MOLECULAR AND CELLULAR BIOLOGY, Oct. 1996, p. 5772–5781 Vol. 16, No. 10
0270-7306/96/$04.00+0

Mad Proteins Contain a Dominant Transcription Repression Domain

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Received 21 May 1996/Returned for modification 23 June 1996/Accepted 23 July 1996

Transcription repression by the basic region–helix-loop-helix-zipper (bHLHZip) protein Mad1 requires DNA binding as a ternary complex with Max and mSin3A or mSin3B, the mammalian orthologs of the *Saccharomyces cerevisiae* transcriptional corepressor SIN3. The interaction between Mad1 and mSin3 is mediated by three potential amphipathic α-helices: one in the N terminus of Mad (mSin interaction domain, or SID) and two within the second paired amphipathic helix domain (PAH2) of mSin3A. Mutations that alter the structure of the SID inhibit in vitro interaction between Mad and mSin3 and inactivate Mad's transcriptional repression activity. Here we show that a 35-residue region containing the SID represents a dominant repression domain whose activity can be transferred to a heterologous DNA binding region. A fusion protein comprising the Mad1 SID linked to a Gal4 DNA binding domain mediates repression of minimal as well as complex promoters dependent on Gal4 DNA binding sites. In addition, the SID represses the transcriptional activity of linked VP16 and c-Myc transactivation domains. When fused to a full-length c-Myc protein, the Mad1 SID specifically represses both c-Myc's transcriptional and transforming activities. Fusions between the GAL DNA binding domain and full-length mSin3 were also capable of repression. We show that the association between Mad1 and mSin3 is not only dependent on the helical SID but is also dependent on both putative helices of the mSin3 PAH2 region, suggesting that stable interaction requires all three helices. Our results indicate that the SID is necessary and sufficient for transcriptional repression mediated by the Mad protein family and that SID repression is dominant over several distinct transcriptional activators.

Max is a small basic region-helix-loop-helix-zipper (bHLHZip) protein that plays a central role in a network of bHLHZip transcription factors (11, 31, 65). This network appears to function in both transcriptional activation and repression. Max contributes to the transcription activation branch of this network by forming heterodimers with members of the Myc family of proto-oncoproteins (12, 53). These Myc:Max heterodimers recognize the E-box-related sequence CACGTG to activate the transcription of synthetic reporter genes (4, 29, 41, 42) and, presumably, cellular Myc-responsive target genes (9, 25, 26a). Transcription repression is mediated by Max heterodimer formation with members of the Mad family. The Mad family (Mad1, Mxi1, Mad3, and Mad4) is a group of related bHLHZip proteins originally identified through interaction cloning screens with Max (7, 34, 76). Like the transcriptional activator Myc, all Mad family members form dimers with Max and bind the E-box sequence CACGTG. It has been demonstrated that Mad1, Mad3, and Mad4 function in this context as transcriptional repressors (7, 34). The opposing transcriptional activities of Myc:Max and Mad:Max have led to the hypothesis that Mad family members antagonize Myc's positive influence on cellular proliferation. This notion has gained support from experiments showing that overexpression of each Mad family member can inhibit cell growth and cellular transformation by Myc and Ras (15, 18, 34, 40, 44, 70). In addition, Mxi1 has been found to be mutated in a small percentage of prostate tumors, suggesting that it and possibly other Mad family members function as tumor suppressors (23). Mad's inhibition of cell growth appears to be due to an increase in the G_1 phase of the cell cycle, because cells that overexpress Mad1 accumulate with a 2 N DNA content higher than control values (16, 55). The inhibitory effect of Mad on proliferation appears tightly linked to its function as a transcriptional repressor, since mutations that block binding to Max or to the transcriptional corepressor mSin3 significantly reduced Mad1's ability to block cell proliferation (40, 55).

Myc expression is primarily associated with proliferation, and its function appears to be required for the G_0 to G_1 transition and for cell cycle progression (for reviews, see references 31 and 50). In addition, as cells exit the cell cycle during differentiation, Myc protein synthesis is often downregulated. In contrast, expression of Mad family members is low in proliferating cells but is induced during differentiation in cells of myeloid, neuronal, and epithelial lineages, suggesting that Mad family members play a general role in the differentiation process (6, 18, 22, 33, 34, 45, 53a, 70). In addition, coimmunoprecipitation experiments have revealed a switch between Myc:Max and Mad1:Max heterocomplexes after the induction of differentiation in myeloid and epithelial cells (6, 33, 34). The patterns of expression of the different *mad* family genes are complex; however, in general, *mad1* and *mad4* RNAs appear to be highest during later stages of terminal differentiation, while *mxi1* is present before and during differentiation (34). Recent evidence suggests that *mad3* is expressed in proliferating progenitor cells prior to differentiation (53a). Given the antiproliferative effect of Mad overexpression (16, 55), it seems most likely that Mad is involved in cell cycle exit rather than providing specific differentiation signals.

Recently, some light has been shed on the mechanism of Mad transcriptional repression by the identification of putative

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mammalian corepressors that interact with all four Mad family proteins (8, 34, 58). The interacting proteins, mSin3A and mSin3B, are related to the *Saccharomyces cerevisiae* corepressor SIN3 (8). The two mammalian proteins are approximately 60% identical to each other and 35% identical to yeast SIN3. The regions of most extensive homology between the yeast and mammalian proteins center around the four paired amphipathic helix (PAH) regions that have been proposed to function as protein interaction surfaces (8, 72). The interaction between Mad family proteins and the mSin3 proteins requires sequences at the amino terminus of the Mad protein and PAH2 of either mSin3 protein (8, 34, 58). The amino-terminal region of the Mad proteins is highly conserved between all family members and across species, and we have referred to it as the mSin interaction domain (SID) (34) (Fig. 1A). Interaction between Mad and mSin3 is strongly correlated with repression, since point mutations in the SID of Mad1 not only disrupt interaction with mSin3 in vitro but also result in loss of repression (8). The same mutations also render Mad1 incapable of inhibiting Myc-Ras cotransformation or cell cycle progression (40, 55). Furthermore, naturally occurring splice variants of the Mxi1 protein (Mxi1-WR) have been identified which lack the SID and which fail to block cotransformation by Myc-Ras (58).

Although, yeast SIN3 does not possess intrinsic DNA binding activity (73), we have shown that Mad:Max:mSin3 binds DNA as a ternary complex (8), suggesting that transcriptional repression by both the yeast and mammalian Sin3 proteins is mediated by sequence-specific DNA-binding proteins which act to tether the corepressors to DNA. Consistent with this hypothesis is the finding the Sin3 can repress transcription in yeast cells when brought to the DNA by the heterologous DNA binding domain of *lexA* (74). Furthermore, recent studies indicate that mSin3 can functionally substitute for the SID of Mxi (54). Here, we report that the SID can function as a transcriptional repression domain when fused to a heterologous DNA binding domain. Moreover, repression by SID is dominant over several strong transcriptional activators and can block the transcriptional and transforming activities of c-Myc.

MATERIALS AND METHODS

Fusion protein construction. All of the fusion proteins used in these studies were constructed by PCR and standard cloning techniques. The PAH2 constructs have amino acids 321 to 337 for ΔhelixA and amino acids 369 to 382 for ΔhelixB (numbering based on the full-length mSin3A) deleted. The entire PAH2 region in VP16 was the original PAH2 (8) clone identified and spanned amino acids 251 to 405 of the full-length mSin3A protein. To construct the GAL fusion protein, we initially amplified sequences encoding amino acids 1 to 147 of the GAL4 DNA binding domain (GALDBD) and cloned them into the expression vector pSP271 (41). This served as the base plasmid for construction of the other fusion proteins. To construct MadN35-GAL or Mad(Pro)N35-GAL, we amplified sequences encoding the amino-terminal 35 amino acids from either pSPMad or pSPMad(L12P/A16P) (8) and fused them in frame to the amino-terminus of the GALDBD. To construct the series of clones with the activation domain of VP16 fused to the carboxy terminus of the GALDBD, we amplified sequences encoding the VP16 activation domain from pVP16 (71) and cloned them in frame at the carboxy-terminal site of pSPGAL, pSPMadN35GAL, and pSPMad(Pro)N35GAL to create pSPGALVP16, pSPMadN35GALVP16, and pSPMad(Pro)N35GALVP16, respectively. The entire coding regions of mSin3 were amplified from pVZmSin3A or pVZmSin3AΔPAH2 (8) and fused in frame to the carboxy-terminal end of the GALDBD in the pSP271 background. The MadMyc and the Mad(Pro)Myc fusions were made by amplifying the human Myc cDNA from pSPMyc (41) and replacing the GALDBD portion of the MadN35-GAL and Mad(Pro)N35-GAL constructs with the amplified Myc sequences. Again, these constructs were in the pSP271 background. For transformation assays, we subcloned MadMyc, Mad(Pro)Myc, and Myc into a cytomegalovirus-based expression vector, pRC/CMV (InVitrogen). The luciferase reporter containing four CACGTG binding sites was constructed by subcloning the sites from M4minCAT (41) into pGL2pro (Stratagene).

Protein interaction assays. The directed two-hybrid assay was performed by transforming the yeast strain L40 with the appropriate plasmids and then assaying for β-galactosidase activity as described previously (71). The immunoprecipitation experiments were performed as described previously (8).

Luciferase and CAT assays. 293 cells, grown in Dulbecco modified Eagle medium (DME) with 10% defined calf serum (Hyclone), were transfected with the amounts of plasmids given in the legend. Thirty-six to 48 h after transfection, cell lysates were prepared, and the luciferase or chloramphenicol acetyltransferase (CAT) assays were done as described previously (13). Transfection efficiencies were normalized with an internal β-galactosidase control.

Transformation assays. Rat1A cells were grown in DME with 10% fetal calf serum (Hyclone) with penicillin-streptomycin. They were transfected with 10 μg of either MadN35-Myc, Mad(Pro)N35-Myc, or c-Myc in pRC/CMV. Forty-eight hours after transfection, cells were split 1 to 10 and placed into medium containing 400 μg of G418 (Gibco/BRL) per ml. Pools of cells were collected and allowed to grow to confluence.

RESULTS

The SID can function as a transferable repression domain. Previous studies had demonstrated that the conserved amino-terminal region found in all four Mad family proteins was required for their ability to associate with mSin3A/B (8, 34, 58). Helical wheel analysis suggested that residues 5 to 21 of Mad1 and Mxi1(SR) could potentially form an amphipathic α-helix (8, 58), and substitution of prolines for leucine and alanine at positions 12 and 16 [Mad1(Pro)] was sufficient to disrupt interaction of Mad1 with mSin3 (8). The SID therefore comprises a highly conserved region that includes this putative α-helix (Fig. 1A). To test if the SID could function as a transferable repression domain, we fused the first 35 amino acids of Mad1 to the GALDBD (amino acids 1 to 147). This fusion construct, MadN35-GAL, was tested for its ability to repress or activate transcription from a minimal promoter derived from the myelomonocytic growth factor promoter. The reporter construct contained sequences from −41 to +61 driving transcription of a downstream luciferase reporter gene and was made responsive to the GALDBD by the inclusion of four consensus GAL4 binding sites proximal to −41 (60). When transfected into the human kidney epithelial cell line 293, the reporter lacking GAL4 binding sites was unresponsive to both the GALDBD and various amounts of MadN35-GAL expression vector (Fig. 1B). However, when the reporter contained four GAL4 binding sites, it was activated threefold by the GALDBD alone and repressed by the MadN35-GAL expression construct. The finding that GALDBD alone activated transcription is consistent with earlier findings demonstrating the presence of a cryptic activation domain encoded between amino acids 97 and 147 of GAL4 (49). The repressive effect increased as the amount of the MadN35-GAL expression vector used in the transfection was increased and, in the most severe case, there was approximately 30-fold repression of transcription relative to GALDBD alone (Fig. 1B). To test if this repression required the binding of mSin3A or mSin3B, we employed a SID containing the proline substitutions, described above, which render the full-length Mad1 protein incapable of binding mSin3 in vitro or in vivo (reference 8 and data not shown). When incorporated into the expression vector, this mutant, Mad(Pro)N35-GAL, failed to repress transcription from the GALDBD-responsive reporter in transfection assays (Fig. 1C). Both the MadN35-GAL and the Mad(Pro)N35-GAL construct were expressed to equivalent levels and bound DNA with similar efficiencies (data not shown), indicating that the loss of repression activity in the mutant protein is not due to gross alteration of its properties. Rather, these experiments suggest that transcriptional repression by Mad1 requires an intact SID capable of recruiting the mSin3 proteins to DNA.

The results with the MadN35-GAL fusions suggested that simply bringing mSin3 to DNA should be sufficient to elicit transcription repression. To test this idea, we fused full-length mSin3A to the GALDBD. This fusion protein was able to

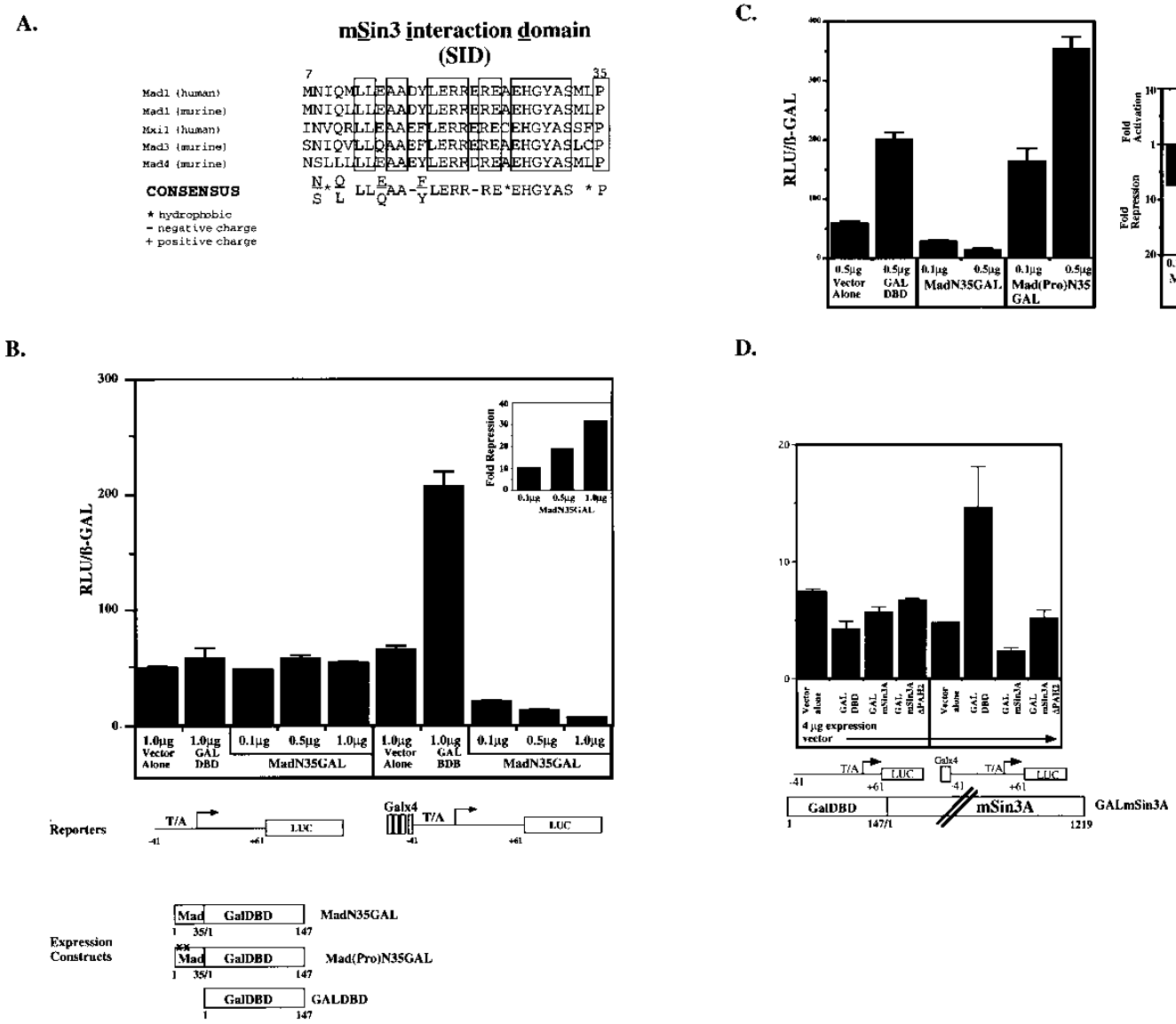

FIG. 1. Transcription repression by the SID. (A) Alignment of the amino-terminal 35 residues of the Mad family proteins with the derived consensus at the bottom. The boxed residues indicate absolute conservation among all family members. (B to D) Transcriptional activity of the diagrammed myelomonocytic growth factor promoters was determined by measuring luciferase (LUC) activity (relative light units [RLU]) after transfection into 293 cells. The expression plasmids are diagrammed at the bottom of the figure and were supplied in the indicated amounts. The first five and second five bars of panel B indicate promoter activity in the absence or presence of GAL sites in the promoter, respectively. The insert shows the fold repression relative to the GALDBD alone. In panel C, the leftmost panel shows the activity of the promoter with GAL sites in the presence of the indicated amount of expression vector. On the right, the activation or repression relative to the GALDBD alone is shown. In panel D, the transcriptional activity of a GAL-mSin3 fusion is shown. The promoters used are shown at the bottom of the panel.

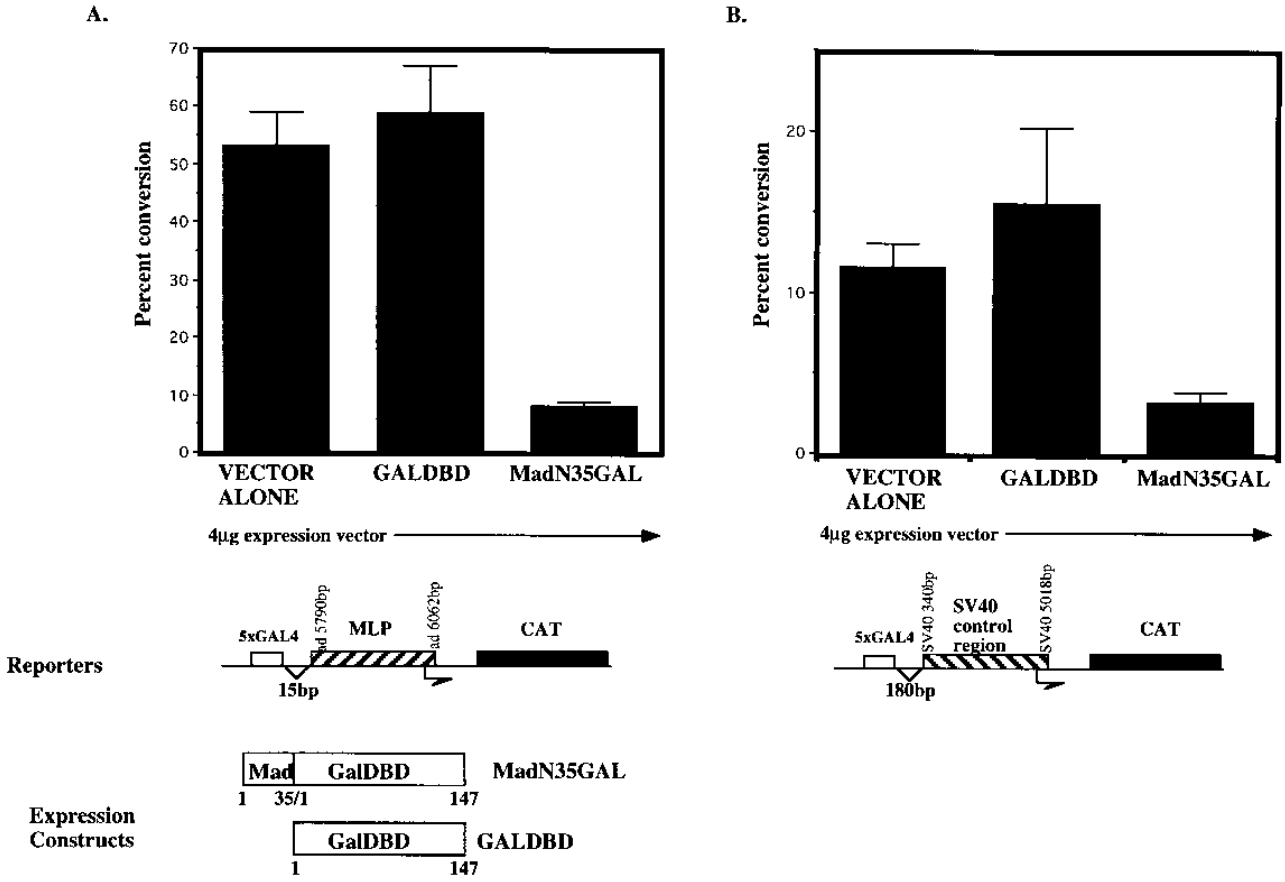

FIG. 2. SID can repress transcription in multiple promoter contexts. The effect of MadN35-Gal overexpression on the transcriptional activity of the adenovirus major late promoter (MLP) (A) and on the SV40 promoter/enhancer (B) is shown. The reporters used in this experiment are diagrammed at the bottom of each figure. The transcriptional activity of the reporter is reported as the percentage of conversion of unacetylated [^{14}C]chloramphenicol to the diacetylated form. There was no effect of MadN35-GAL overexpression in the absence of GAL binding sites in the reporter constructs (data not shown).

repress the level of transcription observed with the GALDBD alone, and the repression required the presence of Gal DNA binding sites in the reporter (Fig. 1D). These results show that mSin3 can repress transcription when brought to DNA by a heterologous DNA binding domain and suggest that mSin3 normally functions by being tethered to DNA by a sequence-specific DNA-binding protein such as Mad1.

Mad1 interacts with the PAH2 domain of both mSin3A and mSin3B, and we have postulated that this interaction brings mSin3A or mSin3B to the DNA and facilitates transcription repression. This model predicts that transcriptional repression driven by targeting mSin3A to the DNA via a heterologous DNA binding domain should be independent of the PAH2 domain. To test this idea, we fused a mutant of mSin3A that lacks the PAH2 domain to the GALDBD and determined if this chimera could repress transcription on the reporters used above. This fusion, GALmSIN3AΔPAH2, repressed transcription as well as GALmSin3A, suggesting that the PAH2 domain serves as a protein docking surface rather than providing sequences required for mSin3A repression (Fig. 1D).

Transcription repression by mSin3 is dominant over activation. To determine if the MadN35-GAL chimera could repress transcription from promoters more complex than the minimal promoter used above, we tested it on the simian virus 40 (SV40) early and adenovirus major late promoters that were made responsive to GALDBD fusions by insertion of GAL4 binding sites. The activation of these promoters is controlled by abundant cellular transcription factors, including SP1, AP1, and USF (26, 27, 46, 57). Upon introduction of MadN35-Gal, we observed repression that was dependent on GAL4 binding sites with different promoter constructs (Fig. 2 and data not shown), demonstrating that MadN35-GAL can repress transcription driven by multiple activators. In addition, the GAL4 binding sites are approximately 500 and 300 bp upstream of the transcription initiation site for the SV40 early and adenovirus major late promoters, respectively, implying that MadN35-Gal can repress transcription when bound a considerable distance from the transcription start site. The ability of the Mad1 SID to repress promoters containing binding sites for different types of activators suggests that repression in this system is dominant over activation.

Mad1 has a cryptic activation domain in its carboxy terminus that is negatively regulated by mSin3 binding to the SID (data not shown). To test whether the SID could abrogate the transcription activation capabilities of other and more potent activators in *cis*, we transferred the amino-terminal 35 amino acids of Mad or Mad(Pro) to GAL-VP16. To mimic the structural organization of the Mad family proteins, we chose to fuse the SID to the amino terminus of GAL-VP16. GAL-VP16 activated transcription of the minimal promoter roughly 3,000-fold. In contrast, the MadN35-GAL-VP16 fusion protein was roughly 50-fold less potent than GAL-VP16 as a transcriptional activator. The loss of GAL-VP16's ability to activate transcription appeared dependent on an intact SID, since the

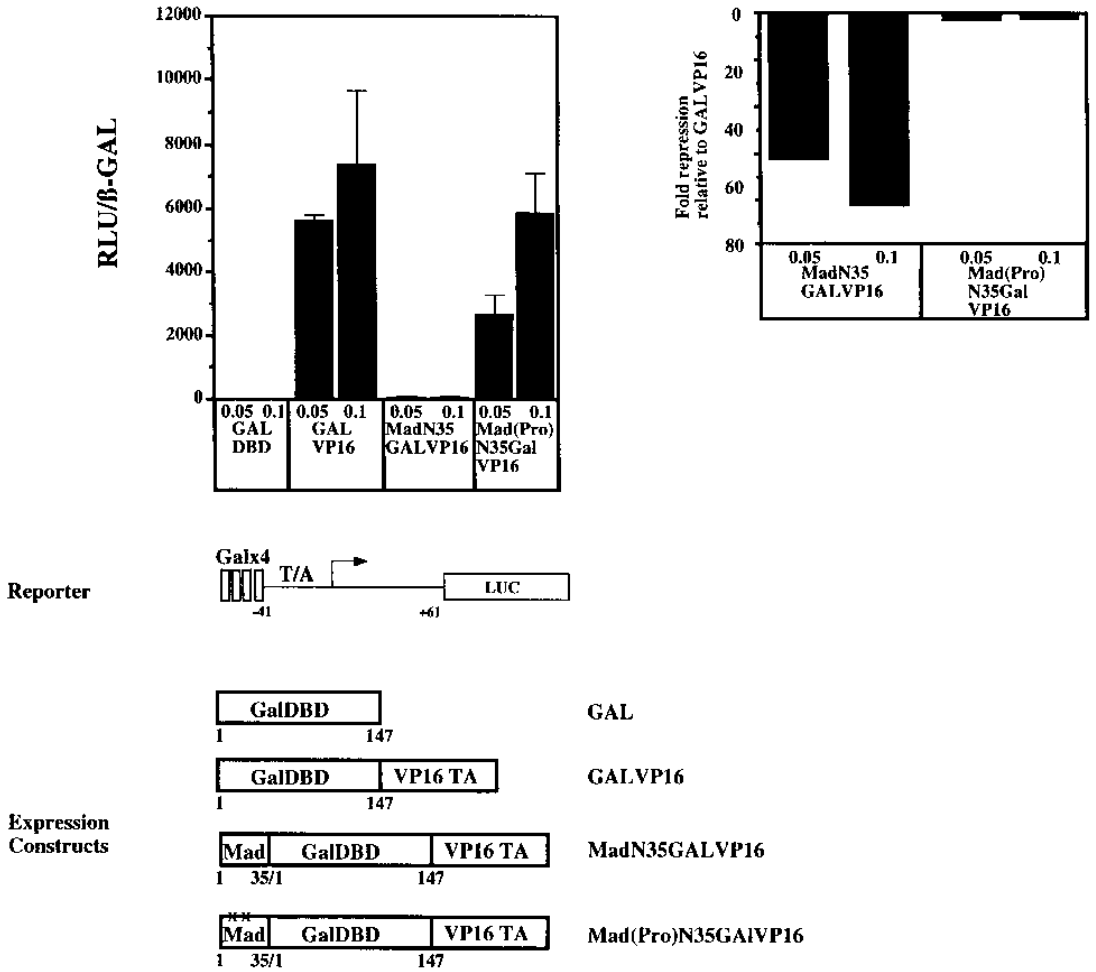

FIG. 3. SID can function as a dominant repressor. Transcriptional activity of the myelomonocytic growth factor promoter with GAL4 DNA binding sites was determined by measuring luciferase activity (relative light units [RLU]) after transfection into 293 cells. The expression vectors used are diagrammed at the bottom of the figure. On the left, the promoter activity in the presence of the indicated amount (in micrograms) of expression vector is shown. On the right, the fold activation or repression relative to that seen with the GALDBD is shown.

fusion protein containing the L12P/A16P mutation, Mad-(Pro)N35-GAL-VP16, had a greatly reduced effect on the activity on GAL-VP16 (Fig. 3). The fusion proteins used in these experiments were all expressed at similar levels and had very similar DNA binding characteristics (data not shown). The results, therefore, suggest that a SID capable of interacting with mSin3A/B can silence transcription activation domains when fused to an activator in *cis* and underscore the dominance of repression over activation.

The SID blocks c-Myc transcriptional activation and transformation. The GAL-VP16 fusion is a well-characterized chimeric transcription factor composed of the DNA binding domain of a yeast protein and the activation domain of a virally encoded transcription factor. To test if the SID domain could repress the activation domain of a naturally occurring transcription factor, we fused the SID to full-length c-Myc (Fig. 4A). To assay transcription from full-length c-Myc and the MadN35-Myc chimeras, we constructed a luciferase reporter construct by inserting four copies of the canonical Myc:Max binding site (CACGTG) into the distal end of the SV40 early promoter. This reporter was activated 10-fold in response to transfected c-Myc expression vectors (Fig. 4B). However, when the MadN35-Myc construct was transfected into these cells, there was a complete abrogation of c-Myc transcriptional activity (Fig. 4B). Again, this loss of activation potential depended on an intact SID, since a fusion protein carrying the L12P/A16P mutation [Mad(Pro)N35-Myc] was as active as wild-type c-Myc (Fig. 4B). To verify that the proteins were all expressed in these cells, we performed immunoprecipitations with Myc-specific antibodies from cells that had been transfected with the expression plasmids and labeled with [^{35}S]methionine (Fig. 4C). Because all of the proteins were highly expressed, we believe that the inability of MadN35-Myc to activate transcription is due to the abrogation of c-Myc transcriptional activity by the attached SID.

It has been shown that the transactivation domain of Myc is required for its function as a transforming oncogene (36, 62). Therefore, Myc's activity as an oncogene is presumed to be tightly coupled to its ability to activate cellular target genes involved in growth control. To determine if MadN35-Myc and Mad(Pro)N35-Myc could influence the expression of endogenous c-Myc target genes, we measured their ability to function as transforming oncogenes by transfecting each construct into Rat1A, a cell line in which ectopic overexpression of c-Myc is sufficient to cause transformation (62). After selection in G418, the bulk populations of stably transfected cells were grown to

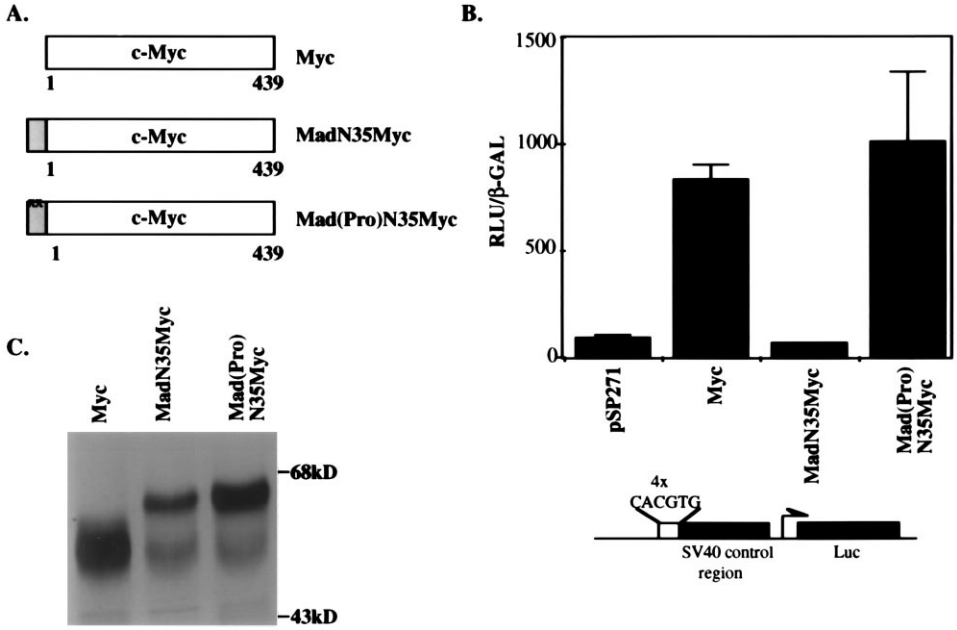

FIG. 4. SID can convert c-Myc into a transcriptional repressor. (A) Reporter and expression vectors used in these experiments. (B) Transcriptional activity of the Myc-responsive promoter in the presence of the expression vectors indicated at the bottom of the panel. (C) [^{35}S]methionine-labeled Myc proteins detected by immunoprecipitation in 293 cells transfected with the expression vectors indicated at the top of the figure. The immunoprecipitates were analyzed on a sodium dodecyl sulfate–12.5% polyacrylamide gel. The dried gel was exposed for autoradiography. RLU, relative light units; Luc, luciferase.

confluence. Cells transfected with c-Myc and Mad(Pro)N35-Myc lost contact inhibition and grew as foci above the monolayer, while cells transfected with empty vector or MadN35-Myc were contact inhibited (Fig. 5).

Immunoprecipitations from the pools of cells showed that the chimeric proteins were expressed to identical levels (data not shown). To test another parameter of transformation, we plated 1,000 cells from each of the stable pools of cells into soft agar. Only the cells expressing c-Myc or Mad(Pro)N35-Myc formed colonies in the soft agar, further demonstrating their transformed phenotype (data not shown). Therefore, the fusion of the Mad1 SID with c-Myc not only abolished c-Myc's ability to activate transcription, but also inactivated it as a transforming oncoprotein. These data suggest that mSin3 can repress transcription in a dominant manner not only from an artificial reporter but from downstream c-Myc target genes.

Both amphipathic helices of PAH2 are required for interaction with Mad. The interaction between the Mad SID and mSin3A/B is dependent on the PAH2 region of the latter proteins (8, 58). The PAH domains of the Sin proteins have been modeled as two amphipathic helixes separated by a loop, and it has been suggested that these regions serve as protein-protein interaction surfaces (72). To determine which of the amphipathic helices of PAH2 (A or B) of mSin3A were required for interaction with the SID, we deleted each helix separately. This was done in the context of the VP16-PAH2 fusion identified as binding Mad1 in a two-hybrid screen (8). These deletion constructs, along with LexA-Mad, were used to transform the yeast strain L40, which carries a LexA-responsive lacZ reporter. No interaction between LexA-Mad and either of the PAH2 deletion mutants was observed, even though the fusion proteins were expressed to the same level, and interaction between full-length VP16-PAH2 and LexA-Mad was easily detectable (data not shown and Fig. 6B and C). To confirm this result, each of the PAH2 constructs was in vitro translated and tested for interaction with in vitro-translated Mad1 by coimmunoprecipitation with anti-Mad antiserum. No detectable interaction above background between Mad and either of the deletion mutants was observed under conditions in which Mad interacted with full-length VP16-PAH2 (Fig. 6B). These results indicate that both helices of PAH2 are necessary to form the structural unit recognized by the SID.

DISCUSSION

A transferable repression domain. We define the SID as the amino-terminal 35 amino acids of the Mad1 transcriptional repressor that can function as a transferable repression domain. Point mutations in the Mad1 SID abolish mSin3A or mSin3B binding (8). These mutant Mad proteins also fail to repress transcription. These observations suggest that repression requires the interaction between the SID and endogenous mSin3 proteins present in 293 cells. Indeed, we have shown that mSin3 is a stable, nuclear protein expressed in a wide variety of different cell types (8a) and that mSin3 itself has intrinsic repression activity when tethered to DNA (Fig. 1). However, we cannot, at this point, formally rule out the possibility that SID-mediated repression requires interaction between the SID and another, as yet unidentified, factor(s).

By analogy to the yeast SIN3 protein, which does not possess any detectable DNA binding activity (73), we have proposed that repression by Mad:Max heterodimers is mediated by the recruitment of mSin3 to DNA to form a ternary complex. We had previously shown that these three proteins form such a ternary complex on the Mad:Max CACGTG binding site in vitro (8). Furthermore, it has been demonstrated that Mad:Max can repress transcription in yeast cells in a manner that is dependent on the formation of a DNA-binding ternary complex with yeast Sin3 (35a). We believe the SID-GALDBD fusion proteins function in a similar manner, by recruiting mSin3 to the DNA. Supporting this idea is our finding that the

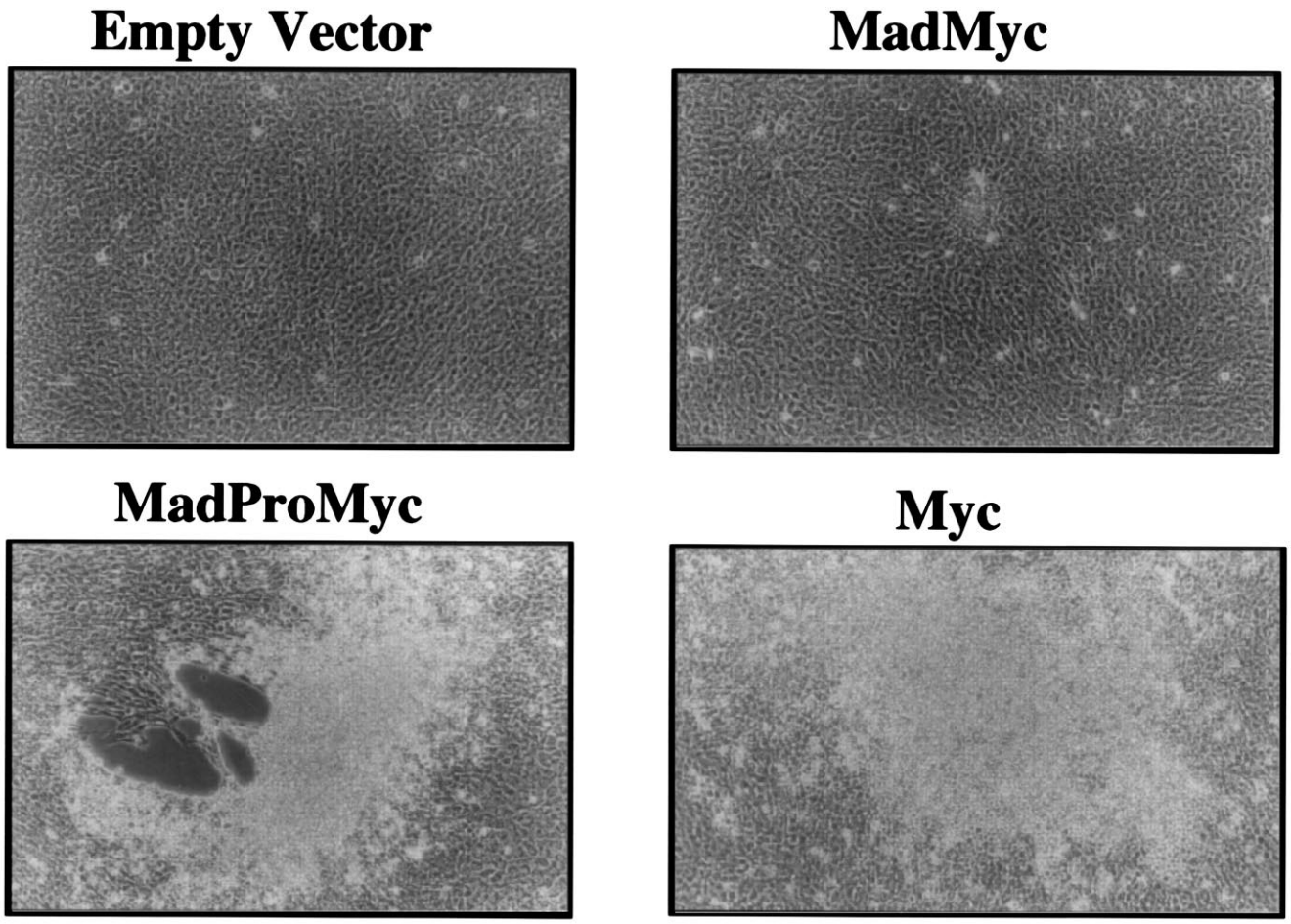

FIG. 5. SID can repress c-Myc's ability to transform Rat1A cells. Photographs of representative areas from monolayer cultures of the stably transfected cells are shown at magnification ×18. The expression vector used is indicated at the top of each panel.

GAL-mSin3 fusion also represses transcription in a manner dependent on the inclusion of GAL sites in the promoter (Fig. 1D). In addition, repression was independent of the PAH2 domain (Fig. 1D), suggesting that the primary function of PAH2 is to serve as an interaction domain for transcription repressors like the Mad family. Given that mSin3A is a relatively abundant protein and is expressed in cells in which none of the Mad family members are expressed (8a), it seems likely that other repressors will utilize PAH2 to tether mSin3A to DNA and drive repression.

The SID overrides multiple activators. Our experiments with different promoters demonstrate that the Mad SID can repress transcription in multiple contexts (Fig. 1 and 2). The promoters used here contain binding sites for different types of ubiquitously expressed activators, including SP1, AP1, and USF, which contribute to the basal levels of transcription detected from the reporter genes. Therefore, the repression induced by SID is dominant over some or all of these activators. Since these factors have multiple and different types of activation domains (1, 2, 20, 38), it is likely that the dominant effect of SID is not restricted to one specific activation domain. This conclusion is further supported by the fact that repression is observed with the SID fused either to GALVP16 or c-Myc, implying that SID can override potent activation domains in the same protein (Fig. 3). In this regard, it is interesting that the Mad1 protein has an activation domain in its carboxy terminus that is masked by the presence of the SID in its natural context (unpublished data). Therefore, under conditions in which the activity levels of the mSin3 proteins are reduced, Mad or other proteins that utilize mSin3 as a corepressor may shift their transcription activity from repression to activation. The alternative splicing of Mxi1, resulting in loss of the SID, provides one example of how such regulation could occur (58).

The fusion of the SID to c-Myc abolished the latter's ability to activate transcription (Fig. 4). These experiments were carried out with synthetic reporter genes, but the finding that the SID-Myc fusion can no longer transform cells suggests that it fails to activate at least some of c-Myc's natural targets (Fig. 5). The control experiment utilizing a SID containing two proline substitutions demonstrates that it is the repression activity that is important in blocking transformation and not simply a general loss of Myc function due to the fusion of a heterologous domain. Earlier deletion mutagenesis studies of the c-Myc activation region, as well as domain swap experiments, had suggested a strong correlation between transformation and Max-dependent transcriptional activity (3, 62). Our findings further confirm that abrogation of Myc's transcriptional activity blocks its function as an oncogene. Recent results indicate that Myc can also function as a transcriptional repressor in certain contexts (47, 52); however, the relationship between Myc-mediated transcriptional repression and transformation is

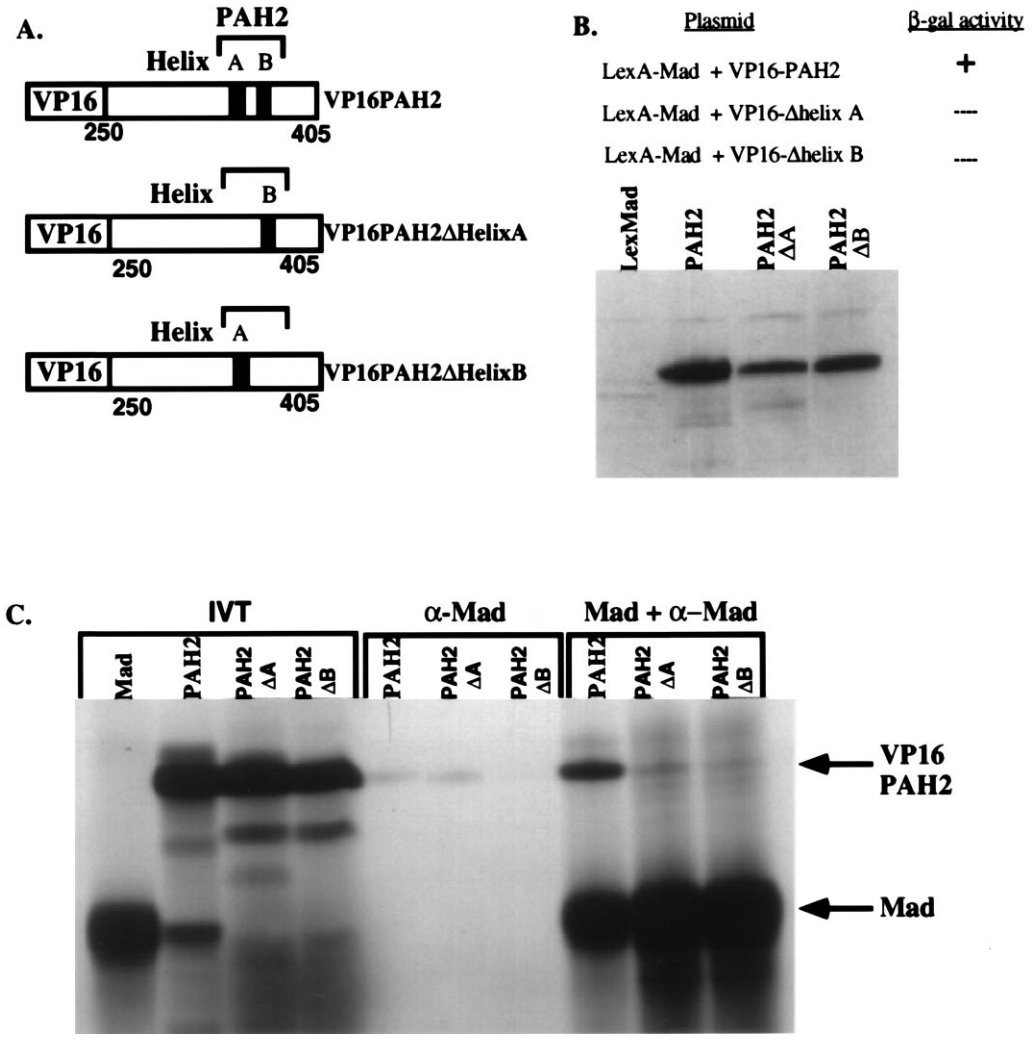

FIG. 6. mSin3A requires both helix A and helix B of PAH2 for interaction with Mad. (A) VP16 fusion constructs used in these experiments. (B) β-Galactosidase activity of L40 yeast strains transformed with the indicated plasmids. Protein levels of VP16-PAH2 and the two deletion mutants in yeast whole-cell lysates were determined by Western blotting (immunoblotting) with an antiserum specific for mSin3A. (C) Radiolabeled proteins synthesized in vitro with reticulocyte lysate programmed with RNAs encoding the proteins indicated at the top of the panel. The labeled proteins were separated on a sodium dodecyl sulfate-polyacrylamide gel either untreated (IVT) or after immunoprecipitation in the presence or absence of Mad protein with an antibody specific for Mad1 (α-Mad) (6) as indicated. The positions of the VP16-PAH2 and Mad proteins are shown.

not clear. Currently, we do not know if fusing the SID of Mad1 to c-Myc affects its native transcription repression function.

The ability of the SID of Mad1 to inhibit both c-Myc's transcriptional and transforming activities is of particular interest, because previous results showed that Mad1 can block Myc-dependent cell cycle progression and Myc-Ras cotransformation (40, 44, 55). c-Myc has been shown to possess three separable but interdependent activation domains (36): one resembles the acidic activation domain of VP16, the other is rich in glutamine and proline residues similar to the activation domain of SP1, and the third is unique. The findings that GAL-SID-mSin3 complex can abrogate the activity of several distinct activation domains as well as repress transcription from minimal and more complex promoters suggest that an element shared by all promoters is a target for repression.

Possible mechanisms of mSin3 repression. There appear to be at least three common mechanisms by which transcription repression is achieved, all of which are likely to impinge directly on the basal transcription apparatus and thereby the formation of functional preinitiation complexes. Repressors like MOT1 and DR1, which do not bind DNA specifically, appear to repress transcription by modulating the number of functional preinitiation complexes formed (5, 75). Transcription repressors that bind DNA specifically either make direct contact with components of the basal transcription machinery (21, 24, 28, 56, 69) or utilize a corepressor to mediate their action. Repression by Mad falls into the latter category and is similar to that observed for the *Drosophila* HLH protein Hairy (51), *S. cerevisiae* proteins α2 and Mig1 (37, 66–68), and thyroid hormone receptor α (17, 32) in that it requires a corepressor for function. Perhaps corepressors make direct contact with the basal machinery to facilitate repression. Furthermore, recent experiments showing that several *S. cerevisiae* proteins thought to be primarily involved with transcription repression are components of the mediator complex of the RNA Pol II holoenzyme (30, 39, 48, 59, 64) raise the possibility that the holoenzyme itself mediates the action of repressors or corepressors. Another possibility is that the influence of mSin3 on

the basal machinery is indirect. This would be consistent with our finding that the GAL-SID-mSin3 complex can repress transcription even when the binding site is distant from the initiation site (Fig. 2). Perhaps the mSin3 repression complex functions in a manner analogous to, but opposite from, that of the Swi-Snf complex (14, 19, 35, 43) and alters nucleosome or chromatin structure to establish a "negative chromatin domain" that either blocks the accessibility of the initiation site by the basal machinery and activators or blocks the communication between upstream activators and initiation site components. A related possibility is suggested by recent work demonstrating that the mammalian homolog of the yeast RPD3 protein is a histone deactylase (63). Since in yeast cells RPD3 has been shown to genetically interact with SIN3 (61), it is possible that the mammalian homologs of these proteins associate to influence chromatin structure by deacetylation of histone substrates. Now that cDNAs encoding some of the components of the repression machinery are available, it should be possible to answer some of these questions directly.

Biological implications. The ability of the SID of Mad1 to block multiple transcriptional activities may also provide a role for Mad1 function in differentiating cells that have previously downregulated c-Myc expression (6, 33, 34, 45, 70). Since c-Myc is absent in such cells it can be argued that there would be no need to repress Myc:Max target genes. However, all of the Myc targets identified to date appear likely to be regulated by multiple activators, both at the CACGTG binding sites as well as at other transcription factor binding sites within the promoter (9, 10, 25). Therefore, it is possible that Mad:Max binding results in a more general repression of a Myc target than could be achieved by the downregulation of c-Myc alone. A more rigorous test of this idea will require a careful examination of the effect of Mad-SID repression in promoters containing different combinations of proximal and distal enhancers.

All four known Mad family proteins from several species possess a SID that mediates interaction with mSin3, suggesting that SID repression is likely to function in widely diverse biological contexts (8, 34, 58). The demonstration that the SID domain can reverse the transforming capacity of the c-Myc oncogene and block the activity of other linked transactivation domains opens the possibility that the SID of Mad1 can be used more generally to study gene expression. By linking the relatively small (35-residue) SID of Mad1 to specific DNA binding domains or to well-characterized transcriptional activators, it should be possible to assess the biological effects of repression of specific genes. For example, fusion of the SID of Mad1 to other dominant nuclear oncogenes might be used to probe their function and reverse their oncogenic potential.

ACKNOWLEDGMENTS

We thank Achim Leutz and Arnold Berk for plasmids and Edward Giniger, David Stillman, and Susan Mango for helpful discussions and critical reading of the manuscript.

This work was supported by NCI grant RO1CA53718 (R.N.E.) and an NIH postdoctoral fellowship to C.D.L.

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