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Association of p53 Binding and Immortalization of Primary C57BL/6 Mouse Embryo Fibroblasts by Using Simian Virus 40 T-Antigen Mutants Bearing Internal Overlapping Deletion Mutations

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To more precisely map the immortalization and p53 binding domains of T antigen, a large series of overlapping deletion mutations were created between codons 251 to 651 by utilizing a combination of *Bal* 31 deletion and oligonucleotide-directed mutagenesis. Immortalization assay results indicated that amino acids (aa) 252 to 350, 400, and 451 to 532 could be removed without seriously compromising immortalization, although the appearance of immortal colonies was delayed in some cases. Western immunoblotting experiments indicated that the p53 binding capacities of T antigen produced by mutants missing aa 252 to 300, 301 to 350, 400, or 451 to 532 were only slightly reduced relative to that of wild-type T antigen. Within the limits of this deletion analysis, the immortalization and p53 binding domains appear to be colinear and, in fact, may represent two aspects of the same domain. This deletion analysis eliminates the entire zinc finger domain (aa 302 to 320), a small portion of the leucine-rich region (aa 345 to 350), and a large portion of the ATP binding domain (aa 451 to 528) as participants in p53 binding or in the immortalization process. The results also show that removal of T antigen amino acids within the region 451 to 532 appears to alter the capacity of newly synthesized but not older T antigen and p53 molecules to form complexes.

The simian virus 40 (SV40) T antigen is a predominantly nuclear, multifunctional protein that plays a pivotal role both in lytic growth of the virus and in transformation of cells in culture (20, 55, 72, 73). Genetic and biochemical evidence indicates that specific portions of the T-antigen molecule contain (1, 3, 16, 34–36, 50, 57, 63, 74) or are essential for (7, 8, 10, 11, 18, 46, 50, 51, 62, 64) individual T-antigen functions. Among the properties of T antigen is the ability to bind at least six cellular proteins. These proteins include DNA polymerase α (16, 26, 60), Ap-2 (49), p185 (36), hsp73 (57), and the tumor suppressor gene products Rb (10), p107/120 (17–19), and p53 (39, 43). Regions of T antigen required for binding these cellular proteins, with the exception of Ap-2, have been identified. Rb and p107/120 binding requires the integrity of T-antigen amino acids (aa) 105 to 114 (10, 18). DNA polymerase α can bind to two independent regions of T antigen: aa 1 to 82 (16) and aa 83 to 708 (21). The p185 protein binds to an N-terminal fragment of T antigen spanning aa 1 to 121 (36). Binding of hsp73 requires T-antigen aa 1 to 178 (57). The region of T antigen required for p53 binding apparently differs in in vitro and in vivo assays. Analysis of binding between T-antigen proteolytic fragments and p53 suggests that amino acids beyond 517 are not required for in vitro binding (58). However, the region of T antigen involved in p53 binding as defined by genetic analysis lies between T-antigen aa 346 and 626 (71, 78). Several mutations that alter or delete amino acids within the hydrophobic region between 571 and 589 are defective in p53 binding in vivo (54, 71, 78). These results indicate that in vivo binding may be sensitive to conformational alterations in T antigen.

Interruption of either Rb or the p53 binding capacity alters specific transforming activities of T antigen. Deletion or point mutations within the Rb binding region (aa 105 to 114) reduce the capacity of cells expressing the altered protein to form dense foci (10, 35), to grow to high saturation density (72), or to grow as anchorage-independent clones in semisolid medium (5, 6, 72). Immortalization, however, is not affected by absence of Rb binding (5, 72). In contrast, mutants producing T antigens that fail to bind p53 (71) or that bind p53 inefficiently (41, 42) are defective for both dense focus formation (54) and immortalization in some cell types (41, 54, 67, 71, 78).

Recently we showed that individual growth properties of transformed cells are associated with specific segments of T antigen (71, 72). Mutants producing T antigens missing amino acids between 1 and 250 or beyond 626 immortalized C57BL/6 mouse embryo fibroblasts (B6MEF) as efficiently as did wild-type T antigen. This result suggested that the region from aa 251 to 626 contains all of the functions required for immortalization. More recently, the N-terminal boundary of the immortalization domain has been redefined as aa 347 (78). The ability to bind p53 localizes to this same region of T antigen. It is not known whether this segment of T antigen contains two independent domains, one for immortalization and a second for p53 binding, or alternatively, whether these functions are colinear and represent a single functional domain. Precise localization of the immortalizing and p53 binding regions has been limited by the paucity of mutants that produce stable T antigens with alterations within the broad segment of the protein containing these activities.

The investigations reported here address the relationship between p53 binding and immortalization by using a large series of internal in-frame deletion mutants that remove segments of the region between aa 251 and 651 of T antigen.

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We show that aa 252 to 350 and 451 to 532 can be removed without seriously affecting either property. However, interruption of the 351-to-450 segment prevents both p53 binding and immortalization without substantially reducing the steady-state level of T antigen in cell lines expressing the mutant proteins. We show, in addition, that interruption of the segment including aa 533 to 650 prevents p53 binding and immortalization. On the basis of these and our previous results, we conclude that T-antigen aa 1 to 350, 451 to 532, and 627 to 708 are not involved in either p53 binding or immortalization.

MATERIALS AND METHODS

Plasmid constructions. (i) *Bal* 31 exonuclease deletion mutagenesis. pPVU-0 (35) plasmid DNA was linearized within the codon for residue 437 of T antigen by using the restriction enzyme *Pvu*II. The linearized plasmid DNA was treated for various lengths of time with exonuclease *Bal* 31 to sequentially remove nucleotides from the *Pvu*II cleavage site. The remaining single-strand extensions at the ends of the *Bal* 31-digested DNA molecules were filled in by using Klenow polymerase and all four deoxynucleoside triphosphates. Phosphorylated *Eco*RI 10-mer linkers were ligated to the blunt-ended DNA population. Excess linkers were removed by digestion with *Eco*RI. The DNA population then was recircularized by treatment with T4 DNA ligase. The ligation mixture was transfected into competent *Escherichia coli* DH5α.

(ii) Oligonucleotide-directed mutagenesis. Oligonucleotide-directed mutagenesis reactions were performed according to the instructions provided with the Amersham oligonucleotide-directed mutagenesis kit. The Amersham kit utilizes mutagenesis reactions based on the method of Eckstein et al. (53, 68, 69). The vector used in all mutagenesis reactions was pGC2ESV(RI$^-$). To create the phagemid pGC2ESV, the *Nco*I (nucleotide 37)-to-*Bam*HI (nucleotide 2533) fragment of SV40 strain VA45-54 containing the origin of replication, promoter, and entire early coding region was cloned into the pGC2 vector (52). The SV40 strand present as the positive strand of the phagemid runs 3′ to 5′ from the *Nco*I site to the *Bam*HI site and has the sense of late mRNA (SV40 negative strand). The unique *Eco*RI site present in the pBR322 backbone of the pGC2ESV vector was removed by digestion with *Eco*RI, Klenow polymerase fill-in, and blunt-end ligation using T4 DNA ligase. Oligonucleotides synthesized in the Pennsylvania State University Macromolecular Core Facility were used in all mutagenesis reactions. Separate oligonucleotide-directed mutagenesis reactions were used to insert *Eco*RI 8-mer linkers following T-antigen codon 250, 350, 370, 450, 550, or 650 or to insert *Eco*RI 10-mer linkers following T-antigen codon 300, 400, 500, or 600. Mutagenic oligonucleotides were designed such that the T_H of each arm extending from the linker sequence was 41 to 43°C, and the procedure took into account the differences in nucleotide sequence between SV40 strain VA45-54 (37a) and the published sequence for strain 776 (73).

(iii) DNA sequence analysis. Products from all *Bal* 31 deletion mutagenesis, oligonucleotide-directed mutagenesis, and subsequent fragment exchange reactions were analyzed by dideoxy sequencing (56). Briefly, sequencing reactions were performed on double-stranded templates as follows. Templates were prepared by the cetyltrimethylammonium bromide DNA precipitation method (12). Templates were alkali denatured and sequenced with the Sequenase version 2.0 sequencing kit (United States Biochemical) as specified by the manufacturer. Oligonucleotides synthesized in the Pennsylvania State University Macromolecular Core Facility were used as primers in all sequencing reactions. Products of sequencing reactions were resolved on 6% polyacrylamide buffer gradient sequencing gels (59), using a model S2 sequencing gel apparatus (Bethesda Research Laboratories) and gel reagents obtained from National Diagnostics. Following electrophoresis, the gels were fixed in 10% acetic acid–methanol, dried, and exposed on Kodak XAR-5 film at room temperature.

(iv) Fragment exchange. To construct in-frame deletion mutations of desired size and location, a fragment exchange procedure was used. *Eco*RI-*Bam*HI restriction digestions were performed on two different plasmid constructs derived either from the *Bal* 31 exonuclease deletion mutagenesis or the oligonucleotide-directed mutagenesis reactions. Plasmid DNA (approximately 2 μg) used in the reactions was produced by the rapid plasmid boiling technique described below. The restriction enzyme digestion products were resolved on a 1% TAE–agarose gel. The DNA fragment containing the T-antigen coding sequence 5′ of the linker (large fragment) of one plasmid construct and the DNA fragment containing the T-antigen sequence 3′ of the linker (small fragment) of the other plasmid construct were excised from the gel and recovered by using the Geneclean (Bio 101) procedure. The large and small DNA fragments were ligated and transfected into *E. coli* DH5α by using standard techniques.

(v) Insertion of the neomycin resistance gene into plasmid constructs. Wild-type or mutant plasmid DNA was prepared by the rapid plasmid boiling technique described below. For each mutant construct, approximately 2.5 μg of plasmid DNA was digested with *Bam*HI to linearize the plasmid at its single *Bam*HI site. A neomycin resistance gene bounded by *Bam*HI cleavage sites was derived from the construct pMSV-neo (24). In pMSV-neo, the neomycin resistance gene is under control of the murine sarcoma virus long terminal repeat and is positioned between an *Eco*RI and a *Bam*HI site. The unique pMSV-neo *Eco*RI site, lying approximately 400 bp upstream of the murine sarcoma virus 5′-long terminal repeat promoter, was converted to a *Bam*HI site by a fill-in/*Bam*HI-linker addition reaction. The 3.2-kb *Bam*HI-linkered neomycin resistance gene cassette was then cloned into the unique *Bam*HI site of pBR322 (pBR322-MO:NEO:*Bam*HI). The neomycin resistance gene cassette was isolated from pBR322-MO:NEO:*Bam*HI plasmid DNA by digestion with *Bam*HI. The restriction enzyme digestion products were resolved on a 1% TAE–agarose gel. The 3.2-kb fragment containing the *Bam*HI-linkered neomycin resistance gene cassette was excised from the gel and recovered by the Geneclean procedure. Approximately 150 ng of *Bam*HI-digested rapid plasmid DNA preparation and 110 ng of *Bam*HI-linkered neomycin resistance gene cassette were ligated by standard techniques. The products of the ligation reactions were transfected into competent *E. coli* DH5α. Transformants were selected on kanamycin (50 μg/ml)-containing L-agar plates. Typically, 5 to 50 colonies were obtained from each transfection.

Cells and cell lines. Primary B6MEF were prepared from 12- to 16-day-old C57BL/6 embryos as described previously (70) and were used between the second and fourth passages for immortalization assays. B6S-cl7 is a clonally derived spontaneously immortalized B6MEF cell line (72). All primary cells and cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μg of streptomycin per ml, 100 μg of kanamycin per ml, 100

U of penicillin per ml, 0.03% glutamine, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.15% $NaHCO_3$, and 10% fetal bovine serum (FBS) (10 × 1 DMEM) unless indicated otherwise. FBS was heat inactivated for 45 min at 56°C.

Immortalization assay. Immortalization assays were performed essentially as described previously (70). One day prior to transfection, primary B6MEF were seeded at a density of 10^5 cells into 75-cm^2 tissue culture flasks containing 20 ml of 10 × 1 DMEM. Transfection was accomplished by the calcium phosphate precipitation method (28, 75) by using 1 μg of CsCl gradient-purified wild-type or mutant DNA and 10 μg of B6MEF carrier DNA per flask. B6MEF carrier DNA was prepared as described previously (27, 70). After 16 to 24 h of exposure to the precipitate, the medium was removed from the flasks and replaced with 10 × 1 DMEM. Thereafter, the medium in the tissue culture flasks was changed twice weekly. Throughout the assay, incubation temperature was 37°C. Colonies were counted after 3 to 8 weeks. At the time of counting, colonies were picked for clonal expansion into cell lines.

In this assay, the cell density at the time of transfection was sufficiently low to prevent the formation of a monolayer before nonimmortalized cells became senescent. Before and after transfection, the cells were incubated in medium containing 10% FBS. Therefore, the only intentional selective pressure was for escape from senescence (immortalization).

Rapid plasmid DNA preparation. Rapid plasmid preparation was essentially a modification of the Holmes protocol (33). Ten-milliliter Circlegrow overnight cultures were centrifuged at 2,000 × *g* for 10 min at room temperature. The bacterial pellets were resuspended in 700 μl of STET buffer (8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris [pH 8.0]) containing 10 μl of 50-mg/ml lysozyme. The bacterial suspensions were boiled for 45 s, and the debris was pelleted by centrifugation at 15,600 × *g* for 15 min at room temperature. The pellets were removed with sterile toothpicks, and an equal volume of isopropanol was added. The mixtures were incubated for 5 min at −70°C and then centrifuged at 15,600 × *g* for 15 min at 4°C. The resulting crude plasmid DNA pellets were resuspended in 100 μl of 3 M sodium acetate–0.5 mM EDTA (pH 6.0). The suspensions were centrifuged at 15,600 × *g* for 15 min at 4°C. The supernatants were collected, and 2 volumes of 100% ethanol was added to each. The mixtures were incubated for 5 min at −70°C and then centrifuged at 15,600 × *g* for 15 min at 4°C. The resulting plasmid DNA pellets were dissolved in 100 μl of distilled water (dH_2O) to which was added 50 μl of 7.5 M ammonium acetate and 300 μl of 100% ethanol. The mixtures were incubated for 5 min at −70°C and then centrifuged at 15,600 × *g* for 15 min at 4°C. The plasmid DNA pellets were air dried for 10 min. The plasmid DNA pellets were dissolved in 20 μl of dH_2O. Typical yield was approximately 5 μg of plasmid DNA per preparation.

Generation of G418-resistant cell lines. For the generation of G418-resistant cell lines, a neomycin resistance gene was added to each mutant construct as described above. One day prior to transfection, B6S-cl7 cells were seeded at a density of 10^5 cells into 75-cm^2 tissue culture flasks containing 10 × 1 DMEM. Transfection was accomplished by the calcium phosphate precipitation method (28, 70, 75), using approximately 2.5 μg of wild-type or mutant rapid plasmid DNA containing a neomycin resistance gene and 10 μg of B6MEF carrier DNA per tissue culture flask. After 16 to 24 h of exposure to the precipitate, the medium was removed and replaced with 10 × 1 DMEM. At 4 days posttransfection, the medium was removed and replaced with 10 × 1 DMEM supplemented with G418 (500 μg/ml) to select for G418-resistant transformants. Thereafter, the medium was changed twice weekly with 10 × 1 DMEM supplemented with G418. After 3 weeks, G418-resistant colonies were picked and clonally expanded into cell lines.

Antibodies. Anti-T antibodies used in immunoprecipitation and Western immunoblotting experiments included PAb901 (71b, 74) and a rabbit anti-D2 (23, 29) polyclonal antibody (a kind gift from D. Lane). The mouse monoclonal antibody PAb901 recognizes a denaturation-resistant epitope contained within aa 684 to 698 of T antigen (71b). The PAb901 epitope is located C terminal to all deletions created; thus, PAb901 reactivity confirms that a given deletion mutation produces an in-frame protein product.

Anti-p53 antibodies used in immunoprecipitation and Western blotting experiments included PAb421 (31), PAb240 (25), PAb246 (76), and a rabbit anti-p53 polyclonal antibody (Chemicon). PAb421 reacts with a denaturation-resistant C-terminal epitope (31) found in both mutated and wild-type p53. PAb240 also reacts with a denaturation-resistant epitope (aa 213 to 217 of mouse p53) found in both mutated and wild-type p53 of all vertebrate species (65). The PAb240 epitope is conformationally exposed in nondenatured mutated p53 and conformationally hidden in the nondenatured wild-type p53 (25). PAb246 reacts with a denaturation-sensitive epitope (aa 88 to 109 of mouse p53). PAb246 is preferentially reactive with nondenatured wild-type mouse p53 (22, 66). The rabbit anti-p53 polyclonal antibody was obtained by using human p53 as an immunogen; however, the serum is also reactive with both wild-type and mutant mouse p53. P3N3 is a nonspecific mouse myeloma ascites fluid used as a negative control and to preclear cell lysates in immunoprecipitation experiments.

Secondary antibody used in Western blotting experiments was goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) (Sigma). The goat anti-mouse antibody is directed to the Fc portion of mouse IgG and is conjugated to the HRP enzyme.

Immunoprecipitation. Cells were grown to near confluence in 25-cm^2 tissue culture flasks. The cell monolayers were washed twice with prewarmed phosphate-buffered saline, pH 7.4 (PBS), starved for 1 h at 37°C in methionine-free DMEM supplemented with 2% dialyzed FBS, and then radiolabeled for 2 h at 37°C in 1 ml of methionine-free DMEM supplemented with 100 μCi of [^{35}S]methionine and 2% dialyzed FBS. Following two washes with ice-cold Tris-buffered saline (pH 7.4), cell monolayers were incubated for 30 min at 4°C with 1 ml of lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.01 mg of aprotinin per ml). Cell debris was pelleted by centrifugation at 15,600 × *g* for 15 min at 4°C. Ten microliters of P3N3 and 20 μl of 50% protein A-Sepharose beads (Sigma) were added to each cell lysate. The mixtures were incubated overnight at 4°C with rotation and then centrifuged at 15,600 × *g* for 15 min at 4°C. The supernatants were collected. After quantitation of the radioactivity in 10-μl aliquots in a liquid scintillation counter, the extracts were adjusted with lysis buffer to yield between 3 × 10^6 and 1 × 10^7 cpm/500 μl for a given experiment. Three microliters of monoclonal antibody-ascites fluid or 200 μl of monoclonal antibody-tissue culture supernatant fluid and 10 μl of 50% protein A-Sepharose beads were added to each 500 μl of extract. Extract volumes were adjusted to 800 μl with lysis buffer. The mixtures were rotated for 2 h at 4°C. Immune complexes were pelleted by

centrifugation at 15,600 × *g* for 15 min at 4°C. The immune complexes were washed three times with SNNTE buffer (50 mM Tris, 5 mM EDTA, 0.5 M NaCl, 5% sucrose, 1% Nonidet P-40 [pH 7.4]) and one time with radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1% deoxycholic acid [pH 7.4]). The immune complexes were resuspended in 30 μl of loading buffer (80 mM Tris, 2% SDS, 100 mM dithiothreitol, 10% sucrose, 0.01% bromphenol blue, 1.45 M β-mercaptoethanol [pH 6.8]). Proteins were dissociated from the protein A-Sepharose beads by incubation for 5 min at 100°C. Immunoprecipitates were subjected to electrophoresis in SDS–10% polyacrylamide gels (38). Standard ^{14}C-protein molecular weight markers (New England Nuclear) were included for comparison. Following electrophoresis, the gels were fixed in isopropanol-acetic acid-dH_2O (25:10:65), fluorographed with Amplify (Amersham), dried, and exposed on Kodak X-Omat film at −70°C.

Western blotting. Cells were passaged 1:2 into a 75-cm^2 tissue culture flask containing 10 × 1 DMEM 1 day prior to protein extraction. Cell monolayers were rinsed twice with cold Tris-buffered saline (pH 7.4). Cell lysis was accomplished by addition of 1.5 ml of lysis buffer (see above) to each flask, followed by incubation for 30 min at 4°C on a rocking platform. Cell debris was pelleted by centrifugation at 15,600 × *g* for 10 min at 4°C. Protein concentrations were determined by the Bradford assay (Bio-Rad kit). Each immunoprecipitation contained an equivalent amount of protein extract (typically 1 to 2 mg). Immunoprecipitation reaction volumes were adjusted to 1 ml with lysis buffer. Immunoprecipitation reaction conditions and SDS-polyacrylamide protein gel running conditions were essentially the same as described above.

Protein transfer to polyvinylidene difluoride (PVDF) membranes (Millipore) was accomplished by using a Transphor II electrophoresis unit (Hoefer). Transfers were performed at 1.0-A constant current for 3 h at room temperature. PVDF membranes were blocked with blocking buffer (PBS [pH 7.4], 10% Carnation lowfat milk) for 30 min at room temperature on a rocking platform. PVDF membranes were probed with primary antibody in blotting buffer (1 mM EDTA, 10 mM Tris, 100 mM NaCl, 1% Carnation lowfat milk) for 2 h at room temperature on a rocking platform. Primary antibodies were used at the following concentrations: PAb901 ascites fluid, 1:2,500; PAb421 ascites fluid, 1:5,000; and PAb240 tissue culture supernatant, 1:30. PVDF membranes were rinsed three times (for 5 min each time) at room temperature with rinse buffer (1 mM EDTA, 10 mM Tris, 100 mM NaCl). PVDF membranes were probed with secondary antibody-HRP (1:30,000 dilution) in blotting buffer for 1 h at room temperature on a rocking platform. PVDF membranes were rinsed three times (for 5 min each time) at room temperature with rinse buffer. One milliliter each of ECL (Amersham) reagents 1 and 2 were mixed and evenly distributed over the PVDF membranes. The ECL reagent mixture was aspirated after 1 min, and the membranes were placed between two pieces of 3M write-on transparency film and exposed to Kodak X-Omat film at room temperature. Typical exposure times ranged from 1 min to 2 h.

RESULTS

The experiments reported here were conducted in order to more precisely map the immortalization and p53 binding domains of T antigen. To determine whether the two activities could be dissociated genetically, a large series of deletion-linker insertion mutations were introduced into the T-antigen coding region and used to construct in-frame deletion mutants spanning the T-antigen segment believed to contain the two activities.

TABLE 1. Linker positions in *Bal* 31 and oligonucleotide-directed deletion and insertion mutant constructs: 5′ ends available for construction of in-frame deletion mutants

Deletion terminus[a]	Last intact SV40 codon before linker[b]	Sequence 5′ to linker[c]	Reading frame match[d]
4068	250	5′ TTG CCA GGT gg	2
3918	300	5′ AGT TTT GAA ccg	3
3831	329	5′ TTT GCT GAC ccg	3
3812	335	5′ AA AAA ACC Acc g	1
3806	337	5′ CC ATA TGC Ccc g	1
3799	339	5′ C CAA CAG GCc cg	2
3780	346	5′ GTT TTA GCT ccg	3
3768	350	5′ AAG CGG GTT gg	2
3760	352	5′ T GAT AGC CTc cg	2
3750	356	5′ CAA TTA ACT ccg	3
3732	362	5′ ATG TTA ACA ccg	3
3723	365	5′ AAC AGA TTT ccg	3
3715	367	5′ T AAT GAT CTc cg	2
3708	370	5′ CTT TTG GAT gg	2
3678	380	5′ TCT ACA GGC ccg	3
3676	380	5′ T ACA GGC TCc cg	2
3675	381	5′ ACA GGC TCT ccg	3
3656	387	5′ AA GAA TGG Acc g	1
3651	389	5′ TGG ATG GCT ccg	3
3637	393	5′ T GCT TGG CTc cg	2
3636	394	5′ GCT TGG CTA ccg	3
3622	398	5′ T TTG TTG CCc cg	2
3621	399	5′ TTG TTG CCC ccg	3
3618	400	5′ TTG CCC AAA ccg	3
3565	417	5′ C ATT CCT AAc cg	2
3564	418	5′ ATT CCT AAA ccg	3
3563	418	5′ TT CCT AAA Acc g	1
3553	421	5′ A AGA TAC TGc cg	2
3551	422	5′ GA TAC TGG Ccc g	1
3522	432	5′ AGT GGT AAA ccg	3
3520	432	5′ T GGT AAA ACc g	1
3510	436	5′ ACA TTA GCA ccg	3
3509	436	5′ CA TTA GCA Gcc g	1
3509	436	5′ CA TTA GCA Gcc gg	2
3509	436	5′ CA TTA GCA Ggg	3
3468	450	5′ TTA AAT GTT gg	2
3318	500	5′ AGG GAT TAT cgg	3
3168	550	5′ AGG CCC AAA gg	2
3018	600	5′ GAG TGG AAA ccg	3
2868	650	5′ GAT GGT GGG gg	2

[a] Numbers indicate the last authentic SV40 nucleotide before the *Eco*RI linker, using the numbering system of Tooze (73).

[b] Numbers indicate the last authentic SV40 amino acid encoded by sequences before the *Eco*RI linker.

[c] SV40 codons 5′ of the *Eco*RI linkers. SV40 sequences are listed in uppercase letters; *Eco*RI linker sequences are listed in lowercase letters. Note that in some cases, nucleotides of the *Eco*RI linker can substitute for deleted SV40 nucleotides to encode an authentic SV40 amino acid.

[d] Numbers indicate N-terminal ends which can be paired with C-terminal ends. Matching ends with the identical number produce an in-frame protein product.

Mutant constructs. Deletion mutations were created by combining appropriate DNA fragments isolated from two different linker-addition mutants contained in the *Bal* 31 deletion/oligonucleotide-directed mutagenesis library summarized in Tables 1 and 2. Resulting mutant T antigens contain two to four additional amino acids encoded by the *Eco*RI linker at the point of each deletion (Tables 1 to 3). All

TABLE 2. Linker positions in *Bal* 31 and oligonucleotide-directed deletion and insertion mutants; 3′ ends available for construction of in-frame deletion mutants

Deletion terminus[a]	First intact SV40 codon after linker[b]	Sequence 3′ to linker[c]	Reading frame match[d]
4067	251	aat tcc GGG TTA AAG 3′	3
3917	301	a att cgg ATG TGT TTA 3′	2
3862[e]	320	aat tcg gAG CAT TAT 3′	3
3767	351	aat tcc GAT AGC CTA 3′	3
3707	371	aat tcc AGG ATG GAT 3′	3
3617	401	a att cgg ATG GAT TCA 3′	2
3508	438	aat tcg gCT GCT TTG C 3′	3
3508	438	aa ttc cCT GCT TTG C 3′	1
3508	438	a att ccg gCT GCT TTG C 3′	2
3507	438	aa ttc ggT GCT TTG CT 3′	1
3502	440	aat tcg gTG CTT GAA T 3′	3
3501	440	aa ttc ggG CTT GAA TT 3′	1
3498	441	aa ttc ggT GAA TTA TG 3′	1
3488	444	a att cgg GGG GGG AAA 3′	2
3486	445	aa ttc ggG GGG AAA GC 3′	1
3467	451	aat tcc AAT TTG CCC 3′	3
3456	455	aa ttc ggG GAC AGG CT 3′	1
3431	463	a att cgg GTA GCT ATT 3′	2
3422	466	a att cgg GAC CAG TTT 3′	2
3381	480	aa ttc ggA GGG GAG TC 3′	1
3379	481	aat tcg gGG GAG TCC A 3′	3
3376	482	aat tcg gAG TCC AGA G 3′	3
3373	483	aat tcg gCC AGA GAT T 3′	3
3370	484	aat tcg gGA GAT TTG C 3′	3
3357	488	aa ttc ggA GGT CAG GG 3′	1
3356	488	a att cgg GGT CAG GGA 3′	2
3355	489	aat tcg gGT CAG GGA A 3′	3
3351	490	aa ttc ggG GGA ATT AA 3′	1
3350	490	a att cgg GGA ATT AAT 3′	2
3336	495	aa ttc ggG GAC AAT TT 3′	1
3335	495	a att cgg GAC AAT TTA 3′	2
3325	499	aat tcg gGG GAT TAT T 3′	3
3317	501	a att ccg TTG GAT GGC 3′	2
3313	503	aat tcg gAT GGC AGT G 3′	3
3311	503	a att cgg GGC AGT GTT 3′	2
3279	514	aa ttc ggC CTA AAT AA 3′	1
3243	526	aa ttc ggA GTC ACC AT 3′	1
3221	533	a att cgg GTG CCT AAA 3′	2
3167	551	aat tcc GAT TAT TTA 3′	3
3016	602	a att cgg (A)[f]AG AGA TT 3′	2
2867	651	aat tcc GAG AAG AAC 3′	3

[a] Numbers indicate the first authentic SV40 nucleotide after the *Eco*RI linker, using the numbering system of Tooze (73).

[b] Numbers indicate the first authentic SV40 amino acid encoded by sequences after the *Eco*RI linker.

[c] SV40 codons 3′ of the *Eco*RI linkers. SV40 sequences are listed in uppercase letters; *Eco*RI linker sequences are listed in lowercase letters. Note that in some cases nucleotides of the *Eco*RI linker can substitute for deleted SV40 nucleotides to encode an authentic SV40 amino acid.

[d] Numbers indicate C-terminal ends which can be paired with N-terminal ends. Matching ends with the identical number produce an in-frame protein product.

[e] End was obtained from another *Bal* 31 deletion mutagenesis library produced in this laboratory.

[f] Point mutation that occurred during oligonucleotide-directed linker insertion mutagenesis procedure.

deletion mutations were created such that the appropriate reading frame was maintained beyond the point of each deletion. Small deletions (typically 50 aa or less) were generated with the hope that perturbation of protein conformation would be minimized. Smaller deletions also allow more discrete mapping of functional domains. The deletions created (Fig. 1) span the entire region encoding the immortalization and p53 binding domains. Deletion mutants are designated by the amino acid deleted from T antigen. For example, *dl*252-300 denotes a deletion mutant that produces a T antigen lacking aa 252 through 300.

Immortalization of primary B6MEF. Primary B6MEF were used for immortalization assays. The choice of B6MEF as a cell system for investigating immortalization by T antigen was based on three considerations. First, primary cells are unlikely to have acquired cellular mutations that might complement T-antigen functions missing in mutant proteins. For instance, it was recently demonstrated that selection of spontaneously immortalized cell lines of BALB/c mouse embryo fibroblasts by the 3T3 protocol uniformly yielded cell lines with altered p53 genes (32). It is expected that such cell lines can be transformed by T antigens that are unable to bind p53 or to alter its metabolism. Second, the B6MEF population senesces rapidly in culture. B6MEF can be passaged approximately four times before senescence ensues and the cells die. When B6MEF are seeded into culture vessels at low cell density, they do not divide enough times to form colonies. It is not necessary, therefore, to use a dominant selectable marker to eliminate nontransfected cells. Additionally, because of the limited number of divisions, a monolayer does not form in flasks seeded sparsely. Thus, dual selection for immortalization and dense focus formation is avoided. The impact of dual selection on genetic analysis of transforming functions has been discussed previously (72). Third, spontaneously immortalized colonies that can be expanded into cell lines very rarely appear.

Primary B6MEF were seeded into tissue culture flasks at a sufficiently low density to allow nonimmortalized cells to undergo senescence before confluence was reached. Cells that became immortal proliferated to form colonies, which were picked and expanded into cell lines for further analysis. A mutant construct was said to immortalize efficiently if the number of colonies arising during an experiment was at least 20% of the number arising with a wild-type construct and the colonies readily expanded into continuous cell lines. The 20% value reflects the level of variation between experiments in the number of colonies resulting from transfection with a wild-type construct (data not shown). For immortalization assays, duplicate flasks were transfected with each mutant construct. At least two independent experiments were performed for each mutant construct. As indicated in Table 3, some mutants produced colonies much more slowly than did the wild-type T-antigen-encoding construct. The results of the assays are presented in Table 3 and summarized in Fig. 2a.

Removal of aa 252 to 300 resulted in an immortalization frequency indistinguishable from that of wild-type T antigen. Removal of aa 301 to 350 resulted in the appearance of immortalized colonies at an average of 44% of the wild-type level. Smaller deletions within the 301-to-350 segment also immortalized well. All deletion mutants that produced T antigens missing amino acids between residues 350 to 451, with the exception of *dl*400, did not immortalize primary B6MEF at a detectable frequency. In the T antigen produced by *dl*400, aa 400 is replaced by three amino acids encoded by the *Eco*RI linker at the site of the deletion (*dl*400 therefore contains two amino acids inserted adjacent to an amino acid substitution). Removal of aa 451 to 532 resulted in an averaged immortalization frequency that was 35% of the wild-type T-antigen level. Smaller deletions within the 451-to-532 segment also immortalized well. Thus, all deletion mutants that produced T antigens missing amino acids N

TABLE 3. Immortalization of B6MEF by mutants with overlapping in-frame deletions between codons 251 and 651 of the T-antigen coding sequence

DNA	Amino acid lesion[a]	No. of immortal colonies/flask[b]				Colonies expandable[c]	p53 binding[d]
		Expt 1	Expt 2	Expt 3	Expt 4		
pPVU-0		46, 54 (1.00) 3	35, 37 (1.00) 3	39, 45 (1.00) 3	25, 33 (1.00) 3	Yes	Yes
Carrier		0, 0 (0.00) 8	0, 2 (0.03) 8	0, 2 (0.02) 8	1, 2 (0.05) 8	No	No
*dl*252-300	251 I,R 301	32, 40 (0.72) 3	33, 40 (1.01) 3			Yes	Yes
*dl*301-350	300 P,N,S 351	4, 42 (0.46) 4	14, 16 (0.42) 6			Yes	Yes
*dl*301-319	300 P,N,S,E 320	15, 21 (0.36) 6	12, 15 (0.38) 6			Yes	Yes
*dl*330-350	329 P,N,S 351	8, 19 (0.27) 5	9, 10 (0.26) 8			Yes	Yes
*dl*347-350	346 P,N,S 351			7, 14 (0.25) 7	4, 9 (0.22) 8	Yes	Yes
*dl*301-370	300 P,N,S 371			0, 3 (0.04) 8	2, 4 (0.10) 8	No	No
*dl*330-370	329 P,N,S 371			0, 0 (0.00) 8	1, 2 (0.05) 8	No	No
*dl*347-370	346 P,N,S 371			0, 0 (0.00) 8	2, 3 (0.09) 8	No	No
*dl*357-370	356 P,N,S 371			0, 4 (0.05) 8	1, 1 (0.03) 8	No	No
*dl*363-370	362 P,N,S 371			0, 1 (0.01) 8	1, 1 (0.03) 8	No	No
*dl*366-370	365 P,N,S 371			0, 2 (0.02) 8	0, 3 (0.05) 8	No	No
*dl*336-494	335 T,E,F,G 495			1, 3 (0.05) 8	0, 2 (0.03) 8	No	No
*dl*351-400	350 G,I,R 401			0, 0 (0.00) 8	0, 1 (0.02) 8	No	No
*dl*354-400	353 R,I,R 401			0, 2 (0.02) 8	1, 2 (0.05) 8	No	No
*dl*369-400	368 R,I,R 401			0, 0 (0.00) 8	1, 2 (0.05) 8	No	No
*dl*382-400	381 R,I,R 401			0, 0 (0.00) 8	1, 1 (0.03) 8	No	No
*dl*400	399 R,I,R 401	20, 29 (0.49) 5	12, 27 (0.40) 6			Yes	Yes
*dl*371-443	370 G,I,R 444			0, 0 (0.00) 8	0, 1 (0.02) 8	No	No
*dl*390-450	389 P,N,S 451			0, 0 (0.00) 8	2, 3 (0.09) 8	No	No
*dl*395-450	394 P,N,S 451			0, 1 (0.01) 8	0, 2 (0.03) 8	No	No
*dl*401-450	400 P,N,S 451			0, 0 (0.00) 8	1, 2 (0.05) 8	No	No
*dl*401-436	400 P,N,S 437			0, 0 (0.00) 8	1, 1 (0.03) 8	No	No
*dl*419-450	418 P,N,S 451			0, 1 (0.01) 8	0, 1 (0.02) 8	No	No
*dl*437-450	436 P,N,S 451			0, 2 (0.02) 8	1, 1 (0.03) 8	No	No
*dl*434-444	433--445			0, 0 (0.00) 8	0, 0 (0.00) 8	No	No
*dl*451-601	450 G,I,R,K 602			2, 2 (0.05) 8	2, 2 (0.07) 8	No	No
*dl*451-532	450 G,I,P 533	14, 34 (0.48) 5	6, 10 (0.22) 9			Yes	Yes
*dl*451-500	450 G,I,P 501	25, 31 (0.56) 6	9, 11 (0.28) 9			Yes	Yes
*dl*451-494	450 G,I,R 495	5, 11 (0.16) 6	7, 9 (0.22) 9			Yes	Yes
*dl*451-489	450 G,I,R 490	8, 9 (0.17) 6	7, 8 (0.21) 9			Yes	Yes
*dl*451-465	450 G,I,R 466	9, 15 (0.24) 6	7, 9 (0.22) 9			Yes	Yes
*dl*451-462	450 G,I,R 463	8, 13 (0.21) 6	7, 15 (0.31) 9			Yes	Yes
*dl*501	500 R,N,S 502	8, 16 (0.24) 5	11, 14 (0.35) 6			Yes	Yes
*dl*501-550	500 R,N,S 551	5, 8 (0.13) 8	4, 7 (0.15) 9			No	No
*dl*601-650	600 P,N,S 651	5, 6 (0.11) 8	4, 8 (0.17) 9			No	No

[a] Authentic SV40 amino acids adjacent to the deletion. Also shown are the amino acids (single-letter code) encoded by *Eco*RI linker sequences.
[b] The first two numbers are the numbers of colonies from duplicate flasks in each experiment. The number in parentheses is the level relative to that of the wild-type SV40 T-antigen-encoding plasmid pPVU-0, used as a positive control in each experiment. The last number indicates the number of weeks required for colonies to appear.
[c] Entries indicate whether the colonies arising during an immortalization assay could be expanded into cell lines.
[d] Ability of a mutant T antigen to bind p53 (visualized by Western blot analysis).

terminal to residue 351 or missing amino acids between residues 450 to 533 immortalized primary B6MEF at or near wild-type levels. All deletion mutants that produced T antigens missing amino acids between residues 350 to 451 and residues 532 to 651 did not immortalize primary B6MEF.

p53 binding by mutant T antigens in immortal and G418-resistant cell lines. Western blot analysis was used to assess the ability of the mutant T antigens to physically associate with mouse p53 for two reasons. First, the stabilization and consequent accumulation of high levels of p53 are a hallmark of SV40-transformed cells. Second, it has been shown that maturation of both T antigen and p53 must occur before protein complexes can form (4, 37). Therefore, the steady-state level of T antigen-p53 protein complexes in cells constitutively expressing mutant T antigens seems the most appropriate measure of the ability of the two proteins to interact in a manner reflective of the transforming properties of T antigen.

The ability of the mutant T antigens to complex p53 was examined by using clonally derived immortal C57BL/6 mouse cell lines for those mutants that retained immortalizing capacity. Mutant T antigens that could not immortalize B6MEF were examined for p53 binding by using clonally derived G418-resistant B6S-cl7 cells constitutively expressing the T polypeptides.

Since it was recently observed that spontaneously immortalized mouse cell lines frequently have altered p53 genes (32), it was necessary to show that the cell line chosen for expression of nonimmortalizing T antigens did not contain such an alteration. Three observations (data not shown) indicate that the spontaneously immortalized B6S-cl7 cell line expresses wild-type p53. First, detectable amounts of p53 could not be immunoprecipitated with monoclonal antibodies directed against wild-type p53 (PAb246), against mutant p53 (PAb240), or against both mutant and wild-type p53 (PAb421) from extracts of 2-h [^{35}S]methionine-pulse-labeled cells. This result suggested that the p53 protein is unstable. Second, p53 was stabilized after infection by

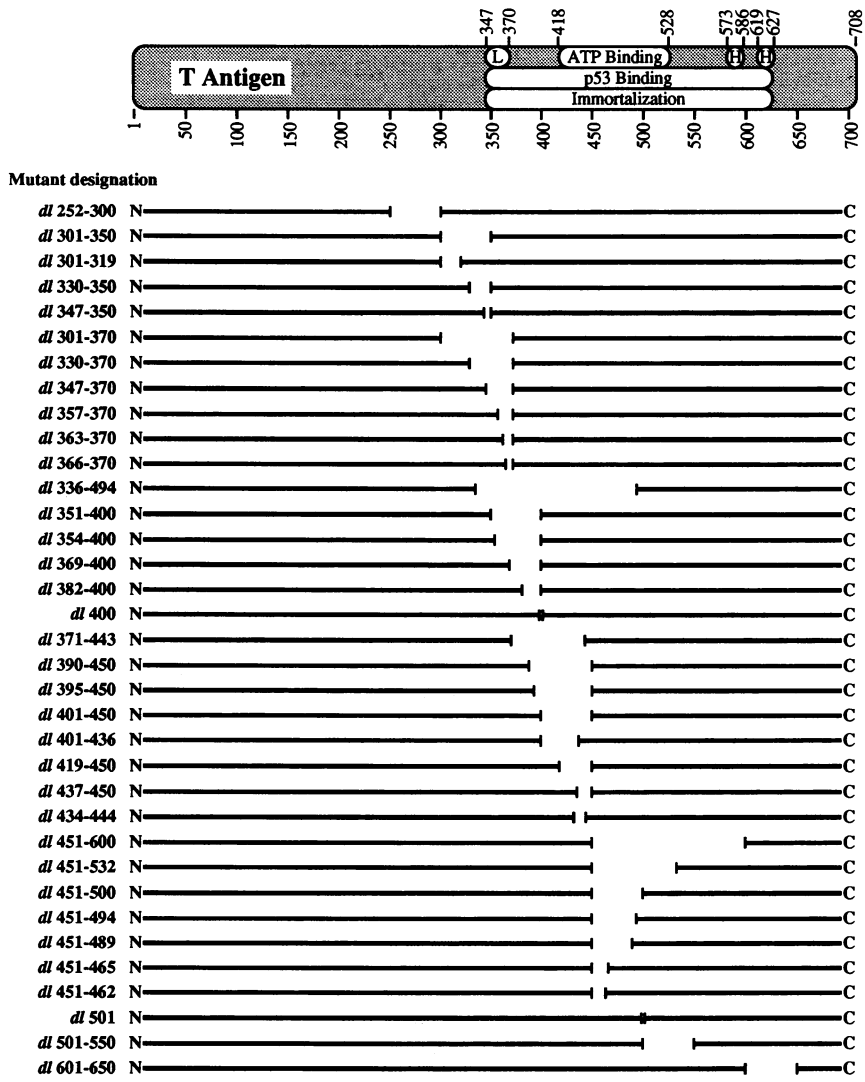

FIG. 1. Constructs used. Deletion mutants constructed for this study are shown graphically beneath a functional map of T antigen. Only the functional domains which overlap with the putative immortalization domain are shown. L, leucine-rich region; H, hydrophobic-rich region(s). The numbers above and below the representation of T antigen indicate amino acid positions. For each mutant, lines represent amino acids present and gaps represent amino acids missing.

SV40, as the protein was readily detectable in radiolabeled infected-cell extracts. The p53 in these infected B6S-cl7 cells was immunoprecipitated by PAb246 and PAb421 but not by PAb240. Finally, T antigen coprecipitated with the p53 extracted from the infected cells. Comparison of the steady-state levels of p53 in the B6S-cl7 cells, their parental cell population B6MEF, a B6S-cl7 cell line expressing wild-type T antigen (pPVU-0:NEO), and a B6MEF cell line immortalized by wild-type T antigen (pPVU-0) is shown in Fig. 3. By this analysis also, the steady-state level of p53 in B6S-cl7 was low, presumably reflective of protein instability, and was similar to the level that accumulates in the parental B6MEF population. Expression of wild-type T antigen in both cell types led to equivalent increased accumulation of p53, and the p53, as expected, complexed with T antigen. Thus, the p53 in the B6S-cl7 cell line fulfilled three major criteria for wild-type protein: apparent instability, stabilization in T-antigen-expressing cells, and immunological reactivity. In addition, as can be seen in Fig. 3, the T antigen-p53 protein interactions seen in a wild-type T-antigen-immortalized cell line and a B6S-cl7 cell line constitutively expressing wild-type T antigen were equivalent. This cell line was, therefore, an appropriate background in which to express nonimmortalizing mutant T antigens for the purpose of assessing their ability to complex with p53.

Extracts from exponentially growing cells were immunoprecipitated with anti-p53 (Fig. 4) or anti-T (Fig. 2) rabbit polyclonal antibodies and then resolved on SDS-polyacrylamide gels. Separated proteins were transferred to PVDF membranes and probed with a cocktail of anti-T (PAb901) and anti-p53 (PAb421 and PAb240) mouse monoclonal antibodies. Blotted proteins were visualized by using goat anti-mouse IgG-HRP secondary antibody and the ECL chemoluminescence system (Amersham).

Before drawing conclusions concerning the levels of T antigen-p53 protein complexes in cell lines immortalized by deletion mutants, it was important to confirm that a series of T-antigen-immortalized cell lines accumulated equivalent

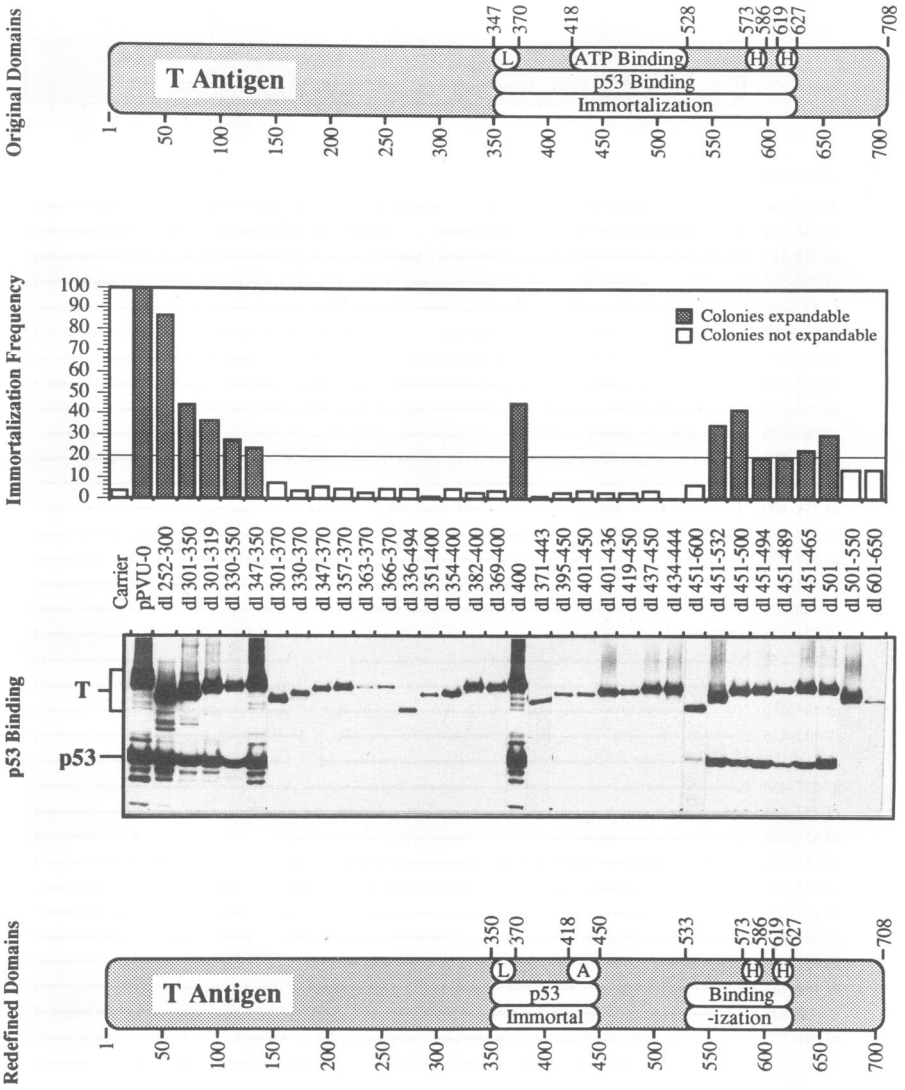

FIG. 2. (a) Relationships among p53 binding, immortalization, and other functional domains of T antigen. Shown at the top is a map of the functional domains of T antigen which overlap the putative immortalization domain as delineated prior to this study. The data in Table 3 are summarized in the immortalization frequency graph. Shaded bars represent colonies which could be expanded into cell lines. Open bars represent colonies which could not be expanded into cell lines. Twenty percent or above is classified as wild-type frequency of immortalization. The ability of mutant T antigens to bind p53 is shown in the Western blot below the immortalization frequency graph. G418-resistant clonally derived cell lines were used for constructs which could not immortalize. Clonally derived cell lines developed from immortalization assays were used for constructs which could immortalize. For the Western blot analysis, 1.5 mg of total cellular extract was immunoprecipitated with anti-T (anti-D2) polyclonal rabbit serum. Separated proteins were transferred to PVDF membranes and probed with a cocktail of anti-T (PAb901) and anti-p53 (PAb421 and PAb240) monoclonal antibodies. Secondary antibody was goat anti-mouse IgG-HRP. Detection was with the ECL chemoluminescence system. Shown at the bottom is a map of the redefined immortalization and p53 binding domains along with other overlapping functional domains. L, leucine-rich region; A, remainder of the ATP binding site; H, hydrophobic-rich region(s). (b) Overexposure of a section of the Western blot shown in panel a. When the blot is overexposed, p53 binding is not seen for those mutants which do not immortalize.

levels of p53. This demonstration was necessitated by the nature of B6MEF cultures. These primary populations contain a variety of cell types. Since the T-antigen-immortalized cell lines are clonally derived, the possibility that cell clones might arise from cells that are genetically programmed to express different levels of p53 could not be ignored. The results in Fig. 4 showed that the steady-state level of p53 was equivalent in all of the immortalized B6MEF cell lines. The

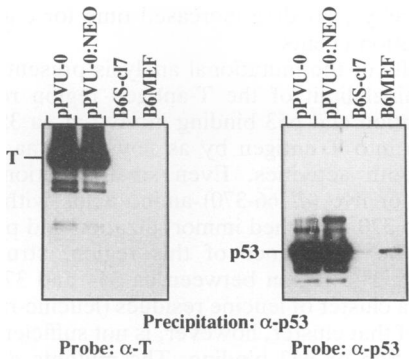

FIG. 3. Comparison of the T antigen-p53 protein interaction seen in a wild-type T-antigen-immortalized cell line (pPVU-0) and a B6S-cl7 cell line constitutively expressing wild-type T antigen (pPVU-0:NEO). For the Western blot analysis, 1.5 mg of total cellular extract was immunoprecipitated with anti-p53 polyclonal rabbit serum. The blot on the left was probed with PAb901; the blot on the right was probed with a cocktail of PAb421 and PAb240. Detection was as described in the legend to Fig. 2a. Extracts prepared from the parental cell line B6S-cl7 and B6MEF were included for comparison.

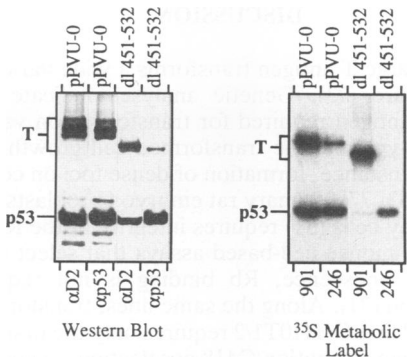

FIG. 5. Comparison of results obtained with Western blotting and ^{35}S metabolic labeling experiments. For the Western blot analysis, 1.5 mg of total cellular extract was immunoprecipitated with anti-T (αD2) or anti-p53 (αp53) polyclonal rabbit serum. Separated proteins were probed and detected as described in the legend to Fig. 2a. For the ^{35}S-metabolic labeling analysis, 5×10^6 cpm of total cellular extract was immunoprecipitated with anti-T (PAb901) or anti-p53 (PAb246) monoclonal antibody.

results in Fig. 2a showed that all mutant T antigens isolated from the immortalized B6MEF cell lines had bound p53, although the amount of T antigen bound to p53 varied. Results equivalent to those seen in Fig. 4 and Fig. 2a were obtained by using additional, independently isolated immortal cell lines expressing the mutant T antigens (data not shown). It was determined in control experiments that the extraneous bands seen on the Western blots in Fig. 2a, 3, 4, and 5 were immunoreactive proteolytic degradation products of T antigen and p53 (data not shown).

Coimmunoprecipitation of T antigen and p53 by anti-T polyclonal antibody also was examined for clonally derived G418-resistant cell lines constitutively expressing mutant T antigens that did not retain immortalizing capacity (Fig. 2). In separate experiments (Fig. 3), the levels of T antigen-p53 protein complexes in extracts of a B6MEF cell line immortalized by wild-type T antigen (pPVU-0) and in extracts of a G418-resistant B6S-cl7 cell line constitutively expressing wild-type T antigen (pPVU-0:NEO) were observed to be equivalent. Also, in the case of mutant T antigens that retained their ability to immortalize, the levels of T antigen-p53 protein complexes were compared in the immortalized

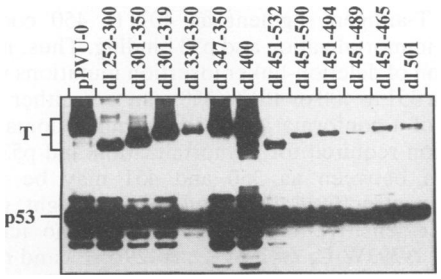

FIG. 4. Coprecipitation of p53 and T antigen from extracts of clonally derived immortalized cell lines expressing mutant T antigens. For the Western blot analysis, 1.5 mg of total cellular extract was immunoprecipitated with anti-p53 polyclonal rabbit serum. Separated proteins were probed and detected as described in the legend to Fig. 2a.

cell lines and G418-resistant cell lines constitutively expressing the mutant proteins and were observed to be equivalent in all cases (data not shown). Thus, the direct comparison of T antigen-p53 protein complexes between immortalized and G418-resistant cell lines (as appears in Fig. 2) is appropriate.

The results presented in Fig. 2 show that no mutant T antigen was so unstable that it could not be detected by Western blot analysis. Mutant T antigens migrated at their expected sizes in the Western blotting experiments. Immunoreactivity of mutant T antigens with PAb901 in the Western blotting experiments confirmed that all were in-frame protein products. T antigen produced by deletion mutants 252-300, 301-350, 301-319, 330-350, 347-350, 400, 451-532, 451-500, 451-494, 451-489, 451-465, and 501 bound p53. No p53 binding was observed in the case of deletion mutants 301-370, 330-370, 347-370, 357-370, 363-370, 366-370, 336-494, 351-400, 354-400, 382-400, 369-400, 371-443, 395-450, 401-450, 401-436, 419-450, 437-450, 434-444, 451-600, and 601-650. The results of the Western blot analysis shown in Fig. 2 confirmed that mutant T antigens that were capable of immortalizing B6MEF bound p53, while those which were defective in immortalization capacity did not bind p53.

Maturation of T antigen and p53. It has been suggested that maturation of both T antigen and p53 is a necessary event for the formation of T antigen-p53 protein complexes (4, 37). The maturation of T antigen and p53 was assessed by comparing the levels of T antigen-p53 protein interaction seen in Western blotting (older proteins) and [^{35}S]methionine metabolic labeling experiments (newly synthesized proteins). An example of a T-antigen mutant which appears somewhat defective in maturation is shown in Fig. 5. At the end of a 2-h [^{35}S]methionine pulse in metabolic labeling experiments, wild-type T antigen is fully capable of binding p53, while *dl*451-532 is apparently incapable of binding p53. However, when the steady-state interaction of T antigen and p53 is observed via Western blot analysis, *dl*451-532 appears to be fully capable of binding p53. With this in mind, some deletion mutants used in this study appear to mature more slowly than wild-type T antigen or, alternatively, have an effect on the maturation of p53 itself within the cell.

DISCUSSION

The SV40 large T antigen transforms a wide variety of cell types in culture (73). Genetic analyses indicate that the portion of T antigen required for transformation varies with both the cell type and the transformed cell growth property selected. For instance, formation of dense foci on continuous rat cell lines (63, 77), primary rat embryo fibroblasts (71), and baby rat kidney cells (51) requires integrity of the Rb binding region. Yet in mouse cell-based assays that select solely for escape from senescence, Rb binding is not required for immortalization (71). Along the same lines, transformation of the mouse cell line C3H10T1/2 requires only the first 121 aa of T antigen in transformation/G418 coselection assays (63, 77), while transformation of the continuous rat cell line REF52 requires aa 1 to 626. These combined results indicate strongly, as has been suggested previously (71), that individual cell types or cell lines can complement one or more T-antigen functions. The assessment of T-antigen transforming activities in a variety of cells and assay systems and parallel investigation of cellular complementing functions will be necessary to fully understand the transformation process.

Our investigations have focused on transformation of B6MEF cells. Using the B6MEF system, we (71, 72) and others (78) have shown a correlation between immortalization and p53 binding. We showed that removal of the first 127 aa, aa 127 to 250, or aa 626 to 708 from T antigen did not reduce its immortalizing or p53 binding capacity (71, 72). Manfredi and Prives (47) showed that SV40 T-antigen aa 337 to 708 were sufficient in the context of polyomavirus large T-SV40 T antigen hybrid proteins for p53 binding. Immortalization was not investigated in that case, since the N-terminal segment of polyomavirus large T antigen alone will immortalize (2, 30). Zhu et al. (78), using deletion mutants, showed that aa 168 to 346 of SV40 T antigen could be removed without compromising immortalization or p53 binding. The results of the present study are in complete agreement with these previous reports and delimit the boundaries of the regions required for both activities further.

Removal of aa 252 to 300 did not diminish either immortalizing capacity or p53 binding. Removal of the segment containing aa 301 to 350 reduced the immortalizing capacity of *dl*301-350 by only 56%. Removal of aa 451 to 532 or of smaller blocks of residues within that segment also did not prevent immortalization or p53 binding, indicating that neither function requires integrity of this region of T antigen. Modest decreases in immortalization were, however, consistently observed with T antigens with smaller internal deletions of amino acids between residues 300 and 350 and between residues 451 and 532. The modest decreases in immortalization frequency do not correlate with either the steady-state level of T antigen or the steady-state level of T antigen-p53 protein complexes (Fig. 2a). However, the decreases in immortalization frequency appear to correspond to the time of appearance of colonies (Table 3). We speculate that the particular deletion and consequent insertion of amino acids encoded in the linker may disturb one or more functions involved in altered cell growth properties that could enhance either integration of the plasmid containing the T-antigen coding sequence or expression of T antigen during early events in the immortalization pathway. The B6MEF cultures, since they are derived from whole embryos, contain a variety of cell types. Individual cell types are expected to senesce at different time points in culture. Perhaps the somewhat reduced frequencies of immortalization observed reflect transformation of cells that survive longer, thereby providing increased time for completion of immortalization events.

The results of the mutational analysis presented here set the N-terminal limit of the T-antigen region required for immortalization and p53 binding in vivo at aa 351. Further penetration into T antigen by as few as 19 aa (*dl*347-370) abolishes both activities. Even small deletions of eight (*dl*363-370) or five (*dl*366-370) amino acids within the segment 350 to 370 abolished immortalization and p53 binding, indicating the importance of this region, structurally or functionally. The region between aa 344 and 370 is distinguished by a cluster of leucine residues (leucine-rich region). Retention of that cluster, however, is not sufficient for either immortalization or p53 binding. The mutants *dl*382-400 as well as *dl*395-450 and the mutants with smaller deletions within that 55-aa segment all contain the leucine-rich region, yet none immortalize or bind p53.

The four deletions that span the segment aa 351 to 400 all end at aa 400. It was possible that aa 400 is critical to the structure of this portion of T antigen or to a function required for immortalization and p53 binding. Alternatively, the insertion of the linker-encoded amino acid sequence Arg-Ile-Arg adjacent to aa 401 in the T antigen produced by *dl*354-400, *dl*369-400, and *dl*382-400 could distort a critical structure or function. These possibilities are diminished by the observation that *dl*400, which produces a T antigen in which aa 400 is replaced by Arg-Ile-Arg, both immortalizes and binds p53 efficiently.

The finding that aa 400 can be removed without compromising immortalization or p53 binding in mouse cells is consistent with recent reports. Lin and Simmons (42) found that amino acid substitutions at residue 400 (Lys→Arg or Lys→Glu) had little effect on T-antigen complex formation with monkey, human, or mouse p53. Several other amino acid substitution mutations within the segment from aa 388 to 411 also had little or no effect on the resulting T antigen-p53 protein interaction. However substitutions made at T-antigen aa 402 (Asp→Glu, Asp→Asn, or Asp→His) eliminated p53 binding in monkey and human cells and substantially reduced the level of p53 binding in mouse cells. This finding suggested that T-antigen aa 402 may function as a contact point with p53. Subsequently, Lin and Simmons (41) showed that the 402-aa substitution mutants were also defective in dense focus formation assays, using secondary human cells and slightly defective in dense focus formation assays utilizing secondary mouse embryo cells. The close correspondence in that system between p53 binding and certain aspects of transformation is consistent with our present findings.

Although certain amino acid substitutions surrounding aa 400 may be tolerated, removal of as few as 14 aa (*dl*437-450) from the T-antigen segment aa 401 to 450 coordinately abrogated immortalization and p53 binding. Thus, in general, introduction of deletion-linker insertion mutations within the segment aa 351 to 400 or 401 to 450 results in either structural distortion of a conformationally determined domain or loss of a function required for immortalization and p53 binding. The region between aa 350 and 451 may be unusually sensitive to structural distortion. Six of eight groups of temperature-sensitive mutants (45) alter amino acids within (*ts*357R-K, *ts*393W-C, *ts*422W-C, *ts*429 P-L, and *ts*438A-V) or immediately adjacent to (*ts*453P-S) that T-antigen segment. The pleiotropic effects of these substitutions and the lability of the T antigens at nonpermissive temperature suggest conformational sensitivity of this region. Within this region is a highly conserved stretch of residues (aa 418 to 528) (3) with structural similarity to an ATP-binding fold.

The T-antigen region between aa 532 and 651 was represented in the genetic analysis presented here only by fairly large deletions. Nonetheless, the results indicated that removal of T-antigen segments aa 451 to 600, 501 to 550, and 601 to 650 abolished immortalization and p53 binding, indicating that integrity of these regions is needed for both activities. Previous investigations showed that the last 82 aa of T antigen could be removed without reducing immortalizing or p53 binding activity (71). Therefore, integrity of the T-antigen segment aa 533 to 626 appears to be necessary for these activities.

Located within the segment aa 533 to 626 are two very hydrophobic stretches of residues (aa 571 to 589 and 619 to 627). Several mutations that alter or delete amino acids within the hydrophobic region between 571 and 589 have been shown defective in p53 binding in vivo (54, 71, 78) and also to be defective for immortalization of B6MEF cells (71, 78) and transformation of REF52 cells (54, 77). It was shown that many of these mutants produced T antigens which were altered in multiple biochemical attributes. They were defective in oligomerization and had shorter half-lives, decreased ATPase activity, and altered levels and patterns of phosphorylation (9, 67, 71). It was concluded that the segment aa 571 to 589 was important for regulating higher-order structural and functional relationships in T antigen (67). The region between aa 619 to 627 displays a hydrophobicity profile similar to that of the segment aa 571 to 589. The aa 619-to-627 region of T antigen has also been shown to be important for the maintenance of higher-order structural relationships in T antigen (39a). Mutations that alter or delete amino acids within this hydrophobic region have been shown to be defective in immortalization of B6MEF (71). Similar mutants have been shown by Lin et al. (40) to have altered monoclonal antibody reactivity profiles and increased sensitivity to trypsin and to be defective in nonspecific binding of double-stranded DNA and oligomerization.

A comparison of the intensities of the p53 bands in Fig. 4 and 2a indicates that only a fraction of the p53 in the cells is bound to T antigen. For instance, more p53 was immunoprecipitated with anti-p53 polyclonal antibody from extracts from cell lines expressing T antigens missing amino acids from the 301-to-350 region than was immunoprecipitated from the same extracts with the anti-T-antigen polyclonal antibody anti-D2. This result indicated, as has been shown previously (13–15, 48), that p53 need not remain bound to T antigen to achieve stability, as reflected by accumulation to high steady-state level. Whether T antigen alters the stability of p53 by direct interaction or by altering cellular processes that affect p53 accumulation is not certain.

As with most deletion mutant-based analyses, the results presented here are most valuable for identifying T-antigen regions that are not needed for immortalization or p53 binding. These results, along with previous findings (47, 71, 72, 78), show that the T-antigen segment aa 252 to 350, aa 400, and the segment containing aa 451 to 532 can be removed without abrogating either of these activities. The coordinate loss or retention of both properties by this large set of internal deletion mutants, which produce T antigens that reach readily detected and often wild-type steady-state levels in cells constitutively expressing the mutant proteins, strongly suggests that the central region of T antigen contains a p53 binding-dependent immortalizing activity. More detailed genetic analysis of the regions whose alteration by deletion of amino acids prevents function is needed to identify the amino acids critical for immortalization and for p53 binding in vivo.

There is an apparent discrepancy between the results reported here and those reported by Schmieg and Simmons (58) with respect to the region of T antigen required for p53 binding. Schmieg and Simmons reported that a 46K proteolytic fragment of T antigen spanning aa 131 to 517 was capable of binding p53 in vitro, while our own results and those of others (54, 67, 78) suggest that sequences between aa 532 and 627 are necessary for in vivo p53 binding. A number of scenarios could be envisioned to rationalize the differences seen between the two systems, the simplest of which is that there are fundamental differences in the requirements for in vitro and in vivo p53 binding by T antigen.

Residues 533 to 626 may play a role in holding T antigen in a correct conformation to allow p53 binding to occur. Evidence for this hypothesis may be seen in a report by Tack et al. (67), in which it was shown that a T-antigen substitution mutant (Pro-584→Leu) which maps to the aa 533-to-626 region is defective for multiple biochemical properties, including p53 binding, oligomerization, phosphorylation, and ATPase activity. A deletion mutant producing a T antigen missing residues between aa 532 to 626 may result in a polypeptide in which the p53 binding site is either disrupted or conformationally hidden, preventing p53 binding. However, if this whole region is removed, as in the 46K proteolytic T-antigen fragment, the p53 binding site again becomes accessible and therefore p53 binding can occur. If this hypothesis is correct, a peptide spanning residues 351 to 450 should be able to complex p53 in vitro.

Residues 533 to 626 may interact directly via protein folding with aa 351 to 450 to form the active site for p53 binding by T antigen. Deletion of residues in the aa 533-to-626 region could make the T antigen-p53 complex so weak that a functional in vivo interaction leading to immortalization is not achieved, and in addition, the complexes do not survive the extraction process. When similar complexes are formed in vitro, there is no extraction process to survive and no biological phenomenon to observe (immortalization). Under those conditions, a mutant T antigen or a proteolytic fragment of T antigen may be functionally equivalent to wild-type T antigen.

Perhaps the most attractive possibility is that there is an as yet undefined domain in T antigen important for the appropriate functional maturation of the protein (and or p53) and that residues 533 to 626 are required for this activity. It has been shown that maturation of both T antigen and p53 is a necessary event for the formation of T antigen-p53 protein complexes (4, 37). Deletion mutants producing T antigens missing residues between aa 532 and 626 may result in a polypeptide which cannot mature correctly and therefore cannot bind p53 in vivo. The 46K proteolytic fragment was isolated from wild-type mature T antigen which was therefore fully capable of binding p53 in vitro.

The combined results from several laboratories indicate that T antigen contains two independent immortalizing regions (see reference 44 for a review). Results from other laboratories have shown convincingly that N-terminal fragments of T antigen as short as aa 1 to 147 (61) or 1 to 137 (2) efficiently immortalize rat or mouse cells, respectively. In contrast, Thompson et al. (72) have demonstrated an immortalizing activity located between aa 250 and 708. In the B6MEF system, short N-terminal T-antigen fragments including the segment aa 1 to 147 as contained in plasmid pSRT147NS (kindly provided by L. Sompayrac) do not generate colonies that can be expanded into cell lines (71a). The inability of N-terminal T-antigen fragments to immortalize B6MEF may be related to the unusually short life span of these primary cells in culture. We do not know as yet either the precise T-antigen functions that are sufficient for immor-

talization or the time frame in which those functions must operate in order for primary cells in culture to escape senescence. It remains possible that the rapid senescence of B6MEF in culture precludes immortalization operating through the pathway utilized by the N-terminal immortalizing region. The results presented here address the relationship between a C-terminal immortalizing region of T antigen and p53 binding. These results suggest that the mechanism of immortalization that is interrupted by mutations in the regions aa 351 to 450 and 533 to 626 depends on binding of the cellular tumor suppressor gene product p53. Investigations are in progress to determine whether either or both of these regions are sufficient for p53 binding in vivo or immortalization.

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