Particle and genomic characteristics of a new member of the Ascoviridae: Diadromus pulchellus ascovirus

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A new member of the family Ascoviridae, Diadromus pulchellus ascovirus (DpAV), has been found in the lepidopteran nymphs of Acrolepiopsis assectella parasitized by the hymenopteran wasp Diadromus pulchellus. Virions have the standard features of the ascovirus group; each particle is about 220 nm long and 150 nm wide. They are multilayered, with two clear 7-nm-thick outer layers and one 15-nm-thick inner layer surrounding an electron-dense core (155 × 110 nm). However, the flattened rice-grain shape and fragility of the DpAV particles are unlike that of known ascoviruses infecting Noctuidae species. They form large vesicles containing virions in infected cells. The DpAV genome is about 116 kb long and has a circular and relaxed structure. It contains 6–8 repeated and interspersed sequences of 494 bp. The structural and genomic features of DpAV suggest that this virus belongs to an ascovirus sub-family different from that containing the ascoviruses previously found to infect species of Noctuidae (Federici et al., 1991).

Introduction

Members of a recently discovered group of viruses, the Ascoviridae, cause chronic and fatal diseases in the larvae of Lepidoptera infected with species of Noctuidae (Federici et al., 1991; Federici, 1993). They appear to be vectored among lepidopteran hosts by parasitoid wasps at oviposition (Hamm et al., 1985). The viral particles are large (130 × 400 nm), enveloped, allantoid to reniform or bacilliform in shape, with a complex symmetry. The virions contain at least 12 polypeptides and have a linear double-stranded DNA about 130–180 kbp long. The pathology of ascovirus disease is marked by a milky-white discolouration of the larvae, which is due to the accumulation of numerous virion-containing vesicles. Cytopathology begins with hypertrophy of the infected cell nucleus and invaginations of the nuclear membrane. The nuclear membrane becomes fragmented and sheets of the cytoplasmic membrane assemble throughout the cell and coalesce, partitioning the cell into clusters of vesicles. The vesicles then dissociate from each other and accumulate in the tissues, which are disrupted as the disease progresses.

The DNA polymerase of Spodoptera ascovirus has recently been identified and characterized (Pellock et al., 1996). Phylogenetic analyses of its amino acid sequence revealed conserved motifs characteristic of the δ-DNA polymerases found in other DNA viruses, such as baculoviruses, herpesviruses, adenoviruses and entomopoxviruses (Braithwaite & Ito, 1993). However, analysis of this gene supports the placement of insect ascoviruses in a separate virus family distantly related to the previously cited DNA virus families.

The present report describes a new member of the Ascoviridae, Diadromus pulchellus ascovirus (DpAV). This virus was found in all tissues of pupae of the leek-moth, Acrolepiopsis assectella, parasitized by the hymenopteran wasp Diadromus pulchellus. Structural analysis revealed that the virions are shaped like a flattened rice-grain. They contain a circular DNA genome of 116 kb containing several 494 bp repeats, and features that are similar to the genomes of baculoviruses, herpesviruses and adenoviruses.

Methods

Materials. D. pulchellus is a solitary hymenopteran endoparasitoid of the leek-moth, A. assectella (Lepidoptera), which infests Allium species. The hymenopteran strain used in these experiments was bred in bulk, on host pupae following the technique of Lecomte & Thibout (1984). Briefly, the parasitoid wasps were reared in cages at 25 °C, 60 ± 10% relative humidity (RH) during the 16 h light period, and at 15 °C, 70 ± 10% RH during the 8 h dark period. Twenty-four-hour-old pupae of A. assectella
were presented to *Diadromus* females each day for oviposition. The host strain was from standard stock bred in the laboratory (Auger & Thibout, 1983). The hymenopteran strain was established from imago wasps harvested in September 1990 in the locality of Antibes (southern France).

**Virus purification.** Virus was purified from two sources: 3–4-day-old parasitized nymphs which had been dissected to extract the hymenopteran larvae, and non-parasitized nymphs artificially inoculated using glass pins infected with DpAV-containing haemolymph from 3–4-day-old parasitized nymphs. The procedure was optimized by checking several extraction and purification media and buffers at various concentrations, pH and with or without NaCl and non-ionic detergents (Triton X-100, NP40). These trials indicated that media containing amines, such as Tris, PIPES, MOPS and HEPES, or salts such as sodium phosphate and sodium citrate buffers, or sonicatation all destroyed the DpAV particles. A variety of centrifugation conditions on sucrose or CsCl gradients were also tested. The particles purified from parasitized nymphs were found to be more fragile than those obtained from artificially infected nymphs.

The ascovirus particles were prepared using nymphs artificially infected with DpAV. Viral particles were homogenized in distilled water containing 0·1 mM PMSF. Cell debris was eliminated by filtration through glass wool followed by centrifugation at 4 °C for 10 min at 2000 g. The resulting supernatant fraction was layered onto a 20–55% (w/v) sucrose gradient in distilled water and centrifuged at 4 °C for 1 h in a TST41–14 rotor (Kontron) at 72000 g. The virion band was collected, diluted 5-fold with distilled water and centrifuged at 4 °C for 1 h at 110000 g. Finally, the viral genome was purified by treatment with proteinase K in 10 mM Tris-HCl (pH 7·5), 100 mM EDTA, 0·5% SDS at 37 °C for 1 h followed by standard phenol-chloroform extraction.

**Purification, cloning, sequencing and alignment of viral DNA fragments.** *PstI, Smal* and *HindIII* fragments of the digested viral genome were separated on low-melting-point agarose gels (0·5%) and stained with ethidium bromide (EB). DNA fragments were eluted using a Qiagen Kit and their concentrations were estimated on agarose gels. Fragments were sub-cloned with the M13mp18 (20-mers) from each newly sequenced 500 bp were used to proceed along the cloned fragments (Strauss et al., 1983). Specific oligonucleotides (lanes 4 and 9), *DraI* (lanes 5 and 10) and *PvuII* (lanes 6 and 11) of the CITI2 program (Dessen et al., 1990) and have been deposited in EMBL under accession numbers X85001-X85007.

**Quantification of purified virus.** The virus fractions purified on the sucrose gradient were quantified by dot blot assay with cloned solutions of the DpAV DNA genome and cloned virus fragments as controls. Sample blot assays and quantification by DNA hybridization were carried out as previously described (Bigot et al., 1991), using cloned DpAV fragments as probes.

**Virus transmission by inoculation.** The biological conservation, the rate of infectivity of purified DpAV virus and the presence of DpAV in tissues were assayed by infecting healthy *A. assectella* nymphs with sterile glass pins dipped in dilutions of purified DpAV virus or tissue extract. About 0·1–0·2 µl was injected into each nymph. The nymphs were then incubated under breeding conditions for 4 days. DpAV infection was checked by hybridizing squash-blots of infected nymphs (Anxolabéhère et al., 1988) with virus probes (see above), or by gel electrophoresis of total DNA from each infected nymph digested with restriction enzymes. The biological conservation and rate of infectivity were checked using ten replicates for each virus dilution (1·10−10).

![Fig. 1. Restriction patterns of total DNA from healthy (lanes 2–6) and parasitized (lanes 7–11) A. assectella nymphs on a 1% agarose gel. Total DNA was digested with *Avil* (lanes 2 and 7), *Avill* (lanes 3 and 8), *BglII* (lanes 4 and 9), *DraI* (lanes 5 and 10) and *PvuII* (lanes 6 and 11). Molecular masses are in kb and correspond to *HindIII* (lane 1) and *BglII* (lane 12) digests of *λ*-phage DNA. The gel was stained with EB.](https://www.microbiologyresearch.org/article/1140)
Transmission electron microscopy (TEM). Entire lepidopteran nymphs were dissected in insect Ringer's solution and the pieces placed in modified Karnovsky's fixative at 4 °C (McDowell & Trump, 1976). Fixed tissues were stored in 1-butanol for several days, dehydrated in an ethanol series and embedded in Epon resin. Ultra-thin sections (0.2 μm) were cut and contrasted with uranyl acetate and lead citrate. Carbon-coated grids were floated on a drop of virus suspension or haemolymph, stained by floating on uranyl acetate for 1 min, dried and examined by TEM.

Results

Features of the viral particles infecting A. assectella nymphs parasitized by D. pulchellus

Total DNA digests of healthy and parasitized nymphs (4 days after oviposition) of the host A. assectella were separated on agarose gels (Fig. 1). Only the total DNA from parasitized A. assectella nymphs contained numerous repeated fragments which did not correspond to repeated chromosomal sequences of the lepidopteran genome. Data from several different restriction enzyme digests indicated that the total size of these repeated fragments was about 120 kb. The low total molecular mass of these repeated fragments and their rapid development in the parasitized A. assectella nymphs suggested that infection by a microbiological agent occurred during the embryonic and larval instars of the D. pulchellus endoparasitoid.

Haemolymph from parasitized larvae was placed on a carbon grid to view the microbiological agent under the electron microscope. Large vesicles containing allantoid-shaped virions (Fig. 2A), each enclosed in a membrane, were amplified in the same PCR tube using the paired primers 5′ GGAAA-CCTGGGCACTCAT 3′/5′ GAATACGGCTCGCAGAACGC 3′ and 5′ ACAACCCAGCCATCCACGTATCCCTGTCACCACACTCTC 3′. Each PCR was performed on 50–100 ng total DNA from each wasp tested. The hymenopteran DNA was dissolved in 10 mM Tris-HCl (pH 9), 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, with 150 μM each of dATP, dCTP, dGTP and dTTP (per 0.1 μM of the first primer pair, and per 0.25 μM of the second primer pair) in a 100 μl reaction volume with 3 U Taq polymerase (Appligene). Each PCR was carried out in a programmable thermal controller (Perkin-Elmer) for 30 cycles. The cycle was 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. At the end of the 30th cycle extension was allowed to proceed at 72 °C for 10 min.
seen. Most of the vesicles and the virions were partially or almost completely degraded, and only the envelope membranes could be seen, indicating that they were very fragile (Fig. 2B). However, there were intact virions in all the grids containing squashed vesicles (Fig. 2C). Pictures of these virions without their envelope membranes show their structure (Fig. 2D). Each allantoid particle was about 220 nm long and 150 nm in diameter. It was composed of two clear 7-nm-thick

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**Fig. 3.** TEM of the DpAV particle showing features in sections. (A) Gut cells from 3-day-parasitized *A. assectella* nymphs; (B) gut cells from healthy *A. assectella* nymphs; (C) vesicle containing ascovirus particles; (D, E and F) details of the DpAV particle sectioned at different angles. (D) Lengthwise section; (E) transverse section; (F) second transverse section. vcp, Vesicle containing DpAV particles; n, nucleus; m, microvilli; lg, lumen gut. In (C) arrows indicate DpAV particles cut across their width and along their length with large arrows indicating DpAV particles cut across their breadth or seen intact in this view. Bars correspond to (A and B) 2 µm; (C) 200 nm; (D, E and F) 50 nm.

**Fig. 4.** (A) TEM of purified DpAV particles. ve, Viral envelope; dmvp, dissociated material of the virion particle. Bar corresponds to 100 nm. (B) Restriction patterns of DpAV genome from purified particles (lanes 2–5) on a 1% agarose gel. The viral DNA was digested with *Ava*I (lane 2), *Avb*II (lane 3), *Bgl*II (lane 4) and *Dra*I (lane 5). Molecular masses are in kb and correspond to *Hin*dIII digests of λ-phage DNA (lanes 1 and 6). The gel was stained with EB.

**Fig. 5.** PFGE analysis of the DpAV genome from total DNA from 4-day-parasitized *A. assectella* nymphs (lanes 2–5). The nucleic acids were separated on 1% agarose gels stained with EB (lanes 1, 2 and 4) and analysed by Southern blots (lanes 3 and 5) hybridized with the 939 bp *Sma*I DpAV fragment (final blot washing 0.5 x SSC, 65 °C). Total DNA was undigested (lanes 2 and 3) or digested with *Not*I (lanes 4 and 5). Molecular masses are in kb and correspond to undigested plus *Bgl*II-digested λ-phage DNA (lane 1) and yeast chromosome (lane 6).
outer layers and a 15-nm-thick inner layer surrounding an electron-dense core (155 × 110 nm). No reticulations were observed on the surface of the virion. Except for the reticulations, all the features corresponded to those of the ascovirus-like particles (Federici, 1993).

Transverse sections of 3-day-parasitized nymphs after oviposition were checked by TEM for the presence of ascovirus particles. The infected cells in the epithelium under the cuticle, adipocytes and gut were greatly altered (Fig. 3A, B). The nuclei were partially or completely disrupted and large vesicles containing ascovirus particles were observed. Examination of the particles in these vesicles showed virion sections at different angles. Most were about 45 nm thick and 150–220 nm long (Fig. 3C, small arrows). Less abundant particles (Fig. 3C, large arrows) had features similar to those in haemolymph from parasitized larvae. These features were not compatible with the ascovirus particle being allantoid in shape. Views showing the length (Fig. 3D), thickness (Fig. 3E) and width (Fig. 3F) of virions led to a 3D view of the ascovirus particle which we consider to be shaped like a flattened rice grain.

Particles were prepared by sucrose-gradient centrifugation and immediately plated onto carbon grids as they were very fragile. TEM (Fig. 4A) showed that the purified particles were the same as those in the haemolymph. However, as for the haemolymph, most of the purified virions plated on grids were partially degraded and only the envelope membranes could be seen (Fig. 2B). We tried to improve the quality of the purified virions by using fixatives such as glutaraldehyde, formaldehyde, or dimethyl suberimidate at different steps in the purification. None of these agents improved the quality of the particles. Fixing, using Karnovsky’s fixative or any of the ones given above, destroyed the purified particles (the free virion).

The effect of the plating method for TEM was checked using diluted solutions (calibrated by dot blot hybridization) of purified virus (8 × 10⁸ to 0.008 virions/ml) to inoculate healthy A. assectella nymphs with an infected glass pin. Controls were inoculated with sterile water. Squash-blot analysis of the inoculated nymphs showed that as few as about ten particles yielded 80–90% virus infection. Inoculation with about 0.1–0.01 purified virion per inoculation resulted in several cases of infection suggesting that a single virus might be enough to cause virus development. These experiments indicate that DpAV particles are very fragile and very labile in the presence of standard buffers and fixatives. However, the virions purified on a sucrose gradient were infectious, indicating that most of the degraded particles seen in TEM were damaged during the plating step.

DNA was purified from these particles and digested with various restriction enzymes (Fig. 4B). The side-by-side com-
As the ascoviruses were in cytoplasmic vesicles, the presence of the DpAV genome in the 4-day-parasitized nymphs was checked by PFGE using total DNA extracts. The presence of a virus fragment in a digest was confirmed by comparison with the restricted pattern from total DNA extracts of healthy nymphs. Hybridization with each of the five cloned restriction enzyme fragments to Southern blots from PFGE gels of undigested parasitized host DNA showed that the viral genome was present as one large smeared band of about 49 kb (Fig. 5, lane 3). DNA digested by SfiI had a similar pattern, but a single 116 kb fragment was obtained by digestion with NotI. Digestion with Cpol or Nhel gave several bands with a cumulative molecular mass of about 116 kb (data not shown). It is worth noting that during PFGE migration, a circular DNA molecule migrates more quickly than a linear molecule of similar molecular mass. Moreover, these molecules have a different flexibility. These features explain why the circular molecule migrated faster and as a smeared band. The results obtained with the undigested DpAV DNA therefore indicate that the viral genome is circular, contains no SfiI restriction site and one NotI, five Cpol and seven Nhel restriction sites. The total DNA of a parasitized host was also centrifuged (100000 r.p.m., 4 h, 20 °C, TNV100 rotor, TL100 Beckman centrifuge) on a 1% CsCl-EB gradient. No bands corresponding to circular superhelical DNA were obtained, suggesting that the DpAV genome is circular and not superhelical.

Complementary studies of this feature were performed by PFGE and standard agarose gel electrophoresis. Theoretically, when DNA is digested by restriction enzymes E1 and E2 producing x and y fragments, respectively, the number of fragments produced by double-digests should be x + y if the DNA is circular and x + y – 1 if the DNA is linear. Single digests with NotI and Nhel restriction enzymes produced 1 and 6 fragments, respectively, and double-digests gave 7 fragments (Fig. 6, lanes 3, 4 and 5). In accordance with this, a single digest with the restriction enzyme AsnI produced 16 fragments and the Nhel-AsnI double-digest gave 22 fragments (Fig. 6, lanes 2, 3 and 6). Similar results were also obtained from digests with other pairs of restriction enzymes (data not shown), confirming that the DpAV genome is circular.

The five cloned SfiI and Smal DpAV fragments were hybridized with DNA digests from healthy and parasitized nymphs. The 617, 694 and 738 bp SfiI and 939 bp Smal fragments were not repeated in the DpAV genome. However, Southern blots with the 737 bp Smal fragment (Fig. 7) indicated that this fragment was repeated 6–8 times in the DpAV genome. Two other DpAV Hinfl fragments (1522 and 1597 bp; accession numbers X85006 and X85007) containing similar sequences were cloned and sequenced (Fig. 8). Comparison of these three fragments defined a 494 bp consensus motif that was 77% conserved, repeated 6–8 times, and interspersed throughout the 116 kb DpAV genome. The sequences of these repeats were analysed on both strands and

**Fig. 7.** Southern blot analysis of total DNA from A. assectella nymphs digested with Accl (lane 1), AspHI (lane 2), Avol (lane 3), BamiHI (lane 4), BglII (lane 5), Ddel (lane 6) and DraI (lane 7), and separated on a 1% agarose gel. The blot was probed with the cloned 737 bp Smal fragment which contained one of the 494 bp interspersed sequences of the DpAV genome (final blot washing 0.5 x SSC, 65 °C). Molecular masses are in kb on the right-hand side.

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\begin{array}{cccccc}
1 & 2 & 3 & 4 & 5 & 6 \\
\hline
9.5 & 6.6 & 4.3 & 2.2 & 2.0 & 0.6
\end{array}
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Features of the DpAV genome

Five small fragments of the DpAV genome were cloned and sequenced from PstI- (617, 694 and 738 bp; EMBL accession numbers X85001-X85003) and Smal- (737 and 939 bp; EMBL accession numbers X85004 and X85005) digested DNA. These fragments were used as probes on Southern blots. The probes did not hybridize to total DNA digests from uninfected nymphs, indicating that the viral genome was absent from healthy hosts. This was confirmed by the absence of amplified fragments from PCR using internal primers of the 694 bp PstI and the 939 bp Smal fragments (data not shown).
revealed two imperfect palindromes with inverted repeats located at positions 34–55/56–76 and 74–146/386–454 in the consensus sequence. Two types of transcription factor DNA-binding motif were found in the 494 bp repeats. The first corresponded to two overlapping motifs of ubiquitous factors, the mammalian NFI CS7 (position 336–350; Faisst & Meyer, 1992) and the fungal PUT2 UAS1 (343–350; Siddiqui & Brandriss, 1988). The second type of motif corresponded to two well-conserved DNA binding sites (XGGAYGT) of the adenovirus E1A enhancer factor (Yoshida et al., 1989) located in positions 194–200 and 437–444, and one less well matched in position 203–209. The four nucleotides 3′ of the two motifs in positions 194–200 and 437–444 were also conserved and gave a 11 bp consensus motif, 5′ AGGACGTGTTG 3′. This motif was also similar to the 5′ (T/A)(T/A)CGNGTR 3′ motif found in sequences upstream of several early genes and in homologous repeated regions of several baculoviruses (Blissard & Rohrmann, 1990).
of the faces is flattened. The second is the absence of reticulation on the surface of the virion. However, this fine feature of the virion might be present in DpAV, but not be observable because of the fragility of the purified particles. The third is the fragility of the particles in the standard buffers, salts, detergents and fixatives used for virus purification. This fragility may be caused by the proteases present in the parasitized nymphs and secreted by the wasp larvae and their teratocytes during development (Dahlman, 1990). However, the purified particles remain infectious, so it is likely that plating particles onto the carbon grids used for TEM might also cause some degradation.

The DpAV genome is about 116 kb long and PFGE data indicate that the native DpAV genome is circular. Data obtained by DNA ultracentrifugation on CsCl-EB gradients show that it is not superhelical. However, the DpAV virion may have a genomic superhelical structure that is not detected in our experiments if the DpAV genome was nicked during DNA purification. It has been suggested that the genomes of Heliotis virescens ascovirus (HAV), Trichoplusia ni ascovirus (TAV) and Spodoptera frugiperda ascovirus (SAV) are linear (Federici et al., 1990), but these ascoviral genomes were not studied by PFGE. The DpAV genome contains 6–8 interspersed repeated sequences of 494 bp. Sequence analyses revealed that these repeats contain two imperfect palindromes and similar enhancer motifs of the ubiquitous and virus early transcription factors. Six 500–800 bp homologous regions (hr) have also been described in the genome of several multiple nucleocapsid nuclear polyhedrosis baculoviruses (Pearson et al., 1992; Kool et al., 1993 for review). The sequences of these baculovirus hrs also contain structurally similar palindromic regions and DNA-binding sites for early transcriptional enhancer factors. These sequence features are implicated in viral DNA replication in baculoviruses (Pearson et al., 1992; Kool et al., 1993) and in the early expression of viral genes (Guarindo & Dong, 1994; Douglas et al., 1995). This suggests that the DpAV 494 bp repeats might also be involved in DNA replication. Multiple replication origins have also been reported for herpes simplex virus type 1 and Chilo iridescent virus. These repeats might also modulate the expression of the early viral genes. Such similarities of the DpAV genome with those of the baculovirus and herpesvirus genomes do not imply that these viruses are phylogenetically related as they are only structural and putative functional similarities and might result from evolutionary convergence. However, the particle and genomic differences between DpAV and the known ascoviruses indicate that this virus should be placed in an ascovirus sub-family different from that containing the previously described ascoviruses infecting Noctuidae species (Federici et al., 1991).

We thank G. Devauchelle and G. Crozier for their help throughout the investigation. This work was supported by grants from the C.N.R.S. (URA1298), the M.E.N.E.S.R. ACC-SV7 and BIOTECHNOCENTRE.

References


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Received 5 August 1996; Accepted 6 January 1997