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Cell-Free Translation of Simian Virus 40 16S and 19S L-Strand-Specific mRNA Classes to Simian Virus 40 Major VP-1 and Minor VP-2 and VP-3 Capsid Proteins

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Simian virus 40 capsid proteins VP-1, VP-2, and VP-3 have been synthesized in wheat germ and reticulocyte cell-free systems in response to either poly(A)-containing mRNA from the cytoplasm of infected cells or viral RNA purified by hybridization to simian virus 40 DNA linked to Sepharose. All three viral polypeptides synthesized in vitro are specifically immunoprecipitated with anti-simian virus 40 capsid serum. VP-2 and VP-3 are related by tryptic peptide mapping to each other but not to VP-1. The most abundant class of L-strand-specific viral mRNA, the 16S species, codes for the major capsid protein. The relatively minor 19S class directs the cell-free synthesis of VP-1, VP-2, and VP-3. Whether the 19S RNA represents more than one distinct species of mRNA is not yet clear. VP-1 mRNA can be isolated from the cytoplasm, detergent-washed nuclei, and the nuclear wash fraction. The mRNA from the nuclear wash fraction is enriched for VP-2 mRNA when compared to other viral or cellular polypeptides.

An understanding of the relationship between the simian virus 40 (SV40) RNA classes and the viral capsid proteins VP-1, VP-2, and VP-3 has been largely sought through techniques involving mRNA isolation and translation in cell-free systems. To date, the following observations have been made about the translation of the L-strand-specific classes of SV40 mRNA. SV40 16S mRNA, the most abundant viral mRNA class, codes for VP-1, the major capsid polypeptide, whereas SV40 19S mRNA codes, in addition, for another viral polypeptide originally termed the X polypeptide (25), which was later found to be identical to the minor viral capsid polypeptide VP-2 (C. Prives, unpublished data). However, the origin of VP-3 was not well defined; this product was never clearly identified among the cell-free products of mRNA from infected cells or even of viral RNA purified by hybridization to SV40 DNA-Sepharose.

In the present study, we have improved the conditions for the in vitro synthesis and identification of the viral proteins. Through the use of RNA fractionation on sucrose gradients, an efficient translation system and specific immunoprecipitation of cell-free products with antisera directed against SV40 capsid proteins, we have been able to identify VP-1, VP-2, and VP-3 among the cell-free products of RNA from SV40-infected cells and to assess the approximate size and subcellular distribution of their mRNA's.

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MATERIALS AND METHODS

Cells and viruses. The standard wild-type SV40 used in this laboratory is a plaque-purified stock of strain 777 grown from a limiting dilution (15) in the BSC-1 line of African green monkey kidney cells. Radioactively labeled SV40 virus was prepared as described for normal virus preparation (15), except that infected cells were grown in the presence of [^{35}S]methionine (20 μCi/ml) in medium containing one-tenth the normal amount of methionine.

RNA preparation. BSC-1 cultures (10^8 cells) were infected with SV40 (50 PFU/cell). Approximately one-fifth of the cultures were routinely labeled with [5,6-^{3}H]uridine (50 μCi/ml) from 40 to 48 h postinfection, after which cells were washed three times with ice-cold buffer containing 0.01 M Tris (pH 7.5) and 0.14 M NaCl, before lysis in Nonidet P-40 (NP40) extraction buffer (0.1 M NaCl, 0.005 M Tris, pH 8.5, 0.003 M $MgCl_2$, and 0.5% NP40), using 2 ml per 10^7 cells. Cell extracts were centrifuged at 2,000 × *g* for 5 min at 4°C in a clinical centrifuge to remove nuclei which, in some cases, were saved for further fractionation. After a second centrifugation at 10,000 rpm in a Sorvall centrifuge for 10 min at 4°C, the supernatant was subjected to phenol-chloroform extraction (21), followed by ethanol precipitation to obtain cytoplasmic RNA. In experiments where nuclear fractions were desired, the nuclei obtained after the first low-speed centrifugation were washed twice in NP40 extraction buffer and then subjected to a modification of the detergent wash procedure first described by Penman (21). The nuclear pellet was suspended in 5 ml of a solution containing 0.006 M NaCl, 0.006 M Tris-hydrochloride (pH 7.5), 0.0025 M $MgCl_2$, 2% NP40, and 1% sodium deoxycholate. The suspension was gently blended in a Vortex mixer and then centrifuged at 2,000 rpm in a

clinical centrifuge for 5 min at 4°C. The supernatant (nuclear wash fraction) was subjected to a similar phenol-chloroform extraction as for the cytoplasmic RNA preparation. The washed nuclear pellet was subjected to an extraction procedure used by Hirt (10), in which the nuclei were suspended in a solution containing 1.0 M NaCl, 0.001 M EDTA, and 0.6% sodium dodecyl sulfate (SDS) for 16 h at 4°C. The lysed nuclei were centrifuged at 30,000 rpm for 45 min in a Beckman 30 rotor, and the resulting supernatant was also subjected to phenol-chloroform extraction as described.

RNA from cytoplasm or nuclear fractions was subjected to two successive rounds of oligo(dT)-cellulose chromatography (2). The poly(A)-containing RNA was then either added directly to the translation system or subjected to preparative hybridization and/or sucrose gradient sedimentation.

Preparative hybridization. SV40 DNA-Sepharose (100 μg of DNA/ml of Sepharose) was prepared and poly(A)-containing RNA was hybridized to and eluted from the immobilized DNA as previously described by Gilboa et al. (9).

Sucrose gradient sedimentation. Poly(A)-containing RNA or hybridization selected RNA were dissolved in buffer containing 37.5% formamide (Fluka), 0.1% SDS, 0.001 M EDTA, 0.01 M NaCl, 0.01 M Tris-hydrochloride (pH 7.5), and approximately 1,000 to 5,000 cpm of ^{32}P-labeled purified 28S and 18S rRNA markers. After being heated at 40°C for 10 min, the RNA solution was layered onto a 17-ml gradient of 15 to 30% (wt/vol) sucrose in SDS buffer (0.1 M NaCl, 0.01 M Tris [pH 7.5], 0.001 M EDTA, 0.5% SDS). Gradients were centrifuged at 26,000 rpm for 22 h at 20°C in a Beckman SW27.1 rotor.

Translation. Cell-free systems were prepared from wheat germ or reticulocytes. The wheat germ system was essentially that described by Roberts and Paterson (28) with previously described modifications (24). The reticulocyte system was prepared and used as described by Pelham and Jackson (20). In either system, reaction volumes were 25 μl (or multiples thereof), containing 10 to 20 μCi of [^{35}S]methionine, 0.5 to 1 μg of mRNA, and all other components as published.

Immunoprecipitation. Immunoprecipitations were carried out by a modification of a previously described procedure (16). Reaction mixtures (50 μl each) were diluted to 200 μl in a solution containing 0.1 M NaCl, 0.01 M Tris (pH 7.5), and 0.5% SDS. After incubation at 37°C for 30 min, 50 μl of a solution of bovine serum albumin (5 mg/ml) was added, followed by 15 μl of rabbit antiserum prepared against SDS-dissociated SV40 capsid proteins and the incubation continued at 20°C for 1 h. At the end of the incubation period, 50 μl of a 10% suspension of heat-inactivated, formaldehyde-fixed *Staphylococcus aureus* (13) was added, and the suspension was kept at 20°C for a further 30 min. Bacterial pellets containing the adhering immunoglobulin complexes were precipitated by centrifugation at 3,000 × *g* for 2 min and washed four times in 0.5 ml of buffer containing 0.02 M Tris (pH 7.5), 0.15 M NaCl, 0.01 M EDTA, and 0.05% NP40. After the final washing, the pellet was suspended in 50 μl of electrophoresis sample buffer (24) and heated at 100°C for 1 min. The samples were centrifuged at 3,000 × *g* once more, and the supernatants were carefully removed and saved for SDS-polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. Reaction mixtures or immunoprecipitates in electrophoresis sample buffer were heated at 100°C for 1 min and applied to 10 to 20% gradient or 10% simple discontinuous slab polyacrylamide gels prepared as described (26). Electrophoresis was generally carried out for 3 h at 150 V run at constant voltage. Gels were fixed and stained with Coomassie brilliant blue (24) and either autoradiographed directly or fluorographed (4) using Kodak SB-5 film.

Tryptic peptide mapping. Viral capsid polypeptides synthesized in vitro in response to mRNA from SV40-infected cells were immunoprecipitated with anti-SV40 capsid serum and subjected to electrophoresis in 10% polyacrylamide slab gels. Individual polypeptides in the unfixed, dried gel were located by autoradiography. The method for tryptic digestion, as well as two-dimensional electrophoresis and ascending chromatography on thin-layer silica gel plates was as previously described (23).

Materials. Rabbit antisera directed against SDS-dissociated SV40 capsids or gel-purified viral polypeptides were generous gifts from H. Ozer and H. Kasamatsu, respectively. Wheat germ was obtained from the Bar Rav Mills, Israel. [^{35}S]methionine (500 to 1,000 Ci/mol) and [3,6-^{3}H]uridine (40 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, England. Calf liver tRNA was purchased from Boehringer-Mannheim, Germany.

RESULTS

Purification and translation of SV40 mRNA. SV40 does not inhibit the synthesis of host mRNA (17) or proteins (33), and viral mRNA, late in lytic infection, does not comprise more than 10% of the cellular mRNA (22). Therefore, to assess which proteins are encoded by viral mRNA sequences we have used the technique of hybridization selection in which mRNA from infected cells was annealed to SV40 DNA-Sepharose. SV40 DNA immobilized on nitrocellulose filters has proved useful for isolation of viral late mRNA classes (25, 26). More recently, SV40 DNA-Sepharose was shown to have advantages for translation of SV40 late (9) and early (24) mRNA classes. SV40 19S and 16S mRNA's were prepared by hybridization of total mRNA to SV40 DNA-Sepharose followed by sucrose gradient sedimentation of the virus-specific RNA (Fig. 1A). The 19S and 16S fractions were separately pooled, concentrated, and translated in the wheat germ system (Fig. 1B). 16S mRNA directs the synthesis of VP-1 and several small polypeptides. The wheat germ system is known to prematurely terminate polypeptide chains, and at least some of the products synthesized which are smaller than VP-1 may well be the result of this.

19S mRNA directs the cell-free synthesis of

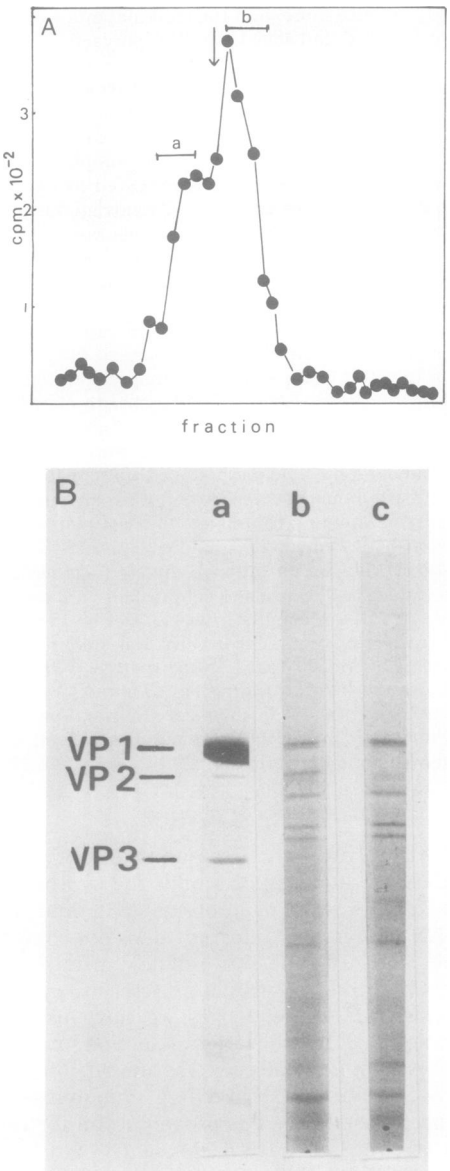

FIG. 1. *(A) Sucrose gradient profile of hybridization-selected RNA. [^{3}H]uridine-labeled SV40 RNA (from 10^8 cells) selected by hybridization to SV40 DNA–Sepharose was subjected to sucrose gradient centrifugation as described in the text. A total of 35 fractions were collected and 25-μl portions were counted by liquid scintillation. The arrow denotes the position of the ^{32}P-labeled 18S rRNA internal marker. The heavier portion of the gradient is on the left; a and b refer to 19S and 16S RNA fractions, respectively, which were separately pooled and precipitated. (B) Cell-free products of SV40 16S and 19S mRNA. The 19S and 16S SV40 RNA pools from the gradient shown in A were each added to a wheat germ translation system which had virtually no endogenous protein-synthesizing activity (not shown). Autoradiograms are of (a) purified [^{35}S]methionine-labeled SV40 virions; (b) products directed by SV40 19S mRNA (1 μg); and (c) products directed by SV40 16S mRNA (1 μg).*

products which comigrate electrophoretically with VP-1 and VP-2. In addition, a small amount of a product which comigrates with VP-3 is occasionally detected among the cell-free products of the 19S peak (Fig. 1B). To more clearly establish the in vitro synthesis of VP-3, we have recently utilized the reticulocyte translation system (20) and immunoprecipitation techniques to identify viral products.

Translation of mRNA fractionated by sucrose gradient sedimentation. The general distribution of viral mRNA into two sedimenting species does not preclude the existence of more than two viral mRNA classes with slight differences in their sedimentation properties. To investigate this possibility, we have fractionated mRNA from infected cells on a sucrose gradient which was collected into a large number (45 to 50) of fractions. Each fraction was precipitated separately and translated in the reticulocyte lysate system. When the products of the mRNA in the individual fractions were analyzed by SDS gel electrophoresis, it was clear that this approach is useful for assessing the size of the mRNA's for a large number of different proteins. The sedimentation profile of total mRNA late in lytic infection is an amorphous bimodal peak, the lighter portion very likely representing the viral 16S class and the rest representing the pool of cellular and viral mRNA, of which the cellular mRNA is probably predominant (Fig. 2A).

Nevertheless, among the fractions translated (no. 16 to 25 of 45 total fractions) it can be seen that the mRNA's of several different polypeptides are quite sharply defined in their sedimentation profile (Fig. 2B). Among these are products which comigrate electrophoretically with both the VP-2 and VP-3 markers, in which both clearly peak in one (and the same) fraction (Fig. 2B; slot e, corresponding to fraction 19). It can also be seen from this figure that the VP-1 mRNA is clearly the most abundant mRNA late in lytic infection. Its great quantity has raised the possibility that VP-1 synthesized in response to 19S mRNA may well be the result of contamination with the far more abundant viral 16S mRNA class. However, closer analysis suggests that this is not the case.

Immunoprecipitation of the cell-free products of mRNA from infected cells. To further identify the viral proteins among the cell-free products of the separate RNA gradient fractions, the protein synthesis reaction mix-

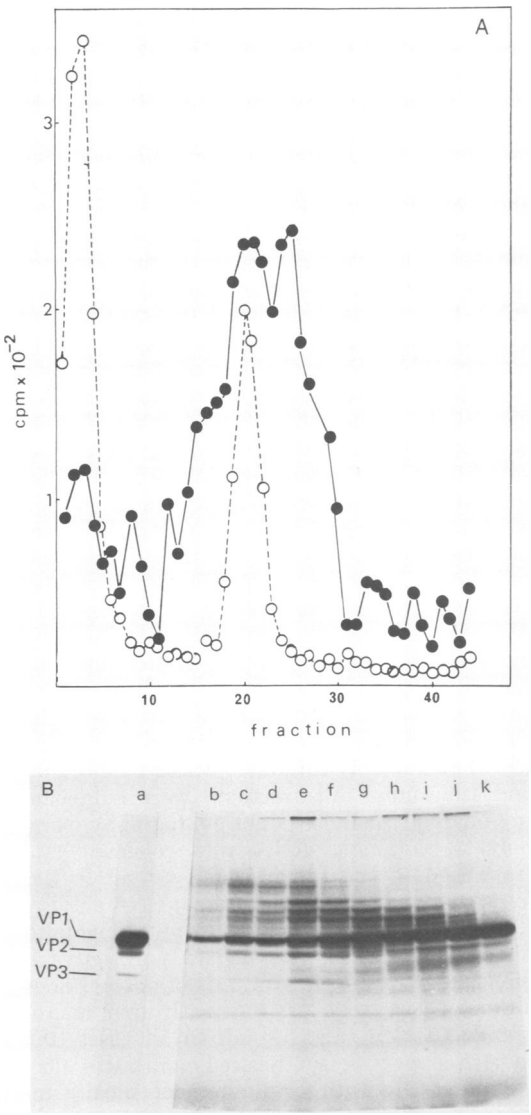

FIG. 2. *(A) Sucrose gradient profile of poly(A)-containing RNA from SV40-infected cells. [^{3}H]uridine-labeled poly(A)-containing mRNA from 10^8 cells was isolated and subjected to sucrose gradient centrifugation as described in the text. A total of 45 fractions were collected, and the positions of the ^{32}P-labeled internal 28S and 18S rRNA markers were determined by direct Cerenkov radiation counting. Portions (10 μl each) of the fractions were counted by liquid scintillation, and the remainder of each fraction was ethanol precipitated with 5 μl of calf liver tRNA as carrier. The heavy portion of the gradient is on the left. (B) Cell-free products of mRNA from individual gradient fractions. RNA fractions in A were precipitated, and one-fifth of each was added to reticulocyte translation system reaction mixtures (50 μl). Aliquots (5 μl each) of each were analyzed directly on 10 to 20% gradient SDS-polyacrylamide gels. Autoradiograms are of (a) [^{35}S]methionine-labeled SV40 virus and (b-k) cell-free products of fractions 16 to 25, respectively. The 18S internal marker peak was in fraction 20 (slot f).*

tures were subjected to immunoprecipitation with anti-SV40 capsid serum (anti-C serum). From Fig. 3A it can be seen that proteins comigrating with VP-1, VP-2, and VP-3 are immunoprecipitated with anti-C serum. Preimmunization control serum did not react with any of the cell-free products. It is interesting that an immunoreactive product whose molecular weight was estimated to be approximately 60,000, by comparison with known marker proteins in the same gel, is reproducibly synthesized in response to mRNA which cosedimented with the mRNA for VP-1.

Densitometer tracings of the different viral polypeptides in the autoradiograms were made, and the amount of each polypeptide was estimated by calculating the area of each peak (Fig. 3B). From the sedimentation profile of the viral protein products, it could be seen that VP-2 and VP-3 both appear to be directed by mRNA which has a defined peak slightly heavier than the 18S mRNA internal marker. Thus, it appeared that these two proteins appeared to be encoded by mRNA species which have very similar sucrose gradient sedimentation properties. It could also be seen that the VP-1 directed by the 19S RNA is not the result of simple contamination by the 16S RNA class. mRNA with a similar size distribution as the VP-2 and VP-3 19S mRNA's appears also to code for VP-1. Possible explanations for this are dealt with below.

Tryptic peptide fingerprints of VP-1, VP-2, and VP-3 synthesized in vitro. It has been reported that SV40 VP-2 and VP-3 share common sequences which differ from those of VP-1 (8, 29). This is consistent with data derived from the known DNA sequences of the late region in which VP-2 and VP-3 have overlapping sequences and are read in a different frame from VP-1 (6). We wished to ascertain that this is also the case for VP-1, VP-2, and VP-3 synthesized in vitro in response to infected cell mRNA. It had been previously shown that VP-1 synthesized in vitro has a similar tryptic fingerprint to virion VP-1 (25, 26, 29) and, similarly, that the tryptic map of VP-2 synthesized in vitro resembles that of virion VP-2 (29; C. Prives, unpublished data). The immunoprecipitated viral polypeptides synthesized in vitro were isolated by polyacrylamide gel electrophoresis, digested with trypsin, and subjected to two-dimensional separation by electrophoresis in the first phase and ascending chromatography in the second. It

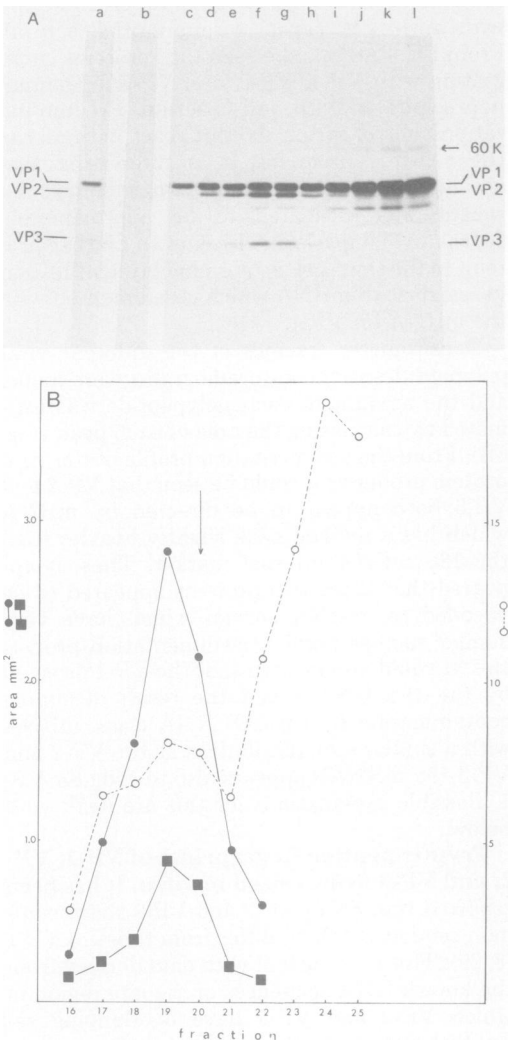

FIG. 3. *(A) Immunoprecipitates of cell-free products of mRNA from sucrose gradient fractions. Translation products from gradient fractions depicted in Fig. 2A were immunoprecipitated with antiserum as described in the text and then subjected to electrophoresis on SDS 10% polyacrylamide slab gels. Autoradiograms are of (a) [^{35}S]methionine-labeled SV40 virus; (b) control rabbit serum immunoprecipitate of products of total mRNA which was subjected to sucrose gradient centrifugation in Fig. 2A; and (c-l) anti-SV40 capsid serum immunoprecipitates of gradient fractions 16 to 25 depicted in Fig. 2A, respectively. (B) Plot of densitometer tracings. Quantitated densitometer tracings of VP-1 (○), VP-2 (●), and VP-3 (■) peaks from autoradiograms of immunoprecipitates in A. Fraction numbers are as described in legend to Fig. 2A. The arrow marks the position of the internal 18S rRNA marker in the gradient.*

could be seen that this observation also extends to the cell-free products (Fig. 4). The VP-1 tryptic digest shows five to seven major [^{35}S]methionine-containing tryptic peptides. VP-2 and VP-3 have very similar fingerprints, both containing two major [^{35}S]methionine-containing peptides. The VP-1 fingerprint is clearly different from those of VP-2 and VP-3.

Cellular localization of SV40 late mRNA classes. The original purpose of these experiments was to assess whether poly(A)-containing RNA from nuclei will direct the synthesis of viral polypeptides. A problem in isolating nuclear mRNA is the probability of contamination with the far more abundant cytoplasmic mRNA classes. To reduce this likelihood, nuclei were washed twice in NP40 extraction buffer and subjected to the detergent wash procedure described by Penman (21). The combined use of a weak ionic detergent with a nonionic detergent has been reported to remove the outer nuclear membrane including a specifically associated class of structures resembling ribosomes (11). This should greatly reduce the likelihood of cytoplasmic contamination associated with the nuclear fraction.

RNA was extracted from the cytoplasm, the washed nuclei, and also the nuclear wash portion which presumably contains nuclear membranes and ribosomes. All fractions were subjected to oligo(dT)-cellulose chromatography, and poly(A)-containing RNA was collected and translated in the reticulocyte system. It was found that, whereas most of the mRNA activity for VP-1, VP-2, and VP-3 is cytoplasmic, both nuclear and nuclear wash mRNA's are active in directing the cell-free synthesis of polypeptides although relatively less efficiently (per microgram of RNA) (Fig. 5A-D). In all cases, the major polypeptide synthesized comigrates electrophoretically with VP-1 and is specifically immunoprecipitated with rabbit anti-SV40 capsid serum. The pattern of polypeptides synthesized was similar in all cases with one clear exception; the nuclear wash fraction mRNA directed the synthesis of proportionally much more VP-2 than the nuclear portion or the cytoplasmic portion. Immunoprecipitation of the products of the three types of mRNA with anticapsid serum yielded confirmatory results (Fig. 5E-H). The autoradiograms of the immunoprecipitates were subjected to densitometer analysis, and the areas of the peaks corresponding to VP-1, VP-2, and VP-3 were estimated. Table 1 shows the relative quantities of VP-1 and VP-2 synthesized by the three fractions and their ratios. The VP-2-to-VP-1 ratio in the nuclear wash fractions was considerably greater than that in the nuclei or

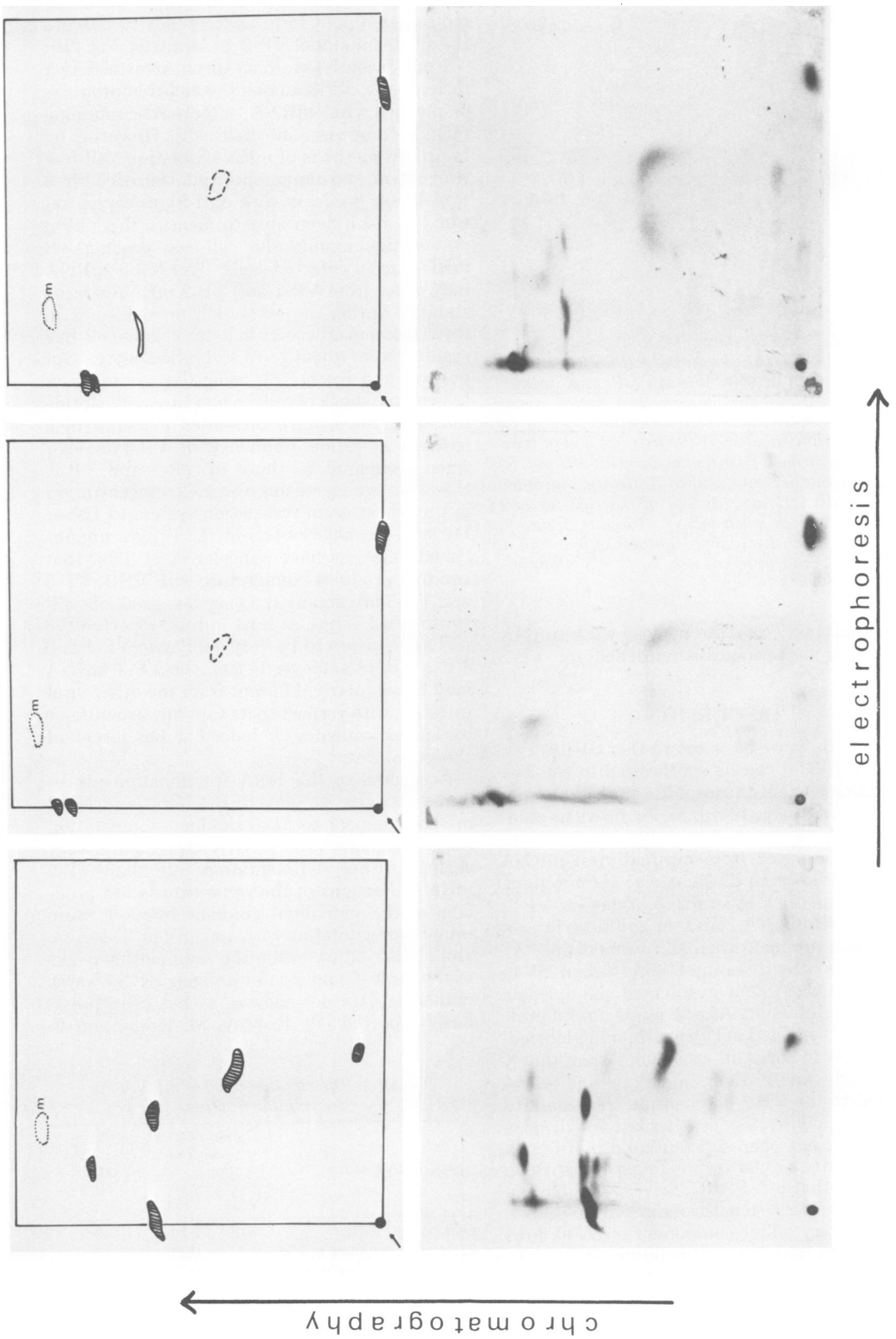

FIG. 4. *Tryptic fingerprints of VP-1, VP-2, and VP-3 synthesized in vitro. Autoradiograms of two-dimensional tryptic peptide maps of purified VP-1, VP-3, and VP-2 from left to right, respectively. The diagrams at the top are tracings of the autoradiograms below. The "m" spot denotes the position of the internal dinitrophenyl-lysine marker.*

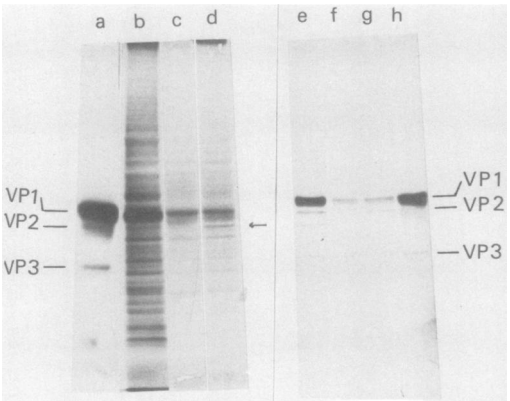

FIG. 5. *Cell-free products of mRNA from different cell fractions. Poly(A)-containing mRNA from different cell fractions of SV40-infected cells was isolated and translated in the reticulocyte translation system (50-μl reaction volume). Autoradiograms are of (a) [^{35}S]methionine-labeled SV40 virus; (b) 5 μl of reaction mixture primed by cytoplasmic mRNA (1 μg); (c) 5 μl of reaction mixture primed by detergent-treated nuclear mRNA (1 μg); (d) 5 μl of reaction mixture primed by nuclear wash mRNA (1 μg). (e, f, and g) Anti-SV40 capsid immunoprecipitates of b, c, and d, respectively, on a different gel; (h) [^{35}S]methionine-labeled SV40 virus.*

the cytoplasm. Thus, the nuclear wash mRNA appears to be specifically enriched for VP-2 mRNA.

DISCUSSION

In this study, we have shown that SV40 VP-1, VP-2, and VP-3 can be synthesized in cell-free translation systems in response to mRNA from SV40-infected cells. In this and in previous studies (25), the SV40 16S and 19S species were separately isolated from purified viral mRNA and each translated in the wheat germ system. It was found that 16S mRNA codes for VP-1, whereas 19S mRNA codes, in addition, for another viral product, originally termed the "X" polypeptide which was not observed in SV40 virions but was found in infected cell extracts late in lytic infection. After a more careful purification and analysis of [^{35}S]methionine-labeled, purified SV40 virus it was found that the X polypeptide was present in virions and corresponded to the VP-2 polypeptide. The identity of the X polypeptide synthesized in vitro as virion VP-2 has been substantiated by the fact that they have similar tryptic fingerprints which differ from that of VP-1 (C. Prives, unpublished data). As can be seen in the figures in this paper, VP-2 is a very minor component of SV40. Furthermore, we have repeatedly observed that VP-1 has a tendency to be converted from the 45,000 molecular weight form to a 40,000 to 42,000 molecular weight form which tends to obscure the small amount of VP-2 present (e.g., Fig. 2B).

Until recently, we have been able to detect little, if any, VP-3 among the cell-free products of purified viral mRNA or poly(A)-containing mRNA from virus-infected cells. However, by improved methods of mRNA isolation, cell-free translation, and immunoprecipitation of cell-free translation products with anti-SV40 capsid serum, we have been able to identify this virion polypeptide among the cell-free products of mRNA from infected cells. The VP-3 mRNA may differ from VP-1 and VP-2 mRNA's in its stability during the extraction procedure or in its translation efficiency in heterologous cell-free systems from wheat germ and reticulocytes. Opperman and Koch (18), using the technique of hypertonic shock of cells to measure polypeptide initiation rates, have presented evidence of a reduced initiation frequency of VP-3 in vivo when compared to those of VP-1 and VP-2. However, by increasing the KCl concentration in the wheat germ translation system to 120 or 140 mM we have observed (C. Prives, unpublished data), as have Wheeler et al. (35), that cell-free products comigrating with VP-1, VP-2 and VP-3 are among the very few products still synthesized. Thus, at least in this respect, VP-3 mRNA appears to be very similar to VP-1 and VP-2 mRNA's. Nevertheless, the VP-3 mRNA may be singularly different from the other viral mRNA's with respect to its capping structure or its leader sequence, if indeed it has either of these features.

According to the RNA fractionation procedures we have employed, the VP-3 and VP-2 mRNA's appear to have similar sedimentation rates, although VP-3 mRNA may have a very slightly slower sedimentation rate than VP-2 mRNA. Portions of the same translation products of the individual gradient fractions were immunoprecipitated with anti-SV40 T serum, and a clear difference in the sedimenting peaks of the large T and small t antigen mRNA's was obtained (data not shown), as has been previously reported (19; E. May, M. Kress, and P.

TABLE 1. *Distribution of viral mRNA's in subcellular fractions*

mRNA source	Capsid polypeptide area (mm^2)		VP-2/VP-1 ratio ($\times 10^2$)
	VP-1	VP-2	
Cytoplasm	9.09	0.81	9
Detergent-washed nuclei	1.11	0.11	10
Nuclear wash fraction	1.22	0.35	29

May, Nucleic Acids Res., in press). Thus this RNA gradient fractionation is sensitive enough to detect differences of approximately 250 nucleotides, the putative size difference between the two early mRNA's (3). Barring conformational differences of the VP-2 and VP-3 mRNA's not detected in these nondenaturing conditions, the two major capsid mRNA's are very close in size.

In polyoma, there are three major L-strand mRNA classes with sedimentation coefficients of approximately 16S, 18S, and 19S which code for VP-1, VP-3, and VP-2, respectively (12, 30). Our data suggest that there is not an analogous 18S SV40 VP-3 mRNA. That there are only two major size classes of SV40 L-strand-specific mRNA as originally described by Weinberg et al. (34) has recently been confirmed by using more refined techniques. Bratosin et al. (5), upon examination of SV40 mRNA's by R-loop electron microscopy, have demonstrated that there are two major species of L-strand-specific cytoplasmic mRNA's with clearly defined positions of their leader and body sequences on the viral genome. Lai et al. (14), using S-1 nuclease analysis, have also shown that there are 16S and 19S body classes with some heterogeneity in the sequences of the leader position of the 16S and 19S RNAs. However, size variations in the leader sequences are not sufficient to affect the sedimentation properties of either of the two RNA classes. Furthermore, S-1 analysis of individual sucrose gradient fractions using similar sedimentation conditions to those we have used for the experiments described herein have again shown that there are only two major size classes of L-strand mRNA (C.-J. Lai, personal communication). These observations and our own findings raise the question of how the VP-3 is initiated internally at least 300 nucleotides from the 5′ terminus of the 19S mRNA molecule. If more than one 19S mRNA exists, what differences in their structures are responsible for altering the accessibilities of their initiation sites?

According to our experiments, VP-1 appears to be synthesized in response to 19S mRNA as well as to 16S mRNA. There are several possible explanations for this. (i) VP-1 is initiated internally on the 19S mRNA (which of course could also account for the synthesis of VP-3 in response to the 19S size class of viral mRNA). However, there are clear demonstrations in other virus systems, notably polyoma, which argue against this. After sucrose gradient sedimentation under RNA-denaturing conditions, polyoma 19S mRNA codes, in the main, for VP-2, despite the fact that, as with its SV40 counterpart, it contains VP-1 and VP-3 coding sequences (30, 31). (ii) SV40 16S mRNA has an unusual association with SV40 19S mRNA resulting in a portion of it cosedimenting with the larger species. This is unlikely, because repeated attempts to isolate 19S mRNA free of VP-1 coding properties after denaturing formamide sucrose gradients have failed (C. Prives, unpublished data). (iii) There are three 19S mRNA species, each coding for a specific viral polypeptide. If this is the case, it will be discernible only by direct sequence analysis of 19S mRNA, because most RNA size techniques do not discriminate between different molecules in the 19S size class. However, heterogeneity of SV40 19S mRNA leader sequences has been demonstrated (14) which could result in different coding properties of 19S mRNA's. (iv) The SV40 19S mRNA which is known to be rather unstable may be converted to a form which has an accessible initiation site for VP-1 during the translation process. We consider this a likely possibility because when we isolated cytoplasmic RNA which sedimented more rapidly than the 18S rRNA marker and subjected it to a second sucrose gradient centrifugation followed by translation of the individual fractions as performed in Fig. 3, most of the VP-1 mRNA activity resediments in a heterogeneous peak of less than 18S. This suggests that the VP-1 mRNA activity in this region of the gradient is not an intact 19S mRNA molecule. Experiments to test this on the comparative kinetics of VP-1 and VP-2 synthesis in response to 19S mRNA are underway.

Studies to ascertain the subcellular distribution of viral mRNA's have yielded an interesting observation. It appears that mRNA coding for VP-1 can be isolated from both nuclear wash and nuclear fractions and that the nuclear wash RNA in addition is particularly enriched for VP-2 mRNA. Penman (21) has described a technique for specifically removing cytoplasmic tabs and unbroken cells from preparations of nuclei by the combined use of ionic and nonionic detergents. Electron microscopic studies (11) showed that this procedure removes the outer nuclear membrane and a class of associated ribosomes. That mRNA isolated from this fraction was particularly enriched for VP-2 mRNA could indicate that this viral RNA species has a special affinity for membrane-bound ribosomes. Although the exact position of the VP-2 on the viral genome has not yet been determined by amino-terminal sequence analysis, the SV40 DNA sequence (7, 27) has yielded a very likely position of the initiation ATG codon for this polypeptide (36). If the deduced sequence is correct, then the N-terminal portion of VP-2 is unusually hydrophobic, being exceptionally rich in alanine and lacking basic residues in the first 98 amino acids. This suggests a possible inter-

action of VP-2 with membranes which may well be the explanation for the enrichment of VP-2 mRNA in the nuclear wash fraction.

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