Multiple Murine Double Minute Gene 2 (MDM2) Proteins Are Induced by Ultraviolet Light*

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The mdm2 (murine double minute 2) oncogene encodes several proteins, the largest of which (p90) binds to and inactivates the p53 tumor suppressor protein. Multiple MDM2 proteins have been detected in tumors and in cell lines expressing high levels of mdm2 mRNAs. Here we show that one of these proteins (p76) is expressed, along with p90, in wild-type and p53-null mouse embryo fibroblasts, indicating that it may have an important physiological role in normal cells. Expression of this protein is induced, as is that of p90, by UV light in a p53-dependent manner. The p76 protein is synthesized via translational initiation at AUG codon 50 and thus lacks the N terminus of p90 and does not bind p53. In cells, p90 and p76 can be synthesized from mdm2 mRNAs transcribed from both the P1 (constitutive) and P2 (p53-responsive) promoters. Site-directed mutagenesis reveals that these RNAs give rise to p76 via internal initiation of translation. In addition, mdm2 mRNAs lacking exon 3 give rise to p76 exclusively, and such mRNAs are induced by p53 in response to UV light. These data indicate that p76 may be an important product of the mdm2 gene and a downstream effector of p53.

The mdm2 oncogene is a determinant of embryogenesis (1, 2), tumorigenesis (3, 4), and cell cycle progression (5, 6). The effects of MDM2 on these processes depend, in part, on its ability to inactivate the p53 tumor suppressor protein. For example, mice with a homozygous deletion of the mdm2 gene die during embryogenesis, and this deficiency is rescued if p53 is also deleted (1, 2). In human tumors, the homologue of MDM2 is overexpressed most often in the subset lacking inactivating mutations in the p53 gene (7). Thus, high levels of MDM2 may be redundant with mutational inactivation of p53. Following exposure of cells to γ-radiation, MDM2 inactivates the G1 block mediated by p53 (5). In some cell types, MDM2 can inhibit apoptosis mediated by p53 (8). MDM2 binds to p53, inhibiting the transcriptional activation function of p53 (3, 9) and stimulating degradation of p53 (10, 11). Recently, MDM2 has been shown to bind to another tumor suppressor protein, p19ARF (12, 13). The ability of MDM2 to stimulate degradation of p53 is inhibited by p19ARF, indicating that the interaction of p19ARF and MDM2 may be an important determinant of cell cycle progression.

In addition to interacting with p53 and p19ARF, MDM2 binds to and alters the function of other proteins that regulate cell division. For example, overexpression of MDM2 stimulates the activity of the E2F transcription factor (14) and reverses an arrest in the cell cycle mediated by either the retinoblastoma protein (15) or a related protein, p107 (6). These effects of MDM2 do not depend on the presence of p53. Further evidence for p53-independent functions of MDM2 was provided by Lundgren et al. (4), who generated mice expressing high levels of MDM2 in the mammary gland. The mammary epithelial cells of these mice developed polyplody, and this effect of MDM2 was also seen in mice lacking p53. Mammary tumors arose in mice overexpressing mdm2, but the dependence on p53 was not measured since mice lacking p53 die before mammary tumors would have developed. Sigalas et al. (16) found that alternatively spliced mdm2 mRNAs were overexpressed in some human tumors. Introduction of these mRNAs into NIH3T3 cells resulted in morphological transformation, even though a subset of them encoded MDM2 proteins that could not bind p53. Therefore, some of the oncogenic effects of MDM2 are independent of p53.

Several MDM2 proteins are detectable in cells overexpressing MDM2, including human tumor cells and transformed murine cell lines (17, 18). Olson et al. (17) hypothesized that these proteins arise through alternative splicing, proteolytic processing, or post-translational modification. It has not been clear whether these proteins arise through mechanisms used in the normal regulation of mdm2 expression. Multiple MDM2 proteins can be translated from single mRNAs in rabbit reticulocyte lysates, indicating that internal initiation may be a mechanism whereby multiple MDM2 proteins are expressed in cells (19). In fact, there is evidence that enhanced translation accounts for the overexpression of multiple MDM2 proteins in some human tumors (18). In the DM3T3 cell line, in which the mdm2 gene is amplified, multiple MDM2 proteins and mRNAs are expressed (17, 20). The most abundant protein in DM3T3 cells (p90) is the well characterized mdm2 gene product that binds to p53 and inhibits its function (3, 9, 10, 11). The second most abundant protein (p76) does not bind p53 (17, 19), but does bind p19ARF (13). The primary structure and source of p76 have not been established.

Here we show that p76 is a bona fide product of the mdm2 gene, as it is expressed in normal wild-type MEFs, but not in mdm2-null fibroblasts. Expression of p76 is induced by UV light in a p53-dependent manner. Using epitope mapping and site-directed mutagenesis, we show that p76 appears to be the product of translational initiation at codon 50 (AUG). Both

* This work was supported in part by Cancer Center Support Grant CA-07175 (to the McArdle Laboratory for Cancer Research) and by National Institutes of Health Grant CA-70718 (to M. E. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by NCI Predoctoral Grant CA-09135 from the National Institutes of Health.

§ Supported by Predoctoral Grant GM-07215 from the National Institutes of Health.

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This paper is available online at http://www.jbc.org
internal initiation and alternate splicing can give rise to p76 in cells. The p53-responsive internal promoter of mdm2 is induced by UV light, and a fraction of the induced RNA is spliced such that it lacks exon 3 and the first two AUG codons. In this alternatively spliced mRNA, AUG codon 50 (referred to hereafter as AUG 50) is the first initiation codon. We provide evidence that the increase in this alternatively spliced mRNA accounts for the induction of p76 expression in response to UV light. Identification and characterization of p76 are important because it is likely to share some oncogenic functions with p90.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Wild-type MEFs were obtained from Arnold J. Levine. MEFs lacking p53 or both p53 and MDM2 were provided by Guillermoina Lozano (21). All three strains of fibroblasts were derived from 14-day-old embryos with a mixed Sv129 × C57BL/6 genotype. DM3T3 cells are transformed murine fibroblasts that contain amplified copies of the mdm2 gene (20). C127 is a nontransformed murine cell line expressing wild-type p53 (22). The (10/3) cell line lacks p53 and was derived from 14-day-old embryos of BALB/c mice (23). COS-1 cells are African green monkey kidney cells expressing the large T-antigen of SV40. All cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum (Summit).

**Plasmids and Mutagenesis**—Several plasmids expressing mdm2 were used to generate MDM2 proteins in reticulocyte lysates. pGEM1F, pGEM1X2, and pGEM1B (19) were kindly provided by Moshe Oren. pGEM1F expresses an mRNA containing exons 1–12; pGEM1X2 contains exons 2–12; and pGEM1B contains part of exon 3 and exons 4–12. Derivatives of pGEM1F were made that contained mutations at either ATG 50 (Fp50) or ATG 62 (Fp62) and at both ATGs 50 and 62 (Fp50,62). The template for site-directed mutagenesis of the mdm2 coding region was pGEM1X2 (19). Single- or double-base changes were achieved using two oligonucleotides, both of which contained the desired base change (s).

The oligonucleotides were hybridized to a denatured circular plasmid and extended using T7 polymerase (25). The reaction was repeated 12 times. For each preparation, an AcI fragment containing the mutation was transferred from pGEM1X2 to pGEM1F.

For transfection of mammalian cells, the cDNAs described above were transferred to the EcoRI site of the vector pCMV5 (26), which contains the cytomegalovirus promoter, a polyadenylation signal, and the SV40 origin of replication. A similar eukaryotic expression vector with a cDNA containing exons 1, 2, and 4–12 of mdm2 (pOCD) was obtained from Moshe Oren. The cDNAs encoding F, X2, and D mRNAs were transferred from pGEM1X2 to pGEM1F.

**Transfection and Translation**—For transfection and translation in vitro, 1 µg of each cDNA was linearized and added to the transcription extract (Stratagene). RNAs were incubated with rabbit reticulocyte lysate (Stratagene) in the presence or absence of [35S]methionine (Amersham Pharmacia Biotech). Immunoreactive proteins were revealed by ECL (Amersham Pharmacia Biotech) following the manufacturer’s suggestions.

**RT-PCR**—RNA was isolated using Tri Reagent (Molecular Research Center, Inc.). Five µg of total RNA was reverse-transcribed using oligo(dT) (Promega) as a primer, and the cDNA product of this reaction was amplified by PCR. The amount of cDNA added to each PCR was normalized using glyceraldehyde-3-phosphate dehydrogenase cDNA as the standard. Glyceraldehyde-3-phosphate dehydrogenase amounts were measured by limited PCR using the primer pair 5′-ACCACAGTCATGCTCATCA and 5′-TCCACACCTGTTTGTGTA. Two primer sets were used to amplify mdm2. One set amplifies only RNAs initiated at the constitutive promoter, and the second set amplifies RNAs initiated at both the constitutive and p53-responsive promoters. A backward primer complementary to mdm2 exon 4 (5′-GCTCCAAGCGACTTTACAACCTTCACT) was end-labeled with [γ-32P]ATP using T4 kinase (New England Biolabs Inc.). This primer was paired with either a primer in exon 1 (5′-CGTGAAGGGTCCCCGAATGC) or a primer in exon 2 (5′-TGGCCAGGCGGGAGACCGAC) in PCRs using Taq polymerase. DNAs were amplified for 25 cycles (94 °C for 30 s, 59 °C for 30 s, and 72 °C for 45 s), which was determined to be within the exponential range of amplification of mdm2 in these samples. To quantify the different mdm2 transcripts, several reactions were performed in the presence of increasing amounts of an engineered internal standard (31). This standard contained sequences complementary to the primer pairs used and was amplified with efficiencies similar to those of the mdm2 cDNAs. The source for the standard was an mdm2 cDNA lacking exon 3 isolated from C127 cells following amplification of first-strand cDNA with a primer in exon 1 (5′-AGCGGGCGCCCTCAAGGT) and a primer that spans the junction of exons 4 and 5 (5′-TAAATTCTTGATAGGAATGGT). The PCR product was cloned using the Topo TA kit (Invitrogen) and sequenced. The internal standard was created by amplifying exons 1–4 of this mdm2 clone using a backward primer containing an insertion of 20 random base pairs (shown in boldface; 5′-GGCTCCACCCGGAGCTTTACAACCTTCCCTGATCGACCTAAAACCAATG). Following amplification of cDNAs from untreated and UV-irradiated C127 cells, the products were separated on 5% nondenaturing polyacrylamide gels and quantitated on a PhosphorImager (Molecular Dynamics, Inc.). To determine the amount of each mdm2 transcript, the logarithm of the amount of the internal standard was plotted against the logarithm of the ratio of the internal standard to the mdm2 transcript (31). When the ratio is 1, the log of the ratio is zero, and the amount of transcript is equal to the amount of standard. From the known quantity of the standard, the amount of each transcript was calculated.

**RESULTS**

Two MDM2 Proteins Are Present in Mouse Embryo Fibroblasts—DM3T3 cells, which are transformed and contain multiple copies of the mdm2 gene (32), express multiple proteins that react with antibodies to MDM2 (17). We compared the number of immunoreactive proteins in DM3T3 cells with wild-type MEFs, p53-null MEFs, and p53/mdm2-null MEFs (Fig. 1). Both DM3T3 cells and wild-type MEFs expressed at least four proteins that immunoreacted with the 2A10 monoclonal antibody. All four of these proteins were also evident in p53-
null embryo fibroblasts, but only two appeared in p53/mdm2-null fibroblasts. Therefore, two of the immunoreactive proteins in wild-type mouse embryo fibroblasts are products of the mdm2 gene. These same two proteins were over-represented in the lysate from DM3T3 cells. The larger MDM2 protein expressed in DM3T3 cells has previously been designated "p90," whereas the smaller has been referred to as "p76" (17, 33). Our result indicates that both p90 and p76 arise from normal alleles of the mdm2 gene.

Synthesis of Two MDM2 Proteins Is Induced by UV Light—Previously, we showed that exposure of the C127 murine cell line to UV light results in a p53-dependent increase in mdm2 expression (29, 34). Western blot analysis with a polyclonal antiserum indicated that expression of two immunoreactive proteins was increased (34). To determine whether the smaller protein induced by UV light was similar to the p76 protein expressed in DM3T3 cells, we compared the mobilities of the proteins in DM3T3 cells with those in C127 cells before and after UV light (Fig. 2). The major form of MDM2 induced in C127 cells migrated with p90 and the minor form with p76. Both proteins were expressed in cells lacking p53 ((10)3), but neither protein was induced following exposure of these cells to UV light.

Pulse-chase analysis of C127 cells before and after UV light (Fig. 3) demonstrated that de novo synthesis of both p90 and p76 accounted for their increase in response to UV light. In the absence of UV light, the relative ratio of p90 to p76UV was ~4.0 (Fig. 3). Two h following exposure of C127 cells to a UV dose of 4 J/m2, the relative increase in p90 was 2.3-fold greater than that of p76UV, such that the ratio was 9.2. The p76UV protein does not appear to be a proteolytic product of p90 since it did not accumulate during the chase period (Fig. 3). Furthermore, both proteins have a half-life of ~10 min after exposure of cells to UV light. These data led us to conclude that the faster migrating protein, p76UV, was an MDM2 protein whose synthesis was induced by UV light.

Evidence That p76UV Lacks the N terminus of p90—To further characterize p76UV, we compared the mobility of p76UV to those of MDM2 proteins synthesized in rabbit reticulocyte lysates. mRNA was transcribed from the constitutive (P1) and p53-responsive (P2) promoters of mdm2 differed by the presence of exon 1, which is noncoding (Fig. 4A). Both mRNAs gave rise to multiple proteins when translated in rabbit reticulocyte lysates (Fig. 4B) (19). The mechanism by which the multiple proteins are expressed is proposed to be internal initiation since multiple AUG codons exist in the mdm2 coding sequence, and none is in an optimal ribosome-binding sequence (20, 35). The proteins are postulated to be translated from AUGs 1 (and/or 6), 50, and 62 (19). Two of the proteins translated from each mRNA migrated with mobilities similar to those of the MDM2 proteins expressed in UV-treated C127 cells (Fig. 4B). The protein that migrates the same as p90 is thought to initiate at AUG 1 (and/or 6), and the protein that migrates the same as p76UV is thought to initiate at AUG 50 (19). p76UV also migrated with a protein synthesized from an mRNA, designated B, in which AUG 50 is the first start codon (Fig. 4B, compare lanes 3 and 4). The p76 protein in DM3T3 cells has been shown to lack the ability to bind p53, as has an exogenously expressed MDM2 protein lacking amino acids 1–49 of p90 (26). To test whether p76UV could co-immunoprecipitate with p53, we used two different p53-specific antibodies whose epitopes do not overlap (Fig. 4B). Whereas p90 co-immunoprecipitated with p53 when either antibody was used, p76UV did not co-immunoprecipitate with p53 (lanes 5 and 6). These results indicate that p76UV may lack the N terminus of p90 since the p53-binding site is at the N terminus of MDM2 (28).

The epitopes of several monoclonal antibodies raised against human MDM2 have been determined (Fig. 5A) (28). Since the sequences of the human and murine MDM2 proteins are highly conserved, we used these antibodies to determine the domains of MDM2 present in p76. Monoclonal antibody 4B2 recognizes amino acids 19–50 of human MDM2 for binding (17, 28). The 4B2 antibody recognized murine p90, but not p76UV (Fig. 5B), indicating that p76UV lacks N-terminal sequences that contribute to binding by 4B2. A monoclonal antibody (3G5) with an epitope between amino acids 59 and 89 of human MDM2 recognized both p90 and p76UV, as did two monoclonal antibodies (2A10 and 4B11) that recognized epitopes in the middle (amino acids 294–339) and C-terminal (amino acids 383–491) portions of human MDM2, respectively (Fig. 5B). These results indicate that p76UV lacks the N terminus of p90.

Translation of p76UV Appears to Initiate at AUG 50—There are several potential AUG initiation codons that are conserved between mouse and human MDM2 at codons 1, 6, 50, 62, and 102 (3). Initiation of translation at either AUG 1 or 6 gives rise to a protein the size of p90 (19, 28). AUG codons at positions 50, 62, and 102 could serve as initiation codons for smaller MDM2 proteins that cannot bind p53. To identify the codon likely to serve as the initiation codon for p76UV, we compared the immunoreactivity of p76UV with that of MDM2 proteins synthesized in rabbit reticulocyte lysates (Fig. 6A). Labeling the reticulocyte lysate allowed resolution of the faster migrating species into two distinct bands (Fig. 6A). The slower migrating protein in the doublet (p74IVT) has been presumed to arise from AUG 50 (19), and the faster migrating protein in the doublet (p74IVT) has been presumed to initiate at AUG 62 (19). Thus, we asked whether any of the monoclonal antibodies used in Fig. 5B could distinguish between p76IVT and p74 IVT (Fig. 6A). As expected, all the antibodies recognized p90. Only the 3G5 antibody distinguished between proteins initiated at AUGs 50 and 62. The assumptions that p76IVT initiates at AUG 50 and p74 IVT has been presumed to initiate at AUG 62 (19). Thus, we asked whether any of the monoclonal antibodies used in Fig. 5B could distinguish between p76IVT and p74 IVT (Fig. 6A). As expected, all the antibodies recognized p90. Only the 3G5 antibody distinguished between p76IVT and p74 IVT (lane 4). In fact, the protein thought to initiate at AUG 50 (p76IVT) had identical immunoreactivity to p76UV. Neither p76IVT nor p76UV protein was precipitated by 4B2, but both were precipitated by 3G5, 2A10, and 4B11. The epitope for 3G5 on human MDM2 has been mapped to within amino acids 59 and 89, and leucine 66, tyrosine 67, and glutamine 69, which are conserved between murine and human MDM2, are part of the epitope for 3G5 (36). However, it is not known whether 3G5 would be expected to distinguish between proteins initiated at AUGs 50 and 62. The assumptions that p76IVT initiates at AUG 50 and that p74 IVT initiates at AUG 62 had to be tested before the identity of p76UV could be known.

To determine whether the p76IVT and p74 IVT proteins initiate from AUGs 50 and 62, respectively, we used site-directed
mutagenesis to generate cDNAs in which the ATG codon at position 50, 62, or 50 and 62 was changed to ATC. The cDNAs contained exons 1–12 so that p90 would serve as an internal control for translation. The mutant cDNAs were transcribed, and the resulting mRNAs were translated in reticulocyte lysates. Translation of RNAs containing single mutations resulted in synthesis of p90 and two other proteins (Fig. 6A, lanes 9 and 10). Mutation of AUG 50 resulted in loss of the slower migrating protein in the doublet (p76IVT), but revealed a new protein with mobility intermediate between those of p76IVT and p74IVT (lane 9). Mutation of AUG 62 resulted in expression of proteins identical in mobility to those expressed from pGEM1F (lane 10). Mutation of both AUGs 50 and 62 resulted in loss of p76IVT, but not p74IVT (lane 9). Mutation of AUG 62 resulted in expression of proteins identical in mobility to those expressed from pGEM1F (lane 10). Mutation of both AUGs 50 and 62 resulted in loss of p76IVT, but not p74IVT (lane 9). The protein with identical immunoreactivity to p76UV (p76IVT) initiated at AUG 50 since p76IVT was lost upon mutation of AUG 50 alone and in combination with AUG 62 (lanes 9 and 11). The protein that did not bind to 3G5 (p74IVT) is not a product of internal initiation at AUG 50 or 62 since it was not lost upon mutagenesis. This protein may be the product of either degradation or translation from AUG 102. AUG 62 is not used as an initiation codon for p74IVT, but it is used for the protein of intermediate mobility between p76IVT and p74IVT because this protein is translated from RNA mutated at AUG 50, but not from RNA mutated at AUGs 50 and 62 (lanes 9 and 11). This protein does not appear to be translated from the wild-type RNA, indicating that “leaky scanning” gives rise to translation of this protein only in the lysate programmed with RNA containing AUC 50.

The results described above indicate that p74IVT is not initiated at AUG 62, so we have no evidence that 3G5 can distinguish between proteins initiated at AUGs 50 and 62. To determine whether the 3G5 antibody could distinguish between proteins initiated at AUGs 50 and 62, we immunoprecipitated proteins expressed from the mRNA designated F50 in which AUG 50 had been changed to AUC (lane 8). The p90 protein was also recognized by 3G5 when AUG 50 was altered to AUC (F50) (lane 8), but the smaller MDM2 proteins were not recognized, even though they were more abundant than p90 in the lysate (lane 6). This result indicates that 3G5 does not recognize MDM2 initiated at AUG 62, which is present in this translation (lane 6). An MDM2 protein with the same apparent mobility as p76UV was synthesized from an mRNA in which AUG 50 is the first methionine (B) and was recognized by 3G5 (lane 12). Together, these mapping experiments indicate that the MDM2 protein initiated at AUG 50 is recognized by 3G5, whereas the one initiated at AUG 62 is not. Hence, it is likely that p76IVT, which is recognized by 3G5 (Fig. 5B), initiates at AUG 50.

FIG. 3. p90 and p76UV have similar half-lives. UV-treated and untreated C127 cells were labeled with [35S]Express for 30 min and then incubated for 0, 20, 40, or 80 min in medium containing an excess of unlabeled methionine. Equivalent amounts of total cell protein were immunoprecipitated twice sequentially with antibody 2A10. Following the first immunoprecipitation, the samples were boiled, cooled, diluted 2-fold with lysis buffer, adjusted to 2.6% Triton X-100, and reincubated with the 2A10 antibody and protein A-Sepharose.

FIG. 4. Evidence that p76UV lacks the N terminus of p90. A, schematic of ATG codons within the first six exons of mdm2. Potential initiation codons for p76UV are in exons 4–6 of mdm2. B, p76UV does not bind p53. C127 cells were exposed to a UV dose of 4 J/m² and harvested 2 h later. A lysate was incubated with MDM2 antibody 2A10 or p53 antibodies 421 and CM5, and the resulting immunoprecipitates were analyzed by Western blotting as described in the legend to Fig. 1. Lane 1, products of in vitro translation of mdm2 RNA containing exons 1–12 (F); lane 2, products of in vitro translation of mdm2 RNA lacking exon 1 (X2); lane 3, products of in vitro translation of mdm2 RNA lacking AUGs 1 and 6 (B); lane 4, products of immunoprecipitation of C127 cell lysate (100 μg) with MDM2 antibody 2A10; lane 5, products of immunoprecipitation of C127 cell lysate (1.5 mg) with p53 antibody 421; lane 6, products of immunoprecipitation of C127 cell lysate (1.5 mg) with p53 antibody CM5.


Both Alternative Splicing and Internal Initiation Can Give Rise to a Protein the Size of p76UV in Intact Cells—There is strong evidence that internal initiation of mdm2 mRNAs gives rise to multiple MDM2 proteins in rabbit reticulocyte lysates, but it has not been determined whether internal initiation of mdm2 mRNAs occurs in cells. Smaller proteins could also arise through alternative splicing of exons 3–5 of mdm2 since any one of three AUG codons could become the first initiation codon (Fig. 4A) (37). Deletion of exon 3 alone would place AUG 50 as the first methionine, and RNAs lacking exon 3 have been detected in cells expressing multiple MDM2 proteins (20).

To determine whether full-length and alternately spliced mRNA species lacking exon 3 were capable of directing synthesis of a protein with mobility similar to that of p76 in intact cells, we transfected mammalian cDNA expression plasmids, which contain the SV40 origin of replication (24), ensuring high levels of expression of the MDM2 proteins. The two normally spliced mRNAs initiated at the P1 promoter (containing exons 1, 2, and 4–12; D), and an artificially engineered mRNA (lacking exon 3) (29). mRNA lacking exon 3 was not detected in RNA from C127 cells using primer extension with a radiolabeled primer complementary to exon 4. Since p76 is expressed in C127 cells in the absence of exposure to UV light, it seemed reasonable to hypothesize that internal initiation was the mechanism through which this protein was expressed in these cells. However, if an mRNA lacking exon 3 were expressed at levels undetectable by the primer extension assay, it might still be a source for the small amount of p76 made. To test whether unirradiated C127 cells expressed mdm2 mRNA lacking exon 3, we used RT-PCR to amplify cDNAs containing exons 1 and 4 of mdm2. Two species were cloned and sequenced. One contained exons 1–4; the other contained exons 1, 2, and 4. Therefore, both internal initiation and alternative splicing are possible mechanisms for expression of p76 in unirradiated C127 cells.

An Alternatively Spliced RNA Initiated at the Internal Promoter of mdm2 mRNA Is Expressed in C127 Cells—Previously, we showed that the most abundant mdm2 RNA in C127 cells is initiated at the P1 promoter and contains exon 3 (29). mRNA lacking exon 3 was not detected in RNA from C127 cells using primer extension with a radiolabeled primer complementary to exon 4. Since p76 is expressed in C127 cells in the absence of exposure to UV light, it seemed reasonable to hypothesize that internal initiation was the mechanism through which this protein was expressed in these cells. However, if an mRNA lacking exon 3 were expressed at levels undetectable by the primer extension assay, it might still be a source for the small amount of p76 made. To test whether unirradiated C127 cells expressed mdm2 mRNA lacking exon 3, we used RT-PCR to amplify cDNAs containing exons 1 and 4 of mdm2. Two species were cloned and sequenced. One contained exons 1–4; the other contained exons 1, 2, and 4. Therefore, both internal initiation and alternative splicing are possible mechanisms for expression of p76 in unirradiated C127 cells.

An Alternatively Spliced RNA Initiated at the Internal Promoter of mdm2 mRNA Is Induced in Response to UV Light—The normally spliced mdm2 mRNA induced by UV light (X2) is an inefficient template for translation of p76 (Fig. 7). Since p76 is induced in C127 cells by UV light, we investigated whether some of the transcripts initiated at the P2 promoter were spliced to lack exon 3. A sensitive, quantitative, radioactive RT-PCR assay was designed to detect RNAs containing and lacking exon 3. The amount of each mRNA was determined before (Fig. 9A) and after (Fig. 9B) exposure of C127 cells to UV light. Two sets of primers were used. One set, in exons 1 and 4, amplified only mRNAs containing exon 1 (initiated at the P1 promoter). The other set, in exons 2 and 4, amplified mRNAs initiating at both the P1 and P2 promoters. The primer in exon 4 was end-labeled, and reactions were performed with increasing amounts of an internal standard so that the amount of each mdm2 mRNA species could be quantified. By subtracting the amount of mRNA initiated from the P1 promoter from the total, the amount of mRNA transcribed from the P2 promoter could be calculated. In untreated C127 cells (Fig. 9A), most of the mdm2 mRNA initiated at the P1 promoter and contained exon 3. Twenty-five percent of the mRNA synthesized from the P1 promoter was alternatively spliced and lacked exon 3. Little normally spliced or alternatively spliced mRNA was expressed from the P2 promoter in the absence of DNA damage. Two h following exposure to a dose of UV light of 4 J/m², there was no

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3 L. J. Saucedo, unpublished results.
change in the amount of either RNA initiated from the P1 promoter (Fig. 9B). However, the amount of mRNA transcribed from the P2 promoter and containing exon 3 was increased 14-fold. At the same time, the amount of mRNA initiated from the P2 promoter and lacking exon 3 was increased 3-fold. These results indicate that some or all of the increase in p76UV expression following exposure to UV light is mediated by alternative splicing of an induced RNA.

**DISCUSSION**

We have shown here that the p76 MDM2 protein is synthesized in normal cells. Alternatively spliced mdm2 mRNAs lacking exon 3 express p76, but not p90, and such mRNAs are induced by UV light. Synthesis of p76 is initiated at an AUG codon at position 50 of the coding sequence for p90, and p76 lacks the N-terminal domain required for interaction with p53. However, the protein retains many of the structural motifs of p90, including the nuclear localization signal, nuclear export signals, acidic domain (20), and RING finger (38). It is likely that p76 shares some functions with p90, and its presence in normal cells indicates that it may mediate some of the physiological functions of MDM2.

The ability of MDM2 to stimulate the degradation of p53 requires that MDM2 bind p53 (10, 11). However, the level of p76 may influence the stability of p53 because p76 could bind factors that regulate the process. For example, p76 binds the p19ARF protein, which inhibits the ability of p90 to stimulate degradation of p53 (13). An increase in the amount of p76 relative to p90 might free p90 to be more effective at stimulating degradation of p53. Alternatively, it is possible that p76 is a dominant-negative inhibitor of the ability of p90 to stimulate degradation of p53 since p76 might titrate factors required for that function of p90. We have preliminary evidence that the second model is correct. Overexpression of p76 antagonizes the
A multiple MDM2 proteins are overexpressed (7, 18). The human independent of p53 (4, 14, 15). In a subset of human tumors, the oncogenic effects of MDM2 are mediated by mechanisms other than p53 for degradation. Evidence suggests that some of ligase activity of MDM2 (40). Perhaps p76 targets a protein identified HECT domain, which is required for the ubiquitin functions of MDM2. For example, p76 retains the recently determinants of cell survival.

Dissection of the functions of p76 may reveal p53-independent functions of MDM2. For example, p76 retains the recently identified HECT domain, which is required for the ubiquitin ligase activity of MDM2 (40). Perhaps p76 targets a protein other than p53 for degradation. Evidence suggests that some of the oncogenic effects of MDM2 are mediated by mechanisms independent of p53 (4, 14, 15). In a subset of human tumors, multiple MDM2 proteins are overexpressed (7, 18). The human gene has AUG codons at positions 1, 6, 50, 62, and 102 (3), and a human p76 protein is expressed in normal fibroblasts. It may be that p76 is overexpressed in tumors and contributes to development of cancer. Identification of any oncogenic properties of p76 is important because therapeutic interventions are being designed to disrupt the interaction between p90 and p53. In tumors overexpressing p76, such therapies may not be effective.

Acknowledgments—We thank Moshe Oren for mdm2 cDNA constructs; Arnold J. Levine for monoclonal antibodies to p53, MDM2, and T-antigen; Angie Teresky for wild-type mouse embryo fibroblasts; Guillermina Lozano for p53-null and p53/mdm2-null mouse fibroblasts; and Bill Sugden for COS-1 cells. The technical expertise of Marisa Holubar and Amy Prevost is greatly appreciated. We gratefully acknowledge Nancy Thompson and Kit Nolan for advice on typing monoclonal antibodies and John Petrini for advice on site-directed mutagenesis. We appreciate stimulating conversations about this work with Marilyn Kozak, Moshe Oren, and Jeff Ross and insightful comments on the manuscript by Chris Bradfield, Susan Mendrysa, and Jeff Ross.

REFERENCES
1. Montes de Oca Luna, R., Wagner, D. S., and Lozano, G. (1995) Nature 378, 203–206
2. Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995) Nature 378, 206–208
3. Oshiro, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L., and Vogelstein, B. (1992) Nature 358, 80–83
4. Lundgren, K., Montes de Oca Luna, R., McNeil, Y. B., Emeric, E. P., Spencer, B., Barfield, C. R., Lozano, G., Rosenberg, M. P., and Finlay, C. A. (1997) Genes Dev. 11, 714–725
5. Chen, C.-Y., Oliner, J. D., Zhan, Q., Fornace, A. J., Vogelstein, B., and Kastan, M. B. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 2684–2688
6. Dubs-Potrzebowski, M. C., Tocque, B., and Wasylyk, B. (1995) Oncogene 11, 2445–2449
7. Leach, F. S., Tokino, T., Meltzer, P., Burrell, M., Oliner, J. D., Smith, S., Hill, D. E., Sidransky, D., Kinzler, K. W., and Vogelstein, B. (1993) Cancer Res. 53, 2231–2234
8. Haupt, Y., Barak, Y., and Oren, M. (1996) EMBO J. 15, 1596–1606
9. Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. (1992) Cell 69, 1237–1245
10. Haupt, Y., Maya, R., Kozak, A., and Oren, M. (1997) Nature 387, 296–299
11. Rubenstien, M. H. G., Jones, S. N., and Vousden, K. (1997) Nature 387, 299–303
12. Zhang, Y., Xiong, Y., and Yarbrough, W. G. (1998) Cell 92, 725–734
13. Pommerantz, J., Schreiber-Aguas, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlew, L., Lee, H.-W., Cordon-Cardo, C., and DePinho, R. A. (1998) Cell 92, 713–723
14. Martin, K., Troupe, D., Hagemeier, C., Suresen, T. S., La Thangue, N. B., and Krouskides, T. (1995) Nature 375, 691–694
15. Xiao, Z.-X., Chen, J., Levine, A. J., Moghaddam, N., Xing, J., Sellers, W. R., and Livingston, D. M. (1998) Nature 375, 694–698

4 M. Holubar and M. E. Perry, unpublished results.
16. Sigalis, I., Calvert, A. H., Anderson, J. J., Neal, D. E., and Lunec, J. (1996) *Nat. Med.* 2, 912–917
17. Olson, D. C., Marechal, V., Momand, J., Chen, J., Romocki, C., and Levine, A. J. (1993) *Oncogene* 8, 2353–2360
18. Landers, J. E., Haines, D. S., Strauss, J. F., and George, D. L. (1994) *Oncogene* 9, 2745–2750
19. Barak, Y., Gottlieb, E., Juven-Gershon, T., and Oren, M. (1994) *Genes Dev.* 8, 1739–1749
20. Fakharzadeh, S. S., Trusko, S. P., and George, D. L. (1991) *EMBO J.* 10, 1565–1569
21. McMasters, K. M., Montes de Oca Luna, R., Pena, J. R., and Lozano, G. (1996) *Oncogene* 13, 1731–1736
22. Sherley, J. L. (1991) *J. Biol. Chem.* 266, 24815–24828
23. Harvey D. M., and Levine, A. J. (1991) *Genes Dev.* 5, 2375–2385
24. Gluzman, Y. (1981) *Cell* 23, 175–182
25. Papworth, C., Bauer, J. C., Braman, J., and Wright, D. A. (1995) *Strategies* 9, 3–4
26. Haines, D. S., Landers, J. E., Engle, L. J., and George, D. L. (1994) *Mol. Cell. Biol.* 14, 1171–1178
27. Chu, G., and Sharp, P. A. (1981) *Gene* (Amst.) 13, 197–202
28. Chen, J., Marechal, V., and Levine, A. J. (1993) *Mol. Cell. Biol.* 13, 4107–4114
29. Saucedo, L. J., Carstens, B. C., Seavey, S. E., Albee, L. D., and Perry, M. E. (1996) *Cell Growth Differ.* 9, 119–130
30. Lane, D. P., Stephen, C. W., Midgley, C. A., Sparks, A., Hupp, T. R., Daniels, D. A., Greaves, R., Reid, A., Vojtesek, B., and Picksley, S. M. (1996) *Oncogene* 12, 2461–2466
31. Kirchmaier, A., and Sudgen, B. (1996) *J. Virol.* 70, 1766–1775
32. Cahilly Snyder, L., Yang-Feng, T., Francke, U., and George, D. L. (1987) *Somatic Cell Mol. Genet.* 13, 235–244
33. Hinds, P. W., Finlay, C. A., Quarrin, R. S., Baker, S. J., Fearon, E. R., Vogelstein, B., and Levine, A. J. (1990) *Cell Growth Differ.* 1, 571–580
34. Perry, M. E., Piette, J., Zawadowski, J. A., Harvey, D., and Levine, A. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 11623–11627
35. Kozak, M. (1984) *Nature* 308, 241–246
36. Bottger, A., Bottger, V., Garcia-Echeverria, C., Chene, P., Hochkeppel, H.-K., Sampson, W., Ang, K., Howard, S. F., Picksley, S. M., and Lane, D. P. (1997) *J. Mol. Biol.* 269, 744–756
37. Montes de Oca Luna, R., Tabor, A. D., Ebner, G., Hulboy, D. L., Worth, L. L., Coleman, M. S., Finlay, C. A., and Lozano, G. (1996) *Genomics* 33, 352–357
38. Boddy, M. N., Freemont, P. S., and Borden, K. L. B. (1994) *Trends Biochem. Sci.* 19, 198–199
39. Zeigler, A., Jonason, A. S., Lefell, D. J., Simon, J. A., Sharma, H. W., Kimmelman, J., Remington, L., Jacks, T., and Brash, D. E. (1994) *Nature* 372, 773–776
40. Honda, R., Tanaka, H., and Yasuda, H. (1997) *FEBS Lett.* 420, 25–27
41. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY