Two Unique RNA Species of the Nodavirus Black Beetle Virus

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Two-dimensional fingerprinting of RNase T₁-derived oligonucleotides of the two individual RNA segments of the Nodavirus black beetle virus indicates that each RNA species possesses a distinct nucleotide sequence. Species 1 RNA has a genome complexity of approximately 3,000 nucleotides, and species 2 RNA is composed of approximately 1,500 nucleotides. Submolar amounts of oligonucleotides apparently derived from a third virus-specific RNA were also detected in black beetle virus RNA preparations.

The Nodaviridae are a recently classified family of small RNA viruses of invertebrates that possess a bisegmented genome (15, 15a). The type species of this family is Nodamura virus, which has two virion RNA species of $\approx 1 \times 10^6$ and 0.5×10^6 daltons, both of which are required for infectivity (16, 17, 19). Nodamura virus RNA lacks 3'-terminal polyadenylic acid (18) but has activity in in vitro translation systems. Translation of the larger RNA yields a 105,000-molecular-weight (105K) protein, and the smaller RNA codes for a 43K protein (20). Mature virions contain a major capsid protein of 40K and two minor proteins of 39K and 43K.

Black beetle virus (BBV), which was isolated from *Heteronychus arator* (13) and has properties very similar to those of Nodamura virus, is a candidate member of the Nodaviridae. Analyses of the structural components of BBV have established that there is one major capsid protein of 40K, and a small-molecular-weight (5K) polypeptide of unknown function. In addition, small amounts of two other proteins have been detected, including a possible capsid protein precursor (8, 9, 14). In vivo and in vitro protein synthesis studies have shown that the virus codes for a large protein of 110K, which is a candidate RNA polymerase, a capsid protein precursor, and an 8 to 10K polypeptide of unknown function (5, 6, 8, 12).

There are two single-stranded virion RNA species comprising the genome of BBV with sedimentation coefficients of 22S and 15S, corresponding to molecular weights of 1.0×10^6 (species 1) and 0.5×10^6 (species 2), respectively. These RNAs are packaged in the same virus

particle (14). Species 1 RNA codes for the 110K protein and species 2 for a capsid protein precursor (6, 12). Although such data are compatible with the virus possessing two RNA species with unique nucleotide sequences, they do not provide direct evidence. The purpose of this communication is to provide such evidence and thus demonstrate that the two RNA species of BBV are unrelated.

MATERIALS AND METHODS

Cells and virus. *Drosophila melanogaster* cells were grown as monolayers at 28°C in Schneider *Drosophila* medium (revised) (GIBCO Europe Ltd., Uxbridge, United Kingdom) as previously described (5). BBV for cell infection was prepared from the initial stock by three serial passages at low multiplicity of infection through *Drosophila* cells and was stored at -70°C.

Infection of cells and virus purification. Confluent 80-cm² monolayers of cells were infected with 1.5 PFU/cell at 28°C. The inoculum was removed after a 1-h adsorption period, and the cells were overlaid with 5 ml of Schneider phosphate-deficient medium (supplemented with 2% dialyzed fetal calf serum plus antibiotics) containing 100 μ Ci of ³²P (Amersham International Ltd., Amersham, United Kingdom) per ml. After 4 days, the cells were disrupted by three freeze-thaw cycles to release the virus, which was purified by differential centrifugation and sucrose velocity gradients (5).

Purification of labeled viral RNA species. Labeled RNA was extracted from the purified virus by digestion of the protein for 1 h with 500 μ g of proteinase K (The Boehringer Corp. Ltd., Lewes, East Sussex, United Kingdom) per ml in TNE buffer (10 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA). The protein was removed by extraction with 2 volumes of TNE-equilibrated phenol for 10 min. After separation of the phases by centrifugation, the aqueous phase was removed, and the phenol phase and interphase were reextracted with buffer. The pooled aqueous phases were further extracted with phenol. NaCl was added to the aqueous phase to a final concentration of 0.3 M,

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and the RNA was precipitated overnight at -20°C after the addition of 2.5 volumes of ethanol. The RNA precipitate was collected by centrifugation at $2,000 \times g$ in an MSE Chilsbin for 15 min, washed four times with ethanol and twice with ether, and dissolved in water after drying under vacuum for 2 h in a desiccator.

Radiolabeling and purification of cellular RNA. *Drosophila* cells were seeded at $\approx 10^6$ cells per 80-cm^2 plastic flask (Nunc Plastics, Kamstrup, Denmark) in Schneider growth medium (10% fetal calf serum) and maintained overnight at 28°C . The cell sheet was then washed with 5 ml of Schneider phosphate-deficient medium and reincubated at 28°C in 5 ml of this medium containing 5% dialyzed fetal calf serum and $100 \mu\text{Ci}$ of ^{32}P per ml until the cells were confluent (48 h). After labeling, the cell sheet was washed with 5 ml of sterile phosphate-buffered saline (Dulbecco A [pH 7.3]), and the cells were scraped off into an additional 5 ml. The cells were pelleted at $1,000 \times g$ for 5 min, washed twice in sterile phosphate-buffered saline, and then suspended in TNE buffer (pH 7.4). After lysis of the cells with 1% nonionic detergent (Nonidet P-40), the nuclei were removed by centrifugation ($3,000 \times g$ for 15 min), and the RNA was purified from the supernatant by using proteinase K and phenol extraction as described above.

Sucrose gradient resolution of viral RNA species. Purified RNA was dissolved in 0.1 M sodium acetate (pH 5.0) for sucrose gradient centrifugation. The RNA was layered onto a 12-ml 5 to 25% (wt/wt) sucrose gradient in 0.1 M sodium acetate and centrifuged at $80,000 \times g$ for 16 h at 10°C in an MSE $6 \times 14\text{-ml}$ swing-out rotor. The gradient was harvested by downward displacement, and the RNA-containing fractions were determined by assaying for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer. The fractions corresponding to RNA 1 and RNA 2 were precipitated separately with ethanol and redissolved in water.

Analytical gel electrophoresis of viral RNA species. RNA species were resolved by electrophoresis in 1% (wt/vol) agarose gels in 50% (vol/vol) formamide by using a horizontal, submerged gel system as previously described (3).

Resolution of RNase T_1 -derived oligonucleotides by two-dimensional polyacrylamide gel electrophoresis. The digestion of ^{32}P -labeled RNA by RNase T_1 and the resolution of oligonucleotides by two-dimensional polyacrylamide gel electrophoresis were done by the procedures developed by De Wachter and Fiers (7) as modified by Clewley and associates (4).

Extraction and RNase A analyses of T_1 -derived oligonucleotides. Oligonucleotides in polyacrylamide gels were located by autoradiography, and gel plugs containing resolved oligonucleotides were punched out with a cork borer. Each gel plug was placed in a sterile minivial, and radioactivity was determined by Cerenkov counting. Selected samples were incubated overnight in 0.5 ml of distilled water at 4°C , after which time the water was removed, the gel slice was crushed, and 1 ml of 1 M NaCl was added for a further overnight incubation. The two aqueous supernatants were combined, and insoluble acrylamide was removed by centrifugation. Oligonucleotides were recovered by centrifugation after the addition of 0.25 ml of 4 M NaCl, 2 ml of isopropanol, and 50 μg of carrier tRNA and

subsequent incubation at -20°C overnight. Between 60 and 80% of the radioactivity was recovered. The precipitated materials were lyophilized and digested with 0.005 ml of RNase A (2 mg/ml) in 0.1 M ammonium bicarbonate (pH 8.0). The resulting products were then resolved by the thin-layer chromatography procedure described by Volckaert and Fiers (21) as modified by Clerx-Van Haaster and associates (2).

RESULTS

RNA species of BBV. The RNA extracted from purified preparations of ^{32}P -labeled BBV was resolved in a sucrose gradient, and two major peaks of radioactivity were observed (Fig. 1). Fractions of the gradient were pooled to obtain each RNA species as free as possible from the other RNA species. A sample of each fraction was analyzed by agarose gel electrophoresis to determine its degree of purity (Fig. 2). Species 1, the larger molecular weight RNA, was found to contain contaminating amounts of species 2 and a minor RNA component (arrow in Fig. 2). Species 2, the lower molecular weight RNA, contained minor amounts of species 1 and two RNA bands migrating faster than species 2. The origin of these extra bands is discussed later.

Two-dimensional gel electrophoresis of oligonucleotides obtained from individual RNA species of BBV. The two ^{32}P -labeled RNA species isolated from a sucrose gradient were digested with RNase T_1 , and the digests were resolved by two-dimensional gel electrophoresis and autoradiography (Fig. 3A and B). In addition, a mixture of the two RNAs was digested with RNase T_1 and analyzed (Fig. 3C). For reference purposes, the oligonucleotides are numbered in Fig. 3C.

The RNase T_1 -derived oligonucleotides of

FIG. 1. Sucrose gradient fractionation of BBV RNA. Centrifugation conditions are described in the text. Fractions 10 to 12 and 14 to 16 were pooled to give RNA 2 and RNA 1, respectively.

FIG. 2. Electrophoresis of ^{32}P -labeled BBV RNA in a denaturing agarose gel. RNA from sucrose gradient fractions 14 to 16 (lane 1) and 10 to 12 (lane 2) was analyzed as described in the text. The arrow indicates a minor component present in RNA 1.

species 1 RNA were more numerous than those of species 2, indicating the greater nucleotide complexity of species 1 RNA. Each autoradiograph had oligonucleotide spots of two different intensities. With the exception of spots 1 to 5 and 11, the other faint spots in the species 2 pattern mimic the well-pronounced spots in the species 1 fingerprint, and vice versa. We conclude that the faint spots in the species 2 fingerprint are derived from contaminating species 1 RNA in the species 2 RNA preparation. Again with the exception of spots 1 to 5 and 11, the faint spots in the species 1 fingerprint are derived from contaminating species 2 RNA.

The large RNase T_1 oligonucleotides unique to each RNA by this analysis are indicated by arrows in Fig. 3A and B. There are 15 oligonucleotides unique to species 1 and 7 oligonucleotides unique to species 2. There are no apparent large oligonucleotides shared by both RNAs. The two RNA bands migrating faster than species 2 RNA in Fig. 2 (lane 2) are likely to be

FIG. 3. Oligonucleotide fingerprint analysis of BBV RNA and uninfected *Drosophila* cell RNA. (A) RNA from fractions 14 to 16 of a sucrose gradient. The arrows indicate those oligonucleotides unique to species 1 RNA. (B) RNA from fractions 10 to 12 of a sucrose gradient. The arrows indicate those oligonucleotides unique to species 2 RNA. (C) Coelectrophoresis of a mixture of RNAs 1 and 2. The unique oligonucleotides are numbered for reference. Oligonucleotides 1 through 5 and 11 are of unknown origin; 6, 7a and b, 8, 12, 14, 16a and b, 17, 18, 20, 21, and 22 are derived from species 1 RNA; 9, 10, 13, 15, and 19 are derived from species 2 RNA. Oligonucleotides 23 through 26 are unassigned. (D) RNA isolated from uninfected *Drosophila* cells. Electrophoresis in the first dimension is from left to right at pH 3.5 in a 10% gel, and from bottom to top at pH 8.3 in a 22% gel in the second dimension. The lower cross is the xylene cyanol dye marker, and the upper cross is the bromophenol blue dye marker.

specific degradation products of species 2 RNA, since no unique oligonucleotides can be assigned to them. There is no evidence for the presence of a polyadenylic acid tract in either of the fingerprints.

The faint spots 1 to 5 are present in the species 1 fingerprint, but only in very low amounts in the species 2 fingerprint. They are present in submolar quantities compared with the oligonucleotides unique to species 1. Oligonucleotide 11 is present in submolar amounts in the species 2 fingerprint but is roughly equally represented in the species 1 fingerprint. To determine if these oligonucleotides are derived from cellular RNA either contaminating the virus preparation or packaged by the virus, total intracellular [³²P]RNA from uninfected *Drosophila* cells was prepared and fingerprinted (Fig. 3D). It is clear that oligonucleotides 1 to 5 are not present in the fingerprint of *Drosophila* intracellular RNA. Oligonucleotide 11 may be derived from *Drosophila* RNA as it migrates in an area of the gel in which there are many *Drosophila* oligonucleotides.

If oligonucleotides 1 to 5 are not derived from species 1 or 2 RNAs, they may originate from the third faint RNA band detected in purified virions (Fig. 2, lane 1 [arrow]). The origin of this RNA is not presently known.

Complexity analysis of BBV RNAs. The nucleotide complexities of BBV species 1 and 2 RNAs were calculated after elution of selected oligonucleotides from the gel and determination of their pancreatic RNase composition. For each fingerprint, two oligonucleotides (6 and 7a and b for species 1; 10 and 19 for species 2) were eluted

from gel plugs, recovered, and digested with pancreatic RNase. The digestion products were resolved by thin-layer polyethyleneimine two-dimensional chromatography as described earlier. For each digest, the phosphate-specific activity was deduced from the label recovered in the guanine-containing nucleotide. Thus, for an oligonucleotide yielding AG, one-half of the recovered AG label was taken to be the phosphate-specific activity. The moles of each nucleotide in a digest were then calculated by dividing their counts by the phosphate-specific activity and multiplying by the nucleotide size. The complexity of each RNA species was determined by the formula: complexity of RNA = (input cpm × oligonucleotide chain length)/cpm in oligonucleotide (1).

The results obtained for the four selected oligonucleotides are shown in Table 1. The approximate complexity of species 1 RNA was determined to be 3,129 nucleotides, a result in agreement with the theoretical complexity of 2,967 calculated, assuming a molecular weight of 1.0×10^6 and an average nucleotide molecular weight of 337. The complexity of species 2 RNA was shown to be 1,480 nucleotides, again in good agreement with the predicted complexity of 1,484, assuming a molecular weight of 0.5×10^6 .

DISCUSSION

The two RNA segments of BBV can be distinguished by oligonucleotide fingerprinting, providing further evidence of a segmented genome for this virus. Nucleotide complexity measure-

TABLE 1. Pancreatic RNase digests of selected BBV T₁ oligonucleotides

Oligonucleotide	Composition	Chain length	cpm	Complexity of RNA ^a
Species 1				
6	1AG, 3A ₃ U, 3A ₂ U, 2AU, 1A ₃ C, 3AC	37	2,007	3,268
7a and b ^b	1A ₃ G, 1AG, 2A ₃ U, 3A ₂ U, 4AU, 1A ₃ C, 4A ₂ C, 2AC	51	3,023	2,990
				mean = 3,129
Species 2				
10	1AG, 2-3U, 1AU, 1A ₃ C, 2A ₂ C, 1AC, 1C	19	1,140	1,490
19	1AG, 3U, 1AU, 2A ₂ C, 1AC, 1C	16	974	1,469
				mean = 1,480

^a In nucleotides, determined from formula:

$$\text{Complexity} = \frac{\text{input cpm of RNA} \times \text{chain length of oligonucleotide}}{\text{cpm of oligonucleotide}}$$

The input cpm was 177,288 for species 1 RNA and 89,432 for species 2 RNA. These figures are corrected for radioactive decay. The counts per minute of the oligonucleotides were those obtained on initial counting of the whole gel plug.

^b Mixture of two oligonucleotides.

ments indicate that the length of species 1 RNA is about 3,000 nucleotides and that of species 2 about 1,500 nucleotides. This is in good agreement with the molecular weight determined by sucrose gradients and gel electrophoresis measurements of $\approx 1.0 \times 10^6$ and 0.5×10^6 .

It is unlikely, therefore, that the two RNA segments contain significant common sequences.

The presence of a bipartite genome confers on these viruses the potential ability to undergo recombination by genome reassortment between different virus subtypes or between different virus species of the Nodaviridae. Whether this occurs in the field is unknown. Potentially, it may be possible to generate reassortants between BBV and Nodamura virus in the laboratory, as has been achieved for various bunyaviruses (10, 11).

The oligonucleotides present in submolar quantities (1 to 5) are apparently derived from virus-specific or virus-induced cellular RNA, as they are not found in RNA from uninfected *Drosophila* cells. If they are virus specific, they may reflect some sequence heterogeneity in species 1 RNA. Unfortunately, it was not possible to recover sufficient quantities of these oligonucleotides to perform sequence analyses on them. Alternatively, they may be due to packaging by the virus of a viral intracellular RNA, for instance, negative-stranded RNA or a replicative intermediate. If this is the case, the likely candidate source of these oligonucleotides is the RNA band visible in Fig. 2 (lane 1), migrating more slowly than the species 2 RNA. It is unlikely that these oligonucleotides are derived from a third genome segment, as they are present in 1/10 of the molar quantity of species 1 and 2, which are equimolar.

Oligonucleotide 11 migrates in an area of the gel to which many *Drosophila* intracellular RNA T₁ oligonucleotides would be expected to migrate. Thus, it may be derived from a contaminating *Drosophila* RNA, although this is unlikely, as other oligonucleotides derived from this putative RNA would be expected to be apparent. Oligonucleotide 11 may therefore also be derived from the RNA species giving rise to oligonucleotides 1 to 5.

Resolution of these questions will have to await further molecular characterization of the replication cycle of the Nodaviruses.

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