Homologous recombination between the inverted terminal repeats of defective transposon TCp3.2 causes an inversion in the genome of *Cydia pomonella* granulovirus

Hugo M. Arends and Johannes A. Jehle

State Education and Research Center for Agriculture, Viticulture and Horticulture (SLFA), Biotechnological Crop Protection, Breitenweg 71, 67435 Neustadt/Weinstr., Germany

In this study, a new mutant of the *Cydia pomonella* granulovirus (CpGV), which shows spontaneous inversion of a transposable element during in vivo replication, is described. CpGV-MCp4 is a natural mutant of CpGV-M, containing the transposable element TCp3.2, which originated from the genome of the host *C. pomonella*. During in vivo cloning studies of CpGV-MCp4, a mutant called CpGV-MCp4inv was isolated. CpGV-MCp4inv shows heterogeneity in the genome area of transposon insertion. Restriction mapping, PCR analysis and subsequent sequence analysis gave strong evidence that an inversion of TCp3.2 is caused by homologous recombination between the long inverted terminal repeats (ITRs) of the transposon. This finding demonstrated that extensive homologous repeat regions such as the ITRs of transposons cause inversions by homologous recombination during in vivo replication. The observed in vivo inversion between the ITRs can be considered as a model for the contribution of repeated sequences in the genome rearrangement of baculoviruses and a source for genetic heterogeneity among different baculoviruses and baculovirus genotypes.

**Introduction**

*Cydia pomonella* granulovirus (CpGV) is a baculovirus that is highly pathogenic for the codling moth (*C. pomonella*) (Crook, 1991). CpGV belongs to the family *Baculoviridae* and has a double-stranded circular DNA genome. The complete genome of CpGV-M1 (cloned Mexican isolate) was recently sequenced and has a genome size of 123 500 bp (Luque et al., 2001). Other characterized isolates, e.g. CpGV-E (English isolate) and CpGV-R (Russian isolate), show only small genotypic differences compared to CpGV-M (Crook et al., 1985, 1997; Harvey & Volkman, 1983).

Recently, two mutants of CpGV-M (CpGV-MCp4 and CpGV-MCp5) were isolated by in vivo cloning from the susceptible hosts *C. pomonella* and *Cryptophlebia leucotreta* (Jehle et al., 1995, 1998). Both mutants harbour insect host transposable elements that horizontally escaped into the CpGV-M genome. CpGV-MCp5 contains transposon TC4.7, which originated from the genome of *C. leucotreta*. TC4.7 is 4.7 kb long, has 29 bp inverted terminal repeats (ITRs) and encodes a defective putative transposase gene. CpGV-MCp4 harbours transposon TCp3.2, which originated from the genome of *C. pomonella*. TCp3.2 is 3.2 kb long, has ITRs of 756 bp and also has a defective transposase gene due to a frame shift mutation within its open reading frame (ORF). Both transposons belong to the superfamily of *Tc1*/mariner-like transposable elements and are integrated at a TA dinucleotide; this is typical for this transposon family (Radice et al., 1994; Robertson, 1995; Doak et al., 1994). Both transposons stably integrated into the genome of CpGV and were not lost during further passages. TCp3.2 integrated into a non-coding region between two ORFs, lef-2 and ORF35Ra or ORF41 and ORF42, as described in Luque et al. (2001) and Jehle et al. (1997). The TA integration site is part of the putative TATA box promoter sequence of the possibly early-transcribed gene ORF35Ra. The biological activity of CpGV-MCp4 is similar to that of CpGV-M; the lethal concentrations of both mutants are not significantly different viruses (Jehle et al., 1995).

In this study, we report the spontaneous inversion of the transposable element TCp3.2 and provide evidence that this was caused by homologous recombination between the ITRs within the genome of CpGV-MCp4. To our knowledge, this is the first report of an inversion of a genomic region within a baculovirus by homologous recombination under in vivo conditions. Recently, genomic sequencing of baculovirus
genomes provided evidence that recombination between homologous regions (hrs) and homologous genes may contribute to genome rearrangements during baculovirus evolution. The observed recombination between the ITR sequences is a useful model to study the sequence requirements and the frequency of recombination-dependent rearrangements of baculovirus genomes.

**Methods**

**Virus and larval stock.** Viruses used in this study derived from an *in vivo* cloned genotype of *C. pomonella* granulovirus (CpGV-M) (Tanada, 1964). Insertion mutant CpGV-MCp4 was isolated from an infection experiment of *C. leucotreta* larvae with CpGV-M and CrleGV-CV3 and a subsequent *in vivo* cloning procedure in *C. pomonella* larvae, as described previously by Jehle et al. (1995).

CpGV-MCp4inv was isolated after *in vivo* cloning of CpGV-MCp4 in the fifth instar *C. pomonella* larvae using the limiting dilution method described by Smith & Crook (1988).

**Virus and DNA isolation.** Infected fifth instar *C. pomonella* larvae were ground in 0.05 M Na2CO3 at 37 °C for 30 min. The solution was neutralized to pH 8.0 with 1 M HCl and was subsequently treated with 45 μg/ml RNase A at 37 °C for 10 min. To disrupt the virions, the solution was incubated in 1% SDS, filtered through miracloth and centrifuged at 10000 g for 15 min. The virus band was recovered and washed twice in distilled water.

DNA was purified by dissolving occlusion bodies in 0.05 M Na2CO3 – 0.0 M Na2CO3 at 37 °C for 30 min. The solution was neutralized to pH 8.0 with 1 M HCl and subsequently treated with 45 μg/ml RNase A at 37 °C for 10 min. To disrupt the virions, the solution was incubated in 1% SDS for 1 h at 37 °C and treated with 250 μg/ml proteinase K. The solution was extracted twice with a TE-saturated phenol:chloroform:isoamyl alcohol mixture (25:24:1 (v/v/v)), then once with a chloroform:isoamyl alcohol mixture (24:1 (v/v)) and dialysed against TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0).

**Restriction analysis.** Virus DNA (0.6 μg) was digested with restriction enzymes as recommended by the supplier (Gibco BRL). The restriction fragments were separated on a 0.8% TAE agarose gel using a current of 5 mA/cm for 16 h. The gel was then stained with 0.5 μg/ml ethidium bromide.

**PCR analysis for the orientation of the transposon.** The ITRs and bordering regions of transposon TCP3.2 were amplified using 0.1 ng of a mixture of CpGV-MCp4 and CpGV-MCp4inv genomic DNA. PCR was performed under standard conditions using recombinant Taq DNA polymerase (Gibco BRL). The following primers were used: LB (left border), 5′ TTAGTACAGTAGATTTAGTTCT; LI (left internal), 5′ AGGTTATTGGTGTGTTCT; RB (right border), 5′ TGGTGACGAGGGGACAGAAG; and RI (right internal), 5′ AGACCCGAATTAAGACCATCAGAAG. After 30 cycles of amplification, each cycle comprising 1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C, the PCR products were separated on a 1% TBE agarose gel.

**Cloning and sequencing of the ITRs.** The ITRs of CpGV-MCp4inv were amplified by PCR using the primer combinations LB and RI for the left ITR and RB and LI for the right ITR. PCR products were cloned in *Escherichia coli* using the pGEM-T Easy Vector system (Promega). Plasmid DNA was prepared using the NucleoSpin kit (Machery–Nagel) and sequenced using dye terminator cycle sequencing kit (Promega). The position of the *HindIII* restriction site within the *BglII* restriction fragment E (Jehle et al., 1997; Luque et al., 2001). During further *in vivo* cloning of CpGV-MCp4, a mutant genotype, named...
Inversion of transposon TCp3.2 in CpGV

Inversion of transposon TCp3.2 in CpGV-MCp4

In order to determine whether transposon TCp3.2 inverted, a PCR test was developed to detect the orientation of TCp3.2 in CpGV-MCp4inv. Based on known sequences of CpGV-MCp4, PCR primer pairs were designed to specifically amplify the left and right border sequences for each orientation of the transposon. If transposon TCp3.2 had inverted, it was expected that the exchange of primer combinations used to amplify the CpGV-MCp4 border sequences would also result in positive PCR reactions.

As shown in Fig. 3, the corresponding PCR analyses produced specific fragments of sizes that were expected arithmetically for both genotypes. This result proved that transposon TCp3.2 had inverted within the genome of CpGV-MCp4.

TCp3.2 inverted by homologous recombination between the ITRs

From the previous results, it was not clear which mechanism was involved in the inversion of the transposon. Since TCp3.2
Fig. 4. Recombination model of TCp3.2 in CpGV-MCp4 resulting in the inversion of TCp3.2 in CpGV-MCp4inv. As indicated by sequence analysis of the PCR products obtained using CpGV border-specific primers (LB or RB) and internal transposon-specific primers (LI or RI), the difference between the two ITRs of CpGV-MCp4 and CpGV-MCp4inv is restricted to the two most internal mismatching nucleotides. The positions and orientations of the PCR primers, LB, LI, RI and RB, are indicated as line arrows. The ITRs and the putative transposase gene are indicated as open box arrows and grey box arrows, respectively. Mismatching nucleotides (residues 1–7) are indicated by the numbering at the top and the positions (bp) at the bottom.

is a mobile element, it could be excised from the CpGV-MCp4 genome and then inserted again in an inverted orientation. Though the TCp3.2 copy in CpGV-MCp4 encodes a defective transposase gene, the transposon could have been transactivated by a functional transposase encoded by another TCp3.2 copy present in the host genome. Alternatively, the inversion could have been caused by internal homologous recombination between the ITRs of the transposon. The final result of these possible events is not the same: after an excision-mediated inversion, the entire transposon inverts, whereas after recombination-mediated inversion, some central part may invert, leaving the terminal ends of the ITRs at their original location.

The left and right ITRs of TCp3.2 are 756 bp long and mismatch in seven positions dispersed through the ITR (Fig. 4). The position of these ITR-specific nucleotides in CpGV-MCp4inv allowed us to determine whether the complete transposon or only its central parts inverted during replication. In the case of an excision-mediated complete inversion, it was expected that all ITR-specific nucleotides would change their position. For a recombination-mediated internal inversion, it was expected that at least a few of the specific nucleotides in MCp4inv would keep their original position.

In order to identify the potential mechanism involved in the inversion of TCp3.2, the ITRs in CpGV-MCp4inv were sequenced and compared with those in CpGV-MCp4. The CpGV-MCp4inv left and right ITRs were amplified by PCR using primers LB and RI and RB and LI (Fig. 4), cloned and sequenced. As shown in Fig. 4, the only differences between the sequences of the ITRs of CpGV-MCp4 and CpGV-MCp4inv lies in the two most internal-specific nucleotides of the ITR sequence. In CpGV-MCp4inv, the CpGV-MCp4-specific residues A$^\text{173}$ and A$^\text{2811}$ were replaced with T$^\text{173}$ and C$^\text{2811}$, which are the inverse complements of A$^\text{2587}$ and C$^\text{2587}$. On the other hand, CpGV-MCp4-specific residues A$^\text{2811}$ and C$^\text{2587}$ of the right ITR were replaced in CpGV-MCp4inv with T$^\text{2811}$ and T$^\text{2587}$, which again are the inverse complements of A$^\text{173}$ and A$^\text{597}$.

These analyses demonstrated that only the central part of the transposon (not the entire transposon), including the putative transposase gene and parts of the ITRs, inverted. Since ITR-specific nucleotides (those at positions 1–5) did not change their position, the homologous recombination most probably took place between sequences located between the fifth and the sixth mismatching nucleotides of the ITRs (Fig. 4).

**Discussion**

In this study, the spontaneous inversion of the mobile genetic element TCp3.2 in the genome of CpGV was characterized. TCp3.2 changed its orientation during the replication of CpGV in larvae of the codling moth. These analyses provide clear evidence that the inversion of TCp3.2 is caused by homologous recombination between the extended ITR sequences of 756 bp, which flank the transposon on the left- and right-hand side rather than by an excision-mediated inversion.

To our knowledge, this is the first report that demonstrates transposon inversion in a baculovirus genome under *in vivo* conditions using infected larvae. Inversion of a transposable element in a baculovirus genome was described by Martin & Weber (1997) using an *in vitro* assay. In this study, it was...
demonstrated that a pair of inverted repeat IS50 elements (derived from the bacterial transposon Tn5), which were cloned into the polyhedrin gene locus of Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV), inverted by homologous recombination between its IS50 repeats. They suggested that these recombination events were strictly dependent upon AcMNPV-mediated DNA replication and required a minimum of 300 bp identity within the ITR. Regarding the minimum length of identity required for homologous recombination, the in vivo experiments of this study are in good agreement with the in vitro results of Martin & Weber (1997). TCP3.2 has ITRs of 756 bp in length that are inverted, whereas inversion was never observed for transposon TCI4.7, which contains only 29 bp long ITRs.

The integration of a transposable element in a baculovirus genome can be an additional source of genetic heterogeneity. Theoretically, a baculovirus genome could harbour two, or even more, transposon copies. Homologous recombination between these copies could lead to inversions or deletions of genomic regions between the transposable elements. Transposons are also known to contain high-affinity binding sites for transcription factors. Integration of a transposable element in a baculovirus genome is generally thought to increase the frequency and sequence requirement for recombination.

Insertions of host transposable elements into baculovirus genomes are an occasional event. However, the observed recombination-mediated inversion between the transposon-specific ITRs might serve as a model for the involvement and contribution of any repeated sequence to baculovirus genome rearrangements. In general, baculovirus genomes are characterized by the presence of repeated ORFs (e.g. hr genes) and hrs (Kuzio et al., 1993; Crook, 1997). The potential involvement of baculovirus bro genes or hrs in intragenomic recombination has been suggested in earlier studies. Analyses of the distribution of bro genes in different strains of Bombyx mori NPV (BmNPV) indicate that intraspecific recombination between these genes causes an active redistribution of sequences within the genome of BmNPV (Lopez Ferber et al., 2001). Comparative analyses of the genomes of the closely related group I NPVs, AcMNPV, BmNPV and Orgyia pseudotsugata MNPV (OpMNPV) demonstrated that the organization of ORFs and hrs was generally conserved. A few dissimilarities due to inversions of genomic regions between these baculovirus genomes are observed. The OpMNPV ORF1–10 region has an inverse orientation compared to the hrs in BmNPV (ORF130–135 and 1–3) and AcMNPV (ORF1, 2, 4–6 and 8–10). Also, BmNPV ORF24 (fgf) has an inverse orientation compared to its homologues in AcMNPV (ORF32) and OpMNPV (ORF27). Interestingly, both inverted regions are flanked by hrs: AcMNPV ORF1–10 are flanked by hr1 and hr1a and ORF24 of BmNPV is flanked by hr2-L and hr2-R (Ayres et al., 1994; Ahrens et al., 1997; Gomi et al., 1999). Another example of inversion of a genetic region in the virus Tellus is found for Helicoverpa armigera single nucleopolyhedris NPV (HaSNPV) ORF61–67. This HaSNPV genomic region is flanked by hr3 on its left-hand side and has an opposite orientation compared to hrs in Spodoptera exigua MNPV (ORF88–93), AcMNPV (ORF65–71) and BmNPV (ORF53–58) (Chen et al., 2001; IJkel et al., 1999).

This study demonstrates that recombination between homologous ITRs can result in spontaneous inversions within the CpGV genome during in vivo infection. These results provide further evidence that the presence of extended homologous sequences, such as long ITRs or hrs and repeated genes, may play a significant role for genome rearrangement by homologous recombination within baculovirus genomes. The CpGV genome appears to lack extended hr sequences and has only a limited number of repeated genes (Luque et al., 2001). Therefore, the mutant CpGV-MCP4 carrying the transposon TCP3.2 appears to be a useful model to study the frequency and sequence requirement for recombination-dependent inversion and genome rearrangement in CpGV.

This work was supported by a grant of the Deutsche Forschungsgemeinschaft (JE 245/2-2).

References


Received 14 December 2001; Accepted 25 March 2002