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Engineering of an *Oryctes* Baculovirus Recombinant: Insertion of the Polyhedrin Gene from the *Autographa californica* Nuclear Polyhedrosis Virus

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SUMMARY

A cotransfection method has been developed for the generation of recombinant *Oryctes* baculoviruses. The permissive coleopteran cell-line DSIR-HA-1179 was transfected with a mixture of *Oryctes* baculovirus DNA (strain PV505) and a transfer vector. The transfer vector, a pUC8-based plasmid, contained the polyhedrin gene from the *Autographa californica* nuclear polyhedrosis virus, flanked by *Oryctes* baculovirus DNA from the *Hind*III fragment N. *Oryctes* baculovirus does not form plaques with DSIR-HA-1179 cells. Therefore an endpoint dilution method was used to screen for, recover and purify recombinants. There was no phenotypic character that could be used for detecting recombinants, so a dot blot assay was used to screen infected cultures for the presence of recombinants. A recombinant generated by this method contained the entire polyhedrin gene inserted at 98.03 map units from the designated start of the *Oryctes* baculovirus physical map. No evidence of transcription or translation of the polyhedrin gene was obtained.

Transfection has become a very useful tool in the molecular genetics of viruses because it allows genetic changes, made using precise *in vitro* methods, to be inserted easily into the viral genome by recombination. For baculoviruses, the only transfection systems developed have been for *Autographa californica* nuclear polyhedrosis virus (AcNPV) in either *Spodoptera frugiperda* or *Trichoplusia ni* cell lines (Potter & Miller, 1980; Burand *et al.*, 1980), for *Bombyx mori* NPV in its homologous cell line BM-N (Maeda *et al.*, 1985) and for *Lymantria dispar* NPV in *L. dispar* cells (Gonnet & Devauchelle, 1987). Most transfections are performed with the *S. frugiperda* cell line IPLB-SF-21, which has a higher transfection efficiency. The major use for transfection systems with baculoviruses has been to generate recombinants. Recombinant AcNPVs expressing a wide range of foreign genes under the control of the highly active polyhedrin promoter are the best examples of this (Smith *et al.*, 1983; Pennock *et al.*, 1984; Luckow & Summers, 1988).

Transfection does not appear to extend the host range of baculoviruses (Burand *et al.*, 1980) so each virus will require its own transfection system to be developed. This paper describes optimization of transfection for *Oryctes* baculovirus DNA in the coleopteran cell line DSIR-HA-1179. The CaCl₂ method (Graham & van der Eb, 1973) was used as the basis of the procedure, with temperature, incubation time and CaCl₂ concentration all being optimized. In addition this paper describes the production of the first recombinant *Oryctes* baculovirus.

Oryctes baculovirus is best known for its successful control of the coconut palm rhinoceros beetle, *Oryctes rhinoceros* (L.), a serious pest of coconut palms (Bedford, 1981). It is a member of a small group of baculoviruses (subgroup C) which do not produce inclusion bodies. *Oryctes*

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Table 1. *The effect of various incubation conditions on the transfection rate of Oryctes baculovirus DNA for Ha cells*

Incubation conditions			Transfection results			
Time (min)	Temp (°C)	CaCl ₂ (M)	No. of wells transfected	Wells infected (%)	Corrected* infection rate (%)	Infections per µg DNA/5 × 10 ⁵ cells
20	20	0.22	192	11.4	12.1	40 a†
20	20	0.3	384	21.6	24.3	81 bc
20	20	0.37	192	25.5	29.4	98 bc
20	10	0.3	192	12.5	13.3	44 a
20	20	0.3	384	21.6	24.3	81 bc
20	30	0.3	192	27.6	32.3	108 c
10	20	0.3	192	18.7	20.7	69 ab
20	20	0.3	384	21.6	24.3	81 bc
30	20	0.3	192	25.5	29.4	98 bc
40	20	0.3	192	16.6	18.1	60 ab

*Transfection rate is corrected for the fact that with an increasing proportion of the wells being infected an increasing number will be due to multiple transfection events.

†Values sharing the same letter are not significantly different ($P > 0.05$) from each other.

baculovirus is one of only two members of this subgroup which have been studied in any detail in addition to their detection by electron microscopy of thin sections of insect tissues. For this reason it is difficult to generalize about the group except that all members lack an inclusion body. *Oryctes* baculovirus morphogenesis differs from that of other baculoviruses in two other respects, however: the nucleocapsid is never found without an envelope; virus particles which bud from the cell surface acquire a second membrane as they pass through the plasma membrane (Crawford & Sheehan, 1985).

Oryctes baculovirus is infectious for a number of scarab beetles as well as its primary host. Three of these beetles are serious insect pests: *Costelytra zealandica* and *Heteronychus arator* attack pasture in New Zealand (Crawford *et al.*, 1985a); *Papuana hubneri* feeds on tropical root crops such as taro and cassava in many islands of the South Pacific (Zelazny *et al.*, 1988). *Oryctes* baculovirus is non-occluded so therefore it is inactivated rapidly in the soil (Zelazny, 1972). As a consequence, transmission of the disease amongst these soil-dwelling insects by the virus would not be sufficient to make the virus a serious candidate for their microbial control. If, on the other hand, *Oryctes* baculovirus were occluded like subgroup A and B baculoviruses it would survive very well in the soil (Jaques, 1975) and might be an ideal microbial control agent for these pests. AcNPV has been shown to break down in the mid-gut of *H. arator* (A. M. Crawford & C. M. Sheehan, unpublished data). The long term goal in understanding the molecular biology of *Oryctes* baculovirus, and the reason for developing the transfection method described in this paper, is to be able to engineer the virus genetically so that it will occlude itself and become a useful control agent for these other scarab pests.

Oryctes baculovirus, strain PV505, was used for all experiments. Virus was grown in the coleopteran cell line DSIR-HA-1179 (Ha cells) derived from the black beetle *H. arator* (Crawford, 1982), and its DNA was purified as previously described (Crawford *et al.*, 1985b). The transfections were also carried out with the Ha cells. Ha cells were maintained in Schneider's *Drosophila* medium (Gibco) containing 10% foetal calf serum and 50 µg/ml gentamicin.

The optimum incubation time, CaCl₂ concentration and incubation temperature for transfecting Ha cells with *Oryctes* baculovirus DNA were determined empirically (see Table 1). It was also found that the transfection rate was significantly increased ($P < 0.001$) when the DNA was precipitated by adding CaCl₂ directly to the flask containing the cells rather than

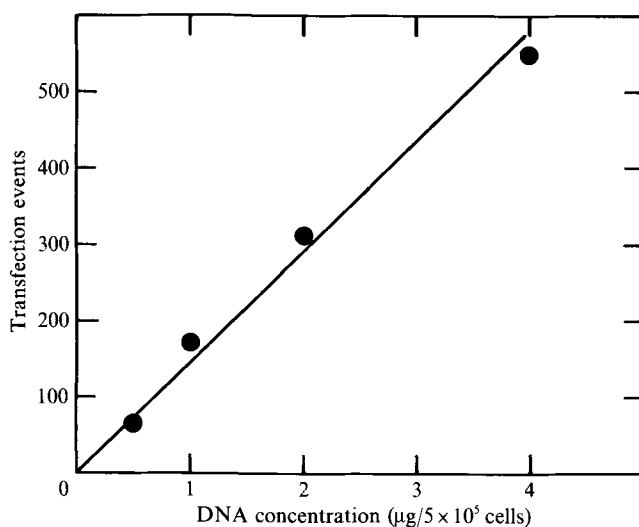


Fig. 1. The dose-response relationship between DNA concentration and transfection. The transfection method used for this experiment was the final method described in the text. Carrier DNA (pUC8) was adjusted so that a total of 11 µg of DNA was always used per 5×10^5 cells.

forming the precipitate in a separate tube and adding to the cells after precipitation (data not shown). The method gave a linear dose-response curve for DNA concentrations between 0.5 and 4.0 µg/ 5×10^5 cells (Fig. 1). Treatment of cells with pancreatic DNase I (Sigma)-digested viral DNA did not yield any infections. The final method adopted for transfections is described below.

A confluent monolayer of Ha cells in a 25 cm² tissue culture flask (containing 5.0×10^5 cells) was rinsed twice with TBS buffer (10 mM-Tris-HCl pH 7.2, 0.85% w/w NaCl). DNA (1 µg of viral DNA and 10 µg of either carrier or transfer vector DNA dissolved in 0.4 ml TBS buffer) was then added. After a period of 5 min 80 µl of 2 M-CaCl₂ was then added to precipitate the DNA (final CaCl₂ concentration equal to 0.3 M). The cells were then incubated for 15 min at 30 °C with occasional rocking to ensure the cells remained covered by the small volume of liquid. The addition of CaCl₂ causes the normally well attached cells to float off. Approximately 150 ml of Schneider's medium was then added to the now floating cells and the solution was evenly distributed into the wells of 10 microtitre plates (Costar no. 3596). Each of the 960 wells contained approximately 500 cells in 150 µl of medium. After a 2 week incubation at 30 °C, each well was examined using an inverted light microscope for the characteristic c.p.e. of an *Oryctes baculovirus* infection.

When recombinants were being generated by cotransfection the cells were harvested and a sample of the infected cell suspension was spotted on to a nylon membrane (Amersham Hybond-N) using a suction manifold. The membrane was air-dried and then placed on top of filter paper (Whatman 3MM), dampened with an alkaline salt solution (0.5 M-NaOH, 1.5 M-NaCl) for 15 min to disrupt the cells and release ssDNA for binding to the membrane. After irradiation for 5 min on a u.v. transilluminator, the membrane was washed in $2 \times$ SSC, 0.1% SDS for a further 10 min. The recombinant virus was screened for by employing DNA hybridization as previously described (Crawford *et al.*, 1985b) using the *Eco*RI fragment I from AcNPV cloned into pUC8 as a probe. This method is similar to that recently published by Fung *et al.* (1988) but was developed independently of this work.

Experience has shown that if 20 to 40% of 960 wells contain infected cells, it is probable that at least one of those wells contains a stable recombinant. In the initial screening some of the wells showed evidence of hybridization indicating a recombinant which on further endpoint cloning could not be detected. These false positives could be due to carry over of the transfer vector, or as a result of only one cross-over event occurring giving allelic duplication rather than replacement.

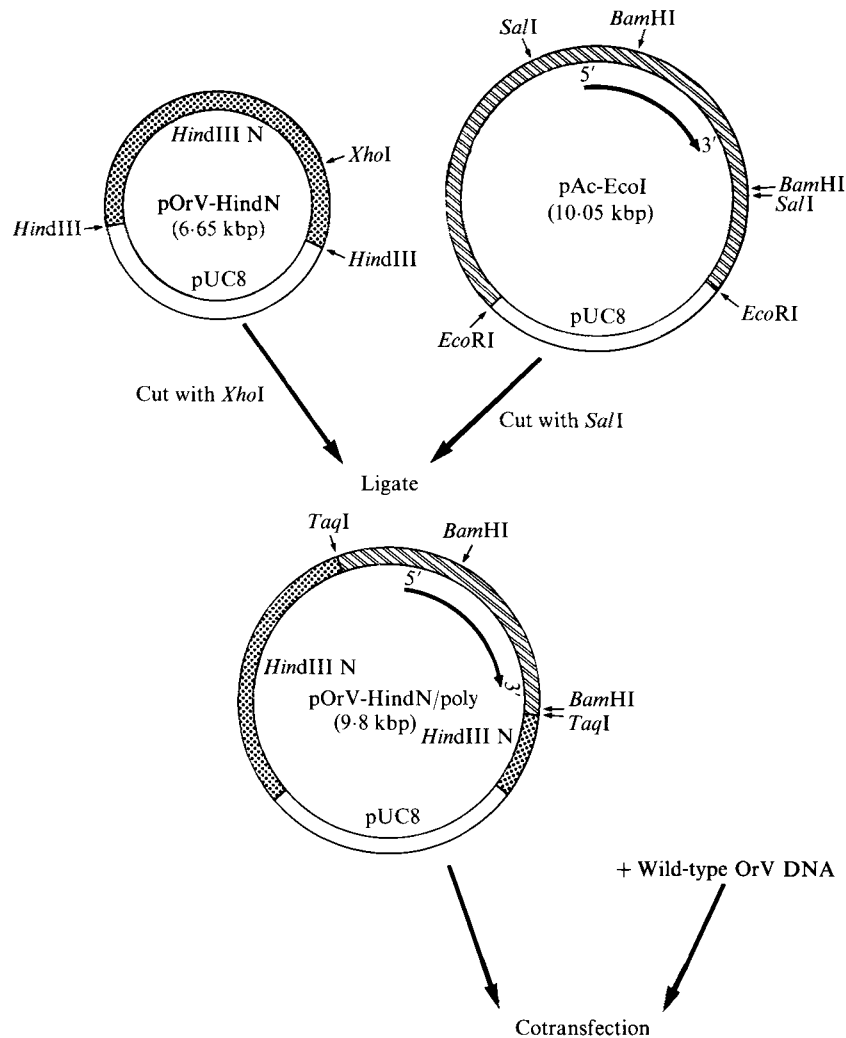


Fig. 2. Construction of the transfer vector pOrV-HindN/poly used in the cotransfection of Ha cells. A pUC8 plasmid containing the *HindIII* N fragment of *Oryctes* baculovirus (OrV) (map units 96.06 to 99.13; see Crawford *et al.*, 1985b) was cut at a unique *XhoI* site within the *HindIII* N fragment. A 3.15 kb *SalI* fragment from the AcNPV *EcoRI* I fragment which encoded the entire polyhedrin gene including the promoter region was ligated into the *XhoI* site. The manipulations described above used standard methods (Maniatis *et al.*, 1982), the host bacteria being JM109.

These recombinants would be unstable because the inserted gene could have been deleted by homologous recombination. It was found that three rounds of cloning by endpoint dilution gave a single and stable recombinant genotype.

The construction of the transfer vector is shown in Fig. 2. The reason that the *HindIII* N fragment was chosen for use in the transfer vector was that our restriction enzyme analysis of *Oryctes* baculovirus (Crawford *et al.*, 1986) showed that a restriction site was lost in this region. This indicated that the region might not be essential for viral replication and so might make a useful insertion site for foreign DNA. Attempts were also made to insert DNA in a region where no variation could be detected amongst geographical isolates. A second transfer vector using the *HindIII* fragment D as a flanking sequence was used but no recombinants were obtained (data not shown).

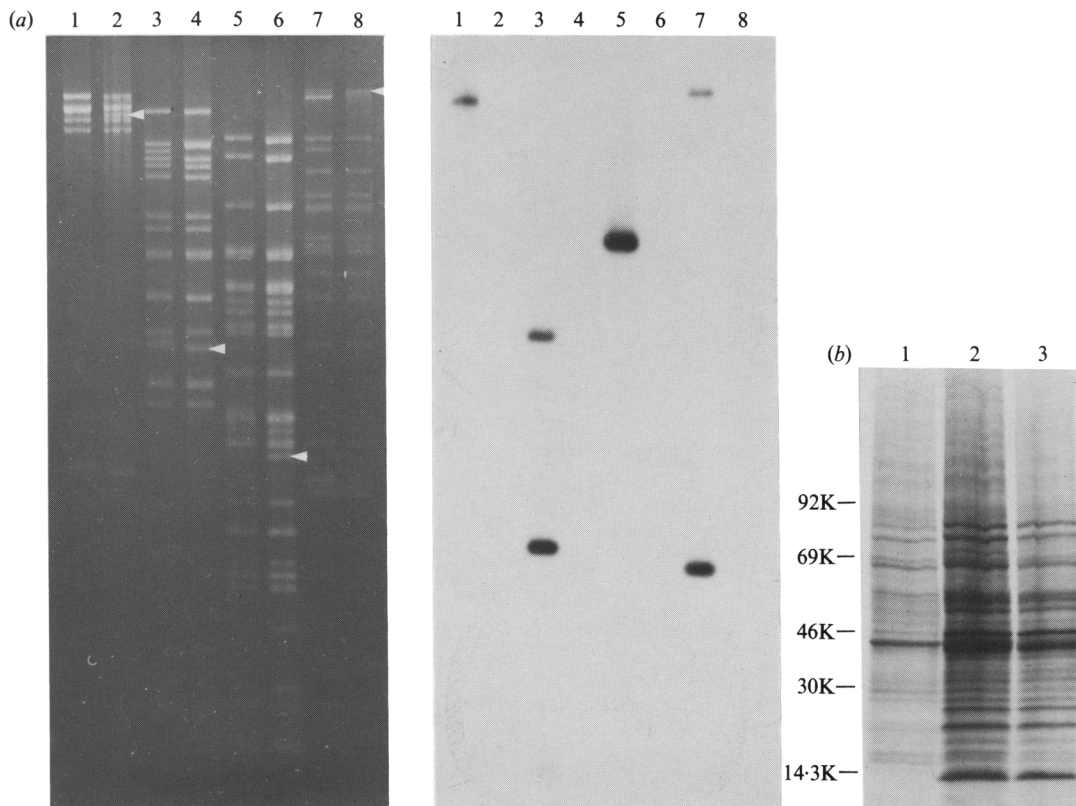


Fig. 3. (a) A comparison of the restriction endonuclease digestion patterns of wild-type and recombinant (rec) *Oryctes* baculovirus DNA. Lane 1, rec + *Pst*I; lane 2, wild-type + *Pst*I; lane 3, rec + *Hind*III; lane 4, wild-type + *Hind*III; lane 5, rec + *Eco*RI; lane 6, wild-type + *Eco*RI; lane 7, rec + *Bam*HI; lane 8, wild-type + *Bam*HI. The white arrowheads point to the four wild-type restriction fragments that have been altered by the insertion of the polyhedrin fragment. Beside the gel an autoradiogram is shown of a Southern blot of the agarose gel using the AcNPV *Eco*RI fragment I as the labelled probe for DNA hybridization. The blot is the same size and in the same orientation as the gel. (b) An autoradiogram of a polyacrylamide gel of [35 S]methionine-labelled proteins from Ha cells either uninfected (lane 1) or infected with wild-type (lane 2) or rec (lane 3) *Oryctes* baculovirus. The pulse was from 8 to 24 h post-infection following a 2 h incubation in methionine-free medium. Each lane contains labelled proteins from 1×10^4 cells.

Cotransfection of the *Hind*III fragment N transfer vector with wild-type *Oryctes* baculovirus DNA using the methods above resulted in the formation of a stable recombinant. Restriction enzyme and Southern blot analysis of the new virus (Fig. 3a) showed that the *Sal*I fragment from AcNPV that was in the transfer vector had been inserted within the *Hind*III N fragment of *Oryctes* baculovirus at 98.03 map units. The *Hind*III fragment N, *Bam*HI fragment A, *Eco*RI fragment T and *Pst*I fragment C, all of which span the insertion site at 98.03 map units, were all altered (see white arrowheads Fig. 3a). Southern blot analysis of the restriction digest showed hybridization to AcNPV fragments that should have been present in the insert (e.g. *Hind*III fragments V and T) indicating that the correct fragment had been inserted.

The *Sal*I fragment from AcNPV contained the complete sequence of the polyhedrin gene including all of the promoter region (Hooft van Iddekinge *et al.*, 1983). Despite this, no difference in phenotype between the recombinant and the wild-type virus could be detected. Both viruses grew to similar titres in cell culture (data not shown) with identical cytopathic effects. Polyhedrin transcripts could not be detected in Northern blots of RNA purified from Ha cells infected with the recombinant (see Fig. 4a).

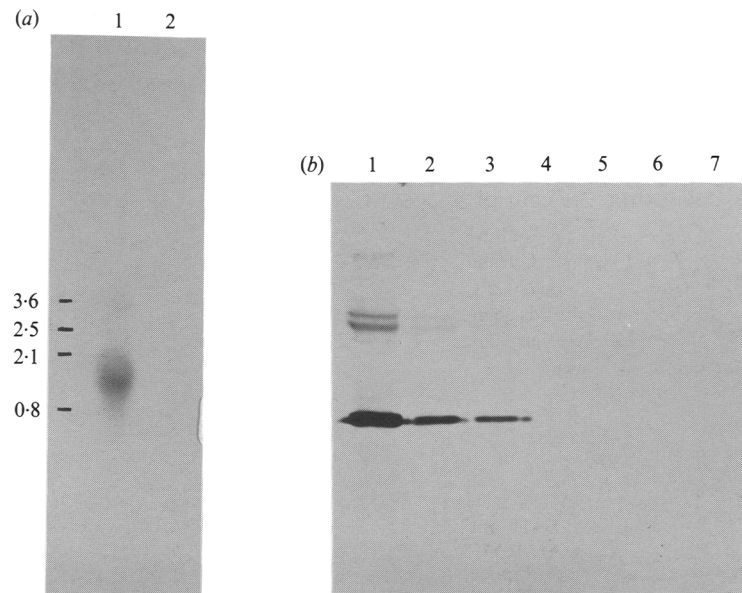


Fig. 4. (a) Transcription of the polyhedrin gene in Ha cells infected with recombinant *Oryctes* baculovirus. Total cellular RNA was prepared using the method of Chirgwin *et al.* (1979) from either 5×10^6 *S. frugiperda* cells collected at 48 h after infection with AcNPV (lane 1) or 5×10^6 Ha cells collected at 24 h after infection with recombinant *Oryctes* baculovirus (lane 2). Northern blots were made as previously described (Guilford & Forster, 1986). The probe used to detect the polyhedrin transcripts was a cloned *Bam*HI F fragment of AcNPV DNA labelled with 32 P by nick translation. Size markers are indicated in kb. (b) A Western blot to detect AcNPV polyhedrin in Ha cells infected with the recombinant *Oryctes* baculovirus. Proteins from Ha cells either uninfected (lane 7) or infected with recombinant and collected at 48 h (lane 6), or 24 h after infection to which 1000 (lane 1), 100 (lane 2), 10 (lane 3), 1 (lane 4) or 0 (lane 5) polyhedra per 100 cells had been added were separated on a 12% polyacrylamide gel and blotted on to nitrocellulose. The Western blot method was that of Andrew *et al.* (1987) and used rabbit polyclonal antisera prepared against AcNPV polyhedra. Each lane contained proteins from 10^3 cells.

When protein synthesis was examined by pulse-labelling cells with [35 S]methionine, no change in the pattern of proteins, resolved on a one-dimensional polyacrylamide gel, could be found between the wild-type and recombinant virus (Fig. 3*b*). In order to detect polyhedrin synthesis a Western blot was also performed. No polyhedrin was detected in *Oryctes* baculovirus recombinant-infected Ha cells. A range of polyhedra added to Ha cells established that the detection limit for AcNPV polyhedra on this western blot was 10 polyhedra/100 cells.

Our result is similar to that of Carbonell *et al.* (1985) who found that the β -galactosidase gene under the control of the AcNPV polyhedrin promoter was not expressed in dipteran or mammalian cells but was abundantly expressed in the permissive *S. frugiperda* cell line. We have extended the work of Carbonell *et al.* (1985) by incorporating the polyhedrin gene into a baculovirus capable of replication in a non-lepidopteran cell line rather than use AcNPV which will only replicate in lepidopteran cells. However, in both cases no activity could be detected.

The polyhedrin promoter is clearly not useful as a highly active promoter in *Oryctes* baculovirus. The recombinant will however be useful as a genetically 'tagged' virus in studies which monitor the establishment and spread of virus following the field release of infected adult beetles into pest populations of *O. rhinoceros* in which the virus is already endemic. The major technical difficulty delaying these studies has been that the released virus could not be easily distinguished from the endemic virus; the recombinant solves this problem.

Other studies of transfection of baculovirus DNA (Potter & Miller, 1980; Burand *et al.*, 1980) have used AcNPV DNA in either *S. frugiperda* or *T. ni* cells. The transfection rate obtained in these two cell lines varied considerably despite the use of the same DNA source. It is not

surprising therefore that the *Oryctes* baculovirus DNA/Ha cell system should also have a different transfection rate. Our best transfection rate, 108 transfection events/ μ g of viral DNA, was up to 30-fold less than that for AcNPV DNA in TN 368 cells (Burand *et al.*, 1980) and 350-fold less for AcNPV DNA in *S. frugiperda* cells (Potter & Miller, 1980). Despite the relatively low rate of transfection obtained by our method we have shown it can be used for the generation of *Oryctes* baculovirus recombinants which it is hoped will lead to the generation of more effective virus isolates for the control of scarab pests.

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