Analysis of very late gene expression by *Autographa californica* nuclear polyhedrosis virus and the further development of multiple expression vectors

Ulrike Weyer, Stuart Knight and Robert D. Possee*

NERC Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford OX1 3SR, U.K.

The consequences of locating the polyhedrin gene coding sequences and the p10 promoter at heterologous positions within the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) genome were investigated. Positioning the polyhedrin or β-galactosidase coding sequences under the control of the p10 gene promoter via the use of the new transfer vector, pAcUW1, resulted in viable recombinant viruses able to produce high levels of each non-fused gene product at the appropriate time. Polyhedra were also produced by the virus with the p10 promoter–polyhedrin hybrid gene and appeared normal in thin sections. Therefore the combination of polyhedrin promoter and coding sequences is evidently not essential for efficient expression of this protein. The p10 promoter can serve this function equally well. Viruses with the p10 promoter and β-galactosidase coding sequences placed upstream from the polyhedrin gene in either orientation produced large amounts of β-galactosidase protein in infected cells, thus demonstrating that the p10 promoter can function at an alternative position within the virus genome. A second transfer vector, pAcUW2B, was constructed, with a copy of the p10 gene promoter placed upstream and in opposition to the polyhedrin gene. This mediates the insertion of any foreign gene under the control of the p10 promoter while preserving normal p10 gene expression. The advantages of these constructs over the conventional vectors presently used to express foreign genes in insect cell systems and their utilization in the production of virus insecticides are discussed.

Introduction

The insect baculoviruses *Autographa californica* (Ac) and *Bombyx mori* (Bm) nuclear polyhedrosis viruses (MNPV) have been used as versatile high level expression vectors of prokaryotic and eukaryotic genes (reviewed by Luckow & Summers, 1988; Miller, 1988; Fraser, 1989; Maeda, 1989). Their success owes much to the unique nature of the baculovirus replication cycle, which involves the sequential expression of virus-encoded genes in four, temporally distinct phases (Carstens *et al.*, 1979; Kelly & Lescott, 1981; Miller *et al.*, 1983; Guarino & Summers, 1986). The first three stages result in the production of infectious virus particles which invade other cells, either in culture or within the insect larva and thus disseminate infection. In the final, very late phase of gene expression, virus particles are occluded into crystalline proteinaceous structures called polyhedra. These serve to protect the virus in the environment after release from the infected insect. Throughout the very late phase, from about 18 h post-infection (p.i.), two virus-encoded gene products are expressed in copious quantities. These are the 29K polyhedrin and 10K p10 proteins. The polyhedrin protein constitutes the major component of the polyhedra whereas the p10 protein is thought to play some role in the morphogenesis of these structures (Vlak *et al.*, 1988; Williams *et al.*, 1989). Both gene products are dispensable for the production of virus particles (Smith *et al.*, 1983a; Vlak *et al.*, 1988), which means that they can be replaced with foreign coding sequences in the expression vector systems (Smith *et al.*, 1983b; Vlak *et al.*, 1988). The polyhedrin promoter has been utilized for the expression of many foreign genes but the potential of the p10 promoter remains largely unexplored; it has only been used to express a reporter gene, namely β-galactosidase (*lacZ*) as a fusion protein (Vlak *et al.*, 1988; Williams *et al.*, 1989) and a phosphotransferase gene which inactivated neomycin (Gonnet & Devauchelle, 1987).

It is of considerable interest to determine what factors are responsible for the efficient activity of the polyhedrin and p10 promoters and in consequence, to facilitate the
design of improved expression vectors, particularly those where promoters are duplicated to permit the syntheses of multiple foreign gene products (Emery & Bishop, 1987). The polyhedrin gene promoter of AcMNPV appears to consist of a 49 nucleotide 5' non-coding leader sequence (Matsuura et al., 1987; Rankin et al., 1988) and a short sequence of 15 to 20 nucleotides upstream from the transcription initiation site (Possee & Howard, 1987; Rankin et al., 1988). The AcMNPV p10 promoter comprises a 70 nucleotide 5' non-coding leader sequence and a sequence of about 30 nucleotides upstream from the mRNA start site (Weyer & Possee, 1988, 1989; Qin et al., 1989). A consensus sequence of 12 nucleotides spans the mRNA start sites of the AcMNPV polyhedrin and p10 genes and is found in all late and very late baculovirus gene promoters (Rohrmann, 1986; Wilson et al., 1987). It is intriguing that although the two promoters share the same general organization they do not have significant sequence homology. Only the consensus sequence spanning the mRNA start sites and the AT-rich nature of the 5' non-coding regions make the polyhedrin and p10 promoters in any way comparable structures.

The effects of other sequences on very late gene expression, which may act over a longer distance, have not been investigated fully. There is some evidence that homologous region 5 (Cochran & Faulkner, 1983) may play some role in the function of the p10 promoter (Qin et al., 1989). However in other studies (Rankin et al., 1988; U. Weyer et al., unpublished data) it has been concluded that this element has little effect on the polyhedrin promoter. It is also unclear whether the position of the two promoters within the virus genome has any effect on their activity; this might be a factor if long-range enhancer elements play a role in their efficient function. Rankin et al. (1988) demonstrated that by reversing the polyhedrin promoter a twofold increase in activity resulted. However, to date, the effect of moving these very late promoters to other locations within the virus genome has not been studied.

In this report we have investigated two issues. Firstly, we studied whether the combination of the polyhedrin promoter with the polyhedrin coding sequence has significance in the high level expression of this gene which occurs very late in infection. This was investigated by removing the polyhedrin coding sequences, placing them under the control of the p10 promoter and determining whether the recombinant virus was capable of high level expression of the polyhedrin protein and subsequent formation of mature polyhedra. Secondly, we investigated the question of whether the position of the p10 very late promoter within the virus genome affects its activity and, as a consequence, whether multiple expression vectors utilizing this promoter can be constructed.

Methods

Viruses and cells. Spodoptera frugiperda cells (PLB-SF21) (Vaughn et al., 1977), AcMNPV (C6) and viruses containing the lacZ gene were propagated as described previously (Possee, 1986; Possee & Howard, 1987).

Construction of plasmid transfer vectors. Standard plasmid manipulation techniques were used throughout these experiments (Maniatis et al., 1982).

(i) pAcUW1. The AcMNPV EcoRI P fragment containing the complete p10 gene (Rohel et al., 1983; Smith et al., 1983; Kuze et al., 1984; Lübben & Doerfler, 1984) was inserted into the EcoRI site of pUC8/6 (a pUC8 derivative retaining only the EcoRI site in the polylinker; Possee, 1986) to derive pAcEI-P. This was digested with BglII and Mulu to remove the p10 promoter and 152 nucleotides of the p10 coding sequence. This region was replaced with an Mulu-BgllI-excisable fragment (208 nucleotides) from the deletion mutant pAcpl0+1 (Weyer & Possee, 1988) which contains the complete p10 promoter and first nucleotide of the translation initiation codon. The resulting transfer vector was designated pAcUW1 (Fig. 1).

(ii) pAcUW1-lacZ. The plasmid pCH110-BgLl (Possee & Howard, 1987) was digested with BglII and BamHl to release a 3.7 kbp fragment containing the Escherichia coli lacZ gene and simian virus 40 (SV40) polyadenylation signals. This was inserted into the BglII site of pAcUW1 to produce pAcUW1-lacZ.

(iii) pAcUW1-PH. A cassette containing only the coding sequences of the AcMNPV polyhedrin gene was constructed. The plasmid pUC8/6/8 (Possee, 1986), containing the EcoRI I fragment, was digested sequentially with EcoRV (92 nucleotides upstream from the polyhedrin ATG) and Bal 31 exonuclease to remove the 5' non-coding sequences of the polyhedrin gene; these were replaced with a BglII linker. The 3' non-coding sequences of the polyhedrin gene were removed in a similar fashion by making deletions from a Smal site (90 bp downstream from the coding sequence) in pUC8/6/8 and replacing them with a BglII linker. One mutant was selected from each deletion series and used to reassemble a variant of pUC8/6/8 (pPH1), where the polyhedrin-coding sequences were excisable with BglII. These were then inserted into pAcUW1 to derive pAcUW1-PH (Fig. 1).

(iv) pAcUW2-lacZ. The plasmid pUC8/6/8 was digested with EcoRV and a synthetic BglII linker was inserted at this position to derive pUC8/6/8-BglII. The construct pAcpl0+1 (Weyer & Possee, 1988) was digested with Smal and a BamHl linker was inserted. Digestion of this modified plasmid with BglII and BamHl released a 230 nucleotide fragment containing the p10 promoter. This promoter was inserted at the BglII site of pUC8/6/8-BglII in tandem (pUC8/6/8-p10A) or in the reverse orientation (pUC8/6/8-p10B), relative to the polyhedrin promoter. The lacZ gene (described above) was inserted into each of these variants to derive pAcUW2-lacZ(A) or -(B) (Fig. 6).

(v) pAcUW2 (A) and -(B). The plasmid pCH110-BglII was digested with EcoRI, and treated with the Klenow fragment of E. coli DNA polymerase prior to ligation with BglII linkers. The ligation reaction mix was digested with BglII and BamHl and a 577 bp fragment containing the 3' end of the lacZ coding sequence and SV40 polyadenylation signals was isolated. This was inserted at the BglII site of pUC8/6/8-BglII, in both orientations, and the resulting plasmids were modified further by inserting the BglII-BamHl-excisable p10 promoter fragment (230 nucleotides) used for the construction of pAcUW2-lacZ(A) or -(B). Therefore these plasmids contained the p10 promoter, a BglII insertion site, the 3' end of the lacZ gene and SV40 polyadenylation signals inserted at the EcoRV site upstream from the polyhedrin ATG translation start codon (Fig. 8).

Construction and purification of recombinant viruses. The preparation of infectious virus DNA and cotransfection protocols for the derivation of recombinant viruses have been described previously (Possee, 1986;
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Results

Expression of the polyhedrin and $\beta$-galactosidase genes by the p10 promoter

The consequences of expressing the polyhedrin coding sequences at an alternative location within the AcMNPV genome were investigated by making use of an expression system based on the p10 promoter, capable of producing non-fused proteins.

The transfer vector pAcUW1 (Fig. 1) was constructed to permit insertion of foreign genes into the virus genome under the control of the p10 promoter, in lieu of p10 coding sequences. This vector retained the S' leader of p10 and the first nucleotide of the ATG translation initiation codon, thus mimicking the promoter structure of pAcYM1, a high yielding polyhedrin promoter-based vector (Matsuura et al., 1987). To produce a recombinant virus expressing the polyhedrin gene at a different site within the virus genome the vector pAcUW1-PH (Fig. 1) was constructed and cotransfected with AcRP23. lacZ DNA (Possee & Howard, 1987). The resulting p10-negative virus, designated AcUW1-PH, contained the polyhedrin gene under control of the p10 promoter at the p10 locus and the lacZ gene under polyhedrin promoter control at the polyhedrin locus. The arrangement of the polyhedrin coding sequences relative to the p10 promoter is shown in Fig. 2.

The lacZ gene from E. coli is an example of a foreign gene which has been expressed to high levels by the polyhedrin promoter as an unfused protein (Pennock et al., 1984; Possee & Howard, 1987). Therefore to serve as a control the lacZ coding sequences were inserted into pAcUW1 to derive pAcUW1-lacZ (Fig. 1). This transfer vector was cotransfected with wild-type AcMNPV DNA to produce the recombinant, p10-negative virus AcUW1-lacZ with the lacZ gene under p10 promoter control at the p10 locus.

Analysis of protein expression by recombinant viruses

Recombinant viruses were used to infect S. frugiperda cells and protein synthesis was analysed by pulse-labelling with $^{3}$H-leucine at various times after infection, followed by fractionation in polyacrylamide gradient gels. Fig. 3 summarizes the results obtained with AcUW1-lacZ (p10-negative, lacZ-positive). Fig. 3(a) shows a gel stained with Coomassie blue. In AcUW1-lacZ-infected cells, p10 protein was undetectable, whereas $\beta$-galactosidase accumulated to high levels very late after infection (24 to 48 h p.i.). An autoradiograph of the same gel (Fig. 3b) demonstrated that $\beta$-galactosidase synthesis in AcUW1-lacZ-infected cells continued until at least 48 h p.i. Fig. 3(b) demonstrated also that the traces of $\beta$-galactosidase and polyhedrin proteins seen in the stained gel (Fig. 3a) at 4 to 8 h p.i. were due to contamination from the virus inoculum, since they were not radio-labelled.

Fig. 4 illustrates the data obtained after infection of cells with the recombinant virus AcUW1-PH, which contains the polyhedrin-coding sequences under the p10 promoter and inserted at the p10 locus. This virus also contains the lacZ gene in the polyhedrin locus. Fig. 4(a) shows a Coomassie blue-stained gel. Polyhedrin expressed under the control of the p10 promoter accumulated to a high level at very late times after infection which appear to be similar to those obtained in wild-type AcMNPV-infected cells (Fig. 4a, lane wt/48). The autoradiograph of the same gel (Fig. 4b) shows that in AcUW1-PH-infected cells the kinetics of polyhedrin protein synthesis were similar to the rate of $\beta$-galacto-
sidase protein synthesis under the control of the polyhedrin promoter.

**Structural features of AcUW1-PH**

Cells infected with AcUW1-PH and observed using light microscopy revealed apparently normal polyhedra within the nuclei (data not shown). This conclusion was substantiated by viewing thin sections of infected cells using an electron microscope. Fig. 5 shows sections from cells infected with AcMNPV and with AcUW1-PH. The polyhedra produced by both viruses packaged virus particles in the normal way. Observation of the polyhedron membrane (Vlak et al., 1988; Williams et al., 1989) was variable in cells infected with each virus and appeared to be dependent upon the angle of section.

**Construction of a virus with two copies of the p10 promoter**

The function of the AcMNPV p10 promoter and its utility as an expression system was further investigated by constructing a virus with a copy of the p10 promoter at another position within the virus genome. This served to determine whether the normal location of the p10 promoter was critical for its high level of activity and secondly, whether it would be possible to derive multiple expression vectors utilizing this promoter. A suitable position for insertion of a foreign gene was considered to be the *EcoRV* site located 92 nucleotides upstream from the polyhedrin ATG translation initiation codon, since it has been shown that insertions of 8 to 3052 nucleotides at this site did not interfere with polyhedrin promoter activity or viability of the virus (Emery & Bishop, 1987; Possee & Howard, 1987). The p10 promoter was inserted
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Fig. 3. Temporal expression of proteins in AcUW1-lacZ-infected cells. S. frugiperda cells were infected with recombinant or wild-type AcMNPV and pulse-labelled at various times (4 to 48 h) after infection with [3H]leucine for 1 h. Extracts were analysed in a 10 to 30% polyacrylamide gel, stained with Coomassie blue (a), treated with EN3HANCE, dried and exposed for autoradiography (b). M, Mr markers; wt, AcMNPV; (-), uninfected cells; c, AcRP23. lacZ-infected cells (not radiolabelled); β-gal, β-galactosidase; PH, polyhedrin.

Fig. 4. Temporal expression of proteins in AcUW1-PH-infected cells. Infected cells were radiolabelled and analysed as described in Fig. 3. (a) Coomassie blue-stained gradient gel; (b) autoradiograph of the same gel.

in both orientations at this position as a 230 nucleotide fragment, together with the β-galactosidase gene in tandem [pAcUW2-lacZ(A)], or inverted [pAcUW2-lacZ(B)], relative to the polyhedrin gene (see Methods and Fig. 6). These plasmids were coprecipitated with infectious DNA from an AcMNPV mutant with a deletion of the entire polyhedrin coding sequence (Bishop et al., 1988) and used to transfet S. frugiperda cells. Recombinant viruses designated AcUW2-lacZ(A) or -(B) were isolated which contained the wild-type p10 and polyhedrin genes and in addition, the lacZ gene under p10 promoter control upstream of the polyhedrin gene in both orientations as shown in Fig. 6.

Analysis of protein synthesis in AcUW2-lacZ

Protein synthesis in AcUW2-lacZ-infected cells was monitored as described above. Fig. 7(a) shows a stained polyacrylamide gradient gel, analysing proteins from AcUW2-lacZ(B)-infected cells. The β-galactosidase pro-
Fig. 5. Thin sections of *S. frugiperda* cells infected with AcMNPV (a) and AcUW1-PH (b) after 48 h and examined using the electron microscope. P, polyhedron; VP, virus particle. Bar markers represent 0.5 μm.
very late gene expression within the virus genome has significance for the high level expression of these genes.

A transfer vector, pAcUW1, containing the complete 5' non-coding leader sequence and the first nucleotide of the p10 translation start codon was constructed to enable heterologous DNA to be inserted under the control of the p10 promoter in lieu of the p10 coding sequences. This vector was used to derive two recombinant viruses, AcUW1-lacZ and AcUW1-PH, which expressed the lacZ and polyhedrin coding sequences respectively, as non-fused proteins. Analysis of protein expression in cells infected with AcUW1-lacZ showed that β-galactosidase was expressed to high levels similar to those expected for p10 synthesis in AcMNPV-infected cells. The polyhedrin coding sequences were also expressed effectively when placed under the control of the p10 promoter in AcUW1-PH. The levels and kinetics of polyhedrin protein expression in cells infected with this recombinant were comparable to those obtained in AcMNPV-infected cells.

Electron microscopy showed that occlusion bodies produced in AcUW1-PH-infected cells contained numbers of virus particles similar to those produced in AcMNPV-infected cells. We were unable to make any conclusions concerning the role of the p10 protein in the formation of the envelope surrounding the polyhedron (Vlak et al., 1988; Williams et al., 1989) because the observation of this structure was variable in our studies.

These data demonstrate that the polyhedrin gene does not have to be expressed under the control of the
polyhedrin promoter and at its natural location to facilitate the production of mature occlusion bodies. Furthermore, the use of pAcUW1 in conjunction with AcUW1-lacZ or AcUW1-PH will permit the insertion of any foreign gene with its own translation initiation codon at the p10 locus by selecting for white (lacZ-negative) or polyhedrin-negative viruses respectively.

The p10 promoter, although functional at the same time in the infection cycle as the polyhedrin promoter, does have a substantially different nucleotide sequence. It is not possible using the limited sequence data available to determine whether both have evolved from the same ancestor. However both the initiation sites for p10 and polyhedrin transcripts have been mapped within a common core sequence, ATAAG (Kuzio et al., 1984; Lübert & Doerfler, 1984; Howard et al., 1986; Rankin et al., 1988), which has been found in all the late and very late genes of AcMNPV analysed so far. Mutation of this core sequence abolished the activity of both the polyhedrin and the p10 promoter (Possee & Howard, 1987; Rankin et al., 1988; Qin et al., 1989; Weyer & Possee, 1989) suggesting that the ATAAG motif plays an important role in the regulation of the p10 and polyhedrin very late gene expression.

This study has also shown that the p10 promoter can function if moved to a different site within the virus genome, namely upstream of the polyhedrin gene. In the recombinant virus AcUW2-lacZ(B), high levels of β-galactosidase protein were expressed from the p10 promoter located at this position. The expression of the polyhedrin gene was not affected by this insertion. There was no significant difference between alternative arrangements of the p10 and polyhedrin promoters with respect to each other, i.e., in tandem or in opposition. To date it is not clear whether long-range cis-acting elements such as the five homologous regions found in the AcMNPV genome (Cochran & Faulkner, 1983) have an effect on activity of the p10 promoter. Our data suggest that if these structures are required for high expression of the very late genes, it is not important that the promoter is located in its normal position. It will be interesting to examine further the factors which are involved in the regulation of the very late p10 and polyhedrin genes.

A further consequence of these studies was that a universal transfer vector, pAcUW2, could be developed for the insertion of foreign genes, expressed under p10 promoter control, at a site upstream of the polyhedrin gene. Recombinant viruses can be identified by forming polyhedron-containing plaques, after cotransfection with polyhedrin-negative virus DNA. This vector has potential in the development of genetically engineered virus insecticides. Recently a programme has been initiated with the aim of improving the effectiveness of their action by incorporating genes encoding functional insecticidal products which might have a deleterious effect on the insect and hasten its death (Bishop et al., 1988). The use of one of the traditional transfer vectors based on the polyhedrin promoter for the construction of such recombinants would result in polyhedrin-negative viruses. However it is preferable that recombinant viruses used as insecticides should express the polyhedrin gene for the production of viral inclusion bodies, since these are necessary for the persistence of the virus in the environment and therefore the infection of subsequent generations of insects. The use of the p10 promoter presents an alternative since the p10 coding sequences may be replaced with a foreign gene without affecting
the formation of polyhedra (Vlak et al., 1988; Williams et al., 1989). However the p10 gene product must have some role in virus replication in vivo and it may be wise to preserve its integrity in engineered virus insecticides. Therefore, by using pAcUW2 it will be possible to insert a foreign gene into the virus genome, upstream of the polyhedrin gene and thus leave the normal pl0 gene unaffected. We are currently evaluating this vector for this purpose.

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References


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