A Small RNA Virus with a Divided Genome from *Heteronychus arator* (F.) [Coleoptera: Scarabaeidae]

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**SUMMARY**

Some properties of a small RNA virus isolated from larvae of *Heteronychus arator* [Coleoptera: Scarabaeidae] are described. The virus develops in the cytoplasm of gut and fat body cells and is 30 nm in diameter. The sedimentation coefficient of virus was 137S and the buoyant density was 1.33 g/ml. The single stranded RNA was isolated as two species which sedimented at 22S and 15S, corresponding to mol. wt. of 100 and 5 × 10⁶ respectively. The base ratios of the two RNA species were 22S: A = 29.0, C = 24.1, G = 22.8, U = 24.1; 15S: A = 26.4, C = 23.8, G = 24.7, U = 25.2. There was one major polypeptide, mol. wt. 40000 and two minor polypeptides in the virus. The virus was infective for *Galleria mellonella* and some other insects, but was not infective for mice. No evidence was obtained to suggest that the two RNA species were present in different particles.

**INTRODUCTION**

The black beetle, *Heteronychus arator* has become a major pest of improved pasture in New Zealand since it was first detected in 1937 (Todd, 1959). A small isometric virus was isolated from 25% of a larval population of *H. arator* near Auckland (Longworth & Archibald, 1975), and the virus was found in pupae and adult beetles. The virus was transmitted by injection to larvae of *Galleria mellonella* and other Lepidoptera, and a fatal paralysis ensued after 10 to 30 days; the virus was shown to develop in the cytoplasm of gut and fat-body cells of black beetle larvae.

A preliminary study of the properties of the virus showed striking similarities with Nodamura virus (Newman & Brown, 1973); in particular both viruses contain two species of RNA. The present paper describes some properties of the *H. arator* virus.

**METHODS**

*Virus production.* 12-day-old *Galleria* larvae were injected with 10 μl of a virus preparation containing 0.3 mg/ml of virus and 1000 international units of penicillin/ml. The larvae were reared at 25 °C for 10 to 30 days and dead larvae were collected and stored at −15 °C. Virus was labelled by inoculating *Galleria* larvae 8 days p.i. with 15 μCi ³²P-phosphate on 3 successive days.

*Virus purification.* Infected larvae were macerated whilst frozen and homogenized in 0.1 M-phosphate buffer, pH 7.6. The homogenate was clarified at 10000 g for 30 min and virus was sedimented from the supernatant fluid at 100000 g for 2 h. The pellet was resuspended in phosphate buffer, clarified at 5000 g and then centrifuged for 3 h in a 20 to 50%
w/w, sucrose gradient in the same buffer at 110,000 g. The virus band was removed and two further cycles of gradient sedimentation were performed.

Pre-formed cesium chloride gradients from 1.2 to 1.4 g/ml were prepared in 0.1 M-phosphate buffer, pH 7.2. Sedimentation was for 6 h at 110,000 g and extinction and refractive index of 0.5 ml fractions were determined.

Sedimentation coefficients were determined in a Spinco Model E analytical ultracentrifuge; 1 mg of virus in 0.1 M-phosphate buffer, pH 7.6, was centrifuged at 29,500 rev/min in an AND rotor with temperature controlled at 20 °C. Turnip yellow mosaic virus (TYMV = 113S) was used as an internal standard.

Polyacrylamide gel electrophoresis of virus polypeptides. Acrylamide gel electrophoresis was carried out according to the method of Shapiro, Vinuela & Maizel (1967). The mol. wt. of the structural proteins of the Heteronychus arator virus were determined by a modification of the procedure of Weber & Osborne (1969) described by Longworth, Payne & Macleod (1973). The gels were stained in 0.2 % Coomassie brilliant blue in methanol:acetic acid: water (7.5:1:22.5) for 20 min and destained overnight in the same solvent. The gels were scanned in a Pye Unicam SP1809 densitometer. For mol. wt. determinations the following standards were used: bovine serum albumin 68,000, ovalbumin 43,000, lactate dehydrogenase monomer 36,000, trypsin 23,300 and cytochrome c 12,384.

Polyacrylamide gel electrophoresis of the virus RNA. The virus RNA was analysed in 2.4 % polyacrylamide gels containing 0.6 % agarose and 6 M-urea. RNA suspended in 50 μl of electrophoresis buffer (Loening’s buffer, pH 7.8) containing 2 M-urea was electrophoresed in 9 cm gels at 50 V for 2.5 h. The unstained gels were analysed directly in a densitometer at 260 nm or at 570 nm after staining with 0.01 % methylene blue in 0.02 M-sodium acetate, pH 7.0, for 10 min.

The gels were destained overnight in a large excess of distilled water. For mol. wt. determinations, Galleria ribosomal RNA (26S, 17S and 4S) was included as a marker.

Sedimentation analysis of the virus RNA. The RNA was extracted from purified virus with phenol alone, with phenol-SDS or with pronase-SDS. Phenol-extracted RNA was first precipitated by adding 2 vol. ethanol at -20 °C and after 2 h the precipitate was collected at 4000 g, dissolved in 0.1 % SDS-0.1 M-acetate, pH 5.0, and then subjected to sedimentation analysis. Pronase-SDS treated virus samples were centrifuged directly on 5 to 30 %, w/w, sucrose gradients made in 0.1 % SDS-0.1 M-acetate, at 70,000 g for 16 h. Relative sedimentation coefficients of the labelled virus RNA species were estimated by comparison with unlabelled Galleria ribosomal RNA centrifuged in the same gradients.

Determination of the chemical composition of the virus. The presence of RNA was established by the orcinol reaction of Mejbaum (1939) and protein concentration was determined by the Folin method of Lowry et al. (1951) as modified by Eggstein & Kreutz (1955).

Analysis of base composition. Precipitated, labelled virus RNA samples were hydrolysed together with unlabelled Galleria ribosomal RNA in 0.35 N-KOH at 35 °C for 18 h, then adjusted to pH 4.0 with 1 N-HCl. The nucleotides were separated by the paper electrophoresis method described by Davidson & Smellie (1952) using 0.13 M-formic acid-ammonium formate buffer, pH 3.5. Electrophoresis was continued for 24 h at 300 V. U.v. absorbing zones were cut from the papers and counted in a Packard liquid scintillation counter, Model no. 3320, in vials containing PPO–POPOP in toluene with Triton X-100.

Infectivity of the virus and virus RNA species. Samples of the two RNA species collected from sucrose gradients were inoculated separately and in combination into groups of Galleria larvae. Extracts of dead larvae were then examined for the presence of virus using the immunodiffusion technique.
A divided genome in Heteronychus virus

The infectivity of the *H. arator* virus for mice was tested by intraperitoneal injection of 100 µg virus into 24-h-old animals; after 5 days each was injected with ³H-uridine and after 10 days extracts of tissue were examined for the presence of virus in sucrose gradients.

**Electron microscopy.** Ultrathin sections of gut and fat body of *Galleria* infected with *H. arator* virus were prepared and examined as described by Harrap & Robertson (1968). Purified virus preparations were negatively stained with 1 % uranyl acetate and specimens were examined on a JEM 100B at an accelerating voltage of 80 kV.

**RESULTS**

**Electron microscopy**

Ultrathin sections of *Galleria* larvae infected with the *Heteronychus arator* virus by injection showed extensive development of virus in the cytoplasm of midgut columnar cells. The nuclei were unaffected. In cells at an advanced stage of infection (Fig. 1) large crystalline arrays of virus particles were commonly observed, though in many cells, virus particles occurred in large numbers generally distributed throughout the cytoplasm. Comparable samples of fat body showed only limited virus development and whilst the virus replicates in both tissues, it would seem that the midgut is the primary target tissue. Negatively stained preparations of virus particles (Fig. 2) had a mean diam. of 30 nm.

**Chemical composition of the virus**

The virus gave a positive orcinol reaction and was estimated to contain 28·2 % RNA. Using the Folin test, a second sample was estimated to contain 71·8 % protein. The *E*₅₆₀ was calculated to be 4·15/mg. These tests were performed using yeast RNA and bovine serum albumin as standards.

**Sedimentation analysis of virus particles**

The virus sedimented in sucrose gradients in a single band and the average yield of virus per larva was 0·75 *E*₂₆₀ units. The sedimentation coefficient of purified virus was estimated to be 137S, relative to that of turnip yellow mosaic virus (113S). Only one component of 137S was resolved and there was no dependence on concentration in the range 2 to 10 *E*₂₆₀ units per ml.

In caesium chloride gradients, purified virus which had been stored at −15 °C for some time, but which was still infective for *Galleria* larvae gave a complex profile with a small peak in the region of 1·34 g/ml and other peaks in the density range 1·37 to 1·50 g/ml. Freshly purified virus, and virus treated with 0·05 % formaldehyde gave a single peak at 1·33 g/ml (Fig. 3) and there was no evidence of breakdown of virus. Freshly purified virus which was further centrifuged on caesium chloride gradients was still infective for *Galleria* larvae. Samples of virus prepared in 0·1 M-phosphate buffer, pH 7·6, and in 1·6 M-NaCl, were centrifuged on sucrose gradients and similar amounts of virus were recovered from each, suggesting that Cl⁻ ions were not responsible for breakdown of virus on the caesium chloride gradients.

Samples of virus were held for 24 h at a range of pH from 3 to 10 and then centrifuged on sucrose gradients in 0·1 M-phosphate buffer, pH 7·6. Each preparation gave a prominent light scattering band at the same position on each gradient and when examined in the electron microscope, the virus particles were apparently undamaged. The virus appears stable therefore over this pH range.
Fig. 1. Cytoplasm of mid-gut cell of *Galleria mellonella* showing accumulations of the *Heteronychus arator* virus.

Fig. 2. Electron micrograph of *H. arator* virus particles negatively stained with uranyl acetate.

The virus nucleic acid

When the virus was extracted with either phenol, or with phenol-SDS, two RNA species were obtained when the aqueous layer was centrifuged on sucrose gradients (Fig. 4a). A similar profile was obtained when RNA extractions were performed at pH 7·2 and pH 8·5. Both species of RNA were hydrolysed to slowly sedimenting molecules by pre-treatment
A divided genome in *Heteronychus* virus

Fig. 3. Sedimentation of *H. arator* virus in a cesium chloride gradient buffered with 0.1 M-phosphate, pH 7.2; the virus equilibrated at a density of 1.33 g/ml: ■■, extinction; ••, density.

with 0.1 μg ribonuclease per ml (Fig. 4b). By reference to the sedimentation coefficients of the *Galleria* ribosomal RNA markers the two virus RNA species were estimated to be 22S and 15S. In most phenol extractions the relative amounts of 22S and 15S RNA would indicate that they are present in 1:1 proportions.

Treatment of virus with pronase-SDS for 15 min also released the two RNA species (Fig. 5a), and when samples were analysed on sucrose gradients, it appeared that relatively more 15S RNA was released. Increasing the period of pronase incubation released more 22S RNA however, and after a 60 min incubation the profile was similar to that produced by extraction with phenol SDS, although still not in 1:1 proportions (Fig. 5b).

The two RNA species were also analysed on 2.4% polyacrylamide/agarose gels (Fig. 6) and by comparison with the *Galleria* RNA markers, values similar to the above were obtained for the two species. These relative sedimentation coefficients correspond to mol. wt. of 1.0 and 0.5 × 10^6 (Spirin, 1964).

These values could be affected by possible conformational differences in the two RNA species, however, formaldehyde and other denaturing treatments may not equally denature all RNAs (Kolakofsky, Boy de la Tour & Delius, 1974), and this possibility was not examined further in the present study.

The base composition of the two RNA species was determined using nucleic acid extracted from ^32^P-labelled virus. The two species were separated by one cycle of sucrose gradient sedimentation then each was recycled through two further gradients to ensure maximum separation of one species from the other. The base composition of the two species was clearly different, and both are quite distinct from the two RNA species of Nodamura virus (Table 1). The GC content for the 22S and 15S species of Nodamura virus was 55 and 51% and for *H. arator* virus was 47 and 49% respectively.
Fig. 4. (a) Sedimentation of $^{32}$P-labelled \textit{H. arator} virus RNA and \textit{G. mellonella} RNA in a 5 to 30 \%, w/w, sucrose gradient showing the distribution of radioactivity and absorbency. Sedimentation was for 16 h at 70,000 g. (b) Sedimentation of a similar preparation after pre-treatment with 0.1 \(\mu\)g ribonuclease/ml: \textbullet ---, virus RNA profile; \textbullet ---, \textit{Galleria} RNA profile.

\textbf{Polyacrylamide gel electrophoresis of virus polypeptides}

One major polypeptide, mol. wt. 40,000 \(\pm\) 1200 and two minor bands of 44,750 and 32,750 mol. wt. were obtained by polyacrylamide gel electrophoresis on 10 \% SDS gels and the profile was similar when 8 M-urea was included in the gels and urea and 2-mercaptoethanol were included in dissociation of the virus.
Fig. 5. (a) Sedimentation of *H. arator* virus RNA prepared by pronase digestion of virus for 15 min, in a 5 to 30 %, w/w, sucrose gradient. (b) RNA prepared by pronase digestion for 60 min.

**Serological relationships**

Samples of the *H. arator* virus were tested for relationships with Nodamura virus and carnation ringspot virus with negative results (J. F. E. Newman, P. R. Fry, personal communications). The *H. arator* virus propagated in *Galleria* was serologically identical to the original isolate from *H. arator* larvae.

Samples of serum were collected from 20 cattle on the site from which the infected *H. arator* were obtained and these were tested for antibodies to the *H. arator* virus with negative results.
Fig. 6. Polyacrylamide gel electrophoresis of *H. arator* virus RNA. The RNA was electrophoresed in 9 cm gels for 2.5 h at 50 V.

Table 1. **Base composition of Nodamura virus** and **Heteronychus arator** virus RNAs

<table>
<thead>
<tr>
<th>Virus</th>
<th>RNA species</th>
<th>Adenylic acid</th>
<th>Cytidylic acid</th>
<th>Guanylic acid</th>
<th>Uridylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodamura</td>
<td>22S</td>
<td>22.4</td>
<td>27.0</td>
<td>27.6</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>15S</td>
<td>24.9</td>
<td>28.2</td>
<td>22.8</td>
<td>24.1</td>
</tr>
<tr>
<td><em>H. arator</em></td>
<td>22S†</td>
<td>29.0 ± 0.25</td>
<td>24.1 ± 0.46</td>
<td>22.8 ± 0.38</td>
<td>24.1 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>15S‡</td>
<td>26.4 ± 0.89</td>
<td>23.8 ± 0.99</td>
<td>24.7 ± 0.70</td>
<td>25.2 ± 0.61</td>
</tr>
</tbody>
</table>

† Average of four determinations.
‡ Average of three determinations.

**Infectivity of the two RNA species**

The two RNA species were injected separately and in combination into *Galleria* larvae in 15 μl amounts at concentrations of 0.25 mg/ml. Most of the treated larvae pupated normally and those larvae which died were examined serologically for the presence of virus with negative results.

In a further experiment, *Galleria* larvae were each injected with 12 μg of each RNA species alone and in combination and subsequently with ³H-uridine. Larvae were extracted 14 days after injection and the extracts analysed for radioactivity after fractionation on sucrose gradients. No radioactivity was found in the fractions containing an unlabelled virus marker.

**Infectivity of virus for mice**

Day old mice were injected intraperitoneally with freshly prepared virus and after 5 days were further inoculated with ³H-uridine. Extracts of the mice were made after a further 5 days and the extracts were analysed for radioactivity. No radioactivity was found in fractions from sucrose gradients containing the unlabelled virus marker. The behaviour of the treated mice over the 10 day period was similar to that of a control group; no signs of paralysis were noted.
A divided genome in *Heteronychus* virus

Table 2. *Comparison of properties of Nodamura and* *H. arator* *viruses*

<table>
<thead>
<tr>
<th></th>
<th>Nodamura virus</th>
<th><em>H. arator</em> virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter</td>
<td>29 nm</td>
<td>30 nm</td>
</tr>
<tr>
<td>$s_{20\text{,}w}$</td>
<td>135</td>
<td>137</td>
</tr>
<tr>
<td>Density in CsCl</td>
<td>1.34 g/ml</td>
<td>1.33 g/ml</td>
</tr>
<tr>
<td>Capsid proteins</td>
<td>One major: $35 \times 10^9$</td>
<td>One major: $40 \times 10^9$</td>
</tr>
<tr>
<td>RNA</td>
<td>Two species: $1.0 \times 10^6$ $0.5 \times 10^6$</td>
<td>Two species: $1.0 \times 10^6$ $0.5 \times 10^6$</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The *Heteronychus arator* virus shows striking similarities to Nodamura virus (Table 2). The two viruses have a similar size, density and sedimentation coefficient, and both have a divided RNA genome; the mol. wt. of the two RNA species were $1.0$ and $0.5 \times 10^6$ daltons.

Nodamura virus is unusual for an insect virus in that it has a wide host range, including mammals; the *H. arator* virus also appears to have a wide host range within insects but it does not replicate in mice. The two viruses are serologically unrelated. Both viruses were relatively unstable, and stored, purified *H. arator* virus was degraded in neutral caesium chloride gradients.

The consistent isolation of the *H. arator* virus RNA species in 1:1 proportions would suggest that both are encapsidated in the same virus particle, and their dissimilar base compositions would further suggest that the 15 S RNA is not derived by cleavage of the 22 S molecule. Both RNA species were particularly stable and successive sucrose gradients of the same 22 S RNA preparation gave no indication of conversion to 15 S molecules. Neither phenol-SDS nor pronase-SDS extractions gave molecules larger than 22 S. Newman & Brown (1973) obtained evidence of differential release of 22 S and 15 S RNA from Nodamura virus with phenol alone, or with phenol-SDS. J. F. E. Newman (personal communication) reported that with phenol alone the release of both RNA species was pH dependent. This was not the case with the *H. arator* virus, and the only indication of differential release of the two species was with pronase-SDS which produced substantially more 15 S RNA than 22 S. There are, however, sufficient similarities between the two viruses to suggest that they should form a distinct sub-group of the small RNA viruses of insects.

The replication of Nodamura virus in invertebrate tissue culture systems has been reported (Bailey, Newman & Porterfield, 1975) and should the *H. arator* virus replicate equally readily in invertebrate cells, it will be possible to investigate problems of genetic reassortment with small RNA viruses. The existence of two insect viruses containing two species of RNA and with wide experimental host ranges suggests possibilities of genetic reassortment between such viruses and the consequent hazards if they were used in a biological control programme.

The cryptogram for the *H. arator* virus is R/1:1.5/28:S/S:1/0.
REFERENCES


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