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Biosynthetic Properties of a Polyoma Nucleoprotein Complex: Evidence for Replication Sites

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Under normal growth conditions, *all* of the newly synthesized polyoma deoxyribonucleic acid (py DNA) that could be extracted from infected mouse cell cultures by the Triton procedure of Green, Miller, and Hendler was in the form of a 55*S* nucleoprotein complex. Inhibition of protein synthesis by cycloheximide reduced the sedimentation rate of the polyoma complex synthesized during the first hour after addition of the drug to 25 to 35*S*. Since the 55*S* and the 25 to 35*S* complexes each contain closed circular 20*S* py DNA, it is suggested that the slower complex contains less protein per DNA molecule and that there is normally a small or unstable pool of protein available for binding to newly replicated py DNA. In the presence of cycloheximide, the newly formed 25 to 35*S* complex was not derived from preexisting 55*S* complex. Thus, some py DNA which was not solubilized by the Triton method served as a template for replication. Further evidence for the existence of polyoma replication sites is provided by the demonstration that, during the inhibition of protein synthesis, a class of newly replicated py DNA can be solubilized by the sodium dodecyl sulfate procedure of Hirt, but not by the Triton method. It is postulated that continuous protein synthesis is required to release py DNA from replication sites in the form of a Triton-extractable nucleoprotein complex.

It was recently shown by Green et al. that newly replicated polyoma deoxyribonucleic acid (py DNA) can be extracted from infected mouse cells in the form of a 55*S* nucleoprotein complex (9). Complete polyoma virions sediment at 240*S* (23). The extraction procedure entails the use of a nonionic detergent (Triton X-100) rather than the customarily used anionic detergent sodium dodecyl sulfate (SDS). The latter permits the separation of free 20*S* py DNA from cellular DNA (11). Employing the Triton extraction method, White and Eason obtained a similar nucleoprotein complex from simian virus 40 (SV40)-infected African green monkey kidney cells (22). In both studies, *all* of the viral DNA that was Triton-extractable sedimented as a fairly homogeneous nucleoprotein complex. The only other evidence for the complex being a specific one was based on reconstruction experiments, wherein purified viral DNA was put through the Triton extraction procedure in the presence of infected-cell extracts and was recovered with an unaltered sedimentation coefficient.

This report provides further evidence for the specificity of the 55*S* polyoma complex by demonstrating that its formation is strictly dependent on continuous protein synthesis. The question of whether the py DNA in the 55*S* complex can serve as a template for viral replication was also examined. Some py DNA is shown to be replicated from templates which are not solubilized by the Triton method. This and other evidence for the existence of viral replication sites will be presented.

MATERIALS AND METHODS

Cell culture and virus. A small-plaque strain of polyoma virus was used in these experiments (5). Polyoma virus was isolated from infected secondary mouse embryo cultures by the method described by Crawford (4). Virus stocks were concentrated by centrifugation and purified by one cycle of banding in CsCl equilibrium density gradients. Cultures of secondary mouse embryos were grown at 37 C in 90-mm plastic petri dishes (Nunc) containing Dulbecco's modified Eagle's medium plus 10% calf serum. When confluent, the cells were infected at a multiplicity of 10 to 20 plaque-forming units per cell, and were then incubated at 37 C in 5 ml of medium containing 1% horse serum.

Pulse labeling experiments. DNA was labeled for 1 to 2 hr by the addition of 5 to 10 μCi of ^{3}H-thymidine per ml (New England Nuclear Corp., ca. 15 Ci/mmole) to the growth medium at approximately 24 hr after infection. Unless otherwise specified, cycloheximide (Sigma Chemical Co.) was used at a concentration of 10 μg/ml. In the pulse-chase experiments, the radioactive medium was removed by aspiration, the

cells were washed once with warm tris(hydroxymethyl)aminomethane (Tris)-saline solution, and then warm medium was added back to the cultures. No unlabeled thymidine was used. In experiments designed to measure semiconservative replication of py DNA (10), 5-bromodeoxyuridine (BUdR) at 1.3×10^{-5} M and 5-fluorodeoxyuridine (FUdR) at 6×10^{-5} M were added 2 hr after the cultures had been returned to nonradioactive medium. This was found to be sufficient time to permit the exhaustion of radioactive pools.

Preparation of cell extracts. Duplicate plates were generally used for each assay. The cells were extracted either by Hirt's SDS procedure (11) or by the Triton method (9). Both methods permit the separation of viral DNA from cellular DNA. The SDS extracts were cleared of cellular DNA by centrifugation for 30 min at 20,000 rev/min in an SW39 or SW50.1 rotor (Beckman Instruments, Inc.); Triton extracts were cleared by centrifugation for 15 min at $1,000 \times g$.

Density gradient centrifugation. Neutral and alkaline sucrose gradients for analytical runs were prepared and analyzed as described previously (9). Neutral gradients were centrifuged for 120 min at 36,000 rev/min at 5 to 8 C in an SW39 or SW50.1 rotor; alkaline gradients (*p*H 12.5) were centrifuged for 150 min. Preparative neutral gradients were centrifuged for 150 min at 36,000 rev/min in an SW41 rotor. Up to 1 ml of sample was layered onto 11 ml of a 5 to 20% sucrose gradient. Samples for density measurements were mixed thoroughly with a solution of CsCl such that the final density was 1.75 g/cc in a volume of 4.2 ml. The solutions contained 0.01 M Tris-0.01 M ethylenediaminetetraacetate, *p*H 7.9. They were centrifuged for 63 hr at 34,000 rev/min at 20 C in an SW50.1 rotor. Samples were collected from the bottom of the tubes onto Whatman paper squares and were acid-precipitated in the same manner as for the sucrose gradients. During the collection of fractions, some drops were collected into tubes and analyzed for refractive index (Bausch & Lomb refractometer). These measurements were converted to density with the aid of published tables (19).

RESULTS

Sensitivity of py DNA synthesis to cycloheximide. As described previously (9), no free py DNA was extracted from infected mouse cells by the Triton method. All of the py DNA was in the form of a relatively homogeneous 55*S* complex with protein. Previous reports have demonstrated that cycloheximide interferes with the synthesis of py DNA and SV40 DNA, even when added after replication has begun (2, 14, 15). The following experiments demonstrate that the inhibition of protein synthesis by cycloheximide interferes with the formation of the polyoma 55*S* complex.

The data presented in Fig. 1 demonstrate that synthesis of py DNA and synthesis of cellular DNA have approximately the same sensitivity to cycloheximide. The kinetics of inhibition is shown in Fig. 1A, and the dose response, in Fig. 1B.

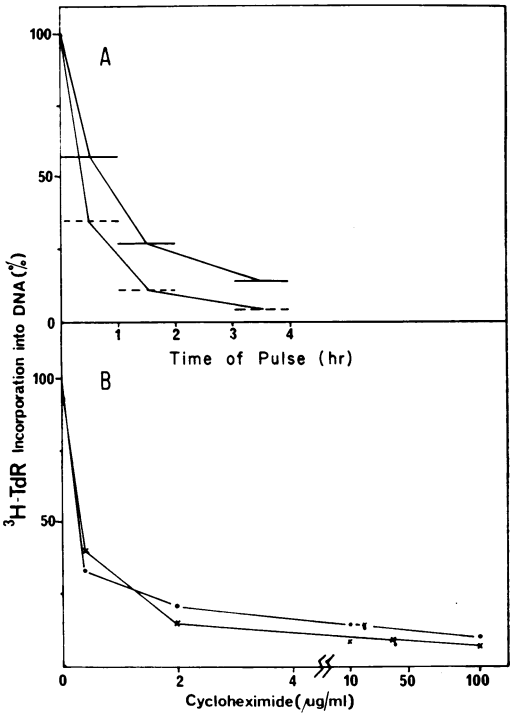

FIG. 1. *Inhibition of polyoma and cellular DNA synthesis by cycloheximide. (A) Kinetics of inhibition. At 24 hr after infection (t = 0), cycloheximide (10 μg/ml) was added to all but the control plates. The cultures were pulse-labeled with ^{3}H-thymidine (10 μCi/ml) for 1-hr intervals thereafter. Samples were prepared by the Triton extraction method, and portions of the resuspended pellet (cellular DNA) and the supernatant fluid (viral DNA) were precipitated and washed on Whatman paper squares with trichloroacetic acid. Solid and dashed lines indicate the duration of the ^{3}H-thymidine pulse and the relative rates of cellular and polyoma DNA, respectively. The rates of polyoma and cellular DNA synthesis in the absence of cycloheximide are taken as 100%. (B) Dose response. At 24 hr after infection, cycloheximide was added to cultures at concentrations ranging from 0 to 100 μg/ml. The cells were labeled with ^{3}H-thymidine (5 μCi/ml) from 26 to 28 hr after infection. Triton extracts were prepared and analyzed for polyoma DNA (●) and cellular DNA (×) as above.*

At the dose used for the kinetic study (10 μg/ml), the rate of protein synthesis was reduced to less than 10% of the control within 1 hr after addition of cycloheximide. This was determined by measuring the incorporation of ^{3}H-leucine into acid-precipitable material during 30-min pulses (data not shown).

The newly replicated, Triton-extractable py DNA synthesized during the first and second hours after the addition of cycloheximide is

apparently associated with less protein than is py DNA synthesized in the absence of the drug. In the presence of cycloheximide, most of the viral complex sedimented at 25 to 35*S*, as compared with 55*S* in the absence of the drug (Fig. 2). That the Triton-extracted material labeled in the presence of cycloheximide is actually py DNA was demonstrated by examining its sedimentation behavior in alkaline sucrose gradients. Over 80% of the ^{3}H-DNA sedimented at 53*S* (characteristic of form I py DNA), and the remainder sedimented at 16 to 18*S* (form II py DNA; 7, 20, 21). Typical sedimentation profiles are shown in Fig. 3. In contrast to the results of Branton et al. (2), cycloheximide did not prevent ring closure of py DNA synthesized in the presence of the drug.

These data lead to the conclusion that there is either a relatively small pool of protein available for binding to py DNA or that the pool is very unstable. It should be noted, however, that under normal growth conditions there is a sufficient pool of protein to cause *all* of the Triton-extractable py DNA to sediment at 55*S*. We shall return to this point in the Discussion section.

Polyoma complex after removal of cycloheximide. It was next asked whether the proteins synthesized after removal of cycloheximide would show preferential binding either to newly replicated py DNA (Fig. 4B) or to incomplete polyoma complex (25 to 35*S*) which had been formed in the presence of the drug (Fig. 4A). In Fig. 4A, py DNA was labeled with ^{3}H-thymidine from 60 to 120 min after the addition of cycloheximide. The cells were then washed and incubated in nonradioactive medium containing the drug. After 1 hr, the Triton-extractable polyoma complex still sedimented at 25 to 35*S*. A further 3-hr chase in medium lacking cycloheximide produced a broadening of the sedimentation profile and the appearance of a small amount of 55*S* polyoma complex. I cannot be certain that this 55*S* complex was derived from the 25 to 35*S* material present prior to the chase. The main point is that the great majority of the 25 to 35*S* complex failed to become 55*S* complex in 3 hr after removal of the drug. (The rate of protein synthesis recovered to 50% of control levels within 1 hr after removal of cycloheximide.)

In contrast, the experiment described in Fig. 4B demonstrates that nearly all of the Triton-ex-

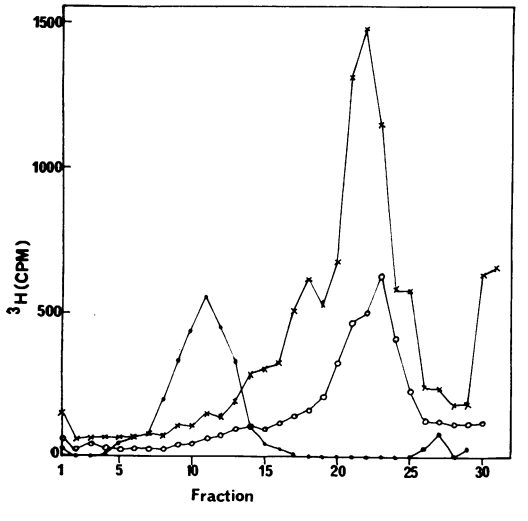

FIG. 2. *Effect of cycloheximide on the sedimentation of newly synthesized polyoma complex. Infected cells were labeled with ^{3}H-thymidine from 0 to 60 min (×) and from 60 to 120 min (○) after the addition of cycloheximide (10 μg/ml). Infected cells not exposed to the drug (●) were labeled with one-fifth the concentration of ^{3}H-thymidine (2 μCi/ml). Equal portions of the Triton extracts were centrifuged through neutral sucrose gradients.*

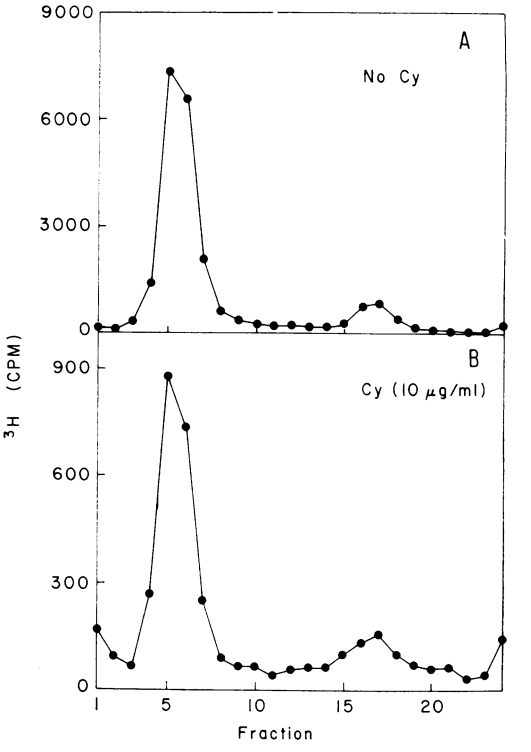

FIG. 3. *Analysis of Triton extracts in alkaline sucrose gradients. Infected cells were pulse-labeled for 2 hr with ^{3}H-thymidine (5 μCi/ml), either in the absence (A) or 2 hr after the addition (B) of cycloheximide (10 μg/ml). Marker py DNA (not shown) was run separately.*

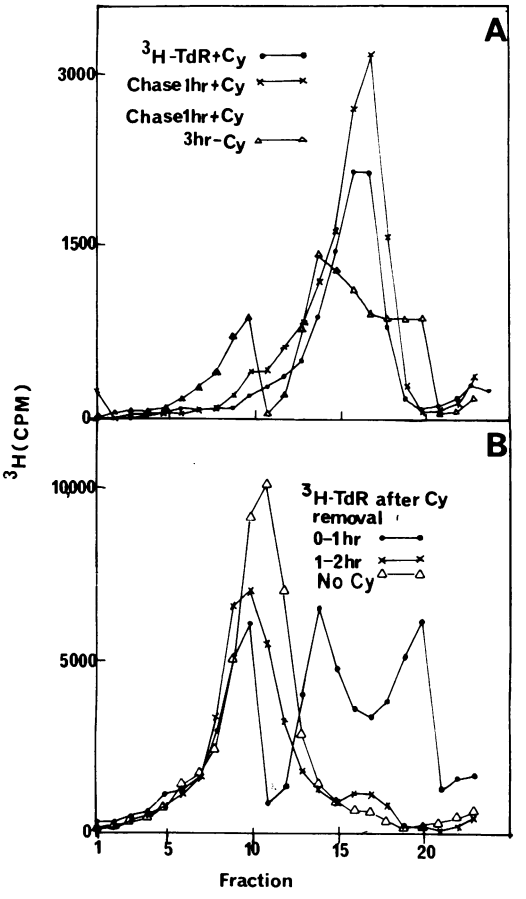

FIG. 4. *Preferential binding of protein to newly synthesized polyoma DNA. (A) Cycloheximide (10 μg/ml) was added to three plates of infected cells (t = 0). The cultures were labeled with ^{3}H-thymidine from t = 1 to 2 hr. Two plates were washed and incubated in unlabeled medium containing cycloheximide until t = 3 hr. One plate was washed free from the drug and incubated an additional 3 hr (t = 6 hr). Triton extracts were prepared at t = 2 hr (●), 3 hr (×), and 6 hr (△). (B) Parallel plates were incubated from t = 0 to 2 hr with cycloheximide. The drug was removed by washing, and the cultures were labeled with ^{3}H-thymidine from t = 2 to 3 hr (●) and 3 to 4 hr (×). A control culture was labeled without being exposed to the drug (△). Triton extracts were prepared and analyzed in neutral sucrose gradients.*

tractable py DNA synthesized from 1 to 2 hr after removal of cycloheximide sedimented at ~55S. It is thus apparent that there is a preferential binding of newly synthesized protein to py DNA synthesized after removal of the inhibitor rather than to py DNA that existed as a 25 to 35S complex while the drug was still present. The specificity of the addition of this protein to newly synthesized py DNA provides additional evidence for the natural occurrence of the 55S complex, and suggests that the interaction of the protein with py DNA may play an important role in viral development (see Discussion).

Stability of 55S polyoma complex in cycloheximide. In the previous experiment (Fig. 4), it was shown that slowly sedimenting polyoma complex (25 to 35S) was formed during the first hour after addition of cycloheximide (10 μg/ml). The results presented in Fig. 5 permit the conclusion that the 25 to 35S complex did not result from the rapid degradation of 55S polyoma complex due to the presence of the drug. In Fig. 5 A, polyoma-infected cells were labeled for 1 hr with ^{3}H-thymidine, chased for 2 hr with nonradioactive medium, and then treated either with cycloheximide, FUdR, or both for an additional 4 hr. Extracts were prepared by the Triton method and were analyzed by centrifugation in neutral sucrose gradients. The profiles indicate that a slight decrease in the sedimentation rate of the 55S polyoma complex may have occurred, for all samples treated with cycloheximide, and a small amount of heterodisperse material, appeared in the region of 30 to 45S. FUdR alone had no effect on the stability of the 55S complex. In the presence of cycloheximide, FUdR did not reduce the amount of 30 to 45S material formed. The appearance of the 30 to 45S material was therefore most likely due to partial breakdown of 55S complex caused by the inhibition of protein synthesis by cycloheximide. The stability of most of the preformed 55S complex during exposure to cycloheximide, however, precludes the possibility that the 55S complex is an unstable intermediate in the formation of 25 to 35S material.

The experiment described in Fig. 5B serves as a control to show that the small amount of heterodisperse complex (30 to 45S) which arises in the presence of cycloheximide is not due to the incorporation of residual ^{3}H-labeled precursors to DNA which remained in the cells after the 2-hr chase. Cultures were pulse-labeled for 1 hr with ^{3}H-thymidine and were chased for periods of 0, 2, or 4 hr. Then cycloheximide was added and the cells were incubated for an additional 4 hr. The amount of 30 to 45S material in the Triton extracts was the same for samples chased for 2 or 4 hr in nonlabeled medium, and was considerably greater in the sample which was not chased prior to addition of the drug (Fig. 5B). This indicates that the ^{3}H-labeled precursors to DNA are exhausted in less than 2 hr, and substantiates the conclusion drawn above.

Evidence for a polyoma replication site. From

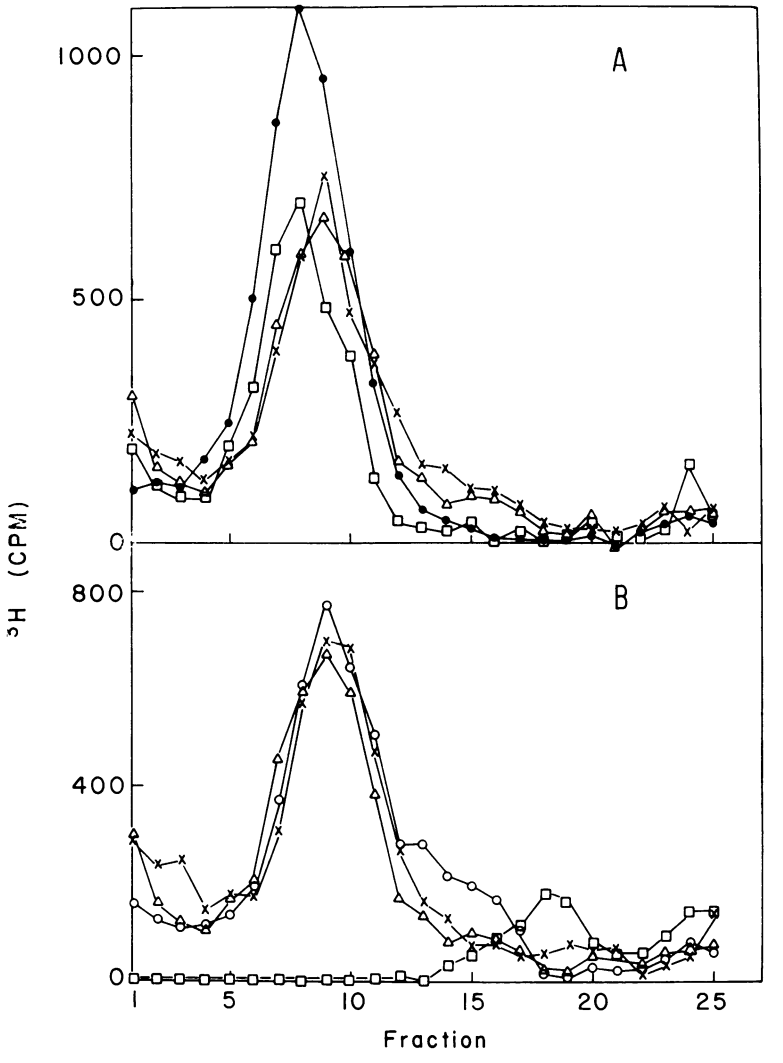

FIG. 5. *Effect of cycloheximide and FUdR on the 55S polyoma complex. (A) Infected cells were pulse-labeled for 1 hr with ^{3}H-thymidine and chased for 2 hr in unlabeled medium (●). Cultures were then chased for an additional 4 hr in the presence of: △, cycloheximide (10 μg/ml); □, FUdR (6 × 10^{-5} M); or ×, cycloheximide plus FUdR. Triton extracts were prepared and analyzed by neutral sucrose gradient centrifugation. (B) Parallel plates were pulse-labeled as above, and were then chased in unlabeled medium for 0 (○), 2 (△), or 4 hr (×) prior to the addition of cycloheximide and a further 4-hr incubation. One culture was labeled with ^{3}H-thymidine from 60 to 120 min after the addition of cycloheximide (□). Triton extracts were prepared and analyzed by neutral sucrose gradient centrifugation.*

the above experiments, it is evident that, whereas py DNA-protein complex sedimenting at 25 to 35*S* is formed in the presence of cycloheximide, the existing 55*S* polyoma complex is rather stable. The ^{3}H-DNA in the 55*S* complex did not detectably function as a semiconserved template for the production of 25 to 35*S* complex. This result suggested the possibility that the DNA in the 55*S* complex was unable to serve as a template for viral DNA synthesis. If this were true, the actual "replication pool" must be nonextractable by the Triton method, because under normal physiological conditions this method extracts only 55*S* complex.

To explore this question further, the following Meselson-Stahl type of experiment (17) was performed. It specifically examined whether prelabeled ^{3}H-DNA in 55*S* complex could "re-

cycle" as a template (incorporate BUdR) in the presence of cycloheximide, yet persist as a Triton-extractable 55*S* complex. The presence of cycloheximide insured that no new 55*S* complex was formed from some pool of py DNA that is not Triton-extractable (see Fig. 2). After the onset of polyoma replication, cells were pulse-labeled with ^{3}H-thymidine for 1 hr, and then chased in nonradioactive medium containing BUdR in the absence (Fig. 6A) or presence (Fig. 6B) of cycloheximide. The incorporation of BUdR into ^{3}H-DNA ("recycled" molecules) in the Triton-extractable 55*S* complex was compared with the incorporation of BUdR into total 20*S* py ^{3}H-DNA extracted by Hirt's SDS procedure (11). The recycled DNA molecules were detected by virtue of their increased buoyant density in CsCl, which resulted from the incorporation of BUdR. In Fig. 6C, 25 to 35*S* py complex was labeled with ^{3}H-thymidine in the presence of cycloheximide, and was assayed for its ability to incorporate BUdR during a chase after removal of cycloheximide. Again, duplicate plates were extracted with either Triton or SDS.

Preparative sucrose gradients were run on the Triton and SDS extracts, and samples were acid-precipitated. The profiles are depicted in Fig. 6. Appropriate fractions from the six gradients were pooled, as indicated by the brackets in Fig. 6, and were subjected to analysis by CsCl equilibrium density gradient centrifugation (Fig. 7). Each of these pooled samples contained at least 80% form I py DNA, based on sedimentation analyses in alkaline sucrose gradients (data not presented). Incorporation of BUdR into py DNA increased the buoyant density by 0.05 g/cc, the same amount found by Hirt for semi-conservatively replicated py DNA (10).

The radioactivity in the recycled, heavy-light (HL) DNA and in the nonreplicated (LL) DNA for each of the six samples is summarized in Table 1. The percentage of HL DNA extracted by Triton (55*S* polyoma complex) and by SDS (20*S* py DNA) was quite similar when BUdR was incorporated in the absence of cycloheximide (Fig. 6A). However, when the synthesis of the 55*S* polyoma complex was blocked by cycloheximide during the incorporation of BUdR, the percentage of recycled 20*S* py DNA molecules in the SDS extract was three times greater than that in the Triton-extracted 55S complex (Fig. 6B). The slower-sedimenting material from this Triton extract was not analyzed because it was shown to result from the breakdown of 55*S* complex (see Fig. 5).

Figure 7C shows that the Triton-extractable 25 to 35*S* polyoma complex formed in the pres-

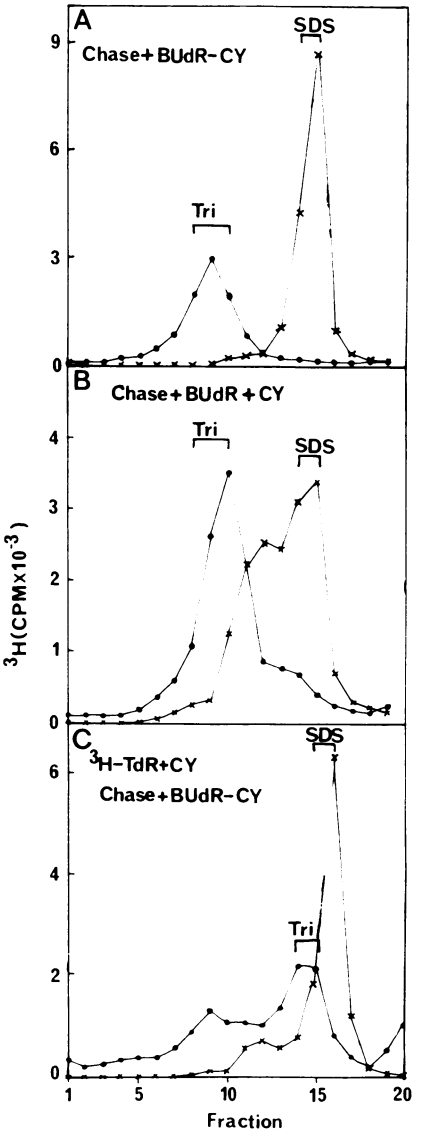

FIG. 6. *Preparative neutral sucrose gradients. (A) Infected cultures were pulse-labeled for 1 hr with ^{3}H-thymidine and chased for 3 hr in medium containing BUdR. (B) Pulse-labeled cultures were chased for 3 hr in medium containing BUdR and cycloheximide. The latter was added 15 min prior to adding BUdR. (C) Infected cells were labeled from 60 to 120 min after the addition of cycloheximide. They were then washed free from the drug and chased for 2.25 hr in medium containing BUdR. Duplicate cultures from parts A to C were either extracted by the Triton method (●) or the SDS method (×), and the supernatant fluids were sedimented through neutral sucrose gradients. The brackets designate the fractions from the six gradients which were separately pooled and analyzed in CsCl equilibrium gradients (Fig. 7).*

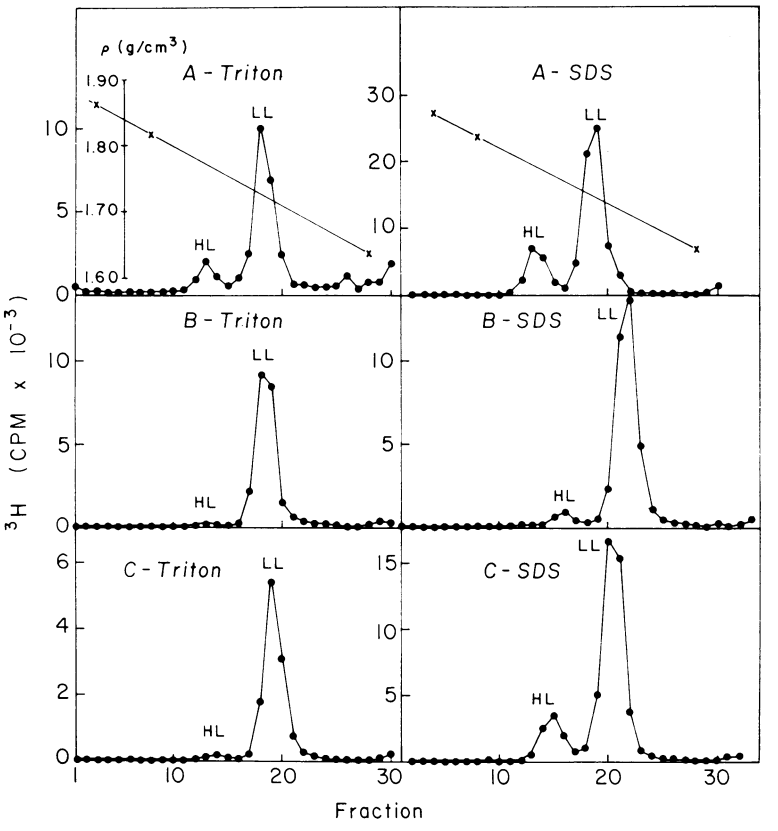

FIG. 7. *CsCl density gradient equilibrium centrifugation. Each of the six samples described in Fig. 6 was centrifuged to equilibrium in CsCl. Panels A to C are consistent with experiments A to C as described for Fig. 6.*

TABLE 1. *Solubilization of recycled polyoma DNA (HL) by Triton vs. SDS*

Expt[a]	Extraction method	HL (counts/min)	LL (counts/min)	Percent HL
A	Triton	4,425	21,848	16.8
	SDS	16,614	58,702	22.1
B	Triton	501	21,256	2.30
	SDS	2,318	32,512	6.66
C	Triton	471	11,041	4.09
	SDS	8,611	40,773	17.4

[a] Experimental conditions are described in the legend to Fig. 6.

ence of cycloheximide contained one-fourth as much recycled (HL) py DNA as did the 20*S* py DNA extracted by SDS. It should be noted that, in contrast to Fig. 7B, incorporation of BUdR occurred in the absence of cycloheximide. Since it was shown earlier (Fig. 4A) that the 25 to 35*S* complex continues to sediment at that rate after removal of cycloheximide, it would appear that, once the nucleoprotein complex has formed, the DNA becomes a relatively inefficient template for further replication.

The three- to fourfold differences in the solubilization of recycled py DNA by Triton and SDS in Fig. 7B and C are minimal estimates for two reasons: (i) the SDS extract contains probably all of the nonreplicated (LL) py ^{3}H-DNA found in the Triton extract, and (ii) there is trailing of radioactivity from LL DNA into the HL region of the CsCl gradient.

From the data shown in Fig. 6B, the Triton-extractable ^{3}H-DNA amounted to 87% of the total py ^{3}H-DNA extracted by SDS. (The heavy shoulder for the SDS-extracted material was shown to be py DNA by alkaline sucrose gradient analysis. I have no explanation for this abnormal profile.) Correcting for the contribution of HL DNA that was Triton-extractable, the SDS extract must contain a small "replicated pool" of py DNA molecules (13% of the total py ^{3}H-DNA), which is not Triton-extractable and which contains 36% HL DNA. This value is to be compared with the less than 2.3% HL DNA found in the Triton-extractable 55*S* com-

plex. Thus, the DNA in the 55*S* complex did not serve as a template and remain as a Triton-extractable 55*S* complex by preferential binding to the limited pool of protein caused by cycloheximide. This experiment does *not* exclude the possibility that the DNA in the 55*S* complex can act as an efficient template for replication. However, if it does so in the presence of cycloheximide, most of the newly synthesized DNA becomes nonextractable by the Triton method.

Parts A and B of the pulse-chase experiment described in Fig. 6 were repeated with one modification. The entire Triton and SDS extracts (supernatant fluids) were analyzed in CsCl equilibrium density gradients without prior fractionation by sedimentation through sucrose gradients. The results were essentially identical to those presented in Table 1, confirming the conclusion that the py ^{3}H-DNA that recycled in the presence of cycloheximide was SDS-extractable, but not Triton-extractable. The recycled py DNA molecules therefore must have been in the Triton pellet fraction, sedimentable by centrifugation at 1,000 × *g* for 15 min.

In the following experiment, a variety of extraction conditions were used to analyze which factors play a role in the solubilization of py DNA that recycles in the presence of cycloheximide. As in Fig. 6B, polyoma-infected cells were pulse-labeled for 1 hr with ^{3}H-thymidine, and were then transferred to nonradioactive medium containing cycloheximide and BUdR. Cleared extracts were prepared and analyzed by equilibrium density gradient centrifugation in CsCl. As in Fig. 7, essentially complete separation was achieved for recycled (HL) DNA and nonreplicated (LL) DNA. The results are summarized in Table 2.

It is apparent that the solubilization of recycled py DNA (HL) is dependent on both the nature of the detergent and the ionic strength. Whereas Triton in 0.2 M NaCl extracted only 8.9% of the HL DNA capable of being extracted by Hirt's method (0.6% SDS, 1.0 M, NaCl), Triton in 1.0 M NaCl extracted 105%, Sarkosyl in 0.2 M NaCl extracted 126%, and deoxycholate in 0.2 M NaCl extracted 54% of the HL DNA. A mild, nonionic detergent, Nonidet, extracted only 5% of the HL DNA in 0.2 M NaCl. Repeated freeze-thaws of the Triton extract in low salt (sample 4, Table 2) promoted the release of 27% of the HL DNA. Since this procedure is known to disrupt intact nuclei, the solubilization of recycled py DNA is dependent on more than the physical disruption of the nuclear membrane. The results support the hypothesis that the recycled py DNA synthesized in the presence of cycloheximide remains associated with nuclear membrane.

DISCUSSION

The pulse-chase experiment summarized in Table 1 demonstrates several points and raises at least as many questions. It is apparent that semiconservative polyoma replication (recycling) occurs in the presence of cycloheximide. The recycled py DNA can be solubilized by Hirt's SDS extraction method (11), but very little of it was extractable by the Triton method (9). When the chase is carried out in the absence of cycloheximide, both extraction methods solubilize nearly the same fraction of recycled viral DNA. To account for these results, I propose that there are sites for py DNA synthesis, probably on the nuclear membrane (3, 13), and that continuous protein synthesis is required for the release of viral DNA from these replication sites. The py DNA is released from the sites in the form of a Triton-extractable nucleoprotein complex. The need for *continuous* protein synthesis to

TABLE 2. *Extraction conditions for the solubilization of recycled polyoma DNA (HL)*[a]

No.	Extraction conditions	HL (counts/min)	LL (counts/min)	HL/total (%)	HL/HL SDS (%)
1	0.6% SDS, 1.0 M NaCl	2,643	26,087	9.2	100
2	0.25% Triton, 0.2 M NaCl	234	14,119	1.6	8.9
3	0.25% Triton, 1.0 M NaCl	2,777	38,936	6.7	105
4	0.25% Triton, 0.2 M NaCl; freeze-thaw three times	715	19,787	3.5	27
5	0.5% Deoxycholate, 0.2 M NaCl	1,435	16,353	8.1	54
6	0.5% Nonidet, 0.2 M NaCl	133	9,685	1.4	5.0
7	0.5% Sarkosyl, 0.2 M NaCl	3,210	30,606	9.5	126

[a] Experimental conditions were the same as those described in the legend to Fig. 6B. The Sarkosyl extract (No. 7) was cleared of cellular DNA under conditions used for SDS extracts; samples 2 to 6 were cleared under conditions used for Triton extracts (see Materials and Methods). Sample 4 was extracted by the standard Triton Method, and then was frozen and thawed three times prior to the clearing spin.

release recycled DNA may be due to the small or unstable pool of protein that is used for forming the 55*S* nucleoprotein complex (see Fig. 2).

An interesting paradox emerges from the two ways I have examined polyoma replication in the presence of cycloheximide. On the one hand, pulse-labeling experiments indicated that newly synthesized viral DNA was Triton-extractable in the form of a 25 to 35*S* complex. On the other hand, pulse-chase experiments showed that after a second cycle of replication (as measured by the incorporation of BUdR into py DNA which had incorporated ^{3}H-thymidine during a previous round of replication) the recycled viral DNA was extractable by SDS, but not by Triton. The following models are examples of how this apparent discrepancy can be formally explained.

(i) Temporal exclusion: In the pulse-chase experiment, nearly all of the ^{3}H-DNA may leave the replication sites during the 2-hr chase prior to the addition of cycloheximide and BUdR. Owing to the limited availability of protein required for the release of py DNA from the sites, a small fraction of newly replicated DNA can be released in a Triton-extractable form, but prelabeled viral DNA cannot enter the replication sites until vacancies occur. By this time, insufficient protein is available for the recycled DNA to become Triton extractable, even though it replicates.

(ii) Separate replication sites: Pulse-labeled py DNA replicates at sites A and B; recycled py DNA molecules replicate only at site B. Under the conditions of limited protein synthesis caused by cycloheximide, only those DNA molecules which replicate at site A become Triton extractable.

Is it possible that, in the presence of cycloheximide, the pulse-labeled Triton-extractable py DNA reflects mostly repair synthesis, whereas the pulse-chase experiment with BUdR measures semiconservative replication? This seems very unlikely, because the pulse-labeled DNA enters only the 25 to 35*S* polyoma complex and not the 55*S* complex. Prior to the pulse, the cells contain a much greater amount of 55*S* complex than 25 to 35*S* complex (*unpublished data*), so there could not be a significant amount of ^{3}H-thymidine incorporation due to random repair of these Triton-extractable complexes.

It is not known whether the inhibition of py and cellular DNA synthesis by cycloheximide is due to the inhibition of protein synthesis or to some side effect of the drug. Nevertheless, it is apparent that the formation of the 55*S* polyoma nucleoprotein complex is dependent on continuous protein synthesis. During the first hour after adding cycloheximide, nearly all of the newly synthesized, Triton-extractable polyoma nucleoprotein complex sedimented at 25 to 35*S*. It would appear that there is either a rather small pool of protein available for binding to py DNA, or that the protein pool is very unstable. Yet, there is sufficient protein to form a fairly homogeneous 55*S* complex with *all* of the newly replicated, Triton-extractable py DNA under normal growth conditions. These results suggest that there is a stoichiometric relation between py DNA and the protein(s) included in the 55*S* complex. This might occur if the concentration of viral DNA governed the rate of synthesis of these proteins, or if the concentration of these proteins regulated the rate of viral DNA synthesis, or if both were true. It is of interest to note that mutants of bacteriophages ϕX174 and λ which are defective in the production of complete head structures are also unable to synthesize mature progeny DNA molecules (6, 12, 16).

The nature of the protein(s) in the polyoma 55*S* complex is as yet uncertain. Preliminary data (M. H. Green, *unpublished data*) suggest that most of the protein is the major polyoma capsid protein, P2 (18). This conclusion is based primarily on electrophoretic analyses in polyacrylamide gels. A 46*S* nucleoprotein complex has been obtained by mild alkaline degradation of SV40 virions (1, 8). This complex contains the three minor polypeptides (VP4, 5, and 6) of the virion (8). Both the naturally occurring polyoma 55*S* complex and the synthetic SV40 complex have the interesting property of serving as a relatively inefficient template for ribonucleic acid synthesis by the *Escherichia coli* ribonucleic acid polymerase when compared with purified viral DNA (8; M. H. Green, *unpublished data*).

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ADDENDUM IN PROOF

It has recently been reported that a polyoma DNA-protein complex sedimenting at 25*S* can be extracted

from infected mouse cells by a method utilizing 0.25% sodium deoxycholate (P. Bourgaux and D. Bourgaux-Ramoisy, Nature [London] **235**:105–107, 1972). In the presence of puromycin, newly replicated py DNA apparently had less protein associated with it, in support of conclusions presented herein. It should be noted, however, that 0.5% deoxycholate has been shown to degrade the Triton-extractable 55s py DNA-protein complex to a 20 to 25*S* form (9).

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