The region upstream of the Spodoptera exigua multicapsid nucleopolyhedrovirus (SeMNPV) ubiquitin gene contains four near-identical 68-bp-long palindromic repeats. This region, named Sehr6 and located at map unit (m.u.) 88 of the SeMNPV genome on pSeEcoRI-2.2, showed structural homology to previously identified homologous regions (hrs) in a number of other baculoviruses. Hrs function as enhancers of transcription and as putative origins (oris) of baculovirus DNA replication. Five additional hrs (Sehr1–Sehr5) 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 Sehr6. Sehr1–Sehr6 were dispersed on the SeMNPV genome at m.u. 80, 30.0, 38.5, 51.0, 77.0 and 88.0, respectively.

Sequence analysis of these hrs confirmed the presence of palindromic repeats, highly similar to those found in pSeEcoRI-2.2. The number of palindromes varied from one (Sehr4) to nine (Sehr1) per hr. The Sehrs are all present in non-coding regions of the SeMNPV genome and also contain multiple putative transcription recognition sequences. Plasmids containing either of the Sehrs replicated in an SeMNPV-dependent DNA replication assay. The Sehrs 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 hrs in this process is highly specific.
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 \( hr \) 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-\( hrs \) 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 \( hrs \) in the genome of SeMNPV are described and their replication competence in SeMNPV- and AcMNPV-infected insect cells is investigated. The specificity of \( hrs \) 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
Homologous regions of Spodoptera exigua MNPV DNA

Fig. 1. (a) Genomic location of hrs on the genome of SeMNPV with XbaI and PstI restriction sites. The location and direction of transcription (arrows) of the SeMNPV polyhedrin (ph), helicase, ubi and p10 gene, and the location of the SeMNPV non-hr type origin, are shown. (b) Location of the pSeEcoRI-2.2 fragment between map units 87 ± 2 and 89 ± 0 on the XbaI restriction map of SeMNPV DNA. The black boxes represent the position of the palindromic repeats P1–P4 within the hr on the pSeEcoRI-2.2 fragment. (c) Nucleotide sequence of the 1 ± 3 k b SalI–EcoRI fragment within fragment pSeEcoRI-2.2. Restriction sites are indicated in italics. Palindromic repeats P1–P4 are underlined. The asterisk (*) represents a mutation in P1 that disrupts the BglII site. The direct repeats DR1a, DR1b and DR1c are double underlined. The CGATT motif is in bold and marked with a \( \text{U} \), 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 on the pSeEcoRI-2.2 fragment. A stop ORF Xbl187.
50 °C with RB-33, which was end-labelled with [γ-32P]ATP 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, pT7Z 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 pSeEcoRI-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 Xbal-C with HindIII and KpnI, and subsequent isolation of the 5-7 kb HindIII–KpnI restriction fragment and cloning into pUC19. Plasmids pSeXbal-H and pSeXbal-N were taken from the Xbal-library of SeMNPV in pUC19 (Heldens et al., 1996). Clone pSeBstI-5.6 was obtained after digestion of cosmid 17 DNA (Heldens et al., 1996) with BstNI and PstI. Plasmid pSeBstI-M was obtained by digestion of SeMNPV ODV DNA with PstI and insertion of the PstI-M fragment into PstI-digested pUC19. Clone pSeSpeI-6.3 was obtained after digestion of cosmid 22 DNA (Heldens et al., 1996) with PstI and SpeI, and insertion of a 6-3 kbp fragment into PstI- and Xbal-digested pUC19. Achr5 was present on pAchrHindIII-L (Kool et al., 1993). All plasmids were amplified in E. coli DH5α and JM101 (ΔDam) strains.

■ Replication assay. The assay to test the replicative ability of the Sehr and Achr plasmids was as previously described by Heldens et al. (1997) for the SeMNPV non-hr. In short, 10⁶ 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 TCID₅₀ 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 10⁵ infected cells (Summers & Smith, 1987) and resuspended in 60 µl H₂O. One aliquot (10 µl) was digested with HindIII to linearize the plasmid DNA. A second aliquot (10 µl) was digested with HindIII and DpnI. The use of DpnI allows the discrimination of input (DpnI-sensitive) from replicated plasmid DNA (DpnI-insensitive) (Kool et al., 1993). After agarose gel electrophoresis, the DNA was transferred to a nylon membrane filter (Hybond-N) and hybridized to a 32P-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 dyeodeoxy chain termination based protocol (Sanger et al., 1977). Sequence analyses were carried out using UWGGC 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 pSeEcoRI-2.2

Analysis of sequences upstream of the SeMNPV ubi gene region on fragment pSeEcoRI-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. 1b) 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 palindromic of 68 bp in length, of which each of the last three repeats (P2–P4) were centred around a BgIII site. The BgIII 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. 1c). The organization of such repeat motifs in the SeEcoRI-2.2 region is comparable to that of other baculovirus hrs (Lu et al., 1997). This suggests that the identified palindromic repeats might represent an SeMNPV hr-type ori (Sehr).

Within eukaryotic oris 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 oris. Several such sequences were identified in the region encompassing the putative Sehr (Fig. 1c; 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 (p143) genes (Lu & Carstens, 1993) was present four times in this Sehr. 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. 1c; 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 SalI–EcoRI 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 hrs

The presence and dispersed occurrence of hrs 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 pSeEcoRI-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 Xbal-B, Xbal-C Xbal-H, Xbal-N, PstI-M (Fig. 1a), Spel-G and SeI-E (not indicated). Two fragments, Xbal-B and Xbal-H, are adjacent fragments on the physical map of the SeMNPV genome and represent pSeEcoRI-2.2. These hybridization data and comparison with the location of the respective fragments
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suggest that hr-like sequences occur dispersed on the SeMNPV genome as well.

Further subcloning of fragments XbaI-C, SpeI-G and SstI-E and hybridization with RB-33 revealed (data not shown) that putative SeMNPV hrs are present on fragments pSeBstl-5.6, pSeCHK-5.7 and pSeSpeI-6.3, derived from PstI-C, XbaI-C and XbaI-B, respectively. Further hrs were found on pSePstl-M and pSeXbaI-N. The hrs are numbered Sehr1–Sehr6 according to their relative position on the physical map of the SeMNPV genome (Fig. 1a).

Sequence analysis of the fragments containing Sehr1–Sehr5 confirmed the presence of clusters of P repeats, which were highly homologous to those found in Sehr6. The number of P repeats present varied per hr ranging from one in Sehr4 to nine in Sehr1 (Fig. 2, Table 1). The sequences of Sehr1 to Sehr5 were examined for the presence of the putative transcriptional motifs and DRs found in Sehr6 (Fig. 1c, 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 Sehr1, and in front of two out of the seven P repeats in Sehr5. The DR2 motif, characterized by the consensus TCATcGCAAAaATAGATTTGAGCAGAATacaA- AACT, was not present in the other hrs. The DR1 motif identified in Sehr6 was not present in Sehr1–Sehr5.

**Alignment of Sehr repeats**

When all 32 P repeats in Sehr1–Sehr6 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 BgIII site at the core–loop region of the palindrome. The BgIII 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 hrs of AcMNPV, OpMNPV, LdMNPV, Spodoptera littoralis NPV (SpliNPV), CfMNPV or AgMNPV (data not shown).
Table 1. Number and types of sequence motifs found within Sehr1–Sehr6

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

<table>
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<tr>
<th>Motif</th>
<th>hr1</th>
<th>hr2</th>
<th>hr3</th>
<th>hr4</th>
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<tr>
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<td>0</td>
<td>0</td>
<td>2</td>
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<tr>
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<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
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<td>4 (2)</td>
<td>2</td>
<td>4 (2)</td>
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<td>2</td>
</tr>
<tr>
<td>Early trans. ini. site</td>
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<td>3 (2)</td>
<td>3</td>
<td>5 (1)</td>
<td>0</td>
<td>4 (3)</td>
</tr>
<tr>
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<td>5</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Poly(A)</td>
<td>5 (3)</td>
<td>3 (1)</td>
<td>5 (2)</td>
<td>6 (5)</td>
<td>6 (4)</td>
<td>3 (2)</td>
</tr>
<tr>
<td>CGATT motif</td>
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<td>7 (4)</td>
<td>7 (3)</td>
<td>5 (2)</td>
<td>5 (3)</td>
<td>10 (5)</td>
</tr>
</tbody>
</table>

Replicative ability of Sehs

The ability of all Sehr-containing fragments to replicate was tested in an SeMNPV-dependent DNA replication assay (Fig. 4). All Sehrs 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, pSeXbal-F1 (lane 10), did not replicate in the assay. Sf-AE-21 cells transfected with pSeEcoRI-2.2 (lane 1) and pUC19 (all lanes) alone were included in the assay in order to identify the background of input DNA for DpnI digestions. All hr-containing SeMNPV fragments (lanes 2–7) replicated at a lower level than the non-hr type ori present on pSeXbal-F2 (lane 11) (Heldens et al., 1997). Based on the replicative ability of pSeCHK-5.7 (Sehr4, lane 4) and of pSeXbal-H (Fig. 1a; 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 Sehrs in DNA replication

To test the specificity of the hrs for SeMNPV DNA replication factors, we investigated whether AcMNPV could recognize and replicate SeMNPV hr oris and SeMNPV the AcMNPV hr oris. Sf-AE-21 cells were thus transfected with Sehr6 or Achr5 and infected with AcMNPV or SeMNPV, respectively. The results show that Sehr6 replicates only in the presence of SeMNPV (Fig. 5, lane 2) and not in the presence of AcMNPV (Fig. 5, lane 1). Conversely, Achr5 did not replicate.
in the presence of SeMNPV (Fig. 5, lane 4), suggesting that the DNA replication is \textit{hr}-specific. These results contrast with the observation that the SeMNPV non-\textit{hr} \textit{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 (\textit{hrs}) in other baculoviruses. This region (Sehrö) contained four palindromic repeats (P1–4), two direct repeats (DR) and a number of other motifs which could be involved in regulation of transcription. Sehrö also replicated in an SeMNPV-dependent DNA replication assay. This strongly suggests that this SeMNPV sequence is an \textit{hr} (Lu et al., 1997).

Hybridization of a conserved oligonucleotide sequence of these P repeats of Sehrö with the SeMNPV genome led to the identification of five additional \textit{hr}-regions in the genome, numbered Sehr1–Sehr5 according to their relative position on the SeMNPV physical map (Heldens et al., 1996). The Sehrs described here are interspersed throughout the genome in a similar fashion to other baculoviruses. Even an \textit{hr} with only a single P repeat (Sehr4) was identified by Southern hybridization, making the presence of additional \textit{hrs} of this type unlikely. The presence of multiple \textit{hrs} in baculoviruses may provide redundancy of \textit{oris} to ensure DNA replication.

A single repeat is a minimal requirement for plasmid-dependent DNA replication (Leisy et al., 1995). All plasmids containing an \textit{Sehr} 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 \textit{XbaI}-F, which contained a non-\textit{hr} \textit{ori} (Heldens et al., 1997). It is possible that other \textit{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...
the six SehRs 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 SehR1, SehR5 and SehR6, but not in SehR2–SehR4. The SehR regions also contained a number of other motifs and repeats which may be involved in either DNA replication and/or enhancement of transcription of SehRs. Preliminary experiments indicate that SehR6 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 SehRs contained a near-identical 68-bp-long palindromic repeat with no sequence homology with other known baculovirus hrs (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 hrs of other baculoviruses. Both the length of the repeat and the unique sequence may contribute to the specificity in replication-competence of the SehRs for SeMNPV. Sequence alignment of all SeMNPV hrs 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, BglII, 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 EcoRI site is present in the hr (Cochran & Faulkner, 1983). Mismatches are found in all identified palindromic repeat-containing baculovirus hrs. 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 AhChr1 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 hrs (Habib et al., 1996).

In contrast to the SeXbal-F2 non-hr ori (Heldens et al., 1997) the replication of SehRs is highly specific. This is unlike the situation with AcMNPV hrs when tested with other baculoviruses. Plasmids containing AhChr5, 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, Chfr1-containing plasmids could not replicate in the presence of AcMNPV in Sf21 cells (Xie et al., 1995) as is the case for SehRs. hr SplNPV does replicate in AcMNPV-infected cells, but not in SeMNPV-infected cells (Faktor et al., 1997). These data suggest that AcMNPV hrs are often more promiscuous than hrs 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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