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Modulation of p53 Protein Expression during Cellular Transformation with Simian Virus 40

WOLFGANG DEPPERT,†* MARTINA HAUG, AND THOMAS STEINMAYER

Department of Biochemistry, University of Ulm, D-7900 Ulm, Federal Republic of Germany

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We analyzed the relation of metabolic stabilization of the p53 protein during cellular transformation by simian virus 40 (SV40) to (i) expression of the transformed phenotype and (ii) expression of the large tumor antigen (large T). Analysis of SV40-*tsA*28-mutant-transformed rat cells (tsA28.3 cells) showed that both p53 complexed to large T and free p53 (W. Deppert and M. Haug, Mol. Cell. Biol. 6:2233–2240, 1986) were metabolically stable when the cells were cultured at 32°C and expressed large T and the transformed phenotype. At the nonpermissive temperature (39°C), large-T expression is shut off in these cells and they revert to the normal phenotype. In such cells, p53 was metabolically unstable, like p53 in untransformed cells. To determine whether metabolic stabilization of p53 is directly controlled by large T, we next analyzed the metabolic stability of complexed and free p53 in SV40 abortively infected normal BALB/c mouse 3T3 cells. We found that neither p53 in complex with large T nor free p53 was metabolically stable. However, both forms of p53 were stabilized in SV40-transformed cells which had been developed in parallel from SV40 abortively infected cultures. Our results indicate that neither formation of a complex of p53 with large T nor large-T expression as such is sufficient for a significant metabolic stabilization of p53. Therefore, we suggest that metabolic stabilization of p53 during cellular transformation with SV40 is mediated by a cellular process and probably is the consequence of the large-T-induced transformed phenotype.

p53 is a cellular protein involved in the control of proliferation in normal cells (for reviews, see references 21 and 30). p53 also has an oncogenic potential: transfection of p53 expression vectors into primary cells resulted in immortalization (13) and, in cooperation with an activated v-*ras* oncogene, in full transformation (6, 25). Therefore, abnormal expression of p53 seems to be causally related to cellular transformation. Abnormal expression of p53 in transformed cells often is manifested by increased steady-state levels of the protein (for reviews, see references 3, 21, and 30). Such elevated levels may result from an increase in p53 mRNA abundance or from increased metabolic stability of the p53 protein or both (22, 23, 26). Whereas increased p53 mRNA levels are observed only in certain transformed cell lines (22, 23, 26), increased metabolic stability of the p53 protein seems to be a characteristic alteration of p53 expression in transformed cells. Although the molecular basis underlying the process of metabolic stabilization of p53 in transformed cells is not known, most often it does not seem to result from mutagenic activation of the p53 gene but rather to be a posttranscriptional or posttranslational event (22, 23, 26). However, mutagenic activation of the p53 gene resulting in expression of metabolically stable mutant p53 proteins has also been described (12, 19, 31).

p53 was first detected in simian virus 40 (SV40)-transformed cells, in which it forms a stable complex with the transforming protein of SV40, the large tumor antigen (large T; 15). In such cells, p53 levels are highly elevated as compared with those in their untransformed counterparts, and p53 in complex with large T exhibits a metabolic stability similar to large T itself (16, 17, 22). These findings suggested that formation of a complex of large T with p53 may be the cause of the increased metabolic stability of p53 in SV40-transformed cells (for a review, see reference 3). This view was supported by experiments described by Linzer et al. (17) which demonstrated that metabolic stabilization of p53 depends on the expression of a functional large T. However, formation of a p53 complex with a transforming protein could not explain the increase in metabolic stability of p53 in many other transformed cells: except for formation of complexes with large T of SV40 and several other papovaviruses (for a review, see reference 3) and with the adenovirus E1b 58K tumor antigen (33), no other complexes of p53 with viral or cellular transforming proteins have been reported. Furthermore, we have provided evidence that SV40-transformed cells also contain p53 molecules which are not complexed to large T (free p53) but are metabolically stable, like p53 in non-SV40-transformed cells, e.g., MethA cells (5). This finding suggested that at least the free p53 in SV40-transformed cells might become stabilized by a cellular process. This process should be independent of stabilization of p53 by formation of a complex with a viral tumor antigen, like SV40 large T, and might reflect a common cellular mechanism for p53 stabilization during transformation.

In this study, we further explored this hypothesis. We analyzed large T and p53 expression in SV40-*tsA*-mutant-transformed and SV40 abortively infected mouse and rat fibroblasts. Our data show that expression of a metabolically stable p53 in SV40-*tsA*-mutant-transformed cells correlates with the expression of a functional large T and the expression of the transformed phenotype. In normal mouse BALB/c 3T3 cells abortively infected with SV40, large-T expression as such is not sufficient to stabilize either p53 in complex with large T or the free p53 to an extent similar to

* Corresponding author.
† Present address: Heinrich-Pette-Institute for Experimental Virology and Immunology at the University of Hamburg, D-2000 Hamburg, Federal Republic of Germany.

that observed in SV40 stably transformed cells, i.e., p53 stabilization correlates with stable transformation. Therefore, we suggest that metabolic stabilization of p53 is an additional cellular step induced during transformation by SV40.

MATERIALS AND METHODS

Cells. BALB/c mouse 3T3 cells, Swiss mouse 3T3 cells, mouse NIH 3T3 cells, rat F111 cells, and rat FR3T3 cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal calf serum. SV40-mutant-*tsA*28-transformed rat embryo fibroblasts tsA28.3 (24) were cultured in DMEM supplemented with 10% fetal calf serum.

Abortive infection. Normal mouse and rat fibroblasts were infected with wild-type SV40 (strain 776) at multiplicities of infection (MOIs) between 1 and 100, as described below, and analyzed 24 or 48 h postinfection (p.i.). MOIs for abortive infections were determined by infecting BALB/c mouse 3T3 cells with purified SV40 diluted with DMEM plus 5% fetal calf serum in consecutive 1:10 dilution steps. A MOI of 1 in abortive infections was defined as the dilution of SV40 at which more than 95% of the infected cells were positive for SV40 large-T expression as judged by immunofluorescence analysis. It is important that the MOI determined in this way does not correspond to the MOI of the same virus preparation determined on permissive monkey TC7 cells.

Labeling of cells and in situ cell fractionation. A detailed description of the cell fractionation procedure, as well as a characterization of extracts and structures, has been given elsewhere (11, 34–36). Briefly, cells grown on petri dishes (9 cm) were washed with KM buffer [10 mM morpholinepropanesulfonic acid (MOPS; pH 6.8), 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 mM dithiothreitol (DTT), 10% glycerol] and lysed in KM buffer containing 1% Nonidet P-40 (NP-40) (nucleoplasmic extract). Nuclear structures still attached to the substratum (NP-40 nuclei) were extracted with KM buffer without DTT and EGTA and containing 100 μg of DNase I per ml (no. D-18530; Serva, Heidelberg, Federal Republic of Germany) for 15 min at 32°C. The DNase buffer was then adjusted to 2 M NaCl, 5 mM DTT, and 1 mM EGTA, and the incubation was continued for 30 min at 4°C (chromatin extract). Nuclear matrices were solubilized in TK buffer (40 mM Tris hydrochloride [pH 9.0], 25 mM KCl, 5 mM DTT, 10% glycerol) containing 1% Empigen BB (Albright and Wilson, Ltd.) for 60 min at 4°C. All buffers contained 30 μg of aprotinin (200 kIU; Trasylol; Bayer) per ml, and immediately after fractionation, phenylmethylsulfonyl fluoride was added to yield a concentration of 1 mM. The nucleoplasmic and nuclear matrix extracts were made 150 mM in NaCl and adjusted to pH 8.0 by the addition of 100 mM Tris hydrochloride (pH 8.0). NP-40 was added to the Empigen BB extract (nuclear matrix extract) to yield a final concentration of 2%. The chromatin extract was desalted on a small Sephadex G-25 column. These treatments helped to prevent degradation and denaturation of large T and p53 in the various extracts and allowed their quantitative recovery from the extracts by immunoprecipitation. Before fractionation, cells were labeled with [^{35}S]methionine (100 μCi per plate in 1 ml of methionine-free DMEM) or ^{32}Pi (200 μCi per plate in 1 ml of phosphate-free DMEM) as described previously (5, 7) and below. Whole-cell extracts were prepared by lysing the cells on the culture dish with lysis buffer (120 mM NaCl, 5 mM DTT, 50 mM Tris [pH 8.0], 1% NP-40).

Antibodies, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and Western blotting. SV40 large T and large T-p53 complexes were immunoprecipitated with monoclonal antibody PAb 108, which recognizes a non-denaturation-sensitive determinant at the amino-terminal end of large T (10). p53 was immunoprecipitated with monoclonal antibody PAb 122 (9).

All extracts were cleared by centrifugation at 130,000 × *g* for 30 min at 4°C. Extracts from SV40-transformed or SV40 abortively infected cells were then sequentially precipitated, first with 10 μl of PAb 108 ascitic fluid and then with 200 μl of settled protein A-Sepharose, as described previously (5). After the removal of immune complexes bound to protein A-Sepharose by centrifugation at 2,000 × *g*, immunoprecipitations with PAb 108 were repeated twice. Cleared extracts were then reprecipitated with 10 μl of PAb 122 ascitic fluid followed by 200 μl of settled protein A-Sepharose. Whole-cell extracts of the various mouse and rat fibroblast cells were directly precipitated with PAb 122 (uninfected cells) or PAb 108 (SV40 abortively infected cells). Samples were processed for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and analyzed on SDS-polyacrylamide gels as described previously (5, 35).

Western blot (immunoblot) analysis of steady-state levels of large T was performed exactly as described previously (11, 34).

RESULTS

Expression and metabolic stability of p53 in SV40-*tsA*-mutant-transformed cells at permissive and nonpermissive growth temperatures. SV40-transformed cells, in addition to p53 complexed to large T, contain free p53, which like the complexed form of p53 is metabolically stable (5). This suggested that the free form of p53 in these cells, like p53 in non-SV40-transformed cells (e.g., MethA cells), becomes stabilized by means other than a direct interaction with large T. In a first approach to determine whether the metabolic stability of free p53 in SV40-transformed cells correlates with the expression of the transformed phenotype, we analyzed the expression and metabolic stability of p53 in cells transformed by an SV40 mutant coding for a temperature-sensitive large T (*tsA* mutant). Specifically, we wanted to know whether free p53, which should be metabolically stable in cells expressing the transformed phenotype at the permissive growth temperature (5), would become metabolically unstable in these cells after they had reverted to the normal phenotype at the nonpermissive temperature, as is p53 in other normal cells (5, 22, 24). We chose to analyze the rat cell line tsA28.3 (4, 24). tsA28.3 cells are fully transformed at the permissive growth temperature (32°C) but revert to the normal phenotype after shift to the nonpermissive temperature (39°C) (4, 24). These cells are particularly suited for such an analysis since after shift to the nonpermissive growth temperature, they shut off large-T expression (4, 24). Therefore, any possible influence of even heat-inactivated large T on the metabolic stabilization of free p53 at the nonpermissive temperature can be ruled out since p53 expression under these growth conditions is completely under cellular control.

tsA28.3 cells were analyzed for p53 expression at the permissive and nonpermissive growth temperatures. Growing cells kept at the permissive (32°C) or nonpermissive (39°C) temperature for 3 days were pulse-labeled (1 h) and pulse-chase-labeled (1-h pulse and 2-h chase) with [^{35}S] methionine and subfractionated as described previously (5,

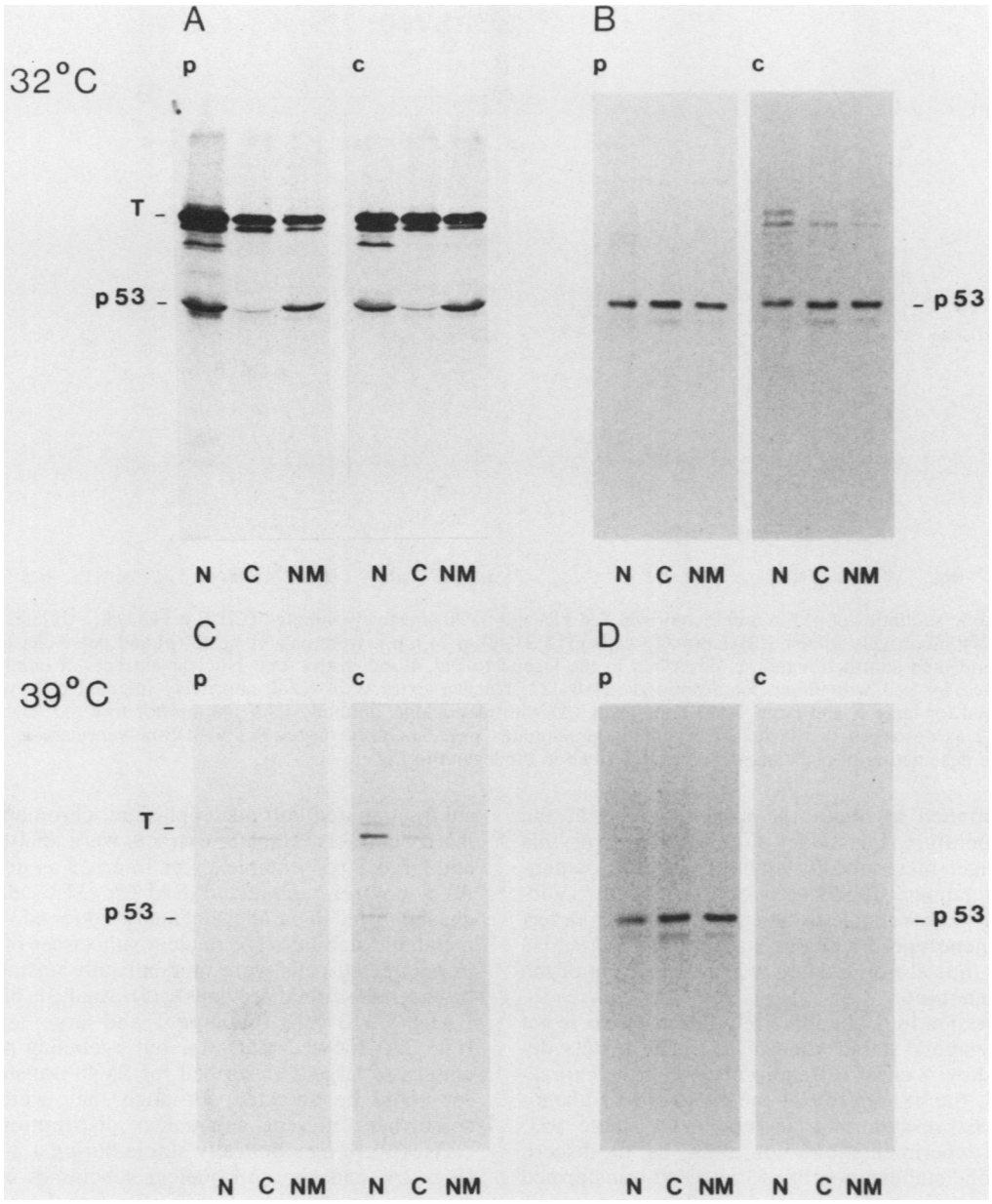

FIG. 1. Metabolic stabilities of p53 in tsA28.3 cells kept at the permissive (32°C) or nonpermissive (39°C) growth temperature. tsA28.3 cells kept at 32 or 39°C for 3 days were pulse-chase-labeled with [^{35}S]methionine (1-h pulse followed by a 2-h chase in normal growth medium) at the respective growth temperatures. Pulse-labeled (p) and chased (c) cells were subfractionated as described in the text. Nuclear extracts were sequentially immunoprecipitated, first for large T and large T-p53 complexes (A and C) with monoclonal antibody PAb 108 and then for free p53 (B and D) with monoclonal antibody PAb 122, as described in the text. The immunoprecipitates were analyzed on an 11.5% SDS-polyacrylamide gel, followed by fluorography. Nuclear extracts were the nucleoplasmic extracts (lanes N), chromatin extracts (lanes C), and nuclear matrix extracts (lanes NM). A small amount of large T can be seen in the nucleoplasmic extract of cells pulse-chase-labeled at 39°C (panel Cc, lane N). This large T was synthesized during the chase period because of an unavoidable slight drop in temperature during the change from the labeling to the chase medium.

34–36) and in Materials and Methods. Nuclear subfractions, comprising the nucleoplasmic extract, the chromatin extract, and the nuclear matrix extract, were analyzed for large T and large T-p53 complexes and for free p53 by sequential immunoprecipitations with the large-T-specific monoclonal antibody PAb 108 (10) and the p53-specific monoclonal antibody PAb 122 (9), respectively, as described previously (5) and in Materials and Methods (Fig. 1). In cells kept and labeled at 32°C, *tsA28* mutant large T and large T-p53 complexes exhibited a subnuclear distribution typical for SV40-transformed cells (Fig. 1A). Both large T and p53 in complex with large T were metabolically stable. As had been shown previously for other SV40-transformed rat cells (5), free p53 was present in all nuclear subfractions of tsA28.3 cells (Fig. 1B). As expected, this p53 was also metabolically stable. In tsA28.3 cells kept at the nonpermissive temperature (39°C), large-T expression was drastically reduced (Fig. 1C), as had been reported previously (4, 24). In contrast, p53

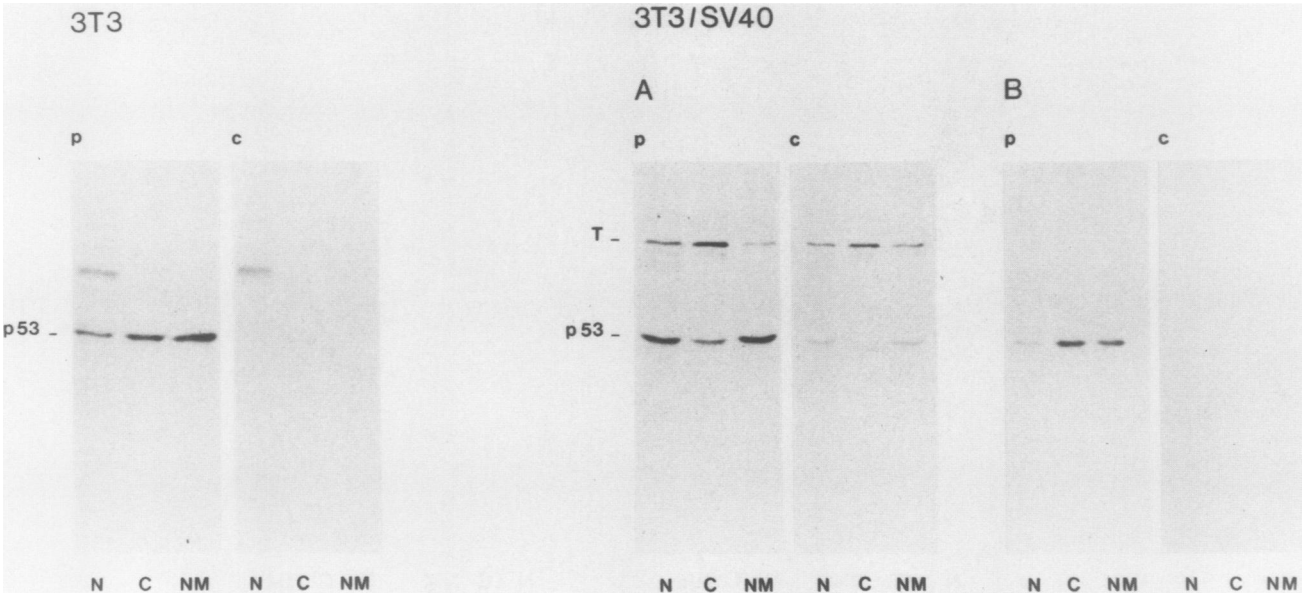

FIG. 2. Metabolic stabilities of p53 in uninfected BALB/c 3T3 and SV40 abortively infected BALB/c 3T3 cells. Uninfected BALB/c 3T3 cells (3T3) and SV40 abortively infected BALB/c 3T3 cells (3T3/SV40) at 24 h p.i. were pulse-labeled (p) and pulse-chase-labeled (c) with [^{35}S]methionine and then subfractionated as described in the legend to Fig. 1 and in the text. Nuclear extracts of uninfected cells were immunoprecipitated for p53 with monoclonal antibody PAb 122. Nuclear extracts of SV40 abortively infected cells were sequentially immunoprecipitated for large T and large T-p53 complexes (A) with monoclonal antibody PAb 108 and for free p53 (B) with monoclonal antibody PAb 122 as described in the text. The immunoprecipitates were analyzed on an 11.5% SDS-polyacrylamide gel, followed by fluorography. The designation of nuclear extracts is described in the legend to Fig. 1.

expression continued at about the same level as at the permissive temperature (Fig. 1D; cf. Fig. 1B). However, this p53 was no longer metabolically stable (Fig. 1D), demonstrating that metabolic stabilization of free p53 in SV40-transformed cells is reversible if the cells themselves revert to the normal phenotype. Metabolic stabilization of free p53 in these cells is thus closely related to the expression of the transformed phenotype.

Large-T expression in SV40 abortively infected cells is not sufficient for metabolic stabilization of p53. The results described above show a clear correlation between the expression of large T, the expression of the transformed phenotype, and the expression of a metabolically stable p53. However, the experiments did not allow us to discern whether metabolic stabilization of p53 in SV40-transformed cells is the direct consequence of large-T expression or indirectly results from the expression of the transformed phenotype. This question is difficult to analyze in SV40 stably transformed cells. Such cells are usually obtained after applying selective conditions because transformation by SV40 is rather inefficient (for reviews, see references 18 and 37). Therefore, in SV40-transformed cells it is difficult to discern between effects of large T itself and secondary, cellular effects contributing to the expression of the transformed phenotype.

For these reasons, we analyzed normal BALB/c 3T3 cells abortively infected with wild-type SV40 for the expression of p53. These cells are not stably transformed but transiently express a functional large T. Therefore, it should be possible to monitor any direct influence of large T on p53 expression and stabilization. Parallel cultures of semiconfluent BALB/c 3T3 cells infected with SV40 at a MOI of 10 (see Materials and Methods for definition of MOI in abortively infected cells) were pulse-labeled (1 h) or pulse-chase-labeled (1-h pulse and 2-h chase) with [^{35}S]methionine at 24 h p.i. and subfractionated into nucleoplasmic, chromatin, and nuclear matrix extracts. Nuclear extracts were analyzed for large T and large T-p53 complexes or free p53 as described above. As a control, uninfected BALB/c 3T3 cells were pulse-chase-labeled in parallel and analyzed for p53 expression and metabolic stability. The nuclear subclasses of p53 expressed in uninfected cells were metabolically unstable (Fig. 2), as has been described previously (5). Analysis of SV40-infected BALB/c 3T3 cells for large T and large T-p53 complexes (Fig. 2A) showed that after pulse-labeling all nuclear subclasses of large T described for SV40-transformed cells (5, 35) could be detected, although they were present in a somewhat different subnuclear distribution. These subclasses were metabolically stable during a 2-h chase period (Fig. 2A, panel c). All nuclear subclasses of large T were found to be complexed with p53 after the pulse-label (Fig. 2A, panel p). However, to our great surprise, this p53 was not metabolically stable, since it was hardly detectable in pulse-chase-labeled cells (Fig. 2A, panel c). Free p53 was also present in SV40 abortively infected BALB/c 3T3 cells and, like p53 in uninfected 3T3 cells, could be demonstrated only in pulse-labeled cells (Fig. 2B, panel p). Identical results were obtained when BALB/c 3T3 cells infected with SV40 were analyzed 48 h p.i. with SV40 (data not shown). Since more than 95% of the infected cells were found to express large T at both times p.i. when analyzed by immunofluorescence microscopy (data not shown), these data can be interpreted only as indicating that in these cells neither formation of a p53 complex with large T nor large-T expression as such is sufficient for metabolic stabilization of p53 to a similar extent as in SV40 stably transformed cells (5, 17).

p53 is metabolically stable in SV40-transformed cells developed from SV40 abortively infected cells. Both free p53 and p53 in complex with large T were found to be metabolically stable in a variety of SV40-transformed cells (5) but were

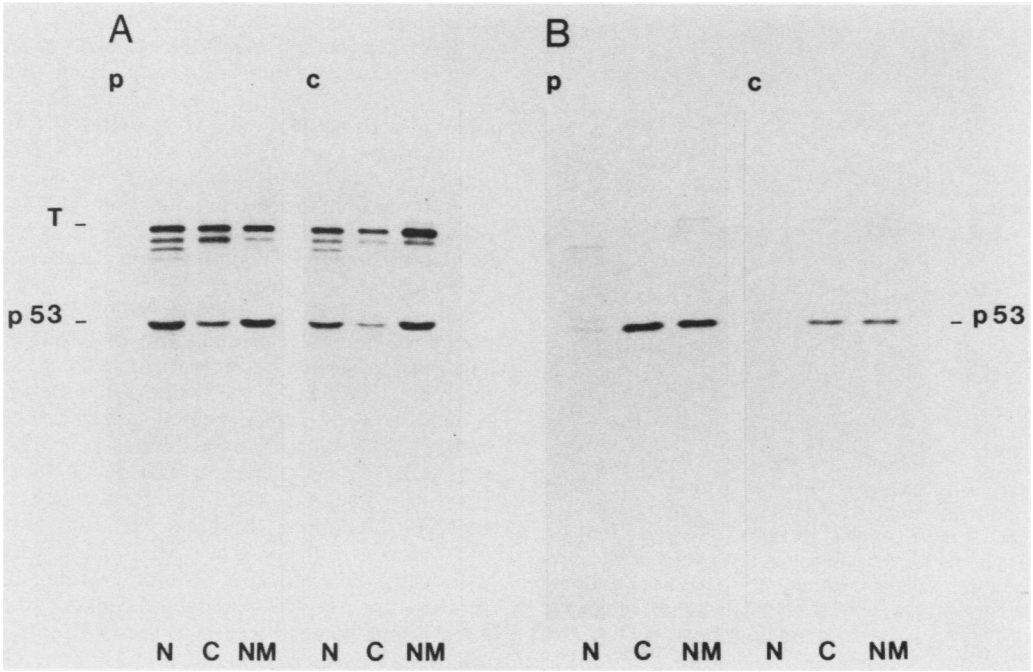

FIG. 3. Metabolic stabilities of p53 in SV40 stably transformed BALB/c 3T3 cells developed from abortive infections. In an experiment parallel to the one described in the legend to Fig. 2, SV40 abortively infected BALB/c 3T3 cells were kept in culture until foci of SV40 stably transformed cells had developed. Foci were picked and expanded. Transformed cells (SV3T3 cells) were pulse-labeled (p) and pulse-chase-labeled (c) with [^{35}S]methionine and then subfractionated as described in the legend to Fig. 1 and in the text. Nuclear extracts were sequentially immunoprecipitated for large T-p53 complexes (A) with monoclonal antibody PAb 108 and for free p53 (B) with monoclonal antibody PAb 122 as described in the text. The immunoprecipitates were analyzed on an 11.5% SDS-polyacrylamide gel, followed by fluorography. The designation of nuclear extracts is described in the legend to Fig. 1.

metabolically unstable in SV40 abortively infected mouse BALB/c 3T3 cells expressing a functional large T (see above). An explanation for this finding might be that, in addition to large-T expression, a cellular process induced during transformation is responsible for metabolic stabilization of p53 in SV40 stably transformed cells. If this is the case, SV40 stably transformed cells developed from SV40 abortively infected BALB/c 3T3 cells should express metabolically stable p53. To test this hypothesis, parallel cultures of BALB/c 3T3 cells were infected with SV40 and either analyzed for the expression and stabilization of p53 at 24 and 48 h p.i. as described above or kept in culture for the development of foci of SV40 stably transformed cells. Analysis of abortively infected cells gave the same results as those shown in Fig. 2 (data not shown). After about 2 weeks, foci had developed in the parallel cultures of abortively infected BALB/c 3T3 cells (about 10 to 20 foci per 10^6 cells). Foci were picked and expanded and then analyzed for the expression of large T, large T-p53 complexes, and free p53 as described above. Typical results are shown in Fig. 3. Analysis for large T and large T-p53 complexes in these stably transformed cells (SV3T3 cells) demonstrated the presence of metabolically stable p53 complexed to large T in all nuclear subfractions (Fig. 3A). In addition, free p53 present in the chromatin and nuclear matrix fractions of these cells also showed strongly increased metabolic stability (Fig. 3B) compared with that of p53 in 3T3 cells or in SV40 abortively infected cells (cf. Fig. 2).

Analysis of p53 stabilization in SV40 abortively infected cells expressing increasing amounts of large T. Although more than 95% of the SV40 abortively infected BALB/c 3T3 cells analyzed in the experiment for which results are shown Fig. 2 expressed large T, as judged by immunofluorescence analysis for nuclear large T (data not shown), it could be argued that the amount of large T expressed in these cells was too low to accomplish metabolic stabilization of p53. We therefore determined the average amounts of large T expressed in BALB/c 3T3 cells abortively infected with SV40 at different MOIs and compared them with the average amount of large T expressed in the SV40-transformed BALB/c mouse tumor line mKSA, a cell line known to express a high amount of large T (11, 35).

Large T present in whole-cell lysates of identical numbers (10^6) of BALB/c 3T3 cells abortively infected with SV40 at a MOI of 1, 10, or 100 for 24 or 48 h and of logarithmically growing mKSA cells was recovered by immunoprecipitation with monoclonal antibody PAb 108, and steady-state levels were determined by Western blotting as described previously (11, 34). At a MOI of 100, SV40 abortively infected BALB/c 3T3 cells contained at least as much large T as did mKSA cells, both at 24 and 48 h p.i. (Fig. 4). A significant amount of large T was already present in cells infected at a MOI of 10, i.e., the MOI used in the experiment for which results are shown in Fig. 2.

To determine whether the amount of large T present in SV40 abortively infected cells has any influence on the metabolic stabilization of p53, we analyzed the metabolic stabilities of p53 complexed to large T in BALB/c 3T3 cells abortively infected at a MOI of 1, 10, or 100 at 24 h p.i. The cells were labeled for 30 min with [^{35}S]methionine, and whole-cell lysates were analyzed either directly after the pulse or after a chase period of 2 h. The metabolic stabilities of free p53 in uninfected BALB/c 3T3 cells and of p53 complexed to large T in mKSA cells were analyzed in

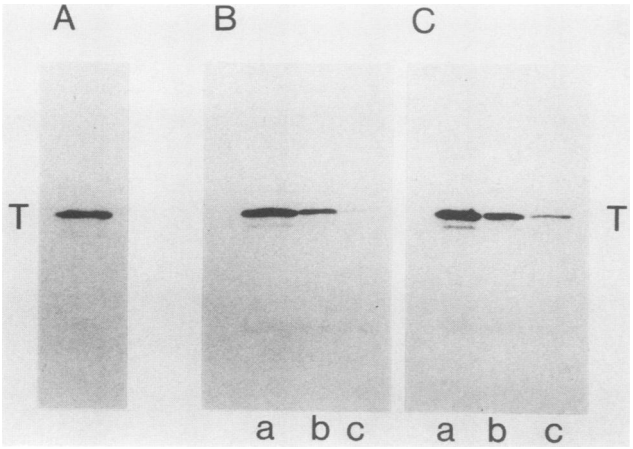

FIG. 4. Comparison of steady-state levels of large T in mKSA cells and SV40 abortively infected BALB/c 3T3 cells. Whole-cell extracts of 10^6 mKSA cells (A) and 10^6 SV40 abortively infected BALB/c 3T3 cells (B and C) were immunoprecipitated with monoclonal antibody PAb 108, and the immunoprecipitates were analyzed by Western blotting and detection of large-T immune complexes with ^{3}H-labeled protein A followed by fluorography, as described previously (11, 34). BALB/c 3T3 cells were infected with SV40 at a MOI of 100 (lanes a), 10 (lanes b), or 1 (lanes c) and analyzed 24 h (panel B) or 48 h (panel C) p.i.

parallel. p53 in 3T3 cells was unstable (Fig. 5A), whereas p53 complexed to large T in mKSA cells was as stable as large T itself (Fig. 5B). No significant stabilization of p53 complexed to large T was observed in BALB/c 3T3 cells abortively infected with SV40 at any MOI analyzed (Fig. 5C). Similar results were observed when abortively infected cells were analyzed at 48 h p.i. (data not shown). These findings thus support our conclusions that metabolic stabilization of p53 in SV40-transformed cells is not a primary consequence of formation of a p53 complex with large T but requires a secondary cellular event.

Kinetic analysis of metabolic stabilities of p53 and complexed p53 in uninfected and SV40 abortively infected BALB/c 3T3 cells, respectively. Since complexed p53 in SV40 abortively infected cells in the experiments for which results are shown in Fig. 2 and 5 seemed to exhibit a slightly higher metabolic stability than did either free p53 in uninfected BALB/c 3T3 cells (Fig. 2 and 5) or free p53 in SV40 abortively infected cells (Fig. 2), we performed a kinetic analysis. Uninfected BALB/c 3T3 cells and BALB/c 3T3 cells infected with SV40 at a MOI of 100 were pulse-labeled with [^{35}S]methionine for 30 min. Whole-cell lysates were analyzed for free p53 (uninfected cells) or p53 complexed to large T (infected cells) either directly after the pulse-label or after chase periods of 30, 60, and 120 min. The results (Fig. 6) demonstrate a slight increase in the metabolic stability of p53 complexed to large T in SV40 abortively infected BALB/c 3T3 cells, with a half-life of about 30 min compared with a half-life of about 20 min for free p53 in uninfected BALB/c 3T3 cells. This increase, however, is minimal

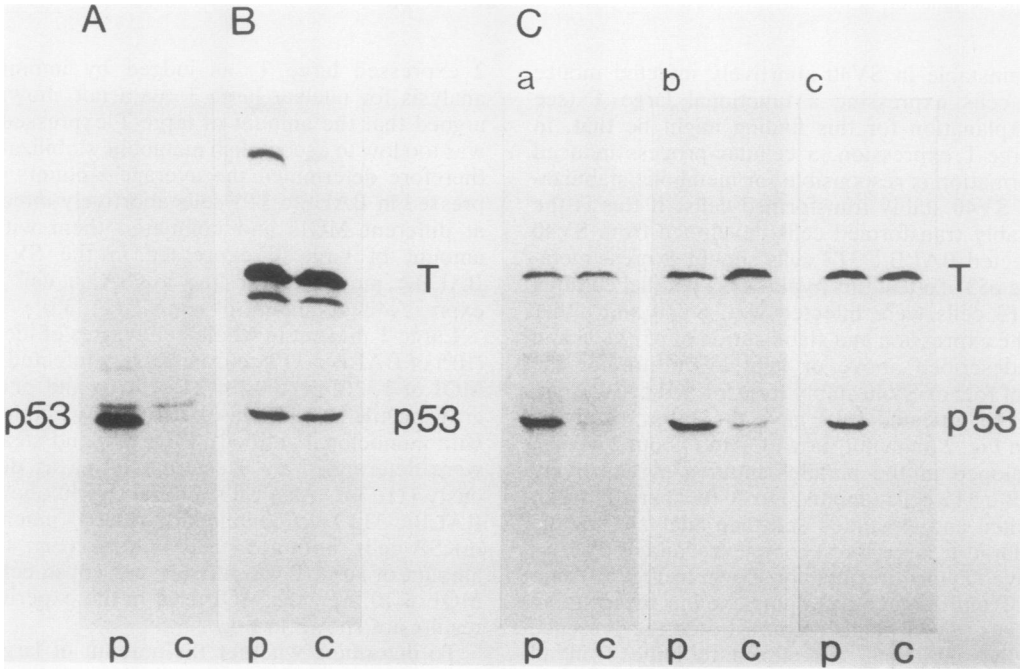

FIG. 5. Influence of amounts of large T expressed in SV40 abortively infected BALB/c 3T3 cells on metabolic stabilization of p53 complexed to large T. BALB/c 3T3 cells were abortively infected with SV40 (C) at a MOI of 1 (a), 10 (b), or 100 (c) and pulse-labeled (p) or pulse-chase-labeled (c) with [^{35}S]methionine (30-min pulse and 2-h chase) at 24 h p.i. as described in the text. Whole-cell extracts were immunoprecipitated for large T-p53 complexes with monoclonal antibody PAb 108, and the immunoprecipitates analyzed on an 11.5% SDS-polyacrylamide gel, followed by fluorography. The gels were exposed for 7 (panel a), 2 (panel b), or 1 (panel c) day to normalize for the different amounts of radiolabeled complexed p53 in various pulse-labeled cell extracts. As a control, the metabolic stabilities of free p53 in uninfected BALB/c 3T3 cells (A) and p53 complexed to large T in SV40 stably transformed BALB/c mouse cells (mKSA) (B) were analyzed in parallel. The cells were pulse-chase-labeled as described above, and whole-cell extracts were immunoprecipitated for p53 with monoclonal antibody PAb 122 (panel A) or for large T-p53 complexes with PAb 108 (panel B).

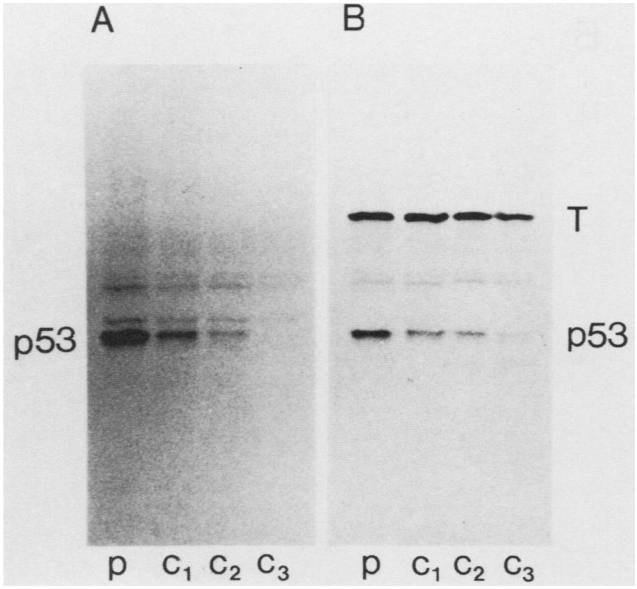

FIG. 6. Kinetic analysis of p53 degradation in uninfected and SV40 abortively infected BALB/c 3T3 cells. Uninfected BALB/c 3T3 cells (A) and BALB/c 3T3 cells abortively infected with a MOI of 100 (B) were pulse-labeled (p) and pulse-chase-labeled (c_1 to c_3) with [^{35}S]methionine (30-min pulse and 30-min [c_1], 60-min [c_2], or 120-min [c_3] chase). Whole-cell lysates were immunoprecipitated for p53 with monoclonal antibody PAb 122 (panel A) or for large T-p53 complexes with monoclonal antibody PAb 108 (panel B). The immunoprecipitates were analyzed on an 11.5% SDS-polyacrylamide gel, followed by fluorography.

compared with the drastic increase in the metabolic stability of p53 complexed to large T in SV40 stably transformed cells, in which the half-life is between 12 and 24 h (17; our unpublished data).

Metabolic stabilization of complexed p53 in various fibroblast cell lines abortively infected with SV40. The results reported above are in marked contrast to a previous report by Linzer et al. (17), who in similar experiments demonstrated that p53 complexed to large T in SV40 abortively infected BALB/c 3T3 cells is metabolically stable. To resolve this discrepancy we asked whether specific properties of the cells analyzed might be responsible for the differences in our results and those obtained by Linzer et al. (17). Therefore, we compared the metabolic stabilities of p53 and p53 in complex with large T in, respectively, uninfected and SV40-infected mouse BALB/c 3T3 cells (Fig. 7A), Swiss mouse 3T3 cells (Fig. 7B), mouse NIH 3T3 cells (Fig. 7C), rat F111 cells (Fig. 7D), and rat FR3T3 cells (Fig. 7E). The cells were pulse-labeled (1-h pulse) and pulse-chase-labeled (1-h pulse and 2-h chase) with [^{35}S]methionine and extracted with lysis buffer (see Materials and Methods). Cellular extracts were immunoprecipitated for p53 with monoclonal antibody PAb 122 (panels a) or for large T and large T-p53 complexes with monoclonal antibody PAb 108 (panels b). p53 in all uninfected fibroblast cell lines was metabolically unstable (Fig. 7, panels a). However, differences were observed in the metabolic stabilities of p53 in complex with large T after abortive infection: p53 complexed to large T in mouse BALB/c 3T3 cells (Fig. 7A, panel b) was metabolically unstable, as described above (Fig. 2 and 5), whereas p53 complexed to large T in SV40 abortively infected Swiss mouse 3T3 (Fig. 7B, panel b) or NIH 3T3 (Fig. 7C, panel b) cells exhibited an increased metabolic stability when compared with that in uninfected cells (Fig. 7B and C, panels a, respectively). p53 in complex with large T in both SV40

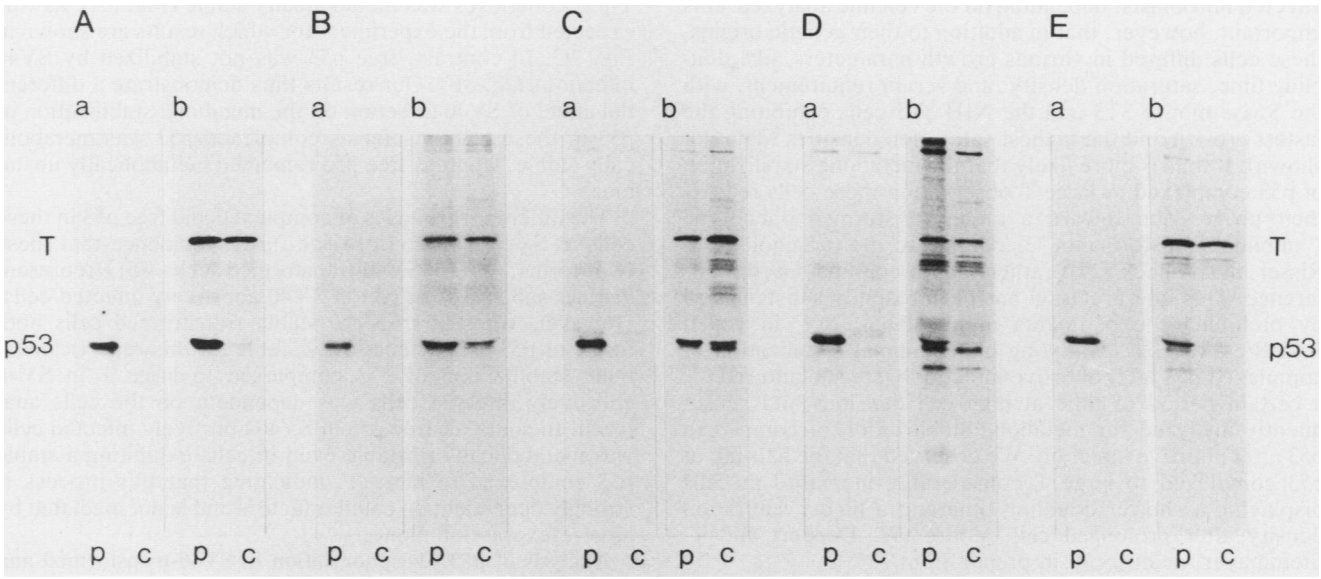

FIG. 7. Analysis of various mouse and rat fibroblast cell lines for metabolic stabilities of p53 in uninfected cells and of p53 complexed to large T after abortive infection. Uninfected cells (a) and SV40 abortively infected cells at 24 h p.i. (b) were pulse-labeled (p) (1-h pulse) and pulse-chase-labeled (c) (1-h pulse and 2-h chase) with [^{35}S]methionine, and whole-cell extracts were prepared as described in the text. Extracts of uninfected cells (panels a) were immunoprecipitated for p53 with monoclonal antibody PAb 122, and extracts of SV40 abortively infected cells (panels b) were immunoprecipitated for large T and large T-p53 complexes with monoclonal antibody PAb 108. The immunoprecipitates were analyzed on an 11.5% SDS-polyacrylamide gel, followed by fluorography. (A) BALB/c mouse 3T3 cells; (B) Swiss mouse 3T3 cells; (C) mouse NIH 3T3 cells; (D) rat F111 cells; (E) rat FR3T3 cells.

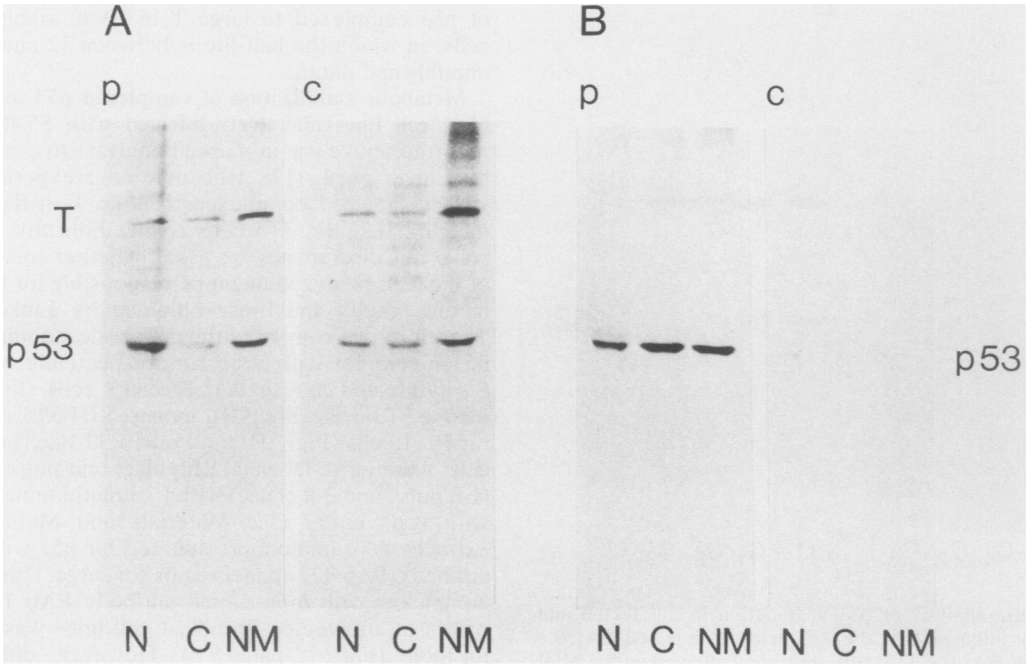

FIG. 8. Metabolic stabilities of complexed and free p53 in SV40 abortively infected mouse NIH 3T3 cells. NIH 3T3 cells abortively infected with SV40 were pulse-labeled (p) and pulse-chase-labeled (c) with [^{35}S]methionine and subfractionated as described in the legend to Fig. 1 and in the text. Nuclear extracts were sequentially immunoprecipitated for large T and large T-p53 complexes (A) with monoclonal antibody PAb 108 and for free p53 (B) with monoclonal antibody PAb 122 as described in the text. The immunoprecipitates were analyzed on an 11.5% SDS-polyacrylamide gel, followed by fluorography. The designation of nuclear extracts is described in the legend to Fig. 1.

abortively infected rat fibroblast cell lines was metabolically unstable, as was p53 in the uninfected cells (Fig. 7D and E). These data demonstrate that p53 in complex with large T might become stabilized to various degrees in abortively infected fibroblasts, depending on the cell line analyzed. It is important, however, that in addition to their genetic origins, these cells differed in various growth parameters, like doubling time, saturation density, and serum requirement, with the Swiss mouse 3T3 and the NIH 3T3 cells exhibiting the fastest growth and the highest saturation densities (data not shown). It thus is more likely that the metabolic stabilization of p53 complexed to large T observed in these cells reflects their progression toward a more transformed phenotype ("minimal transformants" according to the terminology of Risser and Pollack [27]) rather than a cell-line-specific difference. This interpretation has been recently substantiated by preliminary experiments in our laboratory, in which BALB/c 3T3 cells exhibiting only minimal stabilization of complexed p53 after abortive infection were subcultured for a certain period of time at high cell densities and subsequently analyzed for metabolic stabilization of complexed p53 after abortive infection. We observed that the half-life of p53 complexed to large T considerably increased in cells displaying a shorter doubling time and a higher saturation density after prolonged cell culture (W. Deppert and T. Steinmayer, manuscript in preparation).

Analysis of metabolic stability of free p53 in SV40 abortively infected NIH 3T3 cells. We further analyzed whether in cells that show, upon abortive infection with SV40, metabolic stabilization of p53 in complex with large T, free p53 also becomes metabolically stabilized. The comparative analysis of various fibroblasts cell lines (Fig. 7) had shown that p53 in complex with large T was most stable in abortively infected NIH 3T3 cells. Therefore, NIH 3T3 cells abortively infected with SV40 at a MOI of 10 were pulse-chase-labeled at 24 h p.i., subfractionated, and analyzed for large T, large T-p53 complexes, and free p53, as described above. p53 in large T-p53 complexes was metabolically stable (Fig. 8A), as was expected from the experiment for which results are shown in Fig. 7C. In contrast, free p53 was not stabilized by SV40 infection (Fig. 8B). Our results thus demonstrate a differential effect of SV40 infection on the metabolic stabilization of p53 in these cells insofar as complexed p53 was metabolically stable, whereas free p53 remained metabolically unstable.

The different responses of complexed and free p53 in these cells to SV40 infection provide further evidence that these two forms, as in SV40-transformed cells (5), represent distinct subclasses of p53 in SV40 abortively infected cells. However, whereas in SV40 stably transformed cells both forms of p53 are metabolically stable (5; this report), metabolic stabilization of p53 complexed to large T in SV40 abortively infected cells was dependent on the cells analyzed. In contrast, free p53 in SV40 abortively infected cells was metabolically unstable even in cells exhibiting a stable p53 complexed to large T, indicating that this process is strongly dependent on cellular factors and is not mediated by large-T expression alone.

Analysis of p53 phosphorylation in SV40-transformed and SV40 abortively infected cells. The different metabolic stabilities of p53 in BALB/c 3T3 cells stably transformed or abortively infected with SV40 suggested differences either in the molecular properties of the p53 expressed in these cells or in the cellular mechanism for p53 degradation (8). In a first approach to distinguish between these alternatives, we analyzed the in vivo phosphorylation of p53 in SV40 stably

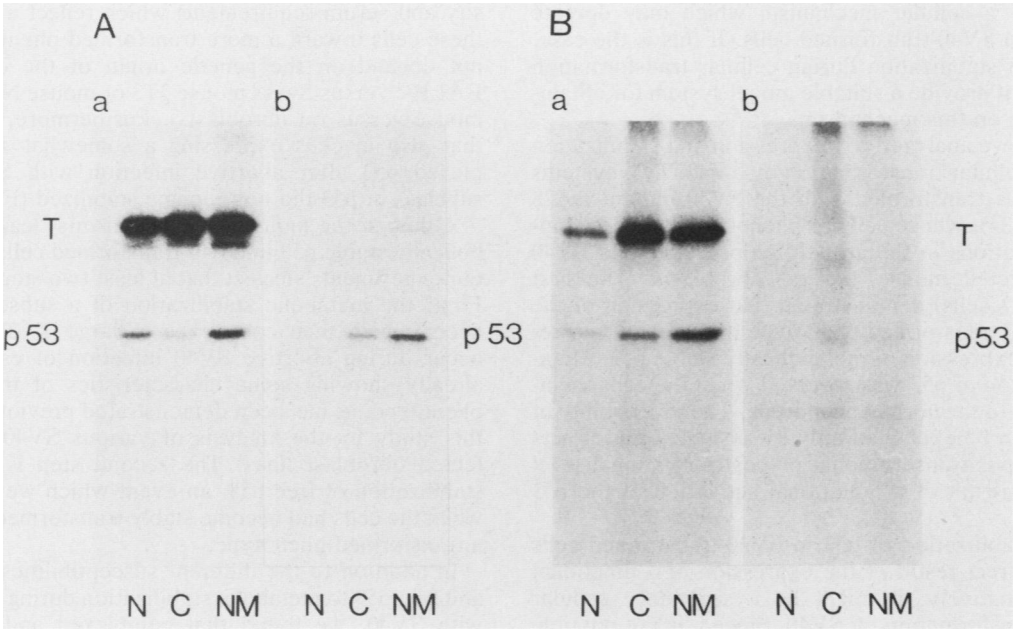

FIG. 9. Phosphorylation of complexed and free p53 in SV40 stably transformed and SV40 abortively infected BALB/c cells. SV40 stably transformed BALB/c 3T3 cells (A) (SV3T3 cells; Fig. 3) and SV40 abortively infected BALB/c 3T3 cells at 24 h p.i. (B) were labeled for 3 h with $^{32}P_i$ (200 μCi per plate per ml in phosphate-free DMEM) and subfractionated as described in the text. Nuclear extracts were sequentially immunoprecipitated for large T and large T-p53 complexes (a) with monoclonal antibody PAb 108 and for free p53 (b) with monoclonal antibody PAb 122 as described in the text. The immunoprecipitates were analyzed on an 11.5% SDS-polyacrylamide gel, followed by autoradiography with an intensifying screen. The designation of nuclear extracts is described in the legend to Fig. 1.

transformed and SV40 abortively infected BALB/c 3T3 cells since it has been reported that an increase in phosphorylation of p53 can be observed in transformed cells expressing a metabolically stable p53 (2, 28, 32). Parallel cultures of SV40-transformed BALB/c 3T3 cells (SV3T3 cells; Fig. 3) and of BALB/c 3T3 cells 24 h after infection with SV40 were labeled for 3 h with $^{32}P_i$, subfractionated, and analyzed for large T, large T-p53 complexes, and free p53, as described above. The results of the sequential immunoprecipitations of nuclear subfractions from SV3T3 cells are shown in Fig. 9A. As previously reported (for a review, see reference 3; 28, 32), both large T and p53 in complex with large T are phosphoproteins (Fig. 9A, panel a). Furthermore, free p53 in these cells was also found to be phosphorylated (Fig. 9A, panel b). p53 complexed to large T in SV40 abortively infected cells was also phosphorylated (Fig. 9B, panel a), whereas no free p53 could be demonstrated in these cells by using phosphate label (Fig. 9B, panel b). Since both forms of p53 in SV40 abortively infected cells, p53 in complex with large T and free p53, were metabolically unstable (Fig. 2 and 5), these data indicate that there is at least not a direct correlation between metabolic stabilization and general phosphorylation of p53. The data demonstrate, however, that complexed and free p53 in SV40 abortively infected cells differ in their degree of phosphorylation and, therefore, clearly represent distinct forms of p53.

DISCUSSION

Although there is clear evidence that oncogenic activation of p53 can occur by mutagenic activation (12, 19, 31), the abnormal expression of p53 in many transformed cells seems to be due to a posttranscriptional or posttranslational event (22, 23, 26; this report). In this regard, the activation of p53 in these cells as an oncoprotein is mediated by a different mechanism, as is known for the activation of many other cellular transforming proteins which become activated by mutations in either the somatic or regulatory regions of their genes (for a review, see reference 1). The oncogenic activation of p53 by posttranscriptional or posttranslational metabolic stabilization of the p53 molecule may thus represent a novel mechanism by which cellular proteins involved in the control of cell proliferation may be altered to become oncoproteins.

Very little is known about this mechanism. p53 was discovered by its ability to form a tight complex with the transforming protein of SV40, large T (for a review, see reference 3). Since p53 in complex with large T is metabolically stable, whereas free p53 in normal cells is not (5, 22, 28), it has been assumed that formation of a complex with large T stabilizes p53 (for a review, see reference 3). Although there are many examples of metabolically stable p53 molecules in transformed cells which are not complexed to a transforming protein (for a review, see reference 30), this assumption has been further supported by the finding that a metabolically stable p53 is also complexed to the adenovirus E1b 58K tumor antigen (33). However, in a previous study we have provided evidence for two distinct subsets of metabolically stable p53 in SV40-transformed cells, one complexed to large T and the other existing in an uncomplexed free form (5). Similarly, Zantema et al. (38) provided evidence that formation of a complex of p53 with the adenovirus E1b 58K tumor antigen depends on the adenovirus serotype used for transformation and is not related to metabolic stabilization of p53. These findings suggested that in SV40-transformed cells also, formation of a complex of large T and p53 is at least not the only mechanism for p53 stabilization but rather that stabilization of p53

is achieved by a cellular mechanism which may operate similarly in non-SV40-transformed cells. If this is the case, analysis of p53 stabilization during cellular transformation with SV40 might provide a suitable model system for obtaining information on this mechanism.

In this study we analyzed p53 expression and stabilization in relation to cellular transformation by SV40. Two systems were used: cells transformed with the SV40 mutant *tsA28* (tsA28.3 cells; 24), whose cellular phenotype can be manipulated by alterations in the growth temperature, and SV40 abortively infected mouse and rat fibroblasts. The first system (tsA28.3 cells) demonstrated that expression of the large-T-induced transformed phenotype in these cells correlated with the expression of metabolically stable p53. Metabolic stabilization of p53 was reversed when the cells themselves reverted to the normal phenotype. The reversibility of this process can be explained only by assuming that it acts on p53 at the posttranscriptional or posttranslational level and is not the result of a mutational activation of the p53 gene.

Metabolic stabilization of p53 in SV40-transformed cells could be the direct result of the expression of a functional large T. Alternatively, it might be a secondary cellular response to transformation by SV40. Since it is not possible to distinguish between these alternatives in cells already stably transformed with SV40, we analyzed nonpermissive cells abortively infected with wild-type SV40. These cells transiently express large T, but are not transformed, and retain their normal morphology. Only a minor fraction of these cells become stably transformed (for reviews, see references 18 and 37). Although all cells expressed large T, our analyses gave a surprising result: in contrast to previously published data (17), we found that large-T expression in abortively infected BALB/c 3T3 cells did not lead to a significant metabolic stabilization of p53, be it p53 in complex with large T or free p53. It is important for our argument that metabolic stabilization of p53 in these cells was not observed even under conditions in which the cells expressed as much large T as did the SV40-transformed cell line mKSA, which is known to overexpress large T (11). We thus conclude that large T does not directly mediate the metabolic stabilization of p53. In addition, our data demonstrate that formation of a complex of large T and p53 is not as such sufficient to stabilize p53 but rather that it reflects a physical property of both molecules whose biological role in cellular transformation still has to be determined. In contrast, our data strongly indicate that metabolic stabilization of p53 in SV40-transformed cells involves a cellular mechanism(s) which is induced during transformation with SV40. This assumption was further supported by our finding that in SV40 stably transformed cells developed in parallel from SV40 abortively infected BALB/c 3T3 cells, both p53 in complex with large T and free p53 were metabolically stable.

In following up our observation that formation of a complex of large T with p53 did not significantly stabilize the complexed p53 in BALB/c 3T3 cells, we analyzed various SV40 abortively infected fibroblast cell lines. We showed that in only two of the five mouse and rat fibroblast lines analyzed, large-T expression resulted in some metabolic stabilization of p53 complexed to large T suggesting a direct effect of large-T expression or complex formation or both on p53 stability in these cells. This observation, however, deserves some comment: preliminary studies in our laboratory show that metabolic stabilization of p53 complexed to large T in SV40 abortively infected cells correlates with changes in various growth parameters (e.g., saturation density and serum requirement) which reflect a progression of these cells toward a more transformed phenotype and does not depend on the genetic origin of the cell lines (i.e., BALB/c versus Swiss mouse 3T3 or mouse NIH 3T3 cells or mouse versus rat fibroblasts). Furthermore, it is important that also in cells expressing a somewhat stabilized complexed p53 after abortive infection with SV40, the free subclass of p53 did not become stabilized (Fig. 8).

Although the molecular mechanism(s) leading to a metabolically stable p53 in SV40-transformed cells is not known, our experiments suggest that at least two steps are required. First, the metabolic stabilization of a subset of p53 must occur which then complexes to large T. This step might occur during abortive SV40 infection of established cells already showing some characteristics of the transformed phenotype, as has been demonstrated previously (17) and in this study by the analysis of various SV40 abortively infected fibroblast lines. The second step is the metabolic stabilization of free p53, an event which we observed only when the cells had become stably transformed and exhibited a transformed phenotype.

In addition to the different susceptibilities of complexed and free p53 to metabolic stabilization during transformation with SV40, we found that complexed and free p53 also differed in phosphorylation. Although both forms of p53 in SV40 abortively infected BALB/c 3T3 cells were metabolically unstable, the complexed but not the free form could be metabolically labeled with $^{32}P_i$, indicating that general phosphorylation of p53 is not responsible for its metabolic stabilization. These data, however, do not exclude the possibility that phosphorylation of p53 at a specific site (30) may lead to metabolic stabilization.

It is generally assumed that the low frequency of SV40 transformation after abortive infection results from inefficient or silent integration of the SV40 DNA into the cellular genome (see, e.g., reference 14 and literature cited therein). The data reported in this study suggest an additional requirement for SV40 transformation. Provided that metabolic stabilization of p53 is an important step in SV40-induced cellular transformation (20), our results strongly suggest that a cellular mechanism(s) mediating p53 stabilization must be activated before the cells express a transformed phenotype. As our data show, activation of this mechanism does not directly correlate with the expression of large T but becomes manifested during the process of transformation. Therefore, a selective process within the abortively infected cell population has to be postulated. Selection could either be for cells in which this mechanism is already active or those in which it becomes activated as a consequence of large-T expression. Alternatively, it could also be hypothesized that the large T expressed in most of the abortively infected cells is not capable of inducing the transformed phenotype. Selection would then favor cells expressing a "transformation-competent" large T capable of inducing the transformed phenotype and thus metabolic stabilization of p53. This assumption would also explain the finding that in SV40-*tsA*-mutant-transformed cells at the permissive temperature both the expression of the transformed phenotype and the expression of metabolically stable p53 seemed to be under the control of large-T expression (Fig. 1). Although these considerations are purely speculative, they may provide a base for further experimental analysis of cellular transformation by SV40.

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