Repression of Myc-Ras Cotransformation by Mad Is Mediated by Multiple Protein-Protein Interactions[1]

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Abstract

Mad is a bHLH/Zip protein that, as a heterodimer with Max, can repress Myc-induced transcriptional *trans*-activation. Expression of Mad is induced upon terminal differentiation of several cell types, where it has been postulated to down-regulate Myc-induced genes that drive cell proliferation. Here we show that Mad also blocks transformation of primary rat embryo fibroblasts by c-Myc and the activated c-Ha-Ras oncoproteins. Mad mutants lacking either the basic region, the leucine zipper, or an intact NH_2-terminal protein interaction domain fail to inhibit Myc-Ras cotransformation. These results indicate that the repression of cotransformation requires DNA-binding and is mediated by multiple protein-protein interactions involving both Max and mSin3, a putative mammalian corepressor protein. With increasing amounts of the cotransfected *myc* gene, the numbers of transformed foci are reduced and the ability of Mad to inhibit focus formation is attenuated. Moreover, cell lines derived from such foci constitutively express both Myc and Mad proteins. Whereas Bcl-2 can significantly increase the numbers of transformed foci by enhancing the survival of *myc-ras*-transfected cells, it does not counteract the repressive effects of Mad on transformation, suggesting that Mad affects the growth properties rather than the viability of cells. Taken together, our results demonstrate that Mad is capable of antagonizing the biological effects of Myc and thereby suggest that Mad could function as a tumor suppressor gene.

Introduction

The members of the *myc* oncogene family (c-*myc*, N-*myc*, and L-*myc*) encode transcription factors that have been implicated in the control of proliferation of both normal and neoplastic cells (reviewed in Refs. 1–4). The *myc* genes are consistently found activated in several types of human tumors as a result of gene amplification or chromosomal translocation. Furthermore, all three *myc* genes can transform established fibroblasts such as Rat-1A cells and cooperate with an activated *ras* gene in the transformation of primary REF[3] (5–8). The *myc* genes also cause various types of malignancies in transgenic mice when constitutively expressed under the control of tissue-specific promoters (reviewed in Ref. 9). In addition to *ras, in vivo* tumorigenesis by *myc* is accelerated by simultaneous overexpression of either *raf, pim-1, pim-2, bmi*-1 or *cyclin*D (10–15). However, none of these genes has yet been shown to cooperate with *myc* in transformation of cultured cells.

The control of gene expression as well as cell proliferation by the Myc proteins may be modulated by a network of other nuclear proteins including Max (16), Mad (17), Mxi1 (18) and other more recently identified Mad-related proteins.[4] All these proteins share homologous DNA binding and dimerization interfaces consisting of a basic region contiguous with a helix-loop-helix-leucine zipper structure (for the organization of Mad, see Fig. 1*A*). Only Max can form DNA-binding homodimers, whereas heterodimerization with Max is a prerequisite for the known activities of both Myc and Mad family members (19–22). In addition to Max, Mad can also associate with the recently discovered mSin3 proteins (23), which appear to be murine homologues for the *Saccharomyces cerevisiae* general repressor Sin3. Both mouse and yeast Sin3 proteins contain four paired-amphipathic helix domains that may mediate protein-protein interactions (24). Indeed, paired-amphipathic helix-2 is required for the association of mSin3 with the NH_2-terminus of Mad (23).

While Myc:Max heterodimers stimulate transcription through promoters containing CACGTG or related sequences, repression of transcription is observed with both Max:Max homodimers and Mad:Max heterodimers (17, 25–27). This suggests that Mad might also down-regulate the *in vivo* target genes of Myc and thereby oppose Myc function. The different roles of Myc and Mad are further emphasized by their distinct expression patterns; expression of Myc is associated with cell proliferation (1) and that of Mad with cell differentiation (22, 28, 29).[5] The opposite effects of Myc and Mad in transcriptional regulation prompted us to determine whether Mad can also repress cotransformation by Myc and Ras, and if so, what the molecular basis for repression is.

Results

Mad Represses Cell Transformation by Myc and Ras. To examine the effects of the Mad protein in the Myc-Ras cotransformation assay, secondary cultures of REF were transfected with the activated c-Ha-*ras*^{Val12} oncogene together with c-*myc* in the presence or absence of *mad*. The

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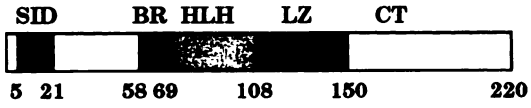
[3] The abbreviations used are: REF, rat embryo fibroblasts; CAT, chloramphenicol acetyltransferase; DOC, sodium deoxycholate.
[4] P. Hurlin, C. Quéva, P. J. Koskinen, E. Steingrimsson, N. Jenkins, and R. N. Eisenman, manuscript in preparation.
[5] P. Hurlin, K. P. Foley, D. E. Ayer, R. N. Eisenman, D. Hanahan, and J. M. Arbeit, submitted for publication.

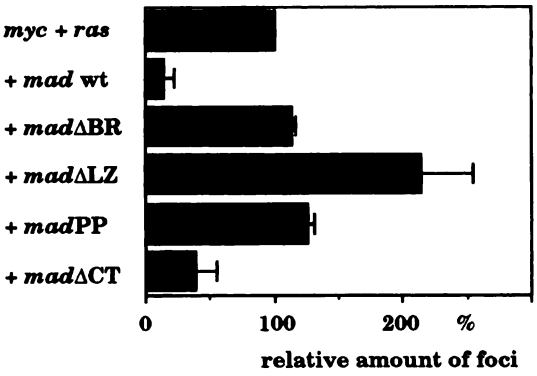
A.



B.

Plasmids transfected	Transformed foci at 14 days Exp. 1	Exp. 2	Exp. 3	Exp. 4
myc + *ras*	102	87	17	190
+ *mad* wt	3	15	2	15
+ *mad*ΔBR	118	96	ND	219
+ *mad*ΔLZ	259	152	ND	ND
+ *mad*PP	134	101	21	ND
+ *mad*ΔCT	14	47	8	81

C.



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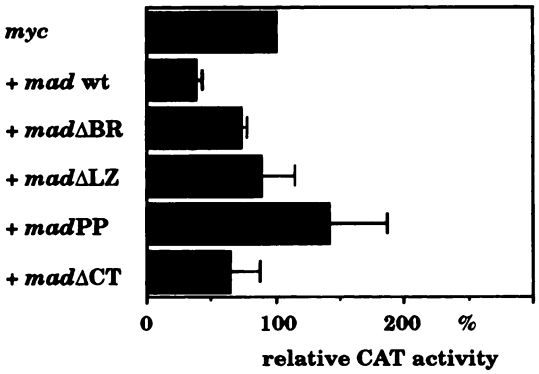


Fig. 1. Effects of wild-type and mutant Mad proteins on Myc-Ras cotransformation. *A*, a diagram of Mad with its functional domains. The numbers refer to amino acid positions. *SID*, mSin3 interaction domain; *BR*, basic region; *HLH*, helix-loop-helix; *LZ*, leucine zipper; *CT*, carboxyterminus. *B*, REF cultures were transfected with 3 μg of c-Ha-*ras*^{Val12} together with 2 μg of *myc* and 3 μg of either wild-type (*wt*) *mad* or one of the *mad* mutants, split once onto six plates and scored 2 weeks later for numbers of transformed foci. *ND*, not determined. *C*, the four independent experiments listed in *B* are summarized here with the average amounts of foci relative to those obtained by Myc and Ras alone. *Bars*, mean deviation. *D*, REF cultures were transiently transfected with 3 μg of pM4-minCAT together with 2 μg of *myc* and 3 μg of either wild-type (*wt*) *mad* or the indicated *mad* mutant. Before assaying for CAT activities, the samples were normalized according to both their protein content and β-galactosidase activities. Results from three separate transfection experiments with duplicate samples were combined to show the average CAT activities relative to those obtained by Myc alone. *Bars*, mean deviation.

concentrations of the transfected *myc* and *ras* genes used in the assay were initially optimized to obtain maximal amounts of transformed foci (data not shown). When the transformed foci were counted 2 weeks after transfection, it became evident that coexpression of *mad* significantly inhibited Myc-Ras cotransformation, with the numbers of foci in individual experiments being reduced by 83 to 97% (Fig. 1*B*). Furthermore, the repression was dependent on the amount of *mad* used in the transfection (data not shown).

To determine which regions of Mad are involved in the observed inhibition of Myc-Ras cotransformation, several mutants of Mad (see Fig. 1 and "Materials and Methods") were included in the assay. Results from four separate cotransformation experiments are summarized in Fig. 1, *B* and *C*. With the MadΔBR mutant, which lacks the basic region required for DNA binding but interacts with Max and mSin3 (17, 23), the numbers of foci were very similar to those obtained in the absence of Mad. This indicates that the ability to bind DNA is required for the repression of transformation by Mad. No foci at all were obtained when wild-type *mad* or the *mad*ΔBR construct was coexpressed with either *myc* or *ras* alone (data not shown), confirming that, in each case, overexpression of both *myc* and *ras* was necessary for focus formation.

The MadΔLZ mutant lacks the leucine zipper and is, therefore, unable to bind DNA as a heterodimeric complex with Max, although it is still capable of associating with mSin3 (23). As with MadΔBR, MadΔLZ did not repress (Fig. 1, *B* and *C*). By contrast, the numbers of transformed foci were slightly up-regulated, most likely due to exceptionally high transfection efficiencies observed with transfection mixes containing the *mad*ΔLZ gene construct (data not shown). However, we cannot exclude the possibility that MadΔLZ could have additional effects on transformation, possibly by sequestering factors that normally limit the effects of Myc.

In the MadPP mutant, leucine 12 and alanine 16 of Mad have been replaced with proline residues. This double mutation disrupts a putative α-helical structure in the NH_2-terminus of Mad that is required for association of Mad with mSin3 but does not affect formation of DNA-binding complexes with Max (23). Again, no repression was observed with the MadPP mutant, indicating that the mSin3 binding domain of Mad is essential for inhibition of transformation. By contrast, the MadΔCT mutant lacking the COOH-terminal sequences downstream of the leucine zipper reduced the numbers of transformed foci, but about 4-fold less efficiently than wild-type Mad. This result suggests a less critical role in inhibition for the COOH-terminus of Mad.

We have demonstrated by immunoprecipitation analyses that all the *mad* constructs are expressed at equivalent levels in transfected cells (data not shown). In addition, all of the proteins that were examined for their subcellular distribution (wild-type Mad, MadΔCT, and MadPP) were found to be nuclear localized (Ref. 22 and data not shown). Therefore, the inabilities of the Mad mutants to block Myc-Ras cotransformation were not due to failures in their expression.

The Inhibition of Transformation by Mad Is Related to Its Ability to Antagonize Transcriptional Regulation by Myc. We next wanted to determine whether the observed effects of wild-type Mad and the mutants in the cotransformation assay correlate with their abilities to repress Myc-mediated transcriptional *trans*-activation. For this purpose, REF were transfected with *myc* and *mad* expression vectors, together

A.

Plasmids transfected	Transformed foci at 12 days		
	Exp. 1	Exp. 2	Exp. 3
myc + ras	20	7	13
+ mad	2	1	4
+ bcl-2	521	115	115
+ mad + bcl-2	ND	41	11

B.

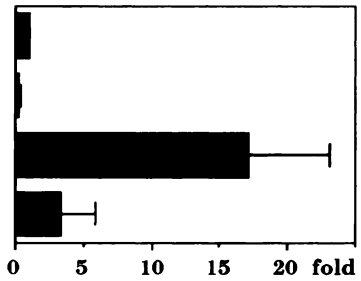


Fig. 2. Effects of Bcl-2 on Myc-Ras cotransformation in the absence or presence of Mad. *A*, REF cultures were transfected with 3 μg of c-Ha-*ras*^{Val12}, 2 μg of *myc* and 3 μg of *mad* and/or *bcl*-2, and the numbers of foci were scored 12 days later. *ND*, not determined. *B*, the three independent experiments listed in *A* are summarized here with the average amounts of transformed foci relative to those obtained by Myc and Ras alone. *Bars*, mean deviation.

with the pM4-minCAT reporter construct (25). This construct contains a 4-fold reiteration of the CACGTG binding site recognized by both Myc:Max and Mad:Max complexes. The transfected cells were harvested 48 h later and analyzed for their β-galactosidase and CAT activities. Similar to the results obtained from the cotransformation assay, significant repression of Myc-induced *trans*-activation was detected only with wild-type Mad and to a lesser extent also with the MadΔCT mutant (Fig. 1*D*). Taken together, our results with the Mad mutants strongly suggest that repression of both Myc-mediated *trans*-activation and Myc-Ras-induced transformation is mediated through distinct activities of Mad, including its binding to DNA, Max, and mSin3.

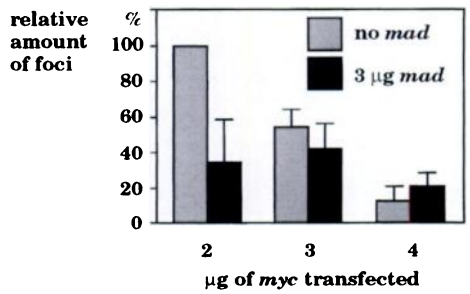
Bcl-2 Enhances Focus Formation by Myc and Ras but Cannot Rescue Repression by Mad. Overexpression of Myc has been shown to sensitize cells for apoptosis (30, 31). Therefore, one possible explanation for the reduced numbers of transformed foci in the presence of Mad is that Mad, by itself or in synergy with Myc, enhances the rate of cell death. Since Bcl-2 has been shown to protect cells from Myc-induced apoptosis (32, 33), we tested the effects of Bcl-2 in the cotransformation assay in the absence or presence of Mad.

As shown in Fig. 2, coexpression of *bcl*-2 with *myc* and *ras* resulted in a striking 10- to 25-fold increase in the numbers of transformed foci, most probably due to improved survival of *myc-ras*-transfected cells. Here it should also be noted that in the presence of *bcl*-2, the transformed foci appeared much more rapidly. For this reason, the numbers of foci were scored at 12 days after transfection rather than the usual 14 days. Although the overall transformation efficiency was clearly enhanced by Bcl-2, Mad still repressed focus formation by an average of 90%, as also observed in the absence of Bcl-2 (Fig. 2).

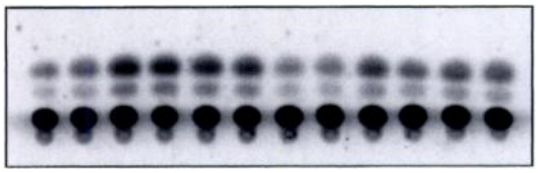
A.

Plasmids transfected		Transformed foci at 14 days		
myc	*mad*	Exp. 1	Exp. 2	Exp. 3
2	–	308	55	87
2	3	219	8	15
3	–	181	24	52
3	3	175	ND	23
4	–	21	3	22
4	3	39	10	29

B.



C.



rel. act.	1.0	3.6	2.4	0.7	1.5	1.5
myc	–	2	4	–	2	4
mad	–	–	–	3	3	3

Fig. 3. Effects of *myc* concentration in the transformation and *trans*-activation assays. *A*, REF cultures were transfected with the indicated amounts (μg) of *myc* and *mad* expression vectors together with 3 μg of c-Ha-*ras*^{Val12}, and the numbers of foci were scored 2 weeks later. *ND*, not determined. *B*, the three independent experiments listed in *A* are summarized here with the average amounts of foci relative to those obtained by Myc and Ras alone. *Bars*, mean deviation. *C*, REF cultures were transiently transfected with 3 μg of pM4-minCAT together with the indicated amounts (μg) of *myc* and *mad* expression vectors. Shown are both the actual and relative (*rel. act.*) CAT activities assayed from normalized samples.

It has been shown previously that Bcl-2 can cooperate with Ras to transform primary REF, if the suppressive effect of neighboring cells is prohibited by neomycin selection (34). Even then, the foci appear with a longer latency than with the combination of Myc and Ras. To demonstrate that the enhanced transformation efficiencies observed here were not merely due to cooperation of Bcl-2 with either Myc, Ras, or Mad, all these combinations as well as Bcl-2 alone were tested in the transformation assay. At 2 weeks after transfection, no foci were detected (data not shown), again confirming that, under our conditions (*i.e.*, in the absence of drug selection), the appearance of transformed foci was strictly dependent on cooperation between Myc and Ras.

Negative Effects of Mad Are Attenuated by Increasing Amounts of Myc. In experiments in which we titrated the amounts of the transfected *myc* DNA, we observed that the numbers of transformed foci were dependent not only on the levels of *mad*, but also of *myc*. In fact, increasing amounts of *myc* resulted in significantly reduced transfor-

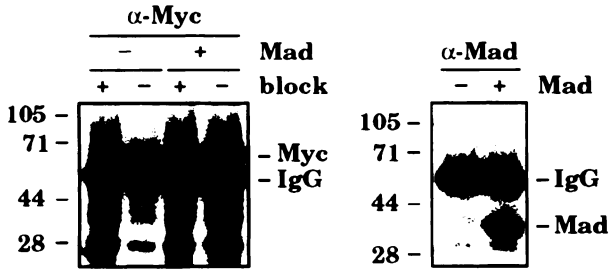


Fig. 4. Expression of Myc and Mad proteins in transformed cell lines. Cell lines were established from transformed foci of cells transfected with *myc* +/− *mad* genes. These cell lines were analyzed for their abilities to express Myc and Mad proteins by α-Myc and α-Mad immunoprecipitations, followed by Western blotting with the same antisera. The specificity of the α-Myc immunoprecipitation was confirmed by blocked samples treated with α-Myc antiserum that had been preincubated with the corresponding antigen.

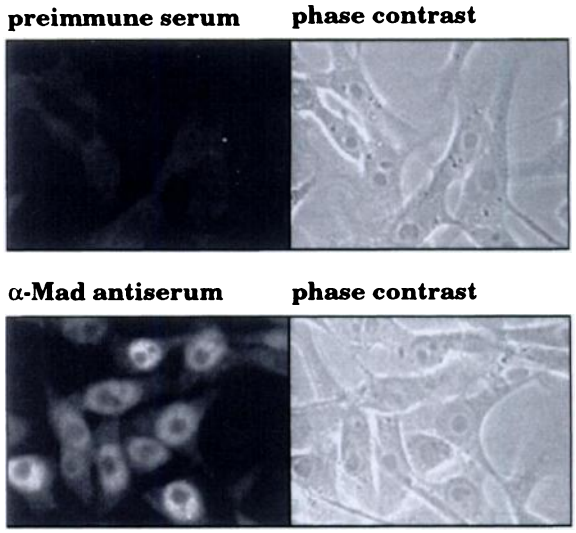


Fig. 5. Localization of the Mad protein in a *myc-mad*-coexpressing cell line. Shown are indirect immunofluorescence staining results with either preimmune serum or α-Mad antiserum from an REF cell line overexpressing both *myc* and *mad* genes and phase-contrast micrographs of the same fields of cells.

mation efficiencies (Fig. 3, *A* and *B*). For example, the numbers of foci with 4 μg of the *myc* expression vector were only 10% of those obtained with 2 μg of the same construct. Furthermore, this reduction in focus formation was accompanied by a gradual loss of repression by Mad relative to that detected at lower concentrations of *myc* (Fig. 3, *A* and *B*). By contrast, Max inhibited transformation at any *myc* dose tested (data not shown). Additional amounts of *ras* DNA did not increase the numbers of foci, indicating that *ras* was not limiting in the cotransformation assay (data not shown).

Since increasing amounts of *myc* resulted in decreased numbers of transformed foci, we considered the possibility that this was due to an even higher rate of apoptosis than with lower doses of the gene. Indeed, coexpression of *bcl*-2 was able to rescue the negative effects of *myc* and increase the numbers of foci close to those observed with lower *myc* doses in the presence of *bcl*-2 (data not shown). Another explanation for the decreased numbers of foci was obtained from *trans*-activation studies. The Myc-mediated stimulation of transcription from the pM4-minCAT reporter construct was diminished by a higher *myc* dose (Fig. 3*C* and data not shown), correlating with the reduced ability of *myc* to cotransform cells. The negative effects of Mad on Myc-mediated *trans*-activation were also attenuated (Fig. 3*C* and data not shown), possibly due to increased competition by Myc for binding to the reporter gene.

We established cell lines from the transformed foci and analyzed them for their abilities to express Myc and Mad proteins. With low doses of the *myc* gene cotransfected with wild-type *mad* or one of the mutants, only the nonrepressive Mad mutants were coexpressed with the Myc protein at detectable levels (data not shown). However, at higher *myc* doses, we were able to establish cell lines that expressed significant amounts of both Myc and the wild-type Mad protein (Fig. 4). Such cells, derived from either independent or pooled foci, displayed a transformed morphology indistinguishable from those of cells transfected with *myc* and *ras* in the absence of *mad* (data not shown). Furthermore, no differences were detected in the growth patterns of *myc*-overexpressing cells in the presence of either low or high serum whether or not the cells coexpressed *mad* (data not shown). Moreover, results from our immunofluorescence studies (Fig. 5) indicate that the stably overexpressed Mad protein localizes to the nucleus as expected from previous cell fractionation studies (22) with a pattern very similar to that observed for Myc and Max (19).

Discussion

Our present results using the REF cotransformation assay indicate that Mad can efficiently antagonize the oncogenic effects of Myc, resulting in a dramatic decrease in the number of transformed foci otherwise observed in the presence of the c-*myc* and the activated c-Ha-*ras* oncogenes. Furthermore, under conditions where repression by Mad was most striking, we were not able to establish any cell lines with detectable levels of the Mad protein. This suggests that overexpression of Mad is incompatible with Myc-Ras cotransformation, as observed previously for Max (35). Thus, the foci obtained from *myc-ras-mad* transfected cultures were derived from cells where either the *mad* gene had not been stably integrated or where its expression was shut down. Similar results with Mad and its close relative Mxi1 were recently reported by Lahoz *et al.* (36).

When the primary cells are transfected with higher than optimal concentrations of c-*myc*, the repressive effects of Mad, but not of Max, are attenuated. These results suggest that there may, indeed, be functional differences between Max:Max homodimers and Mad:Max heterodimers, due for example to distinct protein-protein or protein-DNA interactions. It is also possible that the increasing amounts of Myc result in the squelching of some nuclear factors that are essential for repression by Mad, but not by Max. In any case, with higher doses of the *myc* gene cotransfected with *mad*, we have detected significant levels of expression of both Myc and Mad proteins in cell lines derived from transformed foci. The stably overexpressed Mad displays the expected molecular size as well as a nuclear localization pattern indistinguishable from that observed for Myc and Max. Furthermore, no obvious differences in either growth or morphology of Myc-overexpressing cells have been detected, whether or not they coexpress Mad. These

results suggest that high levels of Myc can allow cells to tolerate the antiproliferative effects of Mad or that Mad can protect cells from the potentially harmful consequences of Myc overexpression.

To address the mechanism(s) of Mad-mediated repression, we tested several mutants of Mad in the Myc-Ras transformation assay. Results from these experiments strongly suggest that repression of transformation requires multiple protein-protein and protein-DNA interactions. Mad mutants with deletions of either the DNA-binding basic region or the leucine zipper required for association with Max are incapable of inhibiting transformation. Thus, binding of Mad:Max heterodimers to DNA is necessary but not sufficient for the observed repression. Deletion of COOH-terminal sequences of Mad downstream of the leucine zipper also partially relieves repression, leaving open a possibility of an as yet unidentified binding site in the COOH-terminus of Mad for a corepressor protein. However, this large deletion may also have a more general effect on protein configuration and thereby on Mad function. Importantly, no repression at all is observed with an NH_2-terminal double proline mutation that disrupts a putative α-helical structure required for the association of Mad with mSin3. This indicates that binding of mSin3 (or even some other as yet unknown factor) to the NH_2-terminus of Mad is also essential for the ability of Mad to antagonize Myc function. Although the requirement for multiple protein-protein interactions for inhibition of transformation correlates well with the ability of Mad, Max, and mSin3 to form DNA-binding ternary complexes *in vitro* (23), we still lack formal proof that these are the only interactions required for Mad function.

Our conclusions from transformation assays with wild-type Mad and the Mad mutants are very similar to those obtained from *trans*-activation assays, carried out both in the primary REF (this study) and in the more established NIH3T3 cells (17, 23). Thus, in both assays, the basic region, the leucine zipper, and an intact NH_2-terminal mSin3-binding domain of Mad are required for inhibition of Myc activities. These results are consistent with the idea that the negative effects of Mad are primarily mediated via transcriptional repression. Furthermore, they indicate that Mad interferes with Myc-Ras transformation by an active repression mechanism, in contrast to a more passive inhibition merely involving occupation of DNA-binding sites shared by Myc:Max and Mad:Max heterodimers. Although all the Mad mutants (except for MadΔLZ) can sequester endogenous Max into partially inactive complexes, it is unlikely that Max is limiting in our *trans*-activation and transformation assays. Otherwise, we should have observed at least some repression with MadΔBR, the mutant that associates with Max but is unable to compete with Myc:Max heterodimers for binding to DNA.

According to our results, Bcl-2 can dramatically increase the numbers of Myc-Ras-transformed foci. Furthermore, the foci reach a detectable size 2 days earlier than in the absence of Bcl-2. Thus, it seems as if, under our normal assay conditions without Bcl-2, most of the *myc-ras*-transfected cells die far before being able to form foci. Therefore, it seems likely that the numbers of foci counted from the plates cotransfected with *bcl*-2 more closely reflect the amounts of cells that originally incorporated the *myc* and *ras* genes than those detected on the plates lacking *bcl*-2.

Although the presence of Bcl-2 can significantly enhance focus formation by Myc and Ras, it is not able to antagonize the repressive effects of Mad on transformation. Thus, even though the actual numbers of transformed foci increase upon the addition of Bcl-2, a similar reduction in transformation efficiency by Mad is observed whether or not Bcl-2 is present. Although it is formally possible that Mad could cause cell death via a Bcl-2-independent pathway, it seems more likely that Mad does not have any significant influence on the viability of cells, either positive or negative, but rather affects their growth properties. This is also supported by the observation that Mad can repress transformation by Myc and Ras, but not by E1A and Ras (36). Future investigations should reveal whether Mad functions by arresting cells at a specific stage of the cell cycle, such as the G_1-S boundary, where the effects of Myc are believed to take place.

Taken together, our results from both transformation and *trans*-activation assays support a model in which the ability of Mad to block Myc-Ras cotransformation of primary cells is a reflection of the transcriptionally antagonistic activities of Myc and Mad. If the Myc:Max heterodimers and the putative Mad:Max:mSin3 ternary complexes compete for binding to the same or overlapping target genes, then the relative concentrations of the Myc and Mad family members, all of which appear to be tightly regulated, are expected to determine which of the antagonistic transcriptional pathways is initiated. Moreover, this model suggests that Mad also inhibits biological activities of Myc other than cell transformation. Further support for a more general antiproliferative role for Mad has been obtained from recent studies with NIH3T3 cells stably transfected with the CSF-1 receptor.[6] There the Myc-dependent entry of quiescent cells into the cell cycle is blocked by coexpression of the wild-type Mad protein but not by mutants unable to bind to Max or mSin3. It is likely that a similar inhibition of Myc function also occurs during the initiation of terminal differentiation when expression of Mad is induced. Furthermore, the human *mad* gene has been localized to chromosome 2 at p13 in a region possibly involved in neoplasia (37). This, together with the observed negative effects of Mad on Myc-Ras cotransformation, raises the possibility that Mad may inhibit the proliferation-promoting function of deregulated Myc in early stages of neoplastic progression and thereby act as a tumor suppressor protein.

Materials and Methods

Mutagenesis and Subcloning. The *mad*ΔCT construct was prepared from the full-length *mad* cDNA (17) using PCR mutagenesis. The MadΔCT protein lacks the COOH-terminal amino acid residues 155–221 downstream of the leucine zipper. The other Mad mutants, MadΔBR lacking the basic region, the MadΔLZ lacking the leucine zipper, and the MadPP with leucine 12 and alanine 16 being replaced with proline residues, have been described previously (17, 23). For REF transfections, the wild-type and mutant *mad* cDNAs as well as the *bcl*-2 cDNA (38) were transferred to the pLTRpoly vector (39).

REF Transformation Assays. REF were prepared from 13-day-old Fischer rat embryos, grown in DMEM supplemented with 10% FBS and passaged once before transfecting them by the calcium phosphate precipitation technique (40). The transfection mixes included 2–4 μg of pLTR-Tc-

[6] M. Roussel and D. E. Ayer, unpublished data.

myc (41) and 3 μg of each pGEJ(6.6) expressing the activated c-Ha-*ras*^{Val12} oncogene (35), pLTR-*mad*, or the corresponding mutants and/or pLTR-*bcl*-2. One μg of the CMV-β-galactosidase vector (pCH110; Pharmacia LKB) was also added to control for the transfection efficiencies (42). To obtain a total of 12 μg of DNA, appropriate amounts of the empty pLTRpoly vector were added. The transfected cells were split once in a 1:6 ratio and grown in DMEM supplemented with 5% fetal bovine serum. Medium was replenished every third day, and transformed foci were scored at 12 to 14 days after transfection.

***Trans*-Activation Assays.** Secondary cultures of REF were transfected as for the transformation assays, but now the transfection mixes were split onto two 60-mm dishes, and the c-Ha-*ras* expression vector was replaced with the pM4-minCAT reporter (25). This construct contains four copies of the CACGTG sequence upstream of a minimal promoter linked to the CAT gene. The cells were harvested two days after transfection, normalized for both their protein content and β-galactosidase activities, and then assayed for CAT activities using the thin-layer chromatographic method (43).

Protein Analyses. To detect Myc and Mad proteins, stably transfected cells established from transformed foci were lysed in AB buffer [20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.5% NP40, 0.5% SDS, and 0.5% DOC] containing 0.5% aprotinin. Sonicated cell lysates were cleared by centrifugation and immunoprecipitated with α-Myc (44) or α-Mad (Santa Cruz) antiserum in the presence of protein A-Sepharose particles. Immunoprecipitates were washed twice with AB buffer, once with high salt buffer [10 mM Tris-HCl (pH 7.4), 2 M NaCl, 1% NP40, and 0.5% DOC] and twice with RIPA buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 0.1% SDS, and 1% DOC] and were analyzed by SDS-polyacrylamide gel electrophoresis, followed by Western blotting using the same antisera as above and the ECL detection system of Amersham.

Immunofluorescence. Cells stably overexpressing both Myc and Mad were grown on coverslips, fixed with 3.5% paraformaldehyde in PBS for 20 min, and permeabilized with 0.2% NP40 in PBS for 5 min. Staining was carried out with α-Mad antiserum (22), followed by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson Laboratories). The stained samples were then analyzed by confocal microscopy.

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Note Added in Proof

While this paper was under review, two related reports were published. Västrik *et al.* (J. Cell Biol., *128:* 1197–1208, 1995) provide further evidence for the inhibition of Myc-Ras cotransformation by Mad. Schreiber-Agus *et al.* (Cell, *80:* 777–786, 1995) demonstrate that Mxi1 also interacts with mSin3 and that (similarly to our results with Mad) this interaction is required for the inhibition of Myc-Ras cotransformation by Mxi1.

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