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Cooperation of Simian Virus 40 Large and Small T Antigens in Metabolic Stabilization of Tumor Suppressor p53 during Cellular Transformation

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Metabolic stabilization of the tumor suppressor p53 is a key event in cellular transformation by simian virus 40 (SV40). Expression of the SV40 large tumor antigen (large T) is necessary but not sufficient for this process, as metabolic stabilization of p53 complexed to large T in abortively SV40-infected cells strictly depends on the cellular systems analyzed (F. Tiemann and W. Deppert, J. Virol. 68:2869–2878, 1994). Comparative analyses of various cells differing in metabolic stabilization of p53 upon abortive infection with SV40 revealed that metabolic stabilization of p53 closely correlated with expression of the SV40 small t antigen (small t) in these cells: 3T3 cells do not express small t and do not stabilize p53 upon infection with wild-type SV40. However, ectopic expression of small t in 3T3 cells provided these cells with the capacity to stabilize p53 upon SV40 infection. Conversely, precrisis mouse embryo cells express small t and mediate metabolic stabilization of p53 upon infection with wild-type SV40. Infection of these cells with an SV40 small-t deletion mutant did not lead to metabolic stabilization of p53. Small-t expression and metabolic stabilization of p53 correlated with an enhanced transformation efficiency by SV40, supporting the conclusion that at least part of the documented helper effect of small t in SV40 transformation is its ability to promote metabolic stabilization of p53 complexed to large T.

The tumor suppressor p53 plays a pivotal role during carcinogenesis (3, 18, 19, 21). In its wild-type (wt) form, p53 is one of the key elements in maintaining genetic stability (23, 24, 29, 49). Mutant p53 has lost this function but may have acquired properties of a dominant oncogene (7, 8, 26). Because of rapid turnover, levels of wt p53 in normal cells are low (36, 37). However, after exposure of cells to genotoxic agents, p53 levels rise because of metabolic stabilization of the p53 protein, and the function of wt p53 as "guardian of the genome" becomes activated (25). Very often, mutant p53 in transformed and tumor cells, acting as a dominant (or dominant-negative) oncogene, also is expressed at elevated levels because of metabolic stabilization of the p53 protein (7, 8, 14, 22). Thus, metabolic stabilization of the p53 protein appears to be one of the most prominent means for activating p53 functions, both as a tumor suppressor in its wt form and as a dominant oncogene in its mutant form.

Accumulation of wt p53 due to metabolic stabilization also is a key event in simian virus 40 (SV40) transformation (5, 6). The majority of p53 in SV40-transformed cells is complexed to the major transforming protein of this DNA tumor virus, the SV40 large tumor antigen (large T) (27, 28). However, as shown previously, metabolic stabilization of p53 complexed to large T is not simply the result of the physical interaction of these proteins but rather is an SV40-induced, active cellular process (6, 9, 10). In this regard, the interaction of p53 with large T differs strikingly from its interaction with the oncogenic E6 protein of human papovaviruses types 16 and 18, which leads to rapid degradation and thus functional elimination of p53 (44). We therefore concluded that, in contrast to E6-mediated inactivation, metabolic stabilization and the ensuing accumulation of p53 during S40 transformation represent a mechanism to activate a function of wt p53 which enhances the transforming potential of SV40. This conclusion was strongly supported by our recent finding that nonpermissive mouse and rat cells, in which p53 became metabolically stabilized after abortive infection with SV40, were transformed with a higher efficiency than were infected cells in which p53 remained unstable (46). Furthermore, precrisis BALB/c mouse embryo fibroblasts (pMEFd17 cells) were transformed by SV40 with a significantly higher frequency than were either freshly immortalized, 3T3-like cells derived from pMEFd17 cells (FTE cells) or authentic BALB/c 3T3 cells (47). Since immortalized cells are considered to be "pretransformed" (42), these results were rather unexpected. Analysis of p53 stability in these cells, however, revealed that p53 complexed to large T was stable in pMEFd17 cells after SV40 infection, whereas it remained unstable in FTE and 3T3 cells (47), further corroborating the correlation between metabolic stabilization of p53 and SV40 transformation efficiency.

The availability of a matched pair of cells, differing in their responses to SV40 with regard to transformation susceptibility and p53 stabilization (precrisis mouse embryo fibroblasts [pMEFd17 cells] and immortalized cells established from pMEFd17 cells [FTE and 3T3 cells]) provided a tool for analyzing the molecular basis for these differences. Here we show that the major difference between these systems is in the expression of SV40 small t antigen (small t), which is expressed in pMEFd17 cells, but not in FTE and 3T3 cells, infected with SV40. We demonstrate that expression of small t in abortively SV40-infected cells closely correlates with metabolic stabilization of p53 and with the transforming potential of SV40. This further supports our hypothesis that metabolic stabilization of p53 during SV40 transformation is a cellular event actively

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induced by viral genes and not a mere corollary of SV40 transformation.

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MATERIALS AND METHODS

Cells. Precrisis mouse embryo fibroblast d17 cultures (pMEFd17) were derived from primary BALB/c mouse embryo fibroblasts as described previously (46, 47). pMEFd17 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% newborn calf serum and used for experiments between passages 3 and 5. BALB/c 3T3 cells (1), FTE cells (47), the SV40-transformed BALB/c 3T3 cell line SV3T3 (clone 4) (9), normal Fisher rat fibroblast F111 cells (13), and Fisher rat fibroblast FR3T3 cells (39) were grown as described previously (46, 47). COS cells (15) were grown in DMEM supplemented with 10% fetal calf serum (FCS).

Establishment of BALB/c 3T3st cell lines. (i) Vectors. pw2t is an SV40 small-t expression vector (4) and was kindly provided by K. Rundell, Northwestern University, Chicago, Ill. The vector phyg mediates resistance to hygromycin (45) and was kindly provided by H. Abken, University of Bonn, Bonn, Germany.

(ii) Transfection. BALB/c 3T3 cells were transfected with 18 μg of pw2t and 2 μg of phyg as described previously (50). After 2 weeks of selection with 200 μg of hygromycin B (Boehringer) ml^{-1}, hygromycin-resistant colonies were picked and expanded into cell lines (BALB/c 3T3st) for further analyses.

Abortive infection. SV40 *dl*2111 was kindly provided by J. Feunteun. SV40 *dl*2111 carries a deletion of 269 bp between nucleotides 4558 and 4826 within the large-T intron and therefore is small-t negative (12). Abortive infection of the cells with wt SV40 and SV40 *dl*2111 was performed exactly as described previously (46, 47). A multiplicity of infection (MOI) of 1 corresponds to a dilution of the virus stock at which about 90% of the infected cells were positive for large-T expression by immunofluorescence microscopy analysis.

Immunocytochemical staining of small t. SV40-infected cells grown on coverslips were fixed 48 h postinfection (hpi) for 15 min at 4°C with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) containing 1 mM Ca^{2+}-Mg^{2+}, rinsed twice with PBS, and permeabilized for 10 min with acetone at 24°C. The cells were then rinsed again twice with PBS and incubated with the monoclonal antibody PAb280, which is specific for small t (33) (hybridoma supernatant diluted with PBS containing 10% FCS), overnight at 4°C. The cells were rinsed twice with PBS and further incubated with a horse anti-mouse immunoglobulin-biotin conjugate (Vektor Laboratories Inc., Burlingame, Calif.) (10 μg/ml in PBS containing 10% FCS) for 45 min at 24°C. Staining was performed with Avidin-Cy3 (Dianova, Hamburg, Germany) at a 1:100 dilution in PBS containing 10% FCS for 1 h at 24°C.

Cloning in soft agar. Uninfected and SV40-infected cells were plated in four parallel assays at 10^5, 10^4, and 10^3 cells per 35-mm-diameter culture dish in DMEM containing 20% FCS and 0.3% (wt/vol) agar (Bacto-agar; Difco Laboratories) onto a bottom layer of 0.5% (wt/vol) agar in DMEM. Colonies were scored 14 days after plating and designated positive when they were larger than 20 cells. Average values are presented.

Radiolabeling, extraction, immunoprecipitation, SDS-PAGE, and Western blotting (immunoblotting). Equal numbers of subconfluent cells were pulse-labeled (15- to 60-min pulse) and pulse-chase-labeled (15- to 60-min pulse and four different periods of chase) with [^{35}S]methionine-cysteine (Translabel; ICN) in 2 ml of methionine- and cysteine-free DMEM containing 5% dialyzed FCS per dish (100-mm diameter). To enable simultaneous lysis, the cells were labeled during time intervals defined by the chase periods. Cells were washed twice with ice-cold PBS and extracted for 30 min at 4°C with lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM dithiothreitol, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 30 g [200 kIU] of aprotenin [Trasylol; Bayer] per ml, 50 M leupeptin, pH 8.0). Extracts were cleared by centrifugation for 30 min at 12,000 × *g* and 4°C.

SV40 large T, SV40 large T-p53 complexes, SV40 17kT (51), and SV40 small t were immunoprecipitated with the monoclonal antibody PAb419 (16). p53 was immunoprecipitated with the monoclonal antibody PAb421 (17). All antibodies were used in excess over the respective antigens. Immunoprecipitation and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were carried out as described previously (46, 47). Labeled proteins were visualized by fluorography. SV40 large-T and p53 bands were quantitated by densitometric scanning.

mRNA and cDNA analysis. Total RNA isolation and first-strand cDNA synthesis of 1 μg of total RNA were done according to standard protocols (43). PCR was performed as described previously with primers EN5 (5′-TGCAAG GAGTTTCATCCTGATAAAGG-3′) and HM2 (5′-TTTTAGAATTCAGGC CTACAGTGTTTTAGGCACACTGTACTCATTC-3′) (51). SDS-PAGE analysis of amplified DNA and silver staining were done as described previously (51).

RESULTS

Precrisis and established mouse BALB/c fibroblasts differ in SV40 small-t expression. To establish the molecular basis for the different behaviors of precrisis and immortalized BALB/c mouse fibroblasts with regard to metabolic stabilization of p53 upon SV40 infection (47), we first compared the expression of SV40 early proteins in BALB/c 3T3 cells and in pMEFd17 cells after SV40 infection. Figure 1A and B show that infection of 3T3 and pMEFd17 cells, respectively, with SV40 resulted in the expression of similar levels of large T. Large T was metabolically stable in both cell types, which led to equivalent steady-state levels of large T in both types of cells (47). This excluded the possibility that a dosage effect from a difference in large-T expression was responsible for the difference in p53 stabilization between SV40-infected 3T3 cells (Fig. 1A) and SV40-infected pMEFd17 cells (Fig. 1B).

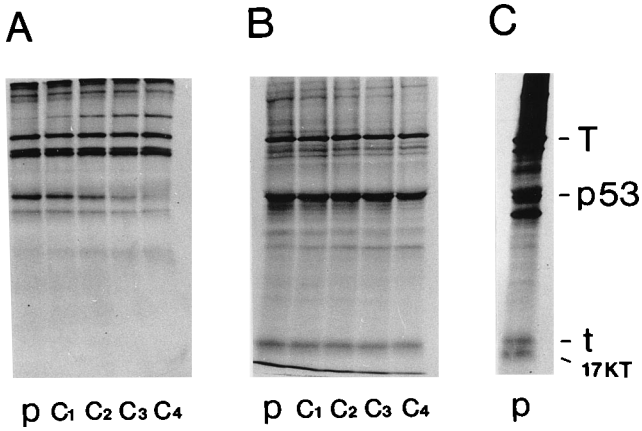

FIG. 1. Expression of SV40 early proteins in BALB/c 3T3 and pMEFd17 cells after infection with SV40. Equal numbers of subconfluent BALB/c 3T3 cells (A) and pMEFd17 cells (B) abortively infected with wt SV40 (MOI of 1) at 48 hpi were pulse-labeled (p) and pulse-chase-labeled (c_1 to c_4) with 100 μCi of [^{35}S]methionine-cysteine (20-min pulse and 15-min [c_1], 30-min [c_2], 60-min [c_3], and 90-min [c_4] chases). COS cells (C) were labeled with 100 μCi of [^{35}S]methionine-cysteine for 1 h. Whole-cell extracts were immunoprecipitated for large T, large T-p53 complexes, SV40 17kT, and SV40 small t with the monoclonal antibody PAb419. Immunoprecipitates were analyzed on an SDS–12.5% polyacrylamide gel. Labeled proteins were visualized by fluorography.

A comparison of panels A and B of Fig. 1 revealed that abortively infected pMEFd17 cells expressed an additional SV40-specific protein of about 19 kDa which was not seen in immunoprecipitations of cellular extracts from 3T3 cells. This protein was identified as SV40 small t by tryptic peptide analysis (data not shown). Neither pMEFd17 nor 3T3 cells expressed the large-T-related 17kT protein expressed in stably SV40-transformed cells (51) (i.e., in the COS cells shown in Fig. 1C). Immortalized FTE cells, established from pMEFd17 cells in our laboratory (47) by the original 3T3 protocol (1), showed the same expression pattern of SV40 early proteins as did 3T3 cells (see Fig. 4), indicating differences in SV40 early gene expression between abortively infected precrisis and 3T3 cell-like established mouse cells, as well as between those and stably SV40-transformed cells.

SV40 small t cooperates with SV40 large T in metabolic stabilization of p53. SV40 small t exerts a so-far not clearly defined auxiliary role in SV40 transformation (2, 32, 35, 41). The correlation between SV40 small-t expression and metabolic stabilization of p53 complexed to large T in the cellular system described above raised the question whether small t, in abortively SV40-infected cells, might mediate metabolic stabilization of p53 in complex with large T. If small t indeed cooperated with large T in metabolic stabilization of p53, one would expect that, upon SV40 infection, constitutive expression of small t in 3T3 cells should lead to metabolic stabilization of p53 in complex with large T. Conversely, upon infection

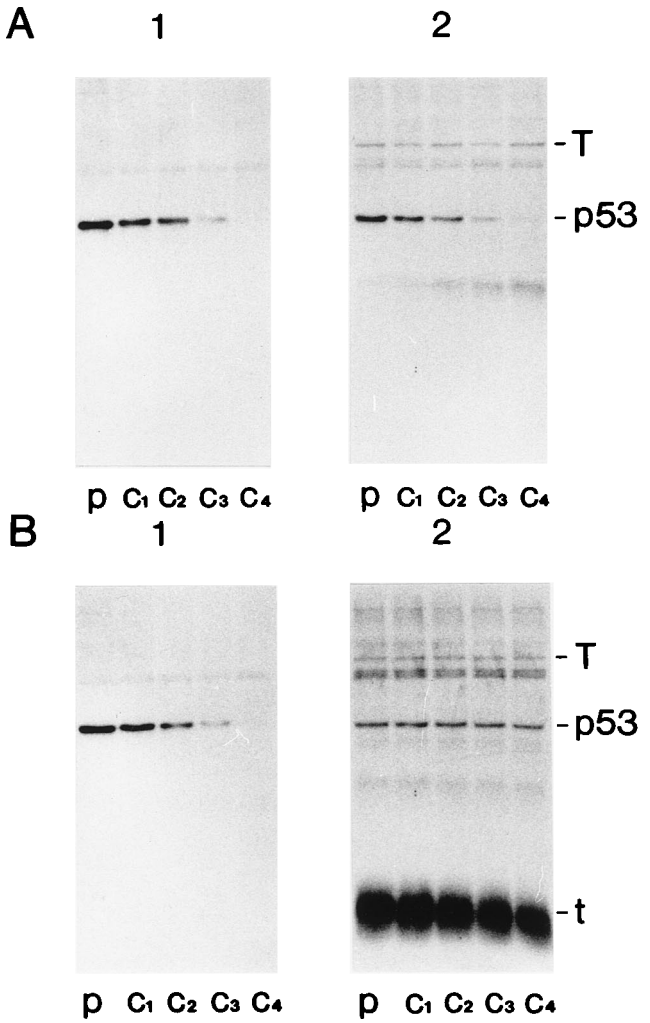

FIG. 2. Constitutive expression of small t in BALB/c 3T3 cells leads to metabolic stabilization of p53 complexed to large T upon SV40 infection. BALB/c 3T3 cells were transfected with the SV40 small-t expression vector pw2t. After hygromycin selection, resistant colonies were picked and expanded into cell lines (BALB/c 3T3st) (see Materials and Methods). Uninfected BALB/c 3T3 cells (A, panel 1), uninfected BALB/c 3T3st cells (B, panel 1), and abortively infected BALB/c 3T3 (A, panel 2) and BALB/c 3T3st (B, panel 2) cells (MOI of 1) at 48 hpi were pulse-labeled (p) and pulse-chase-labeled (c_1 to c_4) as described in the legend to Fig. 1. Whole-cell extracts were immunoprecipitated for large T, large T-p53 complexes, and small t with the monoclonal antibody PAb419 (panels 2). p53 was immunoprecipitated with the monoclonal antibody PAb421 (panels 1). The immunoprecipitates were analyzed by SDS–12.5% PAGE followed by fluorography.

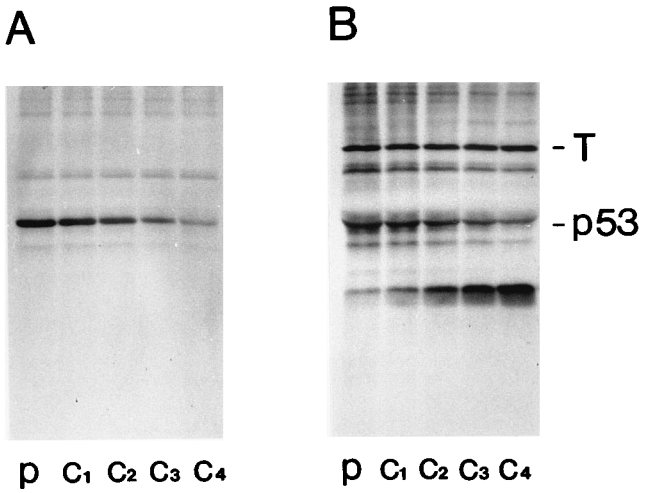

FIG. 3. p53 in complex with large T remains metabolically unstable in pMEFd17 cells infected with the SV40 small-t deletion mutant virus SV40 *dl*2111. Uninfected pMEFd17 cells (A) and pMEFd17 cells abortively infected with SV40 *dl*2111 (MOI of 1) (B) at 48 hpi were pulse-labeled (p) and pulse-chase-labeled (c_1 to c_4) as described in the legend to Fig. 1. Whole-cell extracts were immunoprecipitated for large T, large T-p53 complexes, and small t with the monoclonal antibody PAb419. p53 was immunoprecipitated with the monoclonal antibody PAb421. The immunoprecipitates were analyzed by SDS–12.5% PAGE followed by fluorography. The prominent band below p53 in panel B is a 35-kDa cellular protein, cross-reacting with the monoclonal antibody PAb419 (16), which is not related to p53.

of pMEFd17 cells with an SV40 small-t deletion mutant virus, the p53 in these cells should remain metabolically unstable.

To explore this possibility, we stably transfected 3T3 cells with the small-t expression vector pw2t (4), resulting in stable expression of small t in the resulting 3T3st cells (Fig. 2B, panel 2). Expression of small t in 3T3st cells as such did not affect the metabolic stability of the p53, as it exhibited the same turnover as p53 in the parental 3T3 cells (Fig. 2, panels 1). However, upon infection with SV40, the p53 complexed to large T in 3T3st cells became metabolically stabilized (Fig. 2B, panel 2).

In the reverse experiment, pMEFd17 cells were infected with the SV40 small-t deletion mutant virus SV40 *dl*2111 (see Materials and Methods). As expected, p53 in complex with large T remained metabolically unstable (compare the p53 stabilities in Fig. 3A and B) in these cells, like p53 in 3T3 cells abortively infected with wild-type SV40 (Fig. 1A). These data thus conclusively demonstrated that large T and small t cooperated in metabolic stabilization of p53 during abortive infection of these nonpermissive cells.

This stringent correlation between SV40 small-t expression and metabolic stabilization of p53 complexed to large T during SV40 abortive infection was not restricted to the cellular systems described above. Analysis of a panel of different established mouse and rat fibroblasts (Fig. 4) demonstrated that p53 complexed to large T was metabolically stable when such cells expressed small t and was unstable when small-t expression

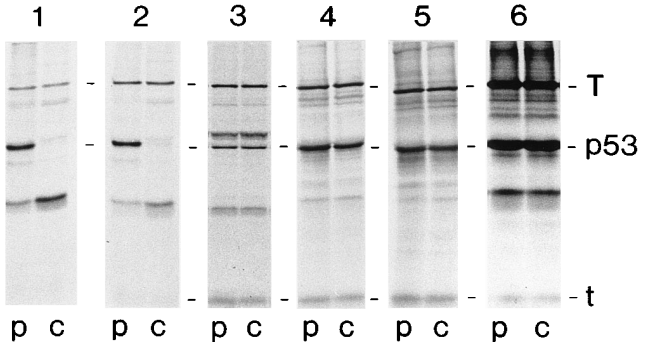

FIG. 4. Correlation between SV40 small-t expression and metabolic stabilization of p53 during SV40 abortive infection in several mouse and rat fibroblasts. Equal numbers of FR3T3 cells (lanes 1), FTE cells (lanes 2), F111 cells (lanes 3), Swiss 3T3 cells (lanes 4) and pMEFd17 cells (lanes 5) abortively infected with wt SV40 (MOI of 1) at 48 hpi, and SV40-transformed SV3T3 cells (lanes 6) were pulse-labeled (lanes p) and pulse-chase-labeled (lanes c) with 100 μCi of [^{35}S]methionine-cysteine (20-min pulse and 120-min chase). Whole-cell extracts were immunoprecipitated for large T, large T-p53 complexes, and SV40 small t with the monoclonal antibody PAb419. The immunoprecipitates were analyzed by SDS–12.5% PAGE followed by fluorography.

could not be detected (Fig. 4). The analysis of different cells shown in Fig. 4 in addition revealed that the difference between pMEFd17 cells and 3T3 or FTE cells, respectively, in small-t expression and metabolic stabilization of p53 was not an a priori consequence of immortalization, since other immortalized cells, like Swiss mouse 3T3 cells (Fig. 4, lanes 4) and rat F111 cells (Fig. 4, lanes 3) expressed small t and mediated metabolic stabilization of p53 complexed to large T.

SV40 small-t-mediated p53 stabilization in abortively SV40-infected cells directly correlates with SV40 transformation efficiency. The identification of small t as an important component of the viral strategy to stabilize p53 allowed us to more directly address the question of the biological relevance of this process for the transforming capacity of SV40. Table 1 demonstrates that wt SV40 was able to transform pMEFd17 cells with an efficiency about 10 times higher than that with 3T3 or FTE cells when transformed cells were selected by anchorage-independent growth in soft agar, which is considered to be the most stringent criterion for in vitro transformation (40). If small-t-mediated metabolic stabilization of p53 in pMEFd17 cells after abortive SV40 infection relates to the enhanced transformation efficiency of SV40 in these cells, one should expect a drastic reduction in SV40 transformation efficiency after infection of these cells with the SV40 small-t deletion mutant virus *dl*2111. Table 1 indeed demonstrates that neither infection of pMEFd17 cells nor infection of 3T3 or FTE cells with the SV40 small-t deletion mutant virus *dl*2111 resulted in colonies of transformed cells with this stringent transformation assay. This result thus further strengthens the correlation between small-t expression, metabolic stabilization of p53, and efficient transformation of these cells by SV40.

Posttranscriptional control of SV40 early gene expression in abortively SV40-infected cells. As shown above (Table 1), the SV40 small-t deletion mutant virus *dl*2111 was unable to generate transformed colonies in soft agar after infection of either pMEFd17, 3T3, or FTE cells. On the other hand, abortive infection of 3T3 or FTE cells with wt SV40 led to such transformants, despite the fact that these cells did not detectably express small t after abortive infection with SV40 (Fig. 1). To resolve this apparent contradiction, we asked whether perhaps small-t expression might be restricted to only a few abortively infected 3T3 cells, which then could form a reservoir for cellular transformation by SV40. On the other hand, small t expressed in only a minor fraction of such cells would not be detectable in bulk cell analyses for small-t expression, e.g., immunoprecipitation of whole-cell extracts as shown in Fig. 1. Comparative single-cell analyses of pMEFd17 and 3T3 cells, abortively infected with SV40, for the expression of small t by using immunofluorescence staining of the cells with the small-t-specific monoclonal antibody PAb280 (33) indeed revealed that virtually all SV40-infected pMEFd17 cells displayed small-t staining (Fig. 5A), whereas only few (approximately 0.1%) of the infected 3T3 cells could be stained for small t (Fig. 5B). In support of our assumption that such cells could form a reservoir for efficient SV40 transformation, we observed that cells in all colonies of SV40-transformed 3T3 cells established from abortively SV40-infected 3T3 cells via colony formation in soft agar expressed small t (and 17kT). As an example, Fig. 6A demonstrates that the SV3T3 cells (lane 2), derived from abortively SV40-infected 3T3 cells, express small t. Figure 6A also shows that the large-T-related 17kT protein is expressed in SV3T3 cells but not in either abortively SV40-infected 3T3 cells or abortively SV40-infected pMEFd17 cells (Fig. 1). This suggested that stable transformation of 3T3 cells with SV40 is accompanied by a change in SV40 early gene expression. To determine the level controlling small-t and 17kT expression in 3T3 cells, we analyzed SV40 early mRNA expression in 3T3 cells and compared it with that in pMEFd17 cells and in SV3T3 cells. Since small-t mRNA in SV40-transformed cells is difficult to visualize by Northern (RNA) blot analysis (51), SV40-specific mRNA was analyzed after reverse transcription and PCR amplification, as described previously (51). Assuming similar levels of amplification of SV40-specific cDNA, Fig. 6B, lanes 2 and 3, shows that 3T3 and pMEFd17 cells contained approximately similar levels of mRNAs for small t, large T, and the recently identified SV40 17kT protein. However, neither small t nor 17kT was detectably expressed at the protein level in SV40-infected 3T3 cells (Fig. 6A). On the other hand, stably transformed SV3T3 cells expressed these proteins, although they did not contain enhanced levels of small-t mRNA (Fig. 6B, lane 4). Therefore, we assume that expression of small t and 17kT in abortively SV40-infected 3T3 cells is controlled at the level of translation. Such a translational control also seems to apply for 17kT expression in SV40-infected pMEFd17 cells. However, this translational block seems to be alleviated in a few abortively infected cells, as shown for the expression of

TABLE 1. Transformation efficiencies of wt SV40 and the SV40 small-t deletion mutant virus SV40 *dl*211 in precrisis BALB/c MEFd17 cells, BALB/c FTE cells, and BALB/c 3T3 cells

Cells[a]	Half-life of p53[b]	Colonies of transformed cells in soft agar[c] with the following no. of cells input:			Efficiency of colony growth (%)
		10^5	10^4	10^3	
pMEFd17, uninfected (control)	20 min	0	0	0	0
pMEFd17, wt SV40 infected	>90 min[d]	100	8	1	~0.1
pMEFd17, SV40 *dl*2111 infected	60 min	0	0	0	0
FTE, uninfected (control)	15 min	0	0	0	0
FTE, wt SV40 infected	30 min	8	1	0	~0.01
FTE, SV40 *dl*2111 infected	30 min	0	0	0	0
BALB/c 3T3, uninfected (control)	15 min	2	0	0	0.002
BALB/c 3T3, wt SV40 infected	40 min	14	1–2	0	~0.01
BALB/c 3T3, SV40 *dl*2111 infected	40 min	2	0	0	0.002
SV3T3	>24 h	—[e]	—	600–800	60–80

[a] For infected cells, the MOI was ~1.
[b] The half-life of p53 was determined by graphic evaluation of densitometer scans of radiolabeled p53 from pulse-chase experiments shown previously (46, 47) and in Fig. 1 to 3.
[c] Average values are presented (see Materials and Methods).
[d] As determined from Fig. 1. The actual half-life is >8 h.
[e] —, not evaluable.

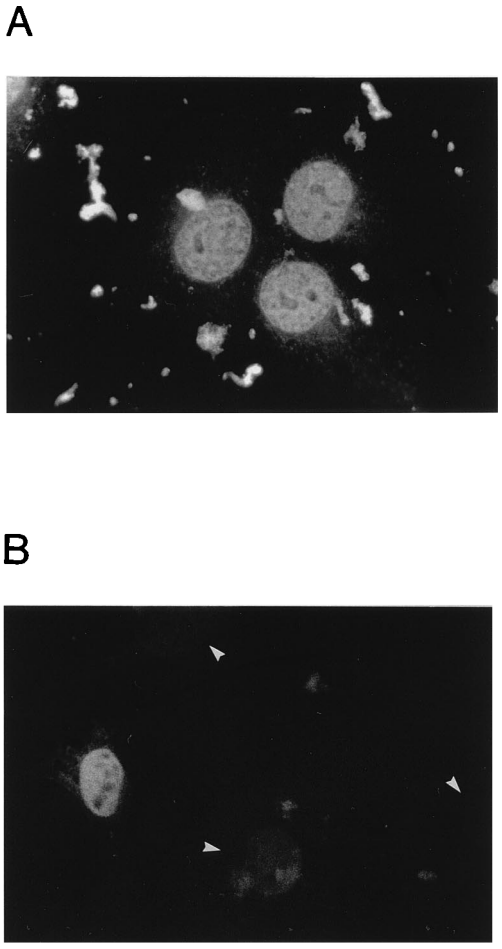

FIG. 5. Immunocytochemical staining of small t in abortively SV40-infected BALB/c pMEFd17 (A) and 3T3 (B) cells. Cells were seeded on coverslips, infected with wt SV40 (MOI of 1), and fixed at 48 hpi. Staining was performed with the small-t monoclonal antibody PAb280 (33) as described in Materials and Methods. The arrowheads in panel B indicate SV40-infected 3T3 cells which could not be stained for small t with PAb280.

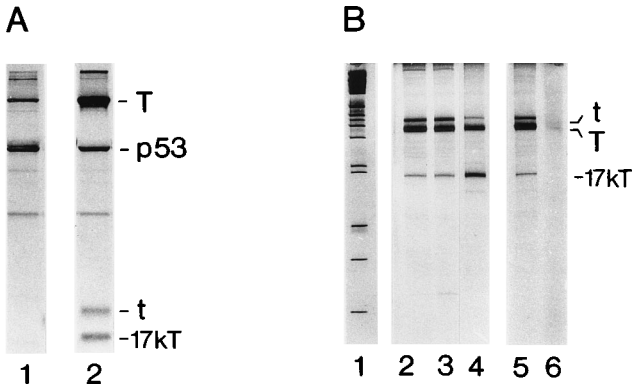

FIG. 6. (A) Analysis of small-t expression in SV40-infected BALB/c 3T3 cells and in BALB/c 3T3-derived stably SV40-transformed cells (SV3T3) at the protein level. Equal numbers of subconfluent BALB/c 3T3 cells (lane 1) and SV3T3 cells (lane 2) were labeled with 100 μCi of [^{35}S]methionine-cysteine for 1 h. Whole-cell extracts were immunoprecipitated for large T, large T-p53 complexes, 17kT, and small t with the monoclonal antibody PAb419. The immunoprecipitates were analyzed by SDS–15% PAGE followed by fluorography. (B) Analysis of small-t expression in various SV40-infected and -transformed cells at the transcriptional level. Total RNA was isolated from abortively infected BALB/c 3T3 (lane 2) and pMEFd17 (lane 3) cells at 48 hpi, SV3T3 cells (lane 4), and COS cells (lane 5). After synthesis of first-strand cDNA, PCR was performed with SV40 primers EN5 and HM2 (see Materials and Methods). Amplified DNA was separated by SDS-PAGE and visualized by silver staining. Lane 1, lambda DNA *Bst*EII digest; lane 6, negative control (H_2O).

small t in 3T3 cells (Fig. 5), which could account for the expression of small t as well as of 17kT in stably SV40-transformed cells.

DISCUSSION

The data presented in this paper established a strict correlation between the expression of the SV40 early proteins large T and small t and the metabolic stabilization of p53 during abortive infection of nonpermissive rodent cells. Furthermore, small-t expression and metabolic stabilization of p53 correlated with an enhanced transformation potential of SV40. The cooperation of small t and large T in metabolic stabilization of p53 thus identifies a new function of small t during SV40 transformation which seems to relate to the helper function of small t in SV40 transformation (2). It is important to note that ectopic expression of small t in 3T3st cells as such did not alter the metabolic stability of p53 but required the concomitant expression of large T (Fig. 2). This situation is different from that with adenovirus infection, in which metabolic stabilization of p53 is mediated by the 12S E1A gene product and is independent from complex formation with the E1B 55-kDa tumor antigen (31). We therefore conclude that SV40-induced metabolic stabilization of p53 is mediated by a cellular pathway, which requires primarily the expression of large T and, in addition, the cooperation of small t with large T, at least in the nonpermissive cells analyzed here. So far, nothing is known about this pathway, but the systems described here should allow its identification. Small t exerts at least two independent functions: (i) transactivation of cellular genes (30) and (ii) modulation of phosphatase 2A activity by binding to the catalytic subunit of phosphatase 2A and substituting for the regulatory B subunit (48). We favor the hypothesis that small t and large T cooperate in inducing or repressing a cellular factor that is involved in controlling p53 turnover, rather than that there is a simple alteration of p53 phosphorylation by the interaction of small t with phosphatase 2A. This is supported first by the finding that small-t expression in 3T3 cells as such did not alter p53 turnover and second by our finding that phosphorylation of p53 from SV40-infected pMEFd17 cells and SV40-infected 3T3 cells is at least qualitatively indistinguishable (data not shown). Our assumption that a virus-induced (or repressed) cellular factor, rather than altered phosphorylation of p53, is responsible for the stabilization of p53 complexed to large T in SV40 abortive infection is further supported by our finding that during productive infection of permissive monkey cells, small t is not required in this process, as p53 also becomes metabolically stabilized after infection of such cells with the SV40 small-t deletion mutant virus *dl*2111 (unpublished observation). One possible explanation of this finding would be that the high level of large T expressed in lytically infected cells can substitute for the small-t requirement. However, we previously noted that high-level expression of large T in abortively SV40-infected 3T3 cells as such was not sufficient to induce metabolic stabilization of p53 complexed to large T (9), again emphasizing the requirement for a cellular factor in this process. Regarding the cooperative role of large T and small t in metabolic stabilization of p53 complexed to large T, it is interesting that the amino-terminal 82 amino acids are common to large T, 17kT, and small t (51). Since large T

and small t seem to exert a common function required for transformation which maps to their amino-terminal regions (34), and since all SV40-transformed cells in addition express the SV40 17kT protein, which basically represents a truncated amino-terminal fragment of large T (51), it is tempting to speculate that the amino-terminal region common to all three SV40 tumor antigens might play a critical role in metabolic stabilization of p53. Further analysis of p53 stabilization in 3T3 cells stably transfected with defined small-t mutants (35) should answer this question and provide insight into which small-t function is required for metabolic stabilization of p53. Such studies are currently under way in our laboratory.

Considering that metabolic stabilization of p53 is a viral function, the correlation between metabolic stabilization of p53 and enhanced transformation efficiency observed in this study and in previous studies (46, 47) implies that wt p53 in SV40 transformation is not simply inactivated by complex formation but that, instead, stabilized p53 is used as an important cofactor in SV40 transformation. This appears to be an unusual role for wt p53. However, since wt p53 complexed to large T has lost at least some of the properties of free wt p53, such as sequence-specific transactivation of p53-responsive genes (11, 38), by this criterion it behaves like a mutant p53. To explain the cooperative function of p53 in SV40 transformation, one thus could hypothesize that wt p53 in a complex with large T might exert properties of a gain-of-function mutant p53. However, since the molecular basis for gain-of-function mutations in p53 is not yet known, it so far is not possible to test this hypothesis at the molecular level. Alternatively, by complexing with large T, p53 might activate a function of large T which is important for cellular transformation. Metabolic stabilization of p53 then would increase the level of such p53-complexed, transformation-competent large T.

As an important side aspect of our studies, we found that various rodent cells differed in the expression of the SV40 early proteins small t and 17kT, although all cells expressed the corresponding mRNAs. Our assumption that expression of these proteins is controlled at the level of translation is supported by our finding that a minority of SV40-infected 3T3 cells expressed small t (and probably also 17kT), indicating that the mRNA coding for these proteins, and present in these cells (Fig. 6B), is functional. Since all SV40-transformed cells derived from abortively infected 3T3 cells, such as the SV3T3 cells shown in Fig. 6A, expressed small t and 17kT, we assume that such cells form a reservoir for efficient transformation by SV40. The restriction of small-t expression in 3T3 (and FTE) cells to only a small percentage of these cells also explains the apparent paradox that these immortalized cells are transformed by SV40 with a lower efficiency than precrisis pMEFd17 cells. The molecular basis for the postulated translational control of small-t (and 17kT) expression in 3T3 and FTE cells is not yet known. However, in the light of our finding that expression of small t in abortively infected nonpermissive cells correlated with metabolic stabilization of p53, it is noteworthy that expression of small t was affected during immortalization of precrisis mouse embryo fibroblasts by the 3T3 scheme (3T3 and FTE cells). Since the p53 pathway very often is a target during immortalization of precrisis cells (20, 47), this points to the interesting possibility that translational control of small-t expression might reflect a cellular alteration relating to alterations in the p53 pathway of these cells.

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