VIROLOGY 208, 328–335 (1995)

Passage of *Autographa californica* Nuclear Polyhedrosis Virus through the Midgut Epithelium of *Spodoptera exigua* Larvae

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Received March 29, 1994; accepted December 2, 1994

A special recombinant of *Autographa californica* multicapsid nuclear polyhedrosis virus (*Ac*NPV) was designed to study the early histopathological events of baculovirus infection in *Spodoptera exigua* larvae. This recombinant contained a *Drosophila melanogaster* heat shock 70 promoter driving an *Escherichia coli* β-galactosidase (Lac-Z) reporter gene to monitor the presence of early viral gene expression and a second reporter gene, the *E. coli* β-glucuronidase (GUS) gene, under control of the very late *Ac*NPV p10 promoter to monitor viral replication. In *S. exigua* larvae, permissive *Spodoptera* spp. cultured cells, and nonpermissive *D. melanogaster* cultured cells early viral gene expression was indicated by the appearance of Lac-Z as early as 3 hr p.i. Late viral gene expression was indicated by the appearance of GUS and occurred only in the permissive cultured cells and larvae. Early and late viral gene expression could be detected simultaneously using differential enzyme histochemistry. Analysis of infected *S. exigua* larvae revealed that midgut columnar cells and, at a low frequency, midgut regenerative cells were the primary sites of infection. Parental nucleocapsids were apparently transported through columnar cells to underlaying regenerative cells before virus replication and progeny production. Infection of tissues beside the midgut epithelium was not detected prior to viral replication within the midgut, suggesting that infection of the midgut is an important prelude to systemic infection.

INTRODUCTION

The Baculoviridae are a family of DNA viruses that mainly infect larvae of Lepidopteran insects. Polyhedra are ingested by the larvae and dissolve in the gut lumen releasing occluded virus particles. These virus particles pass the peritrophic membrane of the midgut and enter columnar cells by membrane fusion of the viral envelope with the microvillar membrane of these cells (Kawanishi *et al.*, 1972). Granados and Lawler, (1981) reported that in *Trichoplusia ni* larvae parental nucleocapsids, within the columnar cells, can be transported to the nucleus where the virus replicates, resulting in progeny virus production, or alternatively, nucleocapsids may be transported directly to the basal side of the columnar cells where they bud into the intercellular space and the hemocoel. Systemic infection is thought to be mediated by virions and infected hematocytes circulating within the hemocoel or through the tracheal system (Granados and Lawler, 1981; Engelhard *et al.*, 1994). We were interested in determining whether parental nucleocapsids of *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) were transported through the midgut epithelium of *Spodoptera exigua* larvae or whether the midgut epithelium was the primary target tissue for infection.

This paper presents histochemical data on primary and secondary infection of *S. exigua* larvae by *Ac*NPV. A recombinant *Ac*NPV was constructed containing two reporter genes to enable us to detect early and late viral gene expression within individual cells. The *Escherichia coli* β-galactosidase (Lac-Z) gene under control of the *Drosophila melanogaster* heat shock 70 promoter (HSP) reports early viral gene expression, indicating successful virus entry, uncoating, and early transcription. The *E. coli* β-glucuronidase (GUS) gene under control of the *Ac*NPV late p10 promoter reports late viral gene expression and hence successful virus replication. Using this recombinant we were able to follow the progression of infection and distinguish primary from secondary infected cells within the midgut epithelium.

METHODS

Insect cells

The *Spodoptera frugiperda* cell line IPLB-Sf-21 (Sf-21; Vaughn *et al.*, 1977) and the *S. exigua* cell line UCR-Se-1 (Se-1; Gelernter and Federici, 1986) were used as permissive cell lines for *Ac*NPV replication. The *D. melanogaster* cell line Dm-1 is nonpermissive for *Ac*NPV replication (Carbonell *et al.*, 1985; Rice and Miller, 1986). All cell lines were cultured at 27° in TNM-FH medium (Hink, 1970) supplemented with 10% fetal bovine serum.

Virus

The E2 variant of *Ac*NPV (Smith and Summers, 1978) was used as wild-type virus. *Ac*NPV/MKn1 (also named

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0042-6822/95 $6.00

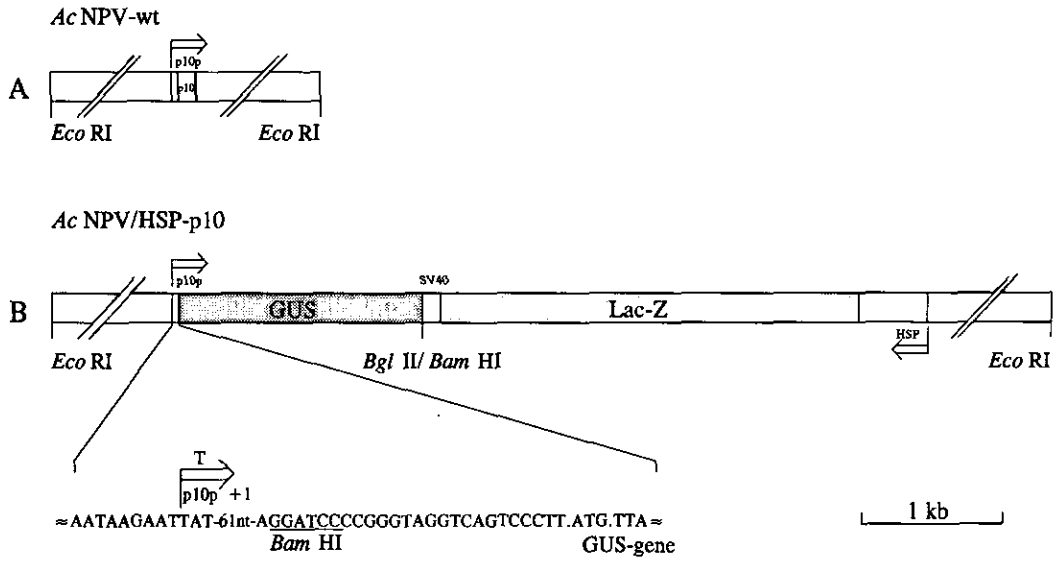

FIG. 1. (A) Schematic representation of the *Eco*RI-P fragment of wt *Ac*NPV DNA, containing the p10 gene. (B) Schematic representation of the corresponding region as found in *Ac*NPV recombinant *Ac*NPV/HSP-p10. The inserted cassette contains the *D. melanogaster* heat shock 70 promoter in front of the *E. coli* Lac-Z gene followed by the SV40 transcription terminator. The p10 coding sequence has been replaced by the *E. coli* GUS gene (p10p, the p10 promoter). The transcription start site of the p10 promoter at position −64 is indicated and the position +1 marks the A of the p10 ATG.

*Ac*NPV/HSP-p10) was constructed using a pAcAS3 (Vlak *et al.*, 1990)-derived transfer vector to insert both reporter genes in the *Ac Eco*RI-P fragment (Fig. 1A). In this vector the *E. coli* Lac-Z gene was under the control of the *D. melanogaster* HSP and terminated with a SV40 terminator to enable detection of early viral gene expression. The HSP–Lac-Z cassette was inserted immediately downstream from the p10 gene. The second reporter gene, *E. coli* GUS gene, was placed under control of the p10 promoter (Fig. 1B). The *E. coli* GUS gene was derived from pB1101.2 (Jefferson, 1987). The *Sst*I site at the 3′-end of the GUS gene was mutated into a *Bgl*II site and subsequently the GUS gene was cloned as a *Bam*HI–*Bgl*II fragment into the *Bam*HI site of pAcAS3. The baculovirus p10 gene is only expressed after virus replication and is not essential for the production of progeny virus (Vlak *et al.*, 1988). The recombinant virus was grown in Sf-21 cells and isolated as extracellular virus. The virus concentration in the cell culture medium was determined in tissue culture infectious dose 50% ($TCID_{50}$) units on Sf-21 cells as described by Summers and Smith (1987). To obtain polyhedra produced *in vivo*, infected Sf-21 cells were harvested 5 days p.i. and fed to fourth instar *S. exigua* larvae. Polyhedra were isolated from deceased larvae and used to infect second instar larvae for subsequent enzyme histochemical analysis.

Infection of cultured insect cells

The expression of Lac-Z and GUS as a function of infection time by *Ac*NPV/HSP-p10 was determined in all cell lines. Prior to infection 10^6 cells in 1 ml were allowed to attach to the bottom of 35-mm petri dishes. At time point zero the medium was removed and the virus was added in 1 ml to the cells with a m.o.i. of 10 $TICD_{50}$ units per cell. The virus was allowed to adsorb for 1 hr after which the cells were washed once with phosphate-buffered saline (PBS) and incubated in the culture medium. Samples were fixed at various intervals until 48 hr p.i. *Ac*NPV/HSP-p10-inoculated cells were analyzed simultaneously for Lac-Z and GUS expression. Five hundred cells per sample were scored for activity; the experiment was repeated three times and two petri dishes per time point were used in every experiment. To investigate the role of viral replication for the expression of the reporter enzymes Sf-21 cells were incubated after infection in medium containing 5 μg/ml aphidicolin to block viral DNA replication (Thiem and Miller, 1989).

Larvae, infection, and dissection

Larvae were reared, infected, and dissected as previously described by Flipsen *et al.* (1993). Second instar larvae were allowed to feed on droplets containing 10^8 polyhedra/ml. The ingested volume (0.33 ± 0.13 μl) contained enough polyhedra to cause 100% mortality (Smits, 1987). Using this dose a high number (20 to 200) of isolated foci originating from a single infected cell were observed in the midgut epithelium. Increasing this dose resulted in overlapping foci from multiple single infected cells at an early stage in the infection (6 hr p.i.; data not shown). Only larvae that had taken up the suspension within 15 min were used for further experiments. Indigotine blue was used to monitor the uptake of the polyhedra suspension. The larvae were dissected at 0.5, 3, 4, 6, 8,

9, 10, 12, 14, 16, 18, 20, 22, 24, and 36 hr p.i. to separate the midgut and adherent tissues from the cuticula.

Fixation

Cultured insect cells and isolated midguts were fixed for 2 hr at 0° by immersion in 0.01% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in phosphate citrate buffer (0.1 *M* Na_2HPO_4, 9.7 m*M* citric acid, pH 7.2) containing 1.5 m*M* calcium chloride. Complete larvae were fixed after they had been pinned down and incised in the longitudinal direction. After fixation the specimens were washed three times for 5 min in PBS.

Enzyme detection

β-Glucuronidase and β-galactosidase enzyme activity was detected by incubating the specimens first (cells, midguts, or larvae) for 2 hr at 27° in a reaction mixture of 3 m*M* $K_3Fe(CN)_6$, 3 m*M* $K_4Fe(CN)_6$, 10 m*M* EDTA in PBS, pH 7.5, containing 1.2 m*M* 5-bromo-4-chloro-3-indolyl-glucuronic acid (X-gluc) giving a blue color as a result of β-glucuronidase activity (GUS expression; Gallie *et al.*, 1992). Subsequently, the specimens were incubated for 2 hr at 27° in the reaction medium (EDTA was replaced by 1 m*M* $MgCl_2$, Bondi *et al.*, 1982) in the presence of 1.2 m*M* 6-chloro-3-indolyl-β-D-galactoside (Red-gal), a red precipitate indicating β-galactosidase activity (Lac-Z expression).

Eukaryotic β-galactosidase and β-glucuronidase are lysosomal enzymes and have a pH optimum of approximately 5.5; the prokaryotic *E. coli* β-galactosidase and β-glucuronidase have a pH optimum of 7.5 (Bondi *et al.*, 1982). To detect endogenous enzyme activity larvae were incubated in the appropriate reaction medium containing X-gluc or Red-gal and a pH of 5.5 for 6 hr at 27°.

Embedding, sectioning, and staining

For light microscopy cultured insect cells were mounted in glycerol and covered by a glass coverslip. Isolated midguts were embedded in LR-Gold as described previously by Flipsen *et al.* (1993). Serial sections of 2 μm thick were cut from the embedded midguts with a Reichert Ultracut S microtome and mounted on glass slides. The sections were studied in serial sequence to identify infected cells and verify connections between infected cells. To rule out possible interference of the blue GUS stain with the light green tissue stain, sections were first examined unstained using phase-contrast microscopy. Thereafter, the sections were stained with light green to facilitate easy identification of the neighboring tissue by bright-field microscopy. Light green staining was carried out by covering the sections with a 0.1% (w/v) solution of light green in 1% (v/v) acetic acid for 5 to 15 min on a hot plate at 80°. The staining time depends on the degree of resin polymerization that varies among different samples. Overstain of light green was removed by rinsing the sections in hot water until a desirable contrast was obtained. The sections were then washed with demineralized water, dried, and embedded in Microcover (Baker) and covered by a coverslip. Photographs of the sections were taken with a Leitz Laborlux S microscope and photographs from the preembedded larvae and the embedded midguts were taken with a Wild M3Z stereo microscope, both using a Kodak Ektar 25 color negative film.

RESULTS

Characterization of *Ac*NPV/HSP-p10 infection in insect cells

Recombinant *Ac*NPV/HSP-p10 was constructed to allow detection of early and late viral gene expression in insect tissues using enzyme histochemical techniques. In this recombinant the GUS gene was inserted downstream of the *Ac*NPV p10 gene promoter to report late viral gene expression. A cassette consisting of the HSP followed by the Lac-Z gene and a SV40 terminator (Zuidema *et al.*, 1990) was inserted downstream of the p10 locus to report early viral gene expression (Fig. 1). To test the detection system cultured insect cells were infected in three replicate experiments with recombinant *Ac*NPV/HSP-p10 and expression of Lac-Z and GUS genes was monitored by scoring the percentage of cells that showed enzyme activity as function of time. Since no differences were found between the three replicate experiments the data were pooled and graphically presented in Fig. 2. In Sf-21 and Se-1 cells, both permissive for *Ac*NPV, the expression of Lac-Z was detected as early as 3 hr p.i. At 7 hr p.i. all cells were positive for Lac-Z expression and no significant difference was observed between the cell lines. At 18 hr p.i. the first expression of GUS was detected in both cell lines. The percentage of GUS-positive cells increased more rapidly in Sf-21 cells than in Se-1 cells, but finally reached a maximum of approximately 80% positive cells at 42 hr p.i. in both cell lines. As the reduction of GUS-positive Sf-21 cells at 42 hr p.i. was within the standard deviation the curve was fitted by hand at this point. Polyhedra were observed only in Sf-21 and Se-1 cells that expressed GUS.

When aphidicolin was used to block DNA replication a similar increase of Lac-Z positive cells was observed in the inoculated Sf-21 cells. However, no GUS activity could be observed in these cells until 48 hr p.i. (the end of the experiment).

In *D. melanogaster* cells, which are nonpermissive for *Ac*NPV, only early viral gene expression (Lac-Z) was observed. The relative proportion of infected cells equaled that of permissive cells. The increase in percentage of positive cells with time was delayed compared to permissive cells, reaching 100% at 12 hr p.i. (Fig. 2). No GUS expression or polyhedra production could be detected in these cells at any time.

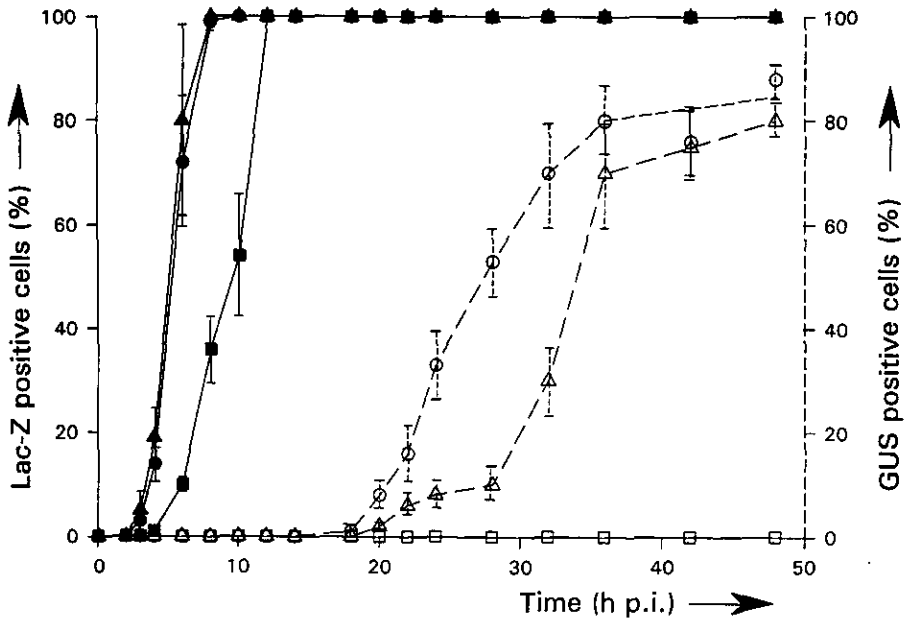

FIG. 2. Percentage of Lac-Z (——)- and GUS (- - - -)-positive *Ac*NPV/HSP-p10-infected cultured insect cells as a function of time. The vertical lines present the standard deviation of the corresponding values. Sf-21 Lac-Z expression ●, GUS expression ○, Se-1 Lac-Z expression ▲, GUS expression △, Dm-1 Lac-Z expression ■, GUS expression □.

From the data obtained by infecting cultured insect cells we concluded that onset of early viral gene expression and late viral gene expression can be distinguished using the recombinant *Ac*NPV/HSP-p10. By employing the different substrates the expression of GUS and Lac-Z can be distinguished within the same specimen.

Primary infection of *S. exigua* larvae with *Ac*NPV/HSP-p10

Prior to the analysis of *Ac*NPV/HSP-p10-infected second instar *S. exigua* larvae, the endogenous β-galactosidase and β-glucuronidase enzyme activities in larval tissue were investigated. Endogenous β-galactosidase activity was found in uninfected insects after 6 hr of incubation in the conventional substrate reaction mixtures with a pH of 5.5. The endogenous activity was restricted to the collar region of the midgut, the pericardial region, and in the dorsal aorta (data not shown). Endogenous activity of β-glucuronidase could not be detected. No endogenous enzyme activity was detected in uninfected and wild-type *Ac*NPV-infected larvae upon incubation in the substrate reaction mixtures at pH 7.5 for 6 hr. In the experiments to follow, enzyme activities were hence recorded using reaction mixtures at pH 7.5 and an incubation time of 2 hr.

Lac-Z was detected as early as 3 hr p.i. in columnar cells of the dissected midguts from the infected larvae (Fig. 3A). At 6 hr p.i. regenerative cells also showed expression of Lac-Z (Fig. 3B). These regenerative cells were always associated with infected columnar cells and appeared in a low (7%) percentage of the infected foci. This percentage remained the same until approximately 14 hr p.i. (Table 1). Infection of a single isolated goblet cell was seen only once at 10 hr p.i. and was considered to be a rare event.

Secondary infection of *S. exigua* larvae with *Ac*NPV/HSP-p10

The first GUS-expressing columnar cells, indicative of virus replication, were detected at 12 hr p.i. (Fig. 3C). GUS expression was found in approximately 10% of the infected columnar cells at this time. This number increased rapidly thereafter until approximately 80% of the primary infected columnar cells showed GUS activity by 24 hr p.i. (Table 1). At 16 hr p.i. the number of infected regenerative cells (13%) in the infected foci had increased compared to the number of Lac-Z-positive regenerative cells (7%) in the infected foci before GUS expression was observed. Only in conjunction with GUS-positive columnar cells were multiple Lac-Z-positive regenerative, columnar, and goblet cells observed (Fig. 3D) as a result of secondary infection. No GUS activity was observed in the goblet cells during the course of the study.

GUS expression was first observed in regenerative cells at 16 hr p.i. Only five GUS-positive regenerative cells were found in the 12 larvae examined at this time and these were always neighbored by infected Lac-Z- or GUS-positive columnar cells. At later stages in infection it could not always be determined whether the progeny virus was produced by the columnar or regenerative cell.

Infection of cells associated with the basal membrane

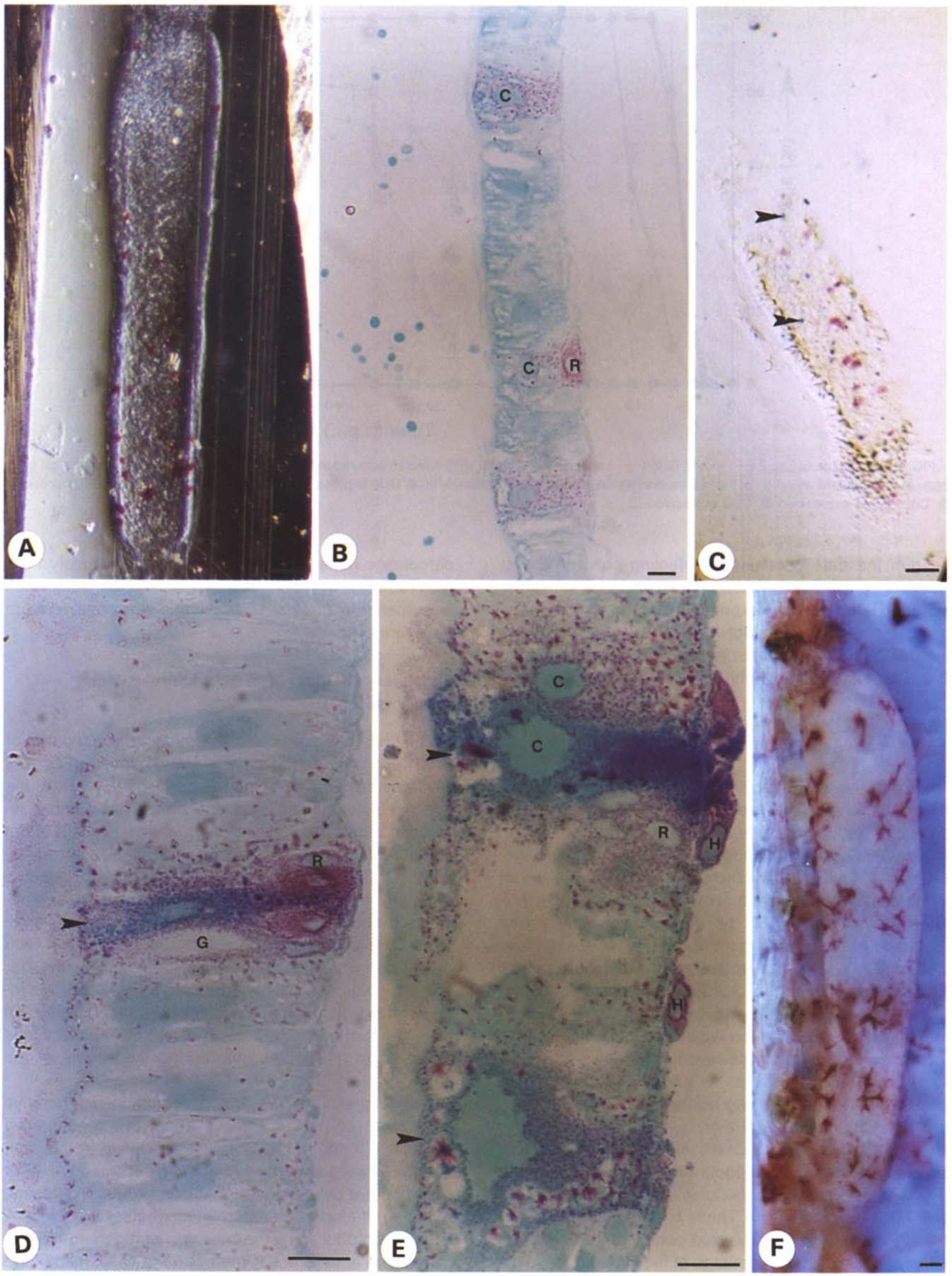
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TABLE 1

Infection Kinetics of *Ac*NPV/HSP-p10 in the Midgut Epithelium of *S. exigua* Larvae[a]

Time (hr p.i.)	Percentage of infected foci where GUS activity was absent and Lac-Z-positive regenerative cells were present	Percentage of infected foci with GUS-positive columnar cells	Percentage of infected foci with GUS-positive columnar cells and Lac-Z-positive regenerative cells
4	0	0	0
6	8 ± 3	0	0
8	8	0	0
10	7 ± 9	0	0
12	5 ± 7	10 ± 6	0
14	10	17	6
16	4 ± 4	43 ± 8	13 ± 10
20	3 ± 3	65 ± 4	36 ± 18
24	0 ± 0	83 ± 10	63 ± 17

[a] The percentage was calculated relative to the number of infected foci (Lac-Z positive; $n > 100$). The standard deviation was calculated only when five or more larvae were examined.

or in the hemocoel was only observed close to sites where GUS activity was present in the midgut epithelium (Fig. 3E). Among these infected cells were muscle and tracheal cells, and hematocytes. Until 20 hr p.i. Lac-Z-positive hematocytes were not found by analysis of the hematocytes in the dissection fluid or by inspection of the remaining tissues after isolation of the midgut (data not shown). Up to 36 hr p.i. the infection was mainly restricted to tissues connected to the infected site of the midgut (Fig. 3F).

DISCUSSION

The chimeric recombinant virus, *Ac*NPV/HSP-p10, was constructed to study early events in *Ac*NPV infection of *S. exigua* larvae. This recombinant allowed us to distinguish between early and late stages of viral infection and to discriminate between primary and secondary infection.

In cultured permissive (Sf-21 and Se-1) cells and Sf-21 cells treated with aphidicolin, onset of early viral gene expression is marked by expression of the Lac-Z gene as early as 3 hr p.i. In cells (*D. melanogaster*) nonpermissive for *Ac*NPV this early gene expression is observed from 4 hr p.i. Lac-Z expression can thus be detected in all cell lines prior to the onset of virus DNA replication and extracellular virus production, which occur from 6 to 8 hr p.i. onward (Tjia *et al.*, 1979; Carstens *et al.*, 1979). From 18 hr p.i. onward GUS expression was found in the permissive cells (Sf-21 and Se-1). Expression of the GUS gene was not recorded in the nonpermissive *D. melanogaster* cells and Sf-21 cells treated with aphidicolin, whereas Lac-Z was expressed in all cultured cells examined. This result is consistent with the current understanding that late viral gene expression requires genomic DNA replication (Rohel *et al.*, 1983; Smith *et al.*, 1983). It is also consistent with the report of Carbonell *et al.* (1985) and Rice and Miller (1986) that *Ac*NPV is unable to complete replication in *D. melanogaster* cells or to express very late viral genes (Morris and Miller, 1993).

Expression of Lac-Z occurred in primary target cells within 12 hr following ingestion of *Ac*NPV/HSP-p10 by second instar *S. exigua* larvae. Midgut columnar cells were the main targets for primary infection but also regenerative cells underlying target columnar cells were infected by the parental inoculum. Transport of nucleocapsids directly through targeted columnar cells apparently occurred since some regenerative cells were infected and produced Lac-Z a long time before any GUS expression (and thus virus replication) had occurred in the columnar cells. Lac-Z activity in the regenerative cells at 6 hr p.i. reflects an infection of these cells several hours earlier. This would be well before any viral replication has been reported. The frequency of infection of regenerative cells through the columnar cells remained stable until 12 hr p.i., when the first GUS expression was observed. After GUS expression, indicating that viral

FIG. 3. Enzyme histochemical demonstration of Lac-Z (red) and GUS (blue) expression in the midgut of *Ac*NPV/HSP-p10-infected *S. exigua* larvae. The foregut is located at the top in all figures. The sections in B, D, and E are stained with light green. The gut lumen in these panels is at the left-hand side. (A) Lac-Z expression at 3 hr p.i. in an embedded midgut of a second instar larvae. (B) Section of the midgut epithelium of a larvae dissected 6 hr p.i. Infected columnar cells (*C*) and one infected regenerative cell (*R*) can be seen. (C) Lac-Z and GUS (arrowheads) expression at 12 hr p.i. in an embedded midgut of a second instar larvae. (D) Section of a midgut at 16 hr p.i. in which a GUS-positive (blue, arrowhead) columnar cell is surrounded by Lac-Z (red)-positive regenerative cells and a Lac-Z-positive goblet cell (*G*). (E) Similar site as in D at 24 hr p.i. At this time infection can also be seen in cells associated with the basal membrane like the hematocytes (*H*). (F) Lac-Z expression in third instar larvae 36 hr p.i. The expression is mainly located in the trachea connected to the midgut. The bars in A, C, and F represent 100 μm, in B, D, and E the bars represent 10 μm.

replication has occurred, more cells surrounding the GUS-positive cell became infected. Secondary infection of regenerative cells is thereby distinguishable from primary infection of regenerative cells.

The possibility of infection of differentiating cells that were already in contact with the gut lumen (Baldwin and Hakim, 1991) at the moment of inoculation cannot be ruled out. However, because we never observed *isolated* infected regenerative cells it is unlikely that these differentiating cells were scored as regenerative cells. Regenerative cells infected with primary inoculum were always observed to be in close contact with infected columnar cells. The ability to infect multiple cells with a single virion may be the payoff for packaging multiple nucleocapsids within a single envelope. Polyhedra-derived virions reared in *S. exigua* larvae exist predominantly as three to six nucleocapsids per virion. When a multinucleocapsid virion enters a columnar cell individual nucleocapsids apparently can enter the nucleus and infect the columnar cell or be transported through the cell to infect an underlying regenerative cell.

Infection was not observed in tissues other than the midgut epithelium until 12 hr p.i. It is thus unlikely that infectious parental nucleocapsids pass the midgut epithelium and basal membrane to infect other larval tissues. Horton and Burand (1993) showed that the envelopes of polyhedra-derived virus particles fuse with the microvilli membrane of columnar cells. This is consistent with our observation that the virus enters the midgut epithelium through columnar cells. Granados and Lawler (1981) detected free virions in the hemocoel at an early stage of infection (0.5 to 2 hr p.i.) and proposed primary infection of hematocytes in the hemocoel by parental virus. In our experiments primary infection by the parental virus was limited to the midgut epithelium and we could not find infected hematocytes or other tissues until 20 hr p.i. using the Lac-Z reporter gene. Infection of nearby tissues was detected *only* after GUS expression in the midgut epithelium. This infection was restricted to hematocytes, muscle cells, and tracheal cells associated with the basal membrane until 24 hr p.i. Infection proceeds in the tracheal elements as shown at 36 hr p.i. These results indicate that viral replication in the midgut epithelium preceded infection of other larval tissues. Passage of parental viral nucleocapsids through the midgut basal membrane cannot be excluded. However, as no infection of any other larval cells or tissues is observed next to the midgut epithelium until the virus has replicated and progeny virus has invaded the basal membrane, this direct transport of virus particles does not contribute to the infection of the larvae and is thus considered irrelevant for the systemic infection.

The midgut epithelial cells produce the basal membrane. Upon viral replication the transcription and translation of the host cell is disturbed. Replication of virus within the midgut epithelium may thus interfere with the maintenance and integrity of the basal membrane and hence facilitate the passage of virions. Infected hematocytes and tracheal elements are thought to play a role in the further transport of the virus to other tissues (Keddie *et al.,* 1989; Engelhard *et al.,* 1994). Hence, infection of these cells may result in progressive infection of the larvae. The relative importance of these routes for the spread of the infection as compared to the role of circulating extracellular virus in the hemocoel will be the subject of another study (Flipsen *et al.,* submitted).

The first expression of GUS in columnar cells was found at 12 hr p.i. The expression of GUS at 12 hr p.i. coincides with virus budding from the basal columnar cell membrane of *Ac*NPV-infected *T. ni* larvae (Adams *et al.,* 1977; Granados and Lawler, 1981). However, using immunolocalization, p10 could be found in midgut epithelium of *S. exigua* larvae from 32 hr p.i. onward (Flipsen *et al.,* 1993). The difference in time to detect p10 expression shows that the GUS reporter system provides a much more sensitive detection method than immunolocalization for late viral gene expression. The GUS expression in the epithelial cells could be recorded 6 hr earlier than the first GUS expression in cultured insect cells, suggesting an increased speed in viral replication or expression of very late *Ac*NPV genes in midgut epithelial cells.

ACKNOWLEDGMENTS

The authors thank Ine Derksen-Koppers and Joop Groenenwegen for their technical assistance, Marga Knoester for help in constructing recombinant *Ac*NPV/HSP-p10, and Rob Goldbach for continued interest. We are particularly grateful to Loy E. Volkman for ample discussion and critical reading of the manuscript. This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Technology Foundation (STW).

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