# RECOMBINATION BETWEEN GENETICALLY MODIFIED AND UNMODIFIED *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRUS IN *TRICHOPLUSIA NI* LARVAE

A.T. MERRYWEATHER-CLARKE*, M. HIRST, R.D. POSSEE

NERC Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford, OX1 3SR, UK

*Received June 27, 1994*

**Summary.** – *Trichoplusia ni* larvae have been injected with a mixture of wild-type *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and a mutant derivative, AcRP8.UW1.lacZ, which lacks the polyhedrin gene, and has the p10 gene replaced by the *Escherichia coli* beta-galactosidase gene. Following plaque assay of the haemolymph and subsequent staining for beta-galactosidase activity and scoring for polyhedra, recombinant plaques were identified and the recombination frequency estimated as 6.6%.

**Key words:** *Autographa californica* multiple nuclear polyhedrosis virus; DNA recombination

## Introduction

Baculoviruses have been used as insect pest control agents since the last century (Entwistle and Evans, 1985). They offer an attractive alternative to the use of chemical insecticides because of the narrow host range of particular virus isolates, which permits targeting of specific pests. The principal disadvantage of baculoviruses, however, is that they are slow to kill the insect host; depending on environmental conditions and the size of the insect larva, a week may elapse between infection of the insect and death. In this period, considerable damage to the crop may result (Entwistle *et al.,* 1988). The delay in killing the host is a consequence of the complex baculovirus life cycle, which has evolved to produce large quantities of progeny virus in the course of the infection. This process initiates after polyhedra are ingested by a susceptible larva. In the alkaline conditions of the midgut, the polyhedra dissolve, releasing infectious, enveloped virus particles which enter the cells lining the gut. Progeny virions bud from these cells and spread the virus thoughout the rest of the insect via successive cycles of infection. Within virus-infected cells, more polyhedra are formed in the nuclei after release of the budded virus. The insect eventually dies when most tissues in the host contain virus polyhedra (reviewed by Volkman and Keddie, 1990).

Considerable efforts are now being devoted to improving baculovirus insecticides using genetic modification techniques. The most widely adopted approach has been to insert foreign sequences encoding insect-specific toxins, hormones or enzymes, under the control of strong virus gene promoters, into the virus genome. Subsequent infection of the host insect with the recombinant virus results in synthesis of the foreign protein and a reduction in the time taken to kill the host insect. The baculovirus commonly used for these experiments is the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) (reviewed by Possee *et al.,* 1993). While laboratory-based studies have been encouraging, they need to be supported by field-trials to confirm the effectiveness of the genetically modified baculoviruses in the environment. A concern, however, is the possibility of recombination between a modified virus and naturally occuring baculoviruses, with unknown potential to affect the host range and/or virulence of the virus. Recombination between different baculoviruses has been documented in cell culture and insects (Smith and Summers, 1980; Summers *et al.*, 1980; Croizier and Quoit, 1991; Croizier *et al.,* 1988; Kondo and Maeda, 1991), and one study has observed heterologous recombination between baculoviral DNA and a transfer vector based on the AcMNPV polyhedrin promoter containing adenovirus 2 DNA (Xiong *et al.,* 1991).

*Corresponding author

However, these studies have not addressed the risk of transferring foreign genes *in vivo* from a recombinant baculovirus to an uncharacterized baculovirus with a different host range. For this to happen, both parental viruses would have to co-replicate in the same cell of an insect host. We have initiated an experimental programme to assess the frequency of this phenomenon. The most likely scenario in the field use of viral insecticides is that they will be used in areas containing the homologous isolate in the natural population. This native virus will probably have an uncharacterized host range, with a concomitant risk associated with the introduction of a foreign gene. Therefore, we have initially concentrated on recombination between homologous viruses. In this study, we have estimated the recombination frequency between a genetically modified AcMNPV and wild type AcMNPV, in order to assess the risk of recombination *in vivo*. The major difficulty in studying the process of recombination is how to distinguish recombinant from parental viruses. To solve this problem, we constructed a recombinant AcMNPV, designated AcRP8.UW1.lacZ, which lacks the complete polyhedrin gene promoter and coding region but has the *E. coli* beta-galactosidase coding sequence inserted *in lieu* of the p10 coding region, under the control of the p10 gene promoter.

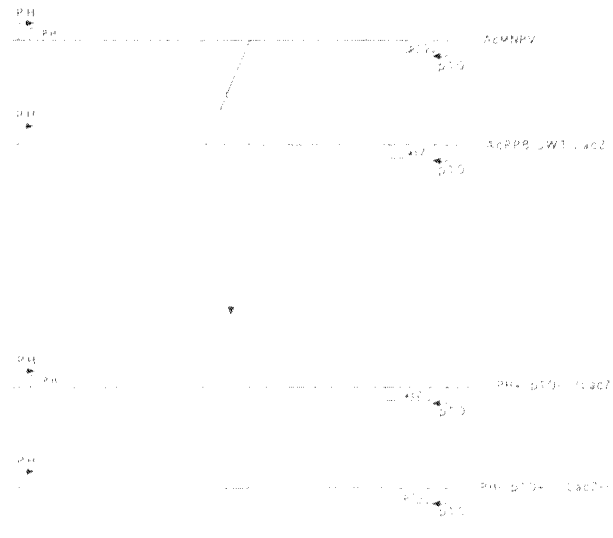

**Fig. 1**
**Genetic organization of AcMNPV and AcRP8.UW1.lacZ**
PH, polyhedrin locus, promoter and gene; p10, p10 locus, promoter and gene; lacZ, beta-galactosidase gene; (+) = positive; (-) = negative.

## Materials and Methods

*Viruses and cells.* *Spodoptera frugiperda* cells (IPLB-Sf21) (Vaughn *et al.*, 1977), AcMNPV (C6 strain, Possee, 1986; Possee *et al.*, 1991), AcRP8 (polyhedrin-negative, p10-positive, Merryweather *et al.*, 1990) were propagated as described previously (King and Possee, 1992).

*Plaque titrations, virus DNA purification and cotransfections* were performed as described King and Possee (1992).

*Trichoplusia ni larvae* maintained in the laboratory on semi-synthetic diet (Hunter *et al.*, 1984).

*Recombination experiments.* Infectious virus DNA, purified from AcRP8 was mixed with the p10-based transfer vector, pAcUW1.lacZ (Weyer *et al.*, 1990), in the presence of lipofectin and used to cotransfect *Spodoptera frugiperda* cells. Two days post-transfection, the progeny virus was harvested and titrated in a plaque assay. Recombinant viruses were detected by adding X-gal to each cell culture dish before counter staining with neutral red (Possee and Howard, 1987). The transfer of the lacZ coding region to the parental virus was indicated by the production of a blue, polyhedrin-negative plaque. Representative plaques were isolated and retitrated four times to ensure genetic homogeneity. The selected virus was designated AcRP8.UW1.lacZ (polyhedrin-negative, p10-negative/lacZ positive) and amplified in *S. frugiperda* cells to provide working virus stocks. The genetic organisation of this virus is shown in Fig. 1.

Unmodified AcMNPV (polyhedrin-positive) produces distinctive crystalline occlusion bodies (polyhedra) in virus-infected cells. However, it was necessary in this experiment to have a polyhedrin-negative phenotype to serve as a genetic marker. The inability of the AcRP8.UW1.lacZ to produce polyhedra prevented us using the oral route to initiate infection in susceptible insects; instead, we coinjected *T. ni* larvae with a mixture of budded, non-occluded AcRP8.UW1.lacZ and AcMNPV. This compromise simply by-passed the gut barrier and placed the virus particles directly into the haemocoel, which is their normal target after release from gut cells infected with occluded virus released from polyhedra in the midgut.

Infectious, budded virus stocks of AcRP8.UW1.lacZ and AcMNPV were mixed in appropriate proportions to provide a secondary stock with equal titers of both viruses. This was confirmed by titration in *S. frugiperda* cells. Thereafter, $2 \times 10^4$ PFU of the mixture, or virus-free cell culture medium, were injected into the haemocoel of 20 late 3rd instar *T. ni* larvae. Five days after injection, most insects displayed symptoms which were typical of virus infection (cream colouration and softening of the cuticle). Haemolymph was extracted from these insects and analyzed for the presence of infectious virus using a plaque assay. Recombinant plaques were identified by staining with X-gal and neutral red, isolated and retitrated twice to confirm their phenotype.

## Results and Discussion

The results of the co-infection experiment are shown in Table 1. Parental viruses were readily identified as polyhedrin-positive/colourless plaques (212) or polyhedrin-negative/blue plaques (133). Putative recombinant plaques were identified as polyhedrin-positive/blue plaques (35) or poly-

**Table 1. Numbers of plaques obtained from mixed infection of T. ni larvae and plaque purification**

| Initial plaque phenotype | Before plaque purification | Results of plaque purification | | Recombination | |
|---|---|---|---|---|---|
| | | Mixed phenotype | Unstable | Recombinant | Frequency |
| PH+/p10+(lacZ-) | 212 | | | | |
| PH+/p10-(lacZ+) | 35 | 12 | 13 | 10 | 2.5% |
| PH-/p10-(lacZ+) | 133 | | | | |
| PH-/p10+(lacZ-) | 26 | 3 | 6 | 17 | 4.2% |

PH, polyhedrin; p10, p10 gene; lacZ, beta-galactosidase; (+) – positive; (-) – negative.

hedrin-negative/colourless plaques (26). To ensure that the polyhedrin-positive/blue plaques were not simply cells co-infected with both parental viruses, they were isolated and retitrated twice in insect cells. Table 1 shows that of the 35 plaques originally isolated, 12 were a consequence of mixed parental virus infection, 10 were true recombinants, and 13 were not viable and would not plaque on retitration. These viruses may have been the result of a single crossover event between two viral genomes, thus representing two virus genomes essentially joined together, which would be unstable. The 26 polyhedrin-negative/colourless plaques were retitrated to ensure that their phenotype was not a consequence of failure to recognize immature polyhedra, or due to insufficient beta-galactosidase in virus-infected cells to give a blue colour. Only 3 of the recombinant plaques were subsequently confirmed as being of parental phenotype; 6 plaque isolates were unstable and lost on retitration. The genetic organization of the baculovirus recombinants derived by co-infection of insect larvae was confirmed by analysis of DNA isolated from virus-infected cells (data not shown).

When considering the outcome of the recombination experiments the potential recombination events have to be evaluated. The polyhedrin and p10 gene loci are separated by 114319 an 19575 nucleotides (Ayres *et al.*, 1994). Recombination could occur on both sides of these loci, thus transferring both the defective polyhedrin and lacZ genes from AcRP8.UW1.lacZ to AcMNPV and the intact polyhedrin and p10 genes in the other direction. These events would not be recognized in our screening procedure. Alternatively, the intact polyhedrin gene could be transferred from AcMNPV to AcRP8.UW1.lacZ, producing a polyhedrin-positive/lacZ-positive virus phenotype; lacZ could also be transferred to AcMNPV to derive the same phenotype. Calculating the recombination frequency as the number of recombinant plaques expressed as a percentage of the total number of plaques observed, we determined that 2.5% of the virus progeny had undergone this modification. Finally, the defective polyhedrin gene in AcRP8.UW1.lacZ could recombine at the polyhedrin locus in AcMNPV to produce a polyhedrin-negative phenotype also lacking the lacZ gene; the same phenotype would be generated if the intact p10 gene replaced lacZ in AcRP8.UW1.lacZ. It was determined that 4.2% of the virus plaques had undergone either of these recombination events, and the overall recombination frequency was 6.6%. Table 1 shows that 212 progeny of the wild-type phenotype were observed, and 133 progeny of the AcRP8.UW1.lacZ phenotype, following plaque assay of haemolymph from infected larvae.

Huang *et al.* (1991) found that during serial passage in tissue culture of a mixture of wild-type AcMNPV and an engineered, polyhedrin-negative virus expressing lacZ from the polyhedrin promoter, the titer of the wild-type AcMNPV reached approximately 1.7-fold higher titer than the polyhedrin-negative recombinant per passage. These results suggest that selection pressure operates in favour of the wild type virus compared with the polyhedrin-negative, lacZ-positive recombinant. The ratio from Table 1 of wild type to engineered virions is 1.6, which concurs with that obtained by Huang *et al.* (1991). If positive selection pressure operates on wild type AcMNPV, there would be a tendency for viruses to retain the polyhedrin gene. However, as discussed above, polyhedra do not apparently play a part in infection of tissue culture or insect tissues other than the gut. While occluded virus is far more efficient at infecting larvae when administered *per os,* there is no reason to assume that the presence of polyhedrin will confer any advantage to budded virions when injected into the haemolymph of a larva, or in tissue culture. However, the data presented in Table 1, and the results of Huang *et al.* (1991) suggest that selection, either for polyhedrin-positive virus or against recombinant virus, is in operation, both in *T. ni* larvae following injection of budded virions, and in tissue culture. The increase in titer of wild type AcMNPV over polyhedrin-negative, lacZ-expressing virus may indicate a negative selection pressure acting against the genetically engineered recombinant producing lacZ, as opposed to a positive selection pressure acting in favour of polyhedrin-positive

wild type virus, in insect haemolymph or tissue culture. The actual process of genetically engineering a baculovirus may be responsible for a reduction in fitness resulting in negative selection against a recombinant. It is commonly seen in our laboratories that viruses expressing foreign genes have slightly elevated $LD_{50}$ values compared with those of their wild-type counterparts (unpublished data). Moreover, Wood *et al.* (1993) report that the presence of the *E. coli* beta-galactosidase gene in polyhedrin-negative AcMNPV considerably increases the $ST_{50}$. Obviously, more extensive experiments would be necessary in order to comment significantly on selection pressure in insect larvae, but these results provide initial evidence that latent or persistent infections of wild type viruses in insects infected with recombinant baculoviruses may have considerable selective advantage over the genetically engineered insecticides, and this must be investigated. It should be noted that in initial release experiments of recombinant viral insecticides, it could be considered advantageous that the potentially unpredictable genetically engineered virus may be selected against.

Having determined the frequency of recombinants between two AcMNPV viruses with different phenotypes, we intend to investigate the frequency between AcMNPV and other viruses, particularly those which have a different host range to that of AcMNPV. Summers *et al.* (1980) observed a recombination frequency in excess of 7% between RoMNPV and AcMNPV following mixed infection of *T. ni* cells in culture, mapping crossover sites using 21 restriction enzyme sites as markers. This is of the same order of magnitude as our value of 6.6% for the *in vivo* recombination frequency of AcMNPV and its mutant AcRP8.UW1.lacZ. Croizer and Quoit (1981) observed somewhat higher recombination frequencies of over 50% between AcMNPV and GmMNPV following a single passage by inoculation of a mixture of the viruses into *G. mellonella* larvae, and between 80 – 90% after 5 passages through insects, using 7 restriction enzyme sites as markers to map sites of recombination. In both studies, the recombinants were the result of crossover of genetic material in two sites only, so that only a single fragment of "foreign DNA" was acquired by each virus. The naturally occuring AcMNPV-RoMNPV recombinants extracted by Smith and Summers (1980) from wild isolates of RoMNPV were the result of recombination events between 4 and 6 sites. Summers *et al.* (1980) found that in 6 out of 7 AcMNPV-RoMNPV recombinants analyzed, crossover had occurred at a site within the polyhedrin gene and flanking regions. This was also true of both recombinants described by Smith and Summers (1980). These results suggest that there may be preferred sites or hot spots for recombination. Neither of the two AcMNPV-GmMNPV recombinants mapped by Croizier and Quoit (1981) was the result of a crossover event in this region.

This is the first documented evidence of exchange of foreign genes between baculoviruses *in vivo*, since all other studies have involved either naturally occurring virus mutants or recombinants, or cell culture (Smith and Summers, 1980; Summers *et al.*, 1980; Croizier and Quoit, 1981; Croizier *et al.*, 1988; Kondo and Maeda, 1991; Xiong *et al.*, 1991). Our motivation for performing this research is to determine the likelihood of a naturally occuring virus acquiring a foreign gene, expressed by a genetically engineered baculovirus insecticide, following a recombination event. That there is a need for such information is demonstrated by the apparent existence of a region exhibiting an increased tendency to recombination in the *Eco*RI I fragment (Summers *et al.*, 1980), considering that this region containing the polyhedrin promoter is commonly chosen for the insertion of insecticidal genes into the viral genome. Future studies will concentrate on recombination at or between the loci at which the foreign genes are to be inserted, usually the polyhedrin or p10 sites. This will enable a comparison of recombination frequencies between AcMNPV and other baculoviruses with the results presented here. This will provide us with data on recombination frequencies which are relevant to an assessment of the risks involved in releasing genetically engineered insecticides into the environment, and bring us a step closer to determining the probability of the genetically engineered insecticide losing its foreign gene in a recombination event with other baculoviruses.

**Acknowledgements.** This work was funded by the Department of the Environment. We thank Mr T. Carty for *T. ni* larvae.

## References

Ayres, M.D., Howard, S.C., Kuzio, J., Lopez-Ferber, M., and Possee, R.D. (1994): The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology* **202**, 586–605.

Croizier, G., and Quoit, J.M. (1981): Obtention and analysis of two genetic recombinants of baculoviruses of lepidoptera, *Autographa californica* Speyer and *Galleria mellonella* L. *Annals de Virologie de l'Institut Pasteur* **132**, 3–18.

Croizier, G., Croizier, L., Quoit, J.M., and Lereclus, D. (1988): Recombination of *Autographa californica* and Rachiplusia ou Nuclear Polyhedrosis Viruses in *Galleria mellonella* L. *J. gen. Virol.* **69**, 177–185.

Entwistle, P.F., and Evans, H.F. (1985): In G.A. Kerkut and L. Gilbert (Eds): *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*. Pergamon Press, Oxford, pp. 347–412.

Entwistle, P.F., Cory, J.S., and Doyle, C. (1988): An overwiev of insect baculovirus ecology as a background to field release of a genetically manipulated nuclear polyhedrosis virus. In W. Klingmuller (Ed.): *Risk Assessment of Delibrate Release*. Springer, Berlin-Heidelberg-New York, pp. 72–80.

Huang, Y.-S., Bobseine, K.L., Setzer, R.W., and Kawanishi, C.Y. (1991): Selection kinetics during serial culture passage of mixtures of

wild-type *Autographa californica* nuclear polyhedrosis virus and its recombinant Ac360-β-gal. *J. gen. Virol.* **72**, 2653–2660.

Hunter, F.R., Crook, N.E., and Entwistle, P.F. (1984): Viruses as pathogens for the control of insects. In J.M. Grainger and J.M. Lynch (Eds): *Microbial Methods for Environmental Biotechnology.* Academic Press, New York-London, pp. 323–347.

King, L.A., and Possee, R.D. (1992): *The Baculovirus Expression System: A Laboratory Guide.* Chapman and Hall, London.

Kondo, A., and Maeda, S. (1991): Host range expansion by recombination of the baculoviruses *Bombyx mori* nuclear polyhedrosis virus and *Autographa californica* nuclear polyhedrosis virus. *J. Virol.* **65**, 3625–3632.

Merryweather, A.T., Weyer, U., Harris, M.P.G., Hirst, M., Booth, T., and Possee, R.D. (1990): Construction of genetically engineered baculovirus insecticides containing the *Bacillus thuringiensis* subsp. kurstaki HD-73 delta endotoxin. *J. gen. Virol. 71*, 1535–1544.

Possee, R.D. (1986): Cell-surface expression of influenza virus haemagglutinin in insect cell using a baculovirus vector. *Virus Res.* **5**, 10233–10248.

Possee, R.D., Sun, T.-P., Ayres, M.D., Hill-Perkins, M., and Gearing, K.L. (1991): Nucleotide sequence of the *Autographa californica* nuclear polyhedrosis virus 9.4 kbp *Eco*RI-I and -R (polyhedrin gene) region. *Virology* **185**, 229–241.

Possee, R.D., Hirst, M., Jones, L.D., Bishop, D.H.L., and Cayley, P.J. (1993): Field tests of genetically engineered baculoviruses. In D.J. Beadlle, D.H.L. Bishop, L.G. Copping, G.K. Dixon, and D.W. Holloman (Eds): *BCPC Monologue No. 55, Opportunities for Molecular Biology in Crop Production.*

Smith, G.E., and Summers, M.D. (1980): Restriction map of *Rachiplusia ou* and *Rachiplusia ou-Autographa californica* baculoviruses recombinants. *J. Virol.* **33**, 311–319.

Summers, M.D., Smith, G.E., Knell, J.D., and Burand, J.P. (1980): Physical map of *Autographa californica* and *Rachiplusia ou* Nuclear Polyhedrosis Virus recombinants. *J. Virol.* **34**, 693–703.

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J., and McCawley, P. (1977): The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *In vitro* **3**, 213–217.

Volkman, L.E., and Keddie, B.A. (1990): Nuclear polyhedrosis virus pathogens. *Seminar in Virology* **1**, 249–156.

Weyer, U., Knight, S., and Posse, R.D. (1990): Analysis of very late gene expression by *Autographa californica* nuclear polyhedrosis virus and the further development of multiple expression vectors. *J. gen. Virol.* **71**, 1525–1534.

Wood, H.A., Trotter, K.M., Davis, T.R., and Hughes, P.R. (1993): *Per os* infectivity of preoccluded vrions from polyhedrin-minus recombinant baculoviruses. *J. Invert. Path.* **62**, 64–67.

Xiong, G., Schorr, J., Tjia, S.T., and Doerfler, W. (1991): Heterologous recombination between *Autographa californica* nuclear polyhedrosis virus DNA and foreign DNA in non-polyhedrin segments of the viral genomes. *Virus Res.* **21**, 65–85.