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# Specificity of multiple homologous genomic regions in *Spodoptera exigua* nucleopolyhedrovirus DNA replication

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**The region upstream of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) ubiquitin gene contains four near-identical 68-bp-long palindromic repeats. This region, named Se*hr*6 and located at map unit (m.u.) 88 of the SeMNPV genome on pSe*Eco*RI-2.2, showed structural homology to previously identified homologous regions (*hr*s) in a number of other baculoviruses. *Hr*s function as enhancers of transcription and as putative origins (*ori*s) of baculovirus DNA replication. Five additional *hr*s (Se*hr*1–Se*hr*5) were identified on the SeMNPV genome by Southern blot hybridization with an 18-bp-long oligonucleotide complementary to a sequence conserved within the arms of the four palindromic repeats of Se*hr*6. Se*hr*1–Se*hr*6 were dispersed on the SeMNPV genome at m.u. 8·0, 30·0, 38·5, 51·0, 77·0 and 88·0, respectively. Sequence analysis of these *hr*s confirmed the presence of palindromic repeats, highly similar to those found in p*SeEco*RI-2.2. The number of palindromes varied from one (Se*hr*4) to nine (Se*hr*1) per *hr*. The Se*hr*s are all present in non-coding regions of the SeMNPV genome and also contain multiple putative transcription recognition sequences. Plasmids containing either of the Se*hr*s replicated in an SeMNPV-dependent DNA replication assay. The Se*hr*s were unable to replicate in an AcMNPV-dependent DNA replication assay. This was in contrast to the previously observed SeMNPV non-*hr* type *ori*, which replicated in the presence of both AcMNPV and SeMNPV. These data suggest that the replication of SeMNPV and the role of *hr*s in this process is highly specific.**

## Introduction

DNA replication is a key process in the multiplication of DNA viruses which involves *cis*- and *trans*-acting elements (Kornberg & Baker, 1992). Baculovirus DNA replication has been predominantly studied for *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and *Orgyia pseudotsugata* MNPV (OpMNPV) (see Lu *et al.*, 1997, for review). Within the AcMNPV genome two types of *cis*-acting elements have been identified as putative origins of DNA replication (*ori*). These *ori*s were able to replicate transiently when transfected into insect cells in the presence of AcMNPV, which provided the five essential *trans*-acting factors, IE-1, DNA polymerase, LEF-1, LEF-2, LEF-3, and the anti-apoptotic factor P35 (Kool *et al.*, 1994).

The first type of *ori* is represented by the homologous regions (*hr*s), which are found dispersed over the AcMNPV genome (Cochran & Faulkner, 1983; Pearson *et al.*, 1992; Kool *et al.*, 1993). AcMNPV *hr*s are characterized by the presence of two to eight repeats of a 72-bp-long sequence with an internal 28-bp-long imperfect palindrome with an *Eco*RI site at its centre. *Hr*s have been identified in the genomes of a number of baculoviruses such as OpMNPV (Ahrens *et al.*, 1995), *Bombyx mori* NPV (BmNPV) (Majima *et al.*, 1993), *Lymantria dispar* MNPV (LdMNPV) (Pearson & Rohrmann, 1995), *Choristoneura fumiferana* MNPV (CfMNPV) (Xie *et al.*, 1995) and *Anticarsia gemmatalis* MNPV (AgMNPV) (Garcia-Canedo *et al.*, 1996). Therefore, the presence of *hr*s can be considered a characteristic feature of baculoviruses.

In AcMNPV and OpMNPV, *hr*s also act as enhancers of early gene expression when placed in *cis* to immediate-early and delayed-early promoters. The efficiency of enhancement

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The relevant nucleotide sequence data are available in the GenBank nucleotide sequence database under accession number AF054872.

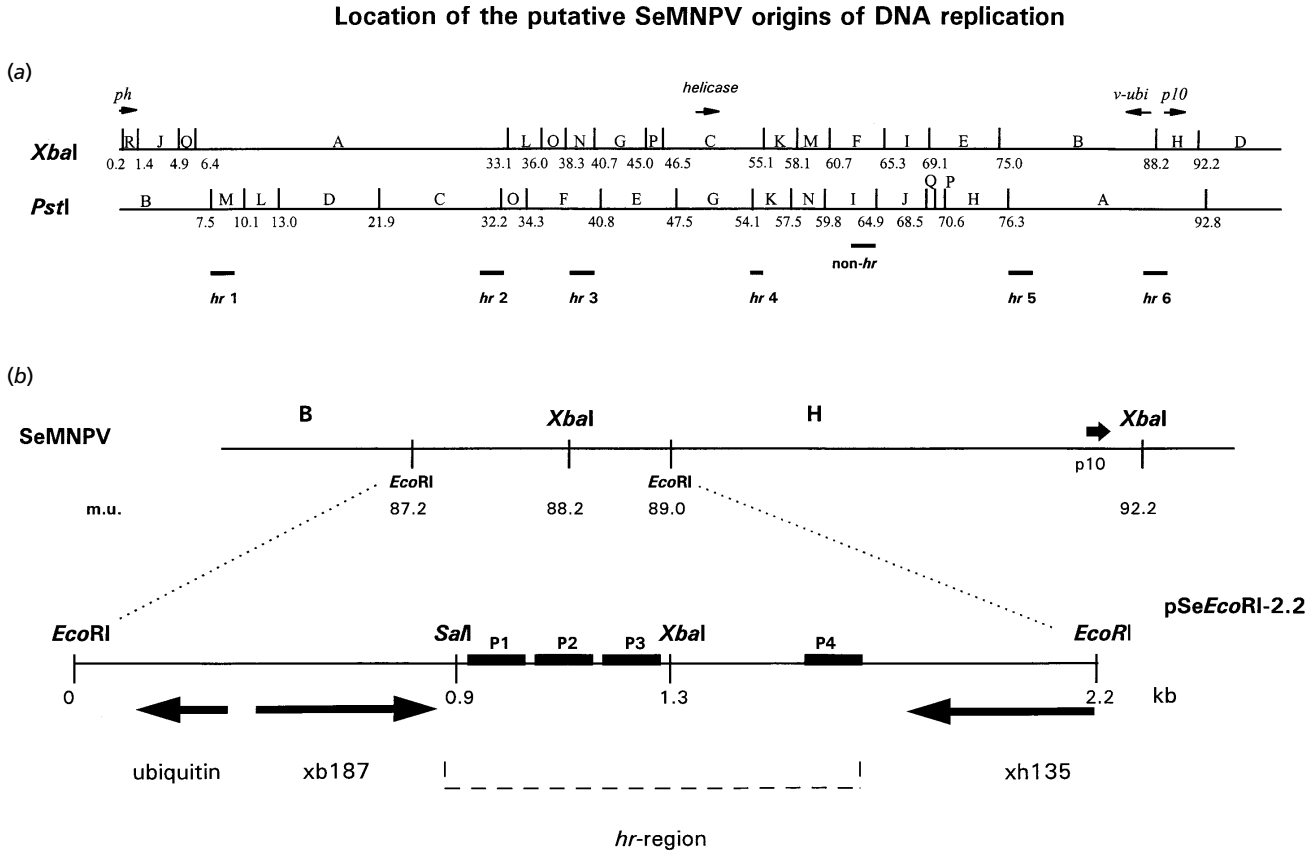


Fig. 1. For legend see facing page.

depends on the presence of the baculovirus regulatory immediate-early gene product IE1 (Guarino & Summers, 1986; Theilmann & Stewart, 1993). AcMNPV IE1 binds as a dimer to the palindromic sequences of an *hr* (Rodems & Friesen, 1995). Interaction of IE1 with these sequences is essential for *hr*s to function as transcriptional enhancers (Leisy *et al.*, 1995). The 28 bp core of the palindrome acts as *ori*, whereas additional flanking sequences are required for enhancer activity (Leisy *et al.*, 1995; Habib *et al.*, 1996).

The second type of putative baculovirus *ori* does not contain *hr*-related sequences (non-*hr ori*), but direct repeats and AT-rich regions resembling eukaryotic *ori*s (Kool *et al.*, 1993; DePamphilis, 1993). Only one copy of such a non-*hr ori* was found in the AcMNPV genome (Kool *et al.*, 1994; Lee & Krell, 1994). Non-*hr ori*s have also been identified in the genomes of OpMNPV and *Spodoptera exigua* MNPV (SeMNPV) (Pearson *et al.*, 1993; Heldens *et al.*, 1997). Enhancing activity of non-*hr*s has not been demonstrated yet. Up to this point, it is unclear what the role or relative contribution of either of these two types of putative *ori*s is in baculovirus DNA replication *in vivo*. The mechanism of baculovirus DNA replication is enigmatic, although a rolling circle model has been proposed (Leisy & Rohrmann, 1993).

SeMNPV is a member of the family *Baculoviridae* and has a double-stranded circular DNA genome of approximately 130 kb (Murphy *et al.*, 1995; Heldens *et al.*, 1996). SeMNPV infects only a single host insect, the beet army worm *S. exigua*, and is successfully applied as a biological insecticide against this pest insect (Smits & Vlak, 1994). A detailed restriction map and an overlapping cosmid library of SeMNPV DNA have recently become available (Heldens *et al.*, 1996). Several SeMNPV genes have been identified such as those encoding polyhedrin, *p10*, *rr1* (ribonucleotide reductase) and *ubiquitin* (van Strien *et al.*, 1992, 1996, 1997; Zuidema *et al.*, 1993). The genetic organization of the SeMNPV genome appeared to differ considerably from that of AcMNPV and OpMNPV (van Strien, 1997; Ayres *et al.*, 1994; Ahrens *et al.*, 1997). In this report, the identification and characterization of *hr*s in the genome of SeMNPV are described and their replication competence in SeMNPV- and AcMNPV-infected insect cells is investigated. The specificity of *hr*s may be one factor involved in the specificity of SeMNPV DNA replication.

## Methods

■ **Cells, virus, plasmids and cosmids.** *S. frugiperda* (*Sf*-AE-21) (Vaughn *et al.*, 1977) and *S. exigua* (Se-IZD-2109) cells (a gift from B. Möckel) were cultured in TNM-FH medium (Hink, 1970) supplemented with 10% foetal calf serum (FCS). The SeMNPV-US isolate (Gelernter & Federici, 1986) and the AcMNPV E2 strain (Smith & Summers, 1978) were produced using *S. exigua* fourth instar larvae. Routine cell culture

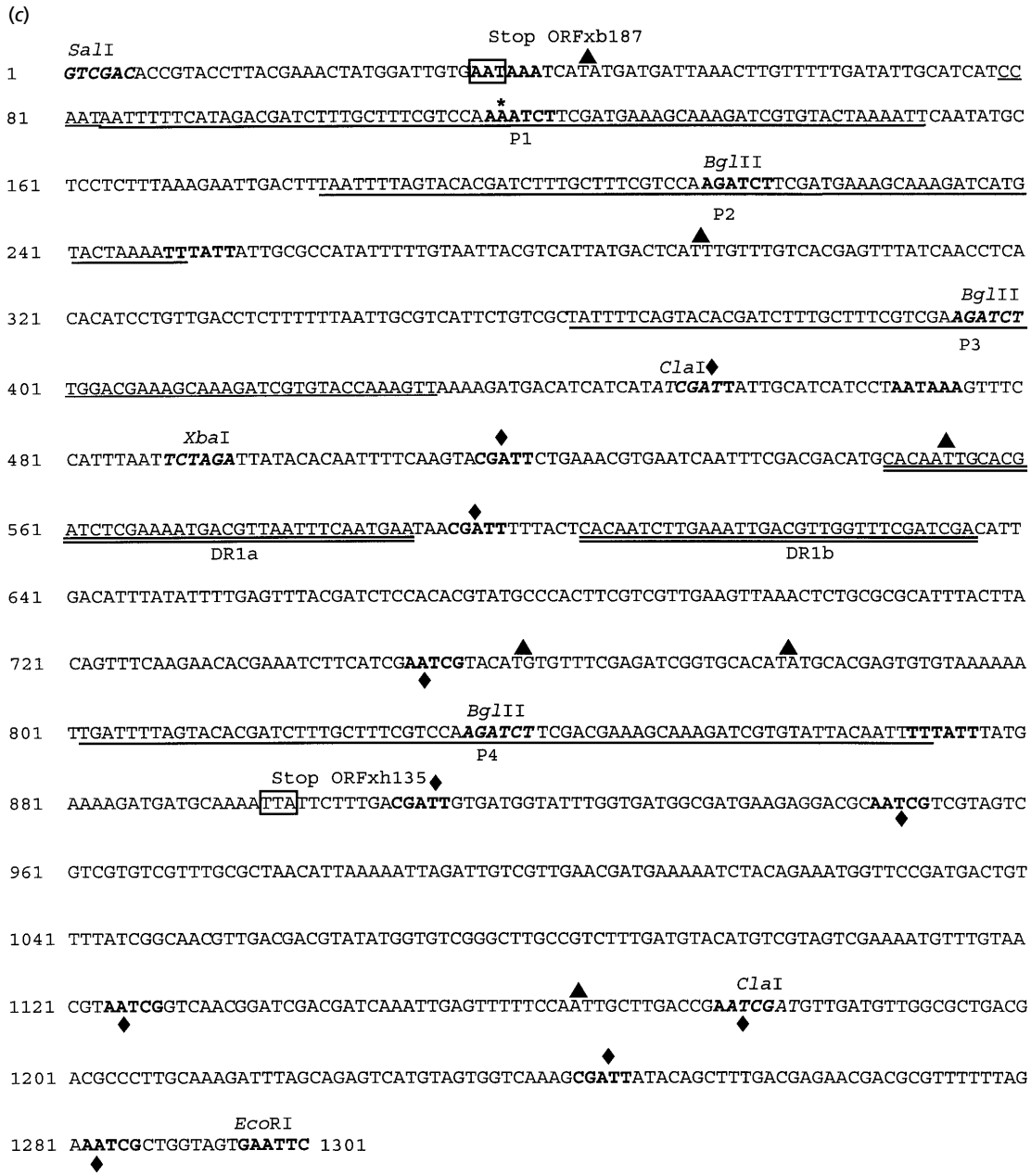


Fig. 1. (*a*) Genomic location of *hr*s on the genome of SeMNPV with *Xba*I and *Pst*I restriction sites. The location and direction of transcription (arrows) of the *Se*MNPV polyhedrin (*ph*), helicase, *ubi* and *p10* gene, and the location of the *Se*MNPV non-*hr* type origin, are shown. (*b*) Location of the pSe*Eco*RI-2.2 fragment between map units 87·2 and 89·0 on the *Xba*I restriction map of SeMNPV DNA. The black boxes represent the position of the palindromic repeats P1–P4 within the *hr* on the pSe*Eco*RI-2.2 fragment. (*c*) Nucleotide sequence of the 1·3 kb *Sal*I–*Eco*RI fragment within fragment pSe*Eco*RI-2.2. Restriction sites are indicated in italics. Palindromic repeats P1–P4 are underlined. The asterisk (*) represents a mutation in P1 that disrupts the *Bgl*II site. The direct repeats DR1a, DR1b and DR1c are double underlined. The CGATT motif is in bold and marked with a ◆, above or below the sequence depending on whether the motif is present on the forward or complementary strand. Putative poly(A) signals are in bold. The CANNTG (MLTF/USF) motif is underlined.

maintenance and AcMNPV and SeMNPV infection procedures were carried out according to published procedures (Summers & Smith, 1987; van Strien *et al.*, 1996; Heldens *et al.*, 1996). The SeMNPV plasmid and cosmid libraries were described previously (Heldens *et al.*, 1996).

■ **Southern blot hybridization.** SeMNPV DNA, isolated from occlusion body-derived (ODV) viral DNA, was digested with various restriction enzymes, separated in a 0·7% agarose gel and transferred to Hybond-N nylon membrane (Southern, 1975). An 18-bp-long oligonucleotide, RB-33 (5′ TAC ACG ATC TTT GCT TTC 3′), was made based on a conserved sequence within the P repeats within Se*hr*6 (see below, Fig. 3). The membrane was hybridized overnight in Church buffer (0·25 M sodium phosphate, pH 7·2, 7% SDS, 1% BSA, 1 mM EDTA) at

50 °C with RB-33, which was end-labelled with [$^{32}$P]dATP using T4 kinase (Gibco BRL). The blot was washed once at room temperature followed by an incubation step for 30 min at 50 °C in Church buffer to remove unbound label and primer. The blot was exposed to Kodak XAR film.

■ **Construction of *hr*-containing plasmid clones.** SeMNPV DNA fragments were cloned into either pUC19, pTZ19 or pBluescript KS(+), and transformed into *Escherichia coli* DH5α using standard techniques (Sambrook *et al*., 1989). DNA isolation, purification, digestions with restriction enzymes (Gibco BRL), agarose gel electrophoresis and Southern blotting were carried out according to standard procedures (Sambrook *et al*., 1989).

Plasmid pSe*Eco*RI-2.2, containing the SeMNPV ubiquitin gene, was described previously by van Strien *et al*. (1996). The fragments that hybridized to RB-33 were cloned and analysed. Clone pSeCHK-5.7 was obtained after digestion of SeMNPV fragment *Xba*I-C with *Hin*dIII and *Kpn*I, and subsequent isolation of the 5·7 kb *Hin*dIII–*Kpn*I restriction fragment and cloning into pUC19. Plasmids pSe*Xba*I-H and pSe*Xba*I-N were taken from the *Xba*I-library of SeMNPV in pUC19 (Heldens *et al*., 1996). Clone pSeB*Pst*I-5.6 was obtained after digestion of cosmid 17 DNA (Heldens *et al*., 1996) with *Bam*HI and *Pst*I. Plasmid pSe*Pst*I-M was obtained by digestion of SeMNPV ODV DNA with *Pst*I and insertion of the *Pst*I-M fragment into *Pst*I-digested pUC19. Clone pSeP*Spe*I-6.3 was obtained after digestion of cosmid 22 DNA (Heldens *et al*., 1996) with *Pst*I and *Spe*I, and insertion of a 6·3 kbp fragment into *Pst*I- and *Xba*I-digested pUC19. Ac*hr*5 was present on pAc*Hin*dIII-L (Kool *et al*., 1993). All plasmids were amplified in *E. coli* DH5α and JM101 (*Dam*$^+$) strains.

■ **Replication assay.** The assay to test the replicative ability of the Se*hr* and Ac*hr* plasmids was as previously described by Heldens *et al*. (1997) for the *Se*MNPV non-*hr*. In short, $10^6$ *Sf*-AE-21 cells were transfected with 1 μg SeMNPV or AcMNPV *hr*-containing plasmids and infected 16 h later with the respective MNPVs with a m.o.i. of 1 $TCID_{50}$ unit per cell. Plasmid pUC19 (1 μg) was added as control for the amount of plasmid DNA retrieved after extraction. The cells were harvested 48 h post-infection (p.i.) (AcMNPV) or 72 h p.i. (SeMNPV). Total DNA was isolated from $10^6$ infected cells (Summers & Smith, 1987) and resuspended in 60 μl $H_2O$. One aliquot (10 μl) was digested with *Hin*dIII to linearize the plasmid DNA. A second aliquot (10 μl) was digested with *Hin*dIII and *Dpn*I. The use of *Dpn*I allows the discrimination of input (*Dpn*I-sensitive) from replicated plasmid DNA (*Dpn*I-insensitive) (Kool *et al*., 1993). After agarose gel electrophoresis, the DNA was transferred to a nylon membrane filter (Hybond-N) and hybridized to α-$^{32}$P-labelled pUC19 to detect plasmid sequences (Sambrook *et al*., 1989).

■ **Nucleotide sequencing.** The nucleotide sequence of the cloned fragments was obtained by sequencing overlapping subclones of the fragments and/or by a primer walking strategy using standard and custom-designed oligonucleotide sequence primers. Sequencing was carried out at the Core Facility for protein and DNA Chemistry at Queen's University in Canada using the dideoxy chain termination based protocol (Sanger *et al*., 1977). Sequence analyses were carried out using UWGCG computer programs (Devereux *et al*., 1984) and MEGALIGN for Windows (DNASTAR). The relevant nucleotide sequence data are available in the GenBank nucleotide sequence database under accession number AF054872.

## Results

### Sequence analysis of pSe*Eco*RI-2.2

Analysis of sequences upstream of the SeMNPV *ubi* gene region on fragment pSe*Eco*RI-2.2 (m.u. 87·2–89·0) revealed the presence of four homologous repeats. These repeats were located in a non-coding region of about 900 bp (Fig. 1*b*) flanked upstream by ORFxb187 (van Strien *et al*., 1997) and downstream by ORFxb135, both of unknown function. The four homologous repeats (P1–P4) contained a near-perfect palindrome of 68 bp in length, of which each of the last three repeats (P2–P4) were centred around a *Bgl*II site. The *Bgl*II site in P1 was imperfect. Further analysis of the region between palindromes P3 and P4 showed the presence of two direct repeats (DR1a and DR1b), 47 bp in length (Fig. 1*c*). The organization of such repeat motifs in the Se*Eco*RI-2.2 region is comparable to that of other baculovirus *hr*s (Lu *et al*., 1997). This suggests that the identified palindromic repeats might represent an SeMNPV *hr*-type *ori* (Se*hr*).

Within eukaryotic *ori*s the processes of transcription and replication are often tightly linked (Heintz *et al*., 1992). This is reflected by the presence of multiple transcription factor binding sites and transcription initiation sites near or within eukaryotic *ori*s. Several such sequences were identified in the region encompassing the putative Se*hr* (Fig. 1*c*; Fig. 2). The CGTGC motif (or its inverse), which is an important early transcription initiation site in AcMNPV DNA polymerase (Tomalski *et al*., 1988) and helicase (*p*143) genes (Lu & Carstens, 1993) was present four times in this Se*hr*. Two CGTGC motifs were clustered in a 50 bp segment located 350 bp upstream of the 5′ end of P1 (not shown). One CGTGC motif was located 20 bp upstream of the 5′ end of P4. Three consensus polyadenylation signals (AATAAA) were present near the 3′ end of P2, P3 and P4. Those at the 3′ end of P2 and P4 were in an antigenomic orientation (Fig. 1*c*; Fig. 2). Six copies of the MLTF/USF motif (CANNTG) (Carthew *et al*., 1985) and two copies of a CCAAT motif (Benoist *et al*., 1980) were present within the 1300 bp *Sal*I–*Eco*RI region. Ten copies of a less well characterized motif, 5′ CGATT 3′ or its inverse (Lee & Krell, 1994), were also present within an 800 bp region downstream of P3.

### Identification and sequence analysis of additional SeMNPV *hr*s

The presence and dispersed occurrence of *hr*s appears to be a common feature of baculovirus genomes. An 18-bp-long oligonucleotide fragment, RB-33, was designed based on a conserved region within palindromes P1–P4 in pSe*Eco*RI-2.2 and hybridized to SeMNPV DNA restriction fragments to identify other *hr* regions on the SeMNPV genome. Several oligonucleotide-specific hybridization signals were observed, which were subsequently mapped to restriction fragments *Xba*I-B, *Xba*I-C *Xba*I-H, *Xba*I-N, *Pst*I-M (Fig. 1*a*), *Spe*I-G and *Sst*I-E (not indicated). Two fragments, *Xba*I-B and *Xba*I-H, are adjacent fragments on the physical map of the SeMNPV genome and represent pSe*Eco*RI-2.2. These hybridization data and comparison with the location of the respective fragments

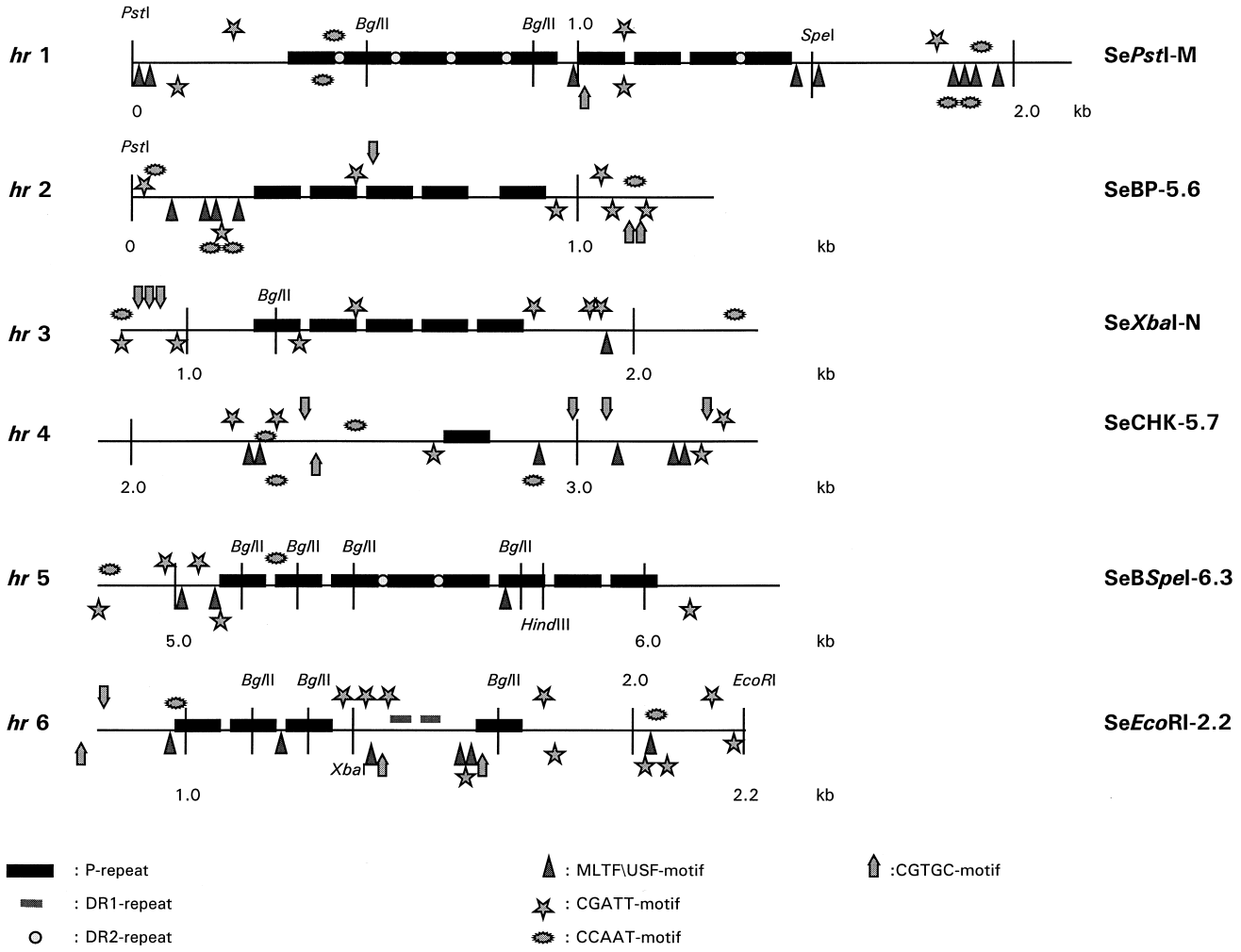


Fig. 2. Organization of palindromic repeats (P), direct repeats (DR) and other motifs within Se*hr*1–Se*hr*6.

suggest that *hr*-like sequences occur dispersed on the SeMNPV genome as well.

Further subcloning of fragments *Xba*I-C, *Spe*I-G and *Sst*I-E and hybridization with RB-33 revealed (data not shown) that putative SeMNPV *hr*s are present on fragments pSeB*Pst*I-5.6, pSeCHK-5.7 and pSeP*Spe*I-6.3, derived from *Pst*I-C, *Xba*I-C and *Xba*I-B, respectively. Further *hr*s were found on pSe*Pst*I-M and pSe*Xba*I-N. The *hr*s are numbered Se*hr*1–Se*hr*6 according to their relative position on the physical map of the SeMNPV genome (Fig. 1*a*).

Sequence analysis of the fragments containing Se*hr*1–Se*hr*5 confirmed the presence of clusters of P repeats, which were highly homologous to those found in Se*hr*6. The number of P repeats present varied per *hr* ranging from one in Se*hr*4 to nine in Se*hr*1 (Fig. 2, Table 1). The sequences of Se*hr*1 to Se*hr*5 were examined for the presence of the putative transcriptional motifs and DRs found in Se*hr*6 (Fig. 1*c*, Fig. 2). A size limit of 500 bp was set to either side of each *hr* in the analysis of sequence motifs. A novel direct repeat sequence element (DR2), 37 bp in length, was found in front of five out of the nine P repeats observed in Se*hr*1, and in front of two out of the seven P repeats in Se*hr*5. The DR2 motif, characterized by the consensus TCATcGCtAAAaATAGATTTGACgCAATacaA-AACT, was not present in the other *hr*s. The DR1 motif identified in Se*hr*6 was not present in Se*hr*1–Se*hr*5.

## Alignment of Se*hr* repeats

When all 32 P repeats in Se*hr*1–Se*hr*6 are aligned, a consensus sequence is derived (Fig. 3), which could form a perfect hairpin. None of the individual P repeats contains the consensus sequence. Twenty-six nucleotides are absolutely conserved, of which an AAAGCAAA stretch (right arm sequence) is most notable. The SeMNPV P repeats are further characterized by the presence of a *Bgl*II site at the core–loop region of the palindrome. The *Bgl*II site is sometimes imperfect. Variations occur at the top and the bottom of the putative hairpin. Alignment of the consensus sequence or each of the individual SeMNPV P repeats failed to show sequence homology with *hr*s of AcMNPV, OpMNPV, LdMNPV, *Spodoptera littoralis* NPV (SpliNPV), CfMNPV or AgMNPV (data not shown).

**Table 1.** Number and types of sequence motifs found within Se*hr*1–Se*hr*6

Palindromes with TACACGATCTTTCTTTC, DR1 with TGAACGTTAATTTC and DR2 with TAGATTTGAC consensus repeat. AcMNPV early transcription signal CGTGC (Tomalski *et al.*, 1988), MLTF/USF motif CANNTG (Carthew *et al.*, 1985).

| Motif | *hr*1 | *hr*2 | *hr*3 | *hr*4 | *hr*5 | *hr*6 |
|---|---|---|---|---|---|---|
| P repeat | 9 | 5 | 5 | 1 | 8 | 4 |
| R1 repeat | 0 | 0 | 0 | 0 | 0 | 2 |
| R2 repeat | 5 | 0 | 0 | 0 | 2 | 0 |
| CCAAT motif | 5 (3) | 4 (2) | 2 | 4 (2) | 2 | 2 |
| Early trans. ini. site | 2 (2) | 3 (2) | 3 | 5 (1) | 0 | 4 (3) |
| MLTF/USF | 14 | 5 | 1 | 6 | 3 | 6 |
| Poly(A) | 5 (3) | 3 (1) | 5 (2) | 6 (5) | 6 (4) | 3 (2) |
| CGATT motif | 6 (2) | 7 (4) | 7 (3) | 5 (2) | 5 (3) | 10 (5) |

## Replicative ability of Se*hr*s

The ability of all Se*hr*-containing fragments to replicate was tested in an SeMNPV-dependent DNA replication assay (Fig. 4). All Se*hr*s were able to replicate in the presence of SeMNPV as helper-virus (lanes 2–7). The replication was *hr*-specific, since plasmid pUC19 (lane 8) and an SeMNPV fragment without a putative *ori*, pSe*Xba*I-F1 (lane 10), did not replicate in the assay. *Sf*-AE-21 cells transfected with pSe*Eco*RI-2.2 (lane 1) and pUC19 (all lanes) alone were included in the assay in order to identify the background of input DNA for *Dpn*I digestions. All *hr*-containing SeMNPV fragments (lanes 2–7) replicated at a lower level than the non-*hr* type *ori* present on pSe*Xba*I-F2 (lane 11) (Heldens *et al.*, 1997). Based on the replicative ability of pSeCHK-5.7 (Se*hr*4, lane 4) and of pSe*Xba*I-H (Fig. 1*a*; results not shown), both containing a single P repeat, it was concluded that the replication assay was highly sensitive and that the presence of a single palindromic sequence was sufficient for replication activity.

## Specificity of Se*hr*s in DNA replication

To test the specificity of the *hr*s for SeMNPV DNA replication factors, we investigated whether AcMNPV could recognize and replicate SeMNPV *hr ori*s and SeMNPV the AcMNPV *hr ori*s. *Sf*-AE-21 cells were thus transfected with Se*hr*6 or Ac*hr*5 and infected with AcMNPV or SeMNPV, respectively. The results show that Se*hr*6 replicates only in the presence of SeMNPV (Fig. 5, lane 2) and not in the presence of AcMNPV (Fig. 5, lane 1). Conversely, Ac*hr*5 did not replicate

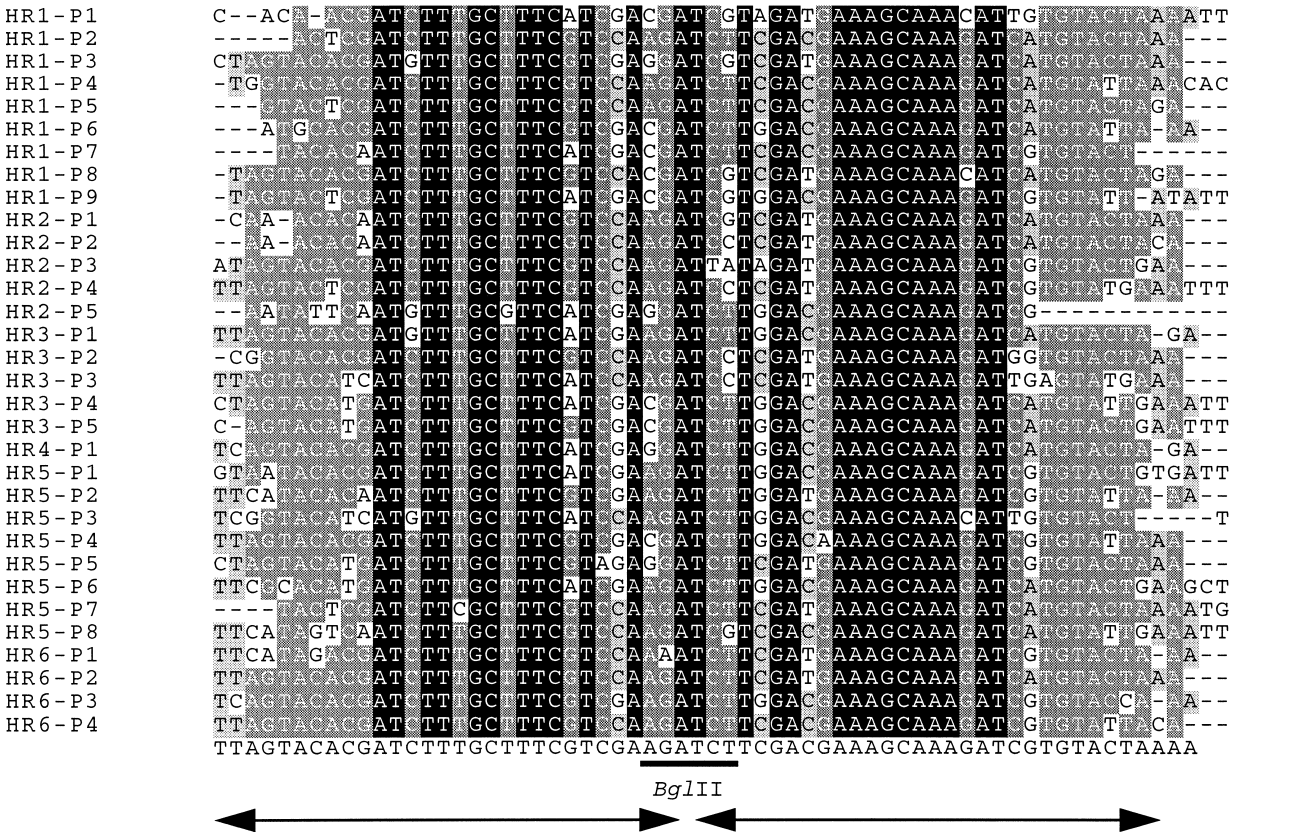


Fig. 3. Alignment of all 32 palindromic repeats from Se*hr*1 to Se*hr*6 and deduced Se*hr* consensus sequence. Sequences are presented in the forward and reverse (r) orientation. The differences in grey shading above the consensus sequence indicate the level of conservation, from black (completely conserved) to light (less conserved) shading.

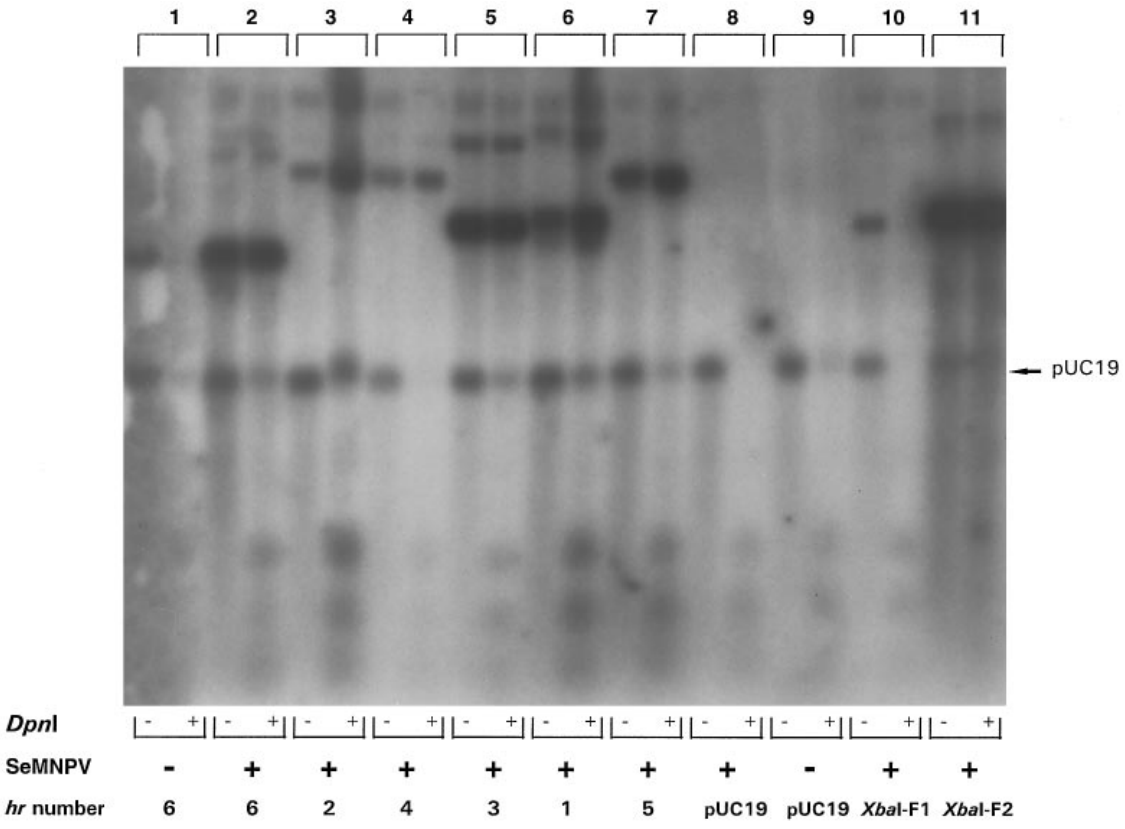


Fig. 4. Transient replication assay of putative *hr*-containing SeMNPV fragments in *Sf*-AE-21 cells infected with SeMNPV. *Sf*-AE-21 cells ($5 \times 10^4$) were transfected with 0·5 μg of SeMNPV plasmid and pUC19 as a internal control. Plasmid pSe*Eco*RI-2.2 (Se*hr*6) (lane 2), pSeB*Pst*I-5.6 (Se*hr*2) (lane 3), pSeCHK-5.7 (Se*hr*4) (lane 4), pSe*Xba*I-N (Se*hr*3) (lane 5), pSe*Pst*I-M (Se*hr*1) (lane 6), pSeP*Spe*I-6.3 (Se*hr*5) (lane 7), pUC19 alone (lane 8), pSe*Xba*I-F1 (lane 10) and pSe*Xba*I-F2 (Se non-*hr*) (lane 11) transfected *Sf*-AE-21 cells followed by SeMNPV infection. Plasmids pSe*Eco*RI-2.2 (Se*hr*6) (lane 1) and pUC19 (lane 9) were without SeMNPV infection. The arrow indicates the position of pUC19, which served as an internal control for DNA yields obtained and for the efficiency of *Dpn*I-digestions. The + or − sign below the lanes indicates whether the sample was digested with *Hin*dIII (−) or with both *Hin*dIII and *Dpn*I (+). Southern hybridization was carried out using $^{32}$P-labelled pUC19 as a probe.

in the presence of SeMNPV (Fig. 5, lane 4), suggesting that the DNA replication is *hr*-specific. These results contrast with the observation that the SeMNPV non-*hr ori* was able to replicate to a significant level in the presence of AcMNPV as helper virus (Heldens *et al*., 1997).

## Discussion

Analysis of a 1·5 kb region flanking the SeMNPV ubiquitin gene (van Strien *et al*., 1996) revealed the presence of sequence motifs structurally reminiscent of homologous regions (*hr*s) in other baculoviruses. This region (Se*hr*6) contained four palindromic repeats (P1–4), two direct repeats (DR) and a number of other motifs which could be involved in regulation of transcription. Se*hr*6 also replicated in an SeMNPV-dependent DNA replication assay. This strongly suggests that this SeMNPV sequence is an *hr* (Lu *et al*., 1997).

Hybridization of a conserved oligonucleotide sequence of these P repeats of Se*hr*6 with the SeMNPV genome led to the identification of five additional *hr*-regions in the genome, numbered Se*hr*1–Se*hr*5 according to their relative position on the SeMNPV physical map (Heldens *et al*., 1996). The Se*hr*s described here are interspersed throughout the genome in a similar fashion to other baculoviruses. Even an *hr* with only a single P repeat (Se*hr*4) was identified by Southern hybridization, making the presence of additional *hr*s of this type unlikely. The presence of multiple *hr*s in baculoviruses may provide redundancy of *ori*s to ensure DNA replication.

A single repeat is a minimal requirement for plasmid-dependent DNA replication (Leisy *et al*., 1995). All plasmids containing an Se*hr*s were replication-competent in transient replication assays (Fig. 3), whereas numerous SeMNPV DNA-containing plasmids were found to be negative in the assay with the exception of SeMNPV *Xba*I-F, which contained a non-*hr ori* (Heldens *et al*., 1997). It is possible that other *hr*-like sequences occur, but these have not yet been discovered in the 70 kb sequence presently available for SeMNPV (R. Broer, unpublished results). Comparison of the replicative activity of

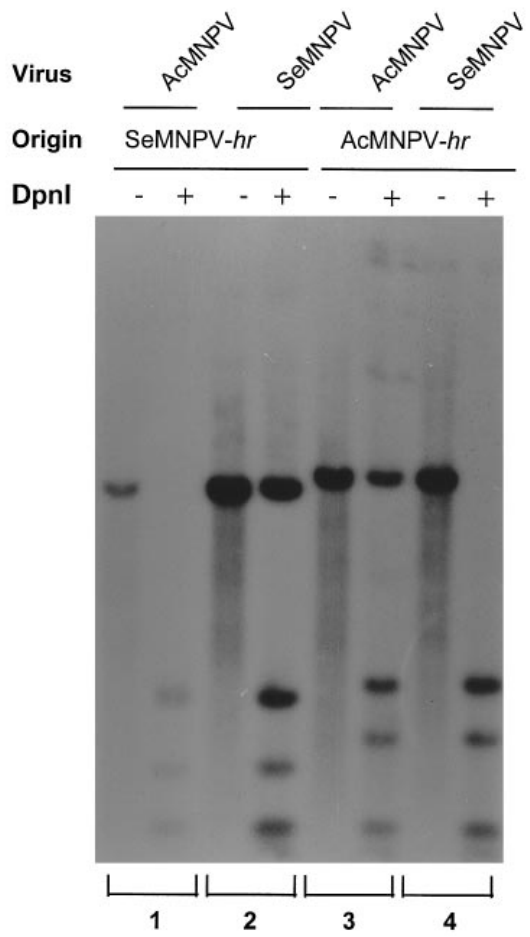


**Fig. 5.** Specificity of SeMNPV *hr* activity in transient replication assays. *Sf*-AE-21 cells were transfected with pAc*Hin*dIII-L (Ac*hr*5) followed by SeMNPV infection (lane 4), pAc*Hin*dIII-L (Ac*hr*5) followed by AcMNPV infection (lane 3), pSe*Eco*RI-2.2 (Se*hr*6) followed by SeMNPV infection (lane 2) or pSe*Eco*RI-2.2 (Se*hr*6) followed by AcMNPV infection (lane 1). The + or − sign above the lanes indicate whether the sample was digested with *Hin*dIII (−) or with both *Hin*dIII and *Dpn*I (+).

the six Se*hr*s did not reveal a positive correlation between the number of P repeats and the replicational signals observed.

Motifs DR1 and DR2 may be candidates for modulation of DNA replication and/or transcription since they occur in Se*hr*1, Se*hr*5 and Se*hr*6, but not in Se*hr*2–Se*hr*4. The Se*hr* regions also contained a number of other motifs and repeats which may be involved in either DNA replication and/or enhancement of transcription of Se*hr*s. Preliminary experiments indicate that Se*hr*6 acts as an enhancer of SeMNPV *ie-1* expression (D. A. Theilmann & E. A. van Strien, unpublished results). Mutational analysis of these motifs could provide more insight into the role that these sequences might play in DNA replication and enhancement of transcription.

All Se*hr*s contained a near-identical 68-bp-long palindromic repeat with no sequence homology with other known baculovirus *hr*s (Fig. 3). This palindrome is much larger than that of AcMNPV (28 bp) and CfMNPV (36 bp), but the structure with a central core sequence resembling a restriction enzyme recognition site is highly similar to *hr*s of other baculoviruses. Both the length of the repeat and the unique sequence may contribute to the specificity in replication-competence of the Se*hr*s for SeMNPV. Sequence alignment of all SeMNPV *hr*s indicate that there is a highly conserved octamer sequence (AAAGCAAA) in one of the arms of each repeat. The functional significance of this conserved octamer box is unclear.

Individual P repeats are characterized by the presence of a (degenerate) restriction site, *Bgl*II, at the centre of the repeat (Fig. 3) and located at the bulge region of the putative stem–loop. A similar situation exists in AcMNPV where a degenerate *Eco*RI site is present in the *hr* (Cochran & Faulkner, 1983). Mismatches are found in all identified palindromic repeat-containing baculovirus *hr*s. Rasmussen *et al.* (1996) investigated whether these mismatches allowed a palindromic repeat to form a cruciform structure, which could enable binding of the baculovirus transactivator IE-1. However, it was found that IE-1 did not bind to a 42 bp perfect and imperfect hairpin structure derived from Ac*hr*1 under cruciform-forming conditions, suggesting that the cruciform structure does not have a direct role in IE-1 binding. IE-1 did, however, bind to the 42 bp perfect and imperfect oligonucleotides with equal affinity under normal non-cruciform-forming conditions, indicating that the conserved AcMNPV palindromic mismatches do not affect IE-1 binding. Maybe the binding of IE-1 to the palindrome is insufficient to enhance transcription, but is required for replication, underscoring the bifunctionality of baculovirus *hr*s (Habib *et al.*, 1996).

In contrast to the Se*Xba*I-F2 non-*hr ori* (Heldens *et al.*, 1997) the replication of Se*hr*s is highly specific. This is unlike the situation with AcMNPV *hr*s when tested with other baculoviruses. Plasmids containing Ac*hr*5, for example, replicate when transfected into OpMNPV-infected Ld652-Y cells (Ahrens *et al.*, 1995) or CfMNPV-infected Cf-124-T cells (Xie *et al.*, 1995). However, Cf*hr*1-containing plasmids could not replicate in the presence of AcMNPV in Sf21 cells (Xie *et al.*, 1995) as is the case for Se*hr*s. *hr* SpliNPV does replicate in AcMNPV-infected cells, but not in SeMNPV-infected cells (Faktor *et al.*, 1997). These data suggest that AcMNPV *hr*s are often more promiscuous than *hr*s of other viruses except when they are transfected into SeMNPV-infected cells, whereas SeMNPV DNA replication is a highly specific process.

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