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A functional p53-responsive intronic promoter is contained within the human *mdm2* gene

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ABSTRACT

The *mdm2* gene is a target for transcriptional activation by the p53 tumor suppressor gene product. Previous work has revealed that the mouse *mdm2* gene contains two promoters: one is located upstream to the gene and is active in the absence of p53, the other resides within the first intron and requires p53 for transcriptional activity. To determine whether this unique promoter activation pattern is biologically important, we investigated the structure and function of the corresponding region of the human *mdm2* (*hmdm2*) gene. We report here that the *hmdm2* gene also contains an intronic, p53-dependent promoter. The structural features of this promoter are highly conserved between mouse and man, as opposed to the lack of conservation of the first exon. This promoter is triggered *in vivo* in the presence of activated wild type p53, leading to the production of novel mRNA species. The intronic *hmdm2* promoter contains two tandem p53 binding elements. Deletion analysis suggests that optimal promoter activity requires the simultaneous binding of p53 to both elements; this may serve to prevent premature triggering of the promoter by p53.

INTRODUCTION

The p53 tumor suppressor protein is a sequence-specific transcription factor, which can activate genes containing p53-response elements (reviewed in 1–3). Several p53 target genes have already been identified and their relevance to various aspects of p53 function is being extensively investigated.

One of the best studied p53 targets is the *mdm2* gene, first identified as a putative proto-oncogene (4). Transcription of *mdm2* is strongly induced by activated wild type (wt) p53, under a variety of conditions (5–11). On the other hand, *Mdm2* proteins actually interfere with p53's transcriptional activity (12,13). Such inactivation is achieved through the formation of tight complexes between p53 and Mdm2 (6,12,14,15), which directly conceal p53's transactivation domain (16) and, in addition, may compromise its sequence-specific DNA binding (17). The ability of mdm2 proteins to interfere with the activity of p53 may account, at least in part, for the tumorigenic effect of overexpressed *mdm2*, as suggested by the analysis of human tumors (14,18–21) as well as by tissue culture experiments (22). In normal cells, the induction of *mdm2* by p53 has been proposed to maintain a negative feedback loop, whereby the activity of p53 is kept in check and is terminated in a regulated manner (5,6,11,23).

Detailed analysis of the mouse *mdm2* gene revealed a unique mode of activation by p53. In all other p53-responsive genes described to date, transcriptional activation by p53 does not affect promoter usage; irrespective of whether the p53 response elements are found upstream or downstream to the transcriptional start sites, the same promoter is utilized both before and after induction of p53 activity (17,24–27). Consequently, the transcripts induced by activated p53 are identical to those made constitutively, at much lower levels, prior to p53 activation. In contrast, the mouse *mdm2* gene actually contains a distinct p53-dependent promoter (P2), residing within the first intron of the gene (7,8). The normal *mdm2* promoter (P1), located upstream to the regular exon 1, is active at basal constitutive levels even in the absolute absence of p53 (7). The intronic promoter, however, is practically silent under such conditions, and is specifically and strongly turned on only in cells which are induced to express activated wt p53 (7).

The triggering of the intronic promoter by wt p53 leads to the production of a novel class of mouse *mdm2* transcripts. These transcripts lack the first exon of the normal mRNA, resulting in a substantially shorter 5′ untranslated region. The full *in vivo* significance of this promoter switch awaits further elucidation. However, *in vitro* translation experiments strongly suggest that the p53-inducible, shorter transcripts possess an altered translation potential, qualitatively and quantitatively (7). The net outcome is that these p53-inducible transcripts can direct the more efficient synthesis of full length Mdm2 protein. While it remains to be established in which ways the multiple species of Mdm2 polypeptides (28) differ with respect to their function, it is conceivable that a shift in the amount and profile of Mdm2 proteins could modulate the biological responses of the cell following p53 induction.

Interestingly, the intronic murine *mdm2* promoter (5,8) contains two adjacent p53 response elements (p53-REs), each of which shares only a limited degree of homology (17/20 nucleotides) with the p53-binding consensus site defined by El-Deiry and co-workers (29). Lower consensus homology is often reflected in reduced p53 binding (17,30,31). Thus, it is conceivable that the p53 binding affinity of each individual *mdm2* p53-RE alone is not very high.

So far, the unique activation of an alternative promoter by p53 has been described only in the mouse *mdm2* gene. If this feature

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is biologically significant rather than coincidental, one may expect it to be conserved across species. Therefore, the corresponding region of the human *mdm2* (*hmdm2*) gene was isolated and assayed for the presence of a p53-responsive promoter. We report here that, like its murine counterpart, the human *mdm2* gene also contains an intronic p53-inducible promoter. The structural features of the intronic p53-dependent promoter, as well as of the resultant mRNA, are highly conserved between species. This contrasts with the very low degree of conservation of the first exon. The intronic *hmdm2* promoter is specifically triggered in cells subjected to elevated wt p53 activity.

The evolutionary structural and functional conservation of the intronic *mdm2* promoter argues strongly in favor of its biological importance. Significantly, a single p53-RE was not sufficient for efficient activation of the promoter by wt p53; this may serve to ensure the proper timing of *mdm2* induction by p53.

MATERIALS AND METHODS

Cells, plasmids and transfections

H1299 human lung carcinoma cells were grown in DMEM, supplemented with 10% fetal calf serum (FCS). Reporter plasmids pGL2hmdm2-PX-luc and pGL2hmdm-HX-luc were constructed by excising the *Pst*I–*Xho*I and *Hin*dIII–*Xho*I fragments, respectively, from the amplified *hmdm2* DNA (Fig. 4A), and subcloning them into pGL2-Basic (Promega). Plasmids pLTRcGval135 (32), pCMVp53wt, pCMVp53m and pCMVp53dl (35) were described before. Plasmid pGL2mdm2-NA-luc contains the *Apa*I–*Nsi*I fragment of the mouse *mdm2* gene, including the entire p53-dependent promoter (8), in front of the luciferase gene in pGL2-Basic.

Transient transfections by the calcium phosphate method were as described previously (17). Luciferase assays were performed according to standard procedures, with the aid of a Turner Designs luminometer.

Generation of the HL135 cell line

To generate a stable clone of HeLa cells overexpressing a temperature sensitive p53 mutant, cells were co-transfected with 3 μg of pLTRp53cGval135 (32) and 0.3 μg of pSV2neo DNA. Cells were maintained at 37.5°C throughout the transfection and selection steps. Forty eight hours post-transfection, cells were subjected to G418 (500 μg/ml) selection.

Drug resistant clones were picked and analysed for p53 expression at 32 and 37.5°C by indirect immunofluorescent staining using the p53 specific monoclonal antibody PAb248, as previously described (36). HL135 cells were maintained in DMEM, supplemented with 10% FCS.

Amplification and DNA sequence determination

A genomic fragment of ~1050 bp was amplified from human DNA, using the following pair of primers:
O1 5′-CCTGTGTGTCGGAAAGATGG-3′
O3 5′-CTGCCTCGAGTCTCTTGTTCCGAAGCTGG-3′
O1 and O3 correspond to positions 40–59 and 388–369, respectively, of the *hmdm2* cDNA sequence described by Oliner *et al.* (14). O3 also contains an added *Xho*I site near its 5′ end.

Where indicated, use was also made of primer O2 5′-TAGACCTGTGGGCACGGACGCA-3′. O2 is located within the putative

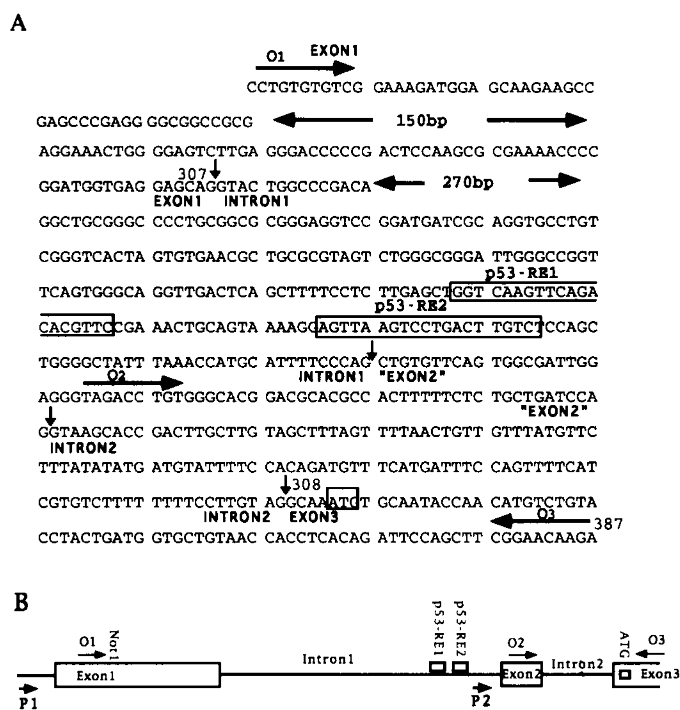

Figure 1. The human *mdm2* genomic DNA region around the potential p53-responsive promoter. (**A**) Partial sequence of the amplified *hmdm2* gene segment. Intron–exon boundaries are indicated by vertical arrowheads. Exon 1 is as defined by Oliner *et al.*, (14). The first coding exon is denoted exon 3, by homology with the corresponding third exon of mouse *mdm2*. In fact, at least in certain types of human cells, it appears to be the second exon of the major *hmdm2* transcript (see below). The position of the segment homologous to mouse *mdm2* exon 2 is indicated as 'exon 2'. The figure also shows the two p53 response elements (p53-REs), as well as the location of the three oligonucleotide primers (O1–O3) employed for PCR reactions and of the first in-frame ATG. Nucleotide positions for the 3′ end of exon 1 and the 5′ end of exon 3 are according to Oliner *et al.* (14). (**B**) Schematic representation of the region detailed in (A). The gene segment employed for comparison between the human and mouse *mdm2* genes in Figure 2 is underlined.

hmdm2 exon 2, whose presence was suggested by homology with exon 2 of mouse *mdm2* (see Fig. 2A).

Direct sequence determination of amplified DNA was done with primers O1, O2 and O3, as well as additional primers corresponding to intron 1 sequences.

Sequence-specific DNA binding assays

DNA binding was analyzed by the McKay assay (37), as described previously (17). End labeled DNA restriction fragments were mixed with an extract of 32°C Clone 6 cells, followed by immunoprecipitation with a mixture of the p53-specific monoclonal antibodies PAb421 and PAb248. The DNA was released from the protein A Sepharose beads and electrophoresed through a 5% denaturing polyacrylamide gel.

Protein analysis

For Western blot analysis, HeLa and HL135 cells were plated at 6×10^5 cells per 6 cm dish. Cells were maintained at either 32 or 37.5°C for 24 h as indicated. Cells were lysed in protein sample

A

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GATCGCAGGTGCCTGTCGGGTC----ACTAGTGTGAACGCTGCGCGTAGT h
GGTGACAGGTGCCTG----GTCCCGGACTCGCCGGGATGCGGC-----TT m

CTGGG-CGGGATTGGGCCGGTTCAGTGGGCAG-GTTGACTCAGCTTTTCC h
CCGGGACGGG--TGGGACTGGTC--TGGGCCGAGTTGACTCAGCTCTTCC m

                  p53-RE1
TCTTGAGCTGGTCAAGTTCAGACACGTTCCGAAACTGCAGTAAAAGGAGT h
TGTGGGGCTGGTCAAGTTGGGACACGTCCGGCGTCGGCTGTCGGAGGAGC m

      p53-RE2                   TATAA
TAAGTCCTGACTTGTCTCCAGCTGGGGCTATTTAAACCATG---CATTTT h
TAAGTCCTGACATGTCTCCAGCTGGGGTTATTTAAACGCTGCCCCGTTTC m

                     EXON2
CCCAGCTGTGTTCAGTGGCGATTGG---------AGGGTAGACCTGTGGG h
CGCAGCCGTCTGCTG-GGCGAGCGGGAGACCGACCGGACACCCCTGGGGG m

-----CACGGACGCACGCCACTTTTTCTCTGCTGATCCAGGTAAG      h
ACCCTCTCGGA-TCACCGCGCTTCTCCTGCGGGCCTCCAGGTAAG      m

HOMOLOGY = 78%
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B

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p53-RE1    G G T C A A G T T C A G A C A C G T T C
           | |   | | | | | | | | | | | |   | | | |
CONSENSUS  R R R C W W G Y Y Y R R R C W W G Y Y Y
           | |     | | | | | |   | | | | | | | | |
p53-RE2    A G T T A A G T C C T G A C T T G T C T
```

Figure 2. Detailed sequence analysis of the genomic DNA region around the p53 response elements. (**A**) Comparison between human and mouse genomic *mdm2* segments. The region serving for this comparison is underlined in Figure 1B. The two p53 response elements (p53-REs), the putative TATAA box, and the second exon as defined for mouse *mdm2* (7) are indicated. Dots indicate sequence identities. (**B**) Comparison between the two p53-REs of the *hmdm2* gene and the p53-binding consensus sequence (29).

buffer, separated by SDS–PAGE (9% gel), subjected to Western blotting using anti-Mdm2 rabbit polyclonal serum (6) and visualized with the ECL system (Amersham).

Reverse transcriptase–polymerase chain reaction analysis

Reverse transcriptase–polymerase chain reaction analysis was performed on total cellular RNA, extracted by the RNAzol method. PCR reactions were carried out for 25 cycles, with the aid of a GeneAmp 9600 thermal cycler (Perkin Elmer). Each cycle consisted of the following incubation steps: 95°C for 30 s, 58°C for 30 s and 72°C for 1 min. The primer combinations were O1+O3 or O2+O3. The PCR products were separated on a 1.6% agarose gel.

RNase protection

RNase protection was performed essentially as described previously (7), except that digestion was for 3 min at room temperature. The probes are described in the respective figures.

RESULTS

The human *mdm2* gene contains a highly conserved intronic region

In order to determine whether the human *mdm2* (*hmdm2*) gene contains an intronic p53-dependent promoter, the pertinent region was cloned. Two oligonucleotide primers (O1 and O3, see Materials and Methods) were employed for PCR amplification of human genomic DNA. O1 corresponds to a sequence located near the 5′ end of the most abundant reported *hmdm2* transcript, whereas O3 resides a short distance downstream to the first in-frame ATG codon (14; see Fig. 1). The amplified band was ~1050 bp long (data not shown). The region spanned by the two primers within the published *hmdm2* cDNA is ~350 bp (14). Hence, the amplified genomic region may comprise up to 700 bp of intronic sequences.

A partial nucleotide sequence of the genomic DNA region contained between the two primers is shown in Figure 1A. The nucleotide positions of the 3′ end of exon 1 and of the 5′ end of the first coding exon are as defined by Oliner *et al* (14). The first coding exon of the human *mdm2* gene is highly homologous to exon 3 of the mouse *mdm2* gene (7). Hence, it will be denoted hereafter *hmdm2* exon 3, even though its actual position within the major *hmdm2* transcript is probably different (see below).

Figure 1A also indicates the position of the two putative p53-response elements (p53-RE1 and p53-RE2), as well as of the segment homologous to mouse *mdm2* exon 2. These features are described in more detail in Figure 2. A schematic diagram of the *hmdm2* genomic DNA region is presented in Figure 1B.

Sequence comparison with the corresponding region of the mouse *mdm2* gene identified a stretch of 271 bp (in the human gene; underlined in Fig. 1B) displaying an overall 78% sequence identity. The comparison between the murine and human sequences is shown in Figure 2A. The two adjacent p53-REs (boxed) are particularly highly conserved; p53-RE1 and p53-RE2 are 85% and 90% identical, respectively, between mouse and man. Furthermore, *hmdm2* p53-RE1 and p53RE-2 conform to the 20 bp p53 consensus binding site (29) in 18 and 17 positions (out of 20), respectively (Fig. 2B), which is quite reminiscent of their murine counterparts (5,8).

Another conserved feature of the *mdm2* intronic region is an AT rich stretch located downstream to p53-RE2 (Fig. 2A, 'TATAA'). The position of this element suggests that it serves as a TATAA box for the p53-dependent promoter (7).

Another remarkably conserved segment encompasses the murine *mdm2* exon 2, including the splice junctions (Fig. 2A), suggesting that this will also be the second exon of human *mdm2* mRNA. Surprisingly, analysis of *hmdm2* cDNA indicates that it is actually spliced out, at least in certain cell types (14; see below),

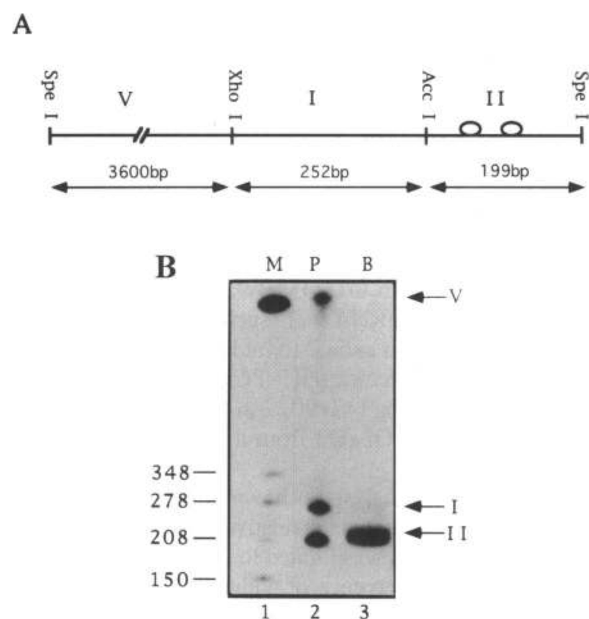

Figure 3. The genomic DNA region containing the putative p53-REs binds sequence-specifically to p53. A plasmid containing a genomic *hmdm2* DNA region within pBluescript II KS+ was digested with a combination of *Spe*I, *Acc*I and *Xho*I and end-labeled. The mixture of end-labeled DNA fragments was then reacted with an extract of Clone 6 (32°C) cells, containing a high amount of active wt p53. Immune complexes were precipitated, and the bound DNA released and separated on a polyacrylamide gel (see Materials and Methods). (**A**) Schematic diagram of the plasmid and the probes. V = vector (pBluescript II KS+). I and II refer to adjacent restriction fragments derived from the human *mdm2* gene. Circles indicate the p53-REs. (**B**) Autoradiogram of the DNA binding assay. P = initial non-reacted radiolabelled DNA digest (1% of the amount taken for each binding reaction). B = bound DNA, eluted from p53 immune complexes. V, I and II are as in (A). M = molecular size markers (in nucleotides).

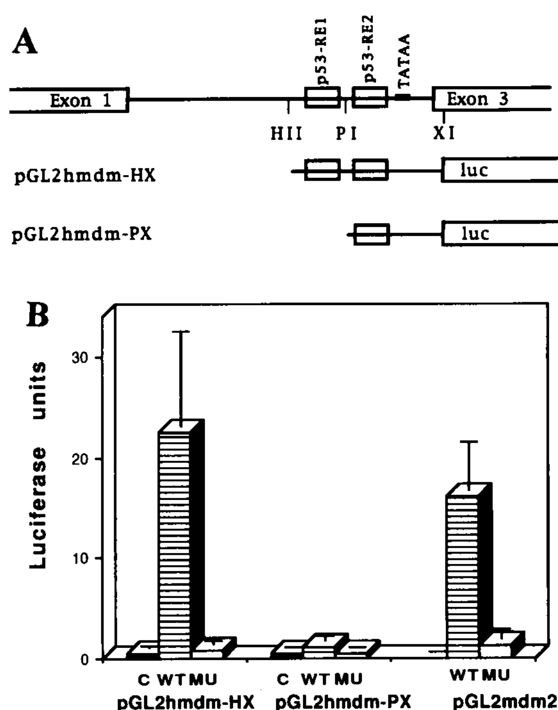

Figure 4. A p53-inducible promoter resides within the first intron of the *hmdm2* gene. (**A**) Schematic map of the reporter plasmids used for promoter analysis. Each plasmid contains the indicated region of the *hmdm2* gene, placed in front of a firefly luciferase reporter gene (luc). (**B**) Luciferase activity of the plasmids depicted in (A). H1299 human lung carcinoma cells (5×10^5 per 60 mm dish) were transfected with a combination of the indicated luciferase reporter plasmid (1 µg/dish) and 0.3 µg/dish of either pCMVp53wt, encoding wild type mouse p53 (WT), or pCMVp53m, encoding the murine p53gly168ile234 mutant (MU), or no p53 plasmid (C). Extracts were prepared 36 h later, and assayed for luciferase activity. The values are presented in arbitrary machine units.

resulting in a transcript which fuses exon 1 directly to exon 3. The relevance of this region for *hmdm2* expression is discussed later.

The *hmdm2* intronic region is specifically bound by p53

We next sought to find out whether the intronic *hmdm2* region can bind p53 sequence-specifically. A plasmid including this region was subjected to a DNA binding assay, based on the selective precipitation of p53-DNA complexes by p53-specific monoclonal antibodies (17). As seen in Figure 3, the DNA fragment which contains the two p53-REs was brought down specifically in a complex with active wt p53, whereas an adjacent fragment was not. Thus, the putative p53-responsive intronic region of *hmdm2* can indeed bind efficiently to wt p53.

The intronic p53-binding region of *hmdm2* can function as a p53-dependent promoter

The intronic region of *hmdm2* possesses several features suggestive of a p53-responsive promoter. Moreover, in addition to the two p53-REs and the presumptive TATAA box, stretches immediately upstream and downstream to these elements are also well conserved (Fig. 2A). We therefore tested the ability of this region to operate as a p53-responsive promoter. Two constructs were made, containing different segments of this region in front of a luciferase reporter gene (Fig. 4A). Plasmid pGL2hmdm-HX-luc contains both p53-REs, whereas pGL2hmdm2-PX-luc retains only a single p53-RE (p53RE-2).

Each construct was transfected into p53-deficient H1299 cells, either alone or together with expression vectors for murine wt p53 or tumor-derived mutant p53. As shown in Figure 4B, both reporter constructs exhibited comparable levels of basal promoter activity (C). Co-expression of wt p53 (WT) markedly enhanced the activity of pGL2hmdm-HX-luc, resulting in a 50-fold stimulation. Mutant p53 (MU) was largely incapable of activating this promoter, bringing about only a 2-fold stimulation. The extent of stimulation by wt p53 was similar to that obtained with a reporter plasmid containing the p53-dependent intronic promoter of mouse *mdm2* (Fig. 4B, pGL2mdm2). Hence, the p53-binding region located within the *hmdm2* intron can indeed operate as a tightly regulated p53-responsive promoter.

It is noteworthy that construct pGL2hmdm2-PX-luc, while possessing basal promoter activity similar to that of pGL2hmdm-HX-luc, was only very weakly induced by wt p53 (less than 3-fold, Fig. 4B). Hence, p53-RE2 alone is not sufficient for optimal activation by wt p53.

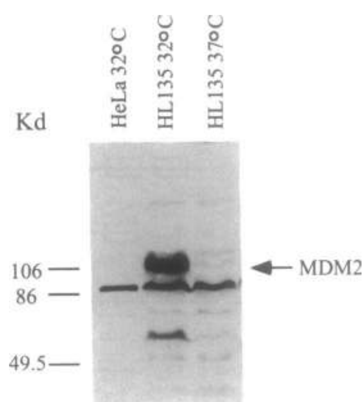

Figure 5. Induction of hMdm2 proteins in HL135 cells at 32°C. Total cell extracts were prepared from HL135 cells maintained at 32 or 37.5°C for 24 h, as well as from HeLa cells growing at 32°C. Proteins were resolved by SDS–PAGE and subjected to Western blotting, using an anti-Mdm2 polyclonal antibody (6). The arrow indicates the position of the major hMdm2 product. Positions of molecular weight markers are also indicated.

The intronic p53-responsive promoter is functional *in vivo*

To determine whether the putative p53-responsive promoter is functional in the context of the chromosomal *hmdm2* gene, we generated a human cell line conditionally overexpressing wt p53.

HeLa cells were stably transfected with the temperature-sensitive p53 mutant p53val135 (32). The resultant cell line, HL135, overexpresses p53val135 protein (not shown). Upon shift to 32°C, this protein assumes a wt conformation (32), accompanied by its translocation from the cytoplasm into the nucleus of HL135 (Y.H., data not shown).

The ability of p53 to transactivate *mdm2* in HL135 at 32°C was tested by Western blot analysis. As seen in Figure 5, there was indeed a pronounced increase in the amount of full length hMdm2 protein (arrow) at 32°C. A smaller hMdm2 product, migrating at about 60 kDa, was also greatly elevated. Hence, HL135 can serve as a good system for the analysis of p53-dependent *hmdm2* transcription.

Mouse *mdm2* contains two non-coding exons (7). In contrast, the major transcript described by Oliner *et al.* (14) appears to contain only one non-coding exon. Notably, the nucleotide sequence of this exon is very different from exon 1 of murine *mdm2* mRNA (14). In contrast, there is a remarkable conservation of short stretches corresponding to the splice donor and acceptor sites of mouse *mdm2* exon 2 (7; boxed in Fig. 2A), suggesting that the *hmdm2* gene could also encode a potential exon 2 in the same position.

Furthermore, the transcriptional start site of the p53-inducible mouse *mdm2* mRNA resides within exon 2, very close to its 5′ end (7). This, together with the presence of a candidate TATAA box a short distance upstream to the 5′ end of the potential *hmdm2* exon 2, strongly suggested that the human p53-inducible transcript may also initiate at a similar position.

To elucidate the repertoire of *hmdm2* transcripts present in HL135 cells before and after the induction of wt p53 activity, a reverse transcriptase–polymerase chain reaction (RT–PCR) was performed on total cytoplasmic RNA from cells maintained at either 37.5 or 32°C. Reverse transcription was primed with oligo (dT), and products were amplified with a combination of either primers O1+O3, or O2+O3 (see Figures 1A and 6A for positions of primers on *hmdm2* genomic DNA and cDNA, respectively).

The predicted RT–PCR products are shown in Figure 6B. With primers O1+O3, a transcript containing all three exons will yield a product of 428 bp (267+71+90). A transcript fusing exon 1 directly to exon 3, as reported by Oliner *et al.* (14), will generate a product of 357 bp (267+90). On the other hand, a transcript driven by the intronic promoter, initiated downstream to exon 1, will not be detected with O1+O3. By analogy with the murine p53-inducible *mdm2* mRNA (7), such transcript is likely to contain a fusion between exon 2 (or at least most of exon 2) and exon 3. The size of the predicted RT–PCR product generated with O2+O3 should be 137 bp (47+90). An identical product should also be generated with O2+O3 from a transcript containing all first three exons.

The actual RT–PCR analysis is shown in Figure 6C. To allow a semi-quantitative reflection of the relative abundance of individual transcripts, amplification was limited to 25 cycles. An increase to 30 cycles led to the emergence of additional bands, presumably representing relatively rare transcripts (data not shown; see below).

Several conclusions can be drawn from Figure 6C. First, in the absence of functional wt p53 (37.5°C), the predominant product of O1+O3 is one of 357 bp (lane 3), combining exon 1 directly with exon 3. Hence, if a full length *hmdm2* mRNA (exons 1+2+3; Fig. 6B, mRNA 1) is produced at all under those conditions, it does not reach significant levels. The relative abundance of the 357 bp PCR product was rather low, suggesting a limited amount of the corresponding transcripts. Such *hmdm2* transcripts are in most likelihood produced from the P1 promoter, located upstream to exon 1. Exon 1 thus appears to be spliced directly to exon 3, skipping the putative exon 2 (Fig. 6B, RNA 2). This is consistent with the structure of the major class of *hmdm2* mRNA described by Oliner *et al.* (14).

Second, similar transcripts continue to be produced also following the activation of wt p53 at 32°C (lane 2). Hence, the upstream promoter P1 remains active; moreover, there even is a modest increase in the amount of P1-derived transcripts.

Third, and most importantly, HL135 cells exposed to activated wt p53 (lane 4) exhibited clear evidence of an abundant transcript fusing exons 2 and 3 (PCR product of 137 bp). The fact that a corresponding band of 428 bp was not seen with O1+O3 (lane 2), suggests that this transcript lacks exon 1. The most likely origin of this mRNA would be through transcription initiation at the intronic p53-dependent promoter P2. By contrast, a 137 bp band was undetectable with 37.5°C RNA (lane 5). Hence transcripts of type 3 (Fig. 6B), which predominate in presence of activated wt p53, are rare in its absence.

In addition to the expected PCR products, a larger band was seen with O2+O3, particularly at 32°C (lane 4). The size of this product, ~250 bp, suggests that intron 2 has not been spliced out of the corresponding transcript. No higher band compatible with an unspliced intron 2 was obtained with O1+O3 (lane 2), arguing that these transcripts do not contain exon 1. Hence, as for the fully spliced major *hmdm2* mRNA present at 32°C (mRNA type 2), this incompletely spliced transcript is also likely to be initiated at P2.

Overall, the RT–PCR analysis suggests that, prior to the induction of wt p53 activity, the main if not only functional *hmdm2* promoter is P1, located upstream to the gene. Following wt p53 induction, P1 continues to be active, at somewhat elevated rates. However, the intronic P2 now starts functioning as well, eventually

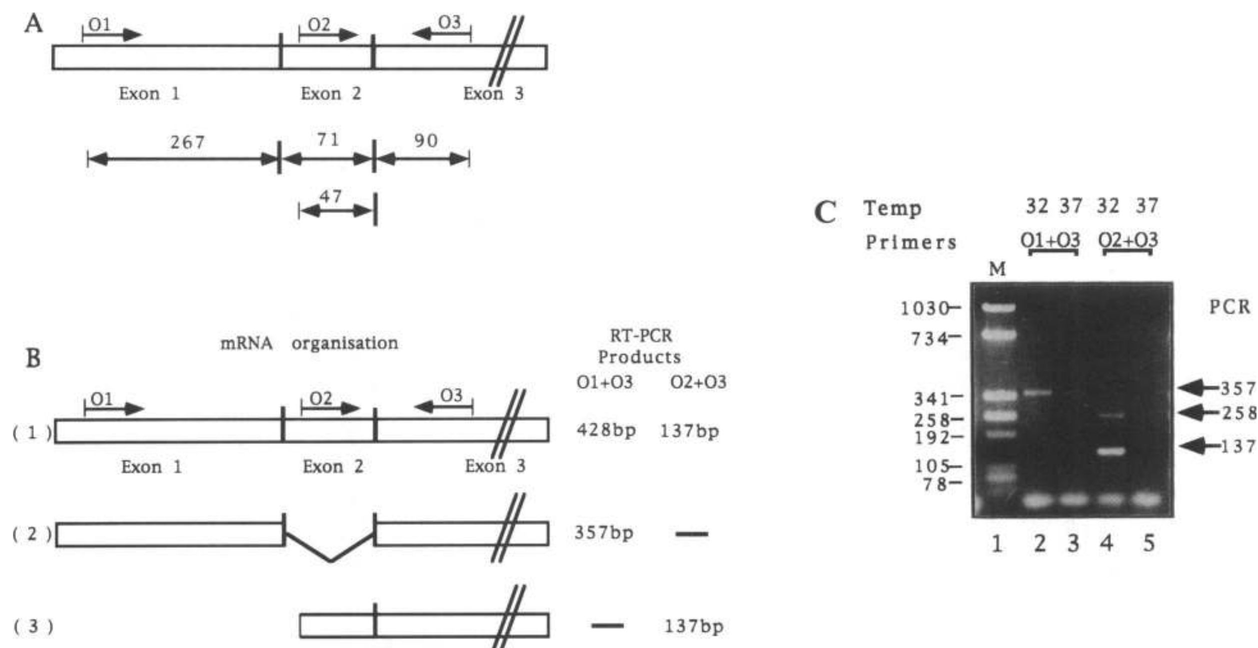

Figure 6. RT–PCR analysis of *hmdm2* transcripts in HL135 cells. RNA was prepared from HL135 cells maintained at either 32 (wt p53 conformation) or 37.5°C (mutant conformation). RNA was reverse transcribed and the products amplified by PCR as described in Materials and Methods, using primer combinations O1+O3 or O2+O3. (**A**) Positions of the three oligonucleotide primers along the putative full length *hmdm2* mRNA. Numbers indicate calculated distances (in base pairs). (**B**) Structure of the three main types of predicted *hmdm2* transcripts and sizes of the corresponding RT–PCR products that should be generated with each pair of primers. No product is expected for transcripts which can hybridize with only one of the two primers in a given pair. RNA (3), emanating from the p53-dependent intronic promoter P2, is projected to start in close vicinity to the 5′ end of exon 2. (**C**) Agarose gel analysis of RT–PCR products. Numbers on the left indicate sizes (in base pairs) of molecular size markers (pBluescript II KS+ DNA digested with Sau3A). Arrows on the right indicate the positions and sizes of the main actual PCR products.

becoming the predominant *hmdm2* promoter. Hence, the prominent increase in overall *hmdm2* mRNA is primarily a result of P2 activation.

To further confirm and extend the RT–PCR data, as well as identify the exact start site of the P2-derived transcripts, RNase protection was performed. In the first set of experiments, the probe was based on *hmdm2* cDNA (Fig. 7A). This cDNA was obtained by RT–PCR, using RNA of HL135 cells maintained at 32°C, and primers O2+O3. Figure 7A illustrates the expected protection products. Any transcript carrying a fusion of exon 2 and exon 3 (i.e. mRNA species 1 and 3 in Fig. 6B) should give rise to a band of 128 nucleotides. On the other hand, transcripts lacking exon 2 (e.g. mRNA 2 in Fig. 6B) will yield a shorter fragment of 81 nucleotides.

The actual data is shown in Figure 7B. At 37.5°C, only a faint band of 81 nucleotides was seen, with no evidence for a 128 nucleotide band. Hence, in the absence of wt p53, exon 2 is essentially absent from HL135 *hmdm2* mRNA. The predominant p53-independent *hmdm2* transcript therefore appears to be initiated at P1, subsequently being spliced directly to exon 3. This is consistent with the RT–PCR data, as well as with the major type of *hmdm2* cDNA cloned from various sources (14; Y. Barak and M. Oren, unpublished).

By contrast, RNA from HL135 cells maintained at 32°C displayed two main bands. One was identical to the 81 nucleotide band seen at 37.5°C, albeit stronger. This is compatible with the continued activity of P1, generating transcripts which fuse exons 1 and 3. In addition, however, a much stronger band of 128 nucleotides was obtained. Its size indicated that it represented an mRNA species containing exons 2 and 3. In conjunction with the RT–PCR data, it is clear that this abundant transcript lacks exon 1, and is therefore likely to emanate from the internal promoter P2. The RNase protection assay, performed with excess radiolabelled probe, is expected to be quantitative. Thus, P2-derived transcripts comprise over 90% of the total amount of *mdm2* mRNA made in HL135 cells overexpressing activated wt p53.

In a second set of RNase protection experiments, the probe was derived from an authentic *hmdm2* cDNA clone. Like in other published *hmdm2* cDNA clones, this cDNA has exon 1 followed immediately by exon 3. The results (data not shown) confirmed that at 37.5°C, the only transcript detectable in HL135 cells was one fusing exon 1 to exon 3; in contrast, cells at 32°C contained a much more abundant mRNA, which protected only exon 3 and is likely to represent a P2-derived, exon 2 + exon 3 species.

Finally, RNase protection was performed with a probe based on *hmdm2* genomic DNA (Fig. 8A). A transcript containing a fusion of exons 1 and 3 should protect only the exon 3 component of the probe, yielding a band of 81 nucleotides. On the other hand, the major product of the internal P2 promoter, containing exons 2 and 3, should also protect the exon 2 component of the probe. Exon 2 is 71 nucleotides long (Fig. 2A). However, if the P2-derived transcripts initiate either upstream or downstream to the 5′ end of exon 2, the corresponding protected band should be longer or shorter, respectively.

As seen in Figure 8B, RNA of cells maintained at 37.5°C yielded a single detectable band. This band, identical in size to the one obtained with the cDNA probe (Fig. 7), represents protection of exon 3 only. As expected the same band, albeit much more

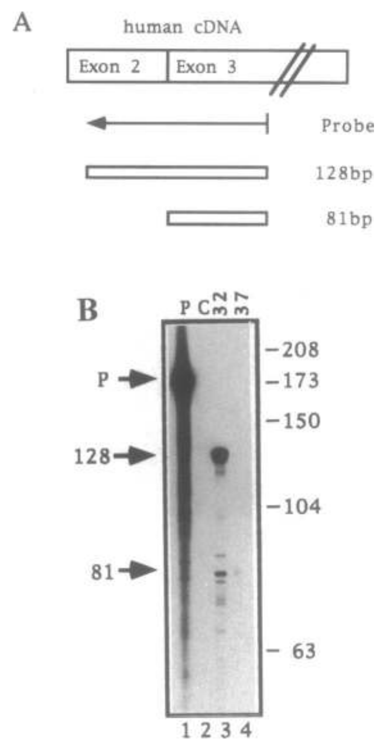

Figure 7. RNase protection analysis of *hmdm2* mRNA–cRNA probe. (**A**) Structure of the probe and sizes of expected protection products. The template for the probe was obtained through an RT–PCR reaction, using RNA of HL135 cells maintained at 32°C, and primers O2+O3. Primer O3 contains, at its 5′ end, nine nucleotides (including an *Xho*I site) which are not homologous to hmdm2 mRNA. The probe contains also 42 nucleotides from the plasmid vector. (**B**) Actual protection products, obtained with RNA of HL135 cells maintained at either 32 or 37.5°C. P = undigested probe. C = reaction containing yeast tRNA instead of HL135 RNA. Sizes of DNA markers (in nucleotides) are indicated on the right side.

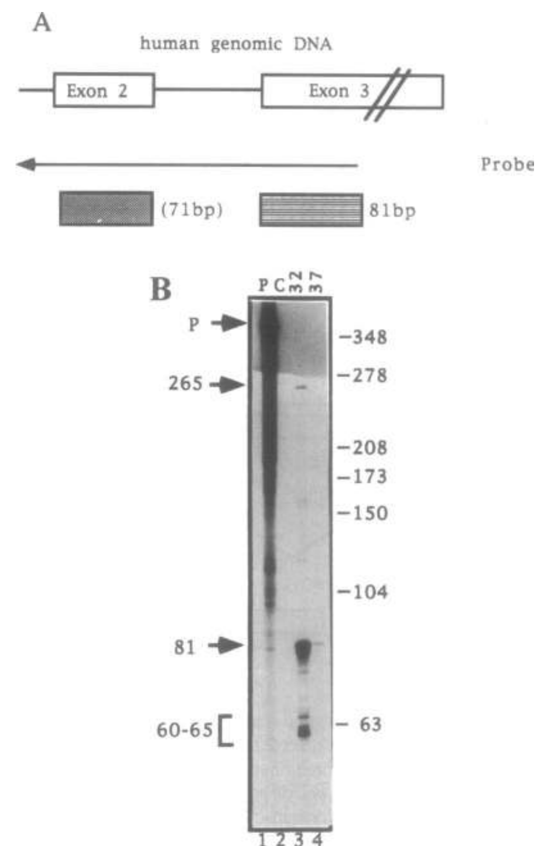

Figure 8. RNase protection analysis of *hmdm2* mRNA–genomic probe. (**A**) Structure of the probe and sizes of expected protection products. The template for the probe was a fragment of *hmdm2* genomic DNA (see Fig. 1). The indicated size of 71 bp is for a transcript initiated exactly at the 5′ end of exon 2 (as well as for a transcript containing exon 1 spliced to exon 2). Initiation within exon 2 or immediately upstream to exon 2 will result in a protected band of slightly shorter or longer size, respectively. (**B**) Actual protection products. Details as in Figure 7.

abundant, was also seen at 32°C. In addition, however, there was a cluster of protected bands ranging in size between 60 and 65 nucleotides. Figure 7 argues that the abundant transcript at 32°C is an exon 2 + exon 3 fusion. Hence, this cluster must represent protection by exon 2 sequences. The protected bands are shorter than 71 nucleotides. The corresponding transcripts therefore appear to initiate within a short stretch located 6–11 nucleotides downstream to the formal 5′ end of exon 2. Taken together, the data show that the predominant class of *hmdm2* transcripts at 32°C is composed of molecules emanating from the intronic P2 promoter, and containing most of exon 2 spliced to exon 3.

It is noteworthy that a less abundant, larger protected fragment of 265 nucleotides is also seen at 32°C (Fig. 8B). Such size corresponds exactly to protection by incompletely spliced transcripts retaining the entire second intron. Evidence for the existence of such molecules was also provided by RT–PCR (see above).

In conclusion, activation of wt p53 in HL135 cells turns on a cryptic intronic promoter, located downstream to exon 1 of the *hmdm2* gene. This changes not only the abundance of *hmdm2* mRNA, but also the structure of the predominant class of transcripts.

DISCUSSION

This work describes the identification of a p53-dependent intronic promoter in the human *mdm2* gene. Evidence for the existence and *in vivo* activity of such promoter (P2) is based on functional assays with reporter gene constructs, as well as on the analysis of actual *hmdm2* mRNA species present in p53-overexpressing human cells. P2 shares a variety of structural and functional features with the intronic promoter identified previously in the murine *mdm2* gene (7,8). The high degree of conservation argues strongly that the activation of a cryptic promoter by p53, and the resultant production of novel *mdm2* transcripts, are biologically significant.

The comparison between the mouse and human *mdm2* intronic promoters highlights an interesting feature. In both species, P2 activity is driven by a tandem pair of p53-REs, each of which exhibits rather imperfect homology (17/20 in three cases, 18/20 in the fourth case) with the canonical p53-binding consensus site (29). It therefore appears likely that this particular combination of two relatively weak p53 binding sites is crucial for the proper regulation of *mdm2* expression. One interesting possibility could be that this is meant to prevent the premature activation of *mdm2*. If the induction of *mdm2* transcription by p53 indeed acts as a negative feedback mechanism, it is conceivable that such mechanism should come into effect only after other target genes have already been induced by the increased levels of wt p53 activity. Genes of the latter type might be regarded as direct positive

mediators of p53 function. The following scenario is therefore proposed: upon activation of wt p53, the protein will first bind to p53-REs for which it possesses a relatively high avidity. The far upstream element of *Waf1*, which falls into the latter category (24), is a likely example. In the case of genes like *Waf1*, the binding of p53 to a single p53RE may suffice to activate the gene. Consequently, an increase in the amounts of the corresponding transcripts may be attained very shortly after the activation of the p53 pathway. On the other hand, *mdm2* may be triggered only when the cell contains a relatively large amount of wt p53, which will allow enough p53 molecules to bind simultaneously to both its p53-REs. Productive binding may rely on stacking interactions between p53 tetramers bound at each of the two sites (33). This could create a time window where genes like *Waf1* are already on, while *mdm2* is still off, and allow p53 to activate productively its positive effector genes. The subsequent further rise in p53 will then gradually turn on also *mdm2*, whose products will accumulate to high enough levels, interact with p53 and eventually terminate the p53-mediated signal. Experimental confirmation of this scenario will require a detailed kinetic analysis. Nevertheless, it is consistent with notions raised by earlier studies (e.g. 11).

The structure of the 5′ region of *hmdm2* mRNA was studied in detail by RT–PCR and by RNase protection. The composite picture suggested by these studies is depicted schematically in Figure 9, in comparison to mouse *mdm2*. In HL135 cells devoid of wt p53 activity (37.5°C), the only detectable class of transcripts is initiated from P1, at a site (I1) corresponding to the 5′ end of exon 1. This is then spliced directly to exon 3, skipping the predicted exon 2. In that respect, the main p53-independent *hmdm2* mRNA (at least on the background of HeLa cells) differs from its earlier described mouse counterpart, which contains all three exons (7). The reason for this difference is unclear. It is possible, however, that an *hmdm2* retaining both exon 1 and exon 2 might be translated at an unacceptably low efficiency. This may not be the case for mouse *mdm2*, which has a different and significantly shorter exon 1.

It is noteworthy that the exact splicing pattern may vary among individual cell types, as was previously reported for mouse *mdm2* (4,7). In fact, in addition to the predominant p53-independent *hmdm2* mRNA containing exon 1 spliced to exon 3 (14), analysis of human *mdm2* cDNA clones also revealed the existence of minor species where exon 2 is spliced to exon 3, as well as rare molecules which may even retain part of the first intron (J. Oliner and B. Vogelstein, unpublished observations).

Following the activation of wt p53, there is a marked change in the composition of *hmdm2* transcripts. There is a several-fold increase in the amount of the 'constitutive', P1-derived transcripts, suggesting a mild enhancer-like effect of the intronic p53-REs on the upstream P1 promoter. The relative translation potential of the P1 transcripts, relative to the P2-derived ones (see below) is still unknown. Therefore, the effect of this enhancement on the pattern of Mdm2 polypeptides remains to be established.

Much more dramatically, however, there emerges a class of new transcripts, whose presence is undetectable at all in the absence of wt p53. The prototypic *hmdm2* mRNA of this class is initiated slightly within exon 2, and is then spliced to exon 3, the first coding exon. Expression of these transcripts is driven by the otherwise cryptic P2, which now becomes the major active *hmdm2* promoter. In addition, there exists a fraction of partially spliced transcripts, also presumably initiated from P2, but retaining intron 2.

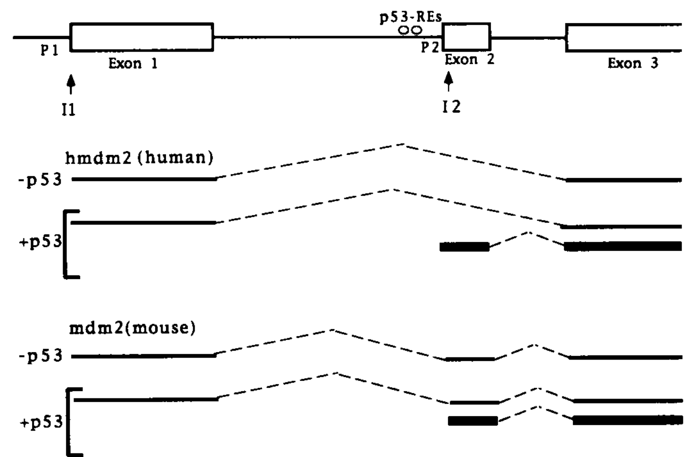

Figure 9. Schematic comparison of the 5′ regions of human and mouse *mdm2* transcripts. The figure depicts the structure and splicing patterns of the main transcripts present in the absence (–p53) or presence (+p53) of activated wild type p53. A scheme of the corresponding genomic region is shown on top. P1 and P2 refer to the two alternative p53 promoters; I1 and I2 represent the corresponding transcription initiation sites. Information on human *mdm2* mRNA is based on HL135 cells (this paper); other cell types may vary with regard to the repertoire and relative abundance of specific splice variants. Information on mouse *mdm2* mRNA is from Barak *et al.* (7). See text for further details.

The relatively high conservation of exon 2, which contributes the 5′ UTR of the p53-dependent *mdm2* transcripts (7), contrasts strongly with the striking lack of conservation of exon 1 (4,14). It is plausible that the 'constitutive' *mdm2* transcripts possess a 5′ UTR which is unsuitable for the conditions prevailing upon wt p53 overexpression. Earlier work, based on mouse *mdm2* (7), indicated that the p53-induced P2 transcripts may have a different translation potential than their P1-derived 'constitutive' counterparts. The need to generate a different type of *mdm2* transcripts, with a shorter 5′ end and possibly less subject to translational repression, could imply that the presence of high levels of wt p53 alters the efficiency or specificity of the translational machinery. One could propose that activated p53 interferes with the translation of the 'constitutive' *mdm2* mRNA; under conditions of p53 overexpression, it may therefore be necessary to generate a different type of *mdm2* transcript, which is refractory to this interference. It is tempting to speculate that the p53 protein itself may play a role in regulating the translation of *mdm2* mRNA. For instance, p53 may bind selectively to the long 5′ untranslated region (UTR) of the P1 transcripts and interfere with their translation, whereas the P2 transcripts, possessing a much shorter 5′ UTR, may be spared from such translational repression. That p53 may act as a direct translational modulator is suggested by recent observations, relating to the expression of the cdk4 kinase (34). It will be of great interest to determine whether p53 indeed affects differentially the translation of the 'constitutive' and the p53-induced *mdm2* transcripts.

In conclusion, overexpression of wt p53 gives rise to a new class of *hmdm2* transcripts. These transcripts lack entirely exon 1, similarly to the p53-inducible murine *mdm2* transcripts. Instead, they possess a much shorter 5′ UTR, derived mainly from exon 2. Hence, as is the case for their murine counterparts (7), they are

likely to possess an altered translational potential. The conservation of the promoter switch, as well as of the structure of the p53-dependent transcripts, argues strongly that this altered translational potential may be crucial for the proper regulation of *mdm2* function in the presence of activated wt p53.

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