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

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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]

Received July 27, 1993; revised version accepted September 2, 1993.

Max is a ubiquitous basic region-helix-loop-helix-zipper (bHLHZip) protein that forms homodimers as well as heterodimers with the Myc family of bHLHZip proteins, including c-Myc, N-Myc, and L-Myc. Max appears to be an obligate heterodimeric partner for Myc in mediating its functions as both a specific DNA-binding transcriptional activator and a transforming protein (for review, see Dang 1991; Blackwood et al. 1992a; Marcu et al. 1992; Meichle et al. 1992). Although Max preferentially dimerizes with Myc, it can also form homodimers and, as such, represses transcription and inhibits transformation by Myc (Blackwood and Eisenman 1991; Prendergast et al. 1991; Amati et al. 1992, 1993; Kretzner et al. 1992; Prendergast et al. 1992; Gu et al. 1993). Both hetero- and homodimers recognize the same set of E-box-related DNA sequences, although Myc:Max complexes bind with higher apparent affinity than Max homodimers (Blackwood and Eisenman 1991; Prendergast et al. 1991; Berberich and Cole 1992; Berberich et al. 1992; Kato et al. 1992; Littlewood et al. 1992; Ayer et al. 1993). In vivo, Max protein is constitutively expressed, highly stable, and appears to be more abundant than Myc (Blackwood et al. 1992b; Littlewood et al. 1992). In contrast, Myc expression is highly regulated, and Myc protein is rapidly degraded after transitory dimerization with Max. These findings have led to a model in which

the balance between activation by Myc:Max heterodimers and transcriptional repression by Max homodimers are dependent on the rate of synthesis of the rate-limiting partner, Myc (Amati et al. 1992; Blackwood et al. 1992a; Kretzner et al. 1992).

Recently, Max has been found to heterodimerize with proteins other than Myc family members. Different interaction cloning strategies employing the Max protein led to the identification of two novel bHLHZip proteins, Mad (Ayer et al. 1993) and Mx1 (Zervos et al. 1993). Although unrelated to Myc, outside of their bHLHZip regions, these two proteins display ~40% identity to each other and can be considered members of the same family (Ayer et al. 1993; Zervos et al. 1993). In vitro studies demonstrate that Mad and Mx1 heterodimerize with Max and bind to the same CACGTG E-box sequence recognized by Myc:Max heterodimers and Max homodimers. As was the case for Myc, Mad:Max heterodimers displayed greater relative binding activity than Max homodimers. Neither Mad nor Mx1 forms homodimers, binds DNA alone, or interacts with a series of other bHLHZip proteins tested (Ayer et al. 1993; Zervos et al. 1993). Also, because Myc and Mad can compete with each other for limiting concentrations of Max in DNA-binding assays, they appear to have approximately the same relative binding affinities for Max (Ayer et al.

1993). Therefore, in terms of their dimerization with Max and DNA-binding activities, Mad and Mx1 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 construct containing a minimal promoter with upstream CACGTG-binding sites (Amati et al. 1992; Kretzner et al. 1992; Amin et al. 1993; Gu et al. 1993). Transcriptional activation by Myc required its amino-terminal region as well as an intact bHLHZip domain and was dependent on interaction with Max (Amati et al. 1992; Kretzner et al. 1992; Amin et al. 1993; Gu et al. 1993). Mad, however, cooperated with Max to repress transcription in a similar assay. Consistent with the results of in vitro experiments showing competition of Myc and Mad for Max, transcriptional activation by Myc is suppressed by increasing the amount of Mad; 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 Mx1 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 Mx1) 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 Mx1 has been shown to be widely expressed and induced upon induction of myeloid differentiation (Zervos et al. 1993; A. Zervos, pers. comm.), neither Mx1 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 monocyte/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 1. Induction of *mad* mRNA following TPA treatment. Northern blot analysis of poly(A)⁺ RNA from untreated U937 cells or U937 cells treated with TPA for 3 hr in the presence or absence of 10 μ g/ml of cycloheximide (CYCLO). (Top) RNAs that hybridized to the human full-length Mad cDNA; (bottom) RNAs that hybridized to the human β -actin cDNA. The molecular mass of the RNA markers are shown in kb.

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 Mx1 are ~40% identical at the amino acid level, it is possible that the Mad antiserum could cross-react with Mx1; however, our Mad antiserum showed only marginal cross-reactivity with in vitro-translated Mx1 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-

Figure 3. Comparison of the proteolytic digestion products from in vitro-translated and in vivo synthesized Mad. [³⁵S]-Methionine-labeled Mad protein from either TPA-treated U937 cells (IP) or reticulocyte lysate programmed with Mad cDNA (IVT) were digested with the indicated amount of V8 protease. The digestion products were analyzed on a 20% SDS–polyacrylamide gel. The arrows indicate peptides common to the in vitro-translated Mad protein and the Mad protein immunoprecipitated from U937 cells. The positions of the molecular mass markers are shown.

Figure 4. Half-life determination of p35 Mad. U937 cells treated with 5 nM TPA for 16 hr were pulse-labeled with [35 S] methionine for 30 min. Labeled cells were either lysed (pulse) or transferred into fresh media containing excess cold methionine for the indicated amount of time (chase). Following lysis and immunoprecipitation with anti-Mad serum, the labeled proteins were analyzed on an SDS-polyacrylamide gel. (Pulse + block) Pulse-labeled cell lysates immunoprecipitated with α -Mad serum blocked with GST-Mad. The positions of the molecular mass markers are shown.

phoprotein (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.

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 [35 S]-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

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 [35 S]-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.

Figure 6. Association of Mad and Max in vivo. Max proteins were immunoprecipitated under high- or low-stringency conditions from TPA-treated U937 cells that had been labeled with [35 S]methionine as described in Materials and methods. The labeled 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 reprecipitated with anti-Mad serum. (+ block) Immunogen-blocked anti-Max serum used in the initial immunoprecipitation. The positions of the molecular mass markers are shown.

tate were eluted with ionic detergents (high-stringency conditions) and reprecipitated with anti-Mad serum. Only the 35-kD polypeptide was precipitated with the secondary Mad antiserum, demonstrating that it was Mad. Mxi-1 mRNA has been shown previously to be induced during TPA treatment of U937 cells (Zervos et al. 1993). However, we detected no obvious association of Mxi-1 and Max in this experiment. As in vitro-translated Mxi-1 protein migrates 2–3 kD faster than Mad on SDS-polyacrylamide gels (data not shown), it may have been obscured by the nonspecific protein bands in the anti-Max immunoprecipitates.

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—

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 [35 S]methionine labeling and immunoprecipitation at various times following TPA treatment of U937 cells (Fig. 7A–C). The two Max proteins did not change either in amount or in their ratio

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

Mad:Max complexes in differentiating cells

during the time course of differentiation. In contrast to the previously reported down-regulation of Myc mRNA following TPA treatment of these cells (Einat et al. 1985), Myc protein synthesis appeared to increase slightly 6 hr after TPA treatment but returned approximately to the level found in untreated cells 24 and 48 hr after induction. Mad synthesis was low in untreated cells, but an induction of Mad protein was detectable as early as 2 hr after TPA treatment. The rate of Mad protein synthesis continued to rise and reached its maximal level 48 hr after TPA treatment. This level of Mad protein remained unchanged as the time of TPA exposure was increased from 48 to 96 hr (data not shown).

To correlate the relative rates of synthesis of these proteins with U937 differentiation we measured two parameters that are characteristic of their differentiation from promonocytes to monocytes and ultimately macrophages (Hass et al. 1989). First, we monitored cell-cell adhesion and attachment to the substratum (Fig. 8). Following TPA treatment, the U937 cells attached to the substratum (24 hr), began to clump (48 hr), and eventually extended pseudopodia and adopt a flattened macrophage-like morphology (72 hr), consistent with a previous report (Hass et al. 1989). Second, differentiating U937 cells have an increased ability to produce oxygen-derived free radicals and thereby have an increased capacity to reduce nitroblue tetrazolium (NBT) (Harris and Ralph 1985). Our clone of U937s displayed a significant increase in the number of cells with blue/black formazan deposits, indicative of NBT reduction, as the differentiation time course proceeded. By 48 hr ~20% of the cells were positive (data not shown). Thus, induction of Mad protein synthesis correlates with events characteristic of an early stage of differentiation along the monocyte/macrophage pathway.

Analysis of the immunoprecipitates shown in Figure 7 demonstrated that Myc, Max, and Mad proteins are all

Figure 8. Morphological changes that accompany U937 differentiation. U937 cells were treated for the times indicated with 5 nM TPA and photographed under 20 $\times$ magnification.

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 antiserum directed against Max. The proteins in the anti-Max immunoprecipitate were eluted and reprecipitated with antisera against Mad and Myc. These anti-Myc and anti-Mad immunoprecipitates were analyzed on a SDS-polyacrylamide gel. (+ block) Immunogen blocked anti-Max antiserum was used in the initial immunoprecipitation.

present during the first 48 hr of U937 differentiation. However, because Myc and Mad function as heterocomplexes with Max, we also determined the levels of each heterocomplex during U937 differentiation. Low-stringency immunoprecipitations were performed using anti-Max serum from [35 S]-methionine-labeled cells at various times after TPA treatment. The proteins in these immunoprecipitates were eluted with ionic detergents, reprecipitated with antisera specific for Myc and Mad, and analyzed on an SDS-polyacrylamide gel (Fig. 9). The level of the Myc:Max heterocomplex peaked 6 hr after induction and returned to untreated levels by 24 hr. However, 48 hr after TPA induction we were unable to detect any Myc in the anti-Max immunoprecipitates. In contrast, the Mad:Max complex was detected as early as 2 hr after induction, and its level continued to rise until 48 hr after TPA addition. Forty-eight hours after induction of differentiation, only Mad:Max complexes were detectable. Therefore, as U937 cells differentiated along the monocyte/macrophage pathway there was a shift in the relative amount of the Myc:Max and the Mad:Max heterocomplexes.

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; Rabbitts 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 *mx1* 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- β (TGF- β) 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 nec-

essarily 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 stabi-

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 Gilchrist 1992), including myeloid cells (Craig et al. 1993), indicating that continual endogenous 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 1×10^8 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 blotter (Stratagene). The filter was probed overnight with 3×10^6 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 2.5×10^5 and 5×10^5 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). Initially, rabbits were immunized with GST fused to the 181 carboxy-terminal amino acids of Mad. 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. 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 1×10^7 cells/ml in methionine-free media. Typically, 1×10^7 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 μ Ci/ml of [35 S]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 μ l of anti-Max crude serum, 5 μ l of anti-Mad ammonium sulfate-purified serum or 10 μ g of affinity-purified anti-c-Myc antibody, and 30 μ l 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 0.5×10^8 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).

Acknowledgments

We thank Q. Lawrence for expert technical assistance and support, S. Collins, C. Laherty, K. Foley, M. Groudine, and P. Hurlin for comments on the manuscript, K. Tietje for help with the initial immunoprecipitations, and A. Zervos for communication of unpublished work. This work was supported by a postdoctoral fellowship from the Leukemia Society of America to D.E.A. and a grant from the National Institutes of Health, National Cancer Institute (R01CA57138) to R.N.E.

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

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Genes Dev. 1993, **7**:

Access the most recent version at doi:[10.1101/gad.7.11.2110](https://doi.org/10.1101/gad.7.11.2110)

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