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Cell-Free Translation of Messenger RNA of Simian Virus 40: Synthesis of the Major Capsid Protein

(wheat-germ extracts/oligo(dT)–cellulose/gel electrophoresis/peptide analysis/ RNA·DNA selective hybridization)

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ABSTRACT **Extracts of wheat germ are capable of synthesizing the major capsid protein of simian virus 40. Poly(A)-containing RNA from BS-C-1 cells infected with simian virus 40 directed the synthesis of a novel polypeptide that migrates in polyacrylamide gels together with the major capsid polypeptide of simian virus 40, VP-1. The patterns of the major tryptic peptides of purified VP-1 and the novel polypeptide synthesized *in vitro* were identical after two-dimensional paper electrophoresis. The novel polypeptide was not synthesized in response to poly(A)-rich RNA from uninfected cells or from virus-infected cells treated with cytosine arabinoside. Messenger RNA from infected cells purified by selective hybridization to DNA of simian virus 40 directs the synthesis of a major polypeptide of electrophoretic mobility similar to that of VP-1 of simian virus 40. This approach should prove useful in identifying additional products specified by DNA tumor viruses.**

Depending on the host cell, the oncogenic simian virus 40 (SV40) can produce either a lytic response accompanied by the production of viral progeny, or cell transformation. In either case, the number of changes wrought in the cell by viral infection appears to be too numerous to be directly accounted for by the relatively small (3×10^6 daltons) viral genome (1, 2). It follows that these changes may represent a pleotropic response initiated by one or very few viral gene products. One decisive way to identify a virus-specific product is by means of the cell-free synthesis of proteins directed by purified viral genetic material. Unlike many viruses, SV40 does not depress cellular RNA and protein synthesis, and the viral capsid structural proteins do not represent more than 10% of the total cellular spectrum of polypeptides even late in lytic infection (3, 4). However, in this report we will show that by combining a sensitive cell-free translation system from wheat-germ extracts (5) with relatively simple methods of purification of viral messenger RNA (mRNA) based both on the presence of polyadenylate [poly(A)] sequences (6, 7) and selective hybridization to SV40 DNA (8), it is possible to synthesize *in vitro* the major SV40 capsid protein, VP-1. The cell-free translation of oncogenic virus mRNA, selected by hybridization, can provide the basis for identifying additional virus-specified products in permissive and transformed cells.

Abbreviations: SV40, simian virus 40; SDS, sodium dodecyl sulfate; ara C, cytosine arabinoside.

MATERIALS AND METHODS

Cells and viruses

A plaque-purified stock of SV40 (strain 777) was grown in the BS-C-1 line of African green-monkey kidney cells as described (9). For production of radioactive virus, [^{35}S]methionine (3 μCi/ml of culture medium; 170 Ci/mmol, The Radiochemical Center, Amersham, England) was added 24 hr after infection to cells in medium containing 7.5 μg/ml of methionine and 2% calf serum. The labeled virus was purified by two consecutive bandings in CsCl density gradients (9) and dialyzed against 0.01 M Tris·HCl buffer, pH 7.9 containing 0.15 M NaCl. The purified virus was dissociated by heating at 100° for 1 min in a solution containing 0.05 M Tris·HCl, pH 6.8, 1% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, and 10% glycerol before SDS-polyacrylamide gel electrophoresis as described below for cell-free product analysis.

Preparation of poly(A)-containing RNA

Total BS-C-1 cellular RNA was extracted from 50 cultures of BS-C-1 cells (4×10^6 cells per culture) 48 hr after infection with plaque-purified SV40 at 100 plaque-forming units per cell, by a described procedure (10). Briefly, this procedure involved several successive phenol–chloroform–isoamylalcohol extractions followed by $LiCl_2$ precipitations. High-molecular-weight RNA (ribosomal and mRNA species) was then subjected to oligodeoxythymidylate [oligo (dT)]–cellulose chromatography (6). RNA retained by oligo(dT)–cellulose was assumed to contain poly(A) sequences. RNA from uninfected cultures was derived from the same cell passage as that utilized for virus infection.

Preparation of virus-specific RNA by hybridization to SV40 DNA

Two out of 50 cultures of infected BS-C-1 cells were labeled with [^{3}H]uridine from 40 to 48 hr after infection (10 μCi/ml of culture medium; 29 Ci/mmol; The Radiochemical Center, Amersham, England). The RNA was extracted from the combined labeled and unlabeled cultures, purified on oligo(dT)–cellulose columns, and dissolved in 1 ml of hybridization buffer (8, 10) [0.75 M NaCl, 0.05 M Tris·HCl, pH 7.4, 0.5% (w/v) SDS, 50% (v/v) formamide] for each 10 cultures extracted. Labeled RNA (1 ml) was then hybridized with 20

μg of plaque-purified SV40 DNA, component 1, immobilized on a nitrocellulose membrane filter, as described (10). For recovery of the hybridized RNA, each filter was incubated for 1 hr at 37° in 1 ml of elution buffer (10) (90 volumes of 100% formamide, 9 volumes of distilled water, and 1 volume of SDS buffer; final pH adjusted to 8.4). The eluted RNA was precipitated out of elution buffer by the addition of 1/10 volume of 1 M sodium acetate, pH 4.5, two volumes of ethanol, and 5 μg/ml of wheat-germ transfer RNA (tRNA). Finally, the virus-specific RNA was again subjected to oligo (dT)–cellulose column chromatography, before its addition to the cell-free translation system.

Cell-free translation in extracts of wheat germ

Extracts from wheat germ (50% "Hard Winter," 40% "Mexican," 10% "Nanasith") kindly supplied by Mr. Shildhaus of the "Bar-Rav" Mill, Tel-Aviv, Israel, were prepared and preincubated (5). Protein synthesis was assayed exactly as described by Roberts and Paterson (5). Six microcuries of [^{35}S]methionine (175 Ci/mmol, The Radiochemical Center, Amersham, England) were added per 100 μl of reaction volume. The optimal concentration of magnesium acetate for cell-free translation was found to be between 2.5 and 3.0 mM.

Product identification

SDS–Polyacrymalide Gel Electrophoresis. Samples (100-μl) of the reaction products of protein synthesis were treated with 1 μg of pancreatic RNase in the presence of 0.01 M EDTA for 20 min at 37°. Then 50 μl of a solution containing 0.15 M Tris·HCl, pH 6.8, 3% SDS, 3% 2-mercaptoethanol, and 30% glycerol was added, and the samples were heated at 100° for 1 min. Portions (10-μl) of the treated samples were subjected to electrophoresis at 150 V for 4 hr through slab gels containing a 10–20% polyacrylamide gradient prepared as described (11). Gels were stained with Coomassie Brilliant Blue, dried, and exposed to x-ray film (Kodak RP54) for 48 hr.

Peptide Analysis. The products of the cell-free translation were run in preparative 10% polyacrylamide slab gels. The labeled polypeptides were recovered from the gels by a published procedure (12) except that the extraction of the gel was in the presence of 1% NH_4HCO_3, 0.1% SDS, and 1 mM 2-mercaptoethanol for 18 hr. The purified polypeptide was oxidized with performic acid as described (13) and twice digested with 20:1 (w/w) trypsin (chymotrypsin-free, Worthington) for 4 hr at 37°. The peptides were subjected to two-dimensional high-voltage (3 kV) paper electrophoresis at pH 6.5 for 50 min in the first dimension and at pH 3.5 for 60 min in the second dimension.

RESULTS

Translation of poly(A)-containing RNA in wheat-germ system

Total RNA prepared from uninfected or SV40-infected BS-C-1 cells was purified by oligo(dT)–cellulose column chromatography. Only material that was bound to oligo(dT) was active in promoting incorporation of [^{35}S]methionine into acid-insoluble material when tested in a wheat-germ cell-free extract (Fig. 1). Both crude and unbound RNA fractions were only slightly active under similar conditions. RNA bound to oligo(dT)–cellulose stimulated polypeptide synthesis over a range of concentrations that were similar to values obtained

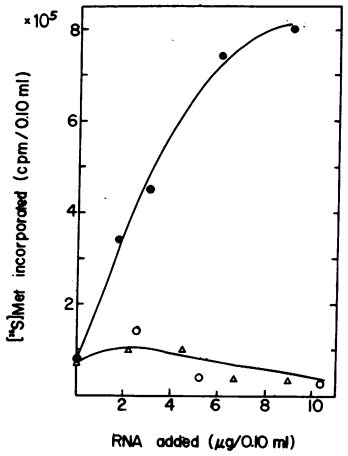

FIG. 1. Protein synthesis directed by RNA from SV40-infected cells in wheat-germ extracts. (●——●) RNA retained by oligo(dT)–cellulose; (O——O) total cellular RNA; (Δ——Δ) RNA not retained by oligo(dT)–cellulose. The specific activity of the [^{35}S]methionine was 175,000 cpm per pmol. Values on ordinate should be multiplied by 10^5.

in this system for tobacco mosaic virus and globin mRNA (5). Furthermore, equivalent amounts of RNA from infected or uninfected BS-C-1 cells stimulated protein synthesis to approximately the same extent in the wheat-germ system. Thus, in a typical experiment 3 μg of oligo(dT)-bound BS-C-1 RNA resulted in the incorporation of 2.9 pmol of [^{35}S]methionine, while 3 μg of oligo(dT)-bound RNA derived from SV40-infected cells resulted in the incorporation of 3.8 pmol of [^{35}S]methionine.

Nature of product

Polyacrylamide Gel Electrophoresis. The products of the cell-free translation of mRNA from uninfected and SV40-infected cells in the wheat-germ system were analyzed by polyacrylamide gel electrophoresis. The RNAs derived both from infected and uninfected cultures directed the synthesis of several polypeptides with molecular weights up to approximately 60,000 (Fig. 2*c–f*). However, the RNA from infected cells gave rise to a major novel band that has the same electrophoretic mobility as the main SV40 capsid protein (VP-1) obtained after dissociation of purified SV40 virions. The molecular weight of the marker VP-1 was estimated to be in the range of 47,000–49,000, by comparison with migration of standard proteins (Fig. 2*a* and *b*). This is somewhat larger than the previously reported values of 42,000–45,000 daltons (14, 15, 3). In two separate experiments the amount of the novel polypeptide was estimated to comprise approximately 5–10% of the total proteins synthesized *in vitro*. In addition to the novel polypeptide described above, another less-intense band was also seen consistently in the gel autoradiogram of products directed by mRNA from SV40-infected cells. This second polypeptide, which had an estimated molecular weight of approximately 35,000, did not appear when mRNA from uninfected cells was used. It is also of interest to note that some polypeptides directed by RNA from infected cells are made in decreased amounts relative to those directed by mRNA from uninfected cells.

The *in vitro* product was compared with the proteins synthesized in infected cells. Extracts of infected and uninfected

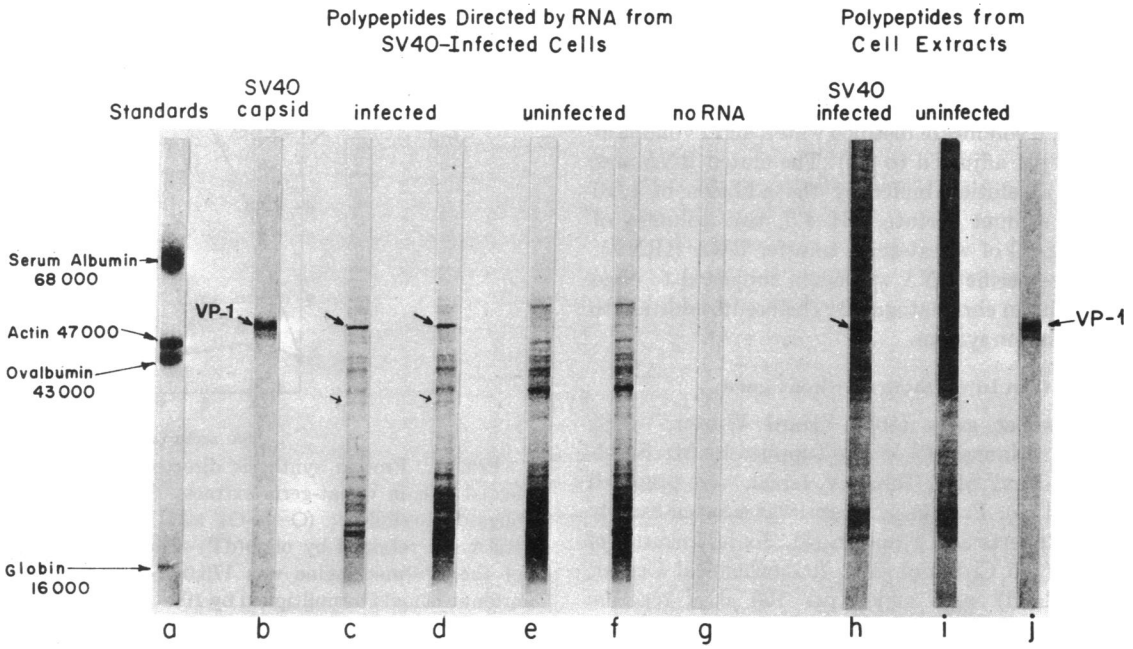

FIG. 2. SDS–polyacrylamide gel electrophoresis of polypeptides directed by RNA from SV40-infected cells. (*a*) Standard proteins stained with Coomassie Brilliant Blue; (*b,j*) proteins from purified SV40 virions used as marker, stained with Coomassie Brilliant Blue; (*c,d*) autoradiogram of [^{35}S]methionine-labeled polypeptides directed by RNA from SV40-infected cells (5 μg of RNA per 100 μl of reaction volume); (*e,f*) autoradiogram of [^{35}S]methionine-labeled polypeptides directed by RNA from uninfected cells (5 μg of RNA per 100 μl of reaction volume); (*g*) autoradiogram of [^{35}S]methionine-labeled reaction mixtures in the absence of added RNA; (*h*) polypeptides from cells 48 hr after SV40 infection, stained with Coomassie Brilliant Blue; (*i*) polypeptides stained with Coomassie Brilliant Blue from uninfected cells.

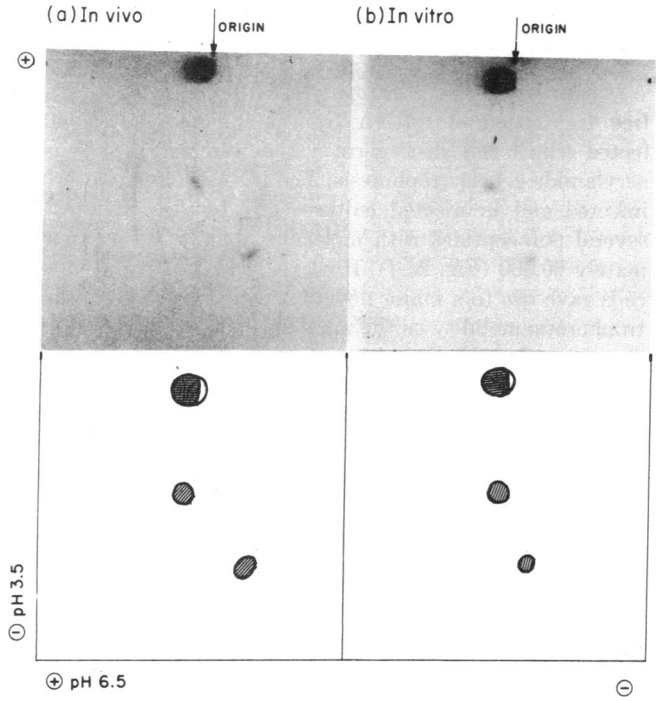

FIG. 3. Two-dimensional electrophoresis of tryptic peptides. (*a*) [^{35}S]Methionine-labeled tryptic peptide autoradiogram of VP-1, from purified SV40 virions (5×10^5 cpm of protein subjected to tryptic analysis; exposed for 20 hr). (*b*) [^{35}S]Methionine-labeled tryptic peptide autoradiogram of the major novel product directed by mRNA from SV40-infected cells (5×10^4 cpm of protein subjected to tryptic analysis; exposed for 7 days).

cells were dissociated in SDS-containing buffer and subjected to the same polyacrylamide gel electrophoresis procedure. A major novel band appears in extracts of infected cells which migrates to the same position as that of the main polypeptide synthesized *in vitro*, and the SV40 VP-1 marker (Fig. 2*h* and *i*).

Peptide Analysis. VP-1 and the major novel polypeptide synthesized *in vitro* were purified by preparative slab-gel electrophoresis, eluted from their respective gels, and subjected to tryptic analysis. The major virion polypeptide yielded three main [^{35}S]methionine-labeled peptides after two-dimensional high-voltage paper ionophoresis (Fig. 3*a*). Additional minor spots have been observed after prolonged exposure; their identities have not been established. Similar treatment of the isolated cell-free translation product also revealed three major radioactive peptides (Fig. 3*b*) whose mobilities in two dimensions, relative to those of the *in vivo* products, were identical. Analysis of the tryptic digestion of the cell-free product has not been sufficiently sensitive to detect the minor peptides mentioned above. From the agreement of the three major tryptic peptides, as well as their similar gel electrophoretic mobilities, we conclude that the major novel polypeptide synthesized in response to RNA from infected cells is the SV40 capsid protein, VP-1.

Polypeptides synthesized in response to RNA from infected cells treated with cytosine arabinoside

Cytosine arabinoside (ara C) is an inhibitor of DNA synthesis. When it is added to cells at the time of SV40 infection, it inhibits the production of viral capsid protein, VP-1 (4, 16),

while having no effect on the appearance of the T antigen, which is an early expression of virus infection (16). It was, therefore, of interest to see whether the *in vitro* product identified as VP-1 by peptide analysis would be synthesized in response to RNA from infected cells treated with ara C. Ara C treatment of the infected cells greatly diminished the capacity of the extracted RNA to direct the *in vitro* synthesis of the novel polypeptide identified as VP-1 (Fig. 4*b* and *c*). Whether the residual gel band represents another polypeptide with the same mobility as VP-1 or reflects an incomplete inhibition by the ara C of the VP-1-specific RNA, remains to be determined. The second novel gel band mentioned previously was not observed when RNA from infected cells treated with ara C was added to the wheat-germ extract. With these two exceptions, the general pattern of polypeptides directed by RNA from SV40-infected cultures was approximately the same whether or not the cells were treated with ara C after virus infection.

Cell-free synthesis of polypeptides directed by mRNA purified by hybridization to SV40 DNA

The cell-free translation of RNA that has been purified by selective hybridization to SV40 DNA should produce a product that is truly virus-specified. Therefore, RNA from SV40-infected cells, purified initially by oligo(dT) chromatography, was further purified by hybridization to SV40 DNA, component 1 (see legend to Fig. 4*d* for data on the specificity of the hybridization procedure). The RNA recovered from the hybrid complex with SV40 DNA was again subjected to oligo (dT)–cellulose chromatography and tested for its ability to direct protein synthesis in the cell-free wheat-germ system. Response to the hybridized RNA in the wheat-germ extract was comparable to that seen with an equivalent amount of nonhybridized, oligo(dT)-bound RNA; thus, addition of approximately 1 μg of hybridized RNA resulted in the incorporation of 1.0 pmol of [^{35}S]methionine into acid-insoluble material. Gel autoradiography of the products synthesized in response to the virus-selected RNA revealed a predominant band as well as several minor bands of lower molecular weight. The electrophoretic mobility of the major polypeptide was identical to that of purified SV40 VP-1 (Fig. 4*d*).

DISCUSSION

The experiments presented herein demonstrate the cell-free translation of RNA coding for the major virion protein of SV40. This conclusion is based upon the findings that mRNA from SV40-infected cells directs the synthesis of a novel polypeptide which, by mobility in polyacrylamide gels and two-dimensional peptide analysis, is identical to SV40 VP-1. The novel polypeptide was not synthesized in response to mRNA from uninfected cells or from cells treated with ara C under conditions that inhibit production of VP-1. Furthermore, virus-specific mRNA selected by specific hybridization to SV40 DNA directs the cell-free synthesis of a polypeptide whose electrophoretic mobility was similar to that of SV40 VP-1. The detection of a product representing a small proportion of a range of polypeptides was greatly facilitated by the use of the wheat-germ translation system, which exhibits extremely low levels of endogenous protein synthesis (5). The use of the *Escherichia coli* coupled transcription-translation system appears to be less advantageous for the cell-free translation of polyoma DNA since only incomplete products were synthesized (17).

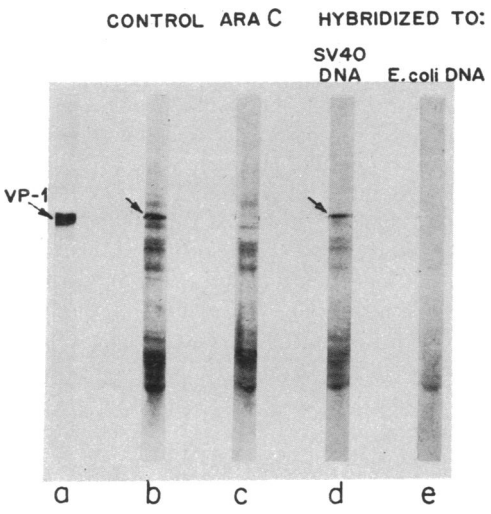

FIG. 4. Autoradiograms of SDS–polyacrylamide gel electrophoresis of polypeptides directed by RNA from SV40-infected cells. (*a*) [^{35}S]Methionine-labeled SV40 virions used as marker. (*b*) [^{35}S]Methionine-labeled polypeptides directed by 5 μg of RNA from SV40-infected cells. (*c*) [^{35}S]Methionine-labeled polypeptides directed by 5 μg of RNA from SV40-infected cells treated with ara C. Ara C (10 μg per ml of medium) was added to cultures immediately after virus adsorption. (*d*) [^{35}S]Methionine-labeled polypeptides directed by RNA extracted from SV40-infected cells which was purified by hybridization to SV40 DNA. Under the hybridization conditions used, 7.5% of oligo-(dT)-bound RNA from SV40-infected cells was specifically hybridized to and eluted from SV40 DNA, while 0.29% hybridized to *E. coli* DNA (0.19% of RNA from uninfected BS-C-1 cells hybridized to SV40 DNA). 1 μg of selected RNA was added per 100-μl reaction volume. (*e*) [^{35}S]Methionine-labeled polypeptides directed by RNA purified as in (*d*) except that the nitrocellulose filters (see *Methods*) contained 20 μg of *E. coli* DNA.

One difference with reported findings that we have observed during these experiments is the apparent molecular weight of VP-1, which has been reported to be in the range of 42,000–45,000 (14, 15, 3). In contrast, we have consistently observed VP-1 to be somewhat larger than ovalbumin (43,000 daltons) or actin (46,000 daltons), as estimated by its relative mobility through SDS–polyacrylamide gels. It is not clear whether the apparent discrepancy in the molecular weight of VP-1 arises from differences in the techniques used or from a natural variation in the populations of SV40 studied. Regardless of these discrepencies, the *in vitro* product and the virion VP-1 studied in this report are seemingly identical in size and peptide composition.

Perhaps the most promising aspect of these results is the translation of RNA purified by hybridization to SV40 DNA. This selected RNA directed the cell-free synthesis of a major polypeptide of the same electrophoretic mobility as SV40 VP-1. Although its positive identification has yet to be confirmed by peptide analysis, the similar electrophoretic mobilities of VP-1 from purified virions, VP-1 synthesized in response to mRNA from infected cells, and the predominant polypeptide directed by the SV40 hybridized mRNA strongly indicate that these three products are the same.

Several other bands are also seen after polyacrylamide gel-electrophoresis of the products directed by SV40 hybridized RNA. Some of these may represent prematurely terminated

SV40 VP-1 polypeptide chains, as has been reported for other translation systems (18, 19). In addition they may represent other virion structural components or even possibly non-structural viral proteins. Several distinct species of SV40-specific RNA have been described, including those that appear early in infection, high-molecular-weight heterogeneous RNA of nuclear origin, and 16S and 19S RNA sedimenting species of cytoplasmic origin (8). It will be of interest to determine which products are specified by each of these classes of SV40 RNA. In addition it may be possible to exploit the system described in this paper for studies on the mechanisms by which SV40 genes are expressed, and for the ultimate identification of virus-specified products in cells transformed by tumor viruses.

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