Replicative activity of histone-deficient SV40 chromatin

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ABSTRACT.

Histone-deficient SV40 chromatin, selectively radiolabeled in the DNA following the addition of cycloheximide to infected monkey cells, was compared with the normal 55S viral chromatin for its ability to serve as a template for a subsequent round of replication. After the removal of cycloheximide, the 26S histone-deficient SV40 chromatin was converted to apparently normal 55S chromatin. During this conversion, the chromatin which sedimented at 26-40S failed to replicate whereas the 44-55S chromatin contained a large fraction (28%) of newly replicated DNA molecules. Thus, the DNA in the 26S histone-deficient 40S chromatin cannot replicate without the prior and/or concomitant addition of protein which increases its sedimentation rate to 41-55S. Nevertheless, when compared with normal 55S viral chromatin, the histone-deficient SV40 chromatin had nearly a 3-fold greater probability of functioning as a template for a subsequent round of replication.

INTRODUCTION.

While it is apparent that histones play a role in the organization of eukaryotic cell DNA into nucleosomes, it is not clear whether this structure is involved in the function of chromatin as a template for transcription or replication. We have utilized the intracellular chromatin formed by SV40 virus to examine the relation of protein content to template activity for replication. During the replication of polyoma and SV40 DNA in productively infected cells, virtually all of the non-encapsidated viral DNA is associated with a discrete amount of protein (1,2). Recent studies have demonstrated that the polyoma and SV40 DNA-protein complexes have a structure closely resembling that of cellular chromatin, with nearly all of the associated protein being histones organized in the form of

nucleosomes (3-9). In the presence of inhibitors of protein synthesis, e.g., puromycin and cycloheximide, ongoing viral DNA synthesis is rapidly inhibited and considerably less protein (~50%) associates with the newly formed DNA (10,11). The resultant DNA contains fewer superhelical turns than are present in the DNA of virions or normal SV40 chromatin (11). In this study we examine the capacity of the histone-deficient SV40 chromatin to function as a template for a subsequent round of replication.

MATERIALS AND METHODS.

a. Virus and cell culture. Monolayer cultures of the TC-7 subline of CV-1 monkey cells (12) were grown and infected with SV40 virus (10 pfu/cell) as described previously (13).

b. Labeling and isolation of SV40 nucleoprotein (chromatin). SV40 infected cell cultures were labeled either with ^3H -thymidine (50 $\mu\text{Ci/ml}$) or ^{14}C -thymidine (1 $\mu\text{Ci/ml}$) at the indicated times post infection. Histone-deficient SV40 chromatin was selectively pulse-labeled with ^3H -thymidine added 2 hr after the addition of cycloheximide (10 $\mu\text{g/ml}$). In experiments designed to measure the replicative template activity of viral chromatin, ^3H -thymidine and cycloheximide were removed by washing the cultures twice with tris buffer saline. The plates were incubated for 30 min in unlabeled medium containing cycloheximide at 10 $\mu\text{g/ml}$. They were then washed twice and fresh medium containing 5-bromodeoxyuridine (BUdR, $1.3 \times 10^{-5}\text{M}$) and 5-fluorodeoxyuridine ($6 \times 10^{-5}\text{M}$) was added. The cultures were incubated at 37°C for designated periods, and SV40 chromatin was isolated by the Triton extraction method (1,13). The lysing mixture contained 0.4 M NaCl, thereby ensuring greater than 90% recovery of labeled viral DNA (13).

c. Analytical and preparative sucrose gradients. SV40 chromatin was sedimented through 5-20% sucrose gradients and fractions were collected and analyzed as described previously (14). The sucrose contained 0.4 M NaCl. Analytical runs were centrifuged for 90 min at 45,000 rpm at 4°C . A Spinco SW 50.1 rotor was utilized. Fractions were collected from the bottom of the tubes.

d. Buoyant density analysis of DNA in CsCl gradients. SV40 chromatin, purified by sucrose gradient centrifugation, was centrifuged to equilibrium in CsCl gradients and analyzed as described previously (15). The high CsCl concentration (1.75 g/ml) was sufficient to separate the DNA from the protein. Samples were centrifuged for 73 hr at 30,000 rpm at 21°C in a Spinco SW 50.1 rotor. For the alkaline CsCl gradient (Fig. 4), viral chromatin was incubated for 15 min at 21°C in 0.5 N NaOH prior to the addition of CsCl (1.75 g/ml) in 0.5 N NaOH. Refractive index measurements were made with a Bausch and Lomb refractometer immediately after the fractions were collected.

RESULTS.

African green monkey kidney cells (line TC-7) were infected with SV40 virus and pulse-labeled with ^3H -thymidine in the presence or absence of cycloheximide. SV40 chromatin was extracted by the Triton method (1) and analyzed for sedimentation rate by sucrose density gradient centrifugation (Fig. 1A). The viral chromatin that was synthesized in the presence of cycloheximide sedimented at ~26S as compared with the 55S chromatin formed in the absence of the drug (10,11,16). More prolonged treatment with cycloheximide reduced the rate of viral DNA synthesis by greater than the 90% value obtained in this experiment, but did not further reduce the sedimentation coefficient of the newly synthesized chromatin (our unpublished data). From its buoyant density in CsCl, we estimate that the 26S chromatin contains half the normal amount of protein (data not shown). Within 3 hr after removal of cycloheximide, most of the 26S chromatin was converted to 55S (Fig. 1B), as shown previously (11), and the resultant chromatin contained a normal ratio of protein to DNA (data not shown).

To determine whether the protein-deficient SV40 chromatin can function as a template for replication, infected cells were labeled with ^3H -thymidine in the presence of cycloheximide as above. The cells were washed free of cycloheximide and ^3H -thymidine, then incubated in the presence of 5-bromo-deoxyuridine (BUdR) for 30 or 60 min. At these times SV40 chromatin was extracted from the cells and the samples were sedi-

Figure 1. Sedimentation analysis of SV40 chromatin synthesized in the presence or absence of cycloheximide. (A) The sample contained SV40 chromatin prepared from infected cultures labeled either with ^{14}C -thymidine (1 $\mu\text{Ci/ml}$) from 24-45 hr p.i. ($\square-\square$) or with ^3H -thymidine (50 $\mu\text{Ci/ml}$) from 41-42 hr p.i. ($\bullet-\bullet$). The latter culture contained cycloheximide from 39-42 hr p.i. (B) The sample contained SV40 chromatin labeled with ^3H -thymidine as above ($\bullet-\bullet$) but prepared 3 hr after transferring the culture to fresh medium containing no cycloheximide or ^3H -thymidine. The ^{14}C -labeled chromatin present in the gradient ($\square-\square$) was prepared as described above.

mented in preparative sucrose gradients (Fig. 2). With increasing times after removal of cycloheximide, the SV40 chromatin attained

Figure 2. Preparative sucrose gradients containing SV40 chromatin from cells recovered from cycloheximide treatment. SV40 infected cultures were treated with cycloheximide from 41-44 hr p.i. and labeled with ^3H -thymidine from 43-44 hr p.i. The cultures were washed and transferred to fresh medium containing BUdR (see Materials and Methods) for periods of 30 min (A) or 60 min (B), at which times SV40 chromatin was isolated. The entire extracts were sedimented through 5-20% sucrose gradients, 0.2 ml fractions were collected into plastic tubes, and aliquots were analyzed for acid precipitable ^3H -DNA.

a more rapid and heterogenous sedimentation profile, gradually increasing from 26S to 55S. Fractions containing fast (41-55S) and slow (26-40S) sedimenting chromatin from the two sucrose gradients were pooled as indicated in Fig. 2, and the DNA in each sample was analyzed for buoyant density in CsCl gradients. The fast sedimenting chromatin obtained at 30 and 60 min after removal of cycloheximide contained 20% (Fig. 3B) and 28% (Fig. 3D) respectively, labeled DNA with a density greater than that of the "fully light" DNA (major peak). In contrast, no "heavy"

DNA was present in the slow complexes obtained at 30 min (Fig. 3A) and at 60 min (Fig. 3C) after removal of cycloheximide. Other experiments have indicated that BUdR does not significantly affect the sedimentation rate of SV40 chromatin (our unpublished data). Apparently, the 26S histone-deficient SV40 chromatin cannot replicate without the prior and/or concomitant addition of protein. The structure of the chromatin that is required for the initiation of viral DNA replication is currently under investigation.

As a control for the previous experiment, it is necessary to establish that the 41-55S "fast chromatin" was actually

Figure 3. Buoyant density analysis of "fast" and "slow" SV40 chromatin from infected cells recovered from cycloheximide treatment. The pooled fractions from the preparative sucrose gradients shown in Fig. 2 were analyzed by equilibrium density gradient centrifugation in CsCl gradients. (A) "Slow chromatin" from Fig. 2A; (B) "fast chromatin" from Fig. 2A; (C) "slow chromatin" from Fig. 2B; (D) "fast chromatin" from Fig. 2B.

derived from the 26S "slow chromatin" after the removal of cycloheximide. Otherwise, it could be argued that a residual pool of ^3H -thymidine was utilized for the preferential synthesis of 41-55S chromatin after the removal of cycloheximide. An aliquot of "fast chromatin" taken from the pools shown in Fig. 2A, was centrifuged to equilibrium in alkaline CsCl. As seen in Fig. 4, the ^3H -DNA had the same density as ^{14}C -labeled SV40 DNA obtained from cells which had not been exposed to BDUr. Since the ^3H -DNA strands were not heavier than the ^{14}C -DNA, they did not contain BUdR and were thus synthesized prior to its addition, i.e., in the presence of cycloheximide. This

Figure 4. Buoyant density analysis of denatured SV40 DNA from infected cells recovered from cycloheximide treatment. An aliquot of the SV40 "fast chromatin" pool (see Fig. 2A) was analyzed for buoyant density in an alkaline CsCl gradient. The ^3H -labeled chromatin ($\bullet$ — $\bullet$) was mixed with ^{14}C -labeled chromatin ($\square$ — $\square$) as marker (prepared as described in Fig. 1). The mixture was treated with 0.5 N NaOH, then centrifuged to equilibrium in alkaline CsCl.

experiment supports the above conclusion that the addition of protein to the 26S complex is a requirement for the replication of SV40 DNA in this chromatin.

The relative "template activity" of the histone-deficient SV40 complex was assessed by measuring the percentage of viral ^3H -DNA, labeled for 1 hr in the presence or absence of cycloheximide, that increased in density during a subsequent 6 hr incubation of the cells in medium containing BUdR. The Triton extracted viral DNA was analyzed for buoyant density as described in Fig. 3, and the results are summarized in Table 1. After 1 hr in the presence of BUdR, the SV40 DNA that was synthesized in the presence of cycloheximide had a nearly 3-fold greater frequency of replication ("template activity") than the DNA which had been synthesized in the absence of the drug. The difference in template activities diminished during the period from 1-3 hr after the addition of BUdR. During the subsequent 3 hr interval (from 3-6 hr) chromatin labeled in the presence or absence of cycloheximide had the same template activities, i.e., 9% (39%-30%) of the "cycloheximide DNA" replicated as compared with 8% (23%-15%) of the "normal" DNA.

From the results presented earlier (Fig. 1), most of the

TABLE 1. Relative Template Activity of Histone-Deficient SV40 Chromatin.

Time (hr) in BUdR	Template Activity of DNA Synthesized:*	
	- Cycloheximide	+ Cycloheximide
1	6	16
3	15	30
6	23	39

* Values are listed as the percentage of ^3H -DNA, labeled in the presence or absence of cycloheximide, which increased in density due to the incorporation of 5-bromodeoxyuridine.

Six cultures were pulse-labeled with ^3H -thymidine from 42-43 hr post-infection (p.i.) with SV40. Cycloheximide (10 $\mu\text{g}/\text{ml}$) was added to three plates at 40 hr p.i. At 43 hr p.i., the cultures were washed and transferred to medium containing BUdR (see Methods). SV40 chromatin was extracted at the designated times thereafter, and the DNA was analyzed for buoyant density in CsCl gradients.

histone-deficient viral chromatin was converted to "normal" 55S chromatin within 3 hr after the removal of cycloheximide. The fact that the template activities of the DNA formed in the presence or absence of cycloheximide became equal at this time is therefore an expected result. We conclude that the histone-deficient SV40 chromatin has a much greater probability of functioning as a template for replication than does the normal 55S viral chromatin.

DISCUSSION.

Histone-deficient SV40 chromatin was selectively labeled following the addition of cycloheximide to infected cells (10, 11). Upon removal of this inhibitor, newly synthesized proteins associated with the 26S viral chromatin, converting it to apparently normal 55S chromatin. In this study, we have investigated the capacity of the histone-deficient 26S chromatin to function as a template for replication after the removal of cycloheximide. Two major conclusions were reached: 1) the 26S histone-deficient SV40 chromatin cannot replicate without the prior and/or concomitant addition of protein which increases its sedimentation rate to 41-55S, and 2) the histone-deficient SV40 chromatin functions as a template for replication with nearly a 3-fold greater probability than the normal 55S chromatin during the first hour after the removal of cycloheximide.

The first conclusion rests on the assumption that the conversion of the 26S chromatin to 41-55S chromatin depends upon the addition of protein. This assumption is based on the following observations: 1) the increase in the rate of sedimentation occurs only after the removal of the inhibitor of protein synthesis, 2) the 55S chromatin which is derived from the 26S chromatin has a normal protein to DNA ratio, whereas the 26S complex has approximately half the normal amount of protein, 3) conversion of the 26S chromatin to 55S restores the normal superhelicity of the SV40 DNA. The introduction of superhelical turns probably occurs during the formation of nucleosomes 4) or similar nucleoprotein structures.

Several important questions emerge concerning the structure of the template which is utilized for replication. Whereas

the 26S chromatin apparently cannot replicate without the addition of some protein, it remains to be determined whether this protein must be added before or during the replication process. If it adds during DNA synthesis, then the 26S chromatin can initiate replication. Alternatively, if the protein must bind to the 26S chromatin before it can begin to replicate, it could then be concluded that some difference in the structure of the histone-deficient chromatin prevents it from initiating DNA synthesis. One known structural modification of the SV40 DNA molecule conferred by a high density of superhelical turns is the appearance of unpaired bases (17-21). It is of interest to determine whether local denaturation also occurs in chromatin containing DNA with a high superhelical density, and whether the unpaired bases occur in the vicinity of the replication origin.

The data in Table 1 indicate that, within 3 hr after removal of cycloheximide, 30% of the DNA molecules in the histone-deficient chromatin replicated. During this same period, essentially all of the 26S viral chromatin was converted to 55S chromatin. It may thus be concluded that replication per se is not a prerequisite for this conversion which, as stated earlier, probably involves the assembly of nucleosomes.

Given that the 26S histone-deficient viral chromatin cannot replicate without the addition of protein, it may appear paradoxical that this chromatin is selected as a template for replication with a greater probability than is the normal 55S chromatin (see Table 1). The following two working models are presented to help clarify this point.

- 1) In comparison to the 55S chromatin, the histone-deficient chromatin might have a greater affinity for some enzyme or factor required for the "initiation" of DNA replication, e.g. the T-antigen (22). The assay which we used to assess template activity for replication demanded that the ^3H -DNA increase detectably in density as a result of the incorporation of 5-bromodeoxyuridine. The term "initiation" as used herein must therefore refer operationally to any step which selects a given DNA molecule as a template for replication and which precedes the formation of detectably heavy ^3H -DNA.

- 2) Alternatively, the 26S chromatin may replicate with

a greater probability because the normal 55S chromatin is more rapidly removed from a replication pool. We have recently demonstrated that newly replicated SV40 DNA becomes relatively inactive as a template for replication within 5 hr after it was synthesized (15). The work of Garber, et al (23) suggests that this template inactivation results from the efficient and rapid packaging of SV40 chromatin into virions.

The above models are obviously not mutually exclusive, nor are they intended to include all possible explanations of the data. Further studies with histone-deficient SV40 chromatin will hopefully provide greater insight into the relation between chromatin structure and the many processes involved in its function as a template for replication.

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