Pattern of Protein Synthesis in Monkey Cells Infected by Simian Virus 40

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After infection of several permanent monkey cell lines by simian virus 40 (SV40), four additional protein bands can be detected by simple sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell extracts. These bands appear only after the onset of viral deoxyribonucleic acid (DNA) synthesis, and inhibitors of DNA synthesis prevent their appearance. Three of them correspond to three previously identified capsid components, VP1, VP2, and VP3. The fourth protein band, which does not correspond to a previously identified virion component, is induced by SV40 infection of CV-1 and BSC-1 cultures but not by infection of MA-134 cultures.

The simian virus 40 (SV40) genome contains 3×10^6 daltons of deoxyribonucleic acid (DNA), an amount which could code for six to ten average-sized proteins (2). In addition to its small circular DNA, the SV40 virion contains one major polypeptide of molecular weight 43,000 (VP1), and five minor polypeptides with molecular weights of 32,000 (VP2), 23,000 (VP3), 14,000 (VP4), 12,500 (VP5), and 11,000 (VP6) (4). Some of these polypeptides presumably are viral-coded, but proof of this presumption is presently lacking.

The eclipse period for the lytic infection of African green monkey cells by SV40 is about 24 hr, and virus is synthesized for several days before complete cell lysis occurs. Within the first 10 hr of infection, virus-specific ribonucleic acid (RNA) (1) and virus-induced T and U antigens can be readily detected (11). Between 12 and 18 hr after infection, viral DNA synthesis begins, and by 24 hr, V antigen, associated with assembled capsids, can be detected in the nuclei (11). In addition to the appearance of the virus-specific antigens, many enzymes concerned with DNA synthesis increase in activity during the early stage of viral replication (3). Altogether, more changes are known to occur after SV40 infection than are likely to be the direct result of synthesis of new virus-specified proteins.

Unlike many viruses, SV40 does not repress the synthesis of host cell RNA and proteins, and so far no inhibitor has been found that inhibits host cell macromolecular synthesis without simultaneously inhibiting viral replication. It is primarily for this reason that so little is known about the synthesis of the SV40-specified proteins, the synthesis of virus-induced host proteins, or the

assembly of virus particles. As each lytically infected cell produces between 10^5 and 10^6 physical virus particles and contains about 1 ng of protein, it follows that late in infection between 0.5 and 5% of the cell protein should be viral. The relatively large yield of virus suggests that it should be possible to detect at least the major viral protein by using a relatively simple fractionation of the cell constituents. Recently it has been reported that new protein complexes could be detected by polyacrylamide gel electrophoresis after the infection of CV-1 monolayers by SV40 (6, 7).

It is presumed that at least one of the SV40-specified proteins is required for the transformation of nonpermissive cells (3); thus, there is considerable interest in identifying and isolating the proteins which are specified by SV40. We have approached the problem of identifying the SV40-specified proteins by comparing those proteins made in productively infected cells with those synthesized in mock-infected cells. This comparison was accomplished by labeling the proteins made before, and at various times after SV40 infection, with radioactive ^{35}S-methionine. The labeled cultures were disrupted in sodium dodecyl sulfate (SDS) and dithiothreitol and then fractionated by electrophoresis on SDS-polyacrylamide gels. For this purpose we have utilized slab-type polyacrylamide gels (13) on which a large number of samples may be subjected to electrophoresis under identical conditions. The advantage of this technique is that a single radioactive isotope may be used for labeling all samples, and the resulting fractionation may be demonstrated by autoradiography, which permits

a much greater resolution of individual proteins than the technique of slicing and counting.

By the above technique, we have found four new protein bands which appear in SV40-infected monkey cells shortly after the onset of viral DNA synthesis. Results similar to ours have been obtained by G. Walter (*personal communication*).

MATERIALS AND METHODS

Cells and virus. Monolayers of CV-1 (TC7 subclone of J. Robb), BSC-1 (W. Eckhart), and MA-134 cells (J. Pagano) were cultivated in Dulbecco's modified Eagle medium (Grand Island Biological Co.) supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) on plastic petri dishes (6 or 9 cm; Nunc, 4-Shore, La Jolla, Calif.) at 37 C in a CO_2 incubator. The SV40 virus stock (strain 777, W. Eckhart, Salk Institute) used in these experiments was prepared by low-multiplicity passage of a plaque-purified seed virus stock on subconfluent CV-1 cells. The viral stock had a titer of 2×10^8 plaque-forming units (PFU)/ml on BSC-1 cells.

Preparation of ^{35}S-methionine-labeled SV40. The ^{35}S-methionine-labeled viral stock used as a marker for polyacrylamide gel electrophoresis was prepared by labeling an infected culture of MA-134 cells 24 to 36 hr after infection with high-specific-activity methionine and purifying the virus as previously described (10).

Preparation of ^{35}S-methionine-labeled cell extracts for SDS-polyacrylamide gel electrophoresis. Subconfluent cultures were infected with SV40 (0.5 ml/6-cm plate, 2×10^8 PFU/ml, ca. 100 PFU/cell) or mock-infected for 1 hr at 37 C. At various times after infection, the cultures were washed with methionine-free Dulbecco's medium and pulse-labeled with ^{35}S-methionine (24 μCi/plate, in 2.0 ml medium at 1.2 Ci/mmole). The incorporation of methionine was approximately linear between 10 min and 12 hr under these conditions. After labeling, the cultures were washed twice with Dulbecco's medium, scraped into 12-ml conical centrifuge tubes, and pelleted in a clinical tabletop centrifuge for 5 min at 4 C. Before centrifugation, 300 μg of phenylmethyl sulfonylfluoride (PMSF, Calbiochem) was added per ml of cell suspension to inhibit protease activity. The cell pellets were disrupted by two cycles of freezing and thawing, suspended in 50 to 200 μliters of SDS sample buffer (0.0625 M tris(hydroxymethyl)aminomethane, pH 6.8; 2% SDS, Serva; 10% glycerol; 0.001% bromophenol blue; 0.1 M dithiothreitol), and disrupted by heating at 100 C for 10 min. From 2 to 20 μliters of each of these samples was applied separately to individual sample wells of a slab-type polyacrylamide gel and analyzed by electrophoresis by using the method of Studier (13). Sample volumes were chosen so that each sample well of a gel received an equal amount of radioactivity (ca. 100,000 counts per min per well, 10 to 50 μg protein). The gels were dried and then autoradiographed on Kodak Royal X-omat medical X-ray film. Some of the gels were rehydrated after autoradiography and stained with Coomassie

brilliant blue. Bovine serum albumin (Pentax, Ill.), 68,000 daltons; ovalbumin (Sigma), 45,000 daltons; carboxypeptidase A (Worthington), 24,000 daltons; and cytochrome *c* (Nutritional Biochemical Corp.), 12,000 daltons, were used as molecular weight standards.

Fingerprinting of ^{35}S-methionine-labeled peptides. An SV40-infected CV-1 culture was labeled with ^{35}S-methionine from 33 to 37 hr after infection as described above. The SDS-dithiothreitol-disrupted extracts of these cultures were fractionated on an SDS-polyacrylamide slab gel, and the areas of the gel corresponding to the positions of the major capsid protein, IVP1, and the major cell protein (designated X in the appropriate figures) as determined from the autoradiogram were cut out and eluted with 0.1% SDS. SDS was removed from the extracted polypeptides by several cycles of trichloroacetic acid precipitation in the presence of carrier bovine serum albumin. After performic acid oxidation and trypsin digestion, the resulting peptides were separated by electrophoresis in pyridine acetate, pH 3.5, as previously described (10).

RESULTS

Production of SV40 capsid proteins in lytically infected cells. To examine the patterns of protein synthesis at successive intervals (between 1 and 48 hr) after SV40 infection, virus- or mock-infected cultures were pulse-labeled with ^{35}S-methionine. The total cell extracts, made by SDS and dithiothreitol disruption, were analyzed on SDS-polyacrylamide gels. Using CV-1 cells as the host, we obtained the autoradiograms shown in Fig. 1 and 2. The mock-infected CV-1 cells give a constant pattern (Fig. 1a, b, p, and 2a, b, o) of discrete protein bands upon a heavy background of radioactivity, which is probably due to the presence of many minor components. During the first 15 hr after SV40 infection, very little change is seen in the protein pattern (Fig. 1 and 2, d–f). Between 15 and 18 hr after infection, however, a new band appears (Fig. 1g) which migrates slightly slower than the most conspicuous host cell protein (ca. 40,000 daltons) and comigrates with the major capsid protein from purified virus (VP1, 43,000 daltons). This band is denoted IVP1 for induced viral protein number one. After another 3 hr, two more bands become visible in the infected cell patterns. The larger one (IVP3) comigrates with the minor capsid protein 3 (VP3); the smaller one (IVP3.5) migrates near the front of the 10% acrylamide gels but may easily be seen as a distinct band appearing between VP3 and VP4 in 20% gels (Fig. 2h). IVP3.5 has an approximate molecular weight of 15,000 daltons and does not correspond to any of the six virion proteins reported by Estes et al. (4). A fourth virus-induced band (IVP2, 32,000 daltons), which migrates slightly faster than the 40,000-dalton major cell
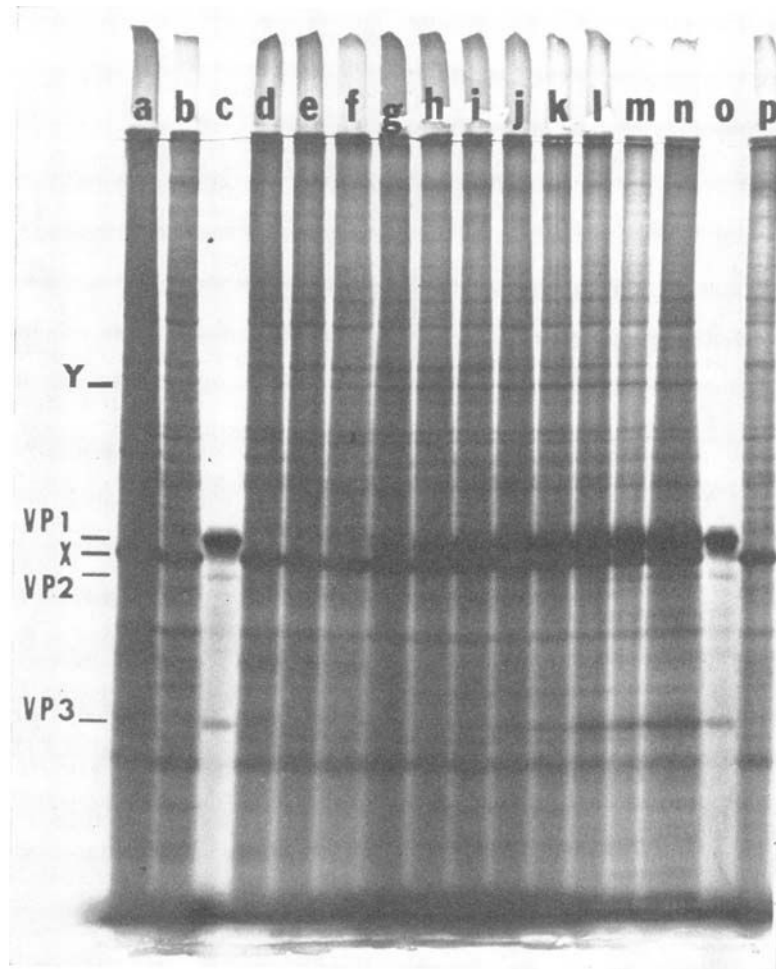
FIG. 1. *Sodium dodecyl sulfate-polyacrylamide* (10%) *gel autoradiogram of extracts from SV40-infected CV-1. Cultures were* ^{35}*S-methionine labeled and dissociated as described in the text. Approximately 100,000 counts/min were applied to each sample well except c and d. In this and in subsequent polyacrylamide gel figures, the approximate positions of the six virion components (VP1-VP6), the SV40-induced component IV P3.5, and the cell bands X and Y are indicated at the left-hand margin. The sample order is (a) mock infection labeled 1 to 6 hr postinfection; (b) mock infection, 24 to 27 hr; (c) purified* ^{35}*S-methionine-labeled SV40 virus; (d–n) SV40-infected cultures labeled from (d) 1 to 6 hr, (e) 6 to 12 hr, (f) 12 to 15 hr, (g) 15 to 18 hr, (h) 18 to 21 hr, (i) 21 to 24 hr, (j) 24 to 27 hr, (k) 27 to 30 hr, (l) 30 to 36 hr, (m) 36 to 42 hr, (n) 42 to 48 hr; (o)* ^{35}*S-methionine-labeled SV40 virus; (p) mock infection, 42 to 48 hr.*

band, can be observed by 24 hr after infection (Fig. 1, j–k). All four virus-induced proteins appear to be made at increasing rates as the infection progresses. Since IVP2, IVP3, and IVP3.5 are made in much smaller quantities than IVP1, and since the background in gels of whole-cell extracts is rather high, it is difficult to determine the time of first appearance of these proteins. However, the above results are consistent with the idea that the synthesis of these four viral components is initiated at the same time, late in the infectious cycle.

The three smallest virion proteins, VP4 (14,000

daltons), VP5 (12,500 daltons), and VP6 (11,000 daltons) comigrate with three rather conspicuous cellular components (Fig. 2) which are synthesized throughout the infectious cycle. Consequently, we would not expect to be able to see the appearance of VP4, VP5, and VP6 unless they were made in very large quantities.

In addition to the four virus-induced bands described above, another band with an approximate molecular weight of 80,000 often appears to increase in relation to the other cellular components after SV40 infection (Fig. 1, Y). The degree
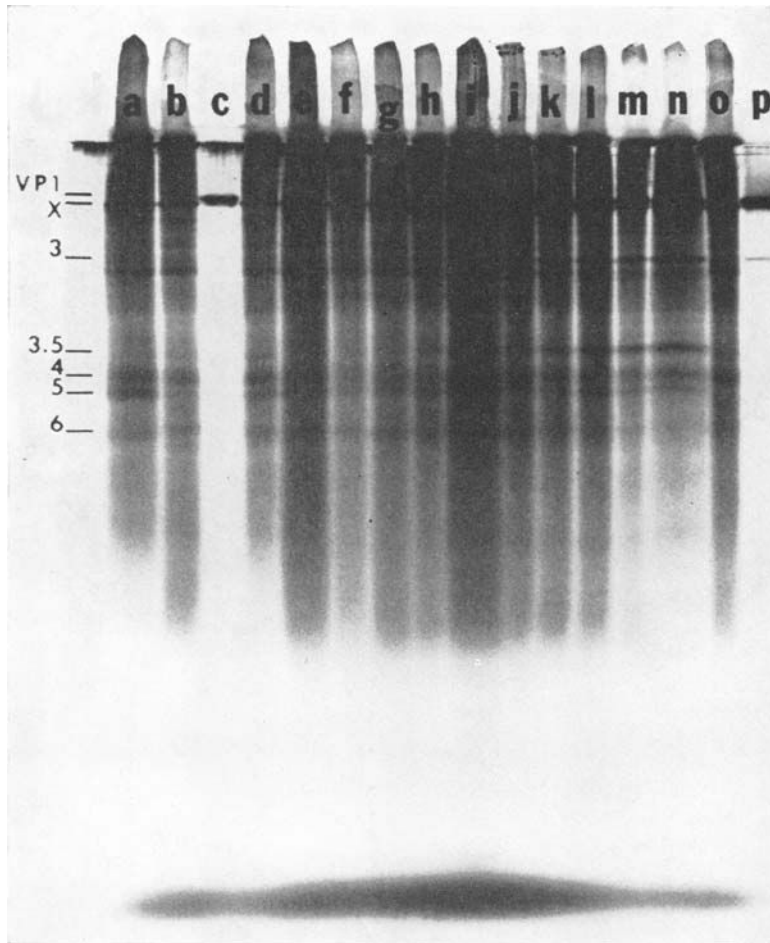
FIG. 2. *Sodium dodecyl sulfate-polyacrylamide (20%) gel autoradiogram of extracts from SV40-infected CV-1. Samples a through n are identical to the respective samples in Fig. 1; (o) mock infection labeled 42 to 48 hr after infection; (p) purified ^{35}S-methionine-labeled SV40 virus. SV40 virion components VP4, VP5, and VP6 are not visible on the exposure selected for the figure, but they could be seen after a longer exposure of the same gel.*

of "induction" of this band is somewhat variable and is not obvious in every infected extract.

After autoradiography, the polyacrylamide gel described in Fig. 1 was rehydrated and stained with Coomassie brilliant blue. IVP1 was clearly visible by staining, and its proportion in relation to the cellular components increased during the 48-hr time course of the experiment. We estimate that the major coat protein (IVP1) accounts for approximately 5 to 10% of the total cellular protein by the time of cell lysis.

Time-course experiments with SV40-infected BSC-1 and MA-134 cultures gave results similar to those reported above for infected CV-1 cultures; at least three of the capsid components were induced at late times after SV40 infection (Fig. 3; BSC time course not shown; see Fig. 4 for BSC pattern). In SV40-infected MA-134 cultures,

however, the noncapsid, virus-induced protein (IVP3.5) was not detected, even after long autoradiographic exposures. However, preparations of virus grown on MA-134 cells often contain small quantities of a protein that behaves like IVP3.5.

In our experience, IVP1, IVP2, and IVP3 appear earliest in infected cultures of MA-134 (at 12 to 15 hr postinfection), later in CV-1 cultures (15 to 16 hr postinfection), and latest in BSC-1-infected cultures (19 to 20 hr postinfection). There is some variation in the exact time of appearance of the capsid proteins from experiment to experiment, depending in part on the state of the monolayer at the time of infection and the multiplicity of infection. In both the BSC-1 and the MA-134 time-course experiments, the onset of viral DNA synthesis was measured by pulse-labeling some of the plates with ^{3}H-thymidine (^{3}H-TdR) at 10
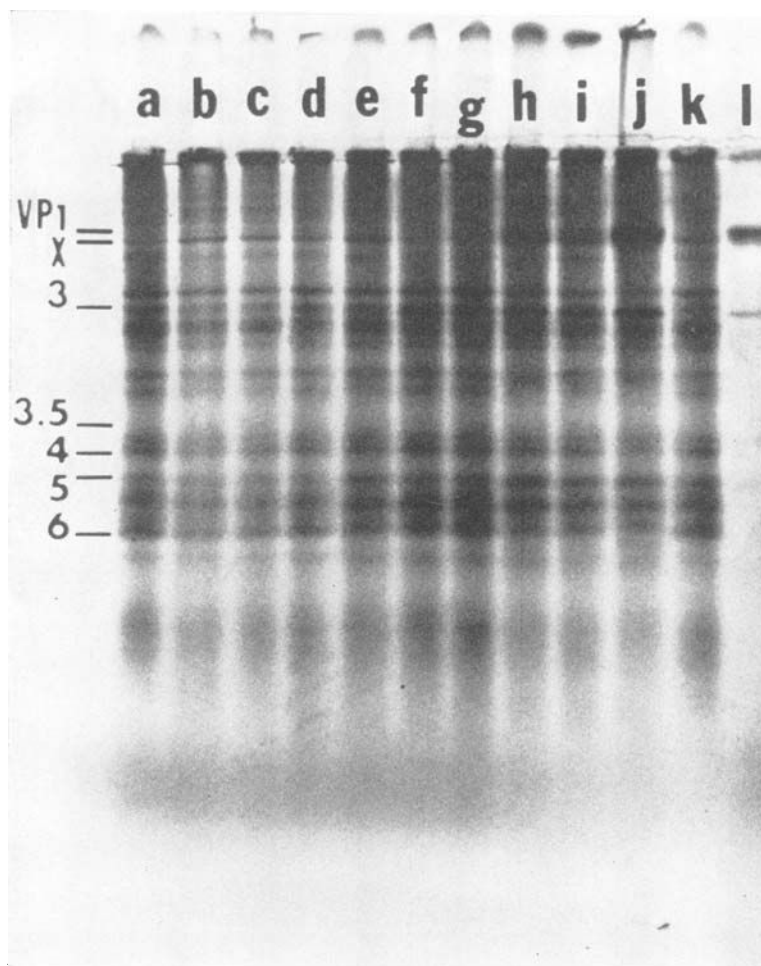
FIG. 3. Sodium dodecyl sulfate-polyacrylamide (20%) gel autoradiogram of extracts from SV40-infected MA-134. Sample order: (a) mock infection labeled 12 to 13 hr after infection; (b–j) SV40-infected and labeled (b) 12 to 13 hr, (c) 13 to 14 hr, (d) 14 to 15 hr, (e) 15 to 16 hr, (f) 16 to 17 hr, (g) 17 to 18 hr, (h) 18 to 19 hr, (i) 19 to 20 hr, (j) 36 to 37 hr; (k) mock infection, 19 to 20 hr; (l) ³⁵S-methionine-labeled SV40 virus.

μCi/plate. Newly synthesized viral DNA [³H-labeled material in Hirt extracts (9) cosedimenting in neutral and alkaline gradients with ³²P-labeled form I SV40 DNA] was detectable in both cell lines just before the appearance of the induced viral proteins IVP1, IVP2, and IVP3 (data not shown).

Tryptic fingerprint of IVP1 and the major viral capsid protein. To rule out the unlikely possibility that the comigration of the induced and virion proteins on SDS-acrylamide gels is fortuitous, we compared tryptic peptide patterns. IVP1 and the neighboring host protein (marked X; 40,000 daltons) were eluted from a preparative SDS-acryl-amide gel of ³⁵S-methionine-labeled infected CV-1 cells. These proteins and similarly labeled, purified virus were digested with trypsin and com-

pared by electrophoresis at pH 3.5 as shown in Fig. 5.

The major tryptic peptides from purified virus are contributed by VP1 (unpublished data) as expected since this protein accounts for 70% of the total virion protein. These peptides match with the major peptides of the induced protein from cells (IVP1), showing that the proteins are the same. Host protein X has a clearly different fingerprint.

Further peptide analyses are in progress to characterize each of the six capsid proteins and to verify the identity of the virus-induced proteins.

Effect of araC and FUdR on the induction of IVP1, IVP2, IVP3, and IVP3.5. Cytosine arabinoside (araC) and 5-fluorodeoxyuridine (FUdR) inhibit the synthesis of SV40 DNA and the formation of
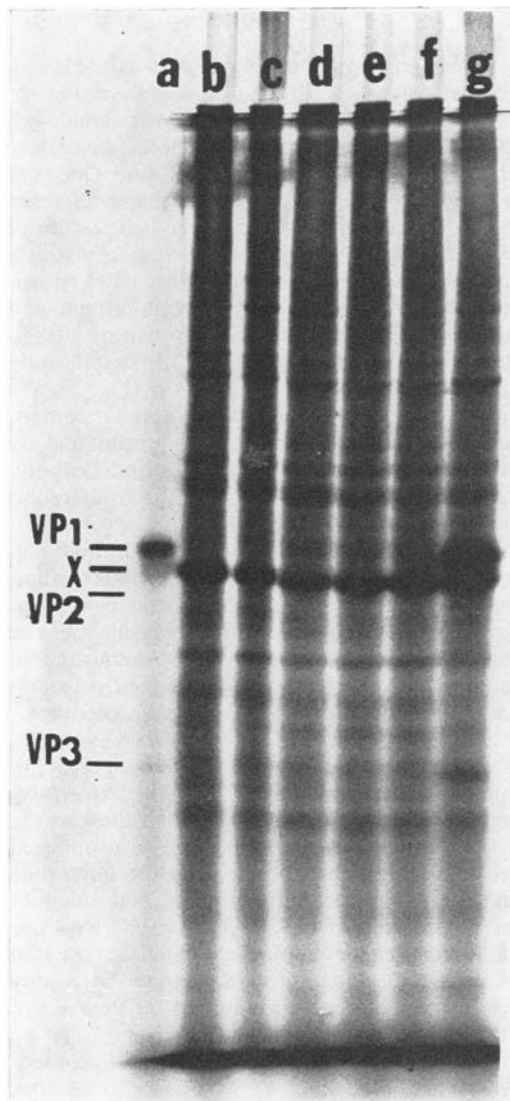
FIG. 4. *Sodium dodecyl sulfate-polyacrylamide (10%) gel autoradiogram of extracts from SV40-infected, cytosine arabinoside (araC)-treated BSC-1. araC Was added at 12.5 µg/ml to the indicated cultures 1 hr after infection and was present throughout the period of labeling. Sample order: (a) purified ^{35}S-methionine-labeled SV40 virus; (b) SV40-infected, araC-treated, labeled 24 to 31 hr after infection; (c) SV40-infected, araC-treated, labeled 31 to 49 hr; (d) SV40-infected, araC-treated, labeled 31 to 32 hr; (e) mock infection, araC-treated, labeled 19 to 20 hr; (f) mock infection, not araC-treated, labeled 19 to 20 hr; (g) SV40-infected, not araC-treated, labeled 31 to 32 hr.*

infectious SV40 virus if they are added to cultures of SV40-infected monkey cells before the onset of viral DNA synthesis (12). This treatment, how-

ever, does not affect the appearance of early virus-induced functions such as SV40 T and U antigens (11, 12). We therefore examined the effect of these inhibitors on the induction of the four SV40-induced proteins.

Cultures of BSC-1 cells in 6-cm petri dishes were either infected with SV40 (100 PFU/cell) or mock-infected as described above. One hour after infection, one-half of the cultures received 12.5 µg of araC per ml. Subsequently, sets of mock-infected or virus-infected cultures, with and without araC, were pulse-labeled with ^{35}S-methionine (24 µCi/plate, 1.2 Ci/mmole) or ^{3}H-thymidine (20 µCi/plate) for consecutive 1-hr intervals. The ^{35}S-methionine-labeled cultures were disrupted in SDS and dithiothreitol as described above for analysis on SDS-polyacrylamide gels. The ^{3}H-thymidine-labeled cultures were extracted by the Hirt procedure (9), and the tritium-labeled DNA was analyzed by sedimentation in neutral and alkaline gradients.

The major SV40 capsid polypeptide was detectable in extracts of BSC-1 cultures labeled from 19 to 20 hr after infection, but no SV40 major capsid polypeptide (IVP1), IVP2, IVP3, or IVP3.5 could be detected in extracts from SV40-infected, araC-treated cultures when these were either pulse-labeled with ^{35}S-methionine 31 to 32 hr after infection or labeled for a relatively long period late in infection (Fig. 4d and e). Tritiated thymidine-labeled material which cosedimented with purified ^{32}P-labeled SV40 DNA in both alkaline and neutral gradients was detectable in Hirt extracts of SV40-infected BSC-1 cultures by 17 to 18 hr after infection; no such material was detected in extracts of araC-treated, SV40-infected cultures labeled 31 to 32 hr after infection. Cytosine arabinoside depressed host DNA synthesis by about 70%.

In a similar experiment to that described above, FUdR (12.5 µg/ml) and uridine (50 µg/ml) were added to a set of mock-infected or SV40-infected MA-134 cultures 1 hr after infection; an identical set of cultures received only 50 µg of uridine per ml. Protein synthesis and DNA synthesis were measured as described above, and results essentially identical to those obtained with araC-treated BSC-1 cells were observed. Neither the major SV40 capsid polypeptide (IVP1) nor IVP2 or IVP3 was detectable in extracts prepared from FUdR-treated, SV40-infected cultures labeled as late as 36 to 37 hr after infection (data not shown). FUdR depressed the level of host DNA synthesis in these experiments by about 95%. In neither the MA-134 nor the BSC-1 experiment were additional viral-induced bands observed due to the presence of araC or FUdR.
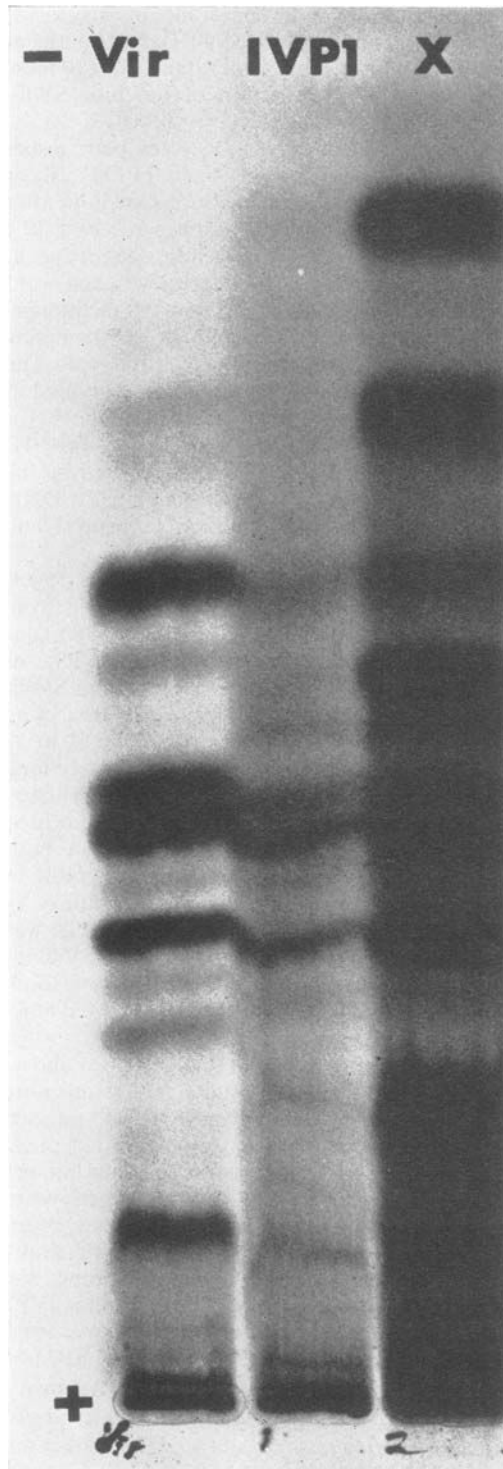
FIG. 5. Tryptic fingerprint of IVP1 from SV40-infected CV-1. The protein components of ³⁵S-methionine-labeled, SV40-infected CV-1 cultures were

DISCUSSION

When an extract of whole cells labeled with ³⁵S-methionine is fractionated (according to molecular weight) on an SDS-polyacrylamide gel, the band pattern obtained is to some degree characteristic of the particular cell line. One very prominent band, which is a convenient reference point for comparing cell extracts, is a feature of all three monkey cell lines as well as of extracts from 3T3 and HeLa cultures. This band constitutes about 10% of the total cell protein and apparently represents a single protein of 40,000-dalton molecular weight as it has a relatively simple tryptic peptide composition (Fig. 5). We suspect that this protein is actin, a major component of many cells (5), as it is precipitated by vinblastine sulfate and comigrates on SDS-polyacrylamide gels with actin purified from rabbit skeletal muscle (unpublished data).

During the first 12 to 20 hr after high-multiplicity infection by SV40, there is no obvious qualitative change in the species of protein being synthesized. It is apparent from this result that the early SV40-induced proteins are synthesized in relatively small amounts and that their detection will require additional fractionation procedures.

Shortly after the onset of viral DNA synthesis, new bands appear in the gel which comigrate with three SV40 capsid proteins. By tryptic fingerprinting, we have identified the largest of these as the major capsid protein. All three virus-induced proteins appear at approximately the same time and are synthesized at increasing rates throughout the late portion of the infectious cycle. By 48 hr after infection, they comprise a significant fraction of the total cellular protein and can be readily detected by autoradiography and, in the case of VP1, by protein staining.

The synthesis of the three major capsid components, VP1, VP2, and VP3, appears to be contingent upon viral DNA synthesis. Viral DNA synthesis was detected in infected cultures before the appearance of the capsid proteins, and none

separated on a preparative sodium dodecyl sulfate-polyacrylamide (10%) slab gel, and the bands corresponding to VP1 and the major cell protein (X) were cut out, eluted, and digested as described in the text. The tryptic peptides were separated by electrophoresis at pH 3.5. Purified ³⁵S-methionine-labeled SV40 (Vir) was also digested and subjected to electrophoresis for comparison; the five most intense bands in the slot marked "Vir" are contributed by the major coat protein (VP1), which comprises about 70% of the virion. Tryptic peptides corresponding to those from VP1 are also found in the major cell band column (X) since IVP1 and X are usually only partially resolved in a 10% gel.

of the capsid components was synthesized in detectable quantities when the infected cells were treated with araC or FUdR before the onset of DNA synthesis. Previous studies have shown only that these inhibitors prevent the synthesis of infectious or immunologically active SV40 capsids (12).

From our present study we cannot determine to what extent SV40 induces the synthesis of capsid components VP4, VP5, and VP6. Frearson and Crawford (8) have recently suggested that the three smallest capsid components of polyoma may be cellular histones. SV40 components VP4, VP5, and VP6 comigrate in SDS-polyacrylamide gels with their polyoma counterparts and with histone fractions isolated from monkey cells and calf thymus (*unpublished data*). Thus it is probable that the SV40 capsid components VP4, VP5, and VP6 are host cell histones. Under the conditions of cell culture that we have employed, proteins with the same mobilities in SDS gels as SV40 VP4, VP5, and VP6 are synthesized in relatively large amounts throughout the entire infectious cycle as well as in mock-infected cultures.

In SV40-infected CV-1 and BSC-1 cultures, we have found a virus-induced protein (IVP3.5) which has not been previously reported. Although this protein does not appear to be induced in SV40-infected MA-134 cultures, a small and variable amount is detectable in preparations of purified virus grown on either CV-1 or MA-134 cells, which may correspond to the trace polypeptide component of purified SV40 reported by Estes et al. (4). The most likely interpretation of these results is that this protein is a proteolytic product of a capsid protein. We are currently testing this hypothesis by obtaining tryptic fingerprints of each of the virion and virus-induced components.

Previous studies of SV40 infection have had to rely primarily on immunological assays to detect the synthesis of virus-induced components in infected cell extracts. The above results demonstrate that a single, simple technique may be used to monitor the synthesis of the individual virion polypeptides in virus-infected cells. With this technique we have shown that three of the SV40 virion proteins begin to be synthesized at a rapid rate shortly after the onset of viral DNA synthesis, a result which suggests to us that VP1, VP2, and VP3 probably are virus-coded. We expect that, in conjunction with additional fractionation procedures, it should be possible to detect and isolate the early virus-coded gene products which are believed to be involved in cell transformation.

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