

## SHORT REPORT

## Mapping of two genes encoding members of a distinct subfamily of MAX interacting proteins: *MAD* to human chromosome 2 and mouse chromosome 6, and *MXII* to human chromosome 10 and mouse chromosome 19

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Both the *MAD* and the *MXII* genes encode basic-helix-loop-helix-leucine zipper (bHLH-Zip) transcription factors which bind Max *in vitro*, forming a sequence-specific DNA-binding complex similar to the Myc-Max heterodimer. Mad and Myc compete for binding to Max. In addition, Mad has been shown to act as a transcriptional repressor while Myc appears to function as an activator. Mxii also appears to lack a transcriptional activation domain. Therefore, Mxii and Mad might antagonize Myc function and are candidate tumor suppressor genes. We report here the mapping of the *MAD* and *MXII* genes in human and mouse by fluorescence *in situ* hybridization (FISH) and by recombination mapping. The *MAD* gene was mapped to human chromosome 2 at band p13 by FISH and to mouse chromosome 6 by meiotic mapping. The *MXII* gene was mapped to human chromosome 10 at band q25 and on mouse chromosome 19 at region D by FISH. There was a second site of hybridization on mouse chromosome 2 at region C, which may represent a pseudogene or a related sequence. The mapping results confirm regions of conservation between human chromosome 2p13 and mouse chromosome 6 and between chromosome 10q25 and mouse chromosome 19D. Human chromosomes 2p13 and 10q25 have been involved in specific tumors where the role of Mad and Mxii can now be investigated.

The *MAD* and *MXII* genes encode proteins that belong to a distinct subfamily of Max interacting proteins (Ayer *et al.*, 1993; Zervos *et al.*, 1993). Max was shown to be a protein capable of specifically interacting with the Myc protein family by forming heterodimers mediated by their basic-helix-loop-helix-leucine zipper (bHLH-Zip) interaction domains (Cole, 1991; Blackwood *et al.*, 1992). The carboxy terminus containing the HLH-Zip domain mediates the protein-protein interaction, while the basic region appears to be required for the formation of a sequence-specific DNA-binding complex (Blackwood & Eisenman, 1991; Prendergast *et al.*, 1991). Binding to Max

was shown to be essential for Myc transcription and transforming activity, while Myc homodimers are inactive (Kretzner *et al.*, 1992; Amati *et al.*, 1993).

Ayer *et al.* (1993) screened a  $\lambda$ gt11 expression library with radiolabeled Max and identified a cDNA encoding a new member of the bHLH-Zip protein family, Mad. Like Myc, Mad does not form homodimers nor interact with other bHLH-Zip proteins tested. Mad preferentially forms heterodimeric complexes with Max demonstrating significantly increased binding activity for CACGTG consensus sequences compared to Max homodimers (Ayer *et al.*, 1993). The Mad-Max heterodimer complex acts as a transcriptional repressor, whereas the Myc-Max heterodimer leads to transcriptional activation, when tested on a simple promoter construct containing a reiterated CACGTG binding site (Ayer *et al.*, 1993). Mad protein has been shown to be induced upon myeloid differentiation and to replace Myc in complexes with Max (Ayer & Eisenman, 1993).

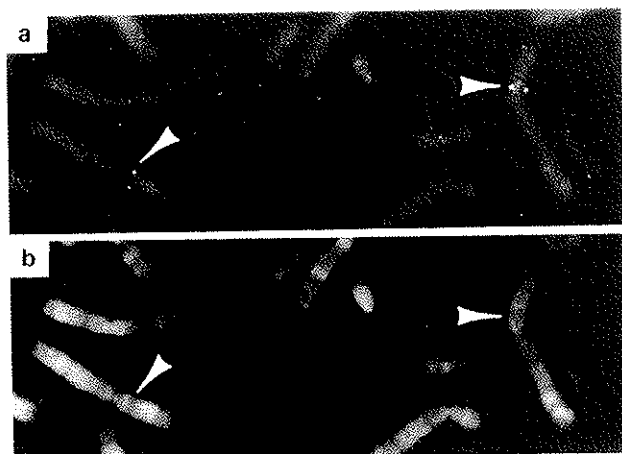
Using a yeast 'interaction trap' or 'two-hybrid' system, Zervos *et al.* (1993) isolated another human protein that interacts specifically with Max, Mxii (for Max interacting protein 1). Mxii contains a bHLH-Zip motif similar to that found in the Myc family of proteins. The similarity between Mxii and Mad is 79%, with identity scores ranging from 43 to 59%, depending on alignment strategy, which suggests an evolutionary relationship (Ayer *et al.*, 1993; Zervos *et al.*, 1993). Mxii in yeast does not associate with itself or with other bHLH-Zip, bZip or bHLH proteins. Mxii is expressed in all normal human tissues and its mRNA is induced dramatically in myeloid cells that are stimulated to differentiate (Zervos *et al.*, 1993). Mxii and Mad may negatively regulate Myc function in two ways: first, by sequestering Max required for binding to target DNA, and second by forming complexes with Max which compete with transcriptionally active Myc-Max heterodimers for the same binding sites and which may negatively regulate Myc-activated genes (Ayer *et al.*, 1993; Ayer & Eisenman, 1993; Zervos *et al.*, 1993).

As the *MAX*, *MAD* and *MXII* genes are potentially involved in tumor suppression, it is of interest to determine the chromosomal location of these genes. *MAX* was previously mapped to human chromosome 14q22-24 and mouse chromosome 12D by *in situ* hybridization

(Gilladoga *et al.*, 1992). We report here the mapping of the *MAD* and *MXII* genes. Three methods, radioactive and fluorescence *in situ* hybridizations (FISH) and recombination mapping, were used to map these genes.

The probe used for the *in situ* hybridization of the *MAD* gene was a 3.2 kb cDNA clone which represents the entire human *MAD* coding region (Ayer *et al.*, 1993). *In situ* hybridization to metaphase chromosomes from lymphocytes of two normal male donors was carried out using the  $^3\text{H}$ -labeled probe to a specific activity of  $2\text{--}3 \times 10^7$  c.p.m.  $\mu\text{g}^{-1}$  as described (Marth *et al.*, 1986). The final probe concentration was  $0.02\text{--}0.05 \mu\text{g} \mu\text{l}^{-1}$  of hybridization mixture. The slides were exposed for 2–4 weeks. Chromosomes were identified by Q-banding and visualized by fluorescence microscopy. A total of 55 metaphase cells with autoradiographic grains were examined. Of 89 sites of hybridization scored, 30 (34%) were located on the proximal portion of the short arm of chromosome 2. The largest number of grains (23) was at band p13. There was no significant hybridization on other human chromosomes. The hybridization was repeated using a biotin-labeled probe for FISH following the protocol described below for the *MXII* probe. The FISH results confirmed the mapping of *MAD* to chromosome 2p13 (Figure 1).

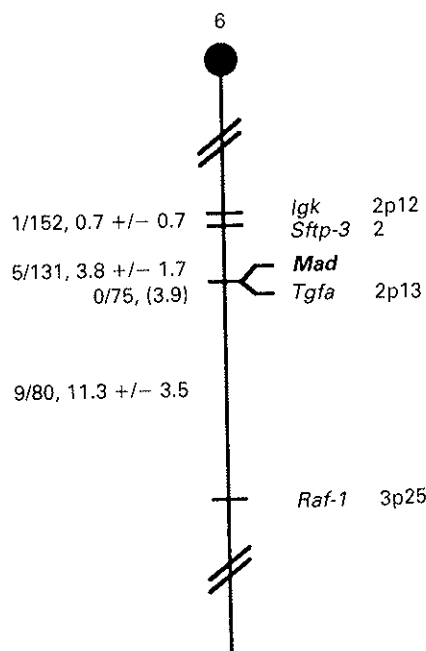
The mouse chromosomal location of *Mad* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J  $\times$  *Mus spretus*)F<sub>1</sub>  $\times$  C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 1300 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland & Jenkins, 1991). A total of 205 N<sub>2</sub> mice were used to map the *Mad* locus. 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 Zetabind nylon membrane (AMF-Cuno). The probe, a 1 kb *NotI*–*EcoRI* fragment of the human *MAD* cDNA, was labeled with [ $\alpha^{32}\text{P}$ ]dCTP using a random primed labeling kit (Stratagene); washing was done to a final stringency of  $1.0 \times \text{SSCP}$ , 0.1% SDS at 65°C. C57BL/



**Figure 1** Human metaphase chromosomes hybridized to a biotinylated 3.2 kb cDNA probe for the *MAD* gene. (a) Hybridization of *MAD* detected with fluorescein on metaphase chromosomes. The probe hybridized to the short arms of both chromosomes 2 at band p13, indicated by the arrows, and identified in (b) by their chromosome banding after staining with Hoechst-actinomycin D

6J and *M. spretus* DNAs were digested with several enzymes and analysed by Southern blot hybridization for informative RFLPs using the human cDNA *MAD* probe. A major fragment of 3.6 kb was detected in *PstI*-digested C57BL/6J DNA and a major fragment of 6.6 kb was detected in *PstI*-digested *M. spretus* DNA. The presence or absence of the 6.6 kb *M. spretus*-specific *PstI* fragment was followed in backcross mice.

A description of most of the probes and restriction fragment length polymorphisms (RFLPs) for the loci linked to *Mad* including immunoglobulin kappa chain (*Igk*), SP-B pulmonary surfactant protein (*Sftp-3*), and *ras*-related fibrosarcoma oncogene (*Raf-1*) has been reported previously (Moore *et al.*, 1992). One locus not previously reported is transforming growth factor  $\alpha$  (*Tgfa*). The probe was a fragment of mouse cDNA kindly provided by Brigid Hogan (Vanderbilt University, Nashville, TN). The probe detected a 6.6 kb *KpnI* fragment in C57BL/6J DNA and a 10.5 kb *KpnI* fragment in *M. spretus* DNA. Recombination distances between loci were calculated in pairwise combination as described (Green, 1981) using the computer program SPRETUS MADNESS. The gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns. The mapping results shown in Figure 2 indicate



**Figure 2** Mouse chromosomal location of *Mad*. The gene was mapped by interspecific backcross analysis. The number of recombinant N<sub>2</sub> animals is presented over the total number of N<sub>2</sub> animals typed, to the left of the chromosome maps, between each pair of loci. The recombination frequencies, expressed as genetic distance in centimorgans ( $\pm$  one standard error) are also shown. The upper 95% confidence limit of the recombination distance is given in parentheses when no recombinants were found between loci. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns. The positions of loci on human chromosomes, where known, are shown to the right of the chromosome maps. References for the map positions of most human loci 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)

that *Mad* is located in the central region of mouse chromosome 6 linked to *Igk*, *Sftp-3*, *Tgfa* and *Raf-1*. *Mad* maps very close to *Tgfa*, distally to *Sftp-3* and *Igk*. These results confirm a region of conservation between human chromosome 2p11-13 and mouse chromosome 6 (Human Gene Mapping 10.5, 1990; Nadeau *et al.*, 1992).

For the mapping of the *MXII* gene, a cDNA probe containing a 2.4 kb insert (pMxi1) was labeled with biotin-11-dUTP by nick translation (Gibco BRL) and used for FISH analysis. The size of the product was determined to be between 200 and 400 bp. Prometaphase chromosome preparations from a normal male were denatured in the presence of 70% formamide at 70°C. The hybridization of pMxi1 was carried out following a modified protocol from Moyzis *et al.* (1987). The hybridization mixture contained 50% formamide, 2 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and 10% dextran sulfate. After overnight incubation at 37°C in a moist chamber, the slides were washed in 4 × SSC, 50% formamide at 40°C. The hybridization signals were detected using a detection system from Vector Laboratories (Burlingame, CA). After a blocking step with goat serum, the biotin label was reacted to fluorescein avidin DCS as primary antibody, then to biotinylated anti-avidin D and finally again to the primary antibody.

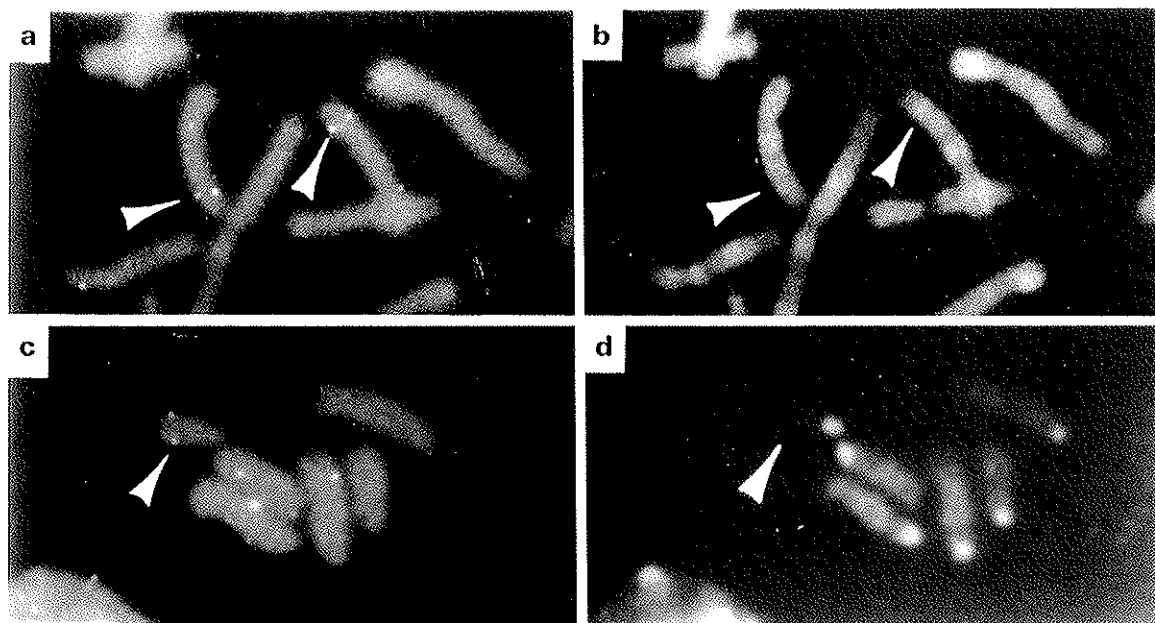
Of 50 human metaphase cells examined after hybridization to a probe for the *MXII* gene, 19 (38%) showed signals on one or both chromosomes 10 at band q25 (Figure 3a and b). There was no significant hybridization to other chromosomes. Thus we conclude that the *MXII* gene maps to human chromosome 10q25.

For the mapping of the mouse *Mxi1* gene, mouse metaphase chromosomes from spleen lymphocytes of a

male C57BL/6J mouse were obtained. A slightly modified protocol of Lemieux *et al.* (1992) was followed for the hybridization. The hybridization mixture contained 50% (vol/vol) formamide, 2 × SSC, 10% (wt/vol) dextran sulfate, 40 mM sodium phosphate, 0.1% NaDodSO<sub>4</sub>, and 1 × Denhardt's solution at pH 7.0. After overnight incubation at 37°C in a moist chamber, the slides were washed once in 2 × SSC, 50% formamide followed by one rinse in 2 × SSC at 37°C. The hybridization signals were detected using a detection system from Vector Laboratories (Burlingame, CA). After incubation with goat anti-biotin antibody, the slides were rinsed in modified phosphate-buffered saline (PBS) (0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 0.15 M Na<sub>2</sub>HPO<sub>4</sub>, 0.15 M NaCl, 0.1% Tween 20, and 0.15% bovine serum albumin). A second incubation with fluorescein-labeled anti-goat IgG and a rinse in modified PBS followed. The chromosomes were banded using Hoechst 33258-actinomycin D staining and counterstained with propidium iodide. The chromosomes and hybridization signals were visualized by fluorescence microscopy, using a dual band pass filter (Omega or Chroma, Brattleboro, Vermont).

Of 146 mouse metaphase cells examined, 26 (17.8%) showed signals on one or both chromosomes 19 at region D (Figure 3c and d). In 10 cells (6.8%) there was a second site of hybridization on chromosome 2 region C. We conclude that the *Mxi1* gene maps to mouse chromosome 19D with a second site of hybridization to chromosome 2C which may represent a pseudogene, or a related DNA sequence. These results confirm a region of conservation between human chromosome 10q24-26 and mouse chromosome 19D (Human Gene Mapping 10.5, 1990; Nadeau *et al.*, 1992).

Our mapping of the *MAD* and *MXII* genes to



**Figure 3** Human (a and b) and mouse (c and d) metaphase cells hybridized to a biotinylated 2.4 kb cDNA probe for the *MXII* gene. (a) Hybridization of *MXII* detected with fluorescein on human metaphase chromosomes. The probe hybridized to the distal long arms of both chromosomes 10 at band q25, indicated by the arrows, and identified in (b) by their chromosome banding after staining with Hoechst-actinomycin D. (c) Hybridization of *Mxi1* detected with fluorescein on mouse metaphase chromosomes. The probe hybridized to chromosome 19 region D indicated by the arrow and identified in (d) by its chromosome banding after staining with Hoechst-actinomycin D

human chromosome 2p13 and 10q25, respectively, points to specific human disorders in which these genes may be implicated. Chromosome 2 band p13 has been involved in several chromosomal translocations associated with Philadelphia chromosome positive precursor B-cell acute lymphoblastic leukemia (Inaba *et al.*, 1991), childhood chronic lymphocytic leukemia (Richardson *et al.*, 1992) and other tumors, such as keratoacanthoma and basal cell papilloma (Mertens *et al.*, 1989). Chromosome 10 is frequently lost in tumor cells from glioblastoma (Bigner *et al.*, 1990), suggesting the presence of a tumor suppressor gene on this chromosome. The common region of deletion or loss of heterozygosity in these tumors was located in 10q24-q26 (Rasheed *et al.*, 1992). There are also several fragile sites on 10q which were demonstrated in von Hippel-Lindau syndrome patients (Neumann *et al.*, 1988) and breast cancer patients (Ochi *et al.*, 1988). Regarding

these findings, the mapping of *MXII* to 10q25 should be of interest as it might play a role as a tumor suppressor gene. For *MAD* the rearrangements previously observed in leukemias do not appear to involve obvious loss of DNA but may nonetheless involve inactivating events. The data reported here therefore provide impetus for a more detailed examination of *MXII* and *MAD* gene structure and its potential alteration in malignancy.

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