Genome Assembly and Particle Maturation of the Birnavirus Infectious Pancreatic Necrosis Virus

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In this study, we have analyzed the morphogenesis of the birnavirus infectious pancreatic necrosis virus throughout the infective cycle in CHSE-214 cells by using a native agarose electrophoresis system. Two types of viral particles (designated A and B) were identified, isolated, and characterized both molecularly and biologically. Together, our results are consistent with a model of morphogenesis in which the genomic double-stranded RNA is immediately assembled, after synthesis, into a large (66-nm diameter) and uninfected particle A, where the capsid is composed of both mature and immature viral polypeptides. Upon maturation, particles A yield particles B through the proteolytic cleavage of most of the remaining viral precursors within the capsid, the compaction of the particle (60-nm diameter), and the acquisition of infectivity. These studies will provide the foundation for further analyses of birnavirus particle assembly and RNA replication.

The *Birnaviridae* family of viruses includes members with a bi-segmented, double-stranded RNA (dsRNA) genome encapsidated within unenveloped, single-shelled icosahedral particles of 60 to 70 nm in diameter. Birnaviruses do not infect mammals, and the family contains three genera: (i) genus *Aquabirnavirus*, including infectious pancreatic necrosis virus (IPNV), which spreads in salmonids and trouts; (ii) genus *Avibirnavirus*, including infectious bursal disease virus (IBDV), which infects young chickens; and (iii) genus *Entombirnavirus*, including *Drosophila* X virus, which infects the fruit fly *Drosophila melanogaster* (14, 19). The 5' ends of both segments of the dsRNA genome of the birnaviruses are bound to a genome-linked protein (VPg) (6, 51, 57). Among the multisegmented dsRNA viruses, these unique properties, among others, made it necessary to classify the birnaviruses in their own family (5), apart from their reovirus relatives (54).

The IPNV particle presents a buoyant density of 1.33 g/cm³ in CsCl (17) and contains both segments of the dsRNA genome (14S) (named segments A and B) (15, 41). Genome segment A (~3,100 bp) involves two partially overlapping open reading frames. The short one encodes a 17-kDa arginine-rich nonstructural protein (42), which, for IBDV, is not required for viral replication but plays an important role in pathogenesis (67). The long one encodes a precursor of a 106-kDa protein (polyprotein [PP]) (NH₂-pVP2-VP4-VP3-COOH), which is cotranslationally cleaved by the viral VP4 protease (29 kDa), generating a precursor of the major capsid protein pVP2 (62 kDa) and VP3 (31 kDa) (20). pVP2 is further processed to generate VP2 (54 kDa) (17). VP3 forms the trimeric subunits that wrap the inner face of the capsid (4, 8). The external surface of IBDV particles is made of trimeric subunits containing variable pVP2/VP2 ratios (9, 40). It has been suggested that

pVP2/VP2 conversion is associated with capsid maturation (9, 17, 48). The uncleaved PP is also detected, although in trace amounts, in highly purified IPNV preparations (43). Genome segment B (~2,900 bp) encodes a minor internal polypeptide, VP1 (94 kDa), the putative viral RNA-dependent RNA polymerase (22). VP1 is found within the virion in two forms: as a free polypeptide, RNA-dependent RNA polymerase-associated activity, and as the genome-linked protein, VPg. As VPg, VP1 is linked to the 5' end of each genome segment by a Ser-5'-GMP phosphodiester bond (6). This bond can be generated in vitro during the guanylation of VP1, yielding VP1pG (13). Interestingly, by electron microscopy, it has been observed that, in vitro, the binding of VPg to the viral RNA confers to the genome a marked propensity to aggregation, where the interactions were found to take place at the end of the segments (6, 51).

The IPNV replication cycle has been mainly studied on continuous cell lines from teleost fishes in culture (66). In Chinook salmon embryo cells (CHSE-214 cells) (37), a single round of virus replication takes approximately 24 h at 15°C. As a result, a characteristic cytopathic effect (CPE) is observed (44), in which apoptosis precedes the pathological changes of necrosis (30–32). Following penetration, viral particles are localized within vesicular compartments in the cytoplasm (11). Early after infection, a putative transcription intermediate (14-16S) is detected between 2 to 4 h postinfection (hpi) (62). After 4 to 6 hpi, viral mRNA species can be identified (62) as well as viral-specific polypeptides, with viral proteins synthesized in the same relative proportions throughout the infective cycle (16). The synthesis of viral genomic RNA in infected cells reaches a maximum level between 8 to 10 hpi, correlating with the detection of genomic dsRNA (62). After 14 to 16 hpi, viral RNA synthesis is diminished. Although the temporal virus-specific synthesis of macromolecules has been characterized, the structures associated with birnavirus replication and genome assembly in infected cells are largely unknown.

In this study, we analyzed the morphogenesis of IPNV

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throughout the infective cycle in CHSE-214 cells by native agarose electrophoresis. Two types of particles (A and B) were identified, isolated, and characterized. Our results are summarized in a model of IPNV morphogenesis during the replication cycle. These studies will provide the foundation for the analysis of birnavirus particle assembly and RNA replication.

MATERIALS AND METHODS

Cells and viruses. Virus stocks (IPNV, strain VR-299) were propagated by inoculating Chinook salmon embryo cells (CHSE-214 cells) (37) at a multiplicity of infection (MOI) of 0.1 to 1 PFU/cell in minimal essential medium (MEM) supplemented with 2% fetal bovine serum (FBS) and antibiotics. Infected cultures were incubated at 15°C until the CPE was evident, and the clarified supernatants were aliquoted and stored at -20°C. Virus fractions were titrated in a plaque formation assay as previously described (34).

Extraction, concentration, and partial purification of IPNV particles. Monolayers of CHSE-214 cells were infected with IPNV at a MOI of 1 to 5 PFU/cell. After 1 h of adsorption at 15°C, cells were washed three times with phosphate-buffered saline, and the inoculum was replaced with methionine- or phosphate-deficient MEM supplemented with 2% FBS and antibiotics, containing 50 μ Ci of either [³⁵S]methionine or [³²P]HPO₄²⁻/ml, respectively. The cells were then incubated at 15°C (this was defined as time zero of infection), and at 2 hpi, 0.5 μ g of actinomycin D/ml was added.

Cultures showing complete CPE were subjected to three freeze-thawing cycles, and cell debris was discarded by centrifugation at 5,000 $\times$ g for 10 min at 4°C. For cultures harvested at different time points during the infective cycle, cells were washed three times with phosphate-buffered saline and incubated for 10 min at 4°C with lysis buffer (3 mM Tris-HCl [pH 8.0], 0.5 mM MgCl₂, 3 mM NaCl), scraped with a rubber policeman, and homogenized with a Dounce at 4°C for best results. The homogenate was centrifuged for 10 min at 5,000 $\times$ g at 4°C, and the pellet was discarded. Nonidet P-40 (1%) was added, since viral progeny remained trapped within cellular debris (44), and the supernatant was incubated for 60 min at 4°C with mild agitation. Viral particles were concentrated by ultracentrifugation at 130,000 $\times$ g for 2 h at 4°C, resuspended in ice-cold 50 mM Tris-HCl (pH 8.0), and then partially purified by ultracentrifugation through a 25% sucrose cushion at 145,000 $\times$ g for 4 h at 4°C. The pellet was resuspended in a buffer containing 10 mM HEPES-KOH (pH 8.0), 2 mM dithiothreitol, and 10% glycerol and stored at -20°C.

The progression of the IPNV infective cycle was monitored by viral dsRNA synthesis and the simultaneous inoculation of CHSE-214 cells (4 to 5 $\times$ 10⁵ cells) with 50 μ Ci of [³²P]HPO₄²⁻/ml. At several time points, the supernatant was discarded, the monolayer was lysed, and the extract was subjected to digestion in buffer K (0.2 M Tris-HCl [pH 8.0], 1% sodium dodecyl sulfate [SDS], 10 mM EDTA, 1.3% [vol/vol] β -mercaptoethanol, 0.7 M NaCl, 160 μ g of proteinase K/ml) at 40°C for 12 h. Total RNA was extracted with phenol-chloroform and ethanol precipitated. Viral dsRNA was resolved by 7% polyacrylamide gel electrophoresis (PAGE) as previously described (27).

In pulse-chase experiments, after virus adsorption, the inoculum was replaced with MEM containing 2% FBS and antibiotics. During the pulse time (4 to 8 hpi), methionine- or phosphate-deficient MEM, containing 50 μ Ci of either [³⁵S]Met or [³²P]HPO₄²⁻/ml, respectively, was added. The monolayers were then washed three times with MEM and placed in isotope-free MEM supplemented with 2% FBS and antibiotics and incubated at 15°C until the indicated harvest time.

Isopycnic CsCl gradient. Partially purified viral particles ([³⁵S]Met- or [³²P]HPO₄²⁻-labeled) were isopycnically banded in a 38% (wt/vol) CsCl solution (in 50 mM Tris-HCl [pH 8.0]) by ultracentrifugation at 195,000 $\times$ g for 18 h in an SW 55 Ti rotor. Two hundred-microliter fractions were collected from the top to the bottom of the tube, and both the radioactivity and the refractive index of each fraction were determined. The buoyant densities were calculated from the refractive index measurements. Radioactive fractions were diluted, and the particles were concentrated by ultracentrifugation at 145,000 $\times$ g for 4 h at 4°C.

TGA electrophoresis. Aliquots of viral particles were mixed with Tris-glycine-agarose (TGA) loading buffer (3 mM Tris-HCl [pH 8.0], 6.6 mM NH₄Cl, 3 mM magnesium acetate, 14 mM potassium acetate, 1 mM dithiothreitol, 10% glycerol, 0.005% bromophenol blue) and subjected to electrophoresis in either 0.8 or 1% agarose gels containing 50 mM Tris and 200 mM glycine at 8 V/cm for 3.5 h at 4°C (26). Gels were dried and exposed to autoradiography. Alternatively, after electrophoresis, viral particles were recovered from fresh gel slices by electroelution at 100 V for 2 to 3 h at 4°C and then dialyzed for 1 h at 4°C against TAE buffer (40 mM Tris-acetic acid, 2 mM EDTA). For the in vitro maturation assays,

aliquots of dialyzed particles A and B were subjected either immediately ($t = 0$) or after an incubation of 24 h at 4°C ($t = 24$) to a second round of TGA electrophoresis. To analyze the polypeptide composition of gel-recovered [³⁵S]Met-labeled particles, the proteins were precipitated with 15% trichloroacetic acid and resolved by SDS-12% PAGE. To analyze the [³²P]HPO₄²⁻-labeled dsRNA content, electroeluates were incubated with buffer K and total RNA was extracted with phenol-chloroform and ethanol precipitated. Viral RNA was resolved on a 7% polyacrylamide gel and autoradiographed (27).

Electron microscopy of IPNV particles recovered from TGA gels. Aliquots of concentrated electroeluates from TGA gels (bands A and B) were fixed for 10 min on copper grids covered with Formvar membranes, previously shaded with carbon. The samples were stained for 10 min with 3% ammonium molybdate, pH 7.0. The particles were observed with a Zeiss electron microscope (Facultad de Ciencias, Universidad de Chile), amplified 50,000 to 80,000 times, and then photographed. The diameters of both particles were estimated by comparison to rotavirus particles (data not shown).

Antiprotease assay. CHSE-214 cells were infected with IPNV in the presence of 50- μ Ci/ml [³⁵S]Met. At 8 hpi, the supernatant was discarded and the monolayers were lysed as described above. Extracts were further incubated for 4 or 8 h at 15°C with 10 mM concentrations of the following antiprotease compounds: iodoacetamide (IAA), phenylmethylsulfonyl fluoride (PMSF), and EDTA. Particles were then extracted and partially purified as described above in the presence of the same concentration of each of the antiprotease compounds. Particles were then identified by TGA electrophoresis.

RESULTS

Isolation and identification of IPNV particles. A native electrophoresis system with polyacrylamide gels in Tris-glycine has been previously utilized to characterize small nuclear particles involved in the splicing of cellular mRNA precursors (35). Later, agarose replaced polyacrylamide for analyzing the assembly of subviral particles of rotaviruses (26). Figure 1A, lane 1, shows the migration of [³⁵S]Met-rotavirus particles in TGA electrophoresis, utilized as a migration control for IPNV particles. As previously reported, two bands were resolved in the gel: double-shelled virions and single-shelled rotavirus subparticles, with corresponding diameters of 80 and 70 nm, respectively (26).

In our initial experiments, we wanted to identify the different viral particles produced during IPNV infection. A single round of IPNV infection in monolayers of CHSE-214 takes 24 h at 15°C (14, 44). To avoid the production of defective interfering particles induced at high MOI (52), infections with virus stocks were performed at low MOI (0.1 to 1 PFU/cell) in the presence of a radioactive precursor ([³⁵S]Met or [³²P]HPO₄²⁻), and monolayers were incubated until a visible CPE was observed (3 to 4 days), indicative of complete virus spread. Viral particles were concentrated, partially purified from cellular supernatants, and resolved by TGA electrophoresis. Figure 1A, lanes 2 and 3, shows the migration of IPNV particles labeled with [³⁵S]Met and [³²P]HPO₄²⁻, respectively. Two discrete bands, designated particles A and B, were identified in the presence of either radioactive precursor, suggesting that both are viral ribonucleoprotein complexes. Such particles could not be detected when supernatants from mock-infected cells were labeled and analyzed under similar conditions (data not shown). The migration of particles A is similar to that of the single-shelled rotavirus particles, indicating that their diameters are comparable. Consistent with its migration, particles B correspond to a diameter of about 60 nm. We cannot discard the possibility that different electrostatic contributions affect the migration of both particles during their separation in the agarose gel. However, these contributions are currently uncertain.

FIG. 1. Isolation and identification of IPNV particles in TGA gels. (A) Radiolabeled and partially purified IPNV particles from culture supernatants were resolved by 0.8% TGA electrophoresis and visualized by autoradiography. Lane 1, [^{35}S]Met-labeled rotavirus particles (strain SA-11) used as migration control. Arrows on the left indicate double-shelled (ds) virions and single-shelled (ss) rotavirus subparticles, corresponding to diameters of approximately 80 and 70 nm, respectively (26). IPNV particles labeled with either [^{35}S]Met (lane 2) or [^{32}P]HPO $_4^{2-}$ (lane 3) are resolved as two bands designated by arrows (particles A and B) on the right. IPNV A and B particles resolved by TGA electrophoresis were recovered from agarose and subjected to electron microscopy analyses. (B) Electron micrograph of structures visualized in particles A. (C) Electron micrograph of structures visualized in particles B. Bar, 100 nm.

Since the particles A and B were resolved under nondenaturing conditions in TGA electrophoresis, concentrated eluates corresponding to bands A and B were processed for electron microscopy (EM) analysis. As a control, a partially purified IPNV preparation (input for TGA gel) was prepared in parallel for negative staining. Figure 1B shows that eluates from band A contain species with the general morphology of a viral particle. However, they are apparently open and permeable to ammonium molybdate staining. The careful observation of different individual particles suggested distinct degrees of openness but similar icosahedral architecture (data not shown). The average EM-estimated diameter for these particles is 66 ± 2 nm.

The viral particles recovered from the band B (Fig. 1C) showed a regular, icosahedral shape and were not permeable to ammonium molybdate staining, which suggests a completely compacted structure. Particles B have an EM-estimated diameter of 60 nm. A partially purified IPNV preparation apparently showed a mixture of both kinds of particles, A and B, but it was difficult to unequivocally distinguish between them (data not shown). Consistent with our observations, populations of different particles have been previously observed for purified birnaviruses (10, 50, 59). These results confirm the different sizes of particles A and B observed by TGA electrophoresis. The appearance of both particles suggests they are late morphogenesis intermediates, in which one probably corresponds to the particle precursor and the other corresponds to the virion.

Identification of IPNV particles obtained during the infective cycle. To identify the particle(s) produced at the different stages of the IPNV infective cycle, infections were carried out in the presence of a radioactive precursor ([^{35}S]Met or [^{32}P]HPO $_4^{2-}$) and then arrested at 8, 12, or 18 hpi. Since a single round of the viral cycle may have slightly different extents between different infections (i.e., 18 to 24 h) (14, 44), we correlated the progression of each infection by monitoring viral dsRNA synthesis at identical time points. Results from TGA electrophoresis showed no particles detectable at 8 hpi for infections performed in the presence of either radioactive precursor (Fig. 2A and B, lanes 8 hpi). In control infections (in the presence of [^{32}P]HPO $_4^{2-}$), no viral genomic dsRNA was detected from infected cells at 8 hpi. Viral RNA was detected at 12 and 18 hpi, as indicated by 7% PAGE (data not shown) (27). At 12 hpi, particles A and B were both identified, and at 18 hpi, they appeared to be accumulated in infected cells, suggesting a continuous assembly (Fig. 2A and B, lanes 12 and 18 hpi). These results indicate that the first IPNV particle is assembled at time points between 8 and 12 hpi during the infective cycle.

To determine the first viral particle produced during the IPNV infection, a narrow period of the infective cycle, 7 to 12 hpi, was monitored. Results showed that particles A become detectable first, at 8 hpi (Fig. 2C and E, lanes 8 hpi), whereas particles B were only identified 2 to 3 h later, at 10 to 11 hpi, with either radioactive precursor (Fig. 2C and E). In both time course experiments, the viral genomic dsRNA was first detected at 8 hpi from infected cells (Fig. 2D and F, lanes 8 hpi). This time point for viral dsRNA synthesis correlates with the identification of particles A by TGA electrophoresis. These results show that during the infective cycle, particles A assem-

FIG. 2. Identification of IPNV particles produced during the infective cycle. CHSE-214 cells were infected with IPNV in the presence of $[^{35}\text{S}]\text{Met}$ (A) or $[^{32}\text{P}]\text{HPO}_4^{2-}$ (B). At 8, 12, or 18 hpi, viral particles were partially purified from cell extracts and resolved by TGA electrophoresis. To determine the time point at which the first viral particle is formed in the presence of $[^{35}\text{S}]\text{Met}$ (C) or $[^{32}\text{P}]\text{HPO}_4^{2-}$ (E), the cells were lysed every hour between 7 and 12 hpi and the viral particles were resolved by TGA electrophoresis. (D and F) To monitor the progression of the viral cycle in the experiments described for panels C and E, respectively, cells were simultaneously infected with IPNV in the presence of $[^{32}\text{P}]\text{HPO}_4^{2-}$ and the monolayer was lysed at identical time points. Viral $[^{32}\text{P}]\text{HPO}_4^{2-}$ -labeled dsRNA was extracted and analyzed by 7% PAGE (27). Control lanes correspond to RNA extracted from $[^{32}\text{P}]\text{HPO}_4^{2-}$ -labeled IPNV particles obtained at 48 hpi. Arrows indicate positions of the segments of viral genomic dsRNA.

ble first, simultaneous with the detection of viral dsRNA. Then, 2 to 3 h later, particles B become identified, probably after the intracellular accumulation of particles A. This sequence of events suggests that particles A are the morphogenesis precursors of particles B. Since both particles A and B are likely constituted of genome and viral proteins, their molecular compositions were analyzed in some detail.

Buoyant density and molecular composition of IPNV particles. To evaluate the buoyant density of the different IPNV

particles, partially purified viral preparations were layered onto CsCl gradients and ultracentrifuged to equilibrium. Consistent with previous results (17, 18), a single peak of radioactivity was obtained at densities of 1.33 g/cm^3 , as shown in Fig. 3A. Fractions containing the peak of radioactivity (fractions 9, 10, and 11) were further analyzed for their particle composition by TGA electrophoresis (Fig. 3B). These fractions were shown to be, in fact, a mixture of both particles A and B. Fraction 9 (1.32 g/cm^3) contained a greater proportion of par-

FIG. 3. Molecular characterization of IPNV A and B particles. (A) Partially purified [³⁵S]Met-labeled IPNV particles were banded in isopycnic CsCl gradients, and both the density and radioactivity of each 200-μl fraction were determined and plotted. (B) Fractions containing the peak of radioactivity (fractions 9 to 11) were subjected to TGA electrophoresis. The migration of A and B particles is indicated by arrows on the left. Upon resolution on TGA gels, particles A and B recovered from the gel were analyzed for [³²P]HPO₄²⁻-labeled dsRNA content by 7% PAGE (C) and for [³⁵S]Met-labeled protein composition by SDS-PAGE (D). The inset shows the densitometric analysis of viral proteins. The migration of viral components is indicated on the left of the panels, and the positions of protein markers are indicated on the right.

ticles B, whereas fraction 11 (1.335 g/cm³) contained more particles A than B. Consistent with a symmetric peak, fraction 10 (1.33 g/cm³), with most of the radioactivity, is composed of nearly equivalent amounts of both particles, indicating their comigration in the gradient and implying the similar buoyant density of particles A and B. The overall higher density of particles A may be explained by a CsCl uptake during the experimental procedure. This uptake would be consistent with

our EM observations where particles A were permeable to ammonium molybdate staining (Fig. 1B). We next went on analyze the macromolecular composition of both viral particles. As earlier expected, both viral particles A and B contained the two segments of viral genomic dsRNA (Fig. 3C). The profile of viral proteins is shown in Fig. 3D. Both populations of particles contain all of the viral structural polypeptides, PP, VP1, pVP2, VP2, VP3, VP4, and the trun-

FIG. 4. Pulse-chase experiments, maturation, and effect of antiproteases. (A) CHSE-214 cells were infected with IPNV as described above. After a [35 S]Met pulse from 4 to 8 hpi, cells were further incubated for chase periods in the absence of label and then lysed at the indicated time points. The viral particles were partially purified and identified by TGA electrophoresis. (B) Radioactive bands from particles A and B, as shown in panel A, were quantified and plotted. Consistent results were obtained from two independent pulse-chase experiments. (C) To

evaluate the maturation of particles A into particles B, the composition of the viral protein precursors varied between them. Quantification of the protein bands from SDS-PAGE (Fig. 3D, inset) indicated that particles A are composed approximately of 1.5% PP and 15% pVP2, whereas particles B contain nondetectable amounts of PP and pVP2. Importantly, particles A were shown to be composed of 30% VP2 and 27% VP3, with a pVP2/VP2 ratio of 0.44, whereas particles B were shown to be composed of up to 35% VP2 and 30% VP3. These values are different than those previously published for birnavirus particles (4, 17, 18), and the differences probably reflect distinct procedures for separation, purification, and analyses. Since both populations of particles A and B contain genomic RNA and the viral structural proteins, these results agree with the notion that particles A are the precursors of particles B. Particle maturation has been associated with postassembly proteolytic cleavage of capsid precursor proteins in other viral families (see Discussion), and it was further investigated for IPNV.

Pulse-chase, maturation experiments, and effect of antiproteases. To test whether particles A are the precursors of particles B, a series of pulse-chase experiments were performed. The viral particles were analyzed by TGA electrophoresis (Fig. 4A), and the bands corresponding to particles A and B were quantified for each time point, as shown in Fig. 4B. With these particular kinetics, particles A became identified at 8 hpi, which correlated with the first detection of viral dsRNA in a control infection (data not shown). Similar to our previous results, the input of radioactivity derived from particles A remained accumulated in infected cells until 12 hpi (Fig. 4A). At 14 hpi, the radioactivity associated with band A diminished 12.5% with respect to earlier time points and a proportional amount of label was detected, as incorporated in the form of particles B, as shown in Fig. 4B. Analysis of later time points indicated that further chase of radioactivity from particles A into particles B is a slow process with a maximum at 48 hpi, where more than 30% of radioactivity was chased in the form of particles B (Fig. 4A and B). These results are consistent with the interpretation that particles A are the morphogenesis precursors of particles B.

To directly prove the relationship between both populations of particles, a cell-free maturation assay was envisioned (Fig. 4C). For this assay, [35 S]Met-labeled IPNV particles A and B were separated by TGA electrophoresis and then recovered

perform an in vitro maturation assay, [35 S]Met-labeled IPNV particles A and particles B (lane 4, input) were recovered by electroelution. Lanes 1 and 2 correspond to particles recovered from bands A and B, respectively, and were run immediately after electroelution ($t = 0$). Lane 3 corresponds to the dialyzed electroeluate of particles A, incubated at 4°C for 24 h ($t = 24$). Arrows on the left of the figure indicate the migration of particles A and B. (D) The effect of antiproteases on particle maturation was tested in a cell-free assay. Cells were infected with IPNV in the presence of [35 S]Met. At 8 hpi, the cell extracts were incubated with 10 mM IAA, PMSF, or EDTA for 4 or 8 h. Partially purified particles were then resolved by TGA electrophoresis. Lanes 2 and 6 correspond to IAA treatment for 4 and 8 h, respectively; lanes 3 and 7 correspond to PMSF treatment for 4 and 8 h, respectively; lanes 4 and 8 correspond to EDTA treatment for 4 and 8 h, respectively; and lanes 1 and 5 correspond to incubation of extracts in the absence of antiproteases for 4 and 8 h, respectively. Arrows on the left of the figure indicate the migration of control particles.

from the gel by electroelution, defining this time point as time zero ($t = 0$). Recovered particles A were incubated at 4°C for 24 h ($t = 24$). We used incubation at 4°C to exclude particle instability due to a higher temperature during the assay. Both $t = 0$ and $t = 24$ particles were then subjected to a second round of TGA electrophoresis. As shown in Fig. 4C, at $t = 0$, particles A (lane 1) or B (lane 2) were clearly identified, with no detectable levels of cross-contamination. Upon 24 h incubation at 4°C, particles A yielded particles B (lane 3). Altogether, these results present strong evidence that particles A are the precursors of particles B. Based on these data, all of the machinery required for viral maturation is probably contained within particles A.

Electroeluted fractions ($t = 0$) corresponding to either particles A or B (Fig. 4B, lanes 1 and 2, respectively) were also tested for infectivity in a plaque formation assay, as previously reported (34). For comparison, the results were normalized by the specific activity of the [35 S]Met incorporated in each recovered band. Particles A gave a titer of 3.6×10^7 PFU/ μ Ci, whereas particles B gave a titer of 9.1×10^8 PFU/ μ Ci, indicating that the maturation of particles A is accompanied by a gain in virus infectivity of particles B. Thus, the viral titer of isolated particles A may correspond to the continuous maturation of virion precursors.

Previous results from this study have suggested the involvement of the proteolytic cleavage of the viral precursors on the maturation of particles A. The birnavirus-encoded protease VP4 has been shown to be a Ser-Lys protease belonging to the family of Lon proteases (3, 56) which is incorporated into virions (4, 42). To test a role of VP4 in particle maturation, compounds that could have antiprotease activity were assayed as inhibitors: IAA, PMSF, and EDTA. IAA is an alkylating agent for Cys and His residues, and PMSF is a specific inhibitor of serine proteases. Since VP4 is not a metalloprotease (14), EDTA is not expected to produce inhibition. CHSE-214 cells were infected with IPNV in the presence of [35 S]Met, and the cells were harvested early at 8 hpi, a time corresponding to the assembly of particles A in the absence of particles B (data not shown). Then cellular extracts were immediately treated for either 4 or 8 h with the antiprotease compounds, and viral particles were identified by TGA electrophoresis. Figure 4D shows that IAA (lanes 2 and 6) and PMSF (lanes 3 and 7) treatments were able to eliminate virion formation at 12 and 16 hpi, respectively, indicating that the maturation of particles A was arrested by protease inhibitors. In contrast, untreated samples incubated either for 8 to 12 hpi (lane 1) or for 8 to 16 hpi (lane 5) yielded particles B from particles A. As predicted, particles A isolated after an 8-h treatment with either IAA (Fig. 4D, lane 6) or PMSF (Fig. 4D, lane 7) did not produce lysis plaques. Thus, they are not infectious. However, consistent with previous titrations, a partially purified IPNV preparation composed of a mixed population of particles A and B gave an average titer of 1.0×10^7 PFU/ μ Ci when purified with IAA and a titer of 1.9×10^7 PFU/ μ Ci when purified in the presence of PMSF. Furthermore, a preparation of particles B purified in the presence of PMSF produced 1.2×10^8 PFU/ μ Ci, indicating that this antiprotease did not affect the infectivity of virions. These results suggest the involvement of the associated VP4 protease in the proteolytic maturation of particles A, since PMSF completely eliminated the production of particles B.

DISCUSSION

In this study, we have isolated, identified, and characterized, both molecularly and biologically, stable particles produced during the infective cycle of IPNV in CHSE-214 cells. A summary of our results in the context of the infective cycle is presented in Fig. 5. The earliest particle, named particle A (provirion), was first detected simultaneously with the viral dsRNA in infected cells, suggesting that viral assembly occurs as soon as dsRNA replication has begun. No prior intermediates could be detected. Particles A showed appearances of an incomplete viral assembly, and although they are composed by the viral genome, their pattern of viral precursor proteins suggested that they correspond to immature intermediates of the morphogenesis. Upon provirion maturation, infectious particles B (virion) are yielded, 3 to 4 h later during the viral cycle. Particles B were shown to exhibit the icosahedral shape and to be constituted mostly by a pattern of mature capsid proteins as well as by viral dsRNA. Maturation of IPNV particles A not only involved the acquisition of infectivity but also, and remarkably, a reduction in the particle diameter, which may involve a coordinated series of conformational changes visualized by a more-compact particle arrangement.

Postassembly maturation has been previously described for other families of unenveloped viruses, with particularities in each case. Unlike IPNV, the morphogenesis of poliovirus particles (a single-stranded RNA virus) takes place through a discrete set of capsid intermediates which assemble the viral genomic single-stranded RNA into an immature precursor provirion and then triggers proteolytic processing of viral precursors (28, 58). Furthermore, provirion maturation is also a requirement for release of the RNA genome into a new host cell. On the other hand, the morphogenesis of nodaviruses would proceed through an uninterrupted set of intermediaries. Following flock house virus infection of *Drosophila* cells, the first detectable assembled products are provirions whose shells are composed of 180 α -precursor protein subunits encapsidating the two positive-stranded genomic RNAs (25). Provirions are labile, and proteolytic maturation is required for infectivity (61), resulting in an increased virion stability (25). Provirion matures through an autocatalytic cleavage of the α -protein, generating the mature coat protein β and the γ -peptide and leading to the rearrangement of the capsid subunits (25). Additionally, as a result of proteolytic maturation, dramatic conformational changes have been described within a procapsid form of the *Nudaurelia capensis* ω virus (N ω V), a single-stranded RNA virus which infects insects of the lepidopteran order. Electron cryomicroscopy and image analysis were used to elaborate a model in which the procapsids are larger, more rounded, and porous and with more domains than the virion of N ω V (7). The large rearrangements were interpreted as subunit movements within the capsid.

For IPNV, we have found that particles A are built of both mature and precursor viral proteins. Among precursor proteins, we were able to identify the polyprotein and pVP2 as associated with immature provirions. It is actually not known why precursor proteins must be cleaved within the immature particle, but one consequence is the higher compaction of the virion, possibly through conformational changes between subunits, resulting in an icosahedral and regular geometry. For

FIG. 5. IPNV infective cycle and morphogenesis. Our model assumes that the viral events such as transcription, either of the positive or negative strand of RNA, translation, or intracellular accumulation of viral proteins have taken place to favor genome RNA replication. Once dsRNA synthesis has been initiated, it immediately triggers its assembly into immature particles A (provirions). As indicated, prior intermediates are unknown. Upon maturation, where both precursor cleavage and compaction of the capsid occur, the infectious virion (particles B) is yielded.

another birnavirus, IBDV, virus-like particles (VLPs) have been formed in heterologous systems (40). Composition analyses of the different kinds of VLP showed variable pVP2/VP2 ratios in which the presence of pVP2 correlated with spherical capsids and VP2 was predominantly found in regular icosahedral

VLPs, indicating an incomplete maturation in the spherical capsids (9, 40, 48). In a recent report, Chevalier et al. have proposed that the efficient maturation of pVP2 requires a precise and favored assembly of the IBDV particle (9). Furthermore, the C-terminal region of pVP2, which is presumably cleaved during capsid maturation, was implicated in the control of subunit interactions and flexibility during the assembly of IBDV VLPs (8, 9). Similarly, from the results shown for IPNV in this study, the presence of pVP2 correlated with immature provirions, whereas mature VP2 correlated with infectious and icosahedral particles. However, we cannot ignore the existence of subpopulations with different or intermediate degrees of maturation within the particle A fraction (Fig. 5). Thus, the postassembly maturation of pVP2 seems to be a common step during the morphogenesis of different members of the birnavirus family.

The maturation cleavage of viral precursor proteins within the capsid may also be a requirement for viral infectivity. In the present study, we have found that, at least, both pVP2 and the viral polyprotein are associated with uninformative IPNV provirions, whereas infectious virions (particles B) lack these protein precursors. In addition, we have found different VP4 and VP4t patterns in both particles (Fig. 3D). By mutagenesis of recombinant proteins, the cleavage sites of birnavirus pVP2 (38, 56) were identified and some of them were later shown to be essential for viral viability, indicating that maturation of IBDV pVP2 is also critical for infectivity (12). On the other hand, the viral polyprotein is processed and cleaved at the pVP2-VP4 and VP4-VP3 junctions by the viral protease VP4 (1, 45, 46, 56), which is a Ser-Lys protease (56) and shares homology to bacterial Lon proteases (3). VP4 has been described as a structural protein for IBDV (4, 40) and IPNV (42). Additionally, truncated forms of VP4 (VP4t) were previously identified in purified IPNV (42) as well as in our preparations of both particles A and B, although a higher proportion of VP4t is associated with infectious virions. Whether VP4t correspond to a postassembly maturation product or to a degradation fragment generated to inhibit VP4 activity after maturation has been completed remains to be elucidated.

Structural information about birnavirus particles has been obtained on both homologous and heterologous systems, but details of the virion morphogenesis remain obscure. The external surface of the particle is formed of trimeric subunits of VP2 (23, 39), and the innermost layer is formed by trimeric subunits of VP3, the viral dsRNA, VP1, and VP4 (4, 18). VP3, as predicted earlier, interacts with both segments of genomic dsRNA through its carboxy-terminal region (33, 64, 65), which also binds VP1 (40, 63, 64). The association of VP3 with viral dsRNA was also observed after extensive low-salt treatment of IPNV virions (29). Furthermore, EM analyses have shown that viral VPg-dsRNA complexes tend to self-associate where the interactions were found to take place at the end of the segments (6, 51). In this scenario, as soon as it is synthesized, the newly replicated VPg-dsRNA may act as an initiation complex to trigger genome assembly by continuously nucleating capsid proteins. VP3 may play a key role in stabilizing the genomic dsRNA, where charged residues at its C terminus seem to be essential for this interaction, and to prompt proper particle assembly (9, 47, 64). In a proposed model for IBDV VLPs, VP3 needs to be activated by either genomic RNA or VP1 to

induce capsid assembly and pVP2 maturation (9, 48). As elucidated in the present study, the assembly of immature provirions seems to be a rapid and favored process. However, postassembly maturation appeared to be slow and inefficient, and it may reflect that only a fraction of particles A are correctly assembled and capable of undergoing the whole pathway of morphogenesis.

In the present study, we were unable to isolate stable morphogenesis intermediates prior to the detection of the replicated dsRNA assembled into particles A (Fig. 5). One possibility is that smaller capsid precursors are labile and/or their structure is sensitive to the conditions applied (i.e., NP-40). For different RNA viruses, RNA replication complexes form on membrane structures derived from diverse intracellular organelles (21, 24, 36, 49, 55, 60). On the other hand, for reoviruses, replication and assembly take place within cytoplasmic inclusions which are not membrane bound but associate with cytoskeletal elements (2, 53, 54). For IPNV, components or subcellular structures associated with viral RNA replication are unknown. However, since viral assembly occurs simultaneously with RNA replication, there must be a temporal and spatial coordination between these events. The existence of capsid precursors would support the vision of a discrete morphogenesis, as revealed for picornaviruses (see above). Another possibility is the existence of a preformed procapsid. However, we favored the model of a continuous morphogenesis for IPNV, initiated by viral dsRNA synthesis, where it promotes particle assembly, immediately nucleating capsid components until the generation of provirions. The morphogenesis of IPNV is later completed, and probably for the other birnaviruses as well, when most of the molecules of viral precursor proteins have been cleaved within the immature capsid, leading to the compaction of the particle and the acquisition of infectivity to yield icosahedral virions (Fig. 5).

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