Pathogenesis Induced by (Recombinant) Baculoviruses in Insects

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Subject headings: Baculovirus / Pathogenesis .

Stellingen

-1- Lateraal transport van baculovirussen door de intracellulaire ruimte van de trachea draagt niet bij tot de verspreiding van het virus naar verschillende organen.

Engelhard *et al.* (1994) *Proc. Natl. Acad. Sci. USA* **91**, 3224-3227.
Dit proefschrift.

-2- De weefselspecificiteit van baculovirussen wordt niet bepaald door aanhechting en binnendringen van virus of door initiële expressie van vroege virale genen.

Dit proefschrift.

-3- Het cel-cel transport in planten van het tomatebronsvlekkenvirus (tospovirus) en cowpeamozaïekvirus (comovirus) is een analoog proces.

Kromelink *et al.* (1994) *Virology* **200**, 56-65.
Goldbach *et al.* (1994) *Arch. Virol.* **9**, 87-97.

-4- Het valt te betwijfelen of de inschatting van de cel-cel transportsnelheid door middel van druk-injecties realistisch is.

Ding *et al.* (1995) *Virology* **207**, 345-353.

-5- De cellulaire afweer bij insekten tot stand gebracht door bloedcellen is hoofdzakelijk hormonaal gereguleerd.

Vinson (1993) *In:* Insect Immunity (Pathak, ed), 103-112.
Stanley-Samuelson (1994) *J. Insect Physiol.* **40**, 3-11.

-6- De vrees voor hondsdolheid in Europa heeft in Frankrijk en België geleid tot een vaccinatie beleid dat zowel overdreven als ontoereikend is.

McNally (1994) *The Ecologist* **24**, 207-212.

-7- De veronderstelling dat niet-infectieuze adenovirus-12-deeltjes worden gevormd in geïnfecteerde BHK21 cellen (Doerfler, 1994) is onjuist omdat deze alleen op analyse van mRNA's is gebaseerd.

Doerfler (1994) *In:* Encyclopedia of Virology (Webster and Granoff, eds) vol 1, 8-12.
Schiedner *et al.* (1994) *J. Virol.* **68**, 5476-5482.

-8- Wetenschappelijke vooruitgang is lang niet altijd op geheel rationele gronden tot stand gekomen.

-9- Het welslagen van gentherapie in de medische wetenschappen zal de acceptatie van genetisch gemodificeerde produkten verhogen.

-10- Het herplaatsingsbeleid van (semi-)overheidsinstellingen maakt dat de kwaliteit van het werk te lijden heeft, aangezien geen mensen met nieuwe ideeën aangesteld worden, en personen die niet optimaal functioneren vaak worden gehandhaafd.

-11- Ter bevordering van de emancipatie zouden de faciliteiten in arbeidsvoorwaarden die met name voor vrouwen gelden, zoals kinderopvang, deeltijdwerk en verlofregeling voor als het kind geboren wordt, ook aan mannen moeten worden aangeboden.

-12- De stabiliteit van een kano zit met name tussen de oren van de kanoër.

Stellingen behorende bij het proefschrift:

Pathogenesis Induced by (Recombinant) Baculoviruses in Insects

Wageningen, 17 mei 1994 Hans Flipsen

Voorwoord

Hier voor je ligt dan het eindresultaat van mijn promotieonderzoek. Na veel zwoegen en het nodige plezier, is het er dan. Rest mij nog hen te bedanken die een steentje hebben bijgedragen aan de tot stand koming van dit proefschrift. Allereerst natuurlijk de mensen van de vakgroep Virologie. Dank voor de gezellige sfeer en gezonde interesse was het een stimulerende omgeving. Hierbij moet uiteraard Rob Goldbach genoemd worden die mij zowel in het onderzoek als op het ijs een steun in de rug was. In het bijzonder wil ik ook de mensen noemen van de 'E.M.' groep. Jan van Lent die altijd met raad en daad klaar stond, Joop voor zijn nimmer aflatende stroom koffie en mijn kamergenootjes Daniella en Marc voor hun bijdrage.

Just Vlak wil ik bedanken voor het doorspitten van mijn hersenspinsels en het steeds weer bij elkaar brengen van een inspirerende en stimulerende groep baculovirologen. Uit deze groep wil ik verder met name Monique van Oers, John Martens en Ruud Mans bedanken voor de constructie van verschillende baculovirus recombinaten. Uiteraard wil ik niet voorbij gaan aan de studenten welke een bijdrage hebben geleverd, met name Arjen, maar ook Elian, Marga en Thea. Voorts wil ik ook de entomologen, Huug Schooneveld, Hans Smid, Peter Smits, Stan de Kort en Paul Harrewijn voor hun bron aan informatie.

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Of course I also like to thank all foreign baculovirologists like Basil Arif, Brain Federici, David O'Reilly, Loy Volkman, David Theilmann, Allen Wood, Carrie Hauxwell and Bob Granados for time and discussion of this research. Natürlich möchte ich auch Dagmar Knebel-Mörsdorf Dank sagen für die Beiträge am Kapitel 5 und ihre Interesse und Begeisterung für die Arbeit. Verder wil ik Dick Peters, Beb van Strien en L. Jane Theilmann voor hun bijdrage aan de tekst bedanken en vele anderen die in op enigerlei wijze hebben bijgedragen in de totstandkoming van dit proefschrift.

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Nec scire fas est omnia

CONTENTS

ACCOUNT

Flipsen, J.T.M., J.W.M. van Lent, R.W. Goldbach and J.M. Vlak (1993). Expression of polyhedrin and p10 in the midgut of AcMNPV infected *Spodoptera exigua* larvae: an immunoelectron microscopic investigation. *J. Invertebr. Pathol.* **61**, 17-23.

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Van Oers, M.M., J.T.M. Flipsen, C.B.E.M. Reusken, E.L. Sliwinsky, R.W. Goldbach and J.M. Vlak (1993). Functional domains of the p10 protein of *Autographa californica* nuclear polyhedrosis virus. *J. Gen. Virol.* **74**, 563-574.

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SAMENVATTING

Baculovirussen veroorzaken een vernietigende infectieziekte in insekten. Deze infectie leidt meestal tot totale verslijming van de gastheer, waarbij de nieuw gevormde virusdeeltjes veilig zijn ingepakt in veelhoekige eiwitkristallen (polyeders of granula). Deze ingepakte virussen zorgen voor de overleving en verspreiding buiten de gastheer. De gastheren van baculovirussen zijn gelukkig beperkt tot één of enkele nauw verwante insektesoorten. De meeste van deze virussen worden aangetroffen in rupsen van Lepidoptera (Schubvleugeligen, o.a. vlinders). Hier kunnen zij economische schade veroorzaken zoals bijvoorbeeld bij de produktie van zijde door zijderupsen. Anderzijds worden baculovirussen aangewend voor de bestrijding van rupseplagen. Zo wordt in de Nederlandse glastuinbouw het '*Spodoptera exigua* kernpolyedervirus' (Spod-X) met succes gebruikt voor de bestrijding van de z.g. 'koppensneller', de rups van de Floridamot (*S. exigua*), die siergewassen aantast. Deze rups is resistent tegen veel gebruikte chemische bestrijdingsmiddelen. Tevens worden baculovirussen gebruikt als producenten van medisch, industrieel en agrarisch nuttige eiwitten.

In verband met deze nuttige toepassingen van baculovirussen is veel onderzoek verricht naar de genetische organisatie van deze virussen met als doel efficiëntere virussen voor biologische bestrijding te ontwikkelen of een hogere produktie van recombinant-eiwitten te bereiken. In verband hiermee werd de nucleotiden-volgorde (erfelijke informatie) van het complete, cirkelvormige dubbelstrengs DNA-genoom van één van deze virussen (het *Autographa californica* kernpolyedervirus; *Ac*NPV) bepaald en zijn de genen erop gelokaliseerd. Van een beperkt aantal van deze genen is de functie inmiddels bekend (hoofdstuk 1). Over het infectieproces in rupsen is echter slechts weinig informatie beschikbaar. Gedetailleerde informatie met betrekking tot de wijze waarop het virus binnendringt, zich in de rups van weefsel naar weefsel verspreidt, welke weefsels bij de infectie betrokken zijn en in welke mate, is van belang voor de verdere ontwikkeling van baculovirussen met betere eigenschappen als biologisch bestrijdingsmiddel en als producent van recombinante eiwitten.

Het hier beschreven onderzoek is bedoeld om aan deze kennisbehoefte tegemoet te komen. Hiervoor werd de infectie van het modelvirus, *Autographa californica* kernpolyedervirus (*Ac*NPV) in *S. exigua* rupsen onderzocht. Omdat het noodzakelijk was het virus in een vroeg stadium van de infectie in de

rups te lokaliseren, is gebruik gemaakt van een speciale baculovirus recombinant die het *Escherichia coli* Lac-Z reportergen bevat, waarvan het produkt met behulp van enzymhistochemische methoden eenvoudig is aan te tonen. Dit gen is in het *Ac*NPV-genoom onder controle van de *Drosophila melanogaster* promoter HSP-70 gebracht. Hierdoor is het mogelijk het virus, vóór het zich vermenigvuldigt, aan te tonen in celculturen en in geïnfecteerde weefsels, waaronder de middendarm van *S. exigua* rupsen (3 uur na infectie; hoofdstuk 2). Met behulp van deze techniek is vastgesteld dat het virus eerst de cylindercellen en (in lagere frequentie) de regeneratieve cellen van het middendarmepitheel infecteert. Door in hetzelfde virus achter de "late" *Ac*NPV-p10-promoter ook het *E. coli* GUS reportergen in te bouwen, kan tegelijkertijd bepaald worden waar het virus zich in de rups vermenigvuldigt. Late virale genen komen immers alleen tot expressie na replicatie van het virale genoom. Hiermee is aangetoond dat het virus zich eerst in de middendarmepitheelcellen vermenigvuldigt, alvorens zich te verspreiden naar andere weefsels (hoofdstuk 2).

Dat in de cellen van de middendarm na infectie ook polyeders en fibrillaire structuren gevormd worden, is door middel van immunogoudlabeling van het virale polyhedrine en p10 aangetoond (hoofdstuk 3). Het voorkomen van deze structuren in het middendarmepitheel van *Ac*NPV geïnfecteerde rupsen was altijd zeer omstreden. Indien rupsen na de infectie niet meer vervellen, en dus geïnfecteerde middendarmepitheelcellen niet meer geheel afstoten, wordt zelfs een zeer hoge produktie van deze late virale structuren waargenomen.

Vervolgstudies tonen aan dat, na vermeerdering van het virus in het middendarmepitheel, de onderliggende weefsels (submucosa) en daarmee geassocieerde cellen worden geïnfecteerd (hoofdstuk 2). Hierbij dringen virussen op een nog onopgehelderde wijze vanuit het middendarmepitheel door de basale membraan heen naar het hemolymph en de cellen van de submucosa. Vanuit het middendarmepitheel, en later ook vanuit de submucosa, worden nieuw geproduceerde virusdeeltjes uitgescheiden in de bloedbaan van de rupsen. Deze virusdeeltjes infecteren de tracheoblasten. Dit zijn de cellen die zich aan het einde van de luchtbuizen (tracheae) bevinden waar deze buizen de weefsels binnengaan (hoofdstuk 4). Het virus kan deze tracheoblasten infecteren omdat hier de basale membraan, die normaal dik is en een fysieke barrière vormt, juist hier zeer dun is. Deze tracheoblasten bevinden zich in alle weefsels, waardoor het virus dus ook snel in alle weefsels binnendringt. Zo is vastgesteld dat alle weefsels in rupsen van het tweede en derde stadium al binnen 36 uur na opname van het virus zijn geïnfecteerd. Dit kan alleen maar worden verklaard door de snelle infectie van

tracheoblasten. Pas in een later stadium van de infectie (92 tot 116 uur na infectie) verslijmen de rupsen.

Vervolgens is voor een aantal virale genen onderzocht waar zij tot expressie komen, in welke weefsels het virus zich vermenigvuldigt en waar polyederproduktie plaatsvindt (hoofdstuk 5). Hiervoor is gebruik gemaakt van *Ac*NPV recombinanten, waarin reportergenen achter vroege (*Ac*NPV-*PE38*, -*ME53*) en late (-p10) promoters is geplaatst (hoofdstuk 5). In zowel de cellen van de tracheae, het vetlichaam, de huid als de hematocyten blijkt het virus zich te vermenigvuldigen en worden polyeders gevormd. Daarentegen wordt de infectie van speekselklieren, buisjes van Malpighi en bekercellen van de middendarm na expressie van vroege virale genen afgebroken. Er vindt in deze weefsels geen virusvermenigvuldiging of polyederproduktie plaats. Het uitblijven van pathologische veranderingen in de buisjes van Malpighi en de speekselklieren samen met de uiteindelijke regeneratie van het middendarmepitheel, biedt de rups, na infectie, de mogelijkheid te blijven eten en leven, waardoor veel meer polyeders worden geproduceerd. Een dergelijk weefselspecifiek afbreken van infectie na binnenkomst en expressie van de eerste virale genen is slechts voor enkele andere virussen beschreven.

De niet-produktieve baculovirusinfectie, waarbij tóch virale genen tot expressie komen, is van belang voor de biologische veiligheid bij het gebruik van recombinant virussen als biologisch bestrijdingsmiddel. Bij infectie van (schijnbaar) ongevoelige insektesoorten met genetisch-gemodificeerde virussen is het mogelijk dat recombinant-eiwit tot expressie komt. De expressie van recombinant-eiwitten kan het gedrag van zowel de gastheren (de plaaginsekten) als de niet-gastheren beïnvloeden. Met de hier ontwikkelde en beschreven testsystemen is het mogelijk om het gedeeltelijke infectieproces in (schijnbaar) ongevoelige gastheren te bestuderen en potentiële gevaren, ten gevolge van genetische modificatie, voor insekten die niet gevoelig zijn voor *Ac*NPV, in te schatten.

Een voorbeeld van genetische modificatie, waarbij met het hier beschreven systeem de pathogenese werd bestudeerd is de deletie van het ecdysteroid-UPD-glucosyltransferase-gen (*egt*) in het *Ac*NPV-genoom. Het *Ac*NPV-*egt* inactiveert ecdysteroiden die het metabolisme en de differentiatie activeren. Deletie van dit gen veroorzaakt een versnelde sterfte van rupsen (bijna 30% sneller). Histopathologisch onderzoek toonde aan dat deze deletie een verstoring van de pathofysiologie tot gevolg heeft, waardoor een degeneratie van de buisjes van Malpighi wordt geïnduceerd (hoofdstuk 6). Deze degeneratie is waarschijnlijk de oorzaak van de versnelde sterfte van rupsen na infectie met de *egt*-deletie mutant.

De in dit proefschrift weergegeven gegevens kunnen een bijdrage leveren aan de ontwikkeling van een veiligere en effectievere bestrijding van insektelarven (waaronder rupsen) met (recombinante) baculovirussen zonder gevaar voor de in ecologisch, emotioneel of economisch opzicht waardevolle insekten. Daarnaast kunnen de gegevens wellicht helpen bij de optimalisering van de produktie van recombinante eiwitten in rupsen via baculovirussen als alternatief voor het gebruik van insektecel-bioreactoren.

CHAPTER ONE

Introduction

General introduction

Animals are affected by microorganisms and viruses and this interaction often results in disease. Baculoviruses are a major disease agent for insects. Baculoviruses have mainly been isolated from Lepidoptera and Hymenoptera (Adams and Bonami, 1991). The host range of members of the baculoviruses family encompasses only insect and crustacean species and is often restricted to one or a few closely related insect species. In nature baculoviruses cause epidemics with high mortality in their insect hosts and consequently regulate insect infestations. Characteristically, after a number of days larvae infected with baculoviruses cease their feeding activity and become morbidund. The infection invariably ends by death and liquification of the larvae. During this process massive amounts of large proteinaceous capsules are produced, which are subsequently dispersed in the environment.

The family of Baculoviridae represents a large group of rod-shaped (baculum = rod) viruses. A number of these viruses have proven useful in the biological control of agricultural insect pests with clear advantages over chemical insecticides (Payne, 1988). Another important feature of baculoviruses is the presence of several genes not essential for virus replication, some of which are controlled by powerful promoters. The coding region of some of these genes was replaced with exogenous genes which lead to the development of the baculovirus expression systems for a variety of economicly valuable eukaryotic proteins, used as vaccines and diagnostics (Luckow and Summers, 1988; Vlak and Keus, 1990).

In recent years baculovirus research has mainly focused on the analysis of their DNA genome structure, gene expression, and functioning, with the ultimate aim to develop more effective insect control agents and improved expression vectors. The double stranded covalently closed DNA genome of the baculovirus type species, *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV), has been completely sequenced, a large number of genes have been identified, and their putative functions established (Ayres *et al.*, 1994). Limited information, however, is available on the process of baculovirus infection in insects (baculovirus pathogenesis). The information available is mainly based on electron microscopical observations providing only a static view of the infection in a restricted part of the infected insect larvae. Information on the infection dynamics, i.e. the route of virus infection in the insect larvae as a whole, on tissue specificity of viral gene expression, and on the response of host tissues to virus infection, is of great importance for the further development of improved baculoviruses for insect control and for the use of insect larvae in the production of recombinant

proteins. The aim of the research described in this thesis therefore is to analyze the pathway of infection and pathogenesis of baculoviruses in insect larvae, notably the infection of *Spodoptera exigua* larvae by *Ac*NPV. In order to achieve this goal, a novel approach was taken and a unique combination of histochemical and molecular engineering techniques were employed to trace the infection *in situ* and to determine the nature of this infection in a variety of tissues with minimal distortion of the larvae.

Baculovirus taxonomy

The family of baculoviruses is subdivided into two genera, as named below, based on the mode of virus particle occlusion in large (1-10 μm) protein capsules (22nd triennial meeting of the Executive Council of the International Committee on Taxonomy of Viruses, 1993, Glasgow). Each genus also has its characteristic pathology.

The genus **Nucleopolyhedrovirus**, encompassing the nuclear polyhedrosis viruses (NPVs), is characterized by the occurrence of large polyhedral capsules (polyhedra) in the nucleus of infected cells. These capsules contain single- (SNPV) or multiple-nucleocapsid enveloped particles (MNPV) embedded in a protein matrix. The major matrix protein of the polyhedral capsules is called polyhedrin. Virus replication and nucleocapsid assembly takes place in the nucleus which results in hypertrophy of the nucleus. At the end of the infectioncycle the nucleus disintegrates and the cell lysates. Infection of the insect host is restricted mainly to the larval stage and affects different tissues, although tissue tropism can vary strongly among the different members of this genus (Adams and McClintock, 1991).

The genus **Granulovirus**, containing the granulosis viruses (GVs) is characterized by the presence of a proteinaceous granule that occludes a single virion with only one nucleocapsid. Upon infection of fat body cells the virus induces cell division before it replicates and granula morphogenesis takes place. GVs also infect the larval stage of their host but their tissue tropism is much more restricted than in the case of NPVs (Tanada and Hess, 1991). For instance, the GV of the Western grapeleaf skeletonizer, *Harrisina brillians*, is the most extreme example, as virus infection is restricted to the midgut epithelium only (Federici and Stern, 1990).

Of the two genera the NPVs and in particular the type member *Ac*NPV (Francki *et al.*, 1991) has been studied most extensively with respect to molecular, biological, and pathological aspects (Adams *et al.*, 1977; Faulkner, 1981; Granados and Federici, 1986; Blissard and Rohrmann, 1990).

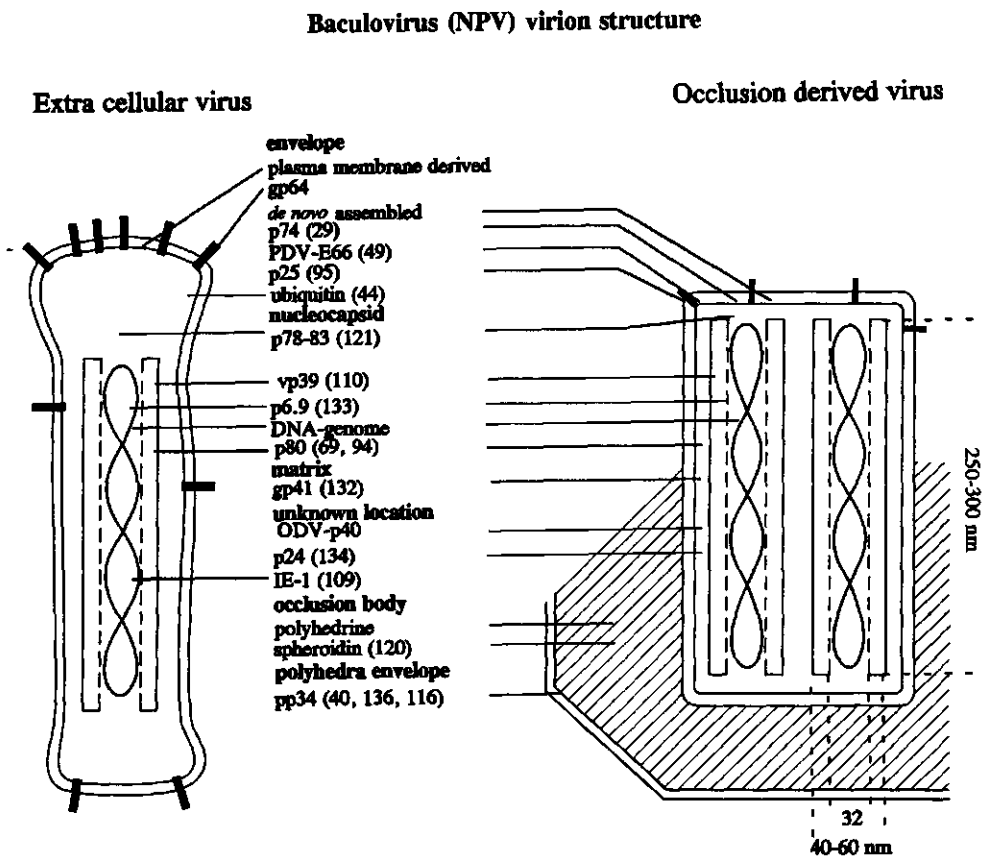


Figure 1: Schematic presentation of extracellular virus (ECV) and occlusion derived virus (ODV) particles of *Ac*NPV. Both are drawn on the scale indicated for the occlusion derived virus. IE-1 is a structural protein in *Orgyia pseudotsugata* NPV but is not found in *Ac*NPV virus particles (L.A. Guarino, personal communication; ODV-p40 Theilmann, personal communication; numbers correspond to references page 97-104).

The baculoviral replicative cycle is distinguished by the synthesis of two functional phenotypes (Fig. 1; 2). One, extracellular virus (ECV) is released from the infected cell by budding and is responsible for the dissemination of infection in larval tissues and cells. The second is synthesized later in the infection cycle and becomes occluded in the polyhedral occlusion body. These latter are responsible for the dissemination of infection in the insect population and has been designated occlusion derived virus (ODV). During infection, progeny viral nucleocapsids are assembled in the nucleus, transported to the cytoplasm and obtain their envelope when passing through the plasma membrane (Fig. 2). These ECVs establish further systemic infection in other cells and tissues of the larval body. The ODVs obtain a *de novo* assembled membrane in the nucleus and are subsequently occluded in polyhedra or granula, which are responsible for the horizontal spread of the virus in insect populations.

Approximately 30 different structural proteins are found in the baculovirus particle (Vlak, 1978; Maruniak and Summers, 1981; Volkman, 1983). Figure 1 presents a schematic drawing of ECV and ODV particles in which structural proteins are indicated that are presently identified on the *Ac*NPV genome. Both phenotypes contain similar nucleocapsids with a diameter of 40 to 60 nm and variable in length from 250 to 300 nm (Adams and McClintock, 1991).

The virions of both phenotypes are surrounded by a lipid membrane. As a consequence of their morphogenesis, major differences can be found in the nature and origin of this envelope, including the lipids and fatty acids and the proteins associated with it (Braunagel and Summers, 1994). The ECV envelope contains the virus coded envelope glycoprotein gp64, which forms the peplomers projected on the ECV particle surface. After adsorptive endocytosis gp64 induces membrane fusion between the viral envelope and the acidified endocytotic vesical (lysosome) membrane (Volkman, 1986). In contrast ODVs contain a number of unique proteins in their envelope of which the function of the p74 protein is best characterized. This protein is essential for oral infectivity of ODVs (Kuzio *et al.*, 1989; Faulkner, 1994) and is thought to be involved in the receptor-mediated membrane fusion between the viral envelope and the microvillar membrane of the midgut columnar cells.

The ODVs are packed into polyhedral proteinaceous capsules of which the matrix is formed by the 33 kDa polyhedrin protein. The polyhedron is surrounded by a polysaccharide rich envelope. The glycocalyx protein (pp34) is an essential component of this envelope (Zuidema *et al.*, 1989).

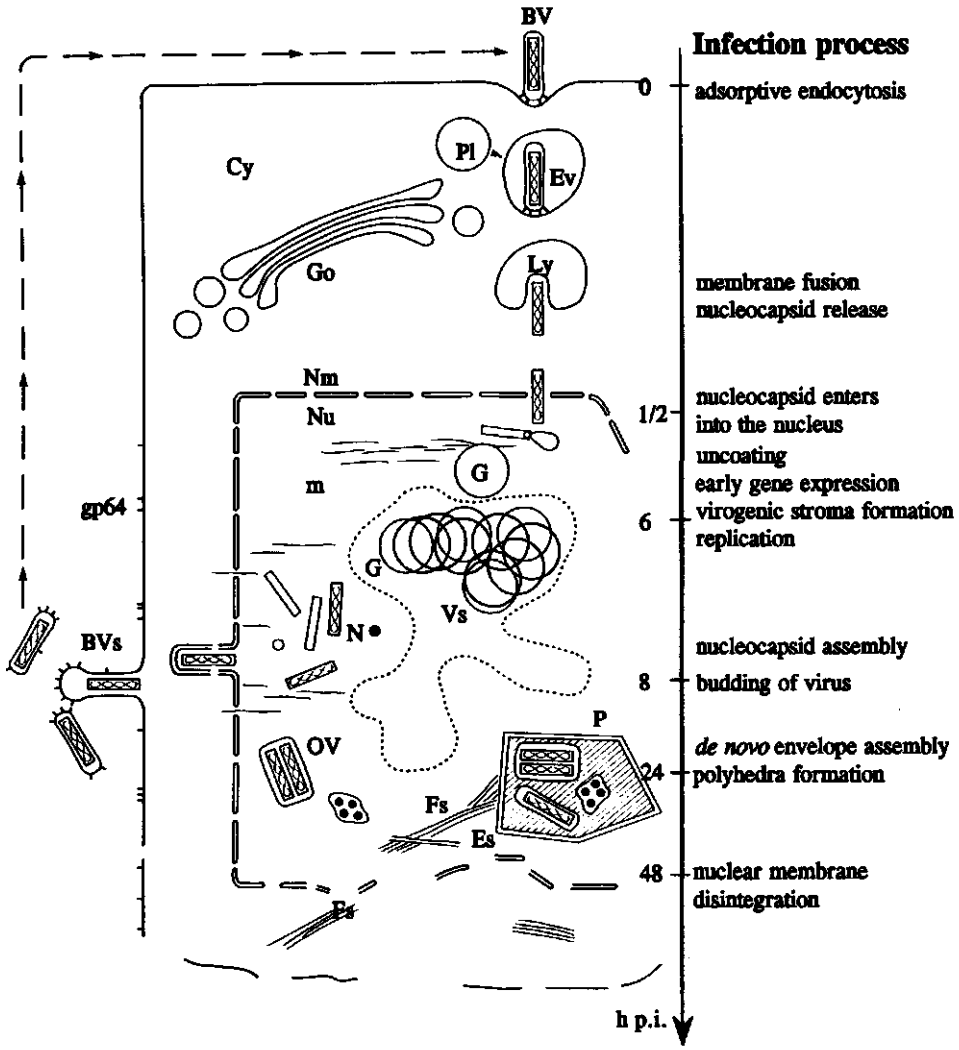


Figure 2: Schematic presentation of the *Ac*NPV infection cycle in an insect cell. The sequence of events is ordered vertically and indicated on the time scale on the right hand side. (ECV=extra cellular virus, Cy=cytoplasm, Pl=pro-lysozyme, Go=golgi zone, Ly=lysozyme, N=nucleocapsid, Nm=nuclear membrane, Nu=nucleus, G=*Ac*NPV genome, Vs=virogenic stroma, m=viral mRNA, OV=occluded virus, P=polyhedra, Fs=fibrillar structure)

Infection process

Insects usually become infected with *Ac*NPV by ingestion of polyhedra during the larval stage. Other mechanisms of infection have been described such as vertical transmission of the virus from adult to offspring (Fuxa and Richter, 1991) and infection through the tracheal system (Kirkpatrick *et al.*, 1994). Normally, the midgut forms the first barrier for successful infection of insects by a baculovirus. Ingested polyhedra dissolve in the alkaline environment of the midgut and the occluded virus particles are released into the gut lumen (Fig. 3 I). In GVs co-occluded 'enhancins' digest 'holes' in the peritrophic membrane through which the virus particles can pass towards the brush border of the midgut epithelium (Fig. 3 II; Derksen and Granados, 1988). Proteins involved in the passage of NPV through the peritrophic membrane have not been identified. ODVs bind to the microvillar membrane of columnar cells by proteinaceous receptors (Horton and Burand, 1993) whereafter the viral envelope fuses with the microvillar membrane (Fig. 3 III). The ODV envelope protein*p2074 a role in either the binding or the fusion of the viral envelope with the microvillar membrane (Faulkner, 1994).

After entry into the columnar cells the parental nucleocapsids are transported to the nucleus (Fig. 3 IV) where the virus replicates and progeny viruses particles are formed (Fig. 3 V). Alternatively, parental nucleocapsids are transported directly to the basal side of the columnar cells where they bud into the intercellular space and through the basal lamina into the hemocoel (Fig. 3 VI; Granados and Lawler, 1981). The basal lamina is a thick layer of proteinaceous material (Locke, 1985) and it is not clear how virus passes this basal lamina. The relative importance of either of these two routes (through the midgut epithelium) for successful systemic infection of larvae is not fully understood (see also chapter 2).

The spread of baculovirus infection in the midgut epithelium and the various cell types involved in this are a matter of debate. Expression of 'late' and 'very late' (see below) viral proteins in the midgut can be an important feature for the design of more efficacious recombinant baculoviruses for insect control. So far, conflicting results have been obtained concerning the formation of late viral structures (polyhedra and the p10-containing fibrillar structures) in midgut epithelial cells (see also chapter 3).

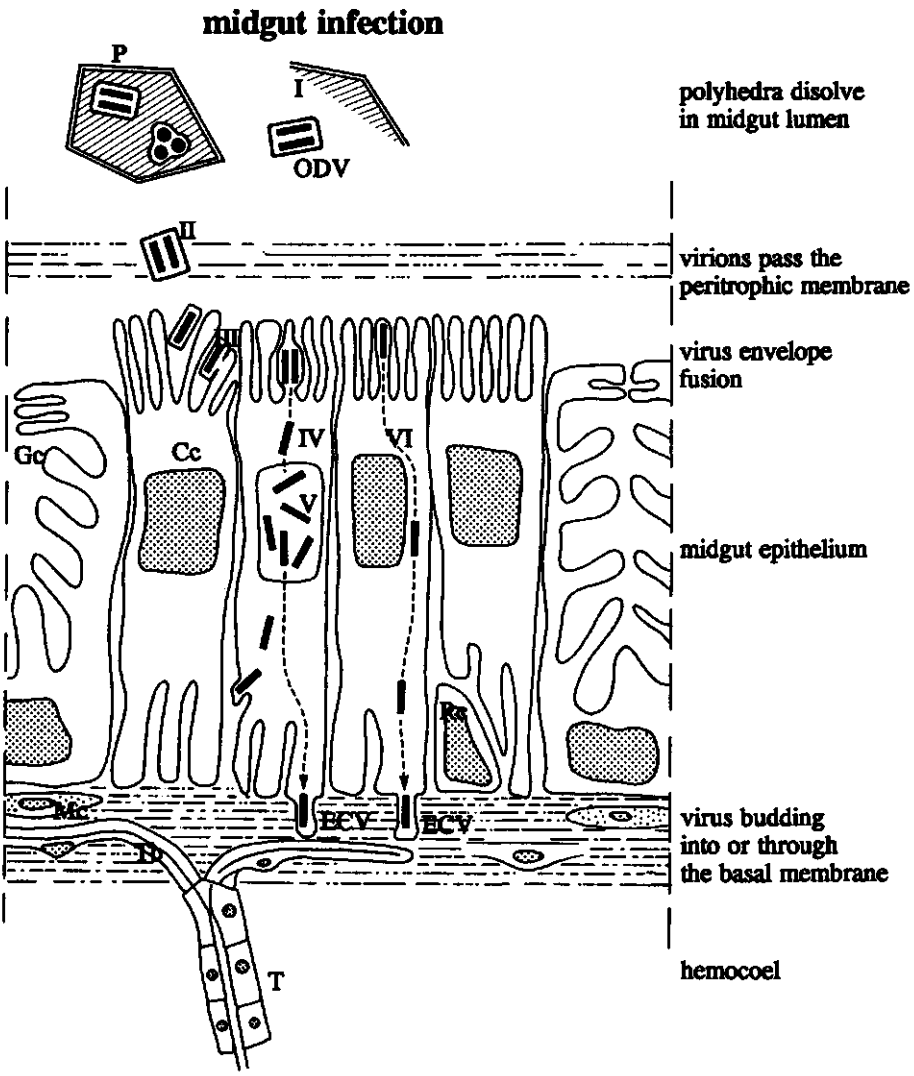


Figure 3: Schematic presentation of the proposed NPV infection processes in the midgut epithelium of an insect larva. (P=polyhedra, ODV=occlusion derived virus, Gl=midgut lumen, Pm=peritrophic membrane, Cc=columnar cell, Gc=goblet cell, Nu=nucleus, Rc=regenerative cell, ECV=extra cellular virus, Bm=midgut basal lamina, Mc=muscle cell, Tb=tracheoblast, Te=tracheal element, He=hemacoel)

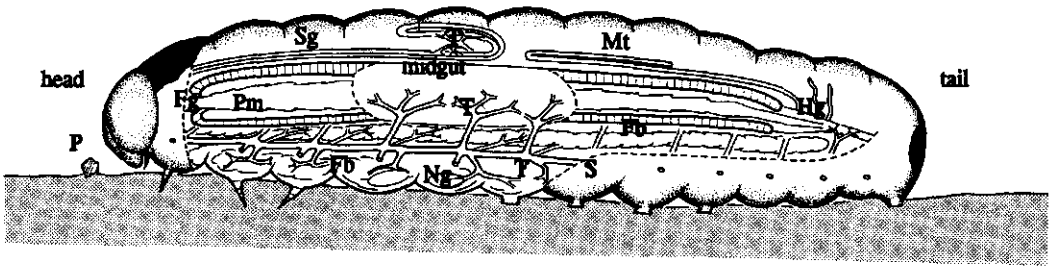


Figure 4: Schematic presentation of a *S. exigua* larva. The larva are opened just behind the head up to the last segments. A section of the midgut is drawn in at the connection of the midgut with the front and hind gut, while in the middle of the midgut the tracheal connection is indicated at the outside. In the early instar larvae the midgut is the major organ, while in the last instar the fat body (Fb) is the major organ. The hemocoel surrounds all tissues and the tracheal system (T) connects nearly all tissues to the stigmata (S) that form the opening of the tracheae in the epidermis. The salivary gland (Sg) is connected to the front gut (Fg) near the mouth pieces. The Malpighian tubules (Mt) secrete their content into the hind gut (Hg). (Ng=neural ganglia; Pm=peritrophic membrane; P=polyhedra)

After passing the midgut epithelium, virions infect cells associated with the midgut basal lamina or, alternatively, virions are released directly into the hemocoel. Different mechanisms for further infection of larval tissues have been proposed: (i) by virions which circulate in the hemocoel (Granados and Lawler, 1981); (ii) by cell-to-cell contact with invading infected hematocytes (Keddie *et al.*, 1989); and (iii) via the tracheal system either by transport of virus particles through the intercellular space of the tracheal elements (Engelhard *et al.*, 1994) or, alternatively, by cell-to-cell contact (Ritter *et al.*, 1982). In view of the anatomy of insect larvae, the transmission of the virus through the tracheal system is elegant as the tracheal system provides a close contact between the midgut and all other organs (Fig. 4) and bypasses the barrier formed by the basal lamina surrounding all tissues. The route by which the virus infects larval tissues determines the speed by which the infection spreads through the insect. This process determines the activity of the virus (speed of kill). It may thus provide possible clues for the genetic improvement of baculoviruses as biological control agents (see also Chapter 4). When the infection has reached a progressed stage, the midgut is often cleared of the infected cells by shedding these cells into the gut lumen or by an apoptotic response. This 'recovery' of the midgut allows the still infected insect larvae to continue feeding and to grow larger before it dies from polyhedrosis.

*Ac*NPV replicates in tracheal cells, fat body, hematocytes, muscle cells, and epidermal cells (Booth *et al.*, 1992). Using classical electron microscopy baculovirus tissue tropism is only identified late after infection when classical signs such as nucleocapsid assembly and polyhedra formation are apparent. This is the normal end product of infection in insect cell lines. Whether or not vital organs such as the Malpighian tubules (the larval kidney homolog) and salivary glands are infected is not known.

The infection *in vivo* can be mimicked by infection of cultured insect cells *in vitro* and this has allowed a detailed study of the infection process initiated by ECVs and ending in polyhedron morphogenesis. Cell lines are homogeneous, but are poorly differentiated. It can, therefore, be questioned whether this infection resembles the infection of the heterogeneous and highly differentiated cells *in vivo*. Nevertheless, much has been learned about the cell pathology of baculovirus infection.

Study of the infection of insect cell lines has revealed that viral genes are expressed in a 'cascade like' fashion; four distinct groups of genes can be recognized. The **immediate early genes** (α) are transcribed by host transcription factors and are thus active independent of viral gene expression. In contrast **delayed early gene** (ß) expression is induced by immediate early viral transcription. Early genes are 'by definition' transcribed before viral DNA replication starts. The early genes include most of the transcription and all DNA replication factors. The **late** (γ) and **very late genes** (δ) are transcribed after DNA replication has started. Use of insect cell lines has indicated that specificity is not determined by processes such as virus binding, entry and uncoating, since *Ac*NPV is able to enter non-permissive cells (Rice and Miller, 1986). Host and tissue specificity may thus be determined at the level of viral gene expression. The reliance on host factors for the expression of immediate early genes may provide a mechanism for host and/or tissue specificity. The question is whether tissue differential infection exists and whether viral gene expression is involved in this tissue specificity (see also Chapter 5).

A number of functionally characterized baculovirus genes play a role in the virus host interaction. Apoptosis is an important defense response to viral infection prohibiting the virus from further multiplication and infection of the entire host larva. Apoptosis can be suppressed or abrogated by one or more viral proteins. These include the *Ac*NPV-*p35* (Clem *et al.*, 1991), the *Cydia pomonella* GV-*IAP1* (Crook *et al.*, 1993), and *Ac*NPV-*IAP2* (Ayres *et al.*, 1994). Other viral gene products are actively involved in the disintegration of cells and larvae. The *Ac*NPV-p10, which is an essential

component of the fibrillar structures (Van der Wilk *et al.*, 1987), is involved in the disintegration of the nucleus (Fig. 2 XII; Van Oers et al, 1993). A serine protease (v-cathepsin) (Rawlings *et al.*, 1992) and a chitinase (Ayres *et al.*, 1994) are probably involved in the liquification of the infected larvae and hence the release of polyhedra and granula.

One of the most intriguing viral proteins involved in the interaction between the virus and the host is the ecdysteroid UDP-glucosyltransferase (*egt*) (O'Reilly and Miller, 1989). This transferase inactivates ecdysteroid hormones and thereby regulates the metabolism and development of the insect larvae (O'Reilly, 1995). Deletion of this gene results in premature death of the infected larvae and is thus an attractive venue to improve baculoviruses for insect pest control (O'Reilly and Miller, 1991). The mechanism responsible for this enhanced speed with which baculoviruses can kill their host is not known (see also Chapter 6).

Scope of the investigation

Despite intensive research in recent years on baculovirus genetic organization, replication *in vitro*, and virus morphology, these studies have not led to a better understanding of the infection process *in vivo*. Many studies on insect pathology caused by baculoviruses have been highly descriptive, on a case-by-case basis, often allowing a static view of the infection process. Understanding of the infection process, such as the route of virus infection in insect larvae, tissue specificity of viral gene expression, and of the response of host tissues to virus infection, is of great importance for the further development of engineered baculoviruses as biocontrol agents and possibly for the use of insect larvae in the production of recombinant proteins. The time after infection when gene expression reaches the highest level, combined with the tissue location of viral protein synthesis are two very useful parameters required for optimizing baculovirus expression vectors. In order to obtain insight into the infection process, advanced knowledge of baculovirus molecular genetics and novel histochemical procedures were used. Marked recombinant viruses containing reporter genes were used (Flipsen *et al.*, 1995) to follow infection through the insect larvae by detection of the onset of viral gene expression in various tissues. Effects of genetic modification on baculovirus pathophysiology can then be determined better, such as the effect of the deletion of the *egt* gene from baculoviruses.

The main questions addressed in this thesis are how and where the baculovirus infection occurs in the midgut epithelium of insect larvae and how the virus spreads quickly to other target tissues. The infection of *Spodoptera exigua* larvae with marked *Ac*NPV recombinants offers a unique opportunity to study the pathological effects of virus infection *in situ* and is taken as a model system. An additional question is whether there is tissue specific replication and viral gene expression in tissues beyond the midgut.

The midgut is the prime target of virus infection after ingestion of polyhedra. Chapter 2 investigates which cells participate in this initial infection, with particular emphasis on the regenerative cells. Various hypotheses exist as to how the virus passes from the initial infection site through the midgut, in particular whether it is essential that virus replication occurs in the midgut before progeny virions radiate out into other organs (Chapter 2). Replication of *Ac*NPV *in vitro* (insect cell lines) leads to the production of fibrillar structures and polyhedra. Whether these late viral structures were produced in the midgut epithelium has been investigated (Chapter 3). The question how the virus invades the interior of the insect larvae is addressed in Chapter 4. Various routes of infection have been proposed, including circulating ECVs and transport via the tracheal system. The use of dual-recombinant baculoviruses to monitor early and late stages of infection in the whole insect simultaneously, appeared to be particularly useful (Chapter 4). After initial replication in the midgut progeny virus targets the tracheal cells, fat body, hematocytes, and outer epidermis as evidenced by the occurrence of polyhedra in these tissues. Other tissues like the salivary glands and Malpighian tubules appear unaffected. The question is whether tissue specificity exists, in particular in regard to early baculovirus gene expression, since sequence motifs occur in these early promoters that are reminiscent of motifs which determine tissue specificity in other biological systems (Chapter 5). Ecdysteroid UDP-glucosyltransferase is involved in the abrogation of normal development of larvae to the benefit of virus. Deletion of the viral *egt*-gene causes early death of the larvae. The use of a marked baculovirus *egt* deletion mutant allowed histopathological analysis to find an explanation for this biological effect in the pathology (Chapter 6). In Chapter 7 the experimental data are discussed and the most likely route of infection is proposed, postulating that the ECVs released into the hemocoel and the susceptibility of tracheoblasts are the most important elements to explain the rapid progress of the disease in this particular system.

CHAPTER TWO

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

This chapter will be published in a slightly modified form as: **Flipsen, J.T.M., J.W.M. Martens, M.M. van Oers, J.M. Vlak and J.W.M. van Lent.** 1995. Passage of *Autographa californica* nuclear polyhedrosis virus through the midgut epithelium of *Spodoptera exigua* larvae. *Virology* **207**, 000-000.

ABSTRACT

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 (HSP) driving an *E. coli* ß-galactosidase (Lac-Z) reporter gene to monitor the presence of early viral gene expression. In this recombinant a second reporter gene, the *E. coli* ß-glucuronidase (GUS) gene was under control of the very late *Ac*NPV p10 promoter to monitor viral replication. In *S. exigua* larvae, permissive *Spodoptera* spp. cell lines, and in a non-permissive *Drosophila melanogaster* cell line early viral gene expression was indicated by the appearance of Lac-Z as early as 3 h p.i. Late viral gene expression was indicated by the appearance of GUS and only occurred in permissive cell lines 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 occurred. Infection of tissues beside the midgut epithelium was not detected prior to viral replication within the midgut. This suggests that infection of the midgut is an important prelude to systemic infection.

INTRODUCTION

The Baculoviridae are a family of DNA viruses that mainly infects 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 into the hemocoel. Systemic infection is thought to be mediated by virions and infected hematocytes circulating in 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 and viral replication.

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* ß-galactosidase (Lac-Z) gene under control of the *Drosophila melanogaster* heat shock-70 promoter (HSP) reports early viral gene expression. This indicates successful virus entry, uncoating of the viral genome, and early transcription. The *E. coli* ß-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 to distinguish primary from secondarily 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 *Drosophila melanogaster* cell line Dm-1 is non-permissive for *Ac*NPV replication (Carbonell *et al.*, 1985; Rice and Miller, 1986). All cell lines were cultured at 27°C 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 (further named, *Ac*NPV/HSP-p10) was constructed using a p*Ac*AS3 (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* heat shock 70 promoter (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 p*Ac*AS3. 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 post inoculation (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.

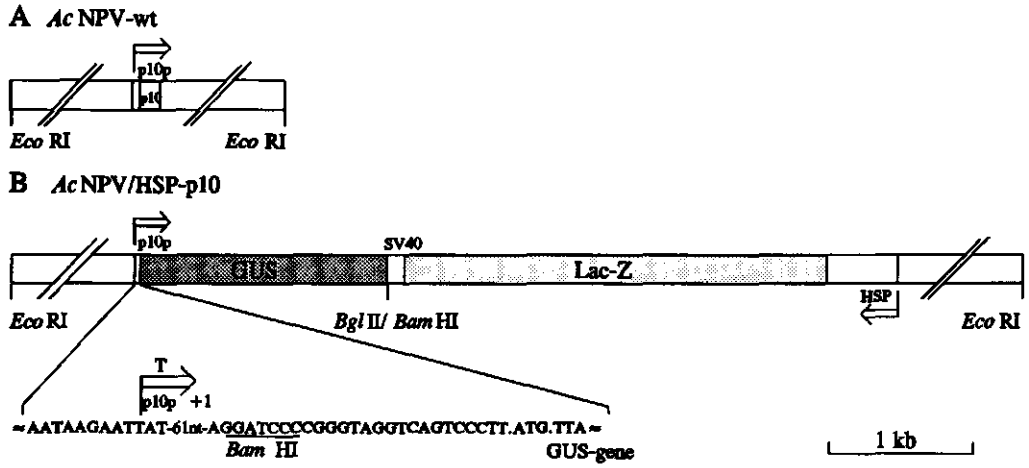


Figure 1: (A) Schematic representation of the *Eco*RI-P fragment of wt-*Ac*NPV DNA, containing the p10 gene. (B)Schematic representation of the corresponding region as found in *Ac*NPV recombinant *Ac*NPV/HSP-p10. The inserted cassette contains the *D. melanogaster* heat shock 70 promoter in front of 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 indicates 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.

Infection of insect cell lines

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 one 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 one ml to the cells with a multiplicity of infection (m.o.i.) of 10 $TICD_{50}$ per cell. The virus was allowed to adsorb for one hour 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 h 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.

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. Aphidicolin blocks 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 per 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 h 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 h p.i. to separate the midgut and adherent tissues from the epidermis.

Fixation

Insect cell lines and isolated midguts were fixed for 2 h at 0°C 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 mM citric acid, pH 7.2) containing 1.5 mM calcium chloride. Larvae were pined on a platform, incised longitudinally and treated with fixative by immersion. After fixation the specimens were washed 3 times for 5 min in PBS.

Enzyme detection

ß-glucuronidase and ß-galactosidase enzyme activity were detected by incubating the specimens (cells, midguts or larvae) for 2 h at 27°C in a reaction mixture of 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, 10 mM EDTA in PBS, pH 7.5 containing 1.2 mM 5-bromo-4-chloro-3-indolyl-glucuronicacid (X-gluc) giving a blue color as a result of ß-glucuronidase activity (GUS expression; Gallie *et al.*, 1992). Subsequently, the specimens were incubated for 2 h at 27°C in the reaction

medium (EDTA was replaced by 1 mM $MgCl_2$, Bondi *et al.*, 1982) in the presence of 1.2 mM 6-chloro-3-indolyl-ß-D-galactoside (Red-gal). A red precipitate indicated ß-galactosidase activity (Lac-Z expression).

Eukaryotic ß-galactosidase and ß-glucuronidase are lysosomal enzymes and have a pH-optimum of approximately 5.5; the prokaryotic *E. coli* ß-galactosidase and ß-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 h at 27°C.

Embedding, sectioning, and staining

For light microscopy insect cells were mounted in glycerol, covered with a glass coverslip and examined under the light microscope. Isolated midguts were embedded in LR-Gold as described 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 a serial sequence to identify infected cells and to verify connections between infected cells. Sections were first examined unstained using phase contrast microscopy. This ruled out any possible interference of the blue GUS stain with the Light Green tissue stain. Thereafter, the sections were stained with Light Green to facilitate 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°C. The staining time depended on the degree of resin polymerization which varied among different samples. In order to remove excessive staining of Light Green, sections were rinsed 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 cover slip.

Photographs of the sections were taken with a Leitz Laborlux S microscope and photographs from the pre-embedded 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 heat shock promoter (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, insect cell lines were infected in three replicate experiments with recombinant *Ac*NPV/HSP-p10. Expression of Lac-Z and GUS genes was monitored by scoring the percentage of cells that showed enzyme activity as a function of time. Since no differences were found between the three replicate experiments, the data are pooled and graphically presented in figure 2. In Sf-21 and Se-1 cells, both permissive for *Ac*NPV, the expression of Lac-Z was detected as early as 3 h post inoculation (p.i.). At 7 h p.i. all cells were positive for Lac-Z expression and no significant difference in percentage of positive cells was observed between both cell lines. At 18 h 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 and reached a maximum of approximately 80% positive cells at 42 h p.i. in both cell lines. As the reduction of GUS positive Sf-21 cells at 42 h p.i. was within the standard deviation, the curve was fitted by hand at this point. Polyhedra were only observed in Sf-21 and Se-1 cells after GUS expression was detected.

Using aphidicolin 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 h p.i. (the end of the experiment).

In *D. melanogaster* cells, which are non-permissive 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 time of the percentage of positive cells was delayed compared to permissive cells, reaching 100% at 12 h p.i. (Fig. 2). No GUS expression or polyhedra production could be detected in these cells at any time.

From the data obtained by infecting insect cell lines we concluded that the onset of early 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 in the same specimen.

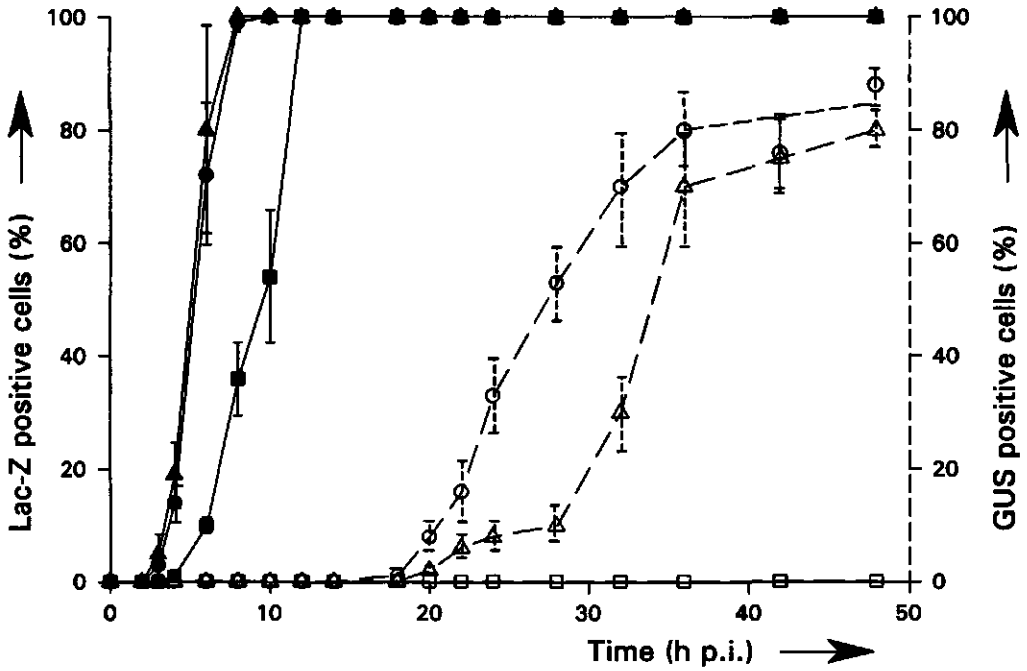
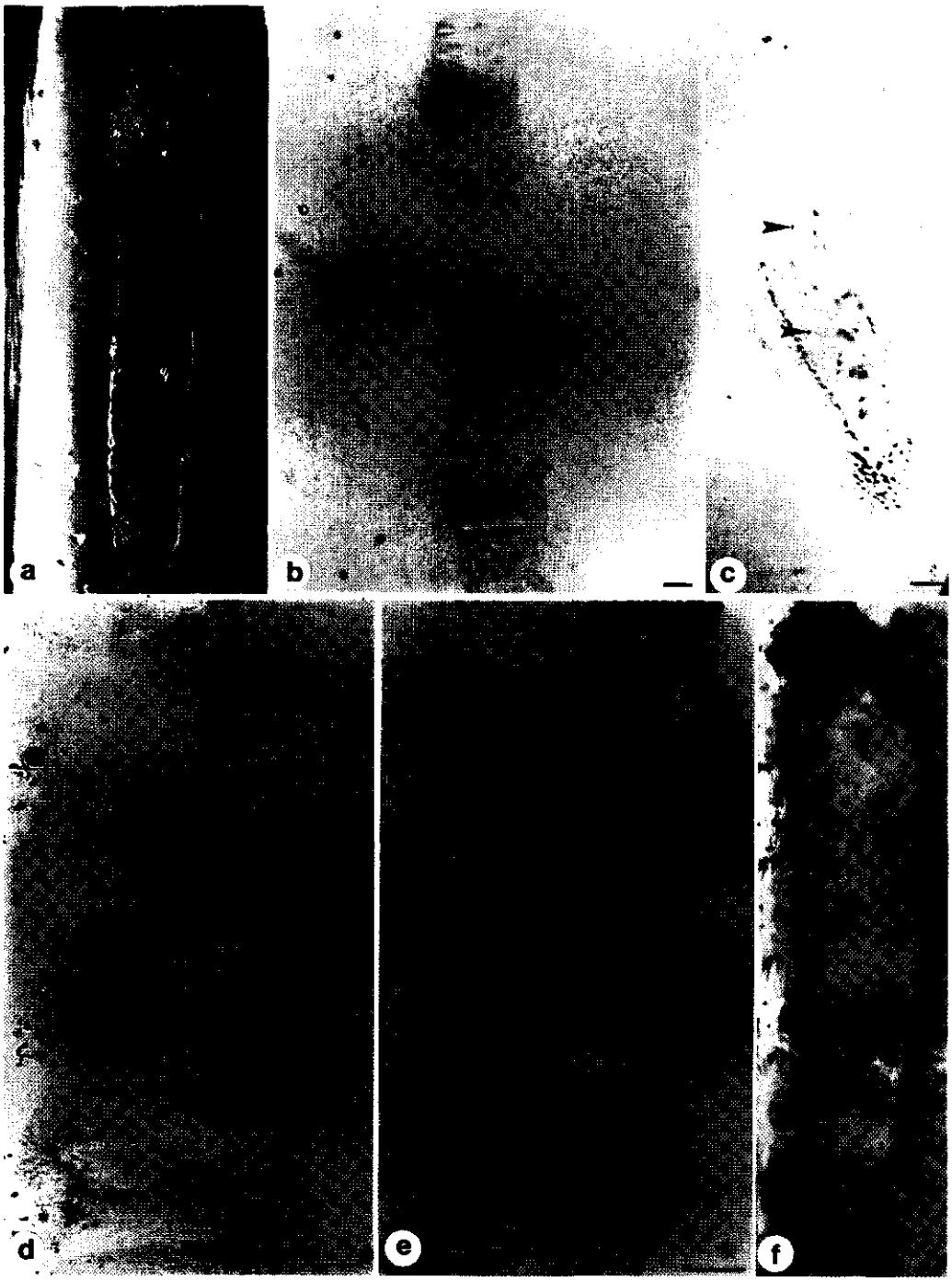


Figure 2: Percentage of Lac-Z (——) and GUS (-----) positive *Ac*NPV/HSP-p10 infected insect cell lines as a function of time. The vertical lines represent the standard deviation of the corresponding values. Sf-21 Lac-Z expression ●, GUS expression ○, Se-1 Lac-Z expression ▲, GUS expression △, Dm-1 Lac-Z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

Figure 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 h p.i. in an embedded midgut of a second instar larvae. [b] Section of the midgut epithelium of a larva dissected 6 h p.i. Infected columnar cells (C) and one infected regenerative cell (R) can be seen. [c] Lac-Z and GUS (arrow heads) expression at 12 h p.i. in an embedded midgut of a second instar larvae. [d] Section of a midgut at 16 h p.i. where a GUS positive (blue, arrow head) 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 h p.i. At this time infection can also be seen in the cells associated with the basal lamina such as hematocytes (H). [f] Lac-Z expression in third instar larva 36 h p.i. The expression is mainly located in the tracheae connected to the midgut. The bar in a, c, and f represents 100 μm, in b, d, and e this bar represents 10 μm.


a
b
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f

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 ß-galactosidase and ß-glucuronidase enzyme activities in larval tissue were investigated. Endogenous ß-galactosidase activity was found in uninfected insects after 6 h 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 ß-glucuronidase could not be detected. No endogenous enzyme activity was detected in uninfected and wild type *Ac*NPV infected larvae after incubation in the substrate reaction mixtures for 6 h at pH 7.5. In the following experiments enzyme activities were hence recorded using reaction mixtures at pH 7.5 and an incubation time of 2 h.

Lac-Z was detected as early as 3 h p.i. in the columnar cells of the dissected midguts from the infected larvae (Fig. 3a). Six h 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 h p.i. (Table 1). Infection of a single isolated goblet cell was seen only once at 10 h p.i. and was considered to be a rare event.

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

Virus replication in columnar cells, as evidenced by GUS positive reaction, was first detected at 12 h p.i. (Fig. 3c). At this time GUS expression was found in approximately 10 % of the infected columnar cells. This number increased rapidly thereafter until approximately 80% of the primarily infected columnar cells showed GUS activity at 24 h p.i. (Table 1). Sixteen h p.i., infected regenerative cells occurred in 13 % of the infected foci. By comparison, an increase in this percentage was observed as the number of infected foci with lac-Z positive regenerative cells was 7 % before GUS expression occurred in the columnar cells. Multiple Lac-Z positive regenerative, columnar and goblet cells were observed as a result of secondary infection (Fig. 3d) only in conjunction with GUS positive columnar cells. No GUS activity was observed in the goblet cells during the course of the study.

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

Infection of cells in the hemocoel associated with the basal lamina or was observed only close to sites where GUS activity was present in the midgut epithelium (Fig. 3e). Infection was seen in muscle in tracheal cells, and in hematocytes. Until 20 h 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 h the infection was mainly restricted to tissues associated with the infected site in the midgut (Fig. 3f).

Table 1

Time (h 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

Table 1: Infection kinetics of *Ac*NPV/HSP-p10 in the midgut epithelium of *S. exigua* larvae. The percentage was calculated relative to the number of infected foci (Lac-Z positive; n > 100). The standard deviation was only calculated when five or more larvae were examined.

DISCUSSION

The chimeric recombinant virus, *Ac*NPV/HSP-p10, was constructed to study early events in *Ac*NPV infection of *Spodoptera 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 in Sf-21 cells treated with aphidicolin, the onset of early viral gene expression was marked by expression of the Lac-Z gene as early as 3 h p.i. In cells non-permissive to *Ac*NPV (*D. melanogaster*) this early gene expression was observed form 4 h 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 starts at 6-8 h p.i. (Tjia *et al.*, 1979; Carstens *et al.*, 1979). From 18 h p.i. onwards GUS expression was found in the permissive cells (Sf-21 and Se-1). Expression of the GUS gene was not recorded in the non-permissive *D. melanogaster* cells and in Sf-21 cells treated with aphidicolin, whereas Lac-Z was expressed in all insect cell lines 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 and to express very late viral genes (Morris and Miller, 1993).

Expression of Lac-Z occurred in primary target cells within 12 h following ingestion of *Ac*NPV/HSP-p10 by second instar *S. exigua* larvae. Midgut columnar cells were the main targets for primary infection. 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. At 6 h p.i. Lac-Z activity in the regenerative cells indicates infection of these cells several hours earlier. This would indicate infection faster then any time reported until now for viral replication. The frequency of infection of regenerative cells through the columnar cells remained stable until 12 h p.i. when the first GUS expression was observed. After this GUS expression, which indicates viral replication, more cells surrounding these GUS positive cells became infected. This provided an indication to separate secondarily infected regenerative cells from the previously, primarily infected 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, it is unlikely that these differentiating cells were scored as regenerative cells because we never observed isolated infected 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 pay-off for packaging multiple nucleocapsids in a single envelope. Polyhedra derived virions reared in *S. exigua* larvae exist predominantly as 3 to 6 nucleocapsids per virion. When a multi-nucleocapsid 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.

Until 12 h p.i., infection was not observed in tissues other than the midgut epithelium. Thus, it is unlikely that infectious parental nucleocapsids pass the midgut epithelium and the basal lamina to infect other larval tissues. Horton and Burand (1993) showed that the envelope of polyhedra derived virus particles fuses with the microvilli membrane of columnar cells. This is consistent with our observation that the virus enters the midgut epithelium through columnar cells. At an early stage of infection (0.5 h to 2 h p.i.) Granados and Lawler (1981) detected free virions in the hemocoel and proposed primary infection of hematocytes by parental virus in the hemocoel. 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 h p.i. using the Lac-Z reporter gene. Infection of nearby tissues was detected only after GUS expression in the midgut epithelium had occurred. This infection was restricted to hematocytes, muscle cells, and tracheal cells associated with the basal lamina until 24 h p.i. At 36 h p.i., infection observed to proceeded in the tracheal elements. 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 lamina cannot be excluded. No infection of any other larval cells or tissues besides the midgut epithelium was observed until the virus had replicated and progeny virus could invade the basal lamina. So direct transport of virus particles did not contribute to the infection of the larvae and was thus considered irrelevant for the systemic infection.

The midgut epithelial cells produce the basal lamina. After viral replication the transcription and translation of the host cells is disturbed. Replication of virus in the midgut epithelium may thus interfere with the integrity of the basal lamina and hence facilitate the passage of virions. Infected

hematocytes and tracheal elements are thought to play a role in the further transport of 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., in preparation).

The first expression of GUS in columnar cells was found at 12 h p.i. This expression of GUS coincides with virus budding from the basal membrane of the columnar cells of *Ac*NPV infected *T. ni* larvae (Adams *et al.*, 1977; Granados and Lawler, 1981). However, using immuno-localization p10 could be found in the midgut epithelium of *S. exigua* larvae from 32 h p.i. onwards (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 immuno-localization. The GUS expression in the epithelial cells could be recorded 6 h earlier than the first GUS expression in insect cell lines. This suggests an increased speed in viral replication or expression of very late *Ac*NPV genes in midgut epithelial cells.

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CHAPTER THREE

Expression of Polyhedrin and p10 in the Midgut of *Ac*NPV Infected *Spodoptera exigua* Larvae

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ABSTRACT

The replication of *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) in midgut epithelium of second instar *Spodoptera exigua* larvae was studied using light and electron microscopy. Microscopical techniques, immunogold labeling and silver enhancement methods were used to localize cytopathic structures in the midgut epithelium of *Ac*NPV infected *S. exigua* larvae. Polyhedra were detected as early as 30 h post inoculation in a few columnar and regenerative cells of the epithelium. Sixty-two h after inoculation large numbers of columnar and regenerative cells showed the full complement of cytopathic structures including polyhedra, fibrillar structures, and electron-dense spacers as normally seen in *Ac*NPV infected fat body and tracheal cells. The polyhedra in the regenerative cells were of a normal size and occluded virus particles. The polyhedra in the columnar cells were relatively small and did not contain occluded virus particles. Infected columnar cells were rejected into the gut lumen, whereas infected regenerative cells had proliferated the midgut epithelium cell layer. Infected larvae that had molted into third instar showed limited signs of infection; only a few columnar and regenerative cells were affected. Since most of the applied promoters for foreign gene expression are derived from *Ac*NPV late genes, the consequences of synthesis of *Ac*NPV late gene products in the midgut of *S. exigua* are discussed with regard to the design and effectiveness of baculoviruses with enhanced insecticidal activity.

INTRODUCTION

Baculoviruses (Baculoviridae; Francki *et al.*, 1991) comprise a large group of insect pathogens which are attractive as control agents of insect pests. The baculovirus *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) has a wide host range infecting at least thirty-two Lepidopteran species (Granados and Williams, 1986). *Ac*NPV has a double-stranded circular DNA genome of about 130 kilobase pairs and may contain about eighty genes (Kelly, 1982; Blissard and Rohrmann, 1990). These genes can be categorized into four classes (immediate-early, delayed-early, late and very-late) according to the order of appearance of the corresponding proteins in infected insect cells. The immediate-early and delayed-early genes are expressed before the onset of DNA replication, whereas the late and very-late genes are expressed after DNA replication has started (Kelly, 1982; Blissard and Rohrmann, 1990). Among the very-late genes polyhedrin and p10 are expressed to a high level. These

proteins are involved in the formation of polyhedra and fibrillar structures respectively (van der Wilk *et al.*, 1987).

The cytopathology of *Ac*NPV infection *in vivo* has been studied using light and electron microscopy (Adams *et al.*, 1977; Granados and Lawler, 1981; Granados and Williams, 1986; Keddie *et al.*, 1989). After dissolution of the polyhedra in the midgut the liberated virions penetrate the peritrophic membrane and enter the midgut epithelium by fusion with the plasma membrane of the microvilli that are located at the apical side of the columnar cells. The nucleocapsids are transported to the nucleus where they are uncoated. Here the viral DNA is replicated and progeny nucleocapsids are assembled. These nucleocapsids move to the basolateral regions of the columnar cells and eventually bud into the haemocoel where they initiate secondary infection. Some controversy exists as to whether regenerative cells that are located along the basal lamina of the midgut epithelium are also infected by parental virus or by progeny virus of the primarily infected epithelial cells (Granados and Lawler, 1981; Keddie *et al.*, 1989). In the secondarily infected tissues (trachea, fat body) another round of replication occurs, but here the nucleocapsids can also acquire a *de novo* assembled envelope in the nucleus. These virions are occluded into polyhedra. The polyhedra are released when the insect disintegrates and transmit the infection to other insects (Blissard and Rohrmann, 1990).

Polyhedra formation and virus occlusion, normally found in secondarily infected tissues, is infrequently observed in midgut epithelial cells. In the nucleus of the *Ac*NPV infected *Trichoplusia ni* midgut epithelial cells. Enveloped nucleocapsids are rare in the polyhedra found in the midgut cells. These polyhedra are comparatively smaller than those found in fat body cells (Granados and Lawler, 1981; Granados and Williams, 1986). Anomalous polyhedron morphogenesis in midgut epithelial cells has also been described for other baculoviruses in insects (Mathad *et al.*, 1968; Summers, 1971; Tanada and Hess, 1976; Falcon and Hess, 1977; Hess and Falcon, 1981). In these instances it was not clear which cell types were affected. Fibrillar structures and electron-dense spacers have only occasionally been reported in midgut epithelial cells after infection with baculoviruses (Falcon and Hess, 1977, Croizier *et al.*, 1980; Hess and Falcon, 1981).

Beet armyworm (*Spodoptera exigua*) is a major pest insect in subtropical areas and in greenhouses (Smits, 1987). The potential of the broad-spectrum *Ac*NPV and highly specific *Se*NPV for control of beet armyworm has been evaluated (Smits and Vlak, 1988). In this paper we investigate the infection

of the *S. exigua* midgut epithelium with *Ac*NPV. Special emphasis is placed on the expression of polyhedra and fibrillar structures in columnar and regenerative cells. Immunoelectron microscopy aided in the detection and localization of polyhedrin and p10 in midgut epithelial cells.

MATERIALS AND METHODS

Virus

The E2 variant of *Ac*NPV (Smith and Summers, 1978) was used. Polyhedra were generated in cultured *Spodoptera exigua* (Se) cells (Gelernter and Federici, 1986). The polyhedra were harvested 4 days after infection, sedimented by centrifugation at 1000 g for 15 min and washed three times with sterile distilled water. To obtain polyhedra free from cellular debris, they were incubated in a solution of 1 % (v/v) Nonidet P40 (Sigma) in phosphate-buffered saline (PBS) at 37°C for 30 min and washed three times with sterile, distilled water. The polyhedra were stored at 4°C until use.

Larvae

Larvae were obtained from a continuous culture of *S. exigua* (Smits, 1987). Eggs were surface-sterilized (Bathon and Gröner, 1977). After hatching the larvae were reared on a semi-artificial diet (Poitout and Bues, 1974) at 28°C.

Inoculation of the larvae

One day old first instar larvae were starved overnight and only larvae that had molted to second instar were used. The larvae were inoculated with *Ac*NPV using the droplet feeding method as described by Hughes and Wood (1986), with droplets containing 10^6 polyhedra/ml. This concentration is sufficient to cause 70% mortality (Smits, 1987). Control larvae were fed on a solution without polyhedra. After inoculation the larvae were placed on artificial diet at 28°C.

Dissection

The larvae were cooled on ice for 5 to 10 min and dissected in longitudinal direction. To make sure the whole midgut was dissected out, the gut was cut through in the fore and hind gut. Infected larvae were dissected at 0, 24, 30, 36, 48, and 62 h post inoculation (p.i.) and were processed for light and electron microscopy. At 36 h p.i. a few infected larvae had molted to the third instar stage; these were also dissected. Mock infected larvae were dissected at 0 h p.i.

Fixation and embedding

Gut tissue was fixed by immersion in 3% glutaraldehyde/2% paraformaldehyde in phosphate citrate (PC) buffer (0.1 M $Na_2HPO_4.2H_2O$, 9.7 mM citric acid, pH 7.2) containing 1.5 mM calcium chloride for 4 to 68 h at 4°C. The tissue was washed twice in PC-buffer for 10 min, dehydrated in ethanol at -25°C, infiltrated and embedded in LR-Gold resin (London Resins Company) at -25°C (Van Lent *et al.*, 1990). The specimens were sectioned with an LKB-V ultratome using a (histo-) diamond knife. Semi-thin sections (1 μm) were mounted on glass slides for light microscopy. Ultra-thin sections (60-80 nm) for electron microscopy were mounted on nickel-grids.

Antisera

Rabbit antiserum against alkali-liberated polyhedrin purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was kindly provided by Dr. M.D. Summers, Texas A&M University, College Station, Texas, USA. Antiserum against SDS-PAGE-purified p10 was a gift of Dr. P. Faulkner, Queens University, Kingston, Ontario, Canada.

Immunogold labeling and silver staining

Immunogold labeling was described previously by van Lent *et al.* (1990). For light microscopy the gold label was enhanced by silver staining for 10 min at 20°C with a 1:1 mixture of initiator and enhancer contained in the Aurion R-gent staining kit (Aurion, Wageningen, Netherlands). The sections were then washed three times for 5 min with distilled water and stained with 0.1% (w/v) toluidine blue in distilled water for 15 min at room temperature. After washing with distilled water, the sections were air dried, mounted in Depex, and covered by a cover slip. Sections were examined with transmitted light or with epi-illumination in a Leitz Laborlux S microscope equipped with a polarization filter block (epipolarization microscopy).

For electron microscopy the labelled sections were stained for 2 min with lead citrate (Reynolds, 1963) and for 4 min with an aqueous solution of 2% (w/v) uranyl acetate, before examination in a Philips CM12 electron microscope.

RESULTS

At different times post inoculation sections of *S. exigua* midguts were screened for the presence of virus induced cytopathic structures, such as polyhedra and fibrillar structures. Twenty out of forty-seven infected second instar larvae molted at approximately 36 h p.i. Both non-molted second instar larvae and molted larvae (third instar) were included in this study. All mock infected second instar larvae had molted to third instar before 36 h p.i.

Control larvae

No virus particles or cytopathic structures were observed in the midgut epithelial cells of mock infected larvae by light and electron microscopy. Immunogold labeling with antisera against polyhedrin and p10 did not result in specific labeling of any structures in the midgut epithelium. This indicated that no baculovirus infection was apparent in the insect stock at the beginning of the experiment.

Infected, non-molted, second instar larvae

At 0 h p.i. no apparent changes were seen in the cells of the midgut epithelium. The first signs of infection were observed in columnar cells at 24 h p.i. The nucleus of the infected cells became enlarged. The coarse chromatin structure was spread out to the periphery of the nucleus and was eventually lost completely while the virogenic stroma appeared. Nucleocapsids were found in the nuclei of these cells and enveloped virions were also observed between the epithelial cells and the basal lamina. However, polyhedrin and p10 could not be detected at this time by immunogold labeling.

Polyhedra and fibrillar structures were observed at 30 h p.i. in the midgut epithelium (Fig. 1). Only a small number of infected columnar and regenerative cells contained these structures at this time. The amount of epithelial cells containing these cytopathic structures as well as the amount of these cytopathic structures per cell increased with time until 62 h p.i. (the end of the experiment). At this time a large number of columnar and regenerative cells contained polyhedra (Fig. 2) and fibrillar structures as visualized by immunogold/silver-staining with polyhedrin and p10 antisera. The polyhedra observed in the columnar cells were small and did not contain virions (Fig. 3A and B). The polyhedra in the regenerative cells were larger than those observed in the columnar cells. In some regenerative cells the polyhedra contained occluded virions (Fig. 4A and B).

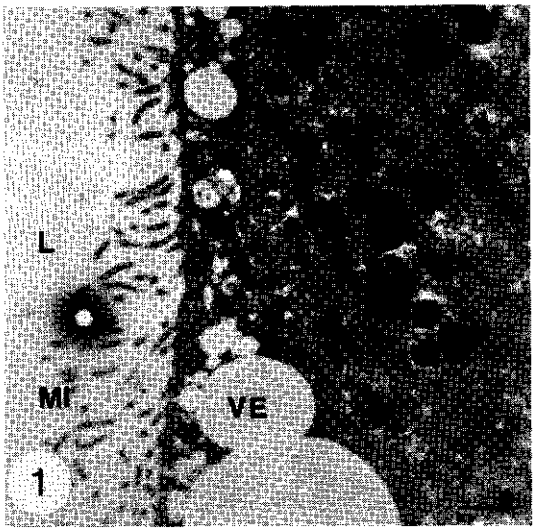

Figure 1: Electron micrograph of a rejected columnar cell of a second instar larva containing polyhedra (P) at 30 h p.i. The cell is vesiculated (VE) and microvilli (MI) are still present at the apical cell surface. L = gut lumen. (Bar represents 1 μm.)

Infected (degenerated) columnar cells were often found rejected from the midgut epithelium (Fig. 2 and 3A) into the gut lumen. Degeneration of these cells was indicated by strong vesiculation of the cytoplasm and loss of microvilli (Fig. 3A). Towards the basal side of these rejected columnar cells, regenerative cells had proliferated into the epithelium often producing polyhedra, fibrillar structures, and electron-dense spacers (Fig. 2, 4A, and B).

Virus nucleocapsids were sometimes present in goblet cells but other cytopathic structures were not seen in these cells (data not shown).

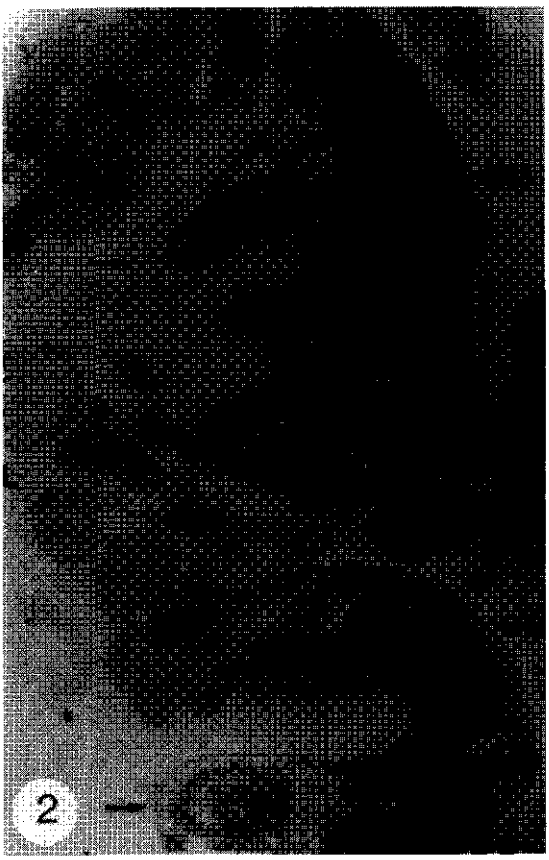

Figure 2: Micrograph showing part of the midgut epithelium of a second instar larva at 62 h p.i. Infected cells can often be recognized by the condensed cytoplasm, the enlarged nucleus, and the lack of the equal spread coarse chromatin structure in this nucleus as seen in healthy cells (arrowhead). Degenerated columnar cells (large arrow) are rejected into the gut lumen (L). Proliferating regenerative cells (R) are present near the basal lamina (BM). Polyhedra are stained black by immunogold silver staining and can be found in regenerative cells and in cells in the haemocoel on the other side of the basal lamina (H). (Bar represents 10 μm.)

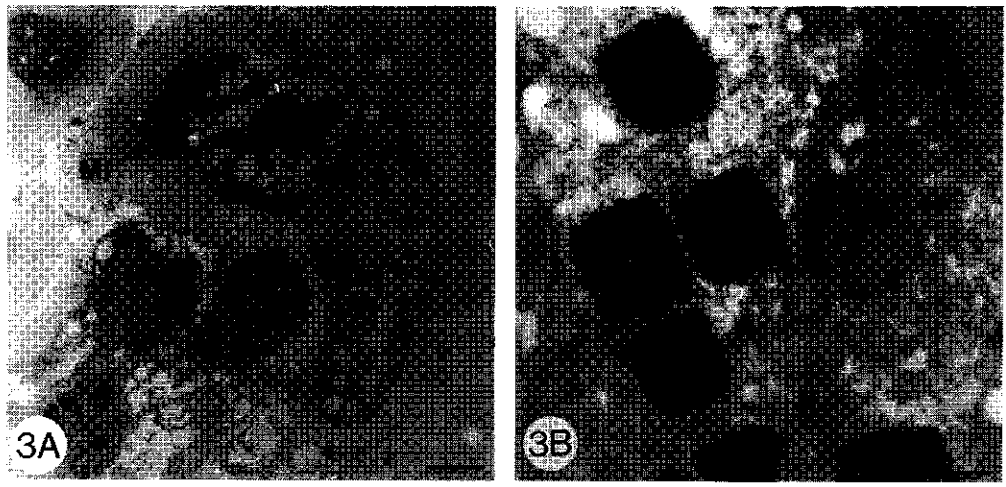

Figure 3: Electron micrographs showing rejected columnar cells containing small polyhedra (3A). Strong vesiculation and loss of microvilli (arrow) indicate the degeneration of these cells. Panel 3B represents a higher magnification of the inset in 3A, showing immunogold labeling of polyhedrin antigens in the polyhedra. Virus occlusion in these polyhedra was not observed. Furthermore, microvilli (MI), nucleocapsids (N), fibrillar structures (F), and electron-dense spacers (E) are present. (Bars represent 10 μm and 0.5 μm respectively.)

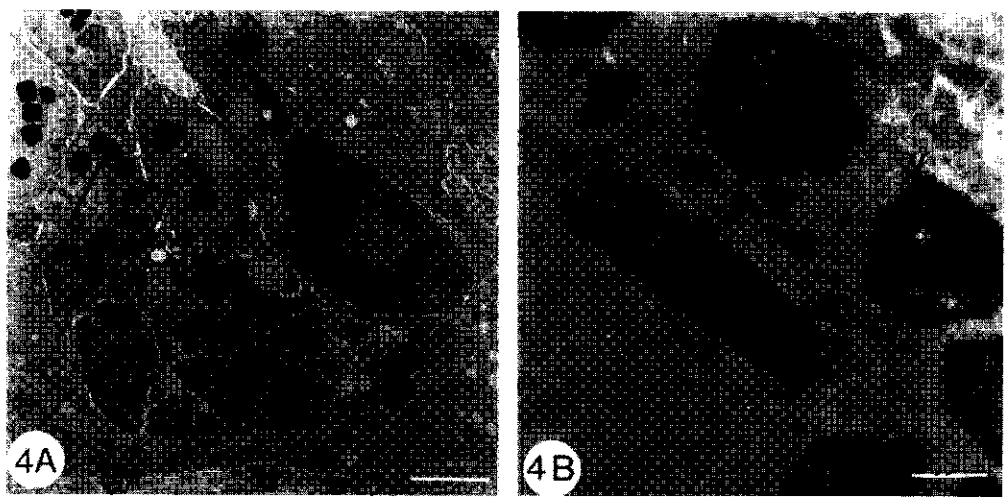

Figure 4: Electron micrographs of regenerative cells proliferating in the epithelium from the basal lamina (BM) towards the gut lumen (direction indicated by arrow). The polyhedra (P) in these cells are large and some occlude virions (V) as can be observed in panel 4B (detail of the inset in 4A). The gold labeling indicates the location of p10 antigens in the fibrillar structure (F). E = electron-dense spacer. (Bars represent 5 μm and 0.5 μm respectively.)

Other cells, containing polyhedra and fibrillar structures, such as tracheal or fat body cells, were distinguished from the gut epithelial cells as the former were positioned on the hemocoel side of the basal lamina (Fig. 2 and 5).

Figure 5: Micrograph showing part of the midgut epithelium of a third instar larva 62 h p.i. Rejected, degenerated columnar cells are present (C). Near the basal lamina (BM) some regenerative cells (R) are situated. Polyhedra (arrows) are stained black by immunogold and silver staining and can be observed in one epithelial cell and in a number of cells in the haemocoel (H). L = Gut lumen. (Bar represents 10 μm.)

Infected, molted third instar larvae

In the midgut of larvae that had molted into third instar at 36 h p.i. the number of infected epithelial cells was strongly reduced in comparison with the second-instar larvae at the same time p.i. Only a few columnar and regenerative cells, either solitary or in small groups, contained polyhedra, fibrillar structures, and electron-dense spacers. The amount of cells containing these cytopathic structures remained low in the midgut epithelium of these larvae until the end of the experiment at 62 h p.i. (Fig. 5). The polyhedra, fibrillar structures and electron-dense spacers in the third instar larvae were morphologically indistinguishable from those seen in the second instar larvae.

DISCUSSION

We have demonstrated the presence of late cytopathic structures (polyhedra, fibrillar structures and electron-dense spacers) in midgut columnar and regenerative cells of second instar *S. exigua* larvae. These structures were observed using light and electron microscopy in *Ac*NPV infected larvae as early as 30 h p.i. Using immunohistochemical staining Keddie *et al.* (1989) observed that the viral glycoprotein gp64 and the major capsid protein were present in both cell types as early as 16 h p.i. The amount of late cytopathic structures in these cells as well as the number of cells infected increased with time.

The polyhedra in the columnar cells were relatively small and did not contain virions. It is possible that these cells are unable to synthesize virion membranes *de novo*, which are considered to be essential for occlusion of the virions. The regenerative cells produced larger polyhedra, and in a number of cells these polyhedra occluded virions. Granados and Lawler (1981) found small polyhedra in the midgut epithelium of *Ac*NPV infected *T. ni* larvae at 24 h p.i., but it was not clear in which cell type these polyhedra occurred. Large, but empty polyhedra were observed in the regenerative cells of *Diparopsis watersi* after infection with *Dw*NPV (Croizier *et al.*, 1980). This suggests that cellular factors are involved in the formation of polyhedra and virus occlusion as these processes apparently differ not only in different cell types of the midgut epithelium, but also in different insect species. This is supported by the observation of Bellonick (1989) who showed that cellular factors are involved in the crystallization of polyhedrin of cytoplasmic polyhedrosis viruses.

Many columnar and proliferating regenerative cells supported the formation of polyhedra, fibrillar structures, and electron-dense spacers in infected *S. exigua* larvae that had not molted by 62 h p.i. Heimpel and Adams (1966) showed that the production of polyhedra in the *T. ni* midgut epithelium could also reach high levels, but Mathad *et al.* (1968) were not able to reproduce the same results. Similar high level production of cytopathic structures was described for the *Dw*NPV infection (Croizier *et al.*, 1980). This was considered to be an exception rather than the rule in NPV infection of lepidopteran insects (Kelly, 1982). In this paper we report massive production of cytopathic structures in the midgut of *S. exigua* infected with *Ac*NPV.

In *Ac*NPV infected second instar *S. exigua* larvae rejection of infected and degenerated midgut columnar cells into the midgut lumen was observed (Fig. 2) at 62 h p.i. Towards the basolateral membrane of these rejected columnar cells regenerative cells had often proliferated into the epithelium. These regenerative cells were also infected, explaining the rapid increase of cells showing production of polyhedra, fibrillar structures, and electron-dense spacers. Similar proliferation of infected regenerative cells was reported for *Dw*NPV infected midgut cells (Croizier *et al.*, 1980). The rejection of infected cells and the proliferation of regenerative cells into the midgut epithelium can be considered as a tissue response to viral infection.

In infected larvae that had molted the number of columnar and regenerative cells producing polyhedra, fibrillar structures and electron-dense spacers was very limited. Comparably low production of polyhedra was also described for *Ac*NPV infection in *T. ni* (Granados and Lawler, 1981), *Estigmene acrea* (Hess and Falcon, 1981) and other NPV infections (Mathad *et al.*, 1968; Summers, 1971; Tanada and Hess, 1976). It is possible that these authors inspected the midguts just after the molt. Falcon and Hess (1977) did report the presence of fibrillar structures and electron-dense spacers in the midgut epithelium of *Ac*NPV and *Se*NPV infected *S. exigua* larvae, but no polyhedra were observed. It is possible that the insect midguts inspected by these authors came from larvae that had molted after infection, but this is not clear from their reports.

After molting of insect larvae a strong decline of infected midgut epithelial cells has been observed in *Bombyx mori* infected with cytoplasmic polyhedrosis virus, flacherie virus and a small DNA virus (Inoue and Miyagawa, 1978). In these cases infected midgut epithelial cells were selectively rejected from the epithelium and replaced by differentiating regenerative cells shortly after the molt. This type of recovery of the midgut from viral infection has also been described for *B. mori* by Yamaguchi (1979) and Choi *et al.* (1989). The replacement of midgut columnar and goblet epithelial cells by differentiating regenerative cells during the molt is considered a normal process in healthy lepidopteran larvae (Vanhaecke, 1984). Massive rejection of midgut epithelial cells during the molt can explain the strong decrease in infected midgut cells in *Ac*NPV infected *S. exigua* larvae after the molt (Fig. 3). Whether this was a selective process or part of the total renewal of midgut epithelium remains unclear.

The relatively high expression of polyhedrin and p10 in *S. exigua* midgut cells holds promise for the engineering of modified baculovirus insecticides with increased insecticidal activity expressing gut-

specific toxins such as *Bacillus thuringiensis* endotoxin (Hoffmann *et al.*, 1988, Martens *et al.*, 1990; Merryweather *et al.*, 1990). The expression of these two major late *Ac*NPV genes in this tissue may also explain the rapid insecticidal effects of a mite neurotoxin expressed under control of the p10 promoter (Tomalski and Miller, 1991) and a scorpion toxin expressed under control of the polyhedrin promoter (Stewart *et al.*, 1991). It remains to be investigated whether other NPVs express polyhedrin and p10 in midgut epithelium.

ACKNOWLEDGMENTS

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CHAPTER FOUR

Systemic Infection of *Spodoptera exigua* Larvae using a Marked *Autographa californica* Nuclear Polyhedrosis Virus Recombinant

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ABSTRACT

Midgut epithelial cells are the primary targets of baculovirus infection of insect larvae (Flipsen *et al.*, 1993, ibid. 1995). From here various routes for the secondary and systemic infection have been proposed to explain the rapid spread of the virus in the insect body. We have used a recombinant *Autographa californica* nuclear polyhedrosis virus, which reports early and late viral gene expression and allows us to follow the spread of the virus *in situ* by histochemistry and (electron) microscopy. The virus was applied orally (as polyhedra) and by injection (as extracellular virus, ECV) into *Spodoptera exigua* larvae, as well as to explanted tissue in culture. Using this recombinant we provide evidence that infection of tracheoblasts at the distal ends of the tracheal system by circulating ECVs and the subsequent invasion of secondary tissues from this focus is the major route of virus spread in the insect body. Quick movement and spread of the virus through the intercellular space of the tracheal system, as has been described recently (Engelhard *et al.*, 1994), did not appear to occur in this insect-virus system, and cannot be considered as a general mechanism of virus spread in insect larvae. The basal lamina surrounding tissues is shown to form an effective barrier against virus invasion. However, tracheoblasts lack such a basal lamina and this explains the infectability of these cells. Enzymatic digestion of this membrane prior to infection resulted in random infection of the various tissues investigated. Hematocytes play a minor role in virus transport and probably serve as an ECV reservoir.

INTRODUCTION

Autographa californica nuclear polyhedrosis virus (*Ac*NPV) is a member of the family Baculoviridae infecting a large number of lepidopteran larvae. Infection of larvae by baculoviruses is a unique process as it involves two genetically identical, but phenotypically distinct viruses each having a unique role in the infection process (Volkman and Keddie, 1990). (i) The occlusion derived virions (ODV) are found embedded in large proteinaceous capsules, known as polyhedra. The ODVs are released from the capsules in the alkaline environment of the midgut lumen, pass the peritrophic membrane and enter the midgut epithelial cells by fusion with the microvilli (Kawanishi *et al.*, 1972; Hortan and Burand, 1993). A primary infection is established in columnar and regenerative cells of the midgut epithelium (Adams *et al.*, 1977; Flipsen *et al.*, 1995). (ii) From here extra cellular progeny virus particles (ECVs) are released into the hemocoel (Granados and Lawler, 1981) or transmitted to

connected tissues such as hematocytes (Keddy *et al.*, 1989) or tracheal cells (Engelhard *et al.*, 1994) to establish a systemic infection. Upon progression of the disease the infected midgut cells are sloughed off into the gut lumen (Keddie *et al.*, 1989; Flipsen *et al.*, 1993). The midgut is regenerated to allow continued feeding of the larva, whereas hematocytes, fat body and epidermis become heavily infected as evidenced from the production of polyhedra in these tissues. Ultimately the insect dies and massive amounts of polyhedra are released.

For the spread of the infection from the midgut epithelium several routes have been proposed: (i) virions pass the midgut basal lamina, are released into the hemocoel, and infect all other tissues directly by penetrating through the basal lamina surrounding the these tissues (Granados and Lawler, 1981); (ii) infection and subsequent circulation of these hematocytes invading other tissues and transmit the virus further by cell-to-cell contact (Keddie *et al.*, 1989); (iii) transmission of infection through cell-to-cell movement of the virus through the tracheal epidermis (Ritter *et al.*, 1982) or (iv) alternatively, as virus particles are transported through the intercellular space of the tracheae (Engelhard *et al.*, 1994).

The first two routes require passage of the basal lamina surrounding all secondary tissues except hematocytes. This proteinaceous layer is impermeable to particles larger that 15 nm in size (Reddy and Locke, 1990) and may thus form a major barrier for virus passage. Baculovirus particles are indeed much larger in size (300 nm long, 50 nm wide; Adams and McClintock, 1991) then the exclusion limit of the basal lamina. In contrast, hematocytes are able to actively penetrate the basal lamina and may infect tissues this way. Transmission of infection through the tracheal epidermis after infection of tracheolar cells requires many rounds of replication before all tissues of the insect will be infected. The fourth route postulates longitudinal extracellular transport to take place in a wide intercellular space of the tracheal epidermis to facilitate spread of virus particles over long distances.

This study set up to investigate the relative importance of these four pathways of virus transport in secondary and systemic baculovirus infection in lepidopteran larvae. This includes the evaluation of the role of the basal lamina in the infection. We have followed the infection of *Spodoptera exigua* larvae *in situ* over time using a marked recombinant *Ac*NPV. This recombinant contained two reporter genes for the concurrent detection of early (HSP driven Lac-Z expression) and late (*Ac*NPV-p10 driven GUS expression) stages of virus infection. This allowed us to follow the progression of the disease simultaneously in various tissues *in situ* (Flipsen *et al.*, 1995). The virus was orally

administered to larvae in the form of polyhedra, by injection of ECVs into the hemocoel, or by incubation of dissected larval tissues in an ECV suspension. Infection of larval tissues was located histochemically and monitored macroscopically in whole mounts and in detail by light and electron microscopy. Our results indicate that the ECVs circulating in the hemocoel are the major resource for systemic baculovirus infection in insects. Apparently the barrier of the basal lamina is circumvented by infection of tracheoblasts which allows the virus to invade other tissues. This route can explain the rapid spread of virus infection in the insect larva.

MATERIALS AND METHODS

Virus and larvae

The E2 variant of *Ac*NPV (Smith and Summers, 1978) was used as wild type virus to construct *Ac*NPV/HSP-p10 (Flipsen *et al.*, 1995a) (Fig. 1). Cell line derived extracellular virus (ECV) and polyhedra were obtained as described by Flipsen *et al.*, (1995). *Spodoptera exigua* larvae were taken from a continuous laboratory culture (Flipsen *et al.*, 1993).

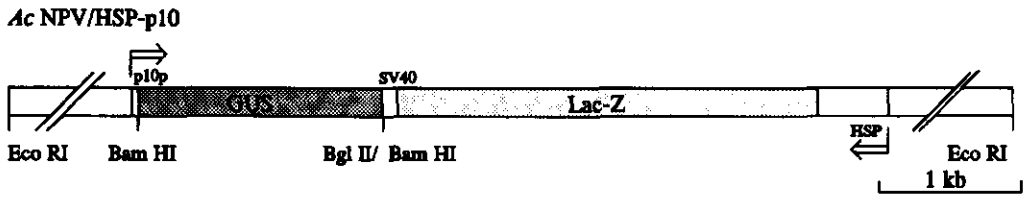


Figure 1: Schematic representation of the reporter gene cassette inserted in recombinant *Ac*NPV/HSP-p10. The cassette contains the *D. melanogaster* heat shock 70 promoter in front of the *E. coli* Lac-Z gene followed by the SV40 transcription terminator. The p10 coding sequence has been replaced by the *E. coli* GUS gene (Flipsen *et al.*, 1995).

Infection and dissection.

Oral infection was achieved by feeding early second instar larvae with droplets containing 10^7 polyhedra per ml (Flipsen *et al.*, 1995). The ingested volume (0.33 ± 0.13 μl; Smits and Vlak, 1987) ensured a mortality of 100%. Infected larvae were dissected at 6, 12, 18, 24, 30, 36, 42, 48, 60 and 72 h post inoculation (p.i.) by a longitudinal insertion at the lateral side of the body (Flipsen *et al.*, 1993). The skin was spread out and pinned down on a solid support. The gut was carefully moved aside and held this way using another set of pins until further processing.

Second instar larvae (24 h post molt) were injected with 20 nl of cell-culture supernatant containing $1.5*10^8$ tissue culture infective dose 50% ($TCID_{50}$) units of *Ac*NPV/HSP-p10 per ml, and dissected at 6, 12, 24 and 48 h p.i. as described above.

For *in vitro* infection of tissues third instar larvae (12 h post molt) were dissected in Hink's medium (Hink, 1970) as described above. A large part of the central nervous system, including the brain and the first two ganglia, was removed. After this, the larval preparations were washed three times with Hink's medium and incubated for 1 h at 4°C in 2 ml medium containing 10^6 $TCID_{50}$ units of *Ac*NPV/HSP-p10 per ml. This incubation was performed at 4°C to minimize (transport) activity in larval tissues. After inoculation the larval preparations were washed three times for 5 min with ice-cold Hink's medium to remove excess virus and then incubated at 27°C for 6 h. Vitality of the residual larval tissues was checked by fluorescence microscopy using 1 μg/ml fluorescein di-acetate (Kasten, 1981). To study the role of the basal lamina in infection, five larvae were dissected as described above and treated with 1.2 U/ml dispase (Boehringer Mannheim, grade II) for 30 min at 27°C to remove this membrane prior to infection. After this treatment the larvae were washed 3 times with Hink's medium and infected as described above.

As a control experiment for the infection with *Ac*NPV/HSP-p10 larvae were orally infected with wild type *Ac*NPV-E2. Intact larvae were injected with and larval preparations were incubated in cell-culture supernatant without ECVs. None of the control larvae or larval preparations showed any GUS or Lac-Z activity throughout the experiment.

Fixation, reporter gene detection, embedding, sectioning, and staining

All specimens described were fixed, washed, and analyzed for enzyme activity as described by Flipsen *et al.* (1995). GUS activity (p10 promoter driven) gives rise to blue coloration and Lac-Z expression is indicated by a red color in infected tissues. Specimens were studied directly using a Wild M3Z stereo microscope or a Leitz Labolux S light microscope.

After enzyme detection larvae were fixed again with a higher concentration of glutaraldehyde. Thereafter they were dehydrated and embedded as described by Flipsen *et al.* (1993). Unstained semi-thin sections (2 μm) were viewed by phase contrast microscopy after which the sections were stained

with Light Green and observed with bright field illumination (Flipsen *et al.*, 1995). For electron microscopy, sections were stained as described previously (Flipsen *et al.*, 1993).

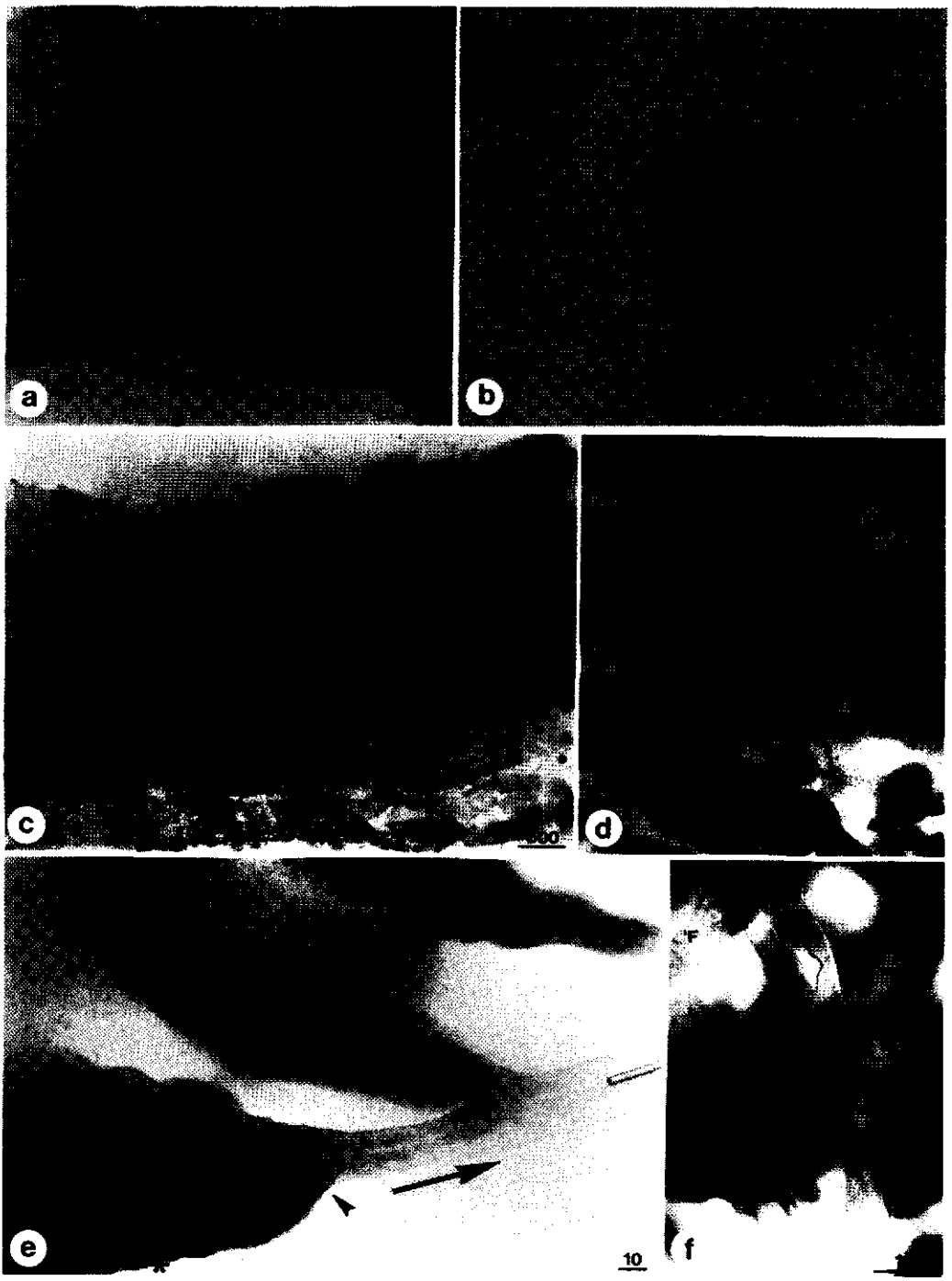
Titration of hemolymph-derived extracellular virus

To compare the infection of orally infected larvae and larvae that were injected into the hemocoel with ECV particles of *Ac*NPV/HSP-p10, the virus titer of the hemolymph of orally infected larvae was determined. Hemolymph from 15 second instar larvae was collected at 30 h p.i. and pooled in a calibrated glass capillary. From this collected sample the virus titer was determined (Summers and Smith, 1987) using IPLB-Sf-21 cells (Vaughn *et al.*, 1977) as a test system.

RESULTS

Upon ingestion of *Ac*NPV/HSP-p10 polyhedra by second instar *S. exigua* larvae the first signs of infection, indicated by Lac-Z activity, were found in midgut columnar cells (Flipsen *et al.*, 1995). Until 24 h p.i. the infection remained restricted to the midgut epithelium and the tissues associated with the midgut basal lamina such as tracheal cells, muscle cells, and hematocytes. From 30 h p.i. the first GUS expression was found in tracheal cells associated with the midgut and in hematocytes scattered in the body cavity (Fig. 2a & b). This suggests that the virus had replicated and that ECVs had been released into the hemolymph. The infected hematocytes were probably released from the midgut basal lamina and circulated in the body cavity. Infected hematocytes attached to the basal lamina of various tissues, but were never observed having penetrated this membrane or having transmitted virus across this membrane to infect other tissues (Fig. 2a & b).

Figure 2: Micrographs showing various steps of the systemic infection and the spread of *Ac*NPV in *S. exigua* larvae after oral infection. (a) Junction site of skeleton muscle (SM) and epidermis (E) with an infected hematocyte (H) at 30 h p.i., (b) Lac-Z (red) and GUS (blue) positive hematocytes (H) attached to the fat body (F) at 60 h p.i., (c) infection in the tracheae (T) indicated by Lac-Z (arrow head) expression at 36 h p.i. Note the uninfected epidermis on the major tracheae and the infected hematocytes between the epidermis and the fat body, (d) detail of c, (e) infection in the tracheal epidermis at 48 h p.i. Polyhedra (P) in the distal end followed by GUS expression (asterisk), nuclear hypertrophy (arrow head), Lac-Z expression (red) and uninfected cells in the major trachea. Direction of movement of the infection front is indicated by the arrow, (f) infection of salivary gland (S) cells connected to the trachea. (M = midgut, Mt = Malpighian tubule). Sizes of the bars are given in μm.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Infection was observed in tracheoblasts and in the cells of distal tracheae irrespective of their connection to the midgut or to fat bodies, the hind gut, the fore gut, salivary glands, and many other tissues. This was evidenced by Lac-Z activity as early as 36 h p.i. (Fig. 2 c and d). At this time cells of the major tracheae were still free of infection. Infection of this tissue progressed from the distal ends towards the main tracheae (Fig. 2e). Late viral infection processes, such as the formation of polyhedra and GUS activity, were detected in cells of the distal ends of tracheae from 42 h p.i. onwards. In cells of the main tracheae earlier stages of infection, evidenced by Lac-Z expression and nuclear hypertrophy, were observed (Fig. 2e). At later times (72 h p.i.) GUS expression and concurrent polyhedra production was also observed in cells of the major tracheae. From 42 h p.i. onwards the infection rapidly radiated out from the junction between the distal tracheae into the neighboring tissues leading to complete infection of the fat body and the epidermis. Infection of salivary glands (Fig. 2f) and the fore gut and hind gut remained restricted to cells directly associated with the tracheae.

These accumulated data suggest that cells of the distal tracheae and the tracheoblasts and not the hematocytes play a key role in the spread of the infection to other tissues. To investigate the mechanism by which cells of the distal tracheae and the tracheoblasts become infected, two approaches were taken. Firstly, the ECV form of *Ac*NPV/HSP-p10 was injected directly into the hemocoel and the route of infection was followed. Secondly, dissected larval preparations were incubated with *Ac*NPV/HSP-p10 ECVs. The role of the basal lamina in preventing infection by extracellular virus was investigated by removing this membrane prior to incubation of larval preparations with this recombinant virus.

Second instar *S. exigua* larvae (n=36) were injected with 20 nl ECVs ($1.5*10^8$ $TCID_{50}$ units/ml). Assuming a hemolymph of about 500 nl (1/4 of the body volume) the ECV concentration in the hemolymph would be approximately $6*10^6$ $TCID_{50}$ units/ml. Under these conditions fifty percent of the larvae treated showed signs of infection by either Lac-Z expression in tissues or in individual cells (n=24), or by liquification within 7 days p.i. (n=12). The ECV titer in the hemolymph of orally infected second instar larvae was determined to be approximately $3*10^6$ $TCID_{50}$ units/ml at 30 h p.i. resulting in 100% mortality after 5 to 6 days p.i. Although injection of ECVs in the hemolymph of larvae resulted in a virus titer in the hemolymph comparable to that of orally infected larvae milder symptoms may still be expected in these injected larvae. This is caused by the continuous virus

production in orally infected larvae, whereby the hemolymph virus titer will increase rapidly. In the virus injected larvae, however, virus production will not contribute to this hemolymph titer until approximately 12 h p.i. This explains why not all virus injected larvae were killed.

Six h post injection of recombinant ECVs in the hemocoel Lac-Z activity was found in hematocytes of these larvae. Infection of cells in the distal tracheae and tracheoblasts was observed from 12 h p.i. onwards. Twenty four h p.i. all infected larvae showed Lac-Z expression in the cells of the distal tracheae (Fig. 3a), in the tracheoblasts and in the hematocytes. At this time the first GUS expression was found in tracheoblasts. Infection of the gut epithelial cells was never observed at any time in these larvae.

Incubation of dissected larval preparations in an ECV suspension resulted predominantly in infection of tracheoblasts (6 h p.i.; Fig. 3 b and c) and a few hematocytes. No infection was observed in midgut cells, muscle cells, salivary glands, Malpighian tubules, fat body etc. When dissected larval preparations were treated with dispase to remove the basal lamina surrounding the various tissues prior to *in vitro* infection, Lac-Z activity was observed simultaneously in cells of the distal tracheae, the tracheoblasts and in the cells of the fat body (Fig. 3 d and e), epidermis and many other tissues.

The histopathological data obtained after oral infection, injection of ECVs into the hemocoel, and infection of the dissected larval preparations are schematically presented in figure 4. The time scale is arranged in such a way that the corresponding infection processes of orally infected larvae, larvae that were injected with ECVs, and of incubated larval preparations were aligned. The sequence of pathological events after ECVs injection is similar to those after oral infection. from approximately 30 h p.i.

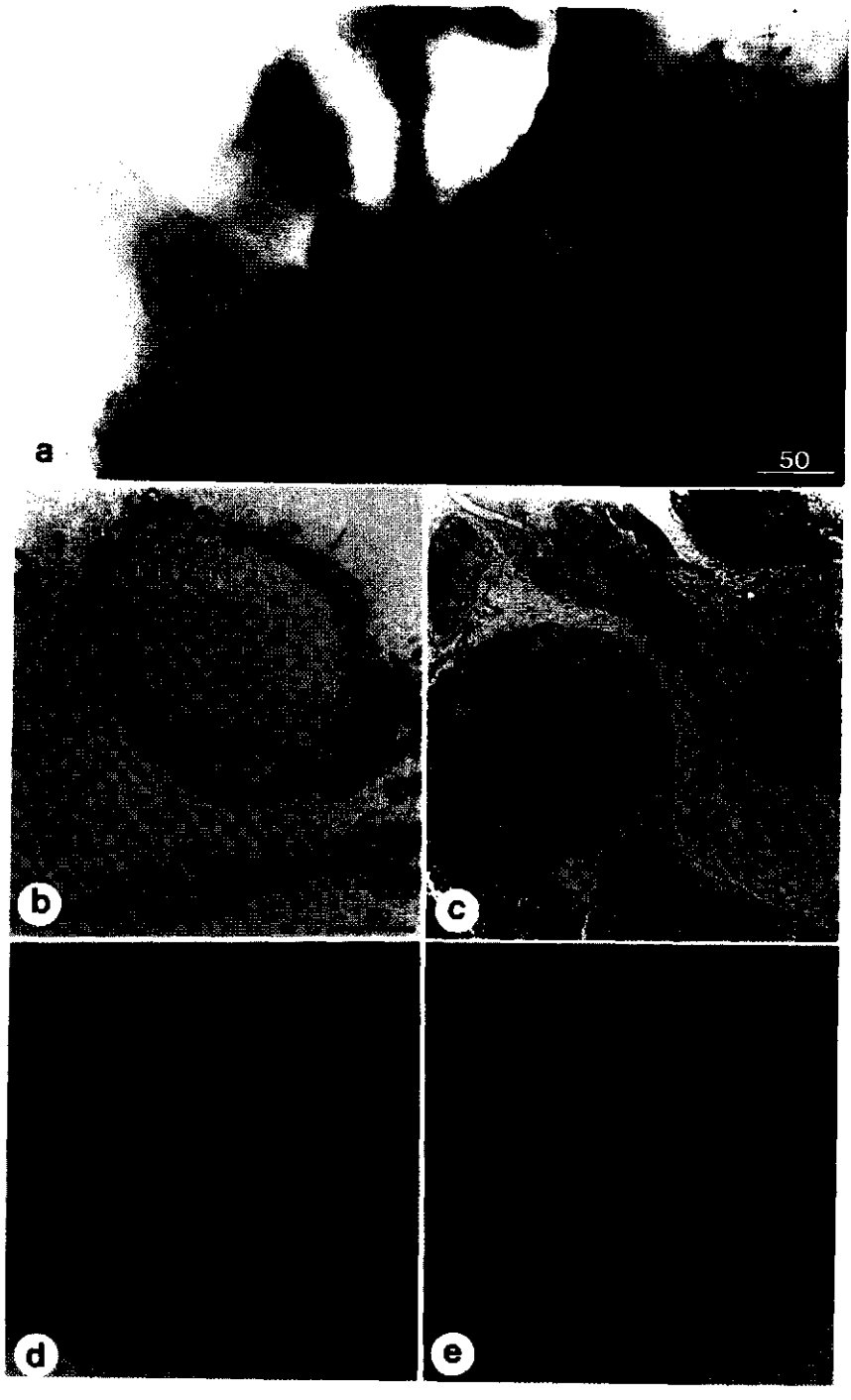
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Figure 3: Micrographs showing the infection of larvae or larval tissues infected in various ways. (a) Infection of a trachea (T) connected to the hind gut (HG) 24 h after injection of ECVs in the hemocoel, (b) infection of a tracheoblast (arrow, red cell) at a similar site of the hind gut (Hg) after incubation of a larval preparation in ECV suspension, (c) Electron microscopical micrograph of the infected tracheoblast (black precipitate) in b, where tracheoli (t) can be seen in the cytoplasm of this infected tracheoblast, (d) Infection of fat body of in a larval preparation that was incubated in a virus suspension after dispase treatment. Infected cells are distributed over the complete fat body whereas in the control (e; without dispase) only tracheoblasts were infected. (T=trachea, F=fat body, E=epidermis, L=gut lumen). Sizes of the bars are given in μm.

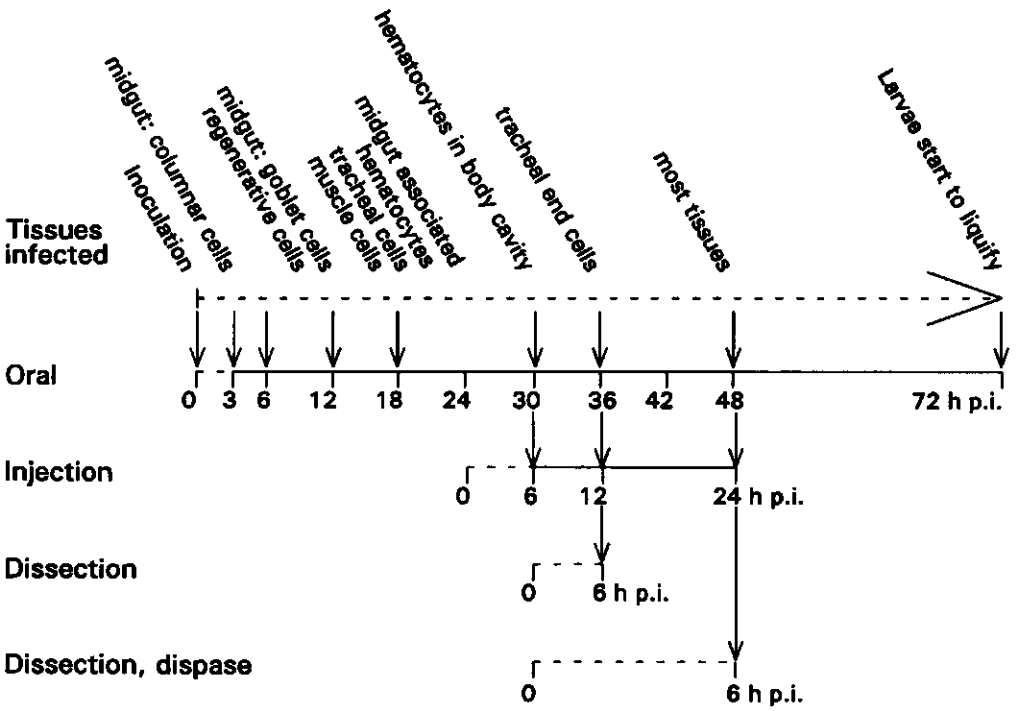

Figure 4: Schematic presentation of the sequence of events associated with infection as observed with the different inoculation methods. The various tissues are placed in the sequence of infection after oral inoculation. The solid line in the time scale indicates Lac-Z activity in the tissues displayed on top of this time scale. The time scales of the non-oral inoculations are aligned to correspond to the orally infected larvae. The vertical arrows indicate the corresponding stages of infection.

DISCUSSION

The tracheal system seems to play an important role in the systemic spread and infection of *Ac*NPV to various tissues of insect larvae. The tracheal epidermis is among the first tissues to be infected after the virus has replicated in the midgut epithelium (Engelhard et al., 1994; Flipsen *et al.*, 1995). We have observed that infection in tissues other than the midgut starts at their junctions with tracheae. Thirty h p.i. a large number of ECVs circulate in the hemocoel. Our results indicate that these particles are capable of infecting the tracheoblasts from the hemocoel side (Fig. 3 and 4). This is demonstrated by the infection of tracheoblasts and cells of the distal tracheae at an early stage after oral infection, by injection of ECVs in the hemocoel and by the selective infection of tracheoblasts after incubation of tissues in an ECV suspension. In accordance with the results obtained with *S. exigua* larvae, injection of extracellular recombinant virus in *Trichoplusia ni* larvae also revealed that both hematocytes and tracheoblasts were equally susceptible to infection by *Ac*NPV-ECVs (Engelhard *et al.*, 1994).

The basal lamina is an important physical barrier for baculovirus infection. Removal of this membrane by dispase leads to a quick and random infection of all tissues (Fig. 3 d and e). The basal lamina of for instance fat body and tracheae has a size exclusion limit of 15 nm particles and larger (Reddy and Locke 1990). The basal lamina surrounding all tissues (except the hematocytes) is highly variable in thickness. For example, Malpighian tubules have a thick basal lamina that will exclude particles of 6 nm and larger (Reddy and Locke, 1990); on the other hand, the basal lamina around the distal ends of tracheae and tracheoblasts is extremely thin or absent (Noirot and Noirot-Timothée, 1980). This may explain the susceptibility of these particular cells to infection by ECVs circulating in the hemocoel. As tracheoblasts often penetrate the basal lamina of various tissues these cells form an ideal route for transport of baculovirus over the basal lamina to these tissues.

Progression of infection through the tracheal epidermis is slow. The infection was observed as a continuous infection front migrating through the tracheal epidermis starting at the distal ends and tracheoblasts (24 h p.i.) moving towards the major part of the tracheae at later times (60 h p.i.) (Fig. 3e). This type of transport and the corresponding infection occurred in the epidermis of tracheae connected to the midgut as well as in the epidermis of tracheae connected to other tissues. The transport of virus particles over a short distance may be explained by the presence of an intercellular

space between the tracheal cells. This space is irregular, wider than 20 nm and intersected by junctions (Locke, 1985). Virus particles with nucleocapsid sizes of 30-35 nm in width and 250-300 nm in length (Adams and McClintock, 1991) may be transported through this labyrinth over a short distance infecting a limited number of tracheal cells.

Spread of infection in the tracheal epidermis of *S. exigua* larvae resembles the infection of tracheal epidermis in *Pseudaletia unipuncta* infected with granulosis virus (Ritter *et al.*, 1982). These authors proposed a cell-to-cell transport mechanism for the progression of infection in the epidermis of tracheae based on electron microscopical observation of a virogenic stroma in the cells, indicative of viral replication. However, the use of reporter genes in our marked *Ac*NPV allowed the *in situ* detection of early viral gene expression prior to virus replication and virogenic stroma formation and thus allowed the detection of the simultaneous infection of tracheal cells. This strongly suggests that virus particles are transported through the extracellular labyrinth of the tracheal epidermis, but only over a short distance. This suggests that transport of virus particles through the tracheae is only a minor route for virus spread.

The rapid infection of cells of the distal tracheae and tracheoblasts in *T. ni* was explained by unidirectional longitudinal transport of virus particles from the midgut towards other tissues over a large distance through the intercellular space of the tracheal epidermis (Engelhard *et al.*, 1994). Our results indicate that infection of tracheoblasts and cells of the distal tracheae can be achieved effectively by virions that circulate in the hemocoel. If longitudinal transport through epidermis of the tracheae occurs, it will be from the distal tracheae towards the major tracheae as described in this paper (Fig. 3 c,d and e) irrespective of the tissue to which the distal tracheae are connected. However, the intercellular space needed for longitudinal transport of such large virus particles as baculoviruses is most probably too small to explain this type of long distance transport (Locke, 1985; Locke, 1991). This type of transport could not be demonstrated using smaller probes (Welling and Paterson, 1985). The presence of a thick basal lamina (Reddy and Locke, 1990) around the major tracheae explains the absence of viral infection at these places (Fig. 2c).

The midgut associated tracheoblasts and hematocytes became infected at an early stage. Together these cells may form the major resource for progeny ECV production resulting in high virus titers in the hemocoel. Infection of hematocytes may occur when they are involved in a 'wound response' at the primary site of infection in the midgut or when they take up virus particles from the hemocoel as

observed after injection of the ECV in the hemocoel. Hematocytes, infected or healthy, move through the body cavity via the hemolymph. They are also able to penetrate the basal lamina of various tissues. Penetration of the basal lamina by infected hematocytes was not observed in our case suggesting that the basal lamina even functions as a barrier for invasion by infected hematocytes.

The use of a marked recombinant *Ac*NPV and *in situ* histochemistry and microscopy has advanced our understanding on how the virus spreads from the primary site of infection (midgut) to other tissues. The progeny ECVs either migrates directly or after one round of replication into the hemocoel. These ECVs can infect cells adjacent to the midgut, such as tracheoblasts, muscle cells, and hematocytes (Flipsen *et al.*, 1995). These cells provide a source of ECV after regeneration of the midgut epithelium. Our results suggest that the major route of systemic infection is by ECVs circulating in the hemocoel and infecting tracheoblasts at the distal ends of the tracheal system. From there virus infection radiates out into the various tissues. We have little evidence to support the view that hematocytes and major tracheae are the general route for the systemic spread of the virus in insect larvae.

ACKNOWLEDGEMENTS

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CHAPTER FIVE

Tissue Specificity and Gene Expression of *Autographa californica* Nuclear Polyhedrosis Virus in *Spodoptera exigua* Larvae.

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ABSTRACT

Upon infection of lepidopteran larvae by *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) many, but not all, tissues are susceptible to virus infection. Factors that determine tissue specificity of *Ac*NPV are not known. Using (immune) histochemistry and electron microscopy we verified whether tissue specificity in *Ac*NPV infection of *Spodoptera exigua* larvae exists. For this, *Ac*NPV recombinants containing reporter genes under control of viral and host derived promoters were used. These recombinants allowed easy monitoring of infection by the constitutive expression of the *Drosophila malanogaster* heat shock 70 promoter (HSP), by early gene expression of the *Ac*NPV immediate-early promoters *PE38* or *ME53*, or by late gene expression of the very late *Ac*NPV p10 promoter. In most tissues these early events were followed by the normal pathophysiological changes associated with late gene expression and polyhedra formation. In midgut goblet cells, salivary glands, and Malpighian tubules the onset of viral gene expression was not followed by very-late p10 expression. No pathological changes or production of viral structural proteins were observed in these tissues. This strongly suggests that infection was aborted after the onset of viral immediate-early gene expression.

INTRODUCTION

Baculoviruses can enter non-permissive cells and initiate early and late genes. However, infectious virus and polyhedra are not formed in these cells (Carbonell *et al.*, 1985; Rice and Miller, 1986; Flipsen *et al.*, 1995a). In permissive cells replication and all subsequent late viral processes occur (Granados and Williams, 1986). These results indicate that tissue specificity and host range of baculoviruses are not necessarily regulated by viral attachment and successful entry of virus particles, but by sequential and regulated viral gene expression.

Gene expression of baculoviruses can be divided into four temporal classes: immediate-early (α), delayed-early (β), late (γ) and very-late (δ) genes (Friesen and Miller, 1986; Kool and Vlak, 1993). Transcription of *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) immediate-early genes is initiated by host factors and do not require previous viral gene expression. Delayed-early viral gene transcription is initiated or enhanced by viral factors. Since it is often difficult to distinguish immediate-early and delayed-early viral genes, they are now both categorized as early genes (Kogan and Blissard, 1994). Late and very-late *Ac*NPV genes are only expressed after the onset of viral

replication (Thiem and Miller, 1989). The reliance of immediate-early genes on host transcription factors may implicate these genes in host and tissue specificity of baculoviruses.

Tissue specificity of baculoviruses in host larvae has always been described on the bases of full replication of the virus leading to nucleocapsid or polyhedra formation (Booth *et al.*, 1992). Whether limited and abortive infection occurs in host tissues, similarly to *Ac*NPV infection of *D. melanogaster* cells (Carbonell *et al.*, 1985; Rice and Miller, 1986), is not known. Nor are factors determining this tissue specificity. The question of tissue specificity is particularly relevant to the pathology of *Ac*NPV in semi- and non-susceptible insects, as well as in relation to the host range of (genetically engineered) baculoviruses.

We have followed the pathogenesis of *Ac*NPV in *Spodoptera exigua* larvae at various stages of infection in a variety of organs in order to find differences in the activity between the constitutive host and viral promoters. In addition, we addressed the question whether viral infection could be aborted in host tissues after expression of immediate-early viral genes. For this, a number of *Ac*NPV recombinants were constructed to follow the infection *in vivo* by histochemical techniques and electron microscopy. These recombinants allowed a comparison of the tissue specific expression of the constitutive *D. melanogaster* HSP-70 promoter, the *Ac*NPV immediate-early genes promoters *PE38* and *ME53*, and the very-late p10 promoter using *Lac-Z* and *GUS* reporter genes. In addition, infected tissues and cells were examined ultrastructurally whereby late viral gene products such as structural proteins were localized using immuno-gold labeling to complement the histological data. The function of *PE38* and *ME53* in the infection process is not fully established, but the presence of a GATA motif (Krappa *et al.*, 1992; Kogan and Blissard, 1994) in their promoter may be important for tissue specific expression (Evans *et al.*, 1988; Orkin, 1990; Abel *et al.*, 1993).

METHODS

Insect cells

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

Recombinant viruses

The recombinant virus *Ac*NPV/HSP-p10 (Fig. 1) is based on the E2 strain of *Ac*NPV (Smith and Summers, 1978) and has been described previously (Flipsen *et al.*, 1995a). This recombinant contains two reporter genes, the *Escherichia coli Lac-Z* gene, which is under transcriptional control of the constitutive *D. melanogaster* HSP promoter, and the *E. coli GUS* gene, driven by the *Ac*NPV p10 promoter.

The recombinant viruses *Ac*NPV/*PE38* and *Ac*NPV/*ME53* were constructed by co-transfection of viral DNA of the *Ac*NPV genotype E (Tjia *et al.*, 1979) and the transfer vectors p*Ac-PE38-Lac-Z*-rev and p*Ac-ME53-Lac-Z*, respectively (Fig. 1). *Ac*NPV/*PE38* contains the *E. coli Lac-Z* gene under the control of the immediate-early *PE38* promoter as an additional insert in the *Eco*RV-site upstream of the polyhedrin gene. *Ac*NPV/*ME53* includes the *Lac-Z* gene under the control of the immediate-early *ME53* promoter at the same location. The transfer vectors were built as follows: The plasmid pBS*Lac-Z* was generated by isolating the *Bam*HI/*Hin*dIII fragment of p*Ac*DZ1-DNA that included the *Lac-Z* gene (Zuidema *et al.*, 1990), and by insertion of this fragment into the vector pBlue-script (KS+). The *PE38* and *ME53* promoter fragments were generated by PCR-amplification (Saiki *et al.*, 1988) using plasmids p*AcHind*-F or p*AcHind*-G as templates and the primers given in figure 1. The 202 bp long *ME53* and the 207 bp long *PE38* promoter fragments were each inserted into the *Xho*I site of pBS*Lac-Z*. The immediate-early promoter/*Lac-Z* cassettes were inserted as *Bam*HI/*Bgl*II fragment into the *Bgl*II site of the p*Ac*UW2 (Weyers *et al.*, 1990). The p*Ac-PE38-Lac-Z*-rev construct includes the *PE38* promoter in opposite orientation to the polyhedrin promoter, whereas the transfer vector p*Ac-ME53-Lac-Z* contains the *ME53* and polyhedrin promoters in the same orientation (Fig. 1).

Cell culture derived recombinant extracellular virus and polyhedra were obtained as described previously (Flipsen *et al.*, 1995a).

Infection of insect cell lines

Cells of the permissive *S. frugiperda* cell line (Sf-21) and the non-permissive *D. melanogaster* (Dm-1) were infected with recombinant virus at a multiplicity of 10 tissue culture infective 50% dose per cell (Flipsen *et al.*, 1995a). The infected cells were subsequently scored for reporter enzyme activity as previously described (Flipsen *et al.*, 1995a). All cell lines were cultured at 27°C in TNM-FH medium (Hink, 1970) supplemented with 10% fetal bovine serum.

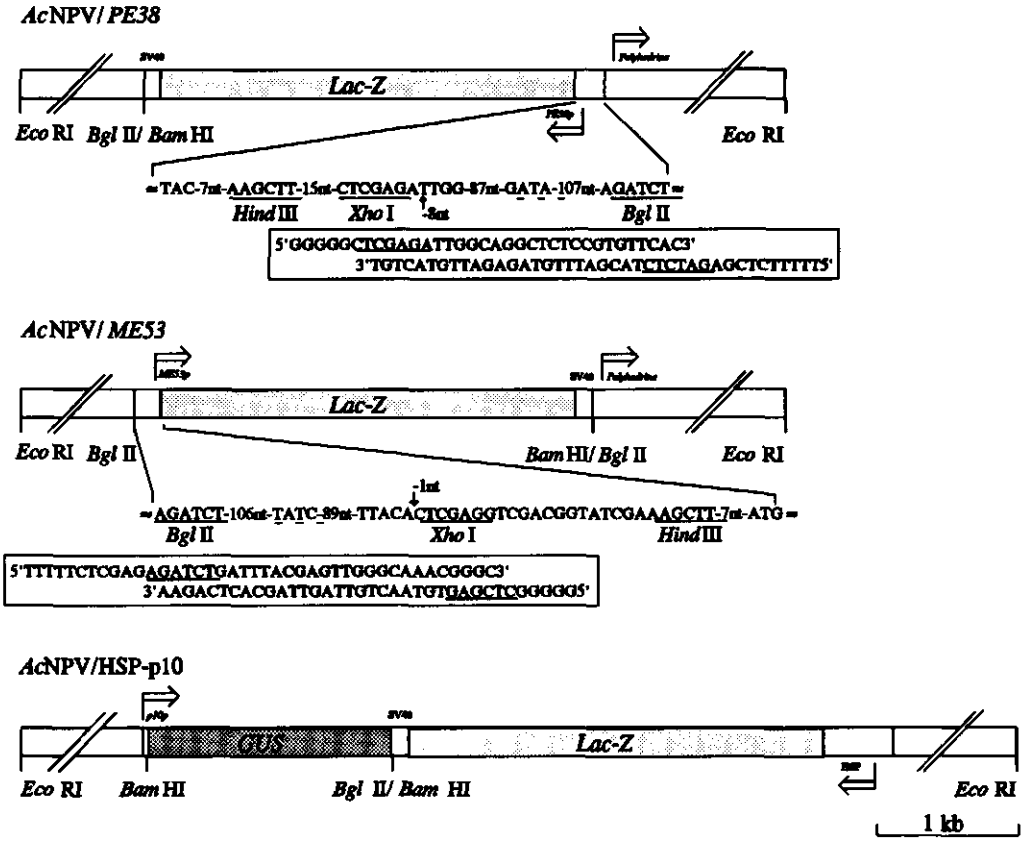


Figure 1: Schematic representation of the reporter gene cassettes present in the recombinant viruses *Ac*NPV/*PE38*, *Ac*NPV/*ME53*, and *Ac*NPV/HSP-p10. In *Ac*NPV/*PE38* the *E. coli* lac-Z gene is placed under control of a second *PE38* promoter and inserted upstream of the polyhedrin promoter in the opposite orientation. In *Ac*NPV/*ME53* the *Lac-Z* gene is placed under control of the duplicated *ME53* promoter and is inserted upstream of the polyhedrin promoter in the same orientation. In the boxes the primers used for PCR amplification of the corresponding promoter fragments are indicated. In *Ac*NPV/HSP-p10 the inserted cassette contains the *D. melanogaster* HSP promoter in front of the *Lac-Z* gene which is followed by the SV40 transcription terminator. The p10 coding sequence has been replaced by the *E. coli GUS* gene (Flipsen *et al.*, 1995).

Infection and histochemical analysis of larvae

S. exigua larvae were obtained from a continuous laboratory culture as previously described (Flipsen *et al.*, 1993). With respect to the lethal dose, the biological activity of the *Ac*NPV genotypes E (Tjia

et al., 1979) and E2 (Smith and Summers, 1978) as compared in a bioassay (Smits, 1987) showed no differences (Flipsen, data not shown).

For histochemical analysis early second instar larvae were inoculated orally with the *Ac*NPV recombinants with the droplet feeding method (Flipsen *et al.*, 1993) using an inoculum of 10^7 polyhedra per ml. Larvae were dissected at 1, 3, 6, 12, 18, 24, 30, 36, 42, 48, 60, 72, and 94 h post inoculation (p.i.) and were subsequently fixed, washed, and incubated for enzymatic detection of the virus (Flipsen *et al.*, 1995a). The enzymatic detection gives rise to a blue and a red color as a result of *GUS* or *Lac-Z* expression, respectively. The preparations were then further fixed with a higher concentration of glutaraldehyde to maintain the ultrastructure. Whereafter the specimens were dehydrated, embedded, sectioned, and studied using light and electron microscopy as described previously (Flipsen *et al.*, 1993; Flipsen *et al.*, 1995a).

Immunogold labeling

Immunogold labeling was carried out as described previously (Flipsen *et al.*, 1993). To locate viral structural proteins, an antiserum directed against alkali-liberated polyhedra-derived viral nucleocapsids was used (Van der Wilk *et al.*, 1987). Western blot analysis revealed that this antiserum was directed against structural proteins *vp39* (the major capsid protein) and *gp41* (the major occluded-virus glycoprotein).

Infection of isolated organs

Salivary glands were infected *in vitro* in order to examine whether the absence of late viral gene expression in tissues such as salivary glands, Malpighian tubules, and goblet cells was due to the limited time of infection of these organs before larvae started to liquify. To this end salivary glands, fat bodies and adhering tracheal elements were isolated from early fourth instar larvae. These organs were infected *in vitro* after treatment with dispase (Flipsen *et al.*, 1995b). The tissues were fixed and stained for enzyme activity at 72 h p.i.

Transcriptional analysis of the *ME53* promoter

Since *Ac*NPV/*PE38* and *Ac*NPV/*ME53* carry a second immediate-early promoter fragment, the question arises whether the duplicated promoter shares the same transcriptional characteristics. Therefore transcriptional activity of the *ME53* promoter in *Ac*NPV/*ME53* was analyzed by

transcriptional mapping. For this Sf-21 cells infected with *Ac*NPV/*ME53* were harvested at various times after inoculation to prepare cytoplasmatic RNA. The 5' ends of the authentic *ME53* transcript and the *ME53/Lac-Z* transcript were mapped by primer extension analysis as previously described (Becker and Knebel-Mörsdorf, 1993). The analyses were performed with a *ME53*-specific 28 bases-long (Knebel-Mörsdorf *et al.*, 1993) and a *Lac-Z*-specific 30 bases-long specific oligo-deoxyribonucleotide primer (5'GTCACGACGTTGTAAAACGACGGCCAGTGC3'). This transcriptional analysis indicated that transcription of the *ME53* gene and of the *ME53/Lac-Z* cassette initiates at the same nucleotide in the same temporal time-frame during the infection.

Mock and wild type *Ac*NPV infected insect cell lines and *S. exigua* larvae, processed and examined as described above, did not show any *Lac-Z* or *GUS* expression.

RESULTS

Previous studies of *Ac*NPV/HSP-p10 infected *S. exigua* larvae showed early gene expression after the virus entered and successfully initiated expression of its genome in midgut goblet cells (Flipsen *et al.*, 1995). The reporter gene (*Lac-Z*) is under control of a host promoter (HSP) which is constitutive active in insect cells (Zuidema *et al.*, 1990). Goblet cells did not show any subsequent very-late viral gene expression, which only occurs when the virus has replicated (Flipsen *et al.*, 1995a). This prompted us to investigate whether or not immediate-early viral gene expression occurred in other tissues and whether immediate-early viral gene expression plays a role in *Ac*NPV tissue specificity in *S. exigua* larvae. Therefore, recombinants containing the promoters of either the *PE38* and *ME53* gene followed by *Lac-Z* as a reporter gene were constructed (Fig. 1).

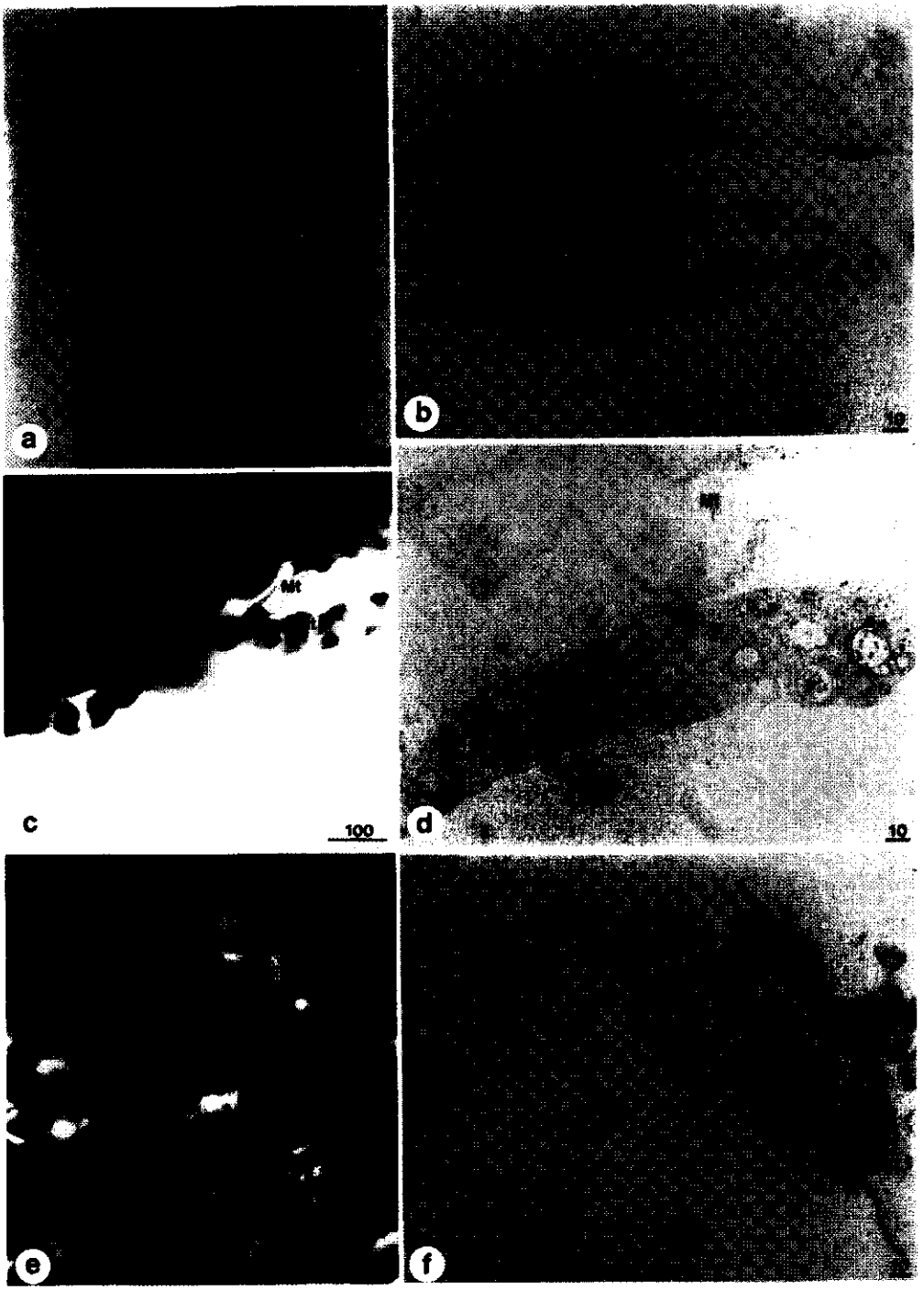
Infection of insect cell lines

Expression of *Lac-Z* from *Ac*NPV/*PE38* and *Ac*NPV/*ME53* was detected in Sf-21 cells as early as 3 h p.i. At 8 h p.i. all infected Sf-21 cells were positive for *Lac-Z* expression. In the non-permissive Dm-1 cells the first expression was detected at 4 h p.i. and the number of positive cells increased until 12 h p.i. when all cells stained positive. These results resemble the expression of *Lac-Z* by *Ac*NPV/HSP-p10 after infection of Sf-21 and Dm-1 cell lines (Flipsen *et al.*, 1995), in which *Lac-Z* expression is driven by the *D. melanogaster* heat shock promoter.

Infection of larvae and histochemical analysis

After inoculation of second instar *S. exigua* larvae with polyhedra of the recombinants *Ac*NPV/*PE38* (Fig. 2, left column) and *Ac*NPV/*ME53* (Fig. 2, right column), *Lac-Z* expression was frequently observed in midgut epithelial columnar cells (from 3 h p.i.). At a low frequency in midgut regenerative cells that were in conjunction with infected columnar cells (from 6 h p.i.), in midgut epithelial cells neighboring the primarily infected cell including goblet cells (from 12 h p.i.), and in cells associated with the midgut basal lamina such as tracheal cells, muscle cells, and hematocytes (from 18 h p.i.; Fig. 2a and b). Subsequently (from 36 h p.i.), *Lac-Z* expression was observed in distal tracheae, fat bodies (Fig. 2e and d), epidermis (Fig. 2f), major tracheae (Fig. 2e and f), skeletal muscle (Fig. 2f), neural glial cells, Malpighian tubules (Fig. 2c and b), and in restricted zones of the salivary glands, fore and hind gut (from 42 h p.i.). The expression of *Lac-Z* in these restricted zones only included cells that were in close contact with the tracheal elements. Similar results were obtained following *Ac*NPV/HSP-p10 infection of *S. exigua* larvae, where *Lac-Z* expression was driven by the heat shock promoter (Flipsen *et al.*, 1995). This indicated that there is no difference in tissue specificity between the constitutive host promoter HSP70 and the virus encoded *PE38* and *ME53* promoters.

Figure 2: Demonstration of *Lac-Z* expression by *Ac*NPV/*PE38* (left column) and *Ac*NPV/*ME53* (right collum) in whole mounts (a, c, and e) and 'Light Green'-stained sections (b, d and f) of larval tissues. (a) *PE38* directed expression at 30 h p.i. in the midgut (M) and midgut associated trachea (T; arrow head), (b) *ME53* expression at 48 h p.i. in tissue associated with the midgut basal lamina such as muscle cells (Ms) as well as expression in a Malpighian tubule (Mt), (c) *PE38* expression in Malpighian tubules at 72 h p.i., (d) *ME53* expression and polyhedra formation (P) in the fat body (F) at 48 h p.i. (e) *PE38* expression in the tracheae (T) and fat body at 72 h p.i. (f) *ME53* expression and polyhedra formation in a major trachea (T), the epidermis (E), and in a skeletal muscle cell (Sm). (Fg=fore gut). Bar size indicated in μm.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Using the *Ac*NPV/HSP-p10 recombinant, *GUS* expression was found in midgut columnar and regenerative cells, hematocytes, tracheal cells, fat body, epidermis, midgut muscle cells, and neural glial cells. Expression of the *GUS* reporter gene was not observed in midgut goblet cells, salivary glands, or Malpighian tubules until the end of the experiment (92 h p.i.). At this time tissues started to disintegrate and larvae showed the first signs of liquification. Detection of viral structural proteins at the same time as *Lac-Z* expression indicated that less than 5% of the *Lac-Z* positive midgut goblet cells and none of the *Lac-Z* positive salivary glands and Malpighian tubules supported late gene expression (72 h p.i.). Table 1 summarizes, compares, and extends these results obtained with the three recombinants with electron microscopic observations.

To verify that, even after a prolonged infection time, no *Ac*NPV very-late gene expression occurred in the *GUS* negative tissues salivary glands and fat bodies (control) were infected *in vitro* with *Ac*NPV/HSP-p10 and analyzed 72 h p.i. *In vivo* 72 h after the initial infection of the salivary glands, (48 h after inoculation) larvae were in an advanced state of disintegration. Even 72 h p.i. no polyhedra formation, *GUS* expression, or nuclear hypertrophy was observed in the *in vitro* infected salivary glands. At 72 h p.i. *in vitro* fat body cells were filled with polyhedra (Fig. 3).

Minor quantitative differences in expression of the various promoters in different tissues were observed. After infection with *Ac*NPV/*PE38* and *Ac*NPV/*ME53* a stronger *Lac-Z* activity was observed in muscle cells, salivary glands, and Malpighian tubules as compared to other tissues and to the *Lac-Z* expression by *Ac*NPV/HSP-p10 in these tissues. Whether these increased levels of *Lac-Z* were due to tissue specific expression levels or due to the absence of viral replication remains unclear.

Table 1

TISSUE	HSP	*PE38* *ME53*	p10	Ultrastructural observation[1]
	h p.i.	h p.i.	h p.i.	
Ectodermal				
Trachea	16	16	30	Polyhedra formation
Epidermis	48	48	60	Polyhedra formation
Neural	60	60	72	Infection is restricted to the neural glial cells surrounding the neurons.
Mesodermal				
Muscle				
Midgut	16	16	60	Formation of a single abnormal polyhedron with occluded virus particles late after infection.
Skeleton	60	60	N.O.	No ultrastructural changes until 72 h p.i.
Hematocytes	16	16	24	Polyhedra formation
Fat body	36	36	48	Polyhedra formation.
Endodermal				
Midgut cells				
Columnar	3	3	12	Formation of virogenic stroma, virus particles, fibrillar structures and small polyhedra which did not occlude virus particles.
Goblet	14	14	N.O.	No polyhedra, fibrillar structure or virogenic stroma formation and only rarely viral structural proteins in the nucleus.
Regenerative	14[2]	14[2]	16	Normal polyhedra formation, although empty polyhedra were also observed.
Malpighian tubules	48	48	N.O.	No polyhedra, fibrillar structure, virogenic stroma formation or nuclear hypertrophy.
Salivary glands	48	48	N.O.	See Malpighian tubules.

1: From 12 h p.i. to 94 h p.i.

2: Expression of the *Lac-Z* reporter gene was observed at earlier time points after infection due direct to passage of nucleocapsids. However, the majority of the infected regenerative cells showed reporter gene expression after viral replication in the primarily infected columnar cells. This infection is noted in this table. (N.O. = not observed)

Table 1: Tissue specificity. Reporter gene expression (*Lac-Z* and *GUS*) and ultrastructural observations in *S. exigua* larvae after infection with *Ac*NPV/HSP-p10, *Ac*NPV/*PE38* and *Ac*NPV/*ME53*.

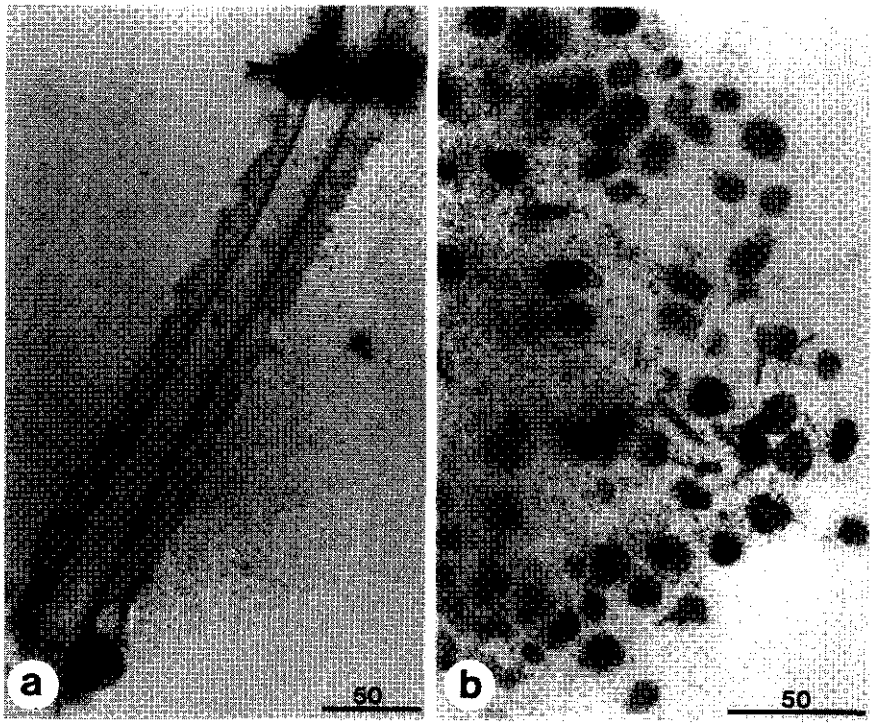

Figure 3: Micrographs showing an *in vitro* *Ac*NPV/HSP-p10 infected salivary gland (a) and fat body (b) at 72 h p.i. The salivary gland shows *Lac-Z* expression in a limited number of cells (arrow head; L=lumen). In the fat body formation of polyhedra (P) was observed. Bar size indicated in μm.

DISCUSSION

Tissue specificity of *Ac*NPV infection with reference to late viral gene expression apparently occurs in infected *S. exigua* larvae. Expression of *Ac*NPV late genes is restricted to tissues such as tracheae, fat body, epidermis, midgut-associated muscle cells, columnar and regenerative cells and hematocytes. Whereas *Lac-Z* expression by immediate-early gene promoters is not only observed in these tissues but also in midgut goblet cells, salivary glands and Malpighian tubules. Thus, tissue specificity of *Ac*NPV infection seems not to be governed by the expression of the immediate-early genes *PE38* and *ME53*.

Vital organs, such as Malpighian tubules, salivary glands, and goblet cells of the midgut epithelium, showed high levels of early viral gene expression as indicated by *Lac-Z*. However, no other pathological effects like nuclear enlargement, viral stroma formation, or production of viral structural

proteins were observed in these tissues *in vivo* (Table 1). *In vitro Ac*NPV infection of salivary glands also revealed no late viral gene expression or other, virus associated, pathological changes. The pattern of p10-*GUS* in *S. exigua* larvae is consistent with the histopathological events of *Ac*NPV infection in *Trichoplusia ni* larvae (Booth *et al.*, 1992). These results suggest that *Ac*NPV-DNA replication and subsequent late gene expression is down-regulated in these tissues.

Abortion of the *Ac*NPV infection after immediate-early gene expression is observed in tissues of endodermic origin, but infection is not always aborted in all these tissues. Midgut columnar and regenerative cells do support viral replication and subsequent very-late viral gene expression. However, occlusion of virus particles is only observed in a limited number of regenerative cells and not at all in the epithelial columnar cells. Apparently, there is also a block late in the infection cycle of these columnar cells preventing occlusion and subsequent reinfection of the midgut epithelium. The midgut epithelium may thus recover from viral infection by rejection of infected cells (Keddie *et al.*, 1989; Flipsen *et al.*, 1993).

The restriction of viral infection to immediate-early gene expression in essential tissues such as the Malpighian tubules, salivary glands, and midgut goblet cells, in conjunction with the recovery of the midgut epithelium, allows the insects to continue feeding, growing, functioning while other tissues such fat body, trachea, epidermis and hematocytes produce viruses. This results in an optimal production of progeny virus.

Deletion of the ecdysteroid UDP-glucosyltransferase gene induced early degeneration of the Malpighian tubules. Hence this gene also plays a role in the tissue specific maintenance of these tubules preventing the early death that normally occurs after infection with these deletion mutants (Flipsen *et al.*, 1995b).

The promoters of most *Ac*NPV immediate-early genes contain regulatory motifs similar to those found in the promoters of *PE38* and *ME53*. Thus tissue specificity is probably not governed by recognition of host transcriptional factors and subsequent expression of immediate-early genes. It cannot, however, be ruled out that specific recognition of other immediate-early gene promoter by host transcription factors plays a role in tissue specificity.

In vitro the GATA motif in the *PE38* gene promoter is recognized by insect proteins (Krappa *et al.*, 1992). In mammals and insects a GATA motif has been demonstrated to play a role in tissue specific expression (Orkin, 1990; Moses *et al.*, 1990; Abel *et al.*, 1993). Whether the GATA motif in the *PE38* or *ME53* promoter is recognized in a tissue specific manner can not be determined from this set of experiments. Mutations and deletion of the GATA motif in the *PE38* and *ME53* promoters driving *Lac-Z* expression may demonstrate tissue specificity.

Thus tissue specificity in baculovirus infection of insect larvae is not determined by viral attachment, viral entry, or initiation of transcription from the viral genome. Neither is it likely that the recognition of host factors of viral immediate-early gene promoters determines this specificity.

CHAPTER SIX

Deletion of the Baculoviral Ecdysteroid UDP-Glucosyltransferase Gene induces Early Degeneration of Malpighian Tubules in Infected Insects

This chapter is submitted in a slightly modified form as: **Flipsen, J.T.M., Mans, R. M.W., Kleefsman A.W.F., Knebel-Mörsdorf, D., and Vlak, J.M.** 1995. Deletion of the baculoviral ecdysteroid UDP-glucosyltransferase gene induces early degeneration of Malpighian tubules in infected insects. *J. Virol. 69, 000-000.*

ABSTRACT

Deletion of the ecdysteroid UDP-glucosyltransferase (*egt*) gene from the *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) genome increases the speed of kill by this virus (O'Reilly and Miller, 1991). Second instar *Spodoptera exigua* larvae infected with the *egt* deletion mutant of *Ac*NPV molt and subsequently resume feeding as mock infected larvae do but are killed more rapidly than wt-*Ac*NPV infected larvae. Wild type and *egt*-minus *Ac*NPV recombinants marked with a *Lac-Z* gene were used to study their pathogenesis in insects. Histopathological investigation revealed that early degeneration of the Malpighian tubules and not the molting *per se* may be the cause of this increased speed of kill by *Ac*NPV.

INTRODUCTION

Baculovirus-encoded ecdysteroid UDP-glucosyltransferase (*egt*) inactivates ecdysteroid hormones in infected insect larvae by conjugating these compounds with glucose or galactose (O'Reilly, 1995). As a result of this inactivation normal development of the *S. frugiperda* larvae such as molting is arrested (O'Reilly and Miller, 1989). Larvae continue to feed and grow, ultimately producing large numbers of polyhedra (O'Reilly and Miller, 1991). Penultimate or final instar larvae infected with an *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) mutant lacking the *egt* gene developed normally (O'Reilly and Miller, 1989). In this case a considerable enhancement of the speed of kill was noticed but the yield of polyhedra was reduced (O'Reilly and Miller, 1991; Eldridge *et al.*, 1992). The mechanism for the accelerated mortality is not understood. One explanation might be that the physiological demands associated with molting are not well compensated in virus infected insects (O'Reilly, 1995). In this paper we provide an pathophysiological explanation for the enhancement in the speed of kill exploiting marked *Ac*NPV recombinants.

METHODS

Virus and larvae

A marked *egt* deletion mutant (*Ac*NPV/RM1) and a marked *Ac*NPV wild-type (*Ac*NPV/*ME53*) were constructed to study the biological effect of the *egt* deletion in insect larvae (Fig. 1). The E2 variant of *Ac*NPV (Smith and Summers, 1978) was used as control wild type virus. Both mutants contain the

E. coli Lac-Z reporter gene needed to follow the infection in the insect (Flipsen *et al.*, 1995a). *Ac*NPV/RM1 was used as the *egt*-deletion mutant, and *Ac*NPV/*ME53* as the marked control (Fig. 1). For the construction of recombinant *Ac*NPV/RM1 the *Ac*NPV-*Pst*I-G fragment containing the entire sequence of the *egt* gene (O'Reilly and Miller, 1989; Ayres *et al.*, 1994) was cloned in pTZ18U (Pharmacia). In the promoter region an *Xba*I, site was introduced by site-directed mutagenesis (Kunkel, 1985). The presence of a *Xba*I site at the 3' end of the *egt* coding sequence facilitated the deletion of the major part of the gene (1.4 kb). The residual *Xba*I sites were made blunt and a blunted 3.4 kb *Pst*I-*Bam*HI fragment, containing the *Lac-Z* reporter gene and the SV40 terminator excised from pAcDZ1 (Zuidema *et al.*, 1990; O'Reilly *et al.*, 1992), was inserted between the *Xba*I sites (Fig. 1). When tested for *egt* activity in infected cells, *Ac*NPV/RM1 was negative (personal communication, dr. D.R. O'Reilly). The second recombinant (*Ac*NPV/*ME53*) contained the same *Lac-Z*-SV40 cassette under the control of the *Ac*NPV-*ME53* promoter (Knebel-Mörsdorf *et al.*, 1993) was inserted upstream of the polyhedrin gene (Fig. 1). This marked *Ac*NPV wild type served as a positive control to follow the infection in insect larvae. Standard cloning and recombinant baculovirus procedures were used (Sambrook *et al.*, 1989; King and Possee, 1992).

Polyhedra were obtained as described by Flipsen *et al.* (1995). *S. exigua* larvae were taken from a continuous laboratory culture as described previously (Flipsen *et al.*, 1993) and infected as described by Flipsen *et al.* (1995). For enzyme histochemical studies, second and fourth instar *S. exigua* larvae were infected with *Ac*NPV/*ME53* or *Ac*NPV/RM1 and sampled at 6 hour intervals from 36 h post inoculation (p.i.) until 96 h p.i. After dissection of larvae, fluorescein di-acetate was applied as a vital stain (Flipsen *et al.*, 1995b). Subsequently the specimens were fixed and processed for reporter enzyme detection to monitor viral infection (Flipsen *et al.*, 1995).

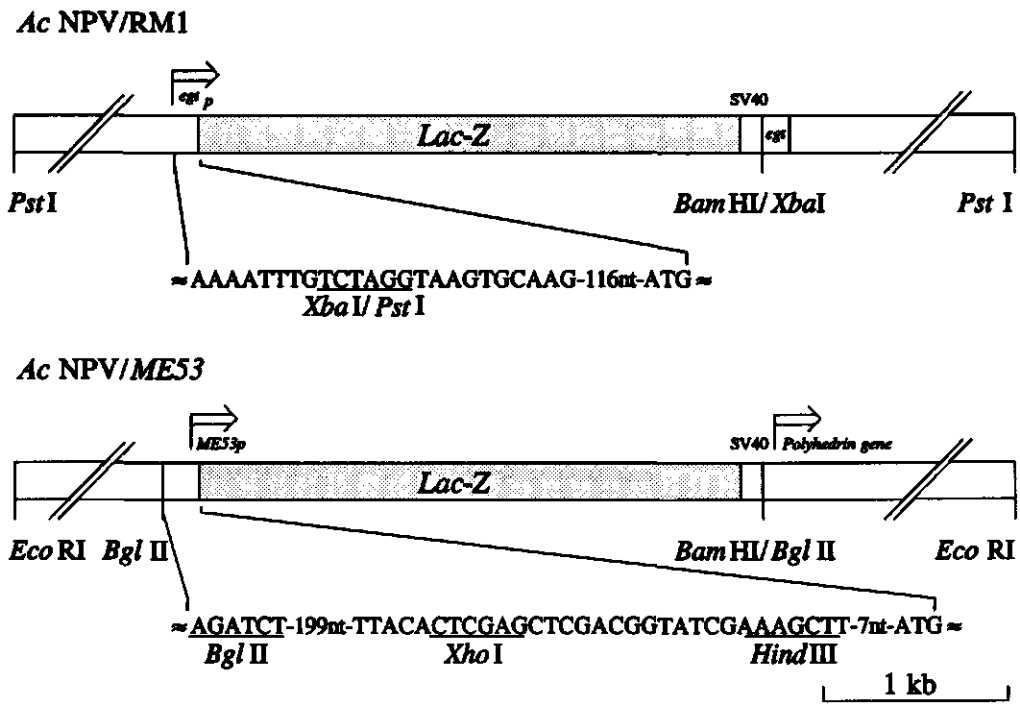

Figure 1: Schematic representation of the location of the *Lac-Z* reporter gene in recombinants *Ac*NPV/RM1 (*egt*-minus) and *Ac*NPV/*ME53* (control). In *Ac*NPV/RM1 the *Lac-Z* gene followed by the SV40 transcriptional termination signal was inserted in the *egt* promoter region. In recombinant *Ac*NPV/*ME53* a cassette containing the *ME53* promoter (6) and the *Lac-Z*-SV40 construct was inserted upstream of the polyhedrin gene (*Eco*RV site) in recombinant *Ac*NPV/*ME53*. The *egt* (egt_p) and *ME53* ($ME53_p$) promoters and the direction of transcription are indicated.

RESULTS AND DISCUSSION

When second instar *S. exigua* larvae were orally infected (Flipsen *et al.*, 1995) with wild-type *Ac*NPV or *Ac*NPV/RM1 (LC_{100}; 10^7 polyhedra/ml), we observed that, unlike with penultimate or final instar *S. frugiperda* (O'Reilly and Miller, 1989) larvae, there was little or no difference in the molting patterns of mock infected, wild type *Ac*NPV or *Ac*NPV/RM1 infected second instars (Fig. 2). Nevertheless, the *Ac*NPV/RM1 virus killed considerably faster than wild-type *Ac*NPV (Fig. 2) similar to the effect reported for fourth instar *S. frugiperda* larvae (O'Reilly and Miller, 1989). This

observation prompted us to investigate the histopathology of baculovirus infection of second and fourth instar *S. exigua* larvae using *Ac*NPV/RM1 and *Ac*NPV/*ME53* as reporters for infection.

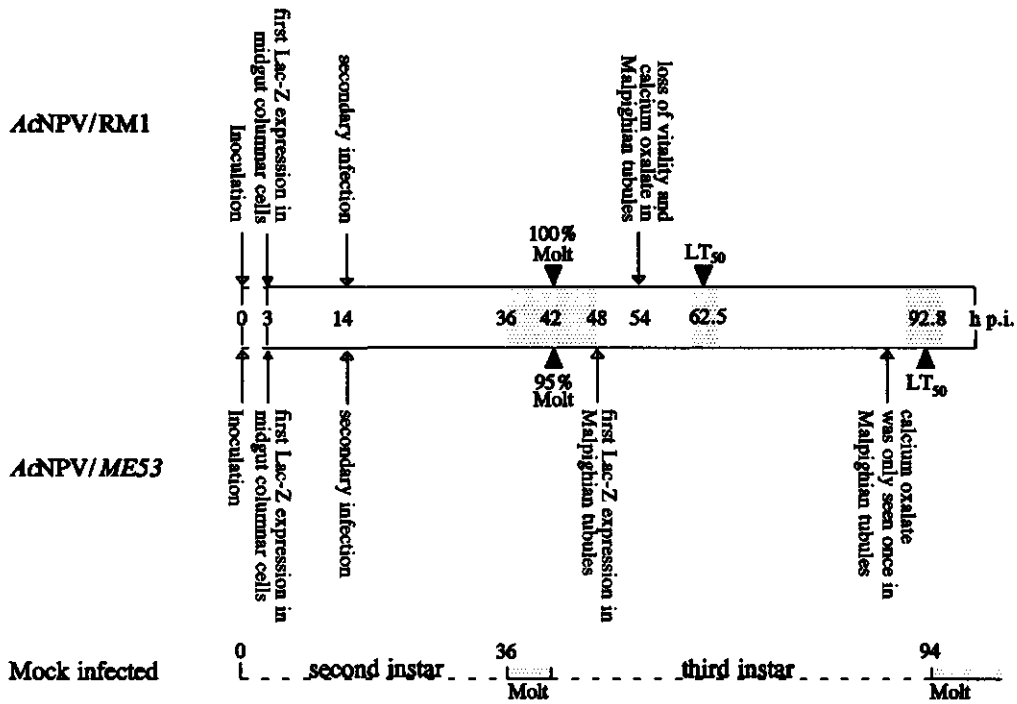

Figure 2: Schematic presentation of developmental and pathological events in second instar *S. exigua* larvae after infection with either *Ac*NPV/*ME53* (control), *Ac*NPV/RM1 (*egt*-minus) with a LC_{100} of 10^7 polyhedra per ml, or mock infected. The grey area represents the time interval in which larvae molted or died (95% significance limits of the LT_{50} assay).

After infection of early second instar *S. exigua* larvae (n > 100) with *Ac*NPV/*ME53* and *Ac*NPV/RM1) these insects molted to third instar and resumed feeding between 36-48 h p.i. From 54 h p.i. onwards *Ac*NPV/RM1 infected larvae showed extensive degeneration of the Malpighian tubules as evidenced by absence of vital stain in these tubules and the presence of severe precipitates, presumably calcium oxalate crystals, throughout the entire length of the Malpighian tubule (Fig. 3a).

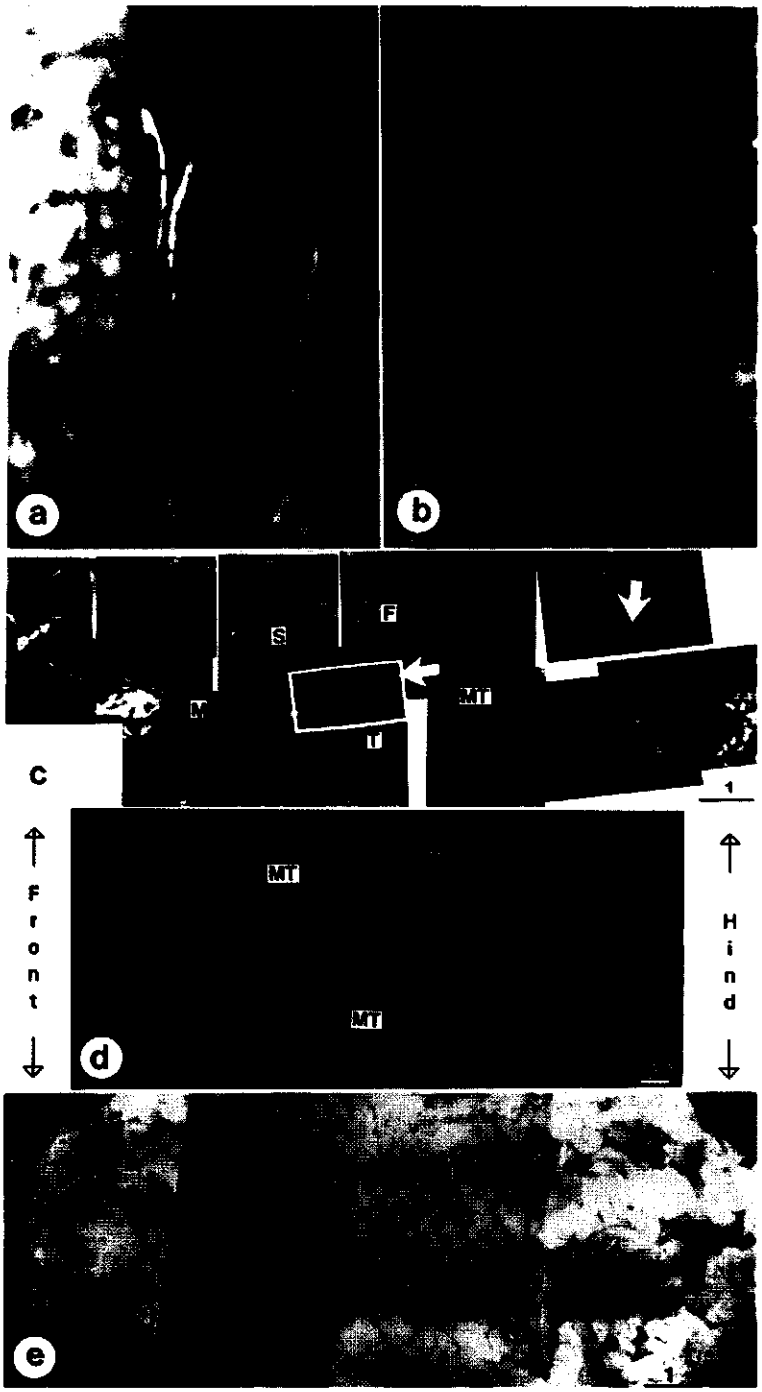
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Figure 3: Enzyme histochemical demonstration of *Lac-Z* expression (red) in the interior organs of a second instar larva of *S. exigua* at 48 h p.i. with (10^6 polyhedra per ml) *Ac*NPV/RM1 (*egt*-minus) (a) and *Ac*NPV/*ME53* (control) (b). Note the absence of staining in the Malpighian tubules (MT) and the presence of presumptive calcium oxalate crystals (arrowhead) in a. Vital stain of the interior of a *Ac*NPV/RM1 infected fourth instar larva (60 h p.i.) showing aggregation of hematocytes (H) on partly degenerated Malpighian tubules (arrow) (c and d). *Lac-Z* expression (arrowhead) in the same larva as presented in c (e). midgut = M; hind gut = HG; epidermis = E; fat body = F; salivary gland = S; tracheae = T. Size bars in a, b and d are in μm and in panel c and e in mm.

Lac-Z expression in the Malpighian tubules of *Ac*NPV/RM1 infected larvae was absent due to the degeneration of these tubules. In contrast, *Ac*NPV/*ME53* infected or mock infected larvae showed normal Malpighian tubules with vital stain and without any precipitate even when larval tissues started to disintegrate (92 h p.i.; n=20) . In Malpighian tubules of *Ac*NPV/*ME53* infected larvae *Lac-Z* expression was observed from 48 h p.i. onwards (Fig. 3b). However, no further pathological changes such as nuclear hypertrophy or polyhedra formation occurred in the tubules after *Ac*NPV/*ME53* infection. It was thus concluded that the absence of the *Ac*NPV-*egt* gene affects the functioning of the Malpighian tubules.

When fourth (penultimate) instar *S. exigua* larvae were infected with *Ac*NPV/RM1 at a low dose, killing only 50% of the larvae, partial degeneration of the Malpighian tubules was apparent from 60 h p.i. onwards as evidenced by the absence of vital stain (Fig. 3c). In this case hematocytes were attached on degenerated sections of the Malpighian tubules suggesting a cellular immune response (Fig. 3d). In these fourth instar larvae infection at this time was restricted to midgut associated tissues (Fig. 3e) and to tracheoblasts and hematocytes scattered throughout the body cavity (data not shown) as marked by *Lac-Z* expression. At this time mock infected and *Ac*NPV/*ME53*-infected insects scattering of the infected hematocytes was not observed. Attachment of multiple hematocytes to the Malpighian tubules or degeneration of these tubules was not observed throughout the experiment in these control larvae. This result supports the view that degeneration of the Malpighian tubules may be responsible for the increased speed of kill in both early and late instar larvae.

Histopathological data presented here suggest a correlation between the absence of the viral-*egt* and early degeneration of Malpighian tubules of *S. exigua* larvae. These organs function as an excretory system in insects analogous to the kidneys of higher organisms. Tubule degeneration, hence malfunction at an early stage are most likely the principle cause of the increased speed of kill of

baculovirus *egt* deletion mutants. Degeneration is not a direct effect of viral infection of the Malpighian tubules in second instar larvae.

Infection of fourth instar larvae with *Ac*NPV/*ME53* did not even reveal any *Lac-Z* expression in the Malpighian tubules at the time of degeneration in the *Ac*NPV/RM1 infected larvae. This provides further evidence that this degeneration of is not correlated to infection of these tubules. In the presence of viral *egt* the Malpighian tubules seem to be somehow protected against early degeneration much to the advantage of the larval life span during infection and for the benefit of progeny virus production. It is conceivable that relative high levels of, or prolonged exposure to, ecdysteroids in larvae infected with *egt* deletion mutants possibly in combination with a hitherto unknown viral factor or host responses to the viral infection initiate the degeneration of the Malpighian tubules.

It is equally possible that the spectrum of ecdysteroids in baculovirus *egt* deletion mutants under these conditions is altered. Ecdysteroids are known to induce a variety of responses in different tissues (Riddiford, 1985; Koolman, 1990), including in the regulation of fluid secretion during differentiation of the Malpighian tubules at metamorphosis (Ryerse, 1978). Fluctuating ecdysteroid titers could be a signal for apoptosis (Robinow *et al.*, 1993). Alternatively, the fluctuation in ecdysteroid titers may activate hematocytes, resulting in a hypersensitive reaction leading to degeneration of Malpighian tubules. The degeneration of a vital organ, such as the Malpighian tubules, must have a major effect on the behavior of the insect larvae. Histopathological studies as described in this paper at early times p.i. in combination with a detailed qualitative and quantitative analysis of the ecdysteroids present or produced in insects infected with *egt* deletion mutants could shed additional light on the mode of action of *egt* on the pathophysiological level.

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CHAPTER SEVEN

Summary and Concluding Remarks

Infection of insect larvae by a baculovirus leads to cessation of feeding and finally to the death of the larva. Under optimal conditions this process may take as little as five days during which the virus multiplies approximately a billion times and transforms 30% of the larval weight into viral products. The key question addressed in this thesis is how virus infection spreads in the insect larvae and how the various tissues and organs respond to infection. The answer to these questions may explain the rapid insect pathogenesis induced by baculoviruses and may provide new leads for the specific engineering of the virus to convert it into an even more efficacious insecticide. In order to obtain such information, a unique explorer system, consisting of a recombinant *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) containing two reporter genes, was designed and used in combination with enzyme-histochemical techniques to record baculovirus infection *in situ* (Chapters 2, 4 and 5). A Lac-Z gene placed behind the constitutive *D. melanogaster* heat shock 70 promoter, is expressed prior to and independently of the viral replication and can thus report the successful entry of virus into cells and tissues. The GUS-gene, placed behind the late viral p10-promoter is only expressed in cells in which virus replication has occurred. This system provided the unique opportunity to distinguish early and late viral infection processes simultaneously in whole insect larvae. Using this baculovirus explorer, the pathway and pathogenesis of *Ac*NPV infection in larvae of the beet army worm (*Spodoptera exigua*) is investigated and described in this thesis.

After ingestion polyhedra dissolve in the alkaline environment of the larval midgut whereby the rod-shaped virus particles are released into the midgut lumen and pass through the peritrophic membrane. The virus particles bind to the microvillar membrane of columnar cells in a receptor-mediated manner (Hortan and Burand, 1993). The viral envelope fuses with this membrane and the nucleocapsids are released into a midgut epithelial cell. The midgut columnar cells and, at low frequency, the underlaying midgut regenerative cells are the primary targets of *Ac*NPV infection (Chapter 2). Some of the parental nucleocapsids are transported directly through the columnar cells and infect nearby regenerative cells. Infected regenerative cells were only found underlaying infected columnar cells. Polyhedra morphogenesis took place in midgut columnar and regenerative cells. It was remarkable that the polyhedra formed in this epithelium were smaller than those formed in other tissues such as the fat body, and that they were often devoid of virus particles. The expression of very late viral genes in this primarily infected tissue may be of interest in the design of engineered virus that can express a gut-specific toxin or protease-inhibitor.

Invasion and infection of tissues other than the midgut epithelium was only recorded after the onset of virus replication in midgut epithelial cells. So, virus multiplication in the midgut cells is an essential prelude for secondary and further systemic infection. Our observations do not support the view that the direct passage of parental virus through the midgut basal lamina, as observed by Granados and Lawler (1981), is a biologically significant route for primary infection of underlying tissues. These authors reported that *Ac*NPV infection of hematocytes in the hemolymph of *Trichoplusia ni* larvae can be established by parental virus passing directly from the midgut lumen through the epithelium, basal lamina, and associated tissues into the hemocoel. Their conclusion was based on the detection of free (infectious) virions in extracts of the hemolymph at 0.5-2 h p.i., but not supported by evidence for an actual *in vivo* infection of other tissues. *In vivo* infection by viruses that directly passed through the midgut epithelium is not likely to occur as these virions lack the characteristics and surface projections of ECVs, including gp64, which are needed to establish secondary infections.

In infected midgut columnar and regenerative cells the full complement of late viral structures (polyhedra and fibrillary structures) was observed depending on the larval stage and the age of the midgut epithelium (Chapter 3). Regeneration of the midgut epithelium of early instar larvae is part of the molting process. As part of this process virus infected cells may be rejected into the gut lumen. During molting, virus infection is eliminated (almost) completely from the epithelium. Elimination of infected midgut cells is not the only way to exclude infection from the midgut epithelium. In *Se*NPV infected *S. exigua* larvae the columnar midgut epithelial cells degenerate after the virus has been transmitted over the midgut epithelium (data not shown). This type of response provides an alternative explanation why late viral structures are not always found in the midgut of nuclear polyhedrosis virus infected lepidopteran larvae.

The requirement of *Ac*NPV to multiply in the midgut epithelium prior to further infection of other larval tissues sets limits to the speed of action that can be achieved by recombinant viruses designed for more effective insect control. Alternatively, the promoters of the very late viral genes, which are expressed in the midgut epithelium, may be used for expression of hormones and toxins in the early phase of larval infection, thus achieving an enhanced speed of kill if successfully targeted for the underlying tissues.

Viral replication in gut epithelial cells is also an important prerequisite to systemic infection of other animal viruses, such as vesicular stomatitis virus, certain retroviruses, and some adenoviruses (Tyler and Fields, 1990). After infection with these viruses, primary infection and the first round of replication takes place in the gut epithelial cells, whereafter progeny virus buds from the cell at the basal site to infect the cells associated with the basal lamina (submucosa). Cells of the submucosa subsequently multiply the virus before it is transported to other tissues. Similarly, after primary infection of the midgut epithelium, secondary *Ac*NPV infection of *S. exigua* larvae is also established in cells of the submucosa, i.e. muscle cells, tracheoblasts, and hematocytes (Chapter 2). These secondarily infected cells, associated with the basal lamina, multiply the virus and release vast amounts of particles into the hemocoel amplifying the virus titer in the hemolymph for further systemic infection (Chapter 4).

Systemic *Ac*NPV infection of other larval tissues is established by virus circulating in the hemolymph. Tracheoblasts of all tissues were the first targets for this circulating virus. From these prior infected cells infection radiated out into neighboring tissues. Larval tissues are physically separated from the hemolymph by a thick basal lamina, that forms an effective barrier to direct virus infection (Reddy and Locke, 1990). However, the basal lamina surrounding the tracheoblasts is very thin (or absent) and can apparently be easily penetrated by baculovirus ECVs. Therefore, by infection of tracheoblasts the virus circumvents the basal lamina and is able to establish further infections (Chapter 4).

A basal lamina is present around all tissues and plays an important role in preventing easy entry of the virus in tissues of *S. exigua* larvae. The composition of the basal lamina and the mechanisms of transport through this lamina offer possibilities to design more effective recombinant viruses. For instance, degradation of this membrane induced by recombinant viruses may enhance the infection and thus the rate kill by the virus. In principle, the feasibility of this approach was shown by the observation that the underlying tissues could be directly infected after treatment of the basal lamina with dispase (Chapter 4).

In the case of *Ac*NPV infected *T. ni* larvae, an alternative route for a fast systemic infection was proposed by Engelhard *et al.* (1994). This route involved transport of virus particles through the intercellular space of the tracheal epidermis. In *Ac*NPV infected *S. exigua* larvae, we observed that

the infection of distal tracheal elements progressed only in a cell-to-cell manner, starting in the tracheoblasts nested in the midgut and in other tissues (Chapter 4). The dimensional proportion of the intercellular space of tracheal elements does not favor long distance transport. Therefore effective systemic infection through the tracheal system is unlikely.

The mode of virus transport proposed by Keddie *et al.* (1989) whereby hematocytes infected at the midgut basal lamina near the primary site of infection carry the infection into other tissues, lacks experimental evidence from the *Ac*NPV-*S. exigua* system. We were unable to find a relation between the infection and replication of the marked *Ac*NPV recombinants in hematocytes and the infection of tissues in contact with these cells (Chapter 4). This observation suggests that infection was not transmitted by hematocytes in a cell-to-cell manner.

It has been shown for flavivirus, measlesvirus, and poliovirus that circulation of virus particles in the blood contributes to the spread of infection to different tissues of vertebrates (Tyler and Fields, 1990). Replication of viruses in hematocytes increases the virulence of these viruses (Tyler and Fields, 1990). In insects the hematocytes may boost the levels of ECVs at later times post infection.

Clem *et al.* (1994) described the infection of *p35* (apoptosis blocking gene) deletion mutants which also lacked the *p94* gene. Differences in infectivity were observed between orally infected larvae which were killed by both wt-*Ac*NPV and the dual deletion mutant, and virus injected larvae which were not killed. These authors suggested that tissue specific apoptosis may be responsible for this difference. This can indeed be the case as infection by either route needs different tissues to support the primary round of virus replication (Chapter 4). In orally infected larvae the virus replicates first in the midgut epithelium and then in cells associated with the midgut basal lamina, whereas primary infection of virus injected larvae is predominantly established in the hematocytes.

Although the majority of larval tissues, such as midgut columnar and regenerative cells, fat body, tracheal cells, hematocytes, and epidermis, supported full replication of the *Ac*NPV, this was not the case in some vital tissues such as midgut goblet cells, salivary glands, and Malpighian tubules (Chapter 5). In these tissues early viral gene expression was observed but this was not followed by late viral gene expression. Using immunogold labeling, to detect late viral structural proteins, revealed these proteins only rarely in midgut goblet cells and never in Malpighian tubules and salivary glands.

The apparent tissue specificity of *Ac*NPV infection *in vivo* is not regulated at the level of receptor binding, virus entry, or uncoating of the genome per se, as successful entry of the virus and initial transcription of the genome did take place in these cells as demonstrated by using *Ac*NPV/HSP-p10. The failure to establish full infection in these tissues must therefore either be regulated at the level of transcription and translation of viral genes or at the level of interaction of viral gene products with host factors. This type of tissue specificity is a rare phenomenon as tissue specificity of viruses is normally thought to be determined by host cell receptors (Tyler and Fields, 1990; Tyler, 1994).

Tissue specificity regulated by selective expression or interaction of viral and host proteins is reported for adenoviruses (Doerfler, 1994) and herpes simplex viruses (Aurelian, 1994). Adenoviruses enter cells, initiate early gene expression, and replicate normally in permissive cells. Ad12 infection of BHK21-cells is abortive. Early genes of Ad12 are expressed in BHK21-cells but viral replication and subsequent late gene expression do not occur. This defect can partially be complemented by expression of the Ad5-E1 region in these cells. The Ad12 will then replicate and transcribe its late genes, but synthesis of the late viral protein (and hence progeny virus) does not occur (Schiedner *et al.*, 1994). So in this case there is a transcriptional and translational control of specificity. In the case of herpes viruses the latent or non-reproductive infection is determined by a viral latency factor and/or the expression of the immediate early gene IE110, the thymidine kinase gene, and the ribonucleotide reductase gene (Aurelian, 1994).

The mechanism determining the tissue specificity of *Ac*NPV in *S. exigua* larvae may be of a similar nature. Expression of <u>one</u> of the *Ac*NPV immediate early genes alone may determine this tissue specificity. The *Ac*NPV-*PE38* and -*ME53* immediate early genes do not seem to be involved in this process (Chapter 5). Sequences that regulate the expression of these genes are found in most immediate early *Ac*NPV promoters and expression patterns homologous to that of *Ac*NPV pe38 and ME53 may be expected. Thus it is unlikely that *Ac*NPV specificity is determined through the expression of immediate early genes by host cell factors.

Having obtained information on the infection pathway and the tissues involved, effects of the deletion of viral genes can be studied *in vivo*. In Chapter 6 the effect of deletion of the *egt* gene from the *Ac*NPV genome was studied. In larvae infected with this recombinant, Malpighian tubules were found to degenerate at an early stage in the infection. This early degeneration most probably causes the

increased speed of kill of *S. exigua* larvae by the *Ac*NPV *egt* deletion mutant. As this degeneration was not observed in uninfected or wild type *Ac*NPV infected larvae, it can be concluded that, during viral infection, ecdysteroid hormones play a role in the degeneration of vital tissues such as the Malpighian tubules. This early degeneration caused by the infection with *egt*-deletion mutants is dictated by the level of ecdysteroid hormones in combination with other hormones, by the viral infection, or by the activation of hematocytes by ecdysteroid hormones (Vinson, 1994). This results in a hypersensitive response.

Design of a baculovirus containing a dual reporter gene system that allows identification of early and late stages of viral infection allowed us to follow baculovirus infection in the whole insect. Such a marked recombinant also allowed the investigation of tissue specificity and the study of the effect of gene deletions with only very little distortion of the insect. Using this recombinant virus, the most likely pathway of infection of *Ac*NPV in *S. exigua* was determined. The virus enters the insect via the midgut columnar cells. At a low frequency parental nucleocapsids can be transported to midgut regenerative cells. The necessity of infection of columnar cells, through which the infecting nucleocapsids are transported, that overlay these regenerative cells is not known. Parental virus does not establish further infection in the insect. Secondary (i.e. systemic) infection occurs only after the virus has replicated in the midgut epithelium. Secondary infection is first established in cells closely associated with the midgut basal lamina close to the primarily infected loci. The secondarily infected cells are muscle cells, tracheoblasts, and hematocytes. How the virus passes the basal lamina is unclear, but it is possible that the infected epithelial cells are not able to maintain this layer. The virus produced in the midgut epithelium starts to circulate in the hemolymph after which infection of other tissues is established. As the virus replicates in, and is released from, the cells associated with the basal lamina, the virus titer of the hemolymph will be enhanced. The circulating virus directly infects the tracheoblasts nested in all tissues. The basal lamina surrounding these cells is thin or absent and does not form a physical barrier for virus passage. In other areas of the tissue this membrane is too thick to be penetrated by virus particles. Transmission of infection by infected hematocytes in a cell-to-cell mechanism does not occur. These infected hematocytes do not pass the basal lamina nor transmit the virus through this membrane. From infected tracheoblasts the infection quickly radiates out into the tissues by cell-to-cell translocation following replication. Infection is not restricted to tissues that support viral replication and subsequent polyhedra morphogenesis, such as fat body, tracheolar cells, epidermis, and hematocytes. ECVs will also enter and initiate early viral gene

expression in apparently non-permissive, vital tissues, such as salivary glands and Malpighian tubules. These vital tissues further remain unaffected by the virus. This allows the infected insect to function, resulting in a high production of progeny virus and polyhedra in the insect.

The information obtained on the pathogenesis induced by *Ac*NPV and the developed reporter virus enables us to evaluate the risks of the use of genetically modified baculoviruses. For example, it allows the study of infection in semi-susceptible and apparently non-susceptible insects and may reveal changes of host range by the virus or potential risks for non-target insects. The study of the effect of *egt* deletion illustrates the further potential the use of these genetically marked baculoviruses to understand the pathogenesis of baculovirus infections in insects.

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CURRICULUM VITAE

Op 18 april 1963 werd Johannes Theodorus Maria Flipsen te Oud- en Nieuw-Gastel geboren. Ze noemden hem Hans. In 1980 behaalde hij een MAVO diploma dat hem toegang verschafte tot het voorbereidend jaar hoger beroepsonderwijs van het toenmalige Dr. Struycken Instituut te Etten-Leur. Na de succesvolle afronding van dit jaar vervolgde hij aan hetzelfde instituut de hogere beroepsopleiding (HLO) tot histo- en cytologisch annalist. Hij rondde deze opleiding in 1985 af met een stage en afstudeeropdracht getiteld Colloïdaal Thorium(IV)hydroxide een Marker voor Elektronenmicroscopie bij het Instituut voor Pathologie van de Erasmus Universiteit te Rotterdam onder de begeleiding van dr. W.C. de Bruijn van de afdeling Elektronenmicroscopie. Zijn grote honger naar kennis zette hem aan tot een vervolgstudie biologie aan de Rijksuniversiteit te Leiden. Hier koos hij de biochemische/moleculair-biologische differentiatie. Hij sloot zijn studie biologie uiteindelijk af met de afstudeeropdracht Detectie van de Genen voor Cyclin/PCNA en Nucleoplasmin in Gist door middel van Hybridisatie met Heterologe Probes bij de vakgroep Genetica van de Lagere Eukaryoten onder begeleiding van dr. B. Zonneveld. Hierna volgde hij een korte stage aan het Laboratorium voor Bloembollen Onderzoek te Lisse, waar hij werkte aan de Histologische Studie van de Infectie van Gladiolekralen door *Fusarium oxysporum* f.sp. Gladioli onder begeleiding van drs. E. Roebroek. In november 1989 kreeg hij zijn doctorandustitel. Hierna was hij voor enige tijd werkzaam als annalist op het Sylvius Laboratorium te Leiden. In mei 1990 werd hij aangesteld als onderzoeker in opleiding op een door de Nederlandse Organisatie voor Wetenschappelijke Onderzoek (NWO) gefinancierd project bij de vakgroep Virologie van de Landbouwuniversiteit te Wageningen. Hier werkte hij onder leiding van dr. ir. Jan van Lent, dr. Just Vlak en prof. dr. Rob Goldbach aan het onderzoek dat resulteerde in de totstandkoming van dit proefschrift.