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Sequence-Specific Transcriptional Activation by Myc and Repression by Max

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The c-Myc oncoprotein, which is required for cellular proliferation, resembles in its structure a growing number of transcription factors. However, the mechanism of its action in vivo is not yet clear. The discovery of the specific cognate DNA-binding site for Myc and its specific heterodimerization partner, Max, enabled the use of direct experiments to elucidate how Myc functions in vivo and how this function is modulated by Max. Here we demonstrate that exogenously expressed Myc is capable of activating transcription in vivo through its specific DNA-binding site. Moreover, transcriptional activation by Myc is dependent on the basic region, the integrity of the helix-loop-helix and leucine zipper dimerization motifs located in the carboxy-terminal portion of the protein, and the regions in the amino terminus conserved among Myc family proteins. In contrast to Myc, exogenously expressed Max elicited transcriptional repression and blocked transcriptional activation by Myc through the same DNA-binding site. Our results suggest a functional antagonism between Myc and Max which is mediated by their relative levels in the cells. A model for the activity of Myc and Max in vivo is presented.

The c-*myc* gene encodes a short-lived nuclear phosphoprotein which is implicated in the normal control of cell growth and cellular differentiation (for reviews see references 11, 27, and 39). Deregulation of c-*myc* expression is associated with the genesis of diverse types of tumors, and enforced high levels of c-*myc* expression elicit transformation of established cell lines or of rat embryo cells, in cooperation with a mutant version of c-H-Ras (11, 27, 38). Despite much data suggesting the importance of Myc in these events, little is known about its mechanism of action. Its nuclear localization and apparent structural similarity to other transcription factors suggest that it may act as a regulator of transcription of other genes (for reviews, see references 13 and 27). The Myc protein contains a DNA-binding basic domain (b) coupled to a helix-loop-helix (HLH) motif adjacent to a leucine zipper (LZ) region; both the HLH and LZ domains are characteristic of protein dimerization domains (25, 30). Two domains in the amino-terminal region of the protein and the b-HLH-LZ domains in the carboxy-terminal region of the protein are conserved across species and among the N-, L-, and c-*myc* gene products (13). The conserved domains in the amino- and carboxy-terminal portions of the protein are absolutely required for transformation (32a, 40), and the amino-terminal region of Myc was shown to be capable of activating transcription when fused to the DNA-binding domain of GAL4, a yeast transcriptional activator (22). Similarly, a LexA-Myc fusion protein also activated transcription of a LexA operator-target gene in yeast cells (26). Identification of the specific DNA-binding site for Myc (7, 20, 24, 34) has allowed additional speculation that indeed Myc can function as a sequence-specific transcriptional regulator. In vitro DNA-binding assays showed that Myc cannot readily form homodimers that bind the specific cognate site (5, 7, 8, 23, 24). This DNA-binding activity is significantly enhanced, however, by the addition of Max, the specific heterodimerization partner of Myc, which was identified on the basis of its ability to dimerize with Myc and on the basis of its similarity to Myc in its b-HLH-LZ domains (8, 33).

Max, unlike Myc, can readily form homodimers that bind specifically to the same DNA-binding site (5, 9, 23, 33). Fusion of Max to the DNA-binding domain of GAL4, however, did not elicit transcriptional activation (23). Furthermore, Max is expressed in quiescent and differentiated cells that do not express Myc (9, 33, 43), and unlike Myc protein and RNA, Max protein and RNA are stable (9, 43). Taken together, these results suggest that Max by itself has a separate function that antagonizes Myc function in vivo. In this report, we show that overexpressed Myc activates transcription in vivo through its cognate binding site, whereas overexpressed Max represses transcription through the same site.

MATERIALS AND METHODS

Plasmid vectors. Plasmid -36PrlCAT, containing 36 bp upstream of the initiation site for the rat prolactin promoter, is derived from the previously described plasmid -36PrlLUC (31). Briefly, the luciferase gene was eliminated by digestion of -36PrlLUC with *Eco*RI and *Hin*dIII. *Eco*RI-*Bgl*II-*Hin*dIII linkers were ligated to the linearized vector, subjected to *Hin*dIII-*Bgl*II digestion, and ligated to the *Hin*dIII-*Bam*HI fragment of pSV2CAT, containing the chloramphenicol acetyltransferase (CAT) gene (17). A multiple cloning site (MCS) from pSP72 (Promega) was inserted immediately upstream of the minimal prolactin promoter between the *Xho*I and *Bam*HI sites (31). Double-stranded oligonucleotides containing various DNA-binding sites were inserted into the *Cla*I site of the MCS. The sequence of the oligonucleotide containing the "E box, Myc site" (EMS) is 5′-CGCCCGGACCACGTGGTCCCTAC-3′; the sequence of the oligonucleotide containing the USF-binding site is 5′-CGGTAGGCCACGTGACCGGG-3′; the sequence of the

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TFE3-binding site is 5′-CGCCTTGCCACATGACCTGC-3′; and the sequence of the oligonucleotide containing the mutated EMS (mut-EMS) is 5′-CGCCCGGACCAAGTG GTCCTAC-3′. In the reporter plasmids used in these studies, the double-stranded oligonucleotides were inserted as tandem head-to-tail repeats oriented as indicated above.

The expression vector for human c-*myc*, pM21, and the human c-*myc* mutants used in this work were previously described (32, 32a, 40). It should be noted that all of the Myc mutants used in this work were previously used to study transformation activity, and no significant change in the expression or the stability of these mutant proteins was detected (32, 32a, 40).

Expression vectors for the human *max* gene and *max* mutants were constructed by cloning the cDNA for human *max* (43) into the MCS of the simian virus 40 (SV40) expression vector pSV7d. Max carboxy-terminal deletion mutant D112-160 was constructed by digesting pBSMax6, a plasmid containing the full-length coding region of the human *max* gene (43), with *Pst*I, followed by insertion of oligonucleotides containing stop codons in three frames. The amino-terminal deletion mutant D1-18 was constructed by converting amino acid 17 from arginine to methionine and amino acid 18 from phenylalanine to valine to create an optimal translation initiation site, using the polymerase chain reaction (PCR). The 5′ primer had the sequence 5′-GAA GAG CAA ACC ATG GTT CAA TCT GCG-3′ (changed nucleotides are indicated in bold type, and the converted amino acid codons are underlined). The 3′ primer and PCR conditions were previously described (43). The PCR product was subcloned into the *Eco*RV site of plasmid pBSII SK(+) (Stratagene) to create pBSMax(D1-18), in which the original initiation methionine was eliminated. Max-VP16 was constructed by linking in frame the acidic carboxy-terminal domain (78 amino acids) of the herpes simplex virus type 1-encoded protein VP16 (41) to the *Pst*I site in the carboxy-terminal region of human *max* cDNA. Plasmid pSJT1193-CRF1, containing the carboxy-terminal domain of VP16, was kindly provided by S. Triezenberg. The Max mutations and the Max-VP16 fusion were verified by sequencing.

In vitro transcription and translation. RNA was transcribed as previously described (21a) from 5 μg of linearized plasmids, using T7 or T3 RNA polymerase. Translation was carried out as previously described (21a), using 35 μl of micrococcal nuclease-treated rabbit reticulocyte lysate (Promega) and approximately 5 μg of in vitro-transcribed RNA.

DNA binding assays of in vitro-translated proteins. The mobility shift DNA binding assays were carried out as previously described (21a). The in vitro-translated proteins (5 μl of the programmed reticulocyte lysate) were preincubated in buffer D (21a) in the presence of 1 μg of poly(dI-dC) for 10 min at 4°C. Double-stranded oligomer (0.2 ng), end labeled with ^{32}P, was added, preincubation at 4°C was continued for an additional 5 min, and then the mixture was incubated for 20 min at room temperature. Protein-DNA complexes were resolved on 5% polyacrylamide gels in 0.5× Tris-borate-EDTA.

Cell culture and transfection. LTK$^-$ cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Cells were transfected by the calcium phosphate method (18). For each transfection, LTK$^-$ cells were plated at 5×10^5 cells per 100-mm-diameter dish. On the following day, the medium was replaced with 9 ml of fresh medium, and 1 ml of calcium phosphate-precipitated DNA was distributed over the cells. After 12 to 18 h of exposure to precipitate, cells were washed with phosphate buffered saline and refed with complete medium. Cells were harvested 48 h after addition of the DNA, and extracts were assayed for CAT activity (17). One-third of the cell extract from one 100-mm-diameter dish was incubated for 8 h at 37°C to assay for CAT activity. This prolonged incubation was required since the basal activity of the minimal prolactin promoter is very low (see reference 31 and references therein). All transfections were repeated at least three times. For normalization of transfection efficiencies, the expression plasmid CMVβgal (2 μg) was included in the cotransfection, after which β-galactosidase activity in the cell extracts was measured (36). No transactivation of plasmid CMVβgal by Myc was observed.

RESULTS

Sequence-specific transcriptional activation by Myc in LTK$^-$ cells. Despite several lines of evidence suggesting that Myc may act in vivo as a transcriptional regulator (for reviews, see references 13 and 27), to date there is no direct evidence that Myc modulates transcription in vivo through its cognate specific DNA-binding site. To study possible sequence-specific transcriptional activation by Myc, we used a transient transcriptional activation assay. Two reporter plasmids that contain tandem repeats of the core Myc-binding site, CACGTG (7, 20, 24, 34), upstream of the minimal rat prolactin promoter linked to the CAT gene were used in this assay (Fig. 1A; see Materials and Methods). The first reporter plasmid, (EMS)PrlCAT, has two tandem repeats of the high-affinity Myc-binding site, termed EMS. This site has the core sequence of the Myc-binding site flanked by palindromic sequences and was shown to bind Myc in vitro with the highest affinity (20). The second plasmid, (USF)PrlCAT, contains three tandem repeats of the USF DNA-binding site from the adenovirus major late promoter that has the core sequence of the Myc site (10, 19, 37). The third reporter plasmid, (μE3)PrlCAT (Fig. 1A), contains three tandem repeats of the TFE3-binding site from the μE3 region of the immunoglobulin heavy-chain enhancer (3, 4, 35). This site, which has the sequence CACATG with one mismatch to the core sequence of the Myc-binding site, was previously shown not to bind Myc in vitro (33). The reporter plasmids were transfected into LTK$^-$ cells with increasing amounts of human c-*myc* expression vector (32a, 40), and the transcriptional activity of the promoter in the reporter plasmids was monitored by the CAT assay (17). Insertion of the EMS, USF, or μE3 sites upstream of the minimal prolactin promoter elicited significant CAT activity even in the absence of exogenously expressed Myc, whereas the PrlCAT plasmid alone did not (Fig. 1B; see also Fig. 3B, 3D, 4C, 4D, and 5A). This activity was also observed in transient transfections in serum-starved cells in which endogenous Myc is expressed at very low levels (2). Therefore, it is likely that this activity is a result of transactivation by ubiquitously expressed endogenous USF, TFE3, TFEB, or similar proteins which can bind the core Myc-binding site as was previously observed (3, 4, 8, 16, 19, 33–35).

Cotransfection of the reporter plasmid (EMS)PrlCAT or (USF)PrlCAT with increasing amounts of Myc expression vector led to a parallel increase in CAT activity (Fig. 1B). CAT activity was consistently greater with (EMS)PrlCAT as a reporter plasmid than when (USF)PrlCAT was used; thus, (EMS)PrlCAT was used as a reporter plasmid in all subsequent experiments. [For typical results obtained from multiple experiments using (EMS)PrlCAT, see Fig. 5A.] This

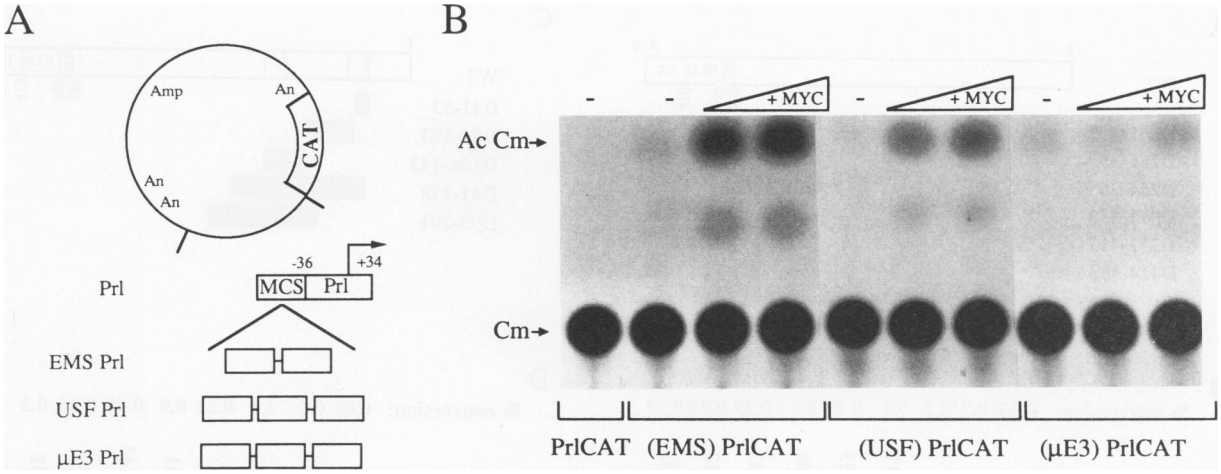

FIG. 1. Sequence-specific transcriptional activation by Myc. (A) Reporter plasmids used in the transient transfection assays. Plasmid -36PrlCAT containing the minimal rat prolactin promoter, which includes 36 bp upstream of the transcription start site, was used as a basic reporter plasmid (31). Tandem DNA-binding sites were inserted immediately upstream of the minimal prolactin promoter at the *Cla*I site within the MCS. Three reporter plasmids were constructed. (EMS)PrlCAT contains two tandem sites of the high-affinity EMS (20), (USF)PrlCAT contains three tandem USF DNA-binding sites from the adenovirus major late promoter (10, 19, 37), and (μE3)PrlCAT contains three tandem repeats of the TFE3-binding sites from the μE3 region in the immunoglobulin heavy-chain enhancer (3, 4, 35). (B) Effects of Myc overexpression on transcriptional activities of the reporter plasmids, as measured by the CAT assay. Increasing amounts (7 and 21 μg) of human c-*myc* expression vector pM21 under the control of the MLV long terminal repeat (32a, 40) were cotransfected with 5 μg of reporter plasmid into LTK$^-$ cells. The total amount of MLV vector in each transfection was kept constant at 21 μg by addition of MLV vector alone. As a control for efficiency of transfection, plasmid CMVβgal (2 μg) was included in each transfection, after which β-galactosidase activity in the cell extracts was measured. The positions of the nonacetylated (Cm) and acetylated (Ac Cm) forms of chloramphenicol are indicated.

transactivation is sequence specific, since only very low level transactivation of the (μE3)PrlCAT reporter plasmid was observed when high levels of Myc expression vector were cotransfected (Fig. 1B). While this work was in progress, it became apparent that an amino-terminally truncated Myc protein can also bind the core sequence of the μE3 site, CACATG, in vitro (1, 6, 23) but with a lower affinity than it can bind the CACGTG core sequence (6). Therefore, it is possible that the weak activity mediated by the μE3 site at high levels of Myc expression reflects weak binding of Myc to this site in vivo. To test this possibility and to establish that transcriptional activation by Myc is indeed sequence specific, another reporter plasmid, (mut-EMS)Prl CAT, was constructed. This plasmid contains two tandem repeats of the EMS with one point mutation in the core sequence (CAAGTG instead of CACGTG; see Materials and Methods). Insertion of two tandem mut-EMS upstream of the minimal prolactin promoter did not elicit any significant increase in CAT activity over the basal level of the minimal promoter (21), and no further activation by coexpression of Myc was observed (Fig. 2). Once again we observed a relatively weak activation by Myc mediated through the μE3 site in comparison with the activation mediated through the EMS (Fig. 2). Taken together, these results, in conjunction with the in vitro DNA-binding properties of Myc, suggest that Myc can bind its cognate DNA site in vivo and activate transcription through this site.

Sequence-specific transcriptional activation by Myc is dependent on the integrity of the b-HLH-LZ domain and on amino-terminal domains. To characterize domains in Myc protein that determine its sequence-specific transcriptional activation activity, various deletion mutants (32a, 40) of the protein were used in transient transfection assays with (EMS)PrlCAT. We first tested mutants with deletions in the carboxy terminus of Myc (Fig. 3A) for the ability to transactivate transcription. Mutant D265-317, which contains a deletion in the nonspecific DNA-binding domain (amino acids 265 to 317) (14), was still able to transactivate; interestingly, we reproducibly found higher levels of activation by this mutant in comparison with wild-type (WT) Myc (Fig. 3A and B). Deletion of amino acids 265 to 353 (D265-353), which deletes a nuclear localization signal (40), reduced activation to about 60% of that observed with WT, while deletion of the basic domain adjacent to the HLH domain (amino acids 265 to 367) abolished transactivation (Fig. 3A and B). This basic region is presumed to be the domain required for specific contact to DNA (7, 8, 33, 34). Mutants

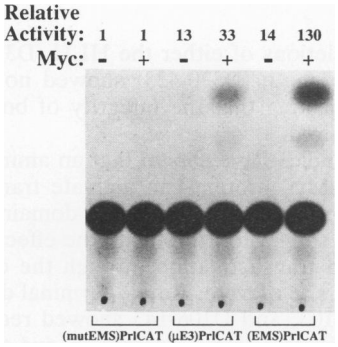

FIG. 2. Inability of a mutated EMS to mediate transcriptional activation by Myc. The effects of Myc overexpression on transcriptional activities of the reporter plasmids were measured by a CAT assay in which 25 μg of MLV vector (−) or 25 μg of the human c-*myc* expression vector pM21 (+) was cotransfected with 5 μg of reporter plasmid into LTK$^-$ cells. The reporter plasmid (mut-EMS)PrlCAT contains two tandem repeats of a mutated EMS as described in Materials and Methods. The reporter plasmids used and their relative activities in the absence or presence of overexpressed Myc are as indicated.

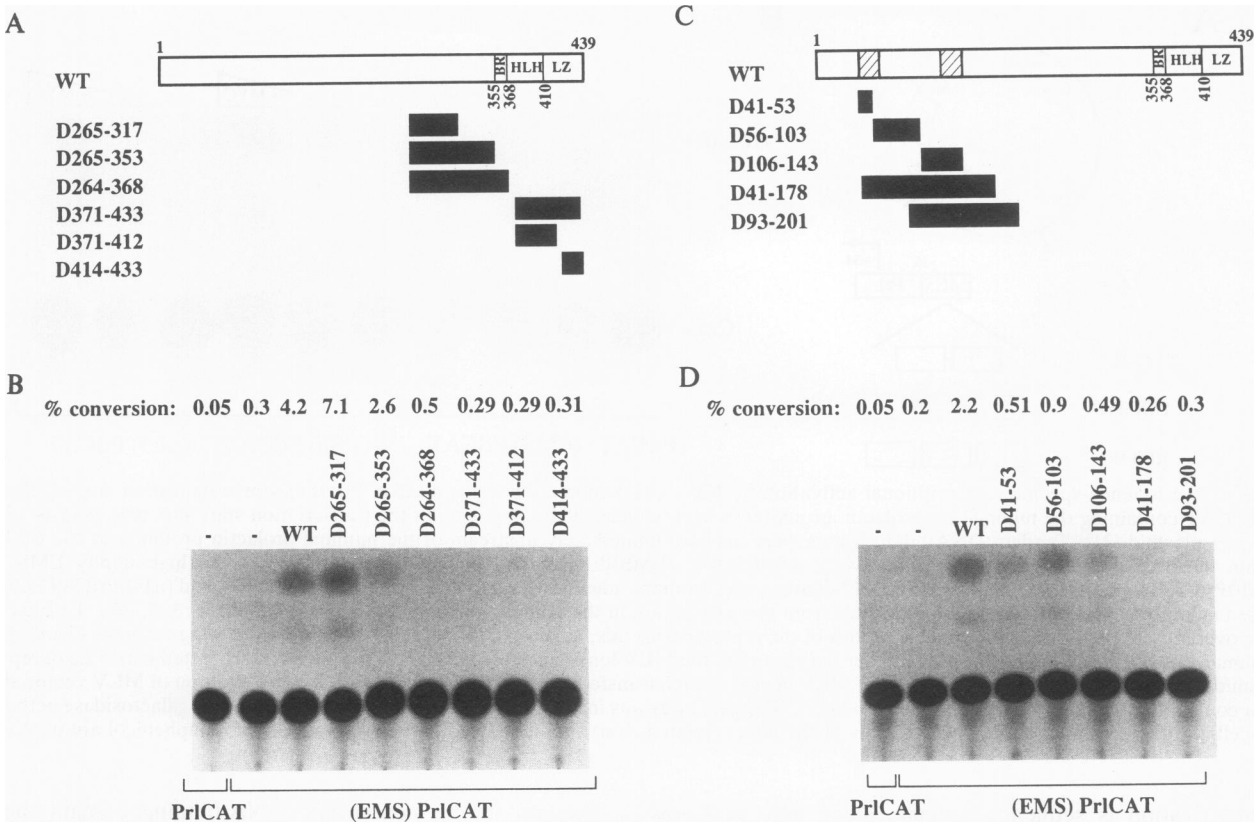

FIG. 3. Evidence that transcriptional activation by Myc is dependent on the basic region, the integrity of both the HLH and LZ dimerization domains, and the amino-terminal domains. (A) Schematic representation of c-Myc protein and carboxy-terminal deletion mutants. WT Myc protein is represented by an open rectangular box, and the basic (BR), HLH, and LZ regions are indicated. Regions that are deleted in mutant proteins are represented by filled boxes. These mutants were previously described and characterized with respect to transformation activity (32a, 40). (B) Transcriptional activity of the carboxy-terminal deletion mutants. Transcriptional activities of mutants depicted in panel A were measured by cotransfection of WT Myc or deletion Myc mutant expression vectors (20 μg) under the control of the MLV LTR with reporter plasmid (EMS)PrlCAT (5 μg), after which a CAT assay was performed as described in the legend to Fig. 1. Percent conversion to acetylated chloramphenicol as measured by liquid scintillation counting of the acetylated and nonacetylated forms isolated from the radioactive spots on the thin-layer chromatography plate is indicated. (C) Schematic representation of amino-terminal deletion mutants. Notation is as in panel A. The hatched boxes represent regions that are highly conserved in all Myc family genes. (D) Transcriptional activities of the amino-terminal deletion mutants shown in panel C, measured as described for panel B. Comparable results were obtained in three or more independent experiments.

containing deletions of either the HLH (D370-413), the LZ (D413-433), or both (D370-433) showed no transactivating abilities, suggesting that the integrity of both dimerization motifs is required.

Previous studies have shown that an amino-terminal portion of the c-Myc protein can activate transcription when linked in frame to the DNA-binding domain of GAL4 (22). We therefore sought to investigate the effect of deletions in this region on transactivation through the cognate specific DNA-binding site of Myc. Amino-terminal deletion mutants D41-53, D56-103, and D106-143 showed reduced transactivation by Myc to various extents in our transient assays (Fig. 3C and D). Reproducibly in multiple experiments, mutants D41-53 and D106-143 had about 25% of WT activity, whereas mutant D56-103 had 30 to 50% of WT activity. Deletion mutants D41-178 and D93-201 showed no transactivation capability.

Sequence-specific repression by Max. Max, the heterodimerization partner of Myc, was shown to form homodimers in vitro that bind specifically to the Myc DNA-binding site (5, 9, 23, 33). To assay the transcriptional activity of Max, increasing amounts of a plasmid containing the human *max* coding region under the control of the SV40 early promoter were cotransfected with the reporter plasmids. In contrast to the transactivation elicited by overexpression of Myc, overexpression of Max inhibited the basal transcription observed with the reporter plasmids containing the Myc-binding sites (Fig. 4C, 4D, and 5A). It should be noted that in order to demonstrate transcriptional repression, higher levels of reporter plasmids were used (10 and 15 μg instead of 5 μg; Fig. 4 and 5A). The levels of the Max expression vector required to elicit repression were much lower than the levels of Myc expression vector required to elicit activation (Fig. 1B, 4C, and 5A). This difference cannot be attributed to the usage of different expression vectors (SV40 early promoter versus the murine leukemia virus [MLV] long terminal repeat), since the two promoters are equally active in LTK^- cells (21), but instead is probably due to the greater stability of Max protein and RNA in comparison to Myc protein and RNA (9, 43). No significant repression by Max was observed when (μE3)PrlCAT was used as a reporter plasmid (2), suggesting that overexpres-

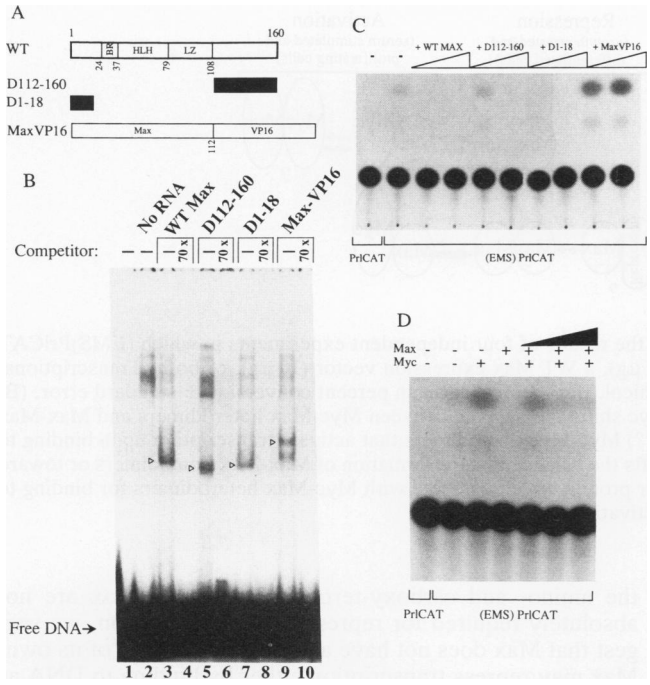

FIG. 4. Transcriptional repression by Max and its carboxy- and amino-terminal deletion mutants and transcriptional activation by Max-VP16. (A) Schematic representation of human Max protein, carboxy- and amino-terminal deletion mutants, and the Max-VP16 chimera. Open rectangular boxes represent WT Max protein and the Max-VP16 chimeric protein; filled boxes represent regions deleted in the mutants. Expression vectors used in transient transfection assays were constructed by subcloning of DNA fragments encoding the full-length Max protein and deletion mutants into the SV40 expression vector pSV7d. (B) Binding of in vitro-translated Max proteins to the EMS oligonucleotide. Double-stranded EMS oligonucleotide end labeled with ^{32}P was incubated with proteins synthesized in vitro and analyzed by the mobility shift DNA binding assay in the absence or presence of a 70-fold molar excess of unlabeled EMS oligonucleotide. Lanes: 1, free probe; 2, unprogrammed rabbit reticulocyte lysate; 3 and 4, WT Max; 5 and 6, Max(D112-160); 7 and 8, Max(D1-18); 9 and 10, Max-VP16. The specific protein-DNA complexes related to the in vitro-translated proteins are indicated by arrowheads. (C) Effects of WT Max, Max(D112-160), Max(D1-18), and Max-VP16 on the transcription activity of (EMS)PrlCAT. The reporter plasmid (EMS)PrlCAT (15 μg) was transfected into LTK$^-$ cells with increasing amounts (3 and 10 μg) of the expression vectors containing WT Max, the carboxy-terminal mutant Max(D112-160), the amino-terminal mutant Max(D1-18), or the chimeric gene Max-VP16. The total amount of the SV40 expression vector was kept constant at 10 μg by addition of plasmid pSV7d alone. (D) Evidence that overexpression of Max inhibits transactivation by Myc. The reporter plasmid (EMS)PrlCAT (10 μg) was transfected into LTK$^-$ cells with either a Myc expression vector (20 μg) alone, a WT Max expression vector (5 μg) alone, or a Myc expression vector (20 μg) together with increasing amounts of a Max expression vector (2, 5, or 15 μg). Comparable results were obtained in three independent experiments.

sion of Max mediates sequence-specific transcriptional repression and indicating that Max is not a general inhibitor of transcription.

To examine whether Max has a specific repression domain, deletion mutants of the carboxy- and amino-terminal regions were constructed (Fig. 4A). The constructed mutants of Max were first tested for the ability to bind specifically to the EMS oligonucleotide. In vitro-translated WT Max and mutants were subjected to a mobility shift DNA binding assay; as shown in Fig. 4B, all of the mutant proteins specifically bound to the EMS site in vitro. The mutants were then used in the transient transfection assay. Both the carboxy- and amino-terminal deletion mutants were able to repress transcription from (EMS)PrlCAT (Fig. 4C). Higher levels of the carboxy-terminal deletion mutant, however, were required to elicit repression in vivo (Fig. 4C). This requirement may be a result of deletion of a nuclear localization signal in the carboxy terminus of Max (23). The relatively small size of this mutant protein could allow entry to the nucleus by passive diffusion as a monomer (15), or the protein could be delivered to the nucleus by interaction with endogenous Max or Myc, but higher levels of the protein would probably be required for efficient delivery. Indeed, it was recently shown that a naturally occurring form of Max lacking the carboxy-terminal region can be localized to the nucleus in the presence of Myc (29). This assumption is also supported by our observation that replacing the carboxy terminus of Max with the transcriptional activation domain of the herpes simplex virus-encoded protein VP16 (41) converts Max to a transcriptional activator (Fig. 4C). Alternatively, the carboxy-terminal region of Max may be actively involved in mediating repression of transcription although it is not absolutely required.

Overexpression of Max inhibits transactivation by Myc. To determine the effect of Max overexpression on Myc-mediated transactivation, Myc and Max expression vectors were cotransfected with (EMS)PrlCAT. We found that increasing amounts of Max expression vector reduced the ability of Myc to transactivate transcription (Fig. 4D and 5A). Reduction in transcriptional activation by Myc was observed in LTK$^-$ cells even when relatively low amounts of Max expression vector were cotransfected with high amounts of Myc expression vector (Fig. 4D).

DISCUSSION

Our findings demonstrate that Myc can activate transcription in a sequence-specific manner. Furthermore, this transactivation is dependent on the specific DNA-binding basic region, the integrity of protein dimerization domains containing the HLH and LZ motifs, and the amino-terminal domains of Myc. Interestingly, deletions of the amino terminus which significantly reduce transactivation include one or two stretches of amino acids that are highly conserved among all members of Myc family genes (Fig. 3C and D) (13). One stretch of conserved amino acids (amino acids 44 to 64) is partially deleted in mutants D41-53 and D56-103, the second conserved stretch of amino acids (amino acids 128 to 144) is completely deleted in mutants D106-143 and D93-201, and both conserved regions are deleted in mutant D41-178. In previous studies by Kato et al. (22), in which the amino-terminal domains of Myc were tested as in-frame fusions with the GAL4 DNA-binding domain, deletion of amino acids 41 to 178 completely abolished the ability of the amino-terminal domain to transactivate transcription, similar to the results obtained in our studies. In both studies, deletion of the nonspecific DNA-binding domain of Myc (amino acids 265 to 317) augmented the level of transactivation. In contrast, deletion of amino acids 106 to 143 in the GAL4 studies did not affect transactivation, whereas the same deletion in the present studies, using the sequence-specific transcriptional activation assay, significantly reduced the transactivation capability of Myc (Fig. 3D). One possible explanation for this discrepancy is that in the

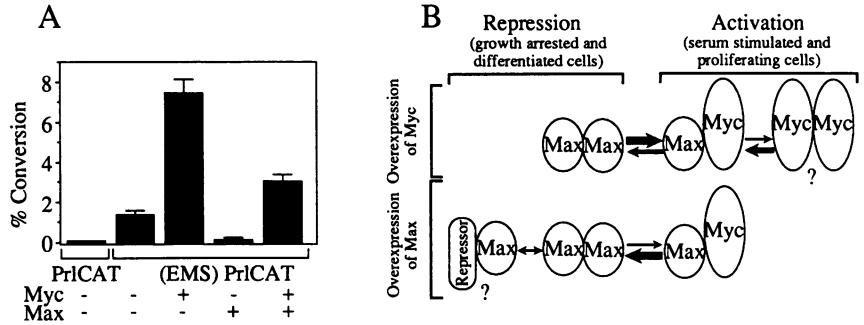

FIG. 5. Transcriptional activities of Myc and Max. (A) Summary of the results of four independent experiments in which (EMS)PrlCAT (10 μg) was cotransfected with either a WT Myc expression vector (20 μg), a WT Max expression vector (10 μg), or both. Transcriptional activity was measured by percent conversion to acetylated chloramphenicol. Bars represent mean percent conversion ± standard error. (B) Model for the activities of Myc and Max in vivo. Overexpression of Myc shifts the balance between Myc-Max heterodimers and Max-Max homodimers toward formation of Myc-Max heterodimers and possibly (?) Myc-Myc homodimers that activate transcription upon binding to their cognate DNA-binding sites. Overexpression of Max in the cells shifts the balance toward formation of Max-Max homodimers or toward formation of putative (?) complexes containing an unidentified repressor protein which compete with Myc-Max heterodimers for binding to the same cognate DNA-binding site and thus prevent transcriptional activation.

sequence-specific transcriptional activation assay, but not in the GAL4 assay, domains in the amino-terminal region of Myc may affect DNA-binding activity or dimerization (5, 23), which is prerequisite for DNA binding and transcriptional activation. Alternatively, it is possible that the integrity of multiple activation domains is required for full transcriptional activation, as was previously suggested (22).

In general, we found a direct correlation between transactivation and cellular transformation by Myc. That is, the amino- and carboxy-terminal domains of Myc that are required for transactivation in our assay have also been shown to be essential for DNA binding and transformation (8, 20, 32a, 40). This correlation, however, was not complete. Although amino-terminal mutants D56-103, D106-143, D41-178, and D93-201, which showed reduced or no transcriptional activation, are similarly unable to transform rat embryo cells in cooperation with EJ-Ras, deletion mutant D41-53, which had significantly reduced transcriptional activation capability in our assay, has been reported to have only a minimal decrease in transforming activity (32a, 40). Thus, further work is required to determine whether sequence-specific transcriptional activation by Myc is absolutely required for cellular transformation. It is still conceivable, however, that this one exception of complete correlation between transcriptional activation and transformation may reflect the use of different cell lines in the transformation assays and in the present studies of transactivation. As mentioned above (see Materials and Methods), all of the mutants used in these studies were previously used for the transformation assays, and their relative levels of expression were shown to be comparable in two cell lines, COS7 and Rat-1 (32, 32a, 40). Thus, although possible, it is unlikely that their relative expression will be different in LTK^- cells, and it is likewise unlikely that our results reflect differences in the level of expression rather than actual activity.

In contrast to Myc, overexpression of Max inhibits transactivation elicited both by ubiquitous transcription factors and by Myc (Fig. 4C, 4D, and 5A). In this respect, the effect of Max on transcriptional activation by Myc is similar to its effect on the transforming activity of Myc; coexpression of high levels of Max along with Myc in the Myc-Ras cotransformation assay was recently shown to inhibit the efficiency of transformation of rat embryo fibroblasts (29). Since both the amino- and carboxy-terminal regions of Max are not absolutely required for repression of transcription, we suggest that Max does not have a repression domain of its own. Max may repress transcription either by binding to DNA as a homodimer and thus blocking transcriptional activation, mediated by Myc and other activators that bind the same site, or alternatively by interacting with another protein that can actively repress transcription in cooperation with Max (Fig. 5B). In support of the first possibility (but without excluding the second) are the observations that Max binds its cognate DNA site in vitro as a homodimer (Fig. 4B and references 5, 9, 23, and 33) and that replacement of the carboxy terminus of Max with the transcriptional activation domain of VP16 converted Max to a transcriptional activator (Fig. 4A and C). This latter observation also suggests that Max can bind the same DNA site as does Myc in vivo.

Antagonism between Myc and Max was previously suggested on the basis of the expression of Max and its protein structure (12). This view is supported by the facts that unlike Myc protein and RNA, Max protein and RNA are stable, and that unlike Myc, Max is constitutively expressed and at relatively high levels in quiescent and differentiated cells (9, 33, 43). On the other hand, Max is required for Myc to bind DNA efficiently (5, 8, 23, 33) and thus is required for sequence-specific transcriptional activation by Myc. Although it seems that sequence-specific transcriptional activation by Myc homodimers is unlikely since Myc homodimers do not form at physiological Myc concentrations and do not avidly bind DNA (5, 23), we observed transactivation by Myc in the absence of a Max expression vector. It is possible, however, that this transactivation occurs by means of a Myc-Max heterodimer; because of the constitutive expression and stability of Max, and considering the comparable steady-state levels of Myc and Max RNAs in proliferating LTK^- cells (42), there is sufficient endogenous Max within the cells to dimerize with the exogenously expressed Myc. Similarly, overexpression of Max results in an excess of Max homodimers which compete with Myc-Max heterodimers for binding to the same DNA-binding site and thus block transcriptional activation (Fig. 4C and D).

Our results, in conjunction with results of others (8, 9, 23), led us to propose a model for the functional interactions of Myc and Max in vivo (Fig. 5B). According to this model, when Myc is expressed at high levels (in serum-stimulated or

proliferating cells), it forms heterodimers with the constitutively expressed Max and activates transcription. Conversely, when the steady-state level of Max is greater than that of Myc (in growth-arrested and differentiated cells), Max forms homodimers or heterodimers with a putative repressor and blocks or actively represses transcription. In addition to regulation at the level of expression, Myc and Max activity may be modulated in different physiological conditions by posttranslational modification. For example, both Myc and Max are targets for casein kinase II phosphorylation, and as was shown recently, phosphorylation of Max inhibits its ability to bind DNA as a homodimer (5).

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ADDENDUM IN PROOF

While this paper was in review, a paper describing similar results and reaching similar conclusions was published [L. Kretzner, E. M. Blackwood, and R. N. Eisenman, Nature (London) **359**:426–429, 1992].

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