The p53–mdm-2 autoregulatory feedback loop

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The p53 protein can bind to a set of specific DNA sequences, and this may activate the transcription of genes adjacent to these DNA elements. The *mdm-2* gene is shown here to contain a p53 DNA-binding site and a genetically responsive element such that expression of the *mdm-2* gene can be regulated by the level of wild-type p53 protein. The mdm-2 protein, in turn, can complex with p53 and decrease its ability to act as a positive transcription factor at the *mdm-2* gene-responsive element. In this way, the *mdm-2* gene is autoregulated. The p53 protein regulates the *mdm-2* gene at the level of transcription, and the mdm-2 protein regulates the p53 protein at the level of its activity. This creates a feedback loop that regulates both the activity of the p53 protein and the expression of the *mdm-2* gene.

The p53 gene product appears to function as a transcription factor (Fields and Jang 1990; Raycroft et al. 1990). The wild-type p53 protein can bind to specific nucleotide sequences termed p53-responsive elements (Bargonetti et al. 1991; Kern et al. 1991; El-Deiry et al. 1992; Fund et al. 1992; Zambetti et al. 1992). When these elements are placed adjacent to a minimal promoter they stimulate expression in a p53-dependent fashion (Farmer et al. 1992; Kern et al. 1992; Seto et al. 1992; Zambetti et al. 1992) both in vivo and in vitro. Mutations in the p53 gene that are commonly found in human carcinomas (Hollstein et al. 1991; Levine et al. 1991) produce p53 proteins that fail to bind to DNA (Kern et al. 1991) and fail to positively regulate the transcription of p53-responsive genes (Farmer et al. 1992; Kern et al. 1992; Zambetti et al. 1992). This coincides with the notion that a tumor suppressor gene in cancerous cells has a loss-of-function mutation. The oncogene products of several DNA tumor viruses bind to the p53 protein (Lane and Crawford 1979; Linzer and Levine 1979; Sarnow et al. 1982; Werness et al. 1990) and block its ability to function as a transcription factor (Farmer et al. 1992; Kern et al. 1992; Mietz et al. 1992; Yew and Berk 1992). In addition, a cellular oncogene product, mdm-2 (Fakharzadeh et al. 1991), has been shown to bind to the p53 protein and eliminate its ability to function as a transcription factor (Momand et al. 1992). These observations are most consistent with the idea that p53 functions as a transcription factor to regulate its tumor suppressor gene activities. The search for cellular genes regulated by the wild-type p53 protein is therefore of considerable interest (Kastan et al. 1992).

The p53–mdm-2 protein complex was originally detected in a cell line containing a temperature-sensitive p53 protein (Martinez et al. 1991; Momand et al. 1992), which behaved like the wild-type p53 protein at 32°C and a mutant p53 protein at 37°C–39°C. The p53–mdm-2 complex was readily detected at 32°C and only poorly observed at 37°C–39°C, even though it was clear that mdm-2 bound well to mutant p53 protein (Hinds et al. 1990). One possible explanation for this observation is that the synthesis of the mdm-2 protein is regulated by the presence of wild-type p53 protein. The results presented here demonstrate that the wild-type p53 protein stimulates increased steady-state levels of *mdm-2* mRNA and mdm-2 protein. The first intron of the *mdm-2* gene contains a p53 DNA-binding site which, when placed adjacent to a minimal promoter, can stimulate a test gene in a p53-dependent fashion. Finally, when additional mdm-2 protein is produced, it binds to p53 and decreases its ability to stimulate the *mdm-2* gene. This then provides an autoregulatory feedback loop for the *mdm-2* gene which, in turn, regulates the transcriptional *trans*-activation activity or function of the p53 protein. This study clearly demonstrates that *mdm-2* is one of the genes that responds to p53 regulation.

Results

mdm-2 protein and mRNA levels are regulated by the p53 protein

The A1 cell line (Finlay et al. 1989; Martinez et al. 1991) is a rat embryo fibroblast cell line transformed by a temperature-sensitive mutant of p53 (codon 135, Ala → Val change) plus an activated *ras* oncogene. At 32°C most of

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the p53 protein is wild type, whereas at 37°C–39°C, most of the p53 protein behaves like the mutant form (Michalovitz et al. 1990; Martinez et al. 1991). It had been noted previously that the level of mdm-2 protein complexed to p53 in these cells was much greater at 32°C than at 39°C (Barak and Oren 1992; Momand et al. 1992). To follow the total pool of mdm-2 protein in these cells, antisera were prepared against purified mdm-2 protein and mdm-2 protein levels were measured by immunoprecipitation. Cells incubated at 32°C or 39°C were labeled with [^{35}S]methionine for 2 hr (the half-life of mdm-2 is ~20–30 min (Olson et al. 1993), so this approaches a steady-state measurement), and soluble protein extracts were prepared from these cells. The extracts were incubated with a control serum that did not react with p53 or mdm-2 proteins, anti-mdm-2 antibodies, or anti-p53 monoclonal antibody PAb421, and the immunoprecipitates were collected and analyzed on SDS–polyacrylamide gels (Fig. 1A). The results demonstrate that anti-mdm-2 sera detected this 90-kD mdm-2 protein in cells at 32°C and coimmunoprecipitated some p53 protein. Similarly, anti-p53 antibodies complexed with p53 and coimmunoprecipitated the 90-kD mdm-2 protein. Little or no mdm-2 protein was found in cells grown at 39°C, and the anti-p53 monoclonal detected mutant p53 protein bound to the heat shock protein at 32°C and 39°C, hsc70, as described previously (Hinds et al. 1987). The steady-state level of mdm-2 mRNA was also examined in A1 cells incubated at 32°C and 39°C. A Northern blot using the mdm-2 cDNA probe to measure mdm-2 mRNA levels shows mdm-2 mRNA species at 32°C but not at 39°C (Fig. 1B). An actin DNA probe (Fig. 1B) was employed for the normalization of RNA levels in these cells. No mdm-2 mRNA was detected at 32°C or 39°C in

a rat embryo fibroblast cell line transformed by a non-temperature-sensitive mutant p53 cDNA clone plus an activated ras oncogene [T101-4 (Finlay et al. 1989)], eliminating the possibility that temperature affected expression of this gene.

Similar results were obtained with a murine cell line (10.1)Val5, which expresses a temperature-sensitive p53 protein but has no endogenous p53 protein, owing to a deletion of the p53 gene in this cell line (Harvey and Levine 1991). The mdm-2 mRNA (Fig. 1C) and protein (data not presented) in these cells were detected at 32°C but not at 39°C. Furthermore, 14-fold more mdm-2 mRNA was made in the (10.1)Val5 cells at 32°C than in (10)1 cells, the parental cell line without the temperature-sensitive p53 gene [Fig. 1C, (10)1 vs. (10.1)Val5 at 32°C]. These data suggest the possibility that the wild-type, but not the mutant, p53 protein can regulate the expression of the mdm-2 gene.

The mdm-2 gene has a p53-responsive element

To search for a p53-responsive element in the mdm-2 gene, various segments of this gene and DNA regions 5′ to the mRNA start site were cloned into an expression vector containing a minimal promoter (the adenovirus major late TATA box and TdT initiator sequence) adjacent to the chloramphenicol acetyltransferase (CAT) gene (Shi et al. 1991). These plasmids were transfected into (10)1 cells, which contain no endogenous p53 protein, and CAT activity was assayed. A 3-kb DNA fragment 5′ to the mdm-2 gene mRNA start site failed to demonstrate any p53-responsive transcriptional elements. A 1-kb DNA fragment containing the first intron of the mdm-2 gene provided a strong induction of CAT
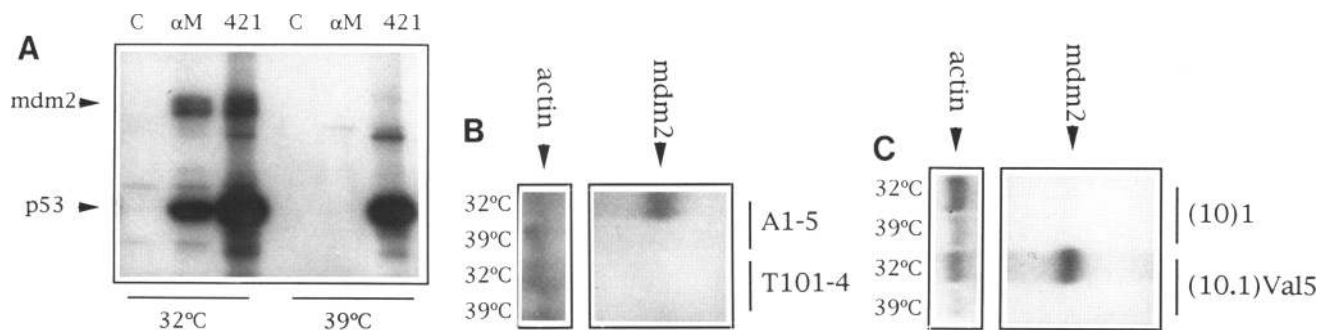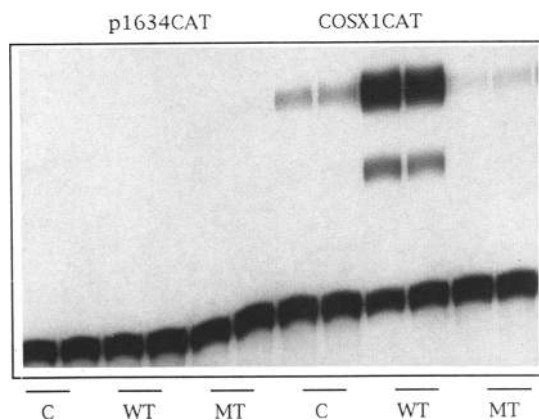

Figure 1. Endogenous levels of mdm-2 are enhanced by the presence of wild-type p53. (A) Immunoprecipitation of mdm-2 and p53 in A1 cells. A1 cells are rat embryonic fibroblasts, which contain a high level of a temperature-sensitive p53 protein. At 32°C, p53 exhibits a wild-type conformation while mutant p53 is predominantly produced at 39°C. The cells were metabolically labeled with [^{35}S]methionine, and cell extracts were precipitated with control sera, PAb419, which reacts with the SV40 T antigen (C), an anti-p53 monoclonal antibody (421), or anti-mdm-2 (αM) antisera. mdm-2 protein was greatly induced at 32°C with wild-type p53 activity. (B) Northern analysis of mdm-2 RNA in A1 cells. Both A1 and control T101-4 cells were grown at either 32°C or 39°C. Total RNA was prepared and analyzed for mdm-2 mRNA level. High levels of mdm-2 transcripts were detected at 32°C in A1 cells. T101-4 contains only the mutant p53, which does not behave in a temperature-sensitive fashion. The quantity of RNA in each lane was normalized by hybridization to an actin cDNA probe (actin). (C) Northern analysis of mdm-2 RNA in (10)1 cells and (10)1 cells with temperature-sensitive p53 [(10.1)Val5]. (10)1 cells and (10.1)Val5 cells were grown at 32°C and 39°C, and RNA was then extracted from these cells. mdm-2 RNA levels were determined by Northern blot hybridization; an autoradiograph of this experiment is presented. These data were quantitated from multiple exposures on the PhosphorImager. High levels of mdm-2 mRNA were found only in cells with wild-type p53 [(10.1)Val5 at 32°C] protein. RNA levels in each lane were normalized to actin mRNA levels (actin).
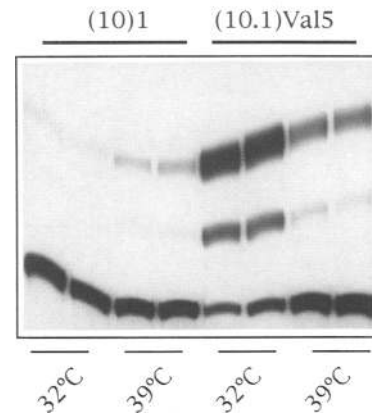
activity only when it was cotransfected into these cells with the wild-type p53 plasmid (Fig. 2, Cosx1CAT, wt). The minimal promoter in the absence of these *mdm-2* DNA sequences (Fig. 2, p1634CAT) did not respond by producing CAT activity when it was transfected into these cells alone, with wild-type, or mutant p53 plasmids. The test plasmid with *mdm-2* gene sequences (Cosx1CAT) gave low CAT activity in the absence of p53 or with a mutant p53 plasmid, but high activity with a wild-type p53 plasmid. The lower levels of CAT activity with the mutant p53 plasmid is not a reproducible finding.

The Cosx1CAT plasmid containing the *mdm-2* DNA sequences was also transfected into the (10)1 cell line [with no endogenous p53 protein present owing to a deletion of the p53 gene (Harvey and Levine 1991)] or the (10.1)Val5 cell line containing the temperature-sensitive p53 mutant. Figure 3 shows that the (10)1 cell line had only low CAT activity at 32°C or 39°C while the same cells with a temperature-sensitive p53 protein had high levels of CAT activity at 32°C but not at 39°C.

Deletion analysis of this 1-kb *mdm-2* DNA fragment using the first intron mapped the p53 wild-type responsive element to 85 bp in the first intron of the *mdm-2* gene (Fig. 4). Deletion of this site made the Cosx1CAT plasmid nonresponsive to wild-type p53 in the (10)1 cells. An 85-bp sequence containing this DNA element, but not the remainder of the *mdm-2* gene, was sufficient to confer p53-dependent expression on this test gene. The sequence of this region of the *mdm-2* gene is presented in Figure 5. A consensus p53 DNA-binding sequence has been described (El-Deiry et al. 1992; Funk et al. 1992), and two imperfect repeats of these consensus

Figure 3. Cosx1CAT is *trans*-activated only by the wild-type p53 protein in temperature-sensitive (10.1)Val5 cells. The (10.1)Val5 line contains a temperature-sensitive p53 mutant that is wild type at 32°C and mutant at 39°C. The parental line (10)1 has no p53 protein. The presence of a 1-kb sequence of DNA from the *mdm-2* gene in (10.1)Val5 cells only stimulates CAT activity at 32°C; where wild-type p53 protein is present.
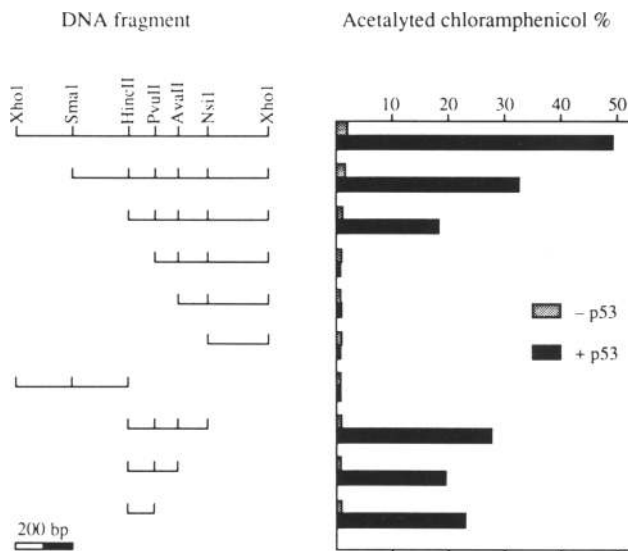
sequences are detected (underlined in Fig.5) in this p53-responsive element. The first repeat contains three mismatches with the consensus sequence, whereas the second repeat contains two differences, including an extra A residue (insertion).

Oligonucleotides that span only the first p53-responsive element or only the second element are able to promote p53-responsive *trans*-activation at a much reduced efficiency (~20% of the 85-bp element). Both elements appear to be needed for a maximum efficiency (results not presented).

Wild-type p53 protein binds to a DNA fragment containing the p53-responsive element

To determine whether the wild-type p53 protein binds to the DNA sequences mapped as the p53-responsive element in the *mdm-2* gene, a McKay assay (McKay 1981), as modified by Kern et al. (1991), was performed. A DNA fragment from the first intron–exon region of the *mdm-2* gene was cleaved with *Hinc*II and end-labeled to detect the DNA fragments. The DNA was incubated with wild-type p53 protein prepared from baculovirus-infected cells (Friedman et al. 1990), and monoclonal antibodies (PAb242, 248, and 421) were added to immunoselect the DNA–p53 protein complexes that formed. The immunoselected DNA–protein complexes were analyzed on a polyacrylamide gel, and the autoradiograph of the gel is presented in Figure 6. Without immunoselection, both DNA fragments are present in equal levels. With p53 protein binding and immunoselection, the DNA fragment with the p53-responsive element is preferentially selected by these antibodies. Leaving out the p53 protein or the antibodies directed against this protein fails to immunoselect the DNA fragment that binds p53 protein. Thus, the p53 protein binds to this p53-responsive

Figure 2. Localization of a p53-responsive element in the *mdm-2* gene. A 1.0-kb fragment from the first intron of the murine *mdm-2* gene was subcloned upstream from the adenovirus major late TATA box/TdT initiation signal–CAT gene (p1634CAT). The resulting plasmid, Cosx1CAT, was transfected into (10)1 cells (p53 negative) either alone (lanes C), with the wild-type p53 (lanes WT), or with mutant KH215 p53 (lanes MT). p1634CAT with no *mdm-2* sequences was also transfected as a control (p1634CAT lanes). Cell extracts were analyzed for CAT activity. Positive *trans*-activation of Cosx1CAT was observed by wild-type p53 but not by the mutant p53 protein.

Figure 4. Mapping the p53-responsive element in the first intron of the *mdm-2* gene. The restriction enzyme map of the 1-kb first intron of the *mdm-2* gene is presented. The different DNA fragments indicated under the map were cloned into the 1634 CAT expression vector, which contains a minimal promoter and a test gene (CAT). These reporters were transfected into (10)1 cells with (solid bars) or without (stippled bars) wild-type p53 expression vectors. The CAT activity produced in these cells is given as a percentage of the chloramphenicol acetylated. The results presented are the average of two experiments. These data are representative of multiple experiments carried out at independent times. This permitted the mapping of the p53-responsive element in the *mdm-2* gene to an 85-bp *Hinc*II and *PvU*II fragment.

DNA element containing the two DNA-binding consensus sites.

mdm-2 can negatively regulate the p53 stimulation of the p53-responsive element in the mdm-2 *gene*
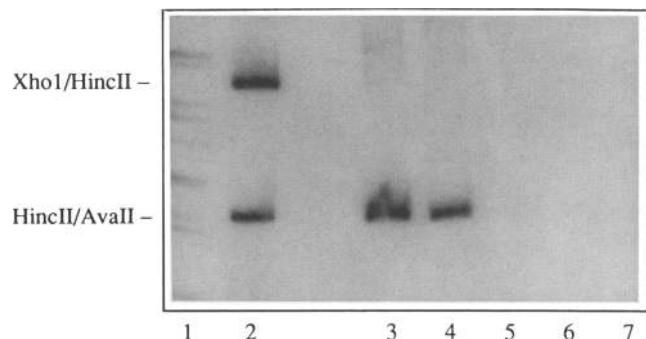
The wild-type p53 protein can bind to an element in the first intron of the *mdm-2* gene and stimulate the expression of that gene. It has been shown previously that the mdm-2 protein can bind to p53 and negatively regulate its stimulation of a known p53-binding element in the

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gactcagctcttcctgtggggct GGTCAAGTTG GGACACGTCC
                                 *        *      *

ggcgtcg gctgtcggag GAGCTAAGTCC TGACATGTCT ccag
                          *          *
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Figure 5. The nucleotide sequence of the p53-responsive element from the *mdm-2* gene. The nucleotide sequence of the DNA fragment (85 bases) mapped in Fig. 4 is given. Two putative p53 DNA-binding elements based on a DNA-binding consensus sequence determined previously (El-Deiry et al. 1992; Funk et al. 1992) are underlined. There are several mismatches in the consensus sequence, which is Pu, Pu, Pu, G, A/T, T/A, C. Py, Py, Py repeated twice with 0- to 13-bp spacing. An asterisk (*) as been placed over a mismatch in the consensus sequence.

gene for the muscle form of creatine phosphokinase (Momand et al. 1992; Zambetti et al. 1992). To determine whether the expression of the mdm-2 protein also acts on the ability of the p53 protein to stimulate the *mdm-2* gene, the following experiment was performed. The *mdm-2*-responsive element in the Cosx1CAT reporter was transfected into (10)1 cells along with wild-type p53, the entire *mdm-2* gene to synthesize mdm-2 protein, or the vector CV001. Figure 7 presents the levels of CAT activity detected in the extracts from these cells. The Cosx1CAT test gene had a low level of activity that was stimulated by either wild-type p53 alone or wild-type p53 cotransfected with the CV001 vector. Neither *mdm-2* nor CV001 stimulated the Cosx1CAT activity. mdm-2 protein expressed in the same cells with p53 wild-type protein and the Cosx1CAT reporter gene blocked the ability of p53 to stimulate the *mdm-2*-responsive element from the *mdm-2* gene (Fig. 7).

It was possible that the *mdm-2* gene in the cosmid was competing for p53-binding sites with Cosx1CAT plasmid; therefore, that p53 protein was limiting in cells containing wild-type p53, the *mdm-2* gene, and the Cosx1CAT reporter plasmid. This possibility was eliminated, however, by demonstrating that the competition for p53 protein binding by an *mdm-2* deletion mutant (*mdm-Δ13*), which contains the p53-responsive element, had only a small effect on the ability of p53 plasmid to

Figure 6. Immunoselection of wild-type p53 protein bound to the DNA fragment with a p53-responsive element. p53 protein can bind to DNA sequences in the *mdm-2* first intron, through which it can stimulate transcription. Labeled DNA fragments from the *mdm-2* gene and the DNA 1-kb ladder (GIBCO/BRL) were mixed and incubated with purified wild-type murine p53 and purified monoclonal antibodies, and were precipitated with protein A–Sepharose. After washing, labeled DNA fragments complexed to the beads were analyzed by polyacrylamide gel electrophoresis. Lanes 1 and 2 represent 2% of the input-labeled 1-kb ladder (lane 1) and a 450-bp *Xho*I–*Hinc*II fragment from the *mdm-2* gene and a 160-bp *Hinc*II–*Ava*II fragment containing the imperfect consensus binding sequences for p53 (see Figs. 4 and 5). Lanes 3 and 4 represent labeled DNA precipitated with p53 protein and a cocktail of monoclonal antibodies 242, 248 (Yewdell et al. 1986), and 421 (Harlow et al. 1981) directed to the p53 protein (lane 3) and with PAb248 alone (lane 4). Lanes 5–7 are control experiments utilizing antibody PAb419 (Harlow et al. 1981), which is not specific for p53 (lane 5), no antibody (lane 6), and no p53 protein (lane 7).

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CosxlCAT	+	+	+	+	+	+	+	+
p53wt	-	+	-	-	-	+	+	+
mdm2	-	-	+	-	-	+	-	-
CV001	-	-	-	+	-	-	+	-
mdmΔ13	-	-	-	-	+	-	-	+
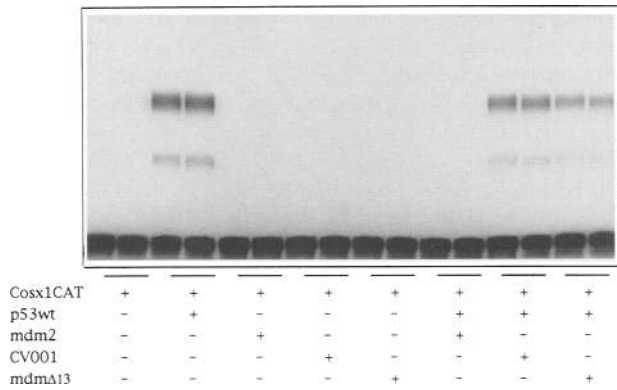
Figure 7. mdm-2 protein can negatively regulate its own expression by inhibiting p53-mediated *trans*-activation. (10)1 cells were transfected with the CosxlCAT *mdm-2* reporter gene and various combinations of the wild-type p53 (p53 wt), *mdm-2*, CV001, which is the vector that carries *mdm-2* or an *mdm-2* deletion mutant, and *mdm-Δ13*, which does not express mdm-2 protein but contains the p53-responsive element. The combinations are given in the matrix, below. While wild-type p53 *trans*-activates the *mdm-2*-responsive elements in the presence of CV001 (vector alone), it fails to *trans*-activate CosxlCAT when the *mdm-2* gene is present and expressed. *mdm-Δ13*, a deletion mutant, does not inhibit p53 *trans*-activation.

activate the CosxlCAT gene (Fig. 7), and this level does not account for the complete lack of p53 activity when the mdm-2 protein is present. Thus, the expression of mdm-2 protein is essential in blocking the p53-mediated *trans*-activation of the *mdm-2* gene.

Discussion

The experiments described here demonstrate that the wild-type p53 protein can regulate the expression of the *mdm-2* gene. In virtually all previous experiments demonstrating that p53 responsive elements are present and can regulate a test gene, the experiments were carried out by employing DNA transfection protocols (Farmer et al. 1992; Kastan et al. 1992; Kern et al. 1992; Zambetti et al. 1992). This places a test gene and the p53 expression plasmid in very high concentrations in a cell, and the test gene is not in the context of a normal chromosomal locus. In the studies presented here, however, wild-type p53 protein was shown to regulate the *mdm-2* mRNA and protein levels from the normal endogenous *mdm-2* gene. The *cis*-acting DNA element in the *mdm-2* gene that binds the p53 protein and makes the gene responsive to p53 protein levels was isolated and identified. It has an imperfect consensus DNA-binding sequence (El-Deiry et al. 1992; Funk et al. 1992), which suggests that there will be some variation in p53 DNA-binding sites and p53-responsive elements. It remains possible that additional p53-responsive elements are present in the *mdm-2* gene and have not yet been identified.

Because mdm-2 protein can combine with p53 and modulate down its activity as a transcription factor (Momand et al. 1992), the regulation of the *mdm-2* gene by

the p53 protein has an interesting consequence. When mdm-2 protein is expressed in a cell where p53 is active, it blocks further p53 function, which results in less mdm-2 being made (see Fig. 8). Thus, the activity of p53 and the levels of mdm-2 in a cell are kept in balance by this autoregulatory feedback loop. Factors that disturb this loop and act to increase mdm-2 levels (via amplification of this gene) (Oliner et al. 1992) or increased mdm-2 activity will promote cell proliferation, whereas factors that alter the ability of p53 protein to stimulate mdm-2 or inactivate mdm-2 activity should lead to growth arrest. It seems likely that additional ways to regulate p53 activity could be mediated through mdm-2 via protein modification, different mRNA splice variants of the *mdm-2* mRNA, or even other proteins that modulate mdm-2 activity. Because of the central role that p53 plays in cancers, it will be important to understand this p53–mdm-2 autoregulatory feedback loop and the factors that control it.

mdm-2 was originally identified as an oncogene that conferred an enhanced tumorigenic potential on cells when the *mdm-2* gene was amplified and overexpressed (Fakharzadeh et al. 1991). The overexpression of *mdm-2* plus the *ras* oncogene results in the cooperative transformation of primary rat embryo fibroblasts in cell culture (Finlay 1993). These biological properties of the mdm-2 protein could be the result of its binding to p53 protein and inactivating the p53 tumor suppressor function. Alternatively, mdm-2 may well have additional functions other than those that result from p53 interactions. The primary amino acid sequence of the mdm-2 protein contains several putative functional domains and motifs such as zinc fingers, an acidic or highly negatively charged region, and nuclear localization signals. It seems likely that mdm-2 will act as a transcription factor by itself or in complex with other proteins. Although it is clear that p53 protein can regulate the levels of mdm-2 protein and, therefore, its putative activity as
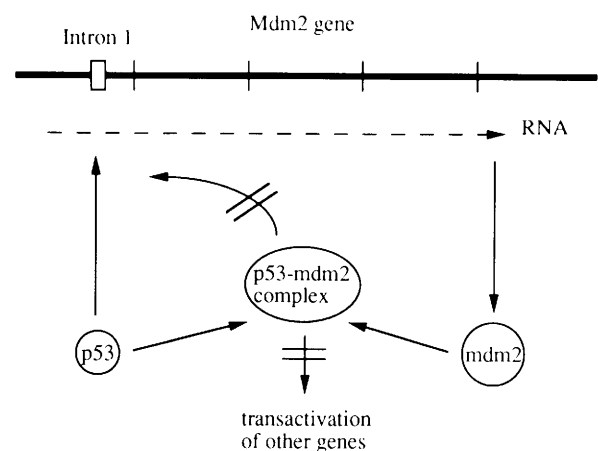
Figure 8. The p53–mdm-2 autoregulatory loop. p53 positively regulates mdm-2 by activating *mdm-2* gene expression. mdm-2, on the other hand, inhibits the *trans*-activating function of p53 after forming a p53–mdm-2 complex.

a transcription factor in a cell, it certainly remains a viable hypothesis that the p53 protein could regulate mdm-2 activity in the p53–mdm-2 protein complex. Such speculations will rapidly become testable when mdm-2-responsive DNA elements are identified and the genes regulated by the mdm-2 proteins are described.

Materials and methods

Cell lines

The A1 cell line is a rat embryo fibroblast-derived line that is transformed with a temperature-sensitive murine p53 mutant clone (codon 135, Ala → Val change) plus an activated ras oncogene (Finlay et al. 1989). Its properties are described in Martinez et al. (1991). The (10)1 cell line is a BALB/c mouse embryo fibroblast cell line that has been immortalized with a passage schedule that keeps the cell line nontransformed for several properties (Harvey and Levine 1991).

Plasmids

The mdm-2 cosmid and the vector for this murine gene CV001 are described in Fakharzadeh et al. (1991). The p53 wild-type and mutant expression vectors are described in Zambetti et al. (1992). The baculovirus p53 vector was a gift from C. Prives (Columbia University, NY), and the procedures for its replication are given in O'Reilly and Miller (1986) and Friedman et al. (1990).

p53–DNA immunoprecipitation assay

Specific binding of p53 to sequences in the mdm-2 gene mediating the transcriptional response was demonstrated by the method of McKay (1981), as modified by Kern et al. (1991). A 610-bp XhoI–AvaII DNA fragment containing sequences from exon 1 and intron 1 of the murine mdm-2 gene was digested with HincII. The resulting 160- and 450-bp fragments (see Figs. 4 and 6) were end-labeled with polynucleotide kinase and [α-^{32}P]ATP as described (Maniatis et al. 1982). Sequences from the 1-kb DNA molecular mass marker (GIBCO/BRL) were labeled in a similar manner.

Sf9 insect cells were infected with a recombinant baculovirus encoding wild-type murine p53 according to a methodology outlined by O'Reilly and Miller (1986) and Friedman et al. (1990). p53 was purified from infected cells by immunoaffinity chromatography as outlined by Momand et al. (1992). Protein was eluted from a column of conjugated monoclonal antibody 421 with a peptide containing the epitope for the antibody. Peptide was removed by dialysis against a solution containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, and 20% glycerol.

Approximately 90 ng of p53 was incubated with 2×10^5 cpm of mdm-2 probe and 1×10^5 cpm of 1-kb ladder, 0.8–1.2 μg of purified monoclonal antibodies, and 95 μl of binding buffer (20 mM Tris-HCl at pH 7.2, 100 mM NaCl, 10% glycerol, 1% NP-40, 1 mM EDTA) at 4°C for 30 min while rotating. A mixture of 1.25 mg of protein A–Sepharose and 12.5 μg of poly [d(I-C)] (Pharmacia) in binding buffer was added to the immunocomplexes at 4°C for 30 min while rotating. Samples were washed twice with 500 μl of binding buffer, suspended in 150 μl of binding buffer, and extracted with phenol–chloroform and chloroform. DNA was ethanol precipitated and loaded onto a 4% polyacrylamide gel. Labeled products were separated by electrophoresis, and the gel was dried and exposed for autoradiography.

DNA transfection

The cells were plated on a 6-cm tissue culture dish and grown in 3 ml of Dulbecco's modified Eagle medium (DMEM) containing 10% FBS. At ~50% confluency, they were transfected with DNA by a calcium phosphate precipitate procedure as described (Graham and van der Eb 1973). A total of 10 μg of DNA (4 μg of construct adjusted to 10 μg with salmon sperm DNA) was used in each transfection. In the case of the mdm-2 inhibition experiment, an equal molar amount of DNA was added for each construct. The transfected cells were incubated at 37°C for ~18 hr, washed with PBS, and refed with 3 ml of the medium. The cells were incubated for an additional 30–40 hr and collected by trypsinization and centrifugation. The cells were resuspended in 100 μl of 0.25 M Tris-HCl (pH 8.0) and lysed by three cycles of freeze–thawing, alternating between a dry ice/ethanol bath and 37°C water bath (5 min at each time and vortex between each cycle). Cellular debris was removed by centrifugation, and the protein concentration was determined by Bradford assay.

CAT assays

CAT assays were carried out as described by Zambetti et al. (1992).

Note added in proof

After this paper was written and submitted for publication, Y. Barak, T. Juven, R. Haffner, and M. Oren published a paper (EMBO J. **12:** 461–468, 1993) demonstrating that mdm-2 mRNA levels were regulated and induced by the wild-type form of the p53 protein.

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