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Two key mutations in the host-range specificity domain of the *p143* gene of *Autographa californica* nucleopolyhedrovirus are required to kill *Bombyx mori* larvae

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***Autographa californica* nucleopolyhedrovirus (AcMNPV) does not replicate in *Bombyx mori* cells (Bm5, BmN). We have shown previously that when a short DNA sequence within AcMNPV ORF95, which encodes the viral helicase P143, is replaced with the collinear region of *B. mori* nucleopolyhedrovirus (BmNPV), AcMNPV gains the ability to replicate in Bm5 cells. To determine the mutational events in the *p143* gene required to allow AcMNPV replication in *B. mori* cells, AcMNPV recombinants produced in Sf9 cells were screened *in vivo* in *B. mori* larvae, which are more permissive to baculovirus infection than *B. mori* cell lines. Eight combinations of mutations were tested and characterization of viral DNA extracted from dead larvae showed that amino acid changes at position 564 and 577 are required to kill *B. mori* larvae.**

Baculoviruses are enveloped viruses with a circular double-stranded DNA genome (Murphy *et al.*, 1995), and are promising bioinsecticides. However, baculoviruses typically possess a restricted host range (Gröner, 1986), a property which is ecologically advantageous but commercially detrimental. An understanding of the molecular basis of baculovirus specificity is thus of great importance to the success of this group of control agents.

Many studies have been done to try to establish the causes of abortive virus replication in non-permissive insect cell lines. Among the most effective approaches are the study of temperature-sensitive mutants (Partington *et al.*, 1990) or mutants with altered infectivities (Clem & Miller, 1994). Analysis of gene expression in heterologous systems (Van Oers *et al.*, 1994) using gene expression reporters (Mukherjee *et al.*, 1995) or replication and transcription assays (Lu & Miller, 1995; Ahrens & Rohrmann, 1996) have permitted the identification of several host-range genes. Blockage of productive baculovirus infection may occur at any point in the virus cycle (McClintock *et al.*, 1986; Wickham *et al.*, 1992; Clem & Miller, 1993; Morris & Miller, 1993; Mukherjee *et al.*, 1995). It has been shown that some of the genes required for baculovirus late expression are cell line-dependent (Lu & Miller, 1995).

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Autographa californica nucleopolyhedrovirus (AcMNPV) and *Bombyx mori* nucleopolyhedrovirus (BmNPV) are closely related to each other, having collinear genome organizations, but their host ranges are different. *B. mori* Bm5 cells are non-permissive for AcMNPV infection (Mukherjee *et al.*, 1995), supporting limited viral DNA replication but failing to produce progeny virus (Kondo & Maeda, 1991). Hence restriction of the AcMNPV infection cycle on Bm5 cells occurs after the onset of DNA replication.

The *p143* gene is the host-range genetic determinant for AcMNPV infectivity in Bm5 or BmN cells (Croizier *et al.*, 1994; Maeda *et al.*, 1993). This gene, essential to AcMNPV DNA replication, has been suggested to encode a helicase (Lu & Carstens, 1991). Helicase activity might be obtained by an association between *p143* and cofactors (Laufs *et al.*, 1997), as occurs in herpesvirus helicase (Challberg, 1996). Helicases (reviewed by Matson & Kaiser-Roger, 1990) are generally multifunctional proteins (Dracheva *et al.*, 1995) and are probably involved in diverse mechanisms during the baculovirus life-cycle.

We have previously shown that the substitution of a short AcMNPV genomic sequence between amino acids 536 and 584 for the homologous BmNPV sequence in the *p143* gene was sufficient to enable AcMNPV to replicate in Bm5 cells and to kill *B. mori* larvae (Croizier *et al.*, 1994). In this region five amino acids differ between AcMNPV and BmNPV sequences. A site-directed mutagenesis experiment was performed to establish the mutations in the AcMNPV *p143* gene required to allow AcMNPV to kill *B. mori* larvae. A 399 bp *Sac*I–*Sau*3AI fragment within the AcMNPV *p143* gene (Figs 1 and 2*a*) was cloned into pUC19 yielding plasmid p9, which acted as a negative control in transfection experiments. This fragment was capable of undergoing recombination at high efficiency,

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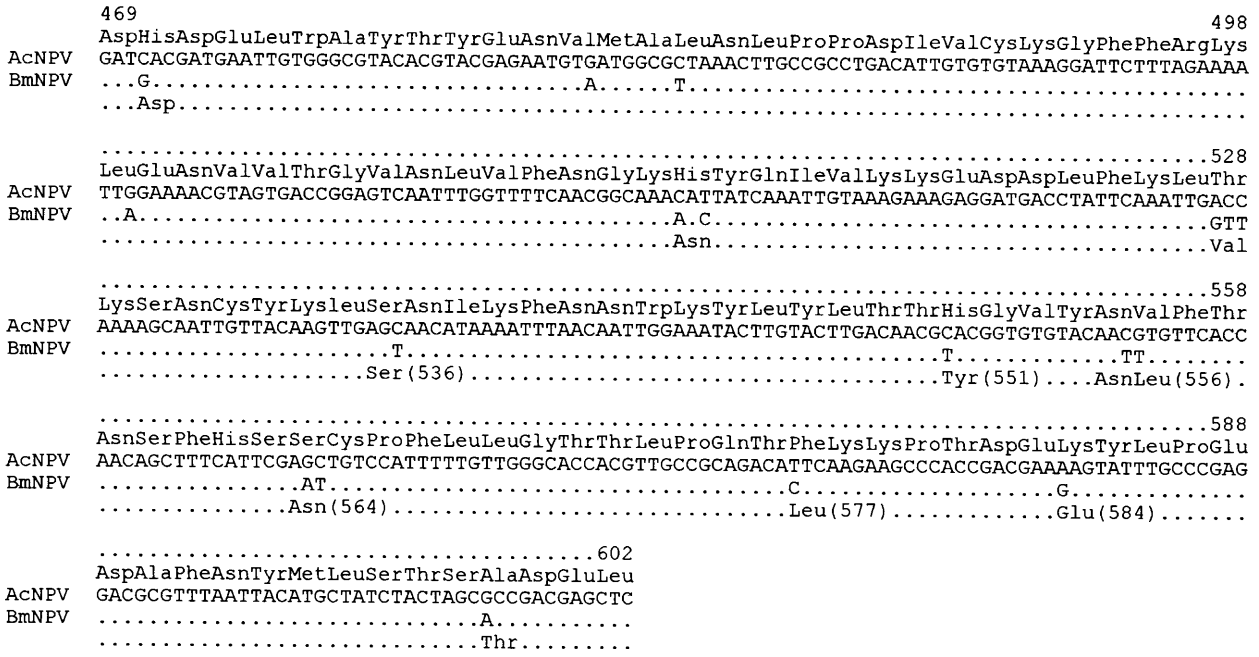

Fig. 1. Nucleotide and amino acid sequence comparison of the *Sau*3AI–*Sac*I fragment of the *p143* gene for AcMNPV (Lu & Carstens., 1991; Ayres *et al*., 1994) and BmNPV SC7 (Croizier *et al*., 1994). Amino acid positions targeted in directed mutagenesis are numbered. Numbering is relative to the initiation codon of the AcMNPV *p143* gene sequence.

but was sufficiently small to permit rapid monitoring of mutations by DNA sequencing. Mutagenesis experiments were performed using the Transformer site-directed mutagenesis kit (Clontech). Oligonucleotide primer sequences used to generate the mutations were as follows.

S536 (AGC) → S (AGT) 5′ GTTAAATTTTATGTTACTCAACTT 3′

H551 (CAC) → Y (TAC) 5′ ACCGTACGTTGTCAAGTAC 3′

V556 (GTG) → L (TTG) 5′ GTTGGTGAACAAATTGTACAC 3′

S564 (AGC) → N (AAT) 5′ CAAAAATGGACAATTCGAATG 3′

F577 (TTC) → L (CTC) 5′ GCTTCTTGAGTGTCTGCGGC 3′

K584 (AAG) → E (GAG) 5′ GGGCAAATACTCTTCGTCGG 3′

Mutated plasmids p1 to p8 (Fig. 2*c*) were sequenced manually with the T7 DNA sequencing kit (Pharmacia) using universal and reverse oligonucleotide primers. Plasmid DNAs were obtained using Qiagen columns. AcMNPV DNA (250 ng) and plasmid (2 μg) were mixed in 600 μl TC100 medium with 8 μl DOTAP reagent and added to 3×10^5 Sf9 cells (*Spodoptera frugiperda* IPLB-Sf21-AE clone 9) per well in a 24-well plate (Boehringer Mannheim). After 5 h the transfection mixture was replaced with fresh TC100 medium supplemented with 10% foetal calf serum. The transfection supernatants collected 48 h p.i. obtained by cotransfection of AcMNPV genome and one plasmid from the p1 to p9 plasmid series failed to infect productively Bm5 cells. Kamita & Maeda (1996) have shown that replication of eh2-AcMNPV, which contains the 572 bp *Sac*I–*Hin*dIII BmNPV fragment of the *p143* specificity domain, in Sf9 cells was dependent on the m.o.i. used for inoculation. Infections of Sf9 cells with eh2-AcMNPV at m.o.i. values of 1 and 0·1 resulted in 900- and 10000-fold reduced titres, respectively, compared with infection at an m.o.i. of 20. Moreover, recombinant virus infectivity on Sf9 and Bm5 cells decreased drastically when the 430 nt BmNPV *Sac*I–*Tth*111I fragment was used in place of the BmNPV *Sac*I–*Hin*dIII fragment (Fig. 2*a*) in cotransfections with wild-type AcMNPV DNA (Croizier *et al*., 1994; data not shown). Bm5 cells, due to poor permissivity for AcMNPV mutated in the region under study, were not suitable to analyse the mutations required to produce an AcMNPV effective P143 protein. The transfection supernatants were tested in more permissive *B. mori* larvae from two strains, BA 200 and the hybrid 200/300. The egg masses were provided by Unité Séricicole (INRA, Lyon, France). Third-instar larvae were injected with 8 μl of Sf9 cell-transfected medium collected 48 h post-infection. Twelve different cell supernatants were injected, including those infected with both parental viruses and the nine Sf9 supernatants resulting from transfection of AcMNPV DNA and each of the nine plasmids (Fig. 2*c*). Dead larvae were processed individually. Smears of dead larvae were analysed by light microscopy using Loeffler's staining to visualize the size and shape of inclusion bodies. Small cuboidal polyhedra approximately 1 μm in diameter were observed in larvae infected with supernatants containing recombinant AcMNPV. These polyhedra were quite different from BmNPV polyhedra, which are not cuboidal and have a diameter greater than 3 μm (data not shown). The two positive controls provided by

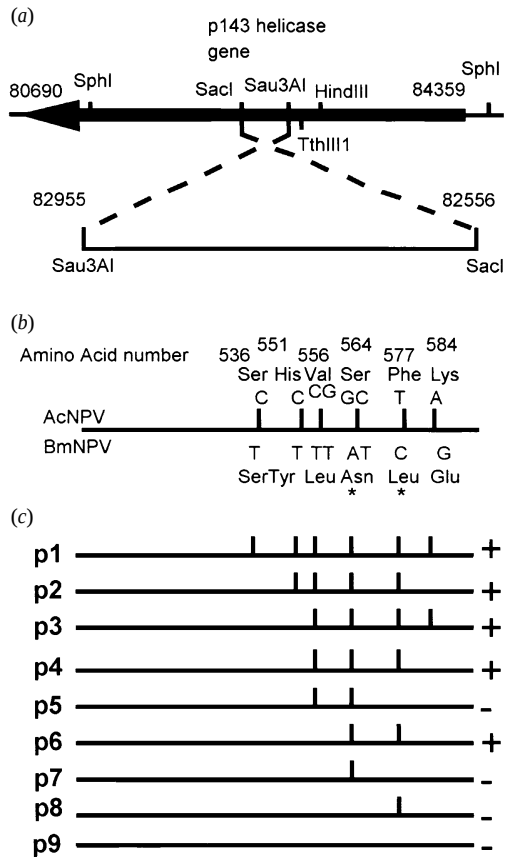

Fig. 2. Representation of the helicase gene region targeted for mutagenesis and of the series of mutated plasmids used to determine essential amino acid residues. (*a*) Genomic location of the host-range region. Restriction sites and helicase gene boundaries are marked (arrow, nucleotides 84359 to 80690; Ayres *et al*., 1994). The *Sac*I–*Sau*3AI AcMNPV fragment used for site-directed mutagenesis is expanded. (*b*) Nomenclature of the mutated positions between the *Sac*I and *Sau*3AI sites. Asterisks indicate the amino acid substitutions required to kill *B. mori* larvae. (*c*) Locations of BmNPV-specific codons introduced into the AcMNPV *Sac*I–*Sau*3AI region to study the effect of mutations on AcMNPV infectivity in *B. mori* larvae. The + and − signs to the right of the plasmid genotypes denote their ability to extend AcMNPV infectivity to *B. mori* larvae following recombination.

Table 1. *B. mori* larval mortality after injection of transfected Sf9 cell supernatants

Genotype	Total no. of injected larvae	No. of dead larvae*	Baculovirus-induced mortality (%)
AcMNPV	51	0 (8)	0
AcMNPV + p1	15	1† (1)	7
AcMNPV + p2	52	8 (10)	15
AcMNPV + p3	15	2 (1)	13
AcMNPV + p4	53	10 (15)	19
AcMNPV + p5	53	0 (6)	0
AcMNPV + p6	53	9 (7)	17
AcMNPV + p7	53	0 (11)	0
AcMNPV + p8	45	0 (4)	0
AcMNPV + p9	15	0	0
TC100 medium	15	0 (2)	0
AcMNPV + p81-15	30	26	87
BmNPV‡	15	15	100

* Numbers in parentheses refer to dead larvae showing no sign of polyhedrosis.
† Viruses purified from the single larva killed by the AcMNPV + p1 supernatant possessed a P3 genotype as shown by DNA sequencing.
‡ Supernatant of BmNPV-infected Bm5 cells.

BmNPV and AcMNPV/p81.15 (a 3279 bp *Sph*I fragment containing most of the BmNPV *p143* gene cloned into pUC19) induced baculovirus mortalities of 100% and 87%, respectively, whereas none of the recombinant viruses containing mutated sequences killed more than 20% of injected larvae (Table 1). Five of eight mutated virus populations induced mortality (Table 1; Fig. 2*c*). The other three transfection supernatants (p5, p7 and p8) and the three negative controls (AcMNPV, p9 and TC100 medium) did not produce any baculovirus-specific infection. Mortality from undetermined causes is indicated in Table 1 (numbers in parentheses).

Virus DNAs were extracted from polyhedra purified from dead *B. mori* larvae by standard methods (Summers & Smith, 1987). *Eco*RI restriction profiles of all the viral DNAs extracted from infected larvae were identical to that of wild-type AcMNPV DNA (data not shown). To confirm that sequences which had been mutated in the plasmids had been incorporated into pathogenic genomes, the 399 bp *Sac*I–*Sau*3AI region involved in the recombination was sequenced on both strands for viral DNAs extracted from at least one dead larva in each group. Mutated bases in viral DNAs were confirmed by sequencing after PCR amplification with primers A and B, described previously (Croizier *et al*., 1994), using the Dye Deoxy Terminator method on a 373 DNA sequencer (Applied Biosystems). We designated the various virus populations arising from transfection of AcMNPV DNA with each of the mutated plasmids according to the plasmid (p1 to p9) from which it originated. Thus, for example, transfection with plasmid p2 produced a recombinant virus genotype designated P2 able to kill *B. mori* larvae. Larvae killed by p2, p3 and p4 supernatants yielded corresponding genotypes. However, viral DNA in polyhedra purified from the single dead larva infected with p1 supernatant was of the P3 genotype. Neither the silent mutation in S536 nor the substitution giving rise to H551 → Y were present in the DNA of this recombinant virus. Virus genomes recovered from dead larvae were apparently pure as shown by the sequence analyses at the mutated positions. Sequences within the *p143* specificity domain in representatives of p2, p3, p4 and p6 populations were not contaminated with any other mutant or wild genotypes. Larvae injected with the p6 supernatant supported replication of the P6 genotype,

showing that the mutations S564 → N and F577 → L are required to enable AcMNPV to kill *B. mori* (Fig. 2*c*). This result is in accordance with the permissivity of *B. mori* larvae towards P2, P3 and P4 infectivity; these genomes contain the two mutations present in P6.

Cotransfection of Sf9 cells with plasmids containing mutations within the AcMNPV *p143* specificity domain and wild-type AcMNPV DNA generates a mixed population composed of AcMNPV recombinant genotypes and wild-type virus. *B. mori* larvae subsequently infected with Sf9 supernatants screen the various genotypes formed by recombination, selecting for those which can replicate in the larvae and eventually kill the insects. The two-step method followed in this work thus consists of creating and then selecting for mutant viruses infectious for a new host.

We have shown that mutations in the AcMNPV *p143* gene which cause only two amino acid substitutions, S564 → N and F577 → L, are required to kill *B. mori* larvae; viruses constructed with plasmids p7 and p8, which specify only one of the two amino acid substitutions, were unable to infect *B. mori* larvae. In the case of infections with supernatant p1, it remains to be established whether the absence of larvae killed by the P1 genotype reflects a low representation of this genotype in the p1 supernatant or a counter-selection of the P1 genotype.

In our experiments enough recombinant DNA was obtained to identify the new genotypes by restriction profile analysis and sequencing and for limited biological assays. Attempts to transfect either Bm5 or Sf9 cells with the recombinant DNAs from dead larvae failed, suggesting that mutant viruses were helped by wt AcMNPV, at least for the packaging of recombinant DNA in virus particles during the initial Sf9 transfection. Whether the differences in infectivity of the new genotypes *in vivo* and *in vitro* are due to differences in tissue susceptibility or simply the better permissivity of *B. mori* larvae remains to be studied.

AcMNPV *p143* is thus fully active in Sf9 cells and defective in Bm5 cells. Two amino acid substitutions, at position 564 and 577 in the AcMNPV *p143* specificity region, are sufficient to yield a P143 protein which is effective in *B. mori* larvae.

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