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Synthesis and Assembly of Simian Virus 40

II. Synthesis of the Major Capsid Protein and Its Incorporation into Viral Particles

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African green monkey kidney cells infected by simian virus 40 were analyzed for the presence of the major capsid protein (capsid protein I) by immunological and radiolabeling techniques. Antisera with different specificities were prepared by immunization with intact or denatured viral particles. Antisera prepared against intact virus reacted by complement fixation with viral particles and with an 8*S* subunit containing the capsid protein I. Antisera prepared against denatured viral particles reacted with unassembled capsid protein(s) as well as with viral particles. These antisera were used to detect 8*S* viral subunits or unassembled viral capsid protein in soluble extracts of infected cells after centrifugation at 100,000 × *g* to remove viral particles. The soluble antigen pool was found to be small during infection with wild-type virus or a temperature-sensitive mutant deficient in the synthesis of viral particles. Pulse-chase experiments, performed at a high multiplicity of infection, also indicated a small pool of nonparticle capsid protein I. Radioactive lysine was incorporated into capsid protein I of virus particles during a 2-hr pulse. A subsequent chase with excess unlabeled lysine resulted in only a slight increase in the radioactivity found in capsid protein I of viral particles. Furthermore, in the same experiments, capsid protein I was incorporated preferentially into empty shells during the pulse with a shift in radioactivity to intact virions during the chase period, indicating a possible precursor relationship between the two types of virus particles.

Two types of viral particles have been purified from simian virus 40 (SV40)-infected monkey cells: intact virions and empty shells. In the accompanying report, it is shown that more than 70 to 75% of the protein in both particles (determined as ^{3}H-lysine) consisted of a polypeptide of 45,000 daltons, designated capsid protein I (11). No studies on the rate of synthesis of capsid protein I or any of the other polypeptides in virus particles have been reported because of the difficulty in resolving them biochemically in the midst of the continued synthesis of cellular proteins (5).

Previous workers have employed immunological methods to detect viral structural proteins (10, 13). However, though the antisera used reacted with purified virions, their reactivity with subvirion structural components had not been assessed. Rarely was it possible, therefore, to determine whether viral proteins were being measured independently of viral particles in infected cells.

In the present study, we have attempted to evaluate the synthesis of the major capsid protein. Antisera prepared against purified SV40 particles and against capsid protein(s) of denatured particles have been used to identify and quantitate the amounts of viral components of various sizes in infected cells. Pulse-labeling and pulse-chase experiments, employing isolation of viral particles on CsCl cushions, have been performed to estimate the proportion of the major capsid protein assembled into viral particles and to determine the relationship of the two types of viral particles to each other.

MATERIALS AND METHODS

Cell line and viruses. The cultivation of Vero cells and virus stocks has been previously reported (11, 14).

Solutions. Unless otherwise noted, all solutions were in 0.01 M $NaH_2PO_4 \cdot Na_2HPO_4$, *p*H 7.2 (NaP).

Preparation of radioactive lysine-labeled infection cells. A 32-oz (0.946 liter) bottle of confluent infected Vero cells was pulse-labeled in lysine-free Eagle's medium with 1% fetal calf serum (GIBCO) as previously described (11). In pulse-chase experiments, the labeling medium was decanted, the monolayer was washed once with 20 ml of Eagle's medium, and the cells were reincubated in 40 ml of Eagle's medium

supplemented with 7.3 mg of unlabeled lysine per ml (100-fold excess over the concentration in Eagle's medium) and 1% fetal calf serum. Cells were harvested in the wash medium (for pulse) or chase medium, centrifuged, and frozen at −20 C.

Determination of radioactively labeled capsid protein I. Viral particles were extracted from infected cells by sonic treatment and isolated on CsCl cushions as previously reported (11). The radioactivity in the major capsid protein of the CsCl fractions was determined by electrophoresis on sodium dodecyl sulfate (SDS)-acrylamide gels as previously described (11).

Preparation of "soluble" fraction from infected cells. A 0.2-ml portion of the supernatant fluid (10,000 × *g* for 10 min) obtained from sonically treated cells was layered onto a 0.2-ml cushion of 15% sucrose in 0.15 M NaCl, NaP in tubes (ca. 0.48 by 4.13 cm) in adaptors (Beckman, Spinco Division) and centrifuged at either 35,000 rev/min for 1 hr (SW50.1 rotor) or 25,000 rev/min for 2 hr (fixed-angle no. 30 rotor) to sediment virus. The sample and the upper half of the cushion were removed by a Pasteur pipette and designated 100,000 × *g* supernatant fluid. The pellet was suspended in an equal volume. In 10,000 × *g* supernatant fluids from sonically treated infected cells labeled with ^{3}H-lysine, approximately 75% of the trichloroacetic acid-precipitable radioactivity was in the 100,000 × *g* supernatant fluid.

Isolation of 8S viral antigen from infected cells. Extracts of cells infected with wild-type SV40 were analyzed by density gradient centrifugation, and complement fixation (CF) antigen was assayed in the fractions with a guinea pig antiserum prepared against virus particles. Sucrose density gradients (5 to 20%) in 0.15 M NaCl, NaP were prepared with a Beckman density gradient former. A 0.2-ml amount (1 to 2 mg of protein) of the 10,000 × *g* supernatant fluid of sonically treated cells was layered on the gradient and centrifuged at 40,000 rev/min for 14 hr at 4 C in an SW50.1 rotor. Fifteen-drop fractions were collected from the bottom through a 22-gauge needle. ^{125}I-labeled rabbit immunoglobulin G (IgG) (7*S*, 160,000 daltons), used as an internal standard, was kindly provided by Michael Mage, National Institutes of Health. A single peak was observed in the gradient slightly ahead of the 7*S* marker, as shown in Figure 1. The peak tubes were pooled and used for immunological studies without further treatment.

Isolation of capsid proteins on acrylamide gels. Capsid proteins were isolated from purified virions or cell preparations for immunological studies by electrophoresis on SDS-acrylamide gels. The proteins were eluted from the gels, after slicing (1.3 mm per slice), by freezing and thawing and subsequent incubation overnight in NaP at 4 C. The capsid proteins were identified by complement fixation with a rabbit antiserum prepared against SDS-treated virus and by their mobilities relative to internal standards of ^{125}I-IgG rabbit heavy chains (50,000 daltons) and light chains (23,000 daltons).

Serology. Complement fixation was performed as previously reported (12). T antigen was determined with a hamster antiserum prepared against virus-free, SV40-transformed hamster tumor. The following antisera were used to detect viral capsid protein(s).

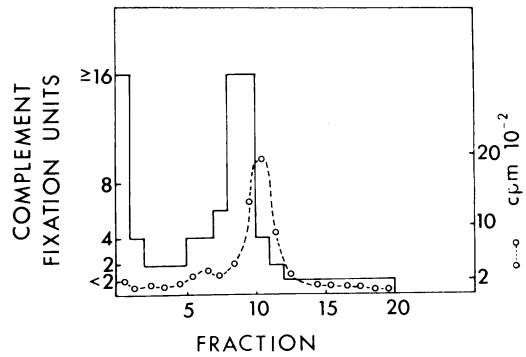

FIG. 1. *Sucrose gradient analysis of infected Vero cells. Cells were harvested at 72 hr after infection at a multiplicity of infection of 1 and extracted as described in the text. The 10,000 × g supernatant fluid was sedimented (right to left) on a 5 to 20% sucrose gradient and drop-collected from the bottom. A 25-μliter sample of each fraction was assayed for antigen by complement fixation with guinea pig antivirus serum diluted 1:200. Marker ^{125}I-IgG (7S) was determined by gamma spectrometry* (O--O).

Antisera to virus particles were as follows.

(i) Guinea pig antiserum to purified viral particles. Purified empty shells of SV40 (strain 777) were prepared by the method of Black et al. (3) and isolated free of contaminants by repeated equilibrium centrifugation in CsCl. Approximately 2 × 10^{4} CF units were injected into the footpads of one adult female NIH strain guinea pig in complete Freund's adjuvant (CFA). All studies were performed with a single bleeding 65 days after injection. No T antibody was detected. This serum was equally reactive with intact virions and empty shells by complement fixation. The serum was generously provided by David Hoggan, National Institutes of Health, and was chosen as a reference serum since the use of empty shells as an immunogen should have avoided the problem of antibodies to virus-induced, nonstructural proteins.

(ii) Baboon antiserum to virus. Baboons were injected once with unpurified SV40 harvested from baboon primary tissue culture cells. The serum had a T antibody titer of 16 and was obtained from the Research Reagents Branch, National Institute of Allergy and Infectious Diseases (V-430-501-050).

Antisera to denatured virus particles were as follows.

(i) Guinea pig antiserum to capsid protein(s). Intact virions of SV40 (strain SV-S) were purified by the method of Uchida et al. (15). After disruption of the virus at alkaline *p*H, the capsid proteins were isolated as a 4*S* peak on a sucrose gradient by the method of Anderer et al. (1). Analysis on SDS-acrylamide gels showed the 4*S* peak to contain predominantly the 45,000-dalton and 23,000-dalton capsid proteins (2, 4, 11). After dialysis and concentration against 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, *p*H 7.4, 300 μg of protein in CFA was injected into the footpads of an adult NIH strain female guinea pig. The pool used was composed of the bleedings from

days 35 and 58 after injection. No T antibody was detected.

(ii) Rabbit antiserum to SDS-virus particles. Empty shells of SV40 (strain SV-S) were purified by the method of Uchida et al. (15). The particles were disrupted at *p*H 10.5 and treated with 1% (w/v) SDS and 0.1% mercaptoethanol. A 150-μg amount of protein was injected subcutaneously three times, at 10-day intervals, into an adult female New Zealand rabbit. The first injection was in CFA, and the latter two in incomplete Freund's adjuvant. The animal was boosted intravenously with 150 μg of protein at day 50 and was exsanguinated 2 weeks later. No antibody to T antigen was detected.

Protein determination. Protein was determined by the method of Lowry et al. (9), with bovine serum albumin as a standard.

Determination of radioactivity. Total and trichloroacetic acid-precipitable radioactivity were determined by liquid scintillation spectrometry as previously reported (11). ^{125}I-IgG was determined with a gamma spectrometer (Packard model 3002).

RESULTS

Reactivity of antisera to viral components. Antisera prepared against viral particles or capsid protein(s) from denatured virus were compared for their reactivities. As shown in Table 1, antisera prepared against viral particles (similar to V antisera reported by other workers) reacted not only with viral particles but also with an 8*S* fraction isolated from infected cells on sucrose gradients (Fig. 1). Further characterization of this 8*S* antigen is presented below. These sera, however, did not react significantly with antigens obtained after denaturing virus at alkaline *p*H or with SDS. Consequently, use of these antisera at appropriate dilution detected viral particles and the 8*S* antigen, and should be specific for the latter in preparations devoid of viral particles. The guinea pig antiserum to viral particles was used for subsequent analysis of infected cells.

On the other hand, the two antisera prepared against denatured virus (*see above*) reacted with purified virus but not with the 8*S* antigen. Moreover, both of these antisera reacted with purified capsid proteins I (45,000 daltons) and II (23,000 daltons) eluted from SDS-acrylamide gels (11) or capsid proteins prepared from alkaline-denatured virions (isolated on sucrose gradients; reference 11). Neither antiserum reacted with 13,000- to 15,000-dalton protein components of the virion prepared by elution from SDS gels.

The guinea pig antiserum to capsid proteins was used at a 1:20 dilution for subsequent analysis of infected cells.

Analysis of infected cells for V antigen. V antigen is first detected with fluoroscent antibody or by CF in permissively infected Vero cells between 16 and 26 hr postinfection. Previous workers have demonstrated that newly synthesized viral particles are present in the nucleus by this time (10). However, it is not clear whether all the V antigen detected is in viral particles. One approach to evaluate this possibility would be to remove viral particles and analyze the extracts for V antigen. As shown in Table 2, more than 99% of amino acid-labeled, purified SV40 virions was sedimented at 100,000 × *g* through a 15% sucrose cushion when assayed as either radioactivity or V antigen. On the other hand, the major portion of material 20*S* or lower remained in the supernatant fluid.

Since virus and viral components were clearly separated by the above procedure, analysis of extracts of infected cells was undertaken. Infected cells were frozen and thawed once and treated sonically. After centrifugation at 10,000 × *g*, the

TABLE 1. *Reaction of antisera with purified viral components*

Antiserum	CF titer of antisera				
	Purified virus[a]	8*S* antigen[b]	SDS capsid proteins[c]		Capsid protein(s), *p*H 10.5[d]
			45,000 daltons	23,000 daltons	
I. Guinea pig antivirus particle.....	1,600	200	<6	<6	8
II. Baboon antivirus.....	500	>50	<6	<6	4
III. Guinea pig anti-10.5 capsid.....	250	<4	400	150	>250
IV. Rabbit anti-SDS-virus particle..	64	<2	160	160	64

[a] CsCl equilibrium purified intact virions [eight complement-fixation (CF) units with antiserum I diluted 1:200].

[b] Sucrose gradient preparation as in Fig. 1 (four CF units with antiserum I diluted 1:50 or 1:200).

[c] Sodium dodecyl sulfate (SDS)-polyacrylamide gel eluate of SDS-treated, purified, intact virus isolated as described in the text (one to four CF units with antiserum III or IV diluted 1:20).

[d] Sucrose gradient fraction (4*S*) of alkaline-denatured virion prepared by the method of Anderer et al. (10 CF units with antiserum III diluted 1:20; reference 1).

supernatant fraction was recentrifuged at 100,000 × *g* as in Table 2. As shown in Table 3, the great majority of the V antigen pelleted at 100,000 × *g* when assayed with antiserum prepared against viral particles. (The distribution of T antigen, a soluble protein, is included for comparison.) Similar results were observed at multiplicities of infection (MOI) between 1 and 70 using three different virus pools. Except for one case (24 hr at the highest MOI), only 2–12% of the V antigen was soluble. Analysis of samples at moderate cell degeneration (not shown) revealed no increase in the proportion of intracellular soluble V antigen.

TABLE 2. *Standards for 100,000 × g fractionation of infected cells*

Prepn[a]	Sedimentation rate	% Of preparation not sedimented at 100,000 × *g*	
		CF antigen[b]	Radioactivity
Intact virion.....	240*S*	<1 to 2	1
Viral DNA I.....	20*S*		82
Capsid proteins....	4 to 5*S*	90	90

[a] Samples were sedimented as described in the text for preparing "soluble" fraction. Intact virions and viral DNA I were purified by standard procedures. Capsid protein was isolated after *p*H 10.5 disruption of purified virions, as described in Table 1.

[b] Determined with guinea pig antiserum to capsid proteins diluted 1:20. CF, complement fixation.

Determination of the nature of the "soluble" V antigen. The V antigen in the 100,000 × *g* supernatant fraction ("soluble fraction") was analyzed to determine whether contamination by viral particles was responsible for the "soluble" V antigen. In three experiments (Table 3, virus pool 13), the presence of viral particles was determined on CsCl cushions. Only 0.8% of radiolabeled intact virions in the 10,000 × *g* supernatant fluid of infected cells remained in the 100,000 × *g* supernatant fluid. On the other hand, 4% of the V antigen in the 10,000 × *g* supernatant fluid remained in the 100,000 × *g* supernatant fluid. These results suggested that a considerable portion of the V antigen in the "soluble fraction" was not in intact virions.

Further evidence that the V antigen in the 100,000 × *g* supernatant fraction was not due merely to residual virus comes from sucrose gradient analysis of infected cell extracts. A soluble V antigen with a sedimentation coefficient of 8*S* was detected (Fig. 1). As can be seen from Table 2, material of less than 20*S* would be expected to be in the "soluble fraction." Consequently, an attempt was made to determine whether all of the soluble V antigen was in the 8*S* component. Although the sucrose gradient analysis of the soluble fraction revealed a single peak

TABLE 3. *100,000 × g fractionation of infected cells*[a]

Virus pool	Multiplicity of infection	Hr postinfection	V antigen[b]		T antigen[c]	
			Total CF units[d]	% Soluble[e]	Total CF units[d]	% Soluble[e]
11	25	2	8	ND[f]	<2	ND
		16	4	ND	<2	ND
		26	16	12	8	67
		64	128	6	32	67
13	1	73[g]	128	6	16	85
		96[g]	256	6	16	67
		112[g]	512	2	16	85
14	70	24	64	33	16	80
		48	1,024	6	64	80
		68	2,048	3	64	80

[a] Infected cells were extracted by sonic treatment and sedimented as described in the text and in Table 2.

[b] Determined with guinea pig antiserum to virus particles diluted 1:200.

[c] Determined with hamster antiserum to SV40 tumor serum diluted 1:20.

[d] Complement-fixation (CF) titer per 25 μliters of 10,000 × *g* supernatant fluid.

[e] (CF titer of 100,000 × *g* supernatant fluid/complement-fixation titer of 10,000 × *g* supernatant fluid) × 100.

[f] Not determined.

[g] Infected cells labeled with ^{3}H-lysine in lysine-reduced (10%) Eagle's medium for 16 to 24 hr before harvest.

of V antigen in the region of 8S, the CF titer (1:4) was so low that other peaks of V antigen could not be ruled out. However, both the 8S and "soluble" V antigen were markedly heat-labile, as shown in Table 4. Incubation at 56 C for 30 min inactivated more than 80% of the V antigen complement-fixation activity of both preparations. V antigen in purified viral particles (both intact virions and empty shells) was not markedly affected by this treatment. Both heat-inactivated preparations were reactive with antiserum prepared against denatured virus. When the two V antigen preparations were treated with SDS and dithiothreitol and subjected to electrophoresis on SDS-acrylamide gels, CF activity (determined with the rabbit antiserum to SDS virus) was detected only in the region of 40,000 to 50,000 daltons in both cases. It would thus appear that the V antigen detected in the 100,000 × g supernatant fluid of infected cell extracts was due predominantly to the presence of the 8S antigen.

Identification of unassembled capsid protein in cells infected with wild-type or temperature-sensitive mutant SV40. A class of temperature-sensitive mutants of SV40 (NTG-2 and NTG-11) has been described (14) which synthesized viral deoxyribonucleic acid but only low titers of V antigen at the restrictive temperature (41 C). However, extracts of these infected cells were appreciably more reactive with antiserum prepared against denatured viral particles. (At the permissive temperature, antigen reactive with sera prepared against both intact and denatured virus was observed.) Sonically treated cell extracts infected with NTG-2 at the restrictive temperature or wild-type virus were analyzed on 5 to 20% sucrose gradients to determine the size distribution of CF antigens. All samples were treated with deoxycholate before centrifugation since preliminary experiments had shown that the CF antigen in NTG-2-infected cells was not solubilized by sonic treatment alone. As can be seen in Table 5, low levels of CF antigen were detectable in the 4S region of the gradient (consistent with unassembled capsid protein) of both types of infected cells. Though this 4S antigen(s) represented all the immunoreactive material in cells infected with the temperature-sensitive mutant, it is a minor proportion of the complement-fixation reactivity in wild type-infected cells when assayed with antiserum prepared against either viral particles or capsid proteins.

In summary, the immunological studies of wild type-infected cells demonstrated only small quantities of 100,000 × g "soluble" capsid antigens either as free capsid protein or assembled into an 8S unit. To evaluate this result further, pulse-chase experiments were performed as detailed below.

Pulse-chase analysis of capsid protein in infected cells. Pulse-chase studies were performed to estimate the size of the pool of precursor material for the major capsid protein found in viral particles. Two approaches were considered: (i) determination of radiolabeled capsid protein in fractions freed of virus (e.g., 100,000 × g supernatant fraction) or (ii) recovery of capsid protein radio-

TABLE 4. *Comparison of 8S and 100,000 × g supernatant "V" antigens*

Prepn	CF antigen titer with	
	Antiserum to virus particles[a]	Antiserum to capsid proteins[b]
8S antigen[c]		
Unheated	16	2
Heated at 56 C for 30 min	2	8
100,000 × g supernatant fluid[d]		
Unheated	64	64
Heated at 56 C for 30 min	8	128

[a] Guinea pig antiserum to virus particles diluted 1:50 or 1:200. CF, complement fixation.

[b] Guinea pig antiserum to proteins diluted 1:20.

[c] Prepared on sucrose gradient as in Fig. 1.

[d] Prepared as in Table 3 from cells infected with virus pool 14 (48 hr postinfection).

TABLE 5. *Sucrose gradient analysis of cells infected at 41 C*[a]

Virus	Fraction	CF antigen titer[b] with	
		Antiserum to virus particles	Antiserum to capsid proteins
NTG-2	Pellet	<2	<2
	8S region	0	0
	4S region	0	10
Wild type	Pellet	128	64
	8S region	20	3
	4S region	6	6

[a] Cells were infected at multiplicity of infection of 1 to 10 and harvested 72 hr postinfection. The cells were extracted by sonic treatment, and the resultant 10,000 × g supernatant fluids were analyzed on 5 to 20% sucrose gradients as described in the text, except that samples were incubated for 10 min at 4 C with 1% deoxycholate in 0.05 M Tris-hydrochloride, pH 8, immediately before centrifugation. Complement fixation (CF) units per 25 μliters adjusted for volume in different pools.

[b] Determined with antisera as in Table 4.

activity in isolated viral particles. We have found that the amount of capsid protein in the 100,000 × *g* soluble fraction was difficult to quantitate accurately because of continued synthesis of cellular proteins of similar size in infected cells. On the other hand, it has been shown previously (11) that viral particles could be isolated free of cellular proteins on CsCl cushions and that the proportion of the major capsid protein in the particles could be determined by SDS-acrylamide gel electrophoresis. Furthermore, a significant quantity of newly synthesized capsid protein could be shown to have been assembled into viral particles within 15 min after addition of radioactive lysine (11). Therefore, the actual incorporation of major capsid protein into purified particles during pulse-chase experiments was chosen as the simplest and most accurate measure of the processing of the major capsid protein. Pulse-chase studies were undertaken to quantitate capsid protein synthesized in 2-hr pulses at different times after infection at high MOI. If the pool of nonvirion capsid was small (as suggested by the immunological analysis), only a minor increase in the total radioactivity in the labeled capsid protein in viral particles would be expected during a subsequent chase of sufficient length.

Preliminary to performing such experiments, it was necessary to verify the efficacy of the conditions of the chase. Cells were labeled for 24 hr with ^{3}H-lysine and the labeling medium was removed. The cells then were infected at an MOI of 50 to 100 infectious units per cell and reincubated for 29 or 35 hr in chase medium composed of Eagle's medium with a 100-fold excess of lysine. There was a decrease in total trichloroacetic acid-precipitable radioactivity in cells during the chase with a corresponding accumulation of radioactivity (non-acid-precipitable) in the medium at a rate of approximately 1% per hr. Approximately 0.1% of uninfected cell protein was indistinguishable from the major capsid protein after purification of extracts on CsCl cushion and gel electrophoresis, as shown in Table 6 (line 1). During the chase after infection, the specific activity in total cell protein and virus-associated, capsid-like protein decreased in a comparable manner. [Parallel cultures mock-infected and reincubated in chase medium behaved in a similar manner (Table 6, lines 6 and 7).] Under these conditions of infection, 4 to 5% of the total acid-precipitable ^{3}H-lysine from infected cells was in virus-associated capsid protein I in a 2-hr pulse at 29 to 31 hr postinfection. Consequently, it appears that radioactive lysine liberated from the breakdown of cellular protein became equilibrated with the extracellular medium and did not become preferentially incorporated into viral capsid protein.

To evaluate the pool of capsid protein in infected cells, cells infected with an MOI of 50 to 100 infectious units per cell were labeled for 2 hr with ^{14}C- or ^{3}H-lysine (pulse). Parallel cultures were pulsed for 2 hr and reincubated in chase medium for an additional 4 to 6 hr (chase). Viral particles were isolated on CsCl cushions, and radioactivity

TABLE 6. *Turnover of intracellular protein*[a]

Samples	Radioactivity in cell protein[b]		Radioactivity in capsid protein[c]	
	Counts/min/mg of protein	% Decrease per hr	Counts/min/mg of cell protein	% Decrease per hr
Preinfection	16.8×10^5		2,380	
Infected + chase				
29 hr	11.0×10^5	2.0	1,080	1.7
31 hr[d]	10.5×10^5	2.0	1,510	1.2
35 hr[d]	9.2×10^5	2.2	1,440	1.1
35 hr	9.0×10^5	2.2	1,120	1.5
Uninfected + chase				
31 hr[d]	11.9×10^5	1.6	905	2.2
35 hr[d]	10.2×10^5	2.1	1,200	1.8

[a] Cells were labeled with ^{3}H-lysine before infection, infected or mock-infected, and reincubated for the times indicated in Eagle's medium supplemented with a 100-fold excess of unlabeled lysine as described in the text.

[b] Trichloroacetic acid-precipitable radioactivity.

[c] Viral particles (or corresponding fractions from uninfected cells) were isolated on a CsCl cushion, and per cent radioactivity at 45,000 daltons was determined on SDS-acrylamide gels with unlabeled marker capsid protein as described in the text.

[d] Chase medium was removed at 29 hr postinfection, and cells were reincubated in lysine-free medium for 2 hr. The 31-hr samples were harvested immediately; 35-hr samples were reincubated for 4 hr in fresh chase medium.

in capsid protein was determined on SDS-acrylamide gels, as shown in Table 7. As expected, the specific activity of total cell protein decreased during the chase. The radioactivity in capsid protein of particles increased, but only slightly, during the chase, varying between 0 to 34% increase (average 13%). Though relatively short incubations in chase medium were employed, sufficient time would have elapsed for nonparticle capsid protein to have been incorporated into the capsid protein of viral particles, since previous experiments indicated incorporation of capsid protein into viral particles within 15 min (11) and the chase periods were two to three times longer than the initial pulse periods.

Since it had been shown previously that ^{3}H-lysine was incorporated preferentially into empty shells in a 2-hr pulse (11), we also compared the radioactivity in capsid protein for the two types of viral particles in these experiments, as shown in Table 8. In all cases, there was a preferential labeling of empty shells during the pulse and a subsequent increase in radioactivity in intact virions with a decrease in empty shells during the chase.

DISCUSSION

SV40-infected Vero cells have been analyzed immunologically and by pulse-chase experiments to measure the synthesis of the major capsid structural protein and to follow its assembly into viral particles. At least three forms of this protein have been detected late in infection: viral particles, an 8*S* antigen (see below), and a low-molecular-weight form compatible with free capsid protein. The great majority of immunoreactive material was in viral particles. Approximately 5 to 15% of the total antigen was in the other two forms, as determined when cells were extracted by sonic treatment and viral particles were removed by ultracentrifugation. Similar results were obtained in other monkey cell lines (CV-1, AH-1, and BSC-1) infected with SV40 (H. L. Ozer, *unpublished data*). This is at best an approximate estimate since 8*S* antigen and capsid protein could not be immunologically evaluated in cell fractions containing viral particles.

An 8*S* antigen, obtained by sedimentation of infected cell extracts on sucrose gradients, was identified immunologically with antiserum prepared against purified viral particles. This antigen is tentatively thought to represent viral capsomeres, based on the fact that its size was compatible with estimates of capsomeres previously

TABLE 8. *Pulse-chase experiment: radioactivity in capsid protein of intact virions and empty shells*[a]

Expt	Time postinfection	Capsid protein counts/min/mg of cell protein in	
		Intact virions	Empty shells
1	29–35 Hr	6,400	8,900
	Pulse	9,560	5,380
	Chase	12,030	4,530
2	29–35 Hr		
	Pulse	1,150	4,900
	Chase	5,275	2,950
3	48–56 Hr		
	Pulse	6,675	15,920
	Chase	12,015	12,985

[a] Samples as described in Table 7.

TABLE 7. *Pulse-chase experiments in infected cells*[a]

Expt	Hr postinfection	CF units ($\times 10^{-4}$)[b]	Cell protein counts/min/mg of protein		Capsid protein[c] counts/min/mg of cell protein		
			Pulse	Chase	Hr of chase		
					0	4	6
1							
A	29–31	2	3.1×10^5	2.6×10^5	15,300	14,860	
B				3.0×10^5		16,470	
2	29–31	2	1.4×10^5	0.9×10^5	6,050	8,220	
3	48–50	4	1.3×10^6	1.1×10^6	22,600		25,000

[a] Cells were infected at a multiplicity of infection of 50 to 100, pulse-labeled with ^{14}C-lysine (0.3 Ci/mmole, experiment 1 and 2) or ^{3}H-lysine (6.5 Ci/mmole, experiment 3). Incorporation of radioactive lysine was linear over the pulse period. Conditions of pulse and chase are described in the text. Duplicate cultures were employed for chase in experiments 1A and B.

[b] Complement fixation (CF) units per culture, determined with guinea pig antivirus particle serum.

[c] Viral particles were isolated on CsCl cushions, and per cent radioactivity in major capsid protein was determined on SDS-acrylamide gels as described in the text.

reported for this virus by electron microscopy (8) and for capsomeres isolated from purified polyoma virus (6). Analysis of the 8S antigen on SDS-acrylamide gels demonstrated the presence of the 45,000-dalton capsid protein as the major immunologically reactive component. Also, heating capsomeres at 56 C destroyed their reactivity with antivirus serum but resulted in the appearance of a new antigen detectible with antiserum reactive with capsid protein from denatured virus particles. It has not yet been determined whether the 8S antigen is a precursor to virus or a breakdown product of previously synthesized virus.

Unassembled capsid protein could be demonstrated best by using a temperature-sensitive mutant of SV40 (i.e., NTG-2 or NTG-11). At the restrictive temperature (41 C), these mutants synthesized little virus or 8S antigen, permitting evaluation of crude cell extracts with sera reactive with capsid proteins. Relatively small quantities of antigen (16 CF units) were observed at 64 hr postinfection at MOI of 1 to 10 at the restrictive temperature (14). Analysis on sucrose gradients of cells infected with this mutant confirmed that this antigen could be isolated as a low-molecular-weight entity. Prior treatment of the sonically treated cell extract with deoxycholate was necessary for optimal recovery. When wild-type virus-infected cell extracts were analyzed in a similar manner, lower levels of capsid protein were detected.

Analysis of antisera prepared against viral particles demonstrated that they contained a spectrum of antibodies at different concentrations, as determined by complement fixation. As previously recognized, the highest titer was directed against viral particles. However, antibodies against 8S antigen and probably against capsid protein were present. Antisera prepared against denatured viral capsid react with viral particles, cell extracts of temperature-sensitive mutants blocked in virus assembly, heat-denatured 8S antigen, and capsid-like antigen from sucrose gradients of infected cells treated with deoxycholate. Since antibodies to viral structural proteins other than the major capsid protein (e.g., capsid protein II) were also present at the dilutions used, it could not be concluded definitely that the same protein was being measured in all cases. Nonetheless, these sera should be useful reagents in dissecting further the steps in viral assembly and ascertaining whether viral structural proteins are synthesized in non-virus-releasing, transformed cell lines.

Pulse-chase experiments, under conditions of high MOI (50 to 100 per cell), supported the immunological data in that only a small pool of nonvirion capsid protein was detected at 29 or 48 hr postinfection. It would appear, therefore, that, under these conditions in which all the cells were producing large quantities of virus, the major capsid protein was rapidly assembled into viral particles. This result was consistent with the finding in the accompanying report that radiolabeled capsid protein was detectable in viral particles within 15 min after addition of radioactive lysine to infected cells. Furthermore, these results indicate that the rate of synthesis of the major capsid protein could be readily determined in such infected cells despite the persistence of cellular protein synthesis by isolating viral particles (as on CsCl cushions employed in these studies).

It is of interest that, at 48 to 72 hr after infection with an MOI of 1, the major portion of capsid protein labeled in a 2-hr pulse was not incorporated immediately into viral particles. In those cases, a twofold increase in labeled capsid protein of viral particles was observed in a 2-hr chase, and a fivefold increase was observed in a 16-hr chase (H. L. Ozer, *unpublished data*). These experiments cannot be considered directly comparable to the ones reported at high multiplicity because of the complications of multiple and asynchronous cycles of virus replication at the low MOI. Nonetheless, they suggest a possible rate-limiting step in virus assembly, and this possibility is being investigated.

Finally, the pulse-chase experiments suggest a model for the assembly of SV40 intact virions. As has been shown previously, capsid protein (possibly in the form of capsomeres) was preferentially incorporated into empty shells rather than intact virions in short pulses (15 min to 2 hr). During the chase period, however, radioactive capsid protein accumulated in intact virions with diminution of radioactivity in empty shells. These results confirm that empty shells were synthesized independently of intact virions and further suggested that empty shells were precursors to intact virions as reported for poliovirus (7). Inasmuch as the population of empty shells isolated on CsCl cushion was found to be morphologically heterogeneous (11), attempts are being made to determine which structure might serve as the possible intermediate.

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