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Regulation of *mdm2* expression by p53: alternative promoters produce transcripts with nonidentical translation potential

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The *mdm2* proto-oncogene product binds to the p53 tumor suppressor protein and inhibits its ability to *trans*-activate target genes. One such target gene is *mdm2* itself, which is therefore considered a component of a p53 negative feedback loop. Two tandem p53-binding motifs residing within the first intron of the murine *mdm2* gene confer upon it p53-mediated activation. We now report that in murine cells p53 activates an internal *mdm2* promoter (P_2) located near the 3′ end of intron 1, resulting in mRNA whose transcription starts within exon 2. P_2 is activated by p53 within artificial constructs, as well as within the context of the chromosomal *mdm2* gene. Activation follows either the introduction of overexpressed wild-type p53 into cells or the induction of endogenous wild-type p53 by ionizing radiation. The upstream, constitutive (P_1) *mdm2* promoter is only mildly affected by p53, if at all. The p53-derived *mdm2* transcripts lack exon 1 and a few nucleotides from exon 2. As the first in-frame AUG of *mdm2* is located within exon 3, the two types of *mdm2* transcripts should possess similar coding potentials. Nevertheless, in vitro conditions, where each of these transcripts yields a distinct translation profile, reflect the differential usage of translation initiation codons. Initiation of translation at internal AUG codons, which occurs also in vivo, gives rise to MDM2 polypeptides incapable of binding to p53. In vitro translation profiles of the various *mdm2* transcripts could be manipulated by changing the amounts of input RNA. Thus, p53 can modulate both the amount and the nature of MDM2 polypeptides through activation of the internal P_2 promoter.

[Key Words: p53; *mdm2*; DNA damage; transcription; translational control]

Received May 13, 1994; accepted June 13, 1994.

The p53 tumor suppressor gene encodes a sequence-specific transcription factor (El-Deiry et al. 1992; Zambetti et al. 1992). p53 is frequently mutated in human tumors (for review, see Harris and Hollstein 1993), leading to the loss of its DNA-binding and/or *trans*-activation properties (Farmer et al. 1992; Kern et al. 1992; Zambetti and Levine 1993). Recent studies suggest that transcriptional activation by p53 accounts for its tumor suppressor function (Pietenpol et al. 1994). The finding that transcription of the Cdk inhibitor *WAF1*, a key player in cell cycle arrest, is activated by p53 (El-Deiry et al. 1993) strongly supports this notion.

The activity of p53 is potentiated in normal cells following DNA damage (Maltzman and Czyzyk 1984; Kastan et al. 1991, 1992; Kuerbitz et al. 1992; Fritsche et al. 1993; Lu and Lane 1993; Zhan et al. 1993). This is achieved, at least in part, through stabilization of the otherwise labile p53 protein, which leads to the accumulation of considerable amounts of p53 and stimulates the activation of its transcriptional targets. The enhanced biochemical activity of p53 may culminate in either growth arrest or apoptosis, two processes that can be evoked experimentally by overexpressed wild-type p53 (Diller et al. 1990; Mercer et al. 1990; Michalovitz et al. 1990; Gannon and Lane 1991; Martinez et al. 1991; Yonish-Rouach et al. 1991). The determinants that dictate the choice between growth arrest and apoptosis in response to p53 activation are poorly understood at the moment; the extent of the damage and the repair capacity of the cells, as well as availability of survival signals, may play a role in the final decision.

The *mdm2* proto-oncogene was originally isolated by virtue of its amplification in a tumorigenic derivative of NIH-3T3 cells (Cahilly-Snyder et al. 1987; Fakharzadeh et al. 1991). Amplification of *mdm2* has since been documented in an array of human malignancies (Oliner et al. 1992; Ladanyi et al. 1993; Leach et al. 1993; Reifenberger et al. 1993; Cordon-Cardo et al. 1994). The oncogenic properties of the *mdm2* gene product have been attributed to its interaction with the p53 protein (Barak and Oren 1992; Momand et al. 1992; Oliner et al. 1992; Barak et al. 1993; Finlay 1993), an interaction that inhibits the biochemical activities of the latter (Momand et al. 1992; Oliner et al. 1993; Zauberman et al. 1993). It was therefore surprising to discover that the *mdm2* gene is

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actually activated by p53 (Barak et al. 1993; Otto and Deppert 1993; Wu et al. 1993). This finding has been taken to imply that *mdm2* is a component of a negative feedback loop whereby p53, once having fulfilled its biochemical tasks, activates *mdm2* whose product in turn renders p53 inactive (Barak et al. 1993; Picksley and Lane 1993; Wu et al. 1993). Activation of the *mdm2* gene by p53 involves the physical interaction of p53 with two imperfect response elements located within the first intron of this gene (Juven et al. 1993; Wu et al. 1993). Interestingly, transfection experiments have indicated that a small genomic DNA fragment encompassing these two p53 response elements (p53 REs) can exhibit p53-dependent promoter activity when coupled to a reporter gene (Juven et al. 1993).

We now report that this activity is attributable to the existence of a functional p53-responsive promoter within the first intron of the murine *mdm2* gene. This promoter (P_2) gives rise to distinct transcripts, which lack the entire first exon and a few nucleotides from the second exon of murine *mdm2*. Moreover, P_2 is activated by p53 not only in artificial reporter plasmids but also within the context of the endogenous, chromosomal *mdm2* gene. Transcription from P_2 appears to be strongly p53-dependent, whereas transcription from the upstream, constitutive *mdm2* promoter (P_1) is only mildly induced, if at all, by excess wild-type p53. The sequence of these two types of *mdm2* transcripts predicts that they should encode identical products. Nevertheless, conditions could be found where synthetic RNA molecules corresponding to each transcript gave rise to distinct in vitro translation profiles, with varying proportions of p53-binding and nonbinding MDM2 proteins. These data imply that p53, through the activation of an alternative promoter, can potentially modulate both the amount and the nature of the MDM2 proteins, thereby adding a further dimension to the complex interaction between these two growth regulators.

Results

The 5′ end of the p53-dependent mdm2 *transcript is located within exon 2*

Using chimeric reporter plasmids, we demonstrated previously that p53 can induce transcription from a murine *mdm2* genomic DNA fragment lacking the 5′-flanking sequences of the gene. This observation could imply the existence of a putative internal promoter (P_2), located within this fragment. This promoter could potentially be associated with the two p53 REs identified within the first intron of the mouse *mdm2* gene (Juven et al. 1993). The minimal internal *mdm2* genomic DNA fragment that still exhibits p53-dependent promoter activity is an *ApaI–NsiI* ~400-bp fragment, encompassing the 3′ part of intron 1, the entire exon 2, and the 5′ half of intron 2 (Juven et al. 1993; see also Fig. 1A). A transcript emanating from P_2 will therefore lack exon 1 but may include exon 3 and possibly sequences from intron 1 and/or exon 2 or alternatively intron 2 (see Fig. 1A). We wished to determine whether overexpression of wild-type p53 gives rise to a discrete transcript initiated within this fragment and to compare the structure of such transcript with that of the full-length *mdm2* cDNA characterized previously (Fakharzadeh et al. 1991). To that end, RNase protection analysis was performed using an antisense cDNA-derived riboprobe extending from position +264 to position +3 of the full-length *mdm2* transcript. Advantage was taken of a series of transformed rat embryo fibroblast (REF) cell lines, stably transfected with a chimeric construct containing a 2.9-kb *Eco*RI genomic DNA fragment from the 5′ part of the *mdm2* gene linked to the bacterial chloramphenicol acetyltranferase (CAT) gene. The 2.9-kb fragment extends from a position of approximately −800 bp (relative to the first nucleotide of exon 1) down to the middle of intron 3 (Fig. 1A). In addition, these cell lines also harbor activated Ha-*ras*, as well as the temperature-sensitive p53 mutant p53val135. Shifting the growth temperature of these cells down to 32°C also leads to p53-mediated growth arrest, as well as a marked p53-dependent induction of CAT enzymatic activity (Juven et al. 1993). This induction reflects the activation of a p53-responsive promoter residing within the cloned genomic *mdm2* DNA segment.

The sizes of the RNA fragments expected to arise as a result of the protection of the riboprobe by RNA from such cell lines are illustrated in Figure 1B. Briefly, mRNA emanating from the upstream *mdm2* promoter described previously (Fakharzadeh et al. 1991), should protect exons 1–3 and yield a fragment of ~262 nucleotides; mRNA initiated anywhere within intron 1 is expected to protect only exons 2 and 3 and yield a fragment of 149 nucleotides; mRNA starting within intron 2 should protect only exon 3 sequences and give rise to a fragment of 66 nucleotides; initiation within exon 2 should result in a protected fragment whose length will be anywhere between 66 and 149 nucleotides.

The actual protection pattern obtained with RNA from the rp-2.9md–CAT#1 cell line is presented in Figure 1C. Two major protected fragments are detected. The larger is ~260 nucleotides long, corresponding in size to a transcript containing exons 1, 2, and 3, probably emanating from the upstream (P_1) promoter (Fig. 1B). The rather low intensity of the band suggests that the 2.9-kb *mdm2* genomic DNA fragment contains elements sufficient for driving basal transcription from P_1. In addition, the failure to induce this band following shift of the cells to 32°C suggests that the transcriptional activity of P_1 is not significantly regulated by p53.

The smaller protected fragment, whose levels are dramatically induced by wild-type p53 at 32°C, is ~147 nucleotides long. This size could be attributable to protection of exon 3 plus almost the entire exon 2. In that case, the corresponding transcript might initiate within exon 2, very close to its 5′ end. The detection of such a transcript would confirm the existence of the putative internal p53-dependent promoter (P_2) and place its position in very close proximity upstream to exon 2.

To determine unequivocally the initiation site (I_2) of the P_2-derived transcripts, RNase protection analysis

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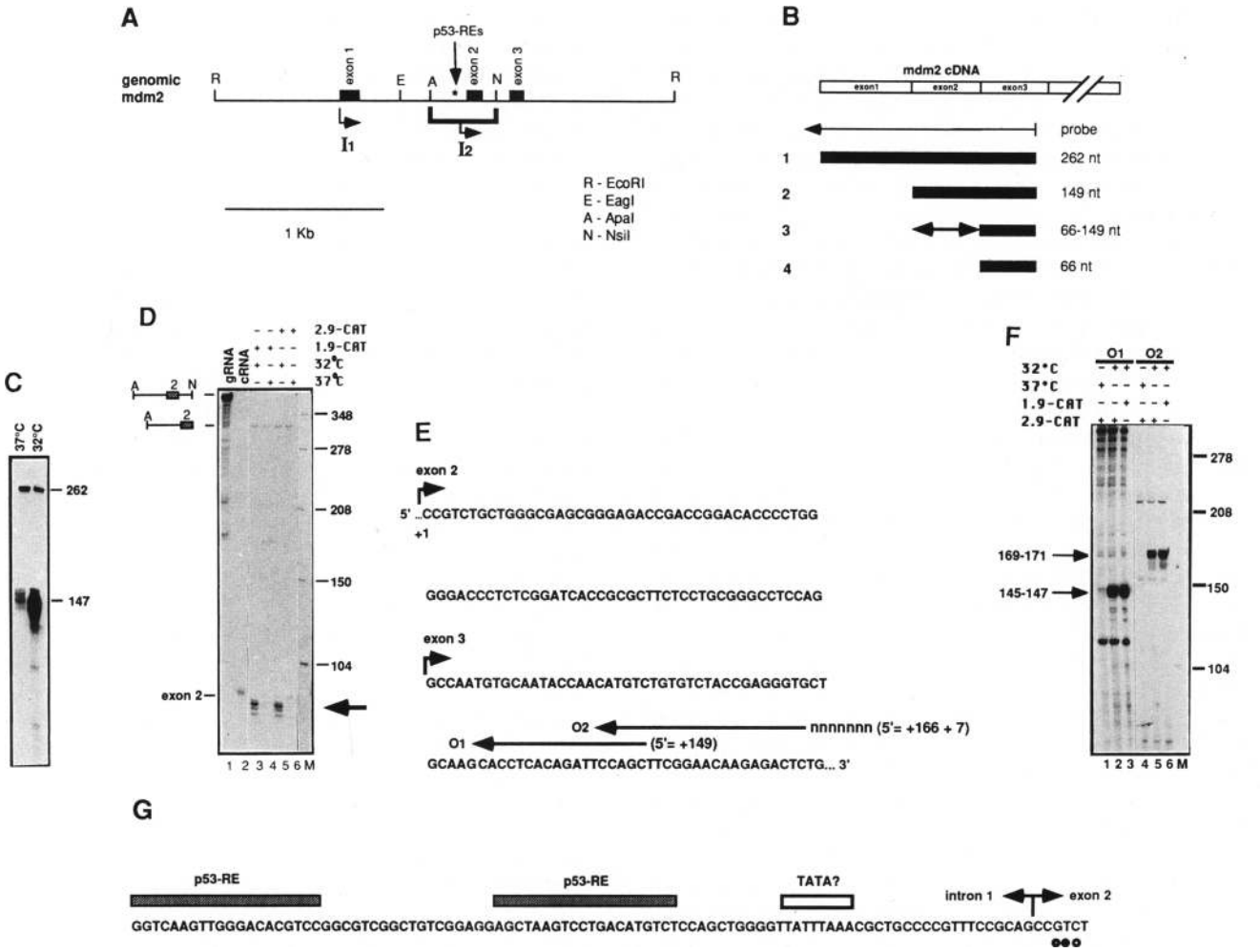

Figure 1. Mapping the internal transcription initiation site (I_2) of *mdm2*. (*A*) Physical map of the upstream region (2.9-kb *Eco*RI fragment) of the murine *mdm2* gene. Locations of exons, major restriction sites, and the p53 REs are indicated. The thick bracket indicates the *Apa*I–*Nsi*I fragment containing the internal P_2 promoter (see text). Arrows mark the two alternative transcription start sites, I_1 and I_2 (see text). (*B*) Illustration of the cDNA riboprobe used for the RNase protection analysis shown in *C*. Composition and sizes (in nucleotides) of potential RNase-resistant fragments are indicated (see text for further details). (*C*) RNase protection analysis of RNA from cell line rp-md2.9–CAT#1. RNA was prepared from cells maintained at either 37.5°C or 32°C and was analyzed using the riboprobe illustrated in *B*. Sizes (in nucleotides) of the main protected fragments are indicated. (*D*) RNase protection analysis of RNA from cell lines rp-md2.9–CAT#1 and rp-md1.9–CAT#1, maintained at either 37.5°C or 32°C. The genomic *mdm2* ~400-bp *Nsi*I–*Apa*I fragment indicated in *A* served as a template for the synthesis of the antisense riboprobe used in this assay. Control reactions included hybridization to sense RNA generated by in vitro transcription of an *Eag*I–*Eco*RI genomic *mdm2* fragment (gRNA, lane *1*), and of full-length *mdm2* cDNA (cRNA, lane *2*). The arrow marks the position of the cluster of major protected fragments (lanes *3*,*5*). Possible structures of additional detectable fragments are depicted at *left*. Sizes of DNA markers (M), in nucleotides, are indicated at *right*. (*E*) Locations of the oligonucleotides employed in the primer extension analysis shown in *F*. (See Materials and methods for precise sequences of primers.) (*F*) Primer extension analysis of RNA from cell lines rp-md2.9–CAT#1 and rp-md1.9–CAT#1, maintained at either 37.5°C or 32°C. Arrows indicate the positions and sizes (in nucleotides) of the major extension products obtained with each primer. Sizes of DNA markers (M), in nucleotides, are indicated at *right*. (*G*) Schematic illustration of the sequence elements preceding the I_2 site in the *mdm2* gene. Indicated are the two p53 REs, a putative TATA box, intron 1–exon 2 junction, and the deduced positions of I_2 (marked by small circles below the sequence). (●) The strongest initiation site; (○) additional, more minor initiation sites.

was next carried out with a radiolabeled antisense riboprobe derived from the *Nsi*I–*Apa*I fragment of the *mdm2* gene (Fig. 1A). When this probe was reacted with RNA from rp-2.9md–CAT#1 cells, a cluster of protected fragments, ~80 nucleotides long, was obtained (Fig. 1D, bold arrow, lane 5). These fragments are slightly shorter than the one resulting from the protection of the entire exon 2 by a synthetic cRNA (Fig. 1D, lane 2). These data corroborated the conclusion that I_2 is located within exon 2, very close to its 5′ end. A similar analysis was performed

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with RNA from cell line rp-1.9md–CAT#1, harboring a 1.9-kb genomic *mdm2* DNA fragment (*Eag*I–*Eco*RI; Fig. 1A). This 1.9-kb fragment begins within intron 1 and, hence, lacks P_1 and I_1; consequently, it is expected to give rise only to transcripts driven by P_2 and initiating at I_2. The results obtained with this cell line (Fig. 1D, lane 3) were essentially identical to those seen with the line containing the intact 2.9-kb region, further confirming that the protected ~80-nucleotide bands represent authentic P_2-derived transcripts. As expected, the abundance of these transcripts was highly temperature dependent; far less RNA was produced in cells maintained at 37.5°C, where p53val135 possesses very little wild-type p53 activity (lanes 4,6).

To establish definitively the position of I_2, primer extension analysis was performed. Two staggered primers, complementary to sequences within the third exon of the *mdm2* gene, were used. The 5′ ends of these primers correspond to positions +149 and +173, relative to the first nucleotide of exon 2 (O1 and O2, respectively, Fig. 1E). As a template for the reverse transcription reaction, we used RNA extracted from the rp-2.9md–CAT#1 and rp-1.9md–CAT#1 lines maintained at either 37.5°C or 32°C. The abundance of the major extension products of both primers (marked by arrows in Fig. 1F) exhibits a clear dependence on the growth temperature of the cells. The sizes of the extended products imply that I_2 is located at about the third nucleotide of exon 2 (Fig. 1G). Control experiments, using a synthetic full-length *mdm2* mRNA as a template for primer extension, ruled out the possibility that the pattern in Figure 1F is attributable to premature termination by reverse transcriptase (data not shown), thereby confirming the deduced position of I_2. It is noteworthy that an AT-rich sequence, which may function as a TATA box, is located ~20 bp upstream of I_2 (Fig. 1G).

In summary, we conclude that the first intron of the mouse *mdm2* gene contains an authentic p53-responsive promoter and that the two adjacent p53-binding sites constitute bona fide promoter–proximal elements. These elements can mediate the induction of a distinct transcript of murine *mdm2*, starting at the third nucleotide of exon 2.

Activated wild-type p53 triggers P_2 within the endogenous mdm2 *gene*

The experiments presented in Figure 1 were performed with chimeric constructs, containing defined segments of the mouse *mdm2* gene. To validate the relevance of these findings, the analysis was extended to the nonmanipulated, endogenous mouse *mdm2* gene in LTR6 cells. The LTR6 cell line was established by transfection of p53-deficient M1 myeloid leukemia cells with the temperature-sensitive p53val135 mutant (Yonish-Rouach et al. 1991). LTR6 cells undergo apoptosis in response to the induction of wild-type p53 activity following a shift to 32°C (Yonish-Rouach et al. 1991, 1993). RNA prepared from LTR6 cells before and after a temperature shift to 32°C was used in an RNase protection assay, in which the radiolabeled riboprobe was synthesized off of *mdm2* cDNA (identical to the probe in Fig. 1B,C). Figure 2A illustrates the fragments expected to arise through protection of the probe by transcripts initiating from either P_1 (262 and 196 nucleotides) or P_2 (147 and 81 nucleotides). The predictions take into account the existence of alternative *mdm2* mRNA splicing variants devoid of exon 3, detected by virtue of their cloning from cDNA libraries (Fakharzadeh et al. 1991; Haines et al. 1994; Y. Barak and M. Oren, unpubl.). These variants are not expected to be observed in the analysis of the *mdm2*–CAT chimeras (Fig. 1), which lack the splice acceptor site at the 3′ end of intron 3.

The results of the RNase protection analysis (Fig. 2B) reveal that in the absence of functional wild-type p53 (parental M1 cells and LTR6 cells maintained at 37.5°C; lanes 3 and 4, respectively), only transcripts initiating at P_1 (denoted F, for the sake of brevity) were detectable. On the other hand, activation of wild-type p53 at 32°C caused a marked induction of P_2-derived transcripts (denoted X2) while not affecting the amount of P_1-derived *mdm2* mRNA (lane 5). This pattern was not affected by exposure of the cells to IL-6, which slows down the p53-induced apoptotic process (Yonish-Rouach et al. 1991). Hence, induction of wild-type p53 activity at 32°C activates transcription from P_2 also in the context of the normal *mdm2* gene. This proves further that P_2 is a physiological p53-responsive promoter.

A similar assay was also performed with RNA from 3T3DM cells, which harbor an amplified *mdm2* gene, and consequently overexpress MDM2 proteins (Fakharzadeh et al. 1991). As seen in lane 2, these cells were found to contain large amounts of the P_1-derived, full-length transcript but no detectable P_2-derived X2 transcripts. This is consistent with the notion that X2 is p53-dependent and that *mdm2* amplification in 3T3DM cells leads to a complete inactivation of p53. It is noteworthy that in all RNA preparations, a relatively constant proportion of transcripts was devoid of exon 3, regardless of the initiation site.

Agents that induce DNA damage, such as ionizing radiation, elicit stabilization and activation of the wild-type p53 protein (Maltzman and Czyzyk 1984; Kastan et al. 1991, 1992; Kuerbitz et al. 1992; Fritsche et al. 1993; Lu and Lane 1993). As a result, the transcriptional activity of p53 is markedly elevated, inducing the expression of target genes such as *gadd45* (Kastan et al. 1992; Zhan et al. 1993), *WAF1* (E. Gottlieb and M. Oren, unpubl.), and *mdm2* (Perry et al. 1993; Chen et al. 1994). To find out whether P_2 activity is induced in a p53-dependent manner also in response to DNA damage, RNase protection analysis was performed with RNA from derivatives of DA-1 cells. DA-1 is an interleukin-3 (IL3)-dependent cell line that expresses wild-type p53 (Gottlieb et al. 1994). We have described previously the inactivation of the endogenous wild-type p53 in DA-1 cells, by means of infection with a retroviral vector encoding a negative-dominant carboxy-terminal fragment of p53. The resultant cell population, termed DIDD, fails to undergo efficient apoptosis in response to either ionizing radiation or

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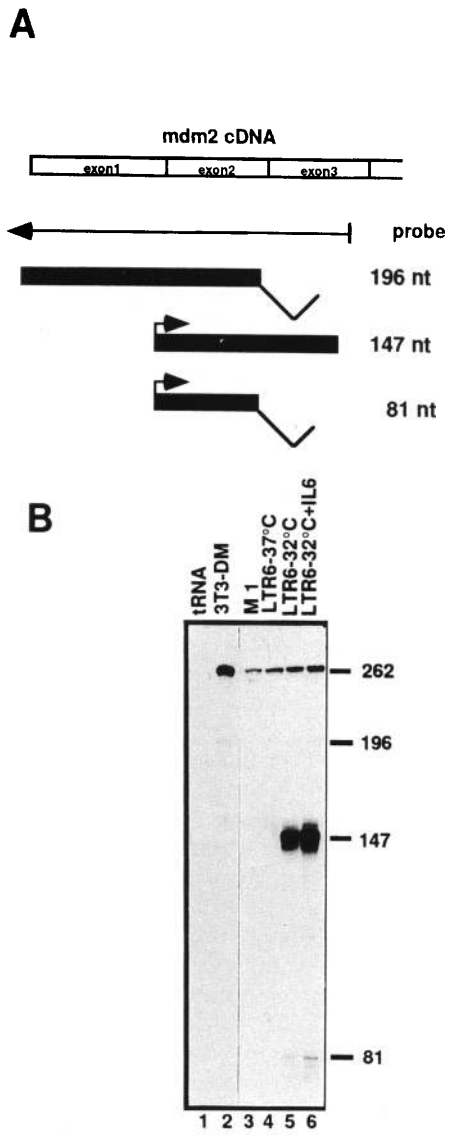

Figure 2. Selective activation of the internal *mdm2* promoter (P_2) by a temperature down-shift of cells expressing p53val135. (*A*) Schematic representation of the cDNA probe used for RNase protection analysis. Composition and sizes (in nucleotides) of potential RNase-resistant fragments are indicated (see text for further details). These include a 262-nucleotide fragment representing hybrids containing full-length, P_1-derived *mdm2* RNA, a 147-nucleotide fragment arising from hybrids containing the p53-inducble, P_2-derived transcript, and fragments of 196 and 83 nucleotides, representing splice variants of both types of *mdm2* transcripts. (*B*) RNase protection analysis. Total cellular RNA was prepared from the cell lines indicated (*top*) which were maintained continuously at 37.5°C except for LTR6 which was shifted to 32°C for 8 hr (lane *5*) or 13 hr in the presence of IL-6 (lane *6*). Each lane represents 40 μg of RNA. The control reaction in lane *1* contained 15 μg of tRNA. Deduced fragment sizes are indicated. Lanes *3–6* represent a longer autoradiographic exposure than lanes *1–2*.

IL-3 withdrawal (Gottlieb et al. 1994). On the other hand, DA1 cells infected with a retrovirus encoding only the neomycin-resistance (*neo*) (DIN) continue to express functional wild-type p53 and retain a normal response to DNA damage and factor withdrawal.

RNA extracted from DIN and DIDD cells 3 hr after exposure to γ-irradiation or sham treatment was analyzed using the *mdm2* cDNA-derived riboprobe described in Figures 1B and 2A. As seen in Figure 3, irradiation of DIN cells resulted in a clear induction of the X2 transcript, giving rise to a series of protected bands of ~147 nucleotides (cf. lanes 2 and 3). In contrast, no induction of X2 was observed in irradiated DIDD cells (cf. lanes 4 and 5), confirming that the activity of P_2 is strictly dependent on the presence of functional wild-type p53. These data establish that P_2 of *mdm2* is a DNA damage-inducible, p53-dependent promoter.

In addition to the activation of the P_2 promoter, irradiated DIN cells also displayed a moderate increase in the amount of P_1-derived F transcripts (cf. lanes 2 and 3). This effect, too, appears to require functional wild-type p53; no parallel induction was seen in DIDD cells (lanes 4,5). The response of P_1 in DA1 cells thus differs from that seen in LTR6 and rp-2.9md–CAT#1 cells, where P_1 appears to be totally unaffected by the activation of wild-type p53 (Fig. 1C and 2B). This could be attributable ei-

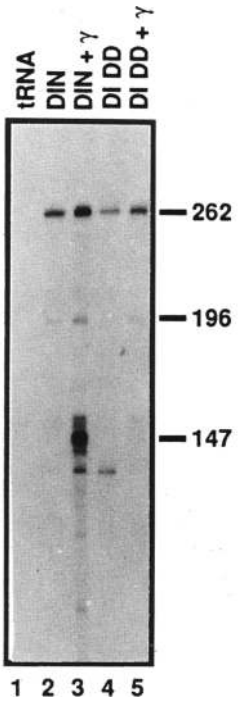

Figure 3. p53-dependent activation of P_2 in cells exposed to ionizing radiation. Total cellular RNA was prepared from DIN and DIDD cells 3 hr after exposure to either 500 rads of γ radiation (+γ) or sham irradiation. RNA (20 μg) was analyzed by RNase protection using the same probe as in Fig. 2A. The control reaction contained 20 μg of tRNA (lane *1*). Deduced fragment sizes are indicated.

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ther to cell type-specific differences or to the different manner by which p53 activation was achieved in each case.

The F and X2 transcripts of mdm2 *display nonidentical in vitro translation profiles*

The first in-frame AUG codon of murine *mdm2* mRNA resides within exon 3 and is therefore present both in the full-length F transcript and in the p53-dependent X2 transcript (Fig. 4A). Consequently, both transcripts formally have an identical coding potential. It is noteworthy, however, that this first AUG resides within the rather unfavorable sequence context CCAAUGU (Kozak 1986). This could potentially lead to inefficient translation initiation at this site and allow at least some of the translated polypeptides to initiate at downstream codons. Analysis of cellular MDM2 proteins has revealed

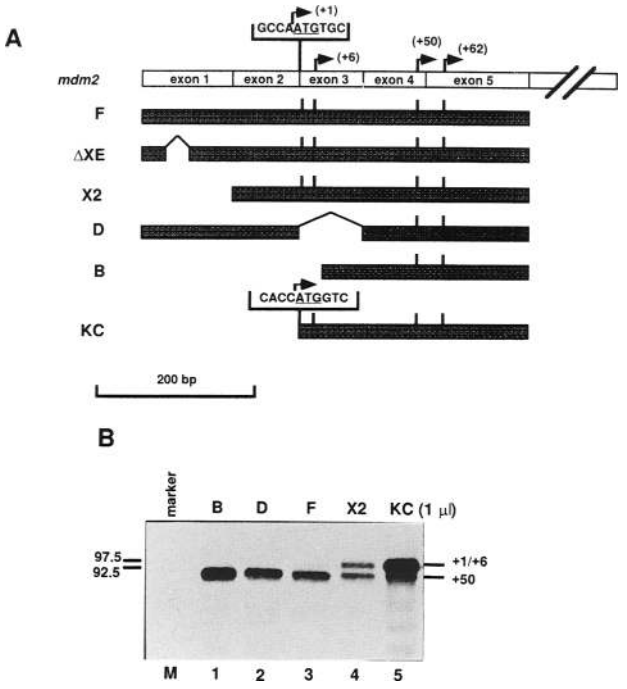

Figure 4. In vitro translation profiles of various *mdm2* transcripts. (*A*) Schematic representation of the 5′ regions of the murine *mdm2* transcripts employed for in vitro translation. Each of the indicated synthetic transcripts was generated through in vitro transcription of a corresponding *mdm2* cDNA-derived plasmid (see text for details). Vertical bars mark the positions of in-frame AUG codons. The DNA sequence of the region specifying the first in-frame AUG, as well as its modified version present in the KC site-directed mutant, is indicated. The 3′ part of the RNA, extending downstream from exon 5, is essentially identical in all transcripts. (*B*) The synthetic transcripts illustrated in *A* were translated in a rabbit reticulocyte lysate, using standard RNA inputs (5 pmoles per reaction). Unless specified otherwise, each lane corresponds to 2 μl of the translation reaction. The deduced amino acid positions of the translation initiation sites are indicated for each polypeptide, along with the sizes of relevant molecular weight markers.

that the first in-frame AUG does serve as an initiation site in vivo. This could be confirmed by direct epitope mapping (Olson et al. 1993), as well as by the ability of the corresponding polypeptide to associate with p53, a property that requires the integrity of the amino-terminal portion of MDM2 (Chen et al. 1993; Oliner et al. 1993). Yet previous studies have also indicated the existence of shorter MDM2 proteins (Barak et al. 1993; Olson et al. 1993), at least some of which could potentially be attributable to translational initiation at downstream AUG codons. It was therefore interesting to compare the translation profiles of the F and X2 transcripts of *mdm2*.

To address this issue, we constructed a series of in vitro transcription vectors, capable of directing the synthesis of different RNA variants of mouse *mdm2*; the structure of the various transcripts and the positions of the first four in-frame AUG codons are illustrated schematically in Figure 4A. In addition to F and X2, these synthetic transcripts included an alternatively spliced variant devoid of of the third exon and thus lacking the first two in-frame AUG codons (D), a 5′ truncated transcript initiating downstream to the second AUG codon (B), and a variant lacking exons 1 and 2, which had been mutated so that its first in-frame AUG now resides within an optimal consensus translation initiation context (KC). A stretch of 15 nucleotides, contributed by the vector, is present at the 5′ end of all these synthetic *mdm2* RNA molecules.

The various synthetic *mdm2* transcripts were next subjected to standard in vitro translation reactions in a rabbit reticulocyte lysate. Two major classes of translation products could be detected in these reactions (Fig. 4B). The B and D transcripts, both of which lack the first two in-frame AUG codons (Fig. 4A), yielded a faster migrating MDM2 protein (Fig. 4B, lanes 1,2). It can therefore be deduced that this form represents translational initiation at an internal AUG codon, presumably the one at position +50 (Fig. 4A). On the other hand, the KC transcript was translated primarily into a slower migrating form of MDM2 (Fig. 4B, lane 5); in addition, it was translated more efficiently than all other species. Both these features are consistent with the fact that KC has been designed to optimize initiation at the first in-frame AUG. On the basis of the patterns obtained with these control *mdm2* transcripts, the slower and faster migrating translation products of *mdm2* can be attributed to initiation at positions +1 (or +6) and +50, respectively.

When the F and X2 transcripts were translated under the same reaction conditions (~5 pmoles of RNA per reaction), a striking difference between the translation products of each was observed (Fig. 4B, cf. lanes 3 and 4). Each of the RNA species gave rise to the same two classes of MDM2 polypeptides. However, although F was translated predominantly into the shorter MDM2 polypeptides, the absence of exon 1 in the X2 transcript greatly facilitated initiation at the first in-frame AUG. Consequently, X2 was translated into approximately equal amounts of the shorter and the longer MDM2 polypeptides. Thus, the p53-dependent (P_2-derived) X2 transcripts of *mdm2* exhibit an altered translation potential,

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despite retaining the same coding region in common with the constitutive (P_1-derived) F transcripts.

In vivo expression patterns of MDM2 protein variants

It was of interest to determine whether the potential for alternative use of translation initiation sites is reflected in the pattern of MDM2 proteins expressed in intact murine cells. To that end, unlabeled extracts from several cell types were immunoprecipitated, resolved by SDS-PAGE, blotted, and probed with a polyclonal serum specific for MDM2. In the cell types examined, the results revealed the presence of a longer MDM2 polypeptide as well as of a faster migrating band (Fig. 5, lanes 1–4,6). The latter band could be resolved further into two separate polypeptides in some of the immunoprecipitated extracts (lanes 1,2). The main MDM2 bands observed in intact cells exhibited electrophoretic mobilities indistinguishable from those of the alternative in vitro translation initiation products (lane 8). This suggests that these polypeptides are generated through the usage of alternative in-frame initiator AUGs, similar to those utilized by the reticulocyte lysate.

As expected from the RNA data shown in Figure 2, much more total MDM2 protein was made in LTR6 at 32°C, in the absence or presence of IL6, than in the same cells or the parental M1 cells at 37.5°C (cf. lanes 3 and 4 with lanes 1 and 2). On the other hand, at least under the conditions used in this experiment, no marked differences were observed with regard to the ratio between the longer and shorter polypeptides in the various cells. Thus, although LTR6 cells contain primarily F transcripts at 37.5°C and X2 transcripts at 32°C (Fig. 2, lanes 4,5), the patterns of MDM2 polypeptides were qualitatively quite similar at both temperatures (Fig. 5, lanes 2,3); at best, there was only a mild increase in the relative proportion of the larger MDM2 polypeptide (initiated at +1) at 32°C. A similar distribution of MDM2 polypeptides was also seen in 3T3DM cells (lane 6), which produce large amounts of the F transcript and practically lack X2 RNA (see Fig. 2B, lane 2). The slower migrating band (~105 kD) observed above the larger MDM2 polypeptide represents nonspecific cross-reactivity and is also brought down by irrelevant control antibodies (data not shown).

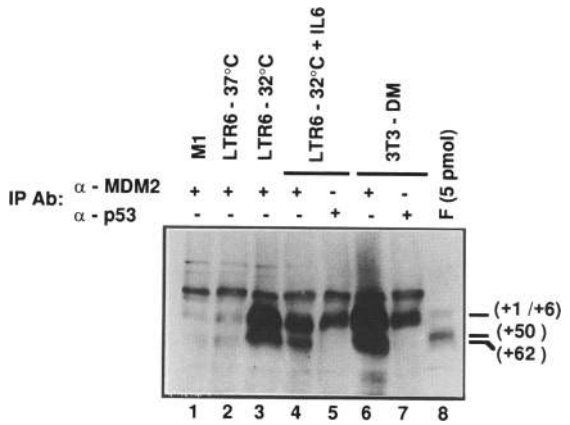

Figure 5. Analysis of MDM2 polypeptides from intact cells. Extracts from cell lines maintained under the conditions indicated at the *top* were immunoprecipitated using either anti-MDM2 polyclonal serum (lanes *1–4,6*) or the anti-p53 monoclonal antibody pAb421 (lanes *5,7*). Immunoprecipitated material, as well as in vitro translation products of the synthetic F transcript (see Fig. 4B, lane *3*), were subjected to Western blotting and probed with anti-MDM2 polyclonal serum. The deduced amino acid positions of the translation initiation sites are indicated for each polypeptide. The 105-kD band observed above the MDM2 polypeptides represents nonspecific cross-reactivity, precipitated also by control antibodies (data not shown).

In addition, we performed a functional analysis of the various MDM2 polypeptides by testing their ability to coprecipitate with p53, employing a monoclonal antibody against the latter in the immunoprecipitation step of the analysis. Based on the localization of the p53-binding site to the most amino-terminal portion of MDM2 (Chen et al. 1993; Oliner et al. 1993), an internally initiated MDM2 protein beginning at the fiftieth in-frame codon is expected not to bind p53. The results (Fig. 5, lanes 5,7) demonstrate clearly that this is the case; only the longer MDM2 polypeptide could be coprecipitated with p53, suggesting that the shorter polypeptide is missing its amino terminus, most likely as a result of internal translation initiation.

In conclusion, the results presented in Figure 5 argue strongly that the various forms of *mdm2* mRNA are subject to alternative translation initiation also in intact cells. However, under these conditions, the first in-frame AUG appears to be employed preferentially also for the translation of the F transcript.

The in vitro translation patterns of mdm2 *depend on the amount of input RNA*

A possible explanation for the quantitative variance between in vitro and in vivo translation patterns of the F and X2 forms of *mdm2* RNA could be a different ratio between the input RNA and limiting components of the translation machinery. Standard in vitro translation reactions employ a large excess of specific RNA relative to the situation in vivo. It is conceivable that the translatability of the various *mdm2* transcripts could be affected by the ratio between the amount of mRNA and that of available translation factors. To address this possibility, in vitro translation reactions were programmed with lower mRNA inputs: 1 or 0.1 pmole per assay. The use of 1 pmole of RNA resulted in a significant improvement of translational initiation at the first AUG of both the F and the X2 transcripts (Fig. 6A, lanes 1,2). Nevertheless, the ratio between full-length MDM2 proteins (initiated at codon +1) and polypeptides initiated at internal AUGs remained significantly higher with X2 than with F. The ΔXE transcript, which differs from F only by the presence of a small internal deletion within the first exon (Fig. 4A), yielded a translation pattern indistinguishable from that of the F RNA (Fig. 6A, lane 3), lending further support for the reproducibility of these results. The products of the D and KC transcripts were both as expected (Fig.

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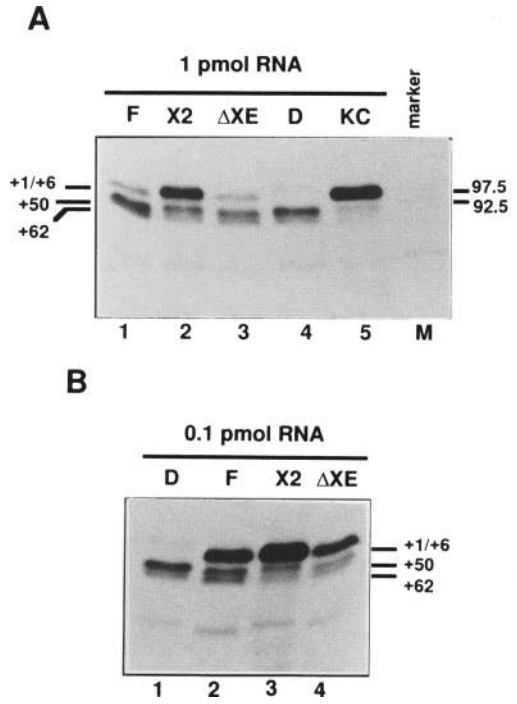

Figure 6. In vitro translation profiles of the F and X2 transcripts of *mdm2* depend on the amount of input RNA. The synthetic transcripts illustrated in Fig. 4A were translated in a rabbit reticulocyte lysate, using the amounts of input RNA indicated (*top*). Each lane corresponds to 2 μl of the translation reaction in *A*, or 5 μl in *B*. (See Fig. 4 for further details).

6A, lanes 4,5), except that KC now yielded almost exclusively the full-length MDM2 protein. A further 10-fold dilution of the RNA in the translation mixture led to an even more efficient initiation at the first AUG of both F and X2, making the difference between the translation profiles of both less pronounced (Fig. 6B). Yet, even under those very nonstandard conditions, more of the full-length protein was produced when the reaction was programmed with X2, relative to F (Fig. 6B, cf. the upper band in lanes 2 and 3). It is noteworthy that in Figure 6 one can also observe a smaller polypeptide that most probably represents initiation at the fourth in-frame AUG (position +62).

These data suggest further that the F and X2 types of *mdm2* transcripts bear a potential for alternative translational initiation, at least when programming a reticulocyte lysate. The manifestation of this potential becomes more pronounced under conditions of RNA excess, suggesting that a titratable factor may be required for efficient initiation at the first AUG. The data presented above suggest that the presence of exon 1 at its 5′ end may render the P_1-derived, F form of *mdm2* mRNA more dependent on the availability of this putative factor.

Discussion

The present study demonstrates that transcription of the mouse *mdm2* gene can emanate from two distinct promoters. This feature confers upon *mdm2* the ability to be strongly inducible by p53, as well as to be expressed at significant levels in the absence of p53.

Activation of murine *mdm2* transcription by p53 is achieved through an internal promoter, whose activity is strictly dependent on wild-type p53. This promoter, termed here P_2, is practically silent in cells that do not express wild-type p53 or express only basal levels of the protein (e.g., unirradiated DIN cells; Fig. 3). However, when the cell is confronted with high levels of wild-type p53, P_2 becomes strongly activated. This activation not only occurs in response to the forced overexpression of transfected p53 but also when the endogenous p53 is induced by DNA damage. Hence, P_2 represents a specific natural target for *trans*-activation by wild-type p53.

In contrast, the constitutive *mdm2* promoter P_1 does not require wild-type p53 to become functional. Consequently, p53-deficient cells express low but clearly detectable levels of *mdm2* mRNA and of the corresponding protein products, the production of which is under the control of P_1. The effect of wild-type p53 overexpression on P_1 varies with the particular cell system. The REF-derived cell line rp-2.9md–CAT#1, as well as the myeloid LTR6 cells, harbor the transfected temperature-sensitive p53 mutant p53val135. In both cases, activation of p53 at the permissive temperature does not exert any measurable effect on transcription from P_1. On the other hand, potentiation of endogenous wild-type p53 by ionizing radiation in DIN cells leads, in parallel with the dramatic induction of P_2, also to a moderate increase in transcription from P_1 (see Fig. 3). Hence, apart from being integral components of the p53-dependent P_2 promoter, the p53 REs located in front of exon 2 also sometimes do function as an intronic enhancer, acting to augment transcription from P_1.

The structural features of P_1, as well as its mode of regulation, still remain to be determined. It is conceivable that P_1 may contain response elements for other signals, unrelated to p53, which may also contribute to modulation of *mdm2* gene expression under various conditions.

An additional p53 target, the *gadd45* gene, also possesses an intronic p53-binding site (Kastan et al. 1992). It would be of interest to determine whether *gadd45*, too, contains an internal p53-activatable promoter or whether its p53 RE serves only as a bona fide downstream enhancer.

The first two exons of murine *mdm2* do not include protein-coding sequences, and the transcript directed by P_2 differs from the constitutive, P_1-directed transcript by the mere absence of 5′ noncoding sequences. Hence, both types of transcripts are expected to generate the same translation products. It therefore appeared intriguing that p53 needs to selectively induce transcription from P_2, rather than merely serve as an enhancer toward the potentiation of P_1. A partial explanation is provided by the in vitro translation experiments (Figs. 4 and 6), which show clearly that the presence of distinct 5′ ends can dictate the differential usage of in-frame translation

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initiation codons. This possibility becomes even more appealing in view of the fact that the products of this alternative translation initiation differ from each other with regard to their ability to bind p53. Thus, initiation at the first (+1) or second (+6) AUG will yield p53-binding polypeptides, whereas initiation at more downstream AUGs should give rise to forms of MDM2 that cannot associate with p53.

In the F transcript, whose synthesis is, by and large, unaffected by p53, initiation at the first AUG codon appears to be particularly context dependent. Under conditions of excess RNA, its translation in vitro yields mainly amino-terminally truncated MDM2 variants, which cannot bind to p53. However, when diluted, this transcript regains the capacity to encode a full-length, p53-binding MDM2 protein. This phenomenon may imply that a titratable factor is required for efficient translation initiation at the upstream AUG of *mdm2* mRNA. Although this requirement appears to be very strict for RNA directed by P_1, it may be more relaxed for the p53-induced X2 transcript. As a result, translation of X2 gives rise to a higher ratio of full-length to amino-terminally truncated MDM2 polypeptides than translation of F, as well as to significantly greater absolute amounts of the full-length forms (see Fig. 4).

The MDM2 protein contains several canonical sequence motifs, such as metal-binding fingers and acidic *trans*-activation-like domains (Fakharzadeh et al. 1991), which are distinct from its p53-binding region (Chen et al. 1993; Oliner et al. 1993). It is conceivable that MDM2 products may fulfill additional biochemical functions, distinct from their role in the p53 autoregulatory feedback loop (Barak et al. 1993; Wu et al. 1993). One may therefore envisage situations where P_1 is activated by signals that bear no direct relevance to p53. If this always resulted in accumulation of p53 binding, full-length MDM2 polypeptides, p53 function could have been compromised even under conditions when this may be disadvantageous for the cell. This problem can potentially be circumvented through the mechanism suggested by our data, namely the usage of internal translation initiation sites to generate amino-terminally truncated MDM2 polypeptides. Such polypeptides may not bind p53 but will retain all other structural domains required for the additional putative functions of the protein.

There is also another mechanism for the production of similar amino-terminally truncated MDM2 proteins. Analysis of mouse *mdm2* cDNA clones from various sources provides compelling evidence for the existence of an alternatively spliced variant, devoid of exon 3 (Fakharzadeh et al. 1991; Y. Barak and M. Oren, unpubl.; see also the cDNA variant designated D in Fig. 4A). A limited amount of such spliced transcripts is also evident in the data presented in Figures 2 and 3 (fragments of 196 nucleotides and 81–83 nucleotides). This transcript can encode only an amino-terminally truncated MDM2 protein, lacking half of its p53-binding domain (Fig. 4B, lane 2), and incapable of binding p53 (Haines et al. 1994; see also Fig. 5). Thus, increased levels of p53 nonbinding MDM2 proteins can potentially be achieved either by providing conditions that favor translational initiation at internal AUG codons or by increasing the proportion of transcripts from which exon 3 is spliced out.

In parallel, our findings also imply a mechanism for enhanced production of full-length, p53-binding forms of MDM2 proteins. Such outcome can be achieved through the preferential activation of P_2, as is the case for cells exposed to the presence of activated wild-type p53. As suggested by the data in Figures 4 and 6, the resultant X2 transcript is translated into full-length MDM2 more efficiently than the constitutive F transcript. There may be conditions where the putative titratable factor necessary for efficient initiation at the first in-frame AUG becomes limiting. Under such conditions, an induced increase in *mdm2* mRNA through P_1 might have resulted in a large number of p53 nonbinding MDM2 polypeptides, incapable of participating in the p53 feedback loop (Barak et al. 1993; Wu et al. 1993). Activation through P_2 will give rise to a form of *mdm2* mRNA that can be translated efficiently into full-length protein even under such less favorable conditions. Hence, P_2 may serve to maintain an efficient p53 feedback loop under a wide spectrum of cellular contexts.

In conclusion, the present study reveals a novel facet of the complex and very dynamic interplay between p53 and *mdm2*. The precise functions of each form of MDM2 protein, as well as the mechanisms that dictate the ratio between initiation at the first AUG and at the more downstream AUGs, still remain to be investigated in more detail.

Materials and methods

Cell lines

Lines rp-md2.9–CAT#1 and rp-md1.9–CAT#1 are derived from primary REFs, transformed by cotransfection of activated Ha-*ras*, the temperature-sensitive p53 mutant p53val135, and a plasmid containing either a 2.9- or a 1.9-kb genomic *mdm2* DNA fragment, respectively, upstream of a promoterless CAT gene (Juven et al. 1993). The 2.9-kb *mdm2* fragment extends from ~0.8-kb upstream of the reported first nucleotide of *mdm2* mRNA to the middle of the third intron (see Fig. 1A). The 1.9-kb fragment was generated from the 2.9-kb fragment by deletion of the 5′ ~1.0 kb (from *Eco*RI to *Eag*I; Juven et al. 1993). 3T3DM is a transformed derivative of NIH-3T3 cells carrying multiple copies of an amplified *mdm2* gene (Cahilly-Snyder et al. 1987). All cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% or 10% FCS.

M1 is a p53-deficient murine myeloid leukemic cell line (Yonish-Rouach et al. 1991). LTR6 cells were derived from M1 by stable transfection of p53val135 (Yonish-Rouach et al. 1991). These cells were maintained at 37.5°C in RPMI medium supplemented with 10% FCS. Where appropriate, cells were shifted to 32°C for 12–13 hr in the presence of 8 ng/ml of IL-6, or for 8–9 hr in the absence of IL-6.

DIN and DIDD are cell pools obtained by retroviral infection of the DA-1 lymphoid line (Gottlieb et al. 1994). DIN cells harbor an integrated provirus expressing *neo*. The retrovirus used for generating DIDD expresses, in addition to the *neo* gene, the carboxy-terminal oligomerization domain of p53; this domain was shown previously to inhibit p53 activity in a dominant-

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negative fashion (Shaulian et al. 1992). Cells were maintained in DMEM + 10% FCS in the presence of IL-3. Where indicated, cells were exposed to 500 rads of radiation from an isotopic cobalt source (+γ).

Ribonuclease protection assays

Assays were performed essentially as described (Melton et al. 1984). Cellular RNA was prepared by the RNAzol method. In vitro transcription plasmids were based on the commercial vector pSL301 (Invitrogen). Radiolabeled antisense riboprobes were generated by transcription with T7 RNA polymerase. DNA fragments used as templates for transcription include a PCR-derived murine *mdm2* cDNA fragment extending from position +264 to position +3 of the published sequence (Fakharzadeh et al. 1991) and an *Apa*I–*Nsi*I murine *mdm2* genomic DNA fragment. Synthetic RNA used as control for hybridization with the probes was derived by transcribing either the full-length *mdm2* cDNA (cRNA) or an ~900-bp *Eag*I–*Xho*I *mdm2* genomic DNA fragment (gRNA). Hybridization was typically carried out overnight at 45–50°C, using 10–40 μg of total cellular RNA and 2×10^5 cpm of the appropriate riboprobe. RNase digestion was performed by incubation with a mixture of RNase A and RNase T1 (Sigma) for 30–60 min at 25–32°C. Nuclease-resistant fragments were resolved on 6% polyacrylamide–urea gels.

Primer extension

Total cellular RNA (20 μg) was hybridized overnight, at room temperature, to end-labeled oligonucleotides (1×10^6 cpm/reaction) in 3 M NaCl. Extensions were carried out for 90 min at 42°C, followed by 30 min at 52°C, using AMV reverse transcriptase and nonradioactive deoxynucleotides. Extension products were resolved on 6% polyacrylamide–urea gels. The sequences of the oligonucleotide primers used are 5′-GCTGGAATCTGTGAGGTG-3′ (O1) and 5′-CTGCCTCGAGTCTCTTGTTCCGAAGCTGG-3′ (O2).

In vitro transcription/translation

The indicated *mdm2* cDNA fragments were constructed into the *Eco*RI site of plasmid pGEM1 (Promega). RNA was transcribed using T7 RNA polymerase, and template DNA was then hydrolyzed using RNase-free DNase (Worthington). Following phenol and chloroform extraction, RNA was differentially precipitated in the presence of ammonium acetate. The RNA concentration in each sample was determined by OD_{260} measurement and normalized to molar concentration according to the calculated size of each transcript. The indicated amounts of RNA were translated in a rabbit reticulocyte lysate (Promega) under the conditions recommended by the manufacturer. Translation products were resolved on 7.5% polyacrylamide–SDS gels.

Antibodies, immunoprecipitation, and Western blotting

Antibodies used include the anti-p53 monoclonal antibody pAb421 (Harlow et al. 1981) and an anti-MDM2 rabbit serum (Barak et al. 1993). Immunoprecipitations were carried out as described previously (Maltzman et al. 1981). Immunoprecipitated proteins were resolved by 7.5% polyacrylamide–SDS gels. Western blots were performed as described (Barak et al. 1993), except that nitrocellulose membranes were used.

Acknowledgments

We thank Dr. D. George for the gift of genomic *mdm2* plasmids and the 3T3DM cell line. This work was supported in part by grant (RO1 CA40099) from the National Cancer Institute, and by grants from the Minerva Foundation (Munich), the National Council for Research and Development, Israel, and the German Cancer Research Center (DKFZ), Heidelberg, the Laub Foundation, and the Leo and Julia Forchheimer Center for Molecular Genetics.

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