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Simian Virus 40 Large T Antigen Contains Two Independent Activities That Cooperate with a *ras* Oncogene To Transform Rat Embryo Fibroblasts

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The simian virus 40 large T antigen immortalizes growing primary cells in culture. In addition, this viral oncoprotein cooperates with an activated *ras* protein to produce dense foci on monolayers of rat embryo fibroblasts (REF). The relationship between independent immortalization and cooperative transformation with *ras* has not been defined. Previously, two regions of T antigen were shown to contain immortalization activities. An N-terminal fragment consisting of amino acids 1 to 147 immortalizes rodent cells (L. Sompayrac and K. J. Danna, Virology 181:412–415, 1991). Loss-of-function analysis indicated that immortalization depended on integrity of the T-antigen segments containing amino acids 351 to 450 and 533 to 626 (T. D. Kierstead and M. J. Tevethia, J. Virol. 67:1817–1829, 1993). The experiments described here were directed toward determining whether these same T-antigen regions were sufficient for cooperation with *ras*. Initially, constructs that produce T antigens containing amino acids 176 to 708 (T176-708) or 1 to 147 were tested in a *ras* cooperation assay. Both polypeptides cooperated with *ras* to produce dense foci on monolayers of primary REF. These results showed that T antigen contains two separate *ras* cooperation activities. In order to determine the N-terminal limit of the *ras* cooperation activity contained within the T176–708 polypeptide, a series of constructs designed to produce fusion proteins containing T-antigen segments beginning at residues 251, 301, 337, 351, 371, 401, 451, 501, 551, 601, and 651 was generated. Each of these constructs was tested for the capacity to cooperate with *ras* to produce dense foci on REF monolayers. The results indicated that a polypeptide containing T-antigen amino acids 251 to 708 (T251-708) was sufficient to cooperate with *ras*, whereas the more extensively truncated products were not. The abilities of the N-terminally truncated T antigens to bind p53 were examined in p53-deficient cells infected with a recombinant vaccinia virus expressing a phenotypically wild-type mouse p53. The results showed that polypeptides containing T-antigen amino acids 251 to 708, 301 to 708, 337 to 708, or 351 to 708 retained p53-binding capacity. The introduction into the T251-708 polypeptide of deletions that either prevented p53 binding (*dl*434-444) or did not prevent p53 binding (*dl*400) abrogated *ras* cooperation. These results indicated that although p53 binding may be necessary for *ras* cooperation, an additional, as-yet-undefined activity contained within the T251-708 polypeptide is needed.

Continued expression of the simian virus 40 (SV40) large T antigen is sufficient to immortalize primary cells in culture (64). Previously we demonstrated that deletions in the central region of T antigen abrogated immortalization. More specifically, T antigens missing amino acids 1 to 127 or 127 to 250 immortalized mouse embryo fibroblasts with wild-type efficiency (62), as did T antigens with internal deletions of amino acids 252 to 300, 301 to 350, 400, or 451 to 532 (28). In contrast, T antigens containing deletions within the segments encompassing amino acids 351 to 450 or 533 to 625 (28) lost immortalizing capacity.

Others showed that N-terminal segments of T antigen containing the first 138 (2) or 147 (54) amino acids were sufficient to immortalize rat embryo fibroblasts (REF). The combined results suggested that T antigen contains two immortalizing activities.

In addition to its ability to immortalize cells independently, large T antigen cooperates with an activated *ras* oncogene to generate dense foci on monolayers of REF. The *ras* oncogene cannot produce dense foci on primary cell monolayers independently of a cooperating oncogene, although it produces foci on monolayers of continuous cell lines efficiently (reviewed in reference 3). It is presumed, therefore, that large T antigen contributes to the immortalization of primary cells during *ras* cooperation assays.

Previously, others have examined various T antigen mutants in *ras* cooperation assays. In those studies, two functions in the N terminus of T antigen, Rb binding and a T-antigen function needed to complement adenovirus E1A mutants which are defective in binding the cellular protein p300, have been implicated in *ras* cooperation (35, 68). Deletion of amino acids 17 to 27 abrogated T antigen's ability to cooperate with *ras* (35, 68) and to complement adenovirus E1A p300-binding-negative mutants. Similarly, a mutant T antigen incapable of binding Rb failed to cooperate with *ras* (40, 68). The identification of a central region of the protein involved in immortalization raised the questions of whether this region also contained a *ras* cooperation activity and whether the same functions within the central portion of T antigen were involved in immortalization and in *ras* cooperation.

We show here that two independent regions of T antigen are sufficient to cooperate with *ras*. Specifically, we show that a T-antigen polypeptide containing amino acids 1 to 147 (T1-147) and a T-antigen polypeptide containing amino acids 251 to 708 (T251-708), but not T polypeptides initiated at amino

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acid 301 or beyond, cooperate with an activated *ras* oncogene to produce dense foci on monolayers of REF. We show also that the T251-708 polypeptide cooperates with adenovirus E1A in dense-focus assays. Moreover, introduction into the T251-708 polypeptide of a mutation that prevents p53 binding abrogates transformation both by T251-708 plus *ras* and by T251-708 plus E1A, suggesting that p53 binding, or a function that is lost simultaneously when binding is abrogated in the mutants tested, is required for cooperative transformation with those viral oncoproteins. However, additional data indicate that p53 binding, if required, is not sufficient for transformation of REF in cooperation with *ras*.

MATERIALS AND METHODS

Plasmids and recombinant virus. The plasmid pPVU0 (27) contains the SV40 enhancer, promoter, origin, and early-region coding sequences from the *Pvu*II site (nucleotide [nt] 272) to the *Bam*HI site (nt 2533) cloned into pBR328 at the corresponding sites. pPVU0 produces functional large T and small t antigens. The plasmid p*dl*2005 contains the genome of the mutant *dl*2005. This mutant contains a 230-bp deletion within the large T intron and does not produce detectable quantities of small t antigen (50). The plasmid py2-70K (y2) has been described previously (1) and contains the SV40 enhancer, promoter, and origin followed by the *Taq*1 (nt 4739)-to-*Bam*HI early-region segment of the mutant y2. The y2 mutation is a deletion removing nucleotides 4499 to 4478. This deletion removes the in-frame methionine codon 109. The T antigen produced by the plasmid y2 is expected to begin with the methionine at position 176.

The plasmids p*dl*536 and pT147NS were generously provided by L. Sompayrac. The p*dl*536 (52) construct contains the genome of the deletion mutant *dl*536 cloned into the *Bam*HI site of pBR322. The *dl*536 deletion removes the splice acceptor site for the large T- and small t-antigen mRNAs. Splice donor sites for both messages are maintained. Thus, p*dl*536 encodes an authentic small t antigen but no large T antigen. The plasmid pT147NS (53) contains the early region of an SV40 mutant containing a stop codon in place of T-antigen amino acid 148. Expression is from the human T-cell leukemia virus type I long terminal repeat and utilizes the splice donor and acceptor from the vector pcDL-SRα (59). In the pT147NS construct, the SV40 sequences between nucleotides 4568 and 5135 have been replaced with the corresponding fragment from a cDNA copy of the large T-antigen mRNA. Thus, pT147NS produces a truncated large T antigen containing amino acids 1 to 147 and does not produce small t antigen.

The plasmid pE1A contains the E1A control and coding sequences from adenovirus type 2 or 5, as described previously (56). The SP72-*ras* construct was kindly provided by A. Levine. This plasmid contains the activated H-*ras* (T24) oncogene (5) cloned into the *Bam*HI site of the vector SP72.

The CAV-IL2 plasmid was kindly provided by David Cosman, Immunex, Seattle, Wash. This plasmid consists of a cDNA copy of the interleukin-2 gene inserted between *Kpn*I and *Bgl*II sites in the vector DC302 (37). The DC302 vector contains an SV40 origin of replication followed by the human cytomegalovirus major immediate-early enhancer/promoter, the adenovirus tripartite leader sequence, a polylinker, an SV40 polyadenylation signal, and adenovirus VA RNA sequences. The CAV-IL2 plasmid was modified by converting the unique *Xho*I site preceding the interleukin-2-coding sequence to an *Mlu*I site (CAV-IL2M). The plasmid pSV2-neo (55) contains the neomycin resistance gene under control of the SV40 enhancer/promoter.

A recombinant vaccinia virus containing a wild-type mouse p53 gene and wild-type vaccinia virus were kindly provided by B. Moss, J. Bennink, and J. Yewdell. The mouse p53 gene, originally cloned and sequenced by Jenkins et al. (23), was introduced into vaccinia virus by using the methodology described by Mackett et al. (33). Vaccinia virus stocks were prepared and titrated on human 143 thymidine kinase-negative cells (44). The vaccinia virus-mouse p53 recombinant produces a p53 protein that is phenotypically wild type in terms of antibody reactivity (38) and its ability to bind SV40 T antigen (17, 38).

Cells and cell lines. Fisher 344 REF were prepared from 14- to 16-day embryos as described previously in detail for mouse embryo fibroblasts (60). The TC7 cell line is a subclone of the African green monkey kidney cell line CV1 (45). The Rat-2 cell line (65) is a thymidine kinase-negative derivative of the established fibroblastoid Rat-1 (6) cell line. Saos-2 is an osteosarcoma cell line that is devoid of p53 (12). Primary cell cultures and cell lines were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 0.075% $NaHCO_3$ 10×1 unless specified otherwise.

Generation of T-antigen fusion protein constructs. The generation of a library of *Eco*RI linker-insertion constructs with *Eco*RI 8-mer linkers (GGAATTCC) immediately following T-antigen codons 250, 350, 450, 550, and 650 or with *Eco*RI 10-mer linkers (CCGAATTCCGG) immediately following codons 300, 400, 500, and 600 in the plasmid pPVU0 was described previously (28). By using the same procedures, 8-mer linkers also were inserted into the T-antigen-coding sequence of pPVU0 immediately following codon 336 or codon 370. The authenticity and location of the linker and surrounding sequences were verified by dideoxy sequencing (47).

In an initial step to producing fusion protein constructs containing portions of T antigen under transcriptional control of the human cytomegalovirus immediate-early enhancer/promoter, a series of plasmids capable of producing segments of T antigen fused to the α peptide of β-galactosidase was generated. Specifically, the pPVU0 plasmids containing *Eco*RI linkers were cleaved with *Bam*HI, and the resulting 3′ recessed ends were filled in by Klenow polymerase. *Eco*RI digestions then were performed to liberate T-antigen segments encoding amino acids 251 to 708, 301 to 708, 337 to 708, 351 to 708, 371 to 708, 401 to 708, 451 to 708, 501 to 708, 551 to 708, 601 to 708, and 651 to 708. These fragments were transferred into a modified BluescriptII SK$^+$ phagemid between the *Eco*RI and *Eco*RV sites. The BluescriptII SK$^+$ phagemid (Stratagene) had been modified by converting the *Nae*I site at nt 330 to a *Bgl*II site and converting the *Pvu*II site at nt 977 to an *Mlu*I site.

Those fragments of the T-antigen-coding sequence, preceded by an *Eco*RI 8-mer linker, joined the *Eco*RI site of the polylinker within the β-galactosidase α-peptide-coding sequence of BluescriptII SK$^+$ in frame. Those fragments preceded by an *Eco*RI 10-mer were introduced into a further-modified Bluescript vector in which the reading frame was adjusted to accommodate the additional nucleotide of the *Eco*RI linker. This adjustment was accomplished by adding an *Aat*II 8-mer (GGACGTCC) linker at the BluescriptII SK$^+$ *Sma*I site. In all cases, the fusion proteins utilized the start codon of β-galactosidase to produce a polypeptide containing the first 7 amino acids of β-galactosidase followed by 31 (8-mer-linked segments) or 34 (10-mer-linked segments) amino acids encoded by the synthetic polylinker in BluescriptII SK$^+$ and 1 or 2 amino acids encoded by the *Eco*RI linker. In the case of the segments linked by an *Eco*RI 8-mer, a serine residue intervenes between the BluescriptII SK$^+$ polylinker- and the T-antigen-encoded amino acids. In the case of the segments linked by an *Eco*RI 10-mer, the amino acids between the *Sma*I site of the BluescriptII SK$^+$ and the first T-antigen codon are Asp Val Arg Ala Ala Gly Ile Arg instead of Gly Cys Arg Asn Ser.

To facilitate their expression in mammalian cells, the *Mlu*I-to-*Bgl*II Bluescript SK$^+$ fragments containing the fusion protein-coding sequences were introduced into the CAV-IL2M vector. Digestion of CAV-IL2M with *Mlu*I and *Bgl*II released the interleukin-2-coding sequence. The *Mlu*I-*Bgl*II fragments from BluescriptII SK$^+$ containing fusion protein-coding sequences were then inserted.

Transient-expression procedures. Transfection of DNA into TC7 cells was accomplished by the DEAE-dextran-chloroquine procedure essentially as described previously (34). Cells were seeded into 75- or 150-cm^2 tissue culture flasks 1 day prior to transfection. For each transfection, DNA (20 or 40 μg) was added to 250 μl of a 2-mg/ml stock solution of DEAE-dextran, and the reaction mixture was brought to 1 ml with Tris-buffered saline to give a final DEAE-dextran concentration of 500 μg/ml. Cell monolayers were rinsed twice with Tris-buffered saline. The DNA mixtures then were placed onto the cells, and the flasks were rocked for 20 min at room temperature. The DEAE-dextran–DNA mixtures were aspirated, and the cell monolayers were rinsed with Tris-buffered saline. Twenty milliliters of DMEM 10×1 containing 100 μM chloroquine phosphate was added to each flask (34). The cultures were incubated at 37°C for precisely 3.5 h. The medium then was replaced with DMEM 10×1, and the cells were incubated at 37°C for 48 h.

Antibodies. Anti-T-antigen antibodies used in immunoprecipitation and Western immunoblot analysis included PAb901 (66), PAb902 (60), PAb419 (22), PAb416 (22), a rabbit anti-D2 polyclonal antibody (16, 29) (a kind gift from D. Lane), and a rabbit anti-T-antigen polyclonal antibody. PAb901 recognizes a denaturation-resistant epitope located between T-antigen amino acids 684 and 698 (61a). PAb902 recognizes a conformation-sensitive epitope contained within the first 82 amino acids of both large T and small t antigens. PAb419 is directed toward a denaturation-resistant epitope within the N-terminal 82 amino acids of large T and small t antigens. The polyclonal anti-D2 antibody was generated against the adenovirus-SV40 hybrid D2 protein, which contains the N-terminal 104 amino acids of the 33-kDa adenovirus type 2 structural protein fused to T-antigen amino acids 115 to 708. Rabbit polyclonal anti-T-antigen antibody was generated against native T antigen (a kind gift from S. Tevethia).

Anti-p53 antibodies used included an anti-p53 polyclonal antibody, PAb421, PAb246, and PAb240. The rabbit anti-p53 polyclonal antibody was purchased from Chemicon. PAb421 (22) is a mouse monoclonal antibody directed toward a denaturation-resistant epitope located in the C terminus of both mutant and wild-type p53 (58). PAb246 recognizes p53 in its native conformation (15, 18). PAb240 (18) recognizes an epitope between amino acids 213 and 217 in denatured mutant and wild-type p53. PAb240 is unique in that its epitope is conformationally exposed in native mutant p53 yet is conformationally hidden in native wild-type p53. Thus, only under native conditions does PAb240 detect only mutant p53.

The monoclonal antibody to gD (II-436-1) (hereafter referred to as anti-gD antibody) recognizes an epitope on the herpes simplex virus glycoprotein D (24, 26). This antibody was used as a negative control in some immunoprecipitations. P3N3 is nonspecific mouse myeloma ascites fluid and was used, unless stated otherwise, for preclearing cell lysates prior to immunoprecipitation and as a negative control in some experiments.

Immunoprecipitation and Western blotting. Immunoprecipitation was performed exactly as described previously (28) except that cells were grown to near confluence in 75-cm^2 flasks and were metabolically labeled with 300 μCi of

[^{35}S]methionine for 50 min. The Western blotting was performed on immunoprecipitates as described extensively previously (28).

Transformation assays. For *ras* cooperation assays, REF were recovered from liquid nitrogen and seeded into a 75-cm^2 tissue culture flask in DMEM 10×1. On the following day the medium in the flasks was replaced. On the next day the cells were passaged at 1:2 or 1:3, depending on the batch of REF. On the following day 75-cm^2 flasks were seeded with 3×10^5 to 5×10^5 cells per flask in DMEM 10×1. Cells were transfected 24 h later by using the $CaPO_4$ precipitation method with 10 μg of carrier DNA (typically calf thymus) and 1 μg of various plasmids containing T-antigen-coding segments plus 1 μg of the SP72-*ras* plasmid. The DNA-calcium phosphate precipitate remained in the medium for 16 to 20 h, after which the medium was replaced with fresh DMEM 10×1. When the cell monolayer became confluent (typically 5 days later), the medium was replaced with DMEM 10×1. The medium was replaced every 3 to 4 days. The medium was changed to DMEM containing 10% fetal bovine serum and 0.15% $NaHCO_3$ (DMEM 10×2) or 0.21% $NaHCO_3$ (DMEM 10×3) as needed to maintain an appropriate pH. Cultures were incubated at 37°C for 3 to 6 weeks. For expansion of colonies into cell lines, dense foci which appeared on the monolayer were picked with cloning pipettes, transferred to wells of a 12-well tissue culture plate, and then transferred to larger vessels as needed. For E1A cooperation assays, REF were transfected similarly with 10 μg of carrier DNA, 1 μg of pE1A, and 1 μg of plasmid containing SV40 T-antigen mutant protein- or fusion protein-coding sequences.

Vaccinia virus infection of transfected Saos-2 cells. Saos-2 cells were seeded into 75-cm^2 flasks. When the flasks were approximately 75% confluent, the cells were transfected with DNA mixtures containing 20 μg of salmon sperm DNA and 5 μg of a construct encoding a truncated T antigen or fusion protein by using the calcium phosphate-DNA precipitation method as described above. Forty-eight hours later, the transfected cells were infected at a multiplicity of infection of 10 with either vaccinia virus or the recombinant vaccinia virus encoding a phenotypically wild-type mouse p53 gene. Six hours later the cells were labeled with [^{35}S]methionine (300 μCi) for 1 h. Cell extracts were precleared with anti-gD antibody and then immunoprecipitated with anti-gD antibody (negative control), PAb901, or PAb246. The immunoprecipitated products were resolved by electrophoresis through sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. The gels were exposed to XAR (Kodak) film for autoradiography.

RESULTS

Two independent regions of T antigen cooperate with an activated *ras* gene to transform REF. Initially, a construct designed to produce a T antigen consisting of amino acids 1 to 147 (T1-147) (53) and a construct designed to produce a nonoverlapping T-antigen segment containing amino acids 176 to 708 (T176-708) (62) were investigated for their abilities to cooperate with *ras*. The constructs were cotransfected with a *ras*-expressing construct into REF cells. The appearance of dense foci on the cell monolayers was monitored over a 3- to 4-week period. The results are shown in Table 1. A construct encoding full-length large T and small t antigens (pPVU0, experiments 1, 3, and 5) or a construct encoding only a full-length large T antigen (p*dl*2005, experiments 2, 4, and 5) generated dense foci efficiently in cooperation with the *ras* oncogene. Introduction of a plasmid (pT147NS) encoding T1-147 did not result in formation of dense foci above the level that developed in cultures transfected with carrier DNA. However, cotransfection of pT147NS with the *ras* oncogene (Table 1, experiments 1 to 4) resulted in efficient formation of dense foci (a level of 0.26 to 0.65 relative to the wild-type T-antigen plasmid level). These results indicated that the N-terminal 147 amino acids of T antigen were sufficient to cooperate with *ras* in producing dense foci on REF monolayers.

Introduction of the y2 plasmid encoding an N-terminally truncated T antigen containing amino acids 176 to 708 (T176-708) did not result in formation of dense foci. However, cotransfection of the y2 plasmid with the activated *ras* oncogene (Table 1, experiments 3 to 5) resulted in the formation of dense foci (a level of 0.17 to 0.21 relative to the wild-type T-antigen plasmid level) on the transfected REF monolayers. These results indicated that the segment of T antigen encompassing amino acids 176 to 708 also was sufficient to cooperate with *ras*.

Individual dense foci were picked from flasks that received pPVU0, y2, or pT147NS plus *ras* DNA in experiment 3 (Table 1) and were expanded into cell lines, and the steady-state levels of the T antigens in cell lines expressing wild-type T antigen, T176-708, or T1-147 then were investigated by Western blot analysis. Representative results appear in Fig. 1. Relative to the level of T antigen in cells expressing the full-length protein, considerably less T176-708 polypeptide, which appears as a doublet, accumulated in the cells cotransformed by T176-708 plus *ras* (Fig. 1A). A protein with an apparent molecular mass of 20 to 22 kDa was immunoprecipitated by PAb902 from an extract of cells cotransformed by T1-147 plus *ras* (Fig. 1B). PAb902 was used to immunoprecipitate T1-147, since the rabbit anti-T-antigen polyclonal antibody did not efficiently recognize the polypeptide (7). The accumulated T1-147 protein was abundant. In multiple experiments (data not shown) the level of T1-147 was equivalent to or greater than that of wild-type T antigen in transient-transfection experiments as well. As a negative control, Western blot analysis of an extract from cells expressing a T-antigen segment that is missing amino acids 1-250 (T251-708) was performed (Fig. 1B). As expected, no band was detected at the position of the T1-147 polypeptide.

When the amount of large T antigen is limited, small t antigen augments its transforming activity in some assays (4). Since the level of T176-708 in cell lines expressing the protein

TABLE 1. Two segments of T antigen cooperate with an activated *ras* oncogene to transform REF

DNA	T antigen(s)[a]	No. of dense foci per flask[b] in expt:				
		1	2	3	4	5
pPVU0 + *ras*	T1-708, t	119, 150 (1.0)	ND[c]	99, 108 (1.0)	ND	120, 157 (1.0)
p*dl*2005 + *ras*	T1-708	ND	206, 207 (1.0)	ND	55, 56 (1.0)	140, 150 (1.05)
p*dl*2005 + *ras* + p*dl*536	T1-708, t	ND	ND	ND	ND	155, 117 (0.98)
pT147NS	T1-147	5, 6 (0.04)	5, 7 (0.02)	0, 0 (<0.005)	0, 0 (<0.009)	ND
pT147NS + *ras*	T1-147	34, 41 (0.26)	112, 133 (0.59)	28, 27 (0.27)	36, 25 (0.65)	ND
y2	T176-708	ND	ND	0, 0 (<0.005)	0, 0 (<0.009)	0, 0 (< 0.004)
y2 + *ras*	T176-708	ND	ND	18, 21 (0.19)	8, 11 (0.17)	27, 32 (0.21)
y2 + p*dl*536	T176-708, t	ND	ND	ND	ND	0, 1 (0.004)
y2 + *ras* + p*dl*536	T176-708, t	ND	ND	ND	10, 22 (0.32)	35, 31 (0.24)
Carrier		1, 2 (0.01)	5, 3 (0.007)	0, 0 (<0.005)	0, 0 (<0.009)	0, 0 (<0.004)
ras		7, 12 (0.07)	3, 4 (0.002)	5, 1 (0.03)	0, 1 (0.009)	3, 1 (0.014)

[a] t, small t antigen-coding sequence present.
[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 with the wild-type T-antigen plasmid (pPVU0 or p*dl*2005) used as a positive control.
[c] ND, not determined.

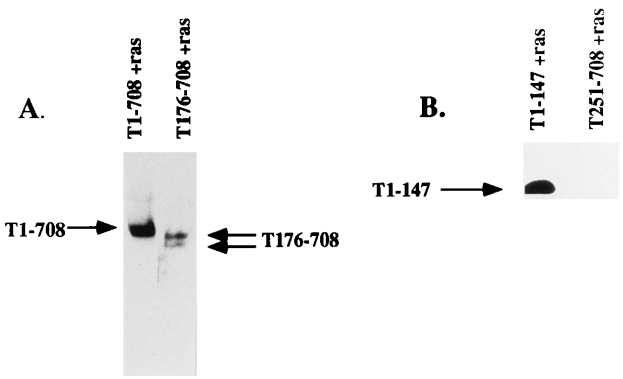

FIG. 1. Steady-state levels of T antigens in cell lines derived from dense foci in flasks receiving a plasmid encoding *ras* and a plasmid encoding T1-708, T176-708, T1-147, or T251-708. For Western blot analysis, equivalent amounts of protein extracts were immunoprecipitated with a rabbit anti-T-antigen polyclonal serum (anti-D2) (A) or with PAb902, which recognizes an epitope within the first 82 amino acids in native T antigen (B). Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with PAb901, which recognizes an epitope in the C terminus of T antigen (amino acids 684 to 698) (A), or with PAb419, which recognizes a denaturation-resistant epitope between T-antigen amino acids 1 and 82 (B). The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G. Detection was with the ECL chemiluminescence system (Amersham). In panel B only the lower portion of the gel is shown. The arrows indicate the positions of the T antigens detected. Their apparent sizes, determined by the migration of molecular weight markers (not shown), were as expected on the basis of the coding sequences introduced.

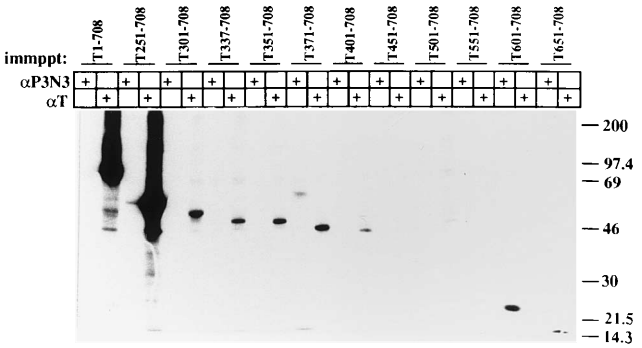

FIG. 2. Synthesis of α peptide–T-antigen fusion proteins. The T-antigen fusion constructs (CAV251-708, CAV301-708, CAV337-708, CAV351-708, CAV371-708, CAV401-708, CAV451-708, CAV501-708, CAV551-708, CAV601-708, and CAV651-708) were transfected into TC7 cells. Forty-eight hours later, the cells were labeled with 300 μCi of [^{35}S]methionine for 50 min. Protein extracts were prepared, precleared with a nonspecific mouse myeloma ascites fluid (P3N3), and immunoprecipitated with PAb901 (αT), which recognizes an epitope in the C terminus of T antigen (amino acids 684 to 698), or with P3N3 (αP3N3). The precipitated proteins were subjected to SDS–10% PAGE and visualized by autoradiography. The designations across the top indicate the T antigen expected in the extract. The boxes indicate whether the extract was immunoprecipitated (immppt) (+) or not (empty box) with the negative control antibody (αP3N3) or PAb901 (αT). The numbers to the right indicate the positions of the molecular weight markers (in thousands).

was low, it was reasonable to question whether the presence of small t antigen would augment formation of dense foci in y2-plus-*ras* cooperative transformation assays. Therefore, REF cultures were transfected with the three plasmids y2, *ras*, and p*dl*536 (a plasmid which encodes only small t antigen) (Table 1, experiments 4 and 5). As controls, other cultures were transfected with the plasmids p*dl*536 and y2 (experiment 5), with a plasmid that encodes only large T antigen (p*dl*2005) plus the *ras* plasmid (experiments 2, 4, and 5), or with the combination of plasmids p*dl*536, *ras*, and p*dl*2005 (experiment 5). The presence of small t antigen did not consistently enhance cotransformation by either full-length T antigen or T176-708. Thus, either the amount of T176-708 that accumulated in the transfected cells was not limiting for *ras* cooperation or small t antigen cannot enhance transformation by large T antigen in this type of assay.

Expression of T-antigen fusion proteins. In order to determine the N-terminal limit of the centrally located *ras* cooperation activity of T antigen, a series of DNA constructs expected to produce T antigens missing increasing lengths of the N terminus was generated. The constructs were designed to produce fusion proteins that utilize the start codon of β-galactosidase to produce a polypeptide containing the first 7 amino acids of β-galactosidase followed by either 31 or 34 amino acids from the synthetic polylinker in the plasmid BluescriptII SK$^+$ and 1 or 2 amino acids from an *Eco*RI linker fused to defined segments of T antigen (28).

To determine whether the T-antigen fusion constructs could produce proteins of the expected sizes, TC7 cells were transfected with each construct, and proteins were labeled 48 h later with [^{35}S]methionine. The proteins were then extracted and immunoprecipitated with PAb901. The results are shown in Fig. 2. T antigens of the expected sizes were detected in extracts of cells transfected with constructs encoding T251-708, T301-708, T337-708, T351-708, T371-708, T401-708, T601-708, and T651-708. The intensity of the T251-708 band was similar to that of the wild-type T-antigen band; however, the intensities of all other fusion protein bands were substantially less. No product was detected in extracts of cells transfected with constructs encoding T451-708, T501-708, or T551-708. The lower intensity or absence of some of the fusion protein bands raised the possibility that the truncated proteins were sufficiently unstable that the labeled products turned over rapidly within the labeling period.

The steady-state level of T antigen in cells transiently transfected with each construct was investigated. The T antigens in extracts of transfected cells were immunoprecipitated with a rabbit anti-T-antigen polyclonal antibody (anti-D2), resolved by polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidene difluoride paper. The protein blot then was probed with PAb901. Figure 3 shows the results. Approximately wild-type levels of T251-708 and T351-708 (Fig. 3A) were detected. The less-abundant products T301-708 and T337-708 also were detected readily (Fig. 3A). In a longer exposure, T371-708, T401-708, and T601-708 could be seen as faint bands (Fig. 3B). Presumably, differences in the abundances of T-antigen polypeptides detected by radiolabeling and by Western blot analyses reflect differences in protein stability. Separately, TC7 cells were transfected with the y2 plasmid, and the steady-state level of T176-708 was examined 48 h later. The results in Fig. 3C show that remarkably little T176-708 protein accumulated. Similar substantive differences in the relative levels of accumulated wild-type T antigen and T176-708 were observed in multiple transient-transfection experiments.

Although it remains possible that the amount of T176-708 visualized by Western blot analysis does not reflect the actual amount of the protein present, this prospect seems unlikely. Because of the polyclonal nature of the rabbit anti-D2 antibody used in the Western blot analysis and its ability to recognize more heavily truncated T-antigen polypeptides (Fig. 3), the possibility that it does not recognize T176-708 efficiently is remote. An additional indication that the intensity of the T176-708 band in Western blots reflects the steady-state level of the protein and not the ability of the polyclonal antibody to im-

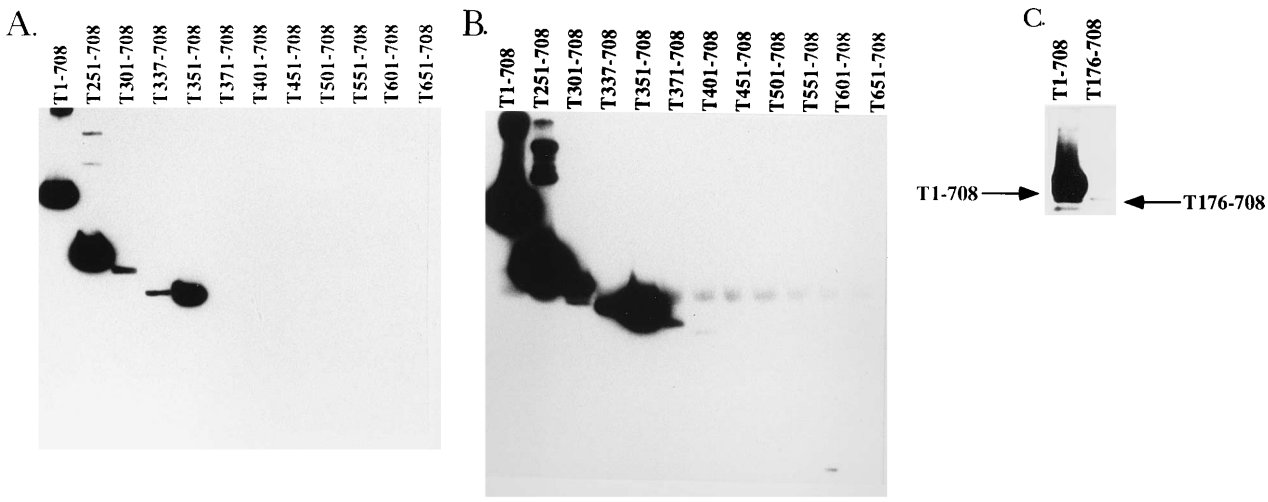

FIG. 3. Steady-state levels of N-terminally truncated T antigens. TC7 cells were transfected with pPVU0 or the fusion protein constructs (CAV251-708, CAV301-708, CAV337-708, CAV351-708, CAV371-708, CAV401-708, CAV451-708, CAV501-708, CAV551-708, CAV601-708, and CAV651-708) (A and B) or with pPVU0 or y2 (C). Forty-eight hours later, cell extracts were prepared. Equivalent amounts of protein were immunoprecipitated with the anti-T-antigen polyclonal antibody anti-D2. The precipitated proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride paper. The blot was probed with PAb901 followed by a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G secondary antibody. Proteins were detected with the ECL chemiluminescence system (Amersham). Panels A and B show different exposures of the same blot (20 min [A] and 2 h [B]).

munoprecipitate it is the observation that the T176-708 polypeptide could not be detected by metabolic labeling (data not shown; also see Fig. 5A) following transient expression, although other truncated proteins were detected without difficulty (Fig. 3). The PAb901 antibody, which was used to immunoprecipitate radiolabeled T antigens, recognizes a denaturation-resistant epitope in the carboxy terminus of the protein. Its recognition is unlikely to be influenced by alterations in protein conformation. This belief is strengthened by recent findings that the PAb901 epitope can be translocated within T antigen (8) or transferred to a heterologous protein (17a) and remain functional.

Ability of T-antigen fusion proteins to cooperate with *ras* to transform REF. Even though some of the fusion protein constructs produced or led to the accumulation of very little or no detectable protein, all were initially tested in *ras* cooperation assays. The steady-state level of T antigen that must be attained to initiate *ras* cooperation is not known. However, a comparison of the levels of wild-type T antigen and T176-708 (Fig. 3C) following transient transfection strongly suggests that very little T antigen is needed to initiate transformation of REF in cooperation with an activated *ras* oncogene (Table 1). In three attempts (data not shown) none of the fusion proteins produced dense foci when transfected alone (in the absence of an activated *ras* oncogene). Table 2 shows the results of two representative assays of cooperation between T-antigen fusion proteins and *ras*. The T251-708 polypeptide cooperated with *ras* to transform REF (a level of 0.31 to 0.52 relative to the wild-type level; also see Tables 3 and 4). None of the fusion proteins T301-708, T337-708, T351-708, and T371-708 (Table 2) and none of the fusion proteins initiating T antigen at or beyond amino acid 401 (data not shown) cooperated with *ras* to produce dense foci. Cotransfection of a construct encoding small t antigen along with *ras* and the fusion protein constructs did not appreciably alter the numbers of dense foci produced by T251-708 or result in focus formation by any of the other fusion protein constructs (data not shown; also see Table 4). Thus, the truncation of T antigen from the N terminus to amino acid 301 (or further) eliminates its capacity to cooperate with *ras*.

Dense foci were picked from flasks that received the *ras* and CAV251-708 plasmids and were expanded into cell lines. Western blot analysis revealed levels of T251-708 in two independent clonally derived cell lines in excess of the level accumulated in cells transformed by full-length T antigen and *ras* (data not shown).

Relationship between *ras* cooperation and p53 binding. We (61) and others (71) showed previously that immortalization of primary B6MEF cells is closely correlated with the ability of T antigen to bind p53. Although *ras* cooperation presumably identifies immortalization activities, the mechanisms involved in independent immortalization of B6MEF and *ras* cooperation in REF need not be the same. Wild-type T antigen may immortalize by binding and sequestering cellular p53; however, in cooperation with the *ras* oncogene, p53 binding may not be essential. Clearly, the T1-147 polypeptide (Table 1) and adenovirus E1A (46), neither of which binds p53, cooperate with *ras* to transform REF. Therefore, it was relevant to determine

TABLE 2. Cooperative transformation between T-antigen fusion proteins and an activated *ras* oncogene

DNA	T antigen(s)[a]	No. dense foci per flask[b] in expt:	
		1	2[c]
Carrier		ND[d]	0, 0 (<0.007)
ras		1.0, 0 (0.007)	2, 1 (0.02)
pPVU0 + *ras*	T1-708, t	ND	ND
p*dl*2005 + *ras*	T1-708	70, 78 (1.0)	83, 68 (1.0)
CAV251-708 + *ras*	T251-708	35, 42 (0.52)	14, 33 (0.31)
CAV301-708 + *ras*	T301-708	0, 0 (<0.007)	1, 1 (0.013)
CAV337-708 + *ras*	T337-708	0, 0	2, 2 (0.026)
CAV351-708 + *ras*	T351-708	0, 0	4, 3 (0.046)
CAV371-708 + *ras*	T371-708	0, 0	0, 0

[a] t, small t antigen-coding sequence present.
[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 with the wild-type T-antigen plasmid (pPVU0 or p*dl*2005) used as a positive control.
[c] The p*dl*536 plasmid, which produces small t antigen, was added to each flask in addition to the plasmids expressing *ras* and truncated T antigens.
[d] ND, not determined.

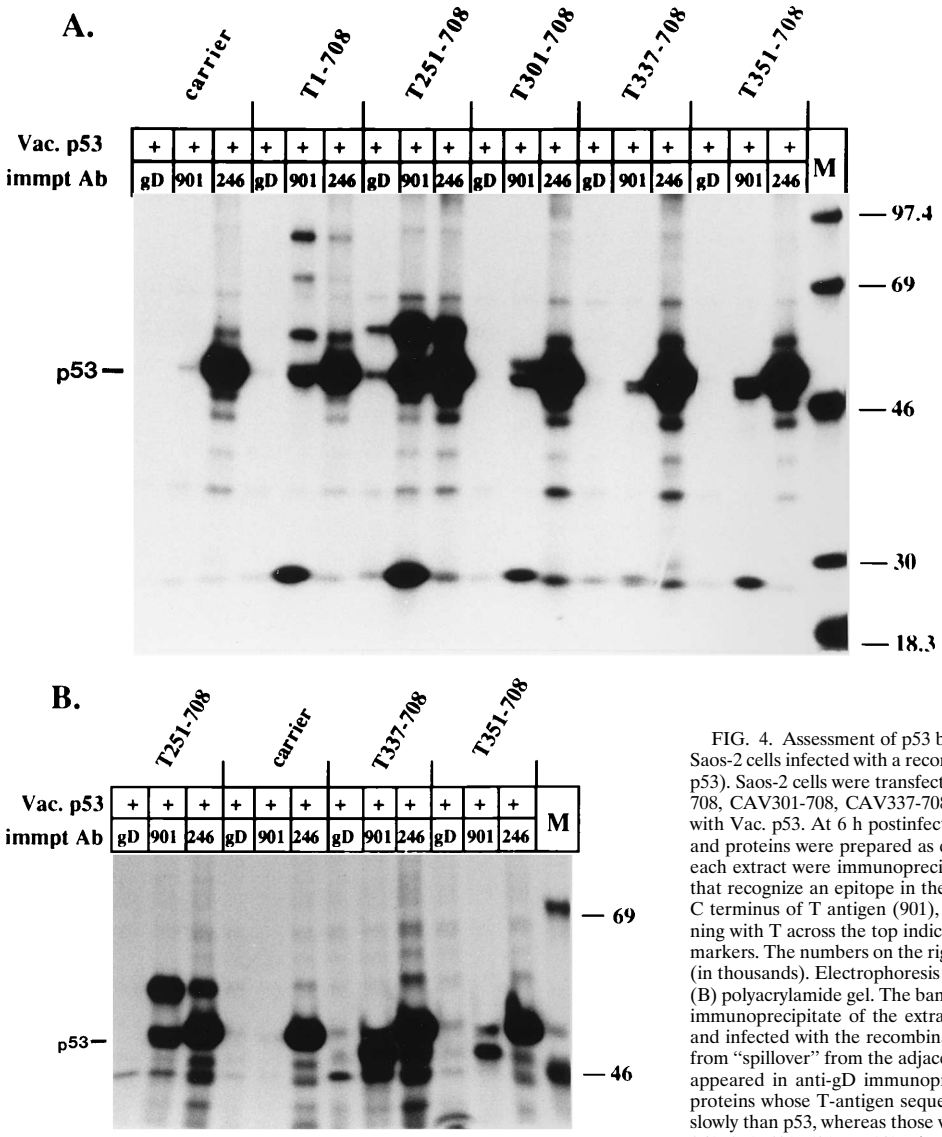

FIG. 4. Assessment of p53 binding by T-antigen fusion proteins expressed in Saos-2 cells infected with a recombinant vaccinia virus encoding mouse p53 (Vac. p53). Saos-2 cells were transfected with carrier DNA or with construct CAV251-708, CAV301-708, CAV337-708, or CAV351-708 and 48 h later were infected with Vac. p53. At 6 h postinfection the cells were labeled with [^{35}S]methionine, and proteins were prepared as described in Materials and Methods. Aliquots of each extract were immunoprecipitated with monoclonal antibodies (immpt Ab) that recognize an epitope in the herpes simplex virus glycoprotein gD (gD), the C terminus of T antigen (901), or wild-type p53 (246). The designations beginning with T across the top indicate the T antigen expected. M, molecular weight markers. The numbers on the right indicate the molecular weights of the markers (in thousands). Electrophoresis was for 16 h at 35 V through a 10% (A) or 7.5% (B) polyacrylamide gel. The bands that appear in the lane containing the anti-gD immunoprecipitate of the extract from cells transfected with CAV251-708 (A) and infected with the recombinant vaccinia virus are presumed to have resulted from "spillover" from the adjacent lane during loading of the gel. No such bands appeared in anti-gD immunoprecipitates in repeated experiments. The fusion proteins whose T-antigen sequences begin at position 251 or 300 migrate more slowly than p53, whereas those whose T-antigen sequences begin at position 337, 351, 371, 401, 601, or 651 migrate more rapidly than p53.

whether the capacities of truncated T antigens to bind p53 correlated with their abilities to cooperate with *ras*. Accordingly, p53-deficient Saos-2 cells were transfected with plasmids expressing T1-708, T176-708, T251-708, T301-708, T337-708, T351-708, T371-708, T401-708, T601-708, or T651-708 and 48 h later were infected with a recombinant vaccinia virus that encodes a phenotypically wild-type mouse p53 protein, as described in Materials and Methods. Six hours later the infected cells were labeled with [^{35}S]methionine for 1 h. Cell extracts were then prepared and immunoprecipitated with the negative control antibody anti-gD, the anti-T-antigen antibody PAb901, or the anti-p53 antibody PAb246.

The results (Fig. 4) show that an equivalent amount of mouse p53 was synthesized in all of the transfected and subsequently infected cultures. In each case a band of radioactivity appeared in the expected position for p53 in the lanes containing the proteins immunoprecipitated by PAb246. No such protein was immunoprecipitated by anti-gD antibody, which was used as a negative control. In PAb901 immunoprecipitates of cells transfected with a construct producing T1-708, bands at the positions expected for full-length T antigen (92 kDa) and for p53 were observed (Fig. 4A). These same bands were observed in lanes containing PAb246 immunoprecipitates of the same extracts, indicating that the two proteins were physically associated. The ability of the mouse p53 to physically associate with T antigen and to be immunoprecipitated by PAb246 confirmed that the p53 encoded by the recombinant vaccinia virus was phenotypically wild type in its T-antigen-binding capacity and protein conformation. Separately we showed that the mouse p53 was not immunoprecipitated by PAb240, which recognizes native mutant but not native wild-type p53 (data not shown). PAb901 immunoprecipitated both p53 and T antigens that migrated appropriately for their expected sizes from extracts of the cultures transfected with constructs producing T251-708 (61 kDa), T301-708 (57 kDa), T337-708 (50 kDa), and T351-708 (48 kDa). These results indicated that each of the T-antigen fusion proteins was capable of binding p53 from cells expressing large amounts of mouse wild-type p53. Since the positions of the T301-708, T337-708, and T351-708 fusion proteins did not differ greatly

from that of p53, the large band of p53 undoubtedly obscured their detection in lanes containing proteins immunoprecipitated by PAb246.

To confirm the conclusion that T337-708 and T351-708 bound p53, a second experiment was performed in which the percentage of acrylamide in the polyacrylamide gel was lowered to increase separation of the proteins. The results appear in Fig. 4B. In the lanes containing the PAb901 immunoprecipitates of extracts from cells transfected with CAV251-708, CAV337-708, and CAV351-708, the fusion proteins were clearly resolved from the p53 bands. Thus, the association between T337-708 and T351-708 antigens and p53 was confirmed.

In each instance of transfection with a T-antigen construct and infection by the vaccinia virus recombinant, several additional bands consistently appeared in lanes containing PAb901 immunoprecipitates (Fig. 4A and Fig. 5). Two possibilities as to their origin were considered: they were either T-antigen breakdown products or vaccinia virus proteins containing a PAb901-cross-reactive epitope.

The results in Fig. 5A address these possibilities. In the lane containing PAb901 immunoprecipitates of extracts from cells transfected with CAV1-708 and infected with wild-type vaccinia virus, the expected full-length T-antigen band (92 kDa) and a p53 band, as well as three other bands of approximately 77, 61, and 24 kDa, were observed. The absence of the 77-, 61-, and 24-kDa bands in PAb901 immunoprecipitates from extracts of cells transfected with carrier DNA and then infected with wild-type vaccinia virus showed that these bands were not vaccinia virus products that contain a PAb901-cross-reactive epitope. While experiments to directly address the origin of the low-molecular-weight proteins were not performed, it seems likely that they represent C-terminal fragments of T antigen produced in the vaccinia virus-infected Saos-2 cells, since they were immunoprecipitated by a monoclonal antibody (PAb901) that recognizes an epitope in the extreme C terminus of T antigen. A protein of approximately 46 kDa was immunoprecipitated by gD, PAb901, or PAb246 from extracts of cells transfected with carrier DNA and then infected with wild-type vaccinia virus. This nonspecifically immunoprecipitated protein also was observed often in cells infected with the vaccinia virus-mouse p53 recombinant.

The analysis of p53 binding by mutant T antigens was extended to include T176-708, T371-708, T401-708, T601-708, and T651-708. The results are shown in Fig. 5. The T176-708 polypeptide was not observed (Fig. 5A). This result is consistent with the low level of T176-708 observed after transient transfection (Fig. 3C). Although the T401-708, T601-708, and T651-708 proteins were detected readily (Fig. 5C), no p53 binding was detected. On a longer exposure of the gel, T371-708 was easily seen; however, there was no evidence of p53 binding (data not shown).

The absence of *ras* cooperation and retention of p53-binding capacity by the T301-708, T337-708, and T351-708 polypeptides suggested at least three possibilities. First, these polypeptides may accumulate to levels that are insufficient to initiate or maintain *ras* cooperation. Since the steady-state levels of T301-708, T337-708, and T371-708 were lower than that of T251-708, this possibility could not be ruled out. It seems unlikely, however, that the lower levels of T301-708, T337-708, and T371-708 relative to T251-708 in transfected cells are sufficient to explain lack of *ras* cooperation, since remarkably low levels of protein following transfection are needed to establish dense foci in cooperation with *ras* (Table 1 and Fig. 3C). Second, p53 binding may not be needed for *ras* cooperation by T251-708. Binding of p53 is not uniformly required for *ras* cooperation. For instance, a nonoverlapping portion of T antigen (T1-147) that cannot bind p53 (see Table 1) and several other oncogene products devoid of p53-binding capacity cooperate with *ras* to transform rodent cells. Third, p53 binding may be necessary but not sufficient for T251-708 to cooperate with *ras*.

Inability of a T251-708 polypeptide containing a lesion that prevents p53 binding to cooperate with *ras* to transform REF. To investigate further the relationship between T-antigen–p53 binding and *ras* cooperation, mutations were introduced into the T251-708-coding sequence. Constructs encoding the T251-708 polypeptide containing a deletion of amino acids 434 to 444 or of amino acid 400 were generated. We showed previously (28) that in the context of the entire protein, the deletion of T-antigen amino acids 434 to 444 abrogated binding of rodent p53 and immortalization of mouse fibroblasts, whereas the replacement of amino acid 400 by the sequence Arg Ile Arg did not. The deletions were introduced into the T251-708-coding sequence by DNA fragment exchange between CAV251-708 and the T-antigen construct *dl*433-444 or *dl*400 (28). In the context of the whole protein, neither mutation compromised the steady-state level of T antigen (28). To assess the steady-state level of the T251-708*dl*434-444 or T251-708*dl*400 polypeptide, TC7 cells were transfected with the corresponding plasmids and the accumulated levels of the T antigens were assessed by Western blot analysis. The results (Fig. 6) showed that although the levels of T251-708*dl*434-444 and T251-708*dl*400 were somewhat decreased relative to the level of T251-708, they nonetheless were equivalent to the level observed in cells transfected with a wild-type T-antigen-encoding plasmid.

The p53-binding capacities of the resulting proteins are shown in Fig. 5B. As expected, p53 binding by T251-708*dl*434-444 was diminished substantially, whereas p53 binding by T251-708*dl*400 was not diminished.

To confirm the expectation that deletion of amino acids 433 to 444 from T251-708 would prevent functional inactivation of p53, T251-708 and T251-708*dl*433-444 were tested for the ability to cooperate with adenovirus E1A in transforming REF. The adenovirus E1A gene region when introduced alone into primary rodent cell cultures induces formation of foci. However, proliferation is not sustained, and the colonies regress with time (41). Cell death results from E1A induction of a p53-dependent apoptosis pathway (31, 41). Cotransfection of cells with adenovirus E1B 55-kDa protein, E1B 19-kDa protein, or a mutant p53 gene of the dominant-negative class blocks induction of this pathway by binding and functionally inactivating wild-type p53 (9). If the T251-708–p53 complexes inactivate p53 function, then T251-708 should cooperate with E1A to transform REF whereas T251-708*dl*433-444 should not. The results of such a test appear in Table 3. The T251-708 polypeptide cooperated efficiently with E1A (a level of 1.3 to 1.4 relative to that of the E1A-plus-*ras* control in experiments 1 and 2; a level of 0.43 relative to that of the E1A-plus-p*dl*2005 control in experiment 3), indicating that p53 lost wild-type function when complexed with T251-708. In contrast, T251-708*dl*433-444 did not cooperate with E1A. As expected, T1-147, which is incapable of binding p53, did not cooperate with E1A.

The effect of abrogation of p53 binding on *ras* cooperation in a dense-focus assay was assessed by utilizing the T251-708*dl*433-444 and T251-708*dl*400 constructs. The results are shown in Table 4. Wild-type T antigen with or without small t antigen efficiently cooperated with *ras* to produce dense foci. The T251-708 polypeptide also cooperated efficiently (a level of 0.76 to 1.97 relative to that of full-length T antigen plus *ras*). Deletion of amino acids 434 to 444 reduced the number of foci

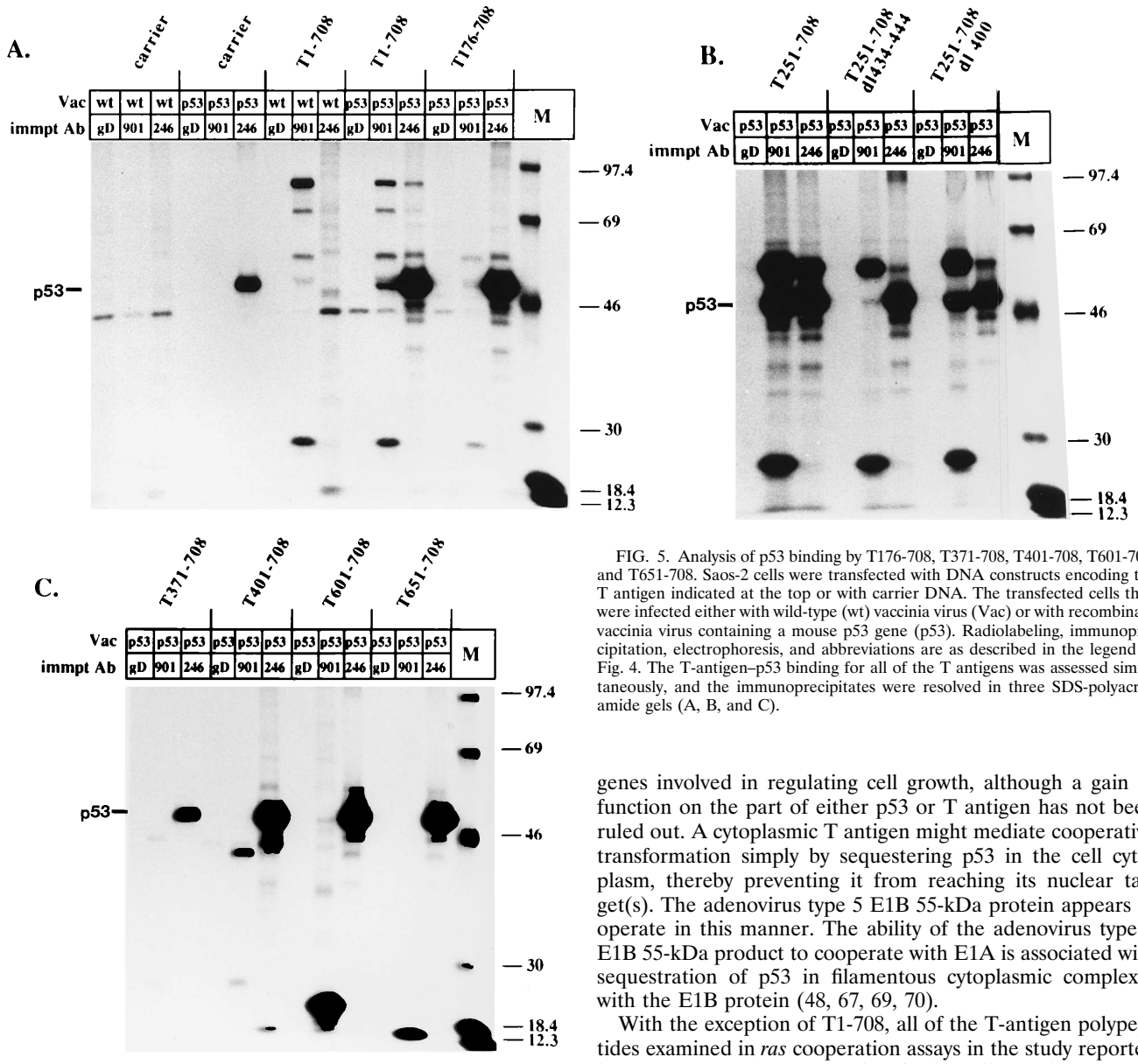

FIG. 5. Analysis of p53 binding by T176-708, T371-708, T401-708, T601-708, and T651-708. Saos-2 cells were transfected with DNA constructs encoding the T antigen indicated at the top or with carrier DNA. The transfected cells then were infected either with wild-type (wt) vaccinia virus (Vac) or with recombinant vaccinia virus containing a mouse p53 gene (p53). Radiolabeling, immunoprecipitation, electrophoresis, and abbreviations are as described in the legend to Fig. 4. The T-antigen–p53 binding for all of the T antigens was assessed simultaneously, and the immunoprecipitates were resolved in three SDS-polyacrylamide gels (A, B, and C).

to a level of 0.006 to 0.047 relative to the wild-type value. Cointroduction of a plasmid encoding small t antigen did not consistently increase the number of foci produced by wild-type T antigen, T251-708, or T251-708*dl*434-444. These results indicated that the ability of the T251-708 polypeptide to cooperate with *ras* was lost coordinately with p53 binding as a result of the mutation. T251-708 containing a replacement of amino acid 400 with the sequence Arg Ile Arg, although retaining p53-binding capacity, did not cooperate with *ras* to transform REF.

Cooperation with *ras* occurs independently of T251-708 subcellular location. The observation that T251-708 cooperated with *ras* and E1A was consistent with the ability of the T-antigen polypeptide to accumulate to large amounts and bind p53 efficiently within transfected cells. In cells transformed by wild-type T antigen, T-antigen–p53 complexes accumulate in the cell nucleus (36) and presumably prevent p53 from transactivating (14, 25, 42) or repressing (32, 49) specific cellular genes involved in regulating cell growth, although a gain of function on the part of either p53 or T antigen has not been ruled out. A cytoplasmic T antigen might mediate cooperative transformation simply by sequestering p53 in the cell cytoplasm, thereby preventing it from reaching its nuclear target(s). The adenovirus type 5 E1B 55-kDa protein appears to operate in this manner. The ability of the adenovirus type 5 E1B 55-kDa product to cooperate with E1A is associated with sequestration of p53 in filamentous cytoplasmic complexes with the E1B protein (48, 67, 69, 70).

With the exception of T1-708, all of the T-antigen polypeptides examined in *ras* cooperation assays in the study reported here are expected to accumulate in the cell cytoplasm. It was important, therefore, to rule out the possibility that retention of p53 in the cytoplasm by binding to a cytoplasmic T251-708 polypeptide, a property that would not be shared with wild-type T antigen, was responsible for the ability of the truncated protein to cooperate with *ras*. If the sole mechanism of the ability of T251-708 to cooperate with *ras* was retention of p53 in the cytoplasm, then the addition of a nuclear localization signal to the T251-708 polypeptide would prevent transformation with an activated *ras* oncogene.

To address this possibility, the SV40 nuclear localization signal was inserted into T251-708. Specifically, a double-stranded oligonucleotide encoding the T-antigen nuclear localization signal contained within amino acids 128 to 132 (Lys Lys Arg Lys Val) flanked by *Eco*RI-compatible single-stranded extensions was inserted in frame at the *Eco*RI linker immediately preceding amino acid 251. The authenticities of the inserted nucleotides and maintenance of the T-antigen reading frame were confirmed by DNA sequencing. Immunofluorescence assays showed that T251-708 accumulated in the cyto-

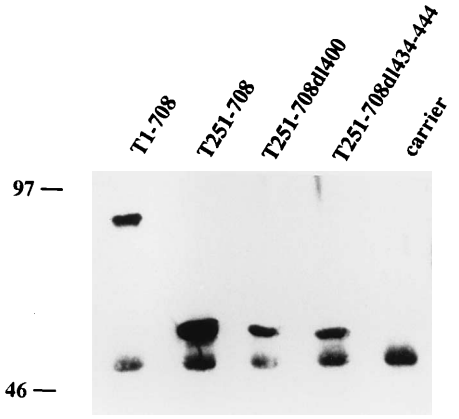

FIG. 6. Steady-state levels of T251-708*dl*400 and T251-708*dl*434-444 in transiently transfected cells. TC7 cells were transfected with plasmids producing either T1-708, T251-708, T251-708*dl*400, or T251-708*dl*434-444 or with carrier DNA. At 48 h posttransfection, cell extracts were prepared and T antigens were immunoprecipitated by an anti-T-antigen polyclonal antibody. Western blot analysis was performed with PAb901 as a probe. The lower band present in all lanes is immunoglobulin G. Numbers at left are molecular weights (in thousands).

plasm. Very little, if any, protein was observed in the nucleus. In contrast, the T251-708 polypeptide containing the nuclear localization signal (T251-708NL) accumulated in the nucleus of transfected cells. Little, if any, T antigen was detected in the cell cytoplasm (data not shown). The results in Table 4 (experiments 3 and 4) show that approximately equal numbers of dense foci were produced when plasmids expressing T251-708 or T251-708NL were cotransfected with the *ras*-expressing plasmid. Thus, the ability of T251-708 to cooperate with *ras* did not depend on sequesteration of p53 in the cytoplasm.

The observation that the T301-708, T337-708, and T351-708 polypeptides, all of which bound p53, did not cooperate with *ras* also indicated that cytoplasmic localization was not sufficient for that transforming activity. This result is in agreement with the findings of van der Heuvel et al. (67). Those investigators showed that retention of p53 in the cytoplasm by the adenovirus type 5 E1B 55-kDa protein also was not sufficient for *ras* cooperation (67).

DISCUSSION

The results presented here demonstrate the existence of two independent regions in T antigen that are sufficient to transform primary cells in cooperation with an activated *ras* oncogene. Both an N-terminal T-antigen segment (T1-147) and a segment devoid of the N terminus (T251-708) cooperated with an activated *ras* oncogene to produce dense foci on monolayers of REF. These foci were readily expanded into cell lines, confirming the retention of immortalizing activity. Both the T1-147- and T251-708-expressing constructs were tested at the same time in multiple *ras* cooperation assays (Table 1) with three independent batches of REF; thus, the conclusion that T antigen contains two separate and independent *ras* cooperation activities is firm.

The duplication of activities within T antigen is not unprecedented. For instance, independent regions of T antigen bind the cellular DNA polymerase α primase (13, 19, 20, 51). Similarly, four regions of T antigen, each of which appears to act independently, cooperate in stimulating cellular DNA synthesis (11). Finally, T-antigen mutants devoid of the N-terminal sequences known to contain a transcriptional transactivator (57) still exhibit transactivating potential (21). Thus, there is clear precedent for duplication of function in T antigen.

The finding of independent *ras* cooperation functions, however, was somewhat unexpected. Previously, others showed that mutant T antigens that cannot bind Rb or complement p300-binding-negative E1A mutants could not transform cells in cooperation with *ras* (35, 68). Both Rb binding and p300 binding also have been implicated in the ability of T antigen to overcome the antiproliferative effects of wild-type p53 in cells cotransformed by an activated *ras* oncogene and a temperature-sensitive p53 gene (35). Yet, most of the mutant T antigens used in those investigations retained the segment consisting of amino acids 251 to 708. Thus, cooperation with *ras* by the activity contained therein would have been expected. Two resolutions to this paradox can be envisioned. It is possible that the deletion of amino acids 17 to 27, which abrogates complementation of E1A p300-binding mutants, or the specific mutations in the Rb-binding site used distort a portion of the region consisting of amino acids 251 to 708 or prevent it from attaining a functional conformation needed for *ras* cooperation. Alternatively, removal of the N-terminal 175 or 250 amino acids may unmask a *ras* cooperation activity that is silent

TABLE 3. A fragment of T antigen containing amino acids 251 to 708 cooperates with adenovirus E1A to transform REF

DNA	T antigen	No. of dense foci per flask[a] in expt:		
		1	2	3
Carrier		0, 0 (<0.029)	1, 0 (0.009)	0, 0 (<0.007)
ras		1, 3 (0.114)	1, 0 (0.009)	ND[b]
E1A		0, 3 (0.086)	6, 9 (0.14)	3, 4 (0.046)
E1A + *ras*		13, 22 (1.0)	60, 46 (1.0)	ND
E1A + CAV251-708	T251-708	27, 22 (1.4)	76, 63 (1.3)	32, 33 (0.43)
E1A + CAV251-708*dl*433-444	T251-708*dl*434-444	2, 3 (0.14)	2, 4 (0.057)	ND
CAV251-708 + *ras*	T251-708*dl*434-444	30, 19 (1.4)	25, 39 (0.60)	ND
CAV251-708*dl*434-444 + *ras*	T251-708*dl*434-444	0, 0 (<0.029)	2, 3 (0.047)	ND
E1A + pT147NS	T1-147	4, 0 (0.114)	ND	3, 3 (0.039)
E1A + p*dl*2005	T1-708	ND	ND	81, 72 (1.0)

[a] 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 E1A-plus-*ras* (experiments 1 and 2) or T1-708-plus-E1A (experiment 3) DNA used as a positive control. Colonies that developed in flasks receiving E1A alone or in combination with a truncated T antigen had E1A morphology, while those that received *ras* had *ras* morphology.

[b] ND, not determined.

TABLE 4. Removal of amino acids 433 to 444 prevents T251-708 from cooperating with *ras* to transform REF

DNA	T antigen(s)[a]	No. of dense foci per flask[b] in expt:			
		1	2	3	4
Carrier		5	0, 0	0, 0	1.0 (0.012)
ras		3	0, 0	1, 0 (0.006)	9, 6 (0.18)
p*dl*2005 + *ras*	T1-708	ND[c]	126, 128 (1.32)	93, 82 (1.0)	39, 45 (1.0)
p*dl*2005 + *ras* + p*dl*536	T1-708, t	206, 208 (1.0)	72, 120 (1.0)	ND	ND
CAV251-708 + *ras*	T251-708	66, 72 (0.33)	19, 24 (0.22)	67, 66 (0.76)	74, 92 (1.97)
CAV251-708 + *ras* + p*dl*536	T251-708, t	72, 67 (0.33)	37, 20 (0.30)	ND	ND
CAV251-708*dl*434-444 + *ras*	T251-708*dl*434-444	2, 3 (0.012)	4, 5 (0.047)[d]	0, 1 (0.006)	ND
CAV251-708*dl*434-444 + *ras* + p*dl*536	T251-708*dl*434-444, t	0, 1 (0.002)	5, 7 (0.063)[d]	ND	ND
CAV251-708*dl*400 + *ras*	T251-708*dl*400	ND	ND	0, 3 (0.017)	11, 6 (0.20)
CAV251-708NL[e] + *ras*	T251-708NL	ND	ND	48, 51 (0.57)	63, 84 (1.73)

[a] t, small t antigen-coding sequence present.

[b] The first two numbers are the numbers of colonies from duplicate flasks counted at 10 days posttransfection in each experiment. The number in parentheses is the level relative to that of p*dl*2005 + *ras* + p*dl*536 (experiments 1 and 2) or p*dl*2005 + *ras* (experiments 3 and 4) used as a positive control.

[c] ND, not determined.

[d] The cultures transfected with CAV251-708*dl*433-444 plus *ras* and those transfected with CAV251-708*dl*433-444 plus *ras* plus p*dl*536 were held for 2 weeks beyond the time that the colonies in the other transfected cultures were counted in order to determine whether slowly developing colonies would appear. The numbers of colonies reported are those counted after the prolonged incubation (16 to 20 days). With prolonged incubation similar numbers of colonies developed in cultures transfected with carrier DNA or with *ras* alone.

[e] NL indicates that the SV40 nuclear localization signal (Lys Lys Arg Lys Val) was added preceding T-antigen amino acid 251 by utilizing an *Eco*RI linker in that position.

in the context of the whole protein. Further analyses will be needed to distinguish between these possibilities.

By virtue of the functions mapped within the two T-antigen regions that cooperate with *ras*, the duplicate *ras* cooperation activities may operate through different mechanisms. A logical starting point for investigating the role of T-antigen activities within the T251-708 polypeptide that are required for *ras* cooperation was p53 binding. Careful consideration of the test system to be utilized was needed. T antigen stabilizes p53 by two mechanisms. Binding of p53 to T antigen extends the half-life of the cellular protein from approximately 20 min (43) to approximately 22 to 24 h (39). In addition, T antigen can induce cellular processes that stabilize p53 in the absence of T-antigen–p53 binding (10, 63). The location of the function(s) required for stabilization of p53 in the absence of T-antigen binding is not known. Thus, failure of a truncated T antigen to bind p53 could either be due to a defect in or distortion of the p53-binding region or, alternatively, might reflect the absence of a p53 stabilization function needed to increase the p53 level to a point at which binding could be demonstrated.

In order to achieve a high and consistent level of p53 expression in cells transfected with T-antigen mutants, a recombinant vaccinia virus capable of expressing phenotypically wild-type mouse p53 was used. Overexpression of p53 in cells by infecting them with the vaccinia virus recombinant encoding mouse p53 provided the means of ensuring that, independent of the T-antigen construct transfected, all cells expressed roughly equivalent amounts of p53. The use of this system also was justified on a second basis. Others (30) have shown that T-antigen mutants with decreased binding capacity for mouse p53 retained immortalizing activity. We reasoned that by investigating p53 binding in cells overexpressing this tumor suppressor product, even weak interactions might be observed. The results indicated that T antigens initiated at residue 251, 301, 337, or 351 retained the ability to bind mouse p53, at least when p53 was overexpressed. In contrast, T antigens initiated at amino acid 401 or beyond did not bind p53. It should be pointed out that this assay determines only the capacity of a T antigen to form a physical complex with p53. Whether the complexed p53 is functionally inactivated cannot be assessed in a system designed for overexpression of wild-type p53.

The finding that removal of amino acids 1 to 250, 1 to 300, 1 to 336, or 1 to 350 did not abrogate p53 binding was consistent with our previous findings. We showed earlier that T antigens with internal deletions of amino acids spanning positions 252 to 350, in the context of an otherwise unaltered T antigen, bound p53 effectively (28). The current finding that amino acids preceding position 351 could be removed entirely suggested strongly that if p53 binding depended upon a conformationally determined site, amino acids between positions 1 and 350 were not needed to maintain that site.

In *ras* cooperation assays, only the T176-708 and T251-708 polypeptides were able to produce dense foci. The introduction into the T251-708-coding sequence of a deletion that abrogated p53 binding prevented *ras* cooperation. This result suggested two possibilities. First, p53 binding may be required for cooperation with *ras*. Such a conclusion would be consistent with the ability of *ras* to cooperate with dominant-negative p53 mutants in transformation of REF (reference 15 and references therein). Second, there may be an activity other than or in addition to p53 binding involved in *ras* cooperation, and that activity may be lost coordinately with p53 binding when the *dl*433-444 or *dl*400 mutation is introduced.

The remarkably low level of mutant T antigen needed for *ras* cooperation strongly suggests that stable T-antigen–p53 complex formation (sequestering p53) is not needed for the central region of T antigen to cooperate with *ras*. The T176-708 polypeptide, which efficiently cooperated with *ras* in cooperative transformation assays, accumulated to very low levels relative to that of wild-type T antigen following transient transfection (Fig. 3C) and upon stable expression in cooperatively transformed cell lines (Fig. 1A). Since T176-708 could bind only a small fraction of the cellular p53 at any one time, it seems unlikely that its ability to cooperate with *ras* depends on its ability to bind p53 stably.

Clearly, if p53 binding was required for *ras* cooperation, then it could not be sufficient to confer that activity. This conclusion is based on two observations. First, the T301-708, T337-708, and T351-708 polypeptides retained the ability to bind p53, yet they did not cooperate with *ras*. Second, the T251-708*dl*400 protein, which retained the ability to bind p53, did not cooperate with *ras*. These findings imply the existence of an activity

that depends upon the integrity of the amino acid sequence between positions 252 and 300 and at position 400 and functions either independently of or in concert with p53 during cotransformation with the *ras* oncogene.

Rigorous proof as to whether p53 binding is involved in cooperation between T251-708 and *ras* will require a more extensive analysis of mutations introduced into the T251-708 polypeptide. Structural distortions of adjacent or even distant regions of T antigen when T-antigen activities are lost coincidentally in mutant proteins cannot be ruled out. Thus, it will be necessary to identify a mutation that abrogates p53 binding without reducing the ability of the T-antigen polypeptide to cooperate with *ras* in order to conclude with confidence that p53 binding is not required.

It seems likely that immortalization and *ras* cooperation by T antigen are related but not identical processes. N-terminal segments of T antigen have been reported to immortalize REF independently of a cooperating oncogene (54). Sompayrac and Danna (54) showed that T1-147 was sufficient to immortalize REF. Similarly, Asselin and Bastin (2) showed that an N-terminal fragment containing only the first 138 amino acids of T antigen immortalized REF. In both of those instances, the T-antigen-coding segment was introduced into cells along with a plasmid conferring resistance to G418, and selection for antibiotic resistance was applied. Thus, immortalization was the sole altered growth property selected. That same T-antigen segment, T1-147, was sufficient to cooperate with *ras*. Similarly, using an assay that selected only for immortalization, we showed previously that two segments encompassing amino acids 351 to 450 and 533 to 626 were essential for retention of immortalization (28), while N-terminal amino acids 1 to 126, 127 to 250, 251 to 300, or 301 to 350 could be deleted without compromising that activity. In that analysis, retention of p53 binding and retention of immortalizing capacity were coordinate. On the basis of those findings and the observation that the same N-terminal T-antigen segment immortalized independently and cooperated with *ras* to transform REF, we expected that a T antigen consisting of amino acids 351 to 708 would both bind p53 and exhibit *ras* cooperation activity. In the study reported here we showed, however, that the truncated T antigens T301-708, T337-708, T351-708, and T251-708*dl*400 all bound p53 but did not cooperate with *ras*. Thus, in contrast to independent immortalization, retention of p53 binding and the ability to produce dense foci in cooperation with *ras* on REF monolayers were not coordinate.

Finally, the separation of the *ras* cooperation activities provides the means of analyzing the functions underlying cooperative transformation by these T-antigen fragments and of determining whether they reflect duplicated or analogous functions and whether their underlying mechanisms of action are the same or different.

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REFERENCES

1. **Anderson, R. W., M. J. Tevethia, D. Kalderon, A. E. Smith, and S. S. Tevethia.** 1988. Fine mapping two distinct antigenic sites on simian virus 40 (SV40) T antigen reactive with SV40-specific cytotoxic T-cell clones by using SV40 deletion mutants. J. Virol. **62:**285–296.
2. **Asselin, C., and M. Bastin.** 1985. Sequences from polyomavirus and simian virus 40 large T genes capable of immortalizing primary rat embryo fibroblasts. J. Virol. **56:**958–968.
3. **Barbacid, M.** 1987. Ras genes. Annu. Rev. Biochem. **56:**779–827.
4. **Bikel, I., X. Montano, M. E. Agha, M. Brown, M. McCormack, J. Boltax, and D. M. Livingston.** 1987. SV40 small t antigen enhances the transformation activity of limiting concentrations of SV40 large T antigen. Cell **48:**321–330.
5. **Boldfarb, M., K. Shimizu, M. Perucho, and M. Wigler.** 1982. Isolation and preliminary characterization of a human transforming gene from T24 bladder carcinoma cells. Nature (London) **296:**404–409.
6. **Botchan, M., W. Topp, and J. Sambrook.** 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. Cell **9:**269–287.
7. **Cavender, J. F., and M. J. Tevethia.** Unpublished data.
8. **Conn, A., H. Lacko, and M. J. Tevethia.** Unpublished data.
9. **Debbas, M., and E. White.** 1993. Wild-type p53 mediates apoptosis by E1A which is inhibited by E1B. Genes Dev. **7:**546–554.
10. **Deppert, W., T. Steinmayer, and W. Richter.** 1989. Cooperation of SV40 large T antigen and the cellular protein p53 in maintenance of cell transformation. Oncogene **4:**1103–1110.
11. **Dickmanns, A., A. Zeitvogel, F. Simmerbach, R. Weber, A. K. Arthur, S. Dehde, A. Wildeman, and E. Fanning.** 1994. The kinetics of simian virus 40-induced progression of quiescent cells into S phase depend on four independent functions of large T antigen. J. Virol. **68:**5496–5508.
12. **Diller, L., J. Kassel, C. Nelson, M. Gryka, G. Litwak, M. Gebhardt, B. Bressac, M. Ozturk, S. Baker, B. Vogelstein, and S. H. Friend.** 1990. p53 functions as a cell cycle control protein in osteosarcomas. Mol. Cell. Biol. **10:**5772–5781.
13. **Dornreiter, I., A. Höss, A. K. Arthur, and E. Fanning.** 1990. SV40 T antigen binds directly to the large subunit of purified DNA polymerase alpha. EMBO J. **9:**3329–3336.
14. **Fields, S., and S. K. Jang.** 1990. Presence of a potent transcription activating sequence in the p53 protein. Science **249:**1046–1049.
15. **Finlay, C. A., P. W. Hinds, and A. J. Levine.** 1989. The p53 proto-oncogene can act as a suppressor of transformation. Cell **57:**1083–1093.
16. **Flint, S. J., L. Y. Wewerka, A. S. Levine, J. Sambrook, and P. A. Sharp.** 1975. Adenovirus transcription. II. RNA sequences complementary to simian virus 40 and adenovirus 2 DNA in AD2+ND1- and AD2+ND3-infected cells. J. Virol. **16:**662–673.
17. **Friedman, P. N., S. E. Kern, B. Vogelstein, and C. Prives.** 1990. Wild-type, but not mutant, human p53 proteins inhibit the replication activities of simian virus 40 large tumor antigen. Proc. Natl. Acad. Sci. USA **87:**9275–9279.

17a. **Fu, T.-M., M. J. Tevethia, and S. S. Tevethia.** Unpublished data.

18. **Gannon, J. V., R. Greaves, R. Iggo, and D. P. Lane.** 1990. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. EMBO J. **9:**1595–1602.
19. **Gannon, J. V., and D. P. Lane.** 1987. p53 and DNA polymerase alpha compete for binding to SV40 T antigen. Nature (London) **329:**456–458.
20. **Gough, G., J. V. Gannon, and D. P. Lane.** 1988. Competition between DNA polymerase α and p53 for binding to SV40 T antigen. Cancer Cells **6:**153–158.
21. **Grunda, M. C., J. M. Zabolotny, J. H. Xiao, I. Davidson, and J. C. Alwine.** 1993. Transcriptional activation by simian virus 40 large T antigen: interactions with multiple components of the transcription complex. Mol. Cell. Biol. **13:**961–969.
22. **Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson.** 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. J. Virol. **39:**861–869.
23. **Jenkins, J. R., K. Rudge, S. Redmond, and A. Wade-Evans.** 1984. Cloning and expression analysis of full length mouse cDNA sequences encoding the transformation associated protein p53. Nucleic Acids Res. **12:**5609–5626.
24. **Jennings, S. R., P. A. Lippe, K. J. Pauza, P. G. Spear, L. Pereira, and S. S. Tevethia.** 1987. Kinetics of expression of herpes simplex virus type 1-specific glycoprotein species on the surfaces of infected murine, simian, and human cells: flow cytometric analysis. J. Virol. **61:**104–112.
25. **Jiang, D., A. Srinivasan, G. Lozano, and P. D. Robbins.** 1993. SV40 T antigen abrogates p53-mediated transcriptional activity. Oncogene **8:**2805–2812.
26. **Johnson, D. C., and P. G. Spear.** 1982. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. **43:**1102–1112.
27. **Kalderon, D., and A. E. Smith.** 1984. In vitro mutagenesis of a putative DNA binding domain of SV40 large-T. Virology **139:**109–137.
28. **Kierstead, T. D., and M. J. Tevethia.** 1993. 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. J. Virol. **67:**1817–1829.
29. **Lane, D. P., and L. V. Crawford.** 1979. T antigen is bound to a host protein in SV40-transformed cells. Nature (London) **278:**261–263.
30. **Lin, J. Y., and D. T. Simmons.** 1991. Stable T-p53 complexes are not required for replication of simian virus 40 in culture or for enhanced phosphorylation of T antigen and p53. J. Virol. **65:**2066–2072.
31. **Lowe, S. W., H. E. Ruley, T. Jacks, and D. E. Housman.** 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell **74:**957–967.
32. **Mack, D. H., J. Vartikar, J. M. Pipas, and L. A. Laimins.** 1993. Specific repression of the TATA-mediated but not initiator-mediated transcription by wild type p53. Nature (London) **363:**281–283.
33. **Mackett, M., G. L. Smith, and B. Moss.** 1984. General method for produc-

tion and selection of infectious vaccinia virus recombinants expressing foreign genes. J. Virol. **49:**857–864.
34. **McCutchan, J. H., and J. S. Pagano.** 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. J. Natl. Cancer Inst. **41:**351–357.
35. **Michalovitz, D., F. L. Fischer, C. Vesco, J. M. Pipas, and M. Oren.** 1987. Activated Ha-*ras* can cooperate with defective simian virus 40 in the transformation of nonestablished rat embryo fibroblasts. J. Virol. **61:**2648–2654.
36. **Montenarh, M.** 1992. Biochemical properties of the growth suppressor/oncoprotein p53. Oncogene **7:**1673–1680.
37. **Mosley, B., M. P. Beckman, C. J. March, R. L. Idzerda, S. D. Gimpel, T. VandenBos, D. Friend, A. Alpert, J. Jackson, J. M. Wignall, C. Smith, B. Gallis, J. E. Sims, D. Urdal, M. B. Widmer, D. Cosman, and L. S. Park.** 1989. The murine interleukin-4 receptor: molecular cloning and characterization of the secreted and membrane bound forms. Cell **59:**335–348.
38. **O'Reilly, D. R., and L. K. Miller.** 1988. Expression and complex formation of simian virus 40 large T antigen and mouse p53 in insect cells. J. Virol. **62:**3109–3119.
39. **Oren, M., W. Maltzman, and A. J. Levine.** 1981. Posttranslational regulation of the 54K cellular tumor antigen in normal and transformed cells. Mol. Cell. Biol. **1:**101–110.
40. **Quartin, R. S., C. N. Cole, J. M. Pipas, and A. J. Levine.** 1994. The amino-terminal functions of simian virus 40 large T antigen are required to overcome wild-type p53-mediated growth arrest of cells. J. Virol. **68:**1334–1341.
41. **Rao, L., M. Debbas, P. Sabbatini, D. Hockenbery, S. Korsmeyer, and E. White.** 1992. The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. Proc. Natl. Acad. Sci. USA **89:**7742–7746.
42. **Raycroft, L., H. Y. Wu, and G. Lozano.** 1990. Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. Science **249:**1049–1051.
43. **Reich, N. C., and A. J. Levine.** 1984. Growth regulation of a cellular tumour antigen, p53, in nontransformed cells. Nature (London) **308:**199–201.
44. **Rhim, J. S., H. Y. Cho, and R. J. Huebner.** 1975. Non-producer human cells induced by murine sarcoma virus. Int. J. Cancer **15:**23–29.
45. **Robb, J. A., and K. Huebner.** 1973. Effect of cell chromosome number on simian virus 40 replication. Exp. Cell Res. **81:**120–126.
46. **Ruley, E. H.** 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature (London) **304:**602–606.
47. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
48. **Sarnow, P., Y. S. Ho, J. Williams, and A. J. Levine.** 1982. Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. Cell **28:**387–394.
49. **Seto, E., A. Usheve, G. P. Zambetti, and A. Levine.** 1992. Wild type p53 binds to the TATA-binding protein and represses transcription. Proc. Natl. Acad. Sci. USA **89:**12028–12032.
50. **Sleigh, M. J., W. C. Topp, R. Hanich, and J. F. Sambrook.** 1978. Mutants of SV40 with an altered small t protein are reduced in their ability to transform cells. Cell **14:**79–88.
51. **Smale, S. T., and R. Tjian.** 1986. T-antigen–DNA polymerase alpha complex implicated in simian virus 40 DNA replication. Mol. Cell. Biol. **6:**4077–4087.
52. **Sompayrac, L., and K. J. Danna.** 1983. Simian virus 40 deletion mutants that transform with reduced efficiency. Mol. Cell. Biol. **3:**484–489.
53. **Sompayrac, L., and K. J. Danna.** 1989. An SV40 mutant oncoprotein has a nuclear location. Virology **171:**267–270.
54. **Sompayrac, L., and K. J. Danna.** 1991. The amino-terminal 147 amino acids of SV40 large T antigen transform secondary rat embryo fibroblasts. Virology **181:**412–415.
55. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1:**327–341.
56. **Spector, D. J., and M. J. Tevethia.** 1986. Identification of a human cytomegalovirus DNA segment that complements an adenovirus 5 immediate early mutant. Virology **151:**329–338.
57. **Srinivasan, A., K. W. Peden, and J. M. Pipas.** 1989. The large tumor antigen of simian virus 40 encodes at least two distinct transforming functions. J. Virol. **63:**5459–5463.
58. **Stürzbecher, H. W., C. Addison, and J. R. Jenkins.** 1988. Characterization of mutant p53-hsp72/73 protein-protein complexes by transient expression in monkey COS cells. Mol. Cell. Biol. **8:**3740–3747.
59. **Takebe, Y., M. Seiki, J. Fujisawa, P. Hoy, K. Yokota, K. Arai, M. Yoshida, and N. Arai.** 1988. SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. Mol. Cell. Biol. **8:**466–472.
60. **Tevethia, M. J.** 1984. Immortalization of primary mouse embryo fibroblasts with SV40 virions, viral DNA, and a subgenomic DNA fragment in a quantitative assay. Virology **137:**414–421.
61. **Tevethia, M. J., J. M. Pipas, T. Kierstead, and C. Cole.** 1988. Requirements for immortalization of primary mouse embryo fibroblasts probed with mutants bearing deletions in the 3′ end of SV40 gene A. Virology **162:**76–89.
61a. **Tevethia, S. S., and D. L. Thompson.** Unpublished data.
62. **Thompson, D. L., D. Kalderon, A. E. Smith, and M. J. Tevethia.** 1990. Dissociation of Rb-binding and anchorage-independent growth from immortalization and tumorigenicity using SV40 mutants producing N-terminally truncated large T antigens. Virology **178:**15–34.
63. **Tiemann, F., and W. Deppert.** 1994. Stabilization of the tumor suppressor p53 during cellular transformation by simian virus 40: influence of viral and cellular factors and biological consequences. J. Virol. **68:**2869–2878.
64. **Tooze, J.** 1981. DNA tumor viruses: molecular biology of tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
65. **Topp, W.** 1981. Normal rat cell lines deficient in nuclear thymidine kinase. Virology **113:**408–411.
66. **Tornow, J., M. Polvino-Bodnar, G. Santangelo, and C. N. Cole.** 1985. Two separable functional domains of simian virus 40 large T antigen: carboxyl-terminal region of simian virus 40 large T antigen is required for efficient capsid protein synthesis. J. Virol. **53:**415–424.
67. **van der Heuvel, S. J. L., T. van Laar, I. The, and A. J. van der Eb.** 1993. Large E1B proteins of adenovirus types 5 and 12 have different effects on p53 and distinct roles in cell transformation. J. Virol. **67:**5226–5234.
68. **Yaciuk, P., M. C. Carter, J. M. Pipas, and E. Moran.** 1991. Simian virus 40 large-T antigen expresses a biological activity complementary to the p300-associated transforming function of the adenovirus E1a gene products. Mol. Cell. Biol. **11:**2116–2124.
69. **Zantema, A., A. M. Fransen, A. Davis-Olivier, F. C. S. Ramaekers, G. P. Vooijs, B. de Leys, and A. J. van der Eb.** 1985. Localization of the EIB proteins of adenovirus 5 in transformed cells, as revealed by interaction with monoclonal antibodies. Virology **142:**44–58.
70. **Zantema, A., P. I. Shier, A. Davis-Olivier, T. van Laar, R. T. M. J. Vaessen, and A. J. van der Eb.** 1985. Adenovirus serotype determines association and localization of the large EIB tumor antigen with cellular tumor antigen p53 in transformed cells. Mol. Cell. Biol. **5:**3084–3091.
71. **Zhu, J. Y., M. Abate, P. W. Rice, and C. N. Cole.** 1991. The ability of simian virus 40 large T antigen to immortalize primary mouse embryo fibroblasts cosegregates with its ability to bind to p53. J. Virol. **65:**6872–6880.