

# Regulation of Transcription Functions of the p53 Tumor Suppressor by the mdm-2 Oncogene

Jiandong Chen, Jiayuh Lin, and Arnold J. Levine

Department of Molecular Biology, Princeton University, Princeton, New Jersey, U.S.A.

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## ABSTRACT

**Background:** Mdm-2, a zinc finger protein, negatively regulates the p53 tumor suppressor gene product by binding to it and preventing transcriptional activation (16).

**Materials and Methods:** Assays for p53 mediated transcription, repression and activation by mutant and wild-type p53 proteins were used to measure the ability of mdm-2 to block each activity.

**Results:** Mdm-2 was able to inhibit all three functions of the wild-type and mutant p53 activities; transcriptional activation by the wild-type protein, transcriptional activation by the mutant p53 protein, and repression by the wild-type protein.

**Conclusions:** The mdm protein binds to the amino terminal portion of the p53 protein and, in so doing, blocks the ability of p53 to interact with the transcrip-

tional machinery of the cell (23). The mdm-2 protein binds to both leucine-tryptophan residues at amino acids 22 and 23, from the amino terminal end of the protein, and in so doing, prevents all p53 functions. The ability of a mutant p53 protein to transactivate a multidrug resistance-1 gene promoter is blocked by mdm-2 and the ability of the wild-type p53 protein to repress transcription of some genes is also blocked by the mdm-2 protein. Thus, all three functions of the p53 protein—transcriptional activation, repression and mutant protein activation—require the p53 amino terminal domain functions and are regulated by the mdm-2 protein in a cell. When mdm-2 is overproduced, resulting in a tumor or transformation of a cell, all of the p53 activities are inactivated.

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## INTRODUCTION

The p53 tumor suppressor gene and its protein product have been shown to be involved as a checkpoint in the cell cycle in response to DNA damage (1–3). The p53 protein is a transcription factor that can regulate a set of genes resulting in cell cycle growth arrest (1–3) or apoptosis (4,5). Transcriptional activation of genes by the p53 protein requires an amino-terminal transcription activation domain and a DNA sequence-specific DNA binding domain. The mutant p53 proteins from human cancer cells have lost transcription factor activity (6–8), suggesting that this activity plays a critical role in the tumor suppressor function of p53. The wild-type p53 protein has also been shown to repress or negatively regulate

transcription from a variety of viral and cellular promoters that do not contain DNA sequences recognized by the p53 protein (9–12). Finally, some mutant p53 proteins, but not the wild-type protein, can transcriptionally activate the multidrug resistance gene-1 promoter (MDR-1) in transient transfection assays (13) and this reflects a “gain of function” phenotype of mutant p53 proteins (14,15).

Recently, p53 has been shown to bind to a cellular protein encoded by the mdm-2 oncogene (16). The mdm-2 gene was originally isolated as a cellular oncogene amplified on a mouse double-minute chromosome. Overexpression of the mdm-2 gene in BALB/c 3T3 cells increased their tumorigenic potential (17). The murine mdm-2 genomic clone can transform primary rat embryo fibroblasts in cooperation with the activated *ras* oncogene (18). Overexpression of the mdm-2 gene also inhibits transcription

Address correspondence and reprint requests to: Arnold J. Levine, Department of Molecular Biology, Princeton University, Princeton, NJ 08544-1014, U.S.A.

activation by p53 (16). Furthermore, amplification of the mdm-2 gene has been observed in several types of human sarcomas (19,20). The mdm-2 gene can be positively regulated by the p53 protein through a p53-responsive DNA element present in its first intron (21,22). This then provides a way to regulate the level of p53 activity via a negative feedback loop (22). Mdm-2 binds to the N-terminal 52 residues of p53 which contain the transactivation domain (23,24). Based upon this, it was proposed that mdm-2 may suppress p53-mediated transcription by blocking its transactivation domain. Thus, the transforming activity of mdm-2 could result from the inhibition of the transactivation activity of p53.

The strong physical association between mdm-2 and p53 suggests that complex formation may be important for the functional interaction between the two proteins. In some experimental systems such as the tumorigenic DM3T3 cell line, where p53 is apparently suppressed by the over-expressed mdm-2 gene, the majority of the p53 protein in the cell is indeed present in a complex with mdm-2 (25). However, there has not always been strict correlation between the detection of the p53–mdm-2 complex and the suppression of p53 function in other systems (18, 26). Therefore, it is not clear whether formation of a detectable complex is necessary for the inhibition of p53 function. The polypeptide sequence of mdm-2 contains a central acidic region and three zinc-finger motifs, suggestive of a role as a transcription factor. These highly conserved domains are dispensable for the binding to p53 protein *in vitro* (23) but they still could encode functions that contribute to or indirectly regulate p53 function. For this reason, a study was initiated to identify the functional domains of the mdm-2 protein that play a role in regulating several of the p53 transcriptional activities.

This report describes the evidence demonstrating that mdm-2 can negatively regulate the wild-type p53 protein activities for transcriptional activation and transcriptional repression. The mdm-2 protein also eliminates the ability of mutant p53 proteins to transactivate the MDR-1 promoter in cells. A panel of mdm-2 mutant proteins was used to demonstrate that binding to the p53 protein by the amino-terminal residues of mdm-2 was sufficient to regulate all of these activities of the p53 protein. Furthermore, p53 point mutations that specifically destroyed the binding of mdm-2 to p53 were employed to demonstrate the critical role of the physical in-

teraction between mdm-2 and p53 in the regulation of the p53 transcriptional activity.

## MATERIALS AND METHODS

### Plasmids

Human mdm-2 deletion mutants  $\Delta 222-325$  and  $\Delta 222-437$  were constructed by PCR amplification of the mdm-2 coding regions between 325 and 491, and 437 and 491 using the following primers: 5'-CGGGATCCCCTTCGTGAGAATTGGC-3' for  $\Delta 222-325$  and 5'-CGGGATCCTTGTTGATTGTCAA-3' for  $\Delta 222-437$ . After amplification, the PCR products were digested with BamHI and inserted into a Bluescript plasmid encoding residues 1–222 of mdm-2. The p53 binding activities of these two mutants *in vitro* were determined as previously described (23). Other mdm-2 mutants used in this study were constructed previously. The cDNA inserts encoding these mutants were isolated from the Bluescript vectors and cloned into the pCMV-neo-Bam vector.

### Transfection and CAT Assay

SAOS-2 cells were maintained in DMEM with 15% fetal calf serum. Transfections and CAT assays were performed as described previously. Briefly,  $5 \times 10^5$  cells were seeded into 10-cm dishes and transfected with indicated amounts of plasmids, total amount of DNA for each transfection was adjusted to 30  $\mu\text{g}$  with salmon sperm carrier DNA. Cells were transfected using the calcium phosphate protocol. Transfected cells were harvested about 56 hr after addition of DNA, and 100  $\mu\text{l}$  cytoplasmic extracts were prepared from each 10-cm plate. CAT assays were performed using 50  $\mu\text{l}$  of extract adjusted to identical protein concentrations in a 150  $\mu\text{l}$  reaction containing 0.8 mM of acetyl-CoA, 0.4  $\mu\text{Ci}$  of  $^{14}\text{C}$ -chloramphenical, and 0.25 M of Tris.HCl, pH 7.5. Conversion of  $^{14}\text{C}$ -chloramphenical to acetylated  $^{14}\text{C}$ -chloramphenical was quantitated using a PhosphoImager (Molecular Dynamics).

### Immunofluorescence Staining

SAOS-2 cells were grown on glass slides. Cells were washed with phosphate-buffered saline (PBS) and treated with 100% methanol at room temperature for 10 min. The cells were treated with PBS with 0.1% Triton X-100 for 10 min and

then incubated with PBS containing 10% normal goat serum (NGS) for 20 min. The cells were incubated with a 1/100 dilution of 2A10 monoclonal hybridoma supernatant for 1 hr, washed with PBS, and incubated with a 1/100 dilution of FITC-conjugated goat-anti-mouse IgG (Boeringer Mannheim). The cells were washed and mounted with a solution of 90% glycerol, 0.15 M NaPO<sub>4</sub> (pH 7.5), and 0.1% p-phenylenediamine. Fluorescent photography was performed using a Zeiss fluorescent microscope and Trix-Pan film.

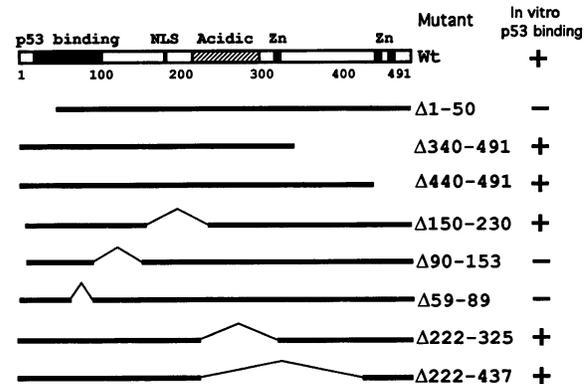
### Metabolic Labeling and Immunoprecipitation

Cells were labeled with <sup>35</sup>S-methionine, and immunoprecipitations were performed as described previously using 300 μl of 4B11 hybridoma supernatant for mdm-2 mutants containing intact C termini and 3F3 supernatant for mutants Δ340–491 and Δ440–491. The immunoprecipitated proteins were analyzed by SDS-PAGE and detected by fluorography.

## RESULTS

### The Expression, Cellular Localization, and Binding of mdm-2 Mutant Proteins to the p53 Protein

To study the functions of the mdm-2 protein in mammalian cells, a human mdm-2 cDNA (23) and several mdm-2 deletion mutants derived from this cDNA (Fig. 1) were subcloned into an expression vector regulated by the human cytomegalovirus promoter (27). These mdm-2 expression plasmids were transiently transfected into the human osteosarcoma cell line SAOS-2, which is devoid of endogenous p53 protein (28) and has no detectable mdm-2 protein. The expression of mutant mdm-2 proteins from these plasmids were confirmed by labeling cells transfected with these plasmids with <sup>35</sup>S-methionine and immunoprecipitation of the soluble cell extracts with monoclonal antibodies directed against mdm-2. The ability of these mutant proteins to bind to the p53 protein was tested previously by using p53 proteins translated in vitro and mixed with the mdm-2 proteins in extracts (23). This analysis was limited to an association tested in vitro. To examine the ability of the p53 protein to bind to these mdm-2 mutants in vivo,

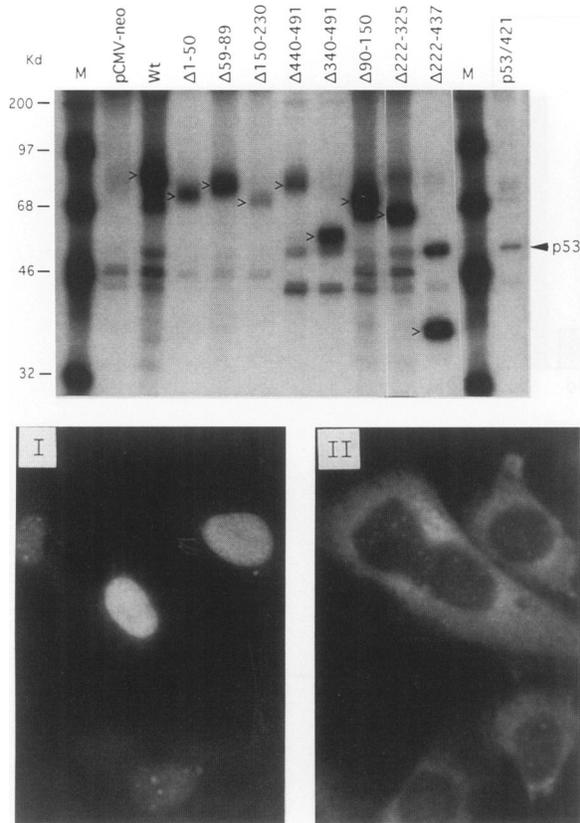


**FIG. 1. Diagram of mdm-2 deletion mutants used in this study**

The top diagram shows the structural features of the mdm-2 polypeptide. The thick solid lines represent the polypeptide regions encoded by the mdm-2 deletion mutants. The thin lines indicate internal regions of the protein that are deleted. The ability of these mutants to bind to p53 in vitro have been determined previously. Major motifs of mdm-2 include: p53 binding domain—19–102; nuclear localization (NLS)—181–185; acidic region—220–296; zinc-fingers (Zn)—305–322, 438–457 and 461–478.

permanent cell lines expressing each mdm-2 mutant protein were established and then transiently transfected with a human wild-type p53 expression plasmid. The cells were then labeled with <sup>35</sup>S-methionine and soluble cell extracts were analyzed by immunoprecipitation using anti-mdm-2 monoclonal antibodies. The results of this analysis are shown in Fig. 2A.

The ability of these mdm-2 mutants to bind to p53 in vivo are consistent with the previous results mapping mdm-2 and p53 protein interactions in vitro. The only exception was mutant Δ150–230 which was not able to bind to p53 protein in vivo although its p53 binding domain is intact and it can bind to p53 protein in vitro. It has been suggested, based on amino acid sequence analysis, that residue 181–185 of the mdm-2 protein may function as a nuclear localization signal (NLS) (17). It was therefore possible that this protein failed to bind to p53, because it did not localize in the nucleus. To test this possibility, immunofluorescence staining was performed using an anti-mdm-2 monoclonal antibody 2A10 and SAOS-2 cell lines selected for stable expression of the mdm-2 mutant proteins. As shown in Fig. 2B, full length mdm-2 is located in the nucleus, however, mutant Δ150–230 is clearly located in the cytoplasm of the cells, suggesting that the region between residue 150 and



**FIG. 2. Expression and cellular localization of mdm-2 mutants**

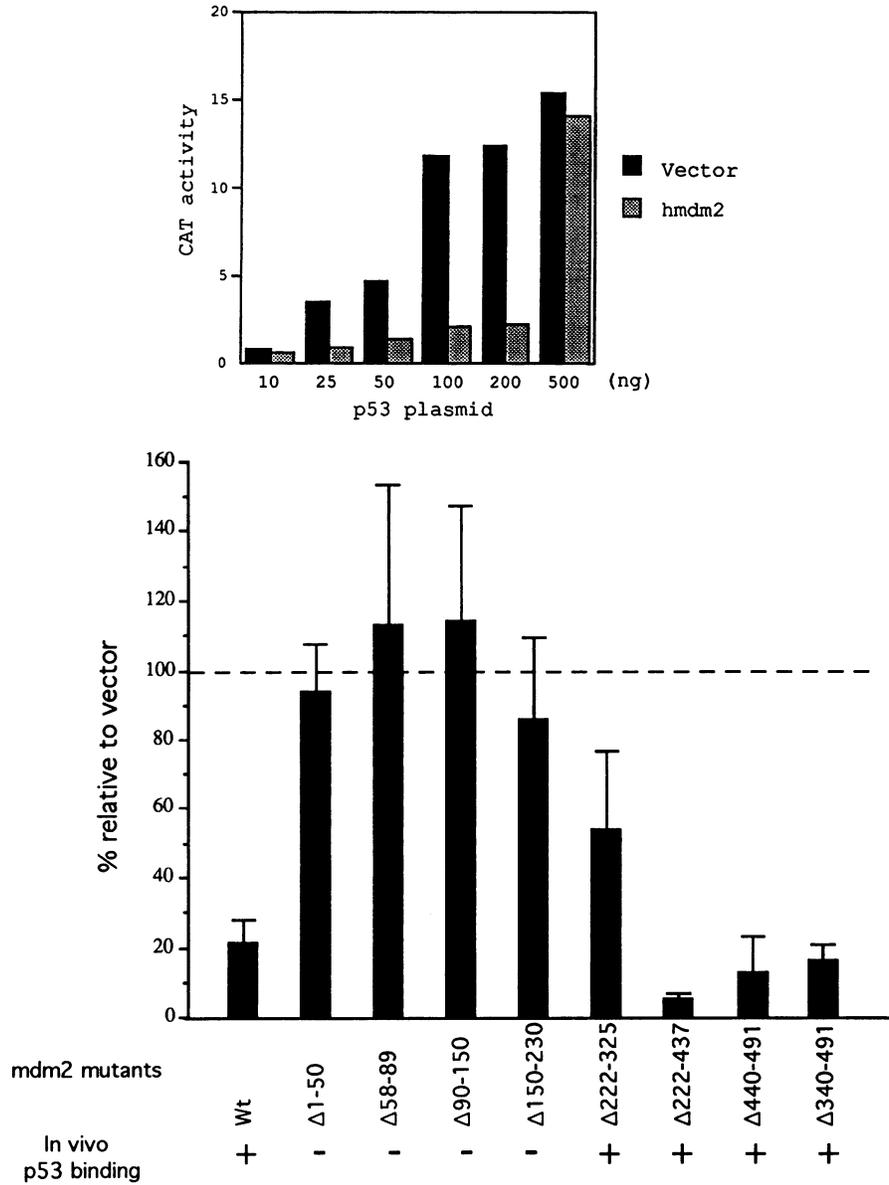
(A) Expression of mdm-2 mutants in SAOS-2 cells and *in vivo* interaction with p53. SAOS-2 cells were transfected with mdm-2 expression plasmids and selected with G418. Pooled drug resistant colonies were then transiently transfected with pC53-C1N3, which expresses wild-type human p53. The cells were labeled with  $^{35}\text{S}$ -methionine and analyzed by immunoprecipitation using the 4B2 or 4B11 monoclonal antibodies against mdm-2. The two doublet bands of varying intensity in each lane, at or below the 43 kD marker, are background bands nonspecifically binding to the antibodies, as shown by the vector transfected cells. Mdm-2 mutants able to bind p53 *in vivo* include  $\Delta 440-491$ ,  $\Delta 340-491$ ,  $\Delta 222-325$ , and  $\Delta 222-437$ . Mutant  $\Delta 90-150$  showed weak interaction with p53. (B) Cellular localization of mdm-2 mutants. SAOS-2 cells were transfected with mdm-2 expression plasmids and selected with G418. Pooled G418-resistant cells were stained with anti-mdm-2 monoclonal antibody 2A10 by immunofluorescence. Shown here is a representative nuclear-localized mutant  $\Delta 440-491$  (I) and a cytoplasmic mutant  $\Delta 150-230$  (II).

230 indeed contains a signal necessary for the proper nuclear transport of mdm-2. These results also suggest that nuclear localization of mdm-2 is necessary for binding to the p53 protein.

### Inhibition of p53-Mediated Transactivation by mdm-2 Mutants

It has been shown previously that cotransfection of a p53 expression plasmid with a cosmid containing the murine mdm-2 genomic clone can result in significant inhibition of p53-mediated transactivation of a reporter plasmid (16). To test the mdm-2 mutant cDNA clones for their ability to inhibit p53 mediated transactivation, the reporter plasmid pCOSX1-CAT was employed. It contains a p53-responsive DNA element derived from the first intron of the murine mdm-2 gene, the adenovirus major late promoter TATA box, an initiator element from the terminal deoxynucleotidyl transferase gene promoter, and the chloramphenicol acetyl transferase coding sequences (22). This reporter plasmid is highly responsive to activation by p53, as demonstrated using increasing levels of p53 plasmid in a titration experiment. As shown in Fig. 3A, significant CAT activity can be detected with as little as 10 ng of p53 expression plasmid and the response is approximately linear with dose. When the full-length human mdm-2 cDNA expression plasmid was included in these transfection assays, a 5- to 8-fold reduction of CAT activity was observed when less than 200 ng of p53 expression plasmid was used (Fig. 3A). The inhibition by the mdm-2 cDNA was reversed when greater amounts of the p53 expression plasmid was used, suggesting that excess p53 expression will overcome the limited amount of mdm-2 protein produced by the expression vector.

Next, the entire panel of mdm-2 deletion mutants was utilized to identify the regions of the mdm-2 protein that were necessary for regulation of p53. The results, shown in Fig. 3B, show that the mdm-2 mutants that bind to the p53 protein and have an intact NLS region are all able to inhibit p53-mediated transactivation. Those mdm-2 mutant proteins that do not bind to p53 protein do not inhibit its activity. These data demonstrate that the central acidic region and the carboxyl zinc-finger motifs of the mdm-2 protein are dispensable for inhibition of p53 mediated transactivation. Therefore, the region of the mdm-2 protein that is essential for *in vivo* inhibition of wild-type p53-mediated transactivation is included in the N-terminal 222 amino acid residues. The ability of the deletion mutants to bind to p53 and localize to the nucleus appear to be essential for the inhibition of p53 mediated transactivation.



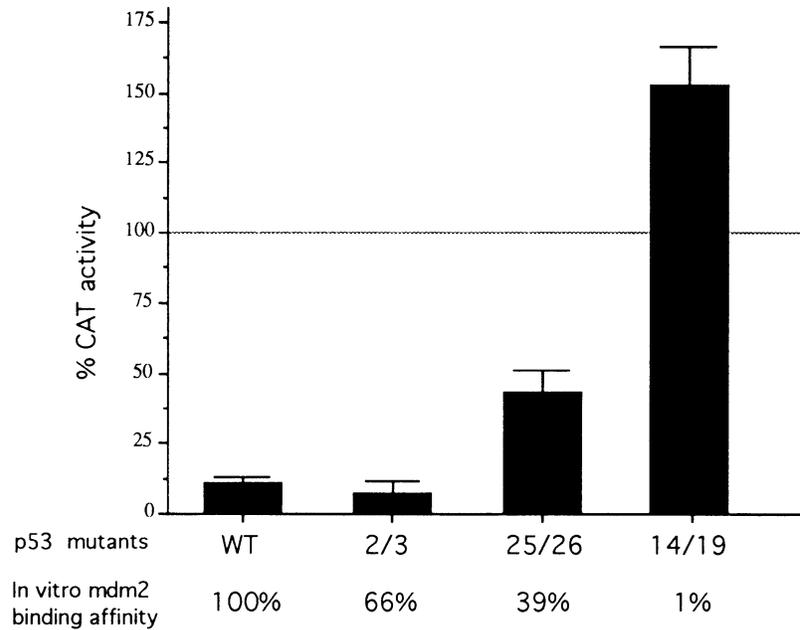
**FIG. 3. Suppression of p53 transactivation by mdm-2 and mdm-2 mutants**

(A) Suppression of p53 transactivation by mdm-2. One microgram of pCOSX1-CAT is cotransfected with indicated amounts of plasmid p11-4 (encoding murine p53) into SAOS-2 cells. Five micrograms of human mdm-2 expression plasmid or pCMV-neo-Bam vector is included in each transfection. CAT activities are shown as percent conversion of substrate. In this experiment, mdm-2 suppressed the transactivation by up to 200 ng of p53 plasmid. (B) Suppression of p53 transactivation by mdm-2 mutants. One microgram of pCOSX1-CAT is cotransfected with 20–50 ng of pC53-SN3 plasmid encoding human p53 and 5 μg of mdm-2 deletion mutant plasmid or vector. The inhibition of transactivation by the mdm-2 mutants are shown as percentage of CAT activity compared with vector. Thus, 100% indicates no suppression, and 20% represents 5-fold suppression. Each mutant was assayed in at least five experiments and the error bars represent standard deviation.

**Mapping the Regions of p53 Required for mdm-2-Mediated Inhibition of p53 Functions**

A set of previous experiments (29) introduced a series of point mutations into the N-terminal

domain of the p53 protein using site-directed mutagenesis. One double point mutation in the p53 gene, containing amino acid 14L-Q and 19F-S mutations, both located in the evolutionarily conserved region I of the p53 protein (30),



**FIG. 4. Suppression of p53 point mutations by mdm-2**

One microgram of pCOSX1-CAT is cotransfected with 50 ng of p53 expression plasmids encoding either human wild-type (Wt) or p53 double-point mutants into SAOS-2 cells. Five micrograms of mdm-2 expression plasmid encoding the suppressive mutant  $\Delta 222-437$  or vector alone are also cotransfected. The CAT activities induced by each p53 plasmid in the presence of vector are set at 100%. Each mutant was analyzed at least five times. Error bars represent standard deviations. Experiments using full-length mdm-2 produced similar results (data not shown). The p53 double-point mutant 2/3 contains 2E-K, 3E-K; mutant 14/19 contains 14L-Q, 19F-S; mutant 25/26 contains 25L-Q, 26L-H substitutions.

was found to be defective for mdm-2 binding *in vitro*, yet retained a 50% level of transactivation activity (29). If mdm-2 binding was necessary for the regulation of p53-mediated transactivation, one would predict that the 14/19 double mutant should no longer be regulated by the mdm-2 protein.

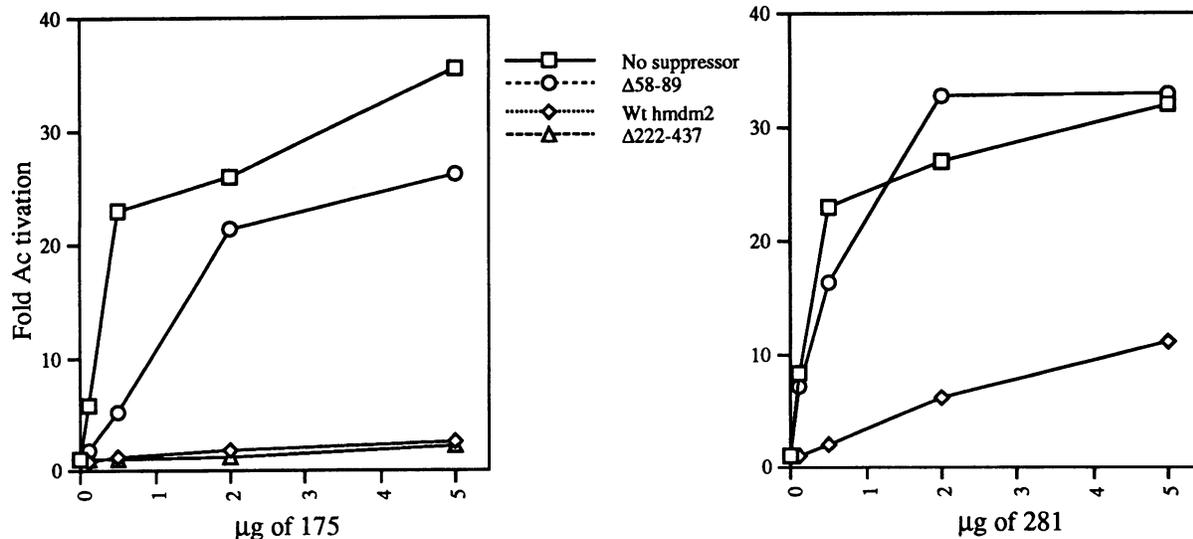
The 14/19 mutant, as well as two other p53 point mutations that showed moderately reduced mdm-2 binding, were cotransfected into SAOS-2 cells along with mdm-2 cDNA expression vectors and a p53 responsive CAT reporter plasmid. The ability of wild-type mdm-2 to inhibit p53 mutant proteins for p53-mediated transactivation was measured. The results, summarized in Fig. 4, showed that mutant 14/19 was completely unresponsive to inhibition by mdm-2. Mutant 2/3, with 66% of mdm-2 binding affinity compared with wild-type p53, was inhibited as much as the wild-type p53 protein. Mutant 25/26, with a 39% mdm-2 binding affinity, was partially blocked by mdm-2. These results demonstrate that the ability of mdm-2 to bind to p53 is essential for the negative regula-

tion of p53 by mdm-2. These data support the hypothesis that one of the roles of mdm-2 in a cell is to negatively regulate the activity of p53 through direct protein-protein interactions.

#### **The Activation of the MDR Promoter by Mutant p53 Is Also Inhibited by mdm-2**

Mdm-2 is known to form complexes with both wild-type and mutant p53 proteins *in vitro* and *in vivo* (19, 23, 31). *In vitro* binding experiments showed that the same N-terminal region of mdm-2 is also involved in the interaction with mutant p53 proteins (J. Chen, unpublished results). This suggested that the interaction between mdm-2 and mutant p53 may be similar to the interaction between mdm-2 and wild-type p53 (i.e., mdm-2 in binding to the N-terminal transactivation domain of mutant p53 would block its ability to transactivate a test gene).

A distinct phenotype of human p53 hot spot mutants are their ability to transactivate the MDR-1 promoter in transient cotransfections of cells in culture (13). The mechanism by which



**FIG. 5. Mdm-2 inhibits mutant p53 activation of MDR promoter**

Five micrograms of pMDR-CAT is cotransfected with indicated amounts of mutant p53 expression plasmids and 10  $\mu$ g of wild-type or mutant mdm-2 expression plasmids. The activation of MDR promoter is quantitated by CAT assays. Each point is the average of two experiments. The results for mutant  $\Delta 222-437$  is from a single experiment. The activation of MDR promoter by both p53 mutants 175H and 281G are suppressed by mdm-2. Mutant mdm-2 defective in binding to p53 does not affect the transactivation significantly.

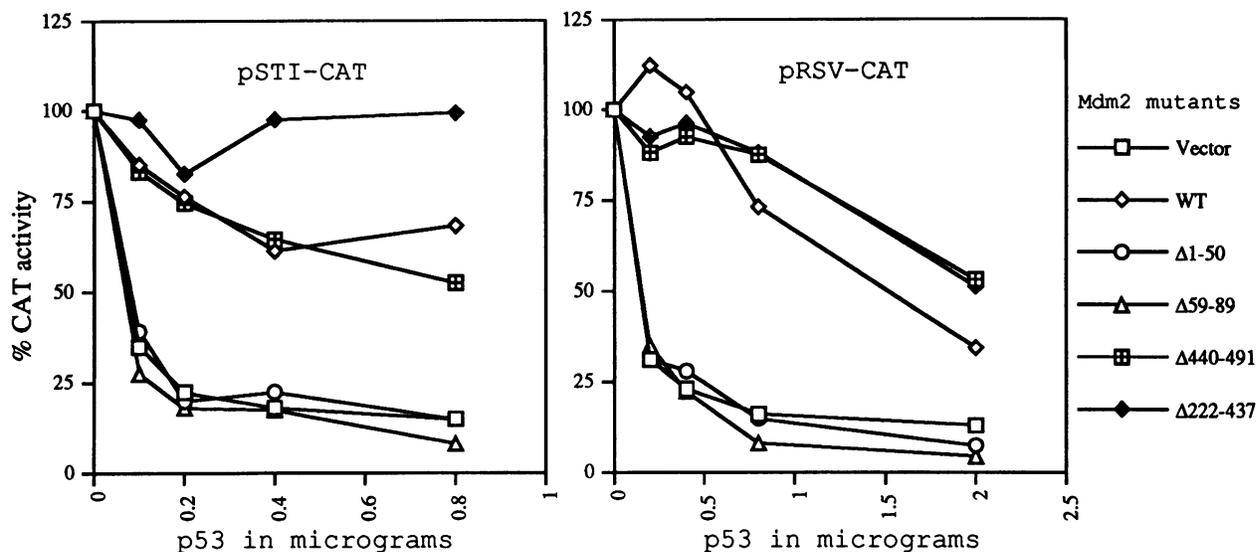
mutant p53 functions is still not understood. To test whether mdm-2 can regulate the transactivation function of mutant p53, we performed transfection experiments in SAOS-2 cells which included the pMDR-CAT reporter, a mutant p53 expression plasmid, and the mdm-2 expression plasmid.

As shown in Fig. 5, mutant p53 175<sup>Arg-His</sup> and 281<sup>Asp-Gly</sup> activated MDR-CAT in a dose-dependent fashion up to 30- to 40-fold in the SAOS-2 cells. This activation was not significantly affected by cotransfection with the mdm-2 deletion mutant ( $\Delta 59-89$ ) defective in its interaction with the p53 protein. However, transfection with a wild-type mdm-2 plasmid or a mdm-2 deletion mutant ( $\Delta 222-437$ ) still capable of binding to p53 protein, resulted in significant inhibition of CAT expression (more than 10-fold reduction for mutant 175 and approximately 5-fold reduction for mutant 281). Similarly, mutants  $\Delta 340-491$  and  $\Delta 440-491$  can bind to mutant p53 in vitro and were able to inhibit mutant p53 (data not shown). Therefore, the transactivation of the MDR promoter by mutant mdm-2 is also negatively regulated by mdm-2. The effect of mdm-2 in this assay is specific for the p53-mediated activation by a p53 mutant protein because the activation of the MDR-1 promoter, using an activated *ras* oncogene, was not affected by mdm-2 (data not shown). Thus, the *ras* acti-

vation of MDR-1 is not mediated through a mutant p53 pathway.

### Relief of p53-Mediated Repression by mdm-2 Protein

The efficient inhibition of p53-mediated transactivation by mdm-2 in transient transfection assays led us to further test the ability of mdm-2 in the regulation of another p53-mediated activity, transcriptional repression of a test gene. Two reporter constructs were utilized for this experiment; pRSV-CAT contains the CAT gene driven by the Rous sarcoma virus long terminal repeat (32), and the second plasmid pSTi-CAT contains several SP1 binding sites, the adenovirus major late promoter TATA box, and the TDT initiator DNA sequences. Consistent with previous reports, the pRSV-CAT expression vector was repressed 10- to 20-fold by wild-type p53 protein (Fig. 6). Cotransfection of pSTi-CAT with a wild-type p53 plasmid also resulted in a 5- to 10-fold reduction of CAT activities (Fig. 6). Cotransfection with a third plasmid, the CMV-neo vector, as well as plasmids encoding mdm-2 mutants  $\Delta 1-50$  and  $\Delta 59-89$  which are defective for p53 binding, had no effect on the repression of these reporter genes by p53 proteins. However, when a full-length mdm-2 expression plasmid or two mdm-2 mutants ( $\Delta 440-491$  and  $\Delta 222-437$ )



**FIG. 6. Relief of p53-mediated transcription repression by mdm-2**

One microgram of pRSV-CAT or 10  $\mu$ g of pSTi-CAT are cotransfected into SAOS-2 cells with the indicated amounts of p53 expression vector pC53-SN3. Each transfection also contained 5  $\mu$ g of mdm-2 expression plasmid or vector alone. The CAT activities in transfections with no p53 are set as 100%. Each point is the average of two assays. Mutants  $\Delta$ 1-50 and  $\Delta$ 59-89 are defective for p53 binding and behaved similar to vector. Wild-type mdm-2, mutants  $\Delta$ 440-491 and  $\Delta$ 222-437 are competent in p53 binding and partially relieved suppression by p53.

that are able to bind to p53 proteins were cotransfected, there was a significant relief of the p53-mediated repression over the entire range of the titration curve. The inhibition of repression by mdm-2 is most evident when lower levels of the p53 plasmids were used. The relief of repression by mdm-2 was observed with both the RSV-CAT and the pSTi-CAT reporter plasmids. These results demonstrate that the mdm-2 protein can block transcriptional repression by the p53 protein and this also requires direct binding between the two proteins.

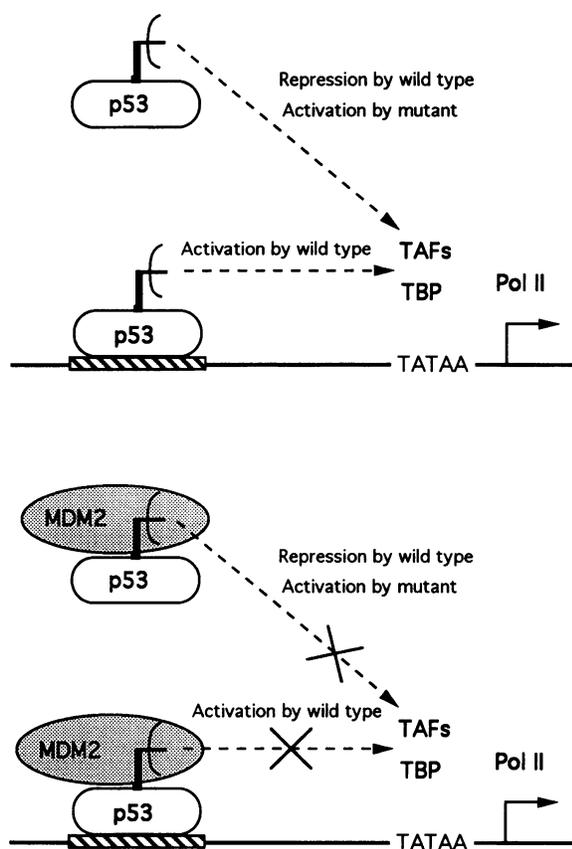
## DISCUSSION

The wild-type p53 protein can positively transactivate the transcription of genes that contain p53-DNA binding responsive elements and negatively represses many genes without such elements (9-12,33-36). The positive regulation of the p21-pic-1 (or WAF-1) gene is thought to mediate the G<sub>1</sub> arrest of cells expressing high levels of p53 protein (1,2,37-39). The p21 gene product binds to the cyclin dependent kinase-2 and inhibits its activity, thus blocking progress through the cell cycle (40,41). While a direct role for transcriptional repression in these processes has not been proven, it has been suggested that p53-mediated transcriptional repression plays a role in initiating

apoptosis (42) in a p53-dependent fashion. Mdm-2 will inhibit both positive and negative regulation of transcription by the wild-type p53 protein (Fig. 7). The third activity of the p53 protein tested here is the ability of mutant p53 proteins to transcriptionally activate the MDR-1 promoter in cells (13,15,43). This is a novel function associated only with mutant forms of p53 protein, but not the wild-type protein, and mdm-2 protein also blocks the ability of mutant p53 to activate the MDR-1 promoter (Fig. 7).

For all of these activities of the mdm-2 protein, the amino terminal domain between residues 1 and 222 (out of 491) are critical for both binding to p53 proteins and inhibiting the p53 activities. This region can be subdivided into a mdm-2 binding domain at residues 1-102 (23) and a putative nuclear localization signal at residues 181-185. The nuclear localization signal is dispensable for binding of mdm-2 to p53 proteins in vitro but not in vivo. The acidic region domain (amino acid residues 220-296) and the zinc-finger region domains (amino acid residues 305-322, 438-457, 461-478) are dispensable for both binding mdm-2 to p53 protein and the inhibition of p53 activities.

Previous experiments demonstrated that two double mutants of the p53 protein, at residues 14 and 19 and residues 22 and 23, each failed to bind the mdm-2 protein. The 22/23 mutant had



**FIG. 7. A possible mechanism of regulation of p53 transcription activities by mdm-2**

The N terminus of p53 may be directly involved in DNA sequence-specific transactivation and sequence-independent transcription repression by wild-type p53 as well as transactivation by mutant p53. These effects may be mediated by interactions with TBP or TAFs using a few critical residues on p53. Mdm-2 interacts with the same critical residues of p53 with high affinity and blocks the interactions of p53 with the transcription machinery. The negative regulation of all three p53 activities are strictly dependent on physical interaction between the two proteins.

lost all of its transcriptional transactivation activity but bound to DNA normally and produced a native protein (29). The 14/19 mutant had 50% of the transcriptional transactivation activity of the wild-type protein and bound to DNA normally (29). This mutant permitted one to ask whether the failure of mdm-2 binding to the 14/19 mutant resulted in a loss of inhibition of p53 transcriptional activity even in the presence of wild-type mdm-2 protein. The p53-14/19 mutant is not responsive to mdm-2 regulation. This p53 mutant should provide an important tool for exploring mdm-2 functions in a cell. These re-

sults reinforce the clear correlation for the need of mdm-2 to bind to p53 to inhibit the latter's activities.

Several experiments have shown that mdm-2 is an oncogene (16-20). Mdm-2 plus an activated *ras* oncogene can transform primary rat embryo fibroblasts (18). Overexpression of mdm-2 can block the ability of wild-type p53 to suppress transformation of cells in culture (18). The mdm-2 genes are amplified and overexpressed in several types of sarcomas (19,20), and overexpression of the mdm-2 has been shown to enhance the tumorigenic potential of cells in nude mice (17). Mdm-2 could act as an oncogene product solely by inhibiting p53 functions or it could also have activities intrinsic to the mdm-2 protein regions not involved in p53 regulation. The fact that amino acid residues 222-491, which encode an acidic domain and the zinc-fingers of the protein, are dispensable for blocking p53 functions suggests additional mdm-2 functions that reside in those domains. In addition, there are several isoforms of mdm-2 proteins in a cell (25), and at least one isoform lacks the amino-terminal epitopes required for mdm-2 to bind to p53 (25). The presence of mdm-2 proteins that fail to bind to p53 also suggests additional functions for mdm-2 in light of the clear requirement for mdm-2 to bind to p53 for it to block p53-mediated functions.

The amino-terminal domain of the p53 protein is required for transcriptional transactivation probably by interacting with the basal transcription factors in a cell (7). Amino acid residues 22 and 23 of the p53 protein appear to play a critical role in this process (29). The mdm-2 protein binds to amino acids 22 and 23 as part of its binding site on the p53 protein, and so it had been suggested that mdm-2 functions by sterically blocking the interaction of p53 with the basal transcriptional machinery (23,24). The fact that mdm-2 must bind to the p53 protein to inhibit its activities, as shown here, supports that hypothesis. An additional test of this hypothesis is that the mutations at amino acid residues 22 and 23 should destroy the ability of a mutant p53 protein to activate the MDR-1 promoter, just as mdm-2 blocks this activity. This is indeed the case. The p53 22<sup>Leu-Gln</sup> and 23<sup>Trp-Ser</sup> mutants are defective for MDR-1 transactivation when they are combined with a 281 p53 mutant protein (J. Lin and A. J. Levine, in preparation). The fact that these predictions are borne out in these experimental tests is most consistent with the idea that mdm-2 sterically blocks p53 interaction

with basal transcription factors. The more complicated hypothesis, that mdm-2 has in its first 222 residues an active repression domain, as was proposed for the adenovirus E1B-55kD-p53 complex (44), remains a formal alternative that will now need to be tested.

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