The relationship of SV40 replicating chromosomes to two forms of the non-replicating SV40 chromosome

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ABSTRACT

SV40 replicating chromosomes were extracted from infected cells using a detergent free extraction method. This procedure also extracts 2 forms of the non-replicating chromosome, one of which corresponds to the well characterized 50-55S SV40 minichromosome. The other is a more compact structure which has a sedimentation coefficient of 80-85S. The replicating chromosomes sediment between the 2 conformations of the mature chromosome. Electron microscopy of the replicating chromosomes suggests an overall conformation that resembles the 50-55S form of the mature chromosome rather than that of the 80-85S structure. Nucleosomes are present on both sides of the replication forks. When the replicating chromosomes were incubated in an *in vitro* DNA synthesis assay all regions of the SV40 genome were synthesized and a significant fraction of the replicating chromosomes completed replication. The progeny chromosomes co-sedimented with the 50-55S chromosomes which were present prior to the incubation. The sedimentation coefficients and relative amounts of the two forms of the mature chromosome were unaffected by the incubation.

INTRODUCTION

The SV40 minichromosome has received considerable attention because of its importance to our understanding of the biology of the virus and its utility as a model of some of the structural features of the eukaryotic chromosome. (1,2). Procedures which extract the SV40 Form I chromosome from the nuclei of infected cells also extract SV40 replicating chromosomes (3) and systems have been developed which support the continued replication of these structures. These *in vitro* replication systems should be useful in extending our knowledge of the structure of the replicating SV40 and cellular chromosomes.

The SV40 Form I chromosome can be isolated from infected cells and virions(4). The complexes from both sources have a sedimentation coefficient of about 55 S in sucrose gradients and have been shown by many authors to consist of superhelical DNA associated with histones in nucleosomes. The nucleosomes appear either as "beads on a string" or closely associated in a 100-120Å fiber depending on the conditions of the experiment (1,2). The 55 S Form I

chromosome is not a completely satisfactory model of viral DNA packaging or cellular chromatin structure. This is because the minichromosome is not sufficiently compact to be contained within a virion and because much of the cellular chromatin, particularly in metaphase, exists as 200-300 Å fibers (5). There is another level of condensation required to convert 100 Å "nucleofilaments" to the structures appropriate for virion assembly in the case of the virus or 200-300 Å fibers in the cellular chromatin.

Recently a more compact form of the SV40 Form I chromosome has been isolated. (6,7). This structure sediments at about 85S (see 6 but also 7) and under certain conditions virtually all the SV40 chromosomes in the nuclear extract are recovered as the fast sedimenting form. It is compact enough for encapsidation and is a roughly spherical particle with a diameter of about 300 Å.

These observations add another dimension to the problem of the replication of the SV40 chromosome. In the experiments presented in this paper we have examined the relationship of the structure of the replicating SV40 chromosome to the two conformations of the Form I chromosome. We have extracted replicating chromosomes and studied them before and after incubation in an in vitro replication system in which replicative DNA synthesis occurs and progeny Form I chromosomes are generated. (8).

MATERIALS AND METHODS

Cells and Virus

BSC-1 cells were obtained from the laboratory of Dr. M. Singer of the National Institutes of Health. They were grown in modified Eagle's No. 2 medium plus 10% fetal calf serum (Gibco). The cells were seeded such that they were approximately 75% confluent 24 hours later at which time they were infected with the 776 strain of SV40 obtained from Dr. R. Martin of the National Institutes of Health. The virus was plaque purified, titered by plaque assay, and cells were infected at an input multiplicity of 30-50 plaque forming units per cell. The infected cells were incubated in modified Eagle's No. 2 medium plus 2% fetal calf serum.

LABELLING PROCEDURES

SV40 Form I chromosomes (non replicating chromosomes) were labelled by incubating infected cells with [^{14}C] thymidine (New England Nuclear) at a concentration of 1 uci/ml from 30 to 42 hours after infection. The replicating chromosomes were labelled with [^3H] thymidine at a concentration of 100 uci/ml for 3 minutes (8). Plates were floated on a 37° water bath during this labelling procedure.

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 Pamphlis, 10mM N2-hydroxyethyl- piperazine -N'-2-ethane sulfonic acid (Hepes) pH 7.5, 5mM KCl 0.5 MgCl₂ 1mM dithiothreitol (DTT) (8). The cells were scraped off the plates in this buffer and lysed in a Dounce homogenizer. The nuclei were collected by centrifugation and resuspended in 0.5 mM NaH₂PO₄ pH 7.5, 0.5 M sucrose, 1mM DTT, a buffer similar to that employed by Edenberg *et al.* (9). All buffers contained 10⁻⁴M tosyl phenyl chloromethyl ketone (TPCK) and 10⁻⁴M tosyl-lysyl-chloromethyl ketone (TLCK). The nuclear suspension was held at 4°C for 20 minutes and then centrifuged at 3000 RPM. The procedure was repeated twice and the supernatants combined. The nuclear extract contained both replicating and SV40 form I chromosomes. Although as much as 40-50% of the Form I chromosomes were found in the cytoplasm after lysis of the cells greater than 95% of the replicating chromosomes were retained in the nuclei. The yield of the replicating chromosomes was obtained by comparing the TCA insoluble [³H] present in a Hirt extract of the nuclear extract with the TCA insoluble [³H] present in a Hirt extract of the corresponding number of whole cells. The extraction procedure of Su and DePamphlis (8) consistently gave yields of less than 10% of the replicating chromosome while the procedure described here released 20-30% of the replicating chromosomes. The nuclear extract contains most of the remaining Form I chromosomes as well.

GRADIENTS

The replicating and non-replicating chromosomes were resolved on 5-30% sucrose gradients in 10mM Tris: HCl pH 7.5, 1mM EDTA, 5 mM KCl centrifuged in a SW41 rotor (Beckman) at 41,000 RPM for 2 hours. These gradients were calibrated using T4 DNA (61S) and SV40 DNA (21S). Alkaline sucrose and CsCl propidium diiodide equilibrium density gradient centrifugation were as previously described. (10)

IN VITRO DNA SYNTHESIS

The *in vitro* replication assay was prepared as described by Su and DePamphlis, (8) except that the cytosol fraction was prepared by lysing mid log phase HeLa cells in 10mM Hepes pH 7.5, 5mM KCl, 0.5 mM MgCl₂, 1mM DTT followed by low speed centrifugation to remove nuclei. The cytosol was clarified by centrifugation at 30,000 xg for 1 hr. Assays consisted of 150 µl of cytosol, 150 µl of nuclear extract from cells labelled for 3 minutes with [³H] thymidine and 100 µl of the additions described by Su and DePamphlis,

(8). When the incubation mixture contained [^{32}P] ATP (50-150 Ci/mmmole, New England Nuclear) unlabelled dATP was omitted from the assay. Reactions were stopped by the addition of EDTA to 20 mM. The incorporation of radioactivity into macromolecules was determined by measuring the trichloroacetic acid insoluble material collected by filtration on GF/A glass fiber filters (Whatman). The results were expressed as a ratio of $^{32}\text{P}/^3\text{H}$. In experiments which required analysis of the viral DNA the samples were adjusted to 1M NaCl, 0.5% sarkosyl and dialyzed in acetylated dialysis tubing against 1M NaCl, 10mM Tris:HCl pH 7.5, 1 mM EDTA to remove unincorporated radioactive precursors.

RESTRICTION ENZYME ANALYSIS OF SV40 DNA FORMED IN VIVO

Form I DNA was isolated by CsCl propidium di Iodide equilibrium density gradient centrifugation. The pooled fractions containing Form I DNA were dialysed in collodion bags against 10mM Tris: HCl pH 7.5 50 NaCl, 1 mM EDTA and Dowex 50 resin (11) and then alcohol precipitated. The DNA was resuspended in a digestion buffer consisting of 10 mM Tris: HCl pH 7.5, 50 mM NaCl, 6.6 mM MgCl_2 and incubated with Hind II and III restriction endonucleases (Bio Labs) for 3 hr. at 37° . The reaction was stopped by the addition of SDS and samples were applied directly to 3.0% acrylamide, 0.5% agarose gels and electrophoresed as described (12). The gel was dried and examined by autoradiography.

ELECTRON MICROSCOPY

Gradient fractions containing viral replicating and fast sedimenting Form I chromosomes were dialyzed in acetylated dialysis tubing against 10 mM Tris : HCl pH 7.5, 5mM KCl, 1 mM EDTA and then concentrated against Sephadex G-150. Samples were spread without further additions over the surface of a hypophase solution containing 5 mM EDTA. Parlodion coated copper grids were touched briefly to the surface of the hypophase, washed in 90% ethanol and rotary shadowed with platinum-palladium. Grids were examined in a Siemens Elmiskop 101 Electron Microscope at 40 KV accelerating voltage. Dimensions were measured from diffraction grating calibrated electron image plates using a computer-coupled tracing device (Neumonics Corp.).

RESULTS

ISOLATION OF REPLICATING SV40 CHROMOSOMES

The initial goal of this work was the preparation of a nuclear extract which would contain replicating SV40 chromosomes (RL chromosomes) capable of continued replication. Extraction buffers which are commonly used to extract the non-replicating SV40 minichromosome (Form I chromosome) contain Triton

X-100 and physiological concentrations of NaCl (2,3). When nuclei from infected cells were extracted with buffers containing the detergent and either no salt or 0.15M NaCl yields of 30-40% of the replicating chromosomes were obtained. These extracts, when added to the in vitro replication system (see Materials and Methods), did incorporate ($\alpha^{32}\text{P}$) dATP into viral DNA. However, extensive nicking of the endogenous Form I DNA was observed in these experiments. In reaction mixtures containing cytosol extract 50% of the Form I DNA present at the beginning of the incubation was converted to Form II after 60 minutes at 30° (the standard incubation conditions). In reactions in which the cytosol extract was omitted the conversion of Form I to Form II DNA was as high as 90%. It seemed likely that the nuclear extract contained nuclease activity. The possibility that the nuclease (s) was released by the Triton X-100 led us to try extraction procedures which did not include the detergent. Extraction of nuclei in a hypotonic buffer consisting of 0.5 mM NaH_2PO_4 pH 7.5, 0.05M sucrose, (9) gave yields of 20-30% of the replicating chromosomes with no loss of endogenous Form I DNA during in vitro replication assays.

GRADIENT ANALYSIS OF REPLICATING AND FORM I CHROMOSOMES

Over the course of this work the replicating and non-replicating chromosomes were analysed many times on sucrose gradients. A representative gradient pattern is shown in Figure 1. There are several features of interest in this profile. There are two peaks of [^{14}C] thymidine labelled chromosomes. Analysis of the DNA extracted from each peak showed that both contained Form I DNA. The sedimentation coefficients of the two peaks are 50 S and 80 S, relative to DNA markers (see Materials and Methods). The 50 S peak corresponds to the now traditional 55 S SV40 Form I chromosome in the DNA packaged into a 100 Å "nucleofilament". The fast moving peak is the compact 300A, form recently described by Christansen and Griffith (6) and Varshavsky et al. (7). The relative amounts of the two forms varied from preparation to preparation, from 70:30 to 30:70 (80S:50 S). This variation was not due to gradient composition. (See figure 6) When aliquots of the same preparation were analyzed on gradients of low (5 mM K^+) or high ionic strength (150 mM K^+) the two peaks appeared in the same proportion on both gradients. Predicatably both forms sedimented slightly faster in the gradient of higher ionic strength (55 S and 85S). The two forms were also seen in gradients of no salt or 0.5 mM MgCl_2 .

Regardless of gradient composition or preparation, the replicating chromosomes always sediment between the two conformations of the Form I

Figure 1. Sedimentation analysis of replicating and Form I SV40 chromosomes.

Infected cells were labelled with (^{14}C) thymidine at 30-42 hours post infection. They were labelled for 3 minutes with (^3H) thymidine. Nuclei were prepared and extracted. An aliquot of the nuclear extract was sedimented on a 5-30% sucrose gradient.

chromosomes, with sedimentation coefficients of 1.2-1.4 times the S value of the slower sedimenting Form I chromosome. This relationship has been observed by other investigators (2,3) and is most probably due to the greater mass of a replicating chromosome. Their observations were made in experiments in which the faster sedimenting conformation of the Form I chromosome was not seen. The relationship holds in our experiments in which a significant percentage of the Form I chromosome population sediments as the compact structure. The suggestion by Hall et al. (3) of some heterogeneity in the profile of the replicating-chromosomes appears to be supported by the pattern in Figure 1.

ELECTRON MICROSCOPY OF REPLICATING CHROMOSOMES

Nuclear extracts were centrifuged on gradients as described in figure 1 and the fractions containing the replicating chromosomes were pooled and dialyzed and concentrated. The samples were spread over 5 mM EDTA pH 7.5 and the grids prepared as described (Materials and Methods). Some of the samples were spread without fixing, others were first treated with formaldehyde and glutaraldehyde as described by Christiansen and Griffiths (6).

Examples of the unfixed replicating chromosomes are shown in Figure 2a,b,c,d. Nucleosomes are apparent as "beads on a string" and are present on either side of the replication fork. The irregularity of their spacing has been discussed (6). In figure 2e and f are shown replicating chromosomes fixed with formaldehyde and glutaraldehyde prior to spreading. The nucleosomes appear bunched together and are present on both sides of the replication forks. Also visible in the fields in figure 2e and f are structures which are 300-350Å in diameter and which may be the structures described by Christiansen and Griffith (6) and Varsharsky et al. (7).

CONTINUED REPLICATION OF THE REPLICATING CHROMOSOMES IN VIVO

The continued replication of the replicating chromosomes was studied in the in vitro replication system devised by Su and DePamphilis (8). The kinetics of [$\alpha^{32}\text{P}$]dATP incorporation into viral DNA are shown in figure 3. As reported by these authors and by Edenberg et al. (9) DNA synthesis is largely over after about 30 minutes of incubation, although there is some continued incorporation for as long as 60 minutes. The conversion of the [^3H] thymidine labelled replicating DNA to Form I DNA was measured at each time point by alkaline sucrose gradient centrifugation of the viral DNA. (Figure 4). The time course of the formation of Form I in vitro is also shown in figure 3. The appearance of the [^3H] thymidine label in Form I DNA lags behind the [$\alpha^{32}\text{P}$]dATP incorporation by about 10 minutes. In the experiment described in figure 3, 26% of the [^3H] thymidine labelled replicating molecules were converted to Form I DNA at the end of 60 minutes.

CONVERSION OF REPLICATING MOLECULES TO FORM I DNA

Figure 4 shows the results of alkaline sucrose gradient analyses of the reaction products before (4a) and after a 60 min incubation (4b). At the outset of the reaction a small amount of [^3H] labelled viral DNA is present as Form I (less than 5%). Most of the [^3H] labelled DNA is found in a broad peak of less than 16S with a clear side peak of 4S. The broad peak contains daughter strands shorter than genome length while the 4S fragments are small "Okazaki" type fragments (13). Fig. 4b shows the gradient pattern of the viral DNA after incubation in a reaction mixture containing [$\alpha^{32}\text{P}$] dATP. Both isotopes appear in the Form I peak. In this particular experiment 29% of the ^3H labelled DNA was converted to Form I DNA. The remaining [^3H] and [^{32}P] labelled DNA is in a peak of about 15 S. There is no 4S peak.

Another aliquot of the reaction products from the experiment described in figure 4b was analyzed on CsCl propidium diiodide buoyant density

Figure 2. Electron microscopy of SV40 replicating chromosomes. Replicating SV40 chromosomes were purified in sucrose gradients. The appropriate fractions were grouped, dialyzed and concentrated. Fixing of some of the samples was by two step procedure of Christiansen and Griffith (6). The chromosomes were spread as described (Materials and Methods). The molecules in a,b,c,d were not fixed, those in e and f were.

gradients. The distribution of the [^3H] labelled DNA at the beginning of the reaction is shown in figure 5a. There is a broad peak in the region of the gradient in which the [^{14}C] marker Form I DNA is found. There is very little [^3H] labelled DNA in the region in which the [^{14}C] labelled marker Form I DNA is found. At the end of the reaction the [^3H] labelled peak in the Form II region has sharpened and there is a significant amount of [^3H] labelled DNA in the Form I peak. The [^{32}P] labelled DNA is in a similar distribution. The coincidence of the [^3H] labelled Form I peak with the [^{14}C] labelled marker Form I peak suggests that the progeny Form I DNA is of the same superhelix density as DNA isolated from virions. This conclusion was confirmed when the [^{32}P] form I DNA synthesized in vitro and

Figure 3. Kinetics of incorporation of $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ into viral DNA and synthesis of SV40 Form I DNA. Nuclear extracts containing $[\text{H}]$ thymidine labeled replicating chromosomes were incubated in the *in vitro* replication system which contained $[\alpha\text{-}^{32}\text{P}]\text{dATP}$. At the appropriated times the amount of TCA insoluble $(\alpha\text{-}^{32}\text{P})\text{dATP}$ incorporated into viral DNA was determined and expressed as the ratio of (^{32}P) to (^3H) . (arbitrary units). The amount of Form I DNA synthesized at each time was measured by analyzing the (^3H) thymidine labeled DNA in alkaline sucrose gradients.

purified on CsCl propidium diiodide gradients was analyzed on agarose acrylamide gels. These gels resolve superhelical SV40 DNA as a function of superhelix density. (14) The progeny Form I DNA had the same superhelix density as Form I DNA isolated from virions. A similar conclusion has been reached by Su and DePamphilis. (8).

Our current view of superhelicity is that it is quantitatively related to the association of the DNA with histones (15). Consequently the Form I chromosome derived from the replicating chromosome has the same histone: DNA ratio as the Form I chromosome isolated from cells. That being so the Form I chromosome synthesized *in vitro* should co-sediment with at least one of the two forms of the non-replicating chromosome described in figure 1.

The results of such an experiment are shown in figure 6. Figure 6a is the gradient pattern of the replicating ($[\text{H}]$ thymidine labelled) and Form I chromosomes ($[\text{C}]$ thymidine labelled) before the *in vitro* incubation. In this preparation there were approximately equal amounts of the two

Figure 4. Analysis of the *in vitro* reaction products on alkaline sucrose gradients.
 a) (^3H) thymidine labelled replicating SV40 DNA at the beginning of the *in vitro* incubation. (^{14}C) thymidine labelled SV40 Form I DNA was added to the gradient.
 b) The (^3H)₂ labelled SV40 DNA at the end of a 60 minute incubation in which [α - ^{32}P]dATP was present in the assay mixture.

conformations of the Form I chromosome. The position of the replicating chromosomes is as in figure 1. The pattern after a 60 minute incubation is shown in figure 6b. It is apparent that there has been a shift of some of the [^3H] thymidine labelled chromosomes to coincide with the slower sedimenting [^{14}C] thymidine labelled Form I chromosome. Analysis on alkaline sucrose gradients of the [^3H] thymidine labelled DNA present in various regions of the gradient indicated that the 50-55 S region contained Form I DNA while the 60-75S and 80-85S regions contained molecules that had not completed replication. (See Fig. 4). Another feature of interest in these gradient profiles is the retention of both the sedimentation properties and the relative proportions of the two conformations of the Form I chromosomes during the *in vitro* incubation. This experiment has been done several times with the same results.

The data from the preceding experiments indicate that the replicating chromosomes function in the *in vitro* replication system and a significant portion of these replicating chromosomes are converted to Form I chromosomes. However, there are several questions about the system which remain unanswered. One of the most important of these is related to the yield of replicating chromosomes in the original nuclear extract and the conversion of only a fraction of these to Form I chromosomes containing Form I DNA. Only 20-30%

Figure 5. Analysis of the *in vitro* reaction products on CsCl propidium di-iodide buoyant density gradients.
a) ^3H thymidine labeled replicating SV40 DNA at the beginning of the *in vitro* incubation.
b) The ^3H labeled SV40 DNA at the end of a 60 minute incubation in which $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ was present. (^{14}C) Form I and Form II SV40 DNA markers were added to the gradients.

of the replicating chromosomes (almost all of the replicating molecules in the cell are found in the nucleus, see Materials and Methods) in the nucleus are obtained in the nuclear extract and only 30% of these are converted to daughter Form I chromosomes. The progeny molecules are derived, then, from no more than 10% of the original replicating chromosome population. It is

Figure 6. Sedimentation analysis of SV40 Form I and replicating chromosomes before and after *in vitro* replication. The nuclear extract containing (^{14}C) labeled Form I chromosomes and (^3H) labeled replicating chromosomes was added to the replication assay. An aliquot was withdrawn, adjusted to 10mM EDTA and held at 4°C for 60 min (a). The remainder was incubated at 30° for 60 min (b). The two samples were then analyzed on sucrose gradients as before.

possible that the replicating molecules present in the extract represent a specific subgroup, perhaps molecules advanced in replication, and that the replication *in vitro* is essentially a "finishing reaction" in which the most advanced structures in that group complete replication.

This possibility can be tested by analyzing the restriction fragments of the Form I DNA present at the end of a incubation in which [α - ^{32}P]dATP

is present. If the in vitro synthesis is essentially the completion of very advanced replicating molecules then the radioactivity should appear principally in the region of SV40 DNA where the termination of replication takes place. This region has been located in the B and G fragments of a Hind II and III restriction endonuclease digest of SV40 (16). However, the observation that the radioactivity is present in all the Hind II and III fragments of the Form I DNA present at the end of the incubation cannot be taken as proof that all regions of the SV40 DNA were involved in the in vitro replication and thus the nuclear extract contained a completely representative population of replicating chromosomes. There is a serious objection to the assumption that the incorporation of the [α - ^{32}P] dATP into form I DNA is the result of "Replicative" DNA synthesis. This is because there is an active "repair type" synthesis activity present in extracts of the sort employed in these studies. In other experiments a mixture of ^3H thymidine labelled SV40 Form I and II DNA was added to an incubation mixture consisting of a nuclear extract, prepared from nuclei from uninfected cells, and cytosol and the appropriate salts and precursors including [^{32}P] dATP. At the end of a 60 minute incubation of 30° all the SV40 DNA was present as Form I DNA which was also labelled with ^{32}P (Birkenmeier, E., and Seidman, M., unpublished data).

These questions were answered in the following experiment based on that of Nathans and Danna (17). An extract from infected cell nuclei was prepared and incubated in the in vitro replication system in the presence of [α - ^{32}P] dATP. Aliquots were removed at various times and mixed with SDS and adjusted to 1 M NaCl. After dialysis against 1 M NaCl to remove the bulk of the unincorporated [α - ^{32}P]dATP the samples were centrifuged to equilibrium on CsCl-propidium diiodide density gradients as described in figure 5. The fractions containing the Form I DNA from each sample were grouped and dialyzed to remove CsCl and propidium diiodide. After concentrating the DNA each sample was digested with the Hind II and III endonucleases and the digestion products separated on acrylamide agarose gel. The autoradiogram of the gel is shown in Figure 7. The Form I which was isolated after 5 minutes of incubation has the radioactivity almost exclusively in the B and G fragments with a trace in the J fragment. These fragments are in the regions of the genome where termination of DNA synthesis occurs. (16). In the 10 minute sample the J fragment is more heavily labelled and radioactivity appears in the F fragment and faintly in the A, C and D fragments. Radioactivity is present in all fragments of the Form I DNA isolated after 20 minutes of in-

Figure 7. Hind II & III restriction endonuclease digestion of Form I DNA synthesized at various times. An *in vitro* synthesis mixture was prepared with [α^{32} P]dATP included. At various times during the incubation the viral DNA was centrifuged on CsCl propidium diiodide gradients (figure 5) the fractions containing the Form I DNA were grouped, processed as described (Materials and Methods), and digested with Hind II & III restriction endonucleases. The products of digestion were separated by electrophoresis and the radioactive bands visualized by autoradiography. The samples are from left to right: Uniformly labelled marker DNA, the 5,10, 20 minute products, marker DNA, 30,40,60 minute products.

cubation. The amount of radioactivity in all fragments continues to increase through the 60 minute course of the experiment. The results of this experiment indicate that all regions of the genome participate in the synthesis in vitro, and thus the in vitro system is not limited to completion of very advanced replicating molecules. In turn this means that the replicating chromosomes present in the nuclear extract are representative of the entire population of replicating chromosomes. The pattern of radioactivity in the digests of the 5 and 10 minute samples argue strongly against the incorporation of [α - 32 P] dATP into Form I DNA being the result of repair type synthesis. While this possibility cannot be completely excluded, if it were the principal mode of incorporation then all fragments would be expected to be labelled at all times of incubation, and this is clearly not the case. The results of this experiment also indicate that the pattern of synthesis in vitro appears to mimic that in vivo, i.e., synthesis is bidirectional and terminates in the B and G region of the SV40 DNA. The minimum time required to complete a relatively young replicating molecule in vitro appears to be between 10 and 20 minutes.

DISCUSSION

The data presented in figures 1 and 2 suggest that the SV40 chromosome replicates in a conformation more closely related to the "55 S" 100 Å nucleofilament structure than to the 300 Å compact form. Although this statement has an intuitive appeal it would stand on stronger ground if the comparison were made under conditions in which all the non-replicating SV40 chromosomes were present as the compact structure. In all our experiments both 80 and 50 S forms appeared in the sucrose gradient regardless of ionic strength of the gradients. This is not in agreement with the results of Varshavsky et al., who have reported a complete conversion of the 80S to the 50S form in gradients of low ionic strength (7). On the other hand, Christiansen and Griffith have reported the stability of the fast sedimenting form in low ionic strength gradients (6). It is possible the variation in extraction procedure is responsible for the discrepancy in these observations. It is also possible that viral coat proteins may be involved in the maintenance of the compact mini chromosome. Whatever the eventual outcome of this point, the description of the compact chromosome, and the likelihood that this structure exists in vivo raises the interesting question of how and if it participates in the replicative cycle of the virus. It is possible that the two forms exist in some equilibrium state in vivo, and replicating chromosomes are drawn from the pool of 50S chromosomes. Alternatively the

compact form may be the dominant species and there might be some active process required for the transition to the open configuration. The factors involved in the transition from one form to the other do not seem to function in the in vitro replication system. The data in figure 6 indicate that there is no net conversion of one form to the other during the incubation. While this might be taken as evidence for the gradient profile representing the equilibrium distribution of the two forms, the failure of any of the progeny Form I chromosomes to sediment as the 80 S form argues against the idea of a dynamic equilibrium between the two forms in vitro. This is interesting in view of the apparent presence in the incubation mixture of histones and other components necessary for the formation of superhelical DNA (see figures 5,6) (15). An elucidation of the factors that control the distribution of the two forms is relevant to the question of how the chromosomes enter replication and what controls the actual numbers of replicating chromosomes at any time. If the compact SV40 chromosome proves to have features in common with the 300 Å cellular chromatin fiber then the questions considered here will have an obvious relevance to the generation and maintenance of that structure and its entry into replication.

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