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## Molecular Analysis of an *enhancin* Gene in the *Lymantria dispar* Nuclear Polyhedrosis Virus

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**A *Lymantria dispar* nuclear polyhedrosis virus (LdMNPV) gene has been identified that encodes a homolog to the granulovirus (GV) enhancin proteins that are capable of enhancing the infection of other baculoviruses. Enhancin genes have been identified and sequenced for three species of GVs but have not been found in any other nuclear polyhedrosis virus to date. The LdMNPV *enhancin* gene is located between 67.6 and 70.1 kbp on the viral genome. Northern and primer extension analyses of viral RNAs indicate that the *enhancin* gene transcripts are expressed at late times postinfection from a consensus baculovirus late promoter. The LdMNPV *enhancin* exhibits 29% amino acid identity to the enhancin proteins of the *Trichoplusia ni*, *Pseudaletia unipuncta*, and *Helicoverpa armigera* GVs. All four proteins contain a conserved zinc-binding domain characteristic of metalloproteases. A recombinant virus (*enhancin::cat*) was constructed in which the LdMNPV *enhancin* gene was inactivated by insertion mutagenesis in order to ascertain the effect of the enhancin protein on viral potency. The bioassay results indicate that disruption of the *enhancin* gene in the LdMNPV results in a reduction in viral potency.**

Nuclear polyhedrosis virus (NPVs) and granuloviruses (GVs) are members of the *Baculoviridae* which infect insects and other arthropods. All baculoviruses have a unique infection cycle in that they produce two infectious forms: a budded virus, which infects different cell types within a single larva; and an occlusion body, a form of the virus that is embedded in a protein structure. Larvae are infected upon ingestion of the occlusion body and release of the viral particles in the alkaline environment of the midgut. In NPVs, many viral particles are occluded in a single intranuclear crystal called a polyhedron, whereas in GVs a single viral particle is found in each crystal, or granule. The polyhedron protects the viral particles from environmental elements and is the viral form that is used for biocontrol of agricultural and forest insect pests (for a review see reference 10).

Enhancin, which has also been referred to as the synergistic or viral enhancing factor, is a protein found in the GV occlusion body that has the ability to enhance the infection of other NPVs (15, 18, 37, 40, 41). The enhancin protein is highly expressed in GVs and makes up approximately 5% of the total protein in the granules (37). It has been localized deep within the capsule matrix, close to the viral envelope (47, 49). Purified enhancin from the *Trichoplusia ni* GV (TnGV) can enhance the infection of the *Autographa californica* NPV (AcMNPV) 2- to 4-fold when fed to *T. ni* larvae and up to 12-fold when fed to other larvae such as those of *Spodoptera exigua* (45). Recently, enhancin genes have also been identified in the *Pseudaletia unipuncta* GV (PuGV) and the *Helicoverpa armigera* GV (HaGV) (32).

Two modes of action have been observed for enhancin proteins. First, the enhancins exhibit proteolytic activity (16, 38) which results in the enzymatic hydrolysis of the peritrophic membrane (12, 45), a barrier against microbial pathogens in the insect midgut. Degradation of the peritrophic membrane by enhancin is believed to allow the virus easier access to the

midgut columnar cells, resulting in the insect's increased susceptibility to viral infection (12). Second, there is an increased fusion of the nucleocapsids with the midgut cells through interaction of the enhancin protein with both the viral envelope and the cell plasma membrane (37, 39, 42, 45).

The *Lymantria dispar* NPV (LdMNPV) is pathogenic to the gypsy moth, a forest and urban tree-defoliating pest in the northeastern United States. The genome of the LdMNPV is approximately 162 kbp in length (29), in contrast to the 133-kbp genome of the prototype baculovirus, AcMNPV. The entire genome of the AcMNPV has been sequenced (2), and although that of the LdMNPV is not as well characterized, several genes which are present in both the LdMNPV and the AcMNPV have been identified (6, 9, 31, 36). The extra 29 kbp of DNA present in the LdMNPV suggests that this virus has the potential to possess several genes which are not present in the AcMNPV. Two genes, the host range factor 1 gene (43) and *G22* (5), that have been identified and characterized for the LdMNPV do not have homologs in the AcMNPV. In this study we extended the characterization of the LdMNPV by cloning and sequencing the *enhancin* gene. Homologs of this gene are present in the GVs but have not been identified in any other NPV to date. We also explored the effect of the LdMNPV enhancin protein on viral pathogenicity by inactivating the *enhancin* gene through insertional mutagenesis.

### MATERIALS AND METHODS

**Cells, virus, and insects.** *L. dispar* 652Y cells were grown as monolayers in Goodwin's IPL-52B medium supplemented with 6.25 mM glutamine and 10% fetal bovine serum. Cell cultures were inoculated with either LdMNPV isolate A21-MPV (35), which produces wild-type polyhedra, or the *enhancin::cat* virus (in which *enhancin* is disrupted by a chloramphenicol acetyltransferase [*cat*] gene). *L. dispar* egg masses were obtained from the U.S. Department of Agriculture's Animal and Plant Health Inspection Service rearing facility at Otis Air Force Base (Mass.). Hatched larvae were reared on a gypsy moth diet (3).

**Viral DNA isolation and Southern blot analysis.** Budded virus was isolated from infected 652Y cells as described previously (4) and used as a source of genomic DNA for restriction analysis. Viral DNA was digested with restriction endonucleases and fractionated on 1.0% agarose-Tris-borate-EDTA gels. Southern blot analysis was performed on nitrocellulose with probes labeled with the nick translation kit (Bethesda Research Laboratories) and [ $\alpha$ - $^{32}$ P]dCTP (NEN).

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**Sequencing.** The sequence of the *enhancin* gene from isolate A21-MPV was determined for both strands by the dideoxynucleotide sequencing method. Plasmid and single-strand M13 DNA templates were sequenced with the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemicals) or the *fmol* DNA sequencing system (Promega) by using the protocols supplied with the kits.  $\alpha$ - $^{35}$ S-dATP was supplied by NEN. Sequence analysis was done with the MacVector program (International Biotechnologies, Inc.).

**In vitro transcription and translation of the *enhancin* gene.** A 4.2-kbp *Sst*II fragment containing the *enhancin* gene was subcloned into pBluescript SK+ (Stratagene) to generate pDB126. The *enhancin* protein was expressed from pDB126 with the T<sub>N</sub>T coupled reticulocyte lysate system and T7 RNA polymerase (Promega) per the directions provided with the kit. The expressed protein was labeled by the addition of [ $^{35}$ S]methionine (NEN). Reaction products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

**RNA isolation and Northern blot analysis.** A21-MPV-infected 652Y cells were harvested at various times postinfection (p.i.). Cytoplasmic RNA was isolated as described by Friesen and Miller (14). RNA was separated on 1.2% agarose gels containing formaldehyde and transferred to nitrocellulose. Northern blot analysis was performed as described by Mahmoudi and Lin (28). A 30-base oligonucleotide (complementary to positions 939 to 968 in Fig. 2) was end labeled with [ $\gamma$ - $^{32}$ P]ATP (NEN) and used as a strand-specific probe to detect the *enhancin* transcripts.

**Primer extension mapping of transcripts.** Primer extension reactions were performed by the method of Crawford and Miller (11). Cytoplasmic RNA was isolated at 48 and 72 h p.i. from 652Y cells infected with A21-MPV. An 18-base oligonucleotide (complementary to positions 132 to 149 in Fig. 2) was used in the reactions after being end labeled with [ $\gamma$ - $^{32}$ P]ATP (NEN). The primer was extended with Moloney murine leukemia virus reverse transcriptase. Primer extension products were fractionated on 6% polyacrylamide-8 M urea gels and visualized by autoradiography.

**Construction of the *enhancin:cat* virus.** A transplacement vector (pDB159) containing the *enhancin* gene disrupted with the *cat* gene was constructed. Transfections were carried out with 2.5  $\mu$ g of A21-MPV viral DNA and 2.5  $\mu$ g of pDB159 plasmid DNA by using the Lipofectin reagent (Bethesda Research Laboratories) as described previously (6). At 7 days p.i., the cells and medium (3 ml) were collected and diluted with 12 ml of fresh medium in a T75 flask. The nonoccluded virus was collected at 7 days p.i. and plaque purified. Approximately 200 plaques were picked and used to infect 652Y cells in P96 plates. To identify recombinant viruses, budded virus (50  $\mu$ l) from the P96 wells was blotted by standard techniques and probed with the 0.7-kbp *Sst*II fragment containing the *cat* gene from pDB4 (8). For further details, see Results and Discussion.

**Bioassay analysis of the *enhancin:cat* virus.** Polyhedra were isolated from 652Y cells infected with either A21-MPV or the *enhancin:cat* virus and were purified and quantitated as previously described (34). Fourth-instar *L. dispar* larvae were infected per os by placing them on a diet containing surface-applied in vitro-synthesized polyhedra (total of  $5 \times 10^6$  polyhedra). The larvae were placed on fresh food after 48 h on the infected diet. Dead larvae were collected and used as a source of in vivo polyhedra for the bioassays. The polyhedra were tested for biological activity by both the diet incorporation (23) and the droplet feeding (21, 22) methods.

In the diet incorporation bioassays, second-instar larvae were infected by placing them on a diet containing various concentrations of polyhedra ( $10^2$  to  $10^7$  polyhedra per ml of diet) and allowing them to feed ad libitum for 48 h. Five groups (20 larvae each) were infected at each virus dose. After 48 h on the diet containing virus, the larvae were removed and placed on a fresh diet for the remaining 12 days of the bioassay. This type of bioassay was repeated in triplicate.

In the droplet feeding assay, neonate larvae (60 larvae per dilution) were allowed to feed for 30 min on droplets containing various dilutions of polyhedra ( $5 \times 10^3$  to  $10^6$  polyhedra per ml of solution). The larvae were then placed on a fresh diet and were monitored for death for up to 18 days. The volume of solution ingested by *L. dispar* neonate larvae in the droplet feeding assay was determined by the method of Kunimi and Fuxa (26).

Dead larvae from both bioassays were removed and counted daily. The 50% lethal concentrations (LC<sub>50</sub>s) and 50% lethal times were determined for each virus by Probit analysis (13) by using the POLO-PC program (LeOra Software, Berkeley, Calif. [33]). The 50% lethal times and 50% lethal doses were also determined for the droplet feeding method by using the ViStat program (version 2.1; Boyce Thompson Institute, Cornell University, Ithaca, N.Y. [20]).

**Nucleotide sequence accession number.** The nucleotide sequence accession number of the sequence contained in this paper is AF019971.

## RESULTS AND DISCUSSION

**Identification of the *enhancin* gene.** The *enhancin* gene was identified during the mapping and sequencing of the gene mutated in LdMNPV few polyhedron mutants. Initially these mutations were mapped to a 4.3-kbp *Bam*HI/*Eco*RI fragment located at 64.5 to 68.9 kbp on the viral genome. Further map-

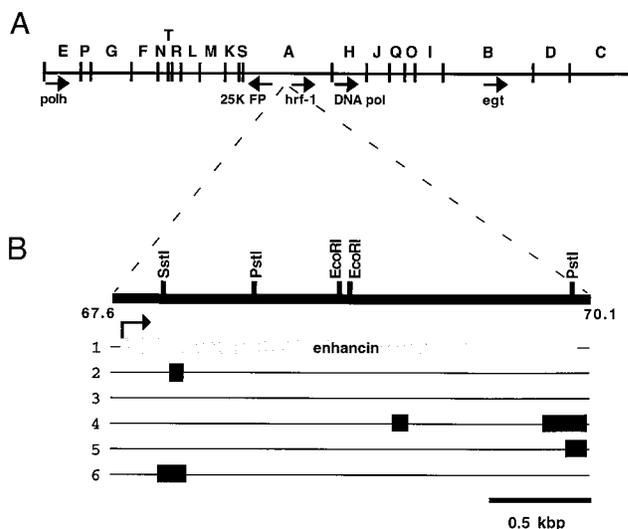


FIG. 1. Genomic location of the LdMNPV *enhancin* gene. (A) *Bgl*II restriction map of the LdMNPV viral genome, with the letter designation of each fragment shown above (6). The locations of other known LdMNPV genes are also indicated. Abbreviations: polh, polyhedrin gene (36); 25K FP, 25K few polyhedron gene (6); egt, ecdysteroid UDP-glucosyltransferase gene (31); DNA pol, DNA polymerase gene (9); hrf-1, host range factor 1 gene (43). (B) The enlarged map indicates the 2,460-bp fragment that encodes *enhancin* (67.6 to 70.1 kbp on the viral genome). Restriction sites used in subcloning and sequencing of the gene are shown. ORF analysis of this fragment is shown in all six reading frames. The black boxes indicate ORFs encoding sequences that are at least 25 aa in length and which begin with an ATG start codon as determined by the MacVector program from International Biotechnologies, Inc. The shaded box indicates the ORF corresponding to the LdMNPV *enhancin* gene.

ping studies revealed that these mutations were within the 25K *FP* gene between 65.3 and 66.0 kbp (6, 7). During the sequencing of the 4.3-kbp fragment, an open reading frame (ORF) which initiated within this fragment and proceeded through the *Eco*RI site located at 68.9 kbp was discovered. The predicted amino acid sequence of this partial ORF was compared with those of other proteins in GenBank at the National Center for Biotechnology Information by using the BLAST network server (1). This ORF exhibited homology to the *enhancin* genes of the TnGV, PuGV, and HaGV. The entire ORF was subcloned on a 4.2-kbp *Sst*II fragment (67.0 to 71.2 kbp on the viral genome) into pBluescript SK+ to generate pDB126, and the DNA sequence of the ORF was determined. The LdMNPV *enhancin* gene is located between 67.6 and 70.1 kbp on the viral genome and is transcribed clockwise with respect to the circular viral genome (Fig. 1A). A partial restriction map and the ORF map of a 2.46-kbp fragment in this region are presented in Fig. 1B, with the largest ORF (2,346 bp in frame 1) corresponding to the *enhancin* gene.

**Characteristics of the nucleotide sequence.** The nucleotide sequence of the 2,460-bp fragment containing the LdMNPV *enhancin* gene is presented in Fig. 2 along with the predicted amino acid sequence of the protein. The *enhancin* ORF begins at nucleotide position 49 and ends at position 2395. The gene could encode a 782-amino acid (aa) protein with a predicted molecular mass of 89,200 Da. Analysis of the sequence upstream of the *enhancin* ORF reveals a potential baculovirus late promoter sequence, TTAAG, beginning 13 bp upstream of the *enhancin* start codon (nucleotides 36 to 40 [Fig. 2]). Potential late baculovirus promoter motifs have also been identified upstream of the *enhancin* genes in the TnGV (ATAAG [18]) and the PuGV (ATAAG [32]). In addition, *enhancin*

1 ATATTAAATATCAATTTGGTTTATTTATATACATTAAGTGGTTACAATGAGCAATACG 4  
 61 ACTACTTTTACGTTGACCGTCTGCCACGACGCTGCCCGTATTTTGGAGCGGAGCGAC 24  
 121 ACCTCGCTGGCGCGGACCAACAATCGCTCCCGTTGGCGTATGTGTGCTCGCGCCACC 44  
 T S L G L H H N R V P L G Y V C S S A T  
 181 ACGATCACCGTACGCGACGCGCGCCATCTCGTTGGCATTTTAAACAACAACCGCACT 64  
 T I T V P S T A A I S L R P L N N N R N  
 241 CTAGAGCGAAGCGTACCGGTGCGAGCACTGGCTCTCTTTACTACACGAAACG 84  
 L E R S V T V A A N S S S S F T H N E T  
 301 TATGTGCGCTTCGCAAGTCGTGTGGTGGCGCGGACGCGGAGGCTACGTCGTCGAGTGT 104  
 Y V P P A D R V V G G D A R G Y V V E C  
 361 ACCGTCAACAATATTGGAGCGCTTGCACCATTACACGACGCTCCACCGACGAGCGG 124  
 T V N N N Y L S V L P H Y T H G L T D E A  
 421 GCGTTCAAAAAGCAATTCGCGCCCTCGACGCAACAACATCTTTGCGTTCTCGAACTC 144  
 A F K N E L R A L D D N Q S F A P L E L  
 481 AAAAACCGCTGCTGTGGTGGCGCGCCGACAAAGCCGAGTGTGCTCGCCCTCGAATTG 164  
 K N A L L L V P P P D K A E L L A L D L  
 541 GGCGCGCTCGACAATTTTACACCACGATCGTGGACAGCTTCGACCACCTGCGGACTG 184  
 G A L D N F Y T T I V D T F D H L I G L  
 601 GTCAACGTGCGGACGACCGCCGACCAAGAACTTTAACAATAAATTTTGGCAAA 204  
 V N V A S D D P A T T R N P N K K Y F C K  
 661 GCCGATTCAAACGCGCTGGCGCTGCTATTCGACCGCAATTCGACGCGGCAACGAGC 224  
 A D S N C G V S F A R V S T R E W T  
 721 GTGAGCATGTCGGCTTACCTGACGCGCGGCACTGGCTGCTGTCGCAAGAACT 244  
 V S M S R Y L Q P R A T N L V L V L H E I  
 781 GGCCACGCTACGATTTCAATTCGTGAGCAACGCGCGCGCTGAACGAGGTGGGAC 264  
 G H A Y D F V S N T P A L N E V W N  
 841 AACGTGCTGGCGCATCGTACCAATGACGATCATGTGCTTCGACGAGCGGAGCGGAC 284  
 N V L A D R Y Q Y D F M S F D E R Q R D  
 901 GCGAGCGTTTACGAAAACGCGAATCGGACCGCATCGAGCGCAACTCGCCGAGCGAATC 304  
 A S V Y E N G R D R V E R N I A E R I  
 961 GCAATCGCGCCCTACGAGAGTGTGCTCTTTTCAAAAATGGCCGCTGATTCACGTGG 324  
 D N R A P Y E S W S F F Q K M A V F T W  
 1021 ATGATGGACACCGATTCGGCGGAGACGATGGCGCGCATCAATCGCCAGTTCAGCGAA 344  
 M M D T D D C G R E T M A R I N R Q F R Q  
 1081 AITAAACTTTCGATTCGAGCCCTCGTACATGCCCACTTTTCGATTTGGTGGTGTCTGCTG 364  
 I K T F D S S P R Y M P T F D W V L L  
 1141 TCGCGGGGACTTCGTCGCTTATTGAAATGATGACGAGTGAATTCATCTGCTGCGA 384  
 S D G D P V P V L K L M Q V E F T S C R  
 1201 GTTTGTCACAACAACGCTGCGACACCTTTTGCACAGAACTTCGTCGTCGCTGCT 404  
 V L S N N N V D T P L H T N S L V R S  
 1261 AAACCGCTCTACTATCCGTCGCAAGCTGATTCGCAATTTGACGCGCTCGCCAAACAAC 424  
 K R V Y Y P V K E L I A N F D A L A N N  
 1321 TACGGTTTCGTCGCGCAAGCAATATTTCGTTGGTGGCGCGGCAAGTCCAGCGCGC 444  
 Y G F V A Q S N Y S L V A P G E V D A R  
 1381 CGCTCTTGGTATACACTGCGACATTCGACGCGCGGACAGATAGCGGACAATTTTT 464  
 A S L V I H C D I E D D A R Q I A G Q F F  
 1441 TACGTTTACGACGCGACCGCTGTTGGCAGTCGCAAACTCGACAACCTCCAATGATG 484  
 Y V Y D G T A T R L V W Q S Q I D N S N R L  
 1501 GTGGTAAACGACATACACGGCGGCTGTACACACTGTTGGCGCGCGCGGCAAAACGG 504  
 V V N D I H A G V Y T M V A P R A H K R  
 1561 TACAGGCTGACTTCGATGAGCGCCCTCGATCGCGCGCTAAACGAACTTTGATTTG 524  
 Y R V Y F D E P A S I A G L N E H L Y L  
 1621 TFCGCGGACACTCGAACCAGCAAGCTTAATTCGACGCGCGCTCGATTCGAGCCCG 544  
 F A D T S K P T R M D E Y E R L D S F  
 1681 GCGGGCGCGCTGCGCGCCACTTTTGGCATTCAAGATTTATACAGACGAAAGT 564  
 A G D R V A A H V L G I N D L Y R A K V  
 1741 ATTTTCGATTTAAAGCCAAACTAATGACGCGGTCACAGTTTTCGAGCACGTTTAAATCG 584  
 I F D F K A K L M S V H S P A S T F N S  
 1801 GGCTACGCAACATCGCGCTTTCGTTTAAACATCAGACGAGCAACTGTACACATTC 604  
 G Y A N M R Y F V L N I R R D Q L Y T P  
 1861 AATCATACGTTTACGGCACGCAAAATTTGTTGGCGCTCATGAGCGTGGACATCGCGCG 624  
 N H T F T G T Q N F V G M S V D I A P  
 1921 GCGCACGATCTCCTCGTTTACCAGCGGCGGACACTCGCTTTCATTTTTTAAACGG 644  
 G D T M S Q G Q D T R F I F L N A  
 1981 AAGAGTCTCAATTTACCGTTCGACCTCTCGGAAAATTTGGTACAGCAACTAATTTGCG 664  
 K S L N F T L N S E N W Y S N T N L P  
 2041 TCCAAAATCAAGTTTCGAAACGAGAAATGGACGAGTGGCGGCTTATTTGTCAGCAAC 684  
 S F A D T S K P T R M D E Y E R L D S F  
 2101 GCATCGGTTTGTATCGCTTCAAAATCCTTAAAGACGCACTGTACTTACGATTCAAA 704  
 A S R L F A F E N H L K D E L Y L T I Q  
 2161 TCTTGGCGGCAAGATTAATCATGAGATTTGATAAATCGCTTTTACCGGCTCAATTT 724  
 S L P D K D Y Y M R L Y N P F L P A H F  
 2221 AAATTACACATTCGACCCGACGCGGTTGAGCAGCATCATGATGGCGGCTGATG 744  
 K F N T F D P T T G S S S I M M A V V I  
 2281 TTTTGTTCGTTCTAGTGGCATTTGGTATCGTTCATTTTGGTGTGTTGCAATCGACGC 764  
 F C F V L V A L V I V F I L V F V N R R  
 2341 GGTAGGCAAGCCGAAAGCGGACGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 782  
 G R Q S P K A A E R A P P L Q R V\*\*\*  
 2401 TACTTCAATTCGACCCGCTGTCAGTACCGCCAGCAGTTCGAGGACGACCGGAC

FIG. 2. Nucleotide sequence of the 2,460-bp fragment containing the LdMNPV *enhancin* gene and the predicted amino acid sequence. The consensus late promoter sequence is shaded, with the late transcriptional start site underlined. The *Sst*I restriction endonuclease site used in construction of the *enhancin::cat* virus is boxed. Oligonucleotides used in transcriptional mapping and primer extension are underlined (see text).

transcripts have been shown to initiate within the HaGV late promoter motif TTAAG (at the nucleotides underlined within this sequence [32]).

Immediately downstream of the LdMNPV *enhancin* gene is the *hrf-1* gene (43), which is also transcribed in a clockwise direction (Fig. 1A). There is no potential polyadenylation signal sequence that can be identified in the 119 bp between the *enhancin* stop codon and the *hrf-1* start codon. This is similar to the TnGV, PuGV, and HaGV, which also lack canonical polyadenylation signal sequences between the *enhancin* gene and the downstream gene (*ORF1*) (18, 32). Despite the similarity between the LdMNPV and the GVs with respect to the

organization of the *enhancin* gene and its downstream gene (*hrf-1* or *ORF1*), the LdMNPV *hrf-1* gene shows no homology to the GV *ORF1* genes.

**Characteristics of the protein sequence.** The LdMNPV *enhancin* gene encodes a shorter protein than the GV *enhancin* genes, with 782 aa for the LdMNPV *enhancin* compared to 901 or 902 aa for the GV *enhancins*. All four proteins show moderate homology at the N terminus and are less homologous to the C-terminal end (Fig. 3A). The TnGV and PuGV *enhancin* proteins are virtually identical, with only 15 residue changes between the proteins for an overall 98% amino acid identity (18, 32). The HaGV *enhancin* protein is less homologous to the other two GV proteins (TnGV and PuGV), with 81% amino acid identity (89% over the first 550 aa and 69% over the last 350 aa) and 90% similarity when conservative residue changes are taken into consideration (Fig. 3B) (32). The LdMNPV *enhancin* protein shows approximately 31% amino acid identity (Fig. 3A) to each of the three GV *enhancin* proteins (32.1% to the TnGV protein, 32.2% to the PuGV protein, and 31.4% to the HaGV protein), with approximately 55% similarity (54.7% to the TnGV protein, 55.2% to the PuGV protein, and 55.6% to the HaGV protein). Overall the four proteins exhibit 29% amino acid identity, with four areas exhibiting greater than 50% amino acid identity (residues 59 to 67 at 78%, 196 to 290 at 51%, 451 to 468 at 58%, and 692 to 704 at 54% [Fig. 3B]).

Comparison of the LdMNPV *enhancin* amino acid sequence with sequences in the BLOCKS database (version 9.0, December 1995 [19]) revealed the presence of a signature pattern characteristic of a zinc-binding domain found within metalloproteases (25, 30). The signature pattern, HEXXH, is sufficient to group a protein into the metalloprotease superfamily. All four of the *enhancin* proteins have this conserved metalloprotease zinc-binding domain (residues 241 to 246 for the LdMNPV) within a larger region of the protein, and all four exhibit 51% amino acid identity in this region (residues 196 to 290 [Fig. 3]). For this type of enzyme, the zinc ion is chelated by the two histidine residues in this sequence and by a third residue, typically a histidine, cysteine, or aspartic or glutamic acid residue, located anywhere from 20 to 120 aa downstream of the HEXXH sequence (for reviews, see references 17 and 24). There are two aspartic acid and two glutamic acid residues between 20 and 120 aa from the HEXXH sequence which are conserved in all four *enhancin* proteins (LdMNPV residues 269, 289, 297, and 302), any of which could function as a third zinc-binding ligand in the *enhancin* proteins (Fig. 3A). In the metalloproteases, the glutamic acid residue within the HEXXH sequence is the catalytic base which polarizes a water molecule involved in the nucleophilic attack of the peptide bond to be cleaved. Recently, it has been demonstrated that the TnGV *enhancin* gene encodes a metalloprotease (27) that is capable of degrading an insect intestinal mucin in the *T. ni* larval midgut (46).

**In vitro transcription and translation of the *enhancin* gene.** To demonstrate that the *enhancin* ORF encoded a protein, the gene was expressed from pDB126 in a rabbit reticulocyte coupled transcription and translation system. Plasmid pDB126 contains the 4.2-kbp *Sst*II fragment (67.0 to 71.2 kbp on the viral genome) and has the *enhancin* gene under the control of the T7 polymerase. Several radiolabeled bands, with apparent molecular masses ranging from 25 to 88 kDa, were visualized after analysis by SDS-PAGE and autoradiography (Fig. 4). The size of the *enhancin* protein is predicted to be 89 kDa from the nucleotide sequence. The smaller bands may correspond to translation initiation at internal methionine-encoding bases within the *enhancin* ORF or could be degradation products of

**A**

LdMNPV	MSNTTFTLTLVLPIT	LPVFLSASDTSGLGH	HNRVPLGYVCSAAT	ITVRS-----TAA	ISLRFLLNNRNLERS	VTVEANSSSSFTTNE	83
TnGV	MS----YKVIVPATV	LPPWLRVGENWIFAR	HRRTVEGVVLPANTK	FRVRADFSRAGFTRP	VIVRLLNNRNTERE	INLNNQWMEVEHAH	86
PuGV	MS----YKVIVPATV	LPPWLRVGENWIFAR	HRRTVEGVVLPANTK	FRVRADFSRAGFTRP	VIVRLLNNRNTERE	INLNNQWMEVEHAH	86
HaGV	MS----YKVIVPATV	LPPWLRVGENWIFAR	HRRTVEGVVLPANTK	FRVRADFAKWGITRP	VIVRLLNNRNTERE	INLNTDQWIEMEHEH	86
LdMNPV	TYVVFADRVVGGDAR	GYVMECTVNNYLSVI	BHYTHGLTDEAAFKN	ELRALDDNQSFAPLE	EKNALLLVPPDKAE	LLALDGLALDNFYTT	173
TnGV	ESVPEVDWLVGKENT	MAEVYFEIDGPHIPL	FVYVFNTRPVEHEKS	EYRQSSSGYCLYLD	L--VCMLVPPASKNA	LLDNVIFELHQFYNE	174
PuGV	ESVPEVDWLVGKENT	MAEVYFEIDGPHIPL	FVYVFNTRPVEHEKS	EYRQSSSGYCLYLD	L--VCMLVPPASKNA	LLDNVIFELHQFYNE	174
HaGV	ECVPEVDWLVGKENT	MAEVYFEIDGPHIPL	FVYVFNTRPVEHEKS	EYRQSSSGYCLYLD	L--VCILVPPASKNV	LLDSDLFELHQFYNE	174
LdMNPV	IVDTFPHLIGLVNVA	SDDPATRNF-NKYYF	CKADNSGVGAAYYDR	NWTAQTNVMSRSLQ	PRATNWLVIHELGH	YDFQFVNTPALNEV	262
TnGV	IINYYDDLCGLVEDP	YADTVDSNLPNKAAF	VKADAGGPGGAYYGP	FWTAPASSNLGDYLR	ISPTNWMVIHELGH	YDFVFTVNT-ILIEI	263
PuGV	IINYYDDLCGLVEDP	YADTVDSNLPNKAAF	VKADAGGPGGAYYGP	FWTAPASSNLGDYLR	ISPTNWMVIHELGH	YDFVFTVNT-ILIEI	263
HaGV	IINYYDDLCGLVEDP	YADTVDSNLPNKAAF	VKADGGGPGGAYYGA	FWTAPASTNLGEYLR	VSPTNWMVIHELGH	YDFVFTVNT-RLIEI	263
LdMNPV	WNNVLADRYQYDFMS	FDERQRDASVYENGN	RDRVERNIAERIDNR	APYESWSPFQKMAVF	TWMDTDCGRETMAR	INRQFRQIKTFDSSP	352
TnGV	WNNSLCDRIQYKWMN	KIKRQQLARVYEN-R	FVNEHNRKVVFHNLH	SPFDNWGFPERLIIF	TWLYNPQRGLDLRN	INHYSRVHATRNSSI	352
PuGV	WNNSLCDRIQYKWMN	KIKRQQLARVYEN-R	RPOKEATIQALIDNN	SPFDNWGFPERLIIF	TWLYNPQRGLDLRN	INHYSRVHATRNSSI	352
HaGV	WNNSCFDRIQYKWMN	KTKRQQLARVYEN-Q	RPOKEAAIQALIDNN	VFPDNWDFEKLSEIF	AWLYNPQRGLDLRN	INHYSRLHAARNPVT	352
LdMNPV	PYMPYFDWVLLSDG	DFVPYLLKLMQVEFTS	CRVLSNNVVDVTFHL	TNSLVRSKRYVYVVK	ELIANFDALANNYGF	--VAQSNYSLVAPGE	440
TnGV	PYPQIWSWLTTSAYD	NFWLYFNLVGVYPAD	FVYVNEHNRKVVFHNLH	LRLALGQSVRYPIK	YIITDFDLVSKNYDI	KQYLESNFDLVIPEE	442
PuGV	PYPQIWSWLTTSAYD	NFWLYFNLVGVYPAD	FVYVNEHNRKVVFHNLH	LRLALGQSVRYPIK	YIITDFDLVSKNYDI	KQYLESNFDLVIPEE	442
HaGV	PYPQIWSWLTTSAYD	NFWLYFNRIGLYPAD	FYINEHNRKVVFHNLH	MRLALGQSVRYPIK	YIITDFDLQKNYDI	KQYLESNFDLVIPEE	442
LdMNPV	V---DARASLVIHCD	IDDARQIAGQFFVYV	DGTRLVWQSQIDNSN	RLVVDNIHAGVYTMV	APRAH-KRYRVYF--	--DEPASIAGLNEHL	522
TnGV	LLDPSQIVGPEPFSVY	LDGNERVFEFSTVADG	DGNERVFEFSTVADG	NMYLVGVGPGVYTLR	APRGKNKRYKLHLAH	SPNEPVHPA--NDHM	530
PuGV	LRQTDLLADVRRVVCV	IDDPSQIVGPEPFSVY	DGNERVFEFSTVADG	NMYLVGVGPGVYTLR	APRGKNKRYKLHLAH	SPNEPVHPA--NDHM	530
HaGV	LRQTDLLADVRRVVCV	IDDPSQIVGPEPFSVY	DGNERVFEFSTVADG	NMYLVGVGPGVYTLR	APRGKDKRYKLHLAH	SPNEPVHPA--NDHM	530
LdMNPV	YLFADTSKPKRLIY	ER-LDSSPAGDRVAA	HVLGIND-LYRAKVI	FDFKAKLMSVHSPAS	TFNSGYAMR--YFV	LNIRRDQLYTFNHTF	608
TnGV	YLLVTPYYPYNTLETY	TPYVNSDLAVD--MA	HLFGSNDRRYVATII	FNPFQIVTVH--LN	NIRAGRENNTLYFE	MVES-NPFGQSQT	615
PuGV	YLLVTPYYPYNTLETY	TPYVNSDLAVD--MA	HLFGSNDRRYVATII	FNPFQIVTVH--LN	NIRAGRENNTLYFE	MVES-NPFGQSQT	615
HaGV	YLLVTPYYPYNTLETY	TRYITSLAID--AA	HLFGT-DRLYVATII	FDLAQIVTVTY--LN	NIRTAGRENNTLYFE	METH-NPFGTSSKF	614
LdMNPV	TGTQNFV---GVMS	VDIAPGDMSSSFHQQ	GDTRFIFLNA--KSL	NFTLNSENWYSNTN	LPSKNQVLET---RM	DECVAVLYANASRLI	689
TnGV	TILEDNPTLRQGYK	FVUVTYSSIR-LNMS	VAGRLLFRRYIFAGG	TTTLMFPNQVLEPN	LFPDGSALNRTLARL	REQAFL-DNYSQLM	703
PuGV	TILEDNPTLRQGYK	FVUVTYSSIR-LNMS	VAGRLLFQDTFLPEG	TTTLMFPNQVLEPN	LFPDGSALNRTLARL	REQAFL-DNYSQLM	703
HaGV	TILEDNPTLRQGYK	FPAVTFSSIR-LHIR	DDNRLMLVQKYLPA	DTLFLMFPNQIVDNN	IFPDGSILITSTYRRI	KEQAFL-ENHKQLL	702
LdMNPV	AFENHLKDELYLTIQ	SL-PDKDYMRLY--	-----	-----	-----	-----	716
TnGV	YIENELRDTIYLASQ	LVDPASDEFVKYYPD	YFRDPHTYVYLFRRF	GLGDFVLLDLQIVPL	LNLATVRIANIQNGP	HSYFDTLYFKVELRD	793
PuGV	YIENELRDSIYLASQ	LVDPASDEFVKYYPD	YFRDPHTYVYLFRRF	GLGDFVLLDLQIVPL	LNLATVRIANNHNGP	HSYFDTLYFKVELRD	793
HaGV	YIENELRDSIYLASQ	FVNSDSNEFLKYYPD	YFRDPHTYVYLFRRF	FDLQVLLLELQIVPI	LNLASVRVGNHNGP	HSYFNTTYLSVEVRD	792
LdMNPV	-----	EFLPAHFKEFTFDPT	TGSSSIMMAVVIFCF	VLVALVIFELVFN	RRGRQSPKAAERAP	FLQRV-----	782
TnGV	TNGAIVFSYSRRGNE	EPTPEHHKFEVYSGY	T-----	-----VELEMREPGN	RLQLIVNKMLDTALE	STQNIIFARITDTQLV	864
PuGV	TNGAIVFSYSRRGNE	EPTPEHHKFEVYSGY	T-----	-----VELEMREPGN	RLQLIVNKMLDTALE	STQNIIFARITDTQLV	864
HaGV	TSGGVVFSYSRNLGNE	EMTHEHHKFEVFKDY	T-----	-----IHLFIQEPGQ	RLQLIVNKMLDTALE	NQCNIIYARLTAQLV	863
LdMNPV	-----	-----	-----	-----	-----	-----	782
TnGV	VGDTSI---EDN--L	VTSINVDCGDDNQK	IRVVEITLKMIAF	901	-----	-----	901
PuGV	VGDTSI---EDN--L	VTSINVDCGDDNQK	IRVVEITLKMIAF	901	-----	-----	901
HaGV	VGEQSI IISDDNDF	VPPRVNCGD---QQ	IRVVEITLKMIAF	902	-----	-----	902

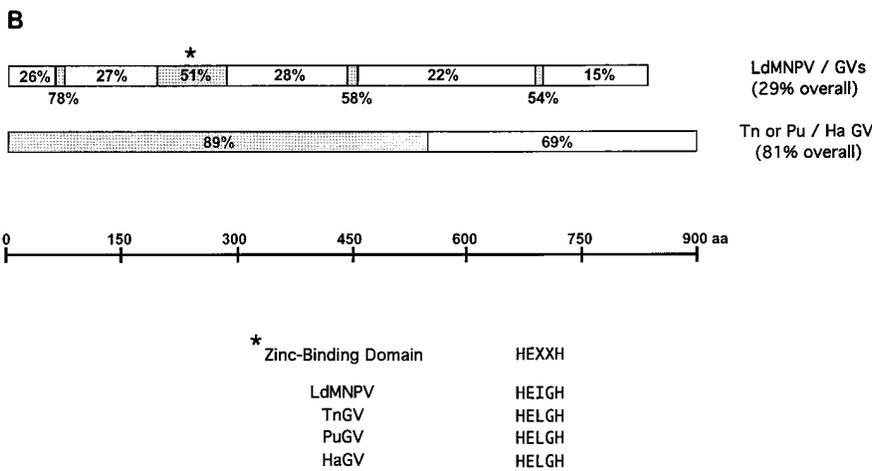


FIG. 3. Alignment of the enhancin proteins with CLUSTAL W version 1.6 (44). (A) Amino acid alignment of the LdMNPV protein with the enhancins of TnGV (18), PuGV (32), and HaGV (32). Shaded boxes indicate identical residues conserved in the four enhancin proteins. Identical residues within the conserved zinc-binding domain of all metalloproteases are in black boxes. (B) Schematic showing the percent amino acid identity (boxed numbers) within regions of the different enhancin proteins. Regions exhibiting the greatest amino acid identity are shaded. LdMNPV enhancin is compared to the three GV proteins, while TnGV and PuGV enhancins are compared with the HaGV protein.

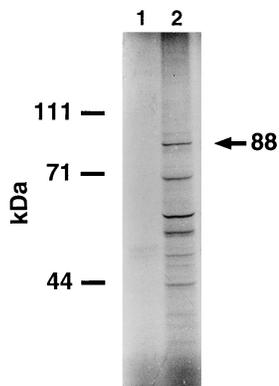


FIG. 4. SDS-PAGE analysis after expression of *enhancin* under the control of the T7 promoter. Shown is an autoradiograph of *enhancin* expressed in a rabbit reticulocyte transcription and translation system and labeled with [<sup>35</sup>S]Met. Lane 1, control plasmid, pBluescript SK+; lane 2, pDB126 expressing *enhancin*. Molecular mass standards are indicated to the left, and the position of the *enhancin* protein is indicated to the right.

the *enhancin* protein itself. No radiolabeled bands were detected for the parent plasmid pBluescript SK+ (Fig. 4).

**Temporal analysis and primer extension mapping of the *enhancin* transcripts.** A 30-base oligonucleotide complementary to nucleotide positions 939 to 968 (Fig. 2) was used as a strand-specific probe to characterize the temporal expression of the LdMNPV *enhancin* gene. The *enhancin* gene is transcribed primarily as a 3.5-kb transcript at late times, 48 and 72 h p.i. (Fig. 5). Two smaller RNA species (1.2 and 1.5 kb) also appear to be expressed at these times, although at lower levels. The gene does not seem to be transcribed at a high level, or else the transcripts are not very stable, since the blot shown in Fig. 5 contained 150 μg of RNA per lane and required a long exposure (3 weeks) for detection of the transcripts. The 3.5-kb transcript is quite large, as the LdMNPV *enhancin* ORF is only 2.3 kbp in length. In comparison, the TnGV *enhancin* gene is expressed as two late transcripts, 2.7 and 3.3 kb in

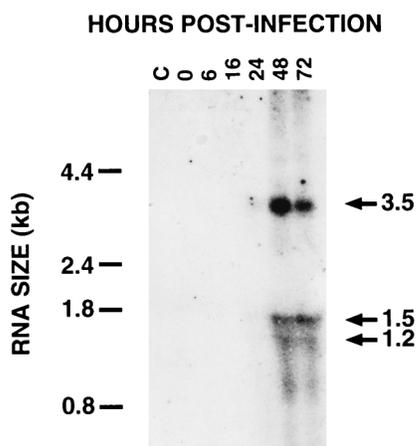


FIG. 5. Temporal analysis of the LdMNPV *enhancin* transcripts. *L. dispar* 652Y cells were infected with LdMNPV isolate A21-MPV, and cytoplasmic RNA was isolated at the times indicated. RNA (150 μg) was separated by formaldehyde-agarose gel electrophoresis, blotted, and probed with a strand-specific oligonucleotide complementary to positions 939 to 968 in the *enhancin* ORF (Fig. 2). RNA from uninfected cells was used as a control (lane C). RNA size standards are indicated on the left, and the sizes of the transcripts are indicated on the right.

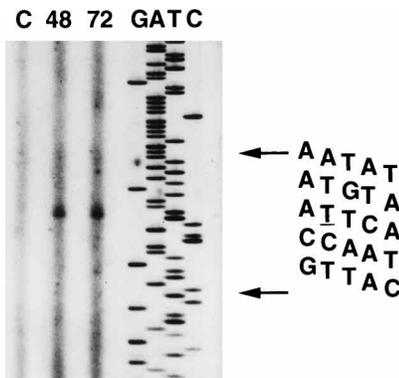


FIG. 6. Determination of the 5' ends of the *enhancin* transcripts. Primer extension analysis was performed for *enhancin* RNAs isolated at 48 and 72 h p.i. RNA (40 μg) was incubated with an end-labeled 18-bp primer that is complementary to the *enhancin* nucleotide sequence at positions 132 to 149 (Fig. 2). The primer was extended with Moloney murine leukemia virus reverse transcriptase. Extension products were fractionated by PAGE and visualized by autoradiography. The sequencing ladder was generated with the same primer. The late transcriptional start site is underlined. C, control.

length, in closer agreement with the size of the 2.7-kbp *enhancin* ORF in this virus (18).

To determine the transcriptional start site of the LdMNPV *enhancin* transcripts, primer extension reactions were performed with an 18-base oligonucleotide that is complementary to nucleotides 132 to 149 within the *enhancin* ORF (Fig. 2). Transcription initiates at the first A residue (position 38 in Fig. 2) within the consensus baculovirus late promoter sequence TTAAG (Fig. 6). Since only one start site was detected, it is possible that the large *enhancin* transcript initiates at this late promoter upstream of the *enhancin* ORF, proceeds through the *enhancin* and *hrf-1* genes, and terminates at a potential polyadenylation signal sequence (AATAAA) that has been identified downstream of the *hrf-1* gene (43). This polyadenylation signal sequence is 3.2 kbp downstream of the *enhancin* late promoter. Analysis of HaGV *enhancin* RNAs by RNase protection assay also indicated that the HaGV *enhancin* gene and the downstream *ORF1* gene may be part of the same bicistronic message (32) and therefore similar to *enhancin* gene transcription in LdMNPV.

**Construction of the *enhancin::cat* virus.** Since purified *enhancin* can increase the potency of the AcMNPV (45), a virus in which the LdMNPV *enhancin* gene was disrupted was constructed, in order to determine if the protein has an effect on LdMNPV potency. Plasmid pDB108 has the 4.3-kbp *Bam*HI/*Eco*RI fragment (located at 64.5 to 68.8 kbp on the viral genome) containing approximately 1.1 kbp of the portion of *enhancin* which encodes the N-terminal end (Fig. 7). It also contains approximately 3.2 kbp of upstream sequence and has an *Sst*I site 223 bp downstream of the *enhancin* ATG start codon (Fig. 2). The insert used to disrupt the gene was a fragment containing the *cat* gene that was isolated on a 0.7-kbp *Sst*I cassette from pDB4 (8). The promoter- and terminatorless *cat* gene was cloned into the *Sst*I site within the *enhancin* gene to generate pDB159. This construct has the *cat* gene inserted at codon 76 in the noncoding orientation with respect to the *enhancin* ORF (Fig. 7). Since the *cat* gene is not translationally fused and in the opposite direction to the *enhancin* ORF, it can be used only as a marker to identify the recombinant virus (through hybridization) and not for studies of gene expression from the *enhancin* promoter. Analysis of the DNA sequence of the insert predicts that a small transcript (approximately 454

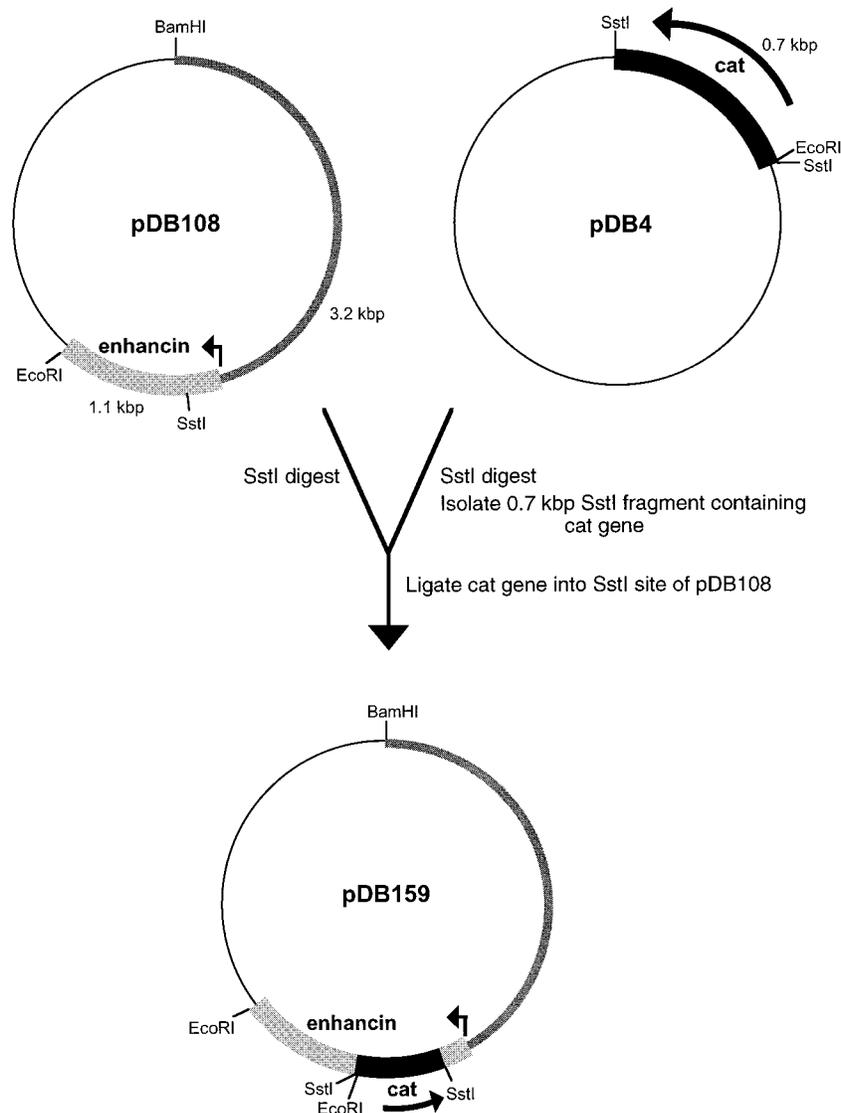


FIG. 7. Construction of the *enhancin::cat* virus. The 4.3-kbp *Bam*HI/*Eco*RI fragment (64.5 to 68.9 kbp on the viral genome) containing the N terminus-encoding portion of the *enhancin* gene was cloned from LdMNPV isolate A21-MPV into pUC18 to generate pDB108. A 0.7-kbp *Sst*I fragment containing the *cat* gene was cloned into the *Sst*I site within the *enhancin* gene to generate pDB159.

bp in length) could still be expressed that initiates at the *enhancin* late promoter and terminates at a consensus polyadenylation signal sequence present in the *cat* gene insert. A small fusion protein containing the first 76 aa of *enhancin* and 9 additional aa (from the *cat* gene insert) could be expressed from the *enhancin* promoter. This fusion protein does not contain the zinc-binding domain and is most likely not active.

The mutant was constructed and verified by Southern blot analysis with the 4.3-kbp *Bam*HI/*Eco*RI fragment as a probe (Fig. 8). Viral DNA from A21-MPV and the *enhancin::cat* virus was isolated and restricted with both *Bam*HI and *Eco*RI. In Fig. 8, lane 1, (A21-MPV), only the 4.3-kbp *Bam*HI/*Eco*RI fragment hybridized with the probe, as expected. The *enhancin::cat* virus (lane 2) has an additional *Eco*RI site at the start of the *cat* gene; therefore, two fragments hybridized in this isolate: (i) a 0.9-kbp *Eco*RI fragment and (ii) a 4.1-kbp fragment from the *Bam*HI to the *Eco*RI site at the beginning of the *cat* gene (Fig. 8). This result confirms that the mutation was transferred to

the viral genome and that the *enhancin* ORF was disrupted with the *cat* gene in this isolate.

**Bioassay analysis of the *enhancin::cat* virus.** Bioassays (diet incorporation and droplet feeding) were conducted with *L. dispar* larvae infected with either A21-MPV or the *enhancin::cat* virus. Both the diet incorporation and droplet feeding methods revealed that inactivation of the *enhancin* gene resulted in a drop in viral potency ranging from 1.4- to 4.0-fold as determined by Probit analysis (Table 1). Although it is hard to determine the exact degree of the drop in potency due to the close  $LC_{50}$ s for both viruses, in all four bioassays the *enhancin::cat* virus was less potent than A21-MPV. The fiducial limits of the  $LC_{50}$ s for A21-MPV and the *enhancin::cat* virus did not overlap in the droplet feeding bioassay. The droplet feeding bioassay was used to verify the results of the diet incorporation bioassays since the former method is more accurate for close  $LC_{50}$ s and yields smaller standard deviations than the diet incorporation method (22). The drop in potency in the four

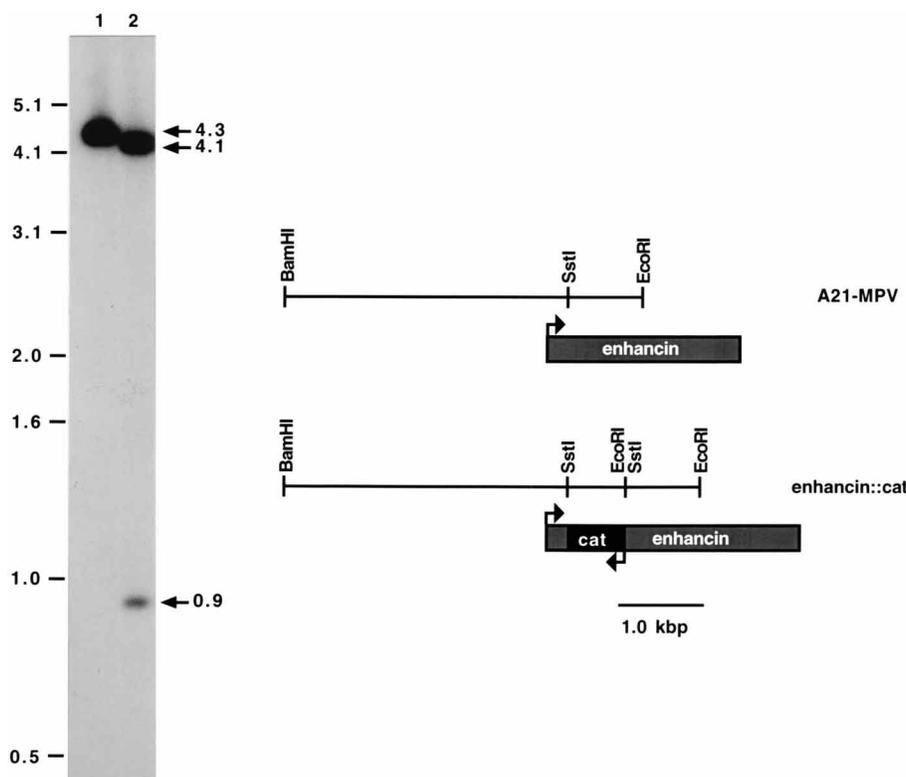


FIG. 8. Southern blot analysis of the *enhancin::cat* virus. Budded-virus DNA isolated from cells infected with either A21-MPV or the *enhancin::cat* virus was restricted with *Bam*HI and *Eco*RI. Digests were electrophoresed on a 1% agarose-Tris-borate-EDTA gel, blotted, and probed with the 4.3-kbp *Bam*HI/*Eco*RI fragment containing the N terminus-encoding portion of the *enhancin* gene. Lane 1, A21-MPV viral DNA; lane 2, *enhancin::cat* viral DNA. DNA size markers are indicated to the left (in kilobase pairs), and the positions of the viral fragments are indicated to the right. Partial restriction maps of both A21-MPV and the *enhancin::cat* virus are shown to the right, with the locations of the *enhancin* ORF and the inserted *cat* fragment indicated.

bioassays was analyzed by the unpaired *t* test ( $P = 0.0125$ ) and by analysis of variance ( $P < 0.05$ ) and was found to be significant. In the droplet feeding method, the volume ingested by neonate *L. dispar* larvae was determined to be  $176.5 \pm 37.1$  nl (data not shown). Based on this volume, the 50% lethal doses were  $17.9 \pm 2.4$  polyhedra per larva for the *enhancin::cat* virus and  $8.7 \pm 1.0$  polyhedra per larva for A21-MPV. Analysis of the time-mortality response showed similar killing speeds for the two viruses (data not shown). Therefore, the *enhancin* protein has an effect on the potency of the virus but not on the killing speed of the virus.

Previously it was reported that LdMNPV is evolutionarily more distant from AcMNPV than are other NPVs, based on

phylogenetic analysis of the known polyhedrin gene sequences from several baculoviruses (48). The finding of an *enhancin* gene in the LdMNPV supports the polyhedrin gene phylogeny. In addition, the presence of the LdMNPV *enhancin* gene may suggest that the LdMNPV is more closely related to the GVs than is the AcMNPV. Since the LdMNPV has an additional 29 kbp of DNA that is not present in the AcMNPV, it is possible that other homologous genes possessed by both the GVs and the LdMNPV will be identified as more sequence data become available.

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TABLE 1. Effect of *enhancin* on the biological activity of LdMNPV

Assay no. <sup>a</sup>	Virus	LC <sub>50</sub> (95% FL) <sup>b</sup>	Ratio <sup>c</sup>
1	A21-MPV	13.5 (7.6–24.0)	1.4
	<i>enhancin::cat</i> virus	19.0 (9.7–38.1)	
2	A21-MPV	24.6 (14.1–43.2)	3.1
	<i>enhancin::cat</i> virus	76.1 (23.6–287.2)	
3	A21-MPV	6.7 (3.9–11.3)	3.2
	<i>enhancin::cat</i> virus	21.2 (11.1–40.9)	
4	A21-MPV	45.4 (31.1–68.6)	4.0
	<i>enhancin::cat</i> virus	181.6 (78.7–232.6)	

<sup>a</sup> Assays 1 to 3, diet incorporation; assay 4, droplet feeding.

<sup>b</sup> Values are numbers of polyhedra per microliter of diet (assays 1 to 3) or solution (assay 4). FL, fiducial limits.

<sup>c</sup> *enhancin::cat* virus LC<sub>50</sub>/A21-MPV LC<sub>50</sub>.

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