GENE EXPRESSION IN *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRUS INFECTIONS EXHIBITING HOST SPECIFIC MODULATION OF OCCLUSION BODY FORMATION

by

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ABSTRACT

Nuclear polyhedrosis viruses (NPVs) are enveloped, rod-shaped insect viruses containing a circular DNA genome (family: Baculoviridae). These viruses, which are being developed as biopesticides, are transmitted via polyhedral inclusion bodies (PIBs) in which viruses are embedded. In order to genetically engineer safe viral pesticides, it is necessary to understand the molecular basis of NPV host specificity and virulence.

This research deals with a host range model (System III) in which Autographa californica multicapsid NPV (AcMNPV) replicates permissively in Spodoptera frugiperda (SF) cells but semipermissively in Bombyx mori (BM) cells. Early in infection, both cell lines displayed similar nuclear hypertrophy, but subsequent stages of cytopathic effect (CPE) were very different. PIBs were produced in SF but not BM cells. Instead, BM cells exhibited a unique CPE characterized by large sac-like bodies at the cell periphery. Virus growth kinetics experiments showed that infectious progeny virus was not produced in BM cells.

Because PIBs were not found in the BM infection, gel electrophoresis was performed to determine whether polyhedrin, the major constituent protein of the PIB, was synthesized. Polyhedrin was not produced. The synthesis of another major late viral protein known as

p10 was also blocked. Thirteen other infected cell-specific polypeptides (ICSPs) were not detected in the semipermissive infection. Most of these appeared to be late ICSPs. Six ICSPs were found only in infected BM cells. Eight ICSPs were produced in both SF and BM infections but at different rates in each cell line. Inhibition of host proteins synthesis occurred in SF cells, but there was no clear evidence for host inhibition in BM cells.

System III demonstrates a strong host range restriction which is manifested late in the BM infection. Additional research on the detailed mechanisms of the restriction should lead to the identification of viral DNA sequences responsible for host specificity and pave the way for the engineering of safe pesticides.

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CHAPTER I

INTRODUCTION

Baculoviruses have long been of interest due to their impact on insect populations. Because of restricted host range and their effectiveness in infecting susceptible hosts, baculoviruses are being considered as potential biological pesticides (2, 22, 23, 25, 94, 97, 99, 100). More recently, interest has further escalated in light of their usefulness as vectors for high-level expression of foreign genes in invertebrate cell lines. The popularity of baculovirus vectors stems from the successful production of large quantities of biologically active products of eukaryotic genes which previously proved difficult using more traditional systems. Although over 400 baculoviruses have been reported in the literature, only a few have been studied at the molecular level. In order to better utilize these viruses as biological control agents and to optimize their use as gene expression vectors, it is imperative that we fully understand the molecular biology of these viruses with respect to their host specificity and virulence.

In this study, I have investigated a host range model system consisting of the prototype baculovirus Autographa californica nuclear polyhedrosis virus (AcMNPV) and the Spodoptera frugiperda (fall armyworm) and Bombyx mori (silkworm) cell lines. I have analyzed cytopathic effect (CPE), virus growth kinetics, and viral protein

synthesis in these two cell lines in order to better understand baculovirus host specificity.

Literature Review

Baculovirus Structure

Members of the family Baculoviridae are rod-shaped, enveloped viruses which possess a single molecule of circular, double-stranded DNA of 80-220 Kb (9, 10). The DNA is packaged in a nucleocapsid which is contained within a lipoprotein envelope. This structure constitutes the infectious virus particle or virion. Following assembly in the nucleus, viral nucleocapsids enter the cytoplasm and bud through the plasma membrane, producing the non-occluded, budded virus (BV) (Fig. 1). Alternatively, nucleocapsids may acquire a membrane de novo while in the nucleus and then become embedded within a paracrystalline protein matrix, forming polyhedral inclusion bodies (PIBs) containing multiple virions. The PIB is composed primarily of polyhedrin, a polypeptide with a molecular weight of approximately 30K. Occluded virions (OV) are responsible for transmitting the virus from host-to-host, whereas cell-to-cell transmission in infected insects and in cultured cells is mediated by budded virus (105). Neutralization studies (110) and studies utilizing immunoblotting methods (105, 107) have shown distinct differences between the proteins of occluded and budded viruses.

Classification

The majority of baculoviruses isolated from several orders of insects and arthropods are nuclear polyhedrosis viruses (NPVs). The

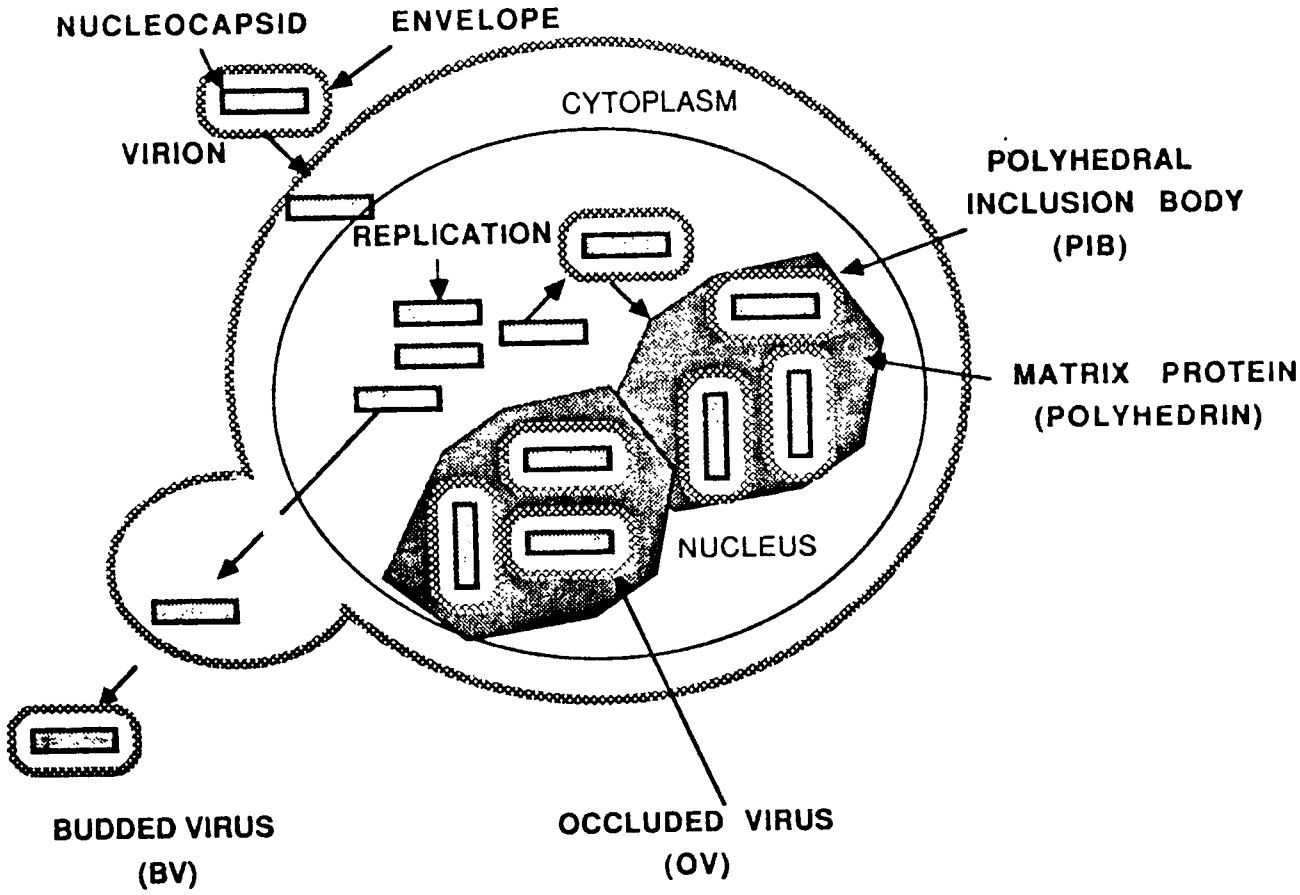


FIGURE 1. Schematic diagram of the baculovirus replication cycle. Following entry of the virus particle into the host cell, the virus uncoats and the nucleocapsid enters the host nucleus where replication occurs. Nucleocapsid of progeny virions are assembled in the nucleus. Budded virus (BV) then acquires an envelope at the nuclear or plasma membrane and then buds through the plasma membrane. Occluded virus (OV) acquires an envelope de novo while in the nucleus and then becomes embedded in a paracrystalline polyhedrin matrix, thus forming the PIB.

term "polyhedrosis" refers to the polyhedral-shaped PIBs which are formed in the nuclei of infected cells. Only one genus (Baculovirus) has been described thus far. Members of this genus have been classified into three subgenera based on morphological differences (Fig. 2). Subgroup A is composed of NPVs. Within this group reside two subgroups: (1) the multiply-embedded viruses (MNPVs), and (2) the singly-embedded viruses (SNPVs). The MNPVs contain many nucleocapsids per envelope, whereas only one nucleocapsid is present in the SNPVs. Most baculovirus isolates belong to the MNPV group. This group has the broadest host range and is the only type which exhibits complete replication in cell culture (33). Subgroup B consists of the granulosis viruses, and subgroup C contains the non-occluded viruses.

In nature, insects become infected by consuming food contaminated with occluded viruses (OVs). The PIBs then dissolve in the insect midgut due to the activity of a polyhedron-associated alkaline protease which is activated by the high pH of the midgut lumen. Released virions infect the gut epithelial cells. As the infection continues, virus is carried to fat body tissues via the hemolymph (36). Late in infection, the fat bodies are glutted with PIBs, resulting in the eventual rupture of the larvae, releasing PIBs into the environment. These PIBs are then ingested by other insects. The PIBs serve to protect the embedded virions from inactivation during the interval between transmission from one host to another. Because most larvae stop feeding within two days of virus ingestion, NPVs are very effective as control agents, and several NPVs are currently registered as biological pesticides (4).

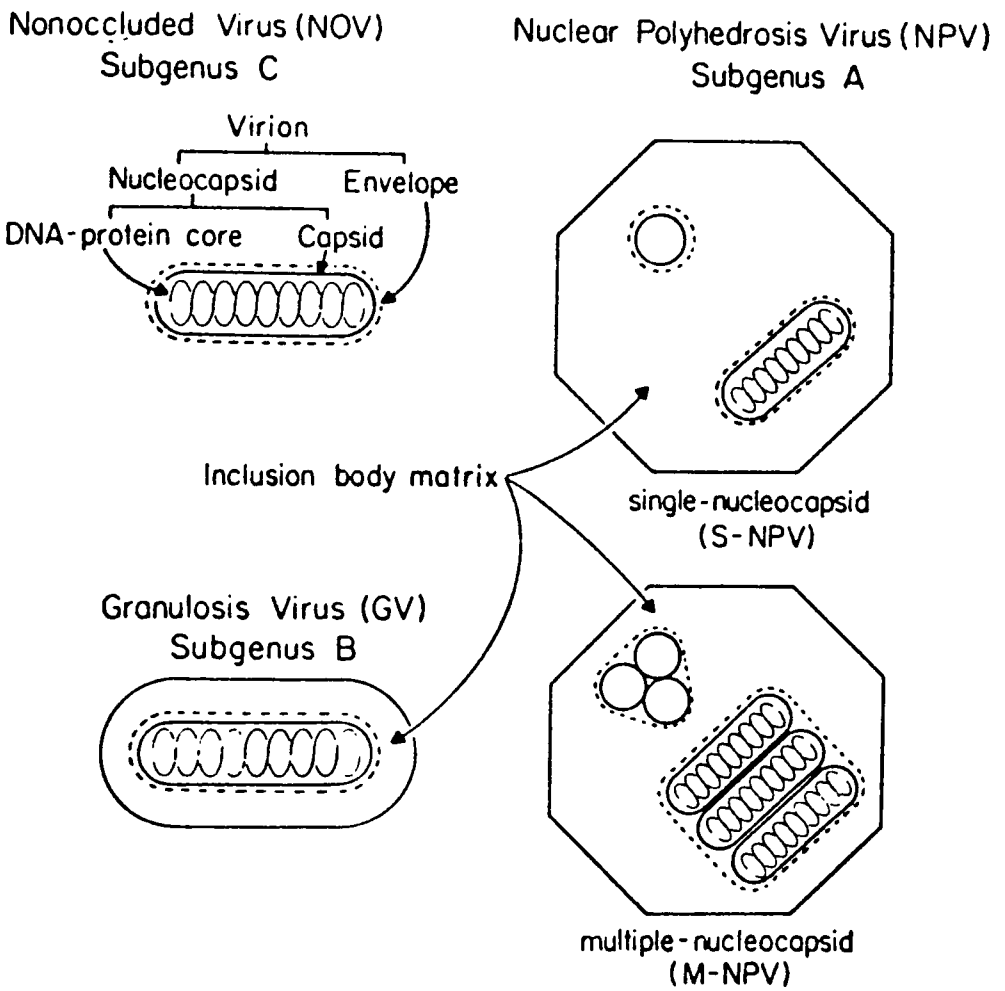


FIGURE 2. Schematic representation of the Baculoviridae. Only one genus Baculovirus has been described. Three subgenera exist. There are two morphological types of viruses: SNPVs in which there is only one nucleocapsid per occluded virion and MNPVs which contain numerous nucleocapsids per virion. (From Bilimoria, S. L., in The Biology of Baculoviruses, R. R. Granados and B. A. Federici, eds. CRC Press, Boca Raton, 1986, with permission.)

Baculovirus Replication

The replication of baculovirus DNA has been widely studied in the past 15 years, and this topic was recently reviewed by Volkman and Knudson (109). Much of this work involved the prototype baculovirus which was originally isolated from Autographa californica, a lepidopteran commonly known as the alfalfa looper. This virus is appropriately called Autographa californica MNPV (AcMNPV). Volkman et al. (108) reported that the budded virus enters the host cell by adsorptive endocytosis and/or fusion at the plasma membrane. The nucleocapsid then enters the nucleus where the nucleoprotein core is released from the capsid (35). There is data to suggest that the viral DNA may be released from the core and then assume a nucleosome-like structure with the aid of host histones. Following adsorption, a series of events occurs beginning with the formation of a virogenic stroma which appears as a dense network structure in the nucleus under the electron microscope. Nucleocapsids then form, followed by envelopment and assembly into polyhedra. The general strategy of baculovirus replication is very similar to that of other large DNA animal viruses such as adeno-, irido-, herpes-, and poxviruses in which early and late phases are recognized (54). At least three phases of AcMNPV gene expression have been distinguished in hosts permissive for AcMNPV replication: (a) an early phase, (b) a late phase, and (c) a very late or occlusion-specific phase.

Viral Protein Synthesis

Several laboratories (114, 63, 19, 12, 115, 48) have documented the kinetics of AcMNPV and other NPV specific protein synthesis. The complexity of baculovirus protein synthesis is evidenced by the sequential and usually transient synthesis of a large number of infected cell-specific polypeptides (ICSPs). Based on their time of appearance and the effect of reversible inhibitors of protein synthesis such as cyclohexamide and inhibitors of DNA replication such as cytosine arabinoside, workers have divided viral protein synthesis into several temporal classes. Although there are minor differences in the way various laboratories designate the classes, the following designations are currently the most widely used. The time in parentheses indicates the first appearance of polypeptides postinfection. The four temporal classes are: alpha or immediate early (2h); beta or delayed-early (6h), which defines the onset of DNA synthesis; gamma or late (10-12h), and delta or very late (15h) (48). The late phases involve the de novo synthesis of intranuclear membranes and the occlusion of virions into polyhedra. During the delta phase, polyhedrin and another infected cell-specific polypeptide known as p10 are synthesized at very high rates with a reduction in synthesis of other ICSPs (114, 63). Except for the α-polypeptides, each subsequent class appears to require the proper expression of the preceding class of proteins (72, 47). This type of expression is known as the temporal cascade model (48).

Several ICSPs including polyhedrin are phosphorylated in AcMNPV and Trichoplusia ni infections, but the role of phosphorylation in

NPVs is not known (63). Stiles and Wood (92) demonstrated the presence of at least nine glycoproteins in AcMNPV-infected cells. Glycoproteins appear to be important in virus attachment and penetration of the host cell (108). Although there is evidence for slow cleavage (19, 12, 48), there is no evidence for rapid post-translational cleavage of viral polypeptides in infected cells.

Virus Genome Organization

Detailed physical maps of a number of baculoviruses have been constructed (70, 86, 95), and genomic libraries are now available (16). Through comparative analysis of these maps, it appears that many baculovirus isolates are minor variants of one another, possessing either deletions or insertions (88, 70, 87). The baculovirus genome is the largest double-stranded circular genome of any virus.

So far, very little information is available concerning the details of replication in these viruses. Wilson and Miller (112) studied DNA-protein complexes in AcMNPV-infected Spodoptera frugiperda cells and showed that the viral DNA acquired a unique nucleoprotein structure with basic, chromatin-associated proteins of 15K and 39K molecular weights. The role of these two proteins in NPV replication is not known. A baculovirus-induced DNA polymerase which has characteristics different from host polymerases has been detected by workers in several laboratories (46, 111). This polymerase was insensitive to aphidicolin and novobiocin but was inhibited by dTTP and N-ethymaleimide which suggests that the polymerase is a gamma-like DNA polymerase (46). Therefore, a virally-encoded DNA polymerase is most likely responsible for baculovirus replication. The mechanisms whereby the

DNA is packaged into nucleocapsids is unknown (51). The baculovirus DNA contains five homologous regions which are widely dispersed on the viral genome (17). Guarino et al. (37) hypothesized that these regions of AcMNPV DNA might serve as origins of DNA replication, but there is no clear evidence for this.

Viral RNA Synthesis

Analysis of the transcription of baculovirus DNA is currently being pursued in several laboratories. Results indicate that baculovirus mRNA contains poly A tails and 5' methylated cap structures in the tradition of eucaryotic mRNAs (13, 103). Transcriptional and translational maps have aided in understanding AcMNPV gene organization and expression (58). Through in vitro translation of hybrid selected RNAs isolated from infected cells, Smith et al. (90) mapped 5 early and 19 late viral polypeptides. Esche et al. (21) mapped 5 early and at least 32 late viral proteins on the AcMNPV genome. With the aid of complementary DNAs (cDNAs), which were synthesized from mRNA through reverse transcriptase, Adang and Miller (1) mapped 11 late AcMNPV infected cell-specific polypeptides.

Early in infection, a host RNA polymerase II transcribes viral genes (30). Host RNA polymerase II is sensitive to α-amanitin. During the course of infection, sensitivity to α-amanitin decreases, and by 24h p.i., α-amanitin no longer inhibits viral RNA synthesis. Therefore, as the temporal cascade proceeds into the late phase of ICSP synthesis, an α-amanitin resistant RNA polymerase is also detected. An early viral gene may encode this polymerase or perhaps some

viral gene product is able to alter an already existent host polymerase.

Rohel and Faulkner (83) characterized at least 50 viral RNA transcripts in AcMNPV. Lubbert and Doerfler (58) were able to map 11 early and more than 90 late viral RNAs on the AcMNPV genome using cloned DNA fragments as hybridization probes in Northern blot experiments. Erlandson and Carstens (20) analyzed early mRNA in baculovirus infections and concluded that early transcription of AcMNPV appears to involve more extensive regions of the genome than those of other DNA viruses.

Late transcripts also map to various locations of the genome (83, 90, 91, 103). Much of the complexity of deciphering baculovirus gene expression is due to the fact that there are many transcripts which have not been linked to translation products. It appears that the control of temporal expression of ICSPs during the course of infection is primarily at the level of transcription (27). The differences in the relative concentrations of various mRNAs may be the result of variability in RNA transcription or processing or perhaps in the transport of individual RNAs.

Regulation of Virus Transcription

Recent analysis of the temporal sequence of AcMNPV RNA transcription revealed individual transcription units composed of overlapping early or late RNAs, or both (27). There are two types of transcriptional units. The first is composed of overlapping early and late RNAs with common 3' termini. The other consists of abundant overlapping RNAs with common 5' ends (82, 104). While studying

transcripts with common 3' ends, Friesen and Miller (28) observed that the smallest RNAs appeared early in infection and were replaced in time by successively larger RNAs which were initiated further upstream. Primer extension analysis suggested that temporal regulation involves the sequential activation of upstream promoters and the coordinate deactivation of downstream promoters. These workers concluded that the transcription of upstream genes suppresses that of downstream genes by a process known as promoter occlusion. Mainprize et al. (61) showed that even though groups of overlapping RNAs occurred throughout the viral genome, only some of them demonstrated the synthesis of larger RNAs with the progression of time.

Previously, there was no evidence for splicing in baculovirus transcripts (58). Recently, however, Chisolm and Henner (15) showed that splicing does occur in RNA transcribed from the IE-1 immediate-early gene of AcMNPV.

Mechanism of the Temporal Cascade

The molecular mechanisms involved in the temporal cascade are not known. Studies are just beginning to identify some viral proteins which may be responsible for regulating the expression of other gene products. Guarino and Summers (38) recently showed that an immediate-early gene product regulated a delayed-early NPV gene. In these experiments, constructs containing the promoter for the 39K (delayed-early, β) gene were expressed in infected but not uninfected cells. Therefore, some immediate-early gene product must be required for the expression of the 39K gene. Additional transient assay experiments indicated that the gene for the putative trans-activating factor was

an immediate-early (α) gene known as IE-1. The IE-1 gene encodes a polypeptide with a molecular weight of approximately 66K. In addition to activating the 39K gene, IE-1 also activates the transcription of several other early AcMNPV genes. Therefore, IE-1 may have a role in regulating β gene expression (39).

Early gene products may also serve to regulate late gene expression. Using transient assays, Knebel et al. (49) showed that the p10 late gene promoter was inactive in uninfected S. frugiperda cells but highly active when the promoter-containing construct was transfected at least 18h p.i., thereby indicating that other viral gene products are necessary for p10 expression. The specific gene products carrying out this regulation are not yet known.

Antisense RNA may be operating in the inhibition of early gene expression. In studies by Friesen and Miller (28), two nonoverlapping genes, extending in opposite directions, code for 35K and 94K polypeptides which are expressed as immediate-early ICSPs. Late in infection, RNAs are transcribed from promoters located several kilobases upstream from the 35K gene. Even though these transcripts completely overlap the 35K and 94K genes, they do not code for any known proteins. This finding suggests that these late RNAs may suppress the early genes (28).

Polyhedrin and p10 ICSPs

Polyhedrin and p10 are the most abundantly synthesized late ICSPs. The mRNAs for these two proteins are synthesized at high rates throughout late infection, even as late as 90h p.i. when nearly all other ICSP synthesis has ceased (82). Thus, the abundancy control of

these polypeptides is most likely at the level of transcription. The polyhedrin gene was the first NPV gene to be sequenced (44). There exists a high degree of conservation among the coding regions of the polyhedrin genes from various baculoviruses. A 12-base sequence located 20 bases upstream of the ATG initiating site was conserved in the polyhedrin genes of various NPVs (56). Kuzio et al. (52) and Lubbert and Doerfler (46) sequenced the p10 gene. Leisy et al. (56) found that the 5' start site of p10 lies within the 12-base pair consensus sequence. In addition, this sequence is conserved in all late NPV genes examined thus far. It has been suggested that this 12-base sequence may serve as a common denominator for all late gene promoters. Recently, potential consensus sequences for early regulatory elements have also been identified (73). These 12-base sequences show similarities to the herpesvirus α-4 binding site and baculovirus hr5 enhancer core sequences, both of which are important in the regulation of genes encoding later classes of proteins.

Polyhedrin and p10 are driven by very strong promoters (91, 1). Given this fact, foreign genes can be inserted into the polyhedrin gene and expressed under the control of this promoter. Within the past few years, a wide variety of genes have been expressed using baculovirus vectors. These include genes for beta interferon (89), influenza hemagglutinin proteins (78), human immunodeficiency virus (HIV) gag and env proteins (45), and E. coli β-galactosidase (77) to name a few. Investigation into the use of vectors utilizing the p10 gene is presently in progress (52).

Homologous Regions of DNA

There is a wealth of literature dealing with repeat sequences in animal DNA viruses (3, 7, 14, 102), but repeat sequences have just recently been discovered in baculoviruses. Cochran and Faulkner (17) were the first to show that AcMNPV contains five homologous regions (hr) which are located throughout the genome. Designated hr1 through hr5, the sequences range in size from 500- to 800-base pairs.

These repeat sequences have been shown to possess enhancer activity (37). Enhancers are repeat sequences of varying length which greatly increase the transcription of genes located upstream or downstream from the enhancer. Oftentimes, these genes are located several kilobases from the active enhancer. The SV 40 enhancer (7), considered to be the prototype enhancer, consists of 72-bp repeats and operates in a host specific fashion. Although the mechanism of enhancer activity is unknown, it has been proposed that enhancers provide DNA binding sites for RNA polymerase II or for host specific factors which induce a conformational change in the DNA (14). Such structural changes might cause the DNA to be more active transcriptionally. Or it is also possible that the host factors, by binding to enhancer sequences, sequester the transcriptional template in a cellular compartment conducive to enhanced transcription (54).

In work by Guarino and Summers (38), the hr5 region of a particular strain of AcMNPV enhanced delayed-early gene expression. These five regions have been sequenced by Guarino et al. (37) and shown to consist of approximately 30-bp repeats, some of which are imperfectly repeated several times. Through deletion analysis of hr5, studies

revealed that one copy of the inverted repeat is sufficient for enhancer activity. In the case of most other enhancers, including those of SV 40, more than one copy is necessary for enhancer activity. In another study, Guarino and Summers (40), demonstrated that late genes can be distinguished from delayed-early genes by their absolute dependence upon a cis-linked enhancer in addition to an immediate early gene such as IE-1 for expression in a transient assay system. Although these results appear to suggest that AcMNPV enhancer activity is a requirement for late gene expression, it is not clear how accurately a transcient expression system mimics the in vivo infection. Considering the host factor requirement, it is possible that baculovirus host specificity may be governed by the activity of one or more of these enhancers.

Host Specificity

Host cells infected with DNA viruses can be classified as either permissive, semipermissive, or nonpermissive (25). In permissive infections, DNA replication is completed and protein synthesis occurs, leading to the production of infectious virus progeny and eventual cell lysis. DNA replication is limited in semipermissive infections. Viral DNA does not replicate in nonpermissive cells (29).

Host specificity studies are necessary to fully understand the ways in which baculovirus gene expression is regulated by virus-host interactions. Such understanding is important for the development of viral pesticides having the desired host specificity and increased virulence. Permissive infections are well characterized at the cellular and organismal level, but very little is known about semi- or

nonpermissive infections. For unknown reasons, good host range mutants have not been isolated. Therefore, semi- or nonpermissive systems have to be used to study host specificity. These studies suggest that the host can restrict the virus at different stages in its replication cycle.

So far, very little information is available on baculovirus host specificity. Most baculoviruses have a narrow host range (47), but AcMNPV has a relatively wide host range. It productively infects at least 33 different species within 10 different families of Lepidoptera (68). Species outside this order are not susceptible to productive infections. Also, NPVs do not replicate in vertebrates (98). Burand et al. (8) showed that the host range of the several baculoviruses tested could not be extended by transfection with viral DNA.

Three host range model systems, designated as system I, II, and III, have been established in our laboratory. In system I, an AcMNPV variant replicated poorly in the semipermissive S. frugiperda cells (31). Carpenter and Bilimoria (11) and Bilimoria et al. (5) studied the infection of TN-368 cells by SfMNPV, and concluded that the primary restriction in the nonpermissive component occurred during the expression of immediate early genes.

McClintock et al. (65) showed that in AcMNPV infections of gypsy moth cells, early but not late ICSPs were synthesized. Typical cytopathic effect was exhibited, but infectious progeny virus was not produced.

Host range restrictions can occur at different points in the viral replication cycle as illustrated above, and it is most certain

that the underlying mechanisms of these restrictions involve complex virus-host interactions. Perhaps baculovirus enhancers will be shown to be important in host specificity through interactions with particular host cell factors. Work is just beginning in the characterization of the hrs (38, 37, 40), but with continued progress, the mechanisms of baculovirus host range may begin to be understood sooner than previously imagined.

Purpose and Scope

This research is based on the initial observation of Summers et al. (96) that only a small percentage of B. mori cells infected with AcMNPV produced polyhedral inclusion bodies at 42h p.i., whereas numerous PIBs were found in S. frugiperda cells. The formation of PIBs is perhaps the most distinguishing feature of the cytopathic effect of permissive SF infections. PIBs appear soon after infection, usually within 24h, and increase in number throughout the course of infection, until finally late in infection, the nucleus is fully laden with PIBs. With the eventual lysis of the host cell, the PIBs are released into the environment of the cell. Such a major disparity in the CPE produced in these two cell lines, signaled an underlying host range restriction in the BM infection and raised important questions as to how the BM cells were able to block some function required for PIB formation. No information is yet available concerning virus restriction or gene expression in BM cells.

In this model system, which I have designated System III, SF cells comprise the permissive component while BM cells constitute the semipermissive component. In order to understand how this host range

restriction might be mediated and to define the stage at which the restriction occurs, it was necessary to analyze virus-induced macromolecular synthesis in both SF and BM cells. In this study, the cytopathology produced in SF versus BM cells was first characterized by monitoring the infected cells by phase contract microscopy throughout the infection. Virus growth kinetics were performed to determine if infectious virus was produced. Due to the lack of PIBs, studies using polyacrylamide gel electrophoresis of ICSPs were conducted to determine whether polyhedrin, the major constituent of the PIB was produced. If studies revealed that polyhedrin, a major late ICSP, was not produced, other late ICSPs might also be restricted. Therefore, in this study I chose to concentrate on the regulation of late ICSP synthesis.

The results obtained in this study reveal the stages at which host range restriction occurs in BM cells and pave the way for additional work aimed at determining the exact molecular mechanisms involved in this restriction. This study also aids in our understanding of semipermissive infections and demonstrates yet another way in which the temporal cascade can be restricted in semipermissive infections. Knowledge gained from System III and that of future work involving semipermissive infections may ultimately lead to the identification of the genes or regulatory sequences governing baculovirus host specificity. Only then can the genes regulating baculovirus host range and virulence be manipulated in a safe and predictable manner for a given insect host.

CHAPTER II

MATERIALS AND METHODS

Cell Growth and Maintenance

Continuous cell lines of Spodoptera frugiperda (SF-IBLB-21) (101) and Bombyx mori (BM-5) (courtesy of J. Vaughn) (32, 80) insect cells were grown in Hink's TNMFH medium (42), supplemented with 10% fetal bovine serum. Both cell lines were grown at 27°C and subcultured at a 1:10 ratio at five-day intervals.

Virus Stock Production

Virus stocks were grown in vitro in SF cells using the procedure of Hink (42). Total virus was harvested by disrupting infected cell monolayers and then sonicating the samples in a Heat Systems Ultrasonicator at 50 W for 10 sec. Preparations were clarified by centrifugation at 2,000 x g for 10 min. The virus-containing supernatant was centrifuged at 33,000 x g for 30 min to remove any contaminating cell debris and PIBs. The pellet, containing budded virus, was resuspended in serum free medium and titrated by the tissue culture infectious dose 50 ($TCID_{50}$) assay of Brown and Faulkner (6) as previously described (11).

The virus was then subjected to plaque purification (113). Virus harvested from individual plaques was designated as passage 1 virus (P-1). This low passage number virus was used throughout the experiments in order to avoid the production of defective virions which

often results from using virus which has been passed through too many infections (26).

Cytopathic Effect

Both SF and BM cells were grown in 25-cm² Corning tissue culture flasks. Following two days of growth, logarithmic phase cells (2 to 3 x 10^5 cells/10 cm² growth surface) were inoculated with AcMNPV at a multiplicity of infection (MOI) of 20 plaque-forming units (PFUs) per cell and rocked gently for two hours at room temperature (23°C-26°C) on a Bellco rocker platform. Control cells were treated with fresh medium only. Following a two-hour adsorption period, virus inoculum was removed. The cells were washed twice with fresh medium to remove unabsorbed virus and then incubated at 27°C. At various times post infection, cells were observed for cytopathic effect by phase contrast microscopy.

Virus Growth Kinetics

Growth Curves

At the logarithmic phase of growth, SF and BM cells, grown in Corning 6-well trays, were infected with virus and washed as described above. At specific times following infection, total virus was harvested by collecting disrupted cell monolayers along with the supernatant, then sonicating the samples to release intracellular virus. $TCID_{50}$ assays were performed in triplicate for each sample taken using SF cells as indicator cells which were scored for PIB production.

Blind-Passage Experiments

SF and BM cells were infected with AcMNPV in Corning 6-well trays as described for the growth curves. However, following adsorption and washing, fresh medium was added and a sample of supernatant was withdrawn to titer the level of virus at time zero. In an identical infection, following washing and the addition of fresh medium, the cells were allowed to continue growing for 48h at 28°C. These cells comprised the passage 1 set of infected cells. After 48h, 500 µl of supernatant was removed from the well, mixed with 1 ml of fresh medium and then added to a fresh batch of cells from which the old medium had been removed. In this procedure, the virus-containing supernatant was diluted 1:2 into medium in an attempt to avoid an excessively high multiplicity of infection which can lead to the production of defective virions. Following a 2h adsorption period, the supernatant inoculum was removed, fresh medium added, and the cells were then incubated for 48h. The same procedure was repeated at each passage. Also at each passage, a sample of supernatant was removed and titered in the $TCID_{50}$ assay to determine the level of budded virus.

Protein Synthesis

Incorporation of Radioactive Isotopes

SF and BM cells were grown and infected in 6-well trays as described. After virus adsorption occurred, spent medium was removed, the cells were washed, and fresh medium was added. At designated times following infection, the cells were washed twice with amino acid-deficient Grace's medium (G-aa) and incubated in G-aa for 2h. Cells were then pulsed for 1h with 50 µCi per well of either

[^{35}S]methionine (800 Ci/mmol; NEN), or a mixture of [^{3}H]-amino acids (250 mCi/mg; ICN) diluted in 0.5 ml of G-aa medium, to ensure the labeling of all proteins, should some be disproportionately methionine rich or poor.

Sample Preparation for PAGE

Following the labeling period, cell monolayers were scraped loose using a rubber policeman and concentrated by centrifugation at 300 x g for 2 min in a microcentrifuge. Cell pellets were washed three times with ice-cold phosphate-buffered saline (PBS). The pellet was re-suspended in Laemmli sample buffer, heated in a boiling water bath for 3 min and stored at -20°C.

Polyacrylamide Gel Electrophoresis and Fluorography

Several types of gel systems were utilized. Initially, a 11% running gel with a 4% stacking gel was used in a Studier gel apparatus (93). Sample aliquots containing 1 x 10^{5} cpm of incorporated [^{35}S]-methionine were subjected to SDS-PAGE using the Laemmli buffer system (53). Electrophoresis was performed at 20-30 mA for approximately 6h. In order to achieve better resolution of the middle to lower molecular weight proteins, 6-15% exponential gradient gels, overlaid with a 4% stacking gel, were used in later experiments. The gradient gels were run using a Hoefer vertical gel assembly with dimensions 15.5 cm x 17.5 cm x 0.075 cm. These gels were loaded with the same amount of incorporated label ([^{35}S]methionine or [^{3}H]-amino acids) and electrophoresed at 20-30 mA, for approximately 5-6h. Following electrophoresis, all gels were processed for fluorography by soaking in

EN^{3}HANCE (NEN) and were then dried under vacuum on Whatman 3MM paper for 5-6h at 75°C and exposed against Kodak X-OMAT x-ray film at -78°C.

The molecular weights of polypeptides were determined using prestained protein markers (12,000-200,000 daltons; BRL). The distances migrated by the standards were marked in the dried gel with [^{3}H] radioactive ink prior to exposure to x-ray film. Infected cell-specific proteins were identified by comparing control versus infected cell lanes. Proteins not present in the control profiles were designated as ICSPs. To determine rates of synthesis for the ICSPs, the fluorograms were scanned with the aid of an E-C densitometer coupled to an Apple IIe microcomputer. The "Zeineh Videophoresis II" electrophoresis reporting integrater program software (Biomed Instruments, Inc.) was used for scanning the gels.

CHAPTER III

RESULTS

Cytopathic Effect of AcMNPV in SF and BM Cells

In order to compare the cytopathology produced by AcMNPV in these cells, infected and control cells were observed by phase contrast microscopy over an extended period postinfection. The CPE produced in each cell line was very different (Figs. 3-6), and these differences became more pronounced during the course of infection.

Infected SF cells began to display nuclear hypertrophy by 24h p.i. A ring zone around the swollen nucleus, a characteristic feature of permissive baculovirus infections (34, 50) was prominent. Approximately one-third of these cells contained several PIBs within each nucleus. At 48h p.i., 92% of the cells contained PIBs (Fig. 3), with an average of 8 PIBs per cell. In addition, 25% of the cells had lysed by this time. All cells contained PIBs at 60h, and 50% of the cells were lysed. With continued incubation, cell nuclei were packed with PIBs, with at least 10-15 per cell, and nearly all cells had lysed by this late in infection.

Infected BM cells began to display CPE by about 24h p.i. with enlarged, darkened nuclei (Fig. 4A) not unlike those seen in the SF infection. Within a few hours, however, cytopathology began to differ greatly from that of SF cells. Dark areas of terminal polarization

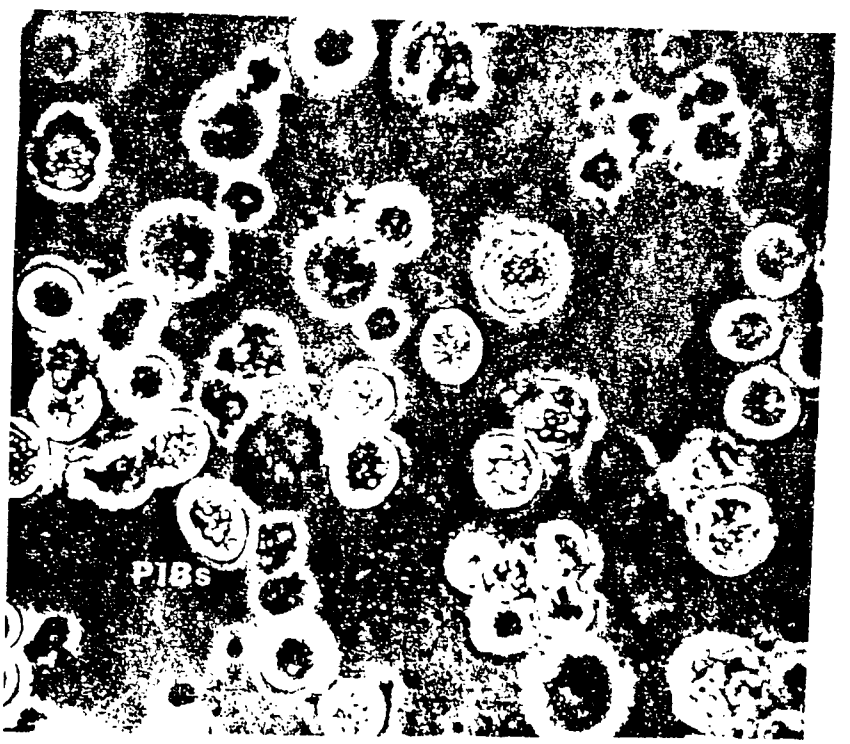
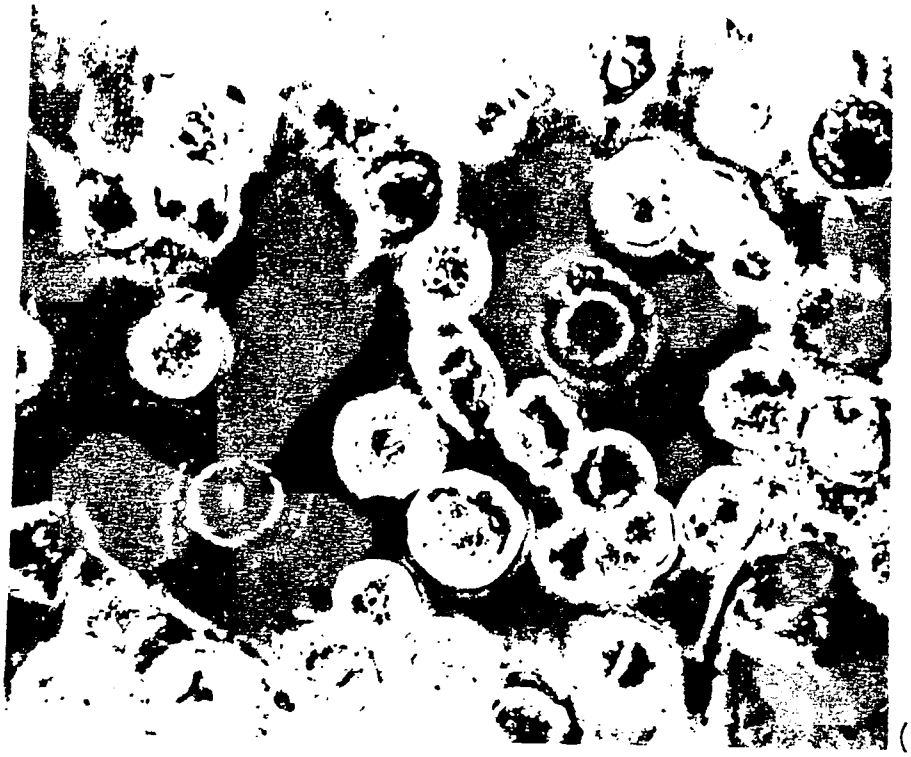


FIGURE 3. Phase contrast micrographs of S. frugiperda cells infected with AcMNPV. SF cells were infected at a MOI of 20 PFU per cell. AcMNPV-infected cells at 48h following infection. Numerous PIBs are present within the host cell nucleus. Magnification (1500X).

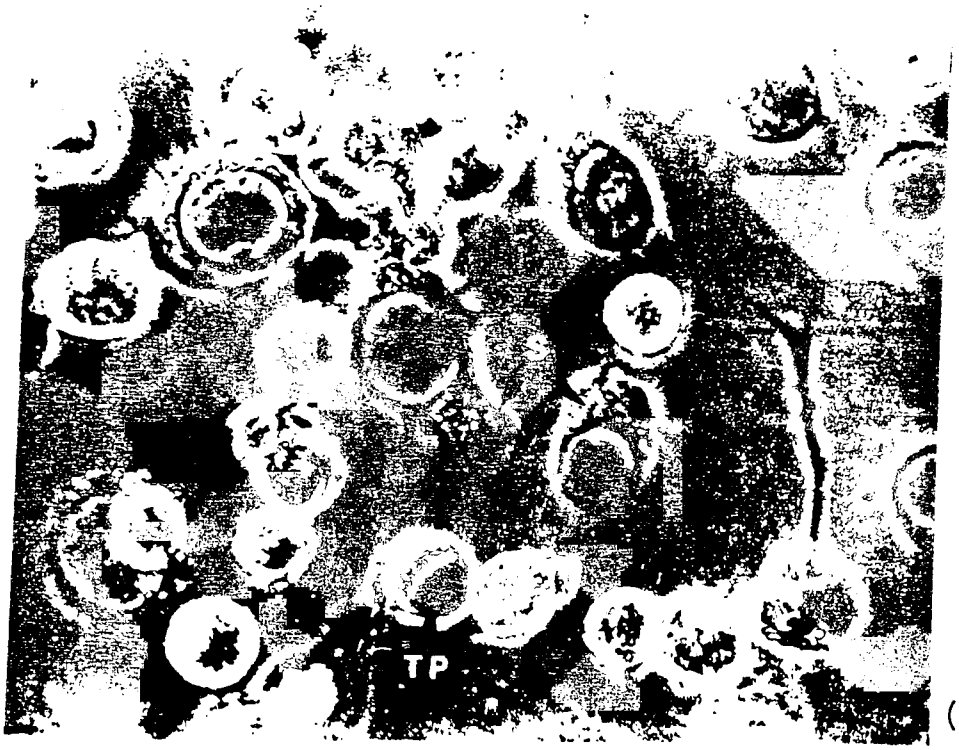


(A)

(B)

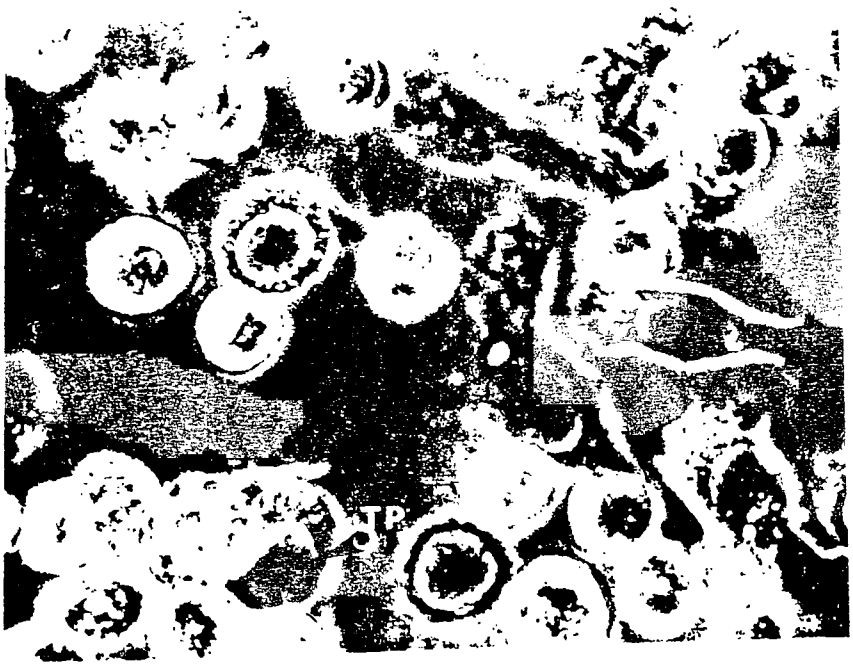
FIGURE 4. Phase contrast micrographs of AcMNPV-infected B. mori cells during initial stages of CPE. (A) At 24h p.i. cells displayed darkened, enlarged nuclei (N). (B) Dark areas of terminal polarization (TP) appeared at 24-30h p.i.

(A)



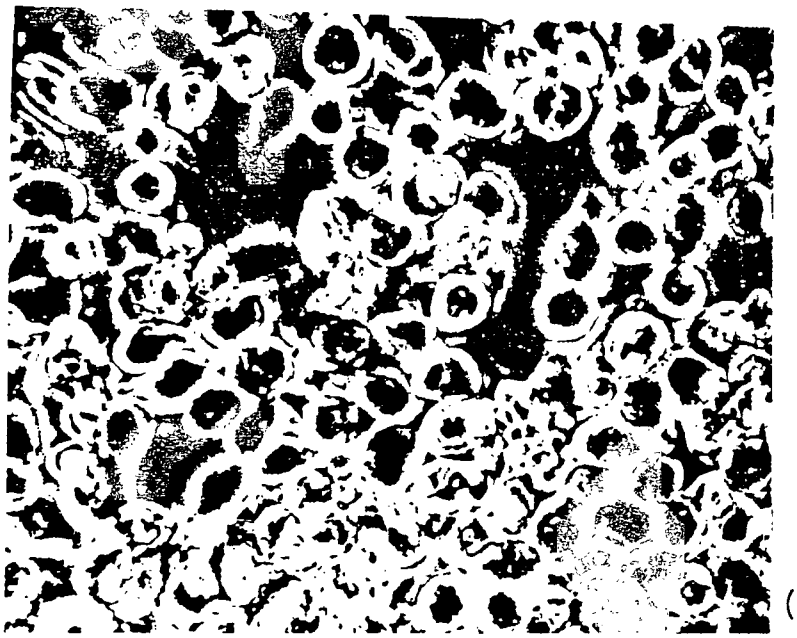
(B)

FIGURE 5. Phase contrast micrographs of the middle-late stages of an AcMNPV infection of B. mori cells. (A) At approximately 48h p.i., the areas of terminal polarization were succeeded by the appearance of unprecedented sac-like bodies (S) at the cell periphery. (B) As the infection progressed, the sac-like bodies extended away from the cell (50-60h p.i.).

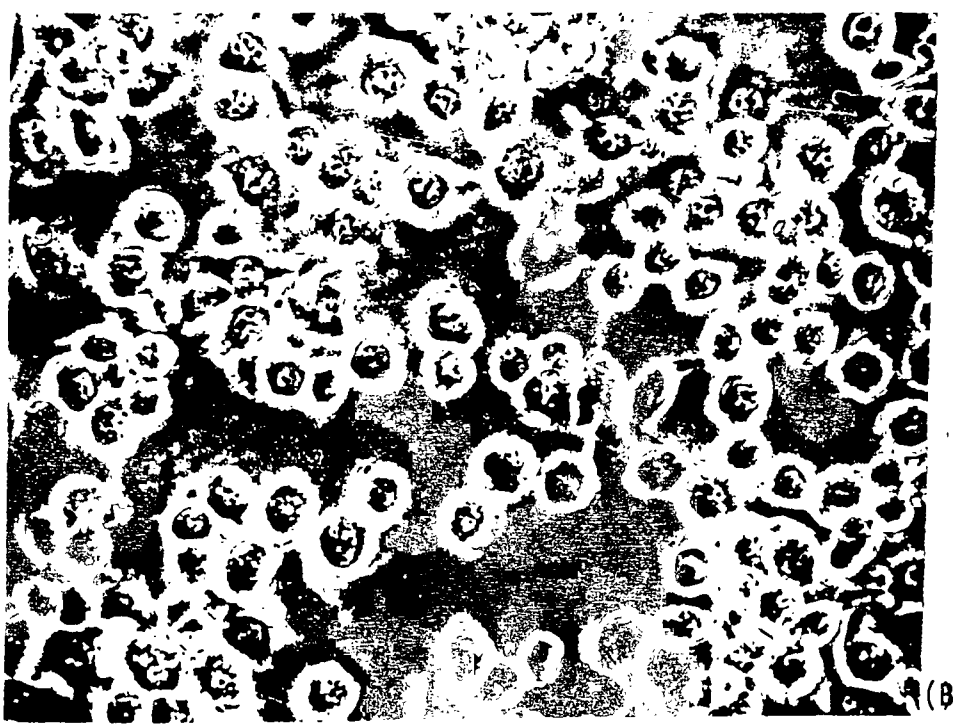


(C)

FIGURE 5. Continued. (C) Further protrusion of sac-like bodies beyond 60h p.i.



(A)



(B)

FIGURE 6. Phase contrast micrographs of uninfected (A) S. frugiperda and (B) B. mori cells.

(TP) began to appear at 24-30h (Fig. 4B). By 48h p.i., these darkened areas had progressed into unique "sac-like bodies" at the cell periphery (Fig. 5A). These structures were visible in 75% of the cells, and during the course of infection, these sac-like bodies protruded even more from the cells (Figs. 5B-5C), but CPE did not change drastically after 48h. Such structures were not seen in SF cells and have never been described in any other baculovirus infections. As expected, PIBs were never seen in infected BM cells, even as late as nine days p.i., whereas Summers et al. (96) observed PIBs in 1% or less of the BM population. Also, in contrast to the SF infection, less than 5% of the BM cells lysed. No CPE of any type was detected in uninfected SF or BM cells (Figs. 6A-6B).

Infectious Virus Yields in the Permissive and Semipermissive Cells

The CPE of an infected cell is a reflection of less tangible virus-cell interactions. To determine if the different cytopathologies were associated with differences in the pattern of infectious virus production, virus growth kinetics were performed for each cell line. As shown in Fig. 7, the total virus titers produced in SF cells showed greater than a 3-log (2,000-fold) increase from time 0 to 40h p.i. In BM cells, there was no significant production of infectious virus even up to 60h p.i. Also, considering that the accuracy of the $TCID_{50}$ assay is approximately ±20% (81), the data suggest that the increase in infectious virus in SF cells is significant, whereas the apparent rise and fall of virus in the BM cells is not.

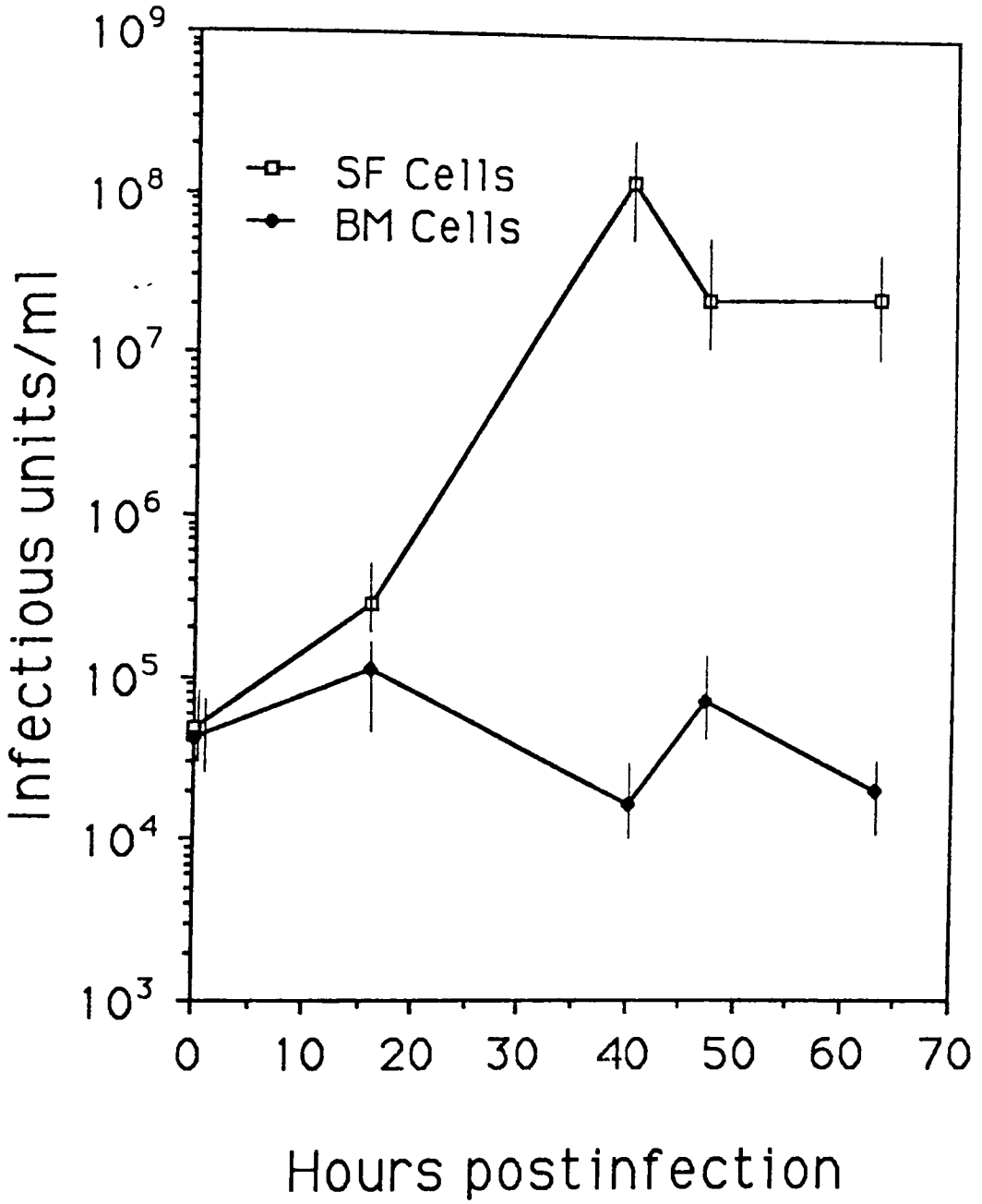


FIGURE 7. Virus growth kinetics in AcMNPV infected S. frugiperda and B. mori cells. The curves represent total virus titers. At specific times postinfection, cell monolayers were disrupted and collected with supernatants. Samples were then sonicated to release intracellular virus, and $TCID_{50}$ assays were conducted to determine the levels of total virus. The data ranges of triplicate samples are indicated at each sampling period.

The blind-passage experiment was performed as a check on the data obtained in the growth curve experiment. In this experiment, at the end of 48h, a portion of the supernatant was taken from the infected cell culture and used to infect a fresh batch of cells. After two days, a supernatant sample was withdrawn and added to a new set of cells. A total of three such passages were conducted. In addition at each passage, a sample of the supernatant was titrated for budded virus. In a serial passage of this sort, if infectious virus is produced at a particular passage, the supernatant used to infect the next batch of cells should contain a high level of virus which will infect these cells and, in turn, lead to the production of infectious virus at this passage. At each step, the supernatant inoculum was diluted 1:2 as a result of its addition to media overlaying the cell monolayer. Even with this dilution effect, the level of input virus was high enough, as shown for the positive control in Fig. 8, to produce a productive infection if the infection was not blocked by some host range restriction.

The data (Fig. 8) indicate that high levels of infectious virus were produced at the first two passages, even though the increase in titer was not as great as in that seen in the growth curve experiment. The slight decrease at the third passage was probably due to a high MOI passage effect (43, 79, 26).

In BM cells, the final virus titers at the first and second passage were below the input level of virus. Considering the fact that there is significant overlap in the ranges of the data, particularly at the second and third passage, the slight increase in titer

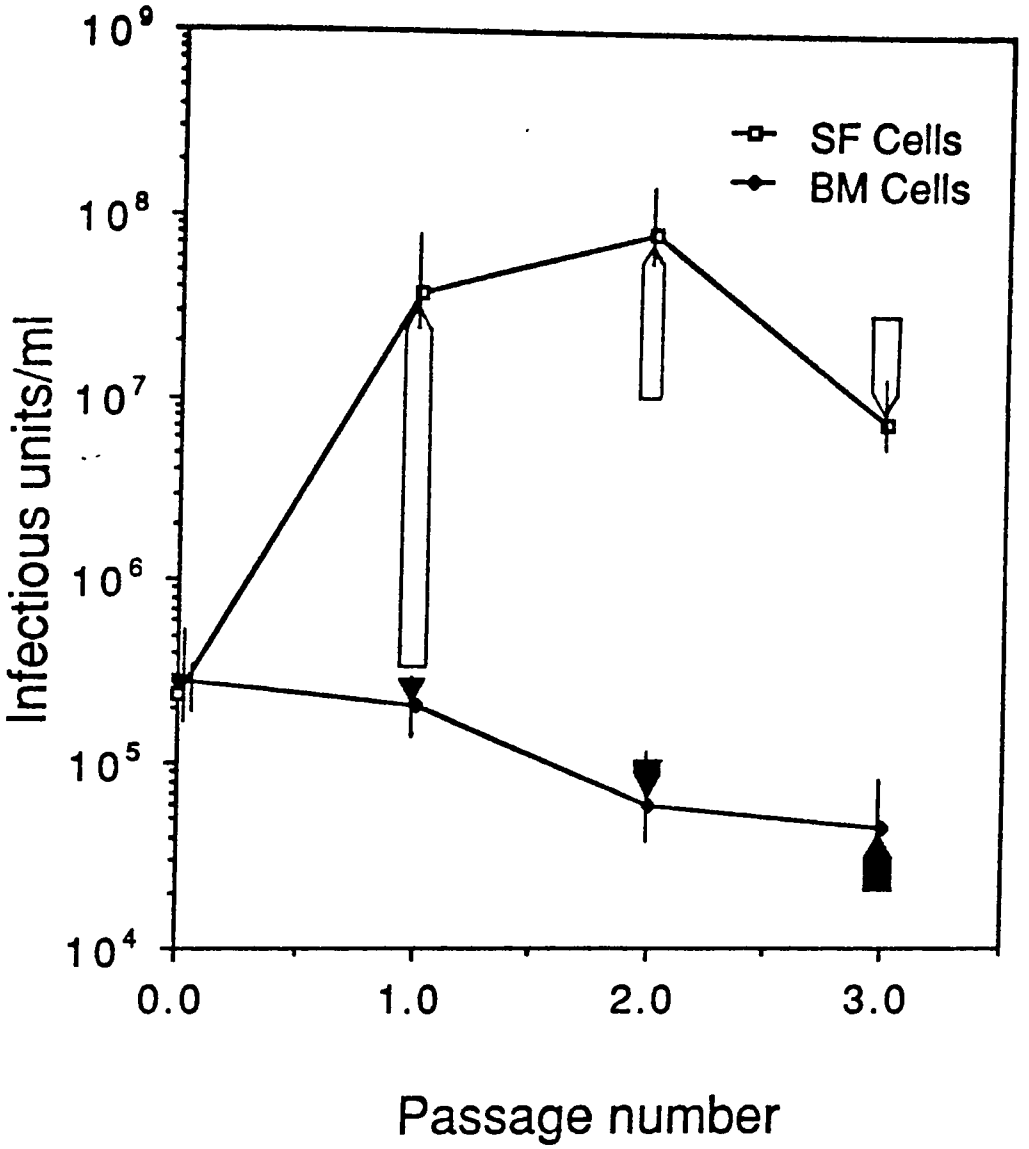


FIGURE 8. Blind-passage experiment of AcMNPV in S. frugiperda and B. mori cells. Cells were grown and infected as described in materials and methods. Following 48h infection (passage 1), a sample of the supernatant was diluted 1:2 in fresh medium and used to infect a fresh monolayer of cells (passage 2). After a 2h adsorption period, the inoculum was removed and fresh medium was added. Following 48h incubation, the supernatant was diluted as before and used to infect another batch of cells (passage 3). Prior to dilution, a sample of supernatant was titered for virus at each passage in the series. Based on the calculated levels of input virus following supernatant dilution, and the mean virus titers (IU/ml) produced at each passage, fold increases or decreases were calculated at each passage and are represented by the bars. The base end of each bar represents the input level of virus while the arrow end represents the mean virus titer (IU/ml) produced at each passage. The ranges of triplicate samples are given for virus titers obtained at each passage.

above the input level at the last passage might be due to sampling error. During the course of the experiment, there was a 9-fold total dilution of the virus. In BM cells, the final virus titer after three passages was no greater than the level resulting from mere dilution. Therefore, the blind-passage data suggest that infectious virus was not made in BM cells and confirm the results of the growth kinetics experiment.

Infected Cell-Specific Protein Synthesis

Because polyhedrin serves as the major component of the inclusion body matrix and PIBs were not produced in the semipermissive infection, SDS-PAGE was performed to determine if polyhedrin was synthesized in BM cells. As seen in Fig. 9, polyhedrin was not synthesized. In addition, p10, the other major late ICSP, was also not produced (Fig. 10). Considering that the two major late ICSPs were blocked in BM cells and assuming that a whole temporal class of ICSPs might be regulated as a group, it was logical to hypothesize that other late proteins might also be blocked. Careful analysis of Fig. 9 showed that numerous other late ICSPs were blocked in the BM infection (Table 1). As seen in Table 1, many ICSPs were produced in either BM cells or SF cells. Altogether, 15 ICSPs were produced in SF but not BM cells. On the other hand, 6 ICSPs were found only in BM cells. Eight ICSPs were produced in BM as well as SF cells, but at different rates in each cell line.

In order to identify each ICSP in Fig. 9 with a temporal class of proteins, an effort was made to correlate these ICSPs with ICSPs in other studies of AcMNPV/SF infections where the proteins have already

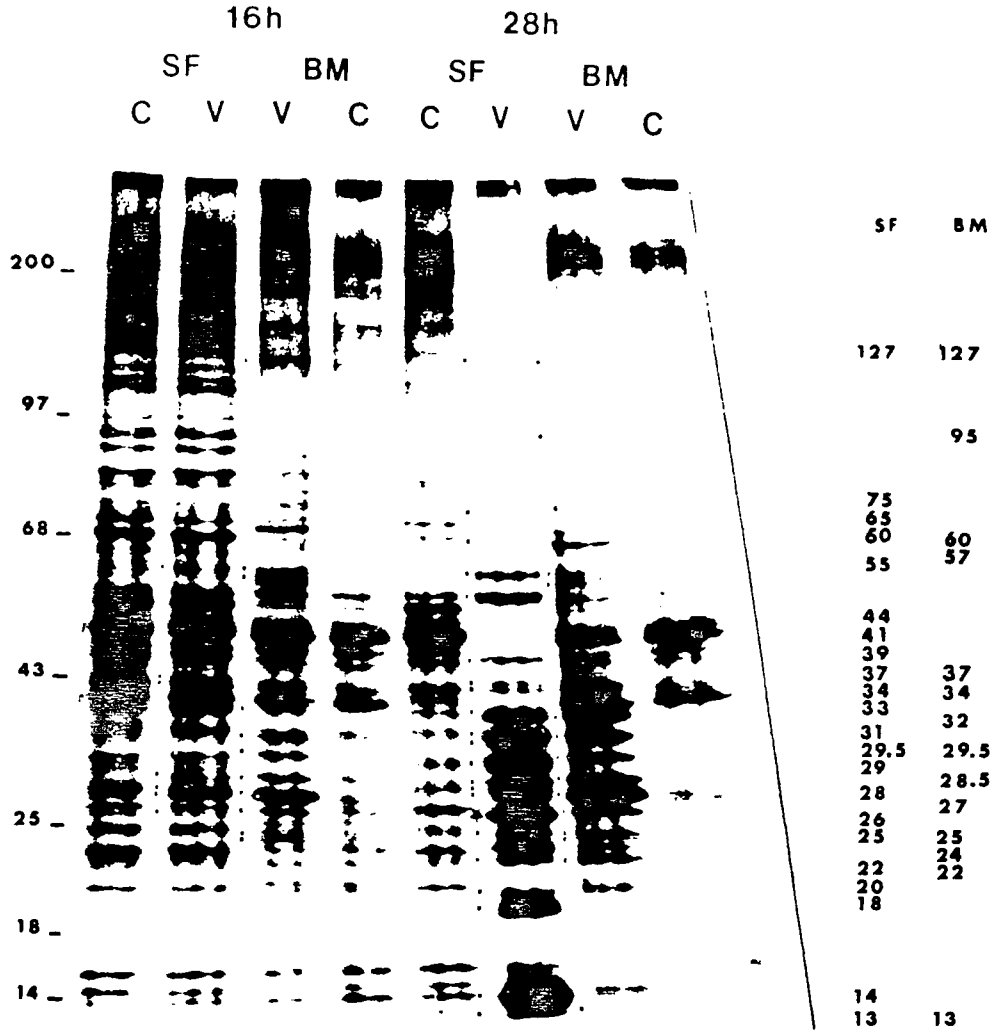


FIGURE 9. A fluorogram of [^{35}S]methionine-labeled polypeptides from AcMNPV infected S. frugiperda (SF) and B. mori (BM) cells at 16 and 28h p.i. Logarithmic phase cells were infected with virus at an MOI of 20 PFU per cell. Cells were washed and starved by incubation in amino-acid deficient Grace's medium (G-aa) for 2h and pulse labeled for 1h with 50 uCi of [^{35}S]methionine (800 Ci/mmol; NEN). At the end of each labeling period, the cells were treated as described in the text, and sample aliquots containing 1x10^5 cpm of incorporated radioactivity were electrophoresed in a 6-15% gradient polyacrylamide gel. Following electrophoresis, the gel was dried and exposed against Kodak X-OMAT x-ray film. Numbers above the lanes indicate the times p.i. at which the samples were radiolabeled. Uninfected control (C) and virus-infected (V) cells were compared at each time-interval tested. The ICSPs in the respective cell lines are indicated by dots with corresponding molecular weights (kilodaltons) indicated on the right. Polyhedrin is indicated by an asterisk. The numbers on the left indicate the molecular weights of standard molecular weight protein markers.

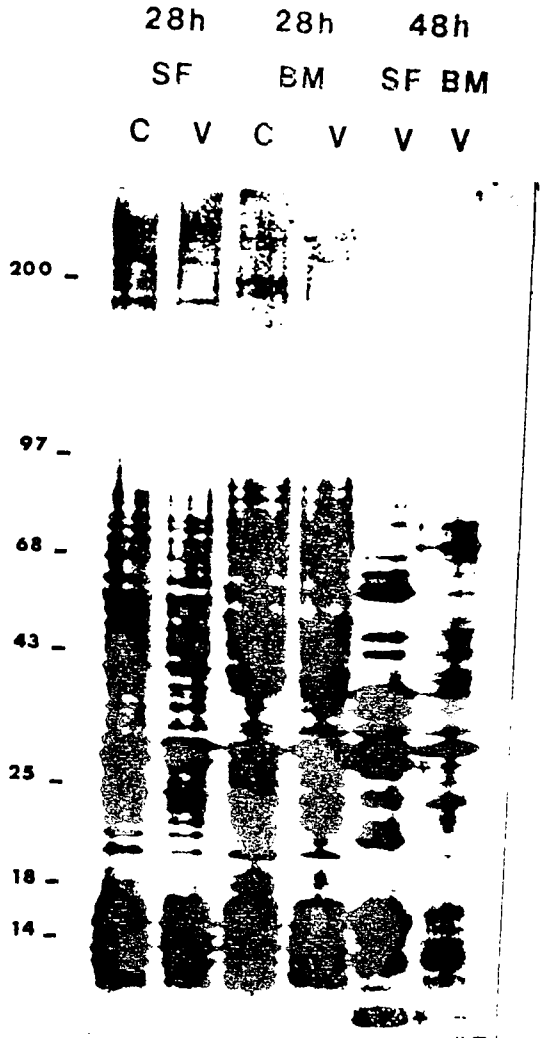


FIGURE 10. SDS-polyacrylamide gel fluorogram of AcMNPV-infected S. frugiperda and B. mori cells, pulse labeled with a mixture of [^{3}H]-amino acids. Cells were infected as described in Fig. 9, and at designated times postinfection, the cells were labeled for 1h with [^{3}H]-amino acids (250 mCi/mg; ICN). Following labeling, the cells were processed and the proteins were analyzed by electrophoresis as described in Fig. 9. The asterisks indicate polyhedrin (26K) and the p10 protein.

TABLE 1

Infected Cell-Specific Proteins Produced in AcMNPV-Infected SF and BM Cells

SF Cells		BM Cells	
16 h	28 h	16 h	28h
	127K	127K	127K
			95K*
	75K* +		
	65K* +		
60K +	60K +	60K +	60K +
		57K*	57K*
	55K*		
44K* +	44K* +		
41K*	41K*		
39K* +	39K* +		
	37K +	37K +	37K +
	34K +	34K +	34K +
	33K* +		
		32K*	32K*
	31K* +		
29.5K	29.5K	29.5K	29.5K
29K* +	29K* +		
		28.5K*	28.5K*
28K* +	28K* +		
		27K*	27K*
26K*	26K*		
	25K	25K	25K
		24K*	24K*
	22K		22K
	20K*		
	18K* +		
	14K* +		
	13K		13K

*Infected cell-specific proteins which are unique to each infection at the times they are detected.
+Indicates structural proteins.

been temporally classified. Depending on the running gel concentration, proteins will migrate with slight differences in mobility from one gel to another. Despite these differences, certain distinguishing patterns of banding can usually be discerned. Thus, it is possible to identify and correlate proteins from one gel to the next. In this manner, the ICSPs produced in System III were correlated with those identified by Wood (114) and Carstens et al. (12). The intensity of the bands in System III and those of the other studies corresponded very well through the course of infection, and thus aided in the identification process. Based on work conducted by Wood (114) and Carstens et al. (12), it was possible to classify most of the ICSPs in System III into a particular temporal class. The temporal class nomenclature varies slightly among workers in this field. For example, Wood (114) used the following classification scheme: early (2h), intermediate (10-16h), transitional (16-24h), and late (beyond 24h). Other workers (19) categorized the AcMNPV replication cycle into early, middle and late phases. To avoid confusion, the classification scheme has been standardized by Kelly and Lescott (48) as the α, β, γ, and δ system. Therefore, based on the times when the ICSPs were synthesized in the systems of Wood (114), Carstens et al. (12), and Stiles and Wood (92), their classification scheme could be translated into the currently used system.

Following this type of analysis, in the group of ICSPs unique to SF cells, the 41K, 29K, and 28K ICSPs were identified as α proteins (Table 2). The 55K, 44K, 31K, 20K, 18K, and 14K ICSPs corresponded with γ proteins, while polyhedrin, of course, is a δ protein. Several

TABLE 2

Temporal Classification of Infected Cell-Specific Proteins Produced in AcMNPV-Infected SF and BM Cells

α ICSPs		β ICSPs		ɣ ICSPs		δ ICSPs	
SF Cells	BM Cells	SF Cells	BM Cells	SF Cells	BM Cells	SF Cells	BM Cells
41K	--	44K	--	60K	60K	26K	--
29K	--	39K	--	55K	--	10K	--
28K	--	33K	--	37K	37K		
25.5K	25.5K	--	--	34K	34K		
				31K	34K		
				29.5K	29.5K		
				25K	25K		
				22K	22K		
				20K	--		
				18K	--		
				14K	--		
				13K	13K		

ICSPs could not be correlated with their counterparts with a great deal of certainty, so they were not assigned to a temporal class. By comparisons with the work of Wood (114) and Carstens et al. (12) and the analysis of structural glycoproteins by Stiles and Woods (92), ICSPs such as the 75K, 65K, 44K, 39K, 33K, 31K, 28K, 18K, and 14K were identified as structural proteins of the virus particle. Therefore, 10 of 13 structural proteins were not found in BM cells.

Quantitative densitometric analysis of the ICSPs in Fig. 9 made it possible to compare the relative rates of synthesis of individual ICSPs. When the rates of synthesis of the ICSPs unique to SF cells were analyzed, it was evident that in the time period between 16 and 28h p.i., the rates increased for the 44K, 39K, and 26K ICSPs (Figs. 11-12). In addition, at 28h many more ICSPs were synthesized which were not made earlier. The ICSPs produced at the highest rates at 28h include the 55K, 44K, 31K, 26K, 18K, and 14K polypeptides. Polyhedrin, the 26K protein, increased dramatically during these few hours as expected. There was no significant change in the rate of synthesis of the 41K, 29K, and 28K problems which were produced at relatively low rates at both times.

In the BM infection, 6 ICSPs were made which were not present in SF cells (Table 1). With the exception of the 95K ICSP, each of them was detected at 16h as well as 28h (Fig. 13). Within this group of proteins, the 32K increased somewhat in its rate of synthesis and the 27K increased dramatically. Nevertheless, these increases were nowhere near those seen in SF cells during this time period. Overall,

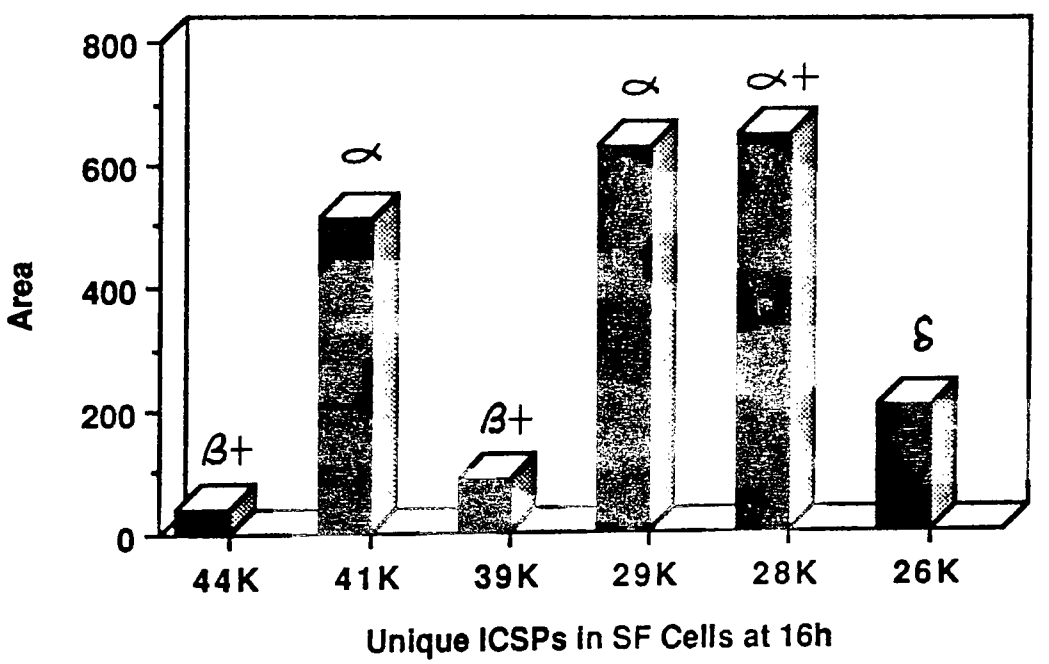


FIGURE 11. Relative rates of synthesis of ICSPs unique to AcMNPV-infected S. frugiperda cells at 16h p.i. The fluorogram in Fig. 9 was scanned with a densitometer (E-C corporation) coupled to an Apple IIe microcomputer. The relative rates of label incorporated in each polypeptide was directly proportional to the intensity of each band. In the scan, each peak represented an individual band in the fluorogram. By densitometric quantitation of the area under each peak, the relative rates of synthesis of the ICSPs was determined. Temporal classes are given for the ICSPs; (+) indicates structural proteins.

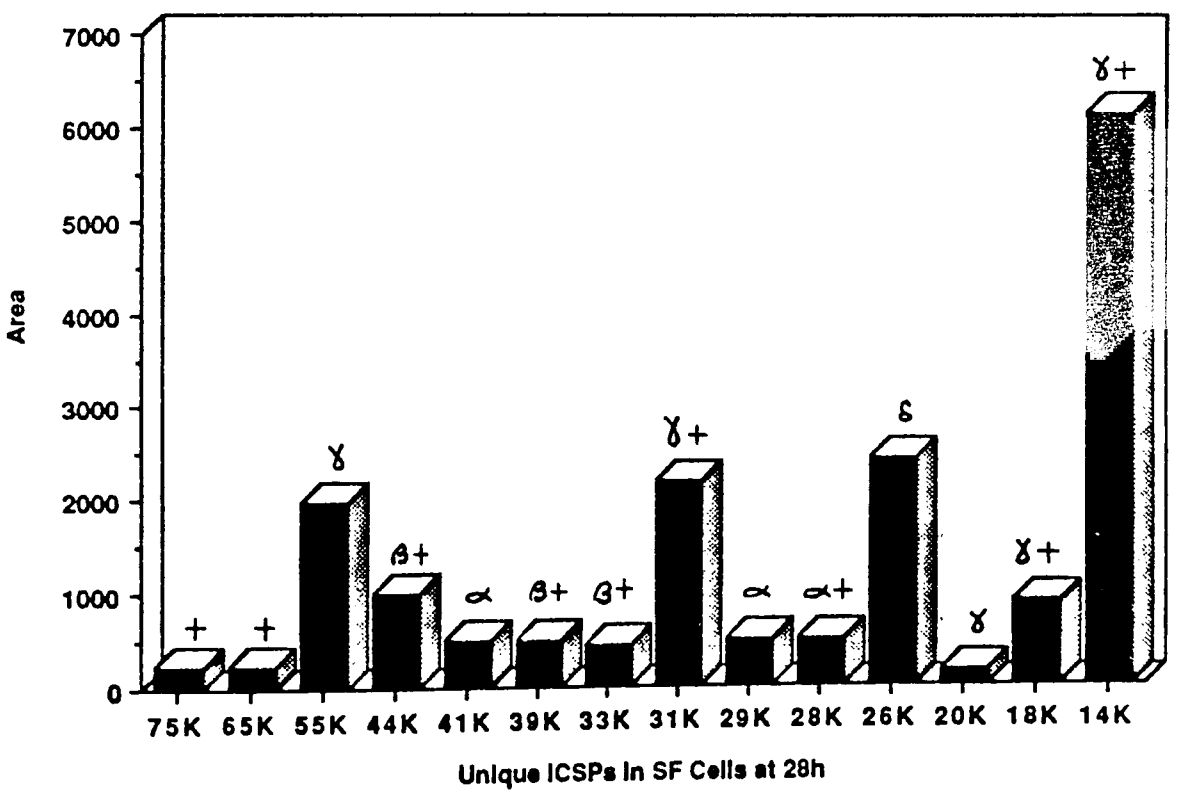


FIGURE 12. Relative rates of synthesis of ICSPs unique to AcMNPV-infected S. frugiperda cells at 28h p.i. Rates of synthesis were determined as stated in the legend for Fig. 9. Temporal classes are given for ICSPs; (+) indicates structural proteins.

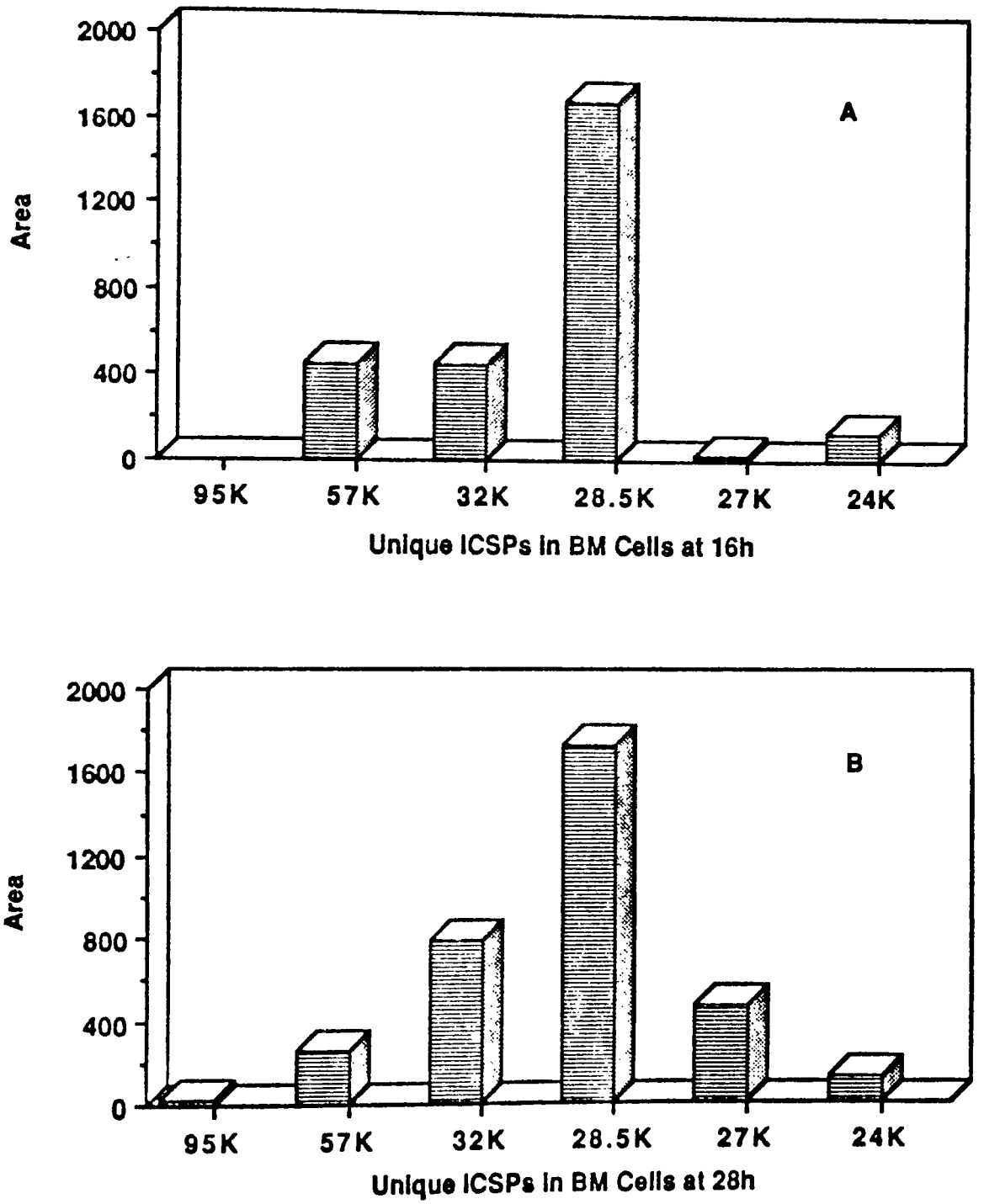


FIGURE 13. Relative rates of synthesis of ICSPs unique to AcMNPV-infected B. mori cells. (A) 16h p.i. (B) 28h p.i. Rates of synthesis were determined as described in the legend for Fig. 9.

the pattern of synthesis for this group of ICSPs was fairly stable from 16-28h p.i., compared to the SF infection.

Viral ICSPs produced in both SF and BM cells exhibited differential rates of synthesis in each cell line (Figs. 14-15). At 16h p.i., the majority of these ICSPs (127K, 37K, 34K, 25K) were found only in BM cells. The 60K and 29.5K ICSPs were detected in SF as well as BM cells, although at slightly lower rates in the permissive cells. However, the pattern of expression was much different at 28h p.i., at which time each of the common ICSPs was expressed in both cell lines. For all of the proteins except one, the rate of synthesis was much higher in SF cells (Fig. 15). The exception was the 22K protein whose rate of synthesis in BM cells was nearly three times that of the permissive infection. In summary, at 16h, these shared ICSPs were produced at higher rates in BM cells, whereas at 28h, higher rates of synthesis were found in SF cells.

Regarding temporal classification of the ICSPs produced in SF as well as BM cells, γ ICSPs include the 60K, 37K, 34K, and 22K polypeptides. The 25K and 13K would be considered as very late (δ) ICSPs based on Wood's data, but in the literature (62), there are only two δ proteins, polyhedrin and p10. Several structural proteins (60K, 37K, 34K) were produced in BM cells. The 60K ICSP corresponds to the 64K envelope protein which serves as an attachment protein important for virus infection of the host (108).

Another interesting feature of the shared ICSPs is that in BM cells, these ICSPs did not increase in their rate of synthesis from 16

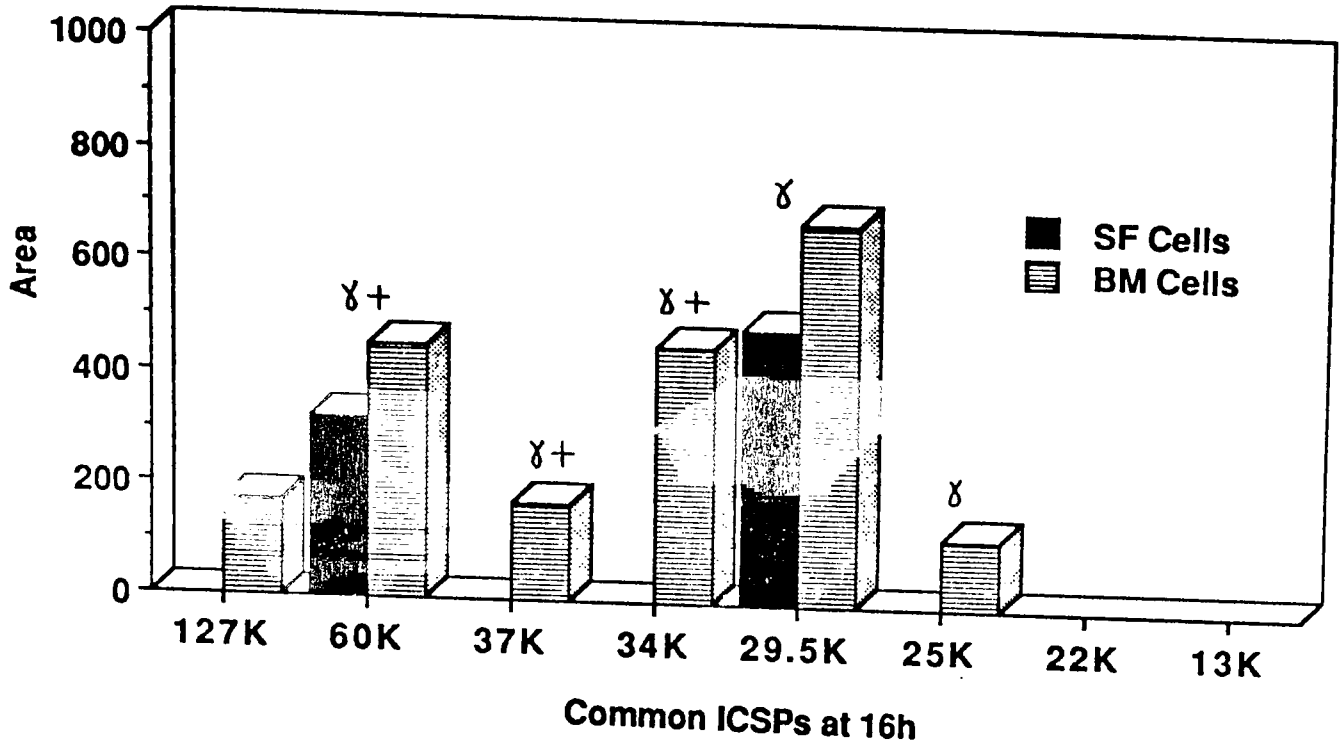


FIGURE 14. Relative rates of synthesis of ICSPs common to AcMNPV-infected S. frugiperda and B. mori cells at 16h p.i. Rates of synthesis were determined as described in Fig. 9. Temporal classes are given for the ICSPs; (+) indicates structural proteins.

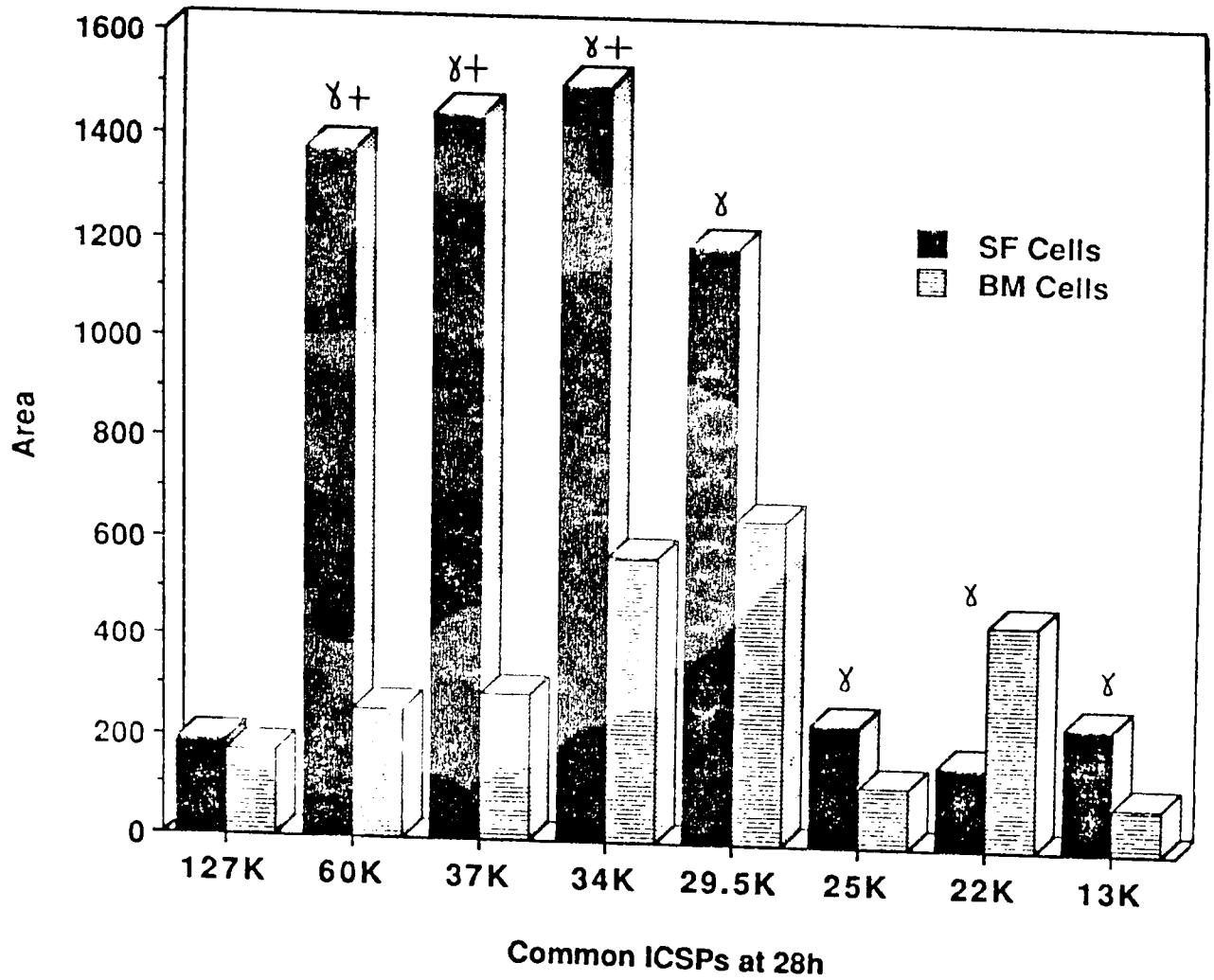


FIGURE 15. Relatives rates of synthesis of ICSPs common to AcMNPV-infected S. frugiperda and B. mori cells at 28h p.i. Rates of synthesis were described in Fig. 9. Temporal classes are given for ICSPs; (+) indicates structural proteins.

to 28h. Except for a few minor fluctuations, the patterns are remarkably similar.

Although the goal of this project was to study late ICSPs and polyhedrin gene regulation, the fact that so many late ICSPs were blocked in BM cells prompted the investigation of earlier time periods to determine if a cascade was operating in this system. As seen in Fig. 16, at 12h, the same set of ICSPs was present in SF cells as was seen in Fig. 9. This gel allowed for better resolution of the cluster of ICSPs in the area of polyhedrin, and clearly shows the absence of polyhedrin in BM cells. For the most part, the ICSPs followed the same patterns of synthesis from 12-24h as they did from 16-28h. Minor exceptions occurred such as the disappearance of the 41K α protein by 24h. Also, a few of the γ proteins (60K, 31K) appeared to be reduced in their synthesis by the latter sampling period. Because most of the ICSPs present at 28h (Fig. 9) were already present at 12h (Fig. 16) and α proteins had decreased or disappeared by 24h, it would appear that the entire infection process was speeded up in this particular experiment. In this case, a decrease in some of the γ proteins would not be unexpected. This accelerated cycle might be due to the infectivity level of the input virus. It has been shown that the MOI not only affects the number of cells infected, but also the timing of replicative events as well (110). Although the same MOI was used in all the experiments, due to the storage of virus stocks, some infectivity may have been lost and virus levels might have been lower in some cases than expected.

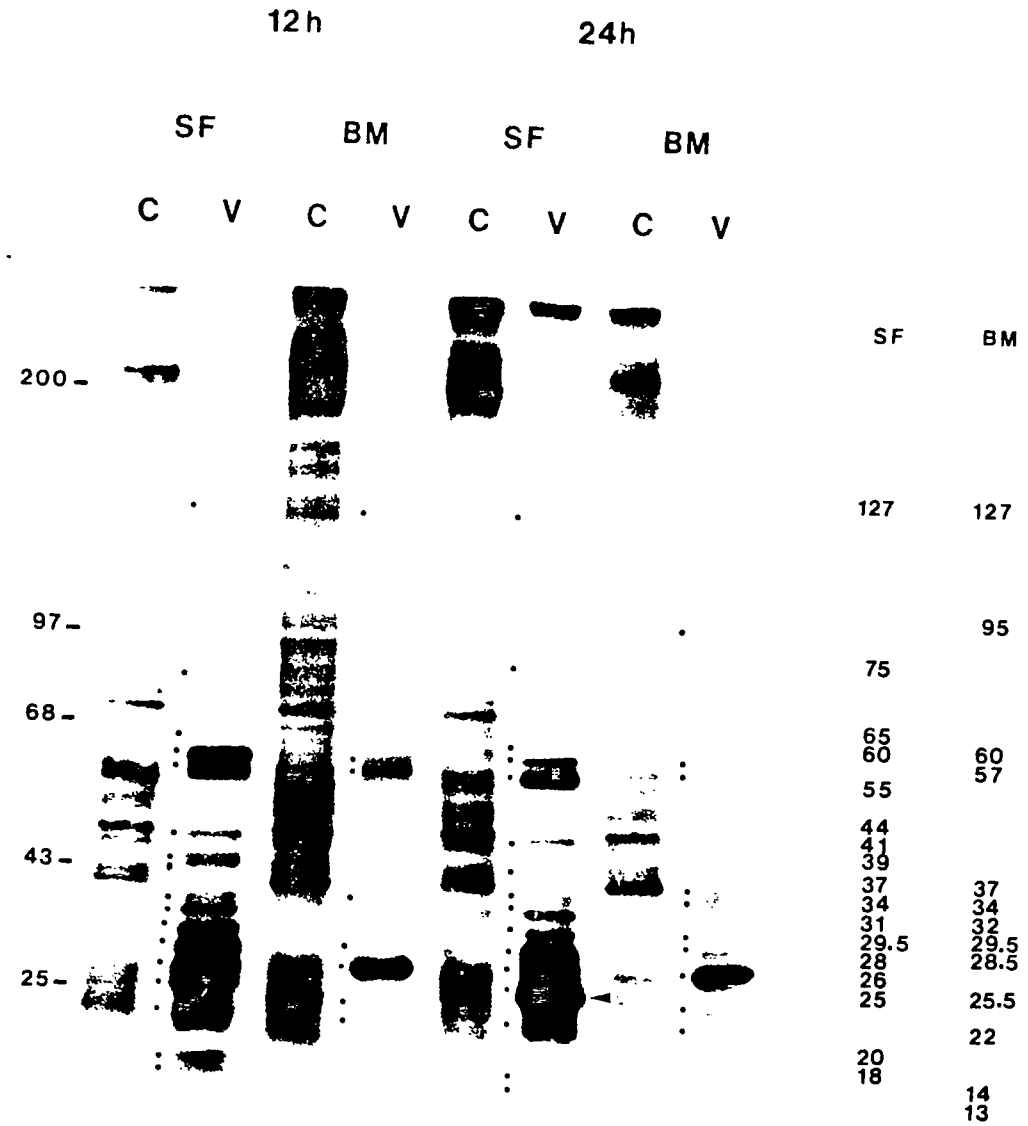


FIGURE 16. Fluorogram of AcMNPV-infected S. frugiperda and B. mori cells pulse labeled with [^{35}S]methionine at 12 and 24h p.i. Cells were grown and infected as described in Materials and Methods. The cells were pulsed for 1h with 50 uCi of [^{35}S]methionine (800 Ci/mmol; NEN). At the end of each labeling period, the samples were processed as described in the text, and sample aliquots containing $1x10^5$ cpm of incorporated radioactivity were electrophoresed in a 11% polyacryalmide gel and processed for fluorography. The numbers on the right indicate molecular weights (kilodaltons) of ICSPs. Molecular weights of protein standards are indicated on the left. Polyhedrin is represented by an arrow in the SF infection (24h p.i.) control cells (C); infected cells (V). ICSPs are indicated by dots.

In the BM infection, the results were in agreement with the previous gel (Fig. 9). Once again, there were no significant differences in the number or rate of synthesis of ICSPs found in these cells from 12 to 24h. The most abundant ICSP (28.5K) appeared to decrease slightly in its rate of synthesis.

After examination of time periods 0 through 48h, the picture of ICSP synthesis was further clarified. As seen in Fig. 17, the same ICSPs were produced as in the other data sets. This gel is useful in defining some of the ICSPs which could not be clearly identified with a particular temporal class in the other gels. For example, the 41K, and 39K ICSPs were present beginning at 4-6h, confirming them as early proteins. Other early ICSPs such as the 29K and 28K proteins appeared by 4-6h and then decreased in their rate of synthesis, with 28K disappearing at approximately 18h. The 44K and 33K ICSPs appeared at about 6h, making them β proteins. Proteins such as the 34K and 37K products showed up at 8-10h and 12h, respectively, placing them at the early end of the δ spectrum. The 31K ICSP can be distinguished as a δ protein at 16h. At 16h, the 29.5K was synthesized and increased throughout infection, characterizing it as a late protein. The envelope 60K protein and 55K ICSPs were also made at 16h onwards, with the probable occlusion 55K ICSP persisting through 48h. The majority of the ICSPs were present at 16-18h, including some (25K, 22K, 20K, 18K, 14K, 13K) which were not present at this time in other gels (Fig. 9). ICSP synthesis appeared to peak at 16-20h. Although the data might tend to suggest that the infection was somewhat slow in the Fig.

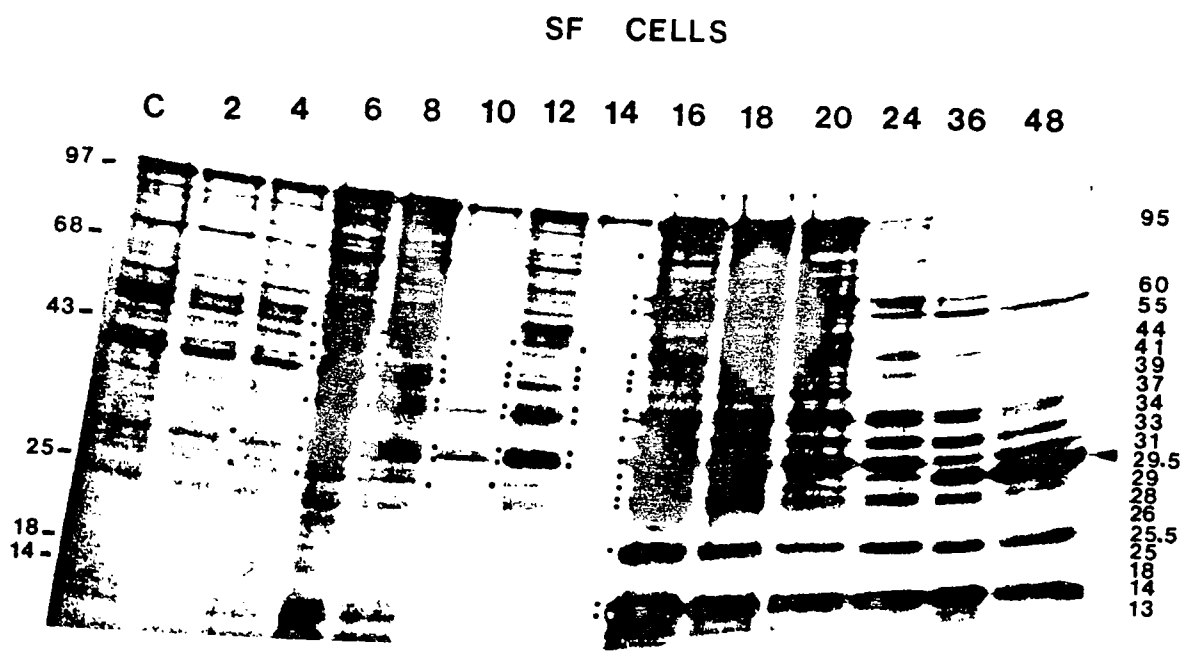


FIGURE 17. Fluorogram of ICSP synthesis in S. frugiperda cells infected with AcMNPV and pulse labeled for 1h at 2-48h p.i. with [^{35}S]methionine. Polypeptides were electrophoresed in a 6-15% gradient gel as described in Fig. 9. The numbers on the right indicate the molecular weights of ICSPs in kilodaltons. Molecular weight standards are indicated on the left. Control cells (C). Dots indicate ICSPs. The arrow indicates polyhedrin.

9 experiment, due to the sampling increments, ICSPs shown only at 28h might actually have been detected at 18h if samples had been taken.

The gel in Fig. 17 depicted an early protein (25.5K) which was first synthesized at about 4-6h p.i. This ICSP is located directly below polyhedrin. There is an early protein in Wood's data in the same position, and this is the only early ICSP which was not detected in SF cells in the gels. This gel also shows the decline of most γ proteins after 36h p.i. In addition, the progressive increase in polyhedrin is best illustrated in this gel. At 48h, polyhedrin was the most abundantly synthesized protein.

For the BM infection (Fig. 18), fewer ICSPs were resolved than on other gels. The major ICSP of infected BM cells (28.5K) was detected. Its synthesis began at 8h and began to decrease in rate by 18-20h. No early ICSPs were detected in this gel. Furthermore, the gel is devoid of late ICSPs which would be detected with increasing rates of synthesis like those seen in the SF infection (Fig. 17).

Inhibition of Host Protein Synthesis

Although inhibition of host protein synthesis did not occur at 16h in SF cells, host synthesis was greatly impaired at 28h (Fig. 9). In SF cells at 28h, the synthesis of approximately 15 host proteins with molecular weights between 40K and 170K was reduced to very low or undetectable levels (Fig. 9). Compared to uninfected SF cells, the synthesis of these host proteins as a group was almost completely inhibited in the infected cells. The synthesis of a major host protein such as the 47K protein was greatly reduced at 28h. It

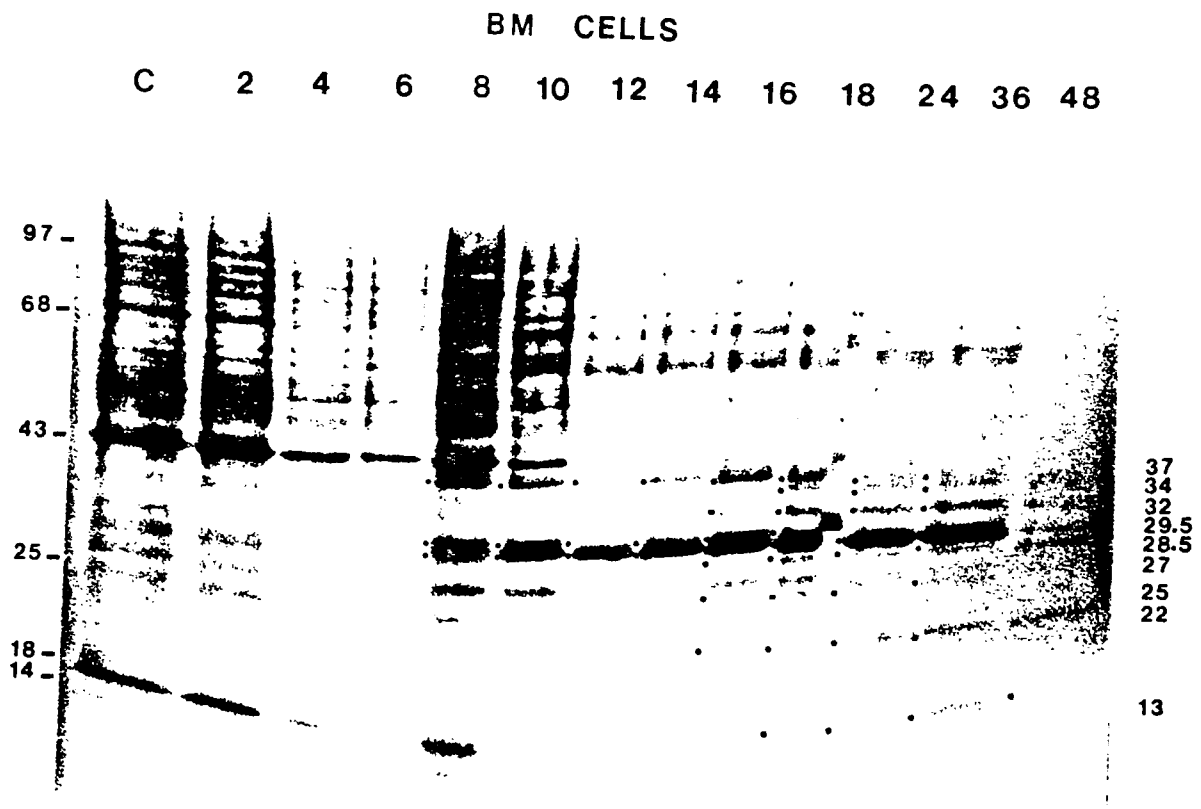


FIGURE 18. Fluorogram of ICSP synthesis in B. mori cells infected with AcMNPV and pulse labeled for 1h at 2-48h p.i. with [^{35}S]-methionine. Polypeptides were electrophoresed as described in Fig. 9. Molecular weights of ICSPs are indicated on the right; molecular weight markers are shown on the left. Control cells (C). Dots indicate ICSPs.

appears that several other host proteins below 40K were also inhibited, yet the masking effect of numerous late viral proteins comigrating in this area of the gel precluded the accurate identification of such host proteins.

In BM cells, however, there was no clear evidence for host inhibition at either 16 or 28h p.i. (Fig. 9). Most notably, the 47K major host protein was synthesized at a high rate in infected BM cells compared to its greatly reduced rate of synthesis in infected SF cells at 28h p.i. (Fig. 9).

CHAPTER IV

DISCUSSION

In this study, I have investigated cytopathic effects, viral growth kinetics, and ICSP synthesis in AcMNPV infections of permissive S. frugiperda and semipermissive B. mori cells. The two infections differed greatly in each of these areas. In cytopathology studies, the permissive cells exhibited a typical pattern and progression of CPE, consisting of darkened, swollen nuclei, numerous PIBs, and eventual lysis of the majority of infected cells. Although BM cells exhibited swollen nuclei early in infection, no similarities existed beyond this stage. The complete absence of PIBs was the most dramatic phenotypic expression of an underlying host range restriction in these cells. The appearance of sac-like bodies and the virtual absence of cell lysis also distinguished the BM infection from that of SF cells or any other baculovirus infection reported thus far.

Observation of extensive pre-polyhedral CPE, even in the absence of inclusion body formation, suggested that infectious virions might still be produced. In studies of polyhedrin deletion mutants (67), it has been shown that BV production is independent of polyhedrin synthesis and that polyhedrin is not required for the envelopment of nucleocapsids or the assembly of infectious virus particles. Therefore, I could not exclude the possibility that infectious virus might be produced just because PIBs were not. However, the data (Figs. 7 and

8) revealed that infectious virions were not produced in the BM infection. The highest titer produced in SF cells was over 2,000-fold greater than that of BM cells (Fig. 7). This difference is even more pronounced than that of other semipermissive infections, such as the 700-fold difference obtained by Carpenter and Bilimoria (11) in the SfMNPV infection of T. ni cells in which CPE was also evident but very few progeny nucleocapsids or progeny virions were produced. In semipermissive gypsy moth cells, McClintock (65) showed that AcMNPV produced classic CPE but failed to yield progeny virus.

The blind-passage experiment confirms the conclusions drawn from the growth curve data. With each passage in BM cells, the level of virus progressively decreased, except for the last passage. Furthermore, the titer at the third passage was no greater than that which would be expected as a result of the 9-fold dilution through the series of passages.

Large increases in virus titer were seen in SF cells at the first two passages (Fig. 8). At the third passage, however, a slight decrease was evident. Because high levels of virus were produced in the first two passages, even with dilution, the MOI was probably close to, or greater than 10-20, which is optimal as a starting inoculum. It has been shown that high MOI serial passage of BVs in cell culture can produce aberrant viruses in the later passages (43, 79, 26).

Based on the infection kinetics and blind-passage data, I conclude that infectious virus was not produced in BM cells. These results are contrary to those of Summers et al. (96) who reported that

infectious progeny virus was produced in an AcMNPV/BM infection. The conclusions of Summers et al., however, were based on an immunoperoxidase detection system utilizing antisera to enveloped nucleocapsids. Time course studies were not conducted. Instead, a single sample conducted at 42h p.i. yielded 10^6 PFU/ml of virus which is a relatively low titer. I do not believe that these results are conclusive for the production of infectious virus. First of all, the detection of viral antigens in infected cells is not synonymous with virus production. In studies involving a persistent infection of SfMNPV in SF cells, McIntosh and Ignoffo (103) showed that while 70% of the cells tested positive for viral antigens, only 0.8% actually produced virus. Secondly, residual virus inoculum may be responsible for low virus titers upon back-titration of culture supernatants. In a follow-up study, using a single-sampling period (64h p.i.), Volkman et al. (106) concluded that BM cells were sensitive to AcMNPV infection based on the presence of enveloped nucleocapsid antigens recognized by the immunoperoxidase assay (96), but less capable of supporting rapid viral replication as indicated by a high percentage of single-cell foci.

Due to the infection kinetics data, the block in ICSP synthesis in BM cells was not unexpected. Although the BM infection failed to produce many of the ICSPs found in SF cells, several novel ICSPs were made in BM cells. It is possible that these proteins are host proteins induced by the virus rather than virus encoded proteins. Other studies involving BmNPV infection of BM cells might help determine if these proteins are of viral or host origin. The function of these

proteins is not known, but one or more of these ICSPs might be responsible for the unprecedented sac-like bodies so characteristic of this infection.

All except one of the ICSPs unique to BM cells were present at both 16 and 28h p.i. (Fig. 9). By contrast, in SF cells, the majority of the unique ICSPs were detected only at the later time or else were synthesized at much greater rates later in infection. This different pattern of ICSP synthesis is probably due to the emergent expression of numerous occlusion-related proteins in SF cells late in infection which does not occur in the BM infection. Reports (12, 114) suggest that maximum virus yields are usually obtained by 24h p.i.; therefore, polypeptides synthesized after this time may be involved in PIB formation. If this is true, it might explain the boost in ICSP synthesis in SF cells between 16 and 28h. In BM cells, however, the pattern of ICSP synthesis remained the same.

Carstens et al. (12) speculated that several late viral ICSPs, designated as 65K, 62K, 39K, 17.5K and 17K in their gel system, which appeared after the initial synthesis of polyhedrin synthesis and coincident with polyhedra formation, may be involved in occlusion. Wood (114) also implicated the 27K, 15.7K and 15K ICSPs as occlusion proteins. In my gel system, the 55K, 37K, 14K, and 13K proteins correlate with the 65K, 39K, 15K and 13K ICSPs, respectively, of Carstens. Furthermore, the 13K protein of System III corresponds to the 15K protein which is considered to be the membrane constituent of the OV (114). Two of these putative occlusion proteins (55K and 14K) were absent in BM cells, but in SF cells (Fig. 12), they were

synthesized maximally late in infection. The two possible occlusion proteins (37K and 13K) found in both cell lines were produced at levels which were only 20-40% of those produced in SF cells. Perhaps these two ICSPs (37K and 13K) are not absolute requisites for PIB production, but rather serve only minor roles in occlusion. Low level expression of these proteins and the failure to synthesize more important occlusion proteins may consequently precipitate the absence of PIBs in BM cells. The 55K and 14K proteins were two of the most abundantly synthesized late proteins found in SF cells (Fig. 15). Therefore, it is possible that they play major roles in virus occlusion.

Even though individual late proteins, with the exception of polyhedrin, have not been definitively correlated with the occlusion process, many late proteins are probably involved, and the regulation of these late proteins is most likely coordinated by the proper expression of early proteins. Many late proteins are apparently blocked in the BM infection. Also, several early (41K, 29K, 28K) ICSPs were not present in BM cells. All but one of the known α ICSPs were identified in the SF cell infection. Perhaps it was produced at an undetectable level or was very transient in its synthesis. In BM cells, only one α ICSP was detected (25.5K). BM cells also restricted several β proteins (44K, 39K, 33K). By comparative analysis and determining the times at which the ICSPs appeared in my gels, I was able to temporally classify all except a few of the ICSPs. Wood did not define a β ICSP class (114), so it is possible that some of the unclassified ICSPs in System III are in fact β ICSPs. Therefore, it

is possible that some β proteins are in fact produced in BM cells. It appears that the block in late ICSP synthesis is the result of concomitant blocks in α and β ICSP synthesis. The fact that some ɣ ICSPs are in fact produced, despite the absence of many α and β proteins, supports the idea of multiple pathways being involved in the regulation of the temporal cascade. Multiple pathways also appear to be operating in System II, previously described in our laboratory (57). My results do not support the postulate of McClintock et al. (65) that the complete set of proteins in each temporal class is required for the induction of subsequent classes. Because blocks occur in each temporal class, the restriction in BM cells is amplified at each phase of the cascade, finally resulting in the restriction of a large number of late ICSPs.

All except one of the ICSPs which were present in both SF and BM cells at 28h were produced at greatly reduced rates in BM cells (Fig. 15). Many of these proteins are virus structural proteins (60K, 37K, 34K). More importantly, 10 structural proteins were completely blocked in BM cells (Table 1). Therefore, the absence of progeny virus most likely results from the inability to assemble virus particles due to the absence of so many structural proteins. The structural ICSP most notable is the 60K ICSP, which is equivalent to the 64K major envelope protein for virus infection of the host, which functions as the attachment site for the host receptor (108). Due to the limited synthesis of this protein in BM cells, virus infectivity probably was severely decreased. It is possible that progeny virions were assembled, but they were not able to function optimally in

secondary rounds of infection; therefore, there were no increases in virus titer at the second and third passages of the blind-passage experiment.

Several nonstructural ICSPs were also synthesized at low rates in BM cells. Peak synthesis of many of the shared ICSPs occurred late in the SF infection (Figs. 14-15). The 22K was the only ICSP synthesized at a higher rate in BM cells at 28h. Perhaps it contributes to the unusual CPE in these cells.

The surge in ICSP synthesis that occurred in SF cells between 16 and 28h did not occur in BM cells. It appears that the BM infection plateaued at 16h p.i. with no subsequent major changes in the pattern of ICSP synthesis (Figs. 13-15). For unique as well as shared ICSPs, there were no major differences between the rates of synthesis at 16 and 28h in BM cells, except for the 22K protein. It is interesting that the appearance of the unusual CPE found in BM cells seems to occur at about 24h p.i. At this time, all other ICSP synthesis has leveled off. Therefore, it is possible that the 22K protein plays a major role in the production of the unusual sac-like bodies found in these cells.

In certain gels (Figs. 16-17), some of the late ICSPs appeared somewhat earlier in infection than they did in the 16 to 28h p.i. gel (Fig. 9). This may result from different infectivity levels of the virus inoculum. It may also be a reflection of the time interval between the 16 and 28h samplings. For example, certain ICSPs may have appeared at 17h p.i., but due to the sampling times, they were not

detected until 28h. However, the overall pattern and progression of ICSP synthesis remained the same in all of the gels.

Host protein synthesis was greatly inhibited at 28h in SF cells. Such inhibition would be expected because the synthesis of major late proteins, such as polyhedrin and p10, would channel macromolecular synthesis in favor of viral products at the expense of cellular proteins. The apparent lack of host inhibition in BM cells was not unexpected due to the fact that so many late ICSPs which comprise the temporal group thought to inhibit host synthesis were not produced.

The function of the various ICSPs are not yet known. Functional mapping studies are just beginning, and several early gene products which regulate other genes have been identified. In my gel system, the 31K protein corresponds to the 39K β protein which has been shown to be activated by the immediate early IE-1 gene (38). This 31K ICSP is not found in BM cells, suggesting that the IE-1 gene may not be active in BM cells. In addition, the expression of the p10 protein, which is blocked in BM cells, has been shown to be regulated by other viral products (50), but the individual proteins involved have not yet been identified. Therefore, it is possible that the lack of p10 results from the lack of other trans-activating proteins which were also blocked in BM cells. The hr5 enhancer is also required for late gene expression in addition to the requirement of two early open reading frames (40). Thus, the block in late ICSPs in BM cells may result from the inability of BM host cell factors to recognize the hr5 enhancer. Studies have shown that a 28K early protein accelerates the expression of two other early genes and numerous late genes in

transient expression assays (18). Based on migration, this ICSP appears to correspond to the 28K or 29K early ICSP in System III. Interestingly, neither the 28K or 29K proteins are made in BM cells. The lack of one of these early proteins may lead to the block in the other two early proteins and many of the late ICSPs which were not found in infected BM cells. Furthermore, the 25.5K ICSP might play a major role in regulating the expression of subsequent classes of ICSPs because it was the only α ICSP detected in BM cells. More work will be needed before the functions of each of the ICSPs is determined, but the pieces are already coming together with advances made only within the past year.

In System III, the absence of certain proteins and/or the differential expression of shared ICSPs may result in this host-range restriction. The data suggest that blocks occur at each phase of the temporal cascade. It is possible that these blocks are regulated at the transcriptional level, possibly by the controlled expression of overlapping transcripts. As shown by Friesen and Miller (27), the sequential activation of upstream promoters leads to the coordinated deactivation of downstream promoters. Accordingly, host polymerases in SF and BM cells may differ in their ability to recognize viral promoters, thereby giving rise to different sets of transcripts in each cell line. The expression of these transcripts would then in turn affect the transcription of neighboring genes. Another mechanism might involve one or more of the AcMNPV enhancers. Host cell factors in concert with viral proteins can regulate viral transcription by binding to enhancers which are host specific. In System III, assuming

that the expression of late proteins in SF cells is contingent upon the formation of transcription complexes involving β viral proteins, specific host cells factors, and enhancers, the BM restriction may be due to the lack of the specific β ICSPs and/or host cell factors. Similar blocks may occur with other classes of ICSPs. Finally, the block in BM cells may be at the translational level with host factors dictating the translational efficacy of particular transcripts.

It has been shown that enhancers are often composed of several domains, for example, three in the case of the SV 40 enhancer (75, 85, 116). For optimal enhancer activity, multiple copies of one or more of the domains is required. It is possible that baculovirus enhancers may also contain similar types of domains although they have not yet been characterized in detail for all of their functional activities. In SV 40, it has been shown that duplication of one or more of the individual domains results in an expansion of the host range of the virus (85). Therefore, when more information is available on AcMNPV enhancers, it also may be possible to manipulate these enhancers in such a way as to broaden or alter their host range.

In this model system, I have shown that AcMNPV produced different CPE in SF versus BM cells. Even though there was marked cytopathology in BM cells, infectious virus was not produced. The lack of progeny virus production probably resulted from the apparent absence of numerous virus structural proteins. The two major late ICSPs, polyhedrin and p10, were completely blocked in BM cells, demonstrating a strong host-mediated restriction. Numerous other late ICSPs were also blocked. It is not altogether clear whether the block in late ICSPs

results from a lack of α and/or β protein synthesis or, for example, some incompatibility between the virus and host involving host cell factors and enhancers. Additional work utilizing metabolic inhibitors will be needed before definite conclusions can be made concerning the possible role of such α and β proteins in regulating the block in late ICSP synthesis in System III. In order to determine the level at which restriction occurs in this model system, Northern blot analysis will also be needed. The only transcripts which have been translated in vitro are polyhedrin and p10. Therefore, with the information currently available, it is not possible to determine if the transcripts for each of the restricted proteins are made in the BM infection. One of the problems is that there are many more transcripts than translation products which makes it difficult to correlate transcripts and their individual products. Additional transcriptional and translational maps must be generated before this problem can be solved. Even then, considerable work will be needed to determine the host cell factors and viral sequences which might be involved in baculovirus host-range restriction. System III should provide a useful model for the elucidation of these host-range phenomena.

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APPENDIX: DEFINITION OF TERMS

AcMNPV	*Autographa californica* multicapsid nuclear polyhedrosis virus
BM	*Bombyx mori*
BV	Budded virus
ICSP	Infected cell-specific polypeptide
IU	Infectious unit
MNPV	Multicapsid nuclear polyhedrosis virus
MOI	Multiplicity of infection
OC	Occluded virus
PFU	Plaque forming units
PIB	Polyhedral inclusion body
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	*Spodoptera frugiperda*
SMNPV	Single nucleocapsid nuclear polyhedrosis virus
SV 40	Simian virus 40