

# Extension of *Autographa californica* nuclear polyhedrosis virus host range by interspecific replacement of a short DNA sequence in the p143 helicase gene

(species specificity/baculoviruses/homologous recombination)

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**ABSTRACT** Recombinant baculoviruses obtained by co-infection of insect cells with *Autographa californica* and *Bombyx mori* nuclear polyhedrosis viruses (AcNPV and BmNPV, respectively) possess a wider *in vitro* host range than either parent virus. To localize the DNA sequences responsible for this species specificity, we used a two-step method of production and selection of recombinant viruses with altered specificity. Sf9 cells, which are permissive for AcNPV, were first cotransfected with genomic AcNPV DNA and a complete or incomplete set of BmNPV restriction fragments. AcNPV–BmNPV recombinants from the Sf9 supernatant were then selected on the basis of ability to replicate in *B. mori* Bm5 cells, which are not permissive for AcNPV. Cotransfection of AcNPV DNA with the 7.6-kbp BmNPV *Sma* I–C fragment was sufficient to produce recombinants able to infect both Sf9 and Bm5 cells. A series of cotransfections with subclones of this fragment defined a 79-nt sequence within the p143 helicase gene capable of extending AcNPV host range *in vitro*. In this 79-nt region, BmNPV and AcNPV differ at six positions, corresponding to four amino acid substitutions. The involvement of the 79-nt region in species specificity control was confirmed by cotransfecting AcNPV DNA and gel-purified polymerase chain reaction products derived from the BmNPV p143 gene. Replacement in the AcNPV genome of three AcNPV-specific amino acids by the three corresponding BmNPV-specific amino acids at positions 556, 564, and 577 of the p143 protein extends AcNPV host range to *B. mori* larvae.

Baculoviruses form a large group of double-stranded DNA viruses infectious to insects (1). Host ranges of several baculoviruses have been documented and found to vary from one susceptible insect species to dozens of species belonging to various families (2–7). Among baculoviruses with the broadest host ranges, *Bombyx mori* nuclear polyhedrosis virus (BmNPV) (2) and *Autographa californica* (Ac)NPV (5) each infect more than 40 different species of Lepidoptera belonging to 11 families, and AcNPV additionally infects a coleopteran cell line (6).

Homologous recombination of DNA sequences between similar baculovirus genotypes is a frequent event which occurs after coinfection or cotransfection of insect larvae and cell lines, under both experimental and natural conditions (8–13). Cotransfection of insect cells with entire baculovirus genomes and subgenomic DNA sequences is an efficient method for localization of temperature-sensitive mutations (14), gene replacement in the baculoviruses expression vector system (15), production of intraspecific recombinants (16), and engineering baculoviruses for pest control (17, 18). AcNPV and BmNPV are very closely related. The general

nucleotide sequence similarity between the two viruses is about 70%, whereas homology between well conserved genes is over 90%. These two viruses are most likely host-range variants of the same virus. Coinfection of insect cells with AcNPV and BmNPV generates new recombinant viruses possessing wider host ranges than either parent (19).

The molecular mechanisms governing baculovirus species specificity are very poorly understood. In lepidopteran cell lines that are semipermissive for AcNPV, a large part of the genetic control of specificity may reside in defective virus–cell molecular interactions which impede full virus cycle development (20, 21). Infection of AcNPV in *Lymantria dispar* cells is arrested by a general shutoff of viral and cellular protein synthesis (22, 23). Although the precise step(s) of AcNPV virus cycle blockage in the semipermissive cells of both *L. dispar* and *B. mori* cells is unknown, it takes place after DNA replication (23, 24). In the present study we surmised that the blockage of AcNPV replication in *B. mori* cells might be overcome by replacing replication-defective AcNPV sequences with replication-competent BmNPV sequences.

To identify replication-defective AcNPV sequences at the nucleotide level, we have devised a two-step method of production and selection of recombinant viruses with altered species specificity. In the first step, recombinants were produced by random homologous recombination between BmNPV restriction fragment(s) and genomic AcNPV DNA after cotransfection of permissive insect Sf9 cells. In the second step, AcNPV–BmNPV recombinant viruses capable of growing in Bm5 cells, which are nonpermissive for parental AcNPV, were recovered. Our first aim was to localize the minimal DNA sequence from the BmNPV genome necessary to replace homologous AcNPV sequences and yield a recombinant virus capable of productive infection in Bm5 cells. A 79-nt region was identified as the minimal sequence requirement. To confirm that alterations in the 79-nt region, located by rescue analysis, were a cause of the observed extended AcNPV host range, we further produced mutant AcNPV capable of productive infection in Bm5 cells and in *B. mori* larvae by recombination with BmNPV polymerase chain reaction (PCR) products corresponding to the 79-nt area. Possible molecular mechanisms in the blockage of parental AcNPV multiplication in Bm5 cells are discussed.†

## MATERIALS AND METHODS

**Cell Lines and Viruses.** *Spodoptera frugiperda* IPLB-SF21-AE (25) clone 9 (Sf9) and *B. mori* Bm5 cells (26) were

Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; BmNPV, *Bombyx mori* nuclear polyhedrosis virus; m.u., map unit(s).

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. X75784).

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used in this study. Cells were maintained at 27°C in TC100 medium supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum. AcNPV-1.2 (11) was propagated in Sf9 cells. The BmNPV isolate was collected in 1986 from diseased larvae found in a silkworm-rearing unit in the French Cevennes. A clonal genotype, BmNPV-SC7, was obtained by three rounds of plaque purification of the BmNPV Cevennes strain on Bm5 cells.

**Infection of *B. mori* Larvae.** The 200 × 300 hybrid *B. mori* was fed on mulberry leaves. Third-instar larvae were injected through a proleg with a 0.45 × 12-mm needle. Each larvae received 8 μl of infectious Sf9 supernatant diluted 1:3 with TC100 medium. Dead larvae were processed individually.

**Virus and DNA Purification.** AcNPV-1.2 virus and viral DNA were obtained from Sf9 cells by standard methods (15). Wild-type BmNPV DNA was initially extracted from inclusion bodies (IBs) purified from dead *B. mori* larvae. BmNPV-SC7 DNA was obtained from infected Bm5 cells either from IBs as already described (10) or from Bm5 cell supernatant. The supernatant was clarified at 2800 × *g* for 15 min, and virus particles were then pelleted at 45,000 × *g* in an SW28 rotor (Beckman). DNA was extracted with phenol after proteinase K digestion (15).

**Recombinant Plasmids and Restriction Map.** AcNPV and BmNPV restriction fragments were cloned in pUC19 by standard methods (27). To construct the BmNPV-SC7 restriction map, a series of labeled fragments were used to probe Southern blots of BmNPV-SC7 *EcoRI*, *HindIII*, *Pst I*, *BamHI*, *Sac I*, and *Apa I* digests and AcNPV *EcoRI* and *Pst I* digests after separation in agarose gels.

**Construction of Recombinant Baculoviruses with Double Specificity.** A two-step method was used to produce and select baculoviruses with double specificity. In the first step, AcNPV DNA was cotransfected with BmNPV DNA restriction fragment(s) in Sf9 cells by lipofection to produce a mixture of wild-type AcNPV and recombinant Ac-BmNPVs. Next, the budded viruses in the Sf9 cell supernatant were plaque purified on Bm5 cells to eliminate pure AcNPV and select Ac-BmNPV recombinants. Ac-BmNPVs were then checked for double specificity by passage on Sf9 cells. In a typical experiment AcNPV DNA (250 ng) and variable amount of plasmids containing BmNPV restriction fragments (1–2 μg) were mixed with 8 μl of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP) lipofectant suspension (Boehringer Mannheim) (8 μl of DOTAP plus 600 μl of TC100 medium plus DNA in 10 μl of Tris/EDTA buffer) and added to Sf9 cells (3 × 10<sup>5</sup> cells per well in a 24-well plate). After 5 hr the medium was replaced with TC100/10% fetal bovine serum. At 72 hr postinfection, the infectious supernatant was harvested, clarified by centrifugation at 2000 × *g* for 5 min, and stored at 4°C before plaquing on Bm5 cells. To plaque purify recombinant viruses, 4.5 × 10<sup>5</sup> Bm5 cells were seeded per well on a 6-well plate and 10, 1, or 0.1 μl of infectious Sf9 supernatant was added to each well.

**DNA Sequencing.** A 500-nt DNA sequence located within the coding region of the p143 gene of AcNPV was amplified by PCR from AcNPV-1.2, BmNPV-SC7, and Ac-BmNPV genomes by using primer A (5'-CACATTTGGCGATGT-GTTAAGTTC-3') and primer B (5'-GGCTATTATTTG-CATACACTACA-3') (28). Viral DNA used as template was purified from 5 ml of supernatant from infected Bm5 cells. After a 35-cycle amplification (94°C for 1 min; 55°C for 1 min; 72°C for 45 sec), PCR products were ethanol precipitated (by addition of 100 μl of 4 M ammonium acetate and 300 μl of ethanol per 100-μl PCR mixture) at room temperature for 10 min, centrifuged at 10,000 × *g* for 30 min, and rinsed with 70% ethanol. These DNAs were sequenced on both strands with fluorescent dye technology, using the Dye Deoxy Terminator method, on a 373 DNA sequencer (Applied Biosystems).

**Production of Recombinants with Modified Host Range by Use of PCR Products Derived from BmNPV Sequences.** Primers C (5'-TATCAAATTGTAAAGAAAGAGGATG-3'), D (5'-ATTAAACAATTGGAAATACTTGTAC-3'), and E (5'-TCGTCGGTGGGCTTCTTGAA-3') and primers A and B were used to amplify BmNPV DNA sequences. The PCR products electrophoresed in agarose gel were purified by extraction of the DNA fragments (133, 299, and 500 nt) by the GeneClean (Bio 101) technique. AcNPV genomic DNA and purified PCR product were cotransfected in Sf9 cells (0.05 μg of PCR product and 0.15 μg of AcNPV DNA for 2 × 10<sup>5</sup> cells). Viruses contained in Sf9 cell supernatants 2 days postinfection were plaque purified in Bm5 cells or injected into *B. mori* larvae.

## RESULTS

**BmNPV-SC7 Restriction Map.** To select restriction fragments used in rescue experiments a restriction map of BmNPV-SC7 was constructed for 10 restriction endonucleases (data not shown). The origin of the BmNPV-SC7 map is the *HindIII* site separating the *HindIII*-B and -L fragments, and the map is oriented as proposed by Maeda and Majima (29) for the related genotype BmNPV-T3. The *Sma I* and *BamHI* maps of clones SC7 and T3 are identical. Numerous differences are observed between BmNPV-SC7 and BmNPV-T3 for *EcoRI*, *HindIII*, and *Pst I* maps.

**Production and Selection of Recombinant Ac-BmNPVs with Double Specificity.** The supernatant of Sf9 cells cotransfected with AcNPV-1.2 and wild-type BmNPV *Pst I* restriction fragments was plaque purified on Bm5 cells. As anticipated, 12 out of 12 clones studied by restriction analysis were recombinant viruses, with unique DNA profiles containing fragments inherited from both parental viruses (data not shown). All 12 plaque-purified recombinant Ac-BmNPVs were infectious when assayed on Sf9 cells.

**Gross Localization of BmNPV Nucleotide Sequences Able to Modify AcNPV Specificity.** Analysis of restriction patterns of the 12 recombinants obtained with a full genomic set of BmNPV *Pst I* fragments indicated that BmNPV DNA sequences were invariably present in two regions of recombinant viruses, in agreement with the findings of Kondo and Maeda (19) (data not shown). The first region was characterized by the fusion of AcNPV *Pst I*-A and -C at 62.3 map units (m.u.), which indicated that BmNPV DNA was present at 62.3 m.u., and the second region by the replacement of AcNPV *Pst I*-N by BmNPV DNA sequences in the 99 m.u. area. To establish whether the BmNPV DNA sequences in both regions were required to alter AcNPV specificity, recombinant Ac-BmNPVs were prepared in Sf9 cells by cotransfections and isolated by plaquing on Bm5 cells. Two BmNPV restriction fragments overlapping the 62.3 m.u. area (*Sma I*-C; Fig. 1) or the 99 m.u. area (*Pst I*-E) were inserted

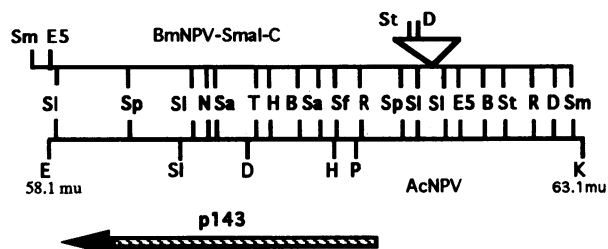


FIG. 1. Comparison of genomic DNAs of AcNPV-1.2 and BmNPV-SC7 in the p143 gene region. AcNPV restriction sites are deduced from sequence data (28). BmNPV restriction sites were located by mapping the cloned *Sma I*-C fragment. B, *Bsm I*; D, *Dra III*; E5, *EcoRV*; E, *EcoRI*; H, *HindIII*; K, *Kpn I*; N, *Nru I*; P, *Pst I*; R, *Rsr II*; Sa, *Sac I*; Sf, *Sfi I*; SI, *Sal I*; Sm, *Sma I*; Sp, *Sph I*; St, *Sty I*; T, *Tth1111*; mu, map units.

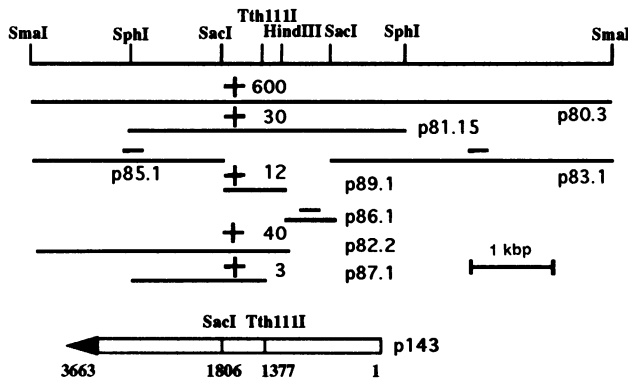


FIG. 2. Efficacy of *Sma* I-C subclones in modifying AcNPV specificity *in vitro*. Eight subcloned fragments were cotransfected individually with AcNPV DNA into Sf9 cells to obtain recombinant virus through homologous recombination. Three subclones (lines labeled -) failed to produce any recombinants in Bm5 cells. The five other subclones (labeled + followed by a titer) produced from  $3 \times 10^3$  to  $600 \times 10^3$  plaque-forming units per ml in Bm5 cells according to their size and their position relative to the *Sac* I-*Tth*111I sequence (titer  $\times 10^{-3}$  is indicated in the figure). The shortest BmNPV DNA insert that produced AcNPV recombinants capable of productive infection in Bm5 cells, p89.1, is 572 nt long. The minimal region of overlapping BmNPV sequence effective for AcNPV specificity alteration is a 430-nt area located within the p143 gene (nucleotides 1377-1806).

into pUC19. The supernatant from Sf9 cells cotransfected with genomic AcNPV and BmNPV *Sma* I-C was infectious

AcNPV	Primer B -->	Tth111 I	460
		ThrGlnSerAlaLy	1391
		.....T.....	15
		.....Leu.....	
BmNPV			
		sAsnGluIleLeuAspHisAspGluLeuTrpAlaTyrThrTyrGluAsnV	1441
		AAACGAAATTTGGATCAGCATGAATTGTGGCGCTACACGTACGAGAATG	65
		.....C...A...G.....	
		.....Thr.....Asp.....	
		alMetAlaLeuAsnLeuProProAspIleValCysLysGlyPhePheArg	1491
		TGATGGCGCTAAACTTGGCCCTGCATTTGTGTGTAAGGATCTTTAGA	115
		.....A.....T.....	
		LysLeuGluAsnValValThrGlyValAsnLeuValPheAsnGlyLysHi	1541
		AAATGGAAAACGTAGTGACCGGAGTCAATTTGGTTTCAACGGCAACA	165
		.....A.....	
		.....As	
		primer C -->	
		sTyrGlnIleValLysLysGluAspAspLeuPheLysLeuThrLysSerA	1591
		TTATCAAATTTGTAAGAAAGAGGATGACCTATTCAAATGACCAAAAGCA	215
		.....C.....GTT.....	
		.....Val.....	
		primer D -->	
		snCysTyrLysLeuSerAsnIleLysPheAsnAsnTrpLysTyrLeuTyr	1641
		ATTGTTACAAGTTGAGCAACATAAAATTTAACAATGGAAATCTTGTAC	265
		.....T.....	
		LeuThrThrHisGlyValTyrAsnValPheThrAsnSerPheHisSerSe	1691
		TTGACAACGACGGTGTGTACAACGTTTCAACCAACAGCTTTCATTCGAG	315
		.....T.....TT.....A	
		.....Tyr.....Leu.....As	
		primer E	
		rCysProPheLeuLeuGlyThrThrLeuProGlnThrPheLysLysProT	1741
		CTGTCCATTTTGTGGCCACCGTTCGCCGACACATTCAGAAGCCCA	365
		.....T.....C.....	
		.....Leu.....	
		hrAspGluLysTyrLeuProGluAspAlaPheAsnTyrMetLeuSerThr	1791
		CCGACGAAAAGTATTTGCCCGGAGGACGGTTAATTACATGCTATCTACT	415
		.....G.....	
		.....Glu.....	
		SerAlaAspGluLeu	
AcNPV	AGCGCCGACGAGCTCAGCATTTATAGAACCTTATCACATCGCCAAAATGTG	1841	
BmNPV	AGCACCCGACGACTC	465	
	.....Thr.....		
	.....Sac I		

FIG. 3. Nucleotide sequence of colinear AcNPV and BmNPV DNAs in the *Tth*111I-*Sac* I region of the p143 gene controlling the AcNPV *in vitro* host range. Primers A and B, which anneal to adjacent sequences of the *Tth*111I-*Sac* I fragment, were chosen on the basis of AcNPV p143 sequence (28).

to Bm5 cells, whereas the supernatant after cotransfection with AcNPV and BmNPV *Pst* I-E was not. Cotransfection of Sf9 cells with AcNPV DNA and a mixture of plasmids containing *Sma* I-C and *Pst* I-E also yielded supernatant infectious to Bm5 cells.

**Comparison of BmNPV *Sma* I-C and AcNPV (58-63.1 m.u.) Restriction Maps.** To subclone from the BmNPV *Sma* I-C fragment and to analyze the restriction profiles of Ac-BmNPV recombinants, a detailed restriction map was constructed for the BmNPV *Sma* I-C area (Fig. 1). A comparison of this map with the map deduced from the AcNPV *Eco*RI-*Kpn* I sequence at 58-63.1 m.u. (28) showed that the two regions were highly conserved. An insertion of 1 kbp containing one site each for *Sty* I and *Dra* III was observed in BmNPV DNA (Fig. 1).

**Localization of BmNPV Nucleotide Sequences Able to Modify AcNPV Specificity.** A variety of subclones of the BmNPV *Sma* I-C fragment were prepared and cotransfected with AcNPV DNA into Sf9 cells (Fig. 2). Supernatants obtained from these cotransfections were tested for infectivity in Bm5

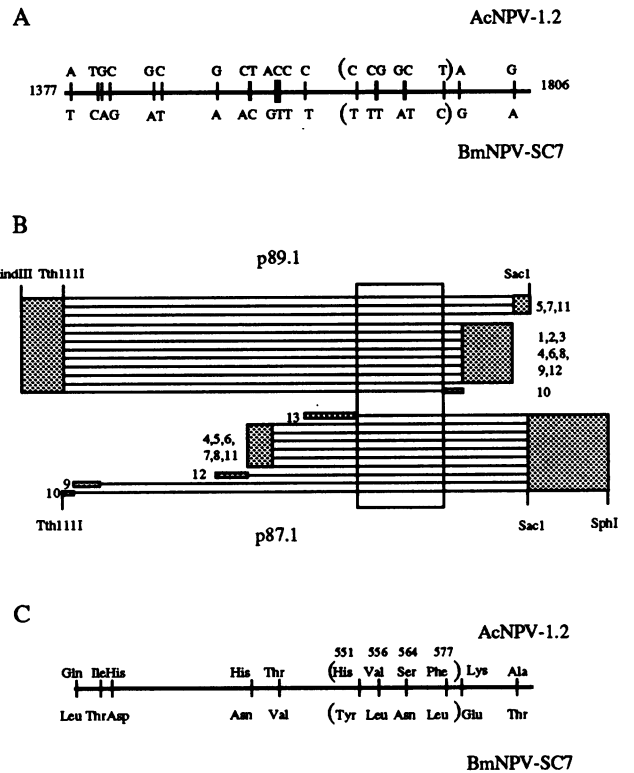


FIG. 4. Sequence analysis of PCR products from 22 progeny viruses obtained by recombination between AcNPV and plasmid p89.1 or p87.1. (A) Distribution of nucleotide differences at 21 positions between the *Tth*111I-*Sac* I fragments of AcNPV-1.2 and BmNPV-SC7 DNA. Nucleotides of the coding strands are shown. (B) Portions of BmNPV-SC7 sequences inherited by AcNPV recombinant infectious for Bm5 by cotransfection with p89.1 (*Hind*III-*Sac* I insert, upper set) or p87.1 (*Tth*111I-*Sph* I insert, lower set). Lines represent BmNPV DNA. Stippled boxes represent regions within which recombination events occurred. The left (*Hind*III-*Tth*111I) and right (*Sac* I-*Sph* I) boxes were not sequenced. The eight internal boxes were sequenced and are devoid of any nucleotide markers distinguishing AcNPV and BmNPV. The open box indicates the 79-nt BmNPV sequence common to the 22 recombinants. This region is indicated with parentheses in C and A. (C) Differences in amino acids between AcNPV and BmNPV sequences in the *Tth*111I-*Sac* I region. The shortest overlapping sequences in B, delineated by the length of unambiguous BmNPV sequence common to clones 10 (from p89.1) and 13 (p87.1), contain the nucleotides T, T, T, A, T, and C (see A) and the amino acids Tyr, Leu, Asn, and Leu specific to BmNPV.

	460	470	480	490	500	510	520	530
AcNPV	T	QSAKNEILDH	DELWAYTYEN	VMALNLPDI	VCKGFFRKLE	NVVTGVNLVF	NGKHYQIVKK	EDDLFKLTKS
BmNPV	.	L.....T..D	.....	.....	.....	.....	.....N.....	.....V..
R1101	.	L.....T..D	.....	.....	.....	.....	.....N.....	.....V..
R1102	.	.....	.....	.....	.....	.....	.....	.....V..
R1103	.	.....	.....	.....	.....	.....	.....	.....V..
R1104	.	.....	.....	.....	.....	.....	.....	.....V..
R1105	.	.....	.....	.....	.....	.....	.....N.....	.....V..
R1106	.	.....	.....	.....	.....	.....	.....	.....V..
R1107	.	.....	.....	.....	.....	.....	.....	.....V..
R1108	.	.....	.....	.....	.....	.....	.....	.....V..
R1109	.	.....	.....	.....	.....	.....	.....	.....V..
R1110	.	.....	.....	.....	.....	.....	.....N.....	.....V..
R1111	.	L.....	.....	.....	.....	.....	.....	.....V..
R1112	.	.....	.....	.....	.....	.....	.....	.....V..
R1201	.	.....	.....	.....	.....	.....	.....	.....V..
R1301	.	.....	.....	.....	.....	.....	.....	.....V..

	540	550	560	570	580	590	600
AcNPV	NCYKLSNIKF	NNWKYLYLTT	HGVYNVFTNS	FHSSCFLLG	TTLPQTFKKP	TDEKYLPEDA	FNYMLSTSAD EL
BmNPV	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1101	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1102	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1103	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1104	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1105	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1106	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1107	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1108	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1109	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1110	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1111	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1112	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1201	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..
R1301	.....	.....	Y...L...	..N.....	.....L...	.....E.....	.....T..

FIG. 5. Amino acid sequences (single-letter code) of recombinants with the double specificity obtained by cotransfection of AcNPV DNA with BmNPV PCR products. Numbering is according to the p143 open reading frame (28).

cells by plaque assay (Fig. 2). All BmNPV-SC7 sequences able to rescue AcNPV infectivity in Bm5 cells encompassed 430 bp delineated by *Tth111I* and *Sac I* restriction sites (Fig. 2).

**Comparison of BmNPV and AcNPV Sequences of the *Sac I-Tth111I* Fragment.** AcNPV-1.2 and BmNPV-SC7 DNAs were amplified with primers A and B and the PCR products were sequenced (Fig. 3). The AcNPV-1.2 *Sac I-Tth111I* sequence was identical to the sequence published by Lu and Carstens (28). BmNPV-SC7 and AcNPV-1.2 sequences differ at 21 positions (Figs. 3 and 4A). Variation in deduced amino acid sequences for the homologous regions of the two viruses occurs at 11 positions (Fig. 4C).

**Analysis of Ac-BmNPV Recombinants Infectious for Sf9 and Bm5 Cells and Identification of the Shortest Common BmNPV Sequence.** Two sets of plaque-purified recombinant Ac-BmNPVs infectious for Bm5 cells, obtained from cotransfection experiments with plasmids p89.1 and p87.1 (Fig. 2), were examined for the presence of AcNPV- or BmNPV-specific bases in the *Tth111I-Sac I* overlap by sequencing PCR products amplified with primers A and B (Fig. 4B). Twelve recombinants were analyzed from cotransfections involving p89.1; one exchange event had occurred between the *HindIII* and *Tth111I* sites (i.e., outside the amplified region) in all 12, while the second exchange had occurred within 12 bp (3 recombinants), 57 bp (8 recombinants), or 78 bp (1 recombinant) of the *Sac I* site inside the amplified region. Likewise, for 10 recombinants derived from cotransfections with p87.1, one exchange had occurred outside the amplified region (between the *Sac I* and *Sph I* sites) in all 10, while the second event had occurred at various positions up to 275 bp from the *Tth111I* site within the amplified region (Fig. 4B).

Six BmNPV-specific bases (from thymine at position 275 to cytosine at position 353 inclusive; see Fig. 3) are present in all 22 of these recombinants and thus define the minimal BmNPV sequence required to enable AcNPV to replicate in Bm5 cells. The minimal sequence corresponds to an acqui-

sition of four BmNPV-specific amino acids (Tyr, Leu, Asn, and Leu; Fig. 4B) by the AcNPV p143 helicase.

**Production of AcNPV Mutants Infectious for Sf9 and Bm5 Cells by Recombination with PCR-Amplified BmNPV Sequences.** Recombinant viruses in the Sf9 cell supernatants, obtained by cotransfection of AcNPV DNA and PCR products (500 nt, primers A and B; 299 nt, primers A and C; 133 nt, primers D and E), were plaque purified on Bm5 cells. Twelve plaque-purified viruses (R1101 to R1112) obtained with the 500-nt PCR product were sequenced (Fig. 5). R1101 contained all 11 BmNPV-specific amino acids, whereas R1105 and R1110, for example, contained 7 BmNPV-specific amino acids. The 12 R11 recombinants possessed BmNPV-specific amino acids at positions 551, 556, 564, and 577.

Supernatants of Sf9 cells cotransfected with AcNPV DNA and either the 299-nt or 133-nt PCR products contained about 200 plaque-forming units per ml when tested on Bm5 cells. These supernatants were directly injected into *B. mori* larvae to recover recombinant viruses. The sequences of the viral DNAs extracted from the polyhedra of two dead larvae are presented in Fig. 5. Six of the seven BmNPV-specific amino acids contained in the 299-nt sequence were present in the recombinant R1201 genome. One out of 10 larvae injected with Sf9 133-nt supernatant also died from patent nuclear polyhedrosis with 0.5- to 1- $\mu$ m polyhedra. The DNA extracted from the polyhedra encoded BmNPV-specific amino acids at positions 556, 564, and 577 and AcNPV-specific amino acids at the eight positions sequenced (Fig. 5). No mortality was observed in the control lots in which TC100 medium or Sf9 cell supernatant from Sf9 cells infected with wild-type AcNPV was injected.

DISCUSSION

The results presented above on the modification of AcNPV specificity demonstrate that the localization of DNA sequences directly implicated in species specificity control can easily be made by a two-step cotransfection method (16). The first step relies on a high level of recombination between

genomes of the "foreign" virus and AcNPV during replication of AcNPV in Sf9. In the second step, recombinant viruses with altered specificity are selected directly in a new host, in this case Bm5 cells. A series of cotransfection experiments is then carried out to determine which foreign DNA sequences are required to modify the AcNPV genome so that it can productively infect the new host. In the present case, a simple replacement of 79 bases or less in the p143 gene, by homologous genetic recombination with BmNPV DNA, transforms AcNPV-1.2 to a variant able to infect Bm5 as well as Sf9 cells. In this short sequence AcNPV-1.2 and BmNPV-SC7 DNAs possess different nucleotides at six positions, and as a consequence four amino acid changes occur. To confirm that substitutions among nucleotides or amino acids at positions 551, 556, 564, and 577 of the AcNPV p143 gene are implicated in AcNPV host-range extension, we cotransfected Sf9 cells with AcNPV DNA and with BmNPV PCR products overlapping this area. Sequence analysis of 14 AcNPV mutants showed that substitution of species-specific amino acids occurred at expected positions. *In vitro*, the 34 recombinants analyzed possess BmNPV-specific amino acids in the 79-nt region. *In vivo*, one of two recombinants possess only three BmNPV-specific amino acids (position 556, 564, and 577). Thus, Leu-556, Asn-564, and Leu-577 appear to be involved in the host range extension to *B. mori* larvae. The individual role of each of the four amino acids *in vivo* and *in vitro* needs to be determined by site-directed mutagenesis. The method we used to visualize the recombination events leading to the replacement of AcNPV sequence by BmNPV DNA in the p143 AcNPV gene is highly efficient.

The 79-nt sequence implicated in AcNPV species specificity comprises nucleotides 1651–1729 in the p143 gene (28). We conclude that AcNPV specificity modification is due to replacement of these nucleotides and that p143 is a host-range gene. The AcNPV p143 allele allows multiplication of wild-type AcNPV in Sf9, whereas those allelic forms of p143 gene modified by recombination with homologous BmNPV sequences—represented, for example, by the 36 recombinant viruses studied here—allow the infection of both Sf9 and Bm5 cells. Allelic control by the baculovirus p143 gene is different from host-range control in vaccinia virus, where modification of specificity is obtained by deletion of a host-range gene (30).

The role of the AcNPV p143 gene is not well known (28, 31). p143 is indispensable and belongs to the helicase superfamily (28, 32). The present results suggest that AcNPV p143 is implicated in a function posterior to the action of the viral DNA polymerase, since the blockage of virus infection in Bm5 cells transfected with wild-type AcNPV DNA occurs after viral DNA synthesis (O.A., F. Odier, L.C., and G.C., unpublished data).

AcNPV host range is broad: half of the lepidopterous species tested so far have been found to be susceptible to AcNPV infection (3, 7). We speculate that one or a few blockages of the AcNPV replication cycle occur at different points, which may be specific to the particular host, during infection in most nonsusceptible insect species or cell lines. One important barrier, for example, may be at the level of virus particle entry into cells; this step is not well understood, although a 64-kDa glycoprotein located in the envelope of the budded virus and a postulated glycoprotein receptor on insect cells might play a role in the specificity of entry *in vitro* and *in vivo* (33, 34). On the other hand, AcNPV particles can penetrate noninsect cells, which apparently lack a specific receptor (35). The AcNPV helicase gene could be one of

many genes controlling species specificity during virus replication. When species not susceptible to AcNPV infection are permissive for other NPVs, the method we have applied here to AcNPV infectivity in Bm5 cells might be used to determine whether some identifiable and surmountable blockage of AcNPV replication occurs in these species.

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