JOURNAL OF VIROLOGY, Mar. 1974, p. 590-595

Vol. 13, No. 3
Printed in U.S.A.

Translation of mRNA from Simian Virus 40-Infected Cells into Simian Virus 40 Capsid Protein by Cell-Free Extracts

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Received for publication 8 November 1973

Messenger RNA was isolated from simian virus 40 (SV40)-infected and mock-infected cells by chromatography on poly(U) sepharose. When added to cell-free extracts from Chinese hamster ovary cells or rabbit reticulocytes, RNA from the infected cells, but not from mock-infected cells, stimulated synthesis of the major SV40 capsid protein. Identification of this species was done by sodium dodecyl sulfate gel electrophoresis, peptide mapping, and immunoprecipitation. The in vitro synthesized capsid protein was slightly different from virion assembled capsid protein, as shown by separation upon chromatography on hydroxylapatite and by minor differences in the peptide map.

The simian virus 40 (SV40) virion contains a major polypeptide (molecular weight of 45,000), two minor polypeptides (molecular weight of 35,000 and 25,000), and three internal histone-like proteins (3, 4, 6, 18). Late in the lytic cycle virion proteins represent 10 to 20% of the total protein synthesis of the cell (2, 18). Viral mRNA's should comprise as much as 20 to 30% of the total mRNA found in cells during this period. Viral RNAs isolated from the cytoplasm late in infection can be resolved into two species, 16*S* and 19*S*, both of which contain at least one poly(A) sequence; they are presumably derived from larger virus-specific RNAs found in the cell nucleus (23, 24). During lytic infection one can detect, in addition to the virion antigen, the T and U antigens in the nucleus. It is not yet clear whether they are encoded by the virus genome (reviewed in ref. 21).

As a first step in defining the function of the SV40 mRNA's, we isolated from infected cells poly(A)-containing RNA. When added to cell-free extracts prepared from either Chinese hamster ovary (CHO) cells or rabbit reticulocytes this RNA directs synthesis of the major SV40 capsid protein. Independently, Prive et al. (C. Prives et al., Proc. Nat. Acad. Sci. U.S.A., in press) have also demonstrated cell-free translation of SV40 mRNA yielding the major capsid protein.

MATERIALS AND METHODS

Virus and cell lines. SV40 strain SV-S was propagated in Vero cells at 38 C at a low multiplicity as previously described (18).

Virus infection. Confluent monolayers of Vero cells (75-cm^2 flask or 150-mm diameter petri dish) were infected at a multiplicity of infection of 5 to 20 PFU/cell as previously described (18). At 44 to 60 h postinfection, cultures were harvested for RNA extraction.

Preparation of labeled virus. Cells were labeled in complete medium from 48 to 96 h postinfection with either [^{3}H]methyl methionine (specific activity 4 Ci/mmol, 20 μCi/ml medium) purchased from New England Nuclear Corp. or [^{14}C]arginine (specific activity 0.3 Ci/mmol, 0.5 μCi/ml medium) purchased from Schwarz-Mann. Virus was purified from the cells by repeated equilibrium centrifugations in CsCl as previously detailed (10).

Extraction of poly(A)-containing RNA. About 5 $\times$ 10^8 cells were washed twice with phosphate-buffered saline and were lysed in a buffer of 0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris, pH 7.4, and 1.5% sodium dodecyl sulfate (SDS). The viscosity of the resultant solution was reduced by shearing briefly in a Dounce homogenizer. The solution was extracted once at room temperature with an equal volume of redistilled phenol plus an equal volume of chloroform, and then twice more with equal volumes of chloroform (20). Sodium chloride was then added to the aqueous phase to a final concentration of 0.4 M and SDS to 1%. The solution at room temperature was layered onto a 5-ml column of poly(U)Sepharose previously equilibrated in the same buffer (5). The column was then washed with several volumes of 50% ethanol in 0.2 M ammonium acetate, and the RNA remaining on the column was eluted with 5 ml of a solution containing 70% formamide and 30% of 0.001 M EDTA. Ammonium acetate was added to the eluate to a final concentration of 0.4 M followed by an equal volume of ethanol. The RNA was precipitated by centrifugation at −5 C in a Beckman SW65 rotor at 40,000 rpm for 4 h. The resultant pellet was resuspended in distilled water and reprecipitated twice more as described above.

The final pellet was lyophilized, and the RNA was dissolved in 200 μliters of distilled water.

Preparation of cell extracts for protein synthesis. Cell extracts derived from CHO cells and rabbit reticulocytes were prepared exactly as described by McDowell et al. (15) and Housman et al. (7).

Cell-free protein synthesis. Conditions for cell-free protein synthesis in CHO extracts were exactly as described by McDowell et al. (15).

Conditions for reticulocyte cell-free synthesis have been detailed by Lodish et al. (13), except that dithiothreitol (0.005 M) replaced 2-mercaptoethanol, and the concentrations of magnesium acetate and creatine phosphokinase used were 0.0012 M and 0.1 mg/ml, respectively. All incubations were at 30 C.

Polyacrylamide gel electrophoresis. Cell-free reactions were terminated by the addition of ribonuclease A (50 μg/ml) followed by a 10-min incubation at 37 C. The products of the reaction were resolved on 7.5% polyacrylamide gels containing 0.1% SDS and 8 M urea as described previously (14); conditions for staining with Coomassie blue and autoradiography of the dried gels also have been described (12). X-ray film was exposed for 4 days; the autoradiograms were scanned with a Joyce Loebel microdensitometer with a wedge in such a way that full-scale pen deflection was 1.49 optical density units.

Hydroxylapatite chromatography. Radioactively labeled proteins (^{35}S) made in cell-free reactions and ^{3}H-labeled SV40 virions were mixed and fractionated together by electrophoresis through polyacrylamide gels. The location of the (^{35}S)labeled products was determined by autoradiography of the dried gels. The labeled (^{35}S-^{3}H) polypeptide migrating in the position of the major capsid protein was eluted from the gel in 0.5% SDS and chromatographed in a hydroxylapatite column as described by Moss and Rosenblum (17).

Immunoprecipitation. Radioactive proteins having a mobility identical to that of the major SV40 capsid protein were eluted from dried polyacrylamide gels in 0.5% SDS. Portions were diluted to 0.2 ml in 0.5% SDS, 0.1 M NaCl, 0.01 M Tris, pH 7.4, and 5 mg of bovine serum albumin per ml, and were incubated for 60 min at 37 C with 20 μliters of a rabbit antiserum to SDS-treated virus particles (19). Subsequently, 0.2 ml of a sheep antiserum to rabbit gamma globulin (kindly provided by M. Mage, National Institutes of Health) was added; the reaction was incubated for 60 min at 37 C, followed by overnight incubation at 4 C, and centrifuged at 10,000 × *g* for 10 min. The immune precipitate was washed three times in 0.1 M NaCl and 0.01 M Tris, pH 7.4, at 4 C and dissolved in 0.5 ml of 1% SDS at 56 C for subsequent determination of radioactivity. (No radioactivity was detectable in the final supernatant.)

Peptide mapping. Radioactive proteins were eluted from dried gels in 0.5% SDS. Urea was added to 6 M, and 1.5 mg of bovine serum albumin was added as carrier. SDS was removed by a published procedure (23), and proteins were recovered by precipitation with 5% trichloroacetic acid. Over 90% of the eluted (^{35}S) radioactivity was recovered by this procedure. After repeated lyophilization to remove residual acid, the protein was digested with 60 μg of TPCK-trypsin (Worthington Biochemicals) in 2.0 ml of 1% ammonium bicarbonate for 4 h at 37 C. After lyophilization the peptides were resolved by paper ionophoresis at pH 3.5 at 40 V/cm for 3 h as detailed previously (11).

RESULTS

Two types of cell-free extracts were used in this study: preincubated extracts from CHO cells and non-preincubated extracts from rabbit reticulocytes. Although the latter extracts synthesize globin and other reticulocyte proteins at a constant rate for at least 1 h, we have been able to detect synthesis of both reovirus (15) and vesicular stomatitis virus proteins (16) in them after addition of the respective viral mRNA's.

The mRNA utilized here was isolated from SV40-infected and mock-infected cells and purified by adsorption to and elution from poly(U) sepharose. Because of the small amount of RNA recovered, we did not attempt to determine the concentration; rather, each preparation was tested at several concentrations, and the optimal concentration was chosen.

CHO extracts. RNA from equivalent amounts of mock-infected or SV40-infected cells stimulated protein synthesis to the same extent (Table 1); the maximal protein synthesis obtained was about one-half that of the maximal synthesis stimulated by bacteriophage Qβ RNA. Reactions incubated with [^{35}S]methionine and containing mock-infected or SV40-infected RNA were analyzed by SDS gel electrophoresis and radioautography. In all cases radioactive protein was found in a diffuse band throughout the gel. However, in the sample

TABLE 1. *Stimulation of cell-free protein synthesis by viral RNA*[a]

RNA	Amt	Counts/min
None		1,193
SV40-infected	1 μliter	3,537
	2 μliters	6,035
	4 μliters	7,408
Mock-infected	1 μliter	3,502
	2 μliters	6,054
	4 μliters	7,641
Qβ RNA	9 μg	15,044

[a] Reactions (25 μliters) contained CHO extracts, [^{3}H]leucine (48 μci/ml; 48 ci/mmol) and the indicated amount of RNA. Poly(A) containing RNA from 5 × 10^{6} SV40-infected or mock-infected cells was dissolved in 200 μliters of H_2O. Reactions were incubated at 30 C for 40 min. A 0.5-ml amount of 0.1 M NaOH was added. After incubation at 37 C for 10 min, 0.5 ml of 10% trichloroacetic acid was added, and the precipitate was collected on fiberglass filters.

containing the SV40-infected mRNA there was a unique new band of mobility precisely like that of the major SV40 capsid marker (vertical line at 5.4 cm, Fig. 1).

An additional experiment showed that this material is the SV40 capsid protein. A cell-free reaction labeled with [^{35}S]methionine and containing SV40 mRNA was mixed with [^{3}H]methionine-labeled virions. After SDS gel electrophoresis and autoradiography, the SV40-specific band at 45,000 molecular weight (detected by [^{35}S] radioactivity) was excised and eluted with 0.5% SDS. This band, as expected, contained the majority (83 to 95%) of the added [^{3}H] radioactivity. After digestion with trypsin, the labeled peptides were resolved by paper ionophoresis at pH 3.5 (Fig. 2). All of the peptides present in the authentic virion protein were found in the product of the cell-free system, although one of the peptides (at 7 cm) was present in the in vitro product in reduced amounts relative to the others. The in vitro product from the reaction primed with SV40

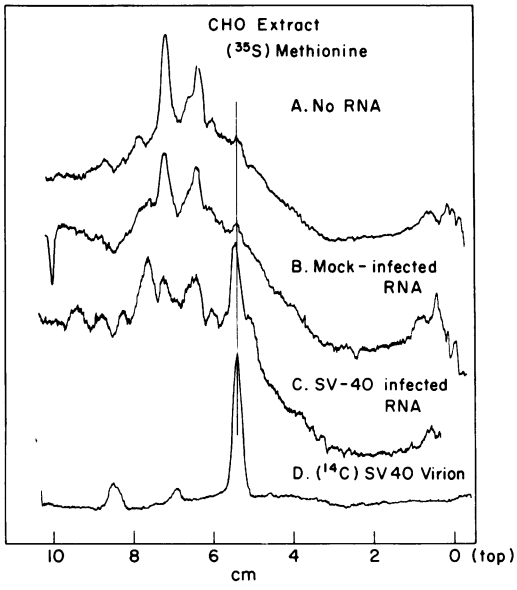

FIG. 1. *Scan of radioautogram of polyacrylamide gel separation of proteins synthesized in CHO extracts. A, No added RNA; B, mock-infected RNA; C, SV40-infected RNA; D, marker (^{14}C) SV40 virion. Reactions (60 μliter) contained 10 μCi of [^{35}S] methionine (100 Ci/mmol) and, where indicated, 4 μliters of RNA (see Table 1). Incubation was at 30 C for 40 min. Portions of 3 μliters were taken for determination of protein radioactivity: A, 5,500 counts/min; B, 8,900 counts/min; C, 9,300 counts/min. The remainder of the reaction was analyzed by gel electrophoresis at 4 mA/gel for 24 H. Shown is the microdensitometer scan of the dried gel (see Materials and Methods).*

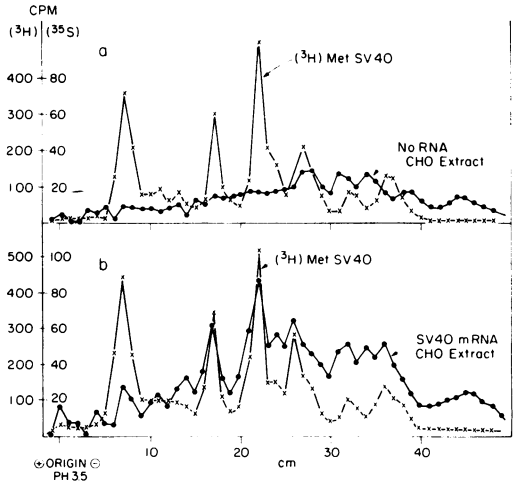

FIG. 2. *Separation of tryptic peptides of SV40 proteins. Top: ^{35}S proteins, synthesized in CHO extract with no added RNA, of gel mobility identical to that of the major SV40 capsid protein. Bottom: analogous ^{35}S proteins synthesized in CHO extract with SV40 RNA. Reactions (240 μliters) of composition identical to those of A and C in Fig. 1 were used. To each was added 20,000 count/min [methyl-^{3}H] methionine-labeled SV40 virions, and each reaction was analyzed on four gels as in Fig. 1. After autoradiography, the region of the major SV40 capsid (45,000 molecular weight) was detected (by ^{35}S radioactivity) and a 2-mm-wide region was eluted. Trypsin digestion and paper electrophoresis techniques were summarized in Materials and Methods.*

mRNA yielded no major peptides not found in authentic virions. Occasionally there were a few tryptic peptides (at 4, 11, 14 cm as shown in Fig. 2c) which did not correspond to viral peptides and which were not produced from reactions without SV40 mRNA. Such small amounts of these were made that it was impossible to determine their nature. The same figure shows the digest of material from the same position in the gel, but from a reaction without SV40 mRNA; it yields a diffuse [^{35}S] profile with no characteristic viral protein peptides.

Reticulocyte extract. Because the extracts used were not preincubated to reduce translation of endogenous reticulocyte mRNA, addition of exogenous mRNA does not stimulate the rate of overall protein synthesis. On the contrary, occasionally, addition of SV40 mRNA (Fig. 3 legend) and other mRNAs such as reo mRNA (15) inhibits incorporation by up to 50%. Nonetheless, analysis of the product of a reticulocyte incubation containing mRNA from SV40-infected cells yields a labeled protein species which comigrates with authentic SV40 capsid, and which is not found in the control

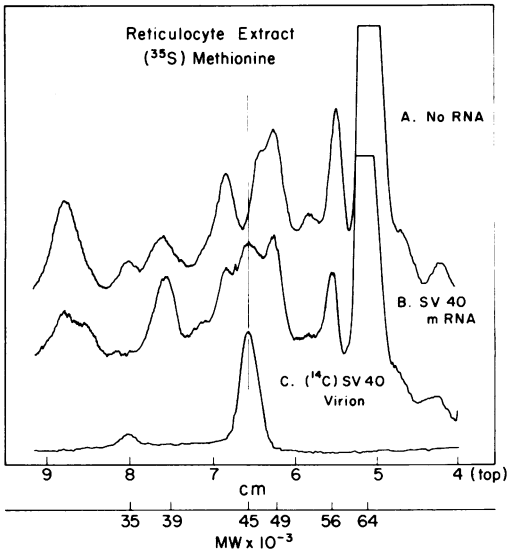

FIG. 3. *Scan of polyacrylamide gel analysis of proteins synthesized in reticulocyte extracts. Reactions (60 μliter) contained 10 μCi of [35S]methionine (102 Ci/mmol) and, in B, 4 μliters of SV40 RNA (Table 1). Incorporation was at 30 C for 50 min. In 3 μliters of reaction A (no RNA) there were 48,000 counts/min and in reaction B, with SV40 rRNA, 40,000 counts/min. Gel electrophoresis was as in Fig. 1. Shown is a scan only of the region from 4 to 9 cm of the gel. Below 9 cm is the enormous peak of globin. Above 4 cm there were no new SV40-specific bands observed. A parallel analysis of a reaction with mock-infected RNA (not shown) yielded results not significantly different from the reaction without RNA. Markers for determination of molecular weights of proteins have been detailed (12).*

reaction lacking SV40 mRNA (Fig. 3). Two types of evidence support the contention that this species is SV40 capsid protein. First, trypsin digestion of the [35S]methionine-labeled species yields a pattern of peptides identical to that of marker [3H]methionine peptide (Fig. 4). As seen previously in the product of the CHO extract, the peptide present at 7 cm is found in the in vitro product in reduced amounts.

Second, the material, after elution from the gel in 0.5% SDS solution, was specifically precipitated with antibody to disrupted SV40 virions (Table 2). Since 60% of purified 45,000-dalton protein from virions was precipitable under these conditions of immunoprecipitation, the data in Table 2 indicate that at least one-half of the material in the 35S in vitro product is antigenically related to the major cpasid.

From neither the CHO (Fig. 1) nor reticulocyte (Fig. 3) reactions could we detect synthesis of proteins which comigrated with either of the minor virion polypeptides (8.5 and 6.9 cm in Fig. 1; 8 cm in Fig. 3), although both reactions yielded small amounts of SV40-specific peptides which did not comigrate with marker virus proteins. We have not studied these bands further.

Hydroxylapatite chromatography. In an attempt to further demonstrate the identity of the capsid protein synthesized in vitro, we analyzed it by chromatography on hydroxylapatite (HAP) in buffers containing SDS. This proce-

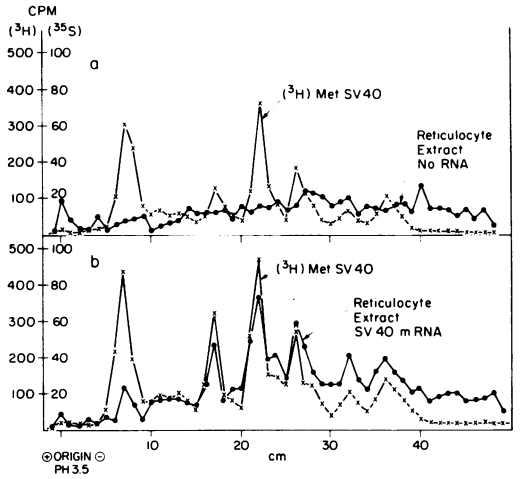

FIG. 4. *Separation of tryptic peptides of SV40-specific proteins made in reticulocyte extracts. Top: [35S] proteins of gel mobility equal to that of virion capsid protein synthesized in reticulocyte extract without added RNA. Bottom: analogous [35S] proteins synthesized in the presence of SV40 mRNA. Reactions (240 μliters) of composition identical to those of A and B (Fig. 3) were mixed with 20,000 count/min [methyl-3H]methionine SV40 virions. Analysis was as in Fig. 2.*

TABLE 2. *Immunoprecipitation of capsid protein synthesized by viral RNA*[a]

Type of RNA used	Input (counts/min)	% in precipitate
No added RNA	747	7.8
	780	5.1
SV40-infected RNA	870	26.6
	563	27.4
	615	28.0

[a] The 45,000-dalton protein synthesized in cell-free extracts from reticulocytes (either with no added RNA or RNA from SV40-infected cells) was isolated by SDS-acrylamide gel electrophoresis and 50 to 75 μliters of this fraction was used in an immunoprecipitation reaction as described in Materials and Methods. Purified capsid protein was 60% precipitable under these conditions.

dure separates proteins on a basis other than molecular weight (17). When [^{3}H]methionine-labeled virion was disrupted in SDS and chromatographed, the protein eluted as a single peak (Fig. 5, inset). Next, ^{35}S-labeled CHO reactions primed with SV40 RNA were mixed with [^{3}H]methionine-labeled virions. The labeled proteins were resolved by gel electrophoresis and, as in the peptide mapping experiments, the region of the major virion coat protein (45,000 dalton region), containing over 85% of the ^{3}H radioactivity, was eluted in 0.5% SDS. It was applied directly to a HAP column. The ^{3}H marker showed a major peak at the expected position, but more heterogeneous labeled material than expected. Presumably this is due to trace amounts of protease acting in the gel or during elution. Of interest was the fact that the in vitro ^{35}S coatlike product eluted three fractions later. When the same region of the SDS gel but from a reaction with no SV40 mRNA was analyzed on HAP, there was only a diffuse profile of ^{35}S radioactivity, with no single large peak (data not shown).

DISCUSSION

This paper shows that poly(A)-containing RNA, isolated from cells late in infection by SV40, can direct the synthesis of a protein which closely resembles the major SV40 capsid protein in cell-free extracts from both CHO and reticulocytes. The in vitro product comigrates on SDS gels with authentic SV40 capsid protein and yields a similar pattern of tryptic peptides to that from authentic capsid protein.

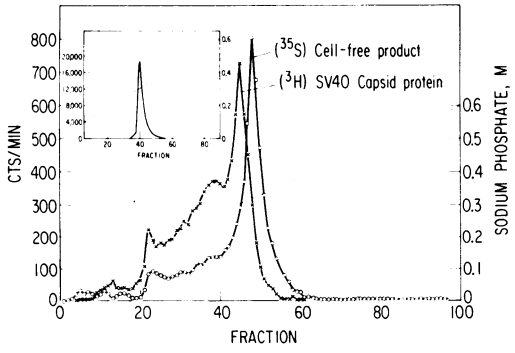

FIG. 5. *Hydroxylapatite chromatography of SV40 proteins. Insert: chromatography of [^{3}H]methionine SV40 virion disrupted in 0.5% SDS. Main figure: [^{3}H]methionine-labeled SV40 virion was mixed with a CHO reaction, labeled with [^{35}S]methionine, and primed with SV40 RNA, exactly as in Fig. 2. The region of the gels corresponding to the SV40 capsid protein was eluted in 0.5% SDS as in Fig. 2 and applied directly to a hydroxylapatite column.*

Two pieces of evidence, however, suggest that the in vitro product is not exactly the same as the protein found in virions. Trypsin digestion of the in vitro product, labeled with [^{35}S]methionine, yields relatively little of one peptide found in the marker [^{3}H]methionine-labeled capsid protein (Figs. 2, 4). Furthermore, the capsid protein synthesized in vitro elutes slightly later than the authentic capsid marker during chromatography on HAP in buffers containing SDS (Fig. 5). During in vivo virus assembly the capsid protein may be modified by loss of some amino acids and thus generate a soluble methionine-containing tryptic peptide (at 7 cm in Fig. 2c and Fig. 4c) from the insoluble tryptic core (not analyzed in Figs. 2 or 4) of the precursor protein. Hence, this peptide (at 7 cm) would not be expected to be found in the cell-free product. Proteolytic cleavage of virus proteins during assembly has been amply documented in other viruses (1, 8, 9). A second possibility is that the virus capsid is methylated at some stage during assembly, resulting in the appearance of the new [^{3}H]methionine-labeled peptide (at 7 cm). Were this the case, this peptide (at 7 cm) again would not be found in the cell-free product.

The techniques detailed in this paper should allow one to determine which of the virus mRNA species found in the cell cytoplasm encodes the capsid protein(s). Finally, by using specific immunological assays one could determine whether the cell-free reactions utilized here synthesize virus T or U antigens. These experiments are in progress.

ACKNOWLEDGMENTS

We thank Oladoyin Desalu and Arthur Ciccone for expert technical assistance.

This work was supported by Public Health Service grants AI-08814 and CA-12708 from the National Institute of Allergy and Infectious Diseases and the National Cancer Institute, respectively. H. F. L. is the recipient of Public Health Service research career development award GM-50175 from the National Institute of General Medical Sciences.

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