A small nuclear RNA, hdm365, is the major processing product of the human mdm2 gene

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ABSTRACT

mdm2 encodes for an E3 ubiquitin ligase targeting constitutively expressed p53 for proteasomal degradation. Several protein isoforms have been described for human MDM2 (HDM2), some of which may correspond to splicing variants detectable by RT–PCR in many tumors. Upon cellular stress, p53 becomes resistant to MDM2 and, in a feedback loop, up-regulates mdm2 transcription. The physiological relevance of stress-induced mdm2 gene activity is not well understood. We describe a small nuclear RNA of 365 bases comprised of the first five hdm2 exons and lacking polyadenylation. hdm365 precedes full-length hdm2 RNA expression after induction by p53 and accumulates to significant levels in the nucleus, detectable at the site of hdm2 transcription and processing only. Considering a 10-fold lower stability and high steady-state levels of the novel RNA species, hdm365 appears to be the major processing product of hdm2 transcripts. hdm365 induction was observed after ectopic expression of p53 and after DNA damaging treatment of tumor cell lines, primary fibroblasts and lymphocytes, and was not related to apoptosis. Corresponding truncated transcripts were observed in hdm2 amplified cells. High stress-inducible expression levels, absence of a corresponding protein, and nuclear localisation of hdm365 suggest a novel RNA-based function for hdm2.

INTRODUCTION

The murine double-minute 2 (mdm2) gene was originally cloned as an amplified gene present on double minute chromosomes in the tumorigenic 3T3DM murine cell line (1). The human homologue, hdm2, mapping to human chromosome 12q13–14, is overexpressed in over 30% of soft tissue sarcomas due to amplification, but amplification is rare in other tumor types, the overall frequency being 7% (2,3). Ectopic mdm2 expression transformation NIH3T3 cells (1), cooperates with activated ras to immortalise rat embryo fibroblasts (4) and, when introduced into murine breast epithelium, uncouples S phase from mitosis and leads to breast tumors with long latency (5,6). HDM2 disrupts the cell cycle between G1 and S phases when overexpressed in untransformed cell lines or normal fibroblasts (7). These oncogenic properties have been attributed to the consequences of interaction between MDM2 protein and the N-terminal transactivation domain of the tumor suppressor p53. As a result, activation of p53 target genes involved in growth arrest and apoptosis is blocked (8–12). In addition, MDM2 serves as an E3 ligase that ubiquitinates p53 and itself (13,14), targeting p53 to the cytoplasm for 26S proteasome-dependent degradation (15–19). Consequently, unrestrained p53 accumulation in mdm2 null embryos results in early lethality if not rescued by co-deletion of p53 (20,21). MDM2 also serves additional, apparently p53-independent functions, since mdm2 transgenic mice show p53-independent altered tissue-specific differentiation (22) and increased tumor incidence (23). This may at least in part be attributed to MDM2 binding of and functional interference with other cell cycle regulatory proteins, including pRb (24), p300 (25), E2F1 (26) and MTBP (27). However, these interactions cannot be completely separated from the communication between p53 and MDM2 since both pRb and p300 have been identified as components of ternary complexes with these two proteins (25,28). MDM2 further bridges p53 and pRb pathways via ternary complex formation with p53 and the alternative ink4a gene product ARF, which binds to its C-terminus (29). ARF expression leads to stabilisation of p53 by a mechanism which may involve increased turnover of MDM2 itself and/or inactivation of the intrinsric ubiquitin ligase activity of MDM2 for p53 (30–32). ARF binding to MDM2 sequesters it to the nucleolus thereby preventing negative feedback regulation of p53 by MDM2 and leading to activation of p53 in the nucleolus (33). In addition to post-translational regulation by ARF and by complex phosphorylations on p53- and ARF-binding domains (34), MDM2 expression is controlled on the transcriptional level. It is transcribed from two different promoters (35–37) leading, in humans, to two alternatively spliced transcripts that differ in their 5′ untranslated regions. Transcription from the first promoter P1 yields a mRNA (L-hdm2) with exon 2 spliced out. Transcription from this promoter is p53 independent. Conversely, transcription from the second promoter P2 is dependent on p53 binding to two p53-responsive elements in intron 1 giving rise to a transcript (S-hdm2) lacking exon 1 but containing exon 2. L-hdm2 contains two upstream open

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reading frames conferring inefficient translation when compared to S-hdm2 (38), which leads to low constitutive levels of HDM2 protein regulating basal p53 levels. Upon activation of p53, transcription from P2 is initiated, resulting in a rapid increase in HDM2 levels to which activated p53 is insensitive due to MDM2 binding site phosphorylation (39–43). Both L-hdm2 and S-hdm2 RNAs encode a 90 kDa full-length HDM2 (p90) protein. In addition, MDM2 and HDM2 proteins of smaller sizes forming multiple complexes with p53 have been identified (44). These differently sized proteins arise through either proteolytic cleavage (45), internal translational initiation (46) or alternative splicing (47,48). Alternatively spliced hdm2 RNAs are present in many tumors, mostly at levels detectable by means of reverse transcriptase–polymerase chain reaction (RT–PCR) only. Among these variant hdm2 transcripts there are frequently aberrantly spliced products (49). Most of them encode proteins that lack the p53-binding domain but retain the ability to transform NIH3T3 cells (48). The different HDM2 protein species localise to different cellular compartments (50). Internally initiated MDM2 (p76) lacking the first 49 amino acids antagonises the ability of MDM2 p90 to stimulate the degradation of p53 and is expressed in a tissue-specific manner in the mouse (51). An alternatively spliced HDM2 protein from non-small cell lung carcinomas lacking the nuclear localisation signal binds and sequesters normal HDM2 protein from non-small cell lung carcinomas lacking the p53-binding domain but retain the ability to transform NIH3T3 cells (48). The different HDM2 protein species localise to different cellular compartments (50). Internally initiated MDM2 (p76) lacking the first 49 amino acids antagonises the ability of MDM2 p90 to stimulate the degradation of p53 and is expressed in a tissue-specific manner in the mouse (51). An alternatively spliced HDM2 protein from non-small cell lung carcinomas lacking the nuclear localisation signal binds and sequesters normal HDM2 to the cytoplasm preventing it from binding to p53 (52). Thus, at least in tumor cells, regulation of p53 stability may be compromised by the presence of variant hdm2 transcripts, supported by the finding that expression of several alternatively spliced, out-of-frame hdm2 transcripts lacking the p53-binding domain correlates with increased levels of wild-type p53 in glioblastoma multiforme (53).

Here, we describe an abundant S-hdm2 derived, truncated transcript resulting from incomplete splicing that precedes full-length S-hdm2 expression in p53 response and that accumulates to significant levels in the nucleus.

### MATERIALS AND METHODS

#### Cell lines and primary cells

All Ewing’s sarcoma family tumour (EFT) cell lines and their p53 gene status have previously been described (54). Cell lines SK-VAL3 and SK-VAL8 expressing temperature-sensitive human p53-138V and a VP16–p53-138V hybrid, respectively, were derived from p53-negative SK-N-MC cells by stable transfection of a CMV promoter-driven expression vector (55). A K562 chronic myelogenous leukemia cell line expressing p53-138V was a gift of N. Tsuchida (Tokyo Medical and Dental University). The hdm2 amplified NB1691 neuroblastoma and Rh18 rhabdomyosarcoma cell lines, and the neuroblastoma cell line SJ-NB7, were kindly provided by P. Houghton and T. Look (St Jude Children’s Research Hospital). NIH3T3 cells were obtained from the American Type Culture Collection (ATCC). The rat embryo fibroblast clone c69 carrying temperature-sensitive p53-135V has previously been described (56). Primary skin fibroblasts were obtained by mechanical disruption from punch biopsies derived from healthy volunteers. Mononuclear cells were obtained from peripheral blood of a healthy donor by Ficoll gradient centrifugation and cultivated for 4 days in the presence of recombinant human IL2. Cell cultures were routinely maintained in RPMI-1640 (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, penicillin (0.1 mg/ml) and streptomycin (0.1 mg/ml) in 5% CO2 at 37°C. X-ray treatment (6 Gy) was performed using a Phillips RT100 irradiation device (1.7 mm aluminium filter, 100 kV) at 12.5 Gy/min. Actinomycin D was used at a concentration of 1 µg/ml to inhibit de novo RNA synthesis.

#### Expression analyses

Extraction of total RNA was performed according to standard procedures. Poly(A)+ RNA was isolated from total RNA by binding to oligo(dT)25 Dynabeads (Dynal; Dynal; Hamburg, Germany) according to the manufacturer’s recommendations. Separation of nuclear and cytoplasmic RNA was performed according to a previously published protocol (57). The following hdm2 probes were generated by PCR: exon 2, primers CTGTGTTCTAGTGCTGGATGGA and GATCAGACGAGAAAGTG; exons 3–5, primers ATGTTGAAAACATTACCAATGGTTCTGG; and CTGTGCTCTTTCACAGAGGCTTG; exons 6–9, primers GATCTACAGGGACATTGTTGAG and GATTCGATGGCTCCTTGATG; exons 10–12, primers GTGACACATTAGTTATAGTTGG and GATCCTATAGACAGGTCAACTAG; intron 5, primers CTGGGCGATGATTATATGACTAAACGATTATAGTATAGTATAGGCTACACAGTACACAGTTCTTGAGTTTGAC. A mouse-specific mdm2 exons 3–5 probe was amplified from irradiated NIH3T3 cells using primers GTGAACATTAGGTGTTGGA and GTCTGCTCTCAGAGGT. A probe for human U2 small nuclear RNA (snRNA) was generated with primers CATCCCTCTCCGCTTCTTGG and TGGAGGTACTGCAATTACAGGAG. Northern blotting was performed according to standard procedures. Analysis of cells transfected with hdm2 expression constructs was performed on DNase-pretreated RNA in order to avoid hybridisation to contaminating plasmid DNA. Signal intensities were quantitated after autoradiography by exposing the filters overnight to a Packard screen and scanning at 50 µm resolution in a phosphorimager instrument (Cyclone Instrument; Packard, Meriden, CT).

For the amplification of hdm2 splicing variants primers TGTCGCAACACTTAGTGCTGT and CTAGGGAAAATGTTAGAC from the ends of the hdm2 coding region were used. HDM2 protein analysis was performed on western blots of 12% Laemmli gels after blotting on either standard PROTRAN BA85 (pore size 0.45 µm) or BA83 (pore size 0.20 µm) cellulose nitrate membrane using antibodies 4B2 and 4B11 (kindly provided by A. Levine, Princeton University, Princeton, NJ) and SMP14 (DAKO, Glostrup, Denmark). For the inhibition of proteasome activity, cells were cultivated overnight in the presence of 10 µM Z-Leu-Leu-Leu-aldehyde (MG-132; Alexis Biochemicals, Lausen, Switzerland). The inhibitor was also present during protein extraction.

#### Cloning of hdm365 RNA

Aliquots of 10 and 50 µg of total RNA from SK-VAL3 cells shifted to 32°C for 8 h were mixed with a formamide-containing sample buffer and separated along with flanking 32P-labelled size markers (50 c.p.m.) in two slots on a 5% polyacrylamide gel containing 8 M urea in 1× TBE for 3 h at...
400 V. One part of the gel (containing 50 μg RNA) was directly exposed to X-ray film and stored at -70°C. The other part of the gel (containing 10 μg RNA) was electrophoretically transferred to Hybond N membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ) in 0.5 × TBE at 35 mA overnight, hybridised to a hdm2 probe and exposed to X-ray film. Subsequently the stored preparative gel and the autoradiographs were aligned according to the marker bands and a gel slice was cut from the preparative gel at the position of the hybridising band on the northern blot. RNA was eluted into 400 μl DEPC-treated water at 45°C overnight. The purified RNA was then cloned by a RNA–DNA ligation-mediated approach modified from the method of Hetzer and Mueller (58). Briefly, after adding 50 ng EcoRI-digested pBluescript SK+ (Stratagene, La Jolla, CA), nucleic acids were ethanol precipitated, dissolved in water and concentrated to a volume of 6 μl. RNA and DNA were ligated using 17 U T4 RNA ligase (Amersham Pharmacia Biotech Inc.) in a 20 μl reaction containing 50 mM HEPES pH 8.0, 20 mM MgCl₂, 20 mM MnCl₂, 3 mM DTT, 0.1 mM ATP, 0.3 ng BSA at 4°C overnight. Aliquots of 2.3 μl of the ligation reaction (containing Mn²⁺ ions) were then subjected to a combined reverse transcription–amplification reaction using 4 U Tth polymerase (GeneCraft, Munster, Germany). Reverse transcription primed by the recessed DNA 3' ends of the EcoRI-digested vector was accomplished by incubation in 20 μl 10 mM Tris pH 8.9, 90 mM KCl, 1 mM dNTP at 60°C for 35 min. Subsequently a preheated PCR master mix consisting of 50.6 μl water, 8 μl 10% PCR buffer (100 mM Tris pH 8.9, 1 M KCl, 15 mM MgCl₂, 500 μg/ml BSA, 0.5% Tween 20), 6.4 μl 10 mM dNTP, 10 μl EGTA (7.5 mM) and 2 μl (20 pmol) of each first round PCR primer was added and PCR performed for 45 cycles (10 cycles of 30 s at 94°C, 30 s at 58°C, 45 s at 72°C, followed by 35 cycles of 30 s at 94°C, 30 s at 50°C and 90 s at 72°C, with a final extension of 5 min at 72°C). First round PCR primers were the hdm2 exon 3 sense oligonucleotide TGGTCAATACCACACTGTCTG and the vector-specific T3 primer AATTAACCCTCACTAAAGGG. An aliquot of 1 μl of the reaction was then subjected to two rounds of nested PCR with hdm2-specific sense primers CACCTACAGTTCAGCTTC and CTGGCAGTGA-TATTAGGC, respectively, and the vector-specific antisense primer CGCTCTAGAACTAGTGGATC. For the determination of the hdm365 5’ end, 5’ RACE was performed using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA) according to the manufacturer’s recommendations. Briefly, adaptor ligated double-stranded cDNA obtained from the eluted RNA by random hexamer primed cDNA synthesis was amplified with the hdm2 exon 3 antisense primers AGTCA-TAA TATAGC (first round) and GAAGCAGTGAATCTGAGGTT (second round) in combination with adaptor-specific oligonucleotides.

All PCR products were size fractionated on agarose gels and cloned into the pGEM-T Easy vector (Promega, Madison, WI).

**Transfections**

For RNA transfections, templates generated by PCR were transcribed in vitro using the MEGAscript T7 kit (Ambion, Austin, TX). In vitro synthesised RNA was treated with 5 U DNase I and purified by phenol/chloroform extraction and ethanol precipitation. RNA quality was monitored on a formaldehyde-containing agarose gel. Aliquots of 50 ng of RNA were transfected using LipofectAMINE Plus reagent (Invitrogen, Groningen, The Netherlands) according to the manufacturer’s instructions.

**RNA fluorescence in situ hybridisation (RNA-FISH)**

RNA in situ hybridisation was performed essentially as described (59). Briefly, cells were fixed in 1% formaldehyde, 5% acetic acid in 1× PBS for 15 min, extensively washed, and digested with 10 μl proteinase K (DAKO) in 50 mM MgCl₂ for 5 min. After repeated washes, cells were dehydrated in a series of ethanol (70, 80 and 96%) and air dried. After denaturation for 5 min at 80°C, hybridisations were performed overnight at 37°C using 10 ng of digoxigenin- or biotin-labelled PCR products in mRNA hybridisation solution (DAKO). For the simultaneous detection of two RNAs or different RNA regions, two differentially labelled (biotin and digoxigenin) PCR probes (10 ng of each probe) were used. Cells were rinsed in PBS and washed with 4× SSC, 0.2% Tween 20 twice at room temperature and twice at 70°C. After blocking with BSA (2% in washing solution), hybridisation signals were detected with anti-digoxigenin–FITC (1:50) (Roche, Indianapolis, IN) or anti-digoxigenin–Cy3 (1:200) (Jackson ImmunoResearch, Westgrove, PA), or anti-biotin–Cy3 (1:200) (Jackson ImmunoResearch) antibodies diluted in blocking solution. DAPI containing mounting medium (Vector, Burlingame, CA) was used for counterstaining of nuclei. FISH signals were evaluated with a Leitz Orthoplan microscope with 100× lens and appropriate filter sets for red, green and blue fluorescence. Pictures were taken with a CCD camera and analysed after background subtraction using the ISIS software (Metasystems, Belmont, MA). For each experiment, between 20 and 50 cells showing intact morphology were randomly photographed and analyzed.

For the simultaneous detection of RNA and DNA sequences, cells were permeabilised after fixation by incubating in preheated target retrieval solution (DAKO) for 30 min at 80°C in order to increase access of the probes to chromosomal DNA. Genomic sequences flanking the hdm2 gene were detected with a biotin-dUTP-labeled YAC probe 747c7 (60) (obtained from a CEPH library, amplified by Alu PCR and labeled by nick translation). The probe was mixed with digoxigenin-dUTP-labelled hdm2 exon-specific PCR products as indicated and the mixture was applied to the cells. After denaturation for 5 min at 94°C hybridisation and detection of signals was performed as described above.

**RESULTS**

**Detection of an hdm2 related small RNA species induced by p53**

During studies on the integrity of the p53 DNA damage response pathway in EFT cell lines upon X-ray treatment (6 Gy) we tested the expression of known p53 transcriptional targets on classical northern blots. For the detection of hdm2 transcripts, an S-hdm2-specific exon 2 probe was used. In addition to full-length S-hdm2 mRNA of ~5–6 kb, induction of a second, small RNA species (hdm365) was consistently observed in the wild-type p53-expressing EFT cell lines WE-68, SAL2 and STA-ET-1, as well as faintly in STA-ET.2.2
carrying a p53-277Y mutation that retains partial transcriptional activity (our unpublished observations; 61) (Fig. 1A). The relative abundances of S-hdm2 and of the small transcript varied between cell lines. Note that in WE-68 cells at 4 h post-irradiation, the signal obtained for hdm365 was even stronger than that for full-length S-hdm2. Reactivity of hdm365 with the exons 3–5 probe suggested the presence of coding sequences in the small RNA variant. Similar results were obtained for wild-type p53-expressing cell lines derived from neuroblastoma and rhabdomyosarcoma and upon treatment with the topoisomerase inhibitor etoposide (VP16), indicating that induction of hdm365 was neither restricted to EFT nor specific to X-ray treatment (not shown). The two RNA species were also detectable at 32°C in temperature-sensitive p53-138V-expressing cells derived by stable transfection from SK-N-MC (SK-VAL3) (55) (Fig. 1) and K562 (not shown) cell lines. This result suggests that hdm365 expression is a direct consequence of p53 activity rather than a secondary result of cytotoxic treatment.

Figure 1B demonstrates that hdm365 expression is not restricted to cancer cell lines since it was also readily detectable in primary human mononuclear cells and in primary skin derived fibroblasts after irradiation. In one of two experiments performed using skin fibroblasts hdm365 even predominated over full-length hdm2 RNA at 2 h post-irradiation. However, when we tested rodent cells—NIH3T3 cells after irradiation and the rat embryo fibroblast clone cl6 carrying the murine temperature-sensitive mutant p53-135V (56) upon shift to 32°C—no transcript corresponding to hdm365 was detected, either with a human exons 3–5 probe exhibiting about 90% homology to rodent mdm2 or with a corresponding murine probe (Fig. 1C). This result indicates that the expression of hdm365 is species specific.

Next we tested if hdm365 is generated by aberrant processing or degradation of full-length hdm2 RNA as a result of apoptosis. SK-VAL8 cells stably express a hybrid temperature-sensitive p53-138V variant in which the p53 N-terminus has been replaced by the HSV VP16 transactivation domain. In contrast to SK-VAL3, SK-VAL8 cells do not die when shifted to the permissive temperature (55). Figure 1D demonstrates that hdm365 was equally induced in both apoptosis-proficient SK-VAL3 and apoptosis-deficient SK-VAL8 cells. Thus, hdm365 expression is directly linked to the transcriptional activity of p53.

Figure 1. Identification of a small S-hdm2 related transcript. (A) hdm365 expression in EFT cell lines upon X-ray treatment. Northern blot with total RNA extracted from the indicated EFT cell lines before and at 4 and 7 h post-irradiation with 6 Gy and hybridised to an hdm2 exon 2 probe. SK-VAL3 cells carrying temperature-sensitive p53-138V and shifted to 32°C for 4 h serve as a positive control. The 70 bp exon 2 probe tends to non-specifically cross-hybridise to 28S and 18S rRNA. (B) hdm365 expression in primary human cells upon X-irradiation. One experiment with mononuclear cells (MNC) and two independent experiments with unrelated primary human skin fibroblast cultures are presented. RNA from SK-VAL3 cells at 32°C is shown for comparison. (C) Absence of expression of a hdm365 analogous RNA species in rodent cells. mdm2 related transcripts were monitored in murine NIH3T3 cells before and 6 h after irradiation (6 Gy) and in the rat embryo fibroblast clone cl6 expressing temperature-sensitive murine p53 at 37 and 32°C. The human hdm2 exons 3–5 probe exhibits 90% homology to the corresponding region on mouse and rat mdm2 RNA. The murine exons 3–5 probe shows significant cross-hybridisation to 28S and 18S rRNA. Note that, in contrast to the corresponding human transcript of ~6 kb, rodent full-length mdm2 RNA is only 3.3 kb long. M, RNA size marker. (D) Detection of the small RNA variant in apoptosis-proficient SK-VAL3 cells and apoptosis-deficient SK-VAL8 cells.
Characterisation of hdm365 structure

Hybridisation of northern blots to strand-specific oligonucleotide probes indicated that hdm365 RNA displays the same orientation as full-length hdm2 transcripts (not shown). Several hdm2 splice variants have previously been described in human tumors that contain at least the ends of the hdm2 coding region (Fig. 4). We therefore argued that hdm365 may possibly represent an aberrant splicing product. RT–PCR using primers from the ends of the HDM2 coding region was performed on RNA extracted from SK-N-MC and SK-VAL3 cells and a cell line expressing the temperature-sensitive p53-143A mutant. Upon activation of p53 by temperature shift to 37°C the permissive temperature a prominent product of 1470 bp was visible on northern blot (Fig. 3B). The RNA eluted from a 5 mm gel slice was cloned and the inserts of 16 independent clones were sequenced. All of them extended into hdm2 exon 5, the 3' end of hdm2-C, respectively, were induced (Fig. 2). However, the RT–PCR approach failed to detect a product corresponding to hdm365, which on the northern blot appeared to be considerably smaller than 1 kb.

Fast migration in formaldehyde–agarose gel electrophoresis may be characteristic of circular RNAs generated during the process of alternative splicing (62). However, using an antisense primer from the 5' end and a sense primer from the 3' end of hdm2 exon 2 for RT–PCR from random hexamer primed cDNA of p53-induced SK-VAL3 cells we were unable to detect any hdm2–specific products that may result from end joining or RNA circularisation (data not shown). Thus, hdm365 was considered to be a linear molecule.

We next tested for polyadenylation of the small transcript as a prerequisite for cloning by 3' RACE. However, as shown in Figure 3A, hdm365 did not bind to oligo(dT) beads, indicating lack of a poly(A) tail.

We therefore chose to use a RNA–DNA ligation-mediated cloning approach. Total RNA from SK-VAL3 cells kept at 32°C for 8 h was separated on a high resolution urea-containing polyacrylamide gel along with flanking radio-labelled size markers. The gel was split into two symmetrical parts, one to perform northern blotting and one for elution of RNA localised in the gel by superimposing the autoradiograph of the corresponding northern blot as detailed under Materials and Methods. On the polyacrylamide northern blot, hdm365 appeared as a single, sharp band corresponding to 360–370 bases compatible with a single RNA species of defined size (Fig. 3B). The RNA eluted from a 5 mm gel slice was ligated to EcoRI-digested vector DNA using T4 RNA ligase. The vector sequences served both the priming of cDNA synthesis via the recessed 3' restriction termini and the subsequent amplification of the hdm365 3' end combining hdm2–specific sense primers (starting from exon 3) with vector–specific antisense primers. It should be noted that this approach was only successful when Tth polymerase was used, which allowed for reverse transcription at 60°C. The requirement for high temperature during the reverse transcription reaction may indicate the presence of stable RNA secondary structures in hdm365. PCR products obtained by this approach were cloned and the inserts of 16 independent clones were sequenced. All of them extended into hdm2 exon 5, the 3' termini clustering around a short inverted repeat sequence at the end of the exon. The longest products ended exactly at the exon 5/6 boundary (Fig. 3C). 5' RACE experiments of the eluted RNA confirmed exon 2 as the starting point of hdm365 RNA. Thus, hdm365 appeared as a fully spliced RNA species extending from hdm2 exon 2 to exon 5 comprising exactly 365 nt, as already inferred from the high resolution northern blot (Fig. 3B). Hybridisation of the northern blot to oligonucleotide probes immediately flanking the exon 5/6 boundary confirmed extension of hdm365 to the end of exon 5 (not shown). The structure of this novel RNA species in comparison to known hdm2 splicing variants is schematically illustrated in Figure 4. The specific architecture of hdm365 is reminiscent of a processed pseudogene. However, on the genomic Southern blot, all hdm2 probes detected only bands of a size predicted for the single copy gene (not shown). Thus, hdm365 is a product of the intact authentic hdm2 gene.
Absence of protein expression from hdm365

Since hdm365 represents a truncated and accurately spliced version of full-length hdm2 it potentially encodes a protein of ~11 kDa comprised of the p53-binding domain only. However, an antibody (4B2), which is directed to the HDM2 N-terminus potentially present in the putative hdm365 product, did not identify any protein of the expected size, either in induced SK-VAL3 cells or in hdm2 gene amplified cell lines used as controls (Fig. 5A). Incubation of cells in the presence of the potent proteasome inhibitor Z-Leu-Leu-Leu-aldehyde (MG-132) in order to stabilise the putative hdm365 product did not result in the appearance of any band around 11 kDa, either in SK-VAL3 cells shifted to 32°C or in SJ-NB7 cells transfected with in vitro synthesised hdm365 RNA (Fig. 5B). Note that the increase in endogenous full-length HDM2 protein in MG-132-treated SJ-NB7 cells serves as a surrogate marker for efficient protein stabilisation. Probing of the western blot with a cytochrome c antibody was performed to control for efficient transfer of small proteins to the membrane. These results demonstrate that hdm365 does not give rise to a protein product. Accordingly, no protein product was obtained from in vitro transcribed hdm365 RNA (data not shown).

Kinetics of hdm365 production

Figure 6 illustrates the kinetics of hdm365 production in comparison to full-length hdm2 RNA after induction of p53 transcriptional activity in SK-VAL3 cells at the permissive temperature. Full-length S-hdm2 RNA was first detectable on the northern blot at 1.5 h after temperature shift and gradually accumulated thereafter. In contrast, hdm365 was already observed 30–60 min after p53 induction, attaining steady-state levels already at 2 h. This result suggests that hdm365 production precedes full-length S-hdm2 expression excluding RNA degradation as the cause of hdm2 RNA truncation. At the time of hdm365 appearance, an intron 5-specific probe detected a faint band larger than the fully spliced hdm2 mRNA and a major band of ~3.4 kb, which did not hybridise to any exon-specific probe and therefore most likely represents the spliced-out intron. As indicated in a cell fractionation experiment both bands were restricted to the cell nucleus, the larger band being recognised by both upstream and downstream exon-specific probes upon overexposure of the
northern blot (data not shown). Therefore, this band most likely represents a long precursor RNA. Thus, hdm365 appears to be generated by incomplete splicing rather than by premature termination of transcription at a potential small hairpin structure at the end of exon 5.

We next investigated the stability of hdm2 transcripts. SK-VAL3 cells were shifted to 32°C and subsequently treated with actinomycin D to block de novo RNA synthesis. Figure 7A illustrates that hdm365 is subject to a much faster turnover than S-hdm2. The calculated half-lives of the two RNA species, as deduced from quantification of hybridisation signals by phosphorimaging, were 20 min and 3.5 h, respectively (Fig. 7B). For S-hdm2 this number corresponded well to values obtained by real time RT–PCR (not shown). In contrast, L-hdm2 RNA monitored in p53-negative SK-N-MC cells using the exons 3–5 probe proved to be highly stable and even slightly increased during a 6 h incubation period in the presence of the transcriptional inhibitor at 32°C. As a complementary approach to assess hdm365 turnover, excluding non-specific effects of actinomycin D on RNA stability, SK-VAL3 cells were incubated at 32°C for 2 or 4 h and then shifted back to 37°C to switch the transcriptional activity of p53 off again. As shown in Figure 7C, hdm365 rapidly disappeared upon cessation of hdm2 P2 promoter activity, while S-hdm2 transcripts persisted for several hours, confirming the pronounced difference in stability between the two RNAs. Since, at 4 h of hdm2 induction, the signal intensity of hdm365 was at most three times weaker than that of S-hdm2 while the half-life was about 10 times shorter (Fig. 7B), hdm365 appears to be the major processing product of primary hdm2 transcripts.

**Subcellular localisation of hdm365**

RNA fractionation experiments indicated that hdm365 RNA is restricted to the nucleus (Fig. 8). This result is consistent with the lack of polyadenylation and of expression of a corresponding polypeptide. In fact, hdm365 accumulated to high levels in the nucleus, visible on the autoradiograph of nuclear RNA hybridised to the exons 3–5 probe already after a few hours of exposure. In contrast, the bulk of fully spliced S-hdm2 RNA appeared to be efficiently transported to the cytoplasm.

For further subcellular localisation of hdm2 RNA, FISH experiments were performed (Fig. 9). In aneuploid SK-VAL3
cells, which are tri- to tetrasonic for chromosome 12, all hdm2-specific probes detected three or four discrete nuclear spots after induction of p53. These spots were only seen in SK-VAL3 cells upon shift to the permissive temperature and not in SK-N-MC cells, and disappeared upon treatment of cells with actinomycin D. Pretreatment of slides with RNase A extinguished the hybridisation signals excluding hybridisation to chromosomal DNA as the source of FISH signals (not shown). All signals obtained with probes from the hdm2 5′ end (exons 3–5) were superimposable with the hybridisation signals for probes from the hdm2 3′ end (exons 6–9) without exception (Fig. 9A–D). Thus, hdm365 and full-length hdm2 transcripts were indistinguishable from each other in RNA-FISH, indicating that they co-localise in the nucleus. FISH signals obtained with a YAC clone (747c7) hybridising to chromosomal DNA flanking the hdm2 gene co-localised with the nuclear spots obtained with the short hdm2 exon-specific probes that monitor hdm2 RNA accumulation (Fig. 9E and F). This result indicated that nuclear hdm2 RNA is concentrated at the site of hdm2 gene transcription. Further, these sites were demonstrated to co-localise with distinct U2 snRNA-containing nuclear speckles indicating the presence of splicing factors at the transcription sites (Fig. 9G and H). RNA hybridisation signals obtained with 5′ terminal hdm2 probes could never be separated from signals obtained with 3′ terminal probes, even at 30 min after shift of SK-VAL3 cells to 32°C, when hdm2 transcripts were first detectable by FISH (not shown). This result is consistent with hdm365 and S-hdm2 being generated from a common precursor that extends at least to exon 9. However, no hybridisation signals were obtained using an intron 5 probe, possibly indicating that intron 5-containing splicing intermediates are not sufficiently stable to allow

Figure 8. RNA fractionation. Nuclear (N) and cytoplasmic (C) RNA were extracted separately from uninduced and induced SK-VAL3 cells and hybridised to the exon probes as indicated. An ethidium bromide (EtBr) stain of the formaldehyde gel showing ribosomal precursor and mature RNAs and a hybridisation to U2 snRNA are shown for quality control and purity of the RNA fractions.

Figure 9. RNA-FISH for hdm2 transcripts in SK-VAL3 cells induced for 4 h at 32°C. (A and B) Co-hybridisation of an exons 3−5 probe (red, A) and an exons 6−9 probe (green, B). (C) Merged picture of (A) and (B). (D) A detail from (C) confirming co-localisation of the two probes. (E and F) Co-localisation of the hdm2 exons 3−5 probe (red) with chromosomal DNA immediately flanking the hdm2 gene (green). (G and H) Association of hdm2 RNA (red) with U2 snRNA containing nuclear speckles (green).
detection by RNA in situ hybridisation (data not shown). Since no other hybridisation signals were obtained with hdm2 exons 3–5 probes than those associated with the hdm2 gene, the bulk of nuclear hdm365 RNA appears to be restricted to the sites of hdm2 transcription and splicing. However, due to the low sensitivity of RNA-FISH, spread of a minor amount of hdm365 RNA to other nuclear compartments cannot be excluded. Among p53-induced genes, including waf1 and gadd45, exclusive accumulation of transcripts at the site of RNA synthesis was specific for hdm2 RNA (Weninger, H., Bartl, S., Watzinger, F., Kovar, H. and Lion, T., manuscript in preparation) although it had previously been observed for certain highly expressed viral and cellular (i.e. heat shock protein) genes (63,64).

**Detection of a constitutively expressed hdm365 homologue in hdm2 amplified cells**

hdm365 expression has so far been observed upon strong activation of the P1 promoter only. A corresponding snRNA may be generated from the P1 promoter as well. However, the longevity and the low steady-state levels of constitutively expressed L-hdm2 are compatible with very low P1 promoter activity. This notion is further supported by the absence of FISH-detectable nuclear RNA accumulations when p53 is inactive (not shown). Thus, a short lived hdm365 homologue expressed from the P1 promoter may remain undetectable due to extremely low abundance. In order to search for p53-independent expression of the small hdm2 RNA variant, we tested hdm2 gene amplified cell lines on the northern blot. The size of the putative P1-derived homologue was predicted to be ~230 bases longer than hdm365 due to the presence of exon 1 in place of exon 2. Figure 10 demonstrates that, in fact, a transcript of 600 bases hybridising to exons 3–5 but neither to exon 2 nor to exons 6–9 was clearly detectable in NB1691 neuroblastoma cells. This band also hybridised to an exon 1-specific oligonucleotide probe and was restricted to the nucleus (not shown). Upon activation of p53 by treatment with the topoisomerase inhibitor etoposide (VP16), hdm365 was normally inducible in these cells. Identical results were obtained in Rh18 rhabdomyosarcoma cells (not shown).

**DISCUSSION**

About 40 different regularly and irregularly spliced variants of hdm2 RNA have previously been defined by RT–PCR only. For only some of them has the potential to encode a protein been demonstrated so far (for a review see 65). However, their relative abundances and physiological relevance remain elusive. Here, we describe a highly abundant truncated but fully spliced hdm2 RNA as the major processing product of hdm2 transcripts. The new variant has been observed in response to ectopic p53 expression and upon induction of endogenous p53 not only in human tumor cell lines, including Ewing’s sarcoma, rhabdomyosarcoma, neuroblastoma and K562 chronic myelogenous leukemia, but also in primary human fibroblasts and lymphocytes. A corresponding transcript was also identified to be constitutively expressed in hdm2 amplified human cell lines. No equivalent RNA species was detectable in rodent cells. Human and rodent mdm2 transcripts generally differ in several aspects: while full-length hdm2 transcripts are ~6 kb long, the corresponding mouse and rat mdm2 transcripts span only 3.3 kb (66). L-hdm2 lacks exon 2 while the corresponding murine transcript retains this exon (37). Thus, despite the important and evolutionarily conserved function of the MDM2 protein, mdm2 RNA structure and possibly RNA-based function appears to be much less well conserved. Nonsense-mediated decay or nonsense-associated altered splicing as the source of this truncated RNA transcript are highly unlikely since they depend on the presence of premature stop codons (for a review see 67). Rather, hdm365 appears to be generated by regular but incomplete splicing since its 3′ terminus coincides with the end of exon 5. We assumed that a short stem–loop structure at the end of exon 5 may be the cause of incomplete splicing since short RNA hairpins as small as 6 nt have been demonstrated to potentially inhibit splicing by interference with early steps in spliceosome assembly in yeast (68). However, mutations relaxing the putative stem structure at the end of hdm2 exon 5 did not affect incomplete splicing of transcripts from an hdm2 minigene (data not shown).

Lack of evolutionary conservation, accumulation around the transcription site and low stability may argue against a cellular function for hdm365. On the other hand, however, hdm365 resembles small non-messenger RNAs in that it lacks polyadenylation and, being restricted to the nucleus, does not encode a protein. The size of 365 bases is well in the range of other snRNAs. The presence of a putative 3′ terminal stem–loop structure is reminiscent of non-polyadenylated histone RNAs and U2 snRNA involved in splicing. snRNAs are assumed to have cellular functions on their own or in complex with proteins that are bound to the RNA and thus form ribonucleoprotein complexes. Non-coding RNAs seem to be particularly abundant in roles that require highly specific nucleic acid recognition without complex catalysis, such as in directing post-transcriptional regulation of gene expression or in guiding RNA modifications. These functions range from dosage compensation and imprinting to RNA modification and the regulation of processing and stability of target mRNAs (for reviews see 69,70). By an EST-like approach tailored for the detection of small RNAs Huttenhofer et al. isolated about 200 novel expressed RNA sequences from mouse brain (71). More than half of them corresponded to new members of small nucleolar RNAs (snoRNAs) that guide RNA ribose methylation and pseudouridylation of rRNA as well as of spliceosomal snRNAs. A large number remain without identified RNA targets; some are expressed in a tissue-specific manner raising
the possibility that they target mRNAs. Interestingly, from 88 novel expressed RNAs from the non-snoRNA type (20 detectable on the northern blot), 30% could be located within known or predicted mRNA or heterogenous nuclear RNA coding regions. Similar to hdm365, more than half of them were part of the open reading frame of mRNAs, the rest being located within the 5' or 3' untranslated regions. The function of these RNAs remains elusive. Most of them did not exhibit any sequence or structural motifs that would have made it possible to assign a specific role to these RNAs (71). The identification of a specific function for such RNAs is extremely difficult and may therefore frequently lag behind the discovery of the respective RNA species by decades, as exemplified for 6S and 7SK snRNAs in bacteria and mammalian cells (for a review see 72). The possibility remains, however, that many non-coding snRNAs do not have any function and are produced by accident. For hdm365, we consider this possibility as highly unlikely due to its striking abundancy, and its inducibility not only in cancer cells, which are notorious for aberrant splicing, but also in primary normal tissue. In fact, considering an ~10-fold difference in the half-life between full-length S-hdm2 and hdm365, steady-state levels of the two RNA species suggest that hdm365 is the major processing product of hdm2 gene transcripts. This is further supported by our finding that, early after p53 induction, hdm365 precedes full-length hdm2 expression. Interestingly, upon irradiation the proportion between hdm365 and S-hdm2 varied significantly between different cell lines. In WE-68 Ewing's sarcoma cells, hdm365 even predominated over full-length hdm2 RNA.

Clues to a possible function of hdm365 may come from its exclusive localisation to the sites of hdm2 RNA synthesis. As indicated by both RNA-FISH and northern blot analysis of nuclear and cytoplasmic RNA, fully spliced hdm2 transcripts accumulate for prolonged periods at the site of transcription and splicing. Such accumulations have previously been observed for highly expressed viral genes and for complex eukaryotic genes. In contrast, hdm2 is not a big gene (~32 kb) and lacks significant complexity. After induction by p53 it is expressed at similar levels to gadd45 RNA and at 3-4 times lower levels than waf1 RNA, which has a similar half-life to S-hdm2 (H. Weninger, unpublished results). However, among these p53-inducible genes nuclear accumulation was specific for hdm2 transcripts only. The RNA in situ hybridisation studies did not allow discrimination between splicing intermediates and fully processed hdm2 RNA species at the transcription site. In addition, we were unable to identify a downstream product of abortive splicing containing intron 5 with exon 6 attached to it. Instead a stable band hybridising exclusively to intron 5 was detected. Thus, the question remains what is happening to the splicing of intron 5 and is this regulatory step controlling levels of hdm2 or immediate neighbouring genes. It is extremely difficult, however, to experimentally address this problem since expression of endogenous S-hdm2 can be separated neither from p53 nor from expression of hdm365. In preliminary transfection studies of in vitro synthesised hdm365 RNA as compared to a nonsense recombinant RNA of similar length no specific effect on basal or induced p53 and HDM2 protein levels could be observed (data not shown). A small increase in nuclear hdm2 and waf1 RNA in response to RNA transfection was associated with the presence of wild-type p53 and not specific to hdm365. Since secondary structure calculations for hdm365 predicted several stretches of potential double-stranded RNA and p53 has a strong RNA:RNA reannealing activity which could stabilise RNA secondary structures (73) we also tested for activation of double-stranded RNA-activated protein kinase, however, with negative results (not shown). Several regulatory RNAs have been demonstrated to serve a cis-acting function only. If this is also true for hdm365, as suggested by the in situ hybridisation studies, providing hdm365 ectopically would not allow us to reveal its function. snRNAs may serve particular roles in the stress response (for a review see 72). While the function of constitutively expressed HDM2 protein in the turnover of p53 is well documented, the reason for feedback up-regulation of hdm2 gene activity upon cellular, p53-inducing stress, when p53 becomes resistant to HDM2 protein-mediated proteasomal degradation, is only poorly understood. Yet, HDM2 overexpressed in the absence of p53 has oncogenic functions which, so far, have been attributed exclusively to its interaction with other cell cycle regulatory proteins. It is intriguing to speculate that hdm365 may contribute to p53 gene function under stress conditions. In order to elucidate such a RNA-mediated role of the hdm2 gene it will be necessary to find ways of separating S-hdm2 and hdm365 expression from each other in situ.

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