Identification and functional analysis of a putative non-hr origin of DNA replication from the *Spodoptera littoralis* type B multinucleocapsid nucleopolyhedrovirus

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A putative non-hr origin of DNA replication was identified in the *Spodoptera littoralis* multinucleocapsid nucleopolyhedrovirus (SpliNPV) genome by transient replication assays. The putative SpliNPV ori was mapped to the Pst-I fragment between 75–1–77−9 map units in the SpliNPV genome. While the DNA sequence of the putative SpliNPV ori aligned with regions within the non-hr ori of *Autographa californica*, *Orgyia pseudotsugata* and *Spodoptera exigua* multinucleocapsid nucleopolyhedroviruses, it has limited DNA sequence identity with these elements. The sequence of the putative SpliNPV non-hr ori fragment contains a unique distribution of imperfect palindromes, multiple direct repeats and putative transcription factor-binding sites. Transient expression assays indicated that the putative SpliNPV ori fragment repressed SpliNPV lef-3 promoter-mediated luciferase reporter gene expression. However, the putative SpliNPV ori fragment itself was capable of directing luciferase expression in the absence of a recognizable baculovirus promoter element in an orientation-independent fashion, suggesting that DNA sequence motifs within its sequence can activate transcription. Gel mobility shift analyses confirmed that proteins within nuclear extracts from both uninfected and virus-infected cells bound with specificity to the putative SpliNPV ori fragment.

Introduction

Baculoviruses are viruses of arthropods with large, rod-shaped virions that contain supercoiled dsDNA genomes in the size range 88–165 kbp (Blissard & Rohrmann, 1990). The most well-characterized baculovirus, *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV), has a genome of 134 kbp and encodes approximately 150 genes (Ayres et al., 1994). AcMNPV contains a set of closely related sequences known as homologous regions (hrs), which are interspersed throughout the genome (reviewed by Lu et al., 1997). The hrs consist of one to eight copies of a repeated sequence composed of 30 bp palindromes flanked by 20 bp direct repeats and separated by approximately 80–120 bp non-repetitive DNA. Some hr sequences have been demonstrated to act as origins of DNA replication in transient replication assays in which recombinant plasmids carrying these elements replicate when introduced into nucleopolyhedrovirus (NPV)-infected insect cells (Kool et al., 1995; Lu et al., 1997). The identification of similar DNA elements in the genomes of other baculoviruses such as the NPVs of *Orgyia pseudotsugata* (OpMNPV; Ahrens et al., 1995), *Bombyx mori* (BmNPV; Majima et al., 1993), *Choristoneura fumiferana* (CfMNPV; Xie et al., 1995), *Lymantria dispar* (LdMNPV; Pearson & Rohrmann, 1995) and *Spodoptera exigua* (SeMNPV; Broer et al., 1998) suggests that hrs perform an essential function during the replication cycle of these viruses. Currently, there is no direct evidence that hrs function as origins of replication in the context of virus infection (Lu et al., 1997). Some hrs from AcMNPV and OpMNPV have been demonstrated to function as cis-acting enhancers of IE-1-mediated early gene expression (Guarino & Summers, 1986; Rodems & Friesen, 1993; Kool et al., 1995; Leisy et al., 1995).

A second type of putative baculovirus origin of replication, referred to as non-hr origins (non-hr oris), has been described in AcMNPV (Kool et al., 1994), OpMNPV (Pearson et al., 1993) and SeMNPV (Heldens et al., 1997). Non-hr oris contain unique palindromic and repetitive sequences that are not found in baculovirus hr sequences and are relatively complex in their
organization. Only one copy of a non-hr sequence was identified in the genome of AcMNPV (Kool et al., 1994; Lee & Krell, 1994). Sequences in the AcMNPV HindIII-K region, also referred to as oriK and located between 84-9-87-3 map units (m.u.) of the AcMNPV genome, support replication of plasmids in transient replication assays (Kool et al., 1994) and become enriched in defective AcMNPV genomes (Lee & Krell, 1994). Deletion analysis of the HindIII-K fragment indicated that the sequences required for optimal replication are contained within a relatively large region within the p94 gene. The function of oriK in vivo is unknown, but its conservation in defective AcMNPV genomes (Lee & Krell, 1994) and in the genome of BmNPV, which is closely related but lacks the p94 gene (Kool et al., 1994), suggests that non-hr elements may play an important role in the replication of NPVs.

Deletion analysis of the OpMNPV non-hr sequence, located within the HindIII-N fragment (70-113 m.u. of the OpMNPV genome), revealed a complex organization, since deletion of any portion of the HindIII-N fragment resulted in reduced replication efficiency, suggesting that sequences affecting ori activity were distributed throughout the fragment. Sequence analysis identified a variety of direct and inverted repeat sequences and palindromic sequences (Pearson et al., 1993). The non-hr sequence of SeMNPV (Heldens et al., 1997) was mapped to 1052 bp within the Xbal F fragment (60-7-62-3 m.u. of the SeMNPV genome). Sequence analysis revealed a unique distribution of six different imperfect palindromes, several polyadenylation consensus motifs, multiple direct repeats and several putative transcription factor-binding sites.

SpliNPV is a member of the Baculoviridae (Volkman et al., 1995) and appears distantly related to AcMNPV and other more extensively studied baculoviruses. Phylogenetic analyses (Hu et al., 1997; Levin et al., 1997; Smith & Goodale, 1998) have suggested that SpliNPV represents a more ancient lineage of NPVs that is distantly related to more commonly studied NPVs that cluster together in a clade referred to as the Group I NPVs (Zamotto et al., 1993). Nucleotide sequence analyses of five SpliNPV genes and their flanking regions [polh (Croizier & Croizier, 1994; Faktor et al., 1997a), egt (Faktor et al., 1995), p10 (Faktor et al., 1997b), lef-3 (Wolff et al., 1998) and lef-8 (Faktor & Kamensky, 1997)] have revealed a number of unique and unusual features about this virus that are not found in other NPVs studied to date. In this study, we have described the identification and characterization of a putative non-hr origin of SpliNPV DNA replication.

Methods

Cells and viruses. Spodoptera littoralis cells (CLS79) were kindly provided by S. Kamita, Department of Entomology, University of California, Davis, CA, USA. Spodoptera frugiperda (Sf9) cells were obtained commercially (PharMingen). Both Sf9 cells and CLS79 cells were cultured according to described procedures (O'Reilly et al., 1992). Spodoptera littoralis type B NPV, isolate M-2 (SpliNPV) was obtained from G. Croizier, Station de Recherches de Pathologie Comparée INRA-CNRS, F-30380 Saint-Christol-les-Ales, France. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (O'Reilly et al., 1992).

Recombinant plasmids. SpliNPV DNA was prepared as described by O'Reilly et al. (1992) and digested with the restriction endonucleases EcoRI, SacI, Xhol, PstI and HindIII (New England Biolabs) individually. The viral DNA fragments were cloned into the EcoRI, SacI, Xhol or HindIII sites of plasmid pUC18 (New England Biolabs) to generate a partial SpliNPV genomic library. Plasmid pE4 was found to contain a 344 bp EcoRI fragment (the ‘E4 fragment’) that enabled the plasmid to replicate in SpliNPV-infected cells by transient replication assays (see below). Plasmids with the E4 fragment in both ‘forward’ and ‘reverse’ orientations were recovered and designated pE4 and pE4R, respectively (Fig. 1a). In the forward orientation, the HindI restriction endonuclease sites within the E4 sequence are proximal to the Smal and Sall sites of pUC18. In the reverse orientation, the HindI sites are distal to the Smal and Sall sites of pUC18. The plasmid pG2L2-Basic was purchased from Promega. The luciferase gene (bp 18-2744) was excised from pG2L2-Basic after cleavage with Smal and Sall, and inserted into pE4 and pE4R, creating the plasmids pE4-luc and pE4R-luc, respectively (Fig. 1a).

The E4 fragment was removed from pE4 by cleavage with EcoRI and sub-cloned into the EcoRI site of pBlueScript II KS (+) (Stratagene) to create a plasmid called pBS-E4. The E4 fragment was then excised from pBS-E4 after cleavage with EcoRV and Smal and cloned into the Smal site of pplef3-luc, a plasmid that contains the lef-3 promoter driving the luciferase gene (Wolff et al., 1998), creating the expression plasmids pE4-lef3-luc (forward orientation) and pE4R-lef3-luc (reverse orientation; Fig. 1b). In the forward orientation, the HindI sites of the E4 fragment are proximal to the 5′ end of the lef-3 promoter element. In the reverse orientation, the HindI sites of the E4 fragment are distal to the 5′ end of the lef-3 promoter element. DNA isolation, restriction digestion, agarose gel electrophoresis and Southern blot analysis were carried out according to standard protocols (Sambrook et al., 1989).

Transient replication assay. Plasmid DNA (5 µg) was transfected into 5 × 10⁶ cells by calcium phosphate precipitation. After 24 h at 27 °C, cells were mock-infected or infected with SpliNPV at an m.o.i. of 10 for 2 h. The infectious media were then replaced by fresh medium and the cultures were further incubated at 27 °C for 72 h. The cells were harvested and the DNA was extracted following the protocol of Sarisky & Hayward (1996). To test for replication in cells, 10 µg DNA was digested in a 100 µl reaction volume overnight at 37 °C, with 30 U HindIII to linearize the plasmid and with 30 U DpnI, which cleaves in the sequence 5′ CAGT 3′ only if the A is methylated. To monitor DpnI activity, 5 µl DpnI reaction mix was removed and incubated simultaneously with 500 ng pUC18 DNA overnight at 37 °C. Complete cleavage of the pUC18 DNA indicated that experimental DNA was also completely digested. After electrophoresis in a 0.7% agarose gel, the DNA was transferred to nylon membranes (Hybond-N; Amersham Life Science) and hybridized with [32P]dCTP-labelled pUC18 plasmid DNA. The plasmid DNA was labelled by random primed PCR according to the protocols specified in the Tag-It kit (Bios). All transfection replication assays were repeated at least three times. Replicated plasmid DNA samples were subjected to partial digestion with HindIII followed by electrophoresis on agarose gels to determine if a ‘step-ladder’ of fragments, indicative of high-molecular-mass concatameric DNAs, was detectable.

DNA sequence analysis. The E4 fragment (in pBS-E4) was sequenced by the dideoxy chain-termination method (Sanger et al., 1977). DNA sequencing reactions were performed with the /smal DNA Sequencing system (Promega) according to the manufacturer’s protocol. Sequences of both strands were obtained by bidirectional sequencing of
plasmid DNA using T3 and T7 primers. Sequences were assembled and analysed with the aid of computer programs from the Lasergene package (DNASTAR). The E4 sequence was compared with DNA and amino acid sequences in GenBank using the BLAST and FASTA network service programs (Altschul et al., 1990). Nucleotide sequence alignments were performed using the Clustal W multiple sequence alignment program (Thompson et al., 1994). Direct and inverted repeat sequences were identified by using the Align program (DNASTAR). A weight matrix search program, MatInspector (Quandt et al., 1995), was used to search for putative transcription factor-binding sites. The helical stability of the E4 fragment sequence was analysed by use of the algorithm Oligo (National BioSciences). The |AG| values across the entire sequence were plotted using Microsoft Excel.

Luciferase assays. Mock-infected and SpliNPV-infected (m.o.i. of 10) Sf9 and CLS79 cells (10⁶) were transfected at 2 h post-infection (p.i.) with 2 μg plasmid-containing luciferase gene, by calcium phosphate precipitation. At 4 h post-transfection, the culture medium was removed, the cells were washed twice with PBS and then provided with fresh medium. Cells were harvested at 48 h post-transfection. The cell pellets were resuspended in 100 μl lysis buffer (25 mM Tris–phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N’,N’-tetraacetic acid, 10% glycerol, 1% Triton X-100) and then incubated at room temperature for 10 min. The Luciferase Assay system (Promega) was used according to the manufacturer’s protocol. Assays were performed using a Liquid Scintillation Counter (model 1410; Wallac). All samples were measured within 2 min of the addition of the luciferase assay reagent.

Preparation of nuclear extracts and gel mobility shift assays. Extracts of nuclear protein were prepared following the methods of Parker & Topol (1984). Protein concentrations of 5–20 mg ml⁻¹ were determined by the method of Bradford (1976), with BSA as a standard of Parker & Topol (1984). Protein concentrations of 5–20 mg ml⁻¹ were determined by the method of Bradford (1976), with BSA as a standard.

Results

Identification of the origin of SpliNPV DNA replication

We screened 25 clones, representing 15% of the viral genome (data not shown) by transient replication assays in our search for a putative SpliNPV origin of DNA replication. Southern blot hybridization revealed virus-dependent replication of one plasmid containing a small SpliNPV EcoRI fragment in both Sf9 and CLS79 cells (Fig. 2a, lanes 4 and 10, respectively). The putative SpliNPV origin fragment was called the ‘E4 fragment’, as it was the fourth EcoRI fragment tested, and the plasmid was subsequently called the pE4. Dmpl-resistant, HindIII-sensitive pE4 plasmids, derived from SpliNPV-infected Sf9 or CLS79 cells, were detected as linearized molecules of approximately 3.0 kbp (2.7 kbp pUC18 + 0.34 kbp insert) by Southern blot analysis. Control experiments, in which DNA extracted from transfected/virus-infected cells was cleaved with HindIII alone or with HindIII + Dmpl, confirmed that Dmpl was unable to cleave the replicated plasmid DNA (Fig. 2h). Other plasmids tested did not generate Dmpl-resistant fragments after co-digestion.
Fig. 2. Transient replication assays. (a) Sf9 and CLS79 cells were transfected with recombinant plasmids containing SpliNPV fragments and with pUC18 (P18) as a control. Plasmids pE4, pX2 and pS3 consisted of pUC18 with cloned SpliNPV EcoRI, XhoI and SacI fragments, respectively. The number to the left of the panel indicates the size (kbp) of replicated pE4 that was linearized after digestion with HindIII and DpnI. The pUC18 plasmid without or with the E4 fragment did not replicate in uninfected Sf9 cells (M1 and M2, respectively). (b) Sf9 and CLS79 cells were transfected with pE4, and then infected with SpliNPV at 24 h post-transfection. Cellular DNA was isolated and then digested with HindIII, with (+) or without (−) DpnI, indicating a lack of virus-dependent DNA replication (Fig. 2a, lanes 5, 6, 8 and 9). The pUC18 vector, without insert, did not replicate in the virus-dependent, transient replication assay (Fig. 2a, lanes 3 and 7). Finally, pUC18 plasmid without or with the E4 fragment did not replicate in uninfected Sf9 cells (Fig. 2a, lanes 1 and 2). Partial digestion of replicated plasmid DNA with HindIII did not result in the production of a ‘step-ladder’ of fragments, indicating that high-molecular-mass concatameric DNA replication products were not produced during replication of pE4 (data not shown).

DNA sequence analysis of the 344 bp E4 fragment revealed a number of perfect and imperfect palindrome sequences, direct repeat sequences (Table 1) and putative transcription factor-binding sites (Fig. 3b). The E4 fragment contains a small perfect palindrome of 8 bp (P1) and a large imperfect palindrome (P2) of 22 bp. There are numerous direct repeats of 6–11 bp (R1–R19) that are found from two to four times within the sequence (Table 1). Analysis of the E4 sequence with MatInspector (Quandt et al., 1995) revealed a number of putative transcription factor-binding sites on both strands (Fig. 3b). This program provides three useful parameters by which putative transcription factor-binding sites may be identified: a ‘matrix similarity’, which indicates the frequency of nucleotide identity between the test sequence and the sequence of a corresponding transcription factor-binding site; a ‘core similarity’, which consists of the four best-conserved consecutive nucleotides of the matrix; and a threshold of ‘minimum matrix similarity’. A large number of consensus transcription factor-binding sites with high similarity (matrix similarity > 0.85; core similarity > 0.95) were well conserved and readily identified.

Two putative stimulating protein-binding sites (SP1; at bp 32–40 and 56–64 of the E4 DNA sequence, respectively) and one putative upstream stimulating factor (USF)-binding site detected on the opposite strand (bp 77–68) were identified on the 5’ site of a 13 bp A+T-rich sequence that was shown to be a region of high helical instability, identified by plotting the
factor I (NFI)-binding sites that overlap on opposite strands (bp 205–222 and 228–211, respectively) and two adjacent putative GATA-binding sites (also found on opposite strands at bp 312–299 and 314–327, respectively) were detected on the 3' side of the A+T-rich sequence. The two NFI sites overlap the large imperfect palindrome (P2). SP1, USF, NFI and NFIII transcription factor recognition-sequence clusters are often found in vertebrate virus origins of replication (van der Vliet, 1996). GATA-binding proteins are ubiquitous eukaryotic transcription factors and have been demonstrated to play a role in modulating transcription of both AcMNPV and SpliNPV early gene promoters (Kogan & Blissard, 1994; Wolff et al., 1998).

The SpliNPV E4 sequence aligned with regions within each of the AcMNPV HindIII-K (bp 1190–1592; Kool et al., 1994; GenBank accession no. M16821), OpMNPV HindIII-N (bp 517–899; Pearson et al., 1993; GenBank accession no. D17353) and SeMNPV XbaI-F (bp 213–598; Heldens et al., 1997) non-hr ori fragments. While the SpliNPV non-hr ori sequence shared 57% sequence identity in the region of alignment with the AcMNPV non-hr ori, and approximately 50% sequence identity with the regions to which it aligned in the SeMNPV and OpMNPV non-hr sequences, no common sequences (other than consensus putative transcription factor-binding motifs) were identified. No sequence similarities to hr oris of other baculoviruses were detected and no open reading frames could be identified in any of the three possible reading frames in the putative SpliNPV non-hr origin fragment.

Functional analysis of cis-acting sequences in the E4 fragment

We investigated the ability of the SpliNPV E4 fragment to act as an enhancer of SpliNPV early gene promoter-mediated gene expression using transient expression assays. In both uninfected and SpliNPV-infected cells, luciferase expression from constructs containing the E4 fragment (pE4-lef3-luc and pE4R-lef3-luc; see Fig. 1b), was lower than luciferase expression from the plef3-luc control plasmid, indicating that the E4 fragment repressed, rather than enhanced, lef-3 promoter-mediated luciferase expression in both SF9 and CLS79 cells (Fig. 1a).

We further tested the E4 fragment for potential ability to promote expression of the luciferase gene. In uninfected cells, transfection of the pE4-luc and pE4R-luc plasmids (Fig. 1a) resulted in basal luciferase gene expression in an orientation-independent manner. Transfection of plasmids into SpliNPV-infected cells resulted in an approximately 10-fold increase in luciferase gene expression and was also orientation-independent (Fig. 4b). However, the level of E4-mediated luciferase expression from pE4-luc and pE4R-luc (i.e. in the absence of the lef-3 promoter) was much lower than SpliNPV lef-3 promoter-mediated luciferase expression (compare Fig. 4a and b). Transient expression assays in SpliNPV-infected SF9 and CLS79
Table 1. Structural motifs within the putative SpliNPV non-hr ori sequence

<table>
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<th>Motif</th>
<th>Sequence</th>
<th>Position (bp)*</th>
<th>Top strand</th>
<th>Bottom strand</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>GAACGTTC</td>
<td>88–95</td>
<td>8</td>
<td></td>
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</tr>
<tr>
<td>P2</td>
<td>CGGGATCTGGCCAGATCgCG</td>
<td>206–217</td>
<td></td>
<td>22</td>
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<tr>
<td>Palindrome sequences†</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>P1</td>
<td>GAACGTTC</td>
<td>88–95</td>
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<tr>
<td>P2</td>
<td>CGGGATCTGGCCAGATCgCG</td>
<td>206–217</td>
<td></td>
<td>22</td>
<td></td>
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<tr>
<td>Repeat sequences‡</td>
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<tr>
<td>R1</td>
<td>CGATGGTGCTT</td>
<td>9–19, 45–55</td>
<td>11</td>
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<tr>
<td>R2</td>
<td>CCGTCGCCG</td>
<td>286–294</td>
<td>44–36</td>
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<tr>
<td>R3</td>
<td>CGTTGCAG</td>
<td>226–232</td>
<td>92–86</td>
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<td>R4</td>
<td>TCGCCCG</td>
<td>221–227, 289–295</td>
<td>47–41</td>
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<tr>
<td>R5</td>
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<td>194–200, 250–256</td>
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<td>R7</td>
<td>GGAGGA/C</td>
<td>64–69, 61–66, 199–204</td>
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<tr>
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<td>CGTTCG</td>
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<td>92–87</td>
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<td>223–228</td>
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<tr>
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<td>285–290</td>
<td>201–196</td>
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</table>

* The base pair positions indicated refer to the nucleotide sequence of the SpliNPV E4 fragment as illustrated in Fig. 3(b).
† Palindromic sequences; P1 and P2 are perfect and imperfect palindromes, respectively. Mismatched bases in the imperfect palindrome P2 are indicated by lower case letters.
‡ Repeat sequences; indicated by R1 to R19.

cells resulted in approximately the same pattern of activity, although the level of E4-mediated luciferase expression was higher in SpliNPV-infected CLS79 cells than in virus-infected SF9 cells. Transient expression assays with pBlue-Script II KS(+) containing the luciferase gene alone (pBS-luc) resulted in only background levels of luciferase expression (Fig. 4b).

Cellular proteins bind to the putative SpliNPV origin sequences

Incubation of nuclear extracts from virus-infected SF9 or CLS79 cells with the intact E4 fragment resulted in the formation of three discrete protein–DNA complexes (Fig. 5a). The addition of increasing amounts of nuclear extract showed that the appearance of each protein–DNA complex was protein concentration-dependent (Fig. 5b). Protein–DNA complex B appeared more readily at low protein concentrations than protein–DNA complexes A and C, and protein–DNA complex A formed a clear, discrete band only at high protein concentrations. The addition of increasing amounts of competitor DNA, in the form of intact, unlabelled E4 fragments, abolished the formation of protein–DNA complexes (Fig. 5c).

To determine the regions of the E4 sequence to which the cellular proteins bind, we conducted gel mobility shift assays with HinfI-cleaved E4 fragment, which generates restriction fragments of 176, 98 and 70 bp (Fig. 3b). The 176 bp fragment contains the A+T-rich region with its overlapping putative NFIII transcription factor-binding site, flanked on the 5’ side by two putative SP1 and one putative USF transcription factor-binding sites. The 98 bp fragment contains two putative NFII-binding sites, and the 70 bp fragment contains two putative GATA-binding sites.

Incubation of nuclear extracts from uninfected or virus-infected cells with the E4 fragment cut with HinfI (176, 98 and 70 bp fragments) also resulted in the formation of three discrete protein–DNA complexes (Fig. 5d, lanes 6–9). Incubation of nuclear extracts from uninfected or virus-infected cells with the gel-purified 98 and 70 bp fragments resulted in the formation of only one protein–DNA complex (Fig. 5d, lanes 2–4). We could not detect a difference in protein–DNA complex formation between the E4 fragment and extracts...
prepared from uninfected or virus-infected cells using the gel mobility shift assay.

**Discussion**

We have identified and characterized a 344 bp *EcoRI* fragment (E4) from the genome of SpliNPV that can support both DNA replication and transcription, as determined by transient expression assays. Several lines of evidence support the conclusion that the E4 fragment contains a non-**hr** type of baculovirus origin of replication.

Firstly, the SpliNPV E4 fragment supported SpliNPV-infection-dependent plasmid replication in transient replication assays. Recombinant plasmids containing the E4 fragment did not replicate in uninfected cells and the parent plasmid (pUC18) did not replicate in uninfected or SpliNPV-infected cells. Thus, replication of the recombinant plasmid in SpliNPV-infected cells was due to the presence of the E4 fragment. Comparison of the E4 nucleotide sequence with sequences in GenBank failed to reveal any similarities with reported baculovirus **lr** sequences and no known baculovirus promoters were detected. Baculovirus early gene promoters, such as the **ie-1** gene promoter, are known to be capable of supporting plasmid replication in transient replication assays (Wu & Carstens, 1996). Thus, the ability of the E4 fragment to support plasmid replication is not due to the presence of an **hr**-like ori or a baculovirus early promoter sequence.

Secondly, the SpliNPV E4 sequence displayed limited alignment with known non-**hr** ori sequences of other NPVs. The E4 fragment shared 57% DNA sequence identity with the AcMNPV non-**hr** element in the region of alignment, which occurred at the 3′ end of the AcMNPV **HindIII**-K fragment. It also shared approximately 50% DNA identity with both the OpMNPV **HindIII**-N fragment and the SeMNPV **XbaI**-F fragment. The region to which the SpliNPV E4 fragment aligned in AcMNPV **oriK** is contained completely within the AcMNPV **EcoRI**-S fragment of **HindIII**-K, which by itself was shown to be unable to support plasmid replication in
AcMNPV-infected cells. Deletion analyses revealed that sequences between 84–9 and 85–9 m.u. of the AcMNPV genome (approximately 1300 bp of the HindIII-K fragment) were required to support plasmid replication. Thus, sequences essential for replication of AcMNPV oriK are distributed over a much larger region of DNA than that to which the SpliNPV E4 sequence aligned. Transient replication assays revealed that deletion clones of OpMNPV HindIII-N were replication-competent only if they contained a central region that spanned bp 1786–2342. The SpliNPV E4 sequence aligned to a location well upstream of this essential region. Thus, while the SpliNPV E4 sequence aligned with sequences within the AcMNPV and OpMNPV non-hr ori elements, the locations of alignment in these elements did not correspond to regions that, by themselves, are replication-competent. The region to which the SpliNPV E4 fragment aligned in the SeMNPV non-hr, however, did lay within an 800 bp Spl fragment that was shown to be replication-competent by deletion analyses (Heldens et al., 1997).

The lengths of NPV non-hr oris are 1052 bp (SeMNPV Xhol-F), 1300 bp (AcMNPV HindIII-K) and 4000 bp (OpMNPV HindIII-N). Thus, the putative SpliNPV non-hr ori is the
shortest baculovirus non-\(hr\) ori identified to date. The lack of common sequence elements within each non-\(hr\) ori (other than consensus motifs of putative transcription factor-binding sites) and the fact that sequences required for replication competence for the AcMNPV, OpMNPV and SeMNPV non-\(hr\) oris are distributed over large regions of DNA suggest that the SpliNPV E4 fragment is, so far, unique among baculovirus non-\(hr\) elements.

Thirdly, the complex structure of direct repeats, palindrome sequences, A—T-rich regions, and putative transcription factor-binding sites in the SpliNPV E4 sequence has much in common with the non-\(hr\) oris identified in AcMNPV, OpMNPV and SeMNPV (Kool et al., 1994; Pearson et al., 1993; Heldens et al., 1997). Fig. 6 displays the distribution of putative transcription factor-binding sites and A—T-rich domains in the four non-\(hr\) oris within the regions of alignment of the SpliNPV sequence with the AcMNPV, OpMNPV and SeMNPV non-\(hr\) oris. We have not shown the numerous direct and inverted repeat sequences that are also found in these elements. While there are common putative transcription factor-binding sites, as well as A—T-rich domains, in the four non-\(hr\) oris, each element has a unique distribution of these sequences. The role of host proteins in the replicative ability of non-\(hr\) oris, and the importance of palindrome sequences, direct repeat sequences and putative transcription factor-binding sites within non-\(hr\) sequences are unknown.

We used gel mobility shift assays to demonstrate that nuclear proteins from both uninfected and SpliNPV-infected cells bound with specificity to sequences within the putative SpliNPV non-\(hr\) ori fragment. Much higher concentrations of non-specific, unlabelled competitor DNA were required to abolish protein—DNA complex formation than unlabelled E4 DNA, suggesting that nuclear proteins bound the E4 fragment with specificity. However, at least 50% of the labelled probe remained unbound in these experiments. This may be due to the vast excess of labelled probe DNA used in the assays or it may be that the reaction conditions used were not optimal for high-affinity binding or perhaps other factors necessary for high-affinity binding were not active in our extracts.

Comparison of the non-\(hr\) oris from AcMNPV, OpMNPV, SeMNPV and SpliNPV with the consensus oris of vertebrate viruses revealed some intriguing structural similarities. Origins of replication among prokaryotes, viruses and multicellular organisms possess a number of common features which variably include simple tri-, tetra- or higher dispersed repetitions of nucleotides, A—T-rich tracts, inverted repeats, initiator protein-binding sites, intrinsically curved DNAs, DNase I-hypersensitive sites and/or binding sites for transcription factors (Boulkas, 1996). Analyses of viral DNA replication in vertebrate cell lines have demonstrated that the oris of most vertebrate viruses consist of two components: (1) an essential core sequence that recruits origin-recognition proteins and specifies the replication initiation site; and (2) auxiliary regions, which contain transcription factor-binding sites and modulate the efficiency of replication initiated at the core (DePamphilis, 1988, 1993, 1996). It is well established that virus replication often depends on sequence elements and proteins that also activate transcription (Herendeen et al., 1989; DePamphilis, 1988, 1996).
Transcription factors are known to play an essential role in replication of vertebrate viruses such as adenovirus (Jones et al., 1987; Pruijn et al., 1988; Mul et al., 1990), simian virus 40 (Cheng & Kelly, 1989) and herpes simplex virus (Nguyen-Huynh & Schaffer, 1998) by recruiting replication proteins to the origin and modulating the efficiency of replication initiation. The SplNPV E4 fragment was able to activate transcription when it was cloned upstream of a reporter gene (luciferase). The absence of a known baculovirus promoter element in the E4 sequence and the observation that expression from the E4 fragment was orientation-independent suggest that the transcriptional activity detected (indirectly as a function of luciferase activity) may have been a consequence of host and/or virus-encoded transcription factors that bind to the E4 element. Alternatively, there may have been an increase in the copy number of the luciferase gene as a result of infection-dependent plasmid replication. Gel mobility shift assays support the contention that there are proteins in nuclear extracts from both uninfected and virus-infected cells that bind specifically to DNA sequence elements in the E4 fragment.

In some cases, however, origins of replication have been demonstrated to act as transcription silencers (Rivier & Rine, 1992). In our transient expression assays, luciferase expression was repressed when the E4 fragment was placed immediately upstream of the lef-3 promoter. It is possible that lef-3 promoter-mediated luciferase expression was reduced due to competition between the E4 fragment and the lef-3 promoter for transcription factors. An alternative explanation might be that it was due to steric constraints placed upon the transcription complex during plasmid replication that render the lef-3 promoter inactive. However, we feel that this is unlikely. Some hr sequences have been demonstrated to act as origins of DNA replication in infection-dependent transient replication assays, as well as cis-acting enhancers of early baculovirus promoters, such as 39K, ie-2, p35 and p145 (Guarino & Summers, 1986; Nissen & Friesen, 1989; Carson et al., 1991; Lu & Carstens, 1993). hr-containing plasmids replicated in AcMNPV-infected cells were shown to consist of high-molecular-mass DNA, possibly in the form of a linear concatamer containing multiple copies of the plasmid (Leisy & Rohrmann, 1993). Despite this replication-dependent change in plasmid DNA structure, hr sequences still enhanced transcription in an orientation- and position-independent manner.

Restriction endonuclease analysis of replicated pE4 plasmid indicated that it did not form high-molecular-mass DNAs, suggesting that it does not form concatamers. Thus, repression of lef-3 promoter-mediated luciferase expression by the E4 fragment is not likely to have occurred as a consequence of steric constraints due to plasmid replication. Moreover, unlike transcription of the AcMNPV lef-3 gene, which reaches its peak at 6 h p.i. and then decreases to a low level by 24 h (Li et al., 1993), the SplNPV lef-3 gene is first detected approximately 4 h p.i. and steadily increases up to 56 h p.i. (Wolff et al., 1998). Thus, the decreased luciferase expression we observed in SplNPV-infected cells was not due to a reduction in lef-3 promoter activity as a consequence of the kinetics of lef-3 transcription. Further characterization of the putative SplNPV non-hr ori will resolve the role of these sequence motifs in viral DNA replication.

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References


of Spodoptera littoralis multicapsid nuclear polyhedrosis virus. Virus Genes 11, 47–52.


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