Polyoma Pseudovirions

I. Sequence of Events in Primary Mouse Embryo Cells Leading to Pseudovirus Production

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Received for publication 11 April 1972

The relationship of the intracellular events leading to the production of polyoma pseudovirions in primary mouse embryo cells has been investigated. Replication of polyoma deoxyribonucleic acid (DNA) began 18 hr after infection. Assembly of viral capsid protein occurred 12 hr later. Intracellular fragments of host cell DNA, of the size found in pseudovirions, were first detected 36 hr after infection. The amount of intracellular 14S host DNA that was produced during infection was seven times greater than the amount of polyoma DNA synthesized. The relative pool sizes of polyoma DNA and 14S DNA at the time of virus assembly may dictate the amounts of polyoma virus and pseudovirus produced.

After the infection of mouse cells with polyoma virus, a group of polyoma-related particles may be produced. The four particles, which have been purified and characterized, are the infectious polyoma virions, defective polyoma virions, polyoma pseudovirions, and empty particles. The infectious polyoma virions consist of the complete polyoma genome encapsidated within polyoma protein coats. The deoxyribonucleic acid (DNA) in these particles is circular, double-stranded, supercoiled, and has a molecular weight of approximately 3×10^6 (8). The defective polyoma virions appear to contain only 50 to 75% of the polyoma genome (4). The production of these defective particles in mouse embryo cells appears to be related to the number of previous serial passages to which the inoculum has been subjected and the high multiplicity of infection. Polyoma pseudovirions contain fragments of host cell DNA encapsidated within the polyoma protein coats (14-16, 22). The DNA fragments have been reported to be heterogeneous in size by some investigators (14, 22). Pseudovirions have been found after polyoma infection of baby mouse kidney cells (14, 22), primary mouse embryo cells (15), and the established mouse cell line, 3T3 (2). Finally, the empty particles are polyoma coats or capsids which lack any detectable DNA.

Pseudovirions appear to be relatively unusual particles in animal virology, having been found thus far only after infection of mouse cells with polyoma or of monkey cells with simian virus 40 (13, 19). However, tobacco mosaic virus pseudo-

virions containing fragments of host ribonucleic acid have been reported (17).

About 10% of the full particles produced after polyoma infection of mouse kidney cells are pseudovirions. When produced in these cells, pseudovirions appeared at a density between that of the infectious virus and that of the empty particles in CsCl equilibrium gradients (22). By using primary mouse embryo cells, a strikingly different result has been found in our laboratory. The infection of primary mouse embryo cells yields full particles, of which approximately 80% or more are pseudovirions. The majority of these pseudovirions have a density similar to the full particles (1.32 g/cm³ in CsCl). Also, the low-molecular-weight DNA extracted from polyoma-infected primary mouse embryo cells is predominantly of host origin, whereas that extracted from baby mouse kidney cells or 3T3 cells after infection is mostly polyoma (Yelton and Aposhian, *manuscript in preparation*).

In the present paper, the relationship of the intracellular events leading to the production of pseudovirions in primary mouse embryo cells infected with polyoma virus has been investigated.

MATERIALS AND METHODS

Virus and cells. The small-plaque variant of the Toronto strain of polyoma virus was used for these experiments. A virus stock was produced by infecting primary mouse embryo cells. The virus used for the experiments reported in this paper has been passed twice in this laboratory at a multiplicity of infection of 0.1 plaque-forming units (PFU)/cell. Hemagglutina-

tion assays were performed in the standard manner by using guinea pig red blood cells (5). Plaque assays were performed by using secondary mouse embryo cells.

Primary mouse embryo cells (Swiss White Webster, Carworth Farms) were grown to confluency in 3.7-liter glass roller bottles at 37 C. Five days after seeding, the cells were infected with polyoma at a multiplicity of infection of about 0.1 PFU/cell. The virus was allowed to adsorb to the cells for 2 hr, and then fresh medium was added.

Cells, whose DNA was labeled with ^3H -thymidine before infection, were prepared by adding to them, 24 hr after seeding, medium containing 2.5 μCi of ^3H -thymidine per ml. Four days later, the medium was removed, and the radioactivity was chased by adding medium containing unlabeled thymidine (50 $\mu\text{g}/\text{ml}$) and deoxycytidine (10 $\mu\text{g}/\text{ml}$). Two hours later, the medium was again removed, and the cells were infected as described above. After the 2-hr adsorption period, complete medium including the chase was added. This is essentially the method used routinely in this laboratory for pseudovirion production and is described in detail elsewhere (15). Cells were labeled postinfection by adding medium containing 5 μCi of ^3H -thymidine per ml immediately after infection.

Cells were harvested by treatment of the monolayers with 0.05% trypsin in 6.0 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.5 for 5 min at 37 C. The cells were pelleted by centrifugation at $250 \times g$ for 10 min, suspended in tris(hydroxymethyl)amino-methane(Tris)-EDTA (0.01 M and 0.01 M) at pH 7.5, and divided into two portions. The low-molecular-weight DNA was extracted from one portion by the sodium dodecyl sulfate (SDS)-1 M NaCl method described by Hirt (10). Cells of the other portion were lysed by five cycles of freezing and thawing, followed by treatment of the lysate with 0.5% deoxycholate (DOC) at 37 C to extract the virus.

Medium. Eagle medium containing 10% calf serum (Gibco) was used (15). ^3H -thymidine (20 Ci/mmol) was obtained from the New England Nuclear Corp. Standard saline citrate (SSC) contained 0.15 M NaCl and 0.015 M Na citrate at pH 7.0. DOC was prepared as a 1% (w/v) solution in 0.01 M Tris buffer.

Gradients. Equilibrium centrifugations were performed by using CsCl isopycnic gradients. Centrifugation was performed at 32,000 rev/min for 22 hr at 25 C with a Spinco SW50.1 rotor.

Preformed alkaline sucrose gradients contained 0.5 M NaCl at pH 12.5. Long gradients (10 to 30% sucrose, 10 ml) were centrifuged at 40,000 rev/min for 15 hr at 4 C in a Spinco SW41 rotor. Short gradients (5 to 20% sucrose, 4.6 ml) were centrifuged at 50,000 rev/min for 2 hr at 4 C in a Spinco SW50.1 rotor.

The gradients were fractionated by puncturing the bottom of the tubes and collecting drops. The radioactivity in each fraction was determined in a Packard Tri-Carb scintillation counter by using a toluene-based scintillation fluid which contained 0.55% of 2,5-diphenyloxazole, 0.01% of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene, 10% water, and 30% Triton X-100 (Rohm and Haas).

DNA-DNA hybridizations. Hybridizations were

performed by using the Denhardt membrane filter technique (6). Hybridizations were carried out at 65 C in $\times 3$ SSC, as described previously (16). The radioactivity bound to each filter was determined in a Packard Tri-Carb scintillation counter by using a toluene-based scintillation fluid containing 0.55% of 2,5-diphenyloxazole and 0.01% of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene.

RESULTS

Confluent monolayers of primary mouse embryo cells were infected with polyoma virus at a multiplicity of 0.1 PFU/cell. At various times after infection, samples were taken. The temporal relationship of some of the intracellular events was determined by analyzing the samples for polyoma component I DNA, pseudoviral DNA, PFU, hemagglutinating units (HAU), and pseudovirions. Samples of mock infected cells were taken at each time and similarly analyzed.

Synthesis of polyoma DNA. The synthesis of polyoma DNA was followed by labeling the infected cells with ^3H -thymidine. At various times after infection, cells were harvested, and polyoma DNA was extracted by using the SDS-1 M NaCl method of Hirt (10). After centrifugation, a sample of the supernatant fluid was sedimented in preformed 5 to 20% alkaline sucrose gradients. The time at which polyoma DNA was replicated and the amount produced was determined by the appearance of radioactive 53S material. Synthesis of polyoma DNA was first detected at 18 hr after infection (Fig. 1). No 53S material was found in the mock infected controls.

FIG. 1. Synthesis of polyoma DNA and fragmentation of host cell DNA. The cells were labeled with ^3H -thymidine after infection. At the indicated times, the low-molecular-weight DNA was extracted from the cells by the method of Hirt (10). The supernatant fluid was sedimented for 2 hr at 50,000 rev/min in a Spinco SW50.1 rotor at 4 C. The total gradient volume was 4.6 ml. The gradient was prepared by using 5 to 20% sucrose dissolved in 0.5 M NaCl and 0.001 M EDTA at pH 12.5.

Production of 16S DNA. The formation of 16S DNA was also followed in cells labeled after infection. This DNA sediments at 16S in alkaline gradients and at 14S in neutral gradients. Evidence will be presented below showing that this DNA is of host origin. The first indication of a discrete 16S peak appeared 36 hr after infection (Fig. 1 and 2). The amount of this fragmented host DNA continued to increase during infection and reached a maximum at about 72 hr after infection. In all cases, control experiments with uninfected cells were performed. No peaks of radioactivity at either 53 or 16S were found in the control experiments. The significance, if any, of the lighter peak of radioactivity that occurred at 18 hr (Fig. 1) is not understood. Occasionally, however, a similar peak was found in extracts from mock-infected cells. The appearance of 16S DNA in infected cells followed the same time course whether the cells were labeled before or after infection.

Under the sedimentation conditions that were used, polyoma component II DNA and 16S pseudoviral DNA would be expected to cosediment in the gradients shown in Fig. 1. To determine if any polyoma component II DNA was present, the supernatant fluids from the SDS-1 M NaCl extracts were sedimented under conditions which separate the circular un-nicked strand of polyoma component II DNA (18S) from pseudoviral DNA (16S). No distinct peak was found at 18S, indicating that significant amounts of component II DNA were not present in the extracts (Fig. 2).

The sharpness of the 16S peak in both Fig. 1 and 2 should be noted, since it is indicative of substantial homogeneity in the size of the host DNA fragments produced. The DNA extracted from pseudovirions produced in primary mouse embryo cells also demonstrates this same homogeneity (P. K. Qasba et al., *In Molecular studies in viral neoplasia, in press.*)

Identification of 53S and 16S DNA by hybridization. To characterize further the radioactive material in the 53 and 16S peaks (Fig. 1), DNA-DNA hybridizations were performed. The radioactive fractions from each peak of the 72-hr postinfection sample were pooled and further purified by centrifugation in alkaline sucrose. The material in each of the resulting peaks was pooled, dialyzed against SSC (pH 7.0), and used in hybridization experiments. The data from these experiments are summarized in Table 1. The 53S material hybridized with unlabeled polyoma DNA at an efficiency comparable to that found with labeled polyoma DNA. As judged by its hybridization with polyoma DNA, less than 6% of the 16S material was polyoma DNA. The 16S material hybridized to mouse embryo DNA with an efficiency comparable to that obtained with

FIG. 2. Analysis of 16S DNA by long alkaline sucrose gradients. Cells were labeled with ^3H -thymidine after infection. At the indicated times, cells were extracted for low-molecular-weight DNA by the method of Hirt (10). The supernatant fluid was sedimented for 15 hr at 40,000 rev/min at 4°C in a Spinco SW41 rotor. The total gradient volume was 10 ml. The gradients were prepared by using 10 to 30% sucrose dissolved in 0.5 M NaCl and 0.001 M EDTA at pH 12.5.

labeled mouse embryo DNA. Thus, the radioactivity sedimenting at 53S in alkaline sucrose represents newly replicated polyoma DNA. DNA sedimenting at 16S represents fragments of newly replicated host cell DNA.

Synthesis of viral particles. The synthesis of polyoma virions, pseudovirions, and empty capsids was studied by using cells labeled with ^3H -thymidine before infection. At the indicated times after infection, a sample of the cells was disrupted by five cycles of freezing and thawing, and the virus was extracted from the samples by using DOC. Each extract was dialyzed against 0.01 M Tris-0.06 M KCl at pH 7.5 to remove the DOC and then assayed for PFU. The remainder of the extract was centrifuged in CsCl equilibrium gradients. Fractions of the gradients were collected, and the radioactivity and HAU of each fraction were determined. Two peaks of HAU, corresponding to full and empty particles, were found at densities of 1.32 and 1.28 g/cm³, respectively. Because the cells were prelabeled and the radioactivity in the medium was removed prior to infection, a peak of radioactivity at 1.32 g/cm³ indicated the presence of pseudovirions. The results are summarized in Table 2. An increase in the empty particles was first detected at 30 hr postinfection. Twelve hours later, an increase of intact virions was detected. Plaque-forming particles (polyoma virus) and pseudovirions ap-

TABLE 1. Identification of 53S DNA and 16S DNA by DNA-DNA hybridization^a

DNA immobilized on filter	Input (counts/min)	Bound (counts/min)	Bound to blank filter (counts/min)	Hybridization	
				Corrected (counts/min)	Input (%)
	53S ³ H DNA				
Polyoma	1,464	854	229	625	42.7
Polyoma	2,928	1,591	244	1,347	46.0
Polyoma	5,856	2,873	148	2,725	46.5
	Polyoma ³ H DNA				
Polyoma	1,320	760	58	702	53.2
Polyoma	2,640	1,733	60	1,673	63.4
Polyoma	6,600	4,142	66	4,076	61.8
	16S ³ H DNA				
Polyoma	3,305	211	78	133	4.0
Polyoma	6,610	366	114	252	3.8
Polyoma	13,220	619	279	340	2.6
	16S ³ H DNA				
Mouse	1,332	417	53	364	27.5
Mouse	1,983	636	46	590	29.7
Mouse	3,305	852	78	774	23.4
	Mouse embryo ³ H DNA				
Mouse	1,801	514	21	493	27.4
Mouse	3,602	826	33	793	22.0
Mouse	7,204	1,511	34	1,478	20.5
Mouse	10,806	2,569	43	2,526	23.4
Mouse	14,408	4,821	51	4,770	33.1
	53S ³ H DNA				
Mouse	2,928	367	244	123	4.2
Mouse	14,640	923	604	319	2.2

^a Nitrocellulose filters were loaded with either 2 μ g of sonically treated, unlabeled polyoma DNA (type I) or with 5 μ g of sonically treated, unlabeled mouse embryo DNA. Specific activities of the DNA were as follows: [³H] DNA of polyoma, 4.73×10^4 counts/min per μ g of DNA; [³H] DNA of mouse embryo, 1.83×10^6 counts/min per μ g of DNA; [³H] DNA of 53S, 8.1×10^3 counts/min per μ g of DNA; [³H] DNA of 16S, 9.25×10^3 counts/min per μ g of DNA.

peared at 42 hr after infection. The synthesis of both pseudovirions and polyoma virus was complete by 96 hr.

Time course of polyoma replication in primary mouse embryo cells. Figure 3 summarizes our data on the events occurring after polyoma infection of primary mouse embryo cells. Synthesis of polyoma DNA was detected 18 hr after infection. This was followed by an increase of the empty particles which began 30 hr after infection. Fragmentation of host DNA to a molecular weight of about 3×10^6 started shortly thereafter, about 36 hr postinfection. Assembly of polyoma virus and pseudovirions began between 36 to 42 hr after infection. By 96 hr, replication was complete.

It should be emphasized that significantly more radioactivity was associated with the 14S host DNA fragments than was associated with the

polyoma DNA molecules (Fig. 3). Because the specific activities of the 14S DNA and the polyoma DNA were about the same (Table 1), the ratio of their radioactivities gives an indication of the relative quantity of each produced. When full particles were first found (42 hr), the ratio of 14S DNA to polyoma DNA was about 3:1; by 96 hr, it was 7:1. The production of 14S DNA in amounts much larger than polyoma DNA appears to be unique to primary mouse embryo cells; it was not found after infection of baby mouse kidney or 3T3 cells (Yelton and Aposhian, *manuscript in preparation*).

DISCUSSION

In this laboratory, about 80% of the full particles produced after infection of primary embryo cells with polyoma virus are pseudovirions. The remainder are polyoma virions. Un-

TABLE 2. *Synthesis of polyoma virions and pseudovirions in primary mouse embryo cells labeled with ^3H thymidine before infection*

Time after infection (hr)	Particle production ^a		Polyoma virions production ^b (PFU)	Pseudo-virion production ^c (counts/min)
	Full particles (HAU)	Empty particles (HAU)		
12	21	54		0.3
18	6	70		0.1
24	5	66	60×10^4	0.2
30	7	106	71×10^4	0.2
36	9	136	78×10^4	0.6
42	18	232	187×10^4	6.8
48	44	308	328×10^4	17.2
54	134	360	$1,130 \times 10^4$	48.9
72	196	364	$1,545 \times 10^4$	74.5
96	390	440	$1,760 \times 10^4$	145.2
120	386	424	$1,690 \times 10^4$	146.7

^a The hemagglutinating units (HAU) found in the 1.32 g/cm³ peak (full particles) and 1.28 g/cm³ peak (empty particles) after equilibrium centrifugation in CsCl of the extract described in (b). The data have been normalized to the HAU from 10^6 cells.

^b Plaque-forming unit (PFU) assay of the extracts obtained after freeze-thaw cycles and deoxycholate treatment of infected cells. Data have been normalized to PFU produced in 10^6 cells. Assays were performed in triplicate.

^c The radioactivity found in the 1.32 g/cm³ peak after equilibrium centrifugation in CsCl of the extract described in (b). The data have been normalized to the radioactivity from 10^6 cells.

FIG. 3. *Relationship of the intracellular events leading to the production of pseudovirions. Infected cells, at the indicated times, were analyzed for polyoma DNA, 14S host cell DNA fragments, full particles, and empty particles. Polyoma DNA synthesis and production of 14S host cell DNA fragments were measured by counting the radioactivity in the 53S and 16S peaks after sedimentation through alkaline sucrose. As a label, ^3H -thymidine (5 $\mu\text{Ci}/\text{ml}$) was added immediately after infection. Hemagglutinating units of the peaks in CsCl equilibrium centrifugation having a density of 1.32 and 1.28 g/cm³ determined the full and empty particles, respectively.*

like the polyoma pseudovirions reported by other laboratories, these pseudovirions cobanded with the full particles at a density of approximately 1.32 g/cm³ in CsCl (P. K. Qasba et al., *In Molecular studies in viral neoplasia, in press*). Because of the differences between these pseudovirions and those produced in other host cells, the time course of polyoma replication and pseudovirus production in primary mouse embryo cells was studied. The results showed that replication of polyoma DNA began by 18 hr after infection and was completed by 48 hr (Fig. 1 and 3). Assembly of viral capsid protein began by 30 hr postinfection, as measured by an increase of empty capsids (Table 2 and Fig. 3). By using fluorescent antibodies, others have shown polyoma capsid protein to be made about 20 hr after infection (3). Intracellular fragments of host cell DNA of the size found in pseudovirions were first detected 36 hr after infection (Fig. 1-3). Production of full particles began by 42 hr postinfection and continued until 96 hr (Table 2 and Fig. 3).

The time course of polyoma replication in primary mouse embryo cells was similar to that found by others using a variety of cell lines (3, 7, 9, 11). However, unique to these primary mouse embryo cells was the large amount of 14S host DNA fragments produced. Not only was a large quantity of 14S DNA made, but this DNA was very homogeneous in size. The reason for this homogeneity is unknown. It may be that the host cell DNA is fragmented into a variety of sizes which nucleases further degrade unless the fragment is protected. Because 14S fragments are about the same size as polyoma DNA, they might be protected by interaction with a viral protein. The data in Fig. 2 support this possibility because, at earlier times, the fragments of host DNA formed a broad peak, but at later times after infection this peak became sharper and sedimented at 14S. Radioactivity at other S values decreased late in infection.

Although others have reported that the percentage of pseudovirions in a polyoma virus preparation varies from 10 to 90%, the usual value is about 10%. With the primary mouse embryo cells, however, we consistently find that 80% or more of the full particles are pseudovirions. This large percentage of pseudovirions is probably related to the large amount of 14S host DNA fragments produced during infection. Because the production of infectious virus mirrored the production of pseudovirions (Table 2), there appears to be no mechanism for selectively encapsidating polyoma DNA. Rather, any intracellular DNA molecule of appropriate size may be packaged. Hence, the relative pool sizes of

polyoma DNA and 14S DNA at the time of virus assembly may dictate the amounts of polyoma virus and pseudovirus produced.

Recently there have been reports of defective viral particles produced during the replication of polyoma (4) and simian virus 40 (12, 18, 20) viruses. It is unlikely that any such defective particles were produced during our studies. The DNA of such defective particles has a sedimentation value slightly less than that of the infectious DNA (53S in alkaline sucrose). The only polyoma DNA produced in our cells was of 53S size; no smaller-sized polyoma DNA was detected. Additionally, in our experiments a low multiplicity of infection was used because such defective particles are formed only after infection at a high multiplicity.

A disadvantage of infecting at a low multiplicity of infection is the lack of synchrony in the infective process from cell to cell. After the first burst of virus, there is also a chance of a second cycle of virus growth caused by infection of previously uninfected cells. If synchrony of infection had not been achieved in our experiments, the effect would have been a delay in the apparent onset of various events. Thus, the times at which we report various events to begin may be later than the time they actually began. The actual sequence of events, however, should not have been affected. Repeated cycles of virus growth do not appear to have been a problem. The curves in Fig. 3 are monophasic, which suggests that only one growth cycle of virus occurred. Also, the virus produced remained cell-associated and was not released into the medium (*unpublished data*). Therefore, it is unlikely that infection of other cells occurred.

The relationship of the intracellular events leading to pseudovirus production reported in this paper not only is of value as information concerning the relationships between polyoma virus and pseudovirus production, but may be of value also in determining the time at which extracts of infected cells should be prepared in attempting *in vitro* cell-free assembly of pseudovirions.

Pseudovirions are of interest because of their potential as gene delivery systems (1, 13-16, 19, 22). It has been demonstrated that polyoma pseudovirions can enter and be uncoated in mouse embryonic or human embryonic cells and that their naked DNA is associated with the nuclear fraction of these cells (16). There is no evidence to suggest that polyoma pseudovirions contain other than random sequences of host DNA. If this is the case, successful transduction experiments will require large numbers of pseudovirions. However, if individual genes can be isolated and

encapsidated, within polyoma coats *in vitro*, the resulting pseudovirions could be used for transduction experiments. The assembly of pseudovirions containing only a single gene would certainly increase the chances of a successful transduction of mammalian cells by an animal virus.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Joanne Buschman, Maybritt Liden, and Ronald Thayer.

This work was supported by Public Health Service contract 70-2082 from the National Institute of General Medical Sciences, grant CA-10497 from the National Cancer Institute, and a grant from the John A. Hartford Foundation, Inc.

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