Characterization of *Mamestra configurata* nucleopolyhedrovirus enhancin and its functional analysis via expression in an *Autographa californica* M nucleopolyhedrovirus recombinant

Qianjun Li,1 Lulin Li,3 Keith Moore,1 Cam Donly,2 David A. Theilmann3 and Martin Erlandson1

Correspondence
Martin Erlandson
erlandsonm@agr.gc.ca

1Saskatoon Research Centre, AAFC-Saskatoon, 107 Science Place, Saskatoon, Saskatchewan, Canada S7N 0X2
2Southern Crop Protection and Food Research Centre, AAFC, London, Ontario, Canada
3Pacific Agri-Food Research Centre, AAFC, Summerland, BC, Canada

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Enhancin genes have been identified in a number of baculoviruses and enhancin proteins are characterized by their ability to enhance the oral infectivity of heterologous baculoviruses in various lepidopteran insects. Here, we describe the putative enhancin gene from *Mamestra configurata* nucleopolyhedrovirus (MacoNPV), only the second NPV in which an enhancin-like ORF has been identified. The putative enhancin gene from MacoNPV has a typical baculovirus late promoter (ATAAG) 15 bp upstream from the ATG codon. The enhancin ORF encodes an 847 amino acid protein with a predicted molecular mass of 98 kDa and contains a conserved zinc-binding domain (HEIAH) common to metalloproteases. The MacoNPV enhancin shows approximately 20 % amino acid identity with other baculovirus enhancins. An *Autographa californica* M nucleopolyhedrovirus (AcMNPV) recombinant, AcMNPV-enMP2, expressing the MacoNPV enhancin gene under control of its native promoter was developed and characterized. Northern blot analysis showed expression of enhancin from 24 through 72 h post-infection. In 2nd-instar *Trichoplusia ni* larvae, the LD$_{50}$ of the AcMNPV-enMP2 recombinant was 2–8 polyhedral inclusion bodies (PIB) per larva, 4–4 times lower than that of AcMNPV E2 wild-type virus (12–4 PIB per larva). At biologically equivalent doses, i.e. LD$_{50}$, the survival time 50 % (ST$_{50}$) of AcMNPV-enMP2 recombinant and AcMNPV E2 wild-type viruses were not significantly different.

INTRODUCTION

Nucleopolyhedrovirus (NPV) and Granulovirus (GV), two genera in the family *Baculoviridae*, are distinguished by their occlusion body (OB) morphology (Blissard et al., 2000). In NPVs, numerous virions are occluded in an OB referred to as a polyhedra or polyhedral inclusion body (PIB). In contrast, for GVs only a single virion is typically occluded in a smaller (0.25–1 μm) OB referred to as a granule. In both PIBs and granules, virion(s) are embedded in a proteinaceous matrix consisting primarily of polyhedrin in NPVs or granulin in GVs. There are several other proteins found in OBs either as structural proteins, calyx or polyhedron envelope protein, or associated with OBs for example, p10, alkaline protease and viral enhancing factor (VEF) or enhancin (reviewed by Funk et al., 1997).

Enhancin is a significant component of the granules of GV and makes up as much as 5 % of the total protein content of the granule (Greenspan Gallo et al., 1991; Roelvink et al., 1995).

Enhancin was originally described from GV granules that showed an ability to enhance the infectivity of NPVs. It was first described from the GV of the armyworm, *Pseudoletia unipuncta* (PsunGV), as an 126 kDa protein, synergistic factor, that was responsible for an increase in larval susceptibility to PsunNPV in mixed infections (Yamamoto & Tanada, 1978, 1980; Zhu et al., 1989). Since then, synergistic activity has been associated with several GVs and enhancin genes have been identified in several GV granules (PsunGV and *Helicoverpa armigera* GV (HearGV) (Roelvink et al., 1995), *Trichoplusia ni* GV (TnGV) (Hashimoto et al., 1991), *Choristoneura fumiferana* GV (CfGV) (NCBI database, accession #AAG33872) and *Xestia c-nigrum* GV (XecnGV) (Hayakawa et al., 1999). However, to date, enhancin has been described in only one NPV, *Lymantria*...
MacoNPV-A isolate was determined (Erlandson, 1990). Recently the complete genome sequence of the baculovirus, Noctuidae), is an important pest of cruciferous oilseed crops in western Canada. A baculovirus, M. configurata (Maco) NPV, has been isolated from field populations of M. configurata larvae and determined to have potential as a biological control agent for this pest insect (Erlandson, 1994; Wang & Granados, 1997). Enhancin facilitates baculovirus infection by disrupting the PM of the midgut thereby allowing virions greater access to the midgut epithelial cells, resulting in the insect’s increased susceptibility to viral infection (Derkens & Granados, 1988; Lepore et al., 1996). There is also evidence that the enhancement effect of the enhancin protein may be achieved through the interaction of enhancin protein with both the viral envelope and the host-cell plasma membrane, thereby increasing the fusion of virions with midgut cells (Tanada, 1985; Tanada et al., 1975, 1980; Wang et al., 1994).

The bertha armyworm, Mamestra configurata (Lepidoptera: Noctuidae), is an important pest of cruciferous oilseed crops in western Canada. A baculovirus, M. configurata, has been isolated from field populations of M. configurata larvae and determined to have potential as a biological control agent for this pest insect (Erlandson, 1990). Recently the complete genome sequence of the MacoNPV-A isolate was determined [155'060 bp encoding 169 putative open reading frames (ORFs) (Li et al., 2002)]. A putative enhancin gene was identified in the MacoNPV genome, which makes it only the second NPV to have an enhancin gene. The contribution of LdMNPV enhancins to the virulence of LdMNPV has been documented (Bischoff & Slavicek, 1997; Popham et al., 2001) and the synergistic effect of GV enhancins has all been demonstrated with NPVs; however, whether an enhancin from an NPV can enhance the virulence of a heterologous NPV is not clear. Here we report on the identification and characterization of the enhancin gene from MacoNPV and an analysis of its synergistic function by its expression in an Autographa californica MNPV (AcMNPV) recombinant.

**METHODS**

**Insects, cells, virus purification and viral DNA preparation.**

A laboratory culture of M. configurata was maintained on a semi-synthetic diet (Bucher & Bracken, 1976), and larvae reared at 21 °C, 60 % relative humidity and a 20:4 light/dark photoperiod. Similarly, a colony of T. ni was maintained on a high wheat-germ artificial diet (Bell et al., 1981) and the larvae reared at 27 °C, 60 % relative humidity, and a 20:4 light/dark photoperiod.

The Spodoptera frugiperda cell line S9 was maintained at 27 °C in Grace’s complete medium (GibcoBRL) supplemented with 10 % foetal bovine serum. Stocks of AcMNPV E2 and AcMNPV-Ac360βGAL (Dickson & Friesen, 1991) viruses were propagated in S9 cells at the specified m.o.i. Infected cells were harvested at various times post-infection (p.i.) and pelleted by centrifugation (3000 g). The cell pellets were re-suspended in TE buffer (0-01 M Tris/HCl, 0-001 M EDTA, pH 7-5) and PIBs were isolated from cells by adding 1/10 vol. of conditioned Pronase (5 mg ml⁻¹ in 0-01 M Tris, pH 7-5, pre-incubated at 37 °C for 2 h) and 10 % Triton X-100, incubating at room temperature for 1 h, and concentrating by centrifugation (3000 g).

Virion purification and viral DNA extraction essentially followed previously described methods (Erlandson, 1990; Li et al., 1997). Briefly, the PIBs were further purified by isopycnic centrifugation on sucrose gradients and the occlusion derived virus (ODV) was released from PIBs by incubation in alkaline PIB dissolution buffer (0-1 M NaCO₃, 0-17 M NaCl, 0-001 M NaEDTA, pH 10.8) and purified by sucrose density-gradient centrifugation. Virus DNA was extracted from ODV using the method of Smith & Summers (1978).

**Cloning the enhancin gene and AcMNPV-enhancin recombinant virus construction.**

The entire MacoNPV enhancin gene is contained within the MacoNPV-A HindIII-J REN fragment, which was previously cloned in a pBS+ vector (Li et al., 1997). Two PCR primers, 5'-CAGGCTACGTTGAGCAAAAT-3' and 5'-CTGCTAGATTTGTTGCGTGCAGCT-3', were designed to amplify the enhancin gene with its intact native promoter. The pBS-MacoHind I plasmid DNA was used as template with these primers to amplify the enhancin gene using Pfu DNA polymerase (Stratagene). The PCR product was digested with Xbal and PstI and cloned into an AcMNPV transfer vector (pEVocc +) which contains a multiple cloning site upstream of the polyhedrin gene (Fig 1) (Dickson & Friesen, 1991). The resulting plasmid, pEVocc +/enMP, was sequenced using two primers designed to sequence the junctions at both ends of the insert.

Transfections were carried out in S9 cells with 2-5 µg of both the transfer vector DNA (pEVocc +/enMP) and AcMNPV-Ac360βGAL viral DNA using the Lipofectin system (GibcoBRL), following the manufacturer’s protocol. At 4 days post-transfection, the cells and medium (2 ml) were harvested and the infectious media used in a plaque assay with S9 cells. White, polyhedra-positive plaques were picked from a background of parental blue, polyhedra-minus plaques and amplified by infecting S9 cells in 25 mm tissue culture plates. Two rounds of plaque isolations were performed to purify the recombinants. The identity of recombinant viruses was confirmed by modified PCR (Malitschek & Scharlt, 1991) using two primers designed to amplify a 2-9 kbp fragment containing the enhancin gene in recombinant virus, whereas in AcMNPV (E2), only a 300 bp fragment was predicted. The enhancin inserts were further confirmed by DNA sequence analysis.

**DNA sequencing and sequence analysis.**

Nucleotide sequences were determined using an ABI Prism BigDye Terminator Cycle Sequencing Kit. For each sequencing reaction, approximately 200 ng of plasmid DNA was subjected to dideoxynucleotide chain termination sequencing and resolved with an ABI 377 DNA Sequencer (PE Biosystems). The generated sequences were analysed with Wisconsin Genetics Computer Group programs (Devereux et al., 1984) and DNASTAR software. Homology searches were carried out with the updated GenBank/EMBL, SWISSPROT and PIR databases, via the NCBI nucleotide and protein database using the BLAST algorithm (Altschul et al., 1990). All percentage identity estimates were based on the percentage of identical amino acid residues for the predicted protein sequences from two complete genes. Multiple sequence alignments were accomplished with DNASTAR, Clustal X and GeneDoc software with default gap penalty (10) and gap length penalty (10) in PAM 250 matrix. For phylogenetic analysis, sequences were aligned using Clustal X and the phylogeny inference package, PHYLIP (version
3.5), was used to estimate phylogenetic relationships. Bootstrap analysis with 100 replicates was used to estimate the reliability of phylogenetic trees.

**RNA purification and Northern blot analysis.** Total RNA was isolated from NPV-infected Sf9 cells, harvested at specific times p.i., using TRIzol reagent (GibcoBRL) following the manufacturer’s protocol. Total RNA (10 μg) was separated on a 1-2 % agarose gel and transferred to Hybond-N+ membranes (Amersham). The 2-6 kb Xbal–PstI fragment from the pEVocc+/enMP plasmid, which contained the *enhancin* gene, was labelled with [α-³²P]dCTP using a random primer DNA labelling system (GibcoBRL) and used as a probe for Northern hybridization analysis (Church & Gilbert, 1984). Briefly, the membrane was pre-hybridized in hybridization buffer (0.5 M sodium phosphate containing 7 % SDS) at 60 °C for 2 h and then hybridized with the labelled probe in the same buffer overnight at 60 °C. After washing twice in 2×SSC buffer at 60 °C for 10 min, twice in 1× SSC containing 0.5 % SDS at 60 °C for 20 min and once in 0.1× SSC at room temperature for 10 min, the membrane was exposed to XAR film (Kodak).

**Insect bioassays.** Polyhedra were isolated from Sf9 cells infected with either AcMNPV-E2 or AcMNPV-enMP2 recombinant virus, purified and quantified as previously described (Li et al., 1997). A dose-response assay was conducted with 2nd-instar *T. ni* larvae using five dose levels, based on a twofold serial dilution series, for each virus. The larvae were infected by allowing them to feed for 12 h on a canola leaf disc (4 mm diameter) treated with known quantities of polyhedra. Larvae that consumed the entire leaf disc were included in the assay and transferred to fresh artificial diet for the remaining 7 days of the bioassay. Larval mortality was assessed daily for the dose-response assays and every 8 h in time-response bioassays used to determine survival time 50 % (ST₅₀). Each deceased larva was checked microscopically to confirm infection. Day 7 mortality data were analysed using SAS-Probit (SAS version 8) to estimate lethal dose 50 % (LD₅₀) values for each virus. The ST₅₀ estimates were derived using the time-mortality model of Vistat (R. P. Hughes, BTI, Ithaca, NY, USA).

**RESULTS**

**Identification and characterization of the MacoNPV *enhancin* nucleotide sequence**

Computer-assisted analysis of the genome of MacoNPV-A identified MacoNPV ORF89 as an *enhancin* homologue (Li et al., 2002). The MacoNPV ORF89 is 2544 bp in length, is located at 50-4–52-0 map units in the HindIII-J REN fragment, and is transcribed clockwise with respect to the circular genome (Li et al., 2002). The *enhancin* ORF encodes a putative 847 aa protein with a predicted
molecular mass of 98,190 Da. The nucleotide sequence of MacoNPV enhancin along with the predicted amino acid sequence of the protein is shown in Fig. 2.

Potential baculovirus consensus late promoter motifs, ATAAAG or TTAAG, have been found in the upstream sequence of all enhancin genes identified thus far. A late promoter motif, ATAAAG, was identified 15 bp upstream of the start codon of MacoNPV enhancin ORF (nucleotides −15 to −19) (Fig. 2).

**Characterization of the putative enhancin protein sequence**

The MacoNPV ORF89, enhancin, encodes a protein of 847 aa (Fig. 2). This is smaller than the 901 aa enhancins of the start codon of MacoNPV enhancin ORF (nucleotides −15 to −19) (Fig. 2).

**Fig. 2.** Nucleotide sequence of the 3·0-kbp region of the MacoNPV genome containing the putative enhancin gene, its upstream promoter elements and the downstream sequences. The consensus baculovirus late promoter motif is indicated (bold and underlined). The predicted amino acid sequence of the putative protein is shown below the coding strand sequence. The putative zinc-binding metalloprotease domain (HEIAH) is shown in a highlighted box. The positions of primers used to amplify the enhancin gene with its native promoter intact are highlighted with grey shading ( ).
of PsunGV, CfGV and TnGV, the 902 aa enhancin of HearGV, and the 867, 898 and 856 aa proteins encoded by XecnGV ORF 152, 154 and 166, respectively (Hashimoto et al., 1999; Kuzio et al., 1999; Popham et al., 1999). BLAST comparisons demonstrated that, overall, MacoNPV enhancin had 19 % and 20 % aa identity to E1 and E2 of LdMNPV, respectively, and 21 % to 23 % aa identities to enhancins of GV s, including HearGV(23 %), TnGV (22 %), PsunGV (22 %), CfGV (22 %), and XecnGV enhancins encoded by ORF 150 (22 %), 152 (21 %), 154 (23 %) and 166 (22 %).

To identify any similarities to potential biologically significant domains or motifs from existing protein families, the MacoNPV enhancin protein sequence was compared with proteins in the PROSITE database (Release 16.45, 30 August 2001). A zinc-binding signature domain, HEXXH, typical of the neutral zinc metalloprotease superfamily (Jongeneel et al., 1989; Murphy et al., 1991; Jiang & Bond, 1992) and similar to that found in LdMNPV and the GV enhancins was found in MacoNPV enhancin at residues 228–232 (HEIAH) (Fig. 2). This zinc-binding signature site also includes a conserved aspartic acid residue 25 aa downstream of the HEXXH sequence (Fig. 3). With the exception of XecnGV ORF 166, a zinc-binding signature typical of metalloproteases was found in all the baculovirus enhancins identified thus far, including enhancins of MacoNPV, LdMNPV, HearGV, TnGV, PsunGV, CfGV, XecnGV ORF 150, 152 and 154 (Fig. 3).

Phylogenetic analysis of enhancin

In order to explore the phylogenetic relationship among NPV and GV enhancins including the most recent NPV enhancin sequence (MacoNPV ORF 89), the amino acid sequences of all known enhancins were aligned using Clustal X (Fig. 3). An unrooted parsimonious tree was generated in Clustal X using the neighbour-joining method and bootstrap analysis, 100 random samples, was preformed using PHYLIP to assess the variability of the phylogenetic tree (Fig. 4). Based on this analysis, enhancins from TnGV, CfGV, PsunGV, HearGV and XecnGV ORF 154 were grouped in one cluster (Cluster I); enhancins from the NPVs, including MacoNPV enhancin and LdMNPV E1 and E2, were grouped along with XecnGV ORF 150, 152 and 166 in another distinct cluster (Cluster II). These two clusters were supported by a high bootstrap value (100 out of 100), indicating the reliability of this separation (Fig. 4). In Cluster I, enhancins from PsunGV, CfGV and TnGV formed a subgroup, while the enhancins from HearGV and ORF 154 of XecnGV formed a second subgroup and this separation was also supported by a high bootstrap value (100 out of 100). MacoNPV enhancin was assigned to cluster II and was grouped together with XecnGV ORF 150 although this subgroup was supported by a low bootstrap value. Except for the separation of LdMNPV E1 and E2 from the rest of the cluster II, the bootstrap separation values of the other enhancins including MacoNPV enhancin were low. Thus MacoNPV enhancin may be related to these XecnGV enhancins but this relationship will require further analysis if and when other NPV enhancin genes are identified.

Characterization of AcMNPV-enMP2 recombinant virus

The MacoNPV enhancin ORF was amplified by Pfu-PCR together with 110 bp of upstream and 92 bp of downstream sequence and cloned into the pEVoCC + vector. In this construct, the enhancin gene was transcribed in the opposite orientation with respect to the polyhedrin gene (Fig. 1). The introduction of the enhancin gene into AcMNPV was accomplished by in vitro transfection in Sf9 cells along with AcMNPV-Ac360βGAL parental viral DNA. The recombinant virus, AcMNPV-enMP2, was selected on the basis of polyhedra-positive, β-Gal-negative plaque phenotype. The insertion of the enhancin gene in the AcMNPV recombinant was confirmed by PCR.

Northern blot analysis was performed to confirm the expression of enhancin mRNA in AcMNPV-enMP2-infected S9 cells. A time-course experiment was done in S9 cells infected with an m.o.i. of 20 TCID50 units per cell. At each time-point p.i., total RNA was extracted, electrophoresed and processed for Northern blot analysis. The MacoNPV-A enhancin gene was expressed from the recombinant virus AcMNPV-enMP2 from 24 to 72 h p.i. as an approximately 2·6 kbp transcript (Fig. 5). As AcMNPV replication initiates at 6–8 h p.i. in Sf9 cells, the initiation of enhancin at 24 h p.i. confirms that it is a late gene as predicted by the late gene promoter consensus sequence at the 5′ end of the gene (Fig. 2). No MacoNPV enhancin-related transcripts were identified in AcMNPV-E2-infected cells.

Insect bioassays

To determine if MacoNPV enhancin could enhance the infectivity of the heterologous virus AcMNPV, the impact of its expression in the recombinant AcMNPV-enMP2 was tested in dose-response assays with 2nd-instar T. ni larvae. The LD50 estimate for the AcMNPV-enMP2 recombinant virus was 2·8 PIB per larva (Table 1). This was 4·4x lower than the LD50 for the wild-type AcMNPV-E2 virus (12·4 PIB per larva) (Table 1). The 95 % fiducial limits of the LD50 estimates for AcMNPV-enMP2 and E2 viruses did not overlap, indicating the LD50 estimates for the two viruses were significantly different and demonstrating that expression of MacoNPV enhancin in the AcMNPV recombinant increased the infectivity of the virus.

The ST50 estimates with biologically equivalent doses were not significantly different for the wild-type and AcMNPV-enhancin recombinant. For example at an LD90 dose
Fig. 3. Alignment of predicted amino acid sequence of all described baculovirus enhancers. The amino acid sequences were aligned in Clustal X and displayed using GeneDoc software. Black shading (□) indicates 100% conserved amino acid residues, medium grey shading (■) 80% or greater conservation and light grey shading (▲) indicates 60–80% conservation. The zinc-binding domain and the aspartic acid residue 25 bp downstream, which constitute the signature identity of metalloprotease, are marked by brackets denoted ** and *, respectively.
(AcMNPV-E2 – 32 PIB per larva; AcMNPV-enMP2 – 12 PIB per larva) the ST50 estimates were 141 ± 2 h and 145 ± 3 h p.i., respectively, for AcMNPV-E2 and enMP2. However, if compared at similar absolute doses the ST50 estimates were significantly different; for example, at 32 PIB per larva the ST50 estimates were 141 ± 2 h and 130 ± 3 h for the AcMNPV-E2 and enMP2 isolates, respectively.

**DISCUSSION**

The identification and characterization of an enhancin gene in MacoNPV-A is only the second report of an NPV with an enhancin-like gene. The MacoNPV ORF89 is 2544 bp in length and encodes an 847 aa protein with a predicted molecular mass of 98 190 Da which contains a zinc-binding domain typical of metalloproteases. The size of the MacoNPV enhancin is intermediate between the smaller E1 and E2 (783–785 aa) enhancins of LdMNPV and the significantly larger enhancins of the GVs (approximately 900 aa). Overall, MacoNPV enhancin has only about 20 % amino acid identity with either the LdMNPV enhancins or the GV enhancins. As was noted previously by Popham et al. (2001) the region of highest homology between enhancins occurs in the N-terminal regions of the protein and particularly between amino acid positions 200–300, which includes the consensus zinc-binding domain (Fig. 3). The zinc-binding domain of MacoNPV enhancin (HEIAH) indicates that this protein has the structural attributes which define a metalloprotease, similar to the other baculovirus enhancins. The region of lowest homology between the enhancins occurred at the C terminus and differences in this region of the molecule account for most of the observed differences in the size of various enhancins described to date (Popham et al., 2001).

A phylogenetic analysis of all the enhancins described thus far from baculoviruses led to the separation of two well-defined clusters (Cluster I and II) (Fig. 4). The phylogenetic tree presented here differs somewhat from that proposed by Popham et al. (2001), in as far as LdMNPV E1 and E2 were previously grouped with XecnGV ORF152 (E2) in one cluster while the other enhancins from GVs were grouped in a second cluster. In our phylogenetic analysis, which includes MacoNPV

**Table 1.** Dose-mortality responses and LD50 values (OB per larva) on day 7 p.i. in 2nd-instar *T. ni* larvae for wild-type (AcMNPV-E2) and enhancin-expressing recombinant (AcMNPV-enMP2).

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>LD50 (95 % CI)</th>
<th>Slope ± SE</th>
<th>Z2/df</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcMNPV-E2 (wt)</td>
<td>12 ± 4 (8–16)</td>
<td>2 ± 0.3</td>
<td>17/13</td>
</tr>
<tr>
<td>AcMNPV-enMP2</td>
<td>2 ± 8 (1–3)</td>
<td>1 ± 0.3</td>
<td>15/13</td>
</tr>
</tbody>
</table>

**Fig. 4.** Unrooted phylogenetic tree of known baculovirus enhancins. The enhancin amino acid sequences were aligned using Clustal X and a neighbour-joining tree was generated. The final unrooted tree was generated using PHYLIP software and bootstrap analysis values are displayed for each tree branch. Numbers on each branch indicate the frequency of a given tree branch after bootstrap analysis (out of 100 replicates).

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**Fig. 5.** Northern blot analysis of enhancin transcripts from Sf9 cells infected with either AcMNPV E2 or AcMNPV-enMP2 recombinant virus. Cells were infected with AcMNPV E2 or AcMNPV-enMP2 (m.o.i. = 20) and harvested at different time-points p.i. Ten µg of total RNA for each time-point was loaded in a lane. The 2-7 kb DNA fragment containing the MacoNPV enhancin gene was 32P-labelled and used as a hybridization probe. Total RNA from uninfected cells (mock lane) served as control. The migration of RNA size standards (kb) are indicated between the panels of the figure.
enhancin, LdMNPV E1 and E2, as well as XecnGV ORF 150 (E1), 152 (E2) and 166 (E4) are grouped together in one cluster (Cluster II, Fig. 4). Interestingly, enhancins in Cluster II fall in a smaller size range (783–867 aa) than the larger GV enhancins in Cluster I (900 aa). This phylogenetic tree would suggest that the enhancins of LdMNPV, MacoNPV and ORF 150, 152 and 166 of XecnGV may have originated from a common ancestor. In our phylogenetic tree the remainder of the GV enhancins group together in Cluster I and the separation of Clusters I and II is well supported by bootstrap analysis (100 out of 100) (Fig. 4). Within Cluster I two subgroups were identified and well supported by bootstrap analysis, including TnGV, CgGV and PsunGV enhancins in one group, and HearGV and XecnGV ORF154 (E3) in a second group (Fig. 4). This is similar to the phylogenetic tree subgrouping from Popham et al. (2001) and thus may indicate that these GV enhancins share a common ancestor.

Potential baculovirus consensus late promoter motifs, ATAAG or TTAAG, have been found in the upstream sequence of all enhancin genes identified thus far. For example, an ATAAG motif has been identified in the upstream sequence of both LdMNPV enhancins (en1 and en2) (Kuzio et al., 1999) and that of PsunGV enhancin (Roelvink et al., 1995). Interestingly, two and three TTAAG motifs have been identified in the upstream sequences of enhancins from TnGV and HearGV, respectively (Roelvink et al., 1995; Hashimoto et al., 1991). There are also one or two late gene promoters in each of the XecnGV enhancins (Hayakawa et al., 1999). The existence of the late gene promoter motif(s) indicates that enhancin is likely expressed late in infection. This has been demonstrated for LdMNPV, in which an E1 enhancin-specific 3·5 kb transcript was detected in infected Ld652Y cells at 48 and 72 h pi but not earlier (Bischoff & Slavicek, 1997). Similarly, in TnGV-infected T. ni larvae, enhancin transcripts were detected at 6 days p.i. but not at 3 days p.i. (Hashimoto et al., 1991). Our Northern blot analysis showed that in AcMNPV-enMP2 (enhancin expression under control of its native promoter)-infected cells, enhancin gene transcription was detected as a late gene (Fig. 5). Thus it is likely that in its native system MacoNPV enhancin is expressed as a late gene as well.

The biological activity of GV enhancins in terms of their ability to increase the efficacy of NPV infection of insect larvae has been well documented. Two mechanisms have been suggested for GV enhancin activity; namely, enhancement of virus–host midgut cell fusion (Kozuma & Hukuhara, 1994) and degradation of peritrophic matrix proteins by metaprotease activity (Lepore et al., 1996; Wang & Granados, 1997). In LdMNPV, both en1 and en2 genes were shown to contribute to virus potency as deletion of either en1 or en2 resulted in an approximately 2-fold decrease in potency (Popham et al., 2001). Deletion of both the en1 and en2 gene resulted in a 12-fold decrease of virus potency when compared to wild-type LdMNPV virus (Bischoff & Slavicek, 1997; Popham et al., 2001). Our bioassay data with the AcMNPV-enMP2 recombinant expressing MacoNPV-A enhancin under the control of its native promoter shows that this gene can impact NPV infectivity. The AcMNPV-enMP2 recombinant was 4·4-fold more potent than AcMNPV wild-type virus based on LD50 estimates. This is in the same range of infectivity enhancement described for the enhancin gene deletion experiments with LdMNPV (Popham et al., 2001). Analysis of time-to-mortality data showed similar speed of kill for the wild-type, AcMNPV-E2 and the enhancin recombinant, AcMNPV-enMP2, when the two viruses were tested at biologically similar doses (i.e. LD90 dose). This result is consistent with previous observations for LdMNPV enhancins (Popham et al., 2001). These authors showed that the ST50 estimates were not significantly different for LdMNPV recombinants in which one or both of the enhancin genes were deleted compared to wild-type virus when tested at LD50 dose rates. However, in the current study, when time-to-mortality was examined at the same absolute dose (32 PIBs per larva) the ST50 for the enhancin recombinant was significantly shorter than for the wild-type virus, 130±3·1 h versus 141±2·9 h p.i., respectively. This observed increase in speed of kill may be the result of a higher effective dose of virus reaching the midgut cells due to the action of MacoNPV enhancin on the peritrophic matrix of the host gut. Thus our results suggest that enhancin functions to increase the amount of initial infection in the host insect midgut rather than increasing the speed of spread of virus infection in the host insect. It would be of interest to determine whether MacoNPV enhancin, as expressed in the AcMNPV-enMP2 recombinant virus, interacts with the intestinal mucus component of T. ni peritrophic matrix in a fashion similar to that of GV enhancins as demonstrated by Wang & Granados (1997).

We intend to pursue this line of investigation in vivo feeding assays with AcMNPV-enMP2 in T. ni larvae and Western blot analysis of PM proteins isolated at various times post-ingestion.

Enhancin genes have been found in several GVs, including PsunGV and HearGV (Roelvink et al., 1995), TnGV (Hashimoto et al., 1991), CgGV (NCBI database) and XecnGV (Hayakawa et al., 1999). Interestingly, all the above GVs belong to those categorized as ‘slow’ GVs in terms of their pathogenicity (Winstanley & O’Reilly, 1999). Granuloviruses in this group have high LD50 values, with pathogenesis being prolonged and mortality occurring as long as 10–20 days after infection. These slow GVs also typically have large genomes (170 kbp or larger) as, for example, in TnGV, HearGV and XecnGV (Winstanley & O’Reilly, 1999). The LdMNPV (161 046 bp) and MacoNPV (155 060 bp) genomes are the two largest NPV genomes sequenced to date (Kuzio et al., 1999; Li et al., 2002), at about 25–30 kb larger than other sequenced NPVs. Both LdMNPV and MacoNPV are also ‘slow killing’, viruses taking upwards of 10 days to kill the
insect at an LD<sub>50</sub> dose (Erlandson, 1990). In the MacoNPV genome several cluster of genes, including enhancin, were found to be homologues of XecnGV genes, indicating some similarity or common ancestry between these baculoviruses. It is possible that baculoviruses with larger genomes have developed different infection strategies to overcome some host-specific barrier, such as a robust host peritrophic matrix structure, and this may have included the incorporation of gene clusters, including possibly enhancin, containing potential virulence factors. Whether there may be complicating fitness costs associated with the replication of larger genomes is open to speculation.

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