

Species- and tissue-specific expression of the C-terminal alternatively spliced form of the tumor suppressor p53

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ABSTRACT

Alternative splicing of the p53 transcript which so far has been demonstrated only in the murine system has been proposed as a general regulatory mechanism for the generation of functionally different p53 proteins. We analyzed by RT-PCR the pattern of p53 mRNAs within the region spanning exons 10 and 11 of the p53 gene in 13 different tissues from two independent mouse strains, in 10 different rat tissues and in six different human tissues. PCR products of the expected sizes, corresponding to the normally spliced and the alternatively spliced p53 mRNAs, were detected in mice. Alternatively spliced mRNA was found at ~25–30% the level of the normally spliced p53 mRNA in most tissues analyzed. In spleen and kidney the proportion of alternatively spliced p53 mRNA was much lower. Surprisingly, examination of p53 mRNAs isolated from 10 different rat tissues and six human tissues within the same region of the p53 gene showed only products of normal size. Although a potential homologous alternative 3' splice site within intron 10 of the human p53 gene is present in the genomic sequence of human p53, the expected corresponding alternatively spliced p53 mRNA was undetectable. These findings imply that the generation of functionally different forms of p53 by alternative splicing of p53 transcripts is a species-specific event, possibly indicating species-specific mechanisms for regulating p53 activities.

INTRODUCTION

The tumor suppressor p53 is a multifunctional protein, exerting a variety of different activities which are all related to protecting the cells of vertebrates against the disastrous consequences of DNA damage (1,2). The so far most prominent activity of p53 is that of a sequence-specific transcription factor that can positively or negatively regulate a set of genes involved in growth control, DNA repair and initiation of apoptosis or cell death (3). In addition, p53 seems to directly 'sense' damaged DNA (4,5) and

promote the annealing of complementary single-stranded RNA and DNA (6,7). RNA re-annealing has very recently been identified as an important activity in p53-mediated translational control of mRNAs with extensive secondary structure (8). DNA annealing might be important for preventing unscheduled recombination promoted by single-stranded DNA intermediates initiating recombination (9,10).

The p53 protein has been structurally and functionally divided into three domains, separated by hinge regions (11–14). The N-terminal 42 amino acids harbor the p53 transactivation domain (15). The central core domain of p53, spanning amino acids 120–290, mediates sequence-specific DNA binding to p53 consensus DNA elements (11–14). The C-terminal domain of p53, comprising amino acids 311–393, contains the nuclear localization signals of p53 (16), the p53 oligomerization domain (17) and a domain mediating the non-sequence-specific interaction of p53 with DNA and RNA (18,19). This domain is also responsible for the complementary single-strand nucleic acid annealing activity mentioned above (6,7). In addition, these domains are targets of an increasing number of viral and cellular proteins (20–22). The C-terminus has outstanding properties insofar as it not only harbors a variety of important properties of p53, as described above, but also represents the major regulatory domain of the p53 molecule, modulating sequence-specific DNA binding of the central core domain of p53 (23) and, together with this central domain, acting as a sensor for damaged DNA (4,5). In line with its regulatory function, the C-terminal domain of p53 contains several phosphorylation sites, thus providing targets for the modulation of p53 activities by cellular kinases (24).

At yet another level of regulation, the alternative use of two exons at the 3'-end of the mouse p53 gene has been proposed (25–27). Alternative splicing of the primary p53 transcript generates two distinct p53 mRNAs and two distinct p53 proteins, the full-length normal splice (NS) p53 and an alternative splice (AS) p53, in which 17 new amino acids substitute for the last 26 C-terminal amino acids of the NS p53 (28,29). Functionally the AS p53 protein differs from the NS p53 protein in that it is constitutively active in sequence-specific DNA binding (30), but lacks the intrinsic annealing activity for single-strand nucleic acids (31). Han and co-workers (25,26) detected the AS p53 mRNA species at ~25–33% of the level of the NS p53 mRNA in

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both normal murine epidermal and carcinoma cells, as well as in mouse 10T1/2 fibroblast cells, mouse liver and testis tissues. Furthermore, it has been found that different levels of NS and AS p53 mRNAs are present at different phases of the cell cycle (26), supporting the idea that alternative splicing might provide an important means for regulating p53 activity.

All vertebrate species analyzed so far, regardless of their evolutionary distance, share a high identity at the protein level, as well as a striking similarity at the level of genomic organization of the *p53* gene (sizes of the exons and the locations of splice junctions; 32). Hence the existence of similar control mechanisms of *p53* gene expression in vertebrates is rather likely. In this study we performed extensive p53 mRNA analyses to determine whether alternative splicing of the p53 transcript, and hence the generation of two functionally distinct p53 proteins, is a general phenomenon in mammalian tissues. Alternative splicing of the p53 transcript was detected in mouse cells and tissues, but not in rat and human. We therefore conclude that alternative splicing of the p53 transcript is a species-specific event.

MATERIALS AND METHODS

Source of tissue specimens

Organs were isolated from BALB/c and DBA mice at days 25 and 40, as well as from Wistar rats at day 40 (Table 1). Professor J. Schmidtke and F. Schnieders (Medizinische Hochschule Hannover, Hannover, Germany) kindly provided us with human tissue specimens from colon, epidermis, kidney, lung and thymoid gland, as well as with three separate testis specimens. Two separate ovary specimens were provided by the Max-Delbrück-Centrum für Molekulare Medizin (Berlin-Buch, Germany).

cDNA analysis

Total RNAs were extracted by the guanidinium-HCl thiocyanate/phenol/chloroform method (33). As a quality check for the prepared murine RNA aliquots of all RNA samples were converted to cDNA using primer 3GAP within the non-translated region of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (34; CGAAAGCTTTAGGCCCTCCTGTTA-TT). Subsequently the resulting cDNA was amplified using primers 3GAP and 5GAP (CGAGGATCCACAGCCGCATCT-TCTTGT) under standard amplification conditions (35), leading to amplification of the full-length GAPDH cDNAs.

Aliquots of all RNA samples were converted to cDNA using a First-Strand-cDNA kit (Pharmacia, Upsala, Sweden) according to the manufacturer's protocol. Locations of the cDNA primers used, p96-1 (human, TGTCAGTGGGGAACAAGAAGTGG), Rp53-3 (rat, GGACTAGCATTGTCTTGTGTCAGC) and Mp53-2 (mouse, AGTCGGATCCAGGGTGGGGGGTGGATA), are shown in Figure 1A.

Amplification

The annealing temperatures were optimized with genomic DNAs. PCR reactions were carried out in principle as described elsewhere (35). Following a 10 min denaturation at 94°C, 30 PCR cycles [denaturation, 0.5 min, 94°C; annealing, 1 min, 58°C (rat and human), 60°C (mouse); elongation, 1 min, 72°C] were performed using the following primer combinations: human, p96-1 and p96-2 (TTGGAAGTCAAGGATGCCAGG) or

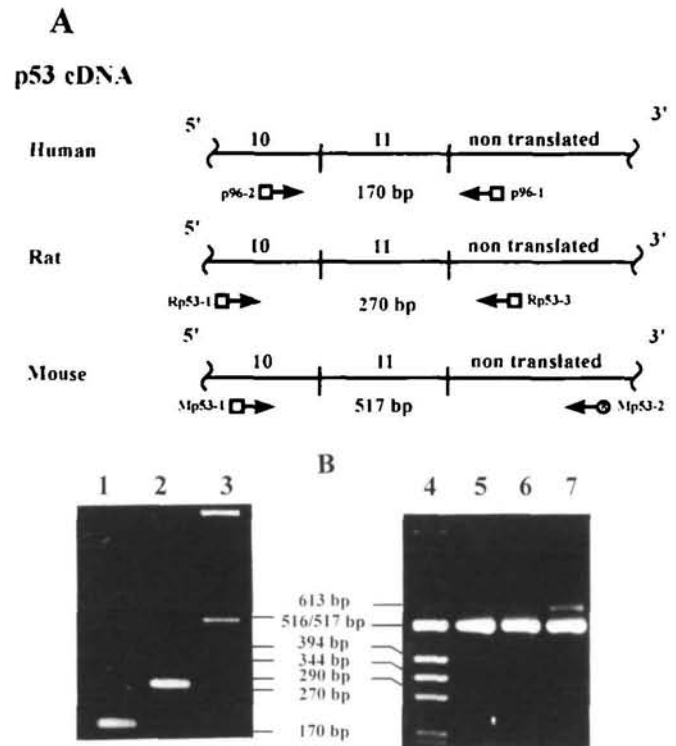


Figure 1. (A) Schematic representation of the homologous p53 cDNA fragments, including exons 10 and 11 and part of the non-translated region of the *p53* gene. RT-PCR analysis of a p53 transcript spanning this region was performed with the indicated pairs of oligonucleotide primers. (B) Analysis of RT-PCR products derived from normally and alternatively spliced p53 transcripts. RT-PCR products were electrophoresed through 2% agarose gels. Lane 1, human testis (170 bp); lane 2, rat testis (270 bp); lane 3, murine kidney (517 bp); lane 6, murine spleen (517 bp); lane 7, murine testis (517 bp). RT-PCR products in lanes 1–6 correspond to the normally spliced p53 transcripts. As a representative of a derivative of the alternatively spliced transcript lane 7 shows an additional PCR product, 613 bp in length, present in the murine testis sample. Lanes 3 and 4, markers.

p96-1 and A4 (GACGGAACAGCTTTGAGGT); rat, Rp53-3 and Rp53-1 (ATGTTCCGAGAGCTGAATGAGG); mouse, Mp53-2 and Mp53-1 (AGTCGAATCAAGGATGCCATGC-TAC) (Fig. 1A). Final extension was at 72°C for 10 min.

The presence of the alternatively spliced p53 transcripts was confirmed by PCR using standard PCR conditions (see above; 30 PCR cycles: denaturation, 30 s, 94°C; annealing, 1 min, 60°C; elongation, 1 min, 72°C) with primer Mp53-1 within exon 10 of the murine *p53* gene and primer Mp96-1 from within the inserted sequence (GCTTTCCTCCTTGATCAAGG).

RT-PCRs specific for the hypothetically included sequence in AS human p53 mRNA were performed with primer p96-4, including the sequence of the 3' boundary of the last exon (TCCCTGACCAATGCTTTGAAGG), and primer p96-2, from within exon 10 (Fig. 2) using standard PCR conditions (see above; 30 PCR cycles: denaturation, 1 min, 94°C; annealing, 1 min, 58°C; elongation, 2 min, 72°C).

PCR product analysis

The amplified PCR products were separated by agarose gel electrophoresis and characterized by restriction digestion.

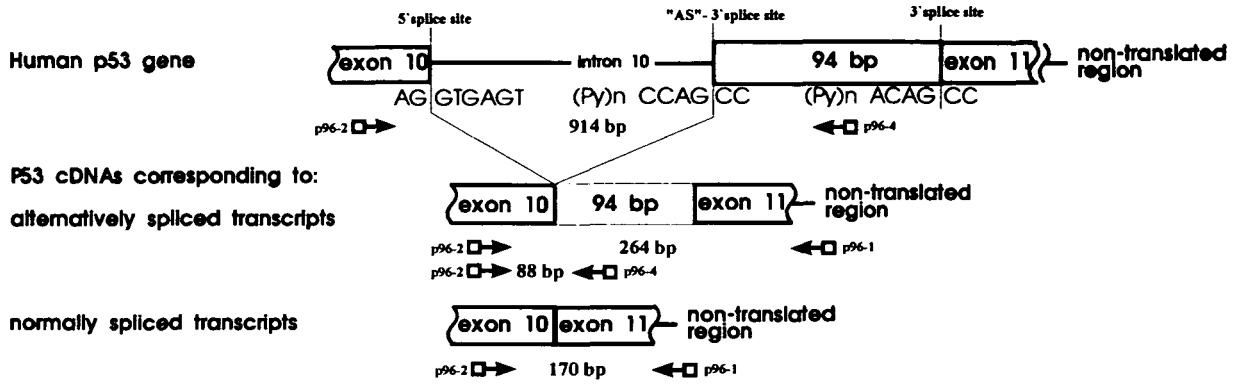


Figure 2. Hypothetical alternative splicing within intron 10 of the human *p53* gene. Alternative splicing would lead to an insertion of 94 bp between exons 10 and 11. In this case RT-PCR using primers p96-2 and p96-1 should generate two separate PCR products 170 and 264 bp in length. RT-PCR using primer p96-4 from the 94 bp sequence and primer p96-2 from within exon 10 in human genomic DNA would yield a PCR product of 914 bp. RT-PCR should lead to amplification of an 88 bp PCR product.

Hybridization using oligonucleotide Mp53-1 as a probe was performed to quantitate the relative amount of the different PCR products by densitometry following standard conditions (36). Ten percent of the total RT-PCR was subjected to electrophoresis through a 2% agarose gel, stained with ethidium bromide and blotted onto N⁺-Nylon membrane (Amersham) in the presence of 0.4 N NaOH. Hybridization with [γ -³²P]dATP-labeled oligonucleotide Mp53-1 was performed for 3 h at 65°C, followed by two washes with 2× SSPE, 0.1% Triton X-100 at room temperature and two washes at 65°C with the same solution. Autoradiographs were analyzed by densitometry.

RESULTS

Tissue-specific splicing of the p53 transcript in mouse tissues

To determine whether alternative splicing of murine p53 transcripts depends on tissue type, mouse strain or sex of the animals we first analyzed the pattern of p53 mRNAs spanning the region of the *p53* gene between exons 10 and 11 in 13 different mouse tissues (Table 1). The tissues were isolated from BALB/c and DBA mice at days 25 and 40, respectively. Total RNA was extracted from tissue specimens using the guanidinium-HCl thiocyanate/phenol/chloroform method (33). As a quality check for the prepared RNA, aliquots of all RNA samples were converted to cDNA using primer 3GAP within the GAPDH gene (34). The resulting cDNA was subsequently amplified using primers 3GAP and 5GAP of the GAPDH gene (see Materials and Methods) and led to amplification of the full-length GAPDH cDNA (data not shown). Aliquots of these RNA samples were then converted to cDNA using primer Mp53-2 within the non-translated region of the *p53* gene, followed by PCR amplification using the same primer and primer Mp53-1 located in exon 10 of the *p53* gene (Fig. 1A). Two PCR products of the expected sizes, 517 and 613 bp in length (25), were detected, corresponding to NS and AS murine p53 mRNA (Fig. 1B, lane 7). With the exception of spleen and liver (discussed below), AS p53 mRNA, as determined by densitometry, was present in these tissues at ~25–30% of the level of NS p53 mRNA, in accordance with the data published by Han and Kulesz-Martin (25,26). The

proportions of these mRNAs did not differ significantly between the mouse strains analyzed and were independent of the sex of the animals. Furthermore, the proportions of alternatively spliced p53 transcripts were similar at days 25 and 40 (data not shown). In contrast, using the same RT-PCR approach we found that the level of alternatively spliced p53 transcripts was much lower in spleen and kidney samples than that determined in all other tissues analyzed (Fig. 1B, lanes 5 and 6). Nevertheless, in these tissues also the presence of the alternatively spliced p53 transcripts was confirmed by PCR, using primers located within exon 10 (Mp53-1) and from within the inserted sequence (M96-1) followed by restriction digestion (*Bcl*I) of the obtained PCR products (data not shown). All data were reproducible in repeated experiments, indicating a tissue-specific splicing of p53 transcripts in mice.

Table 1. Alternative splicing events within intron 10 of the p53 transcripts obtained by RT-PCR analysis of RNAs from various tissue types and species

Origin of transcripts	Mouse		Rat	Human
	DBA	Balb/c		
Colon	+	+	-	-
Small intestine	+	+	-	/
Epidermis	+	+	-	-
Bladder	/	+	-	/
Kidney	o	o	-	-
Testis	+	+	-	-
Liver	/	+	-	/
Lung	+	+	-	-
Thymus	/	+	-	/
Heart	+	+	-	/
Spleen	o	o	/	/
Ovary	/	+	/	-
Brain	/	+	/	/

/, not determined; +, alternative splicing found; o, alternative splicing found at low level; -, alternative splicing not found.

Analysis of rat and human tissues for alternative splicing near the 3'-end of the *p53* gene

Sequence data from Hulla and Schneider (37) provided evidence that the rat *p53* gene differs from other *p53* gene structures. The newly defined rat *p53* gene consists of only 10 exons and 9 introns, because no intervening sequence is present between the region of the rat gene corresponding to exons 6 and 7 of the mouse and human *p53* genes. Thus exons 6 and 7 are combined, forming exon 6 in the rat gene. However, for clarity homologous exon regions of the *p53* genes of mouse, human and rat were assigned the same numbers throughout this study.

Total RNAs from 10 different tissues of Wistar rats (Table 1) were isolated as described above. *p53*-specific RNA was subsequently amplified by RT-PCR using primers of exon 10 (Rp53-1) and from within the non-translated region of the rat *p53* gene (Rp53-3; Fig. 1A). Thirty rounds of PCR were sufficient for amplification of large amounts of PCR products 270 bp in length, as expected for NS *p53* from the published sequence of the rat *p53* gene (37). No further PCR products were detected using this approach (Fig. 1B, lane 2), suggesting that the alternatively spliced *p53* products were expressed at a rather low level, if alternative splicing would occur at all, within this region of the rat *p53* transcript.

Similar experiments were performed to examine *p53* expression in human tissues. RNA from seven different, morphologically normal human tissues was isolated (Table 1) and human *p53* mRNA was converted to cDNA with primer p96-1 from within the non-translated region of the *p53* gene (Fig. 1A). *p53* cDNA spanning exons 8–11 and part of the non-translated region of the *p53* gene (primers A4 and p96-1) and *p53* cDNA including exons 10, 11 and a part of the non-translated region of the *p53* gene (primers p96-1 and p96-2) were amplified in separate reactions. In repeated experiments PCR products of 415 bp (with primers A4 and p96-1; data not shown) or 170 bp (primers p96-1 and p96-2), respectively, were amplified exclusively (Fig. 1B). As both products represent the NS *p53* transcript (38) we did not obtain evidence for the presence of AS *p53* mRNA in human tissue, similarly to rat tissue.

Comparison of the homologous sequences of exons 10, 11 and intron 10 of human, rat and mouse *p53* genes showed a high similarity of the exon–intron boundaries, but differences in sequences and sizes of intron 10 [intron sizes: rat, ~700 bp (37); mouse, 700 bp (39); human, 920 bp (EMBL/GenBank/DDBJ database accession no. X54156)]. Nevertheless, a potential alternative 3' splice site with a short polypyrimidine stretch was detected at almost the same position as the alternative splice site used in the murine *p53* gene, 94 bp further upstream of the 3' splice site of exon 11, within intron 10 of the human *p53* gene (Fig. 2). This sequence perfectly fits the consensus sequence for

3' splice sites (Table 2). A perfect branch point is located 20 bp upstream of this potential 3' splice site. If this potential alternative 3' splice site were activated in human tissue its use should lead to the inclusion of the complete 3' boundary of the last exon within the alternatively spliced transcript (Fig. 2). In this case RT-PCR using primers p96-2 and p96-1 should generate an additional PCR product 264 bp in length (Fig. 2). However, such an additional product was not detected using this approach, as only a product of 170 bp, corresponding to the normally spliced *p53* mRNA (Fig. 2) was found (Fig. 1B, lane 1). To improve the sensitivity of our approach RT-PCR specific for this sequence hypothetically included in an AS human *p53* mRNA was performed using primer p96-4, including the sequence of the 3' boundaries of the last exon, and primer p96-2, from within exon 10 (Fig. 2; EMBL/GenBank/DDBJ database accession no. X54156). PCR conditions were optimized with human genomic DNA and yielded PCR products of the expected size of 914 bp (data not shown). If such an alternatively spliced *p53* transcript were present in the RNA of human tissue, RT-PCR using this set of primers should amplify a cDNA product 88 bp in length (Fig. 2). However, such a product could not be detected. Since products of the expected size were amplified from genomic human DNA, but not from human cDNAs, it is rather unlikely that the failure to detect the 88 bp cDNA product, representing the postulated human AS *p53* mRNA, is due to the PCR conditions. Instead, we conclude that this potential splice site in humans is not used, in a similar fashion as to the corresponding murine splice site.

DISCUSSION

The *p53* protein acts as a superior control element in cellular proliferation. Its multifunctional character is reflected in various biochemical activities (see Introduction), which require tight regulation. In regulating the described activities the *p53* C-terminus plays a prominent role, as it not only regulates the sequence-specific DNA binding of *p53* mediated by the *p53* core domain, but by itself takes part in a variety of biochemical activities, which could be regulated by differential expression of physiologically different variants of *p53* with distinct functional C-terminal ends. An important regulatory element in the C-terminal domain of *p53* is provided by multiple phosphorylation sites, rendering the C-terminus of *p53* a target for a variety of cellular kinases (24). Alternatively, expression of a C-terminally shortened *p53* variant by alternative splicing has been suggested as a means of modulating *p53* activity. In addition to being constitutively active in sequence-specific DNA binding, this AS *p53* is devoid of the activities ascribed to the C-terminal domain (31). Furthermore, by heterocomplex formation with NS *p53*, AS *p53* could alter the functions of full-length NS *p53*.

Table 2. Comparison between splice site sequences of exons 10 and 11 of the murine and human *p53* gene and the consensus sequence for 3' and 5' splice sites (41,42)

	5' Splice sites	3' Splice sites
Consensus sequence	AG/GUAAGU	Y _n CAG/G
Mouse	AG/GUAAGU	AS AAUUCUUUUUUUCAG/CC NS UUCUGUCUCCUAUAG/CT
Human	AG/GUGAGU	'AS' AUUCCGUUGUCCAG/CC NS CUUCUGUCUCCUACAG/CC

Y, pyrimidine; AS, alternative 3' splice site used in the murine *p53* gene; 'AS', potential alternative 3' splice site, 94 bp upstream of the 3' splice site of exon 11, within intron 10 of the human *p53* gene; NS, 3' splice site used normally.

Comparison of the so far available data on *p53* genes from different species revealed that all vertebrate species analyzed share a high identity at the protein level, as well as a striking similarity in genomic organization of the *p53* gene. We therefore expected that alternative splicing, which is recognized as an important means for modulating gene expression (40), would be a general mechanism for the generation of functionally different *p53* molecules and analyzed the expression pattern of *p53* mRNAs in mouse, rat and human tissues. The sizes and sequences of the exons of the respective *p53* genes are quite similar (32). Although the introns vary in length, they interrupt the exons at precisely homologous positions. The occurrence of similar splicing events therefore seemed reasonable. However, in contrast to this assumption, alternative splicing near the 3'-end of the *p53* transcript was only detected in several mouse tissues, but not in corresponding tissue specimens of rat and human origin. In the majority of the mouse tissues analyzed the AS *p53* mRNA species was detected at ~25–30% of the level of NS *p53* mRNA, in accordance with previous findings of Han and Kulesz-Martin (25,26). These transcripts, however, were nearly undetectable in samples of spleen and kidney tissues of mice. Tissue-specific differences in the splicing pattern have been reported for several other genes (36) and could be due to different splicing factors. Tissue-specific alternative splicing is known to play an important role in tissue differentiation, for instance in the gene encoding human neuronal cell adhesion molecules (40). Therefore, our findings could point to tissue-specific functions for the AS *p53* protein in mice.

Screening the known sequences of intron 10 of the human *p53* gene for potential alternative splice sites led to the identification of a candidate splice site at almost the same position as the alternative 3' splice site used in the murine gene. This splice site perfectly fits the consensus sequence for 3' splice sites (41,42; Table 2). Nevertheless, AS *p53* mRNA derived from this site was undetectable, although our approach was sensitive enough technically to detect the alternatively spliced murine product. This is exemplified by the fact that we were able to amplify the corresponding region of the human *p53* gene from human genomic DNA. Thus our failure to detect the much shorter sequence in the corresponding AS cDNA, which should be considerably easier to amplify, strongly suggests that the expected alternative splicing of the *p53* transcript in rat and human is probably a rare event. Alternatively, we cannot exclude the possibility that the resulting transcripts are less stable and therefore undetectable.

Our data reveal evolutionary differences in the generation of variant *p53* proteins by alternative splicing which could be due to changes within the intron sequences, their environment or within splicing factors, avoiding alternative splicing within this region or, alternatively, generating an unstable AS *p53* RNA. Our study focused on alternative splicing near the 3'-end of the *p53* gene of rats and humans only. Therefore, we do not want to exclude the possibility that other alternative splicing events may take place within other regions of the *p53* genes examined. For instance, Matlashewski and co-workers described an altered transcript generated by splicing at an alternative acceptor site in intron 2 of the human *p53* gene (43).

Our results raise important questions as to the biological relevance of alternative splicing of the *p53* transcript in mice. AS *p53* mRNA is detected at a substantial level in most of the tissues analyzed and is translated into a stable AS protein exhibiting quite

different functional properties to the NS *p53* protein (27,31). One has to assume, therefore, that generation of the AS *p53* protein in mice is an important means of regulating *p53* activities. The apparent lack of AS *p53* mRNAs in rat and human tissues thus either indicates functional differences in the NS *p53* proteins between mice and rats and humans or different regulatory mechanisms. The high conservation of *p53* domains within different species argues against the first alternative. In contrast, however, different mechanisms for regulation of wild-type *p53* activities seem to be an intriguing possibility. This possibility is also supported by our previous finding of species-specific phosphorylation of mouse and rat *p53* (44).

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