# Amplification of a gene encoding a p53-associated protein in human sarcomas

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**DESPITE extensive data linking mutations in the p53 gene to human tumorigenesis[1], little is known about the cellular regulators and mediators of p53 function. MDM2 is a strong candidate for one such cellular protein; the *MDM2* gene was originally identified by virtue of its amplification in a spontaneously transformed derivative of mouse BALB/c cells[2] and the MDM2 protein subsequently shown to bind to p53 in rat cells transfected with p53 genes[3,4]. To determine whether MDM2 plays a role in human cancer, we have cloned the human *MDM2* gene. Here we show that recombinant-derived human MDM2 protein binds human p53 *in vitro*, and we use *MDM2* clones to localize the human *MDM2* gene to chromosome 12q13–14. Because this chromosomal position appears to be altered in many sarcomas[5–7], we looked for changes in human *MDM2* in such cancers. The gene was amplified in over a third of 47 sarcomas, including common bone and soft tissue forms. These results are consistent with the hypothesis that MDM2 binds to p53, and that amplification of *MDM2* in sarcomas leads to escape from p53-regulated growth control. This mechanism of tumorigenesis parallels that for virally-induced tumours[8,9], in which viral oncogene products bind to and functionally inactivate p53.**

To obtain human complementary DNA clones, a murine *MDM2* cDNA probe was used to initiate cDNA walking in a human library (see legend to Fig. 1). Sequence analysis of 25 clones revealed several cDNA forms indicative of alternative splicing. The predominant human form is compared with its murine counterpart in Fig. 1. There was an open reading frame extending from the 5′ end of the human cDNA sequence to nucleotide 1,784. Although this signal for translation initiation could not be unambiguously defined, the ATG at nucleotide 312 was considered the most likely position for several reasons. First, the sequence similarity between human and mouse *MDM2* declined dramatically upstream of nucleotide 312. Second, an inverse polymerase chain reaction (PCR) was used in an attempt to acquire additional upstream cDNA sequence[10]. The 5′ ends of the PCR-derived clones were very similar (within 12 base pairs) to the 5′ ends of clones obtained from the cDNA library, indicating that the 5′ end of the human *MDM2* sequence shown in Fig. 1 may represent the 5′ end of the transcript. Third, *in vitro* translation of the sequence shown in Fig. 1, beginning with the methionine encoded by the ATG at position 312, generated a protein similar in size to that observed in human cells (see below).

Comparison of the human and mouse *MDM2* coding regions showed that they were 80.3% identical and shared a basic nuclear localization signal at codons 181 to 185 (ref. 11), several casein kinase II serine-phosphorylation sites[12], an acidic activation domain at codons 223 to 274 (ref. 13), and two metal-binding sites at codons 305 to 322 and 461 to 478, neither of which is highly related to known DNA-binding domains[14].

To determine whether the human MDM2 protein could bind to human p53 protein *in vitro*, a human *MDM2* expression vector was constructed from the cDNA clones (see legend to Fig. 2). RNA transcribed from this vector using T7 RNA polymerase was used to program a rabbit reticulocyte lysate. Although the predicted size of the protein generated from the construct was only 55.2K ($M_r$ 55,200, extending from the methionine at nucleotide 312 to nucleotide 1,784), protein translated *in vitro* migrated at ~90 K. The MDM2 protein was not immunoprecipitated with antibodies against either the C-terminal or N-terminal regions of p53 (Fig. 2, lanes 2 and 3). But when *in vitro*-translated human p53 was mixed with the human *MDM2* translation product, the anti-p53 antibodies precipitated MDM2 protein with p53 (Fig. 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility (MCC[15]) was mixed with p53 and there was no coprecipitation (Fig. 2, lanes 8 and 9). When an *in vitro*-translated His-175 mutant form of p53 was mixed with human MDM2 protein, a similar coprecipitation of MDM2 and p53 proteins was also observed (data not shown).

Polyclonal rabbit antibodies were raised against an *Escherichia coli*-produced human MDM2–glutathione S-transferase fusion protein. The anti-MDM2 antibodies immunoprecipitated p53 when mixed with MDM2 protein (Fig. 2, lane 15) but failed to precipitate p53 alone (Fig. 2, lane 13).

To establish the chromosomal localization of human *MDM2*, somatic cell hybrids were screened, and a human–hamster hybrid containing only human chromosome 12 hybridized to the human *MDM2* probe. Screening of hybrids containing portions of chromosome 12 (ref. 16) with the same probe narrowed the localization to chromosome 12q13–14. Because this region of chromosome 12 is often aberrant in human sarcomas[5–7], southern blot analysis to evaluate whether *MDM2* was genetically altered in such cancers. We found a striking amplification of *MDM2* sequences in several of these tumours (see examples in Fig. 3, lanes 2, 3 and 5). Of 47 sarcomas analysed, 17 showed a 5–50-fold *MDM2* amplification. These tumours included 7 of 13 liposarcomas, 7 of 22 malignant fibrous histiocytomas, 3 of 11 osteosarcomas, and 0 of 1 rhabdomyosarcoma. Five benign soft tissue tumours (lipomas) and seventy-four carcinomas (colorectal or gastric) were also analysed by Southern blotting and no amplification was seen.

We next determined whether this gene amplification was associated with increased expression. Because of RNA degradation in primary sarcomas, only the cell lines could be productively analysed by northern blotting. In the one available sarcoma cell line with *MDM2* amplification, a single transcript of ~5.5 kilobases (kb) was observed (Fig. 4*a*, lane 1). The amount of this transcript was much higher than in a sarcoma cell line without amplification (Fig. 4*a*, lane 2) or in a carcinoma cell line (Fig. 4*a*, lane 3). When purified messenger RNA (rather than total RNA) from the carcinoma cell line was used for analysis, a human *MDM2* transcript of 5.5 kb could also be observed (Fig. 4*a*, lane 4). Expression of the *MDM2* RNA in the sarcoma with amplification was estimated to be at least 30-fold higher than that in the other lines examined. This was consistent with results from western blot analysis. A protein of $M_r$ ~90K was expressed at high levels in the sarcoma cell line with *MDM2* amplification (Fig. 4*b*, lane 3), whereas no expression was evident in two sarcoma cell lines without amplification or in the carcinoma cell line (Fig. 4*b*, lanes 1, 2 and 4). Five primary sarcomas were also analysed by western blotting. Three primary sarcomas with amplification expressed the same sized protein as that in the sarcoma cell line (Fig. 4*c*, lanes 1–3), but no protein was observed in the two sarcomas without amplification (Fig. 4*c*, lanes 4 and 5).

Our results demonstrate that human MDM2 binds to p53 *in vitro* and is genetically altered in a significant fraction of the most common sarcomas of soft tissue and bone[17,18]. It is important to note, however, that amplifications in human tumours

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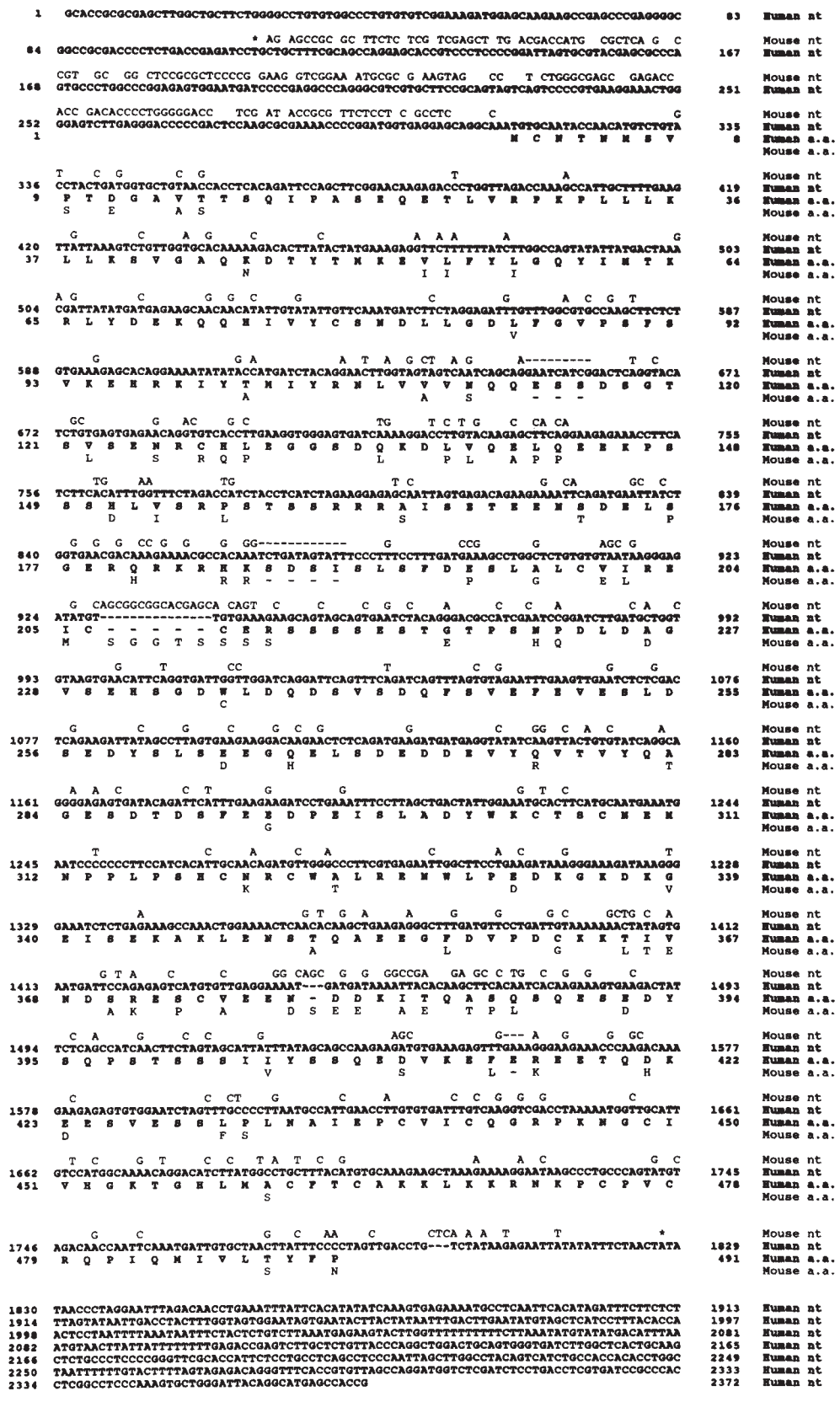

FIG. 1 Human *MDM2* cDNA sequence: human and mouse *MDM2* nucleotide (nt) and amino-acid (a.a.) sequences are compared (single-letter code). The mouse sequence is only shown where it differs from the corresponding human sequence. Asterisks mark the 5′ and 3′ boundaries of the previously published mouse *MDM2* cDNA[2]. Dashes indicate insertions.

METHODS. Polyadenylated RNA isolated from the human colonic carcinoma cell line CaCo-2 was used as a template for the production of random hexamer-primed double-stranded cDNA[22]. The cDNA was ligated to adaptors and then to the lambda YES phage vector, packaged and plated as described[23]. The library was screened initially with a $^{32}$P-labelled [24] mouse *MDM2* cDNA probe (nt 259 to 1,508; ref. 2) and then rescreened with a human *MDM2* cDNA clone containing nt 40 to 702. In total, 25 clones were obtained, partially or totally sequenced, and mapped. The sequence shown is representative of the most abundant class and was assembled from three clones: c14-2 (nt 1–949), c89 (nt 467–1,737), and c33 (nt 390–2,372). The 3′ end of the untranslated region has not yet been cloned. The 5′ end is likely to be at or near nt 1 (see text). The mouse and human amino-acid sequences are compared from the putative translation start site at nt 312 to the conserved stop codon at nt 1,784. This nucleotide sequence has been deposited at the EMBL Data Library, accession number Z12020.

often involve large stretches of the genome, encompassing 300 to 1,000 kb[7,19–21]. Other genes in the *MDM2* amplicon could contribute to, or be responsible for, the growth advantage afforded by the amplification event. Nevertheless, MDM2 is a good candidate for the 'target' of amplification for two reasons. First, MDM2 has oncogenic activity after transfection into NIH3T3 cells[2]. Second, it binds to a protein (p53) with known growth suppressive effects on a wide variety of human tumour types. MDM2 may functionally inactivate p53 in ways similar to those employed by virally encoded oncoproteins such as simian virus 40 T antigen, adenovirus E1B and human papilloma virus E6 (refs. 4, 8, 9). Consistent with this hypothesis, no sarcomas with *MDM2* amplification (of five tested) had any of the p53 gene mutations that occur commonly in other tumours

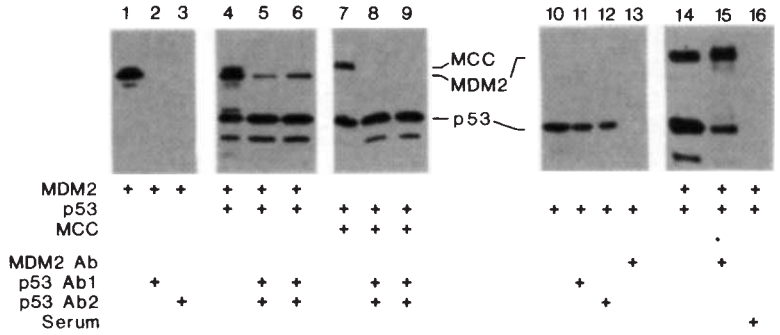


FIG. 2 Coprecipitation of human MDM2 and p53. *In vitro*-translated MDM2, p53 and MCC proteins were mixed as indicated and incubated with p53 Ab1 (monoclonal antibody specific for the C terminus of p53), p53 Ab2 (monoclonal antibody specific for the N terminus of p53), MDM2 Ab (polyclonal rabbit anti-human MDM2 antibodies), or serum (preimmune serum obtained from the rabbit that produced the MDM2 antibody). Lanes 1, 4, 7, 10 and 14 contain aliquots of the protein mixtures used for immunoprecipitation. Bands running slightly faster than p53 are polypeptides produced from internal translation initiation sites.

METHODS. A human *MDM2* expression vector was constructed in pBluescript SK + (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in Fig. 1 from nt 312 to 2,176. A 42-bp black bettle virus ribosome entry sequence[25] was placed immediately upstream of this *MDM2* sequence in order to obtain high expression. This construct, as well as p53 (ref. 26) and *MCC*[15] constructs in pBluescript SK +, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. Lysate (10 μl) containing the three proteins, alone or mixed in pairs, was incubated at 37 °C for 15 min. 1 μg (10 μl) of p53 Ab1 or Ab2 (Oncogene Science) or 5 μl of rabbit serum containing MDM2 antibody or preimmune rabbit serum, were added as indicated. 90 μl RIPA buffer (10 mM Tris, pH 7.5, 1% sodium deoxycholate, 1% NP40, 150 mM NaCl, 0.1% SDS), SNNTE buffer[3], or binding buffer[26] were then added and the mixtures allowed to incubate at 4 °C for 2 h. The three buffers produced similar results, although the coprecipitation was less efficient in SNNTE buffer (containing 0.5 M NaCl; lanes 5 and 8) than in binding buffer (containing 0.1 M NaCl; lanes 6 and 9). Following addition of 2 mg protein A-Sepharose, the tubes were rotated end-over-end at 4 °C for 1 h. After pelleting and washing, immunoprecipitates were electrophoresed on SDS-polyacrylamide gels and the dried gels autoradiographed in the presence of Enhance (New England Nuclear). Rabbits were immunized with a glutathione *S*-transferase (Pharmacia)-MDM2 fusion protein containing human MDM2 from the region corresponding to nt 390–816.

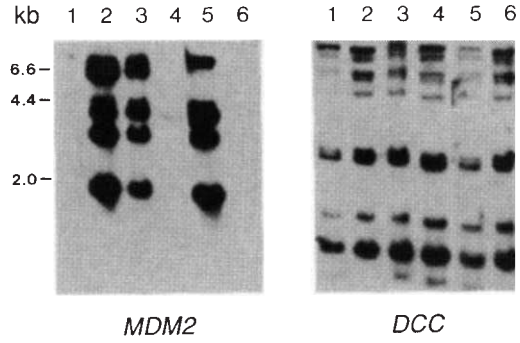


FIG. 3 Amplification of the human *MDM2* gene in sarcomas. DNA (5 μg) was digested with *Eco*RI, separated by agarose gel electrophoresis and transferred to nylon as described[27]. Filters were then hybridized with a human *MDM2* cDNA fragment probe (nt 1–949; Fig. 1) or to a control probe that identifies fragments of similar size (pDCC 1.65; ref. 28). Hybridization was as previously described[29]. DNA was derived from 5 primary sarcomas (lanes 1–4, 6) and one sarcoma cell line (OsA-CL, lane 5). On longer exposure, the same sized *MDM2* fragments were observed in lanes 1, 4 and 6. DNA fragment sizes are shown on the left in kb.

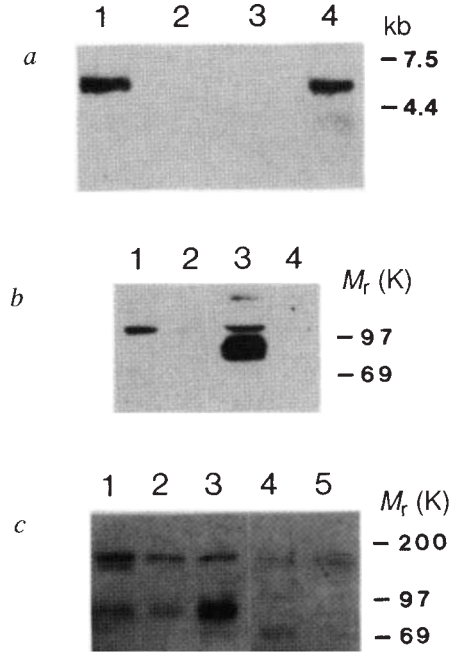


FIG. 4 *MDM2* expression. *a*, Northern blot analysis. RNA was separated by electrophoresis in a MOPS-formaldehyde gel and electrophoretically transferred to nylon filters. Transfer and hybridization were as described[30]. RNA was hybridized to the *MDM2* fragment described in Fig. 3 legend. Total RNA (10 μg) was derived, respectively, from two sarcoma cell lines (OsA-CL, lane 1 and RC13, lane 2) and the colorectal cancer cell line (CaCo-2) used to make the cDNA library (lane 3). Lane 4 contains 10 μg polyadenylated CaCo-2 RNA. RNA sizes are shown on the right in kb. *b*, Western blot analysis of the sarcoma cell lines RC13 (lane 1), OsA-CL (lane 3), HOS (lane 4), and the carcinoma cell line CaCo-2 (lane 2). *c*, Western blot analysis of primary sarcomas. Lanes 1 to 3 contain protein from sarcomas with *MDM2* amplifications, and lanes 4 and 5 contain protein from sarcomas without *MDM2* amplification. Western blots using affinity-purified MDM2 antibody were performed with 50 μg protein per lane as described [31], except that the membranes were blocked in 10% non-fat dried milk and 10% goat serum, and secondary antibodies were coupled to horseradish peroxidase to allow chemiluminescent detection (Amersham ECL). MDM2 antibody was affinity-purified with a pATH-MDM2 fusion protein using methods described in ref. 31. Nonspecific reactive proteins of 75, 105 and 170K are seen in all lanes, irrespective of *MDM2* amplification. MDM2 proteins, of $M_r$ 90K, were observed only in the MDM2-amplified tumours. Protein marker sizes are shown on the right.

(unpublished results with T. Tokino and D. Sidransky). The amplification of *MDM2* provides another provocative parallel between viral carcinogenesis and the naturally occurring genetic alterations underlying sporadic human cancer. □

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# Wild-type p53 activates transcription *in vitro*

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**THE p53 protein is an important determinant in human cancer and regulates the growth of cells in culture[1–3]. It is known to be a sequence-specific DNA-binding protein[4,5] with a powerful activation domain[6–8], but it has not been established whether it regulates transcription directly. Here we show that intact purified wild-type human and murine p53 proteins strongly activate transcription *in vitro*. This activation depends on the ability of p53 to bind to a template bearing a p53-binding sequence. By contrast, tumour-derived mutant p53 proteins cannot activate transcription from the template at all, and when complexed to wild-type p53, these mutants block transcriptional activation by the wild-type protein. Moreover, the simian virus 40 large T antigen inhibits wild-type p53 from activating transcription. Our results support a model in which p53 directly activates transcription but this activity can be inhibited by mutant p53 and SV40 large T antigen through interaction with wild-type p53.**

A DNA-binding immunoassay has been used to screen human genomic clones and show that p53 binds specifically to a region upstream of the transcription start site for the human ribosomal gene cluster (RGC)[4]. We have confirmed and extended this observation by DNase I footprinting and shown that addition of immunopurified p53 to a DNA fragment containing the RGC site leads to strong and specific protection of only the RGC region (Fig. 1). All tumour-derived mutant p53 proteins tested failed to protect this sequence (J.B. *et al.*, manuscript in preparation).

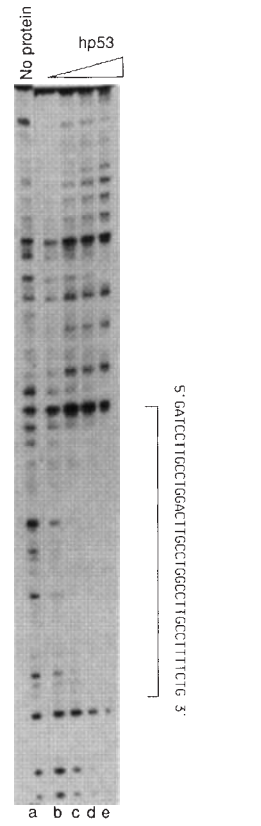


FIG. 1 The p53 protein binds specifically to a site in the human ribosomal gene cluster. DNA binding was assayed in 50-μl volumes containing 40 mM creatine phosphate, pH 7.7, 4 mM ATP, 7 mM $MgCl_2$, bovine serum albumin (0.2 mg $ml^{-1}$) 0.5 mM dithiothreitol, 10 ng carrier plasmid (pAT153) and 10 fmol of 5′ $^{32}$P-labelled DNA fragment containing the ribosomal gene cluster (RGC) p53-binding site[4] and either no protein (lane a) or increasing amounts of wild-type human p53 in increments of 15 ng up to 60 ng (lanes b–e). DNase I treatment of mixtures and processing of samples for electrophoresis on 8% polyacrylamide urea gels has been described[5]. Wild-type p53 was immunopurified from Sf27 cells expressing a recombinant baculovirus, pEV55hwt, using the monoclonal antibody Pab421 crosslinked to Sepharose A[14].

To determine whether p53 can activate transcription *in vitro*, we used as templates the plasmids fos1wt and fos1mt, which contain the human RGC p53 DNA-binding fragment or a mutated RGC fragment respectively (Fig. 2*a*). Three partially purified fractions from HeLa cell nuclear extracts were used as a source of transcription factors[9]. RNA products were analysed by S1 nuclease digestion using specific probes for each construct. Increasing amounts of p53 stimulated transcription from fos1wt (compare lanes 1–4 with lanes 5–8). These reaction mixtures also included a construct containing an abridged adenovirus major late promoter (pMLS; ref. 10) whose transcription was not significantly affected by p53.

The p53 protein activated transcription from another promoter as well (Fig. 2*b*). Plasmids containing either one or sixteen copies of the RGC site, or one mutant RGC site, inserted adjacent to the polyoma virus early promoter to create Py1wt, Py16wt and Py1mt, respectively, were used as templates in transcription reactions. We found that p53 activated transcription of constructs containing the wild-type RGC (lanes 1–9) but not the mutant RGC (lanes 10–12). Diagrams of the templates and the test probe used in these experiments are shown in Fig. 2*c* with the expected S1 nuclease products.

The high incidence of p53 gene mutations in cancer patients suggests that alteration of the normal function of p53 is an important part of the oncogenic process. Therefore it was of interest to examine whether tumour-derived mutant p53 proteins activate transcription. The mutant p53 proteins we chose are defective in both nonspecific and specific DNA binding[4,5,11], so providing an opportunity to confirm that p53 must bind DNA to activate transcription. We compared the ability of wild-type and two tumour-derived mutant p53 proteins with mutations at either amino acid 175 (His 175) or at amino acid 273 (His 273)