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Expression of the influenza virus haemagglutinin in insect cells by a baculovirus vector

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The insect baculovirus *Autographa californica* nuclear polyhydrosis virus (AcNPV) has played a major role in studies on the molecular biology of insect DNA viruses. Recently, this system has been effectively adapted as a highly efficient vector in insect cells for the expression of several mammalian genes. A cDNA sequence of the influenza (fowl plague) virus haemagglutinin gene has been inserted into the *Bam*HI site of the pAc373 polyhedrin vector. *Spodoptera frugiperda* cells were co-transfected with this construct, pAc-HA651, and authentic AcNPV DNA. Recombinant virus was selected by adsorption of transfected cells to erythrocytes followed by serial plaque passages on *S. frugiperda* cells. We have determined the site of insertion of the haemagglutinin gene into the AcNPV genome by restriction enzyme cleavage and Southern blot hybridization analyses using haemagglutinin cDNA as a probe. The influenza haemagglutinin gene is located in the polyhedrin gene of AcNPV DNA. Immunofluorescent labelling, immunoprecipitation and immunoblot analyses with specific antisera revealed that *S. frugiperda* cells produce immune reactive haemagglutinin after infection with the recombinant virus. The haemagglutinin is expressed at the cell surface and has haemolytic capacity that has been activated by post-translational proteolytic cleavage. When chickens were immunized with *S. frugiperda* cells expressing haemagglutinin, they developed haemagglutinin-inhibiting and neutralizing antibodies and were protected from infection with fowl plague virus. These observations demonstrate that the haemagglutinin is processed in insect cells in a similar fashion as in fowl plaque virus-infected vertebrate cells and that it has full biological activity.

Key words: fowl plague virus/influenza virus/*Autographa californica* nuclear polyhedrosis virus vector/recombination in insect cells/recombinant virus/haemagglutinin/influenza vaccine

Introduction

The major constituent of the envelope of influenza virus is haemagglutinin. It induces the formation of neutralizing antibodies and, because of its antigenic variability, it is responsible for the characteristic epidemiology of influenza in man (for review, see Palese and Young, 1983). Haemagglutinin is also of interest because it initiates virus infection by binding to neuraminic acid-containing receptors of host cells and by promoting penetration of the viral genome into the cytoplasm through fusion of the envelope with cellular membranes (Klenk and Rott, 1980; Klenk *et al.*, 1984). The amino acid sequences of many haemagglutinin subtypes have been elucidated (Ward, 1981), and their conformations have been studied by X-ray crystallography (Wilson *et al.*, 1981). As an integral membrane protein haemagglutinin is translated at membrane-bound polysomes, translocated by means of an amino-terminal signal sequence into the lumen of the endoplasmic reticulum and transported from there through the Golgi apparatus to the plasma membrane. In the course of transport, haemagglutinin undergoes post-translational modifications, including removal of the signal peptide, attachment of N-glycosidic oligosaccharide side chains (Keil *et al.*, 1985) and proteolytic cleavage of the precursor HA into the fragments HA_1 and HA_2. The latter modification, which is essential for the realization of the fusion activity, involves the sequential action of two host-dependent enzymes, a trypsin-like endoprotease and carboxypeptidase N that attack on arginine- or lysine-containing cleavage sites (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975; Garten and Klenk, 1983).

In recent years the cloned haemagglutinin gene has been expressed in vertebrate cells by a number of different vectors. We have sought to develop a suitable, efficient expression system in insect cells to study the expression, and in particular the post-translational modifications, of influenza (fowl plague) haemagglutinin.

Among the expression vector systems, the insect baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) has recently been successfully developed: human β-interferon (Smith *et al.*, 1983b), β-galactosidase from *Escherichia coli* (Pennock *et al.*, 1984), human c-*myc* protein (Miyamoto *et al.*, 1985) and human interleukin 2 (Smith *et al.*, 1985) have been expressed in insect cell lines infected with recombinant virus. This expression system has proved to be very valuable because of the efficient polyhedrin promoter of AcNPV. Due to the ability of insect cells to process newly synthesized proteins, biologically active gene products have been obtained. There is evidence that β-interferon has been glycosylated, that the signal peptide of interleukin 2 has been cleaved off and that the c-*myc* protein has been phosphorylated.

Here we describe the construction of a plasmid containing the polyhedrin promoter, part of the polyhedrin coding sequence (Smith *et al.*, 1983a) and the gene for influenza virus haemagglutinin. Upon co-transfection of *Spodoptera frugiperda* insect cells with this plasmid and intact AcNPV DNA, recombinant virus was isolated containing in the polyhedrin sequence of its genome the gene for influenza virus haemagglutinin. When *S. frugiperda* cells were infected with recombinant virus the infected cells produced biologically active influenza haemagglutinin that induced immunity antibodies in chickens.

Results

Construction of the recombinant vector pAc373-HA and of the recombinant virus AcNPV-HA

The strategy for the construction of the insect vector containing the haemagglutinin gene is shown in Figure 1. The haemagglutinin gene of fowl plague virus mutant ts651 was cloned into the *Pst*I site of the plasmid vector pUc8, using the dG-dC tailing procedure (K.Kuroda and H.-D.Klenk, in preparation). This

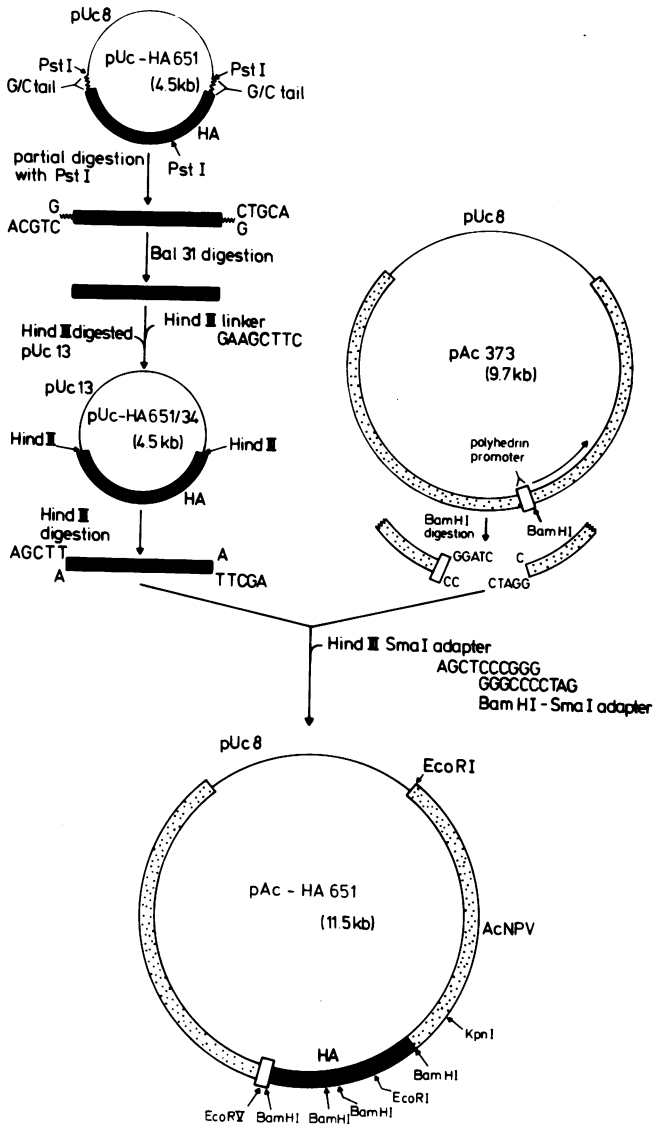

Fig. 1. Construction of the pAc-HA651. Fowl plague virus haemagglutinin and AcNPV sequences are indicated by solid and by dotted bars, respectively. For details see text.

plasmid (pUc-HA651) contains the whole sequence of the haemagglutinin gene, i.e. a non-coding region of 21 nucleotide pairs at the 5′ end followed by the coding region of 1692 nucleotide pairs and a 3′ non-coding region of 29 nucleotide pairs. The construct also carries dG-dC tails of 15 nucleotide pairs at the 5′ end and 21 nucleotide pairs at the 3′ end of the haemagglutinin gene. As the tails were expected to interfere with the expression of the haemagglutinin gene (Riedel *et al.*, 1984), they were removed by *Bal*31 exonuclease digestion. The tailored haemagglutinin gene fragments were cloned into the *Hin*dIII site of pUc13 using *Hin*dIII linkers. The clones which contained the haemagglutinin gene were examined by nucleotide sequence analysis of the flanking regions of the haemagglutinin gene. For further work, one clone pUc-HA651/34 was chosen which had remaining dG-dC residues of one nucleotide pair at the 5′ end and of five nucleotide pairs at the 3′ end. The full length haemagglutinin gene could be excised from this plasmid by *Hin*dIII cleavage and was recloned into the *Bam*HI site of pAc373 using *Bam*HI/*Sma*I and *Hin*dIII/*Sma*I adaptors. The orientations of the

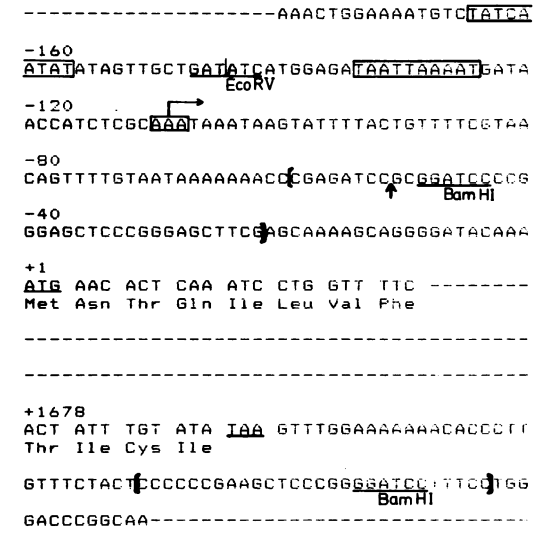

Fig. 2. The nucleotide sequences flanking the haemagglutinin gene in the construct pAc-HA651. The boxes at −165 and at −134 designate the nucleotides that resemble the canonical CAAT and TATA box sequences respectively. The box at −110 marks the bases that code for the 5′ end of polyhedrin mRNA. The brackets at −59 and at +1721 indicate the linker and adaptor regions. The sequences between −21 and −1 and between +1694 and +1720 represent the non-coding regions of the haemagglutinin gene. Nucleotide sequences were determined as described in the text by the method of Sanger *et al.* (1977). See also Materials and methods.

haemagglutinin gene in these clones were determined by double cleavage with the diagnostic restriction endonucleases *Eco*RI and *Eco*RV. Three fragments were produced in this way. The smallest fragment would be 1.3 kbp in length if the haemagglutinin gene was in the *cis* position to the polyhedrin promoter. In contrast, the smallest fragment would be 0.55 kbp in length if the haemagglutinin gene had been inserted in the opposite orientation. For expression studies (see below), one clone, pAc-HA651, was chosen which showed the correct orientation. In this construct the flanking regions of the haemagglutinin gene were sequenced (Figure 2). The nucleotide sequence data proved that the haemagglutinin gene had been correctly cloned into the *Bam*HI site of pAc373. The distance between the start site of polyhedrin mRNA transcription and the initiation codon of the haemagglutinin gene is longer than the respective distance in the original polyhedrin promoter – gene region in AcNPV DNA because of the inserted linker, the adaptors and the non-coding region of the haemagglutinin gene. In the authentic polyhedrin gene that distance amounts to 58 nucleotide pairs and in the pAc373-HA construct it measures 108 nucleotide pairs.

To transfer the haemagglutinin gene into the AcNPV genome, *S. frugiperda* cells were co-transfected with pAc-HA651 DNA and the genomic DNA of authentic AcNPV. Because the progeny virus consisted predominantly of wild-type AcNPV, it was difficult to screen for infected cells lacking polyhedra. The transfected cells were therefore adsorbed to chicken erythrocytes fixed on plastic cell culture dishes in order to pre-select those cells that expressed influenza virus haemagglutinin (see Materials and methods). After release by neuraminidase treatment these cells were then seeded on fresh culture dishes and virus production was allowed to proceed. By this procedure we were able to enrich the recombinant virus by a factor of 10 – 100. The progeny virus was subjected to two plaque purifications, and *S. frugiperda* cells with polyhedrin-free plaques were selected to obtain pure recombinant virus.

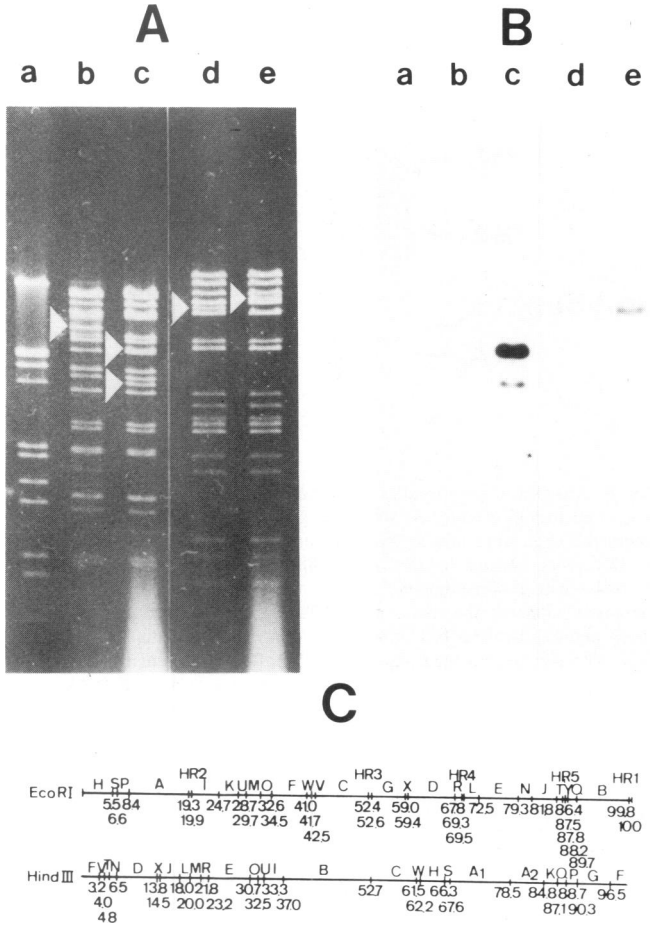

Fig. 3. Analysis of recombinant AcNPV by restriction enzyme cleavage and Southern blot hybridization. Authentic **(b** and **d)** or recombinant AcNPV DNA **(c** and **e)** was digested with *Eco*RI **(b** and **c)** or *Hin*dIII **(d** and **e)** and the digests were separated on a 0.7% agarose gel, followed by staining with ethidium bromide **(A)**. Subsequently the DNA fragments were blotted to a nylon membrane sheet. The blot was hybridized with ^{32}P-labelled haemagglutinin cDNA **(B)**. *Eco*RI/*Hin*dIII-digested λ DNA was used as a size marker **(a)**. The *Eco*RI and *Hin*dIII restriction maps of authentic AcNPV DNA are also shown **(C)** (Lübbert and Doerfler, 1984a).

Analysis of the recombinant AcNPV DNA by restriction enzyme cleavage and Southern blot hybridization

Recombinant AcNPV was propagated, purified and the recombinant AcNPV DNA was extracted as described (Tjia *et al.*, 1979). The recombinant DNA was cleaved with the restriction endonuclease *Hin*dIII or *Eco*RI, and the fragments were separated by electrophoresis on a 0.7% agarose gel in order to identify the site of insertion of the haemagglutinin gene in the AcNPV genome (Figure 3A). The DNA fragments were then transferred to a nylon membrane (Southern, 1975), and the fragments containing the haemagglutinin gene were identified by hybridization (Wahl *et al.*, 1979) to a haemagglutinin gene fragment derived from pUc-HA651/34 which was ^{32}P-labelled by nick translation (Rigby *et al.*, 1977). The results demonstrate that the polyhedrin gene-containing fragment *Eco*RI-H (Figure 3A, lane b, arrow) is absent from the cleavage pattern of the recombinant virus DNA and that two new fragments are generated (Figure 3A, lane c, arrows) which are absent from the cleavage pattern of AcNPV DNA. It is apparent that the two newly generated DNA fragments hybridize with the haemagglutinin DNA (Figure 3B, lane c). AcNPV DNA obviously does not hybridize to this

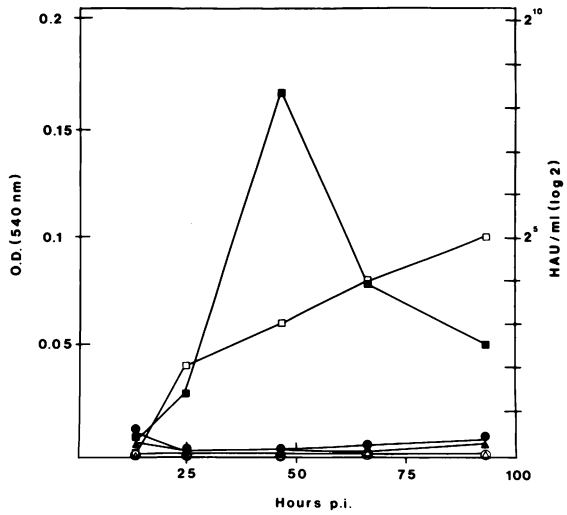

Fig. 4. Production of haemagglutinin in *S. frugiperda* cells. Cells were infected with recombinant (■, □), authentic AcNPV (▲, △), or were mock-infected (●, ○). The haemagglutinin expressed on the cell surface was assayed by haemadsorption activity (■, ▲, ●), and the total amount of haemagglutinin in the cells was assayed by haemagglutinin activity (□,△,○).

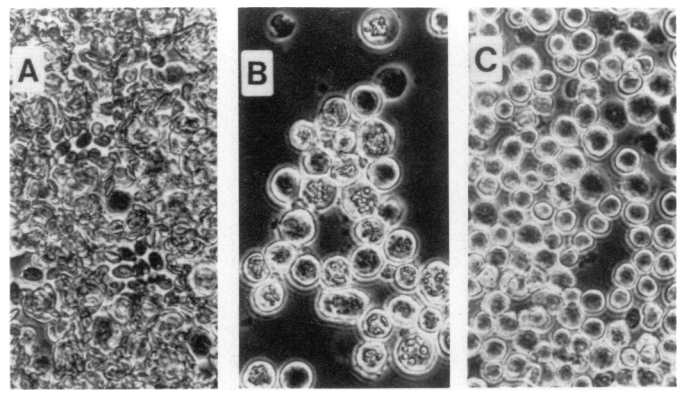

Fig. 5. Haemadsorption of *S. frugiperda* cells after infection with recombinant AcNPV virus. At 2 days after infection with recombinant AcNPV **(A)**, after infection with authentic AcNPV **(B)** or after mock infection **(C)**, haemadsorption was assayed and cell cultures were photographed under the microscope. Cells infected with recombinant virus are loaded with erythrocytes. Cells infected with authentic virus show poyhedra in their nuclei.

probe (Figure 3B, lane b), nor does control λ DNA (Figure 3B, lane a). Similarly, when the recombinant AcNPV DNA is cut with *Hin*dIII, fragment *Hin*dIII-F is not generated and a new fragment arises (Figure 3A, lanes d and e, arrows) which hybridizes to the cloned haemagglutinin gene (Figure 3B, lane e). This result is consistent with the restriction map of the haemagglutinin gene that has an *Eco*RI site but no *Hin*dIII site. The results of this analysis demonstrate that the haemagglutinin gene has been inserted into the polyhedrin gene of AcNPV DNA. So far the site of insertion has not been mapped more precisely.

Expression of influenza haemagglutinin in S. frugiperda cells

After infection with recombinant virus, *S. frugiperda* cells, unlike cells infected with AcNPV wild-type, did not show nuclear inclusions (data not shown). Haemagglutinating activity could be detected in homogenates of such cells starting at ~24 h after inoculation (Figure 4). There was a continuous rise in the haem-

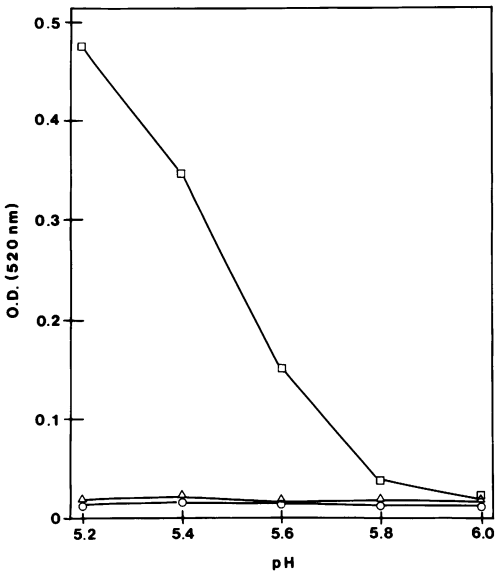

Fig. 6. Haemolytic activity of the haemagglutinin expressed in *S. frugiperda* cells. Cells were infected with recombinant (□) or authentic AcNPV (△), or were mock-infected (○). At 3 days after infection the cells were sonicated, and a suspension containing 64 haemagglutinin units was assayed for haemolytic activity at the pH values indicated.

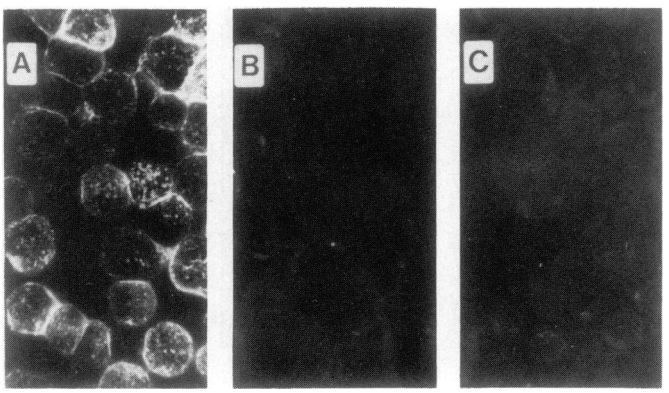

Fig. 7. Immunofluorescent labelling of haemagglutinin produced in *S. frugiperda* cells. Cells were infected with recombinant **(A)** or authentic AcNPV **(B)**, or were mock-infected with PBS **(C)**. After 2 days of incubation, the cells were labelled by indirect immunofluorescence as described in Materials and methods. The exposure time for u.v. photography was 15 s.

agglutination titer up to ~96 h, when most of the cells deteriorated. Haemagglutinating activity was not detectable in the medium. Recombinant virus-infected cells also acquired the ability to haemadsorb. At ~48 h after infection each cell was heavily loaded with erythrocytes (Figure 5). The decrease in the amount of adsorbed erythrocytes observed after 48 h (Figure 4) appeared to be due to a substantial amount of infected cells being detached from the plastic support at this time. Neither cells infected with wild-type AcNPV nor mock-infected cells displayed haemagglutination or haemadsorption activities (Figure 5). These observations indicate that after infection with the recombinant virus haemagglutinin is expressed in *S. frugiperda* cells and that haemagglutinin is transported to the cell surface.

Fusion capacity, the other biological activity of the influenza virus haemagglutinin, depends on low pH and is usually monitored by measuring haemolysis (Maeda and Ohnishi, 1980;

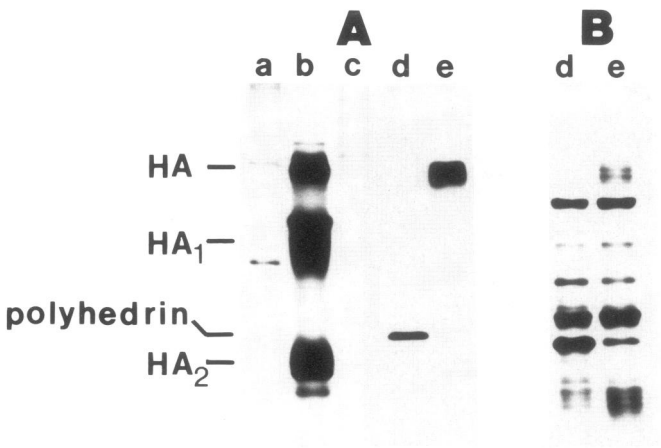

Fig. 8. Analysis of polypeptides by metabolic labelling. MDCK cells were mock-infected **(a)** or infected with fowl plague virus for 5 h **(b)**. *S. frugiperda* cells were infected with authentic **(d)** or recombinant AcNPV **(e)**, or were mock-infected **(c)** for 2 days. The infected cells were labelled by a 2 h pulse with [^{35}S]methionine, and the haemagglutinin was immunoprecipitated and analysed by SDS–polyacrylamide gel electrophoresis as described in Materials and methods. Samples derived from 10^6 cells were loaded onto the gel **(A)**. The lysates of insect cells infected with authentic or recombinant AcNPV were also analysed by gel electrophoresis prior to immunoprecipitation. Samples derived from 10^5 cells were loaded onto the gel **(B)**, and the distribution of polypeptides determined by direct autoradiography after drying the gel.

Huang *et al.*, 1981). A homogenate derived from cells infected with recombinant virus induces haemolysis below pH 5.8, whereas mock- or AcNPV-infected control cells do not (Figure 6). When erythrocytes exposed to homogenates of cells infected with recombinant virus were inspected under the microscope cell fusion was observed (data not shown). The haemagglutinin synthesized in *S. frugiperda* cells can also be directly recognized by antibodies raised against haemagglutinin derived from fowl plague virions, as can be demonstrated by immunofluorescent labelling (Figure 7). Each cell expresses haemagglutinin, and this result is in full agreement with the results of the haemadsorption experiment shown in Figure 5.

The recombinant haemagglutinin was then characterized by SDS–polyacrylamide gel electrophoresis. Figure 8 shows the results of immunoprecipitation experiments in which haemagglutinin synthesized in fowl plague virus-infected Madin–Darby canine kidney (MDCK) cells and in *S. frugiperda* cells infected with recombinant virus have been labelled by a 2 h pulse with [^{35}S]methionine. Haemagglutinin is present in MDCK cells in the cleaved and in the uncleaved forms (Figure 8A, lane b), whereas mainly uncleaved haemagglutinin can be detected in recombinant virus-infected *S. frugiperda* cells (Figure 8A, lane e). The polyhedrin band in the immunoprecipitate obtained from AcNPV-infected control cells (Figure 8A, lane d) resulted from incomplete solubilization of the occlusions in the immunoprecipitation buffer (Miyamoto *et al.*, 1985).

Gel electrophoretic analyses of lysates not subjected to immunoprecipitation allow a comparison of the relative amounts of haemagglutinin synthesized in cells infected with recombinant virus (Figure 8B, lane e) and of polyhedrin synthesized in cells infected with authentic AcNPV (Figure 8B, lane d). The data indicate that haemagglutinin production does not reach the level of polyhedrin synthesis. As has been pointed out already, polyhedrin is not made in cells infected with recombinant virus. The minor band co-migrating with polyhedrin (Figure 8A, lane d,

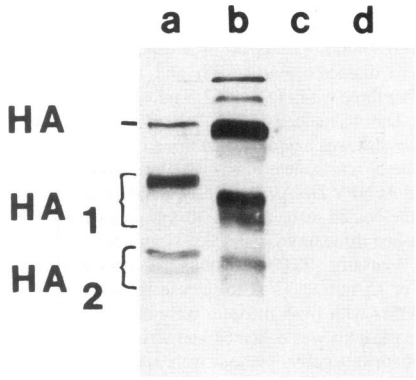

Fig. 9. Analysis of haemagglutinin by immunoblotting. MDCK cells were infected with fowl plague virus **(a)** for 5 h. *S. frugiperda* cells were infected with recombinant **(b)** or authentic AcNPV **(c)**, or were mock-infected **(d)** for 3 days. The polypeptides were separated by SDS – polyacrylamide gel electrophoresis, and then blotted to a nitrocellulose membrane. The blot was analysed with anti-haemagglutinin antibodies as described in Materials and methods. Samples derived from 10^5 cells were loaded onto each lane of the gel.

and Figure 8B, lane e) has been identified as a different protein (Pennock *et al.*, 1984).

When the recombinant virus-produced haemagglutinin was analyzed by the immunoblotting technique it was apparent that the protein was cleaved in *S. frugiperda* cells, even though to a lesser extent than in MDCK cells (Figure 9, lanes a and b). It is also evident that the precursor HA as well as the cleavage fragments HA_1 and HA_2 are more heterogeneous in size and have a lower mol. wt when derived from *S. frugiperda* cells. These differences are most likely due to smaller and incomplete oligosaccharides. It is clear, however, that the haemagglutinin is glycosylated in *S. frugiperda* cells because unglycosylated haemagglutinin would form sharp bands considerably smaller than HA, HA_1 and HA_2 shown in lane b, Figure 9.

The lysates analyzed in lanes a and b of Figure 9 were obtained from the same number (10^5) of MDCK or *S. frugiperda* cells respectively. The relative intensities of the bands indicate, therefore, that the amount of haemagglutinin produced in insect cells is comparable with that obtained in fowl plague virus-infected vertebrate cells.

Immunization of chickens with recombinant haemagglutinin,

To test the immune response to recombinant haemagglutinin, chickens were immunized as described in Materials and methods. The immunized animals produced haemagglutination-inhibiting antibodies at titres of 1:64 – 1:128. The sera neutralized infectivity of fowl plague virus with indices >500. The immunized chickens survived a challenge infection with 10^4 p.f.u. of fowl plague virus 3 weeks after immunization without showing signs of fowl plague, while unprotected control animals died 2 days after infection as expected.

Discussion

We have analyzed recombinants between AcNPV DNA and the influenza virus haemagglutinin gene which express haemagglutinin in insect cell cultures. The amount of haemagglutinin produced in these cells is comparable with that obtained in vertebrate cells infected with fowl plague virus, but it does not reach the levels of polyhedrin synthesis in *S. frugiperda* cells infected with AcNPV. Several eukaryotic genes have already been successfully expressed in insect cells with the aid of this expression vector system, e.g. human β-interferon (Smith *et al.*, 1983b), human c-*myc* protein (Miyamoto *et al.*, 1985) and human interleukin 2 (Smith *et al.*, 1985). A similar vector has been used to produce human α-interferon in silk worms (Maeda *et al.*, 1985). In all instances the insect system-derived proteins have proved biologically active. Similarly, the influenza virus haemagglutinin synthesized under conditions described here is able to induce haemadsorption, cause haemolysis and react with haemagglutinin-specific antibodies. Moreover, the recombinant virus can protect animals against the challenge by fowl plague virus. Thus, in its biological activities the recombinant virus-produced haemagglutinin strikingly resembles authentic fowl plague virus haemagglutinin.

The fowl plague virus haemagglutinin to our knowledge represents the first membrane protein among the recombinant AcNPV-encoded vertebrate proteins. Our data show that the recombinant haemagglutinin is membrane-bound in *S. frugiperda* cells and that it is transported to the cell surface in a similar fashion as it is in vertebrate cells. This observation suggests that the amino-terminal signal peptide of haemagglutinin has been removed, although we have not shown this directly by biochemical techniques. However, at least partial cleavage of a signal peptide could be demonstrated when interleukin 2 was produced in the same expression vector system (Smith *et al.*, 1985).

Moreover, influenza virus haemagglutinin is the first vertebrate polypeptide that has been shown to undergo post-translational proteolytic cleavage in *S. frugiperda* cells. The observation that the recombinant haemagglutinin has haemolytic activity indicates that the endoprotease involved in cleavage attacks the correct peptide bond between the carboxy-terminal arginine of the sequence Lys-Lys-Arg-Lys-Lys-Arg and the adjacent glycine (Garten *et al.*, 1982) since fusion capacity can be expressed only if cleavage had occurred exactly in this position (Garten *et al.*, 1981). It is of general interest that the fowl plague virus haemagglutinin is activated in *S. frugiperda* cells because numerous other vertebrate glycoproteins, notably that of human T cell lymphotropic virus III (Muesing *et al.*, 1985), and a large number of prohormones and proenzymes (Steiner *et al.*, 1980) have similar cleavage sites and might therefore be susceptible to proteolytic activation if expressed in insect cells.

Although the presence of carbohydrates has not been directly demonstrated here, e.g. by methods such as metabolic radiolabelling or glycosidase treatment, the gel electrophoretic analyses indicate that the haemagglutinin is glycosylated. The higher and more heterogeneous electrophoretic mobilities of HA as well as of HA_1 and HA_2 derived from insect cells indicate that the oligosaccharide side chains are not identical to those attached in vertebrate cells. It is known that the biological functions of the haemagglutinin tolerate a relatively high degree of variability in the oligosaccharide content (Romero *et al.*, 1984). On the other hand, however, it is known that the presence of glycosyl groups is required to protect the fowl plague virus haemagglutinin from unspecific proteolytic degradation (Klenk *et al.*, 1974). The glycosylating capacity of *S. frugiperda* cells appears to be sufficient to meet this requirement.

Insect cells do not contain neuraminic acid, i.e. they lack the receptor for influenza viruses. Vector-mediated expression is therefore the only way to produce the haemagglutinin and other myxovirus proteins in these cells. On the other hand, the absence of an influenza virus receptor on insect cells may facilitate the release of the haemagglutinin, especially of its secretory form that lacks the membrane anchor (Gething and Sambrook, 1982). From a biotechnological point of view this may be a considerable

advantage over expression systems in vertebrate cells that all contain neuraminic acid and thus might tend to retain the haemagglutinin.

We have shown that in the recombinant AcNPV the haemagglutinin gene has been inserted into the *Eco*RI-H fragment that encodes the polyhedrin gene (Figure 3). As proposed earlier (Smith *et al.*, 1983b), recombination between the AcNPV genome and the pAc373 construct is mediated by homologous recombination within the polyhedrin gene. We have not yet analysed the exact site of recombination in the recombinant described here. Moreover, it will be interesting to investigate whether homologous recombination is the only possibility of inserting foreign genes into AcNPV DNA or whether other sites in the AcNPV genome can also be utilized, e.g. by heterologous recombination. The homologous recombination in this system appears to be efficient. Perhaps this system offers possibilities to study in detail mechanisms of homologous recombination.

Materials and methods

Cells and viruses

The insect cell line from *S. frugiperda* was propagated in TC-100 medium (Gardiner and Stockdale, 1975), which was modified as previously reported (Carstens *et al.*, 1979) and contained 10% fetal calf serum. The methods employed in virus infection and in the purification of extracellular AcNPV were detailed elsewhere (Carstens *et al.*, 1979; Tjia *et al.*, 1979). Fowl plague virus, strain A/FPV/Rostock/34 (H7N1), was grown in MDCK cells (Rott *et al.*, 1984). Seed stocks were obtained from the allantoic cavity of 11-day-old embryonated chicken eggs (Klenk *et al.*, 1972).

Viral DNA and plasmid DNA

AcNPV DNA was prepared by previously described methods (Lübbert and Doerfler, 1984a,b) with modifications. Cells and cell debris were removed from the growth medium of AcNPV-infected *S. frugiperda* cells by centrifugation at 4000 *g* for 10 min. Subsequently, virions were pelleted by centrifugation at 90 000 *g* for 1 h. The pellet was resuspended in a solution of 10 mM Tris-HCl, pH 7.6, 10 mM EDTA and 1% sodium N-lauroyl sarcosinate. Proteinase K was added to a final concentration of 200 μg/ml, and the mixture incubated for 5 h at 50°C. AcNPV DNA was phenol-extracted directly from the sarcosinate – proteinase K mixture. For transfection the viral DNA was purified by CsCl – ethidium bromide gradient centrifugation as described previously (Lübbert and Doerfler, 1984a). Plasmid DNAs were prepared by the Triton X-100 – lysozyme procedure followed by centrifugation to equilibrium in CsCl – ethidium bromide gradients as described (Doerfler *et al.*, 1979) or by the alkaline lysis method followed by purification on NACS PREPAC (BRL) columns.

Plaque assays with AcNPV on S. frugiperda cells

The methods published previously were applied (Wood, 1977; Tjia *et al.*, 1979).

DNA constructions

Restriction enzymes were purchased from Boehringer (Mannheim), New England Biolabs or Renner (Dannstadt, FRG). Polynucleotide kinase, T4 DNA ligase, the Klenow fragment of DNA polymerase I, exonuclease *Bal*31, calf intestine alkaline phosphatase and *Hin*dIII linkers (GAAGCTC) were obtained from Boehringer (Mannheim). *Bam*HI/*Sma*I (GATCCCCGGG) and *Hin*dIII/*Sma* adaptors (AGCTCCCGGG) were purchased from New England Biolabs. Restriction fragments were isolated from low melting agarose gels (Sea Plaque, Marine Colloids) using NACS PREPAC (BRL) columns and following instructions provided by the manufacturer. DNA was ^{32}P-labelled by nick translation (Rigby *et al.*, 1977). In some experiments a nick translation kit from Amersham was used, and the supplier's protocol was used.

Co-transfection of S. frugiperda cells with AcNPV DNA and the influenza virus haemagglutinin construct

S. frugiperda cells were grown in monolayers to ~3/4 confluency. Subsequently, the cells were transfected jointly with the haemagglutinin gene construct in the pAc373 vector (pAcHA651) and with AcNPV DNA essentially as described by Smith *et al.* (1983a). After incubation with the DNA precipitate, 1 ml of 15% glycerol in HBS was added to the cells. After a 2 min glycerol shock the cells were washed twice with pre-warmed medium and overlayed with fresh liquid medium containing foetal calf serum.

Selection for recombinant AcNPV

A method was devised to enrich for transfected *S. frugiperda* cells that produced recombinant influenza virus haemagglutinin by adsorbing these cells to erythrocytes. Chicken erythrocytes were fixed to plastic cell culture dishes as follows. A volume of 2.5 ml of a poly-L-lysine (mol. wt 120 000, Sigma, Heidelberg, FRG) solution [25 μg/ml in phosphate buffered saline (PBS)] were added to a 5 cm diameter plastic dish and incubated for 15 min at room temperature. After three washings with 2.5 ml of PBS, a 1% chicken erythrocyte suspension in saline was added to the dish and incubated for 30 min at room temperature. The dish was again washed three times with PBS. The *S. frugiperda* cells from one 5 cm diameter dish, which had been jointly transfected with pAc-HA651 and AcNPV DNAs and subsequently incubated for 6 days at 27°C, were added to the coated dish. After a 30 min incubation at room temperature the dish was washed three times with PBS. Neuraminidase from *Vibrio cholerae* (Behringwerke, Marburg, FRG) (0.2 units in 1 ml PBS) was added to the dish and incubated for 15 min at 37°C to release the adsorbed *S. frugiperda* cells. These cells together with fresh medium were added to a new cell culture dish. After 3 days the medium was collected and virus progeny was analysed by plaque assay. Polyhedrin-negative plaques were selected and subjected to a second passage. Cells were infected with the plaque-purified virus and analysed for haemadsorption. Isolates inducing haemadsorption were used as virus stocks.

Nucleotide sequencing at the flanking regions of the haemagglutinin gene in pAc-HA651

Nucleotide sequences were determined by using the chain termination method (Sanger *et al.*, 1977). The pAc-HA651 plasmid DNA was cleaved with *Eco*RV and *Kpn*I and then incubated with the Klenow fragment of DNA polymerase I to generate blunt termini. The smaller DNA fragments were isolated from low melting agarose gels and recloned into the *Sma*I site of bacteriophage M13mp10. The correct recombinant phage, which contained the negative strand of the haemagglutinin gene, was selected after G track nucleotide sequence analysis. The flanking regions at both ends of the haemagglutinin gene were sequenced using this phage DNA and an M13 sequencing primer (New England Biolabs) for the 5′-flanking region or a haemagglutinin-specific primer (a gift of E.-L.Winnacker, Munich), which was homologous to nucleotides 1597 – 1606 of the haemagglutinin gene, for the 3′-flanking region.

Haemadsorption assay

Unless indicated otherwise, at 5 – 6 days after co-transfection or 2 – 3 days after infection with recombinant virus, monolayers of *S. frugiperda* cells were washed once with PBS and a 1% suspension of chicken erythrocytes in saline was added. After 30 min at room temperature the cells were washed three times with PBS and examined microscopically. Results were recorded photographically. Alternatively, adsorbed erythrocytes were lysed by incubation in 0.5% Triton X-100 and the optical density of the released haemoglobin was measured photometrically at 540 nm.

Haemagglutination assay

After the infection with recombinant AcNPV or wild-type AcNPV, *S. frugiperda* cells were scraped off plastic bottles with a rubber policeman and were centrifuged. Pelleted cells (10^6) were suspended in 400 μl of PBS and sonicated for 10 s at 25 W. Haemagglutination was then determined in the homogenate by standard titration techniques.

Haemolysis assay

A volume of 100 μl of a homogenate from infected cells (see above) was mixed with 100 μl of a 1% suspension of chicken erythrocytes in saline and incubated for 15 min on ice and then briefly centrifuged. A volume of 200 μl of 130 mM NaCl, 20 mM sodium acetate at the appropriate pH (cf. Figure 6) was added to the pellet. The mixture was incubated for 15 min at 37°C, centrifuged and the optical density of the supernatant measured at 520 nm (Sato *et al.*, 1983).

Immunofluorescence assay

S. frugiperda cells grown on coverslips were infected with recombinant virus and incubated for 2 days at 27°C. The cells were fixed with formaldehyde and permeabilized with 0.2% Triton X-100 in PBS. The cells were then incubated with a 1:50 dilution of anti-haemagglutinin rabbit serum (a gift of H.Becht, Giessen) for 20 min at room temperature. After washing with PBS, fluorescein isothiocyanate-conjugated donkey anti-rabbit immunoglobulin (Amersham) was adsorbed to the cells for 20 min at room temperature. The excess antibody was washed off with PBS and the cells were then mounted in a 10% solution of 1,4-diazobicyclo-(2,2,2)-octane (Sigma) and a 3.5% solution of Moviol (Hoechst, Frankfurt, FRG). Cells were examined under a microscope equipped with u.v. optics. Results were recorded photographically.

Immunization procedure

For immunization of 30-week-old chickens, insect cells 3 days after infection with recombinant AcNPV were used. In series 1, chickens were injected twice i.m. with ~10^7 cells at an interval of 3 weeks. Two weeks later the birds were challenged by i.m. infection with 10^4 p.f.u. of fowl plague virus. In series 2, chickens were inoculated once i.m. with a cell suspension (~10^7 cells) emulsified in Freund's complete adjuvant and 3 weeks later challenged with the same fowl plague virus concentration as before. Blood samples were obtained before challenge

and the sera were inactivated at 45°C for 30 min. Haemagglutination inhibition and neutralization tests were performed according to standard procedures.

Metabolic radiolabelling and immunoprecipitation

At 2 days after inoculation, *S. frugiperda* cells were washed once with methionine- and peptone-free TC-100 medium and were then incubated for 2 h at 27°C with [^{35}S]methionine (140 μCi/ml) in methionine- and peptone-free medium. When fowl plague virus was grown in MDCK cells, viral proteins were pulse-labelled 5 h after inoculation with [^{35} S]methionine (80 μCi/ml) in reinforced Eagle's medium (REM) lacking methionine. The radiolabelled cells were suspended in the medium and centrifuged. The pellets were solubilized in 400 μl of RIPA buffer (1% Triton X-100, 0.1% SDS, 1% deoxycholate, 10 mM EDTA, 20 mM Tris-HCl, pH 7.6) and the lysate was centrifuged to remove insoluble debris.

A volume of 5 μl of anti-haemagglutinin rabbit serum was added and the mixture incubated for 1 h on ice. Subsequently, 50 μl of fixed *Staphylococcus aureus* (10% suspension in RIPA buffer) was added, and the mixture was incubated for 30 min on ice. The *S. aureus* immune complexes were washed three times with RIPA buffer and suspended in 50 μl of sample buffer. Samples were electrophoresed on SDS–10% polyacrylamide gels and then processed for fluorography.

Immunoblotting

Unless stated otherwise, 3 days after infection with virus, cells were collected with a rubber policeman. The cells were spun down and suspended in PBS. The suspended cells were mixed with an equal volume of 2-fold concentrated sample buffer (10% glycerol, 5% mercaptoethanol, 3% SDS, 62.5 mM Tris-HCl, pH 6.8, bromophenol blue), boiled for 3 min and the lysates then electrophoresed on 12% polyacrylamide gels. The proteins were electroblotted to nitrocellulose membranes (Kyhse-Andersen, 1984). The blot was incubated with a 1:350 dilution of rabbit anti-haemagglutinin serum. The bound antibody was detected using biotinylated anti-rabbit IgG and streptavidin-biotinylated horse radish peroxidase complex (Amersham) following the manufacturer's protocol. 4-Chloro-1-naphthol was used as substrate for the peroxidase (Nakane, 1968).

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