A baculovirus dual expression vector derived from the Autographa californica nuclear polyhedrosis virus polyhedrin and p10 promoters: co-expression of two influenza virus genes in insect cells

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A baculovirus transfer vector, pAcUW3, was developed to facilitate the insertion of two influenza virus genes, those encoding the haemagglutinin (HA) and neuraminidase (NA) membrane glycoproteins, into the Autographa californica nuclear polyhedrosis virus genome in a single cotransfection experiment. The NA gene was inserted in place of the polyhedrin coding sequences under the control of the polyhedrin promoter, whereas the HA gene was placed under the control of a copy of the p10 promoter at a site upstream of and in opposite orientation to the polyhedrin promoter. After infection of Spodoptera frugiperda cells with the recombinant virus, AcUW3HANA, both HA and NA were expressed in the very late phase of infection and were shown to be functional in appropriate assays. Immunofluorescence assays demonstrated their localization at the surface of infected insect cells. The expression of both foreign genes in the recombinant virus was found to be stable for at least 12 passages in cell culture.

Introduction

The baculovirus Autographa californica nuclear polyhedrosis virus (AcMNPV) has been used as a high level expression system for foreign genes (reviewed by Luckow & Summers, 1988; Miller, 1988; Fraser, 1989; Maeda, 1989). Traditionally, the strong polyhedrin promoter, which is active in the very late phase of gene expression (for review see Blissard & Rohrmann, 1990), has been used for the expression of foreign coding sequences inserted in place of the polyhedrin coding region; the polyhedrin protein is not required for the production of virus particles (Smith et al., 1983). Recently, it has been shown that the non-structural p10 protein, which is synthesized at levels similar to those of polyhedrin throughout the very late phase of infection, also is not essential for virus replication (Vlak et al., 1988). The p10 promoter has been utilized to construct expression vectors in which the foreign coding sequences replace those of the p10 gene within the viral genome (Vlak et al., 1988; Williams et al., 1989; Weyer et al., 1990; Vlak et al., 1990). Furthermore, it has been shown that the p10 promoter can also function at a different location within virus DNA. A copy of the p10 promoter placed upstream of the polyhedrin gene is functional, stimulating the production of high levels of β-galactosidase (Weyer et al., 1990) and biologically active Bacillus thuringiensis δ-endotoxin (Merryweather et al., 1990).

Most baculovirus expression vectors mediate the production of one foreign protein in insect cells. A logical progression from this is the co-expression of two or more foreign genes, which might allow the protein products to interact in the cell to form complex structures or participate in enzymic reactions. The simultaneous production of multiple proteins can be achieved by coinfection of cells with two or more recombinant viruses of the conventional type, each synthesizing one foreign protein. For example, influenza virus PB1 and PB2 formed a complex in insect cells after simultaneous infection with two viruses containing one of the two genes (St Angelo et al., 1987), and the three subunits of herpes simplex virus helicase-primase formed a fully active enzyme when co-expressed (Dodson et al., 1989). However, this strategy relies on chance for all the viruses to be in the cell at the same time. Another strategy could involve the step-by-step replacement of two AcMNPV coding regions (e.g. polyhedrin and p10) with foreign sequences using the available single replacement transfer vectors. A disadvantage of this procedure is that it is time-consuming, requiring the preparation of two recombinant viruses. Therefore, the development of a transfer vector that allows the simultaneous insertion of two foreign genes into the virus genome in a single cotransfection experiment would facilitate the construction of dual expression vectors and extend the versatility of the baculovirus expression system.

A dual expression transfer vector (pAcVC2) based on a duplicated polyhedrin promoter has been constructed...
recently (Emery & Bishop, 1987). This vector produces the polyhedrin protein and the lymphocytic choriomeningitis virus nucleoprotein. In a derivative of this vector, pAcVC3, a copy of the polyhedrin promoter in association with polyhedrin transcription termination signals was placed upstream of and in the opposite orientation to the polyhedrin gene without the coding sequences. Foreign coding sequences can be inserted under the control of both the original and duplicated polyhedrin promoters. This vector has been used successfully to co-synthesize the hepatitis B virus core and surface antigens (Takehara et al., 1988), and two bluetongue virus proteins, VP3 and VP7 (French & Roy, 1990). However, in some cases instability of the recombinant virus, resulting in loss or rearrangement of foreign coding sequences, has been observed after subsequent passage in cell culture. This is attributed to homologous recombination between duplicated polyhedrin regulatory sequences (D. H. L. Bishop, personal communication).

In this report, we describe the construction of a dual expression vector (pAcUW3) which has the p10 promoter placed upstream of and in the reverse orientation to the polyhedrin promoter. This configuration was predicted to minimize the possibility of recombination between duplicated AcMNPV sequences. To investigate the properties of recombinant viruses derived from this vector, it was used to co-express the influenza virus haemagglutinin (HA) and neuraminidase (NA) coding sequences. In vertebrate host cells, both influenza proteins are highly glycosylated and form part of the virus lipoprotein envelope. The HAs from two influenza virus strains, A/FPV/Rostock/34 (H7N1) and A/PR/8/34 (H1N1), have recently been expressed in baculovirus single expression vectors (Kuroda et al., 1986, 1989; Possee, 1986). Both studies demonstrated post-translational modification of HA in the insect system and correct insertion of the protein in the plasma membrane. Furthermore, the fowl plague virus HA was shown to induce an immune response in chickens. In this report, we demonstrate the co-expression of HA and NA in insect cells. Both proteins were functional in HA and NA assays and shown to be localized at the cell surface.

**Methods**

*Viruses and cells. Schistosoma frugiperda* cells (IPLB-SF 21; Sf cells) (Vaughn et al., 1977), AcMNPV (C6), and the recombinant viruses AcUW3HANA and AcRP19. HA were propagated as described previously (Possee, 1986; Possee & Howard, 1987).

**Construction of plasmid transfer vectors.** Standard procedures (Maniatis et al., 1982) were used for plasmid manipulations.

(i) pAcRP19. HA. A full-length cDNA of segment 4 RNA from A/PR/8/34 (H1N1) containing the HA gene (1.96 kbp; Possee, 1986) was inserted at the BamHI cloning site of the transfer vector pAcRP19. pAcRP19 contains the polyhedrin 5' leader sequence and the first two nucleotides of the polyhedrin translation initiation codon, but in other respects is identical to pAcRP18 (Matsuura et al., 1987).

(ii) pAcUW3. The transfer vector pAcRP25 (Merryweather et al., 1990) was modified by insertion of a BglII linker at the unique EcoRV site to derive pAcRP25-BglII. A BamHI BglII fragment (230 bp) containing the p10 promoter was derived from the construct pAcpl0+1 (Weyer & Possee, 1988, 1989; Weyer et al., 1990) and inserted at the BglII site of pAcRP25-BglII to form pAcRP25-BglII-p10. A 427 bp BgII-SmaI fragment, containing the polyhedrin and p10 promoters, was removed from this plasmid and inserted into pAcUW2 (B) (Weyer et al., 1990) digested with the same enzymes to remove the 1.17 kb region encompassing the p10 promoter and polyhedrin gene. This new construct, pAcUW3, contained the polyhedrin gene regulatory elements with the p10 promoter, the 3' end of the lacZ gene and simian virus 40 (SV40) polyadenylation signals inserted upstream of and in the opposite orientation to the polyhedrin promoter (Fig. 1). Foreign coding sequences could be inserted at a BamHI site (polyhedrin) or BglII site (p10).

(iii) pAcUW3HANA. The BamHI fragment containing the HA gene was inserted at the BglII cloning site of pAcUW3. Recombinants containing the HA gene in the correct orientation relative to the p10 promoter were isolated and designated pAcUW3HA. Plasmid pNA1 (a gift from I. Jones of this Institute) containing the full-length NA gene from A/NT/60/68 (H3N2) inserted at the PvuII site of pAT153/PvuI1/8 (Jones & Brownlee, 1985) was digested with HindIII, end-repaired with the Klenow fragment of Escherichia coli DNA polymerase and dephosphorylated, and a BamHI linker was inserted. After digestion with BamHI, a fragment, approximately 1430 bp, containing viral cDNA with isolated and ligated into the dephosphorylated BamHI site of pAcUW3HA to form pAcUW3HANA.

**Construction and purification of recombinant viruses.** The transfer vectors pAcRP19. HA and pAcUW3HANA were cotransfected with wild-type infectious DNA as described previously (Possee, 1986; Possee & Howard, 1987). Recombinant viruses AcRP19. HA or AcUW3HANA were identified by screening for polyhedron-negative plaques and purified by four sequential plaque assays.

**Haemagglutination assay and haemadsorption.** Sf cells infected with wild-type AcMNPV, AcRP19. HA or AcUW3HANA (10 p.f.u./cell), or mock-infected cells were tested for haemagglutination activity and haemadsorption at various times after infection as described previously (Possee, 1986).

**NA assay.** SF cells were infected with wild-type AcMNPV or AcUW3HANA (10 p.f.u./cell). At various times after infection, virus-infected and mock-infected cells were harvested, washed and resuspended in PBS. NA assays (5 × 10⁵ cells/assay) were carried out as described by Barrett & Inglis (1985).

**Immunoprecipitation.** SF cells, mock-infected or infected with wild-type AcMNPV, AcRP19. HA or AcUW3HANA (10 p.f.u./cell), were radiolabelled with [35S]methionine (100 µCi/3 × 10⁶ cells) from 19 to 26 h post-infection (p.i.). Cells were mixed on ice with lysis buffer containing 10 mm-Tris–HCl pH 8.0, 1 mm-EDTA, 0.5 mm-NaCl and 4% Zwittergent 3-14 (Calbiochem-Behring). Cell nuclei were removed by centrifugation at 12000 g for 5 min. Virus-specific proteins were immunoprecipitated by incubation with monoclonal antibodies (Mabs) against A/PR/8/34 (H12, H15, H16, H23) or X31 (H3N2) NA (N92, N154, N250) (kindly provided by J. Skehel, NIMR, Mill Hill, London, U.K.), or with anti-X31 rabbit serum (a gift from I. Jones) at 4 °C overnight followed by the addition of 30 µl of 50% Protein A-Sepharose (Sigma) and incubation for 3 h. Precipitates were washed three times in 0.5 x lysis buffer and once in 10 mm-Tris–HCl pH 8.0,
AcMNPV multiple expression vector

A transfer vector (pAcUW3) able to insert two foreign coding sequences into the AcMNPV genome simultaneously was constructed by combining various elements of vectors pAcRP25 (Merryweather et al., 1990) and pAcUW2 (Weyer et al., 1990); see Methods for details of the construction. Fig. 1(a) shows the general organization of pAcUW3; Fig. 1(b) shows the sequence of the polyhedrin and pl0 promoters constructed into this vector. Two foreign coding sequences can be inserted at unique BamHI (polyhedrin) and BglII (pl0) cloning sites. The SV40 polyadenylation signals utilized in this vector have been demonstrated to function efficiently in AcMNPV-infected insect cells (Possee & Howard, 1987).

**Preparation of recombinant virus coexpressing the influenza virus HA and NA genes**

To investigate the potential of vector pAcUW3 to coexpress two foreign proteins, we used it to insert the influenza virus genes encoding HA and NA into AcMNPV. The A/PR/8/34 HA gene was inserted into the BglII site of pAcUW3 under the control of the p10 promoter; the A/NT/60/68 NA gene was then placed at the BamHI site, under the control of the polyhedrin promoter, to derive the transfer vector pAcUW3HANA (Fig. 1). Sf cells were transfected with a coprecipitate of

**Results**

**Construction of the dual expression vector pAcUW3**

A transfer vector (pAcUW3) able to insert two foreign coding sequences into the AcMNPV genome simultaneously was constructed by combining various elements of vectors pAcRP25 (Merryweather et al., 1990) and...
Analysis of protein synthesis by recombinant virus AcUW3HANA

Immunoprecipitation with virus-infected cell extracts was carried out to monitor expression of HA and NA by AcUW3HANA. Sf cells were infected with wild-type AcMNPV, AcUW3HANA or AcRP19.HA, a recombinant virus expressing HA as a single foreign protein under the control of the polyhedrin promoter. The proteins in infected cells were radiolabelled with [35S]methionine and the influenza virus proteins were precipitated with MAbs specific for HA or NA. The results are shown in Fig. 2. By using NA-specific MAbs two diffuse bands were precipitated from AcUW3HANA-infected cells with apparent Mr of about 60K and a faint band of about 65K were detected (lane 5). These two bands were also precipitated from cells infected with the recombinant virus AcRP19.HA (lane 6). HA was not precipitated from AcUW3HANA-infected cells using anti-X31 serum because of the different HA subtypes involved (i.e. X31 is H3, A/PR/8/34 is H1). Neither the NA-specific nor the HA-specific bands were precipitated from wild-type AcMNPV-infected (lane 2) or mock-infected (lanes 1) Sf cells. The presence of a band corresponding to the polyhedrin protein in AcMNPV-infected cells is due to coprecipitation of polyhedra together with the Protein A–Sepharose-linked immune complexes. The origin of the bands of lower Mr present in AcUW3HANA-infected cells (lanes 3 to 5) is not clear and these were not reproduced in repeated experiments.
Functional assays for HA and NA activity in virus-infected cells

SF cells were infected with AcMNPV, AcRP19.HA or AcUW3HANA, harvested at various times p.i., and assayed for HA and NA activity to assess the functional properties and the kinetics of expression of the two proteins (Fig. 3).

In AcUW3HANA-infected cells, significant HA activity was first detected 12 h p.i. It increased at 24 h p.i. to 256 HA units (U)/5 × 10^5 cells and remained constant until 48 h p.i. Uninfected cells or cells infected with wild-type AcMNPV showed no HA activity.

The kinetics of HA expression were similar in AcRP19.HA-infected cells. The level of HA activity in AcRP19.HA-infected cells was about twofold lower than that in cells infected with the dual recombinant.

The NA activity in AcUW3HANA-infected cells was monitored in a colorimetric assay measuring the formation of N-acetylneuraminic acid using fetuin as a substrate. In AcUW3HANA-infected cells, the kinetics of NA expression were very similar to those of HA, with significant activity at 12 h p.i. and maximum expression at 36 to 46 h p.i. Uninfected and AcMNPV-infected cells produced very low background levels of absorbance at 549 nm.

Localization of expressed proteins in the infected cell

HA and NA produced in influenza virus-infected vertebrate host cells are synthesized as precursor molecules with a signal sequence. During their translocation through the endoplasmic reticulum they are extensively glycosylated before incorporation into the plasma membrane of the infected cell. Immunofluorescence studies were carried out to test for cell surface localization of HA and NA in insect cells infected with the recombinant virus AcUW3HANA (Fig. 4). The production of HA and NA in recombinant virus-infected SF cells was demonstrated in fluorescence experiments with acetone-fixed cells by using MAbs specific for HA or NA. In Fig. 4(a) the results obtained with an NA-specific antibody are shown; similar fluorescence was found with an HA-specific antibody (Possee, 1986). Cells infected with wild-type AcMNPV showed very low background fluorescence (Fig. 4b). Cell surface localization of both antigens was shown using unfixed cells and functional assays were carried out 36 h p.i. to demonstrate both HA and NA activity in each of the plaque isolates. We concluded from these results that the recombinant virus AcUW3HANA was viable and that expression of both foreign genes was stable for at least 12 passages in cell culture.

Discussion

This report describes the construction of a new transfer vector, pAcUW3, which mediates the insertion of two foreign genes into the baculovirus genome in a single cotransfection experiment. The vector makes use of a copy of the pl0 promoter inserted upstream of and in the opposite orientation to the polyhedrin promoter. To demonstrate that stable recombinant viruses could be obtained with this vector, two influenza virus genes encoding HA and NA were inserted into AcMNPV in a way such that expression of HA was controlled by the pl0 promoter and that of NA by the polyhedrin promoter. Both HA and NA activities were demonstrated in AcUW3HANA-infected cells and, in comparison with a recombinant virus expressing HA as the only foreign gene (AcRP19.HA), the HA activity was found to be twofold higher. This difference probably was not a result of using the pl0 promoter because previous analyses have shown that Escherichia coli β-galactosidase protein (Weyer et al., 1990) and the B. thuringiensis δ-endotoxin (Merryweather et al., 1990) expressed using each promoter are synthesized to similar levels. The time course of HA and NA expression faithfully mimicked the kinetics of synthesis of the very late proteins in AcMNPV-infected cells.
Fig. 4. Immunofluorescence test of infected SF cells using MAbs against X31 NA (a to c), A/PR/8/34 HA (d) or rabies virus N (5D53) (e and f). (a) AcUW3HANA-infected cells, acetone-fixed; (b) AcMNPV-infected cells, acetone-fixed; (c and d) AcUW3HANA-infected cells, unfixed; (e) AcNPV3-infected cells, acetone-fixed; (f) AcNPV3-infected cells, unfixed.
The presence of the duplicated 230 bp p10 promoter apparently did not interfere with the stability of the virus because both foreign genes were expressed after 12 passages in insect cell culture. The two p10 promoters are positioned in the opposite orientation within the virus genome, and homologous recombination would result in the reversion of sequences between the two copies. It is not known whether such an event would have a deleterious effect on expression of the two foreign genes or the viability of the virus. We did not investigate whether, after several passages, the genome of progeny virus was rearranged compared to the original recombinant virus.

The immunoprecipitation experiments described in this report showed that HA expressed from the dual recombinant AcUW3HANA migrated as bands of the same apparent M, as HA expressed from the single, polyhedrin-based expression vector AcRP19. HA. The dual banding is most likely to be a consequence of variable glycosylation patterns in the very late stages of the virus infection. Glycoproteins produced in the preceding late stage of virus replication using the AcMNPV basic protein promoter (pAcMP1; Hill-Perkins & Possee, 1990) undergo more extensive glycosylation, as evidenced by migration in polyacrylamide gels, than the same protein produced by polyhedrin promoter-based vectors (L. A. King, personal communication).

In influenza virus-infected vertebrate host cells, HA and NA are plasma membrane-bound glycoproteins. Like most membrane proteins, HA contains an N-terminal signal sequence which is cleaved off after translocation across the endoplasmic reticulum membrane, and a C-terminal sequence for anchoring the molecule in the plasma membrane. However, NA is oriented in the membrane in the opposite way to HA; the membrane anchor sequence is located at the N terminus and a signal sequence is not removed (Fields et al., 1981; Blok et al., 1982). Immunofluorescence analysis and a haemadsorption assay showed the presence of both proteins at the surface of AcUW3HANA-infected SF cells, demonstrating that both types of glycoprotein can be expressed in insect cells and incorporated into the plasma membrane.

Although HA and NA expressed from the dual expression vector AcUW3HANA in insect cells most likely are not glycosylated authentically, they were shown to be active in functional assays. The role of protein glycosylation is not yet clear, but several studies have shown that baculovirus-expressed virus antigens can function as immunogens, even though their glycosylation pattern is different from that of the authentic protein (see Possee et al., 1990). For example, fowl plague influenza virus HA expressed in baculovirus-infected insect cell cultures or larvae induces an immune response in chickens capable of protecting the vaccinated animals from virus challenge (Kuroda et al., 1986, 1989, 1990). The coproduction of multiple components of a virus is an attractive prospect for the development of improved synthetic vaccines. Co-synthesized virus proteins could interact in the cell to form subviral particles which might be more immunogenic than single components of the virus particle. Interaction of co-expressed proteins in insect cells has been shown for bluetongue virus proteins, VP3 and VP7. These proteins form core-like particles in infected cells (French & Roy, 1990). It will be interesting to co-express other influenza virus proteins such as the matrix protein and the nucleoprotein together with the major viral antigens HA and NA and investigate whether they can assemble into complex structures with improved immunogenic properties in insect cells. Our new vector, pAcUW3, will greatly assist these studies.

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References


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