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Mlx, a new Max-like bHLHZip family member: the center stage of a novel transcription factors regulatory pathway?

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The Myc proto-oncogene family members have been identified as the cellular homologs of the transforming oncogene of avian retroviruses. They encode central regulators of mammalian cell proliferation and apoptosis, and they associate with the bHLHZip protein Max to bind specific DNA sequences and regulate the expression of genes important for cell cycle progression. The other family members, Mad1, Mxi1, Mad3, Mad4 and Rox (Mnt) antagonize their activities. The Mads and Rox compete with Myc in heterodimerizing with Max and in binding to the same specific target sequences. These Mads:Max and Rox:Max dimers repress transcription through binding to the mSIN3 corepressor protein and by tethering histone deacetylase-containing complexes to the DNA. In a screen for Rox interactors we isolated Mlx, a bHLHZip protein previously identified in a screen for Mad1 interactors. In the present work we extend the known dimerization partners of Mlx by demonstrating its ability to interact with Rox. Moreover, we show that contrary to previous reports Mlx is able to homodimerize and to bind E-box sequences at low concentration levels. The possible role of Mlx in an emerging regulatory pathway and acting parallel to the Max driven network is discussed. *Oncogene* (2000) **19,** 3266–3277.

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Introduction

The transcriptional regulatory proteins encoded by the myc proto-oncogene family have been linked to multiple aspects of eukaryotic cell function, including cell cycle progression, differentiation, and apoptosis. Clinical and biological observations support the implications of these transcription factors in a wide range of neoplasias. c-Myc and N-Myc are involved in Burkitt lymphoma, neuroblastoma and small cell lung carcinoma. The chromosomal alterations observed in these malignancies result in degradation of myc expression, which is normally under tight transcriptional and post-transcriptional control (reviewed in Amati and Land, 1994; Hesketh, 1997). Myc proteins are expressed in proliferating cells and are down-regulated upon cell-cycle withdrawal or differentiation.

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Myc proteins hold a tripartite conserved motif, called basic-Helix–Loop–Helix Leucine Zipper (bHLHZip). This motif is the hallmark of a class of transcription factors which includes the USFs (Gregor *et al*., 1990; Sirito *et al*., 1992, 1994), the TFEs (Beckmann *et al*., 1990; Fisher *et al*., 1991; Hodgkinson, 1993; Zhao *et al*., 1993), Max (Blackwood and Eisenman, 1991; Prendergast *et al*., 1991), and the Mads (Ayer *et al*., 1993; Hurlin *et al*., 1995b; Zervos *et al*., 1993).

The basic region recognizes a canonical CANNTG DNA-binding sequence called E-box (Blackwell *et al*., 1993). The HLH and Zip motifs participate in protein dimerization, a prerequisite for DNA-binding. Max is a widely expressed and stable bHLHZip protein that forms DNA-binding heterodimers with the Myc and Mad proteins (Ayer *et al*., 1993; Blackwood and Eisenman, 1991; Hurlin *et al*., 1995b; Prendergast *et al*., 1991; Zervos *et al*., 1993). Although Myc-Max and Mad-Max heterodimers bind the same E-box-related DNA sequences, they elicit different transcriptional responses. Reporter genes under the control of E-box elements are activated by Myc-Max, while Mad-Max represses their transcription (Amati *et al*., 1992; Ayer *et al*., 1993; Hurlin *et al*., 1995b; Kretzner *et al*., 1992). Thus it appears that Myc activates, while Mad represses, genes involved in promoting cellular proliferation (reviewed in Cole and McMahon, 1999; Dang, 1999; Peters and Taparowsky, 1998).

Recently, two mammalian Mad interactors, mSin3A and mSin3B, homologous to the *Saccharomyces cerevisiae* transcriptional corepressor Sin3p, were identified (Ayer *et al*., 1995; Schreiber Agus *et al*., 1995). They are required in Mad-mediated transcriptional repression (Kasten *et al*., 1996; Roussel *et al*., 1996). Sin3p represses a wide spectrum of genes when fused to a heterologous DNA binding domain. Several lines of evidence suggest that it may function by inhibiting the general transcription machinery, either by direct interaction or through a negatively acting chromatin structure. Its genetical partner, Rpd3p, was shown to exert histone deacetylase activity, suggesting that repression occurs by modification of histones and hence stabilization of chromatin structure (Kasten *et al*., 1997; Taunton *et al*., 1996). Similarly, mSIN3A and mSIN3B are part of high molecular weight protein complexes which contain the mammalian histone deacetylases (Alland *et al*., 1997; Hassig *et al*., 1997; Heinzel *et al*., 1997; Laherty *et al*., 1997; Nagy *et al*., 1997; Zhang *et al*., 1997b).

We and others isolated a new member of the Myc/Max/Mad bHLHZip family when searching for

transcribed sequences of human chromosome 17p13.3. This protein, named Rox (Mnt), heterodimerizes with Max and binds a non-canonical E-box. Rox is able to repress transcription, although in HeLa cells it behaves as an activator. Rox holds a SIN3 interaction domain (SID) and binds the PAH2 regions of both mSIN3A and yeast SIN3p (Hurlin *et al*., 1997; Meroni *et al*., 1997). Rox steady state mRNA is only present in WI-38 quiescent cells, suggesting that Rox might negatively regulate the entry into the cell cycle by repressing genes that should be upregulated to overcome the restriction point. Rox might therefore be considered a Myc antagonist.

In this paper, we describe the isolation of Mlx, a bHLHZip protein, in a screen for new Rox interactors. The same gene was independently identified in a screen for Mad1 interacting proteins (Billin *et al*., 1999). We demonstrate that Mlx is interacting *in vitro* and *in vivo* with Rox. Moreover, contrary to previous reports, we show that Mlx is able to homodimerize and to bind E-box sequences at low concentration levels. Our results and previous reports (Billin *et al*., 1999) suggest that Mlx cooperates with Mad1, Mad4, Rox and possibly other bHLHZip proteins in a novel regulatory pathway parallel to the Max transcription factor controlled network.

Results

Identification of a new bHLHZip protein

To identify new proteins that interact with Rox we performed a yeast two-hybrid assay using a HeLa cDNA library (Gyuris *et al*., 1993; Zervos *et al*., 1993). A fusion between LexA and Rox residues 60–582 was used as bait. This construct lacks the first 59 amino acids of Rox shown to bind SIN3 (Hurlin *et al*., 1997; Meroni *et al*., 1997). This screening allowed us to identify four new Rox-interactors. In this report we describe the cloning and functional characterization of one of these interactors, a new mammalian bHLHZip protein (see Figure 1a). Due to its sequence, size, overall similarity and function features we named the gene BigMax (see below).

From the two-hybrid screening, we recovered four clones corresponding to two independent and overlapping BigMax cDNAs (clones 630, 640, 692 and 694, see Figure 1a). These cDNAs represent the human homolog of murine transcription factor like protein 4 (TCFL4) (Bjerknes and Cheng, 1996). TCFL4 was cloned from the mouse 17q21 syntenic region where BRCA1 was mapped (Friedman *et al*., 1994, 1995). However, these clones lack 162 bp of exon 1 and constitute an alternatively spliced form of the human TCFL4 (isoform BigMax-β, see Figure 1d). A human clone corresponding to complete murine TCFL4 was also isolated through a database search (isoform BigMax-γ, see Figure 1d) (IMAGE clone 488171). Moreover, a third isoform lacking exon 3 and 162 bp in exon 1 (isoform BigMax-α, see Figure 1d) was identified in dbEST (IMAGE clones 488652 and 502642). Nested PCR analysis on cDNA libraries (serum starved WI-38 cells, fetal brain, HeLa and keratinocyte cells) excluded the possibility that the two newly identified splice forms resulted from cloning artifacts. During the preparation of this manuscript, BigMax-β was independently isolated and named Mlx by Donald E Ayer and collaborators in a screen for new Mad1 interactors (Billin *et al*., 1999). For the sake of clarity and to avoid confusion, we decided to adopt their nomenclature and name the BigMax-α, -β and -γ isoforms, Mlx-α, -β and -γ, respectively.

The entire Mlx gene spans approximately 7 kb and is contained in a fully sequenced cosmid (GenBank accession number U34879, (Zhao *et al*., 1996)). It consists of eight exons which can be alternatively spliced to encode the different isoforms (see Figure 1a,d). Interestingly, the two shorter isoforms (-α and -β) use a cryptic AGgcaagc donor site in exon 1A.

We sequenced the three alternative Mlx-α, -β and -γ isoforms and deposited them in GenBank (accession numbers AF213666, AF213667 and AF213668, respectively). The putative initiation codon properly fulfills Kozak's criteria (Kozak, 1984), but no in frame 5′UTR STOP codon was identified in any of the six independent clones we isolated. We also cloned and sequenced the murine Mlx alternatively spliced forms to confirm their existence in rodents (mMlx-α, -β and -γ, GenBank accession numbers AF213670, AF213671 and AF213672, respectively).

The three human isoforms encode polypeptides of 214, 244 and 298 residues, respectively. They share a common carboxyterminal region and a centrally positioned bHLHZip domain. An alignment of the sequence of human Max and Mlx-α is presented in Figure 1a. Like Mlx, the Max gene can be alternatively spliced and encode Max1, Max2, ΔMax and dMax. Max1 is 151 residues long, Max2 contains a nine amino-acid insertion before the basic domain, ΔMax encodes an isoform truncated after the Leucine Zipper and dMax codes for a Max isoform lacking the basic and the first Helix regions (Blackwood and Eisenman, 1991; FitzGerald *et al*., 1999; Makela *et al*., 1992; Vastrik *et al*., 1993).

Homology searches in dbEST revealed a related gene in *Drosophila* (BDGP/HHMI EST Project clones GM14426 and LD05447). The full-length sequence of this gene, which we named dMlx, is deposited in GenBank (accession number AF213669). PCR-based cDNA cloning failed to reveal different isoforms in embryonic, ovarian and disc fly libraries (Figure 1b). The homology is striking in the bHLHZip region (63% identity) and the carboxyterminal tail. The mammalian and insect proteins are 45% identical and 57% similar.

Mlx expression analysis

We analysed the expression pattern of the Mlx gene. It was first assessed by probing multiple adult tissue Northern blots. We detected three different mRNA species, approximately 1.4, 2.4 and 3.3 kb in size. All three species are expressed ubiquitously (see Figure 2a).

Similarly, no specific cell lineage expression of Mlx was detected in the analysis of E9.5 through E14.5 mouse embryos. These results are similar to those obtained on the expression of the other members of the Myc/Max/Mad superfamily (Ayer *et al*., 1993; Hurlin *et al*., 1995a,b, 1997; Larsson *et al*., 1994; Meroni *et al*., 1997; Queva *et al*., 1998; Zervos *et al*., 1993). The overlapping expression of Mlx and Rox in the same

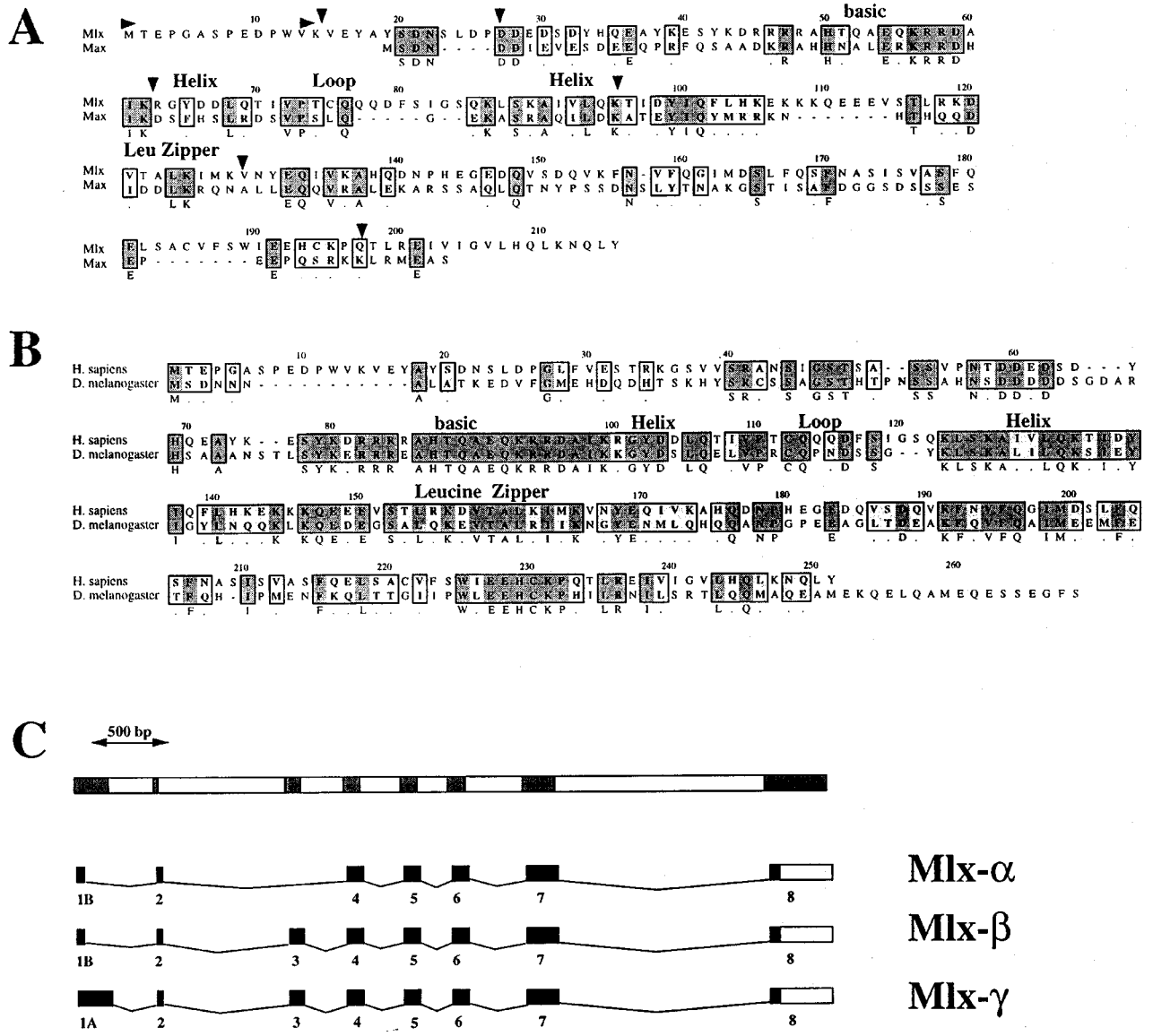


Figure 1 Mlx is a member of the bHLHZip family. Conserved and similar amino acids are boxed. The consensus sequence is indicated below. (**a**) Alignment of human Mlx α and human Max1. The appropriate positions of the bHLHZip domain regions are shown above. The beginnings of clones Mlx 630 and 692 isolated in the two-hybrid screen using Rox 60–582 as bait are indicated by horizontal arrowheads. The positions of introns are denoted by vertical arrowheads. (**b**) Alignment of the human Mlx β and fruitfly dMlx. The approximate positions of the bHLHZip domain regions and of the USF2 activation-like domains are shown above. (**c**) Schematic representation of the Mlx gene structure (top) and of the exon composition of the three Mlx alternatively spliced forms (see below)

tissues and at the same developmental stages is consistent with the possible involvement of Mlx in regulating Rox function (see below).

Although Mlx is ubiquitously expressed, whole-mount RNA *in situ* hybridization experiments, at E9.5 and E10.5, show stronger expression of Mlx in the ventral portion of the central nervous system (Figure 3a,b). Parallel *in situ* hybridization of E12.5 sagittal sections shows a prevalent expression in this tissue (Figure 3c lateral and e medial). Further magnification highlights stronger expression in the dorsal root ganglia and in the segmental bronchi epithelia of the developing lung (Figure 3f). Interestingly, this high expression is not maintained in the surrounding parenchynma. Later in development, high levels of Mlx expression are restricted to the villi of the gut as shown in transversal sections of E14.5 embryos (Figure 3d).

Different authors showed that the steady state mRNA levels of c-Myc, L-Myc, N-Myc, Mad1, Mxi1, Mad3, Mad4 and Rox(Mnt) are tightly regulated through cell cycle and differentiation phases (Armelin *et al*., 1984; Ayer *et al*., 1993; Campisi *et al*., 1984; Kelly *et al*., 1983; Meroni *et al*., 1997; Queva *et al*., 1998). These observations prompted us to investigate Mlx expression profile in synchronized cells. Normal quiescent human lung fibroblasts (WI-38) were obtained by serum starvation for 3 days. The cells were then stimulated to reenter the cell cycle by the addition of 20% fetal bovine serum. Total RNA samples were collected at defined times after serum addition and the expression of the three isoforms of Mlx were monitored by Northern blot analysis. Under these conditions, the cells started to synthesize DNA about 12 h after serum addition and after 24 h most of the cells had entered the S phase. Correct synchronization

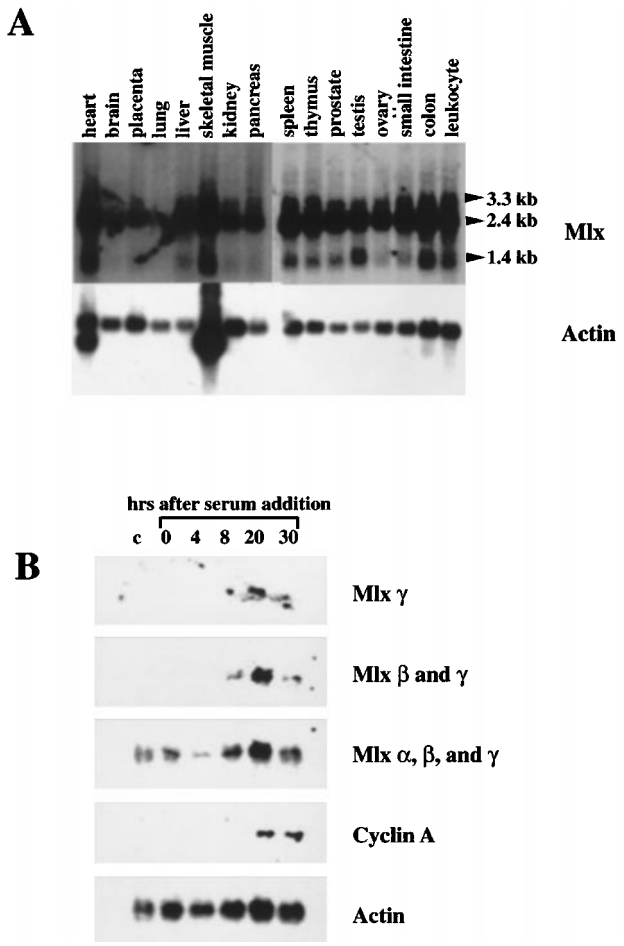


Figure 2 Mlx is ubiquitously expressed in adult tissues, but regulated during the cell cycle. (**a**) Northern blot analysis of adult human tissues probed with a Mlx cDNa fragment. The size of the Mlx mRNA bands are indicated. The normalization with an actin probe is presented below. (**b**) Northern blot analysis of total RNA extracts of synchronized WI-38 fibroblasts: c, cycling cells; 0, serum starved cells; 4, 8, 20, 30 h after serum addition. The Northern blot has been probed with fragments detecting only the γ, the β and γ, and the α, β and γ isoforms mRNAs, respectively (see Results section). Synchronization and normalization controls with an S phase specific probe (Cyclin A) and actin, respectively, are shown in the bottom panels

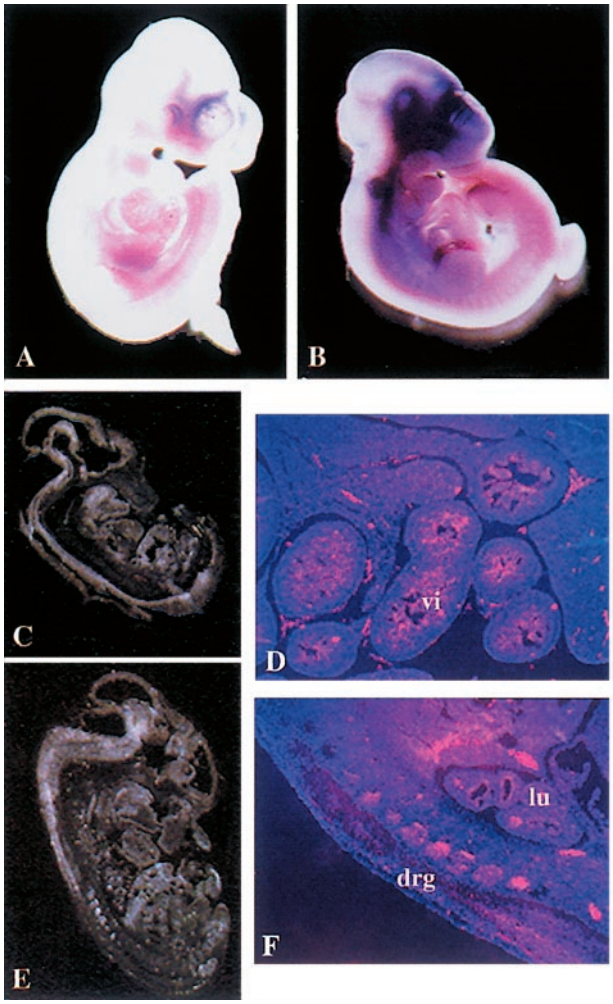


Figure 3 Mlx expression analysis during mouse embryonic development. (**a**) and (**b**) Whole-mount RNA *in situ* hybridization of E9.5 and E10.5 mouse embryos, respectively, showing Mlx expression in the CNS. (**c**) and (**e**) Sagittal sections of whole E12.5 mouse embryos. The white signal, indicating expression, is obvious at the level of the CNS. (**f**) Further magnification reveals expression in the dorsal root ganglia (drg) and in the lung (lu) (red signal). (**d**) Detail of an E14.5 mouse embryo transversal section showing expression of Mlx in the villi (vi) of the gut (red signal)

was assessed by hybridization with the S phase specific Cyclin A (Figure 2b, bottom lane). Different Mlx probes were used to detect either the -γ isoform alone, or the β and γ isoforms, or the α, β and γ isoforms together. These probes are depicted in Figure 1d. They span the last 162 bp of exon 1B, exon 3 and exons 7–8, respectively. Interestingly, the Mlx isoforms β and γ mRNA steady state level peaks in S phase (see Figure 2b). In this regard Mlx shows a unique expression profile among the Myc/Max/Mad/Rox family members.

To test whether Mlx isoforms are tissue specific, a qualitative study of the presence or absence of the three Mlx isoforms was performed by PCR on the Express-Check panel of 62 tissue-specific cDNA libraries (ATCC). We found the three Mlx-α, -β and -γ isoforms throughout the panel (data not shown). These data show that Mlx isoforms, like the Max isoforms, are not tissue specific. The semi-quantitative PCR conditions used in these experiments allowed us to conclude that in a large number of tissues, Mlx-α and -γ are more abundant than the rarer Mlx-β, whereas this ratio is inverted in favor of isoform β in the basal ganglia, liver, placenta and pancreatic islets (data not shown). These results demonstrate that while the isoforms are not tissue specific, their ratio probably is, as previously shown for some of the Max protein isoforms (Makela *et al*., 1992).

Mlx homodimerizes and heterodimerizes with Rox

We tested whether Mlx is able to interact with members of the bHLHZip family using interaction-mating and *in vitro* co-immunoprecipitation approaches.

To investigate Mlx binding specificity, we took advantage of the interaction-mating technology (Reymond and Brent, 1995). Mlx and panels of informative bHLHZip proteins were expressed as fusion proteins with either the LexA-DNA binding domain (bait) or the B42 acidic moiety (prey). A representative mating assay is shown in Figure 4a. Our results confirmed that

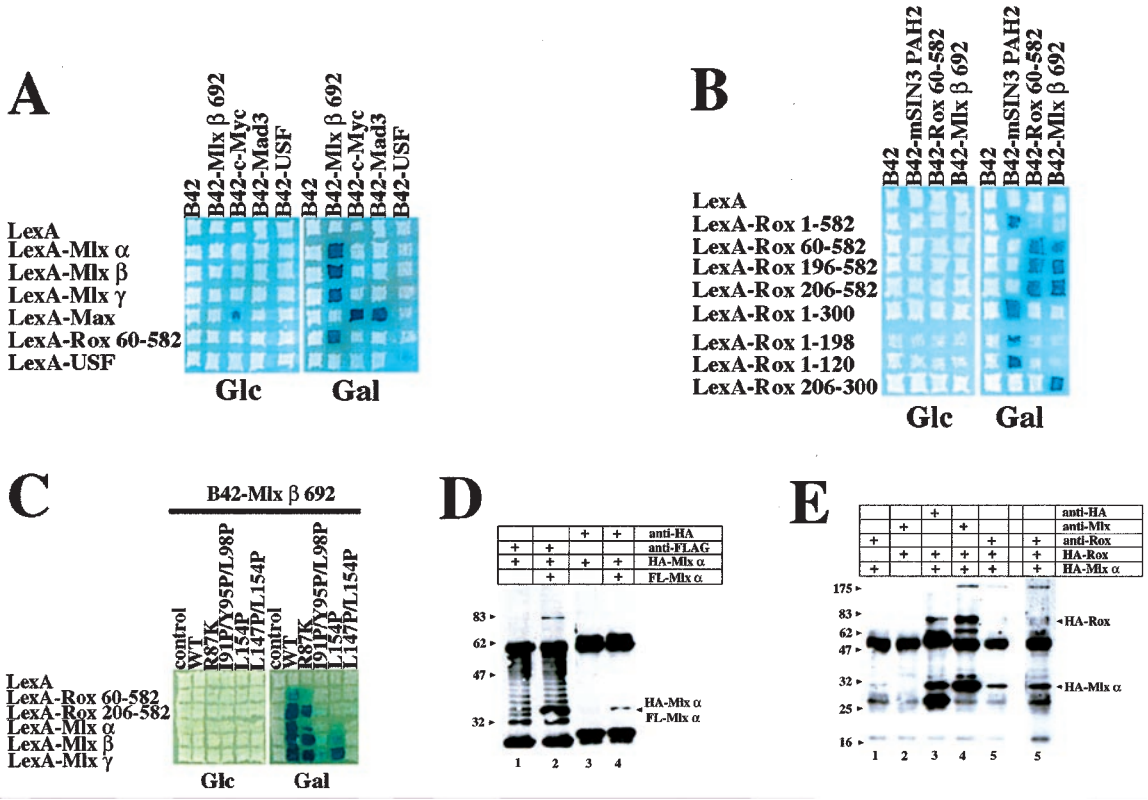


Figure 4 Mlx homodimerizes and heterodimerizes with Rox. (**a**) Interaction-mating assays between strains carrying bHLHZip family members. EGY42 bait strains containing plasmids that expressed LexA fusions to human Mlx α, Mlx β, Mlx γ, Max, Rox 60–582 and USF were mated to EGY48 derivatives that contained B42 fusions to human Mlx β (clone 692), c-Myc and USF, and mouse Mad3. Plates contain either glucose or galactose/raffinose. (**b**) Interaction-mating assays between strains carrying Rox deletion mutants and Mlx β (clone 692). EGY42 bait strains containing plasmids that expressed LexA fusions to Rox deletions were mated to EGY48 derivatives that contained B42 fusions to the mouse mSIN3A PAH2 domain, and to human Rox 60–582 and Mlx β (clone 692). (**c**) Interaction-mating assays between strains carrying Rox or Mlx and Mlx β (clone 692) mutants. EGY42 bait strains containing plasmids that expressed LexA fusions to human Rox 60–582, Rox 206–582, Mlx α, Mlx β and Mlx γ were mated to EGY48 derivatives that contained B42 fusions to human Mlx β mutants (clone 692). The mutants affect either the sequence of the basic region, the helix 1, or the Leucine Zipper domain. See Results section. (**d**) Cos-7 cells were transfected with plasmids expressing HA-Mlx-α and/or FL-Mlx-α as specified on top of the lanes. The cell lysates were immunoprecipitated with anti-HA or anti-FLAG commercially available mAb as indicated. The immunoblot was performed with anti-HA (lanes 1 and 2) or with anti-FLAG (lanes 3 and 4) mAb. Arrowheads on the left show approximate position of the molecular weight markers [kDa]. Arrowhead on the right indicate the position of HA-Mlx-α and FL-Mlx-α. (**e**) Cos-7 cells were transfected with plasmids expressing HA-Rox and/or HA-Mlx-α as specified on top of the lanes. The cell lysates were immunoprecipitated with Rox or Mlx immune sera or with anti-HA mAb as indicated. The immunoblot was performed with anti-HA mAb. Arrowheads on the left show approximate position of the molecular weight markers [kDa]. Arrowheads on the right indicate the position of HA-Rox and HA-Mlx-α, respectively. Two different exposures of lane 5 are presented

Mlx was not interacting with Max, Mxi1, and Mad3, but was heterodimerizing with Mad1 (Billin *et al*., 1999). This result, however, was not confirmed by *in vitro* co-immunoprecipitation (see below). Moreover, we found no interaction with c-Myc, USF and mi. Substantiating the efficiency of the two-hybrid screen we confirm the ability of Mlx to heterodimerize with Rox in interaction mating assays. Interestingly and contrary to previous beliefs the interaction mating experiments showed also that Mlx was readily homodimerizing (see Figure 4a).

To determine which regions of Rox and Mlx are responsible for heterodimerization and homodimerization, we created Rox deletion mutants and Mlx-β mutants: (i) LexA-Rox 60–582 lacks the SID; (ii) LexA-Rox 196–582 and LexA-Rox 206–582 lack the SID and the P/Q rich domain; (iii) LexA-Rox 1–300 lacks the carboxyterminal portion of the protein (C-term); (iv) LexA-Rox 1–198 lacks the bHLHZip domain and the C-term; (v) LexA-Rox 1–120 lacks the bHLHZip domain, the C-term and part of the P/Q rich region; (vi) B42-Mlx-R87K mimicks the basic region dominant negative mutant *Oak-Ridge* of the *microphhtalmia* gene (Hemesath *et al*., 1994); (vii) B42-Mlx-I91P/Y95P/L98P disrupts the first Helix and (viii) B42-Mlx-L154P and B42-Mlx-L147P/L154P change one and two conserved leucine residues of the Leucine Zipper domain, respectively.

The ability of Rox deletion mutants and of the Mlx-β mutants to retain homodimerization and heterodimerization was tested by interaction-mating. The bHLHZip domain of Rox is essential for interaction with Mlx, as LexA-Rox 206–300 is able to interact with B42-Mlx (see Figure 4b). It is important to note that the Rox-Mlx interaction is detectable only in yeast with Rox deletion mutants that lack the SID. This domain interacts with ySIN3 and masks the activation of the two-hybrid reporter genes (Meroni *et al*., 1997). The disruption of the Mlx first Helix or its Leucine Zipper domain decreases or abolishes its capability to homodimerize and heterodimerize with Rox, while modifications of its basic region had no effect (see

Figure 4c). These results confirm the involvement of the first Helix and Leucine Zipper regions in the dimerization of the bHLHZip family members.

To provide independent evidence for Mlx binding specificity, we performed co-immunoprecipitation experiments with bHLHZip family members. *In vitro* translated (IVT) and labeled HA-tagged c-Myc, Max, Mlx, Mad1, Mxi1, Mad3, Mad4 or Rox and FLAG-tagged Mlx were mixed and co-immunoprecipitated in low stringency conditions using anti-Mlx, anti-FLAG or anti-HA antibody. The bound and recovered proteins were separated on SDS–PAGE gels. The results confirm the interaction-mating assays data made exception of Mad1, i.e. the ability of Mlx to homodimerize and heterodimerize with Rox (data not shown).

We confirmed Mlx homodimerization and Rox and Mlx interaction *in vivo*. Cos-7 cells were transiently co-transfected with HA-Rox or FL-Mlx-α and HA-Mlx-α. Cell lysates were immunoprecipitated with anti-HA or anti-FLAG monoclonal antibodies (Figure 4d) and specific sera raised against Rox or Mlx (Figure 4e). The bound and recovered proteins were separated on gel and subjected to immunoblot analysis. In Figure 4d, FL-Mlx-α and HA-Mlx-α were recovered in the anti-HA and anti-FLAG immunoprecipitate respectively (lanes 4 and 2), but not in the controls (lanes 3 and 1) substantiating the ability of Mlx to homodimerize. In Figure 4e, correct expression of the transfected proteins was assessed by immunoprecipitation with a commercially available anti-HA antibody (see Figure 4d, lane 3). HA-Rox was recovered in the anti-Mlx immunoprecipitate as an $\sim$70 kDa doublet (lane 4). Concomitantly, HA-Mlx was detected in the anti-Rox immunoprecipitate as an $\sim$30 kDa band (lane 5).

The results of these *in vivo* and *in vitro* co-immunoprecipitations and interaction-mating experiments suggest that Mlx is not using Max as a partner, unlike any other member of the Myc/Max/Mad family. Moreover, like Max, Mlx is homodimerizing and heterodimerizing with diverse members of the bHLHZip family, Rox (this study), Mad1, and Mad4, the interactions with the Mads however were not confirmed *in vivo* (Billin*et al*., 1999).

Mlx:Mlx and Mlx:Rox dimers bind DNA

Max:Max and Max:bHLHZip heterocomplexes bind to E-box containing sequences as reported in the literature (Ayer *et al*., 1993; Blackwood and Eisenman, 1991; Hurlin *et al*., 1995b, 1997; Meroni *et al*., 1997; Zervos *et al*., 1993). We tested the ability of both Mlx : Mlx and Mlx : Rox dimers to bind to CACGTG-containing DNA sequences by electrophoretic mobility shift assays (EMSA).

Figure 5a shows that a reticulocyte lysate containing IVT HA-Mlx-α or IVT HA-Mlx-γ are able to specifically bind a labeled oligonucleotide containing the CACGTG E-box sequence (lanes 1–5, 8, 11, 13, 14 and 26). This protein-DNA complex can be supershifted in the presence of anti-Mlx serum, but not in the presence of preimmune serum or anti-Max antibodies (lanes 9 and 10, respectively and data not shown), or it can be partially supershifted and mostly abolished in the presence of anti-HA antibody (lanes 6 and 15). Consistent with the presence of Mlx in the DNA-protein complex, we observe that the bandshift produced by expression of Mlx-α migrates faster than the bandshift produced by expression of the longer isoform-γ (compare lanes 1 and 2). Further analysis demonstrates that Mlx-α:Mlx-γ heterocomplexes also bind DNA (lane 7, short exposure). Similarly, the Mlx-β isoform is able to bind CACGTG DNA sequences (data not shown). Alternative splice forms may induce subtle differences in DNA binding as in the case of Max1 versus Max2 (Prochownik and VanAntwerp, 1993), but no obvious changes in E-box binding were observed with Mlx-α, -β and -γ. These results confirm the ability of Mlx to readily homodimerize and bind DNA even at low concentration. In this regard Mlx is similar to Max, as Max was the only member of this transcription factors family shown to homodimerize and bind DNA *in vitro* at low concentration (Ayer *et al*., 1993; Berberich and Cole, 1992; Hurlin *et al*., 1995b, 1997; Zervos *et al*., 1993; Zhang *et al*., 1997a).

Competition experiments revealed that the Mlx-γ:Mlx-γ complexes were also able to recognize the non-canonical CACGCG DNA sequence, although poorly, but not the Myc:Max canonical site CATGTG or the non-canonical sites CACGAG and CATGCG (see Figure 5b) (Blackwell *et al*., 1993). These data suggest that Mlx:Rox complexes would not only be able to regulate CACGTG but also CACGCG operons, with Mlx binding to the CAC and Rox binding to the GYG half site preferentially.

Reticulocytes expressing both HA-Mlx-γ and FL-Rox show three different specific DNA-protein complexes: a fast migrating complex and two slower complexes that almost comigrate (see Figure 5a). Similar results were obtained with the other two Mlx isoforms (data not shown). The faster migrating complex was partially disrupted and partially supershifted in the presence of anti-HA antibody, but no effects were observed in the presence of anti-FLAG antibody (lanes 16–18 and 23–25). These results indicate that the faster migrating complex contains Mlx:Mlx dimers. Besides, the MLR complex (see Figure 5 legend) was disrupted and supershifted in the presence of anti-HA and anti-FLAG antibody, respectively, showing that it contains Mlx:Rox heterodimers (lanes 16–18 and 23–25). Finally, the MAR complex (see Figure 5 legend) was supershifted in the presence of anti-FLAG antibody, but remained unaltered in the presence of anti-HA antibody, showing that this complex contains Max:Rox heterodimers (lanes 16–18 and 23–25). This was confirmed by the observation that an identical MAR complex can be observed in assays containing reticulocyte expressing only FL-Rox (lanes 19–22). As previously published, the reticulocyte lysate provides enough Max to allow the formation of Max/Rox complexes (Meroni *et al*., 1997; Ló Nigro *et al*., 1998). We observed that the two slow migrating bandshifts produced by the expression of Mlx, together with the last 523 residues of Rox, are smaller than the one produced by the expression of Mlx and Rox full-length complexes (compare lanes 17 and 24, 18 and 23, 16 and 25). On the other hand, no differences in the size of the faster migrating bandshift are observed (compare lanes 17 and 24, 18 and 23). These data are consistent with the presence of Rox in both of the slow migrating complexes and with its

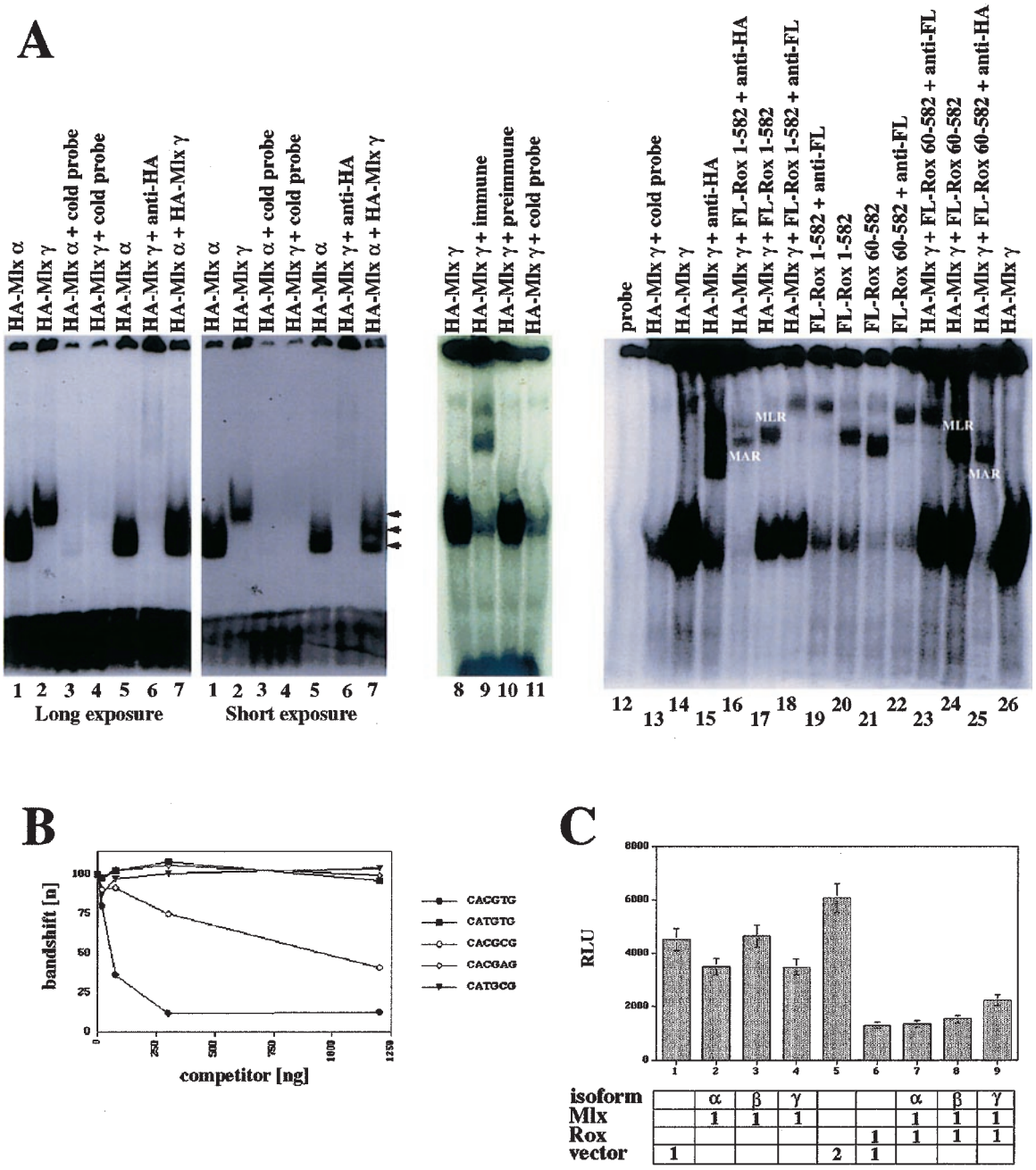


Figure 5 Mlx:Mlx and Mlx:Rox dimers bind E-box sequences. (**a**) The ability of Mlx to bind DNA was tested by EMSA. Reticulocyte lysates expressing Mlx and/or Rox were mixed together with labeled E-box sequence DNA. The following additions were made to the reaction mixtures: 1, 2, 5, 7, 8, 14, 17, 20, 21, 24 and 26, buffer; lanes 3, 4, 11 and 13, 200 ng of unlabeled probe; lanes 6, 15, 16 and 25, 1 μg of anti-HA antibody; lane 9, 5 μl of anti-Mlx antiserum; lane 10, 5 μl of anti-Mlx pre-immune antiserum and lanes 18, 19, 22 and 23, 1 μg of anti-FLAG antibody. 'MLR' and 'MAR' indicate the Mlx:Rox and the Max:Rox containing complexes, respectively. The arrowheads show the position of the Mlx α:Mlx α, Mlx α:Mlx γ and Mlx γ:Mlx γ dimers complexed with the DNA, respectively (**b**) The ability of Mlx:Mlx complexes to bind canonical and non-canonical E-box sequences was tested by EMSA using increasing amounts of CACGTG, CATGTG, CACGCG, CACGAG or CATGCG containing oligonucleotides as cold competitors. DNA-protein complexes were quantified with a PhosphorImager (Molecular Dynamics) and plotted against the various amounts of cold competitors used. (**c**) Mlx moderately represses transcription. HEK293 cells were transiently transfected with pTK81 4x[CACGTG]-luciferase reporter and pCH110 β-Galactosidase reporter, together with the amounts of plasmids indicated below [μg]. The first lane of the table shows the Mlx isoform used in the transfection. Relative luciferase activity (RLU) are shown after β-Galactosidase normalization

absence from the faster migrating DNA-protein complex.

To summarize, the results presented in Figure 5a, confirm the ability of Mlx to both homodimerize and heterodimerize, and indicate that these complexes are able to bind E-box DNA sequences. Moreover, they demonstrate that Mlx, like Max, is able to bind DNA as a homodimer, a feature unshared by c-Myc, L-Myc, N-Myc, Mad1, Mxi1, Mad3, Mad4 and Rox/Mnt at non-extreme concentration levels. The ability of Mlx to homodimerize and bind DNA was not detected in previously published reports (Billin *et al*., 1999). To explain this discrepancy, we can speculate that Mlx post-traductional modifications essential for homodimerization are fulfilled by reticulocyte lysates expressed Mlx.

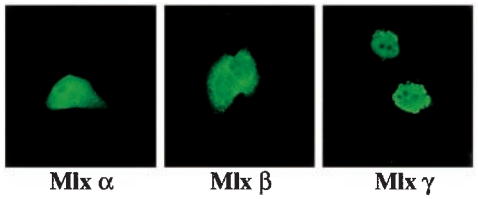
Like Max, Mlx does not affect transcription on its own

We wanted next to understand what was the influence of Mlx on transcription. Previous works have demonstrated that Myc activates or de-represses transcription from a reporter construct with proximal E-box binding sites (Amati *et al*., 1992; Gu *et al*., 1993; Kretzner *et al*., 1992), while Mad1, Mxi1, Mad3, Mad4 repress from the same site (Ayer *et al*., 1993; Gupta *et al*., 1998; Hurlin *et al*., 1995b). We addressed the potential transcriptional activity of the three Mlx isoforms by transiently expressing the genes in HEK293 cells, where no Mlx protein is detectable (Billin *et al*., 1999), together with a luciferase reporter construct containing four reiterations of the CACGTG binding site upstream of a thymidine kinase minimal promoter. Transfections of Mlx-α or -γ resulted in a moderate decrease of the luciferase activity, while transfection with the β isoform had no effect (see Figure 5c, lanes 1–4). The repression of the background level of transcription is consistent with the binding of transcriptionally inert Mlx homodimers to CACGTG. Max was shown to repress transcription presumably with a similar mechanism (Kretzner *et al*., 1992).

In agreement with published reports, overexpression of Rox(Mnt) causes an approximate 5–6-fold decrease in luciferase activity compared with control transfection (lanes 5–6) (Hurlin *et al*., 1997; Meroni *et al*., 1997). The co-expression of any Mlx isoform was not enhancing this repression activity (lanes 5–9), suggesting that Mlx:Rox complexes have similar properties than Max:Rox dimers. However synergy upon transcription activity was observed when Mlx was cotransfected with Mir, a previously unidentified bHLHZip protein (G Merla and A Reymond, in preparation).

Mlx isoforms show different subcellular distribution

We determined the Mlx isoforms subcellular localization by indirect immunofluorescence. HeLa and Cos-7 cells were transiently transfected with a plasmid expressing each of the HA-tagged Mlx isoforms. The results are presented in Figure 6a and summarized in Figure 6b. The two shorter isoforms, Mlx-α and -β, are predominantly cytoplasmic and only partially nuclear, while the Mlx-γ isoform shows a predominantly nuclear localization. The longer isoform differs from both of the two shorter isoforms by the presence of exon 1B, which is 54 residues longer than exon 1A (see Figure 1a,b,c). Two sequences GRAGRARARRGAGRR and PACAKV, resembling a bipartite nuclear localization signal (NLS) and a portion of the c-Myc NLS (PAAKRVKLD), are present in this exon (reviewed in Nigg, 1997). To further assess the importance of these sequences in addressing Mlx-γ to the nucleus, we generated mutant constructs and analysed their subcellular localization. Any tested double amino-acids substitution of consecutively positively charged residues of the GRARARARRGAGRR sequence influences negatively, but not dramatically, the nuclear localization of the Mlx-γ isoform (see Figure 6b). Interestingly, the mutation K68T/V69G, which affects the region with low similarity to the c-Myc NLS, alters the subcellular localization of Mlx-γ. These observations confirm that the basic residue-rich exon 1B encoded amino acids are necessary for the predominantly nuclear localization of Mlx-γ.



B

cDNA	Localization	AA 26-39	AA 64-68
Mlx-α	C>n		
Mlx-β	C>n		
Mlx-γ	N>c	RAGRARARRGAGRR	PACAKV
Mlx-γ-R29G/R31S	N>c	RAGGASARRGAGRR	PACAKV
Mlx-γ-R33G/R34T	N+C	RAGRARAGTGAGRR	PACAKV
Mlx-γ-R38S/R39G	N+C	RAGRARARRGAGSG	PACAKV
Mlx-γ-P64L/A65E	N>c	RAGRARARRGAGRR	LECAKV
Mlx-γ-K68T/V69G	C>n	RAGRARARRGAGRR	PACATG

Figure 6 Mlx isoforms show different subcellular localizations. Subcellular distribution of Mlx α, Mlx β and Mlx γ in HeLa cells. (**a**) A summary of the different subcellular localizations detected, together with the sequence of residues 26–39 and 64–68 of Mlx γ are presented in panel (**b**). N indicates nuclear and C cytoplasmic localization. Hela cells were transfected with cDNAs coding for one of the three HA-tagged Mlx isoforms or HA-tag Mlx γ mutants. Transfected Mlx proteins were revealed 48 h post-transfection using an anti-HA or an anti-Mlx antibody

To test if this basic residue-rich region is sufficient to localize Mlx in the nucleus, we generated fusions between the avian pyruvate kinase, a strictly cytoplasmic protein, and (i) Mlx-γ residues 14 to 93 and (ii) Mlx-γ residues 14 to 147. These peptides contain both the above-described NLS-like sequences. Furthermore, the second peptide also includes the basic region of the bHLHZip domain. The fusion proteins produced localize in the cytoplasm (data not shown), whereas the control fusion protein, between the SV40 T NLS and the pyruvate kinase, results in the relocalization of this kinase into the nucleus. These and the above described results suggest that the exon 1B basic region plays a role in nuclear localization and that it does not contain a *bona fide* NLS. It is conceivable that Mlx-γ translocates into the nucleus after association with other proteins. Either these Mlx interactors possess their proper NLS and Mlx exon 1B increases the affinity for the protein partner, or the formed dimer acts with the participation of exon 1B to create a *de novo* NLS.

We also checked the presence of a piggy-back mechanism of nuclear import by co-transfecting the predominantly cytoplasmic EGFP-tagged Mlx-α isoform together with the nuclear HA-tagged Mlx-γ isoform in HeLa and Cos-7 cells. We reasoned that the ability of Mlx to homodimerize might induce relocalization of one of the isoforms upon presence of the other. Almost two thirds of the cells transfected with Mlx-α show a predominantly cytoplasmic distribution of the protein (35 and 40% of nuclear stained Cos-7 and HeLa cells, respectively). Upon co-transfection with the Mlx-γ isoform, the Mlx-α isoform is relocated preferentially to the nucleus (72 and 90% of nuclear stained Cos-7 and HeLa cells, respectively) substantiating the hypothesis that a piggy-back mechanism is involved in the regulation of the subcellular distribution of the Mlx isoforms.

The slight differences in the subcellular localization of the three Mlx isoforms can account for their differences in transcription assays. Moreover, our data indicate that the ability of Mlx to homodimerize induces relocalization of some isoform upon presence of the other. Furthermore, the basic residue-rich exon 1B is necessary, but not sufficient, for the predominantly nuclear localization of Mlx-γ.

Discussion

In this paper, we described the isolation in a screen for new Rox interactors of the bHLHZip protein, Mlx. It resembles Max in its features and function. This protein was independently identified in a screen for new Mad1 interactors (Billin *et al*., 1999). Mlx does not interact with the Max protein unlike the already known members of the Myc/Max/Mad/Rox family, while it heterodimerizes with Rox (this study), Mad1, Mad4 (Billin *et al*., 1999) and with two previously unidentified bHLHZip proteins (S Cairo, G Merla, A Reymond, manuscript in preparation). We found that, like Max, Mlx binds to E-box DNA as a homodimer. We propose that Mlx might be positioned at the center of a novel transcription-factor network parallel to the Max regulatory pathway.

The ratio between Max partners (c-Myc, L-Myc, N-Myc, Mad1, Mxi1, Mad3, Mad4 and Rox/Mnt) plays a major role in the cell decision to divide, to differentiate or to apoptose (reviewed in Dang, 1999; Eilers, 1999). The stable Max protein binds to the labile Myc proteins to promote cell proliferation. This function is mediated by the activation and/or derepression of specific genes crucial to cell proliferation. These specific targets include α-prothymosin, eIF4E, cad, ornithine decarboxylase, CDC25, the DEAD box gene MrDb, ISGF3 γ, IRP2, QNR-71, RCC1, rcl, cyclin D2, ECA39, and TERT (reviewed in Cole and McMahon, 1999; Dang, 1999; Peters and Taparowsky, 1998). This Myc transactivation and/or derepression is accompanied by loss of expression of some genes, which include H-ferritin, CIP1/WAF1, gas1, cyclinD1, *gadd34*, *gadd45*, *gadd153* and c-Myc itself (reviewed in Claassen and Hann, 1999). Repression is generated, either through initiator elements in the genes basal promoter (Li *et al*., 1994), or indirectly by sequestration (Peukert *et al*., 1997; Shrivastava *et al*., 1993). The different Myc:Max responsive genes were generally identified through expression of exogenous protein and hence critical physiological targets remain unclear. For example, c-Myc null cells misregulate cad and *gadd45*, but no other proposed targets (Bush *et al*., 1998).

On the other hand, Max association to Mad1, Mxi1, Mad3, Mad4 or to Rox/Mnt was shown to block Myc-Ras transformation (Hurlin *et al*., 1995b, 1997; Schreiber Agus *et al*., 1995). It seems likely that this effect is mediated through repression of the genes normally transactivated by the Myc:Max complex. The Mad:Max and Rox:Max complexes were shown to bind E-box DNA sequences and the mSIN3 corepressors, and to repress transcription, at least in part, by tethering histone deacetylases containing complexes to the DNA (Alland *et al*., 1997; Ayer *et al*., 1993; Hassig *et al*., 1997; Heinzel *et al*., 1997; Hurlin *et al*., 1995b, 1997; Laherty *et al*., 1997; Meroni *et al*., 1997; Nagy *et al*., 1997; Schreiber Agus *et al*., 1995; Zhang *et al*., 1997b).

What is the function of the Mlx protein with regard to these highly dynamic processes? It is apparent that Mlx can substitute Max to allow the Mad1, Mad4 and Rox proteins to bind DNA. This might either promote a further level of control over the Max network by regulation of the same target genes, or it may be the tip of the iceberg of a new network of genes required for progression through S phase and/or differentiation. Mlx is expressed broadly like Max, c-Myc, L-Myc, N-Myc, Mad1, Mxi1, Mad3, Mad4 and Rox/Mnt (Ayer *et al*., 1993; Hurlin *et al*., 1995a,b, 1997; Larsson *et al*., 1994; Meroni *et al*., 1997; Zervos *et al*., 1993), but their respective temporal patterns of expression are extremely different. If Max steady state level is constant through the cell cycle (Blackwood *et al*., 1992), Rox is expressed in G0 in WI-38 cells (Meroni *et al*., 1997), while Myc and Mlx expressions peak at the G0-G1 and G1-S transitions, respectively, in these cells (Meroni *et al*., 1997) (this work). Finally Mad3, Mxi1, Mad4 and Mad1 are sequentially induced during differentiation (Queva *et al*., 1998). Hence Mlx might be important in the sequence of events leading to commitment to the cell cycle and the beginning of S phase. First, the repression program is lifted at target sites of Max:Rox complexes, when expression of Myc favors the formation of Myc:Max dimers. Then unbound Rox might be recruited by Mlx to control the expression of other genes and/or to prevent undestroyed Rox from antagonizing Myc function and cell proliferation.

On the other hand studies of E-box DNA binding complexes have revealed that Mad1:Max heterodimers were unlikely to be essential or determining for myeloid differentiation (Ryan and Birnie, 1997). As Mlx interacts with Mad1 and Mad4 (Billin *et al*., 1999), which are expressed late in the differentiation process (Queva *et al*., 1998), we can hypothesize that differentiation of HL60 cells is dependent of Mad1:Mlx heterocomplexes. Future studies will confirm or infirm this possibility. However, if this is the case Mlx might be important for differentiation of specific cell types.

It is even possible that Mlx exert a partially redundant function with Max in specific cell types and/or cellular differentiation stages. For example, the rat nerve growth factor-responsive PC12 cells express a mutant form of Max unable to dimerize. They therefore divide and apoptose through Max/Myc independent pathways (Hopewell and Ziff, 1995). Interestingly enough these particular cells express the rat homolog of Mlx (clone RPCCD51, accession H32587, (Lee *et al*., 1995)), suggesting that PC12 cells are dividing through a Mlx pathway. We are currently investigating this possibility.

Another question concerns the functional role of the Mlx:Mlx homodimers. Like the Max protein, but unlike any other members of the Myc network, Mlx readily homodimerizes at low concentration and binds CACGTG binding sites in EMSA. It has been proposed that the Max:Max homodimers are 'transient states' that exist to accelerate transition from the Myc:Max to the Mad:Max complexes (Yin *et al*., 1998). We can postulate a similar role for the Mlx:Mlx complexes, as we have identified additional Mlx-containing heterodimers (S Cairo, G Merla, A Reymond, in preparation). However, we cannot

exclude that occupation of the target genes by the Mlx:Mlx or the Max:Max homodimers has no physiological function.

Finally, we can speculate a possible role of Mlx in tumorigenesis. Myc family members are activated in a large number of neoplasias (reviewed in Nesbit *et al*., 1999). Consistently, its antagonist, Mxi1, was shown to be a tumor suppressor both in human and rodents (Eagle *et al*., 1995; Li *et al*., 1999; Schreiber Agus *et al*., 1998). Tantalizingly enough, Mlx maps to the gene rich region of 17q21, centromeric to the BRCA1 gene (Friedman *et al*., 1994, 1995). This chromosomal region frequently presents loss of heterozygosity in ovarian (75%), breast (50%) and prostate (40%) cancers (reviewed in Aita *et al*., 1999). BRCA1 is implicated in familial, but rarely in sporadic cases of breast and ovarian cancer (Futreal *et al*., 1994; Miki, 1994). Moreover, sporadic ovarian cancer tumors with allelic deletion of 17q21 region but no BRCA1 mutation were isolated. These findings led to the hypothesis that at least one other tumor suppressor gene, in addition to BRCA1, maps to 17q21 (Vogelstein and Kinzler, 1994). Interestingly, a 400 kb common deletion unit centromeric to BRCA1 and containing the Mlx gene was identified in sporadic ovarian cancer (Tangir *et al*., 1996). We are currently performing mutation analysis to evaluate if the Mlx gene is mutated in these types of tumors.

In conclusion, we have extended the known dimerization partners of Mlx, a new bHLHZip protein, by demonstrating its ability to bind Rox and to homodimerize. Mlx, like Max, is positioned at the center of a network of transcription factors. The importance of this network is emphasized by its conservation through evolution, as we cloned both the Mlx and the Rox fruitfly homologs (see the Results section and E Zanaria and G Meroni, unpublished data). Other members of the Myc/Max/Mad bHLHZip family were previously identified in invertebrates and their biological and biochemical properties were shown to be identical to that of their vertebrate counterparts (Gallant *et al*., 1996; Schreiber Agus *et al*., 1997; Walker *et al*., 1992; Yuan *et al*., 1998).

Materials and methods

Yeast two-hybrid screen and cloning of Mlx

The yeast two-hybrid screen was performed as described in (Zervos *et al*., 1993). Briefly, 1.2 Mio clones of a HeLa cDNA JG4-5 library were screened for interaction with the last 523 residues of the Rox protein (Gyuris *et al*., 1993; Meroni *et al*., 1997). The prey plasmids express the cDNA directionally fused to the B42 activation domain, the SV40 T NLS and a HA tag under the control of the GAL1. Interaction-mating techniques were used to reconfirm interaction after isolation of two-hybrid positive clone (Reymond and Brent, 1995).

The Mlx-β and -γ point mutants used in this manuscript were generated using the Quick-Change mutagenesis kit (Stratagene) and appropriate oligonucleotides.

Co-immunoprecipitation and antibody production

In vitro and *in vivo* co-immunoprecipitations have been previously described (Meroni *et al*., 1997; Reymond and Brent, 1995). The last 88 residues of Mlx fused to GST were expressed in bacteria and purified as published (Reymond and Brent, 1995). Two rabbits were injected intramuscularly at monthly intervals to generate Mlx antisera.

Bandshift assays

The binding reaction was achieved as described in (Meroni *et al*., 1997) using 1 ng of end-labeled probe (5′-GGAAGCA-GACCACGTGGTCTGCTTCC-3′). When specified, unlabeled probe or specific competitors were added at the same time as the labeled probe. Specific sequences of competitors are described in detail in (Meroni *et al*., 1997). For supershift experiments, 1 μg of purified anti-FLAG M2 antibody (Eastman Kodak), 1 μg of purified anti-HA 12CA5 (Boehringer Mannheim), 5 μg of purified anti-Max C-17, 5 μg of purified anti-Max C-17 or C-124 (Santa Cruz Biotechnology) or 5 μg of immune or preimmune anti-Mlx antisera were added to the reaction mix after protein-DNA complex formation.

Indirect immunofluorescence, transfection and transactivation experiments

Indirect immunostaining was performed on paraformaldehyde-fixed cells 3 days posttransfection as explained in (Reymond *et al*., 1999). 12CA5 anti-HA mAb or anti-Mlx anti-sera were used. HEK293 and Hela cells were transfected by calcium phosphate and COS-7 with lipofectamine (Gibco BRL).

For the transactivation experiments 1.5×10^5 cells/35 mm plate were transfected with 1 μg of pTK81 4x[CACGTG]-luciferase reporter (Meroni *et al*., 1997), 1 μg of the expression vector(s) (pCDNA3 modified to include an HA-Tag, Invitrogen) and 100 ng of pCH110 (SV40 promoter driven β-Galactosidase; Clontech) to monitor transfection efficiency. Luciferase and β-Galactosidase were assayed according to the manufacturer's instructions (Promega) 72 h post-transfection. Transactivation assays were performed in triplicate with and without TSA and repeated at least five times.

Expression studies

Human multiple tissues Northern blots (Clontech) were hybridized with clone 692 following the manufacturer's recommendation. Mouse embryo sections were hybridized with ^{35}S-UTP-labeled IMAGE clone 520937 riboprobes as described (Reymond *et al*., 1999). Specimens were viewed and photographed using Hoechst epifluorescence optics combined with darkfield illumination provided by a red light source. Whole-mount *in situ* hybridization of mouse embryos was performed as published in (Riddle *et al*., 1993) using digoxigenin-labeled IMAGE clone 520937 riboprobes. WI-38 human embryo lung fibroblasts were cultured at early passages in DMEM/10% FCS and arrested for 3 days in media with 5% BCS. They were stimulated to re-enter the cell cycle by addition of 20% FCS. RNA was isolated from synchronized cells as described (Chomczynsky and Sacchi, 1987).

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