Identification and functional analysis of the origins of DNA replication in the *Cydia pomonella* granulovirus genome

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The entire genome of *Cydia pomonella* granulovirus (CpGV) was systematically screened for origins of DNA replication, using an infection-dependent DNA replication assay in the granulovirus-permissive *Cydia pomonella* cell line, Cp14R. All seven cosmid libraries that covered the CpGV genome were found to replicate in the assay. A genomic library of 32 overlapping plasmids was subsequently screened. Plasmids that replicated were in turn subcloned into 1–2 kbp overlapping fragments. Eleven subclones replicated, each containing at least one of the 13 single-copy 74–76 bp imperfect palindromes, previously identified in the CpGV genome as possible origins of replication. Genome fragments of 156 bp, each containing one of the 13 palindromes, were cloned to verify replication and provided confirmation that these 13 palindromes are the only origins of replication in the genome. A real-time PCR method was developed for the quantification of DNA replication, which eliminated the need for Southern blotting and hybridization. A set of deletion clones allowed further quantitative characterization of one of the palindromes. The previously proposed non-homologous region origin of replication did not replicate in the assay.

INTRODUCTION

The *Baculoviridae* is a family of invertebrate viruses with large circular, covalently closed, double-stranded DNA genomes (80–188 kb) which are packaged into nucleocapsids. The nucleocapsids are enveloped and embedded in proteinaceous occlusion bodies (OBs). There are two genera of baculoviruses, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Theilmann et al., 2005). The GVs and the NPVs show major differences, not only in the morphology of their OBs but also in their infectivity, tissue specificity and the cytopathology associated with their replication. The NPVs have large polyhedral OBs with diameters of between 0.15 and 15 μm, with single or multiple virions embedded (Theilmann et al., 2005). The GVs have small ovoid OBs which average approximately 0.13 × 0.50 μm and normally contain a single virion (Theilmann et al., 2005). As a group, NPVs have a wider host range than GVs, being isolated mainly from the order Lepidoptera but also from the orders Diptera and Hymenoptera (Theilmann et al., 2005). To date, GVs have been isolated only from lepidopteran larvae (Theilmann et al., 2005).

Within the NPVs, sequences have been identified that function as origins of replication (ori) when cloned into plasmids and transfected into infected cells. In some NPVs, these also function as enhancers of early gene transcription (Cochran & Faulkner, 1983; Guarino & Summers, 1986; Theilmann & Stewart, 1992; Kool et al., 1995; Xie et al., 1995). These regions, called homologous regions (hrs), typically contain one or more copies of an imperfect palindrome sequence and are located in several positions on the genome. They have been identified in most NPVs sequenced to date and found to act as oris in several NPVs using infection-dependent DNA replication assays (Pearson et al., 1992; Pearson & Rohlmann, 1995; Ahrens et al., 1995; Xie et al., 1995; Broer et al., 1998).

In addition, complex structures containing multiple direct and inverted repeats of up to 4000 bp have also been identified that act as oris. These have similar structural characteristics to eukaryotic oris and are only found once per genome; they are therefore called non-bris (Pearson et al., 1993; Kool et al., 1994; Heldens et al., 1997; Habib & Hasnain, 2000; Huang & Levin, 2001). The discovery that serial passage of *Autographa californica* NPV (AcMNPV) results in the appearance of defective interfering particles (DIs), containing a non-ori, is further evidence that non-bris may be involved in genome replication (Kool et al., 1991; Pijlman et al., 2001). A similar finding was reported by Pijlman et al. (2002) for *Spodoptera exigua* NPV (SeMNPV) in Se-301 cells. It has also been suggested that the expansion of non-ori-like regions is not restricted to DI particles. It was found that a 900 bp region...
within the non-\textit{hr} \textit{ori}-like region of \textit{Cryptophlebia leucotreta} GV (CrleGV) was amplified in submolar populations in CrleGV \textit{in vivo}, which resulted in the expansion of the hypervariable region (Jehle, 2002).

Until now, characterization of putative GV \textit{hrs} has not been possible due to the lack of GV-susceptible cell lines. Several GV genome sequences are now available and putative \textit{hrs} and non-\textit{hrs} have been predicted based on the presence of repeated sequences. However, there is little similarity between putative GV \textit{hrs} to date. Some contain palindromic cores, whereas others do not. Sizes also vary from a palindromic singleton of 72 bp in \textit{Adoxophyes orana} GV (AdorGV) (Wormleaton et al., 2003) to large elements of up to 2383 bp in \textit{Plutella xylostella} GV (PlxyGV) (Hashimoto et al., 2000). The only shared feature between some of the putative \textit{hrs} in GVs is their relative positions within the genome. For example, there is often a putative \textit{hr} between the \textit{desmoplakin} and \textit{lef}-3 genes and also between sod and \textit{p74}.

Thirteen imperfect palindromes of 74–76 bp, with similarity to each other, have been identified in the CpGV genome (Luque et al., 2001). Each palindrome appears as a singleton, not occurring as multiple tandem repeats like a genome (Luque et al., 2001). This was a 1.13 kbp region of repeated DNA within the \textit{Pstl-I} fragment [map units (m.u.) 16.0–17.8]. This region includes an AT-rich section comprising six short imperfect direct repeats, followed by three large imperfect direct repeats (Luque et al., 2001).

A method generally used for identifying sequences capable of initiating plasmid replication in infected cells is based on the \textit{DpnI} assay (Pearson et al., 1992). \textit{DpnI} is a restriction enzyme that cleaves the methylated restriction site GATC (Geier & Modrich, 1979). The \textit{DpnI} sites of bacterially produced plasmid DNA are fully methylated and are thus cleaved by \textit{DpnI}. Due to the lack of the appropriate enzymic machinery, \textit{DpnI} sites in DNA replicated in eukaryotic cells are not methylated and are not digested by \textit{DpnI}. Therefore, \textit{DpnI} can be used to differentiate between input-transfected plasmid DNA and plasmid DNA which has replicated in the eukaryotic cells (Peden et al., 1980).

In this paper, the putative origins of replication of a GV are characterized for the first time, using a unique cell line permissive to CpGV infection. A real-time PCR method was developed to eliminate the need for Southern blotting and hybridization and to allow the quantification and comparison of plasmids containing different fragments of CpGV DNA.

### METHODS

#### Virus and cells.

CpGV-M1 (a cloned Mexican isolate) was used (Crook et al., 1997). The \textit{Cydia pomonella} cell line used (Cp14R) was derived from Cp14 cells (Winstanley & Crook, 1993). The cells were propagated in IZD04 medium supplemented with 10% fetal bovine serum (Winstanley & Crook, 1993).

#### Cloning CpGV fragments and PCR products.

A plasmid library covering the entire CpGV genome was produced by cloning specific CpGV restriction fragments into the pBlueScript SK (+) vector (pBSK+) (Stratagene). PCR products were also cloned to bridge adjacent fragments. The 156 bp palindrome-containing fragments and their deleted derivatives over 100 bp were also produced by PCR. Fragments under 100 bp were produced by annealing complementary custom oligonucleotides, by heating to 100 °C for 10 min and cooling to room temperature. The resulting DNA fragments were then cloned as above. Plasmids were amplified in library-efficiency \textit{Escherichia coli} DH5\textit{x} cells (Invitrogen). The inserts were confirmed by restriction endonuclease mapping or sequencing. The cosmids used were made by Crook et al. (1997).

#### Replication assay.

Cp14R cells were seeded at a density of 4 × 10\textsuperscript{5} viable cells per well in six-well cell culture plates. These were incubated at 27 °C for 48 h to allow the cells to attach. The cells were then transfected with plasmid DNA using Cellfectin (Invitrogen) according to the manufacturer’s recommendations. For transfections, 2 μg pBSK+ was used and the amounts of other plasmid DNAs were adjusted to reflect equimolar amounts relative to pBSK+. Three hours later, the transfection mixture was replaced with complete medium. Twenty-four hours later the cells were inoculated with CpGV using an m.o.i. of 0.2. Four hours later the infectious inoculum was replaced with complete medium. After incubation at 27 °C for 5 days, total cell DNA was purified from each well as follows: cells were harvested and centrifuged at 7000 g for 2 min to form a pellet. The supernatant was removed, leaving approximately 20–50 μl behind. The cells were then resuspended and 300 μl cell lysis solution (10 mM Tris, pH 8.0; 0.1 M EDTA; 0.5% SDS) was added to each sample. The cell lysate was then treated with 10 μg RNase A for 5 min at 37 °C. Samples were then cooled on ice for 1 min and 100 μl 10 M ammonium acetate was added to precipitate the proteins. The samples were vortexed vigorously and then centrifuged at 13 000 g for 2 min. The supernatant containing the DNA was precipitated with 2-propanol, washed with 70% ethanol and resuspended overnight in 50 μl TE (10mM Tris, pH 8.0; 1 mM EDTA). An ND-1000 spectrophotometer (NanoDrop) was used to quantify the DNA. Duplicate samples (20 μl) of each DNA were digested with HindIII (10 U) with or without \textit{DpnI} (40 U), in a total volume of 35 μl at 37 °C overnight. They were then treated with 5 μg RNase A for 10 min at 37 °C. To ensure that \textit{DpnI} digestion was complete under these conditions, 20 μl infected cell DNA was mixed with 100 ng plasmid DNA and digested as the other samples. Data from different experiments were normalized with respect to replication levels of a construct containing palindrome 7 (156 bp).

#### Gel electrophoresis, Southern blot and hybridization.

The fragments of digested DNA were separated on 0.7% agarose gels. After separation, the gels were depurinated with 0.25 N HCl for 10 min, denatured in 1.5 M NaCl, 0.5 M NaOH for 30 min, neutralized in 3 M NaCl, 0.5 M Tris pH 7.5 and then blotted onto nylon membrane (Roche). Prehybridization and hybridization reactions were performed using denatured digoxigenin (DIG)-labelled pBSK+ probe, following the manufacturer’s recommendations (DIG luminescent detection kit, Roche).

#### Real-time PCR.

The digested DNA from the replication assay was used in the following real-time PCR method. This method again relied on the \textit{DpnI} resistance of DNA replicated in eukaryotic cells. A
A standard curve of a fivefold dilution of pBSK+ containing a DpnI site. Any plasmid DNA that replicated in the insect (eukaryotic) cells would be resistant to DpnI digestion and produce a PCR product, whereas any bacterially produced DNA would be digested, resulting in no PCR product. As well as distinguishing replicated DNA from non-replicated DNA, this also provided quantifiable data. The sequences of the forward (F) and reverse (R) primers were as follows: pBSK F, 5’-CGACGCGGAGATCAAGTGAAT-3’; and pBSK R, 5’-CGGCGTTCGCGGATCATT-3’.

A standard curve of a fivefold dilution of pBSK+ was made from 2 ng µl⁻¹ down to 0.2 pg µl⁻¹. Approximately 500 ng total cell DNA, digested with either HindIII and DpnI or HindIII alone, was used in the real-time PCR using the SYBR Green PCR master mix (Applied Biosystems). This gave plasmid DNA concentrations within the range of 36–134 ng µl⁻¹ DNA per reaction. Each reaction was set up in triplicate in a 384-well plate with the following components: 2x SYBR Green PCR master mix (10 µl), 1 µM forward primer, 1 µM reverse primer, standard curve DNA or approximately 500 ng digested total cell DNA and water added to 20 µl. Real-time PCR was carried out using the ABI Prism 7900 HT sequence detection system (Applied Biosystems) with standard Taqman cycling conditions (40 cycles of 95 °C for 15 s followed by 1 min at 60 °C). An average of the triplicate results was taken. The quantities of DNA obtained were converted to a ‘percentage replication’ based on the amount of DNA detected from the HindIII- and DpnI-digested DNA (DNA that had replicated in insect cells), divided by the amount of DNA detected which had been digested with HindIII alone (total amount of plasmid DNA).

RESULTS

Optimization of the DpnI assay

The DpnI assay was optimized for the Cp14R cells. These cells take longer to attach and grow than Spodoptera frugiperda (Sf) cells and m.o.i. that can be achieved with CpGV in Cp14R cells are much lower than those achievable in the AcMNPV/Sf system (Winstanley & Crook, 1993). A range of m.o.i. was used to determine the minimum m.o.i. which could be used in this assay. This range was found to be 0.2 (data not shown). An m.o.i. above 0.2 did not result in any increased plasmid replication. The time to collect the cells for the assay was found to be optimal at 5 days post-inoculation.

Preliminary DpnI assays

The first plasmids used in the DpnI assay were SalI-F (m.u. 39.7–45.6) and PstI-J (m.u. 16.0–17.8). The plasmid SalI-F contained one of the 13 CpGV palindromes (pal-7). Using the DpnI assay for CpGV in Cp14R cells and the hybridization technique, SalI-F was found to replicate (Fig. 1). This suggested that the palindrome in SalI-F was a putative ori. We also found that PstI-J, which contains the putative non-hr ori, did not replicate, nor did the larger fragment SalI-B also containing the putative non-hr ori (Fig. 2a, Supplementary Table S1a, available in JGV Online). This suggested that this highly repetitive region of DNA did not act as an ori in this system. The replicating plasmid SalI-F was subcloned into five 1–2 kbp overlapping subclones and the assay was repeated. The subclone containing the palindrome (SalI-F subclone 2) was the only plasmid to replicate.

CpGV genome screening

The entire genome of CpGV was screened for the presence of putative oris using either hybridization or real-time PCR (Figs 2, 3). All seven cosmids in an overlapping library that covers the CpGV genome were found to replicate. These were subcloned into 32 plasmids and 12 were found to replicate in the assay (Fig. 2a, Supplementary Table S1a, available in JGV Online). These were in turn subcloned into 1.4–2.1 kbp fragments (Fig. 2b, Supplementary Table S1b). The resulting 11 fragments that replicated all contained at least one of the 13 single-copy 74–76 bp palindromes previously identified in the CpGV genome as putative oris (Luque et al., 2001) (Fig. 4). The 13 palindromes were each cloned as 156 bp fragments into pBSK+. All of these were used in the DpnI assay and were shown to replicate (Fig. 5). The genome screening confirmed that these 13 palindromes were the only oris in the CpGV genome.

Deletion analysis of palindrome 7

Deletion analysis was used to determine the minimal length of palindrome 7 required to act as an ori. PCR products and oligonucleotides ranging from 156 bp down to the 16 bp core of the palindrome were cloned, with truncations of 10 bp from each end of the palindrome-containing
Fig. 2. Restriction map of CpGV-M1. (a) Cosmid and plasmid library used in transient replication assay. The plasmid library consisted predominantly of cloned restriction endonuclease fragments and also of cloned PCR products which bridged adjacent fragments. Dark-shaded areas show plasmids and cosmids that replicated in the transient replication assay and hatched areas show the plasmids which did not replicate. (b) Position of the 13 palindromes and Pst-I repeated region.

Fig. 3. Real-time PCR assays. pal-7 (156 bp) was used as a standard in each experiment and the relative replication of each plasmid was adjusted according to the standard having a replication of 1. Cp14R cells were transfected with recombinant subcloned plasmids from the CpGV library and with pBSK+. BF, BamHI-F; KG, KpnI-G; SE, SalI-E; SL, SalI-L; ED, EcoRI-D; XD, XhoI-D and SF, SalI-F. Standard error bars are included for the plasmids which were used in triplicate.

Fig. 4. ClustalW alignment (using default parameters) of the 13 CpGV imperfect palindromes. Black shading, 100% identity; dark-grey shading, 80% identity; light-grey shading, 60% identity.
fragment. These were then screened for the presence of putative origins of DNA replication using the hybridization and real-time PCR methods. The entire experiment was repeated in triplicate and the results are shown in Fig. 6. The real-time PCR results show that there was no detectable replication above background (5%) if the palindrome was less than 96 bp long (Fig. 6a). Background was set at 5%, since several preliminary experiments showed there was a small amount of DpnI-resistant DNA (0.7–4.3%) present in all samples, including the negative control of pBSK+. This amount of resistant DNA was not detectable on autoradiographs, possibly due to the plasmid being cleaved successfully by DpnI elsewhere. Autoradiographs of the same data showed a very faint band for the 76 bp palindrome, suggesting that some true replication occurs, which is indistinguishable from background using real-time PCR (Fig. 6b). The entire 74–76 bp palindrome was therefore required for any DNA replication. Replication increased with the size of the palindrome-containing fragment, up to the 1.4 kbp fragment SalI-F subclone 2 (SF2). SF2 replicated to a similar extent as the entire 7.2 kbp SalI-F fragment. The optimal size of the palindrome is therefore between 156 bp and 1.4 kbp.

**Concatenation of replicated plasmids**

Partial digestion of the replicated DNA with a restriction enzyme that cut the input plasmid at a unique site (HindIII) led to the production of a stepladder pattern of fragments (Fig. 7). This is consistent with the DNA being organized as a linear concatemer containing multiple copies of the plasmid. This has also been found with NPV hr-containing plasmids and implies involvement of a rolling-circle mechanism during replication (Leisy & Rohrmann, 1993; Xie et al., 1995).

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**Fig. 5.** Transient replication assay. Cp14R cells were transfected with recombinant plasmids containing each of the 13 CpGV palindromes (1–13) and with pBSK+. Mock-infected cells (mi) were included as controls. The + or - sign below the lanes indicates whether the sample was digested with HindIII (-) or both HindIII and DpnI (+). CpGV+/− indicates whether the cells were infected with CpGV.

**Fig. 6.** Infection-dependent replication assay of deletion clones of palindrome 7. Cp14R cells were transfected with recombinant plasmids containing deletion clones of palindrome 7 and with pBSK+. The number indicates the length of the cloned palindrome. SalI-F subclone 2 (SF2) and SalI-F (SF) were also included. Mock-infected cells (mi) were included as controls. (a) Real-time PCR assay. The dashed line indicates the 5% background cut-off. The assay was performed in triplicate and standard error bars are included. (b) Transient replication assay. The + or - sign below the lanes indicates whether the sample was digested with HindIII (-) or both HindIII and DpnI (+). CpGV+/− indicates whether the cells were infected with CpGV.
investigation is required to determine the requirement for the specific nucleotides within the CpGV hrs.

The CpGV hr regions all consist of single palindromes. Deletion mutagenesis of AcMNPV hr5, which contains six palindromes, indicated that the efficiency of replication of an individual hr was dependent on the number of palindromes present (Pearson et al., 1992). The relative efficiency of replication of a particular hr-containing plasmid increases as the number of palindromes present in that hr increases (Pearson et al., 1992; Leisy et al., 1995). However, the relative level of replication for different AcMNPV hrs appears to be independent of the number of palindromes (Leisy & Rohrmann, 1993). Most of the CpGV plasmids used in the assays contained just one palindrome, except for BamHI-F subclone 5 (BF5), which contained two (palindromes 3 and 4). There was no increase in the amount of replication compared with the plasmids containing one palindrome (Fig. 3). However, a direct comparison to either palindrome 3 or 4 has not been made. The fact that CpGV maintains the palindromes predominantly as singletons and that these sequences have not evolved into multimers suggests that an increased number of palindromes in each hr may not give any replicative advantage to CpGV. More detailed experiments are required to determine the effect of multiple CpGV palindromes.

Although the amount of replication of the CpGV plasmids is quantifiable and comparable, there appears to be relatively little replication of plasmids compared with NPV systems. The amount of replicated plasmid DNA, shown in autoradiographs in NPV assays, usually far exceeded the total amount of DNA from the unreplicated plasmids, even though equimolar amounts were transfected (Pearson et al., 1992, 1993; Pearson & Rohrmann, 1995; Heldens et al., 1997). During the studies with CpGV, we found that the total amount of replicated DNA appeared similar to the total amount of replicated DNA in trans to CpGV. More detailed experiments are required to determine the effect of multiple CpGV palindromes.

**DISCUSSION**

In this paper we located the oris of CpGV, and introduced a real-time PCR method to quantify replicated DNA. Thirteen imperfect palindromes of 74–76 bp, previously identified in the CpGV genome (Luque et al., 2001), were all found to replicate in the infection-dependent DNA replication assay. No other region of DNA in the genome was found to replicate, indicating that these 13 palindromes are the only oris present. The major repeat region in CpGV, which is reminiscent of a non-hr-type ori, was found not to replicate in this assay. The role of this repeat region is yet to be revealed.

Truncations of palindrome 7 showed that a minimum of the entire 76 bp palindrome is required for replication. The amount of replication increased with the size of the fragment and was highest between 156 bp and 1.4 kbp. There is no homology between the CpGV DNA flanking the palindromes, but these specific flanking regions are obviously required for optimal replication.

Previously, investigations using plasmid constructs of AcMNPV hr1a indicated that the central four nucleotides of the core of the palindrome are essential for ori function (Leisy et al., 1995). CpGV palindromes show dyad symmetry throughout the palindrome but are mainly conserved at the ends in the first and last 7 nt (Fig. 6). However, they all have a 20 bp AT-rich core. Further

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**Fig. 7.** Transient replication assay showing concatenation of plasmid DNA. Cp14R cells were transfected with a recombinant plasmid of palindrome 7 and with pBSK+. DNA was partially digested with HindIII. The + or – sign below the lanes indicates whether the sample was digested with DpnI. CpGV +/- indicates whether the cells were infected with CpGV.
(Viswanathan et al., 2003). However, it was not functional as an ori sequence in these cells. A host cell protein has been identified from Spodoptera frugiperda cells (SF9), hr1-BP, which binds very specifically to the AcMNPV hr1 palindromic plus the flanking sequence (Habib & Hasnain, 1996). This protein is required for the enhancer function of hr1. Putative CpGV early genes have been identified based purely on their promoter DNA sequence and homology to NPV genes. An ie-1 gene is present in CpGV but is poorly conserved (27% amino acid identity to AcMNPV IE-1). However, ie-1 genes are not well conserved among baculoviruses in general. Further characterization of CpGV early genes is necessary and studies are required to establish if CpGV hrs act as enhancers of transcription of early genes. If CpGV hrs can act as enhancers of transcription, there may be similar host proteins involved.

The consensus of the 13 CpGV palindromes showed 82% dyad symmetry. The palindromes may have the potential to form secondary structures, such as cruciform (stem-loop or hairpin) structures, through intra-strand base pairing. This may allow initiation of DNA replication via the binding of specific protein factors. Palindromes (or inverted repeats) are a common feature of prokaryotic and eukaryotic control regions, including oris (Pearson et al., 1996). They have been shown to be functionally important for the initiation of DNA replication in plasmids, bacteria, eukaryotic viruses and mammalian cells (Pearson et al., 1996). However, not all palindromes function necessarily as cruciforms. They may act at the RNA level as terminators or attenuators (Pearson et al., 1996). They may also remain in the linear state as DNA, acting as binding sites for protein dimers. This appears to be the case for AcMNPV hr5 palindrome with the regulatory protein IE-1. DNA-binding and antibody-supershift assays indicated that IE-1 bound to a single hr5 palindrome as a dimer (Rodems & Friesen, 1995). It has also been suggested that cruciform formation does not have a direct role in IE-1 binding, as IE-1 did not bind to a single palindrome when annealed under conditions conducive to hairpin formation (Rasmussen et al., 1996).

The origin regions in the herpes simplex virus (HSV) genome all contain AT-rich palindromes with the capacity to form cruciforms (Weller et al., 1985; Lockshon & Galloway, 1986). However, one palindrome (oris) seems to be required in the linear, rather than cruciform, conformation, in the initiation of HSV replication (Lockshon & Galloway, 1988; Deb & Doelberg, 1988). Cruciform structures and easily unwound DNA sequences have also been detected at Epstein–Barr virus oris (Williams & Kowalski, 1993). However, the consensus imperfect palindrome of AcMNPV hr regions did not form a cruciform in vitro, unlike a synthesized perfect palindrome (Rasmussen et al., 1996). It is still unclear whether the palindromes in baculovirus hrs form cruciforms in vivo. However, the formation of cruciforms at hr regions may facilitate local base-pair melting, thereby assisting the process of baculovirus transcription and replication.

With more sequencing data now available, comparisons of the sequences of closely related baculoviruses or different genotypes of the same virus have been made. These have revealed that most differences in the DNA sequence, such as rearrangements, insertions and deletions, are located near hrs. The major differences between Orgyia pseudotsugata NPV (OpMNPV) and AcMNPV are detected near all OpMNPV hrs (Possee & Rohrmann, 1997). The alignment between AcMNPV and Rachiplusia ou multiple NPV (RoMNPV) hrs contains multiple gaps, suggesting that numerous deletions and insertions have occurred in the regions between the palindromic repeats of the hrs (Harrison & Bonning, 2003). This has also been observed between the hrs of Mamestra configurata NPV (McNPV) isolates 90/2 and 96B (Li et al., 2002), and also between the hrs of Helicoverpa armigera single NPV (HaSNPV) and Helicoverpa zea single NPV (HzSNPV) (Chen et al., 2002).

The GV genomes sequenced to date share a higher degree of gene collinearity than NPV genomes (Lange & Jehle, 2003; Escasa et al., 2006), therefore there are no obvious translocations which can be attributed to the position of hrs. CpGV-M1 is the only CpGV genotype to be completely sequenced. However, a region of the Russian genotype CpGV-R3 has been sequenced (Wormleaton, 2000). The Russian CpGV isolate has a 2.45 kbp deletion compared with CpGV-M1 (Crook et al., 1985). The deletion was found to be located 711 bp from palindrome 7 (Wormleaton, 2000). Furthermore, within these 711 bp are multiple insertions and deletions of 1–60 bp. Another example of a rearrangement occurring near a CpGV palindrome is the insertion of the 4.7 kbp transposon TCI4.7 which originated from the Cryptophlebia leucotreta genome. This was found in the mutant CpGV-MCp5, inserted in the 90 bp between palindromes 3 and 4 (Jehle et al., 1995). These observations in CpGV support previous suggestions that hrs are sites of frequent recombination and rearrangement in baculovirus genomes, and that this is also the case for GV genomes. This is the first paper reporting functional oris in a GV. We have demonstrated that the 13 imperfect palindromes within the CpGV genome replicate in an infection-dependent assay and are therefore probably oris. No other area of DNA in the CpGV genome was found to replicate, including the non-hr ori-like region. The entire 76 bp palindrome was needed for any replication, with flanking regions enhancing replication. The CpGV hrs differ from NPV hrs in that they consist of a single palindrome and do not contain direct repeats. Consequently, they are more difficult to detect within the genome sequence. However, as more GV genomes are sequenced, more putative GV hrs may be identified which have characteristics similar to the CpGV hrs.

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