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The Homologous Regions of *Spodoptera litura* (Lepidoptera: Noctuidae) Nucleopolyhedrovirus II Have Both the Function as Origin of DNA Replication and Enhancer

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ABSTRACT. In the genome sequence of the *Spodoptera litura* nucleopolyhedrovirus II (SpltNPVII), seven homologous regions (*hr*s), Sp*hr*1-7, were identified. Each of them composed of three to eight 64-bp highly conserved sequences, and each contained a 24-bp imperfect palindrome. A transient expression assay demonstrated that the expression of SpltNPVII-*ie1* promoter-driven luciferase gene was enhanced between 3- and 13-fold by infection of SpltNPVII in Spli221 cells. Real-time polymerase chain reaction confirmed each of seven *hr*s could function as origin (*ori*) of viral DNA replication. This suggests that these *hr*s are bifunctional, having both *ori* and enhancer activities for transcription. In addition, the potential of seven *hr*s as origins had a significantly positive correlation with the number of their palindromes ($r = 0.847$, Sig: $0.016 < 0.05$), and enhancer efficiency had a significantly positive correlation with the number of characteristic motifs ($r = 0.893$, Sig: $0.007 < 0.01$). The efficiency of replication and enhancement of each *hr* both increased with increasing total numbers of palindromes, repeat sequences, and characteristic motifs. In addition, a single 64-bp highly conserved consensus sequence cannot very good support to the function as origin and enhancer, and require the assistance of other *cis*-elements in *hr*s.

Key Words: homologous region (*hr*), DNA replication origin, enhancer, nucleopolyhedrovirus, *Spodoptera litura*

Baculoviruses comprise a family of invertebrate viruses with large circular, double-stranded, covalently closed, supercoiled DNA genomes ranging in size from 80 to over 188 kb, depending on the viral species (Hilton and Winstanley 2007, Ferrelli et al. 2012). There are four genera of baculoviruses, including *Alphabaculovirus* (lepidopteran-specific nucleopolyhedrovirus [NPV]), *Betabaculovirus* (lepidopteran-specific Granuloviruses), *Gammabaculovirus* (hymenopteran-specific NPV), and *Deltabaculovirus* (dipteran-specific NPV) (Jehle et al. 2006). A unique characteristic of almost all baculovirus genomes studied to date is the presence of homologous repeat regions (*hr*s), the first described being *hr*3 of *Autographa californica* multiple NPV (AcMNPV) (Cochran and Faulkner 1983). These sequences typically contain many AT-rich regions, one or more copies of an imperfect palindrome sequence, some repeat sequences, and other motifs located in several positions on the genome. Within AcMNPV, nine homologous regions contain two to eight repeats centered around *Eco*RI sites (Viswanathan et al. 2003). These function as origins of replication when cloned into plasmids and transfected into infected insect cells and as enhancers of early gene transcription (Olson et al. 2003).

Besides those that function as origins of replication of baculoviruses, another type, non-*hr ori*s, may be involved in genome replication (Kool et al. 1994a). They contain AT-rich regions, direct repeats, and *hr*-unrelated palindromes. Only one non-*hr ori* was found in the AcMNPV genome, and it showed some distant structural homology to consensus eukaryotic *ori*s (DePamphilis 1993, Lee and Krell 1994). Surprisingly, the *p143*-3.2a *ori* of AcMNPV was found to function as an efficient *ori* in mammalian cells without the need for any viral proteins (Wu et al. 2014). Non-*hr ori*s have also been identified in the genomes of *Orgyia pseudotsugata* MNPV (OpMNPV) and *Spodoptera exigua* MNPV (SeMNPV) (Pearson et al. 1993, Heldens et al. 1997). Unlike *hr*s, the non-*hr ori*s of AcMNPV, OpMNPV, and SeMNPV are more complex and unique. Data on the functional role of non-*hr ori*s come from analysis of defective viruses (AcMNPV) isolated after serial passage, in which non-*hr* sequences seem to accumulate (Lee and Krell 1994, Pijlman et al. 2003) and deletion of an *Ssp*I fragment of SeMNPV, which is an essential element of non-*hr ori*s (Heldens et al. 1997).

Baculovirus immediate early gene 1 (*ie-1*), one of six genes required for DNA replication in transient replication assays, encodes a 67-kDa nuclear protein (IE1) that plays crucial roles in viral replication (Kool et al. 1994b, Friesen 1997). Transcription of *ie-1*, together with other promoters, is activated by baculovirus homologous regions (Lin et al. 2010). Thus baculovirus immediate early gene promoters have been proven useful for transient or continuous expression in a multitude of lepidopteran and dipteran cell lines (Jarvis et al. 1996, Pfeifer et al. 1997). In previous work, IE-1, the only early factor of *Bombyx mori* nuclear polyhedrosis virus (BmNPV) mediates *hr* enhancer function in *trans* (Lin et al. 2010).

The *Spodoptera litura* NPV II belongs to *Alphabaculovirus* of baculoviruses (GenBank ID: EU780426.1). The presence of putative origins in the SpltNPVII genome has been determined. In this article, the putative origins of replication of SpltNPVII are characterized for the first time, using a cell line permissive to SpltNPVII infection. We investigated all seven *hr* regions that can function as *ori* in an infection-dependent replication assay. A real-time polymerase chain reaction (PCR) method was used to allow for the quantification and comparison of plasmids containing different *hr* sequences. The results indicated that all *hr*s can serve as origins of SpltNPVII DNA replication. We also demonstrated that SpltNPVII *hr*s can enhance the activity of the SpltNPVII *ie-1* gene promoter with or without viral infection. This suggests that all *hr*s in SpltNPVII are bifunctional, serving as origins of replication and as enhancers of transcription.

Materials and Methods

Viruses and Cells. SpltNPVII strain (Kupriianova et al. 2002) and the cell line TUAT-Spli221 (Spli) (Mitsuhashi 1995) were gifts from Professor Zhu Jiang (Medical College of Soochow University). The cell line was continuously propagated in TC-100 insect medium (AppliChem, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) at 27°C. The details of cell culture methods were as described previously (Summers and Smith 1987).

Bioinformatics Analysis. SpltNPVII homologous regions were surveyed and analyzed using the Genetyx-win software package (parameters: minimum length 20, maximum length 160, and over 75% matching) and compared with genomes of closely related viruses using the nblast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Genetyx software. Correlation analysis was carried out using Statistical Product and Service Solutions (SPSS Inc., Chicago, IL).

Plasmid Construction. Using SpltNPVII DNA as template, the intact fragments (*hr*2: 810, *hr*3: 1,113, *hr*5: 510, and *hr*6: 1,155 bp) were amplified by PCR. *hr*1, *hr*4, and *hr*7, which contain all imperfect palindromes and a varying number of anomalous repeat sequences (*hr*1: 1,327, *hr*4: 1,442, and *hr*7: 1,504 bp), were also amplified. Primers (Table 1) were designed from the SpltNPVII sequence (GenBank ID: EU780426.1). Amplified fragments were subsequently cloned into pUC19, confirmed by direct sequencing (Sanger sequencing, ABI 3730XL), and named pSp*hr*1-7. These were used to determine replication efficiency. The SpltNPVII *ie-1* promoter (~510 bp) was amplified and cloned into the *Xho* and *Hin*dIII sites of pGL3-Basic (Promega, Madison, WI) to generate the plasmid pSp-*ie*1P/*luc*. All *hr* fragments were then subcloned into the pGL3-Basic vector with the *ie-1* promoter, which contains the reporter gene luciferase (*luc*) placed downstream of the multiple cloning sites (*Bam*HI and *Sal*I) generating pSp*hr*1-7/*luc* (Fig. 1).

To explore the nature of *hr*-stimulated replication and enhancement, the typical 64-bp-long conserved repeat sequence of *hr*s, which contains half a *Bgl*II or *Sal*I site in the forward and reverse sequence, respectively (Table 1, pSp64), was synthesized by Sun Biotech Company (Beijing). The synthesized sequence was then cloned into pUC19 (pSp64) and pSp-*ie*1P/*luc* (pSp64-*ie*1P/*luc*) by *Bam*HI and *Sal*I digestion, respectively.

Table 1. Primers used for construction of reporter plasmids

Primer	Sequence (5′–3′)
Sp-*hr*1-F	CGGGATCCACTTTGACGAAGACGCATTTGC (*Bam*HI)
Sp-*hr*1-R	CG GTCGAC AGATGCATCTTCCTTGAAATCG (*Sal*I)
Sp-*hr*2-F	CGAGATCTATAGGCCATAGTCTCGAGAGTAC (*Bgl*II)
Sp-*hr*2-R	CGGTCGACGTGATCTCATCGCGGATGATGAA (*Sal*I)
Sp-*hr*3-F	CGGGATCCACGTCGTAGAGCATAGCTTGGAA (*Bam*HI)
Sp-*hr*3-R	CGCTCGAGAACGGATGCTGTTCGATAGACG (*Xho*II)
Sp-*hr*4-F	CG GGATCC AGAACCTATCGATCGCGAAAC (*Bam*HI)
Sp-*hr*4-R	CG CTCGAG AGTGACTCGATTCTATCGAATG (*Xho*II)
Sp-*hr*5-F	CGGGATCCCGTTACGTACTCGTCATGAGAG (*Bam*HI)
Sp-*hr*5-R	CGGTCGACATCGTGACCGATGACGCCATCGA (*Sal*I)
Sp-*hr*6-F	CGGGATCCCAACGAAACCCATCGTTTCGGC (*Bam*HI)
Sp-*hr*6-R	CGGTCGACTAGTACCACATCGATCAGTGTAG (*Sal*I)
Sp-*hr*7-F	CGGGATCCAGCGACGACGTCGACAGTGTAC (*Bam*HI)
Sp-*hr*7-R	CGCTCGAGCAATCTTGAAGCTGGTATAGAC (*Xho*II)
pSp-*ie*1P-F	GACTCGAGCAGCTCCTTGTGCAAATAGG (*Xho*II)
pSp-*ie*1P-R	TCAAGCTTTACGGCAAACTGAAACAAGACAG (*Hind*III)
Q-PCR-F	GTTGGTAGCTCTTGATCCGGCA (*Dpn*I)
Q-PCR-R	AATAGACAGATCGCTGAGATAG (*Dpn*I)
pSp64-F	GATCTGGCTTTTAGTACTTGATCTTTGCTTTCGTCCACGA TCCTCGATGAAAGCAAACATCAAGTACTAAAAGGATC CCGCCTCGAGG (*Bgl*II)
pSp64-R	TCGACCTCGAGGCGGGATCCTTTTAGTACTTGATGTTTG CTTTCATCGAGGATCGTGGACGAAAGCAAAGATCAA GTACTAAAAGCCA (*Sal*I)

Restriction enzyme sites are underlined.

Luciferase Activity Assay. Spli221 cells were seeded in 24-well plates and allowed to attach at 27°C overnight. Transfection assays were conducted using Lipofectin following the manufacturer's instructions (Invitrogen). Cells were transfected with transfection solution containing 0.3 µg plasmid DNA (pSp*hr*1-7, pSp-*ie*1P/*luc*, pSp64-*ie*1P/*luc*, or pGL_3-Basic, respectively) and 2 µl Lipofectin in a total volume of 25 µl. Four hours later, the transfection solution was replaced with fresh medium supplemented with FBS. If virus infection was required, virus was added to the serum-free medium and left for 1 h. Supernatant was then replaced with complete medium. The cells were harvested at 48 h post-transfection (hpt), and cell extracts were prepared following the instructions with the luciferase assay kit (Promega). Cells were washed twice with PBS and lysed in 200 µl of luciferase cell culture lysis reagent. Protein quantities in lysates were measured using the method of Bradford (Smith 1995). Cell lysate (10 µg) was used to determine the luciferase activity in 50 µl of luciferase assay reagent, and measurements of luciferase activity were taken with a GloMax 20/20 Luminometer (Promega). Luciferase activity is expressed as counts per minute (CPM) in 15 s. Data were collected from triplicate assays of three independent transfections.

DNA Replication Assay. Spli221 cells were seeded at a density of about 1×10^6 viable cells per 35-mm dish. These were incubated at 27°C for 24 h to allow the cells to attach. The cells were inoculated with SpltNPVII or without viral infection. One hour later, the inoculation medium was removed and the cells transfected with plasmid DNA using Lipofectin, according to the manufacturer's instructions (Invitrogen). For transfection, 1 µg *hr*-containing plasmids in 45 µl H_2O and 5 µl Lipofectin was added to 1 ml serum-free medium on the dish. pUC19 DNA (1 µg) was transfected as a control to evaluate the efficiency of prepared total cellular DNA digested with *Dpn*I. Four hours after transfection, the transfection solution was replaced by fresh medium supplemented with 10% FBS. Each transfection contained at least three separate experiments. The cells were harvested 48 h postinfection (p.i.), and SpltNPVII and total DNA were isolated from infected cells as described by Summers and Smith (1987). An ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) was used to quantify DNA. To distinguish input plasmid DNA from plasmid DNA that replicated in insect cells, the DNA was digested with the restriction enzymes *Hin*dIII and *Dpn*I.

Real-Time Quantitative PCR. Digested DNA from the replication assay was used in real-time quantitative PCR (Q-PCR). Q-PCR was carried out as we described previously (Liu et al. 2010). In brief, PCR

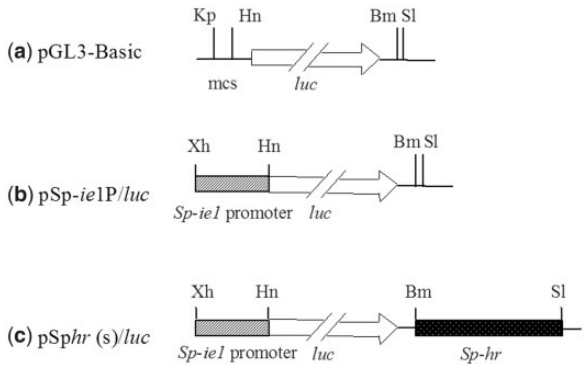

Fig. 1. Diagrams of reporter vector constructs. Schematic representation of the reporter vectors used to assess the enhancement activity of Sp*hr*1-7. The luciferase reporter gene (*luc*) is indicated by arrows. The Sp-*ie*1 promoter and Sp*hr*s are indicated. Multiple cloning sites (mcs) are represented by multiple vertical bars. Key restriction enzyme sites are abbreviated as follows: Kp, *Kpn*I; Hn, *Hind*III; Bm, *BamH*I; Sl, *Sal*I; Xh, *Xho*I.

primers were used to amplify a 314-bp region of the pUC19 vector containing seven *Dpn*I sites (Table 1). Because *Dpn*I cleaves only fully methylated GATC sequences, the DNA replicating in insect cells is resistant to *Dpn*I digestion. To distinguish input plasmid DNA from plasmid DNA that had replicated in insect cells, total DNA was digested with *Hin*dIII and *Dpn*I (Hilton and Winstanley 2007, Yao et al. 2010). Each sample was analyzed three times. A standard curve of 5-fold dilution of the above plasmids was made from 10^4 to 10^8 copies /μl. The Q-PCR results were determined using the standard curve (Bustin et al. 2009).

Results

Identification and Characterization of Sphrs. SpltNPVII, a new species of the genus *Alphabaculovirus*, has a genome of approximately 148 kb, consisting primarily of 149 open reading frames. The genome also has seven homologous regions, designated Sp*hr*1 to Sp*hr*7 according to their relative position on the SpltNPVII genome. SpltNPVII *hr*s comprise many A/T-rich sequences that contain between three and eight highly conserved repeat sequences. All 40 palindromic repeats in Sp*hr*1–Sp*hr*7 are aligned and contain 64-bp-long highly conserved repeated sequences with a 24-bp imperfect palindrome (Fig. 2). The sequence between these imperfect palindromes contains direct or inverted repeats. Furthermore, *hr*1, *hr*4, and *hr*7 contain some variable anomalous repeat sequences. In addition, many other special motifs are present, which may be related to baculovirus genome replication (Table 2).

Enhancement Activity of Sphrs. To determine whether the seven homologous regions of SpltNPVII could improve the activity of the strong *ie-1* promoter, all *hr*s were subcloned into pGL3-Basic with the *ie-1* promoter, and pSp*hr*s-mediated enhancement was examined by measuring the luciferase activity expressed in spli cells with or without infection. The data suggested that all *hr*s acted as strong enhancers under both infected and noninfected conditions. The pSp*hr*s-mediated enhancement of the Sp-*ie1* promoter varied from 2.9-fold to more than 13-fold in infected spli cells. *hr*5 plasmid DNA only stimulated the luciferase activity by 2.9-fold of the control, while *hr*1 and *hr*7 increased luciferase activity by 11.83- and 13.16-fold respectively (Fig. 3). Enhancement by the other four *hr*s varied between 6- and 9-fold. This result suggests that the *hr*s of SpltNPVII stimulated enhanced *ie-1* promoter activity when viral factor(s) were present. In addition, the enhancement efficiencies of *Sphr*s varied from 1.26- (*hr*5) to 6.93-fold (*hr*7) in noninfected cells (Fig. 3), about half that of infected cells.

Replicative Ability of Sphrs. *Dpn*I restriction endonuclease recognition sites (GATC) in *dam*$^+$ bacteria contain fully methylated deoxyadenosine residues, while in eukaryotic cells, these are not methylated (Geier and Modrich 1979). *Dpn*I digests sequences that are fully methylated, so *Dpn*I digestion can be used to differentiate between input plasmid DNA and plasmid DNA that has replicated in eukaryotic cells. The initial experiment indicated that no newly replicated Sp*hr*-containing plasmid DNA was present in noninfected cells. Therefore, the ability of all Sp*hr*-containing fragments to replicate was examined using a SpltNPVII-dependent DNA replication assay. Data suggested that all Sp*hr*s were able to replicate in the presence of SpltNPVII as a helper virus (Table 2). The total SpltNPVII-infected cellular DNA was isolated for real-time PCR. The replication potential of the seven *hr*s varied from 1.52×10^5 copies/μg SpltNPVII-infected cellular DNA (*hr*5) to more than 4.88×10^6 copies/μg DNA (*hr*4), after deducting the control pUC19 copies ($1{,}730 \pm 70$ copies/μg SpltNPVII-infected cellular DNA) in spli cells; Sp*hr*1, *hr*3, and *hr*7 were detected at 9.48×10^5, 6.15×10^5, and 6.98×10^5 copies/μg DNA, respectively; Sp*hr*2 and *hr*6 were detected at lower levels, 3.06×10^5 and 2.05×10^5 copies/μg DNA, respectively (Fig. 4). Therefore, all seven *hr*s were able to function as SpltNPVII DNA origins.

Nondetectable ori and Enhancer Function of a Single 64-bp Highly Conserved Consensus Sequence. To explore the nature of *hr*-stimulated SpltNPVII DNA replication and transcription enhancer activities, a luciferase activity assay and real-time PCR were carried out with a single 64-bp imperfect palindrome. Real-time PCR results showed no detectable replication above background (pSp64: $2{,}015.9 \pm 88.6$; pUC19: $1{,}947.3 \pm 64.9$ copies/μg SpltNPVII-infected cellular DNA). The luciferase activity was not significantly different to the control group (pSp64-*ie*1P/*luc*: $25{,}713 \pm 3{,}572$ CPM; pSp-*ie*1P/*luc*: $23{,}574 \pm 2{,}941$ CPM). Besides the entire 64-bp highly conserved consensus sequence, other *cis*-elements may be required for DNA replication.

Correlations Among Replicative Ability, Enhancer Efficiency, and Sphrs Palindromes. To clarify the mechanism of *hr*s' ability to act as SpltNPVII DNA replication origins and enhancers, a Spearman's correlation analysis of replication ability, enhancer efficiency, and the number of imperfect palindromes or other characteristic sequences of all seven *hr*s (Table 2) was performed. The results showed significant positive correlations between (i) replication potential and the number of palindromes ($r = 0.847$, Sig: $0.016 < 0.05$) and (ii) the numbers of repeat sequences including DR, IR, and R ($r = 0.883$, Sig: $0.008 < 0.01$), and characteristic motifs, including CCAAT, CGATT, and MLTF/USF (Carthew et al. 1985) ($r = 0.821$, Sig: $0.023 < 0.05$), and the total number of palindromes, repetitive sequences, and characteristic motifs ($r = 0.857$, Sig: $0.014 < 0.05$). Correlations also existed between enhancer efficiency and number of palindromes ($r = 0.775$, Sig: $0.041 < 0.05$), and between the numbers of repeat sequences, including DR, IR, and R ($r = 0.821$, Sig: $0.023 < 0.05$), and characteristic motifs, including CCAAT, CGATT, and MLTF/USF motifs ($r = 0.893$, Sig: $0.007 < 0.01$), and the total number of palindromes, repeat sequences, and characteristic motifs ($r = 0.964$, Sig: $0.000 < 0.01$). The *cis*-elements of palindromes, repeat sequences, and characteristic motifs were significantly positively correlated with both replication potential and enhancer efficiency. However, replication potential was most closely related to the number of repeat sequences and enhancer efficiency to characteristic motifs. In addition, no significant Spearman's correlation was observed between the replication potential and enhancer efficiency of Sp*hr*1-7 ($r = 0.239$, Sig: 0.606).

Discussion

In this study, we revealed that SpltNPVII *hr* sequences are involved in both DNA replication and enhancement of transcription. All Sp*hr*s contained a near-identical 64-bp-long highly conserved consensus sequence (total 40; Fig. 2); within this was a 24-bp imperfect palindrome of a similar size to that of AcMNPV (28 bp). Almost half of the 40 imperfect palindromes were centered around a *Pvu*I site. A similar situation exists in AcMNPV, the *hr*s of which contain a degenerate *Eco*RI site (Cochran and Faulkner 1983) and in SeMNPV with its (degenerate) restriction site, *Bgl*II (Broer et al. 1998). Previously, investigations using plasmid constructs of AcMNPV *hr*1a indicated that the central four nucleotides of the palindrome core are essential for *ori* function (Leisy et al. 1995).

A real-time PCR-*Dpn*I method was introduced to quantify nascent DNA. The results confirmed that each of the seven regions may serve as *ori*s of SpltNPVII DNA replication. A transient expression assay demonstrated that the expression of SpltNPVII-*ie1* promoter-driven luciferase gene was enhanced in both infected and noninfected cells. Thus, the seven putative origins of SpltNPVII DNA are bifunctional, but whether they are all active simultaneously *in vivo* remains unclear. In this study, we used a transient replication method based on two steps where the first one is the infection with the baculovirus and the subsequent is the plasmids transfection, which differ from the method of other reports (Wu and Carstens 1996, Carstens 2009). Many experiments conformed our method is more suitable in Bm-N and Spli221 cell (Zhang et al. 1995, Hu et al. 1996). Maybe different experimental approaches are suitable for different baculovirus and cell lines. The role of each as an *ori* and their enhancer activities varied (Figs. 3 and 4), and so which is/are essential and sufficient for DNA replication remains to be determined. A previous report showed that some *hr* regions of

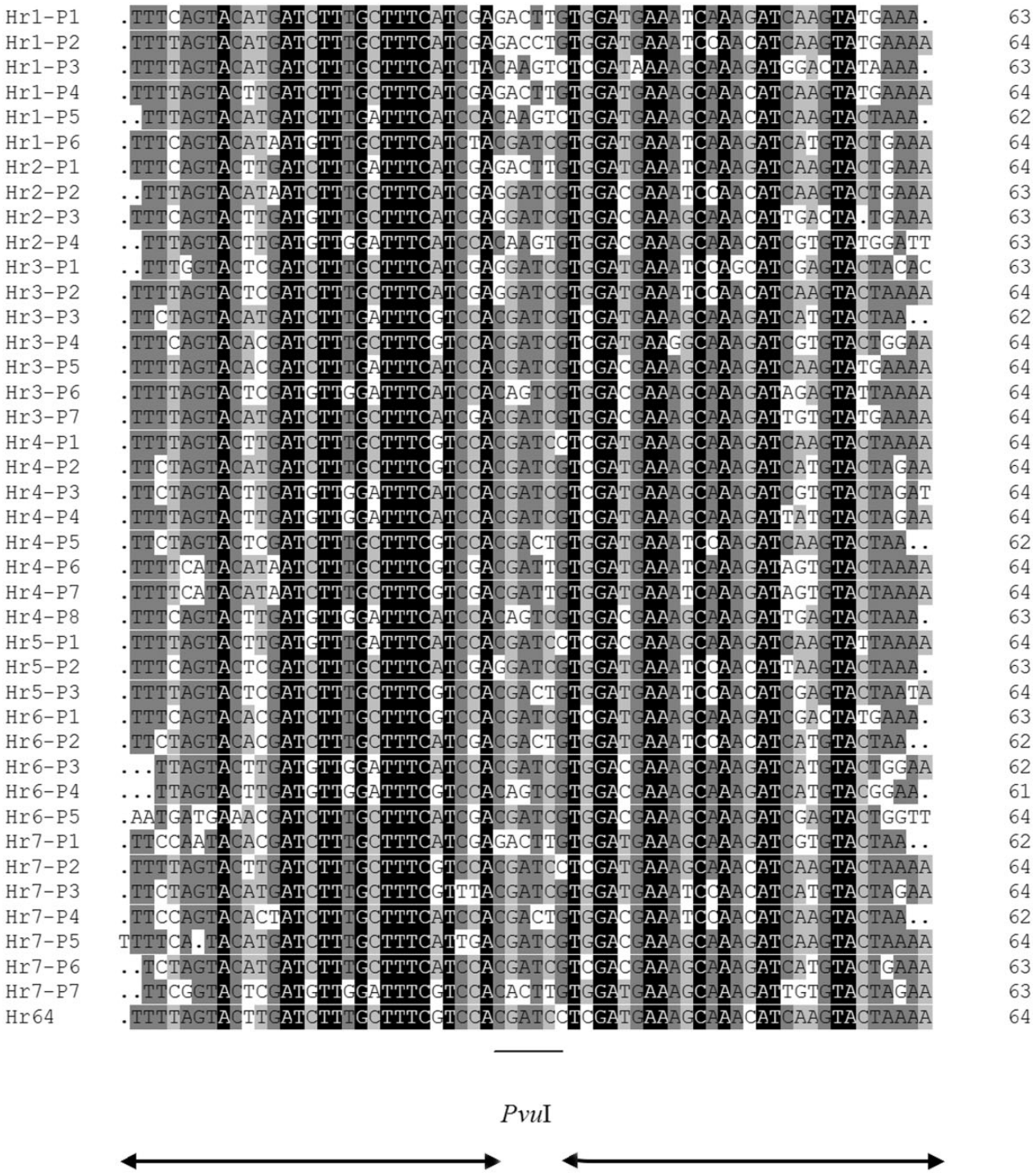

Fig. 2. ClustalW alignment of the 40 SpltNPVII imperfect palindromes. Alignment of all 40 palindromic repeats from Sp*hr*1 to Sp*hr*7 and deduced Sp*hr*s consensus sequences. Sequences are presented in the forward or reverse orientation. Gray shading above the consensus sequence indicates the level of conservation, from black (completely conserved) to light (less conserved) shading.

AcMNPV may be less important or not essential through the production of deletion mutants, each with one or two of the eight *hr*s deleted (Rodems and Friesen 1993, Carstens and Wu 2007). Thus, no one *hr* is essential for AcMNPV DNA replication; however, the presence of multiple *hr* sequences may provide several alternative sites for replication initiation and accelerate the expression of early genes. To determine the function of the highly conserved consensus sequence, one characteristic 64-bp-long sequence was subcloned into a pGL3-Basic vector with the *ie1* promoter or a pUC19 vector. However, no detectable effect was observed, both in terms of enhancer activity and as an origin of replication, meaning that this single 64-bp imperfect palindrome had neither *ori* nor enhancer functions in SpltNPVII-infected cells. The enhancer function of SpltNPVII *hr*s may be relatively weak, maximum enhancement being only 13.16-fold, even in SpltNPVII-infected cells, whereas BmNPV *hr*3 could significantly stimulate the transcription efficiency of the homologous *ie*-1 promoter (>2,400-fold), even though the enhancer ability of BmNPV *hr*3, with only one intact palindrome, was 79-fold (Chen et al. 2004). In addition, a part of AcMNPV *hr*5

containing a single, complete palindrome was sufficient to promote replication, indicating that a single repeat is the minimal requirement for plasmid-dependent DNA replication (Leisy et al. 1995, Pearson et al. 1995). However, transcription enhancement by CpGV *hr* regions, which all consist of a single palindrome, did not increase in *Bam*HI-F subclone 5 with two palindromes (Hilton and Winstanley 2008). Therefore, the single 64-bp highly conserved consensus sequence may not have been sufficient to support replication of the plasmid in SpltNPVII-infected insect cells, or DNA replication may require other *cis*-elements, especially repetitive sequences including DR, IR, and R repeats.

In this study, significant correlations were observed among replication potential, enhancer efficiency, and numbers of imperfect palindrome or other characteristic sequences of all seven Sp*hr*s. The results indicated that efficiency of replication and enhancement of *hr*s increased with increasing numbers of palindromes, repeat sequences, and characteristic motifs. Correlation coefficients indicated that the number of repeat sequences is likely more important to *ori* activity of *hr*s, but enhancer efficiency is most significantly related to the number of characteristic motifs. Indeed, the enhancer efficiency of *hr*s almost exactly tracks the total number of palindromes, repeat sequences, and characteristic motifs; so we speculate that enhancement of *hr*s requires the combination of the presence and distribution of structural motifs in *hr*s. Although the efficiency of *hr*s as *ori*s and enhancers were significantly positively correlated with its total number of special *cis*-elements, no significant correlation was observed between replication potential and enhancer efficiency. The results further suggest that boosting of the efficiency of replication and enhancement were controlled by different *cis*-elements in *hrs* because the *cis*-elements that are most relevant to replication potential are repeat sequences, including DR, IR, and R, while those most important to enhancer efficiency are characteristic motifs, including CCAAT, CGATT, and MLTF/USF. Therefore, more detailed experiments are required to determine whether the effect of multiple SpltNPVII palindromes depends on their numbers and whether direct/inverted repeats and other motifs are involved in the regulation of transcription.

Table 2. Number and types of sequence motifs found within Sp*hr*1–Sp*hr*7

Motif	*hr*1	*hr*2	*hr*3	*hr*4	*hr*5	*hr*6	*hr*7
P	6	4	7	8	3	5	7
DR	4	1	2	1	0	3	1
IR	0	0	1	2	0	1	1
R	6	0	0	8	0	0	7
CCAAT	0	1	0	0	0	0	3
CGATT	1	0	2	1	1	0	3
MLTF/USF	6	1	1	4	0	0	14
Sum of motifs	23	7	13	24	4	9	23

P, imperfect palindromes TTTCATCGAGACTTGTGGATGAAA, nonpalindromic bases are underlined; DR (GATGATGCAATAAAAACAA) and IR (TTGTTTTTATTGC ATCATC) represent direct and inverted repeats, respectively; R represents anomalous repetitive sequences within *hr*1, *hr*4, and *hr*7; MLTF/USF, CANNTG probable RNA polymerase II transcription factor binding site. Sum of motifs, including P, DR, IR, R, MLTF/USF, CCAAT, and CGATT motifs.
(R in *hr*1: ATCTCGAACTTAACGTAGGTTTCAAGGAAAATGCGTTTGTAAAATCTTTTA CA A AAGATTTTTAAAATGTCACG;
R in *hr*4: TTCGGCGAAAGATACATTCTAAAATTTTTGTAAAAATTTTTTAGATAGACTT GATCTTGAAATAGGCATCAAC;
R in *hr*7: CAAGATAACGACATTTGAAAAGATTTTTTATAAAGTTTTAGCAGATGCTTTT CG TCGAAATTAATACTAACTT.)

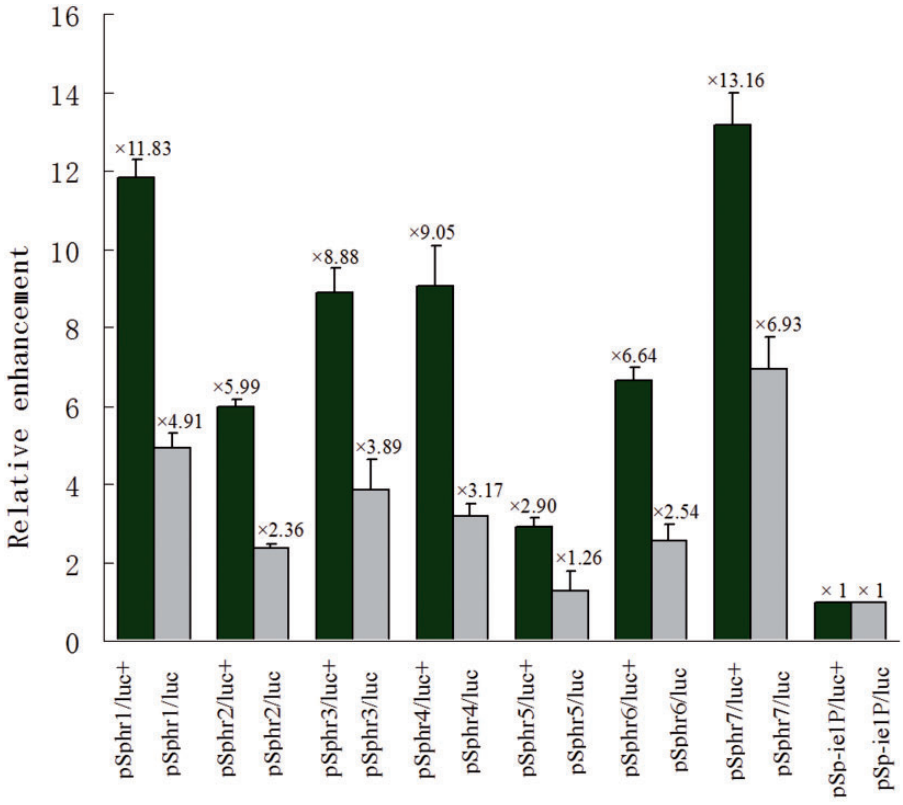

Fig. 3. Enhancement of Sp-*ie1* promoter activity by Sp*hr*1 to Sp*hr*7. Black histograms represent relative luciferase activities of each Sp*hr*-carrying construct in relation to that of the pSp-*ie1*P/luc (*hr*-less) construct in infected cells (m.o.i., 0.5). Gray histograms represent noninfected cells. pSp-*ie*1P/*luc* was used as control. Standard deviations of three independent experiments are presented.

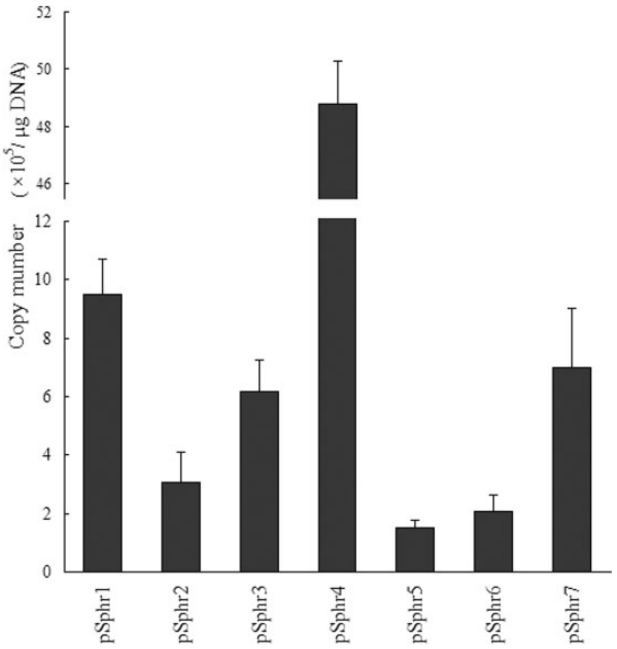

Fig. 4. Replication ability of Sp*hr*1 to Sp*hr*7. Values of the replication potential of each Sp*hr*-carrying plasmid were determined by real-time PCR. The copy number of each plasmid after deduction of the background or incomplete digestion template (pUC19 without Sp*hr*): 1,730 ± 70 copies/μg DNA. Standard deviations of three independent experiments are presented.

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