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B-Myc Inhibits Neoplastic Transformation and Transcriptional Activation by c-Myc

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B-*myc* is a recently described *myc* gene whose product has not been functionally characterized. The predicted product of B-*myc* is a 168-amino-acid protein with extensive homology to the c-Myc amino-terminal region, previously shown to contain a transcriptional activation domain. We hypothesized that B-Myc might also function in transcriptional regulation, although its role in regulating gene expression is predicted to be unique, because B-Myc lacks the specific DNA-binding motif found in other Myc proteins. To determine whether B-Myc could interact with the transcriptional machinery, we studied the transcriptional activation properties of a chimeric protein containing B-Myc sequences fused to the DNA-binding domain of the yeast transcriptional activator GAL4 (GAL4–B-Myc). We found that GAL4-B-Myc strongly activated expression of a GAL4-regulated reporter gene in mammalian cells. In addition, full-length B-Myc was able to inhibit or squelch reporter gene activation by a GAL4 chimeric protein containing the c-Myc transcriptional activation domain. We also observed that B-Myc dramatically inhibited the neoplastic cotransforming activity of c-Myc and activated Ras in rat embryo cells. Because B-Myc inhibits both neoplastic transformation and transcriptional activation by c-Myc, we suggest that the transforming activity of c-Myc is related to its ability to regulate transcription. Whether B-Myc functions biologically to squelch transcription and/or to regulate transcription through a specific DNA-binding protein remains unestablished.

The *myc* family of genes is known to include c-, N-, L-, s-, and, more recently, B-*myc* (2–3, 9, 16). c-*myc* is the best-characterized *myc* gene, although its specific role in normal cell growth and neoplastic transformation has not yet been elucidated. c-*myc* was originally identified as the cellular homolog of the viral oncogene v-*myc* (38, 43). The aberrant expression of c-*myc* appears to contribute to the pathogenesis of several human cancers, most notably Burkitt's lymphoma, in which a chromosomal translocation event presumably causes deregulated, constitutive c-*myc* expression. The c-*myc* gene encodes a short-lived nuclear phosphoprotein whose expression is increased with cellular proliferation (25). Recent evidence suggests that c-Myc may be involved in transcriptional regulation (1, 5, 10, 14, 15, 18, 20, 22, 30), although other studies suggest a role for c-Myc in DNA replication (1, 14, 15) or posttranscriptional regulation of gene expression (31). Mutational analysis of the c-Myc protein has led to the identification of two domains which are critical for the function of c-Myc in neoplastic transformation: an amino-terminal transcriptional-activation domain and a carboxy-terminal specific DNA-binding domain that includes the basic-helix-loop-helix-zipper motif (4–6, 8, 18, 21, 33). The c-Myc helix-loop-helix and leucine zipper dimerization motifs have recently been shown to mediate dimerization to the protein partner Max (5, 6, 32). The Myc family members L-, N-, and s-Myc also contain regions homologous to both the amino- and the carboxy-terminal domains of c-Myc.

B-*myc* is a recently described *myc* gene whose product has not been functionally characterized (2, 3, 9, 16). B-*myc* was first isolated from a rat genomic library on the basis of its homology to exon 2 of c-*myc*. B-*myc* is expressed in multiple tissue types, although the level of expression is highest in the brain (16). In contrast to c-*myc*, which is down-regulated in most adult tissues, B-*myc* is expressed at similar levels in fetal and adult tissues (16). B-*myc* encodes a unique Myc protein of 168 amino acids that has extensive homology to the amino-terminal 168 amino acids of c-Myc (Fig. 1) (2). As noted above, this amino-terminal region of c-Myc is required for neoplastic transformation of rat embryo cells (RECs) in cooperation with an activated *ras* gene (40). In addition, this region of c-Myc has been shown to activate transcription when fused to a heterologous DNA-binding domain (18, 22). The homology of B-Myc to the amino-terminal transcriptional activation domain of c-Myc suggests that B-Myc also functions to regulate transcription. However, the potential role of B-Myc in regulating gene expression is predicted to be novel among Myc proteins, because B-Myc lacks the basic-helix-loop-helix-zipper DNA-binding domain (Fig. 1) found at the carboxy terminus of other Myc proteins (8, 9, 17, 21, 25).

To determine whether B-Myc can interact with the transcriptional machinery, we studied the transcriptional activation properties of B-Myc sequences fused to a heterologous DNA-binding domain in a model system (18, 23). Here, we show that a chimeric protein composed of full-length B-Myc fused to the DNA-binding domain of the yeast transcriptional activator GAL4 (GAL4-B-Myc) functions as a transcriptional activator in mammalian cells. We identified a region at the carboxy terminus of B-Myc, where the B-Myc sequence diverges significantly from that of c-Myc, which appears to be important for the transcriptional activation properties of GAL4-B-Myc. Because B-Myc lacks a recognizable DNA-binding domain, we propose that B-Myc might regulate transcription by competitively binding or squelching

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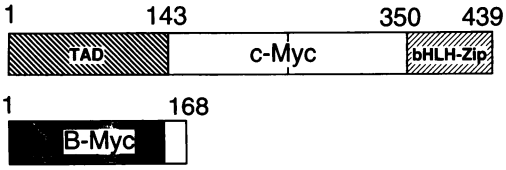

FIG. 1. c-Myc and B-Myc amino acid homology. B-*myc* encodes a predicted 168-amino-acid protein (lower bar) with extensive homology to the c-Myc transcriptional-activation domain (TAD; upper bar). The numbers above each bar indicate amino acid residues. The degree of homology between B-Myc and the c-Myc TAD region is indicated by the degree of shading of the lower bar, with the darkest shading indicating >80% amino acid homology and the lightest shading indicating <50% homology. Note that the B-Myc sequence diverges most from that of c-Myc at the carboxy terminus of B-Myc. bHLH-Zip, the basic-helix-loop-helix-leucine zipper-specific DNA-binding domain of c-Myc.

factors required by other Myc proteins for transcriptional regulation. In support of this model, we found that full-length B-Myc inhibits transcriptional activation by GAL4-c-Myc, but not by GAL4-VP16, a chimera containing the potent transcriptional activation domain of herpes simplex virus protein VP16. These results suggest that Myc proteins regulate transcription through a specific cellular factor or pathway that is distinct from that of acidic activators like VP16. Moreover, we show that B-Myc inhibits the neoplastic transforming activity of c-Myc in RECs. These observations are consistent with the hypothesis that transformation by c-Myc is related to its ability to regulate transcription.

MATERIALS AND METHODS

Plasmid constructions. pSV-B-*myc* (gift from W. Lee) encodes a full-length cDNA copy of B-*myc* (gift from S. Ingvarsson and G. Klein [16]) under the control of the simian virus 40 early promoter. pSV-B(1-120) encodes B-Myc truncated at an internal *Pst*I site (B-Myc amino acid codon 120) under the control of the simian virus 40 early promoter. This plasmid was created by partial digestion of pSV-B-Myc with *Pst*I and insertion of the double-stranded oligonucleotide (5'-AACCTAAGCTTAATGCA-3' and 5'-TTAAGCTTAGG TTTGCA-3') encoding stop codons at the B-Myc *Pst*I site. The full-length B-*myc* *Eco*RI-*Hin*dIII gene fragment from pSV-B-*myc* was subcloned into pBluescript II KS$^-$ (Stratagene), creating pBS-B-*myc*. A *Sma*I-*Sal*I gene fragment containing full-length B-*myc* sequences was retrieved by partial digestion of pBS-B-*myc* with *Sma*I and complete digestion with *Sal*I. This fragment was subcloned into pGALO, which has been previously described (7), to create the fusion gene pGAL4-B-*myc*, encoding GAL4 (amino acids 1 to 147) [GAL4(1-147)] in frame with full-length B-Myc. pGB(1-120) was created by truncating pGAL4-B-*myc* at the *Pst*I site (B-*myc* amino acid codon 120). The GAL4-c-*myc* fusion construct pGM(1-262) has been described elsewhere (18). pGAL4-VP16 and the reporter plasmid pG$_5$E1bCAT (both kindly provided by I. Sadowski and J. Lillie) have been described elsewhere (23). The pCH110 plasmid (Pharmacia), which constitutively expresses β-galactosidase, was used to control for transfection efficiency.

The expression vector pBXL-1, which encodes the DNA-binding domain of the bacterial repressor protein LexA (amino acids 1 to 202), and the vector pLexA-VP16, which encodes the LexA DNA-binding domain fused to the VP16 transcriptional-activation domain (amino acids 413 to 490), have been described elsewhere (27; both were gifts from K. Martin and M. Green). The plasmid pHyg, encoding a gene conferring hygromycin resistance under the transcriptional control of the herpes simplex virus thymidine kinase promoter, has been previously described (42).

Cell culture and transfections. DUKXBII Chinese hamster ovary (CHO) cells were passaged in alpha minimum essential medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The cells were transfected at 50% confluence by the DEAE-dextran technique as previously described (7, 18, 23).

Comparison of transcriptional activation by GAL4 chimeras. To compare the relative potencies of the various GAL4 activator chimeras, 2 μg of DNA from a plasmid encoding a GAL4 activator chimeric protein [GAL4-VP16, GAL4-B-Myc, GB(1-120), or GM(1-262)]; 2 μg of the reporter plasmid pG$_5$E1bCAT, which contains five GAL4 DNA-binding sites upstream of the E1b adenovirus TATA box and the chloramphenicol acetyltransferase (CAT) reporter gene; and 2 μg of the control plasmid pCH110 per 100-mm-diameter plate of CHO cells were transfected. In order to compare levels of reporter gene activation at relatively low amounts of input plasmid, each plasmid encoding a GAL4 activator chimera was cotransfected in 2-ng amounts with 2 μg each of the reporter and control plasmid pCH110. In these experiments, the quantities of transfected plasmid for the chimeras GAL4-VP16 and GAL4-B-*myc* were in the linear range of input GAL4 activator plasmid to CAT activation, and the activator protein levels were below that required to saturate the reporter plasmid GAL4 DNA-binding sites. Cells were harvested 48 h after dimethyl sulfoxide shock as previously described (7, 18, 23).

B-Myc and VP16 competition or squelching experiments. The linear ranges of CAT activation for the GAL4 activator plasmids pGM(1-262) and pGAL4-VP16 were determined by transfecting increasing amounts of each GAL4 activator chimera (from 1 ng to 2 μg) with 2 μg of the pG$_5$E1bCAT reporter construct. The plasmids encoding the GAL4 activator chimeras were then cotransfected within this linear range (at which absolute CAT activities were between 200 and 60,000 cpm) with 3 μg of the competitor plasmid, pSV-B-*myc*, which encodes full-length B-Myc, and 2 μg of the pG$_5$E1bCAT reporter plasmid. pBluescript II KS$^-$ (Stratagene) was added to each plate to keep the total quantity of DNA transfected per plate constant at 6 μg per plate. Cells were processed for CAT assays as described previously (7, 18, 23, 37). Similar transfection experiments were performed with either the expression vector pSV-B(1-120) or pLexA-VP16 as the competitor plasmid.

REC cotransformation assay. RECs were harvested and grown as previously described (7, 40). For cotransformation assays, 2×10^5 RECs in 100-mm-diameter plates were transfected with 5 μg of wild-type c-*myc* plasmid and 5 μg of pEJ*ras* plasmid with Lipofectin (Bethesda Research Laboratories, Gaithersburg, Md.) as previously described (7, 40). To determine the effect of B-Myc on transformation in RECs, 15 μg of pSV-B-*myc* was transfected in addition to 5 μg each of the c-Myc- and EJRas-expressing plasmids. This experiment was repeated with either pSV-B(1-120) or the GAL4 activator chimeras [pGAL4-B-*myc*, pGB(1-120), pGM(1-262), or pGALO] as competitor plasmids with the c-*myc* and pEJ*ras* plasmids. Two to four plates per plasmid combination were used, and transformed foci were counted on days 10, 12, 14, 16, and 18 after transfection.

To determine whether any of the plasmids used in the REC cotransformation assay were toxic to growth, RECs

were cotransfected with each of the competitor plasmids [pSV-B-*myc*, pSV-B(1-120), pGAL4-B-*myc*, pGB(1-120), pGM(1-262), and pGALO] and pHyg by using Lipofectin as described elsewhere (7, 40). Ten micrograms of competitor plasmid was transfected with 1 μg of pHyg per flask in an effort to ensure that all hygromycin-resistant colonies also expressed competitor plasmid. Each transfection experiment was performed in duplicate. Cells were maintained in Dulbecco's modification of Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Forty-eight hours after transfection, hygromycin (12.5 μg/ml) was added to the medium. The medium was changed approximately every 48 h, and resistant colonies were counted 21 days after transfection after being stained with crystal violet.

CAT and β-galactosidase assays. CAT assays were performed by the phase extraction technique with butyryl coenzyme A as described elsewhere (37). β-Galactosidase assays were also performed as previously described (36).

Immunoprecipitation experiments. To estimate the level of expression of each GAL4 activator chimera, immunoprecipitation experiments with an anti-GAL4 antibody (gift from I. Sadowski) in transfected CHO cells were performed. The CHO cells were metabolically labeled with [^{35}S]methionine (200 μCi/ml) as previously described (7, 18, 23).

RESULTS

Transcriptional activation properties of GAL4-B-Myc. To study the transcriptional-activation properties of B-Myc, we constructed a fusion gene encoding the DNA-binding domain of the yeast transcriptional activator GAL4(1-147) fused to full-length B-Myc (pGAL4-B-*myc*). The transcriptional activation properties of GAL4-B-Myc were compared with those of GAL4(1-147) alone, expressed from pGALO, and to those of the GAL4-c-Myc chimera [pGM(1-262)] encoding the GAL4 DNA-binding domain fused to the c-Myc transcriptional-activation domain (amino acids 1 to 262). The strength of transcriptional activation by GAL4-B-Myc was also compared with that of GAL4-VP16, which contains the potent herpes simplex virus VP16 transcriptional activation domain (amino acids 413 to 490). The various GAL4 activator chimeras were transfected into CHO cells with the reporter plasmid pG$_5$E1bCAT, containing five tandem GAL4 DNA-binding sites upstream of the adenovirus E1b TATA box and the CAT gene. To control for transfection efficiency, the eukaryotic expression vector pCH110, which constitutively expresses β-galactosidase, was included in these transfection experiments, and all CAT activities were normalized to β-galactosidase activity (36). To control for variations in protein expression of the chimeric GAL4 proteins, immunoprecipitation experiments were performed and protein quantity was estimated by densitometry of the autoradiograms. Previous immunoprecipitation experiments from our laboratory have shown comparable levels of expression of protein from pGALO and pGM(1-262) (18). We therefore performed immunoprecipitation experiments that compared the expression of pGM(1-262) with those of pGAL4-VP16 and the pGAL4-B-*myc* constructs (Fig. 2). CAT assay results were then normalized to the relative quantities of protein expressed, and the CAT activity obtained with pGALO was arbitrarily assigned an activity value of 1. The results (Fig. 3A) show that GAL4-B-Myc can activate transcription, indicating that B-Myc sequences bound to DNA through a heterologous DNA-binding domain can increase basal transcription from a minimal TATA box. When pGAL4-B-*myc* was transfected in 2-μg and 2-ng amounts, it was approximately 1.5 times more active than the GAL4-c-Myc chimera, pGM(1-262) (Fig. 3A and B).

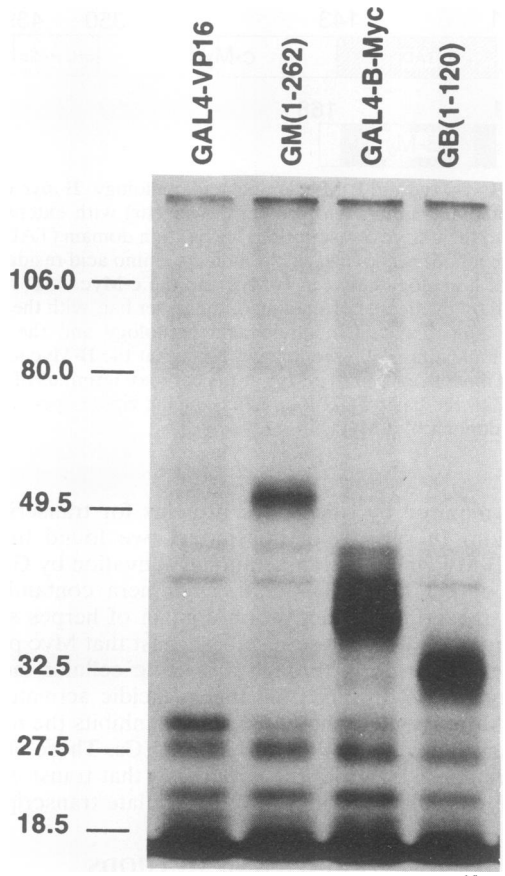

FIG. 2. Protein levels of GAL4 activator chimeras. [^{35}S]methionine-labeled GAL4 chimeric proteins from transfected CHO cells were immunoprecipitated with an anti-GAL4(1-147) antibody and separated on a sodium dodecyl sulfate-10% polyacrylamide gel. The GAL4 activator chimeras are indicated above the lanes, and the positions of prestained molecular markers (in kilodaltons) are indicated in the left margin.

Regions of B-Myc required for activation. To determine which sequences of GAL4-B-Myc made it transcriptionally more active than the comparable GAL4-c-Myc chimera, we truncated GAL4-B-Myc to remove the 48 carboxy-terminal amino acids of B-Myc [GB(1-120)]. This carboxy-terminal region of B-Myc includes the amino acid sequences where B-Myc diverges from c-Myc. We found that GB(1-120) had approximately 0.6-fold less transcriptional activity than GM(1-262) (Fig. 3A and B). The results obtained with this chimera suggest that the 48 carboxy-terminal amino acids are necessary for the increase in the transcriptional activity of GAL4-B-Myc compared with that of GAL4-c-Myc.

Evidence for a Myc-specific cellular target or pathway for transcriptional regulation. Because B-Myc lacks a recognizable DNA-binding motif, we hypothesized that B-Myc could competitively bind or squelch limiting factors required by Myc proteins to regulate transcription. We therefore performed experiments to determine whether full-length B-Myc (lacking the GAL4 DNA-binding domain) could squelch transcriptional activation by GAL4-c-Myc [GM(1-262)] or

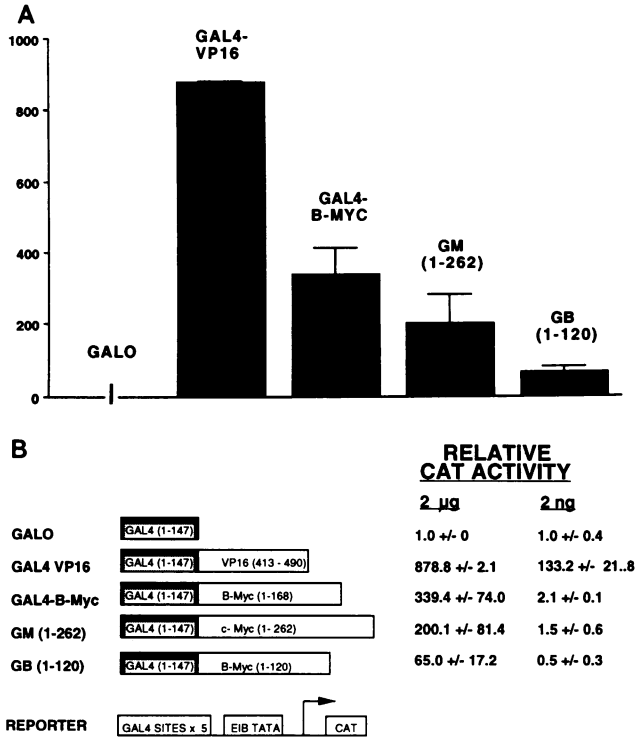

FIG. 3. Relative transcriptional activities of the GAL4 activator chimeric proteins. Relative CAT activities for the GAL4 activator chimeras (2 µg) (A) and a schematic representation of these chimeric activators (B) are shown. Each bar in panel A shows the mean value of CAT activation by each GAL4 activator from two separate transfection experiments; the error bars denote the standard deviations. The CAT activities of the various activators were normalized against that of GALO, which was arbitrarily assigned a value of 1. All values were also corrected for transfection efficiency and protein expression. The GAL4 activator chimeras (B) were also transfected in only 2-ng amounts in order to compare their transcriptional activities at relatively low amounts of input activator plasmid. The results are expressed as the mean CAT activity plus or minus the standard deviation from two separate transfection experiments.

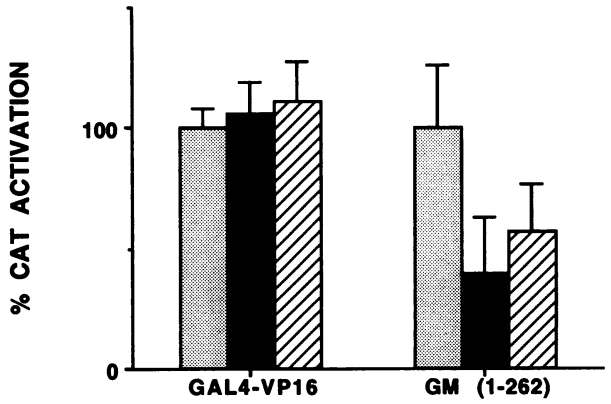

FIG. 4. B-Myc competition with GAL4-VP16 or GM(1-262). The GAL4 activator chimeras GAL4-VP16 and GM(1-262) were cotransfected with the control plasmid DNA (pBluescript KS^-; indicated by light shading), the competitor plasmid pSV-B-*myc* (indicated by dark shading), or the competitor plasmid pSV-B(1-120) (indicated by hatched shading). For each GAL4 activator chimera, the CAT activity with control plasmid DNA was assigned an activity value of 100%. Each bar shows the mean percentage of CAT activation from two to four separate transfection experiments; the error bars indicate the standard deviations. Note that B-Myc significantly inhibits CAT transcription by GM(1-262) ($P = 0.002$ [Student's t test]) but not by GAL4-VP16. Truncated B-Myc, SV-B((1-120), also inhibited CAT transcription by GM(1-262), but to a lesser extent ($P = 0.05$ [Student's t test]). Truncated B-Myc did not inhibit CAT transcription by GAL4-VP16.

GAL4-B-Myc (12, 26–28, 34). The results demonstrate that B-Myc, expressed from pSV-B-*myc*, can effectively inhibit or squelch CAT transcription by GAL4-c-Myc [GM(1-262)] (Fig. 4). These experiments were repeated with truncated B-Myc, pSV-B(1-120), as the competitor plasmid. The results show that truncated B-Myc also squelches transcription by GAL4-c-Myc, although to a lesser extent (Fig. 4).

Both c-Myc and B-Myc have unusual transcriptional activation domains that are characterized by a slightly acidic region rich in glutamine and proline amino acid residues. Thus, we speculated that these Myc proteins might interact with the transcriptional machinery through a unique cellular target(s) or pathway. To characterize further this potential cellular target or pathway, we performed experiments to determine whether B-Myc could squelch transcriptional activation by GAL4-VP16. We found that full-length and truncated B-Myc were unable to squelch transcriptional activation by GAL4-VP16, suggesting that the Myc transcriptional-activation domain interacts with the basal transcriptional machinery through a unique cellular target(s) or pathway not required by the VP16 transcriptional-activation domain in this model system.

Lastly, we sought to determine whether the VP16 transcriptional activation domain could squelch transcriptional activation by GAL4-c-Myc [GM(1-262)] or GAL4-B-Myc. For these experiments, we used the expression vector LexA-VP16, which encodes the LexA DNA-binding domain fused to the VP16 transcriptional-activation domain (amino acids 413 to 490), because previous studies have shown that LexA-VP16 effectively inhibited transcriptional activation by GAL4-VP16 (27). We found that the LexA-VP16 protein inhibits transcriptional activation by GAL4-B-Myc, GAL4-c-Myc, and GAL4-VP16 (Fig. 5). No inhibition was seen when pBXL, which encodes the LexA DNA-binding domain alone, was cotransfected with the GAL4 activator chimeras and the reporter construct, demonstrating that the inhibition observed was specific to the VP16 transcriptional-activation domain (data not shown). These results suggest that the VP16 cellular target or pathway is common to the target or pathway used for transcriptional activation by Myc proteins in this in vivo system. This target or pathway must act distally to the putative target(s) utilized by the GAL4-Myc proteins in this system and could include factors present in the basal transcriptional machinery (see Fig. 7 and see discussion). Of note, LexA-VP16 inhibited transcription by GAL4-VP16 by over 90%. In contrast, LexA-VP16 inhibited transcription by GAL4-B-Myc and GAL4-c-Myc to a lesser extent (Fig. 5).

B-Myc inhibits the cotransforming activity of c-Myc in RECs. Previously, it has been shown that the c-*myc* gene cooperates with an activated *ras* gene (EJ*ras*) to transform RECs (40). To determine whether B-Myc could compete for factors utilized by c-Myc in neoplastic transformation and thereby inhibit this activity, we cotransfected plasmids expressing B-Myc with plasmids expressing c-Myc and EJRas into RECs. The plasmids pSV-B-*myc*, pGAL4-B-*myc*, pSV-B(1-120), and pGB(1-120) were used. Control experiments with pGALO and the B-*myc* deletion mutant [pGB(1-120)] as

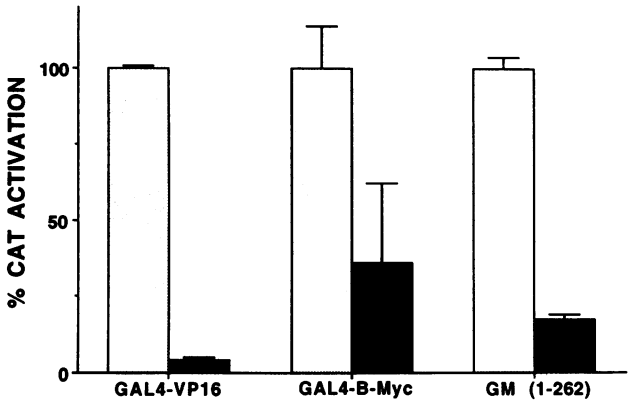

FIG. 5. VP16 activation domain competition with GAL4-VP16, GAL4-B-Myc, and GM(1-262). The GAL4 activator chimeras were cotransfected with the control plasmid DNA (light shading) and with the competitor plasmid LexA-VP16 (dark shading). The CAT activation obtained with the GAL4 activator and control plasmid DNA was assigned an activity value of 100%. Each bar represents the mean percentage of CAT activation from two to four separate transfection experiments; each error bar indicates the standard deviation. Note that LexA-VP16 significantly inhibits CAT transcription by all activators (GAL4-VP16 [$P = 0.03$; Student's t test], GAL4-B-Myc [$P = 0.02$; Student's t test], and GAL4-c-Myc [$P = 0.02$; Student's t test]), although it inhibits GAL4-VP16 to the greatest extent (>90%).

a competitor plasmid were performed. We also determined whether the c-Myc transcriptional-activation domain could inhibit its own transforming activity by cotransfecting the GAL4-c-*myc* chimera [pGM(1-262)] with c-*myc* and EJ*ras*. Our results demonstrate that both B-Myc alone and the GAL4-B-Myc fusion protein dramatically lowered (>95% for GAL4-B-Myc and >75% for pSV-B-Myc) the number of foci formed by c-Myc and mutated Ras in RECs (Fig. 6). In contrast, the plasmid expressing GAL4(1-147) had no effect

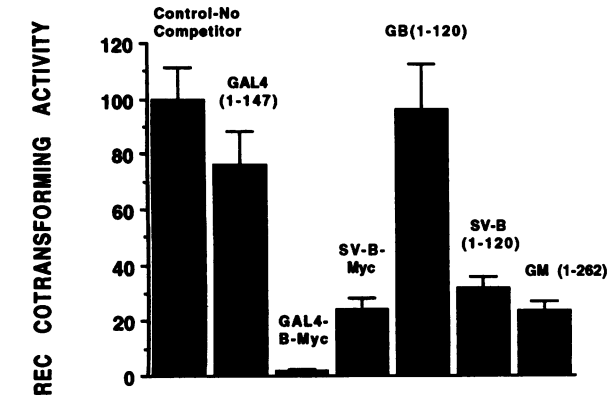

FIG. 6. REC cotransformation assay. REC-cotransforming activity is shown for RECs transfected with plasmids expressing c-Myc and Ras alone and c-Myc, Ras, and competitor plasmids expressing the following proteins: GAL4(1-147), GAL4-B-Myc, SV-B-Myc, GB(1-120), pSV-B(1-120), and GM(1-262). REC-cotransforming activity is defined as the number of transformed foci formed by day 18 and normalized to that of c-Myc and Ras alone, which was assigned a value of 100. Each bar shows the mean cotransforming activity per plate on day 18 from two to four transfection experiments; each error bar shows the standard deviation.

on REC transformation, indicating that the observed suppression in transformation by SV-B-Myc and GAL4-B-Myc was mediated by B-Myc alone. The truncated B-Myc construct, pSV-B(1-120), caused moderate suppression in focus formation (58% fewer foci formed), although the GAL4-B-Myc deletion mutant [GB(1-120)] had no significant effect on transformation. The GAL4-c-Myc fusion construct [pGM(1-262)] produced a moderate suppression in focus formation (60% fewer foci formed; Fig. 6).

To determine whether any of the competitor plasmids used in the REC cotransformation assay were toxic to cell growth and thereby interfered with transformation on that basis, RECs were cotransfected with 1 μg of a plasmid conferring hygromycin resistance (pHyg) and 10 μg of each competitor plasmid [pSV-B-*myc*, pSV-B(1-120), pGAL4-B-*myc*, pGB(1-120), pGM(1-262), and pGALO]. Transfection with each competitor plasmid and pHyg produced a similar number of hygromycin-resistant colonies (average number of colonies per plate, 27). There were no significant differences in the numbers of hygromycin-resistant colonies produced from each combination of competitor plasmid and pHyg (data not shown). This suggests that the inhibition in transformation observed with pSV-B-*myc* and pGAL4-B-*myc* was a specific effect and was not related to a toxic effect inherent in the plasmid constructs or their protein products in RECs.

DISCUSSION

Transcriptional activation by Myc proteins. We observed that when full-length B-Myc sequences were fused to the GAL4 DNA-binding domain, they exhibited strong transcriptional-activation properties in transfected CHO cells. The GAL4-B-Myc fusion protein was transcriptionally more active than the GAL4-c-Myc chimera [GM(1-262)]. GAL4-B-Myc is also more active than GAL4-c-Myc chimeras containing shorter amino-terminal sequences of c-Myc. Specifically, GAL4-c-Myc chimeras containing amino acids 1 to 103 [GM(1-103)] and amino acids 1 to 143 [GM(1-143)] have previously been shown to have activities similar to those of GM(1-262) (18). Both the B-Myc and the c-Myc proteins have unique features characterizing their transcriptional-activating regions. Unlike the VP16 transcriptional-activation domain, which includes highly acidic sequences, B-Myc and c-Myc transcriptional-activation domains include slightly acidic regions rich in glutamine and proline amino acids. The sequence of B-Myc is 76% identical to the amino-terminal 145 amino acids of c-Myc and 67% identical to the first 168 amino acids (Fig. 1). There is no sequence homology to c-Myc in the carboxy-terminal amino acids 143 to 168 of B-Myc (2, 3). As demonstrated by the decrease in the transcriptional activity observed with the deletion mutant [GB(1-120)], the carboxy-terminal region where B-Myc and c-Myc sequences diverge appears to be necessary for the stronger transcriptional-activation properties of the chimera GAL4-B-Myc. However, the region truncated in the mutant GB(1-120) included a portion of the conserved amino acid sequences in B-Myc (amino acids 120 to 142), as well as the divergent sequences (amino acids 143 to 168).

Evidence for a Myc-specific cellular target or pathway utilized to regulate transcription. We speculate that the ability of B-Myc to inhibit transcriptional activation by GAL4-c-Myc is due to sequestration of a unique, limiting cellular target(s) required by Myc proteins to regulate transcription in this model system (Fig. 7). This target could be a single factor or a combination of factors that lead into a

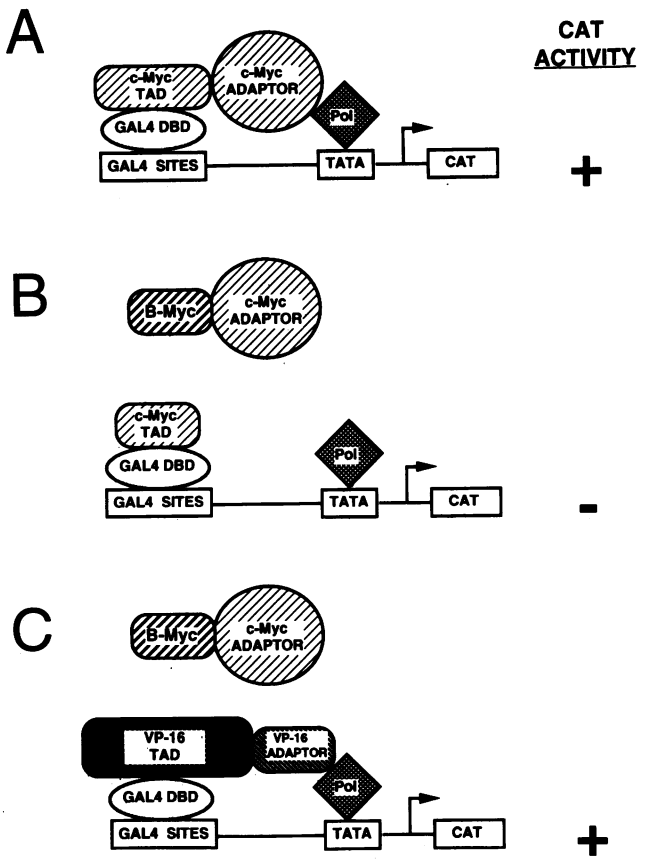

FIG. 7. Model depicting transcriptional activation by GAL4-c-Myc through a unique cellular factor(s) or adaptor. (A) GAL4-c-Myc interacting with the basal transcriptional machinery (indicated by Pol) through a c-Myc specific adaptor. The adaptor is shown interacting with the basal transcriptional machinery at the TATA box and thus driving CAT transcription. (B) B-Myc shown to competitively bind or squelch the adaptor(s) required by GAL4-c-Myc to drive CAT transcription. (C) VP16 adaptor not squelched by B-Myc. This proposed VP16 adaptor must act distally to that utilized by Myc proteins and could include factors within the basal transcriptional machinery, such as TFIIB or TFIID. TAD, transcriptional-activation domain; DBD, DNA-binding domain.

unique pathway that interacts with the basal transcriptional machinery. Because B-Myc was unable to inhibit transcriptional activation by GAL4-VP16, we hypothesize that this target is not required by acidic activators such as VP16 (12, 26–28, 34). Alternatively, GAL4-VP16 perhaps has a higher affinity for this factor(s) and therefore is not squelched by B-Myc, although our data do not support this possibility. Specifically, our results demonstrating that LexA-VP16 inhibited transcriptional activation by the GAL4-VP16 chimera to a greater extent than it inhibited transcription by the GAL4-Myc chimeras is consistent with the idea that Myc proteins utilize a unique target(s). If VP16 had a higher affinity for this putative target required by Myc proteins, it would be expected to inhibit transcription by the GAL4-Myc proteins to a greater extent than the GAL4-VP16 chimera. However, we predicted that VP16 would be able to inhibit or squelch activation by the GAL4-Myc proteins because recent in vitro studies have shown that the VP16 transcriptional activation domain may interact directly with at least two basal transcription factors, TFIIB and TFIID (11, 24, 26, 35, 41). Thus, overexpression of VP16 and subsequent sequestration of these necessary factors would be expected to inhibit transcription by general transcriptional activators that depend on these TATA box-binding factors. In fact, VP16 has been shown to inhibit transcriptional activation by other acidic activators, such as GCN4 and E1A, in experimental systems similar to the system used here (Fig. 7) (11, 27).

B-Myc inhibits c-Myc-mediated transformation of RECs. The c-Myc transcriptional activation domain is necessary for neoplastic cotransformation of RECs (40). Thus, it has been postulated that c-Myc's transforming activity is dependent on its ability to regulate transcription (18). Our results show that B-Myc can inhibit c-Myc's transforming activity in RECs, possibly by competing for factors necessary for c-Myc in the regulation of genes important in cellular growth. Both B-Myc and GAL4-B-Myc inhibited transformation, although GAL4(1-147) had no effect, indicating that this inhibition was mediated by B-Myc. Because GM(1-262) and GB(1-120) produced less transcriptional activation than GAL4-B-Myc in our model system, we predicted that they would inhibit transformation less effectively. Indeed, we observed that the degrees of inhibition by the GAL4 activator chimeras were proportional to their transcriptional activities. SV-B(1-120) inhibited both transcriptional activation by GAL4-c-Myc and neoplastic cotransformation by c-Myc and Ras, although to a lesser extent than full-length B-Myc. Of note, the truncated GAL4-B-Myc chimera GAL4-B(1-120) and the truncated B-Myc construct pSV-B(1-120) had different activities. Specifically, GAL4-B(1-120) had no significant transcriptional activity and likewise did not inhibit transformation by c-Myc and Ras. In contrast, SV-B(1-120) inhibited transcriptional activation by GAL4-c-Myc and also inhibited transformation by c-Myc and Ras. GAL4-B(1-120) may have acted in a manner different from that of SV-B(1-120), because fusion with the GAL4 DNA-binding domain in some way altered the protein's function, possibly by interfering with its native conformation (13).

The REC cotransfection studies with the hygromycin resistance gene failed to show any toxic effect inherent in the competitor plasmids or their protein products, suggesting that the observed inhibition in transformation was a specific effect. Thus, our results further support the hypothesis that c-Myc's transforming activity is dependent on its ability to regulate transcription.

Potential biologic role for B-Myc. DNA-bound B-Myc can activate transcription in a model system and it may also function in vivo to regulate transcription. Since B-Myc lacks the basic region and dimerization motifs found in other Myc proteins (Fig. 1), it may regulate expression of specific genes by interacting with another DNA-binding protein in a fashion analogous to that of VP16, whose site-specific DNA-binding effects on gene regulation are mediated by the Oct-1 protein (26, 39). Finally, B-Myc might also function to squelch transcriptional activation by Myc proteins or other activators that utilize a similar pathway for transcriptional regulation (Fig. 7). B-Myc's localization in the brain, in which DNA replication and cellular division are limited, suggests a potential role for B-Myc in the inhibition of other Myc proteins involved in cellular proliferation. Perhaps the truncated form of L-Myc, which is composed only of a region homologous to the L-Myc transcriptional-activation domain, may also function to inhibit transcriptional regulation by L-Myc (19, 29). Although our studies demonstrate that B-Myc can regulate transcription in a model system and provide evidence for a specific cellular factor(s) utilized by

Myc proteins in transcriptional regulation and transformation, the biologic function of B-Myc remains unestablished.

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