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Nuclear Subcompartmentalization of Simian Virus 40 Large T Antigen: Evidence for In Vivo Regulation of Biochemical Activities

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Simian virus 40 large T antigen (large T) in the early and the late phases of infection differs significantly in its sequence-specific DNA-binding and ATPase activities, indicating that different large-T populations participate in virus-specific events at various stages of the infectious cycle. To further characterize these large-T populations, we have analyzed nuclear subclasses of large T, isolated from their in vivo location, for their biochemical activities. We show that chromatin- and nuclear matrix-associated large-T molecules exhibit different simian virus 40 control region (ORI) DNA-binding and ATPase activities. The association of large T with a certain nuclear substructure, therefore, subcompartmentalizes large-T molecules exerting different biochemical activities. Nuclear subcompartmentalization thus may provide a higher-order level for the regulation of biochemical activities of large T in vivo.

Lytic replication of simian virus 40 (SV40) in monkey cells requires the regulatory action of the SV40 large T antigen (large T). During the early phase of infection, large T affects several host functions, leading to, e.g., the induction of cellular DNA synthesis and the modulation of cellular transcription. Viral replication then proceeds into the late phase, which is defined by the onset of viral DNA synthesis. During this phase large T, in addition, regulates several virus-specific events, such as initiation and maintenance of viral DNA replication, as well as early and late viral transcription (for reviews, see references 7, 28, 42, and 47).

Large T exerts specific biochemical activities necessary for the regulation of viral processes. (i) It binds to nucleotide sequences within the SV40 control region (ORI) (for a review, see reference 28). Specific binding of large T to binding site I is necessary (although not sufficient) for controlling autoregulation of early transcription (27, 29), whereas binding to site II is essential for initiating viral DNA replication (21, 38, 51). (ii) Large T exhibits an ATPase activity which is closely associated with, and indispensable for, the DNA-unwinding function (helicase activity) of large T (for a review, see reference 42). In addition, large T specifically interacts with cellular target molecules to perform its multiple functions in lytic infection. In this regard, the complexing of large T with the cellular proliferation protein p53 (18, 23), DNA polymerase α (10, 26, 40), and the enhancer-binding protein AP2 (24) may be of functional importance. The defined sequence of large-T functions during the course of lytic infection requires that the biochemical activities of large T and its interactions with cellular targets be tightly regulated. It previously has been shown that large T exists in various biochemically defined forms which differ in oligomerization and in complex formation with p53, as well as in various posttranslational modifications (for reviews, see references 26 and 28). Data have been provided demonstrating that these biochemically defined subclasses of large T differ in their biochemical activities (25, 31, 32, 35, 45).

Complex nuclear functions such as DNA replication and transcription are performed in conjunction with structural systems of the nucleus, i.e., the chromatin and the nuclear matrix (for reviews, see references 1 and 48). Therefore, at another level of regulation, large T interacts with target molecules at these structures and, as a consequence, is found to be associated with these structures in transformed cells as well as in lytically infected cells (4, 14, 36, 43, 49). We previously found that, in line with the assumption that these interactions regulate large-T functions, the subnuclear distribution of large T (large T in the nucleoplasm, at the cellular chromatin, and associated with the nuclear matrix) follows a defined pattern during the course of viral infection (36).

To get further insight into the regulation of large-T functions, we have analyzed in vitro biochemical activities of large T (ORI DNA binding toward isolated binding sites I and II, as well as to the wild-type ORI, and ATPase activity) during the early and the late phases of the lytic infectious cycle and have characterized nuclear subclasses of large T, isolated from their in vivo locations, for these activities. We demonstrate that large T extracted at early and late times postinfection (p.i.) differs in its biochemical activities, suggesting that these activities are modulated for the needs of viral replication. Analysis of individual nuclear subclasses of large T for these activities revealed that each nuclear subclass displayed a characteristic profile of biochemical functions. Thus the shift in biochemical activities of large T from the early into the late phase of infection can be explained by the change in subnuclear distribution of large T occurring during the shift from the early phase into the late phase. Our results imply that the different nuclear subclasses of large T participate in different functional activities of large T. We suggest that the interactions of large T with different cellular structures may provide an important mechanism for regulating these activities during lytic replication of SV40.

MATERIALS AND METHODS

Cells and virus. TC7 African green monkey kidney cells (30) were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Monolayer cultures of TC7 cells were inoculated with SV40 strain 776 at an input multiplicity of infection of 10. After an adsorption period of

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2 h, unadsorbed virus was removed and growth medium was added to the cultures.

Whole-cell extraction. Cells were washed three times with phosphate-buffered saline and were then extracted for 30 min at 2°C with lysis buffer (120 mM NaCl, 5 mM dithiothreitol, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 1% Nonidet P-40 (NP-40), 10% glycerol, 10% fetal calf serum [immunoglobulin-free], 1% aprotinin, 50 mM Tris hydrochloride [pH 8.0]).

In situ cell fractionation. A detailed description of the cell fractionation procedure, as well as the characterization of extracts and structures, has been given elsewhere (43, 44). Briefly, monolayer cultures of TC7 cells were washed with KM buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 5 mM dithiothreitol, 10% glycerol, 10 mM MOPS [morpholinepropanesulfonic acid] [pH 6.8]) and were lysed for 30 min at 2°C in KM buffer containing 1% NP-40 and 20% fetal calf serum (nucleoplasmic extract). Nuclear structures, still attached to the substratum, were incubated with 100 μg of DNase I (30 min, 32°C) and then with KM buffer adjusted to 2 M NaCl (30 min, 2°C) (chromatin extract). Finally, the residual protein skeleton was solubilized in a buffer containing 1% Empigen BB (60 min, 2°C) (nuclear matrix extract).

Immunoprecipitation and biochemical assays. All extracts were immediately adjusted to pH 9.0, 200 mM NaCl, and 1% NP-40, were cleared by centrifugation (20,000 × *g*, 30 min, 2°C) and immunoprecipitated for large T with 10 μl of ascites fluid of monoclonal antibody PAb 108 (13) and protein A-Sepharose (4 h, 2°C), and were then subjected to the following biochemical assays.

(i) DNA-binding assay. DNA binding of immunopurified large T was carried out by the method of Hinzpeter et al. (16). Purified immune complexes were washed three times with binding buffer (150 mM NaCl, 1 mM dithiothreitol, 0.5 mM $MgCl_2$, 10 mM MOPS [pH 7.0]). Various amounts of restricted plasmid DNAs containing the complete ORI (pSV/*Hin*dIII DNA) (9) or isolated binding sites I (pON/*Eco*RI-*Sal*I DNA) (33) or II (p1097/*Hin*dIII DNA) (8) in 600 μl of binding buffer were added. Reaction mixtures were incubated for 45 min at 4°C. Bound DNA was separated from free DNA by being washed with a buffer containing 150 mM NaCl–0.5% NP-40–10 mM Tris hydrochloride (pH 7.8) followed by low-salt buffer (10 mM NaCl, 10 mM Tris hydrochloride [pH 7.8]). The two-step elution of bound DNA fragments and of large T and the processing of the eluates for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described previously (37). The absolute amounts of bound SV40 ORI fragments were determined by scanning the silver-stained gels with an Ultroscan densitometer in comparison with restricted marker DNAs of known concentrations electrophoresed on the same gel. The absolute amounts of Coomassie blue-stained large T were determined densitometrically in comparison with bovine serum albumin of known concentrations, as described previously (16).

(ii) ATPase assay. ATP hydrolysis of immunopurified large T was measured as described by Manos and Gluzman (20), with modifications. Briefly, immune complexes were washed two times with a buffer containing 500 mM LiCl–1% NP-40–0.5% mercaptoethanol–100 mM Tris hydrochloride (pH 9.0) and three times with ATPase buffer (75 mM NaCl, 5 mM $MgCl_2$, 0.01% NP-40, 25 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 7.0]). Reaction was started by adding 10 μCi of [γ-^{32}P]ATP (specific activity, 5,000 Ci/mmol)–25 μM unlabeled ATP in 100 μl of ATPase buffer. To determine the specific ATPase activity of large T, three parallel precipitates were incubated for 15, 30, and 45 min at 23°C. After the times indicated, immune complexes were pelleted, and 80 μl of the supernatant was incubated with Norit charcoal solution (7.5% in 50 mM HCl–5 mM H_3PO_4). Charcoal was pelleted, and free $^{32}P_i$ in the supernatant was measured by Cerenkov counting. Background values were determined by analyzing immunoprecipitates from extracts of uninfected TC7 cells and were subtracted. After the assay, large-T immune complexes were eluted from protein A-Sepharose and were subjected to SDS-PAGE.

Western (immuno-)blotting of large T and dot blotting of viral DNA were performed as described previously (36).

RESULTS

Characterization of biochemical activities of large T by using immunocomplexed large T. To compare biochemical activities of large T present in different cellular extracts under standardized experimental conditions, large T was immunoprecipitated with PAb 108 (directed against the extreme N terminus of large T [13]) and protein A-Sepharose. Immunopurified large T then was used for all biochemical assays.

(i) Characterization of ORI DNA-binding activities of large T. We first determined the binding affinities and activities of total large T toward isolated binding sites I and II, as well as toward combined binding sites I and II on the wild-type ORI (see Materials and Methods for descriptions of the respective plasmid DNAs used). TC7 cells lytically infected with SV40 were extracted at 34 h p.i. by whole-cell extraction and were immunoprecipitated for large T. ORI DNA binding was performed by reacting about 0.4 μg of large T with increasing amounts of unlabeled pSV/*Hin*dIII, pON/*Eco*RI-*Sal*I, or p1097/*Hin*dIII DNAs. Bound DNA and reacted large T then were sequentially eluted and analyzed by SDS-PAGE as described previously (16, 37). Absolute amounts of pSV/*Hin*dIII C fragment (containing the wild-type ORI), pON/*Eco*RI-*Sal*I B fragment (containing isolated site I), and p1097/*Hin*dIII C fragment (containing isolated site II) bound by 0.4 μg of large T were subjected to a Scatchard treatment (34), as described previously (15, 16, 37) (Fig. 1). K_d values were obtained as follows: for combined binding sites I and II on the wild-type ORI, about 20×10^{-10} M; for isolated site I, about 50×10^{-10} M; and for isolated site II, about 10×10^{-10} M. These results indicate that large T exhibited affinities in the same order of magnitude for all three DNA fragments analyzed. Scatchard treatment of our binding data also provided the saturating amounts of ORI DNA fragments bound by 0.4 μg of large T. Thus, we were able to determine the percentage of large-T molecules exhibiting specific ORI DNA-binding activity. In repeated experiments, we found that about 45% of the total large-T molecules (calculated per monomers) bound to isolated site I, about 2 to 3% bound to isolated site II, and about 10% bound to combined sites I and II on the wild-type ORI. A detailed analysis of large-T binding to these various SV40 ORI fragments has been published elsewhere (37).

An important consequence of our finding that large T bound to the various ORI DNA fragments with similar affinities is that binding activities of large T can be determined in a one-step binding assay when saturating levels of ORI DNA fragments are used. This is illustrated by the experiment shown in Fig. 2A. A 0.6-μg sample of large T prepared as described above was reacted with the respective DNAs, using saturating levels of DNA. Evaluation of the gels showed that about 38% of the total large-T molecules

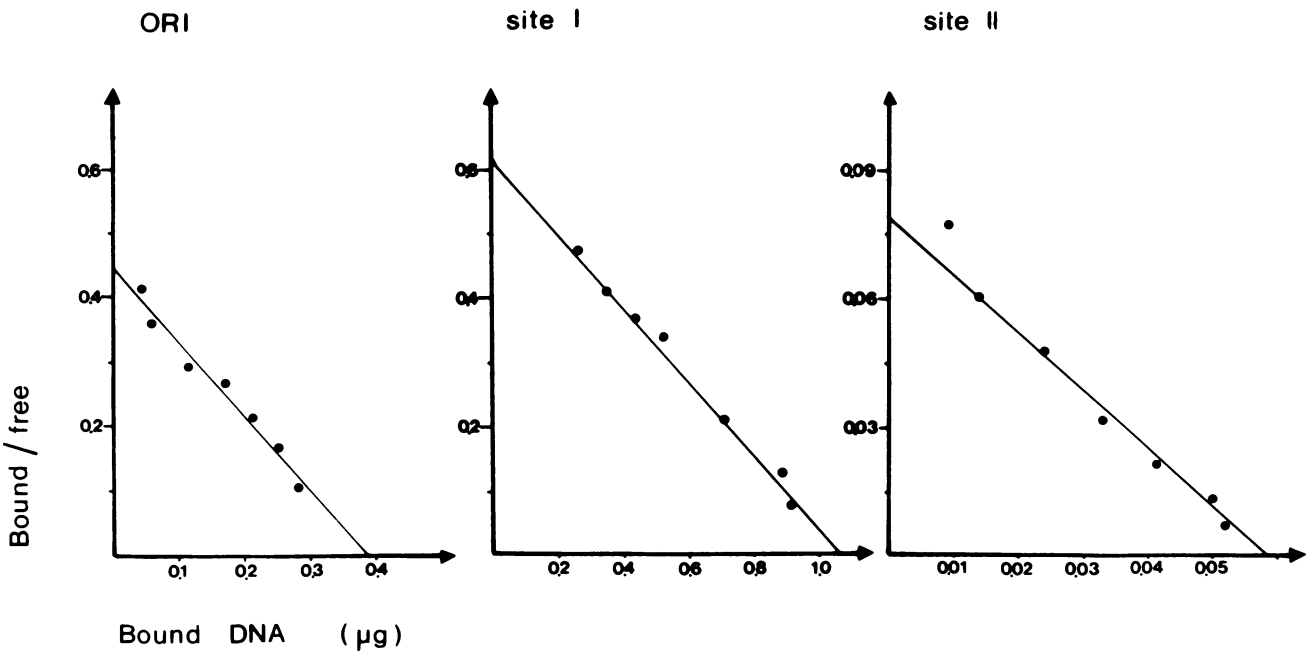

FIG. 1. Scatchard analysis of large-T binding to SV40 ORI DNA fragments. Large T (0.4 μg) was reacted with increasing amounts (1 to 30 μg) of unlabeled pSV/*Hin*dIII DNA (providing the complete ORI), with p1097/*Hin*dIII DNA (1 to 30 μg, providing isolated binding site II), and with pON/*Eco*RI-*Sal*I DNA (5 to 70 μg, providing isolated binding site I), as described in the text. The absolute amounts of ORI DNA fragments bound by large T were determined densitometrically, and the data were subjected to a Scatchard treatment. The ratio of bound to free ORI fragments was plotted against the amount of bound ORI fragments. Least-squares analyses gave straight-line fits with correlation coefficients of 0.97 for the complete ORI, 0.98 for site I, and 0.97 for site II.

exhibited binding activity toward isolated site I, about 3% bound to isolated site II, and about 9% bound to the complete ORI. These data compare well with the DNA-binding activities determined for large T by Scatchard analysis (see above). Therefore, this assay provided a simplified means for the determination of the specific DNA-binding activities of large T.

(ii) Characterization of ATPase activity of large T. To test the large-T intrinsic ATPase activity, we used a modification of the ATPase assay described by Manos and Gluzman (20), as described in Materials and Methods. Immunocomplexed large T, extracted at 34 h p.i., was subjected to the ATPase assay (i) under standard conditions, (ii) after inhibition of the large-T ATPase activity with monoclonal antibody PAb 204 (5), and (iii) after stimulation of the ATPase activity with poly(dT) (11). At the times indicated, the release of free $^{32}P_i$ was measured as described in Materials and Methods, and after being assayed, large T was processed for SDS-PAGE to determine the amounts of reacted large T. Thus, ATP hydrolysis could be directly related to the amount of reacted large T. Figure 2B shows the ATP hydrolysis of 1 μg of large T under the experimental conditions described above. Large-T-catalyzed ATP hydrolysis was proportional to the incubation time and, therefore, allowed the determination of the specific ATPase activity of large T, which accounted for approximately 1.45 μmol/h per mg of large T under standard conditions. This value is in the same order of magnitude as those described by others (12, 39, 45, 46). Similar results were obtained when large T was analyzed in immune complexes with monoclonal antibody PAb KT3, directed against the carboxy terminus of large T (19; data not shown). Therefore, immobilization of large T at its N or C terminus had no detectable effect on its ATPase activity. The addition of poly(dT) stimulated ATPase activity of large T about three- to fourfold, whereas its activity was reduced by about 90% in the presence of monoclonal antibody PAb 204 (Fig. 2B). These values also correspond to data published by others (5, 11).

Comparison of the ORI DNA-binding and ATPase activities of large T in the early and the late phases of infection. Under our experimental conditions, the viral late phase started at between 12 and 16 h p.i. (Fig. 3A). To characterize biochemical activities of large T at the early and the late phases of infection, we have analyzed immunocomplexed large T prepared from whole-cell extracts at 12 and 34 h p.i.

The amount of large T increases significantly during the course of infection (47). Therefore, to obtain approximately equal amounts of large T at both phases of infection, and thus to be able to directly compare the biochemical activities of early-phase and late-phase large T, about 20 times more cells had to be extracted at 12 h p.i. than at 34 h p.i. Large T was subjected to ORI DNA binding at saturating levels of DNA, and, in a parallel experiment, ATP hydrolysis was measured as described above. Figure 3C shows the DNA-binding activities toward the various ORI DNA fragments exerted by 1 μg of large T, and Fig. 3D presents the specific ATPase activities at the respective times p.i. Characteristic differences were found for large T in the early and the late phases of infection. In repeated experiments we found that large-T molecules extracted at the late phase exhibited decreased binding activities toward the complete ORI (−25%) and toward isolated site I (−15%) compared with large T extracted at the early phase. In contrast, the binding activity toward site II increased during the late phase (+130%). Similarly, the ATPase activity of late-phase large T was considerably higher (+70%) than that of early-phase large T (Fig. 3C and D).

Analysis of biochemical activities of nuclear subclasses of

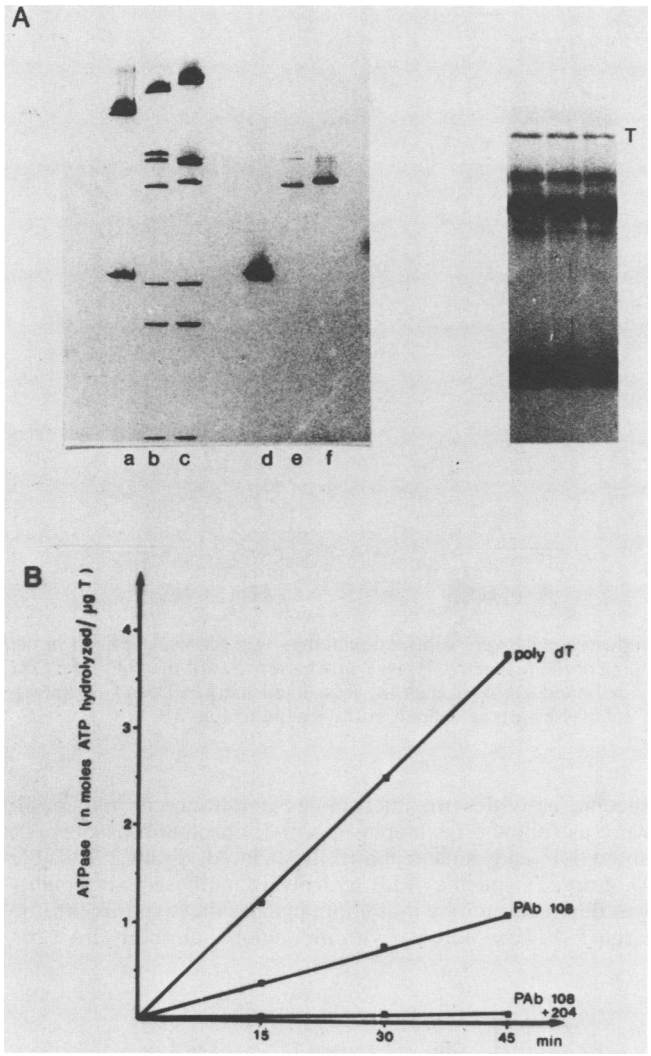

FIG. 2. Characterization of large-T binding to SV40 ORI DNA fragments and of large-T ATPase activity. (A) DNA binding. Large T (0.6 µg per assay) (right panel, from left to right) was reacted with saturating amounts of pSV/*Hin*dIII DNA (ORI), p1097/*Hin*dIII DNA (site II), and pON/*Eco*RI-*Sal*I DNA (site I) as described in the text. Site I (lane d), site II (lane e), and wild-type ORI (lane f) bound DNAs were analyzed by SDS-PAGE and visualized by silver staining. The following restricted marker DNAs were run on the same gel: pON/*Eco*RI-*Sal*I DNA (lane a), p1097/*Hin*dIII DNA (lane b), and pSV/*Hin*dIII DNA (lane c). (B) ATPase assay. Large T (0.6 µg) was assayed for ATPase activity under standard conditions, after being incubated with 10 µl of PAb 204 ascites fluid, and in the presence of 0.1 µg of poly(dT). At 15, 30, and 45 min, the release of free $^{32}P_i$ was determined. ATP hydrolysis is shown for 1 µg of large T.

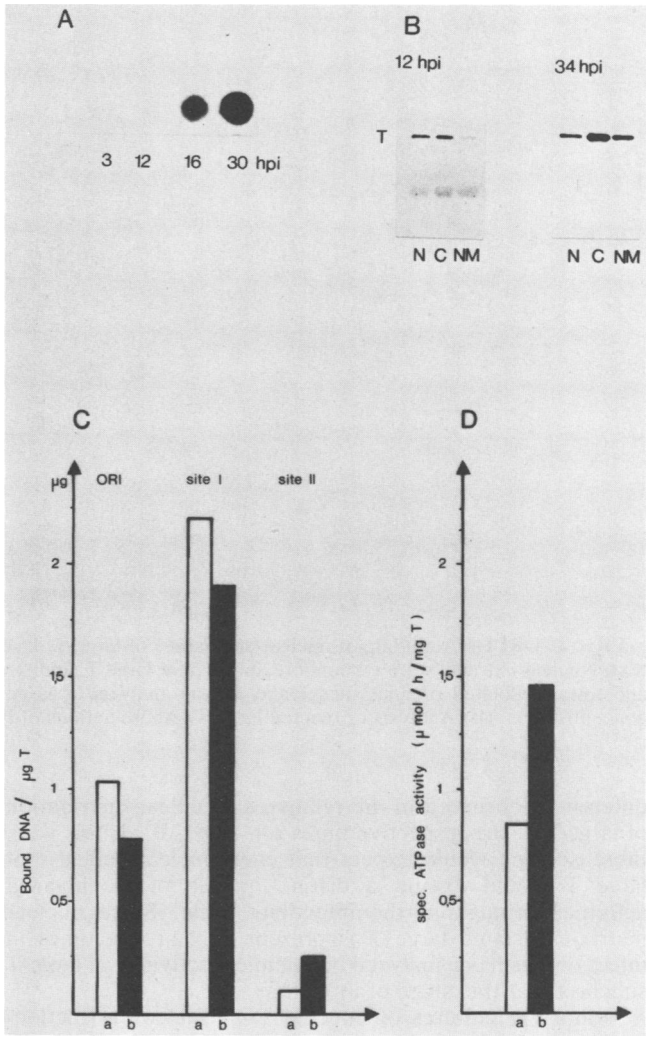

FIG. 3. Comparison of biochemical activities of large T in the early and the late phases of infection. (A) Viral DNA synthesis. Viral DNA was extracted at the indicated times according to the method of Hirt (17), and steady-state levels were determined by dot blotting. (B) Subnuclear distribution of large T. About 4×10^6 cells were subfractionated at 12 and 34 h p.i. as described in the text and were immunoprecipitated for large T. Immunoprecipitates were analyzed by SDS-PAGE, followed by Western blotting and fluorography. Blots of large-T subclasses obtained at 12 h p.i. were exposed for 14 days, and those obtained at 34 h p.i. were exposed for 2 days. Nuclear extracts analyzed were the nucleoplasmic extract (lanes N), the chromatin extract (lanes C), and the nuclear matrix extract (lanes NM). (C and D) Comparison of biochemical activities of large T. About 0.8 µg of large T per assay, obtained by whole-cell extraction at 12 and 34 h p.i., was analyzed for ORI DNA-binding and ATPase activities as described in the text. The relative DNA-binding activities of 1 µg of large T for the complete ORI, for isolated binding site I, and for isolated binding site II (C) and the specific ATPase activities of large T (D) are shown. Values for early-phase large T (12 h p.i., bars a) and for late-phase large T (34 h p.i., bars b) are indicated.

large T. The results described above demonstrate that large T extracted at early or at late times p.i. differs significantly in its biochemical activities in vitro, possibly reflecting its involvement in different viral processes in vivo. Assuming that the biochemical activities of large T are exerted by distinct subpopulations of large T, these results imply that the composition of total large T with regard to these subpopulations changes during the course of infection. We previously have shown that the subnuclear distribution of large T follows a characteristic pattern during the course of infection (Fig. 3B), with a significant change in the relative mass distribution of large T occurring after the switch into the late phase (36). This suggested that the nuclear subclasses of large T perform different functions during viral replication. To support this assumption, we analyzed whether the differences in biochemical activities observed with total large T at early and at late times p.i. (Fig. 3C and D) might reflect the

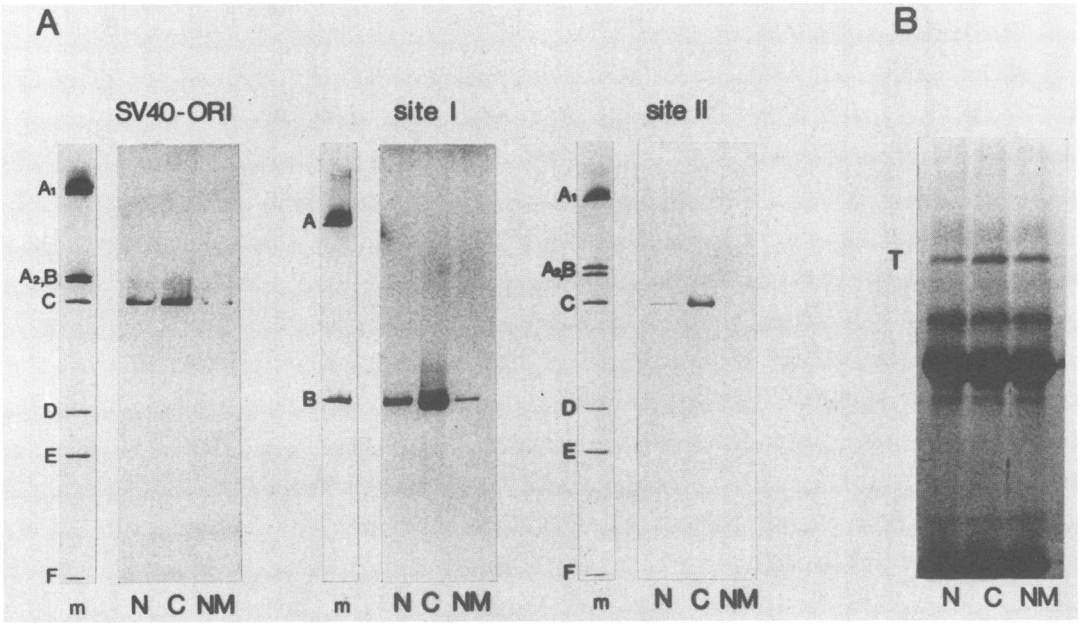

FIG. 4. ORI DNA binding of nuclear subclasses of large T. Lytically infected cells were subfractionated at 34 h p.i. as described in the text. Nuclear extracts were immunoprecipitated for large T, and DNA binding of the respective large-T subclasses toward the wild-type ORI and toward isolated binding sites I and II was analyzed. (A) Analysis of bound DNA. Lanes m, Restricted marker DNAs of known concentrations. (B) Analysis of reacted large T. Abbreviations of large-T subclasses are as defined in the legend to Fig. 3B.

differences observed in the relative subnuclear distribution of large T at the respective times p.i. (Fig. 3B). If this were the case, one would expect that each nuclear subclass of large T would exhibit a distinct profile of biochemical activities throughout the infectious cycle. Since nuclear matrix-associated large T is present only at late times in infection, we have analyzed biochemical activities of large-T subclasses at this stage of infection.

Monolayer cultures of infected TC7 cells were fractionated at 34 h p.i., and nuclear extracts from 8×10^6 cells (nucleoplasmic extract), from 3×10^6 cells (chromatin extract), and from 5×10^6 cells (nuclear matrix extract) were immunoprecipitated for large T. Thus, taking into account the relative mass distribution of large T at 34 h p.i., similar amounts of large T in each subclass could be analyzed.

(i) ORI DNA-binding properties of large-T subclasses. ORI DNA binding of nuclear subclasses was assayed as described above for total large T. Bound DNA fragments and reacted large T in the respective nuclear subclasses are shown in Fig. 4A and B. Gels were quantitatively evaluated as described in Materials and Methods, and the amounts of respective ORI fragments bound by 1 μg of large T were determined (Fig. 5).

Analysis of the relative DNA-binding activities revealed that about 9% of the nucleoplasmic large-T molecules bound to the complete ORI, 20% bound to isolated binding site I, and only 0.8% were able to bind to isolated binding site II. Chromatin-associated large T exhibited a significantly higher DNA-binding activity: about 15% bound to the complete ORI, 60% bound to isolated site I, and about 4 to 5% bound to isolated site II. On the other hand, nuclear matrix-associated large T almost completely lacked ORI DNA-binding activity (Fig. 4A; Table 1).

(ii) ATPase activities of large-T subclasses. ATPase activities of nuclear subclasses of large T were determined as described in Materials and Methods (Fig. 6). The large-T specific activities (in micromoles per hour per milligram) were as follows: nucleoplasmic, 0.7; chromatin associated, about 1.1; and nuclear matrix associated, about 2.7 (Table 1). Large T-specific ATP hydrolysis in these experiments was demonstrated by inhibiting, in a parallel experiment, the large-T ATPase activity with monoclonal antibody PAb 204

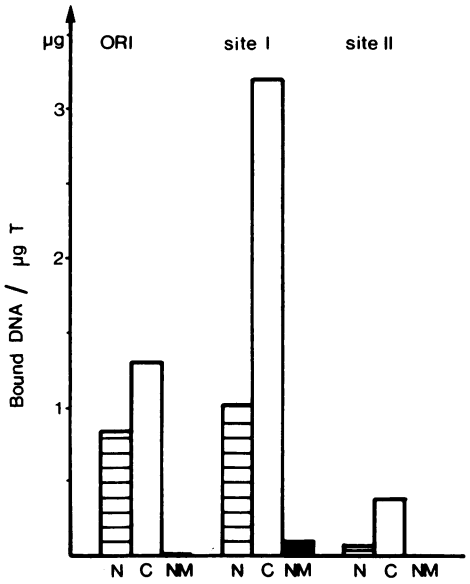

FIG. 5. Quantitative evaluation of ORI DNA binding of large-T subclasses. Absolute amounts of bound ORI DNA fragments and of large T present in the respective subclasses shown in Fig. 4 were quantitatively determined. DNA binding was calculated for 1 μg of large T as described in Materials and Methods. Abbreviations are as defined in the legend to Fig. 3B.

TABLE 1. Comparison of biochemical activities of nuclear subclasses of large T and total large T

T antigen	Activity[a]			
	DNA binding for site:			ATPase
	ORI	I	II	
Subclass[b]				
N	9.3	20.0	0.8	0.7
C	14.5	60.0	4.4	1.1
NM	0.1	1.9	—[c]	2.7
Total				
Calculated[d]	9.4	36.5	2.5	1.5
Experimental[e]	8.7	37.5	2.8	1.45

[a] Specific DNA-binding activity of large T was calculated as the percentage of DNA-binding molecules of large T (calculated for 1 μg of monomers). Specific ATPase activity of large T was calculated as micromoles of ATP hydrolyzed per hour per milligram of large T.

[b] N, Nucleoplasmic; C, chromatin associated; NM, nuclear matrix associated.

[c] —, Not detectable.

[d] Calculation of biochemical activities of total large T from its activities in individual subclasses. At 34 h p.i., approximately 15% of the total large T was found in the nucleoplasm, 55% was associated with the cellular chromatin, and about 30% was bound to the nuclear matrix (Fig. 3B and 4B). Considering this actual proportion of the subclasses relative to total large T and the specific biochemical activities exerted by the individual subclasses of large T, the biochemical activities of total large T were calculated.

[e] Experimental analysis of biochemical activities exerted by total nuclear large T at 34 h p.i. as described in the legend to Fig. 3C and D.

(5) (Fig. 2B). The ATPase activities of all three subclasses then were reduced by about 90% (data not shown). One therefore has to conclude that the nuclear subclasses of large T exhibit different ATPase activities.

Since it is not possible to determine the number of large-T molecules in each fraction exerting ATPase activity, we do not know whether these differences are due to different numbers of large-T molecules in a given subclass exerting the same ATPase activity or whether all large-T molecules in one subclass exhibit a specific activity different from that of another subclass.

(iii) Control experiments. To ensure that the differences observed in the biochemical activities of individual large-T subclasses (Fig. 5 and 6) were not affected by the fractionation conditions employed, we have tested the influence of high salt and Empigen BB on large-T activities as described previously (15).

Lytically infected cells were extracted with lysis buffer (control value), with 2 M NaCl, or with a buffer containing 1% Empigen BB. The corresponding cell lysates were adjusted to immunoprecipitation conditions as for the chromatin or the nuclear matrix extracts (see Materials and Methods), and large T was immunoprecipitated with PAb 108. Then ORI DNA binding toward the complete ORI and toward isolated binding sites I and II and the ATPase activity of immunopurified large T were analyzed. Neither treatment influenced the specific biochemical activities of large T (data not shown).

Another striking argument for our conclusion that individual subclasses of large T exert different biochemical activities was obtained by comparing biochemical activities of total large T obtained by the analysis of large T from whole-cell lysates (Fig. 3C and D) with the activities of total large T calculated from the activities of individual nuclear subclasses (Fig. 5 and 6). By considering the actual subnuclear distribution of large T at 34 h p.i. (Fig. 3B and 4B; 15% nucleoplasmic large T, 55% chromatin-associated large T, and 30% nuclear matrix-associated large T) and the relative biochemical activities exerted by the individual subclasses of large T (Table 1), the biochemical activities of total large T could be determined. These activities added up to approxi-

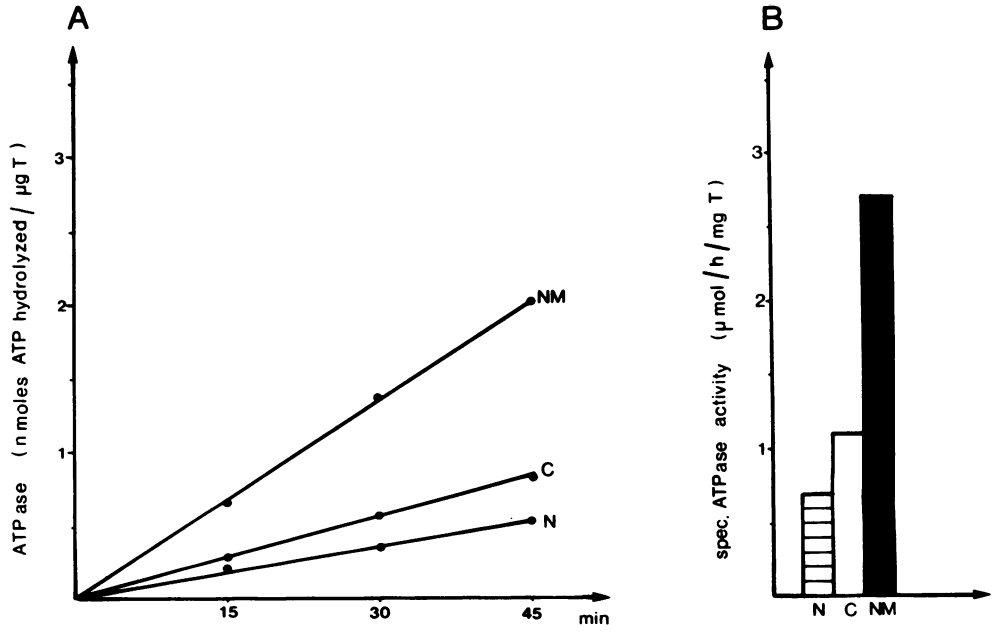

FIG. 6. ATPase activities of large-T subclasses. Lytically infected cells were subfractionated at 34 h p.i. as described in the text, were immunoprecipitated for large T, and were assayed for ATPase activity under standard conditions. (A) ATP hydrolysis was measured at the times indicated and was calculated for 1 μg of reacted large T. (B) The values shown in panel A were given as specific ATPase activities of large T. Abbreviations of large-T subclasses are as defined in the legend to Fig. 3B.

mately the activities determined experimentally for total large T at the same time p.i. (Table 1).

Similarly, assuming the same biochemical activities for large-T subclasses and considering the actual subnuclear distribution of large T at the early phase of infection (Fig. 3B), these activities also added up to the biochemical activities of total large T exerted in the early phase of infection. Thus, the association of large T with nuclear substructures provided nuclear subclasses of large T which exerted distinct biochemical activities.

DISCUSSION

Lytic replication of SV40 is regulated by SV40 large T. Aside from sequence-specific DNA binding (for a review, see reference 28) and from demonstrated ATPase and helicase activities (for reviews, see references 6 and 42), no further biochemical activities have been described for large T so far. Therefore, it is likely that large T performs its regulatory functions indirectly, e.g., by specifically interacting with cellular targets.

Regulatory functions of large T involve the modulation of cellular and viral transcription (for a review, see reference 28) and the induction of cellular DNA synthesis (22). Large T also plays a crucial role in initiation and elongation during viral DNA synthesis (for a review, see reference 42). At least two nuclear structures are implicated as participating in cellular DNA transcription and replication: (i) the chromatin, as the nuclear substructure organizing the DNA, and (ii) the nuclear matrix (for reviews, see references 1 and 48). We recently have shown that in lytically infected cells the subnuclear distribution of large T followed a characteristic pattern during the course of infection (36). During the early phase, large T was present mainly in the nucleoplasm and in association with the cellular chromatin, and only a small fraction was bound to the nuclear matrix. After the switch into the late phase, the relative mass distribution of the three subclasses changed significantly, due to a drastic increase of nuclear matrix-associated large T (Fig. 3B). Considering that large T at the respective stages of infection exerts different functions (for a review, see reference 28), these findings strongly suggested that the in vivo location of large T might play an essential role in the regulation of viral replication.

To obtain further information on the regulation of large-T functions during the lytic cycle, we first compared biochemical activities (ORI DNA-binding and ATPase activities) of total large T, extracted at early and late times of infection. We found significant differences between these large-T preparations (Fig. 3C and D), suggesting that these activities are regulated also in vivo, possibly by the presence of subpopulations of large T. We analyzed the nuclear subclasses of large T, as defined by our in situ cell fractionation protocol, as candidates for such subpopulations. The biochemical analyses of these nuclear subclasses demonstrated a striking correlation between large-T localization and specific biochemical activities. Whereas large-T molecules exhibiting the highest activities for sequence-specific binding to SV40 ORI DNA sequences accumulated at the cellular chromatin, other molecules nearly totally lacking this activity but exhibiting the highest specific ATPase activity accumulated at the nuclear matrix (Fig. 5 and 6B). In comparison with structurally bound large T, nucleoplasmic large T displayed only moderate activities for ORI DNA binding and ATP hydrolysis. This may indicate that this nuclear subclass is not actively involved in viral replication, as was already suggested by our previous finding that large T in SV40 *tsA* mutant-infected cells at the nonpermissive temperature no longer associated with nuclear structures but accumulated in the nucleoplasm (36).

Considering the proportion of large T in each subclass at 34 h p.i. (Fig. 3B) and the DNA-binding and ATPase activities of the respective subclasses (Fig. 5 and 6B), we calculated the actual localization of large-T biochemical activities within the nucleus at late times p.i. We found that about 90% of the large-T molecules able to bind to SV40 ORI DNA sequences were associated with the cellular chromatin. About 40% of the total large-T ATPase activity colocalized with the chromatin subclass, and about 55% colocalized with the nuclear matrix subclass. The finding of a specific subcompartmentalization of biochemical activities of large T within the nucleus suggests a higher-order regulatory mechanism for the control of large-T functions in viral replication in vivo. This is most evident for nuclear matrix-associated large T, the presence of which correlated with the onset of viral DNA synthesis (36). One therefore can assume that this subclass is involved in specific processes during the late phase of viral replication. The finding that this subclass exerts the highest ATPase activity is compatible with the idea that it performs a function in which ATPase activity is necessary. A possible candidate might be the helicase activity of large T, since this activity is closely associated with the large-T-specific ATPase activity (42). Further hints that nuclear matrix-associated large T might regulate specific processes in viral DNA replication were obtained from recent experiments in our laboratory, demonstrating that a significant proportion of replicating viral DNA is also associated with the nuclear matrix (R. Schirmbeck and W. Deppert, manuscript in preparation). Similar colocalizations of T antigens and viral DNA have been observed in other virus systems, too (2, 41, 50), and point to the importance of this nuclear structure for the regulation of virus-specific events.

The predominant biochemical activity exerted by chromatin-associated large T is sequence-specific DNA binding. This corresponds well with large-T functions in cellular and viral transcription as well as in the initiation of viral DNA synthesis (28). Chromatin-associated large T, however, appears to be composed of subpopulations of large T differing in biochemical activities (Fig. 5 and 6B). This complex composition may reflect the complex functions of large T in modulating cellular and viral processes in this nuclear subcompartment. Further studies aimed at isolating the different subpopulations of chromatin-associated large T should yield further information on the functional interactions of large T with chromatin. In this regard it will also be of interest to analyze the molecular mechanisms selecting for large-T molecules associating with different nuclear structures. Posttranslational processes, e.g., oligomerization or specific posttranslational modifications (for a review, see reference 3), might play an important role in mediating the interactions of large T with cellular structures, as well as in determining the functional properties of these large-T populations.

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