A Novel MDMX Transcript Expressed in a Variety of Transformed Cell Lines Encodes a Truncated Protein with Potent p53 Repressive Activity*

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Ravikumar Rallapalli, Gordon Strachan, Brian Cho‡, W. Edward Mercer‡, and David J. Hall§

From the Department of Biochemistry and Molecular Pharmacology and ‡Department of Microbiology and Immunology, Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

The MDMX gene product is related to the MDM2 oncoprotein, both of which interact with the p53 tumor suppressor. We have identified a novel transcript of the MDMX gene that is expressed in a variety of cell lines, and in particular, in growing and transformed cells. This transcript is identical to the published sequence yet it has a short internal deletion of 68 base pairs. This deletion produces a shift in the reading frame after codon 114, resulting in the inclusion of a stop codon at amino acid residue 127 (full-length MDMX is 489 residues). This truncated MDMX protein is termed MDMX-S ("short form"), represents only the p53-binding domain, and appears to bind p53 better than full-length MDMX. The MDMX-S protein can be detected in cell extracts and when overexpressed is much more effective than MDMX in inhibiting p53-mediated transcriptional activation and induction of apoptosis. Since MDMX-S lacks the central and carboxyl-terminal regions contained within full-length MDMX, it is likely to play a key role in the regulation of cell proliferation and apoptosis in a way distinct from MDMX.

The p53 tumor suppressor protein plays an important role in regulating movement through a number of cell cycle checkpoints (1–3, 38). The activity of p53 is in turn modulated through the action of the MDM2 protein which is amplified in a variety of tumors (4–6). MDM2 can associate with p53 (7) and directly inhibit p53's ability to activate transcription of target genes, such as p21. MDM2 performs this function in a number of ways. First, the amino-terminal domain MDM2 binds tightly to the transcriptional transactivation domain of p53 (4–6, 8, 9). Through this interaction, MDM2 blocks the ability of p53 to activate transcription of specific target genes by repressing the formation of the preinitiation complex mediated through the TFIIIE and TATA-binding protein subunits of RNA polymerase II (10, 11). The second blocking activity of MDM2 is that it targets p53 for proteolytic degradation by the ubiquitin-proteasome pathway (12–14, 40). Third, MDM2 also functions to shuttle p53 out of the nucleus and into the cytoplasm, via a nuclear export signal located within the MDM2 protein (33).

While MDM2 was first identified as a protein that inhibits the action of p53 it has recently been shown to affect other components of the G1/S transition. MDM2 was found to associate with both the E2F1 transcription factor (15) and the retinoblastoma tumor suppressor (pRb)3 (16). The interaction with pRb was found to be repressive, in that the association of MDM2 with the carboxyl-terminal domain of pRb inhibited the growth regulatory function of pRb (16). The interaction with E2F1, over the amino terminus of MDM2 and the carboxyl terminus of E2F1, was stimulatory in the sense that MDM2 enhanced the ability of E2F1 to activate target gene expression (15). Thus MDM2 appears to regulate at least three of the important players in the G1/S phase transition: p53, pRb, and E2F1. It has therefore been concluded that MDM2 functions in the fashion of a dominantly acting oncogene, driving cells through the G1/S phase transition, by inhibiting p53 and pRb while helping to enhance the activity of E2F1.

Recently a protein related to MDM2, termed MDMX, has been cloned and partially characterized (17, 18). The region of highest homology between MDM2 and MDMX is within the p53-binding domain at the amino terminus of MDMX. Additionally, a zinc finger domain within the central portion of MDMX and a ring finger domain at the carboxyl terminus are conserved between MDMX and MDM2 (17–19). It has been demonstrated that MDMX associates with p53 and suppresses its activity (17). While MDMX appears very similar to MDM2, it nonetheless has a number of features that are distinct. For example, MDMX is not induced by DNA damaging agents that are known to induce MDM2 (17). This indicates that the MDMX promoter is likely not transcriptionally transactivated by p53. This is distinct from MDM2 which is transactivated by p53 (20, 21). Also, MDMX null mice (both alleles inactivated) are embryonic lethals (22, 23), in a wild-type p53 background. This indicates that if MDMX is expressed, it cannot functionally substitute for the absence of MDM2 with regard to control of p53 activity. However, it should be noted that it has not yet been demonstrated that MDMX is expressed early in development. Thus, MDMX likely functions in ways that are separate from MDM2. Yet the nature of MDMX's role in control of cell proliferation is largely unknown at this time.

To gain insight into the role of MDMX in control of cell cycle, we have explored its regulation. We find that the MDMX gene is expressed as different transcripts. One transcript has a small internal deletion that leads to the introduction of a stop codon following amino acid residue 127. This novel transcript encodes only the p53-binding domain of MDMX and the protein pro-

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§ To whom correspondence should be addressed: Dept. of Orthopaedic Surgery, 501 Curtis Bldg., Thomas Jefferson University, Fax: 215-523-9162; Tel.: 215-503-2035; E-mail: hall@hendrix.jci.tju.edu.

1 The abbreviations used are: pRb, retinoblastoma tumor suppressor; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; β-gal, β-galactosidase; GST, glutathione S-transferase; RT-PCR, reverse transcriptase-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; HFF, human foreskin fibroblast; DHFR, dihydrofolate reductase.
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duced is referred to here as MDMX-S. MDMX-S is better able to suppress p53-mediated transcriptional transactivation and induction of apoptosis, relative to full-length MDMX. MDMX-S expression may therefore play an important role in the regulation of cell proliferation and apoptosis.

MATERIALS AND METHODS

Cell Culture, Plasmids, Transfections, and Apoptosis—All cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum or fetal calf serum. All transfection experiments were initiated in 50% confluent monolayer cultures as described by Aasen et al. (24). Plasmids at a total of 30 μg were transfected by the calcium phosphate procedure (24). The cells were glycerol shocked 5–6 h after DNA addition.

The MDMX and MDMX-S cDNA constructs were cloned into BlueScript KS just 3’ to a FLAG epitope tag sequence. The epitope tag was linked in-frame to the reading frame of MDMX and MDMX-S. For expression studies the tagged MDMX and MDMX-S genes were excised from the BlueScript plasmid and cloned into the eukaryotic expression vector pRC/CMV (Invitrogen). The wild-type p53 cDNA was cloned into and expressed from the pcDNA3 expression plasmid (Invitrogen). The target of p53 was the pRGC linked in-frame to the reading frame of MDMX and MDMX-S. For the script KS just 3’-5’–6’ h after DNA addition.

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The RT-PCR reactions, 1 μg of total RNA from the various cell lines was used in each reaction with primers from the murine sequence (the 5’ primer, gccctctctatgacatgc (spanning amino acid residues 96–102) and the 3’ primer, gtcgtgaggtaggc (spanning amino acid residues 158–163)) and human sequence (the 5’ primer, gccctctctatgacatgc, and the 3’ primer, gtcgtgaggtaggc) (17, 18). The primers for the human sequence represent the same positions as those used for the murine sequence. These primers flank the site of deletion in MDMX. All total RNAs were DNase I treated in the RT reaction prior to the PCR reactions. Additionally, as a control, PCR done in the absence of RT was negative for any ethidium bromide-stained bands (data not shown).

To generate GST fusion proteins, the MDMX and MDMX-S cDNAs were cloned into pGEX-5T (Pharmacia) in-frame with the glutathione S-transferase gene (GST). Fusion proteins were produced as described by Jordan et al. (27). Briefly, the genes were induced with isopropyl-thio-β-D-galactoside. The position of the markers were calculated using the nuclease-treated rabbit reticulocyte lysate, in a total reaction volume of 50 μl. [35S]Methionine at 0.9 MCl/ml was also included in the reactions.

Immunofluorescence—For indirect immunofluorescence, Calu cells were plated on 10-cm tissue culture dishes containing glass coverslips. The cells were transfected with the MDMX or MDMX-S expression plasmids (or the vector control) and 24 h post-transfection the plates were washed once in PBS and then fixed with 4% paraformaldehyde in PBS for 20 min followed by an additional rinse in PBS. To permeabilize the cells, the coverslips were treated with PBS plus 0.2% Triton X-100 for 15 min followed by three 5-min washes in PBS plus 0.2% gelatin, as described by Harlow and Lane (28). The M2 monoclonal antibody was diluted in PBS plus 0.2% gelatin. 50 μl of diluted antibody was added to a 1-μl volume of 5000 dilution of secondary antibody in TBS (goat anti-mouse or goat anti-rabbit conjugated to alkaline phosphatase, Vector Labs). The blots were then stained using the Protoblot system from Promega.

Western Blot Hybridizations—Extracts were first electrophoresed by SDS-PAGE and then the proteins were electrophoretically transferred onto nitrocellulose, the blots were washed in TBST buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20), blocked with 2.5% bovine serum albumin in TBST for 30 min at room temperature, and then incubated with either the M2 anti-FLAG monoclonal antibody (10 ng/ml, WVR/Kodak/IIB) or polyclonal antiserum generated against MDMX-S (1:5000 dilution). The blots were washed three times (10 min each) with TBST then incubated for 30 min at room temperature with a 1:7500 dilution of secondary antibody in TBS (goat anti-mouse or goat anti-rabbit conjugated to alkaline phosphatase, Vector Labs). The blots were then stained using the Protoblot system from Promega.

FIG. 1. PCR of MDMX from NIH3T3 cDNA results in two bands. Primers flanking the start and stop codons of MDMX were used in a PCR reaction with 100 ng of cDNA derived from 1 μg of poly(A)+ mRNA from confluent nontransformed NIH3T3 fibroblasts. The product of the reaction was electrophoresed on a nondenaturing 10% acrylamide gel. The gel was stained with ethidium bromide and photographed (the expected size of MDMX is 1450 base pairs). The position of the markers are shown.

The nuclei were then stained with the DNA dye 4,6-diamidino-2-phenylindole. The coverslips were rinsed three times in PBS, once in deionized water, and then they were dried, mounted, and analyzed by fluorescence microscopy.
RESULTS

Identification of A Novel MDMX Transcript Containing an Internal Deletion—The MDM2 oncoprotein is a known binding partner of a number of cellular factors, namely p53, pRb, and E2F1. In our efforts to study the regulation of these key regulatory factors, we initiated work on the recently identified MDM2-related protein, termed MDMX. To begin studies on MDMX we isolated its cDNA clone by RT-PCR, using primers that flank the site of the deletion in MDMX-S. The products of the reaction were electrophoresed on a 10% polyacrylamide gel. The gel was stained with ethidium bromide and photographed. The positions of MDMX (201 base pairs), MDMX-S (133 base pairs), and the markers are shown.

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**Fig. 2.** The open reading frame of MDMX-S reveals a shortened protein. A, the sequence within MDMX that is deleted in MDMX-S. The brackets define the boundaries of the DNA sequence in MDMX (top line) that is deleted in MDMX-S (bottom line). B, amino acid sequence of MDMX-S. The predicted p53-binding domain that aligns with that of MDM2 is represented by residues 1–107. The sequence underlined and in bold is unique to MDMX-S. C, linear alignment of MDMX and MDMX-S protein sequences. The positions of the p53-binding domain, the zinc finger, the ring finger, the putative nuclear localization sequences (NLS) are outlined in MDMX. D, an RT-PCR reaction was performed on 0.2 and 1.0 μg of cDNA generated from poly(A)+ selected mRNA from confluent NIH3T3 fibroblasts using primers that flank the site of the deletion in MDMX-S. The products of the reaction were electrophoresed on a 10% polyacrylamide gel. The gel was stained with ethidium bromide and photographed. The positions of MDMX (201 base pairs), MDMX-S (133 base pairs), and the markers are shown.
mRNA from confluent nontransformed NIH3T3 fibroblasts in the reaction. It was first reversed transcribed to make cDNA, then used in the polymerase chain reaction with the primers that flank the start and stop codons. The RT-PCR products are shown in Fig. 1. It is apparent from the figure that two bands are produced in the reaction that are very close in size to each other (the expected size is 1450 base pairs). The entire reaction product was then cloned into Bluescript KS and a number of individual isolates were sequenced. While many of the clones produced the expected published sequence (17) a substantial fraction had an internal deletion of 68 base pairs. This deleted sequence results in a change in the reading frame of the protein because it introduces a new translation stop codon just after the sequence encoding the p53-binding domain. The deleted sequence is shown in Fig. 2A and the complete reading frame of this sequence is shown in Fig. 2B.

The first 114 amino acid residues are identical to the published sequence and represent the p53-binding domain (residues 1–100) (17). The change in reading frame results in an insertion of 13 new amino acids followed by a stop codon (Fig. 2, A and B). Fig. 2C shows a comparison of full-length MDMX (489 residues) and the shortened form that we have termed MDMX-S (127 residues). It is clear that MDMX-S lacks the central and carboxyl-terminal domains containing the zinc finger and ring fingers, respectively (17).

To determine the extent of expression of this new form of MDMX-S, PCR primers were generated that flank the deleted sequence and PCR was performed on the original NIH3T3 cDNA pool made from poly(A) mRNA from confluent cells. With these primers, a PCR product corresponding to MDMX would be 201 base pairs in length while a product corresponding to MDMX-S would be 133 base pairs. As seen in Fig. 2D, two bands of 133 and 201 base pairs are seen in the polyacrylamide gel from the products of the PCR reaction, indicating that both MDMX and MDMX-S are expressed as transcripts in these cells.

To ensure that these two bands represent bona fide MDMX and MDMX-S sequences, the bands were excised from the gel, directly cloned via TA Cloning (Invitrogen) into the pCR2.1 plasmid vector, and sequenced. DNA sequence analysis revealed that the 201-base pair band exactly matches the original MDMX sequence while the 133-base pair band exactly matches the MDMX-S sequence containing the internal deletion.

It was next important to determine the extent of expression...
of MDMX and MDMX-S in other cell lines. Total RNA was isolated from nontransformed C3H10T1/2 murine embryonic fibroblasts that were growing, confluent or serum starved for 48 h to arrest in G0/G1 phase of the cell cycle. Total RNA was isolated from the transformed murine lines psi2 (retrovirally transformed), F9 (teratocarcinoma), and SP2/0 (myeloma/hybridoma). Total RNA was also isolated from nontransformed low passage rat vascular smooth muscle cells (A10 cell line) that were serum starved or starved and serum stimulated for 8 h. RT-PCR was performed on 1 μg of the total RNA using the primers that flank the site of the deletion. As shown in Fig. 3, A and B, MDMX-S is expressed as a detectable transcript in all cell lines. In all cell lines, the levels of MDMX remain somewhat constant. However, in serum-starved cells, expression of MDMX-S is barely detectable while in growing cells, and especially those that are transformed, MDMX-S is easily detectable. Further quantitative methods will be needed to determine if MDMX-S expression is actually up-regulated in transformed cells and down-regulated in serum-starved primary cells.

It was of interest to determine if the expression pattern of MDMX-S in human cells mirrors that seen in rodent cells. Using the DNA sequence of human MDMX (18), primers were generated that would flank the deletion in MDMX-S. These primers were then used in an RT-PCR reaction with 1 μg of total RNA from the following human/primate cell lines: COS (SV40 T antigen transformed monkey), HeLa (human cervical carcinoma), Calu-6 (human lung carcinoma), and MCF-7 (human breast cancer). Also, RNA was isolated from nontransformed 4th passage human foreskin fibroblasts (HFFs) that were either serum starved or growing. This is shown in Fig. 4. For comparison, an RT-PCR reaction was also performed on RNA from growing and confluent NIH3T3 fibroblasts and growing C3H10T1/2 fibroblasts, using the mouse specific primers, and the products were run on the same gel. MDMX-S is expressed as a detectable transcript in the RNA from each cell line. In serum-starved skin fibroblasts, MDMX-S transcripts are not easily detectable. However, in growing cells, and those that are transformed, MDMX-S is easily detectable. Further quantitative methods will be needed to determine if MDMX-S is up-regulated in transformed cells and down-regulated in low passage cells. In HeLa cells and MCF7 cells, MDMX-S appears low relative to MDMX. Further experiments will be needed to determine the basis of this difference.

Detection of Endogenous MDMX and MDMX-S Proteins in Cell Extracts—To determine if endogenous MDMX and MDMX-S protein expression could be detected in cell extracts, rabbit polyclonal antibodies were generated against a GST fusion with MDMX-S. These antibodies should recognize both MDMX and MDMX-S. Two separately generated antisera were used in immunoblot experiments. The resulting antibody was directed against the epitope tag followed by incubation with a secondary antibody coupled to alkaline phosphatase. Shown are the developed blots. The bands corresponding to MDMX and MDMX-S are indicated.

Fig. 5. Endogenous MDMX and MDMX-S are detectable in COS cell extracts by immunoblot, using polyclonal antiserum to MDMX-S. 50 μg of COS nuclear and cytosolic extract were electrophoresed by SDS-PAGE and blotted onto nylon membrane. Identical blots were generated and incubated with the different antisera (A, sera number 234; B, sera number 235) generated against MDMX-S. Identical blots were also probed with each preimmune sera. Following incubation with the primary antibody, the blots were incubated with a secondary antibody coupled to alkaline phosphatase. Shown are the developed blots. The bands corresponding to MDMX and MDMX-S are indicated.

Fig. 6. Coupled in vitro transcription/translation of MDMX-S reveals two protein bands. The MDMX-S protein was radiolabeled by coupled in vitro transcription/translation (TnT System, Promega). The resulting [35S]Met/Cys radiolabeled protein was separated by SDS-PAGE followed by immunoblotting. The blot was incubated with the M2 antibody directed against the epitope tag followed by incubation with a secondary antibody coupled to alkaline phosphatase. Shown on the right is the developed blot. The blot was then exposed to x-ray film for autoradiography (the film is shown on the left). The two protein bands of 17 and 27 kDa produced by MDMX-S are indicated.
MDMX, as shown in Fig. 5B, and weakly recognizes MDMX-S. Thus, both MDMX and MDMX-S appear to be expressed as detectable gene products in COS cells.

Detection of an MDMX-S Protein following Translation in Vitro and following Ectopic Expression in COS Cells—To begin functional studies of MDMX-S, the FLAG epitope tag was cloned at the 5′ end of the MDMX-S gene (at the amino terminus of the protein). This tagged MDMX-S gene was then transcribed and translated in vitro using a rabbit reticulocyte lysate, with [35S]methionine/cysteine. The products of the reaction were analyzed by SDS-PAGE, the gel immunoblotted, and the blot probed with the M2 antibody which recognizes the epitope tag. Two protein bands of 17 and 27 kDa were detected in the immunoblot, as shown on the right side of Fig. 6 (“Western”). When the blot was exposed to x-ray film these bands were also detected by autoradiography, as seen on the left side of Fig. 6 ([35S] label). If the 17-kDa form represents the correct size MDMX-S protein as predicted from the open reading frame, then the 27-kDa form is possibly a modified version. We do not yet know the nature of any potential modification although MDMX-S is rich in serine, a potential site for phosphorylation.

The epitope-tagged MDMX and MDMX-S genes were then cloned into the pRC/CMV expression plasmid for use in transfection studies. The plasmids were transfected into COS cells and nuclear and cytosolic extracts were generated at 48 h post-transfection. The extracts (50 μg) were electrophoresed by SDS-PAGE, immunoblotted, and incubated first with the M2 monoclonal antibody and then with a secondary antibody conjugated with alkaline phosphatase (shown in Fig. 7A). It is clear that expression of both proteins can be detected in the extracts, and that by the intensity of staining, both proteins are expressed to roughly equal levels. The distribution of protein between nuclear and cytosolic extracts may be due to overexpression. It is also clear that the ectopically expressed MDMX-S is approximately 27 kDa, equivalent in size to the larger species seen in the in vitro translation reactions from Fig. 6.

Similar transfection experiments were then performed in Calu cells. Unfortunately the ectopically expressed MDMX and MDMX-S could not be detected by immunoblot. Expression could be detected by indirect immunofluorescence, however, as shown in Fig. 7B (the secondary antibody was conjugated to fluorescein isothiocyanate). The control transfected cells (vector only) were completely negative for fluorescein isothiocyanate fluorescence (data not shown). To determine if the level of expression was equal between MDMX and MDMX-S, the fluorescence intensity of a number of fluorescent positive cells was quantitated using a confocal microscope and the Bio-Rad COMOS software program (as in Ref. 41). For each transfection, about 40 individual cells were image analyzed and the relative level of fluorescence intensity measured. Shown in the lower part of Fig. 7B are the results of this quantitation. It is clear that MDMX and MDMX-S protein levels are nearly identical. Thus the data from Fig. 7, A and B, indicate that MDMX and MDMX-S are expressed to equal levels in both COS and Calu cells.

MDMX-S Is a More Potent Repressor of p53-mediated Transcription Than MDMX—To test the function of MDMX-S, its role in p53-mediated transcriptional activation was assessed. MDMX-S and MDMX, cloned into the pRC/CMV expression vector, were used in transfection studies along with a p53 expression plasmid and a p53 target reporter containing multiple p53 consensus binding sites (pRGCAFOS-LucZ) (30). Transfections were first performed in COS cells and promoter activity was monitored at 24 h post-transfection. As shown in Fig. 8A, left panel, p53 expression is able to significantly upregulate transcription from this promoter, to approximately

![Fig. 7. Ectopically expressed MDMX and MDMX-S are detectable in COS cell extracts by immunoblot and in Calu cells by indirect immunofluorescence. A, COS cells were transfected with pRC/CMV vector alone (“control”) (10 μg), pRC/CMV-MDMX (10 μg), or pRC/CMV-MDMX-S (10 μg). Forty-eight hours post-transfection, the cells were lysed and extracts generated. 50 μg of COS nuclear and cytosolic extract were electrophoresed by SDS-PAGE and blotted onto nylon membrane. Blots were generated and incubated with the M2 antiserum specific to the epitope tag. This was followed by a secondary antibody coupled to alkaline phosphatase. Shown are the developed blots. The bands corresponding to MDMX and MDMX-S are indicated. B, Calu cells grown on coverslips were transfected with pRC/CMV-MDMX (10 μg), pRC/CMV-MDMX-S (10 μg), or with the vector alone (10 μg). Forty-eight hours post-transfection, the cells were fixed and processed for indirect immunofluorescence (M2 primary antibody directed against the epitope tag and a fluorescein isothiocyanate conjugated secondary antibody). Shown in the figure are two representative fluorescent positive cells for each transfection. Fluorescein isothiocyanate fluorescence levels per cell were quantitated using the Bio-Rad COMOS software program (Bio-Rad Microsciences Division). The relative fluorescence intensity is an average of 40 positively fluorescing cells (MDMX and MDMX-S) or 40 nonfluorescing cells (vector control).](image-url)
17-fold. This is consistent with the ability of p53 to enhance expression from this promoter. Coexpression of MDMX reduces the level of p53-mediated transcription to 3-fold above the control, consistent with its role in repressing p53 function (17).

Interestingly, MDMX-S was able to reduce the level of p53-mediated transcription much further, to only 50% of the control. This is 6-fold greater than was accomplished by MDMX.

When transfections were next performed in Calu cells, which
are devoid of a functional p53, a more striking effect was seen. Expression of MDMX reduced the level of p53-mediated transaction by one-half. MDMX-S on the other hand was able to reduce the level of p53-mediated transcription to background levels which is 12-fold more than was accomplished by MDMX (Fig. 8, left panel). Taken together, it therefore appears that MDMX-S is a much more potent repressor of p53-mediated transcriptional activation than full-length MDMX.

As controls in these experiments, the pA-FosLacZ reporter construct, lacking the p53-binding sites, was used in similar transfections. As shown in Fig. 8, A and B, right panels, p53 is not able to activate transcription from this promoter. Furthermore, MDMX-S and MDMX have no additional effect on transcription from pA-FosLacZ. This indicates that the effect of MDMX-S on the pRGCD-FOS-LacZ promoter plasmid, containing the p53-binding sites, is dependent on p53. As additional controls in these experiments, MDMX alone or MDMX-S alone had no effect on transcription from either of the target promoters (data not shown).

The biological effect of p53 is to induce both cell cycle arrest in G1 phase and apoptosis. A G1 phase arrest would be evident as a suppression of S phase gene expression, represented, for example, by dihydrofolate reductase (DHFR) and DNA polymerase α (DNA pol α). Thus, overexpression of p53 should down-regulate expression from the DHFR and DNA pol α promoters. This is shown in Fig. 8C, where coexpression in Calu cells of a DHFR promoter-CAT construct or a DNA pol α promoter-CAT construct, along with a p53 expression plasmid results in a significant drop in transcription, compared with the activity of these promoters in the absence of p53. Promoter activity was monitored at 24 h post-transfection when the levels of apoptosis were low. Additionally, RSV-LacZ was co-transfected so that the levels of CAT activity could be corrected for any differences in transfection efficiency or induction of apoptosis. As seen in Fig. 8C, when MDMX-S is coexpressed along with p53, the inhibitory effects of this tumor suppressor are completely abolished, resulting in full or even enhanced promoter activity. Coexpression of MDMX on the other hand only partially restores activity from these promoters. These data are consistent with the results presented above that MDMX-S is a more potent inhibitor of p53 activity than MDMX. Note that throughout these experiments, as in Fig. 8, A-C, MDM2 was found to suppress p53-mediated transcription to the same extent as full-length MDMX (data not shown), similar to what has been previously described (17, 18).

**MDMX-S Inhibits p53-mediated Apoptosis**—The role of MDMX-S in p53-mediated apoptosis was next assessed. Evidence indicates that p53 mediates apoptosis in part through its transcriptional activation domain (37), which is the region likely bound by MDMX and MDMX-S. Thus overexpression of MDMX and MDMX-S should block p53-induced apoptosis. The MDMX-S and MDMX expression plasmids were therefore used in transfection studies along with a p53 expression plasmid and RSV-LacZ. Transfections were performed in COS cells and in Calu cells. β-Galactosidase positive cells were identified by staining and the percent apoptotic cells within this β-gal positive population was determined. As shown in Fig. 9, p53 expression results in a significant increase apoptosis, at 72 h post-transfection, in both cell types. This is consistent with the known ability of p53 to induce apoptosis when overexpressed (1–3, 38). Coexpression of MDMX-S reduces p53-mediated apoptosis by 62% in both Calu cells and COS cells. This is consistent with its ability to strongly suppress p53 transcriptional activity as described above. MDMX coexpression, however, was only able to reduce the level of p53 apoptosis by 32–34% in these two cell types. Therefore, it appears that MDMX-S is a more potent repressor of p53-mediated apoptosis than full-length MDMX.

**Association of p53 with MDMX and MDMX-S**—Binding experiments were next performed to determine if wild-type p53 could associate with both the MDMX and MDMX-S proteins. p53 was radiolabeled in an *in vitro* transcription/translation reaction and then equal quantities of the labeled p53 were applied to small batch columns (100 μl) containing GST, GST-MDMX, or GST-MDMX-S. It is estimated that nanogram quantities of p53 are applied to the columns which contain microgram quantities of the fusion proteins (thus the fusions are in 1,000–10,000-fold excess). The columns were washed extensively with buffer and then the bound material was eluted by boiling the columns in SDS-PAGE sample buffer. The eluate was electrophoresed by SDS-PAGE and the gel exposed to film. As shown in Fig. 10A, it is clear that p53 associates with both MDMX and MDMX-S but does not associate with GST alone. This is consistent with the notion that the amino-terminal domain of MDMX is the p53-binding domain (17, 18). However, it appears from the film that the affinity of p53 for MDMX-S may be higher than for MDMX. In these experiments we consistently see an approximate 8-fold increase in the amount of p53 bound to MDMX-S, compared with MDMX. Fig. 10B shows the Coomassie Blue-stained gel of the fusion proteins from the affinity columns.

As a control for these experiments a mutant p53 was used that fails to associate with MDM2 (39). This mutant has changes at residues 22 and 23 (Leu22 to Gln and Trp23 to Ser).
that result in an abrogation of MDM2 binding (39). As described above, p53(L22Q,W23S) was radiolabeled in an in vitro transcription/translation reaction. Equal quantities of radiolabeled protein were then applied to small batch affinity columns containing GST, GST-MDMX, and GST-MDMX-S. The columns were washed extensively in buffer and then boiled directly in SDS-PAGE sample buffer and electrophoresed by SDS-PAGE. The gel was dried and then exposed to x-ray film for autoradiography (the film is shown). A novel MDMX transcript of the MDMX gene. This form of MDMX, termed MDMX-S, produces a shortened protein comprised of the p53-binding domain of MDMX and lacking the central and carboxyl-terminal regions. This truncation is due to an internal deletion of 68 base pairs within the normal MDMX transcript that leads to the introduction of a stop codon just after the sequence encoding the p53-binding domain at residue 127. This deletion, which may be due to some form of regulated alternative splicing, also changes the reading frame and introduces 13 new amino acids into the carboxyl-terminal end of the truncation. At this time it is not known what role these new residues play in MDMX-S function.

What is perhaps most intriguing about the different forms of MDMX mRNA is that the MDMX-S transcript is expressed in a variety of mouse and human transformed cell lines. In non-transformed quiescent cells such as serum-starved murine embryonic fibroblasts, HFFs, and rat vascular smooth muscle cells, MDMX-S transcripts are difficult to detect by RT-PCR (especially in the low passage HFF and rat cell lines). In proliferating cells, and especially in some of the transformed cells, MDMX-S transcripts are easily detectable. It should be noted that quantitative methods will be needed to determine the exact levels of MDMX versus MDMX-S. That MDMX-S is expressed in proliferating and transformed cells suggests that it may play an important role in the control of cell proliferation.

Examination of the MDMX-S protein reveals that it binds p53 strongly, and with what might be a higher affinity than the binding of MDMX to p53. This finding appears consistent with the biological activity of MDMX-S when it is ectopically expressed in a variety of cell lines. In transient transfection studies, MDMX-S was better able to suppress p53-mediated gene expression, when compared with MDMX. This was shown using a promoter containing multiple p53-binding sites that is able to be transactivated by p53. In a second set of experiments, p53 overexpression was able to down-regulate transcription from the promoters of the cell cycle control genes dihydrofolate reductase and DNA polymerase α. This was likely due to a G1 phase cell cycle arrest or a general repressive effect of wild-type p53 expression. This down-regulation was completely reversed by MDMX-S, while MDMX was not nearly as effective. Finally, MDMX-S was able to block p53-mediated induction of apoptosis to a greater extent than MDMX. These data indicate that MDMX-S blocks the transcriptional/apoptotic effects of p53 to a greater extent than is accomplished by MDMX. Thus, the expression of MDMX-S in transformed cells may represent an important step in neoplastic transformation possibly because it may be more effective at blocking p53 function than full-length MDMX.

Since MDMX-S lacks the central and carboxyl-terminal zinc finger and ring finger domains, it may be under unique regulation. For example, the central and carboxyl-terminal regions may be important for protein-protein interaction, a newly described role for zinc and ring finger motifs (19). In our preliminary studies, MDMX does not appear to bind DNA and hence the zinc/ring fingers do not appear to contribute to that function. The central and carboxyl-terminal domains may aid in targeting MDMX for proteosome-mediated degradation via ubiquitin ligase E3, as appears to be the case for MDM2 (12–14, 29, 40). Given its differences with MDMX, MDMX-S will therefore likely have affects on cell cycle control that are distinct from that of MDMX.

That the MDMX gene is expressed as multiple transcripts is similar to the expression pattern of MDM2, which is expressed as multiple mRNAs, each with varying translation start and stop codons (31, 32). These multiple transcripts produce differ-
ent sized MDM2 proteins (31, 32). MDM2 and MDMX appear to share some features of their gene expression patterns, in that a variety of proteins can be produced from expression of a single gene. This form of regulation of gene expression may be a way to expand the functional capabilities in the MDM2/MDMX gene family.

Another feature of the studies presented here is that MDMX, MDMX-S, and MDM2 migrate on SDS-PAGE significantly higher than their predicted molecular weights. The fact that MDMX-S, and MDM2 migrate on SDS-PAGE significantly lower than their predicted molecular weights is another feature of the studies presented here. MDMX, MDMX-S, and MDM2 migrate on SDS-PAGE significantly higher than their predicted molecular weights, modification at the amino terminus may be the reason why full-length MDMX and MDMX-S contain 14 serines and 5 threonines, it is possible that it is modified by phosphorylation. Since both MDMX-S and MDMX migrate at approximately 10–12 kDa above their predicted values, modification at the amino terminus may be the reason why full-length MDMX and MDMX-S migrate on SDS-PAGE higher than its predicted molecular weight. Current efforts are underway to determine if this is the case.

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