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Physical and Functional Interaction between Wild-Type p53 and mdm2 Proteins

DALE S. HAINES, JOHN E. LANDERS, LINDA J. ENGLE, AND DONNA L. GEORGE*

Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

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The *mdm2* oncogene, which is often amplified in mammalian tumors, produces a number of transcripts that encode distinct protein forms. Previous studies demonstrating that overexpression of the *mdm2* gene can activate its transforming potential, and can inhibit the transcriptional activation function of p53, prompted us to begin to explore possible functional differences among the various mdm2 products. Utilizing a transient transfection assay, we have evaluated four naturally occuring murine mdm2 forms for their ability to inhibit p53-mediated transcriptional activation of reporter genes regulated by p53 response elements. Three of these mdm2 forms were found to physically associate with the wild-type p53 protein and to possess the ability to inhibit its transactivation function. A fourth form failed to exhibit either of these functions. This last mdm2 form lacks the N-terminal protein domain that is present in the other three splice forms examined, pointing to this region as one that is critical for complex formation with the p53 protein. Identifying such differences among mdm2 proteins provides important clues for dissecting their functional domains, and emphasizes that defining the individual properties of these products will be critical in elucidating the overall growth control function of the *mdm2* gene.

A variety of mammalian tumor cells contain amplified copies of genes whose transforming potential is activated by overexpression (16). The *mdm2* (murine double minute) oncogene was originally identified as a gene that is amplified and overexpressed in a tumorigenic derivative of mouse 3T3 cells (3T3DM cell line), with the amplified sequences located on extrachromosomal double minute particles (3). Our initial functional analysis demonstrated that, when overexpressed in immortalized rodent cells, the *mdm2* gene is capable of tumorigenically transforming these cells (11). Subsequent studies revealed that the overexpression of *mdm2* can immortalize primary rat embryo fibroblasts and cooperate with an activated *ras* gene to transform these cells (13). Amplification and overexpression of the *mdm2* gene have been detected in a number of human sarcomas (24, 32), indicating that this oncogene plays a role in human carcinogenesis. Further evidence supporting the conclusion that *mdm2* represents a key factor in growth control pathways comes from experiments demonstrating that mdm2 proteins physically associate with the protein product of the p53 tumor suppressor gene in vivo (2, 20, 31, 34) and can inhibit p53-mediated transcriptional activation (31, 33).

The wild-type p53 gene encodes a nuclear phosphoprotein that exhibits tumor suppressor activity (21, 25). This protein can inhibit oncogene-mediated transformation (1, 10, 14) and, when overexpressed, can block progression through the G_1 phase of the cell cycle (8, 29, 30). This protein has also been implicated as having a direct role in pathways of cellular differentiation (37), apoptosis (6, 27, 28, 38, 44), and maintenance of genome stability (26, 43). Mutation of the p53 gene, with loss of its growth suppressor activity, represents one of the most common genetic events in human cancer (21); however, the biochemical pathways by which p53 functions in the regulation of cellular proliferation remain to be elucidated. Several groups have found that p53 protein has the properties of a transcription factor since it can bind to specific DNA sequences (9, 15, 22) and activates expression of genes containing p53 response elements (12, 15, 23, 45). Similarly, p53 protein can negatively regulate the expression of a number of genes (5, 36, 39), most likely through interaction with basal transcription factors (36). Such studies indicate that p53 functions as part of the cell's transcriptional machinery and likely affects cellular proliferation by modulating the expression of a set of critical growth control genes (41). It is of interest that the *mdm2* gene represents one of the targets of p53-mediated gene regulation (2, 35, 42). The first intron of the *mdm2* gene contains a *cis*-acting p53 response element (42), and elevated expression of p53 leads to an increase in levels of mdm2 mRNA and protein (2, 35, 42). Given that mdm2 proteins can bind to and modulate the transcriptional activation function of p53 protein, it would seem likely that a p53-mdm2 autoregulatory feedback loop (42) maintains a critical mdm2/p53 ratio within a cell and plays a key role in pathways of cellular proliferation control.

An intriguing characteristic of the *mdm2* gene is that it can generate multiple transcripts differing in coding potential. This has been shown by DNA sequence analysis of clones isolated from murine (11) and human (32) cDNA libraries. Such findings raise important questions about possible functional differences among the various mdm2 products. The initial studies demonstrating that overexpression of *mdm2* can both activate mdm2's transforming potential (13) and inhibit the transcriptional activation function of p53 (31) were carried out with a genomic clone encoding the entire *mdm2* gene. Although this type of analysis proved essential for defining a functional link between these two cellular

* Corresponding author. Mailing address: Department of Genetics, University of Pennsylvania School of Medicine, Room 445 Clinical Research Building, 422 Curie Blvd., Philadelphia, PA 19104-6145. Phone: (215) 898-5032. Fax: (215) 573-5892.

factors, it provided no data as to whether all, or only some, of the different mdm2 protein products were responsible for the observed effects.

In work presented here, we have evaluated cDNA clones representing four distinct mdm2 splice forms for their ability to physically associate with p53 and to inhibit p53-regulated gene expression. The data obtained demonstrate that mdm2 splice variants differ in their ability to complex with and modulate the transcriptional activation function of the p53 protein. A comparison of the differences in coding potential present in the mdm2 cDNA forms has provided important information with regard to regions of the mdm2 protein required for interaction with p53. It also emphasizes that characterizing the individual properties of the various mdm2 products will be critical in defining the overall growth control function of the *mdm2* gene.

MATERIALS AND METHODS

Cell culture and plasmid constructs. H1299 cells, a human non-small-cell lung cancer line that contains a homozygous deletion of the p53 gene, were maintained in minimum essential medium (GIBCO/BRL, Gaithersburg, Md.) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO/BRL) and 1% penicillin-streptomycin (GIBCO/BRL).

Sequences corresponding to the alternatively spliced forms of the *mdm2* oncogene used for these studies were described previously (11). A cDNA coding for each of the various forms was subcloned into the *Eco*RI site of the pCMV5 expression plasmid. The MCK-CAT (50-2 plasmid [31]) and p53CON (15) reporter constructs were kindly provided by A. Levine (Princeton University) and W. Funk (University of Texas Southwest Medical Center), respectively. Generation of pCMV5:p53 was performed by cloning a PCR product amplified from the murine wild-type p53 vector, p11-4 (40), into the pCMV5 eukaryotic expression vector with primers spanning the entire p53 coding region.

Cell transfections. H1299 cells were plated at approximately 50% confluency in a 100-mm tissue culture dish and incubated at 37°C for 18 to 24 h. The cells were refed with complete medium and cultured for 1 h prior to the addition of transfected DNA prepared in a calcium phosphate precipitate (18). The amount of DNA (30 μg) for each transfection was kept constant by the addition of an appropriate amount of sheared salmon sperm DNA. Cells were incubated with this precipitate for 18 to 24 h at 37°C, washed with complete medium, refed with complete medium, and incubated at 37°C for an additional 24 h prior to harvest. Transfected cells were rinsed once with wash buffer (40 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 8.0], 150 mM NaCl), resuspended in 100 μl of extract buffer (250 mM Tris-HCl [pH 7.5], 5 mM dithiothreitol, 15% glycerol) and lysed by three freeze-thaw cycles. Supernatant was clarified by microcentrifugation, and the protein concentration of supernatant from each sample was determined by the Bradford method (Bio-Rad Laboratories, Richmond, Calif.).

CAT and luciferase assays. Twenty micrograms of protein derived from transfected extract was analyzed for chloramphenicol acetyltransferase (CAT) (17) and luciferase (7) activity. CAT assays were performed by incubating 20 μg of extract from each transfection with 1.0×10^{-3} μCi of [^{14}C]chloramphenicol (NEN, Boston, Mass.) and 0.5 mM acetyl coenzyme A in a 150-μl 0.2 M Tris-HCl (pH 7.5) solution at 37°C for 1.5 h. Reaction mixtures were extracted twice with equal volumes of ethyl acetate and dried under vacuum. Samples were resuspended in 20 μl of ethyl acetate and spotted onto a silica gel 1b chromatography plate (J. T. Baker, Phillipsburg, N.J.). Chromatograms were developed in 95% chloroform–5% methanol, placed in a 2-methylnaphthaline–0.4% PPO (2,5-diphenyloxazole) solution, dried, and analyzed by autoradiography. Quantitation of CAT activity was performed by determining percent acetylation of [^{14}C]chloramphenicol.

Luciferase assays were performed by adding 350 μl of a 1:1 mixture of a 50 mM glycylglycine (pH 7.8)–30 mM $MgSO_4$–10 mM ATP (pH 7.5) solution to 50 μl of extract containing 20 μg of total protein. Samples were then placed in a Lumat LB9501 luminometer (EG&G Berthold, Nashua, N.H.) for which 50 μl of 1 mM luciferin (pH 6.2) was automatically added to each reaction. A 10-s reading was employed to quantitate the amount of luciferase activity in extracts from transfected cells.

Cell labelling and immunoprecipitations. H1299 cells transiently transfected in 100-mm dishes with constructs encoding p53 and the various alternatively spliced forms of mdm2 were labelled with [^{35}S]methionine (Amersham, Arlington Heights, Ill.) as follows. After the 18- to 24-h incubation with the calcium phosphate DNA precipitate, H1299 cells were washed with complete medium, refed with complete medium, and incubated for 1 h at 37°C. Cells were washed twice with phosphate-buffered saline and refed for 1 h with methionine-free minimum essential medium supplemented with 2% dialyzed fetal bovine serum. [^{35}S]methionine (200 μCi per dish) was then added directly to the medium, and cells were incubated at 37°C for an additional 2 h. At the end of the labelling period, cells were washed once with phosphate-buffered saline, scraped from dishes, and pelleted.

Cell pellets were lysed in TENN buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA [pH 8.0], 0.5% Nonidet P-40, 150 mM NaCl supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, 2 μg of leupeptin per ml, and 1 μg of pepstatin per ml) for 20 min on ice with frequent vortexing. Lysates were then clarified by microcentrifugation, and supernatants from these samples were precleared with protein A-Sepharose (Pharmacia, Piscataway, N.J.). Extracts containing equal amounts of trichloroacetic acid-insoluble counts were incubated for 18 h at 4°C with protein A-Sepharose and mdm2 polyclonal antiserum or the p53 monoclonal antibody PAb421 (both kindly provided by A. Levine). Immunoprecipitated complexes were then pelleted and washed three times with SNNTE buffer (5% sucrose, 1% Nonidet P-40, 500 mM NaCl, 50 mM Tris-HCl [pH 7.5], 5 mM EDTA [pH 8.0]) and once with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.5], 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 150 mM NaCl). Immunoprecipitated proteins were analyzed on 7.5% denaturing polyacrylamide gels and prepared for fluorography with En^{3}Hance (NEN).

RESULTS

The *mdm2* oncogene generates several alternatively spliced mRNA forms. Data obtained in this and other laboratories have demonstrated that the *mdm2* oncogene has an extremely complex expression pattern. Specifically, DNA sequence analysis of *mdm2* genomic and cDNA clones isolated from both mouse (11) and human (32) sources has revealed that the *mdm2* gene can generate at least seven distinct mRNA species. These species differ within their 5′ nontranslated regions as well as within the *mdm2* coding region. To

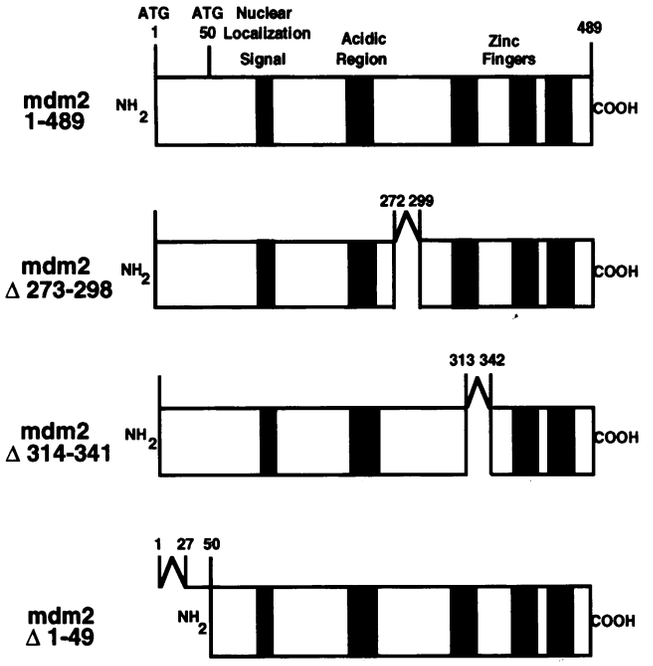

FIG. 1. The *mdm2* oncogene generates several alternatively spliced forms. Diagram of the four cDNAs encoding the mdm2 protein forms examined in this study. Regions which are spliced out of the various mdm2 cDNAs compared with the cDNA encoding the putative full-length form, mdm2:1-489, are indicated and are used to designate each alternatively spliced mdm2 form.

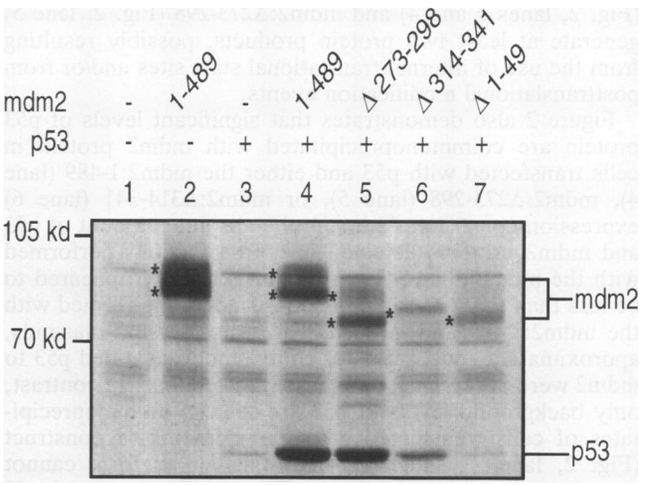

FIG. 2. Analysis of complex formation between alternatively spliced mdm2 variants with p53 by using mdm2 antiserum. H1299 cells were transfected with the p53 expression plasmid (5 μg) and expression constructs encoding the various mdm2 forms (25 μg for mdm2:Δ273-298, mdm2:Δ314-341, and mdm2:Δ1-49; 10 μg for mdm2:1-489). After transfections, cells were metabolically labelled with [^{35}S]methionine and soluble extracts were immunoprecipitated with mdm2 antiserum. Immunoprecipitated mdm2 (indicated with asterisks) and p53 proteins were analyzed by SDS-PAGE as described in Materials and Methods.

begin to explore possible functional differences among the various mdm2 species, we chose to analyze four different cDNAs, isolated from a murine 3T3DM cDNA library (11), that each encode a distinct mdm2 protein product. These four proteins were evaluated for their ability to physically associate with the p53 protein, and to inhibit the transcriptional activation function of p53.

A diagram of the coding potential of the four mdm2 spliced forms analyzed in this study is presented in Fig. 1. The cDNA designated mdm2:1-489 contains the largest known coding region of the murine *mdm2* gene and has the potential to produce a 489-amino-acid polypeptide. The ATG encoding the amino acid designated "1" of the mdm2 species in Fig. 1 is considered to represent the 5'-most signal for translational initiation, as previously discussed (11, 32), in part because the nucleotide sequence similarity between the human and murine genes declines dramatically immediately upstream of this ATG, and there are no in-frame ATGs upstream of this translational initiation signal. The full-length mdm2:1-489 form includes all of the putative functional motifs ascribed to this protein (11), including the following evolutionarily conserved domains: a consensus nuclear localization signal (residues 178 to 182), a highly negatively charged acidic domain (residues 221 to 272), and three potential metal binding domains, or zinc finger motifs (residues 303 to 320, 436 to 455, and 459 to 478).

The cDNA form designated mdm2:Δ273-298 has spliced out a region encoding amino acids 273 to 298. The region absent from this form is located between the acidic domain and the first zinc finger element of mdm2 and contains no recognizable functional motifs. The form termed mdm2:Δ314-341 has spliced out nucleotides encoding residues 314 to 341, which disrupts the potential zinc finger domain between amino acid residues 303 and 320. Finally, in the mdm2:Δ1-49 species, an alternative splicing event has eliminated the first coding exon of the *mdm2* gene. Although this form retains an upstream untranslated exon, the first in-frame ATG that might be used for translational initiation of this sequence corresponds to amino acid 50 of the full-length mdm2 protein. Therefore, this splice variant is referred to as mdm2:Δ1-49.

In vivo association between p53 and products of the alternatively spliced mdm2 mRNAs. Recently, antibodies generated against the human and murine mdm2 proteins have detected multiple mdm2 protein forms in a number of cell lines (19, 34). The different species of mdm2 proteins may result from alternative splicing of mRNAs, utilization of alternative promoters, and/or posttranslational modification events, and initial studies indicate that only a subset of these proteins form a complex with p53 (19, 34). Therefore, we wanted to determine whether the proteins encoded by the various alternatively spliced mdm2 mRNAs would differ in their ability to complex with the wild-type p53 protein.

We chose to evaluate complex formation by an in vivo binding assay. Each cDNA described in Fig. 1 was cloned into the pCMV5 expression vector and cotransfected with a p53 expression plasmid into a recipient cell line, H1299, that lacks endogenous p53. Extracts were prepared from transfected cells metabolically labelled with [^{35}S]methionine, and both the expression and in vivo association of the individual mdm2 forms with p53 were determined by immunoprecipitation analysis with mdm2 antiserum. As shown in Fig. 2, all four mdm2 expression constructs generate detectable levels of precipitable mdm2 protein, with molecular sizes of approximately 75 to 97 kDa, as determined by SDS-polyacrylamide gel electrophoresis (PAGE) analysis. The molecular weights of proteins encoded by these cDNAs are consistent with previous observations documenting the apparent size of mdm2 protein forms (34). Interestingly, both mdm2:1-489

(Fig. 2, lanes 2 and 4) and mdm2:Δ273-298 (Fig. 2, lane 5) generate at least two protein products, possibly resulting from the use of internal translational start sites and/or from posttranslational modification events.

Figure 2 also demonstrates that significant levels of p53 protein are coimmunoprecipitated with mdm2 protein in cells transfected with p53 and either the mdm2:1-489 (lane 4), mdm2:Δ273-298 (lane 5), or mdm2:Δ314-341 (lane 6) expression construct. Although the absolute amount of p53 and mdm2 proteins detected in cotransfections performed with the mdm2:Δ314-341 expression construct appeared to be less than that observed in cotransfection performed with the mdm2:1-489 and mdm2:Δ273-298 expression plasmids, approximately equal ratios of coimmunoprecipitated p53 to mdm2 were observed for all these transfections. In contrast, only background levels of p53 are seen in immunoprecipitates of cells transfected with the mdm2:Δ1-49 construct (Fig. 2, lane 7), indicating that this mdm2 form cannot complex with the p53 protein. The small amount of p53 detected in cells transfected with mdm2:Δ1-49 most likely resulted from binding between transfected p53 product and the endogenous mdm2 protein in the H1299 cells, since the amount of coimmunoprecipitated p53 was similar to that observed in cells transfected with the p53 expression construct alone (compare lanes 3 and 7 in Fig. 2).

The same conclusions were reached when the immunoprecipitation assays were carried out with the anti-p53 monoclonal antibody, PAb421. To illustrate this point, the H1299 recipient cells were transfected with constructs encoding mdm2:Δ1-49 and mdm2:Δ314-341, which produce similar amounts of mdm2 protein (see Fig. 2, lanes 6 and 7). As shown in Fig. 3, the mdm2 protein form encoded by the mdm2:Δ314-341 construct is coimmunoprecipitated with p53 (lane 4). In contrast, the protein form encoded by the mdm2:Δ1-49 cDNA was not coimmunoprecipitated with p53 when the PAb421 antibody was used (lane 5). The absence of a detectable association between p53 and mdm2:Δ1-49 protein was not due to the amount of mdm2:Δ1-49 or p53 produced, since comparable levels of both mdm2 protein forms were detected when the immunoprecipitations were carried out with the mdm2 antiserum (Fig. 3, lanes 9 and 10), and equivalent amounts of p53 were present in extracts of these transfected cells (Fig. 3, lanes 4 and 5). Therefore, the results of these studies show that alternative splicing generates at least one mdm2 protein form which does not complex with the p53 protein.

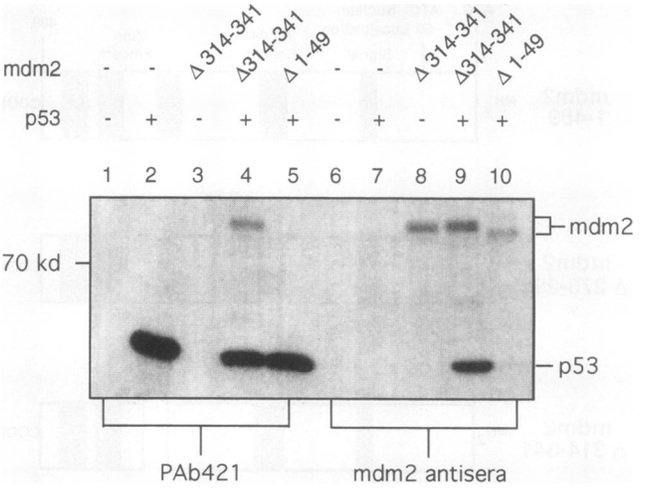

FIG. 3. Analysis of complex formation between alternatively spliced mdm2 variants with p53 by using the p53 monoclonal antibody PAb421. H1299 cells were transfected with 5 μg of the p53 expression plasmid and 25 μg of either the mdm2:Δ314-341 or the mdm2:Δ1-49 expression construct. After transfections, cells were metabolically labelled with [^{35}S]methionine and soluble extracts were immunoprecipitated with the p53 monoclonal antibody, PAb421 (lanes 1 to 5), or mdm2 antiserum (lanes 6 to 10). Immunoprecipitated mdm2 and p53 proteins were analyzed by SDS-PAGE as described in Materials and Methods.

The mdm2 splice variants differ in their ability to inhibit p53-mediated transactivation. Experiments were then carried out to determine whether a correlation exists between the ability of these mdm2 proteins to complex with p53 and their ability to inhibit the transcriptional activation function of p53. To do this, each of the mdm2 protein forms was tested in transient expression assays for its effectiveness in inhibiting the ability of a cotransfected wild-type p53 cDNA to transactivate a reporter gene. For our initial assays, we used the MCK-CAT reporter plasmid (31). This construct contains a minimal p53 response element, derived from the promoter of the murine muscle creatine kinase (MCK) gene, linked to the bacterial CAT gene (31).

As shown in Fig. 4, cotransfection of the MCK-CAT construct with a wild-type p53 plasmid leads to a marked increase in CAT gene expression in recipient H1299 cells (Fig. 4, lanes 1 and 2). When the cotransfections were carried out with the MCK-CAT construct, together with equal microgram amounts of the p53 construct and the mdm2:1-489 construct, there was little effect on CAT activity (Fig. 4, lane 3). However, increasing the mdm2 to p53 cDNA input ratio used for the cotransfections resulted in a dramatic decrease in the levels of CAT activity observed (Fig. 4, lanes 4 to 7). This marked decrease in CAT activity was not observed when control transfections were performed with the p53 construct together with the pCMV5 vector itself (Fig. 5A, lanes 13 and 14), indicating that elevated levels of mdm2 protein are needed to effectively inhibit p53-mediated transactivation of the MCK promoter.

Similarly, when the transient expression assays were carried out with either mdm2:Δ273-298 (Fig. 5A, lanes 7 and 8) or mdm2:Δ314-341 (Fig. 5A, lanes 9 and 10), we detected a marked inhibition of p53-induced CAT activity. As presented in Fig. 5B, cotransfection of increasing amounts of the mdm2:1-489 (lanes 3 to 5), mdm2:Δ273-298 (lanes 6 to 8), and mdm2:Δ314-341 (lanes 9 to 11) splice forms all led to parallel decreases in levels of CAT activity; this is consistent with the idea that the relative ability of the mdm2 proteins to inhibit the p53-mediated transactivation of the MCK promoter is dependent on a critical mdm2/p53 protein ratio within the cell. In contrast, cotransfections carried out with the mdm2:Δ1-49 expression construct did not result in detectable inhibition of p53-induced CAT gene expression (Fig. 5A, lanes 11 and 12). A lack of detectable inhibition of this transactivation function was noted at all of the mdm2:Δ1-49 concentrations tested (Fig. 5B, lanes 12 to 14).

Because there are some variations among reported p53 binding sites, we extended these studies to look at another p53-responsive promoter. The transient expression assays were repeated, in this case with the p53CON-LUC reporter construct. This construct contains the luciferase reporter gene under the transcriptional control of a p53 response element that was isolated by the CASTing (cyclic amplification and selection of target) procedure (15). The results obtained in examining the ability of the various mdm2

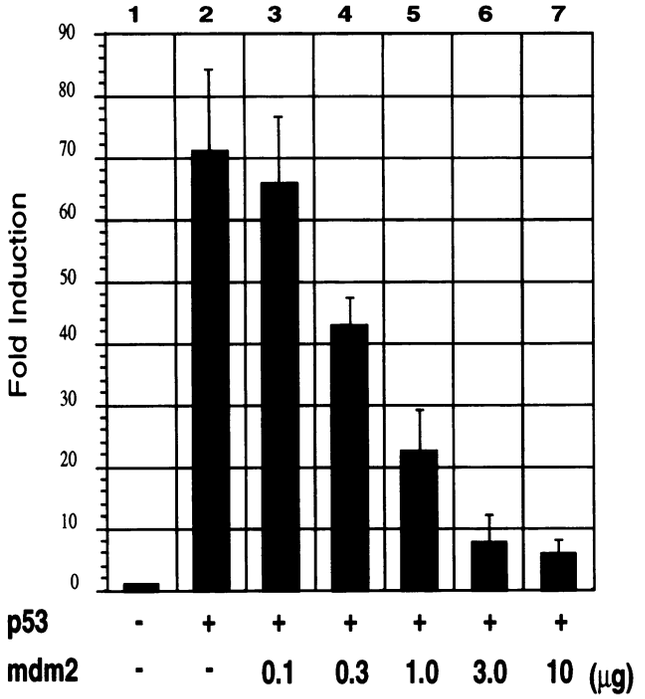

FIG. 4. mdm2:1-489 inhibits p53-mediated transactivation of the MCK promoter. H1299 cells, devoid of endogenous p53 (15), were transfected with 3 μg of the MCK-CAT reporter construct, 0.1 μg of an expression plasmid encoding wild-type murine p53, and the indicated amounts of the mdm2:1-489 expression construct. Extracts were prepared from transfected cells and assayed for CAT activity as described in Materials and Methods. Fold induction (amount of reporter activity relative to that observed in non-p53-transfected cells) was normalized for inhibition of reporter activity observed at similar concentrations with the pCMV5 vector-alone control.

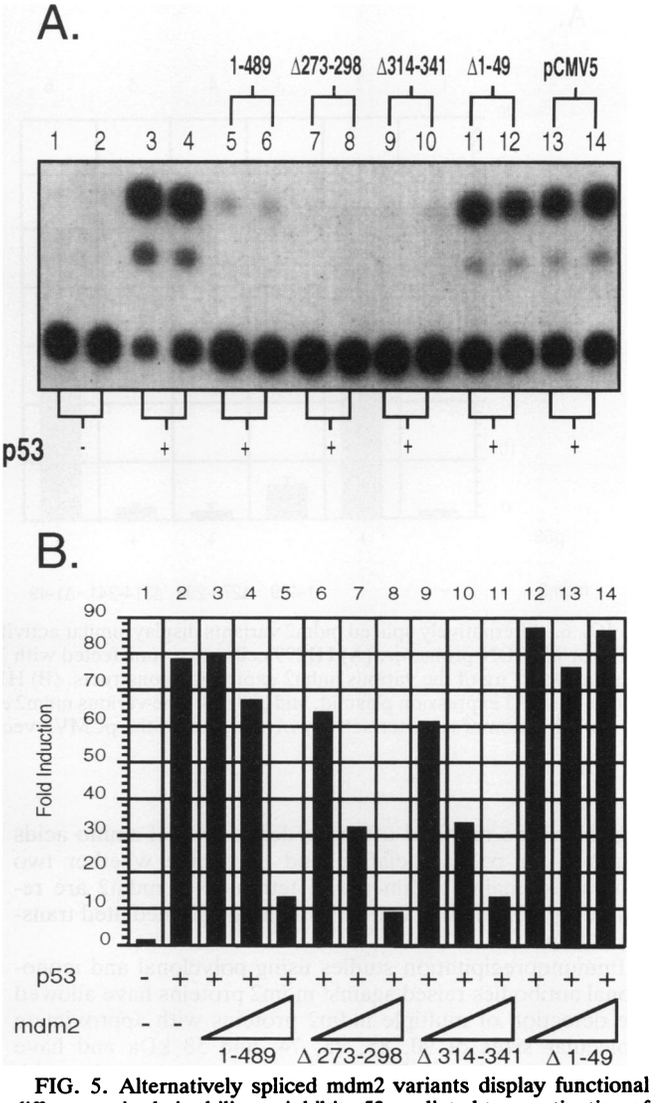

FIG. 5. Alternatively spliced mdm2 variants display functional differences in their ability to inhibit p53-mediated transactivation of the MCK promoter. (A) H1299 cells were transfected with 3.0 μg of the MCK-CAT reporter construct, 0.1 μg of the p53 expression plasmid, and 3 μg of the various mdm2 expression constructs. CAT assays were then performed to measure the amount of reporter activity present in extracts from transfected cells. (B) H1299 cells were transfected with 3 μg of the MCK-CAT reporter construct, 0.1 μg of the p53 expression plasmid, and increasing concentrations (0.1, 1.0, and 10 μg) of the mdm2 expression constructs. Fold induction was normalized for inhibition of CAT activity observed at similar concentrations with the pCMV5 vector-alone control.

protein forms to inhibit the p53-mediated transactivation of this p53CON-LUC reporter construct were consistent with those described above for the MCK-CAT reporter. Representative results obtained with both reporter constructs are compared in Fig. 6A, for the MCK-CAT reporter, and in Fig. 6B, for the p53CON-LUC reporter.

DISCUSSION

To better understand the role of the *mdm2* gene in normal cellular processes and in pathways of oncogenesis, we have begun to define this gene's complex expression pattern and the involvement of its products with the p53 protein. Alternatively spliced products of the *mdm2* oncogene serve as natural reagents for such studies and for the dissection of the functional domains of the mdm2 proteins. As reported here, three mdm2 splice forms, designated mdm2:1-489, mdm2:Δ273-298, and mdm2:Δ314-341, all encode proteins that will complex with the p53 protein and can inhibit p53's transcriptional activation function. In contrast, the product encoded by the splice form mdm2:Δ1-49 did not exhibit the ability either to bind to p53 protein or to inhibit the transcriptional activation function of p53.

The transcript encoded by splice variant mdm2:Δ1-49 is missing exonic material containing nontranslated sequences as well as nucleotides encoding the amino terminus (residues 1 to 49) of the full-length mdm2 protein. Our data, therefore, provide evidence that amino acids 1 to 49 include sites required for mdm2-p53 complex formation, and support the idea that inhibition of p53-mediated transactivation by mdm2 requires physical association with p53. Our conclusions are consistent with findings, presented by two other laboratories, which were based on the construction and analysis of a series of deletion mutants of a cDNA encoding the human homolog of a full-length mdm2 protein. These studies revealed that amino acid residues 19 to 102 (4) and 1 to 118 (33) contain a region that is critical for p53 binding , although the presence of other segments of the mdm2 protein seem to be required for this interaction (4). A further dissection of this

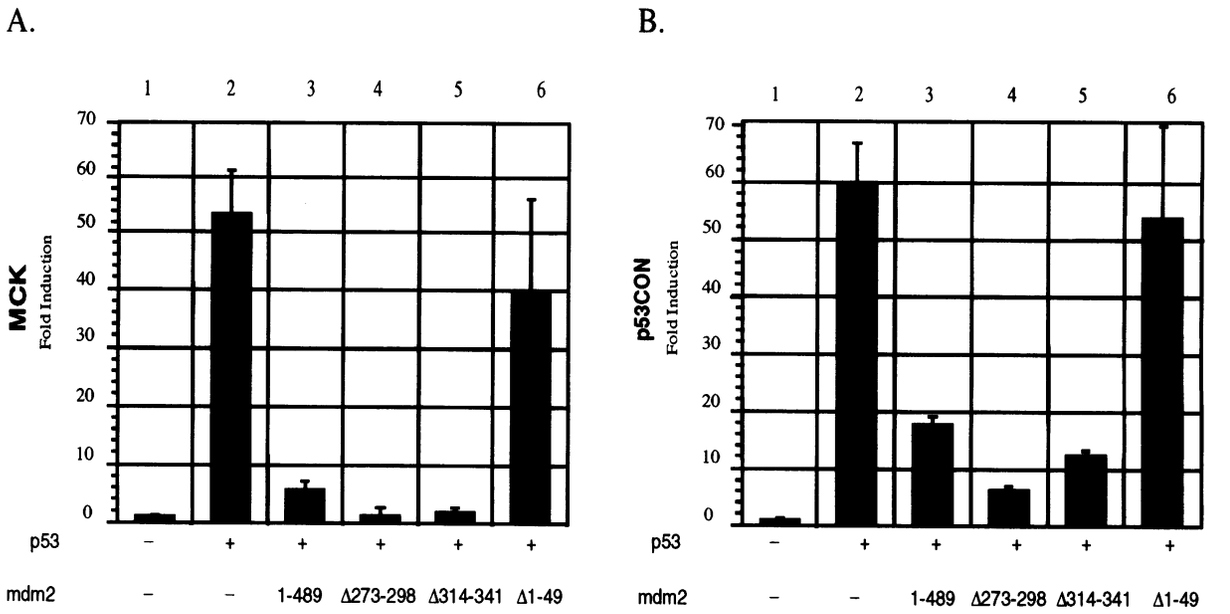

FIG. 6. Alternatively spliced mdm2 variants display similar activities in their ability to inhibit p53-mediated transactivation from either the MCK or p53CON promoter. (A) H1299 cells were transfected with 3 μg of the MCK-CAT reporter construct, 0.1 μg of the p53 expression plasmid, and 3 μg of the various mdm2 expression constructs. (B) H1299 cells were transfected with 1 μg of the p53CON reporter construct, 10 ng of the p53 expression plasmid, and 300 ng of the various mdm2 expression constructs. Fold induction for panels A and B was normalized for the inhibition of reporter activity observed with the pCMV5 vector-alone control.

region will be required to better define critical amino acids required for p53 association and determine whether two separate domains within the N terminus of mdm2 are required for p53 binding and for inhibiting p53-mediated transactivation.

Immunoprecipitation studies using polyclonal and monoclonal antibodies raised against mdm2 proteins have allowed the detection of multiple mdm2 proteins with approximate molecular sizes of 90, 85, 76, 74, and 58 kDa and have implied that only a subset of these proteins complex with p53 (19, 34). The results presented in this report provide evidence that at least some of these different mdm2 protein forms arise from alternatively spliced mRNAs, and have allowed the identification of one mdm2 protein form that does not associate with p53. Additional studies are needed to determine whether other processes, such as posttranslational modification, contribute to the heterogeneity of mdm2 proteins within a cell and whether the pattern of alternative splicing of the *mdm2* gene is regulated in a cell cycle, developmental, and/or tissue-specific fashion. Given that the *mdm2* gene contains an internal p53 binding site and that overexpression of wild-type p53 can upregulate *mdm2* expression (2, 35, 42), a question that arises is whether p53 transactivation of the *mdm2* gene modifies the pattern of alternatively spliced mRNAs that are produced.

The presence of certain structural motifs within the primary amino acid sequence of the mdm2 protein (4) raises the possibility that mdm2 may function as a transcription factor involved in regulating the expression of other genes. Because an mdm2-p53 complex may itself have a direct transcriptional activation or repression function, it will be of interest to determine how the deletion of particular coding regions, such as in the mdm2:Δ273-298 and mdm2:Δ314-341 forms, would affect this function. It is conceivable that different mdm2-p53 complexes may act to regulate the expression of distinct sets of target genes, perhaps by varying the recruitment or the interactions of other transcription factors. Alternatively, the mdm2 splice forms may only differ in cellular pathways that are independent of p53 involvement. To address this question, it will be important to determine whether mdm2 proteins associate with other cellular factors and how p53 association may regulate or affect mdm2-related activities.

Amplification and overexpression of the *mdm2* gene activate its oncogenic potential; overexpression of the *mdm2* gene also results in the inhibition of p53-mediated transcriptional activation. Current models for p53-mediated growth control (41) suggest that the p53 protein, acting as a transcription factor, stimulates or represses a set of target genes involved in the regulation of cellular proliferation and/or cell cycle control pathways. The ability of various mdm2 proteins to bind to p53, and to inhibit the transactivation function of p53, provides one possible explanation for the way in which *mdm2* overexpression could contribute to cellular transformation and tumorigenesis. It remains possible, however, that *mdm2* has oncogenic potential that is independent of its interaction with p53, and further studies are needed to address this question. The existence of multiple mdm2 protein forms presents the challenge of defining the cellular role and oncogenic potential of these related, but different, *mdm2* products.

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