

A baculovirus expression vector derived from the basic protein promoter of *Autographa californica* nuclear polyhedrosis virus

Michele S. Hill-Perkins and Robert D. Possee*

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

The basic protein of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) is associated with virus DNA in virion nucleocapsids and is produced in infected cells during the late phase of gene expression. A transfer vector was constructed containing the β -galactosidase gene, under the control of a copy of the putative basic protein promoter, in place of the polyhedrin gene within the AcMNPV *EcoRI* fragment I. After cotransfection of *Spodoptera frugiperda* cells with the transfer vector and infectious AcMNPV DNA, polyhedrin-negative recombinant viruses were

selected which expressed high levels of β -galactosidase. Radiolabelling of infected cell proteins showed that β -galactosidase was expressed at the same time as the viral basic protein, between 8 to 24 h post-infection, with a peak synthesis at 12 to 15 h. These results demonstrated that the temporal regulation of the basic protein promoter was not affected by its position within the virus genome. Furthermore, a new baculovirus vector system is now available for high level expression of foreign genes at earlier times in infected cells.

Gene expression in baculovirus-infected cells has been subdivided into four temporal phases: immediate early, early, late and very late (Kelly & Lescott, 1981). The highly successful baculovirus vector system utilizes the very late promoter of the polyhedrin gene from the *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV), or from *Bombyx mori* MNPV, to drive expression of foreign genes in infected cells (reviewed by Luckow & Summers, 1988; Miller, 1988). Further developments of this system have made use of the promoter from the AcMNPV p10 very late gene to produce β -galactosidase (from the *lacZ* gene) protein in *Spodoptera frugiperda* cells (Vlak *et al.*, 1988; Williams *et al.*, 1989). The strategy for developing expression vectors from both genes has involved retaining the promoters, while deleting the coding sequences and replacing them with the foreign gene chosen. A consequence of this is that the recombinant viruses lack the polyhedrin or p10 gene products; fortunately both are dispensable for virus replication (Smith *et al.*, 1983; Vlak *et al.*, 1988; Williams *et al.*, 1989). A logical progression from these studies is to make use of other baculovirus gene promoters in expression vectors and thereby extend the versatility of the system.

One such candidate is the gene encoding the basic protein, a structural core protein found closely associated with the virus DNA within the nucleocapsid (Tweeten *et al.*, 1980; Bud & Kelly, 1980). It has an apparent M_r of approximately 12.5K, and is rich in the basic amino acid

arginine. The protein is probably virus-encoded as Wilson *et al.* (1987) identified an open reading frame in the *HindIII* H/*EcoRI* D region of the AcMNPV genome which could encode an arginine-rich 6.9K protein. Similar results were obtained from our own studies (M. S. Hill-Perkins & R. D. Possee, unpublished data). Furthermore, a synthetic peptide of the predicted 6.9K translation product induced antibodies in rabbits which reacted with the basic protein from purified virus particles in Western blot analyses (Wilson, 1988). The discrepancy in the M_r values determined from polyacrylamide gels and the predicted size from the DNA sequence was attributed to the aberrant migration of highly basic proteins in polyacrylamide gels (Wilson *et al.*, 1987). The basic protein is first detected at 6 to 12 h post-infection (p.i.), but is maximally synthesized between 12 to 24 h p.i. and is one of the seven most abundant virus-induced proteins in infected cells (Rohel & Faulkner, 1984). In this report we describe the construction and utilization of an expression vector developed from this gene promoter.

It was considered unlikely that replacing the basic protein gene of AcMNPV with a foreign gene would yield a viable virus recombinant, since this polypeptide appears to be associated intimately with the packaging of virus DNA (Tweeten *et al.*, 1980; Bud & Kelly, 1980). Therefore, it was considered prudent to duplicate the basic protein promoter in an alternative position within the virus genome which would not affect virus replica-

tion. The precedent for this is the duplication and efficient functioning of the polyhedrin promoter within the AcMNPV genome, albeit at a position very close to the original promoter (Emery & Bishop, 1987). The site chosen for the second copy of the basic protein promoter was the region of the polyhedrin gene, where deletion of the polyhedrin coding sequences (Smith *et al.*, 1983) or promoter elements (Possee & Howard, 1987) do not affect virus replication.

The *Hind*III fragment H of AcMNPV DNA (C6 strain; Possee, 1986) was inserted into pAT153, using standard procedures (Maniatis *et al.*, 1982), to give pAcHH. This was digested with *Asp*718 (an isoschizomer of *Kpn*I) to produce three fragments; the smallest of these (364 nucleotides) was sequenced and shown to contain the putative basic protein promoter, transcription initiation site and the 5' end of the coding sequences (Fig. 1 and 2). The following differences were noted between our sequence and that determined by Wilson *et al.* (1987): the nucleotides C, A, T, A, A, T, A and A at positions 263, 251, 245, 241, 232, 223, 218 and 206 respectively in our sequence are not present in that reported by Wilson *et al.* (1987). This fragment was inserted into pUC18 at the *Asp*718 site to produce pBPP (Fig. 2).

The construct pBPP was partially digested with *Dra*I (0.1 to 0.5 units per μ g at 37 °C for 1 h). The linear component of this digestion was resolved in a low gelling temperature (LGT) agarose gel, and purified from the melted agarose (Possee & Kelly, 1988). The linear molecule was then treated with calf intestinal phosphatase (CIP) (Maniatis *et al.*, 1982), and ligated with two complementary, phosphorylated oligonucleotides (5'AAACGGATCC3' and 3'TTTGCCTAGG5') produced using an Applied Biosystems oligonucleotide synthesizer. The recircularized molecules were used to transform *Escherichia coli* JM105 cells, and recombinant plasmids were isolated containing the synthetic oligonucleotide inserted at the single *Dra*I site within the basic protein promoter. Insertion of the oligonucleotides within the basic protein 5' mRNA leader sequence in the desired orientation restored the *Dra*I site and created an additional *Bam*HI site at the normal position of the ATG translation initiation codon (Fig. 1). This plasmid, designated pBPP-Bam (Fig. 2), was digested with *Bam*HI to release a fragment of 340 nucleotides containing the putative basic protein promoter and a small region of the pUC18 polylinker. This fragment was inserted into the *Bgl*II site of pAcRP277 to produce pAcMP1.lacZ. The construct pAcRP277 was derived previously by inserting the *lacZ* gene cassette into pAcRP23+8 (Possee & Howard, 1987) digested with *Bgl*II and *Bam*HI and treated with CIP to remove the polyhedrin promoter (Fig. 2). The transfer vector

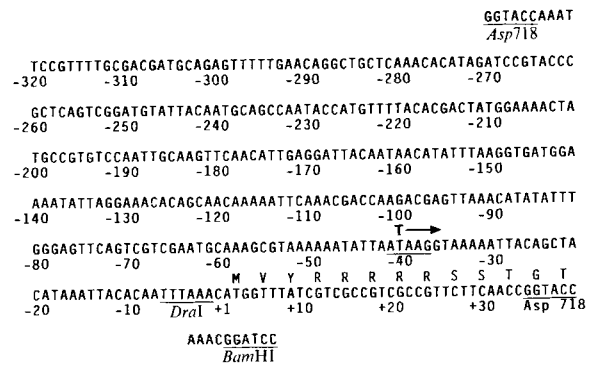


Fig. 1. Nucleotide sequence of the basic protein promoter. The sequence of the 364 bp fragment produced by *Asp*718 digestion of pAcHH is shown. It contains the transcription initiation start site (T) and the 5' end of the basic protein gene-coding sequences; the amino acid residues are indicated above the DNA sequence beginning at +1. The *Dra*I site used to insert the synthetic oligonucleotide and thus create a *Bam*HI site at the normal position of the ATG translation initiation codon is indicated (see Fig. 2 also). The sequence of the oligonucleotide is shown in the bottom line.

pAcMP1 was derived by inserting the 337 bp basic protein promoter fragment, described above, into the *Bgl*II- and *Bam*HI-digested pAcRP23+8.

The transfer vector pAcMP1.lacZ, containing the β -galactosidase gene under the control of the basic protein promoter, was coprecipitated with infectious AcMNPV DNA and used to cotransfect *S. frugiperda* cells (Possee, 1986). The cells were harvested after 2 days and the progeny virus particles were retitrated in a plaque assay. Plaques containing recombinant viruses expressing β -galactosidase were identified by incubating the plates with Xgal (Possee & Howard, 1987). Recombinant plaques turned a blue colour within 1 h incubation at 28 °C; those which lacked polyhedra were isolated and further purified by four rounds of titration in *S. frugiperda* cells. Large stocks of the genetically homogeneous recombinant viruses were obtained by propagation in suspension cultures of the same cells.

To verify the identity of the recombinant virus, DNA was prepared from AcMP1.lacZ (Possee, 1986), digested with *Eco*RI and the fragment containing the *lacZ* gene and basic protein promoter was purified using an LGT agarose gel. This was digested with *Asp*718 to release the promoter fragment and the amino terminus of the *lacZ* gene. This fragment was inserted into M13mp19 for sequencing by the chain termination method (Sanger *et al.*, 1977) to confirm the predicted sequence shown in Fig. 1.

The efficiency of the basic protein promoter in directing foreign gene expression was assessed by inoculating cells with AcMP1.lacZ, AcRP23.lacZ or

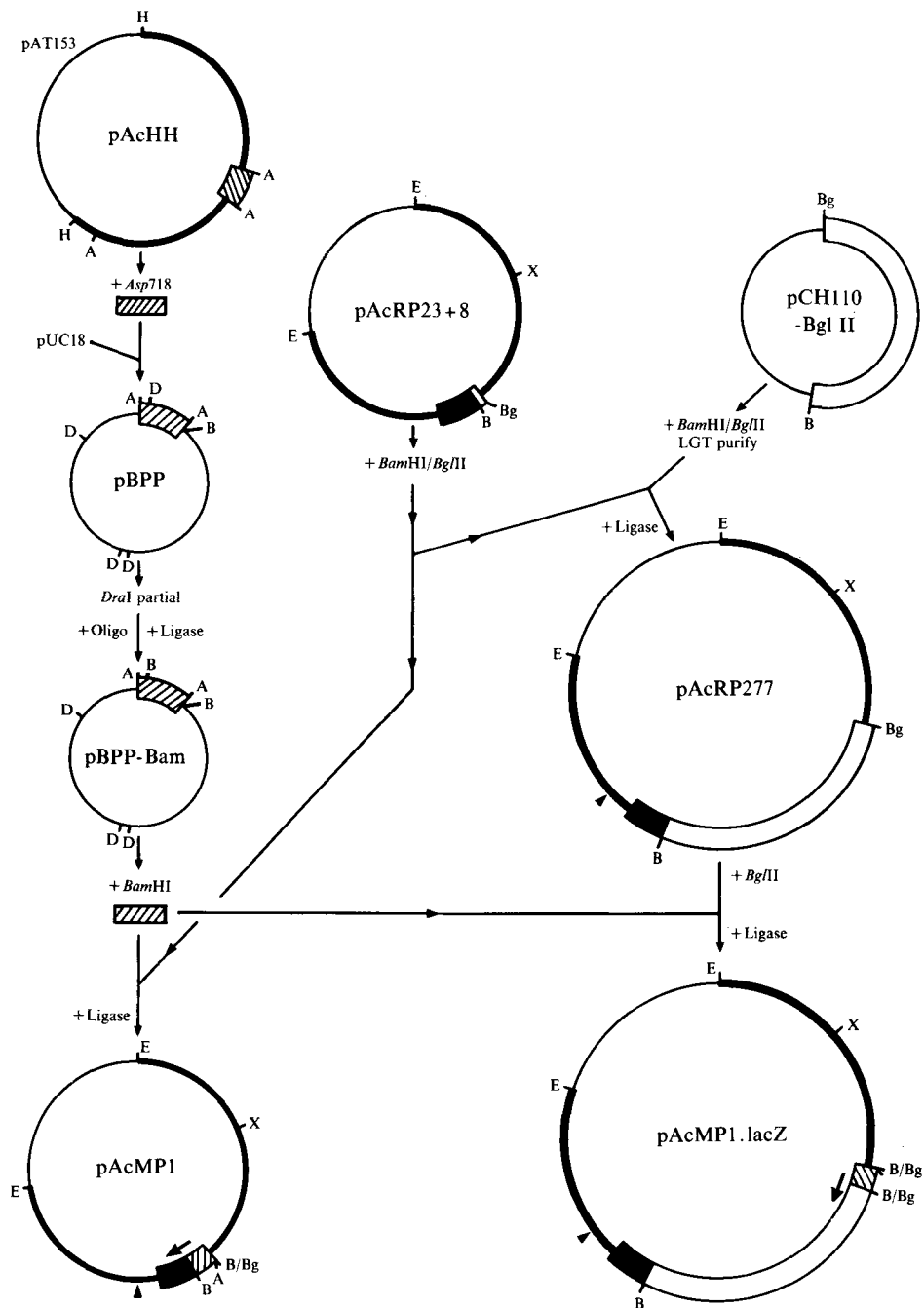


Fig. 2. Construction of the basic protein promoter transfer vector. A schematic representation of the procedures used to derive pAcMP1 and pAcMP1.lacZ is shown. Thick lines represent AcMNPV sequences and thin lines represent the pUC vector, except for pAcHH which contained pAT153. The solid box is the carboxy half of the (out of frame) polyhedrin gene coding sequences and the small open box in pAcRP23 + 8 is the polyhedrin promoter. The *lacZ* gene is depicted by the large open box throughout and the basic protein promoter (sequence shown in Fig. 1) is represented by the hatched box with the direction of transcription indicated by the short arrow; the polyhedrin polyadenylation signal (Howard *et al.*, 1986) is shown by the arrowhead. Restriction enzyme sites: A, *Asp718*; B, *BamHI*; Bg, *BglII*; B/Bg, *BamHI/BglII* fusion; D, *DraI*; E, *EcoRI*; X, *XhoI*. Oligo, synthetic oligonucleotide inserted to modify the putative promoter sequences (see Fig. 1).

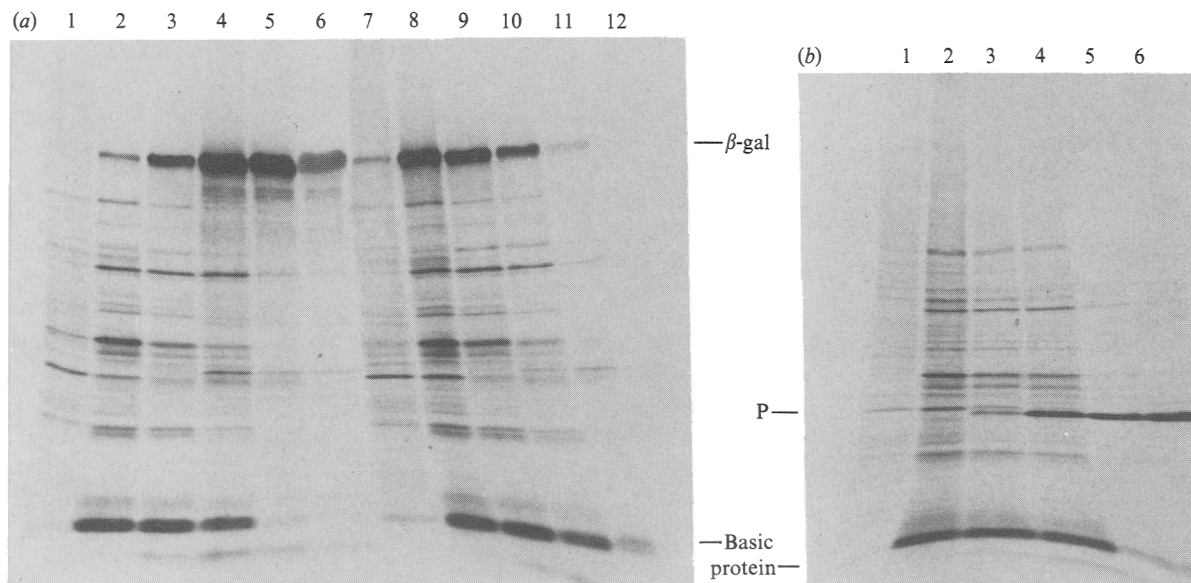


Fig. 3. (a) Expression of β -galactosidase by recombinant viruses. *S. frugiperda* cells were inoculated with 10 p.f.u./cell of AcRP23.lacZ, containing the *lacZ* gene under the control of the polyhedrin promoter (lanes 1 to 6), or AcMP1.lacZ, with the *lacZ* gene under the control of the basic protein promoter (lanes 7 to 12). The cells were incubated at 28 °C until 8, 12, 15, 18, 24 and 30 h.p.i. (lanes 1 to 6, AcRP23.lacZ; lanes 7 to 12, AcMP1.lacZ). Infected cells were pulse-labelled with [14 C]arginine and analysed in a 10 to 30% polyacrylamide gradient gel. β -gal, β -galactosidase; (b) Expression of polyhedrin protein (P) by AcMNPV. Cells were infected with AcMNPV and pulse-labelled at the times described above (lanes 1 to 6).

AcMNPV at a high m.o.i. and pulse labelling with [14 C]arginine (Kelly & Lescott, 1981). Fig. 3(a) shows an autoradiograph of 10 to 30% polyacrylamide gradient gels (Cook *et al.*, 1979) analysing virus-induced proteins in cells infected with AcMP1.lacZ, AcRP23.lacZ or AcMNPV and pulse-labelled for 1 h at various times between 8 and 30 h p.i. The β -galactosidase gene, under control of the basic protein promoter, was first expressed in AcMP1.lacZ-infected cells at 8 h p.i. Thereafter, expression increased rapidly so that between 12 and 15 h p.i. this polypeptide formed a major component of the radiolabelled cell protein. At 18 h p.i. synthesis of β -galactosidase was reduced and by 24 h p.i. very little was being made; at 30 h p.i. it was undetectable. Concurrent with the production of β -galactosidase by AcMP1.lacZ was the synthesis of the virus-encoded basic protein. From the data presented here, together with other similar experiments not shown, we conclude that both the native virus basic protein and the β -galactosidase gene product were synthesised at the same time in infected cells. In contrast, cells infected with AcRP23.lacZ, containing the β -galactosidase gene under the control of the polyhedrin promoter, did not produce detectable amounts of β -galactosidase protein until 12 h p.i. Maximum rates of synthesis were attained by 18 h p.i. and continued until 24 h p.i.; at 30 h p.i. incorporation of the radiolabel declined significantly but

it was clear that the protein was still being produced. Fig. 3(b) features a similar experiment with unmodified AcMNPV; the expression of the basic protein by this virus follows the same kinetics as seen with the previous two virus recombinants. However, the expression of the polyhedrin protein was apparently initiated at 15 h p.i., some 3 h later than that of β -galactosidase under the control of the polyhedrin promoter. Expression of polyhedrin also appeared to continue at very high levels until 30 h p.i.; this is in contrast to the experiment with AcRP23.lacZ, where synthesis at 30 h p.i. had decreased significantly. This may be attributable to the high concentration of β -galactosidase protein in the cell having a toxic effect, or a feedback mechanism inhibiting synthesis. In cells infected with the wild-type AcMNPV the polyhedrin protein crystallizes within the nucleus and is probably unable to affect biosynthetic processes.

To compare the total yield of recombinant protein in cells infected with AcMP1.lacZ and AcRP23.lacZ, extracts from cells infected with these two viruses were analysed in gradient polyacrylamide gels which were stained with Coomassie blue (Fig. 4). These data indicated that although the final yield of β -galactosidase in AcMP1.lacZ-infected cells was lower than that from cells infected with AcRP23.lacZ, it still constituted a major proportion of the cell proteins.

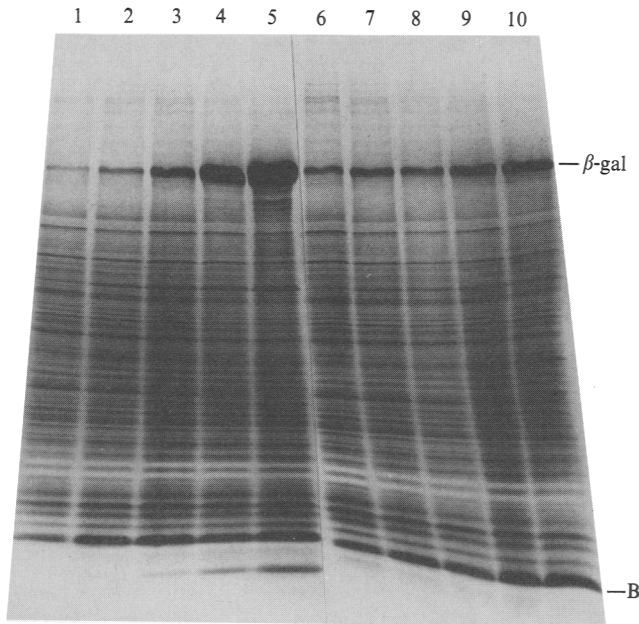


Fig. 4. Accumulated protein expressed by recombinant viruses in infected cells. Cells were infected with AcRP23.lacZ (lanes 1 to 5) or AcMP1.lacZ (lanes 6 to 10), as described for Fig. 3, and harvested at 12, 15, 18, 24 and 30 h p.i. for analysis of intracellular proteins using polyacrylamide gradient gels stained with Coomassie blue. β -gal, β -galactosidase; B, basic protein.

A baculovirus expression vector derived from the AcMNPV basic protein promoter has been produced and used to synthesize high levels of β -galactosidase protein in *S. frugiperda* cells. One advantage of this vector is that foreign proteins may be expressed in infected cells at earlier times (12 h p.i.) than is possible with the standard baculovirus vectors based on the polyhedrin promoter. The amount of foreign protein produced is comparable to that of the normal basic protein gene product; furthermore the kinetics of synthesis are very similar.

The factors controlling the temporal expression of baculovirus genes have yet to be elucidated. It is unclear whether the transition to the very late phase is governed at the level of transcription or translation. Although we have not addressed this issue in these experiments it is worth considering that the synthesis of both the basic and the β -galactosidase proteins decreased significantly by 24 h p.i. However previous reports from Rohel & Faulkner (1984) and Wilson *et al.* (1987) showed that mRNA specific for the basic protein gene was still present in infected cells at high levels after 24 h p.i. This suggests that the polyhedrin-encoding mRNA may be more efficient at binding to ribosomes and in initiating translation. Clearly, more work is required to resolve this subject.

This new vector may also be advantageous when producing proteins requiring extensive post-translational modification (e.g. glycosylation and proteolytic cleavage), since the cell may be better equipped to carry out such processes earlier in infection. Very late in infection the cells are committed to the synthesis of polyhedrin and p10 proteins; apparently neither requires extensive post-translational modification. Although the vectors based on the very late gene promoters certainly appear able to process foreign gene products correctly in the infected cell, there is some evidence that in the case of glycosylation this process does not entirely mimic the normal mammalian cell. For example, the influenza virus haemagglutinin protein produced in insect cells is of a lower apparent M_r than the same product in mammalian cells (Kuroda *et al.*, 1986; Possee, 1986). Kuroda *et al.* (1986) concluded that the oligosaccharide side-chains added to the insect cell-derived protein were not identical to those attached in the vertebrate cells. The vector described in this report will allow comparisons to be made between processing of glycoproteins at different times after infection.

The basic protein expression system will also have advantages when constructing genetically engineered virus insecticides. It will now be feasible to express insect-specific toxins and hormones in the infected insect at earlier times than would be possible with the polyhedrin or p10 expression systems, possibly hastening the effect on the target insect. The vector will also be useful in the design of multiple expression systems using the basic protein, polyhedrin and p10 promoters. It will be possible to express genes in an ordered sequence and thus permit the prior synthesis of proteins which can then play a role in processing those produced later in the infection cycle.

We thank Chris Hatton for expert photography, Val Cooper for producing the synthetic oligonucleotides and Iain Cameron for helpful discussions.

References

- BUD, H. M. & KELLY, D. C. (1980). An electron microscope study of partially lysed baculovirus nucleocapsids: the intranucleocapsid packaging of viral DNA. *Journal of Ultrastructure Research* **73**, 361-368.
- COOK, R. F., AVERY, R. J. & DIMMOCK, N. J. (1979). Infection of chicken erythrocytes with influenza and other viruses. *Infection and Immunity* **25**, 396-402.
- EMERY, V. C. & BISHOP, D. H. L. (1987). The development of multiple expression vectors for high level synthesis of eukaryotic proteins: expression of LCMV-N and AcNPV polyhedrin protein by a recombinant baculovirus. *Protein Engineering* **1**, 359-366.
- HOWARD, S. C., AYRES, M. D. & POSSEE, R. D. (1986). Mapping the 5' and 3' ends of *Autographa californica* nuclear polyhedrosis virus polyhedrin mRNA. *Virus Research* **5**, 109-119.
- KELLY, D. C. & LESCOTT, T. (1981). Baculovirus replication: protein synthesis in *Spodoptera frugiperda* cells infected with *Trichoplusia ni* nuclear polyhedrosis virus. *Microbiologica* **4**, 35-57.

- KURODA, K., HAUSER, C., ROTT, R., KLENK, H.-D. & DOERFLER, W. (1986). Expression of the influenza virus haemagglutinin in insect cells by a baculovirus vector. *EMBO Journal* **5**, 1359-1365.
- LUCKOW, V. A. & SUMMERS, M. D. (1988). Trends in the development of baculovirus expression vectors. *Bio/Technology* **6**, 47-55.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory.
- MILLER, L. K. (1988). Baculoviruses as gene expression vectors. *Annual Review of Microbiology* **42**, 177-199.
- POSSEE, R. D. (1986). Cell-surface expression of influenza virus haemagglutinin in insect cells using a baculovirus vector. *Virus Research* **5**, 43-59.
- POSSEE, R. D. & HOWARD, S. C. (1987). Analysis of the polyhedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus. *Nucleic Acids Research* **15**, 10233-10248.
- POSSEE, R. D. & KELLY, D. C. (1988). Physical maps and comparative DNA hybridization of *Mamestra brassicae* and *Panolis flammea* nuclear polyhedrosis virus genomes. *Journal of General Virology* **69**, 1285-1298.
- ROHEL, D. Z. & FAULKNER, P. (1984). Time course analysis and mapping of *Autographa californica* nuclear polyhedrosis virus transcripts. *Journal of Virology* **50**, 739-747.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, U.S.A.* **74**, 5463-5467.
- SMITH, G. E., FRASER, M. J. & SUMMERS, M. D. (1983). Molecular engineering of the *Autographa californica* nuclear polyhedrosis virus genome: deletion mutations within the polyhedrin gene. *Journal of Virology* **46**, 584-593.
- TWEETEN, K. A., BULLA, L. A., JR & CONSIGLI, R. A. (1980). Characterization of an extremely basic protein derived from granulosis virus nucleocapsids. *Journal of Virology* **33**, 866-876.
- VLAK, J. M., KLINKENBERG, F. A., ZAAL, K. J. M., USMANY, M., KLINGE-ROODE, E. C., GEERVLIEET, J. B. F., ROOSIEN, J. & VAN LENT, J. W. M. (1988). Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10- β -galactosidase fusion gene. *Journal of General Virology* **69**, 765-776.
- WILLIAMS, G. V., ROHEL, D. Z., KUZIO, J. & FAULKNER, P. (1989). A cytopathological investigation of *Autographa californica* nuclear polyhedrosis virus p10 gene function using insertion/deletion mutants. *Journal of General Virology* **70**, 187-202.
- WILSON, M. E. (1988). A synthetic peptide to the predicted 6.9K translation product of the *Hind*III-*H*/*Eco*RI-D region of the AcNPV genome induces antibodies to the basic DNA-binding protein. *Virus Research* **9**, 21-31.
- WILSON, M. E., MAINPRIZE, T. H., FRIESEN, P. D. & MILLER, L. K. (1987). Location, transcription and sequence of a baculovirus gene encoding a small arginine-rich polypeptide. *Journal of Virology* **61**, 661-666.

(Received 8 June 1989; Accepted 30 November 1989)