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N-Terminal Polyhedrin Sequences and Occluded *Baculovirus* Evolution

G. F. Rohrmann[1], M. N. Pearson[1], T. J. Bailey[2], R. R. Becker[2] and G. S. Beaudreau[1]

1 Department of Agricultural Chemistry and
2 Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331, USA

Summary. A phylogenetic tree for occluded baculoviruses was constructed based on the N-terminal amino acid sequence of occlusion body proteins from six baculoviruses including three lepidopteran nuclear polyhedrosis viruses (NPVs), [two unicapsid (*Bombyx mori* and *Orgyia pseudotsugata*) and one multicapsid (*Orgyia pseudotsugata*)]; one granulosis virus (*Pieris brassicae*); and NPVs from a hymenopteran (*Neodiprion sertifer*) and a dipteran (*Tipula paludosa*). Amino acid sequence data for the *B. mori* NPV were from a report by Serebryani et al. (1977) and that for the *O. pseudotsugata* NPVs were reported previously by us (Rohrmann et al. 1979). The other N-terminal amino acid sequences are presented in this paper. The phylogenetic relationships determined based on the molecular evolution of polyhedrin were also investigated by antigenic comparisons of the proteins using a solid phase radioimmune assay. The results indicate that the lepidopteran NPVs are the most closely related of the above group of viruses and are related to these viruses in the following order: *N. sertifer* NPV, *P. brassicae* granulosis virus, and *T. paludosa* NPV. These data, in conjunction with *Baculovirus* distribution and evidence concerning insect phylogeny, suggest that the *Baculovirus* have an ancient association with insects and may have evolved along with them.

Key words: *Baculovirus* – Polyhedrin – N-terminal amino acid sequences – Molecular evolution – Virus evolution

Introduction

The Baculoviridae are a group of large, rod-shaped viruses containing double stranded DNA of 58–110 x 10^6 daltons (Harrap and Payne 1979). Viruses of this family are pathogenic for a crustacean (Couch 1974) and species of several orders of higher insects, particularly the Lepidoptera (Martignoni and Iwai 1977). Most members of the Baculoviridae are characterized by their occlusion in a crystalline protein matrix which is composed of a single virus encoded protein (Van der Beek et al. 1980; Summers et al. 1980) of 25,000–31,000 daltons (Harrap and Payne 1979). These crystals serve to stabilize the virus outside the host insect and are alkali soluble and dissolve and release their virus when they encounter the high pH of the insect midgut. There are three *Baculovirus* subgroups including: subgroup A (Nuclear polyhedrosis viruses, NPVs) in which virions replicate in the nucleus and multiple virions are occluded in each crystal; subgroup B (Granulosis viruses, GVs) which replicate in the cytoplasm and only one virus is occluded per crystal; and subgroup C which is composed of non-occluded strains.

Viruses in subgroup A are present as two morphotypes including a multicapsid morphotype (more than one virion per envelope) and a unicapsid morphotype (one virion per envelope). *Baculovirus* subgroup A, found outside the Lepidoptera (Crustacea, Hymenoptera, Diptera and Trichoptera) are present as the unicapsid morphotype, whereas, within the Lepidoptera both multicapsid and unicapsid morphotypes occur (Martignoni ME, personal communication). In addition, granulosis viruses (subgroup B) are of the unicapsid morphotype and have been reported only in the Lepidoptera (Martignoni and Iwai 1977). This information is summarized in Table 1.

Although *Baculovirus* are genetically diverse, occlusion body proteins (termed polyhedrin and granulin in NPVs and GVs respectively) are highly conserved structurally. Investigations on the serology, tryptic peptides and amino acid sequences of polyhedrins and

Offprint requests to: Dr. George F. Rohrmann

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Table 1. Host Distribution of occluded *Baculoviruses*[a]

	Baculovirus subgroup	Morphotype[b]	No. of species infected
Crustacea			
Decapoda	NPV	U	1
Insecta[c]			
Trichoptera	NPV	U	1
Hymenoptera[d]	NPV	U	25
Diptera	NPV	U	21
Lipidoptera	NPV	U,M	337
	GV	U	103

a From Martignoni and Iwai (1977) and M.E. Martignoni, personal communication

b Morphotype: U, Unicapsid (one nucleocapsid per envelope); M, Multicapsid (multiple nucleocapsids per envelope)

c Two reports indicate NPVs associated with Coleoptera (Koyama 1963; Ryel and Cline 1970). A listing has also been made for a granulosis virus of a Hymenoptera (Smirnoff 1973). Since no evidence for the ability to pass the virus or its tissue specificity are presented in these reports, they are not included in this table. In addition, insects of the order Neuroptera are susceptible in the laboratory to infection with *Lymantria dispar* NPV (Smith et al 1959). However, the incidence of *Baculovirus* naturally occurring in neuropteran populations has not been reported

d Found only in the suborder Symphyta (Sawflies)

granulins indicate the following: all Lepidoptera polyhedrins are related (Rohrmann et al. 1979; Krywienczyk and Bergold 1960, 1961; Rohrmann 1977; Maruniak and Summers 1978; McCarthy and Lambiase 1979); Lepidoptera polyhedrins and granulins appear related (Maruniak and Summers 1978; Summers and Hoop 1980); Hymenoptera polyhedrins are related (Krywienczyk and Bergold 1960); and Lepidoptera and Hymenoptera polyhedrins are related (Norton and Dicapua 1975). Investigations on the relatedness of Diptera and Lepidoptera polyhedrins are limited, however, in one study (Guelpa et al. 1977) no antigenic relatedness was detected between a dipteran and a lepidopteran polyhedrin.

This report compares the N-terminal amino acid sequence from six polyhedrins from the following insect baculoviruses: *Neodiprion sertifer* (unicapsid, subgroup A); *Tipula paludosa* (unicapsid, subgroup A); *Pieris brassicae* (unicapsid, subgroup B); *Orgyia pseudotsugata* (unicapsid and multicapsid, subgroup A); *B. mori* (unicapsid, subgroup A). These viruses are representative occluded *Baculovirus* pathogens of the major insect orders (Lepidoptera, Hymenoptera, and Diptera) and comprise both *Baculovirus* subgroups and both nuclear polyhedrosis virus morphotypes. These data were used to construct a phylogenetic tree. The relatedness of the polyhedrin was further examined using a solid phase radioimmune assay.

Materials and Methods

Viruses. The *T. paludosa*, *N. sertifer*, and *P. brassicae* viruses were the generous gifts of J.C. Veyrunes, D.A. Brown and C.C. Payne respectively.

Preparation of Proteins. All polyhedra were heat-treated at 75°C for 1–2 h to inactivate proteases present in the occlusion bodies. *N. sertifer*, *O. pseudotsugata* and *P. brassicae* occlusion bodies were dissolved by suspending in distilled water and adding 0.1 volume of 1 M Na_2CO_3 – 0.5 N NaCl. These solutions were briefly heated to 50–60°C to facilitate dissolution. The *T. paludosa* NPV was dissolved by suspending the virus in 0.125 M Na_2CO_3, 50 mM dithiothreitol, pH 10.6. This solution was also heated to facilitate dissolution. After dissolving the polyhedra, the solutions were centrifuged at 40,000 x g for 30 min to pellet virions and undissolved polyhedra. The supernatant was dialyzed against 0.01 Tris, pH 8.5 and then lyophilized. All preparations were examined on polyacrylamide gels as previously described (Rohrmann et al. 1980). The gels indicated only one major protein was present and no evidence of degradation was observed. The molecular weights calculated for the proteins were similar to those reported by previous workers (*T. paludosa* NPV, 25,200 (Crozier and Crozier 1977), *N. sertifer*, 25,700 and 29,350 (Crozier and Crozier 1977; Brown et al. 1979), and *P. brassicae*, 28,200 and 27,500 (Crozier and Crozier 1977; Brown et al. 1977)). Reduction, alkylation and N-terminal amino acid sequencing was done as previously described (Rohrmann et al. 1980).

Radioimmune Assay. Proteins were prepared as described above. The antibody, produced as previously described (Rohrmann 1977), was purified by binding it to an affinity column made by reacting 10 mg of polyhedrin from the *O. pseudotsugata* multicapsid NPV (which had been purified on a 3 x 100 cm Bio-Gel A-5m column using 10 mM Na_2CO_3 – 0.05 M NaCl pH 9 elution buffer) to 0.7 g of cyanogen bromide Sepharose (Pharmacia) using manufacturer's instructions. About 100 μg of purified antibodies were labeled using the Bio Rad Enzymobead Radioiodination reagent with about 1.0 mCi ^{125}I using the manufacturer's instructions. A specific activity of 4 x 10^6 cpm/μg was achieved. The solid phase radioimmune assay system was carried out in microtiter plates as described by Nowinski et al (1979) except that labeled antibody was used rather than labeled protein A.

Results and Discussion

Evidence from the N-terminal acid sequences of the lepidopteran, hymenopteran and dipteran occlusion body proteins (Fig. 1) indicates that they are all related. However, in aligning the sequences substantial differences are evident which include many amino acid changes and a number of regions which have been inserted or deleted. Such variation is probably common and is reflected in the differences observed in sizes of a number of these proteins (Crozier and Crozier 1977). Using the method of Fitch (1977) to compare the nucleotide changes necessary to account for the amino acid sequence differences, a preliminary phylogeny based on the partial sequences was determined (Fig. 2).

Such molecular phylogenies reflect the evolution of the whole organism (Wilson et al. 1977). The phylogeny

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LEPIDOPTERA
B. mori (U)                         Pro-Asn-Tyr-Ser-Tyr-(Asn,Pro)-Thr-Ile-Gly-Arg-Thr-Tyr-Val-Tyr-(Asn,Asp)-Lys-Tyr-Tyr-Lys-Asn-Leu-Gly-Gly-Leu-Ile-Lys-Asn-Ala-Lys-Arg-Lys-Lys-His-Leu-
O. pseudotsugata (M)                Pro-Asp-Tyr-Ser-Tyr- Arg-Pro -Thr-Ile-Gly-Arg-Pro-Tyr-Val-Tyr- Asp-Asn -Lys-Tyr-Tyr-Lys-Asn-Leu-Gly-Gly-Val-Ile-Lys-Asn-Ala-Lys-Arg-Lys-Lys-His-Leu-
O. pseudotsugata (U)        Met-Tyr-Pro-Arg-Tyr-Ser-Tyr- Asn-Pro -Thr-Leu-Gly-Arg-Pro-Tyr-Val-Tyr- Asp-Asn -Lys-Tyr-Tyr-Lys-Asn-Leu-Gly-Ala-Val-Ile-Lys-Asn-Ala-Lys-Arg-Lys-Lys-
P. brassicae GV (U)                             Gly-Tyr- Asn-Arg -Ala-Leu-Gly-Pro-Pro- X2-Val-Ile- Asp-Asn -Gln-His-Tyr-Lys- X1- - -Gly-Ala-Val-Leu-Lys-Asp-Val-Lys-His-Lys-Lys-
                                                          Arg -Tyr- X1-Lys-His-Glu

HYMENOPTERA
N. sertifer (U)                     Pro-Asn-Leu-Gly-Tyr- Gln- - -Ser- ' -Ala-Lys-Ser-Tyr-Ile-Tyr- Asp-Asn -Lys-Tyr-Tyr-Lys-Gly-Leu-Gly-Asp-Ile-Ile(Lys)Ser-Ala(Lys)
                                        Ala-Gln

DIPTERA
T. paludosa (U)                     Gln-Asp-Tyr-Gly-Tyr- Glu-Pro -Asn-Val-Asp-Tyr-Pro-Asn-Leu-Tyr- Asp-Arg -Lys-Pro-Tyr-Val-Asp(His)Asp- - -Tyr(Pro)
                                Tyr-Glu-Val-Asn                    Ala(Ser)His-Ala-Gly      Ser Gln
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Fig. 1. N-terminal amino acid sequences for six *Baculovirus* occlusion body proteins. The sequences for *B. mori* and two *O. pseudotsugata* NPVs are from previous reports by Serebryani et al. (1977) and Rohrmann et al. (1979), respectively. The proteins are aligned to demonstrate homologous regions which resulted in gaps designated by ( ), and insertions which are set below the line and indicated by (⌐¬) showing the point of insertion. Tentative identifications are indicated by brackets ( ). Amino acids which were not determined are designated by (–) and unidentified amino acid derivatives by (X). The protein from *P. brassicae* demonstrated two amino acid derivatives: X_1 which appeared as a shoulder to tyrosine and X_2 which appeared as a shoulder to proline when their phenlythiohydantoin derivatives were examined by high pressure liquid chromatography
Abbreviations: U, unicapsid morphotype; M, multicapsid morphotype; GV, granulosis virus

produced from the N-terminal polyhedrin sequence indicates that the lepidopteran NPVs (the *B. mori* unicapsid and *O. pseudotsugata* unicapsid and multicapsid) are the most closely related of the viruses. The *N. sertifer* (Hymenoptera) NPV appears to be more related to the lepidopteran NPV than is the *P. brassicae* (Lepidoptera) granulosis virus. The dipteran NPV (*T. paludosa*) demonstrates the earliest divergence from the other viruses.

In order to compare the relationships established from the N-terminal sequences of the occlusion body proteins to the antigenicity of polyhedrin molecules, we examined, by means of a solid phase radioimmune assay, the ability of antibody made against *O. pseudotsugata* multicapsid virus (Lepidoptera) polyhedrin to bind increasing concentrations of occlusion body proteins from the other viruses (Fig. 3). The results indicate that the *P. brassicae* granulin is more closely related antigenically to the *O. pseudotsugata* polyhedrin than is polyhedrin from *N. sertifer*. This is the reverse of what one would expect from the sequence data. The results for the other proteins were consistent with the phylogenetic tree established from the N-terminal amino acid sequences. The dipteran polyhedrin, which demonstrates the most distant relatedness, bound the antibody in amounts only slightly more than the control protein (0.2% of the total added). The discrepancy between the antigenic and sequence data is likely due to the fact that polyhedrin is not a monomeric antigen and the radioimmune assay may not proportionately quantify amino acid sequence differences (Wilson et al. 1977). Although less likely, it is possible that the portion of the polyhedrins we sequenced, which represent 10–15% of the total molecule, was not representative.

The *Baculovirus* phylogenetic tree which we have constructed, using the N-terminal amino acid sequence of occlusion body proteins, is consistent with *Baculovirus* distribution (Table 1) and appears to be related to the evolution of insects. For example, the Lepidoptera are the most recently evolved order of insects (Reik 1970) and appeared 40–60 x 10^6 years ago and subsequently underwent extensive speciation. Almost 90% of all reported NPVs are from the Lepidoptera (Martignoni and Iwai 1977) and multicapsid NPVs are present only in the Lepidoptera (Table 1). In addition, the N-terminal amino acid sequence (Fig. 1) and antigenicity (Fig. 3) of lepidopteran polyhedrins indicate a close phylogenetic relatedness to one another when contrasted to polyhedrins from the other two insect orders. This concentration of multicapsid and unicapsid NPVs within the Lepidoptera and the phylogenetic relatedness of their polyhedrins indicates that the lepidopteran NPVs have also diverged from one another relatively recently. This suggests that the appearance of the Lepidoptera provided the NPVs with a new environment which facilitated their evolution. Divergence of the lepidopteran NPVs as their host speciated could account for the large number of lepidopteran NPVs and the similarity in their polyhedrin sequences (Table 1).

Granulosis virus evolution is integrally involved with NPV phylogeny. The GVs appear to have diverged from a unicapsid NPV before the divergence of the Hymenoptera virus. The presence of the large number of GVs within the Lepidoptera suggests that they may have

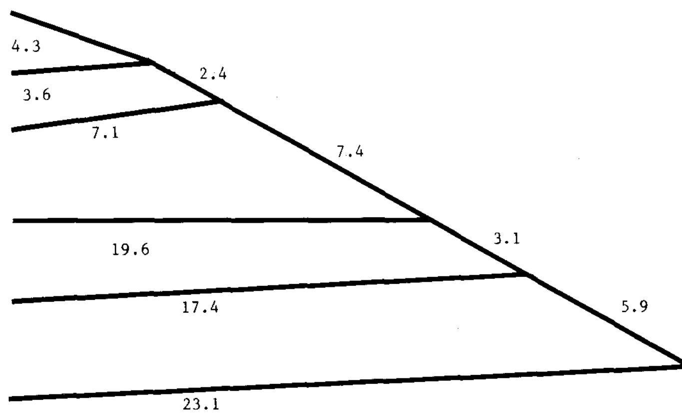

Fig. 2. The molecular phylogeny of six *Baculovirus* polyhedrins based on N-terminal amino acid sequences. The phylogeny was determined using the method of Fitch (1977) with gaps created to align homologous sequences and given a value of 4 nucleotide substitutions per gap. The numbers represent the average phyletic distance in nucleotide substitution between points of branching
Abbreviations: are the same as Fig. 1

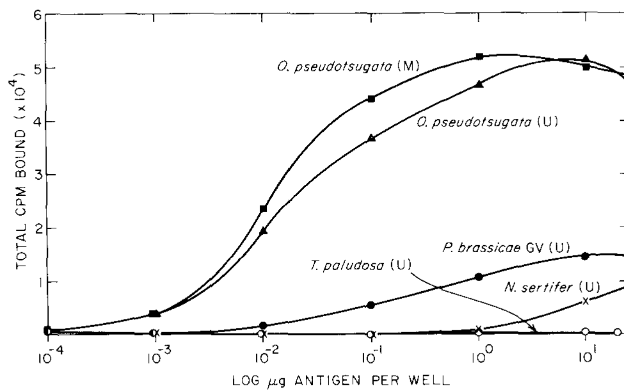

Fig. 3. A comparison of the ability of ^{125}I labeled antibody produced against *O. pseudotsugata* multicapsid NPV polyhedrin to bind occlusion body proteins from four nuclear polyhedrosis visuses and one granulosis virus in a solid phase radioimmune assay. Approximately 136,000 cpm of antibody was used per well. Controls included wells with no antigen and wells with carbonic anhydrase added at concentrations covering the range used in the binding curves. Nonspecific binding of labeled antibody ranged from 0.5–0.7% (747–1082 cpm) of the total counts added. All values are averages of points done in triplicate with the background subtracted. Attempts to enhance the ability of the antigens to bind the labeled antibody by running the reactions in the presence of 0.1% SDS produced no increase in reactivity. In addition, dissolution of the *N. sertifer* NPV polyhedrin without inactivating the protease did not increase the antibody binding
Abbreviations: are the same as Fig. 1

undergone a speciation similar to that postulated for the lepidopteran NPVs.

In contrast to the recent speciation of the Lepidoptera, the Diptera and Hymenoptera are ancient insect orders which were in existence 190–230 x 10^6 years ago (Reik 1970). The presence of only unicapsid NPVs in the Diptera and Hymenoptera (Table 1) and the large phylogenetic distance between these viruses and the lepidopteran NPVs, as indicated by our studies on polyhedrin structure (Figs. 2 and 3), suggests that NPVs have either evolved along with, or have had an ancient relationship with their insect hosts and that the infectivity spectrum of baculoviruses has been confined to their respective order since the divergence occurred. This would be similar to papovavirus phylogeny which has been shown to parallel host evolution (Soeda et al. 1980). The data from the molecular evolution of polyhedrin are not consistent with a recent horizontal invasion by *Baculovirus* of the insect orders because this would result in similar phylogenetic distances between the proteins from the three orders. They are also not consistent with a random exchange of the polyhedrin gene between viruses of different orders as this would blur the phylogenetic relationships.

Investigations on insect phylogeny indicate that the Lepidoptera and Diptera are more closely related to each other than they are to the Hymenoptera (Boudreaux 1979). One method of correlating this insect phylogeny with our proposed *Baculovirus* molecular phylogeny would require the hymenopteran NPV to have originated via a cross-infection from a lepidopteran ancestral NPV. If this cross-infection occurred and once Baculoviruses were established within the susceptible insect orders, they were restricted to their respective insect orders as the molecular phylogeny suggests, one could make the following predictions concerning *Baculovirus* phylogeny: (1) since the multicapsid viruses are a recently evolved morphotype and have evolved from a lepidopteran unicapsid virus, multicapsid viruses will be found only among lepidopteran baculoviruses; (2) since GVs evolved from a unicapsid virus and the evolution of the multicapsid morphotype was an uncommon event in *Baculovirus* evolution, it is unlikely granulosis virus strains which are normally multicapsid will be found; (3) if the Hymenoptera NPV was established via cross-infection by an ancestral NPV after the divergence of the dipteran viruses, it is unlikely GVs will be found in the Diptera or the Hymenoptera; (4) it has been suggested that the Lepidoptera and Trichoptera share a common ancestor

(Boudreaux 1979), if the GVs diverged from the Lepidoptera-Trichoptera insect ancestor, GVs may be found in Trichoptera. To date, (1), (2), and (3) appear to be true (Table 1); (4) could be resolved once the phylogenetic relationships of Trichoptera and Lepidoptera are more clearly understood and the pathology of the Trichoptera has been more closely investigated.

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