Identification of the p53 Protein Domain Involved in Formation of the Simian Virus 40 Large T-Antigen-p53 Protein Complex

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An expression vector utilizing the enhancer and promoter region of the simian virus 40 (SV40) DNA regulating a murine p53 cDNA clone was constructed. The vector produced murine p53 protein in monkey cells identified by five different monoclonal antibodies, three of which were specific for the murine form of p53. The murine p53 produced in monkey cells formed an oligomeric protein complex with the SV40 large tumor antigen. A large number of deletion mutations, in-frame linker insertion mutations, and linker insertion mutations resulting in a frameshift mutation were constructed in the cDNA coding portion of the p53 protein expression vector. The wild-type and mutant p53 cDNA vectors were expressed in monkey cells producing the SV40 large T antigen. The conformation and levels of p53 protein and its ability to form protein complexes with the SV40 T antigen were determined by using five different monoclonal antibodies with quite distinct epitope recognition sites. Insertion mutations between amino acid residues 123 and 215 (of a total of 390 amino acids) eliminated the ability of murine p53 to bind to the SV40 large T antigen. Deletion (at amino acids 11 through 33) and insertion mutations (amino acids 222 through 344) located on either side of this T-antigen-binding protein domain produced a murine p53 protein that bound to the SV40 large T antigen. The same five insertion mutations that failed to bind with the SV40 large T antigen also failed to react with a specific monoclonal antibody, PAb246. In contrast, six additional deletion and insertion mutations that produced p53 protein that did bind with T antigen were each recognized by PAb246. The proposed epitope for PAb246 has been mapped adjacent (amino acids 88 through 109) to the T-antigen-binding domain (amino acids 123 through 215) localized by the mutations mapped in this study. Finally, some insertion mutations that produced a protein that failed to bind to the SV40 T antigen appeared to have an enhanced ability to complex with a 68-kilodalton cellular protein in monkey cells.

The cell-encoded tumor antigen p53 is a phosphoprotein synthesized at higher levels in many transformed cells than in their normal counterparts (7, 26). p53 has been defined as a tumor antigen because animals bearing simian virus 40 (SV40)-induced tumors, animals hyperimmunized with cells transformed with chemicals, or even human patients with breast cancer may produce antibodies against p53 (6, 8, 14, 23, 26). Several lines of evidence demonstrate that p53 can play a role in the process of cell transformation or tumorigenesis. First, expression vectors containing p53 cDNA clones can result in altered growth control of cells producing this gene product in the presence or absence of another oncogene (9, 10, 19, 22, 36, 50). Second, rearrangements and chromosomal translocations of the p53 gene in transformed cells or tumor-derived cell lines have been identified in several different cases (25, 34, 49, 51, 52), resulting in either inactivation or activation of p53 gene products. Third, in SV40- and adenovirus-transformed cell lines, the SV40 large tumor (T) antigen (23, 26) and the adenovirus E1b-58Kd tumor antigen (43) have been shown to be in oligomeric protein complexes with the cellular p53 protein. In each case, these viral tumor antigens are known to be required to transform cells in culture (12, 20, 46). These observations, taken together with the demonstration that p53 in nontransformed cells appears to be regulated with or by events in the cell cycle (21, 29, 30, 32, 39), strongly imply that p53 can be important in regulating growth control.

The levels of p53 in nontransformed or transformed cells are regulated either by the steady-state level of mRNA or by the half-life of the protein itself (35, 40). Although in nontransformed cells the p53 protein has a half-life of about 20 min (35), in SV40- or adenovirus-transformed cell lines it has a much longer half-life (35; T.-H. Tan and A. J. Levine, unpublished results), which results in production of elevated levels of the protein. These high levels of p53 in SV40-transformed cells are regulated in a temperature-sensitive fashion in cells transformed by SV40 temperature-sensitive mutants in the large T-antigen gene (*tsA* mutants) (27). It has therefore been suggested that the T-antigen-p53 complex is responsible for the increased half-life and stability of p53 (35) and may be involved in the maintenance of cell transformation (33).

To explore the structure and relate it to the functions of the p53 gene product, a genetic analysis was initiated that involved site-directed mutagenesis with the cloned p53 cDNA. A p53 cDNA expression vector was used to produce the murine p53 protein in monkey cells. This protein was able to bind to the SV40 large T antigen and was recognized by five different monoclonal antibodies directed against distinct epitopes in the murine p53 protein. A total of two deletion mutations, nine in-frame linker insertion mutations, and three linker insertion frameshift mutations were constructed across the coding region of the p53 cDNA clone. Five of these mutations produced a murine p53 protein that failed to bind to the SV40 T antigen, and these mutations all failed to react with a single specific monoclonal antibody, PAb246. These linker insertion mutations mapped in a discrete region of the protein between amino acids 123 and

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FIG. 1. Physical map of p53 expression vector p11-4 plasmid. p11-4 plasmid contains the *HaeIII*-*ApaI* fragment of pp53-17C plasmid (37) cloned into pSV2-based vector (see Materials and Methods). Arrows indicate the direction of transcription. Symbols: —, pBR322 DNA; □, SV40 DNA; ■, mouse p53 cDNA; ori, the origin of replication in SV40 or pBR322.

215 (out of 390 amino acids). The N-terminal region deletion mutations (amino acids 11 through 33) or carboxy-terminal localized linker insertion mutations (amino acids 222 through 344) produced proteins that bound to the SV40 T antigen and were also recognized and immunoprecipitated by PAb246. Thus, the same p53 mutant proteins failed to bind to or interact with the SV40 large T antigen and PAb246 antibodies. These p53 mutant proteins were recognized by monoclonal antibodies with other specificities and so retained a portion of the p53 native structure. The p53 protein produced by some mutants appeared to bind more tightly to a cellular protein of 68 kilodaltons than did the wild-type p53 protein. Such mutants may be useful in investigating p53 function in normal cells.

MATERIALS AND METHODS

Plasmid constructions, bacterial strains, and oligonucleotides. *Escherichia coli* HB101 (*recA13*, *hsdR*⁻ *hsdM*⁻ *lacY1*) (3) and DH1 (*recA1* *hsdR*⁻ *hsdM*⁺ *endA1*) (15) were used as bacterial hosts for all the DNA transformations.

The p11-4 plasmid was constructed by using the *HaeIII*-*ApaI* fragment of the previously described pp53-17C plasmid (37). This region, containing the coding sequences of p53, was inserted into the *SstI* site of pSV2sst plasmid (a gift from P. Sarnow, Whitehead Institute, Cambridge, Mass.). PSV2sst is derived from pSV2neo (45), with the *neo* gene encoding the aminoglycoside 3'-phosphotransferase II deleted from pSV2neo and replaced with a *SstI* linker. The insertion of the p53 cDNA into that site placed the expression of p53 under the control of SV40 enhancer and promoter (45). Figure 1 shows a representation of this construction.

The octameric *HindIII* linkers (GAAGCTTC or CAAGCTTG) were purchased from either Boehringer Mannheim Biochemicals or Pharmacia P-L Biochemicals. The decameric (CCAAGCTTGG) and the dodecameric (CCCAAGCTTGGG) *HindIII* linkers were synthesized by Pharmacia. Unphosphorylated synthetic oligomers were used for all the linker insertion mutagenesis experiments.

Enzymes and agarose gel electrophoresis. Conditions for restriction enzyme digestions were as recommended by the suppliers except when described. Restriction endonucleases, S1 nuclease, and T4 DNA ligase were obtained from either New England BioLabs or Bethesda Research Laboratories. DNA fragments were resolved in 0.8% agarose (SeaKem) or low-gelling-temperature agarose (Sigma Chem-

ical Co.) gels by using 90 mM Tris-borate (pH 8.3)-2 mM EDTA running buffer.

Construction of in-frame and frameshift linker insertion mutations. To construct BH (i.e., to convert *BalI* to *HindIII*), KH (*KpnI* to *HindIII*), and ScH (*SacII* to *HindIII*) mutations, complete restriction enzyme digestion of p11-4 DNA (Fig. 1) was accomplished by overnight incubation at 37°C. To construct RH (*RsaI* to *HindIII*), NH (*NaeI* to *HindIII*), and SH (*StuI* to *HindIII*) mutations, partial cleavages of p11-4 DNA were carried out at 20°C for various times (1 to 30 min). The reaction mixtures were then adjusted to 25 mM EDTA and heated at 65°C for 5 min to inactivate the restriction endonucleases. The cohesive ends of DNA fragments were made blunt ended by treatment with S1 nuclease in buffer (200 mM NaCl, 50 mM sodium acetate [pH 4.5], 1 mM zinc sulfate, 0.5% glycerol) at 37°C for 30 min (28). Reactions were terminated by adjusting the mixture to 25 mM EDTA. The reaction products were fractionated by electrophoresis through 0.8% low-gelling-temperature agarose (Sigma) gels at 25 V and 4°C for 10 h. The linearized plasmids were recovered by phenol extraction (two times) of the excised band followed by ethyl ether extraction (two times) and ethanol precipitation.

Linker ligation reactions were performed as described by Lathe et al. (24), with the following modification, which is a novel simplified two-step linker insertion procedure. This linker insertion methodology only involves ligation and transformation steps but obviates the removal of unligated linkers, melting, and reannealing of the plasmid described by Lathe et al. (24). The blunt-ended DNA fragments were ligated with excess unphosphorylated linkers directly in 10 μ l of ligation buffer (30 mM NaCl, 30 mM Tris hydrochloride [pH 7.5], 7.5 mM MgCl₂, 1 mM spermidine, 0.25 mM ATP, 2 mM dithiothreitol, 2 mM disodium EDTA, 0.1 mg of bovine serum albumin per ml) containing 2 U of T4 DNA ligase at 4°C for overnight incubation. Because an excess molar amount of the linkers was used, the ends of the DNA fragments tended to be saturated with unphosphorylated linkers. The linear plasmids with one linker on each end and nicked monomers with only one linker would be the major ligation products. Usually about 0.1 to 1.0 μ g of linear plasmid was ligated with 5 μ g of the unphosphorylated linkers. Ligation products were used directly for transformation of *E. coli*. Because the transformation efficiency of nicked circular plasmid DNA is much better than that of linear plasmid DNA, monomers containing only one unphosphorylated linker would be the major transformants. For BH, KH, and ScH mutations, ligation mixtures were then digested with *BalI*, *KpnI*, or *SacII* restriction enzymes, respectively, which cut at unique sites in the p11-4 plasmid prior to the transformation of *E. coli* to reduce the background resulting from self-ligation of the plasmids. Transformation into *E. coli* HB101 or DH1 was as described by Hanahan (15). Ampicillin-resistant clones were screened for the presence of *HindIII*-sensitive (linker insertion) plasmids by *HindIII* enzyme digestion of the plasmids prepared from 4-ml bacterial cultures by using the alkaline lysis method for purification of DNA (2). *HindIII*-sensitive plasmids were further analyzed by extensive restriction enzyme analysis to confirm the positions of the *HindIII* inserts. Mutant plasmids were purified from 1-liter cultures by the alkaline lysis method followed by equilibrium centrifugation in CsCl-ethidium bromide gradients.

Construction of deletion mutations *dIEE* and *dIEB*. To construct *dIEE*, plasmid p11-4 was completely digested with *EcoRV* at 37°C to delete a 68-base-pair DNA fragment. The

linear plasmid was gel purified by gel electrophoresis and blunt end ligated overnight in a ligation buffer (4 mM MgCl₂, 30 mM Tris hydrochloride [pH 8.0], 0.1 mg of bovine serum albumin per ml, 10 mM dithiothreitol, 1 mM ATP) containing 1 U of T4 DNA ligase at 14°C as described by Burns and Beacham (4).

To construct *dIEB*, plasmid *dIEE* was sequentially digested with *BalI* and *EcoRI* to delete a 113-base-pair DNA fragment. The linear plasmid was purified by gel electrophoresis and ligated as described above with an octomeric *HindIII* linker to preserve the reading frame. Prior to transformation, the ligation products were digested with *EcoRV* to reduce the transformation background resulting from plasmid self-ligation. The ampicillin-resistant clones were screened for the presence of plasmids cleaved by *HindIII*. The structure of these *HindIII*-cleaved plasmids was further verified by the presence of a *BalI* site recreated at the junction at which the linker was inserted into the deletion.

Cells and antibodies. The CV-1_{CSH} and COS7 cells (11) and SV40-transformed 3T3 derivative SVT2 cells (1) were provided by M. Perucho, SUNY-Stony Brook, Stony Brook, N.Y., and G. Todaro, Oncogen, Seattle, Wash., respectively. Cells were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 10% heat-inactivated fetal calf serum (Flow Laboratories). Cells were incubated in a water-saturated 5% CO₂ atmosphere. Cells were verified to be free of mycoplasmas by being stained with Hoechst 33258 (American Hoechst Co.).

Monoclonal anti-p53 antibodies, PAb242, PAb246, PAb248 were the gifts of J. Yewdell, Wistar Institute, Philadelphia, Pa. The epitopes recognized by these antibodies have been determined previously (48). The hybridoma cell lines PAb412 and PAb421, which produce monoclonal antibodies against SV40 large T antigen and p53, respectively, were described by Gurney et al. (14) and Harlow et al. (17). The hybridoma cell line RA3-2C2, which produces monoclonal antibody against p53, was obtained from R. Coffman, Stanford University, Stanford, Calif. (5). All the monoclonal antibodies were obtained from culture supernatants of the hybridoma cells. The PAb242 antibody obtained from culture supernatants produced by J. Yewdell may have a different specificity than reported previously. This preparation reacted with mouse p53 from embryonal carcinoma cells, adenovirus-transformed murine cells (C3H-DC3), in which it coimmunoprecipitated the E1b 58-Kd protein (43), and SV40-transformed cells, in which it coimmunoprecipitated the viral T antigen (23, 26). This antibody preparation immunoprecipitated monkey p53 (see Fig. 3 and Table 2) and failed to recognize mouse p53 deleted for amino acids 11 through 33 (*dIEE*) and amino acids 11 through 157 (*dIEB*). This is a similar epitope for PAb242, mapped previously (48). Because this preparation of PAb242 has a specificity possibly different from that previously described (for monkey p53), it will be referred to as PAb242-JY. The human and mouse p53 sequences are closely related at this epitope (residues 9 through 25), and cross-reaction appears possible. Antibodies PAb242-JY and PAb421 react with both mouse and monkey p53 proteins at epitopes located within amino acid residues 9 through 25 and 370 through 378, respectively (48). Antibodies PAb246, PAb248, and RA3-2C2 are specific for murine p53 protein and recognize epitope regions mapped at amino acid residues 88 through 109 for PAb246 and residues 157 through 192 for both PAb248 and RA3-2C2 out of a total of 390 amino acid residues (48).

DNA transfection and protein analysis. Recombinant plas-

mids were introduced into the COS7 cells by the DEAE-dextran method as described by Sompayrac and Danna (44), with the following modifications. COS7 cells 2×10^6 to 3×10^6 were seeded in a 100-mm dish 1 day before transfection. Subconfluent cells were washed twice with 4 ml of serum-free Dulbecco modified Eagle medium and then incubated for 8 h with 8 µg of DNA in 2 ml of transfection medium at 37°C in a CO₂ incubator. The transfection medium was composed of Dulbecco modified Eagle medium with 200 µg of DEAE-dextran (molecular weight, 2×10^6 ; Pharmacia Fine Chemicals) per ml, 2 µM chloroquine diphosphate (Sigma), and 0.05 M Tris hydrochloride (pH 7.3). After transfection, the cells were washed twice with 5 ml of phosphate-buffered saline and then fed with Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. No carrier DNA, glycerol shock, or dimethyl sulfoxide boost was used in this procedure. At 48 to 72 h posttransfection the cells were incubated with methionine-free medium containing 2% dialyzed fetal calf serum for 2 h. The cells were then labeled with 200 µCi of [³⁵S]methionine (New England Nuclear Corp.) in 2 ml of methionine-free medium containing 2% dialyzed fetal calf serum for 4 h. The preparation of cell extracts and the procedures for the immunoprecipitation reactions and polyacrylamide gel electrophoresis were as previously described (27).

RESULTS

p53 expression vector and construction of p53 mutants. The expression vector plasmid for the p53 cDNA clone (p11-4; Fig. 1) contains the p53 coding region under the control of the SV40 enhancer and promoter and utilizes the SV40 small t-antigen mRNA splice site and polyadenylation signal. This vector was shown to produce murine p53 after transfection of monkey cells (CV-1_{CSH} or COS7 cells) by several criteria. First, murine p53 was selectively immunoprecipitated by murine-specific monoclonal antibodies PAb246, PAb248, and RA3-2C2. Second, the murine p53 protein migrates in sodium dodecyl sulfate (SDS)-polyacrylamide gels more rapidly than does the monkey p53 protein. Both murine and monkey p53 proteins were separated and detected in monkey cells after transfection with p11-4 plasmid DNA (see Fig. 3). Furthermore, the murine p53 protein that was produced by p11-4 in monkey cells expressing the SV40 large T antigen bound to the SV40 large T antigen. This could be demonstrated because monoclonal antibodies directed against the murine p53 (but not the monkey p53) coimmunoprecipitated the SV40 large T antigen and vice versa. These results (not presented) confirm identical observations made by Pinhasi and Oren (38). Once the efficacy of the p11-4 expression vector was established, several types of insertion and deletion mutations were introduced into the p53 coding regions of p11-4. For this study, two different deletion mutations were prepared. *dIEE* eliminated a region of the p53 gene between two *EcoRV* sites (Fig. 2), which resulted in a loss of 23 amino acids in the protein near the N-terminal region and a return to the normal open reading frame for the remainder of the gene. Amino acids 11 through 33 were deleted out of a total of 390 amino acids (1 is at the N terminus, and 390 is at the C terminus; Fig. 2). *dIEB* is also an in-frame deletion from *EcoRV* to *BalI*, removing amino acids 11 through 157 of the protein. Nine different linker insertion mutations were made at a variety of restriction enzyme sites in the cDNA (Fig. 2), resulting in insertions of a *HindIII* linker at various positions across the cDNA and retention of the open reading frame. The positions of these insertions are shown in Fig. 2,

FIG. 2. Schematic diagram of the p53 mutant coding sequences. Coordinates are measured in amino acids, with 1 representing the codon for the first amino acid at the N terminus of the protein. Arrowheads indicate the sites of *Hind*III linker insertions. The open bar represents the coding sequence, while the solid bar represents the nontranslated sequence in the frameshift mutations. The number for each linker insertion mutation defines the codon position at which the linker inserts. The space between the open bar for mutants *dIEE* and *dIEB* is the region of the cDNA or protein missing in these deletions.

and the nature of the insertions is detailed in Table 1. Finally, three linker insertions resulting in frameshift mutations were prepared near the carboxy terminus of the protein (NH'₂₂₂, ScH'₃₃₀, and SH'₃₄₄), again with *Hind*III linker insertions at these restriction sites (Fig. 2; Table 1). *Hind*III linkers were used because they introduced a unique site into the vector. All constructions were confirmed by the loss of a restriction site and the gain of a new *Hind*III site (as described in Materials and Methods) which mapped to the correct position in the plasmid, as confirmed by extensive

restriction enzyme mapping analysis. Mutants are named for the restriction sites used in the deletion or insertion and the number of the amino acid in p53 protein that was altered (1 to 390, N to C terminus). Frameshift mutations are indicated by a prime after the enzyme site used.

Analysis of p53 mutants: monoclonal antibody binding and SV40 T-antigen binding. To examine the ability of wild-type murine p53 and mutant p53 proteins to interact with monoclonal antibodies (conformation or epitope change) and to bind with the SV40 large T antigen, the expression vector

TABLE 1. Properties of mutants

Mutation	Site of insertion or deletion	Amino acid position of insertion or deletion	Mutagen (<i>Hind</i> III linker)	Residue change ^a		Reading frame ^b
				From	To	
Linker insertion						
RH ₁₂₃	<i>Rsa</i> I	123	12-mer	Y	SQAWD	i.f.
BH ₁₅₈	<i>Bal</i> I	158	12-mer	A	AQAWA	i.f.
KH ₂₁₅	<i>Kpn</i> I	215–216	10-mer	VP	PSLA	i.f.
RH ₂₁₅	<i>Rsa</i> I	215	12-mer	V	VPSLG	i.f.
NH ₂₂₂	<i>Nae</i> I	222–223	12-mer	AG	APKLGG	i.f.
RH ₂₃₃	<i>Rsa</i> I	233	12-mer	Y	SQAWD	i.f.
RH ₂₈₀	<i>Rsa</i> I	280–281	12-mer	RT	RPKLGT	i.f.
ScH ₃₃₀	<i>Sac</i> II	330	8-mer	R	RSF	i.f.
SH ₃₄₄	<i>Stu</i> I	344	12-mer	A	AQAWA	i.f.
NH' ₂₂₂	<i>Nae</i> I	222–223	10-mer			f.s.
ScH' ₃₃₀	<i>Sac</i> II	330	12-mer			f.s.
SH' ₃₄₄	<i>Stu</i> I	344	8-mer			f.s.
Deletion						
<i>dIEE</i>	<i>EcoRV</i> – <i>EcoRV</i>	11–33				i.f.
<i>dIEB</i>	<i>EcoRV</i> – <i>Bal</i> I	11–157	10-mer			i.f.

^a Abbreviations for predicted amino acid sequence: A, alanine; D, aspartate; F, phenylalanine; G, glycine; K, lysine; L, leucine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

^b Abbreviations: i.f., in-frame; f.s., frameshift.

FIG. 3. Autoradiogram of a polyacrylamide gel of immunoselected p53 protein produced by murine wild-type and mutant expression vectors in COS7 cells. Subconfluent COS7 cells were transfected with 8 μ g each of the indicated plasmid DNA per 100-mm dish by the DEAE-dextran procedure. An 8- μ g portion of salmon sperm DNA was used for mock transfection (panel MOCK). At 72 h posttransfection, the cells were labeled with [35 S]methionine (200 μ Ci/100-mm dish) for 4 h. Equal amounts of the trichloroacetic acid-insoluble labeled proteins were immunoprecipitated with the following antibodies: lane 1, RA3-2C2 anti-mouse p53 monoclonal antibody; lane 2, PAb242-JY anti-p53 monoclonal antibody; lane 3, PAb246 anti-mouse p53 monoclonal antibody; lane 4, PAb248 anti-mouse p53 monoclonal antibody; lane C, a nonspecific control antibody (clone A1-2, anti-adenovirus DNA-binding protein) (41). Lane M contains 14 C-labeled protein molecular weight markers indicated in the figure. Lane m contains SV40-transformed mouse cell (SVT2) extract immunoprecipitated with anti-p53 monoclonal antibody PAb421. The immunoprecipitates were fractionated on a 7.5% SDS-polyacrylamide gel. Numbers on the left represent the molecular weights of the 14 C-labeled protein markers. Symbols: T Ag, SV40 large T antigen; p68, a cellular 68,000-dalton protein; p53_{mk} and p53_{ms}, monkey and mouse p53 (either wild type or mutant), respectively. p11-4 is the wild-type p53 plasmid; KH₂₁₅ and BH₁₅₈ are mutant p53 plasmids.

p11-4 and mutant plasmids KH₂₁₅ and BH₁₅₈ were transfected into COS7 cells. As a control mock transfection, salmon sperm DNA was also transfected into COS7 cell cultures under the same conditions (see Materials and Methods). Since the expression vectors contain an SV40 origin of replication and COS7 cells produce the SV40 large T antigen, these vectors are expected to replicate and synthesize enhanced levels of murine p53 under the *cis*-acting enhancer-promoter control of SV40 signals (Fig. 1). To examine the proteins produced in these cell cultures, the cells were labeled with [35 S]methionine for 4 h at 72 h posttransfection in methionine-free media (see Materials and Methods). Soluble protein extracts were then prepared from the harvested cell pellets, the SV40 large T antigen (detected by PAb412), the murine and monkey p53 proteins (detected by PAb421 or PAb242-JY), or the murine p53 protein (detected by PAb246, PAb248, or RA3-2C2) was immunoprecipitated by different monoclonal antibodies, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. Figure 3 presents an autoradiogram of the gel analysis for this experiment. A comparison of the results for the mock-transfected culture with the results for the cultures transfected by the wild-type p53 expression vector p11-4 demonstrates the following points. (i) p11-4 but not the mock-transfected culture synthesized a 53,000-molecular-weight protein detected by monoclonal antibodies RA3-2C2 (lane 1), PAb246 (lane 3), and PAb248 (lane 4), which react with murine p53 but not primate-specific p53. (ii) PAb242-JY (lane 2) immunoprecipitated the primate p53 in the mock-transfected culture and both the murine and monkey p53 proteins in the p11-4-transfected culture, which are distinguishable by their different molecular weights. (iii) In p11-4-transfected COS7 cells, immunoprecipitation of the murine

p53 (lanes 1, 3, and 4) or the murine and monkey p53 (lane 2) coimmunoprecipitated the SV40 large T antigen (panel p11-4). In the mock-transfected COS7 cells, only PAb242-JY (lane 2) is able to detect primate p53 and coimmunoprecipitate the SV40 large T antigen. (iv) PAb412, which is a monoclonal antibody specific for the large T antigen of SV40, coimmunoprecipitated a primate p53 species in mock-transfected cells and a primate and murine p53 species in p11-4-transfected cells (results not presented). (v) With monoclonal antibody PAb248 (lane 4) in the mock-transfected and p11-4-transfected extracts, two proteins were detected by immunoprecipitation at molecular weights of about 57,000 and 68,000. Different preparations of PAb248 culture medium immunoprecipitated a variable amount (more or less) of these cellular proteins from monkey cells (COS7 or CV-1_{CSH} cells). This apparently represents a monoclonal antibody cross-reaction with cellular proteins. The nature of these proteins is at present unclear.

When COS7 cells transfected with mutant murine p53 plasmids KH₂₁₅ and BH₁₅₈ were analyzed in a similar manner, several different results emerged (Fig. 3). KH₂₁₅ produced a murine p53 of the correct molecular weight that was recognized by antibodies RA3-2C2, PAb242-JY, and PAb248 (lanes 1, 2, and 4) but not by PAb246 (lane 3). KH₂₁₅ p53 protein also failed to bind to the SV40 large T antigen in COS7 cells by the criterion that anti-p53 antibodies failed to coimmunoprecipitate detectable levels of the SV40 large T antigen (panels KH₂₁₅ and p11-4; lanes 1 and 4). SV40 T antigen could be detected in this extract, however, when PAb421 (results not presented) or PAb242-JY (panel KH₂₁₅; lane 2) was used to immunoprecipitate monkey and mouse p53 and coimmunoprecipitate the SV40 large T antigen. Similarly for BH₁₅₈-transfected cell extracts (Fig. 3), PAb246

TABLE 2. Summary of immunoprecipitation results on p53 mutants

Mutation	Mouse p53 protein levels detected by immunoprecipitation with ^a					T antigen binding
	PAb242-JY	PAb246	RA3-2C2	PAb248	PAb421	
<i>d/EE</i>	—	+	+	+	+	+
<i>d/EB</i>	—	—	—	—	+	—
RH ₁₂₃	+	—	+	+	+	—
BH ₁₅₈	+	—	+	+	+	—
KH ₂₁₅	+	—	+	+	+	—
RH ₂₁₅	+	—	+	+	+	—
NH ₂₂₂	+	+	+	+	+	+
RH ₂₃₃	+	+	+	+	+	+
RH ₂₈₀	+	+	+	+	+	+
ScH ₃₃₀	+	+	+	+	+	+
SH ₃₄₄	+	+	+	+	+	+
NH ₂₂₂	—	—	—	—	—	—
ScH ₃₃₀	—	—	—	—	—	—
SH ₃₄₄	—	—	—	—	—	—
P53mk/COS7 ^b	+	—	—	—	+	+
p53ms/p11-4 ^c	+	+	+	+	+	+

^a Symbols: +, detectable; —, nondetectable.^b p53mk/COS7 is endogenous monkey p53 in COS7 cells.^c p53ms/p11-4 is mouse p53 encoded by p11-4 plasmid in transfected COS7 cells.

(lane 3) failed to detect this murine p53 mutant protein, while RA3-2C2, PAb242-JY, and PAb248 (panel BH₁₅₈; lanes 1, 2, and 4) all detected the murine p53 protein. RA3-2C2 (lane 1) and PAb248 (lane 4), while immunoprecipitating the BH₁₅₈ mutant p53 protein, failed to coimmunoprecipitate the SV40 large T antigen. The SV40 large T antigen was present in this extract and bound to the monkey p53 as observed in panel BH₁₅₈, lane 2, in which monkey and mouse p53 and SV40 T antigen are shown. It may be concluded from these experiments that in monkey cells two p53 insertion mutations, KH₂₁₅ and BH₁₅₈, produce murine p53 that can be detected by some p53-specific monoclonal antibodies (RA3-2C2, PAb242-JY, PAb248, and PAb421) but not by other antibodies (PAb246). These mutant p53 proteins also fail to bind to the SV40 large T antigen, or bind at much reduced or nondetectable levels, as determined by a p53-T-antigen coimmunoprecipitation assay (Fig. 3).

A similar analysis with the remainder of the in-frame insertion mutations listed in Table 1 and shown in Fig. 2 demonstrated that RH₁₂₃, BH₁₅₈, KH₂₁₅, and RH₂₁₅ all produced p53 proteins that failed to react with PAb246 and failed to bind to the SV40 large T antigen in a coimmunoprecipitation assay (Table 2). On the other hand, insertion mutations NH₂₂₂, RH₂₃₃, RH₂₈₀, ScH₃₃₀, and SH₃₄₄ all produced murine p53 proteins that reacted with PAb246 and bound detectable levels of SV40 large T antigen. In these experiments the failure of a mutant p53 protein (KH₂₁₅, BH₁₅₈) to bind to a monoclonal antibody (PAb246) or to the SV40 T antigen was not due to proteolysis in the mutant protein extract or reduced levels of the mutant protein, because equal levels of wild-type and mutant p53 proteins were detected by other monoclonal antibodies (RA3-2C2, PAb242-JY, PAb248, and PAb421). The binding of four distinct monoclonal antibodies to mutant p53 protein suggests that at least a portion of the protein is in a native configuration.

Deletion mutants of murine p53. To continue the genetic analysis of murine p53–SV40 large T-antigen interactions,

two deletion mutations of p53 protein (*d/EE* and *d/EB* [Fig. 2; Table 1]) were transfected into COS7 cells. Once again, salmon sperm DNA was used in a transfection of another culture of COS7 cells as a control mock experiment. At 48 h (for *d/EE*) or 72 h (for *d/EB*) posttransfection, the cultures were labeled with [³⁵S]methionine for 4 h in methionine-free medium. Equal levels of soluble protein extracts (normalized for the [³⁵S]methionine incorporation) were immunoprecipitated with a variety of monoclonal antibodies. The proteins in the immunoprecipitates were analyzed on SDS-polyacrylamide gels as previously described. Autoradiograms of these gels are presented in Fig. 4A (*d/EE*) and 4B (*d/EB*).

In mock-transfected COS7 cells (Fig. 4A; panel mock), PAb412 which immunoprecipitated the SV40 large T antigen coimmunoprecipitated the monkey p53 (lane T). Similarly, the p53 antibodies PAb1H5-4 and PAb421 detected monkey p53 and coimmunoprecipitated the SV40 large T antigen (lanes 2 and 3). RA3-2C2 failed to react with monkey p53 (lane 1). In *d/EE*-transfected cells (Fig. 4A, panel *d/EE*), RA3-2C2 detected a murine-specific p53 with a reduced molecular weight (about 46,000), as predicted from the expected size of this deletion with this mutant p53 protein (lane 1). Immunoprecipitation of this mutant p53 protein by the mouse-specific monoclonal antibody RA3-2C2 was accompanied by immunoprecipitation of the SV40 large T antigen and little or no detectable monkey p53 protein (lane 1). Similarly, immunoprecipitation of the SV40 large T antigen in *d/EE*-transfected COS7 cell extracts resulted in the coimmunoprecipitation of the monkey and mouse mutant p53 protein (lane T). PAb242-JY, which detects an epitope between amino acid residues 9 and 25, failed to bind to the *d/EE* mutant (deletes residues 11 through 33), confirming the Wade-Evans and Jenkins (48) mapping of this epitope (Table 2). PAb426, PAb248, RA3-2C2, and PAb421 antibodies all bound to the *d/EE* p53 deletion mutant protein (Table 2). PAb1H5-4 (47), which binds preferentially to the monkey p53 (but detects mouse p53 here) (lane 2), and PAb421 (lane 3) immunoprecipitated the monkey and mutant (deleted) mouse p53 and coimmunoprecipitated the SV40 large T antigen. Clearly, an N-terminal deletion of 23 amino acids (residues 11 through 33 on the map; Fig. 2) has little or no effect upon p53 binding to SV40 T antigen.

When *d/EB*-transfected COS7 cell extracts were analyzed in a similar manner, a different result was obtained. First, only monoclonal antibody PAb421 (Fig. 4B, lane 1) was able to detect the mutant p53 fragment of this p53 protein produced by *d/EB* at a molecular weight of about 23,000. RA3-2C2 (lane 2), PAb242-JY (lane 3), PAb246 (lane 4), and PAb248 (lane 5) all failed to detect this p53 murine mutant fragment of protein (see the 23,000-molecular-weight region of Fig. 4B). This 23,000-molecular-weight fragment is not a degradation product of p53, because PAb421 was not able to detect the same protein fragment in p11-4-transfected COS7 cells (results not presented). Second, this 23,000-molecular-weight p53 fragment made by *d/EB* failed to bind to the SV40 large T antigen. This is based upon the observation that this 23,000-molecular-weight protein failed to be coimmunoprecipitated by PAb412 antibodies (lane T) specific for the SV40 large T antigen or a polyclonal SV40 T antiserum (results not presented), both of which coimmunoprecipitated the monkey p53 protein in the cell extracts (Fig. 4B, lane T). Thus a p53 deletion (*d/EB*) spanning amino acids 11 through 157 that produced a protein fragment of 23,000 daltons, recognized by PAb421 antibodies (epitope at residues 370 through 378), failed to bind with SV40 T antigen and a series of monoclonal antibodies (PAb242-JY, PAb246, PAb248,

FIG. 4. Autoradiogram of a polyacrylamide gel of immunoselected p53 proteins produced by p53 deletion mutant expression vectors in COS7 cells. Subconfluent COS7 cells were transfected with 8 μ g each of either salmon sperm DNA (panel MOCK) or the indicated plasmid DNA by the DEAE-dextran method. At 48 h (*dIEE*) or 72 h (*dIEB*) posttransfection, cells were labeled with [35 S]methionine (200 μ Ci/100-mm dish) for 4 h. Equal amounts of the labeled proteins from cell extracts were immunoprecipitated with the following antibodies: (A) lane T, PAb412; lane 1, RA3-2C2; lane 2, 1H5-4; lane 3, PAb421; lane C, control serum, an anti-hu-myc 12c or anti-mo-myc 12c (anti-myc-peptide serum) (16); (B) lane T, PAb412; lane 1, PAb421; lane 2, RA3-2C2; lane 3, PAb242-JY; lane 4, PAb246; lane 5, PAb248; lane C, control antibody (clone A1-2). The immunoprecipitates were then analyzed in a 12.5% SDS-polyacrylamide gel. p53_{ms} · dIEE and p53_{ms} · dIEB stands for p53 protein produced by the *dIEE* and *dIEB* mutants, respectively. T Ag and p53_{mk} are as described in Fig. 3.

and RA3-2C2) that span epitopes between residues 9 and 192 in the protein.

Three linker insertion mutations were produced (NH'₂₂₂, ScH'₃₃₀, and SH'₃₄₄) (Table 1; Fig. 2) that resulted in a frameshift mutation, deleting the carboxy-terminal end of the p53 protein. In each case, when these plasmids were transfected into COS7 cells and labeled for 4 h with [35 S]methionine in a manner similar to that in the previous experiments, no stable p53 protein was detected in a 4-h labeling of those cell extracts by the five monoclonal antibodies directed against p53 (Table 2). No binding to T antigen was detected when an SV40-specific T-antigen monoclonal antibody PAb412 was used with the same extracts. p53-specific mRNA was produced by these plasmid vectors in COS7 cells, and so it appears that a stable protein (detectable in a 4-h labeling) failed to form or the conformation of the p53 protein prevented antibody from detecting it. All three of these frameshift mutations should contain epitopes recognized by PAb242-JY, PAb246, PAb248, and RA3-2C2, while lacking the PAb421 epitope localized in the C-terminus of the p53 protein (48). These data suggest that N-terminal deletions (residues 11 through 33 and 11 through 157) produce stable detectable proteins in COS7 cells whether (*dIEE*) or not (*dIEB*) the mutant p53 is bound to T antigen while C-terminal deletions (residues 222 through 390, 330 through 390, and 344 through 390) produced unstable proteins or proteins with quite altered conformations in the same COS7 cells. Nothing can be concluded about the abilities of such proteins to bind to the SV40 T antigen.

DISCUSSION

The murine p53 protein is composed of 390 amino acids organized into roughly three regions or domains (37). The N-terminal 75 amino acids are rich in negatively charged residues, with a predicted alpha-helical structure. The next

75 amino acids are quite hydrophobic, with a lower charge density and a high proline content as predicted for a beta-pleated sheet structure. The carboxy-terminal 125 amino acids again have a high charge density of residues rich in basic amino acids, often clustered in groups, in an alpha-helical structure. The protein is rich in proline (about 10% of the residues), with many proline-proline paired dipeptides and a clustering of most prolines between residues 30 and 200 (37). Protein-protein interactions may well be important in p53 function (23, 26, 43), as indicated by the oligomeric protein complexes formed between p53 and SV40 or adenovirus tumor antigens. With that in mind, the goal of the studies presented here was to create a series of p53 mutations in an expression vector and map the regions of the p53 protein important in interactions with these viral tumor antigens. Mutant p53 proteins might fail to interact with the SV40 large T antigen because of an altered epitope or set of amino acid residues that combine directly (make contacts) with T antigen. Alternatively, mutations can result in conformational changes in proteins, leading to poor protein-protein interactions as a result of structural changes. In this case the mutation in the protein may or may not reside in the region of altered conformational change in a protein. Antibody binding can be useful in probing for conformational changes. To examine the structure of p53 wild-type and mutant proteins, five distinct monoclonal antibodies directed against p53 protein, whose epitopes have been mapped (Table 2) (48), were used to probe for possible conformational changes of the mutant p53 proteins studied here. The resultant analysis has produced an interesting correlation, identifying several p53 mutant proteins which failed to bind both a specific monoclonal antibody (PAb246) and the SV40 large T antigen.

Four insertion mutations (RH₁₂₃, BH₁₅₈, KH₂₁₅, and RH₂₁₅) produced p53 murine proteins that failed to bind with the SV40 large T antigen or bound this viral protein with

much reduced efficiency in COS7 cell extracts (Fig. 3; Table 2). These mutations span a contiguous region of protein between amino acids 123 and 215. A deletion mutation spanning this region (*d/EB*, residues 11 through 157) also produced a p53 protein that failed to bind to SV40 T antigen. Mutations mapping on either side of this T-antigen-binding domain (*d/EE*, residues 11 through 33, and *NH*₂₂₂ to *SH*₃₄₄, residues 222 through 344) produced p53 proteins that bound to the SV40 T antigen normally. Each of the five different p53 mutant proteins that failed to bind to SV40 large T antigen also failed to react with the monoclonal antibody PAb246, while the p53 mutant proteins that did bind T antigen (*NH*₂₂₂, *RH*₂₃₃, *RH*₂₈₀, *ScH*₃₃₀, and *SH*₃₄₄) all reacted with PAb246. PAb246 recognizes an epitope between amino acids 88 and 109 (48), while the insertion mutations that encode p53 proteins that fail to bind to SV40 T antigen are localized between amino acids 123 and 215. The simplest interpretation of these observations and this correlation of results is that an alteration in amino acid residues 123 through 215 changes the conformation of residues 88 through 109 and blocks the binding of PAb246 to its epitope. The amino acid residues 88 through 109 and 123 through 215 may normally interact within the folded native protein, and the insertion mutations disrupt this interaction, changing the PAb246 epitope structure. Alternate conformations of p53 proteins detected by monoclonal antibodies have been described previously by Milner (31) and may have significance for growth regulation. This study suggests that the SV40 T-antigen-binding domain on the p53 molecule resides, at least in part, between residues 123 and 215. Equally important in reinforcing this assignment of a protein domain is that mutations between residues 11 and 33 and 222 and 344 permit normal SV40 T-p53 interactions. These results eliminate the idea that any insertion or deletion mutation will change the p53 protein conformation and reduce T-p53 interactions. The facts that these mutations are contiguous on the protein molecule (123 through 215) and each one of them alters the same epitope (88 through 109) for monoclonal antibody PAb246 suggest that a specific region or domain has been identified.

The mutant analysis presented here (Table 2) confirms the placement of the PAb421 epitope near the C-terminal end of p53 protein (residues 370 through 378) (48). The *d/EB* deletion (residues 11 through 157) disrupts the PAb242-JY epitope (residues 9 through 25), the PAb246 epitope (residues 88 through 109), and the PAb248 and RA3-2C2 epitopes (residues 157 through 192), but leaves the PAb421 epitope (residues 370 through 378) intact, also as predicted. Similarly, the observation that *d/EE* (deletion at residues 11 through 33) eliminates the binding of PAb242-JY (epitope at residues 9 through 25) but produces a p53 protein detected by PAb246, PAb248, RA3-2C2, and PAb421 also confirms the assignment of the PAb242 epitope to the region of that deletion (48). Thus, this collection of deletion and insertion mutations have been useful in mapping epitopes and suggesting conformational interactions within the p53 protein.

In mouse cells p53 protein may be associated with a cellular protein of molecular weight 68,000 (13, 38, 42). A protein of similar molecular weight, termed p68, was found to be coimmunoprecipitated with murine p53 expressed in monkey COS7 cells by using a murine p53 expression vector (38). On the basis of those studies, it has been suggested that the p53 protein in a cell (or transfected into a cell) normally associates with a cellular protein, p68, in a fashion analogous to the SV40 or adenovirus tumor antigen-p53 complexes. In this context it is interesting to note that several mutant forms

of murine p53 protein which failed to bind to the SV40 large T antigen were found in association with a 68,000-molecular-weight protein, as detected by their probable coimmunoprecipitation in *KH*₂₁₅ · p53-p68 complexes (Fig. 3, panels *KH*₂₁₅ and *BH*₁₅₈). This might be expected in a p53 mutant with altered binding properties and could even explain the failure to bind to SV40 T antigen (because it is bound tightly to another protein, p68). Additional experimentation and more rigorous criteria for an association of p68 with p53 are required before the meaning of such interactions becomes clear. Equally curious is the variable cross-reaction of a p53-directed monoclonal antibody, PAb248 (Fig. 3, panel mock, lane 4), with two cellular proteins of 57,000 and 68,000 daltons. This observation, which was reproducible with some batches of monoclonal antibody PAb248, must be explored in more detail before anything definitive can be concluded from such cross-reactions with cellular proteins.

The procedure used in this study to demonstrate that p53 bound to the SV40 T antigen was coimmunoprecipitation with monoclonal antibodies directed against SV40 T antigen or p53 epitopes. The association of SV40 T antigen with p53 has also been confirmed by cosedimentation of p53 and T antigen, copurification of these two proteins, and even in vitro association of p53-T complexes (18). In this study, it remains possible that some of the mutant p53 proteins may form weak SV40 T-antigen-p53 protein complexes in solution and that washing procedures (for example) used in the immunoprecipitation test may result in the dissociation of T-p53 complexes. Even if this is the case, such mutant complexes are clearly different from the wild-type p53-T complexes (the control used here), and that is the point under study. Similarly, a 4-h labeling of COS7 cells does not measure the exact steady-state levels of p53 in an extract, but instead measures some function of the rate of synthesis and degradation of p53 (40). The available evidence suggests that p53 bound to SV40 T antigen is more stable than p53 not bound to T antigen (27, 35). In this study p53 mutations in COS7 cells that produced p53 proteins that failed to bind with T antigen were certainly present at readily detectable levels (Fig. 3). There are several different explanations for this observation, including the distinct possibility that the half-life of free p53 protein (not bound to T antigen) in COS7 cells is longer than 4 h. Each transformed cell line has a characteristic half-life (turnover rate) for p53 protein (40), and so it is not possible to predict a priori the relative stabilities of p53 bound to SV40 T antigen or free in COS7 cells.

Finally, this genetic analysis of p53 protein structure and interactions, as probed by the ability of wild-type and mutant p53 proteins to bind to monoclonal antibodies and SV40 T antigen, has outlined a p53 protein domain of some interest. On the basis of the frequency and distribution of amino acids, a hydrophobic, proline-rich, beta-pleated sheet was predicted between residues 75 and 150, followed by a basic and charged region of amino acids between residues 150 and 200 (37). The number of basic residues between positions 75 and 150 (5 of 75 residues) is threefold lower than that found between adjacent residues 150 through 200 (11 of 50 amino acids are basic). Monoclonal antibody PAb246 recognizes an epitope between residues 88 and 109 which is no longer available for antibody recognition when insertion mutations in positions 123 through 215 were constructed and synthesized (Table 2). The amino acid insertions at positions 123, 158, and 215 are conservative (Table 1) but are clearly sufficient to impact upon the conformational structure de-

tected by PAb246 binding in an adjacent site and T-antigen binding in this domain. There remains an N-terminal domain (residues 1 through 75) and a C-terminal domain (residues 265 through 390) that can be and have been altered with less impact upon protein-protein interactions between p53 and the SV40 large T antigen. The function of these domains is presently under study.

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