Rod-shaped nuclear viruses of crustaceans: gut-infecting species

Phyllis T. Johnson¹, Donald V. Lightner²

¹ National Marine Fisheries Service, Northeast Fisheries Center, Oxford, Maryland 21654, USA

² University of Arizona, Environmental Research Laboratory, 2601 E. Airport Drive, Tucson, Arizona 85706, USA

ABSTRACT: There are 6 baculovirus-related nuclear viruses known to attack the hepatopancreatic epithelium of decapod crustaceans, including penaeid shrimp, brachyuran crabs, and anomuran crabs. Four of the 6 viruses are nonoccluded. One of the occluded species is accepted as a member of the Baculoviridae by the International Commission on Taxonomy of Viruses. The 4 available gut-infecting crustacean viruses are described and all 6 are compared to each other and to viruses from insects and arachnids that share some similar characteristics. Morphological similarities, including among others the presence of a truly cylindrical nucleocapsid, a unilateral subapical envelope expansion, and a tail-like appendage that arises from the nucleocapsid and is reflexed within the envelope expansion, indicate that the crustacean viruses are related to one another. In morphological and some developmental attributes, the crustacean viruses, including the occluded species, show affinities to the nonoccluded virus of *Oryctes*, which is currently the type of proposed Subgroup C of the Baculoviridae.

INTRODUCTION

Six rod-shaped, singly enveloped, nuclear viruses are known which attack endoderally derived tissues, mainly the hepatopancreatic epithelium, of decapod crustaceans. They are clearly related to the Baculoviridae, although only one has been sufficiently characterized to allow its acceptance as a member of that family by the International Committee on Taxonomy of Viruses (ICTV) (Matthews 1982). Four of the 6 are nonoccluded.

Study of 1 new virus and 3 others at our disposal adds considerable information about the general nature of the gut-infecting viruses and gives some insight into which developmental and structural characters might indicate degrees of relatedness among them. Development of the species apparently differs, but virions are morphologically similar and unlike virions of lepidopteran nuclear polyhedrosis viruses (NPVs). These viruses are compared to each other, to the remaining crustacean viruses, and to other arthropod viruses that have some attributes in common. Most of the latter viruses are currently assigned to Subgroups A, B, and proposed Subgroup C of the Baculoviridae (Matthews 1982), and to the Polydnviridae (Stoltz et al. 1984, Brown 1986).

MATERIALS AND METHODS

For light microscopy, crab tissues were fixed in Helly's solution (Johnson 1980) and stained by the Feulgen technique, using picric acid and methyl-blue counterstains (Farley 1969); shrimp tissue was fixed in Davidson's fluid (Humason 1967) and stained with hematoxylin and eosin. Primary fixation for crab tissues used in electron microscopy was in cold 4 % glutaraldehyde in 0.1 M, pH 7.4, Sorenson's phosphate buffer. Shrimp tissues were fixed in cold 6 % glutaraldehyde in 0.15 M Millonig's phosphate buffer (Pease 1964), supplemented with 1 % sodium chloride and 0.5 % sucrose. All tissues were postfixed at room temperature in 1 % osmium tetroxide in their respective buffers, dehydrated in ethanol and propylene oxide, and embedded in Spurr's low viscosity embedding medium (Spurr 1969) or in an Epon/Araldite mixture (1:1). Thin sections were stained with uranyl acetate and lead citrate. Negative staining was with phosphotungstic acid. Shrimp tissues were viewed with either a Hitachi H-500, a Phillips 200, or a JEOL 100CX electron microscope; crab tissues were viewed with a Zeiss EM 9S 2 electron microscope.

Terms used in this paper to describe viral structure are defined by Johnson (1988: companion article).

OBSERVATIONS

The 6 viruses that infect the epithelium of the hepatopancreas (midgut gland), and sometimes that of the midgut proper, can be grouped together, depending mainly on nucleocapsid/virion morphology. Early development apparently differs between the 2 species that form occlusion bodies. Three of the viruses, including the occluded ones, are from various species of penaeid shrimp. Two viruses are from the brachyuran portunid crabs *Callinectes sapidus* and *Carcinus mediterraneus*, and one is from the blue king crab *Paralithodes platypus*, which is an anomuran. Most of the viruses have been extensively studied histologically with the light microscope and evidence of infection outside the gut has not been found. It is likely, therefore, that they normally attack only endodermally derived cells.

The shrimp viruses vary considerably in their ability to cause disease, depending on the viral species and the life-history stage of the host (Lightner 1983, Johnson 1984). The viruses from *Callinectes sapidus* and *Paralithodes platypus* are apparently nonpathogenic in juveniles and adults, the only stages that have been studied. The virus from *Carcinus mediterraneus* causes fatal disease in mature crabs (Pappalardo & Bonami 1979, Pappalardo et al. 1986).

The species reviewed here are the occluded virus first found in *Penaeus monodon* (Lightner & Redman 1981), the occluded virus from American and Hawaiian *Penaeus* spp. (Couch 1974a, Brock et al. 1986), the nonoccluded virus of *Callinectes sapidus* (Johnson 1976), and a previously undescribed nonoccluded virus from Alaskan *Paralithodes platypus*.

Hepatopancreatic virus of *Callinectes sapidus* (Baculo-A)

Baculo-A is a nonoccluded virus that infects differentiating cells of the hepatopancreatic epithelium (Johnson 1976, 1983, 1984). Prevalence in groups of crabs collected from Chesapeake and Chincoteague Bays and their tributaries (Maryland and Virginia, USA) usually varied from 4 to 20%; overall prevalence was 6% in 1500 crabs. Baculo-A has not been observed to cause disease in the blue crab. Although infected cells undoubtedly do not perform normal functions, they are almost always few in number and in isolated groups. Baculo-A does not attack stem cells; infection occurs only in those cell populations which are undergoing constant replacement.

Transmission is presumably *per os*. Materials from the hepatopancreas and stomach are enclosed in a peritrophic membrane as they pass through the midgut, and feces are expelled in a string enclosed by

this membrane. It is not uncommon for crabs to eat fecal strings, and containment of virions of Baculo-A within the string would make for more efficient transmission than if virions were released separately into the water. Transmission through cannibalism probably occurs also. The focal nature of infection suggests that virions may pass from one epithelial cell to another, but how they enter epithelial cells from the gut or pass from one epithelial cell to another is unknown.

Infected nuclei were up to 2.3 times normal size. The center of the nucleus was evenly and not strongly Feulgen positive except that occasionally somewhat diffuse and more deeply staining areas occurred there. Usually the center of the nucleus was surrounded by an intensely Feulgen-positive marginal band (Fig. 1). Nucleolar remnants were common, especially in F-cells (Fibrillar cells), which typically have large, multiple nucleoli. The remnants were needle-shaped, spherical, or irregular.

Electron microscopy

Because Baculo-A does not cause disease or grossly visible changes in fresh tissues, infected nuclei could be found only by chance in hepatopancreatic tissues. The few infected nuclei available for ultrastructural study were from 4 crabs. Tissue from 1 crab was tipped into icewater shortly after being immersed in glutaraldehyde, causing damage to cellular components. The most obvious damage was shrinkage and loss of internal cell membranes; positions of plasma membranes remained visible. Tissue from another crab had been embedded in paraffin and was re-embedded for electron-microscopic examination as described by Johnson (1977).

Because of insufficient material, progressive assembly of virions could not be studied. An early infection was found in one properly fixed nucleus. This nucleus had finely fibrillar material through the center and marginated chromatin (Fig. 2). (The fibrillar material had a more granular appearance in infected nuclei from the damaged tissue.) In the early infection virions were scattered at random through the nucleus, and there were no envelope precursors in the central portion, which was presumed to be the viral stroma. Nucleocapsids were cylindrical and squared apically (Fig. 3). A boundary was not visible between core and capsid. The nucleoprotein of the core had distinctly lucent areas. The reticulate appearance was more pronounced in virions from the damaged tissue. Naked (unenveloped) nucleocapsids were not seen except that particles in the re-embedded tissue lacked any indication of envelopes. Envelopes were trilaminar in all other preparations, including the glutaraldehyde-fixed,

Figs. 1 to 4. Baculo-A. Fig. 1. Light micrograph showing infection of both nuclei in a binucleate cell (arrow). Normal nuclei are also present (open arrow). Feulgen. Bar = 10 μ m. Fig. 2. Early infection. Bar = 1.37 μ m. Fig. 3. Virions and long, empty capsids. Note the subapical envelope expansion (arrows), which is also indicated in cross-sectioned virions with an excentric nucleocapsid (arrow). Bar = 106 nm. c: long capsid; e: envelope; nc: nucleocapsid. Fig. 4. Advanced infection. Virions accumulate marginally. Note long, empty capsids in central part (arrows). Bar = 1.37 μ m. nu: nucleolar remnant.

damaged tissues where nuclear and other internal cell membranes were missing. The envelope was 8 to 11 nm thick. It was unilaterally expanded subapically and around the apex of one end of many virions (Fig. 3). The area between the nucleocapsid and the envelope, especially in the expanded portion, was sometimes filled with a dense material. Similar material surrounded nucleocapsids in the re-embedded tissue. A few nucleocapsids in the lightly infected nucleus apparently were not completely enveloped (Fig. 5). Nucleocapsids were 43×240 to 254 nm. Virions were about 60 nm in diameter across the narrowest part and 260 to 300 nm long.

In advanced infections, virions tended to be concentrated along the nuclear margin in more-or-less ordered arrays (Fig. 4). The intensely Feulgen-positive marginal bands visible in nuclei studied with light microscopy (Fig. 1) probably represented similar concentrations of virions. Some cross-sectioned virions in the lightly infected nucleus were each surrounded at a fixed distance by granules that had a strong similarity to ribosomal precursor particles (Alberts et al. 1983) (Fig. 5). Similar granules were scattered in the general area; all were near the nuclear margin and outside the viral stroma. See further description of putative ribosomal precursor particles in the section on MBV.

Several nuclei with advanced infections (all in the damaged tissues) contained long, tubular elements the same width as nucleocapsids and from 240 nm to over $4.5 \mu\text{m}$ long (Figs. 3 and 4). They were presumed to be naked, empty capsids.

Structures that probably represented the nucleolar remnants visible with the light microscope were opaque and without visible internal structure (Fig. 4). They occurred in nuclei from the damaged tissue.

Hepatopancreatic virus of *Paralithodes platypus* (Baculo-PP)

This nonoccluded virus, described here for the first time, was confined to epithelial cells of the hepatopancreas of *Paralithodes platypus*, the blue king crab. Like Baculo-A of the blue crab, it was focal and thus may not be pathogenic, although it probably infected stem cells as well as differentiating cells. Baculo-PP has occurred in at least 2 populations of Alaskan blue king crabs. There was a prevalence of 40% (12/30) at Olga Bay, Kodiak Island, in April 1982 and of 20% (4/20) at the

Pribilof Islands in June 1982. Baculo-PP was not detected in tissues of 25 crabs each from St Matthews Island, July 1982, and St Lawrence Island, September 1982, or in 9 crabs from the Pribilof Islands, February 1983.

Infected nuclei were often hypertrophied but not as markedly as with Baculo-A. The greatest increase in size was less than 1.5 times over normal. Infected nuclei were uniformly Feulgen positive centrally and usually had an intensely Feulgen-positive margin of variable width (Fig. 6). The marginal material often extended inward as a scanty reticulum or as a larger inclusion. Nucleoli were sometimes present in infected nuclei, and small, indistinct, Feulgen-negative inclusions were common. These bodies stained green with a combination of the methyl-blue and picric-acid counterstains.

Electron microscopy

The following description is based on examination of sections of hepatopancreas from a single crab. The tissues had been fixed in Helly's solution, substituting zinc chloride for mercuric chloride. They were stored in 70% ethyl alcohol prior to processing for electron microscopy. Internal cell membranes were missing and definition of other structures was impaired. Identification of material as nucleolar as opposed to chromatin-like, or granular as opposed to fibrillar, was not possible.

Virions were in groups or scattered at random and various nuclear inclusions were present (Fig. 7). Viral particles that appeared to be fully enveloped were associated with many of the smaller inclusions (Fig. 9). The small inclusions perhaps represented the greenish bodies visible in Feulgen-stained tissues examined with the light microscope.

Only enveloped particles were seen (Figs. 8 and 9). The nucleocapsid was cylindrical with squared ends and size 37 to $40 \text{ nm} \times 190$ to 210 nm . There was a suggestion of regularly spaced subunits on or in the nucleocapsid, but how they were arranged could not be ascertained. Virions were 70 nm at their narrowest diameter and 230 to 265 nm long. The boundary between capsid and core was not discernible. The envelope was trilaminar and about 10 nm thick (Figs. 8 and 9). There was a dense substance in the space between the envelope and the nucleocapsid. Some envelopes were expanded unilaterally and subapically,

Fig. 5. Baculo-A. Lightly infected nucleus. Some nucleocapsids are apparently not completely enveloped. Note group of cross-sectioned virions, each of them surrounded by putative ribosomal precursor particles (arrow). Bar = 271 nm

Figs. 6 to 8. Baculo-PP. Fig. 6. Light micrograph of normal and infected (arrow) nuclei. Bar = $10 \mu\text{m}$. Fig. 7. Infected nucleus. Virions are confined to the more lucent area. Bar = $1.37 \mu\text{m}$. V: virion area. Fig. 8. Virions. One has a lateral envelope expansion (arrow). Bar = 93 nm. E: envelope; nc: nucleocapsid

Fig. 9. Baculo-PP. Virions in contact with a small inclusion. One is partially embedded in it (arrow). A cross-sectioned virion has an envelope expansion containing the cross section of a filament (open arrow). Bar = 93 nm. e: envelope; ex: conelike extension of envelope; nc: nucleocapsid

and around the apex. This expansion was flangelike along part of its length (Figs. 8, 9 and 10). Many virions had the envelope extended into a cone at one end (Figs. 9 and 11), and at least sometimes the cone narrowed rapidly into a threadlike tail more than 200 nm long and about 14 nm in diameter, with a very narrow, internal filament extending from the core some distance down the center of the tail (Fig. 11). A dot was sometimes visible in cross-sectioned flanges (Figs. 9 and 10). Presumably it represented a narrow filament similar to the one in the envelope tail.

The cytoplasm of infected and uninfected cells was not in condition for study.

Hepatopancreatic virus of *Penaeus monodon* (MBV)

MBV (Monodon BaculoVirus) is one of the 2 occluded viruses of penaeid shrimp. It sometimes attacks epithelial cells of the anterior midgut proper as well as those of the hepatopancreas. MBV was discovered in adult *Penaeus monodon* reared in Mexico from Taiwanese postlarvae (Lightner & Redman 1981).

MBV, or several strains of MBV and MBV-like viruses, are widespread in the Indo-Pacific and Mediterranean and occur in several penaeid species. Since the first report, infections have been found in cultured populations of *P. monodon* in the Philippines, Taiwan, Malaysia, Hawaii, and Tahiti, in cultured *Penaeus merguensis* from Singapore and Malaysia, in cultured *Penaeus semisulcatus* from Kuwait, Kenya, Israel and Taiwan, and in captive-wild juvenile *Penaeus kerathurus* in Italy (Lightner et al. 1983, 1985, D. V. Lightner unpubl.).

MBV causes disease and sometimes serious mortalities in hatchery-reared postlarval, juvenile, and adult *Penaeus monodon*, *P. merguensis*, and *P. semisulcatus*. Whether it also produces patent disease in larvae is, as yet, unknown, but developing infections have been observed in larvae as early as third stage protozoa. Severe disease is associated with overcrowding, and other conditions, such as heavy gill and surface fouling, are often present in shrimp populations undergoing severe MBV mortalities. The deleterious effect of MBV on its host is due to destruction of hepatopancreatic epithelial cells. MBV is presumably

Figs. 10 and 11 Baculo-PP. Fig. 10. Montage of cross-sectioned virions showing the filament in the lateral envelope expansion (arrows). Bar = 93 nm. Fig. 11. Baculo-PP. Montage of longitudinal sections of virions showing the conelike extension and filament arising from the nucleocapsid. Arrows point to the long slender extension of the cone of one virion. Bar = 93 nm. f: filament

transmitted *per os* by ingestion of free virus and occlusion bodies, and by cannibalism (Lightner et al. 1983).

MBV can be easily diagnosed with the light microscope by observing fresh squashes of the hepatopancreas. If MBV is present, there are multiple, roughly spherical, occlusion bodies in hypertrophied nuclei (Fig. 12). Stains like malachite green help to distinguish occlusion bodies from lipid droplets, normal nuclei, etc. Similarly hypertrophied nuclei, with eosinophilic occlusion bodies, are visible in sectioned and stained tissues. Prior to the appearance of occlusion

Fig. 12. MBV. Light micrograph of hypertrophied nuclei with occlusion bodies (arrows) in a fresh squash of the hepatopancreas. Aqueous 0.05 % malachite green stain. Bar = 10 μ m

bodies, infected nuclei can be recognized in sections because they are slightly hypertrophied and the nucleolus is peripherally displaced or missing. Gross behavioral and morphological signs of MBV disease, and an extended discussion on cytopathological progression of infection, are given by Lightner et al. (1983).

Electron microscopy

Early infection was signaled by margination of chromatin and clearing of the central part of the nucleus, forming the virogenic stroma (Fig. 13). Sometimes the stroma was filled with a finely fibrillar/granular material that was evenly lucent; sometimes finely granular, denser areas were present also. If a nucleolus was present, it was of normal appearance and usually peripheral. Pieces of membrane, and capsids forming in association with membrane, were scattered through the stroma. There were also occasional forming or completely formed nucleocapsids. Later stages had developing and completed occlusion bodies (Fig. 14). If the nucleolus remained by the time of occlusion-body formation, it almost always had lost the fibrillar portion, leaving only the granular compartment, which presumably consisted mainly of ribosomal precursor particles (Fig. 15). Similar particles were spread through the marginal areas of the nucleus not occupied by the stroma. The transformed granular nucleolus was thought to be a part of the virogenic stroma by Lightner et al. (1983), and homologous with the discrete, dense to opaque virogenic stroma produced by lepidopteran NPVs. We now consider that virions migrate from the central stroma to the periphery of the nucleus and that

their close contact with the altered nucleolus, when that structure is present, comes about because they are halted at its boundary. Furthermore, only virions were

associated with the nucleolus; assembly of capsids and nucleocapsids took place in the central virogenic stroma. Membrane fragments occurred in the peripheral areas, but forming or empty capsids did not. Virions were not prevented from passing into areas containing scattered nucleolus-derived granules and sometimes were surrounded at a fixed distance by the granules, as in Baculo-A.

Capsids were never seen without an envelope. Some images suggested that capsids might begin assembly within closely applied fingerlike outpocketings in membrane fragments (Figs. 16 and 21). The envelope of a completed capsid was not in contact with the capsid and was at least sometimes open-ended (Fig. 17). The nucleoprotein that formed the core probably entered the capsid through this open end. Filling of the capsid with nucleoprotein probably began with the appearance of a tenuous filament that extended centrally along the longitudinal axis of the capsid (Figs. 17 and 18). Following this putative initial insertion, filling would have had to be rapid because capsids in more advanced stages of core formation were not seen. The opaque nucleoprotein of the core formed a reticulum and sometimes the central filament was still visible (Fig. 19). Capsids and nucleocapsids were cylindrical and both were ca 42 nm × 246 nm. Virions were about 75 nm × 324 nm. The capsid was not clearly delineated from the core.

Finished viral envelopes usually appeared like a dense layer, ca 16 nm thick, with a fuzzy external margin (Fig. 18). Rarely, limited opaque areas of the envelope were trilaminar in both free and occluded virions (Fig. 20). The envelopes of some virions in a secondary lysosome of a hepatopancreatic cell had an apical cone-shaped projection like that of Baculo-PP (Fig. 19) and in several instances, there were indications that the cones may have been extended into tails, as are the cones of Baculo-PP. Some occluded and free virions in other nuclei, and especially in the cytoplasm of a lysing cell, had the envelope unilaterally expanded subapically and around the apex, and nucleocapsids of a few virions with envelope expansions were bent subapically (Fig. 21). Cross sections demonstrated that part of the envelope expansion was flangelike (Fig. 22). As in Baculo-PP, the dot in the center of cross-sectioned expansions probably represented the cross section of a filament which arose from the apex of the nucleocapsid.

Figs. 13 and 14. MBV. Fig. 13. Early infection. The nucleolus is normal and a dense area is present in the viral stroma, which fills almost all the nucleus. There are pieces of membrane and some forming particles in the stroma (arrows). Bar = 739 nm. nu: nucleolus. Fig. 14. Late infection. Occlusion bodies are present and most virions are marginal or associated with the occlusion bodies. Pieces of membrane are present in the stroma (arrow). Bar = 938 nm. ob: occlusion body; v: virions

Figs 15 and 16. MBV. **Fig. 15.** Transformed nucleolus in an infected cell. It is surrounded by virions. Membrane fragments and forming capsids are present in the virogenic stroma (arrows). Bar = 488 nm. ob: occlusion body, nm: nuclear membrane; nu: nucleolus. **Fig. 16.** Possible early capsid formation within fingerlike outpocketings in membrane (arrows). Bar = 152 nm

Figs. 17 and 18. MBV. **Fig. 17.** Empty capsid and capsid with central filament that probably represents beginning of insertion of nucleoprotein. Bar = 370 nm. c: empty capsid; f: capsid with filament. ob: occlusion body. *Inset* shows a completed capsid with the envelope open at one end (arrow). Bar = 188 nm. **Fig. 18.** Montage of capsids with central filaments (arrows). Bar = 30 nm

and was reflexed within the expanded portion of the envelope (Fig. 21).

The paracrystalline occlusion bodies had an average diameter of about 4.8 μm . They were multiple, more or less spherical, and formed by subunits about 17.7 nm in diameter. Although many virions became embedded in the occlusion bodies, many remained free and were expelled into the lumen of the hepatopancreas in that condition. Occlusion bodies that were released into the gut lumen began to fragment there, but some passed through more or less entire. The nucleus was irregularly shaped in late infection (Fig. 14), and its outer membrane occasionally proliferated to form a complex labyrinth as it sometimes does with BP, the other occluded virus of shrimp.

There were bundles of microfilaments in some infected nuclei (Fig. 22), and also in the cytoplasm. Ultrastructural appearance of the cytoplasm during infection of the nucleus with MBV has been described by Lightner et al. (1983).

The chance finding of a naked, empty capsid, free in the cytoplasm and perpendicular to a nuclear pore (Fig. 23), suggests that entry of MBV into gut cells is probably by fusion of the viral envelope and the plasma membrane, with consequent loss of the envelope, and that uncoating of the genome takes place at the nuclear pores. There was a suggestion of a fine filament, part of it still in the naked capsid, and with its free portion extending through the nuclear pore. The nucleus was not in the early stages of infection. Virions were present, and occlusion bodies had already formed.

Hepatopancreatic virus of American and Hawaiian penaeids (BP)

BP (Baculovirus Penaei) was the first shrimp virus discovered (Couch 1974a) and is the only crustacean baculovirus-related species to be extensively characterized (Summers 1977) and accepted as a member of the family Baculoviridae by the ICTV (Matthews 1982). BP infects various species of American *Penaeus*: *P. duorarum*, *P. aztecus* and *P. setiferus* in the Gulf of

Figs. 19 to 21. MBV. Fig. 19. Virions in a secondary lysosome. Some have cone-shaped envelope extensions, and a central filament can be seen in several nucleocapsids (arrow). Bar = 244 nm. e: envelope; ex: cone-shaped envelope extension. Fig. 20. Occluded virions. One has a lateral envelope expansion and the envelope of another is distinctly trilaminar in part (arrow). Bar = 122 nm. ex: envelope expansion. Fig. 21. Virions in a lysing cell. They have lateral, subapical envelope expansions and a filament arises from the nucleocapsid and is reflexed within the expansion of one (arrow). A forming capsid is present. Bar = 122 nm. c: forming capsid; ob: occlusion body.

Mexico, and *P. vannamei* and *P. stylirostris* in culture facilities along the Pacific coast of Central America and northern South America (Couch 1981, Johnson 1984). It has also been found in Hawaiian *Penaeus marginatus*

(Brock et al. 1986). Our BP-infected specimens were as follows: *P. aztecus*, postlarvae, from a commercial hatchery in Florida; *P. vannamei*, larvae and postlarvae, from a commercial hatchery in Ecuador; and *P. marginatus*, juveniles, collected from shallow beach waters on Oahu, Hawaii. Figures are based on material from *P. marginatus*, except that Fig. 24, inset, is from *P. duorarum*.

BP attacks mainly cells of the hepatopancreatic epithelium, but can also infect midgut epithelium. Its ability to cause disease varies considerably, but it does cause epizootics in larval shrimp in hatcheries (Couch 1981, Lightner et al. 1983).

Couch (1974a, b) has discussed in detail the progression of infection and appearance of infected cells at both light- and electron-microscopic levels, and provided descriptions of the virion. BP infection is diagnosed in the same manner as MBV infection, i.e. by examination of fresh squashes of the hepatopancreas, gut contents, or feces. The BP occlusion bodies visible in such preparations are tetrahedral rather than spherical or irregular as in MBV.

Electron microscopy

Early infection was signaled by margination of remaining host chromatin and clearing of the center of the nucleus to form the virogenic stroma, as in the other gut-infecting viruses. The stroma contained profiles of membrane, many of them circular, immature viral particles, and occasional virions. In later infections virions had a marked tendency to occur next to and within the tetrahedral occlusion bodies, and in a single layer close to the nuclear membrane. Small particles resembling the putative ribosomal precursor particles of nuclei infected with other crustacean viruses were scattered throughout the nucleus (Fig. 24). However, these particles might be subunits of polyhedrin (Couch 1974b).

Naked capsids and nucleocapsids were not seen. Capsids may have developed from small spherical objects, similar to, or the same as 'capsid originators' (Johnson 1988), that were within cup-shaped or spherical pieces of membrane, and nucleoprotein apparently first entered the finished capsid as a central filament (Fig. 24). The entire process of nucleocapsid formation prob-

Figs. 22 and 23. MBV **Fig. 22.** Montage of cross-sectioned virions with lateral envelope expansions (arrows). The occluded one clearly shows the cross section of a filament in the expanded area. Bar = ca 200 nm. **fi:** bundle of microfilaments in the nucleus; **ob:** occlusion body. **Fig. 23.** Naked capsid in the cytoplasm, perpendicular to a nuclear pore. A very fine filament, arising centrally in the capsid, appears to extend through the nuclear pore. Bar = 152 nm. **c:** capsid; **N:** nucleus; **np:** nuclear pore

ably occurred rapidly, because intermediate steps were not present in our material. Nucleocapsids in *Penaeus duorarum* and *P. aztecus* were about 50×270 nm and virions were about 75 nm in diameter (Couch 1974b), but nucleocapsids and virions are significantly smaller in *P. marginatus* (Brock et al. 1986), and slightly larger in *P. vannamei* (Lightner et al. 1985). Envelopes were about 8.5 nm thick, and fit loosely except for occluded virions and ones with envelope expansions, as discussed below. Some virions had a unilateral envelope expansion that was subapical or nearer to one end of the nucleocapsid, which was sometimes slightly bent (Fig. 25). An opaque line within the envelope expansion probably represented a reflexed filament that arose from the end of the nucleocapsid as in Baculo-PP and MBV. The central dot in cross sections of lateral envelope expansions presumably represented that filament (Fig. 25). Negatively stained, naked nucleocapsids were about 50×225 nm in our preparations. They had apical caps and a tail (Fig. 26).

Proliferation of the outer nuclear membrane of infected cells was seldom seen in our material, but such proliferated membranes sometimes formed extensive labyrinths in the preparations studied by Couch (1974b).

BP has double-stranded, circular DNA with a molecular weight of 75×10^6 daltons $\pm 2 \times 10^6$ (Summers 1977).

DISCUSSION

Table 1 lists various attributes of the gut-infecting crustacean viruses and certain other arthropod viruses, both occluded and nonoccluded, that have features in common.

Larsson (1984) has listed a number of other species that share characteristics with some of the crustacean viruses. Table 1 may be referred to in order to find the names or acronyms used for the various viral species mentioned in the text.

Occlusion bodies

We do not believe presence or absence of occlusion bodies in the gut-infecting crustacean species is a major determinant of relatedness. Considering all the

occluded and nonoccluded viruses that belong to, or are related to the Baculoviridae, there is a lack of concordance among the ability to form occlusion bodies and the type of development, morphology of nucleocapsids and envelopes, and the presence or absence of envelope and nucleocapsid modifications. Further, the occluding protein produced differs in species or groups of viruses that form these proteins. The occlusion-body protein of *Tipula* baculovirus cannot be dissolved by techniques used with the lepidopteran and hymenopteran NPVs, and may have a larger cystine residue (Bergoin & Guelpa 1977). Summers (1977) found, and Clerx & Lightner (1985) confirmed, that BP polyhedrin cross-reacts with antisera prepared to polyhedrins and granulins of several insect baculoviruses, and these cross-reactions indicate that BP polyhedrin contains some similar primary sequence(s) and, therefore, common or similar antigenic determinant(s) to those found in polyhedrins and granulins of insect baculoviruses. However, Summers (1977) also found that BP polyhedrin contains different antigenic determinants, and that it differs from insect-virus polyhedrins in various physical properties. For example, size of the major occlusion-body polypeptide of BP differs markedly from the polyhedrin of lepidopteran NPVs (Summers 1977, Clerx & Lightner 1985). Therefore, polyhedrins from BP and the insect viruses are not closely related.

Couch (1974b) remarked on the resemblance between the development of BP, which is occluded, and the nonoccluded virus of *Oryctes*. Bergoin & Guelpa (1977) said that the looseness of its envelope and relative thickness of its nucleocapsid made the occluded *Tipula* virus more like the *Oryctes* virus than any of the other (occluded) dipteran baculoviruses. Also, as Gouranton (1972) pointed out, the ability of granulosis viruses (and some lepidopteran NPVs: Granados 1980) to form occlusion bodies differs according to the cell type infected. He concluded that the ability to form occlusion bodies did not necessarily indicate the viruses in question were closely related.

Development

In all gut-infecting species for which there is information, and like the *Oryctes* virus (Fig. 1 of Huger

Figs. 24 to 26. BP. Fig. 24. Infected nucleus with a fairly advanced infection. Virions are associated with the occlusion body and periphery of the nucleus. To the right there is a capsid with a central filament of nucleoprotein (arrow). There are cup-shaped and circular pieces of membrane, some of them associated with developing viral particles. The viral stroma is filled with granules like ribosomal precursor particles. Bar = 309 nm. ob: occlusion body; V: virion. Inset shows putative capsid originators and an empty capsid. Bar = 64 nm. c: capsid; co: capsid originator. Fig. 25. Virions with envelope expansions. A filament is reflexed within the expansion (arrows). Bar = 185 nm. cu: cup-shaped membrane with developing capsid; ob: occlusion body. Inset is a montage of cross-sectioned virions with envelope expansions, each containing a cross-sectioned filament (arrows). Bar = 77 nm. Fig. 26. Negatively stained naked nucleocapsid. Note apical caps (open arrows) and tail (closed arrows). Bar = 50 nm.

Table 1. Attributes of gut-infecting crustacean viruses and some other viruses with shared characteristics

Viral species	Host	Tissue infected	Occlusion bodies	Envelope modifications/nucleocapsid with tail	Development with membranes	Average dimensions of nucleocapsid ^a	Loose envelope	Vesicles in nucleus	Nucleocapsid a true cylinder	Putative ribosomal precursor particles present	Source
BP (Baculovirus Penaei)	<i>Penaeus</i> spp. (shrimp) (American)	Hepato-pancreas, anterior midgut	Yes	Yes/Yes	Yes	50 × 270	Yes	No	Yes	? ^b	Couch (1974), Lightner et al. (1983)
MBV (Monodon BaculoVirus)	<i>Penaeus</i> spp. (Asian, European)	Hepato-pancreas, anterior midgut	Yes	Yes/Yes	Yes	42 × 246	No	No	Yes	Yes	Lightner et al. (1983)
BMNV (Baculoviral Midgut gland-Necrosis Virus)	<i>Penaeus japonicus</i>	Hepato-pancreas	No	Yes/Yes	No ^c	36 × 250	No	No	Yes	Yes	Sano et al. (1981, 1984)
Tau	<i>Carcinus mediterraneus</i> (crab)	Hepato-pancreas, anterior midgut	No	Yes/Yes	No ^c	68 × 310	No	No	Yes	No	Pappalardo & Bonami (1979), and others
Baculo-A	<i>Callinectes sapidus</i> (crab)	Hepato-pancreas	No	Yes/?	?	43 × 247	No	No	Yes	Yes	Johnson (1976, 1983, 1984)
Baculo-PP	<i>Paralithodes platypus</i> (crab)	Hepato-pancreas	No	Yes/Yes	?	38 × 200	No	No	Yes	?	This paper
<i>Oryctes</i> virus	<i>Oryctes rhinoceros</i> (beetle)	Midgut, fat body, etc.	No	Yes/Yes	Yes/No ^d	45 × 170	Yes	Yes	Yes	No	Huger (1966), and others
<i>Gyrinus</i> virus	<i>Gyrinus natator</i> (beetle)	Midgut	No	No/No	Yes	35 × 150	Yes	No	Yes	No	Gouranton 1972
<i>Chaoborus</i> virus	<i>Chaoborus crystallinus</i> (midge)	Midgut	No	Yes/?	Yes	40 × 218	No	No	Yes	No	Larsson (1984), pers. comm.
<i>Bacillus</i> virus	<i>Bacillus rossius</i> (stick insect)	Midgut	No	??	?	35 × 210	Yes	Yes	Yes	Yes	Scali et al. (1980)
<i>Diabrotica</i> virus	<i>Diabrotica undecimpunctata</i> (beetle)	Hemocytes	No	??	?	52 × 230	No	Yes	Yes	?	Kim & Kitajima (1984)
Braconid virus	Parasitic braconid wasps	Calyx of ovary	No	Yes/Yes	No	40 × 25-250	Yes	No	Yes	No	Stoltz & Vinson 1979
HZ-1	<i>Heliothis zea</i> (moth)	Cell line derived from ovary	No	??	No	40 × 380	Yes	No	Yes	No	Granados et al. (1978), Kelly et al. (1981), Langridge (1981)
<i>Pisaura</i> virus	<i>Pisaura mirabilis</i> (spider)	Hepato-pancreas	No	No/No	No	35 × 300	Yes	No	Yes	No	Morel et al. (1977)
<i>Tipula</i> virus	<i>Tipula paludosa</i> (crane fly)	Hemocytes	Yes	Yes/Yes	?	75 × 220	Yes	No	Yes	?	Bergoin & Guelpa (1977)
Polydnaviridae	Parasitic ichneumonid wasps	Calyx of ovary	No	Yes/Yes	Yes	85 × 330	Yes	No	No	No	Stoltz & Vinson (1978)

^a Measurements, in nm, are based on particles in thin sections, except for Tau and HZ-1, which were negatively stained

^b ? : unknown, unsure, or not stated in the paper and not demonstrated in the included figures

^c Development may eventually be found to take place in association with membrane as well

^d Development known to take place with and without association with membranes

1967) and the crustacean hemocyte-infecting viruses (Johnson 1988), nucleoprotein destined to form the core apparently first enters as a thin filament that extends centrally through the length of the capsid, and which is sometimes visible in the finished nucleocapsid.

Naked capsids of MBV and BP have not been seen within nuclei. There are suggestions that MBV capsids may develop within fingerlike outpocketings in pieces of membrane, but those of BP may be found to have capsid originators like those of the hemocyte-infecting

crustacean viruses (Johnson 1988). Nucleocapsids of Tau (Pappalardo & Bonami 1979, Pappalardo et al. 1986) and BMNV (Sano et al. 1981, 1984) can form in the absence of membrane, but there are indications that Tau may also develop in association with membrane (Fig. 27). We lack information on this point for Baculo-A and Baculo-PP.

Proposed Subgroup C of the Baculoviridae includes all nonoccluded rod-shaped nuclear viruses that lack a polydisperse genome and otherwise meet the criteria for inclusion in the Baculoviridae (Matthews 1982). The

Oryctes virus is presently the designated type of Subgroup C. Quiot et al. (1973) and Crawford & Sheehan (1985) never saw naked particles of *Oryctes* virus in the cultured cells they used in their respective studies, and some have assumed that development with membranes could be considered a criterion for Subgroup C viruses. However, papers based on in vivo observations of the *Oryctes* virus report the presence of naked particles (Huger 1966, 1967 [Fig. 1] Monsarrat et al. 1973a, Payne 1974). If we delete the criterion of development with membrane, all nonoccluded crustacean gut-infecting species can be fitted into Subgroup C. The occluded crustacean viruses cannot be placed in Subgroup A. Not only are there differences in polyhedra, but nucleocapsids of the occluded gut-infecting species form in association with membrane. Morphological similarities of these viruses to *Oryctes* virus suggest that they are also related to Subgroup C. Other species, listed in Table 1, that apparently develop only in association with membrane, are the nonoccluded viruses from *Gyrinus* (Gouranton 1972) and *Chaoborus* (Larsson pers. comm.), and various nonoccluded ichneumonid viruses of the family Polydnviridae (Stoltz & Vinson 1979, Stoltz et al. 1981).

Morphology

The gut-infecting species from crustaceans resemble *Oryctes* virus, viruses of Subgroups A and B, and all the viruses listed in Table 1, except the Polydnviridae, because the nucleocapsid is a true cylinder.

Like the *Oryctes* virus (Stoltz & Vinson 1979), negatively stained nucleocapsids of BP, Tau, and BMNV have distinct caps of different density at either apex. The other crustacean-infecting species have not been negatively stained. The nonoccluded putative baculoviruses that infect calyx cells of braconid wasp ovaries (Stoltz & Vinson 1979), a nonoccluded baculovirus from the calyx of the ichneumonid *Mesoleius tenthredinis* (Stoltz 1981), and the occluded virus from *Tipula* hemocytes (Bergoin & Guelpa 1977), also have broad, moderately to very short nucleocapsids with apical caps. Stoltz & Vinson (1979) refer to the caps of the parasitoid viruses as 'end structures' suggesting that these authors consider the caps homologous to the polarized 'end structures' that occur in some (perhaps all) members of Subgroups A and B (Teakle 1969, Kawanishi & Paschke 1970).

Negatively stained nucleocapsids of BP, BMNV (Sano et al. 1984), Tau (Pappalardo & Bonami 1979), the braconid viruses, *Tipula* virus, and *Oryctes* virus (Monsarrat et al. 1973b, Monsarrat & Veyrunes 1976, Payne et al. 1977), as well as thin-sectioned Baculo-PP, have extensions referred to as 'tails' that arise from one end

Figs. 27 and 28. Tau. Fig. 27. Enveloped capsids are apparently in process of being filled with nucleoprotein (arrows). Some virions have lateral, subapical envelope expansions and are slightly curved. Bar = 250 nm. V: virion. Fig. 28. Enveloped nucleocapsids that are bent to various degrees. Arrows point to ones that are strongly bent subapically. Bar = 455 nm

of the nucleocapsid. It would not be surprising if all the crustacean gut-infecting species were found to have these structures.

Subapical, unilateral envelope expansions are present in thin-sectioned material of all the gut-infecting species. Filaments seen within the expansions in longitudinal and cross sections presumably represent the tails found on negatively stained, naked nucleocapsids. Couch (1974b) found a terminal knoblike cap on a thin-sectioned BP virion, and cone-shaped extensions were present on some thin-sectioned virions of MBV and Baculo-PP. Because the cone of Baculo-PP, and perhaps that of MBV, is sometimes extended into a filament-containing thread, it is tempting to postulate that the various modifications have to do with deployment of the nucleocapsid tail, either naked, as seen in negatively stained nucleocapsids of the various viruses discussed above, or within the envelope, as is apparently the case with Baculo-PP and a member of the Polydnaviridae (Stoltz & Vinson 1979).

Envelope modifications also occur in *Oryctes* virus, the *Chaoborus* virus, and probably in the occluded virus of *Tipula*, for Larsson (1984) noted that nucleocapsids of cross-sectioned virions of the latter species are excentric within the envelope. Further, it will be remembered that the negatively stained nucleocapsid of *Tipula* virus has a tail. Cross-sectioned virions from *Chaoborus* (Larsson 1984) and *Oryctes* (Crawford & Sheehan 1985) sometimes have flanges with cross-sectioned filaments, which closely resemble similar sections of BP, Baculo-PP, and MBV virions. In the case of *Oryctes* virus, Crawford & Sheehan postulated that the lateral bulge holds the nucleocapsid tail. Our experience with the crustacean viruses bears this out.

Payne et al. (1977) believe that the tails seen on negatively stained nucleocapsids of *Oryctes* virus are probably extorted internal components of the nucleocapsid that are usually released when the envelope is removed. They suggest that the distinctiveness of *Oryctes* virus tails may result from their being more easily released than the presumably similar structures of lepidopteran NPVs described by Ponsen (1965), who found that sodium carbonate used to remove NPV virions from occluding proteins also caused removal of the envelope and emergence of a very long threadlike structure from the nucleocapsid. These structures, much longer and thinner than the tails described above, look like filaments that arise from tails on *Oryctes*-virus nucleocapsids that have undergone treatment for release of nucleoprotein (Monsarrat et al. 1975). Extension of tails of crustacean and *Oryctes* viruses in negatively stained material undoubtedly does depend on removal of the envelope. However, prior containment of the tails (or material that will form them) in lepidopteran NPVs as opposed to *Oryctes* and crustacean

viruses is either different or tail formation takes place in a different time frame. The available evidence indicates that while the envelopes of *Oryctes* and the crustacean viruses are still present, the tails are already external to the nucleocapsid and within an expansion of the envelope.

Oryctes virus and some other viruses listed in Table 1 usually have loose envelopes, as opposed to the snug, evenly applied envelopes that surround virions of singly enveloped Subgroup A viruses. (When first formed, the envelope of some Subgroup A viruses is loose: Hughes 1972, Federici 1980.) The envelope of BP virions may be loose, but except for the specialized expansions, the envelopes of the other gut-infecting species are applied closely and evenly around the nucleocapsid.

Bending of capsids/nucleocapsids

In the original material of Tau virus, thin-sectioned nucleocapsids were usually strongly curved medially and the envelope expansion was along the entire concave side of the nucleocapsid (Pappalardo & Bonami 1979). When envelopes were lost during the process of negative staining, the resulting naked nucleocapsids were straight. These facts led Pappalardo & Bonami to propose that there was a lateral structure in the envelope of Tau that extended from either end, and that contracted to cause bending of the nucleocapsids. They postulated that when this structure was ruptured during negative staining, the nucleocapsid was free to become straight. They sometimes saw a lateral structure in the envelope of negatively stained virions which they thought might have served that purpose. Since the original description of Tau, more material has become available. In this material Tau often has straight or only slightly bent nucleocapsids, and some are more curved or bent subapically (Fig. 28), like bent virions of MBV and of BMNV (Sano et al. 1981, 1984). It is possible to relate the lateral structure in Tau's envelope to the filament which we know arises from the nucleocapsid in MBV and Baculo-PP, which is presumably reflexed within the envelope expansion, and which forms the tail seen on negatively stained naked nucleocapsids. We have not seen bending of Baculo-A and Baculo-PP nucleocapsids, but our material of those species is limited.

Other viruses with bent nucleocapsids include the *Gyrinus* virus (Gouranton 1972), the virus of a fire ant, *Solenopsis* sp. (Avery et al. 1977), and the *Pisaura* virus, whose nucleocapsids are bent into a horseshoe shape within an oval, closely fitting envelope (Morel et al. 1977). Among the Baculoviridae, bending of nucleocapsids of the granulosis virus of *Trichoplusia ni* takes

place in cytoplasmic host-derived vesicles (Summers 1971) and Langridge (1981) found bent nucleocapsids in a negatively stained preparation of HZ-1, a nonoccluded virus of cultured *Heliothis* cells.

Whatever the reasons for bending, the capacity to bend is dependent on the architecture of the capsid and core. It would be interesting to test the type and extent of bending that would occur in capsid models based on continuous helices at various pitches, and on the interrupted helices that would occur in the stacked-disc model of the baculovirus capsid proposed by Burley et al. (1982).

Envelopes

It is generally considered that when baculovirus particles are enveloped in the nucleus, the envelopes are assembled *de novo* in the nucleus and are biological unit membranes (Hughes 1972, Stoltz et al. 1973, Stoltz 1981, and many others). Trilaminarity of the envelope is so evident that students of the morphology of baculoviruses are often brought to remark on this in their papers, and Stoltz et al. (1973) stated that envelopes of the fly virus they studied were trilaminar, but nuclear membranes in their preparations were not trilaminar and were thinner. None of the investigators reported using special methods for demonstration of unit membranes, as discussed by Hayat (1970), who remarked that under most circumstances, plasma membranes are more likely to be trilaminar than are internal cell membranes. Among the crustacean viruses, Baculo-A envelopes and internal host-cell membranes were lost in tissues originally embedded in paraffin, but positions of plasma membranes were still visible. Baculo-A and Baculo-PP envelopes were present and trilaminar, internal cell membranes were missing, and positions of plasma membranes were indicated in tissues that had been dropped into icewater during glutaraldehyde fixation, or fixed in Helly's solution and stored in alcohol. These are examples of gross departures from accepted procedures for transmission electron microscopy, but they show that envelopes of these viruses, as well as components of plasma membranes, can be extremely stable.

Other considerations

Negatively stained BMNV capsids are helical, with the spirals at an angle of 37 to 38° to the horizontal axis of the capsid (Sano et al. 1984). We lack other published information on capsid morphology of the crustacean viruses.

The granules that form a marginal band in MBV-

infected nuclei, and surround marginally located MBV, Baculo-A, and BMNV (Sano et al. 1981) virions, are similar to, and most probably are, ribosomal precursor particles (Alberts et al. 1983) released from the disintegrated or altered nucleolus. Similar marginal granules occur in nuclei infected with a hemocyte-infecting crustacean virus (Johnson 1988) and the *Bacillus* virus (Scali et al. 1980). The altered nucleolus in MBV infections, which becomes a mass of presumed ribosomal precursor particles, is similar to that of nuclei infected with adenovirus Type 2 (Phillips & Raskas 1972) except that the granules do not diffuse from the nucleolus in the adenovirus infection.

Oryctes virus occurs regularly in the cytoplasm of intact cells *in vivo* (Huger 1966, Monsarrat et al. 1973a) and *in vitro* (Crawford & Sheehan 1985). Particles the latter authors saw in the cytoplasm had a single envelope, and these gained a second envelope when they budded through the plasma membrane. Among the crustacean viruses, Pappalardo et al. (1986) found virions of Tau in vesicles and free in the ground substance of the cytoplasm of infected cells. Free virions were particularly common close to the plasma membrane, and although viral particles were not seen actively passing through the plasma membrane, these authors thought it likely that such passage did occur and could account for the focal nature of Tau infection. Two other crustacean gut-infecting viruses are known to invade the cytoplasm of heavily infected cells, either by penetrating the nuclear membrane of still intact cells, or by being spilled into the cytoplasm of disintegrating cells. Couch (1974b) reported the presence of BP virions in cytoplasmic vesicles close to the nucleus, and MBV occurs in the cytoplasm of lysing cells.

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