

Characterization of a local isolate of *Bombyx mori* nuclear polyhedrosis virus

Vikas B. Palhan and Karumathil P. Gopinathan

Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560 012, India

Polyhedral bodies of *Bombyx mori* nuclear polyhedrosis virus, BmNPV (BGL) isolated from infected silkworms around Bangalore were propagated either in the cultured *B. mori* cell line, BmN or through infection of larvae. Electron microscopic (EM) observations of the polyhedra revealed an average length of 2 μm and a height of 0.5 μm . The purified polyhedra derived virions (PDV) showed several bands in sucrose gradient centrifugation, indicating the multiple nucleocapsid nature of BmNPV. Electron microscopic studies of PDV revealed a cylindrical, rod-shaped nucleocapsid with an average length of 300 nm and a diameter of 35 nm. The genomic DNA from the PDV was characterized by extensive restriction analysis and the genome size was estimated to be 132 kb. The restriction pattern of BmNPV (BGL) resembled that of the prototype strain BmNPV-T3. Distinct differences due to polymorphic sites for restriction enzyme *Hind*III were apparent between BmNPV (BGL) and the virus isolated from a different part of Karnataka (Dharwad area), BmNPV (DHR).

BACULOVIRUSES are a diverse group of insect-specific viruses, commonly used as biopesticides for selectively controlling insect pests of several agricultural crops. Over the past decade, the biotechnological applications of baculoviruses have been further enhanced by the development of the baculovirus-based expression vectors making use of insect cell lines in culture^{1,2}. This system is widely used to synthesize milligram quantities of soluble, post-translationally modified eukaryotic proteins in their biologically active form. The prototype baculovirus for expression studies has been the *Autographa californica* nuclear polyhedrosis virus (AcNPV) in conjunction with cell lines derived from its insect host, *Spodoptera frugiperda*. Some baculoviruses are also harmful to commercially important insects. For instance, the *Bombyx mori* nuclear polyhedrosis virus (BmNPV) causes grasserie (jaundice) in silkworms and is a major economic loss to the silk farmer. BmNPV is a member of the Eubaculovirinae subfamily of the Baculoviridae³. With the development of high level expression system using AcNPV, a parallel expression system making use of BmNPV was also conceived. The BmNPV-based expression system offers the advantage of economical large scale production of proteins in

silkworm larvae⁴⁻⁷ because they can be reared in large numbers on mulberry leaves or artificial diet, avoiding the high costs of cell culture technology. Being larger in size, *B. mori* larvae provide higher yields of proteins than *Trichoplusia ni* larvae used occasionally in AcNPV-based expression^{8,9}.

To improve our understanding on the baculovirus which infects the silkworm, a local isolate from Bangalore, BmNPV (BGL) was purified and characterized at the structural and molecular levels. Although aetiological and histological studies have been conducted previously, little information was available on the BmNPV genome at the molecular level when this study was initiated. It was therefore considered desirable to have a physical map of the viral genome with respect to various restriction enzymes for comparison to other baculoviruses. Restriction site polymorphism between the genomic DNAs of BmNPV (BGL) and another isolate of the virus from Dharwad area of Karnataka, BmNPV (DHR) has been demonstrated.

Materials and methods

Propagation of BmNPV

The locally isolated BmNPV from infected silkworm larvae collected from silk farms around Bangalore was propagated in larvae or in *B. mori* derived cell line BmN, in culture. For propagation through larvae, *B. mori* larvae (bivoltine race NB₄D₂) in first or second day of 5th instar were fed with 10 μl of purified suspension of polyhedral bodies (10¹⁰ polyhedra/ml in distilled water) and reared in quarantine until advanced stages of infection. For propagating the virus in cultured cell line, BmN (9 \times 10⁶ cells) grown in TC100 medium⁵ in T75 flasks were infected with 1 ml of polyhedra derived virus particles at a multiplicity of infection of 10. After 3-4 days when the cytopathic effects became evident, the extracellular virus was harvested.

Purification of polyhedra from the haemolymph of infected larvae

Five days post infection one of the prolegs of each BmNPV-infected larva was punctured and the oozing

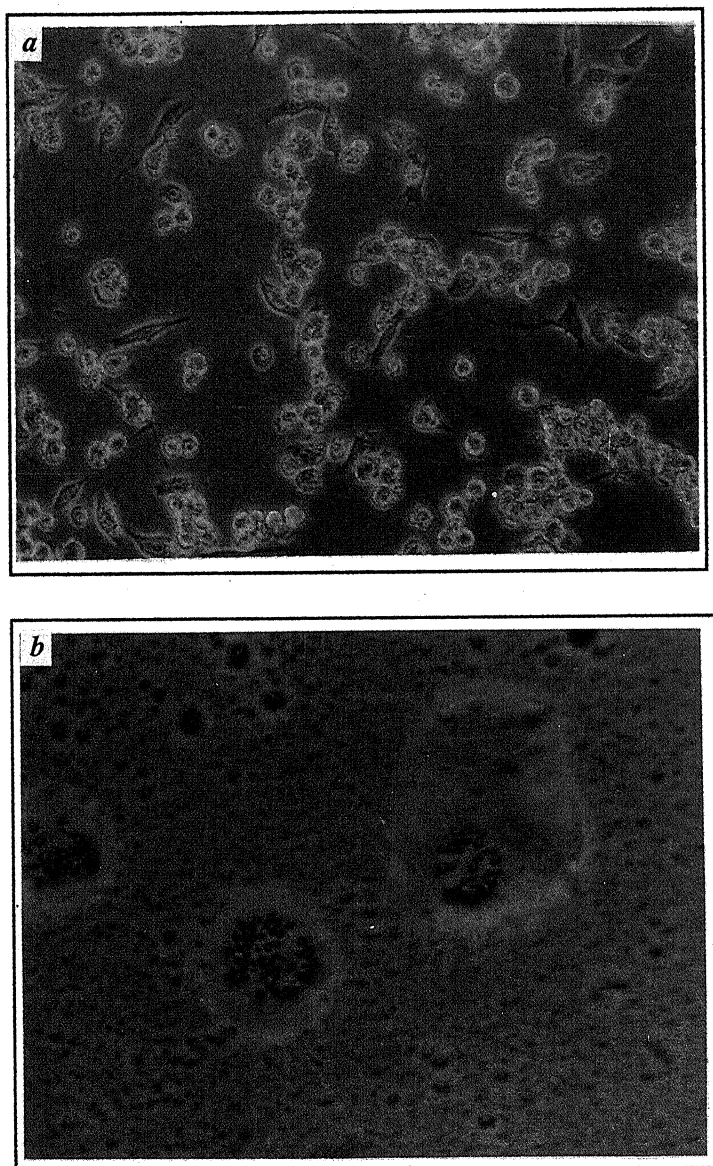


Figure 1. Propagating BmNPV in BmN cell line. The *B. mori* derived cell line BmN was grown in TC100 medium supplemented with 10% FCS. The cells were infected with PDV particles. *a*, Uninfected cells; *b*, Cells infected with BmNPV, 72 h post infection. The infected cells clearly showed the manifestation of cytopathic effects, i.e. accumulation of polyhedral inclusion bodies in the nucleus of each cell.

haemolymph was collected into a tube containing a few crystals of phenylthiourea or dithiothreitol (final concentration, 3 mM) to prevent melanization of the haemolymph. The polyhedra were pelleted by low speed centrifugation (5000 g) for 10 min in a swinging rotor, washed three times with 5 volumes of distilled water until the supernatant was devoid of any turbidity or floating lipids and resuspended in 0.5% sodium dodecyl sulphate (1 ml per insect equivalent) by brief homogenization to disrupt the polyhedra aggregates. The suspension was centrifuged and the pellet was resuspended in a small volume of distilled water (0.5 ml per insect

equivalent). A 10 μ l drop of pure polyhedra suspension was placed on a slide, spread with a cover slip and the purity was examined under a compound microscope. Polyhedra were freeze-dried and stored at 4°C.

Purification of polyhedra-derived virion

The polyhedra-derived virion (PDV) particles were released from polyhedra by solubilizing the occlusion bodies in an alkaline solution. Lyophilized polyhedra (10 mg) were dissolved in 1 ml of dissolution buffer

(0.1 M Na₂CO₃ and 0.05 M NaCl) or about 10¹⁰ polyhedra (several hundred µl as a packed volume) were pelleted and suspended in 4 ml of dissolution buffer. After 1 h at 30°C, the clear opal-coloured solution was centrifuged at 4000 g for 5 min to pellet the undissolved polyhedra and the supernatant was layered on a cushion of 25% sucrose solution containing 5 mM NaCl and 10 mM EDTA. The PDV were pelleted by ultracentrifugation at 100,000 g for 1 h at 5°C and the pellet was resuspended in TE (10 mM Tris, pH 7.5 and 1 mM EDTA). The purified preparation was used for EM studies.

Further purification of the PDV was achieved by banding on sucrose gradients. The PDV pellet obtained from a large scale purification from 10¹⁰ polyhedra was layered on a linear gradient of 20 to 50% sucrose. After centrifugation at 100,000 g for 90 min at 5°C, several viral bands were seen spanning the length of the tube, which were individually harvested. The viral bands were diluted more than 20-fold with distilled water and

pelleted by centrifugation at 100,000 g for 1 h at 5°C. The PDV pellet was soaked overnight in 1 ml TE, resuspended and stored at 4°C or processed for genomic DNA isolation.

Electron microscopic studies

For scanning electron microscopy the samples were fixed in 4% glutaraldehyde in phosphate buffer, pH 7.0 for 4 h and washed extensively with phosphate buffer, pH 7.0. They were dehydrated and mounted on the grid using graphite resin or silver point. The specimen was coated with gold for 3 min. The ultrastructural studies were carried out using a scanning electron microscope (JOEL JSM-840 A) at an operating voltage of 20 kV. Samples of pure PDV were placed on copper grids coated with formvar film and negatively stained with uranyl acetate for 5 min. Specimens were examined in a JOEL 100 C × II transmission electron microscope at 80 kV.

Purification and analysis of genomic DNA

The genomic DNA was isolated from purified PDV as described by Maeda⁵. BmNPV DNA (5 µg) was digested with 10 units of various restriction endonucleases in the appropriate buffer at 37°C for 6 h and analysed by electrophoresis on a 0.7% agarose gel. The DNA bands were visualized on a UV transilluminator after ethidium bromide staining. Using SEQAID II software the size of each fragment was determined by comparing the mobility with that of the standard DNA size markers (λ DNA digested with *Hind*III).

Results and discussion

Polyhedral bodies of BmNPV

BmNPV was routinely propagated in the silkworm (*B. mori*) larvae or in the *B. mori* derived cell line, BmN in culture (Figure 1 a). The infected larvae showed signs of acute infection (fluid accumulation, lethargy, etc.) within 4–5 days and yielded large quantities of polyhedra in their haemolymph which appeared white due to polyhedra accumulation. In cell lines, as the infection established itself the first signs of cytopathic effects were the clumping and detachment of cells which gave rise to 'floaters'. Subsequently the cells lost their shape with the nucleus becoming very large and occupying most of the cellular volume. Giant cells with irregular peripheries were seen infested with polyhedra (Figure 1 b). At late time points post infection, the nuclei of these cells also showed a dense accumulation of polyhedra (average of 70/nucleus). Polyhedra were isolated

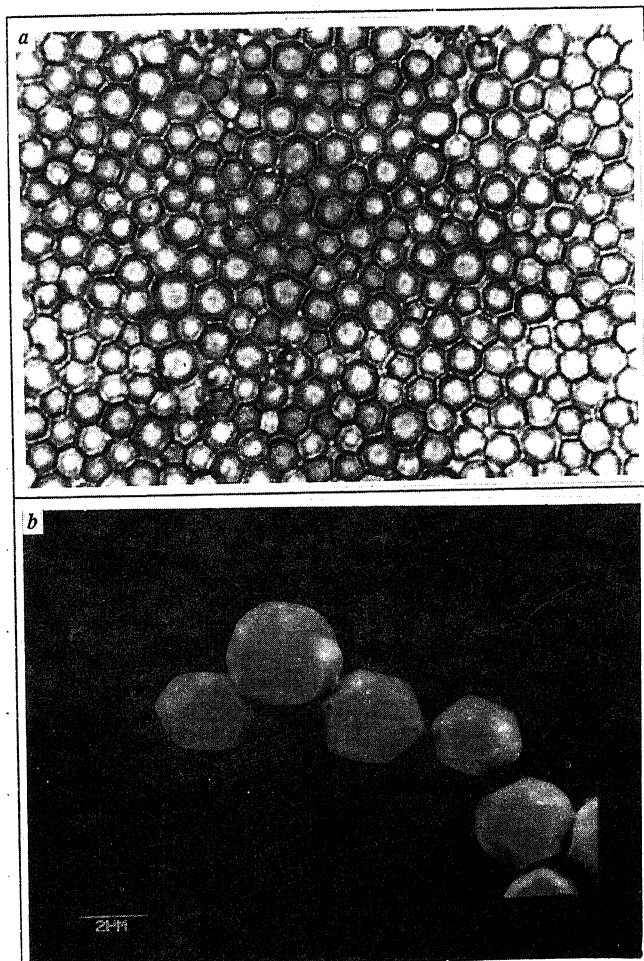


Figure 2. Characterization of BmNPV polyhedra. *a*, A drop of purified and concentrated polyhedra was spread on a slide under a cover slip and observed under a compound microscope at 800 × magnification. *b*, Scanning electron microscopic analysis of BmNPV polyhedra. Pure preparations were scanned to examine the polyhedral shape and symmetry in detail. The average size of polyhedra was 2 µm by 0.5 µm.

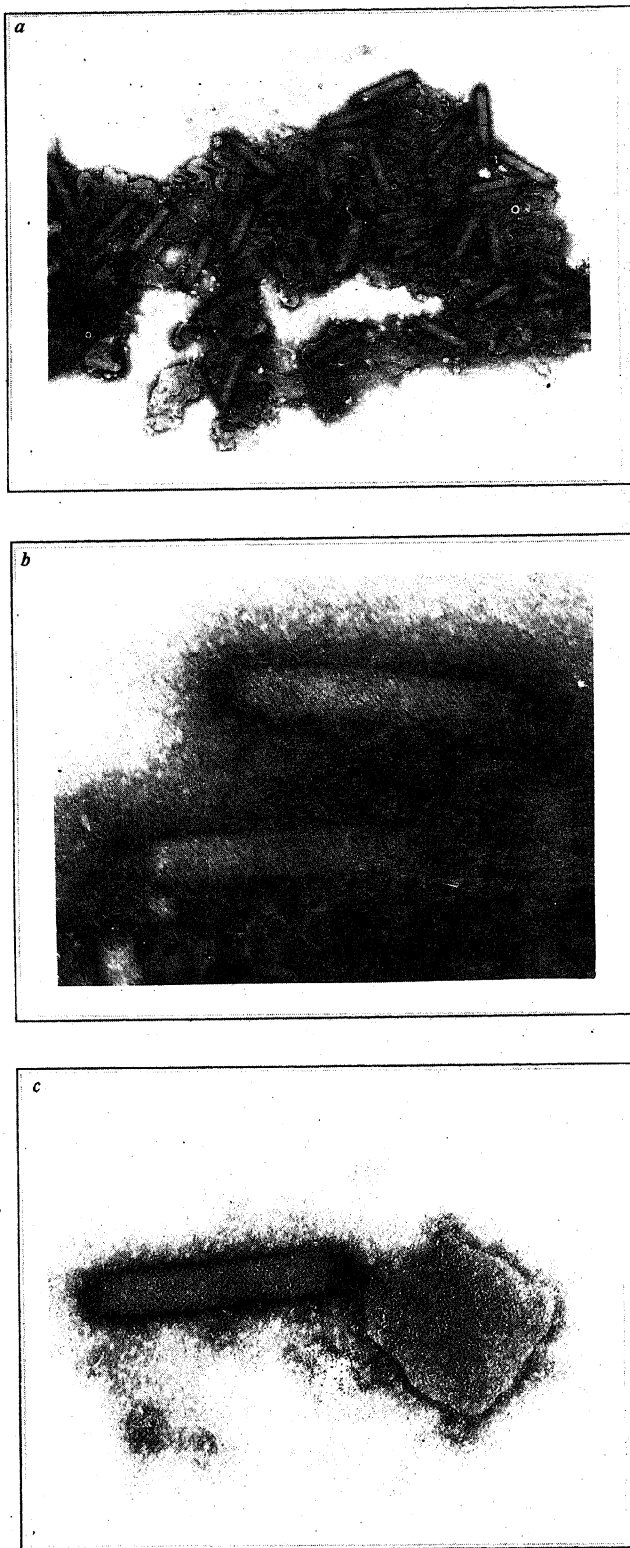


Figure 3. Electron microscopic analysis of purified BmNPV. Purified PDV were spread on a grid, negatively stained with uranyl acetate and examined in a JOEL 100C × II transmission EM. The rod-shaped nucleocapsids can be seen clearly trapped within the remnants of the polyhedral matrix (a). Higher magnification of a single virion revealed the 'nipple-like' triangular projection at one end and the claws in the base plate at the opposite end (b). The nucleocapsid can be seen spreading out of one of the damaged ends of the virion (c).

from the haemolymph of the infected larvae and their purity was examined under the compound microscope. These large inclusion bodies had a uniform, highly refractile, polyhedral appearance (Figure 2 a). Scanning EM of the polyhedra showed perfect polyhedral symmetry in greater detail (Figure 2 b). The polyhedra had an average length of 2 μm and a height of 0.5 μm .

Purification of polyhedra derived virion

PDV were released by dissolving the polyhedra in an alkaline solution and purified by ultracentrifugation through a 25% (w/v) sucrose cushion. When a large scale preparation of PDV was layered on sucrose gradient for purification, several bands were apparent, spanning the length of the tube indicating the presence of virions with multiple nucleocapsids¹⁰. Although BmNPV is considered as the type species of single nucleocapsid polyhedrosis viruses (SNPVs)¹¹, the observed banding pattern suggested that multiple nucleocapsid polyhedrosis viruses (MNPVs) are also present in the BmNPV population.

Electron microscopic analysis of PDV

The purified BmNPV (PDV) pellet was used for transmission EM studies. A group of rod-shaped, cylindrical virions were seen scattered all over the remnants of the polyhedra matrix (Figure 3 a, b). On higher magnification the ultra structure became clearer. Some of the characteristic features were a *cap* or *nipple*-like structure at the apex of each virion and a circle of *claws* in the base plate at the opposite end (Figure 3 b). These dissimilar ends gave a polarity to the virion rods which may be involved in orienting the nucleocapsid for infection of the nucleus¹²⁻¹⁵. In some fields the nucleocapsid could be seen spreading out through one of the damaged ends of the virion (Figure 3 c). Based on several measurements the BmNPV-BGL virion showed an average length of 300 ± 5 nm and a diameter of 35 ± 3 nm.

Molecular characterization of BmNPV DNA

For characterization at the molecular level, genomic DNA was isolated from purified BmNPV particles of the local isolate BmNPV (BGL) and subjected to restriction endonuclease digestion analysis. Typical digestion patterns for a few restriction enzymes are shown in Figure 4 a-c. There were multiple restriction sites on the BmNPV genome for a variety of enzymes. The restriction patterns did not show the presence of any submolar bands which are sometimes found in the preparation of other NPVs¹⁶. Rare cutters like *NotI* and *SfiI* also released a 6.7 kb and 16.4 kb fragment each respectively (Figure 4 b). The molecular sizes of the fragments were

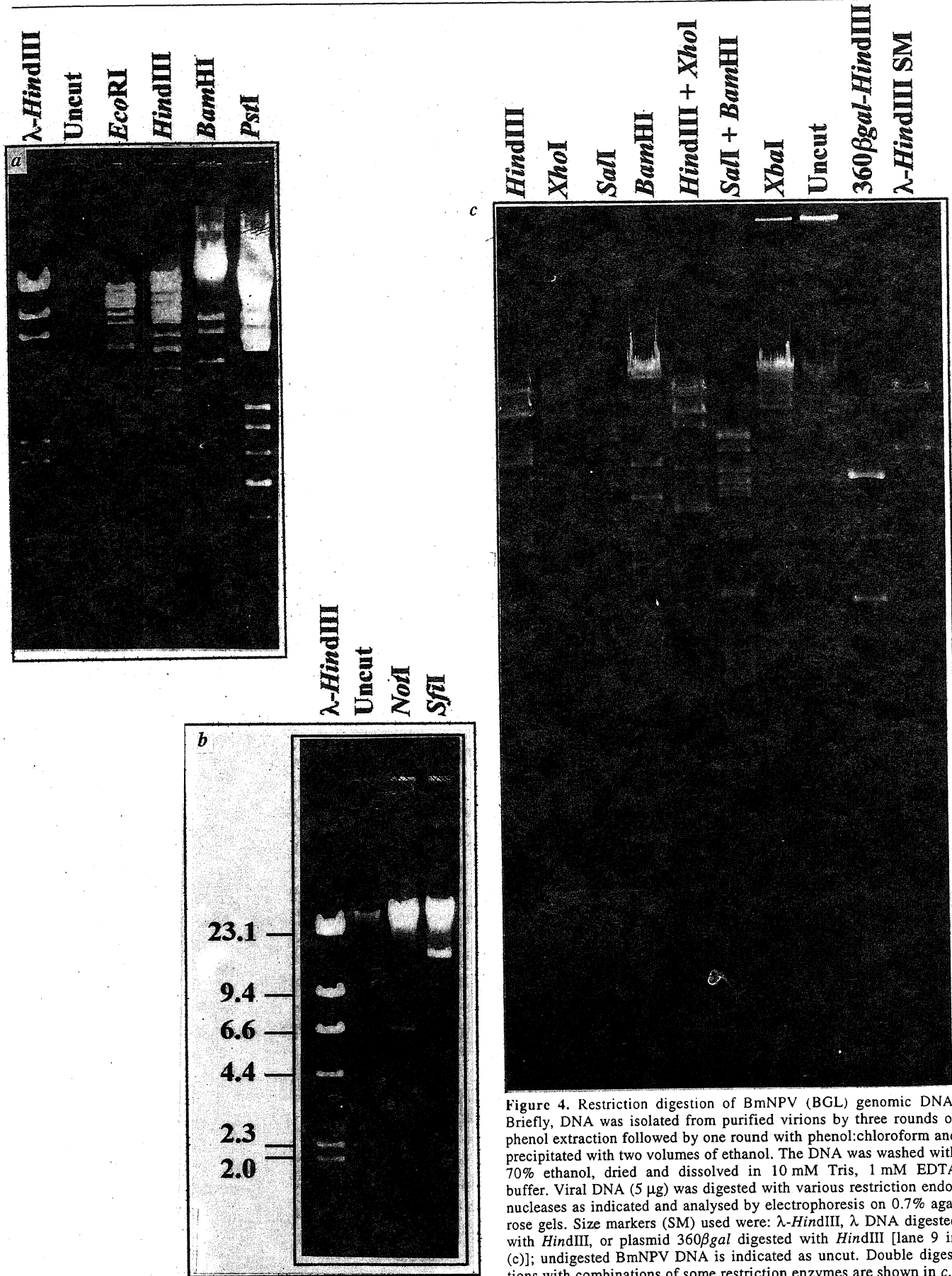


Figure 4. Restriction digestion of BmNPV (BGL) genomic DNA. Briefly, DNA was isolated from purified virions by three rounds of phenol extraction followed by one round with phenol:chloroform and precipitated with two volumes of ethanol. The DNA was washed with 70% ethanol, dried and dissolved in 10 mM Tris, 1 mM EDTA buffer. Viral DNA (5 μ g) was digested with various restriction endonucleases as indicated and analysed by electrophoresis on 0.7% agarose gels. Size markers (SM) used were: λ -HindIII, λ DNA digested with HindIII, or plasmid 360 β gal digested with HindIII [lane 9 in (c)]; undigested BmNPV DNA is indicated as uncut. Double digestions with combinations of some restriction enzymes are shown in c.

RESEARCH ARTICLE

Table 1. Sizes (kb) of BmNPV restriction fragments

Fragment	<i>EcoRI</i>	<i>HindIII</i>	<i>PstI</i>
A	20.9	25.7 ^S	20.2
B	20.9	16.1 ^S	18.5
C	15.5	12.4 ^S	16.2
D	12.4	10.2	14.1
E	13.8	9.6*	12.9
F	11.2	8.7	8.6
G	9.6*	8.3	8.1
H	8.0	7.8	5.9
I	5.6*	6.1	5.7
J	5.3	5.1	5.7
→ K	1.6	5.1	3.8
L	1.5	3.9	2.9
M	1.3	3.3	2.5
N	1.2	2.4	2.1
O	1.15	1.9	2.0
P	1.04	1.8	1.6
Q	0.96	1.2	0.9
R	0.61 ^a	1.1	0.5
S	0.60 ^a	0.7	
Total	133.16	131.4	132.2

Starting with the highest molecular weight, the fragments are labelled in an alphabetical order.

*Polymorphic bands.

^aSince their yield was very low, these low molecular weight bands were visualized on higher percentage gels by overloading.

→ indicates the two missing bands corresponding to the BmNPV-T3 *EcoRI* 'K' (3.9 kb) and 'L' (2.4 kb) fragments¹⁷.

^SRepresents bands of smaller size as compared to T3 fragment digested with the same enzyme.

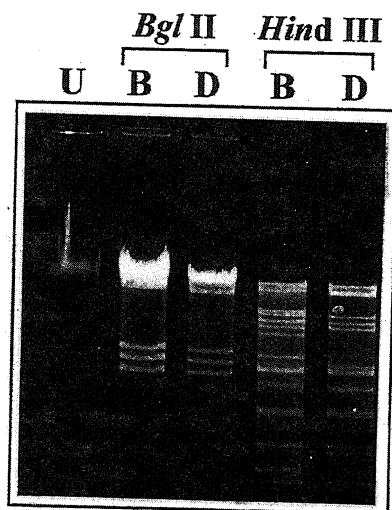


Figure 5. Restriction site polymorphism between two different local isolates of BmNPV - Bangalore (B) and Dharwad (D). Genomic DNA (5 µg) from each isolate was digested with *Bgl*II and *Hind*III and analysed by electrophoresis on a 0.7% agarose gel.

estimated by comparison to the migration of DNA size markers using the SEQAID program. For accuracy in size determination, the electrophoresis of digested samples were analysed on different percentage agarose gels of varying lengths to resolve the larger and smaller fragments better. Resolution between doublets was

achieved by double digestions with combinations of restriction enzymes. The genome size of BmNPV (BGL) was estimated to be about 132 kb by summing the sizes of individual fragments generated by three different restriction endonucleases (Table 1). This estimated size is quite similar to that of the BmNPV-T3 isolate¹⁷ and AcNPV¹⁸.

Construction of a physical map of the BmNPV (BGL) genome using restriction endonucleases was attempted. However, when this study was underway, the restriction map of the BmNPV-T3 genomic DNA with respect to the enzymes *EcoRI*, *Bam*HI, *Pst*I, *Kpn*I and *Sma*I was published¹⁷. The overall restriction pattern of BmNPV (BGL) was similar to that of the BmNPV-T3 isolate, but the *EcoRI* and *Hind*III digestions showed some polymorphic sites (Table 1). For instance, the *Hind*III digestion of BmNPV (BGL) DNA showed that the 'G' equivalent fragment of T3 was lacking. The 'H' fragment was smaller and the 'L' fragment was larger than the corresponding T3 fragments¹⁷ (Table 1). However, since the size distribution of almost all the restriction fragments in single or double digestions was similar in both the local (BGL) isolate and the T3 strain, it was obvious that their physical maps would be identical with minor differences.

The restriction digestion patterns of genomic DNA of BmNPV (BGL) were compared with another BmNPV (DHR) isolated from the infected silkworms of Dharwad area in Karnataka. Restriction site polymorphism was also observed between the genomic DNA of BmNPV (BGL) and the BmNPV (DHR) isolates, particularly with the *Hind*III digestions (Figure 5) whereas the *Bgl*III digestions showed identical patterns with both the isolates. The bands corresponding to the BmNPV (BGL) - *Hind*III 'C', 'G' and 'H' genomic fragments were missing in the DHR-*Hind*III lane. Single base pair substitution or deletion in the recognition sequence of a restriction enzyme present on the viral genome could lead to a change in the restriction pattern of virus isolates. Minor variations were also apparent in *Sal*I and *Eco*RI digestions (data not shown). Such variations due to restriction enzyme polymorphic sites are commonly encountered in large virus samples isolated from infected animals.

Attempts were made to construct a library of BmNPV genomic fragments in the phagemid vector, pTZ18R, with the aim of identifying and cloning new viral genes with strong promoters for developing novel expression vectors. BmNPV (BGL) genomic DNA fragments generated by *Bam*HI, *Eco*RI and *Hind*III digestions were shotgun cloned into pTZ18R linearized with the same enzymes. A partial library of viral genomic DNA with the insert size ranging from 500 to 7 kb has been generated. The 20 clones harbouring 10 different viral genomic fragments (*Bam*HI 'D, E and F'; *Eco*RI, 'I, M, O, Q and R'; *Hind*III 'L' and *Kpn*I 'D') representing approximately 25% of the 130 kb BmNPV genome, have been characterized (data not shown). Some of these

cloned genomic DNA fragments are being exploited for use as diagnostic probes for detecting BmNPV infections at early stages.

1. Luckow, V. E., in *Recombinant DNA Technology and Applications* (eds Prokop, A., Bajpai, R. K. and Ho, C.), McGraw Hill, New York, 1991, pp. 97-152.
2. Miller, L. K., *Curr. Opin. Genet. Dev.*, 1993, **3**, 97-101.
3. Francki, R. I. B., Fauquet, C. M., Knudson, D. L. and Brown, F., in *Fifth Report of the International Committee on Taxonomy of Viruses*, Springer Verlag, New York, 1991.
4. Johnson, R. R., Schimel, D., Iatrou, K. and Gedamu, L., *Biotechnol. Bioeng.*, 1993, **42**, 1293-1300.
5. Maeda, S., in *Invertebrate Cell Systems and Applications* (ed. Mitsuhashi, J.), CRC Press, Boca Raton, 1988, vol. 1, pp. 167-182.
6. Maeda, S., *Annu. Rev. Entomol.*, 1989, **34**, 351-372.
7. Palhan, V. B., Sumathy, S. and Gopinathan, K. P., *BioTechniques*, 1995, **19**, 97-104.
8. Jha, P. K., Nakhai, B., Sridhar, P., Talwar, G. P. and Hasnain, S. E., *FEBS Lett.*, 1990, **274**, 23-26.
9. Medin, J. A., Hunt, L., Gathy, K., Evans, R. A. and Coleman, M. S., *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 2760-2764.
10. King, L. A. and Possee, R. D., in *The Baculovirus Expression System - A Laboratory Guide*, Chapman and Hall, London, UK, 1992, p. 190.
11. Mathews, R. F. F., *Intervirology*, 1982, **17**, 1-199.
12. Bergold, G. H., in *Insect Pathology. An Advanced Treatise* (ed. Steinhaus, E. A.), Academic Press, New York, 1963, vol. 1, p. 413.
13. Teakle, R. E., *J. Invertebr. Pathol.*, 1969, **120**, 433.
14. Summers, M. D., *J. Ultrastruct. Res.*, 1971, **35**, 606.
15. Granados, R. R., *Biotechnol. Bioeng.*, 1980, **22**, 1377.
16. Miller, D. W. and Miller, L. K., *Nature*, 1982, **299**, 562-564.
17. Maeda, S. and Majima, K., *J. Gen. Virol.*, 1990, **71**, 1851-1855.
18. O'Reilly, D. R., Miller, L. K. and Luckow, V. E., in *Baculovirus Expression Vectors - A Laboratory Manual*, W. H. Freeman and Co., New York, 1992, p. 3.

ACKNOWLEDGEMENTS. We thank K. K. Singh, Karnatak University, Dharwad, for help in the characterization of BmNPV (DHR) by providing a bulk stock of haemolymph from virus-infected silkworm larvae from the Dharwad area of Karnataka; G. S. Rajanna of KSSDI, Bidadi for supplying silkworm larvae; N. Prakash for SEM analysis of polyhedra and Rangeshwaran for TEM analysis of PDV. Mini and Asha provided technical assistance in sorting the genomic library clones. This work was supported by grants from the Department of Biotechnology, Govt of India.

Received 10 October 1995; revised accepted 29 November 1995