Small RNA Viruses Co-infecting the Pine Emperor Moth
(Nudaurelia cytherea capensis)

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SUMMARY

Larvae of the pine emperor moth, Nudaurelia cytherea capensis, naturally infected with Nudaurelia $\beta$ virus (N$\beta$V), contained a second serologically unrelated virus which we have called Nudaurelia $\omega$ virus (N$\omega$V). N$\omega$V had a buoyant density of 1.285 g/ml in CsCl and yielded a single major polypeptide of 65000 daltons on gel electrophoresis. The particles of N$\omega$V were morphologically distinguishable from those of Nudaurelia viruses described by others, and tryptic peptide analyses indicated that N$\omega$V protein was distinct from that of N$\beta$V and NeV. Both N$\omega$V and N$\beta$V contained a small stable fraction of particles with buoyant densities of about 1.33 g/ml.

The pine emperor moth, Nudaurelia cytherea capensis (in this paper we have retained the long-entrenched generic name Nudaurelia, although some authorities recently have referred the species cytherea to the genus Imbrasia), is infected in nature by at least five structurally distinct viruses, designated $\gamma$, $\delta$, $\alpha$, $\epsilon$ and $\beta$ (Juckes, 1970, 1974). Of these, Nudaurelia $\beta$ virus (N$\beta$V) has been extensively characterized (Hendry et al., 1968; Tripconey, 1970; Polson et al., 1970; Struthers & Hendry, 1974; Finch et al., 1974). It is the type member of the Nudaurelia $\beta$ virus group, whose members have an undivided single-stranded RNA genome comprising about 11% of the particle mass, and a T = 4 icosahedral capsid of 38 nm diameter consisting of a single protein species with a mol. wt. of about 65000 (Matthews, 1982; Moore & Tinsley, 1982). To date, seven members, and potentially a further nine (Hendry et al., 1984), have been assigned to this group, on the basis of their serological relatedness to N$\beta$V and the size of their capsid proteins (Reinganum et al., 1978; Morris et al., 1979; Moore et al., 1981; Greenwood & Moore, 1981). Owing to the lack of a cell culture system capable of supporting the replication of the Nudaurelia viruses, fresh material is obtained on a seasonal basis from naturally diseased insects and propagated by injecting extracts into symptomless larvae. We report here on the isolation of a new virus from diseased N. cytherea larvae.

Flaccid N. cytherea larvae were collected from pine plantations in the western Cape Province. The presence of N$\beta$V was confirmed serologically and the virus was propagated by collectively homogenizing a number of diseased insects and injecting the clarified extract into symptomless larvae. Other larvae were mock-infected with saline as controls. The former became flaccid within 7 days and were either stored at $-20^\circ$C, or used immediately for virus purification as described by Morris et al. (1979). After centrifugation of the semi-pure virus for 3 h at 23000 r.p.m. into a density gradient of 15 to 45% sucrose in 0.1 M-Tris-HCl pH 7.5 using a Beckman SW25 rotor, the u.v. light-absorbing fractions were collected using an ISCO Model 640 gradient fractionator and UA-5 absorbance monitor.

For further purification, virus in 0.05 M-Tris–HCl pH 7.5 was adjusted to 39% (w/v) with respect to CsCl and centrifuged at 30000 r.p.m. for 16 h in a Beckman SW50 rotor. The tubes were fractionated as described above, and the density of each virus-containing fraction was obtained by determining its refractive index and relating that to density using a standard plot.

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The virus material resolved into two major fractions (A and B, Fig. 1 a) banding at 1.285 g/ml and 1.295 g/ml respectively, and two minor fractions (C and D, Fig. I a) at 1.32 g/ml and 1.33 g/ml respectively. Previously, purified NβV had always banded at 1.295 g/ml (Juckes, 1974). The relative amounts of A, B, C and D on these gradients were not affected by adjusting the CsCl solution to have pH values between 5 and 9. Prolonging the centrifugation time from 16 to 96 h, both in the presence and absence of 1% formaldehyde, did not affect the density distribution, indicating that progressive penetration of the particles by Cs+ ions was not occurring. Only after 96 h centrifugation on a CsCl gradient at pH 10 was there a detectable increase in particles of 1.33 g/ml density (Fig. 1 b), possibly due to a structural alteration permitting facilitated access of Cs+ ions.

Immunodiffusion tests using antisera to NβV, NeV and NyV (all originally prepared by I. R. M. Juckes and kindly supplied by J. Longworth, D.S.I.R., Auckland, New Zealand) confirmed that virus B was NβV (Fig. 2). Virus A yielded a reaction of non-identity with NβV (Fig. 2), and failed to cross-react with NeV in reciprocal immunodiffusion tests using antisera raised to both NeV and virus A. NeV, like virus A, has a buoyant density of 1.285 g/ml and is serologically unrelated to NβV (Juckes, 1979; Juckes et al., 1973). Virus A also failed to react with antisera to either Antheraea virus or Dasychira pudibunda virus (antisera kindly supplied by N. Moore, NERC Institute of Virology, Oxford, U.K.). Virus C reacted with antisera to both NβV and virus A, while virus D reacted only with anti-NβV.

The particles of virus A (Fig. 3a) had a mean diameter of 40 nm, and clearly differed from those of virus B (Fig. 3b) which had a mean diameter of 38 nm and were typical of NβV, with small closely spaced capsomeres and triangular icosahedral faces delineated by grooves in which the negative stain tended to deposit. Virus A also clearly differed from NeV and from the other Nudaurelia viruses described by Juckes (1970). The particles of viruses C and D were indistinguishable from those of A and B, each zone containing particles of both morphologies.

Virus proteins were analysed by electrophoresis at 200 V for 4 h in polyacrylamide gels containing SDS, using a 4% stacking gel and a 10% separating gel (Laemmli, 1970), followed by staining in 0.2% Coomassie Brilliant Blue. Material from both A and B required at least two cycles of isopycnic centrifugation on CsCl before homogeneity was achieved. Each yielded a single major protein species, which migrated to different positions on the gel, neither of which corresponded to that of NeV protein (Fig. 4a). Minor proteins, both larger and smaller than the major species, were observed inconsistently with both A and B. C and D yielded both the
proteins observed with A and B (Fig. 4b), suggesting incomplete separation of C and D on CsCl, and also that A and B possibly share a product–precursor relationship with C and D. C appears enriched with respect to the protein observed with A (compare lanes A and C, Fig. 4b), while D appears enriched with respect to B protein (lanes B and D, Fig. 4b). Protein mol. wt. were estimated using a continuous buffer system, by electrophoresis at 100 V for 4.5 h in 7% polyacrylamide gels in 0.1 M-sodium phosphate buffer pH 7.5 containing 0.1% SDS, using the same buffer at two-thirds strength as tank buffer. Virus was disrupted by boiling for 2 min in full-strength buffer containing 1% SDS, 1% 2-mercaptoethanol and 36% (w/v) urea. With the continuous system, the marker proteins (Sigma) yielded a more significant linear relationship between log mol. wt. versus distance migrated than did the discontinuous system. The protein of A yielded a mol. wt. of 65000 and was clearly larger than NβV protein which yielded a mol. wt. of 61000; both Struthers & Hendry (1974) and Reinganum et al. (1978) also obtained values of 61000 for NβV protein using continuous phosphate-buffered systems.

Fig. 2. Immunodiffusion reactions in agar gel. Wells containing antigen surround the antiserum well. A, Virus A (see Fig. 1); B, virus B; N, NβV; I, mixed antiserum to both viruses A and B.

Fig. 3. Electron micrographs of negatively stained *Nudaurelia* viruses separated by CsCl density gradient centrifugation. (a) Virus particles obtained from zone A (see Fig. 1); (b) virus particles obtained from zone B. Bar markers represent 100 nm.
Fig. 4. Electrophoresis patterns of virus proteins in discontinuous Tris–HCl-buffered polyacrylamide gels containing SDS. In pattern (a), the proteins of NflV (lane N), virus A (lane A) and NeV (lane E) are compared. In pattern (b), the proteins of virus A (lane A), virus B (lane B), virus C (lane C) and virus D (lane D) are compared. Lane (C + D) contained an artificial mixture of viruses C and D. Virus B (lane B) contained a trace of virus A. The marker proteins (lanes M) were phosphorylase B (94000), bovine serum albumin (67000), ovalbumin (43000) and carbonic anhydrase (30000).

Fig. 5. Autoradiographs of the two-dimensional separation of the tryptic peptides of radiolabelled NflV protein (a), NeV protein (b) and NoV protein (c).

The proteins of virus A, NβV and NeV were compared by tryptic peptide mapping. After separation of the proteins by gel electrophoresis, the stained bands were cut from the gel and subjected to iodination with $^{125}$I followed by digestion with trypsin. The peptides were then separated by electrophoresis in the one dimension followed by chromatography in the other (Elder et al., 1977). Autoradiography of the chromatograms yielded distinct patterns for each of the three proteins (Fig. 5), with little detectable correspondence between the positions of any of the radiolabelled peptides.

Our results demonstrate that virus A is distinct from both NβV and NeV, based on their serological behaviour, morphology, and mol. wt. and tryptic peptide maps of the capsid proteins. Also, morphologically virus A resembles none of the other Nudaurelia viruses described by Juckes (1974). We have named virus A Nudaurelia o virus (NoV). NoV is unlikely to be either a precursor or a degradation product of either NβV or NeV, as there is no residual
serological cross-reactivity between NoV and the other two viruses, and (in the case of NoV and NβV) because the relative amounts remained constant over a range of experimental conditions. We conclude that NoV occurred as a co-infecting virus with NβV in the diseased material we collected. However, the possibility of NoV being a satellite or a defective particle has to be borne in mind.

The presence of peaks C and D can be explained by Cs⁺ ion penetration of a proportion of the NoV and NβV particles. The u.v. absorbance spectrum of neither peak indicated an increased nucleic acid content over peaks A or B; however, C and D were serologically related to, and had capsid proteins that co-electrophoresed with, A and B. Particles of intermediate density were not detected, nor did centrifugation in CsCl for extended periods of time generate raised levels of the denser particles. It is of interest that several enteroviruses, including poliovirus, contain a small proportion of stable particles which have a density higher than the 1.34 g/ml of the majority. This is thought to be due to their having an altered protein–RNA interaction resulting in an increased accessibility of the RNA to solute cations such as Cs⁺ (Rowlands et al., 1975; Mapoles et al., 1978).

We were unable with NoV or NβV to repeat the observation of Morris et al. (1979) on the NβV-like Trichoplusia ni virus (TRNV) that pretreatment with formaldehyde resulted in an increase in density to 1.33 g/ml. As seen with the acid-stable enteroviruses whose capsids are impervious to Cs⁺ ions (Mapoles et al., 1978), both NβV (which is stable at pH 3) and NoV have densities that are unaffected by the pH of the gradient or the duration of centrifugation. By contrast, the densities of the acid-labile foot-and-mouth and rhinoviruses increase as the pH is raised or the centrifugation time extended (Rowlands et al., 1975). Apart from the denser variants discussed above, the capsids of NoV and NβV would thus appear also to be impervious to Cs⁺ ions. This impermeability is unaffected by formaldehyde, whereas formaldehyde treatment of TRNV apparently renders the RNA accessible to Cs⁺ ions.

Information concerning the RNA of NoV would be required before attempting to classify this virus. Colorimetric estimations and u.v. analyses indicated that the genome size of NoV is about 60% that of NβV. Although hampered by a shortage of material, sucrose density gradient centrifugation of RNA extracted by the procedure of Daubert et al. (1978) from NoV that had undergone two isopycnic purifications in CsCl, and electrophoresis in composite agarose-acrylamide gels (Dingman & Peacock, 1968), suggested the presence of two RNA species of about 0.9 × 10⁶ and 1.8 × 10⁶ daltons. Work is in progress to clarify this issue.

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REFERENCES


Short communication


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