DNA synthesis by partially purified replicating simian virus 40 chromosomes

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ABSTRACT

We have partially purified replicating simian virus 40 (SV40) chromosomes in a form which allows continued DNA synthesis in vitro. We first prepare a soluble DNA-synthesizing system from SV40-infected monkey cells and then sediment the components through a neutral sucrose gradient of extremely low ionic strength. Replicating SV40 chromosomes isolated from such gradients are capable of continuing DNA synthesis in vitro in the same manner as two crude subnuclear systems we have previously described (4). This indicates that the enzymes and other proteins required for in vitro DNA synthesis are bound to the replicating chromosomes.

INTRODUCTION

The study of chromosome replication in eukaryotic organisms has resulted in considerable information about the topology of replication, and about the involvement of Okazaki fragments and replicon-sized strands as intermediates in the process (for review see reference 3). However, our understanding of the structure of replicating chromatin is still inadequate (13,19), and our understanding of the enzymology of eukaryotic DNA replication is even less adequate (reviewed in reference 3). Despite the identification in eukaryotic cells of several enzymes that may play a role in replication (DNA polymerases, DNA ligase, DNA relaxing enzyme, etc.), so far no single enzyme has been positively identified as required for eukaryotic chromosome replication.

Two approaches have been used in the past to help study the enzymology of eukaryotic chromosome replication: genetic and biochemical. The genetic approach has foundered due to unexplained difficulty in developing clear, non-leaky mutants defective in DNA synthesis (reviewed in reference 3). The biochemical approach has been based on the development of eukaryotic in vitro systems for DNA replication, consisting of intact nuclei and variable amounts of "cytoplasmic" material. Although these systems have provided information about ATP requirements, RNA primers, processing of Okazaki fragments, and other features of eukaryotic replication (reviewed in reference 3), they have not yet

yielded unambiguous information about the role of particular enzymes in replication. There are two reasons for this failure. First, the soluble enzymes of such in vitro systems - enzymes which leak out of nuclei during cell fractionation and which stimulate DNA synthesis when added back to purified nuclei - have proved to be relatively unstable. So far no laboratory has successfully characterized such enzymes although several have tried (2,5,6,11, 12,15). Second, the remaining enzymes required for DNA synthesis in in vitro systems remain bound to the nucleus and cannot be further purified in such a state (15).

The papovaviruses simian virus 40 (SV40) and polyoma offer an alternative approach to studying the structure and enzymology of eukaryotic chromosome replication. With the exception of a viral protein required for initiation of replication (17), these viruses use host-cell enzymes to carry out their replication, and studies of replication intermediates at the replication fork (Okazaki fragment size, RNA primer size and type) suggest that those intermediates are identical for papovaviruses and mammalian cells (reviewed in 3; see also 18). In addition, papovavirus intranuclear DNA is found in the form of a small chromosome - a nucleoprotein complex containing cellular histones - which appears to be analogous in structure to cellular chromosome fibers (1, 9), and papovavirus DNA replication takes place in this chromosome state (1,7, 8,10,14,16,20).

Thus replicating papovavirus chromosomes appear to be excellent models for replicating regions of cellular chromosomes, and viral chromosomes are much smaller and more amenable to analysis than enormous cellular chromosomes. As a first step in taking advantage of these properties of papovavirus chromosomes, we (4) and Su and DePamphilis (16) have recently developed subnuclear in vitro systems capable of synthesizing SV40 DNA in vitro. The first of the systems we developed consists of a thoroughly washed, membrane-free preparation of chromatin that retains the overall structure of greatly swollen nuclei (we have called this material "chromatin bodies"), and the second is a "soluble system" prepared from the first by mild homogenization and pelleting of large debris. Both systems are prepared from monkey cells infected with SV40 and both continue the replication process begun within the whole cell (4).

Both of the previously-developed systems still contain many nuclear proteins. In this paper we show that, even after the replicating SV40 chromosomes contained in the previously-developed systems are separated by neutral sucrose gradient sedimentation from free nuclear proteins and from proteins bound to cellular chromatin, they can continue to synthesize DNA in vitro,

in a way which closely resembles in vitro synthesis by the earlier subnuclear systems. The similarity of the DNA synthetic properties of the partially-purified replicating SV40 chromosomes to the synthetic properties of "chromatin bodies" and the "soluble system" shows that some or all of the replication proteins used by SV40 chromosomes when they are part of "chromatin bodies" are associated with the SV40 chromosomes sufficiently tightly under our isolation conditions to cosediment with them in sucrose gradients. Thus it should prove possible to use replicating SV40 chromosomes to help identify some of the proteins required for mammalian (and viral) chromosome replication.

MATERIALS AND METHODS

Preparation of the "soluble system". BSC-1 cells were infected with SV40 as described (4). In most experiments [^3H]-dThd (20 $\mu\text{Ci/ml}$) was added 6 min before harvesting to predominantly label replicating DNA molecules, and in some experiments [^{14}C]-dThd (0.04 $\mu\text{Ci/ml}$) was added 16 h before harvesting to predominantly label mature molecules.

The "soluble system" was prepared (at 40 h after infection) by a procedure slightly modified from that originally described (all steps at 0-4°C). After the dishes were rinsed twice with ice-cold PBS (110 mM NaCl, 60 mM Na_2HPO_4 , 10 mM KH_2PO_4 , pH 7.2), the cells were resuspended by scraping in 5 ml per 100 mm Petri dish of buffer A' (0.1 M sucrose, 0.2 mM NaKHPO_4 , pH 7.5), and centrifuged at 1800 rpm for 8 min. The pellet from 10-20 plates was resuspended in 1 ml of buffer A', and an equal volume of 0.5% Triton X-100 (adjusted to pH 7.5) was slowly added with mixing. The suspension was then layered over 8 ml of buffer A' and centrifuged in an SW41 rotor at 10,000 rpm for 10 min. The pellet was resuspended in 2 ml of 0.2 mM NaKHPO_4 (pH 7.5), layered and centrifuged as above. The "chromatin bodies" (4) so obtained were resuspended in 1 ml of buffer A', then disrupted in a Dounce homogenizer (Kontes Glass) with no more than 25 strokes of the tight (B) pestle. Large debris was pelleted by centrifugation at 10,000 rpm for 10 min. The supernatant was free of cell debris or nuclei as judged by phase contrast microscopy, and is called a "soluble system", as previously described (4).

Isolation of SV40 chromosomes. One ml of the soluble system was layered over a linear gradient of either 10-30% or 10-40% sucrose in 0.2 mM NaKHPO_4 (pH 7.5), over 1 ml of 80% sucrose in the same buffer, and centrifuged in the SW41 rotor at 40,000 rpm for 120 min at 4°C. Fractions were collected, aliquots were spotted onto paper filters and washed with 1 M HCl, and their radioactivity was determined as previously described (4). Appropriate

fractions were pooled and used for in vitro DNA synthesis.

Determination of SV40 chromosome sedimentation rate. 50S ribosomal subunits (a gift from William A. Held) were included in some gradients like those in the previous paragraph, and were also sedimented in parallel gradients of 10-30% sucrose (with a 1 ml shelf of 80% sucrose) in the higher salt in which ribosomal subunits are normally sedimented (10 mM Tris, 30 mM NH_4Cl , 0.6 mM MgCl_2 , pH 7.4). The ribosomal subunits sedimented about 15% more slowly in the low salt buffer used for SV40 chromosomes than in their normal high salt buffer. Therefore, sedimentation values for SV40 chromosomes were calculated only against 50S ribosomal subunits sedimented under high salt conditions in parallel gradients.

In vitro DNA synthesis. DNA synthesis was carried out as previously described (4). The standard reaction mixture contained 70 mM KCl, 7 mM MgCl_2 , 10 mM HEPES, 100 μM each of three unlabeled dNTP's, the fourth dNTP (α -[^{32}P]-labeled at 2.5-25 Ci/mmmole, New England Nuclear) at 3-6 μM , 5 mM rATP, and 0.35 mM each rGTP, rCTP and rUTP; all at pH 7.8. In addition, variable amounts of sucrose and NaH_2PO_4 were contributed by the sucrose gradient or buffer A'. Soluble extract, when present, constituted 50% of the total reaction mixture. The soluble extract, from HeLa cells, was identical to that described previously as "cytoplasmic extract" (4) and was a gift from Janis M.K. Fraser. The term "soluble extract" allows for the possibility that the active factors may have leaked out of the nuclei during cell disruption instead of being truly cytoplasmic. In some experiments, the incubation was terminated by adding EDTA (to 10 mM) and SDS (to 0.6%), and viral DNA extracted by the method of Hirt (21). The DNA was precipitated and washed with cold ethanol after addition of calf thymus DNA as carrier, dissolved in 0.2 ml of 0.2 N NaOH, spotted onto paper filter discs, dried, washed 4 times in cold 1 N HCl and counted as previously described (4).

Size of DNA synthesized in vitro. Samples of reactions terminated by addition of Sarkosyl (K & K Laboratories, Inc.) to 1% were loaded on 5-30% neutral sucrose gradients (in 1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.1% Sarkosyl, pH 8.0). Centrifugation was at 25000 rpm, 10°C, for 18 h in the SW41 rotor. Fractions were collected and aliquots taken to determine the location of the SV40 DNA; the entire region containing SV40 DNA (20S-26S) was pooled, dialyzed against 10 mM each of Tris, EDTA, and NaCl, pH 7.5, and concentrated by dialysis against 25% Carbowax (Union Carbide) in the same buffer. The volumes were adjusted to 0.3 ml, NaOH was added to 0.2 N, and the samples were layered onto 10-30% alkaline sucrose gradients (0.7 M

NaCl, 2.5 mM EDTA, 0.1% Sarkosyl, titrated to pH 12.1 with NaOH) with a 0.5 ml shelf of 80% alkaline sucrose. Centrifugation was at 30,000 rpm, 10°C, for 17 h in the SW41 rotor. Fractions were collected and 50 µg of calf thymus DNA and 50 µg of BSA added to each before spotting onto paper filter discs, washing with 1 M HCl, and determining radioactivity as described (each sample was counted for at least 20 min).

RESULTS

Partial purification of replicating SV40 chromosomes. When the "soluble system" is prepared (as described in Materials and Methods) from cells labeled overnight with [^{14}C]-dThd (to mark mature SV40 chromosomes) and for a brief time (16 min) with [^3H]-dThd (to mark replicating SV40 chromosomes) and is then sedimented through neutral sucrose gradients in the buffer (0.2 mM Na_2HPO_4) used to prepare the "soluble system", the distributions of acid-precipitable radioactivity and O.D.₂₆₀ shown in Figure 1 are obtained. Considerable radioactivity and O.D. are found at the bottom of the sucrose gradient. This region is frequently somewhat viscous, and it contains both cellular chromatin fragments and aggregated SV40 chromosomes (data not shown). The majority of radioactivity and O.D. are found, however, in broad bands about 1/3 of the way down the gradient. The O.D.₂₆₀ profile and the [^{14}C], overnight prelabel cosediment with a peak value of about 55S, while the [^3H]-pulse prelabel is found in a slightly faster peak of about 61S. [^{14}C] DNA extracted from the 55S region cosediments in neutral sucrose gradients with authentic 21S form I SV40 DNA, while [^3H] DNA extracted from the 61S region sediments in a broad band in neutral sucrose gradients at about 25S similar to authentic SV40 replicating intermediate (RI) DNA (data not shown). We conclude from these observations that the peak of replicating SV40 chromosomes prepared and sedimented under our conditions sediments at about 61S, while the peak of mature SV40 chromosomes sediments at about 55S. Those values must be considered approximate as they have been determined relative to 50S ribosomal subunits run under parallel conditions in high salt buffer. The sedimentation rate of the SV40 chromosomes could not be measured under the same higher salt conditions in which the ribosomal subunits sediment at 50S; under such conditions the SV40 chromosomes aggregate and pellet.

The fact that the O.D.₂₆₀ profile in Figure 1 nearly matches the [^{14}C] profile, but not the [^3H] profile, shows that replicating chromosomes are present in much smaller quantities than mature chromosomes, and that the SV40 chromosome region of this neutral sucrose gradient (fractions 12-24) is

Figure 1. Sedimentation of SV40 chromosomes. A soluble system was prepared from 20 dishes that had been labeled with [^{14}C]-dThd and [^3H]-dThd to mark mature and replicating chromosomes, as described in Materials and Methods. This was sedimented in a 10-30% sucrose gradient for 120 min and aliquots taken to determine radioactivity. The A_{260} was determined on undiluted fractions using a Beckman Model 25 spectrophotometer. $\bullet$ — $\bullet$ ^3H cpm; $\circ$ — $\circ$ ^{14}C cpm; Δ — Δ A_{260} .

not extensively contaminated by non-radioactive material which absorbs light at 260 nm. We are currently testing other fractionation methods for their ability to further purify replicating chromosomes and mature chromosomes without causing loss of in vitro synthetic ability (see below). Gel filtration in Sepharose 4B, using the same low ionic strength buffer, allows full recovery of [^3H] and [^{14}C] radioactivity, of O.D._{260} , and of in vitro DNA synthetic capacity (Waqar, unpublished).

In vitro DNA synthesis. When individual fractions from sucrose gradients like that of Figure 1 were tested for their ability to synthesize DNA in vitro, synthetic capacity was found to cosediment at 61S with replicating SV40 chromosomes (Waqar and Tsubota, unpublished). In order to obtain more material for the experiments reported here, the peak fractions of repli-

cating chromosomes from sucrose gradients like that of Figure 1 were pooled. The data of Figure 2 show that the kinetics and extent of incorporation of dNTP's into DNA by such pooled partially purified replicating SV40 chromosomes are similar to those of "chromatin bodies" and of a "soluble system" from the same preparation of SV40-infected cells. Notice in Figure 2 that the extent of incorporation is measured as the ratio of [32 P] cpm incorporated to [3 H] prelabel. Use of this ratio allows direct comparison between the extents of incorporation for the three systems being tested. As we reported previously (4), incorporation by the soluble system is somewhat less than by chromatin bodies: the difference shown here is smaller than

Figure 2. Kinetics of *in vitro* DNA synthesis: a comparison of 3 subnuclear systems. Chromatin bodies and the soluble system were prepared from 30 dishes, half of which were pulse labeled for 6 min with [3 H]-dThd; aliquots of each were kept on ice while the remainder of the soluble system was sedimented as in Figure 1. The peak fractions of this pulse label (corresponding to fractions 14-21 of Fig. 1) were pooled as replicating SV40 chromosomes. Aliquots of all 3 systems were incubated at 37° in the standard reaction mixture with 3.6 μM α -[32 P]-dTTP (16.8 Ci/mmol), with (open symbols) or without (closed symbols) soluble extract. The DNA was extracted and precipitated as described. Incorporation *in vitro* was normalized to the amount of [3 H] DNA in each aliquot: the [32 P]/[3 H] ratio is shown. O,●: chromatin bodies; V,▼: soluble system; □,■: replicating SV40 chromosomes.

that reported previously, probably as a result of more gentle homogenization during preparation of the soluble system. Incorporation by the partially purified replicating chromosomes is equivalent to that by the chromatin bodies. In all three cases, both the extent of synthesis and the duration of incorporation in vitro are markedly stimulated by the addition of a soluble extract from HeLa cells; this is similar to the stimulation by so-called "cytoplasmic" extracts that has previously been observed both for intact nuclear systems (2,5,12) and for subnuclear systems (4,16). This indicates that there are at least 2 sets of factors required for maximum in vitro replication: those which remain with the replicating DNA during fractionation and those which are easily washed away during the initial stages of preparation.

Reaction conditions were studied in the absence of added soluble extract and found to be similar to the optimal conditions for the previously described subnuclear systems (4). The Mg^{++} optimum is about 7 mM with the standard nucleoside triphosphate concentrations. When either rATP or the other 3 rNTP's or all four rNTP's are eliminated from the standard reaction mixture, the extent of synthesis is reduced by about one half. If the magnesium concentration is then adjusted to 1 mM, the reduction is much less and in fact cannot be demonstrated when rATP alone is omitted. In these properties the replicating chromosomes resemble the "soluble system" and differ from the "chromatin bodies" previously described (4). Preparations of partially purified chromosomes retain their synthetic ability with only slight loss even after storage at -15° for up to 2 months. However, all studies reported here were carried out with fresh preparations.

We were interested in learning whether there was a third set of replication factors, still present in the soluble system but unable to cosediment with the replicating chromosomes. To examine this question, we added aliquots of the pooled top fractions of neutral sucrose gradients like those of Figure 1 to in vitro reaction mixtures, and looked for stimulation of the DNA synthesis carried out by our partially purified replicating chromosomes. In our standard preparations, neither stimulation of DNA synthesis nor any change in the size distribution of the DNA synthesized (see below) could be detected; synthesis carried out in both the presence and absence of the soluble extract mentioned above was unaffected by these pooled top fractions. We did, however, find that if excessive shear were used in preparing the soluble system, the extent of DNA synthesis was reduced from

that seen in standard preparations; in such excessively sheared preparations, there was some stimulation by the top fractions. It is likely that the reduction in DNA synthesis caused by shear is due to damaging the chromosomes, and that the stimulation of incorporation caused by top fractions under these conditions is due to incorporation at nicks or breaks in the sheared chromosomes. We did no further work with the top fractions, and we avoided excessive shear in the preparations reported on here.

Size distribution of DNA synthesized in vitro. DNA synthesized in vitro by the partially purified replicating SV40 chromosomes was extracted and sedimented through neutral sucrose gradients. Both in the presence and absence of soluble extract the DNA synthesized in vitro sedimented somewhat faster than form I DNA, as expected for replicating intermediates of SV40. After longer incubations in the presence of soluble extract, the difference in sedimentation was reduced, similar to our previous findings with chromatin bodies and the soluble system (4) (data not shown).

When DNA synthesized in vitro in the absence of soluble extract is purified on neutral sucrose gradients and then denatured and resedimented in alkaline sucrose gradients (Fig. 3 a-c), some of the radioactive label is in long strands (up to full viral length), but most of the label is found in short strands (Okazaki fragments) whether the incubation was short (2 min; Fig. 3a) or long (20 min; Fig. 3c). There is no chasing of the short strands into longer strands (Fig. 3b). The lack of chasing of short strands into longer ones in the absence of soluble extract is similar to our previous result with the "chromatin bodies" and "soluble system" (4). In those systems, however, about half of the DNA synthesized in vitro was in longer strands and half in Okazaki fragments. We do not know why the partially purified chromosomes incorporate most of the label into short strands, rather than equally into both size classes.

The addition of soluble extract, in addition to stimulating DNA synthesis in vitro (Fig. 2), leads to the chasing of 30% of the label in Okazaki fragments (when normalized to ^3H prelabel) into longer strands, up to full viral size (Fig. 3 d-f). This is consistent with the effect seen in the sub-nuclear systems previously described (4), although the chasing is not as complete. In the presence of soluble extract, a small fraction (2-3%) of the in vitro label is incorporated into form I molecules (covalently closed circles) after incubations of 20 min or more. In recent experiments, we have found that the extent of chasing into long strands and into covalently closed circles (form I molecules) varies markedly with the soluble extract used (H. Edenberg, unpublished); the reasons for this are not known.

Figure 3. Size distributions of DNA strands synthesized *in vitro* by the replicating SV40 chromosomes: effect of soluble extract. Portions of the replicating chromosome preparation used in Figure 2 were incubated in the *in vitro* conditions of Figure 2 for 2 min (a,b,d,e) or 20 min (c,f). Chases were performed by adding 250 μ M unlabeled dTTP after 2 min, and continuing the incubation for 18 min (b,e). Reactions d,e and f contained soluble extract. Reactions were stopped and sedimented as described. The final alkaline gradients are shown. o—o: [3 H] cpm; —●—: [14 C] cpm. Sedimentation of [3 H] DNA was similar for all gradients but is shown only for c and f. The position of 16-18S mature SV40 linear and circular single strands is assumed to coincide with the leading edge of the 3 H distribution, as shown.

Effect of FdUrd pretreatment on replicating chromosomes. Since the proportion of SV40 chromosomes replicating at any one time is very small, we decided to try to increase this proportion by pre-treating the cells in vivo with FdUrd to accumulate replicating intermediates. After a one h treatment with FdUrd in medium with dialyzed serum, replicating SV40 chromosomes were isolated and incubated in vitro. Although the $[^{32}\text{P}]/[^3\text{H}]$ ratio showed that the soluble extract stimulated incorporation by about 11-fold, the pattern of DNA synthesis appeared abnormal in that soluble extract did not stimulate the usual elongation or joining of the short strands into longer ones. This defect in replicating chromosomes isolated from FdUrd-treated cells led us to abandon our attempts to accumulate replicating chromosomes by such treatment.

DISCUSSION

In this paper we describe a method for preparing partially purified SV40 replicating chromosomes which are capable of continuing DNA synthesis in vitro. The replicating chromosomes are largely free of fragments of cellular chromatin as well as soluble nuclear proteins, and are partially separated from mature SV40 chromosomes. These replicating chromosomes, in the absence of any other protein factors, synthesize predominantly Okazaki fragments. The synthesis carried out by these partially purified replicating chromosomes resembles closely, in rate, optimum reaction conditions, and the size of DNA made, the synthesis we have previously described in less pure subnuclear systems (4)

All of our subnuclear systems, as well as another reported subnuclear system (16) and intact nuclear systems (2,5,12) are stimulated by addition of a soluble extract. The stimulation of DNA synthesis by soluble extract (seen in Fig. 2) indicates that, while a set of proteins required for DNA synthesis are present in all 3 subnuclear systems, there are other factors that contribute to replication which are lost during the preparation. This second set of replication factors are apparently not bound (or bound only transiently and less tightly) to replicating chromatin, and thus appear in soluble (extra-nuclear) fractions during early stages of fractionation. In addition to stimulating the rate and extent of DNA synthesis, these factors alter the characteristics of the DNA synthesized; the major effect seen is restoration of the ability to join Okazaki fragments and to make mature, covalently closed DNA. These factors are not species specific: experiments in this paper utilized a soluble extract of uninfected HeLa cells (human) to supplement our SV40 replication system prepared from

BSC-1 cells (monkey). A similar soluble extract from uninfected monkey cells also works well (Edenberg, unpublished).

The fact that replicating SV40 chromosomes isolated as described here synthesize DNA in a manner very much like the "chromatin bodies" and "soluble system" we described earlier (4) demonstrates that the proteins needed for limited DNA synthesis in vitro cosediment with the replicating chromosomes. There is no evidence for a third set of factors which might remain associated with the chromatin bodies and soluble systems but be separated from the replicating chromosomes by sedimentation (i.e., a soluble "nuclear associated" fraction).

The replicating SV40 chromosomes retain the ability to be complemented by soluble extracts in the same fashion as the less pure subnuclear systems, allowing synthesis of full-length DNA chains and a limited amount of synthesis of completed, covalently closed molecules. Thus replicating SV40 chromosomes should prove to be extremely useful in characterizing many of the enzymes and other factors required for replication of SV40 and mammalian cell DNA. In addition, because the ability of these chromosomes to continue DNA synthesis in vitro provides a criterion for establishing that they have not been irreversibly altered, they should also prove to be useful models in structural studies of chromosome replication.

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