JOURNAL OF VIROLOGY, Feb. 1979, p. 753-762
0022-538X/79/02-0753/10$02.00/0
Vol. 29, No. 2

Phosphorylation of T-Antigen and Control of T-Antigen Expression in Cells Transformed by Wild-Type and *tsA* Mutants of Simian Virus 40

CAROL A. F. EDWARDS,[1] GEORGE KHOURY,[2] AND ROBERT G. MARTIN[1]*

Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism and Digestive Diseases,[1] and Laboratory of DNA Tumor Viruses, National Cancer Institute,[2] Bethesda, Maryland 20014

Received for publication 16 August 1978

Chinese hamster lung (CHL) cells transformed by wild-type simian virus 40 (cell line CHLWT15) or transformed by the simian virus 40 mutants *tsA30* (cell lines CHLA30L1 and CHLA30L2) or *tsA239* (cell line CHLA239L1) were used to determine the rates of turnover and synthesis of the T-antigen protein and the rate of turnover of the phosphate group(s) attached to the T-antigen at both the permissive and restrictive temperatures. The phosphate group turned over several times within the lifetime of the protein to which it was attached, with the exception of the phosphate group in the *tsA* transformants at 40°C, which turned over at the same rate as the T-antigen protein. The steady-state levels of the T-antigens (molecular weights, 92,000 [92K] and 17K) and the amount of simian virus 40-specific RNA was also determined in each of the lines. The CHLA30L1 line contained two to three times more early simian virus 40 RNA than the CHLA30L2 line; although neither line formed colonies in agar at 40°C, CHLA30L1 overgrew a normal monolayer at 40°C. The rate of 92K T-antigen synthesis was 1.5 times faster in CHLA30L1 than in CHLA30L2 at 33°C and 4 times faster at 40°C. The different phenotypes of these two presumably isogenic cell lines seem to be related to the levels of the T-antigens. The ratios of the 92K T-antigen to the 17K T-antigen were similar in the two lines. Transformed CHL cell lines, unlike transformed mouse 3T3 cell lines, were found to contain very small amounts of the 56K T-antigen.

The simian virus 40 (SV40) T-antigen has been shown to be a phosphoprotein; only one tryptic peptide from T-antigen is phosphorylated, and only phosphoserine is identified after acid hydrolysis of T-antigen (23). If the phosphate turns over more rapidly than the T-antigen protein, phosphorylation and dephosphorylation may be important to the function of the T-antigen. We have, therefore, determined the rate of turnover of the phosphate group(s) on T-antigen and of the T-antigen protein in cells transformed by both wild type and *tsA* mutants. We have found that in most cases the phosphate group(s) does turn over several times more rapidly than the protein to which it is attached.

Because we have hypothesized that T-antigen expression in SV40-transformed cells is controlled in part by the host and may be influenced by the site of integration of the viral genome within the host (3, 14, 16, 22, 25), we have included in this study two independently isolated cell lines derived from the same parental Chinese hamster lung (CHL) cell line and transformed by the same temperature-sensitive *A30* mutant (cell lines CHLA30L1 and CHLA30L2). The CHLA30L1 cell line forms foci on a normal monolayer of CHL cells at both 33 and 40°C, whereas the CHLA30L2 cell line (like most N-type *tsA* transformants; 4, 13, 20, 25) does not overgrow a monolayer at the nonpermissive temperature of 40°C. On the other hand, neither cell line forms clones in soft agar at 40°C (a more stringent criterion of transformation; 18), although both form clones at 33°C.

CHL cells are semipermissive for SV40, and transformed lines generally shed low levels of free virus (13). The virus recovered from the CHLA30L1 and CHLA30L2 cell lines contains the *tsA30* mutation (25). The Southern blotting techniques (21) showed that the DNA from cultures of each line grown at 33°C includes both a single integrated copy of SV40 DNA and an equivalent amount of free SV40 component I DNA (M. DiLauro and R. G. Martin, unpublished data). Whether the free DNA corresponds to one copy per cell or many copies in a few cells has not yet been determined. The temperature sensitivity of the T-antigen from the two lines is the same when measured by the temperature-dependent loss of complement-fixing activity in

crude lysates (25). The CHLA30L1 line, however, contains higher levels of the temperature-sensitive T-antigen; we therefore suggested (before the t-antigen having a molecular weight of 17,000 [17K] was described) that excessive amounts of the defective *tsA30* T-antigen allowed overgrowth of a normal monolayer by CHLA30L1, but were insufficient to permit growth in soft agar at 40°C. The current study was undertaken to define more precisely the basis for the different amounts of the 92K and 17K T-antigens in the two cell lines and to detect any difference in the phosphorylation of the defective T-antigens.

MATERIALS AND METHODS

Cell lines and media. CHL cells transformed by wild-type SV40 (cell line CHLWT15) and by the SV40 mutants *tsA30* (cell lines CHLA30L1 and CHLA30L2) or *tsA239* (cell line CHLA239L1) have been described previously (5, 6, 13, 25). Cells were grown in Dulbecco and Vogt's modification of Eagle minimal essential medium, supplemented with 10% fetal calf serum (DV10 medium; Flow Laboratories, Rockville, Md.).

Growth and pulse-chase of cells for turnover experiments. A total of 12 or 14 cultures of each cell line were allowed to grow at 33 or 40°C in 150-cm^2 flasks for 2 days. Cells were rinsed twice with methionine-free DV medium containing 1% serum, and then incubated in the same medium for 1 h. Next, cells were rinsed twice with phosphate- and methionine-free DV medium containing 1% serum previously dialyzed to remove phosphate; cells were labeled for 4 h with 100 to 150 μCi of carrier-free ^{32}P per ml and 35 to 75 μCi of [^{35}S]methionine (400 to 500 Ci/mmol) per ml in a total volume of 5 ml of the rinse medium. After the labeling period, two flasks of cells were collected (by scraping) directly for each cell line at each temperature for determination of zero-time points. The remaining flasks were rinsed twice with DV10 and then returned to DV10 medium for various chase periods. DV10 contained a 1,000-fold excess of methionine and approximately a 10^7-fold excess of inorganic phosphate over the amounts in the labeling medium. For CHLWT15 cells, one flask at each temperature was collected at 3, 6, 12, 18, and 24 h; flasks containing the *tsA* transformants were collected at 3, 6, 9, and 12 h.

Collection of cells and preparation of lysates. Cells were rinsed three times with cold Tris-buffered saline, pH 7.4 (2), and scraped off the plastic surface into a total volume of 9 ml of cold Tris-buffered saline. After centrifugation for 10 min at 4,000 rpm in a Sorvall SS-34 rotor, cells were suspended in 4 ml of Tris-buffered saline, pH 8.0, containing 1 mM dithiothreitol, 300 μg of phenylmethylsulfonylfluoride per ml, and 150 μg of L-1-tosylamide-2-phenylethylchloromethyl ketone per ml. After another centrifugation, cells were suspended in 1 ml of lysis buffer (Tris-buffered saline, pH 8.0, containing 0.5% Nonidet P-40, 1 mM dithiothreitol, 300 μg of phenylmethylsulfonylfluoride per ml and 150 μg of L-1-tosylamide-2-phenylethylchloromethyl ketone per ml) and allowed to lyse for 20 min at 0°C. The lysates were centrifuged for 30 min at 5°C and 30,000 rpm in a Spinco type 40 rotor. Samples of the resulting supernatants were removed for determination of (i) the total radioactivity in the lysate, (ii) the incorporation of radioactivity into acid-precipitable material, and (iii) the protein concentration by the method of Lowry (12). Finally, the supernatants were frozen in liquid nitrogen until all the flasks from the various chase periods had been collected.

Immunoprecipitation. Immunoprecipitation of the T-antigen was done with serum from hamsters bearing SV40-induced tumors (lot 4x-0416; Huntingdon Research Center, Brooklandville, Md.); normal hamster serum was used for controls. The lysates (1 ml) were immunoprecipitated with 20 μl of serum for 1 h at room temperature. Next, 400 μl of a 10% (wt/vol) solution of *Staphylococcus aureus* cells prepared in lysis buffer (see above) as described by Kessler (7) was added; the mixtures were incubated at 0°C for 1 h with occasional shaking. The bacteria, carrying the bound hamster immunoglobulins, were collected by centrifugation at 8,000 rpm for 10 min in a Sorvall SS-34 rotor. The pellet was washed three times in 1 ml of lysis buffer each time. After the third wash, the pellet was suspended in 90 μl of sodium dodecyl sulfate (SDS) sample buffer for electrophoresis (11) and heated for 10 min at 56°C. After centrifugation, the pellet was washed with an additional 40 μl of buffer, and the two supernatants were combined and saved for electrophoresis.

The immunoprecipitation for the turnover experiments with CHLA239L1 and the steady-state quantitation of T-antigen were done by using an improved technique which substitutes protein A-Sepharose CL-4B beads for the *S. aureus*. This technique does not change the amount of T-antigen precipitated from a given sample, but does decrease the background in the gels. The technique (19) involved blending in a Vortex mixer 1 ml of cell lysate plus 20 μl of serum and 40 μl of packed beads in the cold for 30 min. The beads were collected by centrifugation and washed five times with lysis buffer (1 ml each time). Then the T-antigen was eluted from the beads with SDS sample buffer as described above.

During the collection of cells, preparation of lysates, and immunoprecipitation, loss of phosphate from T-antigen is expected to be minimal. ^{32}P-labeled T-antigen after immunoprecipitation was found to be stable at pH 2 for 1 h at room temperature and at pH 13 for 1 h at 60°C. The immunoprecipitation conditions yielded quantitative recovery of T-antigen; the amount of anti-T serum was selected to give maximum recovery of T-antigen over an approximate 10-fold range of T-antigen concentration. The ratio of ^{32}P to ^{35}S did not vary among six samples of T-antigen immunoprecipitated with varying amounts of antibody.

SDS-acrylamide gel electrophoresis. Gel electrophoresis was done by the method of Laemmli (11). Separating gels (6% acrylamide) were approximately 125 mm long, and stacking gels (3% acrylamide) were 30 mm long. After running, gels were minced with an Aliquogel Fractionator (Gilson Medical Electronics, Inc., Middleton, Wis.) into fractions of 1 or 2 mm. A 0.2% SDS solution was used as the rinse solution for the fractionator.

The fractions were counted for ^{35}S and ^{32}P, and the numbers were corrected for spillage. In CHLWT15 cells at both 33 and 40°C, the ^{35}S gel background was 8 to 12% of the counts in the fractions including the T-antigen peak, and the ^{32}P gel background was 8 to 18% of the counts. In the CHLA30L1 and CHLA30L2 cells, the ^{35}S gel background was 30 to 60%, and the ^{32}P gel background was 20 to 50%. In the CHLA239L1 cells the ^{35}S gel background was between 8 and 35%, and the ^{32}P gel background was between 10 and 45%. In all cases the relative size of the gel background was the largest at later times in the chase.

RNA extraction and hybridization. Total cellular RNA was extracted by a method described previously (8) from confluent monolayers of CHLA30L1 and CHLA30L2 cells which had been grown at 33 or 40°C for 72 h before extraction. After treatment of the extracts with DNase (50 μg of DNase per ml for 1 h at room temperature), the RNA was extracted twice with phenol-chloroform-isoamyl alcohol (50:49:1) and chromatographed on Sephadex G-100. Concentrated RNAs were annealed to the separated strands of ^{32}P-labeled SV40 DNA (8, 9).

Hybridization of cellular RNA to the separated strands of SV40 DNA. Small quantities (0.6 to 1.2 ng) of the separated strands of ^{32}P-labeled SV40 DNA (specific activity, 4×10^5 to 8×10^5 cpm/μg) were annealed with increasing amounts of total cellular RNA from CHLA30L1 and CHLA30L2 cells grown at 33 or 40°C as described above. The reaction mixtures contained 1.0 M NaCl, 0.05 M phosphate buffer, pH 7.0, and 0.1% SDS in a volume of 0.05 to 2.0 ml. After 36 h of incubation at 68°C, the percentage of duplex molecules in each sample was determined by hydroxyapatite chromatography (8).

Synthesis of T-antigen. A rough estimate of the amount of T-antigen relative to total cellular protein synthesized during the labeling period was made by using the following formula: $T = T^0/C^0$ (equation 1), where T is the amount of T-antigen synthesized during the labeling period relative to total cellular protein, T^0 is the counts per minute of ^{35}S in the T-antigen peak at zero time (start of chase period), and C^0 is the counts per minute of ^{35}S acid-precipitable from cellular lysate at zero time.

In some cases, however, the half-life for turnover of the T-antigen was similar to the length of the labeling period, and the amount of T-antigen observed at zero time (T^0) did not reflect all of the T-antigen synthesized during the labeling period (T_{corr}). Values for T^0 (equation 1) were therefore corrected for the presumed turnover of T-antigen during the 4-h labeling period by using the following formula: $T^0_{corr} = [0.69\, T^0 \cdot \tau]/[\tau_{1/2}(1 - 2^{-\tau/\tau_{1/2}})]$ (equation 2), where T^0_{corr} is the total amount of T-antigen synthesized during the labeling period, T^0 is the observed amount of T-antigen synthesized, as in equation 1, $\tau_{1/2}$ is the half-life for T-antigen or cellular protein turnover, as determined in turnover experiments, and τ is the length of the synthesis period (4 h in these experiments). Similar corrections were calculated for C^0 to give C^0_{corr}, but these were insignificant due to the long half-lives of total cellular protein. When corrected values for T^0 (T^0_{corr}) were used in equation 1, the rates of T-antigen synthesis (T_{corr}) were obtained.

Steady-state levels of T-antigen. Steady-state levels of T-antigen were determined by calculation from the rates of synthesis and turnover and by chronic labeling of cells. These values could then be compared with the relative levels of T-antigen found by complement fixation.

(i) Determination by calculation. Because we measured relative rates of synthesis and of turnover, it should be possible to calculate relative steady-state levels of T-antigen at the two temperatures. For steady state conditions, $dT/dt = 0 = k_s - k_t\,[T]$, where dT/dt is the increase in T-antigen content (per milligram of cell protein in randomly growing cells) with time, k_s is the rate constant for the synthesis of T-antigen, k_t is the rate constant for the degradation of T-antigen and is proportional to $1/\tau_{1/2}$, the reciprocal of the half-life, and $[T]$ is the concentration of T-antigen. Hence the steady-state concentration of T-antigen is: $[T] \propto T_{corr} \cdot \tau_{1/2}$.

(ii) Determination by chronic labeling of cells. Steady-state levels of T-antigen were also approximated by chronically labeling cultures and measuring the ^{35}S incorporated into T-antigen relative to that incorporated into total acid-precipitable material (total cell protein). Four or five cultures of each cell line were allowed to grow at 33 or 40°C in labeled medium. The medium was DV10 with half the usual amount of cold methionine (0.1 mM) and 2 μCi of [^{35}S]methionine per ml. After 3 or 4 days, the cells were harvested, immunoprecipitates were prepared, and gel electrophoresis was performed on the immunoprecipitates. The amounts of ^{35}S in T-antigen and in total cellular protein were calculated.

RESULTS

Turnover of T-antigen. CHL cells transformed by wild-type SV40 (CHLWT15), two independently isolated CHL cell lines transformed by the SV40 *tsA30* mutant (CHLA30L1 and CHLA30L2), and CHL cells transformed by the *tsA239* mutant (CHLA239L1) were used. Experiments to determine the rate of turnover of the phosphate moiety and the protein of T-antigen were performed as described above. Briefly, cells were doubly labeled with ^{32}P and [^{35}S]methionine and then chased for various periods in cold medium. Generally, the cellular protein increased by approximately 40% after the first 12 h of the chase and by 100% after 24 h. After the cells were collected and lysed, the T-antigen was immunoprecipitated with anti-T serum and used for SDS-acrylamide gel electrophoresis. Typical gel patterns obtained from cells collected immediately after labeling are illustrated in Fig. 1. Figure 1B, showing a gel run with an immunoprecipitate made with anti-T serum, has a T-antigen peak around fraction 30; Fig. 1A, a gel with normal hamster serum, has no peak in this region. The T-antigen peak in Fig. 1B contained ^{32}P as well as ^{35}S, indicative of a phosphoprotein. The apparent molecular

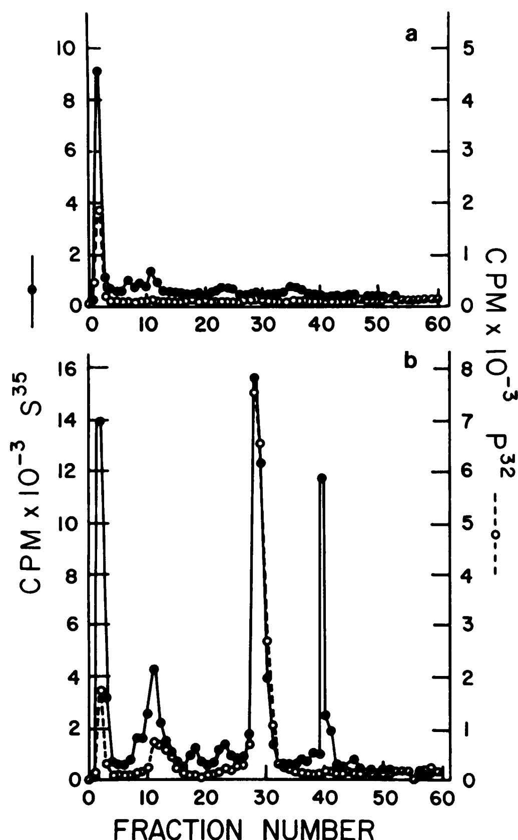

FIG. 1. *SDS-acrylamide gel electrophoresis of immunoprecipitates made with normal hamster serum (a) or anti-T serum (b). Electrophoresis was performed as described in the text by using 6% acrylamide in the separating gels. Migration was from right to left. Gels were minced into fractions of 2 mm each. Those fractions between 20 and 39, however, represent only 1 mm of gel. The immunoprecipitates were obtained from CHLWT15 cells grown at 33°C and collected right after labeling. Symbols:* ●, ^{35}S; ○, ^{32}P. *In multiple other experiments with similar profiles, markers of RNA polymerase, phosphorylase a, and bovine serum albumin indicated that the peak at fraction 30 had an approximate molecular weight of 80K to 84K.*

weight of the T-antigen in these 6% SDS-acrylamide gels was 80K to 84K, as determined by comparison of its mobility with those of RNA polymerase, phosphorylase *a*, and bovine serum albumin. The peak of the 56K "middle" T-antigen, clearly also a phosphorylated protein, is seen in fraction 11, but proteins of 17K molecular weight were not resolved from the front.

The results of a pulse-chase experiment with CHLWT15 cells are shown in Fig. 2. At both 33 and 40°C, ^{35}S incorporated into T-antigen is seen to turn over at a rate similar to the turnover of bulk cellular protein. In contrast, ^{32}P incorporated into T-antigen is seen to turn over much more rapidly. The phosphate appeared to turn over in a biphasic manner at both 33 and 40°C; this effect was found to be reproducible in other turnover experiments with CHLWT15 cells. Least-squares analyses of the turnover of ^{35}S in T-antigen, ^{32}P in T-antigen, and ^{35}S in total cellular protein gave the values in Table 1.

The results of pulse-chase experiments with the three cell lines transformed by *tsA* mutants are shown in Fig. 3. In all three lines at 33°C, the ^{35}S in T-antigen turned over slightly faster than the total cellular protein, but the ^{32}P in T-antigen turned over much faster than the ^{35}S in T-antigen. At 40°C, in contrast, the ^{35}S and ^{32}P in T-antigen turned over at nearly identical rates, both much faster than the ^{35}S in total cellular protein. Within the limits of error for the experiment, the turnover of both ^{35}S and ^{32}P in T-antigen appeared to be linear on a log scale.

The turnover results for CHLWT15, CHLA30L1, CHLA30L2, and CHLA239L1 cells are given in Table 1. The data for CHLWT15 cells represent 24-h chase periods, whereas those for CHLA30L1, CHLA30L2, and CHLA239L1 represent 12-h chase periods; hence the half-lives in Table 1 greater than 24 h for CHLWT15 cells and greater than 12 h for the *tsA* transformants are derived from shallow slopes and subject to considerable error.

In all cases the phosphate turned over very rapidly. At both temperatures, CHLWT15 cells seemed to have a biphasic phosphate turnover, with a break at 6 h. The turnover was three to four times as rapid during the first 6 h of the chase compared with the last 18 h. The phosphate turnover rates for the CHLWT15 cells were about the same at 33 and 40°C. In contrast to the CHLWT15 cells, the *tsA* mutant cell lines gave linear rates of phosphate turnover. At the permissive temperature, the T-antigen phosphate in the three *tsA* transformants seemed to be slightly more stable than the phosphate during a short chase in CHLWT15 cells (approximately 5.5 versus 3 h), but a difference of this magnitude is near the borderline of our estimated experimental error. During a short chase at the restrictive temperature, the T-antigen phosphate turned over at the same rate in all three cell lines.

The ^{35}S-labeled T-antigen protein from CHLWT15 cells turned over at similar rates at both temperatures. In all three *tsA* transformants, the T-antigen protein turned over more rapidly at 40°C than at 33°C, as demonstrated for other *tsA* transformants by Tegtmeyer et al. (24). At the permissive temperature, the CHLA30L1 T-antigen seemed to turn over more slowly than the CHLA30L2 T-antigen (although

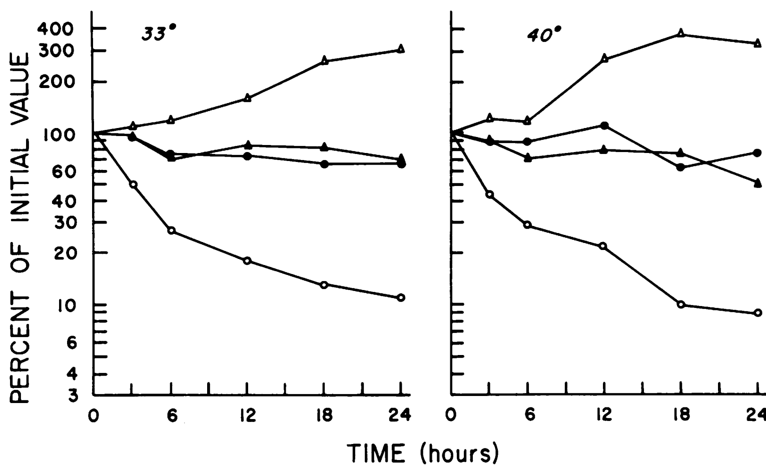

FIG. 2. *Turnover of T-antigen in CHLWT15 cells at 33 and 40°C. Areas under the T-antigen ^{35}S and ^{32}P peaks are plotted versus the length of the chase. In addition, ^{35}S incorporation into, and protein content of, the total cellular lysate are plotted. The points represent percentages of the initial values for ^{35}S in T-antigen (●; initial values, 14,200 cpm at 33°C and 8,300 cpm at 40°C), ^{32}P in T-antigen (○; initial values, 9,900 cpm at 33°C and 3,900 cpm at 40°C), ^{35}S in the total lysate (▲; initial values, 1.6×10^7 cpm at 33°C and 1.7×10^7 cpm at 40°C), and total cold protein in the lysate (△; initial values, 1.14 mg at 33°C and 1.30 mg at 40°C).*

TABLE 1. *Turnover of ^{32}P and ^{35}S in T-antigen and of ^{35}S in cellular protein*

Cell line	Determination based on data from chase period (h)	Half-life (h)					
		T-antigen				Cell lysate	
		33°C		40°C			
		^{35}S	^{32}P	^{35}S	^{32}P	^{35}S at 33°C	^{35}S at 40°C
CHLWT15	0–24	38		55		60	30
	0–6		3.2		3.4		
	6–24		14		9.5		
CHLA30L1	0–12	58	5.7	4.3	3.9	1,100	110
CHLA30L2	0–12	17	5.6	3.9	3.3	100	16
CHLA239L1	0–12	19	5.8	3.8	3.3	40	26

the difference may not be significant because chase periods of only 12 h were used). This result is consistent with the findings of Tenen et al. (25), which indicated more T-antigen complement-fixing activity in the CHLA30L1 line than in the CHLA30L2 line. At the restrictive temperature, the *tsA* protein turned over as fast as the phosphate.

The turnover of total cellular proteins was determined by counting the acid-precipitable ^{35}S in samples of the cell lysates collected at each timepoint. These results are expressed in Table 1 as half-lives for the turnover. We stress that the half-lives for total cellular protein are derived from the extrapolation of very shallow slopes and therefore, particularly in the case of the *tsA* mutants, are not highly reliable. However, in each case, cellular protein turned over more rapidly at 40°C than at 33°C. In CHLWT15 cells, the T-antigen turned over approximately at the same rate as the cellular protein. In contrast, the cellular protein in the *tsA* transformants was more stable than the mutant T-antigen, indicating that the more rapid turnover of the T-antigen was not a reflection of cell death at the higher temperature.

Rate of synthesis of T-antigen. The rate of synthesis of T-antigen (T_{corr}) during the 4-h labeling period in the turnover experiments was calculated as described above. The results (Table 2) indicated a slower rate of synthesis for CHLWT15 T-antigen at 40°C than at 33°C. The CHLA30L1 line was remarkable in that it overproduced T-antigen by fourfold at 40 versus 33°C (a sevenfold increase relative to the CHLWT15 line); in contrast, the CHLA30L2 cell line gave nearly the same rate of synthesis at both temperatures (a 2.5-fold increase relative to the CHLWT15 line). The CHLA239L1 line gave a threefold increase, which was in agree-

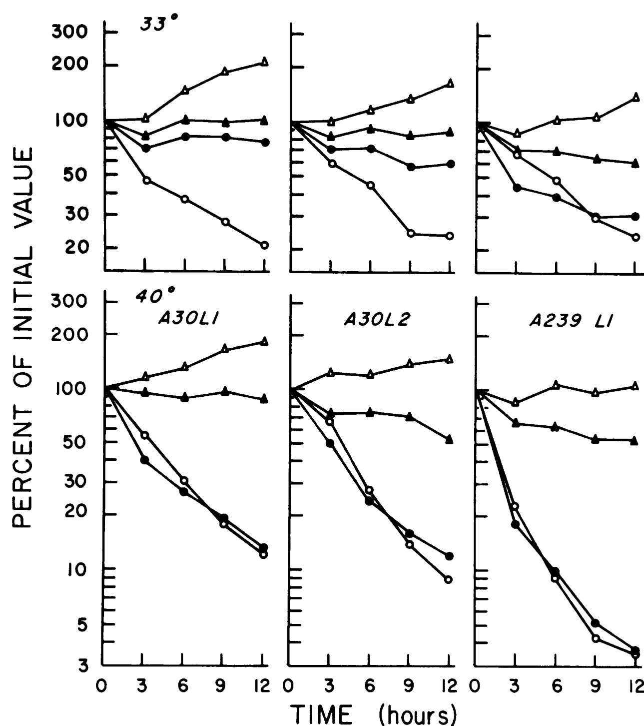

FIG. 3. *Turnover of T-antigen in CHLA30L1, CHLA30L2, and CHLA239L1 cell lines at 33 and 40°C. The data are plotted as in Fig. 2, with the panels showing results at 33°C above those showing results at 40°C. The points represent percentages of the initial values for* ^{35}S *in T-antigen (●; initial values, 26,000 to 37,000 cpm at 33°C and 25,000 to 96,000 cpm at 40°C),* ^{32}P *in T-antigen (○; initial values, 5,000 to 12,000 cpm at 33°C and 2,000 to 5,000 cpm at 40°C),* ^{35}S *in the total lysate (▲; initial values, 5.2 × 10*^{7} *to 6.7 × 10*^{7} *cpm at 33°C and 4 × 10*^{7} *to 7 × 10*^{7} *cpm at 40°C), and total cold protein in the lysate (△; initial values, 1.0 to 1.6 mg at 33°C and 1.0 to 1.3 mg at 40°C).*

TABLE 2. *Rate of synthesis of T-antigen relative to total cellular protein*

Cell line	T-antigen synthesis relative to total cellular protein synthesis (×10^{3})[a]		Synthesis at 40°C/synthesis at 33°C	Synthesis at 40°C/synthesis at 33°C, relative to CHLWT15
	33°C	40°C		
CHLWT15	0.91	0.51	0.56	1.0
CHLA30L1	0.73	2.65	3.6	6.5
CHLA30L2	0.47	0.68	1.4	2.6
CHLA239L1	0.48	0.87	1.8	3.1

[a] Calculated from equation 1, corrected for T-antigen turnover by equation 2.

ment with the results of Alwine et al. (1), which showed a threefold increase in early SV40 mRNA. Thus, all three *tsA* transformants overproduced T-antigen relative to CHLWT15 at 40 versus 33°C, but the increase was two- to threefold greater in CHLA30L1 than in CHLA30L2 or CHLA239L1.

Steady-state levels of T-antigen. The relative steady-state levels of T-antigen in the four cell lines were calculated from the turnover experiments and from experiments in which cells were chronically labeled with [^{35}S]methionine (see above). These results and those from direct measurement of T-antigen by complement fixation activity (25) are given in Table 3. By all three measurements of steady-state levels of T-antigen the *tsA* transformants appear to have relatively less T-antigen at 40°C than 33°C when compared with the wild-type transformant (Table 3). Furthermore, by all three measurements the level of T-antigen in CHLA30L1 at 40°C was greater than that of CHLA30L2.

The difference in the relative ratios of T-antigen at 40 versus 33°C between lines CHLA30L1 and CHLA30L2 varied in the three determinations. By chronic labeling, CHLA30L1 contained 1.4-fold more T-antigen at 40 versus 33°C than did the CHLA30L2 line. This ratio was greater than twofold by complement fixation but reversed in the steady-state calculation.

TABLE 3. *Steady-state levels of T-antigen*

Cell line	Temp (°C)	Level of T-antigen by: Chronic labeling expt		Steady-state approximation		Complement fixation	
		Relative cpm ($\times 10^4$)	40°C/33°C relative to CHLWT15	Arbitrary units	40°C/33°C relative to CHLWT15	Average CF[a] (CF units)	40°C/33°C relative to CHLWT15[b]
CHLWT15	33	4.1	1.0 (0.73)	35	1.0 (0.8)	30	1.0 (0.36)
	40	3.0		28		12	
CHLA30L1	33	4.7	0.29	42	0.33	36	0.78
	40	1.0		11		10	
CHLA30L2	33	4.0	0.21	8	0.41	30	0.34
	40	0.6		2.6		3.3	
CHLA239L1	33	3.6	0.65	8.7	0.49	35	0.27
	40	1.7		4.3		3.0	

[a] Average complement fixation (CF) values were derived from two to four experiments (25).

[b] Values for (CF at 40°C)/(CF at 33°C) were calculated for each of two to four CF determinations. These values were then averaged for each cell type and normalized to the value for CHLWT15 (0.36). The data are taken from Tenen et al. (25).

Because the steady-state calculation is dependent upon the ratio of four experimentally determined parameters (two of which are derived from shallow slopes and subject to considerable error), we are inclined to disregard this discrepancy. It is also possible that some of the discrepancies between the amounts of T-antigen measured here and those obtained by complement fixation are related to the ability of phosphorylated and dephosphorylated T-antigen to fix complement.

Relative amounts of T-antigens. To determine whether the different phenotypes of CHLA30L1 and CHLA30L2 might in some way be related to the middle T-antigen and the "little" t-antigen, immunoprecipitates were made from cells labeled as for the turnover experiments and used for SDS-gel electrophoresis in slab gels containing 10% acrylamide. Immunoprecipitates were prepared from each of the three CHL *tsA* lines, at both the permissive and restrictive temperatures. The gel (Fig. 4) indicated no major change in the relative rates of synthesis of the 92K and 17K antigens at the two temperatures. All of these lines showed virtually none of the 56K antigen. We therefore examined several additional CHL transformed cell lines and repeatedly failed to observe significant levels of the middle T-antigen. However, in five independently isolated SV40-transformed mouse 3T3 cell lines, the 56K band was always prominent. A comparison of wild-type SV40-transformed mouse and CHL cells is shown in Fig. 5. The 92K T-antigen and the 17K t-antigen were seen in approximately the same amounts in the two cell lines, whereas the 56K middle T-antigen was considerably more prominent in the mouse cell line. Tracings of the film with a Joyce-Loebl microdensitometer and counting of tube gels prepared as for the turnover experiments showed approximately equal amounts of the 92K and 56K antigens in the mouse line. The 56K antigen was hardly detectable in the hamster line.

mRNA in the *tsA* transformants. To determine whether the level of SV40-specific RNA was related to the level of T-antigen in the two *tsA30*-transformed cell lines, hybridization experiments were performed with RNA extracted from both cell lines at 33 and 40°C. Increasing concentrations of RNA from each cell line were annealed with ^{32}P-labeled strands of SV40 DNA, and the percentage of duplex molecules was determined as described above. Results presented in Fig. 6 indicate that a hybridization plateau of approximately 50% was reached with two to three times less RNA from cell line CHLA30L1 than from cell line CHLA30L2. This was true at both temperatures and indicates that, for a given amount of total cellular RNA, early SV40 RNA sequences were two to three times more abundant in the CHLA30L1 cells, regardless of temperature. The data in Fig. 6 also suggest that the RNA from CHLA30L2 cells contains at least two populations of SV40-specific molecules, which differ in abundance. The more abundant population was complementary to about 50% of the E strand, whereas the less abundant population(s) represented at least an additional 10% of the E strand. Thus, the annealing at high RNA concentrations of between 50 and 60% of the SV40 E strand was seen with RNA from CHLA30L2 cells, but not with RNA from CHLA30L1 cells. In further experiments

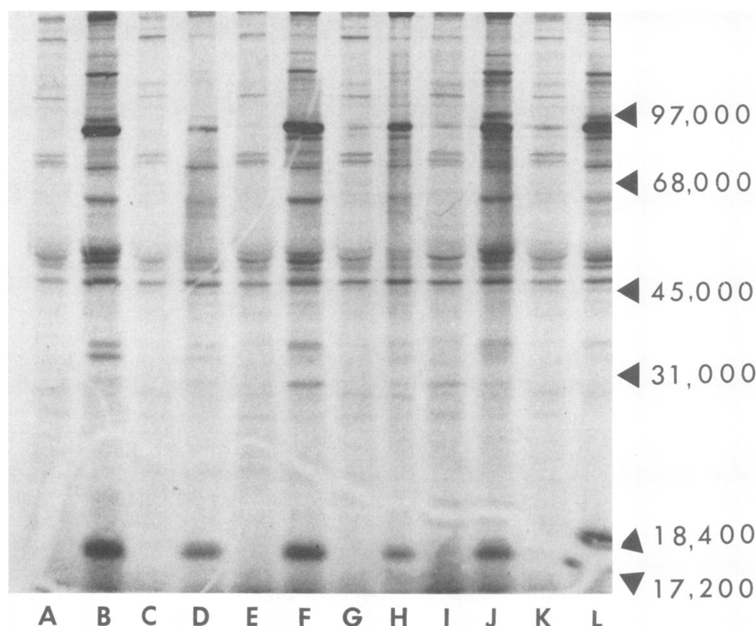

FIG. 4. *Autoradiogram of SDS-acrylamide electrophoretic gel showing* ^{35}*S-labeled immunoprecipitates from CHLA239L1, CHLA30L1, and CHLA30L2 cell lines grown at 33 or 40°C. Electrophoresis was performed as described in the text by using 10% acrylamide in the separating gel. Protein markers were phosphorylase a (97K), bovine serum albumin (68K), ovalbumin (45K), DNAse I (31K), β-lactoglobulin (18.4K), and myoglobin (17.2K). Lanes A, C, E, G, I, and K contained immunoprecipitates made with normal hamster serum, whereas lanes B, D, F, H, J, and L contained immunoprecipitates made with anti-SV40 T-antigen serum. Lanes A through D represent CHLA239L1 cells grown at 33°C (lanes A and B) or 40°C (lanes C and D). Lanes E through H are CHLA30L1 cells grown at 33°C (E and F) or 40°C (G and H). Lanes I through L are CHLA30L2 cells grown at 33°C (I and J) or 40°C (K and L). A crack in the gel displaced the little t-antigen upward in lane L; this does not represent a heavier little t-antigen molecule. In addition to the 92K and 17K peptides, each of the immunoprecipitates with anti-T serum contained distinct bands at approximately 130K and 62K, but not at 56K.*

with the labeled E strands of restriction enzyme fragments of the SV40 genome (unpublished data), we have shown that the abundant RNA from both cell lines is similar if not identical to early SV40 RNA, and that the scarce component in the RNA from CHLA30L2 cells is located at the 5′ end of the early transcript.

DISCUSSION

The reproducible biphasic turnover of T-antigen phosphate in CHLWT15 cells may indicate that two populations of phosphate groups exist. The results of Tegtmeyer et al. (23) showed only one phosphorylated tryptic peptide, with all of the phosphate attached to serine after acid hydrolysis. These results do not exclude the possibility of several phosphoserine residues in one tryptic peptide or the presence of additional acid-labile phosphate groups on that peptide. The linear rate of phosphate turnover observed with the *tsA* transformants might indicate a difference in the sites of phosphorylation or in the accessibility of these sites to phosphatases.

The turnover of phosphate in T-antigen was in all cases very rapid and in most cases faster than the turnover of the T-antigen protein. In this regard, T-antigen appears to be analogous to the phosphorylated nonhistone chromosomal proteins. The nonhistone proteins are most often phosphorylated on serine residues (10) and are highly acidic, as is T-antigen (23; Edwards, unpublished data). Kleinsmith has suggested that the phosphorylation and dephosphorylation of these nonhistone proteins modifies their interaction with chromatin, providing a mechanism for the regulation of gene activity (10). Because it is known that T-antigen both binds to chromatin (17) and functions to decrease the average replicon size in transformed cells (15), it is possible that changes in phosphorylation of the T-antigen affect its binding to chromatin and its ability (direct or not) to cause additional sites to initiate replication.

At both 33 and 40°C the CHLA30L1 line contained approximately two to three times more SV40-specific mRNA than did the CHLA30L2 line. This difference in the amount

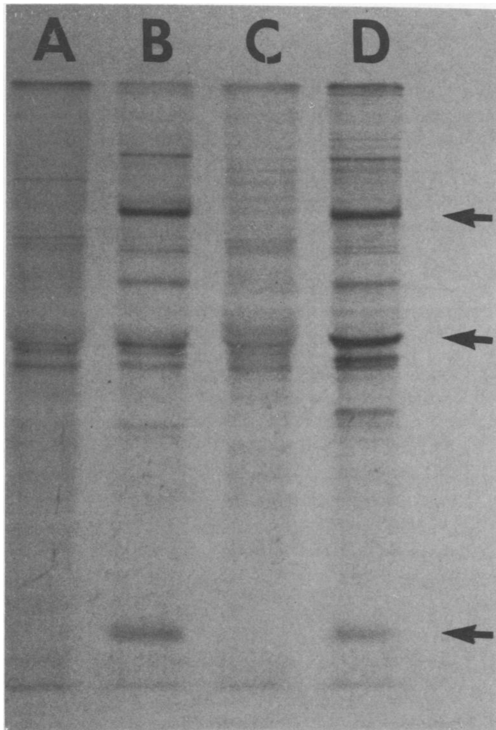

FIG. 5. *Autoradiogram of SDS-acrylamide electrophoretic gel showing* ^{35}S*-labeled immunoprecipitates from CHLWT15 and 3T3WT cells. Electrophoresis was performed as described in the legend to Fig. 4. Protein markers used were phosphorylase a, bovine serum albumin, DNAse I, and myoglobin. The lanes contained immunoprecipitates from CHLWT15 cells with normal serum (lane A) and anti-SV40 T-antigen serum (lane B) and from 3T3WT cells with normal serum (lane C) and anti-SV40 T-antigen serum (lane D). In addition to the 92K, 56K, and 17K protein bands, whose positions are indicatd by the arrows (from top to bottom, respectively), the two immunoprecipitates with anti-T serum also contained bands at approximately 130K and 62K.*

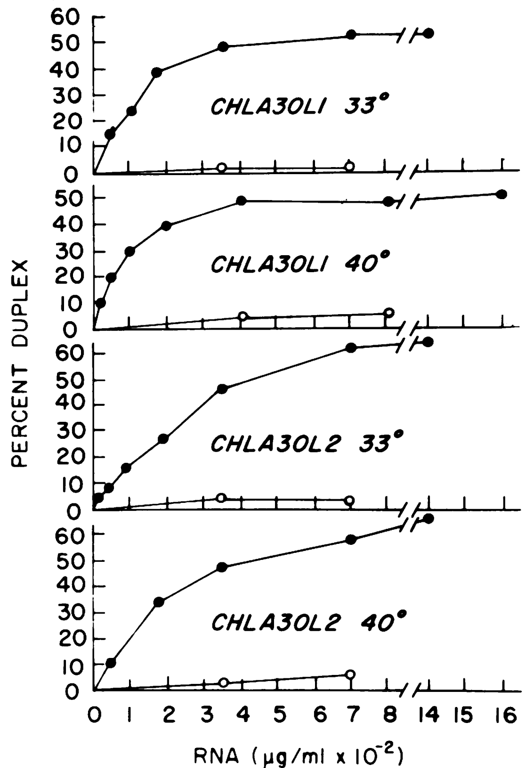

FIG. 6. *Hybridization of* ^{32}P*-labeled strands of SV40 DNA to cellular RNA from CHLA30L1 and CHLA30L2. RNA was hybridized to SV40 DNA as described in the text. Symbols: ●, hybridization of cellular RNA to early-strand SV40 DNA; ○, hybridization of cellular RNA to late-strand SV40 DNA. The amounts of RNA were estimated both from the point at which half of the SV40 specific RNA had reannealed and from the initial slopes of the reannealing curves. For example, in the experiment at 40°C, CHLA30L1 was half reannealed at an mRNA concentration of 80 μg/ml, whereas CHLA30L2 was half reannealed at 170 μg/ml.*

of mRNA was reflected in an approximately 1.5-fold increase at 33°C and a 4-fold increase at 40°C in the rate of T-antigen synthesis (Table 2) in CHLA30L1 versus CHLA30L2. By three independent assays (Table 3), the CHLA30L1 line contained more T-antigen at both temperatures than did the CHLA30L2 line. In two of the three assays the reduction in the amount of T-antigen in CHLA30L2 relative to CHLA30L1 was more striking at 40°C than at 33°C. In all cases the rate of synthesis of the 17K t-antigen appeared to parallel the rate of synthesis of the 92K T-antigen.

From our current results we cannot say whether the increased amounts of the T-antigens and SV40-specific early mRNA in CHLA30L1 versus CHLA30L2 result from the expression of the integrated and/or the nonintegrated genomes in the two cell lines. We favor the hypothesis that the differential expression of T-antigen reflects primarily the expression of the integrated genome, because the two cell lines are uniformly T-antigen positive by immunofluorescence and because the free virus may derive from a small percentage of the cell population, as is the case in polyoma *tsA*-transformed rat cells (26). Because further work will be required to substantiate this hypothesis, it is not possible at the present to determine whether the difference in T-antigen expression in these cell lines is an example of host control of SV40 expression. It is clear, however, that the different phenotypes are not related to the differential expres-

sion of the middle T-antigen. Furthermore, there is a species difference in the expression of the 56K T-antigen; this polypeptide is very predominant in transformed mouse cell lines and greatly diminished in transformed Chinese hamster lines.

LITERATURE CITED

1. **Alwine, J. C., S. I. Reed, and G. R. Stark.** 1977. Characterization of the autoregulation of simian virus 40 gene *A*. J. Virol. **24**:22-27.
2. **Anderson, J. L., R. G. Martin, C. Chang, and P. T. Mora.** 1977. Tumor-specific transplantation antigen is expressed during SV40 lytic infection with wild-type and *tsA* mutant viruses. Virology **76**:254-262.
3. **Basilico, C., and D. Zouzias.** 1976. Regulation of viral transcription and tumor antigen expression in cells transformed by simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **73**:1931-1935.
4. **Brockman, W. W.** 1978. Transformation of BALB/c-3T3 cells by *tsA* mutants of simian virus 40: temperature sensitivity of the transformed phenotype and retransformation by wild-type virus. J. Virol. **25**:860-870.
5. **Chou, J. Y., and R. G. Martin.** 1974. Complementation analysis of simian virus 40 mutants. J. Virol. **13**: 1101-1109.
6. **Chou, J. Y., and R. G. Martin.** 1975. DNA infectivity and the induction of host DNA synthesis with temperature-sensitive mutants of simian virus 40. J. Virol. **15**: 145-150.
7. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **115**:1617-1624.
8. **Khoury, G., J. C. Byrne, and M. A. Martin.** 1972. Patterns of simian virus 40 DNA transcription after acute infection of permissive and nonpermissive cells. Proc. Natl. Acad. Sci. U.S.A. **69**:1925-1928.
9. **Khoury, G., P. Howley, M. Brown, and M. Martin.** 1974. The detection and quantitation of SV40 nucleic acid sequences using single-stranded SV40 DNA probes. Cold Spring Harbor Symp. Quant. Biol. **39**:147-152.
10. **Kleinsmith, L. J.** 1975. Phosphorylation of non-histone proteins in the regulation of chromosome structure and function. J. Cell Physiol. **85**:459-476.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
12. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
13. **Martin, R. G., and J. Y. Chou.** 1975. Simian virus 40 functions required for the establishment and maintenance of malignant transformation. J. Virol. **15**: 599-612.
14. **Martin, R. G., J. Y. Chou, J. Avila, and R. Saral.** 1974. The semiautonomous replicon: a molecular model for the oncogenicity of SV40. Cold Spring Harbor Symp. Quant. Biol. **39**:17-24.
15. **Martin, R. G., and A. Oppenheim.** 1977. Initiation points for DNA replication in nontransformed and simian virus 40-transformed Chinese hamster lung cells. Cell **11**:859-869.
16. **Martin, R. G., M. Persico-DiLauro, C. A. F. Edwards, and A. Oppenheim.** 1977. The molecular basis of transformation by simian virus 40, p. 87-100. *In* J. Schultz and Z. Brada (ed.), Miami Winter Symposium 14. Genetic manipulation as it affects the cancer problem. Academic Press Inc., New York.
17. **Persico-DiLauro, M., R. G. Martin, and D. M. Livingston.** 1977. Interaction of simian virus 40 chromatin with simian virus 40 T-antigen. J. Virol. **24**:451-460.
18. **Risser, R., and R. Pollack.** 1974. A nonselective analysis of SV40 transformation of mouse 3T3 cells. Virology **59**:477-489.
19. **Schaffhausen, B. S., J. E. Silver, and T. L. Benjamin.** 1978. T antigen(s) in cells productively infected by wild type polyoma virus and mutant NG-18. Proc. Natl. Acad. Sci. U.S.A. **75**:79-83.
20. **Seif, R., and F. Cuzin.** 1977. Temperature-sensitive growth regulation in one type of transformed rat cells induced by the *tsa* mutant of polyoma virus. J. Virol. **24**:721-728.
21. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.
22. **Steinberg, B., R. Pollack, W. Topp, and B. Botchan.** 1977. Isolation and characterization of T-antigen negative revertants from a line of transformed rat cells containing only one copy of the SV40 genome. Cell **13**: 19-32.
23. **Tegtmeyer, P., K. Rundell, and J. K. Collins.** 1977. Modification of simian virus 40 protein A. J. Virol. **21**: 647-657.
24. **Tegtmeyer, P., M. Schwartz, J. K. Collins, and K. Rundell.** 1975. Regulation of tumor antigen synthesis by simian virus 40 gene *A*. J. Virol. **16**:168-178.
25. **Tenen, D. G., R. G. Martin, J. Anderson, and D. M. Livingston.** 1977. Biological and biochemical studies of cells transformed by simian virus 40 temperature-sensitive gene *A* mutants and *A* mutant revertants. J. Virol. **22**:210-218.
26. **Zouzias, D., I. Prasad, and C. Basilico.** 1977. State of the viral DNA in rat cells transformed by polyoma virus. II. Identification of the cells containing nonintegrated viral DNA and the effect of viral mutations. J. Virol. **24**:142-150.