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Autographa californica Baculoviruses with Large Genomic Deletions Are Rapidly Generated in Infected Insect Cells

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Defective interfering baculoviruses (DIs) lack considerable portions of the genome, interfere with the replication of helper virus, and cause the so-called "passage-effect" during serial passaging in insect cells and in bioreactor configurations. We investigated their origin by (nested) PCR and demonstrated that DIs lacking approximately 43% (d43) of their DNA are present in low-passage *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV)-E2 virus stocks and in polyhedra, but not in the authentic AcMNPV isolate obtained prior to passage in cell culture. To investigate whether DIs are rapidly generated *de novo* in Sf21 insect cells, a genetically homogeneous AcMNPV bacmid was serially passaged, resulting in the generation of d43 DIs within two passages. AT-rich sequences of up to 66 nucleotides of partly unknown origin were found at the deletion junctions in the d43 DI genomes. These data suggest that the rapid generation of DIs is an intrinsic property of baculovirus infection in insect cell culture and involves several recombination steps.

Key Words: AcMNPV; defective interfering particles; passage-effect; recombination; bacmid; deletion; nested PCR.

INTRODUCTION

The baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) is widely exploited as a safe eukaryotic expression vector in insect cells (King and Possee, 1992). The baculovirus insect-cell expression system usually gives high yields of heterologous proteins, which in many cases are immunologically and biologically similar to their authentic counterpart (Vialard *et al.,* 1995). Engineered baculoviruses are further envisaged as improved bioinsecticides (Cunningham, 1995; Black *et al.,* 1997) and may have potential as vectors for gene therapy (Hofmann *et al.,* 1995).

Baculovirus and recombinant protein production following infection of insect cells is accompanied by the occurrence and accumulation of defective interfering (DI; Huang and Baltimore, 1970) particles (Kool *et al.,* 1991; Wickham *et al.,* 1991). These DIs are generated upon multiple passaging at high multiplicity in insect cells and are responsible for the so-called "passage-effect" observed, among others, in bioreactor configurations (Van Lier *et al.,* 1992). This phenomenon prevents the development and exploitation of stably operated continuous insect cell bioreactor production systems and complicates the scale-up of baculovirus and recombinant proteins *in vitro*. The nature and origin of these DIs are not well understood.

DIs most likely have a replication advantage as a result of their smaller size and the increased density of origins of DNA replication successfully competing for essential *trans*-acting factors (Krell, 1996). After 81 passages of AcMNPV-E2 in Sf21 insect cells, DI genomes were generated and were largely composed of reiterations of a short genomic sequence (Lee and Krell, 1992, 1994), which was shown to contain a putative origin of DNA replication (Kool *et al.,* 1994). *In vivo* activity of this non-*hr* origin of DNA replication has now been demonstrated (Habib and Hasnain, 2000).

Kool *et al.* (1991) observed the accumulation of DIs containing a major genomic deletion of approximately 43% (d43) upon serial passage of AcMNPV-E2 in Sf21 insect cells. In several independent studies, the generation of AcMNPV DIs with a similar deletion was demonstrated (Carstens, 1982; Lee and Krell, 1992; Van Lier *et al.,* 1994; Wickham *et al.,* 1991). The deleted region of the genome contains genes which are considered to be involved in baculovirus DNA replication, such as *lef-1, lef-2, lef-3,* and DNA polymerase (Kool *et al.,* 1995; Lu and Miller, 1995). DIs of *Bombyx mori* NPV (BmNPV), a baculovirus closely related to AcMNPV with respect to gene order and overall gene homology, were also found to contain deletions spanning this region (Hashimoto *et al.,* 1993; Yanase *et al.,* 1998). Thus, the accumulation of baculovirus DIs with major deletions is a general phenomenon.

It is not clear whether the accumulation of AcMNPV d43 DIs is the result of a generation mechanism that involves progressive deletions over time or, alternatively, by specific selection of DIs from a mixed population in

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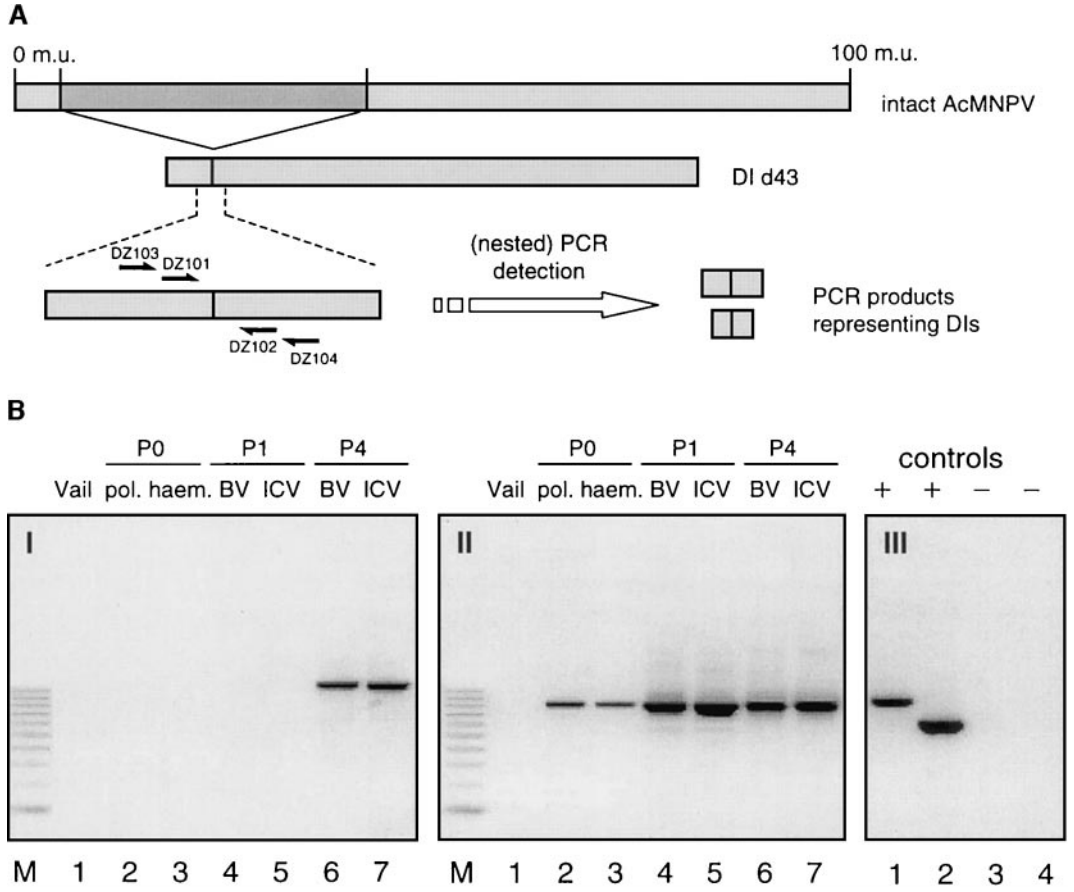

FIG. 1. (A) PCR detection of d43 deletions with (nested) primers flanking the AcMNPV deletion junction site. (B) PCR detection of d43 DIs in DNA preparations of AcMNPV virus stocks. (I) One-step PCR on DNA from polyhedra of AcMNPV-Vail (lanes 1) and on AcMNPV-E2 DNA preparations; insect-derived polyhedra (lanes 2), hemolymph-derived budded viruses (lanes 3), serially passaged BV and intracellular (ICV) viral DNA of the first (P1, lanes 4 and 5, respectively) and the fourth passage (P4, lanes 6 and 7, respectively). (II) Nested PCR on amplification products from the one-step PCR. (III) Plasmid pMK1 used as a positive control template for one-step and nested PCR, giving products of 894 bp (lane 1) and 630 bp (lane 2), respectively. For the negative controls no template DNA was used for the one-step (lane 3) and nested (lane 4) PCR. Lane M contains a 100-bp DNA size marker.

the virus inoculum. We used a sensitive (nested) PCR-based method to detect minor amounts of AcMNPV DIs in low-passage virus stocks and insect-derived polyhedra. DIs may be part of a natural baculovirus population that subsequently predominates in insect cell culture. Alternatively, DIs may be generated solely as an artifact of *in vitro* cell culture. To answer this question, the original isolate of AcMNPV (Vail *et al.,* 1971), which had never been replicated in cell culture, was investigated for the presence of DIs. Furthermore, a recombinant baculovirus, which was generated from a "bacmid," was serially passaged in insect cells. Bacmids are baculovirus shuttle vectors that replicate in *Escherichia coli* as a low-copy plasmid and are genetically homogeneous (Luckow *et al.,* 1993). These bacterial artificial chromosomes (BACs) are stably maintained in well-characterized recombination-deficient *E. coli* host strains (e.g., DH10β). No rearrangements have been observed in BACs after 100 generations of growth (Shizuya *et al.,* 1992). The use of this bacmid resulted in a starting material composed only of intact viral genomic DNA. Our results indicate that DIs are rapidly generated in cell culture and can persist in insects.

RESULTS

AcMNPV DI detection by PCR

A hemolymph preparation derived from fourth instar *Spodoptera exigua* larvae orally infected with a lethal dose of AcMNPV-E2 was defined as passage zero (P0) budded virus (BV) stock. The first round of infection in Sf21 insect cells was initiated with a multiplicity of infection (m.o.i.) of 10. Polyhedra, virus containing hemolymph (P0), and virus serially passaged through Sf21 cells (P1 to P4) were investigated for the presence of DIs by (nested) PCR (Fig. 1). This PCR was specifically designed to amplify junction fragments resulting from deletions of approximately 43% (d43) of the genome on a defined location (Kool *et al.,* 1991). For the BV and intracellular virus (ICV) preparations of P4 only, a PCR product of 1.1

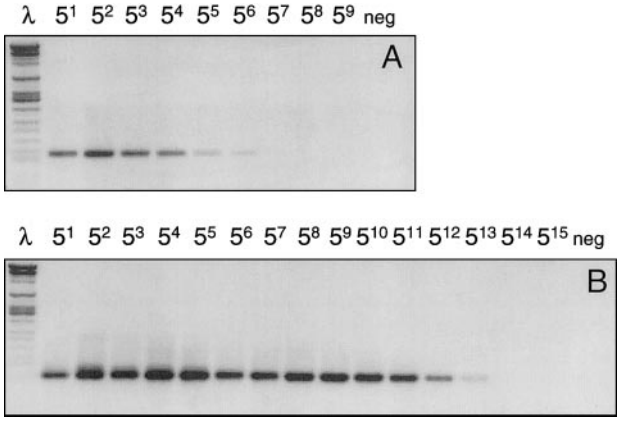

FIG. 2. Sensitivity of one-step (A) and nested (B) PCR for detection of DIs. Fivefold dilution series from 5^1 to 5^{15} of pMK1 (containing an AcMNPV-E2 *Sst*II-BE junction fragment) were tested using primer sets DZ103/104 and DZ101/102, respectively. One microliter reaction product of the one-step PCR was used as template for nested PCR.

kb was amplified (Fig. 1B, I). A nested PCR using nested primers resulted in the generation of single PCR products of 0.8 kb for all samples except the sample in lane 1 (Fig. 1B, II). PCR amplification products from AcMNPV-E2 (polyhedra P0, hemolymph P0, BV P1 and P4, and ICV P1 and P4) were cloned. To investigate the presence of d43 DIs in a natural baculovirus population, a DNA preparation of the original AcMNPV isolate from the alfalfa looper *A. californica* (Vail *et al.,* 1971) was subjected to PCR. The one-step or nested PCR (Fig. 1B, I and II) generated no products.

Plasmid pMK1, containing the *Sst*II-BE deletion junction fragment of AcMNPV (Kool *et al.,* 1991), served as positive control template, giving products of 894 and 630 bp in the one-step and the nested PCR, respectively (Fig. 1B, III). The sensitivity of the one-step and nested PCR was demonstrated by testing a dilution series of pMK1. With the one-step PCR only, the lower detection limit was dilution 5^7, corresponding with approximately 0.6×10^6 molecules (Fig. 2A), whereas the detection limit of the nested PCR was dilution 5^{13}, giving an improvement of at least 10^4-fold, resulting in a detection level between 10 and 100 molecules per 50 μl PCR reaction volume (Fig. 2B).

The quality and authenticity of the DNA templates used for the DI PCR experiments described above was demonstrated by a PCR, specifically amplifying a genomic region of 819 bp in the *Pst*I-E fragment of AcMNPV. This region is located within the major deletion of d43 DIs and is indicative of the presence of intact viral DNA. This PCR proved to be positive for all investigated AcMNPV viral DNA samples, including the authentic AcMNPV isolate (data not shown).

Passage effect upon serial passage of AcMNPV bacmid bGFP

To investigate whether DIs are rapidly generated *de novo* in insect cells, we serially passaged a recombinant baculovirus that was generated from a genetically homogeneous source (bacmid). To facilitate virus titration and to observe changes in recombinant protein production during passaging, the bacmid was equipped with the S65T variant of the green fluorescent protein (GFP) and was designated bGFP. Sf21 insect cells were transfected with bGFP DNA and the transfection supernatant containing BV was defined as the first passage (P1) virus stock and was used to initiate serial passaging with a m.o.i. of 20. Throughout passaging a gradual but definite decrease in virus titer was observed from approximately 10^8 TCID50 units/ml in the early passages to a complete loss of GFP expression from P31 and further on (Fig. 3).

DNA preparations from the serially passaged bGFP-derived virus productions were analyzed for the presence of DIs by PCR. For P2, P21, and P41 of both BV and ICV DNA preparations, a similar set of amplification products was generated with sizes from about 0.5 up to 2.0 kb (Fig. 4). The dominant four PCR products of bGFP (BV P2) with sizes of 1.8, 1.4, 1.3, and 0.5 kb were designated bGFP-A, -B, -C, and -D, respectively, and subsequently cloned.

Sequence analysis of DI deletion junction sites

The PCR amplification products from AcMNPV-E2 (polyhedra P0, hemolymph P0, BV P1 and P4, and ICV P1 and P4; Fig. 1B) and the four abundant PCR products of bGFP (Fig. 4) were cloned and sequenced, and the DNA organization at the junction sites was determined (Fig. 5). The sequences of the six (nested) PCR products from wild-type AcMNPV-E2 (Fig. 1B, II) proved to be identical and revealed the presence of an insertion of 66 nucleotides (AT 67%) at the junction site. The precise origin of this sequence could not be identified, but 21 contiguous nucleotides were identical to a part of *ie-1*, a gene located at nt positions 127198 to 128944 (Ayres *et al.,* 1994).

The four bGFP amplification products were cloned and sequenced and revealed differences in the organization at the junction site. The largest fragment (bGFP-A), rep-

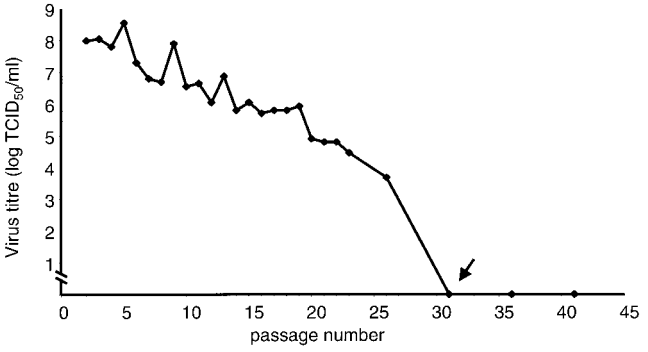

FIG. 3. Titration bGFP virus stocks during serial passage in Sf21 insect cells as measured by GFP expression. The arrow indicates the earliest passage number with loss of GFP expression.

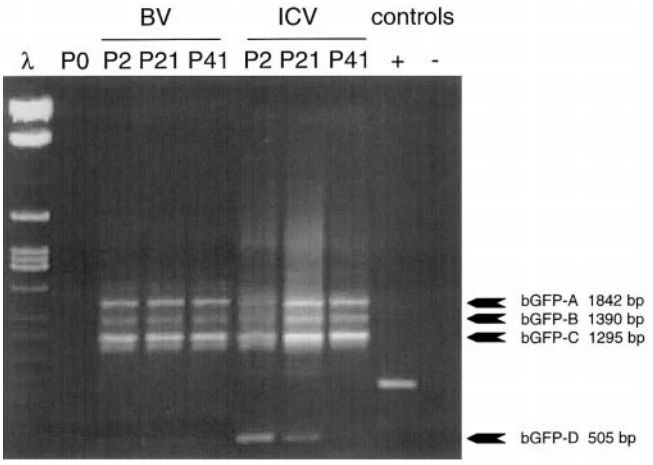

FIG. 4. PCR detection of major deletions in DNA preparations from bacmid bGFP (P0), serial passaged bGFP-derived budded virus (BV), and intracellular virus (ICV). Passage numbers are indicated above the lanes. Cloned amplification products are designated bGFP-A to -D. Positive (+) and negative (−) controls were plasmid pMK1 and no template DNA, respectively.

resenting the smallest deletion, contained an additional 11 bp (AT 100%) at the junction site. The second (bGFP-B) and the third (bGFP-C) product both contained an extended insertion of 48 bp (AT 75%) and 66 bp (AT 82%), respectively. Interestingly, 27 nucleotides of the bGFP-C insertion sequence were identical to a region in the mini-F replicon, which is part of the bacmid vector. Fragment bGFP-D did not have an insertion, but an overlap of two nucleotides (TG) on either side of the junction instead.

DISCUSSION

We investigated whether DIs are present in a natural virus isolate and subsequently accumulate in cell culture or, alternatively, whether their generation is solely an artifact of cell culture. For AcMNPV, the origin of the predominant DI d43 as described by Kool *et al.* (1991) has never been further investigated due to the lack of sensitive detection methods. DI detection by nested PCR generated identical amplification products of 0.8 kb in all the virus stocks of AcMNPV-E2 (Fig. 1B) indicating that this technique is robust and sensitive (Fig. 2). For the virus samples of P4, a product was already generated by one-step PCR only, indicating an increased proportion of d43 DIs as compared to the samples of lower passages. Since polyhedra- and hemolymph-derived virus preparations were found positive, we concluded that d43 DIs were present in the starting material (polyhedra, hemolymph) isolated from infected larvae and subsequently accumulated in cell culture to reach a visible stage by restriction enzyme analysis (Kool *et al.,* 1991). The detection of d43 DIs in AcMNPV-E2 polyhedra from insects indicates that DIs can persist in insects for more than one passage.

The presence of DIs in polyhedra reflects the occurrence of DIs *in vivo*, as was also shown by Muñoz *et al.* (1998). The latter authors demonstrated that in a natural baculovirus population of *S. exigua* MNPV (SeMNPV) deletion mutants, which could not be purely isolated by *in vivo* cloning, acted as parasitic genotypes. In an independent study, Dai *et al.* (2000) showed the presence of deletion mutants with maintained infectivity in the wild-type US1 isolate of SeMNPV by PCR. Serial passage of SeMNPV in SeUCR cells resulted in the prevalence of mutants with a 25-kb deletion, lacking virulence *in vivo* (Heldens *et al.,* 1996). These observations suggest that genetic heterogeneity including the presence of DIs is not uncommon in natural baculovirus isolates.

It is not understood why DIs eventually do not predominate in insects, despite the fact that baculovirus recom-

fragment	left	junction sequence	right
Kool *et al.* (1991)	(2459) CGGC	*TGTT*	GAGT (59268)
AcMNPV-E2 wt	(2627) TCAT	*ATGACGATTGGTTTGCGCGTAATACATATAATGCAACGTCCGATATTATTTGTATTGCATGTTAAT*	AGAT (59305)
bGFP-A.	(3343) TATG	*TTTAATAATTA*	AGTT (59211)
bGFP-B.	(2711) GCGC	*TTTATAGTTTGTGTACATATTTACTGTGTTCAATTTAAAACTCACGTA*	ACGC (59068)
bGFP-C.	(2536) GGCA	*ATATTTTCTGACTAAGTATTTAAAATTTTAGATTGTCACACTAAATAAAAAAAACTTAAATTCACA*	CGCT (59006)
bGFP-D.	(2290) **TATG**	**TG**	**TG**CC (59482)

FIG. 5. Sequence of DI deletion junction sites. Viral sequences on the junction site borders are indicated on the left and right. Insertion sequences are in italics. Numbers refer to positions of viral nucleotides at the border of the junction based on Ayres *et al.* (1994). Overlapping viral sequences are indicated in bold. Underlined sequences indicate continuous stretches of nucleotides identical to parts of the ie-1 gene (AcMNPV-E2 wt) and the mini-F replicon (bGFP-C), respectively.

bination readily occurs *in vivo* and deletions are frequently observed (Muñoz *et al.,* 1997; Hajós *et al.,* 1998; Dai *et al.,* 2000). The preferential maintenance of parental virus *in vivo* might be due to a local low m.o.i. in infected larval tissues or a specific selection barrier against the encapsulation of DIs.

In contrast to these reports, investigation of the authentic wild-type AcMNPV (Vail *et al.,* 1971) by nested PCR did not reveal the presence of d43 DIs (Fig. 1B). However, it cannot be excluded that their presence is below the detection level of this technique (approximately 100 DI molecules per 100 ng DNA). This implies that d43 DIs in the AcMNPV-E2 isolate may have found their origin in the original plaque purification procedure of the several AcMNPV variants (including the E2 strain) in cultured TN-368–10 insect cells (Smith and Summers, 1978) or in subsequent amplifications. PCR detection of major deletions upon transfection and subsequent infection of insect cells with genetically homogeneous bacmid-derived AcMNPV DNA (Fig. 4) support the suggested rapid generation of d43 DIs upon infecting in insect cells. However, possible enhanced instability resulting from the presence of the bacterial expression cassette of the bacmid causing defective genomes cannot be excluded.

Assuming that large genomic deletions are the result of recombination, sequences at the deletion junction sites may contain the remains or imprints of possible intermediate deletion mutants. For AcMNPV-E2, only one PCR product was generated, suggesting that a particular d43 DI had become predominant in the virus inoculum. At least four different PCR products were generated from bGFP, indicating that a variety of DIs with deletions in the same genomic region was generated and maintained upon passaging. Sequence data of the amplification products generated by (nested) PCR revealed in four out of five cases the presence of short AT-rich (67–100%) sequences of (largely) unknown origin (Fig. 5). For bGFP-C, 27 bp out of a total insertion sequence of 66 bp was found identical to a region in the mini-F replicon of the bacmid, which is physically mapped within the d43 deletion and therefore supports a progressive deletion mechanism. In contrast, 21 bp of the deletion insertion sequence of AcMNPV-E2 of 66 bp was identical to the AcMNPV *ie-1* gene, which is not located within the d43 deletion and therefore suggests that several recombination mechanisms are involved in the generation of deletions. We were unable to determine a consensus insertion sequence or evident homology between viral sequences flanking the junction sites. Although, in two cases we found an overlap of 2 bp (bGFP-D) and 8 bp (from a recombinant AcMNPV DI, unpublished results) from sequences flanking the deletion junction site, likely to be the result of homologous recombination.

The presence of insertion sequences at the deletion junction sites of DIs are consistent with the findings of Kool *et al.* (1991), who determined an insertion of four nucleotides (TGTT) in a cloned junction fragment of an AcMNPV DI (Fig. 5). More recently, Wu *et al.* (1999) described the random integration of plasmid DNA into the AcMNPV genome due to co-replication in Sf21 insect cells. Analysis of integration junction sites revealed large genomic deletions and several insertions of short, AT-rich sequences of unknown origin. Analogous to our DI sequences, comparison of the junction insertion sequences did not reveal any consensus sequence or obvious homology between pUC19 and AcMNPV DNA. Although this study (Wu *et al.,* 1999) focused on the integration of foreign sequences into the baculovirus genome, our data show complementary evidence, suggesting that the same recombination mechanisms may be involved.

Homologous recombination between a pair of integrated inverted repeat IS50 elements *in vitro* readily occurred during AcMNPV replication in Sf9 cells (Martin and Weber, 1997). Moreover, homologous recombination at high frequency (Hajós *et al.,* 2000) is an important feature of baculovirus replication. Nonhomologous recombination *in vitro* was shown to occur between foreign and AcMNPV DNA in Sf9 cells (Xiong *et al.,* 1991; Schorr and Doerfler, 1993). These studies enforce the view that the process of baculovirus DNA replication in insect cells involves several mechanisms of hetero- and homologous recombination that may result in the rapid generation of defective genomes.

From this study it was concluded that the rapid generation of DIs is an intrinsic property of baculovirus infection in insect cell culture. We assume that continuous hetero- and homologous recombination of baculovirus DNA in infected insect cells occurs in combination with a selection for mutants which have a replication advantage and are able to multiply faster at the expense of the helper virus. With the development of a specific (nested) PCR strategy for the detection of DIs, a sensitive tool was obtained, which we will further use in experiments to demonstrate whether the rate of DI generation differs among cell lines from various origins and whether the genetic stability of baculovirus in cell culture can be enhanced by genetic engineering.

MATERIALS AND METHODS

Cells, insects, and virus

Spodoptera frugiperda (Sf-AE-21) cells (Vaughn *et al.,* 1977) were maintained at 27°C in Grace's supplemented insect medium (GIBCO BRL) with 10% fetal calf serum (FCS). Routine cell culture maintenance was performed according to established procedures (Summers and Smith, 1987; King and Possee, 1992). Polyhedra of the E2 strain of wild-type (wt) AcMNPV (Smith and Summers, 1978) were propagated in fourth instar larvae of *S. exigua*, which were raised on artificial diet (Smits *et al.,*

1988). Hemolymph was taken from infected larvae by cutting a proleg. The preparation was defined as the passage zero (P0) virus stock and was used for a first round of infection (passage) in Sf21 cells at a multiplicity of infection (m.o.i.) of 10 ($TCID_{50}$ units per cell). Budded virus solutions were titrated using the endpoint dilution assay (Vlak, 1979). Serial undiluted passaging was performed in 25 cm^2 tissue culture flasks (Nunc) by incubation for 2 h of 2.5×10^6 cells with 1 ml of the virus inoculum of the previous passage. The cells were washed with fresh medium and were further incubated in 4 ml of medium for 72 h.

Bacmid-derived virus

The S65T GFP gene from pGreenlantern (GIBCO BRL) was cloned into the MCS of pFastBac1 (GIBCO BRL) as a *Not*I fragment to generate bGFP by transposition according to the Bac-to-Bac baculovirus expression systems manual (GIBCO BRL). Bacmid bGFP was kindly provided by Dr. Ray Harris (Life Technologies) as a glycerolstock, which was subsequently plated and amplified from a single colony. Isolation and transfection of bacmid DNA to Sf21 cells was done according to the Bac-to-Bac baculovirus expression systems manual (GIBCO BRL). The bacmid-derived BV inoculum was defined as bGFP P1 and was used to initiate undiluted serial passaging in Sf21 cells at an initial m.o.i. of 20 $TCID_{50}$ units/cell.

Isolation of viral DNA

Budded virus was purified from infectious supernatant by centrifugation. BV viral DNA and total intracellular (ICV) DNA was isolated as described by Summers and Smith (1987), but viral DNA was purified by ethanol precipitation instead of dialysis.

PCR, cloning, and sequencing

PCR was performed using custom designed (DNAstar Primerselect) and synthesized (GIBCO BRL) primers. The forward primer VW293 for amplification of a part of AcMNPV *Pst*I-E (from position 44013 to 44832, according to Ayres *et al.* (1994)) was 5′ AGTTACAGAGTTTTCCGTGGTTCAG 3′ and the reverse primer VW294 was 5′ CCCCGTTATCGTCAATTTTCTCAAG 3′. Forward DZ103 and nested-forward DZ101 primers for detection of DIs were 5′ GTACCGCAGGTTGAACGTATCTTC 3′ and 5′ CGCTACAACACTCGTCGTTATG 3′, located on the AcMNPV complete genome (Ayres *et al.,* 1994) at positions 1968–1991 and 2125–2146, respectively. Reverse DZ104 and nested-reverse DZ102 primers were 5′ CCACACTTGATGCTAATCTCAAATAC 3′ and 5′ GTACACGCACACCGAGTTGTTTGTC 3′, located at positions 59667–59642 and 59560–59536, respectively. PCR was carried out for 30 amplification rounds in a reaction volume of 50 μl using approximately 100 ng viral DNA template per reaction. For nested PCR, 1 μl of the amplification product from the first round was used as a template for 30 additional amplification rounds. Plasmid pMK1 (6.45 kb) containing the AcMNPV *Sst*II-BE deletion junction fragment in PJD118 as described by Kool *et al.* (1991) was used as a positive control template for the PCR detection of major deletions. Fivefold dilution series until 5^{15} of pMK1 (stock was 166 ng/μl) were made to test the sensitivity of the (nested) PCR method.

PCR products were run in 0.8% agarose gels, purified with Glassmax (GIBCO BRL), and cloned into the pGEM-T vector (Promega Inc.) according to the manufacturer's protocol and using standard techniques (Sambrook *et al.,* 1989). As a DNA size marker, λ-DNA digested with *Eco*RI/*Hin*dIII/*Bam*HI was used. Plasmid DNA was purified using the High Pure Plasmid Isolation Kit (Roche). Automatic sequencing was performed using an ABI prism 310 genetic analyzer (Perkin–Elmer) at the department of Molecular Biology, Wageningen University. Sequence analyses were performed using FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.,* 1997) from the UWGCG computer programs (release 10.0). All plasmids were maintained in DH5α *E. coli* cells.

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