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Isolation and Identification of the Human Homolog of a New p53-Binding Protein, Mdmx

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We recently reported the identification of a mouse cDNA encoding a new p53-associating protein that we called Mdmx because of its structural similarity to Mdm2, a well-known p53-binding protein. Here we report the isolation of a cDNA encoding the human homolog of Mdmx. The ORF of the cDNA encodes a protein of 490 amino acids, 90% similar to mouse Mdmx. The homology between Mdmx and Mdm2 is most prominent in the p53-binding domain and the putative metal-binding domains. The Mdmx protein, which, based on SDS-PAGE, has a MW of 80 kDa, can bind p53 *in vitro*. The human *MDMX* gene is transcribed in all tissues tested, with high levels in thymus. By fluorescence *in situ* hybridization analysis we mapped the mouse *mdmx* gene to chromosome 1 (region F-G) and the human *MDMX* gene to chromosome 1q32. © 1997 Academic Press

INTRODUCTION

The loss of wild-type p53 in human malignancies results in aberrant cell-cycle progression, escape from apoptosis, and enhanced angiogenesis, which all contribute to tumor growth. These biological processes are most likely controlled by p53 through its transcription-regulating function, which, in human tumors, is usually altered due to gene mutations (Hollstein *et al.*, 1991, 1994), but can also take place via cytoplasmic retention (Moll *et al.*, 1992, 1995) and by association with other proteins. Tumor antigens of several DNA tumor viruses have been shown to inactivate the function(s) of p53, by a tight association (SV40-LT, adenovirus type 5 large E1B protein, HBV X-antigen; Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow *et al.*, 1982; Zantema *et al.*, 1985; Feitelson *et al.*, 1993),

by enhanced degradation of the p53 protein (HPV-E6; Scheffner *et al.*, 1990; Werner *et al.*, 1990), or by as yet unknown ways, possibly involving altered oligomerization of p53 (adenovirus type 12 large E1B, adenovirus type 5 E1A; Steegenga *et al.*, 1995, 1996). Importantly, HPV and HBV have been implicated in human cervical carcinoma and liver carcinoma (Srivastava *et al.*, 1992; Ueda *et al.*, 1995), respectively. In addition, some cellular proteins have been found to bind to p53 and influence its properties as a transcription factor. First, the Wilms' tumor protein WT1, a transcription factor itself, is able to modulate p53-regulated transcription (Maheswaran *et al.*, 1993). Recently, the E2F1 and DP1 proteins were also shown to interact directly with p53 and inhibit its transcription-stimulation function (O'Connor *et al.*, 1995; Martin *et al.*, 1995). Last, p53 was found to complex with the Mdm2 protein (Barak and Oren, 1992; Momand *et al.*, 1992). The association of Mdm2 with p53 completely abrogates all transcription-regulating properties of p53 (Momand *et al.*, 1992; Chen *et al.*, 1995). Mdm2 most likely functions by concealing the transactivation domain of p53 (Oliner *et al.*, 1993). Most interestingly, overexpression of Mdm2 has been found in a variety of tumors, in general correlating with the absence of mutations in the p53 gene (Oliner *et al.*, 1992; Reifemberger *et al.*, 1993; Habuchi *et al.*, 1994; Lianes *et al.*, 1994; Corvi *et al.*, 1995). Overexpression of Mdm2 is apparently sufficient for inhibition of the p53 tumor-suppression function. Thus, inactivation of p53 in human tumors can be achieved by mutation, cytoplasmic retention, or overexpression of Mdm2, or by several viral proteins.

We recently reported the identification of a new p53-associating protein. The translation product of this gene, named *mdmx*, shows significant homology with the Mdm2 protein, especially in the p53-binding domain and in the putative functional domains, located in the C-terminal part of the protein. We reported that the murine Mdmx protein binds to p53 *in vivo* and, like Mdm2, inhibits the activation of transcription by p53 (Shvarts *et al.*, 1996). Unlike *mdm2*, which is a p53-

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responsive gene, *mdmx* transcription appears not to be regulated in a p53-dependent manner, at least not after UV irradiation. This might suggest that *mdmx* is not a modifier of p53 after UV irradiation. We now report the isolation of a human cDNA of 2216 bp that encodes the human Mdmx protein of 490 aa. The mouse and human Mdmx proteins have 90% identity at the protein level. We show that the human Mdmx protein binds to p53 *in vitro*. By FISH analysis we mapped the mouse *mdmx* gene to chromosome 1 region F–G and its human homolog to chromosome 1q32. We report the identification of a new member of the *mdm* family that might be involved in human carcinogenesis.

MATERIALS AND METHODS

Northern analysis. A human multitissue Northern blot (Clontech) was hybridized with ³²P-labeled probes according to the manufacturer's manual.

Isolation and sequencing of human MDMX cDNA. A cDNA library from the colonic epithelial cell line T84 in a UNI-Zap-XR vector (Stratagene) was screened with mouse *mdmx* cDNA probe according to the protocol supplied by the manufacturer. pBSK plasmids containing the human MDMX cDNA were obtained with R408 helper phage (Stratagene).

Double-stranded DNA was sequenced by a T7 polymerase sequencing kit and AutoRead Sequencing kit (Pharmacia). The sequence reactions were subsequently analyzed manually on polyacrylamide gel and by the ALF (automatic laser fluorescence) DNA sequencing machine (Pharmacia). All the sequence primers were obtained from Isogene Bio-Science B.V.

Isolation of genomic *mdmx* DNA clones. To obtain genomic mouse *mdmx* sequences, we screened a genomic mouse library derived from 129/OLA mice in EMBL3A phage using the standard procedure (Sambrook *et al.*, 1989). Subclones were generated by digestion with *SalI* and by cloning the fragments into *SalI*-digested pIC20R vector. To obtain human MDMX genomic sequences, library N0. 700 (P1 human), ligated into pAd10SacBII (Francis *et al.*, 1994), was screened with a fragment of approximately 560 bp obtained by RT-PCR on mRNA isolated from the human tumor cell line G401 (Weismann *et al.*, 1987) with a primer set corresponding to nt 460–478/1003–1022. The presence of MDMX-specific sequences in the genomic clones was established by Southern blot analysis with the use of specific cDNA probes (data not shown).

***In vitro* associations between Mdmx and p53.** To obtain *in vitro* translated Mdmx proteins, the full-length coding regions of mouse and human Mdmx were cloned into pcDNA3 vector (Invitrogen). Human p53 protein was translated *in vitro* from the modified pET15b/p53 vector (Shvarts *et al.*, 1996) and the human cJun protein from a pBAT vector (Annweiler *et al.*, 1991) containing the human cJun coding region (pBAT-cJun, a gift from Dr. P. Angel, Karlsruhe, Germany).

***In vitro* transcription/translation** was performed with the use of the coupled transcription/translation system obtained from Promega (Madison, WI) in the presence of [³⁵S]methionine. *In vitro* translated Mdmx proteins were incubated in the presence or absence of bacterially produced p53 proteins (Shvarts *et al.*, 1996) for 30 min on ice. Subsequent immunoprecipitations were performed as described previously (Steegenga *et al.*, 1995) with a mixture of the monoclonal antibodies PAb122/PAb421 directed against p53, the polyclonal rabbit antiserum pAb100 directed against a peptide of mouse Mdmx, or the polyclonal rabbit antiserum pAb55 raised against full-length human Mdmx.

To obtain GST–human Mdmx fusion protein, the total coding region of human Mdmx was cloned into vector pRP259, a modified pGEX expression vector (Smith and Johnson, 1988). Approximately 1 μg of GST–Mdmx, bound to beads, was tumbled for 4 h at 4°C

with labeled *in vitro* translated p53. Subsequently the beads were washed three times with binding buffer. Immunoprecipitated and GST–Mdmx-bound proteins were separated on a 9% SDS–polyacrylamide gel, prepared for fluorography with 22.5% PPO (2,5-diphenylloxazole) in dimethylsulfoxide, dried, and exposed to Kodak XAR-5 film at –80°C.

Fluorescence *in situ* hybridization (FISH). FISH was performed on mouse cells regenerated from a muscle biopt and on human lymphocytes. Metaphase spreads were prepared via the standard procedures. A total of 400 and 150 ng labeled *mdmx* probe for mouse and human chromosomes, respectively, and 50x mouse Cot I and 50x human Cot I DNA (Gibco, Life Technology) was dissolved in 12 μl of a hybridization solution (50% v/v deionized formamide, 10% dextran sulfate, 2x SSC, 1% v/v Tween 20, pH 7.0). Prior to hybridization, the probe was denatured at 80°C for 10 min, chilled on ice, and incubated at 37°C for 30 min allowing preannealing. After denaturation of the slides, probe incubations were carried out under an 20 × 20 mm coverslip in a moist chamber for 45 h. Immunocytochemical detection of the hybridizing probe was achieved with FITC-conjugated sheep-anti-digoxigenin (Boehringer).

For evaluation of the chromosomal spreads a Zeiss epifluorescence microscope equipped with the appropriate filter for visualization of DAPI and FITC fluorescence was used. Digital images were acquired with a high-performance cooled CCD camera (Photometrics, Tucson) and further processed on a computer with the help of the BDS-Image FISH software package (Biological Detection Systems Inc, Rockville, MD).

RESULTS

Molecular Cloning of *mdmx* cDNA

To identify the human MDMX cDNA we screened a cDNA library from the colonic tumorigenic cell line

Construction of human MDMX cDNA

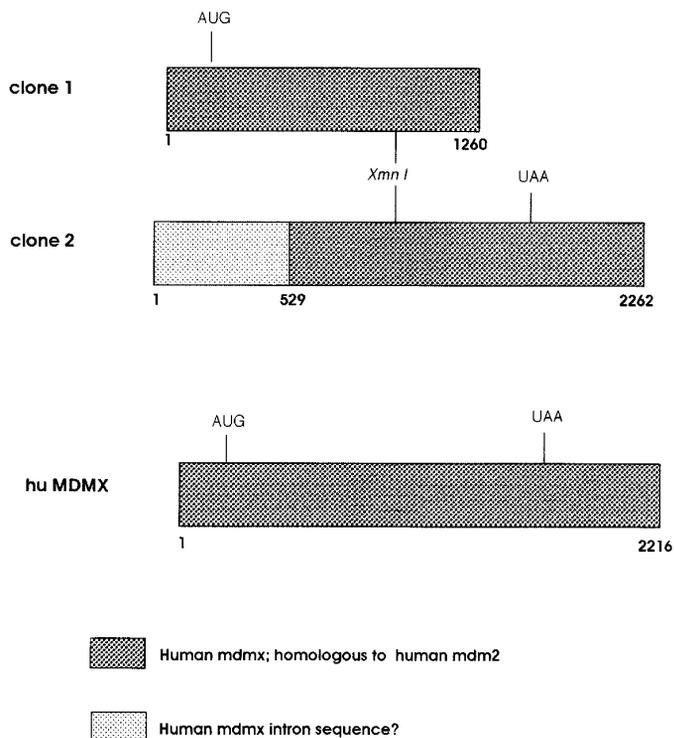


FIG. 1. Schematic representation of human MDMX cDNA clones obtained from a LambdaZap cDNA library from colon carcinoma cell line T84. The *XmnI* site in the middle of the cDNA has been used to obtain the human MDMX cDNA containing the complete open reading frame.

A

1	CGGCACGAGCTAGGATCTGTGACTGCCACCCCTCCCCACCCGGGCTCGGCGGGGGAGC	60
61	GACTCATGGAGCTGCCGTAAGTTTTACCAACAGACTGCAGTTTTCTTACTACCAAATGA	120
	M T	2
121	CATCATTTTCCACCTCTGCTCAGTGTTC AACATCTGACAGTCTTG CAGGATCTCTCTG	180
3	S F S T S A Q C S T S D S A C R I S P G	22
181	GACAAATCAATCAGGTACGACCAAACCTGCGCTTTTGAAGATTTTG CAGCAGCGTG	240
23	Q I N Q V R P K L P L L K I L H A A G G A	42
241	CGCAAGGTGAAATGTTCACTGTTAAAGAGGTCATGCACTATTTAGGTCAGTACATAATGG	300
43	Q G E M F T V K E V M H Y L G Q Y I M V	62
301	TGAAGCAACTTTATGATCAGCAGGAGCAGCATATGGTATATTGTGGGAGATCTTTTGG	360
63	K Q L Y D Q E Q H M V Y C G G D L L G	82
361	GAGAACTACTGGGACGTCAGAGCTTCTCCGTAAGAACCCAAGCCCTCTCTATGATATGC	420
83	E L L G R Q S F S V K N P S P L Y D M L	102
421	TAAGAAAGAATCTTGTCACTTTAGCCACTACTACTACAGATGCTGCTCAGACTCTCGCTC	480
103	R K N L V T L A T G A T T D A A Q T L C A L	122
481	TCGCACAGGATCACAGTATGGATATCCAAGTCAAGACCAACTGAAGCAAAGTGCAGAGG	540
123	A Q D H S M D I P S Q D Q L K Q S A E E	142
541	AAAGTTCCACTCCAGAAAAGAAGTACAGAAAGACGATATCCCCA CACTGCCTACCTCAG	600
143	S T S R K R T S T E D D I P T L S E	162
601	AGCATAAATGCATACATTCTAGAGAAGATGAAGACTTAATTGAAAATTTAGCCCAAGATG	660
163	H K C I H S R E D E D L I E N L A Q D E	182
661	AAACTCTAGGCTGGACCTTGGATTTGAGGAGTGGGATGTAGCTGGCCTGCCTTGGTGGT	720
183	T S R L D L G F E E W D V A G L P W F	202
721	TTTTAGGAAACTTGAGAAGCAACTATACACCTAGAAGTAATGGCTCAACTGATTTACAGA	780
203	L G N L R S N Y T P R S N G S T D L Q T	222
781	CAAAATCAGGATGTGGTACTGCCATTTGTTT CAGATACTACAGATGACTTGTGGT'TTTGA	840
223	N Q D V T A I V S D T T D D L D L N	242
841	ATGAGTCAGTATCAGAGCAGTTAGGTGTTGGAATAAAAGTTGAAGCTGCTGATACTGAAC	900
243	E S V S E Q L G V G I K V E A A D T E Q	262
901	AAACAAGTGAAGAAGTAGGGAAGTAAAGTGACAAAAGGTGATTGAAGTGGGAAAATG	960
263	T S E V G K V S D K K V I E V G K N D	282
961	ATGACTGGAGGACTTAAGTCCCTTAAGTATGATACCGATGTAGAGTGGCTTCTGAGG	1020
283	D L E D S K S L S D D T D V E V T S E D	302
1021	ATGAGTGGCAGTGTACTGAATGCAAGAAATTTAACTCTCCAAGCAAGAGGTACTGTTTC	1080
303	E W Q C T E C K K F N S P S K R Y C F R	322
1081	GT'TGTGGCCCTTGAGGAAGGATGGTATTGAGATTGTTCAAAGTTAACCCATTCTCTCT	1140
323	C W A L R K D W Y S D C S K L T H S L S	342
1141	CCACGTCTGATATCACTGCCATACCTGAAAAGGAAAATGAAGGAAATGATGTCCCTGATT	1200
343	T S D I T A I P E K E N E G N D V P D C	362
1201	GTCGAAGAACCCATTTCCGGCTCTGCTCGTTAGACCTAAAGATGCGTATATAAAGAAAGAAA	1260
363	R R T I S A P V V R P K D A Y I K K E N	382
1261	ACTCCAAACTTTTTGATCCCTGCAACTCAGTGGAAATCTTGGATTTGGCTCACAGT'TCTG	1320
383	S K L F D P C N S V E F L D L A H S S E	402
1321	AAAGCCAAGAGACCTCTCAAGCATGGGAGAACAGTTAGATAACCTTTCTGAACAGAGAA	1380
403	S Q S M T I S S M G E Q L D N L S E Q R T	422
1381	CAGATACAGAAAACATGGAGGATGGCAGAATCTCTGAAGCCATGTAGCTTATGTGAGA	1440
423	D T E N M E D C Q N L L K P C S L C E K	442
1441	AAAGACCAGCAGACGGGAACATTATTCATGGAAGGACGGGCCATCTTGTCACTTGT'TTC	1500
443	<u>R</u> P R D G N I I H G R T G H L V T C F H	462
1501	ACTGTGCCAGAAGACTAAAGAAGGCTGGGGCTTCATGCCCTATTTGCAAGAAAGAGATTC	1560
463	C A R R L K K A G A S C P I C K K E I Q	482
1561	AGCTGGTTATTAAGGTTTATAGCATAATGGTAGTACGAACATAAAAATGCATTTATTC	1620
483	L V I K V F I A	490
1621	AGTTCACCTTACCACATTTATTTGAAAATCAATCCTTTATTTAATTTTATTTCCAACCTGTC	1680
1681	AGAGAATGTTCTTAGGCATCAAAATCCAAGGTAGCTGTAAGAAAAATACTGGAGCTAACAA	1740
1741	ATGAAGAACAGAAAGTAACTGATTAGTCAAATATTAAAGTCCATGGATTAAGTTATGACA	1800
1801	GCAGTCAGGTACATAGTTAGGTGAACCCAAAAGAAAACTCTTGAAAATACTAGATTTCT	1860
1861	TCCATCCACATTTTCAATATTTGAGGTATAATTAACATGATAAAGTGT'TTCTCTCTAACGA	1920
1921	CTTGTAAGAAATCTAGTAACCAACCCAAAAGCAATAGAATGTTTGTGCCACCCCAAAAC	1980
1981	ACTCCCTTCTGCCCTCTTCCAGCAGTCTTCAAGTATTTCAATGGCTCTCCACCCAGTTT	2040
2041	TTTTTTTTTTTTTGCCTTTTTTTTTTTCGGGGGTATAGGGGAGGTCGTGGCCACAGCGT	2100
2101	CTGCTTGTCTTCTGCTCCAGGCTGAAGTGCAGTGAAGTCAAGATTGAGCCACTGCACTCC	2160
2161	AGCCTGGGTGACAGCGGAGACTCCATCTCAGAAAAAATAAAAAAAAAAAAAAAAAAAAAA	2216

FIG. 2. Nucleotide and derived amino acid sequence of human *MDMX*. **(A)** Nucleotide sequence of *MDMX* cDNA and predicted protein sequence. The putative AUG codon is underlined and boldface; putative alternative translation start sites are shown in boldface. Conserved cysteines in the putative zinc-finger and in the RING finger domains together with the core amino acids are given in boldface italics, and a putative nuclear localization signal is underlined. **(B)** Sequence alignment of Mdm-like proteins.

T84 (Stratagene) with a mouse *mdmx* cDNA probe (see Materials and Methods). Two independent phage clones, which remained positive after three rounds of purification, were isolated and purified to homogeneity. Sequence analysis showed inserts of 1260 and 2260 bp (clone 1 and clone 2, respectively, in Fig. 1).

The shortest construct shows a strong homology with the 5' part of the previously identified mouse *mdmx* cDNA (Shvarts *et al.*, 1996), including the putative translation start site. Sequencing of the longest construct revealed that in the 3' part of the insert a stretch of 1143 bp shows a strong similarity with the

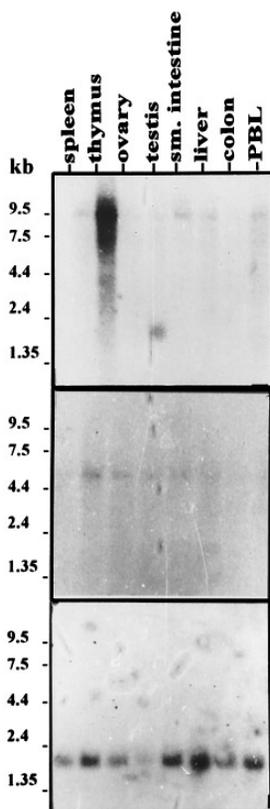


FIG. 3. Expression of *MDMX* mRNA in human tissues. (A) Northern blot containing poly(A)⁺-selected mRNA from human tissues was probed with human *MDMX* cDNA. (B) The same blot was probed with human *MDM2* cDNA. (C) Human β -actin cDNA was used as control for the amount of mRNA loaded. Positions of molecular-weight markers (kb) are indicated.

localization signal of the Mdm2 protein is found at positions 178–183 (RKRHK), in the middle part of the protein, whereas in the human Mdmx protein it is found at positions 442–445 (KRPR) in the C-terminal part.

Expression of *MDMX* mRNAs in Human Tissues

To determine the expression pattern of human *MDMX* mRNA we performed Northern blot analysis on a human multi tissue blot. Figure 3A shows that a 10-kb human *MDMX* transcript is synthesized in all tissues, with the highest amount in the thymus and a very low expression in colon. An additional shorter transcript of approximately 2.2 kb is detectable in testis. We do not know the exact nature of the different mRNAs, but the mRNA expression pattern of the mouse and the human is rather conserved and will be discussed in more detail later. Furthermore, expression of human *MDMX* mRNA was found in all tissues and cell lines tested by RT-PCR (data not shown). We compared the expression of human *MDMX* with that of *MDM2* by reprobing the same blot with an *MDM2* probe (Fig. 3B). The human *MDM2* gene was previously shown to be expressed in a 5.5-kb transcript, first identified by Oliner *et al.* (1992) predominantly in skeletal muscle and liver, with somewhat lower levels

in pancreas and lung (Ladanyi *et al.*, 1993). A 5.5-kb *MDM2* transcript was detected, but we find the *MDM2* gene to be almost equally expressed in all tissues on this blot with only a somewhat higher expression in the thymus, but certainly not as prominent as the *MDMX* transcript.

In Vitro Interaction between Mdmx and p53

We have shown previously that mouse Mdmx can bind p53 *in vivo* (Shvarts *et al.*, 1996). To investigate the *in vitro* interaction between human and mouse Mdmx and human p53, we performed two types of experiments. First, we mixed *in vitro* translated, ³⁵S-labeled Mdmx with bacterially produced and purified p53 protein (see Materials and Methods). After this incubation immunoprecipitations with anti-p53 and anti-Mdmx antibodies were performed.

The results presented in Figs. 4A and 4B demonstrate that in the presence of p53 protein significant amounts of the *in vitro* translated human and mouse Mdmx proteins are coimmunoprecipitated with a mixture of the anti-p53 antibodies PAb 122/PAb 421. Furthermore, it seems that only the upper band of mouse Mdmx, which is probably generated from the first in-frame AUG, interacts with p53, suggesting that the protein lacking the N-terminal part does not bind p53. Although difficult to see in Fig. 4, the same seems to be true for the human Mdmx. To verify the *in vitro* binding association between human Mdmx and p53, a GST–Mdmx fusion protein was coupled to glutathione beads and incubated with ³⁵S-labeled, *in vitro* translated human p53 or cJun as a control. As can be seen in Fig. 4C, the GST–Mdmx protein specifically interacts with human p53 and not with cJun, while GST alone binds neither protein.

We conclude from these results that both human and mouse Mdmx proteins associate with p53 protein *in vitro* and that the binding domain is probably located in the extreme N-terminal part of the Mdmx proteins.

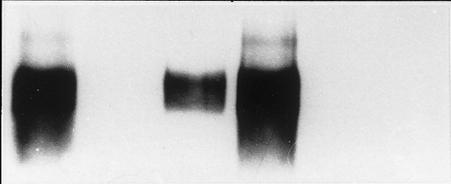
Chromosomal Mapping of the *mdmx* Genes

Amplification of the human *MDM2* gene has been observed in a variety of tumors (Oliner *et al.*, 1992; Reifemberger *et al.*, 1993; Habuchi *et al.*, 1994; Lianes *et al.*, 1994; Corvi *et al.*, 1995). Since the *MDMX* gene might be present in amplicons described in human tumors, we set out to localize the gene. To begin with, the mouse *mdmx* gene was mapped with FISH to chromosome 1, region F–G (Lyon and Searle, 1989; Figs. 5A and 5B). This region is in synteny with a region on the long arm of the human chromosome 1. This possible location of the human *MDMX* gene was verified by FISH analysis with the P1 clone containing human *MDMX* sequences. The human *MDMX* gene was mapped to human chromosome 1q32 (Figs. 5C and 5D). This region was recently reported to be amplified in a subset of liposarcomas

A

proteins Ab's	MMDMX + p53			MMDMX		
	α -MMDMX	+			+	
N.I. mouse		+			+	
α -p53			+			+
						

B

proteins Ab's	HMDMX + p53			HMDMX		
	α -HMDMX	+			+	
N.I. mouse		+			+	
α -p53			+			+
						

C

10% input		GST-HMDMX		GST	
cJun	p53	+ cJun	+ p53	+ cJun	+ p53
					

FIG. 4. *In vitro* association between Mdmx and p53. (A, B) Immunoprecipitations on a mixture of *in vitro* translated, ^{35}S -labeled Mdmx proteins and bacterially produced p53 with a mouse Mdmx-specific polyclonal antibody pAb100 (α MMDMX), a polyclonal antibody recognizing human Mdmx pAb55 (α HMDMX), or a mixture of PAb 122/421 (α p53); nonimmune mouse antibody (N.I. mouse) was used in control immunoprecipitations as indicated. (C) GST-HMDMX fusion protein or only GST bound to glutathione beads was mixed with either *in vitro* translated ^{35}S -labeled p53 protein or cJun protein. After this incubation the beads were washed and bound proteins were separated on SDS-PAGE.

(Szymanska *et al.*, 1996; Forus *et al.*, 1995). The possible amplification of the *MDMX* gene in these tumors is currently under investigation.

DISCUSSION

We report here the isolation and identification of the human counterpart of the mouse *mdmx* gene. The human *MDMX* gene encodes a protein of 490 aa that is 90% homologous to mouse Mdmx (Shvarts *et al.*, 1996). The amino acid sequence predicts a protein with a theoretical molecular weight of 54 kDa. However, on SDS-PAGE human Mdmx was found to migrate as a protein with a molecular weight of approximately 80 kDa. This behavior corresponds to the observation that the Mdm2 protein also runs at a much higher molecular weight on SDS-PAGE than expected from its amino acid se-

quence. We expect the Mdmx amino acid sequence to be as presented here, since upstream from the putative AUG, in-frame stop codons are found. The apparent molecular weight of human Mdmx is somewhat larger than that earlier found for mouse Mdmx. This might be caused by different posttranslational modifications, e.g., phosphorylation, which is known to occur on the Mdm2 proteins.

A sequence comparison between human *MDMX* and human *MDM2* reveals conservation that is strongest in the p53-binding domain located in the N-terminal part of both proteins. Furthermore, putative functional domains in the C-terminal part of the proteins are highly conserved. These domains consist of a zinc finger and a zinc-binding domain called the RING finger. The latter is thought to be involved in protein-protein interaction (Boddy *et al.*, 1994).

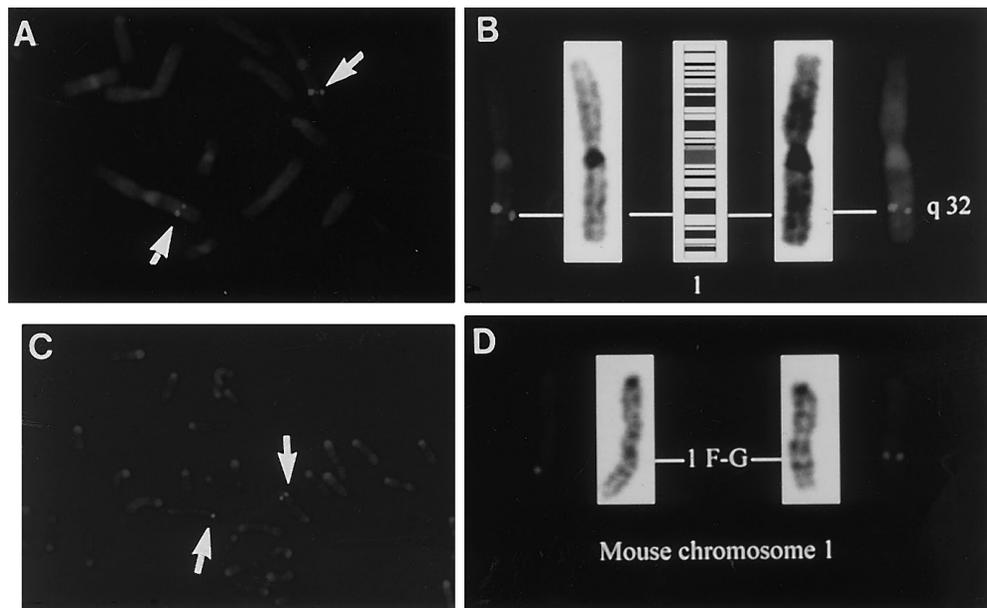


FIG. 5. Chromosomal localization of the *mdmx* genes by fluorescence *in situ* hybridization. (A, C) Mapping of the *mdmx* gene on mouse and human metaphase chromosomes, respectively. Probe DNA, light spots; chromosomes are counterstained with DAPI. (B, D) Giemsa-like banding to identify the chromosome and localize the probe on mouse chromosome 1 F-G and on human chromosome 1q32. At least 20 informative chromosome spreads were used to determine the localization of the *mdmx* genes.

By Northern blot analysis we detect a 10-kb transcript of human *MDMX* in most tissues, with a relatively high expression in thymus. Also mouse thymus was found to express relatively high levels of the murine *mdmx* mRNAs (data not shown). The murine *mdmx* gene is expressed prominently into two long mRNAs of approx. 10 and 7.5 kb in all tested tissues and a mRNA of 2.2 kb, which is highly expressed in testis and to a very low level in other tissues (Shvarts *et al.*, 1996). An *MDMX* mRNA of approx. 2.2 kb is also found in human testis. This mRNA is not detected in the other tissues, but if the expression of this mRNA in the other tissues is as low relative to the longer mRNA as in mouse tissues, it might be a detection problem. Thus, the mRNA expression patterns of the human and mouse *mdmx* genes are rather similar, with the possible exception of the shorter mRNAs in tissues other than testis. It is not firmly established whether the mRNAs detected all contain the coding region here presented. However, we hypothesize that the longer hybridizing RNA band represents not (fully) processed mRNA because of the following reasons. First, the cDNA clone 2 we isolated most likely contains intron sequences, while the library was made from oligo(dT)-

primed mRNAs. Second, an entry in the NCBI EST database of 275 bp (L44283), derived from human thymus RNA, is identical over a stretch of 113 bp with the human *MDMX* cDNA sequence shown here (Fig. 6). From studies on the genomic organization of the mouse gene we know this to be exon 2. Both 5' and 3' of these exon sequences the sequence of the EST diverges from the cDNA sequence (Fig. 6), but now shows similarity with intron sequences of the mouse *mdmx* gene (not shown). These results already suggested that unspliced or not fully spliced *mdmx* mRNAs are expressed. In addition, we performed an RT-PCR assay on mouse thymus mRNA with one primer derived from exon 2 and another from the following intron. A product of the expected length could be detected, again suggesting that (partly) unspliced *mdmx* mRNAs are expressed.

The 5' end of cDNA most likely represents the approximate 5' end of the mRNA, as indicated by 5'-RACE experiments (Shvarts *et al.*, 1996) and because in the mouse genome just upstream of this region promoter activity was found (R.C.A. van Ham and A.G. Jochemsen, unpublished results). From these results we hypothesize that the long mRNAs represent not fully processed mRNA and that the cDNA sequence we

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hu mdmx          1-GGCACGAGCTAGGATCTGTGACTGCCACCCCTCCCCCACC6GGGCTCGGCGGGGAGCGACTCATGGAGCTGCCGTAAG
EST              ATGTGTCATATGTGTGTTTTACTTCTGTCTGGTTGCGCTTTGTGTGAATGCTAAATAGGGAATTTTCTGCATTAGAATAGATGTTATAAATTTTTTTTCTATTATG

hu mdmx  TTTTACCAACAGACTGCAGTTTCTTCACTACCAAAATGACATCATTTTCCACCTCTGCTCAGTGTTCAACATCTGACAGTGCTTGCAGGATCTCTCCTGGACAAATCAATCAG
EST      TTTTACCAACAGACTGCAGTTTCTTCACTACCAAAATGACATCATTTTCCACCTCTGCTCAGTGTTCAACATCTGACAGTGCTTGCAGGATCTCTCCTGGACAAATCAATCAG

hu mdmx  GTACGACCAAACTGCCGCTTTTGAAGATTTTGCATGCAGCAGGTGCGCAAGGTGA
EST      GTAATCATTTTCGGTATTCTAGTTTTTGGTTTTTTTTTTTTTAAATTTTAAAA

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FIG. 6. Alignment of the 5'-part of the human *MDMX* cDNA sequence with an EST from the NCBI database (Accession No. L44283).

now show is a representation of the smaller mRNA of 2.2 kb, although other possibilities cannot be excluded. Thus, although we find the human *MDMX* 10-kb mRNA to be highly expressed in thymus, it remains to be seen whether this has consequences at the protein level. It is not excluded that the unspliced mRNAs also code for Mdmx-like proteins, since in clone 2 an in-frame ATG codon is present just eight amino acids in front of the Mdmx-homology region. It will be interesting to find out whether this relatively high mRNA expression will turn out to be important in the differentiation of T-cells or be reflected in a role of *MDMX* (over)-expression in hematological malignancies, as has been reported for *MDM2* (Bueso-Ramos *et al.*, 1993; Maestro *et al.*, 1995; Zhou *et al.*, 1995). *MDM2* amplification is found in approximately 30% of human sarcomas (Oliner *et al.*, 1992). A recent analysis of liposarcomas shows 50% amplification in 12q14, most probably involving *MDM2*. In several liposarcomas (4/14) amplification around 1q32 has also been found, possibly indicating involvement of *MDMX* (Szymanska *et al.*, 1996). Whether the *MDMX* gene is present in this amplicon is currently under investigation.

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