Identification of Virus-Induced Proteins in Cells Productively Infected with Simian Virus 40

HANS FISCHER AND GERHARD SAUER

Institut für Virusforschung, Deutsches Krebsforschungszentrum, 69 Heidelberg, Germany

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The number and molecular weight of the structural polypeptides of highly purified simian virus 40 (SV40) were determined by polyacrylamide gel electrophoresis. Six different polypeptides were found, two of which (VP1 and VP2) comprise the bulk of the viral capsid proteins. The pattern of protein synthesis in productively infected CV-1 cells was studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Identification of virus-induced proteins in the infected CV-1 cells was achieved in double-labeling experiments by electrophoresis with purified labeled SV40 capsid proteins. Four of these proteins (VP1 and VP4) could be classified as components of the virion because their synthesis occurred after the onset of viral deoxyribonucleic acid (DNA) replication and because they were inhibited by arabinofuranosylcytosine (ara-C). Appearance of two other virus-induced proteins was not prevented by ara-C; one of them did not comigrate in the electrophoresis with purified virion polypeptides, and both could be detected before the onset of viral DNA synthesis. These latter two proteins were classified on the basis of these criteria as nonvirion capsid proteins (NCVP1 and NCVP2).

The genome of the oncogenic simian virus 40 (SV40) is very small and contains information for only six to eight proteins of average size. In a previous paper (6) describing the alterations in the protein pattern of productively infected cells, more induced proteins were detected than could be coded by the viral genome. In addition to the structural proteins, one must account for those proteins which are not part of the virion. Of these nonstructural proteins, tumor antigen (T-antigen) (2), the transplantation and surface antigens (9, 23, 24), and several enzymes (14, 16) have been described.

Attempts have been made to determine which of the proteins in the infected cells are part of the virion and which are nonstructural. To identify the structural proteins in the whole protein pattern of infected cells, we analyzed by means of gel electrophoresis first the polypeptide components of highly purified SV40. We found six structural polypeptides, in agreement with Girard et al. (7), Estes et al. (5), and Hirt et al. (11). These structural components were added as internal markers in double-labeling experiments to preparations of cellular proteins from infected cells. By virtue of identical mobility, some proteins could be tentatively characterized as constituents of the virion. In addition, an inhibitor of deoxyribonucleic acid (DNA) replication, arabinofuranosylcytosine (ara-C), was employed

to block the expression of viral DNA synthesis and of late functions, in particular the synthesis of viral capsid proteins. In these experiments, in which coelectrophoresis with purified virus as an internal marker was combined with inhibition by ara-C of the sequential appearance of particular proteins during infection, it was possible to identify a number of proteins in the infected cells as definite constituents of the virion. By means of exclusion, other proteins which are not part of the virion have been found. From the kinetics of synthesis and differential inhibition by ara-C, two will be shown to be nonstructural, virus-induced proteins.

MATERIALS AND METHODS

Virus and cells. Plaque-purified SV40, strain Rh 911 (8), was propagated in CV-1 cells (12) by low-multiplicity infection [0.1 plaque-forming unit (PFU)/cell]. CV-1 cells were maintained in Eagle basal medium (BME) with twice the concentration of amino acids and vitamins, Earle's balanced salt solution, and 5% fetal bovine serum.

Preparation of SV40 labeled in its coat protein moiety. For radioactive labeling of the structural viral proteins, CV-1 cells grown in large roller production vessels (Bellco Glass Inc., Vineland, N.J.) were infected at a multiplicity of 1 PFU/cell. After a 2-hr adsorption period, the unattached virus was removed by two washings with Hanks solution. Cells were maintained then in BME with onefold concentration

of amino acids and vitamins and 5% fetal bovine serum. At 24 hr after infection, 62 μCi of ^{14}C -labeled amino acids per roller bottle (chlorella protein hydrolysate, specific activity 45 mCi/m atom of carbon; The Radiochemical Centre, Amersham, England) was added.

The virus was harvested 6 to 7 days after infection, when the cytopathogenic effect was well developed.

The technique of SV40 purification was as follows. The virus was harvested by freezing and thawing the cells, cellular debris was removed by low-speed centrifugation, and then 10% polyethylene glycol (PEG) was added. After storage at 4 C for 24 hr, the suspension was centrifuged for 20 min at 3,000 rev/min. Then the pellet was dissolved in $0.1\times$ SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate).

Then, the virus-containing fluid was layered on top of a cushion of saturated KBr (15 ml) and centrifuged for 3 hr at 20,000 rev/min in an SW25.2 rotor. After centrifugation, two bands were formed in the KBr. The lower band, which contained the complete virions, was collected and desalted on a Sephadex G-50 column. A 10.1-ml amount of the virus suspension was mixed with 5.5 ml of a CsCl solution saturated at room temperature and centrifuged into equilibrium in an SW39 rotor at 38,000 rev/min for 20 hr as previously described (17). The peak fraction of the denser of the two bands was used for determination of the virion polypeptides.

Isotopic labeling of proteins. Confluent CV-1 cell cultures (4×10^6 cells per milk dilution bottle) were infected with SV40 at a multiplicity of 20 PFU/cell. After 2 hr of adsorption, the cells were washed twice with Hanks solution and refed with BME with one-fold concentration of amino acids and vitamins and 5% fetal bovine serum. Control cultures were treated likewise except that mock infection was performed with used medium. For isotope incorporation a mixture of ^3H -amino acids (The Radiochemical Centre, Amersham) was added either throughout the duration of the experiments as indicated in each particular case, or, in the case of short-pulse labeling experiments, for 30 min. To each milk dilution bottle (10 ml of medium) was added 50 μCi of the mixture which consisted of equal activities of the following amino acids: L-lysine-4,5- ^3H (nHCl), specific activity 250 mCi/mmol; L-leucine-4,5- ^3H , specific activity 1,000 mCi/mmol; L-valine-2,3- ^3H (n), specific activity 1,500 mCi/mmol. The period of isotopic labeling was terminated by washing the cells three times with phosphate-buffered salt solution (calcium- and magnesium-free phosphate-buffered isotonic saline, pH 7.4). Then the cells were transferred to -20 C.

Inhibition of synthesis of SV40-capsid proteins. Arabinofuranosylcytosine (ara-C; Serva, Heidelberg) was added to the cultures after the period of adsorption or mock infection at a concentration of 20 $\mu\text{g}/\text{ml}$. The cells were exposed to the inhibitor for the entire period of the experiment.

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed with gel columns (0.6 by 5 cm) which contained the acrylamide monomer at a concentration of 7.5%. Conditions for electrophoresis, fixation, and staining of the gels were as described previously (6).

Frozen cells were thawed, scraped from the glass, collected in 2 ml of reticulocyte standard buffer (RSB; 22), and disrupted by sonic treatment (Branson sonifier, step 4, 20 sec). Portions of lyophilized, purified, ^{14}C -labeled SV40 were suspended in 1 ml of RSB without sonic treatment.

To the protein preparations in RSB a solution comprising one-tenth volume was added which contained 1% sodium dodecyl sulfate (SDS), 0.5 M urea in 0.1 M phosphate buffer, and 10% glacial acetic acid. The samples were incubated for 1 hr at 37 C and then dialyzed for 18 hr at room temperature against 3,000 volumes of 0.01 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS, 0.1% 2-mercaptoethanol, and 0.5 M urea (22).

Assay of radioactivity. The gels were cut into 1-mm slices and solubilized for 2 hr in 0.1 ml of H_2O_2 mixed with 1 ml of Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.), and 10 ml of toluene-based scintillator was added (Omnifluor, NEN Chemicals GmbH, 6072 Dreieichenhain, Germany). The vials were kept overnight at 4 C to reduce luminescence, and then radioactivity was measured in a Tricarb liquid scintillation spectrometer 3380 (Packard).

Immunofluorescence. Immunofluorescence was performed as previously described (21). The presence of T-antigen was demonstrated by incubation of the fixed cells with tagged anti-T-serum (Flow Laboratories, Inc., Rockville, Md.). Viral coat antigen (VP) was detected with a tagged serum from monkeys (*Cercopithecus*) immunized with SV40.

RESULTS

Preparation of SV40 capsid proteins. To make sure that the method of virus purification employed here led to complete removal of extraneous cellular proteins, a lysate of ^3H -labeled CV-1 cells was added to the virus-containing fluid. Then a virus purification was performed as described above, and the disappearance of radioactivity from the virus-containing material was determined during the course of purification (Table 1). It may be seen from the result that after completion of the virus purification there was practically no radioactivity detectable. Thus, it appears that the purification results in a virus preparation that is free from contamination with external cellular proteins. The data do not exclude, however, the inclusion of cellular material in the virions, which might take place during the process of virus maturation. The purified virus preparation was degraded, and the ^{14}C -labeled viral capsid proteins were included as internal markers in the preparations of cellular proteins.

As shown in Fig. 1, the pattern of radioactively labeled viral proteins comprises at least six discrete bands (open circles). The first small peak of macromolecular material to the left of VPI is probably undegraded virus, since the use of 8 M urea causes disappearance of the heavy material. Thus, the pattern obtained is similar to the results

TABLE 1. *Purification of SV40 virus and removal of ³H-labeled host cell material*

Preparation	Vol- ume (ml)	Protein concn ^a (μg/ml)	Counts per min per ml ^b	Per cent of orig- inal radio- activity
Supernatant from sonically treated, infected cells after low-speed centrifugation (2,000 × g, 10 min)	155	1,830	36,200	100
PEG precipitation and centrifugation (3,500 × g, 20 min), resuspended in 8 ml of 0.1 X SSC	8	500	27,200	75
Elate after Sephadex G-50 chromatography	15	201	16,800	46
KBr-gradient, lower band after dialysis	3.5	55	6,200	16
CsCl-gradient centrifugation after dialysis (17)	0.65	25	20	0.05

^a Total protein, including radioactively labeled cellular proteins, as determined by the method of Lowry et al. (18).

^b CV-1 cells (1.2×10^7) labeled with ³H-amino acids (see text) were harvested by three cycles of freezing and thawing, suspended in 5 ml of Hanks solution, and combined with 150 ml of virus-containing medium, which was obtained from two Roux bottles 6 days after infection of CV-1 cells with 10 PFU of SV40 per cell.

described by others (5, 7). With decreasing order of molecular weight the peaks have been denoted VP1 to 6. VP1 comprises the bulk of the viral capsid proteins. Both VP2 and VP3 occur in the same quantity, whereas the three minor peaks VP4 to 6 can be detected only in rather small amounts. Estimates of their molecular weight based on their relative mobility compare well with the data reported (5, 7).

Coelectrophoresis of the ³H-labeled proteins from uninfected control cells (closed circles) with the ¹⁴C-labeled SV40 marker proteins (Fig. 1) revealed that the position of the viral proteins, with the exception of VP5, does not coincide with the positions of the cellular proteins—a fact which facilitates identification of the virus-induced proteins among the proteins of infected cells.

Effect of ara-C on protein synthesis in infected and uninfected cells. Ara-C, an inhibitor of DNA synthesis (4, 13) can be used to inhibit specifically "late viral functions," such as capsid proteins, the expression of which depends on replication of the SV40 DNA (3). In contrast, expression of "early SV40 functions" (T-antigen) is unimpaired by the presence of the drug (3). Therefore, we have used ara-C to distinguish between early and late SV40 proteins in productively infected cells.

The prolonged presence of ara-C in CV-1 cell

FIG. 1. Coelectrophoresis of SV40 virion polypeptides labeled with ¹⁴C-amino acids and ³H-amino acid-labeled proteins from uninfected CV-1 cells. Cells were labeled with a mixture of amino acids (5 μCi/ml) for 32 hr and then harvested. ¹⁴C-labeled SV40 protein (26 μg) was applied to the gel. Both the cellular and viral proteins were disintegrated separately, mixed before electrophoresis, and applied to 7.5% polyacrylamide gel as described in text. Direction of migration is from left to right. (●) ³H; (○) ¹⁴C.

cultures does not seem to cause severe damage of the cells. Ara-C (12.5 μg/ml) does not lead to substantial loss of cells even after an exposure for 50 hr (20). No cytotoxic effect could be observed microscopically when the cells were exposed to 20 μg of ara-C per ml for up to 96 hr, whereas 50 μg of ara-C per ml led to cytotoxic effects. The continued presence of 20 μg of ara-C per ml in SV40-infected CV-1 cell cultures had no influence on the number of T-antigen-synthesizing cells, whereas production of capsid proteins was completely prevented, as evidenced by immunofluorescence (Table 2). Thus, previous observations (3) could be confirmed.

In control experiments, the pattern of protein synthesis in mock-infected CV-1 cultures was studied in the presence or absence of ara-C. The presence of ara-C in mock-infected CV-1 cultures led to a slight enhancement of protein synthesis as

TABLE 2. Influence of arabinofuranosylcytosine (ara-C) on synthesis of T-antigen and viral capsid protein

Treatment of cells	T-antigen-positive cells ^a (%)	Viral capsid protein-positive cells (%)
None	100	80
Ara-C (20 μ g/ml) ^b	100	0

^a Multiplicity of infection, 10 PFU/cell. Cells were fixed at 48 hr postinfection.

^b Present for 48 hr (added 2 hr postinfection).

revealed by the increase in the specific activity (dpm/ μ g of protein). The specific activity of protein obtained from SV40-infected CV-1 cells that had been labeled with ³H-amino acids for 24 hr was 321 dpm/ μ g of protein. In contrast, 1 μ g of protein from cells treated likewise, except that 20 μ g of ara-C per ml had been present, incorporated 491 dpm.

Synthesis of structural and nonstructural SV40-induced polypeptides. Labeling experiments were carried out with ³H-amino acid mixtures, which were added to the cultures after adsorption of the virus. Then, the cells were harvested, and to the proteins of the infected cultures, which had been maintained in the presence or absence of ara-C, ¹⁴C-labeled, purified, degraded SV40 capsid proteins were added as internal markers. After electrophoresis the gels were sliced, and the distribution of ³H- and ¹⁴C-labeled proteins was determined.

In Fig. 2 the patterns of proteins synthesized early and late and in the presence or absence of ara-C in SV40-infected cells are compared. We shall consider first the early stage after infection, i.e., 18 hr postinfection (Fig. 2a and b). Up to this time, viral DNA replication has not yet commenced, and "early" viral functions such as T-antigen must be coded for by the incoming viral DNA. In contrast, the expression of "late" viral functions, one of which is the synthesis of capsid proteins, depends on the replication of the viral DNA. Because of this requirement, VP proteins cannot be manufactured earlier than 18 hr postinfection, a time which denotes the onset of viral DNA replication.

In agreement with this expectation, there do not appear (with one exception) at this early stage of infection viral capsid proteins in the infected cultures which would, as separate peaks, assume identical positions with the internal marker proteins. Further evidence for the absence of capsid proteins at this early stage comes from the ara-C-

treated culture, which shows no difference in the protein pattern as compared with the untreated infected culture. There are, however, two remarkable differences from the uninfected control cultures (Fig. 1). First, the synthesis of some low-molecular-weight proteins is inhibited in the infected cultures. Secondly, two peaks appear in the infected cultures (Fig. 2a, b) designated NCVPI, according to the nomenclature used by Summers et al. (22), and NCVP2, which are missing in the uninfected control culture. Peak NCVPI does not display the same electrophoretic mobility as any one of the internal ¹⁴C-labeled viral capsid proteins, whereas NCVP2 apparently has the same position as the VP3 marker protein. However, ara-C treatment (Fig. 2b) does not influence the synthesis of these particular proteins (Fig. 2a to h). In contrast, NCVP2 synthesis seems to be even enhanced after treatment with ara-C for 18 hr (Fig. 2b).

After the onset of viral DNA replication (16) during the late stages of infection, 32 hr to 72 hr postinfection (Fig. 2c to h), the protein pattern in the infected cultures displays considerable alterations. There appears one new peak, the synthetic activity of which increases with increasing time of infection and which shows the same electrophoretic mobility as the VP1 peak, the major constituent of the viral capsid. The synthesis of this protein in infected cells can be completely prevented by addition of ara-C (Fig. 2d, f, h). The same holds true for the peak in the infected cultures corresponding to one of the major components of the viral capsid, VP2. The other constituents, VP3 to 6, however, cannot be detected after prolonged periods of radioactive labeling, so that no clear-cut correlation can be established with the electrophoretic mobility of the corresponding internal ¹⁴C-labeled marker proteins.

The experiments depicted in Fig. 2 were conducted on the basis of long-term labeling periods to achieve labeling of as many proteins as possible. Thus, residual cellular protein synthesis, which might have still been going on, may have contributed to a large background of labeling in the electrophoretic patterns. Conceivably, as the time of infection proceeds, the synthesis of cellular proteins may be reduced or even largely abolished. It seemed to be worthwhile, therefore, to attempt a preferential labeling of viral proteins whose synthesis might be prevailing, by short-pulse labeling experiments. A comparison of the results displayed in Fig. 3 (short-pulse labeling) with those of Fig. 2 (long-term labeling) shows that the major viral capsid proteins VP1 and 2 are still being actively synthesized between 71.5 and 72 hr postinfection, while NCVPI and NCVP2 syn-

thesis seems to have ceased already. It is of particular interest that the short-pulse labeling experiment reveals the VP3 and VP4 peaks (Fig. 3), whose synthesis can be prevented by ara-C. These two proteins did not become clearly apparent after long-term labeling (Fig. 2) because of the presence of various other labeled proteins in the electrophoretic patterns.

DISCUSSION

Our results have confirmed the notion that purified SV40 virions contain at least six different polypeptide chains (5, 7, 11). There is one major component (VP1) which comprises, together with two other components (VP2, VP3), more than 80% of the viral capsid constituents. Three minor components exist in addition to the three larger polypeptide chains (VP1 to 6). The estimated molecular weights of the six polypeptides are also in close agreement with those already reported (1, 5, 7, 11).

If one extrapolates from the size and number of the six polypeptides the number of codons required for their specification, the rather limited coding capacity of the small SV40 genome poses a particular problem, in that almost no cistrons seem to be available other than those necessary to specify the viral capsid proteins. If one accepts the idea that the viral genome, in addition to specifying the various capsid proteins of the virion, also codes for other functions such as initiation and maintenance of transformation, induction of tumor antigen, surface alterations of the infected and of the transformed cells, and induction of cellular DNA synthesis, to quote only some of the functions attributed to the SV40 genome, then one is indeed in a dilemma. Additional space on the viral genome could be provided by one of several possibilities: either the viral capsid polypeptide chains may assume non-structural functions in addition to constituting the viral coat; or some components might be derived from the host cell and become incorporated into the virus capsid. For example, the possibility that particular polymerases might be constituents of the virion cannot be ruled out at present. There is still the possibility, which also cannot be disregarded at present, that minor polypeptide chains arise as cleavage products from the major constituents of the capsid. This possibility would also render more codons available for the specification of some of the biological functions mentioned above.

The purified labeled and disintegrated viral capsid proteins were employed as internal markers in extracts of SV40-infected cells in order to facilitate identification of virus-induced polypeptides

among the various species of cellular proteins. Furthermore, advantage was taken of the observation that treatment of SV40-infected cells with ara-C prevents replication of viral DNA which is required for transcription of "late" SV40 messenger RNA (mRNA) synthesis (20), which, in turn, is necessary for synthesis of viral capsid protein. Similar experiments with an inhibitor of DNA synthesis have also been performed with vaccinia virus (19). By using these criteria it has been possible to identify the major polypeptides which constitute the bulk of the SV40 capsid proteins among the large number of cellular proteins in the infected cell (cf. Fig. 2 and 3). VP1 and VP2 comigrate with the respective proteins obtained from purified virions, can be inhibited by ara-C, and cannot be detected in the infected cells before 36 hr after infection. VP3 and VP4 are detected by short-pulse labeling experiments 72 hr postinfection (Fig. 3). As one would expect, however, it is difficult to identify the other VP proteins which participate in the formation of the viral capsid to a much smaller extent, although the synthesis of a number of proteins appears to be curtailed in the infected cells after treatment with ara-C.

One of the major purposes of the experiments described in this paper was, in addition to the identification of viral capsid proteins in the infected cells, the identification of virus-induced proteins which are not constituents of the viral capsid. These virus-induced polypeptides belong to the so-called "early" class of proteins, which become apparent in the infected cell before replication of viral DNA because the synthesis of their specific mRNA species is independent of DNA replication. Thus, various inhibitors do not prevent synthesis of these particular nonviral capsid proteins. Virus-induced proteins can be classified as "early" proteins in the infected cells only by virtue of the following properties: they do not appear in uninfected cells; their synthesis is unaffected by ara-C; they appear before the onset of viral DNA replication, i.e., before 18 hr postinfection; and further evidence may be derived from their electrophoretic mobility in polyacrylamide gels which might be different from "late" viral proteins. Using these criteria, we have been able to identify two particular NCVP proteins in the productively infected cells. Their rate of synthesis is most pronounced up to 36 hr after infection; it declines sharply, however, during later stages of infection (72 hr after infection). These properties, although they are by no means conclusive, make it likely that we are dealing here, in the case of one of these proteins, with the virus-induced complement-fixing or tumor antigen. It has been

FIG. 2. Time sequence of protein synthesis in SV40-infected CV-1 cells. ^3H -amino acid-labeled proteins of infected cells which were either treated or not treated with ara-C were obtained at different times after infection. They were coelectrophoresed with ^{14}C -labeled purified SV40-capsid proteins. Radioactive labeling and treatment with

ara-C were carried out throughout the periods of time indicated in the graphs. For further details see text. VP1 and VP2 denote the position of viral capsid proteins; nonviral capsid proteins are designated NCV1 and NCV2. (●) ^3H ; (○) ^{14}C .

FIG. 3. Protein synthetic activity in SV40-infected CV-1 cells at 72 hr after infection. Proteins of infected cells which had been labeled with ^3H -amino acids for 30 min before harvesting were coelectrophoresed with ^{14}C -labeled purified SV40-capsid proteins. Treatment with ara-C was carried out for 72 hr. For further details see text. (●) ^3H ; (○) ^{14}C .

shown, in agreement with our observation, that the complement-fixing titer of this antigen declines as the time of infection of cells with SV40 and polyoma virus proceeds (10). This agrees with the observation that at late stages of infection after short-pulse labeling almost no label is found in the position of the NCVP peaks (Fig. 3).

Since "late" viral functions, like synthesis of viral capsid proteins, are missing in SV40 transformed cells, while "early" viral functions, in particular T-antigen, are being synthesized, it is important to determine such early viral functions among the various cellular proteins in transformed cells. This paper has been a first step in this direction, in that the position of two early viral NCVP proteins has been localized in polyacrylamide gels prepared from productively infected cells. Experiments are now in progress to characterize similar alterations in cells transformed by SV40.

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