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Mga, a dual-specificity transcription factor that interacts with Max and contains a T-domain DNA-binding motif

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The basic-helix-loop-helix-leucine zipper (bHLHZip) proteins Myc, Mad and Mnt are part of a transcription activation/repression system involved in the regulation of cell proliferation. The function of these proteins as transcription factors is mediated by heterodimerization with the small bHLHZip protein Max, which is required for their specific DNA binding to E-box sequences. We have identified a novel Max-interacting protein, Mga, which contains a Myc-like bHLHZip motif, but otherwise shows no relationship with Myc or other Max-interacting proteins. Like Myc, Mad and Mnt proteins, Mga requires heterodimerization with Max for binding to the preferred Myc–Max-binding site CACGTG. In addition to the bHLHZip domain, Mga contains a second DNA-binding domain: the T-box or T-domain. The T-domain is a highly conserved DNA-binding motif originally defined in Brachyury and characteristic of the Tbx family of transcription factors. Mga binds the preferred Brachyury-binding sequence and represses transcription of reporter genes containing promoter-proximal Brachyury-binding sites. Surprisingly, Mga is converted to a transcription activator of both Myc–Max and Brachyury site-containing reporters in a Max-dependent manner. Our results suggest that Mga functions as a dual-specificity transcription factor that regulates the expression of both Max-network and T-box family target genes.
Keywords: Brachyury/Max/mesoderm/Mga/Tbx

Introduction

The Myc family of transcription factors plays an important role in the regulation of cell proliferation and differentiation, apoptosis, and embryonic development (Henrikson and Lüscher, 1996). Myc proteins are members of a group of basic-helix-loop-helix-leucine zipper (bHLHZip) transcription factors that require heterodimerization with another bHLHZip protein, Max, for specific DNA binding and transcriptional regulation at E-box sequences. In addition to Myc proteins, Max heterodimerizes with Mad family proteins and the related protein Mnt/Rox (Ayer *et al.*, 1993; Zervos *et al.*, 1993; Hurlin *et al.*, 1995, 1997; Meroni *et al.*, 1997). Transcription assays using reporter genes containing promoter-proximal E-box sites show that Myc–Max complexes activate transcription (Amati *et al.*, 1992; Kretzner *et al.*, 1992) and Mad–Max and Mnt/Rox–Max complexes repress transcription (Ayer *et al.*, 1993; Hurlin *et al.*, 1995, 1997, Meroni *et al.*, 1997). For both Mad and Mnt/Rox proteins, transcriptional repression is dependent on a small N-terminal motif that interacts with corepressor proteins mSin3A and mSin3B related to the yeast protein Sin3p (Ayer *et al.*, 1995; Hurlin *et al.*, 1995, 1997; Schreiber-Agus *et al.*, 1995; Meroni *et al.*, 1997). The mSin3-interaction domain (SID) in the Mad and Mnt/Rox proteins and its associated transcription repression function are required for biological activity of these proteins, as measured by their ability to suppress Myc-dependent cell transformation and proliferation (Koskinen *et al.*, 1995; Schreiber-Agus *et al.*, 1995; Roussel *et al.*, 1996; Hurlin *et al.*, 1997). Taken together, these results suggest that Max-interacting proteins function as a transcription activation/repression system that regulates a common or overlapping set of target genes involved in controlling cell proliferation.

Like the bHLHZip domain, the T-domain is a highly conserved DNA-binding and dimerization motif (Kispert and Herrmann, 1993; Müller and Herrmann, 1997). The T-domain was first identified in the protein Brachyury, a transcription factor that plays an essential role in posterior mesoderm induction and is mutated in the mouse mutant, tailless (Herrmann and Kispert, 1994). A family of proteins has now been identified that contain the T-domain, and is known collectively as the Tbx family. Several members of the Tbx family, like Brachyury, play important roles in the specification, induction and differentiation of mesoderm during embryonic development (Papaioannou and Silver, 1998). For example, inactivation of *Tbx6* by gene targeting in the mouse revealed an essential role in paraxial mesoderm induction during development (Chapman and Papaioannou, 1998) and mutations in *Tbx3* and *Tbx5* are responsible for human disease syndromes that affect limb and other organ systems in which mesoderm induction and/or mesoderm–epithelial interactions are impaired (Bamshad *et al.*, 1997; Basson *et al.*, 1997; Li *et al.*, 1997; Horb and Thomsen, 1999). It has also been recently demonstrated that the T-domain protein VegT is required to establish the earliest stages of germ layer specification in *Xenopus* (Zhang *et al.*, 1998). Thus, T-domain-containing proteins are thought to act as master regulators, directing the transcription of genes essential for a variety of embryonic inductive events.

In this study, we report the identification and charac-

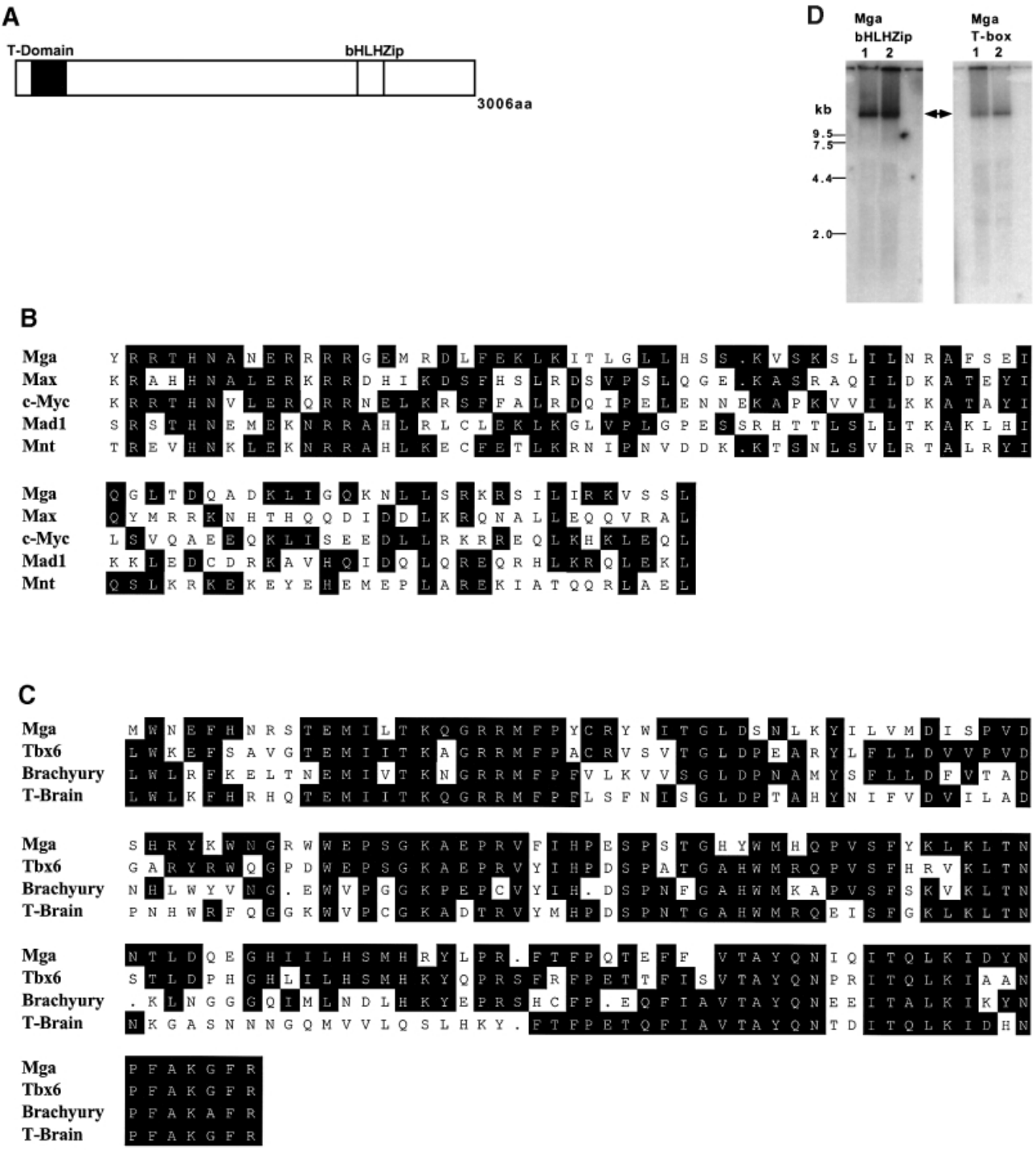

Fig. 1. (**A**) Schematic diagram of Mga. (**B**) Comparison of the murine Mga bHLHZip domain (aa 2365–2443) with those of Max and Max-interacting proteins. (**C**) Comparison of the Mga T-domain (aa 84–259) with several T-domain-containing proteins. Amino acids outlined in black indicate identical positions and serve to highlight conserved positions in distinct murine T-domain proteins. (**D**) Northern Blot analysis of duplicate blots of total RNA prepared from PC12 cells, using probes from either the Mga T-box region or the bHLHZip region as indicated. Lanes 1 and 2 were loaded with 40 and 20 μg, respectively. The T-box and bHLHZip probes hybridize to an ~14 kb RNA (arrows).

terization of a novel Max-interacting protein, Mga, which contains not only a Myc-like bHLHZip domain, but also a T-domain DNA-binding motif. Our data suggest that Mga functions as a dual-specificity transcription factor capable of regulating the expression of both Max-network and T-domain target genes.

Results

Identification of Mga

To identify proteins that interact with Max, a yeast two-hybrid assay was used to screen a mouse embryonic day (e)9.5 and e10.5 cDNA library. Four proteins that contained a bHLHZip region related to the bHLHZip domain of

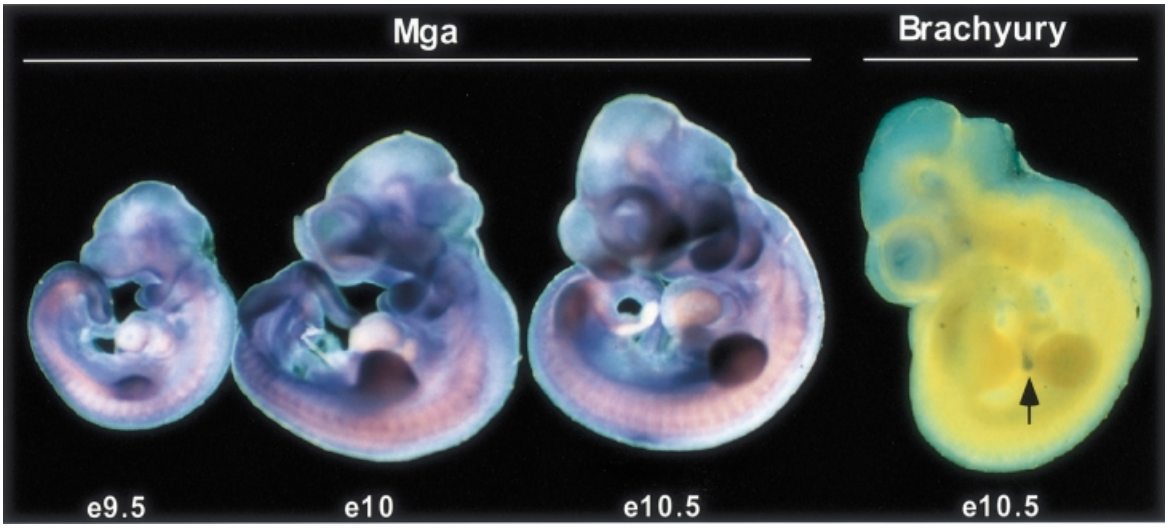

Fig. 2. Whole-mount *in situ* hybridization analysis of Mga expression in the mouse at embryonic day (e) 9.5, e10 and e10.5. Brachyury expression at e10.5, which is limited to the distal tip of the tail (arrow) at this stage, was used as a control. Mga expression is widespread, but with highest levels in the limb buds and branchial arches.

Max, Myc or Mad were identified in this screen. The cloning and characterization of three of these proteins have been described previously (Hurlin *et al.*, 1995, 1997). The characterization of the fourth of these proteins, Mga, is presented here. From the Max yeast two-hybrid screen, several interacting clones were identified that contained cDNAs coding for the same novel bHLHZip DNA-binding, dimerization domain. To isolate a full-length cDNA, the region obtained in the two-hybrid screen was initially used to screen a variety of murine and human cDNA libraries. cDNAs containing an identical bHLHZip region were found using a murine e14.5 kidney library (a generous gift of V.Dixit). An apparent full-length sequence was constructed using several overlapping cDNA clones obtained from sequential screening of the embryonic kidney library. This cDNA, containing an open reading frame (ORF) of 9018 nucleotides and coding for a protein of 3006 amino acids (aa), was named Mga (DDBJ/EMBL/GenBank accession No. AF205935). The cDNA contained a start codon in good context, preceded by numerous stop codons in all three reading frames. A stop codon followed by a polyadenylation signal and sequence indicated the 3′ end of the cDNA. Database comparisons of the full- length Mga sequence revealed not only a bHLHZip region most closely related to c-Myc (Figure 1B; 39% identical to c-Myc), but also strong homology between an N-terminal region in the Mga protein and the T-box DNA-binding domain of the Tbx family of proteins (Figure 1C). An alignment of the Mga T-domain with several Tbx family members, including Tbx6, which it is most similar to (55% identical in the T-domain), is shown in Figure 1C. Mga showed little or no homology with Tbx proteins outside the T-box or with Myc proteins (and related bHLHZip proteins) outside the bHLHZip domain.

After surveying RNA from a number of different cell lines and adult tissues by Northern blot analysis using a Mga bHLHZip probe, the only positive samples found were the rat pheochromocytoma cell line PC12 (Figure 1D) and C2C12 myoblasts (H.Carlson and P.J.Hurlin, unpublished) with each showing a hybridizing signal at ~14 kb. Using duplicate Northern blots prepared from PC12 RNA, we found that separate probes specific for either the Mga T-box region or the bHLHZip region gave a hybridization signal at apparently identical positions (Figure 1D). The size of the mRNA detected by both probes is consistent with the ~14 kb Mga cDNA obtained in library screens (not shown). In addition to these data, we found that the T-box and bHLHZip regions map to the same chromosomal position (Figure 6), providing further evidence that both domains are encoded within a single ORF.

The embryonic expression pattern of Mga in the mouse was examined by whole-mount *in situ* hybridization at e9.5, e10 and e10.5 using probes specific for the bHLHZip (Figure 2) and T-box regions (not shown). Apparently identical patterns of expression were obtained using these two probes (not shown). The general pattern of Mga expression was similar during the embryonic stages examined with highest expression levels in the limb buds, branchial arches and the tail region (Figure 2). In contrast to Mga, Brachyury expression at e10.5 was limited to the distal tip of the tail (Figure 2) as demonstrated previously (Kispert *et al.*, 1995). Notably, the expression pattern of Mga appears to overlap with many other T-box-containing genes, including Brachyury, Tbx2, 3, 4 and 5 (Papaionnou and Silver, 1998). These results raise the possibility that Mga may participate in regulatory pathways controlling mesoderm induction, possibly in concert with other T-domain proteins.

Interaction with Max

To initially confirm the yeast two-hybrid results, *in vitro* co-immunoprecipitation experiments were performed. The yeast two-hybrid library plasmid containing the Mga bHLHZip region was used to produce *in vitro* translated, [^{32}S]methionine-labeled VP16–Mga fusion protein. This protein was incubated with baculovirus-produced Max and immunoprecipitated with Max antisera. The Max antisera efficiently co-immunoprecipitated the VP16–Mga fusion protein in the presence but not the absence of added

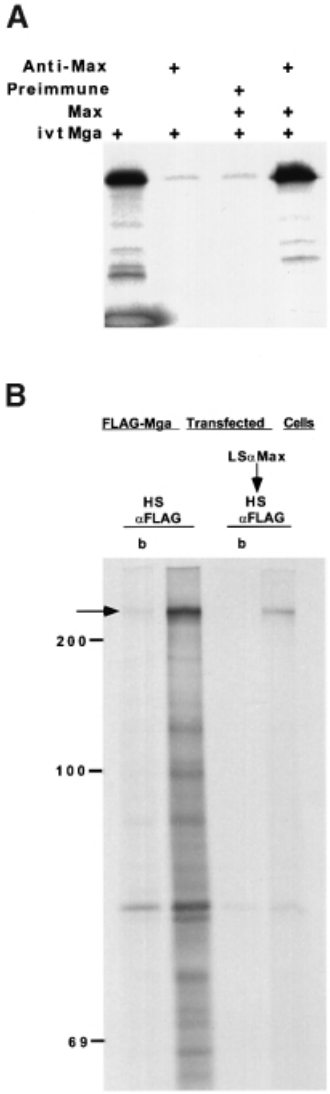

Fig. 3. Mga interacts with Max *in vitro* and *in vivo*. (**A**) The Mga bHLHZip region fused to VP16 was *in vitro* translated (ivt Mga) and interaction with baculovirus-produced Max was tested by performing immunoprecipitations with either Max immune or Max preimmune serum as indicated. (**B**) HEK 293T cells were transfected with pMEFLAG-Mga expressing a FLAG-tagged Mga protein. To identify the expressed FLAG-Mga protein, transfected cells were immunoprecipitated under high stringency (HS) conditions with a FLAG monoclonal antibody or the same antibody blocked (b) by pre-incubation with a FLAG peptide. To test for co-immunoprecipitation between Mga and Max, endogenous Max was immunoprecipitated under low stringency (LS) conditions, the immunoprecipitates were washed in the same LS buffer and then incubated in high stringency buffer to release proteins bound to Max. High stringency eluates were immunoprecipitated with anti-FLAG antibody or immunogen blocked (b) anti-FLAG antibody. The location of FLAG-Mga is indicated with an arrow.

Max (Figure 3A). Additional *in vitro* studies investigating potential interaction between Mga and Myc, Mad and Mnt proteins were negative (data not shown).

To investigate the interaction between Mga and Max *in vivo*, co-immunoprecipitation experiments were carried out in HEK 293 cells transfected with a Flag-tagged version of the full-length Mga cDNA. Transfected cells were metabolically labeled with [^{35}S]methionine and cell extracts were immunoprecipitated with Max antisera or Max preimmune sera under low stringency conditions (Hurlin *et al.*, 1997). The low stringency immunoprecipitations were washed three times with low stringency buffer and co-immunoprecipitated proteins eluted by incubation in high stringency buffer. The eluted proteins were subjected to a second immunoprecipitation with an anti-Flag antibody and analyzed by PAGE (Figure 3B). A high stringency immunoprecipitation of Flag-Mga transfected cell extracts with anti-Flag antibody or immunogen blocked anti-Flag antibody was run on the same gel (Figure 3B). A large protein was detected in the low stringency Max immunoprecipitate that co-migrated with the Flag-Mga protein immunoprecipitated under high stringency conditions (Figure 3B). These results show that overexpressed Mga interacts with Max and strongly suggest that Max and Mga associate *in vivo*.

DNA-binding and transcriptional activities of Mga

The DNA-binding properties of the bHLHZip and T-domain regions of Mga were investigated by electrophoretic mobility shift assays (EMSA) employing either oligonucleotides containing the preferred Myc–Max bHLHZip-binding sequence CACGTG or the preferred Brachyury recognition sequence AATTTCACACCTAGGTGTGAAATT (Kispert and Herrmann, 1993). For binding to CACGTG, a glutathione *S*-transferase (GST) fusion protein containing the Mga bHLHZip domain (aa 2347–2479) or a control GST fusion protein containing a Mga C-terminal region (aa 2771–1940) showing no homology to any known proteins was used. As shown in Figure 4A, the Mga bHLHZip domain alone failed to bind CACGTG, but produced a strong, concentration-dependent band shift when pre-incubated with baculovirus-produced Max.

To test Mga T-domain binding to the preferred Brachyury-binding sequence, the plasmid pCDNAMgaX1-Pst was used to produce an *in vitro* translated N-terminal fragment of Mga containing the T-domain region (N-terminal 568 aa). Mga binding was compared with that of *in vitro* translated Brachyury containing an HA tag (made from plasmid pCS2-Bra5′Ha). Mga bound the Brachyury site (Figure 4B) and both Mga and Brachyury band shifts were supershifted using antisera raised against the N-terminus of Mga or anti-HA antibody, respectively, but not by control antibodies (anti-Mga C-terminus and anti-FLAG, respectively).

Based on these DNA-binding properties, the transcriptional activities of Mga were tested using either an empty reporter plasmid [pGL2pro (Promega)] or ones containing either four promoter-proximal Myc–Max (CACGTG)-binding sites (pGL2M4; Ayer *et al.*, 1995), two Brachyury (T)-binding sites (pGL2T2) or both Brachyury and Myc–Max sites (pGL2T2M4). When transfected into HEK 293 cells, the activity of the empty reporter (pGL2pro) was very low relative to the other reporter plasmids and was not affected by co-transfection of any of the expression plasmids used in this study (data not shown), which include ones expressing Mga, Brachyury, Max and ΔBRMax. ΔBRMax lacks its DNA-binding basic region and functions as a dominant negative by forming heterodimers incapable of binding DNA Myc–Max target sites (Ayer *et al.*, 1993). Consistent with a previous study (Kispert *et al.*, 1995), Brachyury activated transcription of reporters containing Brachyury sites, but

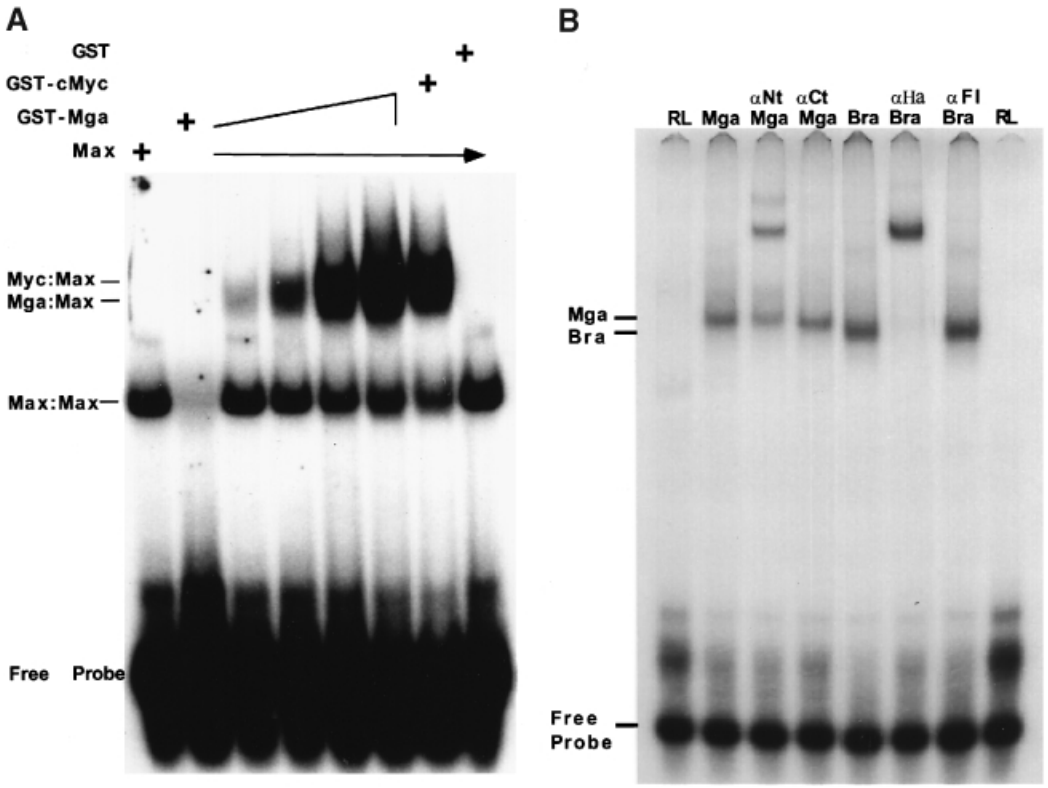

Fig. 4. DNA binding by the Mga bHLHZip and T-domain regions. (**A**) DNA binding by the Mga bHLHZip region to an oligonucleotide containing the preferred Myc–Max-binding site CACGTG was tested using a purified GST fusion protein containing the Mga bHLHZip domain. The Mga bHLHZip fusion protein was incubated with the oligonucleotide in the presence and absence of baculovirus-produced Max and with increasing amounts of Mga fusion protein in the presence of a constant amount of Max (sloped line). A GST fusion protein containing the C-terminal 96 aa of the c-MYC protein (GST–cMyc) was used as a positive control. (**B**) Binding of the Mga T-domain to the preferred Brachyury-binding site AATTTCACACCTAGGTGTGAAATT was tested using an *in vitro* transcribed and translated N-terminal fragment (568 aa) of Mga containing the entire T-domain. For a positive control, *in vitro* transcribed and translated Brachyury containing an HA tag was used. Supershift analysis of Mga complexes was performed using antiserum raised against the N-terminal 268 aa of Mga (αNt) or a C-terminal portion of Mga (αCt). For supershift analysis of Brachyury, anti-HA or anti-FLAG (negative control) antibodies were used.

no activity was observed when it was co-transfected with the pGLM4 reporter containing only proximal CACGTG sites (data not shown). In contrast, transfection of our full-length Mga cDNA repressed reporter transcription from the pGLT2 and pGLT2M4 plasmids, but showed little activity on the pGL2M4 reporter (Figure 5). Thus, repression by Mga was enhanced or dependent on the presence of T-domain-binding sites. Surprisingly, co-transfection of Max with Mga resulted in transcriptional activation of each of the reporters. To determine whether the observed activation was dependent on E-box DNA binding by Mga, ΔBRMax was co-transfected with Mga. ΔBRMax relieved activation of the pGL2M4 promoter (Figure 5A) and partially relieved activation of the pGL2M4T2 reporter (Figure 5C), consistent with the idea that Max-mediated E-box binding by Mga contributes to its activation function on promoters containing Myc–Max sites. However, co-transfection of ΔBRMax functionally substituted for Max in mediating activation of the pGL2T2 reporter (Figure 5B), indicating that Max binding to Mga also has a function independent of its role in DNA binding.

To investigate more rigorously the unexpected ability of Max and ΔBRMax to mediate transcriptional activation by Mga on the pGL2T2 reporter, a series of titration experiments was performed. Using constant amounts of transfected Mga, increasing amounts of Max or ΔBRMax were co-transfected along with the pGL2T2 reporter (Figure 6). A dose-dependent inhibition of Mga repression and a switch to activation were observed. Since neither Max nor ΔBRMax alone had little effect on this reporter, we conclude that Max binding to Mga is necessary for the transcription activation observed. Therefore, we conclude that Max binding to Mga confers a DNA-binding-dependent function (on promoters containing E-box sites) and a DNA-binding-independent function (on promoters containing Brachyury-binding sites). Although complicated, our results indicate that Mga is capable of regulating transcription from promoters containing either Brachyury or Myc–Max-binding sites and suggest that cellular Max levels dictate whether it functions as a repressor or an activator.

Mga inhibits Myc-dependent cell transformation

The DNA-binding and transcriptional activities of Mga suggest that it may impinge on the function of other Max-interacting proteins. We used the Myc+Ras co-transformation assay (Land *et al.*, 1983) as a system to determine whether Mga expression influences the oncogenic activities of Myc. Transfections of primary rat fibroblasts with Myc and Ras expression plasmids were performed with or without the addition of Mga expression plasmid and foci of transformed cells were counted after 2 weeks in cell culture (Figure 7A). In the absence of Mga, transfection of Myc+Ras resulted in the development

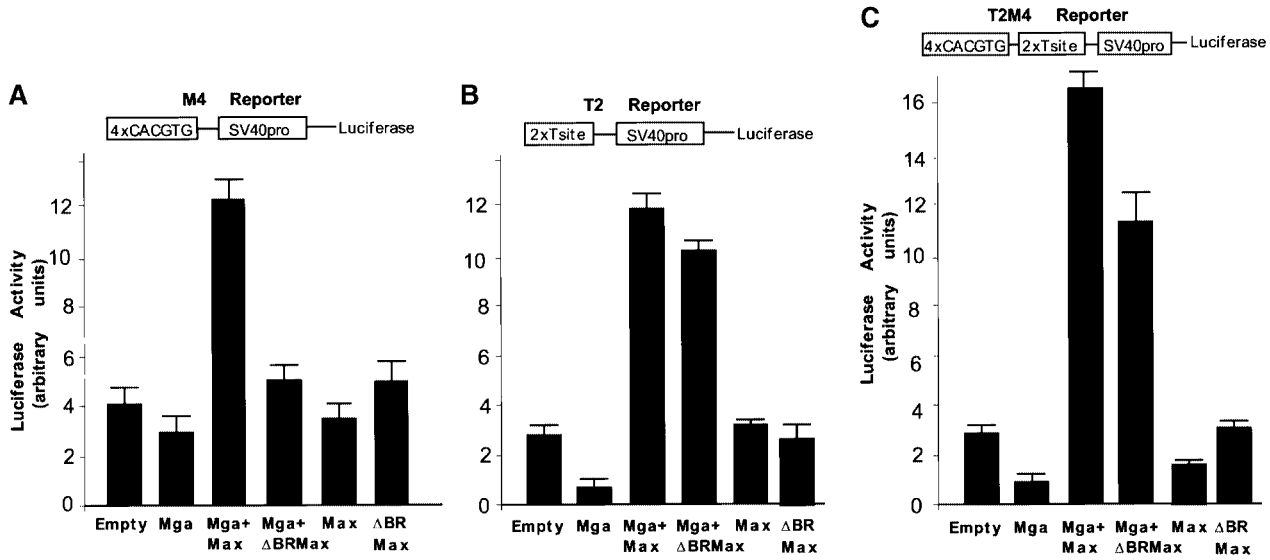

Fig. 5. Transcriptional activities of Mga. The indicated cDNAs in the vector pCS2 were transfected into HEK 293 cells together with the reporter pGLM4 (**A**) containing four promoter-proximal Myc–Max-binding sites, the reporter pGLT2 (**B**) containing two promoter-proximal Brachyury-binding sites (Kispert and Herrmann, 1993), or the reporter pGLM4T2 (**C**) containing four Myc–Max and two Brachyury sites at the same positions as in the pGLM4 and pGLT2 reporters, respectively. The total amount of DNA transfected in each assay was equalized using empty vector (pCS2). Luciferase activity was normalized to β-galactosidase activity produced by a co-transfected plasmid, pCMVβGAL, and measured using the luminescent substrate Galacton Plus (Tropix). Transfections were performed in triplicate and repeated at least two times. Results are represented as mean ± SEM.

of numerous foci. In the presence of transfected Mga, a dramatic reduction in the number of foci in each of three separate experiments was seen. An expression plasmid containing the N-terminal 758 aa of Mga (pCDNA-MgaA2) and thus containing the T-domain and lacking the bHLHZip, failed to reduce the number of foci produced by Myc and Ras. Likewise, co-transfection of Brachyury also failed to reduce the number of foci produced by Myc and Ras. Therefore, these results suggest that the T-domain region of Mga is insufficient for inhibition of Myc/Ras co-transformation and that regions C-terminal (including the bHLHZip region) are required.

The results of our cell transformation assays suggested that the mechanism of suppression by Mga might involve interference with the transcription activation of Myc target genes. We tested this posibility by performing transcription assays in which increasing amounts of Mga expression plasmid were transfected in the presence of a constant amount of transfected c-Myc expression plasmid (Figure 7B). Whereas Myc alone activated transcription 6-fold, co-transfection of Mga resulted in a dose-dependent suppression of activation. We note that suppression of Myc activation by Mga appears less potent than that caused by Mad proteins and Mnt, which also both efficiently suppress Myc-dependent transformation (Hurlin *et al.*, 1995, 1997).

Chromosomal location of Mga

The mouse chromosomal location of Mga was determined using separate cDNA probes specific for either the bHLHZip region or the T-domain of Mga. Interspecies backcross analysis was performed using progeny derived from matings of [(C57BL/6J × *M.spretus*)F1 × C57BL/6J] mice (see Methods in Copeland and Jenkins, 1991). The Mga bHLHZip and T-domain probes localized to the same chromosomal position at the central region of chromosome 2 (Figure 8). This position did not recombine with Epb4.2 in 161 animals typed in common, suggesting that these loci are within 1.9 cM of each other (upper 95% confidence limit).

The central region of chromosome 2 shares a region of homology with human chromosome 15q. In particular, the tight linkage between Mga and Epb4.2 suggests that human MGA lies on human chromosome 15q15. The location of Mga at human chromosome 15q15 was confirmed using fluorescent *in situ* hybridization (K.Sushia and C.Disteche, unpublished). Although there have been several reports of chromosomal translocations or deletions occurring at human 15q15 (Hunger *et al.*, 1993; D'Alessandro *et al.*, 1994), no common disease syndromes linked to unidentified genes appear to map to this position.

Discussion

The Myc, Mad and Mnt bHLHZip proteins possess intrinsic transcriptional activities, but require heterodimerization with the small bHLHZip protein Max for specific DNA binding to E-box sites. Thus, Max functions as an obligate partner for members of a transcription factor network that participate in regulating cell proliferation. We show here that Max interacts with another bHLHZip protein, Mga, which also requires heterodimerization with Max for E-box binding. The Mga bHLHZip region falls within a subclass of bHLHZip proteins that includes Max, Myc, Mad and Mnt proteins. However, Mga is distinctly different from these proteins in that it contains a second highly conserved DNA-binding domain, the T-domain. Although the presence of two DNA-binding domains in the same molecule is not unprecedented [for example several members of the Pax family of transcription factors contain both homeobox and paired-box DNA-binding

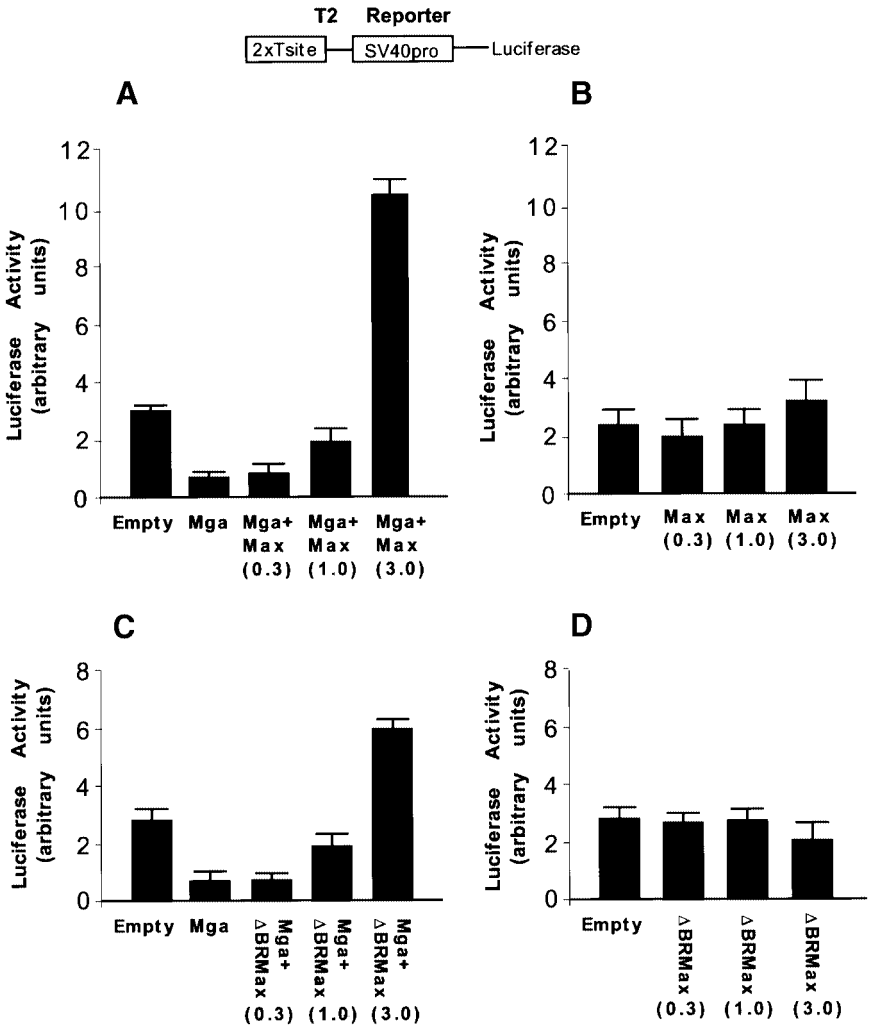

Fig. 6. E-box-independent activities of Max on Mga function. (**A** and **C**) Titrations of increasing amounts (μg shown in parentheses) of transfected Max or ΔBRMax plasmids were performed in the presence of a constant amount of transfected Mga plasmid (5 μg). (**B** and **D**) Titrations of increasing amounts of transfected Max or ΔBRMax plasmids were also carried out in the absence of transfected Mga to measure their Mga-independent activities. Experiments were carried out in triplicate and performed twice. Results are represented as mean ± SEM.

domains (Stuart *et al.*, 1994)], it is nonetheless highly unusual. In an attempt to determine whether the Mga cDNA was an artifact of cloning, both the T-domain and the bHLHZip regions were used as probes to identify chromosomal position (Figure 6), mRNA transcript size, and tissue and embryonic expression patterns (Figures 1 and 2; data not shown). In each situation, apparently identical results were achieved with the separate probes, strongly suggesting that the cloned Mga cDNA represents a naturally occurring gene product. Interestingly, sequencing of the mouse Mga genomic T-box region revealed that Mga does not contain the highly conserved intron–exon structure characteristic of T-box-containing genes (data not shown). Instead, the Mga T-box is contained on a single exon, raising the possibility that the Mga gene evolved from the insertion of a reverse transcribed mRNA of a T-box gene into a gene coding for a Max-interacting bHLHZip protein.

The presence of both a T-domain and a bHLHZip in Mga suggests that its transcriptional and biological activities will be more complex than other members of the bHLHZip and T-domain families. Indeed, the ability of Mga–Max heterodimers to bind to the preferred Myc–Max- and Brachyury-binding sites (Figure 4) raises the possibility that Mga may regulate the same, or an overlapping set of genes regulated by Myc–Max heterodimers, Brachyury and their related proteins. Although we find that Mga exhibits very little net transcriptional activity alone on a reporter plasmid containing only promoter-proximal CACGTG-binding sites (Figure 5A), our results demonstrating that Mga suppresses transcription activation by c-Myc and inhibits Myc-dependent cell transformation (Figure 7) are consistent with the theory that Mga regulates Myc–Max target genes *in vivo*. Our studies of the transcriptional activities of Mga using promoters engineered to contain proximal Brachyury sites and/or Myc–Max sites indicate that Mga has similar and distinct binding site-dependent activities and that it can function as both a transcriptional repressor and activator (Figures 5 and 6). Whereas the transcriptional repression function of Mga appears to be dependent on the presence of Brachyury-binding sites, transcriptional activation was seen with reporters containing either Brachyury- or Myc–Max-binding sites and was dependent on transfected Max (Figures 5 and 6). Therefore, our results indicate that Mga–Max heterodimers mediate the activation function

A

Transfected Plasmid	Number of Foci Exp.1	Exp.2	Exp.3
Myc	0	-	-
Ras	0	-	-
Myc+Ras	46	25	32
Mga	0	0	0
Mga+Myc+Ras	6	7	5
Mga+Ras	0	-	-
Mga+Myc	0	-	-
Brachyury	-	0	-
Brachyury+Myc+Ras	-	28	-
Brachyury+Ras	-	0	-
MgaA2*	-	-	0
Myc+Ras+MgaA2	-	-	36

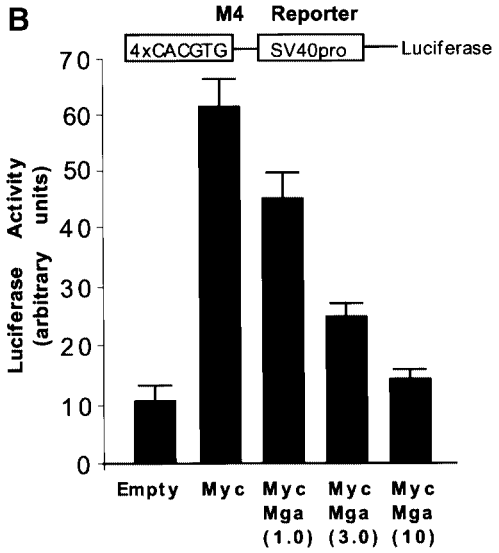

Fig. 7. Suppression of Myc transcription activation and inhibition of Myc-dependent cell transformation by Mga. (**A**) Primary rat embryo fibroblasts were transfected with expression plasmids containing the indicated cDNAs and maintained in medium containing 5% fetal bovine serum for 2 weeks. The total number of foci produced is indicated for each of three independent experiments. MgaA2* is a deletion mutant of Mga containing the N-terminal 758 aa of Mga, which includes the T-domain. (**B**) Transcription assays using the pGL2M4 reporter plasmid co-transfected with a constant amount of c-Myc plasmid and with increasing amounts of Mga (μg shown in parentheses). Results are represented as mean ± SEM.

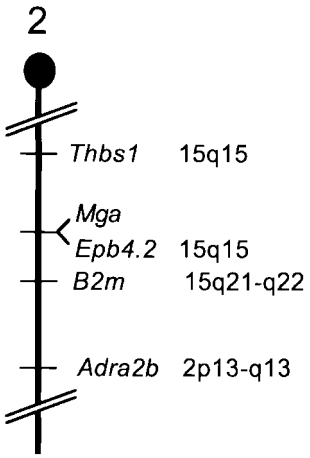

Fig. 8. The *Mga* murine chromosomal position. A partial chromosome linkage map is shown indicating the location of Mga in relation to several linked genes. The number of recombinant F2 animals over the total number of F2 animals typed plus the recombination frequencies, expressed as genetic distance in centimorgans (± SE), is shown for each pair of loci on the left of the chromosome map. Where no recombinants were found between loci, the upper 95% confidence limit of the recombination distance is given in parentheses. The human chromosomal positions for the indicated loci are shown to the right.

of Mga. However, it is difficult to explain why Max, as well as ΔBRMax, a dominant-negative form of Max predicted to extinguish E-box binding by Mga, is also capable of mediating Mga-dependent activation of a reporter containing only Brachyury sites (Figures 5B and 6C). One possiblity is that Max binding to Mga serves the dual function of both generating an E-box-binding heterodimer and simultaneously blocking interaction of a corepressor that also interacts with the bHLHZip region of Mga. Thus, Max binding may provide a mechanism to regulate both target gene specificity and transcriptional activity (whether it functions as a repressor or activator) of Mga. Although Max protein levels are generally thought to be in excess of Myc, there are reports of changes in Max levels during differentiation (Delgado *et al.*, 1995) and apoptosis (Shichiri *et al.*, 1999), and indeed PC12 cells have been found to lack Max entirely (Hopewell and Ziff, 1995). Furthermore, Max levels may be limiting in some cells due to competition with additional Max-interacting proteins for binding to Max. Our results predict that the transcriptional and biological activities of Mga would be modulated under these conditions.

Although several studies have investigated the transcriptional activities of T-domain proteins including Brachyury (Kispert *et al.*, 1995; Casey *et al.*, 1998) and Tbx2 (Carreira *et al.*, 1998), the major focus of studies investigating the function of Tbx family proteins has been their role in embryonic development. The gene encoding the prototypical T-domain protein Brachyury was cloned by virtue of it being responsible for the classic mouse mutant, tailless, in which posterior mesoderm induction is abrogated (for review see Herrmann and Kispert, 1994). The mouse Brachyury phenotype corresponds well to Brachyury expression in posterior mesoderm precursors and ectopic expression of Brachyury induces the expression of mesodermal markers (Cunliffe and Smith, 1992; O'Reilly *et al.*, 1995). Thus, Brachyury appears to fulfill a role as a primary regulator of mesoderm induction during gastrulation. Like Brachyury, several other T-box genes play essential roles in the regional induction and specification of mesoderm during development (Papaioannou and Silver, 1998). Although Mga expression is widespread, highest levels are found in limb buds, branchial arches and tail, regions patterned by mesoderm and mesodermal–epithelial interactions (Figure 2). This expression pattern raises the possibility that Mga may participate in regulating mesoderm induction or differentiation at these sites. Furthermore, since Mga expression overlaps with the expression of several other Tbx genes, complex regulatory mechanisms governing mesoderm induction may exist that utilize multiple T-domain proteins. Indeed, in *Xenopus* regulatory pathways involving *Xenopus* Brachyury (Xbra), and the T-domain proteins VegT and Eomes have been identified that are thought to coordinate the transcriptional regulation of mesoderm specific genes in response to mesoderm signaling proteins such as activin and fibroblast

growth factor family members (Lustig *et al.*, 1996; Ryan *et al.*, 1996; Stennard *et al.*, 1996; Zhang and King, 1996; Horb and Thomsen, 1997).

Although the biological function of Mga has yet to be determined, the presence in Mga of both a T-domain and a Max-interacting bHLHZip domain suggests a role in the coordinate regulation of target genes recognized by both Max-network and T-domain proteins. Thus, Mga may function to integrate regulation of cell proliferation with mesoderm specification/induction during embryonic development.

Materials and methods

Cloning Mga

A yeast two-hybrid screen was carried out using Max as bait as described previously (Hurlin *et al.*, 1995) using a cDNA library derived from 9.5 and 10.5-day-old mouse embryos (Wilkinson and Nieto, 1993). The cDNA fragment recovered in the two-hybrid screen coding for a bHLHZip motif was used to screen a mouse e14.5 kidney cDNA library (a kind gift of V.Dixit). Sequential library screens were performed using cDNA fragments obtained until consensus start and stop sites were identified. Because of the surprising finding of a T-domain DNA-binding domain within the putative full-length ORF, additional library screens were performed to generate multiple independent overlapping cDNA clones covering the entire putative full-length cDNA. The extensive set of independent cDNA fragments was used to confirm the Mga cDNA structure. The putative full-length Mga cDNA was constructed from three separate, overlapping cDNAs (designated X1, N6 and I28) in the plasmid pBC SK+ (Stratagene). The Mga sequence was determined by sequencing the ends of individual cDNAs obtained in library screens and by sequencing nested exonuclease III deletions (Henikoff, 1987) of the putative full-length cDNA. The putative full-length Mga cDNA contained a 9018 bp ORF coding for a protein of 3006 aa. The full-length cDNA was subcloned into the mammalian expression vectors pRCCMV (Invitrogen) and pME (a gift of Y.Shio). A FLAG epitope tag sequence was introduced into the 5′ end of the Mga cDNA by PCR prior to cloning into the pME vector. GST fusion proteins containing the Mga T-domain (pGEX-MgaNT, aa 1–333), the Mga bHLHZip domain (pGEXMgabHLHZip, aa 2347–2479) and a portion of the C-terminus (pGEXMgaCT, aa 2771–1940) were constructed in the vector pGEX2T (Pharmacia).

Interaction and DNA-binding assays

The cDNA fragment of Mga obtained from the two-hybrid screen was *in vitro* transcribed and translated from the library plasmid BTM116 (Hollenberg *et al.*, 1995) and used in co-immunoprecipitation assays with 20 ng of baculovirus-produced Max (Ayer *et al.*, 1993). Anti-Max antisera 8711 was used to immunoprecipitate Max under low stringency conditions (L-buffer: PBS+0.2% NP-40) in the presence of the *in vitro* translated, [^{35}S]methionine-labeled Mga fragment. Immunoprecipitates were washed 4× with L-buffer and analyzed by SDS–PAGE and autoradiography. To test for *in vivo* association between Mga and Max, a FLAG-tagged version of Mga in the vector pME was transfected into HEK 293T cells. Co-immunoprecipitation experiments were carried out as described above using Max antiserum 8711 and anti-FLAG monoclonal antibodies (Sigma).

EMSAs employing an oligonucleotide containing the preferred Myc–Max-binding sequence CACGTG were performed as described previously (Hurlin *et al.*, 1997) using baculovirus Max, and the GST fusion proteins GST–MgabHLHZip and GST–MgaCt. GST–Myc(c96) (Blackwood *et al.*, 1991) fusion protein was used as a positive control. For EMSA of the Mga T-domain and for Brachyury, the preferred Brachyury-binding sequence AATTTCACACCTAGGTGTGAAATT (Kispert and Herrmann, 1993) was used. The Mga T-domain region was *in vitro* transcribed and translated from the plasmid pCDNAMgaX1-Pst. Brachyury was *in vitro* transcribed and translated from the plasmid pCS2-Bra5′HA, containing an introduced HA tag at the 5′ end of Brachyury. Supershift analysis of Mga DNA-binding complexes was performed using polyclonal antisera raised against GST fusion proteins produced from plasmids pGEXMgaNT and pGEXMgaCT (see above). Anti-HA antibodies (BMB) were used to supershift Brachyury complexes.

Luciferase assays

HEK 293 cells were transfected using the calcium phosphate precipitation method (Graham and van der Eb, 1973). The T2 reporter plasmid was constructed by inserting tandem consensus Brachyury-binding sites derived from the plasmid pG.Cat.BS.p2 (Kispert *et al.*, 1995) into the proximal promoter *Xho*I site of pGL2pro (Promega). The T2M4 reporter was constructed by inserting the same Brachyury sites into the *Xho*I site of pGL2M4 (Ayer *et al.*, 1993), containing a 4-fold reiteration of the consensus Myc–Max-binding site CACGTG. The plasmid pCS2 (CMV promoter; D.Turner and R.Rupp, unpublished) was used to express Mga, Max, ΔBRMax and c-Myc. The total amount of plasmid DNA in transfections was adjusted with empty pCS2 plasmid to maintain uniform levels. β-galactosidase and luciferase assays were performed using Galacton Plus (Tropix) and luciferin luminescent substrates and a Tropix TR717 luminometer.

Myc+Ras transformation assay

Transformation assays were performed as described previously (Hurlin *et al.*, 1997) with the exception that 5 μg of pCS2-based Mga and Brachyury expression plasmids were used in co-transfections with Myc and Ras cDNAs.

Northern blotting and in situ hybridization

Northern blots were prepared from total RNA purified from PC12 cells using Trizol (Gibco-BRL). RNA was separated by electrophoresis in a 1% agarose, 2.2 M formaldehyde gel and transferred onto Hybond N+ (Amersham). ^{32}P-labeled probes specific to the Mga T-box and bHLHZip were made from *Bam*HI–*Eco*RI restriction enzyme (New England Biolabs) released inserts of plasmids GST–MgaNT and GST–bHLHZip, respectively. Whole-mount *in situ* hybridization using digoxygenin-labeled probes was carried out as described by Wilkinson and Nieto (1993). Digoxygenin 11 UTP (Boehringer) incorporated antisense probes specific for the T-box region and the bHLHZip region of Mga were produced from plasmids pCDNA1MgaX1 and pCDNA1MgaK32, respectively, using T7 RNA polymerase. A Brachyury probe was made from plasmid pBT1 (a gift from P.Soriano) using T3 RNA polymerase.

Chromosomal localization

Interspecies backcross mapping was performed as described previously (Copeland and Jenkins, 1991). Two separate probes were used: one specific for the Mga bHLHZip region (0.5 kb bHLHZip clone10 fragment from the library plasmid obtained in the yeast two-hybrid screen), and one specific for the Mga T-domain (2 kb fragment from the kidney cDNA library plasmid pCDNA1-A2). A description of the probes and RFLPs for loci linked to *Mga* including thrombospondin (*Thbs1*), adenosine deaminase (*Ada*), β_2-microglobulin (*β2m*), and α_2-adrenergic receptor subtype b (*Adra2b*) has been reported previously (Lawler *et al.*, 1991; Chang *et al.*, 1994). The erythrocyte protein 4.2 (*Ebp4.2*) locus has not been reported previously for this interspecies backcross. Recombination distances and gene order were determined using Map Manager version 2.6.5 (Manley, 1993) by minimizing the number of recombination events required to explain the allele distribution patterns.

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