Molecular Analysis of an enhacin 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) enhacin proteins that are capable of enhancing the infection of other baculoviruses. Enhacin 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 enhacin 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 enhacin gene transcripts are expressed at late times postinfection from a consensus baculovirus late promoter. The LdMNPV enhacin exhibits 29% amino acid identity to the enhacin 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 (enhacin::cat) was constructed in which the LdMNPV enhacin gene was inactivated by insertion mutagenesis in order to ascertain the effect of the enhacin protein on viral potency. The bioassay results indicate that disruption of the enhacin 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).

Enhacin, 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 enhacin 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 enhacin 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, enhacin 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 enhacin proteins. First, the enhacinns 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 enhacin 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 enhacin 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 enhacin 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 enhacin epitope on viral pathogenicity by inactivating the enhacin gene through insertional mutagenesis.

MATERIALS AND METHODS

Cells, virus, and insects. Lymantria 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 enhacin::cat virus (in which enhacin is disrupted by a chloramphenicol acetyltransferase [cat] gene). Ly. 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 Ly. dispar 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 [α-32P]dCTP (NEB).
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. S^32P-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 SstII fragment containing the enhancin gene was cloned into pBluescript SK- (Stratagene) to generate pDB126. The enhancin protein was expressed from pDB126 with the TNT coupled reticulocyte lysate system and T7 RNA polymerase (Stratagene) to generate pDB126. The enhancin protein was expressed from L. dispar gene from pDB4 (8). For further details, see Results and Discussion.

RESULTS AND DISCUSSION

Characteristics of the nucleotide sequence. The nucleotide sequence of the 2,460-bp fragment containing the LdMNVP 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 mapping 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 EcoRI 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 SstII 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 LdMNVP 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.

Identification of the enhancin gene. The enhancin gene was identified during the mapping and sequencing of the gene mutated in LdMNVP few polyhedron mutants. Initially these mutations were mapped to a 4.3-kbp BamHI/EcoRI fragment located at 64.5 to 68.9 kbp on the viral genome. Further mapping 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 EcoRI 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 SstII 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 LdMNVP 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.

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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 enhancins, 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 at the C-terminal end (Fig. 3A). The TnGV and PuGV enhancins are virtually identical, with only 15 residue changes between the proteins for an overall 98.0% amino acid identity (18, 32). The HaGV enhancin protein is less homologous to the other two GV proteins (TnGV and PuGV), with 81.6% amino acid identity (89.0% over the first 550 aa and 69.0% over the last 350 aa) and 90.0% similarity when conservative residue changes are taken into consideration (Fig. 3B) (32).

The LdMNPV enhancin protein shows approximately 31.0% amino acid identity 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 55.0% similarity to the TnGV protein, 55.2% to the PuGV protein, and 55.6% to the HaGV protein. Overall, the four proteins exhibit 29.0% amino acid identity, with four areas exhibiting greater than 50% amino acid identity (residues 59 to 67 at 78.0%, 196 to 290 at 51.0%, 451 to 468 at 58.0%, and 692 to 704 at 54.0% [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 enhancin protein). The TnGV and PuGV enhancin proteins exhibit greater than 50% amino acid identity with the other two GV proteins (TnGV and PuGV), with 81.0% 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 29.0% amino acid identity, with four areas exhibiting greater than 50% amino acid identity (residues 59 to 67 at 78.0%, 196 to 290 at 51.0%, 451 to 468 at 58.0%, and 692 to 704 at 54.0% [Fig. 3B]).

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 SstI 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 the enhancin protein.

The transcribed RNA was prepared from the RNAse inhibitor-containing transcription reaction. The reaction mixture contained the RNAse inhibitor and 500 units of T7 RNA polymerase in standard transcription buffer. The RNAse inhibitor was added to the transcription reaction to prevent degradation of the newly synthesized RNA. The RNAs were separated into two lanes, one for each transcription reaction. The RNAse inhibitor was added to one lane to prevent degradation of the RNA, while the other lane was used to determine the extent to which the RNAse inhibitor protected the RNA. The RNAse inhibitor protected the RNA from degradation, indicating that the RNA was not degraded. The RNA was then transferred to a nitrocellulose membrane and hybridized with a DNA probe specific for the enhancin gene. The DNA probe was labeled with Dolichos biflorus lectin (DBL) and hybridized to the RNA. The DBL was used to probe the RNA to determine the extent to which the RNA was degraded. The DBL was labeled with a radioactive isotope and hybridized to the RNA. The RNA was then visualized using autoradiography. The DBL was hybridized to the RNA, indicating that the RNA was not degraded. The DBL interacted with the enhancin gene and the predicted amino acid sequence. The consensus late promoter sequence is shaded, with the late transcriptional start site underlined (see text).
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. LdMNPV enhancin is compared to the three GV proteins, while TnGV and PuGV enhancins are compared with the HaGV protein.
The enhancin protein is indicated to the right. Molecular mass standards are indicated to the left, and the position of the enhancin protein is indicated to the right.

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 [35S]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.

Fig. 5. Temporal analysis of the LdMNVP enhancin transcripts. L. dispar 652Y cells were infected with LdMNVP 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.

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 LdMNVP 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 LdMNVP 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 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 LdMNVP 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 LdMNVP.

Construction of the enhancin::cat virus. Since purified enhancin can increase the potency of the AcMNVP (45), a virus in which the LdMNVP enhancin gene was disrupted was constructed, in order to determine if the protein has an effect on LdMNVP potency. Plasmid pDB108 has the 4.3-kbp BamHI/EcoRI 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 SstI 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 SstI cassette from pDB4 (8). The promoter- and terminatorless cat gene was cloned into the SstI 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...
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 BamHI/EcoRI fragment as a probe (Fig. 8). Viral DNA from A21-MPV and the enhancin::cat virus was isolated and restricted with both BamHI and EcoRI. In Fig. 8, lane 1, (A21-MPV), only the 4.3-kbp BamHI/EcoRI fragment hybridized with the probe, as expected. The enhancin::cat virus (lane 2) has an additional EcoRI site at the start of the cat gene; therefore, two fragments hybridized in this isolate: (i) a 0.9-kbp EcoRI fragment and (ii) a 4.1-kbp fragment from the BamHI to the EcoRI 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 LC50s for both viruses, in all four bioassays the enhancin::cat virus was less potent than A21-MPV. The fiducial limits of the LC50s 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 LC50s and yields smaller standard deviations than the diet incorporation method (22). The drop in potency in the four

**FIG. 7.** Construction of the enhancin::cat virus. The 4.3-kbp BamHI/EcoRI 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 SstI fragment containing the cat gene was cloned into the SstI site within the enhancin gene to generate pDB159.
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 LdNPV is evolutionarily more distant from AcNPV 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 LdNPV supports the polyhedrin gene phylogeny. In addition, the presence of the LdNPV enhancin gene may suggest that the LdNPV is more closely related to the GVs than is the AcNPV. Since the LdNPV has an additional 29 kbp of DNA that is not present in the AcNPV, it is possible that other homologous genes possessed by both the GVs and the LdNPV will be identified as more sequence data become available.

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REFERENCES


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 BamHI and EcoRI. Digests were electrophoresed on a 1% agarose-Tris-borate-EDTA gel, blotted, and probed with the 4.3-kbp BamHI/EcoRI 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.

TABLE 1. Effect of enhancin on the biological activity of LdNPV

<table>
<thead>
<tr>
<th>Assay no.</th>
<th>Virus</th>
<th>LC$_{50}$ (95% FL)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A21-MPV</td>
<td>13.5 (7.6–24.0)</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>enhancin::cat virus</td>
<td>19.0 (9.7–38.1)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>A21-MPV</td>
<td>24.6 (14.1–43.2)</td>
<td>3.1</td>
</tr>
<tr>
<td>4</td>
<td>enhancin::cat virus</td>
<td>76.1 (23.6–287.2)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>A21-MPV</td>
<td>6.7 (3.9–11.3)</td>
<td>3.2</td>
</tr>
<tr>
<td>6</td>
<td>enhancin::cat virus</td>
<td>21.2 (11.1–40.9)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>A21-MPV</td>
<td>45.4 (31.1–68.6)</td>
<td>4.0</td>
</tr>
<tr>
<td>8</td>
<td>enhancin::cat virus</td>
<td>181.6 (78.7–232.6)</td>
<td></td>
</tr>
</tbody>
</table>

a Assays 1 to 3, diet incorporation; assay 4, droplet feeding.
b Values are numbers of polyhedra per microliter of diet (assays 1 to 3) or solution (assay 4). FL, fiducial limits.
c enhancin::cat virus LC$_{50}$/A21-MPV LC$_{50}$


