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Stabilization of the Large T Protein in Temperature-Independent (Type A) FR 3T3 Rat Cells Transformed with the Simian Virus 40 *tsA*30 Mutant

JEAN IMBERT,[1] PHILIPPE CLERTANT,[2] BÉATRICE DE BOVIS,[1] JACQUELINE PLANCHE,[1] AND FRANÇOISE BIRG[1]*

Unité 119 de l'Institut National de la Santé et de la Recherche Médicale, 13009 Marseille,[1] and Centre de Biochimie du Centre National de la Recherche Scientifique, Université de Nice, 06034 Nice,[2] France

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The stabilities of in vivo [^{35}S]methionine-labeled large T and small t proteins, synthesized in temperature-sensitive (type N) and temperature-insensitive (type A) FR 3T3 rat cells transformed by an early temperature-sensitive mutant of simian virus 40 (SV40), *tsA*30, were analyzed at the permissive and restrictive temperatures. The two polypeptides, detected in greatly reduced amounts in cells of the N type at the restrictive temperature, were also unstable at the permissive temperature. However, both were made in similar amounts and were apparently stable in cells of the A type, irrespective of the temperature. The structures of the viral RNAs present at the permissive temperature were analyzed for transformants representative of each type, and containing a single integration of viral DNA. The two cell lines synthesized transcripts identical to the large T and small t mRNAs identified in SV40-infected monkey cells. Similar amounts of viral RNA were found in A and N transformants in active growth at the permissive and restrictive temperatures, which argued against a control at a transcriptional level. Assay of a defined function of the protein, namely, the binding of nucleotide detected by affinity labeling with periodate-oxidized [α-^{32}P]ATP, clearly showed that the large T proteins from both types of transformants exhibited, at least for that particular biochemical function, the same in vitro temperature sensitivity. In transformants of the A type only could a reduced binding activity be detected in extracts from cells grown at the restrictive temperature. Thus, the temperature-independent behavior of the A transformants may result from an in vivo partial stabilization of the newly synthesized large T protein, probably through interaction with a cellular component(s).

The simian virus 40 (SV40) large T antigen (46) appears to be a complex protein, likely acting at different levels: the initiation of viral DNA replication and the regulation of early viral transcription in infected permissive cells, and the maintenance of the transformed state in stable transformants. The experimental approach used to analyze the latter function was to transform rodent cells at the permissive temperature with viral mutants which were known to express a thermolabile large T protein and were known to be temperature sensitive for the initiation of viral DNA synthesis in permissive monkey cells (39, 41), and then to analyze the expression of transformation characters upon temperature shift experiments (7, 8, 19, 25, 28, 31, 40). This methodology is based on the a priori assumption that when a complex protein appears to be thermolabile in a given cell, or temperature sensitive for a given function, it will also behave in a temperature-sensitive manner for another function in a different cell line.

The results of these experiments appeared to depend to some extent upon the host cell species. Considering only rat fibroblasts, three distinct situations were observed. (i) Cells expressed temperature-dependent properties (14, 31, 36); at high temperature, they appeared negative by immunofluorescence staining for T antigen (14, 28), and only reduced amounts of large T were detected after [^{35}S]methionine labeling (15, 36). (ii) Cells expressed a temperature-sensitive transformed phenotype, but large T was present at the nonpermissive temperature (14, 36). (iii) Cells exhibited a temperature-independent transformed phenotype (14, 31, 36).

The fact that temperature-dependent transformed cells were obtained upon infection with temperature-sensitive mutants (first two situations) is in agreement with the approach outlined

above. The occurrence of temperature-independent transformants, however, was not expected, and many hypotheses have been put forward to explain this situation (14, 36, 46). We have considered the possibility that in these transformants, the large T protein may be under a different transcriptional control or subject to stabilization. The cell lines used were established by M. Rassoulzadegan and co-workers (31), by transforming FR 3T3 rat cells (35) with the *tsA*30 mutant of SV40 (41): the N and A types of transformants thus obtained correspond, respectively, to situations (i) and (iii) described above.

(This work constitutes part of a thesis submitted by J.I. for the Doctorat d'Etat at the University of Marseille-Luminy.)

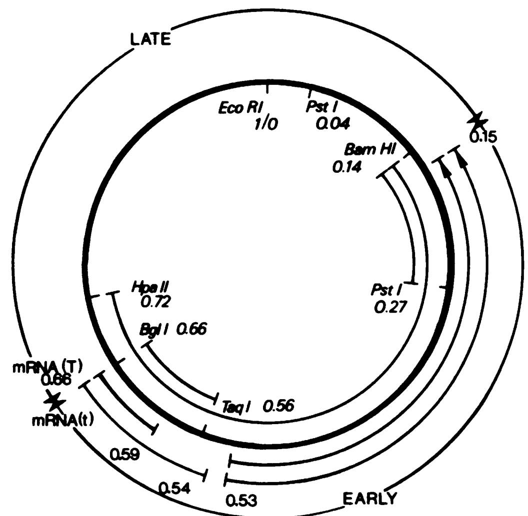


FIG. 1. Restriction enzyme map of SV40 DNA and structures of the early viral mRNAs. The heavy circle represents the SV40 DNA molecule, divided in early and late regions. The positions of some restriction enzyme sites are indicated (13, 32). The *Eco*RI site is taken as the zero of the map. The inner partial circles represent the DNA fragments used in viral RNA mapping, and the outer partial circles show the locations and the structures of the two spliced RNAs coding for large T and small t (5, 13, 32).

MATERIALS AND METHODS

Cells and viruses. The *tsA*30 (41) and the wild-type (WT) SV40 derivatives of FR 3T3 cells (35) have been described previously (31). They were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% newborn calf serum (GIBCO Laboratories). Cells were used at low passage levels (fewer than 10 transfers). WT SV40 strain VA4554 was grown in CV-1 cells in DMEM plus 10% fetal calf serum (Seromed). Virus stocks were made from cells infected at low multiplicities (less than 0.01 PFU per cell).

Purification of SV40 DNA fragments. Viral DNA was prepared from CV-1 cells infected at 10 PFU per cell by selective extraction (16). Form I DNA was purified by cesium chloride-ethidium bromide equilibrium centrifugation, followed by velocity sedimentation in a sucrose gradient, essentially as described previously (12). Subgenomic fragments were obtained by cleavage of purified form I SV40 DNA with the restriction endonucleases *Bgl*I, *Taq*I (Bethesda Research Laboratories), *Hpa*II, *Pst*I (Boehringer Mannheim), and *Bam*HI (Miles Laboratories), using the conditions recommended by the manufacturers. The mixtures of fragments were resolved by electrophoresis in 0.6% Low Gelling Temperature agarose (Marine Colloids) in E buffer (37). The positions of the fragments were visualized by ethidium bromide staining, the bands were cut out, and the agarose was solubilized by treatment with 5 M sodium perchlorate (48). DNA fragments were precipitated with ethanol and used in the hybridization reactions. The various fragments used and their locations on the viral genome are shown in Fig. 1.

Preparation of RNA from transformed cells. Cytoplasmic RNA was isolated from subconfluent cultures maintained at 32°C, as described previously (12). High-temperature preparations were made from cells seeded at a density of 2×10^5 to 3×10^5 per 90-mm dish, allowed to grow for 24 h at 32°C, and shifted to 40.5°C for 72 h. At 24 h before harvest, the cells were medium-changed with prewarmed DMEM containing 10% calf serum. Polyadenylated cytoplasmic RNAs were isolated by double chromatography on oligodeoxythymidylate-cellulose (type III, Collaborative Research) as described previously (3).

In parallel with the RNA preparations, cells were labeled with [^{35}S]methionine (≥1,100 Ci/mmol; Radiochemical Centre, Amersham, England), and the viral polypeptides were identified by immunoprecipitation with anti-SV40 T antigen serum (a serum pool made from hamsters inoculated with Cl_2TSV5 cells [15]).

Preparation of RNA from infected cells. Subconfluent CV-1 cells were infected with WT SV40 at 100 PFU per cell. After 1 h at 37°C, the virus suspension was removed and replaced by DMEM containing 10% fetal calf serum and 20 μg of cytosine arabinoside (Sigma) per ml (5); cytoplasmic RNA was isolated at 17 h after infection as described above.

Analysis of viral RNAs by S1 mapping. S1 mapping (4) was done as described previously (12). Each hybridization reaction contained viral DNA (0.01 or 0.02 μg) or equivalent amounts of a specific fragment, and total cytoplasmic (12.5 to 100 μg) or polyadenylated (1 to 10 μg) RNA in 10 or 20 μl of hybridization buffer [40 mM piperazine-*N*-N′-bis(2-ethanesulfonic acid), pH 6.4 (Sigma), containing 0.4 M NaCl, 1 mM EDTA, and 80% (vol/vol) recrystallized formamide]. Nucleic acids were denatured for 15 min at 85°C and were allowed to reassociate for 3 h at 49°C. Single-stranded nucleic acids were hydrolyzed with 100 to 200 U of S1 endonuclease (Sigma) for 30 min at 37°C. S1-treated hybrids were electrophoresed in a 2% agarose gel (15 by 20 cm) (Marine Colloids) in 0.03 N NaOH containing 1 mM EDTA (12). DNA fragments were transferred to nitrocellulose (0.1 μm pore size, Sartorius) (38) and were hybridized to high-specific-activity [^{32}P]SV40 DNA labeled in vitro by nick translation (34). Specific activities of the probe were in the range

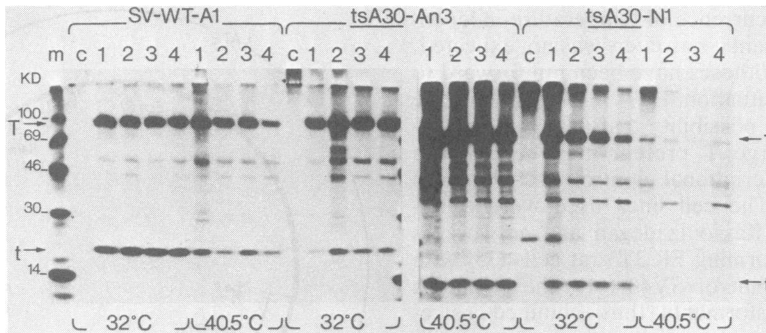


FIG. 2. In vivo stabilities of large T and small t in various transformants grown at 32 and 40.5°C. Cells were labeled for 1 h with [^{32}S]methionine as described in the text, and the immunoprecipitated proteins (17) were analyzed in a 12.5% polyacrylamide gel (21). For each cell line, lanes 1 to 4 at each temperature correspond, respectively, to no chase, 30 min, 60 min, and 90 min of chase in cold medium. Lanes labeled "c" were control immunoprecipitations of cells grown at 32°C and treated with normal hamster serum. Lane "m" contained marker ^{14}C-labeled proteins electrophoresed in parallel; their sizes are indicated. The positions of large T and small t are indicated for each gel. The film was exposed for 7 days.

of 1×10^8 to 3×10^8 cpm/μg. Hybridizations were performed in a large volume (26). Bands were detected by fluorography, using Fuji RX films and DuPont Lightning-Plus intensifying screens (23).

Analysis of viral RNAs by direct transfer to nitrocellulose. RNA samples were denatured with glyoxal/dimethylsulfoxide as described previously (45), except that the denaturation time was lowered to 30 min. Subsequent steps were as described previously.

In vivo stability of the early viral polypeptides. A total of 10^5 cells from each transformant were seeded per 60-mm dish and were allowed to grow for 24 h at 32°C. Half of the dishes were then shifted to 40.5°C for 72 h, and the remaining half were maintained at 32°C for the same time. Cells were incubated in DMEM minus methionine for 1 h and then were labeled for 1 h with 100 μCi of [^{35}S]methionine per dish (1 ml) in the same medium. After being washed with DMEM, the label was chased for increasing time periods (30 to 90 min). The viral polypeptides were immunoprecipitated with anti-SV40 T antigen hamster serum, using *Staphylococcus aureus* cells (Sigma) and the urea technique (17), and were analyzed by polyacrylamide gel electrophoresis (21). The sizes of the immunoprecipitated proteins were determined from the migration of marker ^{14}C-proteins (Radiochemical Centre) included in the same gel.

Covalent affinity labeling of the large T protein. Covalent affinity labeling was performed as described previously (10) by overnight incubation at 0°C of [α-^{32}P]ATP (410 Ci/mmol; Radiochemical Centre), previously oxidized with periodate, in the presence of $NaCNBH_3$, with the following modifications. Every step, except as otherwise noted, was performed at 0°C. Extracts, made by cell lysis at high pH (8.6) and high ionic strength in the presence of detergent, were brought to the optimal conditions for oxidized-ATP labeling by rapid filtration on Bio-Gel P30 (P. Clertant, P. Gaudray, and F. Cuzin, manuscript in preparation). The labeled viral proteins were then immunoprecipitated from the reaction mixture and analyzed by polyacrylamide gel electrophoresis. Quantification of the amounts of proteins was done by Coomassie blue staining of the gel, followed by scanning on a Vernon densitometer. The gel was then dried and submitted to autoradiography; the large T bands were cut out, rehydrated, and counted by Cerenkow radiation.

RESULTS

In vivo stabilities of the early viral polypeptides synthesized in A and N FR 3T3-SV40 *tsA30* transformants. SV-*tsA30*-N1, SV-*tsA*30-An3, and SV-WT-A1 cells were labeled with [^{35}S]methionine at 32 and 40.5°C; the label was chased for increasing time periods; and the immunoprecipitated proteins were analyzed by polyacrylamide gel electrophoresis (Fig. 2). In the WT transformant, large T exhibited similar stabilities at the two temperatures. Large T, small t, and the associated cellular proteins were not only detected in reduced amounts in SV-*tsA*30-N1 cells grown at 40.5°C (15; Fig. 2), but they were also unstable at 32°C. The half-life of large T in the various transformants was estimated by densitometer scanning of the autoradiograms. It was 30 min at 32°C for SV-*tsA*30-N1 cells. In contrast, large T in SV-*tsA*30-An3 cells grown at 40.5°C had a half-life of at least 90 min, a value similar to that obtained in the case of SV-WT-A1 cells. The same experiment, performed on another N transformant (SV-*tsA*30-N2) and on two other A transformants (SV-*tsA*30-A1 and SV-*tsA*30-An6), gave results identical to those described above (data not shown).

The SV40 large T protein is therefore rather unstable in cells of the N type, even at the permissive temperature, but it is made in relatively large amounts and is apparently stable in cells of the A type at the restrictive temperature. However, before we can consider a stabilization

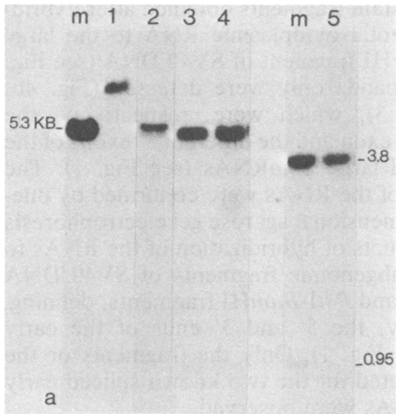
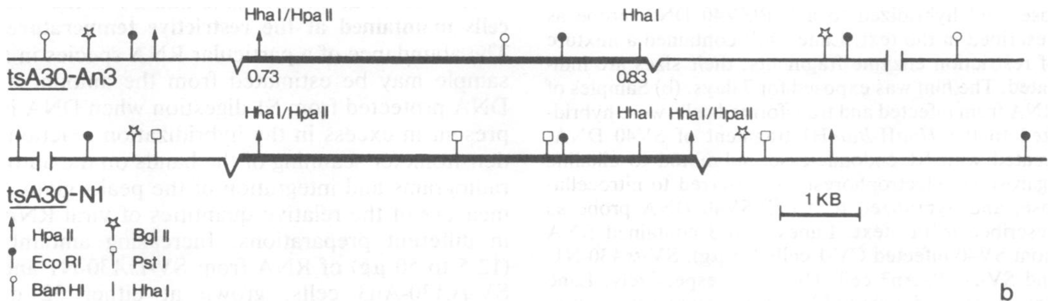


FIG. 3. Restriction enzyme mapping of the viral insertion in SV-*tsA*30-N1 cells. All experimental procedures have been described previously (26). (a) Samples (10 μg) of SV-*tsA*30-N1 DNA were digested with the restriction enzymes *Bgl*II (lane 1), *Eco*RI (lane 2), *Bgl*II + *Hpa*II (lane 3), *Hpa*II (lane 4), and *Hha*I + *Eco*RI (lane 5). The two lanes labeled "m" contained marker SV40 DNA fragments, *Eco*RI-cleaved SV40 DNA (5.3 kb) and *Eco*RI + *Hha*I-cleaved SV40 DNA (only the two largest fragments, 3.8 and 0.95 kb, respectively, are visible). (b) Restriction enzyme maps of the viral DNA sequences integrated in the cellular DNA in cell lines SV-*tsA*30-N1 and SV-*tsA*30-An3. The map of the SV-*tsA*30-An3 insertion is taken from Mougneau et al. (26). SV40 sequences are indicated by a heavy line, and the flanking cellular sequences are indicated by a light line. Broken lines indicate the sites of recombination. The cleavage sites for various restriction endonucleases in the adjacent cellular sequences are also indicated.



of large T in A transformants as a basis for their temperature-independent expression of the transformed phenotype, two interfering hypotheses have to be checked. Transcription could possibly be affected at high temperature, due to a repression in N transformants (positive regulation suggested by Gaudray et al. [15]) or to a derepression in A transformants, as observed previously in lytically infected cells and in transformed cells of other species (2, 11, 18, 33, 36, 39, 42, 44). Alternatively, A transformants could carry a revertant viral genome; however, this hypothesis is made unlikely by the fact that these transformants are obtained at high frequencies under defined transformation conditions (30); they produce virus plaques upon fusion with permissive monkey cells only at the permissive temperature (31).

Representative transformants of the A and N types, namely cell lines SV-*tsA*30-An3 and SV-*tsA*30-N1, respectively, were chosen for a detailed analysis because they both contain a single insertion of SV40 DNA (26; see below).

Restriction enzyme mapping of the viral insertions in SV-*tsA*30-An3 and SV-*tsA*30-N1 cells. The structure of the viral insertion in SV-*tsA*30-An3 cells has been reported; that of the SV-*tsA*30-N1 cells was determined. A restriction enzyme which does not cut the SV40 genome produced a single labeled fragment (Fig. 3a, lane 1). The integration of more than a complete viral genome was suggested by the results of the *Hpa*II digestion, which produced, in addition to two faint fragments, a heavily labeled band which comigrated with unit-length viral DNA (Fig. 3a, lane 4). This was confirmed by digestions of the cellular DNA, either with restriction enzymes introducing multiple cuts in the viral DNA or with a combination of restriction enzymes (Fig. 3a). A map of the integrated SV40 DNA sequences was deduced from these data (Fig. 3b): the cells contain a partial tandem repeat of SV40 DNA, a type of organization often found in SV40-transformed rat cells (6, 26). The structures of the two viral insertions are very similar, as the viral sites of recombination lie in the same region of the viral DNA molecule. A complete early region with its promoters is apparently preserved in both cell lines (46). The two viral insertions, however, are located in different cellular environments, as indicated by the different distributions of some restriction enzyme sites in the adjacent cellular sequences (Fig. 3b).

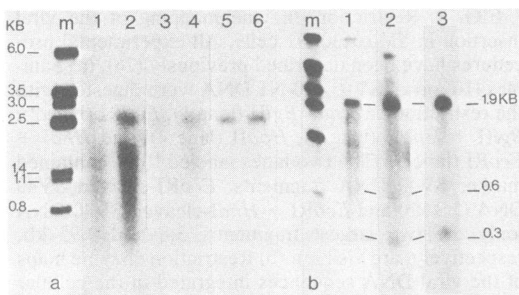


FIG. 4. Identification of the viral RNAs in SV-*tsA*30-N1 and SV-*tsA*30-An3 cells grown at the permissive temperature. (a) Denatured cytoplasmic RNAs from SV40-infected CV-1 cells (25 and 50 μg, lanes 1 and 2, respectively), SV-*tsA*30-N1 (50 and 100 μg, lanes 3 and 4, respectively), and SV-*tsA*30-An3 (50 and 100 μg, lanes 5 and 6, respectively) were electrophoresed on an agarose gel, transferred to nitrocellulose, and hybridized to a [^{32}P]SV40 DNA probe as described in the text. Lane "m" contained a mixture of restriction enzyme fragments; their sizes are indicated. The film was exposed for 7 days. (b) Samples of RNA from infected and transformed cells were hybridized to the *Hpa*II-*Bam*HI fragment of SV40 DNA, treated with S1 endonuclease, submitted to alkaline agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to a [^{32}P]SV40 DNA probe as described in the text. Lanes 1 to 3 contained RNA from SV40-infected CV-1 cells (25 μg), SV-*tsA*30-N1, and SV-*tsA*30-An3 cells (100 μg), respectively. Lane "m" contained marker SV40 DNA fragments (a mixture of *Eco*RI, *Hin*dIII, and *Bam*HI + *Hpa*II fragments). The film was exposed for 3 days.

Identification and mapping on the viral genome of the early viral RNAs synthesized in the two transformants at the permissive temperature. Cytoplasmic RNAs extracted from SV-*tsA*30-N1 and SV-*tsA*30-An3 cells grown at 32°C were denatured with glyoxal/dimethylsulfoxide (45), fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to a [^{32}P]SV40 DNA probe as described above. For both cell lines, a major band of 2.5 to 2.6 kilobases (kb) was detected (Fig. 4a, lanes 3 to 6); a band with the same mobility (Fig. 4a, lanes 1 and 2) was observed with cytoplasmic RNA extracted from SV40-infected CV-1 cells treated with cytosine arabinoside (referred to as early lytic RNA). Only this band, which presumably corresponds to the large T mRNA (29), was detected in these conditions. As similar results (data not shown) were obtained either with total cytoplasmic RNA or with the polyadenylate-containing fraction, most experiments were done with total cytoplasmic RNA.

The determination of the structures of the viral RNAs was done by S1 mapping, using a combination of one-dimensional (alkaline) and two-dimensional (12) agarose gel electrophoresis, together with hybridization to different portions of the SV40 early region. Figure 4b shows the S1-resistant fragments obtained after hybridization of total cytoplasmic RNA to the large *Hpa*II-*Bam*HI fragment of SV40 DNA (see Fig. 1). Three bands only were detected (Fig. 4b, slots 1 to 3), which were, respectively, the common 3′ exon and the different 5′ exons of the small t and large T mRNAs (see Fig. 1). The structures of the RNAs were confirmed by one- and two-dimensional agarose gel electrophoresis of the products of hybridization of the RNAs to different subgenomic fragments of SV40 DNA (*Bgl*I-*Taq*I and *Pst*I-*Bam*HI fragments, defining, respectively, the 5′ and 3′ ends of the early region; see Fig. 1). Only the fragments or the spots expected for the two known spliced early SV40 mRNAs were observed.

Viral RNA in SV-*tsA*30-N1 and SV-*tsA*30-An3 cells maintained at the restrictive temperature. The abundance of a particular RNA species in a sample may be estimated from the amount of DNA protected from S1 digestion when DNA is present in excess in the hybridization reaction; densitometer scanning of the bands on the autoradiograms and integration of the peaks gives a measure of the relative quantities of viral RNA in different preparations. Increasing amounts (12.5 to 50 μg) of RNA from SV-*tsA*30-N1 and SV-*tsA*30-An3 cells, grown at either 32 or 40.5°C, were hybridized to a fixed amount of the *Hpa*II-*Bam*HI fragment of SV40 DNA (Fig. 5b). Viral RNA was detected in all cases, even in SV-*tsA*30-N1 cells grown at 40.5°C for 3 days, in which the amount of [^{35}S]methionine-labeled early viral polypeptides was strongly reduced (Fig. 5a, lane 3). On the contrary, amounts of labeled early viral polypeptides and of viral RNA were similar at the two temperatures for SV-*tsA*30-An3 cells (Fig. 5a, lanes 5 and 6, and Fig. 5b). The maximum variation observed between RNA preparations from SV-*tsA*30-N1 cells grown at the permissive and restrictive temperatures was a twofold reduction (Table 1). These results suggest that the strong reduction in the amounts of [^{35}S]methionine-labeled large T in N transformants does not result from a repression of transcription (15). Similar amounts of viral RNA were found in SV-*tsA*30-An3 cells grown at either 32 or 40.5°C; the variations observed were of the same order of magnitude as in the case of SV-WT-A1 cells (Table 1). Early viral RNA is therefore not overproduced in transformants of the A type, at least under the conditions used here (3 days of growth at 40.5°C), in which a steady-state level may have been reached.

The overall amounts of viral RNA were apparently higher in SV-*tsA*30-An3 cells than in SV-*tsA*30-N1 cells (see Fig. 4 and 5); this was

reproducibly observed and was also true for other A and N transformants (data not shown). On the other hand, examination of some autoradiograms (see, for example, Fig. 4) suggested that there exists a relative preference for the large T mRNA splice in type A cells. However, an integration of the densitometric profiles of the large T and small t 5′ exons, performed as described previously (1), showed that the ratios of the large T to small t mRNAs in the two types of cells (SV-*tsA*30-N1 [2.2 ± 0.2] to SV-*tsA*30-An3 [2.8 ± 0.4]; 7 and 10 independent determinations, respectively) were not significantly different from each other and were similar to the values obtained in the case of SV40-infected monkey cells grown at 32°C (1). This ratio was unaffected by the temperature shift in the case of the two transformants (data not shown), a situation at variance with that of SV40-infected monkey cells, in which the large T to small t mRNA ratio increased with temperature (1). These data suggest that the control of gene expression is not mediated by a differential splicing.

Transformants of the A type synthesize a temperature-sensitive large T protein. It was shown

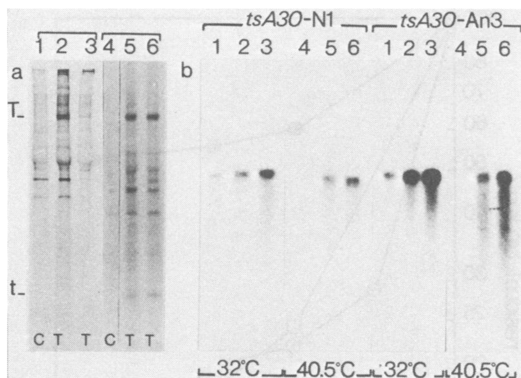


FIG. 5. Viral RNA in SV-*tsA*30-N1 and SV-*tsA*30-An3 cells grown at the restrictive temperature. (a) Immunoprecipitation of the early viral polypeptides from cells grown at either 32 or 40.5°C and labeled for 1 h with [^{35}S]methionine before extraction. Lanes 1 to 6: SV-*tsA*30-N1 cells grown at (1) 32°C, treated with normal hamster serum; (2) 32°C, treated with tumor serum; (3) 40.5°C, treated with tumor serum; and SV-*tsA*30-An3 cells grown at (4) 32°C, treated with normal hamster serum; (5) 32°C, treated with tumor serum; and (6) 40.5°C, treated with tumor serum. (b) Increasing amounts of RNA from cells grown at 32 and 40.5°C were hybridized to a fixed amount of the *Hpa*II-*Bam*HI fragment of SV40 DNA and treated as described in the legend to Fig. 4b. For each cell line, lanes 1 to 3 contained 12.5, 25, and 50 μg, respectively, of RNA from cells grown at 32°C, and lanes 4 to 6 contained the same amounts of RNA from cells grown at 40.5°C.

TABLE 1. Quantification of the amounts of viral RNA in the various transformants grown at 32 and 40.5°C[a]

Cell line	Peak area ratio (40.5°C/32°C) ± confidence interval[b]	n^c
SV-*tsA*30-N1	0.65 ± 0.27	10
SV-*tsA*30-An3	0.92 ± 0.37	8
SV-WT-A1	0.98 ± 0.26	3

[a] Three different RNA preparations were made from cells grown in parallel at the two temperatures.

[b] Ratio (40.5°C/32°C) of the peak areas of the densitometric profiles of the 1.9-kb fragment (S1 mapping) or of the 2.5-kb band (direct transfer). Every ratio was determined for a given amount of total cytoplasmic or polyadenylated RNA. Autoradiograms were not saturated.

[c] Number of independent determinations at each temperature.

previously that the nucleotide binding activity of the large T protein, detected by covalent affinity labeling with periodate-oxidized [α-^{32}P]ATP, is more thermolabile in extracts of cells infected with the *tsA*30 mutant than in extracts of WT SV40-infected monkey cells (10). To exclude any reversion or suppression of the *tsA* mutation in the transformants of the A type, we assayed this activity of large T in extracts of transformed cells grown at either 32 or 40.5°C. After incubation of crude cell extracts with periodate-oxidized [α-^{32}P]ATP in the presence of cyanoborohydride, the large T molecules were purified by immunoprecipitation. An affinity labeling could be observed in comparable amounts for the large T made in SV-WT-A1 cells at the two temperatures (Fig. 6). This labeling could also be detected for the two *tsA*30 transformants, SV-*tsA*30-N1 and SV-*tsA*30-An3, at the permissive temperature (Fig. 6, lanes "T" at 32°C). Although it was greatly reduced, some binding activity remained in SV-*tsA*30-An3 cells grown at 40.5°C; but it disappeared almost completely at high temperature in the case of SV-*tsA*30-N1 cells. Two other transformants, SV-*tsA*30-An6 and SV-*tsA*30-N2, behaved, respectively, like SV-*tsA*30-An3 and SV-*tsA*30-N1 (data not shown). The specificity of the binding reaction was checked as previously described (10), by protection with an excess of cold ATP (Fig. 6, lanes "C" at 32 and 40.5°C). We can therefore conclude that a residual level of a biochemical activity characteristic of large T is still present at the nonpermissive temperature in transformants of the A type but is almost absent in transformants of the N type.

In vitro denaturation experiments, performed

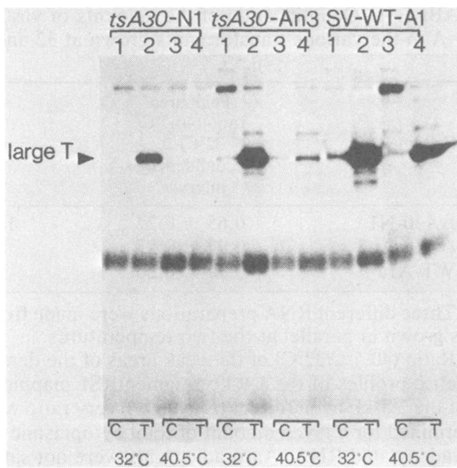


FIG. 6. Affinity labeling of large T with periodate-oxidized [α-^{32}P]ATP in extracts of transformed cells grown at 32 and 40.5°C. Extracts (0.25 ml, made from 5×10^5 cells) were incubated in the presence of oxidized [α-^{32}P]ATP (0.2 μM) and 10 mM $NaCNBH_3$ for 14 h at 0°C. Large T was immunoprecipitated and analyzed by polyacrylamide gel electrophoresis. The labeling incubation was made in the presence (lanes "C") or absence (lanes "T") of cold ATP (1 mM).

on extracts of A and N cells grown at 32°C, clearly showed that the binding activity was equally thermolabile in both types of cells (Fig. 7). Large T exhibited a half-life of 5 min at 37°C in the two *tsA*30 transformants, a value identical to that previously determined for this protein in *tsA*30-infected monkey cells (10). These results exclude the possibility of a reversion or genetic suppression of the *tsA* mutation in the transformants of the A type.

Partial stabilization of the large T protein in transformants of the A type at the restrictive temperature. It was shown previously (10) that under the labeling conditions used here, the oxidized [α-^{32}P]ATP labeling does not measure a steady-state equilibrium, but rather the rate of the binding reaction. Therefore, the specific activity of ATP labeling, which could be estimated (Table 2), represents a measure of the amounts of active large T molecules. From 50 to 70% of the large T molecules were denatured in extracts of A and N cells grown at 32°C, according to the cell line (Table 2). However, after growth at 40.5°C, 16% of the large T molecules were still active in SV-*tsA*30-An3 cells, a percentage quite higher than in SV-*tsA*30-N1 or SV-*tsA*30-N2 cells (data on SV-*tsA*30-N2 cells were included for comparison, as the determinations made on cells on the N type are at the limit of sensitivity of the technique). In transformants of the A type, large T therefore appears to be at least partially stabilized in vivo at 40.5°C; this result is in agreement with the stability of the newly synthesized large T molecules observed in the pulse-chase labeling experiments described above.

DISCUSSION

To account for the occurrence of temperature-insensitive (type A) transformants upon infection of rodent cells with temperature-sensitive mutants of the SV40 *A* group, many hypotheses have been formulated (7, 14, 31, 36, 46) but have not been extensively investigated. Several authors have postulated that large T is involved in the maintenance of the transformed state in temperature-insensitive transformants, and they have suggested that these cells contain higher levels of active large T than do N transformants at the restrictive temperature (7, 11, 36, 44). A higher T antigen level may be obtained either through an overproduction (11) or through a stabilization of the protein (7, 36). We report

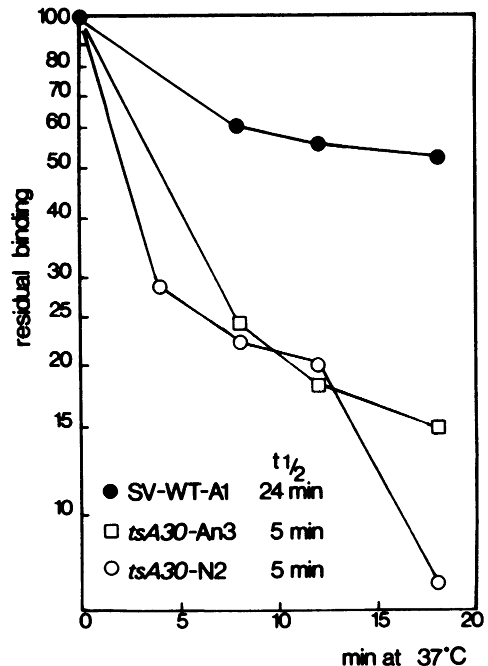


FIG. 7. Thermosensitivity of the nucleotide binding activity of large T in various transformants. Extracts of transformed cells grown at 32°C were incubated at 37°C for increasing time periods and then were assayed at 0°C for oxidized [α-^{32}P]ATP labeling (see Fig. 6 and the text). The large T bands in the polyacrylamide gels were then cut out and counted.

TABLE 2. Characteristics of the oxidized [α-^{32}P]ATP binding activity in various transformants

Extract[a] from:	In vitro half-life (min) of nucleotide binding activity[b]		Amt of large T (a.u.)[c]		Amt of oxidized [α-^{32}P]ATP bound (fmol)		Sp act of oxidized [α-^{32}P]ATP binding (fmol/a.u.)		Relative activity to fully active molecules (%)[e]	
	32°C	40.5°C	32°C	40.5°C	32°C	40.5°C	32°C	40.5°C	32°C	40.5°C
SV-WT-A1	24	ND[d]	4.0	ND	32.7	10.8	8.2	ND	100	ND
SV-*tsA*30-An3	≃5	ND	4.3	0.9	17.8	1.2	4.1	1.3	50	16
SV-*tsA*30-N1	≤5	ND	1.7	<0.4	3.5	0.15	2.1	0.4	26	≤5
SV-*tsA*30-N2	≃5	ND	3.0	0.4	10.1	0.15	3.4	0.4	41	5

[a] All data correspond to the same amount of extract (equivalent to 5×10^5 cells).
[b] See Fig. 7.
[c] Measured by Coomassie blue staining of the gels, and expressed in arbitrary densitometric units (a.u).
[d] ND, Not determined.
[e] Determined as the ratio of the specific activity of the sample to the specific activity of SV-WT-A1 cells at 32°C, assuming that the latter extract contained negligible amounts of denatured inactive large T protein.

here experiments performed to test the relative stabilities of the large T polypeptide in FR 3T3 rat transformants of the A and N types.

We have determined the stabilities of the newly synthesized large T and small t polypeptides in the two types of transformants grown at the permissive and restrictive temperatures, using pulse-chase labeling with [^{35}S]methionine and immunoprecipitation. For three different A transformants, the half-life of large T at the restrictive temperature was comparable to that determined for a WT virus transformant. In contrast, large T in two N transformants exhibited a reduced stability, even at the permissive temperature. The results obtained in the case of the N transformants agree well with the reported thermal instabilities of the T antigen molecules present in various temperature-sensitive transformants of different species (43, 44). Less expected was the observation that the stability of small t appeared to follow that of large T in type N transformants. A coordinate decrease of the large T and small t polypeptides was previously observed upon shift of these temperature-sensitive transformants to the restrictive temperature (15). These results suggest that the amount and the stability of the small t polypeptide, which is not structurally modified by the *tsA* mutation, are regulated in type N cells by a mechanism depending on the activity of the viral *ts* protein. Alternatively, these correlated variations may indicate some hitherto undetected association between the two early viral gene products. The possible mechanisms regulating the expression of small t in transformed cells are obviously open to investigation.

The possibility that the high stability of the *tsA*30 large T molecules in A transformants is only apparent, and either reflects a different transcriptional control or results from the integration of a revertant viral genome, was investigated as follows. Transformants representative of the A and N types were chosen among the different FR 3T3 *tsA*30 transformants previously isolated (31), using as a criterion the fact that they contain a single integration of SV40 DNA sequences. RNA mapping experiments showed that the viral RNAs made in the transformants of the two types were identical, at the permissive and restrictive temperatures, to the two early lytic mRNAs (5). The quantification of the amounts of viral RNA made in A cells grown at 40.5°C, relative to the amounts made at 32°C, did not indicate an overproduction of viral RNA at the restrictive temperature.

Alternatively, the *tsA*30 large T molecule in A transformants may not be a mutant molecule, due to a reversion or suppression of the *tsA* mutation. Indirect arguments against this hypothesis have been published: such transformants may be obtained at a high frequency under defined transformation conditions (30), and rat transformants of the A type, like temperature-sensitive transformants of other species, produce plaques only at the permissive temperature upon fusion with monkey cells (7, 31). As large T is a multifunctional protein (46), the initiation of viral DNA replication in permissive cells, and the maintenance of transformation in nonpermissive hosts, may not exhibit the same thermal stabilities. The binding of nucleotide, detected by affinity labeling with periodate-oxidized [α-^{32}P]ATP, is a defined function of the large T protein, which is known to be temperature sensitive in the permissive host (10) and which can be measured in transformed cells. These experiments showed clearly that the covalent affinity labeling of large T with oxidized [α-^{32}P]ATP was equally temperature sensitive in A and N transformants; the half-life of the nucleotide binding activity was identical, in the *tsA*30 transformants of both types, to that measured for the *tsA*30 large T molecule in infected permissive cells (10). These two lines of results demonstrate

that the *tsA*30 large T molecule in transformants of the A type is a molecule thermolabile for at least one of its biochemical properties.

The *tsA*30 large T molecules in A transformants, however, exhibit a new property, as they are stabilized—at least partially—at the permissive as well as at the restrictive temperatures. The determination of the pool of active molecules (performed by oxidized [α-^{32}P]ATP labeling) and the results of the pulse-chase labeling experiments suggest that this stabilization occurs immediately after the synthesis of the protein, and that this must happen at the permissive as well as at the restrictive temperature. Stabilization is likely to be achieved through association of large T to a cellular component. The cellular p53 protein (9, 20, 22, 24) is a good candidate, as the association between large T and p53 is maintained in temperature-insensitive *tsA* rat transformants grown at the restrictive temperature (D. P. Lane and M. Anderson, unpublished data; see reference 47); alternatively, it appears that p53 is stabilized through association with large T in SV40-transformed mouse cells (27).

However, another line of data should possibly by taken into account in explaining the phenomenon. We have recently shown (16a) that the A and N transformants used in this study could be differentiated by another phenotypic property at the permissive temperature: transformants of the N type show an accumulation of the immunofluorescent nuclear T antigen restricted to the G2 phase of the cell cycle, whereas A transformants are positively stained for T antigen throughout the cell cycle. If this different accumulation of large T were paralleled by a differential transcription of viral RNA and synthesis of the protein through the cell cycle, it would result, on one hand, in larger amounts of viral RNA in transformants of the A type (as only a fraction of the cells in a population of type N cells would contain viral RNA) and, on the other hand, in different apparent stabilities of the large T protein in the two types of transformants. Therefore, stability versus instability, and cycle-dependent versus cycle-independent accumulation of the large T polypeptide, may be two manifestations of the same phenomenon, and they may suggest new experimental approaches to study the A/N paradox.

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