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Subcellular Distribution of Viral Structural Proteins During Simian Virus 40 Infection

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The amounts of simian virus 40 structural polypeptides Vp1, Vp2, and Vp3 in different subcellular fractions at various times after lytic infection were determined by a quantitative immunoblotting procedure. Simian virus 40-infected cells were lysed with a buffer containing Nonidet P-40 to yield a soluble fraction. The Nonidet P-40–insoluble fraction was further fractionated in the presence of deoxycholate and Tween 40 to yield a soluble fraction (cytoskeletal) and an insoluble fraction (Nuc), which is primarily cell nuclei. At 33 h postinfection, the majority of viral structural proteins was found in the cell nucleus, whereas, at 48 to 65 h postinfection, Vp1 was distributed evenly among all cell fractions and Vp2 and Vp3 were found predominantly in the cytoskeletal and Nuc fractions. Thus, not all of the viral polypeptides synthesized in the cytoplasm migrated into the cell nucleus. Throughout infection, the molar ratio (Vp3/Vp2) was rather constant in all subcellular fractions, indicating that the synthesis or processing or both of Vp2 and Vp3 are coordinately regulated. The molar ratio of Vp1/(Vp2 + Vp3) varied among the fractions. The Vp1/(Vp2 + Vp3) molar ratio in the soluble fraction varied during the course of infection; however, constant ratios were maintained in the cytoskeletal and Nuc fractions. Thus, the mechanism which controls the movement of Vp1 to different compartments of the cell appears to be different from that of Vp2 and Vp3. The Vp1/(Vp2 + Vp3) value in the Nuc fraction was similar to the ratio found in virus particles. The constant molar distribution of Vp1, Vp2, and Vp3 in the Nuc fraction throughout infection suggests that there is a specific mechanism which regulates the transport of viral structural proteins. These results support the hypothesis that the structural proteins of simian virus 40 are transported into the cell nucleus in precise proportions.

Very little is known about the mechanism of polypeptide transport into the cell nucleus. The synthesis of proteins takes place in the cell cytoplasm, and the newly synthesized proteins are transported to the nucleus across the nuclear envelope. There seems to exist a specific transport mechanism for nuclear proteins which may be uncoupled to protein translation, since isolated nuclear proteins, both histone and nonhistone proteins, that are injected into the cytosol of cells migrate into the cell nucleus (3, 4, 13, 14, 22, 46). The possible mechanisms can be studied by using simian virus 40 (SV40)-infected cells, since viral structural polypeptides are transported into the cell nucleus for assembly into virus particles.

The genome of SV40 is a circular DNA molecule with ca. 5,200 nucleotides (16, 47). The double-stranded circular DNA is associated with four histones, H2A, H2B, H3, and H4 (35, 42), and this nucleoprotein complex is packaged in a capsid constructed from the virus-coded polypeptides Vp1, Vp2, and Vp3 (1, 19). Tryptic peptide analyses have shown that Vp2 and Vp3 share common tryptic fragments (18, 21, 43) and that Vp2 contains the entire Vp3 polypeptide chain at the C-terminal region (11, 48). It has been postulated that the structure of a virion particle is an icosahedron arranged with 72 capsomers, of which 12 are penton subunits and 60 are hexon subunits (1, 7, 10, 15, 23). As early as 15 h after infection, the viral capsid proteins can be detected by gel electrophoresis of cell extracts labeled with radioactive amino acids (2, 17, 30, 39, 55). These polypeptides are metabolically stable (30, 40, 55) and have been shown to be synthesized at a similar rate in the late phase of productive infection and transported to the nucleus rapidly after their synthesis (51).

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By using polyclonal antisera to Vp1 and Vp3, the cellular location of viral antigens was compared for wild-type and mutant virus-infected cells by immunofluorescence (26, 27). Our study showed that several mutations within the coding sequence of Vp1 alter the subcellular localization of Vp2 or Vp3 or both (27). Thus, we have postulated that Vp1 is involved in the transport of Vp2 or Vp3 or both from the cytoplasm to the nucleus for virus assembly (27). It has been shown that 8S soluble particles, which are composed of about six Vp1 polypeptides, exist in infected cells and that Vp1 polypeptides are rapidly assembled into virus particles (40). Therefore, one possible model for the effect of Vp1 on the intracellular localization of Vp2 or Vp3 or both is as follows. First, newly synthesized Vp1 polypeptides form an 8S particle, complex I, of $(Vp1)_n$ (n = 5 to 6). Second, one molecule of Vp2 or Vp3 or both is complexed to the 8S particle to form complex II, $(Vp1)_n$:Vp2 or $(Vp1)_n$:Vp3. These complexes are transported to the cell nucleus for assembly of newly made viral DNA and host histones into mature virions. At the restrictive temperature, the mutant Vp1 has an altered configuration which interacts differently with other Vp1 polypeptides or with a Vp2 polypeptide, a Vp3 polypeptide, or both thereby affecting their transport. If the transport of viral structural polypeptides occurs in the form of a complex as proposed above, the molar ratio of structural proteins in the nucleus should be constant, independent of the changing total amounts of Vp1, Vp2, and Vp3 in the cell during the course of infection.

In procaryotic systems, the structural proteins of bacteriophages are synthesized in precise proportions in infected cells (44, 45). Structural proteins of animal viruses are also required in precise proportions with respect to each other in virion assembly. Furthermore, the site for virus assembly in eucaryotic cells is confined to different compartments of

cells in a virus-specific manner. The assembly of SV40 takes place in the cell nucleus (20), thus the individual viral polypeptides which are synthesized in the cell cytoplasm must be transported to the cell nucleus for virus assembly. The ratio of structural protein may be regulated at: (i) the level of protein synthesis; (ii) the level of their transport to the site of assembly, i.e., the cell nucleus; or (iii) the level of assembly.

As an approach towards the understanding of the mechanism involved in the regulation of the viral structural protein transport, we have quantitated the amount of viral polypeptides in the soluble (Sol), cytoskeletal (Csk), and nuclear (Nuc) fractions of virus-infected cells, using polyclonal antisera and a quantitative immunoblotting technique (5, 36, 54). We believe that this is the first quantitative study of the viral structural proteins throughout viral infection.

MATERIALS AND METHODS

Cells and virus. The cells used in these studies were TC7 cells, a subline of African green monkey kidney cells. Cells were cultured in 100-mm dishes (Miles Laboratories, Inc.)in Dulbecco modified Eagle medium supplemented with arginine-hydrochloride (0.3 mg/ml), glutamine (1.5 mg/ml), histidine-hydrochloride (20 μg/ml), glucose (5 mg/ml), and 10% (vol/vol) fetal calf serum (Irvine Scientific). Wild-type SV40, strain 776, was obtained by two cycles of plaque purification. Stock SV40 lysate was prepared from infected cultures 10 to 15 days postinfection (p.i.) by three cycles of freeze-thawing. For our studies, subconfluent cultures of TC7 cells were infected at a multiplicity of infection of ca. 5. Cells were infected with virus for 1.5 h at 37°C and then overlaid with culture medium containing 3% fetal calf serum and (per ml): 125 μg of streptomycin sulfate, 31.25 μg of kanamycin, 250 μg of *n*-butyl *p*-hydroxybenzoate, and 625 U of penicillin G. Virus particles were purified as described previously (29) and were kept at the concentration of 1 mg/ml at −20°C in small portions in Laemmli sample buffer (31).

Cell counting. The number of cells per plate was determined either by direct counting in a hemacytometer after trypsinization of cells or by photomicrographic measurements. For photomicrographic measurements, micrographs of three randomly selected fields of each plate of cells were obtained before cell fractionation. The micrographs were taken with a Vario-orthomat camera (Leitz/Opto-Metric Div. of E. Leitz Inc.) attached to an Orthoplan microscope equipped with a X10 phase objective. The projected area of the culture plate on the micrograph was determined from the micrographs of a micrometer (Leitz) photographed at the same magnifications. The number of cells in each micrograph of known area was counted, and the average obtained from the three fields was used to calculate the number of cells in each plate at different times after infection.

Subcellular fractionation. Cells from both uninfected and infected cultures were fractionated into Sol, Csk, and Nuc fractions as previously described (8, 41) with some modifications. After removal of the medium from the culture dishes, the cells were washed twice with TD (1 mM sodium phosphate [pH 7.4], 0.14 M NaCl, 5 mM KCl, 25 mm Tris-hydrochloride) warmed to 37°C. All subsequent isolation steps were performed on ice. The cells were lysed on 100-mm culture dishes with 0.5 ml of TSCM lysis buffer (10 mM Tris-hydrochloride [pH 7.2], 1 mM $CaCl_2$, 1 mM $MgCl_2$, 250 mM sucrose, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) for 3 min on ice. As much of the solubilized cellular material as possible was removed with a pipette, and the insoluble material remaining on the culture dish was scraped off with a rubber policeman. The remaining soluble supernatant was removed after low-speed centrifugation at 2,000 × *g*, and the combined TSCM-soluble material was designated as the cytoplasmic soluble fraction (Sol). The insoluble material contains both nucleus and cytoskeletal components. The pellet was resuspended with RSB buffer (10 mM Tris-hydrochloride [pH 7.2], 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride), and Tween 40 and deoxycholate were added to final concentrations of 1 and 0.5% in 0.25 ml, respectively. The suspension was homogenized by 10 strokes in a Dounce homogenizer equipped with a B-pestle (Kontes Co.). The integrity of the nuclei was monitored by light microscopy. Low-speed centrifugation (2,000 × *g*) separated the solubilized cytoplasmic Csk fraction from the insoluble Nuc fraction, which consisted mainly of nuclei (6, 50). Protein fractions were prepared for sodium dodecyl sulfate (SDS)-gel electrophoresis and immunoblotting as described below.

Protein determination. The amount of protein in each of the Sol, Csk, and Nuc fractions from different times after infection was determined by the method described by Lowry et al. (37).

Antiserum preparation and characterization. Antisera against SDS-denatured SV40 viral polypeptides Vp1 or Vp3 were obtained from New Zealand White rabbits immunized by intradermal and subscapular injections. Preparation of the immunogens, immunization regimen, serum preparation, and characterization have been described previously (26).

SDS-polyacrylamide gel electrophoresis and immunoblotting. Protein samples in 50 mM Tris-hydrochloride (pH 6.8)–10 mM dithiothreitol–2% SDS–10% glycerol–0.001% bromophenol blue were boiled for 3 min and applied to SDS-polyacrylamide gels prepared by the method of Laemmli (31). After electrophoresis, the gels were applied to 0.1-μm (Schleicher & Schuell, Inc.) or 0.45-μm (Sartorius Filters, Inc.) nitrocellulose membranes, and the separated polypeptides were transferred to nitrocellulose as previously described (36). After electrophoretic transfer, the nitrocellulose replicas were incubated with 0.25% gelatin (Sigma Chemical Co.) in buffer A (0.05 M Tris-hydrochloride [pH 7.4], 0.15 M NaCl, 0.1% sodium azide) for 45 min at 37°C. The nitrocellulose sheets were then sealed in plastic bags containing a 1:400 dilution of anti-Vp1 or a 1:200 dilution of anti-Vp3 for the detection of Vp1 or Vp2 and Vp3, respectively. The replicas were incubated in antiserum for 13 to 16 h at room temperature on a rotator and washed extensively with three to five changes of buffer A for ca. 1 to 2 h. We noted that the contaminating anti-Vp1 activity in our anti-Vp3 serum was enhanced with increased reaction time (see below). Although the presence of the anti-Vp1 activity in anti-Vp3 serum did not influence the computation of the amount of Vp2 or Vp3, the length of the incubation time with anti-Vp3 was controlled and did not exceed 16 h. We interpreted this as differences in the binding constant between the anti-Vp1 and anti-Vp3 activities. The nitrocellulose membranes were then transferred to buffer A containing 0.25% gelatin for 10 min, and ^{125}I-labeled protein A was added to ca. 2×10^5 cpm/ml. After 45 min of incubation, the unbound ^{125}I-protein A was removed by washing with three to five changes of buffer A for ca. 1 to 2 h. The membranes were blotted dry between sheets of 3MM (Whatman, Inc.) paper and exposed to X-ray film (XAR-5, Eastman Kodak Co.), using an intensifying screen (Quanta II; Du Pont Co.). For determination of relative mobilities (relative molecular weight [M_r]), marker polypeptides labeled with ^{125}I were electrophoresed with protein samples and blotted to nitrocellulose. Marker

polypeptides and protein A were iodinated by the chloramine-T method described by Hunter and Greenwood (24), except that the reaction was quenched with 0.4 mg of tyrosine per ml instead of sodium metabisulfite. The specific activity of the ^{125}I-labeled polypeptides was ca. 3×10^6 cpm/μg.

Quantitation of viral antigens. The amounts of individual viral polypeptides in cell fractions were determined either by counting the amount of ^{125}I-protein A which was bound to the antibody-antigen complex on immunoblots, using the Beckman 7000 gamma counter, or by densitometric analysis of the antigen bands detected by autoradiography. The antigen bands on protein blots detected by autoradiography were scanned with a double-beam recording microdensitometer (MK IIIC; Joyce, Loebl, and Co., Ltd.). Different exposures of the same blots were obtained, and the ones used for densitometer scanning did not have bands whose intensity exceeded the linear range of film response. For the quantitation of the areas of the curves from the densitometer tracings, the information was entered into a Tektronix 4954 digitizing tablet. The data were then transferred directly to an IBM 3033 computer and analyzed. The radioactivity in or area of antigen bands in lanes contain known amounts of viral polypeptides (see below) served as standards for the calculation of the amount of viral antigens in the samples on the same blot.

RESULTS

Specificity of polyclonal antisera. The specificity of polyclonal anti-Vp1 and anti-Vp3 sera was examined by an immunoblotting technique (Fig. 1). SV40 virions were disrupted by SDS, and the virion polypeptides were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Nitrocellulose replicas containing different concentrations of virion protein were reacted with either anti-Vp1 or anti-Vp3.

The 44-kilodalton (KD) polypeptide, which is the major virion polypeptide, Vp1, was readily detectable in 8 ng of virion protein with a 1:400 dilution of anti-Vp1. Thus, as little as 6 ng of Vp1 was detectable, assuming that viral polypeptides were quantitatively transferred to nitrocellulose. In gel lanes containing 56 and 80 ng of virion protein (Fig. 1, lanes 4 and 5), Vp1-related antigens of 110, 103, and 90 KD were detected. At virus concentrations above 80 ng, additional Vp1-related antigens having M_rs of 50,000 (50K), 39K, 36K, 33.5K, and 19.5K were also detectable (data not shown; see below). Immunoblots containing between 240 and 1,040 ng of SDS-disrupted virion polypeptides showed that anti-Vp3 reacted with the 37-KD (Vp2) and 28-KD (Vp3) polypeptides (Fig. 1, lanes 6 through 10). Vp3 was detected in 240 ng of virion protein, although other experiments showed that Vp3 is detectable in as little as 100 ng of virion protein. More than 300 to 400 ng of virion protein was needed for the detection of Vp2. Thus, ca. 10 ng of either Vp2 or Vp3 antigen is detectable with a 1:200 dilution of anti-Vp3, using this method. We see that a 44-KD antigen, which has the same M_r as virion Vp1, immunoreacted with anti-Vp3. Immunoblots of disrupted virion polypeptides separated by two-dimensional isoelectric focusing gels described by O'Farrell (38) showed that the 44-KD antigen comigrated with Vp1 and possesses identical isoelectric points (data not shown). Since the 44-KD antigen was detected in lanes with more than 560 ng of virion protein, of which 420 ng was Vp1, the anti-Vp1 activity in the anti-Vp3 serum is small.

Quantitation of Vp1 and Vp3 polypeptides by immunoblotting technique. The amounts of 44-KD (Vp1) and 28-KD (Vp3) polypeptides were examined by densitometric tracing and ^{125}I counts in the antigen bands detected by autoradiography (Fig. 2). By both methods of quantitation, in both 3.5 to 120 ng of Vp1 (Fig. 2A) and 12 to 60 ng of Vp3 (Fig. 2B), the amount of bound antibody and ^{125}I-protein A is directly proportional to the amount of antigen immobilized on nitrocellulose. The amount of bound antibody and ^{125}I-protein A was off by ca. 40% when bands containing 240 to 350 ng of Vp1 were immunoblotted. In a separate experiment, up to 100 ng of Vp3 was quantitatively measured by this method.

The effect of protein overloading on the detectability of viral antigens was also tested. A constant amount of viral protein (400 ng) was coelectrophoresed in the presence of increasing amounts of soluble protein isolated from uninfected cells (up to 320 μg), and the viral polypeptides were blotted to nitrocellulose and reacted with anti-Vp3. The ^{125}I counts in Vp2 and Vp3 bands detected in lanes which included up to 320 μg of uninfected soluble protein were identical to those from a lane with virion protein only (data not shown). Thus, viral antigens in cellular fractions containing up to 320 μg of protein can be quantitated by extrapolation from standard curves from virion polypeptides detected on the same immunoblot.

Change in the protein content of subcellular fractions of

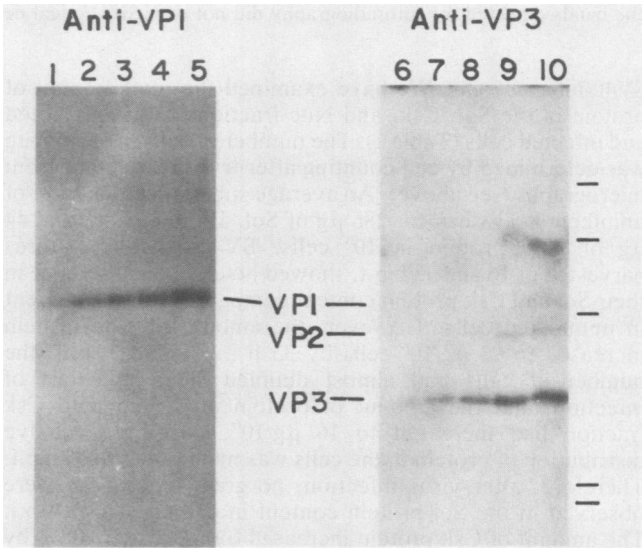

FIG. 1. Detection of viral polypeptides by immunoblots. Increasing amounts of SDS-disrupted virions were separated by SDS-polyacrylamide gel electrophoresis and transferred to a 0.1-μm nitrocellulose membrane by electrophoresis at 150 mA for 2 h, followed by electrophoresis for 10 h at 80 mA. The sheets of nitrocellulose were reacted either with a 1:400 dilution of anti-Vp1 or a 1:200 dilution of anti-Vp3 sera, then reacted with 2×10^5 cpm of ^{125}I-protein A per ml, and exposed to X-ray film (see the text). The amount of disrupted viral protein in each lane is: 1, 8 ng; 2, 16 ng; 3, 32 ng; 4, 56 ng; 5, 80 ng; 6, 240 ng; 7, 400 ng; 8, 560 ng; 9, 800 ng; and 10, 1,040 ng. The amount of Vp1, Vp2, and Vp3 in the virion capsids was determined by planimetry on Coomassie blue-stained polypeptide bands, using bovine serum albumin polypeptide bands of known concentration as standards. In our preparations, Vp1, Vp2, and Vp3 make up 75, 3.75, and 7.5% of the total mass of virion protein, respectively (data not shown). Molecular weight markers were iodinated and transferred to nitrocellulose membrane along with the virus samples. The molecular weight markers (designated by bars at the right side of the figure) are: phosphorylase *a* (92.5K), bovine serum albumin (68K), creatine phosphokinase (40K), soybean trypsin inhibitor (18.4K), and β-lactoglobulin (17.5K).

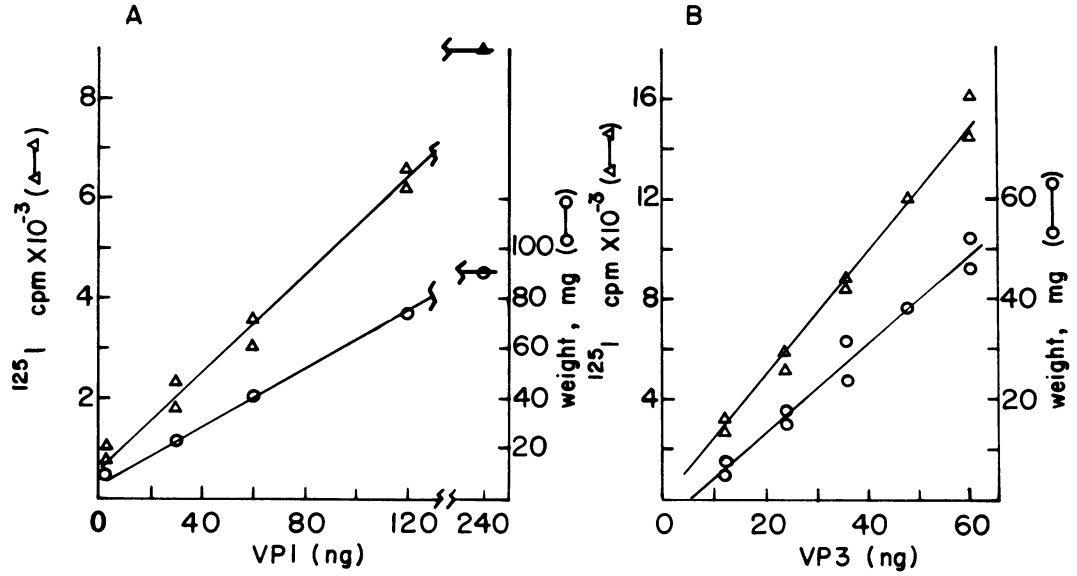

FIG. 2. Antibody-antigen reaction with increasing antigen concentration. Increasing concentrations of SDS-disrupted virion protein were separated on SDS-polyacryalmide gels, transferred to a 0.45-μm nitrocellulose membrane by electrophoresis for 3 h at 250 mA, reacted with ^{125}I-protein A and either anti-Vp1 (A) or anti-Vp3 (B), and exposed to X-ray film as described in the text. The reactivity of the different concentrations of antigens immobilized on nitrocellulose with immunoglobulin G and ^{125}I-protein A was determined by either densitometry tracings (○) or by excision of the bands from the nitrocellulose membrane and counting in the gamma counter (△). For densitometric analysis, the bands detected by autoradiography did not exceed 2 optical density units.

SV40-infected cells. We have examined the total amount of protein in the Sol, Csk, and Nuc fractions from uninfected and infected cells (Table 1). The number of cells in each plate was determined by cell counting after trypsinization or from micrographs (see above). An average subconfluent culture of uninfected cells has ca. 286 μg of Sol, 10 μg of Csk, and 44 μg of Nuc protein in 10^6 cells. SV40-infected cultures harvested at 16 and 33 h p.i. showed no obvious difference in their Sol and Csk protein content compared with the content in uninfected cells. However, the amount of Nuc protein increased to 93 μg/10^6 cells by 33 h p.i. By 48 h p.i., the number of cells had almost doubled since the start of infection, and the amount of protein in the infected Csk fraction had increased to 16 μg/10^6 cells. This relative distribution of protein in the cells was maintained to 65 h p.i. Therefore, after virus infection, no great differences were observed in the Sol protein content in cells up to 65 h p.i. The amount of Csk protein increased by more than 50% (by 48 h p.i.), and the amount of Nuc protein increased by 80% (at 33 h p.i.). Other experiments have shown that an increase in Nuc protein occurs after ca. 30 to 40 h of infection, and the increase (50%) (see footnote in Table I) was not as high as that (80%) seen in this particular experiment (data not shown). However, in all of the experiments, the increase observed in the Csk fraction (ca. 50%) late in infection was reproducible.

Distribution of Vp1, Vp2, and Vp3 in subcellular fractions. The polypeptides in subcellular fractions from uninfected and infected cells harvested at 40 h p.i. were visualized by Coomassie blue staining after SDS-polyacrylamide gel electrophoresis (Fig. 3). The Vp1 band was detectable in the Csk and Nuc fractions, and Vp3 was only barely visible in the nuclear fraction. Thus, the amount of viral polypeptides in each cell fraction was examined by an immunoblotting technique (Fig. 4).

With anti-Vp1 reactions, a band with the M_r of 44K, which is identical to the M_r of mature virion Vp1, is the major antigen detected in all of the fractions as early as 16 h p.i. At 48 and 65 h p.i. (in 2.5 and 2.8 μg of Csk protein in each gel lane, respectively), doublets of 110 and 103-KD and 90 and 19.5-KD bands were detectable in the Csk fraction. When up to 20 μg of Nuc protein isolated at 33, 48, and 65 h. p.i. was used for immunoblotting, bands similar to those detected in the Csk blots were also detectable (Fig. 4B). In addition, other minor polypeptide bands with M_rs of 50K, 39K, 36K, 33.5K, and 19.5K were detectable only in the Nuc fraction. Antigens with identical M_rs were also detected in the lanes containing higher concentrations of Csk protein late in infection (data not shown). These minor Vp1-related polypeptide species, currently unknown in origin, are identical in M_r to those detected in purified virions (see above).

TABLE 1. Amount of protien in subcellular fractions[a]

h p.i.	No. of cells (10^6) per plate[b]	μg of protein per 10^6 cells[c]		
		Sol	Csk	Nuc
0[d]	5.0	286	10	44
16	5.3	257	8	50
33	8.0	228	6	93
48	9.1	283	16	91
65	11.3	300	15	90

[a] In three different experiments, the protein distribution in uninfected cells showed similar values to the ones shown here and did not appear to be dependent on cell density (data not shown). Values for infected cultures were also confirmed in three different experiments. In two experiments, the amount of protein in the Nuc fraction isolated between 30 and 40 h p.i. was 150% of that in uninfected cultures.

[b] The number of cells in each 10-cm plate was determined from micrographs of randomly selected fields of each culture dish before extraction. The values agree with those obtained from trypsinization and counting.

[c] The amount of protein in each of the cell fractions was determined by the Lowry assay.

[d] Uninfected TC7 cultures.

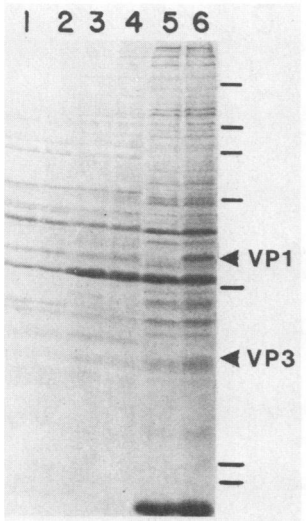

FIG. 3. Coomassie blue-stained polypeptides from Sol, Csk, and Nuc fractions of uninfected and SV40-infected cells. Uninfected (lanes 1, 3, and 5) and SV40-infected (lanes 2, 4, and 6) cultures were fractionated into Sol (lanes 1 and 2), Csk (lanes 3 and 4), and Nuc (lanes 5 and 6) fractions. Protein (70 μg) from each fraction was separated on a 15% SDS-polyacrylamide gel, and the polypeptides were visualized by Coomassie blue staining. The molecular weight markers (designated by bars at the right side of the figure) are: myosin (210K), β-galactosidase (120K), phosphorylase *a* (92.5K), bovine serum albumin (68K), creatine phosphokinase (40K), soybean trypsin inhibitor (18.4K), and β-lactoglobulin (17.5K).

With anti-Vp3 reactions, bands with M_rs of 37K (Vp2) and 28K (Vp3) were found in all three fractions by 33 h p.i. A 44-KD band which comigrates with virion Vp1 was also detected in all of the fractions. Other minor bands were also detectable: a band with an M_r of 24.5K was found in both the Sol and Csk fractions but was more pronounced in the Sol fraction; bands with M_rs of 74.5K and 78K were found in the Csk and Nuc fractions; and a 57K band was found in both the Sol and Nuc fractions. The presence of the 24.5, 74.5, and 78-KD bands in the Csk fraction was confirmed by separate experiments, using a high concentration of Csk proteins (data not shown). In addition, an intense 16.5-KD band was detected in the Sol and Csk fractions, and a 15.5-KD band was found in the Nuc fraction (Fig. 4C and D). The 15.5-KD polypeptide found only in the Nuc fraction is one of the histones, since isolated histones exhibited the immunoreactive band, using this anti-Vp3 serum, and the polypeptides were also detected in the Nuc fraction of uninfected cells (data not shown). In up to 1,040 ng of virion polypeptides, none of these additional Vp3-related antigens were found in purified virions (Fig. 1). The amount of ^{125}I counts in the 16.5-KD polypeptide band was quantitated relative to 37-KD (Vp2) and 28-KD (Vp3) polypeptides in the Sol and Csk fractions of the sample at 65 h p.i. (Fig. 4D). The molar ratio for the 37-, 28-, and 16.5-KD polypeptides in both the Sol and the Csk fractions is 1:2.4:1.5. Similar amounts of the three polypeptides were found in the Sol fraction of the sample at 48 h p.i., also. Thus, a relatively large number of anti-Vp3 reactive 16.5-KD polypeptides are present only in the cytoplasm of infected cells. In all of the immunoblots, using the 1:200 dilution of anti-Vp3 serum, a band of 64 KD was detected in all of the sample lanes. We have previously reported the detection of this 64-KD antigen (28) in both SV40-infected and uninfected samples. At this time, the nature of this reactive antigen is still unresolved.

The amounts of antigen in the 44-KD (Vp1), 37-KD (Vp2), and 28-KD (Vp3) bands in each fraction were quantitated by immunoblotting (Table 2). As infection progressed, the concentration of each viral polypeptide increased in all fractions. At 65 h p.i., Vp1, Vp2, and Vp3 constituted only 0.9, 0.02, and 0.05% of Sol protein, respectively, whereas Vp1, Vp2, and Vp3 constituted 4.5, 0.5, and 1.1%, and 1.4%, 0.07%, and 0.15% of the total protein in Csk and Nuc fractions, respectively. Thus, the concentration of each viral polypeptide was higher in the Csk and Nuc fractions compared with that in the Sol fraction. Vp1, Vp2, and Vp3 make up 1.1, 0.05, and 0.1% of the total protein in the cell at 65 h p.i., and the total amount of Vp1, Vp2, and Vp3 found per 10^6 cells at 48 and 65 h p.i. is 5.56×10^{-11}, 0.37×10^{-11}, and 1.0×10^{-11} mol and 10.4×10^{-11}, 0.54×10^{-11}, and 1.53×10^{-11} mol, respectively. The distribution of viral polypeptides at different times after infection in the Sol, Csk, and Nuc fractions is variable. At 33 h p.i., a large fraction (ca. 70%) of all three polypeptides was found in the Nuc fraction, whereas at 65 h p.i., Vp1 was distributed mostly to the Sol fraction and Vp2 and Vp3 were distributed evenly to all of the fractions. This suggests that the efficiency of viral protein transport to the cell nucleus changes during the course of infection. Alternatively, cell death at later times after infection would result in the release of mature virus particles from the cell nucleus to the cytoplasm and therefore raise the concentration of viral polypeptides in the Sol fraction. We thus believe that the data for 33 and 48 h p.i. are representative of the subcellular distribution of viral polypeptides. We conclude that Vp1 is distributed equally in all fractions, whereas Vp2 and Vp3 are found mostly in the Csk and Nuc fractions.

Molar ratio of viral polypeptides. Throughout infection, the molar ratio of Vp3 to Vp2, Vp3/Vp2, remained approximately constant with an average value of 2.5 in all subcellular fractions (Table 2), indicating that the synthesis and the movement of Vp2 and Vp3 are coordinately regulated. The molar ratio of Vp1 to the sum of Vp2 and Vp3 (Vpl/[Vp2 + Vp3]) differed among subcellular fractions (Table 2). In the Csk and Nuc fractions, the Vp1/(Vp2 + Vp3) ratios of ca. 2 and 4.3, respectively, remained approximately constant independent of time after infection, whereas it was variable in the Sol fraction. If average values for Vpl/(Vp2 + Vp3) are taken as 2 and 8 for the Csk and Sol fractions, then for every 1 Vp2 molecule and 2.5 Vp3 molecules, there are 7 Vp1 molecules in the Csk fraction and 28 Vp1 molecules in the Sol fraction. A relatively high concentration of Vp2 and Vp3 is present in the Csk fraction as opposed to the Sol fraction, suggesting that the mechanism for maintaining and regulating the amount of Vp1 in the different compartments of the cytoplasm differs from that of Vp2 and Vp3. The molar distribution of Vp1, Vp2, and Vp3 in the cell nucleus is constant throughout the progression of infection and is close to that in a virus particle (see legend to Fig. 1), suggesting that there is a selective mechanism which transports the right proportions of Vp1, Vp2, and Vp3 from the cell cytoplasm to the nucleus for virus assembly. Since the Vp1/(Vp2 + Vp3) ratio in the Sol fraction is much higher than that in the Csk or Nuc fraction, this again suggests that some selective mechanism exists which regulates the amount of Vp1, Vp2, and Vp3 in different subcellular fractions.

DISCUSSION

The present study points to three conclusions. (i) There is a selective mechanism for regulating the proportions of Vp1, Vp2, and Vp3 for transport into the cell nucleus for virus

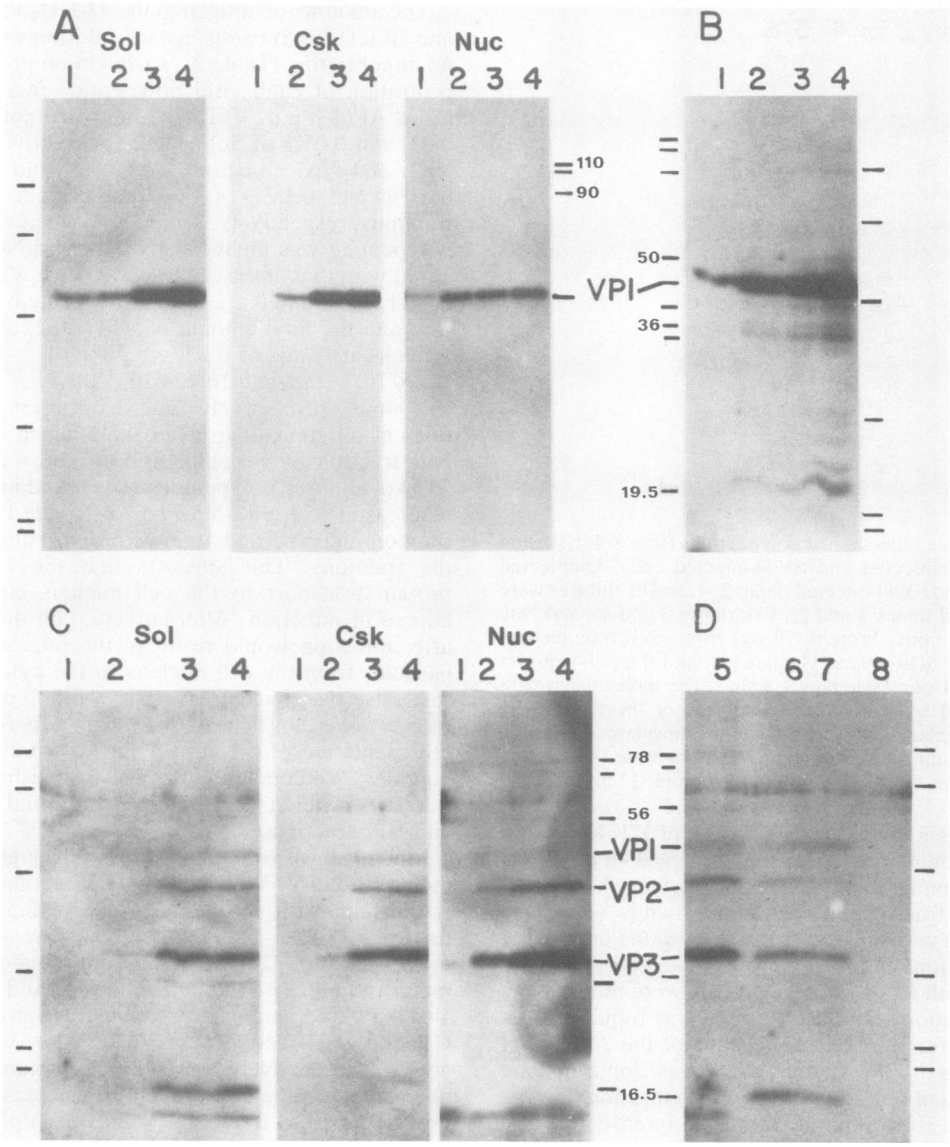

FIG. 4. Detection of viral antigens in Sol, Csk, and Nuc fractions by immunoblots. Protein from Sol, Csk, and Nuc fractions was separated on 12.5% SDS-polyacrylamide gels and transferred to 0.1-μm nitrocellulose membranes as described in the legend to Fig. 1. The blots were reacted with either a 1:400 dilution of anti-Vp1 (A and B) or a 1:200 dilution of anti-Vp3 (C and D). In panels A, B, and C, the fractions were obtained from SV40-infected cells at 16 (lane 1), 33 (lane 2), 48 (lane 3), and 65 (lane 4). Panel B shows the minor Vp1-related bands detectable in high concentrations of protein from the Nuc fractions at 48 h p.i. Panel D shows the anti-Vp3-reacted immunoblot of Nuc (lane 5), Sol (lane 6), and Csk (lane 7) protein of infected cells harvested at 65 h p.i. and of Sol protein of uninfected cells (lane 8). The amount of protein in each lane of the immunoblots are as follows: (A) Sol: 1, 56 μg; 2, 10 μg; 3, 10 μg; and 4, 9 μg. Csk: 1, 4 μg; 2, 2.5 μg; 3, 2.5 μg; and 4, 2.8 μg. Nuc: 1, 25 μg; 2, 4 μg; 3, 2 μg; and 4, 2 μg. (B) 1 through 4, 20 μg per lane. (C) Sol: 1, 280 μg; 2, 253 μg; 3, 335 μg; and 4, 88 μg. Csk: 1, 8 μg; 2, 9 μg; 3, 6.2 μg; and 4, 9.8 μg. Nuc: 1, 100 μg; 2, 80 μg; 3, 99 μg; and 4, 99 μg. (D) 5, 59.4 μg; 6, 88 μg; 7, 4.2 μg; and 8, 45 μg. The positions for Vp1, Vp2, and Vp3, as well as for minor bands detected by immunoblots, are designated by lines between panels A and B and panels C and D. Lines on the right of panel A mark the positions of the 110-, 103-, and 90-KD polypeptides. Bars on the left of panel B represent, from top to bottom, 110, 103, 90, 50, 39, 36, 33.5, and 19.5 KD, and between panels C and D, bars represent, from top to bottom, 78, 74.5, 56, 24.5, and 16.5 KD. The presence of minor antigen bands, some of which are not visible in the reproduced figure, was confirmed by separate experiments, using a high concentration of proteins. Bars on the left of panels A and C and on the right of panels B and D indicate the positions of molecular weight markers; from the top, these are 92.5K, 68K, 40K, 24K, 18.4K, and 17.5K.

assembly. The molar ratio of Vp1/(Vp2 + Vp3) in the Nuc fraction was close to that of mature virions and remained rather constant throughout infection. The molar ratio of Vp1, Vp2, and Vp3 (16.88:1:2.65) in virus particles obtained in this study (see legend to Fig. 1) is somewhat different from the data reported previously (10:1:2.3) (15, 23, 42). In the present study, we used the value obtained from our virus preparation for comparison. (ii) The mechanism which controls the movement of Vp1 to different compartments of cells is different from that of Vp2 and Vp3, for the Vp3/Vp2 ratio was rather constant in all of the cell fractions, but the Vpl/(Vp2 + Vp3) molar ratios varied among the individual fractions. It is unlikely that Vp1 was easily and artifactually released from the cytoplasmic cytoskeletal fraction during

TABLE 2. Amount of Vp1, Vp2, and Vp3 in subcellular fractions[a]

h p.i.	Vp1			Vp2			Vp3			Molar ratios	
	ng/μg[b]	mol (10^{11}) per 10^6 cells[c]	%[d]	ng/μg[b]	mol (10^{11}) per 10^6 cells	%	ng/μg	mol (10^{11}) per 10^6 cells	%	Vp3/Vp2	Vp1/(Vp2 + Vp3)
Sol											
16	0.09	0.06	31								
33	0.56	0.29	19	0.03	0.02	18	0.04	0.04	12	2	4.83
48	3.05	1.95	35	0.07	0.05	14	0.14	0.14	14	2.8	10.26
65	9.08	6.06	58	0.20	0.16	30	0.48	0.50	33	3.1	9.18
Csk											
16	1.56	0.03	16								
33	5.50	0.08	5	1.00	0.02	14	1.81	0.04	14	2	1.33
48	41.39	1.48	27	4.55	0.19	52	9.36	0.53	53	2.8	2.06
65	47.61	1.62	16	5.15	0.21	39	10.43	0.56	37	2.7	2.10
Nuc											
16	0.83	0.11	53								
33	5.62	1.19	76	0.33	0.08	68	0.64	0.21	74	2.6	4.1
48	10.43	2.13	38	0.53	0.13	34	1.04	0.33	33	2.5	4.63
65	13.61	2.72	26	0.71	0.17	31	1.48	0.47	30	2.8	4.25

[a] The data presented in this table represent average values obtained from at least three measurements of each sample. Vp1 and Vp3 were quantitated in two other experiments; the data were in agreement with that of this experiment, whereas Vp2 was only quantitated in samples from this experiment.

[b] Nanograms of viral polypeptide detected per microgram of protein from each fraction used for immunoblotting. For the detection of Vp1, each gel lane contained between 0.5 and 56 μg of protein and between ca. 6 and 280 μg for the detection of Vp2 and Vp3.

[c] Moles of viral polypeptide per 10^6 cells (assuming that all cells are infected).

[d] Percent distribution of viral polypeptides in each of the fractions at different times after infection.

the isolation procedure, since the fraction of Vp1 found in the Sol fraction increases as infection progresses. (iii) The synthesis and the movement of Vp2 and Vp3 are coordinately regulated, for there was a constant Vp3/Vp2 molar ratio of ca. 2.5 in all cell fractions. In addition, the present study indicates that not all of the viral polypeptides synthesized in the cytoplasm migrate into the cell nucleus. At 33 h p.i., ca. 70% of the virion structural proteins are located in the Nuc fraction. By 48 h p.i., only ca. 35% of the total viral antigens are located in the cell nucleus. This also suggests that the efficiency of viral protein transport to the cell nucleus changes during infection.

Late in infection, various Vp1-related antigens of distinct molecular weights are detected in both the Csk and Nuc fractions. Vp1-related polypeptides with the same molecular weights can also be detected in SDS-denatured virions purified by CsCl density gradient centrifugation. Some of these have been reported previously (25, 30, 55). Anti-Vp3 serum detected a substantial amount of a 16.5-KD polypeptide in the Sol and Csk fractions of infected cells, especially late in infection, but not in virus particles or in the nuclear fraction. The role of the Vp1-related polypeptides late in infection or in virions and the 16.5-KD Vp3-related polypeptide in the cell cytoplasm is unclear at this time.

The protein content of the Csk fraction doubled by 48 h after virus infection, and Vp1, Vp2, and Vp3 were highest in that fraction late in infection. In contrast to the Sol fraction of the cytoplasm, the molar ratios of Vp1/(Vp2 + Vp3) in the Csk fraction was low throughout infection. Our preliminary data suggest that viral polypeptides are synthesized in the Sol fraction and then anchored onto a detergent-insoluble fraction after synthesis. Therefore, some mechanism must regulate the anchoring of viral polypeptides to Csk structures in precise proportions after synthesis.

The method used to quantitate specific antigens in the present study measures the accumulated amount of individual viral polypeptide over the course of infection but does not offer any information regarding the rate of synthesis or degradation of the polypeptides. However, one might assume that the value obtained in our study reflects the net polypeptide synthesis, for SV40 polypeptides have been reported to be synthesized at similar rates and to be metabolically stable (30, 40, 51, 55). Thus, our study suggests that after synthesis the structural proteins of SV40 are transported into the cell nucleus in precise proportions. The data presented in this paper agree with our working hypothesis that the transport of viral structural polypeptides occurs in the form of a complex (27). A similar study on the quantitation of viral polypeptides with temperature-sensitive mutants (9, 12, 33, 34, 51–53), as well as with viable deletion mutants (11, 32, 49), will test our hypothesis.

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LITERATURE CITED

1. **Anderer, F. A., H. D. Schlumberger, M. A. Koch, H. Frank, and H. J. Eggers.** 1967. Structure of simian virus 40. II. Symmetry and components of the virus particle. Virology **32**:511–523.
2. **Anderson, C. W., and R. F. Gesteland.** 1972. Pattern of protein synthesis in monkey cells infected by simian virus 40. J. Virol. **9**:758–765.
3. **Bonner, W. M.** 1975. Protein migration into nuclei. I. Frog oocyte nuclei in vivo accumulate microinjected histones, allow entry to small proteins, and exclude large proteins. J. Cell Biol. **64**:421–430.
4. **Bonner, W. M.** 1975. Protein migration into nuclei. II. Frog oocyte nuclei accumulate a class of microinjected oocyte nuclear proteins and exclude a class of microinjected oocyte cytoplasmic proteins. J. Cell Biol. **64**:431–437.
5. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacryl-

amide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
6. **Capco, D. G., K. M. Wan, and S. Penman.** 1982. The nuclear matrix: three dimensional architecture and protein composition. Cell **29**:847–858.
7. **Caspar, D. L. D., and A. Klug.** 1962. Physical principles in the construction of regular viruses. Cold Spring Harbor Symp. Quant. Biol. **27**:1–24.
8. **Cervera, M., G. Dreyfuss, and S. Penman.** 1980. Messenger RNA is translated when associated with the cytoskeletal framework in normal and VSV-infected HeLa cells. Cell **23**:113–120.
9. **Chou, J. Y., and R. G. Martin.** 1974. Complementation analysis of simian virus 40 mutants. J. Virol. **13**:1101–1109.
10. **Christiansen, G., T. Landers, J. Griffith, and P. Berg.** 1977. Characterization of components released by alkali disruption of simian virus 40. J. Virol. **21**:1079–1084.
11. **Cole, C. N., T. Landers, S. P. Goff, S. Manteuil-Brutlag, and P. Berg.** 1977. Physical and genetic characterization of deletion mutants of simian virus 40 constructed in vitro. J. Virol. **24**:277–294.
12. **Cosman, D. J., and M. J. Tevethia.** 1981. Characterization of a temperature-sensitive, DNA-positive, nontransforming mutant of simian virus 40. Virology **112**:605–624.
13. **De Robertis, E. M., R. F. Longthorne, and J. B. Gordon.** 1978. Intracellular migration of nuclear proteins in xenopus oocytes. Nature (London) **272**:254–256.
14. **Dingwall, C., S. V. Sharnick, and R. A. Laskey.** 1982. A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. Cell **30**:449–458.
15. **Estes, M. K., E.-S. Huang, and J. S. Pagano.** 1971. Structural polypeptides of simian virus 40. J. Virol. **7**:635–641.
16. **Fiers, W., R. Contreras, G. Haegeman, R. Rogiers, A. Van de Voorde, H. Van Heuverswyn, J. Van Herreweghe, G. Volckaert, and M. Ysebaert.** 1978. Complete nucleotide sequence of SV40 DNA. Nature (London) **273**:113–120.
17. **Fischer, H., and G. Sauer.** 1972. Identification of virus-induced proteins in cells productively infected with simian virus 40. J. Virol. **9**:1–9.
18. **Gibson, W.** 1975. A peptide comparison of proteins in the simian virus 40 virion. Virology **68**:539–543.
19. **Girard, M., L. Marty, and F. Suarez.** 1970. Capsid proteins of simian virus 40. Biochem. Biophys. Res. Commun. **40**:97–102.
20. **Granboulan, N., P. Tournier, R. Wicker, and W. Bernhard.** 1963. An electron microscope study of the development of SV40 virus. J. Cell Biol. **17**:423–441.
21. **Greenaway, P. J., and D. LeVine.** 1973. Amino acid compositions of simian virus 40 structural proteins. Biochem. Biophys. Res. Commun. **52**:1221–1227.
22. **Gurdon, J. B.** 1970. Nuclear transplantation and the control of gene activity in animal development. Proc. R. Soc. London B **176**:303–314.
23. **Huang, E.-S., M. K. Estes, and J. S. Pagano.** 1972. Structure and function of the polypeptides in simian virus 40. I. Existence of subviral deoxynucleoprotein complexes. J. Virol. **9**:923–929.
24. **Hunter, W. M., and F. C. Greenwood.** 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature (London) **194**:495–496.
25. **Kasamatsu, H., and J. Flory.** 1978. Synthesis of the SV40 viral polypeptide Vp1 during infection. Virology **86**:344–353.
26. **Kasamatsu, H., and A. Nehorayan.** 1979. Intracellular localization of viral polypeptides during simian virus 40 infection. J. Virol. **32**:648–660.
27. **Kasamatsu, H., and A. Nehorayan.** 1979. Vp1 affects intracellular localization of Vp3 polypeptide during simian virus 40 infection. Proc. Natl. Acad. Sci. U.S.A. **76**:2808–2812.
28. **Kasamatsu, H., M. Shyamala, and W. Lin.** 1980. Host antigens in the centriolar region are induced in SV40-infected TC7 cells: SV40 small-t-function requirement. Cold Spring Harbor Symp. Quant. Biol. **44**:243–252.
29. **Kasamatsu, H., and M. Wu.** 1976. Protein-SV40 DNA complex stable in high salt and sodium dodecyl sulfate. Biochem. Biophys. Res. Commun. **68**:927–936.
30. **Kiehn, E. D.** 1973. Protein metabolism in SV40-infected cells. Virology **56**:313–333.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
32. **Lai, C.-J., and D. Nathans.** 1974. Deletion mutants of SV40 generated by enzymatic excision of DNA segments from the viral genome. J. Mol. Biol. **89**:170–193.
33. **Lai, C.-J., and D. Nathans.** 1975. A map of temperature-sensitive mutants of simian virus 40. Virology **66**:70–81.
34. **Lai, C.-J., and D. Nathans.** 1976. The B/C gene of simian virus 40. Virology **75**:335–345.
35. **Lake, R. S., S. Barban, and N. P. Salzman.** 1973. Resolutions and identification of the core deoxynucleoproteins of the simian virus 40. Biochem. Biophys. Res. Commun. **54**:640–647.
36. **Lin, W., and H. Kasamatsu.** 1983. On the electrotransfer of polypeptides from gels to nitrocellulose membranes. Anal. Biochem. **128**:302–311.
37. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**:265–275.
38. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
39. **O'Farrell, P. Z., and H. M. Goodman.** 1976. Resolution of simian virus 40 proteins in whole cell extracts by two-dimensional electrophoresis: heterogeneity of the major capsid protein. Cell **9**:289–298.
40. **Ozer, H. L., and P. Tegtmeyer.** 1972. Synthesis and assembly of simian virus 40. II. Synthesis of the major capsid protein and its incorporation into viral particles. J. Virol. **9**:52–60.
41. **Penman, S.** 1966. RNA metabolism in the HeLa cell nucleus. J. Mol. Biol. **17**:117–130.
42. **Pett, D. M., M. K. Estes, and J. S. Pagano.** 1975. Structural proteins of simian virus 40. I. Histone characteristics of low-molecular-weight polypeptides. J. Virol. **15**:379–385.
43. **Prives, C. L., and H. Shure.** 1979. Cell-free translation of simian virus 40 16S and 19S L-strand-specific mRNA classes to simian virus 40 major Vp-1 and Minor Vp-2 and Vp-3 capsid proteins. J. Virol. **29**:1204–1212.
44. **Ray, P. N., and M. L. Pearson.** 1974. Evidence for post-transcriptional control of the morphogenetic genes of bacteriophage lambda. J. Mol. Biol. **85**:163–175.
45. **Ray, P. N., and M. L. Pearson.** 1975. Functional inactivation of bacteriophage λ morphogenetic gene mRNA. Nature (London) **253**:647–650.
46. **Rechsteiner, M., and L. Kuehl.** 1979. Microinjection of the nonhistone chromosomal protein HMG1 into bovine fibroblasts and Hela cells. Cell **16**:901–908.
47. **Reddy, V. B., B. Thimmappaya, R. Dhar, K. N. Subramanian, B. S. Zain, J. Pan, P. K. Ghosh, M. L. Celma, and S. M. Weissman.** 1978. The genome of simian virus 40. Science **200**:494–502.
48. **Rozenblatt, S., R. C. Mulligan, M. Gorecki, B. E. Roberts, and A. Rich.** 1976. Direct biochemical mapping of eukaryotic viral DNA by means of a linked transcription-translation cell-free system. Proc. Natl. Acad. Sci. U.S.A. **73**:2747–2751.
49. **Shenk, T. E., J. Carbon, and P. Berg.** 1976. Construction and analysis of viable deletion mutants of simian virus 40. J. Virol. **18**:664–671.
50. **Staufenbiel, M., and W. Deppert.** 1982. Intermediate filament systems are collapsed onto the nuclear surface after isolation of nuclei from tissue culture cells. Exp. Cell Res. **138**:207–214.
51. **Tegtmeyer, P., J. A. Robb, C. Widmer, and H. L. Ozer.** 1974. Altered protein metabolism in infection by the late tsB11 mutant of simian virus 40. J. Virol. **14**:997–1007.
52. **Tegtmeyer, P., and H. L. Ozer.** 1971. Temperature-sensitive mutants of simian virus 40: infection of permissive cells. J.

Virol. **8**:516–524.
53. **Tevethia, M. J., and L. W. Ripper.** 1977. Biology of simian virus 40 (SV40) transplantation antigen (TrAg). II. Isolation and characterization of additional temperature-sensitive mutants of SV40. Virology **81**:192–211.
54. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**:4350–4354.
55. **Walter, G., R. Roblin, and R. Dulbecco.** 1972. Protein synthesis in simian virus 40-infected monkey cells. Proc. Natl. Acad. Sci. U.S.A. **69**:921–924.