

Expression of a *Toxoneuron nigriceps* polydnavirus-encoded protein causes apoptosis-like programmed cell death in lepidopteran insect cells

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The polydnavirus *Toxoneuron nigriceps* bracovirus (TnBV) is an obligate symbiont associated with the braconid wasp *T. nigriceps*, a parasitoid of *Heliothis virescens* larvae. Previously, to identify polydnavirus genes that allow parasitization by altering the host immune and endocrine systems, expression patterns of TnBV genes from parasitized *H. virescens* larvae were analysed and cDNAs were obtained. To study the function of the protein from one such cDNA, TnBV1, overexpression of the protein was attempted by using the baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus. Recovery of stable recombinant virus was unsuccessful, with the exception of recombinants with deletions/mutations within the *TnBV1* gene. It was hypothesized that TnBV1 expression was cytotoxic to the *Spodoptera frugiperda* (Sf21) insect cells that were used to produce the recombinants. Therefore, the Bac-to-Bac system was used to create recombinant baculoviruses maintained in *Escherichia coli* expressing either TnBV1 (Ac-TnBV1) or an initiator-methionine mutant [Ac-TnBV1(ATG–)]. Microscopy revealed substantial cell death of Sf21 and High Five cells from 48 h post-infection with Ac-TnBV1, but not with the Ac-TnBV1(ATG–) recombinant virus. Ac-TnBV1-infected Sf21 cells, but not those with parental virus infection, showed an increased caspase-3-like protease activity, as well as increased terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) for breaks in host genomic DNA. Although indicative of apoptosis, blebbing and apoptotic bodies were not observed in infected cells. Transiently expressing TnBV1 alone caused TUNEL staining in High Five cells. These data suggest that TnBV1 expression alone can induce apoptosis-like programmed cell death in two insect cell lines. Injection of Ac-TnBV1 budded virus, compared with parental virus, did not result in an alteration of virulence in *H. virescens* larvae.

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INTRODUCTION

Polydnviruses (PDVs) are obligate symbionts that are associated with hymenopteran parasitoids of lepidopteran larvae (Kroemer & Webb, 2004). The PDV genome is multipartite, made of closed circular DNA, integrated as provirus into the wasp genome and transmitted vertically through the germ line. The viral particles replicate from proviral DNA in the calyx cells of the wasp oviduct and are injected at oviposition, along with venom and ovarian proteins, into the lepidopteran host. During virus replication in the wasp, PDV class I genes are expressed, whereas class II viral genes are expressed in the parasitized host to disrupt its physiology and immune response, to allow successful parasitism.

Although PDVs can infect most larval tissues, the greatest infection occurs in haemocytes (Stoltz & Vinson, 1979; Wyder *et al.*, 2003). Infected haemocytes lose their ability to adhere to a substrate, often show cytoskeleton disruption and may become apoptotic (Schmidt *et al.*, 2001; Lavine & Strand, 2002). All of these events ultimately interfere with encapsulation of the parasitoid egg and larvae. *In vitro* infection of some insect host-cell lines has also been shown to lead to the form of programmed cell death (PCD) known as apoptosis in a subset of the cells, but transformed cell lines recover from this phenotype (Béliveau *et al.*, 2003; Kim *et al.*, 1996).

Toxoneuron nigriceps, a parasitoid of *Heliothis virescens* larvae, is associated with the PDV *Toxoneuron nigriceps* bracovirus (TnBV) (Stoltz *et al.*, 1976; Stoltz & Vinson, 1979). Parasitization of *H. virescens* larvae by *T. nigriceps* leads to suppression of the immune response and developmental arrest of the host larvae, caused by the combined action of TnBV and teratocyte cells from the parasitoid's embryonic serosa (Pennacchio *et al.*, 2001). To identify PDV gene products that contribute to these processes, the expression pattern of TnBV genes from parasitized *H. virescens* larvae was analysed and cDNAs of viral transcripts were obtained (Malva *et al.*, 2004; Falabella *et al.*, 2003). The first TnBV gene identified (formerly named *CnBV1*, but here renamed *TnBV1* to accommodate the ICTV reclassification of TnBV) is 467 nt long, contains one intron and encodes a putative protein of 124 aa with a predicted molecular mass of ~15 kDa (Varricchio *et al.*, 1999; GenBank accession no. Y19010). The deduced amino acid sequence of *TnBV1* does not show a significant level of identity to other proteins. The early and sustained expression of *TnBV1* in fat body and haemocytes of last-instar *H. virescens* larvae (Varricchio *et al.*, 1999; P. Falabella & F. Pennacchio, unpublished data) suggests that it may have a role in immune suppression. Also, detection of *TnBV1* mRNA in the host's prothoracic glands also indicated a possible influence on endocrine and developmental disruption. In order to characterize the function of *TnBV1* in the parasitization of *H. virescens* larvae, we expressed the cDNA in lepidopteran cell lines by using various systems and assessed its effects on cell morphology and viability.

METHODS

Cells, viruses and insect rearing. *Spodoptera frugiperda* (Sf21) cells and *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) strain L1, as well as the various recombinant viruses created, were propagated and maintained as described by O'Reilly *et al.* (1992). High Five cells were cultured in Express Five serum-free medium (both from Invitrogen), complete with glutamine (16 mM) and antibiotic/antimycotic solution at the suggested concentrations. The bacmid strain was maintained as directed by the manufacturer (Invitrogen). The p35 deletion virus (p35 del), also known as vAcAnh (Clem *et al.*, 1991), was maintained as described for AcMNPV but in High Five cells. *H. virescens* eggs were provided by Syngenta and larvae were maintained on a synthetic diet, obtained from Syngenta, at 27 °C.

Construction and identification of recombinant viruses.

Transfer-vector plasmids were constructed to generate AcMNPV recombinants expressing a *TnBV1* cDNA that was selected from a cDNA library of *T. nigriceps*-parasitized *H. virescens* larvae (Varricchio *et al.*, 1999). *TnBV1* was subcloned from pBluescript (Stratagene) into the *XhoI* and *EcoRI* sites of the pSynXIV VI⁺ X3 vector (O'Reilly *et al.*, 1992) to yield pSyn-*TnBV1*. To obtain recombinant virus expressing *TnBV1*, Sf21 cells, at a density of 2×10^6 cells per 60 mm dish, were co-transfected with 1 µg vsynVI-gal or vsynVI-egt(-) viral DNA (O'Reilly *et al.*, 1992) and 2 µg transfer vector pSyn-*TnBV1* plasmid DNA, using DOTAP reagent (Roche). Recombinant viruses were selected by screening for occlusion-positive white plaques and purified between three and seven times (O'Reilly *et al.*, 1992). The selected viral recombinants were amplified to passage 3 and the presence of the *TnBV1* gene was checked by restriction-enzyme digestion, PCR and sequence analysis of viral DNA.

TnBV1 was also cloned into the *XhoI* and *EcoRI* sites of the pFastBac1 vector (Invitrogen). In addition, a *TnBV1*(ATG-) mutant, in which the initiator codon ATG was mutated to ATT, was PCR-amplified from the pSyn-*TnBV1* template by using the *TnBV1*nullIF and the *TnBV1*nullR primers (Table 1). The *TnBV1*(ATG-) PCR product was first cloned into pGEM-T Easy (Promega) and the insert was digested with *XhoI* and *EcoRI* for cloning in the pFastBac1 vector. pFB-*TnBV1* and pFB-*TnBV1*(ATG-) vectors were transformed into DH10Bac *Escherichia coli* cells (Gibco) and positive clones were selected as described by the manufacturer. To obtain recombinant viruses, high-molecular-mass DNA was purified from the selected clones as recommended by the manufacturer and transfected into Sf21 cells. The Ac-*TnBV1* and Ac-*TnBV1*(ATG-) bacmid recombinant viruses were plaque-purified and amplified to passage 3 in Sf21 cells (O'Reilly *et al.*, 1992). The presence of the *TnBV1* gene or its ATG- mutant variant was confirmed by PCR and sequence analysis of viral DNA.

Construction of transient-expression vectors. The expression vector pIE1/153A (Lu *et al.*, 1997; Farrell *et al.*, 1998) was employed for transient expression of recombinant *TnBV1* in insect cells. Primers Tn1F and Tn1R (Table 1) corresponding to the 5' and 3' ends, respectively, of the *TnBV1* ORF with extensions for *NotI* restriction sites were used in PCR amplification, using *Pfu* polymerase (Promega) and the pBluescript plasmid containing *TnBV1* (Varricchio *et al.*, 1999) as template. The reaction yielded a product containing the *TnBV1* ORF flanked by *NotI* restriction sites. This was digested with *NotI* and cloned into the unique *NotI* site of pBluescript (Stratagene). After sequence verification, the fragment was subcloned into the unique *NotI* site of pIE1/153A to yield pIE1/153A.*TnBV1*.

Analysis of insect cells infected with a baculovirus expressing *TnBV1*. Phenotypes of the recombinant virus infections were observed by light microscopy (Leica) under a 40× objective lens,

Table 1. Primers used for subcloning the cDNA for TnBV1

Primer	Sequence (5'→3')
TnBV1nullF	GCGGAATTCTCAAATTTTATCTTGAATAAT
TnBV1nullR	GTCGACAGATCTGCAGCTCGAGTTTT
TnBV1for	CTGGTACTTAGAGTTTTA
TnBV1rev	GTAAAGGGTTACATATCT
Tn1F	TCGAGCGGCCGCAAATTTTATCATGAATAATTATTAT
Tn1R	ATCTGCGGCCGCTTATAAATCTATAACTATGTTAAGGG

analysed by SDS-PAGE and subjected to Western blot analysis, using either High Five or Sf21 cells that were mock-infected or infected with wild-type AcMNPV, parental bacmid or recombinant Ac-TnBV1 or Ac-TnBV1(ATG-) virus at an m.o.i. of 10. Cells were harvested 12–72 h post-infection (p.i.) and processed for SDS-PAGE and Western blot analysis as described by O'Reilly *et al.* (1992). Blots were probed with a 1:10 000 dilution of anti-p10 DNP 4.17 antibody (a gift from G. W. Blissard, Boyce Thompson Institute, Cornell University, NY, USA) and visualized with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories) at a 1:10 000 dilution by using an ECL kit (Amersham Biosciences).

Assessment of expression of TnBV1 in transfected High Five cells by RT-PCR. Transcription of TnBV1 in transfected High Five cells was assessed by RT-PCR. High Five cells were seeded at a density of 4×10^5 cells per well in a 12-well plate (Corning). Plasmid DNA (2 µg) (pIE1/153A or pIE1/153A.TnBV1) and 0.5 µg of a plasmid carrying the green fluorescent protein marker gene, *gfp* (pIE1/153A.GFP; Farrell *et al.*, 1998), along with 2.5 µl Lipofectin (Invitrogen) in Express Five medium, were used to transfect cells according to the manufacturer's instructions. Total RNA was extracted at 36 h post-transfection by using Tri-Reagent (Sigma). For first-strand cDNA synthesis, 5 µg total RNA was used in the SuperScript First-Strand Synthesis system for RT-PCR (Gibco). PCRs were carried out by using one-fifth of the RT product as template with the TnBV1for and TnBV1rev primers (Table 1); PCR products were visualized by gel electrophoresis.

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) assay. The effect of TnBV1 expression in Sf21 cells was analysed by fluorescence-activated cell sorting (FACS) coupled with a TUNEL assay. Sf21 cells (1×10^6) were infected with bacmid or Ac-TnBV1 at an m.o.i. of 10 for 48 h before being fixed, permeabilized (70% ethanol or methanol) and treated for the TUNEL assay according to the manufacturer's protocol (Mebstain Apoptosis kit II; MBL). For each treatment, 10 000 cells were processed by flow cytometry to measure fluorescein isothiocyanate (FITC) labelling. TUNEL-positive cells were gated electronically at 1% of the bacmid-infected control. Cells that fluoresced at an intensity higher than the 1% gate (M1) in the Ac-TnBV1-infected cell population were considered TUNEL-positive. The number of TUNEL-positive cells out of 10 000 was counted in five independent experimental replicates. A generalized linear model with binomial errors, a logit link function and an overdispersion parameter was used to analyse the probability that a TnBV1-infected cell fluoresces at or above the M1 gate for the matched bacmid control. The hypothesis that this probability equals 0.01 was then tested at the $\alpha=0.05$ level with an appropriate *t*-test on the logit scale (PROC GENMOD; SAS Institute Inc., 1999).

The effect of TnBV1 transient expression in High Five cells was analysed by microscopy coupled with a TUNEL assay. High Five cells were seeded at a density of 4×10^5 cells per well in a 12-well plate. Then,

2.0 µg pIE1/153A.TnBV1 plasmid DNA and 0.5 µg of a plasmid carrying the marker gene *gfp* (pIE1/153A.GFP; Farrell *et al.*, 1998), along with 2.5 µl Lipofectin (Invitrogen) in Express Five medium, were used to transfect cells according to the manufacturer's instructions. Cells transfected with 2.5 µg plasmid pIE1/153A.GFP alone were used as a control. TUNEL labelling was assessed in transfected cells and controls by detecting genomic DNA fragmentation via a DeadEnd Colorimetric TUNEL assay kit (Promega). Slides were observed by transmitted light or phase-contrast (Axiophot; Zeiss) and then fluorescence microscopy at 18 and 36 h post-transfection. TUNEL-positive and -negative cells were counted on five microscopic fields at a magnification of $250 \times$, randomly selected from slides obtained in two independent replicates of the experiment. A generalized linear model with binomial error, a logit link and an overdispersion parameter was fitted to the cell counts (PROC GENMOD; SAS Institute Inc., 1999). The hypothesis that the proportion of TUNEL-positive cells was higher in the presence of TnBV1 than in the control at 18 and 36 h post-transfection was tested at the $\alpha=0.05$ level with appropriate contrasts amongst the parameters of the model.

Caspase assay. The effect of TnBV1 expression in Sf21 cells was analysed enzymically by using a caspase-3 activity assay. Sf21 cells were seeded at a density of 9×10^5 cells per 35 mm dish and infected with parental bacmid, Ac-TnBV1 or a mutant AcMNPV lacking a functional p35 anti-apoptotic gene (p35 del) (Clem *et al.*, 1991) at an m.o.i. of 5. After 48 h, cells were washed and processed with an EnzChek Caspase-3 assay kit (Molecular Probes), in the presence or absence of a caspase-3 inhibitor, as described in the manufacturer's protocol. The experiment was carried out five times, using two or three dishes per treatment. Two cell counts from each dish were transformed to their natural logarithm to normalize the residuals and stabilize their variance. The variance of the log counts was analysed through a mixed linear model with random effects for the batches of material submitted to a treatment in any replicate and for each dish, and with fixed effects for recombinant virus, inhibitor level and their interaction (PROC MIXED; SAS Institute Inc., 1999).

Bioassays. LD₅₀ of recombinant budded virus was determined in fourth-instar *H. virescens* larvae infected by haemocoelic injection, as described by O'Reilly *et al.* (1992). Doses of either the bacmid parental or Ac-TnBV1 recombinant virus, ranging from 0.01 to 1000 p.f.u., produced and titrated in Sf21 cells and diluted in TC100 medium (Gibco) supplemented with antibiotics/antimycotics (Sigma), were used for the injection experiments. Control larvae ($n=25$ or 26) were injected with TC100 medium supplemented with antibiotics/antimycotics (1 µl per insect). Mortality was recorded on a daily basis and total mortality was recorded at day 7 post-injection. Experiments were repeated twice. LD₅₀ was estimated from a mixed non-linear model of the probability of death at each dose, which accounted for mortality by injection with uninfected TC100 medium, for the two replicates and for the random effects of the groups of insects submitted to each dose in each replicate (PROC NL MIXED; SAS Institute Inc., 1999).

RESULTS

Selection of an Ac-TnBV1 recombinant virus and its phenotype in baculovirus-infected cells

TnBV1 cDNA was cloned into a transfer-vector plasmid, pSyn-TnBV1, to allow targeting of the gene to the polyhedrin locus of AcMNPV via homologous recombination during infection. In this construct, TnBV1 was placed under a synthetic hybrid viral promoter, with the adjacent polyhedrin gene left under the control of its native promoter. The pSyn-TnBV1 transfer-vector plasmid was co-transfected into Sf21 cells along with the vsynVI⁻-gal or vsynVI⁻-egt(-) parental virus strains (O'Reilly *et al.*, 1992), which lack the polyhedrin gene and therefore cannot produce the occluded form of the virus. Screening for recombinant progeny virus involved looking for viruses following plaque assay of insect cells in culture that could produce the occluded form of the virus. Following selection from several independent experiments, 34 occlusion-positive plaques were passaged to screen for possible recombinant viruses, but only six plaques were purified successfully, whilst the remaining plaques reverted to the parental strain phenotype (data not shown). All of the plaque-purified recombinant viruses were tested by Southern blotting and sequence analysis, but none of them contained the entire TnBV1 cDNA (data not shown). The sustained difficulty in isolating a recombinant that could successfully express TnBV1 led us to suspect that the gene product was toxic to the cells. As the *TnBV1* gene was placed under the control of the very late polyhedrin promoter, expression of a toxic TnBV1 protein would lead to premature cell death and would result in a lack of occlusion-positive plaques.

Given that our attempts to select for an occlusion-positive TnBV1 recombinant AcMNPV virus by using homologous recombination in insect cells were unsuccessful, we first employed the Bac-To-Bac system (Invitrogen) to select the recombinant Ac-TnBV1 viral genome in bacteria. This system allows maintenance and manipulation of an AcMNPV-based genome as a bacterial artificial chromosome (bacmid). TnBV1 cDNA or an initiator-methionine mutant (ATG-) of TnBV1 was first cloned into the pFastBac transfer vector under the control of the polyhedrin promoter. Once selected for in bacterial cells, the Ac-TnBV1 and Ac-TnBV1(ATG-) recombinant viral DNAs were transfected into Sf21 cells and recombinant virus was purified.

The recombinant Ac-TnBV1 and Ac-TnBV1(ATG-) viruses replicated in Sf21 cells and produced titres of progeny virus similar to those of the parental or wild-type AcMNPV viruses (data not shown). A time course of virus-infected Sf21 cells was analysed by SDS-PAGE of whole-cell extracts (Fig. 1a). The predicted 14.8 kDa TnBV1-specific band was not detected in Ac-TnBV1-infected cells (Fig. 1a, lanes 6–9). However, a 10 kDa band was detected in both AcMNPV- and Ac-TnBV1(ATG-)-infected cells at 48 and 72 h p.i. (Fig. 1a, lanes 4, 5, 12 and 13), but not

in Ac-TnBV1-infected cells (Fig. 1a, lanes 8 and 9). The samples were subjected to Western blot analysis and probed with an antibody specific for the baculovirus protein p10, which is expressed as a very late gene in AcMNPV-infected cells. A 10 kDa specific band corresponding in size to the band seen in the Coomassie blue-stained gel was detected in both AcMNPV- and Ac-TnBV1(ATG-)-infected (Fig. 1b, lanes 3, 4, 5, 12 and 13), but not in Ac-TnBV1-infected, Sf21 cell extracts (Fig. 1b, lanes 6–9). The reduction in this very late protein in Ac-TnBV1-infected cells coincided with the time of expected expression of TnBV1 in these cells. We therefore examined whether production of small amounts of TnBV1 hampered progression of baculovirus infection and, consequently, the accumulation of very late viral protein(s).

Cells that were mock- and virus-infected were observed by light microscopy at various times p.i. (Fig. 1c). AcMNPV-infected cells were characteristically swollen with nuclear occlusion bodies visible at 48 h p.i. (Fig. 1c, filled arrows). Bacmid- and Ac-TnBV1(ATG-)-infected cells also appeared swollen at 48 h p.i. but, as the polyhedrin gene was lacking in these viruses, occlusion bodies were not formed. In cells infected with the Ac-TnBV1 recombinant virus, however, the nucleus and cytoplasm appeared to have shrunk by 24 h p.i. and the majority of cells had died by 48 h p.i. (Fig. 1c, open arrows). A similar phenotype was observed in High Five cells (data not shown).

Expression of TnBV1 in baculovirus-infected Sf21 cells leads to apoptosis-like PCD

Loss of cell viability can occur following a toxic insult by triggering general necrotic events or from engaging specific PCD pathways. To assess the nature of the phenotypes observed in Ac-TnBV1-infected cells, we tested bacmid-versus Ac-TnBV1-infected Sf21 cells for evidence of apoptosis-like PCD. DNA fragmentation, due to the endonuclease activation associated with apoptosis, results in an increased number of free 3'-hydroxyl groups at DNA ends, which leads to increased labelling in a TUNEL assay (Gavrieli *et al.*, 1992). Sf21 cells were bacmid- or Ac-TnBV1-infected for 48 h before being labelled by the TUNEL assay. Infected cells were processed by flow cytometry and FITC labelling was measured. On average, 27% of Ac-TnBV1-infected cells fluoresced at a level higher than the 1% gate determined for the corresponding parental bacmid-infected cells ($P=0.0098$, Fig. 2), suggesting that the expression of TnBV1 in AcMNPV-infected cells caused the Sf21 cellular DNA to become specifically fragmented.

To validate further the apoptotic-like PCD effect of TnBV1 expression on Sf21 cells, lysates of Sf21 cells infected with parental bacmid, Ac-TnBV1 or a mutant baculovirus (p35 del) known to trigger apoptosis in Sf21 cells (Clem *et al.*, 1991) were also analysed enzymically for caspase activity by using an EnzChek Caspase-3 assay kit at 48 h p.i., in the presence or absence of an exogenously added caspase inhibitor. Recognition and cleavage of a fluorogenic peptide

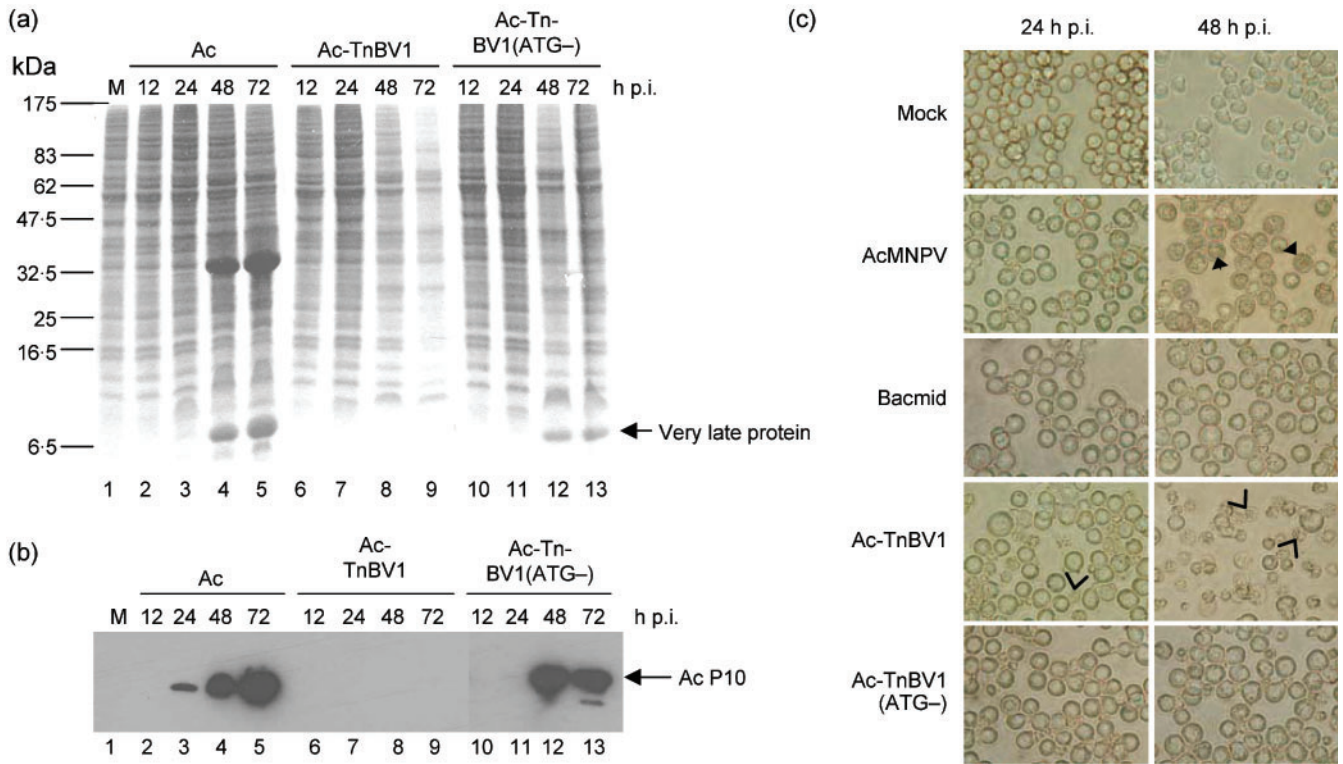


Fig. 1. Expression of TnBV1 alters the phenotype of baculovirus infection of Sf21 cells. (a) Sf21 cells were mock- (M), AcMNPV- (Ac), Ac-TnBV1- or Ac-TnBV1(ATG-)-infected. The cells were harvested for protein-gel analysis from 12 to 72 h p.i. Cell lysates were electrophoresed on 10–20% Tris/Tricine gels and Coomassie blue-stained. (b) Cell lysates from (a) were subjected to Western blot analysis and probed with anti-p10 serum. (c) Sf21 cells were mock-, AcMNPV-, bacmid-, Ac-TnBV1- or Ac-TnBV1(ATG-)-infected before being observed at 24 and 48 h p.i. by light microscopy at a magnification of 400 \times . Filled arrowheads show nuclei with occlusion bodies and open arrowheads indicate cells that have condensed nuclear and cytoplasmic material.

containing the mammalian caspase-3 recognition sequence, DEVD, was measured. Expression of TnBV1 in Ac-TnBV1-infected Sf21 cells resulted in a 23% increase in caspase activity compared with the parental strain bacmid-infected cells ($P=0.0204$, Table 2). Caspase activity from Sf21 cells infected with the p35 del virus, the positive control for activation of apoptosis, was 44% higher than that observed in cells infected with Ac-TnBV1 ($P=0.0003$) or 77% higher than when infected with bacmid alone ($P<0.0001$). The effect of adding caspase inhibitor on the caspase activity of cell lysates differed among the infection types ($P=0.0011$). In the p35 del-infected cell lysates, caspase activity was 70% higher in the absence of the inhibitor than in its presence ($P\leq 0.0001$, Table 2). The effect of the inhibitor was negligible when cells were infected with the bacmid (1% reduction, $P=0.8981$). For cells infected with the Ac-TnBV1 virus, the inhibitor reduced caspase activity significantly (by 26%, $P=0.0124$). Finally, in the presence of inhibitors, none of the virus-infected Sf21 cells showed a significant difference in caspase activity relative to one another.

Transient expression of TnBV1 in High Five cells allows TUNEL staining

As mentioned above, we also noticed toxicity to the High Five cell line following infection with Ac-TnBV1 relative to the parental bacmid virus. To investigate this further, High Five cells were transfected with either the empty pIE1/153A expression vector or pIE1/153A.TnBV1 plasmid, which expresses TnBV1 constitutively in the High Five cell line under the control of a double-enhanced insect-gene promoter (Farrell *et al.*, 1998). To assess transfection efficiency, pIE1/153A.GFP was co-transfected with all samples to allow visual assessment of production of GFP (Chalfie *et al.*, 1994). In any given independent replicate of the experiment for each treatment group, the percentage of High Five cells showing GFP expression was approximately 70% (Fig. 3b). To ensure that TnBV1 was expressed in the transfected cells, RNA was harvested from the transfected cells and TnBV1 transcription was confirmed by RT-PCR. An amplicon of the expected size (298 bp) was obtained (Fig. 3f) in the pIE1/153A.TnBV1 DNA-transfected High Five cells, thereby confirming TnBV1 RNA expression. The

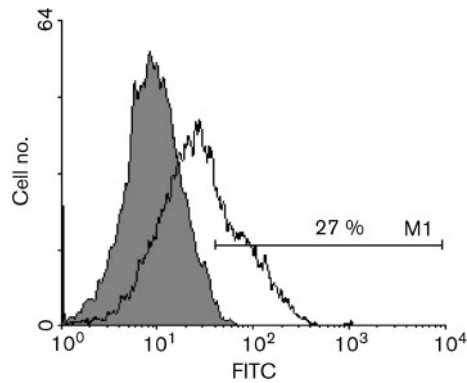


Fig. 2. TUNEL staining and FACS analysis of baculovirus-infected Sf21 cells. Sf21 cells were infected with bacmid or Ac-TnBV1 virus and subjected to a TUNEL assay at 48 h p.i. Samples were analysed by flow cytometry and a representative experiment is shown. The shaded histogram represents the fluorescence intensity of the bacmid-infected Sf21 cell population, whilst the open histogram represents fluorescence intensity of the Ac-TnBV1-infected Sf21 cells. TUNEL-positive cells were gated electronically at 1% of the bacmid-infected control. Cells that fluoresced at an intensity higher than the 1% gate (M1) in the Ac-TnBV1-infected cell population were considered to be TUNEL-positive.

percentage of apoptosis-like PCD in cells, as determined by TUNEL staining and microscopy following transfection with the pIE1/153A.TnBV1 expression construct (Fig. 3d) and the control vector plasmid (Fig. 3c) at different time intervals, is reported in Table 3. Negative-control cells (Fig. 3c) showed a low level of labelling, whereas both DNase-treated positive-control cells (Fig. 3e) and pIE1/153A.TnBV1-transfected cells (Fig. 3d) showed a high level of TUNEL staining. The difference between the controls and TnBV1-transfected cells was statistically significant ($P < 0.0001$) for the data points obtained at 36 h, but not for those obtained at 18 h post-transfection ($P = 0.7826$, Table 3).

Bioassay analysis of TnBV1 expression *in vivo*

We tested whether the expression of TnBV1 increased the virulence of AcMNPV *in vivo* in the natural host of the parasitoid wasp *T. nigriceps* (Viereck). Fourth-instar *H. virescens* larvae were injected into the haemocoel with different doses of budded virus from either the bacmid parental strain or Ac-TnBV1 recombinant, or, in the case of control larvae, with TC100 medium. The LD₅₀ of the Ac-TnBV1 recombinant virus did not differ significantly from that of the parental strain of the virus ($P = 0.3161$, Table 4), indicating that the expression of TnBV1 did not affect the virulence of AcMNPV in *H. virescens*.

DISCUSSION

Cellular organisms have evolved complex, programmed responses to developmental needs and to various stresses, including infection. PCD is a process that is multifaceted and is only beginning to be categorized in all of its complexity. The best-understood cellular-death mechanism is apoptosis. Hallmarks of apoptosis include nuclear condensation and fragmentation, geometric chromatin condensation and cleavage of chromosomal DNA into internucleosomal fragments (Cohen & Duke, 1984). This caspase-dependent phenomenon leads to packaging of dead cells into apoptotic bodies without plasma-membrane breakdown and subsequent removal of the dead cells by phagocytic cells (Kerr *et al.*, 1972).

The role of non-apoptotic or apoptotic-like PCD has, in recent years, begun to be acknowledged in cell-survival and -suicide research (Edinger & Thompson, 2004; Leist & Jäättelä, 2001). In contrast to apoptosis, the mechanisms underlying other types of cell death, such as necrosis, autophagy and paraptosis, are not yet well-categorized. Generally, these processes are caspase-independent, involve extensive cytoplasmic vacuolation and usually lack specific DNA fragmentation (Syntichaki & Tavernarakis, 2002; Edinger & Thompson, 2004; Sperandio *et al.*, 2000; Wyllie & Golstein, 2001).

Table 2. Caspase activity of Sf21 cells infected with recombinant baculoviruses at 48 h p.i.

Caspase-3-like protease activities are expressed as units relative to the activity in mock-infected cells, which was given a value of 1. Sf21 cells infected with the AcMNPV p35 deletion virus (p35 del) were utilized as a positive control. Means and their 95% confidence limits (in parentheses) were computed on the natural logarithmic scale and transformed for presentation.

Virus	Activity with:		Relative reduction (%)	P value*
	No inhibitor	Inhibitor		
Infection with:				
p35 del	15691 (11334, 21722)	9224 (6663, 12770)	70 (43, 103)	<0.0001
Bacmid	8853 (6395, 12256)	8757 (6326, 12123)	1 (-15, 20)	0.8981
Bac-TnBV1	10932 (7897, 15134)	8685 (6274, 12024)	26 (6, 50)	0.0124
Bacmid vs Bac-TnBV1 P value	0.0204	0.9225		

*P value in the absence vs the presence of inhibitor.

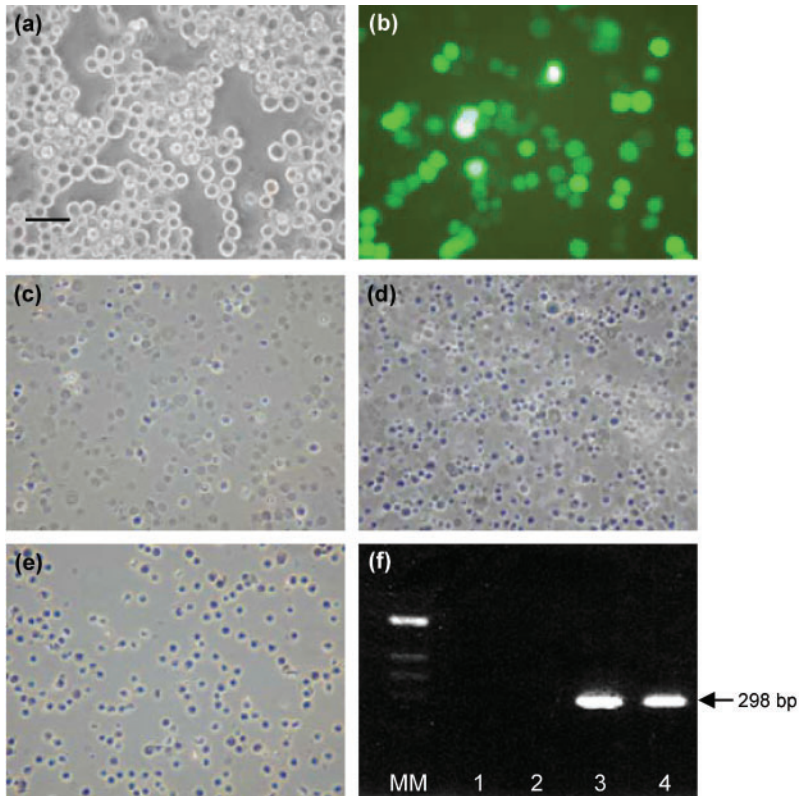


Fig. 3. Transient transfection and TUNEL staining of High Five cells. (a, b) High Five cells transfected with the pIE1/153A transient-expression plasmid carrying GFP (pIE1/153A.GFP) observed under phase-contrast (a) and fluorescence (b) microscopy. Bar, 50 μ m. (c–e) Phase-contrast images of TUNEL-stained High Five cells after transfection with empty pIE1/153A and pIE1/153A.GFP plasmids (4:1) (c), with pIE1/153A.TnBV1 and pIE1/153A.GFP plasmids (4:1) (d) and after DNase treatment (positive control) (e). (f) UV image of ethidium bromide-stained agarose gel loaded with PCR products obtained by using internal primers for TnBV1 sequences with the following templates: 1, water (negative control); 2, total RNA extracted from High Five cells transfected with empty pIE1/153A and pIE1/153A.GFP plasmids (4:1); 3, total RNA from High Five cells transfected with pIE1/153A.TnBV1 and pIE1/153A.GFP plasmids (4:1); 4, TnBV1 cDNA (positive control). MM, Molecular mass marker.

In our initial attempt to overexpress TnBV1, we realized that the expression of this protein in insect cells had a profound effect on their survival. Recombinant AcMNPV virus expressing an intact copy of TnBV1 under the polyhedrin promoter could not be selected for via homologous recombination and screening for occlusion-positive plaques during infection of Sf21 cells. However, when using the Bac-To-Bac system, based on cloning recombinant baculovirus genomes in bacteria, an Ac-TnBV1 recombinant was purified. When cell lysates were screened by SDS-PAGE and Coomassie blue staining, the Ac-TnBV1 recombinant virus did not appear to overexpress a protein of 14.8 kDa, the predicted size of TnBV1 (Fig. 1a). However, marked phenotypic effects were observed in Sf21 cells that were

infected with the Ac-TnBV1 virus when compared with cells infected with either the parental bacmid virus or a recombinant virus, Ac-TnBV1(ATG-), carrying an initiator-methionine mutation (Fig. 1c). Sf21 cells infected with Ac-TnBV1 did not attain the swollen appearance typical of infected cells around 24 h p.i. (Fig. 1c). In fact, Ac-TnBV1-infected Sf21 cells appeared to shrink and had lost viability by 48 h p.i. (Fig. 1c). This truncated infection resulted in a lack of expression of the very late AcMNPV *p10* gene in Ac-TnBV1, but not in Ac-TnBV1(ATG-) infections (Fig. 1b). Thus, the toxic effect of TnBV1 expression within infected Sf21 cells was probably the cause of the inability to obtain an occlusion-positive recombinant virus with an intact copy of TnBV1 by using homologous recombination and selection within insect cells. Occlusion-body formation requires polyhedrin protein production at very late times of infection and, presumably, this was compromised by the premature death of the infected cells. Only viral mutants

Table 3. Percentage (with lower and upper 95% confidence limits) of transfected High Five cells showing TUNEL-positive staining at two times post-transfection

Percentages within the same sampling time were compared by means of relevant contrasts amongst parameters of the generalized linear model.

Time post-transfection (h)	pIE1/153A.TnBV1	pIE1/153A	<i>P</i> value*
18	28 (25, 32)	27 (21, 34)	0.7826
36	51 (48, 55)	27 (23, 32)	<0.0001

**P* value for test of plasmid effect.

Table 4. LD₅₀ of fourth-instar *H. virescens* larvae injected with budded virus from parental bacmid AcMNPV and the recombinant bacmid expressing TnBV1

Virus	LD ₅₀ (p.f.u. ml ⁻¹)	95% confidence limits	
		Lower	Upper
Bacmid	0.31	0.0	0.90
Ac-TnBV1	0.84	0.0	2.43

that had lost the *TnBV1* gene gave rise to occlusion-positive plaques.

Expression of TnBV1 in Ac-TnBV1-infected Sf21 cells led to breaks in the host genomic DNA, providing free 3'-hydroxyl ends that became available for TUNEL staining (Fig. 2) and to an increased caspase-3-like protease activity (Table 2), both indicative of apoptosis. Monitoring of mammalian caspase-3-like activity was chosen following reports that infection of Sf21 cells with the p35 del baculovirus leads to activation of *S. frugiperda* caspase 1 (Ahmad *et al.*, 1997), which is similar in activity to mammalian caspase-3 (Seshagiri & Miller, 1997). Although the phenotype of Ac-TnBV1-infected Sf21 cells did include nuclear and cytoplasmic condensation consistent with apoptosis (Fig. 1c), we did not observe classical 'blebbing' of apoptotic bodies from the plasma membrane, as is seen with p35 del infections (Clem *et al.*, 1991). Transient expression of TnBV1 in Sf21 cells, without baculovirus infection, also resulted in a slight, yet significant, decrease in the number of viable cells calculated by trypan blue exclusion, demonstrating that TnBV1 alone is sufficient to cause this effect (data not shown). Surprisingly, DNA cleavage leading to TUNEL staining was also detected in High Five cells transiently expressing TnBV1 (Fig. 3, Table 3), showing that this phenotype was not unique to Sf21 cells. Tn-368 (Hink, 1970), the *Trichoplusia ni* parental cell line from which the High Five cell line was derived, is the cell line that is typically used to propagate the p35 del virus, as this virus does not cause apoptosis when infecting this cell line (Clem *et al.*, 1991). The Tn-368 cell line has been demonstrated to be resistant to a variety of apoptotic stimuli (Clem & Miller, 1993; Clem *et al.*, 1994) and, to our knowledge, this is the first published report of an apoptotic-like response in a *T. ni*-derived cell line.

Definitive categorization of the PCD pathway engaged by TnBV1 expression in insect cells is difficult. Expression of TnBV1 in insect cell lines caused nuclear and cytoplasmic shrinkage, caspase activation, DNA fragmentation as indicated by TUNEL staining, and membrane permeabilization as defined by trypan blue staining. The absence of blebbing and apoptotic bodies in infected and transfected insect cells, however, prevents the characterization of the TnBV1 effects as classical apoptosis. Apoptosis-like PCD, in various systems, shows degrees and combinations of apoptotic features; chromatin condensation is usually less compact and is also caspase-independent (Leist & Jäättelä, 2001).

Similarly, caspase activation and the absence of cytoplasmic vacuolation prevented the categorization of TnBV1 effects into non-apoptotic PCD pathways, such as necrosis, autophagy and paraptosis. Although TUNEL staining has been described in autophagic *Manduca sexta* fat-body cells (Muller *et al.*, 2004), DNA fragmentation is not typical of non-apoptotic PCD. Caspase-dependent autophagy has been documented in *Drosophila melanogaster* salivary glands, where baculovirus p35 expression inhibited cell death (Kimura *et al.*, 2004), supporting the complexity of

PCD occurring in various systems. Finally, multiple death pathways can be triggered by a single protein, such as the tumour necrosis factor receptor (TNFR1) (Laster *et al.*, 1988; Leist & Jäättelä, 2001). Depending on the cell type, TNFR1 signalling induces classical apoptosis, necrosis or a cytolytic phenotype that resembles apoptosis, but does not include DNA fragmentation. To delineate further the PCD features present in TnBV1-expressing cells, staining and electron microscopy could be carried out in future experiments to examine both chromatin-condensation patterns and the appearance of the organelles.

Despite effects in cell culture, expression of TnBV1 did not affect the virulence of AcMNPV in *H. virescens* fourth-instar larvae following injection of the budded form of the virus into the haemolymph (Table 4). Several factors could be causing this apparent discrepancy between the *in vitro* cell line and *in vivo* bioassay data. Production of budded virus from Ac-TnBV1 infection of Sf21 cells was similar to that caused by wild-type AcMNPV (data not shown). This is probably due to the fact that budded virus is abundantly produced from 12 to 20 h p.i. (Miller, 1997), before the expected onset of TnBV1 expression from the polyhedrin promoter. Any apoptosis-like effects of infected cells following this period would not affect the spread of infection by budded virus in the infected insects. Also, we did not test Ac-TnBV1 in *H. virescens* cell lines to demonstrate apoptosis-like PCD. Therefore, the possibility that TnBV1 expression does not have an effect on cells derived from this caterpillar requires further investigation. However, recent experimental data indicate that granulocytes of *H. virescens* larvae undergo apoptosis, starting 6 h after *T. nigricipes* parasitization (M. Eguileor & F. Pennachio, unpublished data).

Detecting apoptosis of individual cells/tissues in whole infected caterpillars is difficult, but has recently been reported by using TUNEL staining (Clarke & Clem, 2003). It would be interesting to apply this approach to Ac-TnBV1-injected *H. virescens*. Another measure of the effect of TnBV1 expression on the productivity of Ac-TnBV1 versus wild-type AcMNPV infection would be to reintroduce polyhedrin under its own promoter in Ac-TnBV1 and then assess the production of infectious occlusion bodies from each viral infection in *H. virescens* cadavers. If infected cells are undergoing apoptosis-like PCD *in vivo*, thereby truncating infection within the very late phase, fewer occlusion bodies (and/or containing fewer virions, making them less infectious) would be expected to arise from Ac-TnBV1- than from wild-type-injected caterpillars. At present, the role of TnBV1 expression in parasitized *H. virescens* larvae remains elusive and needs to be demonstrated more directly *in vivo*. However, this might be difficult, as the TnBV1 protein may interact with other TnBV proteins during parasitism to exert its function. The timing, level and localization of expression of TnBV1 within the host caterpillar might also need to be replicated faithfully to study its function.

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