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An Essential Domain of the c-Myc Protein Interacts with a Nuclear Factor That Is Also Required for E1A-Mediated Transformation

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Cell transformation by nuclear oncogenes such as c-*myc* presumably involves the transcriptional activation of a set of target genes that participate in the control of cell division. The function of a small evolutionarily conserved domain of the c-*myc* gene encompassing amino acids 129 to 145 was analyzed to explore the relationship between cell transformation and transcriptional activation. Deletion of this domain inactivated the c-*myc* oncogene for cell transformation while retaining the ability to activate transcription of either *myc* consensus binding sites or a GAL4-dependent promoter when the c-*myc* N-terminus was fused to the GAL4 DNA-binding domain. Point mutations that altered a conserved tryptophan (amino acid 136) within this domain had similar effects. Expression of the wt c-Myc N terminus (amino acids 1 to 262) as a GAL4 fusion was a dominant inhibitor of cell transformation by the c-*myc* oncogene, and this same domain also inhibited transformation by the adenovirus E1A gene. Surprisingly, deletion of amino acids 129 to 145 eliminated the dominant negative activity of GAL4-Myc on both c-*myc* and E1A transformation. Expression of the GAL4-Myc protein in Cos cells led to the formation of a specific complex between the Myc N terminus and a nuclear factor, and this complex was absent with the *dl*129-145 mutant. These results suggest that an essential domain of the c-Myc protein interacts with a specific nuclear factor that is also required for E1A transformation.

Mutations that disrupt the regulation or expression level of the c-*myc* gene are frequently found in human and animal cancers (reviewed in references 10, 30, and 32). The c-*myc* gene is a member of a small family of genes with basic, helix-loop-helix, and leucine zipper domains that all encode sequence-specific DNA-binding proteins (9). Myc dimerizes with Max and recognizes the core sequence CACGTG (8, 41) but exhibits somewhat higher affinity for the more extended sequence ACCACGTGGT (4, 19). Despite many years of intensive effort, only one cellular gene (the ornithine decarboxylase, [ODC] gene) which may be directly regulated by c-*myc* (4) and which appears to mediate part of the effect that *myc* expression has on cell growth and differentiation has been identified (38). However, nontransforming mutants of the c-*myc* gene can still transactivate the ODC promoter, and mutants that are defective for transactivation of the ODC gene can still transform (4), so the precise role of ODC in c-*myc* transformation remains unclear. Ectopic expression studies define numerous biological activities of the c-*myc* gene, including transformation, immortalization, blockage of cell differentiation, and induction of apoptosis (13, 30). This bewildering array of biological activities makes the c-*myc* gene one of the most intriguing oncogenes and a challenge to understand how a single gene can manifest so many different effects.

Since the Myc proteins bind to specific DNA sequences, the mechanism of cell transformation can be viewed broadly as that of a transcription factor which binds to target genes and presumably increases transcription. Since few potential target genes are known (4, 15), it is not clear if the diverse activities of c-*myc* result from a common set of effector targets which have distinct effects dependent on cellular context or if different effectors mediate the different responses. Early mutagenesis studies that disrupted the DNA-binding domain eliminated the transforming activity of c-*myc*, consistent with a role for c-Myc as a transcription factor (53). Also consistent with a role for c-Myc as a transcription factor is the observation that the c-Myc N terminus contains a domain which can activate transcription of a synthetic reporter gene. This activity is rather weak (usually 3- to 5-fold) when c-*myc* is assayed on its own binding sites (1, 2, 18, 23, 43, 44) and somewhat stronger (20-fold) when c-*myc* is assayed as a GAL4 fusion (22). Thus, the c-Myc protein has all of the hallmarks of a transcription factor, presumably with a specific function in the progression of cells through the cell cycle.

In lieu of a collection of c-*myc* target genes, the only probe that is useful to investigate the DNA-binding properties of the protein is a synthetic one derived from the enrichment of sequences with the highest binding affinity (ACCACGTGGT or close approximations). The use of this sequence in mobility shift assays with cellular extracts reveals an added complexity in understanding the activation of specific target genes. Numerous cellular proteins that bind to this site can be identified, but c-Myc represents only a very minor species that is usually masked by proteins with greater abundance and/or apparent DNA-binding activity (5, 28). The best-characterized of the proteins that bind to the same sequence as c-Myc are upstream stimulatory factor (USF) (16), TFE3 (3), and TFEB (14), all of which have similar basic, helix-loop-helix, and leucine zipper domains. Subtle differences in DNA recognition specificity probably exist among these proteins, but they all bind avidly to the core CACGTG motif. Thus, for c-*myc* to activate specific targets, there must be a mechanism that allows genes to be distinguished by different potential transcription factors. This might involve currently unrecognized variations in the DNA sequences recognized by each protein (7) or more complex

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features of the target promoters such as the synergistic activities of additional DNA-binding proteins.

The c-*myc* gene is not the only oncogene with a wide range of biological activities linked to cell transformation. The adenovirus E1A gene exhibits a pattern of activities remarkably similar to those of c-*myc* (reviewed in reference 52). Both genes can immortalize primary cells and transform in cooperation with other oncogenes. Moreover, both genes can block cell differentiation, reduce growth factor dependence, and induce apoptosis (11, 29, 52). However, biochemically, E1A and c-Myc are apparently very different. Unlike c-Myc, E1A is not a sequence-specific DNA-binding protein but instead binds tightly to cellular proteins that in turn control phases of the cell cycle (reviewed in reference 52). The best characterized of the E1A-binding proteins is the retinoblastoma protein (Rb), which is reversibly phosphorylated during the cell cycle and participates in the decision to enter into S phase (reviewed in reference 57). E1A binding to Rb dissociates Rb from the transcription factor E2F, which plays a role in cell cycle progression (reviewed in reference 36).

We set out to characterize the function of a small, N-terminal, evolutionarily conserved domain of the c-Myc protein that is essential for cell transformation. This sequence (termed Myc homology box 2 [MB2]) is apparently unique to the Myc family of proteins, and its tight conservation among all *myc* family genes implies that it has a critical role in Myc protein function. It seems highly likely from the conservation of this domain that it provides an essential contact face for another cellular protein with which c-Myc interacts in both normal and transformed cells. The c-Myc protein is known to bind to both p107 and TATA-binding protein (18, 31), but neither of these interactions appears to require MB2. We are interested in defining the function of this domain as a means of understanding how the c-*myc* gene can transform cells as well as control growth and differentiation. We show here that although the MB2 domain is essential for cell transformation, it does not contribute to the ability of c-Myc to activate the transcription of synthetic promoters. Furthermore, this same domain can act as a dominant inhibitor of E1A-mediated transformation and interact with a nuclear factor(s), suggesting that c-*myc* and E1A may have a common cellular effector pathway.

MATERIALS AND METHODS

DNA constructs. The cytomegalovirus (CMV) promoter-driven mouse c-*myc* expression vector used in transformation assays has been described previously (6). Mutations of the c-*myc* gene were constructed by PCR-mediated mutagenesis of the subcloned *Eco*RV-*Sac*II fragment. Mutations were sequenced and exchanged with the wild-type (wt) fragment in the expression vector. The *dl*181-272 mutant was created by fusion of a PCR product with an endpoint at amino acid 272 to the *Pst*I site at amino acid 181. The *dl*181-321 mutant was created by ligation of the *Pvu*II site at amino acid 321 to the *Pst*I site at amino acid 181. The VP16-*myc* fusion was created by first adding an ATG upstream of the VP16 transactivation domain (amino acids 358 to 477), which was kindly provided by D. Olson. This VP16 segment was then fused to the DNA-binding domain of the mouse c-*myc* gene at amino acid 321 (*Pvu*II) and subcloned into the CMV expression vector. The adenovirus type 5 E1A plasmid, p1A, was originally obtained from J. Logan and T. Shenk. The H-*ras*$^{Val\text{-}12}$ oncogene was obtained from Cathy Finlay.

GAL0 (GAL4 amino acids 1 to 147) and GALM (GAL4 amino acids 1 to 147 fused to human c-*myc* amino acids 1 to 262) plasmids were generous gifts of C. V. Dang. The mouse c-*myc* gene and mutated derivatives were introduced into GALM by exchanging a fragment bounded by restriction sites (*Eco*RV-*Sac*II) that are conserved between mouse and human genes. The wt mouse c-*myc* exchange was done as a control for the small number of amino acid differences that exist between human and mouse c-*myc* in this region. Human GALM and the mouse-human chimeric GAL4-Myc gave identical results in all assays.

The reporter plasmid used for assaying transcriptional enhancement contained the chloramphenicol acetyltransferase (CAT) gene driven by a minimal adenovirus E4 promoter and five GAL4 binding sites (a gift of D. Reinberg). To assay transactivation by c-*myc*, we introduced four copies of a Myc consensus binding site (GACCACGTGGTC) between the five GAL4 DNA-binding sites and the minimal promoter. All DNAs used in cell transfections were prepared by cesium chloride banding or by column purification (Qiagen), and at least two different DNA preparations of each construct were independently transfected. For the rat embryo fibroblast (REF) transformation dominant negative analyses, at least four different preparations were tested.

Cell culture and transformation assays. Primary REFs were prepared from 14- to 15-day-old Fisher 344 rat embryos, or frozen ampoules were purchased from BioWhittaker. REFs and Cos-1 cells were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (Gibco). HeLa cells were grown in Dulbecco's minimal essential medium with 10% calf serum. For transformation assays (26), subconfluent REF (2×10^5 to 7×10^5 cells per plate) were fed with fresh culture medium 2 to 4 h before transfection. The designated c-*myc* gene (1 μg) was cotransfected with 1 μg of an activated H-*ras* gene (H-*ras*$^{Val\text{-}12}$) and 18 μg of carrier plasmid (pBluescriptKS$^-$) by calcium phosphate coprecipitation. The cells were washed twice with phosphate-buffered saline and fed with fresh medium 8 to 12 h posttransfection. The cells were refed every 4 to 6 days, and the number of transformed foci was determined 2 to 3 weeks posttransfection. Morphologically transformed cells were visible within 7 to 10 days following transfection. Transformation by the adenovirus E1A gene was determined by transfecting REFs as described above except that 1 μg of p1A was included in place of c-*myc*. Typical transformation assays gave 70 to 100 foci per 8-cm-diameter dish for both c-*myc* and E1A. To assay the effect of various GAL4 constructs on wt c-*myc* or E1A transformation, the transfections were carried out as described above except that the test construct was titered into the transfection in place of carrier plasmid.

Transcriptional enhancement assays. Subconfluent cells were fed with culture medium 1 to 2 h before transfection. The amount of activator plasmid (usually 8 μg) was transfected as described above with 2 μg of reporter construct, 2 μg of pRSV-βGAL, and carrier plasmid (pKS$^-$) for a total of 18 μg. The cells were harvested 2 to 5 days after transfection, and the extract was prepared and analyzed (49). CAT assays were normalized for equivalent transfection efficiency based on β-galactosidase activity. To assess transactivation by the c-*myc* gene, the c-*myc* cDNA and MB2Δ mutant were cloned into an expression vector driven by the murine sarcoma virus long terminal repeat (pEMSV). This was necessitated because the CMV expression vector (and derivatives) had a severe squelching effect on transient CAT assays in our experiments.

Protein complex analysis. The GAL0 or GAL4 fusion expression plasmids (20 μg) were transfected into Cos cells, and nuclear and cytoplasmic extracts were prepared after 48 h (27). HeLa cell nuclear extracts (NEs) were prepared as described previously (12). Protein complexes were detected by electrophoretic mobility shift assays (EMSA) using a GAL4 DNA-binding site (GATCCGGAG TACTGTCCTCCGGATC [56]) that was end labeled with [γ-^{32}P]ATP. Binding reaction mixtures contained 1 to 2 μg of cellular protein extract, 0.2 ng of GAL4 DNA, and 1 μg of poly(dI-dC) in 50 mM KCl–20 mM Tris-HCl (pH 7.5)–5 mM $MgCl_2$–1 mM EDTA–1 mM dithiothreitol. Binding reaction mixtures were incubated for 30 min at 22°C and then electrophoresed on a 4% acrylamide gel in 0.25× Tris-borate-EDTA at room temperature. Rabbit polyclonal antisera to GAL4 and mouse c-Myc proteins were generous gifts from I. Sadowski and S. Hann, respectively. Antibody disruption experiments were performed by preincubation of the cellular extract in binding buffer with 1 μl of a 1:20 dilution of the antiserum on ice for 60 min, followed by the addition of the GAL4 DNA probe and further incubation for 30 min at room temperature. Reconstitution of complexes was achieved by addition of 4 μg of HeLa NE to the Cos extracts and incubation for 20 min at 22°C.

RESULTS

Defining an essential transforming domain of the c-Myc protein. It is likely that transformation by c-*myc* involves the transcriptional activation of specific cellular genes. We tested if the N-terminal transformation domain of c-*myc* could be replaced by a domain from another transcriptional activator, the herpes simplex virus VP16 protein. The VP16 protein transactivation domain can be fused to a number of different DNA-binding domains, and it strongly stimulates transcription when targeted to an appropriate reporter gene (48). Therefore, if the function of the c-Myc N terminus is to interact with general transcription factors, then a VP16-Myc fusion should be an effective transforming protein. The VP16-*myc* fusion gene (Fig. 1) was cloned into a CMV promoter-driven expression vector and assayed for transforming activity by cotransfection with a mutant *ras* gene into early-passage REFs. Compared with wt c-*myc*, transformation by the VP16-*myc* gene was clearly very defective, with only 5 to 8% of the wt c-*myc* foci produced (Fig. 2). In addition to the reduced number, the foci that formed were smaller than those with the wt c-*myc* gene

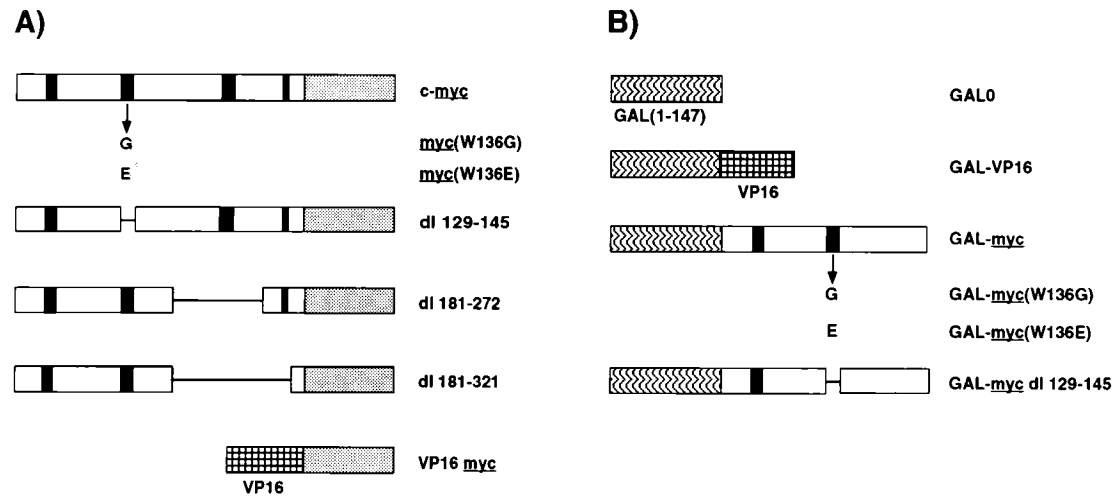

FIG. 1. Schematic diagram of the c-*myc* gene and mutants. The black boxes within c-*myc* represent small domains that are highly conserved in evolution. The stippled area represents the c-*myc* DNA-binding domain. Except where indicated, the c-*myc* genes and mutations were expressed using a CMV promoter vector. The GAL fusion genes were expressed from a simian virus 40 early promoter. GAL-*myc dl*129-145 is referred to as GAL4-MB2Δ throughout the text.

and difficult to establish in long-term culture (not shown). Thus, even though the VP16 transactivation domain is a better transcriptional activator than the N terminus of c-*myc* (22) (see below), it cannot transform when fused to the c-*myc* DNA-binding domain. This finding suggests that the c-*myc* N terminus provides a function required for cell transformation that cannot be replaced by a heterologous transcriptional activation domain.

One of the most highly conserved domains among Myc family proteins encompasses amino acids 129 and 144 of c-Myc, which we will refer to as MB2 since it is the second major conserved domain from the N terminus. Since deletion mutations which include this region eliminate transforming activity (50, 53), we sought to further define the domain by additional mutagenesis. We created three mutant c-*myc* genes which either deleted the 17-amino-acid motif of MB2 (*dl*129-145 or MB2Δ) or replaced the invariant tryptophan residue with glycine (W136G) or glutamic acid (W136E) (Fig. 1). Each mutant gene was cloned into a CMV promoter-driven expression vector and assayed for transforming activity by cotransfection with an H-*ras* oncogene into REFs.

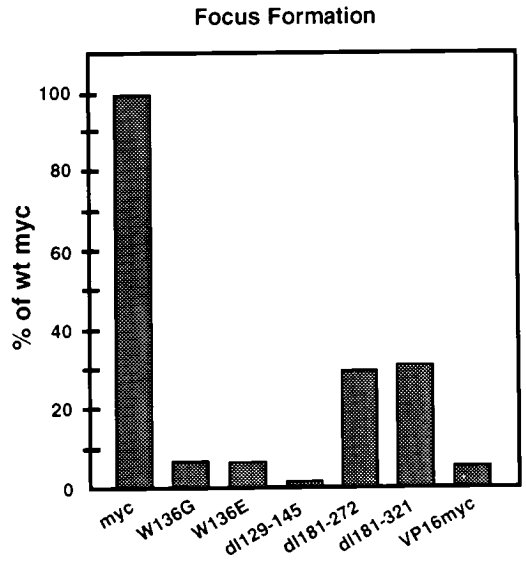

FIG. 2. Transforming activity of wt and mutant c-*myc* genes. The c-*myc* gene and mutants were transfected with an activated H-*ras* gene into secondary cultures of rat embryo cells, and the number of foci was counted after 3 weeks. The number of foci for wt c-*myc* (60 to 80 per dish) was set at 100%, and the transforming activity of each of the mutant genes was normalized to this value. The transformation activity reported is the average of three independent experiments with two plates for each construct.

Deletion of MB2 almost completely eliminated transforming activity compared with the wt protein (Fig. 2). MB2Δ plus *ras* generated only 1 to 5% of the number of transformed foci as wt-*myc* plus *ras*. The point mutations at the conserved W residue were also transformation defective, although their activities were reproducibly better than that of the deletion mutant. W136G and W136E yielded averages of 7 and 9% of wt *myc* foci, respectively. These mutations define a domain within the c-Myc protein that is essential for cell transformation and distinct from DNA binding, similar to what was found in previous studies (50, 53). Other mutants that delete the center of the c-Myc protein (*dl*181-272 and *dl*181-321 [Fig. 1]), which include other evolutionarily conserved domains, were also defective compared with wt c-*myc* but much less defective than the smaller MB2 deletion (Fig. 2). These findings are similar to those of previous reports (50, 53).

Deletion of MB2 does not disrupt transcriptional activation. The lack of transformation by the MB2Δ- and VP16-*myc* mutant genes led us to test directly for the transcriptional activation potential of the MB2 mutants. We first tested these mutations for the ability to transactivate consensus sites recognized by the C-terminal DNA-binding domain of c-*myc*. We used REFs as recipients to approximate the conditions under which the genes are assayed for transforming activity, but HeLa cells gave similar results. As has been reported in other studies, transactivation by the full-length c-*myc* gene gave a reproducible stimulation of transcription (three- to fourfold) from a reporter construct containing four Myc-consensus binding sites. More importantly, the transactivating activity of the MB2Δ mutant was unchanged from that of wt c-*myc*. (In this assay, each plasmid or the expression vector alone was cotransfected with a reporter gene in which four

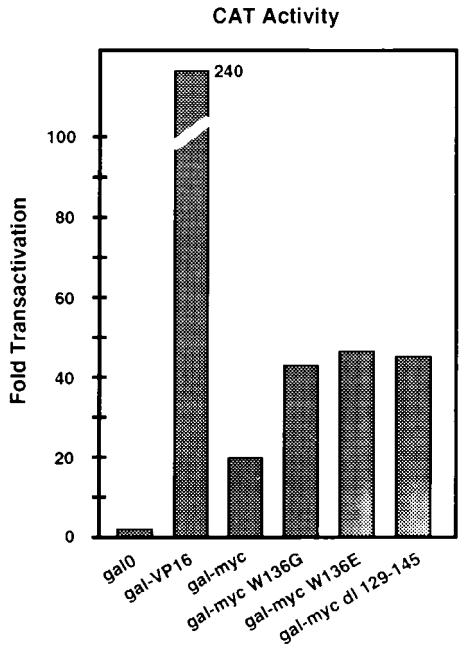

FIG. 3. Transactivation by GAL4-*myc* fusion genes. The GAL4-*myc* fusion genes and controls were transfected into early-passage REFs along with a G5-CAT reporter plasmid. CAT activity was determined and normalized to that of GAL0, which was set at 1.

copies of a Myc consensus binding site were cloned upstream of the E4-CAT gene. The recipient cells were the same early-passage rat embryo cells used for the focus assay. The pEMSV expression vector was used for transactivation studies because we found that the CMV vector suppressed transactivation by any cotransfected plasmid.) The VP16-*myc* fusion was also found to have a transactivating activity equivalent to that of wt c-*myc*. To increase the sensitivity of the transactivation assay, the c-*myc* N-terminus mutants were also tested as GAL4 fusions starting with the GAL4 fusion gene constructed by Kato et al. (22), which contains the GAL4 DNA-binding domain fused to amino acids 1 to 262 of the human c-*myc* gene. Since the c-*myc* mutants described above were derived from the mouse gene, we first showed that replacement of the human c-*myc* sequences in the GAL4 fusion with those from mouse gave equivalent transactivation activity (20-fold) with a GAL4 reporter gene when transfected into REFs (Fig. 3). The MB2 deletion as well as the W136E and W136G mutations were introduced into the same GAL4-*myc* fusion backbone. All of these mutations retained transactivation potential and were reproducibly 1.5- to 2-fold more active than the wt c-*myc* N terminus in this assay (Fig. 3). The observation that MB2 does not encode any of the Myc N-terminal transactivation activity is consistent with the finding that a previous larger deletion (D106-143) also retained all transactivation activity (22). Thus, MB2 does not appear to encode any of the transcriptional activation activity of the c-*myc* N terminus, at least as defined by synthetic promoters. While the data are consistent with separation of transformation and general transcription functions, they do not exclude a function for MB2 in the activation of specific target genes.

Deletion of MB2 eliminates the dominant negative activity of GAL4-*myc* fusions. The simplest hypothesis for the function of MB2 is as a contact site for another cellular protein that participates in cell transformation. If so, then overexpression of the c-*myc* N terminus alone might titrate out this accessory protein into a nonfunctional complex and reduce the transforming activity of the wt c-*myc* gene. As expected, the GAL4-*myc* fusions could dramatically suppress transformation in a dosage-dependent fashion when cotransfected with wt c-*myc* and *ras* in the REF focus assay (Fig. 4). Similar dominant negative effects of GAL-Myc fusions have been described previously (45). Surprisingly, the GAL4-MB2Δ fusion lost all detectable dominant negative function, even though it retains other domains conserved within the *myc* family that have been shown by mutagenesis to impair transforming activity, such as MB1 (50, 53). This result implies that the cellular protein that is hypothesized to bind to MB2 is the major factor that interacts with the N terminus that is both essential and rate limiting for c-*myc* transformation.

A trivial explanation for the MB2-dependent dominant negative effects is that the GAL4 chimeric proteins are expressed at different levels. Hence, GAL4-MB2Δ might not inhibit transformation because it fails to reach a critical concentration. This is unlikely for two reasons. First, when assayed for transcriptional activity in a transient CAT assay, the GAL4-MB2Δ protein is reproducibly more active than the GAL4-Myc fusion (Fig. 3), implying that the protein is produced efficiently. Second, we tested directly for the synthesis and stability of the GAL4 fusion proteins by transient transfection and immunoprecipitation or immunoblotting with GAL4 antibodies (not shown) and by analyzing DNA-binding activity (see below). All proteins were produced at nearly identical levels in these assays, similar to findings of previous reports (22, 45). Similar questions arise for the expression of the c-Myc protein and mutants in the focus assay. Unfortunately, we have been unable to detect the transiently expressed mouse c-Myc protein in REFs over the background of endogenous rat c-Myc protein. However, previous studies of c-Myc mutant expression did not detect major differences in the stability of c-Myc protein mutants (53). Since both wt and MB2Δ proteins can transactivate a synthetic promoter (see above), we infer that they are produced at comparable levels.

GAL4-*myc* inhibits E1A-mediated transformation. The c-*myc* oncogene exhibits many similarities with the adenovirus E1A gene in cell transformation, as discussed in the introduction. Therefore, we were interested in exploring whether the two genes operate through common cellular pathways. The GAL4-Myc fusions provided a tool to assess if a c-Myc-associated factor or c-Myc itself is also required for E1A-mediated transformation. E1A and *ras* were cotransfected into REFs, and focus formation was monitored. This combination of oncogenes transforms REFs efficiently (46), with more than 80 foci per dish. However, when the GAL4-*myc* fusion vector was included in the transfection, focus formation was dramatically reduced in a dosage-dependent fashion (Fig. 5). Focus formation was nearly eliminated (<20%) at the highest dosage (20 μg). In striking contrast, the GAL4-MB2Δ mutant failed to inhibit focus formation by E1A plus *ras* at any of the concentrations tested (Fig. 5). Inclusion of the GAL4 DNA-binding domain alone (without a transactivation domain) in these assays had no effect on focus formation. This finding suggests that the inhibition by GAL-Myc is Myc specific and also does not arise from promoter competition between vectors (Fig. 5). As with the inhibition of transformation by c-Myc described above, we interpret these results to indicate that the GAL4-Myc fusion can titrate out a factor that is essential for E1A transformation. Moreover, the dominant negative inhibition of E1A is dependent on the same MB2 domain that is also essential for c-*myc* transformation and dominant inhibition by

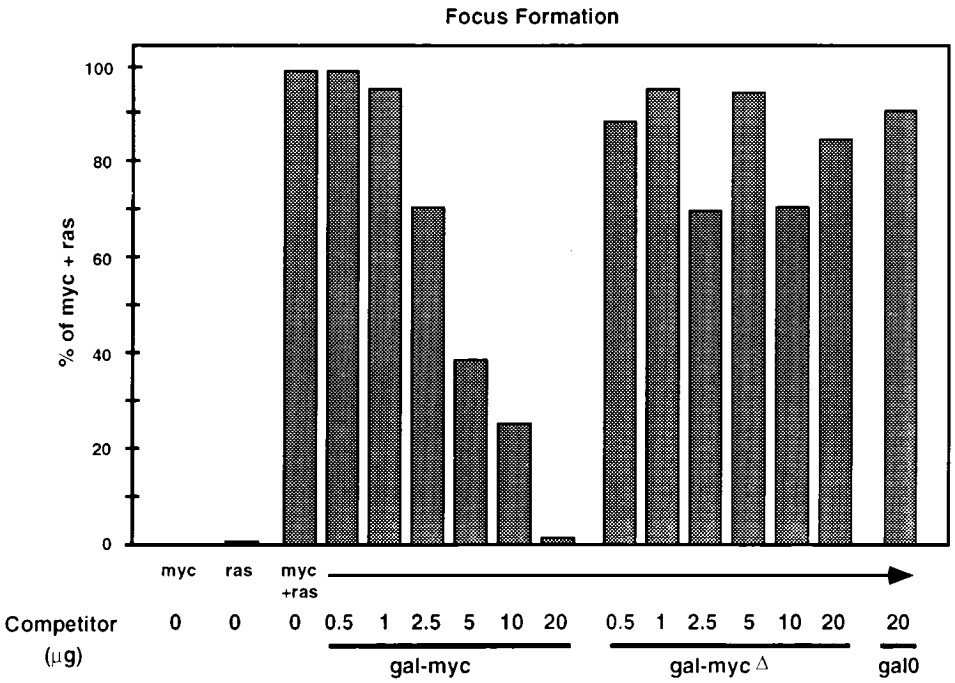

FIG. 4. Dominant negative activity of GAL4-*myc* fusions on c-*myc*-plus-*ras* transformation. The wt c-*myc* and *ras* genes were cotransfected into REFs with the indicated amount and type of GAL4 competitor. Foci were counted after 3 weeks and normalized to the value for wt c-*myc* and *ras* alone as 100%. The data represent averages of five experiments.

Gal-Myc. It is very unlikely that the GAL4-Myc fusions affect the synthesis of the E1A protein because E1A-dependent transactivation of the E4 promoter is unaffected by GAL4-Myc or GAL4-MB2Δ cotransfection (data not shown).

MB2-specific protein complex formation. The role of c-Myc MB2 sequences in cell transformation is likely due to the requirement for a specific interaction with a cellular factor(s). Unfortunately, it has not been possible to analyze the c-Myc

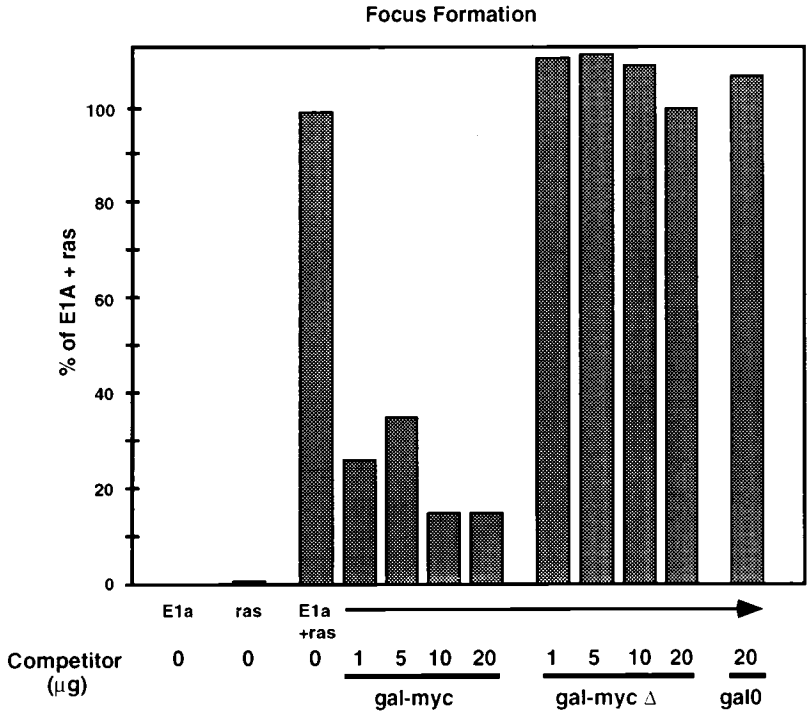

FIG. 5. Dominant negative activity of GAL4-*myc* fusions on E1A-plus-*ras* transformation. The adenovirus E1A and *ras* genes were cotransfected into REFs with the indicated amount and type of GAL4 competitor. Foci were counted after 3 weeks and normalized to the value for E1A and *ras* alone as 100%. The data represent averages of three experiments.

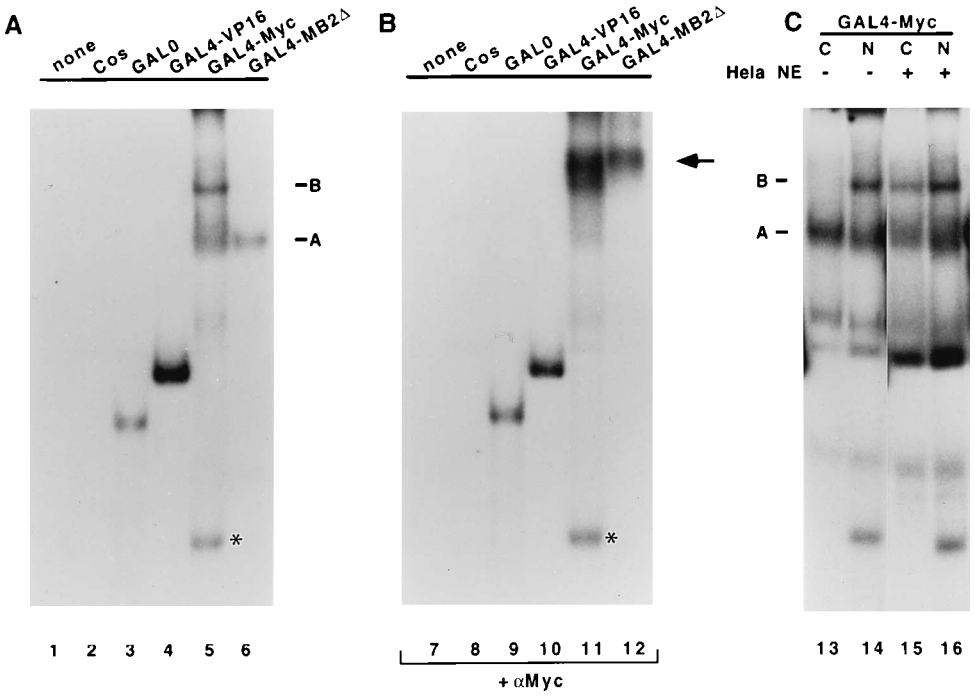

FIG. 6. DNA-binding complexes formed with GAL4 fusions. The GAL4 constructs diagramed in Fig. 1B (GAL0, GAL4-VP16, GAL4-*myc*, and GAL4-*myc dl*129-145 [MB2Δ]) were transfected into Cos-7 cells, and NEs were prepared. Extracts were prepared from untransfected Cos-1 cells as a control (lanes 2 and 8). The GAL4 fusion proteins and complexes were detected by EMSA with a GAL4 DNA-binding-site probe. Free probe was electrophoresed just off the bottom of the gel. Lanes 1, probe alone. Lanes 2 to 6, DNA-binding complexes with 1 μl (~1 μg of protein) of the indicated extract and 0.2 ng of GAL4 DNA probe. Complexes that are specific to the GAL4-Myc fusion protein are labeled A and B. Lanes 7 to 12, DNA-binding complexes with the indicated extract (1 μl), 0.2 ng of GAL4 DNA probe, and 1 μl of Myc antiserum (αMyc). Lanes 13 and 14, DNA protein complexes detected with the cytoplasmic (C) or nuclear (N) extracts (1 μl) from GAL4-Myc-transfected cells. Lanes 15 and 16, DNA-protein complexes detected with either cytoplasmic or nuclear extracts (1 μl) that were preincubated with 1 μl of HeLa NE (4 μg). The supershifted complex (B) formed in the reconstitution (lane 15) comigrated with that found in the transfected Cos cell NE (lane 14), and no additional complex is formed by preincubation of the Cos NE with HeLa NE (lane 16).

protein itself in cellular extracts because of technical limitations such as rarity and lack of solubility (28, 39). Since the GAL4-Myc protein exhibited MB2-specific dominant inhibitory activity, we reasoned that this protein might be a suitable substrate with which to detect complexes with cellular proteins. We therefore transfected the GAL4 fusion constructs into Cos cells and prepared nuclear extracts from GAL0, GAL4-VP16, GAL4-Myc, and GAL4-MB2Δ transfections. The GAL4 proteins were detected by using an EMSA with a GAL4 DNA-binding site. GAL0 gave a single, rapidly migrating DNA binding species that was readily detected over a faint band present in nontransfected Cos cells (Fig. 6, lanes 2 and 3). We assume that this complex is the GAL4 DNA-binding domain alone, and antibodies to GAL4 disrupted this band (not shown). The GAL4-VP16-transfected cells gave a more slowly migrating band (lane 4), consistent with the larger size of the fusion protein, and this band was also disrupted by GAL4 antibodies. The binding of all GAL4-specific complexes to the labeled probe was eliminated by preincubation with excess cold GAL4 DNA (not shown).

Extracts from the GAL4-Myc and GAL4-MB2Δ transfections gave strikingly different DNA-binding complexes compared with GAL0 or GAL4-VP16. The GAL4-Myc fusion exhibited primarily two distinct complexes (labeled A and B; Fig. 6, lane 5) that differed from those observed with GAL0 and GAL4-VP16. In contrast to GAL4-Myc, the GAL4-MB2Δ fusion exhibited only a single predominant complex that comigrated with complex A and no complex with a slower migration (Fig. 6, lane 6). Since the only difference between GAL4-Myc and GAL4-MB2Δ is the 17-amino-acid deletion of MB2, the formation of complex B is apparently dependent on this domain. No complexes with the same migration were observed in the GAL0 or GAL4-VP16 extract (lanes 3 and 4). To confirm that the GAL4-Myc fusion complexes contained the expected protein domains, Myc antibodies were used to demonstrate that both complexes A and B could be disrupted or supershifted (Fig. 6, lanes 11 and 12), whereas the same Myc antibodies did not disrupt either GAL0 or GAL4-VP16 complexes (lane 9 and 10). A faster migrating band specific to the GAL4-Myc extract (* in lane 5) was disrupted by αGAL4 (not shown) but not anti-Myc serum (* in lane 11), so we assume that this is a proteolytic product of the fusion protein that retains the GAL4 DNA-binding domain.

For the two DNA-protein complexes evident with the GAL4-Myc fusion protein, it is likely that complex A is the GAL4-Myc/GAL4 DNA complex alone, whereas complex B involves a ternary complex with cellular factors that interact with the c-Myc domain of the fusion. We tested this both by examining the cellular location of each complex and by testing if the more slowly migrating complex could be reconstituted from NE. To test for the cellular location, both cytoplasmic and nuclear extracts were prepared from GAL4-Myc-transfected cells and the complexes were analyzed (Fig. 6). The cytoplasmic extract contained only complex A (lane 13), whereas the NE contained complexes A and B (lane 14), indicating that the latter is formed only once the fusion protein is imported into the nucleus.

The presence of only complex A in the cytoplasmic extracts allowed us to test if complex B involved the binding of nuclear proteins to A and if these nuclear proteins were present in

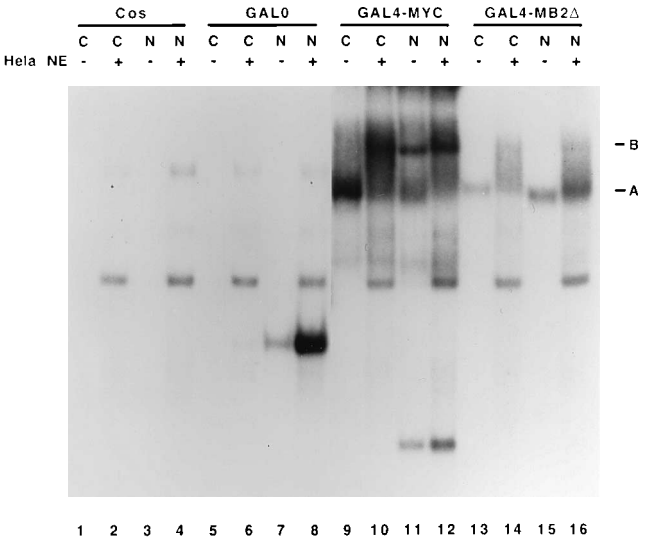

FIG. 7. HeLa nuclear proteins complex specifically with the wt GAL4-Myc fusion. Cytoplasmic (C) and nuclear extracts (N) were prepared from Cos cells that were either mock transfected (Cos) or transfected with the construct indicated (GAL0, GAL4-Myc, or GAL4-MB2Δ). DNA-binding complexes were detected with a GAL4 DNA probe (odd-numbered lanes). Each extract was also preincubated with HeLa NE (4 μg of protein; even-numbered lanes). Cos extracts alone contain very little GAL4 DNA-binding activity (lane 1), whereas HeLa NE forms three prominent complexes alone (not shown) or when added to Cos extracts (lane 2). Reconstitution of complex B is observed by incubation of GAL4-Myc cytoplasm with HeLa NE (compare lanes 9 and 10). No comparable complex is formed with the MB2Δ mutant by incubating HeLa NE with either cytoplasmic or nuclear extracts (lanes 13 to 16). Some supershift of complex A is observed with GAL4-MB2Δ, but dramatically less than with the wt GAL4-Myc fusion, probably reflecting a weak affinity of the mutant protein for the factor. The DNA binding of GAL0 and the fusion proteins was enhanced by the addition of HeLa NE (compare, for example, lanes 7 and 8), probably through a nonspecific effect of protein concentration.

different cell types. An NE from HeLa cells was prepared and then mixed with the cytoplasmic and nuclear extracts from the transfected Cos cells (Fig. 6, lanes 15 and 16). The addition of HeLa NE to the GAL4-Myc cytoplasm yielded a supershifted complex that comigrated with that found in the Cos NE (Fig. 6; compare lanes 14 and 15). Similarly, the addition of HeLa NE to the GAL4-Myc nuclear fraction yielded an increase in complex B and a corresponding decrease in A (lanes 14 and 16). The addition of greater amounts of HeLa NE or chromatographic fractions enriched for the supershift activity leads to the quantitative conversion of complex A into complex B (not shown). These results strongly suggest that complex B arises from the binding of a nuclear factor to the Myc N-terminal domain, and this factor is present in both Cos and HeLa cells. An apparently identical factor is also found in *myc-ras*-transformed REFs (not shown).

The NEs prepared with the mutant GAL4-MB2Δ fusion contained only one specific complex that comigrated with complex A in the wt Myc fusion (Fig. 6, lane 6), suggesting that the MB2 mutation disrupts the interaction with the nuclear factor that forms complex B. To demonstrate this more directly, both cytoplasmic and nuclear fractions were prepared from GAL4-MB2Δ-transfected cells and then tested in the reconstitution assay with HeLa NE. As expected, complex A was found in both the cytoplasmic and nuclear fractions from these cells (Fig. 7, lanes 13 and 15), and the addition of HeLa NE did not reconstitute a complex that comigrated with the B band (Fig. 7, lanes 14 and 16). Some reduced migration of the GAL4-MB2Δ DNA-binding complex was evident, which is likely due to a weak affinity of the mutant for the factor. The supershifted complex was specific for the Myc N terminus since no similar complexes were formed with cytoplasmic and nuclear extracts from GAL0- and mock-transfected cells (Fig. 7, lanes 1 to 8). The only band with migration similar to that of complexes A and B is present in all lanes that include HeLa NE, indicating that this band is due to a HeLa protein that fortuitously binds to the GAL4 probe. This factor is clearly distinguishable in migration from the GAL4-Myc-specific complexes. Thus, MB2 is required for the formation of a specific complex of the Myc N terminus with a nuclear protein. Furthermore, this factor is distinct from previous proteins that have been shown to interact with c-Myc as discussed below.

DISCUSSION

The observation that the c-*myc* gene encoded a DNA-binding domain was a major step toward defining the function of the protein in cell transformation, and it is reasonable to assume that c-Myc functions by transcriptionally activating specific target genes. We have shown in this study that the c-Myc N terminus contains a domain (MB2) that is essential for cell transformation yet does not alter the transcriptional activity of c-Myc on synthetic promoters. At first glance, this finding appears to be inconsistent with the assumption that c-*myc* regulates its target genes at a transcriptional level. Instead, we suggest that this domain may provide a key activity that facilitates the proper selection of target genes in a milieu of similar DNA-binding proteins. In the absence of MB2, c-Myc protein may recognize its target genes very poorly even though it retains the ability to interact with basal transcription factors. If the sole function of the c-Myc N terminus is to induce transcription, then the VP16-*myc* fusion gene would have been expected to be a strong transforming gene, possibly better than c-*myc* itself given the enhanced ability of VP16 to activate transcription (Fig. 3). Since the activities of the VP16-*myc* fusion and MB2Δ mutant in cell transformation were similar, it seems likely that the weak transforming activity that remains in the MB2Δ mutant is the result of a more general transcriptional activation function that maps to other segments of the c-Myc N terminus (2, 17, 22, 25). VP16 and c-Myc may also interact with different basal factors, although v-Myc may differ from c-Myc in this context (33, 45). Previous studies have shown that c-Myc transactivation does not necessarily correlate with transformation (22), and a similar uncoupling of these activities has been reported for c-*jun* (37).

It was surprising to find that MB2 could act as a dominant inhibitor of E1A transformation when expressed as a GAL4-Myc fusion. One interpretation of this result is that E1A requires endogenous c-Myc function, and hence the GAL4-Myc fusion inhibits c-Myc function dominantly. While this possibility cannot be completely excluded, there are several observations that argue against it. First, an independent study has shown that overexpression of the c-Myc C terminus does not inhibit E1A transformation, whereas it does inhibit c-*myc* transformation (35). Furthermore, the c-Myc N terminus (as a C-terminal c-Myc mutant, D414-433) does not have a severe impact on normal cell growth or on cell transformation by the *abl* oncogene, whereas *abl* transformation is inhibited by expression of the c-*myc* basic–helix-loop-helix–leucine zipper domain (51). Thus, *abl* and E1A respond very differently to dominant negative c-Myc assays. The absence of a trivial toxic effect of GAL-Myc fusions on cellular growth has been established previously by testing for growth suppression with antibiotic resistance gene cotransfection (45). A second argument against a requirement for endogenous c-*myc* is that neither

transformation nor immortalization by E1A alters the level or regulation of the c-*myc* gene. Significantly, E1A-immortalized primary cells continue dividing in the absence of serum growth factors, even though endogenous c-*myc* levels fall to undetectable levels (23, 55). Therefore, E1A-immortalized cells do not appear to need c-*myc* function to grow. Furthermore, the c-*myc* gene is regulated normally after transformation of REFs by E1A and *ras*, and it continues to be suppressed by removal of serum growth factors (24). Some studies have shown that E1A can transactivate or derepress the c-*myc* promoter (21, 34, 40, 54), but these studies all used the transfection of an exogenous c-*myc* promoter rather than assessed the expression of the endogenous gene. Thus, there is no evidence that E1A directly regulates, or is dependent on, the c-*myc* gene as part of its transforming or growth-promoting activity. We favor a model in which E1A acts either downstream of c-*myc* or at the same point in a signal cascade.

Further evidence in support of a common factor that interacts with c-Myc and E1A comes from previous work in which an E1A (amino acids 1 to 120)-Myc C-terminus fusion was shown to be an effective transforming gene in the *ras* cooperation assay (42). In this case, the DNA-binding region of c-Myc was fused to the E1A N terminus, and thus the N-terminal function of c-*myc* was directly replaced by E1A. The chimera acquires transforming activity that neither domain has alone. In the simplest interpretation, the DNA-binding domain of c-Myc might interact with the same target genes that it normally recognizes, and the fused E1A domain facilitates transformation. One cellular protein that can interact with both c-Myc and E1A is Rb, but a Myc-Rb complex has been described only for in vitro-synthesized proteins (47). Furthermore, with the Rb-E2F complex as a paradigm (36), one might expect that binding of Rb to c-Myc would inhibit its function and hence the disruption of that interaction would not debilitate c-Myc function as in the MB2Δ mutant (Fig. 2). The c-Myc N terminus has also been reported to bind to the TATA-binding protein, but the domain of interaction does not appear to map to MB2 (20, 31). Very recently, the c-Myc N terminus has been shown to bind to the Rb-related p107 protein (17). The binding site for p107 on c-Myc maps primarily to MB1 rather than MB2, and antisera to p107 and TATA-binding protein do not disrupt or supershift the complexes described in this study (data not shown).

Since mutagenesis of the c-*myc* gene indicates clearly that all of its biological activities require MB2, it is of great interest to identify the cellular factors that interact with this region. Transient expression of the GAL4-Myc fusion protein leads to the formation of a complex (B) that correlates well with the expected factor(s). Complex formation is dependent on the same domain that is required for transforming and dominant negative activities, and the complex can be reconstituted with NEs from at least three different cell types. However, further experiments will be required to determine if the complex that we detect by EMSA is the same cellular factor that is titrated out in the dominant negative assays. It is likely that the formation of complex B involves a distinct nuclear protein(s) because this activity can be chromatographically separated from other proteins in HeLa NEs (data not shown). The role of this factor in transformation will require molecular cloning and an analysis of functional interactions with the c-*myc* oncogene.

What might be the identity and function of the transformation-specific nuclear factor? This factor could either be a DNA-binding protein itself or a cofactor that bridges to other DNA-binding proteins, which would serve two purposes in mediating the activities of c-*myc* and E1A. For c-*myc*, a specific interaction with another DNA-binding protein could provide added specificity in the selection of cellular target genes, which in turn predicts that the target promoters may share common features other than a Myc-binding site. Another aspect of this model is that it would provide a mechanism by which E1A can activate cellular targets in a *myc*-independent fashion. This might explain the overlap in activities between the two otherwise biochemically dissimilar oncogenes. However, we cannot rule out more complicated scenarios in which the dominant negative effects of the GAL4-Myc protein result from interactions with cellular factors that are only indirectly required for E1A transformation. As reviewed in the introduction, our model is supported by the remarkable overlap in functional activities between c-*myc* and E1A. These results emphasize the complex and interrelated nature of c-*myc*- and E1A-mediated transformation and the need to identify the specific factor that interacts with MB2 and other functionally important domains of the c-Myc oncoprotein.

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