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Lymantria dispar Nuclear Polyhedrosis Virus Homologous Regions: Characterization of Their Ability To Function as Replication Origins†

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Homologous regions (*hr*s) were identified in the *Lymantria dispar* nuclear polyhedrosis virus (Ld*M*NPV) genome. A 1.58-kb region surrounding *hr*4 was sequenced and found to have two distinct domains. Domain I (about 600 bp) is composed of seven repeats of about 80 bp including a series of palindromes containing *Mlu*I sites and overlapping *Xho*I and *Sac*I sites. Domain II (about 700 bp) is composed of eight partially repeated sequences of 60 to 100 bp containing a 15- to 25-bp sequence that is 80 to 100% A+T in addition to a 6- to 10-bp palindrome containing an *Nru*I site. Hybridization of a domain I sequence to cosmids containing the Ld*M*NPV genome indicated its presence at eight positions (*hr*1 to -8) on the genome. In contrast, hybridization of domain II indicated that it was present only at the *hr*4 locus. A *Dpn*I-based transient-replication assay was used to determine if subclones of *hr*4 transfected into Ld*M*NPV-infected *L. dispar* cells functioned as replication origins. Subclones of *hr*4 containing either domain I or domain II replicated at very low or moderate levels, respectively. However, when domain I and domain II were linked on the same plasmid, high levels of replication were observed. A 1.4-kb region containing *hr*1 was also sequenced. It lies immediately upstream of the polyhedrin gene and contains six domain I-type repeats. Four-hundred-base-pair regions of domain I repeats from *hr*1 and *hr*4 showed 89% sequence identity. Plasmids containing the *hr*1 domain I replicated at low levels. However, hybrid plasmids in which the AT-rich *hr*4 domain II was inserted adjacent to *hr*1 domain I replicated to high levels, indicating that the AT-rich domain II greatly enhances replication. The orientation and position of domains I and II relative to each other did not have major effects on the levels of replication.

The *Baculoviridae* is a diverse family of insect viruses with large (85- to 166-kb) double-stranded, circular, supercoiled DNA genomes (4). Although several members of this family have achieved widespread use as expression vectors, little is known about the mechanism by which their genomes are replicated. Recently, sequences in the *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (Ac*M*NPV) genome that function as replication origins when cloned into plasmids and transfected into infected cells have been identified (15, 16, 19, 24). These sequences are composed of two to eight repeats of 30-bp imperfect palindromes that are located at seven positions on the Ac*M*NPV genome. The repeats are called homologous regions (*hr*s), and similar sequences are found in the genomes of *Bombyx mori* NPV (21), a virus closely related to Ac*M*NPV, and a more distantly related NPV pathogenic for *Choristoneura fumiferana* (18). In addition, an Ac*M*NPV sequence lacking *hr*s that also functions as an origin in transient-replication assays but replicates less efficiently than *hr*-containing plasmids has been identified (16, 19). *hr*s have also been implicated as enhancers of Ac*M*NPV early gene expression (12, 26). Although *hr*s appear to be important components of the Ac*M*NPV genome, deletion of *hr*5 from the Ac*M*NPV or *B. mori* NPV genome (21, 26) had no apparent effect on the ability of the virus to replicate in cell culture. The *Orgyia pseudotsugata* *M*NPV (Op*M*NPV) has a genome similar in size to that of Ac*M*NPV (about 134 kbp), and major regions of the Op*M*NPV and Ac*M*NPV genomes show colinear patterns of gene organization (10, 20). The Op*M*NPV genome contains repeated sequences about 50% similar to Ac*M*NPV *hr*s, and one of these has been shown to enhance early gene transcription (31). Recently, we found that the Op*M*NPV *hr*s are capable of conferring upon plasmids the ability to undergo Op*M*NPV-infection-dependent replication (unpublished data).

The genome of the *Lymantria dispar* (gypsy moth) *M*NPV (Ld*M*NPV) (29) is about 25% larger (166 kb) than those of Ac*M*NPV and Op*M*NPV and has a substantially higher G+C content of about 60% (22). It has been reported that the Ld*M*NPV genome also contains *hr*s (29). In this report we describe the identification and mapping of *hr*s in the Ld*M*NPV genome. Two of the *hr*s were sequenced, and their ability to act as replication origins in a transient-replication assay was examined.

MATERIALS AND METHODS

Virus, cell line, cosmids, plasmids, and deletion mutagenesis. Ld*M*NPV strain 56-1 was used for infections. Ld*M*NPV cosmids were constructed with a partial *Pst*I or *Cla*I digest of DNA from the Ld*M*NPV clonal isolate CI 5-6 (28) cloned into the cosmid vector pHC79 (14). Both Ld*M*NPV and cosmids were supplied by the U.S. Forest Service Laboratory in Delaware, Ohio.

The *L. dispar* (Ld-652Y) cell line was propagated in TNM-FH medium (30) supplemented with 10% fetal bovine serum, penicillin G (50 U/ml), streptomycin (50 μg/ml; Whittaker Bioproducts), and amphotericin B (Fungizone; 375 ng/ml; Flow Laboratories).

All plasmid subcloning was done in pBlueScribe⁻ (pBS⁻) (Stratagene, Inc.) modified by the addition of a *Bgl*II site (10). The *hr*4-A clones were produced by exonuclease III deletions (13) of a *Pst*I-*Bam*HI digest of a clone containing the *Pst*I-*Eco*RI fragment of *Eco*RI-K (see Fig. 7). The *hr*4-B clones were produced by digesting *hr*4 A2 DNA (see Fig. 2) with *Eco*RI and other selected restriction enzymes, blunting the DNA with S1 nuclease and Klenow, and then religating the DNA (13). pGR80 was produced as described above with an *Nhe*I-*Eco*RI digest of a *Bam*HI-*Eco*RI clone containing *hr*4. pGR83 was produced by digesting the *Bam*HI-*Eco*RI *hr*4 clone (see Fig. 2) with *Xba*I and *Nhe*I and

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          BamHI
1         GGATCCGCCCGGCTCAGACATCGCGCCTCGGCGGACGCCGAGGCCGGAATCGGGTTTG
               XhoI/SacI       MluI          MluI         HincII
59    A    CTCGAGCTCGGCGGACGCCG--------------------GGGTCGATCGGGTTCAAACC-GATGACATCATGCCCAAA
117   B    CTCGAGCTCGGCGGACGCGTAAGATTTTACGCGTCGGCAGGGGTTAACAGAGTTCAAGCC-GATGACATCATGCCCAAA
194   C    CTCGAGCTCGGTGGACGCGTAAGATTTTACGTGCCGGCAGGGGTTAACAGAGTTCAAGCC-GATGAGATCATGCCCAAA
274   D    CTCGAGCTCGGCGGACGCGTAGGATCTTACGCGTCGGTTAGGGTCGATGGAGGTCAAGCC-GATGACATCACGCCTAAA
331   E    -CTGAGCTCGGCGGACGCGTAAGATTT-ACGTGCCGGCAAGGGTCAATCGAGTTCAAACCCGATGACATCACGCCCAAA
428   F    CTCGAGCTCGGCGGGCGCGTAGGATTTTACGTGGCGGCAAGGGTCGATGGAGGTCAAGCC-GATGACATCATGCCTTAA
506   G    CTTGAGCTCGGCGGACGCGTAAGATTTTACGTGCCGGCAAGGGTCAACAGAGTTCAAGCCCGATGAGATCATGCC
                                                        NheI
581        GCCGCGATACTCGTGTCCATGACATCATGCTGCCGTCCATGCTAGCGAGACGGCTCG
                                                                      NruI                        NruI
638   I    TTAGATTTAAATAAAAATAAATTTT--------------GCGAGCACACCAGCTTCGCGAGCCGGCTCGCGC----GCAGGCTCGCGACCGGTGG
714   II   ATTGGATTTTAAAAATTATTT----GGCGTG---CGATAAATTCATTTCCGTGCTCGCGAGCCGGATCGTGT----GCGTGCTCGCGAGCCGGACGAACGAGCCGGTGGC
813   III  TTAAAATCTTTTTAAAATTTATTT-GGCGCGTGCGATTAGACTTATTTCCGTGCTCGCGAACGAGCCGGTGGC    SphI
885   IV   TTAAAATCTTTTTAAAATTTATTTT-GCGCGTGCGATTAGACTTATTTCCGTGCTCGCGAGCGAGCCGGATCGTGTGCATGCTCGCGGCGG
975   V    ATTAAAAATCTTTTTAAAAATA---------CGCGATTAGACTTATTTCCGTGCTCGCGAGCGAGCCGCTGG
1038  VI   ATTGAAATCTTTTAAAAATTATTT----GGCGTGC------CTTATTTCCGTGCTCGCGAGCCGGTCCGTG----TGCATGCTCGCGAACCGGTGG
1120  VII  ATTAAAAATCTTTGAAATTTATTT---------CGATTGGACTTATTGC-GTGCTCGCGAGCCGGCTCGTG----TGCATGCTCGCGGATTTCCTTTGCGTG
                                                       CCGTGCTCGCGAGCCGGCTCG
1194  VIII ATTAAAGATAACGTTCGCTTTAAAAAAAATATTTTTATTGGCGGGCGATTAGTTTAGGCAGAGCGATCCCCGC
                HincII
1308  SacI/XhoI GTTAACAACAACAACATGACCGAAAAGGCTCTGTTTGAAGATTTCGACTTCGCCGACGGC
1368  GAGCTCGAGCGGTGGTGCAACTACGTCGAGACGACGGACGCGAAGCGGCGACGCGTCGAGGAGAAGGCGGCGG
1441  CGGCGGCGTCGATGCTGTTGAACGCCAAACAGCAGTACATTTTCGACTATTTCACGCAAAGGGACTCCTTCGCGCCCGTGTTCGTGAGCGGCAGCCCCGGCACCGGCAAAAGCCGCCCTG
1561  CTGATGGCGCTGCACGAATTC EcoRI
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FIG. 1. Nucleotide sequence of Ld*M*NPV *hr*4 showing alignment of repeated sequences. Domain I repeats are labeled A to G, whereas domain II repeats are labeled I to VIII. Selected unique restriction sites are underlined. Multiple restriction sites present as components of the repeats are indicated as follows: *Xho*I, thin underline; *Sac*I, double underline; *Mlu*I, dashed underline; *Nru*I, dotted underline; *Sph*I, wavy underline; *Hin*cII, thick underline. Gaps are indicated by dashes. Nucleotide sequence positions are indicated to the left.

religating the DNA. Clones in which the insert was religated in the opposite orientation (pGR83) or in which domain I (nucleotide [nt] 1 to 616) from the *hr*4 region was deleted (pGR97) were selected. The *Pst*I-*Sac*II and pGR76 clones were produced by gel purifying the *Pst*I-*Sac*II and *Sac*II-*Fsp*I fragments from the parent 2.3-kb *Pst*I fragment, blunting the DNA by treatment with S1 nuclease and Klenow polymerase, and cloning the DNA into *Sma*I-digested pBS$^-$ (see Fig. 4A). pGR82 is an exonuclease III deletion mutant derived from a *Kpn*I-*Bam*HI digest of the parent 2.3-kb *Pst*I clone. To clone *hr*4 domain II adjacent to *hr*1 domain I, pGR97 (see above) was digested with *Hin*cII to yield a fragment starting from a *Hin*cII site in the polylinker and terminating with the *Hin*cII site at nt 1310 (Fig. 1). This fragment was gel purified, dephosphorylated with calf intestinal phosphatase (New England Biolabs), ligated to *Eco*RI linkers, and digested with *Eco*RI. The resulting fragment was then gel purified and cloned into the *Eco*RI site of pGR82 to yield plasmids pGR85 and pGR86 (see Fig. 4A). *Escherichia coli* DH5α (27) was used for plasmid production. Plasmid and cosmid DNAs used for transfections were purified on Qiagen columns or by a polyethylene glycol purification protocol (see below).

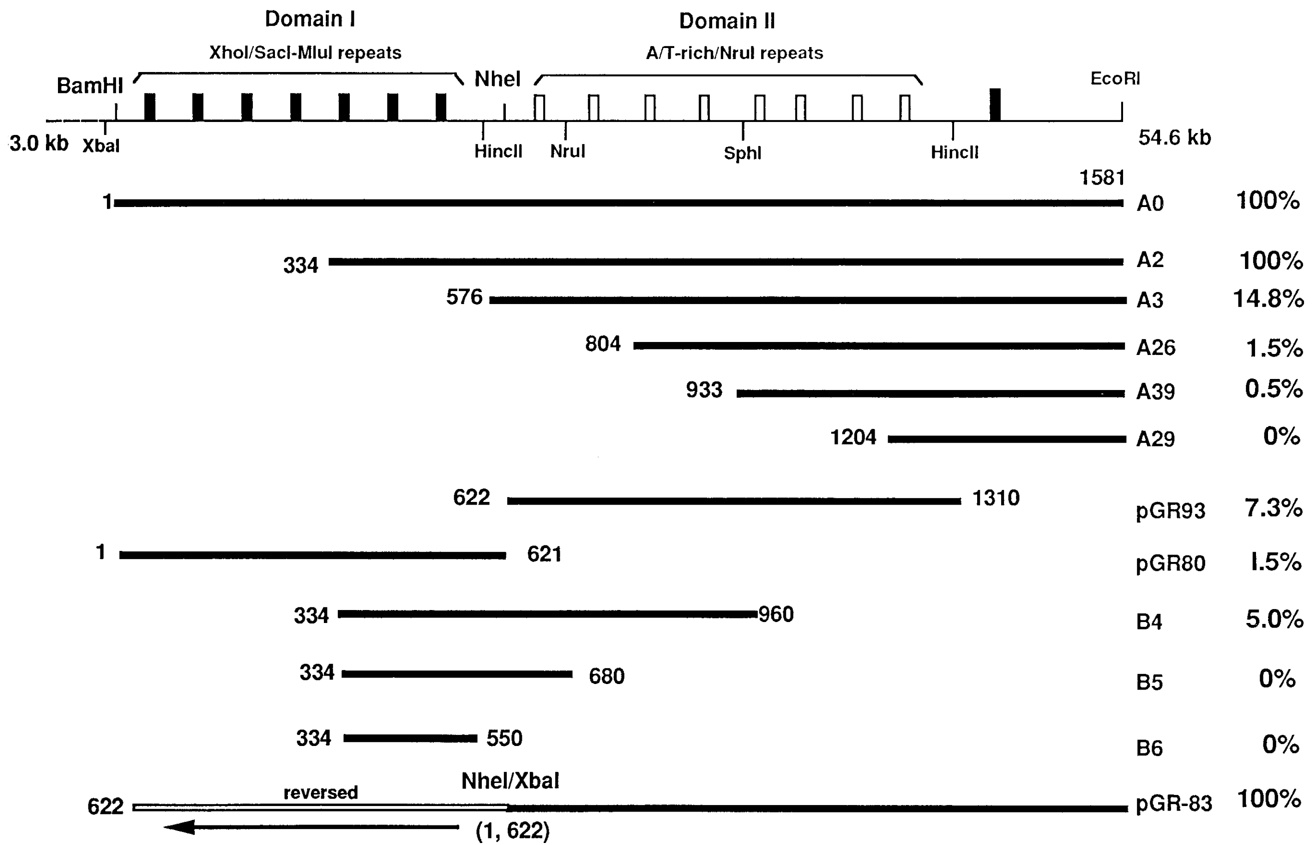
DNA sequence analysis. Sequencing reactions were performed with the Taq DyeDeoxy(TM) Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.) according to the manufacturer's protocol with the exception that the reactions were performed in 5% dimethyl sulfoxide and a higher denaturation temperature (97°C) was used. A Perkin-Elmer Cetus model 480 or model TC1 thermal cycler was used. Reaction mixtures were electrophoresed and analyzed on an Applied Biosystems, Inc., model 373A automated DNA sequencer.

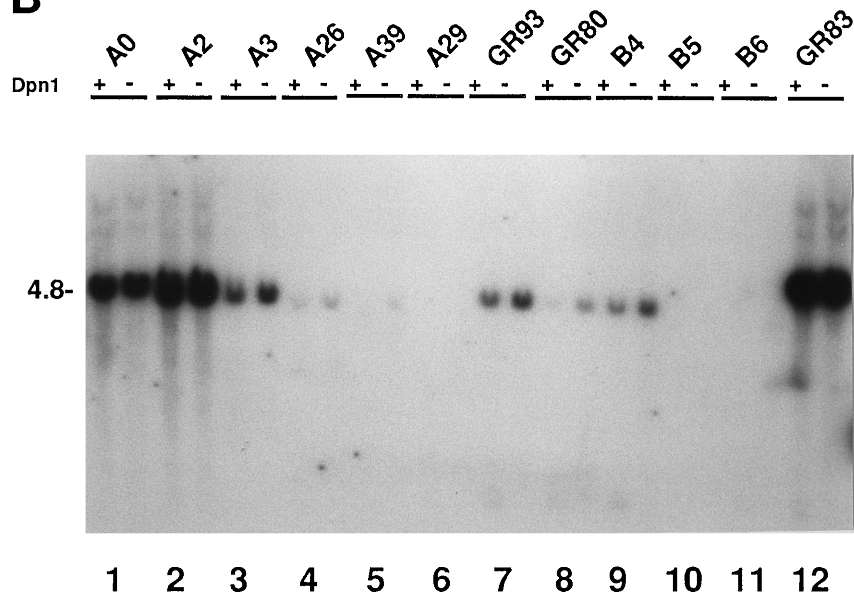
Plasmid templates for sequencing were prepared by a protocol recommended by Applied Biosystems, Inc. It involves sodium dodecyl sulfate (SDS)-alkaline lysis followed by polyethylene glycol precipitation. The nucleotide sequence and the predicted protein sequence were analyzed with the Genetics Computer Group suite of sequence analysis programs (8).

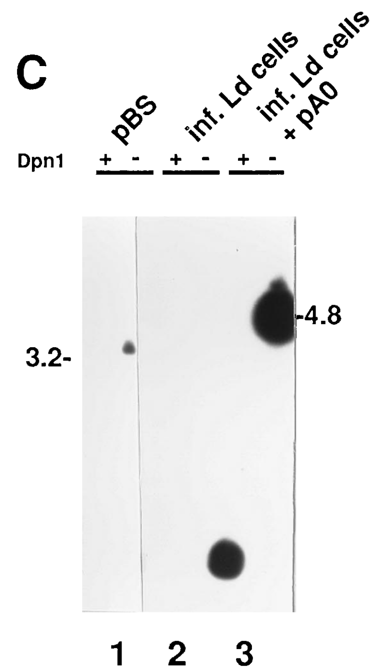
Enzymes and isotopes. Restriction enzymes and DNA-modifying enzymes were purchased from Bethesda Research Laboratories, New England Biolabs, and United States Biochemical and were used according to the manufacturers' instructions. All isotopes were purchased from New England Nuclear, Inc.

Replication assay with virus-infected cells. *L. dispar* cells (1.2×10^6 cells per well in six-well culture plates) incubated at 27°C in TNM-FH medium (30) were transfected with plasmid DNA by using calcium phosphate (11, 30), similar to the protocol of Kool et al. (16). A total of 0.8 μg of pA0 (see Fig. 2A) was used for transfection, and the amounts of other plasmid DNAs were adjusted to reflect equimolar amounts relative to pA0. Four hours later, the transfection mixture was replaced with fresh medium and the cells were incubated at 27°C for 24 h. The cells were then infected with Ld*M*NPV at a multiplicity of infection of 5. Four hours after infection the virus-containing medium was replaced with fresh medium. After incubation at 27°C for 96 h, total cell DNA was purified by resuspending the cells in 450 μl of 10 mM Tris (pH 7.8)–0.6% SDS–10 mM EDTA containing pronase E (Sigma) (2 mg/ml) and incubated at 37°C overnight. The digest was then phenol-chloroform-isoamyl alcohol extracted, ethanol precipitated, and resuspended in 50 μl of TE (10 mM Tris [pH 8], 1 mM EDTA). Duplicate samples (10 μl) of each DNA were treated with *Hin*dIII (10 U) with or without *Dpn*I (40 U) in a total volume of 20 μl at 37°C overnight. Before electrophoresis, all samples were digested for 10 min with RNase A (Sigma, Inc.) (5 μg per sample). To ensure that *Dpn*I digestion was complete under these conditions, 10 μl of infected-cell DNA was mixed with 0.01 μg of pA0 and digested in the same manner as the other samples (see Fig. 2C, lane 3). The digested DNA was electrophoresed through 0.7% agarose gels, blotted onto GeneScreen Plus (Dupont, Inc.), hybridized (16 to 18 h), and washed under

FIG. 2. Infection-dependent replication of deletion clones of *hr*4. (A) Schematic diagram of deletion clones used in the analysis of *hr*4. A restriction map of *hr*4 is shown at the top. Numbers indicate position (in kilobases) on the genome. Domain I is shown with the locations of the *Xho*I-*Sac*I-*Mlu*I restriction sites indicated by solid rectangles. Domain II is shown with the locations of the AT-rich *Nru*I sequences with open rectangles. Selected restriction sites used for constructing deletion mutants are also indicated. The deletion mutants are diagrammed below the map, and the numbers indicate the location of the deletion junction from the sequence shown in Fig. 1. The names of the clones and their relative replication efficiencies are at the right. Relative replication was estimated with a PSI-486 Phosphorimager SI & Imagequant Workstation (Molecular Dynamics) by using the Scanner Control SI-PDSI version 1.0 and Imagequant 4.1 software packages. Values for plasmids showing high levels of replication (A0, A2, and pGR83) were arbitrarily set at 100%. Values for the other plasmids represent signals relative to an average of the values for signals from plasmids A0, A2, and pGR83. Exonuclease III deletions were constructed from the *Bam*HI site by using *Bam*HI-*Pst*I digests of a *Pst*I-*Eco*RI clone of *Eco*RI-K in pBS$^-$. Selected restriction enzyme sites in conjunction with blunt-end ligation were used for constructing the deletions from the right. (B) Replication assay of plasmids from *hr*4. The clones are indicated at the top of the panel for each pair of lanes. Each sample was either treated with *Dpn*I or left untreated as indicated by the plus or minus sign above each lane. The size of the linearized parent plasmid pA0 is indicated beside the lanes at 4.8 kb. (C) Replication controls for panel B and subsequent experiments include pBS$^-$ transfected into Ld*M*NPV-infected *L. dispar* cells (lane 1), DNA from infected *L. dispar* cells (lane 2), and a *Dpn*I digestion control containing pA0 DNA (0.01 μg) mixed with DNA from Ld*M*NPV-infected *L. dispar* cells and digested under standard conditions (lane 3). These controls show that pBS$^-$ transfected into Ld*M*NPV-infected *L. dispar* cells does not replicate (lanes 1), there is no hybridization to Ld*M*NPV-infected *L. dispar* cells (lane 2), and *Dpn*I digestion was complete (lane 3).


A
Domain I
XhoI/SacI-MluI repeats
Domain II
A/T-rich/NruI repeats
BamHI
NheI
EcoRI
3.0 kb
XbaI
HincII
NruI
SphI
HincII
54.6 kb
1 1581 A0 100%
334 A2 100%
576 A3 14.8%
804 A26 1.5%
933 A39 0.5%
1204 A29 0%
622 1310 pGR93 7.3%
1 621 pGR80 1.5%
334 960 B4 5.0%
334 680 B5 0%
334 550 B6 0%
reversed
NheI/XbaI
622 pGR-83 100%
(1, 622)


B
A0 A2 A3 A26 A39 A29 GR93 GR80 B4 B5 B6 GR83
Dpn1 + - + - + - + - + - + - + - + - + - + - + - + -
4.8-
1 2 3 4 5 6 7 8 9 10 11 12


C
pBS
inf. Ld cells
inf. Ld cells + pA0
Dpn1 + - + - + -
3.2-
-4.8
1 2 3

```
PstI     .         .         .         .         .         .         .         .         .        .HincII   .         .
CTGCAGCCGCCGTTCGGCGGATTTCACGCGAGGCAACTACGGCCGCATCTTGTTTTCCCAATTCAAAAAATTTAAATTGGGCAGCACGACGGTGCTGCTGGTCAACGCGGAGAAGAACCA  120
         .         .         .         .         .         .         .         .         .         .         .         .
CGACGAATCGGTGCATCGCTACCGCCGAGAGAGATGGCGCCGGCTGAACGAACGCCTGATCTTCCACCACTACGATTGTTTTCCGAACAAGGCGCGGACGATCGTGGCGGGCTCGTGCCC  240
         .         .    NruI.        .         .         .         .         .         .       SphI        .         .
GTCGCTGGGCGGGGACGCGAGTTTTCGCGAATTGGCGCACGGAGGTCACGGCTTCGGCGAAGAGTCCATGTACACGGACGAGCTGACGCTGTTCTGCATGCACCACAGACTCAAGATGGC  360
         .         .         .     NruI          .         .         .         .         .         .         .         .
CAACACGTTCGCCGAGAATTTGGAAACGCCCGACGATCGCGAGTTTTTCTTGACCAAGCACATCGACGCGGTCGAGCTGCGGTACGTGAGCGTTTGCAACCTGAGGCTGTTGGTGATGGA  480
         .         .         .         .         .         .         .         .         .         .         .         .
CGCGTGCGTCGAGACCGACCGGTTCGACTTTGACTACATCCCCAGACAAGTCCCCGGCCTCGGCTATTTCGACAACGAAGACCTGAGGAGGCGCAGAGGCCATTACAAGAGAACGCTCAC  600
         .         .         .         .         .         .         .         .         .         .         .         .
CAAGCTGGGCCTGTCCAAAGTGATGTCGGACCAGCGCCAATTCGTGACGTCGAAACTGTACGAATACTCCAAGTCTCTAAGTTCGGTGTACGACACAAACGGAAAGCACGCACTGCAAGA  720
         .         .         .     SacII         .         .         .         .         .         .         .         .
TTCCATAAACGACCTGCTGACCTATCACTACTGACCGCGGAATTTAAAACCTTTAGAATTGTAATTATTAATAAACGCTTGTAACCATTATGAAATTGTTTTTTAAACCTTCGCGCGCG  840
         .         .         .    MluI .         .       SacI/XhoI .         .         .         .         .         .
AGCGGGCTCGACCTCGATGATCGACTCCGGCCGACGCGTAAAATCCTACGCGCCCGCCGAGCTCGAGCTTGGGCGTGATGTCATCGGTACAAGCATGATCTCATCGGGCTTGAACCCGAT  960
         .         .     MluI     SacI/XhoI  .         .         .     HincII     . MluI   .     MluI        .
TGACCCTTGCCGGCGCGTAAAATCCTACGCGTCCGCCGAGCTCGAGTTAAGGCATGATCTCATCGGGCTTGAACTCTGTTGACCCTAGCCGACGCGTAAAATCTTACGCGTCCGCCGAGC 1080
SacI/XhoI.         .         .         .         . MluI    .     MluI.     SacI        .         .         .         .
TCGAGTTTGGGCATGATGTCATCGGGCTTGAACTCGATCGACCCTAGCCGACGCGTAAAATCTTACGCGTCCGCCGAGCTCAGTTTAGGCGTGATCTCATCGGGCTCGACCTCCATCGAC 1200
         .         .   MluI  .     SacI .        .         .         .         .      MluI          . MluI    . SacI/XhoI
CCTGGCCGGCACGTAAAATCCTACGCGTCCGCCGAGCTCAGTTTAGGCATGATGTCATCGGGTTTGAACTCGATCGACACCGGCCGACGCGTAAAATCTTACGCGTCCGCCGAGCTCGAG 1320
         .         .         .         .         .         .     FspI          .         .         .         .         .
CTTGGGCGTGATCTCATCGGGCTCGAACCCGATCTCTTTAATATTTGGTATCAATAAAAGTCGATTTGCGCA                                                 1392
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FIG. 3. Nucleotide sequence analysis of Ld*M*NPV *hr*1. The major restriction sites are indicated.

stringent conditions (27). For hybridization, pKS$^-$ DNA labeled with [^{32}P]dCTP was used (9) as the probe. The film was exposed for 16 to 24 h at -80°C with an intensifying screen. Hybridization to locate *hr* sequences on cosmid blots was done as described above.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the following accession numbers: D38306 for *hr*1 and D38307 for *hr*4.

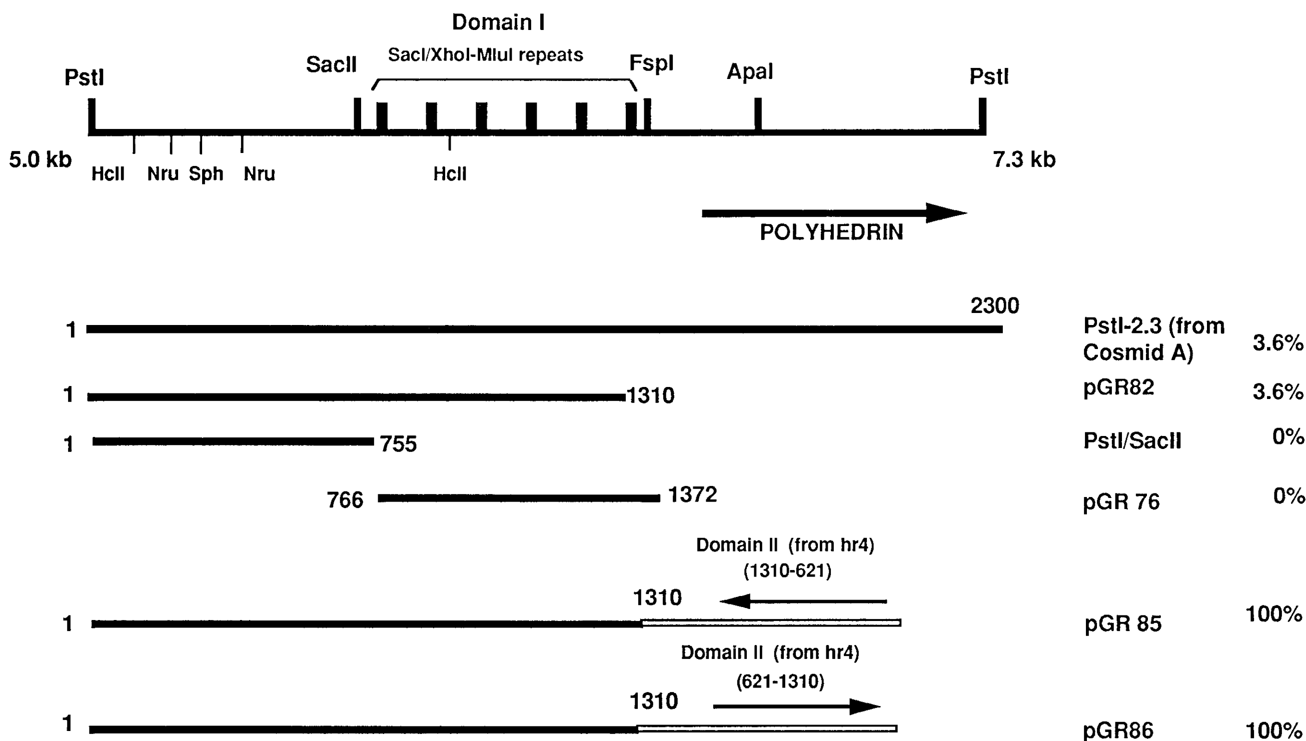
RESULTS

Nucleotide sequence analysis of *hr*4. It was previously reported (29) that the *Pst*I 2.3-kb fragment containing the polyhedrin gene cross-hybridized with four other regions of the genome, including a fragment that corresponds to *Eco*RI-K in Ld*M*NPV strain CI 5-6. Because evidence suggests that *hr*s in Ac*M*NPV may serve as sites for the initiation of genome replication, we examined the ability of plasmids containing the *Eco*RI-K or the *Pst*I 2.3-kb fragment to undergo replication when transfected into Ld*M*NPV-infected *L. dispar* cells. We found that *Eco*RI-K replicated with high efficiency in a *Dpn*I-based replication assay and that the replication origin was located on a 1.58-kb *Bam*HI-*Eco*RI fragment at the right end of *Eco*RI-K (see below). This fragment was sequenced and was found to contain two different repeated domains (Fig. 1). Domain I (nt 1 to 621) is composed of seven repeats of about 80 bp containing 6-bp palindromes characterized by the presence of *Mlu*I sites and overlapping *Xho*I-*Sac*I sites (Fig. 1). Domain II (nt 620 to 1310) is composed of eight partially repeated sequences of 60 to 100 bp, all of which contain a 15- to 25-bp sequence that is 90 to 100% A+T in addition to a 6- to 10-bp palindrome containing an *Nru*I site (Fig. 1). A single domain I repeat in a reversed orientation is found downstream of domain II. Although it contains overlapping *Sac*I and *Xho*I sites and an *Mlu*I site, this repeat is only about 50% related to other domain I repeats.

Characterization of a replication origin in *hr*4 by deletion analysis. *hr*4 was analyzed by a *Dpn*I assay to determine if domain I and II sequences acted as replication origins. *Dpn*I will digest only sequences that are fully methylated. Whereas the *Dpn*I sites of bacterially replicated plasmid DNA are fully methylated and therefore cleaved by *Dpn*I, *Dpn*I sites in DNA replicated in transfected insect cells are not methylated and are resistant to *Dpn*I digestion. Therefore, *Dpn*I digestion can be used to differentiate between input plasmid DNA and plasmid DNA that has replicated in eukaryotic cells (24a). We used this assay to examine replication of plasmids containing subclones of *hr*4 that were transfected into Ld*M*NPV-infected *L. dispar* cells (Fig. 2A and B). The parent *Bam*HI-*Eco*RI clone (A0) and a clone with four repeats deleted from domain I (A2) showed high levels of replication (Fig. 2B, lanes 1 and 2). Deletion of most of domain I (A3; lane 3) led to a reduction of replication efficiency to about 15% of that of the parent clone. Deletion into domain II resulted in a continued decline in replication levels. Plasmids with two (A26; lane 4) and four (A39; lane 5) repeats deleted resulted in replication levels of less than 2% of those of the parent clones. Plasmids with seven repeats deleted failed to replicate (A29; lane 6). A plasmid containing just domain II and lacking the single downstream *hr* (pGR93; lane 7) replicated at levels similar to those of clone A3, which contains both domain II and the single downstream domain I *hr*. A plasmid containing just domain I (pGR80; lane 8) showed trace levels of replication. Clone A2 (lane 2), which shows high levels of replication, was then subjected to deletion analysis. Elimination of the downstream domain I repeat and several of the domain II repeats (B4; lane 9) led to replication levels (about 5%) comparable to those of domain II by itself (lane 7). A clone with all but one domain II repeat removed and a clone with three domain I repeats failed to replicate (B5 and B6, lanes 10 and 11, respectively). When the domain I region was reversed relative to domain II, high levels of replication were evident (lane 12).

The controls for the replication experiment are shown in Fig. 2C and indicate that pBS$^-$ lacking an insert did not replicate when transfected into Ld*M*NPV-infected *L. dispar* cells (lane 1); there was no hybridization of the plasmid to DNA extracted from uninfected *L. dispar* cells (lane 2); and, under our standard conditions, pA0 plasmid mixed with DNA from Ld*M*NPV-infected *L. dispar* cells was completely digested by *Dpn*I (lane 3). An *hr*4-containing plasmid did not replicate in uninfected *L. dispar* cells (data not shown). These results confirm results of similar control experiments done previously with this cell line (23).

Nucleotide sequence analysis of *hr*1. The 1.4-kb region from a 2.3-kb *Pst*I fragment containing *hr*1 was sequenced (Fig. 3). The Ld*M*NPV polyhedrin gene is located at the right end of this fragment (Fig. 4A), and our sequence is contiguous with (5) and overlaps (29) previously reported polyhedrin gene



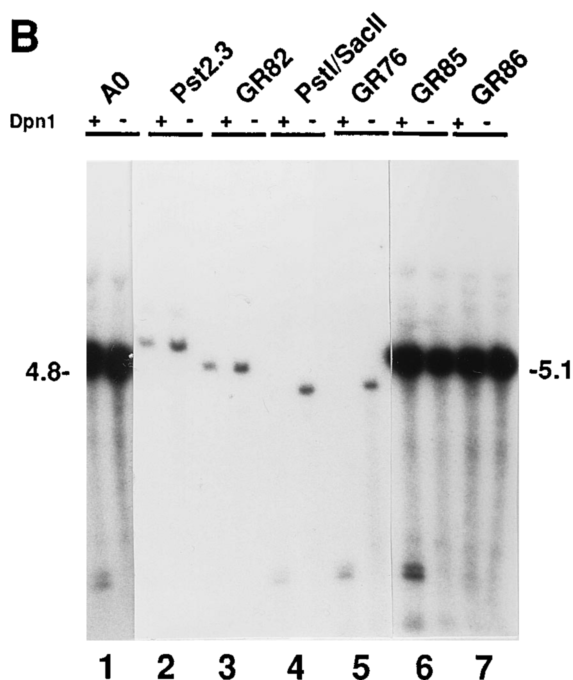


FIG. 4. Infection-dependent replication of deletion clones of *hr*1. (A) Restriction map of *hr*1 is shown at the top. The deletion mutants are diagrammed below the map, and the numbers indicate the locations of the deletion junction from the sequence shown in Fig. 3. The names of the clones and relative replication efficiencies are shown at the right. Replication efficiencies were estimated as described in the legend to Fig. 2, with values for replication of A0, pGR85, and pGR86 arbitrarily set at 100% and the other values calculated relative to an average of the values for A0, pGR85, and pGR86. (B) Replication assay of plasmids containing the sequences shown in panel A. The numbers beside the lanes indicate the sizes of linearized input parent plasmids. For controls, see legend to Fig. 2C.

sequences. A region with six domain I repeats in the orientation opposite to those of *hr*4 was found between nt 850 and 1392 of the sequence (Fig. 3). Although some AT-rich sequences were present upstream of the domain I repeats, sequences with the features of *hr*4 domain II repeats were not present. For the domain I regions from *hr*4 and the *hr*1 reverse sequence, the Genetics Computer Group Gap program aligned a region of about 400 bp containing *hr*1 G, F, E, D, C, and B repeats to *hr*4 repeats C, D, E, F, and G (Fig. 1), with 89% identity (Fig. 5).

Replication analysis of *hr*1. The ability of plasmids containing portions of the *hr*1 region to undergo infection-dependent replication was assayed. The complete *Pst*I 2.3-kb fragment was found to replicate at less than 4% of the levels of the *hr*4 *Bam*HI-*Eco*RI-containing plasmid (Fig. 4B; compare lanes 2 and 1, respectively). Deletion of the polyhedrin gene region did not affect the level of replication (pGR82; Fig. 4B, lane 3). Neither a plasmid containing the domain I sequence (pGR76) with six repeats (nt 766 to 1372) nor a plasmid or a fragment containing the sequence from 1 to 755 (pGR76 and the *Pst*I-*Sac*II fragment, lanes 4 and 5, respectively) replicated. However, when domain II from *hr*4 was inserted adjacent to *hr*1 domain I in either orientation, high levels of plasmid replication were evident (pGR85 and pGR86, lanes 6 and 7, respectively).

Database searches and further characterization of *hr*1 and *hr*4 sequences. Three open reading frames of 251 (nt 2 to 754), 120 (nt 155 to 513), and 207 (nt 583 to 1) amino acids were identified upstream of *hr*1. No convincing homology was identified when these open reading frames were compared

```
                    .         XhoI/SacI .         .         .
ldhr1  55 GATGAGATCACGCCCAAGCTCGAGCTCGGCGGACGCGTAAGATTTTACGC 104
          ||||| |||| |||||| ||||||||||| |||||||||||||||||||
ldhr4 177 GATGACATCATGCCCAAACTCGAGCTCGGTGGACGCGTAAGATTTTACGT 226
                            XhoI/SacI

                    .         .         .         .         .
      105 GTCGGCCGGTGTCGATCGAGTTCAAACCCGATGACATCATGCCTAAACT. 153
          | |||| || ||  |  |||||| || ||||||| |||||||| |||||
      227 GCCGGCAGGGGTTAACAGAGTTC.AAGCCGATGAGATCATGCCCAAACTC 275
           SacI     .         .         .         .    XhoI
      154 GAGCTCGGCGGACGCGTAGGATTTTACGTGCCGGCCAGGGTCGATGGAGG 203
          |||||||||||||||||||||| ||||| | |||  ||||||||||||||
      276 GAGCTCGGCGGACGCGTAGGATCTTACGCGTCGGTTAGGGTCGATGGAGG 325
           SacI     .         .       SacI      .         .
      204 TCGAGCCCGATGAGATCACGCCTAAACTGAGCTCGGCGGACGCGTAAGAT 253
          || || ||||||| |||||||||||||||||||||||||||||||||||
      326 TCAAG.CCGATGACATCACGCCTAAACTGAGCTCGGCGGACGCGTAAGA. 373
                    .         .       SacI      .         .
      254 TTTACGCGTCGGCTAGGGTCGATCGAGTTCAAGCCCGATGACATCATGCC 303
          |||||| | |||| |||||| ||||||||||| ||||||||||||| |||
      374 TTTACGTGCCGGCAAGGGTCAATCGAGTTCAAACCCGATGACATCACGCC 423
              XhoI/SacI         .         .         .         .
      304 CAAACTCGAGCTCGGCGGACGCGTAAGATTTTACGCGTCGGCTAGGGTCA 353
          |||||||||||||||||| |||||| ||||||||| | |||| ||||||
      424 CAAACTCGAGCTCGGCGGGCGCGTAGGATTTTACGTGGCGGCAAGGGTCG 473
              XhoI/SacI         .         .  XhoI/SacI     .
      354 ACAGAGTTCAAGCCCGATGAGATCATGCCTTAACTCGAGCTCGGCGGACG 403
          |  |||  ||||| ||||||| |||||||||||||| ||||||||||||||
      474 ATGGAGGTCAAG.CCGATGACATCATGCCTTAACTTGAGCTCGGCGGACG 522
                    .         .         .    SacI
      404 CGTAGGATTTTACGCGCCGGCAAGGGTCAATCGGGTTCAAGCCCGATGAG 453
          |||| ||||||||| |||||||||||||||  | ||||||||||||||||
      523 CGTAAGATTTTACGTGCCGGCAAGGGTCAACAGAGTTCAAGCCCGATGAG 572

      454 ATCATG 459
          ||||||
      573 ATCATG 578
```

FIG. 5. Alignment of portions of domain I from *hr*1 and *hr*4. The vertical lines indicate sequence identity.

with GenBank release 82.0 by using the TFASTA program. Domain I (nt 117 to 194) (Fig. 1) and domain II (nt 618 to 1190 and 1140 to 1190) repeats were also compared with the GenBank by using the FASTA program. No significant homologies were identified for either of these domains.

The repeated sequences were also examined for the presence of palindromes, which are a feature of the Ac*M*NPV *hr*s. In addition to the short palindromes comprising restriction enzyme sites, a number of longer palindromes were present. These appeared between the repeats of *Xho*I-*Sac*I or AT-rich sites. The most extensive located in *hr*4 (nt 123 to 158; CTCGGCGGACGCGTAAGATTTTACGCGTCGGCAGGG) displays matches of 28 of 36 nt in the palindrome. Likewise, another (nt 284 to 309; CGGACGCGTAGGATCTTACGCGTCGG) contains matches of 22 of 26 nt in the palindrome. In the domain II region, a sequence flanked by two *Nru*I sites (nt 677 to 706; TCGCGAGCCGGCTCGCGCGCAGGCTCGCGA) shows matches of 26 of 30 nt in the palindrome. Because these sequences are components of repeated regions, almost all the repeats contain related palindromes.

Mapping the locations of *hr*s. To map sequences similar to *hr*4 domain I in the Ld*M*NPV genome, a 625-bp *Bam*HI-*Nhe*I fragment containing all of *hr*4 domain I (Fig. 1) was labeled and hybridized under stringent conditions to blots of six overlapping cosmids that encompass the complete Ld*M*NPV genome (Fig. 6). The locations of the cosmids relative to the Ld*M*NPV genome map are shown in Fig. 7. Cosmid A contains two *hr*s; one corresponds to *hr*1 and is present on a large *Bam*HI fragment (Fig. 6A, lane 1) and is located between 5 and 6 kb (Fig. 7) on the genome map just upstream of polyhedrin and is characterized in Fig. 3 and 4. The other *hr* (*hr*2) shows weak hybridization and is located on the *Bam*HI-K fragment (about 1.4 kb) (lane 1) at 15.2 to 16.4 kb (Fig. 7). Sequence analysis of *Bam*HI-K has demonstrated the presence of a sequence of about 50 bp identical to a portion of the domain I repeat (27a). Cosmid B contains two *hr*s located on three *Eco*RI fragments, *Eco*RI-E (10.5 kb), *Eco*RI-H (9 kb), and *Eco*RI-K (4.4 kb) (lane 2). Digestion with both *Hin*dIII and *Eco*RI yielded a 1.2-kb fragment (lane 4) corresponding to 39.7 to 40.9 kb on the map (Fig. 7). Digestion with *Eco*RI and *Bam*HI caused the 9-kb *Eco*RI-H-hybridized fragment to shift to a 1.2-kb fragment (lane 6), demonstrating that the *hr* on *Eco*RI-H is located at the right end of the fragment (36.5 to 39.7 kb) (Fig. 7). Therefore, hybridization spans the *Eco*RI-H–*Eco*RI-E junction and *hr*3 is located between 36.5 and 40.9 kb. The *hr* on *Eco*RI-K is *hr*4 and is cleaved by *Bam*HI to a 1.6-kb fragment (lane 6) at 53 to 54.7 kb (Fig. 7) which is characterized above (Fig. 1 and 2). Cosmid C contains a single *hr*. It overlaps cosmid B and includes the *hr*4-containing *Eco*RI-K fragment located on the 1.6-kb *Bam*HI-*Eco*RI fragment (lane 9). Cosmid D (Fig. 6D) also contains a single *hr* (*hr*5) that is located on *Eco*RI-D (15 kb) (lane 10) and *Bgl*II-J (9 kb) (lane 11). Digestion with *Eco*RI and *Bgl*II yielded a 1.1-kb fragment, indicating that *hr*5 is located at the right end of *Bgl*II-J (96.2 to 97.3 kb), which is immediately upstream of the capsid gene (Fig. 7). Cosmid E (Fig. 6E) contains a single *hr* (*hr*6) that is located on a 10.4-kb *Hin*dIII-*Eco*RI fragment (lane 13) and a 7-kb fragment from a *Hin*dIII-*Eco*RI-*Bam*HI digest (lane 14), placing *hr*6 between 124.6 and 133.2 kb. It is also present on a 3-kb *Pst*I fragment (data not shown). On the basis of a map of the Ld*M*NPV EGT gene (25), this 3-kb *Pst*I fragment is located between 124.6 and 130.5 kb (Fig. 7). Cosmid F has three *hr*s. They are located on a 2.9-kb *Hin*dIII-*Eco*RI fragment (*hr*7) (147.2 to 150.1 kb), a 2.7-kb *Hin*dIII-J fragment (*hr*8) (152.8 to 155.5 kb), and a 21.3-kb *Hin*dIII-*Eco*RI fragment (*hr*1) (lanes 15 and 16). *hr*1 is on cosmid A; results of its analysis are shown above (Fig. 4).

To determine if the domain II repeated region adjacent to *hr*4 is also repeated elsewhere in the genome, a clone containing *hr*4 domain II was constructed by digesting the parent *Bam*HI-*Eco*RI clone (Fig. 2) with *Xba*I (in the polylinker) and *Nhe*I (nt 621) and religating the DNA to produce pGR97. This clone was digested with *Hin*cII, which cut in the polylinker and at nt 1311 (Fig. 1). This fragment (about 700 bp) was gel purified, labeled, and hybridized to the blots used in Fig. 6. No hybridization to any of the blots other than those containing domain I of *hr*4 was observed. Hybridization to the *hr*4 region occurred with the same pattern as hybridization with domain I, indicating that the probe was hybridizing to its own sequence and no other in the genome (data not shown).

DISCUSSION

The Ld*M*NPV *hr*4 sequence, which behaves as a replication origin when cloned into a plasmid and transfected into Ld*M*NPV-infected *L. dispar* cells, is composed of two domains that contain multiple repeating elements. Replication of deletion mutants containing portions of *hr*4 suggests that plasmids containing either domain I or domain II are capable of undergoing limited infection-dependent replication, with plasmids containing domain II replicating somewhat more efficiently than those containing domain I. Removal of more than half of the domain I or II sequences resulted in plasmids that did not replicate. Plasmids with combinations of both domain I and domain II replicate to much higher levels than those with

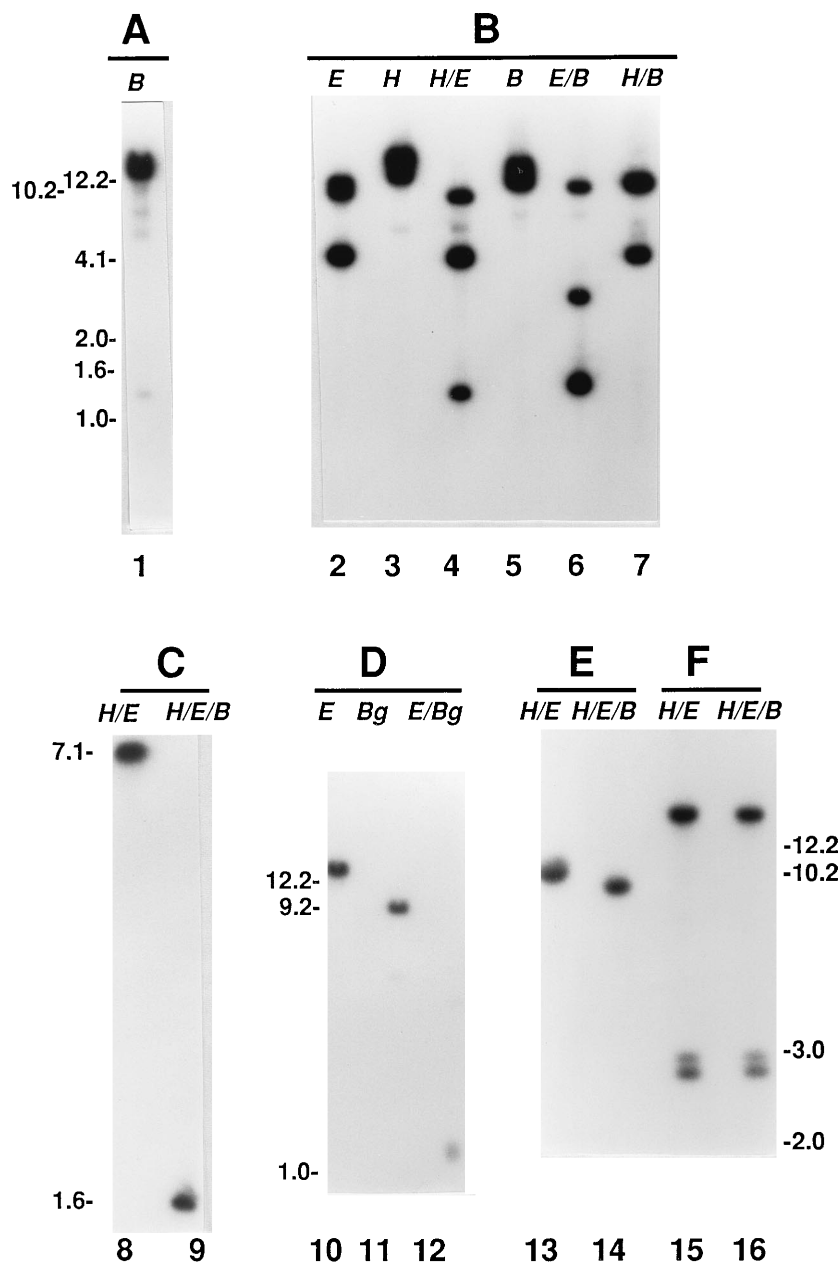


FIG. 6. Mapping the location of *hr*s on the Ld*M*NPV genome by Southern blot analysis. Blots are labeled A to F, and each corresponds to cosmids labeled A to F in Fig. 7. The numbers indicate the positions of selected fragments from a 1-kb DNA ladder size standard. The following abbreviations for restriction enzymes are used: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; and Bg, *Bgl*II.

either domain by itself, and this replication is independent of the orientation of domain I relative to domain II. Similar results were obtained from the analysis of the *hr*1 region. A plasmid containing the complete *hr*1 region plus the polyhedrin gene replicated at low levels. Removal of the polyhedrin gene had no effect on replication. However, when two regions upstream of the polyhedrin region were analyzed, one failed to replicate while the other, containing six domain I repeats, replicated at low levels. Insertion of domain II from *hr*4 in either orientation adjacent to domain I yielded levels of replication similar to those of plasmids containing the complete *hr*4 region. Since *hr*1 is in the orientation opposite to that of *hr*4, this is equivalent to inserting domain II upstream of domain I of *hr*4. These data indicate that efficient infection-dependent plasmid replication in the Ld*M*NPV system requires the presence of both an *hr* sequence and domain II A+T rich repeats. Replication origins that contain a bipartite structure composed of two repeated domains have been well documented (7, 17). A variety of prokaryotic replication origins that contain a repeated region that interacts with an origin-specific binding protein along with a second domain that contains repeats of AT-rich sequences have been described. Once the origin-specific protein is bound, it melts the AT-rich flanking sequences. It is thought that this separation of DNA strands may permit the entry of the replication complex, which is essential for the initiation of replication (17).

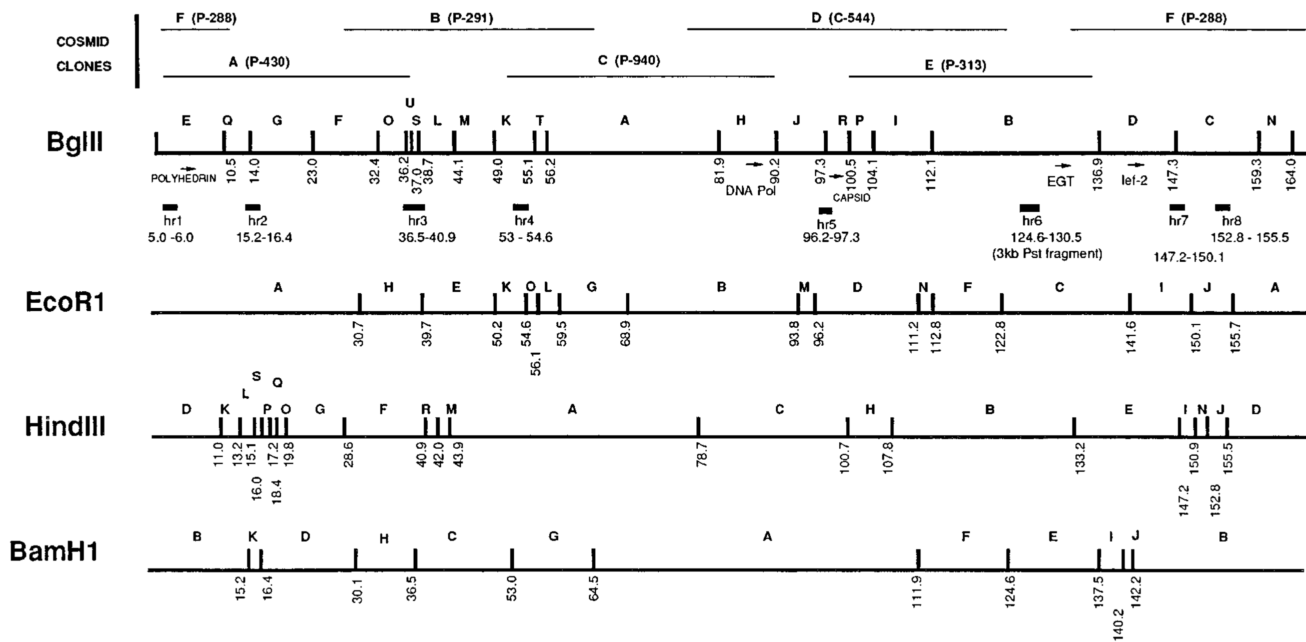


FIG. 7. Map of the Ld*M*NPV genome showing the locations of the *hr*s. The *hr*s are shown as solid boxes below the *Bgl*II map. The portions of the genome contained in cosmids used for these investigations are indicated above the map. The numbers indicate numbers of kilobases from the left end of the *Bgl*II-E fragment. Selected genes are indicated. The polyhedrin gene is described in references 5 and 29; the DNA polymerase, capsid, EGT, and lef2 genes are described in references 1, 3, 25, and 2, respectively.

The presence of *hr*s distributed around the genome has now been reported for Ac*M*NPV (6) and the closely related *B. mori* NPV (21), Op*M*NPV (31), and Ld*M*NPV. In addition, *hr*s similar to those in Ac*M*NPV are also present in the genome of *C. fumiferana* NPV (18). It has been shown that in Ac*M*NPV (12) and Op*M*NPV (31), *hr* sequences enhance the expression of early genes. Enhancer elements that are involved in both transcription and replication have been described in association with eukaryotic origins of replication (7). *hr*s in Ac*M*NPV (15, 24), Op*M*NPV (unpublished data), and Ld*M*NPV (this study) act as replication origins in an infection-dependent assay. However, in contrast to Ac*M*NPV, both Op*M*NPV and Ld*M*NPV require more than the *hr* sequence for efficient levels of replication. Ld*M*NPV requires combinations of both domain I and II repeats for high levels of replication. In Op*M*NPV, sequences in addition to the *hr* that was analyzed were required for efficient replication (unpublished data). Analysis of the ability of the Ld*M*NPV *hr*s to enhance early gene expression is currently under way.

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