Late Replicative Intermediates Are Accumulated During Simian Virus 40 DNA Replication In Vivo and In Vitro

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Simian virus 40 (SV40) replicating chromosomes were extracted from nuclei of infected cells. The chromosomes in the extract were resolved on neutral sucrose gradients, and the extent of replication of the DNA in the chromosome peaks was determined. The extract, in combination with cytosol factors and the appropriate precursors, supports the continued replication of viral DNA. The products of the incubation were mature form I DNA and molecules (after deproteinization) with sedimentation coefficients, in neutral sucrose, of 22S and 29S. The results of our analysis of this system indicate the following. (i) The 22S molecule, which has been described by previous workers, is a relaxed, replicating molecule and is an artifact of the in vitro system. (ii) When the in vitro synthesis is performed at optimal ionic strength (150 mM potassium acetate), the artifactual 22S molecule does not appear. (iii) Late replicative intermediates do accumulate in vivo and in vitro. The major late form accumulated is 91% completed. (iv) The replicating chromosomes can be resolved into two distinct peaks on neutral sucrose gradients. The molecules in these peaks differ in extent of replication. (v) The nuclear extraction procedure preferentially extracts early replicating chromosomes. The relevance of these data to the problem of SV40 and cellular chromosome replication and termination is described.

The replication of the simian virus 40 (SV40) genome has been the subject of many studies which have characterized the major features of the replicative pathway (2, 4, 11, 19, 26, 28). SV40 DNA is replicated bidirectionally from a unique origin (11). The replication time (10 to 15 min) for a single round of synthesis is substantially longer than would be predicted by the rate of eucaryotic chromosome replication (9). It has been suggested that late replicative intermediates accumulate during SV40 DNA synthesis; i.e., there may be a slowdown point(s) late in the replicative pathway (23). At a late stage in replication, but before completion of daughter strand synthesis, the progeny chromosomes separate. The mechanism by which this takes place is unknown and must be solved in the replication of all DNA genomes which employ any variation of Cairns type synthesis (2). After separation, there exists a short-lived, monomer-sized, relaxed, circular intermediate which has a gap of about 50 bases in the newly replicated strand located in the termination region of the molecules (5). The gapped form II is then converted to a covalently closed circular structure with a

superhelix density equal to that of form I DNA found in virions (12).

This picture of SV40 DNA replication is based on studies with deproteinized DNA. However, it is clear that replicating as well as non-replicating viral DNA is organized in nucleosomes by host histones (7, 15, 29). Several in vitro replication systems have been described which support the continued replication of SV40 chromosomes that initiated DNA synthesis in vivo (8, 10, 21, 29, 30, 35). In addition to progeny chromosomes, late replicating molecules accumulate in some of the in vitro systems (8, 21, 30). In particular a deproteinized molecule with a sedimentation coefficient, in neutral sucrose, of 22S has been observed. It was suggested that this structure was the replicative intermediate that exists just prior to separation of the daughter circles (21, 30). Such a "pre-separation" structure would be of considerable interest because of the topological problems associated with the separation of the daughter molecules and the possibility that this process is rate limiting for viral replication.

We have approached this problem by characterizing the replicating molecules present in vivo and those in a nuclear extract containing replicating chromosomes (nucleoproteins). An in vitro synthesis system (29) has been employed

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to support the continued replication of the replicating chromosomes, and the products of the reaction have been characterized. Our data suggest that the 22S molecule is an artifact of the in vitro system. However there are some discrete classes of late replicative intermediates present in vivo and at the end of the in vitro reaction.

MATERIALS AND METHODS

Cells and virus. BSC-1 cells were obtained from the laboratory of M. Singer of the National Institutes of Health. They were grown in modified Eagle medium plus 10% fetal calf serum (GIBCO). Near-confluent cells were infected with the 776 strain of SV40 obtained from R. Martin of the National Institutes of Health. The virus was plaque purified and titrated by plaque assay, and the cells were infected at an input multiplicity of 30 to 50 plaque-forming units per cell. The infected cells were incubated in modified Eagle medium plus 2% fetal calf serum.

Labeling procedures. SV40 form I DNA was labeled by incubating infected cells with [^{14}C]thymidine (New England Nuclear Corp.) at a concentration of 1 $\mu\text{Ci}/\text{ml}$ from 32 to 44 h after infection. Replicating molecules were labeled with [^3H]thymidine at a concentration of 100 $\mu\text{Ci}/\text{ml}$ for 3 min.

Extraction of viral DNA. In those experiments in which viral DNA was extracted directly from cells, the procedure of Hirt was employed (16).

Preparation of nuclei and nuclear extracts. Cells were harvested by washing the plates with ice-cold phosphate-buffered saline (pH 7.5) and then with the buffer described by Su and De Pamphilis (30), 10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (pH 7.5), 5 mM KCl, 0.5 mM MgCl_2 , 1 mM dithiothreitol. The cells were scraped off the plates in this buffer and homogenized in a Dounce homogenizer. The nuclei were collected by centrifugation and suspended in 0.5 mM NaH_2PO_4 (pH 7.5)-0.05 M sucrose-1 mM dithiothreitol, a buffer similar to that employed by Edenberg et al. (10). All buffers contained 10^{-4} M tosyl-phenyl-chloromethyl ketone and 10^{-4} M tosyl-l-lysyl-chloromethyl ketone. The nuclear suspension was held at 4°C for 20 min and then centrifuged at 3,000 rpm. The procedure was repeated twice, and the supernatant fluids were combined. The nuclear extract contained both replicating and non-replicating SV40 chromosomes. The yield of the replicating chromosomes was obtained by comparing the trichloroacetic acid-insoluble ^3H present in the Hirt extract of the nuclear extract with the acid-insoluble ^3H present in the Hirt extract of the corresponding number of whole cells. The nuclear extraction procedure releases 20 to 30% of the replicating chromosomes.

Preparation of cytosol extracts. The procedure of Su and De Pamphilis (30) was used. The cytosol fraction was prepared by lysing mid-log-phase HeLa cells in 10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (pH 7.5)-5 mM KCl-0.5 mM MgCl_2 -1 mM dithiothreitol, followed by low-speed centrifugation to remove nuclei. The cytosol was then clarified by centrifugation at $30,000 \times g$ for 1 h.

In vitro DNA synthesis. The in vitro DNA syn-

thesis assays were performed as described by Su and De Pamphilis (30). Assays consisted of 150 μl of cytosol, 150 μl of nuclear extract from cells pulse labeled for 3 min with [^3H]thymidine, and 100 μl of the additions indicated by those authors. These include the deoxynucleoside triphosphates, ATP, and a final concentration of KCl of 75 mM. When the incubation mixture contained [^{32}P]dATP (50 to 150 Ci/mmol, New England Nuclear Corp.), unlabeled dATP was omitted from the assay. The incubations were done at 30°C and stopped by the addition of EDTA to 20 mM. In experiments in which the reaction products were further analyzed, the samples were adjusted to 1 M NaCl-0.5% Sarkosyl and dialyzed in acetylated dialysis tubing against 1 M NaCl-10 mM Tris-hydrochloride (pH 7.5)-1 mM EDTA to remove unincorporated radioactive precursors. Incorporation of α -[^{32}P]dATP into viral DNA was linear for about 30 min. The kinetics of form I production were similar, although there was a 5- to 10-min lag as compared with the precursor incorporation (N. P. Seidman et al., submitted for publication).

Gradients. The deproteinized products of the reactions were analyzed by neutral sucrose and alkaline sucrose velocity sedimentation and by CsCl propidium diiodide isopycnic banding. The conditions for each of these have been described previously (28). Replicating and non-replicating SV40 chromosomes (nucleoprotein complexes) were fractionated on 5 to 30% sucrose gradients in 150 mM acetate-10 mM Tris-hydrochloride (pH 7.5)-1 mM EDTA and centrifuged in an SW41 rotor (Beckman) at 41,000 rpm for 2 h. These gradients were calibrated with T4 DNA (61S) and SV40 DNA (21S).

Nicking-closing enzyme assays. The partially purified preparation was free of nucleases and was the gift of E. Birkenmeier. Incubations were performed in 10 mM Tris-hydrochloride (pH 7.5)-150 mM KCl-1 mM EDTA for 1 h at 37°C and stopped by the addition of sodium dodecyl sulfate to 0.1%. The products of the reaction were analyzed directly on neutral sucrose gradients.

RESULTS

In all the experiments described here, replicating molecules were labeled by pulsing infected cells with [^3H]thymidine for 3 min. The replicating chromosomes were extracted from isolated nuclei and incubated in the in vitro replication assay. In some experiments, α -[^{32}P]dATP was present in the assay. In one such experiment, aliquots were removed at various times after the incubation began and treated with sodium dodecyl sulfate and 1 M NaCl, and the labeled molecules were analyzed by centrifugation on neutral sucrose gradients (Fig. 1). Prior to incubation in vitro, the replicating molecules sediment as a broad peak of about 29S relative to the [^{14}C]thymidine-labeled SV40 form I DNA marker of 21S (Fig. 1a). Figure 1b shows the gradient profile of the replicating molecules after 10 min of incubation. In addition to the 29S peak there is a distinct 22S peak, which

FIG. 1. Analysis of *in vitro* reaction products on neutral sucrose gradients. Infected cells were labeled for 3 min with [^3H]thymidine, nuclei were prepared and extracted, and the extract was combined with cytosol and precursors, including α -[^{32}P]dATP. After 0 min (a), 10 min (b), and 60 min (c) of incubation, the samples were treated with 1 M NaCl and 0.1% sodium dodecyl sulfate and centrifuged on 5 to 30% sucrose gradients in 10 mM Tris (pH 7.5)-1 mM EDTA-1 M NaCl. ^{14}C -labeled form I DNA (21S) was added as a sedimentation marker.

is reproducibly one or two fractions ahead of the form I DNA marker. Both the [^3H]thymidine-prelabeled and the α -[^{32}P]dATP *in vitro*-labeled replicating molecules are seen in the two peaks, with slightly more ^{32}P labeled in the 29S peak. After 60 min of incubation, the majority of both the ^3H - and ^{32}P -labeled DNA sedimented in the region of the form I marker. The results of this experiment demonstrated that there was a conversion of previously labeled, replicating molecules to structures which sedimented in the region of the form I DNA. It is also apparent that these molecules incorporated α -[^{32}P]dATP *in vitro* and so were actively replicating. In the 60-min gradient, the coincidence of the peak of replicating molecules with the form I peak is somewhat misleading, since only about 30% of the replicating molecules were converted to form I. This was determined by sedimentation of the products of the reaction on alkaline sucrose gradients (data not shown). The presence of the 22S peak (Fig. 1b) seen after 10 min suggested that the replicating molecules observed after 60 min consisted of completed 21S progeny form I and a substantial amount of the 22S structure which could not be resolved in this gradient. In addition there are molecules which are still in the 29S region and a small amount of label in form II. These results are quite similar to those reported by other authors, who have also described the appearance of a 22S peak as one of the reaction products of the *in vitro* system (8, 21). It has been suggested that the molecules in the 22S peak are a late intermediate in SV40 replication, which is formed just prior to segregation of the daughter genomes (8, 21).

To pursue this question, it was necessary to separate the replicating molecules in the assay samples from the progeny form I DNA. This was done by centrifugation of the dialyzed reaction products in equilibrium CsCl propidium

diiodide gradients (28). The fractions in the region of the gradient which contained replicating molecules (Cs II region) were pooled and prepared for analysis on neutral sucrose gradients.

An example of such an analysis is shown in Fig. 2a, which is a neutral sucrose gradient profile of the CsCl-selected replicating molecules from a 60-min incubation. There is a 29S peak as in Fig. 1a and a 22S peak. The lengths of the daughter chains in the molecules in each peak were measured by sedimentation on long alkaline sucrose gradients. The majority of the daughter chains from the 29S peak were about 80% of the full length, whereas those in the 22S peak were about 92 to 95% full length (data not shown). However, the alkaline profiles from both the 29S and 22S peaks indicated a considerable heterogeneity in the lengths of the daughter chains, with a substantial overlap between the molecules from the 29S and 22S peaks. Although these data were not inconsistent with the idea that the 22S peak was a replicative intermediate just prior to separation, the heterogeneity was disturbing. Furthermore, this interpretation contradicts the observation made by Sebring et al. (28). They analyzed replicative intermediates of varying age and found that molecules at all stages of replication had the same sedimentation coefficient in neutral sucrose gradients (about 29S). There seemed to be two explanations for the differing results: either the 22S peak was a genuine intermediate present *in vivo* in concentration too low to be detected, but was accumulated *in vitro*, or it was an artifact of the *in vitro* system.

As part of the effort to establish the structure of the molecules in the 22S peak, a sample of this material was analyzed by electron microscopy. Two types of figures were observed. There were replicative intermediates so advanced in replication that the unreplicated portion could

not be measured. These molecules looked like two circles of relaxed DNA joined at one point. The other class of molecules consisted of replicative intermediates whose unreplicated region DNA was relaxed. The molecules were spread using procedures which did not relax supercoiled form I DNA. It has been demonstrated that the unreplicated DNA in replicative intermediates isolated directly from cells using the Hirt procedure is supercoiled (28). Consequently, it seemed possible that some fraction of the structures in the 22S peak was relaxed, replicating molecules that were produced during the *in vitro* incubation. In turn, this suggested that replicative intermediates which sedimented at 29S could be induced to sediment at 22S by removal of the superhelical turns.

This possibility was tested in the following experiment. The mixture of 29S and 22S molecules shown in Fig. 2a was incubated with a preparation of nicking-closing enzyme (20). The products of the reaction were then analyzed on a neutral sucrose gradient. The results are shown in Fig. 2b. There is a single peak of 22S. These data demonstrated that the conversion of 29S replicative intermediates to a form sedimenting at 22S does not require DNA synthesis and that the 22S molecules were not necessarily intermediates in the replicative pathway.

While these studies were in progress, Champoux demonstrated that at suboptimal salt concentrations the mammalian nicking-closing enzyme would nick but fail to seal superhelical DNA (3). The suboptimal salt concentration in

his experiments (65 mM NaCl) was similar to the optimal KCl concentration in the DNA synthesis assay (75 mM KCl). When the KCl concentration was raised to 150 mM, there was a substantial drop in both the incorporation of radioactive precursors into viral DNA and the generation of progeny form I DNA. However when 150 mM potassium acetate was used, both DNA synthesis and form I production rose. The results of these experiments are shown in Table I. In another experiment the capacity of the molecules present at the end of the standard incubation to continue replication was tested. Additional cytosol and replication cocktail precursors (ATP, etc.) were added to assay samples in 75 mM KCl and 150 mM potassium acetate, and the incubation was continued for another 30

TABLE 1. Comparison of *in vitro* synthesis in different concentrations of KCl and potassium acetate^a

Salt	Concn (mM)	Form I (%)	³² P/ ³ H
KCl	75	30	1.5
KCl	150	19	1.0
Potassium acetate	150	41	2.2

^a ³H-prelabeled replicating molecules were incubated in an *in vitro* synthesis mixture containing α -[³²P]dATP in the designated salt concentration. The incorporation of α -[³²P]dATP into viral DNA was determined and normalized relative to the ³H-labeled viral DNA. The conversion of replicating molecules to form I DNA was determined by analysis on alkaline sucrose gradients of the ³H-labeled molecules after a 60-min incubation.

FIG. 2. Incubation of replicating molecules with nicking-sealing enzyme. Replicating molecules were prepared after a 60-min *in vitro* incubation. They were isolated by cesium chloride-ethidium bromide banding. One-half of the sample was incubated with nicking-sealing enzyme and 0.1% sodium dodecyl sulfate (a). The other half was incubated with nicking-sealing enzyme (b). The reaction products were analyzed on neutral sucrose gradients. ¹⁴C-labeled form I DNA was added after the reactions were stopped. Note change in ³²P scale in (b).

min. An additional 25% of the molecules were converted to form I DNA (to a total of 65% form I) in the 150 mM potassium acetate incubation, while there was no further conversion in the 75 mM KCl (beyond the 30% completion). The inhibitory effect of Cl^- has been reported recently in another replication system and for an *in vitro* translation system (13, 34). In both cases potassium acetate was found to be optimal in the range of 100 to 150 mM.

The effect of the change to 150 mM potassium acetate on the production of progeny form I DNA led us to examine the other products of the reaction. Samples were analyzed after 5, 10, 20, 40, and 60 min of incubation. The neutral sucrose gradients of the replicating molecules which are first selected by CsCl centrifugation (Cs II region) present at 10 and 60 min (Fig. 3a and b, respectively) are quite similar, as were the profiles of the other time points (data not shown). In all gradients the major peak was that at 29S. There was a much smaller (with some variability from experiment to experiment) 22S peak at any particular time. In the 60-min sample, there were also small peaks at 20.5S and 15.5S. These may be degradation products produced during the *in vitro* incubation, as equivalent peaks were not seen in the analysis of replicating molecules extracted from cells by the Hirt procedure. They were not analyzed further. The daughter chains in the 29S and 22S peaks in Fig. 3b were examined by centrifugation on long alkaline sucrose gradients. In Fig. 4a is shown the alkaline sucrose profile of the repli-

cating molecules, labeled in a 3-min pulse *in vivo*, isolated by Hirt extraction and selected from the replicating molecule region of a cesium chloride-propidium diiodide gradient. There was a sharp peak, sedimenting one fraction slower than the 16S linear marker. This peak has a sedimentation coefficient of about 15.5S (about 91% completed). There also appeared to be a somewhat broader peak of about 13.6S (about 68% completed) in addition to the smaller chains. When the daughter chains from the 22S peak (Fig. 3b) were analyzed, a single, very sharp peak, which sedimented coincidentally with the 16S marker, was obtained. The narrowness of the peak indicated a high degree of homogeneity of the daughter strands. The profile of the DNA from the 29S peak revealed the presence of two size classes of daughter chains. Approximately 85% of the [^3H]thymidine label appears in a peak of about 15.5S, whereas the remainder runs at 13.7S. Both peaks are sharp, again suggesting a high degree of homogeneity of chain length in each.

The results of this experiment strongly suggest that a substantial portion of the 22S peak of Fig. 1b and 2a was an artifact of the *in vitro* replication system when operating in suboptimal conditions. When the *in vitro* synthesis was run under optimal conditions, a 22S structure, whose daughter chains are virtually full-length, was formed. The profile of Fig. 3b suggests that this 22S material is not accumulated during the *in vitro* reaction and, thus, it is either fairly short-lived or is a minor product of replication (see

FIG. 3. Neutral sucrose centrifugation of the Cs II region products from an *in vitro* incubation in 150 mM potassium acetate. ^3H -prelabeled replicating molecules were incubated in the synthesis mixture in 150 mM potassium acetate. After 10 min (a) and 60 min (b), the reaction products were centrifuged on CsCl propidium diiodide gradients and the Cs II region (where nicked circular and linear molecules band) molecules were analyzed on neutral sucrose gradients. ^{14}C -labeled form I DNA was added to the samples as a centrifugation marker.

FIG. 4. Long alkaline sucrose gradient analysis of the reaction products from the incubation in 150 mM potassium acetate. An *in vitro* synthesis mixture was prepared with prelabeled replicating molecules and, after a 60-min incubation, the products were treated as described in Fig. 3. The components in the peaks shown in Fig. 3b were analyzed in long alkaline sucrose gradients. (b) Material in the 22S peak. (c) Material in the 29S peak. For comparison, prelabeled cells were extracted by the procedure of Hirt and the replicating molecules were centrifuged in CsCl-propidium diiodide gradients. The Cs II region components were analyzed on a long alkaline gradient (a). The positions of the ^{14}C -labeled 18S and 16S markers are shown.

Discussion). These data also indicate that discrete classes of intermediates do accumulate during the *in vitro* replication (Fig. 4c). These intermediates sediment in neutral sucrose as a single peak of 29S and are seen when replicative intermediates are extracted from cells and analyzed directly on alkaline gradients (Fig. 4a). The correspondence of the peaks in the gradients of Fig. 4a and 4c argues against an artifactual origin for the 15.5S and 13.7S intermediates and supports the conclusion that the same discrete classes of late replicative intermediates accumulate *in vivo* and *in vitro*.

Additional evidence for discrete classes of replicative intermediates was acquired in another, independent experiment. The success of the adjustment of the replication assay to 150 mM potassium acetate led us to try sedimenting the replicating chromosomes on sucrose gradients in that salt. In this experiment, infected cells were labeled with ^{14}C thymidine from 32 to 44 h postinfection and pulse labeled with ^3H thymidine for 3 min at 44 h postinfection. Nuclei were prepared and extracted as described (see Materials and Methods), and the extract was centrifuged directly on a sucrose gradient in 150 mM potassium acetate (Fig. 5). There are two peaks of the ^{14}C -labeled, non-replicating SV40 chromosomes which correspond to the 55S and 85S forms of the mature chromosome. These have been described by Christiansen and Griffith (6), Varshavsky et al. (32), and Seidman et al. (29). The replicating chromosomes also sediment as two peaks. The major peak is quite broad and is between the two conformations of the mature chromosome at about 75S. The minor peak runs ahead of the non-replicating 85S chromosome at about 95S. The resolution of the 95S peak of the replicating chromosomes was not seen when al-

FIG. 5. Centrifugation of replicating chromosomes on a sucrose gradient in 150 mM potassium acetate. Infected cells were labeled for 3 min with ^3H thymidine. Parallel cultures were labeled for 12 h with ^{14}C thymidine. The cells were mixed, and nuclei were isolated. A nuclear extract was prepared and centrifuged on a 5 to 30% sucrose gradient in 150 mM potassium acetate. The top 10 fractions, which contain trichloroacetic acid-soluble radioactivity, are not shown. The arrow marks the position of marker T4 DNA (61S).

iquots of the same nuclear extract were centrifuged on gradients in 5 mM KCl or 150 mM KCl (data not shown). The appearance of the well-defined 95S peak suggests the presence of a discrete class of replicating chromosomes within the population in the nuclear extract. This is in contrast with the results obtained when deproteinized replicating molecules were analyzed on neutral sucrose gradients (Fig. 1a).

The possibility that different classes of replicative intermediates might be distinguished in

the profile in Fig. 5 was tested by centrifugation of the DNA from the pooled fractions of the 95S peak (fractions 4 to 8) and the 75S peak (fractions 10 to 17) on long alkaline sucrose gradients. The 95S peak yields a broad major peak at about 14.3S with a wide shoulder of shorter chains (Fig. 6a). In Fig. 6b is shown the profile of the daughter strands from the 75S peak. There is a single, very broad peak with a center at about 11.5S. Comparison of the last two figures suggests that the 95S peak in Fig. 5 is extensively contaminated with the structures from the 75S peak, which explains the similarity between the wide shoulder in Fig. 6a and the broad peak in Fig. 6b. The results of these experiments argue that there is at least one discrete class of late replicative intermediates in the population of replicating chromosomes in the nuclear extract. These can be partially resolved when the extract is sedimented on gradients which preserve, to some extent, the nucleoprotein nature of the chromosomes. Furthermore, the velocity of a given replicating chromosome is directly related to the extent of replication. Apparently, the balance of mass and superhelix density which operates in the deproteinized replicating molecules to yield a constant sedimentation coefficient regardless of size of the daughter strands (28) is lost when the molecules are analyzed as nucleoproteins.

A comparison of the distribution of replicating chromosomes in Fig. 5 with the distribution of daughter chains in the alkaline gradients of a Hirt extract (Fig. 4a) yields an apparent contradiction. The results of Fig. 4a suggested that there were more older replicating molecules in the Hirt extract than younger molecules. The patterns in Fig. 5 and 6 indicated that the op-

posite was true of the hypotonic extract. To resolve this question, the replicating molecules in the hypotonic nuclear extract were analyzed on long alkaline gradients and compared with DNA prepared by the Hirt procedure. The results are shown in Fig. 7. The profile of the molecules in the hypotonic extract confirms the prediction made by the analyses in Fig. 5 and 6b (compare with Fig. 4a). Although there is a side peak at 15.5S, the dominant component is a broad peak with a center at about 13S. This experiment suggests that the hypotonic extraction procedure does not extract a completely representative sample of the replicating molecules. The hypotonic procedure extracts only 20 to 30% of the replicative intermediates (compared to the Hirt), and at least part of the reason for this lower yield is due to the preferential extraction of younger replicative intermediates.

DISCUSSION

The results of the experiments presented here support the conclusion that intermediates accumulate late in the replicative cycle of SV40 (24). The *in vitro* replication system, because of the lack of initiation, essentially chases the replicating molecules down the replicative pathway. We believe that this system (under optimal conditions) generates products which correspond to those seen *in vivo*. The most prominent of these are form I and the 91% completed form (Fig. 3 and 4). The latter molecule is the dominant, late intermediate formed *in vitro* and has a sedimentation coefficient in neutral sucrose of 29S in agreement with the results of Sebring et al. (28). It is likely then that the 91% intermediate formed *in vitro* is equivalent to the class of late replicative intermediates seen *in vivo*. This cor-

FIG. 6. Long alkaline sucrose analysis of the replicating molecules from the peaks in the replicating chromosome gradient. (a) Alkaline sucrose gradient of the replicating molecules from the 95S peak of Fig. 5. (b) Alkaline sucrose gradient of the replicating molecules from the 75S peak of Fig. 5. The arrows show the position of the 16S SV40 linear marker.

FIG. 7. Alkaline sucrose gradient analysis of replicating molecules in the Hirt extract and in the hypotonic nuclear extract. Parallel, infected cultures were labeled for 3 min with [^3H]thymidine. One set was directly extracted by the Hirt procedure. Nuclei were prepared from the other and extracted as described in the text to release the replicating chromosomes. The samples were directly analyzed on alkaline sucrose gradients. (a) Hirt-extracted replicating molecules. (b) Hypotonic nuclear extract. The ^{14}C -labeled 18S and 16S markers are shown.

respondence suggests that the 91% intermediate accumulated *in vitro* is not a "dead-end" product which arises from inactivation of the replication apparatus. Instead, we consider it probable that intermediates "pile up" at this point *in vitro* for the same reasons this occurs *in vivo*.

In contrast, the data of Fig. 2 argue that the bulk of the 22S peak of Fig. 2a consists of relaxed, replicating molecules which arise artifactually under suboptimal reaction conditions. The demonstration (Fig. 2) that DNA synthesis is not necessary to produce these molecules supports this conclusion and offers a possible mechanism for their production. There is another molecule which sediments at 22S and is seen when the *in vitro* reaction is run in 150 mM potassium acetate. This structure has daughter chains that are essentially full-length (Fig. 3b and 4b) within the limit of resolution of the alkaline gradients. The similarity between the daughter strands in the 22S molecule of Fig. 3b and the full-length linear means that the remaining parental strand duplex region in the molecule is small. This offers a conceptual basis for understanding the similarity of sedimentation coefficient of this molecule and relaxed late replicative intermediates. These two types of molecules sediment in the 22S region of the neutral sucrose gradients, because there is no significant contribution of superhelicity to the conformation of the molecules. In the one case it has been lost and in the other the parental duplex region is too small.

Why does SV40 DNA replication slow down late in the replicative pathway? The most likely explanation is that the process of separation of the daughter molecules is a rate-limiting step. Those factors required for the conversion of the

91% replicated structure to gapped circular monomers (5) may be present in limiting amounts. The consequence of failure of the SV40 daughter molecules to separate is a catenated dimer. Studies on the production of these molecules lead to the conclusion that they arose from replication errors (14, 18). The proportion of SV40 catenated dimers relative to monomers rises in cells treated with cycloheximide (18). This suggests, although does not prove, that protein(s) required for separation may be turned over more rapidly than the other components of the replication apparatus.

The 22S molecule, which was a product of the *in vitro* incubation (Fig. 3b and 4b) and has virtually full-length daughter strands, is probably not a true replicative intermediate. Instead, it is likely that this molecule is the result of the failure of parental strands to separate and has the structure of two interlocked rings with a discontinuity in the daughter strand of each duplex circle. Such a molecule would have a sedimentation coefficient of 21.6S (33), which is in good agreement with our observation. Closure of the daughter strands would yield catenated dimers.

The process of separation requires that a template strand must be cut, unwound relative to the other template strand, and resealed. Our observation that the late (91% completed) replicating chromosomes are not extracted by a procedure which yields younger replicating chromosomes (see Fig. 5 and 7) suggests that whatever the actual mechanism of separation, it takes place in a structure which is resistant to the extraction procedure and, thus, is experimentally distinguished from the younger intermediates. While this structure may be soluble

but too large for extraction, it does not seem unreasonable to suggest that the late replicating chromosomes are anchored within the nucleus, perhaps to membrane components or the nuclear matrix. A similar idea has been proposed by Meinke and Goldstein (25).

What is the relevance of these data and conclusions to the replication of the cellular chromosome? Synthesis of the eucaryotic chromosome is bidirectional and, since there are many internal replicons, replication forks meet, and so the same problem of template strand separation occurs. Since SV40 replication, beyond initiation, is totally dependent on host factors, it seems reasonable to suggest that similar mechanisms would be employed during daughter separation, as well as chain elongation, for both chromosomes. In light of our suggestion that the accumulation of late SV40 replicative intermediates is related to separation, it is pertinent to note that replicon-sized daughter strands accumulate during the replication of cell chromosomes (1, 17, 22). Also, cycloheximide treatment of cells blocks the conversion of the replicon-sized pieces of newly synthesized DNA into high-molecular-weight DNA (27). Thus, there are slowdown points late in the synthesis of cellular replicons, and the reasons for this phenomenon might well be similar to those that underlie the SV40 slowdown. Furthermore, the possibility that chromosome separation may require anchoring to nuclear structures (membrane, matrix, etc.) would be relevant to the longstanding question concerning the relationship between DNA synthesis and the nuclear membrane (9).

Finally, we note the importance of faithful daughter chromosome separation. The SV40 genome is replicated many times during the course of an infection, and a low rate of failure to separate daughter chromosomes (yielding catenated dimers) would not pose a serious threat to the survival of the virus. However, cellular chromosomes replicate only once during the cell cycle. Failure to separate the chromosomes at a termination site would produce interlocked chromosomes. If this condition were not corrected, the result might be strand breakage or an asymmetric distribution of chromosomes during mitosis, which would yield aneuploid daughter cells.

After the completion of this manuscript, the report by Tapper and De Pamphilis appeared (31). Their results and conclusions are in good agreement with those presented here.

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