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EXPLORING A NOVEL VIRUS-HOST INTERACTION IN A BACULOVIRUS-INFECTED INSECT CELL LINE

By

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ABSTRACT

EXPLORING A NOVEL VIRUS-HOST INTERACTION IN A BACULOVIRUS-INFECTED INSECT CELL LINE

By

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Global protein synthesis is shut down at late times post infection (p.i.) in Ld652Y cells derived from gypsy moth infected with baculovirus *Autographa californica* nucleopolyhedrovirus (AcMNPV) in the presence of apparently normal mRNAs. A single gene, host range factor 1 (*hrf-1*) was identified from another baculovirus, *Lymantria dispar* nucleopolyhedrovirus (LdMNPV), that promoted AcMNPV replication in Ld652Y cells. Recombinant AcMNPVs bearing *hrf-1* were constructed that replicated in Ld652Y cells. *hrf-1* was transcribed as an early gene and encoded a novel protein of 25.7 kDa that did not have any motif that might imply its function.

Experiments were carried out to investigate the possible connections between apoptosis and protein synthesis shut down. The apoptosis suppressor AcMNPV P35 was translated prior to protein synthesis shut down and functioned to prevent apoptosis in AcMNPV-infected Ld652Y cells. HRF-1 could prevent protein synthesis shut down even when cells were undergoing apoptosis but HRF-1 could not functionally substitute for P35. The DNA synthesis inhibitor aphidicolin could block both apoptosis and protein synthesis shut down in Ld652Y cells induced by *p35*$^{-}$ AcMNPV but not protein synthesis shut down in wt AcMNPV-infected Ld652Y cells. These data suggested that protein synthesis shut down and apoptosis were separate responses of Ld652Y cells to AcMNPV

infection and that P35 was involved in inducing a second pathway that led to protein synthesis shut down.

A cell-free translation system (cytoplasmic lysate) was established from insect cells and a series of in vitro translation assays were carried out to identify the defect in protein synthesis in AcMNPV-infected Ld652Y cells. Lysate derived from AcMNPV-infected Ld652Y cells at late times p.i. did not display in vitro translation ability but its translation ability could be restored by addition of uncharged eukaryotic tRNA. Our data suggested that the defect of protein synthesis in AcMNPV-infected Ld652Y cells lied in the adaptor ability of a single or a small group of tRNA species and that a common mechanism of protein synthesis shut down was shared in AcMNPV-infected Ld652Y and BmN cells.

To my wife Jingheng Cheng

To my sons Pengcheng (Daniel) and Wanli (Kevin)

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INTRODUCTION

Baculoviruses are a large group of DNA-containing viruses that primarily infect insects. In addition to being used for effective protein expression vectors, baculoviruses have been used as biological insecticides in agriculture and forestry. The molecular biology of host range determination of baculoviruses has been a subject of interest since host range is an important issue in developing and improving baculoviruses as pest control agents. Most baculoviruses have narrow host ranges. This property is considered to be an advantage in terms of safety. However, it is a shortcoming in terms of efficacy and economy since many baculoviruses have to be used in order to control a variety of different pest insects. Thus the efficacy of biological insecticides can be greatly improved if the host range of the baculoviruses can be expanded. On the other hand, host range has to be in good control in order to protect beneficial insects, animals, and even human beings. Understanding the molecular biology of virus-host interaction will give rise to better understanding of the mechanisms for host range determination and thus will improve both efficacy and safety of biological insecticides.

One particular baculovirus, *Autographa californica* nucleopolyhedrovirus (AcMNPV), provides an excellent model for the study of the mechanism of baculovirus host range determination. AcMNPV has the potential to be a wide range insecticide since it has a wider host range than most other baculoviruses with the ability to infect insect

larvae of at least 33 species in 10 families and at least 25 insect cell lines. In addition, AcMNPV can infect many cell lines in a semipermissive fashion. In these semipermissive cell lines, AcMNPV can enter the cells but virus infection is restricted at different stages. One of these semipermissive cell lines, Ld652Y derived from gypsy moth, displays a novel virus-host interaction when infected with AcMNPV . In AcMNPV-infected Ld652Y cells, viral and host mRNAs are transcribed, transported, and are of normal size. However, both viral and host cellular protein synthesis is shut down at late times post infection and no viral progeny are produced. Superinfection of AcMNPV-infected Ld652Y cells with another baculovirus, *Lymantria dispar* nucleopolyhedrovirus (LdMNPV), which normally replicates in Ld652Y cells results in production of AcMNPV viral progeny, suggesting a helper function in LdMNPV that promotes AcMNPV replication in the nonpermissive cell line Ld652Y. Identification of this helper function gene(s) and the defect of protein synthesis will provide clues to the understanding of the mechanisms of protein synthesis shut down and host range determination.

AcMNPV contains a potent apoptotic suppressor gene *p35* and apoptosis is implicated in AcMNPV host range determination. *p35* is not required for AcMNPV replication in *Trichoplusia ni* cell line TN368. However, *p35*$^-$ AcMNPV induces extensive apoptosis in *Spodoptera frugiperda* SF-21 cells and protein synthesis shut down is reported in the apoptotic SF-21 cells. It is of interest to test the possible connections between apoptosis and protein synthesis shut down in AcMNPV-infected Ld652Y cells. Whether they are two separate responses or two phenotypes of a same response will provide insight into the nature of this novel virus-host interaction.

In exploring this novel virus-host interaction, we identified and characterized a single gene in LdMNPV, host range factor 1 (*hrf-1*) that promoted AcMNPV replication in Ld652Y cells. Then I investigated the roles of AcMNPV P35 and LdMNPV HRF-1 in controlling apoptosis and protein synthesis shut down in AcMNPV-infected Ld652Y cells and obtained evidence supporting the hypothesis that apoptosis and protein synthesis shut down are separate responses of Ld652Y cells to AcMNPV infection. Finally, I established cell-free translation systems from insect cells and investigated the defect in protein synthesis in AcMNPV-infected Ld652Y cells by a series of in vitro translation assays. Our data suggested a defect in tRNA in protein synthesis in AcMNPV-infected Ld652Y cells and a same mechanism of protein synthesis shut down shared in another nonpermissive AcMNPV infection.

In this dissertation, chapter 1 is a literature review. Chapter 2 is a manuscript published in Journal of Virology (1996, 70:2221-2229) titled "Identification of a baculovirus gene that promotes *Autographa californica* nucleopolyhedrovirus replication in a nonpermissive insect cell line". Chapter 3 is a manuscript published in Virology (1997, 227:420-430) titled "Characterization of host range factor 1 (*hrf-1*) expression in *Lymantria dispar* nucleopolyhedrovirus- and recombinant *Autographa californica* nucleopolyhedrovirus-infected IPLB-Ld652Y cells". Chapter 4 is a manuscript submitted to Journal of Virology for publication titled "Responses of insect cells to baculovirus infection: protein synthesis shut down and apoptosis". Chapter 5 is a manuscript in preparation for publication titled "Investigation of the defect in protein synthesis in *Autographa californica* nucleopolyhedrovirus-infected Ld652Y cells".

CHAPTER 1

Literature Review

The Biology of Baculoviruses

The Characteristics of Baculoviruses

Baculoviruses are one diverse group of viruses found only in arthropods, mainly in insects. The family *Baculoviridae* is characterized by a rod-shaped, enveloped virion (Harrap, 1972b) containing a circular double-stranded DNA genome (Summers and Anderson, 1972) ranging from 80- 200 kbp (Burgess, 1977). This family contains two genera: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Murphy et al., 1995).

Baculoviruses have a unique biphasic life cycle in which they produce two structurally and functionally distinct forms of viral progeny (Volkman et al., 1976). One is budded virus (BV) which is produced at early stage of infection when progeny nucleocapsids migrate from the nucleus to the cytoplasm and bud through the plasma membrane; the other is occluded virus (OV) which is produced at late stages when progeny nucleocapsids are enveloped by a membrane generated in the nucleus (Braunagel et al., 1996; Braunagel and Summers, 1994; Stoltz et al., 1973) and are embedded in a crystalline protein matrix within the nucleus to form polyhedral inclusion bodies (PIBs).

Baculoviruses are named after the insects from which they are originally isolated. For example, AcMNPV is isolated from alfalfa looper *Autographa californica*. BmNPV

is isolated from silkworm *Bombyx mori*. LdMNPV is isolated from the gypsy moth *Lymantria dispar*. Baculoviruses have been developed as biological insecticides for agriculture and forestry (Bishop, 1989; Podgwaite, 1985; Wood and Granados, 1991). In addition, baculoviruses especially AcMNPV have been developed into vectors for high level expression of heterologous proteins in insect cells (Jarvis et al., 1996; Luckow and Summers, 1988; Miller, 1988; O'Reilly et al., 1992).

In Vivo and In Vitro Infection

AcMNPV is the prototype virus for studying molecular biology of baculoviruses. The biology of baculoviruses is usually represented by AcMNPV. The two forms of progeny have different roles in infection (Volkman and Summers, 1977). In a typical AcMNPV infection, insect larvae ingest PIBs as contaminants of their food. The crystalline polyhedrin matrix is solubilized in the alkaline juice in the midgut of the insects and the embedded virions (OV) are released (Harrap and Longworth, 1974). The released virions enter midgut cells by fusion with the membrane of the microvilli (Granados and Williams, 1986; Wang et al., 1994). This primary infection in the midgut cells results in production of BVs. The newly produced BVs are released from the cells (Keddie et al., 1989) and enter the hemocoel where they are transported via the hemolymph to other tissues in the insect. They can also infect the epithelial cells of tracheoles, spreading the infection along the tracheal network (Engelhard et al., 1994; Flipsen et al., 1995; Kirkpatrick, 1994). Thus OV is responsible for establishing the primary infection and BV spreads the infection to many other tissues within the insect.

Many cell lines have been established that support baculovirus replication (Hink, 1970; Hink, 1979; Hink and Hall, 1989). Infection in cell culture is mediated by budded

viruses and can be considered to occur in three basic phases: early, late and very late (Blissard and Rohrmann, 1990; Friesen and Miller, 1986). BV enters the cells by adsorptive endocytosis (Volkman, 1986; Volkman and Goldsmith, 1985). Nucleocapsids are released in the cytoplasm and migrate to the nucleus presumably through nuclear pores (Granados and Williams, 1986). Infected cells undergo significant changes during the early phase of infection from viral entry to 6 hours post infection (h p.i.). Cytoskeleton rearranges, host chromatin enlarges and is dispersed within the nucleus (Charlton and Volkman, 1991; Lanier et al., 1996; Volkman and Zaal, 1990). Following the early phase is the late phase which extends from 6 h p.i. to approximately 20 to 24 h p.i. Extensive viral DNA replication, late gene expression, and BV production take place in the late phase. A distinct electron-dense structure known as the virogenic stroma forms in the nucleus (Harrap, 1972c; Kelly, 1981) where the progeny nucleocapsids are formed (Bassemir et al., 1983; Fraser, 1986). The newly assembled nucleocapsids leave the nucleus, travel through the cytoplasm (Bassemir et al., 1983; Raghow and Grace, 1974) and bud out through the plasma membrane modified by the viral major glycoprotein GP64 (Blissard and Rohrmann, 1989; Blissard and Wenx, 1992; Oomens et al., 1995; Volkman et al., 1984). During the budding process, the nucleocapsid becomes enveloped by the modified membrane. Logarithmic production of BV occurs from approximately 12 to 20 h p.i. (Knudson and Harrap, 1976; Lee and Miller, 1979). In the mean time, the host gene expression is shut off. A decline in the steady-state levels of host mRNAs begins approximately 12 h p.i. (Ooi and Miller, 1988). Host protein synthesis declines from 18 h p.i. and is completely shut off by 24 h p.i. (Carstens et al., 1979; Dobos and Cochran, 1980; Kelly and Lescott, 1981; Miller et al., 1983). The very late phase is occlusion-

specific phase and begins around 20 h p.i. Membrane envelope segments are generated within the nucleus during this occlusion process as revealed by electron microscopy (Chung et al., 1980; Harrap, 1972c). The origin or mechanism of synthesis of these membranes is not known (Braunagel et al., 1996; Braunagel and Summers, 1994; Hong et al., 1994; Stoltz et al., 1973). Nucleocapsids interact with these membranes and eventually become enveloped (Bassemir et al., 1983; Fraser, 1986). The enveloped nucleocapsids are further occluded to form PIBs. As the PIBs accumulate, the nucleus becomes virtually filled with occlusion bodies that can be seen under the optical microscope.

Gene Organization and Function

AcMNPV consists of a genome of 134 kb that can potentially encode up to 154 genes (Ayres, et al., 1994). One notable feature of AcMNPV genome is the presence of 6 homologous regions (*hr*) (Cochran and Faulk, 1983; Guarino et al., 1986). These *hr*s contain two to eight reiterations of an imperfect palindromic sequence containing an *Eco*RI site at the center of each palindrome. The *hr*s act as enhancers for some early promoters in transient expression assays (Guarino and Summers, 1986b; 1987; Nissen and Friesen, 1989; Rasmussen et al., 1996) and origins for DNA replication (Kool et al., 1993; Leisy et al., 1993; Pearson et al., 1992).

AcMNPV ORFs are usually very closely spaced along the genome. The sequences between many ORFs are extremely A+T rich, which may be related to promoter and transcriptional termination functions (Ayres et al., 1994). There seems to be little or no clustering of functionally related genes, nor of genes belonging to the same transcriptional class (Ayres et al., 1994; O'Reilly et al., 1992).

The sequence of entire AcMNPV genome is available and the functions of many genes have been identified (Ayres et al., 1994; Kool and Vlak, 1993). Among those important structural genes are *polh*, *vp39*, and *gp64*. *polh* is not essential for viral replication in cell culture but is essential for OV formation (Smith et al., 1983a). Polyhedrin is highly expressed late in infection and it is the most common locus for insertion of foreign genes in the baculovirus expression system. *vp39* encodes the major capsid protein which forms the basic shell of the rod-shaped nucleocapsid (Thiem and Miller, 1989a). The major glycoprotein of BV is encoded by *gp64* (also called *gp67*) (Whitford et al., 1989). GP64 is exclusively found associated with the BV envelope and is specifically involved in BV interaction with receptors or the cell surface. Antibodies against GP64 block infectivity but not adsorption (Volkman et al., 1984; Volkman and Glodsmith, 1985). Functional studies of GP64 show that it mediates membrane fusion in a pH-dependent manner, which is essential during viral entry by endocytosis (Blissard and Wenx, 1992; Monsma et al., 1996; Monsma and Blissard, 1995)

Important regulatory genes include *ie-0*, *ie-1*, *ie-2* (also known as *ie-n*). *ie-0* is actually another form of *ie-1* which contains an extra upstream exon (Chisholm and Henner, 1988). *ie-1* encodes a multifunctional regulatory protein transactivating both some early and late gene expression in transient expression assays (Carson et al., 1988; Carstens, 1993; Guarino and Summers, 1986a; 1987; Kovacs et al., 1991a; Lu et al., 1996; Lu and Carstens, 1993; Morris et al, 1994; Nissen and Friesen 1989). IE-2 is a promiscuous transactivator and also regulates its own promoter in transient expression assays (Yoo and Guarino, 1994).

A set of 18 genes have been identified as late gene expression factors (Li et al.,

1993; Lu and Miller, 1995; Morris et al., 1994; Passarelli and Miller, 1993a; 1993b; 1993c; 1994; Passarelli et al., 1994; Todd et al., 1995). Among them, *ie-1*, *ie-2*, *lef-1-3*, *lef-7*, *p143*, *p35*, and *dnapol* are related to DNA replication. The remaining *lef*s, *lef-4-11*, *p47* and *39K*, function either at the level of transcription or at that of mRNA stabilization. *lef-3* has been shown to bind single-stranded DNA (Hang et al., 1995). The predicted sequence of *lef-7* suggests that it is a homologue of herpes virus single-stranded DNA binding protein (UL29) (Lu and Miller, 1995). *dnapol* and *p143* encode polypeptides with sequence motifs shared by DNA polymerases (Tomalski et al., 1988) and DNA helicases (Lu and Carstens, 1991), respectively. *lef-8* (Passarelli et al., 1994) and *lef-9* (Lu and Miller, 1994) encode proteins with motifs found within the two largest subunits of prokaryotic and eukaryotic RNA polymerases. *39K* gene product is a phosphoprotein (pp31) associated with the virogenic stroma (Guarino et al., 1992). P35 suppresses apoptosis in *Spodoptera frugiperda* cells and larvae (Clem et al., 1991; Clem and Miller 1993; Hershberger et al., 1992) as well as in cells and larvae of other organisms (Rabizadeh et al., 1993; Sugimoto et al., 1994). In addition to these 18 *lef*s, a gene, termed very late factor 1 (*vlf-1*), is required for very late but not late gene expression. *vlf-1* encodes a polypeptide with sequence motif characteristic of a family of integrase/resolvases (McLachlin and Miller, 1994).

Some genes appear to be nonessential as judged by the ability of knock-out mutant viruses to replicate in both cell culture and in insects. These nonessential genes may provide some advantage for growth or survival of the virus under specific conditions. For example, the product of the nonessential gene ecdysteroid UDP-glucosyltransferase (*egt*) is secreted from the cell and transfers the sugar moiety from

UDP-sugar to ecdysone, the hormone governing insect molting. This will block the molting of the infected insect host and thus promote viral progeny production (O'Reilly and Miller, 1989).

Gene Expression in Cell Culture

AcMNPV gene expression appears to be primarily controlled at the level of transcription and transcription is coordinately regulated (Erlanderson et al., 1985; Gordon and Carstens, 1984; Rice and Miller, 1986). Transcription of early genes is achieved by cellular RNA polymerase II before the onset of viral DNA replication (Glocker et al., 1993; Grula et al., 1981; Hoopes and Rohrmann, 1991; Huh and Weaver, 1990). Early gene transcription is activated by two other early gene products IE-1 and IE-2 (Guarino and Summers, 1987; Kovacs et al., 1991; Lu and Carstens, 1993; Morris et al., 1994; Nissen and Friesen, 1989; Passarelli and Miller, 1993; Ribeiro, et al., 1994). The transition between the early and late phases of gene expression is marked by a switch from host RNA polymerase II to a novel alpha-amanitin-resistant RNA polymerase activity (Grula et al., 1981; Huh and Weaver, 1990). This novel polymerase is believed to be encoded or modified by the virus (Beniya et al., 1996; Fuchs et al., 1983; Yang et al., 1991). Activation of late and very late genes requires early gene products and depends on the onset of viral DNA replication (Erlandson *et al.*, 1985; Rice and Miller, 1986). These transcripts initiate from a characteristic TAAG motif (Howard *et al.*, 1986; Rohrmann, 1986; Thiem and Miller, 1989b; Wilson *et al.*, 1987).

Most viral genes are transcribed primarily during one phase, however, some genes are transcribed in two or possibly three phases. These genes such as *39K*, *p35*, and *gp64* contain both early and late promoters (Guarino and Smith, 1990; Guarino and Summers,

1986a; Nissen and Friesen, 1989; Whitford et al., 1989). Baculovirus mRNAs are capped (Jun-Chuan and Weaver, 1982) and most appear to be polyadenylated (Friesen and Miller, 1986; Lubbert and Doerfler, 1984; Rohel and Faulkner, 1984). Multiple overlapping transcripts with coterminal 5'- or 3'-ends are a characteristic feature in baculovirus gene transcription (Friesen and Miller, 1985; 1986; Lubbert and Doerfler, 1984 a, b; Oellig *et al.*, 1987; Rankin et al., 1986). Bicistronic and multicistronic transcripts are also observed (Oellig et al., 1987; Passarelli and Miller, 1994; Thiem and Miller, 1989a).

Another striking characteristic of baculovirus gene expression is the lack of RNA splicing. The only RNA splicing event is the transcription of *ie-0* and *ie-1* in which the upstream *ie-0* exon is fused to the *ie-1* open reading frame. This produces protein IE-0 which has a sequence identical to IE-1 except for additional 57 amino acids at its N terminus (Chisholm and Henner, 1988).

Virus-Host Interaction

Host Range Determination

Host range determination has been a subject of interest in the study of molecular biology of baculoviruses since it plays a critical role in the development and improvement of baculoviruses as effective, economic, and safe biological pesticides. Baculoviruses generally have narrow host specificity. Individual isolates normally infect only closely related species. AcMNPV has a relatively wider host range than most other baculoviruses. AcMNPV reportedly can infect at least 33 species of insect larvae in 10 families (Groner, 1986) as well as more than 25 insect cell lines (Hink, 1979; Hink and Hall, 1989). In addition, it can infect at least 26 insect cell lines in a semipermissive

fashion (Possee et al., 1993). Host range determination involves virus-host interaction. Major steps in the AcMNPV replication cycle which may be relevant to host specificity include entry of the virus into the cell, virus early gene expression, DNA replication, virus late gene expression, budded virus formation and release, very late gene expression and PIB formation. AcMNPV can enter most insect cells and express genes under the control of insect or early viral promoters but expression from late and very late promoters is increasingly inefficient (Carbonell et al., 1985; Morris and Miller, 1992; 1993). Thus, the ability of AcMNPV to infect nonpermissive insect cells is not restricted at the step of viral entry to the cells but is probably limited by factors which influence viral DNA replication and/or late gene expression (Morris and Miller, 1993).

A total of 5 genes have been identified that influence the ability of AcMNPV to replicate in specific host cells. Among the 18 *lef*s that collectively transactivate expression from a plasmid containing a reporter gene under late viral promoter control in SF-21 cells (Lu and Miller, 1995; Todd et al., 1995), three of them, *ie-2*, *lef-7*, and *p35* are not required in TN368 cells (Chen and Thiem, 1997; Lu and Miller, 1995). In contrast, another AcMNPV gene, host cell-specific factor 1 (*hcf-1*) is involved in transactivation of late reporter plasmid in TN368 cells but not in SF-21 cells (Lu and Miller, 1995).

lef-7 affects AcMNPV replication in a cell-specific fashion (Chen and Thiem, 1997; Lu and Miller, 1995). *lef-7* is solely responsible for the phenotypes of two mutant AcMNPVs which display much decreased progeny virus production in infected SF-21 cells and SE1c cells derived from *Spodoptera exigua* (Gelernter and Federici, 1986) but production of the mutant viral progeny on TN368 cells is not affected (Chen and Thiem,

1997). It has been shown that *lef-7* stimulates DNA synthesis and the mutant phenotypes result from an inability of the virus to efficiently replicate its DNA in SF-21 and SE1c cells (Chen and Thiem, 1997). *lef-7* encodes a polypeptide with homology to single stranded DNA binding protein (Lu and Miller, 1995) but this function has not been confirmed. The transcriptional activator *ie-2* also stimulates DNA replication (Lu and Miller, 1995) and it is very likely that *ie-2* exerts on AcMNPV the differential host specificity between TN368 and SF-21 cells in a similar way to that of *lef-7*.

The *p35* gene is required to inhibit apoptosis during AcMNPV infection *in S. frugiperda* cells (Clem et al., 1991; Clem et al., 1994; Clem and Miller, 1993; Hershberger et al., 1992). In contrast, *p35* is not required for successful AcMNPV infection of *Trichoplusi ni* cells either in culture or in larvae (Clem and Miller, 1993; Hershberger et al., 1992). Thus the ability of AcMNPV to block an apoptotic response of its host can effectively expand its host range and the presence of functional *p35* can be considered a host range-determining event during AcMNPV replication.

hcf-1 affects AcMNPV replication in both cell line-specific and species-specific fashion (Lu and Miller, 1996). *hcf-1*$^-$ AcMNPV replicates normally in SF-21 cells and exhibits normal infectivity and virulence in *S. frugiperda* larvae (Lu and Miller, 1996). In contrast, replication of *hcf-1*$^-$ AcMNPV in TN368 cells was severely defective and the virulence in *T. ni.* larvae is much decreased (Lu and Miller, 1996). An arrest of protein synthesis is observed by 18 h p.i. in *hcf-1*$^-$ AcMNPV-infected TN368 cells together with a defect in DNA replication and late gene transcription (Lu and Miller, 1996). *hcf-1* is predicted to encode a very cysteine-rich polypeptide with several motifs suggestive of metal ion binding but no extensive homologies to other genes are found (Lu and Miller,

1995).

The putative baculovirus DNA helicase gene, *p143* (Lu and Carstens, 1991), has also been implicated in baculovirus host range determination (Croizier et al., 1993; Maeda et al., 1993). AcMNPV is not able to replicate in BmN cells derived from *Bombyx mori*. In AcMNPV-infected BmN cells, accompanying an atypical cytopathic effect, protein synthesis is dramatically attenuated by 5 h p.i. in the presence of apparently normal mRNAs (Kamita and Maeda, 1993). This inhibition is shown to be induced by the putative helicase gene *p143* of AcMNPV and precluded by the homologous helicase gene in BmNPV (Croizier et al., 1993; Kamita and Maeda, 1993; Maeda et al., 1993). Recombinants containing a 572 bp segment of *p143* from BmNPV in place of the homologous region of the AcMNPV *p143* are able to replicate in BmN cells (Croizier et al., 1993; Maeda et al., 1993). How this segment assists AcMNPV replication in the BmN cells is not known.

Another host range determinant is implied in LdMNPV that is able to expand AcMNPV host range to nonpermissive cell lines Ld652Y and LdFB both derived from *L. dispar* (McClintock and Dougherty, 1987). AcMNPV replication in Ld652Y and LdFB cells is blocked at late phase of infection (McClintock et al., 1986; Guzo et al., 1992). In AcMNPV-infected Ld652Y and LdFB cells, virus can enter the cells, viral DNA is replicated, both viral and host cellular mRNA are transcribed, translated, and of normal size (Guzo et al., 1992; Morris and Miller, 1992; 1993). However, both viral and host translation is shut down at late times p.i. and no viral progeny is produced (Guzo et al., 1992; McClintock et al., 1986). Superinfection of AcMNPV-infected Ld652Y cells with LdMNPV results in production of AcMNPV progeny (Du and Thiem, unpublished data;

McClintock and Dougherty, 1987) suggesting a helper function in LdMNPV to AcMNPV replication in the nonpermissive Ld652Y cells.

The ability of virus to replicate in a host cell depends on the interaction of virus and the host. Virus must compete with their host cell for macromolecular machinery at many levels subsequent to infection. Many viruses establish conditions within the infected cell that enable them to dominate various components of the cellular machinery (Hershey, 1991). The infected cell, for its part, can respond to virus infection by implementing defensive strategies designed to inhibit viral replication, and on the other hand, viruses have, in turn, evolved functions that frustrated the cellular defenses (Hershey, 1991; Schneider and Shenk, 1987).

The nonpermissive AcMNPV infections have demonstrated two major types of virus-host interactions. The ability of cells to undergo apoptotic cell death in response to viral infection constitutes a significant host defense mechanism. Protein synthesis shut down may represent a second host defense mechanism in insect cells against baculovirus infection. Much progress on the study of baculovirus-induced apoptosis has been made such as effects of apoptosis on viral replication (Clem and Miller, 1993; Hershberger et al., 1992), factors involved in the induction and control of apoptosis (Clem and Miller, 1994; LaCount and Friesen, 1997; Prikhod'ko and Miller. 1996), and the function of P35 in the apoptosis signal transduction pathway (Bertin et al., 1996; Bump et al., 1995; Hershberger et al., 1994; Xue and Horvitz, 1995). However, little is known about the mechanism of protein synthesis shut down.

Apoptosis

Apoptosis is programmed cell death, a process by which a cell commits suicide in an orderly fashion. Apoptosis is involved in a wide variety of normal and abnormal organismal processes, including tissue homeostasis, immune system function, embryonic development, cancer, and pathogenesis. The characteristic phenotype includes extensive blebbing of the cell surface, nucleus condensation and fragmentation, degradation of cellular DNA into oligonucleosome-sized fragments, disintegration of affected cells into small membrane-bound vesicles (apoptotic bodies), and premature cell death (Hale et al., 1996).

Research on apoptosis in mammalian cells and developmental programmed cell death in *Caenorhabditis elegans* have led to identification of several important genes involved in the apoptosis pathway (Hale et al., 1996). Genes that encode the interleukin-1β converting enzyme (ICE) family of cysteine proteases (caspase) have a central role in triggering apoptosis. Activation of the caspase in turn activates a series of enzymes that rapidly kill the cell. The caspase induced apoptosis is inhibited by the products of human gene *bcl-2* (Hockenbery et al., 1990; Tsujimoto and Croce, 1986), its relative *bcl-x* (Boise et al., 1993), and its homologue in *C. elegans ced-9* (Hengartner et al., 1992; Hengartner and Horvitz, 1994).

The role of *p35* in blocking apoptosis was discovered during the characterization of a spontaneous mutant of the baculovirus AcMNPV, which triggers but does not block apoptosis in the cell line SF-21 (Clem et al., 1991). *p35*$^-$ AcMNPV also induces apoptosis in BmN cells (Clem et al., 1991) and a functional *p35* homologue is present in the

BmNPV (Kamita et al., 1993), a close relative of AcMNPV. AcMNPV *p35* encodes a stoichiometric inhibitor of caspase (Bump et al., 1995; Xue and Horvitz, 1995). P35 prevents the autoproteolytic activation of caspase from its precursor form and thus blocks caspase-induced apoptosis (Bump et al., 1995; Xue and Horvitz, 1995).

A second baculovirus gene, inhibitor of apoptosis (*iap*), was identified to prevent apoptosis by its ability to functionally replace *p35* in a genetic complementation assay (Birnbaum et al., 1994; Crook et al., 1993). Homologues of *iap* have been found in *Cydia pomonella* granulovirus (CpGV) (Cp-*iap*), *Orgyia pseudotsugata* MNPV (OpMPNV) (OP-*iap*), and AcMNPV (Ac-*iap*). The Ac-*iap* gene was identified only by its homology to the other two *iaps* but it is not funtional. All three *iap* homologues encode a C3HC4 zinc finger as well as two additional Cys/His motifs (baculovirus *iap* repeats) (Birnbaum et al., 1994; Crook et al., 1993) and thus *iap* may control apoptosis directly at the transcriptional level. Cellular homologues of *iaps* with antiapoptotic activities were discovered recently(Clem et al., 1996; Hay et al., 1994; Liston et al., 1996; Rothe et al., 1995). This may suggest that the gene products of *iaps* are normal components of the cellular apoptotic pathway that have been acquired by baculoviruses to block cellular apoptosis during infection.

Many viruses carry genes involved in blocking cellular apoptosis (Teodoro and Branton, 1997). The adenovirus E1B-19K gene prevents apoptosis induced by E1A through a *p53*-dependent mechanism (Debbas and White, 1993; Lowe and Ruley, 1993; Rao et al., 1992). E1B-19K also protects cells against apoptosis stimulated by tumor necrosis factor alpha or anti-Fas antibodies (Gooding et al., 1991; Hashimoto et al., 1991; White et al., 1992). Epstein-Barr virus carries two genes that are involved in protecting

cells from apoptosis: LMP-1 is expressed during viral latency and may act by inducing cellular *bcl-2* expression (Henderson et al., 1991; Martin et al., 1993) and BHRF-1 is expressed during the lytic cycle and shares homology with Bcl-2 (Henderson et al., 1993). African swine fever virus also encodes a protein LMW5-HL containing sequences homologous to Bcl-2 that prevents apoptosis (Neilan et al., 1993). Poxvirus encodes a serine protease inhibitor CrmA which specifically inhibits caspase activity and prevents or delays apoptosis (Tewari et al., 1995; Tewari and Dixit, 1995).

P35 can function in a phylogenetically broad range of organisms to block apoptosis induced by a variety of signals including mammalian cells (Beidler et al., 1995; Martinou et al., 1995, Rabizadeh et al., 1993; Zhong et al., 1993), Drosophila (Hay et al., 1994), and *C. elegans* (Sugimoto et al., 1994). Thus, the pathway or the points at which both P35 and Bcl-2 act is conserved between invertebrates and vertebrates (Hengartner and Horvitz, 1994; Rabizadeh et al., 1993; Vaux et al., 1992). However, *p35* bears no obvious sequence similarity to other proteins required for apoptotic suppression, including the mammalian proto-oncogene *bcl-2*, adenovirus E1B-19K, Epstein-Barr virus BHRF1, Poxvirus CrmA, or baculovirus *iap* gene (Cleary et al., 1986; Crook et al., 1993; Pearson et al., 1987; White et al., 1992).

The molecular mechanisms by which baculoviruses induce apoptosis are not known. Transfection of SF-21 cells with the AcMNPV *ie-1* gene is sufficient to induce apoptosis (Prikhod'ko and Miller, 1996). However, *ie-1* is not the sole factor that induces apoptosis in SF-21 cells since aphidicolin, a DNA replication inhibitor, which blocks viral DNA replication and DNA replication dependent late gene expression but not *ie-1* expression prevents induction of apoptosis by IE-1 (Clem and Miller, 1994; Prikhod'ko

and Miller, 1996). This indicates that a late event is needed for the induction of apoptosis. Other studies using DNA replication deficient temperature mutants revealed that DNA replication or late gene expression is not essential for induction of apoptosis but is essential for full development of apoptosis (LaCount and Friesen, 1997). Studies using RNA transcription inhibitors seem to support the hypothesis that viral trigger of apoptosis in these cells is related to the shutoff of host RNA synthesis since actinomycin D, DRB, and α-amanitin are all inhibitors of RNA synthesis with different modes and all three drugs induce rapid and wide-spread apoptosis in SF-21 cells (Clem and Miller, 1994).

Protein Synthesis Shut Down

Protein synthesis shut down is a common strategy employed by many host cells against viral infection and viruses in turn have evolved methods to overcome this cellular defense (Hershey, 1991; Schneider and Shenk, 1987). In mammals, vaccinia virus is not able to replicate in CHO cells although mRNA is synthesized. Both viral and cellular protein synthesis is shut down at the stage of intermediate viral proteins. A gene from cowpox virus, CHO *hr*, can overcome the protein synthesis shutoff (Ramsey-Ewing and Moss, 1995; Spehner et al., 1988). In herpes simplex virus 1 (HSV-1), protein synthesis is shut down after the expression of α genes in neuronal cells infected with a $\gamma_1$34.5 minus mutant HSV-1 but not wt HSV-1 (Chou and Roizman. 1992; 1994). $\gamma_1$34.5 gene of HSV-1 is able to preclude the protein synthesis inhibition in HSV-1-infected neuronal cells (Chou and Roizman, 1992; 1994). The carboxyl-terminal domain of $\gamma_1$34.5 is homologous to the corresponding domain of *MyD116*, a gene expressed in myeloid

leukemia cells induced to differentiate by interleukin 6 (Lord et al., 1990; McGeoch and Barnett, 1991), and growth arrest and DNA damage gene 34 (GADD34), a gene induced by growth arrest and DNA damage (Fornace et al., 1989). The carboxyl terminus of the Murine *MyD116* gene can substitute for $\gamma_1$34.5 gene to preclude the protein synthesis shutoff (He et al., 1996). Plants activate enzymes in response to virus infection that depurinate 28S RNA thereby inactivating the ribosome and thus inhibiting protein synthesis (Endo and Ysurugi, 1987). In prokaryotes, protein synthesis shut down is observed in some phage exclusions which involve cleavage of the components of the translation apparatus, EF-Tu (Yu and Snyder, 1994) or $tRNA^{lys}$ (Levitz et al., 1990).

The best understood mechanisms of protein synthesis shut down are those mediated by interferon in mammals and other vertebrates (Lengyel, 1982; Samuel, 1991). Interferons are a class of small molecular weight proteins synthesized and secreted in some cell types in mammals and other vertebrates in response to viruses, as well as other stimuli (Lengyel, 1982). Interferon-mediated protein synthesis shut down includes three different pathways (Farrel et al., 1978; Zilberstein et al., 1978). One pathway is mediated by a dsRNA dependent protein kinase PKR that phosphorylates eukaryotic initiation factor 2 α subunit (eIF-2α) and thus inhibiting translation initiation (Chernajovsky et al., 1979). This is the most common pathway and a wide variety of different viruses have developed effective and varied measures to prevent the function of PKR (Samuel, 1991). Adenovirus and HIV-1 produce an RNA, VA RNA (adenovirus) and TAR RNA (HIV-1), which antagonizes the activation of the PKR (Gunnery et al., 1990; Kitajewski et al., 1986; O'Malley et al., 1986). Reovirus produces σ-3 protein (Imani and Jacobs, 1988)

that bind the activator dsRNA and prevent the activation of PKR. Poxvirus produces two proteins, K3L and E3L, to counteract the effects of PKR. The K3L product is a homologue of the α-subunit of eIF-2 and E3L is a dsRNA binding protein. K3L binds to the PKR (Carroll et al., 1993) and E3L binds to the dsRNA activator (Chang et la., 1992; Yuwen et al., 1993) and prevent activation of PKR. The second translational inhibitory pathway is mediated through synthesis of 2'-5' pppApApA (Kerr and Brown, 1977) by the dsRNA/ATP-dependent oligoisoadenylate synthetase E, another interferon-induced enzyme (Farrel et al., 1978; Hovanessian et al., 1977; Zilberstein et al., 1978) which activates RNase L that degrades mRNAs. The third pathway is mediated by a 2'-5'phosphodiesterase (Schmidt et al., 1978). This enzyme degrades the CCA terminus of tRNA, thereby blocking protein synthesis elongation due to tRNA deficiency (Schmidt et al., 1978) which can be reversed by adding tRNA in the cell-free system (Content et al., 1975; Falcoff et al., 1978; Mayr et al., 1977; Samuel, 1976; Sen et al., 1975; Weissenbach et al., 1977; Zilberstein et al., 1976).

Investigation of protein synthesis shut down in baculovirus-infected insect cells has just initiated. The possible function of *p143* is predicted by its homology in sequence to helicase gene but its actual role in this virus-host interaction remains unclear. Limited work has been reported regarding the factor(s) that triggers the protein synthesis shut down in AcMNPV-infected Ld652Y and LdFB cells (Guzo et al., 1991). A factor designated as macromolecular synthesis inhibition factor (MSIF) is found to be produced and secreted from the infected cells in the absence of viral gene activity (Guzo et al., 1991). It is postulated that the MSIF is the AcMNPV GP64 glycoprotein or some

component or complex of this protein (Guzo et al., 1991). However, this hypothesis has not been confirmed and further research on this issue has not been continued. It is the objective of this research project to gain understanding of the nature of this novel virus-host interaction.

CHAPTER 2

Identification of a Baculovirus Gene That Promotes *Autographa californica* Nucleopolyhedrovirus Replication in a Nonpermissive Insect Cell Line

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Abstract

A gene was identified in *Lymantria dispar* nucleopolyhedrovirus (LdMNPV) that promotes *Autographa californica* nucleopolyhedrovirus (AcMNPV) replication in IPLB-Ld652Y cells, a cell line that is non-permissive for AcMNPV. Co-transfection of AcMNPV DNA and a plasmid comprising the LdMNPV gene into IPLB-Ld652Y cells results in AcMNPV replication. The gene maps between 43.3-43.8 m.u. on the 162 kbp genome of LdMNPV. It comprises a 218 amino acid open reading frame and encodes a polypeptide with a predicted molecular weight of 25.7 kDa. The predicted polypeptide is glutamic acid and valine rich and negatively charged, with a pI of 4.61. No protein sequence motifs were identified and no matches with known nucleotide or peptide sequences were found in the AcMNPV genome or database searches that suggest how this gene might function. A recombinant AcMNPV bearing the LdMNPV gene overcomes a block in protein synthesis observed in AcMNPV-infected IPLB-Ld652Y cells. Using Southern blotting techniques, we were unable to identify a homologue in *Orgyia pseudotsugata* nucleopolyhedrovirus, a baculovirus that is routinely propagated in IPLB-Ld652Y cells. This suggests that the LdMNPV host range gene is unique among baculoviruses studied to date. We named this gene *hrf-1* (for host range factor 1).

Introduction

Baculoviruses are large double stranded DNA viruses that infect arthropods and are of interest for use as viral insecticides against Lepidopteran larvae and as eukaryotic gene expression vectors. While understanding of baculovirus genetics and molecular biology has advanced rapidly in recent years (Blissard and Rohrmann, 1990; O'Reilly et al., 1992; Rohrmann, 1992), mechanisms that control baculovirus host specificity are

poorly understood. Understanding these mechanisms is important for assessing potential risks of genetically engineered baculovirus insecticides and for tailoring baculovirus insecticides against specific pest insects. Studies of baculoviruses bearing reporter genes demonstrate that although these viruses do not replicate in non-permissive insect cells, they are able to enter and express some genes (Carbonell et al., 1985; Morris and Miller, 1992). Thus the block in virus infectivity in non-permissive insect cells occurs subsequent to viral entry, uncoating, and early gene expression.

A few baculovirus genes that affect viral host range have been identified. Two virus encoded proteins, P35 and inhibitor of apoptosis (IAP), prevent apoptosis, programmed cell death, in baculovirus-infected cells (Clem et al., 1991; Crook et al. 1993). The deletion of the *p35* gene from *Autographa californica* nucleopolyhedrovirus (AcMNPV) results in premature cell death in infected SF-21 and BmN cells but does not impair virus replication in TN368 cells (Clem et al., 1991) or *Trichoplusia ni* larvae (Clem and Miller, 1993). *p35* is required for efficient AcMNPV replication in both SF-21 cells (Clem et al., 1991; Hershberger et al., 1992) and in *Spodoptera frugiperda* larvae (Clem and Miller, 1993). IAP can functionally substitute for P35 in SF-21 cells (Birnbaum et al., 1994; Crook et al., 1993). *p143*, a baculovirus encoded protein with homology to DNA helicases (Lu and Carstens, 1991), also affects host range. Viruses with expanded host ranges result from recombination between a small region of the DNA helicase genes of *Bombyx mori* nucleopolyhedrovirus (BmNPV) and AcMNPV (Croizier et al., 1994; Maeda et al., 1993). These viruses replicate in both *S. frugiperda* and *B. mori* cell lines. The mechanism by which this region of the DNA helicase expands the virus host range is unknown. A suite of eighteen late expression factors (*lef*s) are required for

expression of late AcMNPV genes in SF-21 cells (Lu and Miller, 1995; Todd et al., 1995). *p35* and *p143* are among these *lefs*. Different *lefs* may be required for virus replication in different cell types, thus affecting virus host range. For example, *lef-7* is not required for AcMNPV replication in TN-368 cells (Lu and Miller, 1995). A new *lef* gene, host cell-specific factor-1 (*hcf-1*) corresponding to AcMNPV ORF70 (Ayres et al., 1994) was identified that is required for late gene expression in TN-368 cells (Lu and Miller, 1995).

Baculoviruses are generally quite host-specific. Infection by most is limited to a single species or few closely related species of insects (Groner, 1986). AcMNPV has a broader host range than many baculoviruses both *in vivo* and *in vitro*, reportedly infecting at least 33 species of Lepidopteran larvae in 10 families (Groner, 1986) as well as over 25 different insect cell lines (Hink, 1979; Hink and Hall, 1989). Although AcMNPV does not infect gypsy moth, *Lymantria dispar* (*L. dispar*), larvae some *L. dispar* cell lines have been established that will support AcMNPV replication (Goodwin et al., 1978; Lynn et al., 1988). However, the cell line IPLB-Ld652Y (subsequently abbreviated Ld652Y) (Goodwin et al., 1978) that is routinely used to propagate the nucleopolyhedroviruses of *Lymantria dispar* (LdMNPV) and *Orgyia pseudotsugata* (OpMNPV), does not support AcMNPV replication. The Ld652Y cell line has been described as semi-permissive for AcMNPV replication because of an observed cytopathic effect and the production of several infected-cell-specific proteins, but no production of infectious virions (McClintock et al., 1986). Infection of Ld652Y cells with AcMNPV results in shut off of both viral and cellular protein synthesis between sixteen and twenty hours post infection (Guzo et al., 1992). However, viral DNA is replicated and viral mRNA from all temporal

classes can be isolated from AcMNPV-infected Ld652Y cells (Guzo et al., 1992; Morris and Miller, 1992; 1993). The amounts of viral DNA isolated from AcMNPV-infected Ld652Y cells at 24 and 36 h post infection (p.i.) are similar to amounts isolated from AcMNPV-infected SF-21 cells (Morris and Miller, 1993). Dot blot analysis of virus DNA in AcMNPV-infected Ld652Y cells over time suggests that AcMNPV DNA synthesis ceases between 20 and 36 h p.i. (McClintock et al., 1986). The amount of viral mRNA isolated from AcMNPV-infected Ld652Y at different times, up to 72 h p.i., is the same or greater than levels of AcMNPV mRNA isolated from infected TN-368 cells at identical times p.i. (Guzo et al., 1992). Reports of AcMNPV replication in AcMNPV-infected Ld652Y cells superinfected with LdMNPV (McClintock and Dougherty, 1987) suggested that LdMNPV could provide a factor necessary for AcMNPV replication in Ld652Y cells. Identification and characterization of this factor is important for understanding the nature of the block for AcMNPV replication in Ld652Y cells.

In preliminary experiments, we determined that co-transfection of AcMNPV and LdMNPV DNA into SF-21 cells resulted in generation of chimeric viruses, comprised of LdMNPV DNA integrated into the AcMNPV genome that could replicate in Ld652Y cells. In this study we have used a transfection assay to functionally map the LdMNPV gene that encodes the factor required for AcMNPV replication in Ld652Y cells. Here we report the physical map location and sequence of the LdMNPV host range gene.

Materials and Methods

Virus and Cell Lines

Spodoptera frugiperda, IPLB-SF-21 (Vaughn et la., 1977), and *Lymantria dispar*, IPLB-Ld652Y cells (Goodwin et al., 1978) were maintained in TC100 medium (Life

Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 0.26% tryptose broth. The gypsy moth cell line, IPLB-Ld652Y, was provided by D. Lynn, USDA-ARS, Beltsville, MD and subsequently adapted for growth in TC100 medium. Viruses used in this study were AcMNPV variant L1 (Lee and Miller, 1978), LdMNPV isolate A21-MPV (Slavicek et al., 1992), and OpMNPV provided by G.W. Blissard, Boyce Thompson Institute at Cornell University, Ithaca, NY. AcMNPV and LdMNPV were propagated in *Trichoplusia ni* larvae and SF-21 cells or *Lymantria dispar* larvae and Ld652Y cells respectively. OpMNPV was propagated in Ld652Y cells.

DNA Isolation

Cosmid and plasmid DNA were prepared using the method of Clewell and Helinski (Clewell and Helinski, 1969) and purified through CsCl gradients or with the Wizard miniprep kit (Promega, Madison, WI) following the manufacturer's instructions. For transfection assays, viral DNA was purified from larval occluded viruses following dissolution with 0.1M $NaCO_3$. DNA from virus plaque isolates was prepared from budded virus using a miniprep procedure (O'Reilly et al., 1992).

Cotransfection Assays

Ld652Y cells were co-transfected with AcMNPV DNA and either intact LdMNPV DNA or cloned fragments of LdMNPV DNA. Cells were seeded 4.5×10^5 cells per 35 mm plate. Cell monolayers were washed 2X with serum-free TC100 medium and overlaid with a final volume of 1.5 ml serum-free TC100 medium. Transfections were by the lipofectin technique (Flegner et al., 1987) using lipofectin from BRL-Life Technologies (Bethesda, MD) and 2.5 μg AcMNPV viral DNA and 2.5 μg each

LdMNPV viral DNA, cosmid or plasmid clone. DNAs were suspended in final volume of 50 µl of sterile Milli-Q H_2O then mixed with 50 µl of lipofectin. The DNA-lipofectin suspension was added dropwise to the cell monolayers and mixed by gentle swirling. Following 2 h incubation at 27°C the lipofectin solution was removed from the cells and replaced with TC100 medium supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY). Cloned LdMNPV DNA comprised a cosmid library of overlapping LdMNPV genomic fragments from isolate A21-MPV (Slavicek et al., in press), cloned into the Supercos vector (Stratagene, Inc., Palo Alto, CA), provided by J. M. Slavicek, U.S. Forest Service, Delaware, OH. Subclones of cosmid C77 were constructed in Bluescript plasmids (Stratagene, Inc., Palo Alto, CA) using standard protocols (Sambrook et al., 1989). The presence of a functional host range gene was scored by the observation of polyhedra in transfected Ld652Y cells and by the presence of infectious budded virus in the cell supernatant. Polyhedra in transfected cells were observed using a Nikon TMS inverted microscope at 200 X magnification. The presence of budded virus was scored by the ability of AcMNPV and co-transfection supernatants to infect SF-21 cells and LdMNPV transfections to infect Ld652Y cells.

Sequencing

A series of overlapping clones were generated by a combination of subcloning restriction fragments into Bluescript plasmids and by the generation of nested deletion clones using exonuclease III (Henikoff, 1984) and mungbean nuclease. The clones were sequenced by dideoxynucleotide chain termination (Sanger et al., 1977) using the Circumvent sequencing kit (New England Biolabs, Beverly, MA) following the

manufacturer instructions. Specific sequencing primers were designed and synthesized (Michigan State University, Macromolecular Structure, Sequencing and Synthesis Facility) to sequence regions we were unable to obtain in both directions by sequencing the various subclones. Both strands were completely sequenced. Sequence data was compiled and analyzed using the Genetics Computer Group (GCG) Sequence Analysis Programs (Devereux et al., 1984). GenBank 91.0, EMBL 44.0, and SWISS-PROT 31.0 databases were searched for sequence homology.

Construction of Recombinant AcMNPV

A recombinant AcMNPV, vAcLdPS, was constructed by first inserting a 2093 bp *Pst*I-*Sst*I (43.3-44.6 m.u.) LdMNPV DNA fragment into the polylinker site of transfer vector pSynXIV VI$^+$ (Wang et al., 1991) to generate the plasmid, pSynLdPS. The recipient virus was vSynVI$^-$gal, a recombinant AcMNPV in which the polyhedrin gene has been replaced with the *lacZ* gene resulting in an occlusion negative virus that forms blue plaques in the presence of X-gal (Wang et al., 1991). vSynVI$^-$gal DNA was linearized by cleavage at a unique *Bsu*36I site within *lacZ*. pSynLdPS and linearized vSynVI$^-$gal DNA were co-transfected into SF-21 cells using lipofectin (Flegner et al., 1987; O'Reilly et al., 1992). White occlusion positive viruses were selected and plaque purified. Correct insertion of the LdMNPV fragment was confirmed by analysis of DNA restriction patterns of the recombinant virus.

Western Blot Analysis

SF-21 cells and Ld652Y cells were infected with wt AcMNPV or vAcLdPS at an MOI of 10. Virus infected cells were harvested at 0, 6, 12, 18, 24 and 48 h p.i. Time zero

was defined as the time when the inoculum was removed and incubation at 27°C was initiated. Mock infection received identical treatment except that cell culture medium was used as inoculum. Mock infected cells were harvested at 48 h p.i. Cells were lysed in 2X disruption buffer (125 mM-Tris-HCl pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.002% bromophenol blue). Proteins were run on 10% SDS polyacrylamide gel (Laemmli, 1970). An amount of protein equivalent to approximately 4 X 10^5 cells (SF-21) or 3 X 10^5 (Ld652Y) were loaded per lane for Coomassie blue stained gels while 2.5 X 10^4 cells (SF-21) or 2 X 10^4 (Ld652Y) loaded per lane for Western blots. Proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Life Science, Arlington Heights, IL) and incubated with antibodies (Towbin et al., 1979). Anti-gp67 monoclonal antibody (Volkman et al., 1984) (provided by L. Volkman, U.C. Berkeley, Berkeley, CA) was used at the dilution of 1:50. Anti-p39 polyclonal antibody (Pearson et al., 1988) (provided by G. Rohrmann, Oregon State U., Corvallis, OR) was used at 1:1000. Secondary antibodies, anti-mouse peroxidase conjugate and anti-rabbit peroxidase conjugate (Sigma, St. Louis, MO), were used at 1:5000. The ECL Western blot detection system (Amersham Life Science, Arlington Heights, IL) was employed for signal detection.

Nucleotide Sequence Accession Number

The nucleotide sequence presented in this report was submitted to GenBank and assigned the accession number LdNPV U38895.

Results

LdMNPV Encodes a Factor That Permits AcMNPV Replication in Ld652Y Cells

AcMNPV does not normally replicate in Ld652Y cells and LdMNPV does not replicate in SF-21 cells. However, AcMNPV replicates in Ld652Y cells superinfected with LdMNPV (McClintock and Dougherty, 1987). The ability of transfected LdMNPV DNA to provide a factor needed for AcMNPV replication in Ld652Y cells was tested by co-transfecting AcMNPV and intact genomic LdMNPV DNA into Ld652Y cells. Cells were observed for the formation of polyhedral inclusion bodies (PIBs). Production of budded AcMNPV was assessed by observation of PIBs in SF-21 cells inoculated with transfection supernatants. Transfection of LdMNPV or LdMNPV plus AcMNPV DNA resulted in the production of PIBs within two to four days, while no PIBs were produced when AcMNPV alone was transfected. Supernatants from the LdMNPV plus AcMNPV transfections, but not AcMNPV or LdMNPV transfections alone, were able to infect SF-21 cells (Table 1). This suggested that the budded viruses produced were AcMNPV and demonstrated that the factor necessary for replication in Ld652Y cells could be provided by transfected LdMNPV DNA.

In order to map the gene encoding this factor, we transfected Ld652Y cells with cloned fragments of LdMNPV along with total genomic AcMNPV DNA. A cosmid library comprising six overlapping fragments of 20-30 kbp each, encompassing the entire LdMNPV genome (Figure 1A), was used in the initial experiments. Virus replication was assessed as described above. Of the six cosmids two (C-12 and C-77), were able to provide the factor that allowed AcMNPV replication in Ld652Y cells (Table 1). When AcMNPV DNA was transfected with all cosmids except either C-12 or C-77, AcMNPV

Table 1. Replication of AcMNPV in Ld652Y Cells Following Transfection of Viral and Cosmid DNAs into Ld652Y Cells

Transfected DNA	Virus Production[a]
AcMNPV	-
LdMNPV	-
AcMNPV + LdMNPV	+
AcMNPV + C-15	-
AcMNPV + C-2	-
AcMNPV + C-12	+
AcMNPV + C-77	+
AcMNPV + C-38	-
AcMNPV + C-64	-
AcMNPV + C-63	-
AcMNPV + All cosmids except C-12	+
AcMNPV + All cosmids except C-77	+
AcMNPV + All cosmids except C-12 and C-77	-

[a]PIBs are produced and transfection supernatants are infectious for SF-21 cells.

Figure 1. Location of Clones Used to Map the LdMNPV Gene in Transfection Assays A: *Bgl*II restriction map of LdMNPV A-21 with scales indicating map units (m.u.) and kbp above. The regions of LdMNPV comprising cosmid clones, C-2, C-12, C-77, C-38, C63, C-64, and C-15, in the overlapping genomic library are shown as lines below the restriction map. B: Restriction map of cosmid C-77 indicating locations of the *Eco*RI, *Bam*HI, and *Hin*dIII sites used for subcloning and the *Bgl*II site at the *Bgl*II A/H junction. Map locations are indicated below the restriction sites in m.u. The restriction fragments comprising individual subclones are depicted as lines below the map, with hatch marks at the restriction sites, and are labeled with lower case letters. The ability of individual clones to promote AcMNPV replication in Ld652Y cells in transfection assays is indicated by a "+" under virus production. Clones unable to promote AcMNPV replication are indicated by "-". Virus replication was assessed by observation of PIBs in transfected Ld652Y cells and the ability of budded virus in transfection supernatants to infect SF-21 cells. C: Restriction maps of the *Eco*RI-*Hin*dIII region, 42.6-46.5 m.u., of LdMNPV. The positions of restriction sites are indicated in m.u. below the maps. Locations of individual subclones and their ability to promote AcMNPV replication in Ld652Y cells is indicated in the same manner as in B. Subclones h and i are named pBSLdSD and pBSLdPD respectively. Abbreviations: E=*Eco*RI, B=*Bam*HI, H=*Hin*dIII, Bg=*Bgl*II, P=*Pst*I, N=*Nar*I, S=*Sst*I, Sa=*Sal*I, X=*Xho*I, and D=*Dra*I.

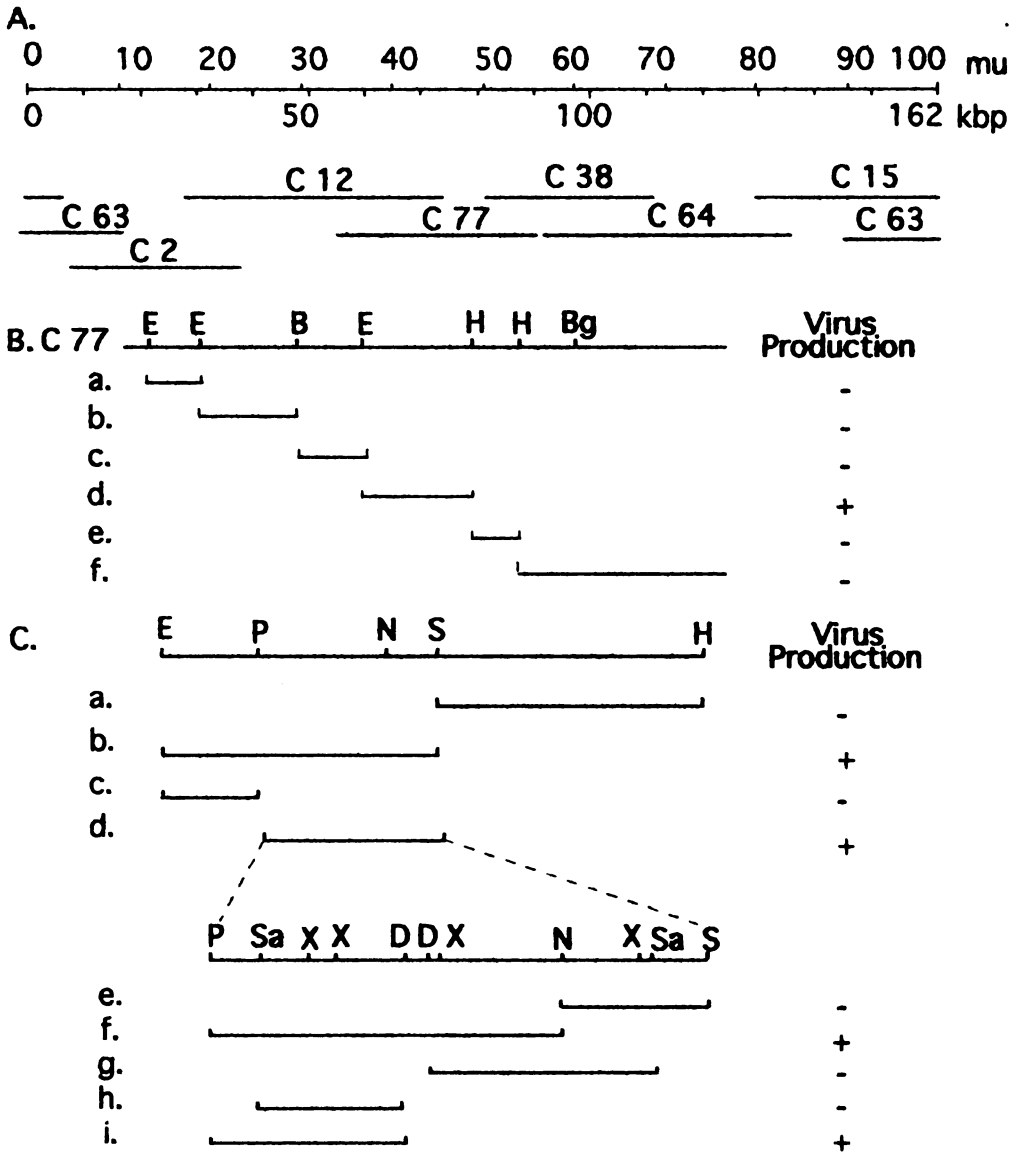


Figure 1. Location of Clones Used to Map the LdMNPV Gene in Transfection Assays

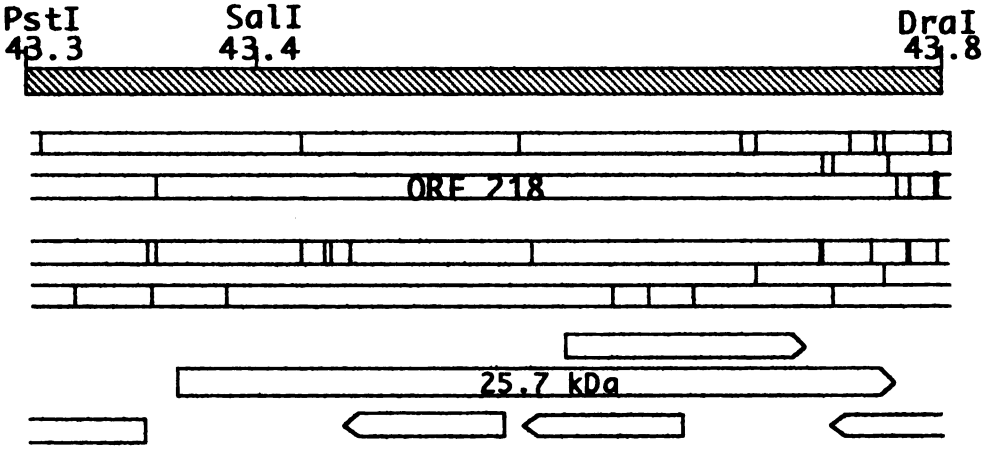
could replicate. However if both were omitted AcMNPV was unable to replicate (Table 1). Since C-12 and C-77 overlap between 33 and 45 m.u. of the LdMNPV genome (Figure 1A), the gene for the factor mapped to the region common to C-12 and C-77. To further localize the gene, we subcloned individual restriction fragments from cosmid C-77 in Bluescript vectors (Figure 1B) and tested each subclone for its ability to promote AcMNPV replication in Ld652Y cells in transfection assays. Only one subclone, a 6.3 kbp *Eco*RI- *Hin*dIII fragment (Figure 1B, d), could provide the required factor. We then tested nine subclones of this fragment (Figure 1C) and were able to localize the gene encoding the factor to a single 835 bp *Pst*I-*Dra*I fragment (Figure 1C, i) that mapped between 43.3-43.8 m.u. This subclone was named pBSLdPD.

The *Pst*I-*Dra*I fragment was sequenced and four open reading frames (ORFs) were identified (Figure 2). The largest encodes a predicted protein of 218 amino acids (25.7 kDa) and is preceded by an initiator sequence, TCAGT (Cherbas and Cherbas, 1993) and followed by a polyadenylation signal, but no TATA box was identified (Figure 2A). Similar initiator sequences have been identified in other baculovirus genes, including the LdMNPV G22 that also lacks a TATA box (Bischoff and Slavicek, 1995).

Microscopic examination of transfected Ld652Y cells revealed that LdMNPV, but not AcMNPV DNA, transfected into Ld652Y cells results in the formation of polyhedra in the cell nuclei (Figure 3, compare panels a and b). A clone with ORF218 truncated (pBSLdSD) did not rescue cotransfected AcMNPV DNA in Ld652Y cells (Figure 3c), whereas the clone (pBSLdPD) containing the complete 218 amino acid ORF along with its 5' flanking region was able to supply this factor (Figure 3d). The three smaller ORFs located between 43.4 to 43.8, as well as their 5' and 3' flanking regions,

Figure 2. Analysis of ORFs and Nucleotide Sequence of the LdMNPV Genome Between 43.3 and 43.8 m.u. A: Diagram of ORFs in six reading frames. The locations of *Pst*I, *Sal*I, and *Dra*I restriction sites, six possible reading frames, and significant ORFs are shown. The locations of stop codons in each reading frame are indicated by vertical lines and the 218 amino acid ORF encoding HRF-1 is labeled. ORFs capable of encoding polypeptides with greater than 49 amino acids are depicted as open arrows, indicating the direction of transcription, below the diagram of the reading frames. *hrf-1* is shaded. B: Nucleotide sequence of LdMNPV A21-MPV, 43.3-43.8 m.u., and the deduced amino acid sequence of *hrf-1*. A predicted polyadenylation recognition signal is double underlined. An arthropod initiator sequence, TCAGT (Cherbas and Cherbas, 1993), is underlined.

A.



B.

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1 CTGCAGCGTGTGTAACTGTACTTGCAATTGCGACCCGTGTCAGTACACGCCAGCGCACAG

61 TGCGGAGCGAACGCGACCAGCGCCGCGCAGCGAGACGAATATCGATTCATTTTATTTAGC

121 GCGCCGACGACCATGGGCATGGACGCCGAGTTTTTCGTGGACGGCGAACGCGTGGACAGC
                M  G  M  D  A  E  F  F  V  D  G  E  R  V  D  S

181 TACAAGTGCACCGGGCGGTGGTCCGCCATCGTCGACGTGCGCCACCGTCCCGCCTTGACG
    Y  K  C  T  G  R  W  S  A  I  V  D  V  R  H  R  P  A  L  T

241 GTGCGCTACCGGTACGAGCGCGGCTACGGCCACTACGCGCTCTTTGTTTATTTTCGACAC
    V  R  Y  R  Y  E  R  G  Y  G  H  Y  A  L  F  V  Y  F  R  H

301 GTGGCCACCGGCATGCTGGAGACGGAGCGCGTGGACGTCGCCGACCGCGACCGCGTCGTG
    V  A  T  G  M  L  E  T  E  R  V  D  V  A  D  R  D  R  V  V

361 CCCGTGCCCGAAGAGTGGCTCGAGTACATCGACGACGACGACGAGGAGCGCGAACGCCAA
    P  V  P  E  E  W  L  E  Y  I  D  D  D  D  E  E  R  E  R  Q

421 GTGGAGGTGTTCGTGTGCATGAAGGGCGACTGCTACGCCCACGACGGCCCCCTGTTCGTC
    V  E  V  F  V  C  M  K  G  D  C  Y  A  H  D  G  P  L  F  V

461 TCCGAGGCGTCCAAATGGTGCAGCCGCGAGCCCGAACACGTTCGCATCAGAGACTCGCCG
    S  E  A  S  K  W  C  S  R  E  P  E  H  V  R  I  R  D  S  P

521 CTCGAGGCCGTGCGACAAATCAAGTGCGCGGAGGACGTGTTCGCGTTCGTCGAAGCGTTC
    L  E  A  V  R  Q  I  K  C  A  E  D  V  F  A  F  V  E  A  F

581 ATGCGGATCGAAGAGGGCGAGTACGCGTGGCGCGATCCGCTTGTGATCGACCAATTAAAC
    M  R  I  E  E  G  E  Y  A  W  R  D  P  L  V  I  D  Q  L  N

641 GACCAAGAACTGGCCTCGATCGAGAAGGTTTTCGCCTCGACGGTGTGGAACTATTATGAA
    D  Q  E  L  A  S  I  E  K  V  F  A  S  T  V  W  N  Y  Y  E

701 AAAGTTAACGCGCGACCGGTTTTGACCTTTGCCGAAAATTATTTGAAGAAATTAAATGAA
    K  V  N  A  R  P  V  L  T  F  A  E  N  Y  L  K  K  L  N  E

761 AGTTTGTAATGTTATTATAGGATCTCGGTGCGTATAAATATTTAATAAAATTT
    S  L  *
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Figure 2. Analysis of ORFs and Nucleotide Sequence of the LdMNPV Genome Between 43.3 and 43.8 m.u.

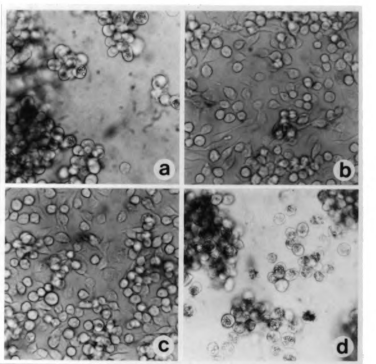

Figure 3. Brightfield Micrographs of Ld652Y Cells Post Transfection Four days post transfection with the following DNAs: (a) LdMNPV, (b) AcMNPV, (c) AcMNPV and pLdSD (Figure 1C, h.), and (d) AcMNPV and pLdPD (Figure 1C, i.).

were present in both clones. Therefore the 218 amino acid ORF encodes the LdMNPV host range factor. We named this gene *hrf-1* for host range factor-1.

The nucleotide and predicted protein sequences of *hrf-1* were compared to known sequences in the GenBank (release 91.0), EMBL (release 44.0), and SWISS-PROT (release 31.0) databases using Fasta and Tfasta algorithms of the Wisconsin User Group GCG sequence analysis programs (Devereux et al., 1984) and no significant identity was observed. A search of the National Center for Biotechnology Information (NCBI) non-redundant protein database employing the Blast algorithm (Altschul et al., 1990) revealed a limited identity with AcMNPV polyhedrin (40% over 32 amino acid residues).

Although no characteristic protein motifs were identified that might suggest how HRF-1 might function, the predicted peptide is rich in glutamic acid (12%) and valine (12%) residues, containing 26 and 25 residues respectively. It also contains 20 (9%) aspartic acid and 18 (8%) arginine residues. The concentration of glutamic and aspartic acid residues contribute to an unusually high negative charge (-16) and an isoelectric point of 4.61. Dividing the net charge of -16 by the number of amino acids, 218, results in a net charge percent of -7.3. Karlin and Brendel (Karlin and Brendel, 1992) statistically analyzed amino acid characteristics of protein sets from humans, *Drosophila*, yeast, *Escherichia coli*, *Bacillus subtilis*, and four herpes viruses, complied from the SWISS-PROT database. Among these data sets less than 5% of the proteins analyzed were more acidic than HRF-1. High net negative charge due to numerous acidic residues has also been observed in proteins that respond to growth arrest and DNA damage (Gadd) (Zhan et al., 1994).

Late Virus Genes Are Translated in Recombinant AcMNPV-Infected Ld652Y Cells

In order to determine if *hrf-1* would facilitate AcMNPV replication in infected, as well as in transfected Ld652Y cells, we constructed recombinant AcMNPV. The *Pst*I-*Sst*I (43.3-44.6 m.u.) fragment (Figure 4A) was cloned into the polylinker site of the transfer vector pSynXIV VI+ (Wang et al., 1991). The resulting plasmid, pSynLdPS, and recipient virus, VsynVI⁻gal (Wang et al., 1991), DNA were transfected into SF-21 cells. A recombinant virus, vAcLdPS, was generated by homologous recombination. Polyhedra are observed in vAcLdPS-infected Ld652Y cells by 24 h p.i., indicating the recombinant is infectious (data not shown). vAcLdPS comprises the entire AcMNPV genome with the addition of the 2093 bp LdMNPV genomic fragment and two synthetic promoters from the transfer vector inserted between 4427 and 4428 bp of the AcMNPV genome (Ayres et al., 1994), adjacent to the polyhedrin gene locus (Figure 4B). The *Pst*I-*Sst*I fragment used for production of this construct contains both *hrf-1* and a gene with homology to entomopox fusolin genes (*fus*) (Cassar and Thiem, submitted) (Figure 4A). We have no evidence that LdMNPV *fus* contributes to AcMNPV replication in Ld652Y cells. The LdMNPV *Dra*I-*Sal*I fragment comprising *fus* (Figure 4A, hatched) does not promote AcMNPV replication in Ld652Y cells in transfection assays (Figure 1C, g). An AcMNPV recombinant bearing only *hrf-1* (Figure 4A, stippled) replicates in Ld652Y cells (Du and Thiem, 1997).

Since the inability of Ld652Y cells to support AcMNPV infection involves a block in protein synthesis (Guzo et al., 1992; Morris and Miller, 1992; 1993), we compared the synthesis of two baculovirus structural proteins, gp67 a peplomer protein found exclusively in the budded form of the virus (Whitford et al., 1989) and p39 the

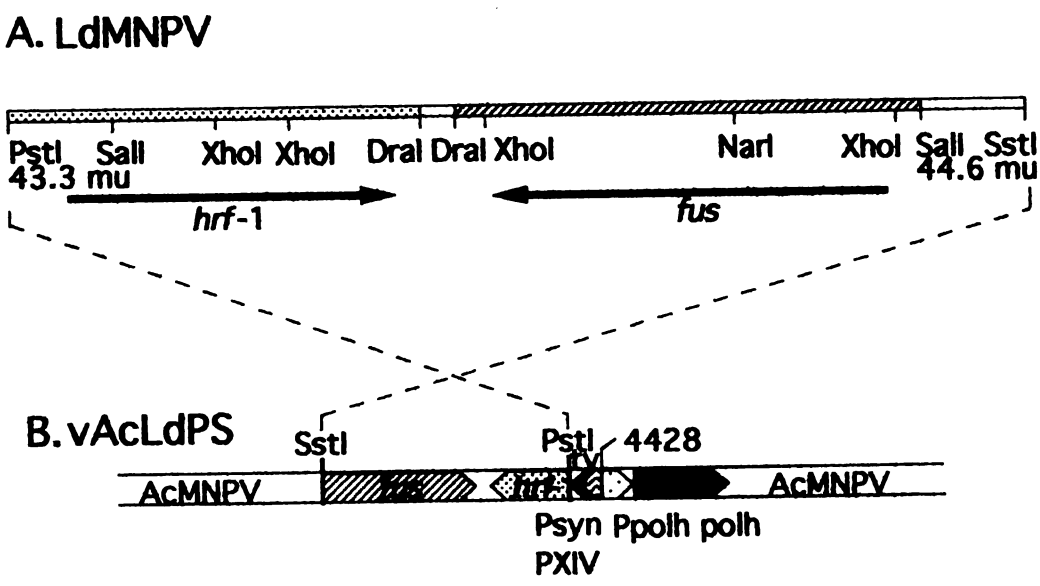

Figure 4. Diagram Showing the Construction of a Recombinant AcMNPV, vAcLdPS A: An expanded restriction map of the LdMNPV *Pst*I-*Sst*I restriction fragment (43.3-44.6 m.u.) showing the location and orientation of *hrf-1* and *fus* with respect to the linearized LdMNPV physical map. The stippled region indicates the location of the smallest restriction fragment able to rescue AcMNPV in transfection assays. A restriction fragment containing *fus* that does not rescue AcMNPV is hatched. B: The polyhedrin gene region of vAcLdPS. The LdMNPV DNA and two synthetic promoters have been inserted adjacent to the AcMNPV polyhedrin gene, nt 4428 (Ayres et al., 1994). The direction of arrows indicate the orientation of genes and promoters. The tandem synthetic promoters derived from the transfer vector, pSynXIV VI+ (Wang et al., 1991), are labeled "tv".

major capsid protein (Thiem and Miller, 1989), in AcMNPV- and vAcLdPS-infected cells by Western blot analysis (Figure 5). SF-21 and Ld652Y cells were infected with AcMNPV and vAcLdPS and harvested at different times p.i. Proteins were separated by SDS-PAGE (Laemmli, 1970) and stained with Coomassie blue or transferred to membranes (Towbin et al., 1979) and incubated with antibodies against gp67 (Volkman et al., 1984) or p39 (Pearson et al., 1988). In SF-21 cells (Figure 5, A-C) gp67 was observed by 12 h p.i. in both wt AcMNPV-infected cells (Figure 5B, I) and in vAcLdPS-infected cells (Figure 5B, II). By 48 h p.i. gp67 production is reduced in cells infected with either virus. p39 is expressed in a similar manner in both AcMNPV- and vAcLdPS-infected SF-21 cells (Figure 5C, compare I and II). However, in Ld652Y cells (Figure 5, D-F) both gp67 and p39 are expressed in vAcLdPS-infected but not in AcMNPV-infected cells (Figure 5E and F, compare panels II and I). In vAcLdPS-infected Ld652Y cells, gp67 expression is observed by 6 h p.i. and diminishes by 48 h p.i. At 48 h p.i. p39 expression is greater in vAcLdPS-infected Ld652Y cells than SF-21 cells (Figure 5, panels C and F). Although polyhedrin antiserum was not used, polyhedrin is observed in all panels that were incubated with antibodies. This is most likely due to non-specific secondary antibody binding, because polyhedrin is present at such high levels at late times p.i.

Evidence That a Gene With Homology to LdMNPV *hrf-1* Is Not Present in the OpMNPV Genome

Since Ld652Y cells are permissive for OpMNPV replication, it seemed likely that OpMNPV might carry a gene homologous to LdMNPV *hrf-1*. To determine if OpMNPV carries a similar gene, we used Southern blots with low stringency hybridization and

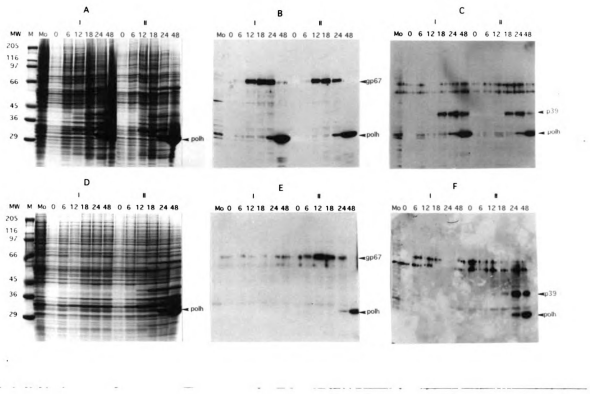

Figure 5. Comassie Blue Stained Gels and Western Blot Analysis of Protein Synthesis A-C: SF-21; D-E: Ld652Y cells. Roman numerals above the panels indicated the virus used for infection; I = wt AcMNPV and II = vAcLdPS. Coomassie blue stained gels are shown in Panel A (SF-21 cells) and D (Ld652Y cells). Western blot analyses with antibodies to gp67 are shown in panels B (SF-21 cells) and E (Ld652Y cells). Western blot analyses with p39 antibodies are shown in panels C (SF-21 cells) and F (Ld652Y cells). Lanes labeled Mo indicate mock infected cells. Arabic numbers above the lanes indicate the time, in hours, after infection that cells were harvested. Lanes MW in Panels A and D are molecular weight standards with molecular weights in kDa indicated to the left of the panels. gp67, p39 and polyhedrin protein bands are indicated by arrows and labeled to the right of individual panels.

wash conditions. The LdMNPV *hrf-1* probe did not hybridize to OpMNPV DNA (data not shown). Based on this study, there does not appear to be an OpMNPV gene with homology to *hrf-1*. Furthermore, OpMNPV was unable to promote AcMNPV replication in Ld652Y cells in transfection assays or when used to superinfect AcMNPV-infected Ld652Y cells (data not shown), suggesting that it does not carry a gene with a similar function.

Discussion

We have identified a unique gene, *hrf-1*, encoding a 25.7 kDa predicted protein that enables AcMNPV to replicate in Ld652Y cells, a non-permissive cell line. Cloned *hrf-1* DNA is able to overcome the block for AcMNPV replication in Ld652Y cells in co-transfection assays. Recombinant AcMNPV carrying *hrf-1* replicate in Ld652Y cells and overcome a block in protein synthesis observed in wt AcMNPV-infected cells. No homologies to genes of known function or characteristic protein motifs that might suggest possible roles for HRF-1 were identified in database searches.

The block for AcMNPV replication in Ld652Y cells involves total inhibition of protein synthesis. When Ld652Y cells are infected with AcMNPV the cells respond by shutting off all protein synthesis, both viral and cellular before 20 h p.i. (Guzo et al., 1992; McClintock et al., 1986). In contrast, viral DNA is replicated and mRNA is transcribed (Guzo et al., 1992; Morris and Miller, 1993). AcMNPV-specific mRNAs are found in both cytoplasmic and nuclear fractions of infected Ld652Y cells and can be translated *in vitro* using rabbit reticulocyte lysates (Guzo et al., 1992). Thus, there are no apparent problems in either transport from the nucleus or in the translatability of viral mRNA in AcMNPV-infected Ld652Y cells. Therefore, it is likely that the block in

AcMNPV replication in Ld652Y cells in some way directly affects protein synthesis.

Recombinant AcMNPV containing reporter genes under the control of either the early AcMNPV ETL or the *Drosophila* hsp70 promoter show low levels of expression in Ld652Y cells (Morris and Miller, 1992; 1993), suggesting that the translation of any genes introduced by AcMNPV-infection in this cell line is minimal. This is puzzling because both late gene transcription and DNA replication occur in AcMNPV-infected Ld652Y cells. Both of these processes require early viral gene products (Lu and Miller, 1995; Todd et al., 1995). Therefore the block in protein synthesis in AcMNPV-infected Ld652Y cells must occur subsequent to the translation of at least some early AcMNPV genes. It is possible that sufficient levels of some viral gene products, essential for late transcription and DNA replication, are translated in the period prior to total protein synthesis shut down. This issue is currently being investigated.

Understanding when and how protein synthesis is blocked in AcMNPV-infected Ld652Y cells could provide clues for the function of HRF-1. Inhibition of protein synthesis in response to virus infection is common in cells infected with a variety of viruses (Schneider and Shenk, 1987). Virus mediated processes include degradation of host mRNA, inactivation of translation factors, and alterations of intracellular ion concentrations in order to favor translation of virus genes. Cells also shutdown protein synthesis in response to viral infection. One of the best characterized cellular responses is the interferon-mediated pathway of vertebrates (Samuel, 1991). Viruses in turn have evolved diverse mechanisms to counteract the cellular response (Samuel, 1991; Schneider and Shenk, 1987) .

The amino acid composition of HRF-1 may be significant. The predicted protein

is unusually highly charged and acidic. This feature is shared with mammalian Gadd proteins (Zhan et al., 1994). All Gadd proteins are negatively charged but, with the exception of GADD45 and MyD116, share no sequence homology (Zhan et al., 1994) . Recently a Herpes Simplex Virus (HSV-1) gene was identified that encodes a protein, ICP34.5, with homology to GADD45 and MyD116 (Chou and Roizman, 1992). Like the Gadd proteins the HSV-1 protein is highly charged. However, unlike Gadd proteins, ICP34.5 is positively, rather than negatively charged. In neuronal cells, this gene overcomes a block in protein synthesis for HSV-1(+17), a variant mutated in this gene. The protein synthesis block in HSV-1(+17)-infected neuronal cells is similar to that observed in AcMNPV-infected Ld652Y cells. How ICP34.5 acts to overcome this is unknown.

Possible explanations for the shutoff of protein synthesis in AcMNPV-infected Ld652Y cells include both viral and cellular mediated processes. Baculoviruses might have a mechanism for shutting down host protein synthesis and selectively maintaining virus protein synthesis. AcMNPV may simply lack a factor, HRF-1, needed to maintain virus protein synthesis in Ld652Y cells. As a consequence all protein synthesis is shut down. Another possibility is that the shut off of protein synthesis in AcMNPV-infected Ld652Y cells may be a cellular defense mechanism against virus infection. Thus the role of HRF-1 may be to circumvent the host's defenses to virus infection and permit a productive virus replication cycle. In baculoviruses there are two different genes, *p35* and *iap*, that are capable of overcoming apoptosis, another cellular defense mechanism (Clem et al., 1991; Crook et al., 1993). The response of Ld652Y cells to AcMNPV infection may represent an additional host defense system against viral infection, the shut off of

protein synthesis. All other baculovirus genes that have been implicated in host range determination, *p35* (Clem et al., 1991; Clem and Miller, 1993), the *p143* helicase (Croizier et al., 1994; Maeda et al., 1993), and *hcf-1* (Lu and Miller, 1995), have roles in DNA replication (Lu and Miller, 1995; Todd et al., 1995). Thus the role of *hrf-1* in host range determination appears to be different from these other genes.

Acknowledgments

We thank Jim M. Slavicek for providing LdMNPV A21-MPV virus and the LdMNPV cosmid library, Dwight Lynn for the IPLB-Ld652Y cell line, Loy Volkman and George Rohrmann for antibodies, and Gary Blissard for OpMNPV. We are particularly grateful to Steven C. Cassar for excellent technical assistance. We also thank Jim M. Slavicek, Steven C. Cassar, and Chi-Ju Chen for critical reading of the manuscript.

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CHAPTER 3

Characterization of Host Range Factor 1 (*hrf-1*) Expression in *Lymantria dispar* Nucleopolyhedrovirus- and Recombinant *Autographa californica* Nucleopolyhedrovirus-Infected IPLB-Ld652Y Cells

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Abstract

We previously identified a gene, host range factor 1 (*hrf-1*), in *Lymantria dispar* nucleopolyhedrovirus (LdMNPV) which promoted *Autographa californica* nucleopolyhedrovirus (AcMNPV) replication in a nonpermissive cell line IPLB-Ld652Y (Ld652Y). A recombinant AcMNPV, vAcLdPS, bearing *hrf-1* controlled by two synthetic baculovirus late promoters was constructed that replicated in Ld652Y cells. In this study, we constructed a new recombinant AcMNPV, vAcLdPD, bearing only *hrf-1* controlled by its own promoter. vAcLdPD replicated in Ld652Y cells in the same manner as vAcLdPS confirming that *hrf-1* alone was sufficient to promote AcMNPV replication in Ld652Y cells. *hrf-1* was transcribed as a delayed early gene in LdMNPV but as an immediate early gene in both recombinant AcMNPVs. Primer extension analysis showed that the initiator sequence TCAGT was used as transcription start site in both LdMNPV and recombinant AcMNPVs. Additional sequencing revealed several regulatory motifs in *hrf-1* upstream region. *hrf-1* transcripts in LdMNPV- and vAcLdPS-infected Ld652Y cells terminated near the polyadenylation signal at the end of *hrf-1* ORF while in vAcLdPD, terminated at a downstream polyadenylation signal at the end of ORF 603. Using Western blot analysis, we detected HRF-1 expression in both recombinant AcMNPV-infected but not in LdMNPV-infected Ld652Y cells.

Introduction

Nucleopolyhedroviruses (NPVs) belong to the family *Baculoviridae*. They are large DNA-containing viruses which infect insects. During their infection cycles, viral genes are activated in a cascade fashion and are expressed in three temporal phases: early, late, and very late (Friesen and Miller, 1986). Early gene promoters resemble those

of the host and many contain the transcription initiator sequence TCAGT (Blissard et al., 1992; Cherbas and Cherbas, 1993; Dickson and Friesen, 1991; Guarino and Smith, 1992). Transcription of early genes is achieved by cellular RNA polymerase II (Hoopes and Rohrmann, 1991; Huh and Weaver, 1990) before the onset of viral DNA replication. Activation of late and very late genes requires early gene products and depends on the onset of viral DNA replication (Erlandson et al., 1985; Rice and Miller, 1986). Late and very late genes are transcribed by a viral-induced α-amanitin-insensitive RNA polymerase (Beniya et al., 1996; Fuchs et al., 1983; Huh and Weaver, 1990). These transcripts initiate from a characteristic TAAG motif (Howard et al., 1986; Rohrmann, 1986; Thiem and Miller, 1989b; Wilson et al., 1987). Multiple overlapping transcripts with coterminal 5'- or 3'-ends are a common feature in baculovirus gene transcription (Friesen and Miller, 1986). Bicistronic and multicistronic transcripts are also observed (Oellig et al., 1987; Passarelli and Miller, 1994; Thiem and Miller, 1989a).

Autographa californica NPV (AcMNPV), is widely used as a eukaryotic expression vector and serves as a model for studying molecular biology of baculoviruses. AcMNPV was originally isolated from the alfalfa looper and is routinely propagated in IPLB-SF-21 cells (SF-21) (Vaughn et al., 1977). *Lymantria dispar* NPV (LdMNPV) is currently used as a control agent for gypsy moth, a serious pest of forest and shade trees in Northeastern United States. LdMNPV is routinely propagated in the gypsy moth cell line IPLB-Ld652Y (Ld652Y) (Goodwin et al., 1978).

AcMNPV is not able to replicate in the gypsy moth cell line Ld652Y (McClintock et al., 1986). Virus can enter the cell and viral DNA is synthesized (Guzo et al., 1992;

McClintock *et al.*, 1986; Morris and Miller, 1992). Viral and host cellular RNAs are transcribed, transported, and of normal size (Guzo et al., 1992; Morris and Miller, 1993). However, protein synthesis is shut off by late times post infection (p.i.) and no viral progeny is produced (Du and Thiem, submitted; Guzo et al., 1992; McClintock et al., 1986). In previous studies, we identified and sequenced a single gene, host range factor 1 (*hrf-1*) in LdMNPV, which was able to promote AcMNPV replication in Ld652Y cells and ensure the production of AcMNPV progeny in cotransfection assays (Thiem et al., 1996). A recombinant AcMNPV, vAcLdPS, bearing *hrf-1*, controlled by two synthetic late promoters, and a second LdMNPV gene, *fus* (Cassar and Thiem, submitted), was constructed that replicated in Ld652Y cells (Thiem et al., 1996). In this study, we constructed a new recombinant AcMNPV, vAcLdPD, bearing only *hrf-1* controlled by its own promoter. We compared virus production in Ld652Y cells infected with these two recombinant AcMNPVs and characterized *hrf-1* expression in LdMNPV- and in recombinant AcMNPV-infected Ld652Y cells.

Materials and Methods

Cells and Viruses

SF-21 cells (Vaughn *et al.*, 1977) and Ld652Y cells (Goodwin et al., 1978) were maintained in TC100 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 0.26% tryptose broth. LdMNPV isolate A21-2 (Bischoff and Slavicek, 1996) was propagated in Ld652Y cells. AcMNPV variant L1 (Lee and Miller, 1978) and recombinant AcMNPVs were propagated in SF-21 cells. Construction of vAcLdPS, the recombinant AcMNPV, was described previously (Thiem et al., 1996). To generate vAcLdPD (Figure 6C), the *Pst*I-*Dra*I fragment which contained only *hrf-1* was

cloned in a transfer vector pAcUW2B (Weyer et al., 1990) to generate the transfer plasmid pAcUWLdPD. This transfer plasmid and DNA from a modified AcMNPV, vSynVI$^-$gal (Wang et al., 1991), in which the polyhedrin (*polh*) gene was replaced by *LacZ*, were cotransfected into SF-21 cells using lipofectin (Flegner et al., 1987; O'Reilly et al., 1992). White occlusion positive viruses were selected and plaque purified. Correct insertion of the LdMNPV fragment was confirmed by analysis of DNA restriction patterns of the recombinant virus. This recombinant AcMNPV was named vAcLdPD.

Comparison of Virus Production

Ld652Y cells were infected with wt AcMNPV, vAcLdPS, or vAcLdPD at a multiplicity of infection (MOI) of 10 plaque formation units (PFU) per cell. After 1 h adsorption, the inoculum was removed and cells were washed twice with incomplete medium and refed with complete medium. Time zero was defined as the time when the inoculum was removed and incubation at 27°C was initiated. To compare occluded virus production, infected cells were collected at 24 and 48 h p.i. Numbers of cells containing polyhedrin inclusion bodies (PIB) were counted using hemacytometer in a total of about 1000 cells and the percentages of cells containing PIB versus total cells were calculated. To compare budded virus production, supernatant of infected cells was collected at 0, 12, 24, and 48 h p.i. and budded virus titers were quantitated as pfu/ml by plaque assays on both Ld652Y and SF-21 cells.

RNA Isolation and PolyA-RNA Selection

Ld652Y cells were infected with LdMNPV at an MOI of 8, vAcLdPS or vAcLdPD at an MOI of 10 and harvested at 0, 6, 12, 18, 24, and 48 h p.i. Mock-infected cells were inoculated with incomplete medium and harvested at 48 h p.i. To block

protein synthesis, cycloheximide was used at 100 μg/ml throughout the time course beginning with a 30 minute pretreatment prior to the addition of inoculum. To block DNA replication, aphidicolin was added at 5 μg/ml following the adsorption period. Both cycloheximide and aphidicolin treated infections were harvested at 12 h p.i. (vAcLdPS and vAcLdPD) or 18 h p.i. (LdMNPV).

Total cellular RNAs were isolated by a single step method (Chomczynski and Sacchi, 1987). Oligo(dT) attached to metal beads (PerSeptive Diagnostics, Inc., Cambridge, MA) was employed to isolate polyadenylated RNA.

Northern Blot Analysis

Seven μg polyA-RNA from LdMNPV-infected or 4 μg polyA-RNA from recombinant AcMNPV-infected Ld652Y cells were used for Northern blot analysis. PolyA-RNA from each time point was separated by electrophoresis on a 1.2% formaldehyde agarose gel and transferred to nylon membranes. The *Pst*I-*Dra*I fragment (Figure 6A) cloned in a Bluescript plasmid (Stratagene, La Jolla, CA) was used as a template to synthesize a strand-specific ribo-probe using α-[^{32}P] CTP (3000Ci/mMol, Dupont NEN, Wilmington, DE) and an *in vitro* transcription system (Promega, Madison, WS). Northern hybridization was performed at 55°C in 50% formamide. An RNA size ladder (Life Technologies, Bethesda, MD) was co-electrophoresed and stained separately as a size marker.

Primer Extension

Total cellular RNAs from mock-, LdMNPV-, vAcLdPS-, and vAcLdPD-infected cells were used for primer extension. A primer, TGCACTTGTAGCTGTCCA,

complementary to the mRNA strand approximately 180 bps downstream from the *Pst*I site (Figure 6A and Figure 11) was synthesized (Michigan State University Macromolecular Structure, Sequencing, and Synthesis Facility, East Lansing, MI), 5'-end labeled using γ-[^{32}P]ATP (3000Ci/mMol, NEN Dupont, Wilmington, DE), and used to prime cDNA synthesis. The primer was annealed to 25 μg total cellular RNA at 30°C overnight and extended with M-MLV reverse transcriptase (Life Technologies, Bethesda, MD). A sequencing ladder was generated using the same labeled primer in a dideoxyribonucleotide-chain termination sequencing reaction (CircumVent, New England Biolabs Inc., Beverly, MA) using cloned LdMNPV *Eco*RI-*Sst*I DNA fragment spanning this region (42.6-44.6 m.u.) (LdMNPV), the transfer plasmid PsynLdPS (Thiem et al., 1996) (vAcLdPS), or pAcUWLdPD as template. PUC18 DNA was cut with *Msp*I and 5'-end labeled as a size marker. Primer extension products and the sequencing ladder were analyzed on 8% polyacrylamide-7M urea sequencing gels.

Nuclease Protection Assay

A 3'-end labeled probe was annealed to polyA-RNAs from mock-, LdMNPV-, and vAcLdPS-infected Ld652Y cells. To generate the probe, a 1.3 kb *Sal*I-*Nar*I fragment (43.4-44.1 m.u.) (Figure 6A) spanning the putative 3'-end region was selectively labeled at the *Sal*I site by filling in the 5' overhanging end of the restriction digested DNA using T4 DNA polymerase and both α-[^{32}P]-dATP and α-[^{32}P]-dTTP (3000 Ci/mMol, Dupont NEN, Wilmington, DE). Two μg polyA-RNA was hybridized to the probe at 59°C overnight followed by mung bean nuclease digestion (1000u/ml, LifeTechnologies, Bethesda, MD) at 37°C for 1 h. Nuclease-resistant fragments were analyzed on an 8%

polyacrylamide-7M urea sequencing gel.

RT-PCR

Twenty μg of total RNA isolated from mock-, vAcLdPS-, and vAcLdPD-infected Ld652Y cells was used to synthesize cDNA with oligo(dT) primer (Promega, Madison, WI) and M-MLV reverse transcriptase (Life Technologies, Bethesda, MD). These cDNA products and DNA from the transfer plasmid, pAcUWLdPD, was used for PCR. PCR was performed 25 cycles (94°C 1 min, 55°C 50 sec, and 72°C 3 min) using two synthetic primers (Michigan State University Macromolecular Structure, Sequencing, and Synthesis Facility, East Lansing, MI), CAGCGAGACGAATATCGA which was 43 nucleotides downstream of the *hrf-1* transcription start site TCAGT and CGATACAAACCAAACGCA which was 2 nucleotides upstream of the polyadenylation signal at the end of ORF 603 (Figure 6C). PCR product was resolved in 1% agarose gel together with DNA size marker and stained with ethidium bromide.

Sequencing

hrf-1 upstream sequence was obtained using synthetic primers, AATTGCAAGTACAGTTAC for the antisense strand and ATTCAACACATTCGACCC for the coding strand (Michigan State University macromolecular structure, sequencing and synthesis facility), and a cloned LdMNPV *Eco*RI-*Sst*I DNA fragment (42.6-44.6 m.u.) as template in a dideoxynucleotide chain termination reaction (Sanger *et al.*, 1977) by employing a Circumvent Sequencing Kit (New England Biolabs, Beverly, MA).

Antibody Preparation

The *Sal*I- *Dra*I fragment (43.4-43.8 m.u.) in *hrf-1* C-terminus (Figure 6A) was ligated to the 3'-end of maltose binding protein (MBP) DNA sequence in a bacterial protein expression plasmid pMAL-c2 (New England Biolabs). The resulting fusion protein was expressed in *E. coli*. This fusion protein contained 40 kDa MBP and 20.6 kDa truncated HRF-1 (*Sal*I-*Dra*I). The highly expressed fusion protein was isolated through amylose column and further purified by eluting from protein gel. 60 μg (0.5 ml) purified fusion protein was emulsified with 0.5 ml Titermax (Vaxcel Inc., Norcross, GA) and used to immunize rabbits by subcutaneous injections. Antiserum was collected every 2 weeks beginning the 4th week after immunization.

Western Blot Analysis

Ld652Y cells were infected with LdMNPV, vAcLdPS, or vAcLdPD at an MOI of 10 PFU as described previously. SF-21 cells were infected with AcMNPV at an MOI of 10 PFU and harvested at 24 hr p.i. Cells were lysed in 2 X disruption buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.002% bromophenol blue). Proteins were run on 10% SDS polyacrylamide gels (Laemmli, 1970). An amount of protein equivalent to approximately 1 X 10^6 cells was loaded per lane. The HRF-1 fusion protein (60.6 kDa) and the truncated HRF-1 (20.6 kDa) cleaved from the fusion protein by Xa factor (New England Biolabs) were used as HRF-1 controls. Following electrophoresis, a parallel gel of vAcLdPS infections was stained with Coomassie blue. For blotting, gels were electrophoretically transferred to Hybond-ECL nitrocellulose membranes (Amersham Life Science, Arlington Heights., IL) in a

transfer buffer containing 20 mM Tris-Cl, 150 mM Glycine (pH 8.3), and 20% methanol and incubated with antibody by standard method (Towbin et al., 1979). Polyclonal antibodies against HRF-1 fusion protein were used at the dilution of 1:100. Secondary antibody, anti-rabbit peroxidase conjugate (Sigma, St. Louis, MO), was used at 1: 100,000. The ECL Western blot detection system (Amersham Life Science, Arlington Heights, IL) was employed for signal detection.

Nucleotide Sequence Accession Number

The GenBank accession number for the nucleotide sequence presented in this report is U38895.

Results

Construction of vAcLdPD

hrf-1 was identified by its ability to promote AcMNPV replication in Ld652Y cells in cotransfection assays (Thiem et al., 1996). A recombinant AcMNPV, vAcLdPS, bearing *hrf-1* was able to replicate in infected Ld652Y cells. vAcLdPS contained an additional LdMNPV gene, *fus* (Cassar and Thiem, submitted), and *hrf-1* expression was regulated by two synthetic late promoters in addition to its own promoter. To rule out the possibility that either *fus* or the synthetic promoters were required for AcMNPV replication in Ld652Y cells, another recombinant AcMNPV, vAcLdPD, was constructed (Figure 6C). vAcLdPD contained only the *hrf-1* gene controlled by its own promoter. Virus production of vAcLdPS and vAcLdPD on Ld652Y cells was compared. For occluded virus production, percentage of cells containing PIB versus total cells was determined at 24 and 48 h p.i. In both recombinant virus-infected Ld652Y cells, 82% of the cells produced PIB by 24 h p.i. and the percentage of cells containing PIB reached

Figure 6. Schematic Diagram of *hrf-1* Context in LdMNPV, vAcLdPS, and vAcLdPD A: LdMNPV; B: vAcLdPS; C: vAcLdPD. For ease of comparison, the conventional orientation of AcMNPV is reversed for recombinant AcMNPVs to show *hrf-1* oriented as in LdMNPV. The location of the synthetic promoters, Psyn and PXIV, from the transfer vector pSynXIV VI+ (Wang *et al.*, 1991), is indicated by hatched lines in vAcLdPS (B). Selected restriction sites are indicated. ORFs are represented by open arrows. Spacing between ORFs and restriction sites is given in bps. The positions of primers and probes are indicated by lines beneath the schematic for LdMNPV (A) or vAcLdPD (C). The restriction fragment used to generate the HRF-1 fusion protein is shaded in the schematic of LdMNPV (A). Labeled ends are indicated by " * ".

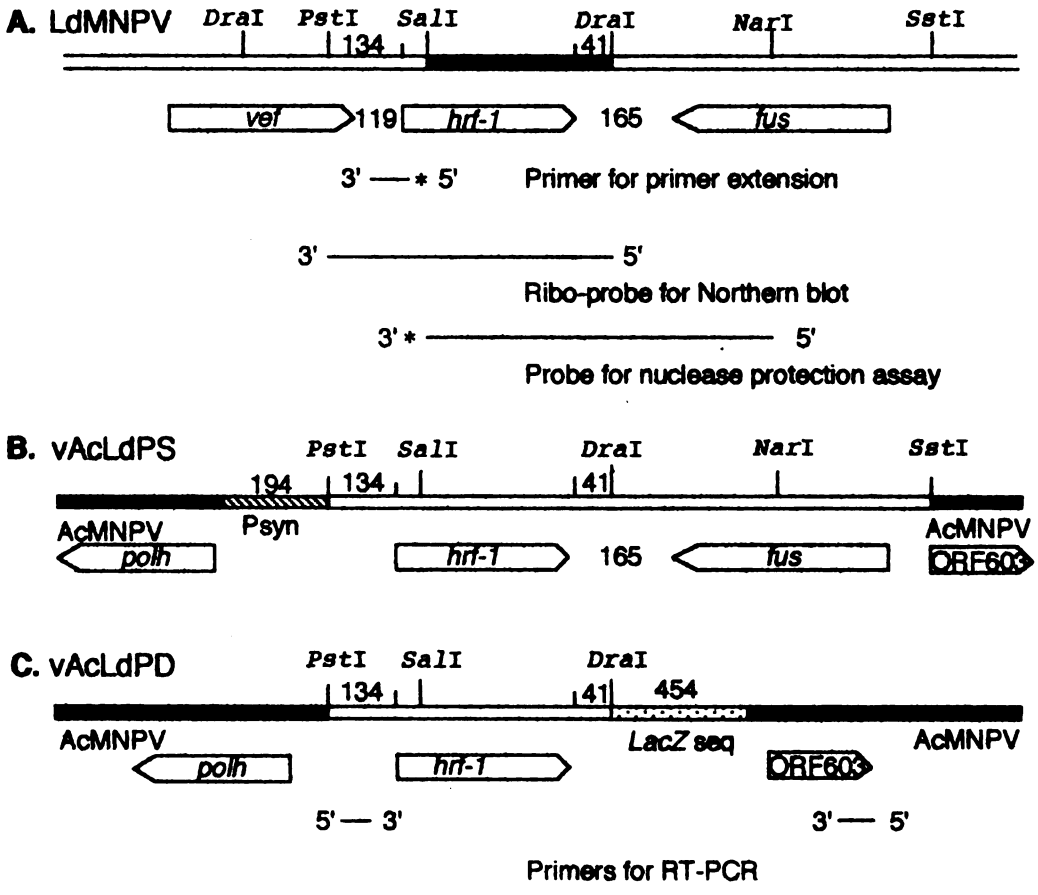

Figure 6. Schematic Diagram of *hrf-1* Context in LdMNPV, vAcLdPS, and vAcLdPD

100% by 48 h p.i. (Table 2). No cells containing PIB in wt AcMNPV-infected Ld652Y cells were observed (Table 2). Budded virus titers in supernatant at different times p.i. were quantitated by plaque assays on both SF-21 and Ld652Y cells (Table 3). In both recombinant virus-infected Ld652Y cells, virus titers remained at about 4-6 X 10^3 pfu/ml due to residual virus inoculum at 0 and 12 h p.i. when titered on either SF-21 or Ld652Y cells. Virus titers of vAcLdPS-infected Ld652Y cells reached 6 X 10^7 pfu/ml at 24 h p.i. and remained the same at 48 h p.i. when titered on SF-21 cells. When titered on Ld652Y cells, titers of vAcLdPS-infected cells reached 5 X 10^7 pfu/ml at 24 h p.i. and increased to 6 X 10^7 pfu/ml at 48 h p.i. Titers of vAcLdPD-infected cells reached 6 X 10^7 at 24 h p.i. and increased to 7 X 10^7 pfu/ml when titered on SF-21 cells and 8 X 10^7 pfu/ml when titered on Ld652Y cells. In contrast, virus titers in wt AcMNPV-infected Ld652Y cells remained at 5-6 X 10^3 pfu/ml throughout the time course when assayed on SF-21 cells and at zero when assayed on Ld652Y cells. These data demonstrated that *hrf-1* alone was sufficient to promote AcMNPV replication in the nonpermissive cell line Ld652Y. The kinetics of recombinant virus replication in Ld652Y cells more closely reflected that of AcMNPV replication on permissive cell lines than LdMNPV replication in Ld652Y cells in which virus progeny is not produced until 48 h p.i. (J. Slavicek, personal communication; McClintock et al., 1987).

The viral context of *hrf-1* in LdMNPV is different from that of recombinant AcMNPVs (Figure 6). In LdMNPV (Figure 6A), *hrf-1* is located between 43.3 and 43.8 m.u., immediately downstream of the virus enhancing factor (*vef*) (J. Slavicek, personal communication) and oriented in the same direction. There were 119 bps between the stop

Table 2. Occluded Virus Production in Ld652Y Cells

	24 h p.i.			48 h p.i.		
	Total cells	Cells with PIB	Percentage	Total cells	Cells with PIB	Percentage
vAcLdPS	1012	826	82%	1045	1045	100%
vAcLdPD	1077	879	82%	1056	1056	100%
AcMNPV	1097	0	0	1074	0	0

Table 3. Budded Virus Production in Ld652Y Cells Assayed on Both Ld652Y and SF-21 Cells

	SF-21				Ld652Y			
	0[a]	12	24	48	0	12	24	48
vAcLdPS	$4X10^{3}$[b]	$6X10^{3}$	$6X10^{7}$	$6X10^{7}$	$4X10^{3}$	$5X10^{3}$	$5X10^{7}$	$6X10^{7}$
vAcLdPD	$6X10^{3}$	$6X10^{3}$	$6X10^{7}$	$7X10^{7}$	$5X10^{3}$	$6X10^{3}$	$6X10^{7}$	$8X10^{7}$
AcMNPV	$6X10^{3}$	$5X10^{3}$	$6X10^{3}$	$6X10^{3}$	0	0	0	0

a H p.i. when supernatants were collected for plaque assays
b Titers in pfu/ml

codon, TAA, of *vef* and the start codon of *hrf-1*. *fus* is located immediately downstream from *hrf-1*, oriented in the opposite direction. The *hrf-1* and *fus* stop codons are separated by 165 bps. In vAcLdPS (Thiem et al., 1996) (Figure 6B), the *Pst*I-*Sst*I fragment containing both *hrf-1* and *fus* was inserted upstream of AcMNPV *polh* at 3.4 m.u. together with two synthetic late promoter sequences, Psyn and PXIV, derived from the transfer vector pSynXIV VI^{+} (Wang et al., 1991). In vAcLdPD (Figure 6C), the *Pst*I-*Dra*I fragment containing the *hrf-1* ORF, 5' (134 bps) and 3' (41 bps) flanking regions and a run of 454 bps of *LacZ* 3'-end sequence derived from the transfer vector, pAcUW2B (Weyer et al., 1990) was inserted upstream of AcMNPV *polh* at 3.4 m.u. This fragment includes the TCAGT initiator motif. Thus, the *hrf-1* locus is identical in both recombinant AcMNPVs. Both vAcLdPS and vAcLdPD have the same *hrf-1* upstream sequences. However, in vAcLdPS, *hrf-1* transcription is also controlled by the two synthetic baculovirus late promoters. The recombinants differ in their sequences at the 3'-end of the *hrf-1* ORF. The sequence in vAcLdPS includes *fus* and thus is identical to LdMNPV at the 3'-end of *hrf-1*. In vAcLdPD, the LdMNPV sequence terminates 4 nucleotides downstream of the *hrf-1* polyadenylation signal.

Transcriptional Analysis

hrf-1 transcription patterns in LdMNPV- and recombinant AcMNPV-infected Ld652Y cells were analyzed by Northern blot analysis (Figure 7) using a strand-specific, ^{32}P-labeled, RNA probe (Figure 6A). In LdMNPV infections, a transcript of approximately 1 kb consistent with the estimated size of *hrf-1*, based on sequence data, was observed at 12, 18, and 24 h p.i. and in aphidicolin-treated cells harvested at 18 hr

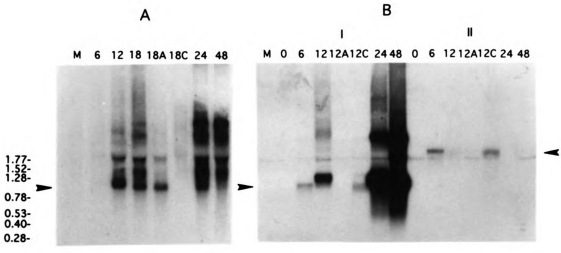

Figure 7. Northern Blot Analysis of *hrf-1* Transcription in LdMNPV- and Recombinant AcMNPV-Infected Ld652Y Cells A: mRNA isolated from LdMNPV-infected Ld652Y cells; B: mRNA from recombinant AcMNPV-infected Ld652Y cells. I = vAcLdPS infections and II = vAcLdPD infections. Numbers and letters at the top of the lanes indicate mRNA isolated from mock-infected (M); LdMNPV-, vAcLdPS, or vAcLdPD-infected Ld652Y cells harvested at 0, 6, 12, 18, 24, and 48 h p.i.; and from aphidicolin (A) or cycloheximide (C) treated virus-infected Ld652Y cells harvested at 12 or 18 h p.i. *hrf-1* transcripts are indicated by arrowheads. The positions of RNA size standards are indicated in kb to the left of the panels.

p.i. (Figure 7A, arrowhead). No transcript was observed at 6 h p.i. or in cycloheximide treated cells harvested at 18 h p.i. The absence of *hrf-1* transcripts in the cycloheximide treated cells indicated that *de novo* protein synthesis was required. Therefore *hrf-1* was classified as a delayed early gene in LdMNPV-infected Ld652Y cells. Larger transcripts of 1.4 and 1.8 kb were observed at 12, 18, 24, and 48 h p.i. The 1.8 kb transcript was also observed as a faint band in aphidicolin-treated cells harvested at 18 h p.i. Two larger transcripts were also prominent at 24 and 48 h p.i. In vAcLdPS, a transcript, approximately 1 kb, was first detected at 6 h p.i. and also observed in cycloheximide treated cells at 12 h p.i. (Figure 7B, I, arrowhead). This transcript diminished after 6 h p.i. A 1.2 kb transcript appeared at 12 h p.i. and was abundant at 24 and 48 h p.i., consistent with initiation from the strong synthetic late promoters. In vAcLdPD, transcript of about 2.0 kb was detected at 6 h p.i. and in cycloheximide treated cells harvested at 12 h p.i. (Figure 7B, II, arrowhead). This transcript diminished after 6 h p.i. Thus in both vAcLdPS- and vAcLdPD-infected Ld652Y cells, *hrf-1* was transcribed as an immediate early gene with no requirement for *de novo* protein synthesis. A faint 1.8 kb band detected throughout the time course was apparently non-specific as it was also observed in mock-infected cells.

Primer extension analysis was employed to map the transcription start sites (Figure 8). A primer complementary to the *hrf-1* coding sequence (Figure 6A; Figure 11) was synthesized and ^{32}P-end-labeled for primer extension experiments. The labeled primer was annealed to RNA isolated from LdMNPV-, vAcLdPS-, and vAcLdPD-infected Ld652Y cells and extended using reverse transcriptase. The extension products

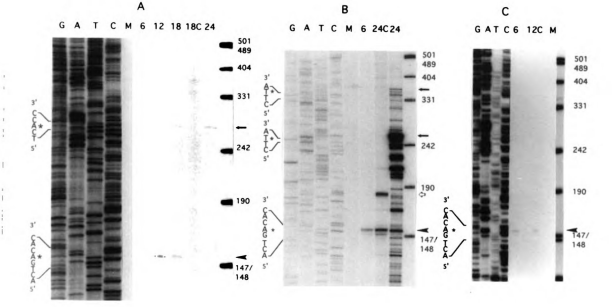

Figure 8. Primer Extension Analysis of LdMNPV-, vAcLdPS-, and vAcLdPD-Infected Ld652Y Cells A: LdMNPV-infected Ld652Y cells; B: vAcLdPS- infected Ld652Y cells; C: vAcLdPD-infected Ld652Y cells. Numbers and letters at the top of the lanes indicate total RNA isolated from mock-infected Ld652Y cells (M), virus-infected Ld652Y cells treated with cycloheximide (18C or 24C), and virus-infected Ld652Y cells at 6, 12, 18, or 24 h p.i. Lanes G, A, T, and C are sequencing reactions using the labeled primer and a cloned LdMNPV DNA template (A), a transfer plasmid pSynLdPS (Thiem *et al.*, 1996) DNA template (B), or a transfer plasmid pAcUWLdPD DNA template (C). The sequence at the start sites is indicated to the left of each panel. Marker sizes are shown to the right of each panel in bp.

were analyzed on a denaturing sequencing gel along with molecular size markers. In LdMNPV infections, two extension products were observed (Figure 8A). The stronger signal was from a 153 nucleotide- band observed at 12 and 18 h p.i. that mapped to the first thymidine in the TCAGT early initiator sequence (Figure 8A, arrowhead). A second, weaker band of 265 nucleotides mapping to thymidine in GGTGA was observed at 18 and 24 h p.i. (Figure 8A, arrow). In vAcLdPS infections, primer extension products mapping to the initiation sequence TCAGT were observed at 6 h p.i. and in cycloheximide treated cells harvested at 24 h p.i. (Figure 8B, arrowhead). A prominent primer extension product mapping to the proximal synthetic promoter, TAAG, was detected at late time (24 h p.i.) (Figure 8B, arrow). For the distal TAAG, only a minor extension product was seen (Figure 8B, arrow). A strong band at 24C (cycloheximide treated) which mapped to the *Pst*I junction used in constructing vAcLdPS was also observed (Figure 8B, open arrow). Additional bands observed at 24 h p.i. might be due to premature termination of cDNA synthesis because of the high GC content of *hrf-1* (69%) (Thiem et al., 1996) and thus possible formation of mRNA secondary structure. In vAcLdPD infections, primer extension product at 6 h p.i. mapped to the TCAGT motif (Figure 8C, arrowhead).

Nuclease protection assays were employed to map the 3'-ends of *hrf-1* transcripts in LdMNPV- and vAcLdPS-infected Ld652Y cells (Figure 9). The probe was a 1.3 kb nucleotide *Sal*I-*Nar*I fragment (43.4-44.2 m.u.), 3'-end-labeled at the *Sal*I site (Figure 6A). The probe was annealed to polyA-RNA isolated from mock-, LdMNPV-, and vAcLdPS-infected Ld652Y cells, digested with mung bean nuclease and analyzed on a sequencing gel. A 620 bp nucleotide major protected fragment was observed in both

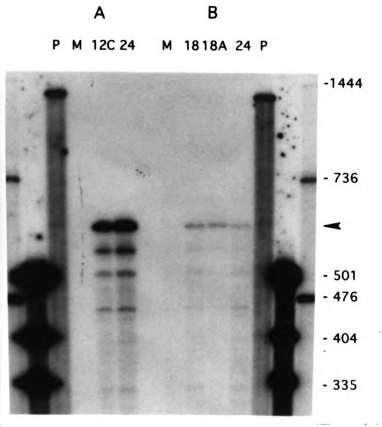

Figure 9. Nuclease Protection Mapping of 3'-Ends of *hrf-1* Transcripts in LdMNPV- and vAcLdPS-Infected Ld652Y Cells A: vAcLdPS-infected Ld652Y cells; B: LdMNPV-infected Ld652Y cells. Numbers and letters at the top of the lanes indicate polyA-RNA isolated from mock-infected cells (M), virus-infected cells at 18 or 24 h p.i., virus-infected cells treated with cycloheximide and harvested at 12 h p.i. (12C), and virus-infected cells treated with aphidicolin and harvested at 18 hr p.i. (18A). P is the probe by itself. Size markers are labeled to the right of the panel in bp. The arrowhead indicates the location of the predominant protected fragment of 620 bps.

LdMNPV- and vAcLdPS-infected cells (Figure 9, arrowhead). This corresponded to the polyadenylation signal identified 34 bps downstream of the *hrf-1* ORF (Thiem et al., 1996).

Since *hrf-1* transcription in vAcLdPD-infected Ld652Y cells also initiated at the TCAGT motif as mapped by primer extension (Figure 8C, arrowhead), it was hypothesized that the larger size of about 2 kb, as detected by Northern blot analysis (Figure 7B, II, arrowhead), was due to transcription through the normal termination site as a result of the truncation of the 3'-end of *hrf-1*. The predicted termination site was near the polyadenylation signal at the end of ORF 603 located downstream of *hrf-1* (Figure 6C). To test this hypothesis, A primer near the *hrf-1* transcription start site TCAGT and a primer adjacent to the ORF 603 polyadenylation signal (Figure 6C) were used for RT-PCR analysis using total RNA isolated from vAcLdPD-infected Ld652Y cells. RNA from mock-, or vAcLdPS-infected Ld652Y cells and DNA from the transfer plasmid, pAcUWLdPD, used to generate vAcLdPD were used as controls. A 1.8 kb PCR product was obtained in the reaction that contained cDNA generated from total RNA of vAcLdPD-infected cells at 6 h p.i. (Figure 10, lane 4) and in the reaction which contained DNA from the transfer plasmid pAcUWLdPD (Figure 10, lane 5) but not in the reactions that contained cDNA generated from either mock-infected cells or vAcLdPS-infected cells (Figure 10, lane 1-3). These data are consistent with the 2 kb transcript detected by Northern analysis (Figure 7B, II, arrowhead) when polyadenylation is accounted for and support the hypothesis that the larger *hrf-1* transcripts observed in vAcLdPD-infected cells are the result of transcriptional read-through.

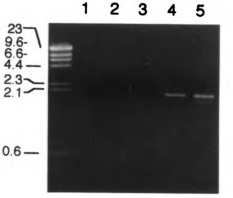

Figure 10. RT-PCR Mapping of 3'-End of *hrf-1* Transcripts in vAcLdPD-Infected Ld652Y Cells Numbers on the top represent cDNA generated from total RNA isolated from mock-infected cells (1), vAcLdPS-infected cells at 6 (2) and 24 (3) h p.i., vAcLdPD-infected cells at 6 h p.i. (4), and DNA from the transfer plasmid pAcUWLdPD (5). DNA size markers are indicated to the left of the panel in kb.

hrf-1 Upstream Sequence

Because the second *hrf-1* primer extension product in LdMNPV-infected cells was mapped beyond the previously sequenced region, additional *hrf-1* upstream sequence was obtained (Figure 11). The second primer extension product was mapped to 211 bps upstream of the *hrf-1* translation start codon and did not correspond to any previously identified initiation sequences. However, two copies of a putative baculovirus early gene regulatory element a/ctcGTGTnc/t (Tomalski et al., 1988) were found in this region. One was located 209 bps the other 130 bps upstream of the *hrf-1* translation initiation codon (Figure 11, double underlined sequence). The distal copy also matched another baculovirus early regulatory motif CGT with the consensus AA/TCGTG/T (Dickson and Friesen, 1991; Nissen and Friesen, 1989) (Figure 11, asterisks). A transcription activation factor NF-1 binding motif, TGGCGG (Caruso et al., 1990), and a transforming growth factor ß1 (TFG-ß1) inhibitory element (TIE) GNNTTGGTGA (Kerr et al., 1990) motif were found 250 bps and 218 bps upstream of the *hrf-1* coding sequence respectively (Figure 11).

HRF-1 Expression

To investigate HRF-1 expression in LdMNPV- and in recombinant AcMNPV-infected Ld652Y cells, a HRF-1-MBP fusion protein was synthesized by cloning the *SalI-DraI* fragment (Figure 6A) into a bacterial protein expression plasmid pMAL-c2 (New England Biolabs) and used to raise antibodies. Because the predicted size of HRF-1 (25.7 kDa) was very close to that of polyhedrin (29 kDa), SF-21 cells infected with AcMNPV and harvested at 24 h p.i. were used as an additional control. Proteins from mock-, LdMNPV-, vAcLdPS-, or vAcLdPD-infected Ld652Y cells harvested at different

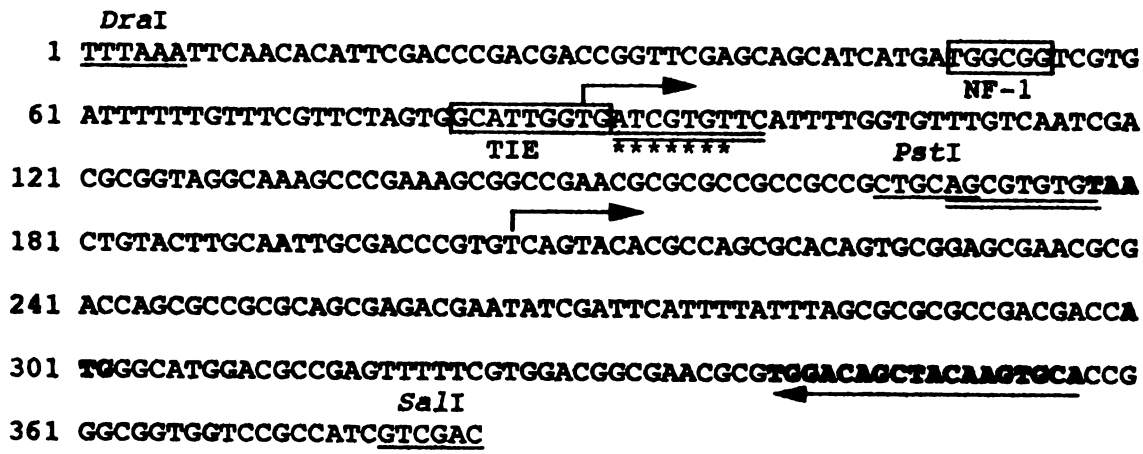

Figure 11. Sequence of *hrf-1* upstream region 240 bps nucleotide sequence upstream and 80 bps nucleotide sequence downstream the *hrf-1* start codon are shown. Selected restriction sites are labeled. The two transcription start sites are indicated by arrows. GTGT motifs are double underlined. The CGT motif is indicated by asterisks underneath. The TIE consensus sequence and the NF-1 binding sequence are boxed. The stop codon of *vef* ORF (TAA) and the start codon of *hrf-1* (ATG) are in bold. The sequence of the primer used for primer extension analysis is in bold and underlined with an arrow indicating direction.

times p.i. and from AcMNPV-infected SF-21 cells were separated by SDS-PAGE (Laemmli, 1978) and stained with Coomassie blue or transferred to membranes (Towbin *et al.*, 1979) and incubated with antibodies against the HRF-1 fusion protein (Figure 12). In vAcLdPS-infected Ld652Y cells, HRF-1 was detected from 6 h p.i. until 48 h p.i. (Figure 12B, arrowhead). These bands appeared in a lower position than the polyhedrin protein band on the Coomassie blue stained gel (Figure 12A, arrow) and no proteins were detected by antisera in the lane containing AcMNPV-infected SF-21 cell lysate (Figure 12B) indicating that these bands represented HRF-1 and not polyhedrin. In vAcLdPD-infected Ld652Y cells, a weak band was detected from 12 h p.i. until late times (Figure 12C, arrowhead). In other experiments, we observed this band as early as 6 h p.i. (Data not shown). This band most likely represented HRF-1 because the size was the same as that of the HRF-1 control and it was not observed in the mock-infected cells. Other bands were apparently nonspecific due to overloading of protein samples since all of them also appeared in mock-infected cells. We were unable to detect HRF-1 in LdMNPV-infected Ld652Y cells (Data not shown).

Discussion

In this study, we demonstrated that LdMNPV *hrf-1* was sufficient to promote AcMNPV replication in Ld652Y cells. Virus production of vAcLdPD-infected Ld652Y cells in which *hrf-1* was controlled by its own promoter was the same as that of vAcLdPS-infected Ld652Y cells in which *hrf-1* was overexpressed and which also carried another LdMNPV gene, *fus*. *hrf-1* expression was differentially regulated in LdMNPV, vAcLdPS, and vAcLdPD. Transcript size and temporal expression of *hrf-1* depended on the viral context of the gene. In LdMNPV- and vAcLdPS-infected Ld652Y

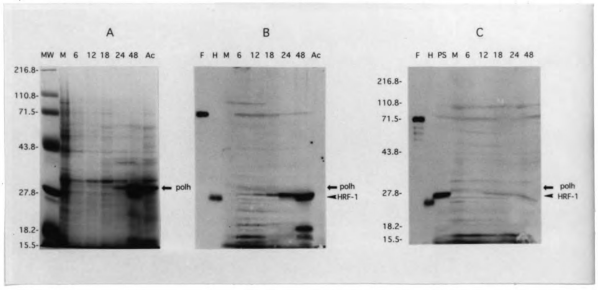

Figure 12. Western Blot Analysis of HRF-1 Expression in Recombinant AcMNPV-Infected Ld652Y Cells A: Coomassie stained parallel gel of vAcLdPS-infected Ld652Y cells; B: Western blots of vAcLdPS-infected Ld652Y cells; C: vAcLdPD-infected Ld652Y cells. Lane labeled M indicates mock-infected Ld652Y cells. Arabic numbers above the lanes indicate the time, in hours, after infection that the cells were harvested. Lane F indicates HRF-1 fusion protein (60.6 kDa). H indicates truncated HRF-1 portion (*SalI-DraI*) (20.6 kDa) cleaved from the fusion protein. Ac represents AcMNPV-infected SF-21 cells harvested at 24 h p.i. PS represents vAcLdPS-infected Ld652Y cells harvested at 24 h p.i. Lane MW is the molecular weight standards with molecular weights in kDa indicated to the left of panels A and C. The position of the polyhedrin protein band is indicated by arrows. HRF-1 bands are indicated by arrowheads.

cells transcripts were approximately 1 kb, as compared to 2 kb in vAcLdPD-infected Ld652Y cells. Abundant 1.2 kb transcripts originated from the synthetic late promoters in vAcLdPS. The 1 kb transcript was consistent with the size of HRF-1 predicted by nucleotide sequence (Thiem et al., 1996) and observed on Western blots (Figure 12). The larger transcripts observed in vAcLdPD-infected cells resulted from transcription through the normal termination site. The 3'-end region truncated in vAcLdPD was important for termination of *hrf-1* transcription. Larger transcripts observed in LdMNPV-infected cells most likely represented transcripts of the gene immediately upstream, *vef*, that extended through *hrf-1*, since no protected 3' fragments larger than those corresponding to the polyadenylation signal following the *hrf-1* ORF were observed (Figure 9). Overlapping transcripts with common 3' ends are often observed in baculovirus infections (Friesen and Miller, 1986). It was also possible that the second primer extension product observed in LdMNPV (Figure 8A, arrow) was of premature termination of cDNA synthesis from *vef* mRNA due to possible mRNA secondary structure.

Transcriptional regulation of *hrf-1* and responses to the inhibitors cycloheximide and aphidicolin differed between LdMNPV-and recombinant AcMNPV-infected Ld652Y cells. *hrf-1* transcription was not observed until 12 h p.i. and was inhibited in the presence of cycloheximide in LdMNPV-infected Ld652Y cells, indicating a dependence on new protein synthesis. In recombinant AcMNPV-infected cells *hrf-1* was transcribed at early times (6 h p.i.) and in the presence of cycloheximide. Since late baculovirus gene expression is contingent on the onset of viral DNA replication, aphidicolin, a DNA synthesis inhibitor, can be used to distinguish early and late genes. When infected cells were treated with aphidicolin, *hrf-1* was not transcribed in recombinant AcMNPV-

infected cells. This inconsistency of responses to aphidicolin was also observed in transcription of other early genes, the *dnapol* gene (Tomalski et al., 1988), *lef-1* (Passarelli and Miller, 1993), and *lef-6* (Passarelli and Miller, 1994). The reason for the inconsistency is not known.

A possible explanation for the difference in temporal regulation of *hrf-1* transcription in recombinant AcMNPV- and LdMNPV-infected Ld652Y cells is the absence of potential regulatory sequences upstream of *hrf-1* in the recombinant AcMNPVs. The delayed expression in LdMNPV-infected cells suggests that these upstream sequences might serve as binding sites for transcriptional repressors in LdMNPV infected cells. Both recombinant AcMNPVs were constructed using the *Pst*I restriction site upstream of the *hrf-1* ORF (Figure 6). Several putative regulatory motifs upstream of the *Pst*I site (Figure 11) could be important for controlling *hrf-1* expression. A GTGT motif, of unknown function, previously identified in the AcMNPV DNA polymerase gene (Tomalski *et al.*, 1988), *ie-1* (Guarino and Summers, 1987), *p35* and *p94* (Friesen and Miller, 1987), and *etl* (Crawford and Miller, 1988), was present in two copies. One of these motifs also matched a CGT motif (Dickson and Friesen, 1991; Nissen and Friesen, 1989) that was required for full *p35* promoter activity during the early phase of infection (Dickson and Friesen, 1991). This motif was also present in other early baculovirus genes including *39K* (Guarino and Summers, 1986), *ie-1* (Guarino and Summers, 1987), and *ets* and *etl* (Crawford and Miller, 1988). Two regulatory sequences previously identified in vertebrate systems were also found in this region. One was a binding site for a nuclear factor belonging to the NF-1 family of transcription factors,

TGGCGG, that was identified in an enhancer of a polyoma virus mutant selected for high efficiency of growth in neuroblastoma cells (Caruso et al., 1990). The other was a transforming growth factor-ß1 (TGF-ß1) inhibitory response element (TIE), GNNTTGGTGA (Kerr et al., 1990), which functions as a transcription silencer for genes regulated by TGF-ß1. Possible roles for these motifs or other upstream sequences in the regulation of *hrf-1* transcription remains to be determined.

Alternatively, the requirement for *de novo* protein synthesis for *hrf-1* transcription in LdMNPV-infected cells may reflect a fundamental difference in the regulation of LdMNPV gene expression as compared to AcMNPV. Additional factors might be required for transcriptional activation of delayed early genes in LdMNPV-infected cells. For example, there are different requirements for new protein synthesis for transcriptional regulation of the ecdysteroid UDP-glucosyltransferase (*egt*) gene in LdMNPV- and AcMNPV-infected cells. Transcription of the LdMNPV *egt* gene in LdMNPV-infected Ld652Y cells required *de novo* protein synthesis (Riegel et al., 1994), but the AcMNPV *egt* gene was expressed immediately after infection without prior protein synthesis in AcMNPV-infected SF-21 cells (O'Reilly and Miller, 1989). Both virus production and temporal patterns of LdMNPV gene expression in Ld652Y cells are delayed relative to AcMNPV gene expression in SF-21 cells (J. Slavicek, personal communication). These differences most likely reflect differences in the virus rather than the host cell, since the temporal appearance of *ie-1*, *ie-n*, *p39* (capsid), and *polh* transcripts are similar in AcMNPV-infected Ld652Y and TN368 cells (Guzo et al., 1992). Furthermore, in Ld652Y cells infected with AcMNPV recombinants bearing *hrf-1* the temporal expression of virus proteins (Thiem et al., 1996) and the appearance of viral

progeny are identical to AcMNPV-infected SF-21 cells.

hrf-1 was expressed at a low level from its natural promoter. In order to detect *hrf-1* transcripts on Northern blots it was necessary to use large quantities of polyA-RNA. HRF-1 could be detected as early as 6 h p.i. in vAcLdPS- or vAcLdPD-infected Ld652Y cells, but only when protein samples equivalent to 1 x 10^6 cells per lane were loaded. In vAcLdPS-infected Ld652Y cells, abundant HRF-1 detected from 12 h p.i. until 48 h p.i. can be attributed to expression from the two synthetic late promoters. However, we were not able to detect HRF-1 expression in LdMNPV-infected Ld652Y cells. The inability to detect HRF-1 in LdMNPV-infected Ld652Y cells may be due to limited sensitivity for low level expression in our assay. It is also possible that HRF-1 was not translated in LdMNPV-infected Ld652Y cells. Work is in progress to disrupt *hrf-1* in LdMNPV and investigate its role in LdMNPV replication on Ld652Y cells.

In AcMNPV-infected Ld652Y cells both viral and host protein synthesis is shut off at late times p.i. (Du and Thiem, submitted; Guzo et al., 1992; McClintock et al., 1986). *hrf*-1 is a novel gene isolated from LdMNPV which replicates in Ld652Y cells that can relieve the block for protein synthesis and ensure production of AcMNPV progeny in Ld652Y cells (Thiem et al., 1996). No motifs have been found in *hrf-1* nucleotide or amino acid sequence that may imply *hrf-1*'s function. Examination of *hrf-1* expression in LdMNPV- and recombinant AcMNPV-infected Ld652Y cells suggested that only low levels of expression are required for its function. Understanding how *hrf-1* functions are important for understanding the host-specificity of baculoviruses. The mechanism responsible for shutting off protein synthesis in AcMNPV-infected Ld652Y

cells and the function of *hrf*-1 in overcoming the protein synthesis shut down in AcMNPV-infected Ld652Y cells are currently being investigated.

Acknowledgments

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CHAPTER 4

Responses of Insect Cells to Baculovirus Infection: Protein Synthesis Shut Down and Apoptosis

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Abstract

Protein synthesis is globally shut down at late times post infection in the baculovirus AcMNPV-infected gypsy moth cell line Ld652Y. A single gene *hrf-1* from another baculovirus LdMNPV is able to preclude the protein synthesis shut down and ensure production of AcMNPV progeny in Ld652Y cells (Thiem, S. M., X. Du, M. M. Quentin, and M. M. Berner, 1996. J. Virol. 70:2221-2229; Du, X., and S. M. Thiem, 1997. Virology 227:420-430). AcMNPV contains a potent apoptotic supressor gene *p35* and protein synthesis shut down was reported in apoptotic insect cells induced by infection with AcMNPV lacking *p35*. In exploring the function of HRF-1 and the possible connection between apoptosis and protein synthesis shut down, a series of recombinant AcMNPVs with different compliments of *p35* and *hrf-1* were constructed and employed in apoptosis and protein synthesis assays. We found that P35 was translated prior to protein synthesis shut down and functioned to prevent apoptosis. HRF-1 prevented protein synthesis shut down even when the cells were undergoing apoptosis but HRF-1 could not functionally substitute for P35. The DNA synthesis inhibitor, aphidicolin, could block both apoptosis and protein synthesis shut down in Ld652Y cells induced by *p35*$^-$ AcMNPVs but not protein synthesis shut down in wt AcMNPV-infected Ld652Y cells. These data suggest that protein synthesis shut down and apoptosis are separate responses of Ld652Y cells to AcMNPV infection and that P35 is involved in inducing a protein synthesis shut down response in Ld652Y cells in the absence of late viral gene expression. A model was developed for these responses of Ld652Y cells to AcMNPV infection.

Introduction

Autographa californica nucleopolyhedrovirus (AcMNPV) is a large DNA containing virus that belongs to the family *Baculoviridae*. It is a model virus for studying the molecular biology of baculoviruses. AcMNPV has a relatively wide host range compared to most other baculoviruses with the ability to replicate in at least 33 species of insect larvae in 10 families (Groner, 1986) as well as more than 25 different insect cell lines (Hink, 1979; Hink and Hall, 1989). However, it is not able to replicate in gypsy moth cell line Ld652Y (Goodwin et al., 1978; McClintock et al., 1986; Morris and Miller, 1992; 1993). When AcMNPV infects Ld652Y cells, virus can enter the cell, both viral and host cellular mRNAs are transcribed and are of normal size (Guzo et al., 1992; Morris and Miller, 1992; 1993) but both viral and host cellular protein synthesis is shut down at late times post infection (p.i.) and no viral progeny are produced (Guzo et al., 1992; McClintock et al., 1986). A single early gene, *hrf-1*, in *Lymantria dispar* nucleopolyhedrovirus (LdMNPV) precludes protein synthesis shutoff and promotes AcMNPV replication in Ld652Y cells (Du and Thiem, 1997; Thiem et al., 1996). *hrf-1* encodes a novel 25.7 kDa protein that has no characteristic motifs that may imply how *hrf-1* assists AcMNPV replication in Ld652Y cells (Thiem et al., 1996).

AcMNPV contains an antiapoptotic gene, *p35* (Clem et al., 1991; Hershberger et al., 1992). The product of the early gene *p35* is required for AcMNPV replication in *Spodoptera frugiperda* cell line SF-21. AcMNPV lacking *p35* induces extensive apoptosis in SF-21 cells and an arrest of protein synthesis was reported in the apoptotic SF-21 cells (Clem and Miller, 1993; Birnbaum et al., 1994). Apoptosis is characterized by plasma membrane blebbing and degradation of chromosomal DNA into

oligonucleosome-sized DNA fragments (Hale et al., 1996). Apoptosis dramatically reduces AcMNPV budded virus production and completely eliminates AcMNPV occluded virus formation in SF-21 cells and thus is considered to be an effective host defense response against viral infection (Clem and Miller, 1993. Hershberger et al., 1992). P35 functions by inhibiting the ICE-like proteases, caspase, activity and thus preventing caspase-induced apoptosis (Bertin et al., 1996; Bump et al., 1995; LaCount and Friesen, 1997; Xue and Horvitz, 1995). This is a central, highly conserved step in the apoptotic pathway (Hale et al., 1996) and *p35* functions in a wide variety of organisms including *Drosophila* (Hay et al., 1994; White et al., 1996), *Caenorhabditis elegans* (Sugimoto et al., 1994; Xue and Horvitz, 1995), and mammalian cells (Beidler et al., 1995; Martinou et al., 1995; Rabizadeh et al., 1993). However, P35 is not always effective, even in insect cell lines. P35 did not prevent apoptosis in AcMNPV-infected Cf-203 cells, although expression of P35 was detected (Palli et al., 1996). AcMNPV-infected SL2 cells also undergo apoptosis suggesting that *p35* is either not expressed or not active in this cell line (Chejanovsky and Gershburg, 1995). A second type of apoptotic suppressor gene, inhibitor of apoptosis (*iap*), has been identified in the baculovirus *Cydia pomonella* granulovirus (CpGV) (Cp-*iap*) and *Orgyia pseudotsugata* M nucleopolyhedrovirus (OpMNPV) (Op-iap) that can substitute for *p35* to prevent apoptosis in a genetic complementation assay in which replacement of *p35* with *iap* prevented apoptosis (Birnbaum et al., 1994; Crook et al., 1993). P35 and IAP do not share any homology in either nucleotide or amino acid sequence (Crook et al., 1993; Friesen and Miller, 1987). It is believed that IAP functions upstream of P35 in the apoptotic pathway.

Shut off of host gene expression is normally observed in permissive AcMNPV infections. A decline in the steady-state levels of host mRNAs begins approximately 12 h p.i. (Ooi and Miller, 1988). Host protein synthesis declines from 18 h p.i. and is completely shut off by 24 h p.i. (Carstens et al., 1979; Dobos and Cochran, 1980). The viral gene product which mediates this host shutoff has not been identified. The protein synthesis shut down in AcMNPV-infected Ld652Y cells, however, was global, earlier (12 h p.i. versus 18 h p.i.) (Guzo et al., 1992), and occurred in the absence of decline of either host or viral mRNA synthesis (Cuzo et al., 1992; Morris and Miller, 1992, 1993). These distinctions suggested that protein synthesis shut down in AcMNPV-infected Ld652Y cells was a cellular response to viral infection.

In exploring the mechanism by which the protein synthesis is shut down and the function of HRF-1 in precluding the protein synthesis inhibition in AcMNPV-infected Ld652Y cells, we initially considered two possibilities. One was that protein synthesis shut down may be a part of the apoptosis response of Ld652Y cells and *hrf-1* encodes another apoptotic suppressor. The other was that protein synthesis shut down and apoptosis might be two separate responses of Ld652Y cells to AcMNPV infection. When we examined AcMNPV-infected Ld652Y cells for hallmarks of apoptosis none were observed. The absence of apoptosis supports the second hypothesis. However, it was possible that P35 prevented apoptosis in AcMNPV-infected Ld652Y cells. The goal of this study was to determine the relationship between these two responses by investigating the roles of AcMNPV P35 and LdMNPV HRF-1 in controlling apoptosis and protein synthesis shut down. Here we present data that support the hypothesis that protein synthesis shut down and apoptosis are separate responses of Ld652Y cells to AcMNPV

infection. Our data also suggest that P35 induces a protein synthesis shut down response in the absence of DNA replication and late viral gene expression in Ld652Y cells.

Materials and Methods

Cells and Viruses

SF-21 cells (Vaughn et al., 1977) and Ld652Y cells (Goodwin et al., 1978) were maintained in TC100 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 0.26% tryptose broth. AcMNPV variant L1 (Lee and Miller, 1978) and the recombinant AcMNPV, vAc/hrf-1 [previously designated vAcLdPD (Du and Thiem, 1997)] were propagated in SF-21 cells. All *p35* deletion recombinant Ac*M*NPVs were propagated in TN368 cells (Hink, 1970). The recombinant AcMNPVs, vΔp35 [previously designated vΔ35K (Hershberger et al., 1992; LaCount and Friesen, 1997)] (Figure 14) , which lacked *p35* and vΔ35K/lacZ (Figure 14) (Hershberger et al., 1992), in which *p35* was deleted and the polyhedrin gene was replaced by *LacZ* were obtained from Paul Friesen (University of Wisconsin, Madison). To construct a recombinant AcMNPV lacking *p35* but carrying *hrf-1*, DNA from a transfer plasmid containing *hrf-1*, pAcUWLdPD (Du and Thiem, 1997), and from vΔ35K/lacZ, were cotransfected into TN368 cells (Hink, 1970), using lipofectin (Flegner et al., 1987; O'Reilly et al., 1992), to generate vΔp35/hrf-1 (Figure 14). To construct a recombinant AcMNPV lacking *p35* but carrying a truncated *hrf-1* ORF, DNA of pAcUWLdPD was cut at the *Sal*I site located in the first one third portion of *hrf-1* coding region, blunt-ended by mung bean nuclease, and religated with T4 DNA ligase. A stop codon was introduced in the resulting plasmid pAcUWPDstop near the original *Sal*I site. DNA from

pAcUWPDstop and DNA from vΔ35K/lacZ, were cotransfected into TN368 cells using lipofectin to generate vΔp35/hrf-1stop (Figure 14). In both cases, white occlusion positive viruses were selected and plaque purified. Correct insertion was confirmed by DNA restriction pattern and Southern blot analyses of the recombinant viruses.

Western Bot Analysis

Ld652Y cells and SF-21 control cells were infected with AcMNPV at multiplicity of infection (MOI) of 10 plaque forming units (PFU) per cell. After 1 h adsorption, inoculum was removed and cells were fed with complete medium. Time 0 was defined as the time when incubation at 27°C was initiated. Mock-infected cells were treated in the same way except that incomplete medium was used as inoculum and cells were harvested at 48 h p.i. AcMNPV-infected Ld652Y and SF-21 cells were harvested at 6, 12, 24, and 48 h p.i. Samples preparation and Western blot analysis were done as previously described (Du and Thiem, 1997; Thiem et al., 1996). An amount of protein equivalent to approximately 5 X 10^5 cells was loaded per lane. Polyclonal antibodies against P35, αp35-NF (Hershberger et al., 1994) were used at the dilution of 1:5000.

Apoptosis Test

Ld652Y cells and SF-21 cells were infected with AcMNPV, vΔp35, or vΔp35/hrf-1 at an MOI of 10. Uninfected SF-21 and Ld652Y cells were treated with actinomycin D as a control. Actinomycin D was added to the medium at a concentration of 1μg/ml after virus inoculum was removed. Infected or treated cells were observed for morphological changes and harvested at 12, 24, and 36 h p.i. Total DNA was isolated, electrophoretically separated in 1.2% agarose gels in the presence of RNase A, and

stained with ethidium bromide (Clem et al., 1991).

Protein Synthesis Labeling Assay

Ld652Y cells were infected at an MOI of 10 with AcMNPV, vΔp35, vΔp35/hrf-1, vΔp35/hrf-1stop, or vAc/hrf-1 (Du and Thiem, 1997). For aphidicolin treatment, Ld652Y cells were pretreated with 5 μg/ml of aphidicolin (Sigma, St. Louis, MO) in DMSO 1 h before the addition of inoculum and infected cells were incubated in medium containing 5 μg/ml of aphidicolin throughout the time course. Mock- or virus-infected cells were labeled with ^{35}S methionine (1000Ci/mMol, Dupont NEN, Wilmington, DE) for 4 hour periods 0-4, 12-16, and 24-28 h p.i. At the beginning of each time period, the medium was removed and 0.5 ml methionine free medium was added to each 35 mm plate followed by addition of ^{35}S methionine. Cells were collected at the end of each labeling period and disrupted in 2X protein sample buffer (125mM Tris-HCl pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.002% bromophenol blue). For those infected cells that were undergoing extensive apoptosis at 24-28 h p.i., small floating vesicles (apoptotic bodies) were collected by gentle centrifugation (500g, 5 min) and resuspended in 0.5 ml methionine free medium and returned to the plates for labeling. Proteins were resolved in 10% SDS-PAGE (Laemmli, 1970) and the gel was stained with Coomassie blue to confirm the equality of loading before autoradiography.

RT-PCR

Ld652Y cells were infected at an MOI of 10 with vΔp35/hrf-1, or vAc/hrf-1 (Du and Thiem, 1997) in the presence or absence of aphidicolin and harvested at 6 and 24 h p.i. Aphidicolin treatment was performed in the same way as described above. Total

RNA was isolated by a single step method (Chomczynski and Sacchi, 1987). Twenty μg of total RNA was used to synthesize cDNA with oligo(dT) primer (Promega, Madison, WI) and M-MLV reverse transcriptase (Life Technologies, Bethesda, MD). These cDNA products were then used for PCR. PCR was performed 25 cycles (94°C 1 min, 55°C 50 sec, and 72°C 3 min) using two synthetic *hrf-1* specific primers (Michigan State University Macromolecular Structure, Sequencing, and Synthesis Facility, East Lansing, MI), CAGCGAGACGAATATCGA near the 5'-end and CGATACAAACCAAACGCA near the 3'-end of *hrf-1* transcript (Du and Thiem, 1997). PCR product was resolved in 1% agarose gel together with DNA size markers and stained with ethidium bromide.

Results

P35 Was Expressed Prior to Global Protein Synthesis Shut Down in AcMNPV-Infected Ld652Y Cells

In preliminary experiments we did not observe apoptosis in AcMNPV-infected Ld652Y cells prior to or following protein synthesis shut down. Since AcMNPV encodes a potent apoptotic suppressor P35, the absence of apoptosis implied that AcMNPV P35 might function to prevent apoptosis. However, no evidence was available that any viral gene was translated in AcMNPV-infected Ld652Y cells. To determine if P35 might prevent apoptosis in AcMNPV-infected Ld652Y cells, we first determined if P35 was translated prior to protein synthesis shut down. Ld652Y cells and SF-21 control cells were infected with AcMNPV and cell lysates were subjected to Western blot analysis with polyclonal antibody against P35. We detected P35 protein in both SF-21 and Ld652Y cells infected with AcMNPV from 6 h p.i. until 48 h p.i. (Figure 13) demonstrating that P35 was translated in AcMNPV-infected Ld652Y cells prior to global

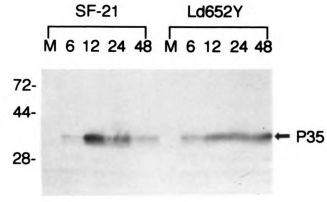

Figure 13. Western Blot Analysis of P35 Expression in AcMNPV-Infected SF-21 and Ld652Y Cells Cell types are indicated on the top of the panel. Arabic numbers above the lanes indicate the time (in hours) after infection that cells were harvested. M represents mock-infected cells. Size markers are shown to the left of the panel in kD. Protein P35 (arrow) is indicated to the right.

protein synthesis shut down. This is the first direct evidence to show a viral gene was translated in AcMNPV-infected Ld652Y cells prior to global arrest of protein synthesis.

P35 Prevented Apoptosis and HRF-1 Could Not Functionally Substitute For P35

We next determined if P35 was functional in AcMNPV-infected Ld652Y cells and if HRF-1 could functionally substitute for P35. If P35 prevented apoptosis, Ld652Y cells infected with AcMNPV lacking *p35* would undergo apoptosis as do SF-21 cells (Clem et al., 1991; Hershberger et al., 1992). If HRF-1 could functionally substitute for P35, cells infected with AcMNPV lacking *p35* but carrying *hrf-1* would not undergo apoptosis. To test this hypothesis, we obtained a *p35* minus AcMNPV, Δp35 (Figure 14, b) (Hershberger et al., 1992), and constructed a recombinant AcMNPV lacking *p35* but carrying *hrf-1*, Δp35/hrf-1 (Figure 14, c). Ld652Y cells and SF-21 control cells were infected with wt AcMNPV, Δp35, or Δp35/hrf-1. Mock-infected cells and actinomycin D treated cells were used as additional controls. Infected or treated cells were observed for morphological changes and DNA was isolated to determine the presence of DNA fragmentation ladders. We observed typical apoptotic plasma membrane blebbing beginning from 12 h p.i. in both SF-21 and Ld652Y cells infected with vΔp35 or vΔp35/hrf-1 and in actinomycin D treated cells but not in wt AcMNPV-infected or mock-infected cells (Data not shown). Total DNA isolated from cells displaying plasma membrane blebbing showed typical DNA fragmentation ladders on agarose gels (Figure 15). These data indicated that P35 prevented apoptosis in AcMNPV-infected Ld652Y cells (Figure 15B, lanes 4-6) and HRF-1 could not substitute for *p35* to prevent apoptosis in either SF-21 (Figure 15A, lanes 10-12) or Ld652Y cells (Figure 15B, lanes 10-12).

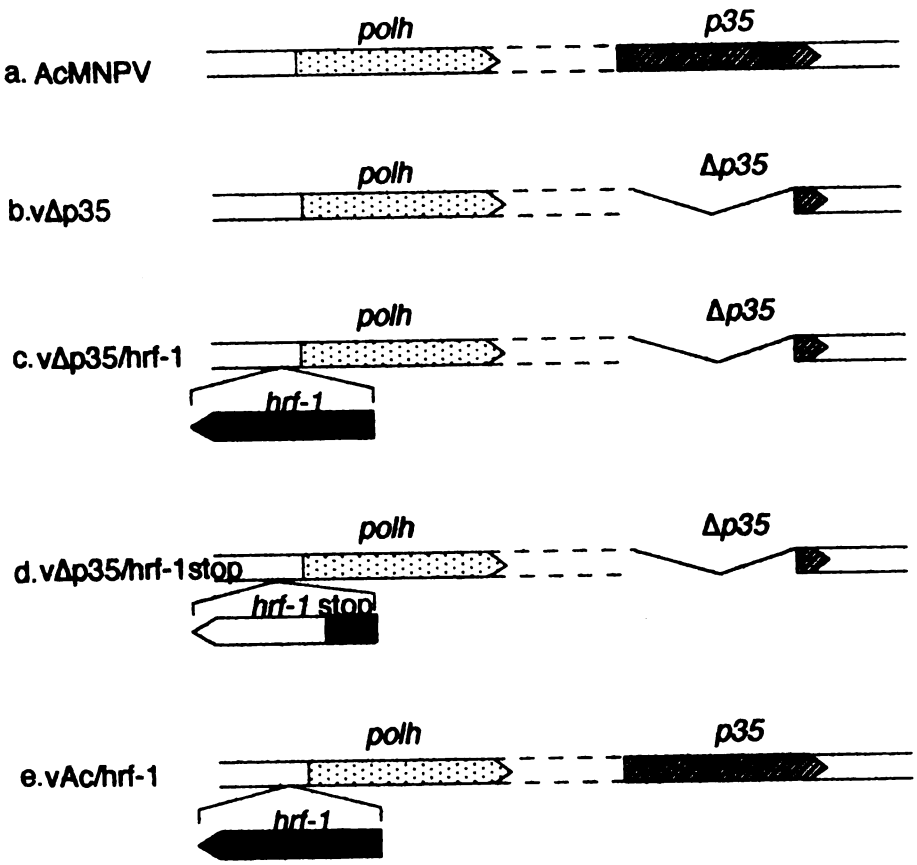

Figure 14. Schematic Diagram Showing the Viruses Used in Apoptosis and Protein Synthesis Assays The positions and orientations of ORFs are indicated by arrows. Deletion of *p35* gene was indicated by indentation line. Truncation of *hrf-1* ORF was indicated by blank arrow.

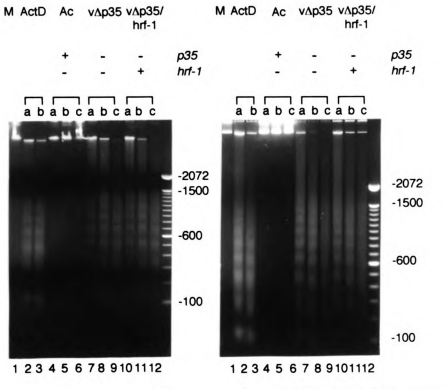

Figure 15. Agarose Gel Showing DNA Fragmentation in SF-21 and Ld652Y Cells A: SF-21 cells infected with wt AcMNPV, vΔp35, vΔp35/hrf-1; B: Ld652Y cells infected with wt AcMNPV, vΔp35, vΔp35/hrf-1. Viruses with or without *p35* or *hrf-1* were indicated by "+" or "-" at the top of the panels. Mock-infected cells, 1; Actinomycin D treated cells, 2-3; Wt AcMNPV-infected cells, 4-6; vΔp35-infected cells, 7-9; vΔp35/*hrf-1*-infected cells, 10-12. a = 12 h p.i or p.t.; b = 24 h p.i. or p.t.; c = 36 h p.i. 100 bp size markers are shown in bp to the right of each panel.

HRF-1 Prevented Protein Synthesis Shut Down Even When Ld652Y Cells Were Undergoing Apoptosis

In order to investigate the roles of P35 and HRF-1 in controlling protein synthesis shut down, we examined protein synthesis in Ld652Y cells infected with recombinant AcMNPVs carrying different compliments of *p35* and *hrf-1* (Figure 14). Another recombinant AcMNPV, vΔp35/hrf-1stop (Figure 14, d), carrying a truncated *hrf-1* ORF but not *p35* was constructed and employed in protein synthesis assays in infected Ld652Y cells as an additional control for HRF-1 function. Protein synthesis in mock- or virus-infected Ld652Y cells was monitored by ^{35}S methionine labeling (Figure 16). We found that protein synthesis was shut down at 12-16 h p.i. in Ld652Y cells infected with wt AcMNPV, carrying *p35* but not *hrf-1*, (Figure 16, compare lanes 4 and 5). In Ld652Y cells infected with vΔp35, carrying neither *p35* nor *hrf-1*, protein synthesis shut down was also observed but delayed since a limited amount of protein synthesis occurred 12-16 h p.i. followed by an arrest by 24-28 h p.i. (Figure 16, lanes 8 and 9). In Ld652Y cells infected with vΔp35/hrf-1, carrying *hrf-1* but not *p35*, protein synthesis shut down was not observed although the protein synthesis ability was reduced at 24-28 h p.i. (Figure 16, lanes 11 and 12). In Ld652Y cells infected with vΔp35/hrf-1stop, carrying a truncated *hrf-1* ORF but not *p35*, protein synthesis shut down was observed and was delayed as in vΔp35-infected Ld652Y cells (Figure 16, lanes 14 and 15; compare with lanes 8 and 9). In Ld652Y cells infected with vAc/hrf-1, carrying both *p35* and *hrf-1*, global protein synthesis arrest was not observed as several proteins, presumably viral, were abundantly synthesized (Figure 16, lanes 17 and 18). These data demonstrated that HRF-1 prevented protein synthesis shut down even when cells were undergoing apoptosis. The reduction of

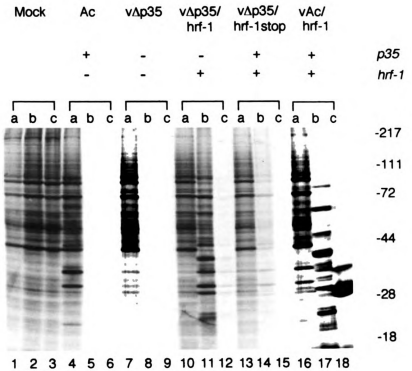

Figure 16. Pulse Labeling Analysis of Protein Synthesis in Ld652Y Cells Infected with Wt or Recombinant AcMNPVs Ld652Y cells were infected with vΔp35, vΔp35/hrf-1, vΔp35/hrf-1stop, and vAc/hrf-1. Viruses with or without *p35* or *hrf-1* were indicated by "+" or "-" at the top of the panel. Mock-infected cells, 1-3; Wt Ac*M*NPV-infected cells, 4-6; vΔp35-infected cells, 7-9; vΔp35/hrf-1-infected cells, 10-12; vΔp35/hrf-1stop-infected cells, 13-15; vAc/hrf-1-infected cells, 16-18. Labeling time periods are indicated by lower case letters. a: 0-4 h p.i.; b: 12-16 h p.i.; c: 24-28 h p.i. Size markers are shown in kD to the left of the panel.

protein synthesis ability at very late times (24-28 h p.i.) in vΔp35/hrf-1-infected Ld652Y cells (Figure 16, lane 12) may be due to the apoptotic cell death of some cells at this very late phase of apoptosis. By 24-28 h p.i., almost all cells had disintegrated into small apoptotic bodies. Some of the cells may have already died. Most apoptotic bodies did not take up the dye trypan blue (data not shown) and displayed translation ability (Figure 16, lane 12). Protein synthesis was shut down in vΔp35/hrf-1stop-infected Ld652Y cells containing a truncated *hrf-1* ORF (Figure 16, lanes 14 and 15) confirming the role of HRF-1 in preventing protein synthesis shut down.

Effects of Aphidicolin On Apoptosis and Protein Synthesis Shut Down

The DNA synthesis inhibitor aphidicolin has been used to distinguish early and late gene expressions, since late but not early gene expression of baculovirus depends on DNA replication. Aphidicolin blocks apoptosis in SF-21 cells induced by *p35*⁻ AcMNPV infections (Clem and Miller, 1994) indicating that apoptosis involves late events. However, aphidicolin did not block protein synthesis shut down in AcMNPV-infected Ld652Y cells (Guzo et al., 1992). This suggested that aphidicolin could be used to discriminate between apoptosis and protein synthesis shut down. In order to separate the roles of HRF-1 and P35 in these responses, Ld652Y cells were infected with AcMNPV, vΔp35, vΔp35/hrf-1, and vAc/hrf-1 in the presence of aphidicolin. Apoptosis was determined by observing morphological changes of the infected cells and protein synthesis was monitored by ^{35}S methionine labeling. We found that aphidicolin blocked apoptosis in Ld652Y cells induced by *p35*⁻ AcMNPV infections as it did in SF-21 cells (Clem and Miller, 1994). Protein synthesis shut down in wt AcMNPV-infected Ld652Y

cells was not blocked by aphidicolin but was much delayed (Figure 17, lanes 5-6; compare with Figure 16, lanes 5-6). In contrast, protein synthesis shut down did not occur in any *p35*⁻ AcMNPV (vΔp35 and vΔp35/hrf-1) -infected Ld652Y cells (Figure 17, lanes 7-12). The ability of aphidicolin to block *p35*⁻ AcMNPV-induced protein synthesis shut down suggested that the protein synthesis shut down response in *p35*⁻ AcMNPV-infected Ld652Y cells involved DNA replication or DNA replication dependent late gene expression. In contrast, protein synthesis shut down appeared to be an early event in wt AcMNPV-infected Ld652Y cells since aphidicolin did not block protein synthesis shut down. The only difference between wt AcMNPV and vΔp35 was the presence or absence of *p35*. These data implied the existence of two pathways that led to protein synthesis shut down, one involving late events and another that is independent of late events that involves P35 as an inducer.

hrf-1 Was Transcribed at Very Low Levels in the Presence of Aphidicolin

Protein synthesis shut down was not observed in vAc/hrf-1-infected Ld652Y cells in the presence of aphidicolin (Figure 17, lanes 12-15). This indicated that HRF-1 prevented protein synthesis arrest (Figure 17, compare lanes 5 and 6 with 14 and 15). However, previous studies employing Northern blot analysis failed to detect *hrf-1* transcription in vAc/hrf-1-infected Ld652Y cells in the presence of aphidicolin (Du and Thiem, 1997). We hypothesized that *hrf-1* was expressed at a very low level in the presence of aphidicolin and this limited expression of HRF-1 was sufficient to prevent protein synthesis shut down induced by P35. To test this hypothesis, a more sensitive method, RT-PCR using *hrf-1* specific primers was employed to detect *hrf-1*

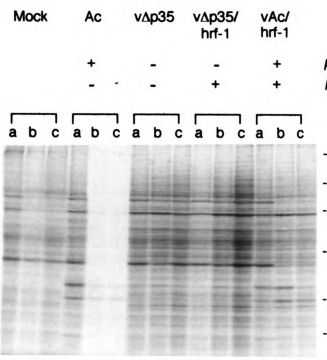

Figure 17. Pulse Labeling Analysis of Protein Synthesis in Ld652Y Cells Infected with Wt or Recombinant AcMNPVs in the Presence of Aphidicolin Ld652Y cells were infected with AcMNPV, vΔp35, vΔp35/hrf-1, and vAc/hrf-1-infected Ld652Y cells in the presence of aphidicolin. Viruses with or without *p35* or *hrf-1* were indicated by "+" or "-" at the top of the panel. Mock-infected cells, 1-3; Wt Ac*M*NPV-infected cells, 4-6; vΔp35-infected cells, 7-9; vΔp35/hrf-1-infected cells, 10-12; vAc/hrf-1-infected cells, 13-15. Labeling time periods are indicated by lower case letters. a: 0-4 h p.i.; b: 12-16 h p.i.; c: 24-28 h p.i. Size markers are shown in kD to the left of the panel.

transcription in vAc/hrf-1-infected Ld652Y cells in the presence of aphidicolin. We detected *hrf-1* specific RT-PCR product in both vΔ35K/hrf-1 and vAc/hrf-1-infected Ld652Y cells in the presence of aphidicolin (Figure 18).

Discussion

The roles of P35 and HRF-1 in controlling apoptosis and protein synthesis shut down in AcMNPV-infected Ld652Y cells were investigated. Our data support the hypothesis that protein synthesis shut down and apoptosis are separate responses of Ld652Y cells to AcMNPV infection. In addition, our data suggest that P35 is involved in a second pathway leading to protein synthesis shut down in AcMNPV-infected Ld652Y cells. These conclusions are based on the following observations: 1) P35 was translated and prevented apoptosis but protein synthesis was still shut down; 2) HRF-1 prevented protein synthesis shut down even when the cells were undergoing apoptosis but it could not substitute for P35 to prevent apoptosis; 3) Aphidicolin blocked apoptosis and protein synthesis shut down in all *p35*⁻ AcMNPV infections but not protein synthesis shut down in wt AcMNPV-infected Ld652Y cells.

Both apoptosis and protein synthesis shut down appear to be common responses of insect cells to baculovirus infections. AcMNPV lacking *p35* induces apoptosis in BmN cells (Clem et al., 1991) and a functional homologous *p35* was identified in *Bombyx mori* nucleopolyhedrovirus (BmNPV) (Kamita et al., 1993). wt AcMNPV induces apoptosis in Cf-203 and SL2 cells where P35 is either not functional or not expressed (Chejanovsky and Gershburg, 1995; Palli et al., 1996). Many DNA viruses carry genes involved in blocking cellular apoptosis (Teodoro and Branton, 1997). The pathways of

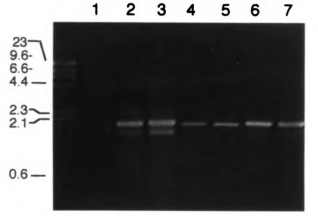

Figure 18. RT-PCR Analysis of *hrf-1* Transcription in vp35/hrf-1- or vAc/hrf-1-infected Ld652Y Cells in the Presence of Aphidicolin Ld652Y cells were infected with vΔp35/hrf-1- or vAc/hrf-1 in the presence of aphidicolin. cDNA was generated from total RNA isolated from mock-infected cells (lane 1), vΔp35/hrf-1-infected cells in the absence of aphidicolin at 24 h p.i. (lane 2), vAc/hrf-1-infected cells in the absence of aphidicolin at 24 h p.i. (lane 3), vΔp35/hrf-1-infected Ld652Y cells in the presence of aphidicolin at 6 (lane 4) and 24 (lane 5) h p.i., vAc/hrf-1-infected cells in the presence of aphidicolin at 6 (lane 6) and 24 (lane 7) h p.i. DNA size markers are indicated to the left of the panel in kb.

apoptosis induction in these virus-infected cells are different but all converge at the activation of caspase (Teodoro and Branton, 1997). For example, adenovirus E1A induces apoptosis through a p53-dependent mechanism which is prevented by the adenovirus E1B-19K (Debbas and White, 1993; Lowe and Ruley, 1993; Rao et al., 1992). E1B-19K also protects cells against apoptosis stimulated by tumor necrosis factor alpha or anti-Fas antibody (Gooding et al., 1991; Hashimoto et al., 1991; White et al., 1992). The AcMNPV transactivator IE-1 is sufficient to induce apoptosis in SF-21 cells (Prikod'ko and Miller, 1996). However, IE-1 does not appear to be the sole factor since aphidicolin, which blocks DNA replication and DNA replication dependent viral late gene expression but not IE-1 expression, blocks induction of apoptosis (Clem and Miller, 1994; this study), indicating a late event is involved in the induction of apoptosis. Experiments using temperature sensitive DNA replication mutants showed that DNA replication or DNA replication dependent late gene expression was not essential for induction but was required for full development of an apoptotic response (LaCount and Friesen, 1997).

Protein synthesis shut down was also observed in other nonpermissive AcMNPV infections in addition to AcMNPV-infected Ld652Y cells. AcMNPV induces dramatic attenuation of protein synthesis by 5 h p.i. and a complete shut down of protein synthesis by 24 h p.i. in BmN cells (Kamita and Maeda, 1993). Transcription of mRNA in AcMNPV-infected BmN cells is apparently normal (Kamita and Maeda, 1993). Protein synthesis arrest is induced by the putative helicase gene *p143* of AcMNPV (Kamita and Maeda, 1993). Replacing a small region of the AcMNPV *p143* with BmNPV homologous sequence prevents protein synthesis shut down (Croizier et al., 1993; Maeda,

et al., 1993). Another example of protein synthesis shut down in baculovirus-infected insect cells involves host cell-specific factor 1 (*hcf-1*) (Lu and Miller, 1995; 1996). *hcf-1* is not required for AcMNPV replication in SF-21 cells but replication of *hcf-1* null mutant in *Trichoplisa ni* cell line TN368 (Hink, 1970) is impaired (Lu and Miller, 1996). Complete shut down of protein synthesis is observed by 18 h p.i. together with defects in viral DNA replication and late gene transcription (Lu and Miller, 1996). The mechanism of protein synthesis shut down in either case is not known. Limited work has been conducted to investigate the inducer of protein synthesis shut down in AcMNPV-infected Ld652Y cells (Guzo et al., 1991). A compound desigated macromolecular synthesis inhibition factor (MSIF) was found in the media in AcMNPV-infected Ld652Y cells that induced protein synthesis arrest in noninfected cells (Guzo et al., 1991). It was postulated that this compound was the AcMNPV GP64 glycoprotein or a component of GP64 (Guzo et al., 1991). However, this hypothesis has not been confirmed.

Our observations on protein synthesis shut down and apoptosis in AcMNPV-infected Ld652Y cells led us to develop a model of the responses of Ld652Y cells to AcMNPV infection (Figure 19). Since protein synthesis shut down and apoptosis appeared to be separate responses, two separate pathways were hypothesized. The specific inducers of both protein synthesis shut down and apoptosis have not been resolved. Although two inducers are shown in our model, we don't exclude the possibility that these two responses share the same inducer (I1 = I2) or that these two responses converge upstream of the pathways (X1 = X2). The apoptosis pathway leads to activation of caspase. This pathway can be blocked by the early gene product P35 which

Figure 19. Diagram Showing a Model of the Responses of Ld652Y Cells to AcMNPV-Infection DNA replication is the point to determine early and late gene expression. Both P35 and HRF-1 are early gene products that are expressed prior to DNA replication. Apoptosis is induced by an unidentified inducer through a mediator X1 and the activation of caspase. P35 blocks apoptosis pathway be preventing activation of caspase (Bump et al., 1995; Xue and Horvitz, 1995). An undefined inducer I2 triggers protein synthesis shut down through mediator X2 and an undefined process Y. Both these two pathways require DNA replication dependent late gene expression and thus can be blocked by DNA synthesis inhibitor aphidicolin. A second signal transduction pathway is induced by P35 that leads to protein synthesis shut down. This pathway does not involve late events and is not blocked by aphidicolin. Both protein synthesis shut down pathways can be blocked by HRF-1 by an unknown mechanism.

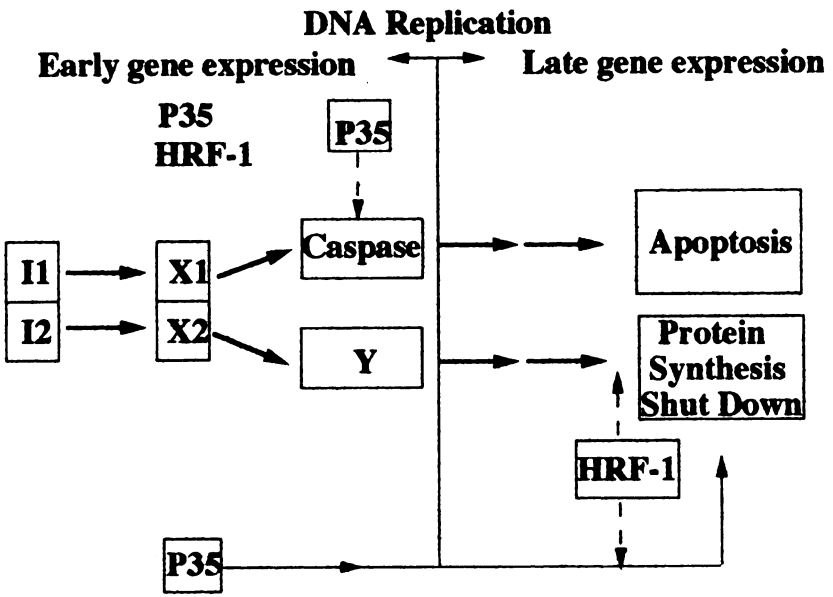

Figure 19. Diagram Showing a Model of Responses in Ld652Y Cells to AcMNPV Infection

specifically acts on caspase. The protein synthesis shut down pathway operates through an undefined mediator Y. This pathway can be blocked by HRF-1 but not P35. Both apoptosis and protein synthesis shut down pathways in *p35*⁻ AcMNPV-infected Ld652Y cells involve DNA replication or DNA replication dependent late gene expression. A second protein synthesis shut down response induced by P35 that does not involve late events was hypothesized. This second protein synthesis shut down response can also be blocked by HRF-1. These two protein synthesis shut down pathways appear to act synergistically. When both pathways were induced, protein synthesis shut down was more extensive and rapid (Figure 16, lanes 4 and 5) than when either one was blocked (Figure 16, lanes 8 and 9; Figure 17, lanes 5 and 6).

Protein synthesis shut down is a common strategy employed by many host cells against viral infection (Hershey, 1991). Most of these systems involve an inactivation of a conserved component of translation apparatus. Viruses in turn have evolved methods to overcome this cullular defense (Hershey, 1991). In prokaryotes, protein synthesis shut down was overved in some phage exclusions which involve cleavage of the components of the translation apparatus, EF-Tu (Yu and Snyder, 1994) or $tRNA^{lys}$ (Levitz et al., 1990). Plants activate enzymes in response to virus infection that depurinate 28S RNA thereby inactivating the ribosome and thus inhibiting protein synthesis (Endo and Ysurugi, 1987). In herpes simplex virus 1 (HSV-1), protein synthesis is shut down after the expression of α genes in neuronal cells infected with a $\gamma_1$34.5 minus mutant HSV-1 but not wt HSV-1 (Chou and Roizman, 1992, 1994). $\gamma_1$34.5 gene of HSV-1 is able to preclude the protein synthesis inhibition in HSV-1-infected neuronal cells (Chou and

Roizman, 1992, 1994). This global protein synthesis shut down reponse of host cells against viral infection is distinct from that of viral host shutoff function of HSV-1 mediated by the virion *vhs* protein (Poon and Roizman, 1997). The best undestood mechamins of protein synthesis shut down are those mediated by interferon in mammals and other vertebrates (Lengyel, 1982; Samuel, 1991). Interferon mediated protein synthesis shut down includes inactivation of eukaryotic initiation factor 2 α subunit (eIF-2α) through phosphorylation by dsRNA dependent protein kinase PKR (Chernajovsky et al., 1979), degradation of mRNA through activation of RNase L (Farrel et al., 1978; Hovanessian et al., 1977; Zillberstein et al., 1978), or degradation of CCA terminus of tRNA by 2'-5' phosphodiesterase (Schmidt et al., 1978). It is likely that global protein synthesis shut down observed in AcMNPV-infected Ld652Y cells is due to inactivation of a component of the protein synthesis machinery and HRF-1 functions to prevent this occurrence. Studies are in progress to analyze the protein synthesis defect in AcMNPV-infected Ld652Y cells and to determine how HRF-1 functions to overcomes the block in protein synthesis. Continuing studies to elucidate these mechanisms will provide insight into the nature of the responses of Ld652Y cells against AcMNPV infection.

Acknowledgments

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CHAPTER 5

Investigation of the Defect in Protein Synthesis in *Autographa californica* Nucleopolyhedrovirus-Infected Ld652Y Cells

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Abstract

Global protein synthesis arrest is observed in AcMNPV-infected Ld652Y cells at late times post infection (p.i.) in the presence of apparently normal mRNAs. A cell-free translation system was established from cytoplasmic lysate of insect cells and in vitro translation assays were carried out to identify the defect in protein synthesis in AcMNPV-infected Ld652Y cells. Lysate derived from AcMNPV-infected Ld652Y cells at late times p.i. did not display in vitro translation ability but its translation ability could be restored by addition of lysate derived from mock-infected cells. The lysate from mock-infected Ld652Y cells was then fractionated and each fraction was tested for its rescue ability. The ribosomal fraction but not the supernatant fraction could rescue; total RNA isolated from mock- or AcMNPV-infected Ld652Y cells at early times but not late times p.i. could rescue; total uncharged tRNA from both calf and yeast but not E. coli could rescue. However, no significant decrease in total tRNA adaptor ability was found in AcMNPV-infected Ld652Y cells at late times p.i. compared to mock- or AcMNPV-infected Ld652Y cells at early times p.i. These data suggest that the defect in protein synthesis in AcMNPV-infected Ld652Y cells lies in a single or a small group of tRNA species. The same in vitro translation assays were applied to other nonpermissive AcMNPV infections in which protein synthesis shut down was also observed. Our data suggest that a common mechanism of protein synthesis shut down is shared in AcMNPV-infected Ld652Y and BmN cells.

Introduction

Autographa californica nucleopolyhedrovirus (AcMNPV) is a member of the family *Baculoviridae*, a large family of double-stranded DNA-containing viruses that

primarily infect insects. AcMNPV serves as a model virus for the study of molecular biology of baculoviruses and has been developed into effective eukaryotic protein expression vectors (Luckow and Summers, 1988; Miller, 1988; O'Reilly et al., 1992) as well as biological pesticides (Bishop, 1989; Wood and Granados, 1991). AcMNPV has a wider host range than most other baculoviruses. AcMNPV is able to replicate in insect larvae of at least 33 species in 10 families (Groner, 1986) and in more than 25 different insect cell lines (Hink, 1979; Hink and Hall, 1989). In addition, it can infect at least 26 different insect cell lines in a semipermissive manner (Possee et al., 1993). One of these semipermissive cell lines, Ld652Y derived from gypsy moth, displays a novel virus-host interaction when infected with AcMNPV (Du and Thiem, 1997; Du and Thiem, submitted; Guzo et al., 1992; McClintock et al., 1986; 1987; Thiem et al., 1996). When AcMNPV infects Ld652Y cells, virus can enter the cells, viral DNA is replicated (McClintock et al., 1986; Morris and Miller, 1992), both viral and host mRNAs are transcribed, transported, and are of normal size (Guzo et al, 1992; Morris and Miller, 1993). However, both viral and host cellular protein synthesis is shut down at late times post infection (p.i.) and no viral progeny is produced (Du and Thiem, submitted; Guzo et al., 1992; McClintock et al., 1986). A single gene, host range factor 1 (*hrf-1*) was identified and characterized in *Lymantria dispar* nucleopolyhedrovirus (LdMNPV) that is able to preclude the protein synthesis shut down and ensure the production of AcMNPV progeny (Du and Thiem, 1997; Thiem et al., 1996). Our previous studies have shown that HRF-1 most likely functions directly on the translation system to maintain the protein synthesis ability (Du and Thiem, submitted).

Protein synthesis shut down is also observed in AcMNPV-infected nonpermissive

cells of other insect species. In AcMNPV-infected BmN cells derived from *Bombyx mori*, infected cells exhibited atypical cytopathic effect (CPE) and protein synthesis was dramatically attenuated by 5 h p.i. and completely shut down by 24 h p.i. in the presence of apparently normal mRNA (Kamita and Maeda, 1993). This inhibition was induced by the putative helicase gene of AcMNPV, *p143*, and precluded by the homologous helicase gene in *Bombyx mori* nucleopolyhedrovirus (BmNPV) (Croizer et al., 1994; Kamita and Maeda, 1993; Maeda et al., 1993). Another gene involved in protein synthesis shut down response is the host cell specific factor 1 (*hcf-1*) of AcMNPV (Lu and Miller, 1995; 1996). AcMNPV *hcf-1* is not required for AcMNPV replication in *Spodoptera frugiperda* cell line SF-21 but *hcf-1*$^-$ AcMNPV replication in TN368 cells derived from *Trichoplusia ni* (Hink, 1970) is greatly impaired (Lu and Miller, 1996). A complete shut down of both viral and host protein synthesis is observed by 18 h p.i. (Lu and Miller, 1996). However, other defects including DNA replication and late gene transcription are also observed in *hcf-1*$^-$ AcMNPV-infected TN368 cells (Lu and Miller, 1996). The mechanisms of protein synthesis shut down remain unkown.

Protein synthesis arrest is a common strategy of cells against a variety of stimuli including viral infection in many other systems (Hershey, 1991; Schneider and Shenk, 1987). Cell-free translation assays have proved to be a very effective way to investigate the protein synthesis control in virus-infected host cells. For example, cell-free translation lysates prepared from vaccinia virus-infected mouse L cells were employed in identification of a P1/eIF kinase inhibitor, encoded by vaccinia virus E3L gene (Chang et al., 1992). Translation assays employing fractionated lysates prepared from uninfected or mengovirus-infected mouse L cells provided evidence of the presence of an inhibitor in

the ribosomes of mengovirus-infected mouse L cells (Pensiero and Lucas-Lenard, 1985). Cell lysates prepared from E. coli cells also contributed to the discovery that elongation factor EF-Tu cleavage was responsible for T4 phage exclusion (Yu and Snyder, 1994). Protein synthesis shut down in AcMNPV-infected Ld652Y cells in the presence of apparently normal mRNA suggested that the block was at translational level. Towards the goal of elucidating the mechanism by which protein synthesis is shut down and the function of HRF-1 in precluding the translation inhibition, we established a cell-free translation system from insect cells and investigated the defect of protein synthesis in AcMNPV-infected Ld652Y cells by a series of in vitro translation rescue assays. Our data suggest that the defect in protein synthesis in AcMNPV-infected Ld652Y cells is at tRNA and that a common mechanism of protein synthesis shut down is shared in AcMNPV-infected Ld652Y and BmN cells.

Materials and Methods

Cells and Viruses

SF-21 cells (Vaughn et al., 1977), Ld652Y cells (Goodwin et al., 1978), TN368 Cells (Hink, 1970), and BmN cells were maintained in TC100 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 0.26% tryptose broth. AcMNPV variant L1 (Lee and Miller, 1978) and *hcf-1*$^{-}$ AcMNPV (Lu and Miller, 1996) obtained from L. Miller (University of Georgia, Athen, GA) were propagated in SF-21 cells.

Preparation of Cell-Free Translation Lysate

Ld652Y cells or BmN cells were infected with AcMNPV at a multiplicity of infection (MOI) of 10 plaque formation units (PFU) per cell. TN368 cells were infected

with *hcf-1*$^-$ AcMNPV at an MOI of 10 per cell. Mock-infected cells were treated in the same way as virus infected except that incomplete medium was used as inoculum. Time zero was defined as the time when the inoculum was removed and incubation at 27°C was initiated. Virus-infected cells were harvested at 8, 12, 16, 20, and 28 h p.i. Mock-infected cells were harvested at 20 h p.i. Cell-free translation lysates were prepared as described by Carroll and Lucas-Lenard (Carroll and Lucas-Lenard, 1993) with minor modification. 4 X 10^7 mock- or virus-infected cells were collected in 50-ml conical tubes and centrifuged at 500g for 3 min. The pellets were suspended in 12 ml ice-cold insect PBS (pH 6.2) (O'Reilly et al., 1992), transferred to a 15-ml conical tube, and again centrifuged for 2 min as above. The pellet was then suspended in 1 ml of cold insect PBS, transferred to a microfuge tube, and repelleted for 10 sec in the microfuge at 14,000g. Cells were kept in PBS in less than 10 min in all above washing steps.

The cell pellet was resuspended in 1 ml lysolecithin lysis buffer (20 mM HEPES-KOH, pH 7.4; 100 mM KAc; 2.2 mM $MgAc_2$; 2 mM DTT). The final volume was about 1.5 ml and 15 µl of 100 X lysolecithin (10 mg/ml) (Sigma, St. Louis, MO) was added and mixed by gentle pipetting. The cells were kept on ice for 50 sec (no longer than 1 min) and centrifuged for 10 sec at 14,000g in the microfuge. The pellet was resuspended in 0.5 ml cell mix (25 mM HEPES-KOH, pH 7.4; 125 mM KAc; 2.8 mM $MgAc_2$; 2.5 mM DTT; 1.25 mM ATP; 0.25 mM GTP; 187 µg/ml creatine phosphokinase; 37.5 mM creatine phosphate; 0.125 mM amino acid mixture minus methionine). The cells were lysed by pipetting them 25 times with a P1000 followed by passing them through a 22-gauge hypodermic needle attached to a 1 ml syringe 10 times. The lysed cells were left

on ice for 5 min, after which they were centrifuged for 20 sec at 14,000g. The supernatant was collected as cell-free translation lysate.

Fractionation of Lysate

To fractionate lysate from mock-infected Ld652Y cells, 0.5 ml of the lysate was ultracentrifuged at 31,000 rpm on a Beckman SW41 rotor for 2.5 hours. The supernatant was collected as ribosomal free supernatant fraction. The pellet was resuspended in 0.5 ml cell mix as the ribosomal fraction.

Isolation of Total RNA

Mock- or corresponding virus-infected Ld652Y cells, BmN cells, or TN368 cells described as above were harvested at 6, 12, and 24 h p.i. Total cellular RNAs were isolated by a single step method (Chomczynski and Sacchi, 1987).

In Vitro Translation Assays

The above lysate (20 μl) was used in in vitro translation assays to compose 80% in the total reaction volume (25 μl). The final reaction contained 20 mM HEPES-KOH (pH 7.4), 100 mM potassium acetate, 2.2 mM magnesium acetate, 2 mM DTT, 1 mM ATP, 0.2 mM GTP, 150 μg/ml creatine phosphokinase; 30 mM creatine phosphate; 20 units RNase inhibitor; 0.1 mM amino acid mixture minus methionine, and 0.4 mCi/ml ^{35}S methionine. For rescue assays with lysate from mock-infected cells, 10 μl of lysate from mock-infected cells was combined with 10 μl of lysate from virus-infected cells. For rescue assays with fractions of mock-infected Ld652Y cells, 10 μl of either supernatant fraction or ribosomal fraction was combined with 10 μl of lysate from AcMNPV-infected Ld652Y cells prepared at 20 h p.i. In all other rescue assays with total RNA or tRNA, 20

μl lysate of virus-infected Ld652Y cells prepared at 20 h p.i. was used. Fifteen μg of total RNA isolated from mock- or virus-infected cells was used for total RNA rescue assays and different amount of uncharged bulk tRNA (4, 8, 16, and 32 μg) from calf liver, yeast, or E. coli (Sigma, St. Louis, MO) was used for tRNA rescue assays. Endogenous mRNAs were the templates for protein synthesis in all in vitro translation assays. Incubations were carried out at 30 °C for 2 h after which 5 μl of the reactions was mixed with 20 μl 2 X protein sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue) and analyzed in 10% SDS polyacrylamide gels (Laemmli, 1970).

Examination of Total tRNA Adaptor Abilities

The adaptor abilities (amino acid acceptance abilities) of tRNA from mock- and AcMNPV-infected Ld652Y cells were determined according to Derwenskus and Sprinzl (1986) with some modifications. Total RNAs from mock- or AcMNPV-infected Ld652Y cells at 6 or 24 h p.i. were first deacylated to eliminate endogenous aminoacyl-tRNA by $CuSO_4$ treatment (Derwenskus et al., 1984). tRNAs were then aminoacylated in 20 μl buffer containing 50 mM HEPES (pH 7.6), 160 mM KCl, 1 mM ATP, 200 μM spermine, 2 mM $MgCl_2$, 50 μg/ml bovine serum albumin, 100 μM DTT, 35 μg total RNA, 20 μM of ^{14}C labeled amino acid mixture containing 15 amino acids (Ala, Arg, Asp, Gly, Glu, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Tyr, Val) (ICN Pharmaceuticals Inc., Costa Mesa, CA). After preincubation for 5 min at 37°C the aminoacylation reaction was started by addition of 14 units of yeast crude aminoacyl-tRNA synthetase (Sigma, St. Louis, MO). Aminoacylation reaction was performed at 37°C for 20 min after which samples were

precipitated with ethanol and the radioactivity retained measured by scintillation counting. The abilities of tRNA to accept amino acids were determined by retained radioactivities which was an indication of the amount of amino acids being charged to the tRNA. As background control, a tube containing only labeled amino acid mixture were performed in vitro aminoacylation together with the samples. The in vitro aminoacylation experiments were repeated three times with three different batches of total RNA preparation. The mean values were calculated and data were analyzed by standard t-test.

Results and Discussion

Establishment of Cell-Free Translation System from Insect Cells

To identify the translation defect in AcMNPV-infected Ld652Y cells, cell-free translation systems were established from both mock- and AcMNPV-infected Ld652Y cells at different times p.i. In vitro translation assays were carried out with these cytoplasmic lysates and protein translation abilities were monitored by including ^{35}S methionine in the in vitro translation reactions. The working hypothesis was that the lysate derived from mock-infected Ld652Y cells should display in vitro translation ability and that from AcMNPV-infected Ld652Y cells at late times p.i. when protein synthesis has been shut down should not. Then we would try to combine the lysates from both mock- and AcMNPV-infected Ld652Y cells and monitor the in vitro translation abilities. If the combined lysates displayed in vitro translation abilities, this may suggest a helper factor in the lysate from mock-infected Ld652Y cells; if the combined lysates did not display in vitro translation abilities, this may suggest a inhibitor in the lysate from AcMNPV-infected Ld652Y cells at late times p.i. By fractionating the lysate from either mock- or AcMNPV-infected Ld652Y cells at late times p.i., and testing each fraction for

its rescue or inhibiting ability, we would be able to identify the helper factor(s) or inhibitor(s). Lysate derived from mock-infected Ld652Y cells and lysates derived from AcMNPV-infected Ld652Y cells at early times p.i. displayed in vitro translation abilities (Figure 20, lanes 1 and 2). Lysates derived from AcMNPV-infected Ld652Y cells at late times p.i. beginning from 12 h p.i. did not display in vitro translation abilities (Figure 20, lanes 3-6). These observations were consistent with in vivo pulse labeling experiments which showed protein synthesis in AcMNPV-infected Ld652Y cells at early times p.i. and protein synthesis arrest at 12-16 h p.i. (Du and Thiem, submitted; Guzo et al., 1992). The different profiles of protein bands in the reactions of lysate from mock- and AcMNPV-infected Ld652Y cells presumably reflected the different composition of mRNAs in these lysates.

In vitro Translation Rescue Assays

Lysates from mock- and AcMNPV-infected Ld652Y cells at late times p.i. were then combined and the in vitro translation abilities were tested. Addition of lysate from mock-infected Ld652Y cells restored the in vitro translation abilities of the lysates derived from AcMNPV-infected Ld652Y cells at late times p.i. (Figure 20, lanes 7-10). To identify the putative helper factor(s) in the lysate derived from mock-infected Ld652Y cells, lysate from mock-infected Ld652Y cells was fractionated by ultracentrifugation and the resulting two fractions were tested for their rescue abilities. The ribosomal fraction that that presumably contained mostly insoluble factors and the ribosomes was able to rescue but the supernatant fraction which presumably contained mostly soluble factors was not (Figure 21, lanes 4 and 5). Moreover, total RNA isolated from mock- and AcMNPV-infected Ld652Y cells at early times p.i. but not late times p.i. could rescue

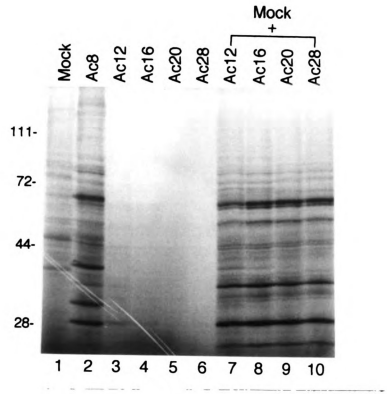

Figure 20. Autoradiogram Showing In Vitro Translation Abilities of Lysates From Mock- or AcMNPV-Infected Ld652Y Cells Protein size markers are indicated to the left of the panel. Mock: lysate derived from mock-infected cells; Ac8, Ac12, Ac16, Ac20, and Ac28: lysates derived from AcMNPV-infected Ld652Y cells at 8, 12, 16, 20, and 28 h p.i.

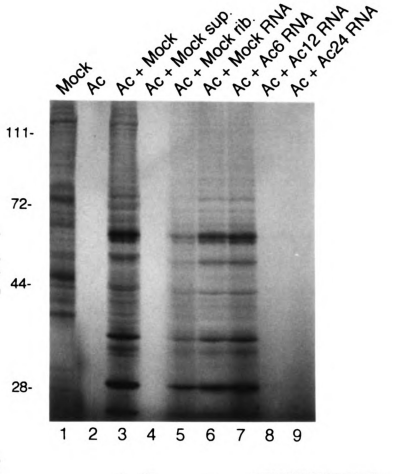

Figure 21. Autoradiogram Showing In Vitro Translation Rescue Abilities of Lysate Fractions and Total RNA Protein size markers are indicated to the left of the panel. Mock: lysate derived from mock-infected Ld652Y cells; Ac: lysate derived from AcMNPV-infected Ld652Y cells at 20 h p.i.; Mock sup.: supernatant fraction from mock-infected Ld652Y cells; Mock rib.: ribosomal fraction from mock-infected Ld652Y cells; Ac6 RNA, Ac12 RNA, and Ac24 RNA: total RNA isolated from AcMNPV-infected Ld652Y cells at 6, 12, and 24 h p.i.

(Figure 21, lanes 6-9).

Since protein synthesis in AcMNPV-infected Ld652Y cells is shut down in the presence of apparently normal mRNA (Guzo et al., 1992; Morris and Miller, 1993), the ability of total RNA to rescue implied a defect of either ribosomal RNA (rRNA), tRNA or some of the RNA required for translation. Uncharged bulk tRNAs from calf liver, yeast and E. coli were tested for their rescue abilities. Both eukaryotic tRNAs either from calf liver or yeast could rescue the in vitro translation ability of lysate derived from AcMNPV-infected Ld652Y cells (Figure 22). The ability to rescue increased with the increasing concentrations of tRNA. However, prokaryotic tRNA from E. coli showed only limited rescue ability at high concentration (Figure 22, lane 15). These data suggested a defect in tRNA in protein synthesis in AcMNPV-infected Ld652Y cells. The inability of prokaryotic tRNA to rescue may reflect an inability of eukaryotic and prokaryotic tRNAs to exchange for each other in the translation reactions.

Examination of the tRNA Adaptor Ability

The function of tRNA in protein synthesis is to carry cognate amino acids and insert them into the elongating protein chains in response to the codon in the mRNA. To fulfill this adaptor role, tRNA must be charged first with amino acids by the charging system during which the cognate amino acid is attached to the tRNA at its 3'-end. Since the tRNAs we used for rescue assays were uncharged, the charging system in AcMNPV-infected Ld652Ycells must be normal, otherwise, the uncharged tRNA could not rescue. Thus the defect most likely lies in the ability of tRNA from AcMNPV-infected Ld652Y cells to accept amino acids.

To investigate the tRNA adaptor ability in AcMNPV-infected Ld652Y cells, total

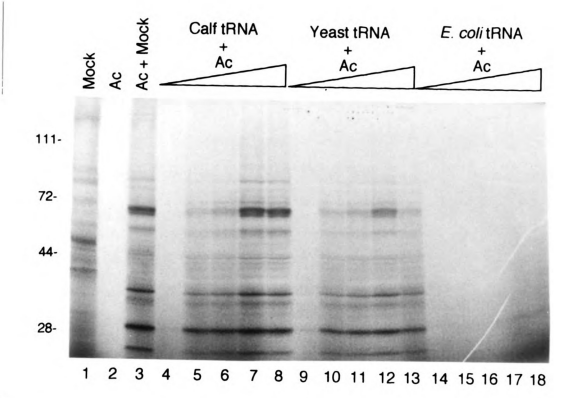

Figure 22. Autoradiogram Showing In Vitro Translation Rescue Abilities of tRNA Origin of tRNA is indicated at the top. Protein size markers are indicated to the left of the panel. Slope indicates the increment of tRNA added (4, 8, 16, 32 μg). Mock: lysate derived from mock-infected Ld652Y cells; Ac: lysate derived from AcMNPV-infected Ld652Y cells at 20 h p.i.

RNA isolated from mock- and AcMNPV-infected Ld652Y cells at early and late time p.i. were charged with ^{14}C labeled amino acid mixture in vitro by crude yeast aminoacyl-tRNA synthetase. The ability of the tRNA to accept amino acids was determined by the amount of labeled amino acids coprecipitated with RNA. Triple experiments were performed with different batches of total RNA and mean values were obtained. Data were analyzed by the standard t-test. A slight decrease of total tRNA adaptor ability was found in AcMNPV-infected Ld652Y cells at late time p.i. from that in mock- or AcMNPV-infected Ld652Y cells at early time p.i. (Table 4). However, this decrease was not significant from that in either mock- ($p = 0.2610 > 0.05$) or AcMNPV-infected Ld652Y cells at early time p.i. ($p = 0.2161 > 0.05$). These data suggest that the defect is not general but restricted to a single or a small group of tRNA species.

A Common Mechanism of Protein Synthesis Shut Down Appeared To Be Shared in AcMNPV-Infected Ld652Y and BmN Cells

To determine whether protein synthesis shut down observed in different nonpermissive AcMNPV infections shared a common mechanism, cell-free translation lysates were prepared from mock- or AcMNPV-infected BmN cells and mock- or *hcf-1*$^-$ AcMNPV-infected TN368 cells. In vitro cell-free translation assays were performed in the same way as for AcMNPV-infected Ld652Y cells. In AcMNPV-infected BmN cells, lysate from mock-infected BmN cells displayed in vitro translation ability (Figure 23, lane 1). Lysate derived from AcMNPV-infected cells at 20 h p.i. was not translation competent, however, its in vitro translation ability could be restored by addition of mock-infected BmN cells (Figure 23, lanes 2 and 3). Total RNA isolated from mock-infected BmN cells (Figure 23, lane 4) and bulk uncharged calf liver tRNA showed limited rescue

Table 4. tRNA Adaptor Abilities

tRNA	Retained Radioactivities (CPM)
Mock	3075.70 +/- 186.66
Ac6	3103.04 +/- 282.57
Ac20	2836.07 +/- 230.51

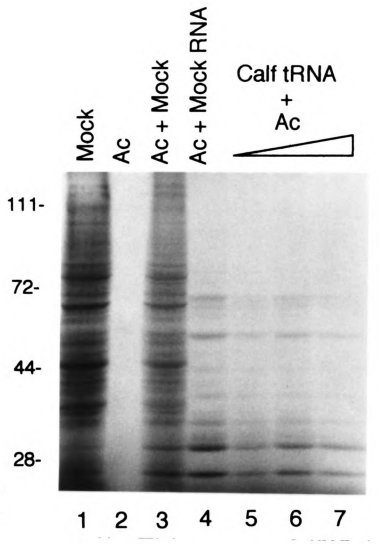

Figure 23. Autoradiogram Showing In Vitro Translation Assays in AcMNPV-Infected BmN Cells Mock: lysate from mock-infected BmN cells; Ac: lysate from AcMNPV-infected BmN cells prepared at 20 h p.i.; Mock RNA: total RNA isolated from mock-infected BmN cells; Slopes indicate the increment of tRNA added (4, 8, 16, 32 μg).

abilities(Figure 23, lanes 5-7). These data suggested that the defect in protein synthesis in AcMNPV-infected BmN cells is probably the same as that in AcMNPV-infected Ld652Y cells. However, we do not know why we observed a reduced rescue ability of both total RNA and tRNA in AcMNPV-infected BmN cells as compared to AcMNPV-infected Ld652Y cells. This may indicate an involvement of another factor.

In *hcf-1*$^{-}$ AcMNPV-infected TN368 cells, however, although in vitro translation ability of virus-infected cells could be restored by combining lysates from mock- and *hcf-1*$^{-}$ AcMNPV-infected TN368 cells, it could not be restored by either total RNA of mock- or virus-infected TN368 cells at early times p.i. or eukaryotic total tRNA (data not shown). These data indicated that the mechanism for protein synthesis shut down in *hcf-1*$^{-}$ AcMNPV-infectedLd652Y cells was different. This was not surprising since the response in *hcf-1*$^{-}$ AcMNPV-infected TN368 cells was quite different from that in AcMNPV-infected Ld652Y and BmN cells. The block in *hcf-1*$^{-}$ AcMNPV-infected TN368 cells involved defects in both DNA replication and late gene transcription in addition to protein synthesis shut down (Lu and Miller, 1996). In AcMNPV-infected Ld652Y and BmN cells, protein synthesis was shut down in the presence of apparently normal DNA replication, and late gene transcription (Guzo et al., 1992; McClintock et al., 1986; Morris and Miller, 1992; 1993).

Protein synthesis shut down has been reported in many systems as a strategy of host cellular defense (Hershey, 1991; Schneider and Shenk, 1987). The best understood mechanisms are those mediated by interferon in mammals and other vertebrates (Lengyel, 1982; Samuel, 1991). Interferon is a class of small molecular weight proteins synthesized and secreted in some cell types in mammals and other vertebrates in response

to viruses, as well as other stimuli (Lengyel, 1982). Interferon mediated protein synthesis shut down includes three different pathways (Zilberstein et al., 1978; Farrel et al., 1978) one of which is mediated by a 2'-5' phosphodiesterase (Schmidt et al., 1978). This enzyme degrades the CCA terminus of tRNA, resulting in minor tRNA deficiency and thereby blocking protein synthesis elongation (Schmidt et al., 1978). This inhibition of protein synthesis can be reversed by adding tRNA in cell-free translation system (Content et al., 1975; Sen et al., 1975; Zilberstein et al., 1976; Weissenbach et al., 1977; Samuel, 1976; Mayr et al., 1977; Falcoff et al., 1978).

Our observation is very similar to that of 2'-5' phosphodiesterase mediated protein synthesis shut down induced by interferon. However, interferon has so far not been reported in insects. We are currently trying to test whether the protein synthesis shut down in AcMNPV-infected Ld652Y cells is due to the same damage in tRNA as in that mediated by interferon. In the meantime, other possible damage in tRNA will be examined through identifying the individual or small group of defective tRNA species in AcMNPV-infected Ld652Y cells. HRF-1 protein is also being prepared in order to test its role in in vitro translation assays and in other functional assays. The fact that a same mechanism may be shared in AcMNPV-infected cell lines of different species suggested that a conserved host cellular defense response against viral infection has evolved in insect.

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