Specific Interaction of Simian Virus 40 Large T Antigen with Cellular Chromatin and Nuclear Matrix during the Course of Infection

REINHOLD SCHIRMBECK[†] AND WOLFGANG DEPPERT^{†*}

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

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We analyzed the subnuclear distribution of the simian virus 40 (SV40) large tumor (large T) antigen during the course of viral infection. Three distinct nuclear subclasses were detected in SV40 lytically infected TC7 cells (large T antigen in the nucleoplasm, at the cellular chromatin, and at the nuclear matrix). During the course of infection the relative subnuclear distribution of large T antigen changed significantly at about the switch from the early to late phase of infection: at early times postinfection, large T antigen was present mainly in the nucleoplasm and at the cellular chromatin, and nuclear-matrix-associated large T antigen was barely detectable. Concomitant with the onset of viral DNA replication, the amount of nuclear-matrix-associated large T antigen increased drastically. During the further course of infection large T antigen accumulated at the cellular chromatin and nuclear matrix, paralleling the increase in viral DNA synthesis. The biological significance of this correlation was corroborated by analysis of cells infected with the SV40 mutant *tsA58* at permissive (32°C) and restrictive (39°C) temperatures. *tsA58* large T antigen failed to initiate viral DNA replication in infected cells kept at the restrictive temperature and also failed to associate with the cellular chromatin and nuclear matrix. By blocking viral DNA synthesis with aphidicolin, an inhibitor of DNA polymerase α , we were able to show that the accumulation of large T antigen at these structures does not result from the binding of large T antigen to viral chromatin but reflects an association with cellular components of the chromatin and nuclear matrix of infected cells.

During lytic infection of monkey cells by simian virus 40 (SV40), the SV40 large tumor (large T) antigen performs a variety of functions. During the early phase, large T antigen is responsible for regulating several cellular processes, including alterations in the steady-state levels of certain mRNAs (26, 27) and induction of cellular DNA synthesis (for a review, see reference 20). During the late phase, large T antigen is necessary to initiate each round of viral DNA replication (37, 40), is involved in the elongation process of viral DNA synthesis, probably by exerting DNA-helicase activity (30-32), and modulates both early and late viral transcription (for a review, see reference 40). Aside from its helicase activity, which seems to correspond to its demonstrated ATPase activity (30), no other enzymatic activity has been unequivocally demonstrated for large T antigen (24, 42). Therefore, the multitude of functions displayed by large T antigen can result only from its regulatory interactions with (i) regulatory sequences on the viral genome (5, 39; for a review, see reference 24) and (ii) cellular components involved in transcription and DNA replication, thereby modulating the cellular transcription and DNA replication machinery for the needs of viral replication and transcription.

Whereas the interactions of large T antigen with regulatory sequences around the SV40 origin of replication have been studied in some detail (for a review, see reference 24), very little is known about its regulatory interactions with cellular components. Large T antigen binds to the cellular proliferation protein p53 (16, 17, 21), but this interaction seems to be more important in cellular transformation by

SV40 than during lytic infection (11, 21). However, possibly as a consequence of regulatory interactions with cellular structures, large T antigen is found in discrete subnuclear locations in both lytically infected and transformed cells: most large T antigen is found in the cell nucleus, where it is present at different nuclear structures (4, 6, 13, 33, 41). In addition, a small amount of large T antigen specifically associates with the plasma membrane (for a review, see reference 3). Whereas the biological significance of the plasma membrane association of large T antigen for viral replication is unknown, it has been demonstrated that mutant large T antigen molecules unable to be transported into the nucleus do not support viral DNA replication, although they retain the specific DNA-binding properties of large T antigen (18). Therefore, these data provide evidence for the importance of nuclear large T antigen for viral replication.

We have previously developed methods which allow the analysis of possible regulatory interactions of large T antigen with different structural systems of the nucleus (33, 34) and have identified three distinct nuclear subclasses of large T antigen in SV40-transformed cells: large T antigen in the nucleoplasm, large T antigen in association with the cellular chromatin, and large T antigen tightly bound to the residual protein skeleton of the nucleus, the nuclear matrix. These subclasses not only differ in their subnuclear location but also in various biochemical and functional parameters (8, 14, 33). It is thus an attractive hypothesis that regulatory interactions of large T antigen with these structures play an important role in modulating cellular functions involved in DNA replication and transcription.

In this study we analyzed the subnuclear distribution of large T antigen in SV40 lytically infected cells during the course of infection. To establish a functional correlation between interactions of large T antigen with different struc-

* Corresponding author.

[†] Present address: Heinrich-Pelte-Institute for Experimental Virology and Immunology, D-2000 Hamburg, Federal Republic of Germany.

tural systems of the nucleus and specific events in viral replication, viral DNA synthesis was measured in parallel. Our data show a correlation between the association of large T antigen with the nuclear matrix and an increased accumulation at the cellular chromatin with the beginning of the late phase of the viral replication cycle, as marked by the onset of viral DNA replication. Our data support the assumption that the association of large T antigen with these structures is important for viral replication.

MATERIALS AND METHODS

Cells and viruses. TC7 African green monkey kidney cells (25) were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Monolayer cultures of TC7 cells were inoculated with SV40 776 or SV40 *tsA* mutant *tsA58* (37) or *tsA28* (36) at an input multiplicity of infection (MOI) of 10. After an adsorption period of 2 h at 37°C (wild-type SV40) or 32°C (SV40 *tsA* mutants), unadsorbed virus was removed and growth medium was added to the cultures.

Extraction of large T antigen. (i) **Whole-cell extraction.** Cells were washed three times with phosphate-buffered saline (140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄ · 2H₂O, 1.5 mM KH₂PO₄ [pH 7.4] [43]) and then extracted for 30 min at 2°C with 1 ml of lysis buffer (120 mM NaCl, 5 mM dithiothreitol [DTT], 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid], 1% aprotinin, 0.5% Nonidet P-40 [NP-40], 10% glycerol, 50 mM Tris hydrochloride [pH 8.0]). For each time point, 4 × 10⁶ cells were extracted.

(ii) **In situ cell fractionation.** A detailed description of the cell fractionation procedure, as well as those for the characterization of extracts and structures, has been given elsewhere (33, 34). Briefly, two plates of confluent TC7 cells (4 × 10⁶ cells) lytically infected with wild-type SV40 or *tsA* virus were washed with KM buffer (10 mM morpholinepropanesulfonic acid [MOPS] [pH 6.8], 10 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 5 mM DTT, 10% glycerol). In the first extraction step, cells were lysed for 30 min at 2°C with KM buffer containing 1% NP-40 and 20% (10 mg of protein per ml) fetal calf serum (immunoglobulin free), which was added to protect large T antigen from proteolytic degradation (nucleoplasmic extract). Nuclear structures (NP-40 nuclei), still attached to the substratum, were digested with 100 µg of DNase I (D-18530; Serva) per ml in KM buffer without EGTA and DTT for 15 min at 37°C. The DNase-buffer was then adjusted to 2 M NaCl, 1 mM EGTA, and 5 mM DTT, and the incubation was continued for a further 30 min at 2°C (chromatin extract). Identical results were obtained when NP-40 nuclei were digested with DNase I for 30 min at 32°C or for 60 min at room temperature. Finally, residual nuclear structures (nuclear matrices) were solubilized in TK buffer (40 mM Tris hydrochloride [pH 9.0], 25 mM KCl, 5 mM DTT, 10% glycerol) containing 1% Empigen BB (Albright & Wilson, Ltd.) for 60 min at 2°C (nuclear matrix extract). All buffers contained 30 µg of aprotinin (200 kIU; Trasylol; Bayer). Immediately after fractionation, phenylmethylsulfonyl fluoride was added to a concentration of 1 mM, and all extracts were adjusted to 150 mM NaCl and pH 9.0. In addition, 2% NP-40 was added to the nuclear matrix extract.

Immunoprecipitation and SDS-PAGE. The nuclear extracts, as well as whole-cell lysates, obtained from two 9-cm dishes (Nunc) of SV40-infected confluent TC7 cells were cleared in a Sorvall centrifuge (30 min, 20,000 × *g*, 1°C). All extracts were then diluted 1:5 with lysis buffer. Large T

antigen was immunoprecipitated by using 10 µl of monoclonal antibody PAb 108 (12) ascites fluid and 200 µl of settled protein A-Sepharose as described previously (8, 42). Immune complexes bound to protein A-Sepharose were removed by centrifugation at 2,000 × *g* and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (8, 42). The lyophilized immune complexes were dissolved in 50 µl of H₂O containing bromophenol blue, 10% glycerol, and 5% 2-mercaptoethanol. After being boiled for 2 min, 5-µl aliquots were analyzed by SDS-PAGE and Western blotting (immunoblotting).

Western blotting. Western blotting of proteins was performed as described by Burnette (2), with modifications. After SDS-PAGE, the gels were incubated for 10 min in equilibration buffer (0.1% SDS, 0.1 mM DTT, 20 mM Tris acetate [pH 8.3]). Proteins were electroblotted with a Transblot apparatus (Bio-Rad Laboratories) onto nitrocellulose paper (BA 85; Schleicher & Schuell) at 60 V for 2 h. The transfer buffer consisted of 0.1% SDS, 20% isopropanol, and 20 mM Tris acetate (pH 8.3). Large T antigen on nitrocellulose blots was identified by detection with antibody (rabbit anti-T serum [9]) and tritiated protein A (44), followed by fluorography. Briefly, nitrocellulose sheets were incubated on a rocking platform for 30 min in 50% isopropanol, for 15 min in TBS buffer (150 mM NaCl, 5 mM Na₂N₃, 1 mM EDTA, 15 mM Tris hydrochloride [pH 7.8]), and then for 10 to 16 h in buffer G (TBS buffer plus 0.1% gelatin) containing 100 µg of bovine serum albumin (immunoglobulin free) per ml. The nitrocellulose was then washed for 1 h in buffer GT (TBS buffer plus 0.1% gelatin plus 0.1% Tween 20) and immersed for 5 h in buffer GT containing rabbit anti-T serum (9) (diluted 1:500). Unbound antibodies were removed by washing the sheets in buffer GT. Antibodies bound to large T antigen were detected by adding [³H]protein A (10 µCi). Bands of ³H-labeled immune complexes were visualized by fluorography after enhancement with 20% 2,5-diphenyloxazol (PPO) in toluol, followed by air drying.

Evaluation of relative amounts of large T antigen. To evaluate the relative amounts of large T antigen obtained from whole-cell lysates and nuclear extracts, 5-µl aliquots of immunoprecipitates were analyzed by SDS-PAGE. The gels were stained with silver (22), large T antigen bands were scanned with a Quick Scan R&D densitometer (Helena Laboratories), and peak areas were determined. In addition, fluorograms of Western blots were scanned as described above, and peak areas were determined. Different exposures for each time course were evaluated. Alternatively, radioactive bands were excised from nitrocellulose sheets, and ³H counts were determined by liquid scintillation analysis. Similar results were obtained with either method. The amounts for each large-T-antigen subclass are presented in arbitrary units.

Extraction and blot hybridization of viral DNA. Viral DNA was extracted from SV40- and *tsA*-infected TC7 cells (one 9-cm dish per time point) by the method of Hirt (15). DNA in the supernatant of the Hirt extract was precipitated with ethanol and suspended in 200 µl of TE buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA). Dot blot hybridization on GeneScreen Plus filters was carried out as described by the manufacturer (New England Nuclear Corp.). Briefly, 40-µl aliquots of DNA samples were adjusted to 0.25 N NaOH (10 min at 2°C) and then diluted with SSC buffer to a final concentration of 0.125 N NaOH and 0.125 × SSC (0.018 M NaCl, 0.0018 M sodium citrate [pH 7.2]). Viral DNA was transferred to a GeneScreen Plus Nylon 66 membrane (New

FIG. 1. Viral DNA synthesis during the course of infection (37°C). TC7 cells were infected with SV40 (MOI of 10) at 37°C. At various times after infection, cultures were analyzed for viral DNA content by Hirt extraction and dot blotting (A) and steady-state levels of SV40 DNA were determined by liquid scintillation counting (B) as described in the text.

England Nuclear) by using a Dot Blot I Minifold apparatus (Schleicher & Schuell) and then hybridized with nick-translated SV40 DNA (specific activity, $>5 \times 10^7$ cpm/ μ g). Nick translation was carried out as described by Maniatis et al. (19). Radioactivity in the blots was determined by autoradiography. To evaluate the amounts of viral DNA, blots were excised and 32 P counts were determined by liquid scintillation analysis.

RESULTS

Subnuclear distribution of large T antigen during the course of the viral replication cycle. To characterize the viral replication cycle under our experimental conditions, TC7 cells were infected with wild-type SV40 at an MOI of 10. At 8, 12, 16, 20, 30, 40, and 50 h postinfection (p.i.), viral DNA was extracted from infected cells as described by Hirt (15) and quantitated by dot blot analysis (see Materials and Methods). The steady-state levels of viral DNA during the course of infection are shown in Fig. 1. A significant increase in the amount of viral DNA was found at 16 h p.i., indicating that under our conditions the viral late phase, marked by the beginning of viral DNA synthesis, started between 12 and 16 h p.i. (see also Fig. 7). Steady-state levels of large T antigen during the course of viral infection were determined in parallel. At each time p.i. two plates of infected cells were extracted with lysis buffer, large T antigen was immunoprecipitated by using the large-T-antigen-specific monoclonal antibody PAb 108 (12), and immunoprecipitates were ana-

lyzed by Western blotting as described in Materials and Methods. The quantitative evaluation of these blots (see Materials and Methods and Fig. 3A) shows a linear increase in the steady-state level of large T antigen up to 40 h p.i.

In a parallel experiment, SV40-infected TC7 cells were subfractionated at 8, 12, 16, 20, 30, 40, and 50 h p.i. by a slight modification of our *in situ* cell fractionation procedure (see Materials and Methods). The NP-40 extract yielding nucleoplasmic large T antigen, the DNase-high-salt extract yielding chromatin-associated large T antigen, and the Empigen BB extract of the residual nuclear matrices yielding nuclear matrix large T antigen (33) were immunoprecipitated for large T antigen. Steady-state levels of nuclear subclasses of large T antigen were analyzed and quantitated as described above. The fluorogram of the Western blot, depicting the subnuclear distribution of large T antigen during the course of infection is shown in Fig. 2. Large T antigen was barely detectable in any subnuclear fraction until 12 h p.i. At that time, i.e., still during the early phase of infection, large T antigen was present mainly in the nucleoplasm and in association with the cellular chromatin. Only a very small amount was found to be associated with the nuclear matrix. During the further course of viral infection, large T antigen accumulated mainly at the cellular chromatin and nuclear matrix. The beginning of the late phase of infection (16 h p.i.; Fig. 1) was marked by a significant increase of nuclear-matrix-associated large T antigen (Fig. 2).

The relative amounts of large T antigen in each subnuclear fraction were quantitatively evaluated, as described in Materials and Methods, from the Western blots shown in Fig. 2 (Fig. 3A). In addition, Fig. 3A shows the steady-state levels of total large T antigen in these cells, obtained from whole-cell lysates in a parallel experiment (see above). It is evident that at each time p.i., *in situ* cell fractionation released about the same amount of total large T antigen as was recovered from whole-cell lysates. Thus, it was possible to analyze the changes in relative mass distribution of large T antigen in each subnuclear fraction during the course of viral infection. The total amount of large T antigen at each time was set at 100%, and the percentage of large T antigen in each subnuclear fraction was determined. The relative mass distribution of nucleoplasmic and nuclear matrix large T antigen changed drastically concomitant with the switch from the early (12 h p.i.) to the late (16 h p.i.) phase of infection (Fig. 3B). At 12 h p.i., approximately 30% of the total large T antigen had accumulated in the nucleoplasm, 60 to 65% was associated with the chromatin, and only about 5% was bound to the nuclear matrix. At 16 h p.i., the relative amount of nucleoplasmic large T antigen had dropped to about 15%, whereas the relative amount of nuclear matrix-associated large T antigen had increased to about 35%. From 20 h p.i., the relative mass distribution of the three subclasses of large T antigen stayed approximately constant during the further course of infection.

Comparison of subnuclear distribution of large T antigen in SV40 wild-type- and *tsA* mutant-infected cells at permissive and restrictive temperatures. The results described above seemed to indicate a correlation between the onset of the late phase of viral replication and the association of large T antigen with the nuclear matrix. To further corroborate this correlation, we compared the subnuclear distribution of large T antigen in SV40 wild-type- and *tsA58* mutant (37)-infected cells at the permissive (32°C) or restrictive (39°C) temperature for the growth of *tsA58* virus (37, 38, 45). TC7 cells were infected with SV40 wild-type or *tsA58* mutant virus at an MOI of 10 at 32°C and kept at 32°C or shifted to

FIG. 2. Subnuclear distribution of large T antigen during the course of infection (37°C). TC7 cells infected with SV40 were subfractionated as described in the text. Nuclear extracts obtained from two dishes (9 cm) of confluent TC7 cells were immunoprecipitated for large T antigen with monoclonal antibody PAb 108. The immunoprecipitates (5- μ l aliquots) were analyzed on 10% SDS-polyacrylamide gels, followed by Western blotting and fluorography as described in the text. Blots of large-T-antigen subclasses at 8, 12, and 16 h p.i. were exposed for 21 days; blots of subclasses at 20, 30, 40, and 50 h p.i. were exposed for 4 days. Nuclear extracts were the nucleoplasmic extract (lanes N), chromatin extract (lanes C), and nuclear matrix extract (lanes NM).

39°C about 2 h after infection. Parallel cultures were analyzed for viral DNA content and subnuclear distribution of large T antigen as described above.

At the permissive temperature (32°C), viral DNA synthesis started between 24 and 36 h p.i. in both wild-type SV40- and *tsA58*-infected cells (Fig. 4A), demonstrating that the course of viral infection proceeded more slowly at 32°C than

at 37°C (40). Lysis of infected cells started at about 100 h p.i. Although cells were infected with wild-type or *tsA* virus at the same MOI, viral DNA replication was less effective in *tsA* virus-infected cells, since these cells at a late time of infection contained only about 50% of the amount of viral DNA found in wild-type-virus-infected cells (Fig. 4A, panel b).

The subnuclear distribution of large T antigen during the

FIG. 3. Quantitative evaluation of large T antigen and large-T-antigen subclasses during the course of infection. (A) Steady-state levels of nuclear subclasses of large T antigen (Fig. 2)—nucleoplasmic (◆), chromatin (●), nuclear matrix (□), and total large T antigen obtained from whole-cell lysates (▲)—were quantitatively evaluated in parallel experiments as described in the text. Amounts of large T antigen are given in arbitrary units (a.u.). (B) Since the same number of cells was analyzed in whole-cell lysates and nuclear extracts, the relative mass distribution could be determined. The amounts of large T antigen (per time point) obtained from whole-cell lysates were designated 100%, and the relative amounts of large T antigen representing the nuclear subclasses were determined: nucleoplasmic (◆), chromatin (●), and nuclear matrix (□).

FIG. 4. Viral DNA synthesis in SV40 wild-type- and *tsA58* mutant-virus-infected cells during the course of infection at 32 and 39°C. TC7 cells were infected with wild-type SV40 or *tsA58* (MOI of 10) at 32°C and either kept at 32°C (A) or shifted to 39°C at about 2 h p.i. (B). (a) At various times after infection cultures were analyzed for viral DNA content by Hirt extraction and dot blotting. (b) Steady-state levels of viral DNA were determined by liquid scintillation counting. Panels A and B were obtained from different hybridizations.

course of infection in wild-type- and *tsA58* mutant-infected cells at the permissive temperature is shown in Fig. 5. Large T antigen exhibited a qualitatively similar distribution during the course of infection both in wild-type- and *tsA* mutant-infected cells. In *tsA* virus infection only about 70 to 80% of wild-type large-T-antigen levels were reached (Fig. 5). As in wild-type-infected cells at 37°C (Fig. 2 and 3A), nuclear matrix association of large T antigen was barely detectable during the early phase of infection (up to 24 h p.i.) but became prominent after the onset of viral DNA replication. During the further course of infection large T antigen accumulated at the chromatin and nuclear matrix.

If cells were kept at the restrictive temperature for *tsA58* mutant virus, no viral DNA was synthesized in *tsA* virus-infected cells (Fig. 4B), whereas viral DNA replication proceeded faster at 39°C than at 32°C in wild-type-infected cells. Viral replication in these cells seemed to be even faster than at 37°C, since lysis of infected cells started at about 44 h p.i., as compared with 65 h p.i. at 37°C. No cytopathic effect was detected in *tsA58*-infected cells kept at the restrictive temperature for as long as 6 days.

At 12 h p.i. wild-type large T antigen exhibited a subnuclear distribution (Fig. 6) typical for cells in the early phase of infection (Fig. 2 and 5), with most large T antigen being associated with the cellular chromatin (approximately 60 to 70%). Again, virtually no large T antigen was found to be

associated with the nuclear matrix at this stage of viral infection. After the switch into the late phase (24 h p.i.), nuclear matrix association of large T antigen again became prominent in wild-type-infected cells. Therefore, the overall subnuclear distribution of large T antigen in wild-type-infected cells at early and late times of infection followed a similar pattern, independent of growth temperature. In addition, the shift in the subnuclear distribution of large T antigen at all growth temperatures strictly correlated with the switch from the early to late phase of viral replication.

Analysis of the subnuclear distribution of large T antigen in *tsA58* mutant-infected cells kept at 39°C (Fig. 6) showed that mutant large T antigen failed to associate with the cellular chromatin at early times p.i. (12 h). After 24 h at the restrictive temperature, chromatin association of mutant large T antigen was still barely detectable, and only a small amount (less than 15%) of mutant large T antigen was present in the nuclear matrix fraction. Thus, most nuclear large T antigen in these cells accumulated in the nucleoplasm. The effect of the restrictive growth temperature on the subnuclear distribution of mutant large T antigen was even more pronounced at 36 h p.i., when more than 95% of nuclear large T antigen was present in the nucleoplasm. At this time, chromatin- and nuclear-matrix-associated large T antigen together accounted for less than 5% of the total large T antigen. This pattern of subnuclear distribution did not

FIG. 5. Subnuclear distribution of SV40 wild-type and *tsA58* large T antigen during the course of infection (32°C). Parallel cultures of SV40 wild-type- and *tsA58*-infected TC7 cells kept under permissive growth conditions (32°C; Fig. 4A) were subfractionated. Nuclear extracts obtained from two dishes (9 cm) of confluent TC7 cells were immunoprecipitated for large T antigen with monoclonal antibody PAb 108. The immunoprecipitates (5- μ l aliquots) were analyzed by SDS-PAGE followed by Western blotting. The blot of large-T-antigen subclasses at 24 h p.i. was exposed for 10 days; the blots of subclasses at 36, 48, 60, and 88 h p.i. were exposed for 2 days. Abbreviations for nuclear extracts are defined in the legend to Fig. 2.

change further in *tsA58* mutant-infected cells kept at the restrictive temperature for as long as 100 h p.i. (data not shown). Experiments similar to those described for SV40 *tsA58* were performed with another SV40 *tsA* mutant, *tsA28* (36), and yielded similar results (data not shown).

Effect of aphidicolin on subnuclear distribution of large T antigen. Our experiments thus far demonstrated a specific accumulation of large T antigen at the cellular chromatin and nuclear matrix of infected cells during the late phase of infection. A likely interpretation of this finding is that these interactions of large T antigen are necessary for specific events in viral replication, since they were not observed in SV40 *tsA* mutant-infected cells kept at the restrictive growth temperature. To preliminarily characterize these interactions, we analyzed whether large T antigen might accumulate at these nuclear structures via binding to viral DNA, since it specifically associates with replicating and transcribing viral chromatin during viral replication (23, 31, 32, 35). The failure to detect large T antigen in the chromatin and nuclear matrix fractions of *tsA* mutant-infected cells at the nonpermissive growth temperature might then have simply reflected the fact that, due to inhibition of viral DNA synthesis, only low quantities of viral chromatin were present in these cells and that large T antigen had lost its ability to bind to viral DNA (45). Alternatively, large T antigen might associate with the cellular chromatin and nuclear matrix mainly via cellular constituents. The latter possibility was suggested by our finding that mutant large T antigen in *tsA58*-infected cells at the restrictive temperature

was unable to associate with the cellular chromatin even at early times p.i., i.e., before the onset of viral DNA replication (Fig. 6). To distinguish between these alternatives, we analyzed the association of large T antigen with the cellular chromatin and nuclear matrix in SV40-infected cells under conditions in which viral DNA replication, and thus accumulation of viral chromatin, was blocked by the addition of aphidicolin, an inhibitor of DNA polymerase α (29). If the association of large T antigen with the chromatin and nuclear matrix is mediated by interactions with cellular constituents rather than by binding to accumulating viral chromatin, it should also occur under these conditions.

Parallel cultures of TC7 cells were infected with wild-type SV40 at an MOI of 10. One culture was treated with 10 μ l of aphidicolin per ml at 14 h p.i.; the other served as a control. After the addition of aphidicolin to the infected cultures, viral DNA synthesis was reduced by about 80% (Fig. 7). Analysis of the subnuclear distribution of large T antigen in both cultures (Fig. 8) demonstrated that blocking viral DNA synthesis with aphidicolin had no detectable effect on the subnuclear distribution of large T antigen. Early in infection (14 h p.i.), large T antigen was present in the nucleoplasm and associated with the cellular chromatin. Only minor amounts of large T antigen were found in the nuclear matrix fraction. After the onset of viral DNA replication the amount of nuclear-matrix-associated large T antigen increased again in both untreated and aphidicolin-treated cultures (Fig. 8, cf. 17 and 19 h p.i.). Therefore, the accumulation of large T antigen at the cellular chromatin and nuclear matrix after the

FIG. 6. Subnuclear distribution of SV40 wild-type and *tsA58* large T antigen during the course of infection (39°C). Parallel cultures of SV40 wild-type- and *tsA58*-infected TC7 cells kept under restrictive growth conditions (39°C; Fig. 4B) were subfractionated. Large T antigen was analyzed as described in the legend to Fig. 5. The blot of subclasses at 12 h p.i. was exposed for 4 days; the blots of subclasses at 24 and 36 h p.i. were exposed for 1 day. Abbreviations for nuclear extracts are defined in the legend to Fig. 2.

switch into the late phase of infection most likely did not result from large T antigen binding to viral chromatin. Therefore, this result strongly indicates that large T antigen in these nuclear subcompartments is associated with cellular rather than viral components.

DISCUSSION

SV40 has long served as a model system for studying cellular DNA replication and transcription because it strictly depends on the cellular DNA replication and transcription machinery for efficient viral replication (for a review, see reference 7). The only viral protein absolutely necessary to support viral replication, large T antigen, aside from a helicase activity in the elongation process of viral DNA replication (30), seems to perform only regulatory functions. This implies that large T antigen at various levels has to modulate cellular processes involved in DNA replication and transcription for the needs of viral replication. It is an attractive but unproven hypothesis that the presence of large T antigen in various subcellular locations in SV40-infected and SV40-transformed cells reflects such regulatory interactions.

In an attempt to correlate the association of large T antigen with nuclear structural systems, especially the cellular chromatin and nuclear matrix, with viral processes during lytic infection, we analyzed the subnuclear distribution of large T antigen in lytically infected monkey cells. Structural systems of the nucleus were analyzed as described in previous studies in which we developed and characterized a method for sequentially extracting cells in situ, leading to the preparation of defined nuclear subfractions (33, 34). Using this method to characterize the association of large T antigen with different structural systems of the nucleus in SV40-transformed cells, we had found that nuclear large T antigen subcompartmented into nucleoplasmic, chromatin-associated, and nuclear-matrix-bound subclasses (33).

FIG. 7. Influence of aphidicolin on viral DNA synthesis. TC7 cells were infected with wild-type SV40 at an MOI of 10. Aphidicolin (10 μ g/ml) was added to the growth medium of one culture at 14 h p.i.; the other culture remained untreated. (A) Viral DNA was extracted from both cultures and analyzed by dot blotting. The upper blot shows the DNA content of untreated cultures; the lower blot shows the corresponding content in aphidicolin-treated cultures. (B) Steady-state levels of viral DNA were determined by liquid scintillation counting. Shown is viral DNA synthesis in untreated (—) and aphidicolin-treated (---) cells. It is noteworthy that the amount of viral DNA at 12 to 14 h p.i. represented input DNA (data not shown).

Application of this method to SV40-infected cells also identified three nuclear subclasses of large T antigen. Detectable levels of these subclasses were already present at 12 h p.i. From then on, the total amount of nuclear large T antigen increased steadily during the course of infection, reaching a plateau at about 50 h p.i. However, the increase in the steady-state levels of large T antigen was different for each nuclear subclass: whereas the amount of nucleoplasmic large T antigen increased only slightly during the course of infection, the amount of both chromatin- and nuclear-matrix-associated large T antigen increased substantially after the switch from the early to late phase of viral infection (Fig. 2 and 3A). This led to a significant change in the relative mass distribution of nucleoplasmic and nuclear-matrix-associated large T antigen between the late early phase (12 h p.i. at 37°C) and the early late phase (16 to 20 h p.i. at 37°C) of infection (Fig. 3B). The proportion of nuclear-matrix-associated large T antigen, which constituted only about 5% of the total nuclear large T antigen at 12 h p.i., rose to about 35% at 16 h p.i., with a concomitant drop in the nucleoplasmic large-T-antigen subclass from 35 to about 15%. It is

FIG. 8. Influence of aphidicolin on subnuclear distribution of large T antigen. In an experiment parallel to the one for which results are shown in Fig. 7, untreated (A) and aphidicolin-treated (B) TC7 cells lytically infected with SV40 were subfractionated. Large T antigen was immunoprecipitated from nuclear extracts and analyzed on 10% SDS-polyacrylamide gels, followed by Western blotting. Abbreviations for nuclear extracts are defined in the legend to Fig. 2.

noteworthy that in wild-type-SV40-infected cells both the increase in the absolute amounts and the shift in the relative mass distribution of the nucleoplasmic and nuclear-matrix-associated subclasses of large T antigen strictly coincided with the switch from the early to late phase of infection, regardless of the growth temperature analyzed (32, 37, or 39°C).

Therefore, it is tempting to hypothesize that these two subclasses play an important role in initiating and maintaining viral replication. The analysis of the subnuclear distribution of large T antigen in SV40 *tsA* mutant-infected cells kept at the permissive growth temperature further supports this hypothesis. *tsA58* mutant large T antigen failed to initiate viral DNA synthesis and also failed to associate with the cellular chromatin and nuclear matrix in cells kept at the restrictive growth temperature, whereas at the permissive temperature viral DNA replication and subnuclear distribution of *tsA* mutant large T antigen followed a similar pattern as in wild-type-SV40-infected cells. This finding not only further stresses the correlation between chromatin and nuclear matrix association of large T antigen and viral replication but also provides additional evidence for a true *in vivo* subcompartmentation of nuclear large T antigen.

A study by Evan and Hancock (10) raised the possibility that certain nuclear oncogene products might be artificially redistributed during nuclear fractionation. Our finding that the subnuclear distribution of wild-type SV40 large T antigen followed a defined pattern during the course of infection regardless of growth temperature and that SV40 *tsA58* mutant large T antigen (differing from the wild-type SV40 large T antigen by only a single amino acid substitution [1]) was distributed strictly in accordance with its functional state renders it very unlikely that large T antigen was artificially distributed into the various subnuclear fractions as a consequence of our cell fractionation.

The subnuclear distribution of large T antigen during the

course of infection reported here is different than the one described previously by Covey et al. (6). These investigators found a decrease rather than an increase in the percentage of nuclear-matrix-associated large T antigen during the course of infection. It is difficult to speculate on an explanation for this discrepancy. However, one obvious difference between our experiments and those performed by Covey et al. (6) was the application of different cell fractionation procedures to obtain the different subclasses of large T antigen. When analyzing the *in vivo* location of nuclear proteins by cell fractionation, one has to be aware that nuclear subfractions can only be operationally defined and the presence of regulatory proteins, like large T antigen, in a certain nuclear subfraction may depend on the fractionation conditions used. Therefore, any definition of nuclear subclasses of large T antigen cannot be based on cell fractionation only but has to be substantiated by other parameters, including the biological and biochemical characterization of the subclasses obtained. We recently addressed this problem in a study aimed at further characterizing the nuclear subclasses of large T antigen in SV40-transformed cells. We were able to demonstrate that these subclasses differ functionally in terms of binding to the SV40 origin of replication-DNA and that the association of large T antigen with the chromatin and nuclear matrix correlates with the expression of the transformed phenotype by SV40 *tsA* transformants (14).

The most striking biological defect of SV40 *tsA58* mutant large T antigen at the restrictive temperature is its inability to specifically interact with regulatory sequences on the viral genome (45). Therefore, we investigated the possibility that the accumulation of large T antigen at the chromatin and nuclear matrix during the course of infection is the consequence of viral DNA accumulating in these nuclear structures and reflects the binding of large T antigen to replicating and transcribing viral chromatin. Although only a small percentage of large T antigen in infected cells has been found to be associated with isolated viral chromatin (23, 32, 35), this interpretation seemed possible since (i) large T antigen might dissociate from viral chromatin during extraction and (ii) a significant proportion of SV40 DNA is not extracted from nuclei by conventional extraction procedures but is tightly bound to the nuclear matrix of infected cells (M. Hiller and W. Depert, manuscript in preparation). However, accumulation of large T antigen at the chromatin and nuclear matrix after the switch into the late phase was not influenced by blocking viral DNA synthesis with aphidicolin, an inhibitor of DNA polymerase α (29). Therefore, this finding strongly suggests a regulatory association of large T antigen with cellular constituents of these structural systems. In this regard, a recent observation by Smale and Tjian (28) may be important. These investigators described evidence for a regulatory interaction of large T antigen with DNA polymerase α during replication of SV40 DNA *in vitro*. Further analyses of the molecular interactions of large T antigen with nuclear structures in SV40-infected and SV40-transformed cells should help to elucidate the molecular mechanism(s) by which large T antigen modulates various cellular processes during lytic infection and cellular transformation.

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