Formation of Cellular Deoxyribonucleic Acid During Productive Polyoma Virus Infection

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It was previously shown that the majority of deoxyribonucleic acid (DNA) made in growing mouse embryo cells productively infected at low multiplicity with polyoma virus is cellular in nature and that some of this cell DNA contains discontinuities in the newly synthesized strand. Evidence obtained indicates the following. (i) Induction of cell DNA synthesis precedes the onset of detectable viral DNA replication by approximately 3 hr. (ii) Double-stranded cell DNA molecules, discontinuous in the newly synthesized strands, arise by direct synthesis (rather than by degradation of a high-molecular-weight precursor) only in the cell DNA replicated after initiation of viral DNA synthesis. (iii) This DNA component is continuously formed throughout the "late" stage of infection and is continuously converted into apparently normal cell DNA of high molecular weight without prior degradation to acid-soluble components.

One of the effects of polyoma virus infection is the disruption of the normal pattern of host cell deoxyribonucleic acid (DNA) replication. At high input multiplicities of infection, the synthesis of cell DNA in productively infected cells is totally blocked, concomitant with the onset of synthesis of progeny viral DNA (27, 30). In contrast, polyoma may also stimulate the formation of cell DNA under conditions of productive infection at low input multiplicity (3, 5, 8, 30, 34, 36, 40) and under conditions of nonproductive infection (14, 27, 28, 39).

The work of Dulbecco et al. (9), Ben-Porat et al. (3), and Branton and Sheinin (5) showed that the cell DNA synthesized "early" in productively infected cells [like that made in nonproductive infection (14, 27)] is normal in its chromatographic behavior on columns of methylated-albumin-kieselguhr. However, a significant proportion of the cell DNA made "late" during productive infection chromatographed on such columns in the pattern of DNA of heterogeneous molecular weight considerably lower than that of normal cell DNA (3, 5). In analyses made by using velocity sedimentation in neutral and alkaline sucrose density gradients and DNA:DNA hybridization (6), it was found that this DNA is comprised of molecules that are double-stranded

but that contain discontinuities in the newly synthesized strand.

In the present work, some of which was described in a preliminary report (Cheevers et al., *Bacteriol. Proc.*, p. 195, 1970), studies on the formation of cell DNA during productive polyoma infection were continued. It was shown that the induction of cell DNA synthesis is an "early" event, in that it precedes the initiation of progeny viral DNA formation by approximately 3 hr and that the cell DNA made "early" is normal. Discontinuous cell DNA molecules, detected by their sedimentation properties in alkaline sucrose (6), were shown to arise by direct synthesis only in the cell DNA replicated after initiation of polyoma DNA synthesis. This newly synthesized DNA was converted into high-molecular-weight cell DNA. It was concluded that the pattern of synthesis of cell DNA is altered "late" in productive infection by a process associated with the replication of viral DNA.

MATERIALS AND METHODS

Procedures for cultivation and infection of mouse embryo cells. Primary explants of mouse embryo cells were grown to confluence in 32-oz (ca. 960 ml) Brockway bottles in medium 1066 (21) supplemented with 10% (v/v) fetal calf serum (Hyland Laboratories, Los Angeles, Calif.) and were subcultured for experiments into 15- by 60-mm petri dishes (Falcon Plastic, Div., Brockway Glass Co., Inc.) at a concentration of 1.5×10^6 to 2×10^6 cells/dish, as previously described (24, 25). Secondary cultures were incubated at

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37 C in an atmosphere of 5% CO₂-95% air for 24 to 36 hr, at which time the cells were approaching the end of exponential growth. Cells were infected with polyoma TSP1 (31) at an estimated input multiplicity of 1 to 10 plaque-forming-units per cell or were mock-infected as previously described (5).

Conditions for labeling preexistent cell DNA. The growth medium of secondary cultures was replaced at least 4 hr after subculture with medium containing ¹⁴C-thymidine (TdR; 0.1 μ Ci/ml; 54 mCi/mole). The cultures were allowed to double at least once, but usually two or three times, in the labeled medium. The cells were then washed with phosphate-buffered saline (PBS; 9) and infected with polyoma virus as described above.

Measurement of DNA synthesis. The synthesis of DNA in uninfected and polyoma-infected cells was measured in terms of incorporation of ³H- or ¹⁴C-labeled TdR (Amersham/Searle) into trichloroacetic acid-insoluble material. The conditions for isotopic labeling of DNA are given with each experiment. After each labeling period, the cultures were washed with ice-cold PBS, and were treated for 5 min with trypsin (Grand Island Biological Co., Grand Island, N.Y.) at 0.1% (w/v) in saline-citrate solution (24) to disperse the cells. The cells were washed with cold PBS and suspended in standard saline citrate (SSC; 18) for measurement of total radioactive trichloroacetic acid-insoluble material or for velocity sedimentation analysis.

Analysis of incorporation of TdR into total DNA. For measurement of the total radioactive acid-insoluble fraction of infected and mock-infected cultures, cell suspensions were lysed by addition of sodium dodecyl sulfate (SDS) to a final concentration of 1% (w/v). This extract was then precipitated with an equal volume of 10% (w/v) trichloroacetic acid, and the labeled acid-insoluble fraction was trapped on membrane filters (Millipore Corp., Bedford, Mass.) and assayed as previously described (8).

Velocity sedimentation analysis of DNA by neutral sucrose density gradient centrifugation. The nature of DNA newly synthesized in mouse-embryo cultures at various times after infection with polyoma virus (or after mock-infection) was determined by velocity sedimentation analysis of unpurified SDS-extracts of cells in neutral sucrose density gradients, as previously described (6; Branton, Cheevers, and Sheinin, *Virology*, *in press*). Cell suspensions were mixed with one-tenth volume of 10% (w/v) SDS in SSC, and the DNA extracts were layered over 22-ml 15 to 30% (w/w) sucrose density gradients in 0.1 M NaCl-0.001 M ethylenediaminetetraacetic acid (EDTA)-0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 7.4-0.5% (w/v) SDS, formed over a 5-ml cushion of 70% sucrose. The extracts were centrifuged in a Spinco SW25.1 rotor at 23,500 rev/min for 15 hr at 25 C. The gradients were fractionated into five-tenths-ml portions by using an Isco model D density-gradient fractionator, equipped with an Isco model UA-2 ultraviolet analyzer for continuous monitoring of optical density at 254 nm. The sedimentation properties of radioactively labeled DNA molecules were assigned on the basis of the properties of the 18S and 28S

ribosomal ribonucleic acid (RNA) components, detected by their ultraviolet absorption.

Velocity sedimentation analysis of DNA by alkaline sucrose density gradient centrifugation. The size distribution of pre-existent cell DNA and of DNA newly synthesized in infected and mock-infected cells was examined by velocity sedimentation analysis in alkaline sucrose density gradients by using the procedures adopted by Sambrook et al. (23). Density gradients ranging from 10 to 30% (w/w) sucrose in 0.5 M NaCl-0.3 M NaOH-0.01 M EDTA were prepared in polyallomer tubes, and 1 ml of 0.5 M NaOH-0.1 M EDTA was layered onto the gradients. A suspension of 5×10^6 to 5×10^8 cells in 0.2 ml of SSC was carefully added to the upper layer; the tubes were sealed in an SW25.1 or SW27 rotor and kept at 4 C for at least 10 hr. The gradients were centrifuged in a Spinco model L2-50 ultracentrifuge at 25,000 rev/min for 5 hr at 4 C and then were fractionated into 0.5- or 1-ml portions as described above.

Representative data obtained by this procedure with normal cells are shown in Fig. 1b. The cell DNA, denatured to single strands by the alkaline conditions, sedimented homogeneously at about 105S. Figure 1a shows the sedimentation characteristics in alkaline sucrose (under similar conditions) of ¹⁴C-labeled DNA extracted from polyoma virions, harvested from mass cultures after many cycles of infection, and purified by the procedure of Blackstein, Stanners, and Farmilo (4). The DNA was separated into two components. On the basis of the known characteristics of sedimentation in alkaline sucrose of the various DNA constituents of polyoma particles (4), the following is concluded. (i) Slowly sedimenting material, near the top of the gradient (fractions 1 to 13), contains the unresolved denatured forms of non-supercoiled viral DNA and pseudovirion cell DNA (4). (ii) The more rapidly sedimenting homogeneous component is composed of supercoiled polyoma DNA molecules in the double-stranded cyclic coil configuration (4, 11, 23, 33, 35, 37). Denatured cell DNA may be separated from all forms of polyoma DNA under the conditions of centrifugation described in Fig. 1.

RESULTS

It was previously shown (6) that in mouse embryo cells, productively infected with polyoma virus at low multiplicity, some of the cell DNA made "late" was not normal. Although double-stranded, this DNA was discontinuous in the newly synthesized strand. It was the objective of the present studies to assess the origin and fate of this DNA, detected by its pattern of heterogeneous sedimentation in alkaline sucrose, and to examine its formation in relation to the induction of cell DNA synthesis and the replication of viral DNA.

Time-course of cell and viral DNA synthesis during productive polyoma infection. To determine the relationship between the induction of cell DNA synthesis and viral DNA replication under the conditions of infection employed, the follow-

FIG. 1. Velocity sedimentation analysis in alkaline sucrose density gradients of polyoma virus DNA and mouse embryo cell DNA. *a*, Polyoma virions (TSP1), labeled in DNA with ^{14}C -TdR by procedures analogous to those already described (27), were purified by the procedure defined by Blackstein et al. (4). Purified virus, suspended in 0.1 M NaCl-0.001 M EDTA-0.01 Tris, pH 7.4, was treated with 1% (w/v) SDS at 60 C for 15 min to release the DNA. The ^{14}C -labeled DNA was analyzed in an alkaline sucrose density gradient as described. Recovery of DNA in such gradients was at least 95%. *b*, Secondary cultures of mouse embryo cells were grown for one generation period in medium containing ^{14}C -TdR (0.1 $\mu\text{Ci}/\text{ml}$; 54 mCi/mmol), and the labeled DNA was analyzed in an alkaline sucrose density gradient as described. Recovery in such gradients was at least 95%.

ing experiment was performed. Polyoma-infected or mock-infected mouse embryo cells were incubated with medium containing ^3H -TdR during various 3-hr intervals after infection, to label newly synthesized DNA. The amount of ^3H -TdR incorporated into total (unfractionated) DNA was determined as described above. The proportions of cell and viral DNA were determined from velocity sedimentation profiles in neutral

sucrose-density gradients, analogous to those shown in Fig. 2.

The ^3H -labeled DNA synthesized during any interval in mock-infected cells cosedimented with ^{14}C -labeled marker cell DNA in a profile similar to that shown in Fig. 2a. The sedimentation characteristics of DNA synthesized in polyoma-infected cells varied, depending upon the time after infection at which this DNA was examined. All of the ^3H -labeled DNA newly synthesized during any interval up to 18 hr postinfection (PI) sedimented as did cell DNA (Fig. 2a). The sedimentation distribution of ^3H -labeled DNA synthesized in infected cells at any time after 18 hr PI was similar to that shown in Fig. 2b. This DNA was comprised of cell DNA, which cosedimented into the 70% sucrose cushion with the ^{14}C -labeled cell DNA marker, as well as supercoiled viral DNA sedimenting at 20S (6; Branton et al., *Virology*, *in press*).

The results of total incorporation studies and calculations of the rate of formation of cell and viral DNA are shown in Table 1. The total incorporation studies showed that the formation

FIG. 2. Representative profiles of DNA of polyoma-infected cells sedimented in neutral sucrose density gradients. Secondary cultures of mouse embryo cells were infected with polyoma virus or were mock-infected as described. At 24 hr PI, the growth medium of (a) mock-infected and (b) infected cultures was replaced with medium containing ^3H -TdR (10 $\mu\text{Ci}/\text{ml}$; 15 Ci/mmol). At 30 hr, replicate cultures (approximately 2×10^7 cells) were harvested by using trypsin treatment. Suspensions of ^3H -labeled cells were mixed with uninfected cells that were incubated for one generation period with medium containing ^{14}C -TdR (0.1 $\mu\text{Ci}/\text{ml}$; 54 mCi/mmol) to provide marker cell DNA. Mixed cell suspensions were treated with 1% (w/v) SDS and were analyzed without further purification by velocity sedimentation in neutral sucrose density gradients as described. Symbols: $\bullet$, newly synthesized ^3H -labeled DNA; $\circ$, ^{14}C -labeled marker cell DNA. Recovery of ^3H -labeled DNA was 95.3 and 97.2%, respectively, for mock-infected cultures and polyoma-infected cultures.

TABLE 1. *Kinetics of synthesis of cell and viral DNA in polyoma-infected cells*

Labeling period (hr PI)	Incorporation of ^3H -TdR into the DNA of					
	Uninfected cells (counts per min per culture) ^a	Infected cells				
		Total DNA counts per min per culture ^a	Cell DNA		Viral DNA	
			Total DNA ^b (%)	Counts per min per culture ^c	Total DNA ^b (%)	Counts per min per culture ^c
0-3	47,435	47,894	100	47,894	Undetectable	Undetectable
3-6	55,762	50,706	100	50,706	Undetectable	Undetectable
6-9	36,307	28,848	100	28,848	Undetectable	Undetectable
9-12	27,794	28,129	100	28,129	Undetectable	Undetectable
12-15	18,004	16,204	100	16,204	Undetectable	Undetectable
15-18	21,609	25,489	100	25,489	Undetectable	Undetectable
18-21	19,426	29,916	97	29,018	3	898
21-24	20,749	39,423	95	37,452	5	1,971
24-27	23,036	67,246	89	59,865	11	7,399
27-30	21,086	73,167	86	62,924	14	10,244

^a Total DNA synthesis was measured in replicate cultures of uninfected and infected cells.

^b For each time interval, the ^3H -DNA newly synthesized in polyoma-infected cultures was analyzed by velocity sedimentation in neutral sucrose density gradients (Fig. 2). The proportion of the total DNA that was viral and cellular was determined from the sedimentation profiles as follows. The cumulative amount of radioactive material sedimenting in the region of polyoma DNA (e.g., fractions 21 to 28, Fig. 2b) and in the region of cell DNA (e.g., fractions 45 to 54, in Fig. 2b) was calculated and expressed as a percentage of the total DNA recovered in each gradient. The recovery of total input DNA in these gradients was essentially quantitative.

^c Values for the amount of ^3H -TdR incorporated into cell and viral DNA components of infected cultures were derived by multiplying the counts per minute incorporated into total DNA for each labeling period by the appropriate percentage of the total contributed by each DNA.

of DNA proceeded at about the same rate in mock-infected and infected cultures up to 12 to 15 hr PI. After this time, the rate of DNA synthesis in infected cultures increased such that, by 27 to 30 hr, it was 3.6 times that in uninfected cells. Calculation of the proportions of cell and polyoma DNA present in infected cells indicated that, whereas an increased rate of DNA synthesis was first observed at 15 to 18 hr PI, viral DNA synthesis was not detected until 3 hr later. Thus, between 15 and 18 hr, polyoma infection resulted in stimulation of only cell DNA formation. The rate of synthesis of cell DNA increased thereafter to a level, at 27 to 30 hr PI, of about three times that of uninfected cells. The rate of formation of viral DNA at the latest time at which it was examined (27 to 30 hr PI) increased by 12-fold over the rate at the time of its initial detection (18 to 21 hr PI).

Kinetics of formation of heterogeneously sedimenting cell DNA during productive polyoma infection. In the foregoing experiments, it was established that induction of cell DNA synthesis precedes polyoma DNA replication in productively infected cells under the conditions of infection used. It was then of interest to examine the pattern of formation of the heterogeneously

sedimenting cell DNA component described above in relation to these two phenomena.

Mouse embryo cells were grown for at least one generation period in medium containing ^{14}C -TdR, to label preexistent normal cell DNA, and were then infected with polyoma virus or were mock-infected. The cultures were incubated with medium containing ^3H -TdR during successive 6-hr intervals throughout one cycle of virus multiplication to label newly synthesized DNA. DNA was then analyzed by velocity sedimentation in alkaline sucrose density gradients (Fig. 3).

Data obtained revealed that the ^3H -DNA newly synthesized in mock-infected cells during any interval throughout the experiment co-sedimented at approximately 105S (Fig. 1b) with the ^{14}C -labeled DNA preexistent in the cells (Fig. 3a). Similar sedimentation profiles were obtained with the ^3H -DNA newly made in polyoma-infected cultures during any interval up to 18 hr PI. These data confirmed those obtained above by using velocity sedimentation analyses in neutral sucrose density gradients (Fig. 2a), indicating that, up to 18 hr PI, only cell DNA is made in polyoma-infected cells. It was also evident that this cell DNA is of the same molecular size as is the normal preexistent cell DNA. Quite different results were

FIG. 3. Representative sedimentation profiles in alkaline sucrose density gradients of DNA of polyoma-infected cells. Secondary cultures of mouse embryo cells, the DNA of which had been labeled with ^{14}C -TdR, were infected with polyoma virus or were mock-infected. At 24 hr PI, medium containing ^3H -TdR (200 $\mu\text{Ci}/\text{ml}$; 19.7 Ci/mmol) was added to a final level of 10 $\mu\text{Ci}/\text{ml}$ to the growth medium of replicate cultures of (a) mock-infected and (b) polyoma-infected cells. At 30 hr PI, cells were harvested by using trypsin treatment, and the labeled DNA was sedimented in alkaline sucrose density gradients as described. Symbols: ●, newly synthesized ^3H -labeled DNA; ○, ^{14}C -labeled preexistent cell DNA. Recovery of ^3H -labeled DNA was 98.5 and 102.7%, respectively, for mock-infected cultures and polyoma-infected cultures.

obtained in the case of DNA newly synthesized after 18 hr PI in virus-infected cells (Fig. 3b). This ^3H -DNA was comprised of three components. One cosedimented with the ^{14}C -labeled preexistent cell DNA (fractions 18 to 25). A second component sedimented homogeneously in the position of form I polyoma DNA (Fig. 1a). The third sedimented heterogeneously, primarily between viral DNA and high-molecular-weight cell DNA (fractions 13 to 17); this component consisted of discontinuous newly synthesized strands of cell DNA (6).

The relative amount of each ^3H -DNA component formed at various periods during the infectious cycle of polyoma virus was determined from alkaline sucrose density gradient profiles (Table 2). The data revealed that the cell DNA which sedimented heterogeneously in alkaline sucrose was formed only after the onset of polyoma DNA replication. Thereafter, it comprised a constant fraction (11 to 12%) of the total DNA made.

Assessment of the origin of heterogeneously sedimenting cell DNA. It was previously observed (6) that the heterogeneous cell DNA component formed in polyoma-infected cells is not derived from the high-molecular-weight DNA preexistent

TABLE 2. Pattern of formation of cell and viral DNA components in polyoma-infected cells

Labeling period (hr PI)	Percentage of ^3H -DNA which sediments in alkaline sucrose-density gradients as ^a		
	Viral DNA	Heterogeneous cell DNA	Normal cell DNA
0-6	Undetectable	Undetectable	100
6-12	Undetectable	Undetectable	100
12-18	Undetectable	Undetectable	100
18-24	4	11	85
24-30	10	12	78
30-36	11	11	78

^a For each time interval noted, the ^3H -DNA newly synthesized in polyoma-infected cultures was examined by velocity sedimentation analysis in alkaline sucrose density gradients (Fig. 3). The proportions of viral DNA, heterogeneous cell DNA, and normal cell DNA were calculated from the areas under specific regions of the sedimentation profiles obtained. For example, these percentages were calculated by using fractions 8 to 12, 13 to 17, and 18 to 25, respectively, for the data shown in Fig. 3b.

in cells at the time of infection. This fact is also evident in Fig. 3b; no ^{14}C -labeled material was detected in the region of alkaline sucrose density gradients containing the heterogeneously sedimenting DNA. The other possibilities considered for the origin of this material were: (i) "late" degradation of the normal cell DNA newly replicated in infected cultures prior to the initiation of viral DNA synthesis, (ii) degradation of the normal cell DNA component newly replicated "late," that is, after the initiation of viral DNA synthesis, and (iii) direct synthesis.

The first hypothesis was examined as follows. Cells with ^{14}C -TdR-labeled DNA were infected with polyoma and were then incubated with medium containing ^3H -TdR between 12 and 16 hr PI. Some of the cultures were harvested at 16 hr to permit detection, by velocity sedimentation in alkaline sucrose, of the normal high-molecular-weight cell DNA newly made during this interval (Fig. 3a). The remaining cultures were incubated an additional 8 hr with medium containing unlabeled TdR. The labeled DNA present after the "pulse-chase" period was similarly analyzed.

The results (Fig. 4) showed that the ^3H -labeled cell DNA newly synthesized in infected cultures prior to the onset of viral DNA replication cosedimented with the ^{14}C -labeled normal cell DNA marker (Fig. 4a) as expected (6; Fig. 3a). This DNA had the same sedimentation properties after the "chase" period (i.e., after initiation of viral DNA synthesis; Fig. 4b) as it did when it was

FIG. 4. Analysis by alkaline sucrose density gradient sedimentation of the stability of normal cell DNA synthesized in polyoma-infected cells prior to formation of progeny viral DNA. Secondary cultures of mouse embryo cells were prelabeled with ^{14}C -TdR and infected with polyoma virus. At 12 hr PI, medium containing ^3H -TdR (200 $\mu\text{Ci}/\text{ml}$; 19.7 Ci/mmol) was added to the growth medium of quadruplicate cultures to a final level of 10 $\mu\text{Ci}/\text{ml}$. At 16 hr, two cultures (a) were harvested. The radioactive medium of the remaining cultures was replaced with unlabeled medium. These cultures (b) were incubated for an additional 8 hr, and the cells were then harvested. Labeled DNA was analyzed by alkaline sucrose density gradient sedimentation as described. Symbols: $\bullet$, ^3H -labeled DNA; $\circ$, ^{14}C -labeled preexistent cell DNA.

examined immediately after it was "pulse-labeled." On the basis of these data, it was concluded that the heterogeneously sedimenting cell DNA made "late" in polyoma-infected cells (Fig. 3b) is not derived by degradation of the apparently normal 105S cell DNA made prior to the onset of synthesis of viral DNA.

To examine the second and third postulated mechanisms of formation of the heterogeneously sedimenting cell DNA, the stability of cell DNA moieties made "late" in polyoma-infected cells (24 to 28 hr PI) was examined in "pulse-chase" experiments. Mouse embryo cells, prelabeled in DNA with ^{14}C -TdR, were infected and incubated with ^3H -TdR at 24 to 28 hr PI. Some cultures were harvested for immediate analysis of the newly made DNA as above. The remaining cultures were incubated with nonradioactive medium until 32 hr PI, and were then examined to determine the fate during the 4-hr "chase" period of the ^3H -DNA newly made between 24 and 28 hr.

The sedimentation profile of the "pulse-labeled" ^3H -DNA made between 24 and 28 hr PI (Fig. 5a) revealed the presence of the three expected DNA moieties (see Fig. 3b). The sedimentation properties of the DNA of infected cells "pulse-labeled" at 24 to 28 hr and incubated for a 4-hr "chase" period (Fig. 4b) showed that the

FIG. 5. Analysis by alkaline sucrose density gradient sedimentation of the stability of DNA synthesized in polyoma-infected cells after the initiation of progeny viral DNA formation. Secondary cultures of mouse embryo cells were prelabeled with ^{14}C -TdR and infected with polyoma virus. At 24 hr PI, medium containing ^3H -TdR (200 $\mu\text{Ci}/\text{ml}$; 19.7 Ci/mmol) was added to the growth medium of quadruplicate cultures to a final level of 10 $\mu\text{Ci}/\text{ml}$. At 28 hr, two cultures (a) were harvested. The radioactive medium of the remaining cultures was replaced with medium containing unlabeled TdR. These cultures (b) were incubated for an additional 4 hr, and the cells were then harvested. Labeled DNA was analyzed by alkaline sucrose density gradient sedimentation as described. Symbols: $\bullet$, ^3H -labeled DNA; $\circ$, ^{14}C -labeled preexistent cell DNA.

^3H -DNA moieties detected after the "chase" were primarily viral DNA and material cosedimenting with the ^{14}C -labeled normal DNA preexistent in the cells before infection. Little of the DNA sedimenting heterogeneously between these two DNA species was detected.

These data indicated that the heterogeneously sedimenting cell DNA newly formed "late" in polyoma-infected cells is not derived by degradation of high-molecular-weight cell DNA made "late" during infection. The results, therefore, suggested that this DNA arises by direct synthesis.

Assessment of the metabolic fate of the heterogeneously sedimenting cell DNA. The data obtained in the "pulse-chase" experiment just discussed (Fig. 5) provide information concerning the metabolic fate of the heterogeneously sedimenting cell DNA component newly made during the "late" stage of polyoma infection. Quantitative aspects of the distribution of radioactivity associated with cell and viral DNA components in "pulse-chase" experiments, analogous to that described in Fig. 5, are shown in Table 3. These data indicated the following: DNA newly synthesized in polyoma infected cultures at 24 to 28 hr PI or at 26 to 30 hr PI was comprised of 11% component I viral DNA, 74% high-molecular-weight cell DNA, and 15% heterogeneously sedi-

TABLE 3. *Quantitative analysis of the stability of DNA components synthesized "late" in polyoma-infected cells^a*

Expt	Period	Total ³ H-DNA recovered (counts/min)	³ H-DNA recovered with the sedimentation properties of						Total DNA (%) in	
			Viral DNA		Heterogeneous cell DNA		Normal cell DNA		Heterogeneous cell DNA lost during chase	Normal cell DNA gained during chase
			Counts/min	Total DNA (%)	Counts/min	Total DNA (%)	Counts/min	Total DNA (%)		
I	Pulse	31,767	3,295	10	5,360	17	23,112	73	14	15
	Chase	30,966	2,792	9	1,038	3	27,136	88		
II	Pulse	35,729	3,905	11	5,050	14	26,774	75	12	11
	Chase	31,948	3,849	12	668	2	27,411	86		
III	Pulse	38,639	4,452	12	5,780	15	28,407	73	10	9
	Chase	40,109	5,225	13	2,010	5	32,956	82		
Avg	Pulse			11		15		74	12	12
	Chase			11		3		86		

^a ³H-DNA in polyoma-infected cultures newly synthesized at 24 to 28 hr PI (experiments I and II) and at 26 to 30 hr PI (experiment III) was examined by velocity sedimentation analysis in alkaline sucrose density gradients (Fig. 5) before and after a 4-hr "chase" period with medium containing unlabeled TdR. The proportions of viral DNA, heterogeneous cell DNA, and normal cell DNA, which comprised the total DNA recovered from each gradient, were calculated (Table 2).

menting cell DNA. After the 4-hr "chase" period, the percentage of viral DNA was unchanged. However, the percentage contribution of the heterogeneously sedimenting cell DNA was decreased from 15 to 3% of the total DNA, whereas the percentage contribution of the high-molecular-weight cell DNA increased from 74 to 86%. Clearly, the radioactivity associated with normal 105S high-molecular-weight cell DNA after the "chase" was augmented by an amount equivalent to that lost from the heterogeneously sedimenting cell DNA formed during the "pulse."

Not all of the radioactive DNA (Table 3) was lost during the "chase" from the region of the gradients designated as containing heterogeneously sedimenting cell DNA. This is to be expected since the three DNA components under study are imperfectly separated under the conditions of sedimentation employed (6). On the basis of sedimentation analyses and DNA:DNA hybridization tests, it may be calculated that, of the DNA sedimenting in the 38 to 105S region of the gradients, approximately 22% is attributable to polyoma DNA and 105S cell DNA. The data (Table 3) actually show that an average of 22.4% of the total DNA sedimented in this region of the gradient after the "chase." Therefore, the data of Table 3 were interpreted as indicating that the newly synthesized heterogeneously sedimenting cell DNA does not remain as such but is converted, either directly or via degradation and reutilization of precursor nucleotides, into high-molecular-weight cell DNA.

To examine the possibility that newly synthesized heterogeneously sedimenting cell DNA was

degraded, the acid-soluble material present in uninfected and infected cells was examined immediately after a "pulse" with ³H-TdR and after varying "chase" periods (Table 4). The radioactivity associated with the acid-soluble fraction of normal and infected cultures rapidly decreased immediately after the addition of unlabeled medium. In the case of infected cells, the radioactivity decreased from over 6,000 counts/min to only 600 counts/min within 15 min, at most, after the addition of unlabeled medium to the cultures. Within 30 min, the acid-soluble radioactive material decreased to a relatively low level of about 2% of that present immediately after the "pulse"; this level remained approximately constant for at least 3.5 hr.

These data reveal no evidence of accumulation, over any interval, of acid-soluble radioactive material that would have been observed if the heterogeneously sedimenting cell DNA, lost during the 4-hr "chase" period (Table 3), had been degraded to acid-soluble components. Furthermore, degradation of the heterogeneously sedimenting cell DNA and its immediate reutilization as nucleotides for synthesis of the high-molecular-weight cell DNA also seem unlikely in view of the data of Table 3, which indicate no increase in the percentage of the total radioactive DNA associated with the viral DNA component after the "chase." If reutilization of degradation products had occurred, one would have expected to observe such an increase in the viral DNA component as well as in the normal cell DNA, since the synthesis of these DNA species would presumably draw on the same precursor pool.

TABLE 4. *Effect of addition of unlabeled medium on the labeled acid-soluble fraction of uninfected and polyoma-infected cells^a*

Time after addition of unlabeled medium (hr)	³ H-labeled trichloroacetic acid-soluble components (counts per min per culture)	
	Uninfected cells	Infected cells
0	2,843	6,715
0.25	435	611
0.5	215	172
0.75	146	156
1	114	139
2	122	107
3	95	113
4	80	85

^a Mock-infected or polyoma-infected mouse embryo cultures were incubated between 22 and 24 hr PI with medium containing 10 μ Ci/ml of ³H-TdR (19.7 Ci/mmole). At 24 hr PI, the labeled medium was replaced with unlabeled medium. At the noted times, replicate cultures were washed with ice-cold PBS; the cells were dispersed with trypsin solution and then lysed with 1% SDS as described. The SDS-lysate was treated with 10% trichloroacetic acid and the acid-soluble fraction (filtrate) was collected by membrane filtration, dried, and analyzed for radioactivity (8).

The data presented in Table 2 showed that the proportion of heterogeneously sedimenting cell DNA formed during any interval of the "late" stage of infection is constant. In view of the data of Tables 3 and 4, the most likely interpretation of this finding was that this DNA is continuously synthesized during the "late" stage of infection and continuously converted into high-molecular-weight cell DNA. To examine this postulate, the following experiment was done. Polyoma-infected mouse embryo cells were incubated with medium containing ³H-TdR at 24 to 28 hr PI to label the newly synthesized DNA. At 28 hr PI, the ³H-labeled medium was replaced with medium containing ¹⁴C-TdR, and the cultures were incubated for an additional 4 hr. The labeled DNA was then sedimented in an alkaline sucrose density gradient.

The results (Fig. 6) showed (Fig. 5b) that very little ³H-labeled DNA was present in the region of the heterogeneously sedimenting cell DNA (fractions 12 to 15) after the 4-hr "chase." However, the ¹⁴C-DNA newly made during the "chase" period contained viral DNA, normal cell DNA, and heterogeneously sedimenting cell DNA in the expected proportions (10, 72, and 18%, respectively). Clearly, new ¹⁴C-labeled heterogeneously sedimenting cell DNA was formed during the period in which previously made ³H-labeled

FIG. 6. Analysis by alkaline sucrose density gradient sedimentation of DNA newly synthesized in polyoma-infected cells during the period of disappearance of previously formed heterogeneously sedimenting cell DNA. At 24 hr PI, medium containing ³H-TdR (200 μ Ci/ml; 19.7 Ci/mmole) was added to a final level of 10 μ Ci/ml to the growth medium of replicate mouse embryo cultures infected with polyoma virus. The cultures were incubated until 28 hr, to pulse-label the newly made DNA. After this time, the ³H-labeled medium was replaced with medium containing ¹⁴C-TdR (0.1 μ Ci/ml; 54 mCi/mmole), and the cultures were further incubated for 4 hr. The cells were then harvested by trypsin treatment, and the labeled DNA was sedimented in alkaline sucrose density gradient as described. Symbols: ●, ³H-labeled DNA; ○, ¹⁴C-labeled DNA.

material was lost. The fact that the synthesis of this ¹⁴C-DNA was not accompanied by the formation of such ³H-DNA supports the data presented above (Tables 3 and 4), indicating that the heterogeneously sedimenting cell DNA is converted to apparently normal cell DNA without prior degradation to acid-soluble components.

DISCUSSION

The demonstration that cells transformed by the oncogenic DNA viruses carry part or all of the virus genome in a form closely associated

with the host genetic material (1, 2, 12, 13, 15, 19, 32, 38) gives added interest to the phenomenon of polyoma-induced initiation of cell DNA synthesis. It is apparent that integration of the viral DNA into the host cell genetic apparatus could occur in association with this event.

In the present study it was shown that, under the conditions of infection employed, the synthesis of cell DNA is stimulated in cells productively infected with polyoma virus about 3 hr before the onset of formation of detectable progeny viral DNA. Two distinct phases of this viral-induced cell DNA synthesis were identified. In the first, which occurs between 15 and 18 hr PI, prior to the onset of viral DNA synthesis, the cell DNA made was normal (double-stranded with both strands sedimenting homogeneously at 105S in alkaline sucrose). In the second phase, which occurs at 18 hr PI, the time at which virus DNA synthesis was first detected, some of the newly synthesized cell DNA exhibited heterogeneous sedimentation in alkaline sucrose. It was previously shown (6) that this DNA is derived from double-stranded DNA, discontinuous in the newly synthesized strand. We found, in the present work, that this heterogeneously sedimenting cell DNA is not formed by degradation of either the DNA preexistent in cells at the time of infection or of normal cell DNA synthesized at any time during infection. The data indicated that this DNA is formed continuously throughout the "late" stage of the productive cycle.

It is now known that the synthesis of DNA in animal cells proceeds in a stepwise fashion. The high-molecular-weight DNA strands of the chromosome are derived by extension and joining of small newly synthesized polynucleotide segments (16, 18, 20, 32; Sheinin, *unpublished data*). A disorientation of this normal sequence of events could explain the results observed in the present study. Thus, the production of low-molecular-weight single-stranded segments of cell DNA in polyoma-infected cells could result either from inactivation of cellular polynucleotide ligase or from the formation of substrate molecules in excess of available functional enzyme. The first hypothesis seems unlikely in view of the observation that the small cell DNA fragments are continuously formed and converted into material of a higher molecular weight. This conclusion is strengthened by the recent finding of Sambrook and Shatkin (23) that polyoma-infected cells contain a large amount of ligase which, at least in the extracted form, is functional. The more likely explanation of the results is that the newly synthesized cell DNA fragments are not immediately available as substrate for ligase. Such a situation might arise from asynchronous initiation of the

synthesis of cell genetic material, either at multiple sites on a single template or on several templates.

Formation in polyoma-infected mouse cells of cell DNA, which carries discontinuous newly synthesized strands, occurs only after the onset of replication of progeny virus DNA. This result is in accord with earlier findings, indicating that aberrant cell DNA synthesis may be temporally related to the formation of structural viral proteins (5), and that the synthesis of discontinuous cell DNA is blocked by inhibition of the maturation of viral DNA caused by cycloheximide (Branton et al., *Virology*, *in press*).

The relevance of the phenomena reported herein to cell transformation elicited by polyoma virus remains of intriguing interest. To proceed to the transformed state, cells must carry out effective chromosomal DNA replication. The data of the present study indicate that expression of the viral replicative function results in disorientation of this process. Since virus DNA is closely associated with the genetic material of transformed cells (23, 38), it might reasonably be anticipated that successful (and irreversible) transformation would occur in association with suppression of the maturation of progeny viral DNA and the concomitant disruption of normal cell DNA replication. This situation does, in fact, occur in cells abortively infected with polyoma virus, some of which do undergo neoplastic conversion (14, 27, 28).

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