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A Virus with a Bisegmented Double-stranded RNA Genome in Rat (Oryzomys nigripes) Intestines

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SUMMARY

Examination of the intestinal contents of free-living Oryzomys nigripes rats by PAGE revealed two sharply defined bands that could be stained by ethidium bromide or by silver nitrate with comparable intensities. The molecules forming these bands were susceptible to digestion by pancreatic RNase A but not by RNase T1 or by DNase I. Their lengths were estimated to be about 2.6 and 1.5 kbp, respectively, by comparison with rotavirus SA11 genome segments. They cosedimented in CsCl gradients at a density of 1.39 to 1.40 g/ml, together with uniform particles approximately 35 nm in diameter with indistinct surface structure. It is suggested that these particles represent an as yet undescribed virus with a bisegmented double-stranded RNA genome, for which the name ‘picobirnavirus’ is proposed.

INTRODUCTION

Viruses with bisegmented dsRNA genomes have been described in animals, plants and fungi (see Matthews, 1982). Those infecting vertebrates are classified in the family birnaviridae (Brown, 1986), of which the most important members are those causing infectious pancreatic necrosis of fish (Hill, 1982; Dobos & Roberts, 1983) and infectious bursal disease of birds (Becht, 1980; Okoye, 1984). They consist of virions 60 to 65 nm in diameter with a skew icosahedral symmetry of T = 13 (Ozel & Gelderblom, 1985) and a buoyant density in CsCl of 1.32 to 1.35 g/ml. Their genome consists of two molecules of dsRNA with molecular size estimates ranging from about 3.3 to 3.8 kbp and 3.6 to 3.9 kbp for the small and large segments respectively.

In the course of testing faecal samples for rotavirus RNA by PAGE we have observed the appearance of two sharply defined bands stainable by ethidium bromide or by silver nitrate in samples from free-living rats, children, pigs, hamsters and guinea-pigs. Characterization of the molecules in a rat sample which formed these bands, and their association with virus particles are described in the present paper.

METHODS

Animals. Free-living Oryzomys nigripes rats were trapped in the municipality of Miracatu in the Vale da Ribeira region of the state of Sao Paulo, Brazil. The animals were bled, killed and the whole intestinal contents were collected and stored at 4 °C, or −20 °C if not processed immediately. Approximately 10% (v/v) suspensions of the intestinal contents in 10 mM-Tris–HCl pH 7.4, 1.5 mM-CaCl2 (Tris/Ca) were clarified by centrifugation at 1000 g for 10 min at 4 °C. Supernatants were stored at −20 °C if not used immediately.

Virus. Simian rotavirus SA11 was grown in MA104 cells in the presence of 10 μg/ml crystalline trypsin (Type IX, Sigma). Infected cultures were harvested when c.p.e. was extensive.
**PAGE.** The technique described by Laemmli (1970) was as used for the detection of rotavirus RNA. Samples of 100 to 400 μl of clarified suspensions of intestinal contents were incubated with 0·1% (w/v) SDS for 30 min at 37°C, extracted with an equal volume of phenol–chloroform (1:1 v/v), centrifuged for 5 min at 15600 g in an Eppendorf microfuge, and the aqueous phase was precipitated with 0·2% (w/v) NaCl and 2·5 volumes of ethanol at −20°C overnight. The precipitates were pelleted by centrifugation for 10 min at 15600 g in an Eppendorf microfuge and dried under vacuum. For routine testing, pellets were dissolved in 10 to 20 μl of sample buffer consisting of 5 M-urea, 3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0·002% (w/v) bromophenol blue. When samples gave a high background with silver staining, pellets were dissolved in 10 μl 5% (w/v) SDS containing 1 mg/ml proteinase K (Type XI, Sigma), and were incubated for 30 min at 37°C, followed by the addition of 10 μl of double concentrated sample buffer and incubation at 56°C before loading onto gels. Electrophoresis was carried out in 7·5% acrylamide gels run either in 15 cm wide, 12 cm high, 0·1 cm thick chambers at 35 mA for 4 h or in 9 cm wide, 7 cm high, 0·1 cm thick chambers at 25 mA for 1·5 h. Gels were stained either with ethidium bromide (0·5 μg/ml in distilled water) for 30 min followed by washing for 1 h in distilled water and photography in transmitted u.v. light, or by silver impregnation as described by Herring et al. (1982).

**Nuclease digestions.** Pancreatic RNase A (5 × crystallized, Sigma) was used after boiling for 15 min, at a final concentration of 40 μg/ml in Tris/Ca, 10 mM-NaCl, 2 mM-EDTA (STE). RNase T1 (Bethesda Research Laboratories) was used at a final concentration of 100 units (U)/ml in STE. DNase (RNase-free, Promega) was used at a final concentration of 100 U/ml in 40 mM-Tris-HCl pH 7·9, 10 mM-CaCl2 and 6 mM-MgCl2. These enzyme concentrations are between five- and 20-fold higher than the minimum amounts, determined in preliminary titrations, that caused complete digestion of control substrates consisting of rotavirus SA11 dsRNA for pancreatic RNase A, yeast tRNA (Type V, Sigma) for RNase T1, and φX174 replicative forms of DNA pre-digested with HaelII (Bethesda Research Laboratories) for RQ1 DNase. Digestion mixtures were incubated at 37°C for 1 h and then dissociated with an equal volume of sample buffer at 56°C for 15 min before applying to the gel.

**Virus purification and buoyant density determination.** A 4 ml aliquot of the clarified intestinal contents suspension was layered onto 1 ml of a 45% (w/v) sucrose solution in Tris/Ca and centrifuged for 1 h at 100000 g in a Beckman SW50.1 rotor. The supernatant was decanted and the pellet was resuspended in 300 μl of Tris/Ca and added to 4·5 ml of the same buffer containing enough CsCl to give a density of 1·4 g/ml. After centrifugation at 160000 g for 16 h at 4°C in a Beckman SW50.1 rotor, gradient fractions were collected and their densities were measured by weighing a 100 μl aliquot of each. They were then dialysed against Tris/Ca and 1 μl of each was added to 10 μl of sample buffer, heated at 56°C for 15 min and subjected to PAGE.

**Electron microscopy.** One volume of 25% (v/v) glutaraldehyde (high grade for electron microscopy) was mixed with nine volumes of distilled water, and 1 drop of this was mixed with an equal volume of the sample under examination. Grids (400-mesh) coated with a thin layer of carbon, previously made hydrophilic by plasma glow ionization, were floated upon the sample mixture for 30 min, then lifted off; the droplet on them was then allowed to dry almost completely (this took a few minutes). They were then quickly rinsed in distilled water, blotted three times and stained for 7 s in 2% potassium phosphotungstate pH 7·0 or saturated aqueous uranyl acetate (about 2%). Photographs were taken at initial magnifications of 50000 or 91000. Magnification was checked using catalase crystals.

**RESULTS**

**Examination of RNA in the intestinal contents of rats**

Examination of the intestinal contents of 30 *O. nigripes* rats by PAGE revealed two distinct electrophoretic bands in two specimens (32889, data not shown; 32890, shown in Fig. 1a, lanes 1, 3 and 4, alongside a preparation of simian rotavirus SA11 in Fig. 1b, lanes 1, 3 and 4). Of these two bands, one was near segment 2 and the other was a little above segment 5 of the rotavirus SA11 genome. In sample 32890 the two bands stained much more intensely than in 32889 (data not shown), but in each the bands were comparable in position and intensity. A very faint band was seen below the slowest migrating band of sample 32890, perhaps derived either from a minor population of RNA with a band that migrated faster, or from an unrelated contaminant. This faint band was not observed in CsCl-purified preparations.

From their electrophoretic mobilities in relation to the genome segments of simian rotavirus SA11 (Both et al., 1982), the lengths were estimated as about 2·6 and 1·5 kbp for the slow and fast migrating components respectively.

**Buoyant density estimation**

CsCl gradient fractions (see Methods) ranged in density from 1·31 to 1·53 g/ml. Analysis by PAGE of 1 μl aliquots of each dialysed fraction revealed that the two bands staining with
Bi-segmented rat virus dsRNA

Fig. 1. Action of nucleases on the electrophoretic bands of rat sample 32890 and on control substrates. (a) Alcohol-precipitated phenol extract of intestinal contents of suspension 32890. (b) Rotavirus SA11 dsRNA. (c) Yeast tRNA. (d) φX174 replicative DNA forms pre-digested with HaeIII. Samples were incubated without enzyme (lanes 1), with pancreatic RNase A (lanes 2), with RNase T1 (lanes 3) or with RQ1 DNase (lanes 4).

comparable intensities in two adjacent fractions sedimented at densities of 1·39 and 1·41 g/ml. Very faint bands were also seen in the two neighbouring fractions with higher and lower densities. Assuming the existence of a peak between the two fractions with the strongest bands, a density of 1·39 to 1·40 g/ml was estimated from the regression line. Similar results were obtained with samples from a guinea-pig and a child (H. G. Pereira et al., unpublished results). Recycling the gradient-purified preparations by CsCl isopycnic centrifugation resulted in cosedimentation of the two bands at densities ranging from 1·38 to 1·40 g/ml.
Bi-segmented rat virus dsRNA

Action of enzymes

Incubation of alcohol-precipitated phenol–chloroform extracts of faecal or intestinal contents samples with proteinase K (Type XI, Sigma) at concentrations of up to 1 mg/ml had no effect on the bands revealed by PAGE. The action of pancreatic RNase A, RNase T1 and RQ1 DNase under the conditions described in Methods is shown in Fig. 1, which includes controls to monitor the activity and specificity of the different enzymes. It is seen that the two bands present in the rat sample were digested completely by pancreatic RNase A but remained unaffected by RNase T1 or by RQ1 DNase. This leads to the conclusion that the two bands were dsRNA.

Electron microscopy

A representative electron micrograph of the CsCl-purified preparation of sample 32890 is shown in Fig. 2(a) in which particles of uniform size, some penetrated by negative stain, are seen. At higher magnification, some particles appear to be intact (Fig. 2b), whereas others show varying degrees of disintegration (Fig. 2b to e). No regular surface structure can be discerned, but the hexagonal outline of occasional particles (Fig. 2e) is suggestive of icosahedral symmetry. Measurement of the diameter of 86 apparently intact particles gave a mean value of 34.75 nm with a standard deviation of 1.55 nm.

DISCUSSION

The observation that the two electrophoretic bands described above were sensitive to RNase A and not to RNase T1 or to DNase I indicates that they were dsRNA. This is supported by the facts that upon migration they formed sharp bands in 7.5% polyacrylamide gels, and that they were stable in crude as well as in partially purified preparations. Their lengths were estimated to be about 2.6 and 1.5 kbp. The suggestion that they represent a bisegmented virus genome is supported by the facts that they were always present in apparently equimolar concentrations (as based on appearance of stained gels), and that they cosedimented in CsCl gradients at a density of about 1.4 g/ml together with uniform virus-like particles with a mean diameter consistent with the observed density, assuming the presence in each of two dsRNA molecules with the size estimates mentioned above.

Presently known animal viruses with bisegmented dsRNA genomes are classified by the International Committee on Taxonomy of Viruses (Brown, 1986) in the family birnaviridae, which includes viruses of birds, fish, insects (Teninges et al., 1979) and molluscs (Dobos et al., 1979). The viruses of infectious pancreatic necrosis of fish and of infectious bursal disease of birds are the most important members of this family in vertebrate hosts (Underwood et al., 1977; see reviews by Becht, 1980; Dobos & Roberts, 1983; Okoye, 1984). They differ in several respects from the virus described in the present paper: their virion diameter is larger (60 to 65 nm), their surface structure is distinct, their buoyant density in CsCl is lower (1.32 to 1.35 g/ml) and their genome segments are larger (about 3.78 and 3.33 kbp).

To our knowledge, no viruses with a bisegmented dsRNA genome have previously been described in mammalian hosts. Our own findings (unpublished results) suggest that agents similar to the one described in the present paper also exist in humans, pigs, guinea-pigs and hamsters. This is based on the appearance of characteristic electrophoretic bands in faecal samples analysed by PAGE, supported in the cases of samples from humans and guinea-pigs by buoyant density determinations and nuclease digestion studies which give similar results to those obtained with the rat agent. Virus particles similar to those described above have been detected in human and guinea-pig samples containing the characteristic electrophoretic bands shown to consist of dsRNA (unpublished results). These findings suggest the existence of a new group of dsRNA viruses, probably of vertebrates, for which the name ‘picobirnavirus’ might be appropriate to indicate their smaller size when compared with birnaviruses.

Fig. 2. Electron micrographs of CsCl-purified 32890 sample fixed in 1.25% glutaraldehyde and negatively stained with potassium phosphotungstate. (a) Low magnification; (b) two particles appear to be coming apart; (c) particles in stages of disintegration; (d) particle penetrated by negative stain; (e) particle partly penetrated by negative stain suggesting icosahedral structure. Bar markers represent (a) 0.2 μm and (b, c, d and e) 0.02 μm.
Viruses with bisegmented dsRNA genomes in hosts other than animals include some of the cryptic viruses of plants (Boccardo et al., 1983) and several fungal viruses (Matthews, 1982) with separately encapsidated genome segments. Genome segment sizes of these viruses differ significantly from those of the agent described here.

Our present results do not enable conclusions to be drawn regarding the prevalence, origin or pathogenic potential of the virus found in rats and possibly in other mammalian hosts. The possibility exists that the two bands detected by PAGE may originate from as yet unknown viruses of enteric microorganisms, or viruses ingested with food and excreted in stools independently of replication in the mammalian host. However, the high staining intensity of the characteristic bands in some of our samples, and the finding that they occurred in faeces of a suckling guinea-pig examined in our laboratory, suggest that this is not the case.

Attempts to propagate the rat agent and other possible agents, detected in our studies, in experimental animals and in cell cultures are in progress.

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REFERENCES


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