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Structural Topography of Simian Virus 40 DNA Replication

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Applying an in situ cell fractionation procedure, we analyzed structural systems of the cell nucleus for the presence of mature and replicating simian virus 40 (SV40) DNA. Replicating SV40 DNA intermediates were tightly and quantitatively associated with the nuclear matrix, indicating that elongation processes of SV40 DNA replication proceed at this structure. Isolated nuclei as well as nuclear matrices were able to continue SV40 DNA elongation under replication conditions in situ, arguing for a coordinated and functional association of SV40 DNA and large T molecules at nuclear structures. SV40 DNA replication also was terminated at the nuclear matrix. While the bulk of newly synthesized, mature SV40 DNA molecules then remained at this structure, some left the nuclear matrix and accumulated at the chromatin.

Complex nuclear functions such as DNA replication and transcription depend on a high level of intranuclear organization for their efficient regulation in eukaryotic cells. As a consequence, such functions are performed in conjunction with structural systems of the nucleus, i.e., the chromatin and the nuclear matrix (for reviews, see references 2 and 48). In accordance, recent data support the hypothesis that eukaryotic DNA replication proceeds in tight association with the nuclear matrix (6, 20, 27). However, the precise relationship between the association of replicating DNA with the nuclear matrix and the specific molecular mechanisms in the process of DNA replication have been difficult to establish because of the complex pattern of replicating genes in eukaryotic cells. The interpretation of the data obtained is further complicated by the fact that variations in nuclear matrix preparation lead to different compositions of the extracted matrices, as well as to different interpretations regarding the specifity of the DNA attachment with this structure (11, 20, 22, 23, 24, 26). To resolve these difficulties, less complex viral systems such as simian virus 40 (SV40) should provide a useful model for probing the intranuclear organization of eukaryotic DNA replication.

Replication of SV40 DNA strictly depends on the enzymatic and regulatory action of the virus-encoded large T antigen, which exhibits specific initiation and unwinding functions (for reviews, see references 8 and 37). All other processes in SV40 DNA replication depend on the host DNA replication machinery but most likely are also modulated by large T for the needs of viral replication. In this regard, formation of complexes of large T with cellular target molecules, such as DNA polymerase α (13, 34) or p53 (for a review, see reference 21), may be of functional importance. At yet another level of regulation, large T interacts with target molecules at the cellular chromatin and the nuclear matrix. As a consequence, large T was found to be associated with these structures in SV40-transformed cells as well as in lytically infected cells (7, 14, 30, 38, 49).

Whereas the basic molecular requirements for SV40 DNA replication have been defined by using in vitro replication assays (12, 18, 25, 40, 47), there is very little information regarding structural cellular requirements in this process. We believe that understanding of such structural requirements might be important for understanding both cellular and large T-dependent mechanisms regulating SV40 DNA replication in vivo. Using the in situ fractionation procedure of Staufenbiel and Deppert (38, 39), we previously showed that the association of large T with these structures follows a defined pattern during lytic infection (30). Furthermore, chromatin- and nuclear matrix-associated large T molecules exhibited different origin of replication (ORI) DNA binding and ATPase activities. This demonstrated that large T molecules exhibiting a specific profile of functions are subcompartmentalized and interact with distinct nuclear substructures (32). These data also suggested that large T interactions with nuclear structures might be important for the regulation of viral replication in vivo. To corroborate this interpretation, we analyzed the subnuclear localization of SV40 DNA in an attempt to demonstrate a functional and coordinated association of both molecules at nuclear structures.

To characterize the subnuclear topography of SV40 DNA replication, we developed an in situ cell fractionation procedure that allows a detailed analysis of the different SV40 DNA replicative intermediates and their in vivo location. Our findings revealed that large T-mediated SV40 DNA elongation as well as termination processes proceed at the nuclear matrix. Newly replicated, mature SV40 DNA molecules then accumulated at the nuclear matrix and the chromatin. Our findings point to a prominent role for nuclear structures in functionally anchoring SV40 DNA and large T molecules during viral replication in vivo.

MATERIALS AND METHODS

Cells and virus. TC7 African green monkey cells and human 293 cells 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.

In vivo radiolabeling of replicating SV40 DNA. Monolayer cultures of TC7 cells (about 10^6 cells) were infected with SV40 as described above. At 36 h postinfection (p.i.), cells were labeled with 200 μCi of [*methyl*-^{3}H]thymidine ([^{3}H]d(Thd) (specific activity, 70 to 85 Ci/mmol; 10 mCi/ml) for the indicated times. In pulse-chase experiments, labeling medium was removed after the labeling period, and the cells were washed with Dulbecco modified Eagle medium supplemented with 50 μM unlabeled dThd. Next, prewarmed

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growth medium containing unlabeled dThd (20 μM) was added, and the cells were incubated at 37°C for the indicated chase periods.

Preparation of cellular extracts. (i) Whole-cell extraction (17). Cells were washed in phosphate-buffered saline and lysed for 5 min at room temperature in TES buffer (1% sodium dodecyl sulfate [SDS], 2 mM EDTA, 20 mM Tris-hydrochloride [pH 7.4]) before being scraped off the dishes.

(ii) Dounce homogenization (41). Cells were washed in a hypotonic buffer containing 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 100 μg of leupeptin per ml, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.8) and scraped off the dishes in the same buffer. The broken cell suspension was agitated in a Dounce homogenizer. The resulting nuclei were separated from the cytoplasm by centrifugation (3,000 × *g*, 5 min) (cytoplasmic extract) and then were incubated in the hypotonic buffer described above for 1 h (4°C). Centrifugation (8,000 × *g*, 10 min) then yielded a supernatant fraction (nuclear extract I) and a pellet consisting of the residual nuclear structures which were lysed in TES buffer as described above (nuclear extract II).

(iii) In situ cell fractionation. The cell fractionation conditions were modified from the in situ cell fractionation protocol of Staufenbiel and Deppert (38, 39). Monolayer cultures were washed with KM buffer (10 mM NaCl, 1 mM $MgCl_2$, 2 mM DTT, 100 μg of leupeptin per ml, 10 mM MOPS [morpholinepropanesulfonic acid] [pH 7.0]) and lysed for 30 min at 2°C in KM buffer containing 0.5% Nonidet P-40 (NP-40) (nucleoplasmic extract). Residual nuclear structures (in situ nuclei), still attached to the substratum, were incubated with non-SV40-cutting restriction endonucleases *Ava*I, *Cla*I, *Mlu*I, *Sal*I, *Xba*I, and *Xho*I (100 U of each per ml) for 30 min at 37°C in restriction buffer (100 mM NaCl, 3 mM $MgCl_2$, 2 mM DTT, 100 μg of leupeptin per ml, 10 mM Tris-hydrochloride [pH 7.4]) and then were extracted with KM buffer adjusted to 1.5 M NaCl (30 min; 2°C) (chromatin extract). The residual nuclear matrices were lysed in TES buffer as described above (nuclear matrix extract).

Extraction and processing of SV40 DNA. Cellular extracts prepared as described above were adjusted to the conditions of TES buffer, and NaCl was added to a final concentration of 1 M. Samples were then incubated for 10 h on ice. Hirt extracts (17) then were centrifuged (20,000 × *g*; 30 min), and the supernatants were treated with protease K followed by extractions with phenol and phenol-chloroform (1:1). SV40 DNA was precipitated with ethanol, dried briefly under a vacuum, and resuspended in TE buffer (0.5 mM EDTA, 10 mM Tris-hydrochloride [pH 7.4]).

Aliquots of SV40 DNA were fractionated on 0.8% neutral agarose gels in running buffer composed of 5 mM sodium acetate, 1 mM EDTA, 0.03% SDS, and 80 mM Tris-hydrochloride (pH 7.5) as described previously (43). Electrophoresis was for 48 h at a constant voltage of 1 V/cm. To detect steady-state levels of viral DNA, we stained gels with ethidium bromide. For fluorographic analysis, gels were dehydrated with acetic acid-methanol (75:25) (4 to 5 h), incubated with 2.5% 2,5-diphenyloxazole (PPO) in acetic acid (12 h), and soaked in water (3 to 5 h). Gels were then dried under vacuum at 50°C and exposed on Fuji X-ray films at −70°C.

Aliquots of SV40 DNA were digested with the restriction endonuclease *Hin*dIII or *Bst*NI according to the supplier's instructions. SV40 DNA fragments were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining as described previously (16, 31), or by fluorography.

Quantitative evaluation of ^{3}H-labeled SV40 form II* DNA. The ^{3}H-labeled SV40 form II + II* DNA band was excised from the agarose gel, denatured in NaOH, and fractionated by electrophoresis in an alkaline agarose gel as described previously (53). The bands on the resulting fluorogram comigrated with single-stranded linear (ssL) and single-stranded circular (ssC) ^{3}H-SV40 DNA standards of denaturated SV40 form II DNA (yielding ssL and ssC) and linearized form III DNA (yielding only ssL). Bands were quantitatively evaluated by densitometry, and the percentage of form II* DNA was calculated as [(ssL − ssC)/(ssL + ssC)] × 100 as described previously (53).

In situ hybridization. In situ nuclei and nuclear matrices were prepared from lytically infected cells as described above, using our standard fractionation conditions. In situ hybridization then was performed using a biotinylated SV40 DNA probe (Enzo Biochem, Inc.). Conditions for hybridization and subsequent detection with Texas red-streptavidin conjugate were as outlined in "Bio-Note 102" (11a). Fluorescence was viewed with a Zeiss photomicroscope II.

In situ SV40 DNA replication. For in situ DNA replication, isolated nuclear structures were prepared from lytically infected cells grown on 5-cm dishes as described in the text. Nuclear structures, still attached to the substratum, were washed in a buffer containing 5 mM K acetate, 0.5 mM $MgCl_2$, 2 mM DTT, and 30 mM HEPES-KOH (pH 7.4) and then incubated with 500 μl of replication buffer (50 mM K acetate, 5 mM $MgCl_2$, 2 mM DTT, 0.05% NP-40, 3 mM ATP, 40 mM creatine phosphate, 25 μg of creatine phosphokinase per ml, 100 μg of leupeptin per ml, 100 μM each UTP, CTP, GTP, dTTP, dATP, dGTP, 20 μM [α-^{32}P]dCTP [20 μCi per dish], 30 mM HEPES-KOH [pH 7.4]). Replication was performed in the presence of nucleosol extract (400 μg of protein per dish) of human 293 cells (see below). Addition of this extract was not a prerequisite for in situ SV40 DNA replication at isolated nuclear structures but increased replication efficiency by a factor of approximately 5 to 10. After the indicated times at 37°C, replication buffer was removed and nuclear structures were lysed with SDS.

Where indicated, in situ nuclear matrices were preincubated with purified mouse control immunoglobulins, mouse anti-large T antibodies from tumor sera, or monoclonal antibody PAb 204 (5) (400 μg per dish) in a buffer containing 5 mM K acetate, 0.5 mM $MgCl_2$, 2 mM DTT, 100 μg of leupeptin per ml, and 30 mM HEPES (pH 7.4) for 20 min at 4°C. The antibodies (100 μg) were also present during the in situ DNA replication assays.

Preparation of nucleosol extract. Nucleosol extracts from human 293 cells were prepared in a manner similar to that of the nucleoplasm preparation during in situ fractionation. The cells were washed in a hypotonic buffer (10 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 100 μg of leupeptin per ml, 20 mM MOPS [pH 7.0]) and then extracted in hypotonic buffer containing 1% NP-40 (30 min, 2°C). Nucleosol extract was separated from nuclear structures by centrifugation (10,000 × *g*; 10 min), passed over a Sephadex G-25 column equilibrated with 50 mM K acetate–30 mM HEPES buffer (pH 7.4), aliquoted, and stored at −70°C.

RESULTS

Characterization of subnuclear distribution of SV40 DNA. (i) Characterization of SV40 DNA replication intermediates. SV40 DNA replication starts on superhelical SV40 form I

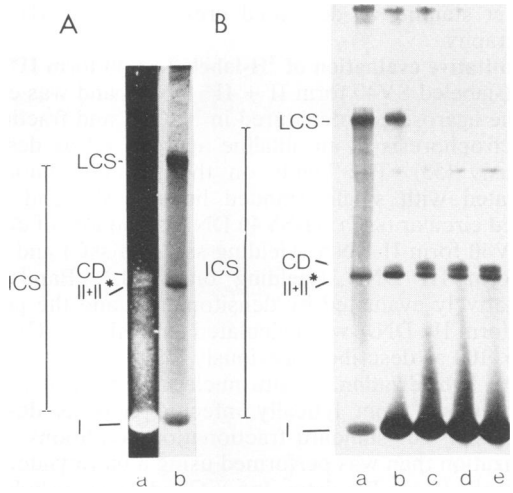

FIG. 1. Analysis of SV40 DNA replication intermediates. (A) TC7 cells were infected with SV40 at a multiplicity of infection of 10. At 36 h p.i., cells were labeled with 200 μCi of [^{3}H]dThd for 5 min. Viral DNA was then extracted by the method of Hirt (17), subjected to neutral agarose gel electrophoresis, and visualized by ethidium bromide staining (lane a) or by fluorography (lane b) as described in Materials and Methods. The positions of SV40 form I DNA (I), SV40 form II + II* DNA (II + II*), catenated dimers (CD), LCS, and ICS are indicated. (B) Lytically infected cells were pulse-labeled with 200 μCi of [^{3}H]dThd at 36 h p.i. for 5 min (lane a), followed by a chase in the presence of unlabeled dThd for 15 min (lane b), 30 min (lane c), 60 min (lane d), and 120 min (lane e). SV40 DNA was extracted from pulse- and pulse-chase-labeled cells by the method of Hirt (17). Aliquots were subjected to neutral agarose gel electrophoresis and visualized by fluorography.

DNA molecules within the ORI. Bidirectional replication from the ORI then generates intermediate Cairns structures (ICS), containing two relaxed loops of nascent DNA, whereas unreplicated parental DNA preserves its superhelical structure. Elongation of nascent DNA chains on ICS proceeds until replication is about 90% completed. At this point replication forks pause, generating the latest Cairns structure (LCS). The separation of the two daughter molecules from the LCS then may occur via two alternative termination pathways. (i) If the remaining parental DNA strands in the LCS were completely unwound during replication, two circular SV40 monomers (SV40 form II* DNA) are separated, each containing a short gap in the nascent DNA strand, localized in the termination region. (ii) If not all helical turns of the parental DNA are removed before the replication forks meet, catenated dimers are formed with the two sibling chromosomes intertwined (for a review, see reference 8).

To characterize the replicative forms of SV40 DNA under our experimental conditions, TC7 cells were infected with SV40 at a multiplicity of infection of 10. At 36 h p.i., cells were labeled for 5 min with [^{3}H]dThd and extracted by the method of Hirt (17). Viral DNA was fractionated by neutral agarose gel electrophoresis (43) and visualized by ethidium bromide staining and fluorography as described in Materials and Methods. Applying these conditions, the mobilities of the various forms of SV40 DNA previously have been characterized in detail (35, 42, 43, 53). The result is shown in Fig. 1A. The most prominent bands in the ethidium bromide-stained gel (lane a), representing steady-state levels of SV40 DNA intermediates, were form I DNA (superhelical), form II + II* DNA (relaxed circles), and catenated dimers (two interlocked SV40 form I molecules [43]). Fluorographic analysis of replicating forms of SV40 DNA (lane b) revealed that labeling of viral DNA for 5 min resulted in radiolabeling of form I DNA, form II + II* DNA, and the LCS, while the majority of the radiolabel was present in the ICS. ICS migrate as a smear between form I and the LCS, since they represent a random population of nascent SV40 DNA at various stages of continued replication (42, 53). Radiolabeled catenated dimers were not detectable.

To analyze the conversion of transient SV40 DNA intermediates during replication into final products, we performed pulse-chase experiments. Lytically infected cells were pulse-labeled with [^{3}H]dThd at 36 h p.i. for 5 min, followed by a chase in the presence of unlabeled dThd. Viral DNA was extracted from pulse- and pulse-chase-labeled cells by Hirt extraction (17) and analyzed as described above. Fig. 1B (lane a) shows the pulse-labeled SV40 DNA intermediates as described in Fig. 1A. During the chase period (Fig. 1B, lanes b to e), radiolabeled ICS significantly decreased within 15 min, accompanied by a concomitant increase of radiolabel in form I molecules. LCS molecules also behaved as expected for transient intermediates in the conversion of ICS in form I and completely disappeared within 30 min. The form II + II* pool was analyzed for form II* DNA content as described by Weaver et al. (53) (see Materials and Methods) and contained about 95% of form II* DNA at the beginning of the chase period (lane b). After 30 min (lane c), form II* DNA decreased to 5 to 10%, thus identifying these molecules as transient intermediates, too. Following the course of catenated dimers, only 0.5 to 1% of catenated dimers were detectable after a 15-min chase. This form accumulated during a further 15-min chase up to about 2% and from then on remained approximately constant during a 2-h chase (lanes b to e). These findings demonstrate that SV40 DNA replication, with regard to termination, in its majority proceeded from LCS via form II* molecules. Thus, these data are in line with the proposed termination pathway of Weaver et al. (53), who showed that under physiological conditions the major termination pathway proceeds via gapped form II* circles into mature form I DNA and that catenated dimers, representing only a minor SV40 DNA population, are not significantly involved in the replication of SV40 DNA.

(ii) Subnuclear distribution of SV40 DNA intermediates. Previous analyses in our laboratory demonstrated biochemical and functional differences for SV40 large T associated with different structural systems of the nucleus in SV40-infected (30, 32) and transformed cells (9, 10, 15, 29), strongly supporting the hypothesis that the various functions of large T in lytic infection and cellular transformation are subcompartmentalized. To correlate the analysis of subnuclear location of SV40 DNA with a functional analysis of large T subclasses, we wanted to apply an in situ fractionation protocol similar to that in our previous studies (38, 39). In addition, in situ fractionation conditions should preserve the structural integrity of the nuclear substructures prepared.

To characterize our in situ fractionation protocol with regard to SV40 DNA extraction, we first compared the initial step of this extraction, i.e., NP-40 lysis of the cells (38), with the method of Su and DePamphilis (41), a method generally used to isolate nuclei and to prepare viral chromosomes. For a detailed description of the extraction conditions, as well as of the precipitation of SV40 DNA from the respective extracts, see Materials and Methods. Lytically infected cells

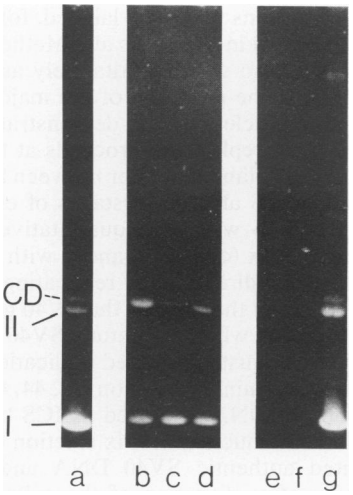

FIG. 2. Analysis of infected cells for soluble and structurally bound SV40 DNA. At 36 h p.i., lytically infected cells were either Hirt extracted (lane a) or subfractionated as described in the text by using Dounce homogenization (lanes b to d) or in situ fractionation (lanes e to g). Viral DNA was precipitated from the respective cellular fractions and analyzed by neutral agarose gel electrophoresis and ethidium bromide staining. Cellular extracts are defined as described in the text. Lanes: a, total cellular Hirt extract; b, cytoplasmic extract; c and f, nuclear extract I; d and g, nuclear extract II; e, nucleoplasmic extract.

were scraped off the culture dishes in a hypotonic buffer (pH 7.8) and agitated in a Dounce homogenizer. The cytoplasmic fraction was separated from the resulting nuclear structures by centrifugation and contained about 30% of viral DNA (Fig. 2, lane b). The nuclei then were incubated in hypotonic buffer for 1 h, releasing a further 30% of viral DNA (Fig. 2, lane c). This extract previously was referred to as "nuclear extract" (41). However, disrupting the residual nuclear pellet with SDS revealed an additional 30% of viral DNA not extractable from the nuclei by applying these conditions (Fig. 2, lane d). In contrast, in situ lysis of the cells, i.e., of cells still attached to the culture dishes, with a similar hypotonic buffer (pH 7.0) supplemented with 0.5% NP-40 resulted in a drastically different pattern of extracted SV40 DNA (Fig. 2, lanes e to g). During the initial cytoplasmic-nucleoplasmic extraction, as well as during incubation of residual in situ nuclei with hypotonic buffer (without NP-40), less than 1% of SV40 DNA was released, whereas the residual nuclei contained about 99% of total SV40 DNA when compared to total SV40 DNA from a Hirt extract of unfractionated cells (Fig. 2, lanes a and g). Thus, the comparison of both extraction procedures showed significant differences in the release of SV40 DNA from nuclei of lytically infected cells. Since structurally bound large T, like SV40 DNA, leaked out of the nuclei when the cells were scraped off the culture dishes and Dounce homogenized (data not shown), we interpret this finding to indicate that this procedure damages the integrity of the nuclei.

To argue against a preparational artifact in the retention of SV40 DNA intermediates in nuclear structures during preparation of in situ nuclei, lytically infected cells were labeled at 36 h p.i. with [^{3}H]dThd and extracted with various modified lysis buffers. These experiments, summarized in Fig. 3, demonstrated that neither adjustment of the hypotonic buffer to pH 8.0 (lanes c) nor omission of Mg^{2+} (lanes d) nor addition of 120 mM NaCl to the initial buffer to generate isotonic lysis conditions (lanes e) had any effect on the qualitative and quantitative association of total SV40 DNA (Fig. 3A) and of replicating SV40 DNA (Fig. 3B) with nuclear structures when compared with our standard conditions for nucleoplasm extraction (see Materials and Meth-

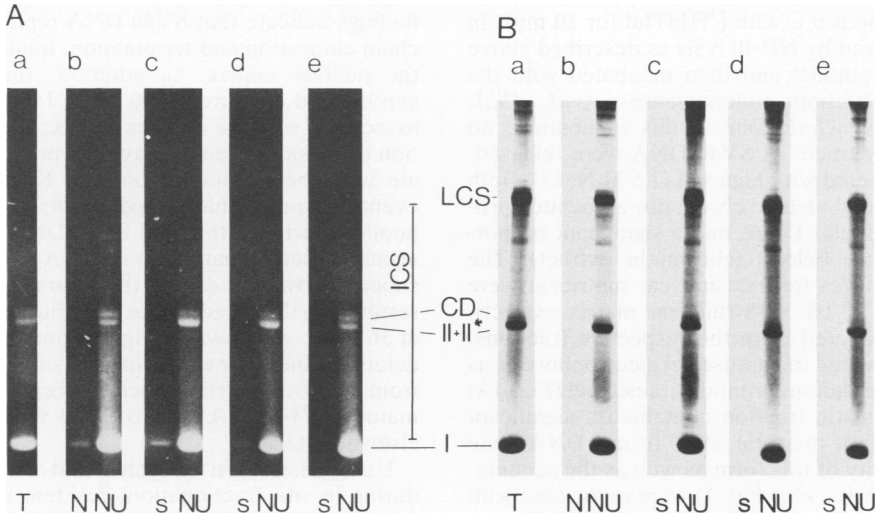

FIG. 3. Characterization of structurally bound SV40 DNA. Lytically infected cells were labeled with 200 μCi of [^{3}H]dThd at 36 h p.i. for 10 min. Parallel dishes were either Hirt extracted (lanes a) or in situ fractionated by using our standard NP-40 lysis of the cells (lanes b) as described in Materials and Methods. In addition, subfractionation was done under modified conditions during the initial NP-40 lysis: adjustment of the lysis buffer to pH 8.0 (lanes c), omission of $MgCl_2$ (lanes d), or addition of 120 mM NaCl (lanes e) to the lysis buffer. Residual nuclear structures were lysed as described in Materials and Methods. SV40 DNA was precipitated from the respective fractions, and aliquots were applied to agarose gels for electrophoresis, followed by ethidium bromide staining (A) or by fluorography (B) of the gels. Nuclear extracts are defined as described in the text: lanes T, total cellular extract; lanes N, nucleoplasmic extract prepared by our standard fractionation procedure; lanes s, soluble nuclear extracts prepared by modified procedures; lanes NU, residual nuclear extracts. SV40 DNA intermediates are marked as defined in the legend to Fig. 1.

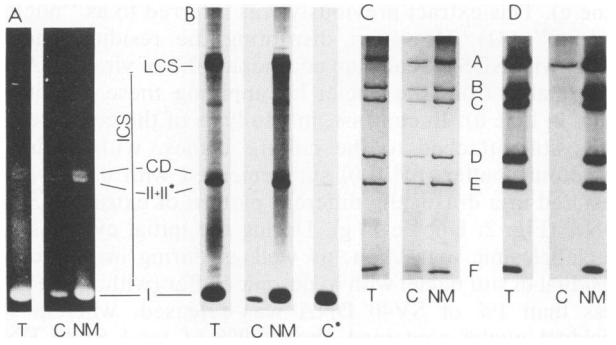

FIG. 4. Subnuclear distribution of mature and replicating SV40 DNA. Lytically infected cells were labeled with 200 μCi of [^{3}H]dThd at 36 h p.i. for 10 min, and in situ nuclei were prepared by our standard NP-40 lysis as described in Materials and Methods. In situ nuclei then were either lysed with SDS (lanes T) or subfractionated into chromatin (lanes C) and nuclear matrix (lanes NM) fractions as described in the text. Nuclear extracts were precipitated for SV40 DNA, and aliquots were subjected to agarose gel electrophoresis, followed by ethidium bromide staining (A) or by fluorography (B). Lane C* represents a long exposure of lane C. SV40 DNA intermediates are marked as defined in the legend to Fig. 1. Aliquots of SV40 DNA from the various nuclear fractions were digested with *Hin*dIII and analyzed by SDS-PAGE, followed by silver staining (C) or by fluorography (D).

ods) (Fig. 3, lanes b). We therefore conclude that nuclear structures, i.e., constituents of the cellular chromatin and the nuclear matrix, play an important role in anchoring SV40 DNA during replication.

To be able to analyze the specific interactions of SV40 DNA with these structures by in situ cell fractionation, DNase treatment of isolated nuclei, used in our standard fractionation procedure to relax the cellular chromatin (38), was replaced by treating in situ nuclei with restriction endonucleases that do not cut SV40 DNA. Lytically infected cells were labeled at 36 h p.i. with [^{3}H]dThd for 10 min. In situ nuclei were prepared by NP-40 lysis as described above (see Materials and Methods) and then incubated with the non-SV40-cutting restriction endonucleases *Ava*I, *Cla*I, *Mlu*I, *Sal*I, *Xba*I, and *Xho*I. During this incubation, no cellular DNA and only traces of SV40 DNA were released. Nuclei then were extracted with high salt (1.5 M NaCl). Both fractions were pooled and yielded chromatin-associated proteins, about 10% of cellular DNA, and a significant proportion of viral DNA (see below) (chromatin extract). The residual nuclear structures (termed nuclear matrices) were lysed by the addition of 1% SDS (nuclear matrix extract). Viral DNA was precipitated from the respective fractions, and aliquots were subjected to agarose gel electrophoresis as described above. The ethidium bromide-stained gel (Fig. 4A) showed that the chromatin fraction contained a significant proportion (10 to 15%) of the total SV40 form I DNA (lane C), whereas the majority of this form as well as the accumulated catenated dimers (see also Fig. 1) were associated with the nuclear matrix (compare lane T with lane NM). Fluorographic analysis (Fig. 4B) revealed that the chromatin fraction also contained a small proportion of radiolabeled SV40 DNA, in its majority consisting of SV40 form I DNA (lanes C and C*), whereas the majority of newly replicated SV40 form I DNA as well as all of SV40-terminating LCS were associated with the nuclear matrix (compare lane T with lane NM). Association of form II* DNA molecules with the nuclear matrix was determined by comparing nuclear matrix and total Hirt fractions of cells labeled for 3 min with [^{3}H]dThd, as described in Materials and Methods. We found that form II* DNA also was quantitatively associated with this structure. Thus, the presence of the major termination intermediates at the nuclear matrix demonstrates that termination of SV40 DNA replication proceeds at this structure. Furthermore, the radiolabeled smear between form I and the LCS, representing ICS at various stages of elongation (42, 53), indicates that ICS were also quantitatively associated with the nuclear matrix (compare lane T with lane NM).

In a population of bidirectionally replicating ICS, radiolabel is found distributed throughout the SV40 genome during brief labeling periods, whereas mature SV40 form I DNA molecules which have just completed replication contain the ^{3}H label only in the termination region (28, 44, 45). To ensure that the radiolabeled DNA identified as ICS by agarose gel electrophoresis in the nuclear matrix fraction (Fig. 4B, lane NM) represented authentic SV40 DNA and not cellular DNA copurifying after digestion of the cellular chromatin with restriction endonucleases (see above) and that the radiolabeled SV40 DNA in the chromatin fraction consisted of newly terminated mature SV40 DNA molecules, aliquots of SV40 DNA from the respective nuclear fractions were digested with *Hin*dIII and analyzed by SDS-PAGE. The silver-stained gels (Fig. 4C) revealed the characteristic band pattern of *Hin*dIII-restricted SV40 DNA. Fluorographic analysis of the gels (Fig. 4D) demonstrated a uniform labeling of the SV40 *Hin*dIII fragments in the nuclear matrix fraction (lane NM), identifying the radiolabeled SV40 DNA molecules as ICS. Furthermore, in comparison with total Hirt-extracted SV40 DNA prepared from unfractionated cells (lane T), this analysis revealed that ICS were quantitatively associated with the nuclear matrix (compare lane T with lane NM). In contrast, the chromatin-associated SV40 DNA molecules (lane C) contained the ^{3}H label only in the termination region (SV40 *Hin*dIII-A fragment), thus demonstrating that only newly synthesized, mature form I DNA was released into the chromatin fraction. In summary, these findings indicate that SV40 DNA replication, with regard to chain elongation and termination, quantitatively proceeds at the nuclear matrix. In addition, the majority of newly synthesized, mature SV40 form I DNA was found to be associated with the nuclear matrix, whereas a small proportion of these molecules leave the nuclear matrix and associate with the cellular chromatin. Kinetic analysis of these events by pulse-chase experiments revealed that the small pool of newly synthesized SV40 DNA directly reflected the accumulation of mature SV40 DNA in the respective nuclear subcompartments during the course of lytic infection and resulted in the steady-state distribution of total SV40 DNA at 36 h p.i. as shown in Fig. 4A and C (data not shown). A detailed kinetic analysis of the subnuclear events leading from nuclear matrix-associated replicating SV40 DNA to mature SV40 DNA and to SV40 virions will be published elsewhere (32a).

Using restriction enzymes that do not cut SV40 DNA during in situ fractionation, residual nuclear matrices contained the bulk of the cellular DNA. To exclude the possibility that replicating SV40 DNA might be associated with the remaining cellular DNA rather than with the residual protein skeleton of the nuclei, we removed the cellular DNA by repeated restriction endonuclease digests and high-salt extractions. Figure 5A shows that nuclear matrices generated by the first restriction enzyme–high-salt treatment contained about 90% of cellular DNA (NM-1). Since high-salt treatment of nuclear structures removes the histones from

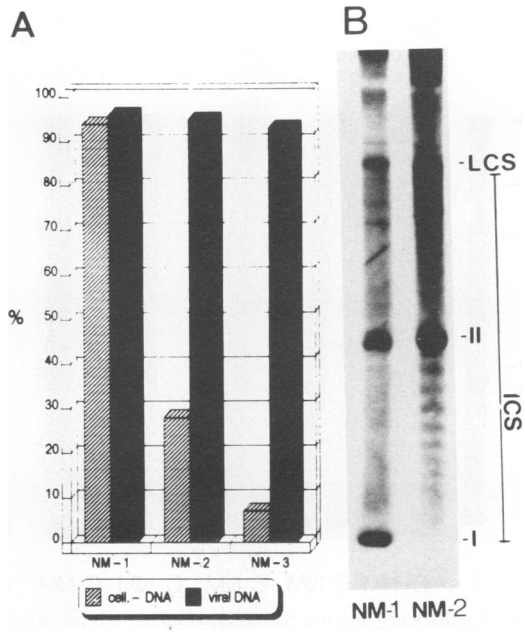

FIG. 5. Quantitative analysis of cellular and viral DNA bound to the nuclear matrix. (A) Monolayer cultures of TC7 cells were labeled with [^{3}H]dThd for 6 h and either lysed directly in a buffer containing 3% SDS and 62.5 mM Tris-HCl (pH 6.8) (control value) or subfractionated as described in the text, yielding our standard in situ nuclear matrices (NM-1). Restriction digest-1.5 M NaCl treatment was repeated once, or twice, on isolated nuclear matrices prepared in parallel (during these incubations, the restriction enzyme concentration was increased to 200 U/ml and the incubation time was raised to 2 h), leading to NM-2 and NM-3 nuclear matrices, respectively. Nuclear matrices then were lysed and analyzed for remaining cellular DNA by liquid scintillation analysis. In a parallel experiment, lytically infected cells were labeled with [^{3}H]dThd at 36 h p.i. for 15 min and subfractionated as described above. Viral DNA was extracted from the residual nuclear matrices by the method of Hirt and precipitated from the respective fractions. SV40 DNA was digested with *Hin*dIII and analyzed by SDS-PAGE, followed by fluorography and densitometric evaluation of the ^{3}H label. The percentage of SV40 DNA remaining at nuclear matrices is shown. (B) SV40 DNA extracted from nuclear matrices NM-1 and NM-2 prepared as described above was subjected to agarose gel electrophoresis, followed by fluorography. SV40 DNA intermediates are marked as defined in the legend to Fig. 1.

the chromatin, cellular DNA now becomes more accessible to restriction enzymes. Thus, the following restriction enzyme–high-salt treatments resulted in the removal of cellular DNA down to about 10% (see lanes NM-2 and NM-3). However, repeated extraction of cellular DNA did not significantly influence the quantitative association of replicating SV40 DNA with the nuclear matrix (compare cellular DNA-rich nuclear matrices [NM-1] and cellular DNA-depleted nuclear matrices [NM-3]). Considering the stringency of the extraction conditions in this experiment, this finding further indicates that replicating SV40 DNA is tightly bound to the residual protein skeleton of the nuclear matrix.

Analysis of nuclear matrix-associated replicative SV40 DNA forms from the above experiment by agarose gel electrophoresis after the first restriction enzyme–high-salt treatment showed the characteristic band pattern of briefly labeled SV40 DNA (Fig. 5B, lane NM-1). However, repeated extraction by restriction enzyme–high-salt treatment generated topological relaxed isomers (topoisomers) from newly replicated SV40 form I DNA (Fig. 5B, lane NM-2). Since such topoisomers were not detectable after the first treatment, the observed relaxation of superhelical SV40 form I DNA must have occurred during the following extractions. Further experiments demonstrated that this relaxation also occurred during incubation of NM-1 matrices with restriction buffer alone (data not shown). We conclude that cellular enzymes, tightly associated with SV40 DNA and/or immobilized at residual nuclear matrix structures, became active during incubation of histone-depleted matrices. Possible candidates for such enzymes are DNA topoisomerases, which are actively involved during unwinding and supercoiling of DNA (for a review, see reference 51). Since almost quantitative extraction of cellular DNA did not alter the quantitative association of replicating SV40 DNA at the nuclear matrix (Fig. 5A), for further experiments, we decided to characterize the interactions of SV40 DNA with cellular DNA-rich nuclear matrices (NM-1).

(iii) Control experiments. We next wanted to analyze the interaction of SV40 DNA with nuclear structures in terms of a functional characterization of replicating SV40 DNA. As a prerequisite, we first tested the effects of temperature, Mg^{2+}, and high salt during nuclear matrix preparation, since these parameters were reported to influence the integrity of nuclear structures as well as the extractability of viral DNA (3, 4, 20, 26, 52).

The results obtained with a wide spectrum of extraction conditions are summarized in Fig. 6. In comparison with our standard fractionation protocol (see Materials and Methods) (lanes b), we found that carrying out the restriction digest at 4°C for 2 h (lanes c) or modifying the initial restriction buffer by omission of $MgCl_2$ (lanes d) or by reduction of NaCl (from 100 to 10 mM) and $MgCl_2$ (from 3 to 0.5 mM) (lanes e) did not significantly alter the subnuclear distribution of total SV40 DNA (Fig. 6A) and of replicating SV40 DNA (Fig. 6B). We further tested the effect of extracting chromatin proteins without prior relaxation of the cellular DNA loops (i) by directly incubating in situ nuclei in a hypotonic buffer (pH 7.4) supplemented with 20 mM lithium diiodosalicylate (20 min, 4°C), a procedure generally used to remove chromatin-associated proteins under low-salt conditions (19, 26) and (ii) by directly incubating in situ nuclei with 1.5 M NaCl (20 min, 4°C). We found that lithium diiodosalicylate extraction resulted in a subnuclear distribution of SV40 DNA (Fig. 6A and B, lanes f) similar to that obtained from nuclease-digested cells. However, direct incubation of in situ nuclei with 1.5 M NaCl resulted in a significant increase of SV40 form I DNA in the chromatin fraction. In contrast, elongating ICS and terminating LCS and form II* SV40 DNA intermediates still remained quantitatively associated with the nuclear matrix even under these drastic extraction conditions (Fig. 6A and B, lanes g).

In summary, our results show that, using a wide spectrum of extraction conditions during in situ preparation of nuclei (Fig. 3) and of nuclear matrices (Fig. 6), the specific interactions of mature and replicating SV40 DNA with nuclear structures are only minimally affected by various conditions, including varying salt and Mg^{2+} concentrations, pH, and temperature. We interpret these findings to indicate that leaving nuclear structures attached to the substratum, i.e., avoiding mechanical forces, preserves their structural integrity and subsequently renders them highly resistant against extraction-induced artifacts. A further hint for the specific interaction of SV40 DNA with nuclear structures was obtained by analysis of structurally bound SV40 DNA by in situ hybridization. Figure 7 shows a very similar pattern of

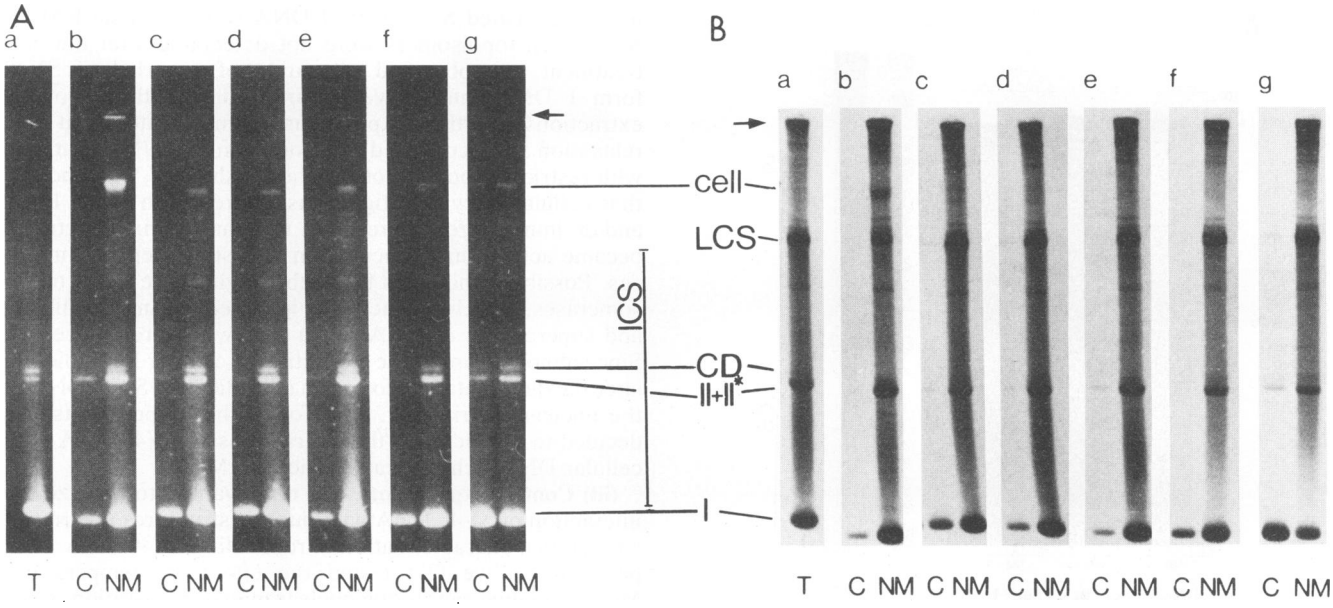

FIG. 6. Characterization of in situ cell fractionation conditions. Lytically infected cells were labeled with [^{3}H]dThd at 36 h p.i. for 10 min, and in situ nuclei were prepared as described in Materials and Methods. In situ nuclei were either lysed with SDS (lanes a) or subfractionated as described in Materials and Methods, using our standard fractionation conditions (lanes b). Alternatively, in situ nuclei on parallel dishes were subfractionated by performing the restriction digest for 2 h at 4°C (lanes c), or omitting $MgCl_2$ during the restriction digest (lanes d), or performing the restriction digest under hypotonic conditions (lanes e). Furthermore, in situ nuclei were directly extracted in a hypotonic buffer supplemented with 20 mM lithium diiodosalicylate for 20 min at 4°C (lanes f) or directly extracted with 1.5 M NaCl (lanes g). SV40 DNA was precipitated from the respective fractions, and aliquots were subjected to agarose gel electrophoresis followed by ethidium bromide staining (A) or by fluorography (B). Nuclear extracts are defined as described in the text: lanes T, total nuclear extract; lanes C, chromatin extracts; lanes NM, nuclear matrix extracts. SV40 DNA intermediates are marked as defined in the legend to Fig. 1. In addition, the positions of the gel load (arrow) and of cellular DNA (cell) are indicated.

SV40 DNA in in situ nuclei (Fig. 7A) and at isolated nuclear matrices (Fig. 7B). SV40 DNA is not randomly distributed at isolated nuclear structures but is organized in clusters of globular structure. Similar globular structures previously were reported to be centers of actively replicating viral and cellular DNA (6, 14, 27, 50). The higher-order SV40 DNA structures seen in Fig. 7 represent unpacked, i.e., replicating, SV40 DNA and not SV40 DNA in virions, since the hybridization signal was not obtained when structures were incubated with DNase, a treatment which does not disrupt SV40 virions but destroys unpacked SV40 DNA (data not shown; see also reference 1). SV40 DNA present in in situ nuclei and at in situ matrices shows a similar pattern of organization, strongly arguing for a functional and highly ordered interaction of SV40 DNA with nuclear matrix components rather than an unspecific entrapment of SV40 DNA within the nuclei during preparation.

Functional and coordinated association of SV40 DNA and large T at isolated nuclear structures. The finding that functionally distinct large T populations (32) as well as biologically dissectable SV40 DNA intermediates are specifically associated with the chromatin and the nuclear matrix (Fig. 4) strongly suggested that large T and SV40 DNA molecules colocalize at nuclear substructures in functional complexes. To test this hypothesis, we analyzed whether isolated in situ nuclei can support SV40 DNA replication in the absence of exogenously added large T. In situ nuclei, prepared 36 h p.i., were incubated under replication conditions (see Materials and Methods), without the addition of exogenous large T and of exogenous SV40 DNA. After the indicated times, replication was stopped and nuclei were extracted with SDS. SV40 DNA was precipitated from the respective fractions and subjected to agarose gel electrophoresis followed by autoradiography. Figure 8A shows that in situ nuclei indeed were able to support SV40 DNA replication under these experimental conditions, as is evident from the time-dependent increase in radiolabeled, mature SV40 form I and II DNA and from the presence of all replicative SV40 DNA intermediates detectable in vivo (compare Fig. 1 with Fig. 8A). However, under our in situ replication conditions, efficient conversion of mature form II DNA into superhelical form I DNA was decreased, indicating that cellular factors involved in this process were extracted and/or destroyed during preparation of in situ nuclei and could not be restored by the addition of cellular extracts during in situ replication, as already seen in previous experiments (45, 46). To further exclude the possibility that incorporation into SV40 DNA intermediates represented DNA repair rather than specific SV40 DNA replication, we analyzed newly synthesized SV40 form II DNA, the major end product of in situ replication, for the distribution of the radiolabel on the SV40 genome at increasing times of replication (compare Fig. 4D). As expected for bidirectional DNA replication, radiolabel was found predominantly (>90%) in the SV40 termination region after brief replication times and migrated toward the origin region during increasing replication times as described previously (28, 44, 45; data not shown).

An important aspect of the in situ replication system described above was that large T-mediated SV40 DNA replication started immediately after replication conditions were applied (data not shown). Since SV40 DNA and large T molecules are immobilized in isolated in situ nuclei, these findings strongly argue for a coordinated and functional association of at least elongation-competent large T-SV40

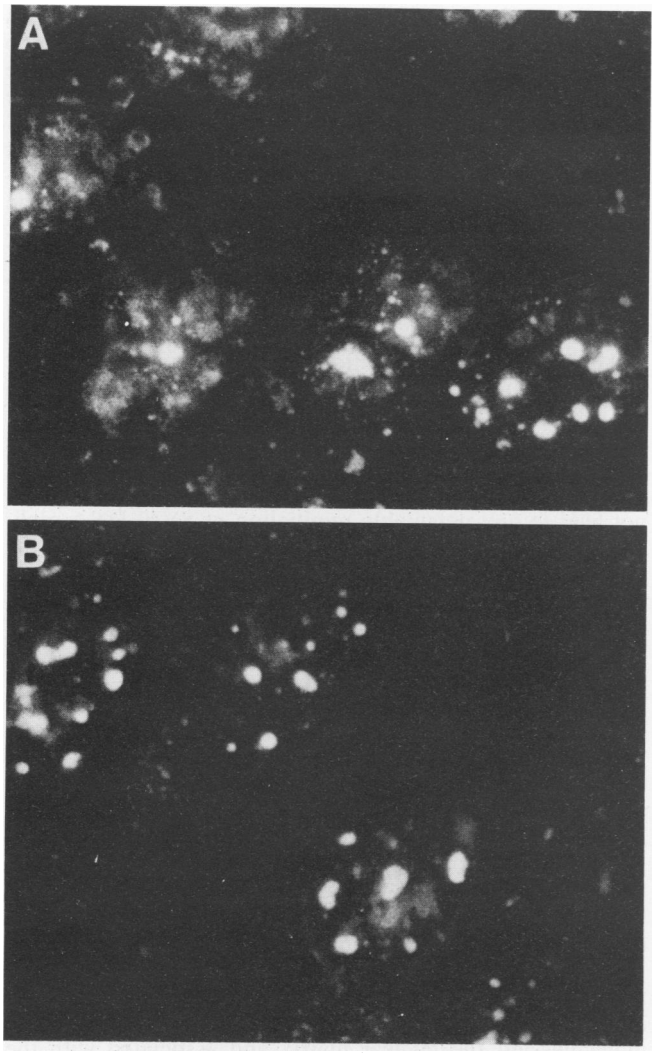

FIG. 7. In situ hybridization of SV40 DNA at isolated nuclear structures. Lytically infected cells were grown on glass coverslips. At 36 h p.i., in situ nuclei (A) or in situ nuclear matrices (B) were prepared, using our standard fractionation conditions (see Materials and Methods). Nuclear structures then were processed for in situ hybridization by using a biotinylated SV40 DNA probe. SV40 DNA was visualized with a Texas red-streptavidin conjugate (see Materials and Methods).

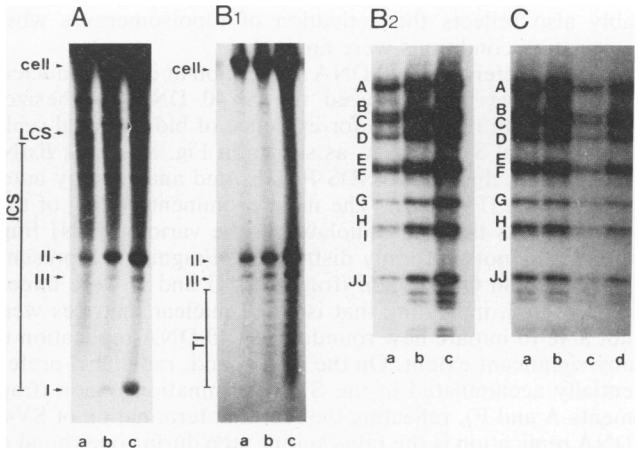

FIG. 8. Demonstration of a elongation-competent complex between SV40 DNA and large T at nuclear structures. (A) In situ nuclei were prepared at 36 h p.i. and replication conditions were applied as described in Materials and Methods. After 10 (lane a), 20 (lane b), and 60 (lane c) min at 37°C, nuclei still attached to the substratum were lysed with SDS. SV40 DNA was precipitated, and aliquots were subjected to agarose gel electrophoresis and viewed by autoradiography. The positions of SV40 DNA intermediates are marked as defined in the legend to Fig. 1. In addition, the positions of linear SV40 form III DNA and of cellular DNA (cell) are indicated. (B) In situ nuclear matrices were prepared 36 h p.i. by using our standard fractionation conditions, and replication conditions were applied for 10 (lane a), 20 (lane b) and 60 (lane c) min (see Materials and Methods). Replication was stopped by the addition of SDS with concomitant lysis of nuclear matrix structures. SV40 DNA was precipitated, and aliquots were subjected to agarose gel electrophoresis and viewed by autoradiography (B_1). Aliquots of SV40 DNA were restricted with *Bst*NI and subjected to SDS-PAGE, followed by autoradiography (B_2). (C) Replication conditions were applied to in situ matrices for 60 min (lane a) or in the presence of unspecific mouse control immunoglobulins (lane b), monoclonal antibody PAb 204 (lane c), or polyclonal anti-large T antibodies from mouse tumor serum (lane d) as described in Materials and Methods. Nuclear matrices then were lysed, and SV40 DNA was precipitated and restricted with *Bst*NI followed by SDS-PAGE and autoradiography as described above. Identical amounts of SV40 DNA were present in the respective nuclear matrix fractions, as demonstrated by silver staining of the gels (data not shown).

DNA complexes (33, 36, 44) at nuclear structures. Data reported in this and in previous studies from our laboratory suggest an important role for the nuclear matrix in functionally anchoring distinct large T and SV40 DNA molecules in replicational complexes, since (i) elongating SV40 DNA intermediates (ICS) were quantitatively associated with the nuclear matrix (Fig. 4); (ii) the nuclear matrix-associated large T population exhibited the highest specific ATPase activity (32), an activity which is closely related to the SV40 DNA elongation function (for a review, see reference 37); and (iii) more circumstantial, the association of large T with the nuclear matrix correlated with the onset of viral DNA replication (30). These correlations strongly suggested that large T-mediated SV40 DNA elongation proceeds at the nuclear matrix. To test this hypothesis, we next analyzed isolated nuclear matrices for their ability to perform SV40 DNA elongation under our in situ conditions. In situ matrices, prepared 36 h p.i., were incubated under replication conditions as described above and in Materials and Methods. After the indicated replication times, nuclear matrices were lysed and SV40 DNA was precipitated and subjected to agarose gel electrophoresis. Figure $8B_1$ shows a time-dependent increase of SV40 form II DNA, the major end product of in situ replication, as seen above for in situ nuclei. Furthermore, linearized SV40 form III DNA and SV40 form I topoisomers were detectable, whereas elongating and terminating SV40 DNA intermediates were not unequivocally detectable because of the smear of radiolabeled cellular DNA. This pattern of SV40 DNA forms, newly replicated at isolated nuclear matrices, strongly resembles the SV40 DNA pattern shown in Fig. 5B, in which topoisomers of newly replicated SV40 form I DNA were generated after incubation of histone-depleted nuclear matrix structures with restriction buffer. Since isolated nuclear matrices used for in situ replication also contained histone-depleted cellular and viral DNA, the generation of SV40 topoisomers (Fig. $8B_1$) prob-

ably also reflects the activation of topoisomerases when replication conditions were applied.

To characterize SV40 DNA replication at isolated nuclear matrices, we first analyzed the SV40 DNA synthesized during in situ replication for evidence of bidirectional replication. Total SV40 DNA, as shown in Fig. $8B_1$, was *Bst*NI restricted, subjected to SDS-PAGE, and analyzed by autoradiography (Fig. $8B_2$). The most prominent finding of this analysis was that the radiolabel in the various *Bst*NI fragments was not uniformly distributed. Fragments representing the SV40 ORI region (fragments G and I) were underrepresented, indicating that isolated nuclear matrices were not able to initiate new rounds of SV40 DNA replication to any significant extent. On the other hand, radiolabel preferentially accumulated in the SV40 termination region (fragments A and F), reflecting the fact that termination of SV40 DNA replication is the rate-limiting step during one round of SV40 DNA synthesis (42, 53). This pattern is most clearly seen in Fig. $8B_2$ (lane a) and was verified by determination of the relative content of radioactive label in the individual SV40 *Bst*NI fragments (data not shown).

We next demonstrated that SV40 DNA elongation at isolated nuclear matrices was dependent on the endogenous, i.e., nuclear matrix-associated, large T population by inhibiting large T-mediated SV40 DNA synthesis through addition of the monoclonal antibody PAb 204 (5), directed against large T, and polyclonal antibodies from mouse SV40 tumor serum to the replication assays. Nuclear matrices were incubated under replication conditions for 60 min in the presence or absence of anti-large T antibodies, and replicated SV40 DNA was analyzed by *Bst*NI restriction and SDS-PAGE (see Materials and Methods). Figure 8C shows that SV40 DNA replication was drastically inhibited by antibody PAb 204 to about 90 to 95% (compare lane a with lane c) and to about 50 to 60% by polyclonal antibodies from tumor serum (lane d), whereas unspecific mouse control immunoglobulins (lane b) did not influence SV40 DNA replication. Since PAb 204 inhibits the DNA elongation function of large T via inhibition of the large T intrinsic ATPase-helicase activity (54), these results directly show large T-mediated in situ replication of SV40 DNA at isolated nuclear matrices. This direct interrelation between the inhibition of large T-mediated in situ SV40 DNA replication and of large T-mediated ATPase-helicase activity was further supported by experiments comparing large T-specific antibody PAb 204 with other anti-large T monoclonal antibodies (e.g., PAb KT3), which did not inhibit the ATPase-helicase activity of large T (54) and also did not inhibit in situ SV40 DNA replication (data not shown).

DISCUSSION

To characterize the structural topography of SV40 replication in vivo, we analyzed the subnuclear distribution of mature and replicating SV40 DNA. Structural systems of the nucleus were prepared by in situ cell fractionation. This method preserved the association of SV40 DNA with nuclear structures under a wide variety of fractionation conditions (Fig. 2 to 6). The association of replicating SV40 DNA with the nuclear matrix was even resistant to direct incubation of in situ nuclei with 1.5 M NaCl, without prior relaxation of cellular DNA loops. Such a treatment, however, significantly damages the structural integrity of residual nuclear structures (3) and largely extracts the nuclear matrix-associated large T subclass (9). Thus, to be able to functionally colocalize SV40 DNA and large T at nuclear structures, we modified our in situ fractionation conditions by using non-SV40-cutting enzymes instead of DNase to relax the cellular chromatin. This procedure preserved the structural associations and biochemical activities of the nuclear large T subclasses, as defined previously (32).

Pulse-labeling experiments with [^{3}H]dThd provided direct evidence that SV40 DNA elongation and termination proceed at the nuclear matrix, since elongating ICS and the major SV40 termination intermediates (LCS and form II* DNA) in the conversion of ICS into mature form I DNA (Fig. 1) (see also references 46 and 53) were quantitatively associated with this nuclear substructure (Fig. 4). The subnuclear assignment of initiation and transcription processes still is circumstantial. Initiation of viral DNA synthesis might also occur at the nuclear matrix, since brief labeling with [^{3}H]dThd (2 min) revealed that already the earliest stages of ICS, containing the radiolabel in the SV40 ORI region, were also associated with the nuclear matrix (data not shown). However, in situ replication at isolated nuclear matrices did not lead to any detectable reinitiation of SV40 DNA replication. Therefore, we cannot exclude the possibility that the very first steps in initiation of SV40 DNA replication are performed at the chromatin and that replicating DNA then is rapidly transferred to the nuclear matrix. Indeed, this possibility would fit well into our previous finding that large T molecules exhibiting ORI DNA binding activity toward binding site II, the best-defined prerequisite for initiation (for a review, see reference 8), accumulate at the chromatin (32). Large T molecules at the chromatin also exhibit binding activity toward binding site I (32), which is essential for the regulation of SV40 early transcription (for a review, see reference 8). This might indicate that SV40 transcription proceeds on chromatin-associated SV40 DNA molecules. This view is further substantiated by our finding that, during the early phase of infection, SV40 DNA in its majority is associated with the chromatin (data not shown).

During brief pulse-labeling times, the majority of newly synthesized, mature SV40 form I DNA remained associated at the nuclear matrix, whereas a small percentage of these molecules left the nuclear matrix and associated with the chromatin (Fig. 4B and D). This pattern of subnuclear segregation of newly synthesized SV40 DNA was observed continuously during SV40 DNA replication (data not shown) and appeared to reflect the accumulation of mature SV40 DNA in the respective nuclear subcompartments at 36 h p.i. (Fig. 4A and C). At 36 h p.i., about 15% of total SV40 DNA was associated with the chromatin and about 85% was associated with the nuclear matrix. Pulse-chase experiments revealed that newly replicated, mature SV40 DNA molecules, once associated with the chromatin and the nuclear matrix, remained at these structures for at least 24 h (data not shown). We conclude that newly replicated SV40 form I DNA molecules after their synthesis enter the pool of total form I molecules at these structures and subsequently no longer are functionally distinguishable from the rest. Within this SV40 form I DNA pool, however, molecules could be differentiated according to their different DNase sensitivity (1), distinguishing between unpacked (i.e., replication- and transcription-competent) SV40 DNA molecules and SV40 virions (data not shown). A detailed analysis of the subnuclear kinetics leading from nuclear matrix-associated replicating SV40 DNA to mature SV40 DNA and to SV40 virions will be published elsewhere (32a).

At 36 h p.i., i.e., at a time when the rate of SV40 DNA synthesis had reached its maximum, about 85 to 90% of total large T (30) as well as about 99% of total replicating and

mature SV40 DNA (Fig. 3) were associated with nuclear structures present in in situ nuclei, pointing to a prominent role for the cellular chromatin and the nuclear matrix in functionally anchoring large T and SV40 DNA molecules during SV40 DNA replication and transcription. Further analyses revealed that biochemically defined large T subclasses (32), as well as functionally defined SV40 DNA intermediates (Fig. 4), were highly organized at nuclear structures. In situ hybridization demonstrated that replication-competent (i.e., unpacked) SV40 DNA was organized in globular structures (Fig. 7), strongly arguing for a highly ordered intranuclear arrangement of replicational SV40 DNA. To show directly that SV40 DNA and large T molecules colocalize at nuclear structures in functionally active complexes, we applied replication conditions toward isolated in situ nuclei, without the addition of exogenous large T and SV40 DNA. Using this in situ replication system, we were able to demonstrate large T-mediated SV40 DNA replication at isolated nuclear structures (Fig. 8A and B). This conclusion was strongly supported by our finding that nuclear matrix-associated SV40 DNA replication could be inhibited by using the large T-specific monoclonal antibody PAb 204 (5) (Fig. 8C). Since PAb 204 inhibits the DNA elongation function of large T by inhibition of the large T intrinsic ATPase-helicase activity (54), these results showed that nuclear matrix-associated large T is directly involved in SV40 DNA elongation. Furthermore, these findings directly relate the in vitro-determined ATPase activity of nuclear matrix-associated large T (32) with large T-mediated SV40 DNA elongation in vivo.

Our finding that in situ SV40 DNA replication, both in isolated nuclei and in isolated nuclear matrices, started without a time lag immediately after replication conditions had been applied strongly suggested that elongation-competent large T-SV40 DNA complexes preformed in vivo were anchored at isolated nuclear structures. This conclusion is supported by preliminary experiments addressing the biological significance of structurally bound replicative SV40 DNA-large T complexes. First, structurally bound complexes were able to support SV40 DNA elongation even in the absence of exogenously added cellular extracts (data not shown). Although replication efficency under these conditions was significantly lower, this finding indicates that components of the cellular DNA replication machinery were directly associated with structurally bound SV40 DNA-large T complexes. The tight interaction of replicational components at nuclear structures was further demonstrated by the fact that in situ replication could be performed in a large volume (500 μl of replication buffer). In such a volume, efficient SV40 DNA replication does not occur in the generally used in vitro replication systems (25, 41) when comparable amounts of input SV40 DNA and large T are applied (data not shown). Thus, analysis of SV40 DNA replication at isolated nuclear structures in situ might provide a tool for understanding the interactions between large T and cellular target molecules in specific processes during SV40 DNA replication in vivo.

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