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Polyoma Virus Complementary RNA Directs the In Vitro Synthesis of Capsid Proteins VP1 and VP2

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Polyoma virus complementary RNA, synthesized in vitro by using highly purified *Escherichia coli* RNA polymerase and nondefective form I polyoma DNA, was translated in a wheat germ cell-free system. Polypeptides were synthesized that comigrated on sodium dodecyl sulfate-polyacrylamide gels with the polyoma capsid proteins VP1 and VP2, although most of the cell-free products were of smaller molecular weights. The VP1-size protein specifically immunoprecipitated with anti-polyoma virus serum, and upon digestion by trypsin yielded [^{35}S]methionine-labeled tryptic peptides that co-chromatographed with the [^{3}H]methionine-labeled tryptic peptides of virion-derived VP1 on both cation-exchange and anion-exchange resins. The VP2-size in vitro product contained all the virion VP2 methionine-labeled tryptic peptides, as shown by cation- and anion-exchange chromatography and two-dimensional fingerprinting on cellulose. We conclude that full-length polyoma VP1 and VP2 are synthesized in response to complementary RNA and consequently that the viral capsid proteins VP1, VP2, and VP3 are entirely virus coded.

The small DNA tumor virus, polyoma, is studied as a model system for eucaryotic DNA replication, for RNA transcription and translation, and for cellular transformation. Genetically, the virus contains a small amount of information encoded in double-stranded, closed circular DNA with a molecular weight of 3.6×10^6. The virus stably transforms certain cell lines and in others successfully replicates to produce progeny. This wide spectrum of biological effects must result in part from the interaction of the viral gene products with the host cell. As a prelude to understanding this interaction, the virus-coded polypeptides of polyoma need to be identified.

Most viruses encode the genetic information for some or all of their structural proteins. The polyoma virus particle contains seven polypeptides (12, 16, 19, 30). The major coat polypeptide (VP1) has a molecular weight of 45,000 and is present at approximately five to six times the amount of two minor virion polypeptides of molecular weights 34,000 (VP2) and 23,000 (VP3). The virus also contains an 86,000-molecular-weight polypeptide (VP0) that is a dimer of VP1 (11, 16) and three small basic polypeptides that have been shown by peptide analysis to be host cell-derived histones (9, 10). VP3 is related to VP2 because all of its tryptic peptides can be found in a digest of VP2 (9, 14, 16). VP1 and VP2 are probably unique because they share few, if any, tryptic peptides.

The productive infection cycle of polyoma virus can be divided into two temporal phases. Before DNA replication, the cytoplasm contains a viral RNA that sediments at 20S (22, 37). It is complementary to about 48% of the "E" strand of polyoma DNA, extending clockwise from unit 72 to 25 (22) on the physical map (15). The only protein detected so far whose appearance is coincident with this early RNA is the polyoma T antigen (data summarized in reference 36). After the initiation of DNA replication, two new viral RNAs can be found in the cytoplasm, a major species that sediments at 16S and a minor species that sediments at 19S (4). These RNAs are complementary to the "L" strand of polyoma DNA (22). The 16S RNA sequences map from units 47 to 25, and the 19S RNA sequences map from 68 to 25. Thus, all the sequences present in 16S RNA are also present in 19S RNA.

In vitro translation of the viral mRNA's can be used to determine the virus-coded proteins of polyoma if the mRNA's are entirely virus coded. Indeed, such experiments have shown that VP2 can be translated from the late 19S mRNA and VP1 from the late 16S mRNA (34). The molecular weights of these proteins, the

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estimated molecular weights of the mRNA's, and the degree of hybridization of the mRNA's to viral DNA are all consistent with the conclusion that VP2 and VP1 are virus coded. However, there is no definitive proof that the viral mRNA's do not contain host RNA sequences. The question as to whether polyoma DNA integrates into the host chromosome during a productive infection is still not resolved (7, 20, 39). Host RNA sequences would not have been detected in the mapping of the mRNA's on polyoma DNA because the hybridization was monitored with labeled viral DNA.

An alternative method for determining the late virus-coded polypeptides is to synthesize in vitro the late RNA sequences from highly purified polyoma DNA grown from nondefective virus stocks and to translate that RNA in a cell-free system. The polypeptides synthesized under these conditions are, by definition, virus coded and the products of the late genes of polyoma. Crawford and Gesteland (8) examined the polypeptides made in a coupled (transcription/translation) *Escherichia coli* cell-free system in which exogenous polyoma DNA was added. They found that some of the polypeptides synthesized in vitro corresponded in size to VP1 and that the cell-free product contained many of the tryptic peptides of VP1. Here we report the use of a noncoupled system using asymmetrically transcribed complementary RNA (cRNA) and a eucaryotic cell-free protein-synthesizing system to determine the late virus-coded polypeptides of polyoma. Experimentally, form I polyoma DNA was transcribed by the *E. coli* RNA polymerase. Over 90% of the RNA synthesized hybridized to the L strand of polyoma DNA and, on the basis of its size, contained all the RNA sequences present in the late 19S and 16S mRNA's. This cRNA was then translated in vitro in the wheat germ cell-free protein-synthesizing system, and the methionine-labeled tryptic peptides of the products were compared with those of the two virion proteins VP1 and VP2. A preliminary description of this work appeared previously (33).

MATERIALS AND METHODS

Polyoma virion proteins and polyoma DNA were purified from the large-plaque polyoma strain A2 (15). The procedures for the growth of the virus, labeling of the virion proteins, and purification of VP1 and VP2 have been described by Hewick et al. (16). Form I DNA was purified by the method of Hirt (18). The viral DNA was routinely checked for the presence of defective molecules by digestion with restriction enzymes. The MRE 600 strain of *E. coli*, an RNase 1 mutant, was obtained from the Microbiological Research Centre, Porton Down, England. *E. coli* RNA polymerase was purified by the procedure of Berg et al. (1), as modified by Mangel (24). Polyoma cRNA was synthesized with slight modification by the procedure of Kamen et al. (21). Reaction mixtures (1 ml) contained: 40 mM Tris-hydrochloride (pH 7.9), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 412 mM KCl, 1 mM each ATP, CTP, GTP, and UTP, 25 μg of form I polyoma DNA, and 42 μg of RNA polymerase. After 3 h at 37°C, 8 μg of DNase (Worthington Biochemicals Corp., Freehold, N.J.) was added, and the mixture was incubated for an additional 5 min. The RNA polymerase was extracted with phenol, and the unincorporated ribonucleoside triphosphates were removed by filtration through Sephadex G-75. The yield was about 100 μg of cRNA. The conditions for in vitro translation in the wheat germ cell-free system, analysis of the products by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and immunoprecipitation have been described by Wheeler et al. (38). The tryptic peptide analyses were performed by the procedures of Hewick et al. (16) for column chromatography and of Wheeler et al. (38) for two-dimensional fingerprints.

RESULTS

Synthesis of cRNA. Polyoma cRNA was synthesized from form I polyoma DNA with the *E. coli* RNA polymerase at high ionic strength (0.412 M KCl). Under these conditions, the RNA is asymmetrically transcribed from the L strand of the DNA (21). After self-annealing in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), over 80% of the cRNA was still sensitive to RNase. The cRNA sedimented in a 5 to 20% sucrose gradient containing 85% formamide (23) with a broad peak at 35S, ranging from 22S to 45S (Fig. 1). Thus, the majority of the cRNA molecules contained a complete copy of the L strand of polyoma DNA, i.e., the late mRNA sequences and those sequences complementary to early mRNA.

In vitro translation of polyoma cRNA. The wheat germ cell-free system was maximized for amino acid incorporation in response to polyoma cRNA. At an RNA concentration of 20 to 60 μg/ml, the optimal ionic conditions are 1.5 mM Mg^{2+} and 40 mM K^+. The cell-free product, when fractionated on SDS-polyacrylamide gels, appeared heterogeneous in size, ranging in molecular weight from 10,000 to 50,000, with the majority of the product less than 25,000 (Fig. 2b). Two of the polypeptides synthesized in vitro comigrated with the polyoma structural proteins VP1 and VP2 (Fig. 2a and b). The polypeptide that comigrates with VP1 is precipitable with antiserum (38) directed against polyoma virions (Fig. 2c). These data imply that polyoma cRNA can direct the synthesis of the polyoma capsid proteins VP1 and VP2. This possibility was further tested by comparing the tryptic peptides

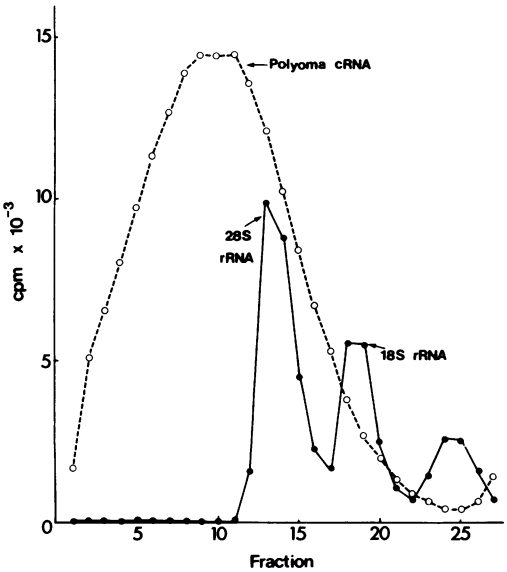

FIG. 1. *Sedimentation of polyoma cRNA on a denaturing sucrose gradient. Tritiated polyoma cRNA was mixed with ^{32}P-labeled rRNA and centrifuged for 17 h at 40,000 rpm in a 5 to 20% sucrose gradient containing 0.01 M Tris-hydrochloride (pH 7.5), 1 mM EDTA, and 85% formamide at 20°C.*

of the in vitro-synthesized product with those of VP1 and VP2.

Tryptic peptide maps. The polypeptides synthesized in the wheat germ cell-free system with polyoma cRNA as message were labeled with [^{35}S]methionine and fractionated on discontinuous SDS-polyacrylamide gels. The two bands comigrating with polyoma VP1 and VP2 were cut out, and the polypeptides were eluted from the gel. The polypeptides were then precipitated, oxidized with performic acid, and digested with trypsin. The resulting peptides were separated on cation- and anion-exchange resins and by two-dimensional fingerprinting on cellulose. For those peptides to be analyzed by ion-exchange chromatography, the appropriate viral protein labeled with [^{3}H]methionine was added prior to oxidation to serve as an internal marker.

(i) Cation-exchange chromatography. The elution pattern from a cation-exchange column of a mixture of authentic VP1 labeled with [^{3}H]methionine and authentic VP2 labeled with [^{35}S]methionine is shown in Fig. 3A. Aside from the flow through, there are three peaks of material from VP1, labeled b, d, and e, and three from VP2, labeled a, b, and c. Peak b, therefore, contains peptides from both VP1 and VP2 that are not resolved on the column. Peaks c and d contain peptides from VP2 and VP1, respectively, and are moderately well resolved. Peaks a and e are well separated from the other tryptic peptides and are unique to their parent proteins.

When the cell-free product that comigrates with VP1 was compared with authentic VP1 by cation-exchange chromatography, the elution patterns were identical (Fig. 3B). There was no material in the position of the VP2-specific peak a or any shoulder on peak d. The peptide elution pattern obtained when the tryptic digest of the VP2-size cell-free product was co-chromatographed with authentic VP1 is more complicated

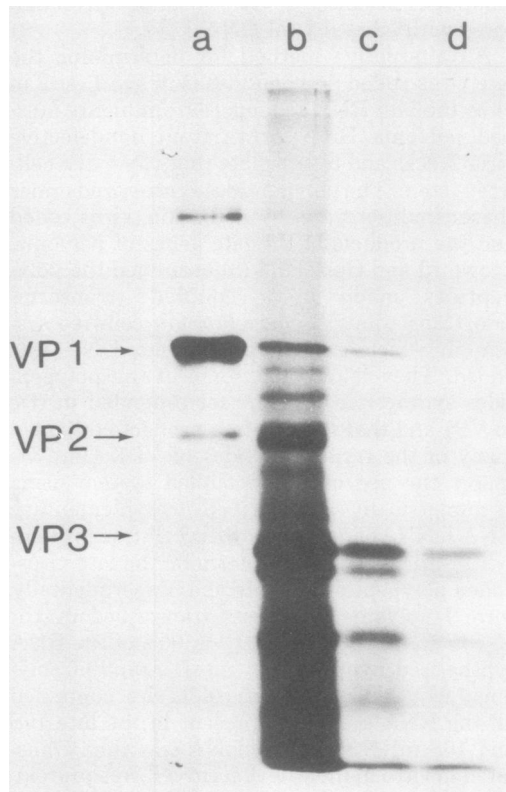

FIG. 2. *Analysis of the translation products of polyoma cRNA on SDS-polyacrylamide gels. Polyoma cRNA (1 μg) was translated in a standard 25-μl reaction mixture (38), including 7.5 μl of wheat germ extract, 15 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (pH 7.0), 40 mM KCl, 1.5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, 50 μM spermine, 250 μM spermidine, and 1 mM dithiothreitol. A total of 19 unlabeled amino acids, omitting methionine, were added to 200 μM, and [^{35}S]methionine was added at 500 μCi/ml. After a 3-h incubation at 22°C, the products were analyzed by SDS-polyacrylamide electrophoresis followed by autoradiography. (a) Purified polyoma virus; (b) 5-μl sample of cRNA-directed cell-free product; (c) sample as (b) after immunoprecipitation with rabbit anti-polyoma serum; (d) sample as (b) after immunoprecipitation with control rabbit serum.*

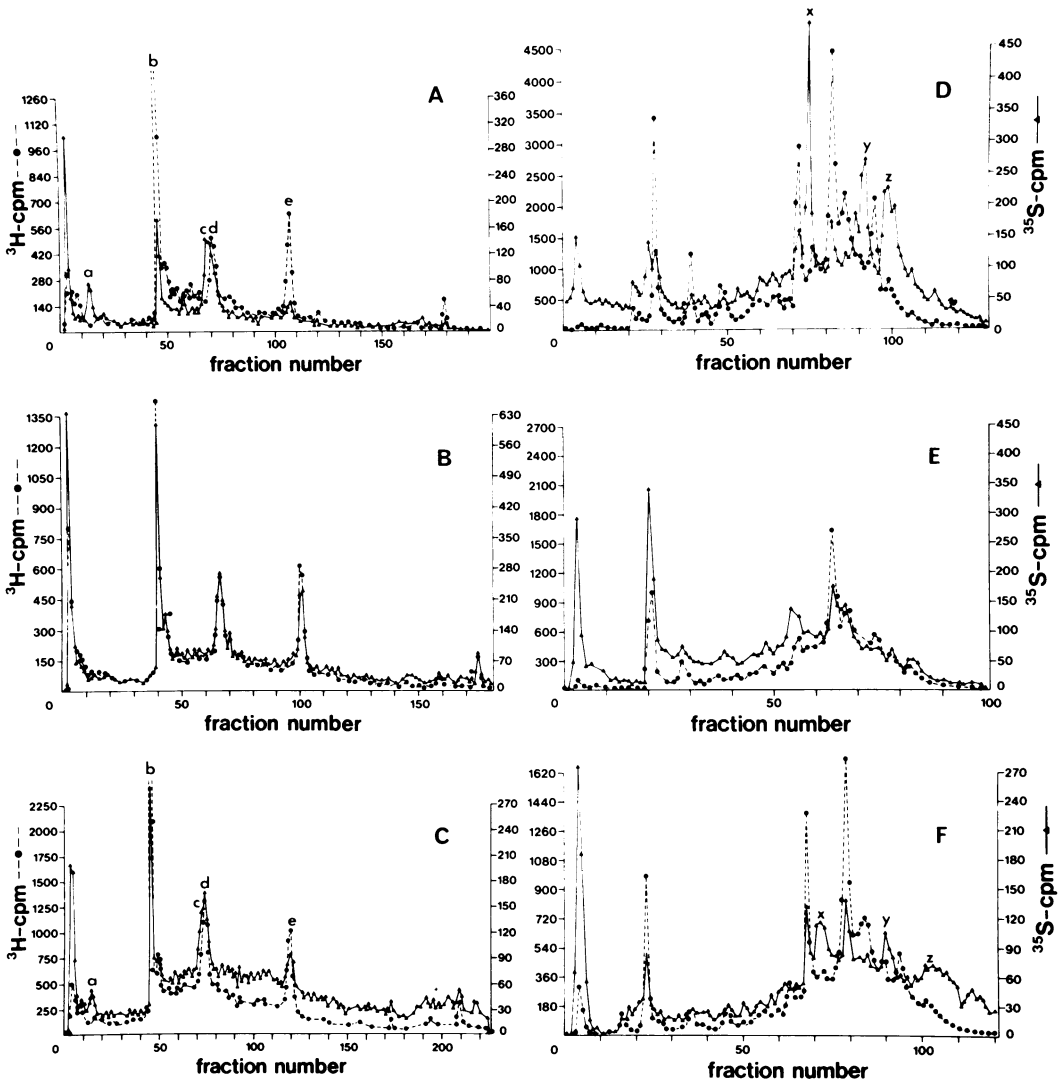

FIG. 3. *Comparison of the methionine-labeled tryptic peptides of VP1 and VP2 and the translation products of polyoma cRNA by cation- and anion-exchange chromatography. Peptides were eluted from the cation column at 50°C with a linear gradient consisting of 125 ml of 0.04 M pyridine formate acid (pH 2.1) and 125 ml of 0.5 M pyridine acetate (pH 3.75), followed by another linear gradient consisting of 50 ml of 0.5 M pyridine acetate (pH 3.75) and 50 ml of 2 M pyridine acetate (pH 5.2), and from the anion column at 50°C with a complex gradient starting with 3% pyridine and finishing with 2.0 M pyridine (pH 5.0), as described by Bornstein (2), except that the volumes of the individual buffers were reduced by half. A mixture of the tryptic peptides of virion VP1 labeled with [^{3}H]methionine and virion VP2 labeled with [^{35}S]methionine was applied to a cation column (A) and an anion column (D). A mixture of the tryptic peptides of virion VP1 labeled with [^{3}H]methionine and the polypeptide synthesized with polyoma cRNA as message, which comigrates on SDS-polyacrylamide gels with virion VP1 labeled with [^{35}S]methionine, was applied to a cation column (B) and an anion column (E). A mixture of the tryptic peptides of virion VP1 labeled with [^{3}H]methionine and the polypeptide synthesized with polyoma cRNA as message, which comigrates on SDS-polyacrylamide gels with virion VP2 labeled with [^{35}S]methionine, was applied to a cation column (C) and an anion column (F).*

(Fig. 3C). There were [^{35}S]methionine-containing peptides at positions a, b, c, d, and e, with c appearing as a shoulder on the leading edge of peak d. These data indicate that polypeptides from this region of the gel include full-length polyoma VP2, because material chromatographing in the VP2-specific positions, a and c, was present in the tryptic digest. In addition, the

data suggest that fragments of VP1 comigrate on gels with VP2 because the VP1-specific peaks d and e were also present.

(ii) Anion-exchange chromatography. Anion-exchange chromatography was also used to compare the methionine-containing tryptic peptides of VP1 and VP2 with their in vitro counterparts synthesized in response to polyoma cRNA (Fig. 3D). In the digest of the two proteins from purified virions, about 8 peaks from VP1 and 10 peaks from VP2 were detected. Of these, the three peaks marked x, y, and z were well separated from the rest and were unique to VP2.

When a tryptic digest of virion protein VP1 and the cRNA-directed product of VP1 size were chromatographed together, their peaks were virtually superimposable (Fig. 3E). The column profile of the digest of viral VP1 and the in vitro-synthesized VP2-size polypeptide shows that the three VP2-specific peaks (x, y, and z) were present (Fig. 3F) in addition to material co-chromatographing with the major VP1-specific peaks. Peaks x, y, and z were not present in the digest of the cell-free product corresponding in size to VP1. Thus, the anion-exchange chromatography data also indicate that authentic VP1 and VP2 are made in response to polyoma cRNA, but that the VP2-size cell-free product contains, in addition, fragments of VP1.

(iii) Two-dimension fingerprints on cellulose. To confirm that VP2 is synthesized with polyoma cRNA as message, tryptic digests of [^{35}S]methionine-labeled virion VP1 and VP2 were compared with a digest of the in vitro-synthesized product that comigrates on gels with VP2 by two-dimensional peptide mapping on cellulose. The peptides were first separated by electrophoresis at pH 2.1 and then by chromatography (38). The characteristic fingerprints of polyoma VP1 and VP2 are shown in Fig. 4A and B, respectively. VP2 contained two major methionine peptides that were unique. The peptide fingerprints of the VP2-size in vitro product made in response to polyoma cRNA contained peptides that migrated in the position of both the VP2 characteristic peptides (Fig. 4C). Their identity was confirmed by mixing a digest of VP2 from purified virions with a similar digest of the VP2-size cell-free product (Fig. 4D). The major virion VP2-specific spots comigrated with two of the in vitro peptides, these spots having become more intense relative to the other peptides. The digest of the VP2-size cell-free product also contained several additional peptides that migrated as VP1-specific peptides. Indeed, when digests of virion-derived VP1 and VP2 were mixed and fractionated, the resulting peptide map (Fig. 4E) was very similar to that of the VP2-size cell-free product. A tracing of the fingerprint in Fig. 4C is shown in Fig. 4F, indicating the origin of the peptides in the cell-free product.

Since the bulk of the cell-free product synthesized with polyoma cRNA as message was smaller than either VP1 or VP2, we also examined this material. Two-dimensional tryptic peptide analysis of the trichloroacetic acid-insoluble material in an unfractionated reaction mixture (Fig. 2b) and of the major polypeptide synthesized in vitro that has a molecular weight of about 22,000 and migrates slightly faster than polyoma VP3 (Fig. 2b) showed that both contained predominantly VP1-derived peptides (data not shown). The finding (Fig. 2c) that several of the smaller polypeptides were immunoprecipitated to a greater extent by the anti-VP1 serum than by the control serum (Fig. 2d) also suggests that they are related to VP1. Thus, the cell-free product contains predominantly fragments of VP1.

This analysis of the polypeptides made in response to polyoma cRNA indicates that the cell-free product includes proteins similar to the authentic capsid proteins VP1 and VP2 in gel mobility, in [^{35}S]methionine tryptic peptide content, and, in the case of VP1, in antigenic properties. We conclude that those two virion proteins are synthesized in vitro and, consequently, that both are entirely virus coded.

DISCUSSION

This work shows that polyoma cRNA can direct the synthesis in vitro of polypeptides apparently identical to the coat proteins VP1 and VP2. Because polyoma cRNA corresponds in sequence to a transcript of the L strand of polyoma DNA, the data imply that the two proteins are the products of the late genes. Furthermore, since the cRNA was synthesized by using highly purified nondefective viral DNA as template, the data also suggest that the two proteins are entirely virus coded, at least as judged by analysis of their methionine-containing tryptic peptides.

Data from other laboratories are also consistent with the above conclusions. All the tryptic peptides in polyoma virus VP3 can also be found in VP2 (9, 14, 16), implying that all of VP3 and part of VP2 are derived from the same sequence of viral DNA. The sum of the molecular weights of VP1 and VP2 indicates that about 40% of the genome codes for these three capsid proteins. This is consistent with the late region comprising 43% of the viral DNA (22) and with the demonstration that the late 19S RNA translates in vitro into VP2 and the late 16S RNA into VP1 (34). Also consistent with this conclusion is

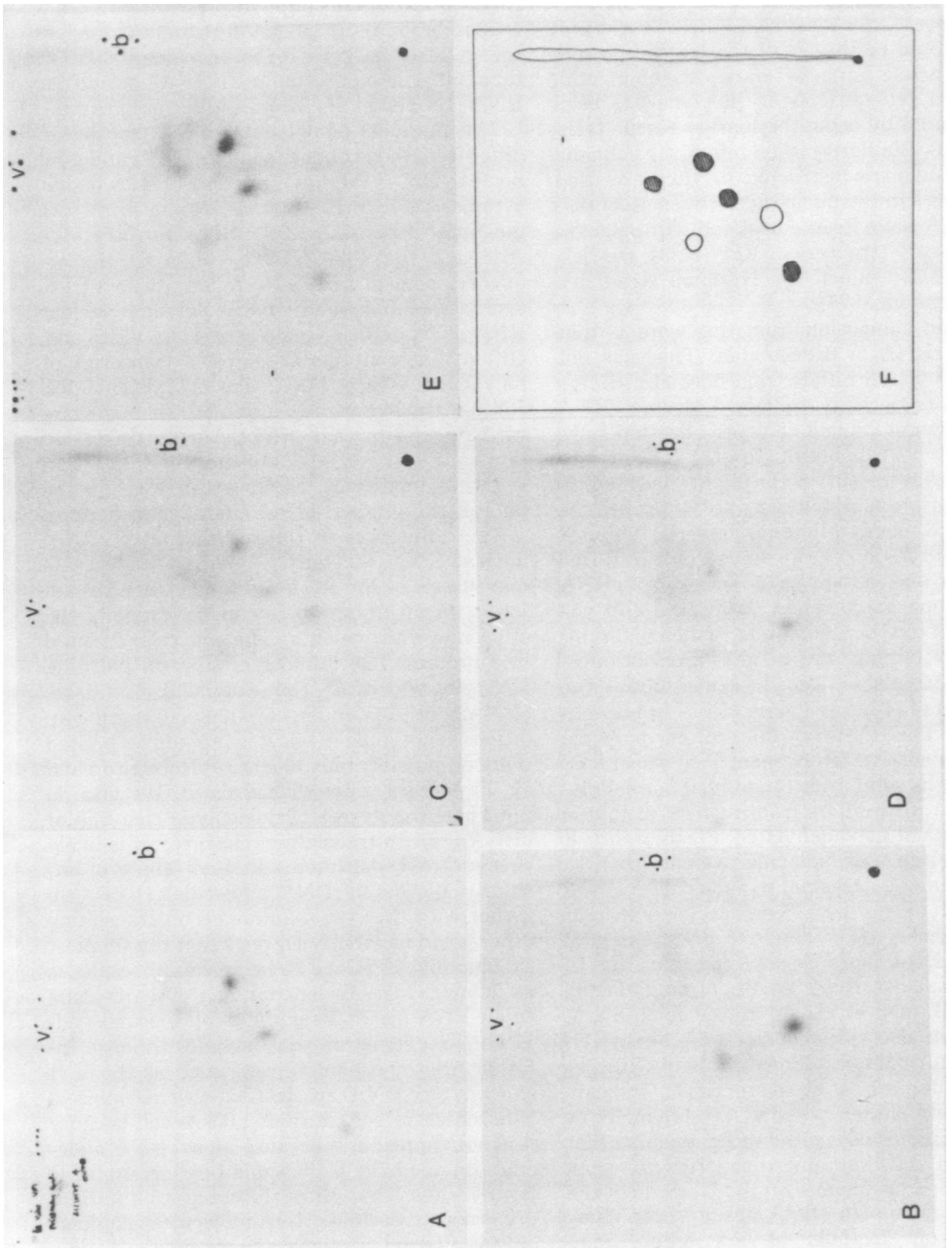

FIG. 4. *Two-dimensional fingerprints on cellulose of polyoma virus proteins VP1 and VP2 and polypeptides made in vitro. Electrophoresis in the first dimension was at pH 2.1 (origin in lower right hand corner, migration toward the cathode); chromatography in the second dimension (bottom to top) was in butanol-acetic acid-water-pyridine (15:3:12:10) (38). Tryptic digests of the following proteins are shown: (A) polyoma virus VP1; (B) polyoma virus VP2; (C) polypeptide made in vitro that comigrates with VP2; (D) mixture (10,000 cpm of each) of (B) and (C), (E) mixture (20,000 cpm of each) of (A) and (B); (F) schematic representation of (C) showing the origin of the different peptides (●, VP1 derived; ○, VP2 derived).*

the observation that late temperature-sensitive mutants of polyoma contain altered two-dimensional peptide maps in VP1 (13). But since revertants were not analyzed, these structural changes could be the result of silent mutations. A direct correlation between peptide changes in VP1 and a genetic locus of polyoma has been demonstrated by using the marker rescue technique (16a). Similarly, when infectious polyoma hybrid genomes constructed in vitro are used (25), changes in the nucleotide sequences of polyoma DNA have been correlated with changes in the primary structure of VP2 (R. M. Hewick, Ph.D. thesis, Council for National Academic Award, London, 1977).

A second conclusion from this work is that polyoma cRNA is translatable. This was not obvious as cRNA differs from normal mRNA's in several respects, as follows. Polyoma cRNA is probably not capped. This is suggested since (i) an activity capable of capping RNA is not known to be present in the highly purified *E. coli* transcription system used to synthesize the cRNA; (ii) *S*-adenosylhomocysteine, an inhibitor of cap methylation in the wheat germ cell-free system (3), does not inhibit translation of cRNA in vitro (our unpublished data); and (iii) the presence of m^7Gp, which under some conditions specifically inhibits the in vitro translation of capped mRNA's (5, 17), does not inhibit the translation of either polyoma cRNA or encephalomyocarditis RNA, but does inhibit polyoma mRNA translation (R. Kamen, T. Wheeler, and A. E. Smith, submitted for publication). Polyoma cRNA also differs from mRNA in that it is probably not polyadenylated. Furthermore, it has a wide range of molecular weights (Fig. 1), most of which correspond to molecules considerably larger than the 19S and 16S cytoplasmic mRNA species (22). The size distribution of polyoma cRNA more closely resembles that of polyoma nuclear RNA isolated from infected cells (21; Kamen et al., submitted for publication), which also varies in size from about 15S to 50S on formamide-sucrose gradients. Recent studies (summarized in reference 32) indicate that the late simian virus 40 (SV40) mRNA's are hybrid molecules containing sequences that are spliced together and thus originate from noncontiguous sequences on the L strand of the viral DNA. Although cRNA has not been characterized from this point of view, splicing may now be another characteristic that cRNA does not share with mRNA.

Although polyoma cRNA is translatable, the optimal in vitro conditions are different compared with other RNAs containing the same sequences. The K^+ optimum for cRNA translation (40 mM) is low compared with a normal mRNA such as actin (80 mM) or polyoma 16S RNA (110 mM) (L. Carrasco, R. Harvey, and A. E. Smith, manuscript in preparation). On a weight basis, more cRNA has to be added to the cell-free system for maximal stimulation than either late polyoma mRNA (38) or nuclear RNA (Kamen et al., submitted for publication).

The products of polyoma cRNA translation differ from those of both viral mRNA's and viral nuclear RNA. We have previously shown that cytoplasmic 16S RNA translates in vitro into polyoma VP1 and the 19S mRNA into VP2 (34). Although the 19S RNA contains all the sequences present in 16S RNA, very little VP1 is synthesized in vitro. Similarly, polyoma nuclear RNA is an efficient messenger in vitro and, although it contains a complete transcript of polyoma L-strand DNA, like cytoplasmic 19S RNA it translates only into VP2. In this work, we have shown that cRNA can be translated into both VP1 and VP2, but that the translation is rather inefficient; as shown in Fig. 2b, the bulk of the product is less than 25,000 daltons.

The differences in the translation of cRNA, nuclear RNA, and mRNA perhaps reflect the importance of the sequences and structures adjacent to an initiation site in determining the efficiency with which a gene will be translated into protein. The number of sites on polyoma DNA at which *E. coli* polymerase initiates cRNA synthesis under high-ionic-strength conditions is not known, but it is probably a limited number, possibly only one (A. J. Robbinson and W. F. Mangel, unpublished data). We also do not know the degree of nucleolytic cleavage of cRNA prior to translation. However, it is likely that cRNA constitutes a limited family of circularly permuted RNA molecules. This, together with the results obtained on translating other polyoma RNAs, suggests that the simplest explanation of the cRNA translation results is as follows. The efficient binding of a ribosome to an RNA occurs near the 5′ end, usually adjacent to a structure that includes the cap. Internal ribosome binding sites such as the VP1 site on 19S RNA are inefficiently recognized. Ribosomes do not translate cRNA well because it is not capped and because most cRNA molecules do not have a ribosome binding site near their 5′ ends. Thus, most ribosomes that initiate protein synthesis on cRNA molecules can do so only at internal positions, and consequently they bind inefficiently.

We do not know why so many ribosomes terminate synthesis prematurely to produce fragments of viral proteins or why such premature termination yields seemingly discrete-size polypeptides. We have obtained a similar result in translating SV40 cRNA which contains the

early mRNA sequences. The largest polypeptide synthesized that is immunoprecipitable by T antibody has a molecular weight of about 60,000 (27, 28, 33). Yet, with the early 20S mRNA from SV40-infected cells as message in the wheat germ cell-free system, a T antibody-precipitable polypeptide with a molecular weight of 94,000 is synthesized (6, 27). This is the apparent molecular weight of in vivo T antigen (6, 35). Thus, with both polyoma cRNA and SV40 cRNA, the major translation products are prematurely terminated fragments, although in the case of polyoma cRNA some full-length viral proteins are synthesized.

Similar experiments on the translation of mRNA (26) and of cRNA, using linked transcription/translation (29, 31), have been reported by using the closely related virus SV40. From all these results, it is clear that the structural proteins VP1, VP2, and VP3 of both polyoma and SV40 are coded for by the viral DNA.

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