ON THE ORIGIN OF THE POLYHEDRAL PROTEIN OF THE NUCLEAR POLYHEDROSIS VIRUS OF *AUTOGRAPHA CALIFORNICA*

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(*met een samenvatting in het Nederlands*)

Proefschrift
ter verkrijging van de graad
van doctor in de landbouwwetenschappen,
op gezag van de rector magnificus
dr. H. C. van der Plas,
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in het openbaar te verdedigen
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STELLINGEN

1.

De genetische code voor het matrix-eiwit van de polyeders, die in de kernen van gekweekte *Spodoptera frugiperda* cellen worden geproduceerd na infectie met het kernpolyedervirus van *Autographa californica*, is vervat in het DNA genoom van dat virus.

Dit proefschrift.

2.

Het is onjuist om te beweren dat resistentie tegen chromaat in *Cephalosporium* zou resulteren in een verhoogde sulfaatopname.

LEMKE, P. A. en BRANNON, D. R. (1972). In: Cephalosporins and Penicillins. Chemistry and Biology (E. H. Flynn ed.). Academic Press, New York; blz. 402.

3.

Een verandering van polyedervorm, als gevolg van de overdracht van een insektevirus naar een andere gastheer, kan slechts dan aan gastheerinvloeden worden toegeschreven, indien wordt uitgesloten dat als gevolg van de infectie niet een ander, latent aanwezig virus wordt geactiveerd.

HUNTER, D. K., HOFFMANN, D. F. en COLLIER, S. J. (1973). J. Invertebr. Pathol. 22: 186–192.
QUIOT, J. M. en BELLONCIK, S. (1977). Arch. Virol. 55: 145–153.
JURKOVIČOVÁ, M. (1979). Proefschrift Landbouwhogeschool Wageningen.

4.

De bedenkingen van Van Vloten-Doting et al. tegen de conclusie van De Jager en Breekland, dat tussen de CPMV-mutanten N123 en N142 complementering binnen de M-component optreedt, gelden evenzeer ten aanzien van hun eigen resultaten met temperatuurgevoelige mutanten van alfalfa mosaic virus.

VAN VLOTEN-DOTING, L. et al. (1980). J. Virol. **46**: 415–426.
DE JAGER, C. P. en BREEKLAND, L. (1979). Virology **99**: 312–318.

5.

Het aantonen van replicatie van het kernpolyedervirus van *Autographa californica* in *Aedes aegypti* cellen, dient vergezeld te gaan van controle experimenten.

SHERMAN, K. E. en MCINTOSH, A. H. (1979). Infect. Immun. **26**: 232–234.

6.

Gesteund door serologisch en/of biochemisch onderzoek zou de veronderstelling van Samyn en Welvaert, dat een door hen in *Chamaecereus sylvestrii* '*aureus*' gevonden bolvormig virus verwant of identiek zou zijn aan het Saguaro cactus virus, duidelijk aan kracht winnen.

SAMYN, G. en WELVAERT W. (1978). Phytopath. Z. **91**: 276–279.

7.

Polyeders van voor insektebestrijding vrijgegeven insektevirussen zouden niet alleen van buiten maar ook van binnen op de aanwezigheid van contaminerende virussen gecontroleerd moeten worden.

HESS, R. T., SUMMERS, M. D. en FALCON, L. A. (1978). J. Ultrastr. Res. **65**: 253–265.
SCOTTI, P. D. en LONGWORTH, J. F. (1978). J. Invertebr. Pathol. **32**: 216–218.

8.

Men dient zich in het algemeen te onthouden van onverwachte en ongevraagde, met lichamelijk contact gepaard gaande hulp aan zich zelfstandig in het verkeer bewegende blinde mensen.

9.

Het verdient aanbeveling om de aanduidingen 'singly embedded' en 'multiply embedded', welke thans in de insektevirologie gebruikt worden om aan te geven of de nucleocapsiden van een kernpolyedervirus in een polyeder alleen, dan wel in groepjes door een membraan zijn omgeven, te vervangen door de termen 'singly enveloped' resp. 'multiply enveloped'.

10.

De drijvende objecten, welke ten behoeve van een nieuwe tak van watersport in de haven van Rotterdam zullen worden gesitueerd, dienen bij voorkeur zodanig te worden afgemeerd dat 's avonds de rode bakboordverlichting naar de walzijde is gericht.

C. P. VAN DER BEEK
Wageningen, 28 mei 1980

CONTENTS

ABBREVIATIONS AND SYMBOLS

AcNPV	*Autographa californica* nuclear polyhedrosis virus
ATP	Adenosine-5′-triphosphate
BamHI	Restriction endonuclease from *Bacillus amyloliquefaciens* H
BML/TC 10	Growth medium for *Spodoptera frugiperda* cells
°C	Degree Celcius
CCMV	Cowpea chlorotic mottle virus
5′-C^{m}CGG-3′ sequence	DNA sequence containing one methylated cytidine residue
Ci	Curie (= 3.7 × 10^{10} desintegrations per second)
CMC	Carboxymethylcellulose
CP-1268	Cell line from *Carpocapsa pomonella*
cpm	Counts per minute
CPV	Cytoplasmic polyhedrosis virus
dAMP	Deoxyadenosine-5′-monophosphate
dCMP	Deoxycytosine-5′-monophosphate
DEAE-	Diethylaminoethyl-
dGMP	Deoxyguanosine-5′-monophosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dpm	Desintegrations per minute
dTMP	Deoxythymidine-5′-monophosphate
EcoRI	Restriction endonuclease from *Escherichia coli* BS 5
EDTA	Ethylenediaminetetraacetic acid
EPV	Entomopox virus
Fig.	Figure
FP	Few polyhedra
g	Force of gravity
GC-percentage	Mol percentage guanosine plus cytosine
GTP	Guanosine-5′-triphosphate
hr(s)	Hour(s)
^{3}H	Tritium
HEPES	2-[4-(2-hydroxyethyl)-1-piperazynyl] enthane sulfonic acid
Hpa II	Restriction endonuclease from *Haemophilus parainfluenzae*
Ig	Immunoglobulin
IPLB-SF-21AE	Cell line from *Spodoptera frugiperda*
ME	Multiply enveloped
min	Minutes
MP	Many polyhedra

mRNA	Messenger ribonucleic acid
Msp I	Restriction endonuclease from a *Moraxella* species
MW	Molecular weight
NP40	Nonidet P40
NPV	Nuclear polyhedrosis virus
OD_{260}	Optical density at 260 nm
Oligo(dT)	Oligodeoxythymidilic acid
PBS	Phosphate buffered saline
PBS-TDS	PBS containing 1% Triton-X-100, 0.5% deoxycholate and 0.1% SDS
poly(A)	Polyadenylic acid
$poly(A)^+$	Poly(A) containing
$poly(A)^-$	Poly(A) lacking
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
S	Sedimentation coefficient in Svedberg units
S_{30}	Supernatant of a 30,000 *g* centrifugation
SDS	Sodium dodecyl sulphate
SE	Singly enveloped
SW	Swinging bucket
T1-RNase	RNase from *Aspergillus oryzae*
TCA	Trichloroacetic acid
$TCID_{50}$	Tissue culture infective dose 50%
Tm	Temperature at which 50% of a double stranded DNA is melted
Tris	Tris (hydroxymethyl) aminomethane
V^-	Property of poxviruses which are not occluded in A-type inclusions
V^+	Property of poxviruses which are occluded in A-type inclusions.

1. INTRODUCTION AND REVIEW OF THE LITERATURE

1.1. General introduction

An increasing resistance of insects against chemical pesticides has necessitated the development of alternative methods to control insect pests. The use of insect viruses (alone or within the frame-work of an integrated pest control programme) is one of those alternatives (356). Members of the family Baculoviridae (nuclear polyhedrosis viruses and granulosis viruses) seem to be most suitable for application because of their high specificity and safety (307), and because of the fact that until now no members of this virus family have been known in vertebrate animals or man.

Large amounts of these viruses are occluded into protein crystals (which are called granula or polyhedra, depending on whether one or many virus particles are occluded in a protein crystal) during their replication cycle in the nuclei of the insect host cells. Because of this property, the virus particles are protected against external influences once they have been liberated into the environment after the death of the insect host. As a consequence they are rather persistent, which decreases the need for repeated applications. An additional advantage of inclusion bodies is that they are easily purified, and stored for a long time without loss of infectivity.

However, because of several arguments, which will be discussed in section 1.3.8. of this chapter, investigators have wondered whether the inclusion body protein is coded for by the virus or by the insect host. When the occlusion body protein would prove to be coded for by the insect host, it would implicate the discovery of a hitherto unknown defence mechanism of insects against viruses. In this thesis experiments are described which prove that the origin of the occlusion body protein of the nuclear polyhedrosis virus of *Autographa californica* lies in the viral genome.

1.2. Properties of the virus

The nuclear polyhedrosis virus of the alfalfa looper, *Autographa californica* (AcNPV), is one of the best characterized members of the Baculoviridae.

As recent studies have indicated that the multiply enveloped nuclear polyhedrosis virus of the cabbage looper *Trichoplusia ni* is a variant of AcNPV, this virus was also included in the description of the properties of the virus. From changes in the amount of conserved restriction enzyme fragments produced by six enzymes, both viruses can be calculated to have a mean sequence homology of 99.96% (281, 322).

1.2.1. *Infection cycle*

As with all nuclear polyhedrosis viruses the nucleocapsid of AcNPV is rod-shaped and surrounded by a lipid containing envelope. The nucleocapsid of AcNPV measures about 220 × 32 nm (252). In the nucleus, which is the site of replication of the virus[1], part of these nucleocapsids acquires an envelope by de novo synthesis. In the case of AcNPV, which is a multiply enveloped type of nuclear polyhedrosis virus, several nucleocapsids are contained within an envelope. Other nucleocapsids are released into the cytoplasm and acquire their envelope by budding through the cytoplasmic membrane (117, 128). These virus particles may infect neighbouring cells, the envelope probably being necessary for infectivity (75, 114). In this way the infection is spread inside the host. The virus particles which are enveloped in the nucleus are occluded in large protein crystals, measuring from 1.1 to 5.3 μm (87, 252, 352), which are called polyhedra. The polyhedra are responsible for the spread of the disease within the alfalfa looper population. When an animal dies, the polyhedra are liberated into the environment. When ingested by a healthy animal, the protein is dissolved in the gut by the alkaline conditions in that organ. The virus particles are liberated from the polyhedra, possibly with the aid of an alkaline protease, and may then infect the gut cells.

A simplified presentation of the replication cycle of the nuclear polyhedrosis virus of AcNPV is given in fig 1. Typical for AcNPV is the formation of large cuboidal inclusion bodies which seem to be located in the cytoplasm (324) and apparently do not contain virus particles (323). The function of these structures is still unknown but, as their formation is independent of the host system in which the virus is multiplied, they probably are induced by the virus.

1.2.2. *Host range and histopathology*

In general nuclear polyhedrosis viruses are quite specific. AcNPV is an exception to this rule. Its host range includes at least 28 insect species from different families (45, 46, 107, 160, 296, 323, 324, 325, 326, 329, 350).

Polyhedra are formed in the hypodermis, tracheal matrix and fat body cells of all hosts, and in the Malpighian tubules, muscle, hemocytes, ganglia, juvenile tissue (imaginal buds), testes, ventral nerve cord, hindgut and midgut of some hosts (323, 326, 350). In this latter respect AcNPV also differs from most nuclear polyhedrosis viruses which generally do not exhibit polyhedra formation in the gut cells, although the virus is multiplied in these cells.

1.2.3. *Multiplication in cell cultures*

AcNPV can be multiplied in several insect cell lines. Its replication has been demonstrated in cell lines of *Spodoptera littoralis* (120), *Porthetria dispar* (2), *Trichoplusia ni* (87, 203, 338), *Spodoptera frugiperda* (94, 327), *Mamestra brassicae* (211), *Amsacta moorei* (103), *Estigmene acrea* (102), *Carpocapsa pomo-*

[1] Two cases of cytoplasmic replication have been reported with nuclear polyhedrosis viruses (84, 284).

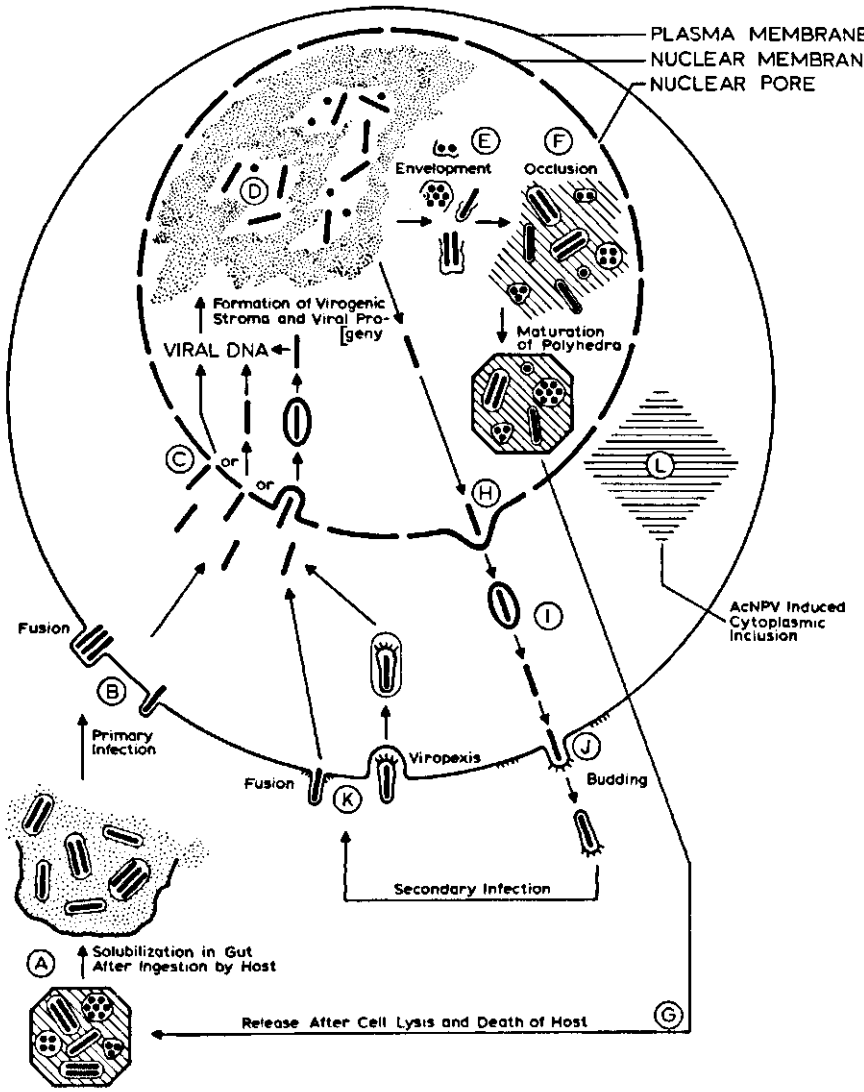

FIG. 1. Simplified infection cycle of AcNPV compiled from data presented in the references 2 and 128. In vivo the infection is initiated by virus particles from ingested polyhedra which are solubilized by the digestive juices from the gut (A). Infection occurs by fusion of the viral envelope with the plasma membrane of the cell (B). The process in which the nucleus is infected by the viral nucleocapsids is not yet clearly established in the case of AcNPV. Three possible processes have been indicated (C). Upon the release of viral DNA a virogenic stroma is formed and progeny viral nucleocapsids are synthesized within this virogenic stroma (D). Part of these viral nucleocapsids are enveloped in the nucleus (E) and subsequently occluded in polyhedral protein (F). Mature (= inclusive of the polyhedral 'membrane') polyhedra are released upon the death of the host (G). Other viral nucleocapsids are released into the cytoplasm by means of vesicles originating from the nuclear membrane (H). These vesicles rupture in the cytoplasm (I) and viral nucleocapsids from these vesicles acquire their envelope by budding through the plasma membrane on sites which have been provided with spikes (J). Virus particles which are released in this way can infect neighbouring cells (and tissue culture cells) by both viropexis and by fusion of the viral envelope with the plasma membrane of the cell (K). The induction of large cuboidal inclusions in the cytoplasm (L) is typical for AcNPV but not for other NPVs.

nella (312), and *Bombyx mori* (312). In *Bombyx mori* cells, however, no polyhedral protein is synthesized and hence virus particles are not occluded. When grown at 32.5 C in *Spodoptera frugiperda* cells, polyhedra are produced but no virus particles are occluded (185). AcNPV has also been shown to penetrate a vertebrate viper cell line (202, 204). A low level of replication in a cell line of *Aedes aegypti* has been claimed by SHERMAN and MCINTOSH (370). It is interesting to note that some of these cell lines are not obtained from natural AcNPV hosts.

The infection of tissue culture cells proceeds in the same way as the secondary in vivo infection depicted in fig. 1 (2, 128). Infections of cell cultures are mostly started with infectious hemolymph from diseased larvae because virus particles which are liberated from polyhedra by weak alkaline solutions are hardly infectious (340). Dissolution of polyhedra in a glycine buffer (351) or in digestive juices from the gut of healthy larvae (328), however, can yield virus which is infectious to cultured cells.

Several parameters of the viral replication in cell cultures have been studied (87, 120, 122, 128, 302, 324, 352). Optimal conditions for polyhedra production and plaque formation have been determined (40, 125, 167, 190, 300, 331, 338), and cheaper tissue culture media have been developed (94, 327).

In addition to the $TCID_{50}$[2] test, plaque assays using semi-solid (124, 126, 338) or solid (41, 42, 165, 353) overlays have been developed and optimized to quantify and clone the virus. A fluorescent dye technique, which is independent of polyhedra formation, is available to quantify the amount of infected cells early in the infection cycle or in cases where no polyhedra are synthesized (259).

Tissue culture derived polyhedra are as infectious as those produced in whole insects (87, 148, 324). Non-occluded virus has been purified from tissue culture fluids and characterized (114, 251, 280, 333, 339, 355). From comparative studies it seems that its properties are practically identical with those of non-occluded virus from whole insects (75, 311, 340).

The use of cultured cells for mass production of AcNPV and other baculoviruses is hampered by the fact that both the amount of polyhedra produced, and the number of virus particles occluded in those polyhedra, are greatly reduced upon prolonged passage of the virus in cultured cells, late passage polyhedra being much less infective than early passage polyhedra (128, 166, 191). For an explanation of this phenomenon see section 1.2.6.

1.2.4. *Nucleic acid*

AcNPV contains double stranded circular DNA. Its molecular weight has been determined by sedimentation (219, 334), electron microscopy (318) and by means of electrophoresis of restriction endonuclease fragments (209, 280, 281, 318, 334). Values between 76 and 104 $\times$ 10^6 dalton have been reported.

The guanine plus cytosine content of the DNA has been determined by quantitative analysis (52), from its buoyant density in CsCl (334) and from its Tm-value (219, 334), yielding GC-percentages from 41.8 to 47.0 %.

[2] $TCID_{50}$ = Tissue Culture Infective Dose 50 %.

The cleavage sites of several restriction endonucleases have recently been mapped on the AcNPV DNA genome (209, 281). From the absence of additional Msp I restriction endonuclease cleavage sites when compared to the amount of Hpa II restriction endonuclease cleavage sites, it can be concluded that no 5′-$C^{m}CGG$-3′ sequences are present (318).

The time course of AcNPV DNA synthesis has been studied in *Spodoptera frugiperda* cells (318) DNA synthesis starts at 5 hrs post infection and reaches a maximum at 18 hrs post infection.

1.2.5. *Proteins*

The structural proteins of both occluded (51, 197, 206, 280, 310) and non-occluded AcNPV (47, 280, 333) have been analysed by polyacrylamide gel electrophoresis. Nucleocapsids were also investigated (310).

Undegrated AcNPV polyhedral protein has a molecular weight of 28,000 to 33,000 dalton (47, 59, 199, 206, 309, 310, 377); its amino amino acid composition has been quantitatively analysed (50, 309), aspartic acid and glutamic acid being the most abundant amino acids.

The time course of AcNPV induced protein synthesis in infected cells has been investigated by both biochemical (47, 54, 162, 354, 377) and serological (312) methods. From the latter report it can also be concluded that AcNPV polyhedral protein is transported very rapidly from the cytoplasm to the nucleus as it was initially detected simultaneously in both the cytoplasm and the nucleus[3]. AcNPV polyhedral protein begins to be synthesized at 10–14 hrs post infection and continues to be synthesized until very late in the infection cycle. Precursor-product relationships (also for the polyhedral protein) are claimed in one report (318), but are not found in other investigations (54, 377). Host protein synthesis is not shut off until very late in the infection cycle.

1.2.6. *AcNPV variants*

As already mentioned in section 1.2.3., both the amount of polyhedra and the amount of virus particles occluded in those polyhedra are greatly reduced upon prolonged passage of the virus in insect cell lines.

Analysis of plaques produced by early and late passage virus has revealed that a strain producing many polyhedra (MP) per cell is replaced by a strain producing only few polyhedra (FP) per cell (123, 246). MP-polyhedra contain many multiply enveloped virus particles, while FP-polyhedra contain only few singly enveloped virus particles or no virus at all (246, 252). This might very well explain the greatly reduced infectivity of the FP-polyhedra (123, 246, 247, 252, 350). Plaque purified variants produce both MP and FP virus when assayed in insects (123, 252) or upon further serial passage in cell cultures (123), suggesting that upon replication of the virus reversible mutations may arise or different phenotypes may be expressed. As the FP-variant produces more extracellular virus than the MP-variant (123) and because it interferes with the

[3] Assuming that protein synthesis only takes place in the cytoplasm and not in the nucleus.

infection of the MP-variant (205), the FP-variant may overgrow the MP-variant in situations where non-occluded virus is used for serial passage of the virus, such as in cell culture systems. When polyhedra are used for the transmission of the virus (as in the situation where insects are infected) the FP-variant is kept at a low level because its polyhedra are much less or not at all infectious. Further support for this 'selection pressure theory' is provided by the fact that the same displacement of the MP-variant by the FP-variant also occurs in the in vivo situation when the virus is passaged through insects by injection of non-occluded virus into the hemolymph, instead of the normal per os route of infection with occluded virus (247). It is not yet known whether this phenomenon is due to selection of spontaneously occuring mutants or to an altered phenotypic expression of the virus. Genotypic variants have been isolated from naturally occuring AcNPV. Plaque purification followed by restriction endonuclease analysis of the viral DNA has resulted in the detection of several strains with slightly different restriction endonuclease fragmentation profiles (184, 280, 281, 318). Even other nuclear polyhedrosis viruses, which were previously thought to be separate virus species, because they were isolated from other hosts, seem to be genotypic variants of AcNPV (208, 281). Some phenotypic changes are associated with these genotypic differences. Serological (22) and biochemical (281) differences have been reported between the virus particles of different variants. Differences can also be detected between the polyhedral proteins of AcNPV and *Trichoplusia ni* ME-NPV (50) and between the infectivities of AcNPV and *Galleria mellonella* NPV (45). Quantitative and qualitative differences in the protein composition of uncloned AcNPV from several in vivo and in vitro sources (197), might also be brought about by the presence of different genotypes in these preparations.

Recently, temperature-sensitive mutants of AcNPV have been induced and assorted into complementation groups (39, 185). Some of these mutants fail to produce non-occluded virus, others are restricted in the formation of polyhedra. A primitive genetic map has been constructed using some of these temperature-sensitive mutants (43).

1.3. Occlusion of virus particles

Ever since the discovery that several groups of insect viruses are embedded in protein crystals, investigators have wondered whether this phenomenon is regulated by the virus or by the host. On the one side this process can be looked upon as a defence mechanism of the host against the virus. The occlusion body protein might on the other hand be a virus specific protective substance, produced as a defence against environmental influences which the virus has to endure outside the host.

In the following sections the properties of invertebrate and other viral occlusion bodies will be discussed. This will provide basic information for the

subsequent summary of arguments which have been used with relation to the origin of the occlusion body protein.

1.3.1. Baculovirus occlusion

The occlusion bodies of baculoviruses have been well characterized. They fall apart in two morphologically distinct groups, the granulosis viruses, in which generally only one virus particle is occluded in each protein crystal, and the nuclear polyhedrosis viruses, where many virus paricles are occluded per occlusion body. Several components take part in the composition of baculovirus occlusion bodies.

– *The virus.* The virus may be multiply or singly enveloped in the case of the nuclear polyhedrosis viruses, and constitutes about 3–5% of the total mass of the polyhedron (28, 227). This figure is not known for the granulosis viruses. Several studies have shown that adequately purified virus particles are serologically not related to the surrounding occlusion body protein in which they are embedded (21, 176, 279, 371).This excludes the possibility that the occlusion body protein is an over-produced structural protein of the virus as in the case of adenovirus, where nuclear crystals are formed as a result of the production of large amounts of core (198) or capsid proteins (37, 115, 347).

Two phenotypically different virus particles are produced during an NPV infection. Those which are excreted by the cells and those which are occluded into polyhedra inside the nucleus (section 1.2.1.). Morphological (2, 117), physical (311), biochemical (280), serological (226, 340) and infectivity differences (339, 340) between these two types of particles have been described. It is not yet known which factor determines whether or not a virus particle is to become occluded. However, the fact that only enveloped virus particles are occluded, suggests that the particles which are provided with such a de novo synthesized envelope inside the nucleus are predetermined to become occluded. Virus particles which, for whatever reason, escape the intranuclear envelopment, may leave the nucleus and become extracellular virus once they have acquired their envelope at the plasma membrane.

– *The 'membrane'.* When polyhedra or granula are dissolved in weak alkaline solutions to liberate the virus particles, a membrane-like structure remains. Several authors have observed these structures in negatively stained (153, 243, 245, 288), shadowed (243, 244, 245) or thin-sectioned preparations (118). Thin sections of intact occlusion bodies reveal an electron-dense layer on the periphery of the crystals (12, 109, 245), of which the formation can be followed in infected cells (118, 191, 305). Isolated polyhedral 'membranes' show a structure which is different from the crystalline lattice of the polyhedral protein (109, 188). The polyhedral 'membrane', however, is not a true biological membrane, as it does not exhibit the double lipid-leaflet structure when observed in ultrathin sections. Chemical analysis has shown that the polyhedral 'membrane' has the same amino acid composition as the polyhedral protein which it surrounds (357).

Proteolytic enzymes do not affect the polyhedral 'membrane'. Neither when it surrounds the intact occlusion body (28), nor in thin sections for the electron microscope (99). From the fact that under certain alkaline conditions the polyhedral protein can be dissolved without disrupting the polyhedral 'membrane' it can be concluded that the polyhedral 'membrane' is penetrable by low molecular weight molecules and probably ruptures because of an osmotic shock (222). The revelation of the exact nature and composition of the polyhedral 'membrane', however, is hampered by its insolubility.

– *The matrix protein.* The main constituent of the occlusion body is the matrix protein in which the virus particles are embedded. When the crystalline array of the matrix protein is solubilized in weak alkaline solutions, high molecular weight subunits can be isolated (28, 109, 188, 260, 267, 277, 306). Analysis under conditions where all non-covalent and S-S-bonds are disrupted, reveals a protein with a molecular weight of 25,000 to 30,000 dalton, depending on the virus and the investigator (38, 47, 50, 58, 59, 79, 80, 106, 111, 153, 168, 169, 170, 199, 200, 206, 228, 234, 260, 308, 309, 310, 321, 360, 377).

The matrix protein is phosphorylated (162, 308, 309). The presence of covalently bound sugars is still a matter of debate (79, 162, 306, 308, 309). Isoelectric points between pH 5.2 and pH 6.5 have been reported (29, 44, 79, 188, 283). The primary structure of the polyhedral protein of *Bombyx mori* NPV has been established (268). The matrix proteins of some NPVs have been found to possess hemagglutinating properties (61, 223, 241, 255). Others failed to agglutinate red blood cells (271). From their amino acid composition (44, 50, 79, 80, 157, 168, 169, 188, 260, 261, 277, 306, 308, 309, 344) and from comparison of their peptide maps (50, 169, 196, 261, 309) it can be seen that baculovirus matrix proteins are very closely related. These relationships are reflected in cross reactions in all serological techniques employed. Cross reactions between inclusion bodies have been observed using the double diffusion technique (48, 61, 62, 63, 111, 177, 206, 248, 260, 279, 371, 372), hemagglutination (61), hemagglutination-inhibition (223), complement fixation (111, 175), immunofluorescence (61), immunoelectrophoresis (61, 279) and radio-immunoassay (55). Inclusion bodies can be solubilized using a variety of solutions (60, 110, 200, 222, 308). Weak alkaline solutions are most commonly used because intact particles can be obtained in this way. The solubility of the inclusion body seems to be dependent on the virus species, *Pterolocera amplicornis* NPV (72) and Tipula paludosa NPV (24, 285) being two of the most resistant viruses. The presence of a reducing substance during the solubilization procedure appears to reveal an extra antigen (60). The time course and cellular location of synthesis of the matrix protein has been investigated by both biochemical and serological techniques (47, 54, 162, 174, 178, 312, 354). According to some authors, there are at least two matrix proteins (188, 266), one of which is also detected in the virus. these two proteins have similar molecular weights but a different isoelectric point and amino acid compostion (266). Whether the protein of the occlusion body, which is also present in the virus, is a contaminant of viral origin or a real part of the surrounding matrix remains to be determined.

– *DNA*. DNA has been reported in virus-free occlusion body protein solutions (82, 262, 264), but no DNA was found in other reports (79, 85). Although the DNA which was found probably represents viral DNA (262, 264), it remains to be determined whether it originates from virus particles which are disrupted during the isolation of the occlusion body protein or whether it is independently occluded with the virus particles during the formation of the protein crystals.
– *RNA*. Several authors have described the presence of RNA in intact occlusion bodies (3, 6, 74, 83, 88, 91, 227, 232, 273) and in virus-free occlusion body protein solutions (85, 121, 227). Another report, however, describes that, in spite of the fact that RNase-treatment of the occlusion bodies has no effect, repeated washings with an acetate buffer containing 0.1% SDS removes nearly all of the original 0.35% orcinol positive material from the occlusion bodies (306). This indicates that RNA, or orther orcinol-positive substances are probably not an intrinsic part of the occlusion body.
– *Enzymes*. A DNase has been reported (358) but its presence was disputed again by a more sensitive technique (86). An alkaline protease has been found in occlusion bodies from in vivo sources (44, 50, 56, 78, 79, 80, 111, 153, 168, 170, 199, 200, 234, 308, 321, 358, 360, 363), but not in all of them (38, 44, 168). The enzyme has been purified and characterized, but its location is still a matter of dispute (234, 321). The enzyme is not found in occlusion bodies from in vitro sources (73, 199, 234, 363), so either the in vitro systems are not capable of synthesizing a viral protease or, which is more likely in view of the fact that tissue culture derived polyhedra are as infective as those from diseased larvae (87, 148, 324), the protease might be a host enzyme which is occluded together with the virus.

A synergistic factor, which enhances the infection of other baculoviruses, has been found associated with the capsule of a granulosis virus (315). Its presence in one strain of the virus but not in another strain (314), and the fact that it is still present after transmission to other hosts (48), suggests that it might be of viral origin. The synergistic factor seems to be composed of proteins and phospholipids (359) and displays an esterase activity (313).
– *Trace elements*. Several elements, other than those involved in the composition of DNA, lipid and protein, have been detected in whole inclusion bodies (82, 89, 90, 92, 100, 132, 133, 272), or in purified matrix protein fractions (82, 132, 227). As all of these studies have been undertaken with only partially purified occlusion bodies, it remains to be determined whether these elements are an intrinsic part of the occlusion body or non-functional substances which have passed the polyhedral 'membrane' or which are accidentally occluded together with the virus. Magnesium, however, may be important in the stabilization of the crystalline lattice structure of the occlusion body protein (109).

Cytoplasmic inclusion bodies have been found associated with some nuclear polyhedrosis viruses (4, 7, 58, 67, 136, 215, 287, 290, 324). Electron microscopic investigations have shown that their structure resembles that of the nuclear polyhedra, but they do not contain virus particles (4, 58, 67, 136, 290). Transmission of these NPVs to another host system still brings about the formation

of these inclusions in some cases (287, 290, 324), but not in others (215). Their structural resemblance to the nuclear polyhedra suggests that they composed of prematurely crystallized polyhedral protein, which in some host systems has not been able to find its way to the nucleus. Biochemical analysis of the cytoplasmic inclusion bodies might verify this hypothesis.

Polyhedra in which no virus particles are occluded are sometimes found in the nucleus too (2, 58, 77, 290, 305). The presence of either only normal polyhedra, or only virus-free polyhedra in one nucleus, suggest that FP-like variants might be involved (section 1.2.6.). Such 'empty' polyhedra are also produced when AcNPV is grown in vitro at 32.5 °C (185).

Fibrillar structures, which are often found associated with the surface of growing polyhedra in infected cells (2, 11, 57, 58, 64, 67, 84, 134, 150, 161, 166, 172, 191, 305) have been thought to represent some kind of precursor to the matrix protein. These structures, however, do not react with antibodies against polyhedral protein (57, 58). This is not in favour of a possible role as a precursor for the matrix protein, but does not exclude another function in the formation of occlusion bodies. The fibrillar structures, however, do react very strongly with natural antibodies from pre-immune serum from rabbits (57, 58, 64), which is very curious.

A special kind of occlusion body is induced by *Oryctes*-virus, a baculovirus which is classified as a non-inclusion type baculovirus. Non-crystalline, virus-containing inclusion bodies, resembling the A-type inclusion bodies of vertebrate pox viruses (section 1.3.4.), have been found in the cytoplasm of *Oryctes*-virus infected cells (212).

1.3.2. *Cytoplasmic polyhedrosis virus occlusion*

Cytoplasmic polyhedrosis viruses (CPVs) contain 10 segments of double stranded RNA. They replicate in the cytoplasm and belong to the Reoviridae family. Just as in the case of the baculoviruses, two types of occlusion can be recognized. The virus particles of most CPVs are occluded in great numbers. Only one CPV had been isolated so far, of which each spherical virus particle is occluded in its own crystal (9).

Unlike the baculoviruses no 'membrane' seems to be present at the surface of the polyhedra (245, 283).

Although the molecular weights of the glycosylated (237) polyhedral proteins are in the same range (25,000–31,000 dalton) as those of the baculoviruses (235, 236, 237), their amino acid composition differs considerably (157).

CPV strains have been isolated that evoke the formation of polyhedra in the nucleus (140, 142, 158, 159, 289). As the virus particles remain restricted to the cytoplasm, no virus particles are occluded in these nuclear polyhedra. In some of these strains only nuclear and no cytoplasmic polyhedra are synthesized. As a consequence all the virus particles of these strains remain non-occluded (158, 159).

1.3.3. *Entomopoxvirus occlusion*

Entomopoxviruses (EPVs), like all poxviruses, contain double stranded DNA. With one possible exception (116) they replicate in the cytoplasm. Apart from the virus containing occlusion bodies (which are often called spherules), virus-free inclusions (named spindles) are found associated with some EPVs. These spindles are generally surrounded by a membrane that appears to be of cytoplasmic origin (23). In several *Choristoneura* species (32, 33, 68, 154) and in *Oncopera alboguttata* (210) they appear as micro-spindles which are also occluded into the spherules, just like the virus. The spindles are serologically not related to the spherules or to the virus (58, 65). They also exhibit a crystalline lattice structure which differs from that of the spherules (210). Their formation may be dependent on the host (257).

Like baculovirus occlusion bodies the spherules are surrounded by a 'membrane' (137). Spherules do not readily solubilize unless reducing substances are present (25). This might be explained by the fact that spherules contain much more sulpur-containing amino acids than the other types of occlusion bodies (26, 180).

A molecular weight of 112,000 dalton has been reported for the occlusion body protein of *Amsacta moorei* EPV (180), but 4 proteins with molecular weights of 97,000 (2 ×), 76,000 and 56,000 dalton are found in an other investigation with the same virus (258). The presence of an alkaline protease (180) might be responsible for the appearance of the additional lower molecular weight proteins reported in 258.

1.3.4. *Occlusion of vertebrate poxviruses*

Some vertebrate poxviruses are also occluded. In general the vertebrate poxvirus occlusion bodies are named A-type inclusions, B-type inclusions being the sites of viral replication. Specific names have been given to the A-type inclusions of fowl pox (Bollinger bodies), extromelia (Marchal bodies), and cow pox (Downie bodies). In contrast with the occlusion bodies of the insect viruses A-type inclusions are not crystalline. Lipid (253) as well as protein (146) were reported to be the main constituent of the A-type inclusion.

Strains which do not incorporate virus into the A-type inclusions (V^- character) have been isolated (146, 155). V^- viruses, however, become occluded in mixed infections with V^+ viruses. Mixed infections of vaccinia virus, which does not produce A-type inclusions, and V^- viruses result in the occlusion of both viruses (147), indicating that vaccinia virus also possesses the V^+ character, although it does not produce any occlusion body protein. Further studies have indicated that V^- viruses lack a polypeptide no. 4^c, which is present in the outer membrane of V^+ viruses and also in vaccinia virus (276). So to become occluded both the occlusion body protein and the polypeptide no. 4^c must be present.

Polyribosomes have been found associated with growing A-type inclusion bodies, but their size is much greater than would be expected for polysomes synthesizing proteins of 27,000 and 28,000 dalton, which are proteins that are found in the A-type inclusions (145).

A-type inclusions are serologically unrelated to entomopoxvirus occlusion bodies (58).

1.3.5. *Adenovirus occlusion*

Adenoviruses are sometimes occluded in nuclear crystalline inclusion bodies (37, 115, 198, 214, 220, 320, 347). These crystals are composed of overproduced viral-structural proteins (37, 115, 198, 347). The occlusion of adenoviruses, however, seems to be an accidental event without functional significance.

1.3.6. *Mixed virus occlusion*

Although not occluded when replicating alone, other viruses can become incorporated into the polyhedral protein of nuclear and/or cytoplasmic polyhedrosis viruses in mixed infections (119, 216, 369). As the occlusion of virus particles is quite specific (no other cell organelles are ever found in polyhedra) this phenomenon might indicate that there is some kind of similarity between the surfaces of the occluded viruses, a situation similar to that of the vertebrate poxviruses, where vaccinia virus, which does not produce A-type inclusions itself, can become occluded in mixed infections, because it possesses the polypeptide no. 4^c on its surface (which is necessary for occlusion).

1.3.7. *Plant virus occlusion*

Several plant viruses induce the formation of inclusion bodies which are composed of host components and/or viral products (194). In some cases virus particles are also occluded in these inclusion bodies. The significance (if any) of this kind of occlusion is not known. One interesting group of isometric DNA-containing plant viruses (caulimoviruses) induces the formation of inclusion bodies in which most of the virus particles are accumulated. Viral DNA is synthesized and virus particles are assembled in these structures (274). In this respect caulimovirus occlusion bodies differ from the previously described occlusion bodies where the virus particles are produced outside the occlusion body. As only few virus particles are found outside these caulimovirus inclusion bodies their function might be more complex than only that of a virus factory. The matrix in which the virus particles are embedded mainly consists of a protein with a molecular weight of 55,000 dalton (275).

1.3.8. *Arguments on the origin of the occlusion body protein*

Several arguments have been used on the origin of the occlusion body protein since the discussion of Bergold in 1947 (27) on this subject. Arguments which have been used in favour of the host are the following:

- In a typical baculovirus infection no polyhedra are formed in the gut cells of the infected larvae (67, 108, 112, 187, 303, 304, 316), although the virus is multiplied and some occlusion body protein may be synthesized (112, 187, 316). From this primary infected tissue the virus particles are excreted into the hemocoel, thereby allowing the infection of other tissues. When polyhedra are formed in the gut cells, such as in the case of the cytoplasmic polyhedrosis viruses (283), and in

TABLE I. Distribution of members of the Pox-, Reo- and Baculoviridae among their plant, vertebrate and invertebrate hosts.

Virus family	Invertebrate Representatives	Vertebrate Representatives	Plant Representatives
Poxviridae	Entomopoxviruses	Poxviruses	not known
Reoviridae	Cytoplasmic Polyhedrosis Viruses	Reoviruses	Wound Tumor Virus
		Orbiviruses	Maize Rough Dwarf Virus
			Mycoviruses
Baculoviridae	Nuclear Polyhedrosis Viruses	not known	not known
	Granulosis viruses		

the case of the nuclear polyhedrosis viruses of hymenoptera (30, 34, 301) and mosquitoes (374, 375, 376), the infection remains restricted to the gut tissue. So at first sight the formation of polyhedra in the gut cells can be interpreted as a defense mechanism of the host against the spread of the virus to other tissues. However, a lot of exceptions to this general rule are known now. Several baculoviruses are able to evoke the formation of polyhedra in the gut tissue, without the spread of the virus to other tissues being affected (4, 7, 113, 143, 144, 149, 181, 182, 286, 319, 323). Furthermore CPV strains have been isolated which are not occluded into polyhedral protein (158, 159). Yet these strains remain limited to the gut tissue. The CPV of *Cutex tarsalis* on the other hand does not remain limited to the gut tissue although it is occluded in these cells (368). The conclusion that can be drawn from these exceptions is, that other mechanisms than the occlusion into polyhedra are involved in the restriction of the spread of the virus to other tissues.

– The formation of polyhedra and granula is restricted to the invertebrates. It occurs with three different invertebrate virus families, while taxonomically closely related viruses of vertebrates and plants do not exhibit the formation of these structures (Table I). The most striking example is provided by the Reoviridae with representatives among plants and vertebrate and invertebrate animals. Although the genome of the invertebrate CPVs does not contain more presumptive mono-cistronic RNA segments than the plant- and vertebrate-Reoviridae, the CPVs are the only genus exhibiting the formation of polyhedra. The reason for this might be on the one hand that the gene coding for polyhedral protein is not present or suppressed in the plant and vertebrate viruses. The restriction of this phenomenon to the invertebrates might on the other hand be due to the fact that it is the invertebrate host that synthesizes the occlusion body protein.[4]

However, if one leaves out the word 'crystal-like' from the definition of a

[4] Note added in proof. Recent experiments of Mertens (378) have indicated that the polyhedral protein of CPVs probably is a virus coded protein. Total and separated CPV type 1 RNA segments were translated in a protein synthesizing system. The product of segment number 10 comigrated with the major polyhedral protein. As a consequence the above mentioned argument has lost its value. The question remains if a similar protein is encoded in segment number 10 of vertebrate- and plant-Reoviridae.

polyhedron (298), polyhedra formation is no longer restricted to the invertebrates as some vertebrate poxviruses are also occluded in proteinous bodies. These vertebrate poxvirus occlusion bodies are not crystalline as in the case of the insect viruses, but they might have a similar role in the dissemination of the virus (section 1.3.4.).

Arguments in favour of the hypothesis that the occlusion body is coded for by the viral genome are:

– The occlusion body protein can be looked upon as a protective substance for the virus. It protects the virus from environmental influences when the host has died and the polyhedra are liberated from its body. And, because non-occluded virus particles are much less infectious when ingested by the larva (270), it apparently also protects the virus on its way to the midgut where the virus particles are liberated from the occlusion body.

The occlusion in protein crystals, however, is not a prerequisite for invertebrate viruses to survive in nature. Several invertebrate virus families are known of which the virus particles are never occluded. In the virus families of which the virus particles are occluded, several examples have been reported of non-inclusion type viruses. In the past few years non-occluded baculo- or baculo-like viruses have been demonstrated in two beetles (101, 135, 233), two crab species (20, 231), a spider (213), a fungus (93), two mites (31, 254), two aphids (163), and a parasitoid wasp (171). Non-occluded reo-like viruses have also been reported (207, 217, 242, 364, 365, 366, 367, 373). So, other ways of dissemination of the virus can apparently be sufficient to ensure survival of invertebrate viruses.

– Spontaneously occurring virus mutants with different shapes of their polyhedra can be isolated and maintained in the same host as the original isolate, indicating that at least the shape of the occlusion body is determined by the virus. Such mutants have been isolated for nuclear polyhydrosis viruses (66, 95, 141, 341), cytoplasmic polyhedrosis viruses (14, 15, 140), and granulosis viruses (13, 294, 295). The presence or absence of virus in the occlusion body (305) as well as the conditions under which the occlusion bodies are formed (277), may also be of influence on the shape, however. Two reports have described an alteration in the shape of the occlusion body upon transmission to another host (144, 250). It was not excluded in these reports, however, that a latent virus might be activated upon the infection with the original virus. This might be verified by electrophoretic comparison of the viral RNA profiles (CPV) and by restriction endonuclease analysis of the viral DNA (NPV).

– Temperature sensitive mutants have been isolated, which are defect in their ability to synthesize polyhedral protein at the restrictive temperature (39, 185). Although it has not been proved that the polyhedral protein itself is temperature sensitive, some viral mechanism in the formation of polyhedra is apparently defect at high temperatures.

– If the occlusion of virus particles into polyhedral protein is a host defense mechanism, one would expect that, for a rather aspecific process like encapsulation, one occlusion body portein would be enough for a particular host to

TABLE II. Sets of viruses originating from one host, possessing different occlusion body proteins.

Viruses		Host	Method	Reference
CPV	NPV	Choristoneura fumiferana	Serology	175, 177
CPV	NPV	Bombyx mori	Amino acid composition	157
CPV	NPV	Bombyx mori	Serology	139
GV	ME-NPV	Trichoplusia ni	Peptide mapping	309
SE-NPV	ME-NPV	Orgyia pseudotsugata	Serology	260
SE-NPV	ME-NPV	Trichoplusia ni	Serology	279
SE-NPV	ME-NPV	Trichoplusia ni	Peptide mapping	50
GV	GV	Pseudaletia unipuncta	Serology	360
CPV	ME-NPV / SE-NPV	Orgyia pseudotsugata	Serology Peptide mapping Amino acid analysis NH_2-terminal amino acid sequence analysis	261
ME-NPV	SE-NPV	Orgyia pseudotsugata	Peptide mapping NH_2-terminal amino acid sequence analysis	261
ME-NPV	SE-NPV	Orgyia pseudotsugata	Electron microscopy	138

CPV: Cytoplasmic Polyhedrosis Virus
NPV: Nuclear Polyhedrosis Virus
GV: Granulosis Virus
ME: Multiply enveloped
SE: Singly enveloped

defend itself. The fact that, in one host, different viruses are occluded into different polyhedral proteins (Table II), is therefore an argument against the host and in favour of the virus.

It can be seen from the table that not only particles from different virus families are occluded into different polyhedral proteins, but even members of the same (baculo)virus family are occluded into different polyhedral proteins.

- A strong argument in favour of the hypothesis that the occlusion body protein is a virus-specific protein, is that it does not change after transmission of the virus to another host system. This has been demonstrated serologically for the nuclear polyhedrosis viruses of *Lymantria dispar* and *Autographa californica* (199) and it has been confirmed by peptide mapping of the polyhedral protein of AcNPV (50, 197). As it is considered to be very unlikely that different insects should have developed exactly the same polyhedral protein as a defense mechanism against one single virus, these data provide very strong circumstantial evidence in favour of the hypothesis that the polyhedral protein is coded for by the viral genome.
- The strongest argument however is provided by recent experiments of Mertens (378) described in note 4 on page 13.

1.4 AIM OF THE INVESTIGATIONS

The aim of this work has been to determine whether the host or the invading

virus could be held responsible for the production of occlusion body protein. Isolation, in vitro translation and determination of the DNA origin of the mRNA for polyhedral protein would elucidate this problem. As a model the nuclear polyhedrosis virus of *Autographa californica* was chosen, because it is one of the best characterized NPVs. The virus was multiplied in a cell line of *Spodoptera frugiperda*. To obtain a genetically pure virus preparation plaque purification was employed (Chapter 2). Attempts to isolate the mRNA for polyhedral protein as a single species were not successful (Chapter 3 and 4). Isolation of viral RNA by means of hybridization with viral DNA (Chapter 5), followed by in vitro translation in a cell free system (Chapter 6), revealed that the mRNA for polyhedral protein is present in the viral RNA population.

2. MATERIALS AND METHODS

2.1. Introduction

Because of the variety of techniques that have been used during the experimental work which is described in this thesis, each chapter will have its own section on materials and methods. Materials and methods of general importance are described in the following sections of this chapter. Special reference is given to the genetical purification of the virus which is used in this study.

2.2. Cells

The cell line used in this study was the IPLB-SF-21AE cell line (332) from *Spodoptera frugiperda*. It was obtained from Dr. Tinsley (N.E.R.C. Unit of Invertebrate Virology, Oxford, England). Cells were grown in polystyrene tissue culture vessels at 27 C in BML/TC10 medium (94) without $NaHCO_3$. They were subcultured once or twice a week by removing the cells from the bottom of the culture vessel by vigorous pipetting, followed by dilution in fresh growth medium.

2.3. Virus

Autographa californica nuclear polyhedrosis virus (multiply enveloped type) which had been multiplied twice in *Estigmene acrea* cells was provided by Dr. Granados (Boyce Thompson Institute for Plant Research, Yonkers, New York, USA). Analysis of the DNA of this virus by means of restriction endonuclease treatment followed by agarose gel electrophoreses revealed the presence of two fragments (EcoRI H and J) which were present in much less than equimolar amounts (334). The presence of these minor fragments was most likely due to the fact that naturally occuring AcNPV consist of a mixture of variants with slightly different restriction endonuclease patterns (184, 280, 281, 318). To obtain a genetically pure virus preparation, plaque purification was employed.

2.3.1. *Plaque purification of the virus*

Polystyrene petridishes (ø 35 mm) were seeded with 10^6 cells in 2 ml growth medium. After 24 hrs the cells were infected with 0.4 ml of appropriate dilutions of the virus. The virus was allowed to adsorb for 1 hr with occasional redistribution of the inoculum. After the adsorption period the virus was removed and 2 ml overlay, consisting of equal volumes of twice concentrated BML/TC10 and 3% agar (Difco Agar purified) in double-distilled water, was poured on the cells and allowed to set. After 4 to 8 days of incubation at 27 C in a humid environ-

ment plaques were examined by an inverted microscope. Thirty free-laying plaques were picked by means of suction into a bended Pasteur-pipette. The small pieces of agar were resuspended in 0.5 ml medium and allowed to stand for at least 12 hrs at 4° C. Then the suspension was centrifuged for 2 min at 8000 *g* and dilutions between 10 and 1000 × were made of the supernatant. With these dilutions new plaques were produced and this whole procedure was repeated three times, taking plaques from the dishes inoculated with the greatest dilution that still produced plaques. The third subsequent plaque of each isolate was resuspended in 5 ml medium and filtrated through a 0.45 µm filter before infection of 25 cm^2 tissue culture flasks.

2.3. *DNA characterization of the 30 AcNPV clones*

DNA was isolated from extracellular virus and analysed with the restriction endonucleases EcoRI and BamHI as described by VLAK and ODINK (334). All 30 clones depicted the same pattern of restriction endonuclease fragments with both enzymes. The minor fragments H and J, which were present in the original isolate, were absent in the EcoRI profile of the 30 AcNPV clones, indicating that other variants had been removed by the plaque purification procedure (Fig. 2). Unfortunately none of these variants was present among the 30 AcNPV clones which were isolated by us. The EcoRI and BamHI pattern of our 30 AcNPV clones is similar to 'L1' of LEE and MILLER (184) and 'Type I' of SMITH and SUMMERS (280). Arbitrarily clone no. 2 was taken as the source of virus in the experiments described in this thesis, because it was one of the clones that consistently produced comparatively large plaques and many polyhedra during the plaque purification procedure. Two or three passages of the virus were made in *Spodoptera frugiperda* cells and the undiluted supernatant of the cells was used as an inoculum for the experiments described in the following chapters. At least 10^8 $TCID_{50}$/ml (but often more) were present in this inoculum.

An additional advantage of the use of a plaque purified virus preparation is the fact that one can start with a many polyhedra variant, which only after several serial passages in vitro will produce lesser polyhedra, as a result of the spontaneous production of the so-called few polyhedra variant (Section 1.2.6).

2.4. CELL INFECTION

About 10^5 cells/cm^2 were seeded and allowed to retain growth for 24 hrs. The medium was removed and replaced by 0.04 ml virus containing medium/cm^2. At least 10 $TCID_{50}$/cell were used to ensure a synchronized infection. After an adsorbtion period of 2 hrs with occasional redistribution of the virus suspension, fresh medium was added without removing the inoculum (± 0.2 ml/cm^2). Infected cells, like healthy cells, were kept at 27 C.

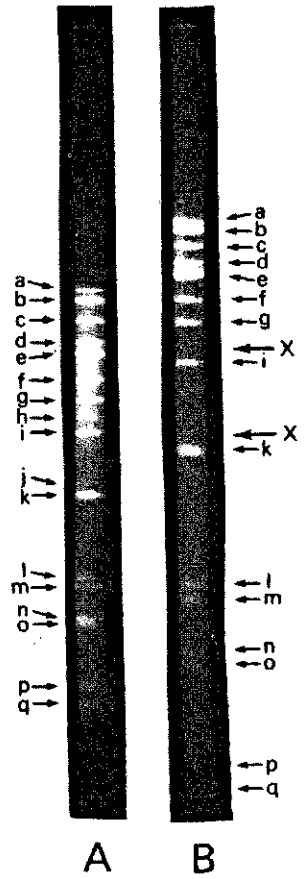

FIG. 2. Genetical purification of *Autographa californica* nuclear polyhedrosis virus. DNA of uncloned (A) and cloned (B) AcNPV was digested by the restriction endonuclease EcoRI and subjected to agarose-gelelectrophoresis in two different experiments (which causes the differences in mobilities between A and B).
X: Missing EcoRI fragments h and j in cloned AcNPV.

2.5. BUFFERS, GLASSWARE AND CENTRIFUGE TUBES

When working with polysomes or RNA, all buffers, sucrose solutions and plastic micropipet tips were autoclaved for 15 min to minimize RNase activity. Glassware was heated for 2 hrs at 150 C and cellulose nitrate centrifuge tubes were left overnight in a strong soap solution and thoroughly rinsed with double distilled water before use.

2.6. Isolation of polyhedra and polyhedral protein

Infected cells were collected by centrifugation (10 min 4000 *g*), resuspended in double distilled water, and disrupted by a series of 15 sec sonification periods at 0 °C. The suspension was diluted with bidest, polyhedra were pelletted at 4000 *g* for 10 min and washed several times with bidest until the supernatant was clear. Polyhedra were then further purified as described by McCarthy and Liu (200) which included incubation for 30 min at 20 °C in each of the following solutions: 4 M Urea, 1 % SDS and 0.5 M NaCl, interrupted by centrifugation at 4000 *g* for 10 min to collect the polyhedra. After the NaCl treatment the polyhedra were washed several times with bidest and stored at −20 °C.

Polyhedral protein was prepared by solubilizing purified polyhedra (heated for 30 min at 70 °C to destroy alkaline protease, if any) in 0.1 M Na_2CO_3, 0.1 M NaCl, pH 11.0. As soon as the suspension became clear the pH was lowered to 8.5 by the addition of dilute HCl. Virus particles and polyhedral 'membranes' were removed by centrifugation at 40,000 rpm for 60 min in a SW50.1 (Beckman) rotor at 5 °C. The supernatant was dialysed overnight against 0.01 M Na_2CO_3, 0.01 M NaCl, pH 8.5.

Protein concentrations were determined as described by Lowry et al. (189) using crystalline bovine serum albumin as standard.

3. ISOLATION AND CHARACTERIZATION OF POLYRIBOSOMES

3.1. INTRODUCTION

To elucidate the origin of the polyhedral protein, attempts were made to isolate its mRNA with the intention to hybridize it to either host- or viral DNA.

In eukaryotic cells protein synthesis takes place in the cytoplasm, in complexes which are composed of mRNA and ribosomes called polyribosomes or polysomes. Recent reports (for reviews see references 1 and 69) have indicated that mRNAs of several eukaryotic cells and their DNA viruses are being processed from large nuclear primary transcripts to functional mRNAs of shorter lengths, which are translated by the polysomes in the cytoplasm, and which may be constructed of non-contiguous regions of the primary transcript by a process that has come to be called RNA-splicing. As we wanted to isolate a translatable mRNA for AcNPV polyhedral protein we decided to use polysomes as our source of mRNA.

Polysomes have been isolated from several lepidopterous tissues or whole insects (10, 249, 342).

The presence of polysomes in lepidopterous tissue culture cells has been demonstrated by DAVIES and HARTIG (71) in a cell-free protein synthesizing system from CP-1268 cells, a cell line derived from the codling moth (*Laspeyresia pomonella*).

In this chapter conditions are determined under which polysomes can be isolated from healthy *Spodoptera frugiperda* cells. A comparison is made between polysomes of healthy and AcNPV infected cells, and attempts are described to isolate polyhedral protein synthesizing polysomes by means of immunoprecipitation with antibodies against AcNPV polyhedral protein, a method which has been successfully applied in the isolation of several eukaryotic (105, 151, 218, 265, 269, 299) and viral mRNAs (218).

3.2. MATERIALS AND METHODS

3.2.1. *Isolation of polysomes*

Cells were removed from the surface of the culture vessel by vigorous pipitting and pelletted at 4 C at 1000 *g* in a Christ table top centrifuge for 10 min. The cells were washed once with ice-cold phosphate buffered saline (PBS) and subsequently lysed by resuspending in lysis buffer (30 mM Tris-HCl, 10 mM KCl, 2 mM magnesium-acetate, 1 % Nonidet P40 (NP40), pH 9.0). A postmitochondrial supernatant was prepared by centrifugation at 12,000 *g* for 10 min. Samples of the postmitochondrial supernatant were layered on isokinetic sucrose gradients prepared for particles with a density of 1.40 g/cm^3 (221, 362).

The gradients were made up in the buffer solution mentioned in the corresponding legends to the figures in SW41 or SW27 (Beckman) centrifuge tubes and centrifuged for the time also indicated in the corresponding legends to the figures Polysome profiles were analysed by pumping the sucrose gradients through an LKB Uvicord II flow analyser (257 mm) at a rate of 0.75 ml/min. The absorbance was monitored on a Servogor type RE514 recorder.

3.2.2. *Preparation of RNase-free immunoglobulins against AcNPV polyhedral protein*

Antiserum against purified polyhedral protein was prepared by injecting rabbits once intravenously with 2 mg polyhedral protein and subsequently twice intramuscularly with 10 mg polyhedral protein emulsified with an equal volume of Freund's complete adjuvant. The injections were given at two-week intervals. Immunoglobulins were purified by $(NH_4)_2SO_4$-precipitation and dialysis against PBS. Fractions of 1 ml were made RNase-free by passing them through a combined CMC-DEAE-cellulose column as described by PALACIOS et al. (229). The effectivity of this procedure was tested by incubating a 2 mg/ml solution of RNase-free immunoglobulins (Ig) with ^{3}H-labelled RNA. After 40 min incubation at 37 C the remaining trichloroacetic acid-precipitable radioactivity was determined. Taking RNA, incubated without immunoglobulins, as a 100% reference point, an Ig-fraction which had passed the CMC-DEAE-cellulose column conserved 99% of the radioactivity, while RNase, present in the original immunoglobulins, completely solubilized the radioactive RNA in 40 min. The titre of the RNase free immunoglobulins was 1/128, when tested against 88 μg polyhedral protein/ml in the Ouchterlony double diffusion test.

3.2.3. *Immunoprecipitation of AcNPV polyhedral protein synthesizing polysomes*

Cells (3.10^7) were seeded and infected with AcNPV 24 hrs later. From 22–24 hrs post infection cells were labelled with 600 μCi 5,6 ^{3}H-uridine in 12 ml growth medium, containing 25 μg cycloheximide/ml, on 300 cm^2 growth area. Twenty-four hrs post infection a postmitochondrial supernatant was prepared as described in section 3.2.1. Polysomes were pelletted by layering the postmitochondrial supernatant on a 27% (w/w in lysis buffer) sucrose cushion and centrifugation for 16 hrs at 26,000 rpm in a SW50.1 (Beckman) rotor at 5 C. The polysome pellet was resuspended in 1 ml immunoprecipitation buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM magnesium acetate, 0.5% NP_{40}). Fractions of 150 μl were incubated for two hrs at 0 C with either 150 μl anti-AcNPV polyhedral protein immunoglobulins or with 150 μl immunoglobulins from preimmune serum. Three immunoglobulin-concentrations (3.45, 0.69 and 0.138 mg/ml) were tested. After the incubation period 0.7 ml protein A-Sepharose suspension (0.25 g in 4.5 ml immunoprecipitation buffer) was added to bind the immunoglobulins and the incubation was continued for another 20 min at 0 C. To remove non-bound substances the protein A-Sepharose spheres were washed by centrifuging them twice through a discontinuous sucrose gradient consisting of 2 ml 0.5 M sucrose on top of 2 ml 1 M sucrose in immunoprecipitation buffer.

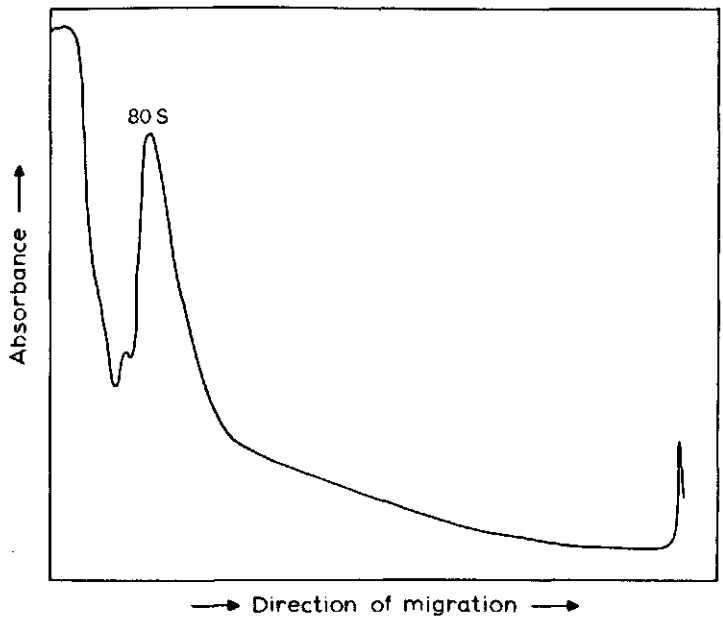

FIG. 3. Sedimentation profile of a postmitochondrial supernatant from untreated cells. Cells (6×10^7) were harvested, washed and lysed in 1 ml lysis buffer. The postmitochondrial supernatant (250 µl) was layered on to a 15–33.5% isokinetic sucrose gradient made up in lysis buffer without NP40 and centrifuged for 2.5 hrs at 82,000 *g* in the SW27.1 rotor at 5 C.

RNA was liberated from polysomes by resuspending the protein A-Sepharose spheres in 0.8 ml immunodissociation buffer (10 mM Tris-HCl, pH 7.5, 20 mM EDTA). After removal of the protein A-Sepharose spheres by centrifugation at 8,000 *g* for 2 min, the radioactivity of the supernatant was determined by mixing samples with 7 ml Hydroluma (Lumac, Amsterdam, The Netherlands), followed by liquid scintillation counting.

3.3. RESULTS

When *Spodoptera frugiperda* tissue culture cells are harvested and a postmitochondrial supernatant is prepared and analysed as described in 3.2.1, only monosomes can be detected (Fig. 3). This phenomenon can either be caused by run-off of the ribosomes from the mRNA or by an endogenous RNase activity in the postmitochondrial supernatant. As run-off ribosomes dissociate under high salt conditions, whereas monosomes produced from polysomes by RNase activity normally do not dissociate under these conditions (195), one should be able to discriminate between these two possibilities by adding 500 mM KCl to the postmitochondrial supernatant. As can be seen in fig. 4, the monosomes dissociate into subunits upon a 500 mM KCl treatment, indicating that run-off was the most likely cause of polysome breakdown.

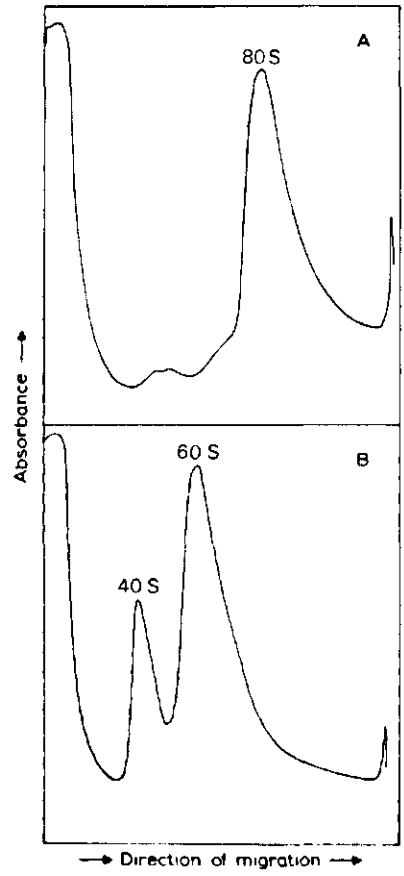

FIG. 4. KCl-treatment of monosomes in a postmitochondrial supernatant from untreated cells. Cells (6×10^7) were harvested, washed and lysed in 1 ml lysis buffer. The postmitochondrial supernatant (250 µl) was directly layered on to a 15 - 33.5 % isokinetic sucrose gradient made up in lysis buffer without NP40 (Fig. 4A). Another 250 µl was made 500 mM with respect to KCl by the addition of solid KCl and subsequently layered on a similar isokinetic sucrose gradient made up in 500 mM KCl containing lysis buffer without NP40 (Fig. 4B). Centrifugation was carried out in the SW41 rotor for 5 hrs at 205,000 *g* at 5 C.

An interesting observation is the presence of two 40 *S* particle populations in postmitochondrial supernatants which are prepared and analysed under low salt conditions (Fig. 4A). These two populations of 40 *S* ribosomal subunits are not well resolved on linear gradients, but under the centrifugal conditions (isokinetic gradients) used by us and by others (127, 282, 337) they can be separated.

3.3.1. *Cycloheximide tratment of cells*

As cycloheximide is known to prevent run-off of ribosomes (17, 346), this antibiotic was added to the cell culture medium, prior to the isolation of the postmitochondrial supernatant. As a result of this, polysomes were detected in

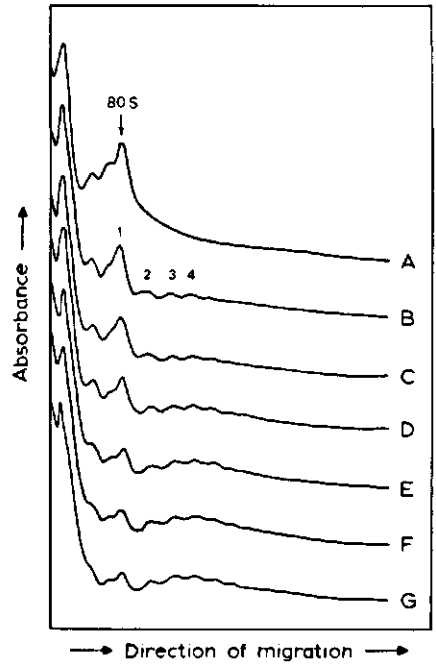

FIG. 5. Effect of the addition of cycloheximide to the cell culture medium at different times prior to the isolation of the postmitochondrial supernatant. To 3×10^7 cells, 50 µg cycloheximide ml of growth medium was added at different times prior to the isolation of the postmitochondrial supernatant. Cells were harvested, washed and lysed in 1 ml lysis buffer. Fractions of the different postmitochondrial supernatants (250 µl) were layered on to 15–33.5 % isokinetic sucrose gradients made up in lysis buffer without NP40. Centrifugation was carried out for 3 hrs at 82,000 *g* in the SW27.1 rotor at 5 C.

A: no cycloheximide added; B-G: cycloheximide added at 0.5, 1, 2, 4, 8, and 16 hrs respectively before harvesting the cells.

Figures indicate the monosomes, disomes, etc.

the postmitochondrial supernatant. Optimum conditions for the addition of cycloheximide were investigated by varying the time of addition to the cell culture medium (Fig.5) as well as the concentration of the cycloheximide (Fig. 6). When cycloheximide was added to the cell culture medium at different times before the harvesting of the cells, an increase in the ratio between polysomes and monosomes could be noticed up to 16 hrs before harvesting the cells. However, when cycloheximide was added 16 hrs before harvesting the cells, the yield of polysomes was less than at other times.

With all the cycloheximide concentrations that we tested a polysome profile was obtained. However, at a concentration of 25 µg/ml the three and four ribosome-containing polysomes seem to be the most abundant species, whereas at the other concentrations the major species are the disomes.

Based on these results we routinely added cycloheximide in a concentration of 25 µg/ml to the cell culture medium, 8 hrs before harvesting the cells, to obtain an undegraded polysome profile.

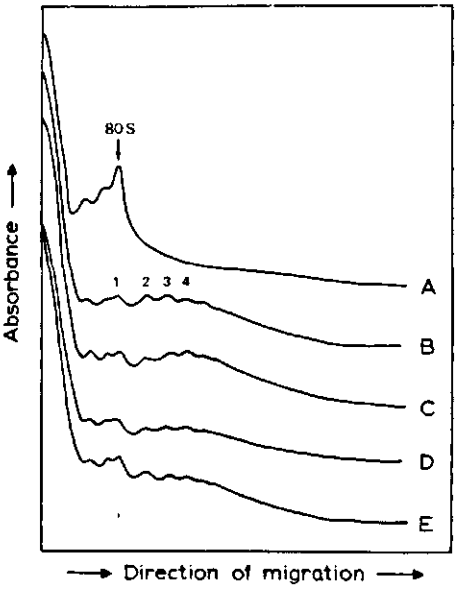

FIG. 6. Effect of the addition of different cycloheximide concentrations to the cell culture medium prior to the isolation of the postmitochondrial supernatant. To 3×10^7 cells cycloheximide was added in different concentrations. After 2 hrs the cells were harvested, washed and lysed in 1 ml lysis buffer, 250 µl fractions of the different postmitochondrial supernatants were layered on to 15-33.5% isokinetic sucrose gradients made up in lysis buffer without NP40. Centrifugation was carried out for 3 hrs at 82,000 *g* in the SW27.1 rotor at 5 C.
A: no cycloheximide added; B-G: cycloheximide added in concentrations of 10, 25, 50 and 100 µg/ml respectively.
Figures indicate the monosomes, disomes, etc.

3.3.2. *High salt treatment of RNase-derived monosomes*

When polysomes from cycloheximide treated cells were incubated with T1-RNase, they were converted to monosomes (Fig. 7A). High salt treatment of these RNase-derived monosomes resulted in a profile as depicted in fig. 7B. Apparently dissociation had taken place, as can be concluded from the disappearance of the monosome peak. However, a clear separation in 40 and 60 *S* subunits as observed in fig. 4B was not obtained. A control experiment in which the monosomes of fig. 4A (which were thought to be run-off ribosomes) were treated with RNase prior to the high salt treatment, resulted in a profile which was identical to fig. 7B, indicating that the preceding RNase treatment probably causes the abnormal dissociation behaviour of the monosomes.

However, the fact that RNase-derived monosomes from *Spodoptera frugiperda* cells are also sensitive to a high salt treatment, makes it impossible to discriminate between RNase-derived and run-off monosomes by this method.

As pretreatment of the cells with cycloheximide (which inhibits run-off by fixing the ribosomes on the mRNA) increases the amount of polysomes, as

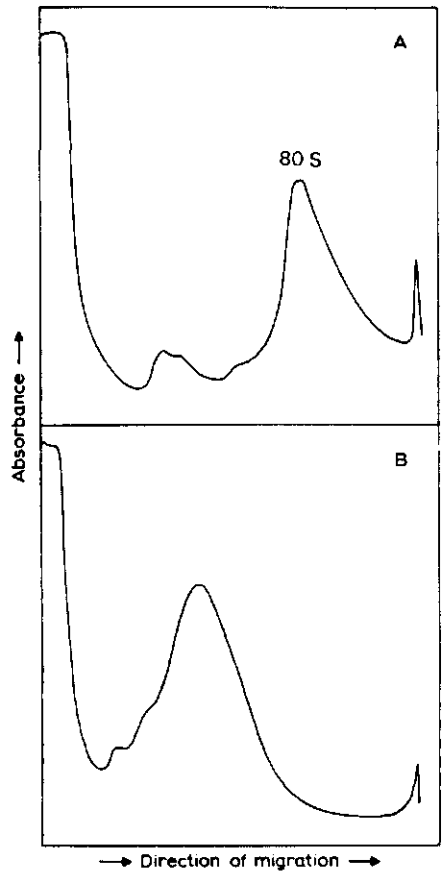

FIG. 7. KCl treatment of monosomes produced by RNase treatment of polysomes in a postmitochondrial supernatant from cycloheximide treated cells. To 6 × 10^7 cells, cycloheximide was added in a final concentration of 25 µg/ml growth medium. After 8 hrs the cells were harvested, washed and lysed in 1 ml lysis buffer. The postmitochondrial supernatant was incubated for 30 min at 0 C with 10 µg/ml T1-RNase. A sample (250 µl) of this incubation mixture was directly layered on to a 15-33.5% sucrose gradient made up in lysis buffer without NP40 (Fig. 7A). Another 250 µl was made 500 mM with respect to KCl by the addition of solid KCl and subsequently layered on a similar isokinetic gradient made up in 500 mM KCl containing lysis buffer without NP40 (Fig. 7B). Centrifugation was carried out in the SW41 rotor for 5 hrs at 205,000 *g* at 5 C.

outlined in the previous section, run-off still remains the most likely cause of polysomal breakdown.

To substantiate this hypothesis further, several RNase inhibitors were tested for their ability to increase the amount of polysomes in cells which were not incubated with cycloheximide. Neither Polyvinylsulphate, nor Diethylpyrocarbonate, nor Macaloid, nor Yeast RNA, nor Rat Liver RNase Inhibitor (kindly provided by Prof. Dr. C. E. Sekeris) improved the polysome yield (results not shown), indicating that active RNAses are not likely to be present in the postmitochondrial supernatant.

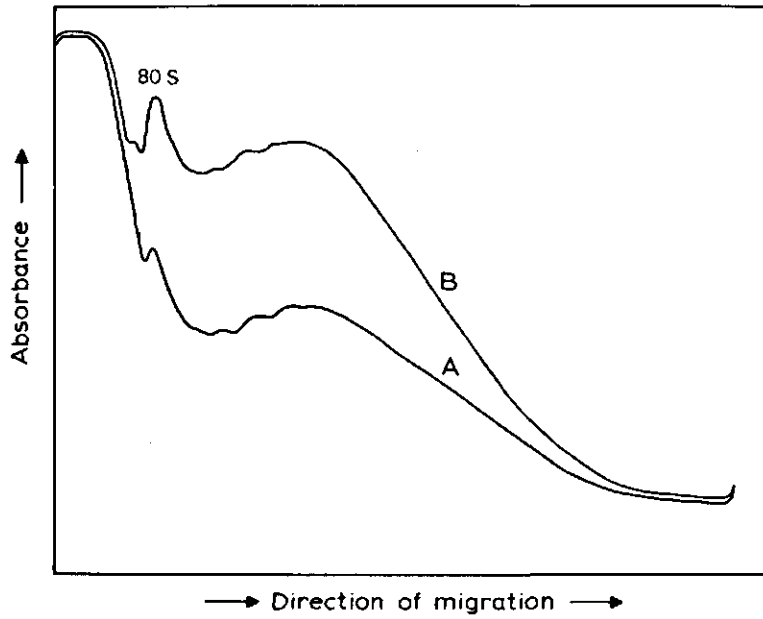

FIG. 8. Influence of the pH on the isolation of the postmitochondrial supernatant from cycloheximide treated cells. To 12 × 10^7 cells cycloheximide was added in a final concentration of 25 µg/ml growth medium. After 8 hrs the cells were harvested, divided into two equal portions and washed with PBS. One portion was lysed in 1 ml lysis buffer pH 7.0 (A); the other portion was lysed in 1 ml lysis buffer pH 9.0 (B). Fractions (500 µl) of the postmitochondrial supernatants were layered on to 15–33.5% isokinetic sucrose gradients made up in lysis buffer with the corresponding pH without NP40. Centrifugation was carried out for 2.5 hrs at 82,000 *g* in the SW27.1 rotor at 5 C.

3.3.3. *Optimum pH and Mg^{2+}-concentration of the lysis buffer*

In experiments to determine the optimum pH of the lysis buffer five different pH values in the range form pH 7.0 to pH 9.0 were tested. The results indicated that an increase in pH resulted in an increase in polysome yield. Fig. 8 shows the sedimentation profiles of polysomes isolated at the two extreme pH values used. As can be seen in this figure, there was no drastic influence on the polysome pattern. However, the yield was greatly increased at pH 9.0.

In a similar kind of experiment six different magnesium acetate-concentrations in the lysis buffer were tested (Fig. 9). When no Mg^{2+} was included in the lysis buffer, a polysome pattern as depicted in fig. 9A was obtained. There was no complete dissociation of the polysomes as would be expected when polysomes were isolated in an Mg^{2+}-free buffer. Cycloheximide might be responsible for this phenomenon as dissociation in an Mg^{2+}-free buffer was much more complete in the absence of cycloheximide (Fig. 10B and C).

When 1, 2, or 5 mM magnesium acetate were included in the lysis buffer and the sucrose gradient, good polysome patterns and a high yield were obtained. Magnesium acetate concentrations of 10 and 25 mM reduced the yield of polysomes in the postmitochondrial supernatant. As satisfactory result were obtained with

1, 2, or 5 mM magnesium acetate, a concentration of 2 mM was chosen arbitrarily for inclusion in the lysis buffer.

In this particular experiment polysomal profiles were analysed by centrifugation in the SW41 rotor which gave a better resolution of polysome profiles than centrifugation in the SW27.1 rotor. As a result of this change, additional shoulders were observed on the heavy sides of the monosomes and disomes at 1 mM Mg^{2+}. These shoulders increased with increasing Mg^{2+}-concentrations, while simultaneously the main monosome and disome peaks decreased. In the

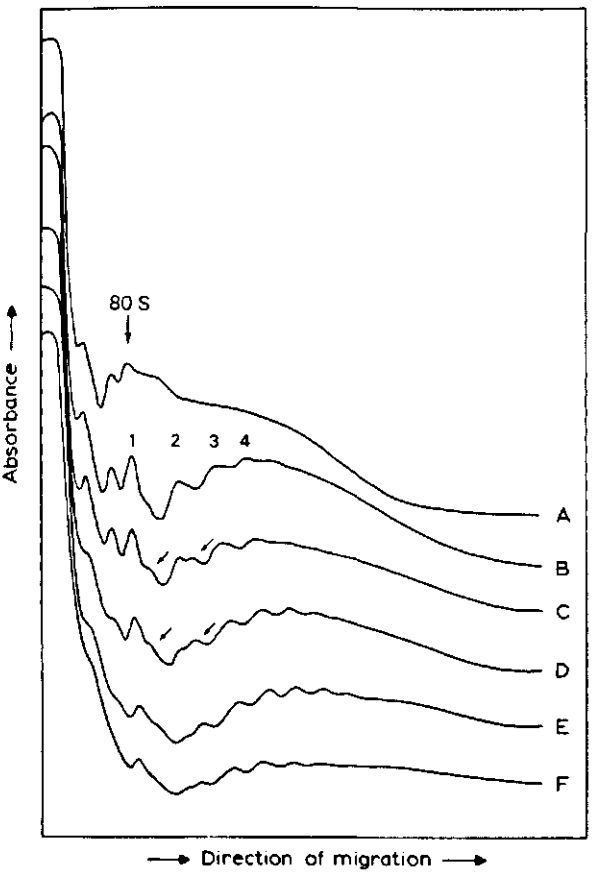

FIG. 9. Effect of different Mg^{2+} concentrations on the isolation of the postmitochondrial supernatant from cycloheximide treated cells. To 6×10^7 cells, cycloheximide was added in a final concentration of 25 µg/ml growth medium. After 8 hrs the cells were harvested, washed and lysed in 0.6 ml lysis buffer containing different magnesium acetate concentrations. Fractions (250 µl) were layered on to 15–33.5% isokinetic sucrose gradients made up in lysis buffer with the corresponding magnesium acetate concentration without NP40. Centrifugation was carried out for 75 min at 205,000 *g* in the SW41 rotor at 5 C.
A: no Mg^{2+}; B: 1 mM Mg^{2+}; C: 2 mM Mg^{2+}; D: 5 mM Mg^{2+}; E: 10 mM Mg^{2+}; F: 25 mM Mg^{2+}.
Figures indicate the monosomes, disomes, etc.
Small arrows indicate shoulders which become higher.

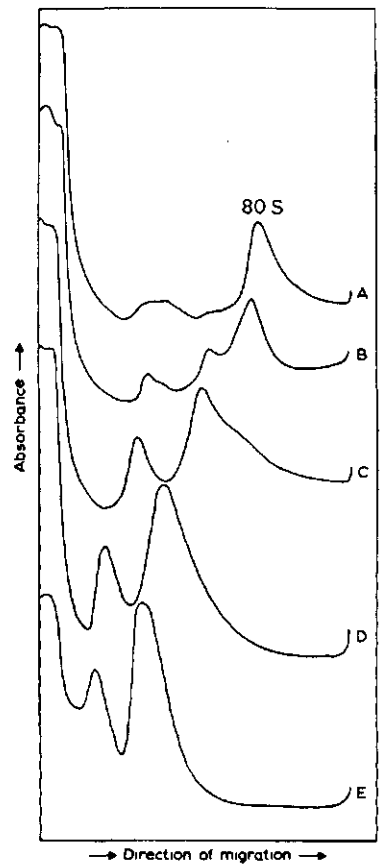

FIG. 10. Sedimentation characteristics of ribosomes and their subunits under different experimental conditions. Cells (6×10^7) were harvested, washed and lysed in 1 ml lysis buffer pH 9.0 (B, C, D, E) or pH 7.0 (A) containing 2 mM Mg^{2+} (A, B, D, E) or no Mg^{2+} (C). Two of the postmitochondrial supernatants were made 500 mM with respect to KCl (D) and 25 mM with respect to EDTA (E) by the addition of the solid chemical. Fractions (250 µl) of the different postmitochondrial supernatants were layered on to 15–33.5% isokinetic sucrose gradients made up in lysis buffer with the above mentioned modifications without NP40. Centrifugation was carried out for 5 hrs at 205,000 *g* in the SW41 rotor at 5 °C.
A: pH 7.0, 2 mM Mg^{2+}; B: pH 9.0, 2 mM Mg^{2+}; C: pH 9.0, no Mg^{2+}; D: pH 9.0, 2 mM Mg^{2+}, 500 mM KCl; E: pH 9.0, 2 mM Mg^{2+}, 25 mM EDTA.

case of the disome the original shoulder even became the main peak. The significance of this observation will be discussed later.

3.3.4. *Sedimentation characteristics of ribosomes and their subunits under different conditions*

During the course of these experiments it was noticed that different pH values and different dissociating conditions altered the sedimentation characteristics of the ribosomes and their subunits. Fig. 10 summarizes these observations.

When a postmitochondrial supernatant was prepared and analysed under low salt conditions at pH 7.0 (Fig. 10A), ribosomes and their subunits sedimented somewhat faster than at pH 9.0 (Fig. 10B). When magnesium acetate was omitted from the lysis buffer and the sucrose gradient, most but not all ribosomes dissociated into subunits (Fig. 10C), which sedimented somewhat slower than subunits isolated under 2 mM Mg^{2+}-conditions (Fig. 10B). High salt conditions (Fig. 10D) further decreased the sedimentation rates of the ribosomal subunits and when ribosomes were dissociated in the presence of 25 mM EDTA (Fig. 10E) the large ribosomal subunits sedimented even at the same speed as the small ribosomal subunit in the untreated postmitochondrial supernatant (Fig. 10B).

3.3.5. *Polysomes from infected cells*

When polysomes are isolated from 24 hr infected cells under optimal conditions and analysed on isokinetic sucrose gradients, a profile similar to that of healthy cells can be observed (results not shown). In both healthy and 24 hr infected cells the polysomes containing 4 or sometimes 5 ribosomes are most abundant.

3.3.6. *Immunoprecipitation of AcNPV polyhedral protein synthesizing polysomes*

As immunoprecipitation of polyhedral protein synthesizing polysomes would be an elegant method to purify the messenger coding for polyhedral protein, a

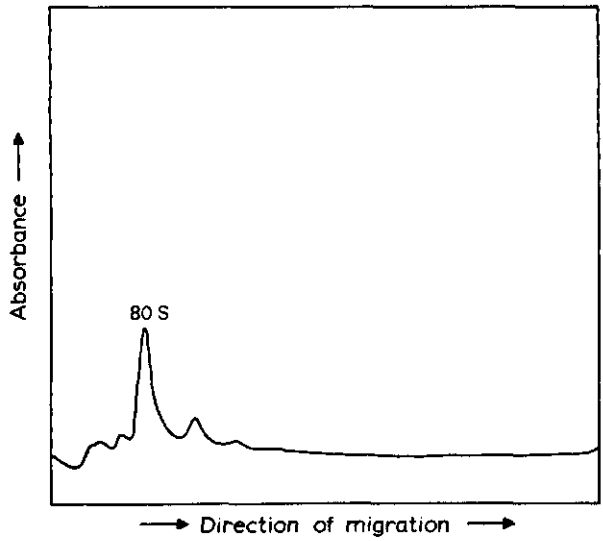

FIG. 11. Polysomal breakdown resulting from pelletting and resuspension. To 6×10^7 cells cycloheximide was added in a final concentration of 25 µg/ml growth medium. After 8 hrs cells were harvested, washed and lysed in 1 ml lysis buffer. The postmitochondrial supernatant was layered on to a 27% (w/w) sucrose cushion made up in lysis buffer without NP40 and centrifuged for 17 hrs at 60,000 *g* in the SW50 rotor at 5 C. Polysomal pellets were resuspended in 1 ml lysis buffer without NP40. A sample of 300 µl was layered on to a 15–33.5% isokinetic sucrose gradient made up in lysis buffer without NP40. Centrifugation was for 75 min at 205,000 *g* in the SW41 rotor at 5 C.

few experiments were performed in order to find out whether this technique was applicable to our system. Following the protocol described in section 3.2.3., which is more or less the standard protocol in these type of experiments, no significant amounts of ^{3}H-uridine labelled material were precipitated from 24 hr infected cells using either pre-immune or anti AcNPV polyhedral protein serum (2 experiments). When the pelletted and resuspended polysomes which were used for these experiments were analysed on isokinetic sucrose gradients, it became evident that during this purification step substantial breakbown of polysomes had occurred (Fig. 11). As the succes of this technique is dependent on the presence of intact polysomes, this breakdown probably was responsible for the poor results of the experiments.

Other attemps to isolate intact polysomes, such as pelletting on a sucrose cushion, pelletting in the presence of emetine (an even stronger run-off inhibitor than cycloheximide), precipitation by high magnesium concentrations (230) and precipitation with ethanol plus magnesium (156) also yielded broken down polysomes or only few polysomes (results not shown).

3.4. Discussion

When postmitochondrial supernatants were prepared from tissue culture cells of *Spodoptera frugiperda* and analysed on sucrose gradients, only monosomes could be detected. From two possible alternatives, run-off and the presence of an endogenous ribonuclease activity, run-off proved to be the most likely cause of polysomal breakdown as the addition of cycloheximide (a run-off inhibitor) to the cell culture medium was able to prevent this breakdown of polysomes (Fig. 5 and fig. 6). Moreover the addition of RNase inhibitors to the lysis buffer did not improve the amount of polysomes.

To obtain a maximum yield of polysomes, the cells were incubated with 25 µg cycloheximide/ml for 8 hours and subsequently lysed in a buffer solution containing 30 mM Tris-HCl (pH 9.0), 10 mM KCL, 3 mM Mg-acetate, 1% NP 40. Incubation for 8 hours with 25 µg cycloheximide/ml is a relatively long period, for which one explanation could be that it takes a long time before an active cylcloheximide concentration has been reached inside the cells. Another possibility might be that there exists a natural pool of monosomes inside these insect cells which, as a result of continuing mRNA synthesis, accumulate into polysomes during the incubation with cycloheximide. However, as cycloheximide inhibits chain initiation as well as chain elongation (225), one would expect at most free monosomes to become mRNA-bound monosomes, which is in contrast with our findings. To elucidate this problem, the time course of inhibition of protein synthesis by cycloheximide should be measured.

The influence of the Mg^{2+} concentration in the lysis buffer on the polysome profiles was examined (Fig. 9). Concentrations of 1 to 5 mM Mg^{2+} gave satisfactory results. Higher concentrations decreased the yield of polysomes, which might be explained by the fact that high Mg^{2+}-concentrations cause precipi-

tation of ribosomes (104, 230). When Mg^{2+} was omitted from the lysis buffer, polysome dissociation was observed. Furthermore, lysis buffers with different pH values, in the range from pH 7.0 to pH 9.0, were tested. The buffer of pH 9.0 proved to give the greatest yield (Fig. 8). In these experiments only an influence on the polysome yield was observed and the distribution of polysomes was not affected. An alteration in the solubility of the polysomes which are pelletted with the nuclei at 12,000 *g* might be responsible for this phenomenon.

The influence of different pH values and several dissociating conditions on the sedimentation characteristics of the ribosomes and their subunits are briefly described in this report (Fig. 10). Conformational changes of the ribosomes and their subunits which are not yet fully understood probably are responsible for these differences (292).

When postmitochondrial supernatants are prepared and analysed under low salt conditions two populations of 40 *S* ribosomal units are observed (Fig. 4A, Fig. 7A, Fig. 10A, B, Fig. 11). This is the first time that this heterogeneity in 40 *S* ribosomal subunits has been demonstrated in insect ribosomal preparations. Using CsCl isopycnic centrifigation SAMESHIMA and IZAWA (263) and VAN VENROOIJ et al (337) showed that these two populations of native 40 *S* ribosomal subunits consisted of up to five classes of particles containing different amounts of associated protein. The different classes of particles are thought to be different intermediates in the formation of the mRNA/40 *S* subunit/Met-$tRNA_f$ initation complex and the associated proteins, which can be released by a high salt treatment, most probably represent initiation factors necessary for the formation of this complex (16).

High resolution centrifugation of polysomes on isokinetic sucrose gradients permits the detection of additional shoulders on the heavy side of the mono- and disome peaks in the polysome profiles (Fig. 9). These shoulders are believed to correspond to a monosome or a disome to which and additional 40 *S* ribosomal subunit is attached at the initiation site of the messenger RNA. They have been observed before in a reticulocyte lysate (183) and can be artificially induced in this sytem by the addition of NaF, which prevents the association of the 60 *S* ribosomal subunit to the initiation complex of mRNA and the 40 *S* ribosomal subunit(129, 130). Anisomycin has the same effect on polysome preparations of Ehrlich ascites tumor cells (336) and of HeLa cells (76).

Polysome profiles from 24 hr infected cells were similar to those from healthy cells, four or five ribosomes containing polysomes being the most abundant class of polysomes. This indicates that either polysomes containing viral RNA are not very abundant or that they have about the same size distribution as polysomes containing host RNA. When translated at the maximum ribosomal packing capacity, the most abundant class of polysomes would be expected to contain a messenger RNA of 8–9 *S* (293), which is too small to code for AcNPV polyhedral protein (molecular weight 30,000 dalton). Taking 114 dalton (309) and 311 dalton$\left(\frac{\text{MW dAMP + dCMP + dGMP + dTMP}}{4} - 16\right)$ as the molecular weights of a mean polyhedral protein amino acid and a mean nucleotide respec-

tively, the minimum size of the mRNA for AcNPV polyhedral protein can be calculated to be $\frac{30{,}000}{114} \times 3 \times 311$ which is about 245,000 dalton. When translated at maximum ribosomal packing capacity, a mRNA of this size would be expected to be translated by 8 or 9 ribosomes (293).

Immunoprecipitation of AcNPV polyhedral protein synthesizing polysomes remained without results, when applied to our system. Polysomal breakdown, which occurs during the purification of the polysomes (Fig. 11), probably was responsible for this failure.

4. ISOLATION AND CHARACTERIZATION OF POLY(A)-CONTAINING RNA

4.1. INTRODUCTION

Very abundant mRNA species can sometimes be purified on the basis of their size (reviewed in 317). As most eukaryotic and viral mRNAs contain a tail of poly(A)-residues at their 3'-terminus, purification can best be started by separating poly(A) lacking RNA (mainly ribosomal RNA and transfer RNA) from poly(A) containing RNA. Late in the infection cycle of AcNPV large amounts of polydral protein are synthesized on behalf of the formation of polyhedra in the nucleus. Accordingly, the mRNA for polyhedral protein would also be expected to be present in large quantities. If so, a purification based on the size of the AcNPV polyhedral protein mRNA would perhaps become possible.

Polysome profiles of infected cells, however, do not reveal the presence of large amounts of polysomes with the expected size for polysomes translating the mRNA for polyhedral protein (See discussion of chapter 3). As host mRNA synthesis is probably not completely shut off during a baculovirus infection (164), there may be a competition between host mRNA species and viral mRNA species for the available ribosomes during the incubation with cycloheximide, which preceeded the isolation of polysomes from both healthy and infected cells. To examine this possibility, poly(A) containing RNA was isolated from healthy and 24 hr infected cells and their size distributions were determined by isokinetic sucrose gradient centrifugation. If large amounts of mRNA for AcNPV polyhedral protein had accumulated in the cytoplasm during the incubation with cycloheximide it should be possible to detect it in this way.

4.2. MATERIALS AND METHODS

4.2.1. *Isolation of poly(A) containing RNA*

Healthy and 24 hr infected cells (about 10^7) were labelled for 2 hrs with 50 µCi 5.6.-^{3}H-uridine (46 Ci/mMol) in 0.5 ml growth medium on 25 cm^2 growth area. A postmitochondrial supernatant was prepared at pH 7.5 as described in section 3.2.1. Poly(A) containing RNA was isolated and deproteinized in a one step procedure on a 1 ml oligo(dT) cellulose column (Type T2, Collaborative Research Inc., USA) as described by KRYSTOSEK et al. (173) and by GIELKENS (96). In brief the postmitochondrial supernatant was mixed with an equal volume of 1 M NaCl, 2 mM EDTA, 1% SDS, heated at 37°C for 5 min, and applied to the oligo(dT) cellulose column. Proteins and poly(A) lacking (poly(A)$^-$) nucleic acids were removed by washing the column with 10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.5% SDS, until samples of the buffer which had passed the column were free of radioactivity. Poly(A) containing (poly(A)$^+$)

RNA which was bound to the column was eluted by washing the column with 10 mM Tris-HCl, pH 7.5. Total radioactivity of the column fractions was determined by mixing samples of the 1 ml column fractions with 7 ml Hydroluma (Lumac, Amsterdam, The Netherlands) followed by liquid scintillation counting. Trichloroacetic acid (TCA) precipitable radioactivity of the high salt fractions was determined as follows. Samples were mixed with 5 ml 5 % TCA and 300 µg bovine serum albumine (in 100 µl H_2O). After 30 min at 0 C the precipitates were collected on Whatman GF/C glass fibre filters, washed 3 times with 5 ml cold 5 % TCA, 4 times with 5 ml 0.5 % cold TCA and twice with ethanol. The filters were dried and the RNA was solubilised overnight at room temperature in 1 ml Soluene 350 (Packard, Bruxelles, Belgium) diluted with one tenth volume of water. Then 7 ml Hydroluma was added and radioactivity was determined by liquid scintillation counting. Counts per minute were converted to desintegrations per minute by the external standard method.

4.2.2. *Size analysis of poly(A)$^+$ RNA*

Poly(A)$^+$ RNA was precipitated from the appropriate oligo(dT) cellulose column fractions by the addition of one tenth volume of 2 M Na-acetate (pH 5.0) and two volumes of ethanol at −20 C for 16 hrs. The RNA was collected by centrifugation at 10,000 *g* for 20 min, dried, dissolved in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.2 % SDS, 1 mM EDTA, and layered on top of 15–36.5 % isokinetic sucrose gradients (221, 362) made up in the same buffer. Centrifugation was for 9 hrs at 40,000 rpm in a SW41 (Beckman) rotor. ^{3}H-Uridine labelled 18 *S* and 28 *S* ribosomal RNA and 4 *S* tRNA were used as sedimentation markers on separate identical gradients.

4.3. Results

4.3.1. *Isolation of poly(A)$^+$ RNA*

Poly(A)$^+$ RNA was isolated from a postmitochondrial supernatant by a simple one step procedure in which the RNA was deproteinized and bound to the oligo(dT) cellulose column in the same buffer. Fig. 12 shows a typical elution profile from such an experiment. Table III summarizes the amounts of bound and unbound material from two experiments. It can be seen that 24 hr infected cells incorporate relatively more radioactivity into poly(A)$^+$ material as compared to healthy cells. The fact that 24 hr infected cells still incorporate radioactivity in poly(A)$^-$ material supports the concluson of Knudson (164) that host RNA synthesis is not completely shut-off during a baculovirus infection.

4.3.2. *Size analysis of poly(A)$^+$ RNA*

Poly(A)$^+$ RNA from healthy and 24 hr infected cells was analysed on isokinetic sucrose gradients. As these gradients exhibit constant velocity sedimentation properties for the particles for which they are constructed, *S*-values of unknown molecules can easily be determined from the *S*-values of known mar-

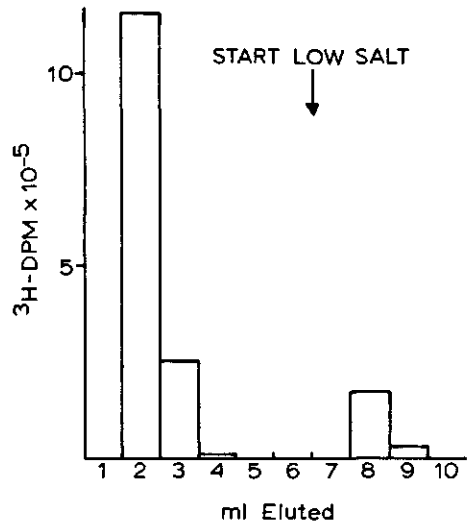

FIG. 12. Oligo(dT) cellulose column chromatography of ^{3}H-uridine labelled cytoplasmic RNA. The radioactivity in the high salt fractions (1–6) is expressed as TCA precipitable dpm and that of the low salt fractions (7–10) as total dpm.

ker molecules. As can be seen from fig. 13 the most abundant poly(A)$^+$ species from healthy cells sediments at somewhat higher *S*-values than the corresponding fraction from 24 hr infected cells. In other experiments, however, this difference was not so pronounced (results not shown). No new abundant mRNA species with an *S*-value corresponding to the size expected for the mRNA for AcNPV polyhedral protein ($\geq$ 12 *S*) seems to be present in the mRNA population from 24 hr infected cells when compared to the mRNA population from healthy cells.

TABLE III. Amounts of poly(A)$^+$ and poly(A)$^-$ RNA in 2 hr labelled healthy and 24 hr infected cells. RNA was isolated as described in section 4.2.1., and expressed as TCA-precipitable dpm for the poly(A)$^-$ RNA and total dpm for the poly(A)$^+$ RNA. No soluble dpm are present in this fraction so the TCA precipitation was omitted.

	poly(A)$^-$ RNA from		poly(A)$^+$ RNA from		% poly(A)$^+$ from	
Experiment number	healthy cells	infected cells	healthy cells	infected cells	healthy cells	infected cells
1	4,661,000 dpm	1,413,000 dpm	228,500 dpm	204,000 dpm	4.7%	12.6%
2	8,351,000 dpm	5,483,000 dpm	789,500 dpm	1,058,000 dpm	8.6%	16.2%

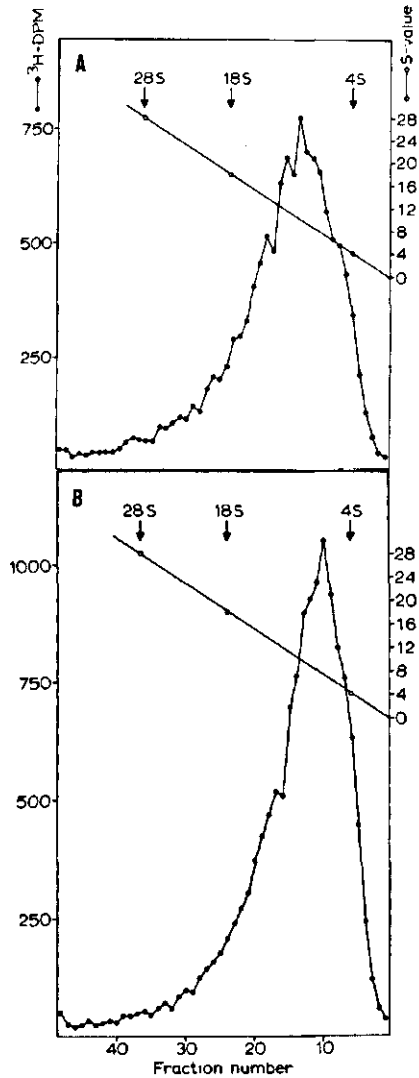

FIG. 13. Size analysis of poly(A)$^+$ RNA on isokinetic sucrose gradients. ^{3}H-Uridine labelled poly(A) containing RNA from healthy (A) and 24 hr infected cells (B) was analysed on 15–36.5% isokinetic sucrose gradients. ^{3}H-Uridine labelled 18 *S* and 28 *S* ribosomal RNA and 4 *S* tRNA were run on separate gradients. Centrifugation was for 9 hrs at 40,000 rpm and 20 C in a SW41 rotor.
(O–O) *S*-value; (●–●) ^{3}H-dpm.

4.4. DISCUSSION

Poly(A) containing RNA was isolated from a postmitochondrial supernatant of healthy and 24 hr infected cells. These RNAs were analysed on isokinetic sucrose gradients to determine whether the mRNA for polyhedral protein was

present as an abundant mRNA species, which could be separated from host mRNA on the basis of its size. However, no new abundant poly(A) containing RNA species could be detected (Fig. 13). Our results generally agree with those of KNUDSON (164), who isolated poly(A) containing RNA from *Spodoptera frugiperda* cells infected with *Spodoptera frugiperda* nuclear polyhedrosis virus. In his study no new abundant mRNA species was found at 24 hrs post infection either. The poly(A) containing RNA population isolated by KNUDSON at 24 hrs post infection sediments at somewhat higher *S*-values than our preparation. This might be explained by the fact that KNUDSON's preparation contained both cytoplasmic and nuclear RNA.

Assuming that a mRNA for AcNPV polyhedral protein is present at 24 hrs post infection (polyhedra formation has already started in our system at that time), our unability to detect it could either mean that is is not present as an abundant mRNA species and may be hidden under host mRNA species or it could mean that it does not contain poly(A), in which case it would have been lost during the isolation procedure.

That a large proportion of the mRNA for polyhedral protein may indeed not contain poly(A) is suggested by the fact that only one third of the virus specific RNA pulse-labelled from 34–36 hrs post infection in *Spodoptera frugiperda* NPV infected *Spodoptera frugiperda* cells is poly(A)$^+$ (164), while the main viral protein synthesized at that time (in AcNPV infected *Spodoptera frugiperda* cells) is the polyhedral protein (47).

The *S*-value of the most abundant messenger from healthy cells is about 9–10 *S* (Fig. 13). Messengers of this size are known to be translated by about 5 ribosomes at the same time, at maximum ribosomal packing capacity (293). As the pentasomes are also one of the most abundant classes of polysomes from healthy cells, this mRNA distribution seems to be a good reflection of the distribution of poly(A)$^+$ RNA in polysomes from healthy cells, and accordingly no breakdown seems to have occurred during the one step isolation procedure.

Looking at the amount of radioactivity incorporated in the poly(A)$^+$ and poly(A)$^-$ fractions of healthy and 24 hr infected cells (Table III) it is striking that the percentage of poly(A)$^+$ material from 24 hr infected cells is much higher than that of poly(A)$^+$ material from healthy cells. From this table it can be seen that this phenomenon is caused by the fact that healthy cells incorporate much more radioactivity in poly(A)$^-$ material. This could mean that in 24 hr infected cells the synthesis or transport to the cytoplasm of poly(A)$^-$ RNA, which is mainly composed of ribosomal and transfer RNA, is inhibited. It could also mean that the turnover of this RNA is much higher.

5. ISOLATION AND CHARACTERIZATION OF AcNPV SPECIFIC RNA

5.1. INTRODUCTION

From the preceding chapters it could be concluded that the isolation of the mRNA for AcNPV polyhedral protein could neither be achieved by immunoprecipitation of AcNPV polyhedral protein synthesizing polysomes (Chapter 3) nor by a fractionation based on a specific size (Chapter 4). As results published by CIBULSKY et al. (50) and MARUNIAK et al. (197) had indicated in the mean time that AcNPV polyhedral protein was likely to be a virus specific protein, it was dediced to isolate virus specific RNA from 24 hr infected cells. Subsequent in vitro translation of these virus specific RNAs (See chapter 6) would indicate whether or not the mRNA for AcNPV polyhedral protein was present in this viral RNA population.

Because host RNA synthesis is not fully shut off during a baculovirus infection (164, chapter 4 of this thesis) DNA-RNA hybridization had to be employed to isolate virus specific RNAs. Apart from KNUDSON (164), who used DNA-RNA hybridization to determine the amount of virus specific RNA in pulse-labelled total, poly(A)$^+$, and poly(A)$^-$ RNA from *Spodoptera frugiperda* NPV infected *Spodoptera frugiperda* cells, there is to my knowledge only one other report concerning baculoviruses in which this technique is used. SOLOMKO et al. (291), working with *Galleria mellonella* NPV, have characterized in vivo synthesized virus specific RNA, isolated by means of hybridization with viral DNA. Total virus specific as well as virus specific polysomal and nuclear RNAs were characterized by means of sucrose gradient centrifugation.

In this chapter experiments are described in which virus specific RNA was isolated from total cytoplasmic RNA by means of hybridization with viral DNA that was covalently coupled to cellulose. Viral RNA isolated in this way was characterized on isokinetic sucrose gradients and by means of polyacrylamide gelelectrophoresis in 98% formamide, which provides fully denaturing conditions.

5.2. MATERIALS AND METHODS

5.2.1. *Labelling of RNA*

RNA which was used for size analysis after hybridization with AcNPV-DNA-cellulose was isolated from about 6×10^7 cells which were labelled for two hours with 400 μCi 5,6-^{3}H-uridine (52 Ci/mMol) in 5 ml growth medium on 150 cm^2 growth area.

5.2.2. *Buffers*

Lysis buffer: 30 mM Tris-HCl (pH 9.0), 10 mM KCl, 2 mM Mg-acetate, 1% NP40.

RNA-extraction buffer: 40 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 % sodium dodecyl sulphate, 2 mM EDTA, 7 M urea.

Hybridization buffer: 100 mM Tris-HCl (pH 7.4), 700 mM NaCl, 1 mM EDTA, 0.1 % sodium dodecyl sulphate.

Wash buffer: 100 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 0.1 % sodium dodecyl sulphate.

SSC: 0.15 M NaCl, 0.015 M Na-citrate (pH 7.5).

TE-buffer: 10 mM Tris-HCl (pH 7.0), 1 mM EDTA.

PBS-TDS: 10 mM phosphate buffer (pH 7.2), 0.9 % NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulphate.

5.2.3. *RNA-extraction*

Cells were removed from the surface of the culture vessel by vigorous pipetting and pelletted at 1000 *g* for 10 min. Cells were washed once with ice-cold phosphate buffered saline (PBS), except when ^{3}H-uridine labelled RNA was isolated; then cells were washed three times with PBS. Cells were disrupted by resuspending them in lysis buffer. Nuclei were removed by centrifugation at 4000 *g* for 10 min. The supernatant was mixed with an equal volume of twice concentrated RNA extraction buffer. This solution was extracted twice with an equal volume of a mixture of 80 ml phenol, 20 ml RNA extraction buffer, 0.1 g 8-hydroxyquinoline, 96 ml chloroform and 4 ml isoamylalcohol. The resulting two phenol phases were extracted again with an equal volume of RNA extraction buffer. The two water phases were combined and extracted twice with chloroform-isoamylalcohol (24:1). Then the RNA was precipitated at –20 C by adding 1/10 volume of 2 M Na-acetate (pH 5.0) and 2 volumes of ethanol. The RNA was collected by centrifugation at 10,000 *g* for 20 min, dried and dissolved in RNA extraction buffer. Subsequently the whole extraction procedure was repeated.

5.2.4 *Isolation of viral DNA*

Viral DNA was isolated from non-occluded, extracellular virus as described by VLAK and ODINK (334). Cells were removed from the virus containing growth medium by low speed centrifugation. After filtration through a 450 nm filter, virus particles were pelletted by centrifugation at 40,000 *g* for 45 min. The virus pellet was dissolved in a small volume of 50 mM phosphate buffer (pH 7.0), 10 mM EDTA and an equal volume of 4 % sodium lauryl sarcosinate in the same buffer was added. After gentle mixing the virus particles were disrupted by incubation at 60 C for 20 min. Then solid CsCl was added to the lysate to a density of 1.58 g/cm^3 in a volume of 4 ml. This 4 ml DNA containing CsCl solution was layered on top of 4 ml CsCl solution with a density of 1.78 g/cm^3. These discontinuous CsCl gradients were centrifuged for 16 hrs at 35,000 rpm and 10 C in a Spinco R50 Ti rotor. The DNA containing fractions were determined by OD_{260} measurement, pooled, dialysed against TE buffer, and concentrated by dialysis against 30% polyethylene glycol 20,000 in TE buffer.

The DNA was further purified by centrifugation on 10–29.5 % isokinetic su-

crose gradients, prepared according to NOLL (221) and VAN DER ZEYST and BLOEMERS (362). The gradients were made in 50 mM Tris-HCl (pH 8.1), 200 mM NaCl, 0.1 % sodium lauryl sarcosinate, 1 mM EDTA. Centrifugation was for 16 hrs at 18,000 rpm and 5 °C in a SW27 (Beckman) rotor. The DNA containing fractions of the gradients were dialysed against TE buffer and concentrated as mentioned above.
As a final purification step the DNA solution was extracted twice with an equal volume of chloroform-isoamylalcohol (24:1). After boiling the DNA solution for 10 min, the denatured DNA was precipitated at –20 °C by the addition of 1/10 volume of 2 M Na-acetate (pH 5.0) and 2 volumes of ethanol.

5.2.5. *DNA-cellulose*

About 100 µg of viral DNA was coupled to 10 mg diazotized m-aminobenzyloxymethyl-cellulose (Miles, USA), exactly as described by NOYES and STARK (224).

5.2.6. *Hybridization and elution of viral RNA*

Alcohol precipitated RNA was collected at 18,000 *g* for 20 min, dried, and dissolved in twice concentrated hybridization buffer in a concentration of 1 or 2 mg/ml. To this RNA solution, which contained about 5 mg of cytoplasmic RNA in preparative experiments, an equal volume of formamide, purified with the mixed bed ion exchanger Merck nr. V, was added. The AcNPV-DNA-cellulose was suspended in this mixture. Hybridization was for 48 hrs at 37 °C, keeping the cellulose in suspension by rotary shaking.

RNA which was not hybridized, was removed by washing the AcNPV-DNA-cellulose twice with 2 × SSC at room temperature, twice with washbuffer containing 50% formamide at 37 °C for 20 min, and twice again with 2 × SSC.

Hybridized RNA was eluted by resuspending the AcNPV-DNA-cellulose twice in 0.2 ml 10 mM Tris-HCl (pH 7.5) at 90 °C, followed by rapid cooling in an ice-bath.

5.2.7. *Isokinetic sucrose gradients*

To 500,000 cpm of ^{3}H-uridine labelled RNA which was selected on AcNPV-DNA-cellulose and eluted at 90 °C, about 100 µg of unlabelled Cowpea chlorotic mottle virus RNA (kindly provided by Dr. Verduin) was added as an internal marker. This mixture was layered on top of 15–36.5 % isokinetic sucrose gradients (221, 362). Gradients were made in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.2 % sodium dodecyl sulphate, 1 mM EDTA. Centrifugation was for 15 hrs at 36,000 rpm and 20 °C in a SW41 (Beckman) rotor.

Sucrose gradient fractions were mixed with 7 ml Hydroluma (Lumac, Amsterdam, The Netherlands) and radioactivity was determined by means of liquid scintillation counting.

5.2.8. *Polyacrylamide gel electrophoresis in 98 % formamide*

^{3}H-Uridine labelled RNA which was selected on AcNPV-DNA-cellulose and

eluted at 90° C was precipitated in the presence of 20 µg unlabelled Cowpea chlorotic mottle virus RNA by the addition of 1/10 volume of 2 M Na-acetate (pH 5.0) and two volumes of ethanol at –20° C, dried, and dissolved at 37° C for 15 min in a solution of 0.5 g sucrose in 4 ml formamide plus 0.08 ml 1.0 M NaCl according to PEDEN and SYMONS (238). Vertical slab gels (4%, 14 cm long, 1.5 mm thick) were prepared as described by STAYNOV et al. (297). A mixed bed ion exchanger (Merck Nr V) was used to purify the formamide before use. Pre-electrophoresis was at 100 V for 1 hr and electrophoresis was at 100 V for 13 hrs. Gels were stained with 0.05% toluidine blue, 55 mM Na-acetate, 0.1 mM EDTA (pH 5.5) and destained in the same solution without dye. After measuring the migration distances of the marker RNAs the gel was prepared for scintillation autoradiography according to the procedure described by BONNER and LASKEY (36).

5.3. RESULTS

5.3.1. *DNA-RNA hybridization*

DNA was isolated from non-occluded extracellular virus and coupled to diazotized m-aminobenzyloxymethyl-cellulose, as described in the sections 5.2.4. and 5.2.5. ^{3}H-Uridine labelled RNA was extracted from a postnuclear supernatant of either healthy or 24 hr infected cells, and hybridized to 5 mg AcNPV-DNA-cellulose for 48 hrs.

The specificity of the hybridization was investigated by comparing the amounts of RNA from healthy and infected cells which were selected on AcNPV-DNA-cellulose. In three experiments the percentage non-specific hybridization, which is defined as hybridization of AcNPV-DNA-cellulose, with ^{3}H-labelled RNA from healthy cells, was 12.3, 3.7 and 11.5% respectively (Table IV). This indicates that the hybridization procedure was able to remove most of the host RNA.

5.3.2. *Size analysis of viral RNA*

^{3}H-Uridine labelled RNA which was selected on AcNPV-DNA-cellulose was used for size analysis on isokinetic sucrose gradients (Fig. 14).

TABLE IV. Efficiency and specificity of the hybridization procedure, using ^{3}H-labelled RNA from either healthy or 24 hr infected cells and 5 mg AcNPV-DNA-cellulose.

Experiment No.	Input (dpm)	Amount of RNA from 24 hr infected cells selected on AcNPV-DNA-cellulose (dpm)	Amount of RNA from healthy cells selected on AcNPV-DNA-cellulose (dpm)	% non-specific hybridization
1	409,000	3,295	405	12.3
2	571,000	42,880	1,582	3.7
3	5,936,000	763,000	87,800	11.5

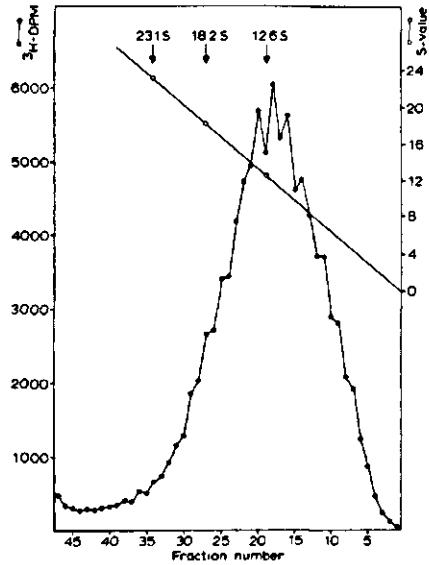

FIG. 14. Size analysis of viral RNA on isokinetic sucrose gradients. ^{3}H-Uridine labelled RNA, selected on AcNPV-DNA-cellulose and eluted at 90 C, was mixed with 100 µg unlabelled CCMV-RNA (RNA 1 + 2: 23.1 *S*, RNA 3: 18.2 *S* and RNA 4: 12.6 *S*) and analysed on 15–36.5 % isokinetic sucrose gradients. Centrifugation was for 15 hrs at 36,000 rpm and 20 C in a SW41 rotor.
(O–O) *S*-value; (●–●) ^{3}H-dpm.

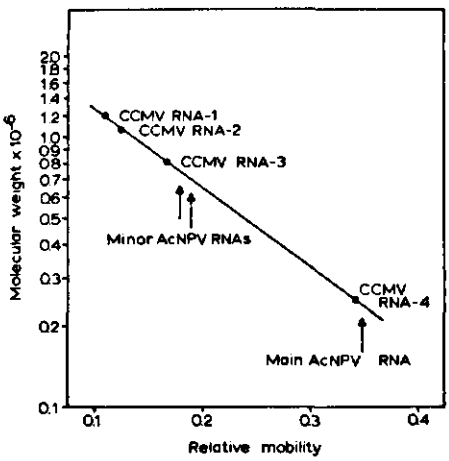

FIG. 15. Size analysis of viral RNA by means of polyacrylamide gel electrophoresis in 98 % formamide. ^{3}H-Uridine labelled RNA which was selected on AcNPV-DNA-cellulose was mixed with 20 µg unlabelled CCMV-RNA (RNA 1: 1.20 × 10^6, RNA 2: 1.07 × 10^6, RNA 3: 0.81 × 10^6, RNA 4: 0.25 × 10^6 dalton) and subjected to polyacrylamide gel electrophoresis as described in section 5.2.8.

Using the *S*-values given by BANCROFT et al. (18) for Cowpea chlorotic mottle virus (CCMV) RNA, which was run on the same gradient, it can be calculated that the majority of the AcNPV RNA species sediments between 10 and 14 *S*.

As analysis under fully denaturing conditions gives a more reliable estimate of RNA sizes, AcNPV RNA was also analysed by means of polyacrylamide gelelectrophoresis in 98 % formamide (Fig. 15). One predominant and two minor RNA species were observed against a faint continuous background of other RNAs. The molecular weights of the discrete RNAs were determined relative to CCMV-RNA, which was co-electrophoresed. Using the molecular weights established by REIJNDERS et al. (256) for CCMV-RNA (RNA 1: 1.20×10^6, RNA 2: 1.07×10^6, RNA 3: 0.81×10^6, RNA 4: 0.25×10^6), the main AcNPV RNA was calculated to have a molecular weight of 240,000 dalton. The two minor AcNPV RNAs have molecular weights of 700,000 and 750,000 dalton respectively.

5.4. DISCUSSION

AcNPV specific RNA was isolated by means of hybridization with viral DNA. Although DNA-RNA hybridization has been used for analytical purposes since many years, it was not until recently that various reports describing a quantitative recovery of analyzable and translatable amounts of hybridized RNA were published. Hybridization in solution, followed by separation of the hybrids on hydroxyapatite (186), by an aqueous polymer two-phase system (192), by exclusion chromatography (240) or by affinity chromatography (53), followed by melting of the hybrid and isolation of RNA on oligo(dT) cellulose columns or by sucrose gradient centrifugation, results in considerable losses of RNA and in total loss of the DNA hybridization probe. Methods using DNA bound to an insoluble support do not suffer from this disadvantage. The filter hybridization technique, originally used analytically only (98), has been adapted for this purpose by binding large amounts of DNA to a filter (201, 343). Other methods involve the binding of DNA to a fine dispersed solid matrix such as agar (35), Sepharose (97, 192) or cellulose (19, 224, 278, 335). We have used the method described by NOYES and STARK (224), which has been used to isolate eukaryotic (5) as well as viral mRNA (8, 49, 81, 131, 152).

We used only cytoplasmic RNA in our hybridization experiments. Nuclei still contain viral DNA which is a competitor in our preparative hybridization procedure. Moreover, primary transcripts (that have to be processed first) have recently been found in nuclei for several eukaryotic and viral mRNAs (1,69). That such processing events may also take place in baculovirus infected cells, may be concluded from the much smaller size of the virus specific polysomal RNAs, as compared to nuclear RNAs in *Galleria mellonella* NPV infected larvae (291). These primary transcripts may also compete in the hybridization procedure. They may also interfere with the size analysis if they are much longer than the functional mRNAs. In addition these primary transcripts may produce non-functional and/or prematurely terminated translation products in the trans-

lation experiments described in chapter 6.

In our hybridization procedure with total cytoplasmic RNA less than 12.5% non-specific hybridization was observed (Table I). Optimalization of the washing procedure might lower this figure (201). As ribosomal RNA and transfer RNA also contribute to the percentage non-specific hybridization, oligo dT-cellulose column chromatography prior to the hybridization procedure may increase the specificity of the procedure for the poly(A) containing viral mRNAs.

Analysis of ^{3}H-uridine labelled RNA, selected on AcNPV-DNA-cellulose, on isokinetic sucrose gradients, shows that the viral RNA isolated at 24 hrs post infection predominantly sediments at 10–14 *S* (Fig. 14). Now that we know the size of the main viral RNA, it can be concluded that a size-dependent separation from host mRNA (as investigated in chapter 4) would have been very difficult if not impossible. Because host mRNA sediments at 8–12 *S* (Fig. 13A) it partially overlaps with the viral RNA population which sediments at 10–14 *S* (Fig. 14).

As analysis under fully denaturing conditions gives a more precise estimate of RNA sizes, polyacrylamide gel electrophoresis in 98% formamide was employed to determine the molecular weight of the viral RNA (Fig. 15). One predominant RNA with a molecular weight of 240,000 dalton and two minor RNAs (molecular weight 700,000 and 750,000 dalton) were detected against a faint continuous background of other RNA species. The predominant 240,000 dalton RNA species might represent the mRNA for polyhedral protein as its molecular weight is just enough to code for a protein with a molecular weight of 30,000 dalton.

6. TRANSLATION OF AcNPV SPECIFIC RNA

6.1. INTRODUCTION

To determine which proteins are coded for by the AcNPV RNA preparation which can be isolated from 24 hr infected *Spodoptera frugiperda* cells by means of hybridization to AcNPV-DNA, in vitro translation in a cell-free system derived from wheat germ was employed.

6.2. MATERIALS AND METHODS

6.2.1 *Cell-free translation of viral RNA*

RNA which was selected on 10 mg AcNPV-DNA-cellulose (prepared as described in section 5.2.5.) and eluted at 90 C, was precipitated at –20 C by the addition of 1/10 volume of 2 M NH_4-acetate and two volumes of ethanol. Yeast RNA (7.5 µg) was added to ensure efficient precipitation. The RNA was collected at 8000 *g* for 4 min in an Eppendorf centrifuge, washed once with 100% ethanol, dried, and dissolved in 20 µl sterile double distilled water.

The reaction mixture for the experiment in fig. 16 contained per 30 µl incubation mixture: 15 µl wheat germ S-30, prepared from floated material according to DAVIES et al (70), 20 mM HEPES (pH 7.5. with KOH), 10 mM Tris (pH 7.5 with acetic acid), 2.9 mM Mg^{2+}, 0.4 mM spermidine-HCl, 140 mM K^+ (130 mM as K-acetate; about 10 mM from KOH and ATP), 37.5 µM of each amino acid except for the labelled one, 2.5 mM ATP (K^+-salt), 0.375 mM GTP (tri-Li-salt), 10 mM creatinephosphate (Tris-salt), 10 µg/ml creatine kinase, 0.5 mM dithiotreitol, 1.5 µl ^{35}S-methionine (1090 Ci/mM; 5.1 mCi/ml, The Radiochemical Center, Amersham) and 2 µl AcNPV mRNA.

The reaction mixture for the experiment in fig. 17 contained per 50 µl incubation mixture: 15 µl wheat germ S-30 prepared from floated material according to MARCU and DUDOCK (193), 26 mM Hepes (pH 7.6 with KOH), 2.5 mM Mg^{2+}, 0.4 mM spermidine-HCl, 116 mM K^+ (70 mM as K-acetate; 36 mM as KCl, and about 10 mM from KOH and ATP), 34.5 µM of each amino acid except for the labelled one, 2.5 mM ATP (K^+-salt), 0.225 mM GTP (K^+-salt), 7 mM creatinephosphate (Na^+-salt), 10 µg/ml creatine kinase, 1.8 mM β-mercaptoethanol, 2.5 µl ^{35}S-methionine (820 Ci/mM; 7.8 mCi/ml; The Radiochemical Center, Amersham) and 10 µl AcNPV mRNA.

Products were analysed on 10% vertical slab gels (14 cm long, 1.5 mm thick) according to LAEMMLI (179). Gels were stained with 0.4% (w/v) Coomassie Brilliant Blue in methanol/acetic acid/H_2O (46:7:47) and destained in methanol/acetic acid H_2O (30:7:63). The gel in fig. 16 was dried on Whatman 3MM paper. The gel in fig. 17 was first processed for scintillation autoradio-

graphy (36) and then dried on Whatman 3MM paper. Bands were visualized by exposure of the dried gels to X-ray film (Kodak, RP Royal X-omat).

6.2.2. *Immunoprecipitation of translation products*

Antiserum against purified polyhedral protein was prepared by injecting rabbits once with 2 mg polyhedral protein intravenously and subsequently twice intramuscularly with 10 mg polyhedral protein emulsified with an equal volume of Freund's complete adjuvant. The injections were given at two week intervals. Immunoglobulins were purified by $(NH_4)_2SO_4$-precipitation. Polyhedral protein specific antibodies were isolated by affinity chromatography on a column to which purified polyhedral protein had been coupled according to the procedure in the descriptive booklet (Pharmacia; CNBr-activated Sepharose 4B). After this procedure the titer of the purified antibodies, as determined by the Ouchterlony double diffusion test was 1/128 when assayed against 1 mg/ml purified polyhedral protein. No precipitation was observed with 0.65 mg/ml purified extracellular virus when assayed against 26 mg/ml purified antibodies, indicating that only polyhedral protein was precipitated by these antibodies.

To 25 µl wheat germ incubation mixture 10 mM EDTA was added and it was diluted twice with an equal volume of 2 × PBS-TDS. This solution was centrifuged for 5 min in an Eppendorf centrifuge at 8000 *g* and 2 µl purified antibodies was added to the supernatant. The antibodies were allowed to react for 18 hrs at 4 C. Then 5 mg protein A-Sepharose (Pharmacia; preswollen in PBS and washed with PBS-TDS) was added to precipitate the immunoglobulins. The incubation was continued for another hour at room temperature, after which the protein A-Sepharose spheres were pelletted at 8000 *g* for 2 min in an Eppendorf centrifuge, washed three times with PBS-TDS, and resuspended in Laemmli sample buffer (20 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate, 10% glycerol, 1% β-mercaptoethanol, 0.001% bromophenol blue). The suspension was heated at 100 C for 2 min before analysis on 10% polyacrylamide gels.

6.3. Results

6.3.1. *Cell-free translation of viral RNA*

AcNPV RNA selected on AcNPV-DNA-cellulose was used in vitro translation experiments in a cell-free protein synthesizing system derived from wheat germ. The system we used was optimized for plant viral mRNAs. Although the conditions may not have been optimal for AcNPV mRNA, a four-fold stimulation of ^{35}S-methionine incorporation into trichloroacetic acid precipitable material was found upon the addition of AcNPV RNA.

RNA from uninfected cells, selected on AcNPV-DNA-cellulose (if any) did not stimulate the wheat germ system.

Analysis of the translation products on polyacrylamide gels revealed many discrete products (Fig. 16D). One of these products with a molecular weight of 30,000 dalton, comigrated with the purified polyhedral protein (Fig. 16C). Pro-

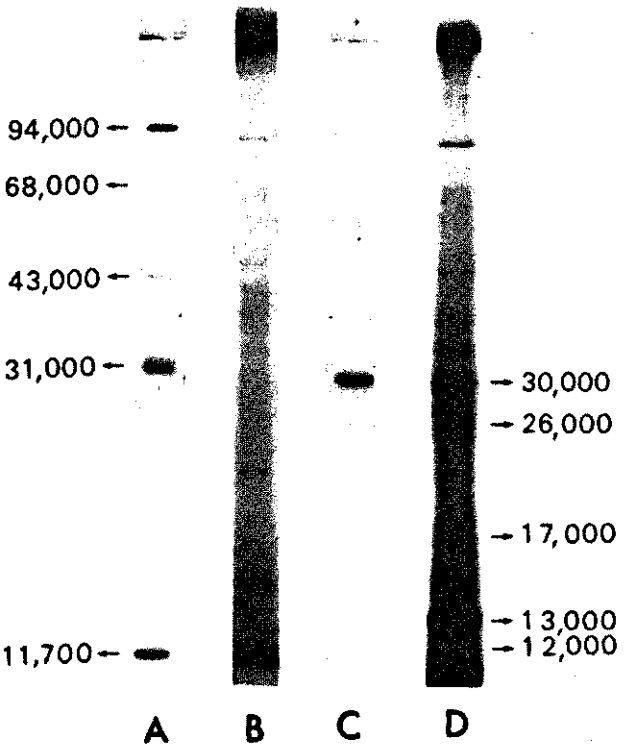

FIG. 16. Cell-free translation of AcNPV RNA. AcNPV RNA isolated at 24 hrs post infection was translated for 1 hr at 30 C in a wheat germ system. Products were analysed on 10% polyacrylamide gels. Gels were stained, dried and subjected to autoradiography. A) Unlabelled, stained marker proteins: Phosphorylase A (MW: 94,000), Bovine serum albumin (MW: 68,000), Ovalbumin (MW: 43,000), DNase (MW: 31,000), Cytochrome C (MW: 11,700). B) Labelled wheat germ system endogenous products. C) Unlabelled, stained purified AcNPV polyhedral protein. D) Labelled wheat germ products synthesized upon the addition of AcNPV RNA.

ducts with molecular weights higher than that of the AcNPV polyhedral protein and some products of lower molecular weight are believed to correspond to endogenous products as they can also be observed in an incubation mixture to which no RNA was added (Fig 16B).

In addition to the 30,000 dalton product, proteins with molecular weights of 26,000, 17,000, 13,000 and 12,000 dalton were observed in this particular experiment. In other translation experiments, however, performed under somewhat different conditions, different sets of low molecular weight proteins were observed in addition to the 30,000 dalton product, which was always synthesized (Fig. 17A).

6.3.2. *Immunoprecipitation of translation products*

To verify whether the 30,000 dalton translation product which comigrated with AcNPV polyhedral protein on polyacrylamide gels was indeed the AcNPV polyhedral protein, immunoprecipitation was employed.

After translation, the incubation mixture was incubated with antibodies against AcNPV polyhedral protein. After the incubation, protein A-Sepharose was used to precipitate the immunoglobulins from the incubation mixture. Analysis of the immunoprecipitates on polyacrylamide gels showed that only the 30,000 dalton product had reacted with the antibodies (Fig. 17). This indicates that the in vitro synthesized 30,000 dalton product and the AcNPV polyhedral protein are likely to be identical.

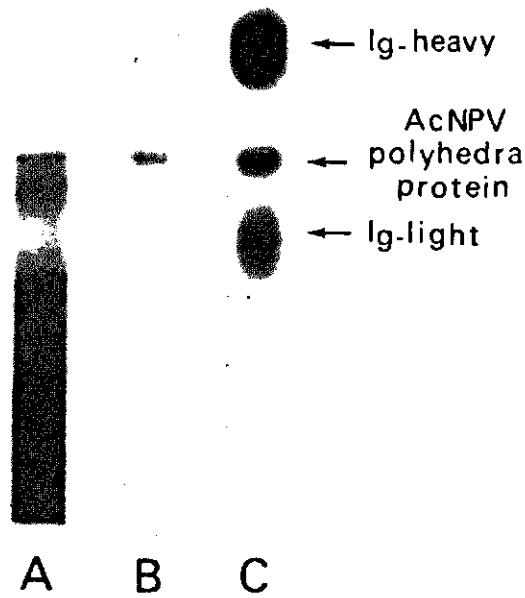

FIG. 17. Immunoprecipitation of cell-free translation products. AcNPV RNA isolated at 24 hrs post infection was translated in a wheat germ system. After the incubation period of 1 hr at 30 C, 25 µl was processed for immunoprecipitation with antibodies against AcNPV polyhedral protein as described in section 6.2.2. The untreated incubation mixture and the immunoprecipitate were stained, processed for scintillation autoradiography, dried, and exposed to X-ray film. A) Labelled wheat germ system products, synthesized upon the addition of AcNPV RNA. B) Labelled immunoprecipitate of the products in track A. C) Stained version of track B; unlabelled AcNPV polyhedral protein was added as an internal marker before electrophoresis, but after the immunoprecipitation.

6.4. Discussion

After translation of preparatively isolated AcNPV RNA in an in vitro protein synthesizing system derived from wheat germ, discrete products were observed upon analysis on polyacrylamide gels (Fig. 16). A 30,000 dalton product comigrated with AcNPV polyhedral protein and was precipitated by antibodies against AcPNV polyhedral protein (Fig. 17), indicating that the in vivo and in vitro synthesized products are similar if not identical.

The 30,000 dalton product was always found but, depending on the conditions in the translation mixture, different sets of low molecular weight proteins were produced (Compare fig. 16 and fig. 17), which is a little bit strange in view of the fact that size analysis reveals only one major RNA species (Chapter 5). An explanation for this phenomenon might be that the low molecular weight proteins are prematurely terminated translation products, which are terminated at different sites (depending on the conditions in the wheat germ system), or products of broken down mRNAs. In this case, however, one would expect them to react with antibodies against AcNPV polyhedral protein, unless the antigenic group is synthesized at the C-terminus of the protein (or when the mRNA or part of the mRNA is translated in an other reading frame). As labelling was from 22–24 hrs infection size analysis included only mRNAs which were synthesized during that period. Messenger RNA species which were synthesized before 22 hrs post infection might also be responsible for the synthesis of the low molecular weight proteins. This hypothesis is supported by the observation of Carsten et al. (47) that apart from the polyhedral protein, some virus induced low molecular weight proteins are synthesized even until very late in the infection cycle.

No products, corresponding to the two minor AcNPV specific RNAs which were seen in chapter 5 (Fig. 15), were observed in the translation experiments. This may, however, be caused by the low concentration of these RNAs.

7. SUMMARY AND GENERAL DISCUSSION

The purpose of this study was to investigate the origin of the polyhedral protein of the nuclear polyhedrosis virus of the alfalfa looper, *Autographa californica* (AcNPV), one of the best characterized viruses of the family Baculoviridae. The present knowledge of the properties of the nuclear polyhedrosis virus of *Autographa californica*, are described in chapter 1.

One of the most striking features of nuclear polyhedrosis viruses is that singly or multiply enveloped virus particles become occluded into protein crystals during their multiplication cycle in the nucleus of their invertebrate host cells. Because of their shape these crystals are called polyhedra. The formation of occlusion bodies is not restricted to the nuclear polyhedrosis viruses. Several other virus families exhibit this phenomenon. The similarities and differences between the occlusion bodies of different virus families are also described in chapter 1 to provide basic information for the subsequent summary of arguments that have been used in favour of either the hypothesis that the occlusion body protein is produced by the host as a defence mechanism against the virus, or the hypothesis that it is produced by the virus to protect itself against environmental influences.

Although previous investigations have provided indirect evidence that the polyhedral protein might be of viral origin (50, 197, 378), our approach resulted in direct proof that this protein is coded for by the viral genome. By isolating the messenger RNA for polyhedral protein and demonstrating that this mRNA is of viral origin, we were able to solve this more than 30 year old (27) problem.

To start with a genetically pure virus preparation, and to avoid as much as possible the formation of the so-called few polyhedra variant (Section 1.2.3.), we have plaque-purified the virus (Chapter 2). Minor variants which were present in the original preparation were removed by this procedure.

Our attemps to isolate the mRNA for AcNPV polyhedral protein have moved along various experimental pathways. Our first approach is reflected in chapter 3, which describes the isolation of polysomes from healthy and infected cells. As AcNPV polyhedral protein is a very abundant protein late in the infection cycle (the whole nucleus eventually becomes filled with polyhedra), one would expect that the mRNA for this protein would also be present in large amounts. Consequently, a certain size class of polysomes would be favoured in infected cells when compared to the polysomal size distribution of healthy cells. No significant differences, however, were detectable between the polysomal profiles of healthy and 24 hr infected cells when analysed on isokinetic sucrose gradients. As a consequence no new abundant mRNA size class would be expected to be translated by polysomes from 24 hr infected cells, although new mRNAs may be present in the existing size classes.

However, some interesting characteristics were discovered when the optimum conditions for the isolation of polysomes were determined.

– Intact polysomes could only be detected when cycloheximide was added to the

cell culture medium while the addition of RNase inhibitors did not increase the yield of polysomes. Run-off is the most satisfactory explanation for the effect of these chemicals on the yield of polysomes.
- The yield of polysomes was increased by using a lysis buffer solution with a high pH (pH 9.0). As high pH values are also infavourable for lysosomal RNases (239), this observation includes the additional advantage of minimizing any residual RNase activity.

Some characteristics which had been observed before in vertebrate systems were also observed in our invertebrate system. They include:
- The existence of two populations of 40 *S* particles when ribosomes are analysed under low salt conditions (Chapter 3: fig. 4A, 7A, 10A, 10B, 11).
- The demonstration of initiation complexes in sucrose gradient profiles of polysomes from cycloheximide treated cells (Chapter 2: fig. 9).
- The difference in sedimentation behaviour of ribosomes and their subunits under various conditions (Chapter 3: fig. 10).

These observations have been described before in vertebrate cell systems but have not yet been reported in any invertebrate system.

Another approach in the isolation of the mRNA for AcNPV polyhedral protein, which involved the immunoprecipitation of polyhedral protein synthesizing polysomes, is also described in chapter 3. Polysomal breakdown, which occurred during the necessary purification procedure of the polysomes, probably was responsible for the negative results obtained with this technique.

As polysomes were induced by the incubation of cycloheximide, which inhibits protein-, but not mRNA synthesis, the possibility remained that a pool of mRNA existed in the cytoplasm for which no ribosomes were available. In that case, size differences in cytoplasmic mRNA populations from healthy and 24 hr infected cells might be detectable after all. When poly(A) containing RNA from healthy and 24 hr infected cells was analysed on sucrose gradients, the size of the mRNA populations corresponded well to that expected on the basis of the size of the polysomes. No new mRNA large enough to code for AcNPV polyhedral protein could be detected in the mRNA population from 24 hr infected cells (Chapter 4).

Isolation and analysis of (on AcNPV-DNA-cellulose selected) viral RNA isolated from 24 hr infected cells resulted in the detection of two minor, and one predominant RNA species with a molecular weight of 240,000 dalton (Chapter 5). This corresponds well to the minimum size expected for the mRNA for AcNPV polyhedral protein (See discussion chapter 3).

In vitro translation of preparatively isolated viral RNA resulted in the synthesis of a protein which comigrated with purified AcNPV polyhedral protein on polyacrylamide gels (Chapter 6). Immunoprecipitation of the translation products followed by polyacrylamide gel electrophoresis demonstrated that the 30,000 dalton translation product represented the AcNPV polyhedral protein.

No translatable RNA could be isolated from healthy cells by selection on AcNPV-DNA-cellulose, indicating that the mRNA for polyhedral protein is a virus-induced mRNA. As there is some aspecific hybridization in our hybridi-

zation procedure the theoretical possibility remains that the AcNPV polyhedral protein is translated from the less than 12.5% host RNA which may still be present in the viral mRNA preparation. Several arguments plead against this hypothesis.

- Size analysis of the viral mRNA population reveals only one mRNA with the right size for AcNPV polyhedral protein. This RNA is present in a quantity which is much greater than 12.5%.
- No attempt could be made to optimize the hybridization – and subsequent washing – and elution procedure. As no preliminary selection was made to remove ribosomal and transfer RNA, the rather high percentage of non-specific hybridization most probably is a reflection of badly removed host ribosomal and transfer RNA rather than badly removed host mRNA.

Selection of mRNA for AcNPV polyhedral protein with restriction endonuclease fragments of AcNPV DNA may show that some fragments are and other fragments are not capable of selecting the mRNA for AcNPV polyhedral protein. This would definitely prove that non-specifically hybridized host mRNA is not responsible for the in vitro synthesis of AcNPV polyhedral protein. This technique will localize at the same time the gene coding for AcNPV polyhedral protein on the physical map of the AcNPV DNA.

Selection of viral mRNAs by means of hybridization followed by in vitro translation provides an excellent way of analysing the time course and size distribution of viral mRNA and protein synthesis in systems (such as ours) where host macromolecular synthesis is not shut off completely during the viral replication cycle. Defects in the viral replication cycle, such as the unability of AcNPV to synthesize polyhedral protein in *Bombyx mori* cells (312), can be classified as a pre- or a posttranscriptional event from the absence or presence of the mRNA for AcNPV polyhedral protein. The same approach would be useful in characterizing temperature sensitive mutations which result in the absence of polyhedra production at the restrictive temperature (39, 185).

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C. P. van der Beek

SAMENVATTING

In deze Nederlandese samenvatting wordt geprobeerd om het in dit proefschrift beschreven onderzoek uit te leggen op een manier die ook voor niet-ingewijden begrijpelijk is. Mensen die wel thuis zijn in de materie kunnen meestal ook de Engelse samenvatting wel lezen en kunnen daar dan ook de in vaktaal uitgedrukte inhoud van het proefschrift terugvinden.

Kernpolyedervirussen worden tijdens hun vermenigvuldiging in de kernen van insektecellen, als krenten in een krentenbol, verpakt in het zogenaamde polyedereiwit. Al heel lang vragen insektenvirologen zich af of dit polyedereiwit door het insekt als afweer tegen het virus gemaakt wordt, of dat het door het virus geproduceerd wordt om zichzelf te beschermen na de dood van het insekt.

In het proefschrift worden experimenten beschreven dit hebben geleid tot het besef dat het polyedereiwit door het virus gemaakt wordt. Door aan te tonen dat de informatie die nodig is voor de produktie van dit eiwit opgeslagen is in het virus, kon worden aangetoond dat het virus verantwoordelijk is voor de aanmaak van het polyedereiwit.

In hoofdstuk 1 worden de eigenschappen van het door ons gebruikte virus beschreven, evenals de vorming van polyedereiwit (en de daaruit resulterende virusbevattende polyeders) bij andere virusfamilies. Ook worden in dit hoofdstuk de argumenten die in het verleden gebruikt zijn om de vorming van polyedereiwit aan de gastheer ofwel aan het binnendringende virus toe te schrijven, behandeld.

Het verwijderen van afwijkende virusvarianten uit het uit de natuur geïsoleerde viruspreparaat, wordt in hoofdstuk 2 beschreven.

De informatie die het virus bij zich draagt om zichzelf te kunnen vermenigvuldigen in de cellen van zijn gastheer, is aanwezig in een bepaalde code, de zogenaamde DNA-code. Van deze DNA-code worden afdrukken gemaakt (boodschapper RNAs) die in bepaalde structuren van de cel (polysomen) omgezet worden in produkten dit het virus nodig heeft om zichzelf te vermenigvuldigen en z'n infectiecyclus te kunnen herhalen. In hoofdstuk 3 wordt de isolatie van deze polysomen beschreven.

Hoofdstuk 4 laat zien hoe uit deze polysomen de boodschapper RNAs werden geïsoleerd en, omdat een nieuwe boodschapper RNA in zieke cellen het boodschapper RNA voor polyedereiwit zou kunnen zijn, werd er gekeken of er verschil bestond tussen de boodschapper RNAs uit gezonde en uit zieke cellen, hetgeen echter niet het geval bleek te zijn.

Gebruik makend van het feit dat de virale boodschapper RNAs afdrukken zijn van de in het virus aanwezige DNA-code, is het mogelijk om d.m.v. een bepaalde techniek (hybridisatie) de boodschapper RNAs die door het virus gemaakt zijn, te scheiden van de boodschapper RNAs die door de cel gemaakt zijn. De isolatie van de virale boodschapper RNAs staat beschreven in hoofdstuk 5.

In hoofdstuk 6 wordt m.b.v. deze virale boodschapper RNAs in de reageerbuis nagebootst, wat er ook in de cel mee gebeurt, nl. het omzetten in produkten die door het virus gebruikt worden voor z'n vermenigvuldiging en de herhaling van z'n infectiecyclus. Eén van deze produkten bleek het polyeder-eiwit te zijn waarmee was aangetoond dat het virus de informatie voor de produktie van dit eiwit bij zich draagt.

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CURRICULUM VITAE

Cornelis Petrus van der Beek werd op 19 oktober 1951 geboren te Rotterdam. In 1970 behaalde hij het HBS-B diploma aan het Charlois Lyceum te Rotterdam, waarna hij zijn studie aan de Landbouwhogeschool te Wageningen begon. Het doctoraal examen in de richting Planteziektenkunde (cum laude), met als verzwaard hoofdvak de Virologie en als bijvakken de Erfelijkheidsleer en de Moleculaire Biologie, werd in 1975 behaald. Van 1975 tot 1979 was hij als wetenschappelijk medewerker in tijdelijke dienst werkzaam bij de vakgroep Virologie van de Landbouwhogeschool te Wageningen.

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