JOURNAL OF VIROLOGY, Aug. 1978, p. 427-431
0022-538X/78/0027-0427$02.00/0

Vol. 27, No. 2
Printed in U.S.A.

NOTES

Polyoma Virus Has Three Late mRNA's: One for Each Virion Protein

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Received for publication 20 March 1978

Polyoma virus mRNA, isolated from the cytoplasm of 3T6 cells late after infection and purified by hybridization to *Hpa*II fragment 3 of polyoma virus DNA, was separated on 50% formamide-containing sucrose density gradients, and the fractionated RNA was recovered and translated in vitro. Analysis of the cell-free products showed that the minor virion protein VP3 was synthesized from an mRNA sedimenting at approximately 18S between the 19S VP2 mRNA and the 16S VP1 mRNA. Other experiments showed that the VP2 and VP3 can be labeled with formyl methionine from initiator tRNA. We conclude that there are three late polyoma virus mRNA's, each directing the synthesis of only one viral capsid protein.

The protein shell of polyoma virus (Py) is composed of one major (VP1) and two minor (VP2 and VP3) capsid proteins with molecular weights of approximately 45,000, 34,000, and 23,000, respectively. Peptide analysis of the two minor proteins has shown that they are related in that all the tryptic peptides present in VP3 are also contained in VP2 (9, 10, 14). By contrast, the major capsid protein VP1 is not related to VP2 since the two proteins share few, if any, tryptic peptides. These results imply that the minor capsid proteins are coded for, in part, by a common nucleotide sequence. A similar relationship probably exists between the capsid proteins of the closely related simian virus 40 (SV40) (21).

Examination of the polypeptides made when Py complementary RNA is translated in cell-free systems (18) and when SV40 DNA is injected into oocytes (8) indicates that the viral capsid proteins are entirely virus coded. Further studies of (i) the minor capsid proteins found in cells infected with specific deletion mutants of SV40 (6), (ii) the proteins synthesized in vitro using a linked transcription-translation system primed with fragments of SV40 DNA (21), and (iii) peptide fingerprints of the minor capsid proteins coded for by the Py mutant *ts*59 before and after marker rescue with fragments of Py DNA (11) all indicate that the sequences common to VP2 and VP3 are located towards the 3'-hydroxyl end of the VP2-coding sequence. Consistent with this, nucleotide sequence studies of SV40 DNA have revealed a sequence at the 3'-hydroxyl end of the region assumed to code for VP2, which could potentially code for VP3 (9a, 20a). The nucleotide sequence data, in agreement with earlier studies on the translation of fractionated viral mRNA (20, 23), also show that the sequences coding for VP1 are present to the 3'-hydroxyl side of the VP2 gene and establish the order of the structural genes to be VP2-VP3-VP1.

Py capsid proteins are synthesized during the late phase of the productive infection cycle at a time when two predominant species of late viral mRNA are present in the cytoplasm (5, 16, 23). The less abundant late mRNA species has a sedimentation coefficient of about 19S and maps between 68 and 25 map units on the L strand of the conventional Py DNA map (5, 16). The major late 16S RNA corresponds to the 3'-half of the 19S RNA (16). When translated in vitro, Py 19S RNA directs the synthesis of VP2, whereas 16S RNA codes for VP1 (23).

We now ask how the minor capsid protein VP3 is synthesized and consider three possible models. First, VP3 could be generated by proteolytic cleavage of VP2; second, the translation of VP3 could be initiated from a second, internal initiation site on the larger (19S) cytoplasmic viral mRNA; or third, VP3 could be synthesized independently from a third, as yet uncharacterized, mRNA.

Whereas each of these models is consistent

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with the data mentioned above, our results on the translation of Py nuclear RNA (R. Kamen, T. Wheeler, and A. E. Smith, Virology, in press) and of purified cytoplasmic viral mRNA (S. G. Siddell, submitted for publication) argue against the first two possibilities. Py nuclear RNA includes transcripts of the entire L strand of the viral DNA (4). The high-molecular-weight transcripts are therefore of the same sense as the late cytoplasmic viral mRNA's and contain sequence information for all the viral capsid proteins. When translated in vitro, however, all size classes of Py nuclear RNA direct the synthesis of capsid protein VP2 but little, if any, VP1 or VP3. Cytoplasmic Py mRNA, purified by preparative-scale hybridization to the *Hpa*II-3 fragment of Py DNA (which corresponds to the 5'-third of the late region) is enriched for the minor late mRNA's. When translated in vitro, under the same conditions used for the translation of nuclear RNA, *Hpa*II-3 DNA-purified Py mRNA directs the synthesis of approximately equimolar amounts of VP1, VP2, and VP3 (Siddell, submitted for publication).

We reasoned from these results that VP3 cannot be generated by cleavage of VP2, nor can it be generated by initiation from an internal initiation site, since were either of these possibilities true, nuclear RNA when translated in vitro would be expected to make VP3 as well as VP2. Instead, we considered it most likely that there is a third late viral mRNA which directs the synthesis of VP3.

If Py VP3 is coded for by a third species of late viral mRNA, it is likely that it would be of intermediate size between 19S and 16S. We tested this possibility by fractionating the *Hpa*II-3 DNA Sephadex-purified mRNA on 50% formamide-containing sucrose gradients (2), collecting a large number of fractions from the gradient, recovering the RNA from each fraction, and translating it in vitro (23, 25). Figure 1

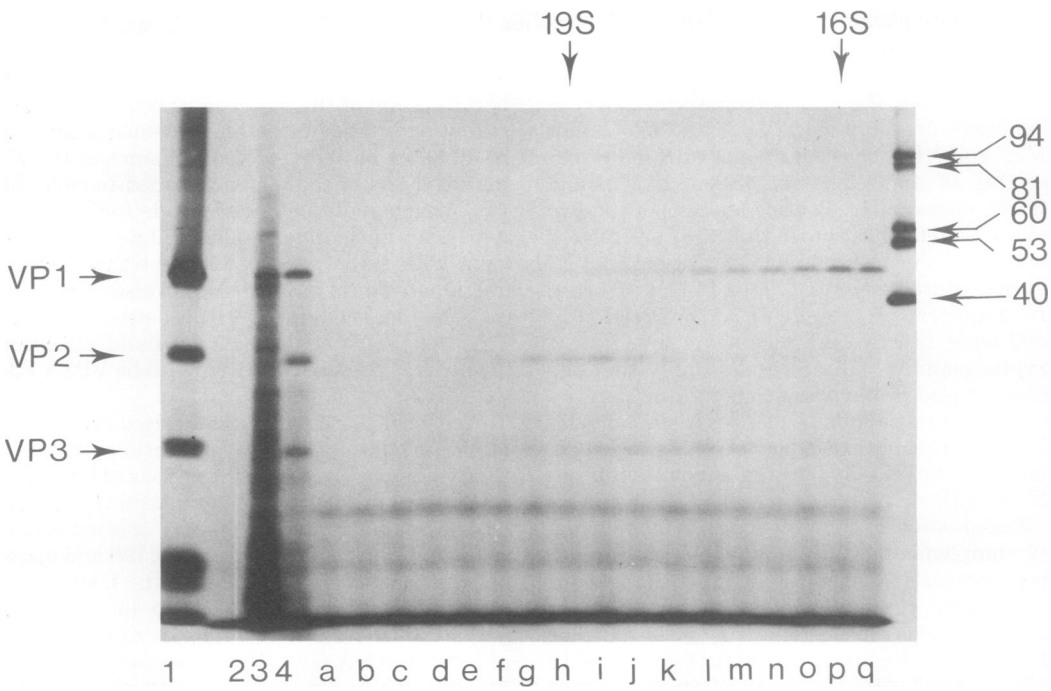

FIG. 1. *In vitro translation of Py late mRNA's after fractionation on 50% formamide-containing sucrose gradients. Total cytoplasmic RNA was isolated from 3T6 cells 28 h after infection (50 PFU/cell) with the A_2 strain of Py virus (25). RNA was purified by hybridization to HpaII-3 Py DNA immobilized on Sephadex as described in detail (Siddell, submitted for publication). About 10 μg of purified viral RNA was sedimented on a 2 to 10% sucrose gradient containing 50% formamide (2, 23). Seventy-five fractions were collected, and the RNA from each fraction was recovered, reprecipitated twice, and resuspended in 20 μl of water; samples were translated in a wheat germ cell-free system as previously described (23, 25). The samples were subjected to electrophoresis on a 15% polyacrylamide gel (25). (1) [^{35}S]methionine-labeled virus; (2) no added RNA; (3) 0.5 μg of total polyadenylic acid-containing, infected-cell RNA before purification; (4) 0.5 μg of HpaII-3 DNA-purified viral mRNA; (a–g) 6 μl of HpaII-3 DNA-purified RNA from fractions 30–46, respectively. ^{3}H-labeled mouse rRNA was analyzed in parallel gradients, and sedimentation was from right to left. The gel was exposed at −80°C for 7 days (17).*

shows the products made using total polyadenylic acid-containing RNA from Py-infected 3T6 cells (track 3), *Hpa*II-3 DNA-purified mRNA before fractionation (track 4), and selected fractions of the same material after gradient centrifugation (tracks a through q). The *Hpa*II-3 DNA-purified mRNA directs the synthesis of three polypeptides, which comigrate with VP1 and slightly ahead of VP2 and VP3 from purified virions. We have presented separately evidence that the proteins made in vitro are VP1, -2, and -3 (Siddell, submitted for publication). In the gel of the gradient-fractionated mRNA products, two peaks of mRNA activity sedimenting at approximately 19S and 16S relative to rRNA markers run in a parallel gradient are apparent. As expected from our earlier results (23), the RNA from the 19S region codes for VP2 and RNA from the 16S region codes for VP1. The VP3-coding activity, however, sediments between the VP1- and VP2-coding activities at approximately 18S. This result clearly shows that the mRNA activities coding for VP2 and VP3 are separable and suggests that they exist as distinct mRNA molecules.

If VP2 and VP3 are synthesized from separate mRNA's, then it follows that they must be initiated independently. We tested this prediction by asking whether both VP2 and VP3 are labeled with methionine from initiator tRNA, $tRNA_F^{Met}$ (12, 24). Figure 2 shows densitometer tracings of an autoradiograph of the products labeled when wheat germ ^{35}S-labeled fMet-$tRNA_F$ was added to the wheat germ cell-free system together with different mRNA's. Tracing B shows that in the absence of added mRNA a peak migrating with an apparent molecular weight of 50,000 is labeled. We do not know the origin of this material, since no major band of this mobility is detected when [^{35}S]methionine is used as the label. Addition of total infected-cell nuclear RNA to the cell-free system generates an additional peak which migrates slightly ahead of Py VP2. When *Hpa*II-3 DNA-purified mRNA is added to the cell-free system, three specific peaks are labeled with formyl methionine, and these comigrate with VP1 and slightly ahead of VP2 and VP3 from purified virions subjected to electrophoresis in an adjacent track.

We have so far not obtained sufficient ^{35}S-formyl methionine-labeled VP2 and VP3 to characterize their N-terminal peptides. Nevertheless, we believe that the radioactive label incorporated into VP1, -2, and -3 in the experiment shown in Fig. 2 was formyl methionine for the following reasons. (i) Under the conditions used to aminoacylate the wheat germ tRNA, only the $tRNA_F^{Met}$ species was acylated (12; data

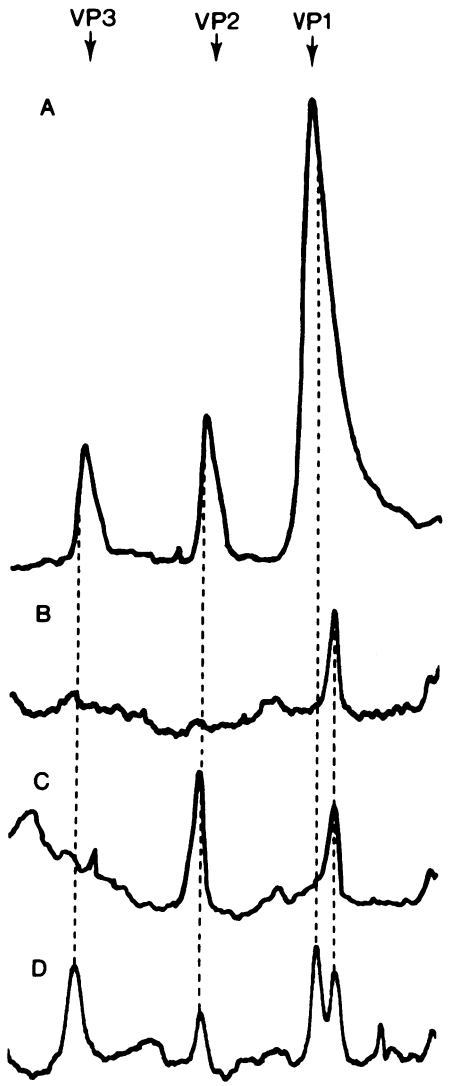

FIG. 2. *Electrophoretic analysis of the fMet-labeled polypeptides synthesized in response to nuclear and cytoplasmic viral mRNA from Py-infected cells. Wheat germ $tRNA^{Met}$ species were fractionated by chromatography on benzoylated DEAE-cellulose (24). The $tRNA_F^{Met}$ was charged by using highly purified E. coli methionyl tRNA synthetase (a gift from C. Bruton) and chemically formylated by a previously published procedure (12). Cell-free extracts (25 μl) contained 5 μg of ^{35}S-labeled fMet-$tRNA_F$ (specific activity, 2×10^5 cpm/μg) and 500 μM non-radioactive methionine. Other components were as described previously (12, 25). The products were separated on a 15% polyacrylamide gel. (A) ^{35}S-labeled virus; (B) no added RNA; (C) 0.5 μg of total nuclear RNA from infected 3T6 cells; (D) 2.0 μg of HpaII-3 DNA-purified mRNA. The gel was exposed at −80°C for 28 days (17), and the developed film was scanned with a Joyce-Loebl densitometer.*

not shown). (ii) By using the chemical method to formylate Met-tRNA$_F$, over 90% of the added label was formylated (12). (iii) Excess unlabeled methionine was added to the cell-free system so that any [^{35}S]methionine deacylated from the small amount of added ^{35}S-labeled Met-tRNA$_F$ was diluted out. Gibson et al. have also concluded that Py VP2 and VP3 can be labeled with formyl methionine from initiator tRNA (referred to in reference 11), and Opperman and Koch, on the basis of experiments in SV40 productively infected cells treated with hypertonic medium, have also deduced that SV40 VP2 and VP3 was initiated independently (19).

The results presented here clearly show that there is a separable mRNA for Py VP3 and strongly argue against a model in which VP3 is a cleavage product of VP2 or a model predicting that 19S RNA has two simultaneously active initiation sites. We interpret the results to mean that the VP3 mRNA is a separate molecular species which is of an intermediate sequence complexity to the late 19S and 16S mRNA's (Fig. 3). However, we recognize that the results presented here cannot exclude the alternative interpretation that the VP3-coding activity results from a different and separable conformation of the 19S VP2-coding mRNA. One argument in favor of the former model is the finding that Py nuclear RNA does not direct the synthesis of VP3 even after similar denaturation and fractionation on formamide-containing sucrose density gradients, conditions which presumably should result in the formation of the putative VP3-coding conformation (Kamen et al., Virology, in press).

Further direct evidence in support of the model shown in Fig. 3 comes from recent results (R. I. Kamen, and J. Favaloro, unpublished data) obtained by sizing on agarose gels the Py DNA which is resistant to S1 nuclease after annealing to Py mRNA (3). Three late mRNA's were identified and mapped. Knowing the probable location of Py DNA of the sequences coding for the viral capsid proteins (11, 16, 23), the map positions and chain lengths determined for the three late mRNA's lead to the same prediction for their coding properties as deduced from the translation experiments. Furthermore, when RNA was taken from each fraction of the sucrose gradient shown in Fig. 1 and characterized using the S1 nuclease method, the intermediate-sized mRNA was found in the fraction active in directing the cell-free synthesis of VP3.

With a knowledge of the likely organization of the genes for the capsid proteins of Py and SV40 it is easy to rationalize the need for three late mRNA's. Since the VP3 initiation site is within the VP2 coding region, any ribosome attempting to initiate synthesis at an internal VP3 initiation site would risk colliding with other ribosomes already in the process of synthesizing VP2. A similar attempt at the internal VP1 initiation site might risk collision with ribosomes translating either VP2 or VP3, since it is now known that in SV40 this region of the DNA is read in two phases which overlap around the N terminus of VP1 (7). The finding that the internal initiation sites of Py mRNA's are inactive is consistent with similar results with many other eucaryotic mRNA's. Indeed, we are not aware of a single, well-characterized exception to the rule that eucaryotic mRNA's have only one active initiation site (22).

Hunter and Gibson (submitted for publication), on the basis of experiments very similar to

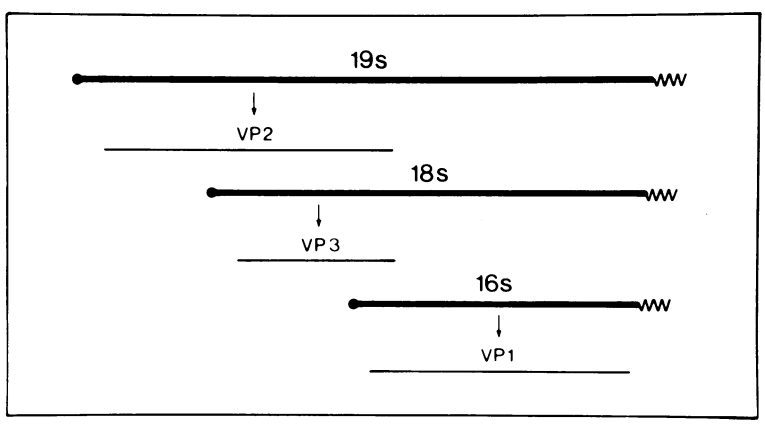

FIG. 3. *Model for the synthesis of Py virion proteins VP1, VP2, and VP3. We propose that the 18S mRNA represents a unique molecular species, but we recognize that the results presented cannot exclude the alternative interpretation that the VP3 mRNA is a conformer of 19S RNA. The circle at the end of mRNA's indicates the presence of a 5' cap and possible leader (1, 15) sequence.* vvV^ *represents polyadenylic acid.*

those described here, have also concluded that there are three late Py mRNA's, which code for the three viral capsid proteins.

We thank R. Kamen and H. Dahl for critical reading of the manuscript, and C. Conway for typing it.

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