Protein Synthesis in Simian Virus 40-Infected Monkey Cells

(structural and nonstructural proteins/gel electrophoresis/polyoma)

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ABSTRACT The proteins of purified Simian virus 40 (SV40) were examined by sodium dodecyl sulfate-acrylamide gel electrophoresis and compared with the polypeptides synthesized in SV40-infected monkey cells. Purified virions contain two major components, with molecular weights of 44,000 and 31,000. Together they make up 83% of the total virion proteins. In addition, the virus contains 12 minor polypeptides, which are believed to be cellular proteins, or peptides derived from proteolytic degradation of the 44,000 molecular weight polypeptide. Pulse-label experiments show that about 90% of the polypeptides synthesized after SV40 infection are host-cell proteins; 10% represent the two major structural components of the virion. A small fraction (about 0.5%) consists of three polypeptides (molecular weights 70,000, 60,000, and 8,000) that are neither part of the virion nor detectable in uninfected cells. They are either virus-induced cellular proteins or, more likely, proteins coded for by the SV40 genome.

Progress in understanding how the small DNA viruses, polyoma and SV40, transform cells now rests on finding out which proteins are specified by the viral genome, and what functions they perform. These proteins can be physically identified by acrylamide gel electrophoresis, in part as structural virus proteins (VP) (1-3) and in part as nonstructural proteins (NVP) present only in infected cells. The identification of nonstructural proteins is hampered by the continuation of host protein synthesis in infected cells, which does not permit resolution of viral from cellular proteins by conventional gel electrophoresis. However, recent developments in acrylamide gel electrophoresis technology, associated with radioautography of the gel and densitometry of the radioautographs, permit a clear identification of proteins synthesized in the cells after infection; they are likely to be specified by viral genes. The study of these proteins, and of those present in virions, helps to determine the number and sizes of the viral proteins. Some of the results presented in this paper were obtained with SV40, some with polyoma virus. We feel that both viruses can be used to build a common picture because the two viruses are so similar physically, biologically, and genetically.

METHODS

Virus and Cells. Large-plaque SV40 was used throughout this study. Vero cells, a continuous cell line isolated from primary cells from African green monkey kidney, and BSC-1 cells, a permanent line of the same origin, were grown in Dul-

becco's Modified Eagle's Medium containing 10% fetal-calf serum and 10% tryptose broth ("medium").

Radioactive Labeling and Purification of Virus. Radioactive SV40 was prepared by infection of confluent BSC-1 cells with a multiplicity of 20 plaque-forming units (PFU)/cell. After a 1-hr adsorption period, medium was added. At 20 hr after infection, this medium was replaced with Earle's solution, containing 10% fetal-calf serum and a mixture of ^{14}C -labeled reconstituted protein hydrolysate (Amersham) (0.5-0.8 $\mu\text{Ci/ml}$). 4 Days after infection, the cells were harvested and virus was purified as described for polyoma virus (4).

Radioactive Labeling of Cells. Cells were infected with a multiplicity of 20 PFU/cell and kept in medium. Before the cells were labeled, the medium was removed, the cells were washed once with Earle's salt solution, and radioactive medium (0.5 ml for a 50-mm petri dish) containing 10% modified Eagles Medium in Earle's salt solution and 20-200 $\mu\text{Ci/ml}$ of ^{14}C -labeled protein hydrolysate was added. After the labeling period, the cells were washed three times with Modified Eagle's Medium, and cold 5% trichloroacetic acid was added to the dish. The cells were scraped off and washed 3 times with cold 5% Cl_3CCOOH and 3 times with cold acetone by sedimentation in a 15-ml conical centrifuge tube. After drying, the cell pellet was dissolved in sample buffer (0.06 M Tris-HCl, pH 6.8-2% sodium dodecyl sulfate), heated for 2 min to 100°, and analyzed on gels. Mock-infected cells were treated identically.

Cell Fractionation. Nuclei and cytoplasm from infected and control cells were prepared by adding 1 ml of a cold 0.5% Nonidet P40 (NP40) solution in isotonic Tris-buffer (0.14 M NaCl-5 mM KCl-0.5 mM MgCl_2 -1 mM CaCl_2 -25 mM Tris-HCl, pH 7.4) to the cells on the petri dish. After 10 min at 4°, the nuclei were pelleted, washed once in Tris-buffer, resuspended in Tris-buffer, and precipitated with an equal volume of cold 10% Cl_3CCOOH . The precipitate was then washed three times with cold 5% Cl_3CCOOH and three times with cold acetone. The cytoplasmic proteins were also precipitated with Cl_3CCOOH and prepared for gel electrophoresis.

Acrylamide Gel Electrophoresis and Radioautography. The methods used in preparing discontinuous acrylamide gels containing sodium dodecyl sulfate (SDS) have been described in detail by Laemmli and Maizel, as were the procedures used for radioautography (5, 6). Briefly, after electrophoresis the gels were sliced longitudinally, dried on paper, and placed under pressure against x-ray film (Kodak, RP, R54). After development, the radioautograph was scanned with a Gilford

Abbreviations: SDS, sodium dodecyl sulfate; VP, viral structural protein; NVP, nonviral-capsid protein.

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Fig. 1. Densitometer recording of SV40 virion polypeptides. Purified SV40 virus labeled with a mixture of [^{14}C]aminoacids was dissolved in sample buffer containing 2% SDS–1% 2-mercaptoethanol. After it was heated for 2 min to 100°, the dissociated virus was electrophoresed on a 10-cm 13% acrylamide gel for 6 hr at 80 V. The dry gel was exposed to x-ray film for 4 weeks to detect all minor components. VP1 and VP3 are overexposed (see *Methods*).

recording spectrophotometer equipped with a linear-transport scanning device. A light source at 600 nm was used. An appropriate exposure time was chosen in which the film gave a linear response to the amount of radioactivity. This was the case up to an A_{600} of 1. Minor virion components could only be detected after an exposure time of several weeks, during which the intensity of the major polypeptides exceeded the limits of linear film response. In order to quantitate all detectable components, two exposures—a short one for the major polypeptides and a long one for the minor components—were necessary.

RESULTS

Virion proteins

SV40 virions labeled with radioactive amino acids were analyzed by polyacrylamide gel electrophoresis in the presence of SDS, by use of a high-pH, discontinuous, Tris-glycine buffer (5, 6), with subsequent analysis of the gels by radioautography. As shown in Fig. 1, two major proteins (VP1 and VP3)[†] and several minor polypeptides, designated by numbers 1–12, can be detected in virions by densitometry of the radioautogram. When the gel is fractionated with an automatic gel fractionator, less resolution is obtained and several of the minor components are not visualized.

The approximate molecular weights of SV40 polypeptides

[†] This nomenclature corresponds to that of Esters *et al.* (3); VP4, VP5, and VP6 correspond to our 10, 11, and 12; VP2 to our 5, 6, and 7.

Fig. 2. Densitometer recordings of radioactive polypeptides from infected and uninfected cells. Confluent Vero cells infected with 20 PFU/cell where labeled with [^{14}C]labeled protein hydrolysate (40 $\mu\text{Ci/ml}$) from 52 to 53 hr after infection. Nuclei and cytoplasm were prepared with NP40 (*Methods*). Mock-infected cultures were treated identically. 13% acrylamide gels were 10 cm long. Duration of electrophoresis was 6 hr at 80 V; (a) total cells; (b) nuclei; (c) SV40 virus; —, infected; ·····, mock-infected.

were determined from their relative migration in SDS gels (7). The coat proteins of adenovirus type 2 (8), polyoma virus (4), and several other proteins of known molecular weight served as markers (Table 1). The two major structural polypeptides VP1 (molecular weight 44,000) and VP3 (molecular weight 31,000) contain together 83% of the total radioactivity of the virion proteins. The other components are present in much smaller quantities (Table 1). In earlier experiments with the SDS-phosphate buffered gel (Walter, G., and Roblin, R., unpublished results), VP3 showed a molecular weight of 24,000, which is significantly lower than that obtained in Tris-glycine buffer.

The minor components 1–4 cannot be eliminated by treatment of the virion proteins with the reducing agent dithiothreitol and alkylation with iodoacetamide in the presence of 8 M guanidine hydrochloride before gel electrophoresis. By this criterion, therefore, polypeptides 1–4 do not seem

TABLE 1. *Molecular weights and relative proportion of SV40 polypeptides*

Component	Molecular weight $\times 10^{-3}$	% of total virion protein*	Estimate of number of polypeptides per virion†
VP1	44	72.0	327
VP3	31	11.0	71
1	170	0.5	0.6
2	126	1.1	1.7
3	94	1.6	3.4
4	67	0.6	1.8
5	41	5.0	
6	38		
7	36		
8	22	1.3	12
9	18	1.2	12.7
10 (VP4)	14	1.0	14.3
11 (VP5)	12	1.0	16.6
12 (VP6)	11	0.9	16.3

* Determined by densitometry of the virus shown in Fig. 1. X-ray film was exposed for 36 hr to measure VP1 and VP3, and 4 weeks for the minor components 1–12.

† Calculated on the assumption that the virion contains a total of 20×10^6 daltons of protein.

to be aggregates. The variable amounts in different virus preparations and their very small proportions suggest that they are minor contaminants.

Qualitatively, the same polypeptide pattern as obtained with SV40 virions (Fig. 1) is found by analysis of empty shells. The ratio of the amounts of VP1 to VP3 is the same for both virions and empty shells, whereas the ratio of VP1 to the minor components 5–7 and 10–12 in some shell preparations is increased 2- to 5-fold, as compared to full particles. The radioactivity present in 5, 6, and 7 increased upon storage of the virus for several weeks at 4°, suggesting that these polypeptides derive, at least in part, from proteolytic degradation of VP1.

Proteins from infected cells

Density determinations of radioautograms of gels obtained with SV40-infected and mock-infected Vero cells are given in Fig. 2. The two major structural polypeptides of SV40, VP1, and VP3, can easily be identified in SV40-infected cells (Fig. 2a). A major polypeptide in uninfected cells apparently

has the same molecular weight as VP1. However, by extension of the electrophoresis time it can be shown that the two components separate, the cellular polypeptide having a slightly smaller molecular weight than VP1. Infected cells also contain small amounts of a polypeptide of molecular weight 8000 (NVP3) that is not found in control cells or in purified virions.

After fractionation of the cells, the polypeptide pattern of the cytoplasm from infected cells differs from the pattern of control cells only with respect to the major structural components VP1 and VP3. In contrast, nuclei from infected cells (Fig. 2b) contain, in addition to VP1 and VP3, two polypeptides (NVP1 and NVP2) that are not present in control nuclei. Their molecular weights are 70,000 and 60,000, respectively. NVP3 is not recognizable in either fraction, perhaps because it becomes acid-soluble in the presence of NP40, which was used to prepare nuclei and cytoplasm. Attempts to detect either NVP1, NVP2, or NVP3 at early times of infection were not successful.

An estimate of the percentage of radioactivity present in VP1, VP3, NVP1, NVP2, and NVP3 after 3 hr of labeling is presented in Table 2. The two major virion polypeptides, VP1 and VP3, contain together 11% of all polypeptides synthesized late in infection. The nuclei are strongly enriched for these two polypeptides, which represent 16% of the total nuclear counts. We have determined that VP1 and VP3 observed in the cytoplasm are largely present in rapidly sedimenting particles (virions and/or empty shells), which might have leaked out of the nuclei during cell fractionation. Both the soluble and the sedimentable nuclear fraction contain VP1 and VP3 in the same ratio as in intact virions or shells. The other polypeptides seen after infection (NVP1, NVP2, and NVP3) are found in much smaller amounts than the structural proteins. They do not exceed 1% of the total polypeptides found in whole cells. The VP1 to VP3 ratio in whole cells and nuclei is similar to that of the virions, but is significantly higher in the cytoplasm. This result might be due to inaccuracy in the measurements, since cytoplasm contains only a small amount of these components. When cells were labeled with radioactive amino acids for only 10 min at a late time of infection, the amount of label in VP1 is at least 3-times greater than in VP3 (Table 2). Pulse-chase experiments demonstrate that these two proteins are metabolically stable.

Results with polyoma virus parallel those with SV40. In polyoma-infected Balb/3T3 cells, we detected the synthesis of four new peptides, two major structural components, closely similar in molecular weights to VP1 and VP3, and two non-

TABLE 2. *Distribution of structural and nonstructural polypeptides in different fractions of infected cells*

	% of total cpm in component*						Molar ratio† VP1/VP3
	VP1	VP2	NVP1	NVP2	NVP3	VP1 + VP3	
Whole cells*	9.2	1.7	—	—	0.4	10.9	5.5
Cytoplasm*	3.5	0.34	—	—	—	3.8	10.5
Nuclei*	14.0	1.7	0.7	0.5	—	15.7	8.2
Whole cells‡ (10 min)	9.5	2.2	—	—	—	11.7	4.2

* Determined by densitometry of experiment described in Fig. 2; densitometry from cytoplasm not shown in Fig. 2

† Calculated on the assumption of molecular weights of 44,000 for VP1 and 31,000 for VP3.

‡ Confluent BSC-1 cells were infected with 20 PFU/cell and labeled 27 hr after infection with ^{14}C -labeled protein hydrolysate (200 $\mu\text{Ci/ml}$) in Earle's salt solution.

structural polypeptides, with molecular weights of 70,000 and 60,000, comparable in size to NVP1 and NVP2. Since the two nonstructural components were labeled between 0 and 12 hr after infection, when the major structural polypeptides were not yet recognizable, they may represent early proteins.

DISCUSSION

SV40 or polyoma DNA, of molecular weight 3.2×10^6 (9), can code for only 190,000 daltons of protein. Thus, a large proportion of the peptides present in virions cannot be primary gene products. They must be either the result of processing, as in poliovirus (10–12), or of breakdown of other proteins, or they represent cellular proteins used for virus formation, or simply contaminants. Peptides 1, 2, 3, and 4 are likely to be contaminants, owing to the small and variable proportions; 5, 6, and 7 seem to be breakdown products of VP1, since they increase in amount during storage of the virus; 8–12 are probably cellular components in the virion's core or superficially adsorbed, since they correspond to peptides of infected cells and do not increase in amount after infection. Polypeptides similar in size to 10, 11, and 12 were found in polyoma virions, and have been shown to be cellular histones (13). The only viral products are VP1 and VP3, possibly the constituents of the hexons and pentons, respectively, since they are found in a constant ratio, which approaches that expected for the two types of protomer, both in virions and in empty shells. The presence of so many nonviral components in the virions invites caution in assessing the significance of enzymatic activities associated with these virions.

Are VP1 and VP3 primary gene products, or do they derive from the cleavage of a larger peptide? The available evidence favors the former hypothesis. Thus, the amount of label is 3-times higher in VP1 than in VP3 after a short labeling period (10 min) and the ratio does not change with longer labeling or after incubation with unlabeled amino acids, showing that the two proteins have similar half-lives. If they came from cleavage of an unidentified precursor, a fraction of VP3 would have to be degraded very rapidly, the other fraction remaining stable; VP3 that enters virions or capsomers could be the stable fraction. This possibility is being investigated by analysis of the distribution of label in VP3 and VP1 after very short pulses. The most likely interpretation is that the two proteins are synthesized independently, at different rates that reflect regulation of gene expression. It cannot be decided whether they are synthesized on the same or different messengers. In late infection with either polyoma virus or SV40, two viral messengers are made, of molecular weights about 800,000 and 600,000 (14) (Weinberg, R., Rudland, P. S., and Ben-Ishai, Z., personal communication); it is not clear which proteins they specify.

Are all five proteins, VP1, VP3, NVP1, NVP2, and NVP3, viral? Since SV40 and polyoma viruses enhance certain enzymatic activities (15, 16), apparently of cellular origin, in the infected cells, some of these proteins may represent such enzymes. This hypothesis is unlikely for several reasons: the increases in enzyme activity are not substantial in actively multiplying cells (such as the Vero cells used here); the number of induced enzymes is more than just two; the new bands do not correspond to detectable preexisting bands. However, this possibility cannot be definitely excluded. If all proteins were viral, they would amount to a total of 213,000 daltons, which exceeds only by about 10% the coding ability of the

viral genome. The discrepancy could be easily accounted for if some of the bands corresponded to glycoproteins, a possibility that has not yet been examined.

Another point in considering the origin of these proteins is their relationship to the number of viral genes detected by studies with temperature-sensitive mutants. Two complementation groups of mutants in late genes appear well established both in SV40 (Kimura, G., personal communication) and polyoma virus (17), and presumably correspond to VP1 and VP3. Early mutants of polyoma virus affect two, possibly three, genes on the basis of complementation and functional differences (18–20); immunologically, three new nonstructural antigens are detected in SV40-infected cells, the T (21–23), U (24), and transplantaion antigen (25). The presence of three nonvirus proteins, two of which are synthesized early in polyoma-infected Balb/3T3 cells, may not represent a fortuitous agreement.

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