JOURNAL OF VIROLOGY, Jan. 1981, p. 92-102
0022-538X/81/010092-11$02.00/0

Vol. 37, No. 1

Detection and Characterization of Multiple Forms of Simian Virus 40 Large T Antigen

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Subclasses of simian virus 40 large T antigen in simian virus 40-transformed and -infected cells separated by zone velocity sedimentation in sucrose density gradients have been characterized. Three forms of large T antigen were distinguished: a 5 to 6S form, a 14 to 16S form, and a 23 to 25S form. These forms appeared to differ biochemically and biologically. Differential labeling experiments suggested that the 5 to 6S form was less highly phosphorylated than the faster-sedimenting forms. The 23 to 25S form which was complexed with one or more host phosphoproteins, as reported recently (D. P. Lane and L. V. Crawford, Nature [London] **268**:261-263, 1979; F. McCormick and E. Harlow, J. Virol. **34**: 213-224, 1980), was prominent in extracts of transformed cells, but was also detected in productively infected cells. Pulse-chase experiments suggested that the 5 to 6S large T antigen is a precursor of the more stable, faster-sedimenting forms of T antigen. Monkey cells infected with a *tsA* mutant of simian virus 40 at 41°C contained only 5 to 6S large T antigen, implying that this form is not active in the initiation of simian virus 40 DNA replication. In pulse-chase, shift-down experiments, DNA replication resumed, and the 5 to 6S large T antigen which had accumulated at 41°C was partially converted at 33°C to a fast-sedimenting form. However, shift-up experiments demonstrated that the fast-sedimenting large T antigen, once formed, remained stable at 41°C, although it was unable to function in initiation. These experiments suggest that different biological functions of large T antigen may be carried out by different subclasses of this protein.

Simian virus 40 (SV40) DNA codes for two early proteins, the large and small tumor (T) antigens (22, 30, 33), which can be immunoprecipitated from extracts of cells productively infected or transformed by SV40, using anti-SV40 tumor serum (38). Although the function of the small T antigen is still uncertain (23), multiple functions in which the large T antigen is essential have been demonstrated, among them the initiation of each round of viral DNA replication, the stimulation of late gene transcription and attenuation of early gene transcription in productively infected cells, and the initiation and maintenance of transformation (reviewed in reference 12). It is not at all clear whether the large T antigen performs these varied functions directly or whether it must interact with host cell factors to fulfill its functions.

Recently, another class of polypeptides with molecular weights of 50,000 to 55,000 (50 to 55K) has been specifically immunoprecipitated from extracts of SV40-transformed (7, 9, 25) and -infected cells (25, 26). An increasing body of evidence suggests that these proteins are probably coded by the host genome (5, 15, 18, 20, 25, 26, 35) and may be induced or stabilized in transformed cells (9, 20, 21). The interaction between these host proteins and the large T antigen could conceivably modify the properties of the host proteins or those of the T antigen itself.

Another possibility which might be considered is that the multiple functions in which the large T antigen is involved may really be performed by different subclasses of large T antigen. This communication describes the identification and characterization of several such subclasses of large T antigen and their possible correlation with some of the functions of this protein.

(A preliminary account of this work was presented at the Imperial Cancer Research Fund DNA Tumor Virus Meeting in Cambridge, England, in July 1979.)

MATERIALS AND METHODS

Cells and virus. SV40-transformed mouse SVT2 (1), hamster THK-1, (3), monkey C2 (10), and human SV80 (39) cell lines have been described previously. Subconfluent CV-1P monkey cells were infected with 1 to 5 PFU of SV40, strain SVS, per cell or with the mutant *tsA58* as described previously (2, 8). All cell lines were cultured in Dulbecco-modified Eagle medium containing 10% calf serum (Boehringer, Mannheim, Germany).

Antisera. Initial gifts of hamster anti-SV40 tumor serum were obtained from H. Fischer, German Cancer Research Center, Heidelberg, Germany, and J.

Gruber, National Institutes of Health, Bethesda, Md. Larger amounts of tumor serum were prepared from hamsters carrying tumors induced by subcutaneous injection of THK-1$_t$ cells and from BALB/c mice injected subcutaneously or intraperitoneally or both with SVT2 cells. Normal sera were obtained from healthy adult mice and hamsters. Rabbit antiserum against isolated, denatured large T antigen was a gift from W. Deppert, University of Ulm, Ulm, Germany. Monkey anti-U serum (19) was obtained from A. M. Lewis, Jr., National Institutes of Health, Bethesda, Md.

Labeling and extraction of proteins. Confluent monolayers were starved for 1 h in methionine- and serum-free medium and then labeled with 100 μCi of [^{35}S]methionine (Amersham Buchler, Braunschweig, Germany) per ml in methionine-free medium. Alternatively, the cells were incubated in phosphate- and serum-free medium for 1 h and labeled for 2 h with 100 μCi of $^{32}PO_4$ (Amersham Buchler) per ml in phosphate-free medium. In some experiments, the cells were labeled for 14 to 15 h with 20 to 30 μCi of [^{3}H] leucine per ml and 40 to 50 μCi of $^{32}PO_4$ per ml in leucine- and phosphate-free medium. At the end of the labeling period, the cells were washed three to five times with cold Hanks balanced salt solution, scraped from the plates, and pelleted. The cells were suspended (10^6 cells per ml) in 50 mM Tris-hydrochloride (pH 8)–120 mM NaCl–0.5% Nonidet P-40 (34), extracted for 1 h at 0°C, and pelleted again. The supernatant was used directly for immunoprecipitation or stored at −70°C in small samples. Reextraction of the nuclear pellet accompanied by ultrasonic treatment failed to yield additional T antigen in significant amounts.

Immunoprecipitation. Immunoprecipitation with anti-SV40 tumor serum and normal serum was performed as described previously (34), except calf serum was substituted for sheep serum. Formaldehyde-fixed *Staphylococcus aureus*, strain Cowan I, was prepared as described previously (13) and stored at −20°C in small samples.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out as described previously (17). Immune complexes were eluted from the washed bacterial pellets by boiling for 3 min in 20 to 50 μl of double-strength sample buffer. Electrophoresis was carried out at 40 mA per gel for 2.5 to 3 h with cooling. The gels were stained for 2 h with 0.1% Coomassie brilliant blue in methanol-water-acetic acid (20:17:3), destained, dried, and autoradiographed or fluorographed on Kodak XR-5 film. Proteins with the following known molecular weights were used to calibrate the gels: β-galactosidase, 130K; *Escherichia coli* RNA polymerase β′, β, and α, 165, 155, and 39K, respectively; phosphorylase *a*, 94K; fructose-6-phosphate kinase, 81K; bovine serum albumin, 68K; glutamate dehydrogenase, 53K; alcohol dehydrogenase, 37K; and soybean trypsin inhibitor, 21.5K.

Zone velocity sedimentation of nuclear extracts. Cell monolayers were labeled and washed as described above, rinsed once with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.8)–5 mM KCl–0.5 mM $MgCl_2$–1 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride (buffer A), and drained. Cells were scraped from the plates in buffer A and disrupted in a Dounce homogenizer by 10 strokes with a tight-fitting Teflon pestle. The nuclei were pelleted, resuspended in buffer A (10^8 nuclei per ml), and incubated for 1 h at 0°C. The nuclei were pelleted, and the supernatant extract (0.2 ml) was layered onto a 5 to 30% sucrose density gradient in buffer A containing 5 mM KCl. Similar results were obtained when the gradients contained 0.5% Nonidet P-40 or 150 mM KCl instead of 5 mM KCl. The gradients were centrifuged in an AH650 (Sorvall) or SW55 rotor (Beckman) for 180 to 190 min at 45,000 rpm and 0°C. SV40 form I DNA (20S) and β-galactosidase (16S) were run in parallel gradients as markers. Fractions were collected from the bottom. Acid-precipitable radioactivity was determined in a 10-μl sample of each fraction, and the rest of the material was analyzed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. In many experiments, 50-μl samples of the extract loaded onto the gradients were immunoprecipitated with tumor serum and normal serum and run on the same gels.

Zone velocity sedimentation of whole-cell extract. After the cells were labeled as indicated in each figure legend, they were washed three times with cold Hanks balanced salt solution, scraped from the plates, and pelleted. They were suspended (10^8 cells per ml) in 50 mM Tris-hydrochloride (pH 8)–120 mM NaCl–0.5% Nonidet P-40 (34), extracted for 1 h at 0°C, and pelleted again. The supernatant was loaded directly onto sucrose gradients (0.2 ml per gradient) and analyzed as described above for nuclear extracts.

RESULTS

Detection of tumor antigens in infected and transformed cells used in this study. SV40-transformed human, mouse, and monkey cells, as well as infected and uninfected monkey cells, were labeled with [^{35}S]methionine. Proteins extracted from each cell line were immunoprecipitated with anti-SV40 tumor serum and normal serum and separated by SDS-polyacrylamide gel electrophoresis. The large T antigen, about 90K (34, 38), and the small T antigen, about 17K (30), were detected in each of the transformed and infected cells except, curiously, this strain of SVT2 (Fig. 1). The large T antigen from SVT2 cells was slightly larger than that from the other cells, as shown previously (31). Limited degradation of large T antigen (34) could not be eliminated entirely and was especially evident in extracts of productively infected cells (Fig. 1).

Host proteins of 50 to 55K (5, 9, 15, 18, 20, 21, 25, 35) were specifically precipitated from these transformed cells by tumor serum. Their relative amounts and apparent molecular weights depended upon the animal species of the transformed cell, as reported recently (7, 20, 25). These proteins were not observed in infected cells in this experiment (Fig. 1), but were detectable in extracts of $^{32}PO_4$-labeled infected

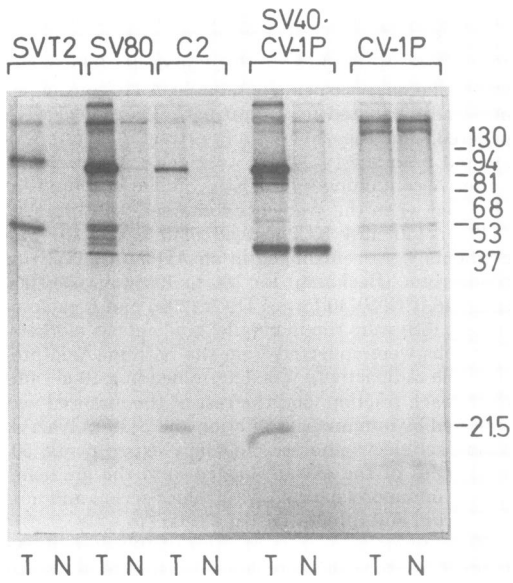

FIG. 1. *SDS-polyacrylamide gel fluorograms of [^{35}S]methionine-labeled T antigens from SV40-infected and -transformed cells. Proteins were immunoprecipitated from whole-cell extracts of SV40-infected (48 h after infection), SVT2, SV80, and C2 cells and uninfected CV-1P cells with normal hamster serum (N) and anti-SV40 tumor serum (T) as indicated. The positions of marker proteins of known molecular weights are shown on the right. The gel contained 12.5% acrylamide.*

cells (see Fig. 3C). Others have also found small amounts of 54K protein in nontransformed cells (11, 20, 26).

Characterization of hamster tumor sera. Some anti-SV40 tumor sera have been shown to react with 55K host proteins specifically (5, 20, 21), whereas other tumor sera which are unable to recognize the host proteins precipitate them as a complex with the large T antigen (18). The tumor serum used in this study was characterized in two ways: (i) its capacity to re-immunoprecipitate denatured host proteins was tested, and (ii) an attempt was made to differentially dissociate the host proteins and the large T antigen from immune complexes.

Whole-cell extracts of $^{32}PO_4$-labeled SV80 cells were immunoprecipitated with anti-SV40 hamster tumor serum. The immune complexes were dissociated from the bacteria by boiling first in sample buffer containing 0.1% SDS (Fig. 2b) and then reboiling the bacterial pellet in sample buffer containing 1% SDS (Fig. 2a) or by boiling the sample as usual (Fig. 2c). Treatment with sample buffer as usual completely dissociated the immune complex, freeing both the host proteins and the large T antigen. Treatment with 0.1% SDS, on the other hand, dissociated only the host proteins from the immune complex. The large T antigen was recovered after treatment with 1% SDS. A preparation containing both host proteins and the large T antigen was diluted and immunoprecipitated a second time with the same tumor serum. The denatured large T antigen was precipitated, but the host proteins failed to react with the tumor serum (Fig. 2d). These results indicate that the tumor sera used in this study had a strong affinity for the large T antigen but only weak affinity, if any, for 55K proteins.

Nuclear extracts were prepared in hypotonic buffer from SV80 cells labeled with $^{32}PO_4$ and analyzed by zone velocity sedimentation in sucrose density gradients. The gradients were fractionated, and acid-precipitable radioactivity was

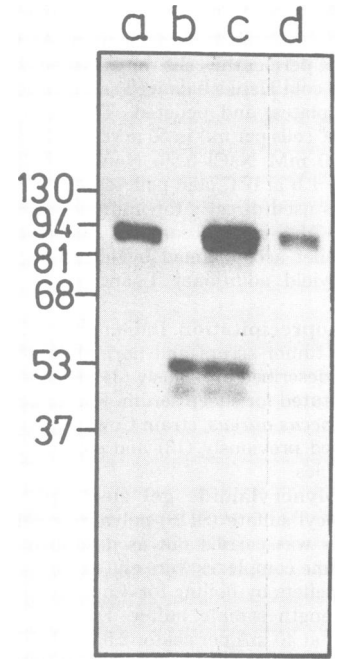

FIG. 2. *Separation of host proteins and large T antigen from SV80 cells by differential dissociation of immune complexes. Whole-cell extracts of $^{32}PO_4$-labeled SV80 cells were immunoprecipitated with hamster tumor serum as described in the text. The immune complexes were dissociated by boiling in sample buffer (c) or in sample buffer containing 0.1% SDS (b), followed by reboiling in 1% SDS (a). A duplicate sample (c) without 2-mercaptoethanol was diluted in 50 mM Tris-hydrochloride (pH 7.5)–150 mM NaCl–5 mM EDTA–0.05% Nonidet P-40 to 0.02% SDS and re-immunoprecipitated with fresh hamster tumor serum (d). The immunoprecipitates were analyzed by electrophoresis in SDS-polyacrylamide gels and autoradiography.*

determined in a sample of each fraction (Fig. 3A). The proteins immunoprecipitated from each fraction with hamster tumor serum were electrophoresed on SDS-polyacrylamide gels; the gels were stained, dried, and autoradiographed. Two peaks of large T antigen were prominent, one at about 14 to 16S (fractions 15 to 17 of Fig. 3B) and another at about 23 to 25S (fractions 12 to 13 of Fig. 3B), which cosedimented with most of the 55K protein. Neither the 55K protein nor the large T antigen was immunoprecipitated with normal hamster serum. These two peaks contained the bulk of the large T antigen in the gradients, as indicated by Coomassie brilliant blue staining and complement fixation (28; A. Dorn and E. Fanning, unpublished data), and were also demonstrated by using extracts from [^{35}S]methionine-labeled SV80 cells and from $^{32}PO_4$-labeled SV40-transformed monkey cells (C2) (data not shown). A complex from transformed mouse cells analogous to the 23 to 25S form has been described recently by McCormick and Harlow (24).

When the nuclear extracts prepared from SV40-infected CV-1P cells labeled with $^{32}PO_4$ were analyzed in the same way, only one peak of large T antigen was found (Fig. 3C). This peak sedimented at about 14 to 16S and also contained most of the 90K protein in the gradient, as indicated by complement fixation (data not shown). The 55K protein was detected in the 23 to 25S region of the gradient, apparently cosedimenting with a heavy shoulder of the main peak of the large T antigen (Fig. 3C). At later times after infection, the 23 to 25S peak containing T antigen and 55K protein was more prominent and could be clearly separated from the 14 to 16S peak (E. Fanning, C. Bıırger, and D. Cörlin, manuscript in preparation).

These results, together with the fact that the tumor serum used did not recognize the 55K protein (Fig. 2), suggested that productively infected cells may also contain a 55K protein-T antigen complex analogous to that in SV80 and C2 cells. Other antisera which do not react with 55K protein, such as rabbit antiserum to the SDS-denatured large T antigen or a monkey anti-U serum (18), gave results indistinguishable from those in Fig. 3 (data not shown).

These results indicate that both transformed cells and productively infected cells contain two forms of phosphorylated large T antigen, the 23 to 25S complex between the 55K protein and the large T antigen, as described recently (24), and a 14 to 16S form.

SV40 large T antigen may be differentially phosphorylated. In addition to the two forms of phosphorylated large T antigen in SV40-transformed and -infected cells (Fig. 3),

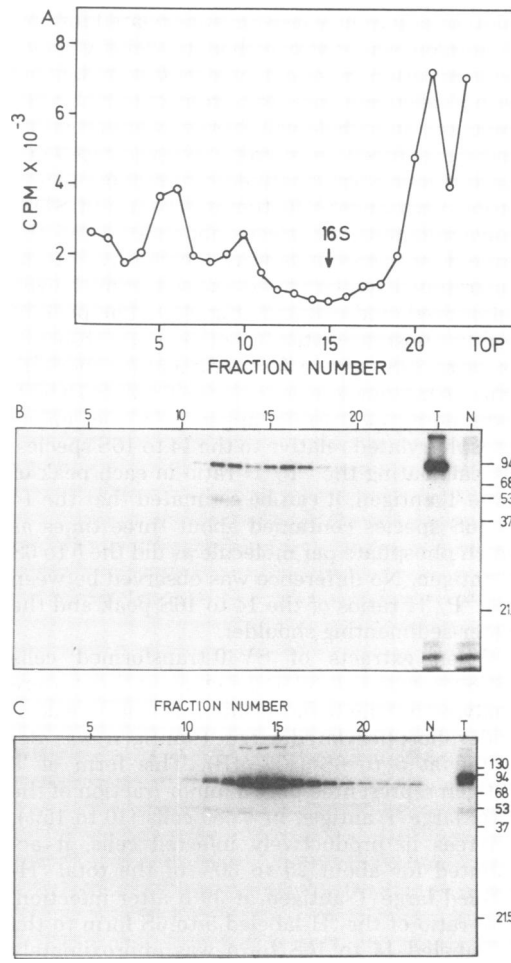

FIG. 3. *Zone velocity sedimentation of an extract from transformed and productively infected cells. An extract from $^{32}PO_4$-labeled SV80 nuclei was analyzed in a 5 to 30% sucrose density gradient as described in the text (AH650, 45,000 rpm, 0°C, 190 min). (A) Acid-precipitable radioactivity was determined in 10-μl samples of each fraction. The arrow indicates the position of β-galactosidase run in a parallel gradient as a marker. (B) Each fraction was immunoprecipitated with anti-SV40 tumor serum and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. An extract from 5 × 10^6 whole cells was immunoprecipitated with normal hamster serum (N) and anti-SV40 tumor serum (T) and run on the same gel. (C) A nuclear extract of SV40-infected CV-1P cells labeled with $^{32}PO_4$ from 45 to 47.5 h after infection was analyzed on a sucrose density gradient as described for (A) and (B). Acid-precipitable radioactivity in 10-μl samples of each fraction was determined (data not shown), and the rest of each fraction was immunoprecipitated with anti-SV40 tumor serum and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The β-galactosidase 16S peak was in fraction 14. The direction of sedimentation is from right to left.*

another apparently less-phosphorylated form of T antigen was distinguished by zone velocity sedimentation. SV40-infected cells were labeled with [^{3}H]leucine and $^{32}PO_4$ from 24 to 39 h after infection, and whole-cell extracts were prepared and centrifuged in sucrose density gradients. Each fraction was analyzed by immunoprecipitation with anti-SV40 tumor serum and SDS-polyacrylamide gel electrophoresis. After the large T antigen bands were located by autoradiography, each band was excised from the gel, solubilized, and counted (Fig. 4A). The peak of highly phosphorylated T antigen was observed at 14 to 16S, with a fast-sedimenting shoulder (Fig. 4A). A new species of large T antigen which sedimented at 5 to 6S appeared to be underphosphorylated relative to the 14 to 16S species. By calculating the $^{32}P/^3H$ ratio in each peak of large T antigen, it can be estimated that the 14 to 16S species contained about three times as much phosphate per molecule as did the 5 to 6S T antigen. No difference was observed between the $^{32}P/^3H$ ratios of the 14 to 16S peak and the faster-sedimenting shoulder.

When extracts of SV40-transformed cells (SV80) labeled with [^{3}H]leucine and $^{32}PO_4$ were analyzed in the same fashion, a small peak of underphosphorylated large T antigen was detected at 5 to 6S (Fig. 4B). This form of T antigen represented only a minor fraction of the total large T antigen in SV80 cells (10 to 15%), whereas in productively infected cells, it accounted for about 25 to 30% of the total ^{3}H-labeled large T antigen at 39 h after infection. The ratio of the ^{3}H-labeled 5 to 6S form to the ^{3}H-labeled 14 to 16S form was approximately the same in SV80 cells and infected cells. Using a different procedure, McCormick and Harlow have also found evidence for an underphosphorylated 5 to 6S form of T antigen in transformed mouse cells (24).

Labeling kinetics of different forms of SV40 large T antigen. Pulse-chase experiments were performed with SV80 cells labeled for 15 min with [^{35}S]methionine or labeled for 15 min and then incubated for 12 h in medium containing a 100-fold excess of unlabeled methionine. Whole-cell extracts were analyzed by zone velocity sedimentation in sucrose density gradients, followed by immunoprecipitation with tumor serum, SDS-polyacrylamide gel electrophoresis, and autoradiography (Fig. 5). Whole-cell extracts from pulse-labeled and pulse-chased cultures were immunoprecipitated with tumor serum and normal serum and electrophoresed on the same gels. Most of the pulse-labeled large T antigen was detected in the 5 to 6S form (Fig. 5A). Since the 50 to 55K host proteins were obscured by nonspecifically precipitated bands,

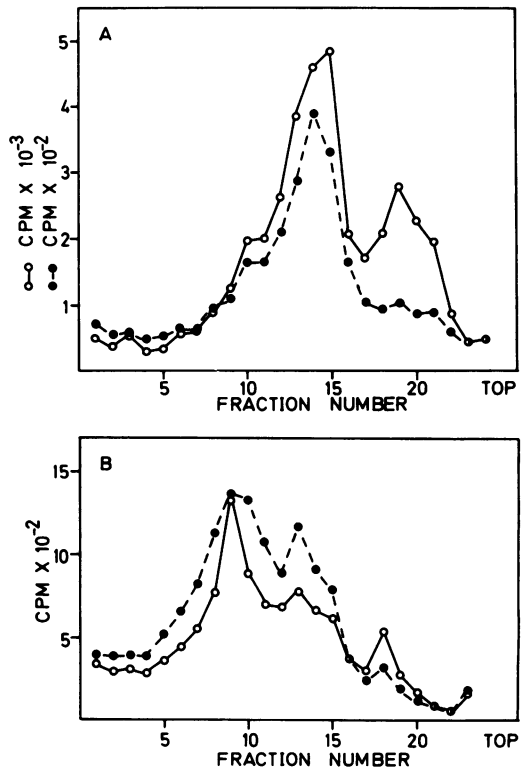

FIG. 4. *Zone velocity sedimentation of [^{3}H]leucine- and $^{32}PO_4$-doubly labeled extract from SV40-infected and -transformed cells. (A) SV40-infected CV-1P cells were labeled with 30 μCi of [^{3}H]leucine per ml and 40 μCi of $^{32}PO_4$ per ml from 24 to 39 h after infection as described in the text. A whole-cell extract from about 10^7 cells was layered on a 5 to 30% sucrose density gradient in 10 mM HEPES (pH 7.8)–5 mM KCl–1 mM dithiothreitol–0.5 mM $MgCl_2$–1 mM phenylmethylsulfonyl fluoride and centrifuged in an SW55 rotor for 3 h at 45,000 rpm and 0°C. Fractions were collected from the bottom and analyzed by immunoprecipitation with anti-SV40 tumor serum, SDS-polyacrylamide gel electrophoresis, and autoradiography as described in the text. The peak of SV40 form I DNA was in fractions 12 to 13. The large T antigen bands (84 to 90K) were excised from the gel, solubilized, and counted. (B) Subconfluent SV80 cells were labeled for 15 h with 20 μCi of [^{3}H]leucine per ml and 50 μCi of $^{32}PO_4$ per ml. A whole-cell extract from about 10^7 cells was analyzed as in (A), except the sucrose gradient contained 150 mM KCl. The peak of SV40 form I DNA was in fractions 11 to 12. Symbols: ○, ^3H; ●, ^{32}P.*

it was not clear whether they were associated with the 5 to 6S form. After a 12-h chase, most of the labeled T antigen appeared in the faster-sedimenting forms (Fig. 5B). The 50 to 55K host proteins could now be easily detected, both in immunoprecipitated whole-cell extract and in the 23 to 25S region of the sucrose gradient (Fig.

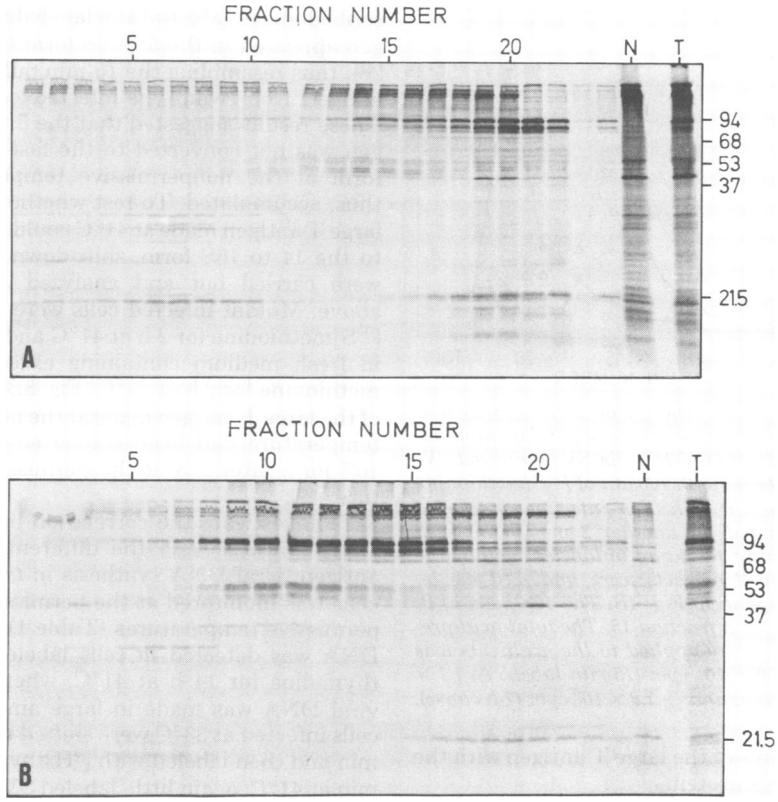

FIG. 5. *Pulse-chase experiment: zone velocity sedimentation of whole-cell extracts from [^{35}S]methionine-labeled SV40-transformed cells. Subconfluent SV80 cells were pulse-labeled with 100 μCi of [^{35}S]methionine per ml for 15 min (A) or pulse-labeled and then incubated with fresh medium containing 1.5 mg of unlabeled methionine per ml for 12 h (B). Whole-cell extracts were analyzed by zone velocity sedimentation, immunoprecipitation with anti-SV40 tumor serum, SDS-polyacrylamide gel electrophoresis, and autoradiography as described in the text. The direction of sedimentation is from right to left. The β-galactosidase 16S peak was in fraction 15. Note that some large T antigen appeared as an 84K degradation product (34, 37), presumably due to the small volume used to extract T antigen in concentrated form before sedimentation. The total acid-precipitable radioactivity applied to the gradients was 4.8 × 10^7 cpm (A) and 3.0 × 10^7 cpm (B).*

5B). The small T antigen, however, was obscured by nonspecific bands in both autoradiograms. This experiment demonstrates a flow of label in the large T antigen from the 5 to 6S form to the fast-sedimenting forms, suggesting a precursor-product relationship between these forms.

Pulse-chase experiments were also carried out with SV40-infected CV-1P cells. Infected cells were pulse-labeled with [^{35}S]methionine for 15 min or pulse-labeled and then incubated with fresh medium containing excess unlabeled methionine for 2 or 7 h. Whole-cell extracts of each culture were subjected to zone velocity sedimentation, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis. The bands of large T antigen (84 to 90K) were located by fluorography, excised from the gels, solubilized, and counted (Fig. 6). Pulse-labeled large T antigen appeared mainly in the 5 to 6S form, whereas after a 2-h chase, most of it was detected in a broad peak between 15 and 25S. This broad peak was still present after a 7-h chase, although a small amount of material was observed at 5 to 6S (Fig. 6). These results suggest that in productively infected cells, as well as in transformed cells (Fig. 5), the 5 to 6S form of large T antigen may serve as a precursor to the more stable fast-sedimenting forms.

Correlation between form and function of large T antigen in *tsA*-infected cells. Mutants of SV40 with a temperature-sensitive lesion in gene *A* are unable to carry out several functions at a nonpermissive temperature, for example, the initiation of new rounds of viral DNA replication (36) and regulation of early gene transcription (32, 38). Analysis of these mutants thus offers the possibility of correlating

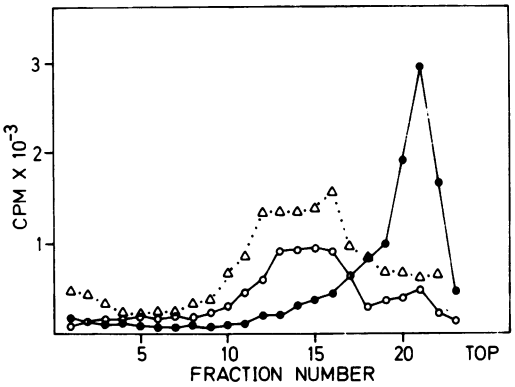

FIG. 6. *Pulse-chase experiment: zone velocity sedimentation of whole-cell extracts of [^{35}S]methionine-labeled SV40-infected CV-1P cells. SV40-infected CV-1P cells were labeled with 100 μCi of [^{35}S]methionine per ml for 15 min at 42 h after infection or labeled and then incubated for another 2 or 7 h in fresh medium containing 1.5 mg of unlabeled methionine per ml. Whole-cell extracts were analyzed as described in the legend to Fig. 4B. The 16S peak of β-galactosidase was in fraction 15. The total acid-precipitable radioactivity applied to the gradients was as follows: ●, 1.2 × 10^7 cpm (15-min label); △, 1.1 × 10^7 cpm (2-h chase); and ○, 7.8 × 10^6 cpm (7-h chase).*

the various forms of the large T antigen with the functions of this protein.

CV-1P cells infected with the mutant *tsA58* were labeled at the permissive temperature for 2 h with [^{35}S]methionine. Whole-cell extracts of each culture were analyzed by zone velocity sedimentation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and fluorography. At 33°C, the labeled large T antigen was observed in both the 5 to 6S and the 14 to 16S forms (Fig. 7), as might be expected from the results obtained with wild-type SV40 (Fig. 4A). When cells infected with *tsA58* were labeled for 2 h at 33°C with [^{35}S]methionine and then shifted to 41°C for a chase, about 70% of the fast-sedimenting large T antigen labeled at 33°C was still present in these forms after 3 h at 41°C, about the same as the fraction of acid-precipitable radioactivity remaining in total cell protein after the chase (75%) (Fig. 7). Thus, the 14 to 16S form, once made at the permissive temperature, seems to be stable at the nonpermissive temperature. Most of the 5 to 6S large T antigen was no longer present after the chase, but it cannot be determined from these results whether the large T antigen was converted to the 14 to 16S form during shift up or degraded completely at 41°C.

In a similar fashion, T antigen was extracted from *tsA58*-infected cells labeled with [^{35}S]methionine for 2 h at 41°C and analyzed as described above. Most of the labeled large T antigen appeared in the 5 to 6S form (Fig. 8A) (16, 28), thus resembling the 15-min pulse-labeled T antigen from wild-type-infected cells (Fig. 6). These results suggested that the 5 to 6S T antigen was not converted to the fast-sedimenting form at the nonpermissive temperature and, thus, accumulated. To test whether the 5 to 6S large T antigen made at 41°C could be converted to the 14 to 16S form, shift-down experiments were carried out and analyzed as described above. Mutant-infected cells were labeled with [^{35}S]methionine for 2 h at 41°C and then chased in fresh medium containing excess unlabeled methionine for 2 h at 33°C (Fig. 8B). About half of the large T antigen made at the nonpermissive temperature had been converted to the 14 to 16S form after 2 h at the permissive temperature.

To investigate the correlation between SV40 DNA synthesis and the different forms of T antigen, viral DNA synthesis in *tsA58*-infected cells was monitored at the permissive and nonpermissive temperatures (Table 1). Little SV40 DNA was detected in cells labeled with [^{3}H]-thymidine for 14 h at 41°C, whereas at 33°C, viral DNA was made in large amounts. When cells infected at 33°C were shifted to 41°C for 30 min and then labeled with [^{3}H]thymidine for 60 min at 41°C, again little labeled SV40 DNA was observed. When infected cells held for 30 min at 41°C were shifted down to 33°C and immedi-

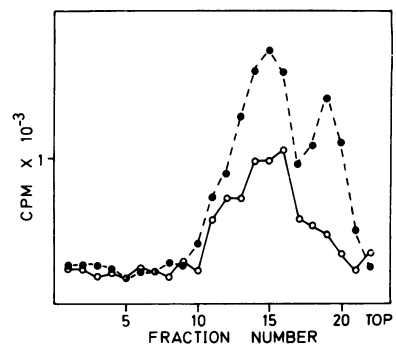

FIG. 7. *Stability of 14 to 16S large T antigen in tsA58-infected CV-1P cells at the nonpermissive temperature. CV-1P cells infected with tsA58 at 33°C were labeled with 100 μCi of [^{35}S]methionine per ml from 71 to 73 h after infection or labeled and then incubated at 41°C with fresh medium containing 1.5 mg of unlabeled methionine per ml for another 3 h. Whole-cell extracts from both cultures were analyzed by zone velocity sedimentation as described in the legend to Fig. 4B. The total acid-precipitable radioactivity applied to the sucrose gradients was as follows: ●, 2.4 × 10^7 cpm (label at 33°C); and ○, 1.8 × 10^7 cpm (chase at 41°C).*

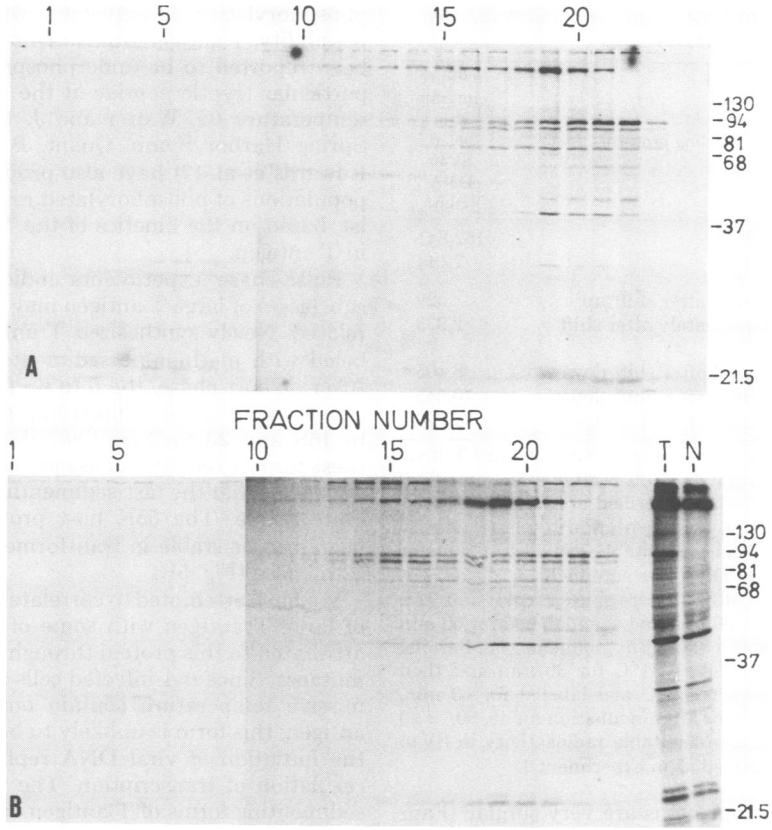

FIG. 8. *Conversion of 5 to 6S large T antigen to a 14 to 16S form in tsA58-infected cells after a shift to the permissive temperature. CV-1P cells infected with tsA58 at 41°C were labeled with 100 μCi of [^{35}S]methionine per ml from 44 to 46 h after infection or labeled and then incubated at 33°C in fresh medium containing excess unlabeled methionine for another 2 h. Whole-cell extracts from both cultures were analyzed by zone velocity sedimentation as described in the legend to Fig. 4B. (A) 2-h label at 41°C; (B) 2-h chase at 33°C. The direction of sedimentation is from right to left. The peak of SV40 form I DNA was in fraction 13. The total acid-precipitable radioactivity applied to the gradients was 4.1 × 10^6 cpm (A) and 3.9 × 10^6 cpm (B).*

ately labeled with [^{3}H]thymidine, some viral DNA synthesis was detected. When [^{3}H]thymidine was added later after the shift down, more labeled viral DNA was found. These experiments indicate that in *tsA58*-infected cells, SV40 DNA synthesis is severely reduced soon after a shift to the nonpermissive temperature and that it begins again after a short time at the permissive temperature, in agreement with earlier reports (36).

DISCUSSION

SV40 large T antigen from productively infected and transformed cells occurs in at least three subclasses separable by zone velocity sedimentation of crude cell extracts in sucrose density gradients. Such subclasses have been observed previously (4, 16, 28, 29), but biological and biochemical differences among them have remained, for the most part, obscure. The apparent sedimentation coefficients of these three subclasses suggest that different states of aggregation of T antigen exist. The 5 to 6S form of large T antigen could correspond to a free 80 to 90K monomer, whereas the 14 to 16S subclass could represent a tetramer or, possibly, a complex with unlabeled host proteins. In agreement with recent observations (24), the 23 to 25S form consists of large T antigen and one or more 50 to 55K host proteins (5–7, 9, 15, 18, 20, 25, 26, 35). These proteins are tightly complexed with the large T antigen; the host proteins from SV80 cells cosediment with the large T antigen and co-immunoprecipitate with it even after sedimentation in 1 M KCl (unpublished data). A comparison of the 55K proteins from infected and transformed cells by two-dimensional gel electrophoresis and V8 peptide mapping sug-

TABLE 1. *SV40 DNA synthesis in tsA58-infected CV-1P cells*

Expt	Labeling protocol	Acid-precipitable ^{3}H-labeled SV40 DNA (cpm)
1[a]	33°C, 14 h	152,841
	41°C, 14 h	7,144
2[b]	41°C, 30 min after shift up	529
	33°C, immediately after shift down	2,933
	33°C, 45 min after shift down	8,405
	33°C, 60 min after shift down	10,345
	33°C, 90 min after shift down	11,876

[a] Infected cells were labeled with 5 μCi of [^{3}H]thymidine per ml from 76 to 90 h after infection. SV40 DNA was extracted as described in the text and analyzed by zone velocity sedimentation in sucrose density gradients (14). Acid-precipitable radioactivity under the peaks of SV40 DNA was summed.

[b] Cells infected at 33°C were shifted to 41°C at 72 h after infection for 30 min and then labeled for 60 min at 41°C with 100 μCi of [^{3}H]thymidine per ml. Parallel cultures were shifted to 41°C for 30 min and then shifted back down to 33°C and labeled for 60 min, either immediately or after incubation for 45, 60, or 90 min at 33°C. Acid-precipitable radioactivity in SV40 DNA was determined as in experiment 1.

gests that these proteins are very similar (Fanning, Burger, and Nowak, manuscript in preparation). In addition to the 5 to 6S and 23 to 25S forms described recently (24), we have consistently observed a 23 to 25S form in extracts of infected cells and a 14 to 16S form in extracts of infected and transformed cells; these differences could possibly result from differences in extraction conditions. It is interesting that three immunologically distinguishable subclasses of large T antigen, one of which corresponds to the 23 to 25S form, have also been found in both infected and transformed cells with monoclonal antibodies (11).

The different forms of large T antigen are apparently differentially phosphorylated (Fig. 4 and reference 24). The 5 to 6S form of T antigen contains less labeled phosphate not only after a long label (Fig. 4), but also after a pulse-label of 15 min (E. Fanning and B. Bukau, unpublished data). These experiments do not exclude the possibility that the apparent underphosphorylation could result in part from cosedimentation of the 5 to 6S form with phosphatase activity. Furthermore, it is not clear whether some of the 5 to 6S T antigen is fully phosphorylated and the rest is not phosphorylated at all or whether all of the 5 to 6S T antigen is modified in only one or some of several possible sites of phosphorylation. In agreement with the second possibility, T antigen from *tsA*-infected cells has been reported to be underphosphorylated in a particular tryptic peptide at the nonpermissive temperature (G. Walter and J. P. Flory, Cold Spring Harbor Symp. Quant. Biol., in press). Edwards et al. (7) have also proposed that two populations of phosphorylated residues may exist, based on the kinetics of the $^{32}PO_4$ turnover in T antigen.

Pulse-chase experiments indicate that the subclasses of large T antigen may be temporally related. Newly synthesized T antigen pulse-labeled with methionine sedimented at 5 to 6S. After a short chase, the 5 to 6S form had been converted to faster-sedimenting forms, both 14 to 16S and 23 to 25S. The label persisted in these forms even after chase periods of 7 to 12 h, indicating that the fast-sedimenting T antigen is quite stable. The 55K host proteins also appeared to be stable in transformed cells after a 12-h chase (Fig. 5B).

We have attempted to correlate the subclasses of large T antigen with some of the functions attributed to this protein through the use of *tsA* mutants. Since *tsA*-infected cells at the nonpermissive temperature contain only 5 to 6S T antigen, this form is unlikely to be functional in the initiation of viral DNA replication or the regulation of transcription. The fact that fast-sedimenting forms of T antigen, once formed at the permissive temperature, persist at 41°C, although the initiation of new rounds of replication is inhibited almost immediately upon shift up (reference 36 and Table 1), means either that the fast-sedimenting forms are also unlikely to be functional in the initiation of replication or that several forms of T antigen cosediment at 14 to 16S, one or more of which is temperature sensitive for initiation.

Alternatively, since neither the fast-sedimenting nor the 5 to 6S form of T antigen was correlated with the initiation of replication, perhaps the transition process itself or a transitory intermediate form of T antigen is essential for initiation. Consistent with this interpretation, both DNA replication and the conversion of 5 to 6S T antigen to the 14 to 16S form resume after a shift down to the permissive temperature. The observation that newly synthesized T antigen binds more tightly to DNA than does older T antigen (27) is also consistent with this idea.

The amount of the fast-sedimenting forms of T antigen may also play a role in the attenuation of early transcription. This function of large T antigen is lost, not immediately, but gradually, after a shift to the nonpermissive temperature (32), just as the amount of fast-sedimenting T antigen gradually declines at the nonpermissive

temperature (Fig. 7). In this way, early transcription would be maximal until sufficient fast-sedimenting T antigen accumulated.

In summary, the large T antigen exists in multiple forms in different relative amounts in SV40-infected and -transformed cells. Evidence has been presented that these forms differ from each other biochemically and that they may be correlated with some of the biological functions of T antigen. This type of analysis may prove to be useful in elucidating the mechanisms by which T antigen carries out its varied functions.

ACKNOWLEDGMENTS

We thank A. J. Levine, A. M. Lewis, Jr., F. J. O'Neill, G. Sauer, and E. Winocour for seed stocks of transformed cells, P. Tegtmeyer for a seed stock of the *tsA58* mutant, W. Deppert, H. Fischer, J. Gruber, and A. M. Lewis, Jr., for samples of antiserum, G. Walter for communicating his results before publication, and F. Gruber for animal care. We are especially grateful to D. Cörlin for excellent technical assistance, M. Hornberger for the construction of laboratory equipment, R. Mettke for the preparation of culture media, I. Lühr and R. Phildius for help with the manuscript, and R. Knippers for his continued interest and support.

This work was supported by the Deutsche Forschungsgemeinschaft through SFB 138/B4.

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