Expression of the *mad* Gene during Cell Differentiation In Vivo and Its Inhibition of Cell Growth In Vitro

Imre Västrik,* Arja Kaipainen,* Tarja-Leena Penttilä,§ Athina Lymboussakis,‡ Riitta Alitalo,‡ Martti Parvinen,§ and Kari Alitalo

*Molecular/Cancer Biology Laboratory, and ‡Transplantation Laboratory, Haartman Institute, University of Helsinki, P.O.B. 21, (Haartmanink. 3), 00014 Helsinki, Finland; §Department of Anatomy, University of Turku, Kiinamyllynk. 10, 20520 Turku, Finland

Abstract. Mad is a basic region helix-loop-helix leucine zipper transcription factor which can dimerize with the Max protein and antagonize transcriptional activation by the Myc–Max transcription factor heterodimer. While the expression of Myc is necessary for cell proliferation, the expression of Mad is induced upon differentiation of at least some leukemia cell lines. Here, the expression of the *mad* gene has been explored in developing mouse tissues. During organogenesis in mouse embryos *mad* mRNA was predominantly expressed in the liver and in the mantle layer of the developing brain. At later stages *mad* expression was detected in neuroretina, epidermis, and whisker follicles, and in adult mice *mad* was expressed at variable levels in most organs analyzed. Interestingly, in the skin *mad* was highly expressed in the differentiating epidermal keratinocytes, but not in the underlying proliferating basal keratinocyte layer. Also, in the gut *mad* mRNA was abundant in the intestinal villi, where cells cease proliferation and differentiate, but not in the crypts, where the intestinal epithelial cells proliferate. In the testis, *mad* expression was associated with the completion of meiosis and early development of haploid cells. In cell culture, Mad inhibited colony formation of a mouse keratinocyte cell line and rat embryo fibroblast transformation by Myc and Ras. The pattern of *mad* expression in tissues and its ability to inhibit cell growth in vitro suggests that Mad can cause the cessation of cell proliferation associated with cell differentiation in vivo.

MEMBERS of the *myc* proto-oncogene family (c-, N-, and L-*myc*) regulate cell proliferation and are commonly activated in various types of neoplasia (for reviews see Alitalo et al., 1992; Blackwood et al., 1992*a*; Evan and Littlewood, 1993; Koskinen and Alitalo, 1993; Västrik et al., 1994). c-*myc* is expressed in most growing cells and it is rapidly induced upon growth stimulation of quiescent cells (Kelly et al., 1983; Waters et al., 1991). The ectopic expression of Myc induces quiescent cells to reenter the cell cycle (Armelin et al., 1984; Eilers et al., 1991). On the other hand differentiation can be induced or inhibited by decreasing or increasing the levels of the Myc proteins, respectively (Griep and Westphal, 1988; Holt et al., 1988; Larsson et al., 1988; Miner and Wold, 1991).

During development, the three *myc* genes have distinct spatial and temporal expression patterns (Downs et al., 1989; Hirvonen et al., 1990; Morgenbesser and DePinho, 1994; Stanton et al., 1992). In general, differentiation or cessation of cell proliferation is associated with downregulation of the *myc* mRNA and protein levels, although in some cases the expression of the *myc* genes can be observed in cells that have ceased proliferation (see Lüscher and Eisenman, 1990). For example, ganglion cells in the inner layer of the neural retina and postmitotic neuronal cells in the mantle layer of telencephalon sustain N-*myc* expression after cessation of proliferation (Hirning et al., 1991; Hirvonen et al., 1990; Mugrauer et al., 1988). In embryonic gut epithelium N-*myc* is expressed in non-proliferating cells covering the villi, while proliferating cells in the crypts express c-*myc* (Hirning et al., 1991).

Myc proteins are thought to function as transcription factors (Blackwood et al., 1992*a*). They have an NH_2-terminal transcription activation domain and a COOH-terminal basic region-helix-loop-helix-leucine zipper domain (bHLHZip) (Kato et al., 1990; Landschulz et al., 1988; Murre et al., 1989), which mediates dimerization with the Max protein and recognition of the DNA sequence CACGTG (Blackwell et al., 1990; Blackwood and Eisenman, 1991). Dimerization with Max is essential for activation of transcription from CACGTG-containing promoters and for the oncogenic activity of the Myc proteins (Amati et al., 1992, 1993). Max is a constitutively expressed bHLHZip protein (Blackwood

Imre Västrik and Arja Kaipainen both contributed equally to this work.

Please address all correspondence to Dr. Kari Alitalo, Molecular/Cancer Biology Laboratory, Haartman Institute, University of Helsinki, P.O.B. 21 (Haartmanink. 3), 00014 Helsinki, Finland. Tel.: 358 434 6723. Fax: 358 434 6348.

and Eisenman, 1991; Västrik et al., 1993). Max can also form homodimers (Kato et al., 1992), which bind to the CACGTG-containing core DNA sequence, but unlike Myc–Max heterodimers, Max homodimers suppress transcriptional activation (Amati et al., 1993). Accordingly, ectopic overexpression of Max suppresses also the oncogenic activity of the Myc proteins (Amati et al., 1993; Mäkelä et al., 1992*a*).

Max can dimerize and bind DNA with at least two additional bHLHZip proteins, named Mad and Mxil, which are not able to form homodimers (Ayer et al., 1993; Zervos et al., 1993). Mad has been shown to repress transcription from CACGTG-containing promoter constructs (Ayer et al., 1993). Although Mxil has not been tested for such repressor activity, it does not seem to activate transcription in a heterologous yeast system (Zervos et al., 1993). Thus, it is likely that the relative abundance of Myc in relation to Mad and Mxil, as well as the level of Max, determine the activity of Myc–Max target genes.

Max protein is ubiquitously expressed and very stable (Blackwood et al., 1992*b*). As Mad and Mxil have been only recently cloned, there is little data on their expression. Interestingly however, it has been shown that at least in certain cells Mad and/or Mxil are induced upon differentiation in vitro. For example, upon induction of monocyte/macrophage differentiation of U937 leukemia cells the expression of both *mad* and *mxil* mRNAs is upregulated while the expression of c-*myc* mRNA declines (Ayer and Eisenman, 1993; Larsson et al., 1994; Zervos et al., 1993). An analogous shift is observed also in the composition of Max heterodimers. While in undifferentiated U937 cells Max is found complexed with c-Myc, differentiation is accompanied by a shift from Myc–Max heterodimers to Mad–Max heterodimers (Ayer and Eisenman, 1993). On the basis of these observations it has been suggested that the switch from Myc–Max to Mad–Max or Mxil–Max complexes represses Myc–Max target genes involved in cell proliferation and maintenance of the undifferentiated state (Ayer and Eisenman, 1993). This raises the interesting possibility that Mad and Mxil may be required for development, because they counterbalance the growth promoting effects of Myc proteins and promote the cessation of cell proliferation associated with differentiation. To further investigate this possibility we have studied *mad* expression during mouse embryonic development and in adult mouse and rat tissues undergoing continuous cell proliferation and differentiation.

Materials and Methods

Cloning of Mouse mad cDNA

The coding region of human *mad* was PCR amplified from cDNA made from K562 leukemia cell RNA using two sets of nested primers flanking the open reading frame of the published human *mad* cDNA sequence (Ayer et al., 1993). The initial PCR was performed with upstream primer 5′-CAT AGC GGG CTC CAC AGC-3′ and downstream primer 5′-AGG AGA CAG CCG CAG TGC-3′. The second PCR was performed with upstream primer 5′GC*T CTA GA*C CCC GGT GCA GAA TGG-3′ and downstream primer 5′G*GA ATT C*AC TCT CTT AGA GAC C-3′. The resulting DNA fragment containing the human *mad* coding region was subcloned into the XbaI and EcoRI sites of pGEM3Zf(+) using the respective sites in the second set of PCR primers (shown in italics), verified by partial sequencing, and used as a probe for screening a mouse cDNA library.

Screening of ~1.5 × 10^6 plaques of a 12-d p.c. mouse embryo cDNA library in the λEXlox vector (Novagen, Madison, WI) with human *mad* cDNA probe resulted in one positive clone containing a 2.5-kb insert. Partial sequencing revealed in the 5′ part of the clone an open reading frame of 226 amino acid residues homologous to the human Mad protein.

RNase Protection Assay

A KpnI fragment, containing 11 bp of the vector sequence and nucleotides 1-297 of the mouse *mad* cDNA was subcloned into the KpnI site of pGEM3Zf(+). An in vitro transcription template was prepared by PCR amplification with the M13 universal and reverse sequencing primers. Mad antisense cRNA was synthesized using T7 polymerase and [^{32}P]UTP. The mouse β-actin cRNA was similarly synthesized from nucleotides 1188-1279 of the published cDNA sequence (Tokunaga et al., 1986). After purification in a 6% polyacrylamide/7 M urea gel, the labeled transcripts were hybridized to 30 μg of total RNA overnight at 55°C. Single-stranded RNA was then digested with RNase T1 and RNase A at 30°C and purified protected fragments were analyzed in a 6% polyacrylamide/7 M urea gel.

Total RNA was isolated from 8–18-d p.c. embryos and 1-d-old mice by guanidium thiocyanate–phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The sample from 8-d p.c. embryos included also the placenta.

Microdissection of Seminiferous Tubules, RNA Isolation, and Northern Blots

Pooled segments of rat seminiferous tubules representing different stages of the spermatogenic cycle were collected by transillumination-assisted microdissection (Parvinen, 1993). Total RNA was isolated as above and 10 μg from each pool was fractionated in 1.2% agarose gels containing formaldehyde, stained in ethidium bromide to visualize the rRNAs, and transferred by blotting to GeneScreen (DuPont) filters. A PCR amplified probe corresponding to nucleotides 215-804 of mouse *mad* cDNA was used for analysis of *mad* expression.

Embryos and Tissues

Mouse embryos were derived from matings of CBA and NMRI mice. Pregnant mice were killed by cervical dislocation and the embryos were transferred immediately via PBS into 4% paraformaldehyde. For paraffin sections (skin, gut, embryos), the embryos and isolated mouse organs were fixed for 18 h at 4°C, dehydrated, embedded in paraffin wax, and cut into 6-μm sections. To make cryostat sections (14-d p.c. embryos, spleen) fresh embryos and organs were immediately embedded in Tissue-Tek (Miles, Inc., Elkhart, IN) and stored at −70°C.

Testes of adult Sprague-Dawley rats were fixed in 10% buffered formalin at room temperature for 24 h, dehydrated, embedded in paraffin wax, and cut into 5-μm sections.

In Situ Hybridization

The mouse *mad* antisense and sense cRNA probes were synthesized from linearized pBluescript II SK+ plasmid (Stratagene, La Jolla, CA), containing an ApaI–PstI fragment of the mouse *mad* cDNA (nucleotides 301-1001) using T3 and T7 polymerases and [^{35}S]UTP (Amersham Corp., Arlington Heights, IL). Similarly, mouse c-*myc* antisense and sense cRNA probes were synthesized from linearized mcxs plasmid (a kind gift of Drs. Ronald DePinho and Nicole Schreiber Agus) containing a 750-bp XbaI–SacI fragment of the mouse c-*myc* in pBluescript SK+ plasmid (Stratagene).

In situ hybridization of paraffin sections was performed as described in Wilkinson et al. (1987*a*,*b*) with the following modifications: (*a*) instead of toluene, xylene was used before embedding in paraffin wax; (*b*) cut sections were placed on a layer of diethyl pyrocarbonate-treated water on the surface of glass slides pretreated with 2% 3-triethoxysilylpropylamine; (*c*) alkaline hydrolysis of the probes was omitted; (*d*) the hybridization mixture contained 60% deionized formamide; and (*e*) the high stringency wash was for 80 min at 65°C in a solution containing 50 mM DTT and 1 × SSC. The sections were covered with NTB-2 emulsion (Kodak) and stored at 4°C. The slides were exposed for 14 d, developed, and stained with hematoxylin. Control hybridizations with sense strand and RNAse A–treated sections did not give a specific signal above background.

Frozen sections of 6 μm were cut onto aminoalkylsilane-pretreated microscope slides. The sections were immediately fixed in freshly prepared 4% paraformaldehyde (PFA)[1] in PBS for 15 min at room temperature (RT), washed in 70% and 100% ethanol, for 5 min each, air-dried for 10

1. *Abbreviations used in this paper*: PFA, paraformaldehyde; RT, room temperature.

min at RT, frozen, and stored at −70°C. Frozen specimens were rehydrated in PBS at RT and treated with 0.5 μg/ml proteinase K (Boehringer Mannheim Corp., Indianapolis, IN), for 5 min at RT. Then the slides were washed with glycine (0.1 M in PBS) for 5 min at RT, postfixed in 4% PFA in PBS, and rinsed with PBS. The sections were then acetylated in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min at RT and rinsed again in PBS. Hybridization was performed as described for paraffin sections.

Cell Culture and Transfections

Rat embryo fibroblast transformation assay was performed essentially as described by Mäkelä et al. (1992*a*), except that a modified calcium phosphate precipitation was used for transfection (Sambrook et al., 1989). The cells were cotransfected with 2 μg of pSV2neo, 7 μg pGEJ(6.6) (Mäkelä et al., 1992*a*), 7 μg pLTR-Tc-Myc and 15 μg of pLTRMax (Mäkelä et al., 1992*a*), pLTRMad or empty vector. pLTRMad was constructed by transferring a PCR-amplified human *mad* cDNA as a HindIII–EcoRI fragment from pGEM3Zf(+) to the respective sites of pLTRpoly (Mäkelä et al., 1992*b*). pLTR-Tc-Myc contains human c-*myc* cDNA in pLTRpoly.

BALB/MK-2 cells were grown in MEM containing 0.05 nM calcium, 10% FCS, and 4 ng/ml EGF (Collaborative Research, Inc., Waltham, MA). The cells were cotransfected with 0.5 μg of SV2neo and 4.5 μg of LTRMad, LTRMax, or empty vector using electroporation. To monitor the transfection efficiency, 0.5 μg of CMVβgal plasmid (MacGregor and Caskey, 1989) was also included. The electroporation was performed as described by Ustav and Stenlund (1991). Briefly, the cells (from 2 subconfluent 10-cm diam dishes per sample) were trypsinized, centrifuged, and resuspended in normal medium, 0.25 ml of cell suspension was mixed with plasmid DNA, 50 μg of salmon sperm DNA was added as a carrier and the mixture was pipetted into an electroporation cuvette with 4-mm gap size. The cell/DNA mixture was subjected to an electric discharge (220 V, 960 μF) using a Gene Pulser with a capacitance extender (BioRad Labs., Hercules, CA). After incubation at room temperature for 5–10 min, the cells were washed with 1 ml of medium. After centrifugation and resuspension, the cells were plated onto 10-cm diam dishes. A small aliquot was plated separately for determination of the transfection efficiency. G418 (0.5 mg/ml) was added to the culture medium 1 d after transfection and drug selection was carried out for ~3 wk. Transfection efficiency was determined one day after transfection by in situ staining of cells for β-galactosidase activity (Ustav and Stenlund, 1991) and was found to be similar in different samples of the same experiment.

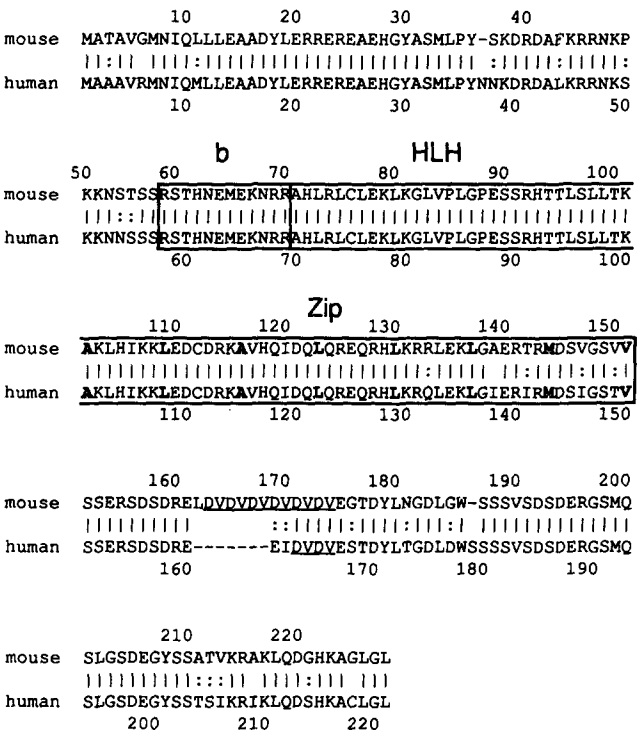


Figure 1. Alignment of the amino acid sequences derived from mouse and human Mad cDNAs. The cDNA for mouse Mad contains an open reading frame of 226 amino acid residues which is shown in the figure, aligned with the 85.5% identical human Mad sequence. The basic region (*b*), the helix-loop-helix (*HLH*) and the leucine zipper (*Zip*) are boxed, the amino acid residues that form the hydrophobic heptad repeat of the leucine zipper are indicated in bold. Note that due to the expansion of the DV diamino acid peptide motif (*underlined*), the mouse sequence is five amino acids longer than the human one. The nucleotide sequence data is available from EMBL/GenBank/DDBJ under accession number 83106.

Results

Isolation and Analysis of Mouse mad cDNA

To study the expression of *mad* mRNA in developing mouse embryos, we isolated *mad* clones from a cDNA library made from RNA of 12 d mouse embryos (see Materials and Methods). The 2.5-kb Mad cDNA insert contained an open reading frame encoding a polypeptide of 226 amino acid residues with a predicted molecular mass of 25 kD. The predicted mouse Mad protein showed 85.5% amino acid sequence identity with its human homologue (Fig. 1). This homology is highest in the central part of the molecule encompassing a bHLHZip DNA-binding and dimerization interface. A curious difference between the mouse and human amino acid sequences is the diaminoacid peptide of aspartate and valine, which is repeated six times in the mouse Mad (residues 163-174), but only twice in the human protein (Ayer et al., 1993).

mad Expression in Developing Mouse Embryos

Total RNA from mouse embryos of different gestational ages was analyzed by RNase protection and found to contain *mad* mRNA through days 8–18 p.c. of development (Fig. 2 *B*). A slight increase of the *mad* mRNA levels occurred between days 10 and 14 p.c. (Fig. 2 *B*). Sections of mouse embryos of 8–18 days p.c. were analyzed by in situ hybridization to localize the expression of *mad* in more detail. No *mad* RNA was seen on days 8–10 p.c. with this approach. On days 11, 12.5, and 14 p.c. *mad* signal was evident in the liver and the mantle layer of the brain (Fig. 3). In the liver *mad* expression was maximal on day 12.5 and decreased subsequently, correlating with the time course of hepatic hematopoiesis. On day 18 p.c. expression was also found in the inner layer of neural retina in the developing eye (Fig. 4, *A–C*) and in the developing epidermis and whisker follicles (*D–F*).

Expression of mad in Adult Mouse Tissues

A Northern blot containing polyA+ RNA from several adult mouse tissues and organs was hybridized with the *mad* probe and analyzed by autoradiography. Strong signals from ~5 kb and 3 kb mRNAs were obtained from the spleen and testis, and lower levels were detected in all other tissues tested (Fig. 2 *C*). In the testis the *mad* probe recognized also additional, shorter mRNA forms of ~2.3 kb and 1.3 kb. The 2.3-kb mRNA was about equally abundant with the 5-kb and 3-kb mRNAs, while the 1.3-kb transcript was barely detectable.

To assign *mad* transcripts to cells and tissues, mouse tissue sections were hybridized in in situ with the labeled *mad* cRNAs. In the spleen the signal was detected in the red pulp

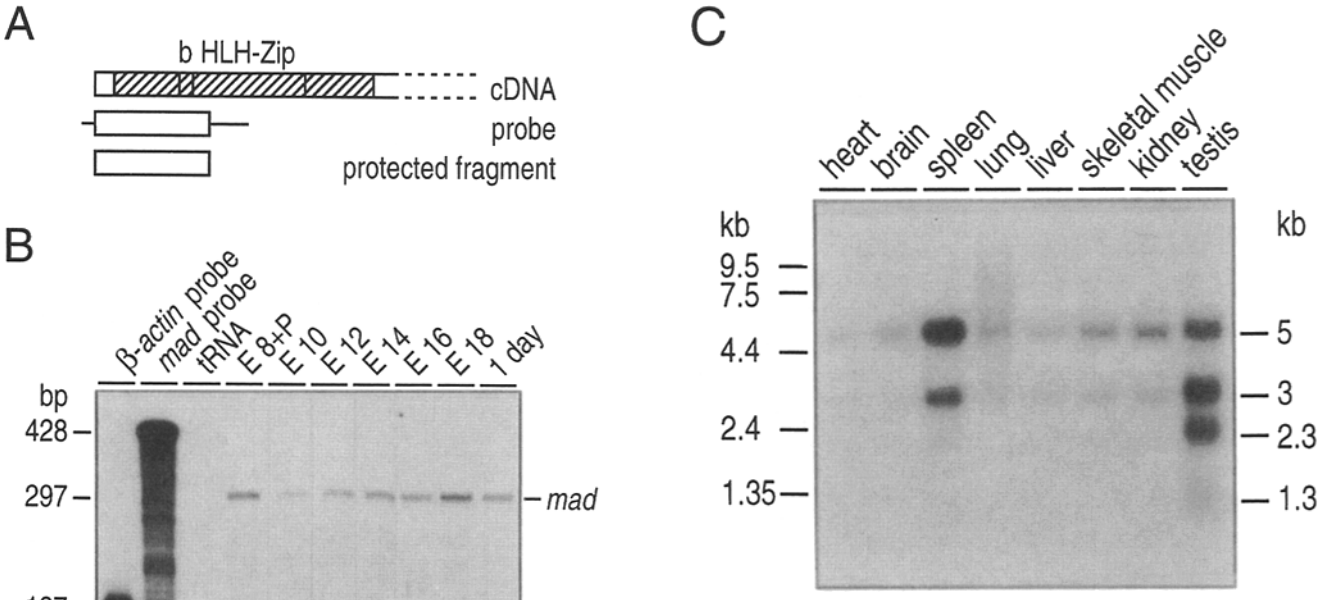
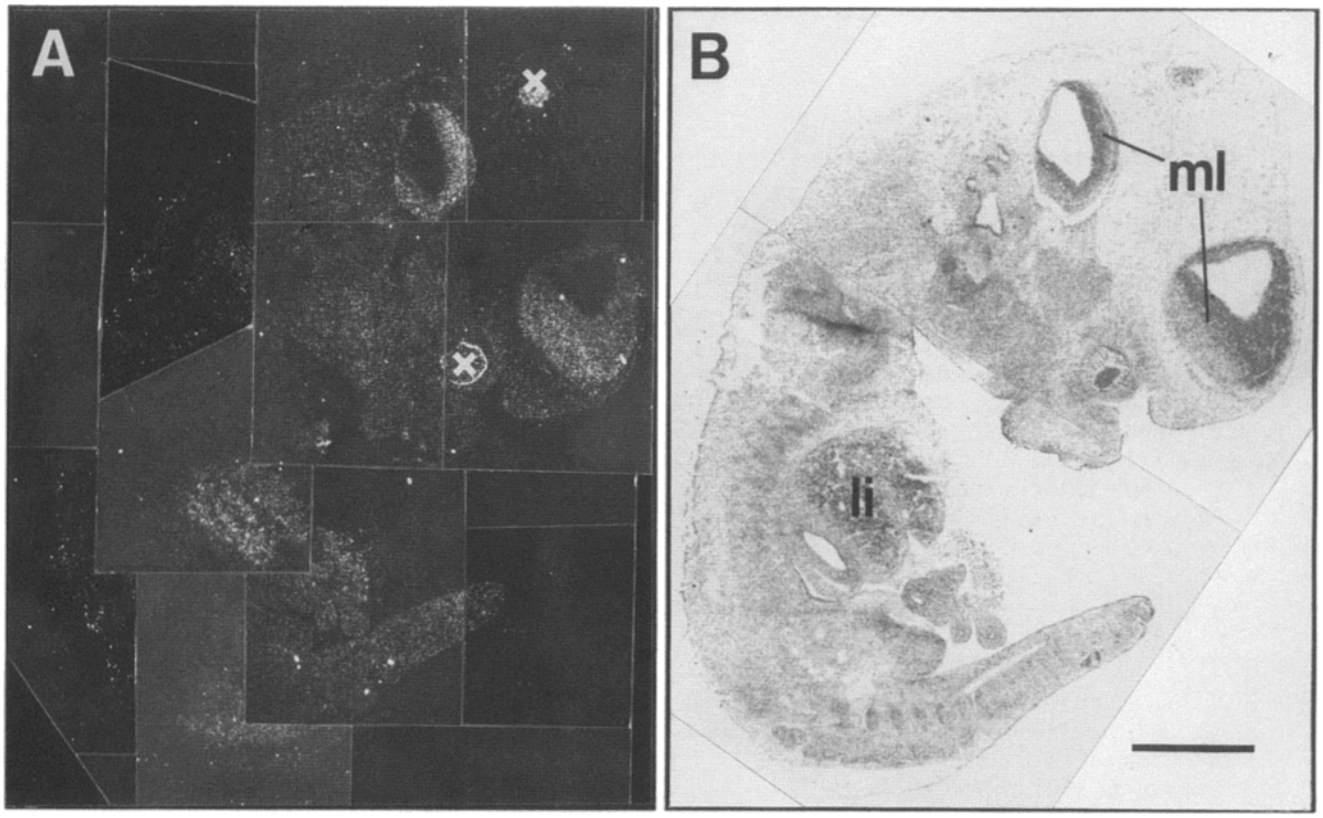


Figure 2. Expression of *mad* mRNA in mouse tissues. (*A*) A schematic diagram of the *mad* cDNA, RNase protection probe and the protected fragment. Striped region indicates the open reading frame. (*B*) RNase protection analysis of RNA isolated from mouse embryos of the indicated gestational ages (*E8-E18*) and from a newborn mouse (*1 day*). Sample E8+P contains also the placenta. The sizes of the probes and the protected *mad* fragment are given in base pairs; β-actin was used as a control. (*C*) A Northern blot containing polyA+ RNA from the indicated tissues of adult mice was hybridized with the mouse *mad* probe. Positions of RNA size markers and the sizes of the *mad* RNAs are indicated.



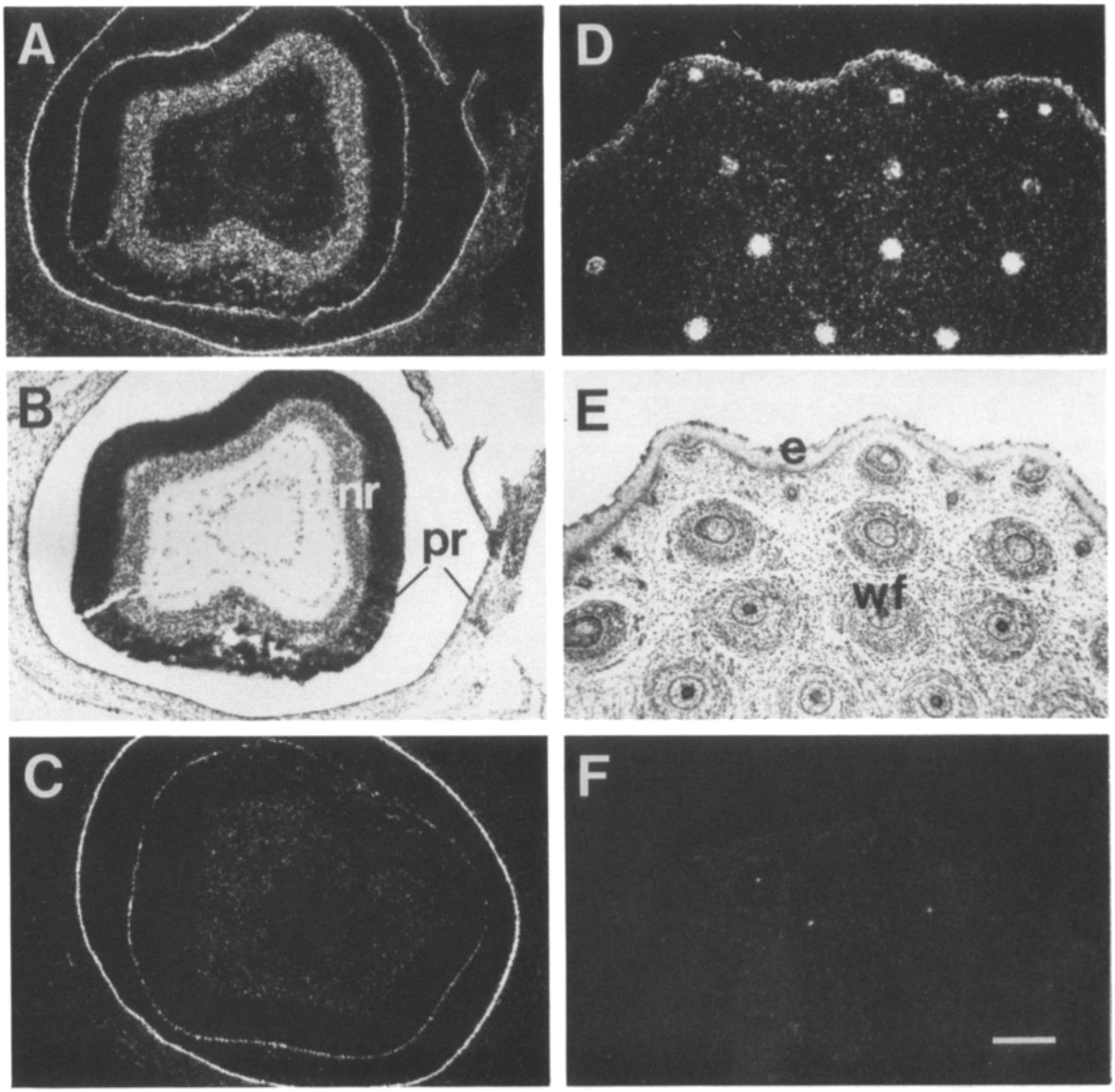


Figure 4. mad expression in the developing eye and snout. Dark- (*A*) and bright-field (*B*) photomicrographs of an 18-d embryo showing *mad* expression in the inner layer of the neuroretina (*nr*) of the eye. (*C*) The signal from the pigmented retina (*pr*) is unspecific, as can be seen from the corresponding section hybridized with the sense probe. (*D* and *E*) In the developing snout, signal is detected in the epidermis and in the inner epithelial aspect of the whisker follicles (*wf*), whereas the (*F*) sense probe gives no signal. Bar, 0.1 mm.

in clusters of cells beneath the capsule (Fig. 5, *A* and *B*). These areas contained developing hematopoietic cells of myeloid origin, as assessed by their positivity with the Leder stain (Fig. 5 *C*, *arrow*). Furthermore, mouse bone marrow contained Mad-positive cells (data not shown).

As in the 18-d p.c. embryos, *mad* mRNA was also present in the epidermis of adult skin. However, unlike in the embryos, the signal in adults was discontinuous, being present in the outer, more differentiated cells at sites where the epidermis was thicker (data not shown). In newborn mouse skin the *mad* signal was stronger, but the pattern was similar (Fig. 5, *D* and *E*). Several cell layers could be distinguished, allowing the determination of the stage of differentiation of the keratinocytes according to their stratified position between the basement membrane and the surface of the epidermis. In higher magnification a strong signal was de-

Figure 3. In situ hybridization of *mad* mRNA in 12.5 d p.c. mouse embryo. Dark- (*A*) and bright-field (*B*) photomicrographs of an in situ autoradiogram are shown. A sagittal section showing signal predominantly in the liver (*li*) and the mantle layer (*ml*) of the brain. Crosses indicate false signals (from e.g., the pigmented retina of the eye; see also Fig. 4). Bar, 1 mm.

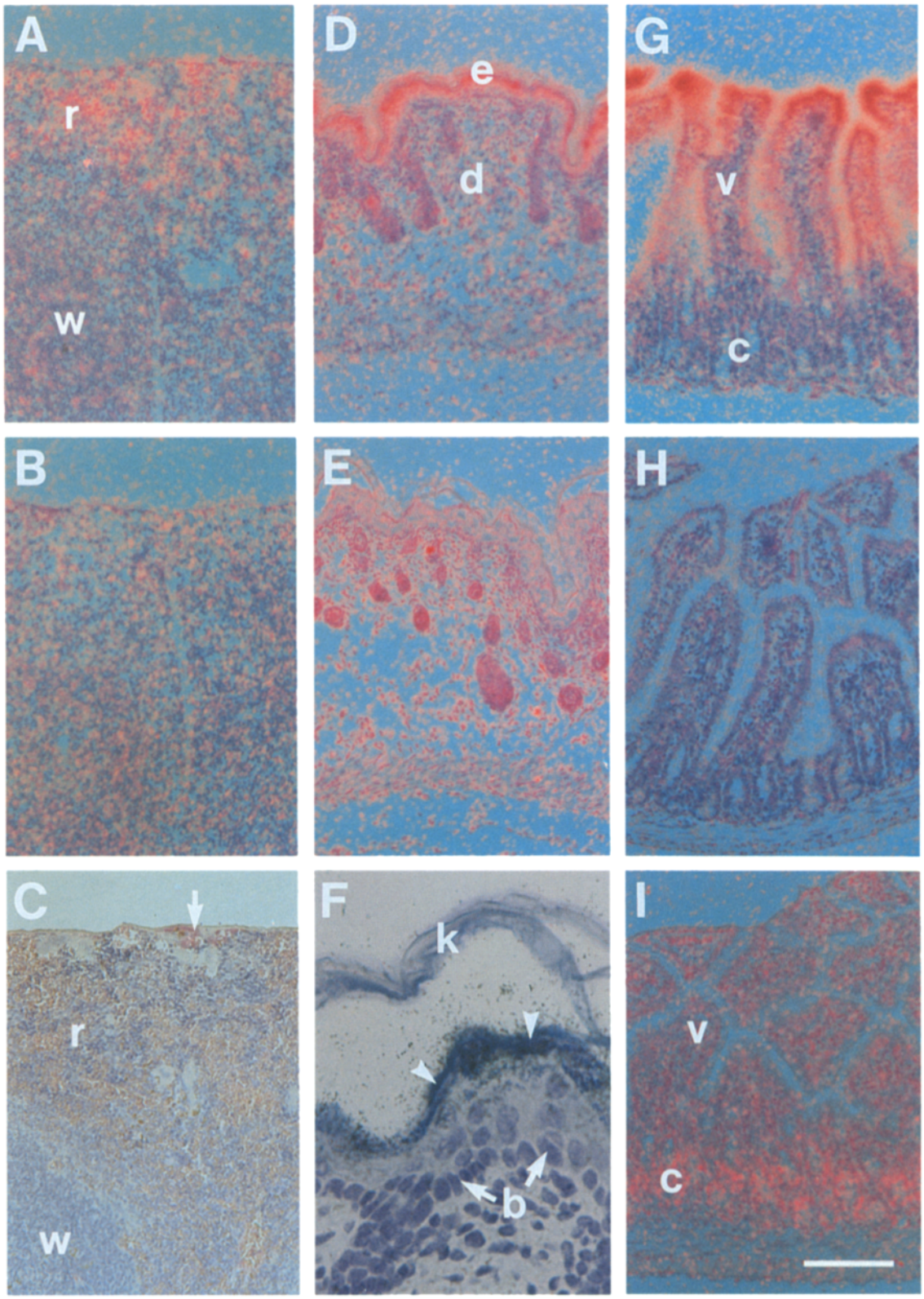


Figure 5. mad mRNA in spleen, skin, and gut. Photomicrographs of adult spleen hybridized with antisense (*A*) and sense (*B*) *mad* cRNA. (*C*) Leder-staining of a section of the same region of spleen with the cells of myeloid origin staining red (*arrow*). Lower (*D* and *E*) and higher (*F*) magnifiations of skin of a newborn mouse showing *mad* expression in the outer layers of epidermis (pointed with *arrowheads*). Small intestine of adult mouse hybridized with antisense (*G*) and sense (*H*) *mad* probes. In the crypts no specific hybridization is detected, while signal increases in the epithelial cells towards the lumen of the gut, being very strong in the tips of the villi. The signal for c-*myc* cRNA is confined to the crypts (*I*). Abbreviations: *r*, red pulp; *w*, white pulp; *e*, epidermis; *d*, dermis; *b*, basal cell layer; *k*, keratinized squames; *c*, crypt; *v*, villus. Bars: (*A–E*) 0.1 mm; (*G–I*) 0.025 mm for *F*.

tected in the epidermal keratinocytes in the outer, differentiated layers of cells, whereas the basal cell layer was negative for *mad* (Fig. 5 *F*).

Particularly interesting results suggesting an involvement of *mad* in the regulation of cell proliferation and differentiation was obtained from sections of the small intestine. The intestinal epithelium has a high turnover rate with typical kinetics of proliferation and differentiation related to its unique architecture. Cells proliferate in the bottom parts of the crypts and migrate up to the villi, where they are shed into the gut lumen. As the cells move upwards, they differentiate, senesce, and finally undergo programmed cell death before being disposed (summarized by Wright and Alison, 1984). In situ hybridization of sections of adult small intestine revealed strong *mad* RNA expression in the gut epithelium. A clearcut gradient of expression was evident in the intestinal villi: the epithelium of the intestinal crypts was negative, but a gradient of increasing signal extended towards the lumen of the gut, the tips of the villi being most intensely decorated with the autoradiographic grains (Fig. 5, *G* and *H*). Control hybridization with the sense probe gave no signal over background (data not shown). Interestingly, the c-*myc* gene was expressed in the crypts containing the proliferating cells (Fig. 5 *I*), as has been described by Hirning et al. (1991).

Stage-dependent Expression of mad in the Testes

Spermatogenesis in the seminiferous epithelium comprises three main phases: spermatogonial multiplication, meiosis, and spermiogenesis. Development from spermatogonia to spermatids is regulated by Sertoli cells in a cyclic fashion. Each stage of the seminiferous epithelium has a defined content of spermatogenic cells at a certain phase of development. Along the seminiferous tubule, the stages follow each other in a wave-like fashion. Different stages can be dissected as morphologically identifiable segments and prepared for biochemical analysis (for a review see Parvinen, 1993). Northern blotting and hybridization of total RNAs from such segments of rat seminiferous tubules showed that *mad* is expressed in a cyclic, stage-dependent manner. As in unfractionated mouse testicular RNA, the *mad* probe detected multiple RNA species (Fig. 6 *A*), although the 5-kb and the 2.3-kb transcripts were less prominent. The 1.3-kb mRNA was not detectable, which is probably due to the use of total RNA instead of polyA+ RNA. The level of the 3-kb transcript was constant in stages I-V, but decreased in stages VI-VIII, and then gradually returned back to the initial levels in stages IX-XIV. The level of the 5-kb RNA was regulated similarly to the 3-kb transcript, but the 2.3-kb RNA form was detected only in stages VI-VIIab, where the levels of other *mad* transcripts were decreased.

Because each stage contains spermatogenic cells in at least four different phases of maturation in addition to Sertoli cells, in situ hybridization of sections of adult rat testes was used to identify the cells expressing *mad* mRNA. A summary of *mad* expression in rat seminiferous epithelium is shown in Fig. 6 *B*. The expression was first detected in pachytene spermatocytes in stage X (data not shown). The signal increased through the following stages (Fig. 7, *D* and *D'*), was detected also during meiotic divisions in stages XIII-XIV (Fig. 7, *E* and *E'*) and in the resulting round spermatids until stage VI (Fig. 7, *A*, *A'*, and *C*, *C'* and data not shown). No specific hybridization was observed in stages VII-IX (Fig. 7, *A* and *A'* and data not shown). Control hybridization with *mad* sense probe gave no specific signal (Fig. 7 *B*).

Mad Antagonizes the Transforming Activity of Myc and Inhibits Colony Formation by a Mouse Keratinocyte Cell Line

Mad has been shown to antagonize transcriptional activation of promoter constructs by Myc (Ayer et al., 1993). Because we found induction of *mad* expression in association with the cessation of cell proliferation and differentiation, we wanted to test whether Mad can inhibit cell growth and repress the growth promoting activity of Myc. We chose the Ras–Myc rat embryo fibroblast cotransformation assay to answer the latter question. Addition of Mad expression construct to Ras–Myc cotransfections reduced the number of transformed foci (Fig. 8 *A*). The extent of this effect was similar to that obtained with the addition of similar amounts of a Max expression construct cloned into the same vector.

To determine whether Mad can inhibit cell proliferation, we employed the colony formation assay used previously for p53, pRB, and p107 (Zhu et al., 1993 and references therein). As *mad* was expressed in differentiated keratinocytes in the skin, we chose to use the mouse epidermal keratinocyte cell line BALB/MK-2 (Weissman and Aaronson, 1983) for these studies. The neomycin resistance plasmid was cotransfected together with Mad or Max expression plasmids or respective empty vector, and the number of neomycin resistant colonies was scored. As shown in Fig. 8 *B*, transfection of BABL/MK-2 cells with either Mad or Max expression construct decreased the number of neomycin resistant colonies, indicating that both Mad and Max have a negative effect on cell growth. Consistent with this, we were also unable to detect Mad expression in the pooled colonies after neomycin selection, although the same plasmid yielded a Mad polypeptide in transient transfection assay of BOSC23 cells (Pear et al., 1994; data not shown). Taken together, these data strongly suggest that cell culture causes selection against Mad expression and that Mad, like p53, pRB, and p107 proteins in a similar assay, inhibits cell growth.

Discussion

We have cloned the mouse *mad* cDNA and used it to study *mad* expression in embryonic and adult mouse tissues. The deduced amino acid sequence of mouse *mad* was 85.5% identical with the corresponding human sequence, the most significant difference being an extended diaminoacid repeat found in its carboxyl-terminal part. We found that *mad* is highly expressed in non-proliferating, terminally differentiated cells in certain tissues. Two particularly clear examples of this were the suprabasal layers of the epidermis and the epithelium of gut villi. We also show that, consistent with its effects on Myc-induced transactivation (Ayer et al., 1993) and transformation (Lahoz et al., 1994; the present results), Mad inhibits cell growth in vitro. Mad could thus be involved in the cessation of cell proliferation associated with terminal differentiation in vivo.

As in human cells, also in mouse and rat, *mad* is expressed as two RNA transcripts of ~5 kb and 3 kb (Ayer and Eisen-

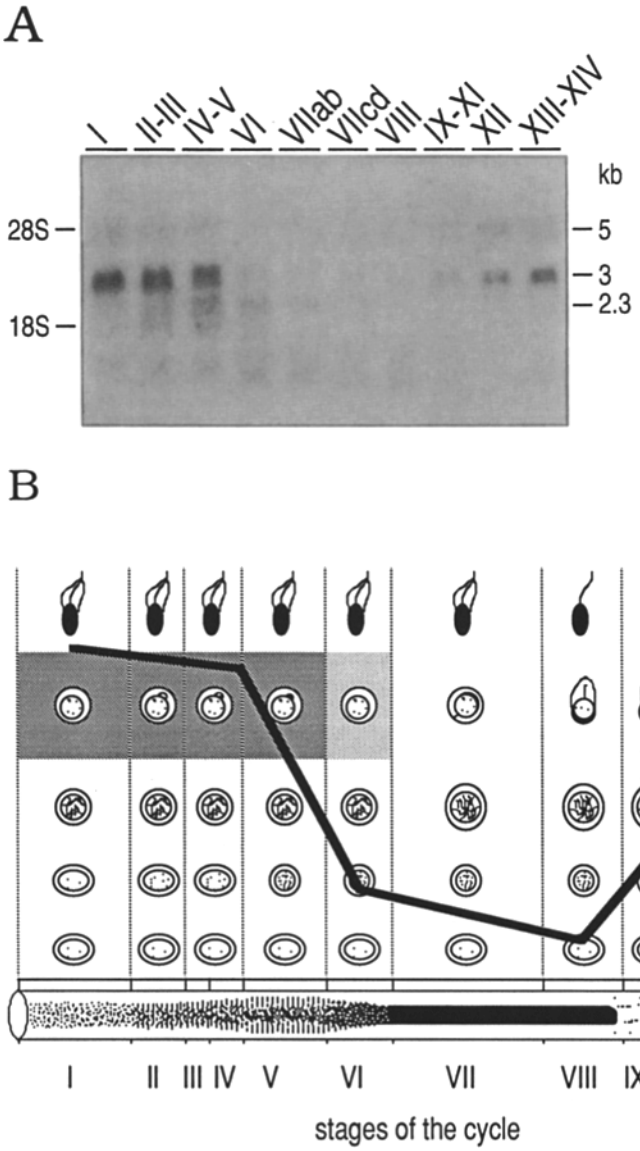


Figure 6. Stage-dependent expression of *mad* mRNAs in the rat testes. (*A*) Northern blotting hybridization of total RNAs from different segments of rat seminiferous tubules representing the indicated stages of the cycle of the seminiferous epithelium. The sizes of the *mad* mRNA bands are indicated. (*B*) A schematic summary of *mad* expression in the seminiferous epithelium during the different stages of spermatogenesis. The cell types expressing *mad* are shown in stippled boxes (*sparse spotting* indicates lower expression). The black line indicating the relative levels of 3 kb *mad* mRNA in different stages was obtained by densitometric scanning of the Northern blot shown in panel *A*.

man, 1993; Larsson et al., 1994). In the testis additional forms of 2.3 kb and 1.3 kb were detected. Currently the coding capacities of these different mRNA forms are unknown. The major diamino acid repeat difference between the human and mouse Mad amino acid sequences could be the result of alternative splicing. However, the sequence of *mad* cDNA encoding the diaminoacid repeat consists of an imperfect six-nucleotide repeat. It is thus possible that the number

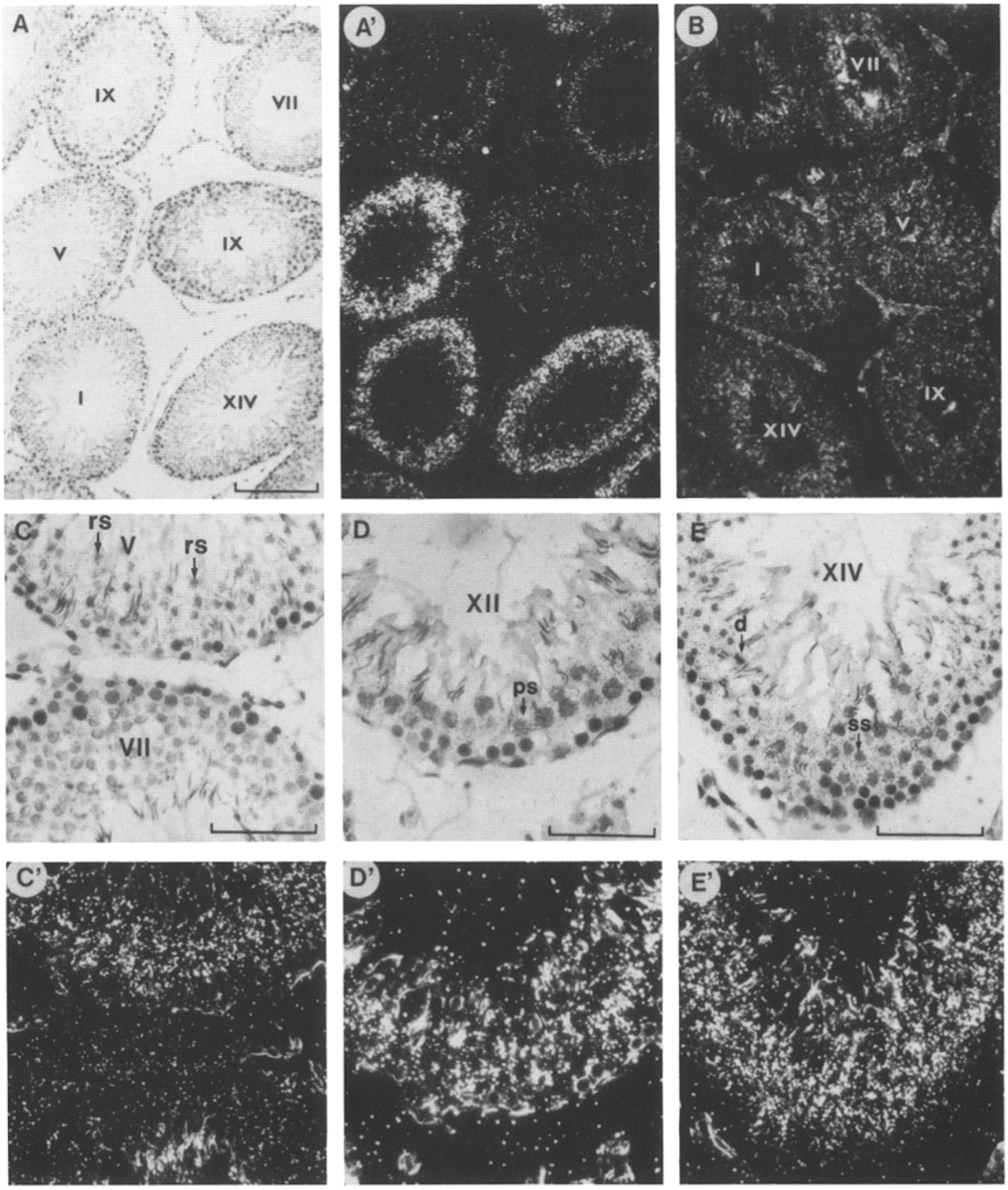


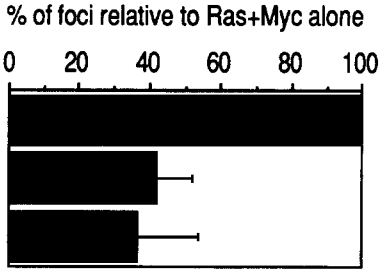
Figure 7. In situ hybridization of *mad* mRNA in adult rat testes. Bright- (*A*, *C*, *D*, and *E*) and dark-field (*A'*, *C'*, *D'*, and *E'*) photomicrographs are shown. Strong hybridization signals are seen in stages I, V, XII, and XIV, whereas in stages VII and IX the signal is at the level of background (*A* and *A'*). High magnifications of stage V and VII (*C* and *C'*), XII (*D* and *D'*) and XIV (*E* and *E'*) tubules show grain accumulation over pachytene spermatocytes (*ps*), spermatocytes in the second meiotic division (*d*), secondary spermatocytes (*ss*), and postmeiotic round spermatids (*rs*). Control hybridization with sense probe (*B*). Bars: (*A*, *A'*, and *B*) 0.1 mm; (*C*, *C'–E*, *E'*) 0.05 mm.

of these repeats differs between mouse and man due to frequent mutations of these repetitive elements during DNA replication or crossing over. These mechanisms are thought to cause expansion or contraction of so-called microsatellite DNA consisting of such repeats (for a review see Kunkel, 1993).

Induction of *mad* and its close relative *mxil* has been observed upon in vitro differentiation of HL-60, U937, and ML-1 leukemia cell lines (Ayer and Eisenman, 1993; Larsson et al., 1994; Zervos et al., 1993). Here we have shown that *mad* is expressed in fetal liver and in adult spleen, organs active in hematopoiesis. As assessed by the Leder-staining,

A

Transfected constructs	Total number of foci			
	Exp.1	Exp.2	Exp.3	Exp.4
Ras+Myc	20	11	40	24
Ras+Myc+Mad	6	6	25	5
Ras+Myc+Max	3	7	27	0



B

Transfected constructs	Total number of colonies			
	Exp.1	Exp.2	Exp.3	Exp.4
Neo	26	63	65	96
Neo+Mad	16	38	32	54
Neo+Max	19	17	31	34

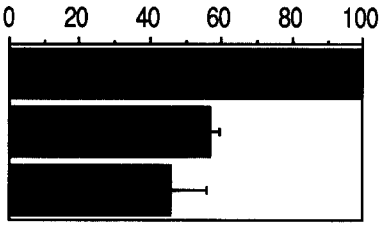


Figure 8. Mad suppresses transformation of rat embryo fibroblasts by Myc and Ras and inhibits growth of a mouse keratinocyte line. (*A*) Number of transformed foci obtained from cotransfection of rat embryo fibroblasts with the indicated expression constructs. The column chart shows the relative effect of Mad and Max on Myc-Ras cotransformation expressed as percent of transformed foci obtained with Myc and Ras only. (*B*) Number of colonies after transfection with neomycin resistance gene (*Neo*) and the indicated expression constructs, followed by neomycin selection. The column chart shows the relative effect of Mad and Max on colony formation expressed as percent of colonies obtained with Neo only. The bars beside the columns denote the standard error.

mad was expressed in myeloproliferative areas of the spleen, but also in certain other cells that we were unable to identify. This and the expression of *mad* in human bone marrow and peripheral blood leukocytes (unpublished observations of the authors) support the involvement of *mad* in the differentiation of certain hematopoietic cell lineages.

Epidermal keratinocytes form a multilayered structure with cell proliferation confined to the basal cell layer. As the cells move upwards, they cease proliferation, differentiate, form keratinized squames, and finally flake off from the surface of the epidermis (for a review see Zinkel and Fuchs, 1994). As demonstrated by in situ hybridization, *mad* expression is associated with the differentiation of epidermal keratinocytes being most prominent in the outer, more differentiated cells. A similar expression pattern was seen also in the inner epithelial aspect of the whisker follicles which are organized topologically similarly to the outer layers of epidermis. Thus, if the *mad* mRNA levels reflect the expression of the protein product, the Mad protein could contribute to the differentiation of keratinocytes. In comparison, N-*myc* is expressed in the proliferating cells of the germinative zones of hair follicles, but not in the epidermis of newborn mice (Mugrauer et al., 1988). Also, c-*myc* and L-*myc* genes have been reported to be expressed in skin (Hirvonen et al., 1990; Semsei et al., 1989), but the exact pattern of their expression is not known.

Gut epithelium provides another system, where proliferation and differentiation take place in a spatially restricted compartment. Cells divide in the crypts and as they stop proliferating, they are pushed towards the tips of the villi where they are finally shed into the gut lumen (summarized by Wright and Alison, 1984). The expression of c-*myc* in the gut epithelium correlates with cell proliferation: the signal is detected in the crypts while the villi are negative (Hirning et al., 1991). This expression pattern fits very well with current ideas about Myc as an inducer of cell proliferation. Somewhat surprisingly, the non-proliferating cells of the epithelial lining of villi have been reported to express N-*myc* (Hirning et al., 1991). Similar examples of N-*myc* expression in non-proliferating cells are found for example in the ganglion cell layer of neural retina and mantle layer of the developing telencephalon (Hirning et al., 1991; Hirvonen et al., 1990; Mugrauer et al., 1988). Yet the ability of N-Myc to induce cell proliferation, transformation, and tumorigenesis in transgenic mice is very similar to that of c-Myc (Cavalieri and Goldfarb, 1988; Dildrop et al., 1989; Rosenbaum et al., 1989; Schwab et al., 1985; Yancopoulos et al., 1985). However, the expression of *mad* in these tissues and its ability to suppress at least some functions of N-*myc* (Lahoz et al., 1994) may explain how N-*myc* expression can be uncoupled from proliferation.

Yet another example of induction of *mad* expression during differentiation was observed during spermatogenesis. During the multi-step process of spermatogenesis the primitive type A spermatogonia, the precursor stem cells, can either undergo renewal or differentiate into intermediate and B type spermatogonia. Type B spermatogonia divide once (in stage VIII), and then enter the prophase of meiosis as preleptotene spermatocytes (for a review, see Parvinen, 1993). Spermatocyte development continues through the leptotene, zygotene, and pachytene stages of meiotic prophase, which is followed by two rapid cell divisions resulting in formation of round spermatids, the earliest haploid postmeiotic cells. The expression of *mad* was observed in pachytene in stage X, increased in the following stages and continued through the meiotic divisions and in the resulting haploid secondary spermatocytes, where it decreased in stage VI. In contrast, the expression of c-*myc* is confined to type A and B spermatogonia and preleptotene spermatocytes in the earlier phases of spermatogenesis, with no detectable expression in

the phases where *mad* was expressed (Wolfes et al., 1989). However, nothing is known about the expression of the other *myc* genes during spermatogenesis.

Consistent with the expression of *mad* in non-proliferating, terminally differentiated cells in several tissues, we present also evidence that the ectopic expression of Mad in a mouse epidermal keratinocyte cell line inhibits cell growth as measured by the colony formation assay. Similar effects have recently been observed in two other cell lines tested (unpublished results of the authors). However, it should be noted that the colony formation assay does not allow us to conclude whether the negative effect of Mad on cell growth is due to a block of the cell cycle or due to a loss of cell viability. The ability of Mad to repress transcriptional (Ayer et al., 1993) and transforming (Lahoz et al., 1994; the present results) activities of the Myc proteins suggests that Mad can inhibit cell proliferation by competing with the Myc proteins and downregulating their activity. Thus Mad could contribute to terminal cell differentiation by inducing a cessation of cell proliferation.

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