

## Mlx, a Novel Max-like BHLHZip Protein That Interacts with the Max Network of Transcription Factors\*

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**Mad:Max heterodimers oppose the growth-promoting action of Myc:Max heterodimers by recruiting the mSin3-histone deacetylase (mSin3-HDAC) complex to DNA and functioning as potent transcriptional repressors. There are four known members of the Mad family that are indistinguishable in their abilities to interact with Max, bind DNA, repress transcription, and block Myc + Ras co-transformation. To investigate functional differences between Mad family proteins, we have identified additional proteins that interact with this family. Here we present the identification and characterization of the novel basic-helix-loop-helix zipper protein Mlx (Max-like protein x), which is structurally and functionally related to Max. The similarities between Mlx and Max include 1) broad expression in many tissues, 2) long protein half-life, and 3) formation of heterodimers with Mad family proteins that are capable of specific CACGTG binding. We show that transcriptional repression by Mad1:Mlx heterodimers is dependent on dimerization, DNA binding, and recruitment of the mSin3A-HDAC corepressor complex. In contrast with Max, Mlx interacts only with Mad1 and Mad4. Together, these findings suggest that Mlx may act to diversify Mad family function by its restricted association with a subset of the Mad family of transcriptional repressors.**

heterodimer combinations can recognize the same E-box motif (CACGTG), suggesting that they reciprocally regulate the same target genes. Several lines of evidence suggest that Mad family proteins play an important role in counteracting the growth-promoting activity of Myc. Both Mad family members and Mnt can efficiently block Myc + Ras co-transformation of rat embryo fibroblasts (6, 9, 14–16), and cause cells to be blocked in the G<sub>1</sub> phase of the cell cycle (17, 18). In addition, the *mxi1* gene maps to a region on chromosome 10q that is frequently mutated in human cancers, and *mxi1* null mice show increased susceptibility to tumorigenesis (19, 20).

The opposing activities of Myc and Mad are manifest during the transition from proliferation to differentiation (2, 21). Myc mRNA and protein expression is associated with cellular proliferation and is typically down-regulated during differentiation (22, 23). By contrast, Mad1 is expressed at low levels in proliferating cells but is induced during the differentiation of several distinct cell lineages *in vitro* and *in vivo* (14, 16, 24–27). Max protein abundance is not highly regulated, suggesting that it is continually available to complex with either Myc or Mad (28, 29). Isolation of Max heterocomplexes demonstrated a shift from Myc:Max to Mad1:Max heterocomplexes during chemically induced differentiation of a myeloid leukemia cell line and primary human keratinocytes (24, 30). We have proposed that this switch in heterocomplexes is important in regulating cell cycle exit during differentiation, presumably by down-regulating Myc-dependent target genes required for cell proliferation (24). These data suggest that the switch from Myc:Max to Mad:Max heterodimers has functional consequences *in vivo*.

It is not yet understood what biological roles are played by individual members of the Mad family and what biochemical mechanisms provide specificity to Mad family function. *In vitro* studies cannot distinguish functional differences between Mad family members. For instance, each family member is able to repress transcription by targeting the mSin3A corepressor complex to DNA (31–33), heterodimerizing with Max to bind CACGTG binding sites, and blocking Myc + Ras co-transformation (1). However, analysis of *mad* family mRNA expression patterns in developing embryos and the phenotypes of mice with null mutations in either *mad1* or *mxi1* suggests that the *in vivo* functions of the Mad family members are not completely redundant. The expression patterns of *mad* family and *mnt* mRNAs are unique and complex; although transcripts for *mnt* and *mad3* are detected in proliferating cells, *mxi1* and *mad4* transcripts are expressed early in the differentiation process, and *mad1* transcripts appear later in differentiation (9, 14, 16, 27). Mice null for *mad1* or *mxi1* are viable but show increased proliferation in precursor cell populations of the spleen and prostate; these effects were most pronounced in granulocytic cluster-forming colonies derived from *mad1*-deficient mice, whereas *mxi1*-deficient mice displayed hyperplastic growth in

The basic-helix-loop-helix-zipper<sup>1</sup> (BHLHZip)<sup>1</sup> protein Max is an essential component in a transcription factor network that functions to regulate cell growth and differentiation (1, 2). Max can form DNA binding heterodimers with at least three different families of BHLHZip proteins: the Myc family of proto-oncogenes (c-Myc, N-Myc, and L-Myc), (3–5) the Mad family (Mad1, Mxi1, Mad3 and Mad4) (6–8), and Mnt (9). Myc:Max heterocomplexes function as transcriptional activators (10–12), whereas Mad:Max (6, 7, 13) and Mnt:Max heterocomplexes function as transcriptional repressors (9). All three

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<sup>1</sup> The abbreviations used are: BHLHZip, basic helix-loop-helix leucine zipper; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; HDAC, histone deacetylase; Mlx, Max-like protein-X; SID, Sin3 interaction domain; p.c., post-coitus; CMV, cytomegalovirus.

the splenic white pulp and prostatic epithelium (20, 34). Together these findings indicate that the activity of Mad family transcriptional repressors may be differentially regulated depending on the cell type or stage in the cellular differentiation program.

To elucidate potential functional differences between the Mad family of transcription factors, we have identified additional members of this transcriptional network that interact with Mad1. Here we present the isolation and characterization of Mlx, a novel Max-like BHLHZip protein. Mlx, like Max, is a stable, widely expressed protein. In addition, Mlx forms heterodimers with Mad1 that are capable of interacting with the mSin3A corepressor and repressing transcription. However, unlike Max, Mlx forms heterodimers with only select members of the Mad family (Mad1 and Mad4). We propose that Mlx diversifies the functional capabilities of the Mad family of transcription factors by interacting with only a subset of Mad proteins.

#### EXPERIMENTAL PROCEDURES

**Two-hybrid Screen and Cloning**—Yeast two-hybrid screening was performed as described previously using a VP16 library constructed from mRNA isolated from mouse embryos at 9.5 and 10.5 days p.c. (31, 48). 80 VP16 fusion clones were chosen for characterization. All 80 VP16 fusion clones failed to show a positive two-hybrid readout when tested for interaction with LexAMad1 in a wild-type *Saccharomyces cerevisiae* strain. However, when a subset of these clones was tested with LexAMad1 in a *sin3* strain, all yielded  $\beta$ -galactosidase levels similar to those observed when they were tested for interaction with LexAMad1 (L12P/A16P) in a wild-type background (data not shown). Therefore, it is likely that the 80 VP16 fusions interacted with wild-type Mad1 in the two-hybrid analysis, but these interactions were masked because endogenous yeast SIN3p repressed the *lacZ* reporter gene. Full-length cDNAs encoding human and mouse Mlx were isolated from an HL60 cDNA library and an embryonic stem cell library, respectively. Mlx cDNAs were subcloned by standard methods into the mammalian expression vector pRC/CMV (Invitrogen). The mutant of Mlx lacking the leucine zipper (Mlx $\Delta$ LZ) was produced by polymerase chain reaction amplification and lacks amino acids 133–161. Mlx $\Delta$ LZ is completely defective for dimerization with Mad1 (data not shown). In  $\Delta$ BRMlx, Glu-84 and Gln-85 of the basic region of Mlx were mutated to glycine and proline, respectively. These mutations abolish Mlx DNA binding (data not shown). VP16 fusions to Mad3, Mad4, N-Myc and L-Myc, and LexAMax were kindly provided by Dr. Peter Hurlin (Fred Hutchinson Cancer Research Center, Seattle, WA). VP16Mad1 and LexAMlx were constructed by amplifying the Mad1 or Mlx cDNA by polymerase chain reaction and cloning the products into pBTM116 or pVP16 (48), respectively. Multiple tissue Northern blots (CLONTECH) were probed with the full-length Mlx cDNA, which had been labeled by random priming (Life Technologies, Inc.). Blots were washed with 0.1 $\times$  SSPE (18 mM NaCl, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.1 mM EDTA), 0.1% SDS for 30 min at 65 °C. *In situ* hybridization was performed as described with <sup>35</sup>S-labeled antisense probes against *mad1*, *max*, and *mlx* (27).

**Electrophoretic Mobility Shift Assays (EMSAs)**—EMSAs were performed by incubating recombinant proteins, typically 1–10 ng, with 0.5 ng of <sup>32</sup>P-labeled probe for 15 min at room temperature. The binding reactions contained 0.5 $\times$  HMO.1 buffer (12.5 mM HEPES, pH 7.5, 5% glycerol, 50 mM KCl, and 0.5 mM dithiothreitol), 0.4 mg ml<sup>-1</sup> bovine serum albumin, 8 mM dithiothreitol, and 0.08% Nonidet P-40. Protein-DNA complexes were resolved on 5% nondenaturing acrylamide gels in 25 mM HEPES, pH 7.5, at 4 °C.

**Protein Expression and Antibody Production**—Rabbit poly-

clonal antisera specific for the N terminus and C terminus of Mlx were generated against GST fusion proteins encoding amino acids 1–76 or 162–244 of Mlx, respectively. Test bleeds were assayed for specific immunoreactivity by low and high stringency immunoprecipitation of radiolabeled *in vitro* translated Mlx. The purification of recombinant GST-BHLHZip Mlx (encoding amino acids 76–162), GST-BHLHZip-Mad3, GST-BHLHZip-Mad4, GST-C92-Myc, Mad1, and Max was based on previously published techniques (6, 7, 49). Max protein was isolated from Sf9 insect cells infected with a Max-expressing recombinant baculovirus (7). All other proteins were expressed in *Escherichia coli*.

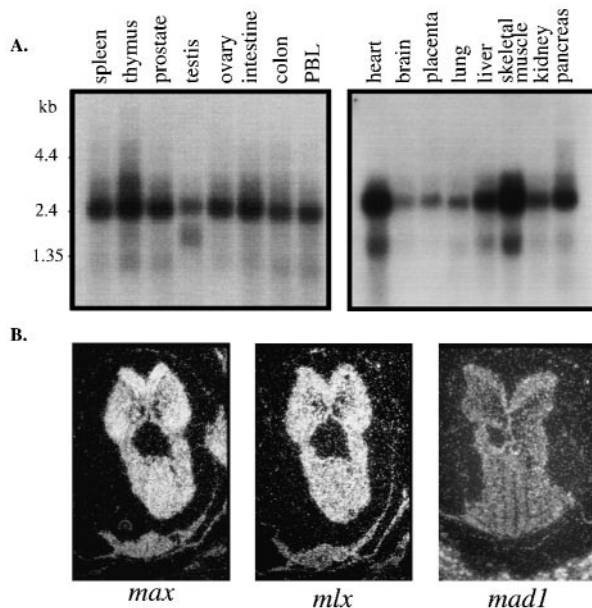
**Cell Culture, Immunoprecipitation, and Luciferase Assays**—Immunoprecipitations were performed as described previously (24). SDS-polyacrylamide electrophoresis gels were transferred to polyvinylidene difluoride membranes and visualized with ECL (Amersham Pharmacia Biotech) using the manufacturer's protocol. Recombinant retrovirus expressing Mlx was made by filling in a *HindIII/XbaI* fragment containing the Mlx-coding region and adding *ClaI* linkers. The linked fragment was cloned in to the *ClaI* site of pSR $\alpha$ -MSV-TKCD8 (50). Virus was produced by transfecting 293 cells with the retroviral construct and a plasmid encoding an amphotrophic helper virus containing a virion packaging  $\psi$ 2 sequence (51, 52). Supernatants containing retrovirus were collected 36 h after transfection and used to infect NIH3T3 cells. Three rounds of infection were performed in the presence of 8  $\mu$ g ml<sup>-1</sup> Polybrene, and cells were assayed for Mlx expression by indirect immunofluorescence. For the half-life experiments, cycloheximide was used at 10  $\mu$ g ml<sup>-1</sup>. To determine if cycloheximide was effective at blocking protein synthesis at this concentration, control cells were treated for 1 h with the drug and then labeled with [<sup>35</sup>S]methionine/cysteine (NEN Life Science Products) for 30 min. Trichloroacetic acid precipitation showed that approximately 95% of the *de novo* protein synthesis was blocked using these conditions.

For luciferase assays, NIH3T3 cells grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (Hyclone), glutamine, and penicillin-streptomycin were seeded onto 6-well dishes at 3  $\times$  10<sup>5</sup> cells in 2 ml of medium/well. Twenty-four h after seeding, cells were transfected using Superfect (Qiagen) in triplicate. Each transfection contained 400 ng of luciferase reporter, 100 ng CMV- $\beta$ -galactosidase, 1  $\mu$ g of expression construct, and carrier DNA to a total of 5  $\mu$ g of DNA. Cell lysates were prepared 24 h after transfection. Luciferase and  $\beta$ -galactosidase activities were assayed according to manufacturers' instructions (Promega, Tropix). To normalize for transfection efficiency, luciferase values were divided by  $\beta$ -galactosidase activity values. Errors reported are the S.E. calculated from experiments performed in triplicate. The luciferase reporter pGL3-CM2 was constructed by inserting four copies of the E-box-containing sequence CCCAGTCGCACGT-GCTGTAGG between the *SacI* and *BglII* sites of pGL3-promoter (Promega).

#### RESULTS

**Identification of Mad1-interacting Proteins Using a Modified Two-hybrid Strategy**—In a previous yeast two-hybrid screen using Mad1 as bait, only the corepressors mSin3A and mSin3B were identified (31). In *S. cerevisiae*, LexAMad1 can repress transcription via interaction with the endogenous SIN3p corepressor complex (35), suggesting that mSin3A and mSin3B were identified because they could displace the endogenous corepressor. Furthermore, because repression by the mSin3A-HDAC1 complex is dominant over activation by VP16 (36), other proteins may have interacted with the LexAMad1 bait but failed to score positively due to a simultaneous inter-



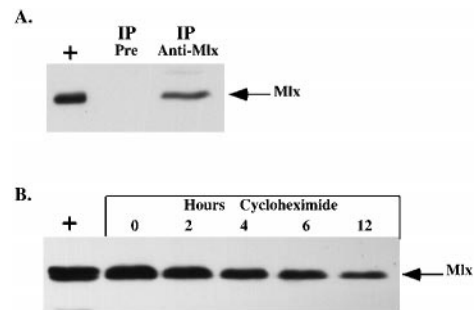


**FIG. 2. Mlx mRNA is ubiquitously expressed in adult tissues and developing embryos.** Northern blotting (A) and *in situ* hybridization (B) were used to determine the expression profiles of Mlx. In B, bright white signal indicates expression of *max*, *mlx*, or *mad1* mRNA expression. Mlx probe detected a 2.4-kilobase message from all adult tissues indicated. Transverse sections from mouse embryos harvested at day 8.5 p.c. were probed with  $^{35}\text{S}$ -labeled antisense probes for Max, Mlx, and Mad1 as indicated. PBL, peripheral blood leukocytes.

Mlx (Fig. 3A). A protein of approximately 30 kDa was immunoprecipitated from high stringency extracts of SW480 colon carcinoma cells. This protein was not detected in immunoprecipitates using preimmune serum. Furthermore, the immunoprecipitated protein is similar in apparent molecular mass to the one produced in 293 cells transfected with an expression vector encoding Mlx. Together, these data confirm that the immunoprecipitated 30-kDa protein is Mlx. A survey of other cell lines showed that HL60, P19, and PC12 cells also contained detectable Mlx protein, whereas NIH3T3 and 293 cells did not (data not shown). These data are consistent with the *in situ* hybridization and Northern-blotting results and suggest that Mlx protein expression is widely distributed although not ubiquitously expressed like Max.

Max is a stable protein with a half-life of at least 6 h (39). In contrast, c-Myc and Mad1 turn over very rapidly, with half-lives of approximately 20 min (24, 40). To determine the stability of Mlx, we measured its half-life in NIH3T3 cells that had been transduced with a Mlx-expressing retrovirus. Because Mlx is labeled poorly *in vivo* using [ $^{35}\text{S}$ ]methionine (data not shown), it was not possible to determine its half-life by performing a pulse-chase experiment. Therefore, the stability of Mlx was determined by blocking protein synthesis with cycloheximide and determining the amount of Mlx that remained at specific time points after cycloheximide addition. Mlx protein was detected by immunoprecipitation and Western blotting following 0, 2, 4, 6, and 12 h of cycloheximide treatment and appears to decay with a half-life of approximately 6–8 h (Fig. 3B). Similar results were obtained with endogenous Mlx in SW480 cells (data not shown). Thus, Mlx is a relatively stable protein and is turned over with kinetics similar to those of Max. This suggests that the composition and function of Mlx heterocomplexes, like that of Max heterocomplexes, might be dictated by unstable protein partners.

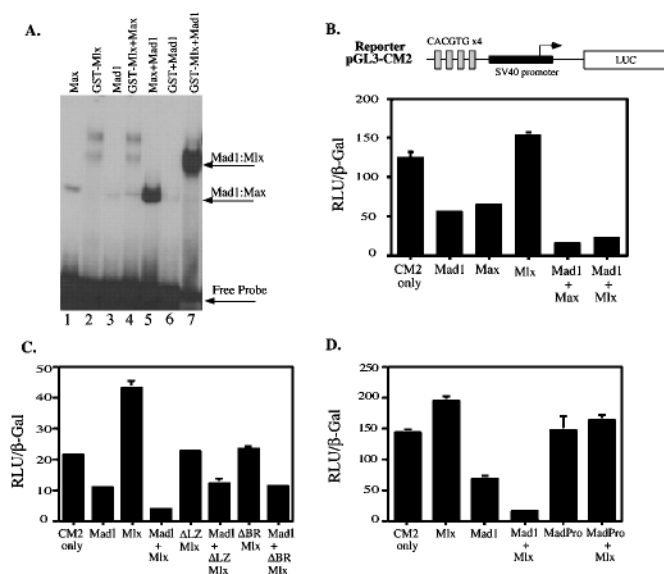
**Mlx Heterodimerizes with Mad1 to Bind DNA Containing E-box Sequences**—Proteins of the Max network bind the DNA recognition element CACGTG as heterodimers (3, 5–7, 9, 41),



**FIG. 3. Mlx is a stable protein in cells.** A, SW480 cell extracts were immunoprecipitated under high stringency with either preimmune serum or an antiserum that recognizes the C terminus of Mlx. Western blots of the immunoprecipitates were probed with the N-terminal Mlx antiserum. In both A and B, + indicates a positive control extract from 293 cells transfected with RC/CMV-Mlx. Pre and anti-Mlx indicate immunoprecipitations (IP) of SW480 cell extracts using preimmune serum or antiserum specific for the C terminus of Mlx, respectively. B, NIH3T3 cells transduced with a Mlx-expressing retrovirus were treated for the times indicated with cycloheximide and then immunoprecipitated and subjected to Western blotting as above.

so we determined whether Mlx also bound DNA with high affinity as a heterodimer. Mutagenesis and structural studies have demonstrated that binding to the CANNTG E-box is conferred by the histidine and glutamic acid residues located at positions 5 and 9, respectively, of the basic region (42, 43). Binding to E-boxes containing a central CG dinucleotide is discriminated by the arginine in position 13 (42). These three amino acids are conserved in Mlx (Fig. 1B), suggesting that Mlx and Mlx-containing heterodimers will also recognize the CACGTG E-box subclass. GST fusion proteins encoding the BHLHZip domain of Mlx, Mad1, and Max were tested by EMSAs for their ability to bind an oligonucleotide probe, CM1, which contains a single CACGTG site. Little DNA binding was detected when Max and Mlx were tested alone or in combination, suggesting that they form heterodimers poorly and do not heterodimerize with one another (Fig. 4A). Consistent with our previous findings, Mad1 was unable to bind CM1 as a homodimer but readily formed heterodimers capable of binding DNA with either Max or Mlx (Fig. 4A, lanes 5 and 7). The binding of Mad1:Mlx to CM1 was specific because it was competed by unlabeled CM1 but not by an unrelated binding site (data not shown). GST alone did not bind CM1, indicating that the GST portion of the Mlx fusion protein did not contribute to DNA binding by Mad1:Mlx heterocomplexes. The amounts of CM1 bound by Mad1:Max and Mad1:Mlx complexes were similar, suggesting that Mad1 has similar affinities *in vitro* for both Max and Mlx and that each heterocomplex binds DNA with similar affinity. Thus Mlx, like Max, requires a heterodimeric partner to bind DNA efficiently.

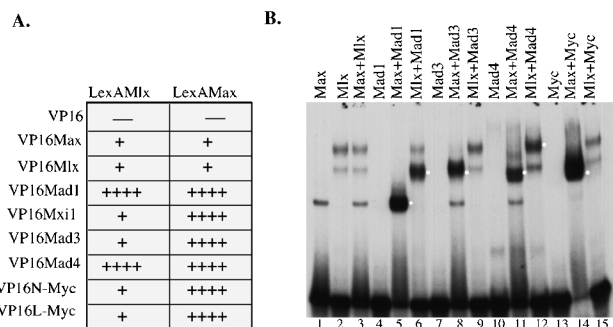
Mad1:Max and Mad1:Mlx heterocomplexes are both capable of specific binding to CACGTG binding sites; however, it is possible that this sequence is not their preferred binding site, raising the possibility that they may bind distinct sites with higher affinities. To address this possibility, we performed a binding site selection experiment using a degenerate pool of oligonucleotides with the sequence  $\text{N}_5\text{CANNTGN}_5$  as the starting material. 5 rounds of selection and amplification were performed with each heterocomplex. Both Mad1:Max and Mad1:Mlx heterocomplexes selected the CACGTG E-box core element as their preferred *in vitro* binding site. Each heterocomplex displayed some preference for the nucleotides flanking the core; however, quantitative gel shift analysis showed that the flanking sequences influence site selection and affinity by less than 2-fold (data not shown). Therefore, Mad1:Max and Mad1:Mlx heterocomplexes bind the same CACGTG site with high affin-



**FIG. 4. Mad1:Mlx heterodimers function as transcriptional repressors.** A, EMSA was performed to determine if Mad1 and Mlx can form heterodimers. CM-1, a labeled probe containing a single CACGTG binding site, was incubated with the indicated recombinant proteins. Complexes were resolved on a nondenaturing 5% acrylamide gel. Positions of the heterocomplexes and free probe are indicated. B, C, and D, transcription assays in transiently transfected NIH3T3 cells were used to determine the transcriptional activity of Mad1:Mlx heterodimers. Cells were transfected with the expression vectors encoding the indicated proteins along with luciferase and  $\beta$ -galactosidase reporters. The luciferase reporter pGL3-CM2, containing four CACGTG E-boxes, is shown. Luciferase and  $\beta$ -galactosidase values were determined 24 h after transfection. B, the ability of Mad1:Max and Mad1:Mlx heterodimers to repress the transcriptional activity of pGL3-CM2 was tested. C, the dependence of Mad1:Mlx repression on the ability of Mlx to dimerize and bind DNA was tested using the mutants  $\Delta$ LZMlx and  $\Delta$ BRMlx, respectively. D, the mutant Mad1(L12P/A16P) MadPro was used to test whether Mad1:Mlx repression requires an interaction between Mad1 and the mSin3-HDAC complex.

ity, suggesting that the target genes regulated by each heterocomplex may be, at least in part, overlapping.

**Mad1:Mlx Heterocomplexes Function as Transcriptional Repressors**—To further examine functional similarities between Mlx and Max, we determined whether Mlx has intrinsic transcriptional properties and whether Mad1:Mlx heterocomplexes can repress transcription in a manner similar to Mad1:Max. Fusion of Mlx to the DNA binding domain of GAL4 showed no alteration in transcription from a reporter gene containing GAL4 binding sites, suggesting that like Max (44), Mlx has no intrinsic ability to activate or repress transcription (data not shown). The effects of Mlx heterocomplexes on transcription were tested using a luciferase reporter gene under control of the SV40 promoter, which was made responsive to Max network proteins by cloning four copies of the CACGTG binding site upstream of the SV40 promoter sequences. When Mad1 or Max was transfected alone, each repressed transcription about 2-fold. Mad1 may be able to repress transcription by interaction with endogenous Max, and overexpression of Max can repress transcription via homodimer formation (1). Mlx by itself was able to activate the reporter as much as 2-fold. This activity is likely to depend on an endogenous binding partner for Mlx, because deletion of the leucine zipper from Mlx abolishes this activity (Fig. 4C). The combination of Mad1:Max and Mad1:Mlx repressed expression from the reporter by about 4-fold below the level of expression seen with reporter alone (Fig. 4B), demonstrating that Mad1:Mlx heterocomplexes function as transcriptional repressors. These data suggest that Mlx itself lacks intrinsic transcriptional activation or repression



**FIG. 5. Mlx heterodimerization and transcriptional repression are restricted to a subset of Max network proteins.** The ability of Mlx to interact with different members of the Max network was determined by directed two-hybrid analysis (A) and EMSA (B). L40 yeast were transformed with either LexAMlx or LexAMax and each of the VP16 fusion proteins indicated. + indicates background  $\beta$ -galactosidase activity. ++++ indicate strong  $\beta$ -galactosidase activity detected following 30 min of incubation at 30 °C. B, the indicated purified proteins were incubated with CM-1. DNA binding complexes were resolved by EMSA. The positions of Max- and Mlx-containing heterocomplexes are marked with white dots.

domains but, like Max, is able to mediate transcriptional repression by Mad1.

To determine whether Mad1:Mlx heterocomplexes require the same molecular interactions to repress transcription as Mad1:Max, we tested Mad1-dependent repression in the presence of Mlx mutants that either lack the leucine zipper ( $\Delta$ LZ), which prevents interaction of Mlx with Mad1 in the two-hybrid assay (data not shown), or contain point mutations in the basic region ( $\Delta$ BR) that abolish its DNA binding activity. Neither of these mutant Mlx molecules were able to repress transcription with Mad1 (Fig. 4C). Therefore, for Mlx to function as a transcription factor, it must be complexed with a binding partner, and this complex must directly bind DNA.

Mad:Max and Mnt:Max heterocomplexes repress transcription by recruiting a large multiprotein complex containing mSin3 and histone deacetylase 1 and 2 (HDAC1 and HDAC2) to DNA (32, 33, 45). To test whether Mad1:Mlx complexes also require the mSin3A-HDAC corepressor to be functional repressors, we utilized a mutant of Mad1, Mad1(L12P/A16P), which is unable to interact with mSin3A-HDAC complex. Compared with wild-type Mad1, Mad1(L12P/A16P) was unable to repress transcription when cotransfected with Mlx or Max (Fig. 4D), indicating a requirement for the mSin3A heterocomplex in Mad1:Mlx transcriptional repression. These experiments show that, like Mad1:Max, Mad1:Mlx represses transcription as a sequence-specific DNA binding heterodimer that recruits the mSin3A/HDAC corepressor complex.

**Restricted Dimerization between Mlx and a Subset of Max Network Proteins**—If Max and Mlx are functionally indistinguishable *in vitro*, how might Mlx diversify the functions of the Mad family *in vivo*? Given its similarity to Max in its BHLHZip region, it seemed possible that Mlx would interact with all members of the Max network. We examined whether Mlx was restricted in its ability to interact with members of the Max transcription factor network. We first tested this hypothesis using directed two-hybrid assays. Mad family proteins can interact with endogenous SIN3p to repress transcription via their SID (Fig. 1A), and hence, all of the Mad-VP16 fusions used in the directed two-hybrid assay lacked the SID. In yeast, LexAMlx showed an interaction with only VP16Mad1 and VP16Mad4 but not with other members of the Max network or with itself (Fig. 5A). As expected, LexAMax showed an interaction with Mad1, Mxi1, Mad3, Mad4, L-Myc or N-Myc VP16 fusion proteins, demonstrating that all the VP16 fusions were expressed and functional. The inability to detect an interaction

between LexAMlx and VP16Mlx confirms the finding shown in Fig. 4A that Mlx, like Max, forms homodimers poorly.

We next tested whether Mlx could form heterocomplexes capable of specific DNA binding with other Max network proteins by EMSAs. As assayed by EMSA, Mlx could form CACGTG binding heterodimers with Mad1 and Mad4 (Fig. 5B, compare lane 2 with lanes 6 and 12). In contrast, no differences in DNA binding were observed when Mlx was incubated in the presence of Max, Mad3, and c-Myc (Fig. 5B, compare lane 2 with lanes 3, 9, and 15). Similar to previously published results, Max showed heterodimerization and specific DNA binding with Mad1, Mad3, Mad4, and c-Myc (Fig. 5B, compare lane 1 with lanes 5, 8, 11, and 14). Therefore, the results from both the directed two-hybrid assay and EMSA demonstrate that Mlx is more restricted than Max in the protein partnerships it can form with members of the Max network.

#### DISCUSSION

To identify new regulatory partner proteins for Mad1, we devised a yeast two-hybrid screen using a LexAMad1 fusion incapable of binding SIN3p. In a previous two-hybrid screen, only the PAH2 domains of the mSin3A/B corepressors were isolated. This was unexpected as cDNAs encoding Max are represented in the library (data not shown). The small number of positives in the original screen arose because they could compete with endogenous SIN3p for binding to the LexAMad1 bait and counteract transcriptional repression of the reporter gene mediated by SIN3p (35). The strategy described here was adopted to search for additional binding partners that were not found in the original screen. Our current screen identified a known Mad1 binding partner, Max (data not shown), and new potential partner proteins for Mad1.

We have presented a characterization of a new Max-like BHLHZip protein and Mad1 binding partner that we have named Mlx. Mlx shares numerous biochemical and physiological characteristics with Max, suggesting similarities in their function. Mlx and Max proteins are stable, with half-lives greater than 6 h, which contrasts with the short half-lives of their heterodimeric partners, the Myc and Mad transcription factors. This finding suggests that the formation of active Max- or Mlx-containing heterocomplexes will be limited by the synthesis and degradation of their heterodimeric partners. Furthermore, the mRNAs encoding each protein are abundant and expressed in both fetal and adult tissues, suggesting that Max and Mlx are constitutively available to bind to their heterodimeric partners. Both Max and Mlx require heterodimerization with another BHLHZip protein for high affinity binding to the CACGTG E-box elements. To repress transcription, both proteins must form heterocomplexes with Mad1 that are capable of binding DNA, and they must recruit the mSin3-HDAC complex.

The data we present suggest that Mad1:Max and Mad1:Mlx heterodimers are very similar in their biochemical properties. However, the lack of homology between Mlx and Max outside their DNA binding and dimerization domains suggests that the heterodimers will have nonredundant functions. One mechanism by which such functional diversity might be achieved is through targeting different promoters. The loop domains within the BHLHZip proteins Max, upstream stimulatory factor, and PHO4 have been shown to make contacts with the DNA phosphate backbone outside the CACGTG core (42, 46, 47). We hypothesize that the longer loop of Mlx might similarly make contacts outside the core, allowing Mlx-containing heterocomplexes to recognize sites different from Max-containing heterocomplexes. In support of this idea, our binding site selection experiments revealed a difference in the sequences flanking the CACGTG core preferred by each heterodimer. We

are currently investigating the contribution of CACGTG flanking sequences to target gene selection *in vivo*. Alternatively, differences in the functions of the two heterodimers could occur by other mechanisms such as cell type-specific factors that facilitate discrimination of E box flanking sequences *in vivo*. Finally, it is possible that the function of Mlx may partially overlap with that of Max with regard to Mad1 and Mad4 activity.

Mad1 was cloned as a Max-binding protein. Therefore, it has been suggested that Mad1 dimerization with Max is sufficient to explain the effects of Mad1 overexpression, such as cell cycle arrest (18, 24). However, there is little evidence that the biological activity of Mad1 relies solely on dimerization with Max or that the Mad family in general is dedicated only to the direct opposition of Myc activity. The biological functions of Mad1 could be mediated through heterodimeric partners other than Max. We propose that Mad1 function and the activity of other Mad family members may be regulated by dimerization with other partners such as Mlx. Mad1:Mlx dimers may allow the Mad1 repressor to function in the absence of Max or to function differently in particular cell types or in specific stages of the cellular differentiation program.

Given the similarity between Mlx and Max in their dimerization domains, it was surprising that both two-hybrid and EMSAs revealed interactions only between Mlx and Mad1 or Mad4. This restricted dimerization between Mlx and Mad1 and Mad4 implies that these two Mad family members may be more similar in function to one another than they are to Mxi1 and Mad3. Detailed analysis of the spatial and temporal expression patterns of Mxi1 and Mad3 show that these two family members are expressed in proliferating cells and early in the differentiation process. In contrast, Mad4 and in particular Mad1 are expressed later during differentiation. Therefore, Mxi1 and Mad3 may regulate aspects of the differentiation pathway distinct from those regulated by Mad1 and Mad4. However, if Max is continuously available in these tissues, what is the function of Mlx? Although other mechanisms are possible, it seems most likely that Mad1:Mlx and Mad4:Mlx heterodimers will regulate a unique subset of downstream target genes whose expression is required for the later stages of differentiation. We postulate that these targets either are not recognized by Mad1:Max and Mad4:Max heterocomplexes or that under some circumstances Max, although expressed, is not available for heterodimerization with some or all of its partners.

Finally, the similarities between Max and Mlx suggest that Mlx may function as a common dimerization partner of a new transcription factor network. In support of this hypothesis, we have recently identified a novel family of BHLHZip proteins that interact with Mlx.<sup>2</sup> These new BHLHZip proteins function as transcriptional activators, demonstrating that, like the Max network, the Mlx network will have both positive and negative components. We are currently examining the function of the Mlx network in controlling aspects of cell growth and differentiation.

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