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STUDIES CONCERNING SEVERAL LATE GENE PRODUCTS IN INSECT CELLS INFECTED WITH AUTOGRAPHA CALIFORNICA NUCLEAR POLYHEDROSIS VIRUS

by

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The thesis, **Studies concerning several late gene products in insect cells infected with *Autographa californica* nuclear polyhedrosis virus**, by Jorge Vialard contains 125 characters in its title.

The shortened version of the thesis title, containing less than 70 characters is:

Characterization of late gene products in baculovirus infection

ABSTRACT

Infections of insect cells by baculoviruses are characterized by distinct early and late temporal phases with a transition demarcated by the beginning of viral DNA replication. Three processes characteristic of baculovirus infection occur during the late phase: (1) the production of two phenotypically distinct types of virions, (2) the occlusion of one of these virion types in large proteinaceous structures, and (3) the appearance of a novel DNA-directed RNA polymerase activity that is responsible for baculovirus late gene expression. Many of the polypeptide components of the late phase processes remain to be elucidated. The identification and characterization of two baculovirus late proteins are described in this thesis. The first, gp37 or SLP, is a glycoprotein that represents the major component of spindle-shaped crystal structures which are often observed at the nuclear membrane of infected cells. Purified spindle bodies are associated with an alkaline protease activity. The second late protein, p78/83, is a phosphoprotein associated with an end-structure of the nucleocapsids. It forms complexes with other infected-cell proteins and copurifies with the virus-induced RNA polymerase activity through a number of chromatographic steps.

RESUME

L'infection de cellules d'insecte par les baculovirus se caractérise par deux phases temporelles distinctes, l'une précoce et l'autre tardive. La transition entre ces phases est délimitée par le début de la réplication du DNA viral. Trois événements caractéristiques de l'infection à baculovirus adviennent lors de la phase tardive: (1) la formation de deux types phénotypiquement distincts de virion, (2) l'inclusion d'un de ces deux types dans une structure protéinique de grande taille, et (3) l'apparition d'une activité RNA polymérase DNA-dépendante spécifiquement responsable de l'expression tardive de certains gènes viraux. La plupart des composants polypeptidiques impliqués dans les événements de la phase tardive restent à déterminer. L'identification et la caractérisation de deux protéines tardives du baculovirus font l'objet de notre travail. La première, gp37 ou SLP, est une glycoprotéine représentant le composant majeur de structures cristallines en forme de fuseau qui sont souvent observées au niveau de la membrane nucléaire des cellules infectées. Nous avons montré que les structures cristallines fusiformes purifiées et incubées en milieu alkalin présentent une activité protéasique. La seconde protéine tardive décrite, p78/83, est une phosphoprotéine associée à une structure présente à l'extrémité des nucléocapsides. Dans les cellules infectées, p78/83 forme différents complexes en s'associant à diverses protéines; elle est également détectée à travers des étapes chromatographiques successives destinées à purifier l'activité RNA polymérase induite par le baculovirus.

ACKNOWLEDGMENTS

The conviction and perseverance required to produce this thesis would not have existed but for the love and support of my parents, brothers, and sister. I thank them for providing me with unwavering models of inspiration. I am especially indebted to Laurence De Moerlooze for her patience, encouragement, advice, and assistance. Above all, her companionship will always be cherished.

I thank my supervisor and friend, Chris Richardson, for giving me the opportunity to enter into this endeavour and seeing me through it. I also thank him for providing countless moments of insanity when all around him sanity prevailed.

Cathy Iorio is especially commended for carrying out my numerous ill-fated suggestions with minimum resistance. I also thank her for the help and friendship she provided throughout most of the course of this study. The constantly positive spirit and sense of conviction demonstrated by Monique Lagacé was greatly appreciated.

I thank the Banvilles for providing me with a family away from home. The wonderful food, stimulating conversation, and numerous days and evenings spent with the kids will never be forgotten.

The members of the Richardson lab, past and present, have made this entire adventure extremely enjoyable. Their friendships will be sadly missed, but there will be many wonderful memories.

Finally, this work was made possible by financial support from the Natural Sciences and Engineering Research Council, and the Fonds pour la formation de chercheurs et l'aide à la recherche.

PREFACE

In accordance with the guidelines concerning thesis preparation, and with the approval of the Department of Microbiology and Immunology, the experimental portion (Chapters 2-5) of this thesis is presented in the form of original papers. A provision in the guidelines concerning thesis preparation reads as follows:

> The candidate has the option, subject to the approval of their Department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.
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> The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.
>
> Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.
>
> In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers.

With respect to these conditions, I have included as chapters of this thesis, four original papers: two which have been published, and two which have been submitted in a slightly modified format for publication. Chapters 2-5 each contain an Abstract, Introduction, Materials

and Methods, Results, and Discussion section. Chapters 3 to 5 also contain a preface that serves to connect the texts and bridge the manuscripts. A general Introduction (Chapter 1), which is in press for publication as a book chapter, and an overall Conclusion and Summary (Chapter 6) have also been included. The references for all the chapters in the body of the thesis are listed in alphabetical order at the end of the thesis. An addendum containing two published manuscripts that describe some of the techniques used in these studies is found following the References section.

The manuscripts in order of their appearance in the thesis are:

1. Vialard, J.E., Arif, B., and Richardson, C.D. 1995. Introduction to the molecular biology of baculoviruses. In Methods in Molecular Biology. Vol. 10: Baculovirus expression protocols. Richardson, C.D., ed. Humana Press, Totowa, NJ. In press.

2. Vialard, J.E., Yuen, L., and Richardson, C.D. 1990. Identification and characterization of a baculovirus occlusion body glycoprotein which resembles spheroidin, an entomopoxvirus protein. J. Virol. 64, 5804-5811.

3. Vialard, J.E., De Moerlooze, L., and Richardson, C.D. 1994. Characterization of baculovirus and entomopoxvirus spindle proteins. Submitted.

4. Vialard, J.E. and Richardson, C.D. 1993. The 1,629-nucleotide open reading frame located downstream of the *Autographa californica* nuclear polyhedrosis virus polyhedrin gene encodes a nucleocapsid-associated phosphoprotein. J. Virol. 67, 5859-5866.

5. Vialard, J.E., Iorio, C., Lagacé, M., and Richardson, C.D. 1994. Late gene products associated with the nucleocapsid phosphoprotein (p78/83) and RNA polymerase of *Autographa californica* nuclear polyhedrosis virus. Submitted.

The manuscripts included in the addendum are:

1. Vialard, J., Lalumière, M., Vernet, T., Briedis, D., Alkhatib, G., Henning, D., Levin, D., and Richardson, C. 1990. Synthesis of the membrane fusion and hemagglutinin proteins of measles virus, using a novel baculovirus vector containing the ß-galactosidase gene. J. Virol. 64, 37-50.

2. Richardson, C.D., Banville, M., Lalumière, M., Vialard, J., and Meighen, E.A. 1992. Bacterial luciferase produced with rapid-screening baculovirus vectors is a sensitive reporter for infection of insect cells and larvae. Intervirol. 34, 213-227.

As attested to by the thesis supervisor (Dr. Christopher Richardson), I was responsible for all the research described in Chapters 2 to 5 and preparation of this thesis with the following exceptions:

Leonard Yuen performed the data bank search described in the Results section of Chapter 2.

Christopher Richardson performed the immunofluorescence studies presented in Figure 5 of Chapter 2.

Caterina Iorio purified the virus-induced RNA polymerase activity described in Figures 3, 4, and 5 of Chapter 5.

Monique Lagacé and Caterina Iorio were responsible for the production of the LEF-8 antibodies and subsequent immunoblot analysis described in Figures 3, 4, and 5 of Chapter 5.

Laurence De Moerlooze purified the *Choristoneura biennis* entomopoxvirus occlusion bodies and performed subsequent electron microscopy studies on them (presented in Figure 3 of Chapter 3). She also performed the PepTag protease assays described in Figure 6 and the amino acid sequence alignment described in Figure 4 of Chapter 3.

Laurence De Moerlooze and I were equally responsible for purification of spindle bodies described in Chapter 3 and the writing of the manuscript.

The section of Chapter 1 entitled "Baculoviruses as Expression Vectors and Engineered Insecticides" was written by Basil Arif.

The baculovirus life cycle figure presented in Chapter 1 was generated by Cathy Iorio.

Figure 3 of Chapter 1 depicting the baculovirus genome was created by Darius Bilimoria.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. I have shown that an open reading frame in the *Autographa californica* nuclear polyhedrosis virus genome shares significant homology to the open reading frame encoding an abundant protein present in purified *Choristoneura biennis* entomopoxvirus occlusion bodies.

2. I demonstrated that the baculovirus protein (SLP or gp37) is produced late in baculovirus infection in relatively small amounts.

3. I have shown, by tunicamycin treatment of cells infected with recombinant baculoviruses overexpressing gp37 and the entomopoxvirus homologues, that both proteins are glycosylated.

4. I have shown that the majority of gp37 is associated with the nucleus of baculovirus-infected cells and that the migration of gp37 to the nucleus is a relatively slow event.

5. I have shown that gp37 is associated with spindle-shaped bodies that are often associated with the nuclear membrane of *Autographa californica* nuclear polyhedrosis virus-infected cells.

6. I have demonstrated that gp37 and the entomopoxvirus homologue are the major components of their respective purified spindle bodies.

7. I have demonstrated that solubilization of purified gp37 and the *Choristoneura biennis* entomopoxvirus homologue in alkaline conditions resembling those of the insect midgut, results in proteolytic cleavage at the carboxyl terminus of both proteins. A dye-linked peptide protease assay was subsequently employed to study the proteolytic activity.

8. I have generated a mutant baculovirus that contains an interruption in the gp37 ORF. I have used this virus to infect insect larvae and cells in culture, and have demonstrated that gp37 is not essential to the viral life cycle in either infection.

9. I have identified a baculovirus protein (p78/83) that is expressed late in infection as a pair of polypeptides of 78 and 83 kDa.

10. I have shown by immunoprecipitation of short-term labeled infected cells, that the 78-kDa protein is a precursor of the 83-kDa form.

11. I have demonstrated that both forms of the protein are equally distributed in cytoplasmic and nuclear fractions of baculovirus-infected cells.

12. I have shown through radiolabeling and phosphatase treatment of p78/83 that the 83-kDa protein represents the phosphorylated form of the 78-kDa polypeptide.

13. I have demonstrated that p78/83 copurifies with both types of baculovirus virions, occluded and budded. Treatment of the purified virions with detergent to remove the virus envelopes demonstrated that both forms of the protein are associated with the nucleocapsid.

14. I have shown, by immunoelectron microscopy, that p78/83 localizes to an end-structure of mature virions embedded within occlusion bodies and to regions surrounding the virogenic stroma where nucleocapsid assembly is thought to occur.

15. I have demonstrated that p78/83 forms a relatively stable complex(es) with various proteins in the nucleus of baculovirus-infected cells.

16. I have confirmed the association of p78/83 with the nucleocapsid by demonstrating that it coimmunoprecipitates with p39, the major baculovirus capsid protein, in partlially denaturing conditions.

17. I have shown that p78/83 is present in a complex with pp31, a nuclear matrix-associated baculovirus phosphoprotein.

18. I have demonstrated that p78/83 copurifies through several chromatographic steps with a baculovirus-induced RNA polymerase activity. The presence of pp31 in the fractions containing RNA polymerase activity mirrors that of p78/83.

TABLE OF CONTENTS

RESEARCH OBJECTIVES AND THESIS OUTLINE

Although the molecular biology of the baculoviruses has gained substantial attention since the advent of the baculovirus/insect cell expression system, many of the proteins encoded by this complex virus have only recently been identified and the majority have not been characterized. The recent release of the entire genomic sequence of AcNPV (Ayres *et al.*, 1994) should greatly facilitate these studies. The baculoviruses are complex viruses that encode a large number of genes, many of which are homologous to insect host genes. These homologues appear to be required for suppression of host processes or are used for replication of the virus. The late phase of baculovirus infection is characterized by three events: (1) the appearance of a virus-induced RNA polymerase required for transcription of the late and very late gene classes; (2) the production of two types of virions which are required, independently, for the infection of cells within an insect and for horizontal (insect-to-insect) transmission; and (3) the production of occlusion bodies (OBs) late in infection for the protection of occluded virions outside of the infected insect. This thesis focuses on the identification and characterization of baculovirus late proteins and their involvement in events of the late phase of infection. The thesis is presented as a collection of manuscripts describing the identification, biochemical characterization, cellular localization, and functional analysis of two baculovirus late proteins.

Chapter 2 reports the identification of a baculovirus protein (SLP or gp37) homologous to an abundant protein which is present in entomopoxvirus occlusion bodies. We describe its expression, and demonstrate that this protein is glycosylated and copurifies with baculovirus polyhedra. In Chapter 3 we extend these studies to demonstrate its rate of synthesis, cellular localization, and presence in nuclear membrane-associated crystal structures. We also describe the solubilization of these crystal structures and report a protease activity associated with the purified crystals.

Chapter 4 presents the identification and characterization of a phosphoprotein (p78/83) which is associated with baculovirus nucleocapsids. We describe its expression, synthesis,

cellular localization, and association with an end-structure of the nucleocapsid. In Chapter 5, this phosphoprotein is analyzed further and we demonstrate its association with other infected-cell proteins and the virus-induced RNA polymerase.

Chapter 6 summarizes the work described in previous chapters and suggests possible functions for the two late proteins in baculovirus infection. It also suggests avenues that may be pursued in future studies of these proteins.

Following the main body of the thesis, an addendum containing two published manuscripts pertinent to the thesis is included. The manuscripts describe the construction of baculovirus expression vectors used in these studies.

CHAPTER 1

Introduction To The Molecular Biology Of Baculoviruses

INTRODUCTION

Over the last 10 years, baculovirus expression vectors have become a very popular and effective means with which to produce recombinant proteins in large quantities (Luckow and Summers, 1988; O'Reilly *et al.*, 1992; King and Possee, 1992; Maeda, 1989a; Kidd and Emery; 1993). Posttranslational modifications of the gene products of these insect viruses closely parallel glycosylation, fatty acid acylation, and phosphorylation in mammalian cells (reviewed in Luckow, 1991). Scale-up of insect cells in culture has also been largely perfected, making purification of large quantities of recombinant proteins a reality (Van Lier *et al.*, 1992). In addition, baculoviruses offer an ecologically acceptable and effective alternative to chemicals for the control of forest and agricultural insect pests (Wood and Granados, 1991; Huber, 1986). Their demonstrated safety as expression vectors and pest management tools is the result of limited host specificity and lack of resemblance to mammalian viruses. The development of the baculovirus expression system was facilitated by the establishment of insect cell lines that support the replication of one subgroup, the nuclear polyhedrosis viruses (NPVs). The ability to propagate baculoviruses in cell culture has also allowed extensive study of their molecular biology (Blissard and Rohrmann, 1990). The model virus in these studies is the *Autographa californica* NPV (AcNPV). Although it was first isolated from the alfalfa looper (*Autographa californica*), it multiplies readily in cell lines derived from both the fall armyworm (*Spodoptera frugiperda*) and the cabbage looper (*Trichoplusia ni*). Most expression vectors are based on AcNPV infection of *Spodoptera frugiperda* cells. However, the production of heterologous proteins in silkworm (*Bombyx mori*; Bm) larvae relies on infection with recombinant BmNPV (Maeda, 1989a). The baculovirus expression system is based on introduction of the foreign gene into nonessential regions of the viral genome through allelic replacement. Production of the recombinant protein is achieved following infection of insect cells or larvae with the newly engineered virus.

CLASSIFICATION

The *Baculoviridae* are a family of double-stranded DNA viruses that infect a variety of arthropods. They can be divided into two subfamilies (Francki *et al.*, 1991): the *Eubaculovirinae* (occluded baculoviruses) and the *Nudibaculovirinae* (nonoccluded baculoviruses). *Eubaculovirinae* infect the larvae of *Lepidoptera*, *Coleoptera*, *Diptera*, *Hymenoptera*, *Neuroptera*, *Siphonoptera*, *Thysanura*, and *Trichoptera*, and even some crustaceans, such as shrimp and crabs (Adams and McClintock, 1991; Couch, 1991). Members of the *Nudibaculovirinae* include the palm rhinoceros beetle (*Orcytes rhinoceros*) virus, the Hz-1 virus, and the cricket (*Gryllus campestris*) virus (Huger and Krieg, 1991). The *Eubaculovirinae* produce crystalline proteinaceous structures called occlusion bodies (OBs) (Figs. 1 and 2), which are absent in the *Nudibaculoviridae*. Virions embedded within these OBs are protected from environmental inactivating factors such as UV light, desiccation, and nucleases. The *Eubaculovirinae* subfamily is made up of two genera (granulosis and nuclear polyhedrosis viruses) which are distinguished by the major protein that constitutes the OB matrix. The granulosis virus OBs are generally small (0.25-0.5 µm), contain a single virion, and are composed of a protein called granulin (Tanada and Hess, 1991). The NPV OBs are much larger (1-15 µm diameter) and are composed of the closely related polyhedrin protein (Rohrmann, 1986). NPV OBs, or polyhedra, usually contain a large number of virions embedded within the matrix. NPVs can be further separated into two subgenera depending on the number of nucleocapsids surrounded by a common membrane; *M*NPVs and *S*NPVs contain multiple and single nucleocapsids, respectively. However, this difference does not seem to be phylogenetically important. Most baculoviruses identified thus far are very host-specific, and the majority of *Eubaculovirinae* have been isolated from larvae of the *Lepidoptera* family. A survey of different baculoviruses with excellent electron photomicrographs can be found in the *Atlas of Invertebrate Viruses* (Adams and McClintock, 1991; Couch, 1991; Tanada and Hess, 1991; Huger and Krieg, 1991).

Figure 1. Life cycle of a baculovirus in an infected insect cell. Two populations of virus are formed: occluded or polyhedron-derived virions (PDV) accumulate in the nucleus and budded virions (BV) mature at the plasma membrane of the host cell. In nature, occlusion bodies serve to protect the virus from the environment (UV light and desiccation); they are ingested by larvae and become solubilized in the gut, releasing virions that attach and fuse with the cells of the midgut. The nucleocapsid is targeted to the nucleus, where replication and transcription occur. BV promotes secondary infection to adjacent cells. The virus spreads to the ovaries, fat bodies, and most endothelial cells via the tracheal system.

Baculovirus Life Cycle

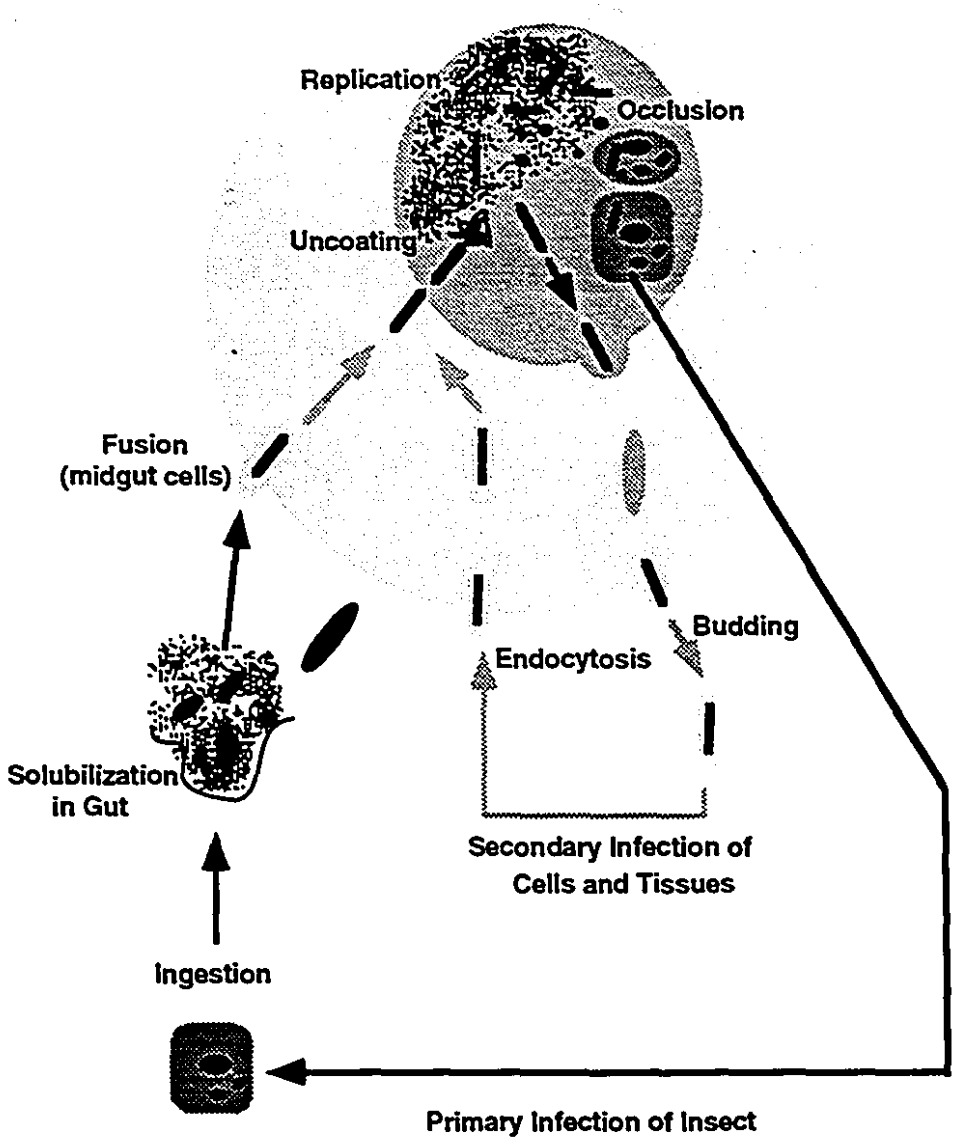


Figure 2. *Autographa californica* nuclear polyhedrosis virus-infected Sf9 insect cells. Various features common to a baculovirus infection in Sf9 cells are indicated. Occlusion bodies or polyhedra (P) containing virions (V) are present in the nuclei. Surrounding each occlusion body is a polyhedral envelope. Replication and assembly of viral nucleocapsids occur in the nucleus in association with the virogenic stroma (VS). P10 is associated with fibrillar structures (FS) which are found both in the nucleus and the cytoplasm. Electron-dense spacers (ES) are associated with FS within the nucleus. ES are believed to be involved in the formation of the polyhedral envelope whereas FS favor lysis of the cell following virus maturation. Spindle bodies (S) that contain gp37 are diamond-like structures that are associated with the nuclear membrane and can sometimes be found in the cytoplasm. Their function is currently unknown.

NATURAL INFECTION OF INSECT LARVAE

Baculovirus infection is characterized by the production of two structurally and functionally distinct types of virions, the occluded or polyhedra-derived virion (PDV) and the extracellular or budded virion (BV). The PDV type, which is responsible for primary infection, is embedded within the matrix of newly formed OBs (Fig. 2) and is required for dissemination in the environment. In a natural infection, larvae ingest PDV-containing OBs that contaminate their food. The alkaline environment of the insect midgut causes the polyhedra to dissolve releasing the embedded virions. The liberated PDVs infect midgut columnar epithelial cells by a process of receptor-mediated membrane fusion (Horton and Burand, 1993). These infected cells produce the BV type, which is required for secondary infection. The BV is responsible for systemic spread within insects and is also the type that infects cells in culture. Cellular entry of the BV occurs through receptor-mediated adsorptive endocytosis (Volkman and Goldsmith; 1985; Charlton and Volkman, 1993). Although it was previously thought that the spread of infection within the insect occurred via hemocytes in the hemocoel (Granados and Lawler, 1981; Keddie *et al.*, 1989), this role has been recently ascribed to cells of the tracheal system (Engelhard *et al.*, 1994). The tracheal system provides access to various tissues, such as the ovaries, fat bodies, and most endothelial cells where both BV and PDV are produced. Studies of baculovirus infection in cell culture have revealed a number of landmark events. Following penetration of the plasma membrane, the nucleocapsids move toward the nucleus by a process that appears to require the formation of actin microfilaments (Charlton and Volkman, 1993). At the nucleus, the nucleocapsids are uncoated, and the DNA is released. At about this time, the nucleus becomes enlarged, and a distinct electron-dense granular structure, called the virogenic stroma, is formed (Fig. 2). This structure is associated with the nuclear matrix and is thought to be the site of nucleocapsid assembly (Granados and Lawler, 1981; Fraser, 1986). Viral transcription and replication may also take place at the virogenic stroma. By 12 hours postinfection, progeny BVs are produced and are released into the extracellular compartment. Polyhedra begin to be formed soon thereafter, and mature PDVs (surrounded by an envelope) become occluded. Feeding

continues throughout infection (5-7 days) during which large numbers of OBs (up to 25% of the dry weight of the insect) accumulate in the infected cells. The production of large numbers of OBs results from hyperexpression of the polyhedrin gene. The polyhedrin protein is generally essential for *in vivo* infections of larvae, but is expendable in infections of cultured cells. Most baculovirus expression vectors exploit this phenomenon by substituting a foreign gene for the coding sequence of polyhedrin. Eventually, the infected insect stops feeding and undergoes several rapid physiological changes. Its cuticle melanizes, the musculature becomes flaccid, and the larva liquefies. Larval disintegration results in release of the OBs, which are subsequently dispersed in the environment. The baculovirus life cycle is summarized in Fig. 1.

VIRUS STRUCTURE AND ASSEMBLY

The AcNPV nucleocapsid is bacilliform in shape, measures 35-40 nm x 200-400 nm and contains a circular, double-stranded DNA genome of approximately 134 kb that has been recently sequenced in its entirety (Ayres *et al.*, 1994). Baculovirus DNA is tightly associated with a protamine-like protein known as p6.9 (Tweeten *et al.*, 1980; Wilson *et al.*, 1987). The resulting DNA-protein complex forms the core of the nucleocapsid. In addition to p6.9, several other genes encoding nucleocapsid proteins have been identified. The most abundant protein in purified nucleocapsids is p39, the major capsid protein (Thiem and Miller, 1989b). Immunoelectron microscopy studies demonstrated its distribution throughout the length of the nucleocapsid (Russell *et al.*, 1991). A similar localization is observed with p24, a minor nucleocapsid protein (Wolgamot *et al.*, 1993). In contrast to the two previous proteins, p78/83, a proline-rich phosphoprotein, is associated with end structures of the nucleocapsids (Possee *et al.*, 1991; Vialard and Richardson, 1993). The precise localization of p87, a fourth nucleocapsid protein, has not been established (Müller *et al.*, 1990).

A model for nucleocapsid morphogenesis proposes that viral DNA is condensed by association with the basic p6.9 protein to form the core, whereas the capsid is assembled independently. The nucleoprotein complex enters the capsid through one end to form the

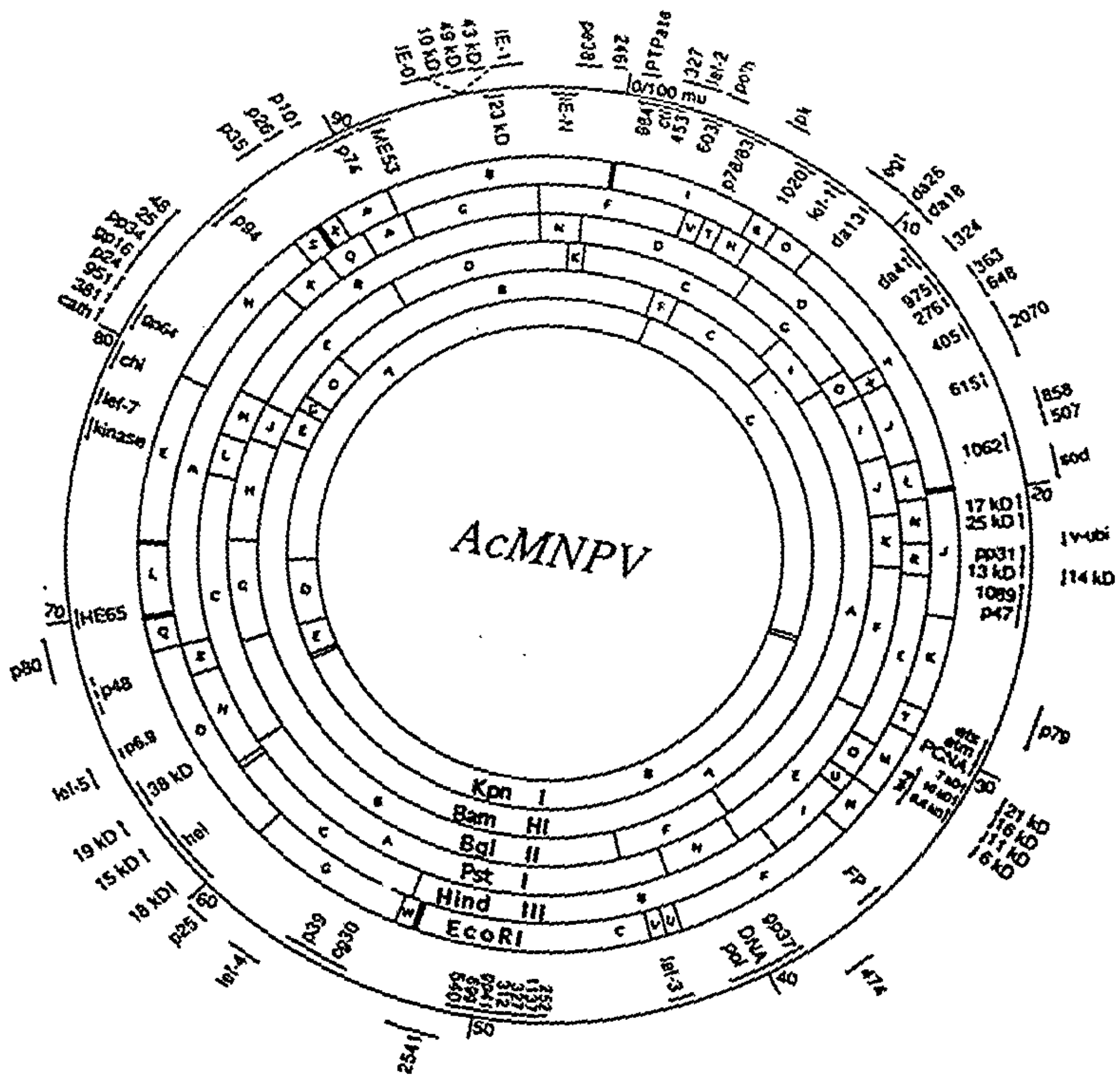
mature nucleocapsid (Fraser, 1986). A baculovirus encoded phosphoprotein, pp31, binds DNA nonspecifically, colocalizes with the virogenic stroma (Fig. 2), and is tightly associated with the nuclear matrix. It may play a role in packaging or, alternatively, in viral transcription and/or replication (Guarino and Smith, 1990; Guarino *et al.*, 1992). Following assembly, nucleocapsids destined to become BV pass through the nuclear membrane and acquire a temporary envelope containing the virus-encoded protein, p16 (Oellig *et al.*, 1987; Gross *et al.* 1993b). This envelope is associated with the BV as it passes through the cytosol, but is lost when the virus buds through the plasma membrane. At the cell surface, the nucleocapsid acquires a looselv fitting envelope that contains the BV envelope glycoprotein, gp67 (Whitford *et al.*, 1989; Blissard and Rohrmann, 1989). This protein, which may be present in peplomer-like structures at one end of the virion, is required for BV infectivity by pH-dependent fusion (Blissard and Wenz, 1992). The nucleocapsids destined to become PDVs remain in the nucleus and acquire a *de novo* envelope of unknown origin. In *M*NPVs, several nucleocapsids may be included within a single tight-fitting envelope. At least three distinct proteins are associated with PDV, but not BV virions. Two of these, p25 and gp41, appear to be associated with the PDV envelope (Russell and Rohrmann, 1993; Nagamine *et al.*, 1991; Whitford and Faulkner, 1992a and b). A third protein, p74, is not essential to viral replication in cell culture, but is required for larval infection following ingestion of OBs (Kuzio *et al.*, 1989). Its precise location is not known.

As previously mentioned, the major component of the OB is polyhedrin, a protein that is highly conserved among the NPVs (Rohrmann, 1986). Surrounding the matrix of the OB is a structure called the polyhedral envelope (PE) or calyx (Fig. 2). This structure has been reported to be rich in carbohydrate (Minion *et al.*, 1979), but also contains a proteinaceous component, called pp34 or PE protein (Whitt and Manning, 1988; Gombart *et al.*, 1989b; Russell and Rohrmann, 1990a). The PE may increase stability of the OB; interruption of the pp34 gene results in the production of OBs that are more sensitive to weak alkali conditions than wild-type OBs (Zuidema *et al.*, 1989). A third gene that is involved in OB formation is p25 or few polyhedra (FP). Insertions of cellular DNA that interrupt this gene result in an FP phenotype (Beams and Summers, 1989).

However, it is not known whether this protein participates in OB formation directly or indirectly. A second hyperexpressed protein, p10, forms fibrous networks in the nucleus and cytoplasm of infected cells (van der Wilk *et al.*, 1987; Williams *et al.*, 1989) (Fig. 2). These p10-containing structures are associated with electron-dense spacers that form in the infected-cell nucleus. The spacers contain pp34 and are thought to be developing PE (Russell and Rohrmann, 1990a; van Lent *et al.*, 1990). An association between p10 and microtubules has also been reported (Volkman and Zaal, 1990). Disruption of the p10 gene results in mutants with varying phenotypes. Some mutants displayed aberrant attachment of PE, which resulted in the production of OBs sensitive to mechanical stress (Williams *et al.*, 1989). Other p10 deletion mutant studies suggest that it is involved in cell lysis late in infection (van Oers *et al.*, 1993). Deletion of the p10 protein prevented release of polyhedra from infected cells, presumably because of impaired nuclear disintegration. A protein called gp37, or SLP, shares homology with a major OB component of another insect virus family, the *Entomopoxviridae*. It forms spindle-shaped inclusions that are found both in the cytoplasm (Dall *et al.*, 1993; Gross *et al.*, 1993a; Vialard *et al.*, 1990b) and associated with the nuclear membrane and may contain a proteolytic activity (Vialard *et al.*, submitted). A review describing studies on baculovirus structural proteins was recently compiled by Rohrmann (1992).

In addition to the structural proteins described above, the baculoviruses encode a number of regulatory proteins. These include a ubiquitin-like factor, Zn/Cu dismutase, protein kinases, PTPase, ecdysosteroid UDP-glucosyltransferase, proliferating cell nuclear antigen (PCNA), DNA polymerase, helicase, chitinase, cysteine protease, and a protein that blocks apoptosis. A summary of the genes encoded by AcNPV that are known at this point is shown in Fig. 3 and Table 1. For a more detailed description of baculovirus genes and references pertaining to these genes, the reader is referred to Ayres *et al.* (1994) and a review by Kool and Vlak (1993).

Figure 3. Genes and open reading frames on the genome of *Autographa californica* nuclear polyhedrosis virus which have been identified to date. The *Kpn*I, *Bam*HI, *Bgl*I, *Pst*I, *Hin*dIII, and *Eco*RI restriction fragments of the circular dsDNA genome of AcNPV are classified using alphabetical letters. The locations of the genes on the 134 kb genome are indicated as positions from 0-100 map units. Further information concerning the genes and their proposed functions is listed in Table 1.



AcMNPV
Kpn I
Bam HI
Bgl II
Pst I
Hind III
EcoRI

Table 1. AcNPV Genes and Open Reading Frames

Table 1. AcNPV Genes and Open Reading Frames.

CLASS[a]	DESIGNATION	DIRECTION[b]	FUNCTION
VL	PTPase (19kD)	R	protein-tyrosine phosphatase
L	ORF 984 (38kD)	L	?
L	ctl (5.6kD)	L	conotoxin
E	ORF 453 (17kD)	L	?
V	ORF 327	R	?
L	lef-2 (24kD)	R	late expression factor
L	ORF 603 (24kD)	L	?
VL	polh (29kD)	R	polyhedrin
L	p78/83	L	nucleocapsid-associated phosphoprotein
VL	pk	R	protein kinase?
?	ORF 1020	L	?
E	lef-1 (31kD)	L	late expression factor
E	egt (57kD)	R	ecdysosteroid UDP-glucosyltransferase
?	da13	L	?
E	da26	R	?
L	da18	R	?
E	da41	L	?
L	ORF 324	R	?
?	ORF 975	L	?
L	ORF 963	R	?
?	ORF 276	L	?
L	ORF 648	R	?
E/L	ORF 2070	R	?
?	ORF 405	L	?
?	ORF 615	L	?
?	ORF 858	R	?
?	ORF 507	R	?
?	ORF 1062	L	?
L	SOD (16kD)	R	Cu/Zn superoxide dismutase
?	17kD ORF	L	?
?	25kD ORF	L	?
L	v-ubi	R	ubiquitin-like
E/L	pp31	L	nuclear matrix-associated protein
E	13kD ORF	L	?
?	14kD ORF	R	?

CLASS[a]	DESIGNATION	DIRECTION[b]	FUNCTION
E	ORF 1089	L	?
E	p47	L	late expression factor
?	p79	R	?
E/L	ets (10kD)	L	?
E	etm	L	?
E	pcna (etl; 28kD)	L	proliferating cell nuclear antigen
?	21kD ORF	R	?
?	7kD ORF	L	?
?	lef-8	L	late expression factor
?	10kD ORF	L	?
?	16kD ORF	R	?
?	11kD ORF	R	?
?	9.6kD ORF	L	?
?	6kD ORF	R	?
L/VL	FP (25kD)	L	few polyhedra phenotype
?	ORF 474	R	?
L	gp37 (spindolin)	L	entomopoxvirus spindolin homologue
E	DNApol (114kD)	L	DNA polymerase
E	lef-3	L	late expression factor
?	ORF 252	L	?
?	ORF 1137	L	?
L	ORF 327	L	?
L	ORF 312	L	?
L	gp41	L	OV associated glycoprotein
?	ORF 699	L	?
?	ORF 540	L	?
?	ORF 2541	R	?
E	cg30	L	zinc finger/leucine zipper
L	p39 (cap)	L	major capsid protein
E	lef-4	R	late expression factor
L	p25	R	OV envelope protein
E	p143 (hel)	L	helicase
?	18kD ORF	R	?
?	15kD ORF	R	?
?	19kD ORF	R	?
L	38kD ORF	L	?
E	lef-5	R	late expression factor

CLASS[a]	DESIGNATION	DIRECTION[b]	FUNCTION
L	p6.9	L	basic DNA binding protein
L	p48	L	?
L	p80	R	capsid associated protein
?	HE65	L	?
?	kinase	L	tyr/ser kinase
?	lef-7	L	late expression factor
?	chi	L	chitinase
L	cath	R	cysteine protease (cathepsin-like)
E	gp64	L	BV major envelope protein
L	ORF 381	R	?
?	ORF 951	R	?
L	p24	R	capsid associated protein
L	gp16	R	nuclear membrane protein
L/LV	pp34	R	polyhedral envelope protein
L	25kD ORF	R	?
L	48kD ORF	R	?
E	p94	L	?
E	p35	R	blocks apoptosis
E/L	p26	R	?
VL	p10	R	cytoplasmic/nuclear fibrous structures
L	p74	L	essential for OB infectivity in larvae
E	ME53	L	zinc finger
E/L	IE-0	R	first exon of IE-1
?	10kD ORF	R	?
?	49kD ORF	R	?
?	43kD ORF	R	?
?	23kD ORF	L	?
E/L	IE-1 (67kD)	R	transactivates early genes
E	IE-N (47kD)	L	modulates IE-1 expression
E	pe38	R	zinc finger/leucine zipper
?	ORF 246	R	?

[a]Time of transcription is classified as early (E), late (L), or very late (VL).
[b] Direction of transcription is indicated rightward (R) or leftward (L) with respect to the 0/100h point in Figure 3.

BACULOVIRUS GENE EXPRESSION AND REPLICATION

Baculovirus gene expression is regulated in a cascade-like fashion where activation of each set of genes relies on the synthesis of proteins from previous gene classes (reviewed in Friesen and Miller, 1986). This temporal regulation allows the grouping of baculovirus genes into three phases during infection: early (E), late (L), and very late (VL). Although most baculovirus genes can be placed into one of the above classes, some may be transcribed in more than one phase. The E genes are transcribed prior to viral DNA replication, whereas the L and VL genes are activated during or after replication. The late classes are not synthesized in the presence of aphidicolin, an inhibitor of DNA replication. The reason for this dependence on viral replication is not yet known. The L genes are activated before the VL genes and are maximally transcribed over a short period of time (between 12 and 24 h postinfection). The VL genes are hyperexpressed following activation of the L genes and remain active well after L transcription has diminished (from 48 h postinfection onwards). The early genes generally encode proteins that are involved in regulatory functions, such as transcription, replication, and modification of host processes. Late genes include BV and PDV structural proteins, whereas VL proteins are those involved in the processes of occlusion and cell lysis.

Baculovirus E genes are transcribed by the host RNA polymerase II. Consequently, transcription from the E promoters is abolished in the presence of α-amanitin, an inhibitor of RNA polymerase II (Huh and Weaver, 1990). The involvement of RNA polymerase II in E gene transcription was demonstrated by accurate initiation of mRNAs in an in vitro transcription system using nuclear extracts from uninfected cells (Hoopes and Rohrmann, 1991). Thus, the E promoters resemble typical eukaryotic RNA polymerase II responsive promoters that contain DNA elements which are recognized by host transcription factors (Krappa *et al.*, 1992; Kogan and Blissard, 1994).

Previously, the E genes were often subdivided into immediate early (IE) and delayed early (DE) components depending on their requirement for viral protein synthesis in transient expression assays where reporter genes were placed under the control of IE or DE promoters and

transfected into insect cells (Guarino and Summers, 1986a). Under these conditions, IE genes do not require viral proteins for their activation and are transcribed in uninfected cells. Activation of the DE genes, however, is dependent on protein products of the IE genes. A distinction between these two gene classes is not observed in infections and both are expressed even in the presence of cycloheximide, an inhibitor of protein synthesis. This suggests that additional factors required for DE promoter activation in transient expression are provided during the initial phase of infection by proteins associated with the virion. For example, IE-1, a transactivator of DE genes, has recently been shown to be a component of the BV (Thielmann and Stewart, 1993).

Most IE proteins identified thus far appear to be involved in the regulation of viral transcription. IE-1 is a 66.9-kDa polypeptide capable of transactivating a number of early and late promoters in transient expression assays (Guarino and Summers, 1986a; Nissen and Friesen, 1989; Guarino and Summers, 1987; Passarelli and Miller, 1993a). IE-1-mediated activation requires the presence of *hr* (homologous repeat) elements in cis with the responsive promoter (Guarino and Summers, 1986b) and has been recently demonstrated to impart its activity through binding to *hr* elements (Guarino and Dong, 1991; Kovacs *et al.*, 1992). The IE-0 protein is a product of alternative splicing, which results from the utilization of an exon 5' to the IE-1 promoter. This results in fusion of 54 amino acids to the N-terminus of IE-1 (Chisholm and Henner, 1988; Kovacs *et al.*, 1991). The resulting mRNA contains its own promoter, which is regulated differently than the IE-1 promoter. Also, the IE-0 transacting functions differ from those of IE-1. For example, in addition to activating a number of genes, IE-1 negatively regulates IE-0 transcription (Kovacs *et al.*, 1991). In contrast, IE-1 expression is stimulated by IE-0 (Carson *et al.*, 1991a). A third IE protein, IE-N (or IE-2), augments IE-1-mediated transactivation moderately, exhibits an autoregulatory activity, and is downregulated by IE-1 (Carson *et al.*, 1988 and 1991b). IE-N contains a zinc finger and a leucine zipper, motifs characteristic of some transcription factors. Two other baculovirus genes, pe-38 and cg-30, also encode these motifs (Krappa and Knebel-Morsdorf, 1991; Thiem and Miller, 1989a). PE38 stimulates IE-N and p143 (helicase) transcription in transient expression assays (Carson *et al.*, 1991b; Lu and Carstens, 1993). A truncated form of

PE38, that does not have stimulatory activity and appears to be the product of alternative transcriptional initiation, has been recently identified (Wu *et al.*, 1993).

Several early genes encode components of the DNA replication machinery. For example, the PCNA (ETL) gene product was shown to be involved in both replication and late gene transcription (Crawford and Miller, 1988; O'Reilly *et al.*, 1989). A mutation in *pcna* produces virus that exhibits delayed replication and late gene expression. In addition, two ts mutants defective in late gene expression have been mapped. One, which is also defective in replication, has a mutation in the p143 (helicase) gene (Lu and Carstens, 1991). The second ts mutant is rescued by wild-type p47, a protein whose function is not yet known (Carstens *et al.*, 1993).

The L and VL genes are under the control of an α-amanitin-resistant RNA polymerase that is induced during infection (Huh and Weaver, 1990; Grula *et al.*, 1981; Fuchs *et al.*, 1983). This polymerase activity is also resistant to tagetitoxin, an inhibitor of insect RNA polymerase III (Glocker *et al.*, 1993). Partial purification of the α-amanitin-resistant activity suggests that its protein composition is different from the three host RNA polymerases (Yang *et al.*, 1991). It is not known whether its components are virus-encoded, host-encoded, or a combination of the two. The specificity of the virus-induced polymerase may be dictated by the unique baculovirus L and VL promoters. They differ from most RNA polymerase II promoters in that they are very compact and do not contain DNA elements, such as the TATA box, present in most eukaryotic promoters. The only DNA element present in all L and VL promoters is a consensus core sequence, TAAG, which contains the transcriptional start site and is essential for activity (Rohrmann, 1986; Wilson *et al.*, 1987; Thiem and Miller, 1989b; Possee and Howard, 1987). This element is present in the promoters of the hyperexpressed VL genes as part of a very well-conserved sequence, TAAATAAGT/AATT. This sequence is responsible for the very high levels of expression observed from the VL promoters (Possee and Howard, 1987). Sequences in the leader region between the TAAG element and the start codon may influence levels of transcription somewhat (Ooi *et al.*, 1989). The factors that interact with the L and VL promoters to regulate transcription are not known, but the development of an in vitro transcription system that utilizes nuclear extracts

obtained late in infection may help in their identification (Glocker *et al.*, 1993). A transient expression system utilizing a mixture of successively smaller fragments of the baculovirus genome that are able to activate L and VL promoters has resulted in the identification of a number of genes encoding late gene expression factors (lef) (Passarelli and Miller, 1993a, b, c, and 1994; Li *et al.*, 1993; Passarelli *et al.*, 1994; Morris *et al.*, 1994). Although some of these genes have not been previously identified (lef 1-8), some have been previously described as regulators of early transcription or replication (ie-1, ie-n, and p143). The predicted amino acid sequence of LEF-8 contains a conserved motif of RNA polymerases (Passarelli *et al.*, 1994) and the protein copurifies with the virus-induced RNA polymerase activity (C. Iorio, personal communication), suggesting that it is a component of the α-amanitin resistant polymerase.

The AcNPV genome contains several regions called homologous repeats (*hrs*) which encompass repeated sequences harboring *Eco*RI recognition sites (Guarino *et al.*, 1986). These elements appear to have two functions. As mentioned above, they act as enhancers for a number of E and L genes when present in plasmids (in transient expression assays) and within the viral genome (Guarino *et al.*, 1986; Rodems and Friesen, 1993; Guarino and Summers, 1988). More recently, they were shown to act as origins of replication for plasmids when co-transfected together with various fragments of the baculovirus genome or introduced into infected cells (Pearson *et al.*, 1992; Kool *et al.*, 1993; Leisy and Rohrmann, 1993). Regions of the baculovirus genome that may encode factors required for replication have also been identified in a plasmid based replication system (Kool *et al.*, 1994). Some genes present in these regions include the baculovirus encoded DNA polymerase, p143 (helicase), and proliferating cell nuclear antigen (PCNA) genes.

BACULOVIRUSES AS EXPRESSION VECTORS AND ENGINEERED INSECTICIDES

Two important features of baculoviruses account for the success of this virus as an expression vector. First, the virus contains a number of nonessential genes that can be replaced

by an exogenous gene. Second, many of these genes, particularly the very late ones, are under the control of powerful promoters that allow abundant expression of the passenger recombinant gene. Most of the baculovirus expression systems make use of the polyhedrin or p10 promoters together with their associated flanking sequences. Both polyhedrin and p10 are nonessential, since deletion of these genes does not affect the replication of the virus in cell culture (Smith *et al.*, 1983a; Weyer *et al.*, 1990). The p6.9 promoter appears to be as efficient as the p10 and polyhedrin promoters and may also be harnessed for recombinant protein expression (Bonning *et al.*, 1994). For reviews concerning the use of baculoviruses in the expression of recombinant proteins important in the pharmaceutical industry and in basic research, the reader is referred to Luckow and Summers (1988) and O'Reilly *et al.* (1992). The same basic principles apply to the utilization of these viruses in pest management strategies where the wild-type virus is ineffective in producing the desirable control of an insect pest. When the virus is used in pest management strategies, a number of important criteria must be observed. In contrast to the use of expression vectors in cell culture where synthesis of polyhedrin in not necessary, the formation of OBs is important for the viral insecticide to survive in nature long enough for the insect to ingest it. Without the protection afforded by the OBs, the virus is quickly inactivated. Although wild-type baculoviruses have been used as insecticides, the lethal dose and time can be improved by genetic engineering (Bonning and Hammock, 1992). A number of candidate genes with potential insecticidal properties have been inserted into baculoviruses, and the engineered viruses have been tested against the target insects. An insect-specific toxin that appears to be effective in enhancing AcNPV as an insecticide is derived from the venom of the North African scorpion, *Androctonus australis* Hector (Stewart *et al.*, 1991; Maeda *et al.*, 1991b; McCutchen *et al.*, 1991). The gene product produced the desired neurologic effects in *Trichoplusia ni* larvae, and reduced both the median survival time of the infected insect and the median lethal dose of virus (Stewart *et al.*, 1991). This modified baculovirus was used recently in a field trial and was shown to be more effective than the wild-type virus in reducing crop damage as a result of its increased lethality (Cory *et al.*, 1994). A toxin (TxP-I) derived from the venom of female mites, *Pyemotes tritici*, was also

shown to be effective against insects. The potential of this toxin was investigated by engineering a cDNA encoding the toxin into AcNPV (Tomalski and Miller, 1991a and b). Larvae infected with the virus carrying the engineered gene became paralyzed during infection. Other genes that have been introduced into baculoviruses for insecticidal purposes include juvenile hormone esterase (*jhe*; 1) (Hammock *et al.*, 1990 and 1993) and an insect diuretic hormone (Maeda, 1989b). Deletion of the ecdysosteroid UDP-glucosyltransferase (*egt*) gene of AcNPV also increased the lethality of the virus by interfering with insect metamorphosis and moulting (O'Reilly and Miller, 1989 and 1991).

In short, the baculovirus expression system has made a great impact in both academic and applied pharmaceutical research. It has become a major workhorse in most expression laboratories.

CHAPTER 2

Identification and Characterization of a Baculovirus Occlusion Body Glycoprotein Which Resembles Spheroidin, an Entomopoxvirus Protein

ABSTRACT

A 37-kDa polypeptide specified by *Autographa californica* nuclear polyhedrosis virus was found to share significant homology with *Choristoneura biennis* entomopoxvirus spheroidin protein, which is the major component of entomopoxvirus occlusion bodies. Antibodies raised against spheroidin cross-reacted with the 37-kDa protein and confirmed its expression in the late phase of wild-type baculovirus infection. Immunoblot analysis and fluorescence microscopy demonstrated that the protein was associated with purified *A. californica* nuclear polyhedrosis virus occlusion bodies and was absent in purified virions. Immunofluorescence studies localized the protein to the periphery of occlusion bodies and the internal membranes of cells infected with wild-type baculovirus. The open reading frame encoding this spheroidinlike protein was inserted into a baculovirus expression vector, and recombinant protein was synthesized under control of the polyhedrin promoter. Studies of the recombinant protein demonstrated that it was heterogeneous in molecular mass as a result of *N*-linked glycosylation. Tunicamycin inhibited carbohydrate addition and yielded proteins of 34 and 33-kDa.

INTRODUCTION

Autographa californica nuclear polyhedrosis virus (*Ac*NPV) is a member of the family Baculoviridae, a group of double-stranded DNA viruses which infect insects from the Lepidoptera, Diptera, and Hymenoptera orders (reviewed in Faulkner and Carstens, 1986). A characteristic of this subgroup of baculoviruses is the occlusion of infectious virions in large proteinaceous structures termed polyhedra (Rohrmann, 1986). The virions contained within these occlusion bodies (OBs) differ from nonoccluded virus (NOV), which bud from the plasma membrane, in both structure and mode of infection (Volkman, 1986). NOVs are responsible for cell-to-cell infection while the occluded virus (OV) is required for insect-to-insect transmission. OBs serve to protect the occluded virions from the outside environment for extended periods of time until they are ingested by insect larvae. The alkaline environment of the insect midgut dissolves the OBs, resulting in the release of virions and subsequent infection of the midgut epithelial cells.

To date, three proteins have been recognized to be associated with the structure of polyhedra. The most abundant component of polyhedra is a 29-kDa protein called polyhedrin, which constitutes the matrix of the OB (Rohrmann, 1986) and accounts for up to 25% of the total protein in virus-infected cells (Smith *et al.*, 1983b). Another protein, p10, is expressed at high levels late in infection (Kuzio *et al.*, 1984) and is also associated with polyhedra. This 10-kDa protein seems to play an important role in morphogenesis of polyhedra since mutants lacking the p10 gene produce fragile OBs (Williams *et al.*, 1989). The third protein, pp34, is a phosphorylated polypeptide associated with the polyhedral envelope (PE), or calyx, a structure which surrounds the mature polyhedron (Gombart *et al.*, 1989a and b; Whitt and Manning, 1988). This envelope is composed primarily of carbohydrate (Minion *et al.*, 1979). A 32-kDa protein, p32, produced by *Orgyia pseudotsugata* NPV (OpNPV) and pp34 have similar properties and exhibit 58% amino acid sequence homology (Gombart *et al.*, 1989b; Oellig *et al.*, 1987). Russell and Rohrmann (1990a) localized p32 to the PE of OpNPV polyhedra by immunogold staining.

In addition to NPVs, several other viruses produce OBs. These include granulosis viruses, cytoplasmic polyhedrosis viruses, and entomopoxviruses (EPVs) (Rohrmann, 1986). Granulosis viruses form cytoplasmic OBs which contain granulin, a protein which is closely related to polyhedrin in both structure and function and bears 50% amino acid homology with polyhedrin. Cytoplasmic polyhedrosis viruses are double-stranded RNA viruses whose OB proteins bear no relatedness to those of baculovirus. Finally, the entomopoxviruses, which infect Lepidoptera and Diptera (Arif, 1984; Kurstak and Garzon, 1977), synthesize a major OB matrix protein called spheroidin (Bilimoria and Arif, 1979). This protein is analogous to polyhedrin but does not possess significant amino acid homology with it. The gene encoding spheroidin in *Choristoneura biennis* EPV (CbEPV) has recently been identified and sequenced; it specifies a polypeptide of 38.5 kDa (Yuen *et al.*, 1990). The observed size of spheroidin is 50 kDa, and it was suggested that this size discrepancy was due to post-translational modifications such as glycosylation. Spheroidin was shown to be extremely rich in cysteine residues and formed high-molecular-weight complexes on sodium dodecyl sulfate (SDS)-polyacrylamide gels. These large species

could be converted to a 50-kDa polypeptide in the presence of 0.5% ß-mercaptoethanol and were shown to have identical patterns of proteolysis when compared with that of the monomer (Yuen *et al.*, 1990).

In this paper we report the discovery and characterization of a 37-kDa glycoprotein that is encoded by a baculovirus and appears to be associated with the PE. As this publication was in progress, another laboratory reported the DNA sequence of this 37-kDa polypeptide (Wu and Miller, 1989). We describe the localization, time of synthesis, and homology of this protein to spheroidin, the major OB component encoded by EPVs.

METHODS AND MATERIALS

Cells and virus.

Spodoptera frugiperda (*Sf*9) insect cells and AcNPV were obtained from Max Summers, Texas A&M University, College Station. Virus and cells were cultured in TNM-FH medium supplemented with 10% fetal bovine serum as previously described (Summers and Smith, 1987).

Construction and isolation of recombinant virus.

A DNA fragment containing the spheroidinlike protein (SLP) open reading frame (ORF) was obtained from AcNPV DNA using the polymerase chain reaction method (Saiki *et al.*, 1988). Two oligonucleotide primers were constructed which consisted of a pair of *Nhe*I endonuclease recognition sites followed by several nucleotides corresponding to sequences near either the 5' or 3' end of the SLP ORF. The nucleotide sequences of the oligonucleotides were as follows: ATG oligo, 5'-GCTAGCGCTAGCAATATGATTGCATTATTA-3' and (transcription termination site oligo) 5'-GCTAGCGCTAGCCTAAATATAATATGT-3'. The resulting polymerase chain reaction fragment was inserted into a baculovirus transfer vector under the control of the polyhedrin promoter. The DNA sequence of the polymerase chain reaction fragment was confirmed by the dideoxy-chain-termination sequencing method (Sanger *et al.*, 1977).

This plasmid was transfected together with wild-type AcNPV DNA into Sf9 cells by using the calcium phosphate precipitation technique (Summers and Smith, 1987). Recombinant virus was plaque purified by the rapid ß-galactosidase screening assay as previously described (Vialard *et al.*, 1990a).

Antiserum.

Polyclonal antiserum from rabbits was prepared against proteins of CbEPV OBs by standard methods (Richardson *et al.*, 1985). The OBs were solubilized by alkali treatment and purified away from virions as previously described (Bilimoria and Arif, 1979). The 50-kDa occlusion body protein (spheroidin) was further purified by ion-exchange chromatography on QA Sepharose (Pharmacia, Uppsala, Sweden) and injected into rabbits with Freund incomplete adjuvant.

PAGE and immunoblots.

Total cellular proteins and purified extracts were subjected to SDS-polyacrylamide electrophoresis (PAGE) by the method of Laemmli (1970). Following electrophoresis the gels were either stained with Coomassie blue or transferred to nitrocellulose membranes; proteins on the nitrocellulose were probed with antibody, and antigen-antibody complexes were detected with radioiodinated protein A (30 mCi/mg; Amersham Canada, Ltd., Oakville, Ontario, Canada) as previously described (Burnette, 1981; Towbin *et al.*, 1979).

Tunicamycin assay.

Sf9 cells were infected with the recombinant virus and subsequently grown in TNM-FH medium containing 10% fetal bovine serum and 10 µg of tunicamycin (Sigma, St. Louis, Mo.) per ml prepared from a 1-mg/ml stock in dimethyl sulfoxide. Proteins were collected 60 h postinfection in electrophoresis sample buffer and separated by SDS-PAGE.

Purification of OBs and virions.

OBs were released from infected Sf9 cells disrupted by treatment with 1% Triton X-100 - 0.01 M Tris hydrochloride (pH 7.0) and several strokes on a Dounce homogenizer. They were then purified on a discontinuous sucrose gradient as previously published (McCarthy *et al.*, 1974), suspended in 0.1% (wt/vol) SDS, washed three times in distilled water, and pelleted each time by centrifugation at 3000 rpm for 5 min. NOVs were purified by the method of Summers and Smith (1987). OVs were purified away from OB proteins by alkali treatment followed by separation on a linear sucrose gradient as previously reported (Bilimoria and Arif, 1979).

Fluorescence microscopy.

Sf9 insect cells were infected with wild-type AcNPV for 72 h in microchamber slides (Nunc, Naperville, Ill). The cells were fixed and incubated for 2 h with a 1:100 dilution of rabbit antiserum directed against spheroidin as previously described (Dreyfuss *et al.*, 1984). This antiserum was previously adsorbed against lysed Sf9 insect cells to minimize background fluorescence. The infected cells, along with the bound primary antibody, were then incubated with a 1:100 dilution of biotinylated donkey anti-rabbit immunoglobulin G serum (Amersham Canada Ltd., Oakville, Ontario, Canada) for 1 h. The biotinylated secondary antibodies were detected by incubating the cells with a 1:100 dilution of fluorescein-conjugated streptavidin for 30 min.

OBs were heat-fixed to glass microscope slides. Some samples were solubilized in a solution of 50 mM sodium carbonate-50 mM sodium chloride for 2 min at room temperature and washed three times with phosphate-buffered saline. Untreated and alkali treated OBs were subsequently fixed with formaldehyde and incubated with antibodies and fluorescent streptavidin as described above.

Spheroidin-antibody complexes were viewed by fluorescence microscopy on a Leitz microscope at X400 to X1000 magnifications with a PL/APO 100 PHACO 3 oil immersion objective.

RESULTS

Homology between AcNPV SLP and CbEPV spheroidin.

The CbEPV spheroidin gene was compared with GenBank sequences, and homology was observed with an ORF found immediately adjacent to the AcNPV DNA polymerase gene (Tomalski *et al.*, 1988). We sequenced this 909-nucleotide ORF and noted that a similar 927-nucleotide ORF had recently been reported by Wu and Miller (1989). The translation products of AcNPV SLP and CbEPV spheroidin genes were aligned and compared by using the Microgenie Sequence Analysis Program (Version 4.0) An overall homology of 39% at the protein level was observed. Although homology was observed throughout the two proteins, several regions of very high amino acid conservation were evident. Five conserved regions were identified and are illustrated in Fig. 1. All of these regions exhibited at least 75% homology between SLP and spheroidin, and where they differed, the general characteristics of the amino acids were retained. This was evident in region 4, which contained a string of 17 amino acids. Four nonidentical residues were apparent, but the general properties of these amino acids were conserved. The most striking example of amino acid sequence conservation was seen in region 5 which spanned 21 positions and contained only a single variation. In this case, the hydrophobic nature of the residue was maintained, ie., a methionine in SLP was replaced by a leucine in spheroidin.

The distributions of proline and cysteine residues throughout the two proteins were highly conserved. SLP contained 18 prolines, and spheroidin contained 19; 11 of these residues were located at the same positions. More remarkably, all 8 cysteines of SLP were paired to eight of the nine cysteines of spheroidin. Potential N-linked glycosylation sites also occurred at similar positions.

Finally, spheroidin was previously demonstrated to possess an N-terminal signal sequence of 20 amino acids. This hydrophobic sequence was proteolytically cleaved during protein maturation (Yuen *et al.*, 1990). SLP contained a similar amino-terminal sequence which may also interact with membranes and be removed during posttranslational processing.

Figure 1. Comparison of the predicted amino acid sequences from the AcNPV SLP gene and the CbEPV spheroidin gene. The vertical lines indicate identical amino acids, and the dots indicate amino acids with similar properties. Potential N-linked glycosylation sites are boxed with solid lines. Regions of high homology are boxed with hatched lines and are specified by number. The hydrophobic N-terminal sequence is underlined.


AcNPV MIAL-LIALFAAIHAPAVRSHGYLSVPTARQYKCFKDGNFYWPDNGDNIP 49
CbEPV MNKLILISLIASLYQVEVDAHGYMTFPIARQRRCSAAGGNWYPVGGGGIQ 50
1
AcNPV DAACRNAYKSVYYKYRALDLESGA--AAS-TAQYMFQQYMEYAAVAGPNY 96
CbEPV DPMCRAAYQNVF-N-KVLNSNGGDVIDASEAANYMYTQDNEYAALAGPDY 98
2
3
AcNPV DDFDLIKQRVVPHTLCGAGSND-RNSVFGDKSGMDEPFNNWRPNTLYLNR 145
CbEPV TNICHIQQRVVPSYLCAAGASDWSIRPFGDKSGMDLP-GSWTPTIIQLSD 147
4
AcNPV YQPV-YQMNVHFCPTAIHEPSYFEVFITKSNWDR-RNPITWNELEYIGGN 193
CbEPV NQQSNVVMELEFCPTAVHDPSYYEVYITNPSFNVYTDNVVWANLDLIYNN 197
5
AcNPV DSNLIPNPGDSLCD-NSLVYSIPVVIPYRSNQFVMYVRWQRIDPVGEGFY 242
CBEPV TVTLRPKLPESTCAANSMVYRFEVSIPVRPSQFVLYVRWQRIDPVGEGFY 247
ACNPV NCADLVF---ET-----L-D-----D-E--C-RY-AQMAKVVRSQLQKHK 273
CBEPV NCVDMKFKYSEGPDEEDIIEPEYEVDNEAECFAYRTNSGNVNVNPLQENK 297
ACNPV -LD-A-R-I-D-HNDEESCWRAR--KSNYSSFFN-P-GF 302
CBEPV YMAYANKAIRNINTHSNGCSRNRNNKNNYNKYYSKTYNYNQNRK 341

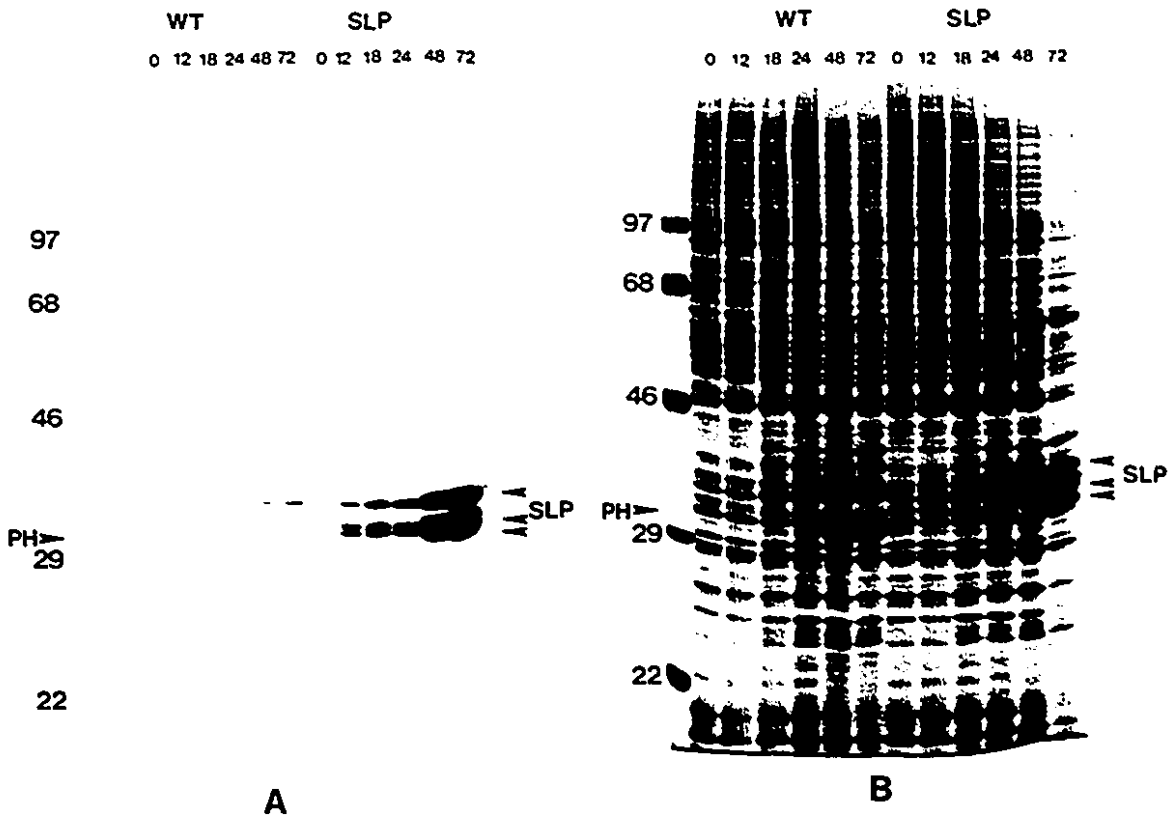
Expression and detection of SLP in insect cells infected with either wild-type or recombinant baculovirus.

Sf9 cells were infected with either wild-type AcNPV or recombinant virus which contained the SLP gene under control of the polyhedrin promoter. Northern (RNA) blot analysis of purified RNA with probes derived from the SLP gene indicated that mRNA was produced between 24 and 48 hours postinfection. This observation was consistent for a gene expressed very late in infection (data not shown). Infected cells were also collected at various times postinfection, and proteins were separated by SDS-PAGE and were either stained with Coomassie blue or transferred to nitrocellulose membranes for immunodetection. The results of this time course study are presented in Fig. 2. In the wild-type AcNPV infection, SLP could not be visualized through Coomassie blue staining. However, a protein band migrating with a molecular mass of 37 kDa could be detected at 18 h postinfection with a polyclonal antiserum directed against spheroidin. This protein continued to be synthesized from 18 to 72 h postinfection and appeared to be produced in relatively low quantities compared with those in cells infected with the recombinant AcNPV. Cells infected with the SLP recombinant virus synthesized three major species which were evident by both staining with Coomassie blue and immunoblot detection. These protein bands migrated with molecular masses of 34, 35, and 37 kDa. We suspected that the smaller protein species might represent underglycosylated precursors of the 37-kDa protein. In addition to virus-derived proteins, the antibody directed against spheroidin reacted weakly with a 53-kDa protein which was present in uninfected cells. This represented a minor protein species which became less abundant late in wild-type baculovirus infection of Sf9 cells.

Tunicamycin-inhibited glycosylation of SLP.

To determine whether SLP was a glycoprotein, we performed experiments with tunicamycin (an inhibitor of N-linked glycosylation). Sf9 cells were infected (in the presence and absence of tunicamycin) with recombinant AcNPV containing the SLP gene. Infected cells were harvested at 60 h postinfection, and cellular proteins were analyzed by SDS-PAGE. The results of this

Figure 2. Immunoblot (A) and Coomassie blue stained gel (B) of total proteins expressed in Sf9 cells infected with either wild-type (WT) or SLP recombinant virus. Total proteins from 10^5 cells were collected at 0, 12, 18, 24, 48, and 72 h postinfection and separated by SDS-PAGE. Panel A is an autoradiogram of an immunoblot prepared from a gel which was a duplicate of the Coomassie blue-stained gel shown in panel B. The immunoblot was probed with polyclonal antiserum directed against CbEPV spheroidin. Bound antibody was detected with protein A conjugated to [^{125}I]. The electrophoretic migrations of the various forms of SLP are indicated with arrows. PH refers to the electrophoretic migration of polyhedrin. Positions of protein mass standards (in kilodaltons) are indicated by numbers.


WT
SLP
0 12 18 24 48 72 0 12 18 24 48 72
97
68
46
PH
29
22
SLP
A
WT
SLP
0 12 18 24 48 72 0 12 18 24 48 72
97
68
46
PH
29
22
SLP
B

experiment are shown in Fig. 3. The addition of tunicamycin to cells infected with the SLP recombinant completely abolished the synthesis of both the 37- and 35-kDa polypeptides. The 34-kDa band remained apparent, and a 33-kDa protein band was also observed. These protein species may reflect other posttranslational modifications and could represent the nonglycosylated SLP polypeptide with and without its signal peptide.

Analysis of virus and OB proteins by using immunoblot detection.

Purified OBs, NOVs, and OVs were solubilized and subjected to SDS-PAGE. The resolved proteins were either stained with Coomassie blue (Fig. 4A) or transferred to nitrocellulose and probed with antibodies directed against the spheroidin protein (Fig. 4B). Although the OV was not totally pure and contained some polyhedrin protein, it failed to react with the polyclonal antiserum used in these experiments. NOVs also failed to react with the spheroidin antibodies, whereas the OBs clearly contained the 37-kDa protein (SLP). The SLP was not a major component of OBs since it was not detected by Coomassie blue staining and was visible only through immunodetection techniques. A minor band migrating slightly faster than the 37-kDa protein was also detected and may represent a different level of N-glycosylation. Antiserum directed against spheroidin recognized two forms of protein in OBs derived from cells infected with CbEPV. Spheroidin protein has been shown to be a 50-kDa monomer which associates via disulfide bonds to form a 100-kDa dimer (Yuen *et al.*, 1990). We also constructed an AcNPV recombinant virus which contained the spheroidin gene of CbEPV. This recombinant virus produced a 50-kDa polypeptide as well as a 100-kDa form when the gene products were subjected to SDS-PAGE and analyzed by immunodetection techniques (data not shown). We conclude from these studies that SLP was associated with the OBs of wild-type AcNPV and had antigenic determinants in common with the spheroidin protein of EPVs.

Figure 3. Analysis of N-linked glycosylation of SLP in Sf9 cells infected with recombinant virus. The figure shows an autoradiogram of an immunoblot (A) and a Coomassie blue-stained gel (B) of total proteins produced by recombinant virus infection in the absence (-) and presence (TM) of tunicamycin. Proteins were collected at 60 h postinfection. The immunoblot was probed with antiserum directed against CbEPV spheroidin and detected with [^{125}I]-protein A. Numbers indicate migration of protein mass standards in kilodaltons.

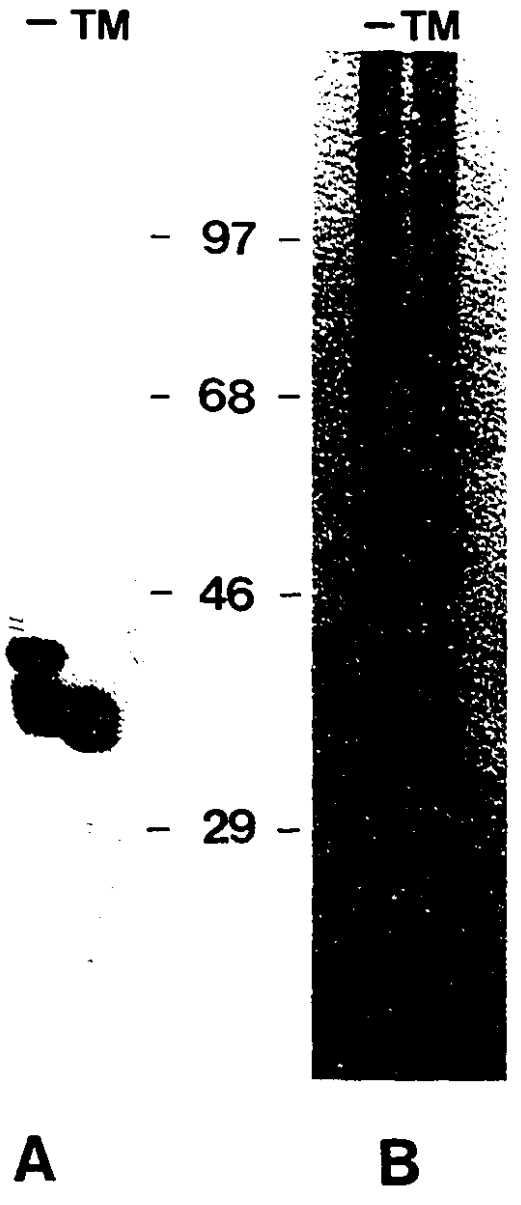
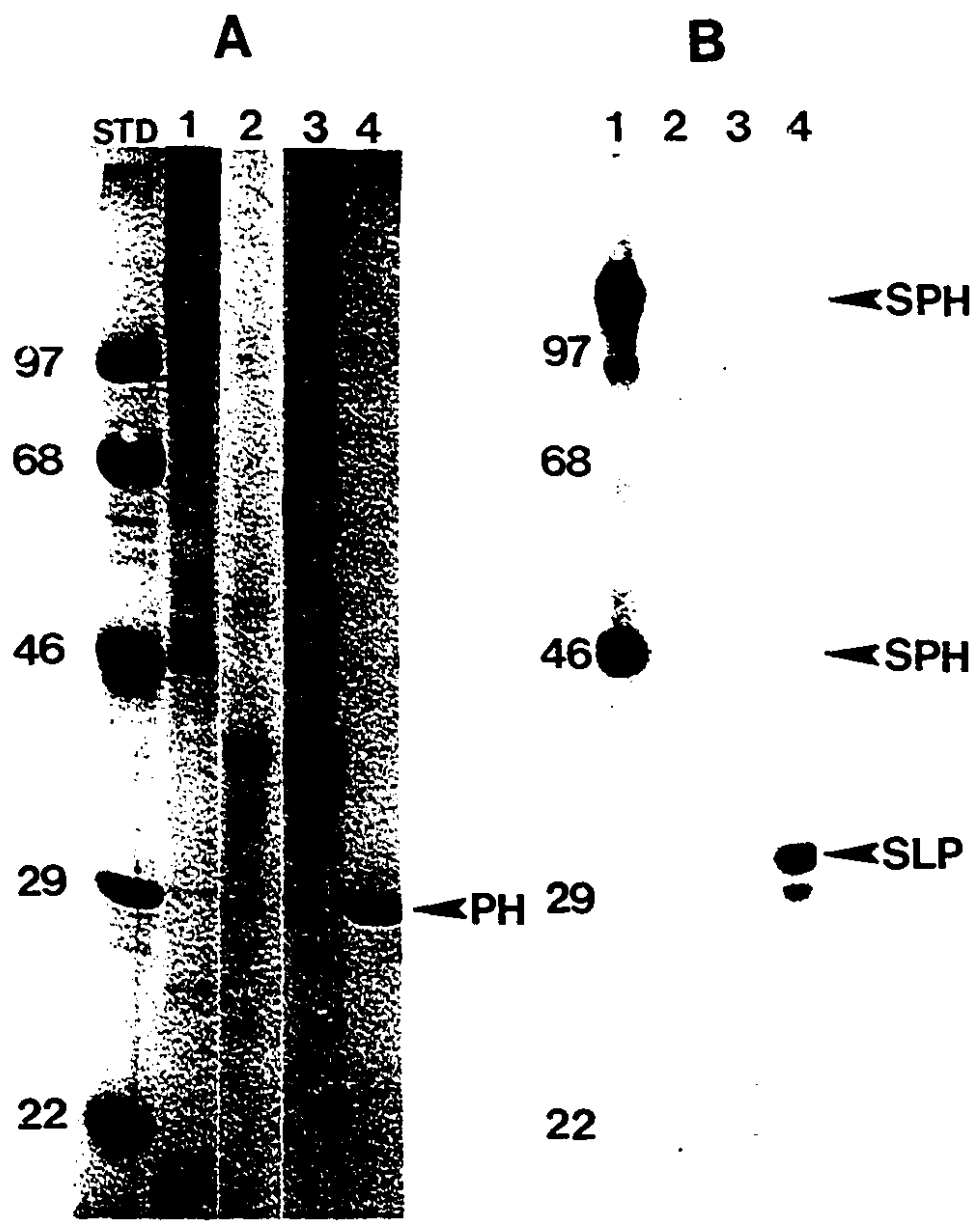

– TM
– TM
97
68
46
29
A
B

Figure 4. Immunodetection of SLP associated with AcNPV occlusion bodies. Virus and OB proteins were separated by SDS-PAGE and detected by Coomassie blue stain (A) or immune autoradiography (B). Lanes: 1, purified CbEPV OB proteins; 2, AcNPV OV proteins; 3, AcNPV NOV proteins; 4, purified AcNPV OB proteins. The immunoblot was probed with antiserum directed against spheroidin and immune complexes were detected with ^{125}I-protein A. SLP, SPH monomer and dimer, and polyhedrin (PH) proteins are indicated by arrows. Numbers indicate size (in kilodaltons) of protein mass standards (lane STD).


A
STD 1 2 3 4
97
68
46
29
22
PH
B
1 2 3 4
97
68
46
29
22
SPH
SPH
SLP

Immunofluorescence studies of AcNPV-infected cells and OBs.

Sf9 cells were infected with wild-type AcNPV and subsequently fixed and incubated with antispheroidin antibodies. Immune complexes were detected with biotinylated antibodies and fluorescent streptavidin. Uninfected Sf9 cells exhibited a small amount of background fluorescence (Fig. 5A), while cell membranes and OBs were clearly labelled at 72 h postinfection (Fig. 5C). Antibodies were localized to the cytoplasm early (12 to 24 h) in infection. However, fluorescent staining was minimal between 0 and 12 h infection and subsequently rose to maximum intensity between 60 and 72 h postinfection. Again, these results indicated that SLP was produced very late in infection. Sf9 cells infected with the SLP recombinant virus produced no OBs, and intense fluorescence was distributed throughout the cytoplasm, plasma membranes, and nuclear membranes (data not shown).

Purified OBs were fixed to glass slides and incubated with antibodies directed against spheroidin. Exterior regions of the OBs were fluorescently labelled in Fig. 6A. Other OBs were subsequently dissolved and permeabilized with sodium carbonate buffer. These solubilized OBs were washed in phosphate-buffered saline and stained with spheroidin antibodies. Alkali treatment converted the normally opaque OBs (Fig. 6B) to transparent sacs which probably represented the PE (Fig. 6D). These sacks were intensely labelled by the spheroidin antibodies (Fig. 6C).

The fluorescent-antibody studies appear to indicate that SLP is synthesized in the cytoplasm and may be transported to the nuclear membrane to become associated with the OBs in the nucleus of infected cells. More specifically, SLP is probably associated with the PE. Further electronmicroscopy and immunogold labeling studies may substantiate this observation.

DISCUSSION

In this communication, we report the identification and characterization of a 37-kDa glycoprotein (SLP) which appears to be associated with the AcNPV OB proteins. We sequenced an ORF immediately adjacent to the AcNPV DNA polymerase gene which encodes a polypeptide

Figure 5. Immunofluorescence localization of SLP in Sf9 cells infected with wild-type AcNPV. The figure shows immunofluorescence (A) and phase-contrast microscopy (B) of uninfected cells and immunofluorescence (C) and phase-contrast microscopy (D) of infected cells at 72 h postinfection. Insect cells were fixed and incubated with antibodies directed against spheroidin as described in Materials and Methods. Magnification, X380

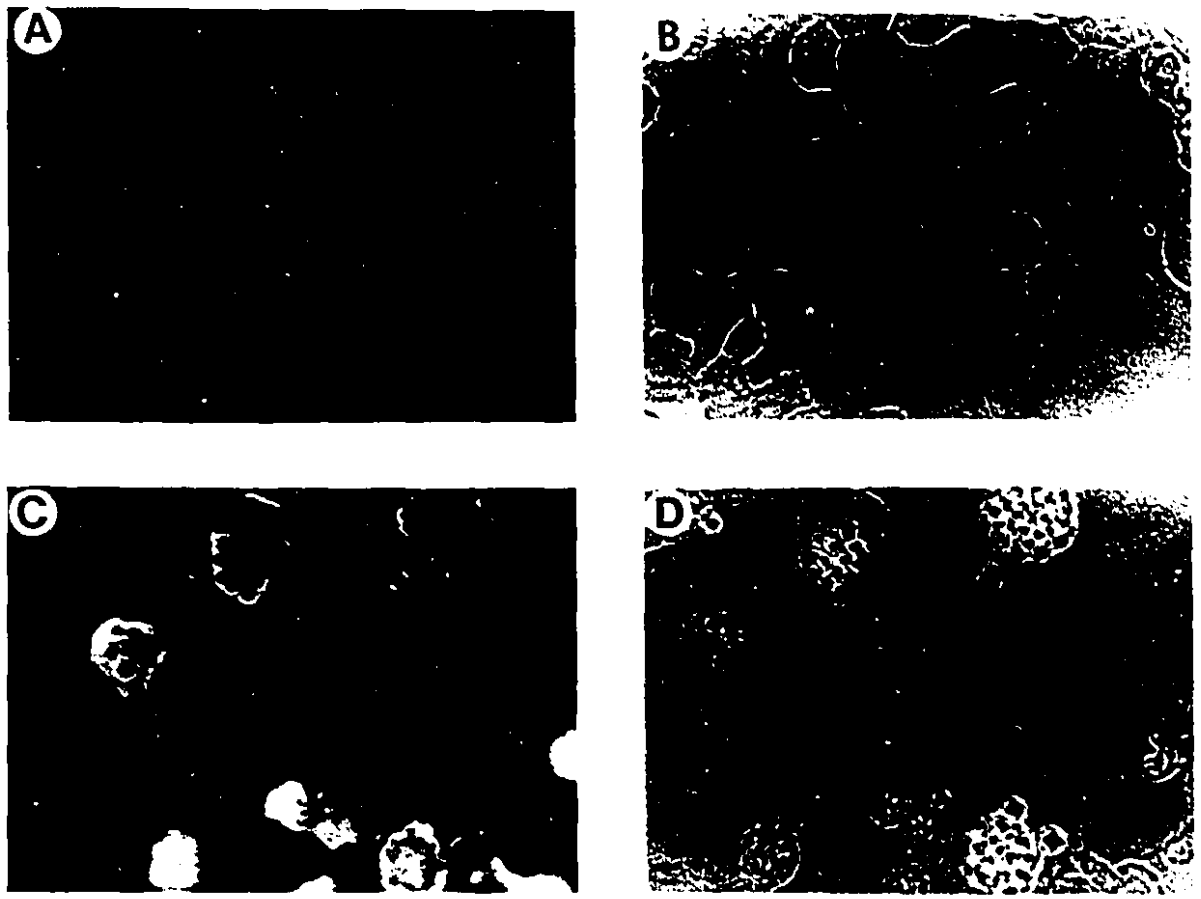
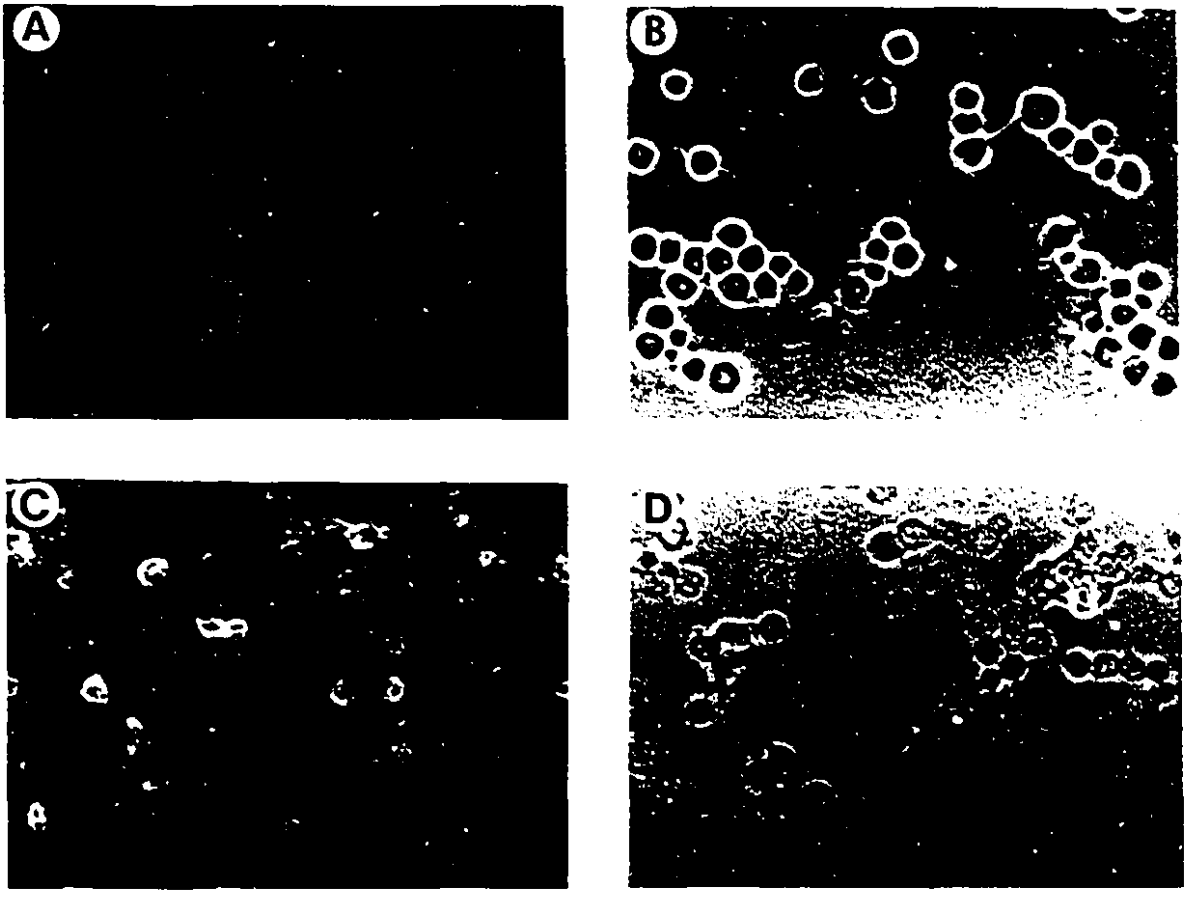

A
B
C
D

Figure 6. Immunofluorescence localization of SLP in purified AcNPV OBs. Immunofluorescence detection (A) and phase-contrast microscopy (B) of purified OBs and immunofluorescence analysis (C) and phase-contrast microscopy (D) of purified OBs treated with sodium carbonate. OBs were heat-fixed and stained as described in Methods and Materials. Magnification, X950.


A
B
C
D

that exhibits 39% amino acid homology to CbEPV spheroidin, the major EPV OB protein. Polyclonal antiserum produced against purified spheroidin reacted with a 37-kDa protein encoded by this ORF, and we demonstrated that this protein was expressed late in infection and localized to the envelope surrounding the OB matrix.

OBs are structures produced by several groups of viruses, including the NPVs, granulosis viruses, cytoplasmic polyhedrosis viruses, and EPVs. Their function is to protect virions embedded within them from the outside environment. Each baculovirus OB is composed of a proteinaceous matrix (Rohrmann, 1986) surrounded by an envelope or calyx (Gombart *et al.*, 1989a and b; Whitt and Manning, 1988) which is composed primarily of carbohydrate (Minion *et al.*, 1979). The major component of AcNPV OBs is polyhedrin (p29), a matrix protein which is hyperexpressed late in the course of infection, as reviewed by Rohrmann (1986).

A second protein (p10) synthesized very late in infection and at high levels seems to be involved in the morphogenesis of the PE (Williams *et al.*, 1989). Fibrillar structures containing p10 associate with electron-dense spacers to form what is thought to be nascent PE (Vlak *et al.*, 1988).

In addition to p10, a phosphoprotein (pp34) is required for the formation of PEs (Zuidema *et al.*, 1989). Like polyhedrin and p10, this protein is also a late gene product. It seems to be associated with the PE via thiol linkages (Whitt and Manning, 1988). Immunogold detection techniques were used to localize p32, an OpNPV protein homologous to pp34 from AcNPV (Gombart *et al.*, 1989a and b), to the envelope surrounding OpNPV polyhedra (Russell and Rohrmann, 1990a). The integrity of the PE and the presence of p32 were maintained even after dissolution of the polyhedra in dilute alkali. As with pp34, dissociation of p32 from the PE required the presence of a reducing agent (Gombart *et al.*, 1989b).

Although SLP and pp34 share several common characteristics, such as molecular mass, timing of expression, and association with the PE, they do not appear to be the same protein. They exhibit different immunoreactive properties and possess different amino acid sequences encoded by separate regions of the baculovirus genome. The predicted amino acid sequence of

p32 shared 58% homology with a translation product (pp34) encoded by an analogous region of the AcNPV genome (Gombart *et al.*, 1989b). These two proteins (pp34 and p32) displayed immune cross-reactivity. In contrast, the amino acid sequence of spheroidin bears no significant homology to that of pp34 or p32. A radioactive probe constructed from the EPV speroidin or SLP gene hybridized to a distinct region of the AcNPV genome. In addition, antibodies directed against spheroidin reacted with the gene product specified by SLP recombinant baculovirus as well as wild-type AcNPV. Therefore, the nucleotide and protein sequences of pp34 and p32 appear to be quite different when compared with SLP (Gombart *et al.*, 1989a and b; Oellig *et al.*, 1987; Wu and Miller, 1989).

The spheroidin antibodies also cross-reacted weakly with a cellular protein. This reactivity disappeared later in infection owing to shut-off of host protein synthesis. Reaction of virus-directed antibodies with cellular proteins is not uncommon (Srinivasappa *et al.*, 1986) and molecular mimicry has previously been observed with a p10-directed monoclonal antibody which cross-reacts with cytoskeletal elements (Quant-Russell *et al.*, 1987). For this reason, antiserum directed against spheroidin was preadsorbed to lysed insect cells to minimize background fluorescence during microscopy studies.

The structure of EPV OBs differs significantly from that of NPV OBs with respect to the major protein component. This is not unexpected since these viruses are not closely related; NPVs replicate in the nuclei of infected cells and produce nuclear OBs, whereas EPVs replicate in the cytoplasm and yield cytoplasmic OBs. The major protein of CbEPV OBs is spheroidin. It is a 50-kDa polypeptide, that is rich in cysteines and capable of forming dimers (Yuen *et al.*, 1990). Although the amino acid sequence of spheroidin does not exhibit significant homology to polyhedrin, it is quite similar to the less abundant SLP found in baculovirus OBs. This amino acid conservation is especially evident in five distinct regions of the two proteins; these may represent functionally or structurally relevant motifs. Conservation of prolines, cysteines, and N-linked glycosylation sites indicates structural homology between the two proteins.

The similarities between the amino acid sequences of SLP and spheroidin suggest that the two proteins were derived from a common ancestor. This hypothesis may be supported by the identification of similar proteins in other OVs in the future. Two distinct possibilities come to mind. The genes encoding these proteins may have been acquired from the host cell genome or they may be introduced by transposition or recombination from other viral genomes. It would be interesting to identify the gene encoding the cellular polypeptide which cross-reacted with the antibodies directed against spheroidin and determine whether it bears significant homology to SLP or spheroidin. On the other hand, the SLP gene could be obtained from other viruses. Acquisition of the SLP gene by AcNPV from a donor EPV could be supported by the codon usage bias observed in the translated product. Codon usage in SLP favors A and T residues at the wobble position of most amino acids (Wu and Miller, 1989). This resembles codon bias in poxviruses whose genomes are A+T rich as opposed to the G+C rich genes of NPVs.

In future studies, our observation that SLP is a protein associated with the PE will be corroborated by immunogold electron microscopy and biochemical separation of the PE from the matrix of the OBs. Finally, the significance of SLP in OB formation may be determined by the deletion of the gene encoding SLP and analysis of this mutated phenotype during baculovirus replication.

CHAPTER 3

Characterization of Baculovirus and Entomopoxvirus Spindle Proteins

PREFACE

The previous chapter described the identification of a 37-kDa glycoprotein (SLP or gp37) expressed late in baculovirus infection that shares significant homology with an abundant entomopoxvirus occlusion body protein. Because of its glycoprotein nature and copurification with baculovirus occlusion bodies, it was suggested that it might be a component of the polyhedral envelope. Immunofluorescence studies of purified baculovirus occlusion bodies appeared to support this hypothesis. This chapter describes further characterization of gp37 expression in infected cells with respect to its synthesis and cellular localization. Immunoelectron microscopy studies were performed to determine the precise localizations of gp37 in infected cells and the entomopoxvirus homologue in purified spheroids. A mutant baculovirus deficient in gp37 synthesis was generated in order to determine its role in baculovirus infection. Recombinant baculoviruses overexpressing gp37 and the 50-kDa entomopoxvirus protein were also generated in order to produce sufficient amounts of the proteins to carry out biochemical analysis and functional studies.

ABSTRACT

Peptide-specific antibodies were generated against amino acid sequences corresponding to *Autographa californica* nuclear polyhedrosis (AcNPV) gp37 and the related 50 kDa protein present in *Choristoneura biennis* entomopoxvirus (CbEPV) occlusion bodies. The antibodies were used in western blot and immunoprecipitation analysis of nuclear and cytoplasmic fractions from baculovirus-infected cells. The majority of gp37 was associated with the nuclear fraction. Immunogold labelling in conjunction with electron microscopy revealed that gp37 was a component of spindle-shaped structures often associated with the nuclear membrane in AcNPV-infected cells. Analysis of purified CbEPV occlusion bodies demonstrated staining of similar structures embedded within the occlusion bodies. AcNPV gp37 and the CbEPV 50-kDa protein were expressed in large amounts using recombinant baculoviruses. Spindle bodies were produced in recombinant-virus infections and could be purified through sucrose gradients. Coomassie blue-stained gel analysis demonstrated that gp37 and the 50-kDa protein were the major component of spindle bodies. In addition, immunoblot analysis of recombinant virus-infected cells treated with tunicamycin revealed that the 50-kDa protein was glycosylated. A proteolytic activity was associated with purified spindle bodies but not with polyhedra obtained from a spindle protein-negative baculovirus, when incubated with dye-linked peptides in alkaline conditions.

INTRODUCTION

Insect virus infections are often characterized by the production of intracellular crystalline structures late in infection. Of these, the most well known are the baculovirus occlusion bodies (OBs) or polyhedra. These large proteinaceous structures are required for horizontal transmission of the virus in natural infections (Blissard and Rohrmann, 1990). Virions become embedded within the OBs and are thereby protected from environmental factors such as UV light and desiccation. The virions are released when OBs are ingested and dissolved in the alkali conditions of the insect midgut. The nuclear polyhedrosis viruses (NPVs), of the Baculoviridae

family, are double-stranded DNA viruses that produce nuclear OBs composed primarily of a 31-kDa protein called polyhedrin (Rohrmann, 1986). This protein is expressed at very high levels late in infection and is not required for viral replication in cell culture. Many of the widely used baculovirus expression vectors rely on replacement of polyhedrin coding sequences with those of the gene of interest (O'Reilly *et al.*, 1992; King and Possee, 1992; Richardson, in press). In addition to the baculoviruses, two cytoplasmic insect viruses produce OBs that are morphologically and functionally similar to NPV polyhedra. The first group is the double-stranded RNA cytoplasmic polyhedrosis viruses, classified within the *Reoviridae* family. They produce OBs composed of a single protein, the 25- to 31-kDa C-polyhedrin (Payne and Mertens, 1983; Belloncik, 1989; Galinski *et al.*, 1994). This protein does not share significant homology with NPV polyhedrin (Rohrmann, 1986). The second group is the entomopoxviruses (EPVs) whose OBs are composed of a 115-kDa protein called spheroidin which again, shows no homology with polyhedrin (Bilimoria and Arif, 1979; reviewed in Arif and Kurstak, 1991; and Goodwin *et al.*, 1991). EPV OBs are also known as spheroids.

A second type of paracrystalline proteinaceous structure has also been described in microscopy studies of some EPV- (Bergoin *et al.*, 1970; Bird, 1974; Milner and Beaton, 1979; Dall *et al.*, 1993) and baculovirus-infected cells (Huger and Kreig, 1968; Adams and Wilcox, 1968; Gross *et al.*, 1993a). Because of their morphological characteristics (sharply pointed ends) they have been referred to as spindle-shaped bodies or spindles. In contrast to OBs, spindle bodies appear to be devoid of virus particles. Their size is highly variable (even within the same species), usually ranging from 0.5 to 12 µm, but spindle bodies up to 25 µm have been described (Arif, 1984). In some EPV infections, they have been subdivided into macro- and micro-spindles, the latter being occluded within spheroids much like virions. Occasionally only spindle bodies become occluded. Macrospindles are found in the cytoplasm and can approach sizes similar to those of spheroids (Goodwin *et al.*, 1991). Spindle bodies have been observed in most EPV infections with the exception of *Amsacta moorei* (Am) EPV and most Diptera-infecting EPVs (Goodwin *et al.*, 1991). Spindle-shaped bodies have also been described in the cytoplasm of

Choristoneura murinana NPV infected cells (Huger and Krieg, 1968). The function of spindle bodies in insect virus infections has not yet been determined.

A previous report described a 50-kDa polypeptide as the major component of *Choristoneura biennis* (Cb)EPV OBs (Yuen *et al.*,1990). The open reading frame (ORF) encoding this protein specified a polypeptide of 38.5 kDa that was erroneously termed spheroidin. The difference in molecular weight (MW) between the predicted protein and its counterpart in OBs was attributed to post-translational modifications, most likely glycosylation. The gene encoding the major OB protein of AmEPV was subsequently found to specify a 115-kDa polypeptide unrelated to the 50-kDa CbEPV protein (Hall and Moyer, 1991; Banville *et al.*, 1992). More recently, nucleotide sequences with homology to the AmEPV spheroidin gene were identified in the CbEPV genome (Hall and Moyer, 1993), suggesting that the 50-kDa CbEPV protein had been previously misidentified as spheroidin. We have previously reported that a 37-kDa glycoprotein (gp37) specified by *Autographa californica* (Ac) NPV shares significant homology with the 50-kDa CbEPV protein (Vialard *et al.*, 1990b). The baculovirus protein was expressed at relatively low levels late in infection and copurified with AcNPV OBs. Homologous genes have also been found in *Heliothis armigera* (Ha) EPV (Dall *et al.*, 1993) and *Orgyia pseudotsugata* (Op) NPV (Gross *et al.*, 1993a). OpNPV gp37 was localized by immunogold labeling to cytoplasmic inclusion bodies and did not appear to be associated with polyhedra (Gross *et al.*, 1993a). The 50-kDa protein encoded by HaEPV was shown by immunofluorescence to be present in cytoplasmic spindle bodies. It represents the most abundant HaEPV infected-cell protein, is not glycosylated, and was suggested to be phosphorylated or myristylated (Dall *et al.*, 1993).

In this report, we describe further biochemical characterization and precise localization of gp37 in AcNPV infected cells, and the 50-kDa protein in CbEPV OBs. We have generated an AcNPV in which the gp37 ORF has been interrupted in order to determine its role in the viral life cycle. Peptide antibodies were raised against the 50-kDa protein of CbEPV and gp37 of AcNPV. Subsequent immunoprecipitation and immunoblot analysis demonstrated that gp37 is present in the cytoplasm, but is primarily associated with the nuclear membranes of AcNPV-infected cells.

Immunoelectron microscopy studies demonstrated that AcNPV gp37 and the CbEPV 50-kDa protein are asscciated with spindle bodies present in AcNPV infected cells or occluded within the CbEPV OBs, respectively. We have also expressed both AcNPV gp37 and the CbEPV 50-kDa protein in insect cells by infection with recombinant baculoviruses and used the purified spindle bodies in alkaline solubilization and protease assays. We have also cloned and sequenced the gene coding for the 50-kDa protein of *Choristoneura fumiferana* (Cf) EPV and compared its deduced amino acid sequence to other EPV and baculovirus homologues.

MATERIALS AND METHODS

Virus, insect cells, and larvae.

CbEPV and CfEPV OBs were supplied by Basil Arif (Forest Pest Managment Institute, Forestry Canada, Sault Ste Marie, Ontario, Canada). *Spodoptera frugiperda* (Sf9) insect cells and AcNPV were obtained from Max Summers (Texas A&M University, College Station). Baculovirus and cells were cultured in Grace's medium (GIBCO/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS).

Construction and isolation of recombinant viruses.

An AcNPV recombinant virus, AcSpinNPV, containing the AcNPV gp37 open reading frame (ORF) under the control of the polyhedrin promoter was generated by homologous recombination and is described in Vialard *et al.* (1990b).

A recombinant baculovirus containing the CbEPV 50-kDa protein (spindle) ORF under the control of the polyhedrin promoter (CbSpinNPV) was constructed as follows. A DNA fragment corresponding to the CbEPV spindle protein ORF was synthesized by PCR from a 2.3-kb *EcoR*I-*Xba*I genomic fragment previously described as encompassing the CbEPV spheroidin gene (Yuen *et al.*, 1990). Two oligonucleotide primers corresponding to the 5' (CGCTAGCAATATGAATAAATTAATAC) and 3' (CGCTAGCTTATTTTCTATTTTG) ends of the CbEPV spindle ORF were used. The underlined nucleotides indicate an *Nhe*I recognition site.

The resulting PCR fragment was inserted into the baculovirus transfer vector pJVETL (Richardson *et al.*, 1992), and the DNA sequence of the PCR fragment was confirmed by the dideoxy-chain termination sequencing method. This plasmid was transfected into Sf9 cells along with wild-type AcNPV DNA by lipofection (GIBCO/BRL, Bethesda, MD). Recombinant virus was plaque purified using the ß-galactosidase screening method as previously described (Vialard *et al.*, 1990a).

An AcNPV in which the gp37 ORF has been interrupted (AcSpin$^-$NPV) was generated by homologous recombination as follows. A *Bam*H I fragment containing the bacterial *lacZ* coding region and SV40 polyadenylation signal (Vialard *et al.*, 1990a) was introduced into the *Bam*H I site of the pSP72 plasmid (Promega, Madison, Wisconsin). The *lacZ* coding region was then placed under the control of the baculovirus IE1 promoter (Guarino and Summers, 1987) by introduction of a PCR fragment corresponding to the IE1 promoter sequences (Richardson *et al.*, 1992) at the *Sma*I site upstream of the *lacZ* ORF. Following restriction with *Hin*dIII and *Bgl*II, the IE1/*lacZ* fragment was purified from an agarose gel following electrophoresis and made blunt by Klenow treatment. It was then cloned into the *Not*I site of the AcNPV *Eco*RI-F genomic fragment (Ayres *et al.*, 1994; Kool and Vlak, 1993) that had been previously cloned into the *Eco*RI site of the pGEM1 plasmid (Promega, Madison, Wisconsin). Co-transfection of this plasmid and wild-type AcNPV DNA into Sf9 cells resulted in a gp37 mutant virus which was purified as described above. Interruption of the gp37 ORF was confirmed by Southern blot analysis and PCR techniques.

Southern blot analysis.

Sf9 cells were infected with AcSpin$^-$NPV at a moi of 5 PFU/cell, medium was collected 4 days postinfection (pi) and centrifuged at low speed to remove cellular debris. Virions were then pelleted from the supernatant by centrifugation for 30 min at 100000xg. The pellet was resuspended in 10 mM Tris-HCl (pH7.5), 10 mM EDTA, 20 mM KCl, 40 µg/ml proteinase K and 1% sarkosyl, and incubated 4h at 50°C. Viral DNA was purified from the resulting supernatant by phenol:chloroform extraction and ethanol precipitation. It was digested with restriction enzymes, electrophoresed on a 0.7 % agarose gel, and transferred onto nitrocellulose by Southern blotting.

The membrane was then hybridized with an AcNPV gp37 probe (PCR fragment described above) by standard procedures (Sambrook *et al.*, 1989).

Purification of occlusion and spindles bodies.

AcSpin⁻NPV OBs, and AcSpinNPV and CbSpinNPV spindle bodies were released from infected Sf9 cells by treatment with 1% Triton X-100/0.2% SDS in 10 mM Tris-HCl (pH 7.4) and several strokes of a Dounce homogenizer. CbEPV OBs were extracted from infected Cf larvae as previously described (Billimoria and Arif, 1979). Spindle body and OB suspensions were dispersed by sonication and then purified through two successive discontinuous sucrose gradients as previously described (McCarthy *et al.*, 1979). OBs and spindle bodies were collected from the gradients, resuspended in sterile water, and pelleted each time by centrifugation at 13,000xg for 10 min. Sodium azide (0.02%) was added to the final supension.

Antisera and immunoblots.

Polyclonal rabbit antiserum raised against CbEPV OB proteins solubilized by alkali treatment (α-SOB) was previously described (Vialard *et al.*, 1990b). Anti-peptide antibodies directed against amino acids 122-136 (α-spCb2) and 327-341 (α-spCb1) of the CbEPV spindle polypeptide, and 53-67 (α-spAc2) and 288-302 (α-spAc1) of the AcNPV spindle polypeptide were prepared by standard methods (Richardson *et al.*, 1985). For subsequent immunoblot analysis, infected cell, purified spindle, and occlusion body proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, and probed with antibodies (Abs) by standard procedures (Harlow and Lane, 1988). Antibody-antigen complexes were detected by addition of alkaline phosphatase-conjugated donkey anti-rabbit immunoglobulin G Ab (Jackson Immunoresearch Laboratories) in the presence of nitro-blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and phenazine methosulfate (Sigma). Alternatively, antibody-antigen complexes were detected with radioiodinated protein A (30

mCi/mg; Amersham Canada, Oakville, Ontario) and exposure to X-ray film (X-OMAT AR; Kodak, Rochester, NY).

Metabolic protein labelling, cell fractionation, and immunoprecipitations.

Sf9 cells infected with AcNPV at a moi of 5 PFU/cell were incubated for 15 min in methionine-free medium at various times pi. Tran^{35}S-label (ICN Biomedicals, Costa Mesa, CA) was then added at 150 µCi/ml for 30 min, after which the labelling medium was removed and the cells were washed with PBS. For pulse/chase studies, cells were labelled at 48 h pi as described above. The labelling medium was replaced with Grace's medium and the cells were collected after incubation for various lengths of time (t_0 corresponding to addition of Tran^{35}S-label). They were then resuspended in extraction buffer (10 mM Tris-HCl [pH 6.5], 140 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 1 mM PMSF and 1 µg/ml aprotinin) at $7X10^3$ cells/ml and incubated 10 min on ice. Cytoplasmic protein fractions were obtained by collecting the supernatant after a 5 min centrifugation at 13000xg. Nuclear protein fractions were acquired by lysis of the pelleted nuclei in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 3 mM $MgCl_2$, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 1 mM PMSF and 1µg/ml aprotinin); NaOH 0.1N was added to both fractions.

Immunoprecipitations were carried out on 100 µl of cytoplasmic or nuclear protein fractions in 1 ml of RIPA buffer as follows. Immunoprecipitations were pre-cleared by incubation with 10 µl of a 1:1 slurry of protein G-sepharose in RIPA buffer followed by a brief centrifugation. The resulting supernatant was then incubated with 2 µl of α-spAc1 for 2 h at room temperature and then 10 µl of protein G sepharose slurry was added. After one hour of incubation, the protein G-sepharose beads were pelleted by centrifugation, washed three times with 1 ml RIPA buffer, and finally resuspended in 2x sample buffer (Laemmli, 1970) prior to electrophoresis through SDS-polyacrylamide gels.

Immunoelectron microscopy.

Purified OBs and infected Sf9 cells (collected at 60h pi) were fixed in 4% paraformaldehyde; 0.5% glutaraldehyde in a phosphate buffer, infiltrated with LR white, polymerized, cut into thin sections, and placed onto nickel grids. Ab staining was performed as described elsewhere (Vialard and Richardson, 1993).

CfEPV spindle protein ORF amplification, cloning, and sequencing.

A DNA fragment corresponding to the CfEPV spindle protein ORF was synthesized by PCR from CfEPV genomic DNA prepared as previously described (Lytvyn *et al.*, 1992). Two oligonucleotide primers corresponding to 5' (AAAGCTTTAAATATCAATTGGTTAAATTCC) and 3' (GGAATTCTACCAATATTTTACTACAACTC) non-coding regions of the CbEPV spindle protein ORF were used. The underlined nucleotides correspond to *Hind* III and *Eco*R V recognition sites in the 5' and 3' primers, respectively. The resulting PCR fragment was inserted into the pUC19 plasmid and the DNA sequence of the PCR fragment was confirmed by the dideoxy-chain termination method on three different clones.

Tunicamycin treatment.

Sf9 cells were infected with recombinant baculovirus at a moi of 5 PFU/cell and10 µg/ml of tunicamycin (Sigma, St Louis, Mo, USA) was added 6 h pi, cells were collected at 48 h pi, resuspended in SDS sample buffer (Laemmli, 1974), and proteins were resolved through SDS-PAGE.

Protease assay.

Protease assays were performed on approximately 5 µg of spindle, spheroidin, or polyhedrin protein as estimated on Coomassie blue-stained polyacrylamide-SDS gel. The spindle bodies or OBs were first solubilized in 170 mM Na_2CO_3 (pH 11.3) for 30 min at room temperature. Then 3 µl of PepTagC1 (Dye-PLSRTLSVAAK) or PepTagA1 (Dye-LRRASLG) (Promega,

Madison, Wisconsin) were added and the samples were diluted with water to a final volume of 15 µl. Assays were carried out overnight at 30°C, followed by addition of 5 µl of 50 mM Tris-HCl (pH 6.8) and 0.8 µl glycerol. The peptides were then subjected to electrophoresis on a 0.8% agarose gel (in 50 mM Tris-HCl [pH 8.0]).

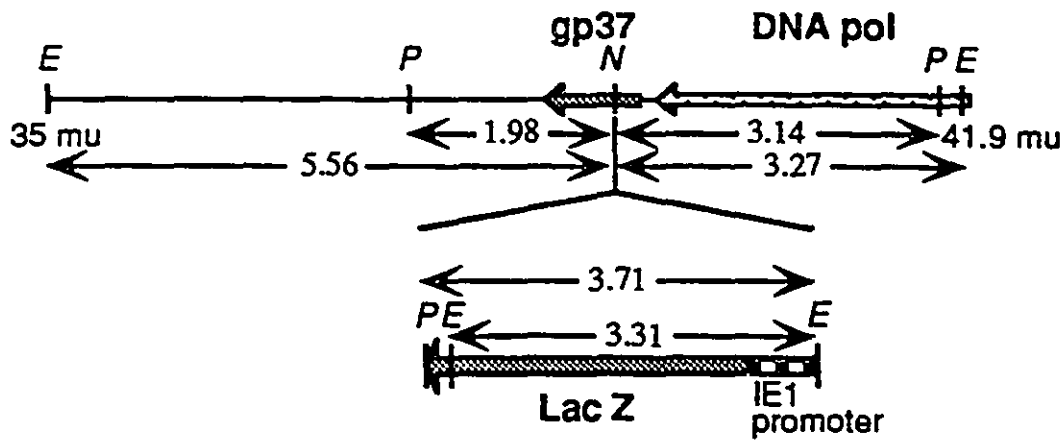
RESULTS

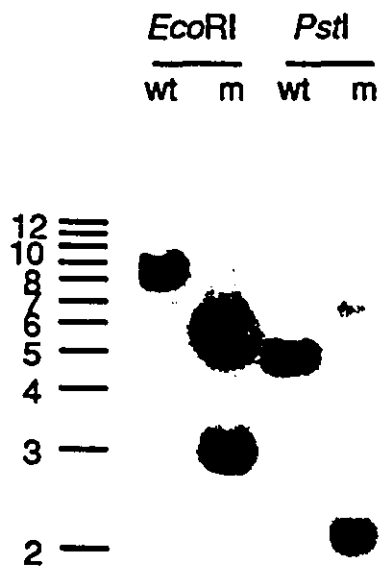
Infection of insect cells and larvae with AcSpin⁻NPV.

In order to determine the function of gp37 in AcNPV infection a mutant virus (AcSpin⁻NPV), in which the gp37 gene was interrupted by insertion of the *lacZ* coding region, was generated through homologous recombination. Plaque purified AcSpin⁻NPV and wild-type virus were used to infect Sf9 cells and viral DNA was prepared from progeny budded virus obtained from the culture medium. Viral DNAs were digested with *Eco*RI and *Pst*I restriction enzymes, and subjected to Southern blot analysis. Interruption of gp37 in AcSpin⁻NPV was confirmed by hybridization of a probe corresponding to the gp37 ORF (Fig. 1). The IE1/*lacZ* insertion in the mutant virus (m) introduced one *Pst*I and two*Eco*RI sites which resulted in hybridization of the gp37 probe to two DNA fragments compared to a single fragment in the wild type (wt) virus. These results were confirmed by PCR analysis of viral DNA using primers corresponding to gp37 sequences on either side of the insertion site (data not shown). Interruption of the gp37 ORF resulted in truncation of the translation product after the first 72 N-terminal amino acids. Absence of the full-length protein was verified by western blot analysis of infected-cell proteins with a peptide antibody directed against the carboxyl terminus of gp37 (α-spAc1; data not shown). We did not observe any differences in infectivity or cytopathic effects of Sf9 cells between the wild-type and mutant viruses. Infection of *Trichoplusia ni* larvae with AcSpin⁻NPV OBs administered *per os* also did not reveal any difference in lethality or morbidity compared with wt AcNPV (data not shown).

Figure 1. AcSpin^{-}NPV construction and Southern blot analysis.

A schematic diagram of the mutational insertion of LacZ is represented on top. Key restriction sites are noted in italics *N*, *Nco*I; *E*, *Eco*RI and *P*, *Pst*I. Southern blot analysis of the mutant AcSpin^{-}NPV (m) and wild-type AcNPV (wt) viral genomes is presented below. Sizes of DNA markers are indicated on left side (in kilobases). Viral DNA prepared from infected cells supernatant was cut with *Pst*I and *Eco*RI and probed with a ^{32}P-labeled gp37 PCR probe.


gp37
DNA pol
E
P
N
P E
35 mu
1.98
3.14
41.9 mu
5.56
3.27
3.71
PE
E
3.31
Lac Z
IE1
promoter

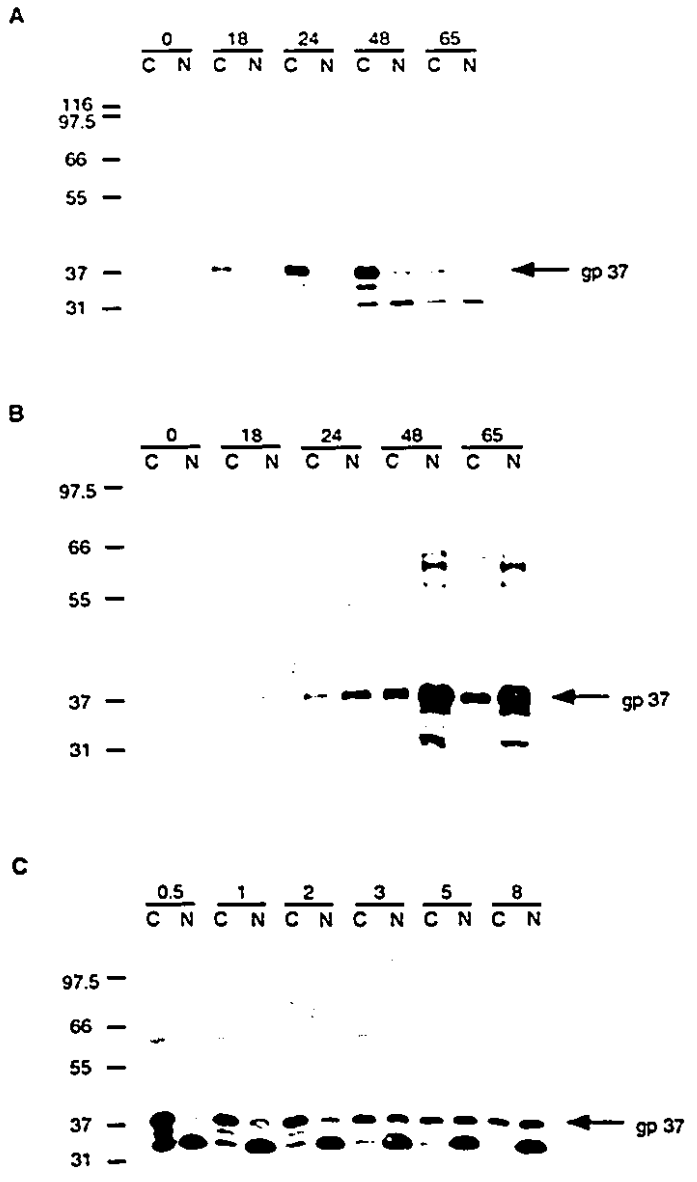

EcoRI
PstI
wt
m
wt
m
12
10
8
7
6
5
4
3
2

gp37 and p50 localization in baculovirus-infected cells and CbEPV OBs.

In order to determine the rate of synthesis and localization of gp37 in AcNPV-infected cells, we prepared cytoplasmic and nuclear fractions from infected cells labelled for 45 min with Tran^{35}S-label at various times pi. Immunoprecipitations were carried out on both fractions with α-spAc1 rabbit polyclonal antiserum. Immunoprecipitated proteins were resolved by SDS-PAGE and the radiolabelled proteins were detected by autoradiography. Labelled gp37 was first detected in the cytoplasm of infected cells18 h pi and reached a maximum rate of synthesis 48 h pi (Fig. 2a). At all time points, the majority of labelled gp37 was found in the cytoplasm. A 33-kDa protein that probably represents polyhedrin (Guarino *et al.*, 1992) was non-specifically precipitated and was detected in both the cytoplasmic and nuclear fractions at 48 and 65 h pi. This protein was also detected when the immunoprecipitations were carried out with preimmune antiserum (data not shown). Western blot analysis of the same fractions probed with α-spAc1 antiserum demonstrated that gp37 was present in equal amounts in both fractions at 18 h pi, but was mainly associated with the nuclear fraction at 48 and 65 h pi (Fig. 2B). These results indicated that there was a migration of the gp37 towards the nucleus during the course of infection. The rate of migration to the nucleus was determined by pulse/chase labelling of infected cells at 48 h pi, followed by immunoprecipitation of the cytoplasmic and nuclear fractions. The autoradiogram from a pulse-chase experiment, in Fig. 2C, demonstrates that the majority of newly synthesized gp37 was present in the cytoplasm 30 min after labelling. However, by 3 h pi approximately half of the labelled gp37 had migrated to the nucleus. The rate of gp37 migration towards the nucleus was relatively slow compared to that of polyhedrin, as demonstrated by the 33-kDa nuclear protein in Fig. 2C. The majority of labelled polyhedrin was present in the nuclear fraction by 1 h pi.

We have previously shown that gp37 (SLP) copurified with OBs through sucrose gradients and suggested that it may be associated with the polyhedral envelope (Vialard *et al.*, 1990b). Subsequently, Gross *et al.* (1993a) demonstrated by immunoelectron microscopy that gp37 of OpNPV was present in cytoplasmic inclusions and not in polyhedra. In order to verify its localization in AcNPV infection, we performed immunogold labelling on infected cells and viewed

Figure 2. Synthesis and intracellular distribution of gp37 at various times postinfection. (A) Autoradiogram of ^{35}S-labeled proteins immunoprecipitated by a peptide-specific Ab (α-spAc1) directed against gp37 and resolved through SDS-PAGE (10% polyacrylamide). (B) Immunoblot analysis of nuclear and cytoplasmic proteins resolved through SDS-PAGE (10% polyacrylamide), and reacted with α-spAc1. (C) Autoradiogram of pulse ^{35}S-labelled proteins immunoprecipitated by α-spAc1. The position of gp37 is indicated on the right. The numbers on the left indicate the position of molecular mass markers (in kilodaltons). The numbers along the top indicate hours postinfection. C, cytoplasmic; N, nuclear.


A
0 18 24 48 65
C N C N C N C N C N
116
97.5
66
55
37
31
gp 37
B
0 18 24 48 65
C N C N C N C N C N
97.5
66
55
37
31
gp 37
C
0.5 1 2 3 5 8
C N C N C N C N C N C N
97.5
66
55
37
31
gp 37

them by electron microscopy. Immunolabelling with α-spAc1 antiserum specifically stained spindle-shaped bodies that were present in the cytoplasm of infected cells and were often associated with the nuclear membrane (Fig. 3A). The micrograph presented in Fig. 3B is representative of those observations and shows the nuclear membrane bifurcating around a spindle-shaped crystal.

The CbEPV homolog of gp37 (50-kDa protein) was reported to be the most abundant protein in purified CbEPV spheroids (Yuen *et al.*, 1990). In order to more precisely localize this protein, CbEPV OBs purified from infected Cf larvae were analyzed by electron microscopy. Treatment with α-spCb2 antiserum followed by immunogold-conjugated secondary antibody revealed that the 50-kDa protein was a component of spindle-shaped structures contained within CbEPV spheroids (Fig. 3C and D).

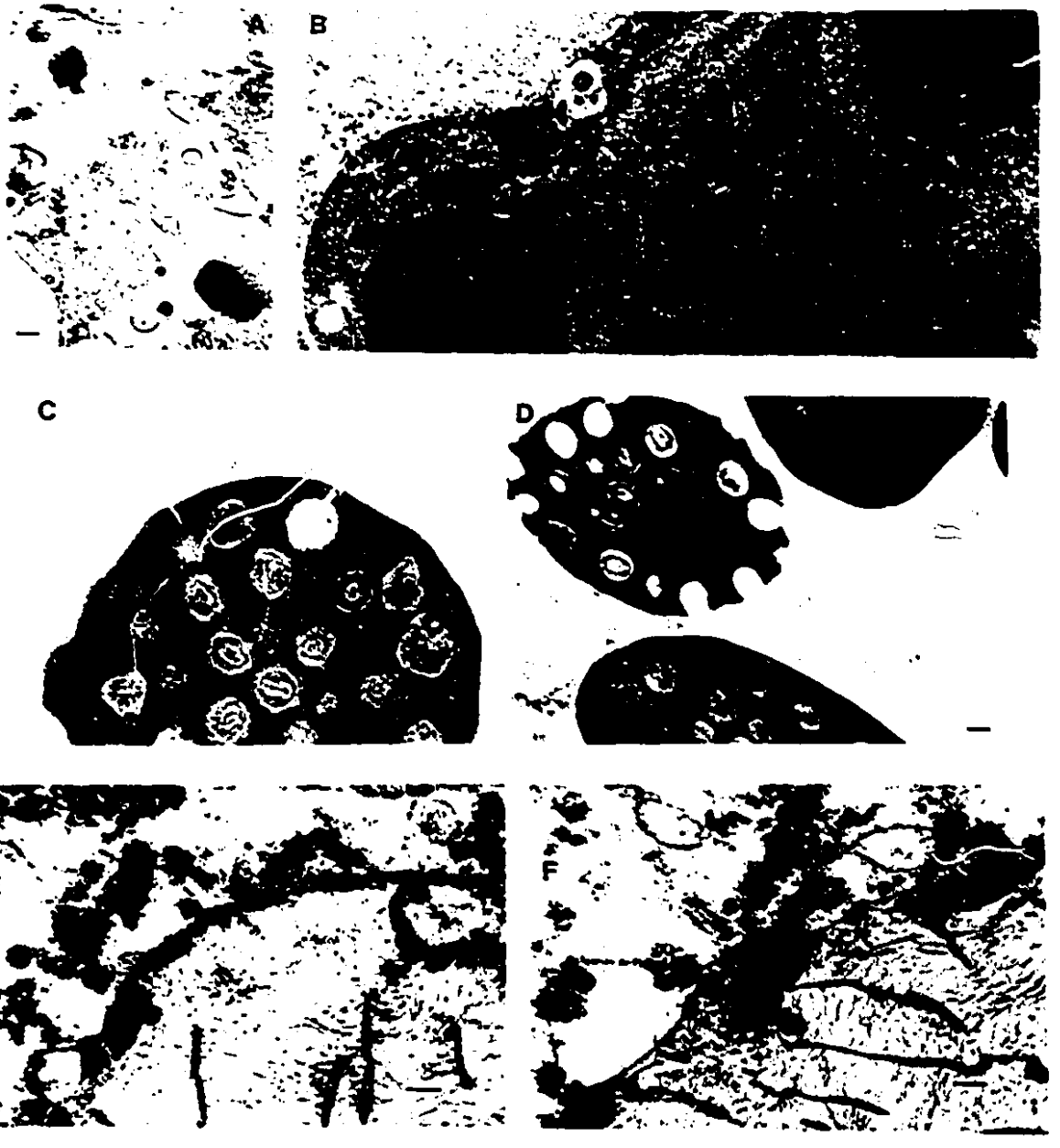
We also constructed recombinant baculoviruses for overexpression of AcNPV gp37 and the 50-kDa CbEPV OB protein (AcSpinNPV and CbSpinNPV, respectively) and viewed the recombinant virus-infected cells by immunoelectron microscopy. In both cases, antisera directed against these proteins (α-spAc1 and α-spCb2) specifically stained a variety of pleiomorphic structures, including a number of spindle-shaped bodies in the cytoplasm of the infected cells. This demonstrated that the overproduced proteins aggregated in spindle-shaped bodies much like their natural counterpart, and that the entomopoxvirus protein can form these structures in baculovirus-infected cells (Fig. 3E and F). The gp37 negative baculovirus (AcSpin$^-$NPV) did not produce spindle bodies (data not shown).

CfEPV spindle gene sequence and alignment with other spindle proteins.

The CfEPV spindle gene was amplified from viral DNA obtained from occluded virions, cloned and sequenced. Its sequence differed from the CbEPV sequence (Yuen *et al.*, 1990) by a deletion of 6 nucleotides (951 to 956 of the CbEPV sequence) and substitutions at 6 others (nt 214: G→A; 220: A→G; 347: C→G; 765: A→C; 927: A→T; 947: G→A). The CfEPV protein has a predicted molecular mass of 38.9 kDa. The amino acid sequence differences resulting from the

Figure 3. Immunogold staining of spindle bodies viewed by electron microscopy.

(A) Staining of a spindle body in the cytoplasm of an AcNPV infected cell (probed with α-spAc1). (B) Spindle body surrounded by the nuclear membrane (indicated by arrow). (C and D) Staining of spindle bodies within purified CbEPV OBs (probed with α-spCb2). (E and F) Aggregation of the AcNPV and CbEPV spindle proteins in Sf9 cells infected with AcSpinNPV (E; α-spAc1) and CbSpinNPV (F; α-spCb2) recombinant viruses. Bar = 0.25 μm.


A
B
C
D
E
F

deletion and substitutions are shown in Fig. 4. We performed an alignment of the CbEPV (Yuen *et al.*, 1989), CfEPV, HaEPV (Dall *et al.*, 1993), AcNPV (Vialard *et al.*, 1990b) and OpNPV (Gross *et al.*, 1993a) amino acid sequences using the PCGene, CLUSTAL multialignment package (Higgins and Sharp, 1988). The resulting alignment is presented in Fig.4. The five sequences are well conserved and regions encompassing amino acids 126-133 and 233-249 of the CbEPV spindle protein sequence are perfectly conserved, suggesting functional importance. However, searches through several databanks for sequences with homology to these polypeptides were unsuccessful. The cysteine residues are very well conserved and similarly spaced in the five spindle sequences suggesting an important role in intramolecular disulfide bond formation and structure of the spindle bodies.

Spindle protein glycosylation, solubilization, and protease activity.

In order to carry out further biochemical analyses of the spindle proteins, spindles were produced in Sf9 cells infected with the recombinant viruses AcSpinNPV and CbSpinNPV. The spindle bodies were purified through discontinuous sucrose gradients. Cells infected with these recombinant viruses were OB negative eliminating possible interference by polyhedra in the purification of spindles. Because the wild-type gp37 gene was present in the recombinant viruses, the AcNPV spindle protein was produced along with the CbEPV protein and they copurified with each other (data not shown). Figure 5A represents a Coomassie blue-stained SDS-polyacrylamide gel of spindles purified through two successive discontinuous gradients. The spindle proteins represent the major polypeptides detected in these preparations. Contaminating AcNPV spindles in the CbSpinNPV fraction were not readily observable by Coomassie blue staining, but could be detected by western blot analysis (data not shown). Four major bands were observed in purified AcSpinNPV spindle preparations. These correspond to the various glycosylated and unglycosylated forms previously described (Vialard *et al.*, 1990b). Two major polypeptides of 50 and 37 kDa were detected in the CbSpinNPV spindle preparations. We have previously shown that AcNPV gp37 is glycosylated (Vialard *et al.*, 1990b) while Dall *et al.*

Figure 4. Comparison of three EPV and two NPV spindle protein amino acid sequences. Deduced amino acid sequence of CfEPV spindle protein, CbEPV p50 (Yuen *et al.*, 1990), HaEPV p50 (Dall *et al.*, 1993), AcNPV gp37 (Wu & Miller, 1989) and OpNPV gp37 (Gross *et al.*, 1993). Alignment was generated by the Clustal algorithm of the PCGene package. Stars indicate perfectly conserved and dots well conserved positions. Conserved Cys residues are boxed. A potential Ser catalytic residue is in bold.

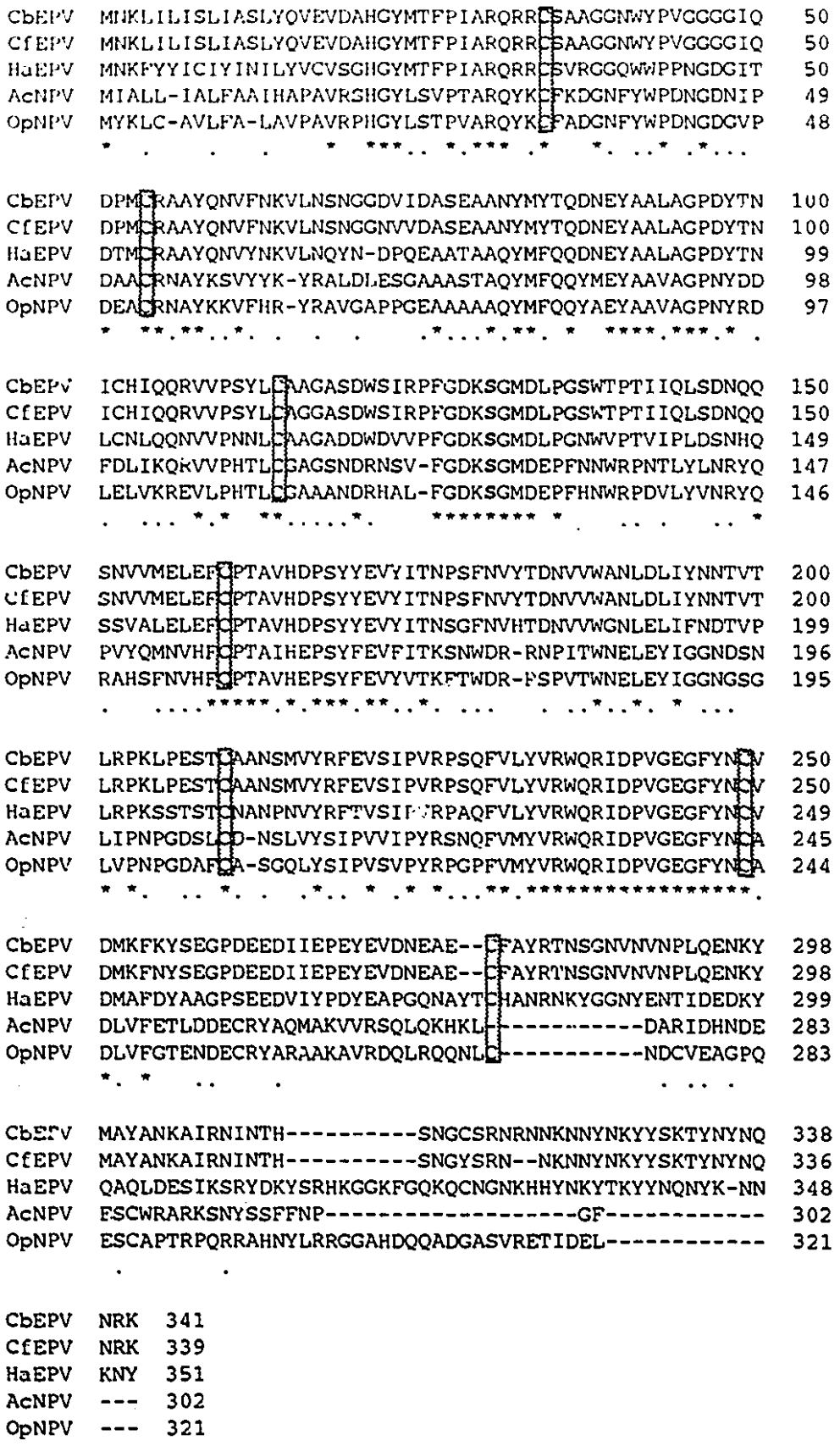
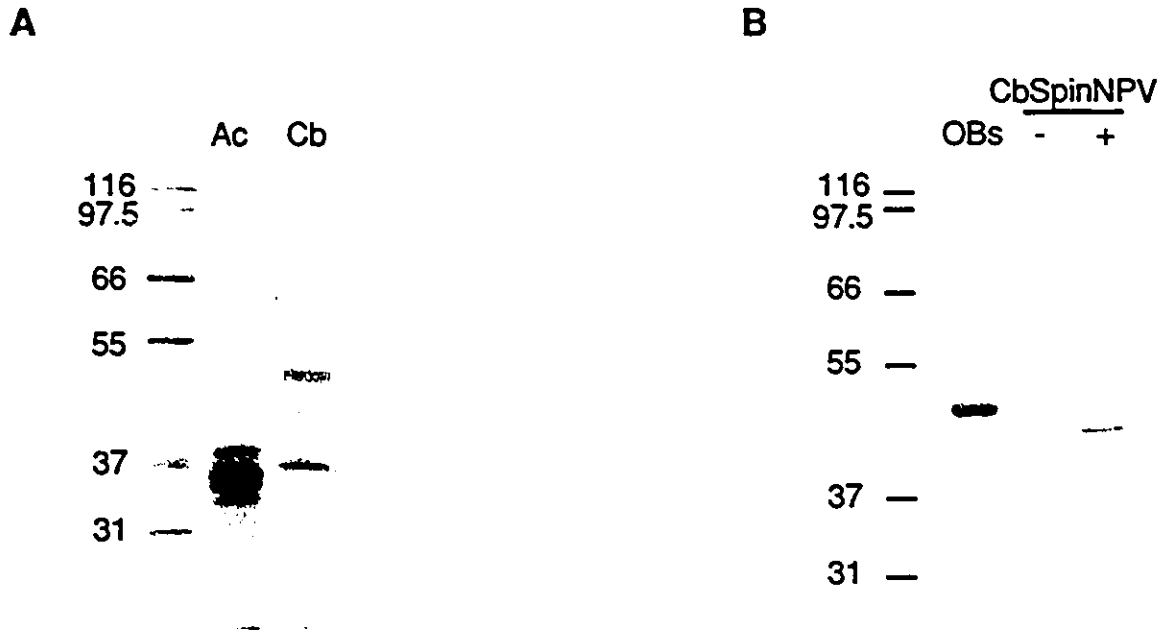

CbEPV MNKLILISLIASLYQVEVDAHGYMTFPIARQRRCSAAGGNWYPVGGGGIQ 50
CfEPV MNKLILISLIASLYQVEVDAHGYMTFPIARQRRCSAAGGNWYPVGGGGIQ 50
HaEPV MNKFYYICIYINILYVCVSGHGYMTFPIARQRRCSVRGGQWWPPNGDGIT 50
AcNPV MIALL-IALFAAIHAPAVRSHGYLSVPTARQYKCFKDGNFYWPDNGDNIP 49
OpNPV MYKLC-AVLFA-LAVPAVRPHGYLSTPVARQYKCFADGNFYWPDNGDGVP 48
CbEPV DPMCRAAYQNVFNKVLNSNGGDVIDASEAANYMYTQDNEYAALAGPDYTN 100
CfEPV DPMCRAAYQNVFNKVLNSNGGNVVDASEAANYMYTQDNEYAALAGPDYTN 100
HaEPV DTMCRAAYQNVYNKVLNQYN-DPQEAATAAQYMFQQDNEYAALAGPDYTN 99
AcNPV DAACRNAYKSVYYK-YRALDLESGAAASTAQYMFQQYMEYAAVAGPNYDD 98
OpNPV DEACRNAYKKVFHR-YRAVGAPPGEAAAAAQYMFQQYAEYAAVAGPNYRD 97
CbEPV ICHIQQRVVPSYLCAAGASDWSIRPFGDKSGMDLPGSWTPTIIQLSDNQQ 150
CfEPV ICHIQQRVVPSYLCAGGASDWSIRPFGDKSGMDLPGSWTPTIIQLSDNQQ 150
HaEPV LCNLQQNVVPNNLCAAGADDWDVVPFGDKSGMDLPGNWVPTVIPLDSNHQ 149
AcNPV FDLIKQKVVPHTLCGAGSNDRNSV-FGDKSGMDEPFNNWRPNTLYLNRYQ 147
OpNPV LELVKREVLPHTLCGAAANDRHAL-FGDKSGMDEPFHNWRPDVLYVNRYQ 146
CbEPV SNVVMELEFCPTAVHDPSYYEVYITNPSFNVYTDNVVWANLDLIYNNTVT 200
CfEPV SNVVMELEFCPTAVHDPSYYEVYITNPSFNVYTDNVVWANLDLIYNNTVT 200
HaEPV SSVALELEFCPTAVHDPSYYEVYITNSGFNVHTDNVVWGNLELIFNDTVP 199
AcNPV PVYQMNVHFCPTAIHEPSYFEVFITKSNWDR-RNPITWNELEYIGGNDSN 196
OpNPV RAHSFNVHFCPTAVHEPSYFEVYVTKFTWDR-PSPVTWNELEYIGGNGSG 195
CbEPV LRPKLPESTCAANSMVYRFEVSIPVRPSQFVLYVRWQRIDPVGEGFYNCV 250
CfEPV LRPKLPESTCAANSMVYRFEVSIPVRPSQFVLYVRWQRIDPVGEGFYNCV 250
HaEPV LRPKSSTSTCNANPNVYRFTVSIPVRPAQFVLYVRWQRIDPVGEGFYNCV 249
AcNPV LIPNPGDSLCD-NSLVYSIPVVIPYRSNQFVMYVRWQRIDPVGEGFYNCA 245
OpNPV LVPNPGDAFCA-SGQLYSIPVSVPYRPGPFVMYVRWQRIDPVGEGFYNCA 244
CbEPV DMKFKYSEGPDEEDIIEPEYEVDNEAE--CFAYRTNSGNVNVNPLQENKY 298
CfEPV DMKFNYSEGPDEEDIIEPEYEVDNEAE--CFAYRTNSGNVNVNPLQENKY 298
HaEPV DMAFDYAAGPSEEDVIYPDYEAPGQNAYTCHANRNKYGGNYENTIDEDKY 299
AcNPV DLVFETLDDECRYAQMAKVVRSQLQKHKL----------DARIDHNDE 283
OpNPV DLVFGTENDECRYARAAKAVRDQLRQQNLC----------NDCVEAGPQ 283
CbEPV MAYANKAIRNINTH----------SNGCSRNRNNKNNYNKYYSKTYNYNQ 338
CfEPV MAYANKAIRNINTH----------SNGYSRN--NKNNYNKYYSKTYNYNQ 336
HaEPV QAQLDESIKSRYDKYSRHKGGKFGQKQCNGNKHHYNKYTKYYNQNYK-NN 348
AcNPV FSCWRARKSNYSSFFNP----------GF---------- 302
OpNPV ESCAPTRPQRRAHNYLRRGGAHDQQADGASVRETIDEL---------- 321
CbEPV NRK 341
CfEPV NRK 339
HaEPV KNY 351
AcNPV --- 302
OpNPV --- 321

Figure 5. Recombinant spindle protein purification and analysis of N-linked glycosylation of CbEPV spindle protein.

(A) Coomassie blue stained gel of purified spindle proteins. Ac, AcNPV spindles; Cb,CbEPV spindles. (B) Immunoblot analysis of CbEPV OBs purified from infected larvae and total proteins produced by CbSpinNPV in the absence (-) and presence (+) of tunicamycin, reacted with α-spAc1.


A
Ac
Cb
116
97.5
66
55
37
31
B
CbSpinNPV
OBs
-
+
116
97.5
66
55
37
31

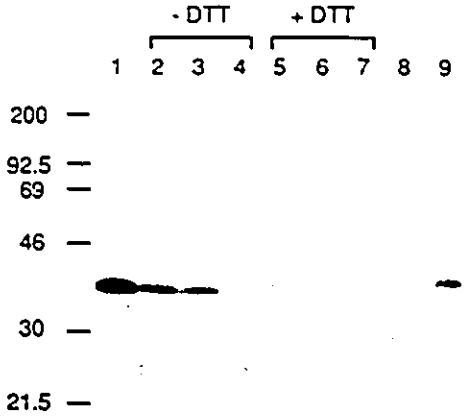
(1993) reported that the HaEPV spindle protein is not. It has also been suggested that the difference in predicted and observed MW of the CbEPV homolog may be due to glycosylation (Yuen *et al.*, 1990). In order to determine the glycosylation state of the CbEPV 50-kDa protein, we treated Sf9 cells infected with CbSpinNPV with tunicamycin and analyzed the proteins by western blot. The CbEPV protein produced in CbSpinNPV-infected cells had a similar mobility through SDS-polyacrylamide as the protein present in purified CbEPV spheroids (Fig. 5B). Following treatment with tunicamycin, a smaller polypeptide (42 kDa) was observed, indicating that the 50-kDa protein was glycosylated.

In natural infections, OBs are dissolved in the alkaline conditions of the insect midgut. In order to determine the effect of high pH on purified spindle bodies, these were incubated in an alkaline buffer in the presence and absence of a reducing agent for various lengths of time. Undissolved material was pelleted by centrifugation, resuspended in electrophoresis sample buffer and resolved by SDS-PAGE. After a 30 min incubation, gp37 was not detected in western blot analysis of undisolved material using α-spAc1 antiserum. The addition of DTT accelerated its solubilization (Fig. 6A). When both dissolved and undissolved spindle-body proteins were analysed by western blot, polypeptides smaller than the full length proteins were also detected (Fig. 6B) indicating that a cleavage of the spindle protein had occurred. Cleavage of the AcNPV spindle protein resulted in detection of a 25-kDa polypeptide which was recognized by α-spAc2. This polypeptide was not recognized by α-spAc1 antiserum suggesting that the cleavage must have occurred at the carboxyl terminus of the protein (data not shown). Cleavage of the CbEPV spindle protein also occurred at the carboxyl terminus and resulted in a polypeptide of approximately 38-kDa recognized by α-spCb2 and not α-spCb1 antiserum (Fig. 6B). These results suggested that a protease activity activated at high pH was associated with purified spindle bodies. In order to further characterize this activity, purified spindle bodies were incubated with two small dye-linked peptides (PepTagA1 and PepTagC1) for various lengths of time and the peptides were resolved through agarose gels. Proteolytic cleavage of these peptides results in changes in their size and charge which alters their electrophoretic mobility. Purified spindle

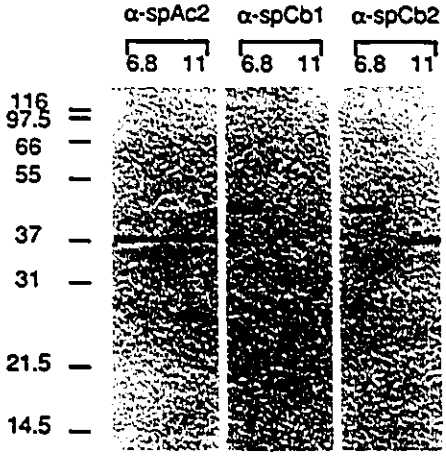
Figure 6. Spindle body solubilization, cleavage, and protease assays.

(A) Purified AcSpinNPV spindle bodies treated for 1 min (lanes 2, 5, and 8), 10 min (lanes 3 and 6) and 30 min (lanes 4 and 7) in alkaline buffer in the presence (+DTT) and absence (-DTT) of a reducing agent. Lane 1: untreated spindle bodies, lane 8: spindle bodies dissolved in alkaline buffer containing 0.2% SDS, lane 9: wt AcNPV-infected cells. After alkaline treatment, undissolved material was pelleted, resolved by SDS-PAGE (10% polyacrylamide), and analysed by immunoblot (reacted with α-spAc1). (B) Immunoblot analysis of purified AcSpinNPV and CbSpinNPV spindle bodies incubated at neutral (6.8) or high (11) pH prior to SDS-PAGE resolution (12% polyacrylamide). AcNPV spindle proteins were reacted with α-spAc2 and CbEPV spindle proteins with α-spCb1 and α-spCb2. (C) Purified AcSpinNPV and CbSpinNPV spindle bodies (equivalent of 5 µg of protein estimated on a Coomassie blue stained gel) were pelleted and both factions (p, pellet; s, supernatant) were incubated in alkaline buffer and then with PepTagC1 for 3 and 16 h at 30°C. The same quantity (5 µg) or ten times less (0.5 µg) of AcNPV and CbEPV purified spindle bodies (spindle) and OBs were dissolved in alkaline buffer and incubated with PepTagC1 for 16 h at 30°C. AcNPV OBs were purified from AcSpinNPV. Samples were then submitted to electrophoresis (0.8% agarose gel). The negative controls (-) are PepTagC1 incubated alone in alkaline buffer. + and - on the left of the gel represent the cleaved (+) and uncleaved (-) forms of the peptide.

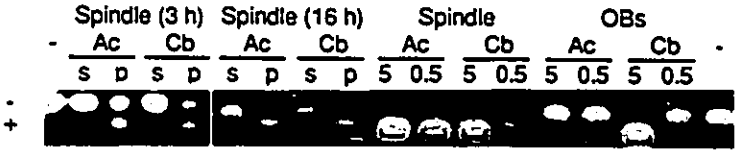
A


- DTT
+ DTT
1 2 3 4 5 6 7 8 9
200
92.5
69
46
30
21.5

B


α-spAc2
α-spCb1
α-spCb2
6.8 11 6.8 11 6.8 11
116
97.5
66
55
37
31
21.5
14.5

C


Spindle (3 h)
Spindle (16 h)
Spindle
OBs
- Ac Cb Ac Cb Ac Cb Ac Cb -
s p s p s p s p 5 0.5 5 0.5 5 0.5 5 0.5
-
+

bodies stored in water were centrifuged and separated into pelleted spindle bodies and supernatant fractions. The pelleted spindle bodies and supernatants were incubated separately in alkaline buffer and the dye-linked peptides were added to both fractions. Cleavage of PepTagC1 incubated with pellets from both AcSpinNPV and CbSpinNPV was detected after a 3 h incubation period and was complete after overnight incubation. The PepTagC1 cleavage did not occur with the supernatants, indicating that the protease activity was associated with the spindle fraction (Fig. 6C). Cleavage occured with 0.5 and 5 µg of both AcNPV and CbEPV spindle proteins (as estimated on a Coomassie blue stained gel), but appeared to be more effective with the AcNPV spindle protein. The other peptide, PepTagA1, was not cleaved by either spindle protein preparation (data not shown). AcNPV OBs prepared from AcSpinˉNPV infected cells and purified parallel to spindle bodies through discontinuous sucrose gradients did not contain an alkaline protease activity, suggesting that it was specific to the spindle bodies. CbEPV OBs purified from infected larvae containing embedded spindle bodies cleaved both PepTagC1 (Fig. 6C) and PepTagA1 (data not shown) indicating that a different and/or additional protease activity was associated with CbEPV OBs.

DISCUSSION

In this communication, we report further biochemical, genetic, and microscopy studies of spindle-shaped bodies which are associated with the nucleus of AcNPV-infected cells or are embedded within CbEPV OBs. We also compared the deduced amino acid sequences of these proteins to two other previously published sequences of spindle proteins as well as a new spindle protein gene obtained from CfEPV.

We have previously reported that the AcNPV gp37 protein copurified with baculovirus OBs (Vialard *et al.*, 1990b). Immunofluorescence labelling of purified OBs solubilized in an alkaline buffer suggested that it may be associated with the polyhedral envelope. However, Gross *et al.*, (1993a) subsequently reported that in the closely related OpNPV, gp37 was associated with cytoplasmic inclusions and not polyhedra. Our labelled-protein immunoprecipitations and western

blot analyses (Fig. 2), indicate that AcNPV gp37 is primarily associated with the nucleus of infected cells. These results were confirmed by immunoelectron microscopy studies that demonstrated its presence in spindle bodies often associated with the nuclear membrane (Fig. 3A and B). Its detection in structures that remained after alkali treatment of OBs (Vialard *et al.*, 1990b) may have resulted from residual gp37 associated nonspecifically with these structures. This is supported by our findings that spindle bodies containing gp37 copurify through sucrose gradients with polyhedra and are also dissolved in alkaline conditions (Fig. 5).

Spindle-shaped bodies have been reported in some baculovirus (Huger and Kreig, 1968; Adams and Wilcox, 1968; Gross *et al.*, 1993a) and EPV (Bergoin *et al.*, 1970; Bird, 1974; Milner and Beaton, 1979; Dall *et al.*, 1993) infected cells, suggesting a common function for these structures in these unrelated insect viruses. Although a function has not been ascribed to spindle bodies, several lines of evidence suggest that they are not essential to baculovirus or EPV infection. They appear to be absent in some EPV species and have been described in only a few baculovirus infections. Attempts to amplify spindle protein coding sequences from AmEPV genomic DNA, using degenerate oligonucleotides corresponding to well conserved regions of published spindle proteins, were unsuccessful (unpublished results). Also, western blot analysis of AmEPV-infected cells with several antisera directed against spindle proteins (described in this report) failed to detect a spindle protein (Banville *et al.*, 1992). These results suggest that AmEPV does not encode a spindle protein. The gp37 gene of AcNPV was suggested to be essential to the viral life cycle (Wu and Miller, 1989). However, we have succeeded in generating a mutant virus (Fig. 1) that encodes a truncated form of the protein. This virus was indistinguishable from wild type virus in infection of both insect larvae and cells in culture. The course of infection in cell cultures (production of budded virus and OBs, cell lysis) was similar for both baculoviruses. Levels of infectivity in larvae and the pathological effects produced were also similar.

The studies described above did not determine whether spindle bodies are required in the initial stages of infection in the insect midgut. Our results demonstrate that AcNPV gp37 and the 50-kDa CbEPV protein are the most abundant components of their respective purified spindles

(Fig. 5A). The fact that spindle proteins aggregate to form crystal structures that dissolve under alkaline conditions resembles the property of viral OBs. In the final stages of baculovirus and EPV infection, the insect disintegrates and OBs containing virions are released into the surroundings. The spindle bodies are presumably also released and may be ingested along with OBs. In some EPV strains, such as CbEPV, CfEPV (Bird, 1974), and *Oncopera alboguttata* EPV (Milner and Beaton, 1979), spindle bodies are occluded within spheroids which ensures their ingestion along with infectious virions. In infections where spindle bodies are not occluded, their ingestion along with OBs may rely on their remaining in close proximity to each other. Preliminary attempts in our laboratory to determine whether spindle proteins are required in the inital stage of infection were unsuccessful. Nevertheless, the spindle protein may influence viral infectivity only in some species of the host insect or under specific conditions.

We have detected an alkaline protease activity which is associated with purified spindle bodies (Fig. 6B and C). It has been previously reported that NPV (Eppstein and Thoma, 1975) and EPV (Bilimoria and Arif, 1979; Langridge and Roberts, 1982) OBs purified from infected insects also contain an alkaline protease activity. However, the activity was probably due to contamination from an insect-specific protease because it was not detected in OBs derived from cell culture (Zummer and Faulkner, 1979). In our experiments, PepTagC1 is cleaved by both AcNPV and CbEPV spindle protein preparations obtained from cell culture and with CbEPV OBs containing spindles. It is not cleaved with cell-culture derived AcSpin$^-$NPV OBs. We can not completely rule out the possibility that the protease activity associated with spindle bodies was due to a contamination with a minor protein associated with these structures. However, activity was not detected in the water in which the spindle bodies were stored nor in AcSpin$^-$NPVs OBs purified in parallel with the recombinant spindle bodies. It is possible that the spindle bodies are composed of more than one protein, although our results indicate that the spindle protein is clearly the major component of these structures (Fig. 5A).

Two recent reports describe a proteinaceous factor contained in the spheroids of *Pseudaletia separata* (Ps) EPV that enhances *Pseudaletia unipuncta* NPV infectivity for Ps larvae

(Xu and Hukuhara, 1992 and 1994). This enhancing factor was purified from alkali-solubilized spheroids by a series of chromatographic steps and resulted in identification of a 38-kDa glycoprotein that retained enhancing activity. Spindle bodies are associated with infection in this EPV (Hukuhara *et al.*, 1990) and SDS-PAGE analysis of purified PsEPV spheroid proteins demonstrates a pattern similar to CbEPV OB proteins. A 115-kDa protein likely represents spheroidin and a 50-kDa polypeptide the spindle protein. Alkali-solubilized OB preparations contained 50-kDa and 38-kDa proteins (Xu and Hukuhara, 1994) similar to the CbEPV spindle body protein pattern presented in Fig. 6B of this report. Thus, the 38-kDa protein that copurified with an enhancing activity (Xu and Hukuhara,1994) may represent the PsEPV 50-kDa spindle protein cleaved under alkaline conditions. We could not verify cross-reactivity between CbEPV spindle protein and PsEPV enhancing factor because the polyclonal serum raised against the latter protein was not available. These observations suggest that spindle proteins may be proteases that function as enhancing factors in insect infections.

Sequence and structure homology searches through databanks did not detect any proteins with significant homology to the spindle protein. It is nevertheless interesting to note that Ser-130 (corresponding to the CbEPV spindle protein) is situated in a block of 8 amino acids conserved in all the sequences. This serine might represent the catalytic residue of a serine protease (Kraut, 1977); H-21 and D-51 being correctly spaced to represent the rest of the catalytic triad. Verification of this hypothesis would require mutation of the Ser residue in the recombinant viruses and testing for protease activity in the spindle bodies purified from the mutant baculoviruses. Weak similarity blocks have also been found between the spindle proteins and two IgA proteases (which also contains a serine catalytic residue) using the BLAST program (Altschul *et al.*, 1990), but the precise nature of the alkaline protease associated with the spindle bodies remains to be determined.

CHAPTER 4

The 1629-Nucleotide ORF Located Downstream of the *Autographa californica* Nuclear Polyhedrosis Virus Polyhedrin Gene Encodes a Nucleocapsid-Associated Phosphoprotein

PREFACE

The two previous chapters described the identification and characterization of a glycoprotein produced late in baculovirus infection that forms spindle-shaped inclusions. This protein appears to be associated with an alkaline protease activity. The following two chapters deal with a second baculovirus protein that is expressed late in infection. In this chapter, the expression, synthesis, and cellular localization of the protein are determined through immunoblot and immunoprecipitation techniques. Posttranslational modifications are investigated by immunoprecipitation of radiolabelled proteins. The association of this phosphoprotein with baculovirus nucleocapsids is determined by biochemical and immunoelectron microscopy techniques.

ABSTRACT

A 78-kDa protein was produced in bacteria from a clone of the 1629-nucleotide open reading frame located immediately downstream from the polyhedrin gene of *Autographa californica* nuclear polyhedrosis virus. The identity of this protein was confirmed by its reactivity with peptide antiserum and amino terminal peptide sequencing after purification from transformed bacteria. The polypeptide was used to produce polyclonal antisera in rabbits. Immunoblot analysis of insect cells infected with the baculovirus indicated that two related proteins with molecular masses of 78 and 83 kDa were synthesized late in infection. Biochemical fractionation studies indicated that both these proteins were present in purified nucleocapsids from budded and occluded virus preparations. Immunoprecipitation of ^{32}P-labeled proteins and treatment of purified nucleocapsids with alkaline phosphatase demonstrated that the 83-kDa protein was a phosphorylated derivative of the 78-kDa protein. Furthermore, immunoelectron microscopy revealed that the proteins were localized to regions of nucleocapsid assembly within the infected cell and appeared to be associated with the end structures of mature nucleocapsids.

INTRODUCTION

Autographa californica nuclear polyhedrosis virus (AcNPV) is a member of the *Baculoviridae*, a family of double-stranded DNA viruses with rod-shaped capsids which infect a number of arthropods. Following infection, these viruses produce two different types of virions (Blissard and Rohrmann, 1990). The extracellular, or budded virus (BV), is required for cell-to-cell transmission and acquires an envelope by budding from the plasma membrane of the infected cell. The occluded, or polyhedra-derived virus (PDV), is required for transmission in the external environment. This viral type acquires its envelope in the nuclei of infected cells and may contain single or multiple nucleocapsids. The PDV is embedded within nuclear occlusion bodies (OBs), also known as polyhedra, which protect them from the environment until they are ingested by a susceptible insect. Once exposed to the alkaline conditions of the insect midgut, the OBs are dissolved, the virions are released, and the infectious cycle proceeds.

Several baculovirus genes encoding virion-associated proteins have been identified previously and are described in a recent review by Rohrmann (1992). Some of these proteins appear to be unique to each type of virion. For example, gp67 (or gp64) is an envelope protein required for membrane fusion and is present only in the BV (Blissard and Rohrmann, 1989; Blissard and Wenz, 1992; Whitford *et al.*, 1989), while gp41 (or p40), a protein whose function is not presently known, has been reported to be localized in the space between the PDV nucleocapsid and its envelope (Ma *et al.*, 1993; Nagamine and Kobayashi, 1991; Nagamine *et al.*, 1991, Whitford and Faulkner, 1992a and b). In addition, the gene encoding a protein (p74) which is required for PDV infectivity has been identified (Hill *et al.*, 1993; Kuzio *et al.*, 1989), but it is not presently clear whether this protein is actually associated with the PDV or is a component of the OBs. Recently, Gross *et al.* (1993b) described a protein (p16) which appears to be associated with a temporary viral envelope in the cytoplasm of infected cells. This envelope is obtained from the nuclear membrane as the nucleocapsids bud into the cytoplasm and is not present in BV or PDV. In contrast to the proteins described above, the nucleocapsid proteins identified thus far are common to both PDV and BV. The major capsid protein, as determined by its abundance and distribution throughout the nucleocapsid, is p39 (Bjornson and Rohrmann, 1992a; Blissard *et al.*, 1989; Pearson *et al.*, 1988; Russell *et al.*, 1991; Thiem and Miller, 1989b). A second capsid protein, p87 of *Orgyia pseudotsugata* NPV and p80, its homologue in AcNPV, have also been described (Lu and Carstens, 1992; Müller *et al.*, 1990). However, this protein is not as abundant as p39 and its function is presently unknown. Recently, a 24-kDa protein associated with both BV and PDV nucleocapsids was identified and it seems to be evenly distributed throughout the nucleocapsids (Bjornson and Rohrmann, 1992b; Gombart *et al.*, 1989b; Oellig *et al.*, 1987; Whitford *et al.*, 1989; Wolgamot *et al.*, 1993). Finally, a small basically charged DNA-binding protein, p6.5-7.9, is associated with both virion types. This protein may be involved in packaging of the viral genome (Maeda *et al.*, 1991a; Russell and Rohrmann, 1990b; Wilson *et al.*, 1987, Wilson and Price, 1988 and 1991).

Recently, a 1629-nucleotide open reading frame (ORF8) from the *Eco*RI-I fragment of AcNPV was sequenced and predicted to encode a proline-rich protein with a molecular mass of 60.6 kDa (Possee *et al.*, 1991). Transcriptional analysis determined that ORF8 is expressed late in infection (Ooi and Miller, 1990; Pham and Sivasubramanian, 1992a; Possee *et al.*, 1991) and may be regulated by transcription from the polyhedrin gene, which is located adjacent to ORF8 (Ooi and Miller, 1990). In addition, ORF8 transcription differs between cells in culture and in different insect tissues; a cellular homologue(s) may also be present in uninfected insect cells (Pham and Sivasubramanian, 1992a). Finally, ORF8 appears to be essential to the viral life-cycle in culture since a mutant virus at this locus could not be propagated in the absence of wild-type helper virus (Possee *et al.*, 1991). In this report we describe the synthesis, biochemical characterization and localization of p78/83, the translated product of ORF8. Polyclonal antibodies were raised against p78 produced in bacteria. Subsequent immunoblot analysis demonstrated that p78/83 is synthesized late in AcNPV infection and is a component of the nucleocapsid. It is present in phosphorylated and unphosphorylated forms. Finally, immunoelectron microscopy studies suggest that p78/83 is associated with an end structure of baculovirus nucleocapsids.

MATERIALS AND METHODS

Cells and virus.

Spodoptera frugiperda (Sf9) insect cells and *Autographa californica* nuclear polyhedrosis virus (AcNPV) were obtained from Max Summers, Texas A&M University, College Station, Texas. Virus and cells were propagated as previously described (Summers and Smith, 1987). Infections were performed by incubating cells with virus at a multiplicity of infection of 5 PFU/cell for 1 hour followed by removal of the viral inoculum. The zero-point of infection corresponded to the addition of virus to cells.

Recombinant bacterial plasmid construction and expression.

A DNA fragment containing ORF8 was synthesized by PCR using oligonucleotide primers corresponding to the 5' (ACGAATCGTAGATATGAA) and 3' (TTAAGCGCTAGATTCTGT) ends of the ORF8 coding sequence (Possee *et al.*, 1991). The PCR product was cloned into the bacterial expression plasmid pT7-7 (Tabor and Richardson, 1985) at the unique *Eco*RI site which had been made blunt by treatment with DNA polymerase Klenow fragment. This cloning procedure resulted in fusion of a met-ala-arg-ile peptide derived from the vector to the amino terminus of the ORF8 encoded protein. The resulting plasmid was introduced into *Escherichia coli* BL21(DE3) and the recombinant gene was expressed by induction with 0.4 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) as previously described (Studier and Moffat, 1986).

Antisera and immunoblots.

Anti-peptide antibodies against amino acids 513 through 527 of the ORF8 translation product (Possee *et al.*, 1991) were prepared by standard methods (Richardson *et al.*, 1985). Polyclonal antibodies (PAbORF8) against the ORF8 protein were prepared by immunization of rabbits with gel purified bacterial p78 as follows. Proteins synthesized by the recombinant bacteria described above were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the band corresponding to p78 was excised and electroeluted from the gel. The partially purified protein was subsequently injected into rabbits for the production of polyclonal antibodies (Harlow and Lane, 1988). For subsequent immunoblot analysis, bacterial and AcNPV-infected insect cells were suspended in SDS-sample buffer (Laemmli, 1970), the proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with antibodies by standard procedures (Harlow and Lane, 1988). Antibody-antigen complexes were detected by addition of alkaline phosphatase-conjugated donkey anti-rabbit IgG antibodies (Jackson Immunoresearch Laboratories) in the presence of the substrates nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and phenazine methosulfate (Sigma).

Metabolic protein labeling.

Proteins were labeled with Tran[^{35}S]-label (ICN) as follows. At various times post-infection, 4 x 10^6 Sf9 cells were incubated in methionine-free medium (Summers and Smith, 1987) for 20 min, followed by the addition of 150 µCi/ml Tran[^{35}S]-label in methionine-free medium. After a 45 min incubation period, the labeling medium was removed and the cells were washed with PBS.

^{32}P-labeling of proteins was performed as follows. At 24 h postinfection, the culture medium was removed and replaced with TNM-FH medium lacking phosphates (Summers and Smith, 1987). Following a 3 hour incubation, 1 mCi/ml [^{32}P]orthophosphate (ICN) in phosphate-free medium was added and the cells were incubated a further 3 hours. The labeling medium was removed and the cells were washed with PBS. The ^{32}P-labeled cells were then incubated in 500 µl lysis buffer (150 mM NaCl, 0.5% NP-40, 50 mM Tris hydrochloride [pH 8.8]) in the presence of 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 4 µg/ml aprotinin as protease inhibitors. Insoluble matter was removed by centrifugation and the lysate containing ^{32}P-labeled soluble proteins was stored at -80°C.

Cell fractionation and immunoprecipitations.

Unlabeled and ^{35}S-labeled proteins were separated into cytoplasmic and nuclear fractions by detergent treatment of cells followed by centrifugation as previously described (Jarvis *et al.*, 1991). Immunoprecipitations were carried out on 100 µl of fractionated lysate or ^{32}P-labeled soluble proteins (above) in 1 ml of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris hydrochloride [pH 7.5]) containing 0.1% bovine serum albumin and protease inhibitors. Immunoprecipitations were cleared with preimmune serum and Immuno-precipitin (Bethesda Research Laboratories) for one hour followed by centrifugation. The resulting supernatant was then incubated with 2 µl of PAbORF8 for a minimum of 2 hours. Antibody-antigen complexes were precipitated by the addition of Immuno-precipitin and incubation for one hour followed by centrifugation. The pellet was washed twice with RIPA buffer

and twice with 10 mM Tris hydrochloride [pH 8.0] prior to SDS-PAGE. The gels were dried and radiolabeled proteins were detected by autoradiography.

Virion and nucleocapsid purification.

BV from infected cell medium was purified through a sucrose gradient as previously described (Summers and Smith, 1987) while PDV was obtained from OBs as follows. OBs were purified from infected cells by detergent treatment and sucrose gradient centrifugation essentially as previously described (Vialard *et al.*, 1990b) with the following modifications. Following cell lysis, occlusion bodies were centrifuged through a 30% sucrose cushion at 10,000 x *g* for 15 min. The pellet was resuspended in lysis buffer (Vialard *et al.*, 1990b) containing 0.2% SDS and OBs were purified from a discontinuous sucrose gradient as previously described (McCarthy *et al.*, 1974). PDV was obtained from solubilized OBs by alkali treatment with 0.17 M Na_2CO_3/0.01 M dithiothreitol followed by centrifugation through a sucrose gradient as described above for BV. Nucleocapsids were purified from BV and PDV by detergent treatment as previously described for PDV (Thiem and Miller, 1989b).

Phosphatase treatment.

PDV was incubated for 4 hours at 37°C in the presence of 3.4 units of calf intestinal alkaline phosphatase (Pharmacia) as previously described (Parthun and Jaehning, 1992). 10 mM NaH_2PO_4 was included in some reactions as a phosphatase inhibitor.

Immunoelectron microscopy.

AcNPV-infected Sf9 cells were collected at 60 h postinfection and fixed in 4% paraformaldehyde/0.5% glutaraldehyde in a phosphate buffer. The cells were infiltrated with LR White, polymerized, cut into thin sections, and placed onto nickel grids. Antibody staining was performed as follows. The grids were placed on drops of PBS containing 1% BSA for 30 minutes followed by a 2 hour incubation with Protein G-sepharose purified PAbORF8 at a 1:500 dilution in

PBS/0.05% Tween-20. They were then washed in PBS/0.05% Tween-20 and incubated for 30 min in a 1:20 dilution of donkey anti-rabbit IgG conjugated to 12-nm gold particles (the use of protein A rather than secondary antibody resulted in non-specific immunogold labeling of various infected cell structures). Finally, the grids were washed with PBS/0.05% Tween-20 followed by water and stained for 5 min with uranyl acetate and 2 min with lead citrate prior to visualization through a Phillips EM-400 microscope.

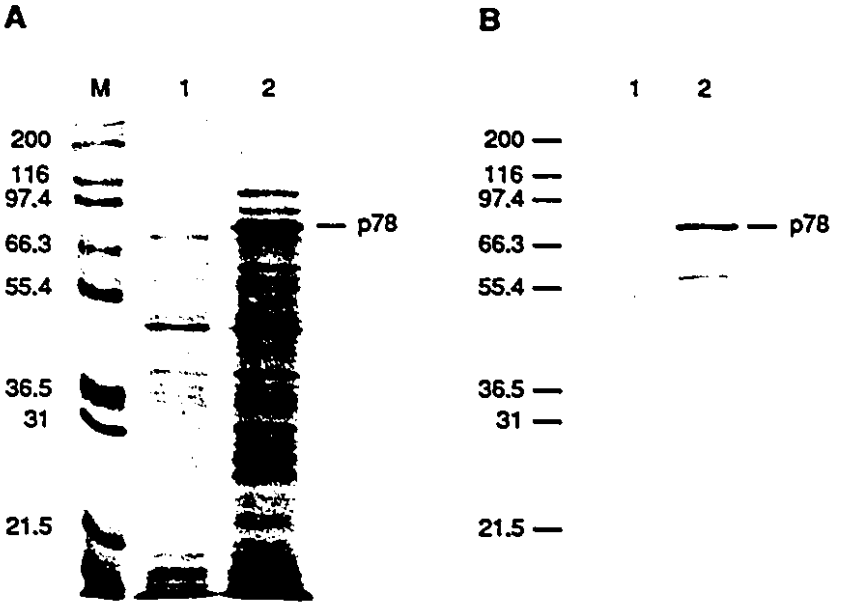
RESULTS

Bacterial expression of ORF8 and production of antibodies against the p78 protein.

The 1629-nucleotide ORF8 located immediately downstream of the AcNPV polyhedrin gene is predicted to encode a 543 amino acid protein with a molecular mass of 60.6 kDa (Possee *et al.*, 1991). This ORF was synthesized by PCR and cloned into the pT7-7 bacterial expression vector under the control of the T7 RNA polymerase promoter. The resulting plasmid was introduced into a bacterial strain which contains an inducible T7 RNA polymerase (see Materials and Methods). Growth of the bacteria in the presence of the inducer (IPTG) resulted in synthesis of a protein with an apparent molecular mass of 78 kDa as determined by its mobility through SDS-PAGE (Fig. 1A). This protein, p78, reacted with peptide antiserum directed against a portion of the ORF8 translation product (Fig. 1B). This antiserum also cross-reacted with smaller proteins which were not related to p78 since they were present in both uninduced and induced cells (Fig. 1B) as well as in bacteria which did not contain the expression plasmid (data not shown). The antibodies did not react specifically with any proteins from baculovirus-infected cells (data not shown). This may have been due to low levels of synthesis of the 1629-nucleotide ORF8 translation product in infected cells.

The 78-kDa protein produced in bacteria was eluted from SDS-polyacrylamide gel and its identity was confirmed by microsequencing the amino-terminus through Edman degradation. The

Figure 1. Expression of the 1629-nucleotide ORF8 of AcNPV in bacteria. A Coomassie blue stained gel (A) and immunoblot (B) of the ORF8 fusion protein (see Materials and Methods) are shown with proteins from bacteria containing ORF8 prior to induction (lane 1) and 4 h after induction (lane 2) with 0.4 mM IPTG. The immunoblot was probed with antiserum raised against a synthetic peptide predicted by the 1629-nucleotide ORF8 sequences. Lane M contains protein molecular mass markers; numbers correspond to sizes in kilodaltons. The position of p78 is indicated.


A
M
1
2
200
116
97.4
66.3
55.4
36.5
31
21.5
p78
B
1
2
200
116
97.4
66.3
55.4
36.5
31
21.5
p78

gel purified protein was subsequently used to raise polyclonal antibodies (PAbORF8) against the full-length p78 protein (see Materials and Methods).

Time-course analysis of p78/83 synthesis and localization in baculovirus-infected cells.

Total proteins from AcNPV-infected cells collected at various times postinfection were resolved by SDS-PAGE, transferred onto nitrocellulose, and subjected to immunoblot analysis with PAbORF8 described above. The results of this time-course analysis are shown in Fig. 2. Two major polypeptides with molecular masses of 78 and 83 kDa were detected late in infection while a pair of smaller proteins appeared very late in infection. The 78-kDa protein was first observed at 10 h postinfection and was present at very low levels until 16 h postinfection. At this point, a dramatic increase in the expression of the 78-kDa protein was observed which coincided with the appearance of an 83-kDa polypeptide. Although the 83-kDa protein was not initially as abundant as the 78-kDa form, by 36 h postinfection they were present in nearly equal amounts. At this time in infection, a pair of smaller proteins (49 and 51 kDa) were detected by PAbORF8. These bands were present in much lower amounts than the 78 and 83-kDa proteins and and may represent products of alternate transcription and/or translation start sites of ORF8. Ooi and Miller (1990) have previously identified several RNAs which contain 5' ends within the 1629-nucleotide ORF8. Translation from these RNAs may result in truncated forms of p78/83. It is also possible that these smaller polypeptides are proteolytic derivatives of full-length p78/83.

The profile of p78/83 synthesis and its intracellular distribution were determined by a combination of immunoprecipitation and western blot analyses. Immunoprecipitations of ^{35}S-labeled proteins from cytoplasmic and nuclear fractions at various times postinfection revealed a pattern of p78/83 synthesis typical of baculovirus late proteins. It was actively produced by 18 h postinfection, reaching a maximum between 24 and 48 h, followed by a substantial reduction in synthesis by 65 h postinfection (Fig. 3A). Under the labeling conditions described in Materials and Methods, only the 78-kDa form was detected and it was predominantly cytoplasmic at all times

Figure 2. Time course analysis of p78/83 accumulation in AcNPV-infected Sf9 cells. Proteins from 2 X 10^5 cells were collected at various times postinfection, separated through SDS-PAGE (10% polyacrylamide), and reacted with PAbORF8. The numbers along the top indicate hours postinfection. The position of the 78- and 83-kDa doublet is indicated on the right; the positions of molecular mass markers (in kilodaltons) are indicated on the left.

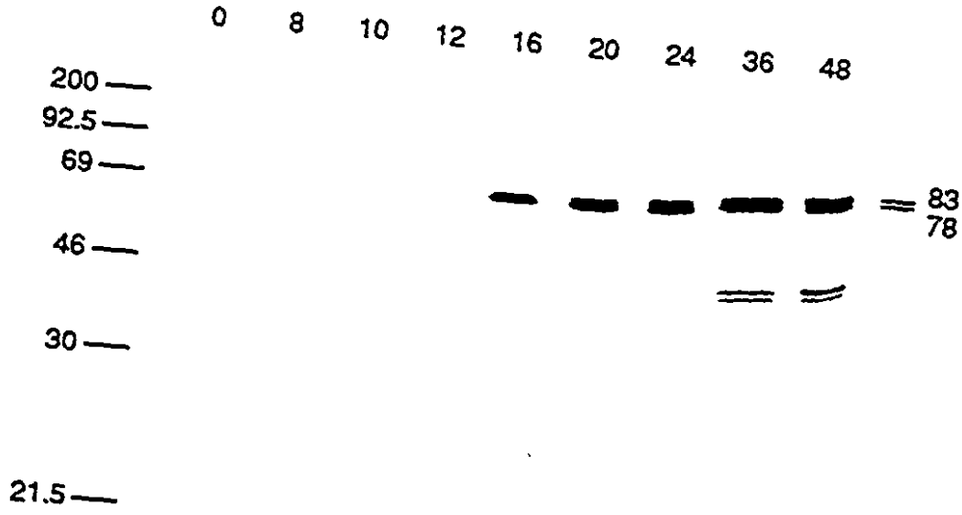
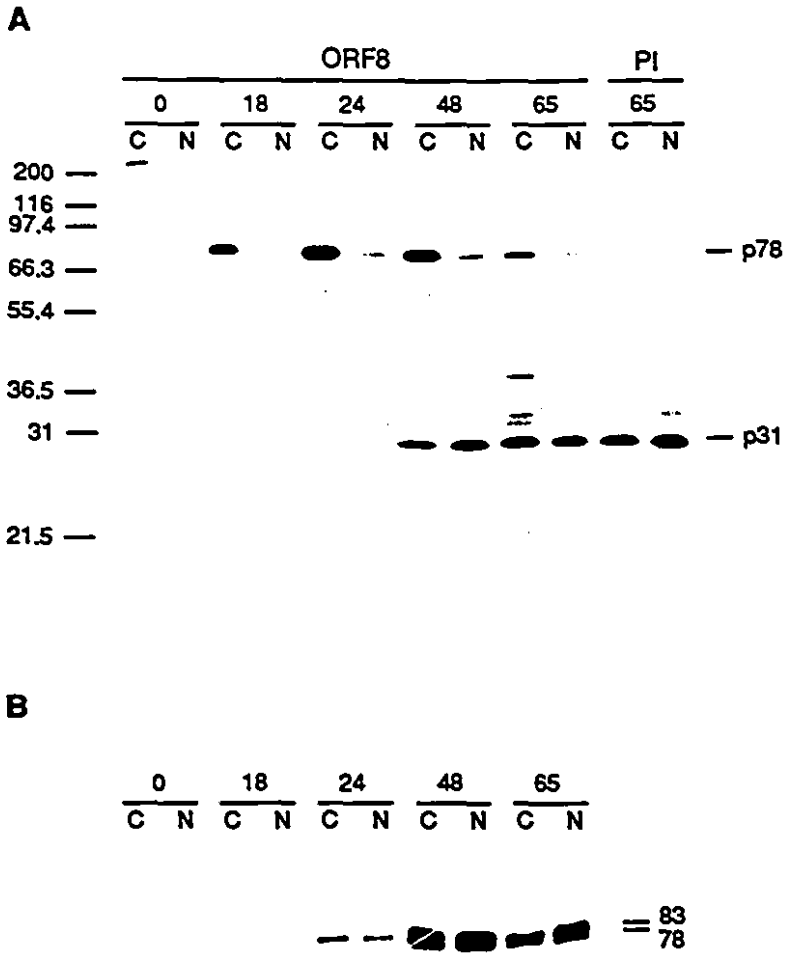

0
8
10
12
16
20
24
36
48
200
92.5
69
46
30
21.5
83
78

Figure 3. Synthesis and intracellular distribution of p78/83 at various times postinfection. (A) Autoradiogram of ^{35}S-labeled proteins immunoprecipitated by PAbORF8 (ORF8) or preimmune antiserum (PI) resolved through SDS-PAGE (10% polyacrylamide). The positions of p78 and p31 are indicated on the right. The numbers along the left indicate the positions of molecular mass markers (in kilodaltons). (B) Immunoblot analysis of fractionated proteins resolved through SDS-PAGE (7% polyacrylamide). The positions of the 78- and 83-kDa forms of p78/83 are indicated on the right. The numbers along the top indicate hours postinfection. C, cytoplasmic; N, nuclear.


A
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C N C N C N C N C N C N
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83
78

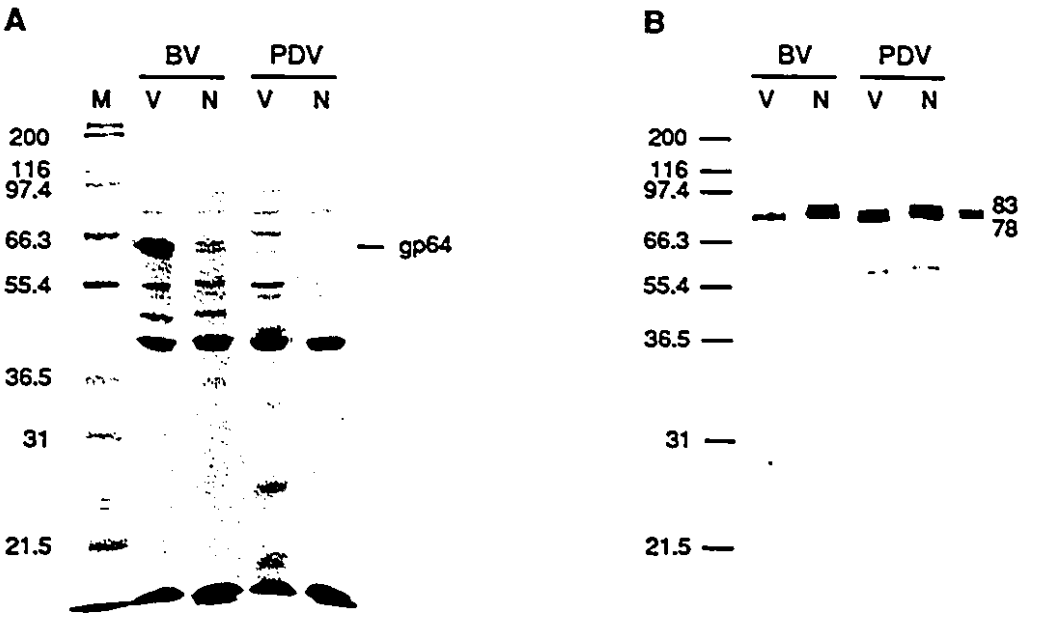
postinfection. Nonetheless, both the 78 and 83-kDa proteins were detected in equal quantities in the cytoplasmic and nuclear fractions when these were analysed by western blotting at later times of infection. At 18 h postinfection, the majority of p78/83 was present as the 78-kDa form in both fractions (Fig. 3B). These results suggested that the 83-kDa protein resulted from a post-translational modification of the 78-kDa polypeptide and that this process was relatively slow since the 83-kDa protein was not detected in the immunoprecipitations of ^{35}S-labeled proteins. Also, when proteins were pulse/chase labeled at 24 h postinfection and immunoprecipitated at various times thereafter, ^{35}S-labeled 83-kDa protein was not detected until two hours after labeling (data not shown).

In addition to p78, a protein of approximately 31 kDa was also observed in the immunoprecipitations (Fig. 3A). However, this protein was precipitated non-specifically by PAbORF8 since it was also present when preimmune serum was used in place of PAbORF8. A similar protein has been previously reported in baculovirus-infected cell immunoprecipitations and may represent the abundant polyhedrin protein (Guarino *et al.*, 1992).

p78/83 is a nucleocapsid protein associated with both the budded and occluded virions.

The identification of ORF8 as a late gene by transcription (Possee *et al.*, 1991) and protein synthesis analyses (above) suggested that it might encode a structural protein. In order to determine the localization of p78/83, BV and PDV were purified from infected-cell medium and solubilized OBs respectively. The purified virions were treated with detergent to remove their envelopes and the virion and nucleocapsid proteins were subjected to SDS-PAGE and subsequent immunoblot analysis with PAbORF8. Removal of envelopes from the nucleocapsids was demonstrated by the absence of several polypeptides (including gp64, the BV envelope glycoprotein) in the detergent-treated virions as determined by analysis of Coomassie blue-stained gels (Fig. 4A). The polyclonal antibodies detected both the 78 and 83-kDa polypeptides in BV as well as PDV nucleocapsids (Fig. 4B). However, p78/83 appears to be a minor constituent

Figure 4. Immunodetection of p78/83 associated with AcNPV virions. Virion (V) and nucleocapsid (N) proteins from BV and PDV were separated through SDS-PAGE (10% polyacrylamide) and were detected by Coomassie blue stain (A) or immunoblot analysis with PAbORF8 (B). Lane M contains molecular mass markers (numbers correspond to molecular mass in kilodaltons). The positions of gp64 and the 78- and 83-kDa forms of p78/83 are indicated.


A
BV
PDV
M
V
N
V
N
200
116
97.4
66.3
55.4
36.5
31
21.5
gp64
B
BV
PDV
V
N
V
N
200
116
97.4
66.3
55.4
36.5
31
21.5
83
78

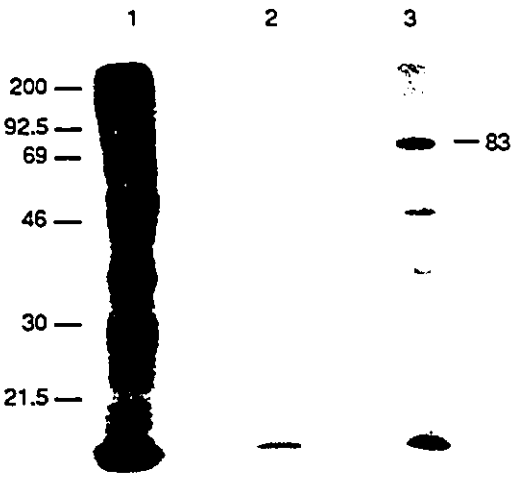
of AcNPV nucleocapsids since a corresponding pair of bands was not readily detected in the protein profiles of purified nucleocapsids stained with Coomassie blue (Fig. 4A). These only became apparent when very large amounts of purified virion or nucleocapsid proteins were resolved by SDS-PAGE (our unpublished observations). The smaller bands observed in the western blot may represent degradation products or truncated forms of p78/83 arising from alternate transcription and/or translation start sites.

Post-translational modifications of p78/83.

The presence of two major polypeptides in the immunoblot analysis of infected cells and purified nucleocapsids suggested that the ORF8 translation product may have undergone post-translational modifications. The predicted amino acid sequence of ORF8 contains several potential phosphorylation and N-linked glycosylation sites. However, p78/83 does not appear to be a glycoprotein since treatment of infected cells with tunicamycin, an inhibitor of N-linked glycosylation, had no effect on the mobility of p78/83 in SDS-PAGE (data not shown).

In order to determine the phosphorylation states of p78/83, infected cell proteins were metabolically labeled with [^{32}P]orthophosphate and immunoprecipitated with PAbORF8. The results of these immunoprecipitations are shown in Fig. 5. Although several phosphorylated proteins were observed in the immunoprecipitations, only one protein band was specific for PAbORF8; this phosphoprotein was not precipitated by preimmune serum. The PAbORF8-specific phosphoprotein migrated through SDS-PAGE somewhat slower than ^{35}S-labeled p78. Western blot analysis of immunoprecipitated ^{32}P-labeled proteins detected both forms of the protein in the immunoprecipitations, but when this membrane was subjected to autoradiography only the 83-kDa protein was detected (data not shown). This result suggested that the 83 and 78-kDa polypeptides represented the phosphorylated and unphosphorylated translation products of ORF8 respectively. In order to confirm this hypothesis, purified PDV was treated with calf intestinal alkaline phosphatase, the proteins were separated through SDS-PAGE, and then subjected to immunoblot analysis. Phosphatase treatment resulted in the disappearance of the

Figure 5. Analysis of p78/83 phosphorylation in AcNPV-infected Sf9 cells at 24 h postinfection. Autoradiogram of ^{32}P-labeled proteins separated through SDS-PAGE (10% polyacrylamide) is shown. Lanes: 1, soluble AcNPV-infected Sf9 cell proteins; 2, proteins immunoprecipitated with preimmune antiserum; 3, proteins immunoprecipitated with PAbORF8. The position of p83 is indicated on the right. Numbers correspond to sizes (in kilodaltons) of molecular mass markers.


1
2
3
200
92.5
69
46
30
21.5
83

83-kDa protein but had no effect on the 78-kDa form (Fig. 6). This effect was prevented in the presence of the phosphatase inhibitor, NaH_2PO_4. These data, taken together with the immunoblot analysis of ^{32}P-labeled proteins, strongly indicate that the 83-kDa protein is a phosphorylated form of p78/83.

Immunoelectron microscopy.

The association of p78/83 with virions and nucleocapsids was verified by immunogold staining of baculovirus-infected cells at 60 h postinfection. PAbORF8-specific staining was observed in areas of nucleocapsid assembly surrounding the virogenic stroma and in mature virions within occlusion bodies (Fig. 7). However, whereas p78/83 appeared to be randomly distributed throughout the areas of nucleocapsid assembly (Fig. 7A), staining of mature virions (within the occlusion bodies) appeared to be specific to the ends of the nucleocapsids (Fig. 7B). Immunogold staining was not observed along the lengths of the nucleocapsids nor in cross-sections through the middle of the nucleocapsids. It was not conclusive from these studies whether both or only one end of the nucleocapsids contained p78/83. Even though p78/83 was shown to be present in both BV and PDV nucleocapsids by western blot analysis (above), immunogold staining of BV nucleocapsids in the cytoplasm or at the plasma membrane was not detected. This may have been due to a combination of the relative paucity of BV as opposed to PDV in infected cells and the requirement for cross-sections to bisect the nucleocapsids at the ends in order for the p78/83 protein to be labeled. Preimmune antiserum and non-specific antibodies did not result in staining of either the nucleocapsids or virions (data not shown).

DISCUSSION

The results of this study reveal that the 1629-nucleotide ORF8 immediately downstream of the polyhedrin gene of AcNPV encodes a protein, p78/83, which is produced late in infection and is a component of both the BV and PDV nucleocapsids. The protein is present in

Figure 6. Alkaline phosphatase treatment of p78/83. Immunoblot of purified PDV subjected to phosphatase treatment and separated through SDS-PAGE (7% polyacrylamide). Presence (+) and absence (-) of calf intestinal alkaline phosphatase (CIAP) and phosphatase inhibitor (INH) are indicated above each lane. The positions of the 78- and 83-kDa forms of p78/83 are indicated on the right. The positions and sizes of molecular mass markers (in kilodaltons) are indicated on the left.

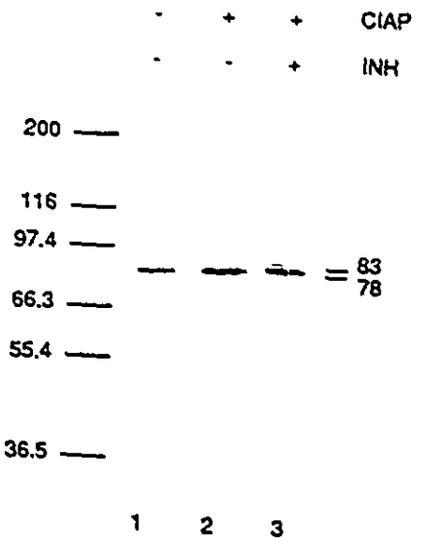
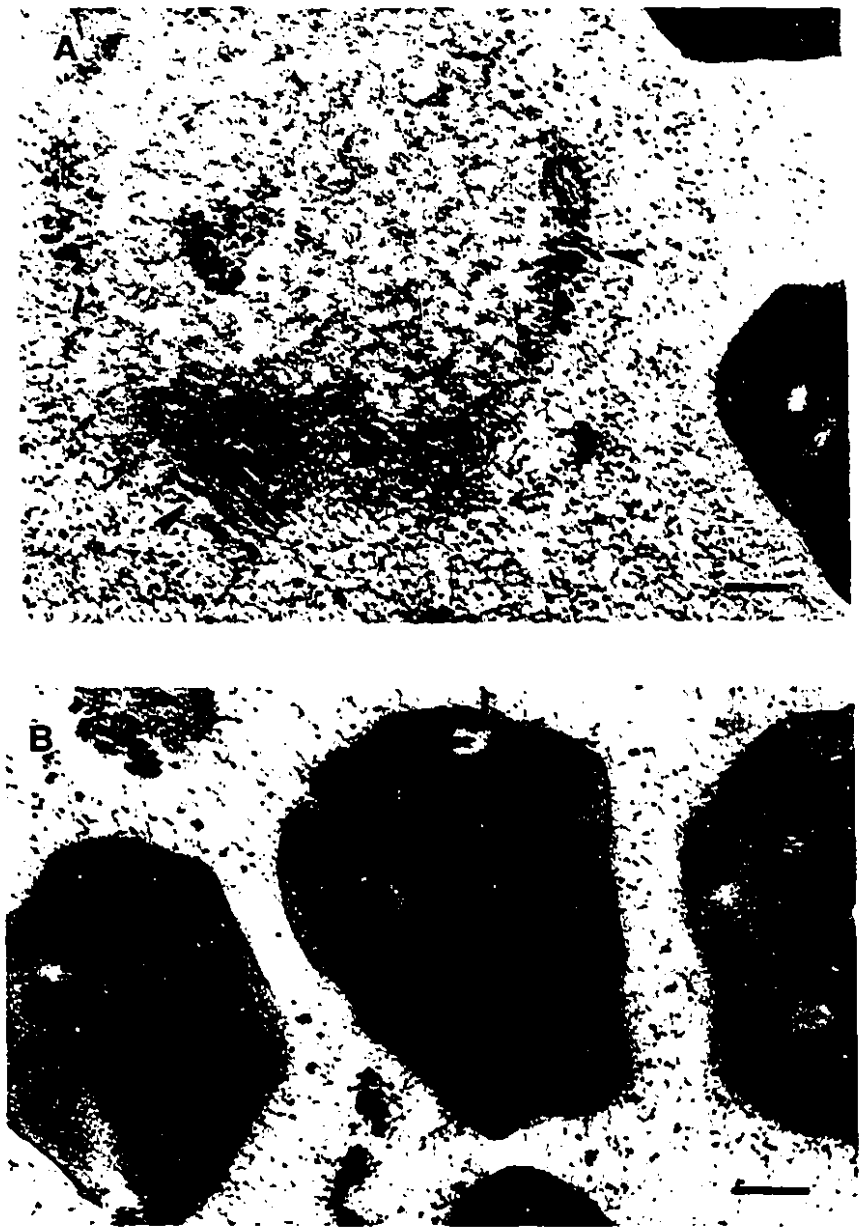

- + + CIAP
- - + INH
200
116
97.4
66.3
55.4
36.5
83
78
1 2 3

Figure 7. Immunogold staining of nuclear structures from infected cells at 60 h postinfection viewed by electron microscopy. (A) Association of p78/83 with areas of nucleocapsid assembly (indicated by arrows) surrounding the virogenic stroma (S). (B) Staining of nucleocapsids within occlusion bodies. The bars represent 0.25 µm.


A
B

phosphorylated and non-phosphorylated forms and appears to be associated with structures at the end(s) of the baculovirus nucleocapsid.

The translated product of ORF8 located in the *Eco*RI-I fragment of AcNPV has a predicted molecular mass of 60.6 kDa (Possee *et al.*, 1991). However, immunoblot analysis of the gene product, demonstrated that it is expressed in AcNPV-infected Sf9 cells as two polypeptides with apparent molecular masses of 78 and 83 kDa in SDS-PAGE (Fig. 2). The large discrepancy between the calculated and observed molecular size does not appear to be due to post-translational modification events. Although the amino acid sequence of ORF8 contains several potential N-linked glycosylation and phosphorylation sites, p78/83 is not glycosylated (our unpublished observations) and the non-phosphorylated form migrates as a 78-kDa protein (Fig. 6). Further evidence for the size of unmodified p78/83 was derived from immunoblot analysis of p78 produced in bacteria (Fig. 1) and an *in vitro* translation system (Pham and Sivasubramanian, 1992b). Thus, the retarded migration of p78/83 in SDS-PAGE appears to be due to the high content and uneven distribution of proline residues in the protein (Possee *et al.*, 1991). The presence of these proline-rich regions may result in an extended, rigid conformation which affects the mobility of p78/83 in SDS-PAGE.

Our virion fractionation results indicate that p78/83 is associated with AcNPV nucleocapsids (Fig. 4). Homology searches through protein data banks resulted in the identification of various proline-rich proteins which generally belong to either transcriptional or structural classes of proteins; p78/83 appears to belong to the latter. Virus encoded transcription proteins associated with the virion are usually required for transcription of immediate-early viral genes (eg. VP16 of Herpes simplex virus). However, the baculovirus immediate-early genes are transcribed by the host cell machinery in the absence of any viral proteins, as demonstrated by the ability of transfected baculovirus DNA to initiate a productive infectious cycle. In addition, it has been recently reported that the presence of the 1629-nucleotide gene has no effect on the levels of transcription from late and very late baculovirus promoters in a transient expression assay (Passarelli and Miller, 1993b). This does not exclude p78/83 from having an effect on other

aspects of baculovirus transcription, such as tissue specificity or timing of gene expression. However, it seems more likely that p78/83 is a structural component of the baculovirus nucleocapsids.

Previous immunogold microscopy studies determined that two other baculovirus proteins, p39 (the major capsid protein) and p24, are distributed throughout the nucleocapsids (Russell *et al.*, 1991; Wolgamot *et al.*, 1993). The precise localization of a third capsid protein, p87, has not yet been determined (Müller *et al.*, 1990). Immunogold staining of AcNPV-infected Sf9 cells revealed that p78/83 is associated with the ends of nucleocapsids in mature virions found embedded within OBs (Fig. 7). Baculovirus nucleocapsids contain distinctive end structures (Adams and McClintock, 1991; Federici, 1986) which are apparently composed of proteins different from the rest of the nucleocapsid (Burley *et al.*, 1982). The appearance of the cap structures is different at each end of the nucleocapsid and have been previously termed the "apicai cap" and "basal structure" (Fraser *et al.*, 1986). These end structures (in particular the "apical cap") have been implicated in various processes, including packaging of the nucleoprotein core (Fraser *et al.*, 1986), initiation of nucleocapsid envelopment within the infected-cell nucleus (Fraser *et al.*, 1986; Kawamoto *et al.*,1977a), and budding through the nuclear and cytoplasmic membranes (Fraser *et al.*, 1986; Kawamoto *et al.*, 1977b). The presence of p78/83 in the areas where nucleocapsid assembly and PDV envelope acquisition occur is consistent with any of the functions described above and may explain the essential requirement for the p78/83 gene in the baculovirus life cycle (Possee *et al.*, 1991).

The significance of p78/83 phosphorylation could not be resolved from this study, but it does not seem to be involved in determining either the localization of the protein within the infected cell or to one of the BV or PDV phenotypes. Both forms of p78/83, phosphorylated and non-phosphorylated, are present in the cytoplasm and in the nucleus (Fig. 3) as well as in both virion types (Fig. 4). Nonetheless, phosphorylation of p78/83 may play a role in nucleocapsid assembly through interactions with the nucleoprotein core or other structural proteins; the amino acid sequence of p78/83 encodes a potential leucine zipper domain which may direct protein-

protein interactions. Alternatively, phosphorylation may be involved in regulating interactions with the envelopes during the maturation of virions.

CHAPTER 5

Late Gene Products Associated with the Nucleocapsid Phosphoprotein (p78/83) and RNA Polymerase of *Autographa californica* Nuclear Polyhedrosis Virus

PREFACE

The previous chapter described the identification and characterization of a baculovirus encoded phosphoprotein which was a component of the nucleocapsids. Following publication of those results, another group reported that the protein was associated with purified virions, but was removed by detergent treatment (Pham *et al.*, 1993). They reported that their results implied localization of the protein to the envelope or the space between the envelope and the nucleocapsid. The studies described in this chapter confirm the association of the p78/83 phosphoprotein with baculovirus nucleocapsids. Its presence in a complex(es) containing the major capsid protein (p39) are demonstrated through co-immunoprecipitation studies. In addition, several other proteins appear to form stable complexes with p78/83. This chapter describes the identification of some of these proteins and suggests possible roles for the previously identified nucleocapsid-associated phosphoprotein.

ABSTRACT

Immunoprecipitation and immunoblot analysis were performed on baculovirus-infected cell proteins with antisera directed against several late proteins produced in baculovirus infection. Antisera specific for p78/83, immunoprecipitated this nucleocapsid-associated phosphoprotein as well as several other proteins at various times postinfection. Further immunoblot analysis of the immunoprecipitated proteins demonstrated that one of these was p39, the major capsid protein. The interaction between p78/83 and p39 was confirmed by a reciprocal immunoblot of proteins immunoprecipitated by p39-specific monoclonal antibodies and the presence of the nucleocapsid-associated phosphoprotein revealed with p78/83 polyclonal antiserum. A 37-kDa protein present in the immunoprecipitations was microsequenced and yielded a sequence that corresponded to the baculovirus nuclear matrix-associated phosphoprotein, pp31. Its identity was confirmed by immunoblot analysis with pp31 polyclonal antiserum. In addition, the baculovirus-induced RNA polymerase activity was purified through several chromatographic steps and was examined for the presence of a number of specific baculovirus proteins. Peptide antibodies raised against a putative component of the polymerase (LEF-8) resulted in the identification of a 96-kDa protein that copurified with the RNA polymerase activity at all chromatographic steps. Finally, p78/83 and pp31 also copurified with the RNA polymerase activity through three successive chromatographic steps.

INTRODUCTION

The baculoviruses are double stranded-DNA insect viruses that infect a large number of invertebrates, particularly members of the Lepidopteran family. They are characterized by a complex, biphasic life-cycle that results in the production of two phenotypically distinct, but genetically similar virion types (reviewed in Blissard and Rohrmann, 1990). The extracellular or budded virus (BV) is produced early in infection and acquires an envelope at the plasma membrane as it exits the infected cell. This type of virus is responsible for systemic spread within the infected insect and is also the form of the pathogen that infects cells in culture. In contrast, the

occluded or polyhedron-derived virus (PDV) is produced late in infection and is required for transmission of infection from one insect to another. PDVs remain in the infected-cell nucleus where they acquire a *de novo* envelope of unknown origin. Eventually, they become embedded within large proteinaceous crystal structures called occlusion bodies (OBs), or polyhedra, that are released upon death and disintegration of the infected insect. OBs protect the embedded virions from environmental factors for extended periods of time. The occluded virions are liberated when OBs are ingested and dissolve in the alkali conditions of the insect midgut.

Although the composition of the envelopes surrounding the two virus types is distinct (Braunagel and Summers, 1994; reviewed in Rohrmann, 1992), the nucleocapsid proteins identified thus far, are common to both. The genes encoding some of the components of baculovirus nucleocapsids have been identified and the localization of their products determined. Immunoelectron microscopy analysis demonstrated that the major capsid protein, p39, is randomly distributed throughout the nucleocapsid (Russell *et al.*, 1991; Pearson *et al.*, 1988; Thiem and Miller, 1989b). A similar distribution was described for a minor capsid protein, p24 (Wolgamot *et al.*, 1993). Recently, we demonstrated that a proline-rich phosphoprotein, p78/83, is localized to an end-structure of baculovirus nucleocapsids (Vialard and Richardson, 1993). A fourth protein, p87 (or p80), has been shown to be a component of nucleocapsids through western blot analysis (Müller *et al.*, 1990; Lu and Carstens, 1992), but its precise localization has not been determined. Finally, a small basic protein, p6.9, that is believed to be responsible for condensing the viral genome for packaging, is tightly associated with baculovirus DNA in both the nucleus and within mature nucleocapsids (Wilson and Price, 1990; Kelly *et al.*, 1983; Tweeten *et al.*, 1980).

Baculovirus nucleocapsid assembly occurs adjacent to and within the virogenic stroma, an electron-dense granular structure believed to be the baculovirus infected-cell counterpart of the nuclear matrix (Young *et al.*, 1993; Wilson and Price, 1988). The nuclear matrix is the structure which remains following detergent treatment, nuclease digestion, and salt extraction of the nucleus. It is free of lipids, DNA, and most proteins, and provides the structural scaffolding for the nucleus (reviewed in Fey *et al.*, 1991). The intimate association of baculovirus nucleocapsids with

these structures is demonstrated by the presence of nucleocapsids within and surrounding the virogenic stroma (Young *et al.*, 1993), and copurification with the nuclear matrix (Wilson and Price, 1988). A model for nucleocapsid assembly suggests that packaging of the viral genome takes place at the virogenic stroma (Fraser, 1986). Immunoelectron microscopy studies demonstrated the presence of the nucleocapsid-associated proteins p6.9, p39, and p78/83 within or at the periphery of the virogenic stroma (Wilson and Price, 1991; Russell *et al.*, 1991; Vialard and Richardson, 1993). A nonspecific DNA-binding phosphoprotein, pp31, has also been detected in the virogenic stroma, particularly in association with filamentous structures believed to be condensed nucleic acids (Guarino *et al.*, 1992). Cofractionation of p6.9, p39, and pp31 with the nuclear matrix after high salt extraction confirmed their association with this structure (Guarino *et al.*, 1992; Wilson and Price, 1988). In addition to providing a scaffolding structure, the nuclear matrix has also been shown to be the site of various cellular processes including DNA replication and transcription (Berezny, 1991; Verheijen *et al.*, 1988). By analogy, the virogenic stroma is believed to be the site of baculovirus transcription and replication. Baculovirus infection is characterized by the production of an a-amanitin-resistant RNA polymerase activity that is required for transcription from late and very late viral promoters (Fuchs *et al.*, 1983; Yang *et al.*, 1991; Huh and Weaver, 1990; Glocker *et al.*, 1993). The polypeptide components of the virus-induced polymerase have not been determined, although some of them might be encoded by recently identified late expression factors (lefs) (Li *et al.*, 1993; Passarelli and Miller, 1993a, b, c, 1994; Morris *et al.*, 1994).

Although many of the viral proteins described above may be components of common structures or mechanisms, the interactions between them have not been determined. In this communication, we report the identification and characterization of protein complexes that contain the nucleocapsid phosphoprotein, p78/83, and a number of baculovirus-infected cell proteins. In addition to forming a complex with the major capsid protein (p39), p78/83 was coimmunoprecipitated with the matrix-associated phosphoprotein, pp31. Also, p78/83 and pp31 were detected in a highly purified fraction of the virus-induced RNA polymerase activity. These

results suggest a number of possible functions for p78/83. Finally, we demonstrate that antibodies directed against the translation product of a gene previously identified as late expression factor (*lef-8*) recognize a 96-kDa protein that copurifies with the virus-induced RNA polymerase activity.

MATERIALS AND METHODS

Cells and virus.

Spodoptera frugiperda (Sf9 and Sf21) insect cells and *Autographa californica* nuclear polyhedrosis virus (AcNPV) were propagated in Grace's medium supplemented with 10% fetal bovine serum as previously described (Summers and Smith, 1987). Unless otherwise indicated, infections were performed by incubating cells with virus at a multiplicity of infection of 5 PFU per cell for 1 hour followed by removal of the viral inoculum. The zero time point of infection corresponded to the addition of virus to cells.

Antisera and immunoblots.

Polyclonal antisera directed against p78/83 (PAbORF8) and gp37 from AcNPV were raised in our laboratory and are described elsewhere (Vialard *et al.*, submitted; Vialard and Richardson, 1993). Polyclonal antiserum against AcNPV pp31 was a gift from Linda Guarino and was previously described (Guarino *et al.*, 1992). Monoclonal antisera against the major capsid protein (Whitt and Manning, 1988) and gp64 (Keddie *et al.*, 1989) of AcNPV were obtained from Loy Volkman. Monoclonal antisera against p24 from *Orgyia psedotsugata* nuclear polyhedrosis virus (Op*M*NPV) were received from George Rohrmann and were previously described (Wolgamot *et al.*, 1993). An antipeptide antibody directed against amino acids 2 through 18 of the deduced LEF-8 sequence (Passarelli *et al.*, 1994) was prepared as previously described (Richardson *et al.*, 1985). For immunoblot analysis, proteins were resolved through SDS-PAGE, transferred onto nitrocellulose membranes and probed with antibodies as previously described (Vialard and Richardson, 1993). Antibody-antigen complexes were detected by addition of alkaline

phosphatase-conjugated anti-rabbit or anti-mouse immunoglobulin G antibodies (Jackson Research Laboratories) and developed in the presence of nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and phenazine methosulfate.

Metabolic protein labeling and immunoprecipitations.

Infected-cell proteins were labeled with Tran[^{35}S]label (ICN) for 45 min, the cells were lysed and proteins collected as previously described (Vialard and Richardson, 1993). Immunoprecipitations were carried out on nuclear extracts (described below) or infected-cell soluble proteins prepared as previously described (Vialard and Richardson, 1993) with the following alterations. Briefly, immunoprecipitations were carried out on 100 µl of nuclear extract or soluble proteins from infected cells in 1 ml of immunoprecipitation buffer. Two different immunoprecipitation buffers were employed: non-denaturing buffer (20 mM Hepes [pH 7.9], 40 mM KCl, 1 mM MgCl, 0.1 mM EGTA, 0.1 mM EDTA, 0.5 mM DTT) and RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris hydrochloride [pH 7.5]). Both buffers contained 1 mM PMSF, 2 µg/ml aprotinin and 1 µg/ml leupeptin as protease inhibitors. Immunoprecipitations were cleared by incubation with protein G-Sepharose and centrifugation. The following amounts of antisera were then added to the supernatant: p78/83, 2 µl; pp31, 2 µl; p39, 20 µl, lef-8, 2 µl. After a two hour incubation at room temperature or overnight at 4°C, the samples were centrifuged briefly to remove debris. Then, 10 µl of a 1:1 slurry of protein G-Sepharose in phosphate-buffered saline were added and the samples were incubated for 1 h at room temperature. The protein G-Sepharose beads were washed three times in 1 ml of immunoprecipitation buffer and once in 1 ml of 10 mM Tris-hydrochloride (pH 7.9) prior to SDS-PAGE. Radiolabeled proteins were detected by autoradiography.

Nuclear extract preparation.

The nuclear extract of log phase Sf21 or Sf9 cells infected with AcNPV at a MOI of 10 was prepared according to Grula *et al.* (1981) with the following modifications. Infected cells were

harvested 40 h postinfection, washed once with PBS, once with hypotonic buffer (10 mM Tris hydrochloride [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM phenylmethyl-sulfonyl fluoride (PMSF), 2 µg/ml aprotinin, 1 µg/ml leupeptin), and resuspended in 5 pelleted-cell volumes of hypotonic buffer. The cells were incubated on ice for 10 min and subsequently lysed with 15 strokes of a type B pestle in a Dounce homogenizer (Wheaton). The homogenate was centrifuged at 4500 x g for 15 min and the pelleted nuclei were resuspended in 1.5 pelleted-nuclei volumes of TGEDP buffer (50 mM Tris hydrochloride [pH7.9], 35% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 2 µg/ml aprotinin, 1 µg/ml leupeptin) containing 0.3 M $(NH_4)_2SO_4$. The nuclei were disrupted by sonication (10 times for 15 seconds with 2 min incubation on ice between each pulse). The nuclear extract was diluted to 3 packed-cell volumes in TGEDP containing 0.3 M $(NH_4)_2SO_4$, and centrifuged at 100,000 x g for 45 minutes to remove insoluble matter. A 10% streptomycin sulfate solution was added to the supernatant to a final concentration of 1.25% and the mixture was incubated on ice for 30 min. The solution was centrifuged at 150,000 x g for 90 min, the supernatant was recovered and was dialysed overnight against TGEDP buffer. Precipitated matter was removed by centrifugation at 170,000 x g for 45 min.

Purification of the baculovirus-induced RNA polymerase.

All purification steps were carried out at 4°C. The columns used in the purification of the baculovirus-induced RNA polymerase were pre-equilibrated with TGEDP buffer containing 25 mM $(NH_4)_2SO_4$. The nuclear extract and column fractions were assayed for nonspecific RNA polymerase activity as described below. Active fractions eluted from each column were pooled and dialysed against TGEDP buffer. The nuclear extract obtained from 1 x 10^9 AcNPV-infected Sf9 or Sf21 cells was applied to a phosphocellulose P11 column (Whatman) and proteins were eluted in a 20 ml linear gradient from 25 mM to 450 mM $(NH_4)_2SO_4$ in TGEDP buffer. The active fractions were combined and applied to a Q Sepharose column (Pharmacia). Proteins were eluted in a 60 ml linear gradient from 25 mM to 400 mM $(NH_4)_2SO_4$ in TGEDP buffer. The active fractions corresponding to the baculovirus-induced RNA polymerase activity were then applied to a DNA

agarose affinity column (Pharmacia) and proteins were eluted in a 40 ml linear gradient from 25 mM to 400 mM $(NH_4)_2SO_4$ in TGEDP buffer. The active fractions were applied to a poly(A)sepharose column (Pharmacia) and the baculovirus-induced RNA polymerase was eluted in a 40 ml linear gradient from 25 mM to 400 mM $(NH_4)_2SO_4$ in TGEDP buffer.

RNA polymerase assays.

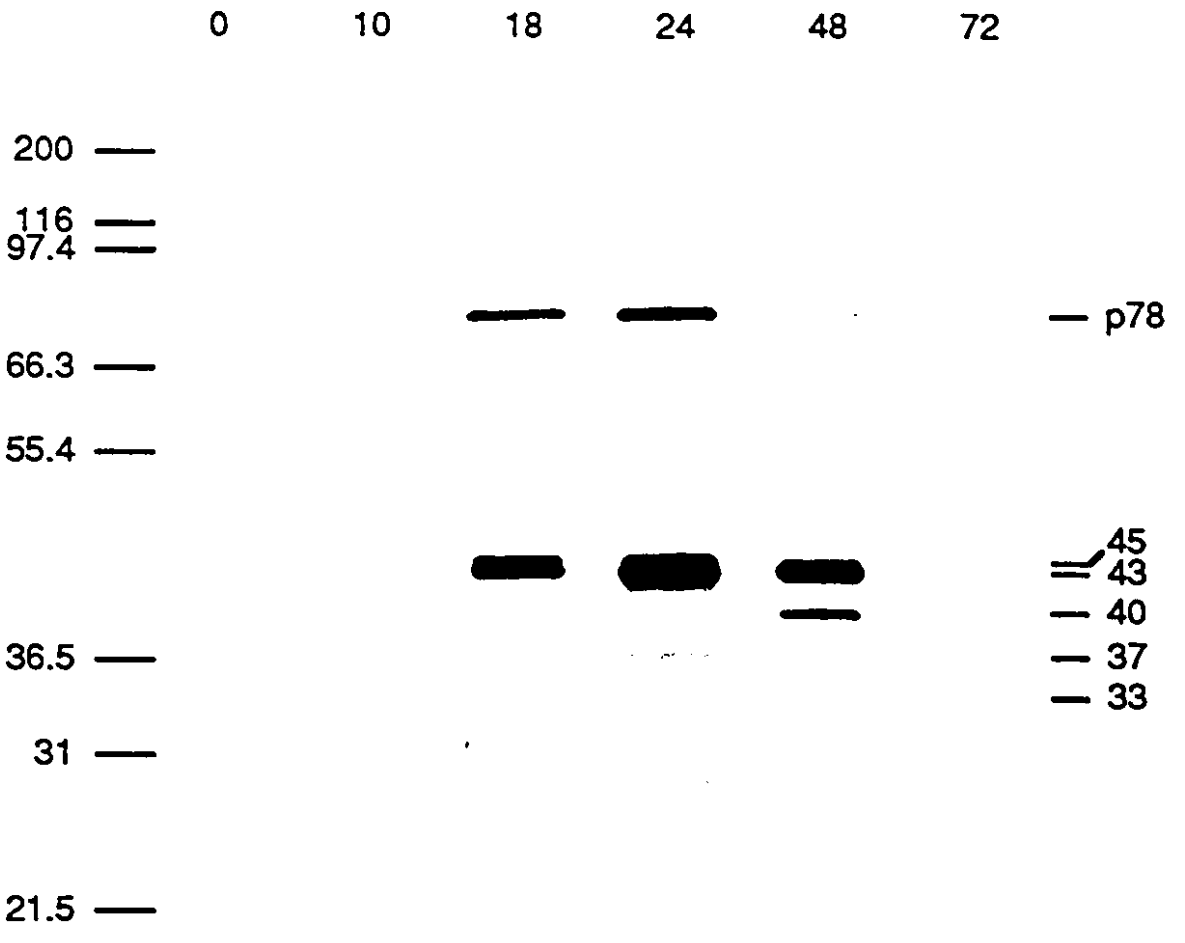
RNA polymerase assays were performed on the nuclear extract and eluted column fractions according to Reinberg and Roeder (1987) with the following modifications. Standard reactions contained 50 mM Tris hydrochloride [pH7.9], 2 mM DTT, 2 mM $(NH_4)_2SO_4$, 4 mM $MnCl_2$, 0.6 mM GTP, 0.6 mM CTP, 0.6 mM ATP, 1 µCi [^{3}H]-UTP, 100 µg/ml herring sperm DNA, and 10 µl of protein fraction in a total volume of 50 µl. Where indicated, 1µg/ml α-amanitin was added to the mixture. The reactions were carried out at 27°C for 30 min and then spotted on 24mm DE81 filter discs (Whatman). The filters were washed with 25 ml of 0.5 M Na_2HPO_4, rinsed in ethanol, and dried. The discs were immersed in Universol scintillation fluid (ICN) and radioactivity counted with a 1219 Rackbeta scintillation counter (LKB).

RESULTS

p78/83 co-immunoprecipitates with a number of infected cell proteins.

Baculovirus-infected Sf9 cells were metabolically labeled and collected at various times postinfection. The cells were lysed and soluble proteins were immunoprecipitated in a nondenaturing buffer with the polyclonal antiserum raised against the baculovirus nucleocapsid-associated protein, p78/83. The precipitated proteins were resolved by SDS-PAGE. An autoradiograph of the resolved proteins is shown in Figure 1. With the labeling conditions used, only the non-phosphorylated (78-kDa) form of the protein was detected. Additional pulse/chase studies determined that phosphorylation of p78 is a relatively slow event, explaining the absence of the phosphorylated form (83-kDa) under short term labeling conditions (data not shown). As previously shown (Vialard and Richardson, 1993), the 78-kDa protein was detected between 18

Figure 1. Immunoprecipitated protein complexes containing p78/83. Autoradiogram of ^{35}S-labelled proteins immunoprecipitated with antibodies directed against p78/83 at various times postinfection. The numbers along the top indicate hours postinfection. The positions of p78 and other labelled proteins are indicated on the right. Numbers on the left indicate sizes (in kilodaltons) of molecular mass markers.

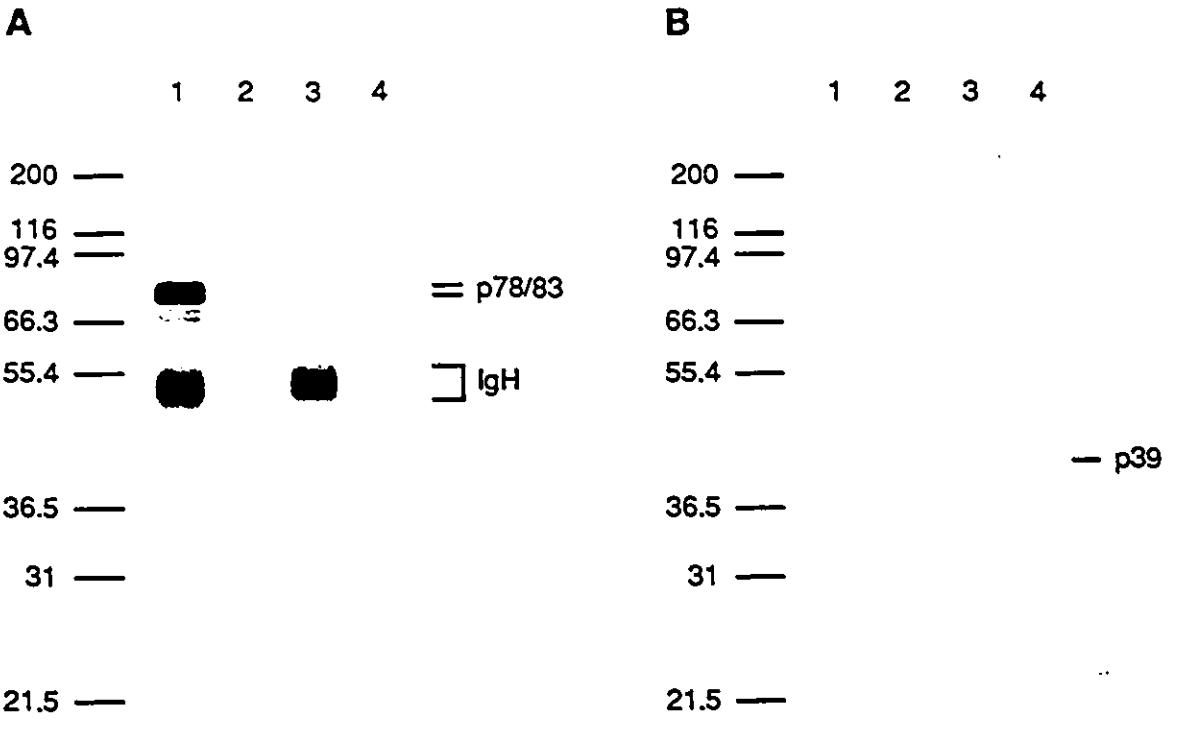

0
10
18
24
48
72
200
116
97.4
66.3
55.4
36.5
31
21.5
p78
45
43
40
37
33

and 48 h postinfection. Several smaller labelled proteins were also detected in the immunoprecipitations from 18 h postinfection onwards. Three polypeptides, with apparent molecular masses of 45, 43, and 37 kDa, were detected in all immunoprecipitations that contained p78 (18 to 48 h). A 40-kDa protein was detected only at the 48 h time point and a protein of approximately 33 kDa was detected at 48 and 72 h postinfection. This last polypeptide probably represents the abundant polyhedrin protein. It was precipitated nonspecifically by pre-immune antiserum (data not shown) and has been detected as a nonspecific contaminant in previous immunoprecipitations (Guarino *et al.*, 1992; Vialard and Richardson, 1993). With the exception of the 33-kDa protein, no other polypeptides were detected at any time point when preimmune antiserum was used in the immunoprecipitations (data not shown).

p39 and pp31 are components of p78/83 immunoprecipitations.

Previous western blot and electron microscopy results demonstrated that p78/83 is a component of the baculovirus nucleocapsid (Vialard and Richardson, 1993). In order to determine whether some of the proteins that coimmunoprecipitated with p78/83 were also components of the nucleocapsid, immunoprecipitations were carried out on nuclear extracts prepared from baculovirus-infected Sf9 cells using antibodies directed against p78/83, the major capsid protein (p39) (Thiem and Miller, 1989b; Pearson *et al.*, 1988; Russell *et al.*, 1991), and another previously identified nucleocapsid protein (p24) (Wolgamot *et al.*, 1993). The immunoprecipitated proteins were resolved through SDS-PAGE, transferred onto nitrocellulose blots and probed with each of the three capsid antibodies. We were not able to detect a protein corresponding to p24 by Coomassie blue stain nor western blot analysis in these immunoprecipitations. However, western blot analysis performed with p78/83 antiserum demonstrated that both forms of the protein (phosphorylated and nonphosphorylated) were present in immunoprecipitations carried out with p39 antibodies (Fig. 2A). They were not detected in immunoprecipitations performed with preimmune polyclonal antiserum or a

Figure 2. Interaction between p78/83 and p39. Immunoblot analysis with p78/83 (A) and p39 (B) antisera of proteins immunoprecipitated from infected cell extracts with four different antisera. The antisera used for immunoprecipitation were: lane 1, p78/83 polyclonal antibodies; lane 2, p39 monoclonal antibodies; lane 3, preimmune polyclonal antibodies; lane 4, nonspecific monoclonal antibodies. The positions of p78/83, p39, and immunoglobulin heavy chains (IgH) are indicated on the right. Numbers on the left represent molecular mass markers (in kilodaltons).


A
1 2 3 4
200
116
97.4
66.3
55.4
36.5
31
21.5
p78/83
IgH
B
1 2 3 4
200
116
97.4
66.3
55.4
p39
36.5
31
21.5

nonspecific monoclonal antibody. Similarly, p39 was detected in immunoprecipitations carried out with the p78/83 antiserum, but not with the two control antisera (Fig 2B). These results indicated that the phosphorylated and nonphosphorylated forms of p78/83 were present in a complex with p39, the major capsid protein.

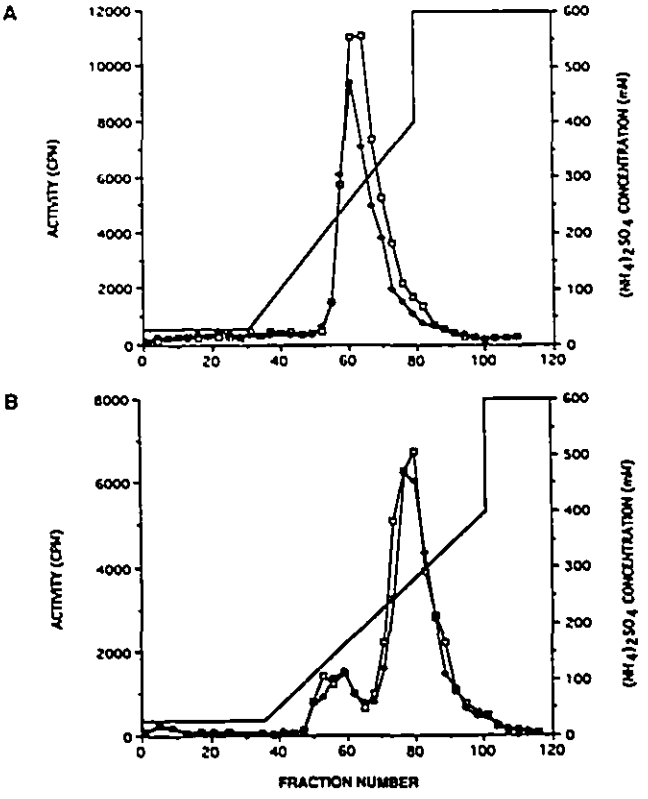
In order to determine the identities of other proteins immunoprecipitated with p78/83, these were transferred onto nitrocellulose membranes and the protein bands were cut from the membranes. The immoblized proteins were treated with cyanogen bromide and the resulting peptides were separated by high pressure liquid chromatography. These were subsequently subjected to Edman degradation microsequencing. A peptide derived from the 37-kDa protein yielded a sequence of 18 amino acids (AKFKTVALKLPVAPSTTE) that corresponded directly with an internal sequence (amino acids 209 to 226) of the nuclear matrix associated phosphoprotein, pp31 (Guarino *et al.*, 1992). Its migration through SDS-PAGE seemed to correspond to that of the most highly phosphorylated form of pp31 (Guarino *et al.*, 1992). We were not able to demonstrate the presence of pp31 in immunoprecipitations performed with the p78/83 antiserum because it migrated close to the immunoglobulin light chains which reacted with the secondary antibody.

p78/83, pp31, and LEF-8 copurify with the virus-induced RNA polymerase activity.

pp31 is a nuclear matrix-associated protein that binds DNA nonspecifically. Its ability to bind DNA suggests that it may be involved in processes such as genome packaging, viral replication, or viral transcription. Western blot analysis and immunoelectron microscopy studies indicated that it was not a component of baculovirus virions (Guarino *et al.*, 1992; our unpublished results). The presence of pp31 in p78/83 immunoprecipitations prompted us to examine other protein fractions that may contain both polypeptides. Viral transcription and replication are believed to take place in the virogenic stroma suggesting that the virus-induced RNA polymerase may be associated with this structure. The virus-induced polymerase was purified from baculovirus-infected Sf21 cells

through a series of chromatographic steps and probed with antibodies directed against several baculovirus proteins, including pp31, p78/83, p39, gp37 (spindle body protein), gp64 (ECV envelope glycoprotein) and LEF-8 (a putative component of the virus-induced RNA polymerase). The *lef-8* gene encodes a predicted polypeptide with a molecular mass of 102 kDa that contains a sequence motif conserved in DNA-directed RNA polymerases of prokaryotes and eukaryotes (Passarelli *et al.*, 1994). The LEF-8 antibodies recognized a 96-kDa baculovirus protein that is expressed late in infection (C. Iorio, unpublished results). In order to confirm that the purified activity belonged to the virus-induced polymerase, chromatographic fractions were assayed in the presence of a-amanitin (an inhibitor of RNA polymerase II) and tagetitoxin (an inhibitor of RNA polymerase III). The nuclear extract from baculovirus-infected Sf21 cells was first resolved through a phosphocellulose P11 column, which resulted in partial purification of all RNA polymerases from other nuclear proteins. Western blot analysis of the fractions containing polymerase activity were probed with antibodies against LEF-8, pp31, p39, p78/83, and gp37. The results indicated that these four baculovirus proteins were still present in the peak fractions (data not shown). However, gp64 (a BV envelope protein) was not detected. The fractions containing RNA polymerase activity were then applied to a Q Sepharose column. At this step, a small peak that eluted before the host polymerases and was not present in parallel purification of RNA polymerases from uninfected cells, was detected (Fig. 3A and B). The fractions corresponding to the novel activity were resistant to both α-amanitin and tagetitoxin, indicating that it did not represent RNA polymerase II or III. Western blot analysis of the fractions eluted from the Q Sepharose column demonstrated that the presence of LEF-8 corresponded directly to the novel activity detected in infected cells (Fig. 3C). Both nucleocapsid proteins (p39 and p78/83) and the matrix associated protein (pp31) also copurified with the virus-induced activity. The spindle body protein (gp37) did not appear to be part of the viral RNA polymerase complex and was eluted after the peak of activity. The fractions containing the viral polymerase were subsequently resolved through a DNA agarose column. Only LEF-8, p78/83 and pp31 were detected in the active fractions eluted from this column; the p39 protein was not present (Fig. 4A and B). The amount of LEF-8 in the active

Figure 3. Q Sepharose column elution profiles of RNA polymerases from uninfected (A) and infected (B) cells, and immunoblot of active fractions (C). (A and B) RNA polymerase activity in the presence (filled box) and absence (open box) of α-amanitin; incorporation of ^{3}H-UMP is indicated on the left. Concentration of $(NH_4)_2SO_4$ in gradient is indicated on the right; fraction numbers are along the bottom. (C) Positions of LEF-8, p78/83, p39, gp37, and pp31 are indicated on the right; load and fraction numbers are indicated along the top; positions of molecular mass markers (in kilodaltons) are on the left.


A
B
ACTIVITY (CPM)
(NH4)2SO4 CONCENTRATION (mM)
FRACTION NUMBER

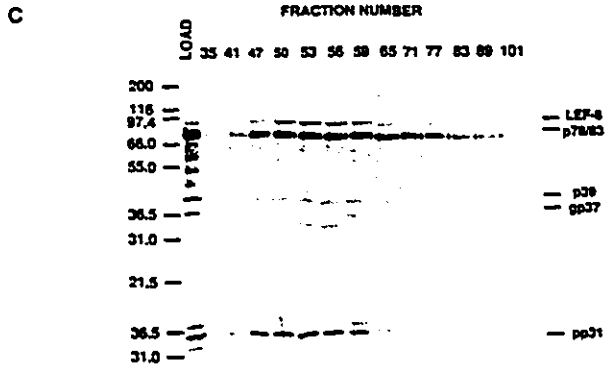
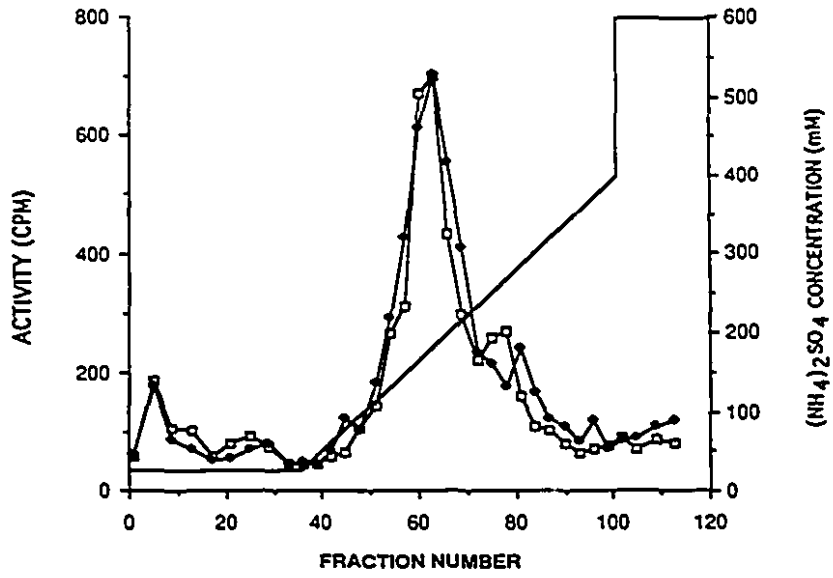
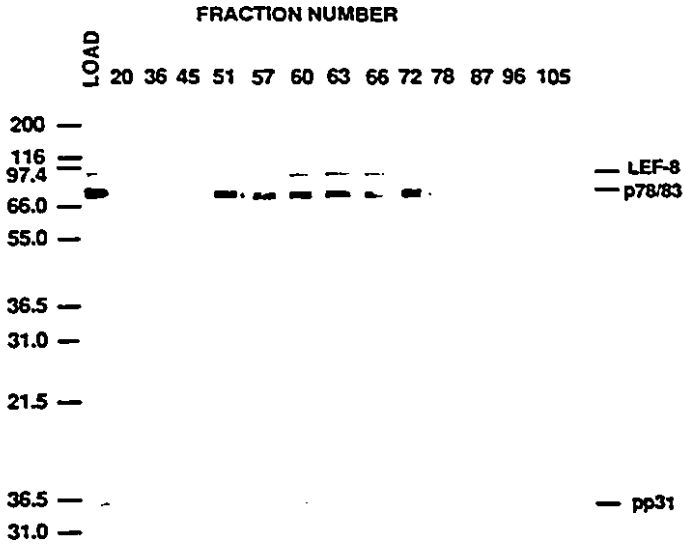

C
FRACTION NUMBER
LOAD
35 41 47 50 53 56 59 65 71 77 83 89 101
200
116
97.4
66.0
55.0
36.5
31.0
21.5
LEF-8
p78/83
p39
gp37
pp31

Figure 4. (A) DNA agarose elution profile of virus-induced RNA polymerase. RNA polymerase activity in the presence (filled box) and absence (open box) of α-amanitin; incorporation of ^{3}H-UMP is indicated on the left. Concentration of $(NH_4)_2SO_4$ in gradient is indicated on the right; fraction numbers are along the bottom. (B) Immunoblot of active fractions. Positions of LEF-8, p78/83, and pp31 are indicated on the right; load and fraction numbers are indicated along the top; positions of molecular mass markers (in kilodaltons) are on the left.

A


800
600
400
200
0
600
500
400
300
200
100
0
0
20
40
60
80
100
120
ACTIVITY (CPM)
$(NH_4)_2SO_4$ CONCENTRATION (mM)
FRACTION NUMBER

B


FRACTION NUMBER
LOAD
20 36 45 51 57 60 63 66 72 78 87 96 105
200
116
97.4
66.0
55.0
36.5
31.0
21.5
36.5
31.0
LEF-8
p78/83
pp31

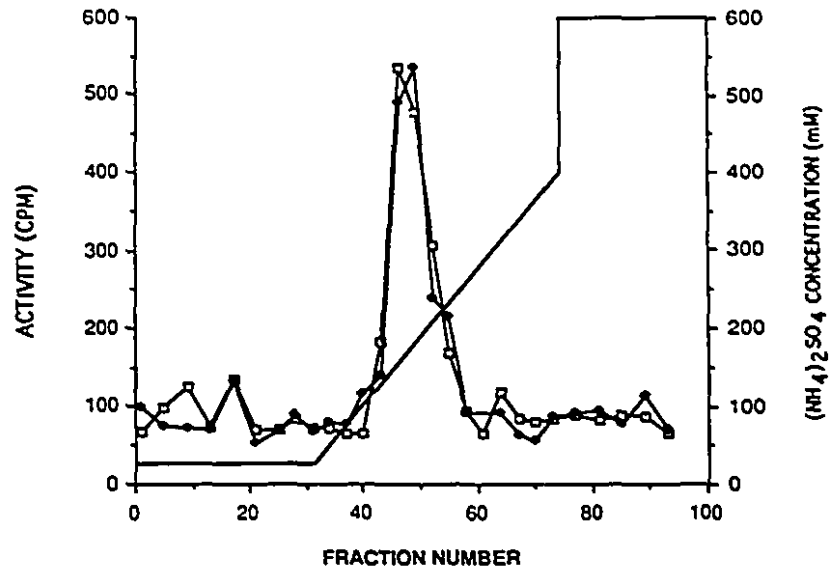
fractions corresponded directly to the level of polymerase activity. The other two proteins, p78/83 and pp31, eluted in the same fractions as the polymerase activity, but their abundance did not correspond completely to the level of activity. The active fractions were purified further through a poly(A)sepharose column. The RNA polymerase activity in the fractions eluted from this final column corresponded only with LEF8; p78/83 and pp31 were eluted slightly ahead of the polymerase activity (Fig. 5A and B) indicating that the association between these proteins and the polymerase had been disrupted. However, p78/83 and pp31 were present in the same fractions suggesting that they remained in association with each other.

DISCUSSION

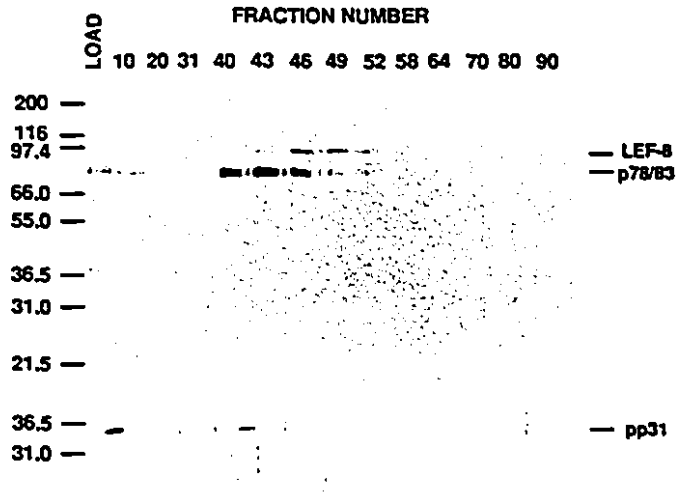
The results of this study demonstrate that the baculovirus nucleocapsid-associated phosphoprotein, p78/83, forms stable complexes with a number of infected-cell proteins (including the major capsid protein and the matrix-associated phosphoprotein) and copurifies with the virus-induced RNA polymerase activity through a number of chromatographic steps. We had previously reported that p78/83 was localized to end-structures of the baculovirus nucleocapsid (Vialard and Richardson, 1993). Detergent treatment of purified virions resulted in removal of the envelope surrounding the nucleocapsids, but had no effect on p78/83. Immunogold staining revealed the presence of this protein at the ends of mature virions embedded within occlusion bodies in the nuclei of infected cells. Staining was also detected in association with nucleocapsids at the periphery of the virogenic stroma, an electron-dense structure that is believed to be the site of various baculovirus processes, including nucleocapsid assembly, replication, and transcription. Another group (Pham *et al.*, 1993) reported that p78/83 was a virion protein associated with the envelope or the space between the nucleocapsid and the envelope. They demonstrated that extensive detergent treatment of purified PDVs resulted in the appearance of p78/83 in the soluble fraction, which led them to suggest that it was not a component of the nucleocapsids. The integrity of the remaining nucleocapsids was not confirmed in that study. Our immunoprecipitation and western blot analysis results confirm a close

Figure 5. (A) Poly(A)sepharose elution profile of virus-induced RNA polymerase. RNA polymerase activity in the presence (filled box) and absence (open box) of α-amanitin; incorporation of ^{3}H-UMP is indicated on the left. Concentration of $(NH_4)_2SO_4$ in gradient is indicated on the right; fraction numbers are along the bottom. (B) Immunoblot of active fractions. Positions of LEF-8, p78/83, and pp31 are indicated on the right; load and fraction numbers are indicated along the top; positions of molecular mass markers (in kilodaltons) are on the left.

A


600
500
400
300
200
100
0
0
20
40
60
80
100
ACTIVITY (CPM)
$(NH_4)_2SO_4$ CONCENTRATION (mM)
FRACTION NUMBER

B


FRACTION NUMBER
LOAD
10 20 31 40 43 46 49 52 58 64 70 80 90
200
116
97.4
66.0
55.0
36.5
31.0
21.5
36.5
31.0
LEF-8
p78/83
pp31

interaction between p39 and p78/83. They were coimmunoprecipitated with antibodies directed against either protein, in the presence of a nondenaturing buffer (Fig. 1) and in the partially denaturing conditions produced by RIPA buffer (Fig. 2). These results suggest that p78/83 and p39 interact directly, supporting the assumption that p78/83 is a component of the nucleocapsid and not the viral envelope or intermediate space. The phosphorylation state of p78/83 did not seem to influence its interaction with p39 since both forms were precipitated with p39 antibodies (Fig. 2).

Microsequencing analysis of the 37-kDa protein detected in p78/83 immunoprecipitations indicated that pp31, the matrix-associated protein, also formed a complex with p78/83. Although a function has not been determined for this protein, it has been shown to bind DNA nonspecifically and copurify with the nuclear matrix after salt extraction (Guarino *et al.*, 1992). This protein is expressed late in infection and has been localized to the virogenic stroma, an electron-dense structure where viral transcription, replication, and virion assembly are thought to take place. We have previously shown that p78/83 is also found at the virogenic stroma late in infection (Vialard and Richardson, 1993). The presence of p78/83 in a complex containing pp31 suggests that these two proteins are involved in a common process. One possible role is packaging of the viral genome. pp31 may act as a scaffolding structure that brings viral DNA and nucleocapsids into close proximity through its dual abilities to bind DNA and interact with p78/83. Alternatively, the presence of pp31 at the virogenic stroma late in infection may indicate a role in baculovirus late transcription (Guarino *et al.*, 1992). We purified the virus-induced RNA polymerase through a number of chromatographic steps and probed the active fractions with antibodies directed against several baculovirus proteins. We raised antibodies against LEF-8, a putative component of the RNA polymerase induced late in baculovirus infection and found that it recognized a 96-kDa protein that was present in the active fractions at all stages of the purification scheme (Fig. 3-5). This result supports the hypothesis that LEF-8 is a subunit of the virus-induced RNA polymerase (Passarelli *et al.*, 1994). The nuclear matrix-associated protein, pp31, copurified with the viral polymerase through three columns and was resolved from the activity only at the last step. A

similar pattern was observed for p78/83, whereas the major capsid protein (p39) was resolved away from the activity in the preceding column. The copurification of pp31 with the purified polymerase suggests that it may be involved in late transcription and supports the hypothesis that it acts as a tethering structure for the baculovirus genome during this process (Guarino *et al.*, 1992). The association of p78/83 with the purified polymerase is less evident since this protein has been previously shown to be a structural component of the nucleocapsid (Vialard and Richardson, 1993). Also, the gene encoding p78/83 was shown to be dispensible for expression from baculovirus late and very late promoters in a transient expression system (Passarelli and Miller, 1993b). Nevertheless, p78/83 may have a structural rather than enzymatic function in association with the polymerase. It has been shown recently that a component of the vaccinia virus-encoded RNA polymerase, RAP94, is required for packaging of the polymerase into virions (Zhang *et al.*, 1994). The life cycle of a mutant virus, in which RAP94 production was repressed, was severely disrupted because newly assembled virions lacked a number of late proteins that are essential for early gene expression. It was suggested that RAP94 forms a complex with proteins required for early gene transcription *in vivo* and targets them to assembling virions. RAP94 copurified with the vaccinia virus RNA polymerase in a fashion similar to p78/83 with the baculovirus-induced RNA polymerase. After three successive chromatographic steps, RAP94 was eventually resolved away from the major activity peak (Ahn and Moss, 1992). The baculovirus nucleocapsid associated-phosphoprotein may have a role similar to that of RAP94. Although baculovirus immediate early gene transcription is not dependent on virion proteins, the presence of these proteins results in a substantial increase in activation of the delayed early genes (Friesen and Miller, 1987; Nissen and Friesen, 1989; Rice and Miller, 1986). This effect is observed in the presence of cycloheximide, an inhibitor of protein synthesis, suggesting that the activation function is associated with a virion protein(s). A protein that may be responsible for immediate activation of the delayed early genes in infections is the *trans*-activator IE-1. This protein was first identified as an early gene activator (Guarino and Summers, 1986a, 1987) and has been recently detected in purified budded virions (Thielmann and Stewart, 1993). It has also been identified as

a late expression factor (Passarelli and Miller, 1993b). The large proline-rich structure of p78/83 may accomodate binding to several proteins (Williamson, 1994). Our immunoprecipitation results (Fig. 1) indicate that it forms a complex with at least four other infected-cell proteins. Its interaction with these proteins may be required for the incorporation of several components into baculovirus virions. The identification of the proteins that form a complex(es) with p78/83 will help to determine the role of the nucieocapsid associated protein in baculovirus infection.

CHAPTER 6

Summary, Conclusions, And Future Prospects

The late phase of baculovirus infection is characterized by three processes that can be related to one another: (1) the production of two types of virions, (2) the occlusion of one of these virion types in the nucleus of the infected cell, and (3) the appearance of a virus-induced RNA polymerase activity that drives transcription from the late gene promoters. Following DNA replication, nucleocapsids are assembled in the nucleus of the infected cell. Some of these migrate out of the nucleus, traverse the cytoplasm, and bud out through the plasma membrane. This BV type is required for cell-to-cell transmission of the virus. The nucleocapsids destined to become PDV remain in the infected-cell nucleus. Eventually they become embedded within large OBs that crystallize in the nucleus. Following the death and eventual disintegration of the infected insect, the OBs are released into the external environment. PDVs are stabilized within the OBs until they are ingested by another insect from contaminated food sources and the OBs are subsequently dissolved due to the alkaline conditions present in the insect midgut. Through the occlusion process, the PDV transmit baculovirus infection from one insect to another. A novel RNA polymerase activity detected late in infection is responsible for transcription of the late and very late baculovirus genes, which encode most of the virion structural proteins, and the abundant polyhedrin and p10 proteins. The results presented in this thesis describe the identification and characterization of two baculovirus late proteins and experiments designed to elucidate their roles in viral replication.

The gene encoding the first late protein, gp37, was found to share significant homology with an entomopoxvirus protein that had been previously identified as a major component of EPV OBs and erroneously named spheroidin (Chapter 2). The baculovirus homologue was produced in relatively low amounts in AcNPV-infected cells and copurified through sucrose gradients with baculovirus OBs. Immunofluorescence studies performed on purified baculovirus OBs that were solubilized in an alkaline solution, revealed staining of structures resembling collapsed OBs. This result suggested that gp37 might be a component of the polyhedral envelope, an alkali resistant structure that surrounds baculovirus OBs (Chapter 2). Subsequent immunoelectron microscopy studies of gp37 in infections with the closely related OpNPV, demonstrated that it was present in

cytoplasmic inclusions and not in OBs (Gross *et al.*, 1993a). In order to clarify the localization of gp37 in AcNPV-infected cells, we analysed its distribution in nuclear and cytoplasmic protein fractions. Our results indicated that gp 37 was primarily associated with the nuclear fraction. Immunoelectron microscopy studies demonstrated that gp37 was present in spindle-shaped bodies that were associated with the nuclear membrane of AcNPV-infected cells (Chapter 3). The spindle-shaped bodies migrated through sucrose gradients in a similar manner as baculovirus OBs and they were also dissolved under analogous alkaline conditions. These characteristics of spindle-shaped bodies explain the immunofluorescence labeling of the empty structures described in the previous study. Staining may have resulted from nonspecific association of solubilized spindle protein with the remaining polyhedral envelopes. In contrast, immunoelectron microscopy studies of purified CbEPV OBs demonstrated that the gp37 homologue was present in spindle-shaped structures embedded within the spheroids. This protein was also shown to be glycosylated (Chapter 3).

Although a function for gp37 was not determined in these studies, it does not seem to be essential to the viral life cycle. We generated a mutant virus in which the gp37 coding sequences were interrupted and no difference in the infection process between this mutant and the wild type virus was observed (Chapter 3). However, the effect of gp37 on infection may be specific to certain insect species or detectable only in certain circumstances. Nevertheless, an alkaline protease activity was detected in association with purified spindle bodies, which are composed primarily of the spindle protein (gp37 or 50-kDa CbEPV OB protein), suggesting a function for this protein during primary infection of the insect midgut cells (Chapter 3). This possibility will be investigated by feeding susceptible larvae purified spindle bodies and OBs (containing PDVs) obtained from the spindle protein negative baculovirus.

The predicted amino acid sequences of a number of baculovirus and EPV spindle proteins were aligned and several well conserved regions were identified (Chapter 3). In particular, a region containing a potential serine protease catalytic residue was present in all the sequences. The

relevance of this sequence with respect to the protease activity might be determined by site-specific mutation of the potential catalytic residue.

The gene encoding the second baculovirus late protein described in this thesis, p78/83, was first identified as a proline-rich polypeptide essential to the virus life cycle (Possee *et al.*, 1991). Immunoblot analysis demonstrated that it was produced in phosphorylated and nonphosphorylated states and that it was evenly distributed in cytoplasmic and nuclear fractions of AcNPV-infected cells (Chapter 4). Its association with nucleocapsids was demonstrated by detergent treatment of purified virions which resulted in the removal of the surrounding viral envelopes. Both forms of p78/83 remained in the protein fraction that represented purified nucleocapsids (Chapter 4). Immunoprecipitation of p78/83 in a stable complex that contained the major capsid protein, p39, confirmed that it was a component of baculovirus capsids (Chapter 5). In addition, immunoelectron microscopy studies demonstrated that p78/83 was associated with the end structures of mature virions embedded within OBs. It was also detected in association with assembling nucleocapsids at the periphery of the virogenic stroma (Chapter 4). The matrix-associated phosphoprotein, pp31, was also immunoprecipitated in a complex with p78/83, suggesting that the phosphoprotein plays a role in attachment of the developing nucleocapsids to the nuclear matrix. Interestingly, both of these proteins (p78/83 and pp31) were detected in association with the partially purified virus-induced RNA polymerase activity (Chapter 5).

The significance surrounding the copurification of p78/83 and pp31 with the RNA polymerase activity was not determined in the studies described in this thesis. However, it may indicate that the role of p78/83 in baculovirus infection is more complex than simply providing a structural element to the nucleocapsid. One possibility is that it acts as a nucleation point for a protein complex that eventually becomes packaged in the virions. A protein that appears to have this function was identified in vaccinia virus (Zhang *et al.*, 1994). Although the virus-induced RNA polymerase is not responsible for baculovirus early gene expression, some of its components may have more than one role and may influence early transcription under certain circumstances. A baculovirus protein, IE-1, which is required for late transcription (Passarelli and Miller, 1993b) as

well as transactivation of early promoters (Guarino and Summers, 1986a; Carson *et al.*, 1991b), is produced throughout the course of infection and was recently reported to be a component of the BV (Thielmann and Stewart, 1993). Immunoblot analysis with antibodies directed against IE-1 might indicate whether it is a component of the purified RNA polymerase. The assignment of a function to p78/83 with respect to the RNA polymerase may be elucidated by the identification of the other proteins detected in a complex(es) with p78/83. The precise role of p78/83 in infection may also be studied through the use of a mutant virus deficient in the production of p78/83. However, since the gene encoding p78/83 is essential to the virus life cycle, a mutant virus can not be generated by standard procedures. One possible strategy is the establishment of a stable cell line expressing p78/83 which could provide a suitable background for the production of a virus defective in p78/83 synthesis. Once purified and amplified, the mutant virus could be used to infect a normal cell line and/or insect larvae and the course of infection monitored. The point at which the viral life cycle was blocked could be determined, providing a clue to the protein's function. Future experiments concerning the role of p78/83 in the viral life cycle with respect to transcription will require further characterization of the components of the virus-induced RNA polymerase. The role of the nuclear matrix in transcription and nucleocapsid assembly will also require further investigation. The reconstitution of the RNA polymerase activity in an in vitro system will help to elucidate the roles of other late proteins in this process.

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ADDENDUM

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Synthesis of the Membrane Fusion and Hemagglutinin Proteins of Measles Virus, Using a Novel Baculovirus Vector Containing the β-Galactosidase Gene

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An improved baculovirus expression vector was developed to expedite screening and facilitate oligonucleotide-directed mutagenesis. This vector contained twin promoters derived from the P10 and polyhedrin genes of *Autographica californica* nuclear polyhedrosis virus. The P10 promoter directed the synthesis of β-galactosidase, whereas the polyhedrin promoter controlled the synthesis of foreign gene products. These two genes recombined with wild-type virus genome to yield recombinants which were polyhedrin negative, produced the foreign gene product, and formed blue plaques when β-galactosidase indicator was present in the agarose overlay. An origin of replication derived from M13 or f1 bacteriophage was also included in the plasmid to permit the synthesis of single-stranded DNA. This template DNA was used to introduce or delete sequences through the process of site-specific mutagenesis. The measles virus virion possesses a membrane envelope which contains two glycoproteins: the hemagglutinin (H) and membrane fusion (F) proteins. The H polypeptide has receptor-binding and hemagglutinating activity, whereas the F protein mediates virus penetration of the host cell, formation of syncytia, and hemolysis of erythrocytes. Genes for these two glycoproteins were inserted into the *Nhe*I cloning site of the modified expression vector described above. The vector and purified wild-type viral DNA were introduced into Sf9 insect cells by calcium phosphate precipitation. A mixture of wild-type and recombinant virus was generated and used to infect Sf9 cells, which were subsequently overlaid with agarose. After 3 days, 0.1 to 1% of the plaques became blue in the presence of β-galactosidase indicator. At least 70% of these blue viral colonies contained the foreign gene of interest as determined by dot blot analysis. Recombinant virus was separated from contaminating wild-type virus through several rounds of plaque purification. Insect cells were then infected with the purified recombinants, and synthesis of H and F proteins was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblot detection and Coomassie blue staining. Glycosylation of the proteins appeared to be impaired somewhat, and the precursor to the F protein was not completely cleaved by the proteases present in insect host cells. On the other hand, both proteins appeared to be active in hemagglutination, hemolysis, and cell fusion assays. Levels of synthesis were in the order of 50 to 150 mg of protein per 10^8 cells.

The baculovirus-insect cell expression system has gained wide popularity as a means of expressing foreign genes for high-level production of relevant proteins (27, 36, 55). Baculovirus expression vectors use the strong, efficient promoter from the polyhedrin gene to direct transcription of the foreign gene. Polyhedrin protein is normally synthesized very late in infection (24 to 72 h postinfection) and can account for 20 to 50% of the total protein made in infected cells. This protein, which is not essential for virus replication, associates with virions in the nucleus to produce protective structures called occlusion bodies. These bodies can easily be discerned by light microscopy (59). The underlying principle behind this expression system relies upon vectors which direct recombination and substitution of the polyhedrin gene with the foreign gene of interest. Recombinant virus is produced, which forms plaques that are occlusion body negative and express the foreign gene product to various degrees.

The most widely used transfer vectors for introducing foreign genes into wild-type baculovirus are pAc373 (50, 51), pE-55 (35), p89B310 (15, 31), pAcYM1 (32), and pVL941 (29). These vectors contain the 5'- and 3'-flanking regions of the polyhedrin gene, the polyhedrin promoter and polyadenylation site, and pUC8 Amp^r for growing the plasmid in *Escherichia coli* bacterial cultures. Insertion of the recombinant gene into the wild-type virus genome relies upon a process of homologous recombination between the flanking sequences of the vector and wild-type DNA. Other vectors consisting of fused polyhedrin and foreign gene-coding sequences have also been developed (28).

Screening recombinant viruses and purifying virus containing the foreign gene away from contaminating wild-type virus can be laborious and time-consuming. This process can involve several rounds of plaque purification by using visual screening or hybridization techniques to detect recombinant plaques. An approach whereby two different promoters controlling β-galactosidase and foreign gene expression recombine as a unit with wild-type viral DNA has proven to be very successful with vaccinia virus (5). Recombinant virus was visualized as blue plaques when β-galactosidase indicator was included in the agar overlay of plaque assays. Recently Pennock et al. (40) have described a baculovirus vector which contains β-galactosidase under control of the polyhedrin promoter together with a unique *Pst*I site for

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insertion of foreign genes under their own promoter control. This vector was used to express chloramphenicol acetyltransferase controlled by the promoter from the long terminal repeat of Rous sarcoma virus (4). Low levels of chloramphenicol acetyltransferase were synthesized in lepidopteran, dipteran, and mammalian cells. Both promoters were tissue specific, and viral replication was host dependent and occurred only in lepidopteran Sf21 cells. Investigators in another laboratory constructed a baculovirus expression vector which contained two polyhedrin gene promoters (10). One promoter controlled synthesis of the normal occlusion body protein, and the other directed synthesis of the nucleocapsid protein from lymphocytic choriomeningitis virus. This vector produced occluded recombinant virus. No baculovirus vector expressing foreign genes under control of the strong polyhedrin promoter together with another promoter controlling β-galactosidase synthesis has yet been described. Such a vector would greatly facilitate the screening and purification of recombinant virus by generating blue recombinant colonies in plaque assays.

Our laboratory has been interested in obtaining large quantities of viral membrane proteins for the purpose of studying virus-host cell interactions. For this reason, we decided to express the two membrane glycoproteins of measles virus in the baculovirus expression system. The membrane fusion (F) and hemagglutinin (H) proteins of measles virus have been cloned and sequenced in our laboratories (1, 43). The H protein is responsible for host cell attachment and confers hemagglutination activity to the virus. The F protein, on the other hand, directs penetration of the host cell by the virus, causes formation of syncytia or giant cells, and mediates hemolysis of erythrocytes. All these activities involve membrane fusion and require processing of a precursor protein (F_0) by a cellular protease (47–49) to yield two disulfide-linked subunits (F_1 and F_2). By analogy, the *env* protein of human immunodefficienty virus is also cleaved by proteases and also possesses membrane fusion activity (26, 30, 33, 53).

The H protein of measles virus was recently expressed in an adenovirus helper-free vector system at levels 65 to 130% of those seen in cells infected with measles virus (2). This system produces functional H protein with accurate glycosylation and cell surface expression. Other paramyxovirus glycoproteins have been expressed in a helper-dependent simian virus 40 (SV40) vector system (38) and the vaccinia virus expression system (39, 54, 60). Levels of expression in these systems are disappointingly low, however. In an attempt to increase the production of recombinant protein, the hemagglutinin-neuraminidase protein of parainfluenza virus type 3 has been synthesized in a baculovirus expression system (7).

In this paper we report the construction of an improved baculovirus expression vector designed to accelerate the screening of recombinant virus and permit oligonucleotide-directed mutagenesis. This vector contained two promoters active very late in infection: the P10 promoter and polyhedrin promoter. P10 is a protein synthesized very late in infection, and it plays some role in the assembly of occlusion bodies (52). The sequences of the P10 promoter and P10 gene product have been reported (21, 24). The P10 promoter has been used in our laboratory to direct the synthesis of β-galactosidase while the polyhedrin promoter controlled the synthesis of the foreign gene products. The two genes, together with their promoters, recombined at high frequency with wild-type viral DNA to yield recombinant virus, which produced blue plaques when infected cells were overlaid with agarose containing β-galactosidase indicator. An origin of replication from f1 bacteriophage was included in the plasmid construction to facilitate the synthesis of single-stranded DNA (ssDNA), which could subsequently be used to introduce mutations with oligonucleotides (34, 46). This vector was used to express the F and H genes of measles virus in Sf9 insect cells. These proteins were produced in large quantities and were biologically active in hemagglutination, hemolysis, and cell fusion assays.

MATERIALS AND METHODS

Cells and Virus. *Spodoptera frugiperda* (SF9) insect cells and *Autographa californica* nuclear polyhedrosis virus (AcNPV) were obtained from the laboratory of Max Summers (Texas A & M University, College Station, Tex.). Cells were cultured in Grace medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum, TC Yeastolate (Difco Laboratories, Detroit, Mich.), lactalbumin hydrosylate, 50 μg of gentamicin sulfate per ml, and 2.5 μg of amphotericin B (Fungizone) per ml in either Falconware T flasks (Becton Dickinson Labware, Oxnard, Calif.) or spinner flasks (Bellco Glass, Inc., Vineland, N.J.) at 28°C by the procedures of Summers and Smith (55).

Escherichia coli DH5 and MC1061 were obtained from David Thomas, Biotechnology Research Institute, Montreal, Quebec, Canada. Bacterial cells were transformed by published methods (13).

Antisera. Antisera were prepared against either oligopeptides or purified proteins by published procedures (44). Rabbit polyclonal antiserum was obtained from the laboratories of Aimo Salmi, University of Alberta, Edmonton, Canada, and Tamas Varsanyi, Karolinska Institute, Stockholm, Sweden. Antiserum directed against the carboxy terminus of the membrane fusion protein of measles virus was prepared from the peptide NH_2-SRPGLKPDLTGTSK SYVRSL-COOH.

Chemicals and reagents. Restriction enzymes were purchased from New England BioLabs, Inc., Beverly, Mass. Oligonucleotides and oligopeptides were synthesized on 380A and 430A synthesizers (Applied Biosystems, Inc., Foster City, Calif.), respectively, at the Biotechnology Research Institute. Radioisotopes (^{125}I-labeled protein A [30 mCi/mg] and [α-^{32}P]CTP [3,000 Ci/mmol]) were from Amersham Canada Ltd., Oakville, Ontario, Canada. Radioactive probes for dot blot hybridizations were synthesized by using the Multiprime DNA-labeling system (Amersham Canada). Endoglycosidase H (endo H) and glycopeptidase F were obtained from Boehringer Mannheim Canada, Dorval, Quebec, Canada. Nitrocellulose paper was supplied by Schleicher & Schuell/Spectrex, Willowdale, Ontario, Canada. "Rainbow" molecular weight standards for proteins came from Amersham Canada. Powdered skim milk came from Carnation, Toronto, Ontario, Canada. African green monkey erythrocytes in Alsevier solution were purchased from IAF Biochemicals, Laval, Quebec, Canada. Bluo-Gal came from Bethesda Research Laboratories, Inc., Gaithersburg, Md. SeaPlaque agarose was obtained from FMC Corp., Marine Colloids Div., Rockland, Maine; Grace insect medium (without hemolymph) and fetal calf serum were obtained from GIBCO/BRL, Burlington, Ontario, Canada.

DNA plasmid and vector constructions. The vector pJV(NheI) was constructed by modifying the parent baculovirus transfer vector pAc373. These steps are summarized in Fig. 1. Purified genomic DNA from AcNPV was digested with *Eco*RI, and the *Eco*RI P fragment (24) was isolated.

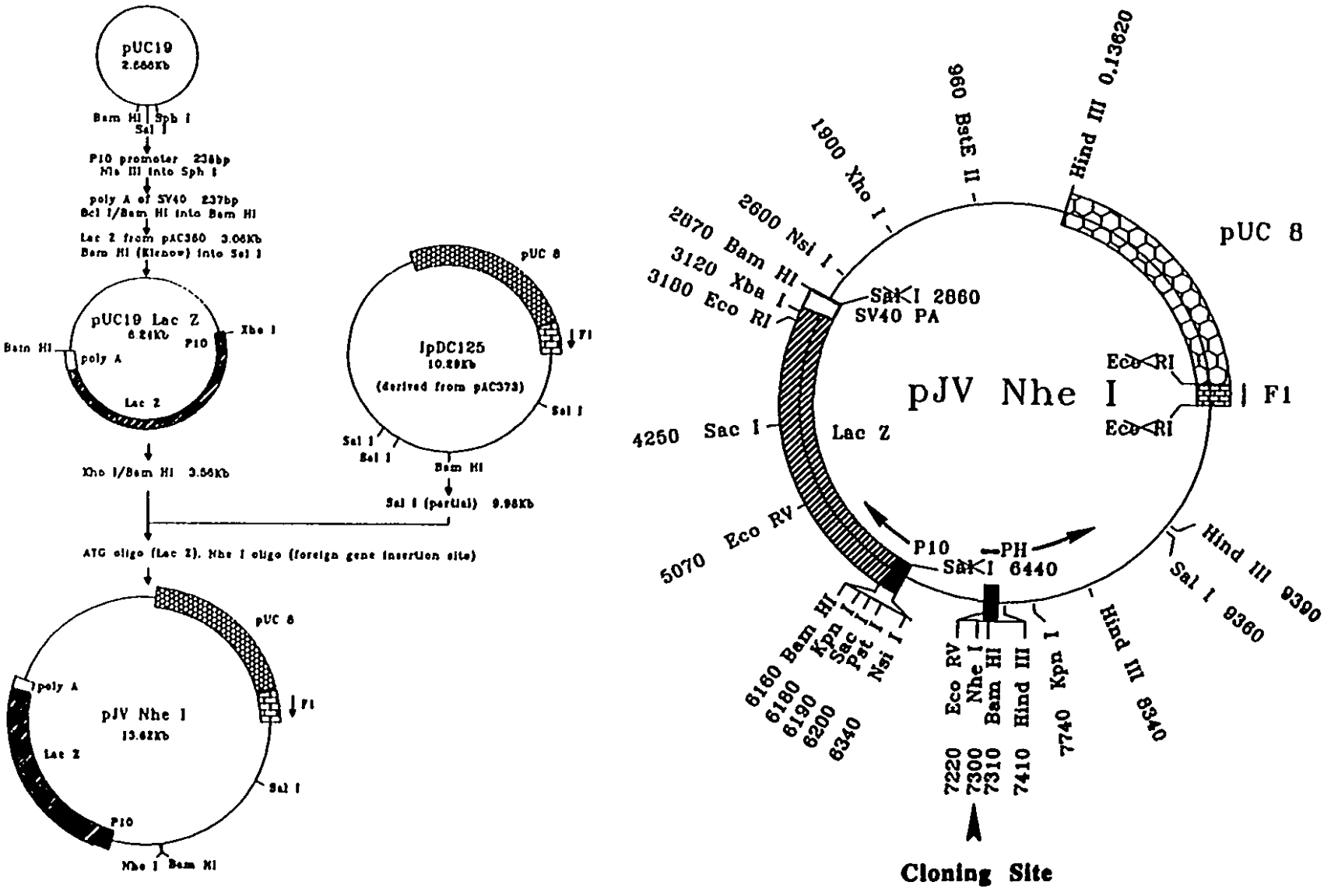


FIG. 1. Construction of the pJV(NheI) baculovirus expression vector. A transcription unit consisting of the P10 gene promoter, the β-galactosidase coding region, and an SV40 polyadenylation signal was synthesized through a series of ligations in the pUC19 shuttle vector. This modified β-galactosidase gene was inserted into the vector IpIDC125 between the two *Sal*I restriction sites lying upstream of the *Bam*HI restriction site. The cloning site for foreign gene insertion was modified to contain a unique *Nhe*I restriction site and an extra 50 nucleotides from the 5′ end of the polyhedrin mRNA to increase levels of expression. Finally, an initiation codon (AUG) was added to the β-galactosidase coding region by using site-directed mutagenesis with a 63-residue oligonucleotide. A map of the restriction endonuclease sites in the vector pJV(NheI) is shown on the right. Locations of the enzymes which cleave the vector in three or fewer positions are indicated as a distance in nucleotides from the *Hind*III site of pUC8. *Eco*RI and *Sal*I enzyme sites which were destroyed during the ligation steps are indicated by crosses. The P10 and polyhedrin (PH) promoters, together with their direction of transcription, are indicated with arrows. Foreign genes were cloned into a unique *Nhe*I restriction site.

This DNA was digested with *Nla*III to produce a 236-nucleotide segment of DNA which contained the promoter and 5′-noncoding region of the mRNA for P10 protein. This *Nla*III fragment was inserted into the compatible *Sph*I site of pUC19. A polyadenylation signal was isolated from SV40 by using the restriction enzymes *Bcl*I and *Bam*HI and ligated into the compatible *Bam*HI site of the previous pUC19 construct. To complete the β-galactosidase transcription unit, the coding region for this enzyme was removed from the vector pAc360 (supplied by Max Summers) by using *Bam*HI. This gene was subsequently inserted into the *Sal*I restriction site of the pUC19 construct by using blunt-end ligation.

Another plasmid, IpDC125, was derived from pAc373 and was formed by including the origin of DNA replication from f1 phage in the vector. This step involved the isolation of a 514-nucleotide fragment from pEMBL8 following digestion of the DNA with *Rsa*I. The f1 origin was inserted into the *Eco*RI site of pAc373 in an orientation which yields ssDNA that is complementary to polyhedrin or foreign gene mRNA. A plasmid with the f1 origin in an opposite orientation (IpDC126) was also constructed but was not used in these experiments. IpDC125 was partially digested with *Sal*I and ligated to the *Xho*I-*Bam*HI fragment of pUC19 LacZ, which contained the transcription unit of β-galactosidase. The orientation of the various fragments was verified by using restriction endonucleases and DNA sequencing across the various junctions.

Additional changes in the modified pAc373 vector described above were made to include a unique *Nhe*I cloning site into which foreign genes could be introduced as well as to create an initiation codon for the β-galactosidase gene. ssDNA was synthesized in *E. coli* CJ236 as described below. Two oligonucleotides [(ATG oligo)ATTTACAATCATGCC TGCAGAGCTCGGTACCATGTGCAGGTCGGATCCCGT CGTTTTACAACG and (*Nhe*I oligo)CAGTTTTGTAATAA AAAAACCTATAAATATTCCGGATTATTCATACCGTC CCACCATCGGGCGTGCTAGCGGATCCTTTCCTGGGA CCCG] were annealed to this ssDNA template, elongated with T4 DNA polymerase, and introduced into competent *E. coli* DH5. Mutant plasmids were identified by mapping with restriction enzymes, and incorporation of the correct oligonucleotide was verified by DNA sequencing.

Oligonucleotide-directed in vitro mutagenesis. The site-directed modifications of the vector described above were performed by using the Muta-Gene in vitro mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.). This technique is based upon the method described by Kunkel et al. (22), in which DNA is synthesized in a *dut ung* double-mutant bacterium to produce nascent DNA which contains a number of uracils in place of thymidine. The uracil-containing strand can be used as a template for in vitro synthesis of an oligonucleotide-primed mutant strand which does not contain uracil. When the resultant double-stranded DNA (dsDNA) is transformed into normal *E. coli* strains, the uracil-containing strand is inactivated and only the non-uracil-containing strand replicates.

Blunt-end ligations. Vector DNA (5 μg) was digested with *Nhe*I, and 5′ protruding ends were filled in with Klenow DNA polymerase. Vector DNA (2 μg) and insert DNA containing the foreign gene (2 μg) were ligated overnight at room temperature in the presence of 0.5 mM ATP and 2 μl (60 U) of T4 DNA ligase. Ligations were repeated the next day for 4 h after the addition of more ligase and ATP.

DNA transfections and plaque assays. Plasmids containing foreign genes were transfected into Sf9 cells together with wild-type viral DNA by using the calcium phosphate precipitation technique (55). Plaque assays were performed as previously described (55) on culture plates (100 by 15 mm). Infected cells were overlaid with 1% SeaPlaque agarose diluted with Grace medium (10 ml per plate). After a 3-day infection the culture plates were overlaid with 1% agarose in Grace medium containing 150 μg of Bluo-Gal per ml (3 ml per plate). The Bluo-Gal was dispensed from a 50-mg/ml solution in dimethylformamide. Blue spots became visible after 6 h.

Isolation of recombinant virus. Plaques which stained blue in the presence of Bluo-Gal were picked with Pasteur pipettes and placed in 1 ml of Grace medium containing 10% fetal calf serum. The virus was allowed to elute from the agarose plug overnight at room temperature. Plaque assays were again performed at 10-, 100-, and 1,000-fold dilutions. Infected cells were overlaid with agarose containing Bluo-Gal, and the blue plaques were picked after 3 days and subjected to plaque purification. Usually three to five rounds of plaque assays were sufficient to generate recombinant virus totally free from contaminating wild-type virus. Isolated recombinant virus was finally amplified in Sf9 cells to yield titers of 10^8 to 10^9 PFU/ml.

Nucleic acid dot blot hybridizations. Microdilution plates containing 24 wells were seeded with Sf9 cells at a density such that they were half confluent. The cells were then infected with either an agarose plug or 50 μl of medium containing recombinant virus and were allowed to incubate for 1 week.

Nucleic acid dot blot hybridizations were performed by a published method (55). Cells in microdilution plates were lysed with sodium hydroxide, neutralized with ammonium acetate, and spotted onto nitrocellulose paper by using a vacuum manifold. These filters were washed in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7]), air dried, and baked at 80°C for 2 h. The samples were prehybridized in 50% formamide–5× SSC–1% glycine–100 μg of denatured herring sperm DNA per ml for 3 h at 45°C. ^{32}P-labeled probes were prepared by using the protocol supplied with the Multiprime nucleic acid kit (Amersham Canada). Probes were prepared from 0.5 μg of purified DNA containing the foreign gene of interest. Hybridizations took place in 50% formamide–5× SSC–1× Denhardt solution–0.3% sodium dodecyl sulfate (SDS)–100 μg of denatured herring sperm DNA per ml for 12 h at 45°C. Nitrocellulose filters were then washed four times with 2× SSC–1% SDS for 10 min at 50°C and finally with 0.2× SSC–0.2% SDS for 5 min at 50°C. The filters were exposed to X-ray film overnight at −70°C.

Polyacrylamide gel electrophoresis and immunoblots. Total cellular proteins were lysed in electrophoresis sample buffer (0.06 M Tris hydrochloride [pH 6.8], 4% SDS, 40% glycerol, 3% dithiothreitol, 0.005% bromphenol blue). DNA was sheared by passage of the sample through a 26-gauge needle 10 times. Samples were applied to 8 or 10% acrylamide gels (acrylamide/bisacrylamide weight ratio, 37.5:1) and subjected to electrophoresis at 100 V overnight by the method of Laemmli (25).

Following electrophoresis, proteins were transferred to nitrocellulose sheets and probed with antibody, and antigen-antibody complexes were detected with radioiodinated protein A (3, 56). The nitrocellulose sheets were incubated for 20 min at room temperature in phosphate-buffered saline (PBS) containing 5% powdered skim milk to block nonspecific binding. The sample was then incubated for 12 h with a 1/100 dilution of antibody in PBS containing 5% milk and

0.05% sodium azide. Filters were washed once with PBS for 10 min, twice with PBS containing 0.1% Triton X-100 for 10 min, and finally again with PBS for 10 min. Radioiodinated protein A (2 μCi) was added to a 40-ml solution of 5% milk–0.05% sodium azide in PBS and incubated with shaking for 2 h. The blots were finally washed with PBS for 10 min, twice with PBS containing 1 M sodium chloride for 10 min, and finally with PBS for 10 min. These nitrocellulose sheets were finally exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens for 6 to 12 h at −70°C.

Analysis of carbohydrates. Proteins were digested with glycopeptidase F or endo H by published methods (45). Cells (2×10^5) containing the appropriate recombinant protein were centrifuged at low speed, and the pellet was suspended in the appropriate enzyme incubation buffer. Incubations with glycopeptidase F were performed by using 100-μl aliquots containing 50 mM Tris hydrochloride (pH 8.6), 25 mM EDTA, 1% Triton X-100, 1% 2-mercaptoethanol, 0.2% SDS, and 0.4 U of enzyme. For endo H digestions, the samples were made up to 100 μl with 0.1 M sodium acetate (pH 5.0), 0.5 M sodium chloride, 1% Triton X-100, 1% 2-mercaptoethanol, 0.2% SDS, and 2 mU of endo H. Incubations were allowed to proceed for 8 h at 37°C. Proteins were precipitated by the addition of 250 μl of cold ethanol, briefly dried under vacuum, and subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis.

Sf9 cells (2×10^5) and Vero cells (2×10^6) were infected with recombinant baculovirus or measles virus, respectively. Tunicamycin was added to the infected cells from a 1 mg/ml stock solution in dimethyl sulfoxide. Sf9 cells were harvested at 60 h postinfection, and Vero cells were collected at 20 h postinfection. The cells were lysed in sample buffer, and proteins were subjected to electrophoresis and immunoblot analysis.

Hemolysis assays. African green monkey erythrocytes were washed twice with 50 ml of PBS and suspended in PBS to give a final 10% suspension. Incubations contained 1.2-ml aliquots of erythrocytes mixed with 200 μl of insect cells (2×10^6 cells) containing the appropriate recombinant proteins or Vero cells (2×10^6 cells) infected with measles virus. Assays were allowed to proceed at 37°C for 12 h, erythrocytes were sedimented by low-speed centrifugation, and the amount of hemoglobin released was quantitated visually or by spectrophotometric measurement at 540 nm.

Cell fusion assays. Monolayers of Sf9 cells in 24-well microdilution dishes were infected with recombinant virus containing the H and F genes at a multiplicity of infection of 3 to 5 PFU per cell. Infections were allowed to proceed for 48 to 96 h in the presence or absence of *N*-acetyltrypsin (0.5 to 2.0 μg/ml). The pH of the medium was varied by adding 0.1 M sodium citrate or PBS buffer. Syncytia formation was monitored under a phase-contrast microscope.

Hemagglutination assays. Hemagglutination titers were determined in the following manner. A 1% suspension of monkey erythrocytes was prepared in PBS. Volumes of 100 μl were aliquoted into 96-well round-bottom microdilution plates. Cells expressing H protein were serially diluted twofold in PBS and added to successive wells in 20-μl volumes. Hemagglutination was allowed to proceed overnight at 4°C.

Sf9 cells infected with H recombinant virus were incubated with monkey erythrocytes and viewed under the microscope with Nomarsky optics. Cells expressing the H protein were cultivated in 24-well microdilution dishes, and 100 μl of a 1% suspension of erythrocytes was added at various times postinfection. The erythrocytes were allowed to adsorb for 2 h at room temperature, and cells were subsequently suspended in Grace medium with a Pasteur pipette. This cell suspension was then observed under the microscope.

RESULTS

Construction of a baculovirus transfer vector containing two very late promoters and the β-galactosidase gene. A baculovirus vector was designed to incorporate features for high-level protein expression, rapid screening of recombinant virus, and effective mutagenesis of foreign genes. The pAc373 vector from the laboratory of Max Summers was modified as outlined in Fig. 1. The additions made to this plasmid are described in detail in Materials and Methods. Briefly, an f1 origin of replication from ssDNA phage was inserted into the *Eco*RI site of pAc373. This addition allows the production of ssDNA which could be packaged by MK07 or R408 helper phage. Oligonucleotides which contained desired mutations or substitutions were annealed to the ssDNA template, elongated with T4 DNA polymerase, and circularized with T4 DNA ligase. A transcription unit containing the promoter for P10 protein, the coding sequence for β-galactosidase, and the polyadenylation signal from SV40 was inserted into the polylinker region of pUC19. The assembled unit was excised and placed between the two *Sal*I restriction enzyme sites located 2,870 and 3,180 nucleotides from the *Hin*dIII site in lpDC125. Removal of this region between the two *Sal*I sites did not appear to affect the replication efficiency of recombinant virus, since titers of 10^8 to 10^9 PFU/ml were routinely obtained. Two final modifications were made by using oligonucleotide-directed mutagenesis. An initiation codon (ATG) was added to the beginning of the β-galactosidase coding region by using a 63-residue oligonucleotide (Fig. 1, ATG oligo) complementary to pAC373 at one end and the β-galactosidase gene at the other. Part of the polyhedrin-coding region and a unique *Nhe*I restriction site were inserted adjacent to the *Bam*HI restriction site of lpDC125 by using a 90-residue oligonucleotide (Fig. 1, *Nhe*I oligo). Fifty nucleotides were inserted at the *Bam*HI site, including the missing 8 nucleotides from the cap leader region of normal polyhedrin mRNA, 33 nucleotides from the coding region of the polyhedrin gene containing an initiation codon which was rendered inoperative by changing it to ATT, and a unique *Nhe*I cloning site. Such modifications purportedly increase the levels of gene expression (28, 29, 31). Oligonucleotide-directed mutagenesis and junctions between the P10 promoter, the β-galactosidase coding sequence, and the SV40 polyadenylation signal were verified by DNA sequencing techniques.

Construction of pJV(*Nhe*I) produced a vehicle which could direct homologous recombination between flanking DNA sequences of the polyhedrin gene in the wild-type virus and similar sequences in the plasmid. As a result, coding sequences for polyhedrin were replaced by a transcription unit containing two promoters which controlled β-galactosidase and foreign gene expression. A similar approach was previously shown to be very successful for the isolation of vaccinia virus recombinants (5). A restriction enzyme map of the completed vector pJV(NheI) is also presented in Fig. 1. The entire vector consisted of 13,620 nucleotides.

Coding sequences for the F and H genes were each inserted into the unique *Nhe*I cloning site of this new transfer vector. Nucleotides from the F gene coding region (1,663 bases) together with five additional nucleotides in

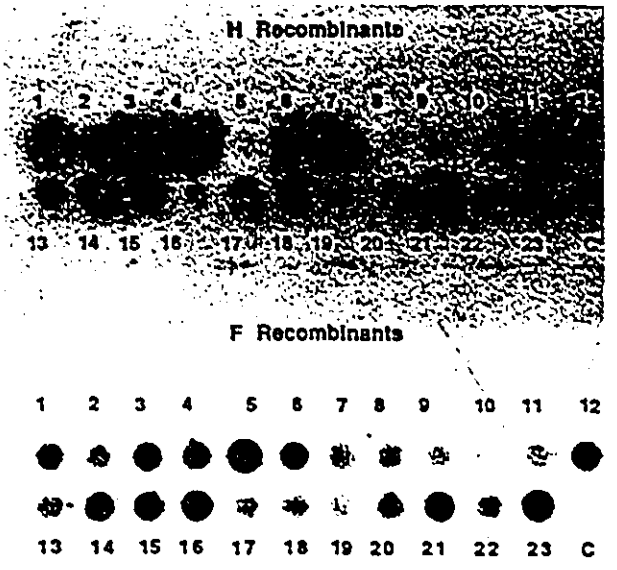


FIG. 2. Dot blot analysis of DNA produced in Sf9 cells infected with virus from the blue recombinant plaques. Agarose plugs were picked from the blue plaques and placed in microdilution wells which contained Sf9 cells. The virus was allowed to propagate for 7 to 10 days. At this time the cells were lysed and DNA was adsorbed to nitrocellulose paper. The nitrocellulose was washed, dried, and hybridized with ^{32}P-labeled probes which were specific to the H and F genes of measles virus. These hybridizations were exposed to X-ray film at −70°C for 8 h. Control cells (C) were infected with wild-type virus.

front of the initiation codon were placed in this plasmid. The H insert (1,911 bases) also contained a few extraneous nucleotides (5 bases) at its 5' terminus along with its amino acid coding region (1,853 bases). The identity and correct orientation of these foreign genes were confirmed by partial DNA sequence analysis and restriction enzyme mapping.

Purification of recombinant baculovirus which contained the H and F genes of measles virus in addition to the β-galactosidase gene. Plasmid DNA [pJV(NheI)] which contained either the H or F gene was introduced into Sf9 insect cells together with purified viral DNA via the calcium phosphate precipitation technique. This transfection was allowed to proceed for 7 days until occlusion bodies were evident. The inoculum was diluted, and recombinant virus was purified from wild-type virus by a series of plaque titrations. At 3 days after infection, each plaque assay was further overlaid with agarose which contained a substrate for β-galactosidase (Bluo-Gal); blue plaques were usually evident after 6 h. The colored plaques continued to increase in intensity over the next day.

Approximately 0.1 to 1% of the plaques were blue, whereas wild-type virus produced white plaques containing occlusion bodies. A number of blue plaques were picked and amplified in microdilution plates which contained Sf9 cells. Lysates of these cells were spotted onto nitrocellulose membranes, hybridized with ^{32}P-labeled probes which were specific for either the F or H gene, and exposed to X-ray film. The dot blot analysis shown in Fig. 2 revealed that 15 of 23 H plaques and at least 18 of 23 F plaques contained both the β-galactosidase gene and the designated foreign gene. Control cells infected with wild-type virus did not hybridize to these probes. Negative blue plaques could be attributed either to lack of growth of virus plaques in culture or to a secondary recombination event in which the foreign gene was deleted from the recombinant.

Blue plaques contained small amounts of contaminating wild-type virus, which often pervaded during isolation of recombinant virus. However, a total of three to five rounds of plaque purification usually produced recombinant virus which did not yield occlusion bodies. The advantage of this screening system is that it permits rapid and efficient selection of recombinant plaques. Plaques produced by using recombinant virus and the pJV(NheI) vector were easier to visualize and could be detected after 3 days as opposed to 5 to 7 days when vectors which did not contain β-galactosidase were used.

Expression of recombinant H and F proteins in Sf9 insect cells. Microdilution plates containing Sf9 cells were infected with either purified recombinant virus or wild-type AcNPV at a multiplicity of infection of 5 PFU per cell. At specific times following infection, 10^5 cells were washed twice with PBS, lysed with SDS-electrophoresis sample buffer, applied to SDS-polyacrylamide gels, and subjected to electrophoresis. Proteins were transferred to nitrocellulose and probed with polyclonal antibody specific for the H or F protein. Specific binding was detected when using ^{125}I-protein A followed by autoradiography. The results of these experiments are presented in Fig. 3A and Fig. 4A. In both cases, H and F recombinant proteins appeared to be synthesized starting at 24 h postinfection and continuing up to 60 h postinfection. Infected cells appeared to be dying by 96 h postinfection.

Two species of H protein appeared to be synthesized. A high-molecular-mass species migrated at 76 kilodaltons (kDa) and comigrated with H protein made by Vero cells which were infected with measles virus. Another protein species migrated with a mobility corresponding to 65 kDa. We suspected that the 76- and 65-kDa species were glycosylated and nonglycosylated forms of the H protein, respectively. Fainter bands which migrated at low molecular masses were most probably degradation products of the H protein. The antiserum appeared to be extremely specific for the H protein, since proteins synthesized in cells infected with wild-type baculovirus failed to react with the antibodies.

Recombinant fusion (F) protein was only partially cleaved in the baculovirus expression system. A range of precursor (F_0) species (56 to 65 kDa) as well as the F_1 subunit (42 kDa) were apparent on the immunoblot autoradiogram shown in Fig. 4A. Polyclonal antisera prepared against the entire F protein failed to react with the small, carbohydrate-rich F_2 subunit (15 kDa) produced in either mammalian or insect cells. Multiple F_0 species could again be explained by differences in glycosylation by the insect cells compared with mammalian cells. The largest F_0 molecules in insect cells migrated at the same rate as F_0 in Vero monkey kidney cells. A smaller F_0 species (Fug) probably reflected incomplete glycosylation within the insect cells and appeared to be the predominant protein synthesized in cells infected with the F recombinant virus. A similar protein (Fug) was also evident in Vero cells at an early stage of infection by measles virus. Since the F_1 subunit of measles virus is not associated with sugars, it migrated exactly at the same rate as F_1 synthesized in mammalian cells. The results for F protein expression in insect cells are not surprising since these cells are known to be deficient in the terminal glycosylases and endoproteolytic enzymes involved in protein processing (19, 20, 36, 41).

Quantities of recombinant protein made in the baculovirus

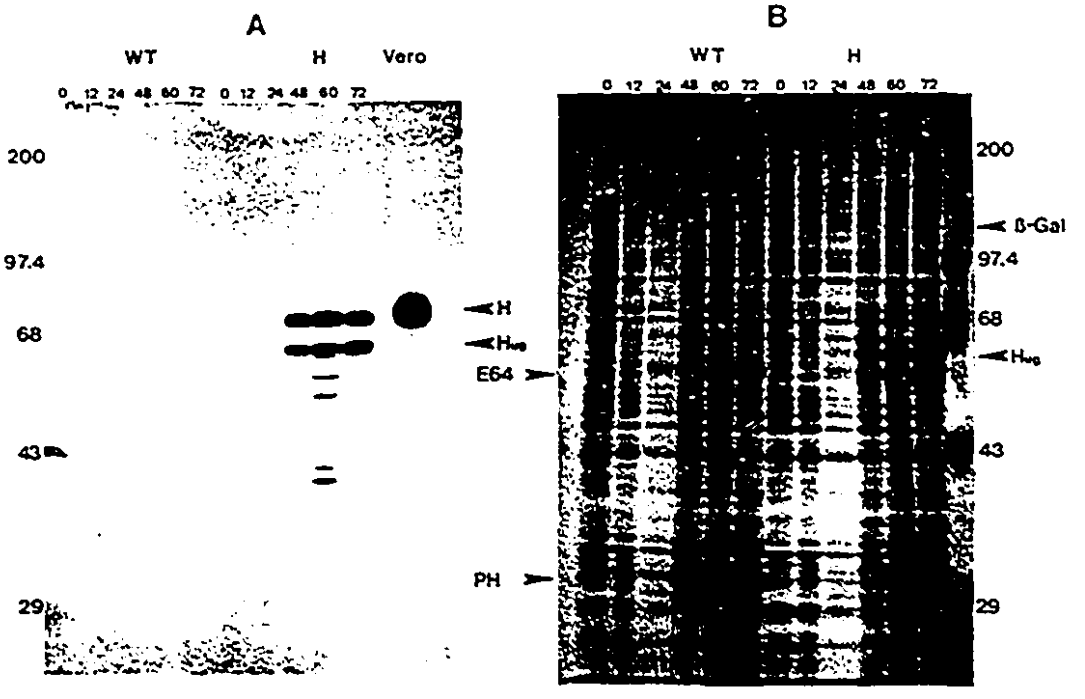


FIG. 3. Immunoblot and Coomassie blue-stained gel of total proteins produced in Sf9 cells infected with either wild-type or recombinant H virus. Sf9 cells were infected with wild-type baculovirus or recombinant virus containing the H gene of measles virus. Total proteins were solubilized by lysing the cells in sample buffer at 0, 12, 24, 48, 60, and 72 h postinfection. A positive control (lane Vero) prepared from 10^6 Vero monkey kidney cells infected with measles virus was also included. These proteins were subsequently separated by SDS-polyacrylamide gel electrophoresis. (A) Autoradiogram of an immunoblot prepared from a gel which was a duplicate of the one shown in panel B. The immunoblot was obtained by electrophoretic transfer of proteins from a polyacrylamide gel to nitrocellulose. Numbers indicate the position of colored molecular mass standards (in kilodaltons) which were transferred from the polyacrylamide gel to the nitrocellulose sheet. This nitrocellulose sheet was probed with rabbit polyclonal antiserum directed against the H protein, and antibodies bound to H protein species were detected by using ^{125}I-labeled *Staphylococcus* protein A. The immunoblot was exposed to X-ray film with an intensifying screen for 12 h at −70°C. (B) Duplicate gel stained with Coomassie blue dye. Protein standards of 200, 97.4, 68, 43, and 29 kDa are indicated by number. The polyhedrin protein (PH) is clearly evident throughout the wild-type infection but is absent in cells infected with the recombinant virus. E64 represents the major envelope protein of the baculovirus AcNPV. The electrophoretic migrations of β-galactosidase (β-Gal) and H recombinant protein products (H, Hug) are indicated with arrows. Hug refers to the unglycosylated precursor protein which migrates with a molecular mass of 65 kDa.

expression system are generally larger than those produced in mammalian systems (27). Proteins on duplicate acrylamide gels to those described above were stained with Coomassie blue and are shown in Fig. 3B and 4B. Proteins corresponding to the unglycosylated forms of H and F_0 could be detected with this dye. At least 20% of the total stained proteins could be represented by H and F polypeptide species through densitometric scanning of gel photographs. Since 250 μg of total protein (from 10^5 cells) was loaded for each lane on the electrophoretic gel, we estimated that the yields of H and F proteins synthesized in this system were in the order of 50 to 150 mg of protein per 10^8 cells. Production of H or F gene products in insect cells remained stable even after five passages of the purified recombinant virus in culture.

Glycosylation of H and F proteins in insect cells. Previous studies indicated that insect glycoproteins were first synthesized and attached to a typical high-mannose oligosaccharide, [Asn]-$GlcNAc_2$-Man_9-Glc_3 (16, 19). These mannose-rich precursors are sensitive to endo H. Further processing of N-linked glycans leads to the formation of proteins with a trimannosyl core ([Asn]-$GlcNAc_2$-Man_3) which is now endo H resistant. Since galactosyl and sialyl transferases are absent in insect cells, the trimannosyl core represents the fully processed oligosaccharide (36).

Four distinct species of F_0 precursor proteins (56 to 61 kDa) were synthesized in insect cells containing the F recombinant virus (Fig. 5). These polypeptides may represent partial glycosylation at the three asparagine sites on the F_2 subunit or may reflect various stages during the processing of the mannose-rich glycoprotein precursor (19, 43). Similar gel patterns were previously observed during immunoprecipitations of the F_0 protein from Vero monkey kidney cells infected with measles virus (43). Three species of F_0 protein were observed in these infected Vero cells. To demonstrate that the heterogeneity of F_0 was in fact due to the presence or absence of carbohydrate, insect cell lysates containing the F protein were digested with either endo H or glycopeptidase F (Fig. 5A). Endo H removes sugars from the high-mannose glycoprotein, leaving one molecule of *N*-acetylglucosamine attached to the asparagine. On the other hand, glycopeptidase F cleaves all types of asparagine-linked N-linked glycans and completely removes all carbohydrate from the glycoprotein.

The top band from the group of four F_0 polypeptides was sensitive to endo H, indicating that it represented the mannose-rich core polypeptide. The other enzyme, glycopeptidase F, completely converted the two larger species to proteins migrating with molecular masses of 56 and possibly 59 kDa. The 59-kDa polypeptide represented the major

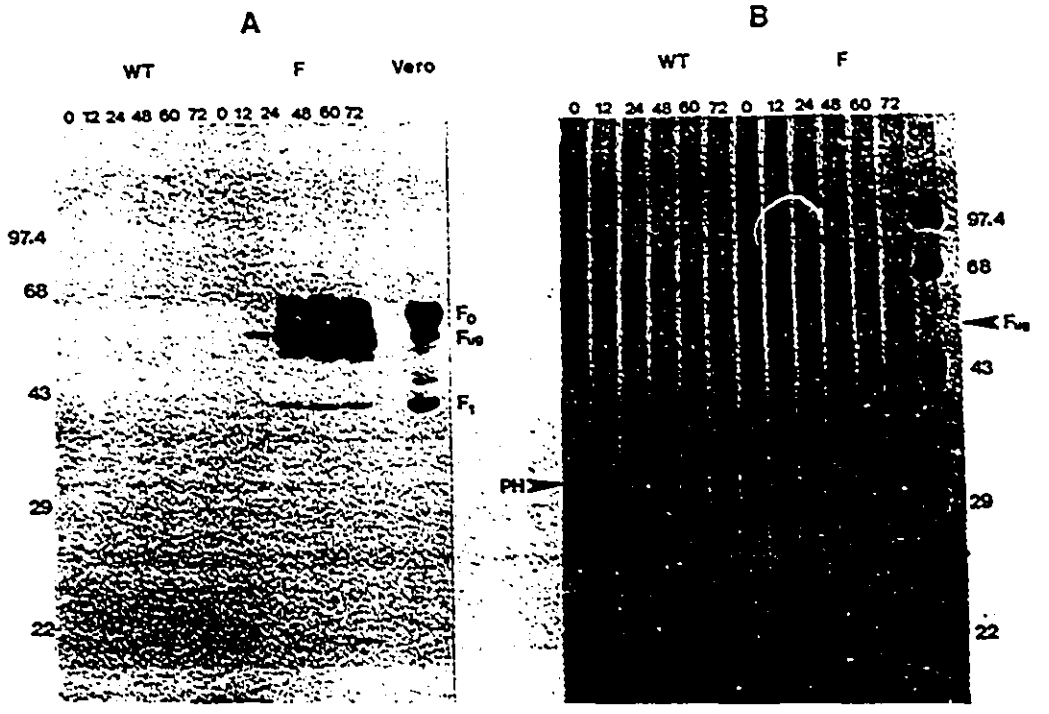


FIG. 4. Immunoblot (A) and Coomassie blue-stained gel (B) of total proteins produced in Sf9 cells infected with either wild-type or recombinant F virus. Experimental procedures were similar to those described for Fig. 3. F protein was detected on immunoblots with a polyclonal antibody directed against the entire polypeptide. The recombinant F_0 precursor protein, F_1 cleavage product, and β-galactosidase proteins are indicated by arrows. Protein molecular mass markers (in kilodaltons) are indicated by numbers. PH refers to the polyhedrin protein of AcNPV. Fug indicates the major unglycosylated species of F_0 in infected cells.

protein synthesized in Sf9 cells infected with F recombinant baculovirus. The cleaved subunit, F_1, was unaffected by glycosidases, since no carbohydrate was attached to this polypeptide (58). Unfortunately, the F_2 subunit was not detected with the polyclonal antisera made available to us.

Addition of tunicamycin (an inhibitor of Asn-linked glycosylation) to Sf9 cells infected with the F recombinant abolished the synthesis of the two largest (61 and 65 kDa) F_0 polypeptides (Fig. 5C). Two unglycosylated species (59 and 56 kDa) may correspond to the unglycosylated F_0 precursor, with and without its signal peptide at the amino terminus. Vero monkey kidney cells normally support the replication of measles virus. Tunicamycin treatment of these mammalian cells prevented the posttranslational cleavage of F_0, reduced glycosylation to yield the 56-kDa polypeptide (Fig. 5C), and also dramatically inhibited the formation of syncytia by infected cells. We hypothesized that the difference in molecular masses between the 59- and 56-kDa F_0 proteins may be due to defective processing of the membrane signal peptide by insect cells. Direct sequencing of the amino termini of these two polypeptides must be performed to substantiate this theory. Finally, further studies involving addition of radioactive sugars, pulse-chase experiments, peptide mapping, and protein sequencing are required to precisely define and identify these multiple F_0 bands.

Two major protein species (68 and 65 kDa) were recognized by antisera directed against the H protein synthesized in insect cells. To prove that the lower band represented the nonglycosylated precursor, we digested proteins with endo H and glycopeptidase F. The results are summarized in Fig. 5B. Endo H appeared to have little effect upon the higher-molecular-mass species, and this indicated that most of the mannose-rich precursor was processed to the trimannosyl core. On the other hand, glycopeptidase F completely converted the high-molecular-mass species to the smaller form. Tunicamycin treatment of infected Sf9 or Vero cells also modified the high-molecular-mass species to the faster-migrating 65-kDa protein (Fig. 5D). These experiments proved definitively that the lower protein band was a nonglycosylated form of the H polypeptide.

Recombinant H and F proteins were biologically functional in hemagglutination, hemolysis, and cell fusion assays. Recombinant H and F proteins were demonstrated to be biologically active in experiments involving cell attachment and membrane fusion assays. African green monkey erythrocytes were incubated with intact Sf9 cells which contained recombinant H protein. Hemagglutination between erythrocytes and these Sf9 cell suspensions was evident, and a reciprocal dilution titer of 2,048 was obtained. On the other hand, wild-type measles virus supernatants produced a titer of 512. Binding of erythrocytes to Sf9 cells which expressed H protein was also observed under the microscope (Fig. 6). Insect cells which contained wild-type AcNPV, or cells which were uninfected, failed to agglutinate erythrocytes. Thus, the recombinant H protein appeared to possess the cell-binding activity characteristic of the hemagglutinin molecule of measles virus.

Hemlyosis of monkey erythrocytes can be produced by measles virus. This activity can be ascribed to the cleaved membrane fusion protein of this virus. Insect cells containing H or F recombinant proteins were frozen and thawed three times and incubated with erythrocytes overnight. This treatment was previously shown to increase the hemolytic activity of measles virus (11). Hemoglobin was released into

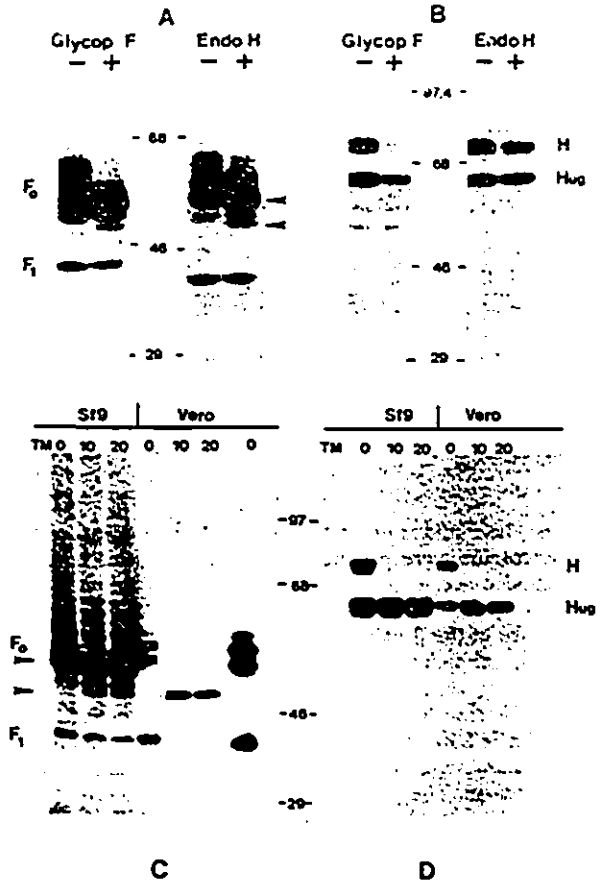


FIG. 5. Analysis of carbohydrate attached to recombinant F and H proteins by using glycopeptidase F (Glycop F), endo H, and tunicamycin (TM). Sf9 cells were infected as described for Fig. 3 and were harvested at 60 h postinfection. Vero monkey kidney cells were infected with measles virus (10 PFU per cell) and were collected at 20 h postinfection. In panels A and B, recombinant proteins from Sf9 insect cells were digested with the appropriate enzyme for 8 h as outlined in Materials and Methods. (A) Effects of endo H and glycopeptidase F digestion upon recombinant F protein; (B) effects of the enzymes upon recombinant H protein. (C and D) Effects of tunicamycin upon glycosylation of F and H proteins, respectively. Tunicamycin was present in the medium of infected Sf9 or Vero cells at concentrations of 0, 10, and 20 μg/ml. F_0 represents the precursor to the mature fusion protein, and F_1 refers to the large subunit of the active molecule. H represents the fully glycosylated hemagglutinin polypeptide, and Hug denotes the unglycosylated species. Numbers refer to the migration of colored molecular mass standards which were transferred to the nitrocellulose sheet from the polyacrylamide gel. Arrows indicate the major (59-kDa) and minor (56-kDa) unglycosylated F_0 species found in Sf9 cells infected with F recombinant virus.

the media from erythrocytes which had been treated with insect cells containing F recombinant virus or insect cells coinfected with H and F recombinant virus. H and F proteins acted synergistically to increase the level of hemolysis. This observation may reflect a requirement for cellular attachment (mediated by H) prior to membrane fusion (mediated by F) to produce an optimal level of hemolysis. Vero cells infected with wild-type measles virus also hemolyzed the erythrocytes. The level of hemolysis produced by the infected Vero cells was much greater than that elicited by insect cells infected with H and F recombinant virus. This observation may reflect a greater proportion of cleaved fusion protein at the surface of mammalian cells infected with measles virus in comparison with insect cells. Unfortunately, addition of exogenous trypsin did not increase the amount of hemolysis due to the presence of recombinant F protein. Insect cells which contained wild-type AcNPV or recombinant H protein had little effect upon the erythrocytes. These results are summarized in Table 1.

Formation of syncytia or giant cells within plaques formed by recombinant F_0 virus were not visible by phase-contrast microscopy. We attempted to demonstrate fusion of insect cells by infecting Sf9 cells with H, F, or a combination of H and F virus in the presence or absence of trypsin (0.5 to 2 μg/ml). The addition of trypsin was previously shown to increase levels of infectivity and membrane fusion activity of paramyxoviruses (14, 47). Formation of syncytia was still not evident under these conditions. The acidity of the cell culture media was then varied from a normal pH of 6.2 to 5.8, 7.0, and 8.0 since the pH requirements for membrane fusion are known to differ between groups of viruses (61). Fusion activity became dramatically apparent at pH 5.8 (Fig. 7C). Expression of F protein by itself was capable of producing polykaryons in insect cells. The addition of H recombinant virus or trypsin did not appear to enhance cell fusion substantially in this case. One might speculate that F protein expressed at the cell surface was previously cleaved en route to the plasma membrane. Also, a receptor for the viral H protein does not appear to be present on insect cells, since Sf9 cells producing recombinant H protein failed to aggregate. Thus, H protein might not be expected to enhance fusion activity between adjacent insect cells. Finally, control experiments with insect cells infected with H recombinant virus (Fig. 7A) and wild-type AcNPV (Fig. 7B) exhibited fewer syncytia and less membrane fusion activity at pH 5.8 when compared with cells infected with the F recombinant (Fig. 7C).

We concluded from these experiments that both recombinant H and F proteins were functional in biological assays for erythrocyte attachment, hemolysis, and membrane fusion.

DISCUSSION

An improved baculovirus expression vector was constructed in our laboratory, and its properties and characteristics are described in this communication. The vector was designed to accelerate the screening of recombinant virus, direct the synthesis of large quantities of protein, and facilitate oligonucleotide-directed mutagenesis of foreign genes. This DNA construct contained two promoters, from the P10 and polyhedrin genes, which are normally active very late in infections produced by AcNPV. The P10 promoter was used to direct the synthesis of β-galactosidase, an enzyme which hydrolyzes the substrate Bluo-Gal (halogenated indoyl-β-D-galactoside) to produce a dark-blue product. The other promoter, which normally regulates the synthesis of polyhedrin, was used to direct the transcription of foreign genes. An origin of replication from f1 phage was also included in the plasmid construction to facilitate the synthesis of ssDNA and subsequent mutagenesis by using complementary oligonucleotides. Both the β-galactosidase and foreign genes together with their respective promoters recombined at high frequencies with wild-type viral DNA to yield recombinant virus. This virus produced blue plaques when infected cells were overlaid with agarose containing β-galactosidase indicator. The vector pJV(NheI) was used to

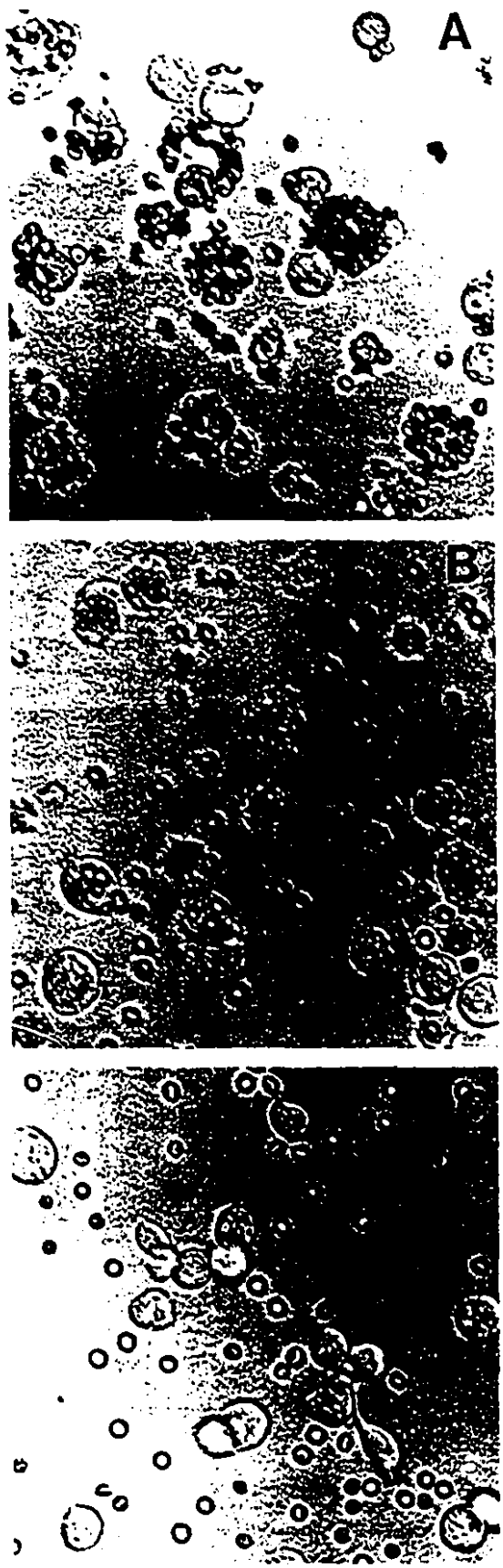


TABLE 1. Hemolysis of erythrocytes by insect cells infected with H and F recombinant baculoviruses

Cell type added to assay[a]	A_{540}[b]
None	0.112
Uninfected Sf9 cells	0.150
Sf9 cells infected with wild-type AcNPV	0.174
Sf9 cells infected with H recombinant virus	0.179
Sf9 cells infected with F recombinant virus	0.320
Sf9 cells infected separately with H and F recombinant virus	0.381
Sf9 cells coinfected with H and F recombinant viruses	0.713
Vero cells infected with wild-type measles virus	1.90

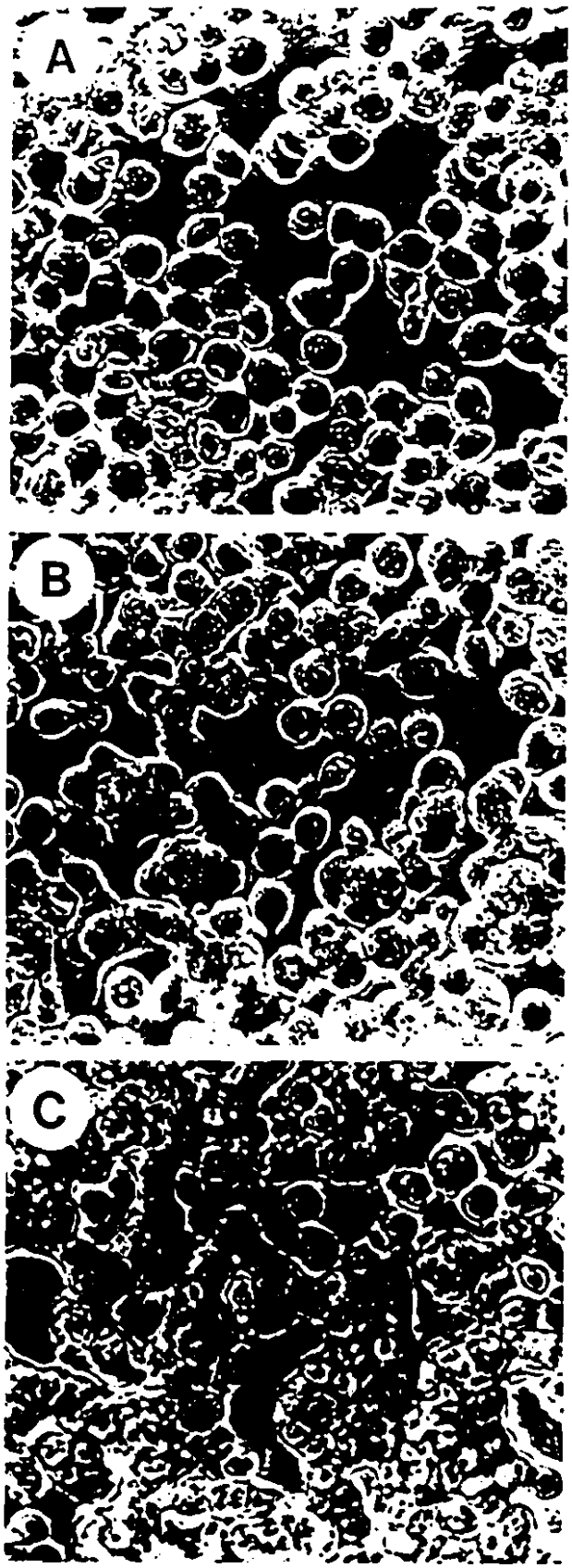
[a] The cells specified were infected with H or F recombinant virus, wild-type AcNPV, or wild-type measles virus. A total of 0.5×10^6 to 1.0×10^6 cells was added to each assay, and hemolysis was allowed to proceed as described in Materials and Methods.

[b] Incubation of erythrocytes with infected cells was allowed to proceed for 12 h at 37°C, and erythrocytes were subsequently sedimented by low-speed centrifugation. Hemoglobin released into the supernatant was quantitated by measuring the A_{540}.

express the F and H proteins of measles virus in insect cells. Both proteins were produced in large quantities and were biologically active in hemagglutination and hemolysis assays.

The use of the β-galactosidase gene for screening baculovirus recombinants was previously suggested but not demonstrated by other investigators. Investigators in this laboratory (40) developed a generalized transplacement vector (pGP-B6874/Sal) which could facilitate the selection of recombinant virus with foreign genes under their own promoter control. The vector was subsequently used to express chloramphenicol acetyltransferase under control of the Rous sarcoma virus long terminal repeat promoter in mammalian and dipteran cells (4). Protein was produced at very low levels in this experiment. Investigators in another laboratory suggested that baculovirus recombinants could be screened by the absence of β-galactosidase activity following transfection of Sf9 cells with a vector containing a foreign gene and recombinant baculovirus DNA expressing the β-galactosidase gene (55). However, this method still yields recombinant plaques which are difficult to visualize. Investigators working with vaccinia virus have previously used β-galactosidase to screen recombinant virus during expression studies (5, 57). Chakrabarti et al. (5) developed a coexpression vector (pSC11) for the isolation of vaccinia virus recombinants expressing hepatitis B surface antigen. This vector contained the hepatitis B surface antigen gene and *lacZ* gene under the control of the separate promoters P7.5 and P11, respectively. These two genes were inserted into the thymidine kinase (TK) locus of vaccinia virus and were bounded by the TK gene-flanking sequences. Tissue culture cells that had been infected with vaccinia virus were trans-

FIG. 6. Hemagglutination of monkey erythrocytes by insect cells expressing recombinant H protein. Insect cells were infected with wild-type or recombinant baculovirus for 70 h. Monkey erythrocytes were added at this time and were allowed to adsorb to infected cells for 2 h at room temperature. Cells were suspended in Grace medium and observed under the microscope with Nomarsky optics at a magnification of ×680. (A) Agglutination experiment performed with insect cells infected with the H recombinant. (B) Control experiment performed with cells infected with wild-type AcNPV. Occlusion bodies within the infected cells are evident and differ in size and density from those in adsorbed erythrocytes. (C) Uninfected cells. These failed to bind erythrocytes.



fected with the plasmid vector, and homologous recombination between wild-type virus and plasmid DNA occurred. TK⁻ recombinants were selected by a plaque assay on a TK⁻ cell line in the presence of 5-bromodeoxyuridine, which inhibited the growth of wild-type virus containing TK. Plaques that were TK⁻ and expressed β-galactosidase as well as hepatitis B surface antigen were shown to stain blue in the presence of indicator. The vaccinia virus expression system has the advantage of positive selection for recombinants in the presence of 5-bromodeoxyuridine. Consequently, fewer rounds of plaque purification are required with the vaccinia virus expression system than for the method developed in our laboratory for baculovirus. However, the presence of β-galactosidase in pJV(NheI) still considerably reduces the time for appearance of viral plaques.

We were concerned that insertion of the β-galactosidase transcription unit into the *SalI* region of pAc373 might disrupt either the function of the polyhedrin promoter or the replication of the recombinant virus. However, this modification appeared to have no effect on the expression of the foreign genes under the control of the polyhedrin promoter. In fact, expression of the H gene in pJV(NheI) was consistently higher than with the pAc373 vector (C. Richardson, unpublished data). Increased levels of expression of pJV(NheI) were due to additional nucleotides inserted in the polyhedrin leader region of pAc373. Since it was difficult to purify recombinant virus from contaminating wild-type virus, we were worried that normal virus might supply some sort of helper or enhancing factor to the recombinant. However, coinfection of recombinant virus with virus-type AcNPV or another recombinant virus generated from pAc373 failed to increase levels of foreign gene or β-galactosidase expression. Recombinant virus also appeared to grow at the same rate as wild-type AcNPV once it was plaque purified. In summary, most blue plaques contained the foreign gene of interest, β-galactosidase activity, as well as the new gene product.

The criteria for optimal expression in the baculovirus system are only now being established. We patterned our expression vector after pVL941 (29), in which the initiation codon of polyhedrin protein was mutated to ATT. The nonfunctional initiation signal for translation was followed by 33 bases from the coding region of polyhedrin mRNA situated next to an *NheI* cloning site. The highest levels of protein expression were previously observed when portions of the coding sequence of the polyhedrin gene were fused in phase with the foreign gene (28). Other investigators have also demonstrated that 8 nucleotides adjacent to the initiation codon of polyhedrin protein are very important for efficient translation of recombinant mRNA (15, 32, 42). These modifications formed a vector which produced mRNA with a 5′ terminus similar to that of polyhedrin; this supposedly confers stability to the molecule. Future vectors will

FIG. 7. Formation of syncytia (giant cells) in Sf9 insect cells infected with the F recombinant. Monolayers of Sf9 cells were infected for 72 h with recombinant baculovirus containing the F gene. Controls were infected with either H recombinant or wild-type virus. Membrane fusion between adjacent cells was not evident at the normal pH of 6.2 found in culture medium. However, when the pH of the medium was shifted to 5.8, formation of syncytia was apparent after 2 h (panel C). Giant-cell formation was less evident in cells infected with H recombinant (panel A) and wild-type (Panel B) viruses. Cells were observed by phase-contrast microscopy at a magnification of ×600.

undoubtedly yield increased levels of expression once factors involved in transcription and translation processes within insect cells and baculoviruses are fully understood.

For the purpose of studying virus-host cell interactions, we decided to express the two membrane glycoproteins of measles virus in the baculovirus expression system. Our results indicated that both proteins were functional in hemagglutination, hemolysis, and cell fusion assays. Levels of protein expression were also impressive, since both H and F proteins could be detected on SDS-gels stained with Coomassie blue. The primary weaknesses of the insect cell expression system lie in the processes of protein glycosylation and cleavage of membrane protein precursors. Both membrane proteins were shown to be only partially glycosylated. These findings are consistent with the fact that insect cells can process the mannose-rich precursor of glycoproteins but lack galactosyl and sialic acid transferases (19, 20, 36). In most cases incomplete carbohydrate addition does not appear to impair the function of the protein. However, the role of sugars in the immunogenicity of proteins produced for vaccine development remains to be ascertained. Cleavage of the membrane fusion protein occurs in the Golgi complex of the infected cell using a host protease (37). Monkey kidney cells appear to cleave this protein efficiently, whereas Sf9 insect cells only partially process the F_0 precursor. Similar situations exist for the HA protein of influenza virus and the envelope protein of human immunodeficiency virus when they are expressed in the baculovirus system (6, 18, 23, 41). A number of mammalian cell lines were also unable to cleave the membrane fusion protein of Sendai virus (14, 47, 48). Another insect cell line, from *Trichoplusia ni*, was demonstrated to cleave F_0 more efficiently, and the activity of this product is currently being investigated in our laboratory (Richardson, unpublished).

We also noted that fusion protein synthesized in Sf9 insect cells appeared to be more active at low pH. This observation is reminiscent of the situation for membrane fusion induced by influenza virus (8, 9, 12). Influenza viruses also penetrate their host cells by membrane fusion, but this process occurs within the cells at the endosomes via a process of receptor-mediated endocytosis where the environment is acidic (pH 5.8). The difference in pH optima for fusion mediated by the fusion proteins expressed by recombinant baculovirus and measles virus was an unexpected observation (17). It is possible that the three-dimensional structure of the F protein differs in the two systems. An acid environment may be more conducive to the unfolding and exposure of the F_1 amino terminus of the recombinant baculovirus protein. It is also possible that membranes of insect and mammalian cells differ substantially in their roles as targets for fusion. These possibilities must be studied further.

Baculovirus vectors have become popular for expressing proteins of academic and industrial relevance. This publication introduces a new type of vector which facilitates faster screening of recombinant virus and efficient synthesis of foreign proteins. Newer generations of vectors which include early promoters and positive selection techniques are currently being developed in our laboratory. Finally, the gene products described in this paper are being studied to understand the early processes of viral infection and lead us toward new avenues in the treatment of viral disease.

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Bacterial Luciferase Produced with Rapid-Screening Baculovirus Vectors Is a Sensitive Reporter for Infection of Insect Cells and Larvae

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Abstract

Bacterial luciferase, derived from a fusion of the luxA and luxB genes of *Vibrio harveyi*, has been expressed at very high levels in caterpillars and insect cells. The coding sequence for luciferase was inserted into vectors developed in our laboratory which were designed to expedite screening of recombinant virus. These vectors contained the β-galactosidase indicator gene under control of immediate early (IE1), early (ETL), or very late (P10) promoters and a cloning site for inserting the fused luciferase gene next to the polyhedrin promoter. Recombinant baculoviruses containing the luciferase gene as well as the β-galactosidase gene could be easily selected when Bluo-gal (β-galactosidase indicator) was included in the plaque assays. Using cells derived from the fall armyworm (*Spodoptera frugiperda*), luciferase was strongly expressed very late in infection (48–72 h). The bacterial luciferase assay was sufficiently sensitive that light production could be detected from an extract of a single cell. In addition, live insects, including the cabbage looper (*Trichoplusia ni*) and saltmarsh caterpillar (*Estigmene acrea*) were infected by mixing recombinant baculovirus into their diet. Cabbage loopers (with an average wet weight of 223 mg) produced at least 195 μg of active luciferase and levels of synthesis peaked between 96–120 h. The results indicate that bacterial luciferase may be used as a reporter of gene expression in insects.

Key Words
Luciferase
Baculovirus
Expression vectors
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Introduction

Evaluation of the species, tissue, and cell tropism of baculoviruses are essential issues to be addressed with regard to the application of virus-based insecticides [1–4]. Highly sensitive reporters are needed to detect the dissemination of viruses and very low levels of infection. Light-emitting sensors containing insect or bacterial luciferase genes have been shown to be at least 100- to 1000-fold more sensitive using film and photomultiplier detection techniques than other reporter systems relying upon radioisotopic or colorimetric assays [5–7]. At present, firefly luciferase has been used as a reporter in a number of viral and eukaryotic systems [6-11], while the heterodimeric ($\alpha\beta$) bacterial luciferase [12–14] coded by luxA and luxB has generally been used for expression in prokaryotes since two separate promoters would be necessary for expression in eukaryotes. Recently however, a monocistronic bacterial luciferase gene has been generated by fusion of the luxA and luxB genes creating an active luciferase with the carboxyl terminus of the α subunit covalently linked to the amino terminus of the β subunit [15–22]. The light-emitting reaction in bacteria involves the oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long-chain fatty aldehyde with the emission of blue-green light.

From another viewpoint, baculovirus/insect cell expression systems have become a popular choice for the production of high levels of recombinant protein [23, 24]. Our laboratory and those of others have spent a great deal of effort in the improvement and refinement of expression vectors for introducing recombinant baculovirus into insect cells [25–32]. We were particularly aware of the difficulty and time involved in the identification and selection of recombinant baculoviruses by many laboratories. Throughout our developmental work we required a standard reporter gene which could be assayed quickly and easily in order to assess levels of gene expression. Since the bacterial luciferase gene from *Vibrio harveyi* was available in our laboratory, we decided to use it as a reporter to test the efficiency of new baculovirus expression vectors which contained a β-galactosidase indicator gene. These vectors facilitated the rapid isolation of recombinant virus containing the luciferase gene which formed blue plaques in the presence of the β-galactosidase substrate Bluo-gal.

Results presented in this publication indicate that cultured *Spodoptera frugiperda* cells (Sf9) infected with recombinant baculovirus produced luciferase at levels which constituted up to 12% of the total cell protein. The catalytic activity of bacterial luciferase could be readily quantitated in coupled enzyme assays which utilized glucose-6-phosphate dehydrogenase and NAD(P)H:FMN oxidoreductase to generate $FMNH_2$. Light emission was quantitated using photometric and film detection techniques. Small quantities of luciferase could be detected early after infection and the assay was sensitive enough to detect a single Sf9 cell by light emission. In addition, recombinant baculoviruses containing the fused bacterial luciferase gene were used to infect cabbage loopers (*Trichoplusia ni*) and saltmarsh caterpillars (*Estigmene acrea*) by introducing recombinant virus into their diet. Light emission could be detected visually or by photographic techniques demonstrating spatial and temporal development of expression in the insects. Luciferase recombinants provide a powerful tool with which to investigate the host range of baculovirus insecticides.

Material and Methods

Cells and Virus

Sf9 insect cells and *Autographa californica* nuclear polyhedrosis virus (AcNPV) were obtained from the laboratory of Max Summers (Texas A & M University,

College Station, Tex., USA) [33]. *T. ni* and *E. acrea* insect larvae were supplied by the Forest Pest Management Institute (Sault Ste. Marie, Canada). *Escherichia coli* DH5α was obtained from Bethesda Research Laboratories (Gaithersburg, Md., USA).

Chemicals and Reagents

Decanal, for luciferase assays, was supplied by Sigma (St. Louis, Mo., USA) and the cofactors FMN and NADH and glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*) came from Boehringer Mannheim (Dorval, Canada). Glucose-6-phosphate (potassium salt) was purchased from Sigma. The *V. harveyi* FMN:NADH oxidoreductase was partially purified and resolved from luciferase while that from *Photobacterium (Vibrio) fischeri* was supplied by Boehringer Mannheim. Standard bacterial luciferase was purified from recombinant bacteria as previously described [21].

Construction of Baculovirus Vectors

Baculovirus vectors which contained a β-galactosidase gene under the control of different baculovirus promoters for purposes of screening recombinant viruses were constructed as follows. DNA fragments containing the promoters from the baculovirus P10 [34], ETL [35], and IE1 [36] genes were synthesized by polymerase chain reaction (PCR) from AcNPV genomic DNA using Taq polymerase [37]. The P10 promoter flanked by *Eco*RV restrictions sites on one end and *Eco*RV/*Bgl*II sites on the other was synthesized by PCR using the two following oligonucleotides: 5'-GATATCGATATCCTCGAGCAAGAAAATAAAACG-3' (5' end oligo), and 5'-GATATCGATATCAGATCTATCATGATTGTAAATAAAATGTAATT-3' (3' end oligo). The ETL promoter was isolated with the following oligonucleotides: 5'-GATATCGATATCAAGCCACACTGGACACGA-3' (5' end oligo) and 5'-GATATCGATATCAGATCTATCATTTTAGCAGTGATTCTAA-3' (3' end oligo). Finally, the IE1 promoter was constructed using the following primers: 5'-GATATCGATATCTGTACTTGTTGTATGCAAATAAAT-3' (5' end oligo), and 5'-GATATCGATATCAGATCTATCATAGTCACTTGGTGGTTCACGATCTT-3' (3' end oligo). The underlined sequences in these oligonucleotides designate *Eco*RV and *Bgl*II restriction sites which were designed to flank the particular promoter region. Each of the promoters (P10, ETL, or IE1) prepared by PCR and digested with the enzyme *Eco*RV was inserted into the *Eco*RV site of a modified pVL941 baculovirus expression vector [25, 26]. The orientation of the fragment was established by dideoxynucleotide sequencing. This plasmid was subsequently cut with the enzyme *Bgl*II. A *Bam*HI fragment containing the β-galactosidase coding region and SV40 polyadenylation signal [25] was subsequently introduced into the compatible *Bgl*II site to yield a plasmid of about 13 kb.

DNA Transfections and Plaque Assays

DNA transfections and plaque assays were performed as previously described [25, 33] on culture plates (100 mm diameter) except that 1 μg linearized AcNPV genomic DNA (Invitrogen, San Diego, Calif., USA) was cotransfected with 5 μg plasmid DNA. Upon recircularizing, this linearized DNA is capable of regnerating the polyhedrin gene. Genomic DNA was almost totally linearized (noninfectious) and produced only a few wild-type plaques containing occlusion bodies when introduced into Sf9 cells. Transfections were performed for 4 h, the medium was changed, and virus was allowed to replicate for 48 h prior to doing plaque assays. Infected cells were overlaid with 5% SeaPlaque agarose containing Bluo-gal as previously described [25]. The proportion of recombinant plaques compared to contaminating wild-type plaques was extremely high (60–80%).

Isolation of Recombinant Virus

Plaques which were occlusion body negative and stained blue in the presence of Bluo-gal were isolated immediately after they became apparent, as previously described [25]. Plaque assays were repeated at 10-, 100-, and 1,000-fold dilutions and blue plaques were again selected. When using linearized AcNPV together with these vectors, two rounds of plaque assays were sufficient to generate recombinant virus totally free from contaminating wild-type virus. Linearized viral DNA reduced the background of wild-type virus and the development of blue color resulted in accelerated identification of viral plaques.

Luminescence Assays for Bacterial Luciferase

Sf9 insect cells infected with recombinant virus containing the luciferase gene were harvested at various times postinfection. Approximately 4×10^5 cells were suspended in 500 μl of PBS, 50 μl was removed for analysis on SDS polyacrylamide gels, and the remainder was lysed either by sonication (3 times for 45 s) or with 1% Triton-X 100. Lysates were analyzed by photometer or in 96-well microtiter plates with X-ray film for detection of luciferase activity. The cell number was determined by Coulter counter prior to lysis and total protein was assayed with the Bio-Rad (Hercules, Calif., USA) protein determination kit. The photometric assay was performed by injection of $FMNH_2$ into the

reaction buffer as previously described [15, 20]. Microtiter plate assays were performed with a coupled enzyme solution in a total volume of 50 µl containing 50 m*M* potassium phosphate (pH 7), 0.2% (w/v) bovine serum albumin, 5 m*M* β-mercaptoethanol, 0.002% decanal, 5 µ*M* FMN, 1 m*M* NAD, 20 m*M* glucose-6-phosphate, 0.1 U of glucose-6-phosphate dehydrogenase, 0.0015 U of NAD(P)H:FMN oxidoreductase and extract containing luciferase. Amido black solution (10% w/v) was placed outside the individual plastic wells to minimize light leakage and reflection into adjacent assays. Microtiter plates were exposed to X-ray film for 2 h prior to development.

Insect larvae were infected by mixing 10^9 PFU of extracellular recombinant virus per milliliter of diet (Bio-Serv, Frenchtown, N.J., USA). Larvae midway through fourth instar (18 days after hatching) were starved for 24 h and then fed twice with 1 ml of diet containing virus, with an interval of 6 h between feedings. The infected larvae were subsequently reared on regular diet and 5 larvae were collected per time point. Luminescence from infected *T. ni* and *E. acrea* caterpillars was assessed by quickly freezing the infected lavae and soaking them in 10 ml of the coupled enzyme buffer described above which contained 1% (v/v) Triton X-100. The larvae, in plastic petri dishes (60 mm diameter), were placed on top of X-ray film and exposed in a light-tight box for 1–4 h. Alternatively, luminescent larvae were photographed directly in a light-tight box using TMAX P3200 black-and-white film (Eastman Kodak, Rochester, N.Y., USA) with a 30-min exposure.

Results

Construction of Baculovirus Expression Vectors

The baculovirus expression vectors pJVP10, pJVETL, and pJVIE1 were constructed as outlined in Materials and Methods. The P10 very late promoter [34], ETL early promoter [35], and IE1 immediate early promoter [36] were synthesized from AcNPV genomic DNA using PCR technology and used to direct transcription of the β-galactosidase gene. The sequences and transcription start sites of these promoters are presented in Table 1. Different promoters were used in order to vary the quantities of β-galactosidase indicator produced in insect cells with the expression vectors developed in our laboratory. Our vectors were designed to expedite the screening of recombinant baculovirus which produced both β-galactosidase and a foreign gene product of interest. The promoters mentioned above directed the synthesis of β-galactosidase, while the synthesis of foreign gene products (e.g., bacterial luciferase) was controlled by the polyhedrin promoter. Indicator and foreign genes were designed to recombine with the wild-type AcNPV genome via the flanking sequences of the polyhedrin gene to yield recombinants which were polyhedrin negative, produced the foreign gene product, and formed blue plaques when Bluo-gal was present in the agarose overlay. Partial restriction maps of the three different vectors are presented in figure 1. Foreign genes can be inserted at either the *Nhe*I or *Bam*HI unique restriction enzyme sites adjacent to the polyhedrin promoter. An *Eco*RV fragment (3 kb in length) which contained the fused luxA/luxB genes of *V. harveyi* luciferase [5, 21] was inserted at the *Nhe*I site of each of the three vectors. The vectors were linearized with *Nhe*I, the ends were rendered blunt with the Klenow fragment of DNA polymerase, and the fused luciferase genes were introduced by ligation. In addition two smaller versions of these plasmids (pETL and pP10) were constructed which contained truncated flanking sequences of the polyhedrin gene (data not shown). These plasmids contain the baculovirus sequences from the *Xho*I (1900) to the *Sal*I (9670/9720) restriction enzyme sites described in figure 1. The smaller expression plasmids (10 kb in size) yielded exactly the same levels of recombinant protein production as the larger vectors (data not shown) and were designed to facilitate the introduction of larger foreign DNA fragments into the cloning sites. However, the luciferase recombinants described in this paper were generated using the larger vectors.

Table 1. Promoter region sequences

Promoter		Sequences				Nucleotides
Polyhedrin	(5')	GATATCATGG	AGATAATTAA	AATGATAACC	ATCTCGCAAA	95
		TAAATAAGTA	TTTTACTGTT	TTCGTAACAG	TTTTGTAATA	
		AAAAAACCTA	TAAAT (3')			
P10	(5')	GCTCGAGCAA	CAAAATAAAA	CGCCAAACGC	GTTGGAGTCT	231
		TGTGTGCTAT	TTTACAAAGA	TTCAGAAATA	CGCATCACTT	
		ACAACAAGGG	GGACTATGAA	ATTATGCATT	TGAGGATGCC	
		GGGACCTTTA	ATTCAACCCA	ACACAATATA	TTATAGTTAA	
		ATAAGAATTA	TTATCAAATC	ATTTGTATAT	TAATTAAAAT	
		ACTATACTGT	AAATTACATT	TTATTTACAA	TC (3')	
ETL	(5')	AAGCCACACT	GGACACGAAA	AAGTCGAAGG	CACTCGCTTC	302
		GATCAATGGA	CAAAGAATCA	ATCGTTCGTA	GGCAACAGAA	
		TGTCGGAAAG	TTTGCATTGG	ATGCGCGGCG	GGTCCAACTT	
		GCCGCAAAAC	TGCGGCGAGT	TCAACGTGGT	GTCCAGCCTG	
		TTGATGTGCA	ACAATACGAT	AATGAAAAAT	TGATAACGCT	
		TGCACGATTG	CAAACATGCA	CGCTCGGTTG	AATAAAAGCT	
		CGCATCGTCG	TCGTAAAATT	AGTTGTATCA	AAGAGCAGCT	
		GCAATTAGAA	TCACTGCTAA	AA (3')		
IE1	(5')	TGTACTTGTT	GTATGCAAAT	AAATCTCGAT	AAAGGCGCGG	366
		CGCGCGAATG	CAGCTGATCA	CGTACGCTCC	TCGTGTTCCG	
		TTCAAGGACG	GTGTTATCGA	CCTCAGATTA	ATGTTTATCG	
		GCCGACTGTT	TTCGTATCCG	CTCACCAAAC	GCGTTTTTGC	
		ATTAACATTG	TATGTCGGCG	GATGTTCTAT	ATCTAATTTG	
		AATAAATAAA	CGATAACCGC	GTTGGTTTTA	GAGGGCATAA	
		TAAAAGAAAT	ATTGTTATCG	TGTTCGCCAT	TAGGGCAGTA	
		TAAATTGACG	TTCATGTTGG	ATATTGTTTC	AGTTGCAAGT	
		TGACACTGGC	GGCGACAAGA	TCGTGAACAA	CCAAGTGACT	
		ATGACG (3')				

Various promoters from the baculovirus of *A. californica* were isolated by PCR and inserted into the vectors shown in figure 1. The transcriptional start sites are underlined. The 5' and 3' ends of the promoters are also indicated.

Screening of Recombinant Baculovirus

Following cotransfection of linearized wild-type AcNPV genomic DNA with the appropriate vectors containing the luciferase gene, plaque assays were performed to isolate recombinant virus away from contaminating wild-type virus. Sf9 cells were infected and overlaid with agarose containing the β-galactosidase substrate Bluo-gal. Blue plaques subsequently appeared in 3 days when using pJVP10, 4 days using pJVETL, and 5 days with pJVIE1. The plaque assays were photographed at 5 days postinfection (fig. 2). Plaques produced by the P10/β-galactosidase transcription unit generally resulted in blue-stained areas which were larger in diameter and more intense in color than those produced with the pJVETL and pJVIE1 vectors. The intensity of the plaques did not increase dramatically over longer incubation times. Only 1 or 2 plaque assays were

required to obtain recombinants free of contaminating wild-type virus, but an additional plaque assay was usually performed to ensure that the recombinant was pure.

Analysis of Recombinant Luciferase by SDS-Polyacrylamide Gel Electrophoresis

Sf9 insect cells were infected with wild-type AcNPV and luciferase recombinants produced with the pJVP10, pJVETL, and pJVIE1 vectors. Cells were harvested at various times postinfection and proteins were fractionated by electrophoresis on SDS polyacrylamide gels and stained with Coomassie blue (fig. 3). Luciferase (80 kD) was clearly evident at 48 h postinfection and polyhedrin (33 kD), whose gene had been replaced by the recombinant gene, was absent in cells infected with recombinant virus. Four different recombinant viruses were isolated with each vector, and the viruses all yielded similar levels of luciferase expression within experimental error. However, recombinant baculovirus produced with the pJVP10 vector clearly synthesized larger quantities of β-galactosidase (110 kD) than virus derived from the pJVETL and pJVIE1 vectors. Our

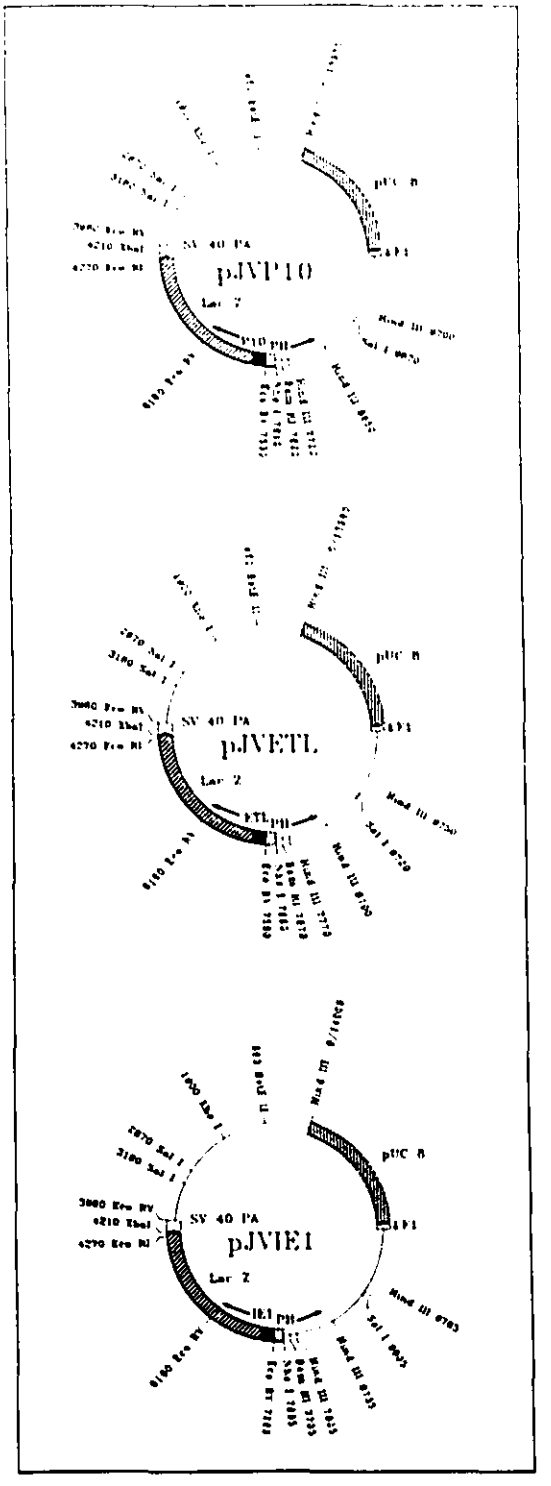

Fig. 1. Restriction endonuclease maps of baculovirus expression vectors designed to expedite the selection of recombinant viruses. These plasmids contain a β-galactosidase gene under control of very late (P10), early (ETL), or immediate early (IE1) promoters and its transcription product is terminated by an SV40 polyadenylation sequence (SV40 PA). In addition, the vectors contain unique *Nhe*I and *Bam*HI sites into which foreign genes can be inserted. The foreign genes are expressed under control of the polyhedrin (PH) promoter. Directions of transcription are indicated with arrows and each plasmid contains an F1 origin of replication which can be used to generate single-stranded DNA. A pUC8 sequence is included which permits the plasmid to grow in *E. coli*. Restriction endonuclease sites are indicated as a distance in nucleotides from the *Hin*dIII site of pUC8.

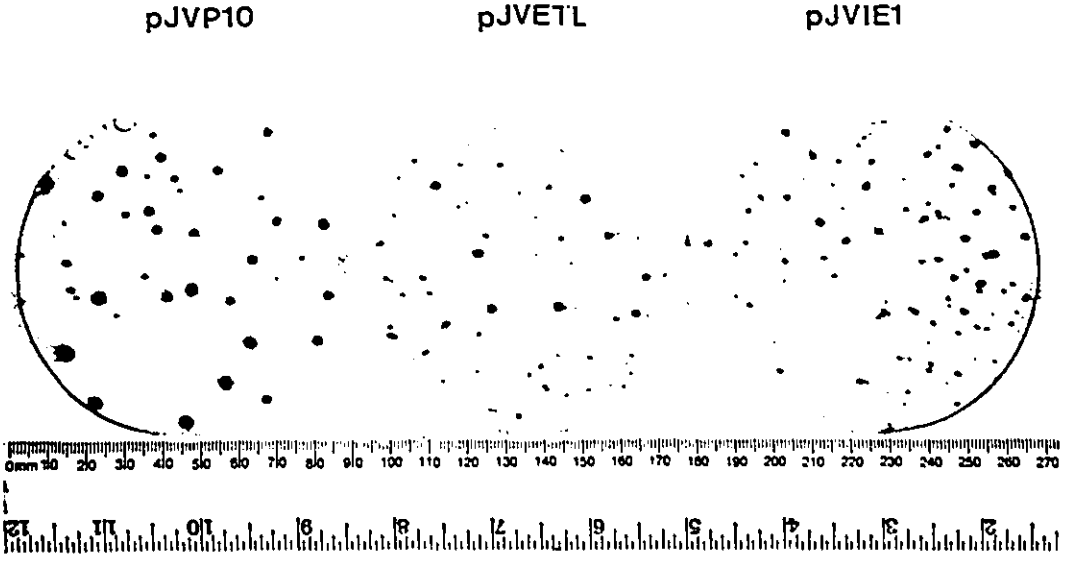

Fig. 2. Recombinant virus plaques formed using the three different expression vectors. Monolayers of Sf9 cells were infected with recombinant luciferase virus which had been isolated after an initial round of plaque purification. Assays were performed as outlined in Materials and Methods and the agarose overlay contained the β-galactosidase substrate Bluo-gal. Plaques appeared at 3, 4, and 5 days using virus produced with pJVP10, pJVETL, and pJVIE1 vectors, respectively. The petri dishes were inverted and photographed at 5 days postinfection.

vector of choice is currently pJVETL, since it minimizes the amount of β-galactosidase produced, which could complicate purification procedures of other recombinant proteins from infected cells. In this study, scanning the stained proteins with a laser densitometer showed that 10–12% of the total infected cell proteins at 72 h postinfection could be attributed to luciferase. We estimated that a yield of 100 mg recombinant luciferase per 10^9 cells was produced using any of the three expression vectors.

Photometric Quantitation of Luciferase Enzyme Activity in Infected Sf9 Insect Cells

Insect cells infected with recombinant baculovirus were harvested at various times postinfection, lysed by sonication and an aliquot of the extract was used in the photometric luciferase assay described in the Materials and Methods. Luciferase activity was quantitated from cells infected with baculovirus prepared with the three different expression vectors and the data are summarized in table 2. Luminescence peaked with samples prepared from the 72-hour infections with all three recombinants and declined slightly with preparations from the 96-hour infection, possibly due to virus-mediated cell lysis. This disintegration of cells was apparent when viewing the cells with a phase contrast microscope. Generally, 30–60 pg of active luciferase was produced per nanogram of total cell protein. More simply, 3.8–7.3% of the total cellular protein was present as active luciferase in our assays. These yields corresponded to a production of 30–46 pg of active

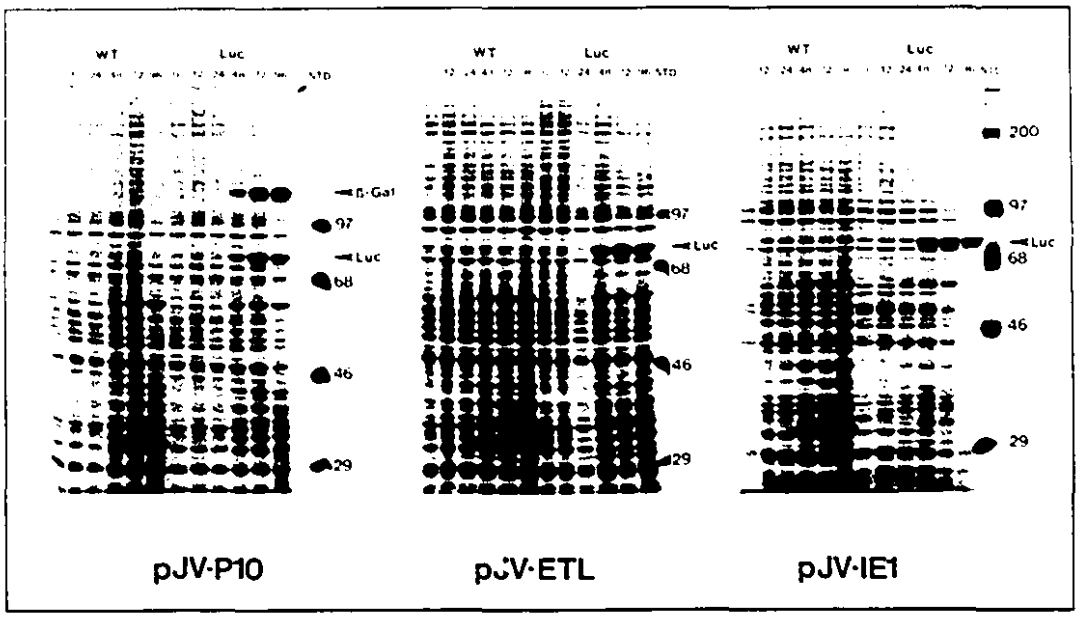

Fig. 3. Coomassie-blue-stained gels of total proteins produced in Sf9 insect cells infected with either wild-type AcNPV baculovirus (WT) or recombinant luciferase virus (Luc). Recombinant virus was produced with each of the pJVP10, pJVETL, or pJVIE1 expression vectors and used to infect Sf9 cells. Proteins were solubilized by lysing the cells in sample buffer at 0, 12, 24, 48, 72, and 96 h postinfection and subsequently separated by SDS-polyacrylamide electrophoresis. Recombinant luciferase (Luc) and β-galactosidase (β-Gal) are indicated by arrows at the right of each gel. Protein standards (STD) of 97, 68, 46, and 29 kD are designated by numbers at the right of each gel.

Table 2. Photometric assays of luciferase activity in Sf9 cells infected with recombinant baculovirus vectors

Time postinfection h	pJVP10		pJVETL		pJVIE1	
	pg/cell	pg/ng protein	pg/cell	pg/ng protein	pg/cell	pg/ng protein
0	0.00	0.00	0.00	0.00	0.00	0.00
12	0.004±0.001	0.009±0.003	0.02±0.005	0.02±0.007	0.01±0.003	0.04±0.02
24	0.31±0.03	0.380±0.03	0.84±0.19	1.02±0.14	0.77±0.10	1.50±0.50
48	22.2±1.3	26.1±2.1	32.7±2.1	35.8±3.5	25.8±8.5	36.3±9
72	36.6±4.0	37.3±2.4	46.0±3.0	59.8±2.9	34.4±8.2	47.2±13
96	32.4±4.2	40.9±3.7	30.0±4.3	51.6±5.9	27.9±3.0	44.6±10

Assays were conducted by photometric injection assay, converted into pg of native luciferase and divided by the number of cells or nanograms of protein extract and assayed as described in Materials and Methods. Errors were calcualted as SD from four experiments.

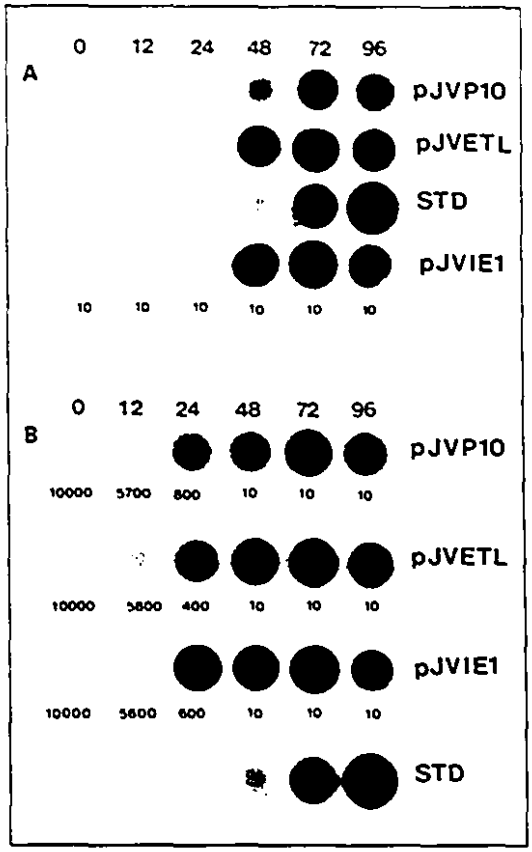

Fig. 4. X-ray film detection assays of luciferase produced in Sf9 insect cells. Microtiter plate assays were performed as outlined in Materials and Methods in the presence of FMN, NAD, glucose-6-phosphase, glucose-6-phosphate dehydrogenase, and NAD(P)H: FMN oxidoreductase. The entire microtiter plate was exposed to X-ray film for 2 h prior to development. **A** Each well contained a lysate prepared from 10 insect cells harvested at 0, 12, 24, 48, 72, and 96 h postinfection. Cells were infected with recombinant luciferase virus prepared with pJVP10, pJVETL, and pJVIE1 expression vectors. Standard (STD) amounts of luciferase (0, 0.01, 0.03, 0.10, 0.31, and 0.94 ng) were included in the assay for estimating the amounts of enzyme produced in insect cells. **B** Similar results except that larger numbers of cells (indicated by the number beneath each well) were assayed at early times of infection in order to determine when luciferase was first synthesized in infected cells.

luciferase per infected insect cell and experimental uncertainty was estimated to be ±12–15%.

Analysis of Luciferase Activity Using a Coupled Enzyme System and Film Detection

In additon to the substrate (decanal), the enzyme luciferase requires the cofactor $FMNH_2$. Since this reduced nucleotide rapidly oxidizes on exposure to air, it was necessary to continuously generate $FMNH_2$ using oxidoreductase and NADH; production of the last cofactor was coupled to the oxidation of glucose-6-phosphate by glucose-6-phosphate dehydrogenase. Aliquots of insect cell lysates from 10 cells were added to enzyme assays in the wells of microtiter plates. These assays were subsequently exposed on X-ray film for 2 h and the results of the experiment are shown in figure 4A. The observed luminescence paralleled the more precise photometric assays presented in table 2. Some luciferase activity could be detected at 12 h postinfection when more cells were added to the assays (figure 4B). This film detection assay offers a convenient method to estimate the amount of active luciferase present in the infected cell using purified recombinant bacterial enzyme as a standard [21]. Quantities as low as 0.1 ng of active luciferase could be detected following 2–4 h exposure to X-ray film.

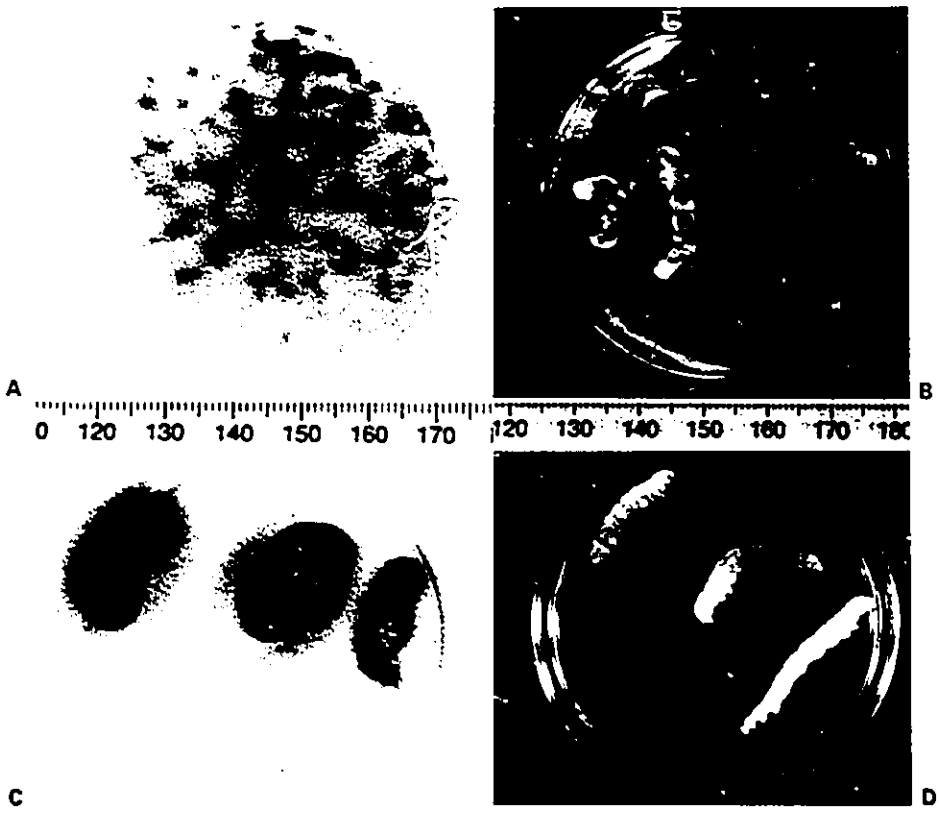

Fig. 5. Luminescence from infected insect cell monolayers and *T. ni* larvae. **A** Virus plaque assays were performed as outlined in Materials and Methods using virus produced with the pJVP10 expression vector containing the luciferase gene. Assays were incubated for 6 days and overlaid with agarose containing glucose-6-phosphate, decanal, NAD, FMN, oxidoreductase, and glucose-6-phosphate dehydrogenase. Culture plates were placed on X-ray film in total darkness for 4 h. **B** *T. ni* larvae were infected with a small amount of food containing luciferase recombinant virus, then collected at different times of infection (0, 12, 36, 60, 96, and 120 h) and placed in the luciferase assay cocktail which contained the coupled enzymes at 1% (v/v) Triton X-100. Larvae were photographed in complete darkness for 30 min using TMAX P3200 black-and-white film (6,400 ASA). **C** Three caterpillars infected with luciferase recombinant virus for 120 h were p[illegible]ed in the luciferase assay cocktail containing 1% (v/v) Triton X-100. The petri plate was exposed to X-ray film in total darkness for 4 h. **D** Black-and-white photograph of the larvae shown in **C** using tungsten lighting.

Demonstration of In vivo Luminescence from Insect Cell Monolayers and Larvae Infected with Recombinant Virus

Insect cell monolayers were infected with the baculovirus recombinants at varying dilutions and were overlaid with agarose containing Bluo-gal. Blue plaques appeared between 3 and 5 days postinfection. At this time, plaque assays were again overlaid with agarose which now contained glucose-6-phosphate, decanal, NAD, FMN, oxidoreductase, and glucose-6-phosphate dehydrogenase. The tissue culture plates were subsequently placed on X-ray film for 4 h. Light emission was detected from indi-

Table 3. Photometric assays of luciferase activity in *T. ni* larvae infected with recombinant baculovirus

Time postinfection h	Luciferase/mg total protein µg	Percent of total protein	Luciferase/larva µg
0	0	0	0
36	0.11±0.05	0.01±0.005	0.7±0.1
60	9.89±3.4	1.0±0.34	96.8±22
96	21.0±5.0	2.1±0.50	195.0±36
120	22.7±3.3	2.3±0.33	149.0±30

Assays were conducted by photometric injection assay, converted into micrograms of native luciferase as described in Materials and Methods. Twenty-five larvae midway through fourth instar (18 days after hatching) were infected with 10^8 PFU of extracellular recombinant virus per milliliter of diet. Five caterpillars were homogenized in 1 ml of 1% TritonX-100 in water at each of the indicated times. Samples were immediately frozen at −20 °C. An aliquot (1 µl) was assayed for activity and the results were averaged. The average wet weight of an infected caterpillar was 223±26 mg. Errors were calculated as SDs between the 5 caterpillars at each time point.

vidual plaques (figure 5A) and the luminescent infected cells correlated well with those which contained β-galactosidase and produced a corresponding blue color.

Cabbage loopers (*T. ni*) midway through fourth instar were deprived of food for 24 h and infected by feeding them recombinant baculovirus (10^8–10^9 PFU) mixed with a small quantity of insect diet twice within 6 h. The infected larvae were subsequently reared on untreated diet. They were harvested at various times postinfection, homogenized, and luciferase was quantitated form aliquots by photometric assay as shown in table 3. Enzyme activity peaked at 96 h and about 0.195 mg of active luciferase was detected in a single infected larva with an average weight of 223 mg. In another experiment, larvae were infected, reared for 3–4 days on diet free of virus, frozen, placed in the luciferase assay cocktail which contained the coupled enzymes, and either photographed in complete darkness (fig. 5B) or exposed to X-ray film for 2 h (fig. 5C). Light was emitted from most regions of the larvae (fig. 5C) when the exposed areas on the film were compared to pictures taken of the caterpillars under tungsten lighting (fig. 5D). This finding is consistent with previous studies concerning the pathology of infections by AcNPV in the larvae of the cabbage looper late in infection [38]. The cabbage loopers usually died by 6–7 days infection. Larvae in the preceding experiments were fed large quantities of extracellular virus. Further experiments using occluded virus would require the administration of smaller quantities of virus and these studies would be a more significant model for infection of larvae in the environment. Similar results were obtained with the larvae of *E. acrea* (data not shown). When homogenates of larvae (solubilized in 1% Triton-X 100) were assayed for active luciferase using photometric detection techniques and standard recombinant luciferase from bacteria [21], it was estimated that each *T. ni* caterpillar produced 195 µg of active luciferase following 96 h infection (table 3).

The preceding results indicate that the luxA/luxB luciferase system is not only an excellent reporter gene in vitro but can also be used to demonstrate expression in vivo.

Discussion

The preceding work describes the construction and development of new baculovirus vectors designed to expedite the screening of recombinant virus which in this case expressed a bacterial luciferase fusion protein. Many baculovirus vectors use the strong promoter from the polyhedrin gene to direct transcription of the foreign gene [23, 24]. These vectors contain the 5'- and 3'-flanking regions of the polyhedrin gene and pUC8 sequences for growing the plasmid in *E. coli* bacterial cultures. Insertion of the recombinant gene into the wild-type virus genome relies upon a process of homologous recombination between the polyhedrin gene flanking sequences in the plasmid and viral DNA. Screening recombinant viruses, and purifying virus containing the foreign gene away from contaminating wild-type virus can be difficult since the recombinant plaques now lack viral occlusion bodies and are often difficult to see. We previously reported the construction of a vector [25] which contained two promoters active very late in infection: the P10 promoter was used to direct the synthesis of β-galactosidase while the polyhedrin promoter controlled the synthesis of foreign gene products. The two genes, together with their promoters recombined at high frequency with wild-type viral DNA to yield recombinant virus, which produced blue plaques when infected cells were overlaid with agarose containing β-galactosidase indicator. The virus produced with this pJV(*Nhe*I) vector synthesized a lot of β-galactosidase which could potentially compete with the foreign gene product for amino acids and also complicate procedures for the purification of the other recombinant protein from infected cells. In this paper we chose immediate early (IE1) and early (ETL) promoters to reduce the amount of β-galactosidase produced. The number of nucleotides between the promoters directing transcription of the lacZ gene and the polyhedrin promoter was also reduced from 900 to 90 nucleotides to maximize the chances that both the β-galactosidase gene and the foreign gene would be introduced together into the recombinant virus. Following cotransfection with DNA from wild-type AcNPV and the new expression vectors, 0.1–1% of all baculovirus plaques contained the β-galactosidase and luciferase genes. However, using linearized AcNPV in the cotransfection process [39, 40] increased the efficiency for selecting recombinant virus to levels of 60–80% since linearized viral DNA is noninfectious. Recombination of linear DNA with the plasmid produces infectious recombinant virus with a small background of contaminating wild-type virus. Smaller (10 kb) vectors with decreased polyhedrin flanking regions have been developed in our laboratory and these plasmids may be more manageable in *E. coli* and should permit the expression of larger genes.

Bacterial luciferase represented an ideal reporter gene for baculovirus infections of Sf9 cells and insect larvae. The assay for this enzyme was extremely sensitive and simple to perform. Quantities of 10–100 pg of enzyme could be detected in photometric and film assays. Microtiter assays and dissected larvae emitted light which could be visualized in a darkroom. This sensitive reporter may permit the investigator to follow the route of virus infection through the gut in an individual host caterpillar. In the laboratory, susceptible cell lines and larvae were monitored for infectivity by extracellular (nonoccluded) recombinant viruses containing the luciferase gene. However, recombinant viruses which produce both occlusion bodies and luciferase may prove of

value when considering the effects of virus-based insecticides upon other nontargeted insects outside the laboratory. In addition, a number of laboratories have utilized *Bombyx mori*, *Heliothis zea*, and *T. ni* larvae to produce relevant proteins [41–43]. Medin et al. [43] demonstrated that cabbage loopers could produce human adenosine deaminase at a yield of 2% of its total protein. Our studies confirm the fact that extremely large quantities of functional recombinant protein can be produced in caterpillars. Such production technology may prove useful in the synthesis of some recombinant proteins. Recently, another laboratory expressed firefly luciferase in insect cells and larvae [10, 44]. These investigators demonstrated high level production of luciferase in tissue homogenates of larvae. In addition, one laboratory recently expressed the click beetle luciferase and demonstrated luminescence in single infected insect cells [11]. Our paper is the first communication demonstrating the use of bacterial luciferase as a reporter in an insect system. The enzyme mechanism is very different from other luciferases with respect to substrate and cofactors, yet results in our laboratory indicate that it is an equally sensitive reporter gene since luminescence could also be detected from single cells expressing the protein. The luxA/luxB gene product was assayed in the presence of a coupled enzyme system which generated the cofactor $FMNH_2$, a requirement for enzyme activity. Bacterial luciferase produced luminescence over longer periods (i.e., hours) compared to firefly and click beetle luciferases which deplete their ATP reserves within minutes. The substrate for the bacterial enzyme (decanal) is also cheaper, permeates the cell better, and is more readily available than the substrate for firefly luciferase (luciferin). Clearly the expression of bacterial luciferase in insect cells is esthetically pleasing but it also has practical applications in assessing the environmental impact of recombinant baculoviruses which are currently being considered for the role of biological insecticides [1–4]. We are presently assaying bacterial luciferase in a wide variety of insect hosts in order to ascertain the uptake and dissemination of baculoviruses in different insect larvae.

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