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The Ability of Large T Antigen To Complex with p53 Is Necessary for the Increased Life Span and Partial Transformation of Human Cells by Simian Virus 40

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Simian virus 40 (SV40) T antigen binds to the tumor suppressor p53 protein, and this association may contribute to oncogenic transformation by the virus. We investigated the importance of this binding on transformation by examining three replication-competent mutants of SV40 (402DE, 402DN, and 402DH). These mutants express T antigens defective in binding to human and monkey p53s but retain some binding with mouse p53. All showed significant reduction in their ability to induce transformed cell foci of two normal human cell lines as well as a slight reduction with mouse embryo cells. Other comparable mutants which express T antigens retaining the ability to complex with p53 were able to induce foci at wild-type levels in both human and mouse cells. Further studies were performed with five T-antigen-positive clones isolated from the few human cell foci that appeared after transfection with 402 mutant DNAs. All five clones reached senescence at about the same point as did the parental untransformed cells. However, six other human cell clones obtained after transfection with DNA from nondefective mutants or wild-type virus were still growing well at more than 10 passages beyond their expected life span. These results suggest that the ability of T antigen to form stable complexes with p53 is necessary for SV40 to extend the life span and partially transform human cells in culture.

Simian virus 40 (SV40) can transform both primary and established cells and induce tumors in laboratory animals (4, 24, 33, 37, 53). Large T antigen of SV40 plays a major role in transformation (4, 21, 37), but the mechanism by which it transforms cells is largely unknown. Although T antigen has several biochemical activities, including ATPase (14, 52), helicase (45), and binding to the viral DNA origin (43, 51), none is required for oncogenic transformation in culture (1, 22, 32, 39). In transformed cells, T antigen complexes with two known tumor suppressor proteins, p53 (25, 29) and Rb, the product of the retinoblastoma susceptibility gene (8), suggesting that these complexes are involved in oncogenic transformation by the virus. Although wild-type p53 is a tumor suppressor protein (2, 6, 9, 12), various mutations activate it to a transforming protein (11, 19). Recent studies from our laboratory (27) showed that transformation of mouse cells by SV40 does not depend on the mutational activation of p53 to an oncogenic form. One possible model for cellular transformation by SV40, therefore, involves, at the minimum, the inactivation or alteration of the normal functions of p53 and Rb by forming complexes with T antigen. Binding of T antigen to these two proteins may prevent their normal suppressor functions and thereby contribute to the process of oncogenic transformation. Recent studies have shown that binding of Rb by T antigen is essential for transformation of established rodent cells but is not necessary for immortalization of primary rodent cells (7, 50). However, binding to Rb is by itself not sufficient to transform and immortalize human or mouse cells in culture (31, 41). Binding of p53 by T antigen has also been suggested by Tevethia et al. (47–49) to also be necessary for transformation by SV40. However, others (44) have demonstrated that in some cases, binding to p53 is not required for the transformation of secondary rat embryo cells.

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Since the role of these complexes in SV40-mediated transformation is unclear, we examined the transforming activity of three replication-competent mutants of SV40 which express T antigens defective in p53 binding (28). Although the formation of stable complexes is not required for virus replication (28), we show here that it is necessary for SV40 to induce transformed foci of human cells and to extend the life span of these cells in culture.

MATERIALS AND METHODS

Plasmids and cells. pBS-SV40 consists of a Bluescript vector (Stratagene) and the entire genome of SV40 (30). Mutations corresponding to single amino acid substitutions in T antigen were generated in pBS-SV40 as previously described (28). Detroit 551 (D.551; ATCC CCL 110) is a line of normal diploid skin fibroblasts, and WI-38 cells (ATCC CCL 75) are normal human diploid lung fibroblasts (17). Mouse embryo cells were prepared from 14-day-old mouse embryos. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (GIBCO or Whittaker) plus penicillin and streptomycin (GIBCO).

Transfection and focus formation assays. For dense focus assays, secondary mouse embryo cells and human cells were seeded at a densities of 5×10^5 and 2×10^5 cells per 60-mm petri dish, respectively. DNA transfections were carried out 1 day later by using a modified calcium phosphate precipitation method (5). Five micrograms of wild-type (WT) or mutant pBS-SV40 DNA and 5 μg of salmon sperm carrier DNA were used per dish. After 16 to 20 h of exposure to the precipitate, the medium was removed from the dishes and replaced with DMEM containing 10% fetal bovine serum. Three days after transfection, the serum concentration was reduced to 2 or 3%, and the medium in the dish was changed every 3 days. Transformed colonies were stained with Giemsa and counted 4 to 6 weeks after transfection.

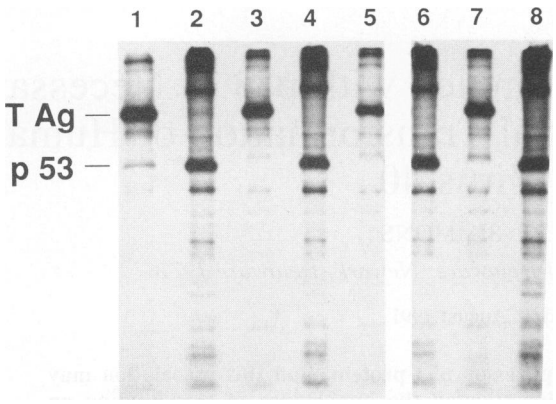

FIG. 1. Association of WT or mutant T antigen with p53 in infected human D.551 cells. Cells were infected with WT or mutant SV40 and labeled with L-[^{35}S]methionine for 16 h. Cell lysates were incubated with either pAb416 (16), a monoclonal antibody to T antigen (lanes 1, 3, 5, and 7), or pAb421 (16), a monoclonal antibody to p53 (lanes 2, 4, 6, and 8). Labeled immunoprecipitated proteins were detected by autoradiography after electrophoresis on 13% SDS-polyacrylamide gels. Lanes: 1 and 2, WT SV40; 3 and 4, 402DE; 5 and 6, 402DN; 7 and 8, 402DH.

Isolation of cloned cell lines and growth assays. Transformed foci of D.551 cells were isolated at 4 to 7 weeks after transfection, and the cells were propagated in culture. Cloned cells were then checked for T-antigen expression by indirect immunofluorescence. For growth curve assays, D.551 cells were seeded at a density of 5×10^4 cells per 60-mm dish. Cell numbers were determined every day for a 6-day period. For estimating the life span of cloned cell lines in culture, cells were grown in DMEM containing 10% fetal bovine serum and routinely subcultured at a 1:5 ratio (36) when confluent. Senescence was recognized by the inhibition of cell growth as described by others (18).

Immunoprecipitation and gel electrophoresis. Cloned cells or cells infected with WT or mutant SV40 were labeled with L-[^{35}S]methionine (200 to 500 μCi/ml) for 16 h. Cells were lysed with Nonidet P-40 lysis buffer (26), and labeled T antigen and associated proteins were immunoprecipitated with anti-T monoclonal antibody pAb416 (16) or pAb419 (16). p53 was immunoprecipitated with p53-specific antibody pAb240 (13), pAb246 (56), or pAb421 (16). Labeled Rb was immunoprecipitated with anti-Rb monoclonal antibody (kindly provided by Ed Harlow). Labeled immunoprecipitated proteins were analyzed by sodium dodecyl sulfate (SDS)-gel electrophoresis and autoradiography.

RESULTS

Complexing of mutant T antigens with p53. We have previously demonstrated that residue Asp-402 in SV40 T antigen is important for the binding to the antioncoprotein p53 (28). Three different mutations at this site (Asp to Glu, Asn, or His) totally prevented the formation of stable complexes with the cellular p53 protein in monkey BSC-1 cells but had no effect on virus multiplication (28). The same three mutations reduced but did not abolish the ability to bind to mouse p53 (28). In this study, we examined the binding of the mutant T antigens to human p53. Figures 1 and 3 show that stable complexes were undetectable, as was the case with monkey p53 (28). Levels of wild-type T-p53 complexes were low, however (Fig. 1, lanes 1 and 2), and we cannot totally exclude the possibility that very small amounts of stable complexes between mutant T antigens and p53 formed in human cells.

Focus formation assays on human cells. SV40 carrying mutations at T-antigen codon 402 replicate at WT levels (28), indicating that the replication activities of the mutant T antigens must be relatively normal. It appears, therefore, that the only defect of these mutants is their reduced binding to p53. Accordingly, the mutants were used to investigate the involvement of stable T-p53 complexes in transformation by SV40. Two normal human cell lines were first tested, D.551 and WI-38. D.551 is a line of normal diploid skin fibroblasts with a limited lifetime of approximately 25 passages from the tissue of origin. The p53 in this cell line is similar to the WT protein, since it is not recognized by pAb240 and it complexes with SV40 T antigen (data not shown). WI-38 cells are normal diploid lung fibroblasts with a finite life span of 50 ± 10 population doublings (17). Cells were transfected at passage 14 with WT or mutant pBS-SV40 DNA (28) by using a modified $CaPO_4$ protocol (5). DNA transfections rather than virus infections were used because (i) more precise estimates were obtained of DNA concentrations than of virus titers and (ii) this approach allowed us to include a nonviable mutant (398LV) in the study. Three days after transfection, the serum concentration was reduced to 2 or 3% to eliminate the appearance of spontaneously transformed cell foci and to prevent the peeling of the monolayer. Transformed cell foci were scored at 4 weeks after transfection (Table 1). All three 402 mutants were significantly reduced in their ability to induce foci of both human cell lines compared with WT DNA. Importantly, other mutant T antigens which harbor mutations at neighboring sites but which bind to p53 normally (28) induced about as many transformed cell foci as did WT antigen (Table 1; Fig. 2A). Similar results were obtained with human WI-38 cells (Table 1; Fig. 2B). The reduced numbers of foci generated with 402 mutant DNAs were not due to a lower transfection frequency because we observed that all DNAs tested had approximately the same transfection frequency in human D.551 cells (Table 2). These results correlate the inability of mutant T antigens to complex with human p53 with a major loss in transforming activity on human cells.

Focus formation assays on mouse cells. Since the 402 mutant T antigens retained some binding activity with mouse p53 (28), we next investigated whether these mutants transformed secondary mouse embryo fibroblasts. The p53 in these cells is similar to the WT protein in that it reacts with pAb246 but not with pAb240 and complexes well with T antigen (data not shown). Table 1 shows that these mutants transformed mouse cells less than half as well as did WT DNA, with the 402DN mutant showing the least activity. Other mutants with changes at sites other than residue 402 transformed at WT levels. There was no apparent correlation between transforming activity and the relative percentage of mouse p53 bound to mutant T antigens in infected cells compared with that bound to WT T antigen (28) (43% for 402DE, 12% for 402DN, and 3% for 402DH). Rather, the transforming activity of these mutants on mouse cells was more closely correlated with the relative percentage of mutant T antigens bound to p53 (28) (21% for 402DE, 8% for 402DN, and 19% for 402DH). One possible explanation of these results is that p53 binding is not involved in the transformation of mouse cells by SV40. This would be consistent with the results of Sompayrac and Danna (44), who investigated the transformation of rat cells by an NH_2-terminal 147-amino-acid fragment of T antigen. How-

TABLE 1. Transforming activities of WT and mutant SV40 DNAs in human and mouse cells

Plasmid[a]	No. of foci/60-mm dish[b]				
	Human D.551 cells		Human WI-38 cells, expt 1	Secondary mouse embryo cells	
	Expt 1	Expt 2		Expt 1	Expt 2
Mock control	0, 0	0, 0		0, 0, 0	0, 0, NT
pSV_2neo			0, 0, NT		
WT	11, 10	18, 15	24, 23, 27	35, 28, 35	33, 45, 44
395HN	NT, NT	20, 18		NT, NT, NT	NT, 46, 45
398LV[c]	8, 6	NT, NT			
400KR	NT, NT	15, 16		NT, NT, NT	NT, 38, 42
401MT	NT, NT	14, 15			
402DE	0, 0	1, 0	1, 0, NT	12, 11, 13	14, NT, NT
402DN	0, 1	2, 1	1, 0, 0	5, 3, 7	7, NT, NT
402DH	0, 0	0, 0	0, 0, NT	12, 11, 7	12, NT, NT
403SA	12, 9	NT, NT	23, 24, 23	27, 28, 30	28, NT, NT
404VL	NT, NT	14, 15		28, 30, NT	NT, NT, NT
407DE	NT, NT	16, 17		40, 36, NT	NT, NT, NT

[a] Mutants are designated by the amino acid residue number which is mutated followed by the substitution, using single-letter codes. The two letters indicate the amino acid before and after the mutational change.
[b] Scored at 4 weeks posttransfection on plates which contained 2×10^5 cells. NT, not tested.
[c] Mutant virus unable to replicate in monkey BSC-1 cells. All others replicate like WT SV40.

ever, because mutations at residue 402 did have a slight effect on transformation of mouse cells (Table 1), we believe that a better interpretation is that T-p53 complexes are involved, but that these mutants are able to transform because some complexes still form.

Characteristics of transformed human cell foci and cloned lines. Since the effect of mutations at residue 402 was more pronounced with human cells than with mouse cells, we further investigated the transformation of human cells by T antigen. In this analysis, we characterized the few human cell colonies that appeared after transfection with 402 mutant DNAs. At 4 weeks posttransfection, there were few or no human cell foci in the plates that received 402 mutant DNAs (Table 1; Fig. 2). The foci were generally smaller than those appearing after transfection with WT or with mutant DNAs encoding T antigens that bind to p53 normally. One to three more foci per plate slowly formed over the next several weeks (by then the foci in all other plates were confluent and uncountable) (Fig. 2A). Foci of D.551 cells were recovered either at 6 to 7 weeks posttransfection (for the 402 mutants) or at 4 to 5 weeks posttransfection (for all others). The foci recovered at these two different times were of about the same size, indicating that these cells had undergone about the same number of doublings after transfection. An early observation was that cells derived from transfection with 402 mutant DNAs grew more slowly than all other cloned cells. Cells were propagated in culture and checked for T-antigen expression by indirect immunofluorescence. Fewer than half (5 of 12) of the cloned cells derived by transfection with mutant 402 DNAs were T antigen positive, whereas all nine of the clones obtained after transfection with control WT, 398LV, or 403SA DNA were T antigen positive. Some of the T-antigen-positive cell clones were then tested by immunoprecipitation for the presence of stable T-p53 complexes. Table 3 shows that whereas a WT, a 398LV-derived, and a 403SA-derived clone contained stable complexes, a 402DE and a 402DN-derived clone contained no detectable T-p53 complexes, as predicted by the effect of mutations at residue 402 (28). Immunoprecipitation results for the 402-derived clones are shown in Fig. 3. One clone (402DE(5)] expressed normal levels of T antigen and p53 but no detectable complexes (Fig. 3, lanes 3 and 4). The other clone (402DN(6)] expressed low levels of T antigen and undetectable levels of p53 and complexes (Fig. 3, lanes 5 and 6). The other three T-antigen-positive 402-derived clones [402DE(2), 402DE(3), and 402DH(3)] reached senescence at the third passage after isolation (in a T-75 flask) (Table 3) and could not be tested.

Determination of the life span of cloned cell lines. Immortalization of human cells by SV40 is quite rare (15, 20), However, SV40 can extend the life span of human diploid cells by about 20 to 40 population doublings (21, 45). To assess the effect of T-antigen mutations on the life span of human cells, we continuously passaged cloned cells (at a split ratio of 1:5) (36) until some of the cell lines reached senescence. We calculated the total number of passages for each clone, starting from the original isolation of D.551 cells (Table 3). Since cells were transfected at passage 14, we calculated from cell numbers that cells recovered from a typical focus were at the equivalence of about passage 22. Based on these calculations, all T-antigen-positive clones generated with mutant 402 DNAs reached senescence at or near passage 25, corresponding to the life span of the original D.551 cells (Table 3). In contrast, cells transformed with WT DNA, mutant 398 DNA, or mutant 403 DNA were still growing well beyond passage 35. We propagated two clones to passage 47 or 48 and one to passage 55 before the experiment was terminated (Table 3).

Figure 4 shows growth curves for some 402 DNA-derived clones as they approached senescence. Two clones of D.551 cells derived by transfection with 402DE were no longer growing at passage 26 (Fig. 4). One clone generated with 402DN was growing slowly at passage 26 and at roughly the same rate as the parental cell line at passage 18. This clone stopped growing at the next passage (Table 3). WT transformants and cells transformed with 403SA were growing well at passage 26 or 27 and dividing faster than the parental cells. These results indicate that the presence of stable T-p53 complexes in these cloned cells contributed to the extension of their life spans.

DISCUSSION

In this report, we showed that three mutants of SV40 coding for T antigens with various substitutions at residue

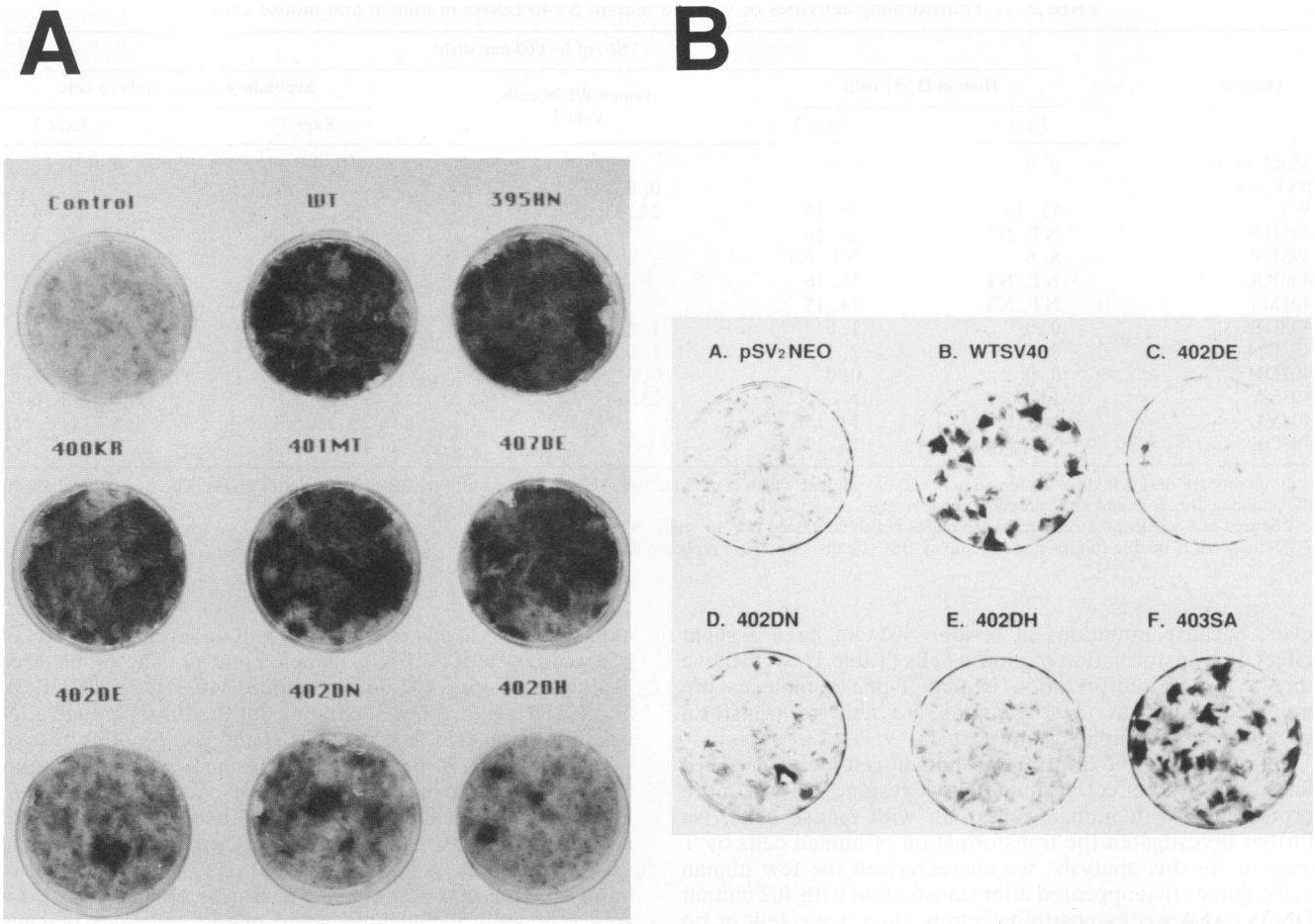

FIG. 2. Morphology of mutant or WT SV40-transformed foci. Cells were transfected as described in Materials and Methods, fixed with methanol, and stained with 5% Giemsa stain 6 weeks after transfection. (A) Background monolayer of human D.551 cells after mock transfection (control) or foci from cells transfected with WT or mutant SV40 DNA, as indicated. (B) Background monolayer of human WI-38 cells after pSV_2neo transfection (control) or foci from cells transfected with WT or mutant SV40 DNA, as indicated. The plates were stained with Giemsa 4 weeks after transfection.

402 and defective in human p53 binding are significantly reduced in their ability to induce transformed foci of two normal human cell lines. This defect is not due to the inability of the mutant T antigens to complex with the product of the Rb gene, as judged by immunoprecipitation assays using anti-Rb antibodies (data not shown). Our results indicate that stable T-p53 complexes play an important role in the formation of human cell foci.

TABLE 2. Transfection frequency of human cells by WT or mutant pBS-SV40 DNA

Transfected DNA	% of T-antigen-positive cells[a]
WT SV40	9
402DE	12
402DN	9
402DH	12
403SA	13

[a] Human D.551 cells were transfected with wt or mutant pBS-SV40 DNA. At 48 h posttransfection, cells were checked for the expression of T antigen by indirect immunofluorescence to determine the percentage of T-antigen-positive cells.

The nature of the few human cell foci that formed after transfection with 402 mutant DNAs is not known. Some of the foci could have consisted of cells that expressed T antigen early on and then lost that expression. The T-anti-

TABLE 3. Comparison of life span of WT or mutant SV40-derived cell lines

Cloned line	T-p53 complexes	Approx life span (no. of passages)
Parental D.551	–	25
WTSV40(2)	NT[a]	>38
WTSV40(4)	+	>35
398LV(1)	+	>39
398LV(2)	NT	>48
402DE(2)	NT	25
402DE(3)	NT	25
402DE(5)	–	27
402DN(6)	–	27
402DH(3)	NT	25
403SA(1)	+	>55
403SA(4)	NT	>47

[a] NT, not tested.

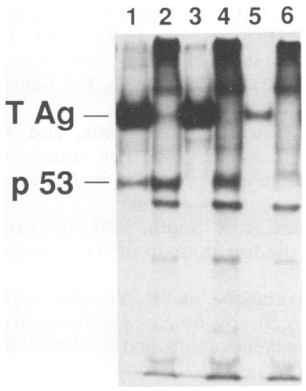

FIG. 3. Association of WT or mutant T antigen with p53 in human D.551 cloned cells. Cells were labeled with L-[^{35}S]methionine for 16 h. Cell lysates were incubated with either pAb416 (16), a monoclonal antibody to T antigen (lanes 1, 3, and 5), or pAb421 (16), a monoclonal antibody to p53 (lanes 2, 4, and 6). Labeled immunoprecipitated proteins were detected by autoradiography after electrophoresis on 13% SDS-polyacrylamide gels. Lanes: 1 and 2, WT SV40; 3 and 4, 402DE(5); 5 and 6, 402DN(6).

gen-positive clones could have formed either because transient T-p53 complexes may have existed or because the other transformation-related activities of the mutant T antigens (such as binding to Rb) are intact.

The role of T antigen-p53 complexes in the transformation of rodent cells is less clear. Previous studies by others have shown that mutant T antigens missing some or all portions of the p53 binding region (amino acid residues 272 to 517 [42]) do not bind p53 and fail to immortalize mouse cells efficiently (47–49). These results are consistent with the finding that the E6 protein of human papillomavirus 16, which binds to p53 (54), can immortalize primary rat cells (23). On the other hand, Sompayrac and Danna (44) have shown that an NH_2-terminal 147-amino-acid fragment of T antigen, expressed under the control of a retrovirus promoter, transforms secondary rat cells as efficiently as does full-length T antigen.

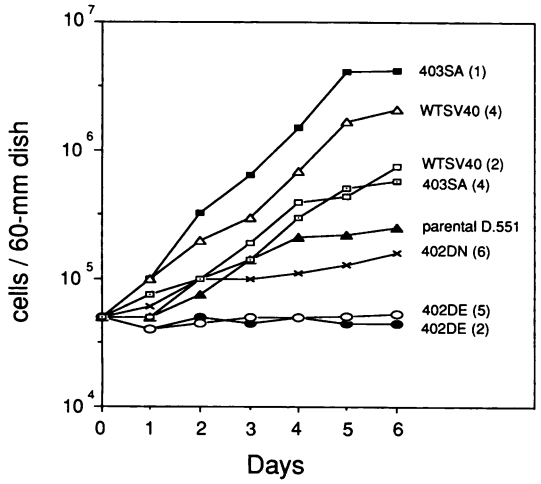

FIG. 4. Growth curves of WT or mutant SV40 DNA-derived clones. Parental or cloned D.551 cells were plated at a density of 5 × 10^4 cells per 60-mm petri dish. Cell numbers were determined every day for a 6-day period. Passage numbers for the clones used: 403SA(1), 26; WTSV40(4), 27; WTSV40(2), 26; 403SA(4), 26; parental D.551, 18; 402DN(6), 26; 402DE(5), 26; 402DE(2), 25.

Our results do not clearly demonstrate the involvement of complexes in the transformation of mouse cells because the 402 mutant T antigens retain a small amount of binding activity to mouse p53. However, these mutations had some effect on focus formation (Table 1), which suggests that in the context of full-length T antigen, and under the control of the SV40 early promoter, T-p53 complexes are necessary for the transformation of mouse cells as they are for human cells. One possible explanation for the discrepancy between our results and those of Sompayrac and Danna (44) is that mouse and rat cells are transformed by SV40 by slightly different mechanisms. However, we think it is more likely that the expression of a truncated T antigen (residues 1 to 147) under the control of a different promoter might abrogate the requirement for p53 binding.

Although SV40 T antigen can immortalize rodent cells, it fails to immortalize human cells as efficiently (15, 20, 38, 46). Wright et al. (55) have suggested that human cells are immortalized only after two stages (mortality stages M1 and M2) are bypassed. The expression of SV40 T antigen overcomes or bypasses the M1 but not the M2 stage (55). As a consequence, the life span of these cells is extended and they continue to divide until M2 is reached, at which point they undergo crisis. Because T antigen can immortalize rodent cells, the bypass of only one stage (corresponding to M1 in human cells) may be required in this case. Our results indicate that stable T-p53 complexes are necessary for the extension in the life span of human cells. This would suggest that the ability of T antigen to form stable complexes with p53 is necessary to bypass or overcome the M1 mechanism in human cells. Furthermore, it also suggests that stable T-p53 complexes may be required at the same stage in the immortalization of rodent cells.

One way in which T-p53 complexes could contribute to the extension of the life span of human cells is through the inactivation or alteration of the normal function of WT p53. Fields and Jang (10) and Raycroft et al. (40) have recently shown that p53 is an activator of transcription. The target genes for p53 are unknown, but conceivably one of them could have growth suppressor function. By binding to p53, T antigen would inhibit this activity and promote cell proliferation. Alternatively, p53 could normally be a suppressor of certain genes which function in cell growth, and this activity could be inhibited or modified by T antigen. Inhibition of p53 function could also come about through protein-protein interactions rather than at the level of gene regulation. WT p53 is loosely associated with the $p34^{cdc2}$ kinase (34, 35), and human p53 is phosphorylated by *cdc2* kinase in vitro (3). This interaction may be functional in the control of cell growth (3). When T antigen binds to p53, it may inactivate this function and promote the extension of the life span of the cells.

Finally, our results suggest yet another model. The transformation of mouse cells by various 402 mutants correlates best with the relative percentage of T antigen in complexes rather than with the relative percentage of WT p53 bound to mutant T antigen (28) (Table 1). This offers the possibility that T-p53 complexes function directly in promoting cell growth and that the inactivation of WT p53 is not of prime importance in transformation by SV40. The presence of large amounts of uncomplexed (and presumably functional) p53 in these mouse cells supports this hypothesis.

In conclusion, we have provided evidence that stable T-p53 complexes are necessary for SV40 to induce foci of

transformed human cells in culture. Furthermore, these complexes appear to be required for the extension of the life span of these cells.

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