

Both *Lymantria dispar* Nucleopolyhedrovirus *Enhancin* Genes Contribute to Viral Potency

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Enhancins are a group of proteins first identified in granuloviruses (GV) that have the ability to enhance nuclear polyhedrosis virus potency. We had previously identified an *enhancin* gene (E1) in the *Lymantria dispar* multinucleocapsid nucleopolyhedrovirus (LdMNPV) (D. S. Bischoff and J. M. Slavicek, J. Virol. 71:8133–8140, 1997). Inactivation of the E1 gene product within the viral genome lowered viral potency by an average of 2.9-fold. A second *enhancin* gene (E2) was identified when the entire genome of LdMNPV was sequenced (Kuzio et al., Virology 253:17–34, 1999). The E2 protein exhibits approximately 30% amino acid identity to the LdMNPV E1 protein as well as the enhancins from *Trichoplusia ni* GV, *Pseudaletia unipuncta* GV, *Helicoverpa armigera* GV, and *Xestia c-nigrum* GV. Northern analysis of viral RNA indicated that the E2 gene transcripts are expressed at late times postinfection from a consensus baculovirus late promoter. The effect of the enhancin proteins on viral potency was investigated through bioassay using two recombinant viruses, one with a deletion in the E2 gene (E2del) and a second with deletion mutations in both *enhancin* genes (E1delE2del). The *enhancin* gene viral constructs were verified by Southern analysis and shown not to produce *enhancin* gene transcripts by Northern analysis. The E2del virus exhibited an average decrease in viral potency of 1.8-fold compared to wild-type virus. In the same bioassays, the recombinant virus E1cat, which does not produce an E1 gene transcript, exhibited an average decrease in viral potency of 2.3-fold compared to control virus. The E1delE2del virus exhibited an average decrease in viral potency of 12-fold compared to wild-type virus. Collectively, these results suggest that both LdMNPV *enhancin* genes contribute to viral potency, that each enhancin protein can partially compensate for the lack of the other protein, and that both enhancin genes are necessary for wild-type viral potency.

Baculoviruses are viruses that are specific to insects and other invertebrates and include two genera, the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs). Both groups of viruses produce virions that are similar in both structure and pathology of infection, though NPVs have members that can package virions singularly or in groups and GVVs can package virions only singularly (8, 10). Another distinction between these viruses is that NPVs produce occlusions that contain many viral particles and GV occlusions contain a single viral particle. Sequence analysis of NPV and GV genomes revealed that the NPVs and GVVs contain many homologous genes (15, 16, 24).

Enhancins are proteins first found in GV occlusion bodies that have the ability to enhance the infection of some NPVs. Also referred to as virus-enhancing or synergistic factors, enhancins were first identified and isolated by Tanada (34, 37; see reference 35 for a review). The enhancin protein of *Pseudaletia unipuncta* GV (PuGV) was found to enhance the infection of *P. unipuncta* (PuNPV) only when both the PuGV protein and PuNPV were inoculated orally, indicating that the midgut is the site of the enhancin activity (38). The enhancin protein purified from the *Trichoplusia ni* GV (TnGV) enhanced *Autographa californica* multinucleocapsid NPV (AcMNPV) infec-

tion in *Helicoverpa zea*, *Spodoptera exigua*, *P. unipuncta*, and *T. ni* by 2- to 14-fold, depending on the host species (41). At present, *enhancin* genes have been sequenced in four GVVs, *Helicoverpa armigera* GV (HaGV) (30), PuGV (30), TnGV (14), and *Xestia c-nigrum* GV (XcGV) (16). The first GV genome to be completely sequenced, XcGV, was found to have four different *enhancin* genes (16). In contrast, the *Plutella xylostella* GV genome lacks an *enhancin* gene (15).

Two functions have been proposed for enhancins, enhancement of virus-host cell fusion and disruption of the peritrophic membrane. The PuGV enhancin was thought to facilitate the uptake of virus particles by increasing the number of virus-membrane fusion events (23, 35, 36, 39). A specific binding site of the TnGV enhancin has been found in the midgut brush border membrane of *P. unipuncta* but not in the brush border membranes of *T. ni*, *H. zea*, or *S. exigua*, all of which demonstrate a response to the TnGV enhancin when infected with AcMNPV (41). The TnGV enhancin was found to damage the peritrophic membrane lining the larval midgut, exposing the gut wall to viral infection (7). Bioassay results of *T. ni* larvae infected with AcMNPV and various concentrations of the TnGV enhancin demonstrated that the major effect of enhancin appears to be an increase in infection efficiency caused by the disruption of the insect peritrophic membrane (11). This enhancin was later found to be a metalloproteinase, which degrades mucin, a major protein constituent of the peritrophic membrane (25, 40).

The *Lymantria dispar* MNPV (LdMNPV) is a baculovirus pathogenic to *L. dispar*, the gypsy moth, a forest and urban

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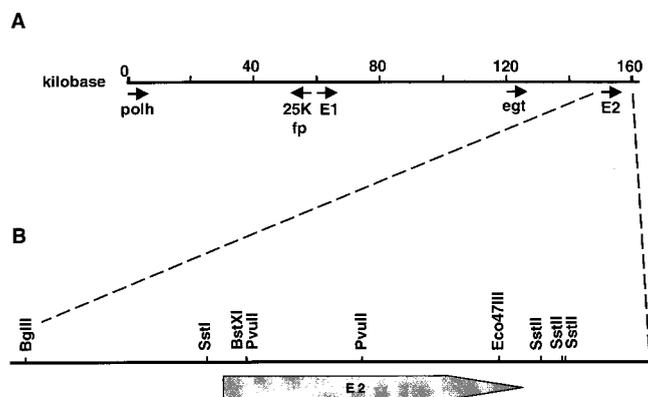


FIG. 1. Genomic location of the two LdMNPV *enhancin* genes. *E1* is located at bp 60268 to 62619, and *E2* is located from bp 155812 to 158178 based on the sequence generated by Kuzio et al. (24). Several other LdMNPV genes are also indicated (A). The enlarged map of plasmid pDB184 indicates the Ca. 6 kb surrounding and encoding the *E2* gene (kbp 155.8 to 158.2). The location of the *E2* ORF and restriction sites used in subcloning of the gene are shown (B).

tree-defoliating pest in the northeastern and some midwestern states. The genome of LdMNPV is significantly larger than that of most other baculoviruses. The genome of the sequenced strain of LdMNPV is 161,046 bases (24), in contrast to 133,894 bases for AcMNPV (2), 128,413 for *Bombyx mori* NPV (12), 131,403 bases for *H. armigera* single nucleocapsid NPV (6), 131,990 bases for *Orgyia pseudotsugata* MNPV (1), and 135,611 bases for *S. exigua* MNPV (19). LdMNPV contains several unique open reading frames (ORFs) as well as ORFs with homology to GV ORFs. The first GV homolog found in LdMNPV was the *enhancin 1* (*E1*) gene, which was also the first *enhancin* gene found in NPVs (5). The LdMNPV *E1* gene product exhibits 29% amino acid identity to the sequenced *enhancin* genes of TnGV, PuGV, and HaGV and contains a conserved zinc-binding domain characteristic of metalloproteases. *E1* gene transcripts are expressed at late times postinfection from a consensus baculovirus late promoter.

A viral isolate lacking a functional *E1* gene was constructed to investigate *enhancin* function. Potency analysis revealed that viral strain without the *enhancin 1* gene was approximately 2- to 3-fold less potent than wild-type viruses, suggesting that the LdMNPV *enhancin* affects viral potency (5). A second *enhancin* gene (*E2*) was recently identified in LdMNPV when the entire genome of isolate 5-6 was sequenced (24). Five other NPVs have been completely sequenced, but no *enhancins* have been found (AcMNPV [2], *B. mori* NPV [12], *H. armigera* NPV [6], *O. pseudotsugata* MNPV [1], and *S. exigua* MNPV [19]). In this study, we characterized the *E2* gene and determined the effect of the *enhancin* genes both individually and collectively on viral potency.

MATERIALS AND METHODS

Cells, virus, and insects. *L. dispar* 652Y cells were grown as monolayers at 27°C in Goodwin's IPL-52B medium (JRH Scientific) supplemented with 6.25 mM glutamine (Gibco-BRL) and 10% fetal bovine serum (Atlanta Biologicals). The LdMNPV isolate A21-MPV (31) served as the parental line for all subsequent viruses. A recombinant virus (E1cat), generated in a previous study (5), that contains the chloramphenicol acetyltransferase (*cat*) gene was also used in this study. *L. dispar* egg masses were obtained from the U.S. Department of

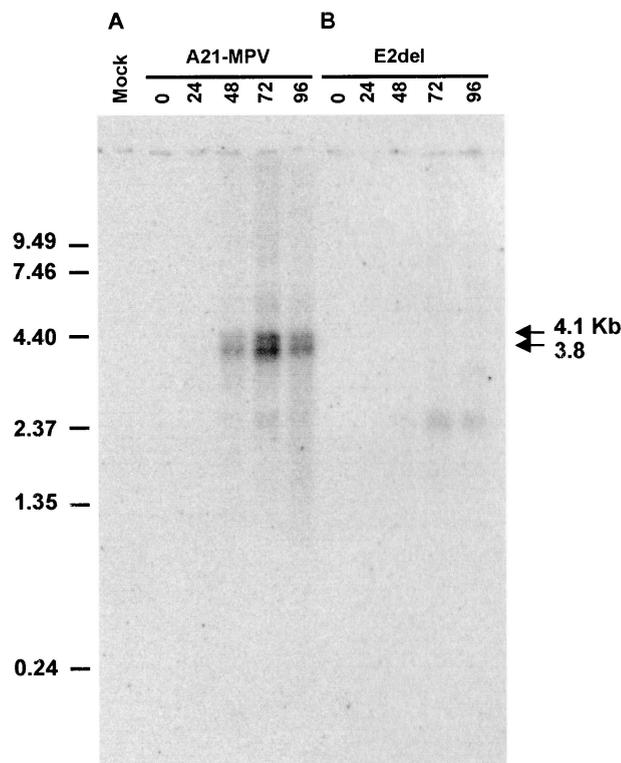


FIG. 2. Temporal analysis of the *enhancin 2* gene transcripts. Cells were infected with A21-MPV or E2del, and cytoplasmic RNA was isolated at the indicated hours postinfection. Poly(A) RNA was isolated from the cytoplasmic RNA, and 1 μ g was separated by formaldehyde-agarose gel electrophoresis, blotted, and probed with a strand-specific oligonucleotide complementary to positions 156708 to 156737 in the *E2* ORF. RNA from uninfected cells was used as a mock infection sample. RNA size standards (in kilobases) are indicated on the left.

Agriculture's Animal and Plant Health Inspection Service rearing facility at Otis Air Force Base (Otis, Mass.). Larvae were reared on gypsy moth diet (ICN) (3).

Northern blot analysis and primer extension mapping of transcripts. Infected Ld652Y cells were harvested at various times postinfection (P.I.). Cytoplasmic RNA was isolated as described by Friesen and Miller (9). Poly(A) RNA was isolated from the cytoplasmic RNA by the PolyATtract mRNA isolation system (Promega), separated on a 1.2% agarose-formaldehyde gel, and then transferred to NitroPlus nitrocellulose transfer membrane (MSI). Northern blots were performed as described by Mahmoudi and Lin (26). Thirty-base oligonucleotides (complementary to bp 61158 to 61187 for the *E1* gene or bp 156708 to 156737 for the *E2* gene [24]) were end labeled with [γ - 32 P]ATP (NEN, Boston, Mass.) and used as strand-specific probes to detect the transcript of interest. Blots were imaged with the Storm 860 phosphorimager (Molecular Dynamics).

Primer extension reactions were performed using the primer extension system with avian myeloblastosis virus reverse transcriptase (Promega). Cytoplasmic RNA was isolated at 72 h postinfection from cells infected with A21-MPV or E2del. A 25-base oligonucleotide (complementary to positions 155841 to 155865 [24]) was used in the reactions after being end labeled with [γ - 32 P]ATP (NEN). Primer extension products were fractionated on 6% polyacrylamide-8 M urea gels, dried, and visualized by phosphorimager.

In vitro transcription and translation of *E2* gene. Plasmid pDB184 (see below) was digested with *Sst*I, and a 2.8-kbp fragment containing the *E2* gene was subcloned into the *Sst*I site of pBluescript to generate pE2SstI. The *E2* protein was expressed from pE2SstI by the TNT quick coupled transcription-translation system (Promega). The expressed protein was labeled with 35 S-EasyTag express protein labeling mix (NEN). Reaction products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Computer programs used for sequence comparison. The amino acids of known *enhancins* available in GenBank were aligned using the Clustal W pro-

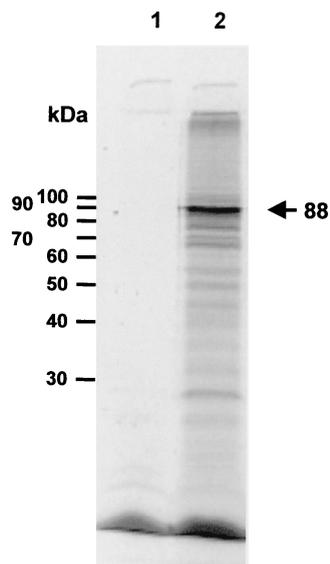


FIG. 3. Analysis of expression of the *enhancin 2* gene. The *enhancin 2* gene was expressed in a rabbit reticulocyte transcription-translation system, and the proteins were separated by SDS-PAGE. Lane 1, Bluescript SK+; lane 2, pE2SstI, expressing E2. Positions of molecular mass standards are indicated to the left, and the position of the *enhancin* protein is indicated to the right.

gram (<http://www2.ebi.ac.uk/clustalw/>). Highlighted comparisons of proteins were produced using the Boxshade program (available at http://www.ch.embl.net.org/software/BOX_form.html). An unrooted phylogenetic tree of known *enhancin* proteins was constructed using the PAUP 3.1 program (33). Bootstrap analysis with 100 replicates was performed to assess the integrity of the phylogeny generated.

Construction and verification of recombinant viruses. An *E2* gene transplacement vector was constructed to generate an A21-MPV recombinant strain lacking the *E2* gene. A vector containing the *E2* gene was constructed by restricting A21-MPV cosmid 15 (4) with *Bgl*II and then religating the fragments. Because there is a single *Bgl*II restriction site within the SuperCos 1 vector (Stratagene), a plasmid was generated, pDB184, containing ca. 6.0 kbp of A21-MPV viral DNA (ca. bp 154.2 to 160, Fig. 1B). A second plasmid with a *lacZ* gene insertion in the *E2* gene was made as follows. Plasmid pDB184 was restricted with *Bst*XI. A 3.8-kbp *Xba*I-*Bam*HI fragment containing the HSP70*lacZ* gene marker was isolated from plasmid p210.1 (provided by American Cyanamid, Princeton, N.J.). The ends of both of the fragments were end filled with T4 DNA polymerase, and the fragments were ligated together to form pDB186. To create a plasmid with a deletion in the *E2* gene, plasmid pDB184 was then restricted with *Pvu*II and *Bgl*II and with *Eco*R47III and *Eco*RI. The 1.7-kbp *Pvu*II-*Bgl*II and 2.2-kbp *Eco*R47III-*Eco*RI fragments were isolated. These fragments were subcloned into the pBluescript vector (Stratagene) *Bam*HI and *Eco*RI sites. The resulting plasmid, pDB185, has a 3.9-kbp insert missing 2.1 kbp (701 amino acids) within the *E2* gene.

A recombinant virus, E2del, with a deletion in the *E2* gene was created in a series of steps as follows. Viral DNA from A21-MPV (5) was cotransfected with plasmid pDB186 to make a virus with a *lacZ* gene insertion in the *E2* gene, vE2*lacZ*. Recombinant viruses were selected based on the presence of blue, occlusion-positive plaque phenotypes. E2*lacZ* viral DNA was then cotransfected with plasmid pDB185. Resulting recombinant plaques had a white, occlusion-positive phenotype. Viral DNA was isolated by the method of O'Reilly et al. (28).

Using the E2del virus as a starting point, a virus, E1delE2del, was generated that lacked functional *E1* and *E2* genes. pDB126 (5) was cut with *Pst*I, and a resulting 1.5-kbp band was isolated containing the C terminus of the *E1* gene (ca. kb 59584 to 62609). This fragment was subcloned into the pBluescript vector *Pst*I site to generate plasmid pDB140. pDB140 was restricted with *Nar*I and end filled with T4 DNA polymerase. A 3.8-kbp *Xba*I-*Bam*HI fragment containing the *lacZ* gene was restricted from plasmid p210.1, the ends were end filled, and the fragment was ligated to the pDB140 fragment to form pE1*lacZ*. To create a plasmid with a deletion in the *E1* gene, plasmid pDB126 (5) was restricted with

*Nar*I and *Bam*HI and separately with *Hinc*II. The 5.3-kbp *Nar*I-*Bam*HI and 0.7-kbp *Hinc*II fragments were isolated, and the 5.3-kbp fragment was end filled. The fragments were then ligated together and the resulting plasmid, pE1del, had a 3.0-kbp insert missing 1.5 kbp (488 amino acids) and a frameshift within the *E1* gene.

Virus E1*lacZ*E2del was generated by cotransfection of E2del viral DNA and pE1*lacZ* plasmid DNA, and blue, occlusion-positive plaques were selected and plaque purified. DNA from the E1*lacZ*E2del recombinant virus was then cotransfected with pE1del plasmid DNA to generate the E1delE2del virus. Two different recombinant viruses of E1delE2del (AE2 and BF6) generated from different transfections and one isolate of E1*lacZ*E2del (5AA) were used for further characterization.

A recombinant virus in which the *E1* gene was replaced in an *enhancin* gene double deletion virus was constructed by cotransfection of E1*lacZ*E2del viral DNA and plasmid pDB126 DNA. White occlusion-positive plaques were selected and plaque purified.

All viruses were verified by restriction enzyme analysis, and those with insertions or deletions in either *enhancin* gene were further verified by Southern blot analysis. Viral DNA restriction enzyme digests were electrophoresed on a 0.7% agarose-Tris-borate-EDTA gel, blotted on Nytran membrane (MSI), and probed with DNA fragments labeled with the nick translation kit (Bethesda Research Laboratories) and [α - 32 P]dCTP (NEN). For viruses with a deletion or insertion in the *E1* gene, the blots were probed using a 735-bp *Bst*XI fragment (bp 60981 to 61761 [24]) that was restricted and then gene cleaned (Q-Bio) from pDB126 (5). Blots containing DNA from recombinant *E2* gene deletion viruses were probed with the 2.1-kbp *Bst*XI-*Eco*R47III fragment deleted from these viruses.

Bioassay analysis of viruses. Fourth-instar *L. dispar* larvae were infected by injection of 5 μ l of budded virus for each test virus (titer of about 5×10^6 tissue culture-infective doses per ml). Larvae were placed on fresh food and allowed to feed until death. Polyhedra were then isolated from insects. The concentration of occluded virus required to kill 50% of the test larvae (LC_{50}) and the mean time to kill 50% of the test larvae (LT_{50}) for all viruses were determined by the droplet feeding method developed by Hughes et al. (18) as described by Slavicek et al. (32) on first-day, second-instar *L. dispar* larvae. Larvae were maintained at $27 \pm 1^\circ\text{C}$ at a photoperiod of 14 h light-10 h dark during the bioassay. LC_{50} values were calculated using Polo-PC (29), and LT_{50} values were determined by the ViStat 2.1 program (17). Statistical comparisons of bioassay data were performed using the StatView program from Abacus Concepts.

RESULTS AND DISCUSSION

Characterization of *E2* gene. The two *enhancin* genes in LdMNPV are located at kbp 60.3 to 62.6 (*E1*) and kbp 155.8 to 158.2 (*E2*) in the genome (Fig. 1A) (24). Analysis of the sequence upstream of the *E2* gene revealed a potential baculovirus late promoter sequence, TTAAG, beginning 28 bp upstream of the *enhancin* gene start codon. Transcription of the *E1* gene in LdMNPV (5) and of the HaGV *enhancin* (30) begins within consensus baculovirus late promoters (TTAAG and TTAAG, respectively, at the underlined nucleotides). All of the other *enhancin* genes identified to date in TnGV, PuGV, and XcGV also contain baculovirus late promoters. To determine the transcriptional start site of the *E2* gene transcript, primer extension reactions were performed with a 25-base oligonucleotide. Transcription initiated at the first T residue within the consensus baculovirus late promoter sequence TTAAG (data not shown). No polyadenylation signal was identified immediately downstream of the *E2* gene. Two consensus polyadenylation sites (AATAAA) are located 280 and 294 bp downstream of the stop codon in the *E2* gene. These sites lie between the end of homologous repeat 8 and the *bro-p* gene. Recent studies on the mechanism of 3'-end formation of baculovirus late transcripts, using an in vitro assay, indicate that 3' ends are formed by termination after transcription of a T-rich region, as opposed to a cleavage-polyadenylation mech-

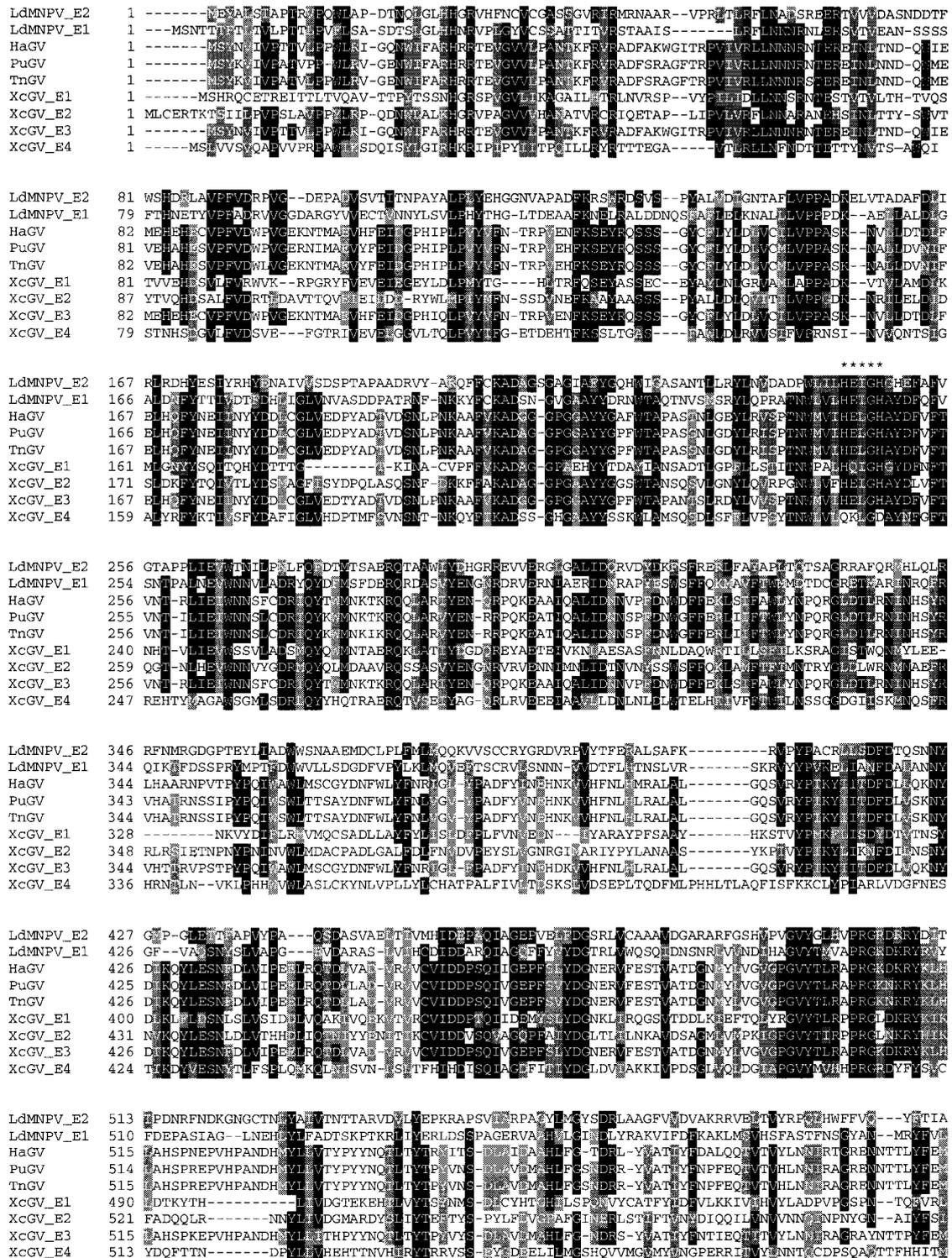


FIG. 4. Aligned amino acid sequences of known baculovirus enhancer genes. Black and gray areas indicate positions with 70% or greater sequence identity or similarity, respectively, among all enhancers. The enhancer sequences aligned include those of LdMNPV, HaGV, PuGV, TnGV, and the four from XcGV. Asterisks indicate the area containing the HEXXH conserved zinc-binding domain of metalloproteases.

anism using a consensus polyadenylation site (21). An earlier study of polyadenylation sites of late transcripts indicated that the sites occurred after a T-rich region a variable distance downstream of consensus polyadenylation sites (42), which

may suggest that the consensus site has a role in the formation of late-transcript 3' ends.

Identification and temporal analysis of E2 gene transcripts. The temporal expression of the E2 gene transcript was inves-

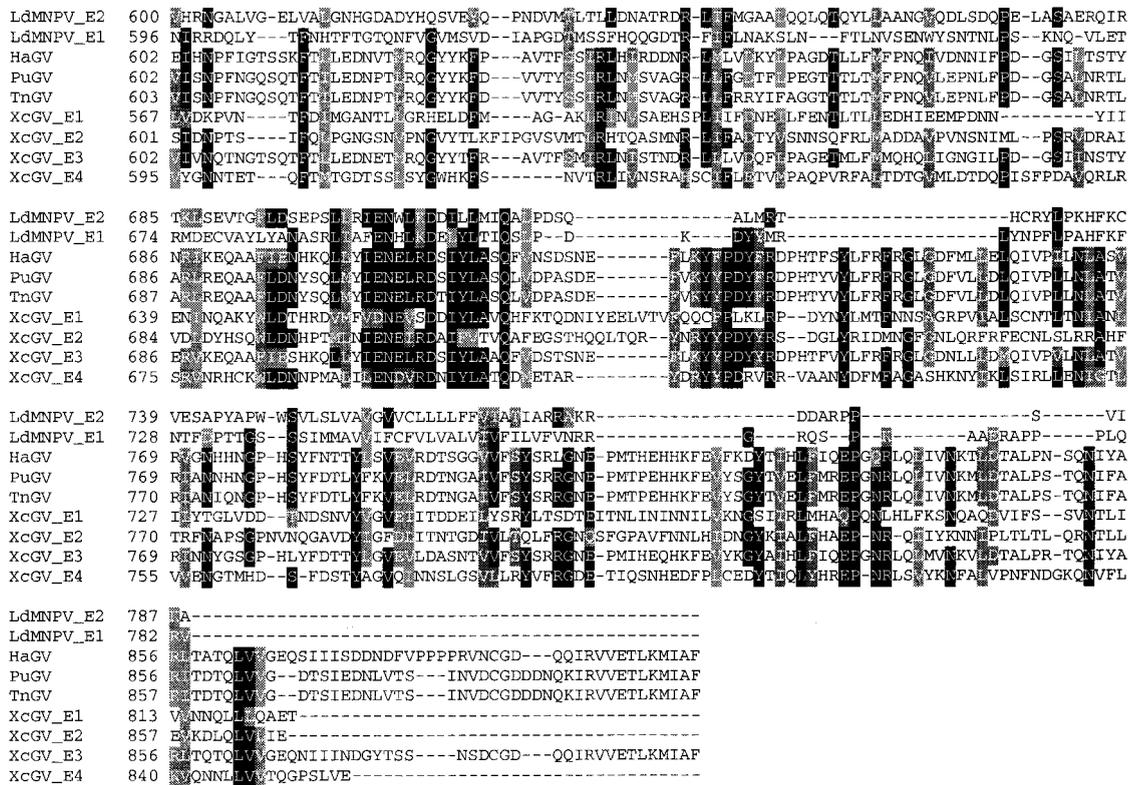


FIG. 4—Continued.

tigated in Ld652Y cells infected with isolate A21-MPV. A primary transcript of approximately 3.8 kb and a secondary transcript of approximately 4.1 kb were found using a strand-specific oligonucleotide probe at late times postinfection 48, 72, and 96 h p.i. (Fig. 2A). No transcripts were evident at the end of the 1-h infection period or at 24 h p.i. The 4.1-kb transcript is more clearly seen in Fig. 8. The lengths of *E2* gene transcripts are longer than expected if the most proximal polyadenylation sites are used. With these sites, transcripts of approximately 2.9 kb would be generated. Further downstream, past the *bro-p* gene, are two consensus polyadenylation sites, and downstream from these sites are several T-rich areas. An *E2* gene transcript terminating near these sites would be approximately 3.9 kb in length, which is the approximate transcript length observed.

The *E1* gene also generated a transcript significantly longer than expected based on the size of the gene (5). The *E1* gene ORF is 2.3 kbp in length, whereas a 3.5-kb transcript is generated. It was speculated that the *E1* gene transcript proceeds through the *hrf-1* gene, which is immediately downstream, and terminates downstream of polyadenylation sites close to the *hrf-1* gene (5). Polyadenylation signal sequences are also absent between the *enhancin* gene and the immediate downstream gene in TnGV, PuGV, and HaGV (14, 30). In addition, analysis of HaGV *enhancin* RNAs indicated that the HaGV *enhancin* gene and the downstream *ORF1* gene may be part of the same bicistronic message (30), and therefore similar to LdMNPV *E1* and *E2* gene transcription. A faint signal for a transcript of approximately 2.4 kb was also found at 72 and

96 h p.i. (Fig. 2A). However, this transcript was also found in cells infected with the E2del virus (Fig. 2B) and not in the mock-infected cells, which suggests that the probe is detecting a viral transcript originating from a different gene.

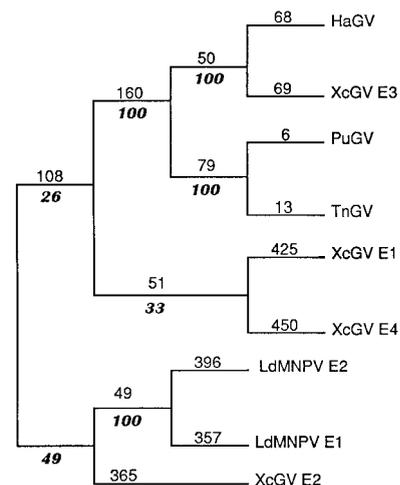


FIG. 5. Phylogenetic tree of known baculovirus enhancins. The most parsimonious tree for known baculovirus enhancins was constructed with the heuristic search algorithm of PAUP. Numbers above the lines in roman type indicate phylogenetic distance, and italic numbers below the lines indicate the frequency of a given cluster after bootstrap analysis (100 replicates).

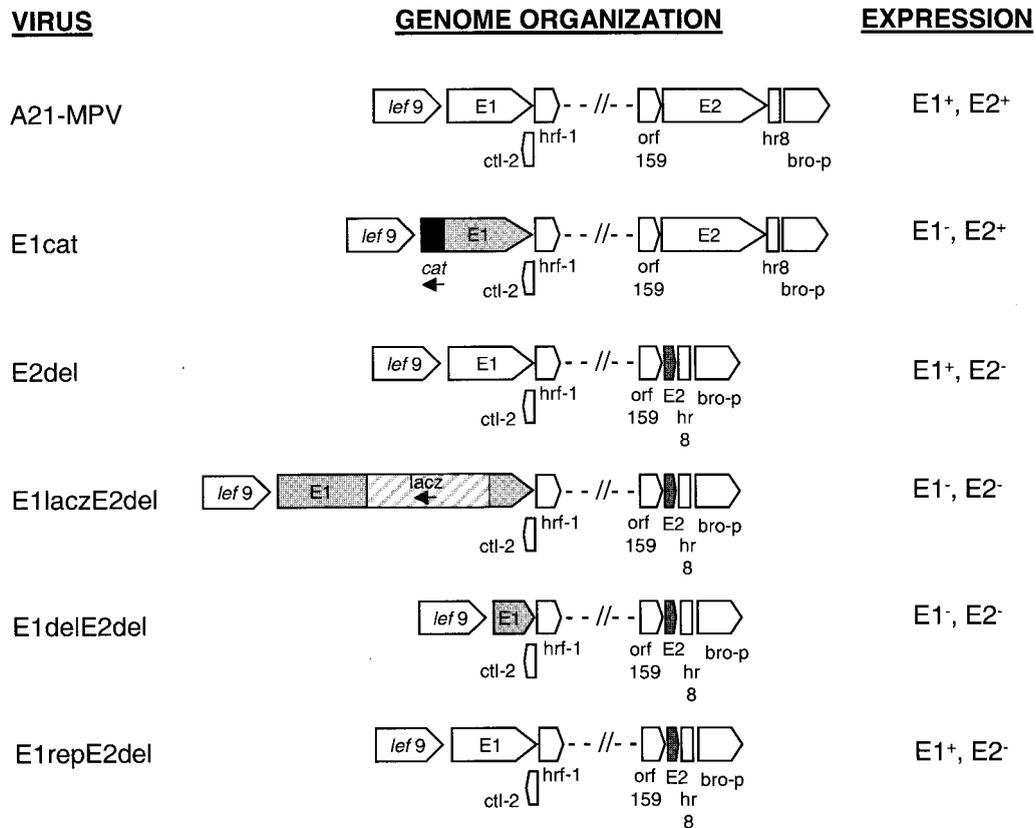


FIG. 6. Schematic representation of A21-MPV and enhancin recombinant viruses. Arrows show the location and direction of ORFs in the regions of the *E1* and *E2* genes (24). The shaded ORFs are those where the enhancin gene has been either deleted or disrupted. The *cat* gene is indicated by a black box, and the *lacZ* gene is indicated by a striped box. Expression of the enhancin gene products is indicated for each virus on the right.

Characteristics of E2 protein and comparison to other enhancins. The *E2* gene could encode a 788-amino-acid protein with a predicted molecular mass of 88,408 Da. A rabbit reticulocyte-coupled transcription-translation system was used to express *E2* to determine if the gene did encode an expressed protein of the predicted size. Plasmid E2SstI, which contained the *E2* gene under the control of the T7 promoter, was used to express the *E2* gene, and Bluescript Sk+ was used as a negative control. A number of bands were produced by pE2SstI, though a major band was seen at 88 kDa, which corresponds to the expected size of the E2 protein (Fig. 3, lane 2). The E2 protein is similar in size to the LdMNPV E1 protein, which contains 783 amino acids. Both LdMNPV enhancin proteins are considerably smaller than the known GV enhancins, most of which range in size from 856 to 902 amino acids. The E1 protein in XcGV is of intermediate size, with a length of 824 amino acids.

Alignment of the known GV and LdMNPV enhancins revealed that the proteins exhibit the greatest homologies in the area bracketed by amino acid positions 200 and 300 (Fig. 4). The region from approximately amino acid positions 440 to 510 is another area of high homology among the enhancins. The LdMNPV E2 enhancin exhibits amino acid identities of about 28, 28, 26, 24, 27, 26, and 26% with the XcGV 1 to 4, HaGV, PuGV, and TnGV enhancins, respectively. There is an overall amino acid identity of about 6% among all the en-

hancin genes compared in Fig. 4. The most heterogeneous region of the enhancins is at the C-terminal end. The LdMNPV E2 protein exhibits an amino acid identity of only approximately 7% with the GV enhancins in the region from amino acid positions 704 to 788. The basis for the difference in the length of the LdMNPV enhancins compared to the GV proteins is within the C-terminal end. The LdMNPV enhancins lack two blocks of amino acids present in the GV proteins within the amino acid regions from approximately 724 to 758 and 807 to 902, relative to the HaGV enhancin. The LdMNPV E1 and E2 enhancins exhibit an amino acid identity of about 30%. The proteins are most conserved within the region from amino acids 1 to 509 (36% amino acid identity), and least conserved in the region from 510 to 788 (21%). The three regions from 203 to 269 (48.5%), 444 to 509 (41.5%), and 700 to 778 (41%) of the LdMNPV enhancins exhibit higher levels of amino acid identity than the other areas of the proteins.

LdMNPV enhancin 2 contains a signature pattern characteristic of a zinc-binding domain found within metalloproteases (22, 27). All of the enhancins sequenced to date except enhancin 4 of XcGV (16) have the characteristic HEXXH zinc-binding site of metalloproteinases. In metalloproteases, the zinc ion is chelated by the two histidine residues in the HEXXH site and by a third residue, typically a histidine, cysteine, or aspartic or glutamic acid residue, located anywhere

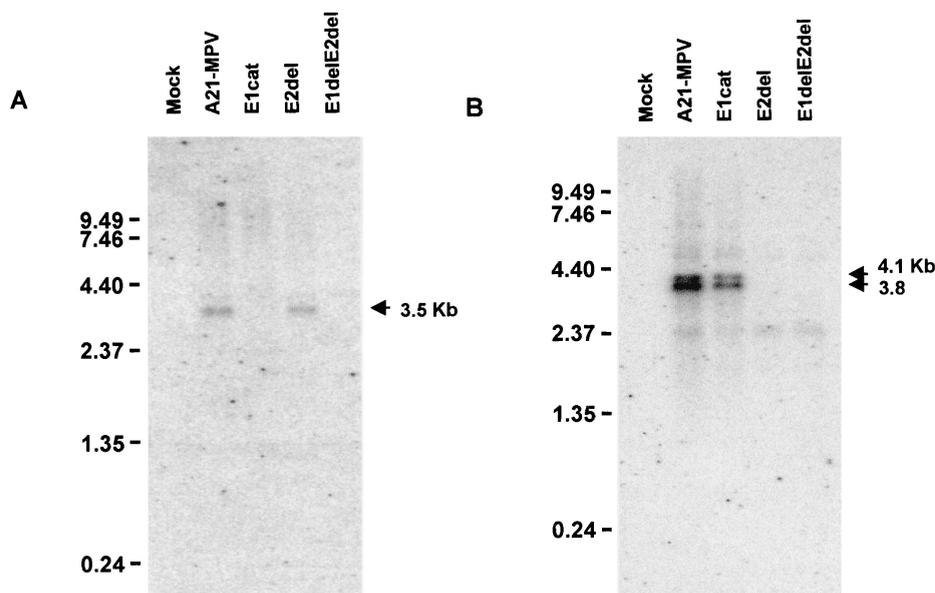


FIG. 7. Northern blot analysis of *enhancin* gene transcripts from cells infected with recombinant viruses. Cells were infected with A21-MPV, E1cat, E2del, and E1delE2del AE2 viruses. Poly(A) RNA was isolated from the cytoplasmic RNA, and 1 µg was separated by formaldehyde-agarose gel electrophoresis, blotted, and probed with a strand-specific oligonucleotide for the *enhancin 1* gene (A) or *enhancin 2* gene (B). RNA from uninfected cells was used as a control (lane mock). Positions of RNA size standards (in kilobases) are indicated on the left.

from 20 to 120 amino acids downstream of the binding site (see references 13 and 20 for reviews). A glutamic acid residue in position 299 (51 amino acids downstream of the binding site) of the LdMNPV E2 gene is conserved among all enhancin proteins (Fig. 4). In addition, most enhancins contain conserved aspartic or glutamic residues at the LdMNPV enhancin 2 positions 291, 307, and 313, which are 43, 59, and 65 amino acids downstream of the binding site, respectively. It is interesting that the XcGV E4 lacks the conserved HEXXH zinc-binding site of metalloproteinases present in the other enhancins (HEI/LGH), and of all the enhancins, this protein exhibits the least homology to LdMNPV E2. The amino acid residues before and after the binding site are well conserved in comparison to the other enhancins, while the binding site (QKLGD) contains only the conserved isoleucine/leucine and

glycine variable residues found in the baculovirus enhancins. This may suggest that the XcGV E4 is not functional and hence would no longer be under evolutionary pressure to conserve amino acids within the protein.

To address the relationships of the LdMNPV and GV enhancins, a phylogenetic analysis was performed with the enhancin protein sequences shown in Fig. 4. An unrooted parsimonious tree was calculated with the PAUP heuristic search algorithm, and bootstrap analysis was performed to assess variability of the phylogeny (Fig. 5). XcGV E1, E3, and E4 and HaGV, TnGV, and PuGV enhancins formed one clade (clade 1), and the LdMNPV E1 and E2 and XcGV E2 enhancins formed another clade (clade 2). Clade 1 contained two subgroups, one consisting of HaGV, XcGV E3, PuGV, and TnGV, and the other of XcGV E1 and XcGV E4. The first

TABLE 1. Dose-response of second-instar *L. dispar* larvae infected per os by droplet feeding with A21-MPV, E1cat, and E2 del

Assay no.	Virus	LC ₅₀					LT ₅₀			
		PIB ^a /ml	95% fiducial limits		Slope	Het. ^b	Difference vs. A21-MPV(fold)	LT ₅₀ (day) ± SE	Slope ± SE	% Mortality
			Lower	Upper						
1	A21-MPV	4.8 × 10 ⁴	1.8 × 10 ³	4.2 × 10 ⁵	0.5 ± 0.1	2.4		7.4 ± 0.3	10.5 ± 2.7	50.0
	E1cat	7.8 × 10 ⁴	2.4 × 10 ³	7.8 × 10 ⁵	0.5 ± 0.1	3.1	1.6	7.8 ± 1.1	13.7 ± 3.9	48.2
	E2del	1.4 × 10 ⁵	8.3 × 10 ³	1.3 × 10 ⁶	0.6 ± 0.1	3.2	2.9	7.3 ± 0.2	26.9 ± 8.1	42.3
2	A21-MPV	3.4 × 10 ⁵	5.2 × 10 ⁴	1.2 × 10 ⁶	0.9 ± 0.1	1.1		8.1 ± 0.3	13.3 ± 4.5	64.7
	E1cat	5.3 × 10 ⁵	1.1 × 10 ⁵	1.6 × 10 ⁶	0.7 ± 0.1	0.4	1.6	8.4 ± 1.1	11.6 ± 3.2	60.0
	E2del	4.0 × 10 ⁵	1.2 × 10 ⁵	1.0 × 10 ⁶	0.7 ± 0.1	0.5	1.2	8.1 ± 0.2	12.6 ± 3.7	78.8
3	A21-MPV	2.4 × 10 ⁵	4.9 × 10 ⁴	8.5 × 10 ⁵	0.9 ± 0.1	1.7		7.9 ± 0.3	8.5 ± 1.6	74.3
	E1cat	9.1 × 10 ⁵	3.8 × 10 ⁵	1.9 × 10 ⁶	0.8 ± 0.1	0.1	3.8	7.6 ± 0.1	25.2 ± 5.4	68.9
	E2del	3.1 × 10 ⁵	1.3 × 10 ⁵	6.2 × 10 ⁵	0.9 ± 0.1	0.8	1.2	8.1 ± 0.2	18.9 ± 3.6	76.5

^a PIB, polyhedron inclusion body.

^b Het., heterogeneity.

TABLE 2. Dose-mortality response of second-instar *L. dispar* larvae infected per os by droplet feeding with A21-MPV and enhancin deletion viruses^a

Bioassay	Virus	LC ₅₀ PIB/ml	95% fiducial limits		Slope	Het.	Difference vs. A21-MPV (fold)
			Lower	Upper			
1	A21-MPV	1.1 × 10 ⁶	5.5 × 10 ⁵	2.0 × 10 ⁶	1.2 ± 0.2	1.00	
	E1delE2del AE2	1.2 × 10 ⁷	6.1 × 10 ⁶	2.6 × 10 ⁷	1.0 ± 0.2	0.39	10.9
	E1delE2del BF6	4.0 × 10 ⁶	1.8 × 10 ⁶	9.8 × 10 ⁶	0.7 ± 0.1	0.73	3.6
	E1lacZ E2del 5AA	1.3 × 10 ⁷	2.7 × 10 ⁶	8.9 × 10 ⁷	0.9 ± 0.2	1.24	11.8
2	A21-MPV	7.3 × 10 ⁴	2.3 × 10 ⁴	1.7 × 10 ⁵	1.0 ± 0.2	0.49	
	E1delE2del AE2	1.0 × 10 ⁶	1.6 × 10 ⁵	3.4 × 10 ⁶	0.6 ± 0.1	0.55	13.7
	E1delE2del BF6	8.3 × 10 ⁵	2.4 × 10 ⁵	2.1 × 10 ⁶	0.8 ± 0.1	0.25	11.4
	E1lacZ E2del 5AA	1.8 × 10 ⁶	2.2 × 10 ⁵	7.7 × 10 ⁶	0.9 ± 0.1	1.24	24.7

^a See Table 1, footnotes *a* and *b*.

subgroup of clade 1 formed two further subgroups of HaGV and XcGV E3 and of PuGV and TnGV. The first subgroup of clade 1 and the two subgroups contained within are well supported by bootstrap analysis. A subgroup consisting of LdMNPV E1 and LdMNPV E2 formed within clade 2, which was well supported by bootstrap analysis. In contrast, the positions of XcGV E1, E2, and E4 within the phylogeny are not well supported by bootstrap analysis. This finding could suggest either that these enhancins originated from a distant ancestor or that each has a different ancestor. It is interesting that while the LdMNPV enhancins exhibit branch lengths comparable to those of XcGV E1, E2, and E4, the subgroup that they form is well supported by bootstrap analysis. The phylogenetic analysis indicates that the HaGV, PuGV, TnGV, and XcGV E3 enhancins originated from a common ancestor and the LdMNPV enhancins came from a common ancestor. To delineate the phylogenetic relationship of the LdMNPV and GV enhancins as well as the relationship of the XcGV E1, E2, and E4 enhancins within the GVs will require identification of and analysis with additional enhancins from NPVs and GVs.

Construction and verification of recombinant viruses. To address the role of the LdMNPV E2 in viral potency, a recombinant virus was constructed that lacked the *E2* gene. In addition, to determine if one *enhancin* gene could compensate for the lack of the other, a virus lacking both *enhancin* genes was generated. The *E2* gene (2,364 nucleotides in length) was subcloned from the genome into a plasmid, pDB184 (Fig. 1B). A *lacZ* gene was inserted into the *Bst*XI site of pDB184, at nucleotide 66 within the *E2* gene ORF, and used to construct

the intermediate virus, E2lacZ. This virus was then recombined with a plasmid containing a deletion of *E2* gene nucleotides 66 to 2168 to produce the virus E2del (Fig. 6). A *lacZ* gene was inserted into the *Nar*I sites of the *E1* gene (2,349 nucleotides in length) at nucleotides 1309 and 1492, and the resulting plasmid, pE1lacZ, was recombined with the E2del virus to generate the virus E1lacZE2del. This virus was recombined with a plasmid containing a deletion of *E1* gene nucleotides 26 to 1492 to produce the virus E1delE2del. To confirm the effect of deletion of both *enhancin* genes, a virus was constructed in which the *E1* gene was replaced. Plasmid pDB126, which contains the *E1* gene, was recombined with E1lacZE2del5AA to give E1repE2del (Fig. 6). The identity of the recombinant viruses was verified by restriction enzyme and Southern blot analysis (data not shown).

Northern analysis was used to verify that the *enhancin* transcripts were not being produced in the double deletion recombinant viruses as well as the single *enhancin* gene deletion viruses (Fig. 7). Poly(A) RNA isolated at 72 h p.i. from mock, A21-MPV, E1cat, E2del, and E1delE2del AE2 viral infections was probed with strand-specific probes for *E1* gene (Fig. 7A) or *E2* gene (Fig. 7B) transcripts. Oligonucleotide sequences that contained little homology were selected in order to differentiate between the *enhancin* gene transcripts. The 3.5-kb *E1* gene transcript is missing in lanes containing RNA isolated from cells infected with E1cat and E1delE2del AE2 viruses, and the 3.8- and 4.1-kb *E2* gene transcripts are missing in lanes containing RNA isolated from cells infected with E2del and

TABLE 3. Time-mortality response of second-instar *L. dispar* larvae infected per os by droplet feeding with A21-MPV and enhancin deletion viruses

Bioassay	Virus	Mortality of ~50%			Mortality of ~80%		
		LT ₅₀ (h) ± SE	Slope ± SE	% Mortality	LT ₅₀ (h) ± SE	Slope ± SE	% Mortality
1	A21-MPV	9.0 ± 0.4	10.1 ± 2.4	46.9	8.2 ± 0.2	16.1 ± 3.1	93.6
	E1delE2del BF6	8.7 ± 0.4	10.6 ± 1.9	40.0	8.9 ± 0.4	8.0 ± 1.6	78.6
	E1delE2del AE2	9.4 ± 0.5	8.9 ± 1.9	48.5	8.2 ± 0.3	11.9 ± 2.6	80.0
	E1lacZ E2del 5AA	8.2 ± 0.3	13.1 ± 3.6	51.5	8.6 ± 0.3	11.1 ± 2.2	75.8
2	A21-MPV	7.3 ± 0.2	19.9 ± 4.6	60.0	7.7 ± 0.1	19.7 ± 3.8	81.3
	E1delE2del AE2	7.7 ± 0.2	16.6 ± 4.1	53.3	7.5 ± 0.2	14.7 ± 3.2	79.2
	E1delE2del BF6	7.8 ± 0.2	19.4 ± 4.7	56.7	7.3 ± 0.1	24.0 ± 5.3	80.8
	E1lacZ E2del 5AA	7.8 ± 0.1	25.0 ± 6.3	47.1	7.5 ± 0.1	18.1 ± 3.3	82.4

TABLE 4. Dose-mortality response of second-instar *L. dispar* larvae infected per os by droplet feeding with A21-MPV and enhancin deletion and replacement viruses^a

Virus	LC ₅₀ (PIB/ml)	95% fiducial limits		Slope	Het.	Difference vs. A21-MPV (fold)
		Lower	Upper			
A21-MPV	1.2×10^5	6.5×10^4	2.1×10^5	1.2 ± 0.2	0.18	
E2del	1.7×10^5	3.2×10^4	6.6×10^5	1.0 ± 0.1	1.66	1.4
E1delE2del AE2	1.6×10^6	3.0×10^5	9.8×10^6	0.7 ± 0.1	2.23	10.0
E1repE2del	2.7×10^5	1.3×10^5	5.2×10^5	0.8 ± 0.1	0.60	2.3

^a See Table 1, footnotes *a* and *b*.

E1delE2del AE2 viruses, as expected, confirming that the *E1* and *E2* genes have been removed.

Bioassay analysis. The biological activity of the recombinant enhancin viruses was determined through bioassay of *L. dispar* larvae. A previously constructed recombinant virus (5) containing a *cat* gene insertion in the *E1* gene was also used in the bioassays. The potency of the E2del virus was found to be consistently less than that of the control virus in three separate bioassays (Table 1). The decrease in potency ranged from 1.2- to 2.9-fold. In agreement with our previous findings, the E1cat recombinant virus exhibited a drop in potency compared to the control virus (Table 1). In this study, the decrease ranged from 1.6- to 3.8-fold, which is in good agreement with the range of 1.4- to 4.0-fold reported in the previous study (5). These results indicate that enhancin 2 had an effect on viral potency and that both enhancin genes encode functional proteins that increase viral potency. Analysis of the time-mortality response showed similar killing speeds for the E2del, E1cat, and A21-MPV viruses (Table 1). Taken together, these results indicate that the enhancins are functioning at the level of viral entry into the insect host and do not affect systemic transmission. E2del, E1cat, and A21-MPV exhibited essentially identical rates of budded virus formation (data not shown), which indicates that the *enhancin* genes are not involved in viral replication. In addition, this finding indicates that a random mutation in a gene involved in DNA replication did not occur in the recombinant viruses. In contrast to the present findings, a decrease in the survival time of 50% of larvae (ST₅₀) was noted in bioassays of mid-fourth-instar *T. ni* larvae infected with AcMNPV in combination with the TnGV enhancin protein (11). Larvae that showed the same mortality with virus and with virus and enhancin had a lower ST₅₀ in the larvae infected with virus and enhancin. Perhaps a dual activity is possible for some enhancin proteins, or the impact on ST₅₀ was a function of the experimental conditions employed.

To determine whether each LdMNPV enhancin protein contributes to viral potency in an additive manner, the contribution of both enhancin genes to viral potency was determined through bioassay of three separately generated viruses with the *E2* gene deleted and the *E1* gene deleted or inactivated by insertion of the *lacZ* gene. In two bioassays, all of the *E1/E2* gene recombinant viruses exhibited decreased potency compared to isolate A21-MPV (Table 2). The decrease in potency ranged from 3.6- to 24.7-fold and averaged 12.7-fold. The drop in potency in these bioassays was analyzed by the unpaired *t* test ($P < 0.0003$) and by analysis of variance ($P < 0.0007$) and found to be significant. As expected, no significant difference in the LT₅₀s was seen for any of the *enhancin* gene deletion

viruses in comparison with A21-MPV (Table 3). This finding corroborates the LT₅₀ results of the single enhancin gene deletion viruses and supports the conclusion that no inadvertent mutations occurred in genes involved in viral replication during the construction of the recombinant viruses. In addition, comparison of the kinetics of budded-virus synthesis of the enhancin gene recombinant viruses with wild-type virus revealed no differences (data not shown). Furthermore, since the same result was obtained with three separately generated viruses with the *E2* gene deleted and the *E1* gene deleted or inactivated, it is unlikely that a random mutation affecting viral potency would have occurred in all three constructs.

To confirm that the large decrease in viral potency was due to deletion of both enhancin genes, a virus was constructed in which the *E1* gene was replaced in an E1lacZE2del recombinant (E1repE2del) and bioassayed. E1repE2del exhibited greater potency than E1lacZE2del and essentially the same potency as E2del (Table 4). In this bioassay, E2del and E1delE2del AE2 exhibited decreases in potency of 1.4- and 10-fold, respectively, which is in good agreement with earlier bioassays (Tables 1 and 2). The finding that the replacement of the *E1* gene restores viral potency to the level of E2del indicates that the drop in potency in E1delE2del was due to the deletion of both *enhancin* genes, as opposed to a mutation in another gene that affects viral potency.

Taken together, the bioassay results indicate that the *E1* and *E2* gene products can partially compensate for the loss of one of the genes, since deletion of the *E1* or *E2* gene decreased viral potency by approximately 2-fold, whereas deletion of both *enhancin* genes decreased potency by approximately 12-fold. The contribution to viral potency by the enhancin proteins does not appear to be additive, since each protein alone is capable of increasing viral potency approximately 10-fold (relative to a virus with no enhancin genes), whereas when both proteins are present, an increase in potency of 12-fold is observed. This suggests that there is a limit to the degree of potency enhancement that the enhancin proteins can effect. Since the total contribution to viral potency of both enhancin proteins together is greater than that of each individually, the presence of both genes makes the virus more competitive than a strain that contains only one. The potency results obtained in this study provide a basis for the presence of two enhancin genes in LdMNPV and possibly for the finding of multiple enhancin genes in XcGV. The mechanism by which the LdMNPV enhancin genes increase viral potency, their site of action, and the means of conveyance to the action site are under investigation.

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