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COMPARATIVE STUDY OF STRUCTURAL PECULIARITIES AND TRANSLATION OF POTEXVIRUS RNAs

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Structural peculiarities of the 3'-end segments of genomic RNA were studied in F potato virus (F-PV) and white clover mosaic virus (WCMV). The methods of affinity chromatography on oligo(dT) cellulose and oligonucleotide mapping revealed a prolonged (up to 210 nucleotides) polyadenyl sequence at the 3'-end of F-PV RNA. A polyadenyl sequence is missing at the 3'-end of WCMV RNA. A study of the translation products of WCMV and F-PV RNAs in a cell-free protein-synthesizing system derived from rabbit reticulocytes showed that polypeptides electrophoretically comigrating with a structural protein of either virus were synthesized alongside high-molecular-weight polypeptides ($M_r \approx 180$-150 kdaltons).

Potexviruses form a very numerous group of plant viruses that have flexible thread-like particles 480-580 nm long [1]. The capsule of these viruses, of spiral symmetry, consists of identical protein subunits of molecular weight 24-28 kdaltons. The virion RNA is a continuous polynucleotide chain of molecular weight 2.1-2.6 Mdaltons. It has been established rather recently that the RNA of some representatives of this group carries at the 5'-end a "cap" of the $m^7G(5')ppp(5')$ type [2-4], while the RNA of X potato virus has a polyadenyl sequence up to 200 nucleotide residues long at the 3'-end [5]. This RNA is an active template in the cell-free protein-synthesizing system [6, 7].

The criteria used for referring viruses to the potex-group are highly arbitrary and are mainly based on the size and form of the viral particles [8]. However, it cannot be precluded that viruses characterized by identical virion morphology may differ essentially in genome structure and the strategy of its expression. It was accordingly deemed useful to compare and characterize the structural peculiarities of the 3'-end regions of RNAs in several different potexviruses and study the peculiarities of their RNA translation in a cell-free protein-synthesizing system.

The viruses studied, besides X-PV, were two, F-PV and WCMV, also referred by Koenig and Leseman [8] to the potex-group on the basis of virion-morphology similarity, Although these viruses were described long ago [9], no data about the structure of the genome and peculiarities of its expression are available.

MATERIALS AND METHODS

Specimens of F-PV were isolated from stramonium (Datura stramonium L.) and a species of tobacco (Nicotiana glutinosa L.) as described previously [10]; WCMV was isolated from beans (Phaseolus vulgaris L., variety Bountiful) by a modification of the method of Miki and Knight [11]. Frozen leaves were homogenized in 0.2 M phosphate buffer, pH 7.8, containing 0.1% sodium sulfate and 0.2% EDTA. The homogenate was centrifuged at 10,000g for 15 min. The supernate was brought to pH 5.0 with 10% acetic acid, kept for 30 min at 4°C, and centrifuged at 10,000g for 10 min to remove cell proteins. Virus was sedimented from the supernate after adding polyethylene glycol (molecular weight 6 kdaltons) to a final concentration of 4% in the presence

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of 0.3 M NaCl. The sediment was spun down at 12,000 rpm for 15 min. Subsequent purification was by differential centrifugation followed by centrifugation in a sucrose gradient (10-40%) in 0.01 M Tris-HCl buffer, pH 7.0, at 25,000 rpm for 3 h (rotor SW27, Spinco L2-75B centrifuge). The RNA of F-PV and WCMV was isolated from purified virus preparations by the method of phenol deproteinization in the presence of sodium dodecylsulfate. A specimen of X-PV RNA was kindly provided by V. K. Novikov.

The nucleoside composition of RNA was determined, after exhaustive enzymatic hydrolysis, by the chromatographic method on a "Varian-8500" high-pressure liquid chromatograph, using hydrolysate of the RNA of a conventional TMV strain as standard. An amount of 20-25 μg RNA was diluted in 20 μliter of buffer, pH 8.4, containing NH_4HCO_3 and $MgCl_2$ of 0.05 and 0.02 M, respectively, 50 μg/ml pancreatic RNase, 50 U/ml RNase T1, 50 μg/ml snake-venom phosphodiesterase, and 20 μg/ml of Escherichia coli alkaline phosphatase, and was incubated at 37 °C for 18 h. The hydrolysate was evaporated dry and analyzed on an Aminex A-7 column.

Affinity chromatography of RNA on oligo(dT) cellulose (Sigma, USA) was done on a 1-ml column by the method described previously [12, 13].

RNA was fractionated by electrophoresis in 2.4% polyacrylamide gel containing 0.5% agarose and 7 M urea, at 200 V (direct current) for 2 h. Molecular weight was determined with the following markers: TMV RNA (M_r 2 Mdaltons) and BMV total RNA containing four species (1, 2, 3, 4) of molecular weights 1.09, 0.99, 0.75, and 0.28 Mdaltons [14].

The oligonucleotide analysis of RNA was done by the method of Pedersen and Haseltine [15]. An amount of 1 μg RNA in 10 mM Tris-HCl buffer containing 1 mM EDTA, pH 8.0, was treated with T_1 RNase (1 U/μg RNA) and E. coli alkaline phosphatase (4×10^{-4} U/μg RNA) for 90 min at 37 °C. The oligonucleotide mixture obtained was labeled at the 5'-end with [γ-^{32}P]-ATP (Amersham, United Kingdom) and phage-T4 polynucleotide kinase. Oligonucleotides labeled with ^{32}P were fractionated by two-dimensional electrophoresis in polyacrylamide gel. Dimension one: 10% gel, 25 mM citric acid, pH 3.5, in the presence of 6 M urea, 1700 V, 2-2.5 h; dimension two: 22.8% gel, 50 mM Tris-borate buffer, pH 8.3, containing 1 mM EDTA, 1200 V, 5-6 h. Labeling of the 3'-end of RNA with ^{32}P was done using 5'-[^{32}P]cytidine diphosphate (Amersham, UK) and phage-T4 RNA ligase by the method of England and Uhlenbeck [16], with some modifications. After introducing the 3'-end label, F-PV RNA was hydrolyzed by T1 RNase (1 U/μg RNA) (50 mM Tris HCl buffer, pH 7.5, in the presence of 10 mM EDTA for 1.5 h at 37 °C), and the hydrolysis products were separated by two-dimensional electrophoresis in polyacrylamide gel as described above.

One-dimensional fractionation of 5'-[^{32}P]-oligonucleotides was done by electrophoresis in a 10% polyacrylamide gel in 50 mM Tris-borate buffer, pH 8.3, in the presence 1 mM EDTA and 7 M urea.

Labeled oligonucleotides were extracted from the gel with a solution containing 1 mM EDTA and 200 μg/ml tRNA for 3 h at 37 °C. The oligonucleotides were then sedimented with ethanol in the presence of 0.3 N sodium acetate and 0.01 M magnesium acetate, pH 6.0, and the sediment was washed with ethanol. It was dried and dissolved in 10 μliters of 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM EDTA.

Sequencing of 5'-[^{32}P]-oligonucleotides was done by the method of Donis-Keller et al. [17] after their partial enzymatic hydrolysis followed by fractionation of the hydrolysis products by electrophoresis in a 25% polyacrylamide gel.

RNA was translated in a cell-free protein-synthesizing system (rabbit reticulocyte lysates) by the method of Pelham and Jackson [18].

Rabbit reticulocyte lysate treated with micrococcus nuclease was obtained from O. N. Denisenko (Institute of Protein, Pushchino). The cell-free system was optimized for the translation of F-PV and WCMV RNA. The incubation mixture (20 μliters) contained 8 μliters of lysate; 20 mM HEPES-KOH buffer, pH 7.6; 2.0 mM magnesium acetate; 140-200 mM potassium acetate; 1 mmole ATP; 0.2 mmole GTP; 15 mmole creatine phosphate; 19 nonlabeled amino acids (0.2 mmole of each); ^{14}C-labeled amino acids (0.3 μCi) or [^{35}S]methionine (1275 Ci/mmole, Amersham, UK) (1 μCi); 3 μg wheat tRNA. The concentration of F-PV RNA varied from 30 to 150 μg/ml. The mixtures were incubated at 30 °C for 60-120 min. Translation products were analyzed by electrophoresis on plates of 10% polyacrylamide gel by the method of Laemmli [19], but the acrylamide–N,N'-methylene bis-acrilamide ratio was 150:1 to prevent gel cracking during drying. The markers were a mixture of carbon-labeled proteins (Amersham, UK), viz: myosin (20,000), phosphorylase (92,500), bovine-serum albumin (68,000), ovalbumin (45,000), carboanhydrase (30,000), and lysozyme (14,300). The gel was stained with Coomassie brilliant blue R-250, dried, and radioautographed on an ORWO HS-11 film.

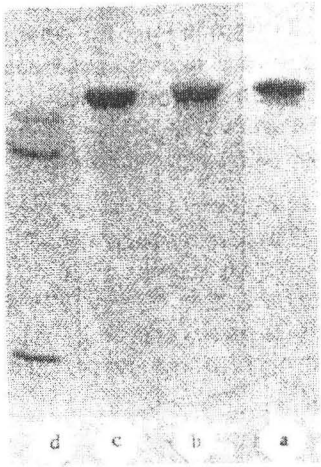

Fig. 1. Electrophoresis of virion RNA of F-PV (c) and WCMV (d) in 2.4% polyacrylamide gel containing 0.5% agarose and 7 M urea. Total BMV RNA (a) and TMV RNA (b) were used as markers.

RESULTS AND DISCUSSION

Chromatography of F-PV and WCMV RNase on an Oligo(dT)-Cellulose Column. The specimens of WCMV and F-PV virion RNA obtained were homogeneous to a satisfactory degree (Fig. 1, c and d). According to polyacrylamide-gel-electrophoresis data, the molecular weights of WCMV and F-PV RNA were 2.0 and 2.5 Mdaltons, respectively.

The nucleoside composition of both RNAs (results not shown) was characterized by a high content of adenosine (31.7% for F-PV RNA and 30% for WCMV RNA), which is typical of potexvirus RNAs in general and suggests the presence of polyadenyl blocks in their structure.

Affinity chromatography showed that in a buffer of high ionic strength 77% of F-PV RNA was sorbed to the oligo(dT)-cellulose column and was eluted from it with water, i.e., it contained lengthy polyadenyl sequences (a poly(A)$^+$ fraction). Under the same conditions, specimens of polyadenylic acid were sorbed up to 99%, while specimens of TMV RNA, not containing a poly(A) sequence, to only 5-10%. As electrophoresis results showed, the poly(A)$^+$ fraction of F-PV RNA did not differ from nonfractionated RNA in migration velocity in polyacrylamide gel, whereas the fraction eluted from the column with high-ionic-strength buffer (poly(A)$^-$ fraction) was a mixture of fragments heterogeneous in size.

In contrast to F-PV RNA, ~83% of WCMV RNA did not attach to oligo(dT)-cellulose in high-ionic-strength buffer. The RNA fraction not uniting with the sorbent was homogeneous in analysis in polyacrylamide gel, and did not differ from the starting WCMV RNA specimen by its electrophoretic behavior. These results indicate that lengthy blocks of poly(A) are missing in WCMV RNA, which distinguishes this virus substantially from the other two representatives, X-PV and F-PV, of the potexgroup. The relatively high content of adenine in WCMV RNA is apparently due to the presence of short intercalary blocks in the viral RNA molecule. One cannot fully preclude that the end block of poly(A) may have been lost as a result of degradation in the process of the isolation, but this seems rather improbable. It should be pointed out that in their early studies Sonenberg et al. [4] did not find any poly(A) sequences in X-PV RNA, in contrast to Morozov et al. [5].

Comparative Analysis of X-PV, F-PV, and WCMV Genomic RNAs by Oligonucleotide Mapping. Oligonucleotide maps of genomic X-PV, F-PV, and WCMV RNAs hydrolyzed by T1 RNase were obtained by two-dimensional electrophoresis of the hydrolysates in polyacrylamide gel. Large, T1-RNase-resistant blocks of electrophoretic mobility characteristic for poly(A)-containing fragments (Fig. 2a, b) were found among the oligonucleotides obtained from F-PV and X-PV, but were not present in WCMV RNA (Fig. 2c).

One-dimensional electrophoresis in 10% polyacrylamide gel in the presence of markers allowed tentative estimation of the size of the poly(A)-containing fragments. They were 210 and 250 nucleotides long in F-PV and X-PV RNA, respectively. To prove that the poly(A)-containing blocks were localized at the 3'-end of the molecule, specimens of F-PV were labeled at the 3' end with [5'-^{32}P]cytidine diphosphate using phase-T4

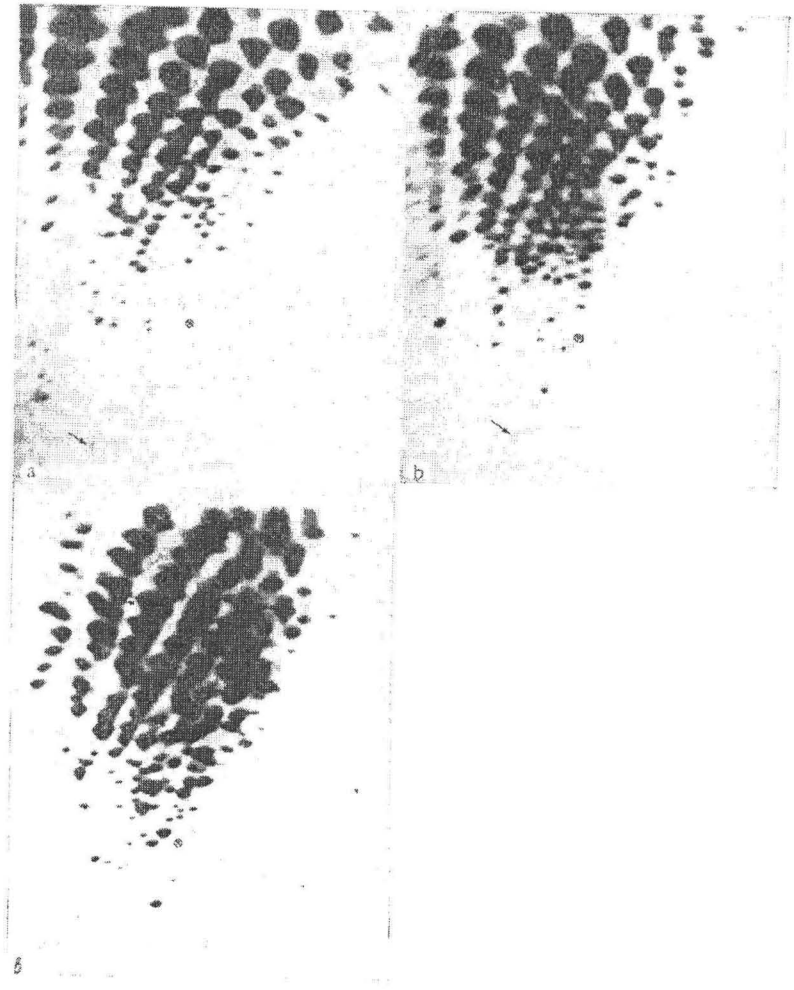

Fig. 2. Oligonucleotide maps of RNA hydrolysates: a) X-PV, b) F-PV, c) WCMV. T1 RNase was used. Left to right dimension 1; bottom to top, dimension 2. Crosses denote position of strains: bromphenol blue (top) and xylene cyanol FF (bottom). Arrows indicate poly(A)-containing fragments.

RNA ligase. Subsequent hydrolysis of the labeled RNA with T1 RNAse and analysis of the hydrolysates by two-dimensional electrophoresis in polyacrylamide gel showed that all of the radioactive label was concentrated in the region of the enriched poly(A) fragments (Fig. 3). To determine the sequence of nucleotides adjacent to the poly(A) block, F-PV RNA was cleaved with T1 RNase and a 5'-end label was introduced into the forming oligonucleotides using [γ-^{32}P]-ATP and polynucleotide kinase. Following electrophoresis in polyacrylamide gel, the poly(A)-containing fragments were eluted and subjected to direct sequencing (Fig. 4). In their primary structure the segments adjacent to the poly(A) block in the F-PV and X-PV RNA proved closely related [5]:

FB-K 5' ... AAUAAU— poly (A) 3'
X-BK 5' ... AAAAAUAUAAAU— poly (A) 3'

Thus, the presence of lengthy poly(A)-containing blocks at the 3'-end of the F-PV RNA molecule relates it with the RNA of X-PV (a typical representative of potexviruses). On the other hand, a 3'-end-poly(A) sequence was absent from the RNA structure of WCMV, which is also classified with the potexviruses and, according to [9], is antigenically related with X-PV.

Translation Peculiarities of F-PV and WCMV RNA in a Cell-Free System from Rabbit Reticulocytes. The cell-free system was optimized for F-PV and WCMV RNA translation. Maximum stimulation of the inclusion of ^{14}C-labeled amino acids into polypeptides was observed at a 140-200 mM potassium acetate and a

Fig. 3

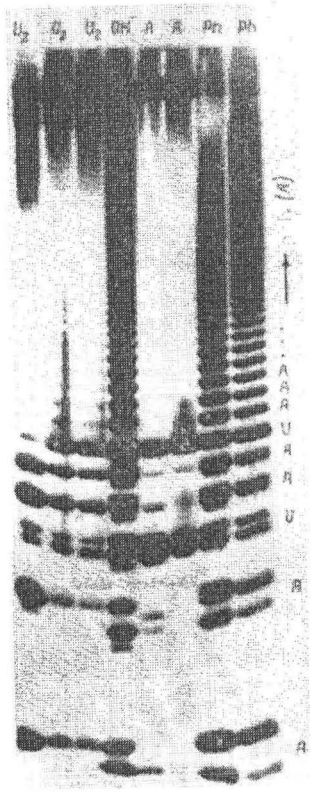

Fig. 4

Fig. 3. Analysis of F-PV RNA labeled at the 3'-end. [^{32}P]-RNA was treated with T1 RNase and the hydrolysis products were fractionated by two-dimensional electrophoresis in polyacrylamide gel. Cross denotes position of stain xylene cyanol FF.

Fig. 4. Determination of primary structure of segment adjacent to the 3'-end poly(A) sequence in F-PV RNA. Lane OH^-, alkaline hydrolysis; lanes U_2, A, and Ph, ribonucleases U2, A, and RNase from Physarium polycephalum, respectively (different concentrations). Right: nucleotide sequence of the F-PV RNA segment, read from the autoradiogram.

2 mM magnesium acetate concentration; the optimal concentration of the virus RNA template under these conditions was 65 μg/ml. Analysis of the F-PV-RNA translation products showed heavy polypeptides of molecular weight ~180, 160, and 150 kdaltons as the basic material. A minor amount of a component of molecular weight ~190 kdaltons was also synthesized (Fig. 5b). Among the low-molecular-weight products of F-PV RNA translation a polypeptide of 26 kdalton was found that in electrophoretic mobility coincided with the structural protein of the virus.

The translation of WCMV RNA (Fig. 5c) resulted in a rather heterogeneous group of products, among which polypeptides of molecular weight 170 and 155 kdaltons were detected. It had previously been shown that in a cell-free system derived from wheat germs, WCMV RNA directs the synthesis of proteins of molecular weight 170 and 140 kdaltons [20]. The system of reticulocyte lysates also contained among the basic WCMV-RNA translation products a low-molecular-weight polypeptide, of 24 kdaltons, that coincided with the structural protein of the virus in mobility.

The RNA of X-PV controlled the synthesis of high-molecular-weight polypeptides, among which products of molecular weight 180 and 145-150 kdaltons were prominent. In contrast to WCMV and F-PV RNAs, however, the genomic RNA of X-PV did not, in the in vitro translation, code for a protein identical in molecular weight to the envelope protein of this virus (Fig. 5a); this is in agreement with other authors' results [6, 7].

If the polypeptides of molecular weight 26 and 24 kdaltons encoded in vitro by F-PV and WCMV RNAs

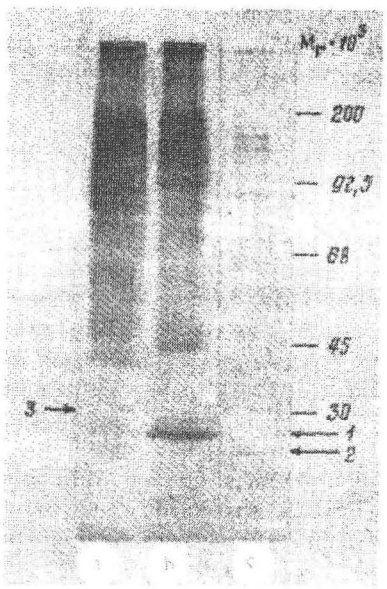

Fig. 5. Electrophoresis of in vitro translation products of X-PV RNA (a), F-PV RNA (b), and WCMV RNA (c) in an 8-20% polyacrylamide-gel gradient containing 0.1% sodium dodecylsulfate. Arrows indicate position of F-PV (1), WCMV (2), and X-PV (3) structural proteins. The numbers correspond to the molecular weight of protein markers.

correspond to the structural proteins of these viruses, it is very probable that specimens of nonfractionated viral RNA include a subgenomic RNA for structural protein. According to the electrophoresis data, the polyacrylamide gels with F-PV and WCMV RNA specimens did not, under the denaturing conditions, contain RNAs that corresponded in size to the subgenomic RNAs of the envelope proteins of these viruses, which, nevertheless, does not fully preclude the possibility that subgenomic RNAs were present in small quantities. Recently the presence of such an RNA has been detected in another potexvirus, the narcissus-mosaic virus [21].

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