NUCLEOPOLYHEDROVIRUS PATHOLOGY IN SPRUCE BUDWORM LARVAE

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Abstract

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Choristoneura fumiferana (Clemens) (Lepidoptera: Tortricidae) multiple nucleopolyhedrovirus (*Cf*MNPV) expressing green fluorescent protein was used to study aspects of nucleopolyhedrovirus infection in the spruce budworm. The temporal and spatial distribution of fluorescence indicated that the virus infected the midgut, entered the tracheal system, and traveled to the epidermis, fat body, and muscles. In contrast to *Autographa californica* (Speyer) (Lepidoptera: Noctuidae) multiple nucleopolyhedrovirus (*Ac*MNPV) infection, hemocytes from infected *C. fumiferana* did not exhibit fluorescence until after *Cf*MNPV had passed from the midgut into the tracheae. Therefore the role of hemocytes may be limited during *Cf*MNPV infection. Also the fluorescence pattern spread from the tracheolar cells to tracheal epithelial cells throughout the tracheal system. Our results indicate that the temporal and spatial events involved in *Cf*MNPV infection of *C. fumiferana* larvae are consistent with those observed in other lepidopteran hosts infected with *Ac*MNPV. Minor deviations between these two systems may be attributed to differences in virulence, infection rate, and possibly host range of the virus.

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Résumé

Le nucléopolyhédrovirus multiple de la Tordeuse des bourgeons de l'épinette (*Choristoneura fumiferana*) (*Cf*MNPV) à protéine fluorescente verte (GFP) a été utilisé pour étudier certains aspects de ce type d'infection chez la tordeuse. La répartition temporelle et spatiale de la fluorescence indique que le virus infecte l'intestin moyen, pénètre dans le système trachéen et se répand dans l'épiderme, le corps gras et les muscles. Contrairement à ce qui se produit dans une infection du nucléopolyhédrovirus multiple de l'*Autographa californica* (Speyer) (Lepidoptera : Noctuidae) (*Ac*MNPV), les hémocytes des tordeuses infectées ne deviennent pas fluorescents avant que le virus *Cf*MNPV ne soit passé de l'intestin moyen au système trachéen. Le rôle des hémocytes est probablement limité dans ce type d'infection. De plus, la fluorescence se répand des cellules trachéolaires aux cellules épithéliales dans tout le système trachéen. Nos résultats indiquent que les étapes du déroulement temporel et spatial de l'infection *Cf*MNPV chez les larves de la tordeuse sont semblables à celles observées chez d'autres lépidoptères infectés du virus *Ac*MNPV. Les différences mineures entre ces deux systèmes peuvent être le résultat de différences dans le degré de virulence, dans le taux d'infection et probablement aussi dans l'éventail des hôtes du virus.

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Introduction

Infection of lepidopterans by the nucleopolyhedrovirus (NPV) type species, *Autographa californica* (Speyer) (Lepidoptera: Noctuidae) multiple nucleopolyhedrovirus (*Ac*MNPV), has been studied in several species, including *Trichoplusia ni* (Hubner) (Lepidoptera: Noctuidae) (Granados and Lawlor 1981; Granados and Williams 1986; Keddie *et al.* 1989; Barrett *et al.* 1998), *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae) (Flipsen *et al.* 1993, 1995), *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) (Washburn *et al.* 1995), and *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) (Kirkpatrick *et al.* 1994). Whether the described scenario of infection is unique to *Ac*MNPV or is shared by other NPVs has not been resolved. In an attempt to verify the universality of this process in lepidopterans, we examined the infection process of *Choristoneura fumiferana* (Clemens) (Lepidoptera:Torticidae) multiple nucleopolyhedrovirus (*Cf*MNPV) expressing green fluorescent protein (GFP) in the infection of the spruce budworm, a major pest of eastern Canadian and northeastern United States forests.

GFP was originally isolated from *Aequorea victoria* (Murbach and Shearer) (Hydrozoa: Campanulinidae), a bioluminescent jellyfish, and has been used successfully as a reporter gene in many heterologous systems. GFP expression results in an intense green fluorescence that is species independent, does not require any cofactors or substrates, and can be visualized in living tissues (Chalfie *et al.* 1994).

Recent reviews have outlined *Ac*MNPV pathogenesis in susceptible hosts (Federici 1997; Volkman 1997). It is generally accepted that ingested polyhedra are dissolved within the high-alkaline environment of the midgut. Released occlusion derived virions fuse with microvilli and enter the midgut epithelial cells. The spread of infection *in vivo* is preceded by the production of budded virus produced early in the infection cycle in the midgut cells and released into the host hemolymph. The precise mechanism of the movement of budded virus from the midgut, across the basal lamina, and into the hemolymph remains unresolved. It appears that *Ac*MNPV budded virus spreads *via* the tracheal matrix using the tracheal system as a conduit for the systemic spread of infection in *T. ni* (Engelhard *et al.* 1994), *H. zea* (Kirkpatrick *et al.* 1994), and *H. virescens* (Washburn *et al.* 1995). To determine whether this pattern of infection is universal among NPV-infected lepidopterans, we examined the temporal sequence of infection in *C. fumiferana* larvae infected with a recombinant *Cf*MNPV expressing GFP under the control of the *Cf*MNPV polyhedrin promoter (Cf*polh*p). We fed 5th-instar larvae 10^4 occlusion bodies per larva (LD_{65}; Ebling *et al.* 1998) and monitored the appearance of fluorescence as an indicator of late gene expression in various tissues using whole mounts, as well as paraffin sections of tissues from *C. fumiferana* larvae over several days.

Materials and Methods

Construction of the Recombinant Virus. Plaque-purified *Cf*MNPV has been described (Arif *et al.* 1984), and all biochemical manipulations followed standard procedures (Sambrook *et al.* 1989). *Cf*MNPV expressing GFP, *Cf*MNPV:Cf*polh*p:GFP, was constructed by amplifying the *Cf*MNPV polyhedrin promoter and cloning it into the GFP vector, pEGFP-1 (Clontech, Inc). The *polh* promoter of *Cf*MNPV was amplified using primer JB4.97 to amplify from the 3′ end (5′-GCCGGATCCAATAATCTGGAATAG-3′). An introduced *Bam*HI endonuclease restriction site is underlined and the altered start site is double underlined. The forward primer, JB5.97 (5′-CAATTGAGCTCAAACAGG-3′), contained an introduced *Sac*I site (underlined). The DNA

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template used was the 2.4-kilobase (kb) *Pst*1-O fragment, part of a plasmid library of *Cf*MNPV. Transfer of the Cf*polh*p:GFP open reading frame (ORF) cassette from pEGFP-1 into our generic transfer vector, pUC18:*egt* (JW Barrett and BM Arif, unpublished data), was achieved by amplifying the area of interest using primers JB25.96 and JB3.97. These primers were designed to amplify the region within pEGFP-1 that contained the multiple cloning sites, the *gfp* ORF, and the polyadenylation sequence. Primer JB25.96, which amplified from the 3′ end, contained an introduced *Sph*I site (underlined), 5′-CGCTTACAATGCATGCCTTAAG-3′. Primer JB3.97, synthesized to amplify from the 5′ end, contained an introduced *Spe*I site (underlined), 5′-GTTATT-ACTAGTCGTACCG-3′. Amplification of the Cf*polh*p:GFP cassette resulted in the synthesis of an approximately 1.2-kb product.

The transfer vector pUC18:*egt* is a derivative of our earlier vector, pFPMI (JW Barrett and BM Arif, unpublished data), a vector centred on the *Cf*MNPV *ecdysteroid UDP-glucosyltransferase* (*egt*) region. It was designed to allow homologous recombination and the production of recombinant viruses. pUC18:*egt* contained a 3.6-kb fragment of the *Cf*MNPV *Hin*dIII-G fragment that was digested with *Pst*I and *Xho*I to release a 358-bp fragment (nucleotides 839–1196 of the *egt* ORF; Barrett *et al.* 1995). Two complementary primers, JB26.96 and JB27.96, were synthesized in such a manner that once the primers had annealed to each other it would result in a 20 nucleotide double stranded fragment containing *Sph*I, *Stu*I, and *Spe*I sites with four base overhangs complementary to the *Xho*I and *Pst*I ends. These ends were modified by altering the fifth base from each end of primer JB26.96 to ensure that they would ligate to *Xho*I and *Pst*I recognition sites but once annealed the sites would no longer be recognized by these enzymes (Fig. 1).

Construction of a *Cf*MNPV-modified virus, expressing GFP under the Cf*polh* promoter, was produced by homologous recombination in the *egt* region using lipofectin (Gibco/BRL, Burlington, Ontario) as outlined in the manufacturer's standard protocol. Transfections were performed using FPMI-CF-203 (CF-203; Sohi *et al.* 1993) cells maintained in Insect Xpress medium (BioWhittaker, Walkersville, Maryland) supplemented with 2.5% fetal bovine serum.

Preparation of the Inoculum. *Choristoneura fumiferana* larvae were reared in our Insect Production Unit (Great Lakes Forestry Centre) on formaldehyde-free spruce budworm diet (McMorran 1965) at 25ºC and 60% RH at a 16L:8D photoperiod. Newly molted 5th-instar larvae were selected and each larva was placed into an individual 1.5-mL centrifuge tube containing a 4-mg diet plug saturated with 10 000 *Cf*MNPV:Cf*polh*:GFP occlusion bodies. Based on our calculations, an individual dose of 10^4 occlusion bodies represents a LD_{65} level of plaque-purified *Cf*MNPV (Ebling *et al.* 1998). The larvae were allowed to feed on the plug for 16 h. Only those larvae which had completely consumed the diet plug were used in all the experiments and were placed into fresh diet cups. The time of transfer onto fresh diet was considered as time 0 h post-feeding. Groups of five larvae were examined and dissected daily for 10 d to follow the course of infection and GFP expression. This time course was replicated three times.

Preparation of Various Tissues. Inoculated larvae were dissected at different times post-feeding. Different tissues and hemolymph were examined and photographed under an Olympus fluorescent microscope using a fluorescein cube with an excitation wavelength of 490 nm and an emission wavelength of 525 nm. Larvae were collected 3–5 d post-feeding and snap frozen in TissuePrep embedding media (Fisher Scientific Co., Toronto, Ontario) with liquid nitrogen. Sections, 7 μm thick, were cut with a microtome and examined under the Olympus fluorescent microscope. The nucleus-specific stain

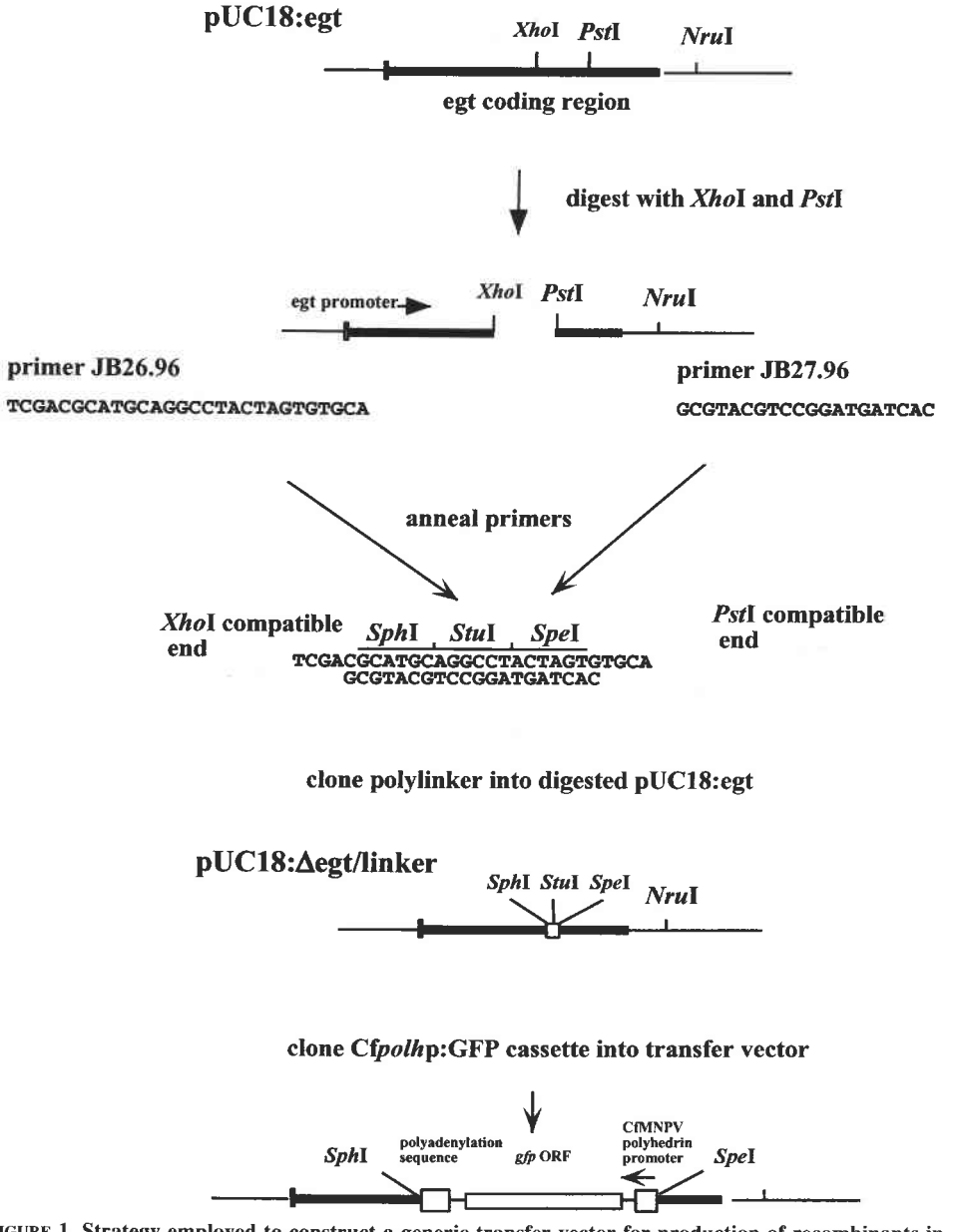

FIGURE 1. Strategy employed to construct a generic transfer vector for production of recombinants in *Cf*MNPV. A 3.6-kb fragment isolated from the *Cf*MNPV genome following digestion with the endonuclease restriction enzyme *Hin*dIII was cloned into the universal cloning vector pUC18. This *Cf*MNPV *Hin*dIII-G fragment contained the characterized *Cf*MNPV *ecdysteroid UDP-glucosyltransferase* (*egt*) coding region (Barrett *et al.* 1995) and the cloned product was named pUC18:*egt*. An artificial multiple cloning site with the unique restriction sites *Sph*I, *Stu*I, and *Spe*I was generated by insertion of annealed primers (JB26.96 and JB27.96) into the *Xho*1 and *Pst*1 restriction sites within the *Cf*MNPV *egt* ORF. The amplified GFP cassette (C*fpolh*p:GFP), containing the GFP ORF under the control of the *Cf*MNPV polyhedrin promoter and the SV40 polyadenylation sequence, was then cloned into the *Sph*I and *Spe*I sites.

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4,6-diamidine-2′-phenylindole dihydrochloride (DAPI) was used to stain the epithelial cells of tracheae. Photographs were taken using Ektachrome 100 film.

Results

Spread of NPV Infection Through Midgut. The first appearance of fluorescence was observed in a small number of midgut epithelial cells in the posterior region 3 d post-feeding. Based on the number of fluorescent cells, we concluded that the infection in *C. fumiferana* started in the posterior region of the midgut and spread toward the anterior end. Cross sections of the midgut collected 3 d post-feeding indicated few fluorescent cells in the anterior portion (Fig. 2A) compared to many at the posterior region of the midgut (Fig. 2B). Within 24 h of the first appearance of fluorescent midgut epithelial cells, *Cf*MNPV infection had spread as indicated by the presence of fluorescent tracheal epithelial cells located adjacent to infected midgut cells (Figs. 2C, 2D). By day 5 post-feeding, larvae started sloughing off GFP-expressing columnar epithelial cells (Figs. 2E, 2F), and by day 6 post-feeding the GFP fluorescence was observed throughout the larvae, except in the midgut epithelial cells (Fig. 2G). Midgut sections stained with acridine orange indicated that the midgut epithelial cells were present but were not fluorescent (Fig. 2H).

Spread of NPV Infection Through Tracheae. GFP fluorescence was observed in tracheal epithelial cells attached to the midgut on day 4 (Figs. 2I, 2J), indicating the passage of the virus from the midgut to the tracheal system (Figs. 2I, 2J). In contrast, little or no fluorescence was observed in the tracheal epithelial cells lining the distal end of the tracheal trunk, indicating that virus spread from the midgut epithelial cells to the adjacent tracheal epithelial cells by physical contact. DAPI staining of the infected tracheal tissue peeled off from the midgut (Fig. 2L) confirmed that all the tracheal epithelial cells that adhered to the midgut were infected (Fig. 2K). The infection then spread throughout the tracheal system between day 4 (Fig. 2M) and day 6 (Fig. 2N) post-feeding. Frozen sections of larvae collected 6 d post-feeding showed that the tracheal epithelial cells lining the tracheae that surround most of the tissues were fluorescent (Figs. 2O, 2P).

Spread of NPV Infection to Various Tissues. Tracheal epithelial cells of the tracheae associated with the midgut (Fig. 3A), fat body (Fig. 3B), epidermis (Fig. 3C), and muscles (Fig. 3D) showed initial fluorescence on day 4 post-feeding. By day 6 post-feeding, the infection was complete, as indicated by the appearance of fluorescence in the fat body (Fig. 3F), epidermis (Fig. 3G), and muscle (Fig. 3H). A small proportion of the hemocytes was fluorescent by day 4 post-feeding (not shown). The number of fluorescent hemocytes increased from day 5 to day 7 post-feeding (not shown). Approximately 50% of the hemocytes were expressing GFP on day 6 post-feeding (Fig. 3E). Silk gland cells (Fig. 3I), Malpighian tubules (Figs. 3J, 3K), and epithelial cells of the main tracheal trunk (Fig. 3L) also showed fluorescence by day 6 post-feeding. At the same time, tracheal epithelial cells that are attached to the ovary (Fig. 3M), testes (Fig. 3N), brain (Fig. 3O), and ventral ganglia (Fig. 3P) also showed fluorescence, but the cells of these organs did not show any fluorescence.

Discussion

In this report we describe the temporal sequence of *Cf*MNPV infection in *C. fumiferana* larvae using a recombinant NPV expressing the GFP under the control of

the *Cf*MNPV polyhedrin promoter. The advantage of utilizing a late promoter, in contrast to using an early or constitutive promoter, is that this strategy allowed us to observe those tissues in which *Cf*MNPV establishes a productive infection and would contribute to the synthesis of occlusion-derived virus. This study is the first rigorous examination of NPV pathogenesis in the spruce budworm and one of the first to outline the NPV infection sequence in a lepidopteran host other than those species infected by *Ac*MNPV.

Our results confirm the importance of the midgut to the initial NPV infection as well as the critical role of the tracheal system in spreading the infection. We observed several differences between *Cf*MNPV and *Ac*MNPV, including a decreased fluorescence in *C. fumiferana* midgut, a later appearance of fluorescence in the hemocytes suggesting a more limited role for the hemocytes in pathogenesis, and a generally slower internal spread of the virus in *C. fumiferana* larvae. When we compare the general level of fluorescence in the midgut of *C. fumiferana* larvae to that in *Ac*MNPV-infected *T. ni* larvae there is a substantial difference. Fluorescence in *T. ni* midguts appears early in the infection cycle and results in strong fluorescence of the midgut epithelial tissue (Barrett *et al.* 1998). In contrast, *C. fumiferana* larvae do not have the same level of fluorescence or number of fluorescent cells, suggesting that the *C. fumiferana* midgut epithelial layer is not infected to the same degree as that of *Ac*MNPV-infected *T. ni* larvae. We also observed differential preference for fluorescence in the posterior end of the midgut (Fig. 2B).

The rapid appearance of fluorescence in the tracheae associated with the midgut and subsequent appearance of fluorescent tracheae supplying the fat body, epidermis, and muscle confirm the importance of the tracheal system as a conduit for the movement of the virus. This is consistent with the observations and hypotheses developed for *Ac*MNPV-infected hosts (Engelhard *et al.* 1994; Kirkpatrick *et al.* 1994; Washburn *et*

FIGURE 2. Spread of *Cf*MNPV infection through the midgut (A–H) and tracheae (I–P). Fifth-instar *Choristoneura fumiferana* larvae were inoculated with 10^4 occlusion bodies of *Cf*MNPV expressing GFP. Larvae were collected 3, 4, and 6 d post-feeding and either frozen (O, P) or embedded (A–H), and cross sections were cut and photographed under a fluorescent microscope. (A) Anterior end of midgut collected 3 d post-feeding, showing a few fluorescent cells. (B) Posterior end of midgut collected 3 d post-feeding, showing more fluorescent cells. (C, D) Cross sections of midguts collected 4 d post-feeding, showing fluorescence in the tracheal epithelial cells lining the tracheae which are adjacent to the infected midgut epithelial cells. Arrows indicate infected midgut epithelial cells. (E, F) Cross sections of midgut collected 6 d post-feeding, showing midgut epithelial cells that are being sloughed off (arrows). (G, H) Consecutive cross sections were photographed unstained (G) or stained with acridine orange (H). (I, J) Tracheae attached to the midgut were pulled off on day 4 post-feeding and photographed. (K, L) The same tracheae were stained with the nucleus-specific dye DAPI (4,6-diamidine-2′-phenylindole dihydrochloride) and photographed under fluorescence (K) and with the DAPI cube (L). (M, N) Entire tracheal system was photographed on day 4 (M) and day 6 (N) post-feeding. (O, P) Frozen sections collected from larvae 6 d post-feeding were photographed exhibiting fluorescent tracheae surrounding most tissues. The scale bar represents 30 μm in A–D, I, and N, 15 μm in E–H, J–L, and O, and 75 μm in M and P. Ep, epidermis; Fb, fat body; Mg, midgut.

FIGURE 3. Spread of *Cf*MNPV infection to various tissues. Fifth-instar *Choristoneura fumiferana* larvae were inoculated with 10^4 occlusion bodies of the recombinant *Cf*MNPV virus expressing the GFP, and the fluorescence in various whole mounts of the tissues was recorded 4–6 d post-feeding. Tracheal epithelial cells present on the surface of the midgut showed fluorescence on day 6 post-feeding (A). Epithelial cells lining the tracheae which are attached to fat body (B), epidermis (C), and muscle (D) were fluorescent by day 4 post-feeding. By day 6 post-feeding, hemocytes (E), fat body (F), epidermis (G), muscle (H), silk gland (I), and Malpighian tubules (J, K) showed fluorescence. Tracheal epithelial cells of the main tracheal trunk (L) and epithelial cells lining the tracheae which are attached to the tissues such as ovary (M), testes (N), brain (O), and ganglia of the ventral nerve cord (P) also showed fluorescence by day 6 post-feeding. The scale bar represents 15 μm in A, C–E, G–I, and O, 30 μm in F and J–N, and 75 μm in B and P. All panels represent whole-mount slides of *C. fumiferana* tissue.

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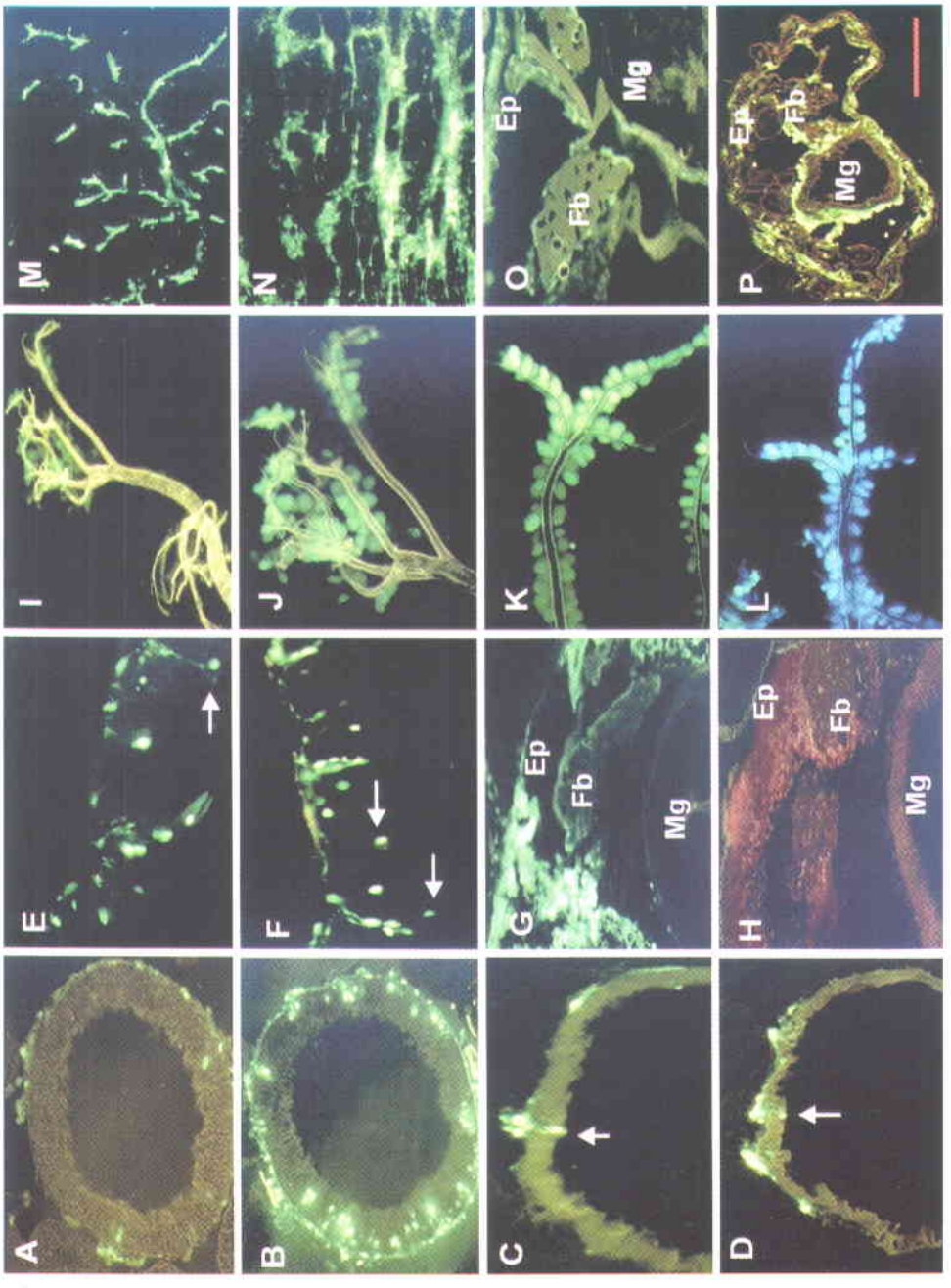

FIG. 2.

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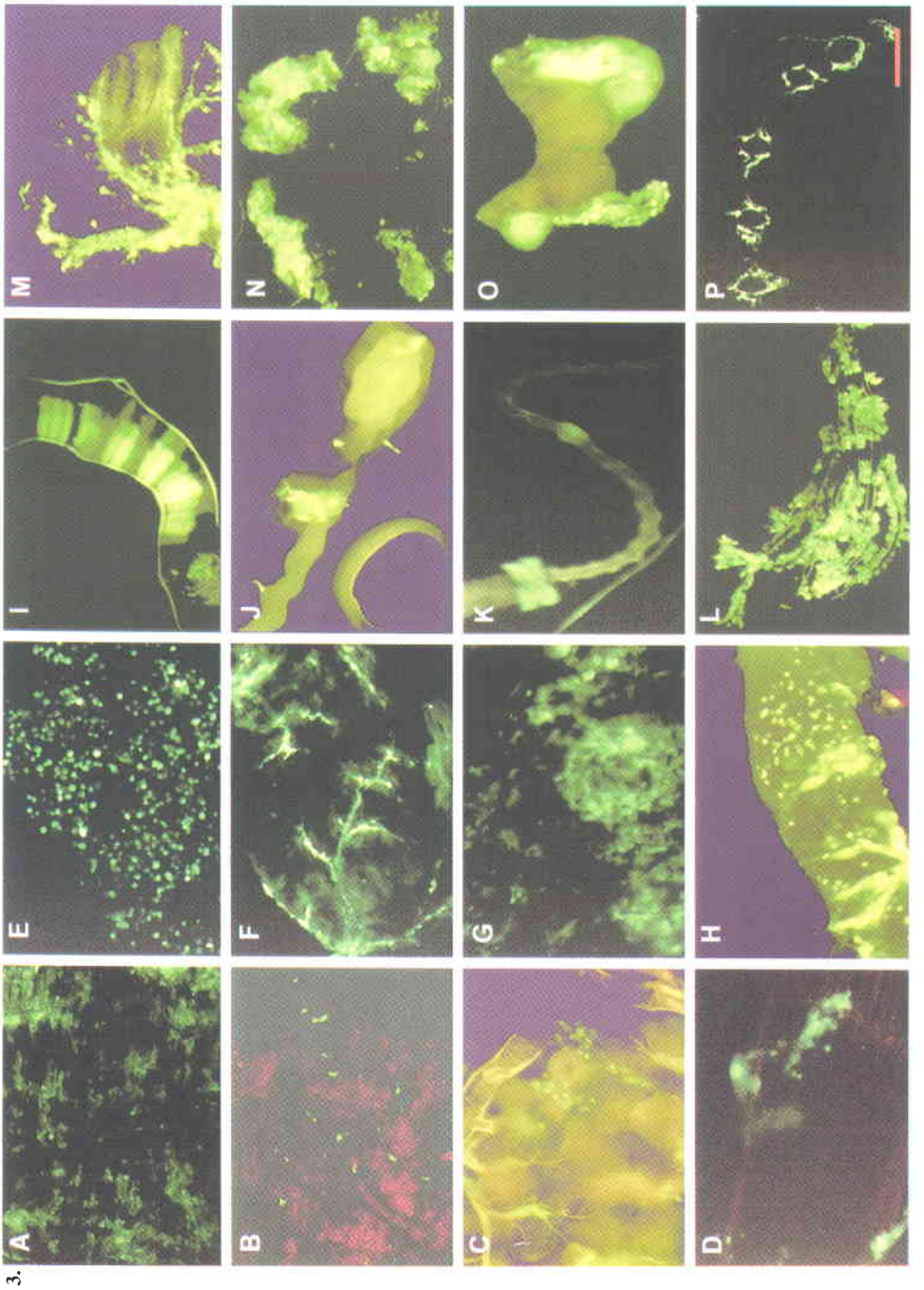

FIG. 3.

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al. 1995). At later times during the infection, the tracheae associated with many other tissues such as ovaries, testes, brain, and ganglia were also heavily fluorescent, even though the organs themselves did not exhibit any fluorescence. This indicates a general infection and migration of the virus through the tracheal system and also suggests that the virus has access to all organs and tissues within the host whether or not that organ or tissue is permissive to infection.

A subtle difference observed in infected *C. fumiferana* larvae was the late appearance of fluorescence in the hemocytes, which contrasts with what was observed in *Ac*MNPV-infected *T. ni* larvae: hemocytes and midgut were infected simultaneously as early as 12 h post-feeding (Barrett *et al.* 1998). In *C. fumiferana* larvae infected with *Cf*MNPV, fluorescent hemocytes were not observed until day 4 post-feeding. In contrast, the first sign of fluorescent midgut epithelial cells was observed 24 h earlier on day 3 post-feeding. This may reflect a change in the role of hemocytes in the progression of infection within the host. The role of hemocytes in *Ac*MNPV-infected hosts was hypothesized to contribute to the rapid spread of the virus (Granados and Lawlor 1981).

Another major difference between *Cf*MNPV and *Ac*MNPV infection pathology was that *Cf*MNPV infection of *C. fumiferana* larvae was generally slower than that reported for *Ac*MNPV infection of susceptible hosts. Although we observed the characteristic pattern of midgut infection followed by sloughing off of infected cells into the midgut lumen, the temporal regulation of these events was more prolonged in *Cf*MNPV-infected larvae.

The difference in the speed of the infection may be related to the virulence of the virus. Studies on *Cf*MNPV infection in cell culture suggest that the natural course of infection, replication, and gene expression are temporally delayed as compared to *Ac*MNPV in SF-21 cells (Liu and Carstens 1993, 1995; Barrett *et al.* 1995, 1996; Li *et al.* 1997). Also, time–response data indicate that when the penultimate (5th) instar of *C. fumiferana* larvae was fed an LD_{95} dose (1.8×10^5 polyhedra) the survival time for 50% (ST_{50}) was 7.8 d and the survival time for 95% (ST_{95}) was 12.9 d (Ebling *et al.* 1998). In contrast, the ST_{50} for the penultimate (4th) instar of *T. ni* larvae, orally inoculated with 15 polyhedra, was approximately 4 d (Washburn *et al.* 1995). Clearly there is a difference in the mode and speed of virus action.

Our aim was to determine whether the *Cf*MNPV infection process followed the general pattern of *Ac*MNPV infection in lepidopterans. Because *Ac*MNPV is virulent and has a wide host range, we tested *Cf*MNPV, a slower acting and less virulent virus with a narrow host range to confirm the infection process. The basic pattern of infection was similar, including the initial infection of the midgut followed by infection of the tracheal system associated with each tissue type. The differences centred on the temporal and spatial progression of the infection, which was slower in the case of *Cf*MNPV, and the reduced role of the hemocytes. It appears that the overall process of NPV infection in lepidopteran species is similar except for minor, subtle differences.

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