"Molecular and biological analysis on the horizontal transfer of insect transposons in Baculoviruses"

Dissertation

zur Erlangung des Grades

"Doktor der

Naturwissenschaften"

am Fachbereich Biologie

der Johannes Gutenberg-Universität

in Mainz

Hugo Martijn Arends

geb. in Utrecht

Mainz, 2004

Decan:

1. Berichterstatter:

2. Berichterstatter:

Tag der mündlichen Prüfung: 29-01-2004

CONTENTS

1. INTRODUCTION 6

1.1. Baculoviruses 6
1.2. Classification and taxonomy of baculoviruses 7
1.3. Baculovirus infection cycle 8
1.4. Gene expression and DNA replication 10
1.5. Transposons 10
1.6. Transposons in nucleopolyhedroviruses 12
1.7. Transposons in granuloviruses 13
1.8. Impact of transposable elements on baculovirus genomes 15
1.9. Are baculoviruses involved in the horizontal transfer of insect transposons? 15
1.10. Aims of the project and outline of the thesis 17

2. MATERIALS AND METHODS 19

2.1. Materials 19
2.1.1. Chemicals and laboratory materials 19
2.1.2. Laboratory equipment 19
2.1.3. Computer programs 20
2.1.4. Virus genotypes 20
2.1.5. Insects 20
2.1.6. Plasmids and bacterial strains 21
2.1.7. Primers 21
2.1.8. Media 22
2.1.8.1. Semi-synthetic insect medium 22
2.1.8.2. Luria Bertani (LB)-medium and LB-agar 23
2.2. Methods 23
2.2.1. Insects and insect cells 23
2.2.1.1. Maintenance of CpDW14R cells 23
2.2.1.2. Preparation of BV from hemolymph 23
2.2.1.3. Infection of *C. pomonella* larvae with BV 24
2.2.1.4. *In vivo* cloning of a virus genotype 24
2.2.1.5. Virus stock production 24

2.2.1.6. Virus capsule purification from larvae 25
2.2.1.7. Virus OB counting 25
2.2.1.8. Bioassays 25
2.2.2. Methods in molecular biology 26
2.2.2.1. Isolation of DNA from occlusion bodies 26
2.2.2.2. Preparation of genomic insect DNA from larvae 26
2.2.2.3. Digestion and agarose gel electrophoresis of DNA 27
2.2.2.4. Mini preparation of plasmid DNA 27
2.2.2.5. Polymerase chain reaction (PCR) 29
2.2.2.6. Sequencing 289
2.2.2.7. Total RNA isolation from insect tissue using TRIzol reagent (Gibco/BRL) 29
2.2.2.8. First strand cDNA preparation 30
2.2.2.9. Quantitative (real time) RT-PCR 30
2.2.2.10. RNA (glyoxal) electrophoresis 31
2.2.2.11. Transfer of DNA/RNA to Hybond-N+ membrane 32
2.2.2.12. Prehybridisation and hybridisation 33
2.2.2.13. Radioactive labelling of DNA hybridisation probe 34
2.2.2.14. DIG-labelling of strand-specific RNA probes 34
2.2.2.15. SDS-PAGE 34
2.2.2.16. Semi-dry blotting 35
2.2.2.17. Immuno detection 36

3. RESULTS 37

3.1. Sequencing of the integration site of TCl4.7 in CpGV-M 37
3.2. Comparison of the CpGV-M1 sequence with the obtained sequences of CpGV-M 40
3.3. Preparation of MCp4, MCp5 and CpGV-M stocks 41
3.4. Isolation and restriction analysis of the spontaneous mutant MCp4inv 43
3.5. Inversion of transposon TCp3.2 in MCp4 44
3.6 TCp3.2 inverted by homologous recombination between the ITRs 45
3.7. Median lethal dose and survival time determination 47
3.8. Virus offspring production 48
3.9. Quantification of the CpGV-M:mutant ratio in the virus offspring. 48
3.10. Competition experiments between CpGV-M and MCp4, and CpGV-M and MCp5 51

3.11. TCp3.2 copies in *C. pomonella* cell lines 57
3.12. Characterisation of the transposase transcripts of transposon TCp3.2 57
3.13. Quantification of transposase transcription..... 60
3.14. Transposase transcription in CpDW14R 64

4. DISCUSSION 66

4.1. The TCl4.7 integration site in CpGV..... 66
4.2. Inversion of transposon TCp3.2..... 67
4.3. Biological characterisation of MCp4 and MCp5 69
4.4. Transposase t32a transcription..... 71
4.5 Risk assessment with regard to transgenes in baculoviruses. 76
4.6 Genetic transformation of insects using transposable elements..... 76

5. REFERENCES 79

SUMMARY..... 93
ZUSAMMENFASSUNG..... 95
DANKWOORD 97
CURRICULUM VITAE .....**Fehler! Textmarke nicht definiert.**
PUBLICATIONS 98

1. INTRODUCTION

1.1. Baculoviruses

Over one million insect species make up more than 80 % of all existing animal species. Insects fulfil numerous functions which make them critically important to the ecosystem. In nature, insect populations are kept in balance by many different factors including viral infections (Fig. 1.1). The effects of insect viruses can vary from minor effects on the health of its host to morbidity in insect populations. Baculoviruses can have very striking consequences on population levels in that they can cause widespread epizootics (Miller, 1996).

(a)  (b) 

Figure 1.1. (a) Healthy and **(b)** CpGV infected larvae of the codling moth, *Cydia pomonella*.

Baculoviruses have been isolated from about 700 arthropod species, primarily from insects of the order of *Lepidoptera* (butterflies and moths), but also from *Hymenoptera* (sawflies) and *Diptera* (flies and mosquitoes) (Blissard *et al.*, 2000). Since in most cases the host range of baculoviruses is restricted to one or a few closely related insect species, there is a considerable interest in these viruses as highly specific and effective bio-pesticides in agriculture and forestry. As pest control agents, baculoviruses do not leave harmful residues in the environment and resistance against the viruses is not observed in the field. Another advantage of these viruses is that they can persist in susceptible insect populations leading to a dynamic control of the pest. The baculovirus used in this project, *Cydia pomonella* granulovirus, is commercially available in many European countries as a bio-pesticide for the control of the codling moth *Cydia pomonella* (Fig. 1.2).

Figure 1.2. The codling moth *Cydia pomonella*.

1.2. Classification and taxonomy of baculoviruses

The genome of baculoviruses (*baculoviridae*) is a large double-stranded supercoiled DNA molecule of 80 to 180 kbp which encodes about 100-200 proteins (Blissard *et al*., 2000). The name of the virus refers to the rod-shaped (*baculum*) nucleocapsids of the virus particles (virion), which are usually 30-60 nm in diameter and 250-300 nm in length (Blissard *et al*., 2000). A unique feature of baculoviruses is the formation of large occlusion bodies (OB) in which the virions are embedded. The crystalline matrix of the OB mainly consists of a 29 kDa protein called polyhedrin or granulin which protects the infectious virion outside the host from physical decay. Corresponding to the morphology of the OB, the family of baculoviruses is divided in two genera, the nucleopolyhedroviruses (NPV) and granuloviruses (GV) (Fig. 1.3a). The NPV produce large (0.15-15 µm) polyhedron shaped OB that contain many virions. The NPV are morphologically divided in two types, SNPV and MNPV, depending on the single (S) or multiple (M) packaging of the nucleocapsids in the virions. The GV have smaller (0.3-0.5 µm) OB called granules that normally contain one virion (Fig. 1.3b).

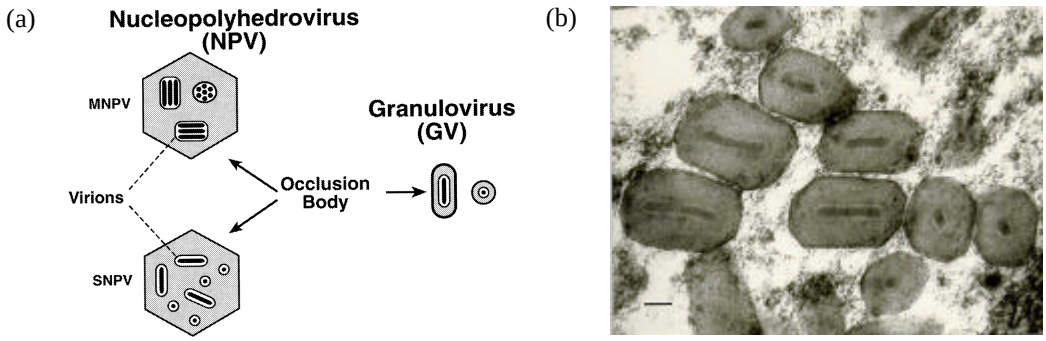


Figure 1.3. (a) Structures of occlusion bodies of nucleopolyhedroviruses and granuloviruses. (modified after Blissard *et al*. (2000)). **(b)** Electron microscopic dissection of a CpGV infected *C. pomonella* cell. Virus occlusion bodies with virions are clearly visible. (Photo: Dr. A. Huger, BBA Darmstadt).

For NPV and GV, two different virion phenotypes are involved in a baculovirus infection cycle, the budded virions (BV) and the occlusion derived virions (ODV). The ODV phenotype is embedded in the polyhedrin matrix of the OB and is needed for the infection in the insect gut epithelium. The second virion genotype, the BV, are generated in a earlier phase of the infection cycle. Its role is to spread the infection from cell to cell within the insect. The nucleocapsids of BV and ODV appear to be identical, the difference between the virion phenotypes is the composition of the envelopes and the associated structures. NPV and GV are not only distinguished by their morphological characteristics, but also in phylogenetic analyses based on sequence data, they represent two well separated clades. Based on phylogenetic data, NPV are

divided in group I and group II NPV (Zanotto *et al*., 1993; Bulach *et al*., 1999; Herniou *et al*., 2001).

One of the best studied GV is the *C. pomonella* granulovirus (CpGV). The complete genome of CpGV-M1 (cloned Mexican isolate) was recently sequenced: it has a size of 123500 bp and contains 143 open reading frames (ORFs) (Luque *et al*., 2001). Other characterised 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).

1.3. Baculovirus infection cycle

For the transmission from insect to insect, baculoviruses have different strategies. The virus can be transmitted vertically within the egg or horizontally via food or on the surface of eggs (Blissard *et al*., 2000). The infection and replication cycle of *Autographa californica* MNPV has been studied intensively and can serve as a model for other baculoviruses. A baculovirus infection starts after ingestion of the OB by an insect larva (Fig. 1.4). OB are commonly present on plant surfaces and in the soil. The OB quickly dissolve in the alkaline environment of the midgut and the ODV are released. The ODV are involved in the primary infection, the passage of the midgut epithelial cells. The first barrier to cross for the ODV is the peritrophic membrane (PM). How the passage takes place is not fully understood yet. In some host insects the pores in the PM are big enough to allow penetration of the virion. In other cases baculoviruses produce enzymes (enhancin) that are capable to create lesions through which virions can pass (Gijzen *et al*., 1995; Hashimoto *et al*., 1991). Nucleocapsids enter the midgut epithelial cells by fusion of the ODV envelope with the microvilliar membrane (Horton & Burand, 1993; Granados & Lawler, 1981). The nucleocapsids are transported to the cell nucleus where transcription of viral genes and replication of the viral genome takes place. The newly formed nucleocapsids are then transported to the basal side of the cell where they bud through the membrane. The now formed second virion phenotype (BV) is responsible for the systemic infection of the insect. Via the hemolymph and the tracheal system, the BV are efficiently spread to other susceptible insect tissue (Granados & Lawler, 1981; Engelhard *et al*., 1994). In the early stage of the secondary infection, infected cells produce BV which subsequently infect other cells. Late in the secondary infection phase, the production of offspring virions is switched from the BV to the ODV phenotype. The ODV are subsequently embedded in a crystalline matrix and OB are formed. At the end of the infection cycle the insect cells and tissues disintegrate and the OB are released in the environment. The disintegration

process is executed by virus encoded proteins like chitinase and cathepsin (Hawtin *et al*., 1995; Slack *et al*., 1995). A further infection cycle can start when the OB are ingested by another susceptible insect. Depending on the baculovirus and the host, the infection cycle requires between 5 and 30 days.

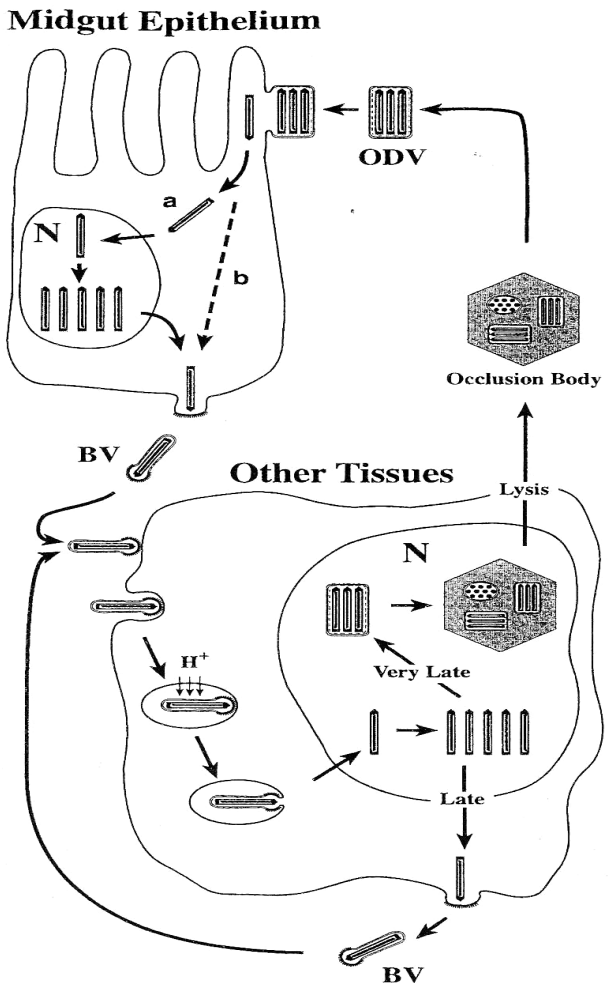


Figure 1.4. The baculovirus infection cycle (modified after Blissard, 1996). **(Top panel)** As illustrated, the infection of the midgut epithelium cells starts with the fusion of the ODV with the plasma membrane. Two possible routes of the nucleocapsids through the ME are indicated (a and b). In route a, which is probably the typical route, the nucleocapsids are first transported to the nucleus where gene expression, DNA replication and the assembly of progeny nucleocapsids takes place. The newly synthesised nucleocapsids are then transported to the basal side of the epithelial cell. In route b, the nucleocapsids bypass the nucleus and migrate to the plasma membrane at the basal side of the epithelium cell where they bud from the cell and acquire a plasma membrane envelope containing fusion proteins. **(Bottom panel)** The BV require this fusion protein for an efficient systemic (secondary) infection. The BV bind to the cells of other tissue and enter by endocytosis. After uptake into the endosome, the acidification triggers the fusion of the viral and endosomal membrane. The released nucleocapsids are then transported from the cytoplasm to the nucleus where gene expression, DNA replication and assembly of progeny nucleocapsids occur. During the early phase of the secondary infection, nucleocapsids bud through the plasma membrane to form the BV which can infect new cells. Late in the secondary infection the ODV are synthesised and are subsequently embedded in a crystalline matrix of polyhedrin to form the OB. After the lysis of the cell, the OB are released into the environment and a new round of infection in another insect can start.

1.4. Gene expression and DNA replication

Baculovirus gene expression is regulated in a cascade-like manner in which each successive phase is dependent on the previous one (Blissard & Rohrmann, 1990). Baculovirus genes are divided into 3 groups, early, late and very late genes, according to their phase of expression. The early genes are transcribed by the host RNA polymerase II and contain transcription motifs which are very similar to initiator motifs of arthropods (Friesen, 1997). Promotors can be categorised as TATA-containing (TATA+), initiator-containing (INR+), or composite (TATA+, INR+) promoters (Novina & Roy, 1996). The (TATA+) promoters contain a 5'-TATAA-3' recognition motif where the transcription complex is assembled. By the (INR+) promoter the transcription is initiated within a conserved 5'-ATCA(G/T)T(C/T)-3' sequence. Most conserved among the early baculovirus genes is the (TATA+, INR+) promoter containing tetranucleotide 5'-CAGT-3' about 20-30 bp downstream from 5'-TATA-3'. In contrast to the early genes, the late genes require other viral encoded proteins for their transcription. Late genes are transcribed by a viral encoded RNA polymerase which recognises a highly conserved 5'-(A/T/G)TAAG-3' motif. For the transcription of the late genes approximately 19 virus-encoded proteins known as late expression factors (LEF) are necessary (Lu *et al*., 1997). The occlusion phase in the viral infection is characterised by hyper-expression of the very late genes *polyhedrin* (or *granulin*) and *p10* (Vlak & Rohrmann, 1985). These two very late genes are not essential for the DNA replication and the BV production. By replacing these genes for genes of interest, it is possible to obtain very high levels of foreign gene expression in insect cell cultures (Smith *et al*., 1983; Martens *et al*., 1995).

Characteristic for baculovirus genomes is the presence of homologous repeat (*hr*) regions distributed around the genome (Cochran & Faulkner, 1983; Majima *et al*., 1993). Functional studies have demonstrated that *hrs* serve as enhancers of early gene transcription (Theilmann & Stewart, 1992; Guarino & Summers, 1996) and as putative origins of DNA replication (Pearson *et al*., 1992; Ahrens *et al*., 1995; Kool *et al*., 1995).

1.5. Transposons

Transposons are DNA sequences that are able to move from one genomic location to another. Since their discovery by Barbara McClintock, transposons have been detected in all organisms in which they have been studied. Depending on the species, 3 to 50 % of the genome consist of transposon sequences or are directly derived from them (Smit, 1999). In the late 1970s

transposons were described as "genomic parasites" or "selfish DNA" since the genomic information carried by transposons is primarily used for self-multiplication without direct benefit to the host (Doolittle & Sapienza, 1980; Orgel & Crick, 1980). At individual level, the mutations introduced by transposons can be deleterious to the organism which contain them. On the other hand, transposons play an important role by lateral gene transfer and by rearranging existing sequences, which are important genetic mechanisms for the evolution of genomes (Britten *et al*., 1997; Capy *et al*., 2000; Miller *et al*., 1997).

Depending on their transposition strategy, mobile elements can be divided into two broad categories, RNA-based transposons (retrotransposons) and DNA-based ones (Berg & Howe, 1989). Retrotransposons are characterised by the presence of long terminal repeats and encode a reverse transcriptase. Retrotransposons move through RNA intermediates and utilise reverse transcription to insert into new genomic locations. Examples of this type of transposons are the *Ty* elements of yeast (Klingsman *et al*., 1988) and the *copia* (Mount *et al*., 1985) and the *gypsy* elements (Rubin, 1983) of *Drosophila*. DNA transposons are characterised by inverted terminal repeats and encode a transposase. There are two main transposition strategies within the group of DNA transposons: conservative and replicative transposition. By conserved transposition the transposon is not duplicated, but is cleaved from the donor site and is integrated into a new genomic location (cut and paste). Examples of this type of transposons are the *P (Rio et al., 1990)*, *hobo* and the *FB* elements (Rubin, 1983) of *Drosophila*, the *Tc1* element of *Caenorhabditis elegans* (Rosenzweig *et al*., 1983) and elements of the mariner family (Robertson and MacLeod, 1993). In replicative transposition the element is copied by a DNA polymerase, one copy remains at the original site, while the other is inserted at the new genomic location. The bacterial elements *Mu* and *Tn3* (Gill *et al*., 1978) perform this kind of transposition.

Transposons can be divided into two functional categories: autonomous and non-autonomous. Autonomous elements are defined as those that can perform the transposition reaction on their own. The activity of non-autonomous elements is dependent upon functions encoded by autonomous elements. In many cases non-autonomous elements are deletion derivatives of autonomous elements (Berg & Howe, 1989). An example is the *Ac-Ds* system in maize: for its transposition the defective *Ds* element is dependent on the transposase encoded by the *Ac* (activator) element (Federoff *et al*., 1989).

1.6. Transposons in nucleopolyhedroviruses

Lepidopteran transposons have also been identified in the genomes of several NPV following serial passage of the virus in insect cell lines (Table 1.1) (for reviews see Friesen, 1993; Jehle, 1996). These transposons were identified when FP (few polyhedra) plaque morphology mutants of AcMNPV and *Galleria mellonella* MNPV (GmMNPV) were analysed. Most of these mutants contained transposon insertions within a gene coding for a late expressed 25-kDa protein (ORF61=Ac61, Ayres *et al*., 1994). This protein appears to have a regulatory role in switching from BV to ODV production during the biphasic viral replication cycle (Beames & Summers, 1989; Harrison & Summers, 1995). Disruption of this gene leads to an increased BV production and a decreased formation of ODV (Jarvis *et al*., 1992). FP mutants seem to have a propagation advantage in insects cell cultures since BV are much more infective for insect cells than ODV (Volkman & Keddie, 1990). Due to this replication advantage, the FP mutants are selected in the virus population under the specific conditions of *in vitro* replication in cell cultures.

Table 1.1. Comparison between different baculovirus transposons (modified after Jehle (1996)).

Transposon	Origin	Length (bp)	Target site	Inverted terminal repeat	Terminal triplet	Open Reading frame	Ref.
Retrotransposons							
TED (AcMNPV)	*T. ni*	7510	AATG	273[1]	-	+	1,2
DNA-transposons							
hitchhiker (GmMNPV)	*T. ni*	579	TTA	39	GGG/CCC	-	3
piggyBac (GmMNPV)	*T. ni*	2746	TTAA	13 + 17	CCC/GGG	+	4
TFP3 (AcMNPV)	*T. ni*	782	TTAA	15	CCC/GGG	-	5
TFP3 (GmMNPV)	*T. ni*	830	TTAA	15	CCC/GGG	-	5
IFP2.2 (AcMNPV)	*S. frugiperda*	2164	GTTTTTC	-	-	?	6
IFP1.6 (AcMNPV	*S. frugiperda*	1565	TTAA	14	CCT/AGG	?	6
E (AcMNPV)	*S. frugiperda*	630	TTAA	14	CCT/AGG	-	7
M5 (AcMNPV)	*S. frugiperda*	290	TTAA	13	CCG/CGG	-	8
TCl4.7 (CpGV)	*C. leucotreta*	4736	TA	29	CAG/CTG	+[2]	9
TCp3.2 (CpGV)	*C. pomonella*	3239	TA	756	CAG/CTG	+[2]	10

[1]Long terminal repeat (LTR), [2]ORFs probably defective. (1) Miller & Miller, 1982; (2) Friesen & Nissen, 1990; (3) Bauser *et al*., 1996); (4) Cary *et al*., 1989; (5) Wang *et al*., 1989; (6) Beames & Summers, 1990; (7) Schetter *et al*., 1990; (8) Carstens, 1987; (9) Jehle *et al*., 1995; (10) Jehle *et al*., 1998.

The isolated AcMNPV and GmMNPV FP mutants contain insertions of about 9 different transposons. These elements vary in size from 290-2746 bp but share some common structural characteristics such as ITRs of 13-39 bp and insertion at a 4 bp TTAA target site (Beames & Summers, 1990). Except for *IFP2* (*piggyBac*), all of these transposons lack an open reading frame to encode a functional transposase. This indicates that most of the DNA-transposons represent non autonomous insertion elements that depend on the gene products of other transposon copies in the host genome (Fraser, 1986).

PiggyBac was successfully used for germ-line transformation of non-drosophilid insects (Handler *et al*., 1998; Tamura *et al*., 2000). In contrast to the other characterised transposons within the FP locus, *hitchhiker* inserted at a TTA target site. The fact that *hitchhiker* has different ITRs and target site specificity than TTAA specific elements, indicates that it is a member of another family of Lepidopteran transposons (Bauser *et al*., 1996). Another well characterised transposon found in a baculovirus genome is retrotransposon *TED* in AcMNPV (Friesen & Nissen, 1990; Miller & Miller, 1982). *TED* is derived from the genome of the cabbage looper *Trichoplusia ni* and is a member of the *gypsy* retrotransposons (Friesen & Nissen, 1990). It spontaneously inserted at 86.7 m.u. of AcMNPV where it disrupted a non essential early gene (Ac94) and altered transcription within the region (Friesen *et al*., 1986). *TED* is 7.5 kbp long, contains 273 bp long direct terminal repeats and three retrovirus-like genes. A capsid protein and an active reverse transcriptase are encoded by *gag* and *pol* (Hajek & Friesen, 1998) and the *env*-like ORF encodes a membrane-bound glycoprotein with retroviral-like properties required for infection (Ozers & Friesen, 1996). Thus, *TED* also exhibits the properties of a retrovirus.

1.7. Transposons in granuloviruses

Recently performed experiments demonstrated that the horizontal transfer of host transposons to baculoviruses is not restricted to the artificial conditions of insect cell cultures but also occurs during infection of insect larvae (Jehle *et al*., 1995). Transposon carrying viruses were isolated from a mixed infection of *Cryptophlebia leucotreta* larvae with CpGV and *Cryptophlebia leucotreta* granulovirus (CrleGV) followed by the isolation of single virus genotypes from the virus progeny in larvae of *C. leucotreta* and *C. pomonella* using an *in vivo* cloning procedure (Jehle *et al*., 1995). Out of 194 *in vivo* cloned CpGV and CrleGV isolates, two mutants were identified which carried insect host transposons that horizontally escaped into the genome of CpGV-M (Fig. 1.5). DNA restriction analysis and sequencing revealed that the transposon in

CpGV mutant MCp5 is 4726 bp long, has inverted terminal repeats of 29 bp and encodes a defective putative transposase gene. This transposon, termed TCl4.7, hybridised to middle repetitive sequences of the genomic DNA of *C. leucotreta.* This indicated that TCl4.7 inserted into the genome of CpGV during the mixed infection in *C. leucotreta* larvae. The other CpGV mutant, termed MCp4, harbours transposon TCp3.2 which is 3239 bp long, has 756 bp long inverted terminal repeats (ITRs) and encodes an intron containing transposase gene which is defective due to a frame shift mutation within its open reading frame (ORF) (Jehle *et al*., 1998). Southern blot hybridisation studies demonstrated that TCp3.2 originated from the genome of *C. pomonella*. The genome of *C. pomonella* harbours about 10 copies of this transposon (Jehle *et al*., 1998). TCp3.2 as well as TCl4.7 belong to the superfamily of *Tc1/mariner*-like transposable elements (Radice *et al*., 1994; Robertson, 1995; Doak *et al*., 1994). This group of transposons is characterised by amino acid sequence homology within the transposase gene (D,D(35)E motif), duplication of a TA target site and inverted terminal repeats ranging from 20-460 bp. They are present in an exceedingly wide variety of genomes including fungi, nematodes, insects, fish and human beings.

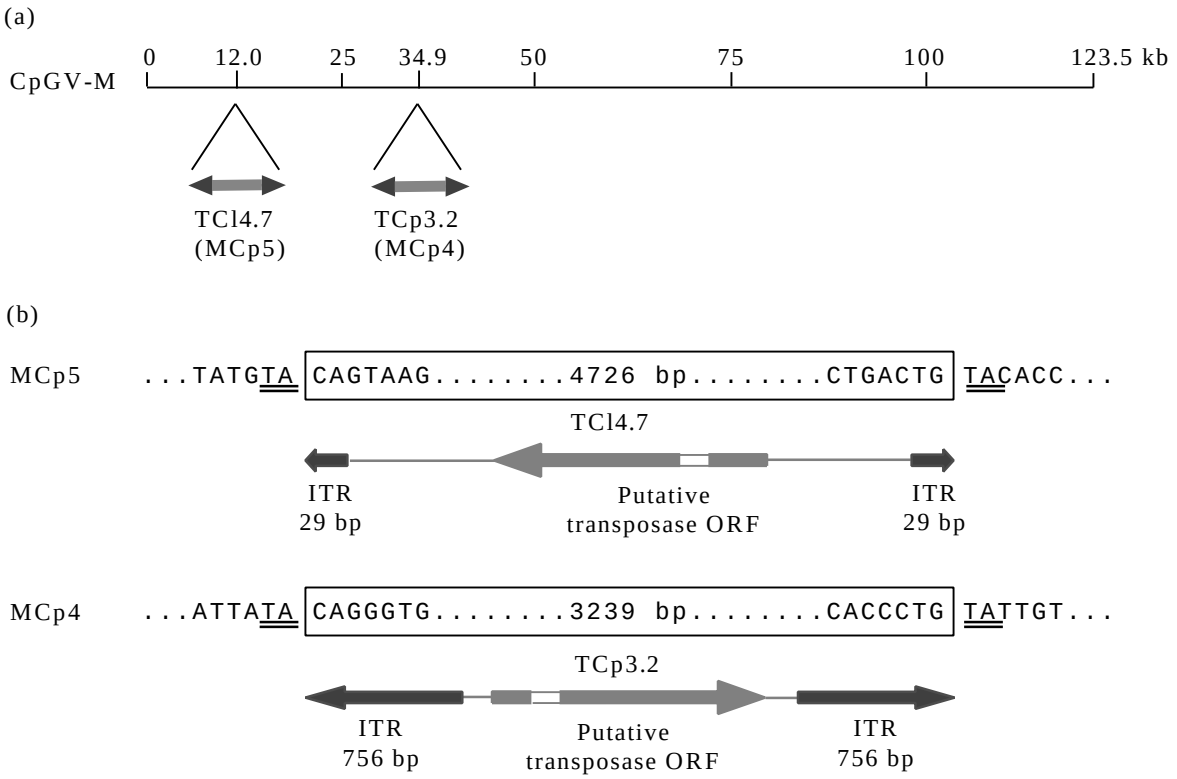


Figure 1.5. Integration sites of TCl4.7 and TCp3.2 into the genome of CpGV **(a)** and a schematic representation of both transposons **(b)**. The duplicated TA target sites are twice underlined. The transposon sequences within MCp5 and MCp4 are boxed. (kb= kilo bases).

The integration site of TCp3.2 is located in a non-translated region downstream of late expression factor 2 (*lef2*) (= Cp41 according to Luque *et al*. (2001)) and upstream of ORF35Ra (= Cp42) (Jehle *et al*., 1997). *Lef2* is a conserved baculovirus gene which is present in all sequenced baculoviruses and is essential for DNA replication (Hayakawa *et al*., 2000; Lu & Miller, 1995; Kool *et al*., 1994). Cp42 is possibly an early transcribed gene with an unknown function. The integration of TCp3.2 occurred at a TA dinucleotide which is part of a putative TATA-box in the promoter region of Cp42. Cp42 is a not conserved gene among the baculoviruses sequenced to date, only *Xestia c-nigrum* granulovirus (XecnGV) and CrleGV have a homologue (Hayakawa *et al*., 1999; Lange & Jehle, unpublished). Both transposons stabily integrated into the genome of CpGV and were not lost during further passages.

1.8. Impact of transposable elements on baculovirus genomes

The integration of host transposons has an impact on the integrity of baculovirus genomes. Depending on the integration site, the effect of transposon insertion can vary from an advantageous to a silent or even a devastating mutation. Disruption of an essential ORF or its regulatory sequence can lead to a non-viable virus mutant, whereas the virus may gain novel genes or regulatory sequences upon transposon integration in an intergenic region. A good example for the gain of novel genes is the insertion of retrotransposon *TED* into the genome of AcMNPV. This transposon introduced three functional genes (*pol, gag* and *env*) into the viral genome (Friesen, 1993; 1996). In evolutionary terms, transposons may be beneficial to baculoviruses because of the acquisition of novel functions which could give the virus instruments to become more competitive. The horizontal transfer also has advantages for the transposon. It enables the element to escape from its host and to invade a new gene pool. Several authors have documented that these transposon-host interactions may have a significant influence on the viral genome and are thus expected to contribute to baculovirus evolution (Blissard & Rohrmann, 1990; Friesen, 1993; Fraser, 1996; Jehle, 1996).

1.9. Are baculoviruses involved in the horizontal transfer of insect transposons?

Analysis of different eucaryotic transposon families have shown that their distribution among species does not follow the phylogeny of the host species. Transposons with high sequence similarity are found in taxonomically distantly related host species (Capy *et al*., 1994). These observations strongly support the concept that transposons are not only transmitted vertically from

generation to generation, but are also able to cross species boundaries (Hurst *et al*., 1992; Kidwell, 1993). There is strong evidence that DNA transposons have been transferred horizontally. The clearest cases of this are the *P* and *mariner* transposable elements of *Drosophila* (Daniels *et al*., 1990; Robertson, 1993; Hagemann *et al*., 1996). Evidence that the more numerous and widely distributed retrotransposons may also be transferred horizontally between species has been more ambiguous. Just recently, it was clearly demonstrated that a long LTR retrotransposon called *copia* has been transferred from *Drosophila willistoni* to *Drosophila melanogaster* within the last 200 years (Jordan *et al*., 1999). The ability of RNA mediated elements to undergo horizontal transfer might be limited by their dependence on host factors. For its replication, LTR retrotransposons are dependent on tRNA priming by the host (Voytas & Boeke, 1993). *In vitro* and *in vivo* studies have proven that *Tc1/mariner*-like elements function autonomously independent of species-specific host factors (Lampe, 1996; Vos *et al*., 1996). This enables these elements to be functional in a broad host range which is essential for horizontal transfer.

Despite the clear evidence that a horizontal transfer of transposons between insect species exists, the possible mechanism involved remains obscure. Different hypotheses to explain this phenomenon have been postulated, but experimental proof is still lacking. In insects, many parasites or pathogens with a broad host range could act as a transfer vector of transposons between species. Phylogenetically closely related strains of the bacteria *Wolbachia* are found in the parasitoid wasp *Nasonia giraulti* and its blowfly host *Protocalliphora* (Werren, 1997). This finding suggests intertaxon transmission between parasites and their hosts as a possible exchange mechanism. A plausible candidate for horizontal transfer in *Drosophila* could be the parasitic wasp *Leptopilina boulardi* which has several hosts (Dupas, 1996). Such parasites may act as a natural syringe transferring genetic material from one individual to another. A potential vector for the transfer of the *P* element between *D. willistoni* and *D. melanogaster* is the mite *Proctolaelaps regalis* (Houck *et al*., 1991).

Baculoviruses are serious candidates to shuttle transposons between insect species (Miller & Miller, 1982; Blissard & Rohrmann, 1990; Friesen, 1993). Beside the fact that many baculoviruses have different hosts, several facets of the their replication strategy and genome organisation permit the spontaneous accommodation of transposons (Jehle *et al*., 1998). Firstly, replication of the double stranded viral DNA genome takes place within the host nucleus which allows the virus and insect DNA to come in close contact giving the transposon a potential chance for horizontal escape (Granados & Williams, 1986). Secondly, baculoviruses are flexible with

regard to the size of genomic DNA packaged during virion assembly. Thirdly, baculoviruses contain non-essential genomic regions where foreign DNA can integrate (Friesen, 1993). Finally, baculoviruses do not necessarily kill their host, they can have abortive infections in non-permissive hosts without complete virus replication (Bilimoria, 1991). Sublethal infections are also reported (Sait *et al*., 1994). This allows the transposon to move from a baculovirus genome back to an insect genome.

1.10. Aims of the project and outline of the thesis

The horizontal transfer of endogenous Lepidopteran transposons into baculovirus genomes occurs during the infection of insect cell cultures and of insect larvae. In this study experiments were performed in order to obtain a better understanding of the rarely observed phenomenon of horizontal transfer of transposons from insects to baculoviruses genomes during natural infections. This study focused on the further characterisation of the recently isolated host transposon harbouring CpGV mutants MCp4 and MCp5. Both mutants harbour insect host transposons that horizontally escaped into the CpGV-M genome. The project started with the characterisation of the integration site of transposon TCl4.7 within the CpGV genome. The DNA sequence of the integration site of TCl4.7 in CpGV-M is analysed and described in 3.1 and 3.2.

Although TCp3.2 stabily integrated into the genome of MCp4, the transposon caused further heterogeneity within the CpGV genome. *In vivo* cloning experiments with MCp4 resulted in the isolation of a new mutant with an altered restriction pattern. In order to identify the genomic changes, the mutant was characterised on molecular level which is described in 3.3-3.6.

Aside from the molecular characteristics, the biological features of the transposon harbouring viruses MCp4 and MCp5 were also determined. From FP mutants it is known that they are frequently selected under the specific conditions of *in vitro* replication. Hence it was investigated whether the selection of MCp4 and MCp5 was based on a possible advantage during *in vivo* replication (3.7-3.10).

Normally, the activity of endogenous transposons is strictly regulated in order to prevent genetic disorder. Different studies, however, showed that transposon activity can be stimulated by genomic stress factors including virus infections. A direct determination of the influence of virus infection on the transposition frequency of TCp3.2 is currently not possible because a suitable

transposition assay for this transposon is not available. Since it is expected that the activity of TCp3.2 depends on the expression level of an active transposase, as it is typical for *Tc1/mariner*-like transposons, the levels of transposase mRNA within the larvae is most probably a good measure for transposon activity. For this reason the transcription of the TCp3.2 transposase t32a in mock and CpGV-M infected larvae and cell line DW14R of *C. pomonella* was analysed in detail (3.11-3.14).

A better understanding of the mechanisms involved in horizontal transfer of insect transposons is not only of fundamental interest, but knowledge about this phenomenon also contributes to a better risk assessment concerning the safety of baculovirus insecticides. Since natural occurring baculoviruses and geneticaly modified baculoviruses play an increasingly important role as bioinsecticides in agriculture, data concerning the genetic stability of baculoviruses are important.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and laboratory materials

Erase-a-Base System	Promega
pGEM®-T Vector System	Promega
1 kb DNA ladder	Gibco/BRL
λ DNA/*Hin*dIII fragments	Gibco/BRL
BenchMark Protein Ladder	Gibco/BRL
Restriction endonucleases	Gibco/BRL
Taq DNA polymerase, recombinant	Gibco/BRL
DNase, Amplification Grade, RNaseOUT	Gibco/BRL
SuperScript RNase H^- Reverse Transcriptase	Gibco/BRL
QuantiTect SYBR Green	Qiagen
Oligo (dT)12-18 primer	Gibco/BRL
Specific PCR primers	MWG-Biotech AG.
T7-RNA polymerase, Sp6-RNA polymerase	Promega
TRIzol Reagents	Gibco/BRL
NucleoSpin® Extract kit, NucleoSpin® Plasmid kit	Macherey Nagel
Hybond-N+, Hybond-P	Amersham Pharmacia
Rediprime II random prime labelling system	Amersham Pharmacia
DIG luminescent detection kit	Roche
ECL Western blotting detection reagents	Amersham Pharmacia
X-ray Film Kodak X-OMAT™ AR	Kodak
Other chemicals	Roth

2.1.2. Laboratory equipment

Bio Rad mini protean II dual slab gel system	Bio-Rad
Semi-Dry-Transfer-Cell, Trans-Blot® SD	Bio-Rad
Intas gel documentation system	Intas

BioPhotometer	Eppendorf
Mastercycler gradient	Eppendorf
DNA Engine Opticon™ System	
PTC-200 DNA Engine™ Cycler	
CFD-3200 Opticon™ Detector	MJ Research

2.1.3. Computer programs

DNAstar version 4	Lasergene
Imagemaster 1D	Pharmacia Biotech
Opticon Monitor™ software	MJ Research
SAS software package	SAS-Institute

2.1.4. Virus genotypes

CpGV-M	(Tanada, 1964)
CpGV-MCp5 (MCp5)	(Jehle *et al*., 1995)
CpGV-MCp4 (MCp4)	(Jehle *et al*., 1995; 1998)
CpGV-MCp4inv (MCp4inv)	(Arends & Jehle, 2002)

2.1.5. Insects

Cydia pomonella larvae	Insect rearing at the Staatliche Lehr- und Forschungsanstalt (SLFA), Neustadt/Weinstrasse Insect rearing at Horticulture Research International (HRI), Wellesbourne, UK	
Cydia pomonella cell line	CpDW14 CpDW14R CpDW15	(Winstanley & Crook, 1993)

2.1.6. Plasmids and bacterial strains

pGEM®-5Zf(+)	Promega
pGEM®-7Zf(+)	Promega
pGEM®-T Vector	Promega
DH5α competent cells	Gibco/BRL

Genotype: F^-ϕ80d*lac*ZδM15 δ(*lac*ZYA-argF) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44λ^- *thi*-1 *gyr*A96 *rel*A1.

2.1.7. Primers

Overview of the used sequencing and PCR primers:

1. Universal sequencing primers:

Sp6 promoter primer	5'-GAT TTA GGT GAC ACT ATA G-3'
T7 promoter primer	5'-TAA TAC GAC TCA CTA TAG GG-3'
M13 forward primer	5'-GTT TTC CCA GTC ACG AC-3'
M13 reverse primer	5'-CAG GAA ACA GCT ATG AC-3'

2. PCR primers for the detection of MCp5, MCp4 and MCp4inv:

PR-MCp5-LB	5'-CTG GTT GGA TGT GGA GTA TGT A-3'
PR-TCl4.7-int	5'-TGC TTC GAC ACA ACA GAG ACA G-3'
PR-MCp4-LB (LB)	5'-TTA GTC AGG TGG ATG GGT TGG T-3'
PR-TCp3.2-int (LI)	5'-AGG TTC ATC TTT GCT GGG TTC T-3'
PR-MCp4-RB (RB)	5'-TGG TGA CGA GGG AGC AGA ATA G-3'
PR-MCp4-RI (RI)	5'-AGA CCC GAA TAA GAG CAT CAG AGA-3'

3. PCR primers to amplify the granulin gene of CpGV-M.

PR-Cp-up	5'-TCA GCT CAA CGC TCA ACT C-3'
PR-Cp-lo	5'-GAC AAA TTG TCA GTT CAC TAG T-3'

4. PCR primers to amplify the t32a transposase.

PR-t32a-lo	5'-GTG CAG CAA TAA ACG ACA AAA CC-3'
PR-t32a-up	5'-AGA CCC GAA TAA GAG CAT CAG AGA-3'

PR-RT-lo	5'-CGC AAA GAA TCT ACC AGG AAC-3'
PR-RT-up	5'-AGT CGT TAT CTT CAA GAC CTT GG-3'

5. Primers used to make a TCp3.2 hybridisation probe

PR-t32-up	5'-GAA GCA TTT GCG ATC TTA GTA-3'
PR-t32END	5'-AAG GAT CCG TAA GCT TTC AAG TTT-3'

2.1.8. Media

2.1.8.1. Semi-synthetic insect medium

The insects at the SLFA were reared on a semi-synthetic diet described by Ivaldi-Sender (1974), the insects at HRI were reared on a similar semi-synthetic diet described by Guennelon *et al*. (1981). The diet according to Ivaldi-Sender was prepared as follows:

Ingredients:

800 ml	bidestilled water
20 g	agar
50 g	corn meal (organic)
50 g	wheat germ (organic)
50 g	brewer’s yeast (organic)
6 g	ascorbic acid
2 g	benzoic acid
2 g	nipagin (hydroxybenzoic acid methyl ester)

Protocol:

- Dissolve nipagin and benzoic acid in some ethanol.
- Dissolve ascorbic acid in a few ml of water.
- Mix bidestilled water and agar and bring to boil in a microwave.
- Add the dissolved benzoic acid and nipagin, the corn meal, wheat germ and brewer's yeast and mix well.
- Wait until mixture has cooled to about 60-70°C before adding the dissolved ascorbic acid, mix again.
- Pour the medium in the rearing plates.

2.1.8.2. Luria Bertani (LB)-medium and LB-agar

For the growth of *E. coli*, LB-medium was used.

Ingredients:

5 g	Bacto Trypton
10 g	Bacto-yeast extract
5 g	NaCl
(15 g	agar)

Protocol:

- Dissolve in 1 liter of bidestilled water and adjust pH to 7.2-7.5 using HCl or NaOH.
- Sterilise by autoclaving for 20 minutes at 121°C.

Depending on the experiment, the following additives were added to the LB-media:

ampicilin	100 µg/ml	(stock solution 10 mg/ml in bidestilled water)
X-Gal	40 µg/ml	(stock solution 20 mg/ml in DMF)
IPTG	40 µg/ml	(stock solution 20 mg/ml in bidestilled water)

2.2. Methods

2.2.1. Insects and insect cells

2.2.1.1. Maintenance of CpDW14R cells

C. pomonella cells were maintained in IZD Lp 04 medium supplemented with 10% FBS (Winstanley and Crook, 1993). The cells were grown at 27°C and passaged every 7-10 days by scraping (split 1:4) with a medium change. The cells were maintained in an antibiotic-free medium except when they were infected with hemolymph. Then, antibiotics were added to produce a final concentration of 50 µg/ml streptomycin, 100 units/ml penicillin, 50 µg/ml gentamicin and 25 µg/ml amphotericin B.

2.2.1.2. Preparation of BV from hemolymph

Infected fifth instar *C. pomonella* were bled by amputating the second proleg four days post infection. The collected hemolymph was diluted 10 times in a serum-free tissue culture

medium in the presence of antibiotics and a small crystal of phenylthiocarbamide to prevent melanisation. The diluted hemolymph was filtered through a 0.45 µm filter and stored in small aliquots at -70°C. The determination of the $TCID_{50}$ titre was done as described by Winstanley & Crook (1993).

2.2.1.3. Infection of *C. pomonella* larvae with BV

Fifth instar *C. pomonella* were infected with the BV isolated from the hemolymph of *C. pomonella* larvae or from the tissue culture medium by injection in the hemolymph. Larvae were numbed with ether for two minutes, sterilised using a 0.4% hyamine solution and injected with 2 µl BV preparation in the second proleg using a micro-injector.

2.2.1.4. *In vivo* cloning of a virus genotype

In vivo cloning was done using the limiting dilution method described by Smith & Crook (1988). Fifth instar *C. pomonella* larvae were inoculated by feeding them a small piece of medium containing a LD_{10} of virus. Only the larvae that had completely ingested the medium within 24 hours were placed on fresh virus free medium and were reared individually at 26°C. Due to the low virus dose, only a small amount of the larvae died from the infection. Virus DNA was isolated from the larval cadaver (2.2.1.5) and the homogeneity of the isolated virus genotype was checked using restriction analysis. The procedure of *in vivo* cloning was repeated until a virus genotype was purified.

2.2.1.5. Virus stock production

In order to produce a virus stock, the viruses were propagated in fifth instar *C. pomonella*. The larvae were infected (as described in (2.2.1.4)) with a LD_{90} of an *in vivo* cloned virus. After larval death, virus from the collected larvae was isolated. DNA was isolated from a part of the obtained virus and the genotype of the virus was analysed using restriction analysis. After enumerating of the remaining virus (2.2.1.7), the virus stock was frozen in small aliquots at -20°C.

2.2.1.6. Virus capsule purification from larvae

For experiments like bioassays and virus DNA isolation it was critically important to have well purified OB. The purest OB could be obtained from *C. pomonella* and *C. leucotreta* cadavers that were harvested just after larval death when they were still whitish.

Protocol:

- Grind infected fifth instar *C. pomonella* larvae in 0.1% sodium dodecyl sulfate (SDS), filter through mira cloth and centrifuge at 10,000 g.
- Resuspend the OB containing pellet in 0.1% SDS, load onto a 30-80% (v/v) glycerol gradient and centrifuge in an ultracentrifuge (40,000 g, 15 min).
- Recover the virus band using a long Pasteur pipette and wash twice in bidestilled water.
- Resuspend capsules in a few ml of bidestilled water and store at -20°C.

2.2.1.7. Virus OB counting

The OB were counted in a dark field with 400 times magnification using a Petroff-Hauser counting chamber (depth 0.01 mm).

2.2.1.8. Bioassays

Fifth instar *C. pomonella* were used to determine the median lethal dose (LD_{50}) and median survival time (ST_{50}),. The bioassays were performed in autoclavable 50-well plates. Larvae were inoculated with diet plugs containing a particular amount of the virus. Only larvae that had completely ingested the diet plug within 24 hours were placed on a fresh virus free medium and were reared individually at 26°C.

The LD_{50} was determined using 5 doses of each virus (0, 1, 10, 25, 50 and 250 OB). About 50 larvae per virus concentration were used in each assay. Mortality was recorded every 2 days until death or pupation of the larvae. Assays were repeated three times for each virus concentration. The data were analysed using probit analysis of the SAS software package.

The ST_{50} was determined by inoculating 50 larvae with a calculated LD_{80} dose. Mortality of the larvae was monitored in 8 hour intervals starting at day 5 post infection until larval death or pupation. The assays were repeated three times for each virus. The data were analysed using survival analysis of the SAS software package.

2.2.2. Methods in molecular biology

2.2.2.1. Isolation of DNA from occlusion bodies

In order to isolate the virus DNA it was important to have OB that were purified on a glycerol-gradient. The following protocol was used for the isolation of DNA from 10 mg OB (about the amount of virus isolated from 3 fifth instar *C. pomonella* larvae). The protocol can be scaled up and down proportionally.

Protocol:

- Resuspend the OB in 500 µl H_2O and add Na_2CO_3 (1M) to a final concentration of 0.05 M, incubate at 37°C for 30 min.
- The solution should be clear now, if not add more 1M Na_2CO_3 and incubate longer.
- Neutralise the solution to pH 8.0 with 1.0 M HCl, check pH with pH-paper.
- Add RNAse A (45 µg/ml) to the solution and incubate at 37°C for 10 min.
- To disrupt the virions, add SDS to a final concentration of 1%. Add proteinase K to a final concentration of 250 µg/ml. Incubate at 37°C for 60 minutes.
- Extract twice with TE saturated phenol/chloroform/isoamyl alcohol (25/24/1 (v/v/v)), then once with chloroform/isoamyl alcohol (24/1 (v/v)).
- Dialyse overnight against TE-buffer, change buffer three times.

2.2.2.2. Preparation of genomic insect DNA from larvae

For the isolation of the total genomic DNA from a single fifth instar *C. pomonella* or *C. leucotreta* larva the following protocol was used. For the isolation of DNA from more larvae the protocol could be scaled up proportionally.

Protocol:

- Homogenise single larva in 200 µl extraction buffer (10 mM Tris (ph 8.0), 100 mM EDTA (pH 8.0), 0.5% SDS and 20 µg/ml RNase A). Incubate at 37°C for 60 minutes.
- Add Proteinase K to a final concentration of 100 µg/ml and incubate at 37°C for 60 minutes.
- Extract 2-3 times with phenol/chloroform/isoamylalcohol (25/24/1).
- Add 0.2 times the total volume of sodium acetate (3 M, pH 5.2) and 2 times the total volume of ethanol (96%). Pellet the precipitated DNA by centrifugating at 10,000 x g.
- Wash pellet twice with ethanol (70%).

- Gently dry DNA pellet on the bench until liquid is just evaporated.
- Dissolve DNA in 100 µl TE.

2.2.2.3. Digestion and agarose gel electrophoresis of DNA

DNA fragments were separated on horizontal 0.8-1.5% agarose gels using TBE (1x) buffer. For separation of baculovirus DNA restriction fragments 0.8% gels in TAE (1x) buffer were used.

Solutions:

TBE (10x): 108 g TRIS, 55 g boric acid, 20 ml EDTA (1 M, pH 8.0) in a total volume of 1 liter.

TAE (10x): 242 g TRIS, 57.1 ml glacial acetic acid, 50 ml EDTA (1 M, pH 8.0) in a total volume of 1 liter.

loading buffer (6x): 0.25% bromophenol blue, 40% sucrose (w/v) in bidestilled water.

Protocol:

- Melt the required amount of agarose in the appropriate buffer in a microwave. Allow the solution to cool to 60°C, pour the gel and let it solidify.
- Prepare the DNA samples by adding the loading buffer and load on the gel.
- Run a mini-gel at 80V (maximum current) for 1 hour. Run a TAE gel containing virus restriction fragments on a maxi-gel at 30V (maximum current) for 10-14 hours.
- After the run stain the gel in an ethidium bromide (0.5 µg/ml) for 30 minutes, destain for 10 minutes in water and photograph on a UV-transilluminator.

2.2.2.4. Mini preparation of plasmid DNA

For the isolation of plasmid DNA from *E. coli* cultures the following protocol was used.

Solutions:

Lysis buffer: 50 mM glucose, 10 mM EDTA, 25 mM Tris/HCl, pH 8.0.

NaOH/SDS solution: 0.2 M NaOH, 1% SDS (solution should be freshly made).

Na-acetate: 3M NaAc, pH 5.2.

Protocol:

- Transfer 1.5 ml of a bacterial overnight culture into an 1.5 ml Eppendorf tube.
- Centrifuge 1 minute in the Eppendorf centrifuge at 5000 x g.
- Resuspend the pellet in 80 µl lysis buffer, and incubate for 5 minutes.
- Add 200 µl NaOH/SDS solution, invert tube 10 times, keep on ice for 5 minutes.
- Add 150 µl sodium acetate solution, mix by inverting (not by vortexing). Keep on ice for 10 minutes.
- Centrifuge for 10 minutes in an Eppendorf centrifuge at max. speed.
- Transfer 400 µl supernatant to a new 1.5 ml Eppendorf tube.
- Add 1 ml ethanol (96%), mix and leave at room temperature for 5 minutes. Centrifuge for 10 minutes in an Eppendorf centrifuge at max. speed.
- Wash the pellet once with 300 µl ethanol (70%). Centrifuge for 5 minutes at max. speed.
- Dry pellet in the speed-vac.
- Dissolve the pellet in 50 µl TE.

2.2.2.5. Polymerase chain reaction (PCR)

The PCRs were performed in a thermocycler (Mastercycler gradient, Eppendorf) with a thermo stable *Taq* DNA polymerase, recombinant (Gibco/BRL). The annealing temperature and the extension time were adjusted to the primers used and to the size of the amplified fragments. The following standard reactions were performed in 0.2 ml PCR tubes:

standard reaction	
5 µl	10 x PCR buffer minus $MgCl_2$
1 µl	10 mM dNTP mixture
1.5 µl	50 mM $MgCl_2$
2.5 µl	Primer1 (10 pmol/µl, 10 µM)
2.5 µl	Primer1 (10 pmol/µl, 10 µM)
1-5 µl	Template DNA
0.25 µl	*Taq* DNA polymerase (5U/µl)
Autoclaved bidestilled water to 50 µl	

standard cyclus	
denature	94°C for 2 minutes
then 30 cycles:	
denature	94°C for 0'30 minute
anneal	55°C for 1 minute
extend	72°C for 1 minute
then:	
7 minutes 72°C	

2.2.2.6. Sequencing

Plasmid DNA and PCR products to be sequenced were purified using the NucleoSpin kit and NucleoSpin Extract kit, respectively (Machery-Nagel). Commercial sequencing was performed by GENterprise company on ABI 373A and ABI 377 automated sequencers (PE Biosystems) using dye terminator cycle sequencing.

2.2.2.7. Total RNA isolation from insect tissue using TRIzol reagent (Gibco/BRL)

TRIzol reagent was used for the isolation of the total RNA from cells and tissue. An advantage of this reagent was that next to the RNA also DNA and proteins could be isolated from the same tissue sample.

Protocol:

- Homogenise fifth instar larvae (100 mg) in a 1.5 ml Eppendorf tube using a micro pistil in 1 ml of TRIzol reagent, incubate for 5 minutes at room temperature.
- Centrifuge at 12,000 x g and 4°C for 10 minutes. Remove the fat containing top layer. Transfer the RNA containing supernatant to a fresh 1.5 ml Eppendorf tube.
- Add 0.2 ml of chloroform, shake tubes vigorously by hand for 15 seconds and incubate for 2-3 minutes at room temperature.
- Centrifuge the samples at 12,000 x g and 4°C for 15 minutes.
- Remove the supernatant and wash the (invisible) RNA pellet with 1 ml of ethanol (75%) by vortexing.
- Centrifuge at 7,500 x g and 4°C for 5 minutes.
- Remove the supernatant, air dry the RNA pellet and dissolve it in RNase-free water. Store the sample at -80°C.

2.2.2.8. First strand cDNA preparation

In order to perform RT-PCR, first strand cDNA was prepared from RNA isolated from *C. pomonella* larvae. Total RNA from larvae was isolated using TRIzol reagent (Gibco/BRL) and was DNase treated (Gibco/BRL) prior to the reverse transcriptase reaction.

Protocol:

- Add the following components to a nuclease-free 1.5 ml Eppendorf tube:
    - 1.0 µl Oligo $(dT)_{12-18}$ primer (500 µg/ml) (Gibco/BRL)
    - 2.0 µg total RNA
    - 1.0 µl dNTP mix (10 mM each)
    - sterile distilled water to12 µl
- Heat the mixture to 65°C for 5 minutes and quickly chill on ice. Centrifuge briefly.
- Add the following components:
    - 4.0 µl 5x first strand buffer
    - 2.0 µl 0.1 M DTT
    - 1.0 µl RNaseOUT (40 units/µl) (Gibco/BRL)
- Mix the contents of the tube gently and incubate at 37°C for 2 minutes and add
    - 1.0 µl SuperScript RT (Gibco/BRL)
- Mix and incubate at 37°C for 50 minutes.
- Inactivate the reverse transcriptase by heating to 70°C for 15 minutes.

2.2.2.9. Quantitative (real time) RT-PCR

Quantification of transposase t32A transcription was done using real time RT-PCR. The reaction and fluorescence detection was performed using the DNA Engine Opticon™ System (MJ Research). QuantiTect SYBR green (Qiagen), a for real time PCR optimised mixture of SYBR-green 1, HotStarTaq DNA polymerase, dNTPs, buffer and $MgCl_2$, was used. The 40 µl reactions were performed in 0.2 ml low-profile strip tubes.

Transcription quantification was done using the following primer combinations: PR-t32a-up/PR-t32a-lo and PR-RT-up/PR-RT-lo (2.1.7). For both primer pairs the same amplification and detection programme could be used. The amplification and subsequent generation of a melting curve was carried out using the following program:

1. Incubate at 94°C for 15'00
2. Incubate at 94°C for 0'30
3. Incubate at 62°C for 0'30
4. Incubate at 72°C for 0'30
5. Plate read
6. Go to line 2 for 49 times more
7. Incubate at 72°C for 7'00)
8. Perform melting curve from 50°C to 95°C: read every 0.5°C: hold for 0'10 between reads
9. End

The data were analysed with the Opticon Monitor™ software.

2.2.2.10. RNA (glyoxal) electrophoresis

RNA electrophoresis was performed as described by Sambrook *et al*. (1989).

Solutions:

glyoxal 6 M:	Deionise stock solution (6 M) through a mixed bed resin (Bio-Rad 501-X8) until pH is at least 5.0.
sodium phosphate buffer (0.1 M):	Mix 3.9 ml of 1 M NaH_2PO_4 with 6.1 ml 1 M Na_2HPO_4, add 90 ml of bidistilled water and check the pH. Adjust to pH 7.0 with either NaH_2PO_4 or Na_2HPO_4.
glyoxal loading buffer:	50% glycerol (v/v), 10 mM sodium phosphate buffer and 0.25% bromophenol blue.

Protocol:

- Thoroughly clean electrophoresis equipment with detergent, rinse with water, incubate with 3% H_2O_2 for 10 minutes and rinse again with bidestilled water.
- Prepare a 1.5% agarose gel in 10 mM sodium phosphate.
- To prepare the RNA samples, mix 5.4 µl 6 M glyoxal, 10.0 µl DMSO, 3.0 µl 0.1 M sodium phosphate and 5.4 µl RNA (up to 10 µg). Incubate at 50°C for 60 minutes and chill on ice. Add 4 µl glyoxal loading buffer to the cooled RNA samples.
- Load the samples on the agarose gel and run at 80 V (maximum current) until the blue dye migrates 2/3 through the gel. Circulate the buffer during the run.

2.2.2.11. Transfer of DNA/RNA to Hybond-N+ membrane

The DNA and RNA fragments separated on agarose gel were transferred to a nylon membrane according to the method developed by Southern (1975). For an efficient transfer of larger DNA fragments it is important to introduce nicks in the DNA strands. Therefore, the gel is incubated in 0.25 M HCl for 5 minutes and subsequently washed in bidestilled water. For RNA blotting this step is not necessary.

Solutions:

denaturing solution:	0.5 M NaOH, 1.5 M NaCl.
neutralising solution:	1 M Tris-HCl, pH7.4, 1.5 M NaCl.
transfer buffer (10x SSC):	0.15 M Na-citrate, pH 7.0, 1.5 M NaCl.

Protocol:

- Incubate the gel for 15 minutes in denaturing solution.
- Rinse the gel with bidestilled water and incubate it for 2 x 15 minutes in the neutralising solution.
-Wet a large piece of Whatmann paper in 10 x SSC and place it on a glass plate. Let the side slips hang out into a tray filled with 10 x SSC. Place 3 Whatmann papers (soaked in 10x SSC) with the size of the gel on top.
- Place the gel on the stack of Whatmann papers.
- Place a pre-soaked (10 x SSC) Hybond-N+ membrane on the gel. Place 3 sheets of Whatmann paper on top of that.
- Place a stack of paper towels on top of the filter papers.
- Place a glass plate and a weight on top of the paper towels.
- Allow the transfer of the DNA or RNA to proceed for 12-24 hours.
- Remove towels and filter papers. Mark slots and position of the marker lane.
- Carefully lift the Hybond-N membrane off the gel.
- Place the membrane with the transferred RNA or DNA side up on a piece of filter paper and let the membrane dry at room temperature.
- Cross link the DNA or RNA to the membrane by baking the membrane for 1 hour at 80°C. Blots can be stored between Whatmann papers for a few weeks at room temperature.

2.2.2.12. Prehybridisation and hybridisation

This method was used for hybridisation of Southern blots with a radioactively labelled DNA probe (Rediprime II random prime labelling system, Amersham Pharmacia). Northern blots were hybridised with a DIG-labelled strand specific probe (Roche).

Solutions:

fragmented salmon sperm DNA: (ssDNA, 5 mg/ml in sterile bidestilled water)

20 x SSC:	175,3 g NaCl and 88,2 g sodium citrate, make up to 1 l with bidestilled water.
100 x Denhardts:	2 g Ficoll, 2 g polyvinylpyrolidone and 2 g BSA, make up to 100 ml with bidestilled water, store in aliquots at -20°C.
hybridisation buffer:	6 x SSC, 5 x Denhardts, 0.01 M EDTA, 0.5% SDS
2 x SSC wash buffer:	100 ml of 20 x SSC and 50 ml of 10% SDS, make up to 1 l with bidestilled water.
0.2 x SSC wash buffer:	10 ml of 20 x SSC and 50 ml of 10% SDS, make up to 1 l with bidestilled water.

Protocol:

- Prewarm the hybridisation oven and the buffers to 68°C and denature the fragmented ssDNA by boiling for 10 minutes (store on ice until further use).
- Carefully place the blot into the hybridisation tube.
- Add 10-20 ml of the pre-warmed hybridisation buffer and 250-500 µl of the heat denatured ssDNA.
- Close the tube, make sure that the rubber ring is properly installed in the lid. Unroll the membrane by gently rolling the tube in your hands. Avoid air bubbles between the blot and the tube.
- Place the tube into the oven and prehybridise at 68°C for two hours.
- Add the heat denatured probe to the tube. Hybridise overnight. Pre-warm the wash buffers to be used the next day at 68°C.
- The next day, remove the hybridisation mix.
- Wash the blot by adding 30-40 ml of 2 x SSC wash buffer and incubate in the oven for 20 minutes.

- Wash once again with a 2 x SSC wash buffer, twice with a 0.2 x SSC wash buffer. Rinse a few times with 2 x SSC to remove the SDS.
- Take the blot out of the tube and dry it on a piece of Whatmann paper.
- Fix the blot to a Whatmann paper with tape, wrap the whole in Saran Wrap before detection.

2.2.2.13. Radioactive labelling of DNA hybridisation probe

The Southern blots were hybridised with a radioactively labelled DNA probe. Random sequence hexanucleotides were used to prime the DNA synthesis on the denatured DNA template (Rediprime II random prime labelling system, Amersham Pharmacia Biotech).

2.2.2.14. DIG-labelling of strand-specific RNA probes

The Northern blots were hybridised with dioxygenin (DIG)-labelled strand specific RNA probes. The dioxygenic-UTP labelling of the RNA was performed by *in vitro* transcription using SP6 and T7 RNA polymerase (Promega). The DIG-labelling was performed as described in the DIG RNA labelling kit (Roche).

2.2.2.15. SDS-PAGE

Proteins were separated according to their molecular weight by SDS polyacrylamide gel electrophoresis. For the experiments the Bio Rad mini protean II dual slab gel system was used. Polyacrylamid gels were prepared as described in Sambrook *et al*. (1989).

Solutions:

Lysis buffer:	50 mM glucose, 10 mM EDTA and 25mM Tris/HCl, pH 8.0.
(4 x) SDS sample buffer:	40 mM Tris/HCl pH 8.0, 4 mM EDTA, 8% SDS (w/v), 40% glycerol (v/v) and 0.0004% bromophenol blue.
staining solution:	325 ml bidestilled water, 125 ml isopropanol, 50 ml glacial acid and 0.2 g Comassie Blue.
destaining solution:	glacial acid (5%) and methanol (7.5%) in bidestilled water.

Protocol:

- Resuspend the protein samples in four times the volume of lysis buffer. Add 0.1 times the volume of β-mercaptoethanol and boil the samples for 10 minutes.
- Add 0.25 times the volume of (4 x) SDS sample buffer.
- Boil 5 minutes and load the gel.
- Run the gel at 75 V (maximum current) until the proteins reach the separation gel, then run at 150 V (maximum current).
- After the run stain the gel in the staining solution for 1 hour, destain in the destaining solution.

2.2.2.16. Semi-dry blotting

In order to prepare Western blots, proteins were separated by SDS PAGE and transferred to an Immobilon-P membrane (Amersham Pharmacia). Blotting was performed using the Transblot SD Semidry Transfer Cell (Bio Rad).

Solution:

continuous buffer: 39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS and 20% methanol.

Protocol:

- Stack 3 in the continuous buffer soaked Whatman-papers (with the size of the gel) on the base of the Semi-Dry Blot Cell.
- Pre-wet the Immobilon-P membrane in methanol for 1 minute, rinse it with bidestilled water and soak the membrane for 15 minutes in continuous buffer. Place it on top of the Whatmann papers.
- Soak the gel in the continuos buffer for 5 minutes and place it on top of the membrane.
- On top of the gel, layer 3 in continuous soaked Whatmann papers
- Dry the electrode plate around the papers and put the other electrode plate on top.
- Close the semi-dry blot apparatus.
- Two mini gels should run for 1 hour at 20V constant, the amperage should be 200-300 mA (use the Bio-Rad Powerpac-200).
- Remove the lid and the upper Whatmann papers. Before removing the membrane, mark the position of the size marker.
- Continue with immuno-detection or dry the membrane and store it at room temperature.

2.2.2.17. Immuno detection

For the immuno-detection of the proteins on the blots the ECL Western blotting kit (Amersham Pharmacia) was used. This kit is based on a non-radioactive method for the detection of immobilised specific antigens with horseradish peroxidase-labelled secondary antibodies.

Solutions:

PBS (pH 7.5): 80 mM Na_2HPO_4, 20 mM $NaH_2PO_4 \cdot 2H_2O$, NaCl 100 mM.

PBS-tween: 0.1% Tween-20 in PBS.

blocking buffer: 5% milk powder (low fat) in PBS-Tween.

Protocol:

- Pre-wet the membrane by soaking it in methanol for 1 minute. then rinse it with bidestilled water and incubate for 15 minutes in PBS-Tween.
- Block the membrane by immersing it in the blocking buffer on an orbital shaker for 1 hour at room temperature.
- Wash the blot 3 times for 5 minutes in TBS-Tween.
- Dilute the primary antibody in PBS-Tween and incubate the blot for 1 hour at room temperature.
- Wash the blot three times for 5 minutes in TBS-Tween.
- Dilute the HRP-labelled secondary antibody in TBS-Tween and incubate with the blot for one hour.
- Wash the membrane once for 15 minutes and four times for 5 minutes in TBS-Tween.
- Perform the detection using detection solution 1 and 2 from the kit.

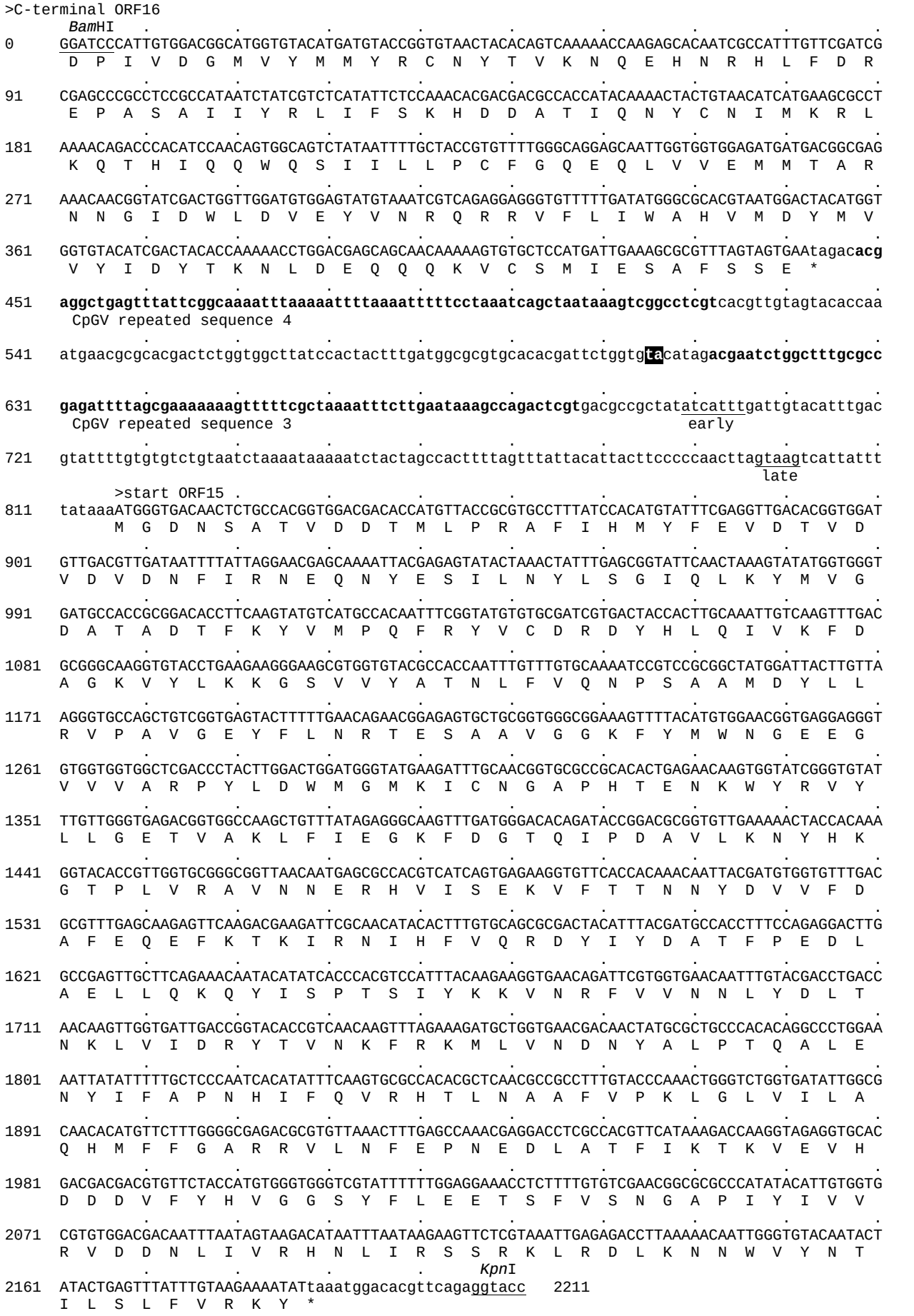
3. RESULTS

3.1. Sequencing of the integration site of TCl4.7 in CpGV-M

The integration of the transposon TCl4.7 within the genome of CpGV-M was mapped by restriction analyses on a *Bam*HI/*Kpn*I fragment corresponding to map unit 10.7-12.9 (Jehle *et al*., 1995). In order to sequence the integration region, this *Bam*HI/*Kpn*I fragment was first cloned in pGEM-7Zf(+) resulting in plasmid pCp5INS (Jehle, 1994). Using the Erase-A-Base system, unidirectional exonuclease III deletion clones were prepared for both nucleotide strands. These clones were sequenced using the M13 forward and M13 reverse primer. After alignment of the obtained sequences, it was determined that the sequenced *Bam*HI/*Kpn*I fragment had a size of 2211 bp (Fig. 3.1). The TA dinucleotide integration site of TCl4.7 was located in a non-translated region between Cp15 (=ORF15) and Cp16 (=ORF16) (Fig. 3.1 and Fig. 3.2) (Luque *et al*., 2001). This intergenic region contains two repeated sequences (repeated sequence 3 and 4) which form imperfect palindromes of approximately 70 bp. These repeats could be CpGV homologous regions (*hrs)* containing only a single copy of a repeated unit (Luque *et al.*, 2001).

The repeated sequences are not the only palindromic sequences in the non translated region between Cp15 and Cp16. The TA dinucleotide of the target site of TCl4.7 is also part of a small palindromic sequence (TG**TA**CA, nt 613-618, Fig. 3.1). Due to these palindromic sequences the transposon integration region is rich in secondary structures. The AT content of the non coding region where TCl4.7 integrated was also determined. With 63.1%, it was somewhat higher than the AT content of the total CpGV-M genome (54.8%).

Figure 3.1.(next page) Nucleotide sequence of the *Bam*HI/*Kpn*I fragment corresponding to map unit 10.7-12.9 of CpGV-M covering the integration site of transposon TCl4.7. The TA integration site is shaded black. The predicted amino acid sequences of ORF15 and ORF16 are indicated by one-letter code below the nucleotide sequence. The early and late promoter sequences of ORF15 are underlined. The CpGV repeated sequences 3 and 4 are in bold face.


>C-terminal ORF16
BamHI
CpGV repeated sequence 4
CpGV repeated sequence 3
early
late
>start ORF15
KpnI

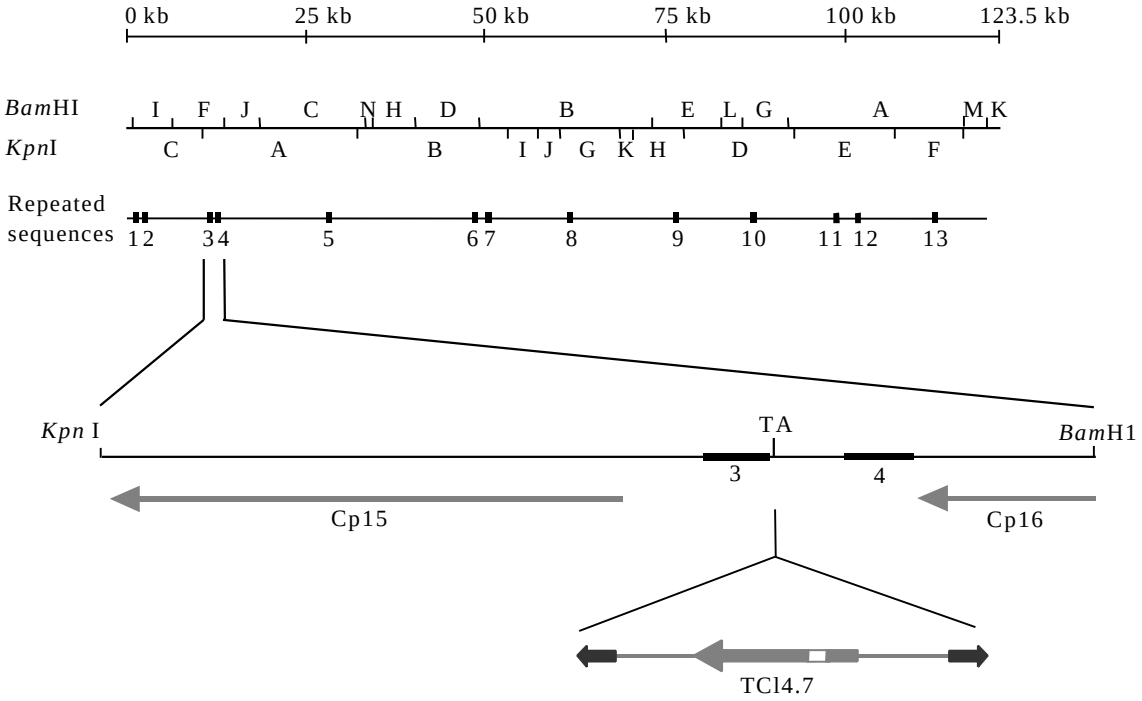


Figure 3.2. Location and organisation of the insertion site of the transposon TCl4.7 within the genome of CpGV. The restriction maps of *Bam*HI and *Kpn*I are indicated (Crook *et al*., 1997). The CpGV repeated sequences are depicted by black bars. The size and the orientation of the identified open reading frames are indicated by grey arrows. The TA dinucleotide is the integration site of TCl4.7.

Cp15 is probably an early and a late transcribed gene. The early promoter of Cp15 is most probably of the INR+ category since a putative early motif 5'-ATCATTT-3' (nt 699-705, Fig. 3.1) without TATA sequence at its 5'-site, is located 118 bp upstream from the ATG start codon (nt 817-819). A putative 5'-GTAAG-3' (nt 797-801) late promoter motif is located 20 bp upstream from the ATG. Cp15 is a conserved baculovirus gene which has homologues in all sequenced baculovirus genomes (Table 3.1). By comparison of the predicted amino acid sequence with conserved domain motifs in the PROSITE database (www.expansy.ch/tools/scanprosite), it was predicted that the protein contains a putative aldo/keto reductase family pattern. Proteins of this family are involved in NADPH-dependent oxidoreductase. The exact function of Cp15 remains unclear. Cp16 is possibly an early transcribed gene with an unknown function. A consensus TATA box and a consensus transcription initiation sequence 5'-CAGT-3' were located 13 and 24 bp upstream from the ATG start codon (Luque *et al*., 2001). Cp16 does have homologues in most of the sequenced NPV and in CrleGV, but does not have homologues in XecnGV and PxGV (Table 3.1). The amino acid sequence of Cp16 was also compared with the PROSITE data base, but no patterns or domains were identified which could predict a distinct evolutionary origin or function.

Table 3.1. Homologues of Cp15 and Cp16 in other sequenced baculoviruses.

	Cp15			Cp16			
Virus	ORF	Size (aa)	promoter	ORF	Size (aa)	promoter	Reference
CpGV	15	457	E,L	16	196	E	1
CrleGV	15	451	L	17	197	?	2
XecnGV	13	453	L	-	-	-	3
PxGV	14	446	L	-	-	-	4
AcMNPV	142	477	E,L	-	-	-	5
BmNPV	118	476	L	-	-	-	6
OpMNPV	139	484	L	31	317	E	7
EppoNPV	124	487	L	-	-	-	8
HaSNPV	9	468	L	-	-	-	9
SpltMNPV	11	439	L	100	321	E	10
SeMNPV	137	460	L	54	364	E	11
LdMNPV	20	483	L	138	291	-	12
MacoMNPV	124	487	L	-	-	-	13

(1) Luque *et al*., 2001; (2) Lange & Jehle (unpublished); (3) Hayakawa *et al*., 1999; (4) Hashimoto *et al*., 2000; (5) Ayers *et al*., 1994; (6) Gomi *et al*., 1999; (7) Ahrens *et al*., 1997; (8) Hyink *et al*., 2002; (9) Chen *et al*., 2001; (10) Pang *et al*., 2001; (11) IJkel *et al*., 1999; (12) Kuzio *et al*., 1999; (13) Li *et al*., 2002.

The DNA sequences of the integration sites of the transposons TCl4.7 and TCp3.2 within the CpGV genome were compared. Only a very low homology between the 40 nucleotides upstream and downstream from the TA dinucleotide target site was found (Fig. 3.3).

Figure 3.3. Sequence alignment of the TCp3.2 and TCl4.7 integration regions in the CpGV-M genome. Homologous nucleotides in the 40 bp up- and downstream of the TA dinucleotide integration sites are shaded grey. The TA target sites are shaded black.

3.2. Comparison of the CpGV-M1 sequence with the obtained sequences of CpGV-M

The sequenced CpGV-M *Bam*HI/*Kpn*I fragment, which contained the TCl4.7 integration site, had a size of 2211 bp. Comparisons of this DNA sequence with the same region in CpGV-M1 (nucleotides 10382-12591) showed that the only difference between the two CpGVs is an additional C-nucleotide in CpGV-M. This nucleotide corresponds to position 12139 in the CpGV-M1 genome which is located within CpGV repeat sequence 4. In the genome of CpGV-M1, 13 repeat sequences were identified (Fig. 3.4) (Luque *et al*., 2001). Twelve of these have a T

nucleotide at position 6, only repeat sequence 4 has a gap at that position. Interestingly, the position of the gap corresponds to the additional C nucleotide in the CpGV-M sequence. This means that the sixth nucleotide in repeat sequence 4 of CpGV-M is a G (complement of C) (Fig. 3.4). With this additional G, the 7 left and right bordering nucleotides of repeat 4 form a perfect palindrome.

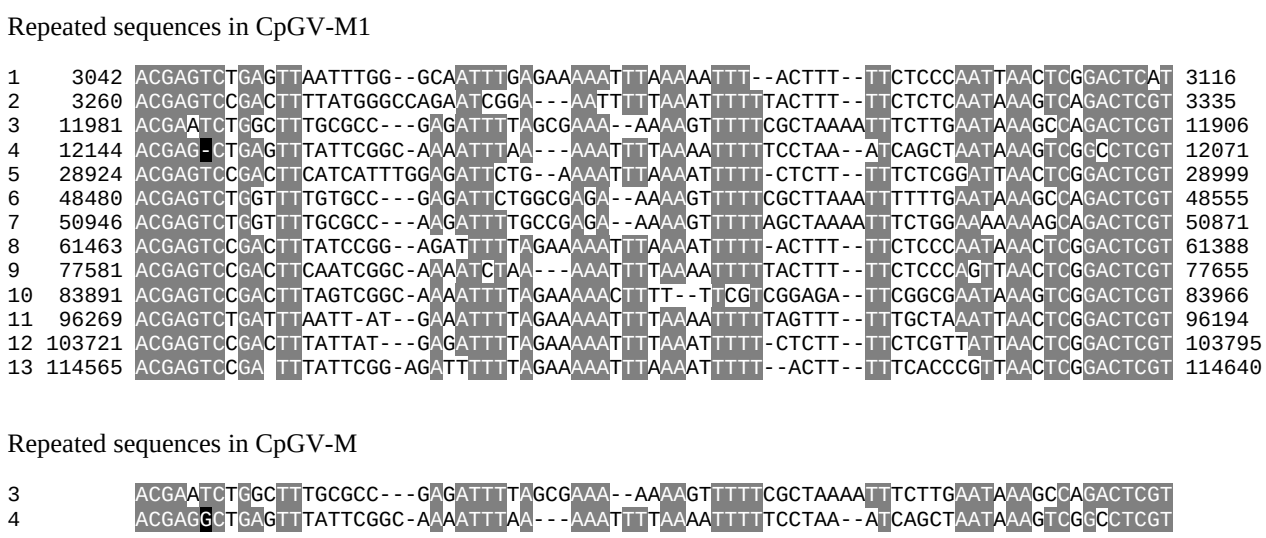


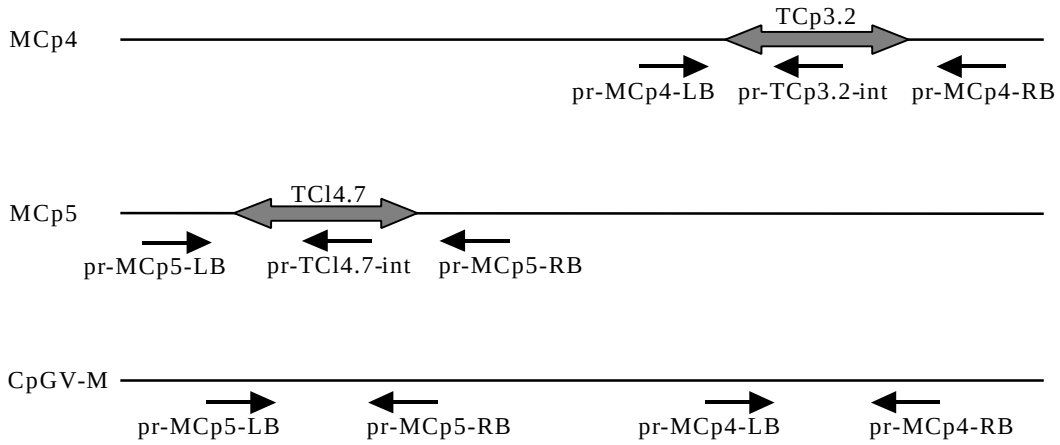
Figure 3.4. CpGV repeated sequences. ClustalW alignment of CpGV dispersed repeat sequences. Nucleotides conserved in at least 10 sequences are shaded grey (Luque *et al.*, 2001). The difference between repeat 4 of CpGV-M1 and CpGV-M is shaded black.

3.3. Preparation of MCp4, MCp5 and CpGV-M stocks

At the onset of this project genetic homogenous virus stocks of the transposon carrying mutants MCp4 and MCp5 and CpGV-M were available. Since they were already stored for a few years, a fresh stock for each virus genotype was prepared. The virus of all three genotypes was propagated in *C. pomonella* larvae. After larval death, OB were purified and their DNA was isolated. Subsequent restriction endonuclease analysis was performed to test the genomic homogeneity of the isolated viruses. A *Bam*HI/*Hin*dIII double digest on the isolated DNA resulted in the restriction profiles specific for all three genotypes. Although no submolar bands were visible on the agarose gel, a PCR analysis was performed to confirm that the prepared virus stocks were not cross contaminated. As demonstrated in Fig. 3.5a, primer pairs were designed which specifically amplified MCp4, MCp5 or CpGV-M fragments. The specific amplification of CpGV-M fragments was achieved using primer pairs that border the transposon integration sites of MCp4 and MCp5. Due to the short elongation time (30 seconds) used in this PCR, only the small CpGV-M specific fragments could be amplified. The much larger MCp4 or MCp5 specific products

(3734 bp and 5237 bp) could not be amplified under these PCR conditions. MCp4 and MCp5 specific fragments were amplified using a transposon and a CpGV-M specific primer (Fig. 3.5a). The arithmetically expected sizes of the PCR fragments using the different primer combinations and virus DNA templates are summarised in Fig. 3.5b. The results of the PCRs on MCp4, MCp5 and CpGV-M DNA are shown in Fig. 3.5c.

a)



b)

Primer pair	Expected size (bp) MCp4	MCp5	CpGV-M
1: pr-MCp4-LB/pr-MCp4-RB	- (1)	495	495
2: pr-MCp4-LB/pr-TCp3.2-int	900	-	-
3: pr-MCp5-LB/pr-MCp5-RB	511	- (2)	511
4: pr-MCp5-LB/pr-TCl4.7-int	-	850	-

The arithmetically expected sizes were for (1): 495+3239=3734bp and for (2): 511+4726=5237bp. Due to the chosen PCR conditions, these fragments were not expected to be amplified.

(c)

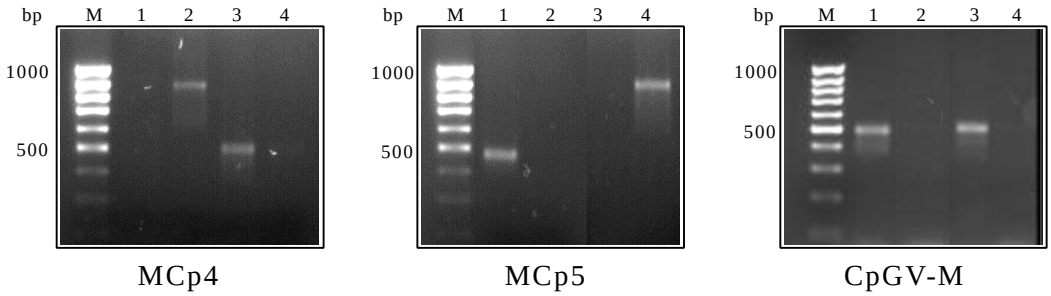


Figure 3.5. PCR to test the homogeneity of the MCp4, MCp5 and CpGV-M virus stocks. **(a)** Binding sites of the primers used for the specific amplification of MCp4, MCp5 and CpGV-M specific fragments (not to scale). **(b)** Arithmetically expected sizes of the PCR products using primer pairs 1-4 and MCp4, MCp5 and CpGV-M DNA as template. **(c)** Results of the PCR reactions using MCp4, MCp5 or CpGV-M DNA as template. Lane M = size standard (100 bp marker), lanes 1-4 correspond to primer pairs 1-4 in Fig. 3.5b. Fragment sizes are indicated on the left.

In case of MCp4 DNA, PCR using the MCp4 specific primers (PR-MCp4-LB/PR-TCp3.2int) resulted in a 900 bp fragment which was expected for MCp4. The PCR with the CpGV-M specific primers (PR-MCp4-LB/PR-MCp4-RB) on the other hand, did not result in an amplification

product, which demonstrated that the MCp4 DNA was not contaminated with CpGV-M DNA. The absence of an amplification product using the MCp5 specific primers (PR-MCp5-LB/PR-TCl4.7-int) together with the 511 bp fragment, which was obtained when the CpGV-M specific primers (PR-MCp5-LB/PR-MCp5-RB) were used, proved that the MCp4 DNA was also not contaminated with MCp5 DNA. PCRs, using the same genotype specific primer pairs as described above, were also performed on the isolated MCp5 and CpGV-M DNA. The results of these reactions demonstrated that the MCp5 and CpGV-M DNA were also not cross contaminated. The prepared MCp4, MCp5 and CpGV-M virus stocks were used for the further infection experiments.

3.4. Isolation and restriction analysis of the spontaneous mutant MCp4inv

MCp4 is a CpGV-M mutant that harbours the host transposable element TCp3.2 at map unit 28.3 (34.9 kb) within the restriction fragment *Bgl*II-E (Jehle *et al*., 1997). During further *in vivo* cloning of MCp4 a mutant genotype named MCp4inv was isolated which showed heterogeneity in the *Hin*dIII restriction site within *Bgl*II-E (Fig. 3.6 and Fig. 3.7).

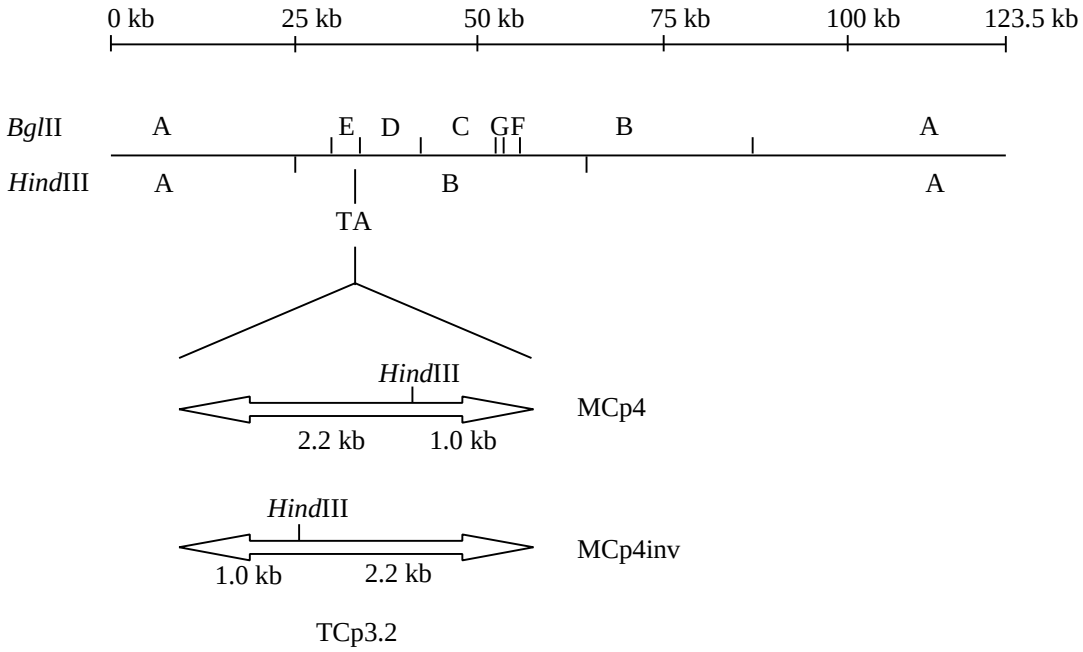


Figure 3.6. *Bgl*II and *Hin*dIII restriction map of CpGV-M. The different orientations of transposon TCp3.2 resulting in CpGV mutants MCp4 and MCp4inv are depicted. The position of the *Hin*dIII restriction site within TCp3.2 is marked. The integration site of TCp3.2 (dinucleotide TA) is located at 34.9 kb.

Transposon TCp3.2 introduced an additional *Hin*dIII restriction site in the genomes of MCp4 and MCp4inv (Fig. 3.6). Since the integration site of TCp3.2 is located within the 40.0 kb *Hin*dIII-B fragment of CpGV-M, an altered *Hin*dIII restriction pattern was observed for the transposon containing mutants. The 40.0 kb fragment was substituted by fragments of 11.6 kb and 31.6 kb in

MCp4 and fragments of 10.4 kb and 32.8 kb in MCp4inv. The *Bgl*II-E fragment of CpGV-M (4.8 kb) fragment was substituted by a 8.0 kb fragment in MCp4 and MCp4inv due to the integration of TCp3.2 into this fragment (Fig. 3.7). The identity of the *Bgl*II pattern of MCp4 and MCp4inv indicated that any difference in the restriction pattern occurred within *Bgl*II-E and is not linked to any insertion or deletion. Restriction endonuclease digests using other enzymes indicated that there was no change in fragments other than *Bgl*II-E and no increase in the total size of MCp4 DNA (data not shown). A DNA double digest using *Bgl*II and *Hin*dIII restrict the *Bgl*II-E fragment for MCp4 and MCp4inv in two genotype specific bands which are 6.8 and 1.2 kb for MCp4, and 5.6 and 2.4 kb for MCp4inv (Fig. 3.7). This specific change of restriction fragment patterns suggested that transposon TCp3.2 inverted within the genome of MCp4 and resulted in genotype MCp4inv.

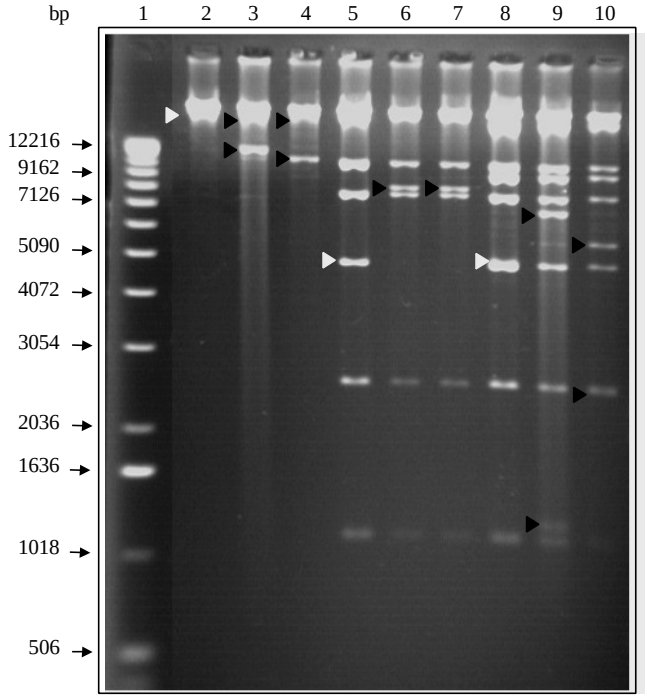


Figure 3.7. Comparative restriction analyses of isolated DNA of CpGV-M, MCp4 and MCp4inv. CpGV-M, MCp4 and MCp4inv DNA is digested with *Hin*dIII, *Bgl*II and *Hin*dIII/*Bgl*II. Lane 1 = size standard (1 kb ladder), lane 2 = CpGV-M x *Hin*dIII, lane 3 = MCp4 x *Hin*dIII, lane 4 = MCp4inv x *Hin*dIII, lane 5 = CpGV-M x *Bgl*II, lane 6 = MCp4 x *Bgl*II, lane 7 = MCp4inv x *Bgl*II, lane 8 = CpGV-M x *Hin*dIII/*Bgl*II, lane 9 = MCp4 x *Hin*dIII/*Bgl*II and lane 10 = MCp4inv x *Hin*dIII/*Bgl*II. Fragment sizes are indicated on the left. The white arrows indicate the CpGV-M restriction fragments into which transposon TCp3.2 integrated. The black arrows indicate the MCp4 and MCp4inv specific restriction fragments.

3.5. Inversion of transposon TCp3.2 in MCp4

In order to determine whether transposon TCp3.2 inverted, a PCR test was developed to detect the orientation of TCp3.2 in MCp4inv. Based on the known sequences of MCp4, PCR primer pairs were designed to amplify the left and right border sequences specifically for each

orientation of the transposon. As demonstrated in Fig. 3.8 and Fig. 3.9, the left and right border sequence of MCp4 was amplified using the left border (LB) and left internal (LI) primers and the right border (RB) and right internal (RI) primers, respectively. In case of an inversion of transposon TCp3.2, it was expected that the exchange of primer combinations used to amplify the MCp4 border sequences would also result in positive PCR reactions. As shown in Fig. 3.8, PCR using primer combinations RB/LI and LB/RI on MCp4inv resulted in a 1250 bp and 2104 bp fragment, respectively. Fragments of these sizes were arithmetically expected for the MCp4inv genotype. This result proved that transposon TCp3.2 had inverted within the genome of MCp4.

(a)

Primer pair	Expected size (bp) MCp4	MCp4inv
1: RB/RI	2454	-
2: LB/LI	900	-
3: RB/LI	-	1250
4: LB/RI	-	2104

(b)

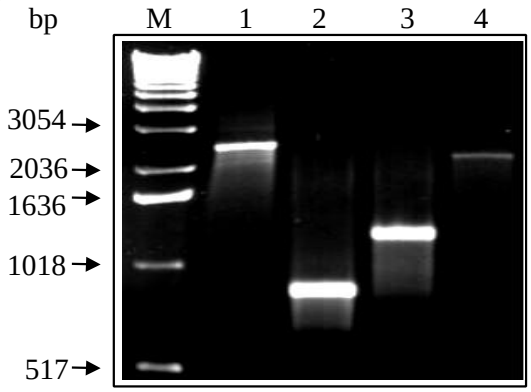


Figure 3.8. PCR analysis of the orientation of transposon TCp3.2 in MCp4 and MCp4inv. **(a)** Arithmetically expected sizes of the PCR products using primers specific for the CpGV left border (LB) and right border (RB) and transposase specific primers left internal (LI) and right internal (RI) (see also Fig. 3.9). **(b)** Results of the PCR reactions using MCp4 and MCp4inv DNA as template. Lane M = size standard (1 kb ladder), lane 1-4 correspond to primer pair 1-4 in Fig. 3.8a. Fragment sizes are indicated on the left.

3.6. TCp3.2 inverted by homologous recombination between the ITRs

From the previous results it was not clear which mechanism was involved in the transposon inversion. Since TCp3.2 is a mobile element, it could be excised from the MCp4 genome and then inserted again in an inverted orientation. Though the TCp3.2 copy in 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: by an excision mediated inversion the entire transposon inverts and by recombination mediated inversion some central part may invert leaving the terminal ends of the ITRs at their original location.

The left and right ITR of TCp3.2 are 756 bp long and do not match in seven positions dispersed over the ITRs (Fig. 3.9). The position of these ITR specific nucleotides in MCp4inv made it possible to determine whether the complete transposon or only its central parts inverted during replication. In case of the 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 may have kept their original position. In order to identify the potential mechanism involved in the inversion of TCp3.2, the ITRs in MCp4inv were sequenced and compared with those in MCp4. The MCp4inv left and right ITR were amplified in the polymerase chain reaction using primers (LB/ RI) and (RB/ LI) (Fig. 3.9), cloned and sequenced. As shown in Fig. 3.9, the only differences between sequences of the ITRs of MCp4 and MCp4inv lies in the two most internal specific nucleotides of the ITRs. In MCp4inv the MCp4 specific A_{473} and A_{597} were replaced by T_{473} and G_{597} which are the inverse complements of A_{2811} and C_{2687}. On the other hand, MCp4 specific A_{2811} and C_{2687} of the right ITR were replaced in MCp4inv by T_{2811} and T_{2687}, which again are the inverse complements of A_{473} and A_{597}.

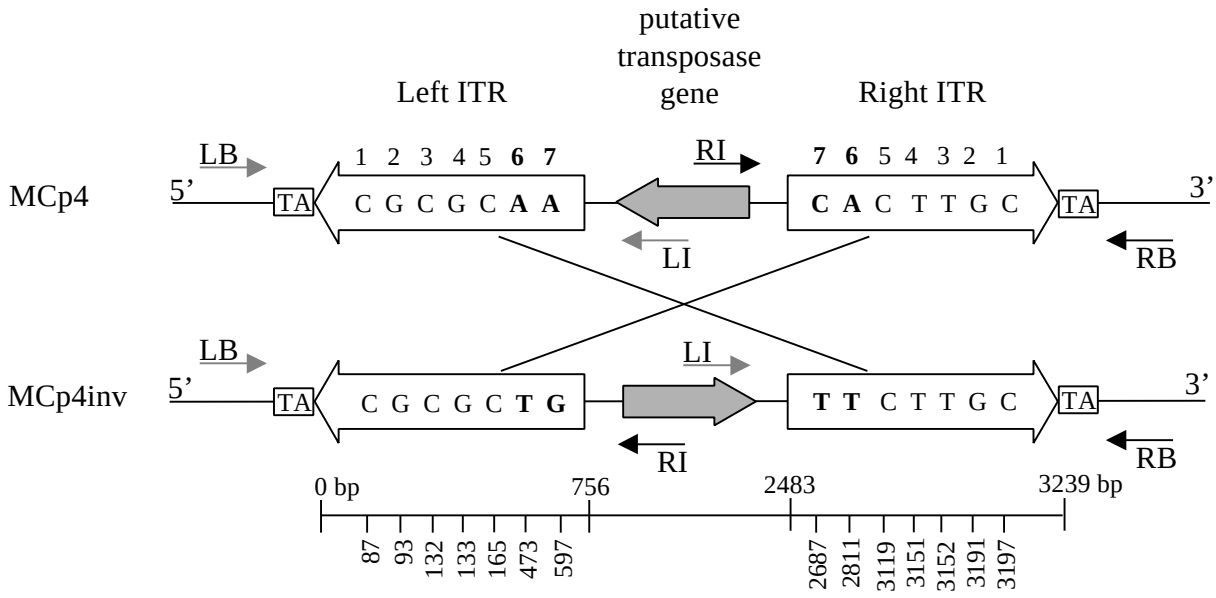


Figure 3.9. Recombination model of TCp3.2 in MCp4 resulting in inversion of TCp3.2 in MCp4inv. As indicated by sequence analysis of the PCR products obtained using CpGV border specific primers (LB, RB) and internal transposon specific primers (LI, RI) difference between the two ITRs of MCp4 and MCp4inv are 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. The mismatching nucleotides (1-7) are indicated by the numbering on the top and the positions (bp) at the bottom.

These analyses demonstrated that not the entire transposon but only the central part including the putative transposase gene and parts of the ITRs inverted. Since ITR specific nucleotides no. 1 - no.

5 did not change their position, the homologous recombination most probably took place between sequences located between the fifth and sixth mismatching nucleotides of the ITRs (Fig. 3.9).

3.7. Median lethal dose and survival time determination

In order to compare the biological activity of MCp4, MCp5 and the parental CpGV-M, the median lethal dose (LD_{50}) and the median survival time (ST_{50}) in fifth instar *C. pomonella* were determined in bioassays. In the lethal dose bioassays groups of about 40 larvae were infected with 5 different doses of virus and the mortality of each group was determined. The experiments were repeated 3 times and using probit analysis the LD_{50} was calculated. There was no significant difference between the LD_{50} values of CpGV-M, MCp4 and MCp5 (Table 3.2). Apparently, the transposon insertions into the CpGV genome had no affect on the amount of virus particles required to initiate the infection.

Table 3.2. Median lethal dose (LD_{50}) for fifth instar *C. pomonella* infected with CpGV-M, MCp4 and MCp5.

Virus	LD_{50}	95% Limits (lower-upper)	Slope	Standard Error	χ^2
CpGV-M	44.69	35.9-54.7	0.74	0.076	92.82
MCp4	35.70	19.1-52.9	0.56	0.089	40.07
MCp5	23.19	13.3-35.9	0.49	0.052	90.11

LD_{50} and confidence limits are given in OB per insect. All χ^2 were significant at P=0.0001.

The survival time bioassays were performed by measuring the time between infection of the larvae (LD_{80}) and their death. The mortality of the about 100 infected larvae was checked every 8 hours. For each virus the experiments were repeated 3 times and the ST_{50} was calculated using survival analysis. In contrast to the LD_{50}, a significant difference in the ST_{50} values between the viruses was observed (Table 3.3). The ST_{50} values of CpGV-M, MCp4 and MCp5 were 180.2 hours, 218.7 hours and 194.5 hours, respectively, demonstrating that the transposon containing viruses killed *C. pomonella* larvae slower than the parental virus. During the bioassays no differences could be observed in the disease symptoms of the *C. pomonella* larvae infected with CpGV-M, MCp4 or MCp5.

Table 3.3. Median survival times (ST_{50}) for fifth instar *C. pomonella* infected with an LD_{80} CpGV-M, MCp4 and MCp5.

Virus	ST_{50}	95% Limits (lower-upper)	Standard Error	χ^2
CpGV-M	180.2	167.8-191.6	0.31	127.6
MCp4	218.7	209.0-228.1	0.24	203.0
MCp5	194.5	185.7-202.7	0.25	226.1

ST_{50} and confidence limits are given in hours post infection. All χ^2 were significant at P=0.0001.

3.8. Virus offspring production

Since a potential selection advantage of a virus may depend on the amount of virus produced, the virus yield of fifth instar *C. pomonella* infected with CpGV-M, MCp4 and MCp5 (LD_{80}) was determined. Twenty cadavers of each type of virus infection were collected, the OB were extracted and counted. CpGV-M, MCp4 and MCp5 infected larvae contained on average 1.09×10^{11} OB/larvae, 1.01×10^{11} OB/larvae and 9.97×10^{10} OB/larvae, respectively. This demonstrated that there is no difference between the OB production for CpGV-M, MCp4 and MCp5 in fifth instar *C. pomonella*.

3.9. Quantification of the CpGV-M:mutant ratio in the virus offspring.

In the next few paragraphs a series of competition experiments are described which were carried out to find out whether CpGV-M had a selection advantage over MCp4 and MCp5. A selection advantage of one of the genotypes could be determined by comparing the virus ratio in the inoculum with the virus ratio in the offspring. In order to determine the genotype ratios, a method was developed to quantify the relative amounts of CpGV-M and mutant in the virus offspring. In this quantification method, the ratio of the CpGV-M:mutant in the virus offspring was determined by comparing the density of the genotype specific restriction fragments with the density of the genotype shared restriction fragments. The CpGV-M:MCp4 ratio in the virus offspring was determined by quantifying genotype specific restriction fragments obtained with *Bam*HI and *Hin*dIII/*Bgl*II digests. Digestion with *Bam*HI resulted in a 5.8 kb specific fragment for CpGV-M. Digestion with *Hin*dIII/*Bgl*II resulted in 6.8 kb and 1.2 kb specific fragments for MCp4 (Fig. 3.10). The CpGV-M:MCp5 ratio in the virus offspring was quantified using a *Bam*HI digest on the isolated virus DNA. This digest resulted in a 7.0 kb specific fragment for CpGV-M and a 11.7 kb specific fragment for MCp5 (Fig. 3.10).

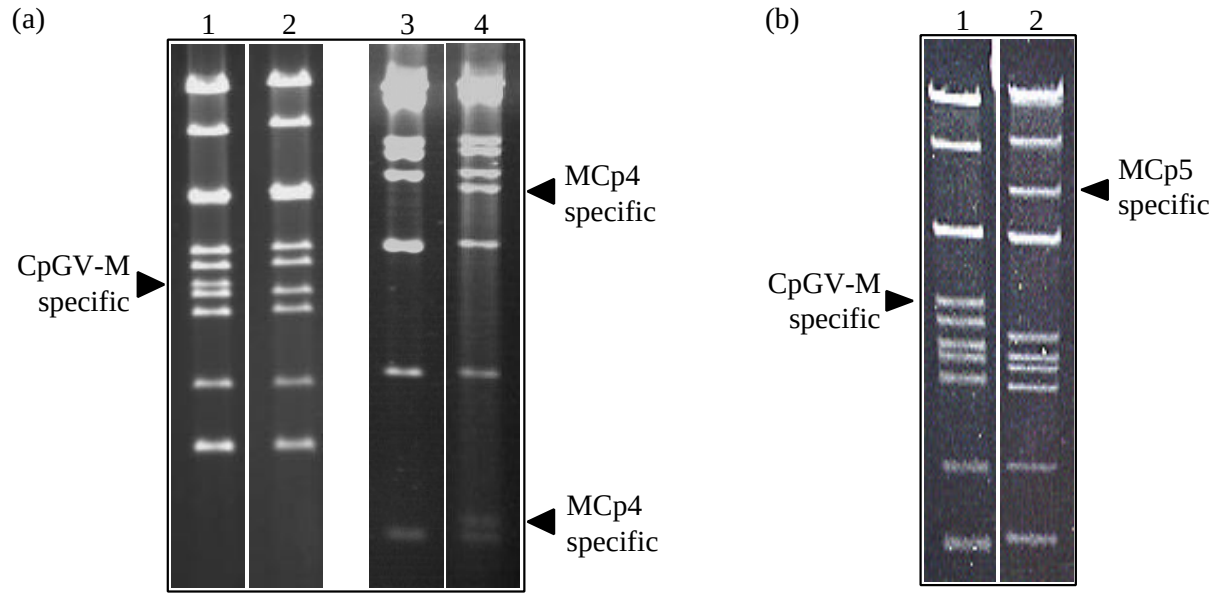
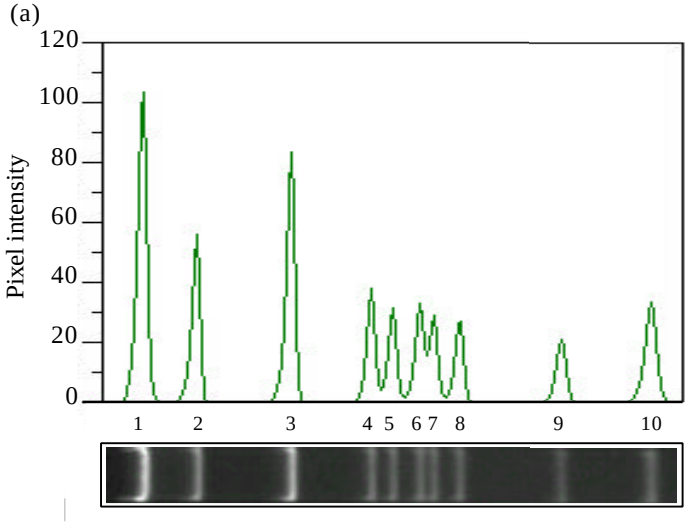


Figure 3.10. CpGV-M, MCp4 and MCp5 genotype specific restriction fragments. **(a)** Digestion with *Bam*HI resulted in a 5.8 kb specific fragment for CpGV-M. Digestion with *Hin*dIII/*Bgl*II resulted in a 6.8 kb and a 1.2 kb specific fragment for MCp4. Lane 1: CpGV-M x *Bam*HI, Lane 2: MCp4 x *Bam*HI, Lane 3: CpGV-M x *Hin*dIII/*Bgl*II, lane 4: MCp4 x *Hin*dIII/*Bgl*II. **(b)** Digestion with *Bam*HI resulted in a 7.0 kb and a 11.7 kb specific fragment for CpGV-M and MCp5, respectively. Lane 1: CpGV-M x *Bam*HI, Lane 2: MCp5 x *Bam*HI. The genotype specific restriction fragments are marked by black arrows.

After digestion of the isolated virus offspring DNA with the selected restriction enzymes, the fragments were electrophoretically separated on agarose gel, stained with ethidium bromide and subsequently photographed under UV light. Using the Imagemaster 1D software, the volumes (the amounts of pixels) of the bands representing the DNA fragments on the photograph were determined. If ethidium bromide evenly binds to the DNA, and if the volumes of the detected bands on the photograph are proportional to the DNA fragment size, the following relation between volume (V), fragment size (L), molarity (M) and a constant factor (C) is expected: V = L x M x C. If this method is valid, the molar ratio of the genotype specific and the genotype shared restriction fragments in the co-infection experiments can be determined. Therefore, it was investigated whether there is a linear relation between fragment size, molarity and quantified volume of the photographed bands. In order to test this assumption, DNA derived from *in vivo* cloned CpGV-M was digested with *Bam*HI and the volumes of the obtained bands were determined using the Imagemaster 1D software (Fig. 3.11a). Since the DNA originated from genotypically homogeneous CpGV-M, the single bands (band 2, 4, 5, 6, 7, 8 and 9) represent fragments in the same molarity (M=1) and the double bands (band 1, 3 and 10) represent restriction fragments in a double molarity (M=2). The volumes of the bands were plotted as

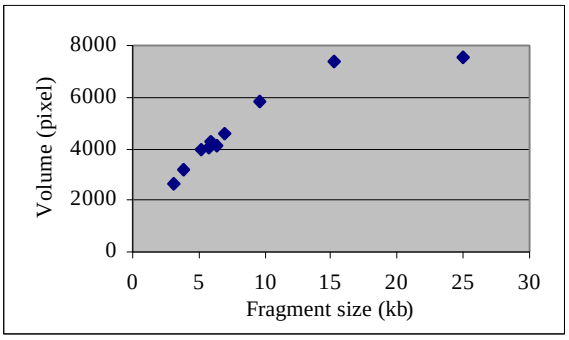
function of the fragment sizes (Fig. 3.11b). The quantified volumes of the double bands were divided by 2 in order to obtain the volume corresponding to the single fragments of these bands (M=1).

(a)



Peak	Fragment (kb)	Volume (pixel)
1	24.4 + 25.4	15072 (7536)
2	15.2	7403
3	9.6 + 9.6	11710 (5855)
4	7.0	4600
5	6.4	4078
6	5.9	4289
7	5.7	4015
8	5.2	3994
9	3.8	3192
10	3.1 + 3.1	5275 (2638)

(b)



(c)

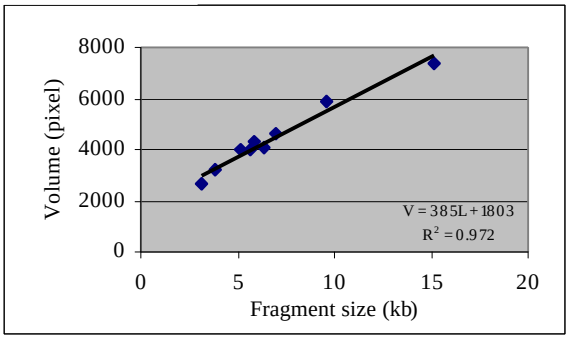


Figure 3.11. Quantification of the volume of CpGV-M/*Bam*HI restriction fragments. **(a)** Determination of the volumes of CpGV-M/*Bam*HI restriction fragments on an ethidium bromide stained agarose gel using the Imagemaster 1D software. The numbers in brackets represent the volumes of the single restriction fragments making up the double bands. **(b)** Size of the CpGV-M/*Bam*HI restriction fragments as a function of its quantified volume. **(c)** Linear regression analysis of the restriction fragment sizes and the quantified volumes. The formula of the regression line and the regression quotient (R^2) are plotted in the graph.

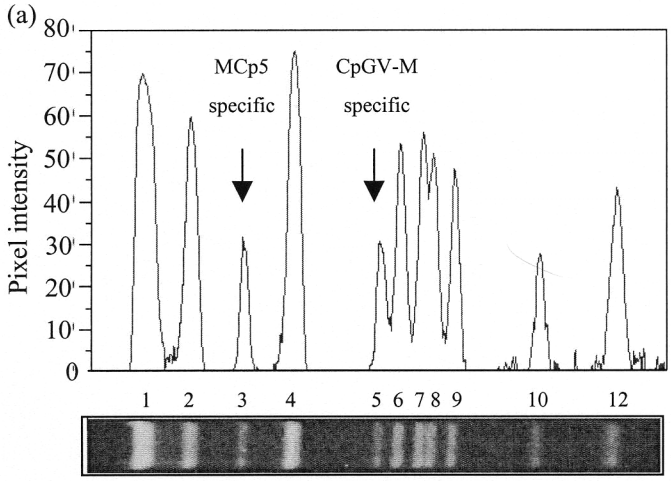
For the smaller fragments, there seemed to be a linearity among the quantified volume, the fragment size and the molarity. Only for the 24.5/25.4 kb double band the quantified volume did not increase proportionally with its size. Linear regression analysis of the CpGV-M/*Bam*HI restriction fragments in the 3-15 kb size range demonstrated a strong correlation (R^2=0.97) between fragment size and volume (Fig. 3.11c). Since the genotype specific restriction fragments to be quantified in the CpGV-M/MCp4 and CpGV-M/MCp5 co-infection experiments were in this size range, the above described quantification method was suitable for the determination of the virus genotype ratios.

3.10. Competition experiments between CpGV-M and MCp4, and CpGV-M and MCp5

The survival analysis demonstrated that MCp4 and MCp5 killed fifth instar *C. pomonella* slower than CpGV-M. However, it was not possible to predict whether the difference in survival times could result in a selection advantage for one of the virus genotypes. In order to compare the propagation efficacy of CpGV-M and the mutants, co-infection experiments of fifth instar *C. pomonella* were performed by oral infection. A selection advantage of one of the genotypes could be determined by comparing the virus ratios in the inoculum with that in the offspring. In order to exclude possible dose effects by the infection, different reciprocal OB ratios of CpGV-M:MCp4 and CpGV-M:MCp5 were applied as inoculum (Table 3.4 and 3.5). Virus OB were isolated from single larvae after they died from the infection. Using the quantification method described in (3.9), the molar proportions of the genotype specific restriction fragments in the virus offsprings were quantified**.**

An example of how the CpGV-M:MCp5 ratio in the offspring of a CpGV-M:MCp5 (5:95) co-infection experiment was determined, is shown in Fig. 3.12**.** The sizes of the restriction fragments were plotted as function of the quantified volumes. Using regression analysis, a regression line for the genotype shared restriction fragments (2, 4, 6-11) was determined; $V=2949L + 8151$ ($R^2=0.94$). Using this formula, volumes were calculated which could be expected for the 7.0 kb and 11.7 kb genotype specific restriction fragments in case of a pure CpGV-M or MCp5 virus offspring (28796 and 42657 pixels, respectively). Since the obtained virus offspring was a mixture of both genotypes, the molar proportion of CpGV-M and MCp5 in the virus offspring was determined by dividing the actual measured volume by the calculated volume. In this example, the CpGV-M genotype makes up 0.56 (16004/28796) and the MCp5 genotype 0.39 (16738/42657) of the virus offspring. Since the virus offspring only consist of CpGV-M and MCp5, the sum of the quantified amounts of CpGV-M and MCp5 should theoretically be 1.0. Although, with 0.95 (0.39 + 0.56) the total amount of actually quantified virus was a little lower, it was shown that the quantification method was fairly accurate.

(a)



Peak	Fragment (kb)	Volume (pixel)
1	24.4 + 25.4	97172 (48586)
2	15.2	53282
3	11.7	16738
4	9.6 + 9.6	64448 (32224)
5	7.0	16004
6	6.4	31291
7	5.9	28375
8	5.7	27153
9	5.2	22652
10	3.8	16479
11	3.1 + 3.1	31332 (15666)

(b)

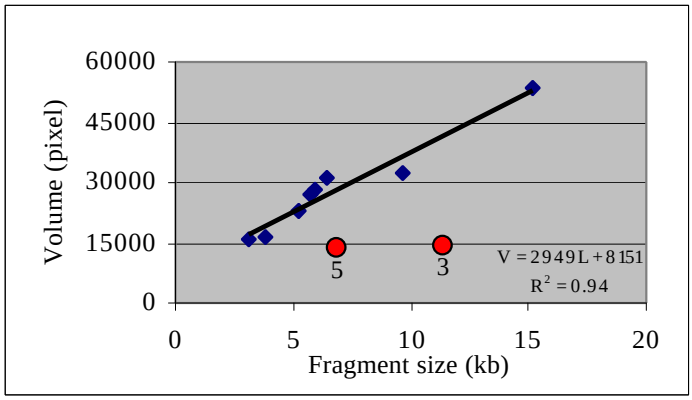


Figure 3.12. Quantification of the CpGV-M:MCp5 ratio in the virus offspring of a co-infection experiment (inoculum: CpGV-M:MCp5 = 5:95). **(a)** Determination of the volumes of the *Bam*HI restriction fragments on an ethidium bromide stained agarose gel using the Imagemaster 1D software. The numbers in brackets represent the volumes of the single restriction fragments making up the double bands. **(b)** Linear regression analysis of the volumes of the CpGV-M and MCp5 genotype shared *Bam*HI restriction fragments. The formula of the regression line and the regression quotient (R^2) are plotted in the graph. The dots represent the 7.0 kb (5) and 11.7 kb (3) CpGV-M and MCp5 genotype specific restriction fragments, respectively.

For every infection ratio in the co-infection experiments, the CpGV-M:mutant ratio in the virus offspring was determined using this method. As shown in Table 3.4, there was a clear difference between the CpGV-M:MCp5 ratios in the inoculum and the offspring. For example, when using an infection ratio of 0.95 MCp5 and 0.05 CpGV-M, only 0.41 of the virus offspring was MCp5, but 0.59 accounted to CpGV-M. This shift towards CpGV-M continued with increasing proportions of CpGV-M in the inoculum. By an inoculum ratio of 0.90:0.10, MCp5 was no longer detectable in the virus offspring. As expected, infections using a ratio of 1.0 CpGV-M or MCp5 genotype resulted in a pure corresponding virus offspring.

Table 3.4. Competition experiments between CpGV-M and MCp5 using different inoculum ratios.

Inoculum ratio CpGV-M:MCp5	Offspring ratio CpGV-M:MCp5	n	SE
1.00:0.00	1.00:0.00	3	n.d.
0.90:0.10	1.00:0.00	4	n.d.
0.60:0.30	0.93:0.07	5	0.029
0.50:0.50	0.88:0.12	8	0.040
0.30:0.60	0.80:0.20	6	0.060
0.10:0.90	0.61:0.39	9	0.072
0.05:0.95	0.59:0.41	3	0.078
0.00:1.00	0.00:1.00	3	n.d.

n= number of analysed larvae, SE= standard error based on the quantification of MCp5, n.d.= not determined.

Table 3.5. Competition experiments between CpGV-M and MCp4 using different inoculum ratios.

Inoculum ratio CpGV-M:MCp4	Offspring ratio CpGV-M:MCp4	n	SE
1.00:0.00	1.00:0.00	3	n.d.
0.90:0.10	1.00:0.00	7	n.d.
0.60:0.30	1.00:0.00	5	n.d.
0.50:0.50	1.00:0.00	6	n.d.
0.30:0.60	1.00:0.00	5	n.d.
0.10:0.90	0.90:0.10	4	0.041
0.00:1.00	0.00:1.00	5	n.d.

n= number of analysed larvae, SE= standard error based on the quantification of MCp4, n.d.= not determined.

A similar, but more dramatic situation was observed in the co-infection experiments using different ratios of CpGV-M and MCp4 OB as inoculum (Table 3.5). When using an infection ratio of 0.90 MCp4 and 0.10 CpGV-M, only 0.10 of the virus offspring was MCp4, but 0.90 accounted to CpGV-M. When the virus inocula contained a ratio of 0.60 or less MCp4, only restriction fragments specific for CpGV-M could be detected in the virus progeny. Infections using a ratio of 1.00 for CpGV-M or MCp4 resulted clearly in an expected pure corresponding virus offspring. These results demonstrated that the transposon containing mutants MCp4 and MCp5 had a tremendous propagation disadvantage compared to wild type CpGV-M when *C. pomonella* larvae were infected perorally.

The previous competition experiment showed that MCp4 and MCp5 are efficiently out-competed by CpGV-M. In order to investigate whether the mutants can be sustained in the virus population, the viruses were serially passaged through larvae. The larvae (Fig. 3.13a, larvae A1) were perorally infected with OB of CpGV and MCp4 or CpGV-M and MCp5 in a 0.10:0.90 ratio. Progeny viruses were used to infect the following passage larvae in two different ways, (i) OB isolated from larval cadavers were used to orally infect next passage larvae (Fig. 3.13a, larvae A2-1 and A3-1), and (ii) BV isolated from the hemolymph of infected larvae was injected into the hemolymph of the next passage larvae (Fig. 3.13a, larvae A2-2 and A3-2).

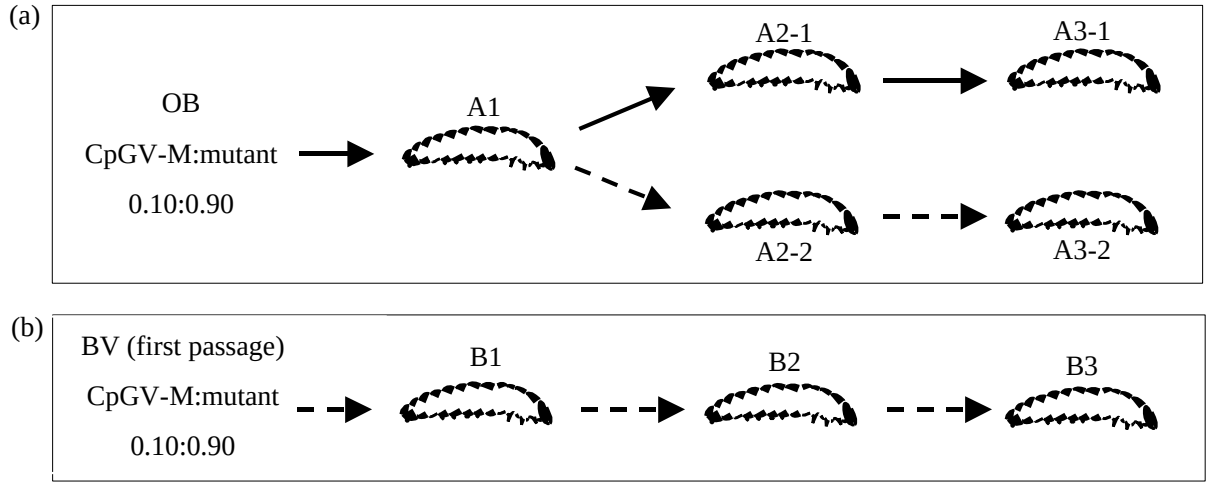


Figure 3.13. Competition experiment between CpGV-M and MCp4, and CpGV-M and MCp5 in *C. pomonella* larvae transferred through several larval passages. **(a)** Larvae (A1) were orally infected with OB (CpGV-M:mutant = 0.10:0.90). Progeny OB were subsequently used to infect the next passage larvae (A2-1 and A3-1). Larvae A2-2 and A3-2 were infected with BV isolated from the hemolymph of infected larvae by injection into the hemolymph. **(b)** Larvae B1 were infected with BV (CpGV-M:mutant = 0.10:0.90) by injection in the proleg. Progeny BV were isolated from the hemolymph and transferred to the next passage larvae (B1 and B2) by injection in the proleg. The line arrow represents the peroral transfer of OB, the dashed arrow indicates the transfer of BV by injection.

DNA was isolated from OB obtained from about 25 pooled larval cadavers and the ratio of CpGV-M:mutant was densitometrically determined as described above. As shown in Table 3.7, the ratio of MCp5 in the virus offspring decreased from 0.90 in the inoculum to 0.46, 0.10 and 0 after 1, 2 and 3 passages, respectively (larvae A1, A2-1 and A3-1). When BV was used in the passage experiment, the ratio of MCp5 had already decreased to 0.10 after the first passage (larvae A2-2) and to undetectable levels after the second passage. Again, for MCp4 similar but more dramatic results were obtained (Table 3.8), after the first OB passage, only 0.10 of the virus offspring was MCp4, but 0.90 accounted to CpGV-M (larvae A1). After the second passage, only restriction fragments specific for CpGV-M could be detected in the virus progeny (larvae A2). When BV were used in this passage experiment, MCp4 was no longer detectable in the virus offspring after the first passage (larvae A2-2). This finding indicated that MCp4 as well as MCp5

can be sustained slightly longer in the CpGV virus mixtures when the viruses are passaged in larvae as OB *per os* rather than as BV by injection.

Table 3.7. Ratio of CpGV-M and MCp5 in the inoculum and the offspring of infected *C. pomonella* larvae described in Fig. 3.13.

Exp.	Larvae	Inoculum	Offspring CpGV-M:MCp5	n	SE
A	A1	OB (CpGV-M:MCp5 = 0.10:0.90)	0.54:0.46	3	0.032
	A2-1	OB from A1	0.90:0.10	3	0.019
	A3-1	OB fromA2-1	1.00:0.00	3	n.d.
	A2-2	BV from A1	1.00:0.00	3	n.d.
	A3-2	BV from A2-2	1.00:0.00	3	n.d.
B	B1	BV (CpGV-M:MCp5 = 0.10:0.90)	0.88:0.12	3	0.009
	B2	BV from B1	1.00:0.00	3	n.d.
	B3	BV from B2	1.00:0.00	3	n.d.

n= number of larvae analysed, SE= standard error based on the quantifications of MCp5, n.d.= not determined.

Table 3.8. Ratio of CpGV-M and MCp4 in the inoculum and the offspring of infected *C. pomonella* larvae described in Fig. 3.13.

Exp.	Larvae	Inoculum	Offspring CpGV-M:MCp4	n	SE
A	A1	OB (CpGV-M:MCp4 = 0.10:0.90)	0.71:0.29	3	0.042
	A2-1	OB from A1	0.98:0.02	3	0.023
	A3-1	OB fromA2-1	1.00:0.00	3	n.d.
	A2-2	BV from A1	1.00:0.00	3	n.d.
	A3-2	BV from A2-2	1.00:0.00	3	n.d.
B	B1	BV (CpGV-M:MCp4 = 0.10:0.90)	1.00:0.00	3	n.d.
	B2	BV from B1	1.00:0.00	3	n.d.
	B3	BV from B2	1.00:0.00	3	n.d.

n= number of larvae analysed, SE= standard error based on the quantifications of MCp4, n.d.= not determined.

Since the detection of genotype specific restriction fragments is not very sensitive, PCR studies were performed to investigate whether traces of MCp4 and MCp5 could be detected in the DNA samples used for restriction analysis (Fig. 3.14). PCR analysis using a transposon specific and a CpGV specific primer (PR-MCp5-LB and PR-TCl4.7-int for MCp5, and PR-MCp4-LB and PR-TCp3.2-int for MCp4, see Fig. 3.5) showed a decreasing intensity of MCp4 and MCp5 specific products in all samples of this passaging experiments (Fig. 3.14). This result suggested

that despite the tremendous propagation disadvantage compared to CpGV-M, traces of MCp5 and MCp4 sustained in the virus population even after three successive passages.

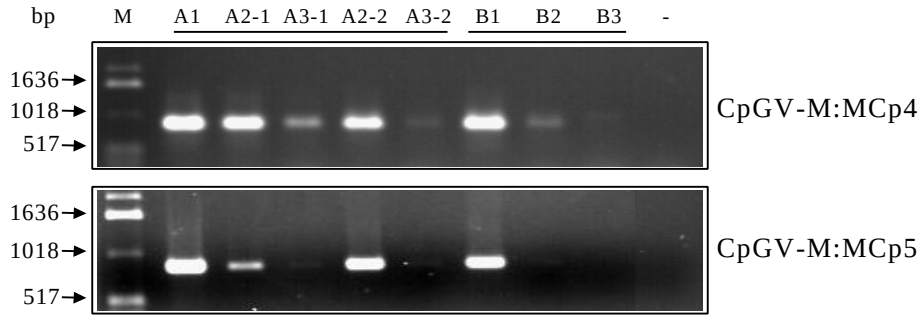


Figure 3.14. PCR analysis to detect MCp4 and MCp5 in the virus progeny of the co-infection experiments. Using PCR primers specific for CpGV-M and transposons TCp3.2 and TCl4.7, MCp4 and MCp5 specific fragments were amplified. Lane M shows the 1 kb marker, lanes A1 to B3 correspond to the larvae in co-infection experiments described in Fig. 3.13 and the last lane (-) shows a negative control containing CpGV-M DNA. Fragment sizes are indicated on the left.

The previous experiments suggested that MCp5 as well as MCp4 viruses were sustained slightly longer in the CpGV population when they were passaged as OB rather than as BV. However, in this experiment the CpGV-M:mutant ratio of BV in the inoculum used to infect larvae A2-2 (Fig. 3.13a) was unknown. In order to investigate how the CpGV-M:mutant ratio changes when BV are passaged, an experiment with a known ratio of BV in the primary inoculum was performed by injecting BV (CpGV-M:mutant = 0.10:0.90) obtained from infected CpDW14R cell lines into the proleg of *C. pomonella* larvae. The infection ratio of 0.10:0.90 was based on determined $TCID_{50}$ values of passage 1 virus, titrated on *C. pomonella* cells. Hemolymph was collected from larvae 4 days p.i. and passaged by injection into fifth instar larvae. BV was passaged three times by injection into larvae (Fig. 3.13b, larvae B1, B2 and B3). Quantification of MCp5 and CpGV-M in the isolated OB fractions showed again that MCp5 was out-competed rapidly by CpGV-M. After one passage the amount of MCp5 in the virus progeny was already reduced to 0.10 and after the second passage to undetectable levels. Again, the outcome of the MCp4/CpGV-M passage experiment was more extreme. After the first passage MCp4 was no longer detectable in the virus progeny. When the isolated DNA was amplified in a PCR, MCp5 and MCp4 were still detectable in the progeny virus population after two passages but disappeared under the PCR detection level after the third passage (Fig. 3.14). The different competition experiments using OB and BV thus ruled out that MCp5 and MCp4 were selected during the mixed infection experiments performed by Jehle *et al*. (1995).

3.11. TCp3.2 copies in *C. pomonella* cell lines

Prior to the experiments to determine t32a transcription in *C. pomonella* larvae and cell lines, an experiment was performed to analyse whether TCp3.2 copies are also present within the genome of the cultured cells which were originally derived from embryonic cells of *C. pomonella*. DNA was isolated from CpDW14, CpDW14R and CpDW15 cells and was digested with *Hin*dIII. For each cell line, 10 µg of DNA were electrophoretically separated on an agarose gel and Southern blots were prepared. As TCp3.2 specific hybridisation probe a [^{32}P]dCTP labelled PCR amplified fragment of the transposase gene, obtained with the primer pair PR-t32-up/PR-t32-END, was used.

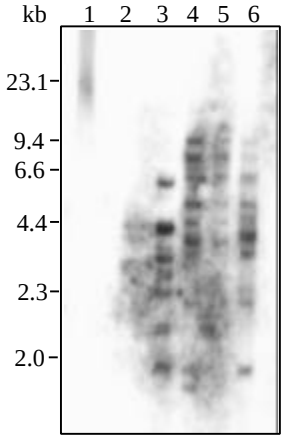


Figure 3.15. Southern analysis to identify TCp3.2 copies in different *Cydia pomonella* cell lines. The genomic DNAs were digested with *Hin*dIII and hybridised with a TCp3.2 specific probe. Lane 1: MCp4 (positive control), lane 2: *C. leucotreta* (negative control), lane 3: *C. pomonella*, lane 4: CpDW14, lane 5: CpDW14R and lane 6: CpDW15. Fragment sizes are indicated on the left.

As shown in Fig. 3.15, TCp3.2 specific signals were found in all tested cell lines. The size and number of bands were almost identical among the three cell lines. The observed band patterns for the cell lines, however, were quite different compared to the band pattern of *C. pomonella* larvae. The number of signals were similar, but for *C. pomonella* larvae more transposon specific signals were identified on DNA fragments of a lower molecular weight compared to the cell lines. As previously demonstrated, no TCp3.2 specific signals were detected in *C. leucotreta* (Jehle *et al*., 1998). These results showed that the *C. pomonella* cell lines as well as *C. pomonella* larvae contain about 10 copies of transposon TCp3.2.

3.12. Characterisation of the transposase transcripts of transposon TCp3.2

The DNA sequence of transposon TCp3.2 was recently analysed by Jehle *et al*. (1998). This study suggested that the TCp3.2 transposase gene (t32a) putatively consists of two exons

separated by an intron of 178 bp. In order to prove this sequence based prediction, the intron region and splicing borders of t32a transcripts were identified in RNA isolated from virus infected and mock infected larvae. Total RNA was isolated from mock and CpGV-M infected larvae and first strand cDNA was prepared by reverse transcription. A PCR was carried out on the prepared cDNA using primer PR-t32a-up and PR-t32a-lo which are located within exon 1 and exon 2 (Fig. 3.16a). The fragment sizes expected for the PCR products are listed in Fig. 3.16b. Since the primers were located beyond the putative intron borders, fragments of spliced and unspliced (pre-mRNA) transcripts could be amplified in this RT-PCR. As shown in Fig. 3.16c, fragments of the expected size (371 bp) for unspliced t32a transcripts were found in CpGV-M and mock infected larvae. Additionally a 172 bp fragment, possibly originating from spliced transcripts, was detected in CpGV-M infected larvae. In the control reactions without RNA or SuperScript in the reverse transcriptase reaction (Fig. 3.16c, lane 3-5) no PCR products were obtained. This demonstrated that the amplified fragments in lane 1 and 2 originated from mRNA transcripts which were reversibly transcribed to cDNA.

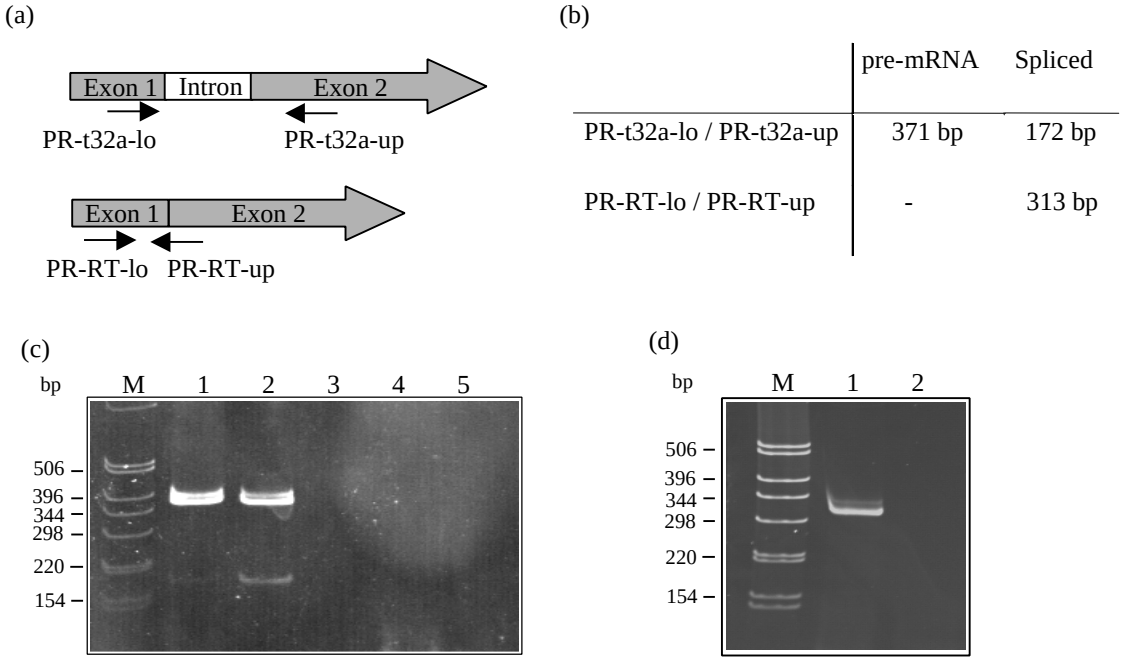


Figure 3.16. RT-PCR on TCp3.2 transposase transcripts. **(a)** Binding sites of the used primers in the RT-PCR. **(b)** Expected size of the PCR amplified transposase fragments using primer the combinations PR-RT-lo/PR-t32a-up and PR-RT-lo/PR-RT-up. **(c)** RT-PCR on RNA prepared from mock and CpGV-M infected *C. pomonella* (*Cp*) larvae using primers PR-t32a-lo and PR-t32a-up. Lane M: 1 kb marker, lane 1: L3 *Cp* larvae, mock infected, lane 2: L3 *Cp* larvae, CpGV-M infected, Lane 3: negative control, no RNA in cDNA synthesis reaction, lane 4/5: negative control, no reverse transcriptase in cDNA synthesis reaction of mock and CpGV-M infected L3 *Cp* larvae. Fragment sizes are indicated on the left. **(d)** RT-PCR on RNA prepared from CpGV-M infected *Cp* larvae using primers PR-RT-lo and PR-RT-up. Lane M: 1 kb marker, lane 1: L3 *Cp* larvae, CpGV-M infected, lane 2: negative control, no reverse transcriptase in cDNA synthesis reaction of CpGV-M infected L3 *Cp* larvae.

The amplified fragments of t32a cDNA were separated on an agarose gel and cloned in *E. coli* using the pGEM-T vector. Inserts from 12 randomly picked clones were sequenced and aligned with the sequence of TCp3.2 found in MCp4 (Jehle *et al*., 1998). As shown in Fig. 3.17, 10 out of 12 randomly picked clones represented cDNAs of unspliced mRNAs, two of them (t32a-11 and t32a-12) represent RT-PCR products of spliced transcripts. The intron appeared to be 199 bp long and had typical eucaryotic splicing borders (GT....AG). The borders corresponded to nucleotides 1347/1348 and 1544/1545 of the TCp3.2 sequence published by Jehle *et al*. (1998), resulting in an intron which was 21 bp longer than predicted there.

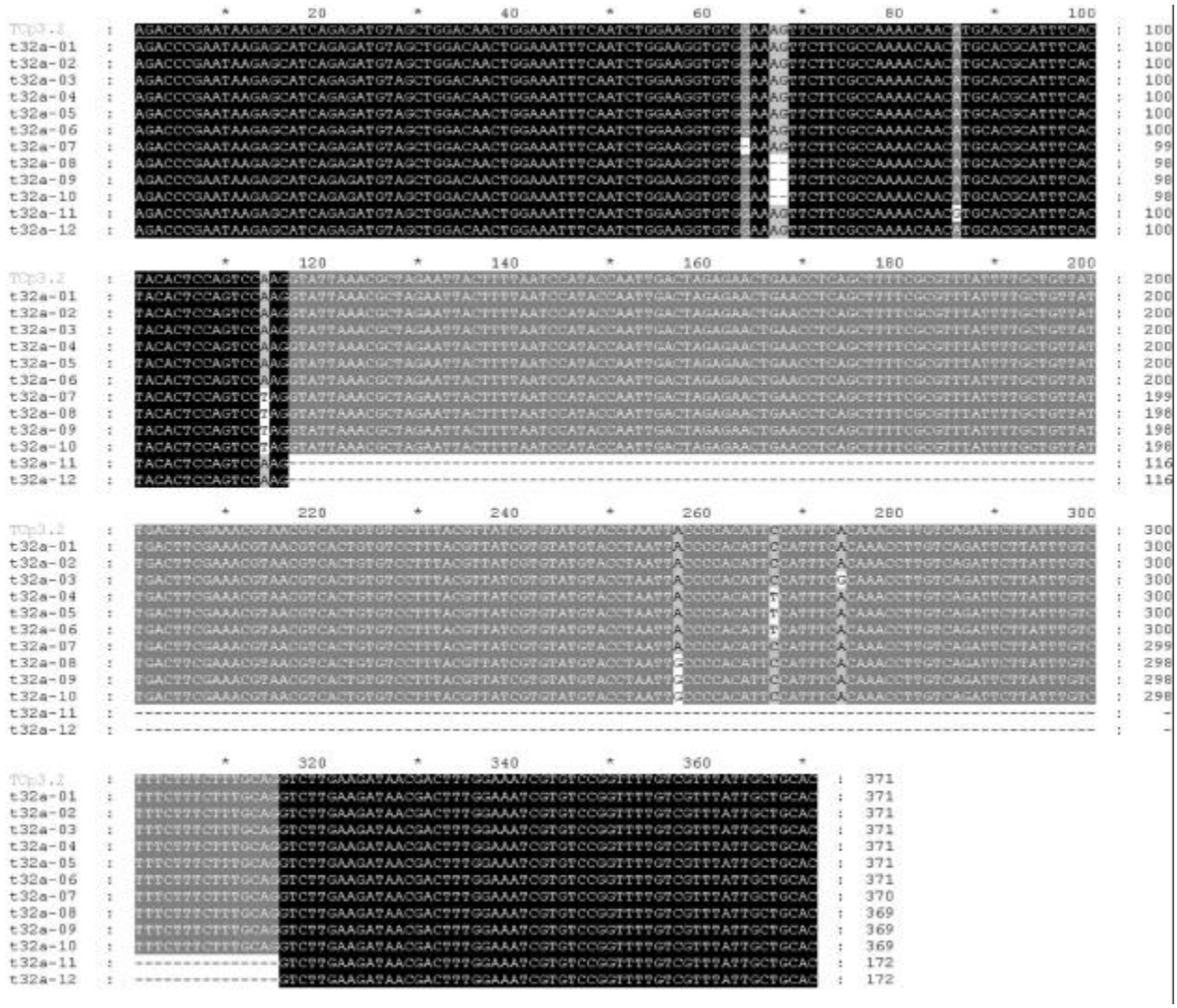


Figure 3.17. Sequence alignment of the intron and bordering regions of RT-PCR products of the transposase t32a obtained from CpGV-M infected larvae (Fig 3.16c, lane 2). t32a-01 - 12 represent RT-PCR products, TCp3.2 shows the t32a sequence found in MCp4. Deletions are marked with (-). Heterogeneities are shaded in grey.

The alignment further showed that only two sequences (t32a-01 and t32a-02) were identical to the t32a sequence present in MCp4 (Jehle *et al*., 1998). Among the transposase cDNAs some heterogeneities caused by nucleotide substitutions or deletions were observed. The

sequence heterogeneity included a A86G substitution for sequence t32a-11 resulting in a valine (GTG) codon instead of methionine (ATG). The A114T substitution was observed at nearly the same frequency among the clones. This change resulted in a codon heterogeneity of CTA (leucine) and CAA (glutamic acid). Three sequences (t32a-08, -09 and -10) had a deletion of an AG dinucleotide at position 67-68, and sequence t32a-07 showed a deletion of a G nucleotide at position 64. The AG deletion caused a frameshift mutation which lead to a translation termination at the TGA stop codon (nt 321-323, Fig. 3.17) in exon 2. The deletion of the G nucleotide cause a frameshift mutation which lead to a transcription termination at the TGA stop codon (nt 62-64, Fig. 3.17) in exon 1. The detection of these non functional transposase transcripts indicated that a substantial amount of the TCp3.2 transposons within the *C. pomonella* genome encoded a defective transposase.

3.13. Quantification of transposase transcription

In Fig. 3.16c, RT-PCR products of 371 bp representing unspliced transcripts of t32a were found in mock infected and CpGV-M infected larvae. In addition, there was an extremely faint 172 bp fragment in the cDNA preparation of mock infected larvae and a considerably stronger band in that of the infected larvae which both represent the spliced t32a mRNA. The different intensities of the PCR signal suggested a variation in t32a transcription and splicing in mock and virus infected larvae. In order to investigate the t32a transcription and expression in mock and CpGV infected larvae, Northern blot analyses of RNA preparations of total larvae using a t32a specific hybridisation probe, and Western blot analyses of protein preparations of total larvae using a T32a specific antibody, were performed (Fig. 3.18 and 3.19). Neither Northern nor Western analysis gave transposase specific signals. This indicated that the t32a transcription and expression were probably too low to exceed the detection levels.

In order to quantify the t32a transcription and subsequent splicing, real time RT-PCR was carried out on mRNA prepared from mock and CpGV-M infected third and fifth instar *C. pomonella*. Total t32a transcription was quantified by using the primer combination PR-t32a-up and PR-t32a-lo, whereas primers PR-RT-up and PR-RT-lo were used for the amplification of the cDNAs from spliced transcripts (Fig. 3.16a). In the latter PCR the upper primer was designed in such a way that it annealed to the 5'-end of exon 2 and to the 3'-end of exon 1, thus annealing only to cDNAs of spliced t32a mRNA but not to those of pre-mRNA or to the background transposon DNA. As shown in (Fig. 3.16d), only a 313 bp product was obtained when these primers were

used in a RT-PCR on CpGV-M infected larvae. No product was obtained in the control reaction where no reverse transcriptase was added to the cDNA reaction. This proved that the primer pair (PR-RT-up/PR-RT-lo) used was specific for the amplification of spliced transposase transcripts.

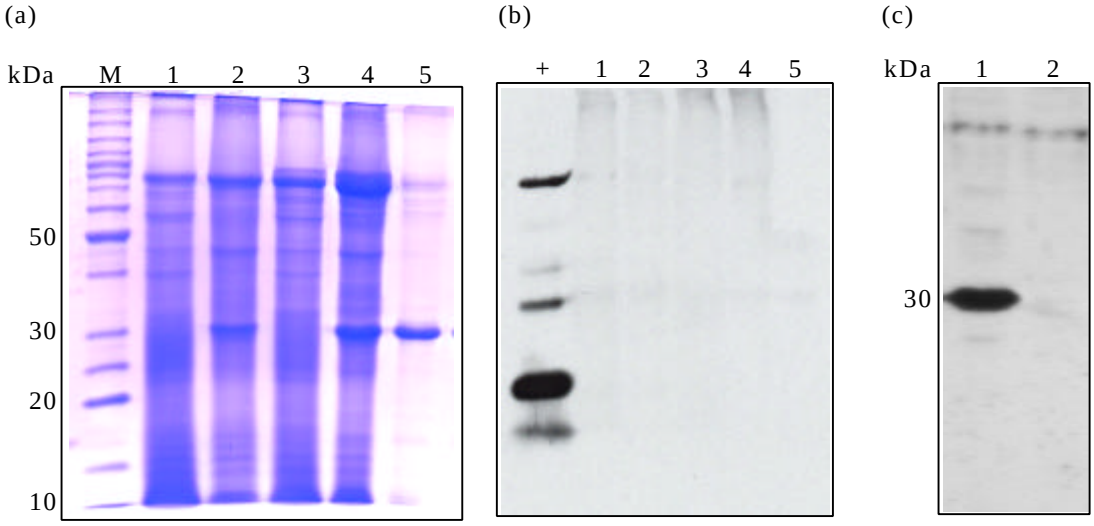


Figure 3.18. Western blot to detect transposase of TCp3.2 in mock and CpGV-M infected *C. pomonella* larvae using a transposase T32a specific antibody (Arends, 1995). **(a)** The protein obtained from *C. pomonella* larvae and CpGV-M occlusion bodies were separated on a SDS PAGE and stained with Commassie Blue. Lane M: Marker, Lane 1: L3 larvae/mock infected, Lane 2: L3 larvae/CpGV-M infected, Lane 3: L5 larvae/mock infected, Lane 4: L5 larvae/CpGV-M infected and Lane 5: CpGV-M OB. Protein sizes are indicated on the left. **(b)** Western blot of SDS gel (a) hybridised with the T32a specific antibody. Lane +: positive control (*E. coli* expressed protein used to raise the antibody), Lane 1: L3 larvae/mock infected, Lane 2: L3 larvae/CpGV-M infected, Lane 3: L5 larvae/mock infected, Lane 4: L5 larvae/CpGV-M infected, Lane 5: CpGV-M. **(c)** Western blot to demonstrate the specificity of the antibody (Jehle *et al*., 1999). Lane 1: a baculovirus expressed T32a (AcT32A in *Sf*21 cells), Lane 2: negative control (AcNPV-PAK6, the parental strain of AcT32A, in *Sf*21 cells).

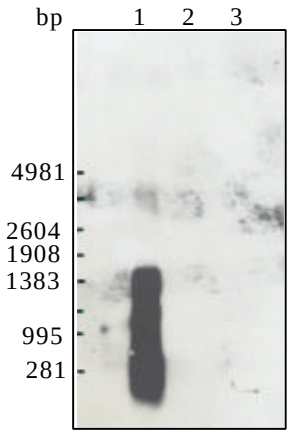


Figure 3.19. Northern blot to detect transposase t32a transcripts in mock and CpGV-M infected *C. pomonella* larvae. DIG-labelled strand specific RNA transcripts were used as a probe. These transcripts were prepared in a transcription reaction from a cloned t32a fragment (pT32A) which was cloned in pGEM-7Zf(+) using SP6 RNA polymerase. Lane 1: positive control, RNA transcripts obtained from the pT32A/T7 transcription reaction, Lane 2: total RNA from mock infected L3 larvae, Lane 3: total RNA from CpGV-M infected L3 larvae**.** Fragment sizes are indicated on the left.

Another control experiment was designed to prove that the specific 172 bp and 313 bp PCR products originated from spliced transcripts and not from possible intron free TCp3.2 copies

within the *C. pomonella* genome. In PCRs performed on RNase treated genomic DNA of *C. pomonella* larvae no fragments of 313 or 172 bp were detected (results not shown), indicating that no TCp3.2 copies without intron were present within the genome of *C. pomonella* and that the products in the RT-PCRs originated from spliced transcripts.

For every quantification reaction of the total transposase transcription, a control measurement was also performed in order to demonstrate that the detected amplified PCR fragments originated from synthesised cDNA and not from contaminating DNA (Fig. 3.20a). A sample in which a first strand synthesis reaction was carried out with the same RNA but no reverse transcriptase served as control. In these control reactions, the quantified transposase template molecules were at least 1000 times lower than in the cDNA containing samples (Fig. 3.20b). This showed that the amount of DNA in the samples was negligible compared to the amount of cDNA template molecules. A melting curve analysis of the PCR products further showed that only specific products with a melting point of 79°C were amplified (Fig. 3.20b). For the quantification of spliced transcripts using primer combination (PR-RT-up/PR-RT-lo) similar control reactions were not necessary since t32a DNA is not amplified by these primers (Fig. 3.16d).

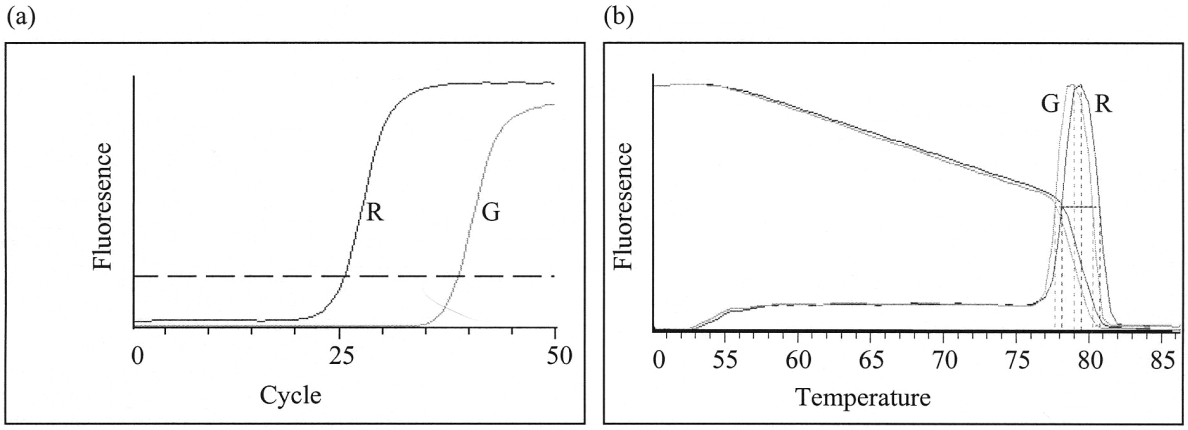


Figure 3.20. A typical real time RT-PCR result for the quantification of total transposase t32a transcription in *C. pomonella* larvae using the primer pair PR-t32a-up/PR-t32a-lo. **(a)** The increase of fluoresence is plotted as a function of the PCR cycles. Red curve (R): Quantification reaction using cDNA synthesised from CpGV-M infected third instar larvae as template, Green curve (G): The control reaction using the same template as above, with the only difference that no reverse transcriptase was added to the cDNA synthesis reaction. **(b)** Melting curve of the PCR products obtained in (a).

The amounts of total and spliced transposase transcripts were quantified by real time RT-PCR in third and fifth instar larvae, which had been mock or CpGV-M (LD_{95}) infected 4 days prior to RNA isolation. With this approach it was possible to determine the potential differences in

the transcription levels among the individual larvae and among different instars. The quantification of transposase transcripts was compared between cDNA of single and pooled larvae which belonged to the same experimental group. As shown in Table 3.9 and Fig. 3.21, there was a good correlation between the quantification of transcript levels of pooled cDNA and the mean of single cDNA for both third and fifth instar larvae. This proved that the determination of the t32a transcription level using real time RT-PCR was very accurate.

Table 3.9. t32a transposase transcripts in mock and CpGV-M infected *C. pomonella* larvae and cell line CpDW14R.

C. pomonella	**Transcripts**	**Inoculum**	**Quantification in pmole[a]**	**Mean (n)[b]; SE**
L3 (single)	Total	Mock	9.59E-08, 5.05E-08, 1.49E-08, 3.58E-08	4.93E-08 (4); 1.71E-08
	Total	CpGV-M	2.17E-07, 2.03E-08, 1.92E-07, 2.17E-08, 5.86E-08	1.02E-07 (5); 4.26E-08
	Spliced	Mock	2.72E-09, 8.59E-09, 1.28E-08, 5.07E-10	6.15E-09 (4); 2.80E-09
	spliced	CpGV-M	1.44E-08, 2.19E-09, 6.53E-08, 7.29E-09, 2.64E-09	1.84E-08 (5); 1.19E-08
L3 (pooled)	Total	Mock	3.49E-08 (4)[c]	
	Total	CpGV-M	1.27E-07 (5)[c]	
	Spliced	Mock	1.36E-09 (4)[c]	
	spliced	CpGV-M	1.19E-08 (5)[c]	
L5 (single)	Total	Mock	4.81E-08, 2.31E-09, 9.73E-09	2.00E-08 (3); 1.42E-08
	Total	CpGV-M	2.21E-08, 5.46E-08, 1.97E-08	3.21E-08 (3); 1.13E-08
	Spliced	Mock	3.61E-09, 4.81E-10, 1.48E-10	1.41E-09 (3); 1.10E-09
	spliced	CpGV-M	9.67E-10, 3.47E-09, 1.52E-09	1.99E-09 (3); 7.60E-10
L5 (pooled)	Total	Mock	2.50E-08 (3)[c]	
	Total	CpGV-M	3.27E-08 (3)[c]	
	Spliced	Mock	1.48E-09 (3)[c]	
	spliced	CpGV-M	2.78E-09 (3)[c]	
CpDW14R	Total	Mock	1.82E-08	
(t=3 days p.i.)	Total	CpGV-M	1.17E-08	
	Spliced	Mock	1.03E-09	
	Spliced	CpGV-M	3.63E-09	
CpDW14R	Total	Mock	2.16E-07	
(t=6 days p.i.)	Total	CpGV-M	7.93E-08	
	Spliced	Mock	2.53E-08	
	Spliced	CpGV-M	5.87E-09	

n= number of tested larvae, SE= Standard Error, L3= third instar, L5= fifth instar.

[a] Pico mole transposase cDNA per first strand synthesis reaction starting from 400 ng total RNA. Each number represents a separate measurement.

[b] Mean amount of transposase for the (n) single larvae.

[c] Mean amount of transposase for each of the (n) pooled larvae.

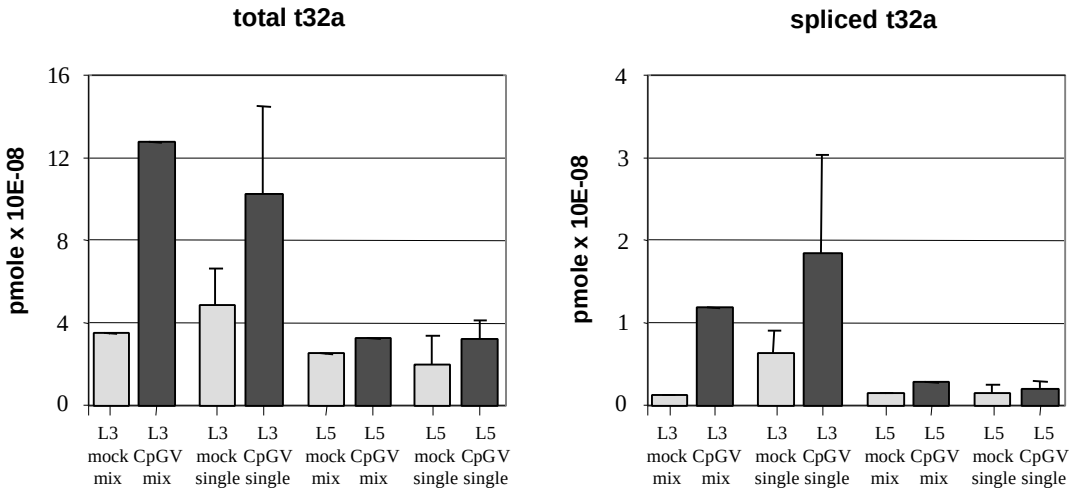


Figure 3.21. Real time RT-PCR of RNA isolated from CpGV-M infected and mock infected third (L3) and fifth (L5) instar *C. pomonella*. RNA was isolated and cDNA was prepared from 4 to 5 individual larvae and the samples analysed either separately (single) or pooled (mix). The bars indicate the standard error.

Comparison of the total transposase transcription demonstrated that the t32a cDNA (transcription) levels were increased about 2-3 times in CpGV-M infected third instar larvae compared to the mock infected third instar larvae (Table 3.9 and Fig. 3.21). For spliced transcripts this difference was about 3-8 times higher, depending on whether pooled cDNA or single cDNA was measured. Comparison between the amount of total t32a cDNA and spliced cDNA in third instar larvae revealed that only about 4-12% of the total t32a cDNA was present as a spliced product in the mock infected larvae. This ratio increased to about 10-20% for CpGV infected larvae. Allthough only a small number of larvae were examined and the measured differences were statistically not significant, these data are a first indication that the virus infection might have an influence on the transposase transcription level in third instar larvae. The differences in transposase transcription and splicing between virus infected and mock infected larvae were not observed in fifth instar larvae. Additionally, the cDNA levels appeared to be generally higher for third instar larvae than for fifth instar larvae, suggesting that that the developmental stage of the larvae had an influence on the endogenous t32a transcription level.

3.14. Transposase transcription in CpDW14R

The transposase t32a transcription and spliced products were also quantified in mock and CpGV-M infected *C. pomonella* CpDW14R cells. Real time RT-PCR was carried out on cDNA prepared from mock and CpGV infected cell cultures three and six days post infection (Table 3.9 and Fig. 3.22). In order to amplify the spliced and unspliced transcripts, the same primer pairs were used as described above. Comparison of the total transposase transcription demonstrated that

three days p.i. the t32a cDNA levels in mock and CpGV-M infected cells were about the same. Six days p.i. however, mock and CpGV-M infected cells showed an increase in transcription of 11.9 and 6.8 times, respectively. For spliced t32a transcripts, three days p.i. the cDNA levels were about the same for mock and CpGV infected cells. This level increased about 24 times six hours p.i. in mock infected cells, whereas it did not change in CpGV infected cells. This finding is in contrast to the situation found in *C. pomonella* larvae where CpGV-M infection stimulated the t32a transcription.

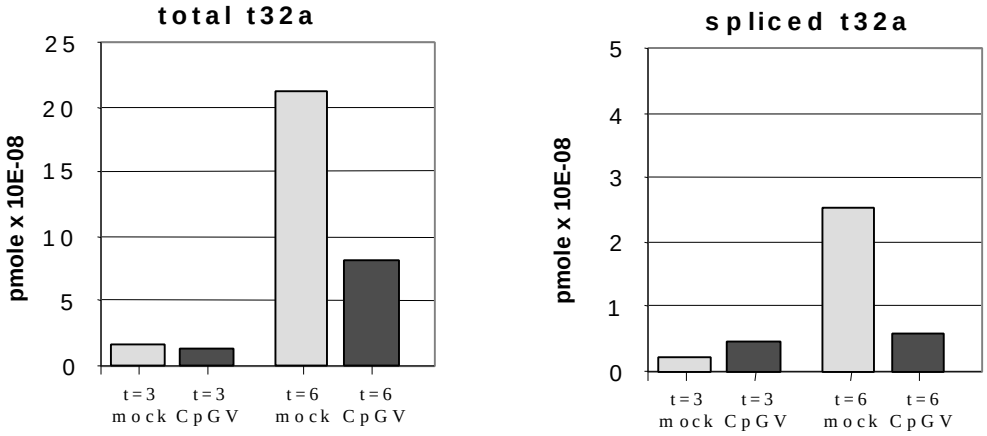


Figure 3.22. Real time RT-PCR to determine the transposase t32a transcription and splicing in CpGV-M infected and mock infected CpDW14R cells. RNA was isolated and cDNA was prepared from cells harvested at t=3 and 6 days post infection.

4. DISCUSSION

4.1. The TCl4.7 integration site in CpGV

The genomic region where transposon TCl4.7 integrated into CpGV was sequenced and characterised. The TA dinucleotide integration site was situated between CpGV repeated sequence 3 and 4 which are located in the non translated region between Cp15 and Cp16 (Luque *et al*., 2001). Repeated sequence 3 and 4 are part of a group of 13 identified repeated imperfect palindromes of about 75 bp which are widely distributed on the CpGV-M1 genome (Luque *et al*., 2001). These sequences are never present as multiple tandem repeats as it is typical for *hr* elements identified in most other baculovirus genomes (Hayakawa *et al*., 2000). Because repeated sequence 3 and 4 are only separated by 90 bp, it remains to be investigated whether these elements actually function as origins of replication or as transcription enhancers (Theilmann & Stewart, 1992; Guarino & Summers, 1996; Pearson *et al*., 1992; Ahrens *et al*., 1995; Kool *et al*., 1995).

Comparison of the obtained 2211 bp of the CpGV-M genome sequence with the corresponding CpGV-M1 sequence, demonstrated that these sequences differ in only one nucleotide. CpGV-M contained one additional G nucleotide within repeated sequence 4. With this extra G, the 7 left and right bordering nucleotides of repeat 4 form a perfect palindrome. It is unknown which consequence this additional nucleotide has on the function of repeated sequence 4.

Due to the presence of the two repeated sequences, the non coding region between Cp15 and Cp16 has a size of 374 bp, which is relatively large for a baculovirus intergenic region. The integration site of TCl4.7 is located 163 bp downstream from the stop codon of Cp16 and 211 bp upstream from the ATG start codon of Cp15. The distances between the integration site and the putative early (93 bp) and late (191 bp) transcriptional start sites of Cp15, are substantial. Whether the transposon integration has an influence on the transcription of Cp15 and Cp16 remains unclear. Like TCl4.7, TCp3.2 also integrated into a non coding region of the CpGV genome (Jehle *et al*., 1997). This non translated region, which is located between Cp41 (*lef*2) and Cp42, is 94 bp long. Like the TCl4.7 integration region, the TCp3.2 integration region is also AT rich (73.6%), but does not contain CpGV repeated sequences. Like most types of transposons, *Tc1/mariner* like elements do not integrate randomly into potential target sites of their host genome. Appropriate target sites may be selected for various reasons. The choice of target site can be based on the

primary DNA sequence but the DNA structure can also have a significant effect. Target choice determinants of the *Tc1* transposon of *C. elegans* were studied. By analysing new insertions in known genes and by determining flanking sequences of endogenous *Tc1* elements present in the genome of *C. elegans***,** an insertion consensus sequence (CAYA**TA**TRTG) was identified (Eide & Anderson, 1988; Mori *et al*., 1988; Korswagen *et al*., 1996). Further deletion mapping and mutagenesis showed that a 4 bp sequence adjacent to the TA was sufficient to make a target site highly preferred (Ketting *et al*., 1997). This is in contrast to the *Himar1* element of the *mariner* family which was isolated from the horn fly *Heamatobia irritans*. *In vivo* and *in vitro* studies on this element showed very little sequence similarity in integration sites beside the TA dinucleotide (Lampe *et al*., 1998; Robertson & Lampe, 1995). The ability of *Himar1* to use a given target TA appeared to be strongly related to the DNA bending of the flanking sequence. The insertion sites of transposon *piggyBac* within different host genomes were also analysed. This demonstrated that no obvious consensus could be detected in the proximal sequences beyond the TTAA target site (Handler *et al*., 1998; Handler & Harrell, 1999). For TCp3.2 and TCl4.7 it is not known which TA target sites are preferred since no sequence information on other integration sites of these transposons is available. Also the sequences of the integration sites of TCp3.2 and TCl4.7 show very little similarity (3.1). It remains unclear whether both elements have similar integration site preferences. The fact that TCl4.7 integrated in between two CpGV repeated sequences and that the TA target site was part of a small palindromic sequence, is probably a first indication that this element has a preference for bent target sequences.

4.2. Inversion of transposon TCp3.2

During the *in vivo* cloning experiments of MCp4, the mutant MCp4inv was identified. Characterisation of the mutant demonstrated the spontaneous inversion of the mobile genetic element TCp3.2 in the genome of MCp4. TCp3.2 changed its orientation during the replication of MCp4 in larvae of the codling moth. The analyses provide clear evidence that the inversion of TCp3.2 is caused by homologous recombination between the extended 756 bp long ITR sequences which flank the transposon to the left and right rather than by an excision mediated inversion (3.4-3.6). This is the first report which demonstrates transposon inversion in a baculovirus genome under *in vivo* conditions using infected larvae. Inversion of a transposon 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 AcMNPV, inverted by homologous

recombination. The authors suggested that these recombination events were strictly dependent upon AcMNPV-mediated DNA replication and required a minimum of 300 bp homology within the ITRs. Regarding the minimum length of homology required for homologous recombination, the *in vivo* experiments of this study are in good agreement with the *in vitro* results of Martin & Weber (1997). Inversion was observed for TCp3.2 which has 756 bp long ITRs, whereas it was never observed for transposon TCl4.7 which contains only 29 bp ITRs.

The integration of a transposon into 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 in between the transposable elements. Transposon inversions in the 5' promoter region can lead to more variation and possibly to altered expression patterns (Kloeckener-Gruissem & Freeling, 1995).

Insertions of host transposons into baculovirus genomes are an occasional event. However, the observed recombination-mediated inversion between the transposon-specific inverted terminal repeat sequences might serve as a model for the involvement and contribution of any repeated sequence to baculovirus genome rearrangements. In general, baculovirus genomes are characterised by the presence of repeated ORFs (e.g. *bro* genes) and homologous regions (*hrs*) (Kuzio *et al*., 1999; Cochran & Faulkner, 1983; Majima *et al*., 1993). The potential involvement of baculovirus *bro* genes and *hrs* in intragenomic recombination have been suggested in earlier studies. Analyses of the distribution of *bro* genes in different strains of BmNPV indicate that intraspecific recombination between these genes cause an active redistribution of sequences within the genome of BmNPV (Lopez Ferber *et al*., 2001). Comparative analyses of the genomes of three closely related group I NPVs (AcMNPV, BmNPV and OpMNPV) demonstrated that the organisation of the 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 homologous regions in BmNPV (ORF130-135, 1-3) and AcMNPV (ORF1,2,4-6,8-10). 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 is 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 neighbouring a *hr* is found for HaSNPV ORF61-67. This HaSNPV genomic region is flanked by *hr3* on its left hand side and has an

opposite orientation compared to homologous regions in SeMNPV (ORF88-93), AcMNPV (ORF65-71) and BmNPV (ORF53-58) (Chen *et al*., 2001; IJkel *et al*., 1999).

The results demonstrate that recombination between homologous ITR sequences can result in spontaneous inversions within the CpGV genome during *in vivo* infection. This provides further evidence that the presence of extended homologous sequences such as long ITRs or *hrs* and repeated genes may play a significant role in the genomic rearrangement by homologous recombination within baculovirus genomes.

4.3. Biological characterisation of MCp4 and MCp5

The integration regions of transposons TCp3.2 and TCl4.7 within the genome of CpGV were sequenced and characterised. In order to investigate which consequences the identified genomic alterations had on the biological fitness of the mutant viruses, biological parameters such as the median lethal dose (LD_{50}), the median survival time (ST_{50}) and the virus production were determined. Additionally, competition experiments were performed to analyse the potential selection constraints on MCp4, MCp5 and the parental virus CpGV-M. These experiments showed, that MCp4 and MCp5 can replicate and produce infective OB in *C. pomonella* larvae. No difference was observed between both the LD_{50} values and the total virus yield of MCp4, MCp5 and CpGV-M, suggesting that the transposon carrying mutants are able to initiate infection and to complete its replication cycle in a normal way (3.7 and 3.8). These findings are in good agreement with earlier LC_{50} determinations, which showed no significant difference between the mutants and CpGV-M (Jehle *et al*., 1995). In contrast, the survival time analyses clearly demonstrated that MCp4 and MCp5 killed *C. pomonella* larvae slower than CpGV-M, the differences between the ST_{50} values of CpGV-M and MCp5 and CpGV-M and MCp4 was about 10 hours and 30 hours, respectively. This indicated that the speed of replication is impaired by the transposon insertions (3.7).

Competition studies were carried out to determine whether the differences in median survival times between CpGV-M, MCp4 and MCp5 affected the ratio of the respective genotypes in the virus yield. Fifth instar *C. pomonella* were co-infected with a mutant and the parental virus. By comparing the CpGV-M:mutant ratio in the virus inoculum with the ratio in the virus progeny it could be shown that MCp4 as well as MCp5 were efficiently out-competed by CpGV-M (3.10). Apparently, the selection of the transposon carrying mutants is significantly disadvantaged

compared to CpGV-M. By performing serial passaging experiments in which OB were inoculated *per os* and BV was inoculated by injection into the hemolymph it was possible to localize the replication disadvantage of the mutants to CpGV-M in the infection cycle (3.10). This experiment proved that the difference in replication efficacy between the mutants and CpGV-M is not caused by differences in their peroral infectivity. The quick out competition of BV suggest that it is probably related to parameters later in the infection process, e.g. the speed of BV replication or to the virus spread. Otherwise, we would not have expected to observe the out-competition of MCp4 and MCp5 when BV was used instead of OB in the competition experiments. It is surprising to see that no or only small differences in the biological parameters can result in extreme differences in competition experiments. This indicates that the "virulence parameters" LC_{50}, LD_{50}, ST_{50} and the virus production are not parameters which reflect the competitivenes of the virus properly.

The disease symptoms of the *C. pomonella* larvae infected with CpGV-M, MCp4 and MCp5 were observed, but no morphological differences during the infection process could be detected. Also the amount of OB progeny produced by MCp4, MCp5 and CpGV-M in fifth instar *C. pomonella* were identical. The only observed difference between the mutants and CpGV-M is the reduced replication rate. The molecular mechanism causing the replication disadvantage of MCp4 and MCp5 is not yet clear. Transposon TCl4.7 of MCp5 integrated in between CpGV repeated sequence 3 and 4 which are located in the non translated region between Cp15 and Cp16 (Luque *et al*.,2001). Since the function of these repeats is unknown, a possible influence of transposon integration in this region is not predictable. The insertion site of TCp3.2 in the genome of MCp4 is located in the non-translated region between Cp41 and Cp42 (Jehle *et al*., 1997; Luque *et al*., 2001). As suggested by Jehle *et al*. (1997), this TA integration site is part of the putative TATA box of the Cp42 early promoter, and could thus probably impair the transcription of this gene. For other transposons inserted into baculovirus genomes it has been reported that they can influence the transcription of viral genes in close proximity to the insertion sites. This has been demonstrated for different baculovirus genes adjacent to transposon insertions (Friesen *et al*., 1986; Friesen & Miller, 1987; Beames & Summers, 1989; Oellig *et al*., 1987; Schetter *et al*., 1990; Lerch & Friesen, 1992). Further experiments will be necessary to analyse whether the insertion of MCp4 within the putative promoter region of Cp42 is linked to the tremendous replication difference observed between CpGV-M and MCp4. If so, this region might be an interesting target for the genetic engineering of CpGV and other GV because it could reduce the competitiveness of the virus without altering other parameters such as infectivity or virus production.

Taken together, the passage experiments using OB and BV for peroral or hemolymph infection of *C. pomonella* larvae clearly ruled out, that MCp4 and MCp5 have any selection advantage over the parental CpGV-M genotype. This finding clearly contrasts the situation of the AcMNPV FP mutants for which was shown that the replication of transposon carrying FP mutants is favoured during subsequent passages through cell cultures (Beames & Summers, 1989).

4.4. Transposase t32a transcription

Experiments were performed to elucidate whether virus infection may have contributed to the horizontal escape of the insect host transposon TCp3.2 into the genome of CpGV-M. Currently, direct determination of the influence of virus infection on the transposition frequency of TCp3.2 is not possible because a suitable transposition assay is not available for this transposon. The activity of TCp3.2 is possibly dependent on the expression level of an active transposase as it is typical for *Tc1/mariner* like transposons. Because the level of transposase mRNA in the larvae is probably a good measure for transposon activity, the transcription of the TCp3.2 transposase t32a was analysed in more detail in larvae of *C. pomonella*. Isolation of total RNA followed by reverse transcription, cDNA cloning and sequencing revealed that t32a indeed contains an intron and which is subsequently spliced. The sequences of 12 randomly isolated cDNA clones also revealed a considerable heterogeneity within the sequences suggesting that more than one of the multiple copies of TCp3.2 transposons present in *C. pomonella* are transcribed (3.12). The analysis of these sequences showed that a substantial proportion of the transposase transcripts cannot be functional due to deletions and frameshift mutations. The nucleotide positions of the splicing borders slightly differ from the borders predicted earlier by Jehle *et al*. (1998). This demonstrated the necessity of RNA based analyses rather than predictions based on DNA sequence only. With the identification of the correct splicing borders it was possible to design a primer (PR-RT-up) which only annealed to cDNA made from mature mRNA rather than primary transcripts of t32a (3.13)**.**

Initially, Northern and Western analyses were carried out with total RNA and protein preparations from virus infected and mock infected larvae. These studies did not provide any evidence of t32a transcription or expression in larvae, suggesting that the levels may be below the detection limit of these methods. Hence, real time RT-PCR was performed to detect and to quantify the t32a transcription in larvae of *C. pomonella*. Conventional RT-PCR suggested a

higher level of t32a splicing in virus infected larvae than in mock infected larvae (3.13). Results from the real time RT-PCR studies on a small number of larvae were in agreement with this observation. Although the level of t32a transcription was rather variable in all tested mock and CpGV-M infected larvae, the average level of spliced transcripts was 3-8 times higher in virus infected larvae. Additionally, the real time experiments suggested a 2-3 fold increase in the total t32a transcription levels in the CpGV-M infected larvae compared to the mock infected larvae (3.13). Due to the small number of analysed larvae and the observed high variability, the differences in t32a transcription need to be interpreted carefully. In order to draw a more solid conclusion about the phenomenon of transcriptional activation by virus infection this experiment should be repeated with a large number of larvae.

The obtained results provided first evidence that CpGV-M infection may influence the transcription of the t32a transposase of transposon TCp3.2 within the genome of third instar *C. pomonella*. It is interesting to note that a difference in transposase transcription was not detected in fifth instar larvae, where the t32a specific cDNA amounts were similarly low in virus infected and in mock infected larvae. Furthermore, the observed variation in transposase transcript levels among the individual larvae demonstrated that the transcription of this gene is not constant and depends on the tested larvae. A similar observation was made for the transcription of the *copia* element of *D. melanogaster* by Arnault & Dufournel (1994). These authors demonstrated that different populations and strains did not respond with the same transcription rates to different temperatures.

The results of the real time PCR showed that for third instar *C. pomonella* the ratio of spliced/total t32a transcripts was higher in the CpGV-M infected then in the mock infected larvae. This indicated that virus infection had an influence on the splicing of the t32a pre-mRNA. Since only spliced mRNA code for a potential active transposase, virus infection seems to influence the regulation of the transposition rate of TCp3.2 also at splicing level. For the *Drosophila P* element it was demonstrated that its activity too is regulated at the level of pre-mRNA splicing. This element is only active in germline cells and not in somatic cells (Laski *et al*., 1986; Rio *et al*., 1986). In germline cells all three introns of the *P* element are spliced accurately resulting in a functional transposase mRNA. In somatic cells the third intron is not removed. This leads to the translation of a truncated protein which is a repressor of transposase activity (Misra *et al*., 1990).

The transposase transcription was also studied in the *C. pomonella* cell line CpDW14R. These experiments showed an increased t32a transcription 6 days p.i. compared to 3 days p.i. for mock and CpGV-M infected cells. This indicated that extended culture time rather then virus infection influenced the t32a transcription in these cells. This finding is in contrast to the situation in *C. pomonella* larvae where CpGV-M infection stimulated t32a transcription. These results indicated that factors other than a virus infection also influenced the transcription of t32a in CpDW14R cells. Experiments performed by Di Franco *et al*. (1992) indicated that several transposable elements can be mobilised by the passage to cell culture. In cultured cells of *Drosophila*, changes in the restriction patterns consistent with various types of rearrangements such as amplification, transposition and excision of the elements of *copia*, *1731*, *412*, *297* and *mdg-4* transposon families were detected. Since CpDW14R cells are very sensitive to changes in the medium composition, the altering medium composition after extended culture time could be a possible stress factor for these cells (Winstanley & Crook, 1993). Because of the slow growth of the CpDW14R cells, the culture media in the flasks need to be refreshed regularly. However, the weekly change probably result in sufficient alteration of the medium composition to cause stress. This elevated stress level in the cells may lead to the activation of transposon TCp3.2. This might be an explanation why both mock and CpGV-M infected cells had a higher transposase transcription 6 days p.i. than 3 days p.i.. For the cells, the poorer medium composition was probably a stronger stress factor than the virus infection.

The induction of transposon activity by viral infection and other stress factors has been studied in several organisms but has been demonstrated for only a few elements (Arnault & Dufournel, 1994; Capy *et al*., 2000). In maize the mobilisation and the insertion of transposable element *Uq* has been reported in plants infected with Barley Stripe Mosaic Virus (Peterson, 1985). In *Drosophila* it was hypothesised that endogenous transposable elements were involved in mutations observed after injection of the avian Rous Sarcoma Virus (RSV) (Gazaryan *et al*., 1984). Yet, it was never proven whether the activity of transposable elements in *Drosophila* was enhanced by a virus infection. There are examples which suggest that beside virus infection other stress factors like heat shock, magnetic field exposure and UV light can also mobilise transposable elements within host genomes of plants, yeast and *E. coli* (Wessler, 1996; Grandbastien, 1998; Ratner *et al*., 1992, Strand & McDonald, 1985; Kupelian & DuBow, 1986; Walbot; 1992; Eichenbaum & Livneh, 1998). Although most of the elements affected by stress factors belong to the retrotransposons, there are also clear examples that DNA transposons respond to environmental stress. For example, laboratory results demonstrated that both somatic and germ

line excision rates of *mariner* elements of *Drosophila stimulans* are effected by the developmental temperature (Chakrani *et al*., 1993; Garza *et al*., 1991; Giraud & Capy, 1996). Also the hybrid dysgenesis syndrome of the *P* and *hobo* elements in *D. melanogaster* is dependent on temperature (Kidwell, 1977; Blackman *et al*., 1987). It has been demonstrated for the maize element *Mu* and the *IS10* element of *E. coli* that UV irradiation activated DNA repair mechanisms which subsequently led to an increased transposition rate of the transposable elements (Walbot, 1992; Eichenbaum & Livneh, 1998).

It also remains to be answered what kind of mechanism could have been involved in the increased TCp3.2 transposase transcription. The enhanced transcription could be a host cell response to cellular stress, but it could also be induced by the RNA polymerase encoded by the virus. How a cell response could influence the transcription remains largely unclear. When faced with stress, organisms may have to induce responses at the physiological and genetic level. It is known that most organisms respond to environmental stress by synthesizing a specific set of heat shock or stress proteins which function as transcription activators of host defence genes (Morimoto *et al*., 1994). In *Drosophila*, detailed analyses of the *copia* and *mariner* regulatory sequences showed the existence of motifs very similar to those of heat shock promotors (Strand & McDonald, 1985; Chakrani *et al*., 1993; McDonald *et al*., 1997). This suggests that after stress the released transcription activators could also induce the activity of transposable elements which contain such regulatory sequences (Capy *et al*., 2000). In most cases the stress proteins induced belong to the heat shock families of the chaperone proteins (Menees & Sandmeyer, 1996). For a herpesvirus infection an increased transcription of ubiquitin, a stress inducible component of a proteolytic system, was also found. Ubiquitin also has been found associated with particles of e.g. the avian leukosis virus, the African swine fever virus and the virions of baculoviruses (Putterman *et al*., 1990; Hingamp *et al*., 1995; Russell & Rohrmann, 1993). It has been suggested that ubiquination can play a role in regulating the transposition of transposable elements. Experiments with the yeast retrovirus-like element *Ty3* indeed indicated that the transposition of this element is sensitive to levels of ubiquitination. (Menees & Sandmeyer, 1996). Since most baculoviruses encode ubiquitin, this protein could induce stress responses in the insect cells which could consequently lead to transposition. Although CpGV-M encode an ubiquitin, it remains hypothetical whether this protein or other cellular stress proteins had an influence on the transcription of the transposase of transposon TCp3.2 within the *C. pomonella* genome. The other possibility for the enhanced TCp3.2 transposase transcription could be based on the effect of the virus encoded RNA polymerase which needs only a very simple late promoter motif. Sequence

analysis of the region upstream from the putative ATG start codon of the transposase gene showed that six consensus late promoter motifs (5'-A/G/T/TAAG-3') are located in the left ITR of TCp3.2 (Table 4.1). Studies on the *vp37* promoter of AcMNPV performed by Thiem & Miller (1989) demonstrated that a TAAG motif which was located as far as 321 bp upstream from the start codon was still functional. Since four of the late motifs in the left ITR are located in closer proximity to the transposase start codon than 321 bp, they could be functional late transcription initiation sites. When this is the case, and the corresponding translation of these RNA transcripts result in functional transposases, virus infection could consequently lead to the activation of transposon TCp3.2. It has been shown that transcription initiation sites located within transposons can be recognised by a virus encoded RNA polymerase. Friesen *et al*. (1986) demonstrated that a single ITR of *TED* which was integrated in the 94K gene of the AcMNPV insertion mutant *FP-DS* directed transcription of new RNAs in both directions. It appeared that the RNA transcription was initiated from a CTTATAAG palindromic sequence which contains a bidirectionally oriented ATAAG motif. Further investigations showed that the ATAAG motif in the ITR must have been recognised by the virus encoded transcription machinery since no transcription was detected from the same site of *TED* elements residing in the *T. ni* host genome (Friesen & Nissen, 1990).

Table 4.1. Positions of the late transcription initiation sites located upstream from the ATG start codon of the transposase gene.

Motif	bp upstream from ATG	Position[a]
1: ATAAG	783	193-197
2: GTAAG	779	197-201
3: GTAAG	311	665-669
4: ATAAG	294	682-686
5: ATAAG	289	687-691
6: TTAAG	213	763-767

[a]The positions are based on the TCp3.2 sequence published by Jehle *et al*. (1998).

Our results clearly demonstrate that the TCp3.2 harbouring mutant MCp4 had a tremendous replication disadvantage compared to CpGV-M. The experiments also indicate a higher level of transposase transcription in CpGV-M infected larvae than in mock infected control larvae. These results suggest that the horizontal escape of the insect host transposon TCp3.2 into the CpGV genome, which resulted in the mutant MCp4, was more likely to be stimulated by virus infection rather than by a selection mechanism of MCp4. Further experiments will be necessary to

elucidate whether the transcription of host copies of TCp3.2 is activated by virus infection. An other interesting question to be answered is whether TCp3.2 is generally activated in all cells or whether it is age and tissue specific.

4.5 Risk assessment with regard to transgenes in baculoviruses.

There is a considerable interest in baculoviruses as a biological control agent for insect pests (Huber, 1986). In order to improve their insecticidal properties, genetically modified baculoviruses have been developed which, for example, express insect-specific toxin or hormone genes (Vlak, 1993). Consequently, the generation of these viruses raised concerns about the risks which are associated with the introduced transgenes. A major concern is the possibility of transfer of the transgenes to naturally occurring baculoviruses or even to other organisms. Some of the clearest examples of horizontal gene transfer are found in insects and involve transposons. Within the last 75 years, the *P* element of *D. willistoni* was horizontally transferred to *D. melanogaster* (Kidwell, 1992). This recent transfer has resulted in the spread of *P* elements to almost every wild population of *D. melanogaster* in the world. The transfer of genes across species boundaries indicates that there is a risk of any transgene being transferred to non target species. Because baculoviruses can harbour additional DNA sequences and because they are in general infective for different hosts, they are good candidates to function as a shuttle vector for genetic information between organisms. In *vivo* and *in vitro* studies have demonstrated that host transposons can escape horizontally into baculoviruses (1.6 and 1.7). A subsequent infection of this baculovirus in another host species could result in the transfer of the transposon to a foreign genome. If a transgene were linked to a transposable element, it could thus undergo horizontal transfer together with the element. Our results support the observation that the horizontal transfer of transposons between species probably is not a rare event. Since baculoviruses are potential transfer vectors, the escape of transgenes from these viruses to insects is a not unlikely scenario.

4.6 Genetic transformation of insects using transposable elements

The introduction of genes of any origin into insects will play an important role in the identification of gene function and a better understanding of insect biology. The development of genetic transformation systems is important in order to study insects of medical and agricultural significance. For stable insect transformation, DNA has to be introduced into the germ cells. Different strategies, including microinjecting, electroporation and biological introduction methods

such as *in vivo* recombination systems, viruses and transposable elements have been developed to genetically transform insects. The first transposable element based transformation system of insects germ cells was based on the *Drosophila melanogaster P* element (Rubin & Sprading, 1982). This system is limited to *Drosophila* as it does not function in insects of other genera (O'Brochta & Atkinson, 1996). Only recently did transformation systems based on transposable elements become available for other dipteran and lepidopteran insects. To date, four transposable elements are available for the genetic transformation of non-drosophilid insects. A list of the insect species which have been transformed by the transposable elements *Minos*, *Hermes*, *Mos1* and *piggyBac* is presented in Table 4.2.

Table 4.2. The genetic transformation of insects using transposable elements (modified after Atkinson *et al.*, 2001).

Transposable element	Family	Original host	Size (kb)	Species transformed	Reference
Minos	Tc1	*D. hydei*	1.8	*D. melanogaster*	Loukeris *et al*., 1995a
				C. capitata	Loukeris *et al*., 1995b
				A. stephensi	Catteruccia *et al*., 2000
Hermes	hAT	*M. domestica*	2.7	*D. melanogaster*	O'Brochta *et al*., 1996
				C. capitata	Michel *et al*., 2001
				S. calcitrans	O'Brochta *et al*., 2000
				A. aegypti	Jasinskiene *et al*., 1998
				C. quinquefasciatus	Allen *et al*., 2001
Mos1	Mariner	*D. mauritiana*	1.3	*D. melanogaster*	Lidholm *et al*., 1993
				A. aegypti	Coates *et al*., 1998
PiggyBac	TTAA	*T. ni*	2.5	*D. melanogaster*	Handler & Harrell, 1999
				C. capitata	Handler *et al*., 1998
				M. domestica	Hediger *et al*., 2001
				B. mori	Tamura *et al*., 2000
				P. gossypiella	Peloquin *et al*., 2000

PiggyBac (IFP2) was the first transposon derived vector which was able to transform a lepidopteran insect. It has been shown to be an effective gene-transfer vector for the silkworm *Bombyx mori* (Tamura *et al*., 2000). *PiggyBac* was originally discovered as the causative agent of FP (few polyhedra) mutations in baculoviruses passaged through a cell line of the cabbage looper *Trichoplusia ni* (Cary *et al*., 1989). *Minos* and *Mos1* belong to the family of *Tc1/mariner* transposable elements. Due to their transposition strategy this type of elements are good candidates as gene transfer vectors. It has been demonstrated that the only protein required to

carry out the transposition of *Tc1 in vitro*, is the transposase protein encoded by the element itself; no host-specific factors are required (Vos *et al*., 1996; Lampe *et al*., 1996). This is in contrast to some other insect transposable elements, that seem unable to transpose to other species due to the requirement of a host encoded protein for the transposition (Beall *et al*., 1994). *Tc1/mariner* transposons have been found in the genomes of all organisms in which they were studied (Robertson, 1995). This wide spread of members of this family of transposable elements is a further evidence that no host specific factors are required. *In vivo* and *in vitro* experiments have shown that *Tc1/mariner* like elements originating from different hosts can jump into heterologous species (Lohe & Hartl, 1996; Garza *et al*., 1991; Gueiros-Filho & Beverley, 1997; Ivics *et al*., 1997; Raz *et al*., 1998).

TCp3.2 and TCl4.7 also belong to the *Tc1/mariner* family. TCl4.7 and TCp3.2 have shown to be able to leave an insect genome and to jump to a virus genome. Due to the nature of this type of elements, complete copies of TCp3.2 and TCl4.7 could also be good candidates as gene-transfer vectors.

5. REFERENCES

Allen, M. L, O'Brochta D. A., Atkinson P. W. & Levesque C. S. (2001). Stable, germ-line transformation of *Culex quinquefasciatus* (Diptera: Culicidae). *Journal of Medical Entomology* **38**, 701-710.

Ahrens, C. H. & Rohrmann, G. F. (1995). Identification of essential trans-acting regions required for DNA replication of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus: *lef-1* is an essential replication gene. *Virology* **207**, 417-428.

Ahrens, C. H., Russell, R., Funk, C. J., Evans, J. T., Harwood, S. H. & Rohrmann, G. F. (1997). The sequence of *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus genome. *Virology* **229**, 381-399.

Arends, H. M. (1995). Anti-transposase antibody binds specifically to a transposase synthesised by a baculovirus expression system: Master project, Laboratory of Virology, Wageningen University, The Netherlands.

Arends, H. M., & Jehle, J. A. (2002). Homologous recombination between the inverted terminal repeats of defective transposon TCp3.2 causes an inversion in the genome of *Cydia pomonella* granulovirus. *Journal of General Virology.* **83,** 1573-1578.

Arnault, C., & Dufournel, I. (1994). Genome and stresses: reactions against aggressions, behaviour of transposable elements. *Genetica* **93,** 149-160.

Atkinson, P. W., Pinkerton, A. C. & O'Brochta DA. (2001). Genetic transformation systems in insects. *Annual Review of Entomology* **46**, 317-346.

Ayres, M. D., Howard, S. C., Kuzio, J., Lopez Ferber, M. & Possee, R. D. (1994). The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology* **202**, 586-605.

Bauser, C. A., Elick, T. A., & Fraser, M. J. (1996). Characterization of *hitchhiker*, a transposon insertion frequently associated with baculovirus FP mutants derived upon passage in the TN- 368 cell line. *Virology* **216,** 235-237.

Beames, B. & Summers, M. D. (1989). Location and nucleotide sequence of the 25K protein missing from baculovirus few polyhedra (FP) mutants. *Virology* **168**, 344-353.

Beames, B., & Summers, M. D. (1990). Sequence comparison of cellular and viral copies of host cell DNA insertions found in *Autographa californica* nuclear polyhedrosis virus. *Virology* **174,** 354-363.

Berg, D. E., & Howe, M. M. (eds.) (1989). Mobile DNA. American Society of Microbiology, Washington.

Bilimoria, S. L. (1986). Taxonomy and identification of baculoviruses. In *The Biology of Baculoviruses, Vol. 1, Biological Properties and Molecular Biology*, pp. 37-59. Edited by RRG & BAF. Boca Raton: CRC Press.

Blackman R. K., Koehler, M. M., Grimaila, R., & Gelbart, W. M. (1987). Mobilization of *hobo* elements residing within the decapentaplegic gene complex: suggestion of a new hybrid dysgenesis system in *Drosophila melanogaster*. *Cell* **49,** 497-505.

Blissard, G. W. & Rohrmann, G. F. (1990). Baculovirus diversity and molecular biology. *Annual Review of Entomology* **35**, 127-155.

Blissard, G. W., Black, B., Crook, N., Keddie, B. A., Possee, R., Rohrmann, G., Theilmann, D. A., & Volkman, L. E. (2000). Family *Baculoviridae*. *In* " Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses" (M. H. V. van Regenmortel, C. M. Fauquet, H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Manilloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle, and R. B. Wicker, Eds.), pp. 195-202. Academic press, New York.

Britten, R. J. (1997). Mobile elements inserted in the distant past have taken on important functions. *Gene* **205**, 177-182.

Bulach, D. M., Kumar, C. A., Zaia, A., Liang, B. F. & Tribe, D. E. (1999). Group II nucleopolyhedrovirus subgroups revealed by phylogenetic analysis of polyhedrin and DNA polymerase gene sequences. *Journal of Invertebrate Pathology* **73**, 59-73.

Capy, P., Anoxlabehere, D. & Langin, T. (1994). The strange phylogenies of transposable elements: are horizontal transfers the only explanation? *Trends In Genetics* **10**, 7-12.

Capy, P., Gasperi, G., Biemont, C., & Bazin, C. (2000). Stress and transposable elements: co-evolution or useful parasites? *Heredity* **85,** 101-106.

Carstens, E. B. (1987). Identification and nucleotide sequence of the regions of *Autographa californica* nuclear polyhedrosis virus genome carrying insertion elements derived from *Spodoptera frugiperda*. *Virology* **161**, 8-17.

Cary, L. C., Goebel, M., Corsaro, B. G., Wang, H. G., Rosen, E. & Fraser, M. J. (1989). Transposon mutagenesis of baculoviruses: analysis of *Trichoplusia ni* transposon *IFP 2* insertions within the FP-locus of nuclear polyhedrosis viruses. *Virology* **172**, 156-169.

Catteruccia, F., Nolan, T., Loukeris, T. G., Blass, C., Savakis, C., Kafatos, F. C. & Crisanti, A. (2000). Stable germline transformation of the malaria mosquito *Anopheles stephensi*. *Nature* **405**, 959-962.

Chakrani, F., Capy, P. & David, J. (1993). Developmental temperature and somatic excision rate of *mariner* transposable element in three natural populations of *Drosophila stimulans*. *Genetical Selective Evololution.* **25,** 121-132.

Chen, X. W., IJkel, W. F. J., Tarchini, R., Sun, X. L., Sandbrink, H., Wang, H. L., Peters, S., Zuidema, D., Lankhorst, R. K., Vlak, J. M. & Hu, Z. H. (2001). The sequence of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus genome. *Journal of General Virology* **82**, 241-257.

Chow, K. C. & Tung, W.L. (2000). Magnetic field exposure stimulates transposition through the induction of DnaK/J synthesis. *Biochemical and Biophysical Research Communication* **270,** 745-748.

Coates, C. J., Jasinskiene, N., Miyashiro, L. & James, A. A. (1998). Mariner transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. *Proceedings of the National Academy of Sciences, U S A* **95**, 3748-3751.

Cochran, M. A. & Faulkner, P. (1983). Location of homologous DNA sequences interspersed at five regions in the baculovirus AcMNPV genome. *Journal of Virology* **45**, 961-970.

Crook, N. E., Spencer, R. A., Payne, C. C. & Leisy, D. J. (1985). Variation in *Cydia pomonella* granulosis virus isolates and physical maps of the DNA from three variants. *Journal of General Virology* **66**, 2423-2430.

Crook, N. E. (1991). Baculoviridae: subgroup B. Comparative aspects of granulosis viruses. In *Viruses of Invertebrates*, pp. 73-110. Edited by E. Kurstak. New York: Marcel Dekker Inc.

Crook, N. E., James, J. D., Smith, I. R. L. & Winstanley, D. (1997). Comprehensive physical map of the *Cydia pomonella* granulovirus genome and sequence analysis of the granulin gene region. *Journal of General Virology* **78**, 965-974.

Daniels, S. B., Peterson, K. R., Strausbaugh, L. D., Kidwell, M. G. & Chovnick, A. **(1990).** Evidence for horizontal transmission of the *P* transposable element between *Drosophila* species. *Genetics* **124**, 339-355.

Di Franco, C., Pisano, C., Fourcade-Peronnet, F., Echalier, G. & Junakovic, N. (1992). Evidence for *de novo* rearrangements of *Drosophila* transposable elements induced by the passage to the cell culture. *Genetica* **87**, 65-73.

Doak, T. G., Doerder, P. F., Jahn, C. L. & Herrick, G. (1994). A proposed superfamily of transposase genes: transposon-like elements in ciliated protozoa and a common "D35E" motif. *Proceedings of the National Academy of Sciences, U S A* **91**, 942-946.

Doolittle, W. F. & Sapienza, C. (1980). Selfish genes, the phenotype paradigm and genome evolution. *Nature* **284**, 601-603.

Dupas, S., Brehelin, M., Frey, F., & Carton, Y. (1996). Immune suppressive virus-like particles in a Drosophila parasitoid: significance of their intraspecific morphological variations. *Parasitology* **113**, 207-212.

Eichenbaum, Z. & Livneh, Z. (1998). UV light induces *IS10* transposition in *Escherichia coli*. *Genetics* **149,** 1173-1181.

Eide, D. & Anderson, P. (1988). Insertion and excision of *Caenorhabditis elegans* transposable element *Tc1*. *Molecular and Cellular Biology* **8**, 737-746.

Engelhard, E. K., Kam Morgan, L. N. W., Washburn, J. O. & Volkman, L. E. (1994). The insect tracheal system: a conduit for the systemic spread of *Autographa californica* M nuclear polyhedrosis virus. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 3224-3227.

Fedoroff, N., Wessler, S. & Shure, M. (1983). Isolation of the transposable maize controlling elements *Ac* and *Ds*. *Cell* **35**, 235-342.

Fraser, M. J. (1986). Transposon-mediated mutagenesis of baculoviruses: Transposon shuttling and implications for speciation. *Annals of the Entomological Society of America* **79,** 773-783.

Friesen, P. D., Rice, W. C., Miller, D. W. & Miller, L. K. (1986). Bidirectional transcription from a solo long terminal repeat of the retrotransposon *TED*: symmetrical RNA start sites. *Molecular and Cellular Biology* **6**, 1599-1607.

Friesen, P. D. & Miller, L. K. (1987). Divergent transcription of early 35- and 94-kilodalton protein genes encoded by the *Hin*dIII genome fragment of the baculovirus *Autographa californica* nuclear polyhedrosis virus. *Journal of Virology* **61**, 2264-2272.

Friesen, P. D. & Nissen, M. S. (1990). Gene organization and transcription of *TED*, a Lepidopteran retrotransposon integrated within the baculovirus genome. *Molecular Cellular Biology* **10,** 3067-3077.

Friesen, P. D. (1993). Invertebrate transposable elements in the baculovirus genome: Characterization and significance. *In* "Parasites and Pathogens of Insects, Vol. 2: Pathogens" (N. E. Beckage, S. N. Thompson, and B. A. Federici, Eds.), pp. 147-178. Academic Press, San Diego.

Friesen, P. D. (1997). Regulation of baculovirus early gene expresion. In 'The Baculoviruses' (L. K. Miller, Ed.), pp141-170. Plenum, New York.

Garza, D., Medhora, M., Koga, A. & Hartl, D. L. (1991). Introduction of the transposable element *mariner* into the germline of *Drosophila melanogaster*. *Genetics* **128,** 303-310.

Gazaryan, K. G., Nabirochkin, S. D., Tatosyan, A. G., Shakhbazyan, A. K. & Shibanova, E. N. (1984). Genetic effects of injection of rous sarcoma virus DNA into polar plasm of early *Drosophila melanogaster* embryos. *Nature* **311,** 392-394.

Gijzen, M., Roelvink, P. & Granados, R. R. (1995). Characterization of a viral enhancing protein from *Trichoplusia ni* granulovirus. *Journal of invertebrate Pathology* **65**, 289-295.

Gill, R., Heffron, F., Dougan, G. & Falkow, S. (1978). Analysis of sequences transposed by complementation of two classes of transposition-deficient mutants of transposition element *Tn3*. *Journal of Bacteriology* **136**, 742-756.

Giraud, T. & Capy, P. (1996). Somatic activity of the *mariner* transposable element in natural populations of *Drosophila simulans*. *Proceedings of the Royal Society of London Biological Sciences* .**263,** 1481-1486.

Gomi, S., Majima, K. & Maeda, S. (1999). Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. *Journal of General Virology* **80**, 1323-1337.

Granados, R. R. & Lawler, K. A. (1981). *In vivo* pathway of *Autographa californica* baculovirus invasion and infection. *Virology* **108**, 297-308.

Granados, R. R. & Williams, K. A. (1986). *In vivo* infection and replication of baculoviruses. In *The Biology of Baculoviruses, Vol.I, Biological Properties and Molecular Biology*, pp. 89-108. Edited by R. R. Granados & B. A. Federici. Boca Raton: CRC Press.

Grandbastien, M. (1998). Activation of plant retrotransposons under stress conditions. *Trends in Plants Science.* **3,** 181-187.

Guarino, L. A. & Summers, M. D. (1986). Functional mapping of trans-activating gene required for expression of a baculovirus delayed-early gene. *Journal of Virology* **57**, 563-571.

Gueiros-Filho, F. J. & Beverley, S. M. (1996). Trans-kingdom transposition of the *Drosophila* element *mariner* within the protozoan *Leishmania. Science* **276**, 1716-1719.

Guennelon, G., Audemard, H., Fremond, J. C. & El Idrissiammari, M. A. (1981). Progrès réalisés dans l'élevage permanent du carpocapse (*Laspeyresia pomonella* L.) sur milieu artificiel. *Agronom.* **1**, 59-64.

Hagemann, S., Haring, E. & Pinsker, W. (1990). Repeated horizontal transfer of *P* transposons between *Scaptomyza pallida* and *Drosophila bifasciata*. *Genetica* **98**, 43-51.

Hajek, K. L. & Friesen, P. D. (1998). Proteolytic processing and assembly of gag and gag-pol proteins of *TED*, a baculovirus-associated retrotransposon of the gypsy family. *Journal of Virology*. **72,** 8718-8724.

Handler, A. M., McCombs, S. D., Fraser, M. J. & Saul, S. H. (1998). The lepidopteran transposon vector, *piggyBac*, mediates germ-line transformation in the mediterranean fruit fly. *Proceedings of the National Academy of Sciences, U S A* **95,** 7520-7525.

Handler, A. M. & Harrell, R. A. (1999). Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. *Insect Molecular Biology* **8**, 449-457.

Harrison, R. L. & Summers, M. D. (1995). Mutations in the *Autographa californica* multinucleocapsid nuclear polyhedrosis virus 25 kDa protein gene result in reduced virion occlusion, altered intranuclear envelopment and enhanced virus production. *Journal of General Virology* **76,** 1451-1459.

Harvey, J. P. & Volkman, L. E. (1983). Biochemical and biological variation of *Cydia pomonella* (codling moth) granulosis virus. *Virology* **124**, 21-34.

Hashimoto, Y., Corsaro, B. G. & Granados, R. R. (1991). Location and nucleotide sequence of the gene encoding the viral enhancing factor of the *Trichoplusia ni* granulosis virus. *Journal of General Virology* **72**, 2645-2651.

Hashimoto, Y., Hayakawa, T., Ueno, Y., Fujita, T., Sano, Y. & Matsumoto, T. (2000). Sequence analysis of the *Plutella xylostella* granulovirus genome. *Virology* **275**, 358-372.

Hayakawa, T., Ko, R., Okano, K., Seong, S., Goto, C. & Maeda, S. (1999). Sequence analysis of the *Xestia c-nigrum* granulovirus genome. *Virology* **262,** 277-297.

Hayakawa, T., Rohrmann, G. F. & Hashimoto, Y. (2000). Patterns of genome organization and content in lepidopteran baculoviruses. *Virology* **278,** 1-12.

Hawtin, R. E., Arnold, K., Ayres, M. D., Zanotto, P. M., Howard, S. C., Gooday, G. W., Chappell, L. H., Kitts, P. A., King, L. A. & Possee, R. D. (1995). Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. *Virology* **212**, 673-685.

Hediger, M., Niessen, M., Wimmer, E. A., Dubendorfer, A. & Bopp, D. (2001). Genetic transformation of the housefly *Musca domestica* with the lepidopteran derived transposon *piggyBac*. *Insect Molecular Biology* **10**, 113-119.

Herniou, E. A., Luque, T., Chen, X., Vlak, J. M., Winstanley, D., Cory, J. S. & O'Reilly, D. (2001). Use of whole genome sequence data to infer baculovirus phylogeny. *Journal of Virology* **75**, 8117-8126.

Hingamp, P. M., Leyland, M. L., Webb, J., Twigger, S., Mayer, R. J. & Dixon, L. K. (1995). Characterization of a ubiquitinated protein which is externally located in African swine fever virions. *Journal of Virology* **69**,1785-1793.

Horton, H. M. & Burand, J. P. (1993). Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. *Journal of Virology* **67**, 1860-1868.

Houck, M. A., Clark, J. B., Peterson, K. R. & Kidwell, M. G. (1991). Possible horizontal transfer of *Drosophila* genes by the mite *Proctolaelaps regalis*. *Science* **253**, 1125-1129.

Huber, J. (1986). Use of baculoviruses in pest management programmes. In: *The biology of baculoviruses, Vol.2, Practical application for insect control*, R. R. Granados and B. A. Federici, pp. 181-202. CRC Press, Boca Raton.

Hurst, G. D. D., Hurst, L. D. & Majerus, E. N. (1992). Selfish genes move sideways. *Nature* **356**, 659-660.

Hyink, O., Dellow, R. A., Olsen, M. J., Caradoc-Davies, K. M., Drake, K., Herniou, E. A., Cory, J. S., O'Reilly, D. R. & Ward, V. K. (2002). Whole genome analysis of the *Epiphyas postvittana* nucleopolyhedrovirus. *Journal of General Virology* **83**, 957-971.

IJkel, W. F. J., van Strien, E. A., Heldens, J. G. M., Broer, R., Zuidema, D., Goldbach, R. W. & Vlak, J. M. (1999). Sequence and organization of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. *Journal of General Virology* **80**, 3289-3304.

Ivaldi-Sender, C. (1974). Techniques simples pour elevage permanent de la tordeuse orientale, *Grapholita molesta* (Lep., Tortricidae), sur milieu artificiel. *Ann. Zool. Ecol. Anim.* **6,** 337-343.

Ivics, Z. D. G., Hackett, P. B., Plasterk, R. H. A. & Izsvak, Z. (1997). Molecular reconstruction of *Sleeping Beauty*, a Tc1-like transposon from fish, and its transposition in human cells. *Cell* **91**, 501-510.

Jarvis, D. L., Bohlmeyer, D. A. & Garcia, A., Jr. (1992). Enhancement of polyhedrin nuclear localization during baculovirus infection. *Journal of Virology* **66,** 6903-6911.

Jasinskiene, N., Coates, C. J., Benedict, M. Q., Cornel, A. J., Rafferty, C. S., James, A. A. & Collins, F. H. (1998). Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the *Hermes* element from the housefly. *Proceedings of the National Academy of Sciences, U S A* **95**, 3743-3747.

Jehle, J. A. (1994). Sicherheitsaspekte der Gentechnologie: Verwandtschaft und Variabilität der Genome des *Cryptophlebia leucotreta* Granulosevirus und des *Cydia pomonella* Granulosevirus: PhD thesis, TU Braunschweig.

Jehle, J. A., Fritsch, E., Nickel, A., Huber, J. & Backhaus, H. (1995). TCl4.7: a novel lepidopteran transposon found in *Cydia pomonella* granulosis virus. *Virology* **207**, 369-379.

Jehle, J. A. (1996). Transmission of insect transposons into baculovirus genomes: An unusual host-pathogen interaction. *In* "Transgenic organsims: biological and social implications" (K. Wöhrmann, J. Tomiuk, and A. Sentker, Eds.), pp. 81-97. Birkhäuser Verlag, Basel.

Jehle, J. A., van der Linden, I. F., Backhaus, H. & Vlak, J. M. (1997). Identification and sequence analysis of the integration site of transposon *TCp3.2* in the genome of *Cydia pomonella* granulovirus. *Virus Research* **50**, 151-157.

Jehle, J. A., Nickel, A., Vlak, J. M. & Backhaus, H. (1998). Horizontal escape of the novel Tc1-like lepidopteran transposon TCp3.2 into *Cydia pomonella* granulovirus. *Journal of Molecular Evolution* **46**, 215-224.

Jehle, J. A., Arends, H. M. & Vlak, J. M. (1999). Molekulare Untersuchungen zum horizontalen Transfer des Insektentransposons TCp3.2 im *Cydia pomonella* Granulovirus- ein Beitrag zur Sicherheitsforschung bei Baculoviren. BMBF-Workshop Biologische Sicherheit, Braunschweig 1998 (185-191).

Jordan, I. K., Matyunina, L. & McDonald, J. F. (1999). Evidence for the recent horizontal transfer of long terminal repeat retrotransposon. *Proceedings of the National Academy of Sciences, U S A* **96**, 12621-12625.

Ketting, R. F., Fischer, S. E. J. & Plasterk, R. H. A. (1997). Target choice determinants of the *Tc1* transposon of *Caenorhabditis elegans*. *Nucleic Acids Research* **25**, 4041-4047.

Kidwell, M. (1977). Reciprocal differences in female recombination associated with hybrid dysgenesis in *Drosophila melanogaster*. *Genetical Research* **30,** 77-88.

Kidwell, M. G. (1993). Voyage of an ancient *mariner*. *Nature* **362**, 202.

Klingsman, A. J. & Kingsman, S. M. (1988). *Ty*: a retroelement moving forward. *Cell* **53**, 333-335.

Kloeckener-Gruissem, B. & Freeling, M. (1995). Transposon-induced promoter scrambling: a mechanism for the evolution of new alleles. *Proceedings of the National Academy of Sciences, U S A* **92**, 1836-1840.

Kool, M., Ahrens, C. H., Goldbach, R. W., Rohrmann, G. F. & Vlak, J. M. (1994). Identification of genes involved in DNA replication of the *Autographa californica* baculovirus. *Proceedings of the National Academy of Sciences, U S A* **91,** 11212-11216.

Kool, M., Ahrens, C. H., Vlak, J. M. & Rohrmann, G. F. (1995). Replication of baculovirus DNA. *Journal of General Virology* **76**, 2103-2118.

Korswagen, H. C., Smits, M. T., Durbin, R. M. & Plasterk, R. H. A. (1996). Transposon *Tc1*-derived, sequence-tagged sites in *Caenorhabditis elegans* as markers for gene mapping. *Proceedings of the National Academy of Sciences, U S A* **93**, 14680-14685.

Kupelian, A. & DuBow, M. S. (1986). The effect of gamma-irradiation on *mu* DNA transposition and gene expression. *Mutational Research* **160,** 1-10.

Kuzio, J., Pearson, M. N., Harwood, S. H., Funk, C. J., Evans, J. T., Slavicek, J. M. & Rohrmann, G. F. (1999). Sequence and analysis of the genome of a baculovirus pathogenic for *Lymantria dispar*. *Virology* **253**, 17-34.

Lampe, D. J., Chuchill, M. E. & Robertson, H. M. (1996). A purified *mariner* transposase is sufficient to mediate transposition *in vitro*. *EMBO Journal* **(19),** 5470-5479.

Lampe, D. J., Grant, T. E. & Robertson, H. M. (1998). Factors affecting transposition of the *Himar1 mariner* transposon *in vitro*. *Genetics* **149**, 179-187.

Laski, F. A. & Rubin GM. (1986). Tissue specificity of *Drosophila P* element transposition is regulated at the level of mRNA splicing. *Cell* **17,** 7-19.

Lerch, R. A. & Friesen, P. D. (1992). The baculovirus-integrated retrotransposon *TED* encodes *gag* and *pol* proteins that assemble into viruslike particles with reverse transcriptase. *Journal of Virology* **66**, 1590-1601.

Li, Q., Donly, C., Li, L., Willis, L. G., Theilmann, D. A. & Erlandson M. (2002). Sequence and organization of the *Mamestra configurata* nucleopolyhedrovirus genome. *Virology* **94**, 106-121.

Lidholm, D. A., Lohe, A. R., Hartl, D. L. (1993). The transposable element *mariner* mediates germline transformation in *Drosophila melanogaster*. *Genetics* **134**, 859-868.

Lohe, A. R., & Hartl, D. L. (1996). Germline transformation of *Drosophila virilis* with the transposable element *mariner*. *Genetics* **143**, 365-374.

Lopez Ferber, M., Argaud, O., Croizier, L. & Croizier, G. (2001). Diversity, distribution, and mobility of *bro* gene sequences in *Bombyx mori* nucleopolyhedrovirus. *Virus Genes* **22**, 247-254.

Loukeris, T. G., Arca, B., Livadaras, I., Dialektaki, G. & Savakis, C. (1995a). Introduction of the transposable element *Minos* into the germ line of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences, U S A* **92**, 9485-9489.

Loukeris, T. G., Livadaras, I., Arca, B., Zabalou, S. & Savakis, C. (1995b). Gene transfer into the medfly, *Ceratitis capitata*, with a *Drosophila hyde* transposable element. *Science* **270**, 2002-2005.

Lu, A. & Miller, L. K. (1995). The roles of eighteen baculovirus late expression factor genes in transcription and DNA replication. *Journal of Virology* **69,** 975-982.

Lu, A. & Miller, L. K. (1997). Regulation of baculovirus late and very late gene expression. In: *The Baculoviruses*, pp. 193-216. Edited by L. K. Miller. New York: Plenum Press.

Luque, T., Finch, R., Crook, N., O'Reilly, D. R. & Winstanley, D. (2001). The complete sequence of the *Cydia pomonella* granulovirus genome. *Journal of General Virology* **82**, 2531-2547.

Majima, K., Kobara, R. & Maeda, S. (1993). Divergence and evolution of homologous regions of *Bombyx mori* nuclear polyhedrosis virus. *Journal of Virology* **67**, 7513-7521.

Martens, J. W., van Oers, M. M., van de Bilt, B. D., Oudshoorn, P. & Vlak, J. M. (1995). Development of a baculovirus vector that facilitates the generation of p10-based recombinants. *Journal of Virological Methods* **52**, 15-19.

Martin, D. W. & Weber, P. C. (1997). DNA replication promotes high-frequency homologous recombination during *Autographa californica* multiple nuclear polyhedrosis virus infection. *Virology* **232**, 300-309.

McDonald, J. F., Matyunina, L. V., Wilson, S., Jordan, I. K., Bowen, N. J. & Miller, W. J. (1997). LTR retrotransposons and the evolution of eukaryotic enhancers. *Genetica* **100**, 3-13.

Menees, T. M. & Sandmeyer, S. B. (1996). Cellular stress inhibits transposition of the yeast retrovirus-like element *Ty3* by a ubiquitin-dependent block of virus-like particle formation. *Proceedings of the National Academy of Sciences, U S A* **93**, 5629-5634.

Michel, K., Pinkerton, A. C., Franz, G., Robinson, A. S., Gariou-Papalexiou, A., Zacharopoulou, A., O'Brochta, D. A. & Atkinson, P. W. (2001). *Hermes*-mediated germ-line transformation of the Mediterranean fruit fly *Ceratitis capitata*. *Insect Molecular Biology* **10**, 155-162.

Miller, D. W. & Miller, L. K. (1982). A virus mutant with an insertion of a copia-like transposable element. *Nature* **299,** 562-564.

Miller, L. K. (1996). Insect viruses. In *Fields virology*, Third Edition, pp. 533-556. Edited by B. N. Fields, D. M. Knipe, P. M. Howley, *et. al.*. Philadelphia: Lippincott-Raven Publishers.

Miller, W. J., McDonald, J. F. & Pinsker, W. (1997). Molecular domestication of mobile elements. *Genetica* **100**, 261-270.

Misra, S. & Rio, D. (1990). Cytotype control of *Drosophila P* element transposition: the 66 kD protein is a repressor of transposase activity. *Cell* **27**, 269-284.

Mori, I., Benian, G. M., Moerman, D. G. & Waterston, R. H. (1988). Transposable element *Tc1* of *Caenorhabditis elegans* recognizes specific target sequences for integration. *Proceedings of the National Academy of Sciences, U S A* **85**, 861-864.

Morimoto, R. I., Tissieres, A. & Georgopoulus, C. (1994). In: *The biology of heat shock proteins and molecular chaperones*, eds. Morimoto, R. I., Tissieres, A. & Georgopoulus, C. (Cold Spring Harbor Laboratory Press, Plainview, New York, pp 1-30.

Mount, S. M. & Rubin, G. M. (1985). Complete nucleotide sequence of the *Drosophila* transposable element *copia*: homology between *copia* and retroviral proteins. *Molecular and Cell Biology* **5**, 1630-1638.

Novina, C. D. & Roy, A. (1996). Core promoters and transcriptional control. *Trends in Genetics* **12**, 351-355.

O'Brochta, D. A., Warren, W., Saville, K. J. & Atkinson, P. W. (1996). *Hermes*, a functional non-drosophilid insect gene vector from *Musca domestica*. *Genetics* **142**, 907-914.

O'Brochta, D. A., Atkinson, P. & Lehane, M. J. (2000). Transformation of *Stomoxys calcitrans* with a *Hermes* gene vector. *Insect Molecular Biology* **9**, 531-538.

Oellig, C., Happ, B., Müller, T. & Doerfler, W. (1987). Overlapping sets of viral RNAs reflect the array of polypeptides in the *Eco*RI J and N fragments (map positions 81.2 to 85.0) of the *Autographa californica* nuclear polyhedrosis virus genome. *Journal of Virology* **61**, 3048-3057.

O'Reilly, D. R., Hails, R. S. & Kelly, T. J. (1998). The impact of host developmental status on baculovirus replication. *Journal of Invertebrate Pathology* **72,** 269-275.

Orgel, L. E. & Crick, F. H. (1980). Selfish DNA: The ultimate parasite. *Nature* **284**, 604-607.

Ozers, M. S. & Friesen, P. D. (1996). The *Env*-like open reading frame of the baculovirus-integrated retrotransposon *TED* encodes a retrovirus-like envelope protein. *Virology* **226,** 252-259.

Pang, Y., Yu, J. X., Wang, L. H., Hu, X. H., Bao, W. D., Li, G., Chen, C., Han, H., Hu, S. N. & Yang, H. M. (2001). Sequence analysis of the *Spodoptera litura* multicapsid nucleopolyhedrovirus genome. *Virology* **287**, 391-404.

Payne, C. C. (1981). The susceptibility of the pea moth, *Cydia nigricana*, to infection by the granulosis virus of the codling moth, *Cydia pomonella*. *Journal of Invertebrate Pathology* **38,** 71-77.

Pearson, M. N., Bjornson, R. M., Pearson, G. & Rohrmann, G. F. (1992). The *Autographa californica* baculovirus genome: Evidence for multiple replication origins. *Science* **257**, 1382-1384.

Peloquin, J. J., Staten, R. & Miller, T. A. (2000). Germ-line transformation of pink bollworm (Lepidoptera: *gelechiidae*) mediated by the *piggyBac* transposable element. *Insect Molecular Biology* **9**, 323-333.

Peterson, P. A. (1985). Virus-induced mutations in maize: On the nature of stress-induction of unstable loci. *Genetical Research* **46,** 207-217.

Putterman, D., Pepinsky, R. B. & Vogt, V. M. (1990). Ubiquitin in avian leukosis virus particles. *Virology* **176**, 633-637.

Radice, A. D., Bugaj, B., Fitch, D. H. A. & Emmons, S. W. (1994). Widespread occurence of the *Tc1* transposon family: Tc1-like transposons from teleost fish. *Molecular and General Genetics* **244**, 606-612.

Ratner, V. A., Zabanov, S. A., Kolesnikova, O. V. & Vasilyeva, L. A. (1992). Induction of the mobile genetic element *Dm-412* transpositions in the *Drosophila* genome by heat shock treatment. *Proceedings of the National Academy of Sciences, U S A* **89,** 5650-5654.

Raz, E, van Luenen, H. G., Schaerringer, B., Plasterk, R. H. & Driever, W. (1998). Transposition of the nematode *Caenorhabditis elegans Tc3* element in the zebrafish *Danio rerio. Current Biology* **8**, 82-88.

Rio, D. S., Laski, F. & Rubin, G. M. (1986). Identification and immunochemical analysis of biologically active *Drosophila P* element transposase. *Cell* **17**, 21-32.

Rio, D. S. (1990). Molecular mechanisms regulating *Drosophila P* element transposition. *Annual Review of Genetics* **24**, 543-578.

Robertson, H. M. & MacLeod, E. G. (1993). Five major subfamilies of mariner transposable elements in insects, including the mediterranean fruit fly, and related arthropods. *Insect Molecular Biology* **2**, 125-139.

Robertson, H. M. (1993). The *mariner* transposable element is widespread in insects. *Nature* **362**, 241-245.

Robertson, H. M. (1995). The Tc1-mariner superfamily of transposons in animals. *Journal of Insect Physiology* **41**, 99-105.

Robertson, H. M. & Lampe, D. J. (1995). Distribution of transposable elements in arthropods. *Annual Review of Entomology* **40**, 333-357.

Rozenzweig, B., Liao, L. W. & Hirsh, D. (1983). Sequence of the *C. elegans* transposable element *Tc1*. *Nucleic acids Research* **11**, 4201-4209.

Rubin, G. M. (1983). In Mobile Genetic elements (J.A.Shapiro, ed) pp 329-351. Academic press, New York.

Sait, S. M., Begon, M. & Thompson, D. J. (1994). The effects of a sublethal baculovirus infection in the Indian meal moth, *Plodia interpunctella*. *Journal of Animal Ecology* **63**, 541-550.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd edn. New York: Cold Spring Harbor Laboratory.

Schetter, C., Oellig, C. & Doerfler, W. (1990). An insertion of cell DNA in the 81-map-unit Segment of the *Autographa californica* nuclear polyhedrosis virus DNA. *Journal of Virology* **64**, 1844-1850.

Slack, J. M., Kuzio, J. & Faulkner, P. (1995). Characterization of v-cath, a *cathepsin* L-like proteinase expressed by the baculovirus *Autographa californica* multiple nuclear polyhedrosis virus. *Journal of General Virology* **76**, 1091-1098.

Smit, A. F.A. (1999) Interspersed repeats and mementos of transposable elements in mammalian genomes. *Current Opinions in Genetic Development* **9**, 657-663.

Smith, G. E., Summers, M. D. & Fraser, M. J. (1983). Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Molecular and Cellular Biology* **3**, 2156-2165.

Smith, I. R. L. & Crook, N. E. (1988). *In vivo* isolation of baculovius genotypes. *Virology* **166**, 240-244.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503-517.

Strand, D. J. & McDonald, J. F. (1985). *Copia* is transcriptionally responsive to environmental stress. *Nucleic Acids Research.* **13,** 4401-4410.

Tamura, T., Thibert, C., Royer, C., Kanda, T., Abraham, E., Kamba, M., Komoto, N., Thomas, J. L., Mauchamp, B., Chavancy, G., Shirk, P., Fraser, M., Prudhomme, J. C. & Couble, P. (2000). Germline transformation of the silkworm *Bombyx mori* L. using a *piggyBac* transposon-derived vector. *Nature Biotechnology* **18,** 81-84.

Tanada, Y. (1964). A granulovirus of the codling moth, *Carpocapsa pomonella L. Journal of Insect Pathology* **6**, 378-380.

Theilmann, D. A. & Stewart, S. (1992). Tandemly repeated sequence at the 3'end of the *IE-2* gene of the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus is an enhancer element. *Virology* **187**, 97-106.

Thiem, S. M. & Miller, L. K. (1990). Differential gene expression mediated by late, very late and hybrid baculovirus promoters. *Gene* **91**, 87-94.

Vlak, J. M. & Rohrmann, 1985, The nature of polyhedrin. *In* "Viral Insecticides for Biological Control" (K. Maramorosh and K. E. Sherman, Eds.), Academic Press, Orlando, pp 489-544.

Vlak, J. M. (1993). Genetic engineering of baculoviruses for insect control. In: J. Oakeshott and M. J. Whitten (eds): *Approaches to fundamental and applied entomology*. Springer-Verlag, New York, pp. 90-127.

Volkman, L. E. & Keddie, B. A. (1990). Nuclear polyhedrosis virus pathogenesis. *Seminars in Virology* **1,** 249-256.

Vos, J. C., Baere, I. D. & Plasterk, R. H. A. (1996). Transposase is the only nematode protein required for *in vitro* transposition of *Tc1*. *Genes and Development* **10,** 755-761.

Voytas, D. F. & Boeke, J. D. (1993). Yeast retrotransposons and tRNAs. *Trends in Genetics* **9**, 421-427.

Walbot, V. (1992). Reactivation of the mutator transposable element of maize by ultraviolet light. *Molecular and Geneneral Genetics* **234**, 353-360.

Wang, H. H., Fraser, M. J. & Cary, L. C. (1989). Transposon mutagenesis of baculoviruses: analysis of *TFP3* lepidopteran transposon insertions at the FP locus of nuclear polyhedrosis viruses. *Gene* **81**, 97-108.

Werren, H. (1997). Biology of *Wolbachia*. *Annual Review of Entomology* **42**, 587-609.

Wessler, S. R. (1996). Turned on by stress. Plant retrotransposons. *Current Biology* **6,** 959-961.

Winstanley, D. & Crook, N. E. (1993). Replication of *Cydia pomonella* granulosis virus in cell cultures. *Journal of General Virology* **74,** 1599-1609.

Zanotto, P. M., Kessing, B. D. & Maruniak, J. E. (1993). Phylogenetic interrelationships among baculoviruses: evolutionary rates and host associations. *Journal of Invertebrate Pathology* **62**, 147-164.

SUMMARY

The horizontal transfer of transposons from insect genomes into the genomes of infecting baculoviruses is a rare event. In previous *in vivo* infection experiments of the Mexican strain of *Cydia pomonella* granulovirus (CpGV-M) in larvae of Lepidopteran host insects, two virus mutants containing a Tc1-like transposon were isolated. Virus mutant MCp4 harbours transposon TCp3.2 which originated from *Cydia pomonella*. TCp3.2 is 3.2 kb long and is flanked by inverted terminal repeats (ITRs) of 756 bp. Mutant MCp5 contains transposon TCl4.7 that escaped from the *Cryptophlebia leucotreta* genome. TCl4.7 is 4.7 kb in size and has imperfect ITRs of 29 bp.

In order to better understand the consequences of the integration of TCl4.7 on the genome of CpGV, the integration site of this transposon was sequenced. It appeared that TCl4.7 integrated in a TA dinucleotide which was located in a non-translated region between two open reading frames (Cp15 and Cp16) with unknown function. Two repeated sequences which form imperfect palindromes of approximately 70 bp were identified in this intergenic region. The obtained 2.2 kb sequence of the CpGV-M genome was compared with the corresponding genetic region of CpGV-M1. These sequences were almost identical, CpGV-M had only one additional nucleotide within one of the palindromic sequences.

Although both transposons stabily integrated into the CpGV genome, it was found that TCp3.2 caused further heterogeneity within the MCp4 genome. During *in vivo* cloning studies of MCp4, a mutant called MCp4inv was isolated. MCp4inv showed heterogeneity in the genomic area of the transposon insertion. Restriction mapping, PCR analysis and subsequent sequence analysis gave strong evidence that TCp3.2 inverted within the MCp4 genome. This inversion was most probably caused by homologous recombination between the long ITRs of the transposon.

The initial *in vivo* cloning studies performed by Jehle *et al*., (1995) resulted in the isolation of two CpGVs harbouring a transposon. It was investigated what may have lead to the isolation of these mutant viruses. The questions addressed included (i) whether the transposon harbouring viruses had a replication advantage over the wild-type and became dominant in the virus population or (ii) whether the activity of the host transposons was stimulated by virus infection. CpGV-M and the mutants were characterised with lethal dose (LD) and survival time bioassays (ST). The LD_{50} values for MCp4, MCp5 and CpGV-M did not differer. The ST_{50} values, however, demonstrated that the transposon containing viruses killed *C. pomonella* larvae slower than

CpGV-M showing that the mutants had a replication disadvantage. In order to compare the replication rate of CpGV-M and the mutants, co-infection experiments of fifth instar *C. pomonella* were performed using a mixture of CpGV-M and mutant viruses as inoculum. By comparing the CpGV-M:mutant ratio of the virus inoculum with the ratio of the virus progeny, it was shown that MCp4 and MCp5 were out-competed quickly by CpGV-M. This further demonstrated a significant selection disadvantage for the transposon carrying mutants compared to CpGV-M.

For a direct measurement of transposon TCp3.2 activity no transposition assay is available. Based on other Tc1-like transposons, however, it is assumed that the transposase is the only protein required for the transposition reaction. Therefore, the level of transposase mRNA within the larvae was used as measure for transposon activity. In order to test whether the virus infection had an influence on the transposition activity, the transcription level of the putative transposase gene of TCp3.2 was investigated in virus infected and mock infected larvae. Transcription levels of host TCp3.2 transposase were determined by quantitativ RT-PCR of cDNA which was generated by reverse transcription of mRNA isolated from *C. pomonella* larvae. The experiments demonstrated that a higher level of transposase transcription was detectable in CpGV-M infected than in mock infected control larvae. This observation gave a first evidence that CpGV-M infection might trigger the activity of transposon TCp3.2 within the genome of *Cydia pomonella*. Our results suggest that horizontal transfer of insect host transposable elements into baculovirus genomes may be induced by virus infection.

ZUSAMMENFASSUNG

Der horizontale Transfer von Insektentransposons in das Genom infizierender Baculoviren ist ein seltenes Ereignis. Zwei Virusmutanten des mexikanischen Stamms des *Cydia pomonella* Granulovirus, die einstmals bei *in vivo* Infektionsversuchen isoliert wurden waren die Basis für diese Arbeit. Beide Mutanten enthalten ein Tc1-ähnliches Transposon. Die Virusmutante MCp4 beherbergt das Transposon TCp3.2, das aus dem Genom von *C. pomonella* stammt, hat ein Länge von 3.2 kb und wird von invertierten terminalen Sequenzwiederholungen von 756 bp flankiert. Die mutante MCp5 enthält das Transposon TCp4.7 welches aus dem *Cryptophlebia leucotreta* Wirtsgenom stammt. TCl4.7 hat ein Länge von 4.7 kb und hat invertierte terminale Sequenzwiederholungen von 29 bp.

Um ein besseres Verständnis für die Folgen der Integration von TCl4.7 auf das CpGV-M Genom zu bekommen, wurde der Integrationsort dieses Transposons sequenziert. Dabei zeigte sich, dass sich TCl4.7 in einen TA-Dinukleotid zwischen zwei offenen Leserastern (Cp15 und Cp16) mit unbekannte Funktionen einfügte. Außerdem wurden wiederholte Sequenzen innerhalb der nicht kodierenden Region identifiziert welche imperfekte Palindrome von ungefähr 70 bp formen. Die erhaltene 2.2 kb der CpGV-M Sequenz wurde mit der übereinstimmender Sequenzregion des komplett sequenzierten CpGV-M1 verglichen.

Obwohl sich beide Transposons stabil in das CpGV Genom einbaute, konnte festgestellt werden dass TCp3.2 weitere Heterogenität innerhalb des MCp4 Genoms verursachte. Während weiterer *in vivo* Klonierungsstudien wurde die MCp4 Mutante, MCp4inv, isoliert. Diese Heterogenität wurde mittels Restriktionanalyse innerhalb der Insertionregion gezeigt. PCR Analyse und nachfolgende Sequenzanalysen lieferten einen überzeugenden Nachweis, dass die Heterogenität durch die Inversion des Transposons TCp3.2 mittels homologer Rekombination zwischen den langen terminalen Sequenzwiederholungen, verursacht wurde.

Da die ursprünglichen *in vivo* Infektionsversuchen in einer relativ hohen Menge von Transposon tragenden Viren resultierte (Jehle *et al*. 1995), wurde nach der Ursache hierfür gesucht. Möglich ist, dass die Transposon-tragenden Viren einen Selektionsvorteil gegenüber den Wildtypviren haben oder dass die Transposition endogener Inzektentransposons durch die Infektion mit Baculoviren begünstigt wird. Für die biologische Charakterisierung der Mutanten wurden die Parameter mittlere letale Dose (LD_{50}) und mittlere Absterbezeit (ST_{50}) bestimmt. Die LD_{50}-Werte für MCp4, MCp5 und CpGV-M waren nicht signifikant unterschiedlich. Die ST_{50}-

Werte hingegen, zeigten dass die Transposon-tragenden Viren *Cydia pomonella* Larven langsamer töteten als CpGV-M. Die Replikationsgeschwindigkeit der Virus-Genotypen MCp4 und MCp5 gegenüber CpGV-M wurde in *C. pomonella* Larven mittels Mischinfektionen bestimmt. Dazu wurde das relative Mengenverhältnis von CpGV-M und den jeweiligen Mutanten in der Infektionsdosis mit dem entsprechenden Mengenverhältnis der Virusnachkommen verglichen. Es konnte gezeigt werden, dass während der Virusreplikation sowohl MCp4 als auch MCp5 rasch heraus konkurriert wurden durch CpGV-M. Diese Ergebnisse demonstrierten dass die Transposon-tragenden Viren einen Selektionsnachteil gegenüber CpGV-M haben.

Um zu untersuchen inwiefern eine Infektion durch Baculoviren die Aktivität endogener Transposons beeinflusst, wurde die Transkription des Transposasegenes von TCp3.2 in mit Virus infizierten und nicht infizierten Larven bestimmt. Die Transkriptionshöhe der Transposase wurde mittels quantitativer RT-PCR bestimmt auf cDNA dass generiert wurde von mRNA dass aus *C. pomonella* Larven gewonnen wurde. Dabei konnte gezeigt werden, dass die Transposase Transkription in mit Virus infizierten Larven höher war als in Kontrolle-Larven. Diese Beobachtungen lieferten Indizien dass CpGV-M Infektion zur Aktivierung des Transposons TCp3.2 innerhalb des *C. pomonella* Genoms führte. Diese Ergebnisse deuten darauf hin dass der horizontale Transfer von Insektentransposons in Baculovirengenome möglicherweise durch Virusinfektion induziert wird.

DANKWOORD

Na 4 jaar met veel plezier aan mijn promotieonderzoek te hebben gewerkt, is het nu de tijd om de laatste pagina van mijn proefschrift te schrijven. Hierin wil ik graag alle mensen bedanken die een bijdrage hebben geleverd aan de succesvolle afronding hiervan.

Om te beginnen wil ik mijn begeleider Xxxxxxxx Xxxxx bedanken voor zijn goede en motiverende begeleiding gedurende dit project. Xxxxxxxx, de grote vrijheid die ik van je kreeg in het uitdenken en uitvoeren van de proeven heb ik zeer gewaardeerd. Verder wil ik Xxxxxx Xxxxxxxxxxx bedanken voor de gelegenheid die ik kreeg om twee maanden in haar lab van Horticulture Research International in Wellesbourne, Engeland te werken. Xxxxxx, ik heb veel geleerd van jouw kennis van granulovirussen en *Cydia pomonella* insecten en celculturen. Je kritische opmerkingen en de goede discussies die we hadden, waren zeer waardevol. Tevens wil ik Prof. Dr. X. X. Xxxxxxx van het Institut für Molekulargenetik und Gentechnologische Sicherheitsforschung en Prof. Dr. X. Xxxxx van het Institut für Mikrobiologie und Weinforschung van de Johannes Gutenberg-Universiteit in Mainz bedanken voor hun referaat.

Verder wil ik Xxxxxxx Xxxxxx van de BBA in Darmstadt en Xxxxxxxx Xxxxxxxx van de SLFA-Neustadt bedanken voor de constante toestroom van 'Apfelwickler' eieren. Ik wil Xxxx Xxxxx bedanken voor het produceren en titreren van de benodigde 'budded viruses', zodat mijn proeven bij het HRI in Engeland efficiënt konden verlopen. Voor de statistische analyse van de bioassay resultaten ben ik dank verschuldigd aan Xxxxxxx Xxxxx van de SLFA-Neustadt. Voor de financiële ondersteuning van dit project dank ik de Deutsche Forschungsgemeinschaft en de Deutscher Akademischer Austauschdienst.

Voor het tot een goed einde brengen van het onderzoek, zijn naast wetenschappelijke-, ook andere factoren erg belangrijk geweest. Voor een goede werksfeer werd in eerste instantie gezorgd door de medewerkers van het vakgebied Biotechnologischer Pflanzenschutz. Ik heb niet alleen genoten van de gezelligheid in het lab, de koffiepauzes en de lunches, maar ook van de “na-het-werk” activiteiten. Xxxxxxx, Xxxxxxx, Xxxx, Xxxxxx, Xxxxxxx, Xxxxxx, Xxxxxx, Xxxxxx, Xxx, Xxxxxxxx, Xxxx, Xxxxxxx, Xxxxxx en Xxxx bedankt!

Verder wil ik mijn ouders bedanken voor hun steun en vooral ook voor hun begrip dat ik naar Duitsland ben gegaan om dit onderzoek te doen.

Naast de resultaten beschreven in dit proefschrift, heeft het lab mij meer opgeleverd. Xxxx, jou wil ik ook hier nog een keer bedanken voor je meer dan met woorden te beschrijven steun, begrip, vertrouwen, geduld, humor en liefde. Ik heb het volle vertrouwen dat ons gezamenlijke vervolgproject "Xxxxxx" minstens zo interessant zal zijn!

PUBLICATIONS

Jehle, J. A., Arends, H. M. & Vlak, J. M. (1999). Molekulare Untersuchungen zum horizontalen Transfer des Insektentransposons TCp3.2 im *Cydia pomonella* Granulovirus- ein Beitrag zur Sicherheitsforschung bei Baculoviren. BMBF-Workshop Biologische Sicherheit, Braunschweig 1999 (185-191).

Arends, H. M. & Jehle, J. A. (2002). Homologous recombination between the inverted terminal repeats of defective transposon TCp3.2 causes an inversion in the genome of *Cydia pomonella* granulovirus. *Journal of General Virology* **83**, 1573-1578.

Arends, H. M. & Jehle, J. A. Analysis of transposon TCp3.2 transposase transcription in *Cydia pomonella* larvae (in preparation).

Arends, H. M., Winstanley, D. & Jehle, J. A. Biological characterisation of two *Cydia pomonella* granulovirus mutants which harbour an insect host transposon (in preparation).