FUNCTIONAL CHARACTERIZATION OF A BACULOVIRUS FIBROBLAST GROWTH FACTOR

by

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B.S., King Mongkut's Institute of Technology Ladkrabang, Thailand, 1997

M.S., Kansas State University, 2001

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Baculoviridae is the only known virus family that encodes genes with homology to vertebrate and invertebrate fibroblast growth factors (*fgfs*), key regulators of developmental processes affecting cell growth, differentiation, and motility. The role of viral *fgfs* during infection is not known.

In this study, we investigated gene regulation and function of the *Autographa californica M* nucleopolyhedrovirus (Ac*M*NPV) *fgf* during infection of permissive insect cells. We demonstrated that the Ac*M*NPV *fgf*, *vfgf*, was transcribed as a 0.6-kb mRNA at early times post infection, but as part of a 1.4-kb bicistronic mRNA at late times. To determine its function, we examined common characteristics between vFGF and other well-characterized FGF homologs. vFGF had strong affinity to heparin, a property important for FGF signaling via an FGF receptor. vFGF was secreted into the extracellular fluid when expressed in insect cells, suggesting that it acts as an extracellular ligand. Finally, vFGF was able to stimulate chemokinesis of different types of insect cells.

We also constructed a recombinant of Ac*M*NPV lacking a functional v*fgf* and analyzed it in two insect cell lines. The kinetics of budded virus production were similar in the parental and *vfgf*-deficient viruses in two cell lines and at both high and low multiplicities of infection. In addition, we observed no obvious differences in the viral DNA synthesis and the protein kinetic profiles of cells infected with the mutant and parental viruses. Finally, coinfection of *vfgf*-containing and -deficient viruses and their passage for several generations did not reveal a consistent growth advantage for either

virus. We propose that vFGF is the signal that directs the motility of uninfected tracheal or blood cells to infected tissues, enabling the virus to infect additional cells and spread systemically in the insect host. This proposal may explain a dispensable role for *vfgf* during virus infection in cell culture; nonetheless, we expect a distinct phenotypic difference between *vfgf*-deficient and *vfgf*-containing viruses during infection in the insect host.

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CHAPTER 1

Literature review

LITERATURE REVIEW

Alternative strategies for pest control are needed to alleviate the excessive uses of chemical insecticides, since long-term usage of these chemicals can result in the development of insect resistance and affect human health and the environment. Using biological control agents like baculoviruses appears to be an ingenious choice, since they are not harmful to humans, other mammals, or beneficial insects and have a restricted host range and high pathogenicity. In addition, in molecular biology research, baculoviruses serve as an invaluable tool for foreign gene expression and as a model to study basic processes in virology.

Baculovirus classification

Baculoviruses belong to the family *Baculoviridae*. Members of this family infect only arthropods, mainly insects in the order Lepidoptera (moths and butterflies) (Adams *et al*., 1991), but also have been shown to infect insects in the orders Diptera (mosquitoes) and Hymenoptera (sawflies), and crustaceans in the order Decapoda (shrimp) (Possee *et al*., 1997; Theilmann *et al.*, 2005).

The family *Baculoviridae* consists of two genera, nucleopolyhedrovirus (NPV) and granulovirus (GV). More recently, it was proposed that *Baculoviridae* can be divided into 4 genera based on host specificity: Alphabaculovirus (lepidopteran-specific NPVs), Betabaculovirus (lepidopteran-specific GVs), Gammabaculovirus (hymenopteran-specific NPVs), and Deltabaculovirus (Dipteran-specific NPVs) (Jehle *et al*., 2006).

The NPVs have been studied more extensively than GVs. The *Autographa californica M* nucleopolyhedrovirus (Ac*M*NPV) is the best-studied NPV, therefore this review will mostly focus on the knowledge obtained from studies with Ac*M*NPV.

Baculovirus genomes and genomic structures

Baculoviruses are enveloped viruses that have large circular double-stranded DNA genomes ranging from 80 to 180 kilobasepairs (kbp) and encode about 80 to 180 genes. A distinct feature of the baculovirus genome is the presence of homologous regions (*hrs*), a repeated region made up of imperfect palindromic sequences flanked with direct repeats, which have been prescribed to function as viral origins of DNA replication and as transcriptional enhancers (Guarino and Summers, 1986; Nissen and Friesen, 1989; Pearson *et al*., 1992; Leisy and Rohrmann, 1993; Kool *et al*., 1995). The approximate size of the nucleocapsid is 30-60 nanometers (nm) in diameter and 250-300 nm in length (Theilmann *et al*., 2005).

Two phenotypes of baculoviruses are produced during infection of cells, occlusion-derived virus (ODV) and budded virus (BV).

ODV resides inside a protective crystalline protein matrix called occlusion bodies or polyhedra. This structure allows virions to withstand the harsh environment for years and thus it ensures efficient horizontal transmission of viruses from insect to insect in the wild. Occlusion bodies of the NPVs range from 0.15 to 15 micrometers (μm) in size and contain more than one ODV embedded in the polyhedrin matrix while, in GVs, their occlusion bodies, granules, are

approximately 0.3 x 0.5 μm and only have a single ODV coated with granulin protein (Funk *et al*., 1997; Theilmann *et al*., 2005).

BV, the other virus phenotype, contains only one nucleocapsid inside an envelope derived from the viral-modified membrane of the host. BV is responsible for spreading infection between tissues inside the insect host and also is highly infectious in cell culture; BV has approximately 1700-fold higher infectivity than that of the ODV (Volkman *et al*., 1976).

Baculovirus attachment and entry

The envelope composition of BV and ODV are not similar and this is related to their roles in infecting different target cells, infectivity under different physiological conditions, and their different mechanisms of entry (Rohrmann 1992; Funk *et al*., 1997).

BV entry

BVs enter host cells by absorptive endocytosis in a low-pH dependent manner (Volkman and Goldsmith, 1985; Hefferon *et al*., 1999; Long *et al.*, 2006). Additionally, detailed studies by Long *et al*. (2006) suggested that BV uses clathrin-mediated endocytic process to enter the cell.

There are two types of BV envelope fusion proteins found in baculoviruses, GP64 and F protein (Pearson and Rohrmann, 2002). These proteins play a major role in the entry process. Sequence analysis indicated that, while only some baculoviruses have GP64, all baculoviruses isolated from lepidopterans and dipterans encode F protein homologs. Surprisingly, F protein was absent in three hymenopteran baculoviruses, *Neodiprion lecontei*, *N.*

sertifer, and *N. abietis* (Garcia-Maruniak *et al*., 2004; Lauzon *et al*., 2004; Duffy *et al*., 2006). NPVs that encode both types of proteins putatively prefer employing GP64 for their entry than F protein (Lung *et al*., 2003). Those that do not have GP64 presumably use F protein to facilitate their entry (Pearson and Rohrmann, 2002).

Ac*M*NPV encodes both GP64, a 59-kilodalton major envelope protein with highly glycosylated and acylated residues, and F protein. It has been shown that GP64 is required for Ac*M*NPV BV infectivity (Monsma *et al*., 1996), and its function could not be replaced by the Ac*M*NPV F protein, *Ac23* (Lung *et al*., 2003). BV acquires GP64 when it buds out through the plasma membrane of the cell. GP64 is synthesized both at the early and late phases of infection, which is not a typical expression pattern for other viral structural proteins (Volkman and Goldsmith, 1985; Blissard and Rohrmann, 1989; Whitford *et al*., 1989; Blissard and Wenz, 1992, Monsma and Blissard, 1995).

ODV entry

The advent of insect cell culture systems permissive for virus replication has made it feasible to study the mechanism of BV entry more easily, while a clear understanding of the process of ODV entry into midgut cells is still lacking due to technical difficulties with *in vivo* studies.

Early electron microscopy studies suggested that ODV entered midgut cells by fusion between the ODV envelope and microvilli of midgut columnar cells (Summers *et al*., 1971; Tanada *et al*., 1975; Adams *et al*., 1991; Federici *et al*., 1997). Later, three per os infectivity genes, *pif1*, *pif*2, and *p74*, were identified as

structural ODV proteins that were required for infectivity of ODV but not for BV. PIF1 and P74, components of the ODV envelope, function as attachment proteins (Kuzio *et al*.,1989; Faulkner *et al*., 1997; Ohkawa 1997; Kikhno *et al*., 2002). Recent *in vivo* studies using fluorescent dequenching assays demonstrated that all three proteins, PIF1, PIF2, and P74 played critical roles in the specific binding of ODV to midgut cells (Haas-Stapleton *et al*., 2004; Ohkawa *et al*., 2005). Interestingly, the absence of any one of them resulted in a defect in ODV binding but did not affect its fusion ability (Haas-Stapleton *et al*., 2004; Ohkawa *et al*., 2005). In addition, ODV entry apparently occurs in the high alkaline milieu of the midgut lumen which contrasts to a typical neutral or low-pH dependent entry pathway used by other enveloped viruses or even by BV of baculovirus (Eckert *et al*., 2001; Smith *et al*., 2004). Because of this, Ohkawa *et al*. (2005) suggested that ODV may utilize a novel mechanism to fuse with the midgut cells or other viral proteins may be involved in the fusion process.

Baculovirus gene regulation

Baculovirus gene regulation is under temporal control and can be divided into 3 phases, early, late, and very late.

Early gene transcription rapidly takes place after the release of the viral genome into the nucleoplasm and is performed by host RNA polymerase II (Fuchs *et al*., 1983; Hoopes and Rohrmann, 1991; Huh and Weaver, 1990a,b). Early transcription can occur in the presence of the protein synthesis inhibitor,

cycloheximide, suggesting that newly-made viral proteins are not required to launch this process.

In contrast, late gene expression is accomplished by the function of a viral-encoded RNA polymerase. Viral DNA replication is a prerequisite as demonstrated by inhibition of late gene transcription in the presence of aphidicolin, a DNA replication inhibitor. While many early genes function in DNA replication and transcription, late and very late genes mostly encode structural proteins. BV is made at late times by budding out through the modified plasma membrane containing GP64. Subsequently, at very late times when the polyhedrin protein is produced abundantly, the virus machinery shifts to produce the ODV phenotype. In contrast to BV, ODV is enveloped and occluded inside the nuclei (Stoltz *et al*., 1973; Tanada and Hess, 1976; Volkman *et al*., 1976). A product of a very late viral gene, P10, is then required for maturation and release of the occlusion bodies from the nuclei (Williams *et al*., 1989; Van-Oers *et al*., 1993, 1994).

Baculovirus genome replication

It is thought that baculovirus genome replication may occur via two pathways, rolling circle or theta mode. Alternate origins of DNA replication can be used including *hr*, *non-hr,* and RNA polymerase II promoters (Okano *et al*., 2006). Viral-encoded genes required for DNA replication in transient replication assays include late expression factor-1 (LEF-1) (a primase), LEF-2 (a primase accessory factor), a helicase, a DNA polymerase, LEF-3 (a single-stranded DNA binding protein, that also transports the

helicase to the nucleus) (Wu and Carstens, 1998 and Chen and Carstens, 2005), and IE-1, a transcriptional transactivator and origin of replication-binding protein (Kool *et al*., 1994; Lu and Miller, 1995; Okano *et al*., 2006). In addition, other viral genes are also required to complete the production of an infectious genome. Alkaline nuclease (*an*) and very late expression factor-1 (*vlf-1*) have been proposed to work in concert during genome processing. AN possesses 5' to 3' nuclease activity to generate 3' single-stranded ends. These ends were predicted to be involved in DNA recombination for completion of a full-sized genome (Okano *et al*., 2004, 2006). VLF-1, a putative tyrosine recombinase, has been shown to be essential for the final stages of DNA packaging as well as for normal capsid assembly (Vanarsdall *et al*., 2006; Okano *et al*., 2006).

Role of actin polymerization during baculovirus infection

It has long been known that during baculovirus infection, actin was accumulated and polymerized inside the nucleus of infected cells and interfering with this process resulted in an aberration of nucleocapsid assembly and infectious progeny production (Charlton and Volkman 1991). After entry, cytoplasmic filamentous actin (F-actin) helps shuttle the nucleocapsid to the nucleus (Lanier and Volkman, 1998). Very recently, a well-defined work from Goley *et al*. (2006) revealed the detailed process of nuclear actin polymerization during Ac*M*NPV infection. These authors demonstrated that a viral factor that played a key role in this process was the P78/83 protein. P78/83 was previously identified as a minor capsid protein that preferentially bound F-actin over G-actin inside the nucleus (Lanier *et al*., 1998). P78/83 contains a motif with homology to Wiskott-

Aldrich syndrome protein (WASP), a factor that binds and activates the Arp2/3 complex, the seven-subunit protein that serves as a nucleation site for actin polymerization and for branched actin filaments (Machesky *et al*., 2001). Upon infection by Ac*M*NPV, monomeric G-actin has been shown to accumulate in the nuclei of infected cells (Ohkawa *et al*., 2002). Work by Goley *et al*. (2006) showed that P78/83 was required to activate Arp2/3 that had been recruited into the nucleus, resulting in the polymerization of recruited G-actin to form nuclear F-actin which is essential for progeny viral production. Mutations in p78/83 that caused dramatic reduction in its nucleation-promoting factor (NPF) activity completely disrupted progeny virus production. Some p78/83 mutants that have moderate NPF activity retained the ability to produce progeny virus but severely impaired its titer. These mutants had severe defects in the pattern of virion assembly including irregular nucleocapsid alignment within an envelope, lack of envelope, or an empty envelope (with no nucleocapsid). This finding thus underlined a critical role of nuclear actin polymerization during baculovirus infection (Ohkawa and Volkman, 1999; Goley *et al*., 2006).

Baculovirus pathogenesis of the insect host

Infection begins when the insect larvae ingest occlusion bodies, subsequently, the high alkaline pH (~11) in the midgut promptly dissolves the polyhedrin matrix, resulting in the release of embedded ODVs. ODV passes the first physical barrier, the peritrophic membrane, a layer made of chitin and mucin lining the gut. A mechanism that ODV utilizes to pass through the peritrophic membrane with approximately 8-9 nm pore size (Terra, 2001) remains controversial.

Many possibilities of how the virus overcomes the peritrophic membrane have been discussed. Washburn *et al*. (1995) suggested that the absence of the peritrophic membrane during molting allowed the virus to access and infect midgut cells but this proposal has been argued against by the fact that the virus is mostly ingested during intermolt stages. Enhancin, a baculoviral-encoded protein, was shown to be a component that degraded the peritrophic membrane and resulted in an increase in virus infectivity (Wang *et al*., 1994). However, enhancin is not present in all baculovirus species suggesting that this protein is not the sole factor to allow bypassing the peritrophic membrane and other strategies may be involved. In addition, Washburn *et al*. (1995) suggested that the presence of the peritrophic membrane did not play an important role in the resistance to virus infection of *Trichoplusia ni* and *Heliothis virescens*, highly permissive hosts to Ac*M*NPV infection, because both species have similar susceptibility during infection at the same developmental larval stage even if the peritrophic membrane was absent in *T. ni* but present in *H. virescens*. In contrast, in some lepidopteran hosts, the optical brightener M2R has been shown to enhance oral infectivity by reducing midgut cell sloughing and disrupting the peritrophic membrane, suggesting that the peritrophic membrane and midgut play important roles in the defense mechanisms of these hosts (Shapiro and Argauer, 1997; Washburn *et al*., 1998; Wang and Granados, 2000).

After crossing the peritrophic membrane, ODV then establishes the primary infection in midgut epithelial cells by fusion between the ODV envelope and microvilli of midgut columnar cells (Summers *et al*., 1971; Tanada *et al*., 1975; Adams *et al*., 1991; Federici *et al*., 1997). The nucleocapsid is then released and travels to the nucleus

where DNA is uncoated and subsequent virus replication occurs. BV is produced, enveloped, and exits from the basal side of midgut cells.

Alternatively, evidence suggested that some uncoated ODV may not enter the nucleus of cells but migrated directly to the basal side of midgut cells, re-enveloped, and shed out. This repackaging strategy has been supported by subsequent studies in which a swift infection, within a few hours after the midgut infection, was detected in tracheal cells. This occurrence is too rapid for the virus to complete a round of replication and assemble into a progeny BV (Granados and Lawler, 1981; Granados and Williams, 1986; Englehard *et al*., 1994; Flipsen *et al*., 1995; Washburn *et al*., 1995, 1999, 2003; Zhang *et al*., 2004). In addition, the repackaging strategy was also supported by the early synthesis of GP64 for the ODV-derived nucleocapsid to acquire a new envelope with GP64 earlier. This strategy provides the virus rapid escape from the midgut to spread infection to the hemocoel because prolonged time in the midgut cells evokes the risk of the virus being eliminated by midgut-sloughing, one of the major host defenses to baculovirus infection (Zhang *et al*., 2004).

Up to this point, the next question is where the BV goes after budding from midgut cells. To date, it remains unknown which is the mechanism and factors that baculoviruses use to go from midgut cells to other cells or tissues and establish a systemic infection. The midgut is lined by a major physical barrier, the midgut basal lamina, an extracellular matrix at the basal side of the midgut. Basal laminae also surround all of the organs of the insect. Treatment of *Bombyx mori* pupae with clostridial collagenase, a basal lamina-degrading enzyme, has been shown to increase susceptibility of its follicular cells to baculovirus infection (Smith-Johannsen *et al*., 1986).

Also, Harrison and Bonning (2001) showed expression of cathepsin, a protease from flesh fly known to degrade basal lamina components, accelerated the killing speed of Ac*M*NPV in *H. virescens*. It remains unknown what strategy baculoviruses utilize to overcome the basal lamina barrier that presumably excludes a particle larger than 15 nm (Reddy and Locke, 1990). Many attempts have been made to unravel this puzzle.

In earlier studies, it was proposed that BV produced from midgut cells may cross the basal lamina directly into the hemolymph and use it as a major route to spread infection to other tissues (Federici, 1997). An alternative mechanism was proposed by Engelhard *et al*. (1994) that the virus uses the tracheal system as a detour to disseminate infection throughout the host. They demonstrated that tracheolar cells that serviced the midgut were infected shortly after midgut cells were infected and subsequently the whole tracheal system was fully infected when larvae were fed with a recombinant Ac*M*NPV expressing *lacZ*. Furthermore, infection of other tissues or organs has been shown to initiate at the area adjacent to tracheal branches (Engelhard *et al*., 1994; Washburn *et al*. 1995, 2003). These observations supported a proposal that the tracheal network is a major route for disseminating virus infection. Nonetheless, it remains to be explained how the virus infects tracheolar cells because these cells are also known to be surrounded by basal lamina.

At very late stages of infection, most tissues and organs are infected and the production of ODV becomes prominent and can be observed by the deposition of occlusion bodies inside in the nucleus of cells. Finally, the infected insect dies, liquefies, and releases billions of occlusion bodies for the next round of horizontal transmission. Liquefaction of the infected host occurs through the functions of two viral-encoded

genes, *chitinase* (*chi A*) and *cathepsin* (*cath*). Deletion of either gene resulted in non-liquefied cadavers, but when the mutants lacking either *chiA* or *cath* were co-infected, disintegration of infected hosts was resumed (Ayres *et al*., 1994; Hawtin *et al*., 1995,1997; Rawlings *et al*., 1992; Slack *et al*., 1995; Thomas *et al*., 1998, 2000; Saville *et al*., 2004).

Fibroblast growth factors (FGFs)

FGF was initially named after the polypeptide isolated from the pituitary gland and brain extract that could induce cell division in mammalian 3T3 cells (Armelin, 1973; Gospodarowicz, 1974). Consequently, many proteins that shared common structures and functions have been grouped together and currently make up a large FGF protein family. FGFs are conserved among eukaryotes spanning from nematodes to humans but have not been identified in unicellular organisms. Surprisingly, *fgf* homologs have been identified in baculovirus genomes. Key features that classify proteins as members of the FGF family include: 1) high similarity in the internal core sequence, a region that determines their binding to FGF receptors (FGFRs), 2) presence of a signal peptide that destines them for secretion where they serve as extracellular ligands, and 3) strong affinity to heparin or heparan-sulfate like glycosaminoglycans (HLGAGs). HLGAGs are required to mediate the interaction between FGF and its receptor and have also been proposed to function to protect FGF from proteolysis. In addition, HLGAGs may serve as a pool for storing and localizing FGF ligands near the cell surface (Faham *et al*., 1996, 1998; Powers *et al*., 2000; Ornitz and Itoh 2001). In general, FGF functions as a signal molecule. Once secreted, it binds to HLGAGs in the extracellular matrix forming

FGF-HLGAGs complexes. The complexes then bind to FGFRs which are transmembrane tyrosine kinase receptors. This interaction leads to receptor phosphorylation and dimerization, resulting in the activation of a downstream signal transduction cascade (Powers *et al*., 2000; Ornitz and Itoh 2001).

Functions of FGFs have been shown to involve a variety of biological processes. FGFs play important roles during embryonic development such as induction of embryonic cell division, formation of germ layers, organogenesis, nervous system development, chemotactic effect on branching morphogenesis of the lung epithelium (Warburton *et al*., 1999), and limb development. FGFs also function in maintaining homeostasis, in particular, wound repair as shown by their roles in the migration of inflammatory cells (Bryd *et al*., 1999), controlling platelet formation (Konishi *et al*., 1996; Zhou *et al*., 1998), proliferation of new endothelial and fibroblast cells, migration of endothelial cells, and regeneration of epithelial cells (Powers *et al*., 2000). Tumorigenesis can arise via malfunction of FGFs controlling cell proliferation, inducing angiogenesis to a growing tumor, and/or inhibiting apoptosis of tumor cells (Powers *et al*., 2000).

FGF homolog in insects

The *Drosophila* tracheal system forms a tubular branching network from tracheal epithelial cells and can be viewed in three major stages including primary, secondary, and terminal branching. In contrast to terminal branching, primary and secondary branching are pre-determined processes.

Branchless (*bnl*), an FGF homolog in *Drosophila*, has been identified as a key factor in controlling tracheal branching and patterning. BNL plays key roles and is required throughout all stages of *Drosophila* tracheal development and morphogenesis. Since tracheal branching does not involve cell proliferation, *bnl* has been shown not to function as a mitogen during this process (Sutherland *et al*., 1996). BNL, secreted from cells surrounding the developing trachea sacs, activates Breathless (BTL), a product of the *btl* gene which encodes the FGFR and is expressed on the developing tracheal cells, and chemoattracts, directs, and specifies the migration and patterning of growing tracheal cells to form specified primary branches (Klambt *et al*., 1992; Reichman-Fried *et al*., 1994; Lee *et al*., 1996). Loss of function mutations in either *btl* or *bnl* resulted in a defect in tracheal branching (Klambt *et al*., 1992; Sutherland *et al*., 1996). BNL activates its receptor in a concentration-dependent gradient manner. Migration of tracheal cells can respond to the BNL source both when the source touches the growing trachea cells or even some distance away (Sutherland *et al*., 1996). Once the primary branches reach the cells producing *bnl*, the expression level of *bnl* dramatically decreases. Migration of tracheal cells to form primary branches occurs rapidly within an hour after expression of *bnl* detection in the neighboring cells, suggesting *bnl* activated pre-existing components rather than regulated gene transcription (Sutherland *et al*., 1996; Shilo *et al*., 1997; Metzger and Krasnow, 1999).

During secondary branching, instead of stimulating cell migration, *bnl* changes its role to induce the expression of another set of genes that are required to complete secondary branching (Lee *et al*., 1996; Sutherland *et al*., 1996). Formation of secondary and terminal branches occurs at the tip of primary and secondary branches,

respectively, and those branches are initiated from the cytoplasmic extension of a single cell.

Unlike primary and secondary branches, terminal branching is regulated by oxygen levels. When larval tissues experience low oxygen conditions (hypoxia), cells of those oxygen-starved tissues are signaled to up-regulate *bnl* expression. BNL then diffuses to nearby tracheal cells, and again functions as a chemoattractant, stimulating migration of terminal branches toward the BNL source in order to oxygenate the hypoxic tissues (Jarecki *et al*., 1999).

During the late larval stage of *Drosophila*, the air sacs of the dorsal thorax are formed to prepare for the transition to the adult tracheal system. Again, *bnl* repeats its role in directing migration of tracheal cells by extending their filopodia and lamellopodia toward the BNL sources (Sato and Kornberg, 2002; Cabernard *et al*., 2005). Initially, Sato and Kornberg (2002) reported that BNL did not function only in tracheal cell migration but also induced cell proliferation during adult tracheal system formation. In contrast, Cabernard *et al.* (2005) found that BNL did function in stimulating migration of tracheal cells to form air sacs, but cell proliferation is not associated with its function nor did it occurr during this process.

Baculovirus FGFs

Baculoviruses are the only viruses that encode *fgf* homologs (Ayres *et al*., 1994; Sutherland *et al*., 1996). To date, thirty-six baculovirus genomes have been sequenced and it appears that all but four contain at least one copy of *fgf*. Thirty-two species that have *fgfs* infect insects in the order Lepidoptera. Among the four viruses in which *fgfs*

are absent, three of them, *Neodiprion leconti* NPV, *N. sertifer* NPV, and *N. albeitis*, infect hymenoptera (sawflies) and one, *Culex nigripalpus* NPV, infects a dipteran (mosquito) (Afonso *et al*., 2001; Garcia-Maruniak *et al*., 2004; Lauzon *et al*., 2004; Duffy *et al*., 2006). Interestingly, these four viruses have different infection patterns from the lepidopteran baculoviruses in which the infection is restricted to midgut eplithelial cells and is unable to spread systemically into the tracheal system and the hemocoel (Federici, 1997; Moser *et al*., 2001).

In this study, we present work done on Ac*M*NPV *fgf*. In chapter 2, we report biochemical characteristics and functional properties of Ac*M*NPV *fgf*, and in chapter 3, we constructed an Ac*M*NPV recombinant lacking a functional *fgf* and characterized it in cell culture. The possible function of Ac*M*NPV *fgf* during infection is discussed.

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CHAPTER 2

Stimulation of cell motility by a viral fibroblast growth factor homolog:

Proposal for a role in viral pathogenesis

ABSTRACT

The *Autographa californica* nucleopolyhedrovirus (Ac*M*NPV) encodes a gene (open reading frame 32) with homology to vertebrate and invertebrate fibroblast growth factors (*fgf*s), key regulators of developmental processes affecting the growth, differentiation, and migration of many cell types. We studied the temporal regulation of the Ac*M*NPV *fgf*, *vfgf*, by Northern (RNA) blot hybridization; *vfgf* was transcribed as a 0.6-kb mRNA at early times but as part of a 1.4-kb bicistronic mRNA at late times. The product of *vfgf*, vFGF, exhibited a number of characteristics that have also been demonstrated for other FGF homologs. vFGF had strong affinity to heparin, a property important for FGF signaling via an FGF receptor. vFGF was secreted into the extra-cellular fluid when expressed in insect cells, suggesting that it acts as an extracellular ligand. Finally, vFGF was able to stimulate migration of several different types of insect cells. We discuss how this activity may be important for its function during virus infection.

INTRODUCTION

Fibroblast growth factors (FGFs) are a family of growth factors widespread in organisms ranging from nematodes to humans; however, they are not present in unicellular organisms (Ornitz and Itoh, 2001; Powers *et al.*, 2000). In general, the structure of FGFs is conserved, and they share a core region of similarity throughout 140 amino acids in length, including 28 highly conserved amino acids, of which a subset interact with the receptor (Ornitz and Itoh, 2001). Most, but not all, FGFs encode a terminal signal peptide and are secreted from cells. FGFs have an array of functions working as mitogens, motogens, and differentiation factors. In addition, they have crucial roles in many developmental processes that include neural cell differentiation, mesoderm induction, limb formation, osteogenesis, angiogenesis, and branching morphogenesis during the development of lungs, seminiferous tubules, and insect trachea. Inappropriate expression of some *fgfs* may lead to the pathogenesis of cancer. FGFs function by binding heparin or heparan sulfate proteoglycans to form oligomers and this complex interacts specifically with FGF receptors (FGFRs), transmembrane tyrosine kinases that are activated upon FGF binding, leading to receptor dimerization and autophosphorylation. The activated FGFR then stimulates signal transduction pathways (reviewed in Powers *et al.*, 2000).

The prototype baculovirus, *Autographa californica* M nucleopolyhedrovirus (Ac*M*NPV), contains a large DNA genome of 133,894 base pairs (bp) encoding about 150 genes (Ayers *et al.*, 1994), and infects insects in the order Lepidoptera. Ac*M*NPV produces two forms of the virus during its replication cycle with identical genetic makeup

and nucleocapsid structure: budded virus (BV) and occluded derived virus (ODV). The BV is produced during the late phase of infection where viral DNA and proteins are packaged to form nucleocapsids that bud through the plasma membrane, thus acquiring an envelope (Adams *et al.*, 1977). The BV spreads systemically within the insect and is also the form of the virus that is used in cell culture. The ODV is produced during the very late phase of infection where nucleocapsids remain in the nucleus, are enveloped, and then are embedded in a matrix that protects the virion in the environment. When the infected insect dies, it releases occlusion bodies that another insect ingests (O'Reilly *et al.*, 1992).

Studies on baculovirus pathology concluded that after infection of midgut cells, tracheal epithelial cells become infected, and then BV appears in the hemolymph followed by the infection of other tissues (Keddie *et al.*, 1989; Trudeau *et al.*, 2001). The hemolymph may be the main route of BV distribution in some infected insects (Adams *et al.*, 1977; Granados and Lawler, 1981). It has been proposed that the tracheal system of the insect serves as a conduit for systemic infection as the virus crosses the basal lamina into the hemocoel of the insect (Engelhard *et al.*, 1994). Other studies suggest that the BV directly passes through the basal lamina into the hemolyph were the virus disseminates (reviewed in Federici, 1997).

Upon infection of insect cell lines with Ac*M*NPV, three major classes of transcripts, early, late, and very late, are apparent (O'Reilly *et al.*, 1992). Early transcripts encode factors necessary for DNA replication and transcription of late viral genes, as well as auxiliary genes that have roles in virus-host interactions. After DNA replication, late and very late genes are transcribed by a viral RNA polymerase activity

(Glocker *et al.*, 1993; Grula *et al.*, 1981; Huh and Weaver, 1990). Translated late and very late products include, but are not limited to, structural proteins.

The Ac*M*NPV open reading frame (orf) 32 has homology to fibroblast growth factors (FGFs) (Ayers *et al.*, 1994), and it is conserved in all baculoviruses that have been sequenced to date that infect insects in the order Lepidoptera (Garcia-Maruniak *et al.*, 2004; Lauzon *et al.*, 2004). However, *fgf* homologs are absent from baculoviruses that infect orders other than Lepidoptera: *Culex nigripalpus* NPV that infects insects in the order Diptera (mosquitoes) (Afonso *et al.*, 2001), and *Neodiprion sertifer* NPV and *N. lecontei* NPV that infect insects in the order Hymenoptera (sawflies) (Garcia-Maruniak *et al.*, 2004; Lauzon *et al.*, 2004). The Ac*M*NPV *fgf*, *vfgf*, was first discovered by sequence homology when the complete nucleotide sequence of Ac*M*NPV was derived (Ayers *et al.*, 1994), however, its function during infection is not known. A study of the BmNPV *fgf* indicated that it was expressed at early times post infection (p.i.) using reverse transcriptase-polymerase chain reaction analysis but the size of the transcripts or temporal expression in the presence of protein and DNA replication inhibitors was not presented (Katsuma *et al.*, 2004). This report also concluded that the BmNPV FGF was secreted and glycosylated, but its functionality was not addressed (Katsuma *et al.*, 2004). In this study we characterize in detail the temporal expression and transcripts of *vfgf*, evaluate biochemical characteristics of its product that correlate with the function of other known FGFs, and assess its properties for chemoattraction of cells. Based on these characteristics, we propose a role for vFGF in viral pathogenesis, but emphasize that other roles are also possible.

RESULTS

Baculovirus *fgfs*

vfgf is present in all baculoviruses sequenced to date that infect lepidopterans, but it is absent in the three baculoviruses that have been completely sequenced to date that infect other insects. Fig. 1 shows an alignment of the predicted polypeptide sequence of *vfgfs* in selected group I and II NPVs and granuloviruses, and compares them to the sequence of the *Drosophila* FGF branchless and a human FGF, highlighting a conserved signal sequence, and a heparin binding-growth factor/FGF family domain (Burgess and Maciag, 1989; Thomas, 1988). Potential N-glycosylation sites in most, but not all, FGFs are indicated.

Northern blot analysis of *vfgf* transcripts

We determined the temporal regulation and overall size of *vfgf* mRNAs by Northern blot analysis. Inspection of sequences upstream of the *vfgf* ORF revealed the presence of a TATA box 29-bp upstream of the translational start codon. We infected cells with Ac*M*NPV at a multiplicity of infection of 20 PFU per cell, purified total RNA at different times p.i., and performed Northern blot analysis using an RNA-specific probe derived from the *vfgf* ORF as described in Materials and Methods (Fig. 2A).

A 0.6-kb transcript was first observed at 3 h p.i. and remained until 12 h p.i. when a probe complementary to the *vfgf* orf was utilized (Fig. 2B). At 24 and 48 h p.i., transcription slightly decreased. Transcription was also observed in the presence of cycloheximide, a protein synthesis inhibitor, indicating that prior protein synthesis was

not required for *vfgf* transcription. This is indicative of early genes, where synthesis is dependent on host proteins and/or viral proteins carried in the virus particle but not on viral gene products that need to be synthesized after virus infection. Similarly, *vfgf* was transcribed in the presence of aphidicolin, a DNA synthesis inhibitor. Late and very late viral genes require viral DNA replication for expression. Thus, since *vfgf* is transcribed at early times p.i. and in the presence of cycloheximide and aphidicolin, it can be designated an early gene. The early 0.6-kb RNA agrees with the predicted size for an RNA corresponding to the 546-nt *vfgf* orf, given that the putative transcriptional start site and polyadenylation signals are fairly close the beginning and end of the orf, respectively.

In addition, two prominent larger late RNAs of 1.4- and 3.1-kb were observed starting at 9 to 12 h p.i. (Fig. 2B). These late transcripts also hybridized to a probe complementary to orf 33 (Fig. 2C). In agreement with late viral genes, these RNAs were not transcribed either in the presence of cycloheximide or aphidicolin. Interestingly, *vfgf* may be transcribed at late times as part of a 1.4-kb bicistronic mRNA, since this mRNA hybridized to probes complementary to orfs 32 and 33. The gene upstream of and in the same direction of *vfgf*, orf 33 (550-nt in length), does not appear to contain a polyadenylation signal at its 3' end and its transcripts may coterminate with the *vfgf* early mRNA using the polyadenylation signal downstream the *vfgf* ORF (17 nts downstream of the translational stop codon for *vfgf*). We do not know the identity of the larger 3.1-kb RNA and did not pursue it any further in this study. Less abundant late mRNAs were also apparent (Fig. 2B and C, lanes 24). The first late transcriptional start sites upstream of the *vfgf* orf are present 202- and 15-nt upstream

of the translational start codons of *vfgf* and orf 33, respectively. Whether these mRNAs utilize either of these late transcriptional start sites was not investigated. We also performed primer extension analysis and observed two early primer extension products that terminated 12- and 13-nt upstream of the translational ATG codon of the *vfgf* orf (results not shown). A short leader sequence starting at 10 nucleotides upstream of the translational start codon was also observed in the BmNPV *fgf* (Katsuma *et al.*, 2004).

vFGF production and secretion

We constructed a plasmid, pHSFGFHA, expressing *vfgf* with an in-frame C-terminal HA epitope tag from the *Drosophila* hsp 70 promoter. SF-21 cells were transfected with pHSFGFHA and protein lysates from either the cell pellet or the culture supernatant were resolved by SDS-PAGE and immunoblotted with anti-HA monoclonal antibody. We found that a protein of about 20-kDa, in agreement with the predicted size for vFGF, was expressed from this plasmid. vFGF was found in the cell lysate but was also secreted into the culture medium (Fig. 3, lanes 1 and 2) consistent with its N-terminal predicted signal sequence. vFGF in the cell lysate may potentially contain vFGF bound to the extracellular matrix. A plasmid containing a viral RNA polymerase subunit (P47) tagged with HA that was not expected to be secreted was used as a control to monitor any contamination in the pellet and supernatant fractions (Fig. 3, lanes 3 and 4). The *fgf* from *Bombyx mori* NPV has also been shown to be secreted (Katsuma *et al.*, 2004).

vFGF affinity to heparin

FGFs bind heparan sulfate proteoglycans in the extracellular matrix and upon cleavage, complexes heparin-bound FGFs bind and activate FGFRs. Previously characterized FGFs bind heparin-Sepharose and elute at high salt concentrations between 1.0 and 2.0 M NaCl (reviewed in Powers *et al.*, 2000). To test whether vFGF had high affinity to heparin like other characterized FGFs, supernatant was collected from SF-21 cells previously transfected with pHSFGFHA, applied to heparin-Sepharose, and bound proteins were eluted with increasing concentrations of NaCl. vFGF routinely started to elute between at 1.0 and 1.2 M NaCl (Fig. 4A), indicating that vFGF conserved the property of binding heparin strongly that is critical for the function of other FGFs.

Transfection of SF-21 cells with a plasmid expressing HA-tagged OpIAP, a baculovirus inhibitor of apoptosis (Birnbaum *et al.*, 1994), a protein that we did not expect to have high affinity for heparin, eluted from heparin primarily in the flow through when a cell lysate was allowed to bind heparin-Sepharose (Fig. 4B).

Chemoattraction properties of vFGF for different cell types

One characteristic of FGFs is their ability to induce cell motility. We tested the ability of vFGF for chemoattraction of SF-21, TN-368, and hemocytes isolated from last instar *T. ni* (Fig. 5). pHSFGFHA or pBluescript was transfected into SF-21 cells and the supernatant containing vFGF or proteins present in the supernatant of vector-transfected cells was partially purified by heparin-Sepharose and used in cell migration

assays. Expression of *vfgf* before and after heparin purification was monitored by immunoblotting using anti-HA antibody (data not shown).

Since vFGF was not purified to homogeneity, we could not add a specific and known amount of vFGF in all experiments. Thus, we generated a dose-response curve for chemoattraction of insect cells for each batch of partially purified vFGF and used the optimal concentration of vFGF in transmigration assays. An example is presented in Fig. 5A. vFGF was not able to induce transmigration of insect cells when low concentrations of the protein (*e.g.*, 0.2 to 1.0 µl, Fig. 5A) were assayed. Optimal concentrations were reached with 2.0 µl of vFGF, and addition of higher concentrations of vFGF (*e.g.*, 10 to 20 µl, Fig. 5A, and results not shown) reduced activity, probably due to diffusion of vFGF preventing the ability of a gradient of FGFs to be established.

We observed that vFGF was able to stimulate transmigration of cells derived from the ovarian cell-derived Lepidoptera cell lines permissive for Ac*M*NPV infection, SF-21 and TN-368 cells, 2.3- and 1.8-fold, respectively, compared to transmigration of these cells to proteins purified from cells transfected with pBluescript (Fig. 5B and C). Similar results have been reported for other better characterized FGFs (Lee *et al.*, 2003; Ritch *et al.*, 2003). Interestingly, vFGF was able to stimulate migration of hemocytes obtained from last instar *T. ni* larvae, consistent with the possibility that vFGF is able to attract hemocytes in the infected host (Fig. 5D). In addition, this vFGF-stimulated cell motility was verified by microscopic examination of cells in the lower transwell compartment (results not shown). In these experiments, the migration observed in the controls may be due in part to secreted cellular FGFs that were purified and to which target cells in the apical transwell insert may be responding.

DISCUSSION

Most FGFs are secreted from cells where they act extracellularly. In addition, they have a high affinity to heparin. Finally, amongst their properties is their ability to function as chemoattractants. These three properties are conserved in the Ac*M*NPV *fgf* in cell culture, since it has a functional signal peptide that allows secretion of the polypeptide (Fig. 3), it binds heparin (Fig. 4), and enhances cell migration (Fig. 5).

A few examples exist of viruses that encode growth factors other than *fgfs* or viruses that affect host *fgfs*. However, to our knowledge, the baculovirus *fgfs* are the only *fgfs* encoded by viruses. The vaccinia virus growth factor, VGF, has homology to epidermal growth factors (Bloomquist *et al.*, 1984; Brown *et al.*, 1985; Reisner, 1985) and is a virulence factor that stimulates cell growth (Buller *et al.*, 1988). Another well-described example is the viral vascular endothelial growth factor, vVEGF, encoded by parapoxviruses (Lyttle *et al.*, 1994; Ueda *et al.*, 2003) that is involved in angiogenesis (Savory *et al.*, 2000). In mice, retroviruses use either WNT or FGF as integration hot spots, activating the genes, and leading to carcinogenesis (Katoh, 2002). Respiratory syncytial virus (RSV) enhances the production of basic FGF, and this may lead to fibroblast activation involved in remodeling the airway epithelium due to chronic inflammatory changes associated with asthma (Dosanjh *et al.*, 2003). Upregulation of basic FGF was suggested to explain prolonged wheezing after RSV infection (Dosanjh *et al.*, 2003). More recently, Raf-induced VEGF augmented Kaposi's sarcoma-associated herpesvirus infection of cells (Hamden *et al.*, 2004).

Branching epithelial morphogenesis is central in vertebrate development of lungs, vasculature, kidneys, and most glands, involving cell migration, division, and attracting more cells to form complex structures apparent in mature organs. In insects, tracheal morphogenesis leads to a network of epithelial tubules that deliver oxygen to every tissue, and *Drosophila* has served as a model system to study the morphological, genetic, and molecular aspects of this process. The *Drosophila* FGF gene, *branchless*, is responsible for controlling tracheal cell migration and the pattern of branching (Sutherland *et al.*, 1996). Its receptor, *breathless*, is expressed on the surface of developing tracheal cells (Glazer and Shilo, 1991; Klambt *et al.*, 1992). Localized activation of the receptor guides the migration of tracheal cells forming primary and secondary branches aided by a number of factors (reviewed in Ghabrial *et al.*, 2003). Terminal branching is regulated by oxygen requirements in cells signaled by branchless (Jarecki *et al.*, 1999). Thus, branchless is an essential mitogen and chemoattractant in the development of the embryonic, larval, and differentiated adult tracheal system (Sato and Kornberg, 2002; Sutherland *et al.*, 1996). The Ac*M*NPV FGF is 42% identical to the FGF core sequences of branchless (amino acids 220 to 409 of branchless). These core sequences encode structural components composed 12 antiparallel β-strands, heparin-, and receptor-binding regions.

Twenty-six baculovirus genomes are available to date and 23 encode *vfgf*. The viruses encoding *vfgf* infect lepidoptera (moths or butterflies), initiating infection in the midgut of the insect larvae. Infection then spreads systemically until the insect dies. Some granulovirus encode more than one copy of *vfgf* (accession number NC 004062; Hashimoto *et al.*, 2000; Wormleaton *et al.*, 2003). The advantage for more than one

copy of *vfgf* with respect to pathogenesis or whether both copies are functional is not known. Furthermore, it would be interesting to find out if the diverse tissue tropisms exhibited by granuloviruses, ranging from midgut restrictions to virus distribution in several tissues (Federici, 1997), are related to *vfgf* function. The baculoviruses that do not encode *fgf* have a smaller genome and are phylogenetically considered to be more ancient and divergent from those that infect Lepidoptera (Garcia-Maruniak *et al.*, 2004; Lauzon *et al.*, 2004). These divergent viruses infect the *Culex* mosquito (Diptera) and *Neodiprion* sawflies (Hymenoptera). More interestingly, these baculoviruses only replicate at the primary site of infection, the epithelial cells of the midgut, and do not spread systemically (Federici, 1997; Moser *et al.*, 2001). Thus, it is possible that *vfgf* is required for efficient spread of infection beyond the midgut or systemically. Thus, baculoviruses infecting lepidoptera may have acquired the *fgf* homologs in order to efficiently invade and replicate in additional tissues.

Given the biological and biochemical characteristics of vFGF presented in this study, possible functions for *vfgf* in viral pathogenesis can be proposed, although alternative functions at other stages of the viral replication cycle are also possible. vFGF may attract uninfected hemocytes to trachea or other infected tissues by chemotaxis, allowing the virus to infect these cells and be spread through the open circulatory system of the insect. Hemocytes are motile cells that migrate towards wound sites or invading organisms for encapsulation. One possibility is that vFGF is used as a lure in which hemocytes are signaled by vFGF in a similar manner to that of chemokine signals that are sent by vertebrate cells upon virus infection to attract immune cells to sites of infection. vFGF may also induce cell proliferation of hemocytes

or other cell types, thus augmenting its replication capacity by increasing the number of progeny virus. Cell proliferation was observed in response to the vaccinia virus virulence factor, VGF, yielding increased numbers of infectious progeny (Buller *et al.*, 1988). Alternatively, vFGF may be serving as a signal for branching and migration of tracheal cells that allow virus accessibility to uninfected tissues.

This study presents the initial functional characterization of the Ac*M*NPV *fgf* and its implications in virus spread by modulating the host signal transduction pathways. We are in the process of characterizing a *vfgf* knockout virus in vitro and in vivo to discriminate amongst its possible roles during virus pathogenesis. Its function as a virulence factor may be useful in augmenting the applications of recombinant baculoviruses used for pest control, including baculoviruses that infect mosquitoes.

MATERIALS AND METHODS

Cells and virus

TN-368 cells (Hink, 1970) derived from the cabagge looper *Trichoplusia ni* and IPLB-SF-21 cells (SF-21) derived from the fall armyworm *Spodoptera frugiperda* (Vaughn *et al.*, 1977) were cultured at 27°C in TC-100 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 0.26% tryptose broth as described previously (O'Reilly *et al.*, 1992). The Ac*M*NPV L1 strain (Lee and Miller, 1978) was propagated and titered in SF-21 cells, and used to infect cells at a multiplicity of infection of 20 plaque-forming units (PFU) per cell (O'Reilly *et al.*, 1992).

Plasmid construction

The plasmid pHSFGFHA contained the *vfgf* orf under control of the *Drosophila* heat shock protein 70 (hsp70) promoter, the influenza hemagglutinin (HA)-epitope tag at the C-terminus of FGF, and a polyadenylation signal derived from the *Orgyia pseudosugata* inhibitor of apoptosis gene (*Opiap*) (Crouch and Passarelli, 2002). First, the *vfgf* orf was amplified by PCR using a plasmid containing viral genomic DNA in this region. The amplified product expanded from the translational start ATG codon to the last codon excluding the translational stop codon, and included an *Xho* I at the 5' end and a *Not* I site at the 3' end to facilitate cloning. Next, the amplified product was cloned into pBluescript at the *Xho* I and *Not* I sites to generate pBSFGF. The HA tag and a translational stop codon were introduced at the C terminus of FGF by annealing two complementary primers (5'-GGCCGCCTACCCATACGACGTCCCAGATTACGCCTAA

CCGC-3' and its complement) containing *Not* I and *Sac* II sites at the ends and cloned into the *Not* I and *Sac* II sites of pBSFGF. The resulting plasmid containing a C-terminally tagged *vfgf*, pBSFGFHA, was cut with *Xho* I and *Sac* I to obtain a fragment with the HA-tagged *vfgf*. Finally, the ends were repaired with T4 DNA polymerase and cloned into the Klenow-repaired *Bsu* 36I sites of pHSGFP (Crouch and Passarelli, 2002) to replace the green fluorescent gene for the HA-tagged FGF, and the resulting plasmid was named pHSFGFHA. The sequence of the *vfgf* insert in pHSFGFHA was verified by nucleotide sequencing.

The plasmids pHSEpiOpIAP and pHSEpiHisp47 have been previously described (Vucic *et al.*, 1997). Briefly, the plasmid pHSEpiOpIAP contains the *Op-iap3* gene with an N-terminal HA epitope tag driven by the *Drosophila* hsp70 promoter. pHSEpiHisp47 contains the Ac*M*NPV *p47* gene with N-terminal HA and poly-histidine tags under control of the hsp70 promoter.

Transfection of cells

In experiments in which vFGF was detected in the cell lysate or supernatant of transfected cells, 2 µg of either pHSFGFHA or pHSEpiHisp47 were introduced into 0.5 x 10^6 SF-21 cells plated on 35-mm cell culture dishes by using 3 µl of a liposome preparation as previously described (Crouch and Passarelli, 2002). Cells were maintained for 4 h at 27°C in the liposome-DNA mix. This mix was then replaced with TC-100 media containing 10% fetal bovine serum and incubated at 27°C. Twenty-four h after addition of DNA, cells were incubated at 42°C for 30 min to induce expression of genes from the hsp 70 promoter. Cells were allowed to express proteins for 4 h at 27°C

before cells and supernatant were harvested. In heparin purification experiments, 6 µg of either pHSFGFHA or pBluescript II SK(+) (Stratagene) were transfected into 3.0 x 10^6 SF-21 cells as described above, but proteins were collected 36 h post-transfection.

Insect rearing and hemocyte collection

T. ni eggs were obtained from Entopath Inc. (Easton, PA). Individual first instar caterpillars were transferred to one-ounce cups containing cabbage looper diet (Southland Products, Inc.) and reared at 27°C with a 12-h light/dark cycle. Caterpillars were allowed to grow to the last instar.

Last instar caterpillars were chilled at 4°C for 30 min before hemolymph extraction. Hemolymph was collected by cutting an anal proleg and allowing the caterpillar to bleed onto a piece of parafilm. Hemolymph was transferred to ice-cold anti-coagulant buffer (4 mM NaCl, 40 mM KCl, 1.7 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES), 146 mM sucrose, 0.1% polyvinylpyrolidone, 8 mM EDTA, 9.5 mM citric acid, and 27 mM sodium citrate) at 1:1 vol/vol ratio and centrifuged at 300 x g at 4°C. Unseparated hemocyte cells were washed twice with ice-cold anti-coagulant buffer and then resuspended in the same buffer. Hemocytes were counted using a hemocytometer and 2 to 3 x 10^4 cells were used in transmigration assays.

RNA isolation

SF-21 monolayers (2 x 10^6 cells per 60-mm diameter dish) were infected with Ac*M*NPV at a multiplicity of infection of 20 PFU per cell. The virus was allowed to adsorb for 1 h at room temperature, and time zero was defined as the time when the

virus was removed and incubation at 27°C commenced. The protein synthesis inhibitor cycloheximide was added to the cells at 100 μg/ml for 30 min before infection and maintained thereafter. The DNA synthesis inhibitor aphidicolin was added at 5 μg/ml following virus adsorption. Total RNA was isolated at selected h p.i. with Trizol reagent (Invitrogen).

Northern blot analysis

Samples of total RNA (20 μg per lane) were electrophoresed on a formaldehyde-1% agarose gel, transferred to a nylon membrane, and hybridized to α-^{32}P-radiolabeled riboprobes. Two probes were generated by first PCR-amplifying v*fgf* from the translational start codon to the translational stop codon and orf 33 from 27,727 to 28,284 nts of Ac*M*NPV (Ayers *et al.*, 1994) (from 6 nts downstream of the translational stop codon to 3 nts upstream of the translational start codon). PCR products were cloned by using the TA Cloning® kit (Invitrogen) and the cRNA probes were synthesized by in vitro transcription using T7 RNA polymerase in the presence of α-^{32}P[UTP].

Immunoblotting

Cell culture supernatant from transfected cells was collected and spun down at 1,000 x g for 5 min to remove residual cells, and proteins in the supernatant were mixed with sodium dodecyl sulfate (SDS)-Laemmli buffer. Attached cells were washed twice with phosphate-buffered saline (PBS), pH 6.2 (Potter and Miller, 1980), and collected with SDS-Laemmli buffer. Proteins from cells and supernatant were resolved by SDS-15% polyacylamide gel electrophoresis (PAGE), transferred to a PVDF membrane

(Pierce) and immunodetected with 1:1,000 dilution of HA.11 monoclonal antibody (Covance), 1:3,000 dilution of goat anti-mouse IgG-horseradish peroxidase (Bio-Rad) and SuperSignal chemiluminescent substrate (Pierce).

Purification of vFGF through heparin-Sepharose

Supernatant from cells transfected with pHSFGFHA or pBluescript was harvested and spun down at 1,000 x g at 4° C to remove residual cells. Proteins in the supernatant of transfected cells were then incubated with heparin-Sepharose^{TM} 6 Fast Flow beads (Amersham Biosciences) previously washed with excess deionized water three times for 4 h at 4°C and constant rotation. Subsequently, the mixture was loaded onto a 2-ml column (BD Bioscience Clontech) and washed with phosphate buffer (10 mM NaH_2PO_4) containing different concentrations of NaCl from 0.15 to 2.0 M as indicated. Each fraction was collected and proteins were detected by immunoblotting. Proteins eluted with phosphate buffer containing 30% glycerol and 1.4 M NaCl and were used in transmigration assays.

Transmigration assays

Cell migration was assessed using Costar transwells with polycarbonate membrane inserts. For migration of SF-21 and TN-368 cells, approximately 3 to 5 x 10^4 cells were loaded onto 8 µM pore-size transwell inserts. Different amounts of heparin-purified vFGF or control proteins (pBluescript-transfected cells) as indicated were added to 24-well plates containing 600 µl of PBS, pH 6.2 or TC-100 lacking serum. For hemocyte migration, approximately 2 to 3 x 10^4 cells were loaded onto 3 µM pore-size

transwell inserts and different amounts of heparin-purified vFGF or control proteins were added to 24-well plates in which each well contained 600 µl of anti-coagulant buffer. The transwell inserts were then transferred to the 24-well plates containing heparin-purified proteins. After 3.5-4 h incubation at 27°C, transwell inserts were removed and cells that migrated were quantified using CellTiter-Glo^{TM} luminescent substrate to measure ATP present according to the protocol provided by the manufacturer (Promega) and viable cells determined with the Wallac Victor^3 1420 Multilabel counter (Perklin Elmer). ATP luminescence-based motility assays are considered more reliable and sensitive than other methods used to enumerate cells (de la Monte *et al.*, 2002). All experiments were performed independently at least three times.

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Figure 1. Alignment of baculovirus FGFs, *Drosophila* FGF branchless (Bnl), and a human FGF. Grey shading shows similarities among proteins, bold-type residues highlight the putative heparin-binding growth factor/FGF family domain, italicized residues indicate putative cleavable signal sequence, underlined residues show putative N-glycosylation sites, numbers in parenthesis refer to amino acids of the corresponding polypeptide, and double slashes indicate the predicted cleavage sites. GenBank database accession numbers for the sequences shown are as follows: *Autographa californica M*NPV (Ac*M*NPV) FGF (NC 001623), *Bombyx mori* NPV (BmNPV) FGF (NC 001962), *Choristoneura fumiferana M*NPV (Cf*M*NPV) FGF (NC 004778), *Orgyia pseudotsugata M*NPV (Op*M*NPV) FGF (NC 001875), *Lymantria dispar* NPV (LdNPV) FGF (NC 001973), *Xestia c-nigrum* GV (XecnGV) FGF (NC 2331), *Plutella xylostella* GV (PxGV) FGF (NC 002593), human FGF 16 (NM 003868), and *Drosophila* FGF Bnl (NM 169875).

Figure 1.


AcMNPV .....MYRLLALVALASMADC//SALLTHITGTSIP
BmNPV .....MYRLLALVTLASMADC//SALLTHITGTSIP
CfMNPV .....MHRLALVVATVASLCAC//RQLDHVTGTQHL
OpMNPV ....MHRLALVVATVAYLCAGHAPL//QHITGTQRL
LdNPV .....MLALLVALALGAHA//F...PLTASTGRL
XecnGV .....MLLQLLIICSCLTCYSMT//(186)TSSSRCELPKVSEVTADDVT
PxGV MLFLFYLFTIVNA//H
Human FGF 16 MAEVGGVFASLDWDLHGFSSSLGN// (44)KLQRGSPTDFAHLKGILRRR
Drosophila Bnl MRRNLRLDWRALALLGALLSFIIAWRGLVSA//(238)TVPQSHLAWTSRK
AcMNPV GQLFINRQFLAVNPDGAVYG.TIESDNVD.....TIFKRVAVDRNRIVI
BmNPV GRLFINRRFLAVNPNGTVYGGTIESDNAD.....TTFKRLAVDRNRIVI
CfMNPV VQVFIHNQYLAVRSNGTIGG..TTHGSMD.....TVLQRVGFSQGRILL
OpMNPV VQVLIHNRYLAVRSDGTVGG.TTYASSLD.....TVLQRIAIAHGRILL
LdNPV VQIGINRQLLTCFANGTVGG.STDERADG.....TAWRRWAAKPREVLI
XecnGV REIKVYSSNKYFYHLRLVDGYVTSLGTLDVIMGDHTRFHKEQLLNETNVF
PxGV YLQSENDLFFCVHKNANVPG.LSRANDESCT..PNVFSIHYSHNGLVINY
Human FGF 16 QLYCRTGFHLEIFPNGTVHG..TRHDHSRFG.....ILEFISLAVGLISI
Drosophila Bnl IQLYIKNRILQILRDGVVNG.TQDENSEFT.....ILQRSTVDVGRIKL
AcMNPV QNAITCVYLCMDRCGQLY.GSKTLSKDCFMREFLEKNNYNTYYKVYD...
BmNPV QNAITCVYLCIDRCGQLY.GSKTLSDDCFMREIMEKNNYNTYYKMYD...
CfMNPV RNAISCMHVCLNRCGAMY.ASIALSSDCILNEVMLEHNYDAMFKIYD...
OpMNPV RNAVSCMYVCLDRCGAMY.ASAALSSDCILNEVMLENNYDVMFKIYN...
LdNPV RSAATCAFVCLDDCGFLYTAAEAPNKECLFVEELSENHYSYLYRVHD...
XecnGV RNTDNCKYLCMDVCGKVY.MSLSYKTDCLVNVIGTTLTSSVYFQFVR...
PxGV KDSNACKYMCLNRCGFLYYSVKFYKQDCVFTTSHFRGIDTLSVKRNN...
Human FGF 16 RGVDSGLYLGMNERGELY.GSKKLTRECVFREQFEENWYNTYASTLY...
Drosophila Bnl QSVATCLYLCMDACGVPY.GSKDFTDDCVFNENMGLQNYNTYSSTYHSQA
AcMNPV RKLTYVALKNDGTPRKLQISKSRKLGKLSVYAMALLKRLSFPIYTSCPN.
BmNPV RKLTYVALKNDGTPRKLQISKGRKLGKFSVYAMTLLKRLSFPIYTSCPN.
CfMNPV GKKTYVALNRDGKPRPVQLPKRRPLRNLRIYAFIMRKPLNYTSVIQCP..
OpMNPV GKKTYVALDNTGNPRRVQLPRQRPLRMMNVYTFIMRIPLNYISVSQCA..
LdNPV RSRIYLALGANGKSRRVVAPVDEPLADHGNPTSVVYREWNSSRDDCATLG
XecnGV HNNYLTVNSDGSLSNSTTRTYRGQVKMDMTENLVLDAYDKKCNMINTNID
PxGV YSDFVATNNYEFIPMSLSAASRLERMKSLISIKFINSTKNEHHKCELGNV
Human FGF 16 KHSDSERQYYVALNKDGSPREGYRTKRHQKFTHFLPRPVDPSKLPSMS..
Drosophila Bnl RRVFYLALNGSGQPRRTQIPASRSLGKLSTYTNAITETVPQERVEQLIAK
AcMNPV .....IKSEIIVRHRKCHV..... .....
BmNPV .....IKNETIVQHRKCHV..... .....
CfMNPV .....KQTKIIKHQKCRLR..... .....
OpMNPV .....KPNKVIRHRKLPLVVQTTLNTISIYIITHV TMLFDHVD..
LdNPV SKRLQAALAFKPRKVCKSRARKPAAAPRTTPPRPTRNANV TLFGEDGSTE
XecnGV DEDEDEDDKDCSSQPTVSSDSSVKNVSYPLTKSVLPTLLF YFVFKNGTFH
PxGV DTNDVSVTCTNYRFNRSEIRYDTHVGYTFNLWDKFLSLIG WYVIDVPDDK
Human FGF 16 .....RDLFHYR ....
Drosophila Bnl NFGANRVKHGVRQLCDTGKPLIELIDVARFKAPPHCSSNT SGSSSSISSS

Figure 2. Northern blot analysis of *vfgf* transcripts. (A) A schematic shows *vfgf* and adjacent open reading frames and the homologous region 2 (*hr*2) where the thick filled arrows indicate the direction of transcription. The transcripts that traverse the region (thin arrows) and their temporal expression (E, early; L, late) are shown above the open reading frames. The size of the transcripts in panels B and C below are indicated in parentheses and to the right of the thin arrows. The dotted arrow indicates that the 5' and 3' ends of the transcript were not defined. The asterisk indicates the approximate location of a polyadenylation signal. Thin arrows below the open reading frames labeled "probe 1" and "probe 2" indicate RNA probes used in Northern blots in panels B and C, respectively. (B and C) Total RNA was extracted from SF-21 cells either mock-infected (mi) or infected with Ac*M*NPV at the time indicated at the top in hours. RNA was extracted from cells treated with cycloheximide (12C) or aphidicolin (12A) at 12 h p.i. The size and position of predominant transcripts are indicated. M, Molecular weight markers.

Figure 2.

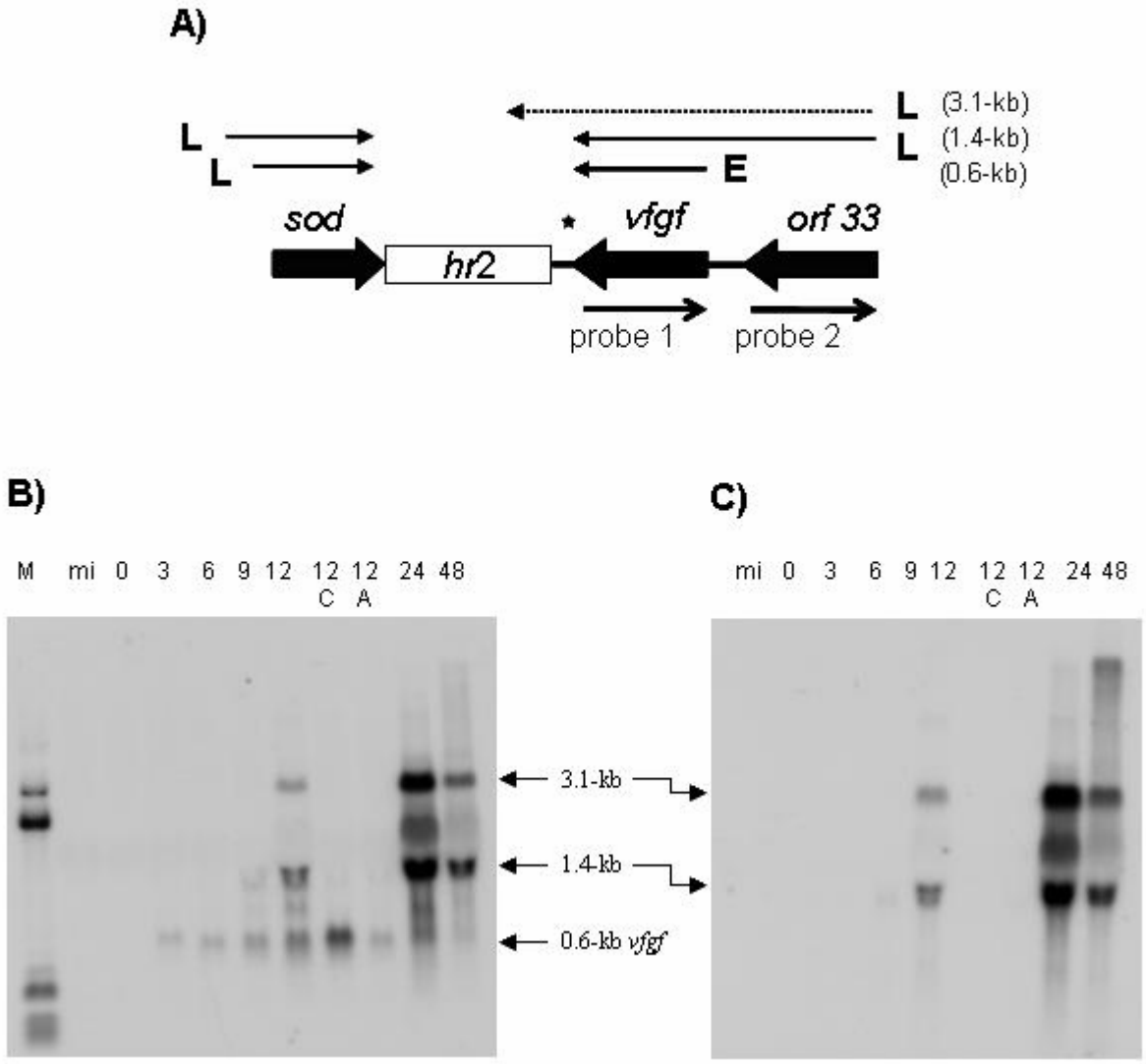

A)
L
L
L (3.1-kb)
L (1.4-kb)
E (0.6-kb)
sod
hr2
vfgf
orf 33
probe 1
probe 2
B)
M mi 0 3 6 9 12 12 C 12 A 24 48
3.1-kb
1.4-kb
0.6-kb vfgf
C)
mi 0 3 6 9 12 12 C 12 A 24 48

Figure 3. Secretion of vFGF. SF-21 cells were transfected with either pHSFGFHA (lanes 1 and 2) or a plasmid expressing HA-tagged P47, pHSEpiHisp47, (lanes 3 and 4). Extracellular proteins present in the supernatant (sup; lanes 2 and 4) or intracellular proteins in the cell lysate (cell; lanes 1 and 3) were resolved by SDS-PAGE, proteins were transferred to a PVDF membrane, and reacted with anti-HA antibody. The migration of vFGF and P47 is indicated by arrows to the right. The asterisk (*) indicates a non-specific immunoreactive band in the supernatant.

Figure 3.

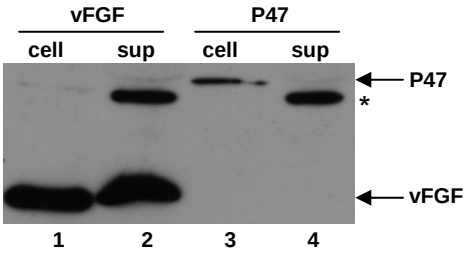


Figure 4. vFGF binds heparin. SF-21 cells were transfected with either pHSFGFHA (A) or pHSEpiOpIAP (B). Proteins in the extracellular fluid (A) or in the cell lysate (B) were collected and incubated with heparin-Sepharose beads. The heparin-bound proteins were washed with phosphate buffer containing different concentrations of NaCl as indicated above each lane. Fractions were collected and detected by immunoblotting with monoclonal anti-HA. Migration of vFGF and OpIAP are indicated to the right of each panel. SM, starting material; FT, flow through.

Figure 4.

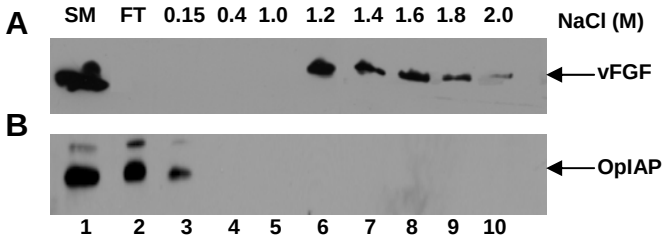
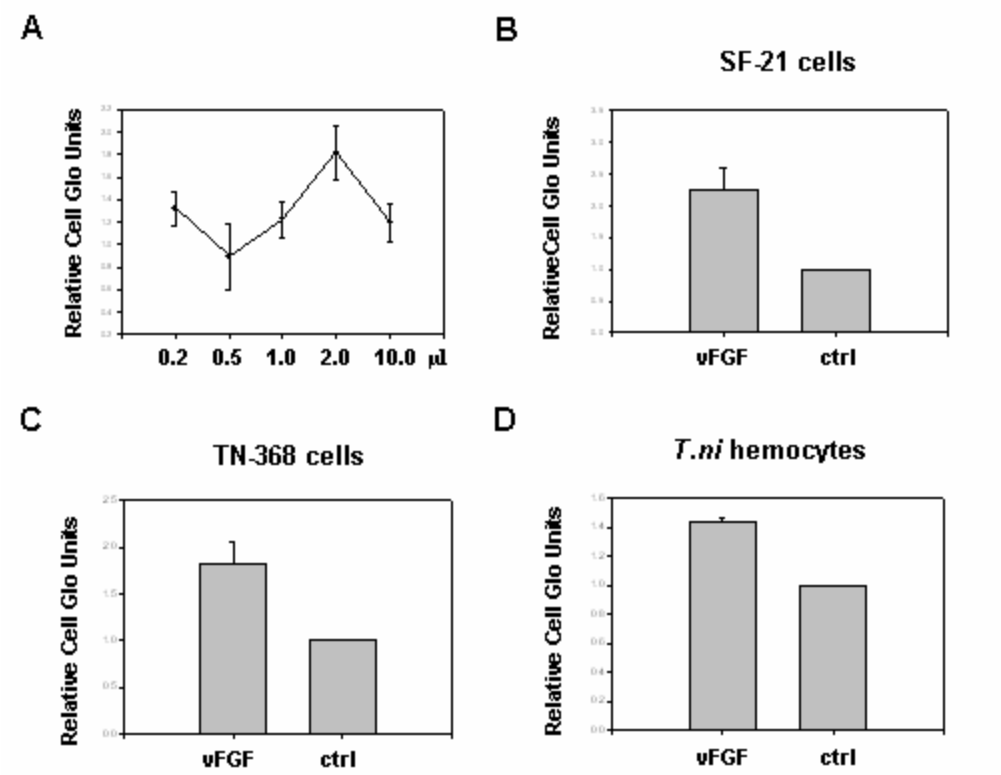


Figure 5. Chemotactic activity of vFGF. (A) Different concentrations of heparin-purified vFGF or control proteins were tested for their ability to enhance motility using TN-368 cells in three independent assays. The relative CellTiter-Glo units of cells treated with vFGF compared to control-treated cells (assigned a value of 1.0) is presented on the ordinate axis. The numbers on the abscissa indicate the volume (μl) of heparin-purified and eluted proteins used. The optimal concentration of vFGF (2 μl) was used to perform transmigration experiments with TN-368 cells (C) below. Similar titration experiments were performed with SF-21 and *T. ni* hemocytes to determine the optimal concentration required for these cells. SF-21 (B), TN-368 (C), or last instar *T. ni* hemocytes (D) were plated in the upper chamber of a transwell and allowed to migrate to vFGF. vFGF and control proteins were obtained from SF-21 cells transfected with pHSFGFHA (vFGF) or pBluescript control (ctrl), respectively, and proteins in the extracellular fluid were partially purified by heparin-Sepharose. An optimal amount of heparin-purified vFGF, as determined by titration of vFGF in transmigration assays, was added to the lower compartment of the transwell. After 3.5 to 4 h, a measurement of viable cells that had migrated to the lower compartment was obtained by CellTiter-Glo. The bars above each column indicate the mean ± standard error of at least three independent experiments.

Figure 5.


A
Relative Cell Glo Units
0.2
0.5
1.0
2.0
10.0 µl
B
SF-21 cells
RelativeCell Glo Units
vFGF
ctrl
C
TN-368 cells
Relative Cell Glo Units
vFGF
ctrl
D
T.ni hemocytes
Relative Cell Glo Units
vFGF
ctrl

CHAPTER 3

Analysis of a baculovirus lacking a functional viral fibroblast growth factor homolog

ABSTRACT

Baculoviruses encode *fibroblast growth factor* (*vfgf*) homologs whose function during virus infection is unknown. We constructed a recombinant bacmid of *Autographa californica* M nucleopolyhedrovirus (Ac*M*NPV) lacking a functional v*fgf* and characterized it in two insect cell lines. The kinetics of budded virus production were similar in the parental and *vfgf*-deficient viruses in both cell lines at both high and low multiplicities of infection. In addition, no obvious differences were observed between the mutant and parental viruses in protein or DNA synthesis. Finally, coinfection of *vfgf*-containing and deficient viruses and passage for several generations did not reveal a consistent growth advantage for either virus.

INTRODUCTION

Fibroblast growth factors (FGFs) function during embryogenesis and in differentiated tissues, stimulating processes as diverse as cell motility, cell proliferation, differentiation, tissue repair, and establishing metastasis (Ornitz and Itoh, 2001; Sato and Kornberg, 2002). FGFs are conserved among vertebrate and invertebrate organisms. However, in viruses, *fgf* homologs have only been identified in baculoviruses. Viral *fgfs* (*vfgfs*) share a number of properties common to better-characterized *fgfs*, such as structural features (Ayres *et al.*, 1994; Detvisitsakun *et al.*, 2005; Katsuma *et al.*, 2004), stimulation of cell motility (Detvisitsakun *et al.*, 2005), extracellular secretion (Detvisitsakun *et al.*, 2005; Katsuma *et al.*, 2004), and affinity for heparin (Detvisitsakun *et al.*, 2005).

Using a bacmid of Ac*M*NPV that replicates in *Escherichia coli*, we deleted *vfgf* and evaluated its replication in two insect cell lines permissive for Ac*M*NPV infection, SF-21 and TN-368. We did not find any obvious differences between viruses encoding and lacking *vfgf* with respect to budded virus production, synthesis of viral proteins and shut-off of viral and host proteins, or genome replication. Coinfection of SF-21 cells with viruses lacking and encoding *vfgf* indicated that *vfgf* did not confer a consistent advantage for virus replication after several passages.

RESULTS

Construction of AcBAC-vfgfKO

We investigated the effects of deleting the Ac*M*NPV *vfgf* on virus replication in cell culture. We constructed three bacmids, AcBAC-vfgfKO, AcBAC, and AcBAC-vfgfRep, in which *vfgf* had been deleted, was unaltered, or reintroduced at the polyhedrin (*polh*) locus under the control of its own promoter, respectively (Fig. 1A). In addition, all the viruses carried the green fluorescent protein (*gfp*) gene under the *Drosophila* heat shock protein (hsp) 70 promoter at the *polh* locus and an unaltered *polh* (Fig. 1B). We confirmed the presence or absence of *vfgf* (primer pairs P1-P2 and P7-P8), the presence of *polh*, *gfp* (results not shown), and zeocin (primer pairs P1-P2 and P3-P4), and the correct location of each gene within the bacmids by polymerase chain reaction (PCR) amplification using primers within or outside the modified regions (primers P1, P3, P5, and P8; Fig. 1 C and D). We also confirmed transcription of *vfgf* in AcBAC-vfgfRep by Reverse Transcriptase (RT)-PCR (Detvisitsakun and Passarelli, unpublished results).

We previously reported that *vfgf* was transcribed as an early monocistronic message and as a late bicistronic message commencing upstream of ORF 33 (Detvisitsakun *et al.*, 2005). We tested if late mRNAs were transcribed through ORF 33 by RT-PCR, and we detected similar levels of late messages in both AcBAC and AcBAC-vfgfKO (results not shown). This indicates that the mutation of *vfgf* did not appear to affect transcription through ORF 33.

Infection of SF-21 cells with AcBAC-vfgfKO, AcBAC, and AcBAC-vfgfRep

AcBAC-vfgfKO, AcBAC, and AcBAC-vfgfRep DNA was extracted from *E. coli* cells and used to transfect SF-21 cells. Budded virus was collected at several times post transfection and titered. We did not observe significant differences in the amount of infectious virus obtained after transfection with the three bacmid DNAs (Fig. 2A). Furthermore, the production of budded virus indicated that *vfgf* was not an essential gene in cell culture.

We obtained high titer stocks of AcBAC-vfgfKO, AcBAC, and AcBAC-vfgfRep and infected SF-21 cells at 5 plaque forming units (PFU) per cell in a single-step growth curve. Comparison of the kinetics of infectious budded virus production at several times post infection (p.i.) did not reveal any drastic differences throughout the time course of infection (Fig. 2B).

We next thought that performing multiple-step growth curves would allow us to distinguish subtle differences in virus replication among the viruses. To this end, we infected SF-21 cultures with AcBAC-vfgfKO, AcBAC, or AcBAC-vfgfRep at a multiplicity of infection (MOI) of 0.01 PFU/cell and collected virus in the media at several times p.i. Although the titers at the onset of the experiment were slightly different, the yields of budded virus at every time point after the initial 12 h were similar and all viruses achieved equivalent titers by 96 h p.i. (Fig. 2C). Similarly, bacmids containing or lacking *vfgf* had similar kinetics and virus production levels as vHSGFP (Clarke and Clem, 2002), an Ac*M*NPV recombinant containing *gfp* under control of the *Drosophila* hsp 70 promoter (Fig. 2B and C). In addition, we did not observe any differences in plaque size

or other obvious phenotypic difference in SF-21 cells infected with AcBAC-vfgfKO, AcBAC, or AcBAC-vfgfRep (results not shown).

Infection of TN-368 cells with AcBAC-vfgfKO, AcBAC, and AcBAC-vfgfRep

We also performed single- and multiple-step growth curves in TN-368 cells infected with AcBAC-vfgfKO, AcBAC, and AcBAC-vfgfRep at a MOI of 5 and 0.01 PFU/cell, respectively. TN-368 cells are more susceptible to AcMNPV infection than SF-21 cells and certain genes necessary for virus replication in SF-21 cells have been shown to not be necessary in TN-368 cells (Chen and Thiem, 1997; Clem and Miller, 1993; Lu and Miller, 1995). Thus, we explored whether the vfgf deletion virus had any effects on virus replication in TN-368 cells. Analogous to the results observed with SF-21 cells infected with vfgf-containing and -deficient viruses, infectious virus production throughout a time course of infection was comparable between viruses encoding or lacking in vfgf (Fig. 2D and E).

Protein and DNA synthesis in SF-21 cells infected with AcBACvfgfKO, AcBAC, and AcBACvfgfRep

The protein synthesis kinetics of AcBAC-vfgfKO, AcBAC, and AcBAC-vfgfRep during infection of SF-21 cells were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of pulse-labeled proteins with ^{35}S-methionine and -cysteine. Protein synthesis profiles were similar in the timing and levels of synthesis and shutoff of viral and host proteins (Fig. 3A). We also examined the pulse-labeled proteins in the supernatant and did not detect any obvious differences

(results not shown). The presence or absence of secreted vFGF was not evident; this may be due to low levels of vFGF production or its accumulation on the surface of cells via association with heparin sulfate proteoglycans.

Viral DNA replication of AcBAC-vfgfKO, AcBAC, and AcBAC-vfgfRep in SF-21 cells

Although we did not see any apparent differences in budded virus production among cells infected with AcBAC-vfgfKO, AcBAC, or AcBAC-vfgf, we investigated the kinetics of viral DNA synthesis. SF-21 cells infected with AcBAC-vfgfKO, AcBAC, or AcBAC-vfgfRep at an MOI of 5 PFU/cell showed similar kinetics in the accumulation of viral DNA from 12 to 96 h p.i. (Fig. 3B).

Serial passage of AcBAC and AcBAC-vfgfKO coinfected cells

Thus far, viruses lacking *vfgf* had no apparent defect in budded virus production, DNA or protein synthesis, or cytopathic effects (results not shown). Consequently, we thought that *vfgf* may confer an advantage for virus replication in cell culture that was not apparent in the assays discussed above. Other mutant baculoviruses that show no conspicuous phenotypic differences when compared to the wild-type virus have been shown to have a disadvantage or advantage for growth in competition experiments (McLachlin *et al.*, 2001; Passarelli and Miller, 1994). We coinfected SF-21 cells with AcBAC-vfgfKO and AcBac at an MOI of 0.05 PFU/cell for each virus for a total MOI of 0.1 PFU/cell, passaged the viruses for several generations, extracting viral DNA and tittering budded virus after each passage. Viral DNA was digested with restriction

endonuclease enzymes that generated a restriction length polymorphism that could identify each virus after resolving the DNA by gel electrophoresis and hybridizing with a labeled fragment of DNA that was represented equally between the two viruses (Fig. 4A).

We found that after passing AcBAC and AcBAC-vfgfKO together 6 times in two independent experiments, we did not observe a consistent trend of virus outgrowth for either AcBAC or AcBAC-vfgfKO (Fig. 4B and C). Thus, from these experiments, we conclude that the presence of *vfgf* does not confer a selective replication advantage to Ac*M*NPV under these conditions.

DISCUSSION

The *vfgf* gene is conserved in all baculoviruses sequenced to date that infect insects in the order Lepidoptera (Garcia-Maruniak *et al.*, 2004; Lauzon *et al.*, 2004; Willis *et al.*, 2005), but its function is not known. We have proposed functions in virus dissemination in vivo, although other roles for *vfgf* during virus replication can be envisioned (Detvisitsakun *et al.*, 2005), but these functions may not be apparent in cell culture. However, we cannot rule out the possibility that *vfgf* may be important in other cell culture systems or other physiological conditions. In this study, we were not able to discern drastic differences in the production of infectious budded virus in SF-21 or TN-368 cells, or in protein or DNA synthesis profiles in SF-21 cells. In virus competition assays, we did not detect a consistent advantage for virus replication for viruses encoding or lacking *vfgf*. In this experiment, there is the possibility that viruses with *vfgf* provided vFGF in trans for cells infected with viruses lacking *vfgf*, making it difficult to observe an advantage, if any, after 6 passages. Also, it is possible that additional passages could have made virus growth advantage more apparent. We also note that all of the experiments described here were carried out using culture media containing fetal bovine serum (FBS) that may contain a number of growth factors. Although it is unlikely that they may affect our results, we cannot rule out this possibility. We are presently evaluating the pathogenesis of AcBAC-vfgfKO in insects where phenotypic differences between *vfgf*-null and *vfgf*-containing viruses may be more apparent.

MATERIALS AND METHODS

Cells and viruses

The cell line IPLB-SF-21 (SF-21) (Vaughn *et al.*, 1977) derived from the fall armyworm, *Spodoptera frugiperda*, and TN-368 cells derived from the cabbage looper, *Trichoplusia ni*, were grown at 27°C in TC-100 medium (Invitrogen) supplemented with 10% FBS (Invitrogen) and 0.26% tryptose broth as previously described (O'Reilly *et al.*, 1994). The Ac*M*NPV-based bacmid, bMON14272 (Invitrogen), derived from Ac*M*NPV E2 was maintained in DH10B cells as previously described (Luckow *et al.*, 1993). Bacmids were grown in SF-21 or TN-368 cells as specified according to the directions for Ac*M*NPV replication (Luckow *et al.*, 1993). vHSGFP is a recombinant of Ac*M*NPV L1 with *gfp* adjacent to the *polh* locus and has been previously described (Clarke and Clem, 2002).

Construction of AcBAC-vfgf-KO

A transfer plasmid was constructed by first amplifying using PCR the Ac*M*NPV region containing v*fgf* using bMON14272 (Invitrogen) DNA and oligonucleotide primers that amplified from 26,139 to 28,407 nt (Ayres *et al.*, 1994) and the product was cloned into pBluescript (Stratagene). Positions -2 and +3 with respect to the ATG at the translation start site of v*fgf* were altered by site-directed mutagenesis to generate an *Nco* I site. This plasmid was then digested with *Nco* I to create a 450-bp deletion of the N-terminus of the v*fgf* coding sequence, removing 82.4% of the ORF. Then, a 462-bp EM7 promoter-controlled zeocin gene cassette from pEM7/Zeo (Invitrogen) for selection in *E. coli* was inserted at the *Nco* I site to replace v*fgf*. This transfer vector contains

821-bp of 5' flanking sequence to *vfgf* that comprises part of the adjacent ORF 33 and the promoter of v*fgf*, and 1,129-bp of 3' flanking sequence to *vfgf* that contains 98-bp of the C-terminus of the v*fgf* ORF, the putative *vfgf* and ORF 33 poly A signal (Detvisitsakun *et al.*, 2005), the *hr2*, and part of *sod*.

A bacmid with a deletion within *vfgf*, AcBAC-vfgfKO, was constructed by recombination in *E. coli* using a modified method as that described by Bideshi and Federici (Bideshi and Federici, 2000). The transfer vector described above was digested with *Nde* I and *Nsi* I resulting in a linear 2.2-kbp fragment containing the zeocin cassette plus the v*fgf* flanking regions. The fragment was then isolated and transformed into *E. coli* BJ5183 containing bMON14272. Cells were then incubated in SOC (Invitrogen) media for 4 h, plated onto Luria-Bertani agar containing 50 µg/ml kanamycin and 30 µg/ml zeocin, and allowed to grow at 37°C for a minimum of 36 h. Colonies resistant to kanamycin and zeocin were selected. The presence of the zeocin cassette (primer pairs P3-P4 and P1-P2) and the absence of the intact v*fgf* ORF (primer pair P7-P8) in bacmid DNA were confirmed by PCR analysis (Fig. 1).

Construction of bacmids with *gfp* and *polh* genes

The Ac*M*NPV bacmid bMON14272 (Invitrogen) lacks *polh*. Both *gfp* and *polh* were inserted into AcBAC-vfgfKO and AcBAC, referred hereafter as wild-type, by Tn7-mediated transposition (Luckow *et al.*, 1993) using a donor plasmid. The donor plasmid was constructed by amplifying *polh*, its promoter, and termination sequences from a plasmid containing the *Eco* RI-I region of Ac*M*NPV, pRI-I, from 4,355 to 5,729 nt (Ayres *et al.*, 1994). The PCR fragment was then cloned into pFastBacHTB (Invitrogen) in

which the 246-bp *polh* promoter has been removed to generate pFastBac-*polh*+. Then, a cassette with the *Drosophila* hsp 70 promoter driving *gfp* was inserted into pFastBac-*polh*+ to generate pFastBac-*polh*+*gfp*+.

To construct a repair virus of AcBAC-vfgfKO, AcBAC-vfgfRep, a donor construct was made by PCR-amplifying a region from 27,008 to 27,790 nt (Ayres *et al.*, 1994) which includes the v*fgf* promoter, *vfgf* ORF and polyadenylation signal using bMON14272 DNA as a template. The PCR fragment was then cloned into pFastBac-*polh*+*gfp*+ resulting in pFastBac-*polh*+*fgf*+*gfp*+.

AcBAC with *polh* and *hsp* promoter-driven *gfp* was generated by transforming MAX Efficiency® DH10Bac™ competent *E. coli* (Invitrogen) with pFastBac-*polh*+*gfp*+. Transformed cells were incubated at 37°C for 4 h and plated onto Luria-Bertani agar containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal, and 40 µg/ml IPTG as described in the Bac-to-Bac® Baculovirus Expression System Manual (Invitrogen). White colonies resistant to kanamycin and gentamicin were selected and bacmid DNA confirmed by PCR for the presence of *gfp* and *polh* (results not shown).

AcBAC-vfgfKO and AcBAC-vfgfRep containing *polh* and a *hsp* 70 promoter driven-*gfp* cassette were also generated by Tn7-mediated transposition in which DH10B *E. coli* competent cells containing AcBACvfgfKO and helper plasmid, pMON7124 (Invitrogen), were transformed with pFastBac-*polh*+*gfp*+ and pFastBac-*polh*+*fgf*+*gfp*+, respectively. Transformed cells were incubated at 37°C for 4 h and plated onto Luria-Bertani agar containing 50 µg/ml kanamycin, 25 µg/ml zeocin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal, and 40 µg/ml IPTG. White colonies resistant to

kanamycin, gentamicin and zeocin were selected and bacmid DNA was confirmed by PCR for the presence of *gfp* and *polh* (results not shown). In addition, the location of insertions in all bacmid genomes was confirmed by PCR analysis using primers outside the region of transposition or recombination.

Bacmid DNA was purified using the Large-construction kit (Qiagen) and used to transfect SF-21 cells by liposome-mediated transfection as described elsewhere (Crouch and Passarelli, 2002). Bacmids were grown as described above.

Time course of virus production in cultured cells

SF-21 cells were either transfected with 1 µg of bacmid DNA or infected at MOI of 5 or 0.01 PFU/cell with budded virus produced and amplified from bacmid DNA transfections. Budded virus in the media was collected at different times post transfection or p.i. Virus titers were determined by $TCID_{50}$ endpoint dilution in SF-21 cells (Luckow *et al.*, 1993). Growth curves in TN-368 cells followed the same procedure except that viruses were amplified and titered in TN-368 cells.

Protein synthesis

SF-21 cells (1×10^6 cells in 35-mm dishes) were infected with AcBAC-vfgfKO, AcBAC-vfgfRep, or AcBAC at MOI of 10 PFU/cell. At 1 h prior to each time point, the cells were pulse-labeled for 1 h with 25 µCi tran ^{35}S (MP Biomedicals) in methionine- and cysteine-free medium, SF-900II (Invitrogen), washed with phosphate buffer saline (PBS), and incubated at 27 °C for 1 h with 0.5 ml of PBS. PBS containing labeled proteins was then collected and used as the extracellular fraction. The cells were then

lysed with 1% Nonidet P-40. Samples were then subjected to SDS-PAGE and fluorography as previously described (O'Reilly *et al.*, 1994).

Viral DNA replication

To examine the time course of intracellular viral DNA replication, SF-21 cells (1 x 10^6 cells per 35-mm diameter dish) were infected at a MOI of 5 PFU per cell with AcBAC, AcBAC-vfgfRep, or AcBAC-vfgf-KO. The virus was allowed to adsorb for 1 h at room temperature, and time zero was defined as the time when the virus was removed and incubation at 27°C commenced. At selected times, cells were harvested and DNA was extracted as previously described (Clem *et al.*, 1991). DNA samples (3 µg) were denatured with NaOH to a final concentration of 0.4 N in a total of 50 µl and spotted onto a Zeta–Probe nylon membrane (BioRad) using a Bio-Dot manifold (BioRad). DNA on blots was hybridized to the *gfp* gene that was α-^{32}P-radiolabeled by random priming using hexadeoxynucleotides (Promega) and Klenow (New England Biolabs, Inc.) as described for the NEBlot Kit (New England Biolabs, Inc.). Radioactive spots were quantitated by densitometry using the Alpha Innotech AlphaImager 2200 version 5.5.

Serial passage of viruses

SF-21 cells were initially co-infected with AcBAC-vfgfKO and AcBAC at an MOI of 0.05 PFU/cell for each virus and a total MOI of 0.1 PFU/cell. Supernatants were collected at 96 h p.i. and virus titers were determined by $TCID_{50}$ end point dilution. For all subsequent 5 passages, the cells were infected with a total MOI of 0.1 PFU/cell of the previous passage. Viral DNA was isolated from each passage as described

elsewhere (O'Reilly *et al.*, 1994) and analyzed by Southern blot hybridization. DNA from AcBAC-vfgfKO and AcBAC were digested with *Sac* I and *Nco* I prior to electrophoresis and blotting. Transferred DNA was hybridized to a 0.5 kbp *Pst* I-*Nsi* I labeled fragment (27,778 to 28,293; Ayres *et al.*, 1994) upstream of *vfgf* (Fig. 4A) as described above. Densitometric analysis was performed as mentioned above.

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Figure 1. Strategy for construction of AcBAC-vfgfKO, AcBAC, and AcBAC-vfgfRep and their confirmation. A. A region of the transfer plasmid used to construct the *vfgf*-null bacmid, AcBAC-vfgfKO, by homologous recombination is shown (top). A schematic of the site of insertion at the *vfgf* locus and its relative location to the polyhedrin (*polh*) loci in the bMON14272 bacmid is indicated (bottom). Open arrows indicate the location and direction of transcription of ORFs. The zeocin gene under EM7 promoter control was used to substitute for *vfgf* leaving 98-nt of the *vfgf* C-terminus and *vfgf* promoter region (Pr). B. Insertion of *polh* and *gfp* to construct AcBAC-vfgfKO, AcBAC-vfgfRep (left) and AcBAC (right) are illustrated. Gentamicin, *polh*, and *gfp* flanked by TN7 sequences were incorporated into AcBAC-vfgfKO by transposition. To construct AcBAC-vfgfRep, *vfgf* under control of its native promoter was included in the region used for transposition. C. The strategy used to confirm the bacmids by PCR is depicted by indicating the primers (arrows) used and their approximate location. D. Confirmation of bacmids by PCR. Bacmid DNA indicated at the top was used with the primers shown at the bottom and the products were resolved by agarose electrophoresis. The migration of DNA markers (M) is shown to the left in kilopasepairs (kbp).

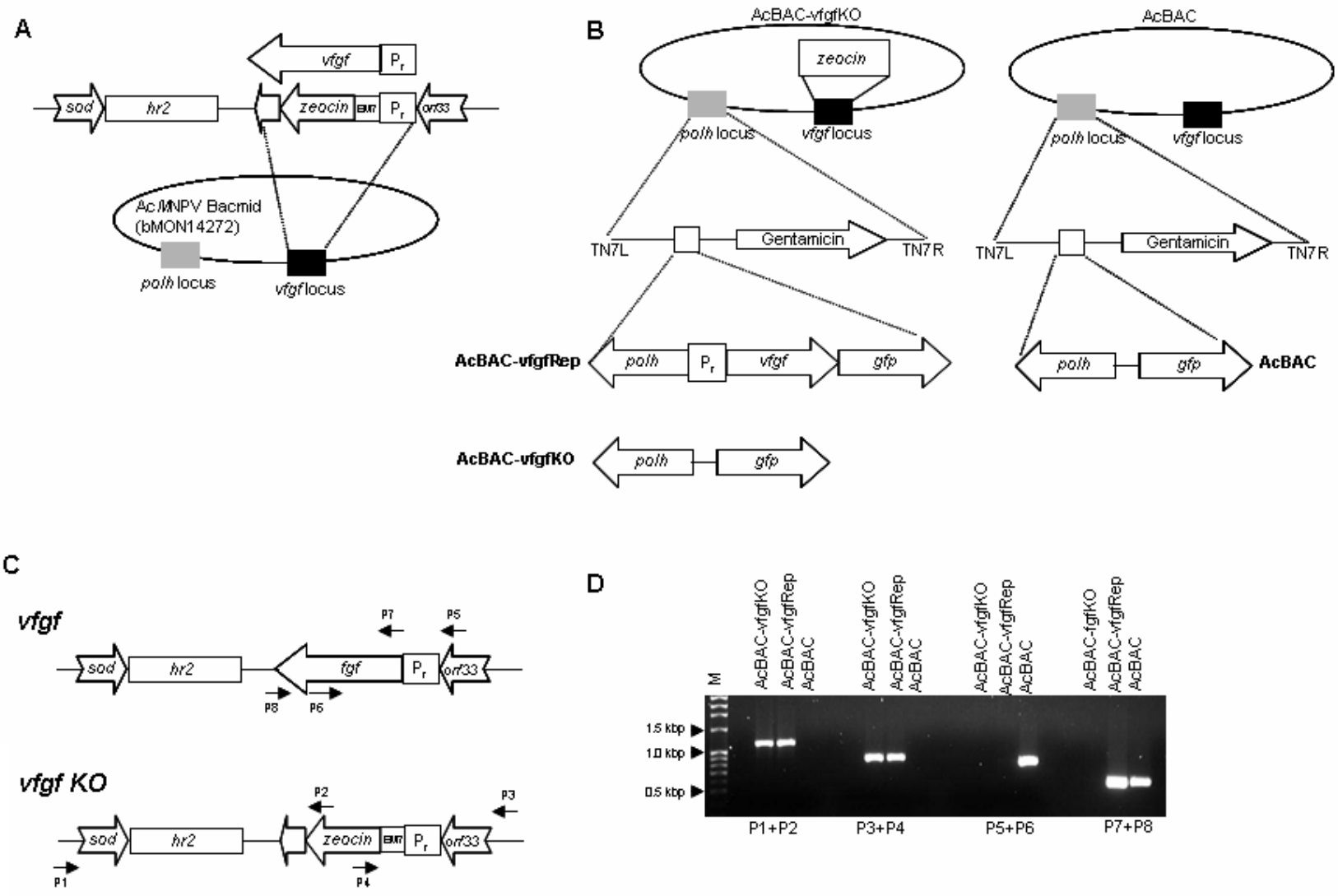

A
vfgf
P r
sod
hr2
zeocin
EM7
P r
orf33
AcMNPV Bacmid
(bMON14272)
polh locus
vfgf locus
B
AcBAC-vfgfKO
zeocin
polh locus
vfgf locus
AcBAC
polh locus
vfgf locus
TN7L
Gentamicin
TN7R
TN7L
Gentamicin
TN7R
AcBAC-vfgfRep
polh
P r
vfgf
gfp
polh
gfp
AcBAC
AcBAC-vfgfKO
polh
gfp
C
vfgf
P7
P5
sod
hr2
fgf
P r
orf33
P8
P6
vfgf KO
P2
P3
sod
hr2
zeocin
EM7
P r
orf33
P1
P4
D
M
AcBAC-vfgfKO
AcBAC-vfgfRep
AcBAC
AcBAC-vfgfKO
AcBAC-vfgfRep
AcBAC
AcBAC-vfgfKO
AcBAC-vfgfRep
AcBAC
AcBAC-fgfKO
AcBAC-vfgfRep
AcBAC
1.5 kbp
1.0 kbp
0.5 kbp
P1+P2
P3+P4
P5+P6
P7+P8

Figure 1.

Figure 2. Time course of AcBAC, AcBAC-vfgfKO, and AcBAC-vfgfRep budded virus production in SF-21 and TN-368. A. Bacmid DNA (1 μg) was extracted from *E. coli* cells and used to transfect SF-21 cells. Budded virus was collected at the indicated times post transfection and titered in SF-21 cells. B to D. SF-21 or TN-368 cells were infected with the indicated viruses at an MOI of 5.0 PFU/cell (B and D) or at an MOI of 0.01 PFU/cell (C and E). Budded virus in the media was collected at several times p. i. and titered in the same cell line. Bars at each time point indicate standard error.

Figure 2.

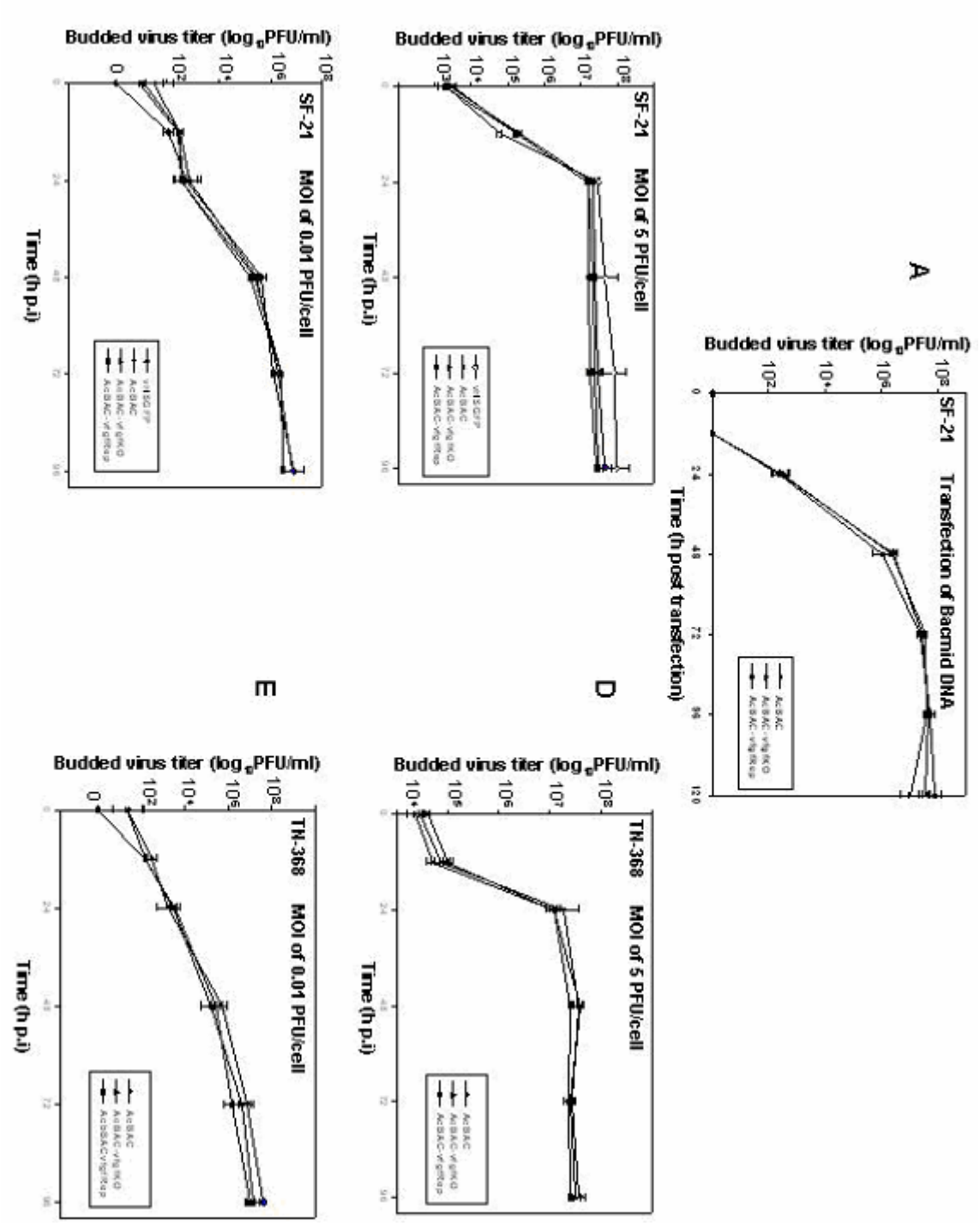

A
SF-21 Transfection of Bacmid DNA
Budded virus titer (log10PFU/ml)
Time (h post transfection)
AcBAC
AcBAC-vIgKO
AcBAC-vIgRap
D
SF-21 MOI of 5 PFU/cell
TN-368 MOI of 5 PFU/cell
E
SF-21 MOI of 0.01 PFU/cell
TN-368 MOI of 0.01 PFU/cell
Time (h p.i.)
vISGFP
AcBAC
AcBAC-vIgKO
AcBAC-vIgRap

Figure 3. Kinetics of protein synthesis and viral DNA replication of AcBAC-vfgfKO-, AcBAC-, or AcBAC-vfgfRep-infected SF-21. A. SF-21 cells were either mock-infected (mi) or infected with AcBAC-vfgfKO-, AcBAC-, or AcBAC-vfgfRep at a MOI of 10 PFU/cell. At the indicated times p.i., proteins were pulse-labeled and extracted. Proteins were resolved by SDS-PAGE and visualized by fluorography. Sizes of protein markers are indicated to the left in kilodaltons. B. SF-21 cells were infected at an MOI of 5 PFU/cell with AcBAC-vfgfKO, AcBAC, and AcBAC-vfgfRep and DNA was extracted at several times p.i. and hybridized to radiolabeled *gfp* DNA (top). The intensity of radioactivity in each dot blot was quantitated by densitometry and is shown (bottom).

Figure 3.

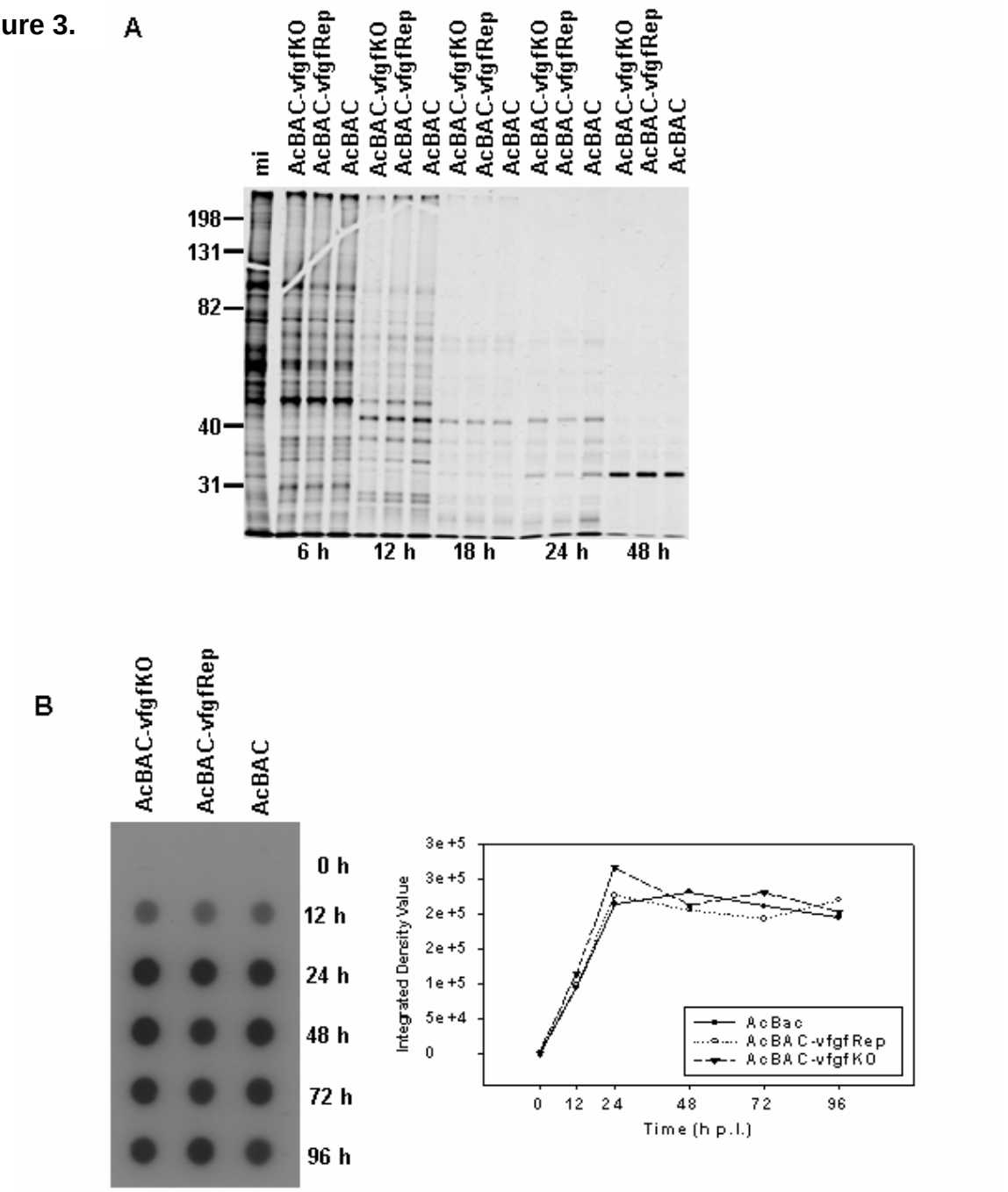
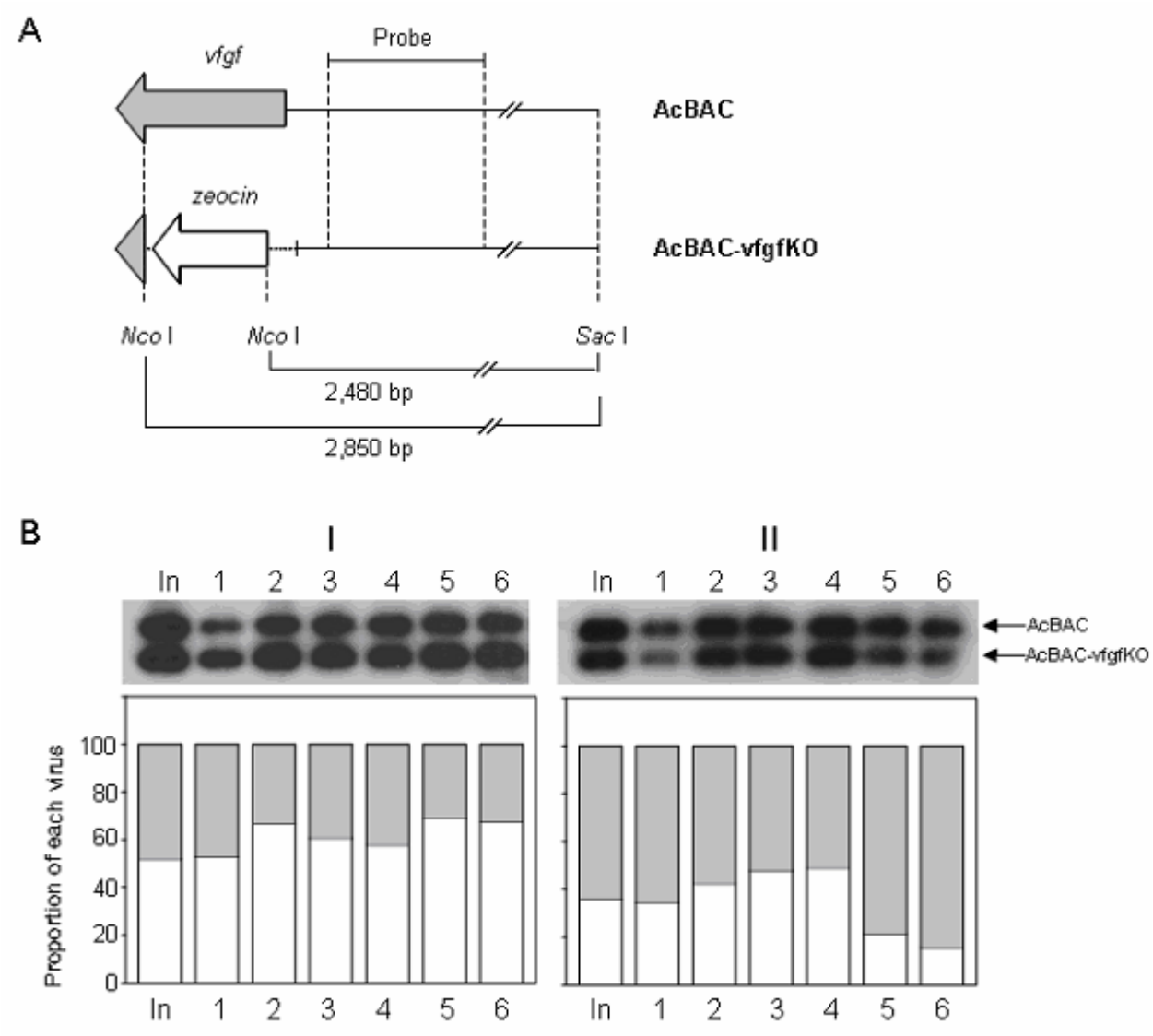

A
mi
AcBAC-vfgfKO
AcBAC-vfgfRep
AcBAC
198
131
82
40
31
6 h
12 h
18 h
24 h
48 h
B
AcBAC-vfgfKO
AcBAC-vfgfRep
AcBAC
0 h
12 h
24 h
48 h
72 h
96 h
Integrated Density Value
3e+5
3e+5
2e+5
2e+5
1e+5
5e+4
0
0
12
24
48
72
96
Time (h p.i.)
AcBac
AcBAC-vfgfRep
AcBAC-vfgfKO

Figure 4. Coinfection of SF-21 cells with AcBAC-vfgfKO and AcBAC. A. A schematic diagram of the *vfgf* region in AcBAC and AcBAC-vfgfKO illustrating the *vfgf* and zeocin ORFs (open arrows) and the approximate locations of *Nco* I and *Sac* I restriction endonuclease recognition sites. The probe that hybridizes to a region of the same size in *Nco* I and *Sac* I digested AcBAC and AcBACvfgf-KO DNA is indicated at the top and the expected restriction fragments are indicated at the bottom. B. Autoradiographs of Southern blots from two independent experiments (I and II) showing the restriction length polymorphism between AcBAC and AcBAC-vfgf-KO DNA digested with *Nco* I and *Sac* I and hybridized to the labeled fragment shown in panel A after each passage indicated at the top (top panels) In, input. Densitometric quantitation of labeled fragments is shown below the corresponding Southern blot. Each passage shows the proportion of each virus, AcBAC (grey bars) and AcBAC-vfgf-KO (white bars) with respect to total (100%).

Figure 4.


A
vfgf
Probe
AcBAC
zeocin
AcBAC-vfgfKO
Nco I
Nco I
Sac I
2,480 bp
2,850 bp
B
I
II
In 1 2 3 4 5 6
In 1 2 3 4 5 6
AcBAC
AcBAC-vfgfKO
Proportion of each virus
100
80
60
40
20
0
In 1 2 3 4 5 6
In 1 2 3 4 5 6

CHAPTER 4

Conclusions

CONCLUSIONS

Fibroblast growth factors (*fgfs*) are encoded by invertebrate and vertebrate organisms and have diverse functions including regulating cell proliferation, migration, and differentiation (Powers *et al*., 2000; Ornitz and Itoh 2001). Interestingly, among viruses, *fgf*s are only present in baculoviruses (Ayres *et al*., 1994), but prior to this work, their function during virus infection was not known. Almost all baculoviruses encode at least one copy of *fgf*. Baculoviruses that encode *fgf* infect insects in the order Lepidoptera (moths and butterflies), initiate infection in the midgut epithelial cells and then spreading systemically until the insect dies. In contrast, baculoviruses that do not encode *fgf* infect insects in the orders Diptera (mosquitoes) and Hymenoptera (sawflies), but, noticeably, their infection is restricted to the midgut epithelial cells and does not spread systemically (Afonso *et al*., 2001; Garcia-Maruniak *et al*., 2004; Lauzon *et al*., 2004; Duffy *et al*., 2006). The different patterns of virus infection fit the hypothesis that the viral *fgf*, *vfgf,* plays an important role for efficient spread of infection beyond the midgut.

Thus, we began our journey to uncover the significance of *vfgf* by studying *vfgf* from Ac*M*NPV, the best-studied baculovirus, and demonstrated that Ac*M*NPV *fgf* shared both structural and biochemical characteristics with other *fgfs*. Most FGFs contain a signal peptide that destines them for secretion. Once secreted, FGFs bind heparan sulfate proteoglycans on the cell surface and upon cleavage of FGF and heparan sulfate, these complexes bind and activate FGF receptors, triggering a signal transduction cascade (Powers *et al*., 2000; Ornitz and Itoh 2001). Besides Ac*M*NPV *fgf*

having sequence homology to other known *fgfs*, our results showed that the predicted Ac*M*NPV *fgf* signal sequence was functional, secreting the Ac*M*NPV *fgf* product, Ac*M*NPV FGF, outside the cell. We next showed that Ac*M*NPV FGF had high affinity to heparin comparable to other characterized FGFs, suggesting that Ac*M*NPV FGF binds heparan sulfate proteoglycans on the cell surface *in vivo*. In addition, Ac*M*NPV *fgf* was expressed at early times post infection, suggesting its function was involved in the early process of infection.

It is possible to envision that baculoviruses acquired *fgfs* from their insect hosts. Knowledge obtained from insect homologs of *fgfs* and its receptor, *Drosophila branchless* (*bnl*) and *breathless* (*btl*), respectively, granted us a favorable clue to study the function of v*fgfs*. BNL functions as a chemoattractant in controlling tracheal cell migration and establishing a branching pattern (Sutherland *et al*., 1996; Sato and Kornberg, 2002). To test whether Ac*M*NPV FGF had chemotactic activity, we performed three-dimensional transmigration assays and observed that Ac*M*NPV FGF was able to stimulate migration of SF-21 and TN-368 cells, insect cells permissive for baculovirus infection, as well as *Trichoplusia ni* hemocytes.

In the insect host, after the infection takes place in midgut epithelial cells, tracheal cells are infected. It has been proposed that baculoviruses use the tracheal system as a route to spread infection from the midgut into other tissues (Engelhard *et al*, 1994; Washburn *et al*., 1995). To this end, we proposed that the virus uses vFGF as a chemoattractant to stimulate migration of uninfected tracheal cells or hemocytes toward infected tissues or cells in order to spread infection throughout the host.

Based on our initial finding and hypothesis, Katsuma *et al*. (2006b) identified the orthologs of *btl* receptor on cells derived from *Bombyx mori* and *Spodotera frugiperda* and called them *bmbtl* and *sfbtl*, respectively. They also showed that *bmbtl* was expressed in the midgut and trachea and moderately expressed in hemocytes of *B. mori* larvae. These authors performed transmigration assay and found that expression levels of *bmbtl* and *sfbt*l in *S. frugiperda*-derived cells affected migration of these cells upon activation by BmNPV FGF, suggesting, like Ac*M*NPV FGF, that BmNPV FGF had the chemotactic activity which was mediated by the BmBTL and SfBTL receptors.

Next, we constructed a recombinant Ac*M*NPV lacking a functional *fgf* and examined whether Ac*M*NPV *fgf* played a role during infection in cell culture. We found that this mutant did not have an obvious defect in viral DNA synthesis and budded virus production, nor did it affect the kinetics of viral protein production. In addition, coinfection of Ac*M*NPV *fgf*-containing and –deficient viruses did not reveal any selective advantages for virus replication. Therefore, we concluded that Ac*M*NPV *fgf* is not required for virus replication in cell culture under the conditions tested. It is possible that the effect of Ac*M*NPV *fgf* may not be exerted in the absence of a chemotactic gradient during infection of a cell monolayer.

In contrast, Katsuma *et al*. (2006c) demonstrated that budded virus production was reduced in the *B. mori*-derived cells and *B. mori* larvae infected with a BmNPV *fgf*-deficient virus. They further showed that this mutant caused a delay in death of larvae both when the virus was fed orally or injected intrahemocoelically. However, this mutant did not have different infectivity from a virus with *fgf*.

We anticipate the phenotypes of an Ac*M*NPV *fgf*-deficient virus will be more apparent during infection of insect hosts. Because of the early expression of Ac*M*NPV *fgf* and the hint that baculoviruses that do not encode *fgf* have restricted infection of midgut cells, we hypothesize that the requirement for Ac*M*NPV *fgf* will be more prominent during the spread of infection from the midgut to trachea. Thus, obvious increases in the lethal time or dose of Ac*M*NPV *fgf*-deficient virus may be expected during per os rather than intrahemocoelic infection where the virus can bypass the midgut. Alternatively, the lethal dose of an Ac*M*NPV *fgf*-deficient virus may remain unchanged similar to that of the BmNPV *fgf*-deficient virus (Katsuma *et al*., 2006b). In this scenario, Ac*M*NPV *fgf* is primarily responsible for disseminating the infection instead of enhancing virus infectivity.

The phenotypic differences between Ac*M*NPV *fgf*- and BmNPV *fgf*-deficient viruses during infection of cultured cells may be explained by a few reasons:

- Differences in the native properties and expression levels between Ac*M*NPV *fgf* and BmNPV *fgf*

Despite highly conserved amino acids between these two proteins, there are distinct differences in the post-translational modifications. Ac*M*NPV *fgf* does not have predicted N-glycosylation sites and its product appeared to be an unglycosylated protein and was secreted at low levels into the culture medium (Katsuma *et al*., 2006a; Lehiy and Passarelli, unpublished results). In contrast, BmNPV *fgf* has two predicted N-glycosylation sites and both sites appeared to be glycosylated. In addition, BmNPV FGF was shown to be secreted efficiently while an unglycosylated mutant had defects in secretion (Katsuma *et al*., 2006a; Cain

and Passarelli, unpublished results). Whether the pattern of glycosylation affects vFGF activity between Ac*M*NPV *fgf* and BmNPV *fgf* remains unknown.

- Differences in virus-host interactions:

Ac*M*NPV and BmNPV are phylogenetically closely related but they have completely different host ranges. While BmNPV has only been known to infect *B. mori*, Ac*M*NPV infects more than 30 lepidopteran species (Granados and Williams, 1986). This may in part affect the requirements for and roles of *vfgf* during infection of different hosts.

Our studies on Ac*M*NPV *fgf* described a functional conservation of *fgf*s and revealed a novel mimicry of a cellular development factor to a virulence factor. The novel function of vFGF as a virulence factor will be useful in augmenting the applications of recombinant baculoviruses as pest control agents.

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CHAPTER 2

Stimulation of cell motility by a viral fibroblast growth factor homolog:
Proposal for a role in viral pathogenesis

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Virology 336, 308-317.

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