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Characterization of the mRNA's for the Polyoma Virus Capsid Proteins VP1, VP2, and VP3

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Polyadenylated cytoplasmic RNA from polyoma virus-infected cells can be translated in the wheat germ system to yield all three polyoma virus capsid proteins, VP1, VP2, and VP3. The translation products of RNA selected from total cytoplasmic RNA of infected cells by hybridization to polyoma virus DNA showed a high degree of enrichment for VP1, VP2, and VP3. The identity of the in vitro products with authentic virion proteins was established in two ways. First, tryptic peptide maps of the in vitro products were found to be essentially identical to those of their in vivo counterparts. Second, the mobilities of the in vitro products on two-dimensional gels were the same as those of viral proteins labeled in vivo. VP1, VP2, and VP3 were all labeled with [^{35}S]formylmethionine when they were synthesized in the presence of [^{35}S]formylmethionyl-tRNA$_f^{met}$. We determined the sizes of the polyadenylated mRNA's for VP1, VP2, and VP3 by fractionation on gels. The sizes of the major mRNA species for the capsid proteins are as follows: VP2, 8.5×10^5 daltons; VP3, 7.4×10^5 daltons; and VP1, 4.6×10^5 daltons. We conclude that all three viral capsid proteins are synthesized independently in vitro, that all three viral capsid proteins are virally coded, and that each of the capsid proteins has a discrete mRNA.

For both simian virus 40 (SV40) and polyoma virus it has been shown that the genetic information for the capsid proteins is only expressed late in infection after the onset of viral DNA synthesis (8, 11). Several lines of evidence (15, 23, 26, 32, 34) indicate that the genes for the capsid proteins comprise approximately half the genome and are organized in tandem as a single unit, such that the cistron for the minor capsid proteins is to the 5′ side of that for the major capsid protein. The minor capsid proteins of both SV40 and polyoma virus are organized such that VP2 completely overlaps VP3, with the proteins sharing a common C-terminal region (6, 9, 13, 17, 32, 34). The major capsid protein, VP1, does not share any amino acid sequences with VP2 or VP3 (9, 13, 14, 17, 32), although in the case of SV40 the 3′ end of the gene for VP2 and VP3 overlaps the 5′ terminal region of the VP1 gene by about 120 nucleotides, albeit in a different reading frame (10, 31).

The major species of late virus-specific mRNA found in the cytoplasm of papova virus-infected cells are 19S and 16S in size, with 16S being the predominant species (1, 22, 36). The 19S species corresponds to an almost complete transcript of the late region, whereas the 16S species maps in the distal half of the late region (1, 22, 35). The exact site of initiation of late transcription on the genome has not been determined, but the primary transcripts of the late region in the nucleus appear to be RNA molecules which are at least as long as a complete copy of the entire late strand (1). These long transcripts are subsequently processed in the nucleus to yield RNA molecules predominantly of the 19S size class. It has recently been shown that both the 19S and 16S mRNA's of SV40 have 100 to 200 base leader sequences on their 5′ ends which come from a region to the 5′ side of the minor capsid protein cistron (3). Thus, in the mature 19S mRNA some sequences between the leader and the coding region for VP2 are absent (C.-J. Lai, R. Dhar, and G. Khoury, Cell, in press). The SV40 16S mRNA may be generated from the 19S mRNA by a cytoplasmic processing event (4). Overlapping species of virus-specific 19S and 16S cytoplasmic RNAs have been observed in polyoma virus-infected cells and have been mapped in positions analogous to those of their SV40 counterparts (22). So far it has not been determined whether polyoma virus mRNA's have leader sequences, but it seems likely that this will be the case. For both polyoma virus and SV40, it has been shown that the 16S mRNA programs the synthesis of VP1 (34), while the SV40 19S mRNA has been shown to code for a protein,

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VPX, which is probably identical to VP2 (30). To date, the mRNA activity for VP3 of neither virus has been identified. We report here that all three capsid proteins of polyoma virus can be synthesized in vitro and that each capsid protein is coded for by a separate mRNA.

MATERIALS AND METHODS

Virus stocks and cell culture. Wild-type polyoma virus (stock 725) and a clone of Swiss 3T6 cells were used for all experiments as previously described (15).

Isolation of RNA. Cytoplasmic RNA was isolated from infected cell cultures and fractionated into polyadenylate [poly(A)]-containing and poly(A)-deficient classes by oligodeoxythymidylic acid-cellulose chromatography as described (15). For these experiments, cells infected with wild-type polyoma virus at a multiplicity of about 20 PFU/cell were harvested 60 to 70 h postinfection at 37°C.

Translation RNA. Poly(A)-containing and poly(A)-deficient RNAs were translated in the wheat germ system as previously described (20), with the use of [^{35}S]methionine (New England Nuclear Corp., NEG 009H; specific activity, >300 Ci/mmol) at a final concentration of 250 μCi/ml or a uniformly ^{14}C-labeled amino acid mixture (Amersham Searle, CFB 104, specific activity 54 mCi/matom) at 50 μCi/ml. The final concentration of poly(A)-containing RNA was 100 μg/ml, and that of poly(A)-deficient RNA or total cytoplasmic RNA was 375 μg/ml.

Selection of virus-specific RNA. Total cytoplasmic RNA was annealed to polyoma virus DNA covalently coupled to aminoethyl-cellulose (27) at a final concentration of 5 to 10 mg/ml in 50% formamide, 0.6 M NaCl, 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminomethane-sulfonic acid, pH 7.5, 1 mM EDTA, 0.2% sodium dodecyl sulfate (SDS) at 37°C for 3 h with continuous agitation. In a typical experiment, after heating to 80°C for 1 min, 1 mg of RNA was hybridized at 5 mg/ml to 2 mg of DNA cellulose, equivalent to 100 μg of sonically disrupted polyoma virus DNA. After hybridization, the DNA cellulose was pelleted by centrifugation for 2 min at 37°C in a microcentrifuge, and the supernatant fluid was aspirated. The pellet was resuspended and washed for 30 min in 1 ml of hybridization buffer. This procedure was repeated twice more before elution. The polyoma virus-specific RNA was eluted from the pelleted DNA cellulose with two successive 0.25-ml washes of 98% formamide–0.1% SDS by heating to 60°C for 30 s. The two elution supernatant fluids were pooled and made 0.2 M in potassium acetate, pH 5.2, and the RNA was precipitated at −70°C with 20 μg of calf liver tRNA carrier by addition of 1 ml of ethanol. The RNA was collected by centrifugation, reprecipitated, and finally lyophilized from 200 μl of water. The lyophilized RNA was taken up in 20 μl of water and stored in liquid nitrogen. One-twentieth of this RNA was used in each 10-μl translation reaction. Where indicated, this virus-specific RNA was fractionated by oligodeoxythymidylic acid-cellulose chromatography, and one-twentieth of both the poly(A)-containing and the poly(A)-deficient classes was translated.

Size fractionation of RNA. RNA was fractionated on 2% acrylamide-0.5% agarose gels exactly as described by Hunter and Garrels (21). In the experiment shown in Fig. 7A, 50 μg of poly(A)-containing RNA from infected cells was run in two adjacent tracks for 4.5 h at 50 V; in that shown in Fig. 7B, 25 μg was run in a single track for 6 h at 50 V. In the experiment in Fig. 8, polyoma virus-specific RNA was selected from 3 mg of infected-cell cytoplasmic RNA and fractionated into poly(A)-containing and poly(A)-deficient classes, and each class was run in a separate track for 6 h at 50 V. The tracks were cut into 2.5-mm slices, and the poly(A)-containing RNAs were recovered from the slices as described (21). Poly(A)-deficient RNA was extracted from gel slices in identical fashion, but was isolated as follows from the aqueous phase by chromatography on cellulose instead of oligodeoxythymidylic acid-cellulose. The aqueous phase following chloroform extraction was made 35% in ethanol and passed over 0.15-ml cellulose (Whatman CF 11) columns equilibrated with 35% ethanol–65% 0.1 M NaCl–0.05 M Tris-chloride, pH 7.5–5 mM EDTA. The columns were washed with 2 ml of this buffer, and the RNA was eluted with 0.5 ml of the same buffer without ethanol. The RNA was precipitated and prepared for translation as described (21).

Polyacrylamide gel electrophoresis. The techniques used for analytical and preparative SDS-polyacrylamide gel electrophoresis were as described (13). Two-dimensional gel electrophoresis was carried out as follows. Samples were prepared by heating to 100°C for 3 min in the presence of 2% SDS, 10% 2-mercaptoethanol, 0.05 M Tris-chloride, pH 7.0, and 10% glycerol. Once cooled, 20 μl of this material was combined with 36 mg of urea, 5 μl of pH 8-10 ampholines (40% solution, wt/vol), and 20 μl of a solution containing 8% Nonidet P-40 and 10% 2-mercaptoethanol (vol/vol in water). The basic two-dimensional gel technique used was that of Garrels and Gibson (12), but the following modifications were introduced for these studies: (i) electrofocusing gels were cross-linked with diallyltartardiamide in the ratio 1:5.7 (diallytartardiamide-acrylamide), as suggested by Baumann and Chrambach (5), and (ii) electrofocusing gels contained 2% pH 6-8 and 2% pH 3-10 ampholines. After electrofocusing, the second-dimension SDS-polyacrylamide gels were run, stained, and processed for fluorography as described by Laskey and Mills (24). Fluorograms were photographed with the use of a contrast-enhancing system to be described elsewhere (W. Gibson, manuscript in preparation).

Tryptic peptide mapping. Tryptic peptide mapping was carried out essentially as described by Deppert and Walter (7). For the peptide maps shown in Fig. 3, a 200-μl in vitro reaction programmed with polyoma virus-specific RNA was run on a 12% polyacrylamide–0.5% diallyltartardiamide cross-linked slab gel 2 mm thick. For the in vivo-labeled proteins, the nuclei from two 9-cm plates of polyoma virus-infected 3T6 cells labeled with 1 μCi of [^{35}S]methionine per ml in methionine-free medium from 24 to 36 h postinfection at 37°C were taken up in 500 μl of solubilizing buffer, sonically treated, and run on a 2 mm thick 12% acrylamide–0.32% bisacrylamide cross-linked slab gel. The protein bands were extracted from the gels after autoradiography, as described (7). The VP1 bands

were processed directly for tryptic digestion. The VP2 and VP3 bands from the preparative gels of both the in vivo- and the in vitro-labeled proteins were repurified, after several cycles of lyophilization to remove NH_4HCO_3, by electrophoresis on a 12.5% acrylamide–0.15% bisacrylamide cross-linked slab gel. The isolated proteins were subjected to tryptic digestion and two-dimensional separation essentially as described (13). For the peptide maps in Fig. 4, a 100-μl in vitro reaction, programmed with poly(A)-containing RNA from polyoma virus-infected cells, was labeled with a mixture of ^{14}C-labeled amino acids and subjected to electrophoresis on a 12% acrylamide–0.5% diallyltartardiamide cross-linked gel with the inclusion of viral capsid proteins as internal markers. For the in vivo-labeled VP1, the nuclei from two 9-cm plates of polyoma virus-infected 3T6 cells, labeled with 3 μCi of a ^{14}C-labeled amino acid mixture per ml for 12 h at 36 h postinfection when the cytopathic effect was apparent, were used as discussed above. The VP1 band was cut out of the stained gel and treated as described (13).

RESULTS

Translation of infected-cell RNA. Total cytoplasmic RNA from polyoma virus-infected cultures late in infection and mock-infected cultures was fractionated into poly(A)-containing and poly(A)-deficient classes by chromatography on oligodeoxythymidylic acid-cellulose. The two RNA classes were translated in the wheat germ system in the presence of [^{35}S]methionine, and the products were analyzed by SDS-polyacrylamide gel electrophoresis as shown in Fig. 1. Comparison of the in vitro products from infected-cell poly(A)-containing RNA (track C) with marker viral proteins (track A) shows that proteins comigrating with the polyoma virus capsid proteins VP1, VP2, and VP3 were synthesized, although the band comigrating with VP3 is not clearly resolved from a host protein. These proteins are absent from the in vitro product from mock-infected cell poly(A)-containing RNA (track B), which suggests that they are of viral origin. Although most of the total mRNA activity of the cytoplasm was found in the poly(A)-containing RNA class, translation of the poly(A)-deficient RNA yielded a small number of proteins (tracks D and E). We have identified the major protein migrating slightly faster than VP1 as actin and have discussed the nature of the poly(A)-deficient RNA coding for actin at length (21). The proteins running near the front of the gel are probably histones, since it is well documented that histone mRNA's lack poly(A) (2). Track E shows that some fraction of the mRNA for the proteins comigrating with VP2 and VP3 appears to be poly(A) deficient. There is rather little poly(A)-deficient mRNA activity for the protein comigrating with VP1.

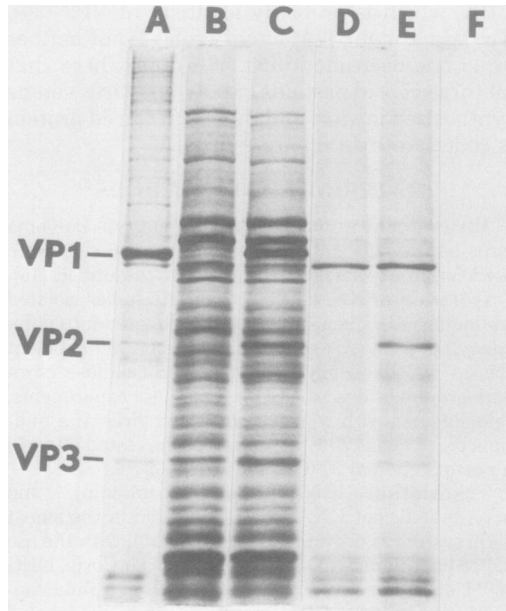


FIG. 1. *In vitro translation of cytoplasmic RNAs from infected and uninfected cells. Cytoplasmic RNA was isolated from polyoma virus-infected and mock-infected cells, fractionated into poly(A)-containing and poly(A)-deficient classes, and translated in the wheat germ system in the presence of [^{35}S]methionine, all as described in Materials and Methods. A 1-μl amount of each in vitro reaction was solubilized and run on a 14% acrylamide–0.37% bisacrylamide gel. The gel was fixed, dried, and autoradiographed. Shown here are (A) ^{14}C-amino acid-labeled marker proteins from polyoma virus-infected cell nuclei, and products of in vitro translation reactions containing: (B) poly(A)-containing RNA from mock-infected cells, (C) poly(A)-containing RNA from infected cells, (D) poly(A)-deficient RNA from mock-infected cells, (E) poly(A)-deficient RNA from infected cells, and (F) no added RNA.*

Translation of polyoma virus selected RNA. To prove that the proteins comigrating with VP1, VP2, and VP3 were indeed virally coded, we selected polyoma virus-specific mRNA's by hybridization of total cytoplasmic RNA to polyoma virus DNA covalently coupled to cellulose (27). The selected RNA was translated in the wheat germ system, and the gel analysis of these products is shown alongside the translation products from total infected-cell cytoplasmic RNA (Fig. 2). As indicated by the marker track (track A), the product from RNA selected from infected-cell cytoplasmic cell RNA (track C) is highly enriched for the proteins comigrating with VP1, VP2, and VP3 by comparison with the product of unselected RNA (track B). Another band lying between VP2 and VP3 is also accentuated in the product of selected RNA, and this protein is almost certainly

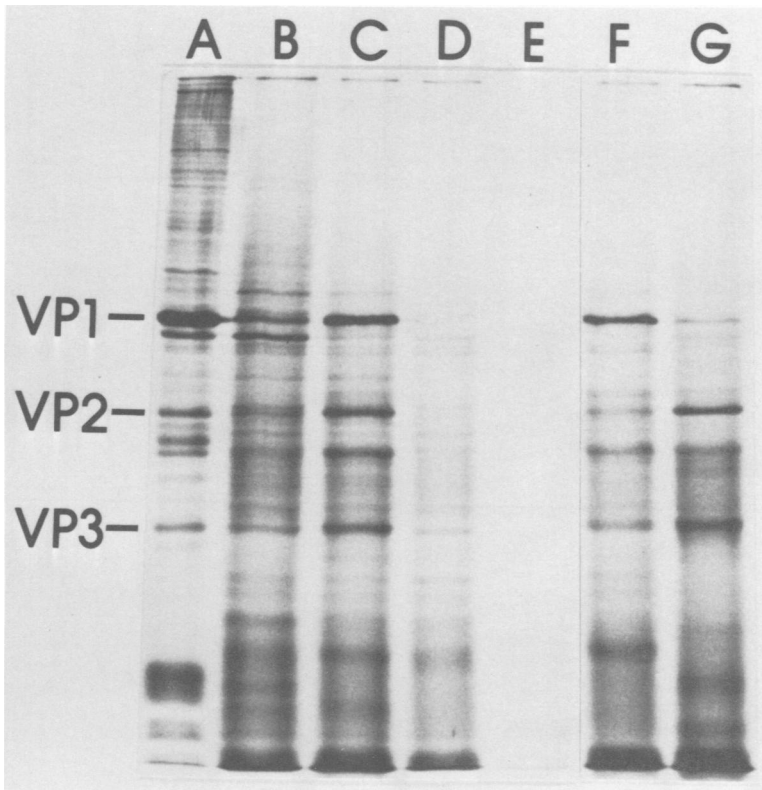
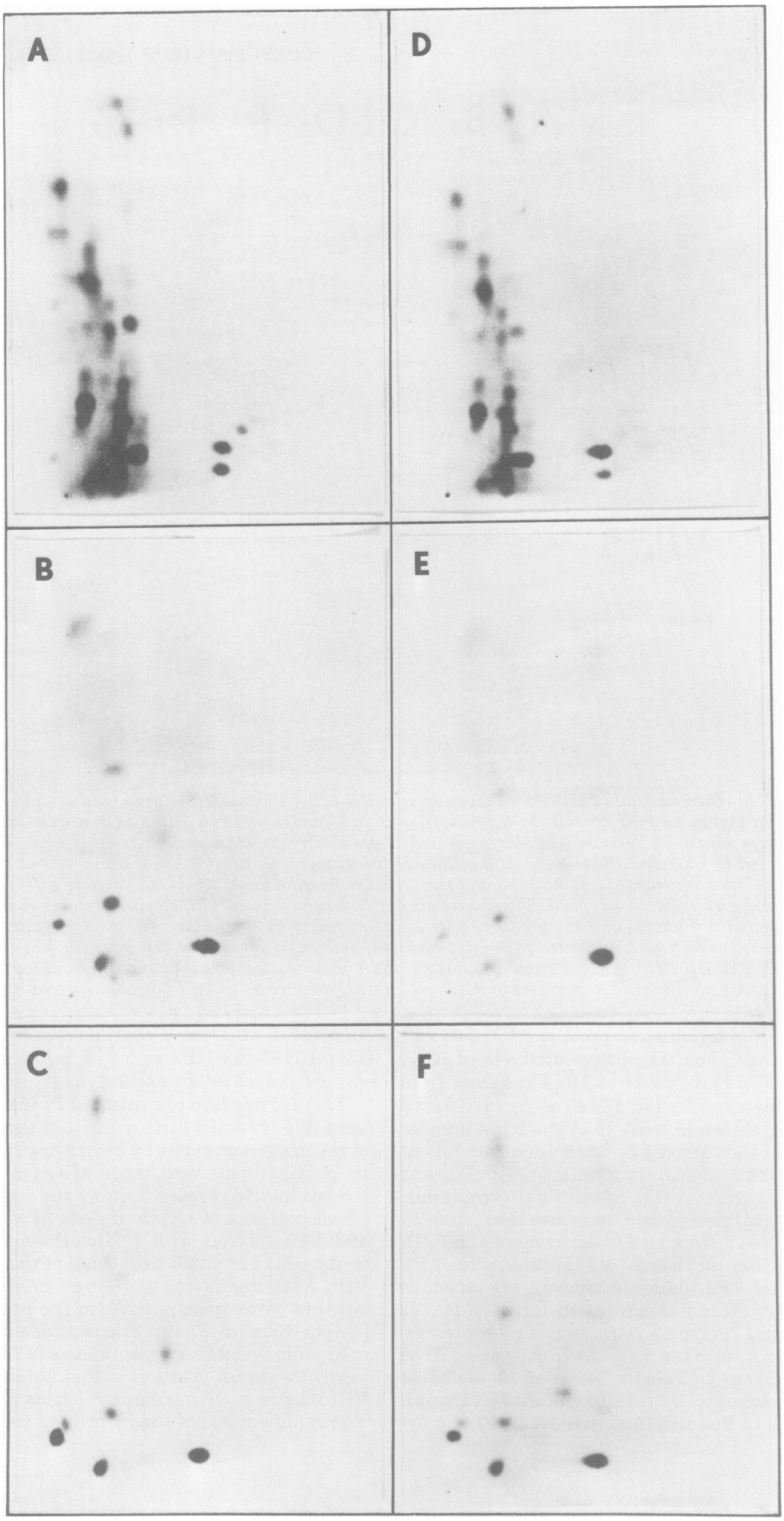


FIG. 2. *In vitro translation of polyoma virus-specific RNA. RNA was selected from total cytoplasmic RNA of infected and mock-infected cells by hybridization to polyoma virus DNA cellulose and translated in the wheat germ system as described in Materials and Methods. Where indicated, the virus-specific RNA was fractionated by oligodeoxythymidylic acid-cellulose chromatography prior to translation. A 2-μl amount of each translation reaction was solubilized and run on a 12% acrylamide–0.32% bisacrylamide gel. The gel was fixed, dried, and autoradiographed. Shown here are (A) ^{14}C-amino acid-labeled marker proteins from polyoma virus-infected cell nuclei, and the products of translation reactions containing: (B) total cytoplasmic RNA from infected cells, (C) RNA selected from cytoplasmic RNA from polyoma virus-infected cells (polyoma virus-specific RNA), (D) RNA selected from cytoplasmic RNA from mock-infected cells, (E) no added RNA, (F) polyoma virus-specific poly(A)-containing RNA, and (G) polyoma virus-specific poly(A)-deficient RNA.*

a premature termination product related to VP1 (see below). The translation products of RNA selected on polyoma virus DNA cellulose from the total cytoplasmic RNA of mock-infected cells are shown in track D. Although a number of faint bands are visible, there is no enrichment for proteins comigrating with VP1, VP2, and VP3. Selection of RNA from total cytoplasmic RNA from infected cells on mouse DNA coupled to cellulose did not lead to enrichment of mRNA activity for the bands comigrating with VP1, VP2, and VP3 (data not shown). We conclude that these proteins are coded for by mRNA's containing polyoma virus-specific sequences. Fractionation of the polyoma virus-specific RNA into poly(A)-containing and poly(A)-deficient classes, followed by translation, showed that almost all of the mRNA activity for VP1 is polyadenylated, whereas a considerable fraction of the mRNA's for VP2 and VP3 isolated in this way are poly(A)-deficient (tracks F and G).

Tryptic peptide mapping of in vitro products. We obtained further proof of the identity of the in vitro products by comparing their tryptic peptide maps with those of virion proteins labeled in vivo. Figure 3 shows the two-dimensional patterns of tryptic digests of VP1, VP2, and VP3 labeled with [^{35}S]methionine in infected cells compared with those of the putative VP1, VP2, and VP3 synthesized in vitro from polyoma virus-specific RNA in the presence of [^{35}S]methionine. The tryptic peptide map of the methionine-containing peptides of VP1 labeled in vitro is almost identical to that obtained from VP1 labeled in vivo (compare panels A and D). The completeness of the identity of the in vitro


A
D
B
E
C
F

product with VP1 is evident when the full array of tryptic peptides is examined by use of proteins labeled with a mixture of ^{14}C-labeled amino acids (Fig. 4). The peptide maps of the putative VP2 and VP3 synthesized in vitro are almost identical to their in vivo counterparts (compare panel B with panel E and panel C with panel F). We can conclude that these two proteins are related to the minor capsid proteins, but, given the lack of methionine-containing peptides unique to VP2, we cannot be certain that they are identical to VP2 and VP3.

Two-dimensional gel analysis of in vitro products. To establish the identity of the in vitro products with VP2 and VP3, we analyzed the translation products by separation in two-dimensional gels. Figure 5 shows a comparison of proteins synthesized in vitro with those synthesized in vivo. Five predominant labeled proteins were found in the nuclear fraction of polyoma virus-infected cells by this procedure (panel A). Two of these (actin and "x") were also present in the nuclear fraction of mock-infected cells (panel B). We have designated the other three predominant proteins in the infected-cell preparation as VP1, VP2, and VP3 on the basis of the following criteria: (i) none is present in mock-infected cells; (ii) the relative positions of each of these spots are appropriately shifted when *ts*59-infected cells are similarly analyzed (e.g., *ts*59 VP1 has a lower molecular weight and *ts*59 VP2 and VP3 both have slightly

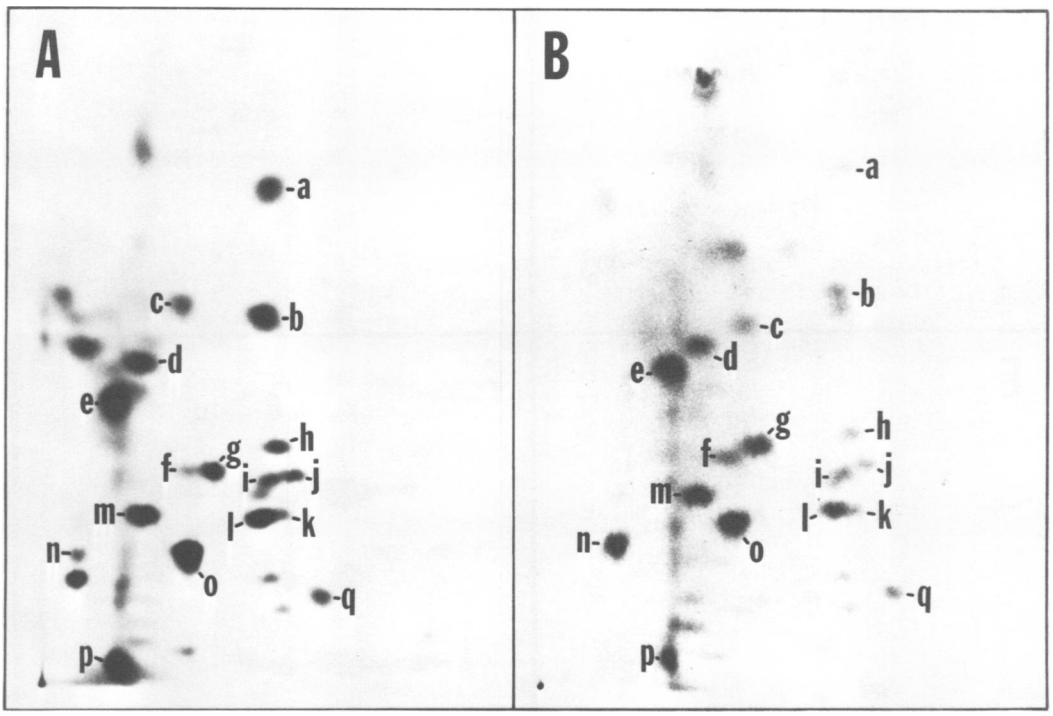


FIG. 4. *Tryptic peptide maps of ^{14}C-amino acid-labeled VP1 synthesized in vivo and in vitro. Tryptic digests were prepared from VP1 labeled with a ^{14}C-amino acid mixture either in infected cells or by translation of poly(A)-containing RNA from polyoma virus-infected cells in the wheat germ system as described in Materials and Methods. The digests were separated in two dimensions as described in the legend to Fig. 5. (A) VP1 labeled in vivo, and (B) VP1 labeled in vitro.*

FIG. 3. *Tryptic peptide maps of [^{35}S]methionine-labeled polyoma capsid proteins synthesized in vivo and in vitro. Tryptic digests were prepared from VP1, VP2, and VP3 labeled with [^{35}S]methionine either in infected cells or by translation of polyoma virus-specific RNA in the wheat germ system as described in Materials and Methods. The digests were separated in two dimensions on thin-layer cellulose plates. In the autoradiograms shown, the origin is in the lower left-hand corner. Electrophoresis was from left to right toward the cathode, and chromatographic development was from the bottom to the top. (A) VP1 labeled in vivo, (B) VP2 labeled in vivo, (C) VP3 labeled in vivo, (D) VP1 synthesized in vitro, (E) VP2 synthesized in vitro, and (F) VP3 synthesized in vitro.*

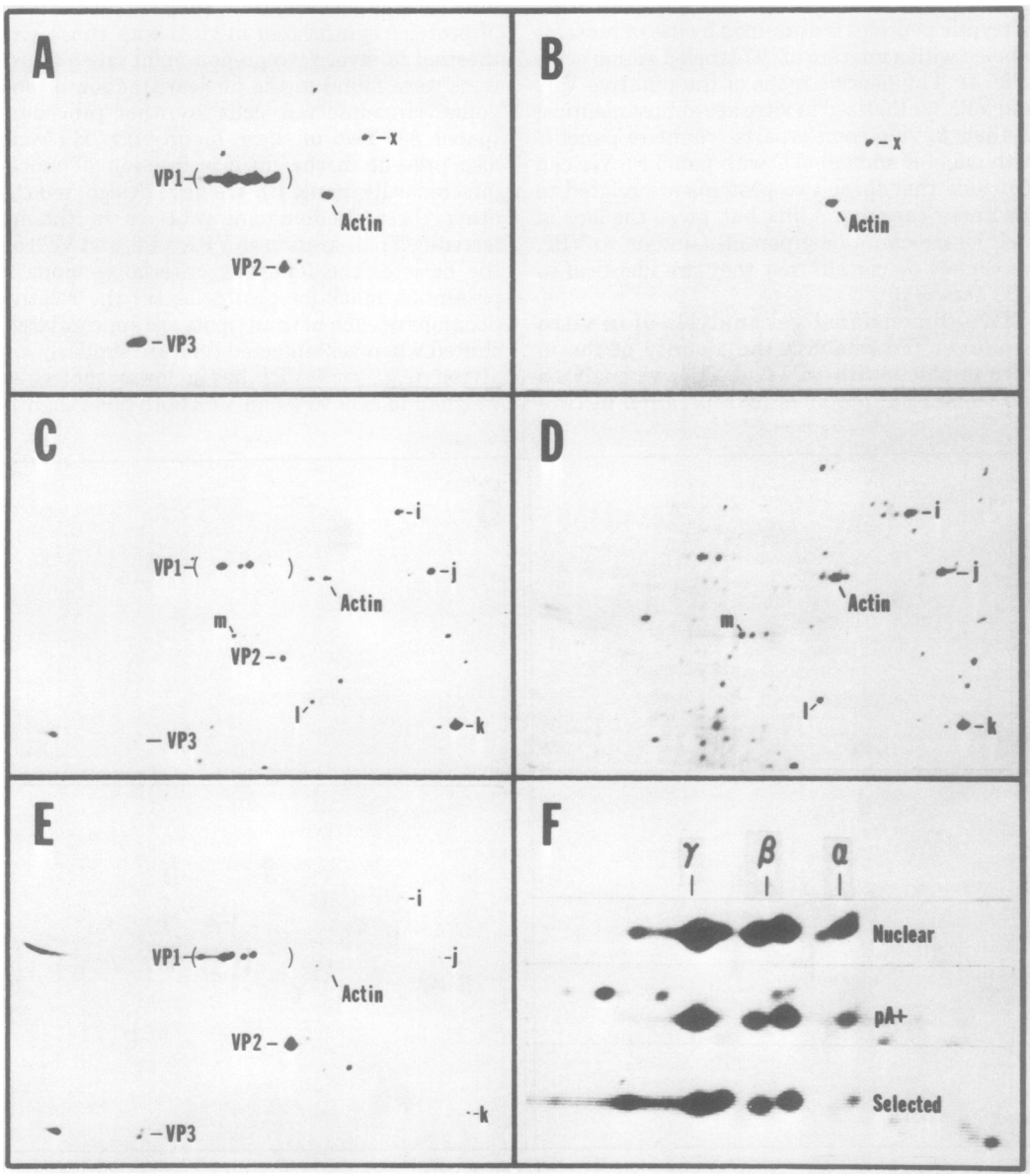


FIG. 5. *Two-dimensional gel analysis of polyoma virus capsid proteins synthesized in vivo and in vitro. Samples were analyzed on two-dimensional gels as described in Materials and Methods. The samples in A and B were nuclear proteins of infected and uninfected cells respectively, labeled with a mixture of* ^{14}*C-amino acids in the same way as the cells used for preparing VP1 for peptide mapping (see Materials and Methods and 15). For the in vitro products, 2 μl of reactions containing the indicated RNAs and [*^{35}*S]methionine were analyzed. All the gels were subjected to fluorography. Shown here are (A)* ^{14}*C-amino acid-labeled nuclear proteins of infected cells, (B)* ^{14}*C-amino acid-labeled nuclear proteins of uninfected cells, (C) [*^{35}*S]methionine-labeled in vitro products of poly(A)-containing RNA from infected cells, (D) [*^{35}*S]methionine-labeled in vitro products of poly(A)-containing RNA from uninfected cells, (E) [*^{35}*S]methionine-labeled in vitro products of polyoma virus-specific RNA, and (F) enlargements of the VP1 regions in parentheses in panels A, C, and E. In panel E, VP2 and VP3 appear as double spots. This type of isomerization is sometimes found with VP2 and VP3.*

higher molecular weights than their wild-type counterparts; see 15); and (iii) the Coomassie brilliant blue-stained VP3 spot fluoresces pink when illuminated with intense incandescent light—a diagnostic feature of this viral protein (see 16).

As shown in panels C and E of Fig. 5, proteins migrating to the same positions as authentic VP1, VP2, and VP3 were present among the products synthesized in vitro by use of either cytoplasmic poly(A)-containing RNA from polyoma virus-infected cells (panel C) or virus-specific RNA selected by hybridization to polyoma virus DNA (panel E). In addition to VP1, VP2, and VP3, a number of other proteins are translated on unselected, infected-cell RNA (e.g., spots i-m), including several also seen in the gels of nuclear proteins (e.g., actin). Comparison of the products from selected RNA (panel E) with those from unselected RNA (panel C) clearly demonstrates the expected enrichment for VP1, VP2, and VP3 when polyoma virus-specific RNA is used (compare intensities with those of spots i-m). Two additional proteins were also enriched in the selected RNA. One of these can be seen to the left (more basic) of VP3, and the second appears about one-third of the distance from VP2 to spot "k." Since neither of these proteins was detected among the in vitro translation products of RNA from mock-infected cells (panel D), and since both were enriched by use of polyoma virus-specific RNA, we conclude that they are polyoma virus-specific polypeptides. However, since they have no apparent counterparts in vivo (panel A), we suggest that they may represent either premature termination products or improperly processed forms of VP1, VP2, or VP3.

Finally, it should be noted that polyoma virus VP1 resolves in this gel system into at least three doublet pairs (panel F). Using the convention that α denotes the most acidic member of a group of closely related spots, we have provisionally designated the VP1 doublets as α, β, and γ. The more basic partner in each pair migrates detectably faster in these gels than its more acidic partner. Further, if these differences reflect post-transcriptional modifications rather than differences in the primary amino acid sequences, then such modifications must also happen in vitro since the in vitro products exhibit the same heterogeneity (panel F). An apparently similar charge heterogeneity has been reported for intracellular SV40 VP1 (28) and for VP1 molecules present in purified polyoma virus virions (18).

Independent synthesis of VP2 and VP3 in vitro. Although both VP2 and VP3 were synthesized in vitro, the exact origin of VP3 was not clear. Since VP3 is completely overlapped by VP2 and probably shares a common C terminus with VP2, it was possible that VP3 could have arisen from VP2 by proteolytic processing, such that the N-terminal region of VP2 was lost. Alternatively, VP3 could have been synthesized de novo by an independent initiation event. We distinguished between these possibilities by labeling the in vitro products in the presence of [^{35}S]formylmethionyl-tRNA$_f^{met}$, a specific N-terminal label (19). Gel analysis of in vitro products synthesized from polyoma virus-specific RNA in the presence of [^{35}S]formylmethionyl-tRNA$_f^{met}$ showed that all three viral capsid proteins were labeled (Fig. 6, track A). Further analysis of VP1 showed that the label was present in a single tryptic peptide, verifying that the [^{35}S]methionine was not incorporated into internal positions. Therefore, we conclude that VP2 and VP3 are synthesized independently in vitro. This conclusion is supported by the finding that inhibitors of proteolysis have no effect on the relative amounts of VP2 and VP3 synthesized in vitro. In addition, the ratios of VP2 and VP3 made from poly(A)-containing and poly(A)-deficient RNAs are very different (see Fig. 1 and 2), which is inconsistent with a mechanism in which VP2 arises from VP3 by processing.

Further experiments to determine the nature of the mRNA's for VP1, VP2, and VP3 showed that the synthesis of all three proteins in the wheat germ system was strongly inhibited by 80 μM m^{7}GTP (compare tracks B and C, Fig. 6). This suggests that the mRNA's for all three proteins are capped.

Size of the mRNA's for VP1, VP2, and VP3. Having established that VP3 was synthesized independently of VP2 in vitro, we wished to determine whether VP3 was synthesized from the same mRNA as VP2 by an internal initiation, or whether VP3 had a separate mRNA. To do this, we sized total cytoplasmic poly(A)-containing RNA from polyoma virus-infected cells on composite agarose-acrylamide gels. RNA was recovered from individual gel slices and translated in the wheat germ system. These translation reactions were analyzed by SDS-polyacrylamide gel electrophoresis, which is shown in Fig. 7A. The major mRNA activity for VP2 can be seen in fractions 7 to 9 and the major activity for VP3, in fractions 8 to 11. Thus, although the distributions of mRNA's for VP2 and VP3 overlap, it is clear that VP2 and VP3 have separate mRNA's. A second example of the separation between the mRNA's for VP2 and VP3 is shown in Fig. 7B, where the resolution in this region of the RNA gel was greater than for the gel in Fig.

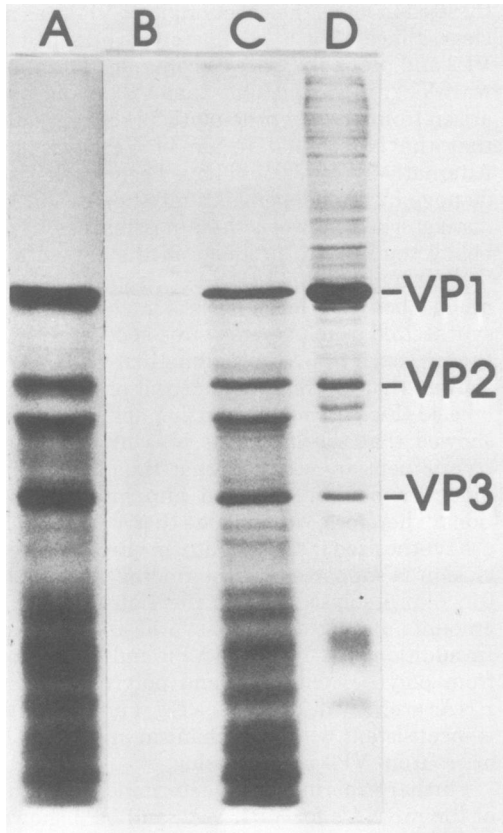


FIG. 6. *In vitro translation of polyoma virus-specific RNA in the presence of [^{35}S]formylmethionyl-tRNA$_f^{met}$. Polyoma virus-specific RNA was translated in the presence of either [^{35}S]formylmethionyl-tRNA$_f^{met}$ (6 × 10^5 cpm/10-μl reaction) and 0.5 mM methionine or [^{35}S]methionine in the wheat germ system as described in Materials and Methods. Where indicated, 80 μM m^{7}GTP was present. A 2-μl amount of each reaction was solubilized and run on a 12% acrylamide 0.32% bisacrylamide gel. The gel was subjected to fluorography. Because of intensity differences, track A, although from the same gel, was exposed 10 times as long as tracks B–D. Shown here are the products of translation reactions containing: (A) polyoma virus-specific RNA and [^{35}S]formylmethionyl-tRNA$_f^{met}$; (B) polyoma virus-specific RNA, [^{35}S]methionine, and m^{7}GTP; (C) polyoma virus-specific RNA and [^{35}S]methionine; and (D) ^{14}C-amino acid-labeled marker proteins from polyoma virus-infected cell nuclei.*

7A. In Fig. 7A, it can also be seen that the mRNA for VP1 is smaller than the mRNA's for VP2 and VP3. In addition to the major mRNA's for the capsid proteins, there are other potential mRNA species for VP2 and VP3. For instance, in fractions 18 to 21, there is an mRNA for a protein comigrating with VP2, and in fractions 18 to 22 there is an mRNA for a protein comigrating with VP3.

In the experiment described above, the identification of VP1, VP2, and VP3 rested solely on their electrophoretic mobilities in the SDS-polyacrylamide gel. To confirm that these protein assignments were correct, we repeated the sizing experiment on poly(A)-containing polyoma virus-specific RNA. The results shown in Fig. 8 confirm that the major mRNA activity for VP2 is larger than that for VP3. The mRNA for the virus-specific band lying between VP2 and VP3 comigrates with that for VP1. This finding, together with the labeling of this band with [^{35}S]formylmethionine (see Fig. 6, track A), strongly suggests that this protein is a premature termination product of VP1 generated in the wheat germ system. This band is not apparent when polyoma virus-specific RNA is translated in the mRNA-dependent reticulocyte lysate.

We have also sized the poly(A)-deficient mRNA's for the polyoma virus capsid proteins. In this case, we could not use the gel fractionation system to size total cytoplasmic poly(A)-deficient RNA because of its low capacity. Sucrose gradient sizing analysis showed that most of the poly(A)-deficient mRNA's for VP2 and VP3 were smaller than 20S. We were able to size the poly(A)-deficient mRNA species present in polyoma virus-specific RNA by gel analysis. There was a broad distribution of mRNA's for both VP2 and VP3, with little activity larger than 8 × 10^5 daltons and the peak being at about 5.5 × 10^5 daltons (data not shown).

DISCUSSION

A critical part of this work was the characterization of the polyoma virus-specific in vitro products. We showed that proteins comigrating with VP1, VP2, and VP3 were synthesized in the wheat germ system in response to mRNA from infected cells but not uninfected cells. These three proteins were the major products when polyoma virus-specific RNA, selected by hybridization to polyoma virus DNA, was translated in the wheat germ system. The three in vitro products in question were shown to be identical to the polyoma virus capsid proteins VP1, VP2, and VP3 by several criteria. First, peptide maps of the methionine-containing tryptic peptides were shown to be essentially identical to those derived from VP1, VP2, and VP3 labeled with [^{35}S]methionine in vivo. In addition, as expected, the maps of VP2 and VP3 were very similar. In fact, VP2 does not have any major methionine-containing peptides in addition to the four it has in common with VP3, even though VP2 is 10,000 daltons larger than VP3. This suggests that the

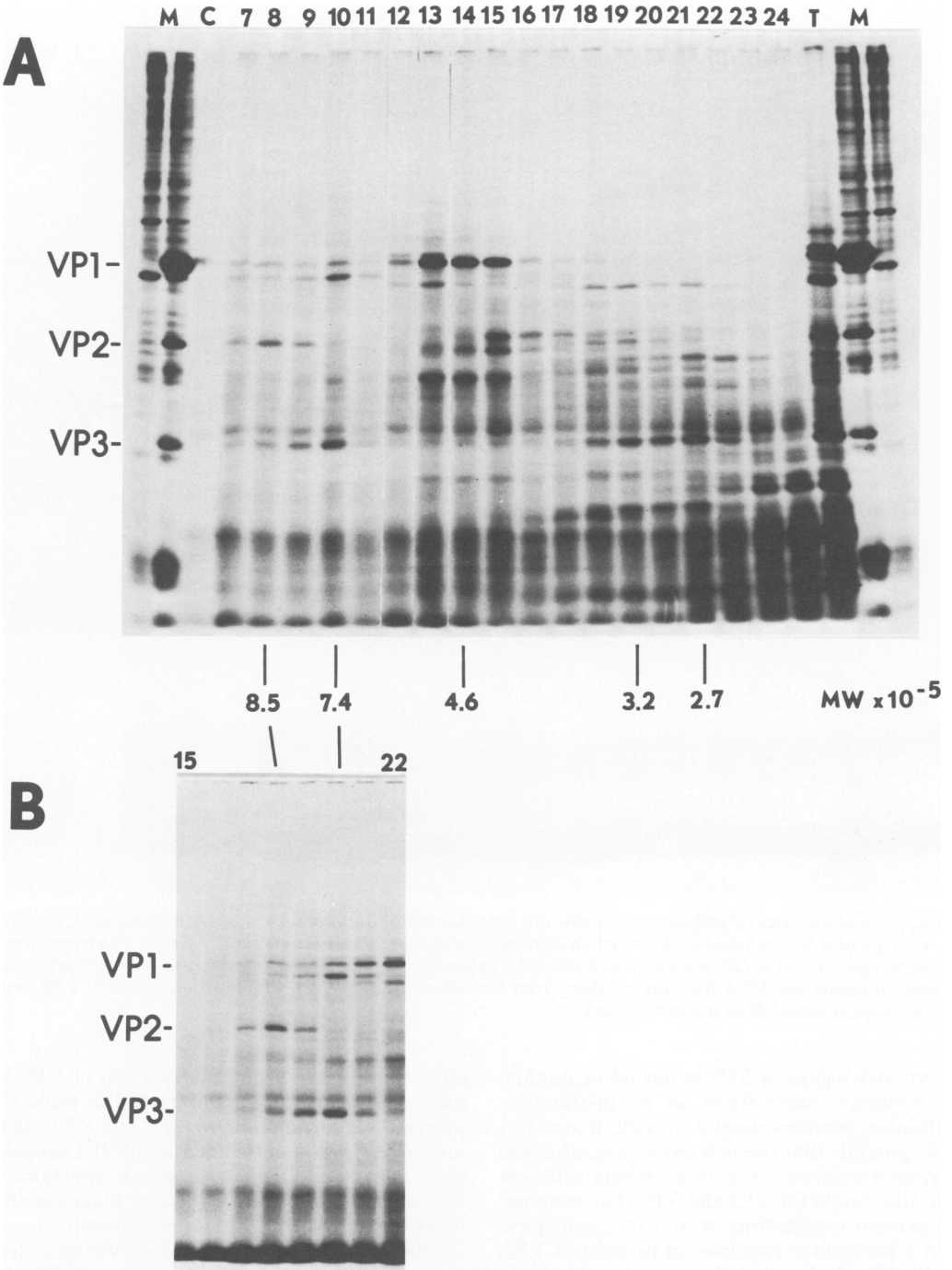


FIG. 7. *Size analysis of poly(A)-containing mRNA's from polyoma virus-infected cells. Poly(A)-containing RNA from infected cells was fractionated on a composite 2% acrylamide–0.5% agarose gel as described in Materials and Methods. The gel was cut into 2.5-mm slices, and the RNA was extracted from each slice. One-tenth of the RNA from each fraction was translated in a 10-μl reaction in the wheat germ system. A 2-μl amount of each reaction was run on a 12% acrylamide–0.32% bisacrylamide gel, which was subjected to fluorography. The track numbers refer to the RNA fractions, numbered from the top of the RNA gel. The track labeled C contained a control incubation to which no RNA was added. The T track contained the in vitro products of unfractionated poly(A)-containing RNA. The M tracks contained marker proteins from infected and uninfected cell nuclei, with the infected cell proteins being on the inside in each case. The approximate molecular weights of various peaks of mRNA activity are given. These are based on the molecular weight of the 16S mRNA for VP1 (22), the molecular weight of the mRNA's for the nonmuscle actins (21), and the molecular weight of the 19S polyoma virus-specific mRNA (22). A and B show two examples of fractionation of poly(A)-containing RNA from infected cells. The resolution of the mRNA's for VP2 and VP3 is better in B.*

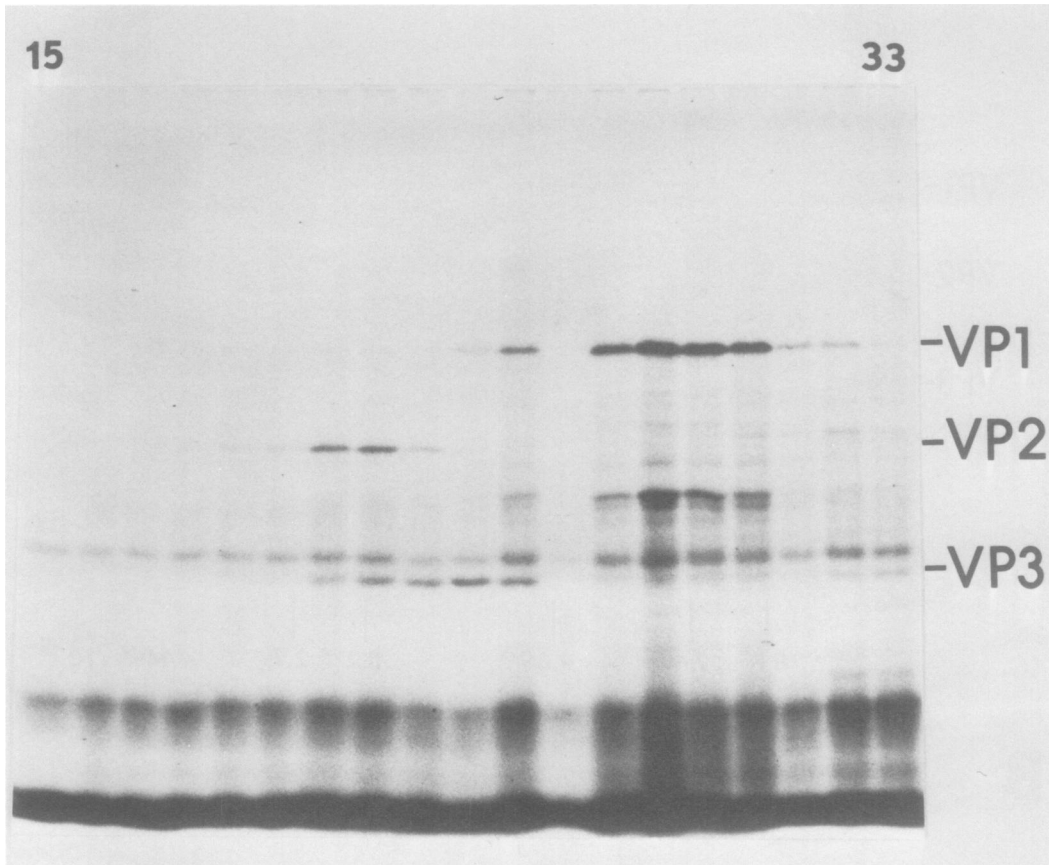


FIG. 8. *Size analysis of polyoma virus-specific poly(A)-containing mRNA's. Polyoma virus-specific RNA was selected and fractionated as described in Materials and Methods and the legend to Fig. 7. The translation products were run on a 12% acrylamide–0.32% bisacrylamide gel, which was subjected to fluorography. The numbers indicate the RNA fraction numbers from the top of the RNA gel. The positions of VP1, VP2, and VP3 were ascertained from a marker track.*

N-terminal region of VP2 is devoid of methionine residues. Since there are no methionine-containing peptides unique to VP2, it was formally possible that the two proteins synthesized in vitro overlapped, but in a fashion different from that found for VP2 and VP3. For instance, the protein comigrating with VP3 could have been a premature termination product of VP2. The low level of radioactivity incorporated into VP2 and VP3 when a mixture of ^{14}C-labeled amino acids was used in vitro made it technically difficult to perform tryptic peptide analysis. As an alternative means of proving the identity of the in vitro products, we showed by two-dimensional gel separations that they have the same charge/size characteristics as authentic VP2 and VP3 labeled in vivo. As has also been shown by others (18), VP3 is a comparatively basic protein whereas VP2 is more nearly neutral. Presumably, the unique N-terminal portion of VP2 is somewhat acidic and counteracts the basic C-terminal region common to VP2 and VP3. As a further indication of the identity of VP1 synthesized in vitro, we have been able to precipitate the in vitro product with antisera monospecific for polyoma virus VP1 (data not shown).

The proof that these proteins are virally coded rests on the finding that VP1, VP2, and VP3 can all be synthesized on RNA molecules selected on the basis of their ability to hybridize to polyoma virus DNA covalently linked to cellulose. By contrast, the mRNA activities for VP1, VP2, and VP3 are not enriched when cytoplasmic RNA from infected cells is hybridized to mouse DNA coupled to cellulose, although the conditions of hybridization would not allow efficient selection of mRNA's representing "single copy" cellular sequences. Wheeler et al. (37) have also

shown that the mRNA's for VP1 and VP2 are enriched by selection on polyoma virus DNA, and they concluded that VP1 and VP2 must be, at least in part, virally coded. Mangel et al. (25) have shown that polyoma virus complementary RNA, synthesized with *Escherichia coli* RNA polymerase, directs that synthesis of VP1 and VP2 in vitro. Similarly, we have obtained synthesis of VP1, VP2, and VP3 by translation in the mRNA-dependent reticulocyte lysate of complementary RNA synthesized with wheat germ RNA polymerase (T. Hunter, unpublished data). Given this evidence, and the fact that the major species of polyoma virus-specific RNA detectable late in infection do not appear to contain host sequences (1, 22), it seems unlikely that any part of VP1, VP2, or VP3 is host coded.

Hybridization studies have shown that there are two classes of polyoma virus-specific mRNA present in the cytoplasm of infected cells late in infection—19S and 16S (1, 22). The 19S mRNA, comprising an almost complete transcript of the late region has been shown to program the synthesis of VP2 in vitro (37). The 16S mRNA, which is the major species and corresponds to the 3′ half of the late region (22), is an active mRNA for VP1 (34). Up to the time this work was started, the mRNA for VP3 had not been identified. The finding that VP2 and VP3 are both labeled in vitro in the presence of [^{35}S]-formylmethionyl-tRNA$_f^{met}$, a specific N-terminal label, indicates that both proteins are initiated and synthesized independently, although they share a common C terminus (15). Therefore, either VP2 and VP3 are both synthesized from the same 19S mRNA molecule, with VP3 being initiated internally, or else there are separate mRNA's for VP2 and VP3. Our characterization of the sizes of the mRNA's for VP1, VP2, and VP3 suggests that the latter possibility is correct. Using the estimate of 8.5×10^5 daltons for the mRNA for VP2, which corresponds to the largest late cytoplasmic transcript identified by Kamen and Shure (22), and 4.6×10^5 daltons as the size of the 16S mRNA for VP1, we estimate the size of the mRNA for VP3 at 7.4×10^5 daltons. The difference between the sizes of the VP2 and VP3 mRNA's is approximately 300 nucleotides. This information could encode a 10,000- to 12,000-dalton protein sequence, which, perhaps coincidentally, is the apparent molecular weight difference between VP2 and VP3. Although it is formally possible that the peaks of mRNA activity for VP2 and VP3 represent isomers of the same 19S mRNA, this seems unlikely. A comparison of our gel sizing system with a totally denaturing gel system has shown that, in general, the mobilities of mRNA's in the nondenaturing gel system are a true reflection of their size (21). Secondly, Kamen has recently observed a third species of polyoma virus-specific late RNA migrating at about 18S, which could be identical to our VP3 mRNA activity (22; R. Kamen, personal communication). Thirdly, Siddell and Smith (33), using a different sizing technique, have also observed a difference in the size of mRNA's for VP2 and VP3.

We propose that the late region of polyoma virus is expressed as depicted in Fig. 9. There are three separate late mRNA's, each of which expresses the initiation site nearest its 5′ end. Polyoma virus thereby conforms to the rule that potentially polycistronic mRNA's are functionally monocistronic in eucaryotic cells. Although

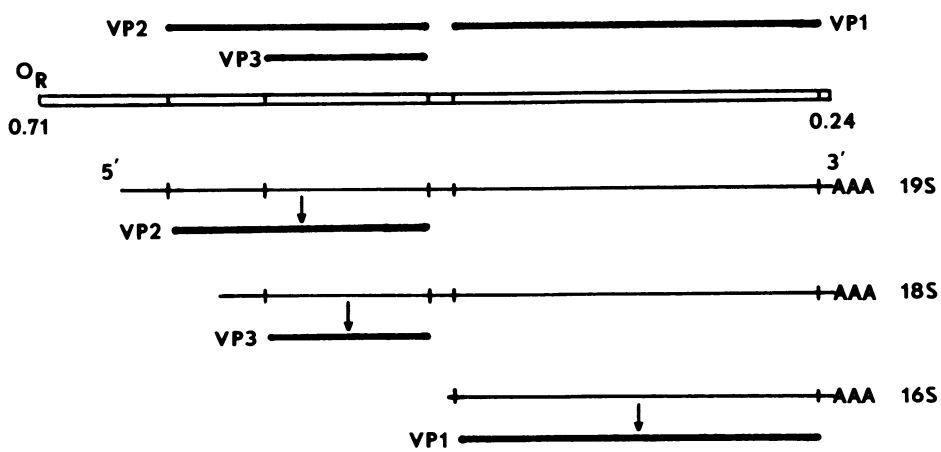


FIG. 9. *Model for the expression of the late region of polyoma virus. The DNA corresponding to the late region of polyoma virus is shown as an open bar with the vertical marks indicating approximate gene boundaries. The thin lines represent virus-specific cytoplasmic mRNA's, and the thick lines represent viral capsid proteins.*

there is no direct evidence, all three mRNA's may have leader sequences, originating from another part of the late region, spliced to their 5′ ends. The inhibition of synthesis of VP1, VP2, and VP3 by m^7GTP suggests that all three mRNA's are capped. To date, we do not know whether the C-terminal end of the minor capsid protein cistron overlaps the N-terminal end of the VP1 cistron as is the case for SV40 (10, 31).

Some fraction of the mRNA's for VP2 and VP3, as isolated from the cell, appears to be poly(A)-deficient. By chromatography on polyuridylic acid-Sepharose, we have concluded that most of these mRNA's truly lack poly(A) and do not simply have shorter poly(A) tails (21). These poly(A)-deficient mRNA's do not appear to be the result of leakage of unadenylated polyoma virus-specific transcripts from the nucleus into the cytoplasm during cell fractionation, since we have been able to find poly(A)-deficient mRNA's for VP2 and VP3 functionally associated with polysomes. Although it is true that unadenylated polyoma virus-specific transcripts from the nucleus of infected cells can be translated to yield VP2 (A. Smith, personal communication), these RNAs are at least 28S in size, whereas the cytoplasmic poly(A)-deficient mRNA's appear to be mostly smaller than 19S. It is possible that these RNAs arise by fragmentation of 19S and 18S polyadenylated mRNA's within the silent VP1 gene during isolation of polyoma virus-specific mRNA's, since the ratio of VP2 and VP3 synthesized on poly(A)-containing and poly(A)-deficient polyoma virus-specific RNAs is about equal (see Fig. 2, tracks F and G). In the unselected poly(A)-deficient mRNA from either the polysomes or total cytoplasm of infected cells, however, there appears to be an excess of VP2 mRNA (see Fig. 1, track E), whereas the poly(A)-containing cytoplasmic mRNA has an excess of VP3 mRNA (see Fig. 7). This situation may arise by selective intracellular fragmentation of the VP2 mRNA. In this regard, we have observed two minor species of poly(A)-containing mRNA of about 3.2×10^5 and 2.7×10^5 daltons, which may be mRNA's for VP2 and VP3, respectively (see Fig. 7). Although we have not characterized these mRNA's, they may represent VP2 and VP3 mRNA's which arise by fragmentation of the 19S and 18S mRNA's and subsequent cytoplasmic readenylation.

We are not certain whether the minor capsid proteins of SV40 are expressed in a manner similar to those of polyoma virus, although the differential efficiencies of translation of VP2 and VP3 in SV40-infected cells subjected to increasing extracellular salt concentrations strongly suggest that discrete mRNA's for SV40 VP2 and VP3 do exist (29). Up until now, in vitro synthesis of the SV40 minor capsid proteins has not been convincingly demonstrated. We have recently, however, observed the synthesis of SV40 VP2 and VP3 from poly(A)-containing SV40-specific RNA, and we are currently sizing their mRNA's.

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