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Preliminary characterization and partial cloning of the genome of a baculovirus from *Penaeus monodon* (PmSNPV = MBV)

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ABSTRACT: Monodon baculovirus (MBV), an occluded virus related to the Baculoviridae, which is enzootic in Southeast Asia in both wild and farmed *Penaeus monodon* populations, was isolated and purified. Virions were rod-shaped, enveloped, 265 to 282 nm in length and 68 to 77 nm in diameter, with unique appendages at each extremity. Singly enveloped nucleocapsids were 250 to 269 nm in length and 62 to 68 nm in diameter, showed a 'cross-hatched' surface appearance, and a double-layered 'cap' structure closing its ends. Subunits of the occlusion body were 22 to 23 nm in diameter and had an icosahedral-like shape. These subunits (polyhedrins) exhibited at least 1 major polypeptide of 58 kDa by SDS-PAGE. The extracted nucleic acid was a very large supercoiled molecule, as shown by electron microscopy, and was demonstrated to be DNA by restriction enzyme digestion. BamHI restriction fragments obtained after agarose gel electrophoresis were separately cloned in the system pUC18-DH5α. Subsequently, restriction maps were established for the inserts, which ranged from 8.5 to 2.8 kbp (kilo base pairs) corresponding to the cloned BamHI restriction fragments. By its characteristics, we propose to include this virus in the subgroup A, subtype SNPV of the Baculoviridae family, and to name it PmSNPV according to the guidelines set forth by the International Committee on Taxonomy of Viruses.

INTRODUCTION

Baculoviruses pathogenic to Crustacea have been reported as non-occluded (subgroup C) and occluded (subgroup A) genera (see reviews from Johnson & Lightner 1988 and Brock & Lightner 1990). MBV (Monodon baculovirus) was originally described in *Penaeus monodon* (Lightner & Redman 1981, Lightner et al. 1983). It constitutes an important disease problem in cultured *P. monodon*, particularly in Southeast Asia. MBV-type baculoviruses have been recently reported in several species of the genus *Penaeus* (Lightner & Redman 1992). Data on the geographical distribution of the disease, its prevalence, the lesions induced and the ultrastructure of the virions and occlusion bodies have been very well documented in the review by Brock & Lightner (1990). However, no data on the characterization of this agent have been available to date, except for morphological data derived from TEM sections of the infected cells.

In fact, this situation is similar for the other baculoviruses isolated from marine crustacea (some from 15 years ago), where only ultrastructural descriptions were given. In contrast to the insect baculoviruses, little data exist on the physicochemical characteristics of the crustacean baculoviruses. The one exception to this is the data for BP isolated from *Penaeus duorarum*, for which the circular structure of the genomic DNA and the relative molecular weight (M_r) of the polyhedrin were demonstrated (Summers 1977). Compared to insect baculoviruses, the relative paucity of results could be explained partially by the absence of marine crustacean cell-lines and by the problems associated with purification of viruses from infected tissues.

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We report here our results on the purification of MBV, its preliminary characterization and the partial cloning of its genome.

MATERIAL AND METHODS

Infected animals. For this study we used previously frozen (stored at –70 °C) hepatopancreata (about 0.2 g each) from 4 small juvenile *Penaeus monodon*, which were obtained from Taiwan (provided by Dr Frelier, Texas A&M University).

Purification procedure. Four hepatopancreata were homogenized in 30 ml of TN buffer (Tris 0.02 M, NaCl 0.4 M, pH 7.4) using a Potter (glass/teflon) tissue blender and clarified 10 min at 2000 × *g*.

The pellet, after being resuspended, was layered onto a 40 to 65 % sucrose gradient and run for 30 min at 25 000 rpm (112 000 × *g*) in an AH629 Sorvall rotor, in an attempt to purify the occlusion bodies (OB).

The supernatant fluid was then pelleted for 30 min at 20 000 rpm (48 000 × *g*) in an SS34 Sorvall rotor. The pellet was resuspended in TN buffer, layered onto a 15 to 40 % (W/W) sucrose gradient and centrifuged at 25 000 rpm (106 000 × *g*) for 1 h in a TH641 Sorvall rotor. The gradient was removed from the centrifuge tube with an Auto-Densiflow density gradient fractionator (Buchler), and fractions (1 ml) were collected and analyzed with an ISCO fraction collector coupled with an ISCO UA5 UV monitor, which recorded the optical density at a wavelength of 254 nm. Fractions of interest were diluted, pelleted at 35 000 rpm (208 000 × *g*) in TH641 rotor for 1 h, and resuspended in 0.5 ml of TN buffer. The fraction containing enveloped virions was treated at room temperature for 10 min with 0.02 % (final concentration) of Triton X100 before being layered onto a preformed 25 to 45 % CsCl gradient (W/W) and centrifuged for 30 min at 35 000 rpm (208 000 × *g*) in TH641 rotor. The gradient was extracted as previously described, and fractions of interest were diluted in TN buffer and pelleted for 30 min at 35 000 rpm (208 000 × *g*). Final pellets were resuspended in 300 µl of TN buffer.

Electron microscopy. The analysis of fractions obtained at the different steps of the purification process was done by negative staining of the suspensions with 2 % sodium phosphotungstate (PTA), using collodium-carbon coated grids. Extracted viral nucleic acid was rotationally shadowed with platinicum-palladium after spreading it onto carbon coated grids, according to the method of Delain & Brack (1974).

SDS-PAGE. For polypeptide separation, 12 % SDS-polyacrylamide vertical gels were used according to a previously published protocol (Bonami et al. 1992). The run duration was 1.5 h at a constant current of 20 mA. Bovine serum albumin, egg albumin and trypsinogen, of 66, 45 and 24 kDa, respectively, were used as molecular weight (MW) markers. Gels were stained with Coomassie blue or silver (Morrissey 1981).

Nucleic acid extraction. Extraction of nucleic acid was performed as previously described (Bonami et al. 1990), except that Proteinase K and Sarkosyl treatments were longer (double time) and the final nucleic acid pellet was resuspended in 0.1 × TE buffer (Maniatis et al. 1982).

Agarose gel electrophoresis. Gels with 0.8 to 1 % agarose in TBE buffer (Maniatis et al. 1982) were used, and stained with 0.5 µg ml^{-1} ethidium bromide incorporated in the gel. Phage Lambda HindIII and

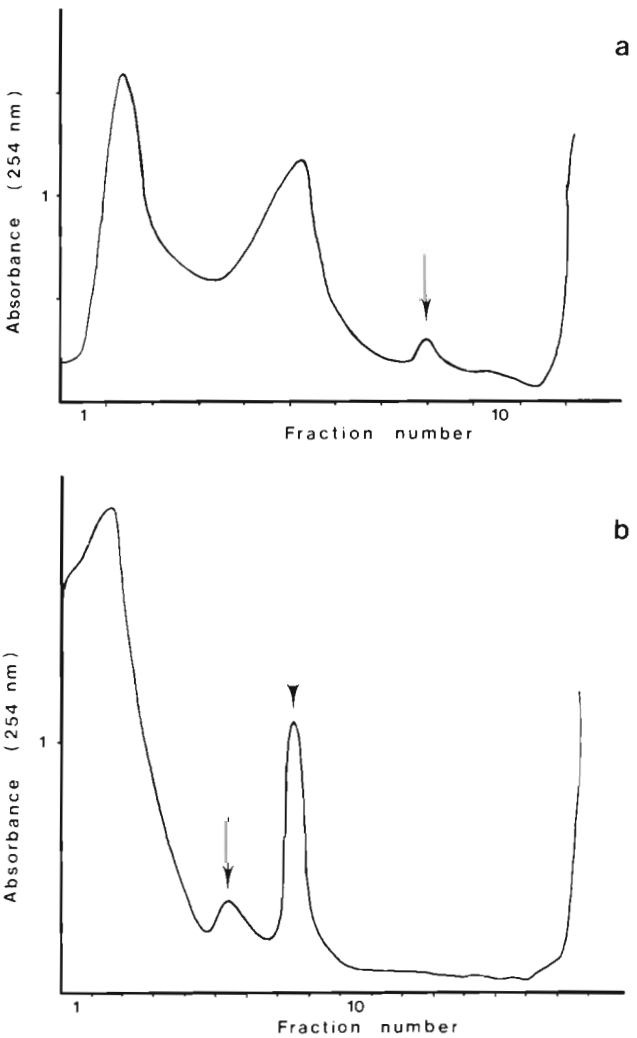
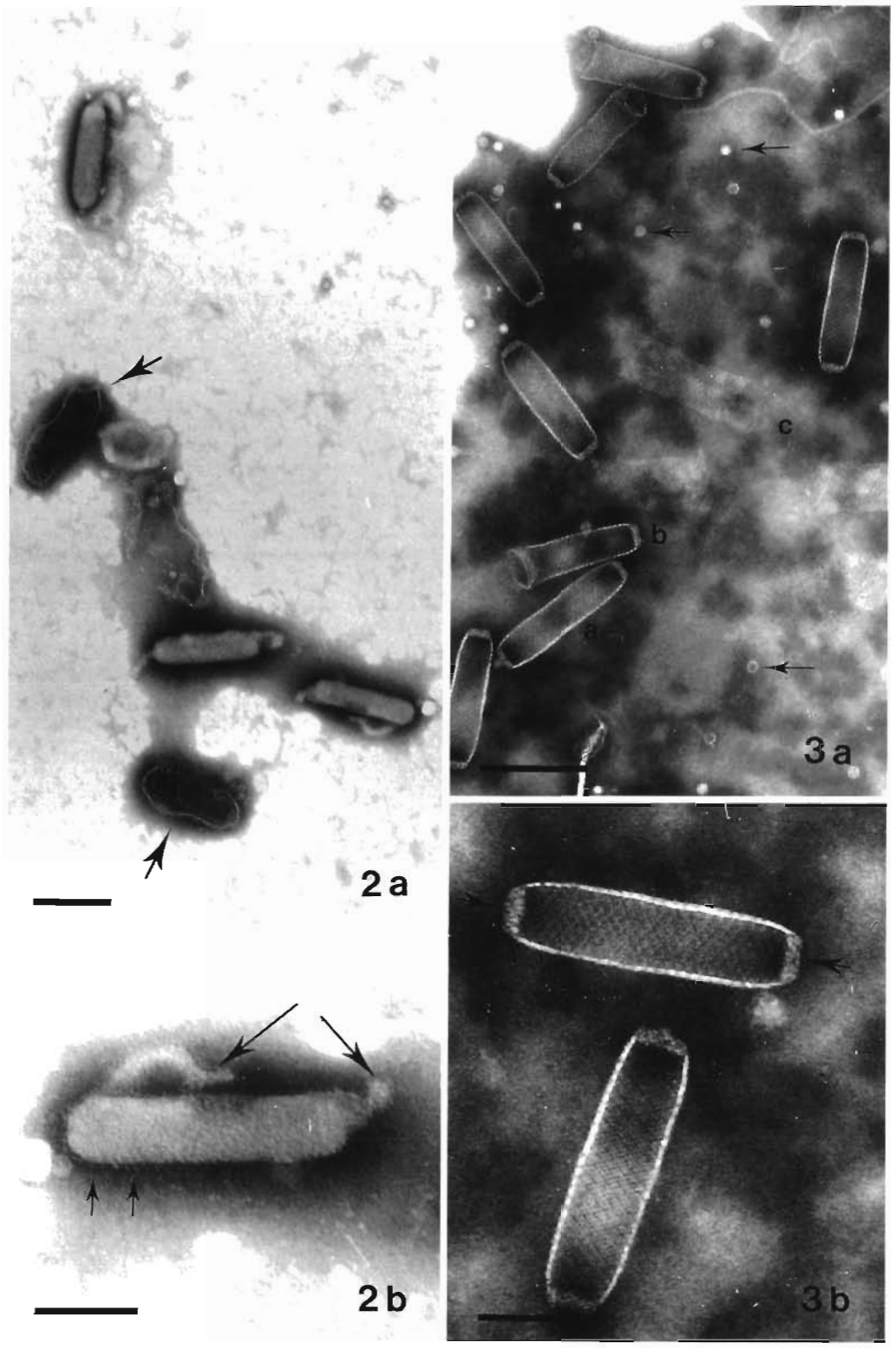


Fig. 1. (a) Purification of MBV, sucrose gradient 15 to 40 %. Arrow: peak corresponding to full enveloped virions. (b) CsCl gradient (25 to 45 %); fractions of the first peak contain envelopes and debris; the second (arrow) corresponds to nucleocapsids, the third (arrow head) to OBS



Figs. 2 and 3. MBV virions. Fig. 2. (a) Negative staining of enveloped MBV particles before treatment with Triton X100; the envelope of 2 particles is disrupted, showing the inner component, the nucleocapsid (arrows). TEM. Scale bar = 200 nm. (b) Higher magnification of an enveloped virion. The spikes on the envelope surface (short arrows) and the appendages at the 2 extremities (long arrows) are clearly evidenced. TEM. Scale bar = 100 nm. Fig. 3. (a) Purified MBV nucleocapsids showing the different degradation steps: full nucleocapsids (a), one extremity open (b) and collapsed empty structures (c); the preparation is contaminated with OBS (arrows). PTA. Scale bar = 200 nm. (b) Higher magnification of nucleocapsids. Note the caps at the extremities (arrows) and the 'cross-hatched' surface. Scale bar = 100 nm

HindIII-EcoRI DNA digests were used as M_r markers. Bands of interest were excised from the gel, and the DNA recovered directly using the Geneclean II Kit (Bio 101, Inc.). Restriction enzymes (Boehringer Mannheim) were used according to the manufacturer's instructions.

Library construction and screening. BamHI restriction fragments of the viral DNA were separated by gel electrophoresis and subsequently ligated using T4 DNA ligase (Boehringer Mannheim) into the BamHI site of pUC 18. Transformation was performed using competent *Escherichia coli*-DH5α cells. Transformed plasmid screening was done by agarose gel electrophoresis from alkaline lysis minipreps with and without prior digestion with restriction enzymes.

RESULTS

Virus purification

The pellet from the first clarification was resuspended and layered onto the 40 to 65 % sucrose gradient (modified from the procedure used by Summers 1977a) in an attempt to purify the OB. However, no band of OB was obtained, but virions were found in the first (upper) layer of the gradient; they were pooled with the virion-rich pellet from the first centrifugation step. The resulting fraction was then centrifuged through a 15 to 40 % sucrose gradient. Fully enveloped virions were found in fractions 8 and 9 (Fig. 1a), and a larger amount of virions was found in the pellet. All these fractions were pooled and, after treatment with Triton X100 to disrupt the envelopes, they were centrifuged through the 25 to 45 % CsCl gradient. Three bands were obtained (Fig. 1b): the first (fractions 1 to 5) was found to contain envelopes, the second (fractions 6 and 7) contained nucleocapsids, and the third (fractions 8 and 9) was composed of OB subunits (OBS = polyhedrin).

Ultrastructure of virions and polyhedrins

Before treatment with Triton X100, the majority of virions were enveloped; only a few showed a disrupted envelope surrounding the nucleocapsid and free nucleocapsids were scarce (Fig. 2a). Intact particles were 265 to 282 nm in length and 68 to 77 nm in diameter. The envelope surface appeared to be composed of small, uniformly sized granular structures interspersed with small spikes which were particularly apparent at apices of intact particles (Fig. 2b) and in PTA-penetrated particles. Each extremity of the envelope exhibited appendages which are believed to be envelope extensions (Fig. 2b).

After disruption of the virion envelope with Triton X100 and purification in CsCl gradient, the nucleocapsids (Fig. 3a) showed rows of surface structures, spaced about 6 nm apart, and arranged in a helical pattern. The arrangement of these structures formed an angle of ca 55° with the long axis of the nucleocapsids, giving the nucleocapsid surface a 'cross-hatched' appearance (Fig. 3b). The non-

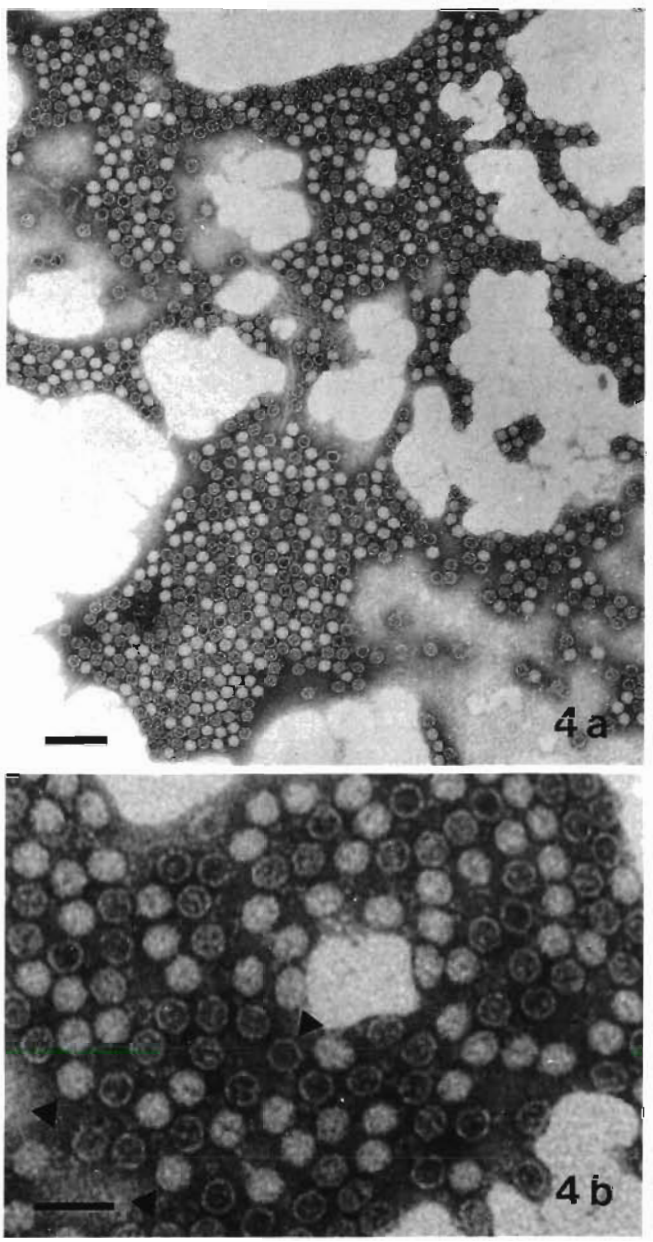


Fig. 4. (a) Negative staining of purified polyhedron subunits (OBS) of MBV resembling full and empty virus-like structures. TEM. Scale bar = 100 nm. (b) Higher magnification of the OBS, some exhibiting clearly a 6-sided or icosahedral structure (arrow heads). Scale bar = 50 nm

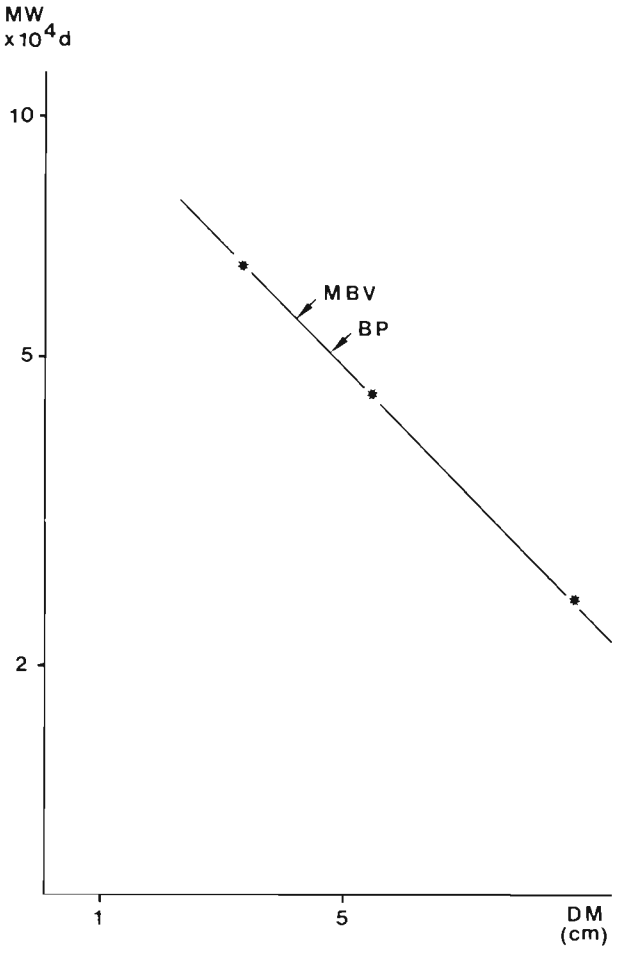


Fig. 5. M_r estimation of the MBV polyhedrin (only the major polypeptide is here plotted) compared with BP polyhedrin. (*) MW markers; DM: distance of migration; MW expressed in daltons

enveloped nucleocapsids were 250 to 269 nm in length and 62 to 68 nm in diameter. Each extremity was closed with a double-layered structure, or cap, 16 to 18 nm in thickness (Fig. 3b). Some nucleocapsids exhibited at one extremity a protruding braided-like structure interpreted as the nucleoprotein (not shown). Some disrupted nucleocapsids possessed only 1 closed extremity, while others lacked these caps and were obviously empty and collapsed (Fig. 3a).

The polyhedrin subunits of the MBV were 22 to 23 nm in diameter, and resembled full and empty (PTA penetrated) icosahedral virus particles (Fig. 4a), as indicated with different relative degrees of PTA penetration. The OB subunits in profile were grossly rounded, but some were obviously 6-sided (Fig. 4b).

SDS-PAGE of polyhedrin

After staining with Coomassie blue, only 1 major polypeptide was present in the gel. Compared with

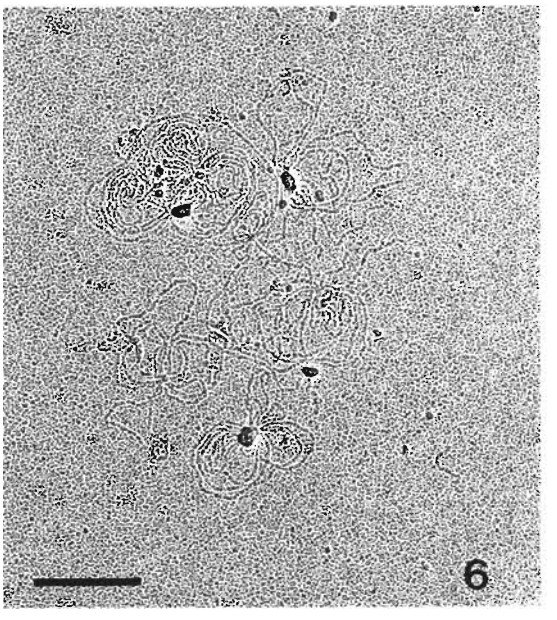


Fig. 6. Supercoiled DNA molecule forming the MBV genome. Platinum-palladium rotational shadowing. TEM. Scale bar = 0.5 µm

MW markers, its size was estimated at 58 kDa (Fig. 5). Two minor polypeptides were present, which were best demonstrated when the gel was silver stained; these very faint bands have an M_r of about 49 and 47 kDa, respectively.

Structure of nucleic acid

The nucleic acid was extracted from the final nucleocapsid fraction. A small portion was used for TEM visualization after spreading of the DNA onto a grid and rotational shadowing. Large, obviously supercoiled molecules with an estimated size of several thousand base pairs were found in the preparation (Fig. 6). Unfortunately, none of the molecules were sufficiently relaxed to permit their measurement in order to determine the total M_r of the genome.

Restriction pattern of viral genome

The remaining nucleic acid was digested with BamHI endonuclease and electrophoresed in a 1 % agarose gel (Fig. 7). Five bands were visualized (A to E, by convention in decreasing size) with an estimated size (compared to M_r markers) of about 21 or more, 9, 6.5, 3.5 and 2.8 kbp (kilo base pairs), respectively; the first (A) could contain 2 or 3 high M_r bands. Because of the small amount of DNA available, it is possible that additional bands of lower M_r were not visible in the gel.

Table 1. Number of restriction sites and insert size of cloned BamHI restriction fragments of the PmSNPV genomic DNA

	EcoRI	EcoRV	HindIII	KpnI	PstI	BglII	XbaI	HincII	SalI	SacI	Size (kbp)
B	2	3	1	1	0	4-5	1	3	0	1	8.5
C	5	2	3	nd	0	1	0	2	0	0	6
D	1	1	0	1	2	1	1	1	0	0	3.5
E	2	nd	3	nd	0	0	2	1	0	0	2.8
										Total	20.8

nd: not done

Cloning of the BamHI digested genomic pieces

Because the large size of DNA fragments in the A band, only the DNA in Bands B to E was recovered from the gel using the Geneclean II kit. Each band was subsequently ligated into the BamHI site of the plasmid pUC18 to produce 4 libraries.

After an initial screening of the 4 different libraries, numerous clones were selected which contained a recombinant plasmid with 2 BamHI sites and an insert of a similar size to the BamHI restriction fragments used for cloning (i.e. Bands B to E). Some other clones were found which contained a recombinant plasmid with only 1 BamHI site.

Characterization and mapping of the inserts

A preliminary study using restriction enzymes has shown that all the inserts with a size corresponding to the cloned restriction fragments were found in the 2 possible orientations. Table 1 lists the insert size and the number of restriction sites located in each. Restriction maps, with the HindIII site of the pUC18 as origin, were constructed for each insert listed in Table 1 (Fig. 8).

All of the cloned fragments with only 1 BamHI site present on the recombinant plasmids which have been investigated to date, do not exhibit restriction patterns similar to those shown by the inserts in Fig. 8. Their sizes range from 1 to 6.2 kbp, corresponding to a total of 32.7 kbp of additional cloned viral genome. These inserts are likely to have been derived from the remaining viral genome (i.e. random pieces of the BamHI-digested genome which were present throughout the agarose gel).

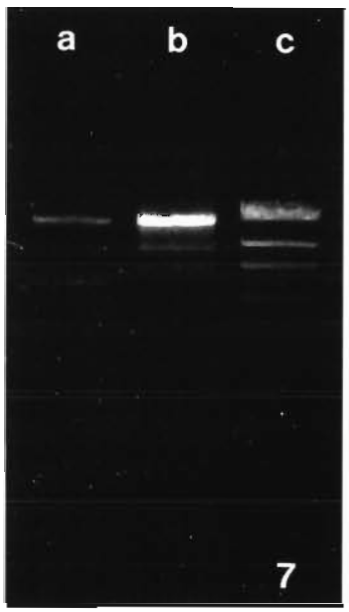


Fig. 7 Restriction pattern of the viral DNA in 1 % agarose gel electrophoresis after BamHI digestion (Lane b). Lane a: Lambda DNA EcoRI/HindIII digest; Lane c: Lambda DNA HindIII digest

DISCUSSION AND CONCLUSION

Based on ultrastructural characteristics in TEM sections, MBV (Monodon baculovirus), pathogenic for the shrimp *Penaeus monodon*, was recognized as a probable baculovirus when it was originally discovered (Lightner & Redman 1981, Lightner et al. 1983). Characteristics of MBV which support that conclusion include the rod-shaped enveloped particles, 324 × 74 nm in size, which develop in the nuclei of target cells, and many of which are occluded in paracrystalline intranuclear inclusion bodies. However, despite the importance of the MBV to the shrimp culture industry in Asia, to our knowledge, all attempts to purify and characterize the agent in other laboratories have been unsuccessful.

The purification procedure used here was not ideally adapted for MBV. In particular, the gradient values and the length of centrifugation runs should be modified to improve the purification technique. However, because we were limited by the amount of MBV-infected shrimp tissue, no improvement was possible. Indeed, after several purification attempts from MBV-infected shrimp with varying degress of infection and differing qualities of preservation, our

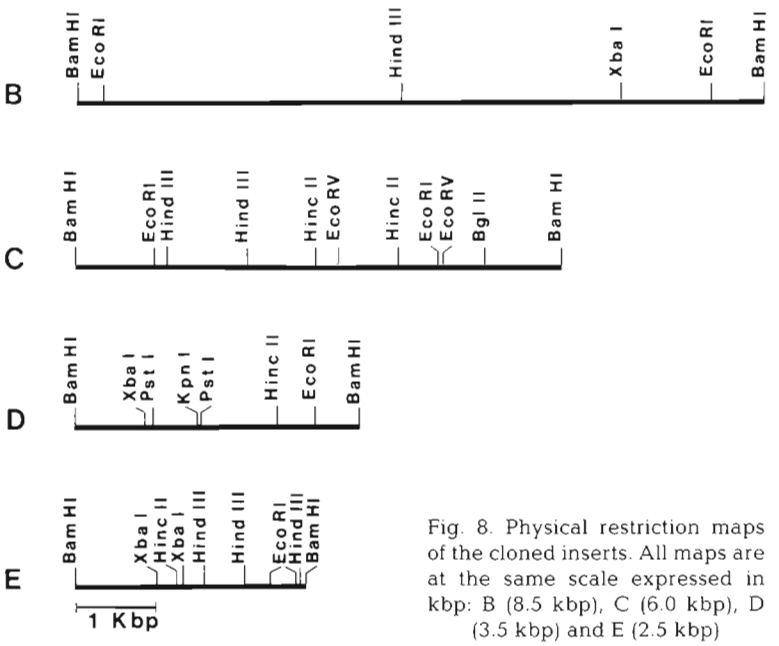


Fig. 8. Physical restriction maps of the cloned inserts. All maps are at the same scale expressed in kbp: B (8.5 kbp), C (6.0 kbp), D (3.5 kbp) and E (2.5 kbp)

opinion can be summarized as follows: the success in this particular purification depends on the 'quality' of the starting sample, i.e. upon the level of infection, the age or the physiological stage of the animals sampled, and the freezing and storage techniques used to preserve the samples prior to laboratory processing procedures.

Morphological studies of MBV revealed here demonstrate that negatively stained enveloped particles contain 1 capsid per envelope. Further, it was found that MBV enveloped particles possess characteristic appendages at each extremity that resemble those described for some non-occluded baculovirus (Pappalardo et al. 1986, Huger & Krieg 1991). The nucleocapsids, by their superficial helical structure and by the 2 layered 'caps' that enclose the extremities, resemble τ (Tau) virus nucleocapsids from *Carcinus mediterraneus* (Pappalardo et al. 1986, Huger & Krieg 1991) and those of the *Oryctes rhinoceros* baculovirus (Monsarrat 1978). Likewise, some MBV nucleocapsids were found to release a braided-like structure interpreted as the nucleoprotein, a feature which was also described in the above mentioned papers. According to these properties of the particle, MBV may be more closely related to the non-occluded crustacean baculoviruses than to the occluded type A insect baculoviruses.

The MBV occlusion body subunits (OBS) were found to be approximately 18 (Johnson & Lightner 1988) to 20 nm in diameter (Chen et al. 1989), as measured in TEM sections. When isolated and negatively stained, they exhibit a mean diameter of 22 nm, and are, to date and to our knowledge, the largest OBS reported in the 3 virus families that exhibit polyhedrons, i.e. Baculoviridae, Reoviridae (cytoplasmic polyhedrosis virus group) and Poxviridae (Entomopoxvirinae) (Kurstak & Garzon 1977, Payne & Harrap 1977, Summers 1977b, Adams & McClintock 1991, Couch 1991, Goodwin et al. 1991, Tanada & Hess 1991). We have no real hypothesis to explain why some OBS appear empty after negative staining, except to suppose a hollow inner structure; these subunits cannot be interpreted as virus particles because: (1) the OB degradation in non-purified preparations shows clearly by TEM, the OBS are derivated from the occlusion bodies, (2) their size corresponds approximately to the size reported in sections, (3) similar features were found during the study of another type of shrimp baculovirus (BP) isolated from *Penaeus vannamei* (Bonami et al. unpubl.). Because of their 6-sided profile in negatively stained preparations, the MBV OBS seem to possess a cubic (perhaps icosahedral) symmetry, which could explain how the OBS become arranged to give a tridimensional crystalline structure. This structure of the MBV OBS is not very different from the model proposed by Harrap (1972) for the structure of the protein molecules of the lattice, which could have the shape of 6-armed nodal units.

The MW of the MBV polyhedrin major polypeptide (58 kDa), is approximately double that of insects, which range from 25 to 33 kDa (Matthews 1982), and is much closer to the BP polyhedrin polypeptide (50 kDa) as determined by Summers (1977a), but determined in our experiment to be 50.5 kDa for purified OBS (Bonami et al. unpubl.) of a BP-type virus when comigrated with MBV polyhedrin (Fig. 5). Assuming that our polyhedrin preparation was not contaminated with viral particles (as no virions were found by TEM of the purified preparation), we have no explanation for the minor polypeptides found in SDS-PAGE. Whether these minor polypeptides represent host contaminants, viral envelope contaminants or structural components of the polyhedron is not known. Recently, Chang et al. (1992) have reported a value of 62 kDa for the polyhedrin issued from a *Penaeus monodon*-type baculovirus (MBV). We hypothesize that these minor differences in the different MW determinations could reflect the diversity of these baculovirus diseases. In fact, we hypothesize that different MBV-type diseases as well as different BP-type diseases could exist, which could carry similar gross characters (OB shape) but differ by some minor changes (virion and OBS sizes, polyhedrin MW).

The freshly extracted MBV nucleic acid appeared

supercoiled in TEM; this means the genome should be a circular molecule. Digestion with BamHI indicates that it is dsDNA. By summation of the M_r of the BamHI digested fragments, the total DNA M_r reaches at least 43 kbp; but since the A band appears strongly stained when compared to the other bands (Fig 7), it suggests that the A band is a multiple band. Moreover, because the small amount of viral DNA extracted, bands of small M_r were not evidenced. Hence, the MBV genomic size could fall within the size range of 80 to 160 kbp (i.e. about 58 to 110 $\times$ 10^6 Da) similar to the dsDNA size of insect baculoviruses (Matthews 1982). To date, we consider that only 53.5 kbp of the viral genome was cloned (20.8 kbp corresponding to the B to E fragments cloned and 32.7 kbp from the random pieces).

Cloning of the BamHI DNA restriction fragments (pieces B to E) allowed characterization of these fragments by restriction enzyme digestion and the determination of a partial restriction map. However, cloning of this genome using another restriction enzyme, such as EcoRI, could lead to a complete map of the genome by obtaining new pieces which overlap those described here.

The characteristics described above, and those published earlier (Lightner & Redman 1981, Lightner et al. 1983, Johnson & Lightner 1988) allow us to propose this agent as a member of subgenus SNPV (single nucleocapsids are enveloped with many virions incorporated per inclusion body), genus NPV (nuclear polyhedrosis virus) of the Eubaculoviridae subfamily (Matthews 1982, Francki et al. 1991); according to the actual nomenclature of this Baculoviridae family, we named it: PmSNPV. Hence, PmSNPV is now the most thoroughly characterized of the crustacean baculoviruses. Similar MBV-type viruses have been reported in numerous other penaeid species, including *Penaeus merguiensis, P. penicillatus, P. plebejus, P. esculentus, P. semisulcatus, P. kerathurus,* and *P. vannamei,* and in a wide geographic distribution from the Indo-Pacific coasts of Asia, Australia, Africa, Red Sea (Kuwait) and Mediterranean Sea (southern Europe and Israel) (Brock & Lightner 1990). This isolate, PmSNPV, could constitute a reference 'strain' from which to investigate the relatedness of other MBV-type viruses, particularly by comparison of the restriction profiles of specific genomic areas. This could be done by PCR amplification, starting from selected primers in our cloned BamHI-digest pieces.

Probes will be prepared from the different cloned pieces obtained after BamHI digestion of the genome. They will allow us, in the near future, to know whether or not the virus is vertically transmitted as well as horizontally; investigations are in progress. This knowledge and the increased diagnostic sensitivity provided by these probes will lead to better control of the disease, which appears to be of considerable economic importance, at least in Southeast Asia where MBV is enzootic in wild and farm raised populations of *Penaeus monodon*.

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