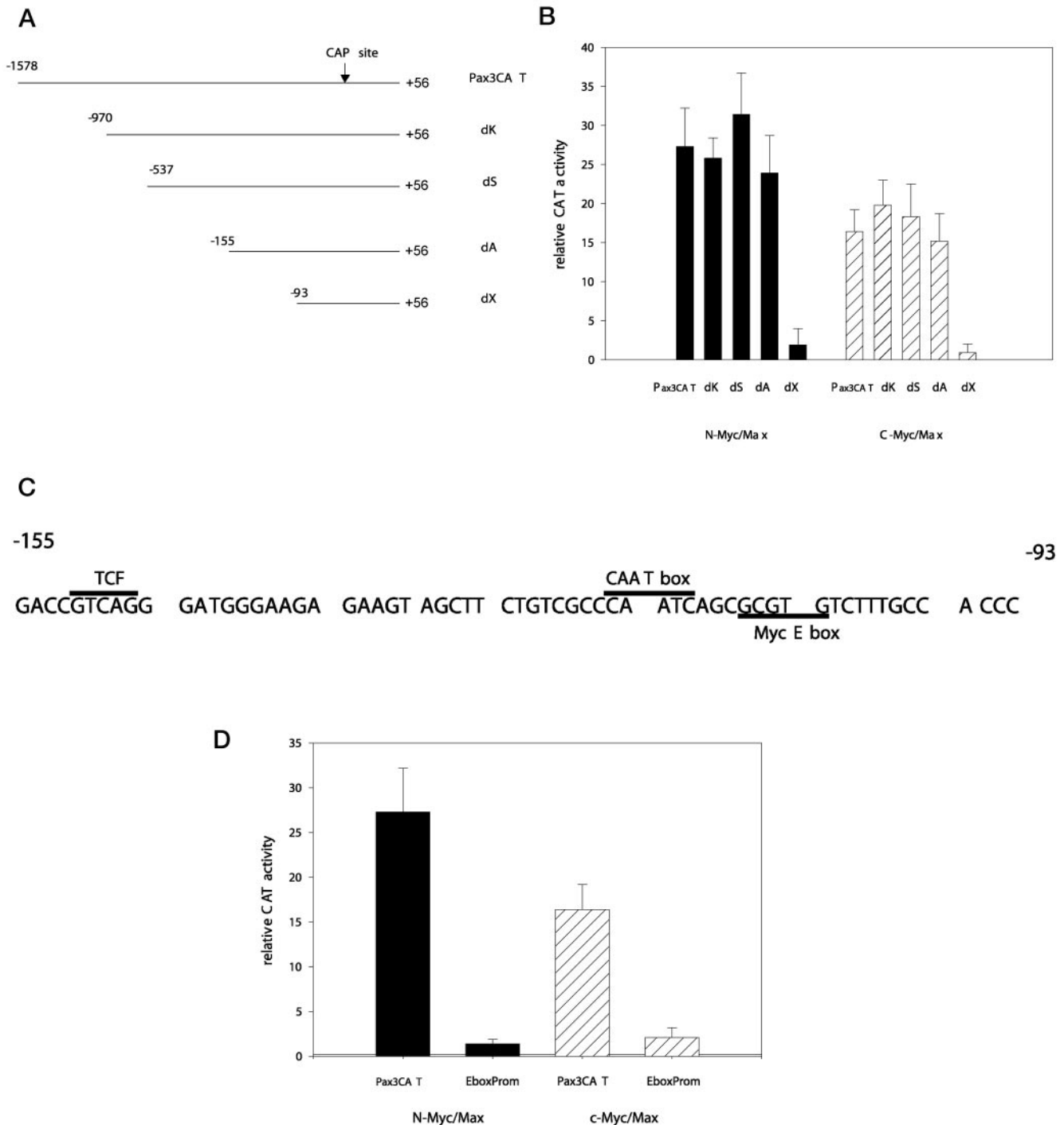


FIG. 2. Identification of the sequences responsible for Myc-Max regulation of Pax-3 promoter activity. *A*, schematic diagram of the region of the Pax-3 gene near the start site of transcription (CAP) to show the constructs tested for Myc-dependent activation. *B*, graph showing the level of relative CAT activity in NIH3T3 cells co-transfected with either 2 μ g of Pax3CAT (bp -1578 to +56) or one of the Pax-3 promoter deletion constructs, dK (bp -970 to +56), dS (bp -537 to +56), dA (bp -155 to +56), dX (bp -93 to +56), and either N-Myc and Max expression vectors (4 μ g each) or c-Myc and Max expression vectors (4 μ g each). The level of Myc-Max activation was then expressed relative to the level of CAT activity observed in cells transfected with Pax3CAT and a null expression vector. This was arbitrarily set at 1. Values represent the mean of four independent experiments \pm S.E. (all values are normalized for pCMV- β -gal activity). *C*, sequence of the Pax-3 promoter from bp -155 to -93 upstream of the transcription start site. The locations of transcription factor binding sites identified in this region are underlined. *D*, graph showing the level of relative CAT activity in NIH3T3 cells cotransfected with either 2 μ g of Pax3CAT or the Pax-3 promoter construct (EboxProm) containing a mutated E box site together with either N-Myc-Max or c-Myc-Max (4 μ g each). Values represent the mean of four independent experiments \pm S.E. (all values are normalized for pCMV- β -gal activity).

gen), and RNA concentrations were determined by slot-blot hybridization. Conventional RT-PCR was carried out as described by Reeves *et al.* (26), with gene-specific primers for Pax-3 (5'-GGAATACAAAGAGAG-AACCCG-3' and 5'-CTTCATCTCACTGAGGTGCG-3') and the housekeeping gene cyclophilin (5'-TTGGGTGCGCTCTGCTTCGA-3' and 5'-GCCAGGACCTGTATGCTTCA-3'). For Taqman quantitative RT-PCR

analysis, all equipment and reagents were supplied by Applied Biosystems. Taqman PCR primers and probes were designed using Primer Express software. (Pax-3 forward primer, 5'-GAGTGAGCGGAGCCT-CTGCAC-3'; reverse primer, 5'-AGGTGGTTCTGCTCCTGCG-3'; Taqman probe, 5'-AGGCTCCGTATTGACTCTGAACCTGATTAC-3'). Primers and probes for the housekeeping gene 18 S ribosomal RNA

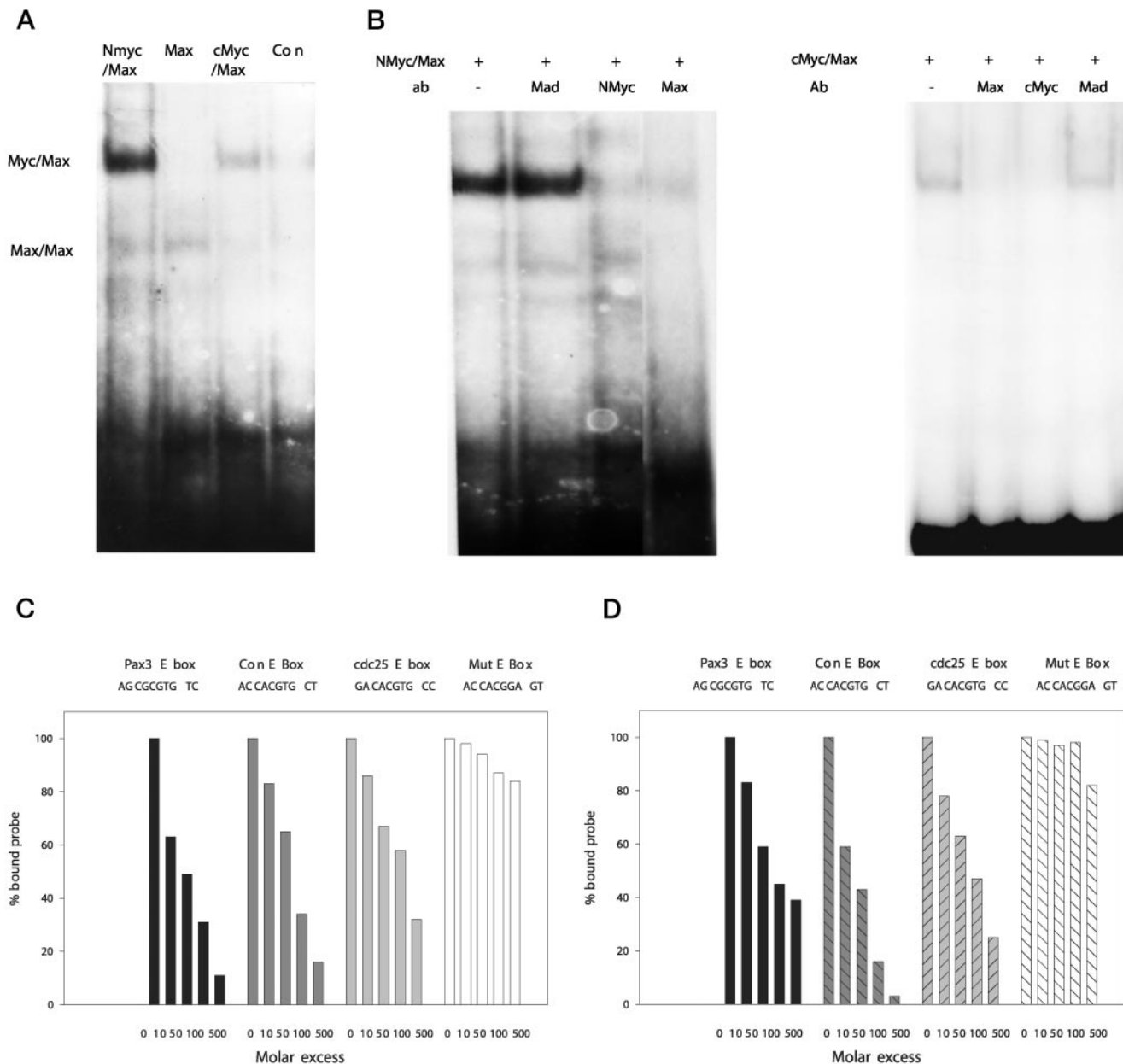


FIG. 3. N-Myc-Max and c-Myc-Max bind to the Pax-3 E box site *in vitro*. A, EMSA in which lysates of COS-7 cells transfected with N-Myc-Max, Max, c-Myc-Max, or an empty expression vector (Con) were incubated with a radiolabeled oligonucleotide containing the Pax-3 E box site. B, lysates from N-Myc-Max- and c-Myc-Max-transfected COS-7 cells were incubated with a radiolabeled oligonucleotide containing the Pax-3 E box site either alone or in the presence of specific antibodies (ab) to N-Myc, c-Myc, Max, or Mad. C and D, comparison of N-Myc-Max (C) and c-Myc-Max (D) binding affinities by competition experiments. Binding to the radiolabeled Pax-3 E box sequence was competed with unlabeled Pax-3 E box sequence (Pax-3 E box), the consensus E box site (Con E box), the *cdc25* E box site (*cdc25* E box), and a mutant E box site (Mut E Box) at 0-, 10-, 50-, 100-, and 500-fold molar excess. Binding of uncompeted Pax-3 E box oligonucleotide was set to 100%.

were supplied by Applied Biosystems. Probes were labeled at the 5'-end with a 6'-carboxyfluorescein reporter dye and at the 3'-end with a 6'-carboxytetramethylrhodamine quencher dye. To test the primer pairs, conventional RT-PCR was used as described previously (26), and amplified products were confirmed by direct sequencing of the PCR. For Taqman PCR, cDNA was synthesized using 200 ng of total RNA in a 10- μ l reaction volume using RTGold reagents according to the manufacturer's instructions. Taqman PCR was performed with 1 μ l (equivalent to 10 ng of input RNA) of cDNA template in a 25- μ l PCR containing 100 nM primers, 50 nM probe in Universal Taqman master mix. Cycling conditions were 50 °C for 2 min, 95 °C for 10 min followed by a 40-cycle amplification cycle of 95 °C for 15 s and 60 °C for 1 min on the Applied Biosystems PRISM 7700 sequence detection system. Samples were analyzed in duplicate and normalized to the measurement of the housekeeping gene as described by Bustin *et al.* (31).

DNA Cloning—The Pax-3 promoter (–1578 to +56 bp) was amplified from MF1 mouse genomic DNA by PCR using the primers 5'-GAGCT-

CTAATGCTCCTCC-3' and 5'-GGTGACGAGGCAGGAAC-3' and Accu-Taq (Sigma). The amplified fragment was cloned into pGem-T Easy (Promega) and sequenced. The Pax-3 promoter fragment was excised from pGem-T Easy with *Sph*I and *Sal*I and subcloned into pCATBasic (Promega) to create Pax3Cat or into pGL3Basic (Promega). Truncated promoter constructs were made by digesting Pax3CAT with *Sph*I and either *Kpn*I, *Sal*I, *Ava*II, or *Xma*I. The digested vectors were then purified and religated to form dK (–970 to +56 bp), dS (–537 to +56 bp), dA (–155 to +56 bp), and dX (–93 to +56 bp). All clones were then sequenced to verify the construct. All deletion constructs used were able to drive expression of a reporter gene. The expression vectors containing N-myc (pmiwNmyc) and Max (pmiwMax) were a kind gift of H. Kondoh. The c-myc cDNA clone was obtained from M. Green and subcloned into pMiv. The vectors pBpuro and pBpuro cMycER were a kind gift from G. Evan.

Site-directed Mutagenesis—The putative E box sequence in the Pax-3 promoter was mutated from CGCGTG to CCGCGG using two-step PCR

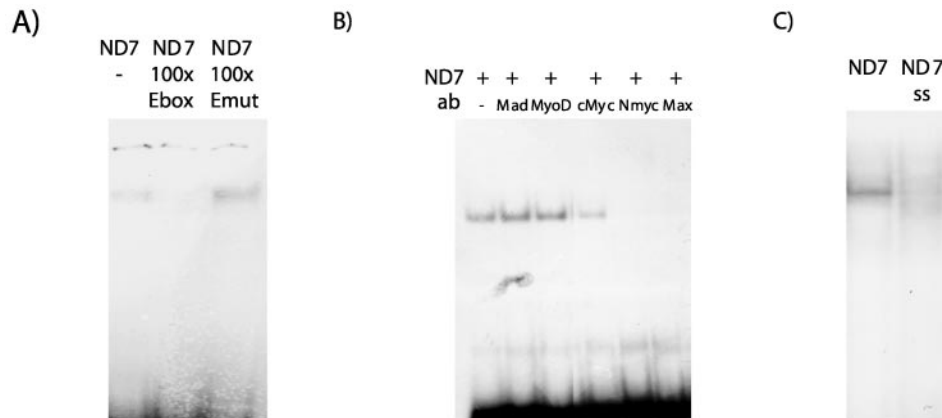


FIG. 4. **Myc-Max binding to the Pax-3 E box site in neuronal cells.** A, EMSA showing nuclear extracts (5 μ g) from dividing ND7 cells incubated with a radiolabeled oligonucleotide containing the Pax-3 E box site either alone (ND7) or together with an excess of cold E box oligonucleotide (ND7 100 \times Ebox) or with an excess of cold mutant E box sequence (ND7 100 \times Emut). B, EMSA showing that Pax-3 E box binding in dividing ND7 is reduced by the addition antibodies raised against N-Myc, Max, and c-Myc. Nuclear extracts from ND7 cells were incubated with radiolabeled E box probe either alone (–), or with antibodies against MyoD, Mad, N-Myc, c-Myc, or Max. C, EMSA showing the decline in E box binding in ND7 cells upon serum starvation. Nuclear extracts (5 μ g) were prepared from dividing ND7 cells (ND7) and from serum-starved ND7 cells (ND7 ss) and incubated with the radiolabeled Pax-3 E box probe and analyzed by EMSA.

mutagenesis using the following primers: 5'-CCCAATCAGCCGCGGTCTTTGCCAC-3' and 5'-GTGGCAAAGACCGCGGCTGATTGGG-3'. The sequence of all clones was verified by sequencing.

Electrophoretic Mobility Shift Assays—Oligonucleotides for use in electrophoretic mobility shift assays were annealed by heating to 85 $^{\circ}$ C for 3 min and labeled using [γ - 32 P]ATP and polynucleotide kinase (Promega). The oligonucleotides used were the Pax-3 E box Myc sequence (5'-GCCCCAATCAGCGCGTGTCTTTGCCAC-3'), a consensus E box Myc sequence (5'-GGAAGCAGACCACGTGCTCTGCTTCC-3'), the *cdc25* E box sequence (5'-ACTACACACGTGCCACCACACCCAA-3'), and a mutant E box Myc sequence (5'-GGAAGCAGACCACGGAGTCTGCTTCC-3'). Nuclear extracts were made using the method described by Dignam *et al.* (30). Electrophoretic mobility shift assays were carried out as previously described (26). Competitions were performed using a 10-, 50-, 100-, and 500-fold excess of unlabelled oligonucleotide, which was incubated with the nuclear extracts prior to the addition of the probe. To confirm the identity of the retarded complex, nuclear extracts were also incubated on ice for 4 h with 2 μ l of 1 μ g/ml specific antiserum prior to the addition of the probe.

Cell Cycle Analysis—For cell cycle distribution, cells were pelleted and resuspended in 1 ml of 0.1% Triton X-100, 0.1% sodium citrate, and 50 μ g/ml propidium iodide and incubated at 4 $^{\circ}$ C for 1 h. DNA content was analyzed by flow cytometry (32) using a Becton Dickinson FACS-Calibur flow cytometer. The analysis of cells in late G₁ was confirmed by arresting cells at the G₁/S border with Aphidicolin, and the analysis of G₂/M cells was confirmed by arresting cells with hydroxyurea. For cell synchronization, cells were seeded onto 8.7-cm² Petri dishes at a density of 2×10^4 . Cells were then serum starved by the addition of DMEM plus 1 mM glutamine media for 24 h. Serum was then added back for 1, 3, 6, 12, and 18 h, and samples were analyzed for DNA content, c-Myc and N-Myc protein levels, and Pax-3 mRNA levels.

RESULTS

Regulation of Pax-3 Promoter Activity by N-Myc and c-Myc—To determine whether Pax-3 expression is regulated by N-Myc, the 5' promoter region of the murine Pax-3 gene (bp –1578 to +56) was amplified by PCR from mouse genomic DNA and sequenced. This region of the Pax-3 promoter has been shown by Natoli *et al.* (33) to be sufficient for the correct induction of Pax-3 expression *in vivo*. For our experiments, the Pax-3 promoter (bp –1578 bp to +56) was then cloned upstream of the reporter gene CAT in the vector pCATbasic (Pax3CAT). The Pax-3 promoter construct was then transiently transfected into the fibroblast cell line NIH3T3 together with an expression vector containing either the full-length cDNA of the mouse N-myc gene or the full-length cDNA of the c-myc gene. CAT activity was measured 48 h later. All transient CAT assay values were normalized with a cotransfected cytomegalovirus- β galactosidase plasmid. We found that co-transfection

of the Pax-3 promoter construct with either N-Myc or c-Myc led to the induction of Pax-3 promoter activity. N-Myc induced an 18-fold increase, whereas c-Myc induced an 8-fold increase in Pax-3 promoter activity (Fig. 1, A and B). Co-transfection of the Pax-3 promoter with N-Myc and Max or c-Myc and Max led to a further increase in Pax-3 promoter activity. In contrast, co-transfection of Max alone with Pax-3CAT led to a small inhibition in Pax-3 promoter activity (Fig. 1, A and B). In addition, when the amounts of expression vectors for N-Myc-Max and c-Myc-Max were increased in a fixed Myc/Max ratio, expression of the Pax-3 promoter was further increased (Fig. 1C), suggesting that members of the Myc family of transcription factors can activate Pax-3 promoter activity *in vitro*. The expression of pCATbasic was unaffected by the addition of increasing amounts of N-Myc-Max or c-Myc-Max.

Myc Regulation of Pax-3 Expression Is Dependent upon Sequences in the Promoter of Pax-3—Having shown that the Myc family of transcription factors is capable of regulating Pax-3 expression in transient transfection assays, we next investigated whether this regulation was dependent on the direct binding of the Myc proteins to sites within the Pax-3 promoter. To test this, a series of truncated Pax-3 promoter constructs derived from the Pax-3CAT promoter construct (bp –1578 to +56) were generated (Fig. 2A). All truncated promoter constructs were able to direct basal gene expression, and we therefore examined the ability of N-Myc-Max and c-Myc-Max to activate each truncated construct. We found that all of the truncated Pax-3 promoter constructs were activated in the presence of both N-Myc and Max and c-Myc and Max, apart from the smallest construct, dX (–93 to +56 bp), which was no longer activated by either N-Myc-Max or c-Myc-Max (Fig. 2B). This suggests that the sequences between –155 and –93 bp are essential for Myc regulation of Pax-3 expression. Computer analysis of this region of the Pax-3 promoter, using MatInspector, version 2.2, based on Transfac 4.0, revealed an inverted Myc E box binding site (CGCGTG) located –110/–105 bp upstream of the transcription start site (Fig. 2C). Mutation of this site from CGCGTG to CCGCGG abolished the ability of N-Myc and Max or c-Myc and Max to activate Pax-3 promoter activity (Fig. 2D).

The Pax-3 E Box Motif Is Bound by Myc and Max—To determine whether N-Myc-Max or c-Myc-Max heterodimers can bind to the E box site from the Pax-3 promoter, whole cell extracts from COS-7 cells transiently transfected with expression vec-

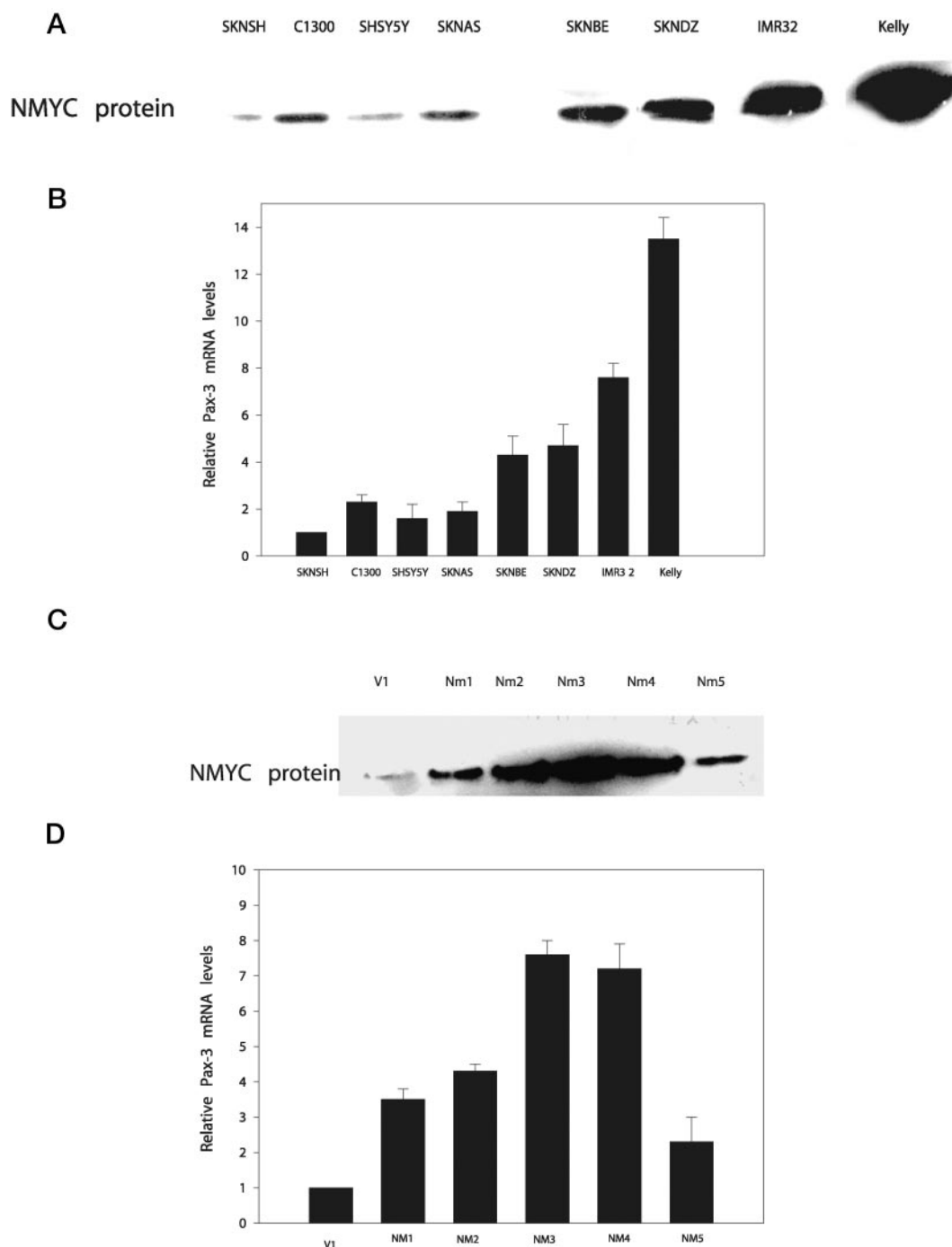


FIG. 5. *Pax-3* mRNA levels are elevated in *NMYC*-amplified neuroblastoma cell lines. *A*, Western blot analysis to determine the level of NMYC protein expressed in the neuroblastoma cell lines SK-N-SH, C1300, SHSY-5Y, SK-N-AS, SK-N-BE, SK-N-DZ, IMR-32, and Kelly. *B*, quantitative RT-PCR analysis of *Pax-3* mRNA expression in neuroblastoma cell lines. Values are the mean of three independent experiments \pm S.E. Levels of *Pax-3* mRNA are expressed relative to the level of *Pax-3* expression detected in SK-N-SH cells, which is set to 1. All values have been normalized to the measurement of 18 S rRNA. *C*, determination of *Pax-3* mRNA levels in N-Myc-transfected mouse neuroblastoma cells. ND7 cells were co-transfected with expression vector pMiSV-N-myc and pcDNA3.1. Five individual clones resistant to G418 were selected and named NM1 to -5. The cell lines were then analyzed for N-Myc protein expression by Western blot analysis (*C*) and for *Pax-3* mRNA expression by quantitative RT-PCR (*D*). Values are the means of three independent experiments \pm S.E. Levels of *Pax-3* mRNA are expressed relative to the level of *Pax-3* expression detected in the ND7 cell line transfected with an empty expression vector (V1), which is set to 1. All values have been normalized to the measurement of 18 S rRNA.

tors containing N-Myc, c-Myc, and Max were incubated with a radiolabeled oligonucleotide containing the Pax-3 E box sequence, and DNA binding was assessed by an electrophoretic mobility shift assay (34). In COS-7 cells transfected with an empty expression vector, little binding to the Pax-3 E box site was observed. In COS-7 cells transfected with N-myc expression vectors, no E box binding above that seen in control cells

was observed (data not shown). However, when COS-7 cells were co-transfected with N-Myc and Max, one major retarded complex was seen together with a very weak high mobility complex (Fig. 3A). In COS-7 cells transfected with both N-Myc and Max, the major retarded complex was disrupted by both N-Myc and Max antibodies, suggesting that this major E box complex represents N-Myc-Max binding to the Pax-3 E box

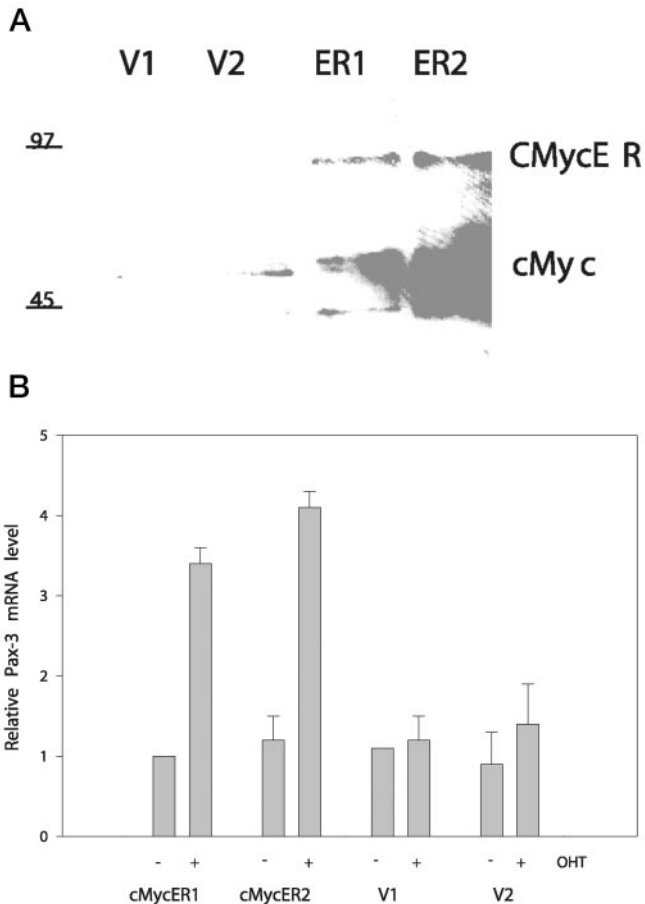


FIG. 6. Expression of c-MycER in ND7 cells. A, Western blot analysis of cell lysates prepared from two vector-transfected cell lines (V1 and V2) and from two c-MycER-transfected cell lines (ER1 and ER2). B, quantitative RT-PCR analysis of RNA extracted from the cell lines V1, V2, c-MycER1, and c-MycER2. The cells were treated with or without OHT (100 nM) for 4 h prior to harvesting. Values are the mean of three independent experiments \pm S.E. Levels of Pax-3 mRNA are expressed relative to the level of Pax-3 expression detected in V1 cells without OHT treatment. This is set to 1. All values have been normalized to the measurement of 18 S rRNA.

sequence. The weaker complex, however, was disrupted only by antibodies against Max, suggesting that this weak E box binding represents Max homodimer binding (Fig. 3B). The faster migrating E box complex was also observed in lysates from COS-7 cells transfected with Max-containing expression vectors, which is consistent with this complex representing Max homodimer binding. We also found that c-Myc, like N-Myc, could not bind to the E box motif alone, but, when transfected with Max, binding was observed. These experiments suggest that Max homodimers and N-Myc-Max and c-Myc-Max heterodimers can bind to the Pax-3 E box motif *in vitro*.

To compare the binding affinity of N-Myc-Max and c-Myc-Max for the Pax-3 E box motif, competition assays were performed using the consensus E box Myc sequence (CACGTG), the E box site from the *cdc25* gene, or a mutant E box site. In these experiments, the radiolabeled Pax-3 E box oligonucleotide was used as a probe with extracts from COS-7 cells, transfected with either N-Myc-Max or c-Myc-Max, and incubated with a 10-, 50-, 100-, or 500-fold molar excess of unlabeled oligonucleotide. The addition of a 500-fold excess of the cold Pax-3 E box site reduced binding by 85 and 61% with N-Myc-Max and c-Myc-Max, respectively (Fig. 3, C and D). The consensus E box motif and the E box site from the *cdc25* gene were able to effectively compete out N-Myc-Max and c-Myc-Max

binding, although both the consensus E box and the *cdc25* E box site were more effective in binding out c-Myc-Max rather than N-Myc-Max binding. An oligonucleotide containing a mutated Pax-3 E box site was not able to effectively compete out N-Myc-Max or c-Myc-Max binding to the probe (Fig. 3, C and D). These experiments demonstrate that both N-Myc-Max and c-Myc-Max heterodimers can effectively bind to the E box motif in the Pax-3 promoter, although N-Myc-Max heterodimers appear to bind to the Pax-3 E box site with greater affinity than c-Myc-Max.

Examination of Myc-Max Binding to the Pax-3 E Box Site in Neuronal Cells—Having shown that transiently transfected N-Myc-Max and c-Myc-Max heterodimers can bind to the Pax-3 E box motif in COS-7 cells, the Pax-3 E box sequence was examined for potential binding to Myc-Max heterodimers in the ND7 cell line. To examine this, nuclear extracts were prepared from the ND7 cell line. These cells proliferate indefinitely in the presence of serum, but upon serum starvation, the cells undergo cell cycle arrest and morphologically differentiate into a mature neuronal-like phenotype. Dividing ND7 cells express Pax-3 mRNA, but upon differentiation, the level of Pax-3 declines. We found when nuclear extracts from dividing ND7 cells were incubated with a radiolabeled oligonucleotide containing the Pax-3 E box site and analyzed by EMSA, one low mobility E box-binding protein was observed (Fig. 4A). Moreover, this binding was specific, since E box binding was competed out by an excess of oligonucleotide containing the E box site but not by an oligonucleotide containing a mutant E box consensus sequence. Furthermore, the addition of antibodies raised against N-Myc and Max dramatically reduced binding. Interestingly, the addition of antibodies raised against c-Myc also reduced binding (Fig. 4B). Since the c-Myc antibody is specific for c-Myc and does not cross-react with other members of this family (data not shown), this suggests that in dividing ND7 cells, the E box site within the Pax-3 promoter is bound by a combination of N-Myc-Max and c-Myc-Max complexes. Western blot analysis confirmed that ND7 cells express both N-Myc and c-Myc proteins (see Fig. 7). In contrast, antibodies against Mad or MyoD, had no effect on E box binding (Fig. 4B). Interestingly, in serum-starved ND7 cells, little c-Myc-Max binding to the E box site was observed (Fig. 4C).

Expression of Pax-3 mRNA in NMYC-amplified Neuroblastoma Cell Lines—Having shown that N-Myc and c-Myc can regulate Pax-3 promoter activity, we next examined the possibility that Pax-3 expression may be regulated by members of the Myc family of transcription factors *in vivo*. If Pax-3 is a target gene of N-Myc, then expression of Pax-3 mRNA would be expected to be altered in cell lines encoding an amplified NMYC gene. We therefore analyzed the expression of Pax-3 mRNA in a range of neuroblastoma cell lines containing either a single copy NMYC gene (SK-N-SH, C1300, SHSY-5Y, and SK-N-AS) or an amplified NMYC gene (IMR-32, Kelly, SK-N-DZ, and SK-N-BE) (2). We found, as shown in Fig. 5A, that all cell lines expressed detectable levels of NMYC protein, with the highest levels of NMYC being detected in Kelly, IMR-32, SK-N-DZ, and SK-N-BE cell lines. These are the cell lines with an amplified NMYC gene. Quantitative RT-PCR analysis revealed that levels of Pax-3 mRNA correlated with levels of NMYC protein, with Kelly and IMR-32 cells expressing the highest levels of Pax-3 mRNA; SK-N-BE and SK-N-DZ cell lines expressing intermediate levels of Pax-3 mRNA; and the lowest levels of Pax-3 mRNA being detected in the neuroblastoma cell lines with a single copy NMYC gene (SK-N-SH, C1300, SHSY-5Y, and SK-N-AS) (Fig. 5B).

To investigate this link between N-Myc and Pax-3 mRNA expression further, the mouse neuroblastoma cell line ND7 was

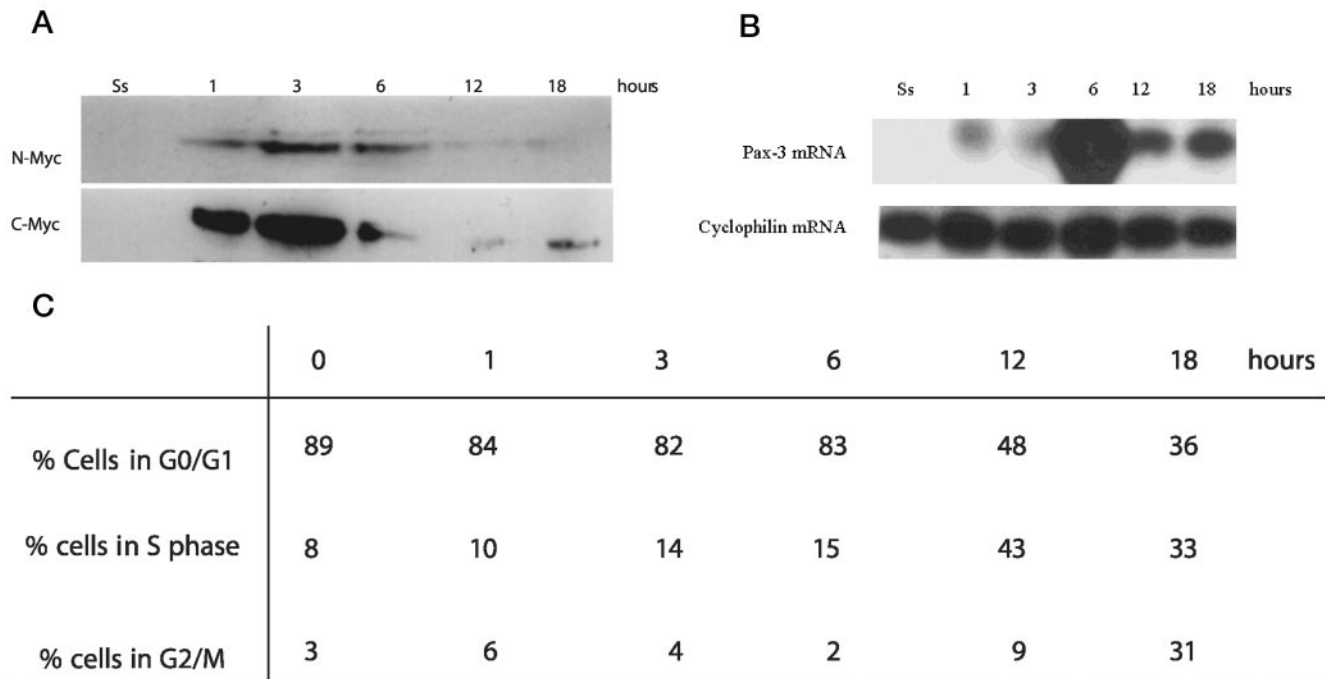


FIG. 7. **Pax-3 mRNA expression oscillates during the cell cycle.** ND7 cells were synchronized by serum starvation for 24 h. The cells were then released from the cell cycle block by the addition of serum, and cells were analyzed 0, 1, 3, 6, 12, and 18 h after the addition of serum for the level of N-Myc and c-Myc protein expression by Western blot analysis (A), for Pax-3 and cyclophilin mRNA expression by RT-PCR (B), and for the percentage of cells in G₀/G₁, S, or G₂/M phase at the indicated time points (C).

transfected with an expression vector containing a full-length N-myc cDNA. Five independent G418-resistant cell lines were selected by stable transfection and grown up for further analysis. We found that in the cells transfected with the N-myc expression vector (NM1–5), the level of N-Myc protein was 2–7-fold higher than that observed in control cells (V1), which were stably transfected with the empty expression vector (Fig. 5C). Pax-3 mRNA levels were also increased in the cell lines overexpressing N-Myc, with the highest levels of Pax-3 mRNA being detected in the cell lines NM2, NM3, and NM4. These cell lines also express the highest levels of N-Myc protein (Fig. 5D). These results suggest that the ectopic expression of N-Myc leads to an elevation in endogenous Pax-3 mRNA expression. Since we have shown that c-Myc can also bind to the Pax-3 E box motif, albeit with lower affinity than N-Myc-Max heterodimers, we next investigated whether Pax-3 mRNA levels were increased in cell lines ectopically expressing c-Myc. However, despite repeated attempts, we failed to produce cell lines constitutively overexpressing c-Myc. However, ND7 cell lines expressing c-MycER, a conditional form of c-Myc, were established (Fig. 6), and these cell lines were then examined to determine whether c-Myc activation induced by the addition of OHT led to an increase in Pax-3 mRNA levels. We found that in the activated c-MycER cell lines, Pax-3 mRNA levels did increase (Fig. 6B), supporting the hypothesis that Pax-3 expression is regulated by members of the Myc family of transcription factors *in vivo*.

Pax-3 mRNA Expression in Neuronal Cells Is Cell Cycle-dependent—Pax-3 mRNA expression was also examined under conditions where the expression of Myc proteins is known to be modulated. Both c-Myc RNA and protein levels are known to oscillate during the cell cycle (35); we therefore compared the levels of Myc expression and the levels of Pax-3 mRNA during the different phases of the cell cycle in the ND7 cell line. To do this, ND7 cells were synchronized by serum starvation for 24 h. The cells were then released from this cell cycle block by the

addition of serum back to the cells. Samples were taken at 0, 1, 3, 6, 12, and 18 h after the addition of serum back to the cells and analyzed for DNA content by flow cytometry, analyzed for c-Myc and N-Myc protein expression by Western blot analysis, and analyzed for Pax-3 mRNA level by RT-PCR (Fig. 7). We found that in growth-arrested ND7 cells, no expression of N-Myc or c-Myc protein could be detected (Fig. 7). However, within 1 h of the addition of serum to the growth-arrested cells, levels of N-Myc and c-Myc protein rose, and the expression of both N-Myc and c-Myc peaked at 3 h after serum addition, before progressively declining through S phase and G₂/M (Fig. 7A). Interestingly, Pax-3 mRNA levels also oscillated through the cell cycle. No expression of Pax-3 mRNA was detected in the growth-arrested cells; however, within 1 h of the addition of serum, low levels of Pax-3 mRNA were detected, and by 6 h after serum addition, high levels of Pax-3 mRNA were detected. Levels of Pax-3 mRNA levels then subsequently declined as the majority of the cells entered S phase (Fig. 7B). Interestingly, the peak in Pax-3 expression lagged behind the peak in N-Myc and c-Myc protein expression by ~1–3 h, which would be consistent with Pax-3 expression being regulated by N-Myc or c-Myc.

Mutation of the Myc E Box Site Changes Pax-3 Cell Cycle Expression—To determine whether members of the Myc family of transcription factors are responsible for the cell cycle-dependent expression of Pax-3 mRNA, the 5' promoter region (bp –1578 to +56) of the Pax-3 gene was fused upstream of the reporter gene luciferase in the vector pGL3Basic. ND7 cells were transiently transfected with this construct (Pax3-Luc) together with pCMV-β-gal and synchronized by serum starvation. The transfected cells were then released from their cell cycle block by the addition of serum, and samples were taken at 0, 1, 3, 6, 12, 18, and 24 h after serum addition and analyzed for luciferase activity, β-galactosidase activity, and DNA content. We found, as shown in Fig. 8, that the expression of Pax3-Luc peaked 6 h after serum addition, before expression decreased

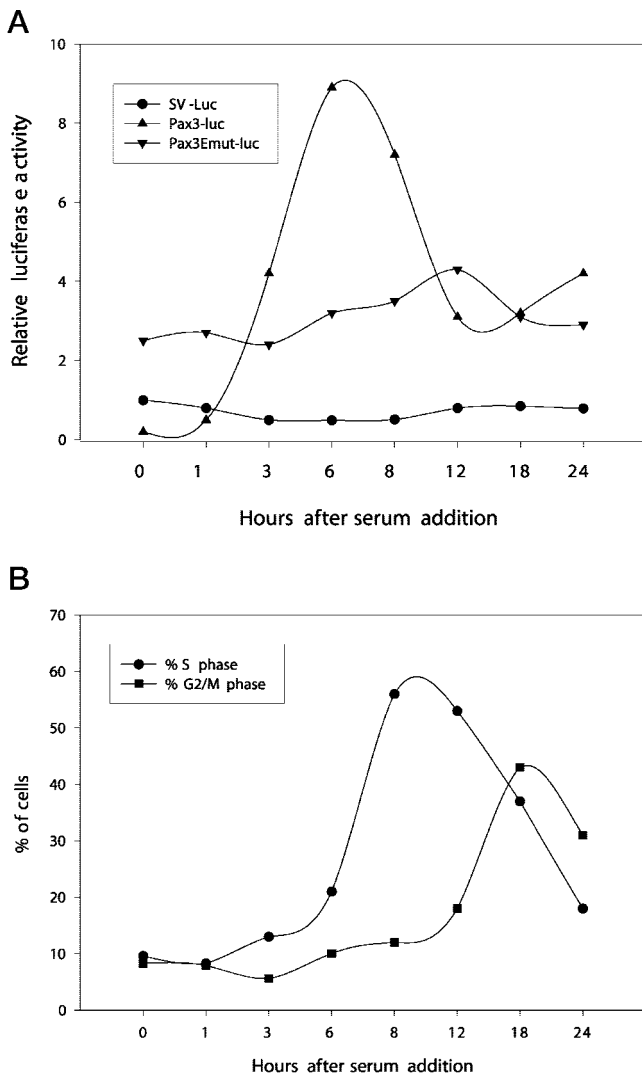


FIG. 8. Induction of the Pax-3 promoter through the cell cycle. The reporter plasmids Pax3-Luc and Pax3Emut-Luc containing either the wild type or mutant E box site, respectively, were transfected with pCMV- β -gal into ND7 cells. The cells were placed in 0% serum for 24 h after the removal of the calcium phosphate precipitates. The cells remained in 0% serum media for 24 h to induce quiescence, at which point serum was added back to the cells (time 0). At the indicated time points, cells were removed for cell cycle analysis by flow cytometry and for the determination of luciferase and β -galactosidase activity. A, luciferase values for a representative experiment (normalized for β -galactosidase activity). The reporter plasmid SV-Luc was also analyzed as a control. B, percentage of cells in S phase and G₂/M phase at the indicated time points.

through S phase. This pattern of cell cycle expression corresponds precisely with the time course of expression of endogenous Pax-3 mRNA during the cell cycle and suggests that all of the necessary elements involved in the cell cycle expression of Pax-3 are located within the region bp -1578 to +56 of the Pax-3 promoter. In contrast, no change was observed in the expression of pSV-Luc, as previously reported by Slansky *et al.* (36) (Fig. 8A) or in pGL3basic expression (data not shown). To determine whether the E box Myc sequence at -110 bp of the Pax-3 promoter is required for the cell cycle expression of the reporter construct Pax3-Luc, the Pax-3 promoter bearing the mutant E box site was fused upstream of the luciferase gene in pGL3Basic, and the expression of this construct was compared with the wild type promoter through the different phases of the cell cycle. Interestingly, we found that the level of luciferase activity in the growth-arrested cells transfected with

the mutant Pax-3 promoter was higher than that observed in cells transfected with the wild type promoter, suggesting that the E box site in the wild type promoter has an inhibitory effect on Pax-3 expression in growth-arrested cells. We also found that the expression of the mutant Pax-3 promoter construct did oscillate during the cell cycle (Fig. 8A); however, both the level of induction and the timing of expression were different in cells transfected with the mutant promoter compared with the wild type promoter. In cells transfected with the mutant promoter, a small peak in luciferase activity occurred 10–12 h after the addition of serum, coinciding with the onset of S phase. This compares with a peak in luciferase activity 6 h after the serum addition in cells transfected with the wild type promoter. These results suggest that the E box binding site located at -110 bp upstream of the transcription start site in the Pax-3 promoter plays an important role both in the repression of Pax-3 expression in growth-arrested cells and in the induction of Pax-3 expression in the G₁ phase of the cell cycle.

DISCUSSION

In this report, we have identified the developmental control gene Pax-3 as a novel transcriptional target of the Myc family of transcription factors. We have shown in transient co-transfection assays that both N-Myc and c-Myc enhanced the activity of the Pax-3 promoter by 18- and 8-fold, respectively. The transcriptional activation of the Pax-3 promoter was further enhanced by the co-transfection of N-Myc and c-Myc with their dimerization partner Max. The ability of N-Myc or c-Myc to activate the Pax-3 promoter in the absence of transfected Max probably reflects the fact that NIH3T3 cells express endogenous Max, which may form heterodimers with the transfected Myc proteins. The transfection of Max, in the absence of N-Myc or c-Myc, in contrast, led to a small reduction in Pax-3 promoter activity. Since Max proteins have been reported not to interact with the Sin3 transcriptional repressor proteins (37), Max probably inhibits Pax-3 promoter activity by competing for the Pax-3 E box site with endogenous Myc-Max heterodimers.

The Myc-Max-Mad network of transcriptional regulators has been shown to modulate gene expression through a subset of E box motifs, which have the core consensus sequence CACGTG (9, 10). Most of the Myc specific E box elements identified to date are found downstream of the transcription initiation site either within the first or second intron or in the 5'-untranslated region or within the protein coding sequence (15, 16, 38, 39). Only the *EIF-4E* (40), *LDH-A* (41), and *ISGF3 γ* (17) genes contain E box sites within the 5' promoter region. Deletion and mutagenesis experiments have shown that the Pax-3 gene contains an inverted E box sequence CGCGTG (CACGCG) in the 5' promoter region that is responsible for Myc regulation of Pax-3 promoter activity. This inverted E box sequence differs by 1 bp from the consensus E box sequence CACGTG. However, we found that both c-Myc-Max and N-Myc-Max complexes bind to the Pax-3 E box site. This is consistent with site selection studies by Blackwell *et al.* (42), who found that c-Myc-Max heterodimers could bind *in vitro* to a set of variant E box sites including CACGCG, CATGTG, CATGCG, CACGAG, and CAACGTG, which are referred to as noncanonical E box sequences. Although c-Myc-Max complexes were shown in general to bind with lower affinity to these noncanonical sites than to the consensus site, Blackwell *et al.* (42) did show that c-Myc-Max complexes bound to the noncanonical site CACGCG almost as well as to the Myc E box consensus site. Furthermore, chromatin immunoprecipitation experiments have revealed that the most common *in vivo* Myc binding sites are noncanonical E box sequences (39). Grandori *et al.* (39) also demonstrated that noncanonical E box sequences could, when cloned in a forward or reverse orientation upstream of the thymidine ki-

nase promoter, confer Myc-dependent transcription on a reporter gene.

In ND7 cells, DNA binding studies showed strong binding to the Pax-3 E box motif. Moreover, E box binding was reduced by antibodies directed against N-Myc, c-Myc, and Max, suggesting that in ND7 cells, the E box motif is bound by a combination of N-Myc-Max and c-Myc-Max. Both N-Myc and c-Myc are expressed in ND7 cells. Interestingly, the level of Myc-Max binding to the E box motif decreased in nondividing ND7 cells. This is consistent with previous reports that show that N-Myc and c-Myc expression is confined to mitotically active cells. Interestingly, however, in serum-starved cells, we could not detect any Mad/Max binding to the E box site. This may be due to the very low activity of Mad in serum-starved ND7 cells or due to the fact that Mad-Max complexes are distributed into many distinct complexes in nondividing cells, which might not be detected individually (34). This suggestion is based on the findings that Mad1 interacts with Sin3 proteins *in vivo* and Sin3 binds to a number of additional proteins such as histone deacetylases and NCo-R.

Studies by Malynn *et al.* (43) have demonstrated a major degree of functional redundancy between the N-Myc and c-Myc proteins. They were able to show that mice (c-myc^{n/n}) in which the c-myc coding sequences had been replaced with N-myc coding sequences survived into adulthood and reproduced. However, there were differences between c-myc^{n/n} mice and controls; the overall survival of postnatal c-myc^{n/n} mice was modestly reduced compared with wild type, the average weight of c-myc^{n/n} mice was also lower than controls, and dystrophy of skeletal muscles was observed in a subset of c-myc^{n/n} mice. These differences observed between c-myc^{n/n} mice and wild type mice suggest that subtle differences between N-Myc and c-Myc exist. This is consistent with the fact that although there is considerable amino acid sequence homology between N-Myc and c-Myc, there are regions unique to N-Myc. Interestingly, we found that N-Myc-Max complexes bound with higher affinity to the Pax-3 E box site than c-Myc-Max. Differences in DNA binding specificity between c-Myc-Max and N-Myc-Max has been previously reported by Prochownik and VanAntwerp (44), who demonstrated that c-Myc-Max and N-Myc-Max exhibited distinct preferences for 5'- and 3'-flanking dinucleotides. N-Myc-Max bound preferentially to the core consensus when flanked at the 5'-end by GA and at the 3'-end by CT, whereas c-Myc-Max preferred AC at the 5'-end and GT at the 3'-end (44). Interestingly, the Pax-3 E box site is flanked by a 5'-GA and a 3'-CT, the dinucleotides preferred by N-Myc-Max. It will be important to determine whether *in vivo* the E box motif in the Pax-3 promoter is bound preferentially by N-Myc-Max or c-Myc-Max. A number of other potential N-Myc target genes have been identified. These include *activin A* (45), *NCAM* (46), *Ndr1* (47), and insulin-like growth factor receptor (48); however, the sequence motifs responsible for N-Myc-dependent regulation of these genes have yet to be identified.

The finding that Myc-Max heterodimers can bind to the E box site in the Pax-3 promoter at least *in vitro* raises the possibility that Pax-3 expression *in vivo* may be regulated by members of the Myc family of transcription factors. In support of this hypothesis, we have shown that Pax-3 mRNA expression was elevated in a number of NMYC-amplified neuroblastoma cell lines. The elevation of Pax-3 mRNA expression in neuroblastoma cell lines with an amplified NMYC gene is intriguing; however, only a limited number of neuroblastoma cell lines have been examined, and it will be important to determine the extent of this correlation and the contribution that Pax-3 makes to the pathogenesis of neuroblastoma by examining Pax-3 mRNA levels in a large series of primary neuroblastoma

cells. It is interesting to note, however, that Pax-3 mRNA has also been reported in other neural crest lineage tumors including melanomas (49, 50), where Pax-3 expression has been shown to be essential for tumor cell survival (50). However, whether amplification of Myc in these tumors correlates with increased Pax-3 expression remains to be determined. In our experiments, we also detected elevated levels of Pax-3 mRNA in a series of cell lines ectopically expressing N-Myc. Interestingly, attempts to create ND7 cell lines ectopically expressing c-myc from a constitutive promoter consistently failed. Cell lines were produced, however, using the conditional form of c-Myc, cMycER. Moreover, the activation of c-MycER by OHT led to increased Pax-3 mRNA expression. This suggests that the ectopic expression of c-Myc, like N-Myc, can induce increased expression of Pax-3 mRNA.

If Pax-3 is an *in vivo* target gene of the Myc family of transcription factors, then Pax-3 expression should not only be elevated in cell lines overexpressing Myc, but Pax-3 mRNA levels should also be modulated under conditions where expression of Myc is altered. We found that Pax-3 mRNA expression did oscillate during the cell cycle, as does N-Myc and c-Myc expression. Moreover, whereas a peak in c-Myc and N-Myc expression was observed 3 h after the addition of serum, early in G₁, a peak in Pax-3 mRNA levels was observed 6 h after serum addition. This lag between the increase in Myc expression and Pax-3 expression is consistent with the suggestion that Pax-3 is a target gene for the Myc family of transcription factors and with previous reports that show that the expression of other Myc target genes such as *cad* (15) are also induced like Pax-3 in late G₁. Using reporter constructs, we were also able to show that all of the necessary cis-acting elements required for Pax-3 cell cycle expression are contained within the 5' region of the Pax-3 promoter from bp -1578 to +56. Interestingly, mutation of the E box site within the Pax-3 promoter significantly altered the pattern of expression of the reporter construct in cycling cells. In cells transfected with the mutant promoter, a peak in luciferase activity was not observed until S phase. Furthermore, the magnitude of the peak was much smaller than observed with the wild type promoter, suggesting that Myc binding proteins play an important role in modulating the cell cycle-dependent expression of Pax-3. We also observed that the level of expression of the mutant Pax-3 promoter was substantially higher than the wild type promoter in the growth-arrested cells. This finding implies that the E box motif in the Pax-3 promoter is bound by inhibitory factors in growth-arrested cells. Such E box binding inhibitory factors may comprise the Mad family of proteins, which have been shown to be highly expressed in growth-arrested cells and may therefore play a role in the down-regulation of Pax-3 expression in nondividing cells. Together, these data suggest that Pax-3 cell cycle expression is modulated through the noncanonical E box motif in the promoter of Pax-3; however, further experiments that show that dominant negative myc mutants (51, 52) abolish Pax-3 cell cycle-regulated expression are required to directly implicate Myc in the cell cycle-dependent expression of Pax-3.

What is the functional significance of Myc regulation of Pax-3 expression? Pax-3 has been suggested to play roles in cell proliferation, differentiation, and/or in cell migration. The finding that Pax-3 expression is modulated by the Myc and the observation that Pax-3 expression is cell cycle-regulated further support a role for Pax-3 in early proliferation events during embryogenesis. The link between Pax-3 and the cell cycle is intriguing, since recent studies by O'Wiggin *et al.* (53) have shown that Pax-3 strongly interacts with the gene product of the retinoblastoma tumor suppressor gene pRB (53). The pRB

family of proteins, which include pRB, p107, and p130, are negative regulators of cell cycle progression. In quiescent cells, pRB is unphosphorylated and interacts with the transcription factor E2F. This interaction inhibits the transcription of E2F-responsive genes, which are essential for cell cycle progression (for a review, see Ref. 54). When cells are stimulated with serum, pRB is phosphorylated and releases free E2F, which is then able to activate its target genes and promote cell cycle progression. Pax-3 also preferentially associates with the unphosphorylated form of pRB, and this association between Pax-3 and pRB, as with E2F, inhibits the ability of Pax-3 to activate its target genes. Thus, both Pax-3 mRNA expression and transactivation ability appear to be cell cycle-dependent, strongly suggesting a role for Pax-3 in cell cycle progression and/or in coordinating cell proliferation and differentiation events.

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