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Mad3 and Mad4: novel Max-interacting transcriptional repressors that suppress c-*myc* dependent transformation and are expressed during neural and epidermal differentiation

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The basic helix–loop–helix-leucine zipper (bHLHZip) protein Max associates with members of the Myc family, as well as with the related proteins Mad (Mad1) and Mxi1. Whereas both Myc:Max and Mad:Max heterodimers bind related E-box sequences, Myc:Max activates transcription and promotes proliferation while Mad:Max represses transcription and suppresses Myc dependent transformation. Here we report the identification and characterization of two novel Mad1- and Mxi1-related proteins, Mad3 and Mad4. Mad3 and Mad4 interact with both Max and mSin3 and repress transcription from a promoter containing CACGTG binding sites. Using a rat embryo fibroblast transformation assay, we show that both Mad3 and Mad4 inhibit c-Myc dependent cell transformation. An examination of the expression patterns of all *mad* genes during murine embryogenesis reveals that *mad1*, *mad3* and *mad4* are expressed primarily in growth-arrested differentiating cells. *mxi1* is also expressed in differentiating cells, but is co-expressed with either c-*myc*, N-*myc*, or both in proliferating cells of the developing central nervous system and the epidermis. In the developing central nervous system and epidermis, downregulation of *myc* genes occurs concomitant with upregulation of *mad* family genes. These expression patterns, together with the demonstrated ability of Mad family proteins to interfere with the proliferation promoting activities of Myc, suggest that the regulated expression of Myc and Mad family proteins function in a concerted fashion to regulate cell growth in differentiating tissues.

Keywords: differentiation/Mad family/Max/Myc/transcriptional repression

Introduction

The basic helix–loop–helix–leucine-zipper (bHLHZip) protein Max is thought to play a critical role in the function of a biologically important group of transcription factors. First identified as a heterodimerization partner for Myc family proteins (c-, N- and L-Myc), Max was subsequently shown to form homodimers as well as heterodimers, and both types of complexes were found to be capable of specifically binding CACGTG or related E-box sequences (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991; Blackwell *et al.*, 1993). Recent structural studies demonstrate that Max dimerization is mediated by folding of the HLHZip regions of both partners into a parallel four helix bundle. The contiguous basic regions then make symmetrical major groove contacts at the DNA binding site (Ferré-D'Amaré *et al.*, 1993).

Because Myc proteins do not homodimerize, heterodimerization with Max is required for specific DNA binding by Myc, and for transcriptional activation of promoters located proximal to the binding sites. This transcriptional activity requires, in addition to the bHLHZip regions of Myc and Max, the N-terminal transcriptional activation domains of Myc (Amati *et al.*, 1992; Kretzner *et al.*, 1992; Amin *et al.*, 1993; Gu *et al.*, 1993). Max itself is thought to be transcriptionally inert (Kato *et al.*, 1992). Overexpression of Max results in suppression of Myc:Max mediated transcription, cell transformation, and tumorigenesis, probably through competition between transcriptionally active heterodimers and inactive Max homodimers for common DNA binding sites (Kretzner *et al.*, 1992; Mäkelä *et al.*, 1992b, Prendergast *et al.*, 1992; Amati *et al.*, 1993a; Gu *et al.*, 1993; Koskinen *et al.*, 1994; Lindeman *et al.*, 1995).

It has been demonstrated that functions of the c-Myc protein in cell transformation and in apoptosis are dependent on its interaction with Max (Amati *et al.*, 1993b; Mukherjee *et al.*, 1992; Harrington *et al.*, 1994). The notion that Max is a necessary cofactor for many of the functions of Myc proteins is supported by findings that Max is expressed in most, if not all, cell types (Blackwood and Eisenman, 1991), and that homozygous deletion of the *max* gene in mice appears to lead to very early [average day 6.5 post coitus (p.c.)] embryonic lethality (R.DePinho, personal communication). Furthermore, Max is a highly stable protein that is synthesized throughout the proliferating cell cycle, as well as during G_0, and in many differentiating cells (Blackwood *et al.*, 1992; Ayer and Eisenman, 1993; Larsson *et al.*, 1994). In contrast, Myc has a short half-life, is induced following the G_0 to G_1 transition and maintained throughout the cell cycle, and downregulated during differentiation in many cell types (for reviews see Lüscher and Eisenman, 1990; DePinho *et al.*, 1991; Marcu *et al.*, 1992). Heterocomplexes containing Myc and Max are found in cycling cells (Blackwood *et al.*, 1992). These results have suggested a model in which synthesis of Myc is rate-limiting in the switch from constitutively expressed inactive Max homodimers to transcriptionally active Myc:Max heterodimers (Amati *et al.*, 1992; Blackwood *et al.*, 1992; Kretzner *et al.*, 1992).

The presence of Max during quiescence and differentia-

tion, when Myc is downregulated (Blackwood *et al.*, 1992; Ayer *et al.*, 1993; Larsson *et al.*, 1994), raised the possibility that additional proteins might exist that associate with Max. Using protein interaction screens, two novel but related bHLHZip proteins, Mad and Mxi1, were found to interact specifically with Max (Ayer *et al.*, 1993; Zervos *et al.*, 1993). Like Myc, neither Mad nor Mxi1 exhibits specific DNA binding on their own. However, heterocomplexes of Mad or Mxi1 with Max recognize the same DNA binding sites as Myc:Max complexes. It was further demonstrated that Mad:Max represses transcription through the same binding sites that Myc:Max activates transcription, and can antagonize transcriptional activation by Myc (Ayer *et al.*, 1993). Thus, Mad and Mxi1 may antagonize Myc function *in vivo*, a notion that has received support from the findings that these proteins block cotransformation by Myc and Ras (Lahoz *et al.*, 1994; Koskinen *et al.*, 1995; Västrik *et al.*, 1995), and that ectopic Mad expression can block cytokine mediated cell cycle entry of quiescent cells (M.Roussel, D.Ayer and R.Eisenman, unpublished data). Recent experiments have demonstrated that Mad and Mxi1 interact with mSin3A and B, mammalian homologues of the yeast transcriptional corepressor Sin3. Mutations in the N-terminal mSin3 interaction domain in Mad result in inhibition of mSin3 binding as well as transcriptional repression activity. Therefore, at least one mechanism of Mad repression may be mediated by its interaction with a conserved corepressor (Ayer *et al.*, 1995; Schrieber-Agus *et al.*, 1995).

Initial studies examining Mad expression revealed that only very low mRNA and protein levels are present in proliferating myeloid leukaemic cells (Ayer and Eisenman, 1993). This is in contrast to *mxi*1 mRNA, which is expressed at relatively high levels in proliferating myeloid cells (Zervos *et al.*, 1993; Larsson *et al.*, 1994). However, upon differentiation of these cells, *mad* RNA and protein, and to a lesser extent *mxi*1 mRNA are induced, apparently as an immediate early response to treatment with differentiating agents (Ayer and Eisenman, 1993; Zervos *et al.*, 1993; Larsson *et al.*, 1994). *mad* RNA and protein were also found to be induced upon differentiation of primary human foreskin keratinocytes (Hurlin *et al.*, 1994, 1995). In both the myeloid lines and primary keratinocytes, a shift from Myc:Max to Mad:Max complexes occurs during differentiation. The switch in heterocomplexes is thought to reflect a transcriptional switch from activation to repression of common target genes, possibly leading to cessation of proliferation (Ayer and Eisenman, 1993). These expression patterns and the demonstrated ability of Mad and Mxi1 to suppress Myc dependent transformation are consistent with a potential function of Mad and Mxi1 as tumour suppressors. Indeed, a recent study has detected allelic loss and mutation at the *mxi*1 locus in prostate cancers (Eagle *et al.*, 1995).

Although the identification and initial characterization of Mad and Mxi1 suggest that these proteins function in a similar fashion to antagonize Myc activities, little is known about the size of this family and how the expression patterns of Mad family members relate to their presumptive roles as inhibitors of cell growth. In this paper we report the identification and characterization of two novel bHLHZip proteins related to Mad (Mad1) and Mxi1, and examine the relationship between the expression patterns of Mad family genes and Myc family genes during neural and epidermal differentiation.

Results

Identification of Max-interacting proteins related to Mad and Mxi

To identify novel Max-interacting proteins we performed a yeast two-hybrid screen of a mouse cDNA library prepared from day 9.5 p.c. and 10.5 p.c. embryos (Vojtek *et al.*, 1993; Hollenberg *et al.*, 1995) using a reporter strain expressing a LexA–Max9 fusion protein. From ~1.5×10^7 transformants screened, 67 clones were recovered that contained candidate Max9-interacting proteins; these clones tested positive for both growth on medium lacking histidine and for β-galactosidase activity (Vojtek *et al.*, 1993). Sequence analysis and comparison with the combined PIR, GENPEPT and SWISSPROT databases revealed that among the clones identified were cDNAs encoding five known Max-interacting bHLHZip proteins: c-, N- and L-Myc and Mad and Mxi1. In addition, multiple independent copies of four previously unreported cDNAs were identified which encoded proteins containing the consensus amino acids for the bHLHZip motif. Two of the proteins identified, which we have designated Mad3 and Mad4, are closely related to Mad (now called Mad1) and Mxi1. These proteins are described in this paper. The other two clones identified show no relationship to any previously identified proteins outside of the bHLHZip region, and will be described elsewhere.

The *mad*3 and *mad*4 partial cDNAs recovered from the two-hybrid screen were used to screen a mouse embryonic stem cell cDNA library (Chen *et al.*, 1994). Putative full-length cDNAs were obtained containing consensus sites for initiation of translation for a long open reading frame, a 3′ untranslated region and a polyadenylated tail. The open reading frames for *mad*3 and *mad*4 encode predicted proteins of 206 and 209 amino acids, respectively. *In vitro* translation of mRNAs generated from the *mad*3 and *mad*4 cDNAs produces proteins that migrate with apparent molecular weights of 29 and 32 kDa, respectively (Figure 1B). An alignment of the Mad3 and Mad4 open reading frames with those of murine Mad (now designated Mad1) and Mxi1 (K.Foley, personal communication) is shown in Figure 1A.

Members of the Mad family contain two highly conserved regions: a central region encompassing the bHLHZip domain, and an N-terminal region (Figure 1A). The N-terminal homology overlaps the region of Mad1 that was previously demonstrated to mediate interaction of Mad1 and Mxi1 with mSin3 proteins (SID: *S*in-*I*nteraction *D*omain, Figure 1A; Ayer *et al.*, 1995; Schreiber-Agus *et al.*, 1995). Disruption of this region interferes with both the transcriptional and biological activities of these proteins (Ayer *et al.*, 1995; Koskinen *et al.*, 1995; Schreiber-Agus *et al.*, 1995). Interestingly, a highly conserved block of amino acids lies immediately adjacent to the minimal SID, and is encoded by a separate exon (K.P.Foley and R.N.Eisenman, unpublished data). Based on helical wheel modelling, this block of amino acids forms a contiguous amphipathic helix with amino acids that extend from the N-terminus and overlaps the minimal SID (data not shown and Ayer *et al.*, 1995). The

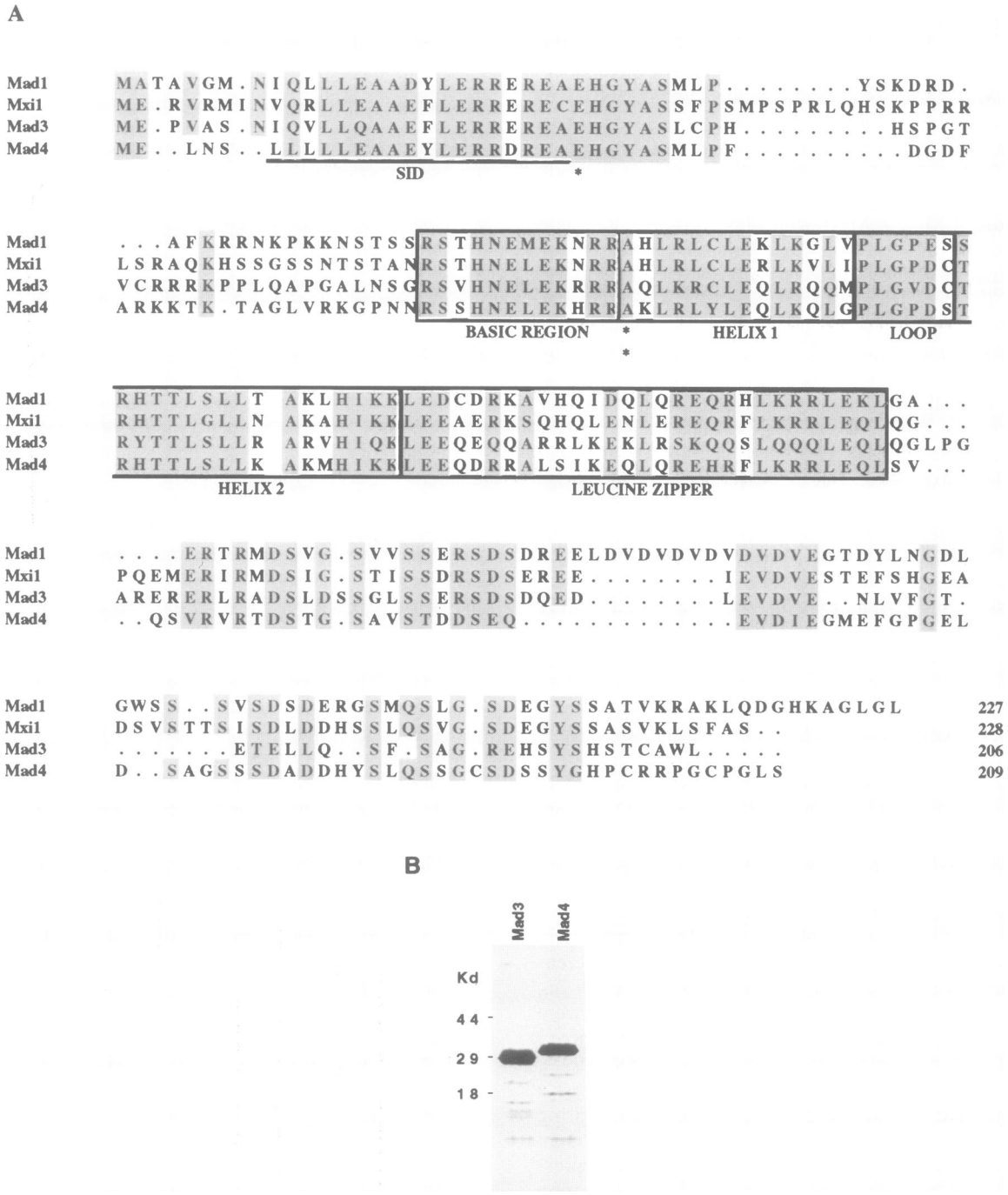


Fig. 1. Amino acid sequence comparison of murine Mad1, Mxi1, Mad3 and Mad4 proteins. (**A**) The sequence alignments were generated using the Pileup program (Genetics Computer Group, Madison, WI). The Sin3-interacting domain (SID) is underlined and BHLHZip regions are boxed. Amino acids that are conserved in at least three of the Mad family proteins are shadowed. The number of amino acid residues is shown at the end of each sequence. * denotes the amino acid position where ΔNtMad4 begins. ** denotes the amino acid position where ΔNtMad3 begins. (**B**) *In vitro* transcribed and translated Mad3 and Mad4 cDNAs.

most divergent regions of Mad family proteins are between the N-terminal region containing the SID and the bHLHZip region, and in the C-terminus (Figure 1).

Mad3 and Mad4 interact with mSin3A and mSin3B

To determine whether the presumptive SID domains of Mad3 and Mad4 mediate interaction with mSin3 proteins, a series of GST fusion proteins containing Mad3 and Mad4 were prepared and used in *in vitro* interaction assays. For both Mad3 and Mad4, GST fusion proteins were used which contained either the full-length protein (FL), or an N-terminal deletion (ΔNt) which removed the SID (see Figure 1A). The GST–Mad3 and –Mad4 fusion proteins were mixed with *in vitro* translated Max, mSin3A or mSin3B that had been labelled with [^{35}S]methionine. Proteins bound to the fusion proteins were recovered on glutathione–Sepharose beads under low stringency conditions (PBS/0.4% NP-40), and analysed by SDS–

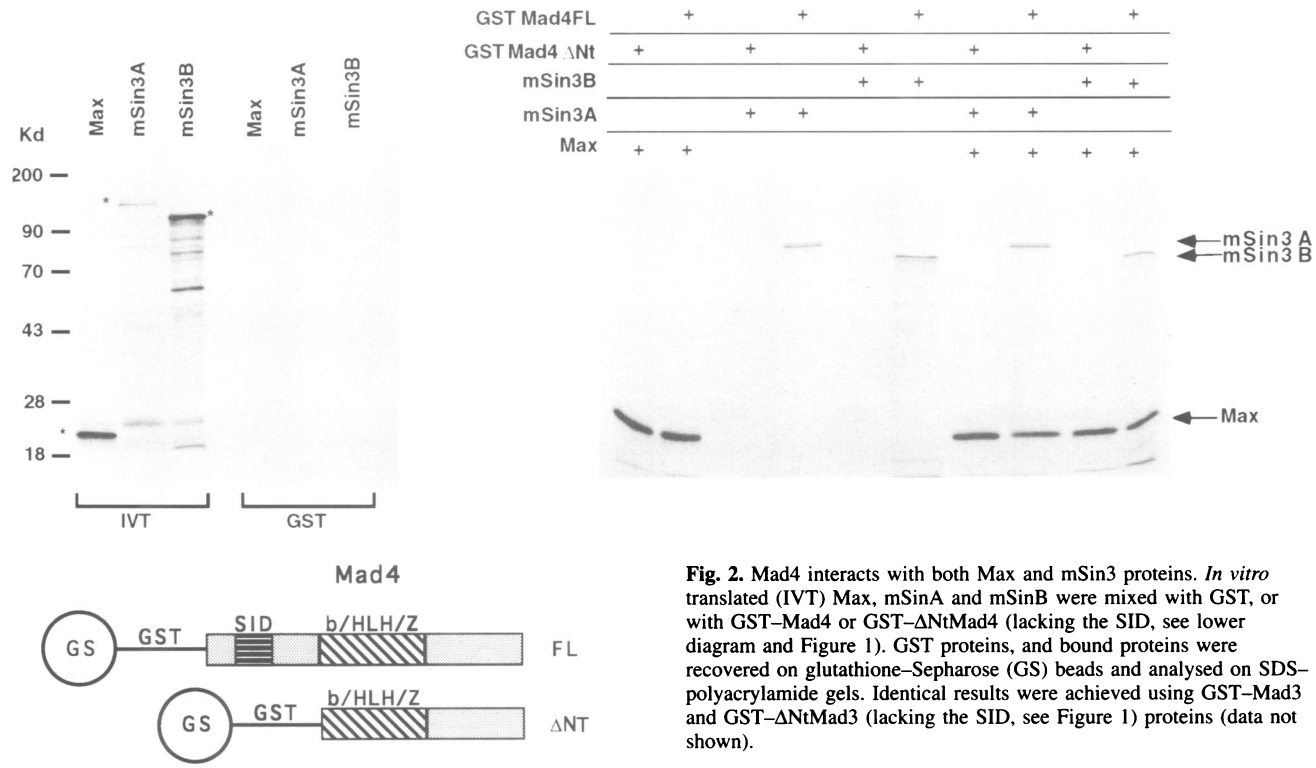


Fig. 2. Mad4 interacts with both Max and mSin3 proteins. *In vitro* translated (IVT) Max, mSinA and mSinB were mixed with GST, or with GST–Mad4 or GST–ΔNtMad4 (lacking the SID, see lower diagram and Figure 1). GST proteins, and bound proteins were recovered on glutathione–Sepharose (GS) beads and analysed on SDS–polyacrylamide gels. Identical results were achieved using GST–Mad3 and GST–ΔNtMad3 (lacking the SID, see Figure 1) proteins (data not shown).

polyacrylamide gel electrophoresis. Figure 2 shows results from an experiment testing interactions with GST–Mad4 fusion proteins. Whereas *in vitro* translated Max, mSin3A and mSin3B proteins showed no interaction with GST alone, each of these proteins interacted with GST fusion proteins containing full-length Mad3 or Mad4 (Figure 2 and data not shown), as previously demonstated for Mad1 (Ayer *et al.*, 1995). Furthermore, both Max and mSin3 proteins are recovered when mixed simultaneously with the full-length proteins (Figure 2 and data not shown). This latter result is consistent with a previous study showing that Mad1, Max and an mSin3 protein can form a ternary complex (Ayer *et al.*, 1995). In contrast to the full-length proteins, Mad3 and Mad4 lacking the N-terminal SID-containing region interact with Max, but not with mSin3 proteins (Figure 2 and data not shown). These results indicate that all of the Mad family members share the ability to interact with both Max and mSin3 proteins, and that interaction with mSin3 occurs through a highly conserved N-terminal domain.

Mad3:Max and Mad4:Max bind the sequence CACGTG and repress transcription

Heterodimeric complexes containing Max and either Mad1 or Mxi1 bind strongly to the preferred Myc:Max binding sequence CACGTG (Blackwell *et al.*, 1990; Ayer and Eisenman, 1993; Zervos *et al.*, 1993). To determine whether Mad3:Max and Mad4:Max complexes interacted with CACGTG, gel shift assays were performed using an oligonucleotide (CM1) containing the CACGTG sequence. GST fusion proteins containing full-length Mad3 and Mad4 proteins were mixed with baculovirus-produced Max (Ayer *et al.*, 1993) in the presence of the CM1 oligonucleotide. Whereas Max by itself forms a homodimeric complex on the CM1 oligo, neither Mad3 nor Mad4 proteins by themselves bound the CM1 oligo (Figure 3A). When equal amounts of GST–Mad3 or GST–Mad4 were mixed with Max, a shifted complex was observed in each case. No such shift was seen if the same amount of GST was instead mixed with Max, indicating that the Mad3 or Mad4 portion of the fusion protein was required for the appearance of the shifted complex. The presence of Max in the shifted complex is indicated by the ability of anti-Max antibodies to supershift the complex (Figure 3A). The weak binding to CM1 of Mad4:Max, compared with Mad3:Max, may reflect a lower affinity of interaction between Mad4 and Max or an intrinsically weaker affinity of the complex for DNA. These differences in activity may relate to differences in amino acid sequence within the bHLHZip regions of Mad3 and Mad4 (see Figure 1A). We have also examined binding to several 'non-canonical' Myc:Max binding sites (i.e. a series of E-box related and unrelated sequences identified as lower affinity binding sites; see Blackwell *et al.*, 1993) and found that Mad3:Max and Mad4:Max also recognize these sequences with lower affinity (data not shown).

We next wanted to determine the transcriptional activities of Mad3:Max and Mad4:Max complexes at promoters containing proximal CACGTG binding sites. It is well established that c-Myc can function to activate transcription as a c-Myc:Max heterodimer at promoters containing CACGTG (Amati *et al.*, 1992; Kretzner *et al.*, 1992; Amin *et al.*, 1993; Gu *et al.*, 1993). Our previous studies examining the transcriptional activities of Mad indicate that it represses transcription (Ayer *et al.*, 1993). We used the same reporter plasmid, pM4MinCAT, to test the transcriptional activities of Mad3 and Mad4. As has been observed previously (Kretzner *et al.* 1992; Ayer *et al.* 1993), expression of Max alone repressed the background levels of transcription (Figure 3B and C), presumably by binding transcriptionally inert homodimers to CACGTG. Expression of c-Myc activated transcription over endo-

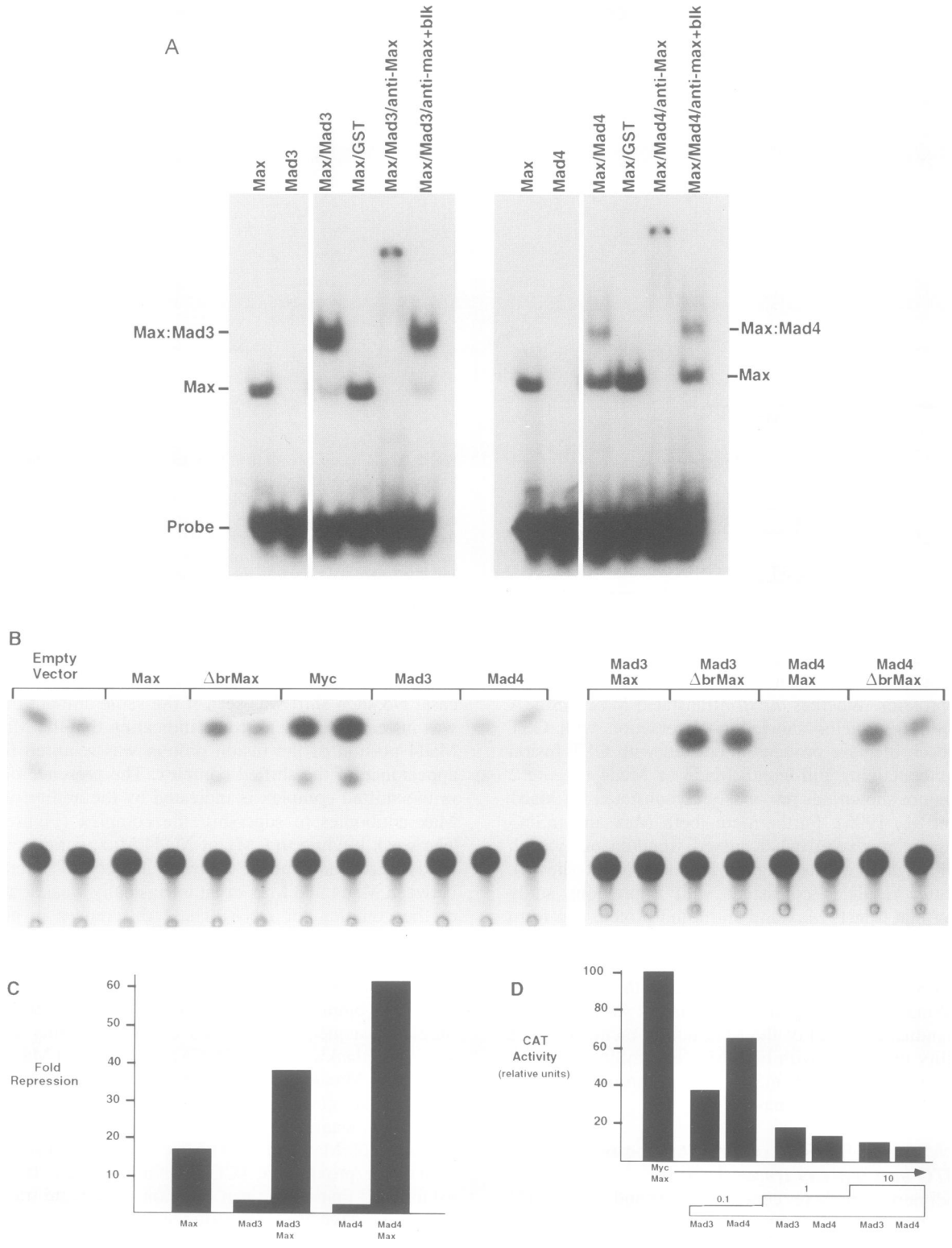


Fig. 3. Mad3:Max and Mad4:Max heterodimers bind CACGTG and repress transcription. (**A**) Electrophoretic mobility shift assays using the CACGTG-containing CM1 oligonucleotide probe, and the indicated proteins. 'Block' denotes pre-incubation of the Max antisera with immunogen before adding it to the reaction. (**B**) Results from a representative CAT assay, performed in duplicate, showing the transcriptional activities of the indicated proteins when analysed alone, and the activities of Mad3 and Mad4 when transfected together with Max9, or ΔBRMax9 expression plasmids. Similar results were achieved in four independent experiments. (**C**) CAT activities for the indicated transfections in (B) were quantitated by PhosphoImage analysis and averaged. Fold repression was determined by dividing the averaged CAT activities measured from the transfected empty vector with that resulting from transfection of the indicated plasmids. (**D**) Results from a representative CAT assay (not shown), in which increasing amounts (μg) of Mad3 and Mad4 plasmids were titrated in the presence of constant Myc and Max plasmids (3 μg each). Each transfection was performed in duplicate and the CAT activity quantitated by PhosphoImage analysis. The average CAT activity for the indicated transfections, relative to that for Myc:Max, is shown. Similar results were achieved in each of two experiments performed.

genous levels (Figure 3B, Kretzner *et al.*, 1992; Ayer *et al.*, 1993). In contrast, expression of Mad3 alone or Mad4 alone repressed transcription (Figure 3B and C). Whereas repression was strongly enhanced when Mad3 or Mad4 were transfected together with Max (Figure 3B and C), co-transfection of either Mad3 or Mad4 with a mutant Max protein lacking the basic region abrogated repression. These results indicate that, despite the apparent differences in binding to CACGTG as a heterodimer with Max (Figure 3A), the extent of transcriptional repression by Mad3 and Mad4 is very similar and is mediated through association with Max. Furthermore, since transcriptional repression by Mad1 requires an intact SID (Ayer *et al.*, 1995), and Mad3 and Mad4 also interact with mSin3 through a homologous region, it is likely that transcriptional repression by Mad3 and Mad4 is also mediated through their SIDs.

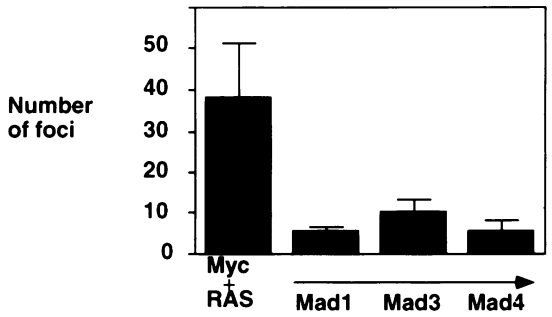
To determine whether repression by Mad3 and Mad4 antagonizes activation by c-Myc, a series of titration experiments were performed. As shown in Figure 3D, in the presence of constant amounts of transfected Myc and Max, both Mad3 and Mad4 repress transcription in a concentration dependent manner. Complementary experiments, in which increasing amounts of c-Myc were titrated in the presence of constant Mad3 or Mad4 and Max, demonstrated concentration dependent activation by c-Myc (data not shown). These results demonstrate that, like Mad1 (Ayer *et al.*, 1993), both Mad3 and Mad4 antagonize the transcriptional activities of c-Myc.

Mad3 and Mad4 suppress c-Myc dependent transformation

The opposing transcriptional activities displayed by c-Myc and the Mad3 and Mad4 proteins suggested that they might possess antagonistic biological activities. It has been recently shown that Mad1 and Mxi1 suppress transformation of primary rat embryo fibroblasts caused by co-transfection of c-*myc* with an activated c-Ha-*ras*^{Val12} oncogene (Lahoz *et al.*, 1994; Koskinen *et al.*, 1995; Schreiber-Agus *et al.*, 1995; Västrik *et al.*, 1995). The effects of Mad1, Mad3 and Mad4 proteins on co-transformation by Myc-Ras are shown in Figure 4. For these experiments, secondary cultures of rat embryo fibroblasts (REFs) were transfected with c-Myc and c-Ha-Ras^{Val12} expression vectors at concentrations previously optimized to obtain maximal numbers of transformed foci (Koskinen *et al.*, 1995). REFs transfected with c-Myc and c-Ha-Ras were also transfected with either Mad1, Mad3 or Mad4 expression vectors. Two weeks after transfection, the numbers of morphologically transformed foci were determined. The numbers of foci obtained in each of three independent experiments are shown, and graphically represented in Figure 4. The average numbers of foci induced by c-Myc and c-Ha-Ras were reduced by 85, 73 and 86% by Mad1, Mad3 and Mad4, respectively. Thus, all Mad family proteins dramatically interfere with c-Myc dependent cell transformation.

Chromosomal positions of Mad3 and Mad4

The mouse chromosomal locations of *Mad3* and *Mad4* were determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J×*Mus spretus*) F1×C57BL/6J] mice. This interspecific backcross



	Number of Foci		
	Expt. 1	Expt. 2	Expt. 3
Myc +Ras	58	32	24
+Mad1	6	7	4
+Mad3	15	10	6
+Mad4	1	8	7

Fig. 4. Mad3 and Mad4 suppress Myc dependent transformation of rat embryo fibroblasts (REF). REFs were transfected with c-Myc and c-Ha-Ras^{Val12}, in the absence or the presence of either Mad1, Mad3 or Mad4 expression vectors, and the numbers of foci were determined 2 weeks later. The table shows results from three independent experiments. The average numbers of foci are summarized in the graph. Bars = mean deviation.

mapping panel has been typed for >1800 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M.spretus* DNAs were digested with several enzymes and analysed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using mouse cDNA probes for Mad3 and Mad4. RFLPs of 4.3 and 5.1 kb, generated by digestion with *Sac*I (see Materials and methods), were used to follow the segregation of the Mad3 and Mad4 loci, respectively, in backcross mice. *Mad3* mapped in the central region of chromosome 13, 0.7 cM distal to *Gpcr15* and 1.6 cM proximal to *Il9*, while Mad4 mapped on the proximal chromosome 5, 1.6 cM distal to *Il6* and 1.5 cM proximal to *Gpcr1*. Interestingly, a second site of hybridization for Mad4 was seen on chromosome 2 region C at or near where a second site of hybridization for Mxi1 was previously detected (Edelhoff *et al.*, 1994). The gene order and the estimated distances for Mad3 and Mad4 and the flanking loci (in centimorgans ± standard error) are shown in Figure 5.

The central region of mouse chromosome 13 shares regions of homology with human chromosome 5q (summarized in Figure 5). In particular, Fgfr4 has been mapped to human 5q33-qter. The tight linkage between Mad3 and Fgfr4 in mouse suggests that Mad3 will reside on 5q in humans. Similarly, the close linkage of Mad4 and Fgfr3 on mouse chromosome 5 suggests that in humans, Mad4 will reside on human chromosome 4p (Figure 5). The observation that the Mad3 and Mad4 genes are each tightly linked to a fibroblast growth factor receptor, an

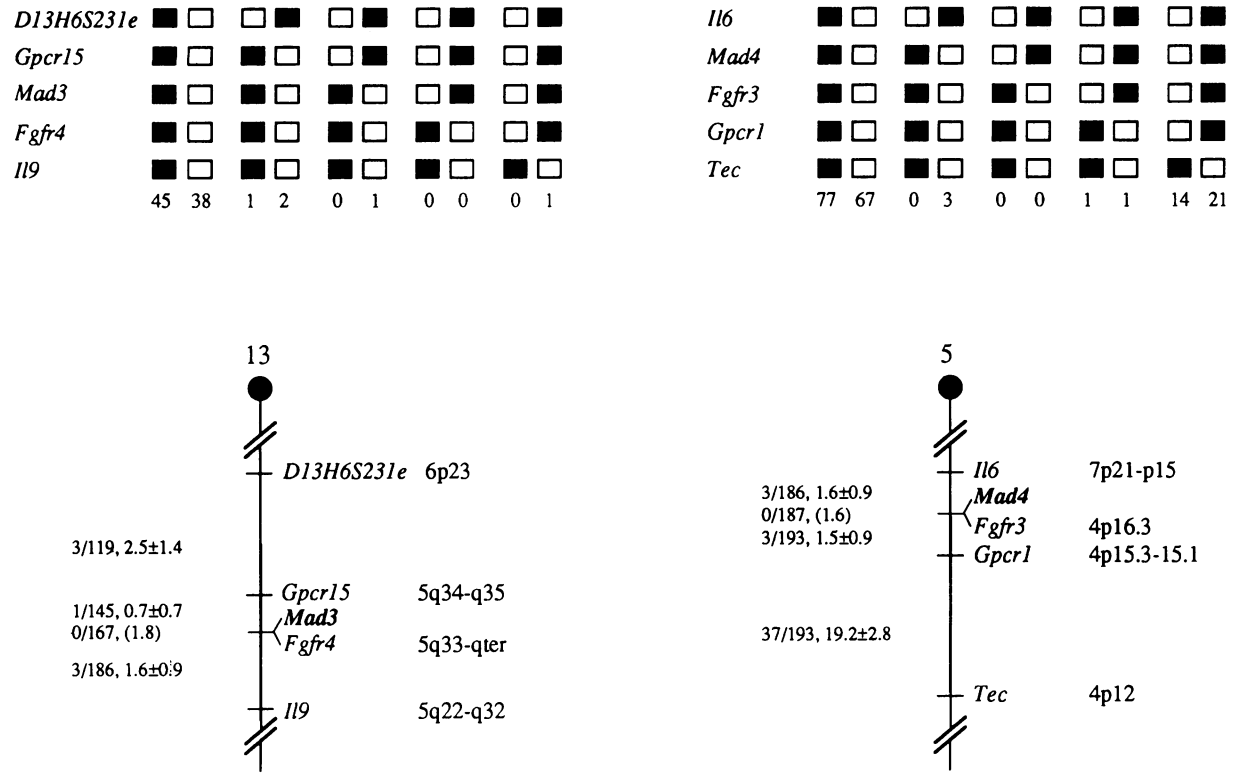


Fig. 5. Mouse chromosomal positions of *Mad3* and *Mad4*. The segregation patterns of *Mad3*, *Mad4* and flanking genes in backcross animals that were typed for all the loci are shown at the top of the figure. More animals were typed for individual pairs of loci. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J×*M.spretus*) F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of an *M.spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. Partial chromosome linkage maps showing the location of *Mad3* and *Mad4* in relation to the linked genes are shown at the bottom of the figure. The number of recombinant F_2 animals over the total number of F_2 animals typed plus the recombination frequencies, expressed as genetic distance in centimorgans (±1 standard error), is shown for each pair of loci on the left of the chromosome maps. Where no recombinants were found between loci, the upper 95% confidence limit of the recombination distance is given in parentheses. No double crossover events were observed for the markers analysed. The positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of the loci cited can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H.Welch Medical Library of the Johns Hopkins University (Baltimore, MD).

interleukin and a G-protein-coupled receptor gene, raises the possibility that they originate from a large chromosomal duplication event. Unlike the mouse, however, human Il6 is not linked to Fgfr3 or Gpcr1, indicating that a later rearrangement has split up this linkage in humans.

Expression patterns of Mad and Myc family members during neural differentiation

To begin to address the function of the Myc-Max-Mad network *in vivo*, we have examined the expression patterns of different members of the network during murine embryogenesis, as well as in adult tissues. Since *mad3* and *mad4* were isolated from a murine embryonic library, we have focused on expression of each of the *mad* family members and of c-*myc* and N-*myc* during embryogenesis.

In situ hybridization analysis was performed on sections of mouse embryos at 8.5, 9.5, 10.5, 11.5, 12.5, 14.5 and 17.5 days p.c. using antisense ^{35}S-labelled riboprobes. In general, the expression of the four *mad* genes and c-*myc* and N-*myc* is not restricted to specific cell lineages or stages of development (data not shown). However, it appears that expression of the *mad* family members correlates with differentiation in a variety of cell lineages. A similar relationship between differentiation and expression of *mad1* has emerged from experiments performed using tissue culture cells (Ayer and Eisenman, 1993; Hurlin *et al.*, 1994, 1995; Larsson *et al.*, 1994). Because of the association with differentiation, we focus here on the expression patterns of *mad* and *myc* family members in the developing central nervous system and the epidermis, tissues where differentiated cells emerge in a well organized fashion.

In the developing vertebrate spinal cord, neurons are generated in a specific sequence both with respect to time and position along the dorso–ventral axis (Nornes and Carry, 1978). The neural tube at embryonic day 10.5 p.c. is functionally divided into two major regions; the ventricular zone (VZ) and the intermediate zone (IZ) (for nomenclature, Boulder Commitee, 1970). The ventricular zone consists of actively dividing precursors of differentiated neurons and glia (Nornes and Carry, 1978). As they differentiate, these precursors exit from the cell cycle, and migrate away from the ventricular zone and into the intermediate zone. The first neural progenitors differentiate in the ventral part of the neural tube and give rise to the presumptive motorneurons. c-*myc* transcripts were found within a subset of cells in the proliferative ventricular zone, as well as in differentiating cells at the ventral portion of the intermediate zone (Figure 6A). In addition, c-*myc* transcripts were detected in the roof plate and in the

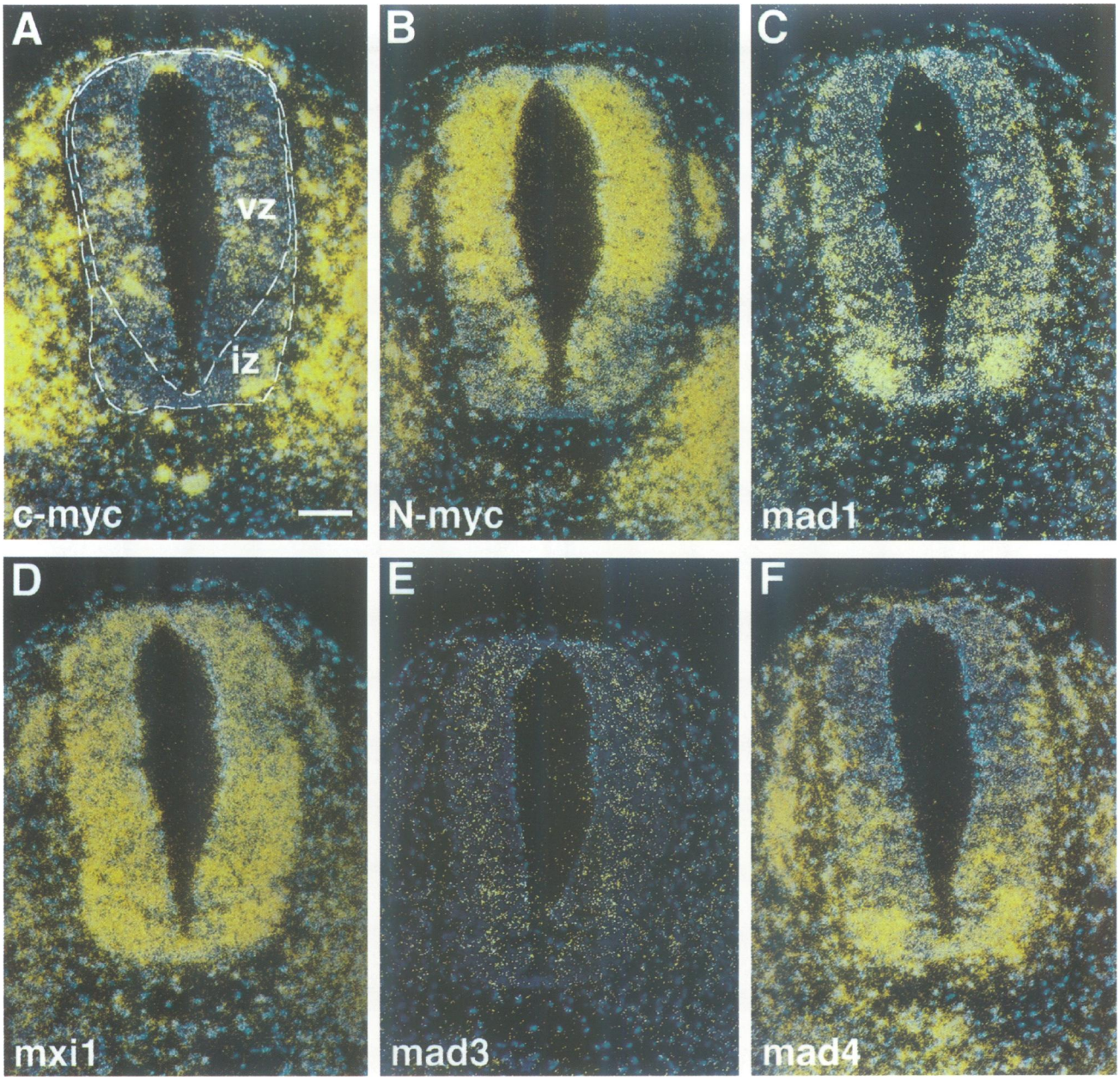


Fig. 6. Expression of Mad family members and c- and N-*myc* in the developing spinal cord. Paraffin sections of embryos at day 10.5 p.c. were hybridized with the indicated antisense riboprobes. The ventricular zone (VZ) and intermediate zone (IZ) define the proliferative and differentiative compartments in the neural tube at this stage of development, respectively, and are outlined with the dotted line in (A). Bar, 80 μm.

neural crest (Figure 6A). N-*myc* is expressed principally in the proliferating cells of the ventricular zone. The signal extends into the intermediate zone, albeit at reduced levels (Figure 6B), confirming previous results (Mugrauer *et al.*, 1988; Wakamatsu *et al.*, 1993). Thus, at 10.5 p.c., N-*myc* expression is restricted primarily to proliferating neural progenitors. *mxi*1 transcripts are also found in the ventricular zone, where they overlap with both c-*myc* and N-*myc*. However, *mxi*1 is expressed at highest levels in cells accumulating in the intermediate zone (Figure 6D). Expression of *mad*1 and *mad*4 are maximal in regions where N-*myc* expression is lowest: *mad*1 and *mad*4 are detected most strongly in differentiating cells of the intermediate zone at the ventral part of the neural tube, and weakly in the ventricular zone (Figure 6C and F). A weak signal for *mad* 3 was detected in cells at the perimeter of the ventricular zone and was absent in cells close to the lumen at 10.5 p.c. (Figure 6E). Thus, *mad*3 appears to be transiently expressed in a subpopulation of neural progenitors beginning to exit the cell cycle and differentiate. Alternatively, *mad*3 could be expressed at a specific phase of the cell cycle as there was a good correlation between the pattern of expression of *mad*3 and the localization of the nuclei in S phase in the outermost region of the ventricular zone (not shown; Rakic, 1972).

Later in development, from 11.5 to 14.5 p.c., the ventricular zone becomes attenuated and eventually disappears as its cells differentiate and the intermediate zone becomes prominent (Nornes and Carry, 1978). At 11.5 and 12.5 p.c., the expression patterns of the *mad* family

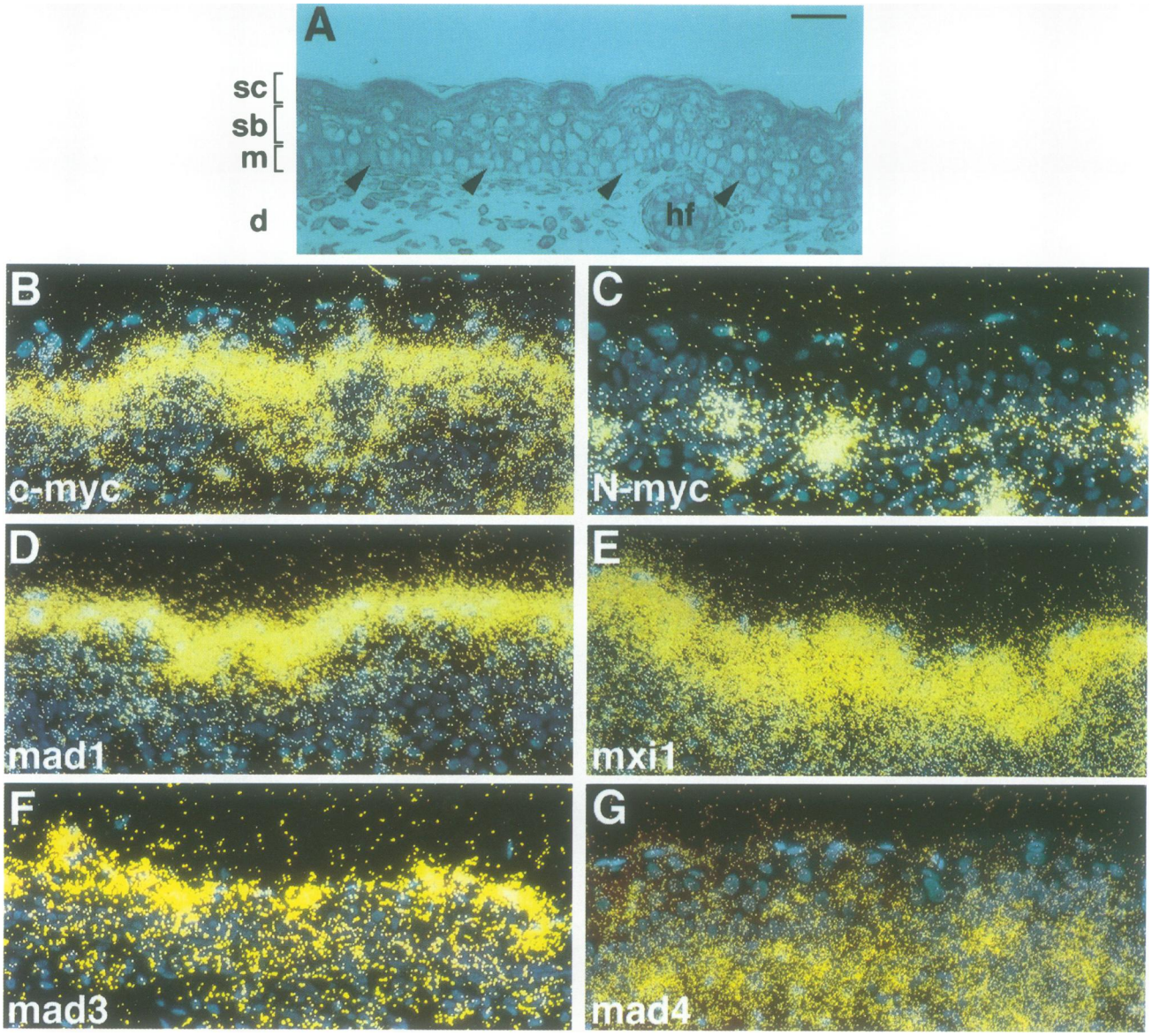


Fig. 7. Expression of Mad family members and c- and N-*myc* in the developing skin. Paraffin sections of embryos at day 17.5 p.c. were hybridized with the indicated antisense riboprobes. (**A**) A section stained with Toluene blue showing the dermis (d) and a hair folicle (hf) and the individual layers of the epidermis at this stage of development; m, Malpighian layer; sb, suprabasal layers; sc, statum corneum. The arrowheads point to the border of the dermis and epidermis where the Malpighian layer begins. Bar, 35 μm.

genes and c-*myc* and N-*myc* are very similar to 10.5 p.c. (data not shown). *mad*1, *mxi*1 and *mad*4 are highly expressed in the intermediate zone (not shown) and present at a reduced level in the ventricular zone that mostly persists in the dorsal part of the neural tube. In contrast, *mad*3 transcripts are only detected at the periphery of the ventricular zone. At 14.5 p.c., cell proliferation is nearly complete. *mad*1, *mxi*1 and *mad*4 are expressed throughout the spinal cord. Their expression is reduced at the outermost periphery of the neural tube containing the most differentiated neurons. *mad*3 transcripts are no longer detectable (not shown). Interesting to note that at this later stage, c-*myc* and N-*myc* transcripts are found in regions containing differentiating post-mitotic neurons, as has been reported previously (Grady *et al.*, 1987; Mugrauer *et al.*, 1988; Wakamatsu *et al.*, 1993).

Expression of Mad and Myc family members in the developing epidermis

At day 14.5 p.c., two layers of cells comprise the dorsal lateral epidermis, with production of fully differentiated squames not yet being apparent (Jackson *et al.*, 1981). At this stage, c-*myc*, *mad*1 and *mxi*1 transcripts are detected, but N-*myc*, *mad*3 and *mad*4 transcripts are not (not shown). As differentiation progresses, the epidermis becomes further stratified, such that defined layers of proliferating and differentiating cells become apparent (Montagna *et al.*, 1974 and references therein). At 17.5 p.c., the lateral back epidermis consists of approximately five cell layers (Figure 7A). Together, the basal cell layer and the first suprabasal cell layer comprise the proliferating cell compartment, which at this stage is referred to as the Malpighian layer (Figure 7A, m). Cells of the Malpighian layer growth

arrest and differentiate concomitant with their migration to the second and third suprabasal cell layers (Figure 7A, sb), and finally to the outermost layer, the statum corneum (sc). Aggregates of dermal papilla anlage cells (Figure 7A, arrow), which provide the inductive signal for hair follicle development (Pisasarakit and Moore, 1986), are readily apparent at this stage adjacent to primary hair germs and developing hair follicles (Figure 7A, hf). Whereas c-*myc* expression is confined primarily to the proliferative Malpighian layer of the epidermis and to the dermal papilla and primary hair germ cells in the dermis (Figure 7B), N-*myc* transcripts appear confined to primary hair germ cells only (Figure 7C). This latter observation is consistent with a previous study (Mugrauer *et al.*, 1989). *mad*1 transcripts are detected in cells just above where c-*myc* is expressed (Figure 7D). These cells appear to be cell cycle arrested, differentiating cells of the suprabasal layers, and not the proliferating cell layers of the Malpighian layer. This is consistent with the expression pattern of *mad*1 in the adult epidermis (Hurlin *et al.*, 1995; Vastrik *et al.*, 1995). Similar to the situation in the neural tube at 10.5 p.c., *mxi*1 expression is not restricted to differentiating cells of the epidermis (Figure 7E). Instead, *mxi*1 is readily detected in the proliferating cell compartment, and its expression extends into the first differentiating cell layers, but decreases in the uppermost layer(s) of the epidermis (Figure 7E). *mad*3 expression was detected only in the uppermost differentiated cell layers underneath the stratum corneum (Figure 7F). Finally, *mad*4 transcripts are found in the dermis and hair follicles, as well as in some differentiating cells in the upper layers of the epidermis (Figure 7G). Thus, in the developing epidermis, expression of c-*myc*, *mxi*1, *mad*1 and *mad*3 is regulated in a differentiation-specific manner.

Discussion

An antagonistic relationship between Myc and Mad proteins

In this study, we have identified and characterized two Max-interacting bHLHZip proteins, Mad3 and Mad4, that are related to Mad (Mad1) and Mxi1 (Ayer *et al.*, 1993; Zervos *et al.*, 1993). We show that Mad3:Max and Mad4:Max repress transcription through binding to the same E-box sequences that mediate Myc:Max activation (Figure 3), as was previously demonstrated for Mad1 (Ayer *et al.*, 1993). The presence of a highly conserved region in the N-terminus of Mad3 and Mad4 required for interaction with mouse homologues of the yeast transcriptional corepressor Sin3 (SID, Figures 1 and 2), and for repression of transcription by Mad1 (Ayer *et al.*, 1995; Schrieber-Agus *et al.*, 1995), strongly suggests that transcriptional repression by Mad proteins is accomplished through a common mechanism. These results, combined with the observation that Mad3 and Mad4 significantly inhibit Myc dependent cell transformation (Figure 4), places them in a family of transcription repressors with the remarkable feature of being able to modulate the transforming activities of Myc (Lahoz *et al.*, 1994; Koskinen *et al.*, 1995; Schrieber-Agus *et al.*, 1995; Västrik *et al.*, 1995).

It is well established that Myc normally functions as a key regulator of cell proliferation, and when deregulated, can contribute to tumorigenesis *in vivo*. The ability of Mad proteins to suppress the transforming activities of Myc in cell culture systems leads to the prediction that expression of Mad family proteins may serve an important negative regulatory role in governing the biological activities of Myc *in vivo*. Our recent studies examining differentiation and tumorigenesis in the epidermis and colon indicate that *mad*1 expression is normally induced concomitant with growth arrest and differentiation in these tissues, and that loss of *mad*1 expression accompanies progression to invasive, poorly-differentiated cancers (Hurlin *et al.*, 1995; J.Arbeit, in preparation). Although the functional relationship between loss of *mad*1 expression and epithelial tumorigenesis has yet to be established, the known affects of these proteins on cell growth, and the differentiation-specific expression pattern of *mad*1 in the adult epidermis and colon (Hurlin *et al.*, 1994, 1995; Västrik *et al.*, 1995), support the notion that loss of *mad*1 expression is involved in malignant progression. The recent finding that allelic loss and mutation of *mxi*1 occurs in prostate cancers (Eagle *et al.*, 1995), provides the first evidence of disruption of a Mad family gene in tumours. It is notable that, based on the mouse chromosomal positions, the predicted human syntenic regions for Mad3 and Mad4 (Figure 5) are candidate regions for the presence of genes associated with a number of different tumour types. These include distal 5q deletions associated with acute myelogenous leukaemia, acute non-lymphocytic leukaemia and myelodysplastic syndrome (5q syndrome) (Westbrook and Le Beau, 1993) (Mad3) and bladder carcinoma (Elder *et al.*, 1994) (Mad4). In light of this, it will be important to establish relevant systems to study the relationship between tumorigenesis and the expression patterns of Mad3 and Mad4, and to examine the genetic loci of the various Mad family members in candidate tumours.

The Myc:Max:Mad network and differentiation

The opposing transcriptional activities exhibited by Myc and Mad family proteins suggest that their antagonistic biological activities are a manifestation of differential regulation of common target genes. If true, then regulation of the relative levels of Myc and Mad family members, and thus the composition of Max complexes, may be a principle mechanism determining the biological activities of this network of proteins. In examining the expression patterns of the *mad* and *myc* family genes during murine embryogenesis, we sought to identify tissues and biological settings where these genes, and the Max-interactor network may function. We found that *myc* and *mad* family genes are expressed in a compartmentalized fashion in tissues that exhibit a cellular architecture defined by populations of proliferating cells and growth-arrested differentiating cells. Clear examples of compartmentalized expression include the developing central nervous system at day 10.5 to 12.5 p.c. (Figure 6 and data not shown), the epidermis at day 17.5 p.c. (Figure 7), as well as the neural retina, limb buds and developing bone (C.Quéva, P.J.Hurlin and R.N.Eisenman, in preparation). As has been previously reported (Mugrauer *et al.*, 1988; Downs *et al.*, 1989; Wakahatsu, 1993), we found that c-*myc* and N-*myc* expression are generally, but not absolutely, associated with the proliferating compartments of tissues where differentiation is occurring (Figures 6 and 7 and data not shown). In

contrast, expression of *mad* 1, *mad*4, and to a lesser extent *mad*3, are generally restricted to cells undergoing differentiation (Figures 6 and 7 and data not shown).

*mxi*1 is unique among the *mad* genes in that it is expressed in both proliferating and differentiating cell compartments in the developing spinal cord and epidermis (Figures 6 and 7), as well as a variety of other tissues (not shown). These results are consistent with previous reports showing expression of *mxi*1 in both proliferating and differentiating myeloid leukaemia cell lines (Zervos *et al.*, 1993; Larsson *et al.*, 1994). Thus, *mxi*1 is typically expressed in proliferating cells simultaneously with c-*myc* and/or N-*myc*. In both the neural tube at day 10.5 p.c. and the epidermis at day 17.5 p.c., *mxi*1 expression overlaps c-*myc* expression, such that it extends into the compartments containing differentiating cells. This expression pattern predicts that an immediate consequence of c-*myc* downregulation during differentiation in these tissues would be an increase in the Mxi1:c-Myc ratio, and a shift in the heterodimer ratio to favour Mxi1:Max over c-Myc:Max. The induction of *mad*1, which appears to occur concomitant with downregulation of c-*myc* during differentiation in the developing spinal cord and epidermis (Figures 6 and 7), as well as in the adult epidermis (Hurlin *et al.*, 1995), suggests that both Mxi1:Max and Mad1:Max heterodimers may be present at early stages of differentiation. In support of this interpretation, we found that a rapid switch from c-Myc:Max to Mad1:Max heterodimers occurs during the differentiation of human keratinocytes in culture (Hurlin *et al.*, 1994, 1995). However, the lack of specific Mxi1 antisera has precluded a study of Mxi1 expression. As differentiating cells migrate to more suprabasal layers in the epidermis at 17.5 p.c., *mxi*1 is downregulated, *mad*3 transcription is induced, and *mad*1 continues to be expressed (Figure 7). This sequence of *mad* gene expression during epidermal differentiation differs somewhat from that seen during differentiation in the developing spinal cord at 10.5 p.c. In the spinal cord at 10.5 p.c., *mad*3 expression is downregulated during differentiation, *mad*1 and *mad*4 are induced, and *mxi*1, although expressed in both the ventricular and intermediate zones, is upregulated in the intermediate zone (Figure 6). Despite these differences, these tissues appear to be similar with respect to the induction or upregulation of different *mad* genes during differentiation.

How might the expression patterns observed for the Mad and Myc family genes relate to their function? Numerous studies have implicated Myc proteins as key regulators in the differentiation programmes of a variety of cell types. Evidence of a role for Myc in differentiation is based primarily on the observations that its expression is typically downregulated upon induced differentiation of cells in culture, and that ectopic expression of c-Myc can inhibit differentiation of several different cell types (for review see Lücsher and Eisenman, 1990; DePinho *et al.*, 1991; Marcu, 1992). The importance of tight control over *myc* expression in tissues undergoing differentiation is emphasized by the ability of deregulated *myc* expression to not only inhibit differentiation, but promote tumorigenesis. Although Myc proteins are highly regulated at the level of transcription, protein synthesis and degradation (for review see Spencer and Groudine, 1991), the ability of Mad proteins to suppress Myc dependent cell proliferation (Figure 4, Lahoz *et al.*, 1994; Koskinen *et al.*, 1995; Schreiber-Agus *et al.*, 1995; Västrik *et al.*, 1995; M.Roussel, D.Ayer and R.Eisenman, unpublished data) predicts that expression of Mad proteins provides an additional mechanism to regulate Myc. Furthermore, the induction or upregulation of *mad* genes and proteins in differentiating tissues suggests that Mad proteins may function in concert with downregulation of Myc in initiating and/or maintaining differentiaiton programmes. However, *myc* gene expression is not always restricted to proliferating cells, but is also found in post-mitotic differentiating cells (Figure 6, for review see DePinho, 1991). These results have been difficult to reconcile, based on the demonstrated growth promoting activities of Myc. We found that *mad*1, *mxi*1 and *mad*4 are co-expressed with c-*myc* in post-mitotic differentiating cells residing in the ventral intermediate zone and the roof plate of the neural tube at 10.5 p.c. (Figure 6), as well as in other post-mitotic cells in many other tissues and at different developmental stages (C.Quéva, P.J.Hurlin and R.N.Eisenman, in preparation). Co-expression of *myc* and *mad* genes may result in competition between Myc and Mad proteins for functional Max heterodimers. Thus, sufficiently high levels of Mad proteins might be expected to provide a growth inhibitory affect, overriding the growth promoting activities of Myc. Furthermore, Myc functions that are independent of Max would be expected to predominate in such a situation. Recent evidence suggests that Myc may act as a transcriptional repressor at initiator elements in a Max independent manner (Roy *et al.*, 1993; Li *et al.*, 1994). Therefore, co-expression of Myc with Mad family proteins could potentially permit a specific subset of Myc functions (e.g. transcriptional repression). On the other hand we do not know whether Myc protein has any function in differentiated cells since, in the cases examined, it has been shown to be predominantly confined to the cytoplasm (Craig *et al.*, 1993; Wakamatsu *et al.*, 1993), and is presumably inactive.

It is interesting that the Max-interactor network appears to be in some ways analogous to other transcription factor networks that regulate target gene expression through mechanisms involving differential dimerization. In the case of neurogenesis and myogenesis, cell fate and differentiation decisions are regulated by dimerization between different combinations of bHLH components, resulting in positive or negative acting complexes (for reviews see Jan and Jan, 1993; Weintraub, 1993; Lassar and Munsterberg, 1994). Implicit in the functioning of these networks is the notion that cell fate and differentiation decisions are governed by the relative levels of the constituent members, all of which require heterodimerization with a constitutively expressed cofactor for DNA binding and target gene activation. Even though members of these networks exhibit some redundant functions, a hierarchial relationship exists with respect to their specific roles in myogenesis and neurogenesis. This hierarchial relationship is determined, at least in part, by their temporally regulated and tissue specific expression patterns during development. The temporal aspect to their regulation appears to be important for the precise coordination of phenotypic transitions governed by these bHLH networks. In the case of the Max-interactor network, dedicated repressors (Mad proteins) may be functionally analogous

to HLH proteins that lack a basic region (e.g., Id; Benezra *et al.*, 1990) and provide a negative regulatory role in the myogenic and neurogenic pathways. However, whereas many of the components of these bHLH networks are restricted to defined tissues, members of the Max-interactor bHLHZip network are expressed in a wide variety of tissues and cell types. Thus, the coordinated expression and concerted action of Max-interacting proteins may provide a more general mechanism for the control of cellular transitions from proliferation to differentiation.

Materials and methods

Isolation of Mad3 and Mad4

A yeast two-hybrid screen was performed essentially as previously described (Vojtek *et al.*, 1993; Hollenberg *et al.*, 1995). A yeast reporter strain was constructed that contained the plasmid pBTM116-Max9. This plasmid contains the entire Max9 (Blackwood *et al.*, 1991) open reading frame fused in-frame to the LexA DNA binding domain. A mouse embryonic (day 9.5 and 10.5 p.c.) library of cDNA fragments fused to VP16 (Hollenberg *et al.*, 1995) was used to transform the reporter strain. Approximately 1.5×10^7 transformants were screened for the ability to grow on His^- medium, and for LacZ expression (β-galactosidase activity). Ten of the 67 positive clones isolated were tested for non-specific interaction by mating them with a yeast strain containing LexA-Lamin (Vojtek *et al.*, 1993). Because only one of the ten clones tested positive in this assay, plasmids containing the positive cDNAs were rescued from all of the original clones, and sequenced using an Applied Biosystems automated sequencing apparatus. cDNA sequences were compared with the combined PIR, GENPEPT and SWISSPROT databases.

Putative full-length *mad*3 and *mad*4 cDNAs were isolated from a mouse AB1 embryonic stem cell library (Chen *et al.*, 1994) using the cDNA fragments recovered from the two-hybrid screen as probes. The *mad*3 and *mad*4 cDNAs were cloned into the plasmid pBS (Stratagene) and both strands sequenced.

In vitro binding assays

The Mad3 and Mad4 full-length open reading frames, and *mad*3 and *mad*4 cDNAs beginning at amino acid position 32 and amino acid position 65, respectively (each lacking the N-terminal SID region, but containing the HLHZip region) were cloned into the pGEX-2T vector, and GST fusion proteins produced and purified as recommended by the manufacturer (Pharmacia). For GST fusion interaction assays, GST–Mad3 and Mad4 proteins were incubated at 4°C for 1 h with various combinations of [^{35}S]methionine labelled *in vitro* translated Max (Blackwood and Eisenman, 1991), mSin3A or mSinB (Ayer *et al.*, 1995) proteins in L-Buffer (phoshate-buffered saline and 0.4% NP-40). Proteins were then recovered on glutathione–Sepharose beads, washed four times with L-Buffer at 4°C, and analysed on SDS–polyacrylamide gels.

CAT assays

NIH 3T3 cells were transfected with plasmids (pSP) containing cDNAs under the control of the SV40 early region promoter and enhancer. Transfection efficiencies were normalized using a co-transfected β-Gal expressing plasmid, and CAT assays were performed as previously described (Kretzner *et al.*, 1992; Ayer *et al.*, 1993). CAT assays were quantitated using a Molecular Dynamics PhosphoImager.

Rat embryo fibroblast transformation assays

Rat embryo fibroblasts (REF) were prepared from 13-day old Fischer rat embryos, grown in DMEM supplemented with 10% fetal bovine serum (FBS) and passaged once before transfecting them using the calcium phosphate precipitation technique (Chen and Okyama, 1987). For REF transfections, the *mad*3 and *mad*4 cDNAs were transfered to the pLTRpoly vector (Mäkelä *et al.*, 1992a). The transfection mixes included 2 μg of pLTR-Tc-*myc* (Koskinen *et al.*, 1994), 3 μg of pGEJ(6.6) expressing the activated c-Ha-*ras*^{Val12} oncogene (Mäkelä *et al.*, 1992b) and 3 μg of either pLTR*mad*1 (Koskinen *et al.*, 1995), pLTR*mad*3 or pLTR*mad*4. One microgram of the CMV-β-Gal vector pCH110 (Pharmacia) was included to control for transfection efficiency (Geballe and Mocarski, 1988). To obtain a total of 12 μg of DNA, appropriate amounts of the empty pLTRpoly vector were added. The transfected cells were split in a 1:6 ratio and grown in DMEM supplemented with 5% FBS. Medium was replenished every 3 days, and transformed foci scored 14 days after transfection.

Interspecific mouse backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J×*M.spretus*) F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 F_2 mice was used to map the *Mad*3 and *Mad*4 loci (see above for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described (Jenkins *et al.*, 1982). All blots were prepared with Hybond N^+ nylon membrane (Amersham). For probes, *mad*3 and *mad*4 cDNAs were labelled with [α-^{32}P]dCTP using a random priming labelling kit (Stratagene). Southern blots were washed to a final stringency of 0.2× SSCP, 0.1% SDS, 65°C. For *Mad*3, fragments of 4.5 and 0.5 kb were detected in *Sac*I digested C57BL/6J DNA and fragments of 4.3 and 0.5 kb in *Sac*I digested *M.spretus* DNA. The presence or absence of the 4.3 kb *M.spretus* specific fragment was followed in backcross mice. For Mad4, a 2.2 kb *Sac*I fragment was detected in C57BL/6J DNA and a 5.1 kb fragment in *Sac*I digested *M.spretus* DNA. The presence or absence of the 5.1 kb fragment was followed in backcross mice.

Probes and RFLPs for loci linked to *Mad*3 and *Mad*4 have been described. These include G-protein-coupled receptors 1 and 15 (*Gpcr*1 and *Gpcr*15) (Wilkie *et al.*, 1993), Dek (*D13H6S231e*), interleukins 6 and 9 (*Il6* and *Il9*), fibroblast growth factor receptors 3 and 4 (*Fgfr*3 and *Fgfr*4) (Avraham *et al.*, 1994) and Tec kinase (Mano *et al.*, 1993). Recombination distances were calculated as described (Green, 1981) using the computer programme SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

In situ hybridization

The following plasmids, each containing full-length murine cDNAs were linearized, and used as templates to make antisense RNA probes: pc-Myc, pN-Myc, pVZ1Max9, pMuMad, pMuMxi, pBSMad3 and pBSMad4. Riboprobes specific for murine c-*myc*, N-*myc*, *max*, *mad*1, *mxi*1, *mad*3 and *mad*4 transcripts were synthesized using the appropriate RNA polymerases in the presence of both [^{35}S]CTP and [^{35}S]UTP (NEN).

The protocol for *in situ* hybridization was described in Quéva *et al.* (1992). After deparaffinization and hydration, 5 μm sections were incubated in 0.1 M glycine, 0.2 M Tris–HCl, pH 7.4 for 10 min at room temperature, treated with 1 μg/ml proteinase K (Boehringer Mannheim) for 15 min at 37°C and further fixed in 4% paraformaldehyde in PBS. The slides were subsequently washed in PBS, acetylated and dehydrated. Prior to hybridization, the probe were diluted to 50 000 c.p.m./μl in the hybridization buffer [50% formamide, 0.3 M NaCl, 20 mM Tris–HCl (pH 7.9), 5 mM EDTA, 10% dextran sulfate, 1× Denhardt's solution, 0.5 mg/ml *Escherichia coli* tRNA and 100 mM DTT]. Hybridization was performed 65°C for 16 h. Thereafter, the slides were washed in 4× SSC, 10 mM DTT for 1 h at room temperature, and in 50% formamide, 0.15 M NaCl, 20 mM Tris–HCl (pH 7.9), 5 mM EDTA, 100 mM DTT at 68°C for 30 min. The sections were subsequently treated with 20 μg/ml of RNase A for 30 min to 1 h at 37°C, incubated 15 min at 65°C in 2× SSC and 15 min at 65°C in 0.1× SSC. The slides were dehydrated and dipped in Kodak NTB2 emulsion diluted 1:1 with 0.6 M ammonium acetate. After 2 weeks exposure at 4°C, the slides were developed, stained with the Hoechst dye 33258 (bisbenzimide) to visualize nuclei, and mounted with a mixture of 2 g of Canada Balsam and 1 ml of methylsalicylate. Sections were examined under dark-field and epifluorescence illumination with a Zeiss microscope (axioplan).

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