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Mutation of the Serine 312 Phosphorylation Site Does Not Alter the Ability of Mouse p53 To Inhibit Simian Virus 40 DNA Replication In Vivo

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Two mutations were introduced into the wild-type mouse p53 gene by oligonucleotide-directed mutagenesis. These mutations substituted alanine or aspartic acid for serine at position 312, which is constitutively phosphorylated. Phosphopeptide mapping of the mutant proteins, expressed in COS cells, confirmed the loss of phosphorylation at position 312. There were no changes in the ability of the mutant p53s to express the conformation-dependent epitope for monoclonal antibody PAb246 or to participate in complexes with the simian virus 40 (SV40) large T antigen. Replication of a plasmid containing the SV40 origin of replication was inhibited in COS cells by wild-type p53 and both of the phosphorylation site mutants with equal efficiency. A transforming mutant of p53, encoding valine at position 135, did not inhibit SV40 DNA replication in COS cells.

p53 is a short-lived nuclear phosphoprotein which is present at low levels in normal cells and at high levels in a variety of tumors and transformed cell lines (for recent reviews, see references 34 and 41). The protein forms stable complexes with the large T antigen of simian virus 40 (SV40) (40, 42, 43) and the E1b 58K protein of adenovirus type 5 (62) and was initially thought to be a cellular target of these transforming proteins. Murine p53 was postulated to be an oncogene of the nuclear type on the basis of its ability to rescue primary cells from senescence (32, 33, 58), to cooperate with an activated *ras* gene to fully transform primary rat embryo fibroblasts (15, 33, 52), and to transform established cell lines to a tumorigenic phenotype without a significant change in cell morphology (14, 37, 82). However, recent evidence has shown that wild-type p53 is not a transforming protein (20, 29). Only certain p53 mutants have oncogenic potential and, even so, perform less well in transformation assays than other well-characterized nuclear oncogenes (15, 52). These mutant proteins differ from the wild type in that they generally do not bind T antigen or express the conformation-dependent epitope recognized by monoclonal antibody PAb246 but do form complexes with members of the hsp70 family of stress proteins and have longer half-lives than the wild-type protein (20, 28, 72, 74). There is now growing evidence that the wild-type p53 may have a suppressor function in transformation. For example, rearrangements which occur at a high frequency in the p53 gene during Friend virus-induced erythroleukemia and which lead to loss of p53 function appear to confer a selective advantage on erythroid cells (9, 27, 49, 50, 59). Mutations in the p53 gene have also been found in human colorectal carcinomas (3) and osteogenic sarcomas (45). Moreover, wild-type p53 has been demonstrated to suppress transformation by *ras* and activated p53 or E1A in cotransfection experiments (19). This is thought to explain, at least in part, the mechanism of transformation by SV40, in which sequestration of p53 by T antigen could block the suppressor function of p53 (23).

The normal function of p53 is unknown. However, several lines of evidence indicate that it is a cell cycle-related protein which may regulate cell growth at the level of DNA replication. For example, mitogenic stimulation of quiescent 3T3 cells is followed by a significant rise in p53 mRNA and protein immediately preceding initiation of DNA synthesis (57). Moreover, microinjection of anti-p53 antibodies into the nuclei of quiescent cells blocks serum-stimulated DNA synthesis and the transition back into the cell cycle (47). Others have reported that quiescent cells have a different antigenic form of p53 from that of actively growing cells (48) and that the expression of p53 antisense RNA results in extensive impairment of cell growth (65). At the biochemical level, mouse p53 can block the association between T antigen and DNA polymerase α (21, 22). Moreover, mouse p53 (but not human p53) specifically inhibits SV40 origin-dependent DNA replication, both in vivo (6, 71) and in vitro (70, 80). This inhibition has been shown to occur during assembly of the preinitiation complex (80), where p53 reduces both the binding of T antigen at site 2 of the SV40 origin and the ability of T antigen to unwind this DNA (70, 80).

p53 is phosphorylated at multiple sites. It does not appear to be a protein kinase itself, although the T antigen-p53 complex has associated protein kinase activities (79). Analyses of the phosphorylation sites of mouse p53 by Edman sequencing (2, 60) and phosphopeptide mapping (46) have identified serine residues 312 and 389 as phosphoacceptors. In addition to these sites, there is a cluster of mainly phosphoserine residues (and a lesser amount of phosphothreonine residues) close to the N terminus (2, 46, 60). The role of phosphorylation in modulating the function of p53 is unclear, but two of these sites may be important for transformation. The potential involvement of phosphorylation at serine 389 in transformation was inferred from its differential phosphorylation in SV40 *ts*A58-transformed cells at nonpermissive and permissive temperatures (2, 60). Increased

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phosphorylation of serine 312 was also observed in SV40-transformed cells compared with nontransformed cells (46).

To investigate the functional significance of p53 phosphorylation, we have introduced two mutations at the codon for serine 312 in the mouse p53 gene by oligonucleotide-directed mutagenesis. We chose to study serine 312 because it is the major site of p53 phosphorylation in SV3T3 cells (46). The mutants encode alanine to test the effects of loss of phosphorylation and aspartic acid to mimic constitutive phosphorylation. Since p53 has profound effects on SV40 DNA replication and might therefore modulate the activity of a T-antigen homolog(s) in cellular DNA replication, we chose to test the effects of these mutations on replication of an SV40 origin-containing plasmid in COS cells. We have included in our study a mutant p53 which encodes valine in place of alanine at position 135 since this mutant displays unusual and interesting behavior in DNA replication assays in vitro (80).

MATERIALS AND METHODS

Plasmid construction. Plasmid pGp53VS (20.3 kilobases [kb]) is the parent plasmid for all of the p53-expressing plasmids described below. It was constructed by transferring a 13-kb *Bgl*II fragment from plasmid pUL8R6 (52, 82), which was generously provided by R. A. Weinberg, into the *Bam*HI site of the vector p*neo*MLV (35). pGp53VS contains the entire murine genomic p53 DNA and has one sequence difference from wild-type p53 (20) at amino acid position 135, where valine is encoded in place of alanine (see below). The vector contains the neomycin phosphotransferase gene and the SV40 origin of replication; the p53 gene is linked in the sense orientation to the 5′ long terminal repeat of the vector. These features and the restriction sites used in construction of the p53 mutants are shown in Fig. 1.

Plasmid pUCBam5.7 was constructed by cloning the 5.7-kb *Bam*HI fragment of pGp53VS into plasmid pUC18, oriented such that the 5′ region of the p53 DNA was located at the 5′ end of the β-galactosidase gene.

Plasmid pGp53delBam was constructed by deleting the 3.1- and 5.7-kb *Bam*HI fragments encoding exons E1 to E6 of the p53 gene.

Oligonucleotide-directed mutagenesis and DNA sequencing. Oligonucleotides were synthesized by using a Biosearch 8750 automated DNA synthesizer and were purified by polyacrylamide gel electrophoresis. Those which were used for mutagenesis were as follows, with the mutant bases given in lowercase letters.

(i) 5′-CACGTCTTCgCCAGCTGGC-3′. This oligonucleotide has one mismatch and was used to change the valine codon at position 135 (located toward the 5′ end of exon 5) to alanine, thereby restoring the wild-type murine p53 sequence (20) and destroying a site for *Hph*I.

(ii) 5′-CAAGCGCCgCgCCCCCGC-3′. This oligonucleotide, which has two mismatches, was used to replace the serine 312 codon (located toward the center of exon 9) with an alanine codon, and it also generated a restriction site for *Bst*UI.

(iii) 5′-CAAGCGCCgaTCCCCCGC-3′. This oligonucleotide has two mismatches; it was used to replace serine 312 with aspartic acid, and it also generated a restriction site for *Sau*3A.

Mutagenesis involved the transfer of the appropriate restriction fragments into M13mp19. For mutagenesis of valine 135, the 2.0-kb *Xho*I-*Bam*HI fragment (encoding exons E2 to E6) (Fig. 1) was transferred to mp19 such that the coding strand was in the single-stranded phage DNA. To construct the mutations at position 312, the 1.2-kb *Bam*HI-*Hin*dIII fragment (encoding E7 to E9) (Fig. 1) was cloned in mp19 such that the noncoding strand was in the single-stranded phage DNA. Recombinant M13 phage were purified by centrifugation on CsCl step gradients (83), and single-stranded template DNA for mutagenesis was prepared by phenol extraction and ethanol precipitation of the purified phage.

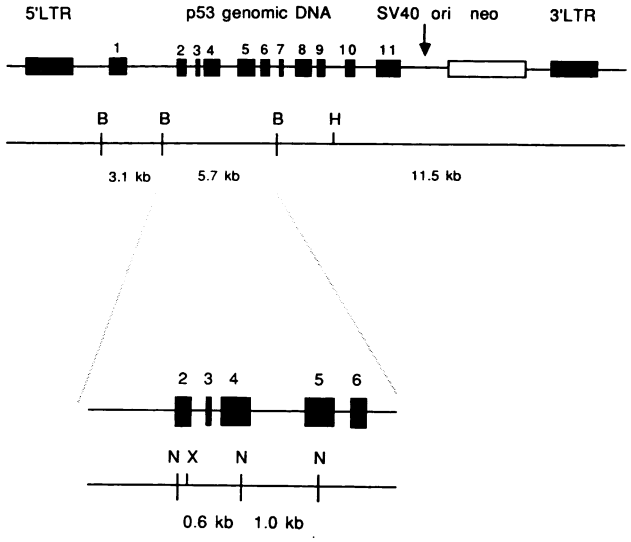

FIG. 1. p53-expressing plasmid pGp53VS. Construction of the plasmid is described in Materials and Methods. The diagram shows the plasmid linearized at the *Eco*RI site in the vector (35). The open box represents the neomycin gene; the hatched boxes are the long terminal repeats (LTR) of the vector; the filled boxes (numbered 1 to 11) are exons 1 to 11, respectively, of the mouse p53 gene; and the arrow indicates the position of the SV40 origin of replication. The positions of restriction cleavage sites for *Bam*HI (B) are shown together with the *Hin*dIII site (H) used for shuttling exons 7 to 9 between the expression plasmid and mp19. The sizes of the *Bam*HI restriction fragments in pGp53VS are given, with the expanded region being the 5.7-kb *Bam*HI fragment which was transferred into pUC18. The positions of sites for *Xho*I (X) and *Nco*I (N) and the sizes of the *Nco*I fragments are shown in the expanded region. The diagram is not drawn to scale.

Mutagenesis was carried out by using an oligonucleotide-directed in vitro mutagenesis system (Amersham Corp.), which employs the procedures developed in the laboratory of F. Eckstein (51, 63, 75, 76). The desired mutations were identified by DNA sequencing, by using the dideoxy-chain termination method of Sanger et al. (61) and employing oligonucleotide primers complementary to sequences in the p53 gene. The remaining p53 sequences (both exon and intron sequences) in the mutated M13 clones were also determined to ensure that only the desired changes had been made.

The fragment containing the alanine 135 change was returned to the parent expression plasmid in two steps, both involving partial restriction digests and purification of the appropriate fragments. In the first step, the 1.0-kb *Nco*I fragment (encoding E4 to E5 and alanine 135) was removed from the M13 vector and substituted for the homologous valine 135-encoding fragment in pUCBam5.7 to generate plasmid pUCala135. The 5.7-kb *Bam*HI fragment was then taken from pUCala135 and substituted for the homologous

TABLE 1. Plasmids encoding and expressing wild-type or mutant mouse p53 proteins

Plasmid[a]	Amino acid encoded at:	
	Position 135	Position 312
pGp53AS	Alanine	Serine
pGp53AA	Alanine	Alanine
pGp53AD	Alanine	Aspartic acid
pGp53VS	Valine	Serine
pGp53VA	Valine	Alanine
pGp53VD	Valine	Aspartic acid

[a] The amino acids encoded at positions 135 and 312 are given in the last two letters, respectively, of the plasmid name. Thus, pGp53AS encodes alanine at 135 and serine at 312, etc. Plasmid pGp53AS encodes the wild-type p53.

fragment in pGp53VS to generate pGp53AS, now encoding wild-type murine p53 (Fig. 1; Table 1).

In order to simplify transfer of the Ala-312 and Asp-312 mutations back into the p53 gene, *Bam*HI-*Hin*dIII fragments encoding either alanine or aspartic acid at position 312 were excised from M13 and substituted for homologous DNA in plasmid pGp53delBam. The remainder of the p53 sequences were then returned to the pGp53delBam plasmids encoding mutations at position 312 as a purified 8.8-kb fragment following partial digestion of pGp53AS or pGp53VS with *Bam*HI. At each stage of reconstruction, transfer of the mutations was monitored by restriction mapping. After the final stages of reassembly, the DNAs were resequenced in the regions encompassing the mutation sites.

A total of six p53-encoding plasmids were generated. Their descriptions and notations are given in Table 1.

Cells, transfections, and establishment of cell lines. CV-1 cells, COS cells, and their derivatives were grown in Dulbecco-Vogt modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS; Hyclone) and incubated at 37°C in a humidified 10% CO_2 atmosphere.

Cells were seeded and transfected with a total of 20 μg of plasmid DNA per 10-cm plate as described by Chen and Okayama (8). Following transfection, the cells were rinsed once with isotonic Tris-buffered saline (TBS) and glycerol shocked (to facilitate DNA uptake) by rinsing the plates for 1 min with 20% glycerol in TBS. The plates were then rinsed twice more with TBS prior to replacing the DMEM plus 10% FCS. To obtain lines which stably expressed mouse p53, cells were transfected with pGp53VS or its derivative plasmids, and the medium was replaced 24 h later with DMEM plus 10% FCS containing Geneticin (G418; GIBCO Diagnostics) at 400 μg/ml. The medium was then replaced every 2 to 3 days. After 14 days, pools of G418-resistant colonies or single colonies were trypsinized and replated. G418 selection was not maintained. Cell lines were analyzed for murine p53 expression as described below.

Radiolabeling of cells. Cells were seeded at a density of 10^6/10-cm plate 8 h before labeling. For [^{32}P]phosphate labeling, the cells were rinsed three times with TBS and once with phosphate-free DMEM and then labeled for 16 h in 4 ml of phosphate-free DMEM supplemented with 10% dialyzed FCS and containing carrier-free [^{32}P]orthophosphoric acid (ICN Pharmaceuticals Inc.) at a concentration of 0.5 mCi/ml. A similar procedure was used for labeling cells with L-[^{35}S]methionine and L-[^{35}S]cysteine; in this case, the final rinse was with methionine- and cysteine-free DMEM and the cells were labeled for 16 h in 4 ml of methionine- and cysteine-free DMEM supplemented with 5% undialyzed FCS and containing Tran^{35}S label (ICN Radiochemicals; specific activity, 1,200 Ci/mmol) at a concentration of 50 μCi/ml.

Cell lysis, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis. To prepare cells for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, they were washed three times with ice-cold TBS and lysed in 1 ml of Nonidet P-40 (NP-40) buffer (0.15 M NaCl, 1% NP-40, 10 mM sodium phosphate [pH 7.0], 1% Trasylol, 50 μM leupeptin, 1 mM dithiothreitol). The lysates were clarified by centrifugation at 20,000 × *g* for 1 h at 4°C.

The following monoclonal antibodies were used as hybridoma supernatants. PAb122 (24), PAb246 (84) (kindly provided by J. Yewdell, Wistar Institute of Anatomy and Biology, Philadelphia, Pa.) and RA3-2C2 (10, 56) all recognize p53 specifically. PAb108 (25) is a monoclonal antibody to the SV40 large T antigen, and NS1 (used as a control) (4, 39) is a nonsecretor murine myeloma line. Immunoprecipitation involved incubation of 200 μl of lysate with an equal volume (excess) of hybridoma supernatant for 1 h on ice. PAb246 and RA3-2C2 required additional 20-min incubations with rabbit anti-mouse immunoglobulin G and goat anti-rat immunoglobulin M antibodies (2 μg per immunoprecipitate), respectively, to facilitate binding to *Staphylococcus aureus* protein A. For immunoprecipitation of ^{32}P-labeled proteins, the incubations were carried out in the presence of 100 μg of RNase A (Sigma Chemical Co.) per ml. Immune complexes were then removed by adsorption to protein A-Sepharose beads (20 μl of a 1:1 [wt/vol] suspension in NP-40 buffer) with gentle shaking for 1 h on ice. The pellets were washed four times with NP-40 buffer and solubilized in SDS sample buffer.

Proteins were resolved by SDS-polyacrylamide gel electrophoresis in 10% gels (acrylamide/bis ratio of 38:1) in the presence of 0.1% SDS. Autoradiography was carried out at −70°C with preflashed XAR-5 film (Eastman Kodak Co.) and intensifying screens. ^{35}S-labeled bands were intensified by impregnating the gels with diphenyloxazole; fluorography was then carried out at −70°C with preflashed XAR-5 film. Exposure times are given in the figure legends.

Two-dimensional phosphopeptide mapping. Proteins were extracted from polyacrylamide gels and oxidized as described previously (5) by using 20 μg of RNAse A as the carrier. Digestion of p53 with trypsin was performed for 4 h at 37°C in 50 mM NH_4HCO_3 (pH 7.2 to 7.5) with the addition of L-1-tosylamido-2-phenylethylchloromethyl ketone-treated trypsin (Worthington Diagnostics) at 1-h intervals at a trypsin/carrier ratio of 1:50 (wt/wt) to minimize contaminating chymotryptic activity. Peptides were separated in two dimensions on 100-μm cellulose thin-layer plates by electrophoresis at pH 1.9 for 40 min at 1,000 V and by chromatography (31). Plates were exposed to preflashed XAR-5 film at −70°C with intensifying screens. The exposure times are given in the figure legends.

Protein half-life determination. Plates (10 cm) containing 10^6 cells were labeled as described above for 1 h with [^{35}S]methionine and [^{35}S]cysteine. Following labeling, the radioactive medium was removed and the cells were washed twice with TBS. The plates were then refed with DMEM plus 10% FCS (chase medium) and incubated for 0- to 60-min time periods. At the end of the chase period, cell lysates, immunoprecipitations, and SDS-polyacrylamide gel electrophoresis were carried out as described above.

In vivo DNA replication assay. For the in vivo SV40 origin-dependent DNA replication assays, replication of the SV40 origin-containing plasmid pSV2*neo* (69) was measured. In general, COS cells, COS cell lines expressing

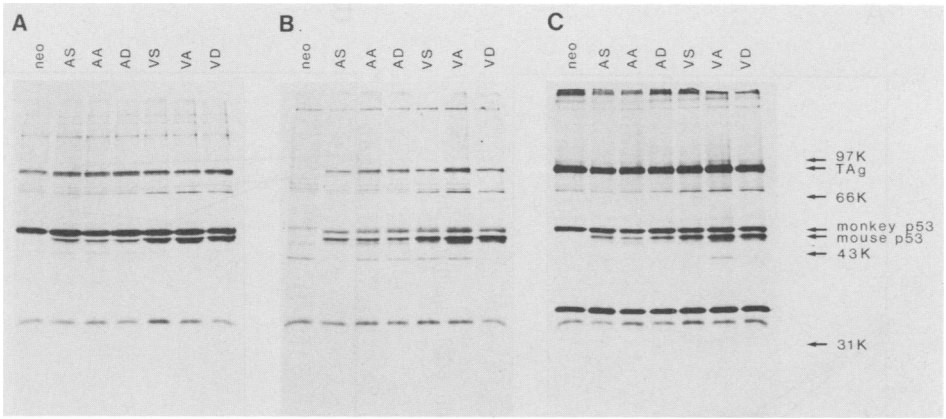

FIG. 2. Expression and characterization of wild-type and mutant p53 proteins. The experimental details are given in Materials and Methods. COS cells were transfected with each of the six wild-type or mutant p53-encoding plasmids (Table 1) or the vector p*neo*MLV as the control and were selected for G418 resistance. Mass cultures of cells and individual colonies were isolated for use in this and the following experiments. Pools of G418-resistant cells were labeled with [^{35}S]methionine and [^{35}S]cysteine, and the proteins were immunoprecipitated and resolved by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels. The antibodies were as follows: PAb122 (α-p53) (A), PAb246 (α-p53) (B), and PAb108 (α-T antigen) (C). Lanes: neo, p*neo*MLV; AS, pGp53AS; AA, pGp53AA; AD, pGp53AD; VS, pGp53VS; VA, pGp53VA; VD, pGp53VD. The positions of mouse p53, monkey p53, T antigen (TAg), and markers are indicated. The exposure time for fluorography was 24 h.

murine p53, or CV-1 cells (as T-antigen-minus controls) were seeded at 5×10^5 cells per 10-cm plate in DMEM plus 10% FCS. On the following day, the cells were transfected by using the procedure described above with 15 μg of pUC18 DNA and 5 μg of pSV2*neo* DNA. The pUC18 DNA served three purposes: (i) as carrier plasmid DNA to give a total amount of 20 μg per transfection (8); (ii) as a negative control in each transfection, since it does not have the SV40 origin and is therefore not replicated; and (iii) as an internal standard with which to gauge the recovery of plasmid DNA after the extraction procedure (see below). At 16 to 24 h after transfection, the cells were glycerol shocked as described above and the medium was replaced. The medium was also changed at 48 h after the glycerol shock. At 72 h after the glycerol shock, the plasmid DNAs were isolated by a modification of the procedure of Hirt (30) and linearized with excess *Bam*HI. The parent and replicated daughter molecules were distinguished by their differential sensitivity to the restriction endonuclease *Dpn*I, for which unmethylated DNA isolated from mammalian cells is not a substrate (53). The DNAs were then fractionated in 0.8% agarose gels and transferred to nitrocellulose (Nytran; Schleicher & Schuell, Inc.) by using a modified procedure (67) of the original method of Southern (68). The filters were probed with pSV2*neo* which had been labeled with ^{35}P by using the random priming method of Feinberg and Vogelstein (17, 18) and exposed to preflashed XAR-5 film at −70°C with intensifying screens (pSV2*neo* also has homology with pUC18). The autoradiographs were quantified by densitometry. The final values were expressed as the ratio of the 6.06-kb pSV2*neo* fragment (replicated DNA) to the 0.96-kb fragment from pUC18 (unreplicated internal standard DNA).

RESULTS

Oligonucleotide-directed mutagenesis of mouse p53. Construction of the parent plasmid pGp53VS, which expresses mouse p53 (encoding valine at position 135) from a genomic insert (52, 82), is described in Materials and Methods. We chose to use a genomic clone, rather than a cDNA, because the presence of introns has been shown to elevate expression of p53 severalfold and significantly enhance biological activity (29, 54; unpublished observations).

Mutations were introduced into the p53 coding sequence by oligonucleotide-directed mutagenesis, as described in detail in Materials and Methods. The first change, Ala-135, replaced valine at position 135 with alanine to restore the wild-type p53 sequence. Two other changes were made, both at the codon for the serine 312 phosphorylation site. One mutation, Ala-312, which replaced serine with alanine, a structurally very similar but nonphosphorylatable amino acid, was constructed to test for the loss of phosphorylation at this site. The other, Asp-312, was constructed to test the effects of constitutive phosphorylation by placing a negatively charged group such as aspartic acid at this position.

Sequence analysis showed that there were no changes in the p53 genes other than the desired mutations (data not shown). A total of six p53-expressing plasmids were constructed, with serine, alanine, or aspartic acid at position 312 and each with valine or alanine at position 135 (Table 1).

Expression and characterization of the mutant p53 proteins. COS cells were transfected with the wild-type and mutant p53-encoding plasmids or with the vector p*neo*MLV as the control. After 14 days of G418 selection, pools of G418-resistant colonies (each pool was derived from about 100 colonies) or individual clones were isolated. To examine mouse p53 expression and its interaction with other proteins, the cells were labeled with [^{35}S]methionine and [^{35}S]cysteine. After lysis, the p53 proteins were immunoprecipitated and separated by SDS-polyacrylamide gel electrophoresis.

COS cells transfected with each of the six p53-encoding plasmids expressed mouse p53; SDS-polyacrylamide gel electrophoresis of the proteins immunoprecipitated with monoclonal antibody PAb122 clearly resolved the mouse p53 from the endogenous monkey p53 (Fig. 2A). There were no differences in mobility between the wild-type p53 and each of the mutant proteins. Equal steady-state levels of the three Ala-135 p53 species (Fig. 2A, lanes AS, AA, and AD) were observed in the G418-resistant pools; individual colonies expressing varying levels of p53 were also isolated (data not shown). In these mass cultures of cells, there was five times

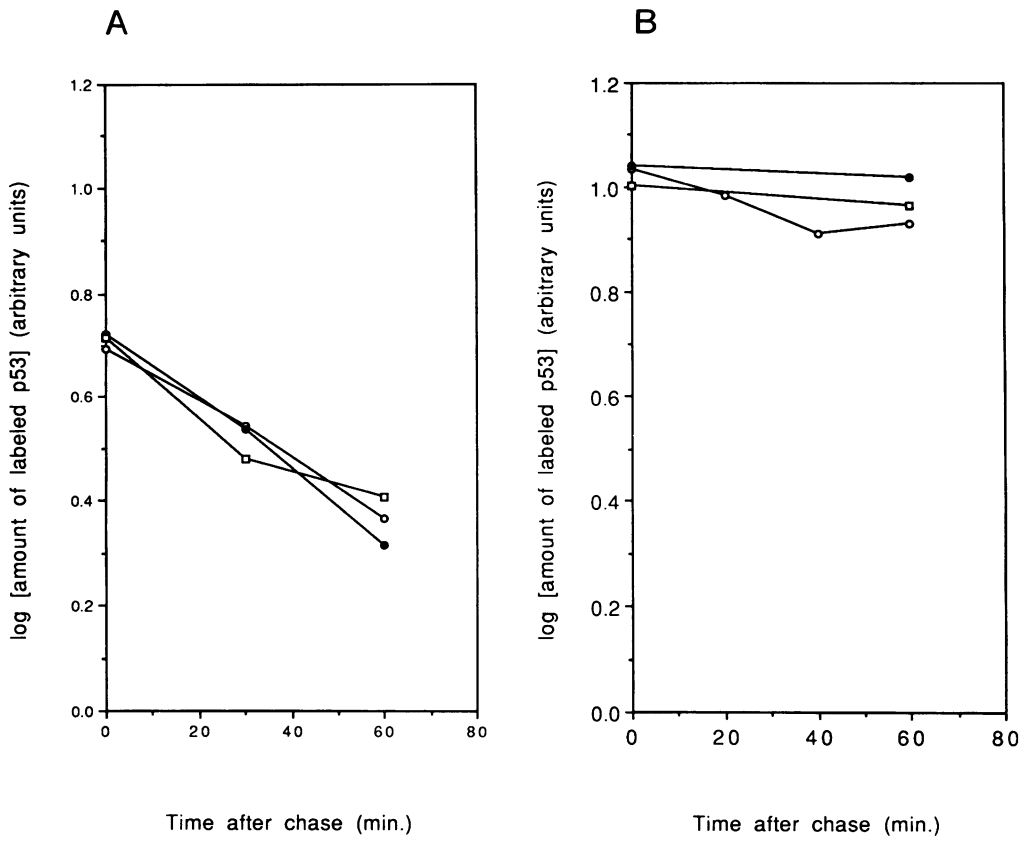

FIG. 3. Stabilities of wild-type and mutant mouse p53 proteins in COS cells. Pulse-labeling of COS cells expressing mouse p53 with [^{35}S]methionine and [^{35}S]cysteine, followed by chase with unlabeled methionine and cysteine, was carried out according to the procedure described in Materials and Methods. Autoradiographs of the labeled proteins resolved by SDS-polyacrylamide gel electrophoresis were quantitated by densitometry. (A) Decay curves for the p53 proteins encoding alanine at position 135; (B) decay curves for the p53 proteins encoding valine at position 135. Proteins encoding serine, alanine, or aspartic acid at position 312 are represented by open circles, closed circles, and open squares, respectively.

less mouse p53 than monkey p53. Similarly, equal amounts of the Val-135 p53 species (lanes VS, VA, and VD) were seen and, although all of the p53s were expressed from the same construct, the Val-135 proteins were present in higher amounts than those encoding Ala-135 (the mouse p53/monkey p53 ratio was about 1:1). p53 encoding valine at position 135 has been reported to be more stable than wild-type p53 (29). We therefore measured the half-lives of the mouse p53 proteins expressed in these cells. Pulse-chase experiments showed that proteins with alanine at position 135 had half-lives in the range of 50 to 60 min (Fig. 3A), whereas the p53 proteins with valine at position 135 had half-lives of greater than 2 h (Fig. 3B). Mutation of codon 312 had no effect on protein stability. These results are consistent with the observed steady-state levels of the mouse p53 proteins in COS cells.

Wild-type mouse p53 and each of the five mutant proteins (Fig. 2B, lanes AS, AA, AD, VS, VA, and VD), but not the monkey p53 (lane neo), have the epitope for mouse p53-specific monoclonal antibody PAb246. Moreover, both monkey p53 and T antigen were present in PAb246 immunoprecipitates (or RA3-2C2 precipitates [data not shown]) only when mouse p53 was present, indicating that the wild-type and the mutant mouse proteins can complex with T antigen and/or monkey p53. In agreement with these findings, both monkey and mouse p53s coimmunoprecipitated with T antigen (Fig. 2C). The data also showed that there was considerably more p53 precipitated than T antigen when anti-p53 antibodies were used (Fig. 2A and B, mouse p53); this suggested that not all of the p53 and T antigen were associated. Similarly, PAb108 (α-T antigen) precipitated considerably more T antigen than mouse or monkey p53 (Fig. 2C). Sequential immunoprecipitations using PAb122 followed by PAb108 (or vice versa) confirmed the presence of uncomplexed p53 and T antigen (data not shown). There were no qualitative or quantitive differences between the association of the wild-type mouse p53 and any of the mutants with the monkey p53-T antigen complex (other than the higher levels of the Val-135 proteins discussed above). When p53 expression was examined in individual clones, profiles similar to those described for the mass cultures of cells were observed (data not shown).

Phosphorylation of wild-type and mutant p53 proteins. To confirm that the oligonucleotide-directed mutations had indeed altered the serine 312 phosphorylation site, COS cells expressing the wild-type or mutant p53 proteins were labeled in vivo with [^{32}P]orthophosphate; the p53 proteins were immunoprecipitated and resolved by SDS-polyacrylamide gel electrophoresis, as described above (data not shown). The proteins were then eluted from the acrylamide gel, oxidized, digested with trypsin, and separated in two dimensions. The tryptic phosphopeptides of mouse p53 are shown in Fig. 4A. T1A and T1B were both previously identified as having phosphoserine at position 312; T2A and T2B are both

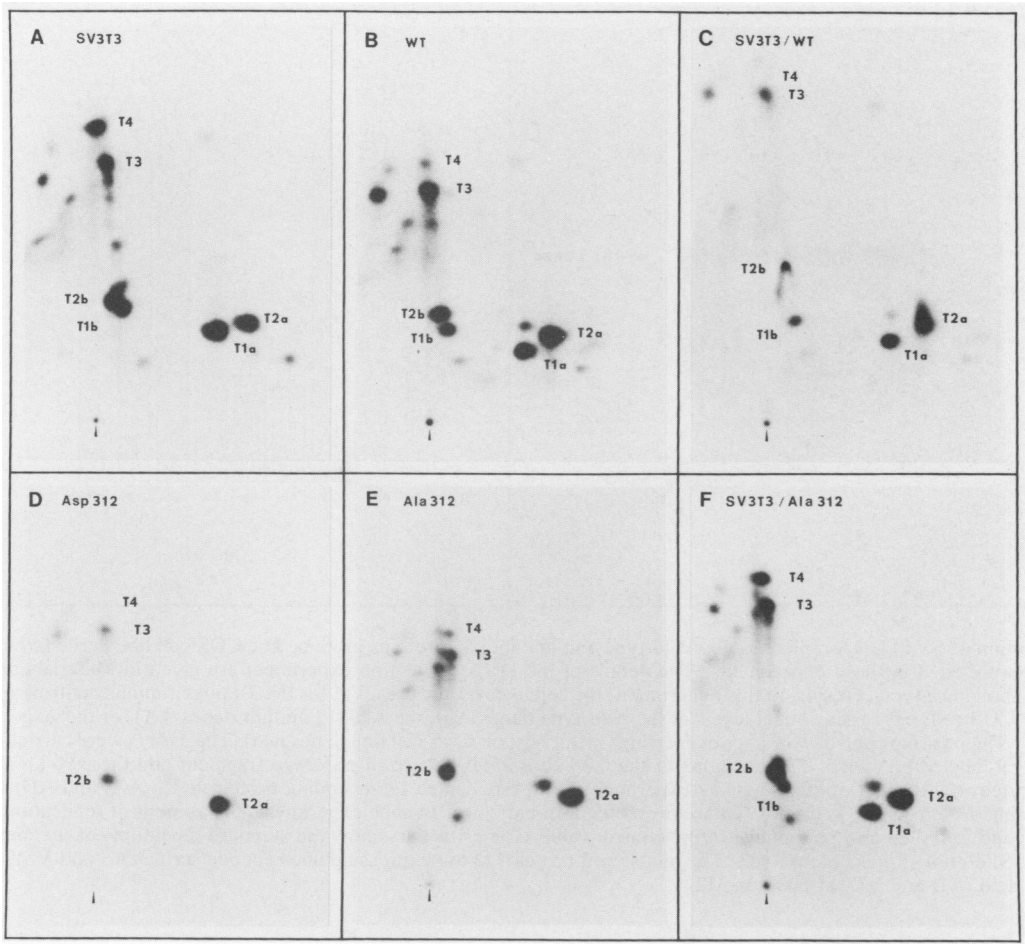

FIG. 4. Digestion of p53 proteins with trypsin. ^{32}P-labeled proteins extracted from SDS–10% polyacrylamide gels were oxidized, digested with trypsin, and resolved by electrophoresis at pH 1.9 in the horizontal dimension (anode at the left) and by chromatography in the vertical dimension, as described in Materials and Methods. The origin is indicated with a vertical arrowhead. (A) p53 from SV3T3 (mouse) cells; (B) mouse p53 from COS cells transfected with pGp53AS (Ser-312); (C) mixture of the phosphopeptides from panels A and B; (D) mouse p53 from COS cells transfected with pGp53AD (Asp-312); (E) mouse p53 from COS cells transfected with pGp53AA; (F) mixture of the phosphopeptides from panels A and E. The Cerenkov counts per minute loaded and exposure times, respectively, were: 9,000 cpm, 1 day (A); 1,000 cpm, 5 days (B); 400 cpm, 9 days (C); 500 cpm, 5 days (D); 8,000 cpm, 1 day (E); and 9,000 cpm, 1 day (F).

phosphorylated at position 389; and a cluster of phosphorylations at the N terminus (between residues 1 and 37) give rise to T3, T4, and the minor phosphopeptides which migrate in their vicinity (46). All of these trypsin-digested phosphopeptides were present in wild-type mouse p53 from COS cells (Fig. 4B). This was confirmed when a mix of the p53 phosphopeptides from SV3T3 and COS cells was analyzed (Fig. 4C). There were no new major phosphopeptides present. Both the Asp-312 and Ala-312 mutant p53 proteins lacked phosphopeptides T1A and T1B (Fig. 4D and E, respectively), although all of the other phosphopeptides were present (Fig. 4D to F). The T1 and T2 phosphopeptides migrate in very close proximity. Therefore, to be sure that T1A and T1B, and not T2A and T2B, were absent from the phosphorylation site mutants, phosphopeptides T2A and T2B were eluted from the Ala-312 and Asp-312 thin-layer plates (Fig. 4E and D, respectively), mixed with phosphopeptides T1A and T1B, or T2A and T2B from the SV3T3 plate (Fig. 4A), and separated as described above. The results confirmed that phosphopeptides T1A and T1B were absent from the Ala-312 and Asp-312 mutants (data not shown). These observations are consistent with loss of phosphorylation at position 312. Similar results were obtained when the mouse p53s were expressed in rat 208F fibroblasts (data not shown).

Inhibition of SV40 DNA replication in vivo by wild-type and mutant mouse p53 proteins. The wild-type and phosphorylation site mutant p53 proteins were tested for their ability to inhibit the replication of DNA containing an SV40 origin of replication in COS cells. COS cell line derivatives expressing mouse p53 (either as G418-resistant pools or individual colonies which expressed levels of p53 approximately equal to that of the pools of cells) were seeded in duplicate and transfected with 5 μg of pSV2*neo* (69), which contains the SV40 origin. Included in each transfection as an internal standard was 15 μg of a pUC18 DNA which is not replicated in COS cells. The control cells lacking mouse p53 were either COS cells alone (data not shown) or COS cells which had been transfected with the vector plasmid p*neo*MLV and selected for G418 resistance. CV-1 cells were used as control cells lacking T antigen. A typical autoradiograph showing the recovery of replicated pSV2*neo* DNA is shown in Fig. 5A; the results from five experiments, each done in duplicate, are summarized in Fig. 5B as a bar chart. Plasmid

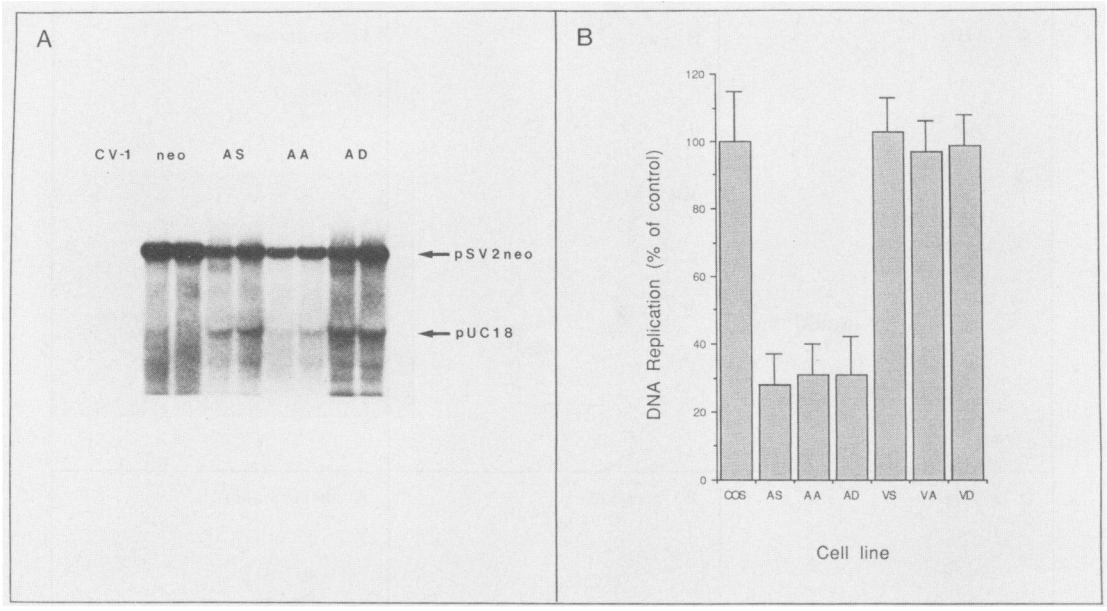

FIG. 5. Inhibition of SV40 DNA replication by wild-type and mutant p53 proteins in vivo. The COS cell line derivatives described in the legend to Fig. 2 were used in these experiments. The details of the DNA replication experiment are given in Materials and Methods. (A) Autoradiograph showing a typical result. In this experiment, the cell lines used were CV-1 (as the T-antigen-minus control) and G418-selected mass cultures of COS cells expressing equal levels of the wild-type (lanes AS), the Ala-312 mutant (lanes AA), or the Asp-312 mutant (lanes AD) mouse p53s. The positive control was a nonexpressing G418-resistant COS cell line (lanes neo). The assays were carried out in duplicate. The probe was ^{32}P-labeled pSV2*neo*. The positions of the 6.06-kb *Bam*HI-linearized pSV2*neo* fragment and the 0.96-kb *Dpn*I fragment of pUC18 are indicated. (B) Results of five duplicate experiments with the alanine 135-encoding proteins (AS, AA, and AD) and five with the valine 135-encoding proteins (VS, VA, and VD) are presented as a bar chart. In each experiment, the amount of replication was determined by densitometry and is shown as a percentage of the control value. The error bars show the standard deviations of the measurements. The control cells (COS) do not express mouse p53. The mouse p53 proteins in the expressing lines encode serine (AS and VS), alanine (AA and VA) or aspartic acid (AD and VD) at position 312.

pSV2*neo* was clearly replicated in the COS cells (Fig. 5A, lanes neo) but not in the T-antigen-minus control CV-1 cells (Fig. 5A, lanes CV-1). No replicated plasmid DNA was recovered from COS cells which had not been transfected with pSV2*neo* DNA or their mouse p53-expressing derivatives (data not shown); the replicated DNA recovered in these experiments was therefore generated only from the transfected plasmid. Wild-type mouse p53 (Fig. 5A and B, lanes AS) reduced the level of pSV2*neo* DNA replication in COS cells, as did the two phosphorylation site mutants (Fig. 5A and B, lanes AA and AD), each typically by about 70%; no significant differences in the efficiencies of these proteins in blocking replication could be discerned. In one clonal cell line where the ratio of wild-type mouse p53 to monkey p53 was 0.4, the amount of inhibition was about 90% (data not shown), which indicated that the level of inhibition was dependent on the level of mouse p53.

A transforming mutant of p53, encoding valine in place of alanine at position 135, and two derivatives of this mutant having alanine or aspartic acid at position 312 were also tested in the replication assay (Fig. 5B, lanes VS, VA, and VD, respectively). Even though each of these mutant p53 proteins was present at much higher levels in the mass cultures of cells as compared with the Ala-135 p53s (Fig. 2), no significant inhibition of pSV2*neo* replication was evident. In four individual clones in which the level of mouse p53 ranged from a mouse p53/monkey p53 ratio of 3.0 to 0.1, again no inhibition of DNA replication was observed (data not shown).

DISCUSSION

We previously identified serine 312 as the major site of phosphorylation of p53 in SV40-transformed mouse 3T3 cells (46). In the present study, we constructed two mutants, by using oligonucleotide-directed mutagenesis, to investigate the functional significance of p53 phosphorylation at this site. The first mutant replaced the serine at position 312 with alanine, which is structurally similar but cannot be phosphorylated. The other change introduced aspartic acid at this site to mimic constitutive phosphorylation. Others have successfully used these amino acids to simulate phosphorylation and dephosphorylation (36, 77).

We expressed the wild-type and mutant proteins in COS cells, which allowed us to resolve the mouse and endogenous monkey p53 species on SDS gels for subsequent analysis of phosphopeptides and protein-protein associations. This was particularly useful because we could characterize the structural and functional properties of the p53 proteins in the same cells. Tryptic phosphopeptide mapping showed that the phosphorylation of wild-type mouse p53 in these cells was very similar to the normal pattern of p53 phosphorylation in mouse cells. The exception (which was also seen in the mutant proteins) was that the N-terminal peptide T4 appeared to be underphosphorylated when expressed in COS cells (Fig. 4). The absence of phosphopeptides T1A and T1B from the mutant proteins confirmed the identity of serine 312 as a phosphorylation site. (However, the possibility that mutation of serine 312 prevents phos-

phorylation at serine 310 has not been ruled out.) None of the other phosphorylations were affected by these mutations, indicating that they are independent of phosphorylation at position 312.

Finlay et al. (19) have shown that activating mutations can result when wild-type p53 is introduced into some cell lines. The p53 expressed in COS cells in our experiments was determined to be wild type, on the basis of the presence of the epitope for PAb246, the ability to complex with T antigen, the lack of association with hsc70, the short half-life, and, most importantly, the ability to inhibit SV40 DNA replication (two laboratories have shown independently that transforming mutants of p53 are unable to block SV40 DNA replication [6, 80]).

The valine 135 mutant p53 has been reported to transform cells in cooperation with *ras* (15, 52). This mutant is unusual. Unlike other transforming species of p53, it appears to be involved in complexes with T antigen and clearly has the epitope for monoclonal antibody PAb246 (Fig. 2). However, like other transforming p53 proteins, it has a significantly longer half-life than the wild-type protein (Fig. 3). These differences in p53 stability are discernible in the presence of T antigen (which is thought to stabilize p53, at least in part [34]) and are consistent with the previous observation that the change of alanine to valine at position 135 contributes to the stabilization of p53 (29). Therefore, other factors appear to affect p53 stability in addition to complex formation with T antigen (12, 13, 26).

Mouse p53 has been shown to specifically inhibit replication of DNA containing an SV40 origin of replication both in vivo and in vitro (6, 80). These observations and a recent report that p53 can suppress cellular transformation (19) are consistent with evidence that p53 behaves as a cell cycle-modulated regulator of DNA synthesis. We therefore used inhibition of SV40 DNA synthesis as an assay with which to examine alterations in the function of p53 resulting from changes in its phosphorylation state, since inhibition most likely reflects the normal cellular function of p53. Our data showed that the replication of SV40 DNA was significantly reduced in COS cells which stably expressed mouse p53 (Fig. 5), thereby confirming previous observations of others (6, 80). In mass cultures of cells (in which the mouse p53/monkey p53 ratio was 1:5), the level of inhibition was typically 70%. The degree of inhibition appeared to be dependent on the amount of mouse p53 in the cell; we were able to observe 90% inhibition in a clone expressing higher levels of mouse p53 (with a mouse p53/monkey p53 ratio of 0.4 [data not shown]). This is in contrast to the results of Braithwaite et al. (6), who reported a lack of correlation between p53 levels and inhibition of DNA synthesis, but is consistent with the p53 concentration dependence for inhibition in vitro observed by Wang et al. (80). The greater than 95% inhibition consistently observed by Braithwaite et al. (6) may have been the result of using the very strong cytomegalovirus early region promoter-enhancer element to drive p53 expression; our study employed a murine leukemia virus long terminal repeat as the promoter. It is possible that there is a saturating level of p53 above which increases in the p53 concentration do not further significantly inhibit replication. In our case, since we were observing 70 to 90% inhibition, we might not have achieved this critical level. Moreover, on the basis of the findings of Tack et al. (73), it is possible that monkey p53 (which is present at elevated levels in COS cells) may itself stimulate replication and therefore antagonize the effects of the mouse p53. There are other factors which could have contributed to the lower levels of inhibition in our studies. For example, the results shown in Fig. 2 and our data from sequential immunoprecipitates (data not shown) show that there is free p53 and T antigen in the COS cells, and therefore not all of the p53 available in the cell might be involved in inhibition at any one time. In addition, we chose to use pools of G418-resistant cells for a major part of this analysis because this would correct for differences in the levels of expression in individual clones and therefore provide better comparisons. While screening individual clones, it was clear that there was a wide variation in the levels of exogenous p53 expressed. Therefore, in some cells there might not be enough mouse p53 to efficiently block replication. The 70% inhibition is therefore an average of the levels in each of the mixed populations of cells.

A transforming mutant of p53, encoding valine at position 135 in place of alanine, had no effect on DNA replication (Fig. 5), even when expressed at a level three times greater than the endogenous monkey p53 (data not shown). This observation is consistent with the reported lack of inhibition of SV40 DNA replication in vitro by this mutant (80). However, unlike others (80), we could not see inhibition of replication when the cellular concentration of the valine 135 mutant was considerably lower (as low as 10-fold below the monkey p53 concentration [data not shown]). Our data are also in agreement with those of Braithwaite et al. (6) since the valine 135 mutation lies within the region of amino acids 132 to 149, the deletion of which also results in the loss of the ability to block DNA replication.

Inhibition of SV40 DNA replication by mouse p53 proteins encoding alanine or aspartic acid in place of the phosphorylated serine at position 312 was indistinguishable from the behavior of wild-type p53 (Fig. 5). Therefore, phosphorylation of serine 312 appears not to be involved in the inhibition of SV40 DNA replication by mouse p53. Site-directed mutagenesis of phosphorylation sites has proved to be a successful approach in understanding the importance of phosphorylation in regulating a number of proteins involved in growth control. Examples include some members of the tyrosine kinase family such as *src*, *lck*, and the epidermal growth factor and insulin receptors, for which site-directed mutagenesis of phosphorylated residues has established their key roles in regulating the molecular function of the proteins and the phenotypes of the cells in which they are expressed (1, 7, 16, 38, 44, 55). However, in some cases, site-directed mutagenesis has indicated that phosphorylation sites do not have a regulatory function (11, 78, 81). In the case of the SV40 large T antigen, oligonucleotide-directed mutagenesis has shown that certain phosphorylations regulate some but not all of the activities of this pleiotropic protein (64). Similarly, mutation of the serine 89 phosphorylation site of E1A affects repression and transformation but not *trans* activation (66).

Our observations indicate that the state of phosphorylation of serine 312 does not affect inhibition of SV40 DNA replication by mouse p53. It is possible that phosphorylation-dependent effects do not appear under the conditions used here. For example, the possible contribution of monkey p53 to this process is unknown. Tack et al. (73) have shown that monkey p53 is associated with a subfraction of T-antigen molecules (during SV40 lytic infection) that is highly active in replication. Therefore, monkey p53 could diminish the inhibitory effect of mouse p53 by stimulating replication and perhaps conceal the effect of phosphorylation. This could be resolved with the availability of a monkey cell line (permissive for SV40 replication) which does not express endogenous p53. It is also possible that phosphorylation

serves some other purpose. In this respect, Carroll and co-workers (2, 60) have suggested that serine 312 is involved in a phosphodiester linkage, rather than a single phosphate monoester, as would be characteristic of regulatory phosphorylation. Therefore, this phosphorylation could play a structural or catalytic role, for example, in an active site as an intermediate in a double displacement reaction. It will be interesting to test whether phosphorylation of p53 at serine 312 is involved in regulating other activities of p53, for example, cooperation with other oncogenes in transformation by mutant p53 proteins or inhibition of transformation in the case of wild-type p53.

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