The *Trichoplusia ni* granulovirus helicase is unable to support replication of *Autographa californica* multicapsid nucleopolyhedrovirus in cells and larvae of *T. ni*

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Baculovirus DNA helicases are essential for replication and are determinants of host range. Helicases of *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and *Trichoplusia ni* granulovirus (TnGV) differ markedly, although both viruses replicate efficiently in the cabbage looper, *T. ni*. To determine whether the TnGV helicase (P137) could support replication of AcMNPV in *T. ni* cells or larvae, the native AcMNPV helicase gene (*p143*) was disrupted and substituted with *p137*. P137 did not support replication when synthesized by the P143-deficient AcMNPV. Moreover, P137 did not inhibit AcMNPV replication when co-synthesized in the presence of the AcMNPV P143. These results suggest that although TnGV and AcMNPV replicate efficiently in *T. ni*, specific protein–protein or protein–DNA interactions between baculoviral helicases and viral-specific factors which form the replicase complex are required for virus replication. A novel and rapid method for disrupting AcMNPV genes in *E. coli* using the commercial Bac-to-Bac AcMNPV baculovirus expression vector is described.

Introduction

The family *Baculoviridae* consists of viruses reported most commonly attacking larvae of species belonging to the order Lepidoptera (Martignoni & Iwai, 1986; Volkman et al., 1995). Although the virions of all baculoviruses are similar in their structural and physical properties, members of this group, even those that attack the same host, can vary markedly in host range, tissue tropism and pathobiology. For example, *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and *Trichoplusia ni* granulovirus (TnGV) replicate efficiently in larvae of the cabbage looper, *T. ni*. However, after the colonizing phase of replication in the midgut epithelium, AcMNPV replicates in all major tissues, whereas TnGV replication is restricted to the fat body (Federici, 1993). In addition, AcMNPV is capable of replicating in species of many lepidopteran families, whereas the host range of TnGV appears to be restricted to members of the family Noctuidae.

The molecular basis for the differences in tissue tropism and host range of AcMNPV and TnGV is unknown. However, recent studies of putative baculoviral helicases suggest that these enzymes are one of the key determinants of host range (Lu & Carstens, 1991; Lu & Miller, 1995; Kool et al., 1995; Ahrens & Rohrmann, 1996; Heldens et al., 1997). For example, changes in a few amino acids, or even in only one, in the AcMNPV helicase (P143) enable AcMNPV to replicate in *Bombyx mori*, a host which does not support AcMNPV replication normally (Maeda et al., 1993; Croizier et al., 1994; Kamita & Maeda, 1997; Argaud et al., 1998). In addition, although the AcMNPV and *Bombyx mori* (Bm)NPV helicases are 96% identical (Kamita & Maeda, 1997), the AcMNPV helicase inhibits BmNPV replication completely in *B. mori* cells (Kamita & Maeda, 1993). Although studies of putative NPV DNA helicases have shown that they are involved in replication and host range determination, nothing is known about the function of GV helicases. At present, the putative helicase (P137) of TnGV is the only GV helicase described (Bideshi et al., 1998). Its amino acid sequence differs significantly (26·4–29·8% identical and 44–54% similar) from NPV homologues. Despite the marked differences between TnGV P137 and AcMNPV P143, both function effectively during replication of their encoding genomes in larvae of the cabbage looper, *T. ni*. These observations raise the questions of whether *p137* encodes a
functional helicase that can support replication of AcMNPV in *T. ni* cells and larvae, and if so, whether P137 can affect the host range and tissue tropism of AcMNPV.

In the present study, we show that P137 was unable to support replication and occlusion, in either *T. ni* cells in *vitro* or in larvae, of a recombinant AcMNPV deficient in native helicase function. In addition, we show that co-synthesis of TnGV P137 and AcMNPV P143 did not inhibit replication of AcMNPV in *T. ni* cells or larvae. These results provide evidence that host and AcMNPV proteins which combine to form the replication complex cannot do so when P137 is substituted for P143. We also describe a rapid method for disrupting AcMNPV genes in *E. coli* using a recombinant AcMNPV (bacmid) capable of replicating in this bacterial host. Compared to conventional methods using virion plaque purification in insect cells, the bacmid system reduces significantly the time required for obtaining AcMNPV recombinants. This method could be particularly useful for recovering recombinants with deletions in genes essential for virus replication and pathogenesis because such recombinants are unlikely to be purified from insect cells or larvae in the absence of a helper virus.

### Methods

**Virus, bacterial strains and transformation.** The Bac-to-Bac AcMNPV Baculovirus Expression (bacmid) system (Gibco BRL) was used to express the putative TnGV helicase gene (p137). Site-specific recombination was performed in *E. coli* BJ5183 (recBC delBC) (Hanahan, 1983; Chartier *et al.*, 1994), which was kindly provided by M. Mehtali (Gene Therapy Department, Transgene SA, Strasbourg, France). Plasmid constructs were amplified in *E. coli* DH5α or in *E. coli* DH10BAC (Gibco BRL). Bacterial cells were transformed by electroporation using an Electro Cell Manipulator (BTX Electroporation System) set at 2-5 kV, 30 µF, 120 Ω. Transformants were allowed to recover in SOC medium (Ausubel *et al.*, 1994) for a minimum of 1 h before plating onto selective media.

**AcMNPV bacmids expressing polh and p137 under control of the p143 promoter.** The polyhedrin gene (polh), which is the last gene deleted from the AcMNPV bacmid expression vector, was re-introduced by site-specific transposition using the transfer vector pFastBac (Gibco BRL) (Fig. 1A). To delete the polyhedrin promoter in pFastBac, the plasmid was digested with *SnaB* and *BamH*, filled with dNTPs and Klenow and ligated to generate pFBd2. The 1.4 kb EcoRV–Asel fragment of AcMNPV (nucleotide positions 4425–5818; Ayres *et al.*, 1994) containing the polh gene was cloned into the filled HindIII site in pFBd2 to generate pFBdP-4 (Fig. 1B).

The p143 promoter was obtained by PCR with Vent (exo−) DNA polymerase (New England Biolabs) and primers (Genosys) Acpro-1 (5’ggcgaaatGTCACGTAAACTCGTGGG 3’, nucleotide positions 84639–84659) and Acpro-2 (5’ GTTGCGTATCTTTCTGTTA 3’, nucleotide positions 84360–84340), and cloned in the EcoRI and SstI sites in pFastBac. The nucleotide sequence was confirmed by the dideoxy-nucleotide chain termination method (Sanger *et al.*, 1977) using a Sequenase version 2.0 kit (United States Biochemical).

The region of the TnGV genome encoding P137 (positions 528–4509) (Bideshi *et al.*, 1998) was cloned downstream from the p143 promoter in pFastBac to generate pHHUP-5. The 4 kb EcoRI–PstI

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**Fig. 1.** Schematic illustration of plasmid constructs used to evaluate the function of TnGV P137. (A) pFastBac, the vector used for transposition of genes into the AcMNPV bacmid. The relative locations of the polyhedrin promoter (Ppolh) and multiple cloning sites (MCS) are shown. (B) pFBdP-4, the plasmid derived from pFastBac containing the intact AcMNPV polyhedrin gene (polh). To generate pFBdP-4, the polyhedrin promoter (Ppolh) was deleted from pFastBac by digestion with *SnaB* and *BamH*, after which the plasmid was circularized by blunt-end ligation. A 1.4 kb EcoRV–Asel fragment containing the AcMNPV polyhedrin gene (polh) was then cloned into the blunt-ended HindIII. (C) pHH7, the plasmid containing both TnGV p137 and AcMNPV polh. This plasmid was generated by cloning a 4 kb EcoRI–PstI fragment containing the coding sequence for the TnGV helicase (P137) under control of the AcMNPV helicase promoter (Pp143) into the same sites in pFBdP-4. (D) pHHN-6, the plasmid used to disrupt p143 in the AcMNPV bacmid in the bacterial host, *E. coli* BJ5183.
A.

P143 probe

ΔP143 probe

B.

MW (kb)

AcHB
MH5
AcHB
21
AcHB
MH5
AcHB
21

1 2 3 4 5 6 7 8 9

Fig. 2. Southern blot analysis showing disruption of the AcMNPV p143 gene. (A) Schematic illustration of the EcoRI-D fragment of AcMNPV containing the p143 gene. The appropriate restriction sites, relative location of the chloramphenicol resistance gene (chlR), primers used for PCR (P143-1, P143-2 and Acpro-1), and probes (P143, ΔP143, CHL) used in the analysis are shown. (B) PCR products obtained using the Acpro-1 and P143-2 primers probed with the ΔP143 (a) or CHL (b) probes. DNAs of the AcHB21 bacmid, which contained the native helicase gene (p143) and the sequence encoding the TrnGV helicase (P137) fragment of pHUP-5 was cloned into the same position in pFBdP-4 to generate pHH-7 (Fig. 1C).

Site-specific transposition using pFBdP-4 and pHH-7, and selection of recombinant AcMNPV bacmids AcBacR (p143 null, kanR, chlR) and AcHB-21 (p143 null, p137 under control of the p143 promoter, polhR, kanR, chlR) were performed according to the manufacturer’s protocol (Gibco BRL).

### DNA and RNA probes.

The 1.2 kb BspHI–XmnI fragment of pBCSK(−) (Stratagene) containing the chloramphenicol resistance gene (CHL probe), coding sequences of p143 (P143 probe), the 2.0 kb BamHI–NruI fragment of p143 (AP143 probe) containing the N-terminal coding region of P143 and the AcMNPV genomic DNA were labelled using a Dig DNA labelling and Detection kit (Boehringer Mannheim) according to the manufacturer’s protocol. Synthesis of the radiolabelled TrnGV p137-antisense RNA probe and p143-antisense RNA probe using pAcBS-2 (see below) was performed as described by Bideshi et al. (1998).

### Disruption of the p143 gene in E. coli BJ5183.

The AcMNPV p143 open reading frame (Lu & Carstens, 1991) was obtained by PCR with primers (Genosys) 143-1 (5′ ccggatcc ATGATTCAGTTCAAGTCATTTTATTACATTTTT 3′) and 143-2 (5′ tgcgaattc GCCGCTGTCCGAGAGATGGGC 3′) initiating 3870 nucleotides downstream of the translation start codon, cleaved with EcoRI and BamHI, and ligated into the same sites in pBlueScript SK(+) (Stratagene) to generate pAcBS-2. The chloramphenicol resistance gene in pBCSK(−) was obtained as a filled 1.2 kb BspHI–XmnI fragment and ligated into the filled SalI–XhoI sites in pHUHP-5 to generate pHCC-1. The 1.9 kb NdeI–HindIII fragment in pAcBS-2, which lacked more than half of the N-terminal coding sequences of P143, was cloned into the filled Xhol and HindIII sites in pHCC-1 to generate pHHN-6 (Fig. 1D).

The E. coli BJ5183 strain was transformed with AcHB-21, which contains polhR, and the sequence encoding the TrnGV P137 under control of the AcMNPV p143 promoter. The resulting strain (E. coli–AcHBM) was transformed with the 3.4 kb EcoRI fragment in pHBNN-6 containing the CHL-disrupted AcMNPV p143 gene (Fig. 1D). Bacterial cells were recovered overnight in SOC and plated onto LB agar with 30 µg/ml kanamycin and 15 µg/ml chloramphenicol (LB-KC). Plates were incubated at 37°C for a maximum of 72 h. Disruption of the p143 gene in an AcHB-21 bacmid recombinant (AcHBHM5) that was resistant to kanamycin and chloramphenicol was confirmed by PCR analysis and Southern blot hybridization with the CHL, P143 and AP143 probes.

### Transfection of insect cells and detection of viral DNA.

DNAs were purified using a Nucleobond AX kit (Clontech). Cells of Trichoplusia ni (BTI-TN-5B1-4; Invitrogen), Spodoptera frugiperda (SF21 and SF9; Pharmingen) and Spodoptera exigua SEC and SE1 (Gelernter & Federici, 1986), were transfected with approximately 2 µg of AcBacR, AcHB-21, AcHBHM5, 2 µg of AcHBHM5 and 4 µg of a plasmid containing the AcMNPV EcoRI-D fragment with the p143 gene (Ayres et al., 1994), or with a mock control, using Cellfectin liposome reagent (Gibco BRL).

Cell cultures were incubated at 28°C for 3–6 days, after which media were collected and used to infect insect cells. To maintain proliferation of the monolayers of mock- and AcHBHM5-transfected cells, aliquots of the cell culture were transferred to fresh medium.

The presence of recombinant AcMNPV bacmid DNA was detected by PCR. DNA was isolated from insect cells or budded virions as described by O’Reilly et al. (1994), and then the 1.6 kb region of the
AcMNPV genome containing the p35 gene (Ayres et al., 1994) was amplified by PCR using primers P35-1 (5’ TGGCGTCGACAGGTT-TATATTCTT 3’, nucleotide position 115999) and P35-3 (5’ GCA-TTACAAGTAGAATTCTACTCGTAAAG 3’, nucleotide position 117598).

■ RNA analysis. RNA samples were collected from BTI-TN-5B1-4 cells transfected or infected with AcBacP⁺, AcHB-21 and AcHBMH5 using the TRIzol reagent (Gibco BRL). Five μg of RNA was dot-blotted onto a nylon membrane and hybridized with the radio-labelled p137and p143-antisense RNA probes, and with the digoxigenin-labelled AcMNPV genomic probe.

■ Insect inoculation. Larvae of T. ni were grown on a semi-defined medium (Shorey & Hale, 1965). Two μg of AcHB-21 or AcHBMH5 DNA was mixed with 6 μl of Cellfectin reagent and 300 μl of Grace’s insect cell culture medium (Gibco BRL). Three separate injections of ten early fourth instar larvae, each with 3 μl of the mixture, were performed within a 5 min period using a Microapplicator model M microinjector (Instrumentation Specialties Company, Inc.). Ten larvae were mock-transfected with Grace’s insect medium. In addition, 2 ml of cell culture medium from transfection experiments with AcHB-21, AcHBMH5 or mock-transfected BTI-TN-5B1-4 cells was collected 6 days after incubation and spun at 15000 g for 20 min and pellets were resuspended in 300 μl of Grace’s insect medium. Twenty larvae were microinjected with 3 μl of each suspension.

Results  
AcMNPV p143 gene-disruption in E. coli BJ5183

Colonies resistant to kanamycin and chloramphenicol were isolated after E. coli-AcHBM cells were transformed with the 3·4 kb EcoRI fragment in pHHN-6 (Fig. 1D) that contained the disrupted p143 gene. The results of PCR analysis and Southern blot hybridization using the CHL, P143 and ΔP143 probes (Fig. 2A) of the recombinant AcHBMH5, which contained the disrupted p143 gene and was resistant to kanamycin and chloramphenicol, are shown in Fig. 2B. The 3·4 kb PCR products originating from the AcHBMH5 bacmid using the Acpro-1 and P143-2 primers [Fig. 2B (a, b)] hybridized with the CHL probe, but not with the ΔP143 probe, whereas the 4·2 kb PCR product from AcHB-21, obtained with the same primers, hybridized with the ΔP143 probe, but not with the CHL probe [Fig. 2B (a, b)]. These results demonstrate that the disrupted p143 and intact p143 was present in, respectively, AcHBMH5 and AcHB-21.

The location of the AcMNPV 11·1 kb EcoRI-D (Ayres et al., 1994) and EcoRI–EcoRV (5·0 kb) fragment containing the intact p143 gene of AcHB-21 was confirmed with the P143 probe [Fig. 2B (c, d)]. Disruption of p143 with the chloramphenicol gene in AcHBMH5 resulted in a truncated EcoRI–EcoRV fragment (4·3 kb) that hybridized with the P143 probe [Fig. 3B (d)], but not with the ΔP143 probe [Fig. 2B (f)]. In addition, the CHL probe hybridized with the EcoRI-D and EcoRI–EcoRV fragments of AcHBMH5 but not with AcHB-21 [Fig. 2B (g, h)].

The presence of the helicase gene of TnGV in AcGVH5 and AcHB-21 was confirmed by hybridization using the Tn-p137 antisense DNA probe, as described by Bideshi et al. (1998) [Fig. 2B (i, j)].

TnGV p137 is unable to substitute for AcMNPV p143 function

When TnGV p137 was substituted for AcMNPV p143 in AcHBMH5, no occlusion bodies were observed in BTI-TN-5B1-4, SF21, SF9, SE-C or SE-1 cells, even as late as 12 days post-transfection (data not shown). The morphology and growth of cells transfected with AcHBMH5 were similar to those of the mock-transfected control. In both, the cells were completely confluent by 4–6 days post-transfection. Cells transfected with AcHB-21 or AcBacP⁺ produced polyhedra within 3 days, and the cell density was approximately 2-fold lower than cells transfected with AcHBMH5 due to the spread of infection, which limited cell proliferation.

In addition, occlusions were not observed in cells treated with medium collected from the initial transfection with AcHBMH5 (data not shown). However, occlusions were observed after 5–8 days in cells co-transfected with AcHBMH5 and a plasmid containing AcMNPV p143, indicating that the latter gene was expressed, and P143 supported AcMNPV replication.

Using the P35-1 and P35-3 primers, designed to amplify a 1·6 kb fragment containing the p35 (Ayres et al., 1994) gene of AcMNPV, AcHB-21 and AcHBMH5 DNAs were detected in BTI-TN-5B1-4 cells from 3 and 5 days post-transfection (Fig. 3, lanes 2–5). However, the 1·6 kb PCR product was not detected 12 days post-transfection of cells with AcHBMH5 (Fig. 3A, lane 6). In addition, the 1·6 kb fragment was not detected in the supernatant of cell transfected with AcHBMH5 (Fig. 3B, lanes 1–4), indicating a lack of budded virions. The 1·6 kb fragment was obtained by PCR from similar preparation of supernatants of cells transfected with AcHB-21 (Fig. 3, lane 5).
AcHB-21, co-expressing p143 and p137 (Fig. 5), replicated just as well as AcBacP+ in BTI-TN-5B1-4, SF21, SF9, SE1 and SEC cells. Polyhedra were observed in BTI-TN-5B1-4 cells within 4 days post-transfection, and 4–6 days in SF21, SF9, SEC and SE1 cells (data not shown). Infection of new cultures of these cells with culture media from cells infected during the initial transfection resulted in virion occlusion within 3 days of treatment (data not shown).

Discussion

Our experiments with T. ni cells and larvae showed that TnGV p137 could not substitute for AcMNPV p143, nor did it interfere with AcMNPV replication when co-produced with AcMNPV P143. These results indicate that P137 was either not transported to the nucleus or, if it was, that it did not interact with AcMNPV proteins and/or host proteins that form a functional replisome. The significant divergence of the amino acid sequences between TnGV P137 and AcMNPV P143, which are only 29% identical and 53% similar (Bideshi et al., 1998), provides a possible explanation for the failure of P137 to substitute for, or interfere with, AcMNPV P143. Assuming that complex interactions are required to form a functional replicase complex, results obtained with the P143 helicase proteins of AcMNPV and BmNPV support this possibility. Even though these helicases are 95% identical (Kamita & Maeda, 1997), AcMNPV is unable to replicate efficiently in B. mori cells. In addition, AcMNPV P143 inhibits replication of BmNPV in B. mori cells (Kamita & Maeda, 1993). Minor amino acid substitutions in AcMNPV P143, however, resulted in AcMNPV replication in these cells. Thus, if such minor differences can interfere with replication, it is not surprising that the major differences between TnGV P137 and AcMNPV P143 prevent their substitution, or that P137 does not interfere with P143 function.

The results obtained in our substitution experiments were consistent with how helicases function in the replication of viral DNA. Viral DNA replication generally involves a set of highly ordered protein–DNA and protein–protein interactions that lead to the assembly of a functional replicase complex. Among these interactions is the recruitment of DNA polymerase by the pre-replicative helicases–primase complex at the origin(s) of DNA replication. The complexity of these interactions in large DNA viruses is illustrated by the pre-replicative complex of herpes simplex virus type 1 (HSV-1). In this virus, the origin-binding protein (UL9), a DNA helicase (Bruckner et al., 1991), binds to the HSV-1 genome with the concomitant binding of the helicase–primase components (UL5, UL8, UL52) and the single-stranded DNA-binding protein ICP8 (Liptak et al., 1996). The UL5 protein requires UL52 for helicase and primase activity (Dodson et al., 1989; Dodson & Lehman, 1991). Similar protein–protein interactions are likely to occur in baculoviruses. However, with the exception of the AcMNPV and Orgyia pseudotsugata

### Co-expression of p143 and p137 does not inhibit AcMNPV replication

Dot-blot analysis of RNAs collected from cells infected with AcBacP+, which lacked TnGV p137, and AcHB-21, which contained the sequences encoding the TnGV P137 under control of the p143 promoter, showed that the p143 gene was expressed during virus replication in BTI-TN-5B1-4 cells (Fig. 5 A). The p137 gene was also detected in BTI-TN-5B1-4 cells infected with AcHB-21 (Fig. 5 B).
MNPV replication proteins (Kool et al., 1994, 1995; Lu & Miller, 1995; Ahrens & Rohrmann, 1996), the proteins involved in baculovirus replication complexes remain unknown. Based on its deduced amino acid sequence, TnGV P137 is probably one of these proteins, but more definitive evidence is required before such a conclusion can be drawn.

Finally, in the present study the utility of a method for the rapid disruption of AcMNPV bacmid genes in E. coli BJ5183 was demonstrated (Hanahan, 1983; Chartier et al., 1996). As AcMNPV strains with non-temperature-sensitive lethal mutations cannot be recovered by plaque purification techniques in the absence of helper virus, this method could prove practical for inactivating genes such as p143 or cis-elements such as hr sequences (Leisy et al., 1995; Pearson & Rohrmann, 1995; Kool et al., 1993; Ayres et al., 1994) that may be essential for virus replication and pathogenesis.

The accompanying paper (Bideshi & Federici, 2000) reports the DNA-independent ATPase activity of the TnGV DNA helicase.

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References


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P143 helicase is required for AcMNPV replication in vivo


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