Pivotal Role of the Non-\(hr\) Origin of DNA Replication in the Genesis of Defective Interfering Baculoviruses

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The generation of deletion mutants, including defective interfering viruses, upon serial passage of \(Spodoptera exigua\) multieapsid nucleopolyhedrovirus (\(Se\)MN\(PV\)) in insect cell culture has been studied. Sequences containing the non-homologous region origin of DNA replication (non-\(hr\) ori) became hypermolar in intracellular viral DNA within 10 passages in \(Se\)301 insect cells, concurrent with a dramatic drop in budded virus and polyhedron production. These predominant non-\(hr\) ori-containing sequences accumulated in larger concatenated forms and were generated de novo as demonstrated by their appearance and accumulation upon infection with a genetically homogenous bacterial clone of \(Se\)MN\(PV\) (bacmid). Sequences were identified at the junctions of the non-\(hr\) ori units within the concatemers, which may be potentially involved in recombination events. Deletion of the \(Se\)MN\(PV\) non-\(hr\) ori using RecE/RecT-mediated homologous ET recombination in \(Escherichia coli\) resulted in a recombinant bacmid with strongly enhanced stability of virus and polyhedron production upon serial passage in insect cells. This suggests that the accumulation of non-\(hr\) ori upon passage is due to the replication advantage of these sequences. The \(non-hr\) ori deletion mutant \(Se\)MN\(PV\) bacmid can be exploited as a stable eukaryotic heterologous protein expression vector in insect cells.

Baculoviruses are large enveloped, circular double-stranded DNA insect viruses which are widely used as bioinsecticides in agriculture and forestry and can be genetically engineered to improve their effectiveness (2, 18). More recently, baculoviruses were shown to have potential as gene delivery vectors for gene therapy (12, 32, 45) or as vectors for surface display of cell lines (36). Yet, their major application to date is as a viral vector for the expression of heterologous proteins in insect cells (19, 36). The prototypic and most intensively studied baculovirus, \(Autographa californica\) multieapsid nucleopolyhedrovirus (AcMN\(PV\)), has been primarily used as an expression vector, while other baculoviruses may become exploited as well, especially when appropriate cell lines are available.

A major drawback in the large-scale production of baculoviruses as bioinsecticides or for heterologous protein production is the so-called passage effect. This effect is notable as a significant drop in production after prolonged virus passage in insect cell culture (reviewed by Krell [25]) and is a result of the accumulation of defective interfering particles (\(DIs\)) (20). These \(DIs\) are rapidly generated in cell culture (39) and become predominant after prolonged passages, meanwhile interfering with the replication of intact helper virus. However, the mechanism of the generation of \(DIs\) is still enigmatic, and the sequences involved are unknown.

\(DIs\) have retained \(cis\)-acting elements essential for baculovirus DNA synthesis, such as origins of replication (ori) (25). Transient virus-mediated plasmid replication assays demonstrated that baculovirus homologous regions (hrs) (21, 22, 28, 37), as well as baculovirus early promoter regions not containing \(hr\) sequences (49), have a putative ori function. In addition, these assays showed that other non-\(hrs\), with structural similarities to eukaryotic oris, may have an ori function (11, 14, 23, 38). Their ori activity in vivo was recently demonstrated but was unfortunately not compared to the ori activity of hrs (7). Strikingly, AcMN\(PV\) \(DIs\) were enriched in such a non-\(hr\) ori (26, 27). This suggests a prominent role of baculovirus non-\(hr\) oris in the generation of \(DIs\).

For large-scale or continuous production of heterologous eukaryotic proteins by use of the baculovirus expression system in insect cell bioreactors, the passage effect is a major obstacle. For prevention of the negative consequences of the passage effect, a genetically stable viral genotype is highly demanded. This may be achieved by selection for viruses with enhanced stability or higher polyhedron (or recombinant protein) production (42) or, alternatively, by site-directed mutagenesis of viral sequences putatively involved in the generation and/or maintenance of \(DIs\). Therefore, we chose the non-\(hr\) ori sequence as a target for mutagenesis studies.

Compared to AcMN\(PV\) infections in widely used cell lines such as \(Sf21\) and \(Sf9\), the generation and predominance of \(Spodoptera exigua\) MN\(PV\) (\(Se\)MN\(PV\)) deletion mutants (including \(DIs\)) in various \(S. exigua\) cell lines occurs significantly faster (3, 11). This virus-cell system thus provides a better model system than AcMN\(PV\) to study the passage effect, the mechanism of DI generation, and the pivotal role of non-\(hr\) ori sequences therein.

In this paper we have studied the rapid passage effect during serial passage of wild-type \(Se\)MN\(PV\) in the established \(S. exigua\) cell line \(Se\)301 (9). To monitor the generation of DI genomes over passage and to study the role of the non-\(hr\) ori sequences in this process, a full-length infectious clone of
SeMNPV propagated in *Escherichia coli* (bacmid) was constructed and used in serial passage experiments. This revealed the pivotal role of the non-*hr* ori in the genesis of DIs and led to the generation of a recombinant SeMNPV bacmid with enhanced stability in cultured insect cells.

**MATERIALS AND METHODS**

**Cells, insects, and virus.** The *S. exigua* cell line Se301 (8, 9) was donated by T. Kawarabata (Institute of Biological Control, Kyushu University, Kyushu, Japan) and was propagated at 27°C in Grace’s supplemented medium ( Gibco BRL) containing 10% fetal calf serum (Gibco BRL). Fourth-instar *S. exigua* larvae were infected by contamination of artificial diet with 4 × 10^7 SeMNPV-US1 (5) polyhedra per larva (43). Hemolymph was collected as previously described (17) and was defined as the passage zero (P0) budded-virus (BV) inoculum to initiate serial passage in cultured Se301 cells. Serial undiluted passages was carried out as previously described (39). Infectious BV titers were determined using the endpoint dilution assay (47).

DNA isolation, Southern hybridization, colony lift, molecular cloning, and sequencing. Intracellular viral (ICV) DNA and BV DNA were isolated as previously described (44). Digested viral DNA was run overnight in ethidium bromide-stained agarose gels, and Southern hybridization was performed using standard methods (40). A colony assay was run (40) to isolate the cloned submolar 5.3-kb fragment using the same probe as described above. Automatic sequencing was performed using an ABI prism 310 genetic analyzer (Perkin–Elmer) using 6U of T4 DNA ligase. DNA isolation, Southern hybridization, colony lift, molecular cloning, and sequencing were performed as described.

**AcNPV bacmids.** The bacmid vector for direct cloning of SeMNPV was constructed by PCR using the Expand long-template PCR system (Roche). PCR products (927 bp) of the SeMNPV non-*hr* ori were made with reverse primer DZ127, 5′-CATCGATGCTACGGTGAATCCGGAG-3′ (nucleotides [nt] 84027 to 84080 [16]), and forward primer DZ128, 5′-CCTTGAGTTCCTTTTCTTGTG-3′ (nt 83122 to 83192; purified using a High Pure PCR purification kit (Roche); and digoxigenin labeled overnight. Hybridization and colorimetric detection with nitroblue tetrazolium–bromo-4-chloro-3-indolyl phosphate (Gibco BRL) were performed according to the manufacturer’s recommendations. Hypermorphic viral XbaI bands were cut from the gel, purified with Glassmav (Gibco BRL), and cloned into pUC19 by electromediation of E. coli DH5α using standard methods (40). A colony assay was run (40) to isolate the cloned submolar 5.3-kb fragment using the same probe as described above. Automatic sequencing was performed using an ABI prism 310 genetic analyzer (Perkin–Elmer) using 6U of T4 DNA ligase. DNA isolation, Southern hybridization, colony lift, molecular cloning, and sequencing were performed as described.

**SeMNPV-US1 bacmid.** The bacmid vector for direct cloning of SeMNPV was constructed by PCR using the Expand long-template PCR system (Roche). Custom made primers (Gibco BRL) were designed using DNAstar PrimeSelect and were based on the sequence of AcNPV transfer plasmid pVL1393 (29), which was the backbone of the transfer vector pMON14272 used to construct the SeMNPV bacmid bMON14272 (30). Primers DZ123 (5′-CCTTGCAGGCTATTTCTTGCCAGAATTC-3′) and DZ128 (5′-GCTTCGAGTTCCTTTTCTTGTG-3′) were oppositely directed to se-quence flanking the BglII cloning site of pVL1393 and contained additional BsaI restriction sites (italics) at their 5′ ends for circulatization. DZ114 (5′-CATTTACTCGAAAACACTGTACACTTCGTCAAAATAAATGAC-3′) was included by two 5′-CAT CGAGGCTATTTCTTGCCAGAATTC-3′ and 5′-GCTTCGAGTTCCTTTTCTTGTG-3′ restriction sites are included in the primers (underlined). The 3′ and 5′ ends of the primers were determined using the Expand long-template PCR system (Roche) according to the manufacturer’s protocol, giving a product of 1.036 bp using a High Pure PCR purification kit (Roche). PCR was performed using 2 μl of plasmid DNA as template, phenol–chloroform extracted, and ethanol precipitated. Approximately 0.5 μg of PCR product was used for transformation of electrocompetent E. coli DH10β containing both SeBAC10 and homologous recombination helper plasmid pBAD-αpY.

**Reconstitution of the SeMNPV polyhedrin gene by pFastBAC1 donor plasmid.** The polyhedrin gene in SeMNPV bacmids, designated SeBAC10Δnonhr, was confirmed by Psil digestion and PCR. The genomic Prl-1 fragment of SeBAC10 (7,017 bp) was expected to be 286 bp bigger in SeBAC10Δnonhr, giving a fragment of 7,303 bp (Fig. 4). PCR was performed with forward primer DZ127 and reverse primer DZ128 as previously described. The PCR product of 1,213 bp was cloned into pGEM-Teasy (Promega) and completely sequenced, revealing that recombination had occurred precisely at the anticipated locus via the 5′-flanking nucleotides.

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RESULTS

Serial passage of SeMNPV in Se301 insect cells. SeMNPV-US1 was serially passaged 25 times in the S. exigua cell line Se301 with BV from infectious hemolymph, defined as P0 inoculum. A decrease of polyhedrin production was observed after fewer than five passages, indicating a dramatic passage effect. ICV DNA was purified and subjected to XbaI (Fig. 1A) digestion. A rapid reduction of the major genomic XbaI-A fragment was observed (Fig. 1A). At the same time, a novel XbaI fragment of about 9 kb became more abundant and was cloned and sequenced. This fragment was already present in the P1 DNA and appeared to consist of the remnants of the XbaI-A fragment as a result of a 26.5-kb deletion (from nt 15301 to 41759), according to the complete genome sequence of SeMNPV (16). The occurrence of mutants with deletions in this particular genomic region is a common phenomenon of SeMNPV infection in cell culture, but these deletions do not compromise BV or protein production (3, 10). In vivo, such deletion mutants also exist and can act as parasitic genotypes (33).

Analysis of hypermolar bands. Hypermolar fragments accumulated in Se301 cells from P10 onwards, and they were visualized as XbaI restriction fragments of 2.6 and 3.0 kb in agarose gels (Fig. 1A). From P15 onwards also, bands of 4.1, 5.5, and 7.0 kb and more became hypermolar. The abundant 2.6-, 3.0-, and 4.1-kb XbaI bands were cloned and sequenced, and it was found that the XbaI sites on either side of the cloned inserts corresponded to the SeMNPV XbaI restriction site at position 82132, according to the complete sequence of SeMNPV (16). Most interestingly, both the 2.6- and 3.0-kb fragments contained the entire SeMNPV non-hr origin of DNA replication (nt 83286 to 83932 [11]) and a junction of sequences flanking this non-hr ori (Fig. 2). The borders and the junction of the 4.1-kb fragment appeared to be identical to those of the 2.6-kb fragment (Fig. 2B). The difference in size is a consequence of a duplicated non-hr ori present in this 4.1-kb fragment. Note-worthy is the presence of an overlapping stretch of 9 bp at the junction site in the 2.6- and 4.1-kb fragment (Fig. 2B), which in the complete SeMNPV genome is present on either side of the non-hr ori, leaving 2.6 kb in between. Because of the presence of a junction site and the same XbaI (position 82132) on either side of the fragments, it was concluded that the hypermolar fragments must exist in the ICV DNA preparation either as DNA minicircles or as tandem repeats in a larger concatenated form.

To investigate whether the other hypermolar bands of 5.5 kb, 7.0 kb, and more also contained the non-hr ori, a Southern blot was made with a non-hr ori probe. The result (Fig. 1B) showed that these fragments hybridized strongly to the probe, and therefore it was concluded that a range of molecules of different sizes containing the SeMNPV non-hr ori predominated upon serial passage.

In addition to the non-hr ori containing the genomic XbaI-F fragment of 6.6 kb, an unexpected additional band of 5.3 kb hybridizing to the non-hr ori probe (Fig. 1B [see also Fig. 3]) became submolar from P15 onwards. Sequencing revealed that the 5.3-kb fragment consisted of two joined, but distantly located, sequences from the SeMNPV genome (Fig. 2B). The ends of the fragment corresponded to XbaI sites at positions 82132 and 119846, respectively. The junction between the two fragments was formed by an overlapping sequence stretch of 19 bp, containing multiple GTC repeats, located at positions 86426 to 86446 and 118807 to 118780. The presence of this 5.3-kb band in the wild-type SeMNPV DNA was confirmed by Southern hybridization.

Replicative-form hypermolar 2.6- and 3.0-kb XbaI fragments. To investigate whether the abundant XbaI fragments of 2.6 and 3.0 kb exist as minicircles or as tandem repeats in a larger concatenated form, ICV DNA of P10 (at a stage when only the 2.6- and 3.0-kb bands were abundant) was subjected to partial digestion with XbaI, using increasing amounts of restriction endonuclease during digestion for 20 min. Hybridization was performed with the same non-hr ori probe as described above (Fig. 3). The partial XbaI digests of P10 viral DNA showed a stepladder of multimers of the 2.6- and 3.0-kb bands. This suggests that the accumulation of the SeMNPV non-hr ori occurs via high-molecular-weight concatemers of tandem repeats of different sizes. This is likely to be the case not only for the 2.6- and 3.0-kb fragments but also for the 4.1-, 5.5-, 7.0-kb, and larger fragments from P15 onwards.

Serial passage of SeMNPV bacmids in Se301 insect cells. A genetically homogeneous SeMNPV bacmid (SeBAC10) and a derived non-hr ori deletion mutant (SeBAC10anohr) were constructed (Fig. 4) to determine whether non-hr ori concatemers are generated de novo in cell culture or preexist and become selectively amplified and whether virus stability might be enhanced by deletion of this non-hr ori.

Prior to serially passaging the bacmid-derived BVs in cell culture, the polyhedrin gene was reintroduced. After transfection of Se301 cells, the BV-containing supernatant was defined as the P1 virus stock and was used to initiate serial undiluted passage. ICV DNA was purified and digested with
Similar to SeMNPV-US1 (this study and references 3 and 10), deletions in XbaI-A occurred for both bacmids SeBAC10ph and SeBAC10phΔnonhr (Fig 5). The deletion in SeBAC10phΔnonhr was mapped as a junction overlap of 3 nt (AAC) from 20162, 20163, or 20164 to 36396, 36397, or 36398, spanning open reading frames (ORFs) 17 to 35. From P6 onward, a small hypermolar XbaI fragment of 3.0 kb was visible in DNA preparations of SeBAC10ph (Fig. 5A). This fragment was cloned and sequenced and appeared to contain the non-hr origin of DNA replication and a junction sequence (Fig. 2c) also observed with the SeMNPV-US1 wild type (Fig. 2B). In contrast, the analysis of ICV DNA from SeBAC10phΔnonhr-infected cells did not reveal any accumulation of hypermolar fragments (Fig. 5B). SeBAC10phΔnonhr BV titers remained at higher levels throughout the entire period of serial passaging than those of SeBAC10ph and SeMNPV-US1 wild type (Fig. 6A). Polyhedron production of SeBAC10phΔnonhr remained constant for at least 20 passages, in contrast to SeBAC10ph (Fig. 6B). These results demonstrated that absence of the non-hr ori strongly increased the stability of the SeMNPV genome in Se301 insect cells.

**DISCUSSION**

The rapid accumulation of DI particles with DNA containing hypermolar non-hr oriis appears to be an artifact of serial passage of SeMNPV in Se301 cells and the major cause of the decrease of virus and polyhedron production. Ultimately, these hypermolar molecules form the majority of the viral DNA. By partial digestion, we showed that these molecules exist as high-molecular-mass concatemers, in agreement with a supposed rolling-circle mechanism for baculovirus DNA replication (24, 28, 35, 50). Their rapid multiplication, together with previous data from in vitro replication assays (11), provides support for the view that the SeMNPV non-hr ori might be a genuine origin of DNA replication.

In order to elucidate whether these non-hr ori concatemers were newly formed in Se301 insect cells or, alternatively, only

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**FIG. 2.** Schematic overview of the genetic organization of hypermolar and other non-hr ori hybridizing bands compared to the complete SeMNPV genome. (A) Genetic organization of the genomic DNA with nucleotide positions according to the complete SeMNPV genome (16). Arrows represent the respective ORFs. Solid and light-grey boxes refer to sequences on either side of XbaI (Xb) 83132, containing SspI (S), PstI (P), EcoRI (E), and XhoI (Xh) sites. The non-hr ori is presented as a hatched box between the two SspI sites (11). (B) Genetic arrangement of hypermolar 2.6-, 3.0-, and 4.1-kb fragments of SeMNPV-US1 (Sewt) and a nonhypermolar cohybridizing 5.3-kb fragment (genomic fragment of a SeMNPV deletion mutant) in the Southern blots. Nucleotide positions and sequence overlaps and insertions are indicated at the junction sites. (C) Genetic arrangement of the hypermolar 3.0-kb fragment of SeBAC10ph, containing two junctions.
accumulated from a genetically heterogeneous wild-type isolate, a full-length infectious clone (bacmid) of SeMNPV was constructed. Such a bacmid could be stably maintained as a single-copy bacterial artificial chromosome in E. coli DH10β and used as a starting material in passage experiments in insect cells (30, 39). Transfection of Se301 insect cells with SeBAC10ph (with a reintroduced intact polyhedron gene) showed normal polyhedron production, but serial passage again resulted in a decrease of viral titers and polyhedron production and the rapid accumulation of non-hr ori containing molecules. Since the non-hr ori could be removed from the viral DNA without affecting virus replication, we must conclude that this non-hr ori is not essential. The two minor ORFs 85 and 86 within the non-hr are also nonessential. These ORFs do not have known baculovirus or other homologues, and it is unknown whether they are transcriptionally active. Furthermore, deletion of this non-hr ori strongly enhanced the genomic stability in cell culture. Transfection of Se301 cells with the non-hr ori deletion mutant bacmid SeBAC10phΔnonhr gave normal polyhedron production and high viral titers (Fig. 6), which were maintained for up to at least 20 passages. Together with an unchanged restriction profile from P1 to P20, these results indicate that this recombinant has an enhanced stability in cell culture compared to both wild-type SeMNPV and the parental bacmid SeBAC10ph. The increased overall stability is probably...
not due to an increase in intrinsic stability of the SeMNPV genome by itself, but rather a consequence of the lack of a cis-acting element (non-hr ori) that has a strong replicative advantage during baculovirus DNA replication.

Our findings with SeMNPV are in line with the results from Lee and Krell (26, 27), who demonstrated that AcMNPV DIs at P81 largely consisted of reiterations of about 2.8 kb of the standard genome, which was later shown to contain an active non-hr origin of DNA replication (7, 23). In addition, previous work on AcMNPV showed that DIs could also be enriched in hrs (21). These observations suggest that reiteration and predominance of baculovirus hrs but particularly non-hr oris, which are complex structures comprising multiple direct and inverted repeats, are more common phenomena upon multiple passage and may contribute to a rapid passage effect. Single copies of non-hr oris, which resemble eukaryotic oris based on structural similarities (4), have been identified in many other baculovirus genomes by transient-replication assays (Orgyia pseudotsugata NPV [38] and Spodoptera littoralis MNPV [14, 15]) or based on sequence and structural similarity only (Bombyx mori NPV [24], Busura suppressaria NPV [13], and Cydia pomonella granulovirus [31]). The conservation of non-hr oris in baculovirus genomes implies an important (biological) role in virus replication and may be related to viral latency and persistence in insect populations. The resemblance to eukaryotic oris suggests that baculoviruses may have obtained these sequences from the host genomes to be able to replicate in the insect without the requirement for virus-encoded replication factors.

At the junctions of non-hr oris concatenated molecules and junctions of major genomic deletions, sequence overlaps of 1 up to 19 bp were found, potentially involved in the causative recombination mechanism. The sequence of the additional 5.3-kb fragment in the Southern blots showed a 19-bp overlap at the deletion junction, consisting of multiple GTC repeats (Fig. 2B). These GTC repeats of up to 27 bp were found scattered throughout the SeMNPV genome on both strands and were more frequent than expected on a random basis. For the 2.6-kb Xbal fragment concatemers, a 9-bp TTGACGTCG overlap from flanking sequences was found at the junction site (Fig. 2B). Also, this repeat was found more frequently (12 times) in the genome than expected on a random basis (<1). The 9-bp overlap implies that the concatemers of the 2.6-kb fragment were generated during serial passage by looping and subsequent excision (homologous recombination) of non-hr oris containing genomic DNA, followed by continued replication and consequent concatenation of this intervening region. The same 9-bp overlap was found at the junction of the 4.1-kb fragment, which contains a duplicated non-hr ori (Fig. 2). This suggests that the 4.1-kb fragment was generated from the 2.6-kb molecule by another recombination event and rapidly became hypermolar because of the presence of two copies of the non-hr ori. This hypothesis is consolidated by the appearance of the 4.1-kb fragment in passages later than the 2.6-kb fragment (Fig. 1A). For the other junctions, smaller overlaps (1 or 3 bp) or even insertions were found, which was also demonstrated for AcMNPV DIs in an earlier study (39), suggesting that the same recombination mechanisms are involved.

The strategy of deletion of sequences that have a replicative advantage, accumulate upon serial passage, and interfere with virus and protein production (e.g., non-hr oris) may now be applied for other baculoviruses as well and will contribute to the solution of problems associated with large-scale applications using baculovirus expression vectors for protein production in insect cells. In further studies we plan to map the sequences in the SeMNPV non-hr ori involved in the generation of DIs in more detail by reintroducing mutant SeMNPV non-hr oris in SeMNPV bacmids. In addition, we want to investigate whether the deletion of the non-hr ori affects baculovirus persistence in vivo.

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