A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation

Donald E. Ayer and Robert N. Eisenman
Division of Basic Sciences A2-025, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104 USA

Mad is a basic-helix-loop-helix-zipper protein that heterodimerizes with Max in vitro. Mad:Max heterodimers recognize the same E-box-related DNA-binding sites as Myc:Max heterodimers. However, in transient transfection assays Myc and Mad influence transcription in opposite ways through interaction with Max: Myc activates while Mad represses transcription. Here, we demonstrate that Mad protein is induced rapidly upon differentiation of cells of the myeloid lineage. The Mad protein is synthesized in human cells as a 35-kD nuclear phosphoprotein with an extremely short half-life ($t_{1/2} = 15-30$ min) and can be detected in vivo in a complex with Max. In the undifferentiated U937 monocyte cell line Max was found complexed with Myc but not Mad. However, Mad:Max complexes began to accumulate as early as $2$ hr after induction of macrophage differentiation with TPA. By $48$ hr following TPA treatment only Mad:Max complexes were detectable. These data show that differentiation is accompanied by a change in the composition of Max heterocomplexes. We speculate that this switch in heterocomplexes results in a change in the transcriptional regulation of Myc:Max target genes required for cell proliferation.

[Key Words: Transcriptional regulation, bHLHZip protein, homodimers, heterodimers, monocyte/macrophage differentiation]

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Max and DNA-binding activities, Mad and Mxi1 behave similarly to Myc.

Despite the similarities between Myc and Mad, examination of their transcriptional activities revealed striking differences. When tested in transient transfection assays in mammalian or yeast cells, overexpression of Myc resulted in activation of transcription from a reporter similar to Myc. Consistent with the results of in vitro experiments showing competition of Myc and Mad for Max, transcriptional activation by Myc is suppressed by increasing amounts of Myc and, conversely, repression by Mad is abrogated by increasing amounts of Myc [Ayer et al. 1993]. Both Myc activation and Mad repression are blocked by overexpression of a dominant interfering Max protein, providing further support for the idea that Max mediates the in vivo effects of both Myc and Mad [Mukherjee et al. 1992; Prendergast et al. 1992; Amati et al. 1993; Ayer et al. 1993]. Although Mxi1 was not tested for repressor activity, it does not activate transcription in a heterologous yeast system [Zervos et al. 1993].

The results described above raise the possibility that Max mediates both positive (through interaction with c-Myc, N-Myc, and L-Myc) and negative (through interaction with Mad, and possibly Mxi1) transcriptional effects on E-box-containing target genes. The ability of Mad to compete with Myc for binding to Max and to repress Myc transcriptional activation suggests that Mad could oppose the biological functions of Myc. Functional antagonism between Myc and Mad would be expected to depend on the relative abundance of these two proteins as well as the level of Max. Although RNA for Mxi1 has been shown to be widely expressed and induced upon injection of myeloid differentiation [Zervos et al. 1993; A. Zervos, pers. comm.], neither Mxi1 nor Mad protein expression has been examined previously. We therefore characterized Mad protein in vivo, and examined its interaction with Max as a function of differentiation.

Results

Characterization of Mad proteins in vivo

Our initial attempts to identify Mad protein in proliferating cells were unsuccessful. However, because myc mRNA levels are often, but not always, down-regulated during differentiation [for review, see Marcu 1992], and down-regulation of Myc appears to be critical for terminal differentiation [e.g., Coppola and Cole 1986; Larsson et al. 1988; Miner and Wold 1991], we decided to examine Mad expression in differentiating cells. U937 is a human monoblastic leukemia cell line that can be induced to differentiate along the monocytic/macrophage pathway by treatment with phorbol esters (Harris and Ralph 1985). To determine whether mad mRNA was induced during U937 differentiation, poly(A)⁺ RNA was prepared from untreated cells or cells treated with TPA for 3 hr in the presence or absence of the protein synthesis inhibitor cycloheximide. Poly(A)⁺ RNA was then analyzed for mad induction by northern blotting (Fig. 1).

The mad cDNA probe hybridized to RNAs of 3.5 and 7 kb, and the amount of both RNA species increased in response to TPA. By normalizing to a β-actin internal control, we determined that the 3.5-kb mRNA was induced approximately fivefold. This induction occurred in the presence of the protein synthesis inhibitor, suggesting that it was an immediate response to TPA treatment and did not require de novo protein synthesis.

To determine whether Mad protein was also induced following TPA treatment of U937 cells, we performed immunoprecipitations from lysates prepared from metabolically labeled U937 cells treated with TPA for 16 hr. The antiserum used in these experiments was generated by immunizing rabbits with a glutathione-S-transferase–Mad fusion protein [GST–Mad; see Materials and methods] and was initially tested on Mad protein synthesized in vitro. Mad protein synthesized in vitro migrates with an apparent molecular mass of 35 kD and was precipitated by the Mad antiserum (Fig. 2A). Although nearly undetectable in untreated cells [see Fig. 7, below], TPA treatment of U937 cells allowed the immunoprecipitation of two polypeptides with an apparent molecular mass of 35 kD and a third of 44 kD by the anti-Mad serum (Fig. 2B). The proteins from the in vitro translation and putative Mad proteins from U937 cells were apparently recognized specifically because they were not precipitated with preimmune serum or antiserum that...
Figure 2. Characterization of anti-Mad serum. Reticulocyte lysate programmed with Mad cDNA (A), TPA-treated U937 cells (B), or both (C) were labeled with [35S]methionine and immunoprecipitated with the anti-Mad serum. The precipitated proteins were analyzed on SDS-polyacrylamide gels. (Pre) Preimmune serum was used in the immunoprecipitations. (GST-Mad or GST) anti-Mad serum was blocked with either purified GST-Mad or GST protein, respectively. The positions of the molecular mass markers are shown in kD.

had been blocked with the immunizing GST–Mad fusion protein. In contrast, precipitation of these proteins was not inhibited by GST protein alone, indicating that epitopes unique to the Mad portion of the immunizing fusion protein were being recognized. The very similar electrophoretic mobilities of the 35 kD polypeptides and in vitro-translated Mad suggests that the former are mad-encoded polypeptides. Because the 44 kD protein was localized to the cytoplasm (Fig. 5, below), and did not associate with Max in vivo (Fig. 6, below), we did not examine it further in this study. Nonetheless, it is possible that this protein might transiently associate with Mad or Max in the cytoplasm.

In vitro-translated Mad protein and the 35 kD doublet from U937 cells migrate closely but not identically on SDS–polyacrylamide gels (Fig. 2C). To confirm that the immunoprecipitated proteins from U937 cells were related to Mad, both of the proteins in the 35 kD doublet were compared with in vitro-translated Mad protein using one-dimensional peptide mapping with V8 protease (Fig. 3). The two protein preparations possessed an array of comigrating proteolytic fragments indicating that they are highly related in primary sequence. We concluded, therefore, that the 35 kD doublet immunoprecipitated from U937 cells corresponds to Mad protein encoded by our cloned cDNA and characterized in our previous work (Ayer et al. 1993). Because Mad and MxiI are ~40% identical at the amino acid level, it is possible that the Mad antiserum could cross-react with MxiI; however, our Mad antiserum showed only marginal cross-reactivity with in vitro-translated MxiI protein [data not shown].

Myc and Max have very different stabilities. Myc is degraded very rapidly (Hann et al. 1985; Rabbitts et al. 1985; Lüscher and Eisenman 1988), whereas Max is extremely stable (Blackwood et al. 1992b). To determine the stability of Mad, U937 cells were treated with TPA for 16 hr to induce its synthesis and then pulse-labeled with [35S]methionine. The pulse-labeled cells were chased with an excess of nonradioactive methionine for increasing lengths of time. The amount of labeled Mad protein remaining at each time point of the chase was determined by immunoprecipitation. As above, pulse-labeled Mad immunoprecipitated as a doublet (Fig. 4).

The upper polypeptide was degraded with a half-life of 15–30 min. Conversion of the faster migrating polypeptide of the Mad doublet to the slower migrating form suggested a precursor–product relationship between the two. The mobility of both polypeptides was altered by phosphatase treatment indicating that Mad is a phos-
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data demonstrate that Mad is synthesized in vivo as a 35-kD highly unstable nuclear phosphoprotein.

Mad and Max associate in vivo

We have demonstrated previously that Mad and Max can form heterodimers in vitro (Ayer et al. 1993). However, before any role can be postulated for Mad:Max heterodimers it is important to demonstrate that the two proteins associate in vivo. To address this question, we performed immunoprecipitations using anti-Max serum under low-stringency conditions (i.e., in the absence of ionic detergents) where Myc:Max heterodimers have been shown to be maintained (Blackwood et al. 1992b). In addition to Max, multiple polypeptides immunoprecipitated with the Max antiserum under these low-stringency conditions (Fig. 6). However, two proteins of 70 and 35 kD were not detected when the Max antiserum was first blocked with immunogen, suggesting that they were precipitated by virtue of their association with Max. The protein of ~70 kD was identified previously as c-Myc (Blackwood et al. 1992b; also see Fig. 9, below). To positively identify the 35 kD protein as Mad, the proteins bound to the Max low-stringency immunoprecipi...

Figure 5. Subcellular localization of p35 Mad. Cytoplasmic (C) and nuclear (N) extracts were prepared from TPA-treated U937 cells metabolically labeled with [35S]-methionine as detailed in Materials and methods. Labeled proteins were immunoprecipitated with antiserum directed against either Max (α-Max) or Mad (α-Mad) and analyzed on a 15% SDS-polyacrylamide gel. [N + block] Immunoprecipitation with antiserum that had been blocked with its cognate immunogen. The positions of the molecular mass markers are shown.

Because Mad is a bHLHZip protein that functions as a transcriptional repressor when complexed to Max (Ayer et al. 1993), we have assumed that it is a nuclear protein. However, the putative nuclear localization signal in the Mad protein has only limited similarity to the consensus determined for bipartite nuclear localization signals (Dingwall and Laskey, 1991; Ayer et al. 1993). To directly demonstrate that Mad is nuclear we fractionated [35S]-methionine-labeled TPA-treated U937 cells into cytoplasmic and nuclear fractions and performed immunoprecipitations using antibodies specific for Max or Mad proteins (Fig. 5). Consistent with previous results, Max was detected primarily in the nuclear fraction (Blackwood et al. 1992b). The majority of the labeled Mad protein was also detected in the nuclear fraction (Fig. 5). Other unidentified proteins in the 44-kD range were also present in the cytoplasmic fractions. Taken together, our

phosphoprotein [data not shown]. However, at this time we cannot rule out the possibility that post-translational modifications other than phosphorylation are responsible for the distinct migration of the two Mad species. Such modifications may account for the lack of exact comigration between in vivo- and in vitro-translated Mad proteins (Fig. 2C).

We have determined that Mad protein is degraded with similar kinetics in another cell type of the monocyte/macrophage lineage where Mad is induced during differentiation (ML-1 cells treated with TPA) and in NIH-3T3 fibroblasts transfected with a Mad expression construct [data not shown]. Therefore, the rapid turnover of Mad is not unique to U937 cells or myeloid differentiation per se but appears to be an intrinsic property of the Mad protein.
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and therefore presumably Mad:Max complexes—do not
dissociate appreciably under these conditions (E.M.
Blackwood, unpubl.). Taken together, our findings
strongly suggest that Mad:Max heterocomplexes
were not formed after lysis but pre-exist in vivo.

The ratio of Myc:Max to Mad:Max heterocomplexes
changes during monocyte/macrophage differentiation

Mad mRNA was induced as an immediate response to
TPA treatment of U937 cells [Fig. 1], suggesting that
Mad may play a role early during differentiation in an
attempt to better understand the role of Mad in myeloid
differentiation, we examined the synthesis of the Mad,
Max, and Myc proteins by [35S]methionine labeling and
immunoprecipitation at various times following TPA
treatment of U937 cells [Fig. 7A-C]. The two Max pro-
teins did not change either in amount or in their ratio

Figure 6. Association of Mad and Max in vivo. Max proteins
were immunoprecipitated under high- or low-stringency con-
tions from TPA-treated U937 cells that had been labeled with
[35S]methionine as described in Materials and methods. The la-
beled proteins were analyzed on an SDS–polyacrylamide gel. [+] block] α-Max used in the immunoprecipitation blocked with
immunogen. The two lanes at right contain proteins eluted
from low-stringency anti-Max immunoprecipitates and repre-
cipitated with anti-Mad serum. [+] block) Immunogen-blocked
anti-Max serum used in the initial immunoprecipitation. The
positions of the molecular mass markers are shown.

Figure 7. Synthesis of Myc, Max, and Mad during U937 differ-
entiation. U937 cells were treated with TPA for the times in-
dicated and metabolically labeled with [35S]methionine for 1 hr.
Labeled proteins were immunoprecipitated from cellular ly-
states under high-stringency conditions with antiserum directed
against Mad [A], Max [B], or Myc [C] and analyzed on SDS–
polyacrylamide gels. [+] block] Immunoprecipitation with an-
tiserum blocked with its cognate immunogen.

It is possible that the Mad:Max heterocomplexes were
formed after cell lysis. However, blocking the anti-Max
serum with Max protein prevented coprecipitation of
Mad and suggests that significant amounts of subunit
exchange did not occur during immunoprecipitation.
Furthermore, previous work has shown that Myc:Max—

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Figure 9. Myc and Mad complexed to Max during U937 differentiation. U937 cells were treated with TPA for the time indicated and then metabolically labeled with \(^{35}\)S-methionine. Labeled proteins were initially immunoprecipitated under low-stringency with antisera directed against Max. The proteins in the anti-Max immunoprecipitate were eluted and reprecipitated with antisera specific for Myc and Mad. These anti-Myc and anti-Mad immunoprecipitates were analyzed on a SDS-polyacrylamide gel (+ block). Immunogen blocked anti-Max antisemr was used in the initial immunoprecipitation.

Discussion

Regulated expression of Mad

We have identified the Mad protein as a phosphorylated, nuclear-localized 35-kD doublet synthesized in a variety...
of cells (see below). Because this protein doublet shares antigenic determinants with in vitro-translated Mad [Fig. 2] and has the identical proteolytic peptides as bona fide Mad protein, synthesized from the cloned Mad cDNA [Fig. 3], we conclude that it represents the in vivo Mad translation product. We have also demonstrated that Mad protein has a half-life on the order of 15–30 min [Fig. 4], which is in the same range as Myc [Lüscher and Eisenman 1988] but much shorter than Max, which possesses a $t_{1/2}>18$ hr (Blackwood et al. 1992b). The pulse-chase analysis indicates a precursor-product relationship between the lower and upper bands of the doublet, suggesting that some form of post-translational modification may mediate the rapid turnover of Mad [Fig. 4]. To date, all proteins tested that heterodimerize with Max have extremely short half-lives [Hann et al. 1985; Rabbits et al. 1985; Ramsay et al. 1986; De Greve et al. 1988; Lüscher and Eisenman 1988; Blackwood et al. 1992b]. This may allow rapid changes in Max heterodimer complexes necessary for Myc-, Max-, or Mad-regulated biological processes.

Analysis of Mad RNA and protein indicated very low levels of expression in a wide range of tissues or cell types. However, upon induction of differentiation in the human myeloid leukemia cell line U937, we detected mad RNA and protein as rapidly as 2 hr after TPA treatment. Because mad RNA induction occurs in the absence of de novo protein synthesis, it is likely that mad levels are augmented as a direct response to treatment with the differentiation inducer. By analogy with myc and other immediate early mRNAs [Kelly et al. 1983; Almendral et al. 1988], the superinduction of mad RNA in the presence of cycloheximide suggests that the mad message may be unstable. RNAs hybridizing with the Mx1 probe were also found to be induced upon TPA treatment of U937 cells, although substantial amounts of mxi1 RNA are already present in undifferentiated U937 cells as well as many other cell types [Zervos et al. 1993; A. Zervos, pers. comm.].

The induction of Mad protein was not restricted to U937 cells but was also detected upon differentiation of the human ML-1 myeloid cell line, a murine erythroleukemia cell line and human primary keratinocytes [P. Hurlin, unpubl.]. We believe that the increase in Mad expression is very likely related to general differentiation events rather than to TPA treatment per se because cell types that do not differentiate in response to TPA also do not show significant Mad induction following TPA treatment [data not shown]. Furthermore, the induction of macrophage differentiation in ML-1 cells by transforming growth factor-$\beta$ (TGF-$\beta$) and transferrin also resulted in increased synthesis of mad mRNA [data not shown].

The absence of Mad expression in many proliferating cells, its rapid and apparently direct induction upon differentiation of cell types as diverse as macrophages and keratinocytes, and the very short half-life of Mad protein suggest that Mad expression is probably highly regulated in mammalian cells. However, as we have not been able to detect Mad protein in differentiating myogenic cells or adipocytes, it is clear that Mad expression is not necessarily linked to all differentiation events. In addition, Mad is expressed at relatively high levels in the human promyelocytic cell line HL60, and its levels do not change when they are induced to differentiate [data not shown]. The function of Mad may be restricted to a specific commitment event that has either been bypassed or has already occurred in these cells. Alternatively, cells that do not induce Mad may express other Max-interacting proteins that carry out functions related to those of Mad but are specific for other differentiation pathways.

A Max heterocomplex switch during differentiation

Our previous study using bacterially expressed or in vitro-translated Mad protein demonstrated that Mad alone does not homodimerize or bind DNA but was capable of forming sequence-specific DNA-binding heterocomplexes with Max [Ayer et al. 1993]. Here, we show that in differentiating U937 cells the 35-kD nuclear Mad protein can be specifically coimmunoprecipitated with Max under conditions in which the in vitro Myc:Max and Mad:Max heterocomplexes are known to be stable [Fig. 6]. Therefore, it is highly likely that Mad and Max are associated in vivo.

The rapid induction of Mad during differentiation and the existence of in vivo Mad:Max complexes prompted us to determine which proteins were present and interacting as a function of U937 cell differentiation. Immunoprecipitations carried out under high-stringency conditions demonstrated that Myc, Mad, and Max proteins were readily detectable up to 48 hr after treatment with inducer [Fig. 7]. Max levels were essentially constant while Mad expression was induced and increased over time as noted above. The amount of Myc protein associated with Max did not change appreciably from that observed in uninduced cells through 24 hr following TPA treatment. During the differentiation time course, increasing amounts of Mad were found associated with Max, in parallel with the total increase in Mad expression. By 24 hr both Myc and Mad were found in association with Max. However, at 48 hr, Myc protein could no longer be detected in Max complexes while the amount of Mad associated with Max increased to its highest level [Fig. 9]. A similar switch to Mad:Max complexes has also been observed during TPA-induced differentiation of primary keratinocytes [P. Hurlin, unpubl.].

Because Max is likely to be in relative excess in vivo [E.M. Blackwood, unpubl.] and Mad and Myc have been shown to compete for binding to Max in vitro, it is surprising that the 48-hr complexes appeared to exclude Myc. It is possible that at this time Max protein is sequestered through interaction with another unidentified protein. Mx1 is a potential candidate, as it is also induced upon U937 differentiation [Zervos et al. 1993]. Although analysis of low-stringency anti-Max immunoprecipitates does not show any obvious specifically associated proteins other than Myc and Mad [Fig. 6], it is possible that other Max partners are present but obscured by the high background. Alternatively, the specific interactions between these proteins might be stabl-
lized or destabilized through post-translational modifications of any of the partners. The above models depend on the limited availability of Max. However, it is possible that late in the differentiation program, when no Myc:Max heterocomplexes are detectable, Myc is sequestered by another, as yet unidentified, heterodimeric partner or localized to another cellular compartment (Craig et al. 1993). Therefore, there are several mechanisms that could explain the preferential formation of Mad:Max complexes in the presence of Myc protein. Whatever the mechanism, it is important to note that Myc is no longer present in a Max complex and is therefore unlikely to function in terms of DNA-binding and transcriptional activation (Blackwood and Eisenman 1991; Prendergast et al. 1991; Amati et al. 1992, 1993; Mukherjee et al. 1992).

Myc expression is generally thought to be incompatible with terminal differentiation, as its constitutive overexpression can block the differentiation of a number of cell types (Coppola and Cole 1986; Maruyama et al. 1987; Freytag 1988; Larsson et al. 1988; Hoffman-Libermann and Libermann 1991; Miner and Wold 1991; Chisholm et al. 1992). However, Myc has been observed to be expressed in several differentiating systems (Dotto et al. 1986; Endo and Nadel-Ginard 1986; Jaffe et al. 1988; Bernard et al. 1992; Younus and Gilchrest 1992), including myeloid cells (Craig et al. 1993), indicating that continual endogeneous Myc expression is not necessarily incompatible with differentiation. The dissociation of Myc:Max complexes observed here may provide a means by which Myc function can be uncoupled from expression and therefore explain the paradoxical finding of differentiation in the presence of persistent Myc expression.

What are the consequences of the Max heterocomplex switch during differentiation? The switch from Myc:Max to Mad:Max complexes at 48 hr occurs concomitantly with increased aggregation and adherence of U937 cells as they differentiate to macrophages (Fig. 8). Therefore, it is likely that key morphological and biochemical changes related to differentiation are taking place during and after the heterocomplex switch. We favor the possibility that at least some of these changes may be related to altered target gene expression as a result of the switch in Max heterodimers. Transient transfection studies, using an artificial reporter construct containing E-box-binding sites have shown that Myc activates while Mad represses transcription in a Max-dependent manner (Ayer et al. 1993). Myc activity in transformation, apoptosis, and inhibition of differentiation requires the Myc protein to have an intact transcriptional activation domain and an intact DNA-binding and dimerization motif (bHLHZip) (Stone et al. 1987; Freytag et al. 1990; Smith et al. 1990; Bar-Ner et al. 1992; Evan et al. 1992). Therefore, several aspects of Myc biological function are likely to occur through its transcriptional activity and could, consequently, be reversed or regulated by accumulation of Mad:Max complexes.

Although a number of candidate genes have been suggested as potential targets for Myc:Max, there is as yet no conclusive evidence that Max-associated complexes actually bind to and regulate expression of these genes in vivo. Nonetheless, it is noteworthy that several of the potential target genes encode cell-surface proteins involved in adherence and recognition (Bernards et al. 1986; Inghirami et al. 1990), as well as proteins likely to be involved in differentiation and proliferation (Benvenisty et al. 1992; Jansen-Dürr et al. 1993; Reisman et al. 1993). We speculate that the heterocomplex switch from Myc:Max to Mad:Max, in turn, drives a transcriptional switch from activation to repression of a set of target genes involved in the maintenance of the undifferentiated state and possibly in early events related to the cessation of proliferation. These ideas will be tested more directly by examining the effects of ectopic Mad expression as well as determining the nature of the genes that are primary targets of the Myc, Max, and Mad transcription complexes.

Materials and methods

mRNA purification and Northern blotting

Total RNA was prepared from 1 x 10⁶ U937 cells for each treatment as described (Chomczynski and Sacchi 1987). Poly(A)+ RNA was purified using microcrystalline oligo(dT)–cellulose according to the vendor's instructions (New England Biolabs). Ten micrograms of the once selected RNA was fractionated on a formaldehyde–agarose gel and transferred to Zetabind nylon membrane (Cuno Laboratory Products) using a pressure blottedter (Stratagenetec). The filter was probed overnight with 3 x 10⁶ cpm/ml using either a random-primed Mad cDNA probe or a human β-actin probe. The filters were washed using high-stringency conditions. The final wash was at 65°C for 30 min in 0.1% SSC and 0.1% SDS.

Cell culture

The U937 cell line was grown in RPMI supplemented with 10% fetal calf serum (GIBCO-BRL) and maintained at a density between 2.5 x 10⁵ and 5 x 10⁶ cells/ml. To induce differentiation, TPA was added to 5 nM in DMSO. For cells treated with TPA for 48 hr, the nonadherent cells were pelleted and resuspended in media with fresh TPA after 24 hr.

Antibodies

The anti-Max (8711) and anti-Myc (3134) sera have been described previously (Blackwood et al. 1992b). To generate anti-Mad serum (1650), a New Zealand white rabbit was immunized with a bacterially expressed and purified GST–Mad fusion protein (Ayer et al. 1993). Because this fusion protein was produced in low yield in bacteria, we have performed subsequent injections with GST fused to the last 81 amino acids of Mad. This fusion protein produced in low yield in bacteria, we have performed subsequent injections with GST fused to the last 81 amino acids of Mad. Before its use, the anti-GST antibodies were absorbed on a GST column.

Immunoprecipitations

Cells were pelleted at 500g for 5 min and resuspended at 1 x 10⁷ cells/ml in methionine-free media. Typically, 1 x 10⁷ cells were used per immunoprecipitation. Following a 20-min incubation at 37°C and 5% CO₂, the cells were labeled for 45 min...
with Express label [250 μCi/ml of [35S]methionine [DuPont-NEN >1000 Ci/mmol], pelleted, and washed once with cold PBS. For high- and low-stringency precipitations the cells were lysed by sonication on ice in antibody buffer or L buffer (Blackwood et al. 1992b), respectively, and the lysates were cleared by centrifugation at 14,000g at 4°C. For the time course experiments, lysates were normalized for TCA precipitable counts. The proteins were precipitated for 60 min at 4°C with either 10 μl of anti-Max crude serum, 5 μl of anti-Max ammonium sulphate-purified serum or 10 μg of affinity-purified anti-c-Myc antibody, and 30 μl of a 50/50 slurry of protein A-Sepharose (Sigma). Following the precipitations, the high-stringency immunoprecipitates were washed once with antibody buffer, once with RIPA buffer, once with high salt buffer and once with RIPA buffer. The low-stringency immunoprecipitations were washed four times with L buffer. Bound protein was released from the protein A by boiling in SDS sample buffer and analyzed on SDS-polyacrylamide gels. Myc and Mad were released from the low-stringency anti-Max immunoprecipitates with antibody buffer and reprecipitated under high-stringency conditions. To prepare cytoplasmic and nuclear extracts from U937 cells, whole cells were swelled for 10 min in hypotonic buffer (25 mM HEPES at pH 7.5, 10 mM MgCl₂, 10 mM KCl) and lysed with 40 strokes of a Dounce homogenizer (A pestle). Nuclei and remaining intact cells were pelleted at 500g for 5 min. The pellet was resuspended in hypotonic buffer and subjected to another 40 strokes with the Dounce. Nuclei were pelleted and lysed in antibody buffer. Before precipitation, the cytoplasmic fraction was adjusted to antibody buffer concentrations of detergents and salt.

In vitro translations and V8 mapping

CiteMad (Ayer et al. 1993) RNA was synthesized with T7 RNA polymerase, and Mad protein was synthesized using rabbit reticulocyte lysate following the manufacturer’s (Promega) instructions. p35 Mad was immunoprecipitated from 0.5 × 10⁹ U937 cells that had been treated with 5 nM TPA overnight. Following fractionation by SDS-PAGE, the bands containing p35 Mad and immunoprecipitated in vitro-translated Mad were excised from the gel and subjected to V8 mapping as described [Bigler and Eisenman 1988].

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