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Activation Domains of L-Myc and c-Myc Determine Their Transforming Potencies in Rat Embryo Cells

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Members of the Myc family of proteins share a number of protein motifs that are found in regulators of gene transcription. Conserved stretches of amino acids found in the N-terminal transcriptional activation domain of c-Myc are required for cotransforming activity. Most of the Myc proteins contain the basic helix-loop-helix zipper (bHLH-Zip) DNA-binding motif which is also required for the cotransforming activity of c-Myc. L-Myc, the product of a *myc* family gene that is highly amplified in many human lung carcinomas, was found to cotransform primary rat embryo cells with an activated *ras* gene. However, L-Myc cotransforming activity was only 1 to 10% of that of c-Myc (M. J. Birrer, S. Segal, J. S. DeGreve, F. Kaye, E. A. Sausville, and J. D. Minna, Mol. Cell. Biol. 8:2668–2673, 1988). We sought to determine whether functional differences between c-Myc and L-Myc in either the N-terminal or the C-terminal domain could account for the relatively diminished L-Myc cotransforming activity. Although the N-terminal domain of L-Myc could activate transcription when fused to the yeast GAL4 DNA-binding domain, the activity was only 5% of that of a comparable c-Myc domain. We next determined that the interaction of the C-terminal bHLH-Zip region of L-Myc or c-Myc with that of a Myc partner protein, Max, was equivalent in transfected cells. A Max expression vector was found to augment the cotransforming activity of L-Myc as well as that of c-Myc. In addition, a bacterially synthesized DNA-binding domain of L-Myc, like that of c-Myc, heterodimerizes with purified Max protein to bind the core DNA sequence CACGTG. To determine the region of L-Myc responsible for its relatively diminished cotransforming activity, we constructed chimeras containing exons 2 (constituting activation domains) and 3 (constituting DNA-binding domains) of c-Myc fused to those of L-Myc. The cotransforming potencies of these chimeras were compared with those of full-length L-Myc or c-Myc in rat embryo cells. The relative cotransforming activities suggest that the potencies of the activation domains determine the cotransforming efficiencies for c-Myc and L-Myc. This correlation supports the hypothesis that the Myc proteins function in neoplastic cotransformation as transcription factors.

The *myc* proto-oncogenes encode a family of nuclear proteins whose deregulated expression appears to be involved in the pathogenesis of several human cancers (2, 6, 11, 19, 48). Members of this family include c-, L-, N-, and s-Myc, all of which contain amino-terminal amino acid sequence homologies as well as strong homologies in the carboxy-terminal basic helix-loop-helix zipper (bHLH-Zip) regions (4, 10, 24, 31, 35–37). B-Myc, a family member whose cDNA was isolated from rats, is a 168-amino-acid protein that lacks a bHLH-Zip domain but contains extensive homology with the c-Myc transcriptional activation domain (3, 23, 25). The various motifs found in the Myc family of proteins suggest that they participate in the regulation of gene expression (24, 36). Nevertheless, several members of the *myc* family have the ability to transform primary rat embryo cells in culture in cooperation with an activated *ras* gene (5, 17, 30, 32). Specifically, L-*myc*, which was first isolated from a human small-cell lung cancer, in which it was detected by the presence of gene amplification (38), can cotransform cells in culture (5). The L-*myc* gene encodes multiple nuclear proteins with a complex pattern of translational initiation and phosphorylation, from alternatively processed mRNAs (18, 20, 27, 33, 43).

Previous studies have demonstrated that the ability of L-Myc to transform rat embryo cells with an activated *ras* gene was markedly diminished (1 to 10%) relative to c-Myc (5). The molecular basis for this difference, however, was not understood. Molecular dissection of c-Myc has identified an amino-terminal 143-amino-acid domain and a carboxy-terminal 120-amino-acid domain which are essential for transforming activity (44, 49). Additional studies have uncovered biochemical functions for these domains and thus provide a preliminary view of c-Myc function (13). The amino-terminal 143 amino acids can activate transcription when linked to a heterologous DNA-binding domain and thus constitute a potential transcriptional activation domain (25). The carboxy-terminal 120 amino acids contain a nuclear targeting sequence and a specific DNA-binding domain with the bHLH-Zip motif (7–9, 13, 16, 21, 22, 26, 27, 39, 40). The bHLH-Zip region of c-Myc is unable to homodimerize strongly in vitro (47) or in transfected cells (14) but can heterodimerize with a recently discovered Myc-associated protein, termed Max or Myn (8, 9, 39).

Recent studies indicate that Max can interact with members of the Myc family of proteins in cells (8, 9, 26, 39, 50). In addition, full-length c-Myc produced in bacteria can bind to a core nucleotide sequence [CAC(G/A)TG] only when heterodimerized to the Max protein (26). In contrast to sequences of c-Myc, those of Max do not activate transcrip-

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tion when linked to the DNA-binding domain of the yeast transcriptional activator GAL4 (26). These studies suggest that c-Myc binds to DNA specifically only in the presence of a partner protein and that Myc, but not its partner, provides the activation domain that interacts with the transcriptional machinery (12, 26).

In the present study, we sought to identify the molecular properties of L-Myc that correlate with its transforming activity in rat embryo cells. The ability of GAL4-linked L-Myc amino-terminal sequences to activate transcription of a reporter plasmid construct was found to be substantially weaker than that of homologous c-Myc sequences. Furthermore, the carboxy-terminal DNA-binding domains of L-Myc and c-Myc are functionally equivalent. Specifically, we demonstrate that the bHLH-Zip domain of L-Myc interacts with that of Max in cells equivalently to the bHLH-Zip domain of c-Myc. Similarly to the c-Myc DNA-binding bHLH-Zip domain, bacterially produced L-Myc DNA-binding domain binds specifically to the core DNA sequence, CACGTG, in the presence of bacterially produced Max protein. In addition, Max can augment the transforming activity of both L-Myc and c-Myc. The transforming potencies of chimeras composed of c-Myc and L-Myc in rat embryo cells correlate with the presence of the c-Myc N-terminal activation domain but not with the C-terminal DNA-binding domain of either L-Myc or c-Myc, although the C-terminal domains are required for activity. These results suggest that the relatively weak transforming activity of L-Myc in rat embryo cells is due to its amino-terminal activation domain.

MATERIALS AND METHODS

Cell culture and transfection. Primary rat embryo cells were harvested from 14-day-old Fisher rat embryos as described elsewhere (30, 32). Briefly, the embryos were removed from amniotic sacs and minced in a 0.05% trypsin solution. The suspension was stirred at room temperature for 15 min and then was centrifuged at 1,000 × *g* for 5 min. The supernatant containing the cells was decanted and mixed with Dulbecco's minimum essential medium containing 20% fetal bovine serum. Cells (5×10^5) were plated in T-75 flasks and cultured in Dulbecco's minimum essential medium with 10% fetal bovine serum in preparation for transfection. For cotransformation assays, each T-75 flask of rat embryo cells was transfected with plasmids by using Lipofectin (Bethesda Research Laboratories) as described elsewhere (14). For transcriptional activation assays, rat embryo cells were similarly transfected with activator and reporter plasmids. Cell extracts were harvested 48 h after transfection by three cycles of freezing and thawing and used for chloramphenicol acetyltransferase (CAT) assays as described previously (25, 34).

Chinese hamster ovary (CHO) cells were cultured in alpha minimum essential medium with 10% fetal bovine serum and transfected with DEAE-dextran as previously described (25, 34). CHO cell extracts were harvested 48 h after dimethyl sulfoxide shock and chloroquine treatment for CAT or β-galactosidase assay (34). Metabolic labelling of transfected CHO cells with [^{35}S]methionine was performed as previously described (14). Immunoprecipitations with anti-GAL4 antibody were performed as described elsewhere (14).

Plasmid constructions. The following plasmids have been described previously (14, 25, 26): pGALO, pGALM, pNLVP, GM(1–262), GM(262–439), VP(6–262), G5E1B CAT, VPMax(8–112), and GMax(8–112). Plasmids encoding the GAL4 DNA-binding domain fused to L-Myc sequences were constructed as follows: an L-*myc* *Eco*NI (mung bean nuclease-treated)–*Bst*EII fragment from the cDNA of the unspliced long form of L-*myc* (20) was inserted into pGALM, creating GLM2; an L-*myc* *Bst*EII–*Eco*RI fragment from the same L-*myc* cDNA was ligated with the *Hin*dIII-*Bst*EII fragment of pGALM and the *Hin*dIII-*Eco*RI fragment of pGALO, creating GLM3. The plasmid pNLVPLM(LZ), which encodes a hybrid protein of the VP16 activation domain fused to L-Myc amino acids 242 to 365, resulted from ligating the *Pst*I-*Xba*I fragment from GLM3 and the *Xba*I-*Hin*dIII fragment from pGALO with the *Hin*dIII-*Nsi*I fragment of pNLVP. GM(350–439) encoding GAL4 fused to c-Myc amino acids 350 to 439 was derived from ligation of the c-*myc* *Xho*I-*Nsi*I fragment from *myc* D262–350 (49) with pGALO.

A Max expression vector driven by the Rous sarcoma virus (RSV) long terminal repeat (LTR) was constructed by inserting a full-length *max* cDNA *Sac*I-*Hin*cII fragment [from a *max* *Eco*RI cDNA in pBSIIKS(−) (Stratagene)] into the corresponding sites in an RSV expression vector between the RSV LTR and a simian virus 40 polyadenylation signal (gift from B. Lee). The *max* cDNA, which encodes p21 Max, was provided by E. Blackwood and R. Eisenman (8).

L-Myc and c-Myc expression vectors driven by Moloney leukemia virus (MLV) LTR were as described elsewhere (5, 49). Chimeric genes encoding fusions of L-Myc and c-Myc were constructed by exchanging exons. The chimera MLVC2L3 (encoding c-Myc amino acids 1 to 262 fused to L-Myc amino acids 165 to 365) was constructed by inserting an *Hpa*I-*Nsi*I fragment of L-*myc* (20) into the MLV-c-*myc* vector cut with *Bgl*II (Klenow fill-in) and *Nsi*I (49). MLVL2C3 (encoding L-Myc amino acids 1 to 165 fused to c-Myc amino acids 262 to 439) resulted from ligating an L-*myc* exon 2 (*Eco*RI fragment) linker-adapted *Bam*HI-*Eco*RV fragment with the MLV-c-*myc* vector *Bgl*II (Klenow fill-in) and *Bam*HI fragment (49).

A bacterial expression vector, pDS-LMyc242–364, producing a hexahistidine L-Myc (amino acids 242 to 364) [L-Myc(242–364)] was constructed by ligating a *Pst*I-*Eco*RI L-*myc* fragment into the pDS-MCS vector (1). Similar plasmid vectors producing c-Myc (amino acids 342 to 439) [c-Myc(342–439)] or Max were as previously described (26).

Bacterially synthesized L-Myc protein. The hexahistidine L-Myc fusion protein produced in *Escherichia coli* with the pDS-L*myc*242–364 vector was partially purified over a nickel-agarose column (Qiagen) as described elsewhere (1, 26). Hexahistidine L-Myc(242–364) was produced at a low level and was only about 10% pure after affinity chromatography, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (29). Similarly, hexahistidine Max and c-Myc(342–439) were purified as described elsewhere (26).

CAT assay. Transfected cell extracts were prepared as described above, and aliquots were used for CAT assays as described elsewhere (25, 45).

EMSA and photocross-linking. Bacterially produced proteins were used in electrophoretic mobility shift assays (EMSAs) as described elsewhere (26). The estimated amounts of protein in a final reaction volume of 20 μl [1 μg of poly(dI-dC), 0.5 μg of denatured sheared salmon sperm DNA, 10 mM Tris-HCl (pH 7.4), 80 mM NaCl, 1 mM dithiothreitol, and 5% glycerol] were as follows: Max (5 ng), c-Myc(342–439) (2 μg), and L-Myc(242–364) (0.5 μg). A 282-bp *Hin*dIII-*Bam*HI (Klenow fragment DNA polymerase

fill-in with [^{32}P]dCTP) radiolabelled DNA fragment containing the core site CACGTG was used as probe for EMSA with Max, L-Myc, and c-Myc DNA-binding domains. We found empirically that this 282-bp probe (49a), rather than a smaller probe, is able to resolve c-Myc/Max or L-Myc/Max heterodimers by EMSA. This 282-bp probe contains a core CACGTG sequence that is 26 bp from an intrinsically bent AT-rich sequence (the vector was a gift from T. Kerppola and T. Curran).

For photocross-linking, DNA-binding reactions were assembled as described for EMSA with a radiolabelled (by Klenow fill-in) 20-bp duplex DNA probe bearing a palindromic sequence, 5′-TCGAGACCACGTGGTCTCGA-3′, in 1.5-ml microcentrifuge tubes (26). Protein concentrations were as described for EMSA above. The tubes were placed directly onto a short wavelength UV light table (Fotodyne) and irradiated for 30 min. Aliquots were heated in Laemmli sample buffer and then analyzed by SDS-PAGE (29).

Metabolic labelling of transformed rat embryo cells and immunoprecipitation. Cells transfected with a mutated *ras* gene and L-*myc*, c-*myc*, C2L3, or L2C3 were labelled with [^{32}P]orthophosphate (1 mCi/ml) for 2 h as described elsewhere (20). L-Myc and C2L3 ^{32}P-labelled proteins were immunoprecipitated with a rabbit polyclonal anti-L-Myc peptide (amino acids 223 to 242 in the carboxy-terminal end of L-Myc) antibody as described elsewhere (20). Control immunoprecipitations were performed with preimmune serum. Metabolically labelled c-Myc and L2C3 were immunoprecipitated with an anti-c-Myc rabbit polyclonal antibody as described elsewhere (14).

RESULTS

The L-Myc amino-terminal domain activates transcription more weakly than that of c-Myc. In order to identify molecular determinants affecting L-Myc transforming activity, we first sought to determine the transcriptional activation potency of the L-Myc amino-terminal region that is homologous to the c-Myc activation domain. Fusion genes that produce hybrid proteins of the GAL4 DNA-binding domain fused to the amino-terminal L-Myc amino acids 1 to 186 (GLM2) or to the carboxy-terminal L-Myc amino acids 186 to 365 (GLM3) were constructed (Fig. 1A). The fusion genes were expressed in CHO cells along with a reporter CAT gene construct linked to five GAL4 DNA-binding sites. The ability of these hybrid L-Myc proteins to activate CAT gene transcription was measured in comparison to GM(1–262), a hybrid protein that contains the c-Myc transcriptional activation domain (Fig. 1B). GLM2 consistently stimulated CAT activity above the background produced by the GAL4 DNA-binding domain alone (Fig. 1B). However, compared with c-Myc amino acids 1 to 262 linked to GAL4 [GM(1–262)], L-Myc amino acids 1 to 186 in GLM2 activated transcription at a level of only 5% of that of c-Myc in CHO cells. GLM3 displayed no activity above the background of GALO (Fig. 1B). In primary rat embryo cells, GM(1–262) activated transcription through GAL4 sites, but the absolute CAT activity per plate of transfected cells was very low and was only 6% of that measured in CHO cells. GLM2 and

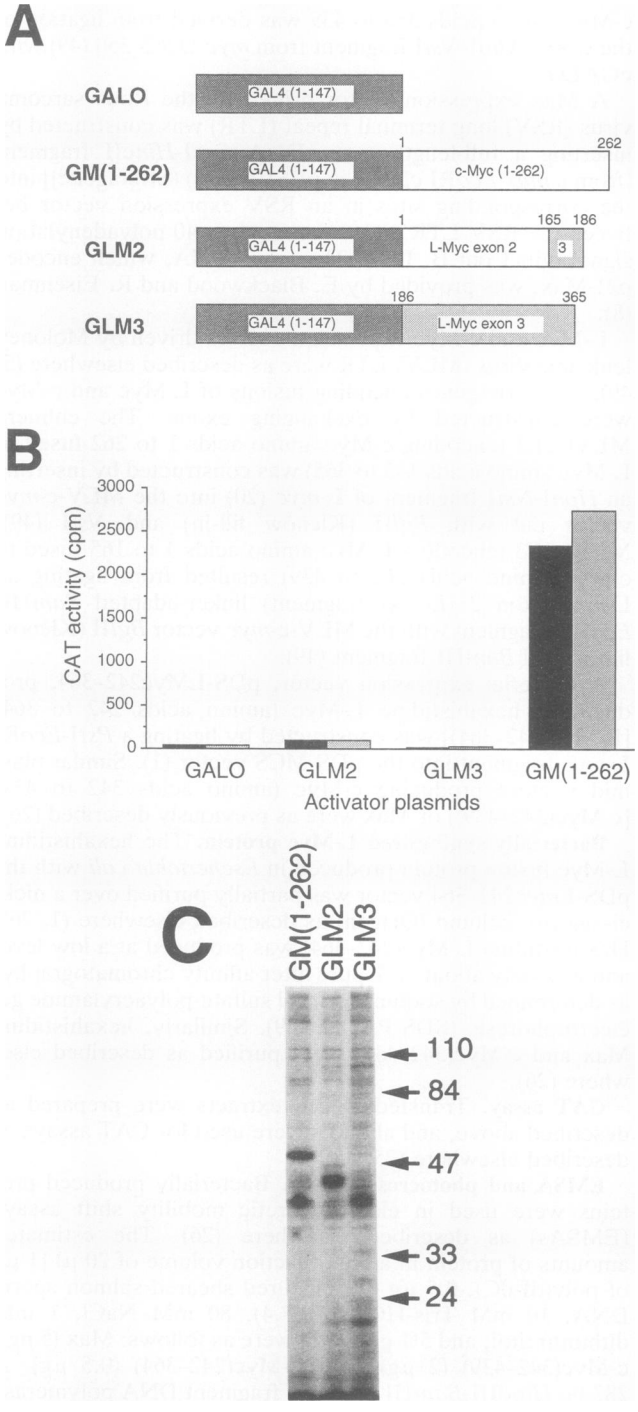

FIG. 1. Relative activities of GAL4 chimeric proteins containing L-Myc or c-Myc sequences. GAL4 chimeric protein expression vectors (2 μg of plasmid DNA per plate) were cotransfected into CHO cells with a reporter plasmid, G5E1bCAT (2 μg of plasmid DNA per plate), and CAT activities were measured. (A) Schematic representations of GAL4 chimeric proteins. The DNA-binding domain of GAL4 (amino acids 1 to 147) is shown alone or fused to c-Myc or L-Myc amino acids, which are shown above the bars. (B) CAT activities of GAL4 chimeras shown in panel A. Pairs of vertical bars with different shadings represent CAT activities (averaged from two separate plates of transfected cells) from two separate transfection experiments. (C) Immunoprecipitation that used anti-GAL4 antiserum of [^{35}S]methionine-labelled CHO cells transfected with GAL4 chimeric protein expression vectors. GAL4 chimeric proteins are indicated above each lane. Positions of prestained molecular mass markers (in kilodaltons) are shown in the right margin.

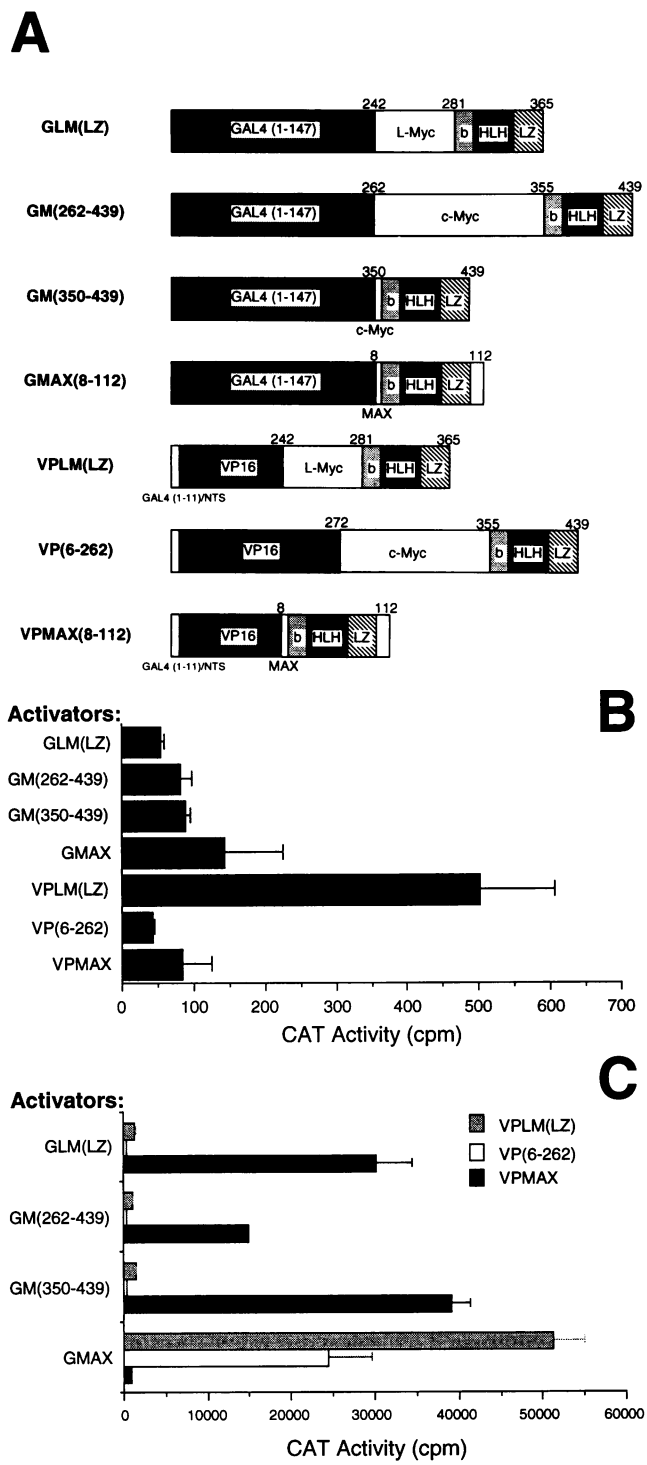

FIG. 2. Intracellular interactions between the oligomerization domains of Max and those of either c-Myc or L-Myc. (A) Schematic representations of GAL4 DNA-binding domain or VP16 activation domain chimeric proteins containing Max, c-Myc, or L-Myc sequences (amino acids are indicated above the bars). (B) CAT activities stimulated by cotransfecting constructs (2 μg of DNA per plate) encoding proteins shown in panel A and the reporter G5E1bCAT (2 μg of DNA per plate). (C) CAT activities stimulated by combinations of constructs (see panel A) demonstrate intracellular interactions between the bHLH-Zip domain of Max with those of c-Myc as well as L-Myc. For these experiments, 2 μg of plasmid DNA encoding GAL4 chimeras, 4 μg of plasmid DNA encoding GLM3 yielded no detectable transcriptional activation above background in primary rat embryo cells, presumably because of the limitation of the CAT assay.

By using anti-GAL4 antibody, immunoprecipitation of transfected [^{35}S]methionine metabolically labelled CHO cells demonstrates comparable steady-state levels of the various GAL4 fusion proteins (Fig. 1C). Thus, the differences in activities among the various GAL4 fusions are not likely to be due to variations in hybrid protein expression. These data suggest that the amino-terminal L-Myc domain linked to a heterologous DNA-binding domain can activate transcription only weakly, compared with the c-Myc activation domain.

Equivalent intracellular interactions of L-Myc and c-Myc bHLH-Zip domains with Max. In order to determine potential functional differences between the oligomerization domains of L-Myc and c-Myc, we studied the intracellular association of the bHLH-Zip domain of the Max with that of c-Myc or L-Myc. This previously described assay utilizes a CAT reporter gene construct, the expression of which is dependent on functional GAL4 protein. Specific interaction between two chimeric proteins, one containing the GAL4 DNA-binding domain and the other containing a potent transcriptional activation domain, reconstitutes GAL4 function, resulting in stimulation of CAT activity.

Expression vectors that express chimeric proteins (Fig. 2A) of the GAL4 DNA-binding domain or the herpes simplex virus VP16 activation domain fused to the bHLH-Zip domains of L-Myc, c-Myc, (14, 26), or Max (26) were constructed. Background CAT activities resulting from each of the individual chimeric proteins are graphed in Fig. 2B. Each GAL4 chimeric protein was tested in combination with chimeras of the VP16 activation domain fused to the L-Myc (pNLVPLM[LZ]), c-Myc [VP(6–262)], or Max (VPMAX) bHLH-Zip domain (Fig. 2C). The bHLH-Zip domain of either c-Myc [in GM(262–439) or GM(350–439)] or L-Myc [in GLM(LZ)] in GAL4 hybrid proteins appears to interact equivalently with VP16-Max hybrid protein (Fig. 2C). It is notable that inclusion of c-Myc amino acids 262 to 349 in the GAL4 hybrid GM(262–439) appears to decrease potency, compared with GM(350–439), to interact with VPMAX. The converse experiments with the GAL4-Max hybrid protein also demonstrate the interaction of the L-Myc or c-Myc [VP(6–262)] bHLH-Zip domain with that of Max. Homo- or heterodimerization between L-Myc and c-Myc bHLH-Zip domains was undetectable. These experiments suggest that the L-Myc and c-Myc dimerization domains, although not identical in length in the chimeras used, interact equivalently with the bHLH-Zip domain of Max in mammalian cells.

Truncated L-Myc binds CACGTG with Max. Although L-Myc and c-Myc each can oligomerize with Max in vitro (8, 40) or in cells and are homologous to one another in the basic region, it has not been established that L-Myc is able to bind to the same DNA sequence bound by c-Myc. We have produced a truncated L-Myc protein in *E. coli* and previously produced a similar c-Myc protein and Max (26). These hexahistidine fusion proteins were partially purified over a nickel affinity column (1, 26). c-Myc and L-Myc each were tested alone and in combination with the Max protein for

VP16 chimeras, and 2 μg of the reporter DNA G5E1bCAT were cotransfected into each plate of CHO cells. Note that the horizontal scales are different in panels B and C. Error bars represent standard deviations from quadruplicate experiments.

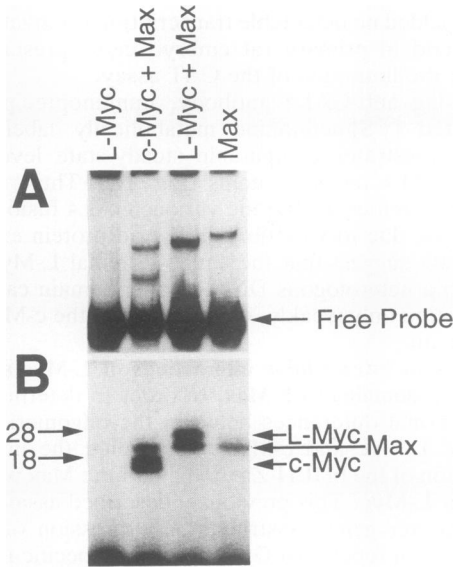

FIG. 3. Specific DNA-binding activities of c-Myc, L-Myc, and Max. (A) Bacterially produced c-Myc (amino acids 342 to 439) or L-Myc (amino acids 242 to 364) bHLH-Zip domain was mixed with bacterially produced Max (amino acids 9 to 151) and tested for the ability to bind to the core sequence CACGTG by EMSA. Proteins in reaction mixtures are indicated above each lane. L-Myc protein did not bind DNA unless mixed with Max protein. Truncated c-Myc can bind DNA as homodimers as well as heterodimers with Max as previously reported (26). (B) Protein-radiolabelled DNA mixtures as in panel A were subjected to UV light-induced photocross-linking. Proteins cross-linked to radiolabelled DNA probe bearing the core sequence CACGTG were separated on an SDS–15% polyacrylamide gel. Positions of prestained molecular mass markers (in kilodaltons) are given on the left.

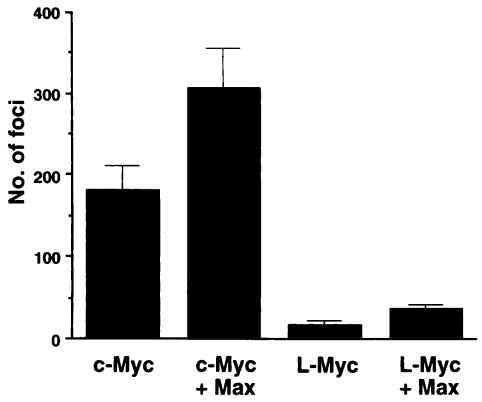

FIG. 4. Augmentation of cotransforming activities of c-Myc and L-Myc by a *max* expression vector. A *max* expression plasmid (10 μg of DNA per plate) driven by the RSV LTR was cotransfected into primary rat embryo cells with a mutated *ras* gene (5 μg of EJ*ras* DNA per plate) and either a c-*myc* or an L-*myc* expression plasmid (10 μg of DNA per plate). The numbers of transformed foci observed at 18 days after transfection are shown from quadruplicate experiments. Error bars represent standard deviations.

their ability to bind to a radiolabelled oligonucleotide bearing the core sequence CACGTG by EMSA. Whereas truncated c-Myc or Max could bind to the oligonucleotide as previously reported (26), L-Myc alone displays no DNA-binding activity (Fig. 3A). The addition of Max to truncated c-Myc or truncated L-Myc resulted in heterodimers that shifted the radiolabelled oligonucleotide to distinct positions (Fig. 3A). Both truncated c-Myc/Max and truncated L-Myc/Max heterodimers displayed faster mobilities than Max/Max homodimers.

In order to confirm these findings, we employed UV photocross-linking to demonstrate the presence of Max, truncated c-Myc, or truncated L-Myc in DNA-bound complexes. DNA-binding reactions were carried out in EMSA buffer, and the mixtures were irradiated with UV light to induce cross-linking of radiolabelled DNA to protein. Photocross-linked radiolabelled polypeptides were visualized by autoradiography after SDS-PAGE. Truncated L-Myc protein alone displayed no photocross-linked product (Fig. 3B). Max protein displayed a photocross-linked 22-kDa band, as previously reported (26). In the presence of Max and truncated c-Myc, photocross-linked bands corresponding to Max and truncated c-Myc were detected. It is notable that truncated c-Myc bands are more intense than Max bands, since there is an excess of truncated c-Myc which could bind DNA as homodimers and therefore c-Myc contributes to two populations. A doublet of bands corresponding to truncated c-Myc presumably resulted from a heterogeneity in the c-Myc protein (Fig. 3B). In contrast, since truncated L-Myc protein bound DNA only in the presence of Max, the ratio of photocross-linked Max to truncated L-Myc was about 1:1, a stoichiometry that suggests bound heterodimers.

Max augments cotransforming activities of L-Myc and c-Myc. It has been reported previously by Prendergast et al. (39) that Max can augment the cotransforming activity of c-Myc. Here, we sought to determine whether L-Myc-cotransforming activity can also be augmented by Max. A Max expression vector was cotransfected with L-*myc* or c-*myc* expression vectors along with a mutated *ras* gene driven by its own promoter. We confirm the observation that Max can augment the cotransforming activity of c-Myc (Fig. 4), but Max had no cotransforming activity of its own (data not shown). Similarly, the cotransforming activity of L-Myc can also be augmented twofold by exogenous Max (Fig. 4). These results suggest that endogenous Max may be limiting in these transformation assays and that exogenous Max can augment the cotransforming activities of either L-Myc or c-Myc. At higher input levels of *max* expression plasmid DNA but not control plasmid DNA, we have observed an inhibition of cotransformation by both L-Myc and c-Myc (3a); however, because we have not measured Max protein levels in these experiments, interpretations of the results are tentative.

c-Myc N-terminal sequences determine transforming potencies of hybrid L-Myc and c-Myc proteins. Since experiments with the GAL4-Myc fusions suggest that the L-Myc amino-terminal domain activates transcription more weakly than c-Myc, we hypothesized that the relative transforming activities of L-Myc and c-Myc are determined by the respective amino-terminal activation domains. The preceding data suggest that the carboxy-terminal domains of c-Myc and L-Myc function equivalently to heterodimerize with Max (or other potential partner proteins) in order to bind specific DNA sites. This hypothesis predicts that the amino-terminal domain determines the strength of transformation by chimeric proteins consisting of amino-terminal domains of either L-Myc or c-Myc fused to either the L-Myc or the c-Myc carboxy-terminal domain.

In order to test this hypothesis, we constructed chimeric

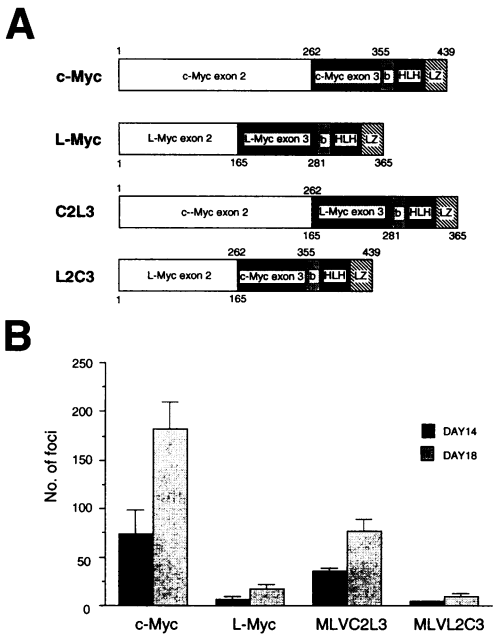

FIG. 5. Cotransforming activities of c-Myc, L-Myc, and chimeric c-Myc/L-Myc proteins. (A) Schematic depiction of c-Myc, L-Myc, and chimeric c-Myc/L-Myc proteins. Amino acids for c-Myc are given above the bars, and those of L-Myc are given below the bars. (B) Focus formation activities of Myc proteins in the presence of an activated *ras* gene. Error bars represent standard deviations from quadruplicate experiments. Numbers of foci 14 and 18 days posttransfection are shown. For these experiments, 5 μg of EJ*ras* plasmid DNA per plate and 10 μg of plasmid DNA encoding c-Myc, L-Myc, C2L3, or L2C3 per plate were cotransfected.

L-*myc* and c-*myc* genes by exchanging exons 2 (constituting activation domains) and 3 (constituting the DNA-binding domain) between c-*myc* and L-*myc*. The two chimeric proteins (L2C3 [L-Myc exon 2 and c-Myc exon 3] and C2L3 [c-Myc exon 2 and L-Myc exon 3]) produced by such fusion genes read in frame across exon-exon splice junctions (Fig. 5A). Plasmids encoding L2C3, C2L3, L-Myc, or c-Myc (each driven by the MLV LTR) were transfected along with an activated *ras* gene into rat embryo cells to assay their transforming activities. The numbers of transformed foci counted at both day 14 and day 18 after transfection were highest for c-Myc (Fig. 5B). C2L3 displayed significantly higher transforming activity than either L2C3 or L-Myc. In order to determine the presence of L-Myc, c-Myc, C2L3, and L2C3 proteins in transformed cells, transfectants were maintained until 45 days posttransfection to allow outgrowth of transformed cells. The transformed cells were then metabolically labelled with [^{32}P]orthophosphate, and immunoprecipitation with either an anti-c-Myc or anti-L-Myc antibody was performed. Both L-Myc and C2L3 were detectable with an anti-L-Myc antibody (see Materials and Methods), while c-Myc and L2C3 were detectable with the anti-c-Myc antibody. In each case, there was a specific immunoprecipitated phosphoprotein band with an apparent M_r of 60,000 to 66,000 (data not shown). Thus, these data suggest that the c-Myc amino-terminal domain determines the higher transforming potencies of c-Myc and C2L3, compared with those of L-Myc and L2C3.

DISCUSSION

It has been speculated that the efficiency of rat embryo cell focus formation by L-*myc* compared with that of c-*myc* may be due to an intrinsic property of the L-Myc protein, recipient cell type, or possibly a requirement for additional genetic events (5). Although we did not specifically address the last two possibilities, our results strongly suggest that the principal cause of the difference between L-*myc* and c-*myc* in the efficiency of focus formation lies in the amino-terminal domain of the proteins. The L-Myc amino-terminal domain, when fused to GAL4, exhibited an ability to activate transcription that is much weaker than that of the homologous region of c-Myc.

It is notable that the L-Myc amino-terminal region encoded by exon 2 is 165 amino acids smaller than the 252 amino acids encoded by c-*myc* exon 2. L-Myc lacks regions homologous to c-Myc amino acids 33 to 41, which is glutamine rich, and amino acids 72 to 105 within the c-Myc transcriptional activation domain (25). It appears likely that these differences could account for the difference in the ability of GAL4 fusions of L-Myc and c-Myc to activate transcription; however, there are additional significant differences between L-Myc and c-Myc outside the c-Myc activation domain amino acids 1 to 143. These differences may also contribute negatively to L-Myc activation potency. A growth factor-regulated MAP kinase site, Ser-62, has been identified in the c-Myc transactivation domain and is required for potent transactivation by GAL4–c-Myc fusions (46). Since this site is conserved in L-Myc and is also phosphorylated in vivo (43), the amino acid sequences outside this putative GSK-3 or MAP kinase site may also play an important role in determining the potency of transcriptional activation by Myc proteins. Moreover, whether Rb, which binds to the Myc activation domain in vitro, is able to regulate either the L-Myc or the c-Myc activation domain in vivo is not yet clear (42).

We examined the ability of the L-Myc carboxy-terminal bHLH-Zip region to interact with Max and to bind specifically to DNA in order to determine whether the amino-terminal activation domain alone accounts for the weak transforming activity of L-Myc. Using an intracellular assay to detect specific protein-protein interactions (14), we found that the bHLH-Zip domain of L-Myc behaves equivalently to that of c-Myc in binding to the bHLH-Zip domain of Max. Max-Max interaction was not detectable in this assay, as reported and discussed previously (26). Moreover, a bacterially produced L-Myc protein containing its bHLH-Zip domain can specifically bind to the core sequence CACGTG in the presence of purified Max protein. The finding that L-Myc/Max dimers can bind CACGTG substantiates the model of how bHLH proteins distinguish between related CANNTG sites (15, 37). This model predicts that any bHLH protein which contains an Arg residue (underlined in the following) in the basic region conserved sequence Glu-Arg-X-Arg-Arg (X, other amino acids) can bind to the core sequence CACGTG. This model, however, does not exclude the possibility that transcription factors bind to more than one distinct DNA sequence. For example, USF, which binds CACGTG, has been suggested to bind to another non-CANNTG sequence termed INR (41).

In contrast to truncated c-Myc, which can bind to CACGTG as a homodimer (26, 28), truncated L-Myc protein alone had no specific DNA-binding activity at the L-Myc protein concentrations used. Although truncated c-Myc can form homodimers in vitro, we could not detect c-Myc or

L-Myc bHLH-Zip homodimerization intracellularly. Thus, this in vitro difference between truncated c-Myc and L-Myc is unlikely to account for biological differences between the two proteins. In fact, we were able to demonstrate that Max can augment the cotransforming activities of both L-Myc and c-Myc. This observation suggests a functional interaction between Max and both Myc proteins in cell transformation. We cannot, however, exclude the possibility that other Max-like proteins may differentially affect the transforming activities of c-Myc and L-Myc.

We sought to localize the biological differences between L-Myc and c-Myc by constructing genes that express hybrid proteins consisting of heterologous amino-terminal and carboxy-terminal domains of L-Myc or c-Myc. These hybrids were tested for their ability to transform rat embryo cells in cooperation with a mutated *ras* gene. We found that full-length c-Myc and a chimera containing the c-Myc amino-terminal domain linked to the L-Myc carboxy-terminal domain (C2L3) were much more efficient in focus formation than either L-Myc or the chimera containing the L-Myc amino-terminal region (L2C3). It is not clear, however, why C2L3 transforming activity is less than that of wild-type c-Myc. Nevertheless, these results indicate that the amino-terminal domains of these Myc proteins contribute significantly to the efficiency of focus formation in rat embryo cells.

The results presented here demonstrate a correlation between the transcriptional activation potency of Myc proteins and their ability to cotransform rat embryo cells. These observations strongly support the hypothesis that Myc proteins function as regulators of gene transcription. Since rat embryo cells are heterogeneous and it is possible that the transcriptional activation domains are cell type specific, the difference between L-Myc and c-Myc focus formation activities may lie in the ability of the activation domains to function in specific cell types. Thus, if the L-Myc activation domain functions only in a small specific population of cells found in rat embryo cells, then the number of foci formed would be limited by the quantity of these cells. Nevertheless, the c-Myc activation domain appears to be more potent than that of L-Myc in both rat embryo cells and in CHO cells. Further evidence to support our simplified model of c-Myc and L-Myc activities in neoplastic transformation will require the identification of genetic targets of Myc proteins that are involved in transformation. The availability of such targets will permit the relative transactivation potencies of native L-Myc and c-Myc to be tested and directly correlated with their neoplastic cotransforming activities.

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