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The ornithine decarboxylase gene is a transcriptional target of c-Myc

CONCHA BELLO-FERNANDEZ, GRAHAM PACKHAM, AND JOHN L. CLEVELAND*

Department of Biochemistry, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38105

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ABSTRACT **Constitutive c-*myc* expression suppresses cell cycle arrest, promotes entry into S phase, and results in the growth factor-independent expression of ornithine decarboxylase (ODC; EC 4.1.1.17). The ODC gene contains a conserved repeat of the Myc binding site, CACGTG, in intron 1. In this report, we demonstrate that c-Myc is a potent transactivator of ODC promoter–reporter gene constructs in fibroblasts that requires the CACGTG repeat. These sites conferred Myc responsiveness on heterologous promoter constructs, suggesting that ODC is regulated by Myc at the level of transcription initiation. Analysis of deletion and point mutants of c-*myc* revealed that domains required for transactivation of the ODC promoter did not include the leucine zipper of the Myc protein. This suggests that Myc may interact with transcription factors other than Max to transactivate the ODC gene.**

The c-*myc* protooncogene is a key regulator of cell growth and differentiation. c-*myc* expression is tightly associated with growth in multiple cell types and is universally induced by mitogens and down-regulated by growth-inhibitory agents (1). Removal of Myc protein appears necessary for withdrawal from the cell cycle, since enforced c-*myc* expression promotes cell cycle progression (2, 3). Myc-family proteins contain several motifs characteristic of known transcription factors, including proline-, glutamine-, and acid-rich regions, a basic–helix–loop–helix (B-HLH) motif, and a leucine zipper (LZ) (1). Myc has sequence-specific DNA-binding activity (4, 5) and binds with high affinity to the "E-box" motif, CACGTG, through its association with a B-HLH-LZ partner, Max (6). Moreover, c-Myc is capable of transactivating reporter genes having a repeated array of synthetic CACGTG motifs in mammalian cells (7). By contrast, Max functions as a repressor of transcription in these assays; repression can be relieved by cotransfection of c-*myc* expression vectors (7).

The identification of genes that are direct targets of Myc has remained elusive. We have reasoned that promotion of cell cycle progression by Myc is due to transcriptional activation of gene targets which are required for entry into S phase. One of the enzymes necessary for progression into S phase is ornithine decarboxylase (ODC; EC 4.1.1.17), the rate-limiting enzyme of polyamine biosynthesis. Specific inhibitors of ODC enzyme activity, or polyamine depletion, arrest cells in G_1 (8). Enforced c-*myc* expression in interleukin 3 (IL-3)-dependent murine myeloid cell lines results in constitutive, IL-3-independent, expression of ODC RNA (2, 9). Multiple copies of the ODC gene occur in mammalian genomes, but only one directs enzyme synthesis (10). The murine ODC gene contains two potential Myc binding sites, CACGTG, within the first intron (Fig. 1*A*). These binding sites are conserved in mammals (Fig. 1*B*). In this report we show that Myc is a potent transactivator of ODC transcription.

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MATERIALS AND METHODS

Cells. NIH 3T3 clone 7 fibroblasts were maintained as described (14).

Transfections and Chloramphenicol Acetyltransferse (CAT) Assays. Calcium phosphate-mediated transfections and CAT assays were performed by standard protocols. Transfections were normalized for total DNA content by using pUC18, and 10–20 μg of cytosolic extract was used for each CAT assay.

Promoter CAT Constructs and Expression Plasmids. The murine ODC-CAT gene fusion constructs pODC CAT and pODCΔCAT (14) were kindly provided by Philip Coffino (University of California, San Francisco). Site-directed mutants of the two Myc binding sites [MB1 (+232 to +238) and MB2 (+271 to +277)] were created by PCR of a 414-bp *Sma* I fragment (−133 to +281) containing both MB1 and MB2 and mutated the Myc binding site from CACGTG to CACCTG to give ODCΔCATΔSMB1.M1 and ODCΔCATΔSMB2.M1. The wild-type *Sma* I fragment was also cloned to give ODCΔCATΔS. This construct lacks a 120-bp *Sma* I fragment downstream of MB2 (Fig. 1*A*). The products of the amplification reactions were also used as a template in a second round of PCR amplification to create the double mutant ODCΔCATΔSMB1/MB2.M1.

Double-stranded oligonucleotides containing the core and flanking sequences of MB1 (TGCGGCCACGTGTCGCGA) and MB2 (AGGGGACACGTGGCCCGG) were ligated into pBLCAT2, which contains a minimal herpes simplex virus 1 thymidine kinase (TK) promoter (15). Mutant oligonucleotides having a single mutation in each of the Myc binding sites (CACCTG) were cloned in the same way. In addition, a double-stranded oligonucleotide (ODC MB1/MB2), containing ODC nt +228 to +281 (Fig. 1*B*), spanning both of the CACGTG motifs, was cloned as above. The structures of these constructs were confirmed by sequencing.

The human c-*myc* expression plasmid pM21 (driven by the long terminal repeat of Moloney murine leukemia virus) and its deletion derivatives, the murine c-*myc* expression plasmid pSV2c-myc, the point mutants of the c-*myc* LZ domain, and expression vectors containing point mutations within the human c-*myc* basic region and helix I have been described (5, 16, 17).

***In Vitro* Transcription/Translation and Electrophoretic Mobility-Shift Assays.** The transcription plasmids pHLmyc0/1 and pVZ21-MAX were a gift of R. Eisenman (Fred Hutchinson Cancer Center, Seattle). Linearized plasmids were transcribed *in vitro* and the RNA produced was translated in rabbit reticulocyte lysate (Promega). Dimerization of programmed reticulocyte lysates and the reaction conditions for complex formation were as described (18). Double-stranded oligonucleotide probes (0.25 ng) spanning MB1 and its mutant derivative were as above.

Abbreviations: ODC, ornithine decarboxylase; CAT, chloramphenicol acetyltransferase; LZ, leucine zipper; B-HLH, basic–helix–loop–helix; MB*n*, Myc binding site *n*; IL-3, interleukin 3; TK, thymidine kinase.

*To whom reprint requests should be addressed.

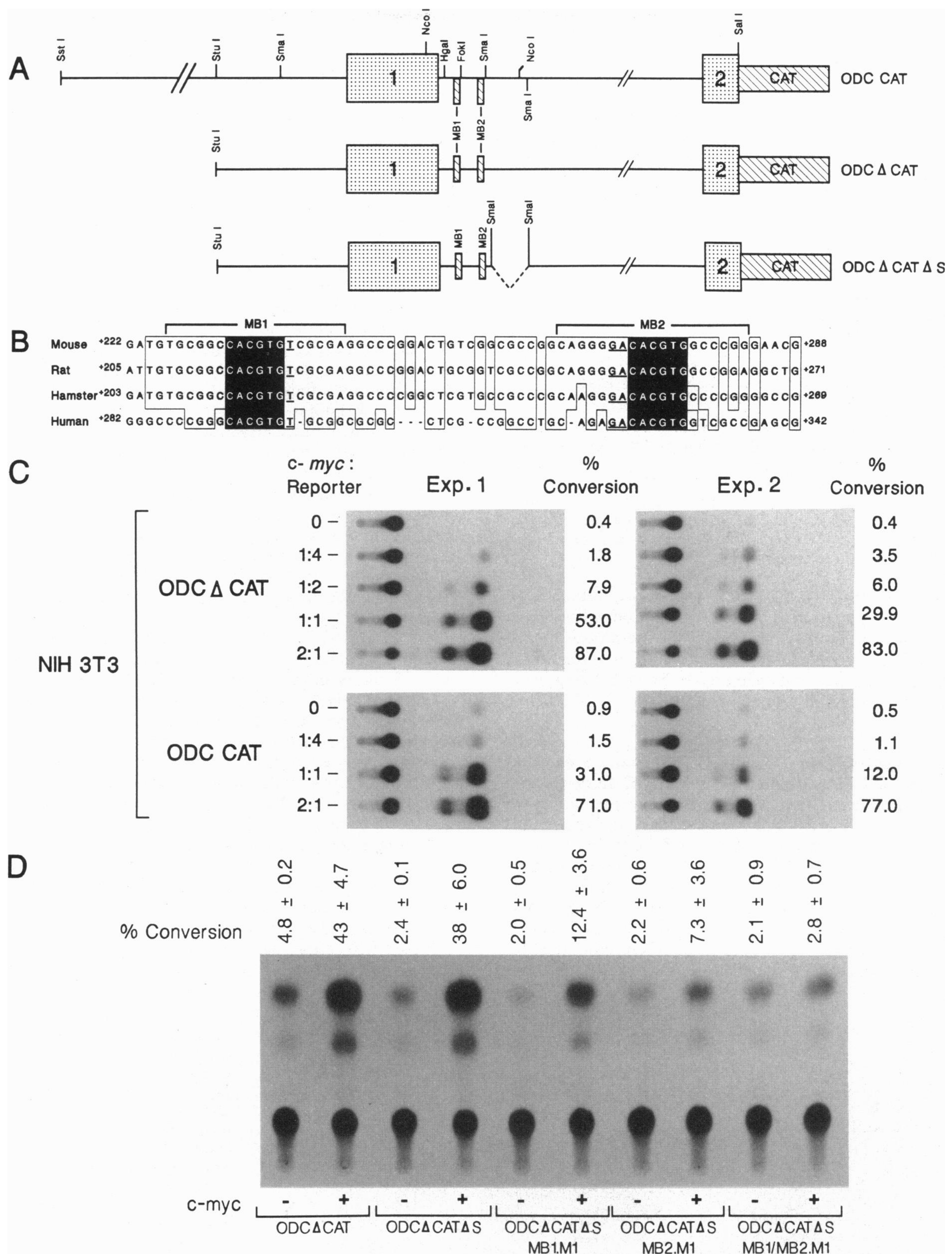


FIG. 1. Myc transactivates the ODC promoter and requires MYC binding sites in intron 1. (*A*) Murine ODC–CAT gene fusion constructs. Relevant restriction sites and the position of the Myc binding (MB) sites are shown. (*B*) Myc binding sites in ODC intron 1 are conserved in mammals (refs. 11–13 and EMBL database entry CCODCPRM). The conserved Myc binding sites are shown in reverse print, and conserved residues which flank this E-box are underlined. Conserved sequences in this region are boxed. Numbers indicate position of this element relative to the major ODC RNA cap site. (*C*) Standardized transient cotransfection titration experiments of ODC CAT, ODCΔCAT, and c-*myc* expression plasmid pM21. Five micrograms of ODC CAT or 8 μg of ODCΔCAT was used in NIH 3T3 cells. Molar ratio of c-*myc* plasmid to CAT reporter construct is shown. Percent conversion [chloramphenicol acetyltransferase (CAT) activity] for each transfection in two experiments is shown. (*D*) Representative CAT assay in NIH 3T3 cells of ODC promoter–CAT constructs having site-directed mutations in MB1 and MB2. Site-directed mutants were in a derivative of ODCΔCAT, ODCΔCATΔS, which lacks a 120-bp *Sma* I fragment. The mutant of MB1 is named ODCΔCATΔSMB1.M1, that of MB2 is ODCΔCATΔSMB2.M1, and the double mutant is ODCΔCATΔSMB1/MB2.M1. Percent conversion is mean ± SD from four experiments.

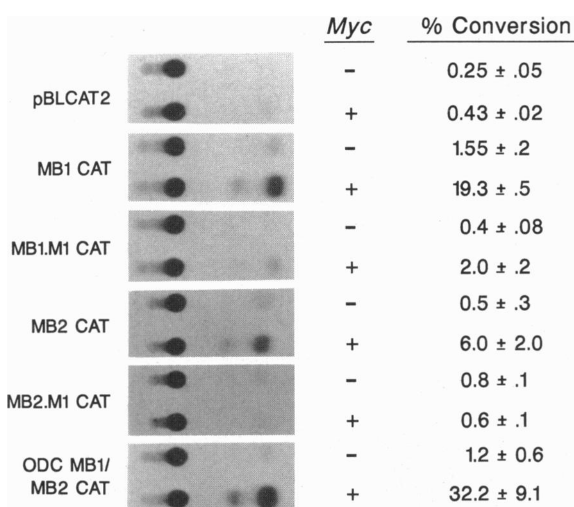


FIG. 2. ODC Myc binding sites promote Myc transactivation from a heterologous promoter. The minimal TK promoter–CAT construct pBLCAT2 (15) and derivatives containing single copies of oligonucleotides MB1, MB2, MB1.M1, MB2.M2, or ODC MB1/MB2 were used as reporter constructs. The autoradiograms are from a representative standardized CAT assay showing the effect of cotransfection of the c-*myc* expression plasmid. Percent conversion is mean ± SD from three experiments.

Protein Analyses. NIH 3T3 cells expressing human Myc derivatives were generated by cotransfection with c-*myc* expression vectors and the plasmid pMCNneoPolyA (Stratagene) and pools were selected with G418. Equal quantities of protein (150 μg) were analyzed by Western blotting using the human Myc-specific monoclonal antibody 9E10 (19).

RESULTS

Myc Transactivates the Murine ODC Promoter and Requires Conserved Myc Binding Sites in ODC Intron 1. To determine whether ODC is a target for Myc regulation we examined the ability of a human c-*myc* expression vector (16) to transactivate two murine ODC–CAT gene fusion constructs [ODC CAT and ODCΔCAT (14)] in transient cotransfection assays. These reporter constructs contain exon 1, intron 1, and a portion of exon 2 of the murine ODC gene and either 264 bp (ODCΔCAT) or ≈2.5 kbp (ODC CAT) of sequence upstream of the murine ODC RNA cap site (Fig. 1*A*). Cotransfection experiments demonstrated that Myc was a potent activator of both ODC promoter constructs in fibroblasts (≈100-fold induction; Fig. 1*C*), and had moderate effects in 32D myeloid cells (3- to 4-fold; data not shown). Since ODCΔCAT was as active as ODC CAT, the Myc-responsive element(s) were within sequences contained in ODCΔCAT. Activation of ODCΔCAT was dependent upon Myc function since transient cotransfection with several constructs expressing mutant forms of Myc failed to promote CAT activity (see below).

To demonstrate that transactivation of ODC specifically required the conserved Myc binding sequences, site directed mutagenesis was performed. The core CACGTG sequence of MB1 and MB2 was altered to CAC<u>C</u>TG in each binding site singularly and in tandem. These mutations greatly reduced the affinity of Myc (see below) for these sites. Mutant binding sites were tested for Myc responsiveness in the context of ODCΔCATΔS (Fig. 1*A*), which is equivalent to ODCΔCAT as a Myc target (Fig. 1*D*). Mutation of MB1 or MB2 alone diminished by a factor of 3–5 the ability of Myc to transactivate the promoter. By contrast, mutation of both binding sites abolished transactivation of this construct (Fig. 1*D*). Therefore, Myc-mediated transactivation of the ODC promoter requires these conserved Myc binding sites.

Myc-Responsive Elements of the ODC Gene Confer Responsiveness on a Heterologous Promoter. The position of the Myc binding sites suggested that Myc could influence ODC promoter–CAT activity by affecting RNA processing, transcription elongation, or transcription initiation. To address the last possibility, oligonucleotides spanning MB1, MB2, or both MB1 and MB2 were cloned 5′ of the minimal TK promoter of pBLCAT2 (MB1 CAT, MB2 CAT, and ODC MB1/MB2 CAT; Fig. 2). The mutant versions of MB1 and MB2 described above were similarly cloned to give MB1.M1 CAT and MB2.M1 CAT, respectively (Fig. 2). c-*myc* cotransfection had little effect upon minimal TK promoter activity yet was an effective transactivator of MB1 and MB2 CAT (13- and 12-fold induction) and was even more effective in transactivating ODC MB1/MB2 (27-fold induction). By contrast, mutation of these Myc binding sites (MB1.M1 and MB2.M1) impaired the ability of Myc to transactivate these promoter constructs.

Binding of Myc to Myc-Responsive Sites of the ODC Gene. To evaluate whether Myc could bind to the conserved Myc-responsive sequences of the ODC gene, human Myc and Max made in reticulocyte lysates were assayed for their ability to bind MB1 probe (Fig. 3*A*). One weak complex was detected with unprogrammed reticulocyte lysates, and this was attributed to endogenous rabbit Max protein, as it could

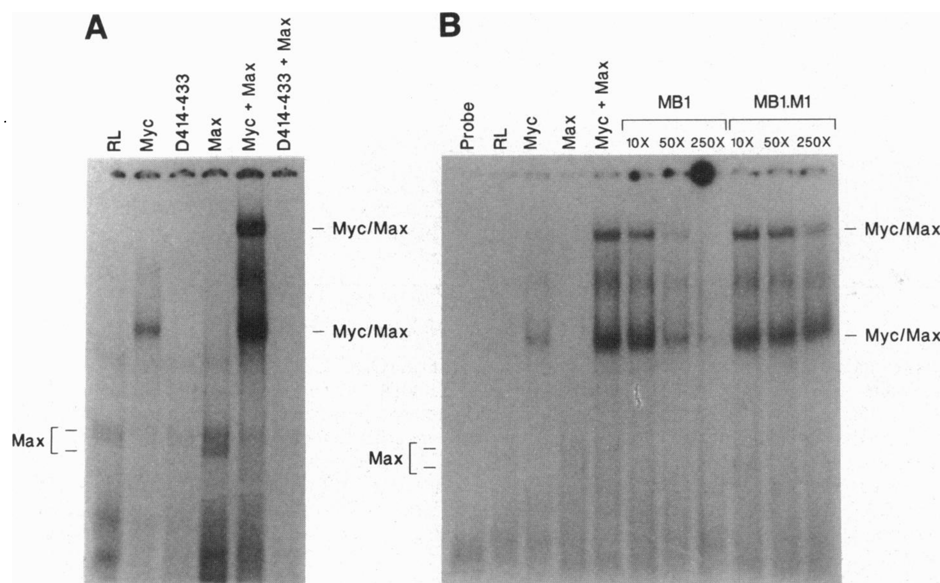


FIG. 3. Myc and Max specifically bind to ODC Myc-responsive sites *in vitro*. (*A*) Binding of Myc, Max, and Myc deletion mutant D414–433 to MB1. Equal quantities of human Myc, Max, and D414–433 synthesized *in vitro* were mixed with an equal volume of unprogrammed reticulocyte lysate (RL) or mixed together in equal proportions, allowed to dimerize for 30 min at 30°C, and then incubated with MB1 probe. (*B*) Specificity of binding of Myc/Max complexes to MB1. DNA sequence specificity was tested by comparative competitions with the indicated molar excess of unlabeled wild-type oligonucleotide (MB1) versus a mutant oligonucleotide (MB1.M1) having a single base-pair change in the core binding sequence (CAC<u>C</u>TG).

be supershifted with Max-specific antiserum (data not shown). Two additional Max-containing complexes were detected with human Max-programmed lysates. Myc was capable of binding as two slower migrating complexes; this binding was presumably dependent upon endogenous rabbit Max, as the same complexes were much more abundant when the probe was incubated with programmed lysates in which Myc and Max had been allowed to dimerize (Fig. 3*A*). All four complexes were specific for the core CACGTG sequence, as they had higher affinity for the wild-type oligonucleotide than for one containing a single base change in this sequence, to CACCTG (Fig. 3*B*). The identity of these complexes was established with supershift experiments with Myc- and Max-specific antibodies (data not shown).

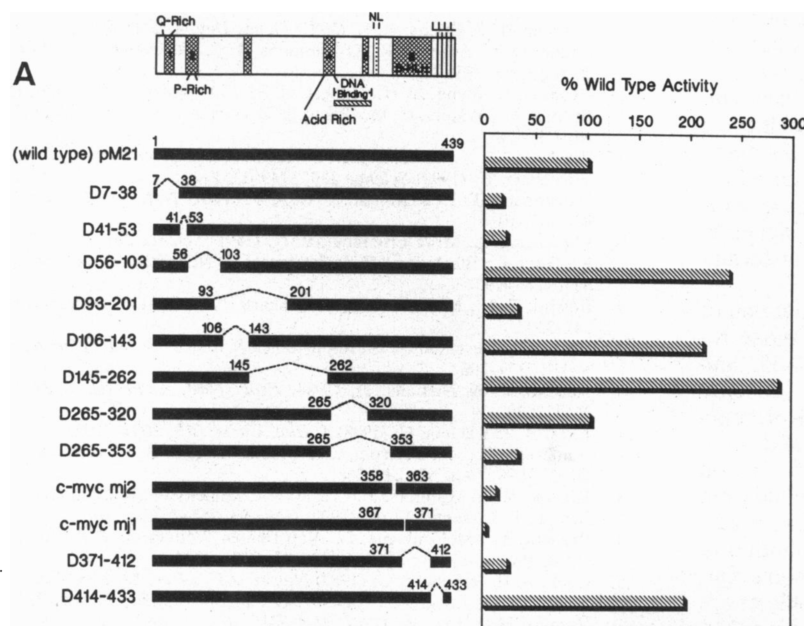
Domains of Myc Required for Transactivation of the ODC Gene. To define the domains of Myc necessary for transactivation of the ODC gene, we tested a series of characterized human c-*myc* deletion mutants (16) and B-HLH and LZ point mutants of human and murine c-*myc* (5, 17) in transient cotransfection assays with ODCΔCAT (Fig. 4*A*). Removal or mutation of several domains shown to be necessary for transformation (such as the B-HLH region; refs. 5 and 16) or which function as transactivation domains (amino terminus, aa 7–53 and 93–201; ref. 20) resulted in loss of activity (Fig. 4*A*). By contrast, removal of other specific regions in the amino terminus (aa 56–103 and 106–143) and portions of the central third of human Myc (aa 145–262) increased transactivating activity of Myc (Fig. 4*A*). The failure of some of these constructs to transactivate and of others to activate ODCΔCAT was not generally attributable to differences in protein levels. Western blot analysis of stably transfected NIH 3T3 cells with 9E10, a monoclonal antibody specific for human Myc, revealed comparable levels of the predicted sizes (16) of Myc polypeptides (Fig. 4*B*). The HLH and LZ deletion mutants could not be detected with this antibody (since the epitope spans this region) but are expressed at comparable levels in COS cells (16).

Interestingly, a deletion (D414–433) which removes Leu^{420} and Leu^{427}, takes Leu^{434} out of context, and compromises transforming activity (16) did not alter Myc transactivation. However, D414–433 Myc protein made in reticulocyte lysates, with or without Max, failed to bind to MB1 (Fig. 3*A*). This suggests that Myc transactivates ODCΔCAT independent of Max.

To evaluate the importance of the specific leucine residues within the LZ domain to ODC transactivation, we tested point mutants of the murine Myc LZ domain (17). Mutants in which Leu^{420} and Leu^{427} were converted to tryptophan and serine, respectively, were as active as the wild-type protein in transactivation (Fig. 5). By contrast, the helix-breaking mutation of Leu^{413} to proline, or mutation of all four leucines (L413–433PWSP), abolished transactivation (Fig. 5).

DISCUSSION

The ODC gene is an appealing transcriptional target for Myc since ODC is also required for G_1 progression and transformation (8, 21). In addition, ODC expression is regulated as an early gene product, follows c-*myc* induction by IL-3 in a



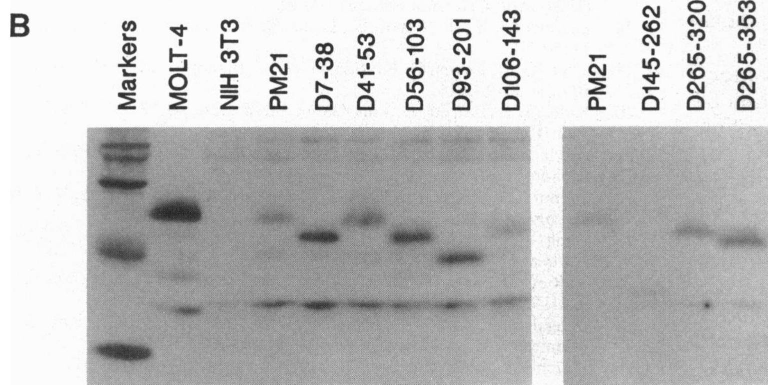


FIG. 4. Domains of Myc required for transactivation of the ODC promoter. (*A*) Domain structure of human Myc is shown above the human Myc deletion and point mutants (5, 16). Bar graph shows the mean data from three experiments using standardized extracts from NIH 3T3 cells transiently transfected with a 2:1 molar ratio of the indicated c-*myc* expression plasmids and the ODCΔCAT reporter. Induction of the ODCΔCAT reporter is shown as percent activity of wild-type (pM21) c-*myc*. (*B*) Myc protein levels in stably transfected NIH 3T3 cells. NIH 3T3 cells were transfected with the indicated constructs and analyzed for Myc protein by Western blot analysis with human Myc-specific monoclonal antibody 9E10. MOLT-4 is a human T-cell line used as positive control, and untransfected NIH 3T3 is the negative control. Size markers (left lane) are 97.4 and 92.5 (doublet), 69, 46, and 30 kDa.

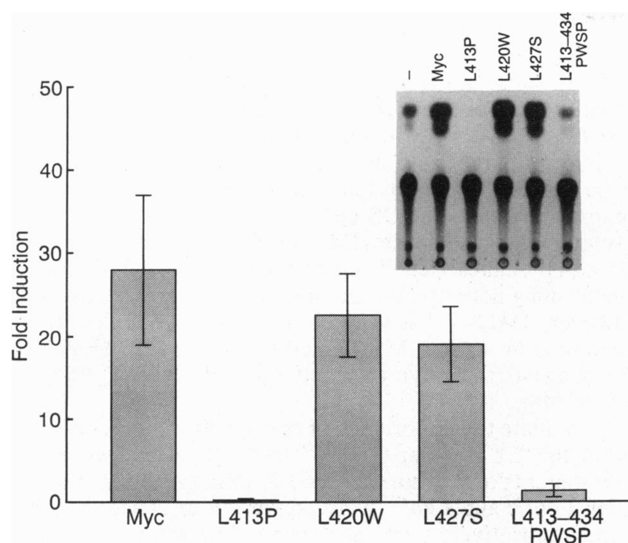


FIG. 5. Analysis of point mutants of the murine Myc LZ domain. NIH 3T3 cells were transfected with the indicated c-*myc* expression plasmids (17) and the ODCΔCAT reporter construct at a 1:1 molar ratio. A representative CAT assay is shown in the *Inset*. Bar graph shows mean fold induction ± SD from three experiments.

temporal fashion, and is relieved of its growth factor dependence when c-*myc* is constitutively expressed in IL-3-dependent myeloid cells (2, 9). In this report we have shown that Myc is a potent activator of the ODC promoter which requires conserved consensus Myc binding sites in ODC intron 1. When cloned upstream of the cap site of a heterologous promoter, these elements retain Myc responsiveness. These criteria suggest that the ODC gene is a Myc transcriptional target and that the ability of Myc to promote cell cycle progression is due, in part, to transcriptional activation of the ODC gene. Since some of the specific domains required for Myc-induced transformation are dispensable for transactivation of the ODC promoter, the induction of ODC appears, however, to be only one component of the *myc* transformation pathway.

Dissection of Myc domains required for transactivation of the ODC promoter revealed that these included those required for transactivation (20) (e.g., D7–38, D41–53, and D93–201), nuclear localization (16) (D265–353), specific DNA binding (5) (basic-region mutant mj2), and protein–protein interaction (6) (helix 1 and 2, D371–412, and mj1). However, in a few cases, regions required for transformation (e.g., D106–143 and D414–433; ref. 16) can be separated from those required for transactivation of the ODC promoter. Recent analyses of these same mutants in differentiation of murine erythroleukemia cells have revealed that some mutants defective in transformation assays of rat fibroblasts are as effective as wild-type Myc in blocking differentiation (22). Therefore, the requirements of specific domains for Myc-mediated transformation may not necessarily reflect those required for growth or activation of gene expression.

The most surprising region dispensable for Myc transactivation of ODC promoter constructs was a large portion of the LZ domain (D414–433), which is required for interaction with Max (Fig. 3*A*) and for transformation (16). Similarly, the specific mutations of Leu^{413}, Leu^{420}, and Leu^{427} all drastically reduce the ability of Myc to inhibit murine erythroleukemia cell differentiation (17), although D414–433 in fact accelerates differentiation of these same cells (22). We have shown that the last three leucines of the Myc LZ domain are dispensable for transactivation of the ODC promoter, whereas the first leucine (Leu^{413}), juxtaposed to the B-HLH domain, is required. This may reflect the nature of the L413P mutation, which introduced a helix-breaking proline, versus those at Leu^{420} and Leu^{427}, which may not break the proposed α-helical structure. The Myc LZ domain is required for formation of homodimers (23), for dimerization with Max both *in vitro* (6) and *in vivo* (24), and for binding of an *in vitro* translated Myc/Max complex to an oligonucleotide containing the CACGTG motif (Fig. 3 and ref. 6). Since the Myc LZ deletion mutant is active in ODC transactivation, we propose that Myc, at least in this context, can function independently of its identified heterodimeric partner. In view of recent findings that B-LZ proteins (Jun and Fos) can interact with B-HLH proteins (MyoD) or B-HLH-LZ proteins (Fip) through their respective dimerization motifs (25, 26), it is possible that Myc may interact with other dimerization partners through HLH–HLH or HLH–LZ interactions to transactivate the ODC gene.

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