Phenotype-Specific Phosphorylation of Simian Virus 40 *tsA* Mutant Large T Antigens in *tsA* N-Type and A-Type Transformants

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To identify molecular differences between simian virus 40 (SV40) *tsA58* mutant large tumor antigen (large T) in cells of *tsA58* N-type transformants [FR(*tsA58*)A cells], which revert to the normal phenotype after the cells are shifted to the nonpermissive growth temperature, and mutant large T in *tsA58* A-type transformants [FR(*tsA58*)57 cells], which maintain their transformed phenotype after the temperature shift, we asked whether the biological activity of these mutant large T antigens at the nonpermissive growth temperature might correlate with phosphorylation at specific sites. At the permissive growth temperature, the phosphorylation patterns of the mutant large T proteins in FR(*tsA58*)A (N-type) cells and in FR(*tsA58*)57 (A-type) cells were largely indistinguishable from that of wild-type large T in FR(wt648) cells. After a shift to the nonpermissive growth temperature, no significant changes in the phosphorylation patterns of wild-type large T in FR(wt648) or of mutant large T in FR(*tsA58*)57 (A-type) cells were observed. In contrast, the phosphorylation pattern of mutant large T in FR(*tsA58*)A (N-type) cells changed in a characteristic manner, leading to an apparent underphosphorylation at specific sites. Phosphorylation of the cellular protein p53 was analyzed in parallel. Characteristic differences in the phosphorylation pattern of p53 were observed when cells of N-type and A-type transformants were kept at 39°C as opposed to 32°C. However, these differences did not relate to the different phenotypes of FR(*tsA58*)A (N-type) and FR(*tsA58*)57 (A-type) cells at the nonpermissive growth temperature. Our results, therefore, suggest that phosphorylation of large T at specific sites correlates with the transforming activity of *tsA* mutant large T in SV40 N-type and A-type transformants. This conclusion was substantiated by demonstrating that the biological properties as well as the phosphorylation patterns of SV40 *tsA28* mutant large T in cells of SV40 *tsA28* N-type and A-type transformants were similar to those in FR(*tsA58*)A (N-type) and in FR(*tsA58*)57 (A-type) cells, respectively. The phenotype-specific phosphorylation of *tsA* mutant large T in *tsA* A-type transformants probably is a cellular process induced during establishment of SV40 *tsA* A-type transformants, since *tsA28* A-type transformant cells could be obtained by a large-T-dependent *in vitro* progression of cells of the *tsA28* N-type transformant *tsA28.3* (M. Osborn and K. Weber, J. Virol. 15:636-644, 1975).

Expression of the simian virus 40 (SV40) large tumor antigen (large T) is required for initiation as well as for maintenance of SV40-induced cellular transformation (for reviews, see references 20, 33, 34, and 44). The functions of large T that are necessary to achieve the complex changes in cellular physiology leading to these events have not yet been delineated in detail, but it has become apparent that they require the interaction of large T with a variety of cellular targets. In this regard, large T can influence cellular gene expression by binding to (10, 30, 52) or by transactivating (14, 34, 53) cellular DNA sequences. In addition, large T seems to directly modulate functions of cellular regulatory proteins since it forms tight complexes with the cellular proliferation protein p53 and the gene product of the retinoblastoma gene pRb (for reviews, see references 18 and 19). At yet another level of regulation, large T interacts with various cellular structures, including different structural systems of the nucleus, and with the plasma membrane (for a review, see reference 3). Given the variety of these rather complex and diverse interactions, one must assume that the interactions are tightly controlled. As an example, only distinct subclasses of large T, differing in their biochemical

activities, interact with the cellular chromatin or the nuclear matrix (13, 42). Furthermore, one must also address the question of whether all interactions of large T with cellular targets reflect intrinsic properties of large T, or, alternatively, whether they require additional cellular factors which modulate the various activities of large T. Evidence for the latter assumption is accumulating since expression of large T as such is not sufficient for mediating cellular transformation but requires additional cellular functions (genes), providing a transforming competence for large T (1, 6, 7, 32, 35). Therefore, analysis of such parameters might lead to the identification of cellular genes involved in controlling SV40-induced cellular transformation.

We recently characterized in some detail a matched pair of SV40 *tsA* mutant *tsA58* N- and A-type transformants which might allow the identification of such cellular functions. These cells were obtained by transformation of normal rat F111 fibroblasts with the temperature-sensitive SV40 mutant *tsA58*, which led to transformants expressing authentic SV40 *tsA58* mutant large T but differing drastically in the temperature sensitivity of their phenotypes. At the permissive growth temperature (32°C), cells of both N-type [FR(*tsA58*)A cells] and A-type [FR(*tsA58*)57 cells] transformants express a transformed phenotype indistinguishable from cells of SV40 wild-type transformants [FR(wt648) cells]. At the nonpermissive growth temperature (39°C),

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FR(tsA58)A (N-type) cells revert to the normal phenotype, reflecting the temperature sensitivity of the mutant large T expressed in these cells. In contrast, FR(tsA58)57 (A-type) cells retain their transformed phenotype. At the nonpermissive growth temperature, the differences in cellular phenotypes between FR(tsA58)A (N-type) and FR(tsA58)57 (A-type) cells correspond well with differences in the properties of the mutant large T proteins expressed in these cells. Mutant large T in FR(tsA58)A (N-type) cells kept at 39°C is no longer able to associate with the cellular chromatin or to bind to the SV40 origin of replication in a sequence-specific manner. In contrast, a significant fraction (approximately 20%) of mutant large T in FR(tsA58)57 (A-type) cells retains these abilities even after prolonged culture at 39°C (32). Both *in vivo* association with the cellular chromatin and *in vitro* binding of large T to the SV40 origin of replication require large T to assume a specific, biologically active conformation (13, 32, 51). Therefore, we hypothesized that, by way of cellular selection processes induced during establishment of these cells as A-type transformants, the mutant large T in FR(tsA58)57 cells became stabilized in a biologically active conformation also at the nonpermissive growth temperature (13, 32).

In this study, we further analyzed the mutant large T proteins in FR(tsA58)A (N-type) and FR(tsA58)57 (A-type) cells for molecular differences when the cells were kept at the nonpermissive growth temperature. It has been demonstrated that phosphorylation of large T plays an important role in regulating its biochemical and biological activities (16, 17, 22–24, 31, 40, 43, 50). Therefore, we asked whether the phosphorylation patterns of mutant large T antigens in FR(tsA58)A (N-type) and in FR(tsA58)57 (A-type) cells exhibited phenotype-specific differences, i.e., whether phosphorylation at specific sites might correlate with the maintenance of a biologically active conformation by the mutant large T protein in FR(tsA58)57 (A-type) cells at the nonpermissive growth temperature. We found specific differences in the phosphorylation patterns of mutant large T antigens in FR(tsA58)A (N-type) cells and in FR(tsA58)57 (A-type) cells when the cells were kept at 39°C, supporting the hypothesis that distinct cellular phosphorylation and/or dephosphorylation processes correlate with the transforming activity of these mutant proteins at the nonpermissive growth temperature. This hypothesis was substantiated by our finding that large-T-dependent *in vitro* progression of *tsA* N-type transformants of different origin (*tsA28.3* cells; 26) to A-type transformants resulted in preservation of the biological properties of the *tsA28* mutant T antigen. In addition, the phosphorylation pattern of the *tsA28* mutant T antigen expressed in these cells was identical to that of *tsA58* mutant large T in FR(tsA58)57 (A-type) cells.

MATERIALS AND METHODS

Cells. Normal Fischer rat fibroblast F111 cells (8) and the SV40 wild-type-transformed F111 cell line FR(wt648) (29) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal calf serum (FCS). Line FR(tsA58)A, an N-type transformant, and line FR(tsA58)57, the corresponding A-type transformant, of F111 cells transformed with SV40 *tsA58* mutant virus (29) were grown in DMEM supplemented with 10% FCS. SV40 wild-type- and *tsA58* mutant virus-transformed F111 cells were kindly provided by Noel Bouck (Chicago). These cells were characterized in detail in previous publications from our labora-

tory (732). Cells were maintained at 32°C and shifted to 39°C only for temperature shift experiments. *tsA28.3* cells are rat embryo fibroblasts transformed by the SV40 *tsA* mutant *tsA28* in a focus assay (26). *tsA28.3/5.0(N)* and *tsA28.3/5.2(A)* cells are N-type and A-type transformants, respectively, derived by *in vitro* progression from *tsA28.3* cells by using soft agar colony assays (15a; this study). These cells were grown in DMEM supplemented with 10% FCS. All *tsA* transformants were occasionally recloned and tested for their N-type or A-type characteristics as described previously (32).

Phosphopeptide analysis. Cells were either routinely grown at 37°C or kept for 2 days at 32 or 39°C before they were labeled for 4 h with 1 mCi of $^{32}\text{P}_i$ (carrier free; Amersham) in P_i -free DMEM supplemented with 5% P_i -free FCS as described recently (28). In each case, the labeling medium was equilibrated at the corresponding labeling temperature before labeling. Whole-cell extracts were then prepared by lysis in sodium phosphate-based lysis buffer (pH 9.0) (28), followed by immunoprecipitation with monoclonal antibody PAb108 specific for SV40 large T (12) or monoclonal antibody PAb122 specific for p53 (11). Immunoprecipitated proteins were purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 1-mm-thick 10% acrylamide gels, extracted from the gels, and oxidized with performic acid as described in previous publications (27, 28).

Phosphopeptides of SV40 large T were prepared by sequential digestion of large T with trypsin and pronase E and then analyzed on cellulose thin-layer plates by electrophoresis at pH 1.9 followed by ascending chromatography in isobutyric acid buffer by use of the protocol published by Scheidtmann et al. (38). Tryptic phosphopeptides of p53 were prepared and analyzed by electrophoresis at pH 8.9 and chromatography in *n*-butanol-pyridine-acetic acid- H_2O (15:10:3:12, by volume) essentially as described recently (28). Phosphorylated amino acids in total digests or isolated peptides were identified after hydrolysis in 6 N HCl for 2 h at 110°C and subsequent two-dimensional electrophoresis in the presence of internal marker phosphoamino acids at pH 1.9 and 3.5 (27).

In comparative analyses of large T phosphopeptides from different cells, approximately similar amounts of radioactive material were loaded onto the thin-layer plates. Alternatively, exposure times were varied. In evaluating the corresponding peptide maps, we considered that, because of changes in the relative abundances of individual phosphopeptides, this procedure resulted in an over- or underrepresentation of certain marker phosphorylation sites (e.g., peptides 6 and 13), whose relative abundances were similar in all large T preparations analyzed. Therefore, changes in relative abundances of individual peptides reported in this study relate to intensities of these marker peptides.

Pulse-chase analysis, cell fractionation, and Western blot (immunoblot) analysis. Cells were pulse-labeled with [^{35}S] methionine and either lysed directly (see below) or chased with growth medium for various periods of time as described previously (5–7) and outlined in the legends of the appropriate figures. Immunoprecipitations were performed as described above from whole-cell extracts (47) or from cellular subfractions prepared by an *in situ* fractionation procedure, yielding nucleoplasmic, chromatin, and nuclear matrix fractions (45, 46), as modified by Deppert et al. (6, 7).

Western blot analysis of large T from unlabeled cells with ^3H -labeled protein A was performed essentially as described recently (13, 32).

FIG. 1. Phosphorylation of SV40 wild-type large T in transformed rat cells. Phosphopeptides of large T from $^{32}\text{P}_i$ -labeled FR(wt648) cells were prepared by sequential digestion of the purified protein with trypsin and pronase followed by two-dimensional analysis by electrophoresis at pH 1.9 and ascending chromatography in isobutyric acid buffer as described in Materials and Methods. The anode is at the left, and the origin is marked with a solid square. Phosphopeptides unequivocally correlating with peptides identified by Scheidtmann et al. (40) and modified by Hoess et al. (15) and Scheidtmann et al. (37) are numbered by using the nomenclature of these authors. Additional or unidentified peptides are marked with letters a to f. P_i indicates the position of free radioactive phosphate.

RESULTS

Characterization of the phosphorylation pattern of large T in FR(wt648) cells. The phosphorylation pattern of SV40 large T has been analyzed in detail in several laboratories, and the sites of phosphorylation on large T have been determined (38, 49). We first compared the phosphorylation pattern of wild-type large T in SV40 wild-type-transformed rat fibroblasts [FR(wt648) cells] with that of wild-type large T expressed in SV40-infected monkey cells, which was established by Scheidtmann et al. (37, 40, 41). We used essentially the methodology established by these authors to allow for a maximal correlation of phosphorylation sites. Figure 1 shows the phosphorylation pattern of wild-type large T in FR(wt648) cells. This pattern was identical to the phosphopeptide pattern of large T extracted from SV40-infected TC7 cells (data not shown). This pattern also is very similar to the one published by Scheidtmann et al. (37, 40, 41) but displays some alterations insofar as additional phosphopeptides were resolved. The nomenclature of individual phosphopeptides in Fig. 1 is such that peptides unequivocally correlating with peptides identified by Scheidtmann et al. (40), and modified by Hoess et al. (15) and Scheidtmann et al. (37), were numbered according to the nomenclature of these authors (peptides 1 to 13), whereas additional peptides were marked with letters (peptides a to f).

Table 1 gives the identification of these peptides and demonstrates that virtually all phosphorylation sites of large T identified so far could be resolved by our analysis. Of particular interest is peptide 6a. Peptide 6a, like peptides 6' and 6, contains phosphorylated Ser-112 as well as an additional, so-far-unidentified phosphorylation site (16a). The

TABLE 1. Phosphopeptides of SV40 large T^a

Peptide	Phosphoamino acid		Phosphorylation site ^b
	Ser	Thr	
1	+	—	Ser-106
2	+	—	Ser-639
4	+	+	Ser-120
			Ser-123
			Thr-124
6	+	—	Ser-112
6'	+	—	Ser-112
6a	+	—	Ser-112 + x
7	+	—	Ser-120
			Ser-123
8	+	—	Ser-677
			Ser-679
9	+	—	Ser-677
			Ser-679
11	+	(+) ^c	Ser-120
			Ser-123
			Thr-124
12, 12a	—	+	Thr-124
13	—	+	Thr-701
a	+	—	N terminus
b	+	—	C terminus
c	+	—	C terminus
d	+	—	N terminus
e, f	+	—	N terminus

^a The list of phosphopeptides is based on two-dimensional analyses of wild-type large T as illustrated in Fig. 1. Peptides corresponding to those identified by Scheidtmann et al. (40, 41) and modified by Hoess et al. (15) and Scheidtmann et al. (37) are numbered according to the nomenclature of these authors (peptides 1 to 13), while additional peptides were marked with letters (peptides a to f). Phosphorylated amino acids in individual peptides were identified as described in Materials and Methods. The presumptive localization of peptides b and c to the C terminus and that of peptides a and d to f to the N terminus of large T is based on our own work (16a).

^b x, a so-far-unknown phosphorylation site in phosphopeptide 6a.

^c (+), trace amounts.

phosphorylation sites represented by the peptides marked a to f in Fig. 1 and listed in Table 1 have not yet been identified unequivocally. Peptides b and c could be localized to the C-terminal region of large T, and peptides a, d, e, and f could be localized to the N-terminal region (16a). These peptides, however, do not result from contaminating proteins and also are not specific for large T extracted from SV40-transformed rat cells, since they were also observed with large T from infected monkey cells (data not shown). When the phosphorylation pattern of large T in these cells was analyzed at 32 and at 39°C (data not shown), very little difference was observed.

Analysis of temperature- and phenotype-dependent phosphorylation of mutant large T proteins in FR(tsA58)A (N-type) and FR(tsA58)57 (A-type) cells. We then analyzed the phosphorylation patterns of mutant large T proteins in FR(tsA58)A (N-type) and FR(tsA58)57 (A-type) cells kept at 32 and 39°C for 3 days. In comparing these phosphopeptide maps, relative abundances of individual peptides relate to the intensities of marker peptides (e.g., peptides 6 and 13), which we found to exhibit similar abundances in all large T preparations analyzed (see also Materials and Methods). Figure 2A and B show the phosphopeptide patterns of mutant large T in FR(tsA58)57 (A-type) cells, kept at 32 (Fig. 2A) and 39°C (Fig. 2B), and reveal that these patterns are rather similar. Some quantitative differences in the relative abundances of individual phosphopeptides were reproduc-

FIG. 2. Phosphorylation of SV40 *tsA58* mutant large T in A-type and N-type transformants of SV40 *tsA58* mutant-transformed F111 cells. FR(*tsA58*)57 (A-type) (A and B) and FR(*tsA58*)A (N-type) (C and D) cells were labeled with $^{32}\text{P}_i$ at 32°C (A and C) or 39°C (B and D), followed by preparation and analysis of large-T-specific phosphopeptides as described in Materials and Methods and in the legend to Fig. 1. Arrowheads indicate peptides exhibiting relative decreases, while arrows point to peptides with relative increases in their abundances in the map of mutant large T from N-type transformants at the nonpermissive temperature. Relative abundances were determined in relation to marker peptides (e.g., peptides 6 and 13) which displayed similar degrees of phosphorylation in all large T proteins analyzed.

ibly observed, probably reflecting the fact that, at the elevated growth temperature, the mutant large T in these cells only partially retained wild-type properties (7, 29, 32). Phosphopeptides 6a, 4, and 8 were slightly reduced in their relative abundances, whereas the abundance of phosphopeptide 12a was more prominently increased, reflecting an apparent underphosphorylation of Ser-120 and Ser-123 (see below). Despite these differences, both patterns closely resembled that of wild-type large T shown in Fig. 1. Figure 2C and D show the phosphopeptide patterns of mutant large T in FR(*tsA58*)A (N-type) cells kept at 32 and 39°C, respectively. Whereas the phosphopeptide pattern of the mutant large T in cells kept at 32°C (Fig. 2C) was indistinguishable from that of wild-type large T (Fig. 1) and that of mutant large T in FR(*tsA58*)57 (A-type) cells (Fig. 2A), some drastic alterations were reproducibly observed in the phosphopeptide pattern of mutant large T in cells kept at 39°C (Fig. 2D). Of these alterations, the most prominent was the almost complete loss of phosphopeptide 6a, representing the so-far-unidentified additional phosphorylation site in the vicinity of Ser-112. Phosphorylation of Ser-112 itself was not altered, since the relative abundance of phosphopeptides 6' and 6, reflecting phosphorylation of Ser-112, was unchanged. The abundance of phosphopeptides 12 and 12a, representing phosphorylation at Thr-124 (40), was markedly increased. This relative abundance of peptides 12 and 12a, however,

most likely does not reflect overphosphorylation of Thr-124, since it was accompanied by a marked reduction in the relative abundance of phosphopeptide 4, containing Ser-120 and Ser-123 in addition to Thr-124, and of phosphopeptide 7, containing Ser-123 and Ser-120 (15, 40, 41). Therefore, it is most likely that the reduction in the abundance of phosphopeptide 4 is due to a specific underphosphorylation of Ser-120 and -123 and one can assume that this underphosphorylation is reflected by the relative abundances of phosphopeptides 12 and 12a. In addition to the selective underphosphorylation of Ser-120 and -123, a strong reduction in the relative abundance of phosphopeptide 8 was observed, reflecting phosphorylation of the carboxy-terminal Ser residues Ser-677 and Ser-679, as well as of phosphopeptides b and c, which localize to the C-terminus of large T (16a). In contrast, phosphorylation of another carboxy-terminal Ser residue, Ser-639, was not affected, since no change in the relative abundance of phosphopeptide 2, representing phosphorylation at Ser-639, was observed. Also, phosphorylation of Thr-701, represented by phosphopeptide 13, was not affected. Changes in the abundances of peptides 1 and 11, as shown in Fig. 2C and D, were not reproducibly observed (see, e.g., Fig. 6C and D) and therefore were not considered relevant.

Analysis of temperature- and phenotype-specific phosphorylation of p53 in FR(*tsA58*)A (N-type) and FR(*tsA58*)57 (A-

FIG. 3. Metabolic stabilities of p53 in F111, in SV40 wild-type-transformed F111 cells, and in cells of F111 *tsA58* mutant N-type or A-type transformants kept at the nonpermissive growth temperature. F111 (*), FR(wt648) (○), FR(tsA58)A (N-type) (□), and FR(tsA58)57 (A-type) (△) cells were pulse-labeled with [³⁵S]methionine for 15 min or pulse-labeled for 15 min and then chased for 15, 30, 60, or 90 min, respectively, at 39°C. p53 was immunoprecipitated from whole-cell extracts and analyzed by SDS-PAGE as described in Materials and Methods. The amounts of radioactive p53 remaining after the chase periods were determined by densitometer scanning and are expressed as percentages of the amount of p53 detected after pulse-labeling.

type) cells. Previous work in our laboratory demonstrated that metabolic stabilization of p53 in SV40-transformed cells is a cellular process that correlates with the establishment and maintenance of the transformed phenotype (5–7). In SV40 *tsA* mutant-transformed cells, large T at the nonpermissive temperature no longer is able to complex p53 (7), yet analysis of the metabolic stability of p53 in FR(tsA58)A (N-type) and in FR(tsA58)57 (A-type) cells kept at the nonpermissive growth temperature demonstrated a close correlation with the phenotype of these cells: p53 in FR(tsA58)A (N-type) cells, like p53 in the parental F111 cells, was rapidly degraded, whereas p53 in FR(tsA58)57 (A-type) cells, like p53 in SV40 wild-type transformed FR(wt648) cells, was metabolically stable even in the absence of any detectable complex of p53 with large T (7). Figure 3 presents the quantitative evaluation of a pulse-chase experiment, analyzing the metabolic stability of total p53 in F111, FR(tsA58)A (N-type), FR(tsA58)57 (A-type), and FR(wt648) cells kept at the nonpermissive growth temperature. p53 in F111 and FR(tsA58)A (N-type) cells exhibited a half-life of about 15 min. p53 in FR(wt648) cells was metabolically stable over the time span analyzed. p53 in FR(tsA58)57 (A-type) cells also showed an increased metabolic stability, although it was not quite as stable as p53 in FR(wt648) cells. Thus, the metabolically stable p53 in FR(tsA58)57 cells might contribute to the maintenance of the transformed phenotype of these cells at the nonpermissive growth temperature (7).

It has been proposed that qualitative and quantitative changes in the phosphorylation of p53 relate to metabolic stabilization of p53 and to expression of the transformed

FIG. 4. Phosphorylation of p53 in F111, in SV40 wild-type-transformed F111 cells, and in cells of F111 *tsA58* mutant N-type or A-type transformants. FR(tsA58)57 (A-type) (A and B) and FR(tsA58)A (N-type) (C and D) cells were labeled with ³²P_i at 32°C (A and C) or 39°C (B and D), while FR(wt648) (E) and F111 (F) cells were labeled at 37°C. Tryptic phosphopeptides of p53 were prepared and analyzed by electrophoresis at pH 8.9 (origin marked with open circle) and ascending chromatography as described in Materials and Methods.

phenotype in SV40-transformed cells. Furthermore, it has been suggested that, in SV40-transformed cells, the enhanced phosphorylation of p53 is mediated by an SV40 large-T-induced cellular kinase (39). Since cells of A-type transformants kept at the nonpermissive growth temperature are phenotypically transformed and express a biologically active mutant large T as well as a metabolically stable p53, one would expect that the p53 in these cells exhibits a phosphorylation pattern closely related to the pattern of p53 in SV40 wild-type transformants. In contrast, p53 in cells of N-type transformants should exhibit a phosphorylation pattern characteristic of p53 in normal cells. Figure 4 shows the phosphopeptide analysis of p53 in FR(tsA58)57 (A-type) (Fig. 4A and B) and in FR(tsA58)A (N-type) (Fig. 4C and D) cells, kept at 32°C (Fig. 4A and C) and at 39°C (Fig. 4B and D), as well as of p53 in FR(wt648) (Fig. 4E) and in F111 (Fig. 4F) cells kept at 37°C. Phosphorylation of p53 in FR(wt648) and in F111 cells was temperature independent, since p53 from cells kept at 39 or 32°C showed phosphorylation patterns closely similar to those of p53 in these cells kept at 37°C (data not shown). Therefore, growth-temperature-induced differences in the phosphorylation pattern of p53 in these cells can be excluded. As reported previously (39), the phosphorylation pattern of p53 in FR(wt648) cells differed significantly from the phosphorylation pattern of p53 in F111 cells, indicating an apparently higher degree of phosphorylation of p53 in SV40-transformed cells. When the cells were kept at 32°C, p53 in both *tsA* mutant-transformed cell lines showed a phosphorylation pattern indistinguishable from the

TABLE 2. Properties of *tsA28* N-type and A-type transformants clonally derived from *tsA28.3* cells by in vitro progression^a

Clone	% Growth in soft agar		Cellular phenotype (morphology)		Large T expression		T/p53 complex		Transformation phenotype
	32°C	39°C	32°C	39°C	32°C	39°C	32°C	39°C	
<i>tsA28.3</i>	5	0	Transformed	Normal	+	—	+	—	N
<i>tsA28.3/5.0(N)</i>	20	0.6	Transformed	Normal	+	+	+	—	N
<i>tsA28.3/5.2(A)</i>	40	40	Transformed	Transformed	+	+	+	—	A

^a In vitro progression of *tsA28.3* cells was performed by soft agar cloning as described in the text. Analysis of cellular phenotype of isolated clones was determined by growth in soft agar, cellular morphology, and actin cable staining.

pattern of p53 in FR(wt648) cells. In contrast, p53 from *tsA* mutant-transformed cells kept at 39°C showed a markedly reduced phosphorylation, regardless of whether cells of the N-type or of the A-type transformants were analyzed. The most pronounced reduction in phosphorylation was an underphosphorylation of phosphopeptides a, b, and f relative to the phosphorylation pattern of p53 in FR(wt648) cells, affecting phosphorylation of Ser residues in rat p53 (28a). Thus, the phosphopeptide patterns of p53 in both *tsA* mutant-transformed cells were closely similar at the elevated growth temperature and appeared to be, in some way, between the phosphorylation patterns of p53 in normal F111 cells and in wild-type-transformed FR(wt648) cells. The phosphorylation of p53 in both *tsA* transformants at the nonpermissive growth temperature thus is independent from the phenotype of these cells and relates neither to the metabolic stability of p53 nor to the functionality of the mutant large T expressed in these cells.

Analysis of biological properties and of phosphorylation patterns of *tsA28* mutant large T in N-type and A-type transformants obtained after in vitro progression of *tsA28.3* cells. A specific difference between cells of *tsA* N-type and A-type transformants at the nonpermissive temperature thus seems to be a different phosphorylation of the mutant large T at specific sites, correlating with the different properties of the mutant large T expressed in these cells. However, the conclusion that these differences are due to specific cellular selection processes depends on the demonstration that such selection processes are not confined to the experimental system analyzed, i.e., that it is possible to follow up such a selection under more defined conditions. Therefore, we set up an experimental system to test whether it is possible to generate, via a large-T-dependent selection process, *tsA* A-type transformants as clonal derivatives from cells of N-type transformants. For a cellular system, *tsA28.3* cells, which are rat embryo fibroblasts transformed by a focus assay with the SV40 *tsA* mutant *tsA28* (26), were chosen. *tsA28.3* cells are fully transformed at the permissive growth temperature but shut off large T expression at the nonpermissive growth temperature and revert to the normal phenotype (4, 6, 26). *tsA28.3* cells were selected by a focus assay, which is less stringent than an agar colony assay. Therefore, we analyzed whether it might be possible to generate A-type transformants by applying growth in soft agar as the more stringent selection criterion. These studies will be published in detail elsewhere (15a). In summary, *tsA28.3* cells were seeded in soft agar at 32 and 39°C. No growth was obtained at 39°C, indicating that, in the absence of large T expression, spontaneous progression of these cells to a temperature-independent phenotype did not occur. About 5% of the cells kept at 32°C grew in soft agar [as compared to about 60% of cells of the wild-type transformant FR(wt648)cells]. Colonies were picked and analyzed for

phenotype and expression of the mutant large T at 32 and 39°C. Only N-type transformants were obtained, but some of them had lost their ability to block large T expression at the nonpermissive temperature. Cells from large T-expressing colonies, growing in soft agar with ~20% efficiency at 32°C, again were subjected to growth in soft agar at 32 and 39°C. Again, about 20% of the cells kept at 32°C grew in soft agar, and cells of all of the colonies obtained at 32°C belonged to the group of N-type transformants. About 0.6% of the N-type transformant cells expressing large T at the elevated growth temperature grew to colonies in soft agar at 39°C. Cells from these colonies were A-type transformants with regard to cellular phenotype at 32 and 39°C and grew with ~40% efficiency in soft agar both at 32 and 39°C. Some properties of the clonal derivatives of *tsA28.3* cells obtained by this large-T-dependent in vitro progression are summarized in Table 2. Progression of *tsA28.3* cells to A-type transformants was not due to reversion of the *tsA28* mutant large T to a wild-type phenotype, since extensive molecular analyses verified that the T antigens expressed in all of these cells corresponded to authentic *tsA28* mutant large T (15a).

Analysis of subnuclear distribution of mutant large T in cells of *tsA28.3* derived N-type and A-type transformants at 32 and 39°C. Our previous analyses of FR(*tsA58*)A (N-type) and FR(*tsA58*)57 (A-type) cells indicated that maintenance of chromatin association of the mutant large T at the nonpermissive growth temperature is important for the maintenance of the transformed phenotype in A-type transformants (7, 32). We therefore compared the subnuclear distributions of wild-type large T in FR(wt648) cells and of the *tsA28* mutant large T in *tsA28.3* cells (25, 26), in *tsA28.3/5.0(N)* (N-type) cells, and in *tsA28.3/5.2(A)* (A-type) cells (Table 2) kept at 32 or 39°C for 24 h. Figure 5 demonstrates that the steady-state levels of wild-type large T in the various subnuclear fractions were rather similar in FR(wt648) cells at both growth temperatures (Fig. 5A and B) as were those of mutant large T in all cells kept at 32°C (Fig. 5C, E, and G). In cells of both N-type transformants kept at 39°C for 24 h, the mutant large T was confined to the nucleoplasm (Fig. 5D and F), indicating a redistribution of the mutant large T due to loss of chromatin and nuclear matrix association, as observed in FR(*tsA58*)A (N-type) cells after shift to the nonpermissive temperature (7, 13, 32). Also, mutant large T in cells of the A-type transformant *tsA28.3/5.2(A)* showed some redistribution after the cells were shifted to 39°C but retained some association with the chromatin and the nuclear matrix (Fig. 5H), which was stable even after prolonged culture of these cells at 39°C (data not shown). *tsA28* mutant large T in these cells thus behaved like *tsA58* mutant large T in FR(*tsA58*)57 (A-type) cells, suggesting that, for *tsA28.3/5.2(A)* cells as well, stabilization of the mutant large T in a biologically active conformation at the nonpermissive

FIG. 5. Subnuclear distribution of wild-type and of *tsA28* mutant large T in cells of *tsA28.3* derived N-type and A-type transformants at 32 and 39°C. Cells grown at either 32°C (A, C, E, and G) or 39°C (B, D, F, and H) were subfractionated into nucleoplasmic (N), chromatin (C), and nuclear matrix (NM) fractions, respectively, followed by immunoprecipitation for large T, SDS-PAGE, and Western blot analysis as described in Materials and Methods. (A and B) FR(wt648) cells; (C and D) *tsA28.3* cells; (E and F) *tsA28.3/5.0(N)* cells (N-type transformants); (G and H) *tsA28.3/5.2(A)* cells (A-type transformants).

growth temperature was responsible for the properties of these cells as *tsA* A-type transformants.

Phosphorylation of *tsA* mutant large T in *tsA28.3/5.0(N)* and in *tsA28.3/5.2(A)* cells at permissive and nonpermissive growth temperatures. We then addressed the question whether or not site-specific phosphorylation of the *tsA28* mutant large T in *tsA28.3/5.2(A)* cells (A-type transformants) and in *tsA28.3/5.0(N)* cells (N-type transformants) at the nonpermissive temperature correlated with the phenotypes of these cells. We analyzed the phosphorylation patterns of the mutant large T proteins from *tsA28.3/5.0(N)* cells and from *tsA28.3/5.2(A)* cells kept at 32 or 39°C. Figure 6A and C demonstrate that the phosphorylation patterns of the mutant large T proteins from both cell types kept at 32°C were identical to each other and to the pattern of wild-type large T

in FR(wt648) cells (Fig. 1). As with mutant large T in FR(*tsA58*)57 cells, only slight differences in the phosphorylation pattern of the mutant large T in *tsA28.3/5.2(A)* cells were observed when the cells were kept at 39°C before labeling (Fig. 6B), indicating the retention of a biologically active conformation of the mutant large T in these cells. In contrast, the phosphorylation pattern of the mutant large T in *tsA28.3/5.0(N)* cells kept at the nonpermissive growth temperature showed similar, drastic alterations as already observed with *tsA58* mutant large T in FR(*tsA58*)A (N-type) cells kept at 39°C, most notably a marked reduction in the relative abundances of peptides 4, 6a, and 7 and an almost complete loss of peptide 8, as well as an increased abundance of peptides 12a and 12 (Fig. 6D). Thus, the mutant large T proteins in two independently derived N-type transformants of completely different origin [FR(*tsA58*)A and *tsA28.3/5.0(N)*] displayed similar changes in their phosphorylation patterns when the cells were kept at the nonpermissive temperature. In contrast, the phosphorylation patterns of both mutant large T proteins in the corresponding A-type transformants were largely similar to that of wild-type large T.

DISCUSSION

Accumulating evidence suggests that phosphorylation plays an important role in regulating the various activities of SV40 large T in viral replication (22–24, 31). One, therefore, might assume that phosphorylation also is important for regulating the transforming activities of large T. Phosphorylation of large T occurs at about 10 different sites, clustered in the aminoterminal (Ser-106 to Thr-124) and the carboxy-terminal (Ser-639 to Thr-701) regions of large T (38, 49). An important aspect reflecting the regulatory role of phosphorylation is that phosphorylation of regulatory-important sites is reversible, leading to sites with a high phosphorylation turnover. This is the case for virtually all phosphorylation sites on large T that are phosphorylated in the nucleus, particularly for Ser-120 and -123 and for Ser-677, whereas phosphorylation on sites phosphorylated in the cytoplasm and on threonine residues is rather stable (36, 41, 48). This pattern fits well into attributing a regulatory function to the phosphorylation at those sites which were differently phosphorylated on the mutant large T proteins in FR(*tsA58*)A cells and in *tsA28.3/5.0(N)* cells kept at 32 or 39°C. With both proteins we found a marked reduction in the phosphorylation of peptides containing phosphorylation sites with high turnover rates (Ser-120 and -123 in the amino-terminal region and Ser-677 and Ser-679 in the carboxy-terminal region) but none of the phosphorylation sites classified as representing stable modifications. It is important, however, and argues for the specificity of the observed changes in phosphorylation of the mutant large T that not all phosphorylation sites exhibiting a high turnover were affected by the temperature shift, since we found that the relative abundance of phosphopeptides 6' and 6, containing Ser-112, remained unchanged after the temperature shift. In addition to these changes, a strong reduction in the phosphorylation of phosphopeptide 6a in mutant large T from cells of the N-type transformants FR(*tsA58*)A and *tsA28.3/5.2(A)* was found. Peptide 6a, in addition to Ser-112, contains an additional, so-far-undescribed phosphorylation site of large T which is currently being analyzed in our laboratory (16a).

Therefore, a reasonable interpretation of our results is that the site-specific alterations in the phosphorylation of the mutant large T proteins in cells of the *tsA* N-type transfor-

FIG. 6. Phosphorylation of mutant large T in *tsA28.3* derived N-type and A-type transformants at 32 and 39°C. Phosphopeptides of large T from *tsA28.3/5.2(A)* cells (A and B) and *tsA28.3/5.0(N)* cells (C and D) labeled with $^{32}\text{P}_i$ at 32°C (A and C) or 39°C (B and D) were prepared and analyzed as described in Materials and Methods and in the legends to Fig. 1 and 2.

mutants FR(*tsA58*)A and *tsA28.3/5.0(N)* at the nonpermissive temperature reflect the biological inactivation of these proteins. This inactivation probably is the result of a conformational change of the mutant proteins at the elevated growth temperature induced by the altered primary structure of the mutant proteins (exchange of Ala-438 to a Val in the *tsA58* mutant large T and of Trp-393 to a Cys in the *tsA28* mutant large T [2, 21]). Although it is difficult to draw conclusions on possible biological roles of individual phosphorylation sites from changes in the phosphorylation pattern of the mutant large T proteins in the N-type cells FR(*tsA58*)A and *tsA28.3/5.0(N)* after shift to the nonpermissive growth temperature, two prominent changes should be addressed.

(i) One change is the marked reduction of phosphorylation of phosphopeptide 6a, representing a so-far-unidentified site in the vicinity of Ser-112. Phosphorylation of this site seems to be important for the transforming activity of large T, since its state of phosphorylation correlated with the expression of the cellular phenotype not only in the *tsA* N-type and A-type systems described in this report, but also in several other cellular systems, where transforming and nontransforming large T proteins were compared (unpublished data).

(ii) Another change is the apparent overphosphorylation of Thr-124. Since phosphorylation of Thr-124 seems to be important for the large T DNA binding and replication activity, i.e., for maintaining large T in a biologically active conformation (9, 22–24, 31, 40, 43, 50), overphosphorylation of Thr-124 does not seem to fit into the pattern of a biologically inactive mutant large T. However, our data strongly suggest that the apparent overphosphorylation of

Thr-124 actually is the result of an underphosphorylation of neighboring phosphoserines, most likely of Ser-120 and -123. Thus, a balanced phosphorylation of Ser-120, Ser-123, and Thr-124 seems to be important for maintaining an active conformation of large T, as suggested previously (9, 40).

A result important for interpreting this phenotype-specific phosphorylation of *tsA* mutant large T in cells of N- and A-type transformants kept at the nonpermissive temperature was that this phenomenon was not restricted to a single set of established *tsA* mutant large T transformants but could be induced by a large-T-dependent *in vitro* progression of *tsA28* N-type transformant cells of completely different origin (*tsA28.3*) to A-type transformant cells. Since the preservation of the biological properties and transforming activity of the *tsA28* mutant large T in the ensuing A-type transformant cells at the nonpermissive growth temperature was accompanied by the preservation of a wild-type phosphorylation pattern of the mutant large T in these cells, induction of a wild-type-like phosphorylation of the mutant large T seems to reflect a more general cellular mechanism in selecting for a transformation-competent *tsA* mutant large T at the nonpermissive growth temperature.

If, indeed, the phosphorylation pattern of large T reflects its conformational state, then the preservation of a wild-type phosphorylation pattern of the mutant large T in A-type FR(*tsA58*)57 and *tsA28.3/5.2(A)* cells kept at the nonpermissive growth temperature would reflect the preservation of a biologically active ("wild-type") conformation of the mutant large T in these cells. An important question, then, is whether the wild-type-like phosphorylation of the mutant

large T proteins in these cells actually is the result of the wild-type-like conformation of the mutant protein or, alternatively, phosphorylation actively induces, and is responsible for, maintaining this conformation. In the first case, the wild-type-like conformation of the mutant large T would be preserved by so-far-unknown means, e.g., by a tight interaction of the mutant large T with a target(s) at the cellular chromatin. The latter interpretation would imply that the phosphorylation state of large T actively controls its conformational state and thus its biological activity. Furthermore, one would have to assume that the activities of the cellular kinases and phosphatases involved in the regulatory phosphorylation of large T are under tight cellular control, allowing for a phenotype-dependent phosphorylation of the mutant large T proteins in the cells analyzed in this study. Although our data, so far, do not allow us to discriminate between these alternatives, we favor the hypothesis that specific phosphorylation of the mutant large T plays an active role in maintaining its biological activity. This interpretation is supported by recent findings in our laboratory that wild-type large T in a flat revertant of SV40-transformed REF 52 (Rev2 cells; 1) exhibits properties and a phosphorylation pattern similar to *tsA* mutant large T in cells of N-type transformants kept at the nonpermissive temperature (6a). However, to further understand the specific and phenotype-dependent phosphorylation of large T, it will be important to characterize the kinases and phosphatases involved in the phosphorylation of large T as well as the regulation of their activities by viral tumor antigens and the cell.

Scheidtmann and Haber recently demonstrated that abortive infection or cellular transformation by SV40 results in specific phosphorylation of the cellular protein p53 at additional sites (39). This additional phosphorylation was large T specific since abortive infection of F111 cells with SV40 *tsA58* mutant virus at the permissive temperature resulted in metabolic stabilization and additional phosphorylation of p53, while infection at the nonpermissive growth temperature failed to induce these changes. Thus, metabolic stabilization and enhanced phosphorylation of p53 would depend on a functionally active large T. Our laboratory provided evidence that p53 is metabolically stable in FR(*tsA58*)57 (A-type) cells, at the nonpermissive temperature but is rapidly degraded in FR(*tsA58*)A (N-type) cells under these growth conditions (7; this study). Thus, the metabolic stability of p53 correlated well with the phenotype of these cells and with our finding that the mutant large T proteins in these cells exhibited either wild-type [FR(*tsA58*)57 (A-type) cells] or mutant [FR(*tsA58*)A (N-type) cells] properties (7; this study). In view of these results, one might have expected a phenotype-dependent phosphorylation of p53 in our cell system. However, phosphorylation of p53 was identical in both of the *tsA* mutant transformed cell lines, exhibiting phosphorylation patterns typical for p53 in SV40 wild-type transformed cells at the permissive growth temperature and a pattern somewhat between that of p53 in normal cells and that of p53 in SV40 wild-type transformed cells at the nonpermissive growth temperature. A temperature-dependent effect on phosphorylation of p53 could be excluded since phosphorylation of p53 in cells of the wild-type transformant FR(wt648) was rather similar at both growth temperatures. Therefore, this finding questions the hypothesis that phosphorylation of p53 in these cells plays a role in metabolic stabilization of p53 and contributes to the transformed phenotype. In fact, the situation in SV40-transformed rat cells would be similar to that in mouse cells,

where it was recently demonstrated that neither metabolic stabilization of p53 nor expression of a transformed phenotype could be correlated with qualitative or major quantitative changes in the p53 phosphorylation pattern (28). In contrast, the phosphorylation pattern of mutant large T correlated well with its function(s) in maintaining the transformed phenotype, the phenotype of p53 with regard to its metabolic stability, and the expression of mutant large T proteins exhibiting either mutant or wild-type properties (7, 32; this study). Therefore, we conclude that specific phosphorylation events allow the mutant large T in SV40 *tsA* mutant A-type transformant cells to assume a wild-type conformation at the nonpermissive growth temperature and that these phosphorylation events are sufficient to modulate the transforming activity of the mutant large T in such a way that it is able to maintain the transformed phenotype.

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