Functional Genomics and RNA interference in an insect DNA virus (Baculovirus) *Autographa Californica*

THESIS

Submitted for the degree of

DOCTOR OF PHILOSOPHY

To

JAWAHARLAL NEHRU UNIVERSITY

NEW DELHI

BY

PRAVEENSINGH B. HAJERI

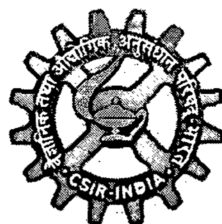
CENTRE FOR CELLULAR AND MOLECULAR BIOLOGY

HYDERABAD 500 007, INDIA

2009

Functional Genomics and RNA interference in an insect DNA virus (Baculovirus) *Autographa Californica*

Praveensingh B. Hajeri

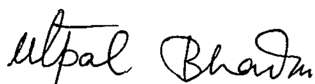


CENTRE FOR CELLULAR AND MOLECULAR BIOLOGY
HYDERABAD 500 007, INDIA

2009

CERTIFICATE

The research work presented in this thesis has been carried out at the **Centre for Cellular and Molecular Biology (CCMB), Hyderabad,** India. This work is original and has not been submitted in part or in full to any other university for other degree or diploma.



Dr. Utpal Bhadra
Supervisor

Praveensingh B. Hajeri
Student

To,

My Dearest

Parents and Brother

Acknowledgements:

I wish to express my sincere thanks to my Supervisor Dr Utpal Bhadra for his precious time and advice during this work and for giving challenging opportunities to work in the emerging field of RNAi. I sincerely appreciate his patience and advise while writing this thesis and his sustained co-operation and encouragement. I would like to extend my gratitude to his family Dr Manika Pal Bhadra for all the suggestions and advise. She was very generous in helping me carry out the microarray work and It was a great learning experience to spend all these years in their lab and every moment was memorable.

I am grateful to my labmates Pallavi, Sumit & Satya who were with me for the last seven long years, sharing my highs and lows and tolerating me. Special thanks to Sumit and Pallavi for all the favors. It was really great talking to Satya about everything and I thank him for supporting me throughout. It is a really difficult task to express my gratefulness in my limited vocabulary. They have gone out of their way to help me and every moment I spent with them was a great learning experience.

I thank Dr Sunit Singh for being so considerate, kind and nice to me. I am grateful for all his brotherly suggestions and favors. He became an important part of our lives in such a short time.

My sincere thanks Dr Manjula Reddy who supported and encouraged me throughout my stay in CCMB. She shared her lab space, and tolerated me for a very long time which would not have been easy for her. She also helped me with fellowship when I needed it the most. That was really a great favor and will be remembered. I thank my well wisher Dr Yogendra Sharma also for being so considerate and offering me fellowship.

I thank Dr Krishnan and Dr Mandar Deshmukh for being so generous, friendly and supportive and for extending their support whenever I asked for. My thanks to Dr.Raghunand, and Dr.Pooran singh for offering all kinds of help.

I thank Mrs Anita Krishnan for all the help she did and being so considerate. My sincere thanks to my labmates Pushpa, venkateshwara Reddy, Santosh , Mehaboob, Madhu, Pranjal , lavanya, Indira, Mamata, Neelima, Deepak, Mahesh, Pramod for being very supportive and it was really nice to spend time with them.

My batch mates Amit, Ashish, Brijesh, Vasanti, Ram, Rupa, Rajkumar, Ganesh, Shoeb , Tiru, Soji, Usha, Shweta, Krishnan, Sahasranshu in CCMB have really made my life easier

here every moment of these years was memorable. I thank AShish, Rajkumar, and Sahasranshu for helping in very critical times and helping me to get over it. My special thanks to Manjunath Joshi, Pavan, Pvan kumar pindi, Satish, Nagabhushan, Gurudatt, Ravi for all their help. I could always count on them for everything.

I thank Mrs Hemalatha, Narasimha and Mrs Saisree for being so helpful and kind to me.

My sincere thanks to my childhood friends Mahesh, Guru, Giri, Subbu, Kapil, Vishwa, Sumanth and Chaitra who found me again after a long time and extending their help unconditionally.

Nothing was possible for me without the support of my family. My dearest parents and little brother Prasanna encouraged me with Great Spirit and I thank god for blessing my life and making me a part of this family.

Synopsis

Synopsis:

RNA interference is a phenomenon by which dsRNAs along with many other protein factors regulate their targets in a sequence specific manner. RNAi is known to be a regulator of the genes and genome which acts thorough a repertoire of small RNAs like siRNAs, miRNAs piRNAs and tasiRNAs etc, their biogenesis and mechanism of action and function are still being investigated but whatever little we know about these processes is enough to highlight their importance in normal functioning of an organism. RNA is an indispensable process for regulating the expression of genes and genome in all organisms, wherever it is found. In addition to their role of gene and genome regulation, they act as a defense mechanism against invasive genetic elements like transposons and viruses. In plants it is well established that RNAi is a major defense mechanism against viruses and viroids. Recently it has been shown that in animals like *C.elegans*, *Drosophila* also RNAi acts as an antiviral immune response. Evidences to support RNAi, as an antiviral immune response in higher animals like vertebrates which have additional adaptive immune response and interferon mediated immune response are accumulating.

In order to counteract host RNAi mediated immune response many viruses have evolved mechanisms to escape/overcome RNAi. They achieve it mainly by encoding suppressors of RNAi. RNAi suppressors can interfere at different steps of RNAi and inhibit them from acting on substrates of viral origin. Many viruses are targeted by miRNAs also and interestingly many viruses exploit host miRNAs in their favor. few viruses like herpes virus, Epstein barr virus, HIV etc encode their own miRNAs and manipulate host genes to inhibit other immune response pathways and/or to induce a period of latency in infection.

Better understanding of the mechanism of RNAi pathway has enabled us to use this as a tool to knock down genes and can be regulated temporally and spatially. Efforts are being made to use RNAi as a therapeutic tool to target diseases caused by over-expression of genes or viral diseases.

A major constraint in using RNAi for therapeutic purposes are to find good targets and to deliver RNAi triggers efficiently. Important features like sequence specificity

and possibilities of regulation of their dose both temporally and spatially, have attracted a wide interest in researchers and a large number of efforts have been undertaken in this regard.

Using RNAi for human therapy is marred with lot of ethical concerns like mode of delivery and possible non-specific effects etc but for livestock like cattle, poultry, shrimps and other economically important insects like silkworms can be engineered and tried to utilize RNAi to induce resistance against viral diseases.

Rationale:

Baculovirus infect many insect species and usually result in death of their hosts. They are highly virulent and show severe pathological symptoms. Many attempts were made to engineer *Bombyx mori* and other insect cells to make them resistant to virus infection but none of them have met complete success. So it was necessary to understand the reasons of such failures. It could be due to employing inappropriate strategy to induce RNAi, or inappropriate choice of targets. It is also possible that these viruses can overcome RNAi targeted against them and therefore could not be controlled by RNAi. so it was necessary to address these issues to unravel the most suitable strategy out of all possible ways to induce RNAi, and to find out viral genes that can be most vulnerable to be targeted by RNAi. These viruses have to be verified whether they have the ability to overcome RNAi with the help of suppressors of RNAi or by any other mechanism.

Basic understanding of the mechanisms underlying the pathogenesis of a virus is an essential thing in order to find remedies for their ill effects. Understanding the role of different genes of Baculovirus will shed light on their basic pathology. So gene involved in processes like viral gene regulation and host gene manipulation by affecting their chromatin organization, replication, transcription or translation are good candidates to study such aspects. General gene regulatory pathways like chromatin modification and DNA methylation regulate not only host genes but genes of viruses infecting them. It is known in many viruses that these aspects influence the expression of viral genes and inhibit their by chemical inhibitors affects viral gene expression and replication. but in baculoviruses such reports and limited and role DNA methylation in regulation of their genes is yet to be studied.

In this thesis we have tried address these issues and our results are divided into 5 chapters. (Chapter 3-7)

Chapter 1: Introduction.

The first part of this chapter gives a brief introduction of RNAi and its role in regulation of genes and its role as an antiviral immune response. Many viruses have the ability to inhibit or overcome RNAi by different ways and we have discussed the mechanisms employed by some of these viruses which are capable of inhibiting or overcoming RNAi. A brief review of reports related to RNAi in other insects and some important aspects of its mechanism, and applications are discussed. In the second half we have reviewed our understanding about Baculoviruses, their genes, genome and infection cycle.

Chapter 2: Materials and General methods.

Provides a detailed view of materials used and General methods followed in this study.

Chapter 3: RNAi can suppress viral genes in AcMNPV infected permissive and non-permissive hosts.

RNAi is widely used as a tool to silence genes and a large number of efforts have been undertaken to use it in antiviral therapeutic applications. The choice of most suitable strategy to induce RNAi is an important aspect and depends on the host cells and targets which are to be silenced.

In this chapter we verified different methods of triggering RNAi in sf9 cells to suppress Baculovirus infection. We employed plasmid based delivery of shRNA & dsRNA, naked dsRNAs and recombinant virus mediated delivery of dsRNA and their efficiency and advantages are discussed. We found dsRNAs were effective when expressed under viral promoters.

Chapter 4: RNAi based Screening and functional genomics of AcMNPV.

In order to effectively suppress a virus by RNAi it is necessary to target the most essential genes of virus. Baculoviruses are well studied among insect viruses and by various methods of mutagenesis many viral genes are found to be essential, but information about which of those genes can be targeted by RNAi more efficiently than others is not known.

In this chapter we performed a RNAi based screening to determine which of the 156 AcMNPV orfs are essential and which of those can be effectively targeted by RNAi. Based on the efficiency of suppression of virus we categorized them into two groups, first group of genes showing more than 60% suppression and second one showing between 40-60% suppression. These genes can be used alone or in combinations as candidates to suppress baculoviruses n RNAi based Strategies.

Chapter 5: RNAi based AcMNPV genome wide screening of putative suppressors of RNAi.

RNAi acts as an antiviral response and many viruses have evolved mechanisms to escape/observe RNAi many efforts were done to suppress baculoviruses in insects and insect cells but none of them assured complete resistance. Moreover RNAi was observed to inhibit viruses only for a brief period with limited efficiency and these viruses appear to overcome the effects of RNAi as the infection progresses. It is possible that these viruses encode suppressors of RNAi to overcome/escape RNAi targeted against them. So we designed Sensor viruses to screen for putative RNAi suppressors in baculoviruses and screened all 156 orfs for their possible role as RNAi suppressors. We could narrow down to a few candidates whose function as suppressors has to be validated.

Chapter 6: Role of bro in AcMNPV infection and Transcription: A Microarray based study.

Baculovirus (AcMNPV) infection causes global shut down of transcription and translation of host genes. Viruses encode many genes which can manipulate the

host gene expression by interfering with transcription, translation, chromatin organization and DNA methylation of host genes.

bro is a viral gene which is known to interact with histones, Laminin, CRM1 etc and might be involved in regulating many host genes. They are also reported to bind to nucleic acid in vitro. It is proposed that bro might have a role in progress of virus infection from one phase to other by regulating specific set of viral genes. In AcMNPV bro is reported to be a nonessential gene unlike BmNPV but when we suppressed bro by RNAi we observed severe reduction in virus infection and production of next generation of viral particles in contrast to earlier observations. Further we examined the expression profile of all 156 orfs of AcMNPV in *bro* knock down cells by microarray. We could find genes involved in replication, transcription, ODV and BV associated proteins and *per-os* genes involved in efficiently infecting insect hosts through oral route, were down-regulated. Many genes which were usually suppressed during normal infection to allow the infection to proceed towards late and very late phases remained up-regulated in bro knock down cells. Genes involved in binding to DNA and having role in DNA damage responses, early gene transactivation etc, were specifically up-regulated. The observation of such specific suppression and up-regulation of particular genes involved in virus infection suggest that the effect on BV production during dsRNA based suppression of BRO is not a nonspecific effect and unlike the earlier report *bro* indeed affects both BV and ODV production.

Chapter 7: Role of AZdC a DNA methyltransferase inhibitor, on Baculovirus infection in sf9 cells.

AcMNPV have a large dsDNA genome and contain 156 orfs which is relatively high for viruses. Genes of many such dsDNA viruses such as Herpes simplex virus etc are known to be affected by host gene regulatory mechanisms like chromatin organization and DNA methylation. However the role of chromatin organization/modifications and DNA DNA methylation pathways in baculoviruses are not well studied.

In this chapter the role of DNA methylation in Baculovirus infection were investigated. We found Baculovirus production was inhibited when cells were treated with Dnmt inhibitors and early stages of infection. Moreover AZdC interferes with viral DNA replication and affects downstream late & very late gene expressions and virus production. It was interesting to see the role DNA methylation in Baculovirus whose hosts have very limited DNA methylation machinery.

Functional Genomics and RNA interference in an insect DNA virus (Baculovirus) *Autographa Californica*

Table of contents

List of Abbreviations......1

List of Figures...... 2

List of Tables...... 3

Chapter 1: Introduction......5.

1. Introduction:...... **5**

1.1. RNAi:...... **5**

1.1.1 History of RNAi...... 5

1.1.2 Natural processes regulated by RNAi:...... 8

1.1.3 siRNA pathway: (fig 1 diversity of RNAi sources)...... 9

1.1.4 miRNAs and miRNA pathway: 9

1.1.5 RNAi as an antiviral response:...... 13

1.1.5.1 RNAi as antiviral response in mammals...... 13

1.1.5.2 miRNA and Virus:...... 15.

1.1.5.3 RNAi as antiviral response in insects:...... 16

1.1.6 RNAi in insects: some special features...... 18

1.1.6.1 Parental RNAi:...... 19

1.1.7 Suppressors of RNAi:...... 20

1.2. Baculovirus: **24**

1.2.1 History of Baculoviruses:...... 24

1.2.2 Baculovirus infection cycle:...... 25

1.2.2.1 Overview:...... 25

1.2.2.2 Infection through gut: (Entry of Baculovirus into midgut cells through gut)

1.2.2.3 Entry of Nucleocapsids into nucleus of Midgut epithelial cells

and Production of Nucleocapsids.. 28

1.2.2.4 Secondary infection of cells:.. 29

1.2.3 Taxonomy:.. 31

1.2.4 Genome:... 33

1.2.4.1 General features.. 33

1.2.4.2 Hrs:... 34

1.2.5 Organization of genes in Baculovirus:.. 35

1.2.5.1 Promoters of viral genes:... 35

1.2.6 Transcription of Baculovirus genes:... 37

1.2.6.1 Early genes:... 37

1.2.6.2 Viral RNA polymerase:.. 38

1.2.6.3 Late & very late genes: 39

1.2.7 Viral DNA replication:... 42

1.2.8 Packaging assembly:... 44

1.2.9 Virus structure:.. 45

1.2.9.1 Nucleocapsids:... 45

1.2.9.2 Budded virus:... 46

1.2.9.3 Occlusion derived virus:.. 47.

1.2.9.4 Occlusion Bodies:... 48

Chapter 2: Materials and General Methods

2.1. Cell lines and medium 51

2.1.1 sf9 cells:... 51

2.1.2 BmN cells:... 51

2.1.3 D.mel2 cells:... 51

2.1.4 Medium of culture: Grace's Medium, TNM-FH and serum free medium.

Schneider's medium, and serum free medium.... 51

2.2. Virus methods.... 52

2.2.1 Wild type virus AcMNPV, BmNPV.... 52

2.2.2 Productions of recombinant viruses.... 52

2.2.3 General method of infection with virus:.... 52

2.2.4 Amplification of virus stock.... 53

2.2.5 Concentration of virus stock.... 53

2.3 Isolation of viral DNA.... 54

2.3.1 Isolation of viral DNA by Phenol, Chloroform method.... 54

2.3.2 Isolation of DNA from purified Budded Virus.... 54

2.3.3 Isolation of viral DNA by High-Pure Viral DNA isolation kit.... 55

2.4. Estimation of Viral titer.... 55

2.4.1 Estimation of viral titer by end point dilution.... 56

2.4.2 Estimation of viral titer by Real Time PCR.... 56

2.4.3 Estimation of virus titer by Plaque assay.... 57

2.5. Plasmid and vectors.... 58

2.5.1 Commercial vectors used in this study.... 58

2.5.2 Plasmids constructed in this study.... 58

2.6. shRNA and dsRNA production by plasmid:.... 58

2.6.1 Plasmid mediated shRNA expression.... 58

2.6.2 Plasmid mediated dsRNA expression:.... 59

2.7. In vitro transcription to produce dsRNAs:.... 59

2.7.1 In-vitro transcription.... 59

2.7.2 In-vitro transcription to produce labeled RNA.... 60

2.7.3 Annealing of dsRNAs.... 60

2.8. Transfection of plasmid and dsRNA.... 60

2.8.1 Transfection of plasmid using Cellfectin reagent 60

2.8.2 Transfection of dsRNA using trans-messenger reagent 62

2.9. Making of stable dsRNA expressing cells: 62

2.9.1 Selection of stable dsRNA expressing lines by transfection and Zeocin selection ....

2.9.2 Selection of stable dsRNA expressing pool of cells by transfection and Zeocin selection 63

2.10. Inhibitors of DNA methyl transferase: 64

2.11. Bacterial expression of BRO gene: 64

2.11.1 PCR amplification of BRO gene 64

2.11.2 Cloning of BRO gene in pET-28a vector 64

2.11.3 Expression of BRO gene 65

2.12. Purification of protein BRO: 65

2.12.1 Quantification of Protein by Bradford method 66

2.13. Raising polyclonal antibodies against BRO: 67

2.13.1 Raising polyclonal antibodies against BRO in rabbits 67

2.13.2 Raising polyclonal antibodies against BRO in mice 68

2.13.3 Raising polyclonal antibodies against BRO in Mice Ascites 68

2.14. Isolation of Total RNA: 68

2.14.1 Isolation of RNA 68

2.14.2 RNA clean up by RNeasy columns 68

2.15. Polymerase chain reaction (PCR): 69

2.15.1 PCR using Taq DNA Polymerase 69

2.15.2 PCR using proof reading DNA polymerases 69

2.16. Reverse transcription PCR: 70

2.16.1 DNase treatment of RNA 70

2.16.2 Reverse transcription of RNA 70

2.17. Cloning of DNA fragments into plasmids: 71

2.17.1 Restriction endonuclease digestion of DNA 71

2.17.2 Ligation of DNA (vector and insert) 71

2.17.3 Preparation of ultra-competent cells 71

2.17.4 Transformation of bacterial cells (Ultra-Competent cells) 72

2.17.5 Screening for recombinant Plasmids 72

2.17.6 Colony PCR 72

2.18 Isolation of plasmid: 73

2.18.1 Isolation of plasmid: by Alkali Lyses method 73

2.18.2 Isolation of Plasmid by Boiling Method 74

2.18.3 Isolation of plasmid by Qiagen plasmid purification system 74

2.19. Phenol:Chloroform extraction of nucleic acids: 75

2.20. Protein methods: 76

2.20.1 Denaturing (SDS) discontinuous gel electrophoresis :(Laemmli method 76

2.20.2 Transfer of Proteins to membranes: 76

2.20.2.1 Wet transfer method 77

2.20.2.2 Semi-Dry transfer method 77

2.20.3 Ponceu staining of membranes: 77

2.20.4 Immunodetection of Proteins: 77

2.21 Silkworm methods: 78

2.21.1 Silk Worm strains 78

2.21.2 Rearing of silkworms 78

2.21.3 Infection of B.mori larvae by injection 79

2.21.4 Genomic DNA isolation of B.mori 79

2.22 Flow cytometric analysis of DNA content in cells. 80

2.23 Microscopy: **80**

2.23.1 Imaging of silkworms and complete tissue culture dish infected with recombinant viruses 81

2.23.2 Imaging of sf9 cells using live cell imaging system: 81

2.23.3 Imaging by confocal microscopes: 82

Chapter-3: RNAi can suppress viral genes in AcMNPV infected permissive and non-permissive hosts.

3.1. Introduction **83**

3.2 Materials and methods: **86**

3.2.1 Cells and medium 86

3.2.2 Silkworm Strains 86

3.2.3 Designing siRNAs and shRNA encoding DNA oligonucleotides 86

3.2.4 Cloning of shRNA encoding DNA oligonucleotides in pHSPshX plasmid vector 86

3.2.5 Construction of plasmid vector to produce dsRNAs 87

3.2.6 Making of Stable lines expressing dsRNAs 90

3.2.7 Production of dsRNAs 90

3.2.8 Transfection of Plasmids and dsRNA 92

3.2.9 Production of Recombinant AcMNPV 92

3.2.10 Recombinant AcMNPV expressing EGFP (vRec-RFP) 92

3.2.11 Recombinant AcMNPV expressing EGFP (vRec-EGFP) 94

3.2.12 Recombinant virus for dsRNA production against RFP (vRecRed-S and vRecRed-SAS) 94

3.2.13 Quantification of Silencing Efficiency by Reverse transcriptase PCR 95

3.2.14 Estimation of silencing Efficiency by microscopy 96

3.3. Results and Discussion: **96**

3.3.1 Infection of sf9 cells with recombinant Baculoviruses....97
3.3.2 Infection of Bombyx mori larvae and BmN cells with recombinant AcMNPV....97
3.3.3 Knockdown of viral genes by vector mediated shRNA delivery....100
3.3.4 Knockdown of Viral genes by transient vector mediated dsRNA delivery....102
3.3.5 Knockdown of viral genes by stable expression of dsRNA....102
3.3.6 Knockdown of viral genes by dsRNA transfection:....106
3.3.7 Knockdown of genes by recombinant baculoviruses:....108
3.3.7.1 Recombinant AcMNPV mediated silencing in sf9 cells:....108
3.3.7.2 Recombinant AcMNPV mediated silencing in Bombyx mori larvae...110

Chapter 4: RNAi based Screening and functional genomics of AcMNPV.

4.1 Introduction:....113
4.2 Materials and Methods:....115
4.2.1 Cells and Medium....115
4.2.2 Recombinant viruses....115
4.2.3 Amplification of orfs and other genes for in vitro transcription....115
4.2.4 Transfection of dsRNA into sf9 cells....125
4.2.5 Infection of transfected sf9 cells with vRecRed-S....125
4.2.6 Estimation of viral Titer....126
4.2.7 Microscopy and imaging of infected cells....126
4.2.8 Analysis of images....126
4.3 Results and discussion:....127
4.3.1 dsRNAs generated against viral orfs, egfp & rfp are not toxic to cells....127
4.3.2 Analysis of off target /nonspecific activity of dsRNAs designed against egfp and rfp
4.3.3 Fluorescence of RFP and viral titer are correlated:....132
4.3.4 RNAi mediated screening for essential genes of AcMNPV in sf9 cells....134

4.3.4.1 Baculovirus core genes in the screening:....................................140

4.3.4.2 Conserved genes in Lepidopteran baculoviruses other than core genes

4.3.4.3 Other novel and uncharacterized genes.......................................141

4.4 Conclusions:..142

Chapter 5: RNAi based AcMNPV genome wide screening of putative suppressors of RNAi.

5.1 Introduction..144

5.2 Materials and methods...145

5.3 Results and discussion:..145

5.3.1 Generation of sensor lines:...146

5.3.2 Outline of experimental strategy of RNai based screening for RNAi suppressors

5.3.3 Putative candidates of RNAi suppressors in AcMNPV...............................146

5.4 Conclusions..151

Chapter 6: Role of bro in AcMNPV infection and Transcription: A Microarray based study.

6.1 Introduction..155

6.2 Materials and methods:...158

6.2.1 Cells medium and virus:..158

6.2.2 Transfection of dsRNA and sample collection...158

6.2.3 RNA isolation and purification..158

6.2.4 RNA Quality Control...158

6.2.5 Labeling and microarray hybridization..158

6.2.6 Hybridization and scanning:..159

6.2.7 Feature Extraction:...159

6.2.8 Microarray Data Analysis:....159

6.2.9 Semi quantitative Estimation of viral transcripts by RT-PCR:....159

6.2.10 Estimation of BRO protein levels by Western blot:....160

6.3 Results and discussion:....160

6.3.1 Silencing of AcMNPV bro gene by RNAi:....162

6.3.2 Microarray analysis of expression of AcMNPV orfs:....162

6.3.4 Analysis of results....162

6.3.4.1 Down-regulated genes:....165

6.3.4.2 Up-regulated genes:....167

6.4 Summary:....169

Chapter 7: Role of AZdC a DNA methyltransferase inhibitor, on Baculovirus infection in sf9 cells.

7.1 Introduction....171

7.2 Material and methods....177

7.2.1 cells and medium....177

7.2.2 Recombinant viruses and infection of sf9 cells....177

7.2.3 Treatment of sf9 cells with AZdC....177

7.2.4 Analysis of Baculovirus replication....178

7.2.5 Microscopy and analysis of images....178

7.3 Results and discussion....179

7.3.1 Effect of AZdC on Baculovirus infection....179

7.3.2 Effect of AZdC treatment at different phases of virus infection....179

7.3.3 Effect of AZdC on Replication of vRec-EGFP in sf9 cells....183

7.4 Conclusions....185

References:....186

List of commonly used Abbreviations.

Abbreviations	Full form
aa	amino acids
AcMNPV	*Autographa californica* multiple nucleopolyhedro virus
AU	*AU:* Arbitrary unit(s)
AZC	Aza Cytidine An inhibitor of DNA methyl transferases
AZdC	5'Aza-2'-deoxy-Cytidine. An inhibitor of DNA methyl transferases
bp	base pairs
bro/BRO	Baculovirus repeated orf
BV	Budded virus
Dnmt	DNA methyltransferase
dsRNA	double stranded RNA
egfp/EGFP	Enhanced green fluorescent protein
GFP	Green fluorescent protein
hrs	hours
ie-1	Baculovirus immediate early gene
lef	Baculovirus Late expression factor
Log2	Logarithm to the base of 2
min	Minutes
miRNA	micro RNA
MOI	Multiplicity of infection
nt	Nucleotides
ODV	Occlusion derived Virus
per os	By oral route
pfu	Plaque forming units
PI	post infection
piRNA	PIWI associated RNA
polh	Baculovirus *polyhedrin* gene
Pol-II	RNA polymerase II
Pol-III	RNA polymerase III
rasiRNA	Repeat associated RNA
RFP	Red fluorescent protein
RNAi	RNA interference
shRNA	short hairpin RNA
siRNA	small interfering RNA
SV-40	Simian virus 40
UTR	Untranslated gene
wt	Wild type
μ	micro 10^{-6}
μg	micro gram

List of figures:

Figure	Title	page
1.1	A diversity of siRNA Sources	10
1.2	miRNA Biogenesis and Action	12
1.3	Infection Cycle of Baculovirus	26
3.1	Classical miRNA and siRNA pathways & various methods toinduce RNAi	84
3.2	A general map of plasmid Vectors constructed for delivering shRNA and dsRNA	88
3.3	Generating dsRNA by *in vitro* transcription	91
3.4	Maps of recombinant AcMNPV	93
3.5	Infection of insect cells (sf9 & BmN) and Bombyx mori larvae with recombinant AcMNPV	98
3.6	Infectionof Bombyx mori larvae with recombiant ACMNPV	99
3.7	shRNA mediated silencing of p35 and gp64 genes	101
3.8		103
3.9	Knockdown of viral genes by stable expression of dsRNA	105
3.10	Knock down of viral genes by dsRNAs	107
3.11	knock down of genes by recombinant AcMNPV mediated dsRNA delivery	109
3.12	Silencing of genes by recombinant AcMNPV mediated delivery of dsRNA in nonpermissive hosts	111
4.1	Analysis of dsRNA toxicity in sf9 cells	129
4.2	Analysis of non specific effects of dsRNAs on viral titer	131
4.3	Infectionof sf9 cells with vRecRed-S and vRecRed-SAS show correlation between RFP levels and viral titer	133
4.4	Viral orfs suppressing RFP by more than 60%	135
5.1	Experimental outline of RNAi based screening for putative suppressors	148
5.2	Putaive Rnai suppressors obtained by Rnai based Screening.	150
6.1	Results confirming the knock downof bro n dsRNA transfected samples	161
6.2	Differential gene expression profiling of all 156 AcMNPV orfs in BRO knockdown cells.	163
6.3	Differential gene expression profiling of all 156 AcMNPV orfs in BRO knockdown cells. Scattered plot of orf showing 1.3 fold difference	164
7.1	AZdC inhibits expression of recombinant gene	181
7.2	AZdC treatment during early phases of infection inhibits recombinant gene expression expressed under viral promoter	182
7.3	AZdC inhibits replication of Baculovirus vRec-EGFP	184

List of Tables:

Chapter	Table	Page	Title
1	1.1	23	List of Suppressors of RNAi
2	2.1	61	A table showing the number of sf9 cells and quantity of DNA used for transfection in suitable tissue culture grade plastic wares
3	3.1	89	List of primers used to amplify specific fragments of viral orfs in both sense and antisense orientation
3	3.2	92	List of primers used for amplifying specific fragments of *egfp* and *rfp*
3	3.3	95	list of primers used in Recombinant Baculovirus construction
4	4.1	116	List of primers and their sequence used to amplify specific regions of each of the 156 orfs of AcMNPV
4	4.2	136	List of genes/orfs showing more than 60% suppression.
4	4.3	138	List of genes showing 40-60% suppression:
5	5.1	152	A List of orfs which show more than 20% difference in levels of RFP expression between vRecRed-S and vRecRed-SAS infected cells after knocking down specific viral orfs
6	6.1	162	Number of genes up-regulated and down-regulated in *bro* silenced cells by 1.5 fold and 1.3 fold

Chapter-1

Introduction

Chapter 1: Introduction

1.1. RNAi:

1.1.1 History of RNAi

RNAi is one of the major discoveries in the modern science which provided a great tool to suppress gene expression which can be regulated temporally and spatially. Fire and Mello for the first time explained the mechanism by which RNAi can be induced in *Caenorhabditis elegans* and won the prestigious Noble prize for their seminal discovery. But

> **"The most exciting phrase to hear in science, the one that heralds new discoveries, is not "Eureka!" but "That's funny..."**
> **-- Isaac Asimov**

There are many scientific reports which have observed the phenomenon of suppression of genes before 1998 but could not explain the reasons behind this. While these reports mentioned their "funny" or "strange" observations, few of them attributed these strange observations to experimental aberrations and/or contamination. But it is because of such strange observations that today we have a magical tool to silence genes saving great amount of efforts to create, loss of function mutants or knock-outs using traditional techniques.

Here we are listing a few such discoveries which preceded the reports of Andrew Fire and Craig Mello.

People working on plants and their pathogens like viruses and viroids have observed that mild infection of plants with viruses or viroids give protection against secondary infection with virulent viruses or viroids (Niblett *et al.* 1978) . Many others have seen that *Agrobacterium* mediated transformation gave transformants showing different levels of gene expression. Integration at more than one place into the genome resulted in varied level of expression of transgenes mostly in lesser levels but most of these results were not published as people were interested in lines

expressing only at higher levels. This process was reversible and when the plants segregate the expression levels reverted back to very high levels. This was thought to be due to 'position effect' although it does not explain the observations completely.

Before RNAi was discovered Antisense mediated silencing was a popular way of silencing genes. Don Grierson produced Tomato plants expressing antisense fragment against a *Polygalacturanase* (PG) enzyme which were having a longer shelf life than wild type, but an interesting observation in these experiments was that even plants expressing sense strand of *Polygalacturanase* gene were showing suppression of PG (Smith *et al.* 1990). This was a case of "Co-suppression" but went without commenting on the mechanism. "Co-suppression" is a phenomenon in which introducing a transgene results in suppression of the transgene as well as homologous endogenous gene.

Matzke M.A et al (Matzke *et al.* 1989) did an interesting observation while transforming Tobacco plants with *Agrobacterium* containing two different T-DNAs having some common sequences between them but different in their coding sequences. They found silencing of only one T-DNA encoded gene which co-related to the methylation of promoter regions when the second T-DNA was transformed. This effect was reversible, as the segregants revert back to normal expression levels. Soon after this two other reports were published in '*Plant cell*' in 1990 one from the group of Rick jorgenson (Napoli *et al.* 1990) and another of JN Mol (van der Krol *et al.* 1990) both trying to increase pigmentation of petunia flowers were surprised to find flowers with lesser pigmentation due to suppression of pigmentation genes but they did not explain the mechanism behind this.

Another significant observation in this field was of Dr David Baulcombe and Harrison (Baulcombe *et al.* 1986; Harrison *et al.* 1987) who found introducing satellite virus fragments into plants resulted in increased protection of those plants against virulent virus infection.

In fact co-suppression like mechanisms was found in many other species as well. Romano et al observed a phenomenon similar to 'Co-suppression' in *Neurospora crassa* called 'Quelling' (Romano *et al.* 1992) where they found suppression of pigmentation gene and the under expression of that gene co related to lower mRNA levels. Pal bhadra *et al* observed an analogous phenomenon in D*rosophila*. They observed suppression in the levels of *Adh* when 6 copies of *white* promoter-*Adh* were introduced in Drosphila (Pal-Bhadra *et al.* 1997).

Thus there were many more reports of this type and most of them were from plants which observed the process of silencing in endogenous genes and also protection against viruses and transposons but none of them could pin point the cause of silencing or the trigger of silencing process.

Guo and colleagues tried to silence *par1* gene in C. elegans and found both antisense and sense strand RNAs were capable of suppressing the target gene (Guo *et al.* 1995). They could not explain it based on the antisense silencing mechanism which was only popular at that time.

These efforts were followed by the work of Andrew fire and Craig Mello who carefully introduced only sense strand, antisense strand and both sense & antisense strands to induce silencing. They found extremely high rate of silencing, when both strands were injected compared to any of those strands injected alone. They also said formation of double stranded RNA was a prerequisite for efficient silencing. This could explain all earlier observations that dsRNA production by introducing transgenes, satellite virus sequences or contamination of dsRNAs in Guo's observations (Guo *et al.* 1995). This was the beginning of the era of RNA silencing which is now used extensively as a tool to silence genes in reverse genetics, basic studies and also in biomedical applications.

1.1.2 Natural processes regulated by RNAi:

RNAi is a phenomenon by which double stranded RNA along with many other protein factors regulates their targets in a sequence specific manner. RNAi is known to be a regulator of the genome which acts through a repertoire of small RNAs like siRNA, miRNA, piRNA, tasiRNA, rasiRNA, scnRNA etc. These small RNAs produced through different biological processes sharing many common factors and regulate their targets in many different ways. Their biogenesis and mechanism of action are studied in much detail. Although we don't understand the mechanism of biogenesis and action of all these small RNAs completely, already known facts are sufficient to highlight their importance in normal functioning of an organism. RNAi is an indispensable process for regulating the expression of genes and genome in most of the organisms wherever it is functional.

1.1.3 siRNA pathway:

A canonical RNAi pathway is triggered by double stranded RNAs. siRNA pathway in general can be triggered by dsRNAs of various length and with perfect or imperfect complementarity. Small RNAs are usually considered to be between 20-30bps. Initially they were considered extra genomic in origin like viruses or RNAs introduced artificially. They were first observed in virus infected or transgene introduced plants (Mello *et al.* 2004), but then many endogenously occurring small RNA were discovered. They might originate from repetitive sequences of the centromere, transposons etc, (Lippman *et al.* 2004) or from converging transcripts. *trans*-acting small RNAs (tasiRNA) were also discovered in plants which originate utilizing miRNA pathway and other small RNA pathways. They regulate specific set of targets. (Vazquez *et al.* 2004; Allen *et al.* 2005). In some organisms like *C.elegans* and plants these small RNA triggers get amplified dependent on RNA dependent RNA polymerase (RdRP).

Recently there are many reports of occurrences of endogenous small RNAs in Drosophila and mice (Chung *et al.* 2008; Czech *et al.* 2008; Ghildiyal *et al.* 2008; Kawamura *et al.* 2008; Okamura *et al.* 2008; Okamura *et al.* 2008; Tam *et al.* 2008;

Watanabe *et al.* 2008). They mainly belong to piRNA, pi-like RNAs, rasiRNAs, 21U-RNAs, scanRNAs and endo-siRNAs. They originate from repetitive regions of the genome in both soma and germ line tissues. Endo-siRNAs are formed from converging transcripts and also from pseudo genes. Endo-siRNAs are not known to modulate expression of genes significantly and/or they might regulate their own parent transcripts (Sandberg *et al.* 2008).

These small RNAs can regulate their targets in different manners depending on the type of small RNA species, they can regulate their targets post transcriptionally by cleaving the targets or by inhibiting translation like miRNAs, they can induce transcriptional gene silencing of their targets by forming a RNA induced Transcriptional gene silencing complex (RITS) . There are some mechanistic differences between TGS in yeast and plants (Grewal *et al.* 2007; Henderson *et al.* 2007; Moazed 2009). In yeast they result in heterochromatinisation of their target but in plants they result in both DNA methylation and heterochromatinisation, **(Figure 1.1)**

1.1.4 miRNAs:

Since their discovery, great amount of efforts have been made to understand their biogenesis and function. They are found in most of the eukaryotes including plants and animals they are very essential components of an organism and necessary to achieve highly regulated expression of targets both temporally and spatially. microRNAs regulate their target genes by various different mechanisms mainly by target mRNA degradation, sequestration and translational repression. In plants many miRNAs exhibit nearly absolute complementarity mainly at the 5' end upstream of the target genes and they cleave their targets. On the other hand animal miRNAs share lesser complementarity with their targets, which is restricted to the 5' end of miRNA (nucleotides ~2-8) i.e., to the 3' end of the target site. This anchoring region is referred as "Seed" region. In the absence of high complementarity, majority of animal miRNAs inhibit translation of their targets rather than catalyzing their cleavage(Olsen *et al.* 1999). Recent studies suggest that

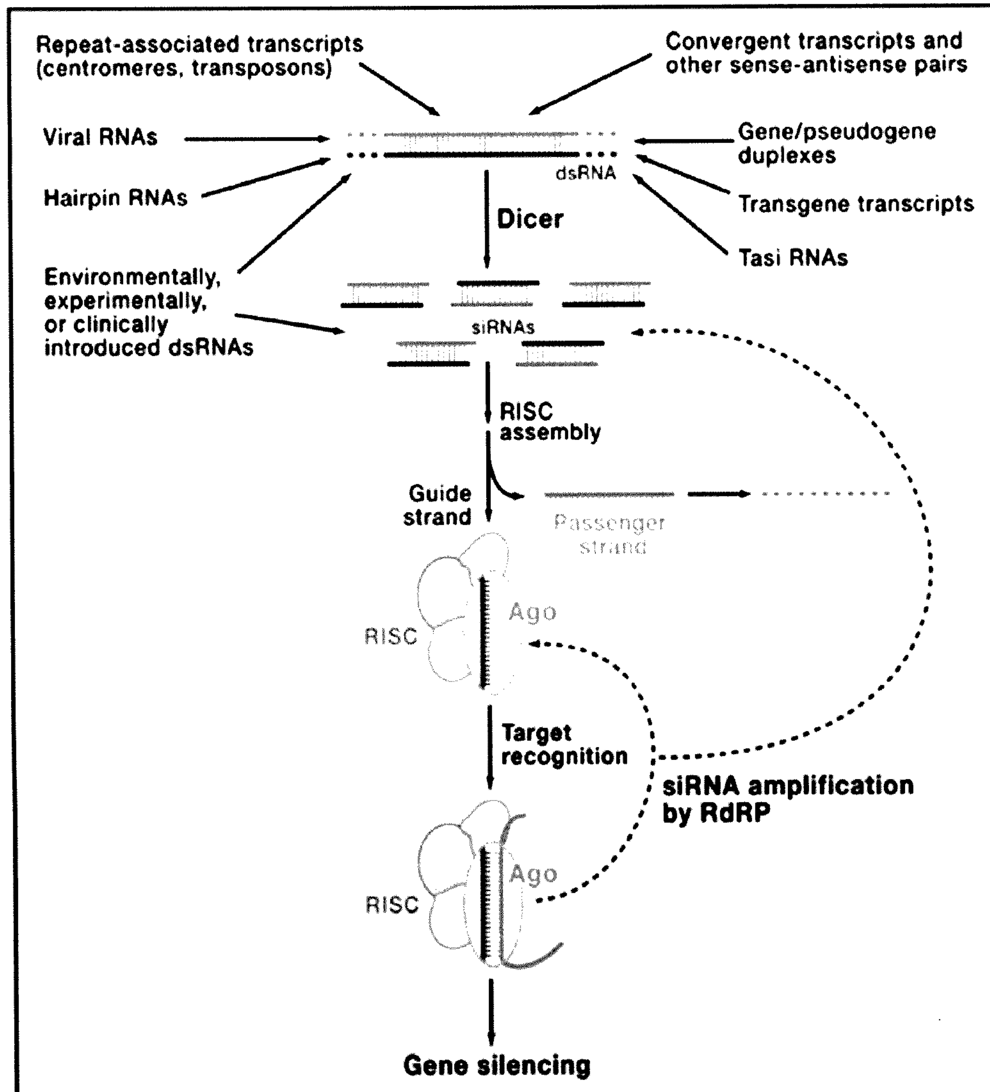


Figure 1.1: A Diversity of siRNA Sources (Adapted from Carthew R.W et al Cell, Volume 136, Issue 4, 642-655)

Several different categories of transcripts can adopt dsRNA structures that can be processed by Dicer into siRNAs. These duplexes can be intra or intermolecular, and although most are perfectly base paired, some (e.g., hairpin RNAs and gene/pseudogene duplexes) are not. An siRNA consists of a guide strand (red), which assembles into functional siRISC, and a passenger strand (blue), which is ejected and degraded. All forms of siRISC contain the siRNA bound to an Ago protein, and many if not most forms of siRISC contain additional factors. Target RNAs are then recognized by base pairing, and silencing ensues through one of several mechanisms. In many species, the siRNA populations that engage a target can be amplified by the action of RNA-dependent RNA polymerase (RdRP) enzymes, strengthening and perpetuating the silencing response.

regulation by miRNAs can direct target mRNA degradation through a pathway that is distinct from small RNA directed endonucleolytic cleavage(Bagga *et al.* 2005; Du *et al.* 2005; Lim *et al.* 2005). In human and *Drosophila* cells, the core components of the miRNA/siRNA silencing pathways along with their respective targets are found to be concentrated in the cytoplasmic foci called "P-bodies". These are the regions where many other proteins are accumulated and actively participate in mRNA degradation reactions like deadenylation, decapping etc. However the localization of miRNAs and their targets to the P-bodies is neither required for endonucleolytic cleavage nor essential for silencing. Recently it is reported that formation of P-bodies is mere consequence but not the causative agent for silencing (Eulalio *et al.* 2007) (**Figure 1.2 RNAi pathway**). These P-bodies also act as temporary storage for some mRNAs, which are later, released into cytosol enabling their translation

It appears that miRNAs may block the translational initiation by relocalizing miRNA programmed RISC and the target mRNA into the P bodies and/or their close proximity (Pillai *et al.* 2005). It is also possible that the mRNAs whose translation is blocked by miRNAs become more susceptible to degradation. It is predicted from bioinformatics and other experimental evidences that nearly 30% of the genes are directly regulated by miRNAs, but miRNAs seem to have influenced the expression of more or less the entire genome (Rehwinkel *et al.* 2006; Kawahara *et al.* 2007). It is reported that nearly 17% of the transcriptome is affected by *Ago-1* depletion. The differences between *in silico* prediction and experimental evidences imply the unexpected complexity and overwhelming diversity in the target selection of miRNA pools. This target selection may not be based only on the 'seed' sequence similarity, but requires other parameters too. RNA editing might play a substantial role in processing and target selection. Many miRNAs and miRNA targets are reported to be edited from adenosine to inosine (A-to-I) for fine tuning of their processing and/or picking up a target (Luciano *et al.* 2004; Kawahara *et al.* 2007) miRNAs which are usually converted into their mature form in the cytoplasm are not restricted to cytoplasm in their action, some miRNA are known to regulate their targets by inducing DNA methylation or heterochromatinization of their targets located in the nucleus (Guil *et al.* 2009).

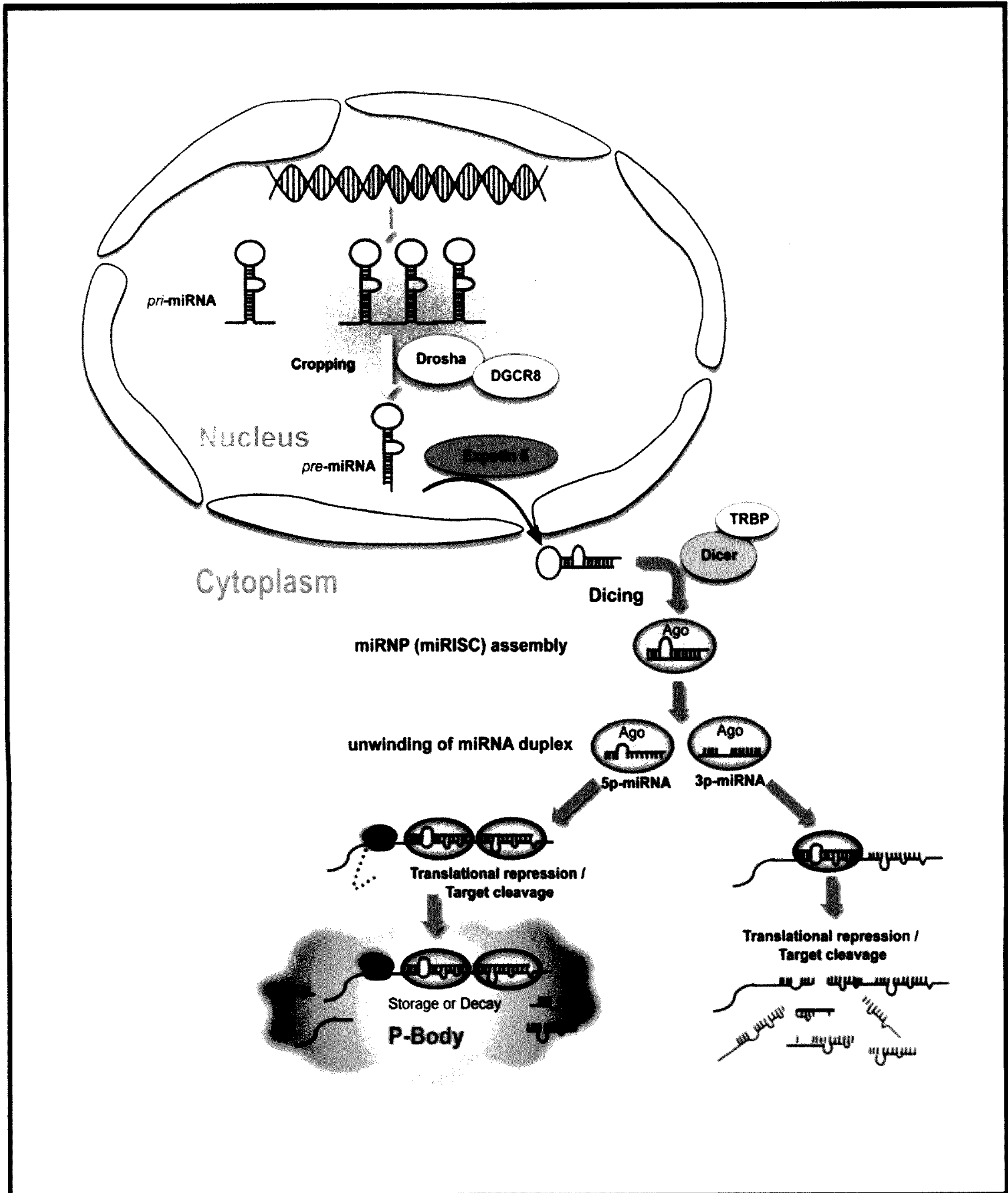


Figure 1.2: miRNA Biogenesis and Action. (adapted from Singh S.K *et al* 2008. FEBS J.275(20):4929-44)

Transcription of miRNA encoding genes produces pri-miRNAs in the nucleus, which are processed by Drosha and DGCR8 to produce pre-miRNA. pre-miRNAs are exported out of the nucleus into cytoplasm by Exportin-5. pre-miRNAs are further processed into miRNA by Dicer with the assistance of TRBP (in mammals). These mature miRNAs get incorporated into miRISC, which retain only the guide strand of miRNA. The miRISC containing guide strand of miRNA can regulate its targets, by suppression of translation and/or degradation in P-bodies or cleave their targets followed by degradation. miRNA targeted mRNAs in the P-bodies can be temporarily stored and released back into the cytosolic pool for their translation.

1.1.5 RNAi as an antiviral response:

RNAi acts as a genome's defense against invasive genetic elements like viruses. It is very well studied and established that RNAi is an antiviral response in plants. There are several lines of evidences to support this fact, **1**. Virus infection of plants results in very strong gene silencing (Lindbo *et al.* 1993). There are also reports about resistance of plants to viral infection which were previously infected mildly by viroids **2**. Artificially induced silencing can inhibit viruses. The efficiency of the RNAi induced artificially was correlated to the level of infection, indicating RNAi does inhibit viruses but the outcome depends on the strength of the RNAi response. **3**. Viruses are known to encode inhibitors of RNAi and removal of inhibitors results in decreased efficiency of infection. **4**. Components of RNAi machinery were seen up regulated in virus infected plants. **5**. Mutants of RNAi machinery are more susceptible to virus infection than their wild type counterparts **6**.RNAi induced artificially or due to virus infection spreads to neighboring tissues which is an inherent property of RNAi in plants and *C.elegans* called Transitive RNAi. Similar observations were done in invertebrates like insects, drosophila and mosquitoes.
There are no known viruses infecting C elegans naturally but infection of FHV (Flock House Virus) and mammalian Vesicular Stomatitis Viruses resulted in production of small RNAs. Mutating RNAi machinery resulted in increased susceptibility to their infection indicating that RNAi is a natural antiviral response against viruses in c elegans and removal of B2 protein which is a suppressor of RNAi resulted in efficient inhibition of viral replication in *C. elegans* (Lu *et al.* 2005; Schott *et al.* 2005; Wilkins *et al.* 2005).

1.1.5.1 RNAi as antiviral response in mammals:

There are different schools of thoughts about the relevance of RNAi as an antiviral response in mammals. One argues against the role of RNAi in mammals merely based on lack of evidences. Although absence of evidence is not the proof of absence (Cullen 2006; Dykxhoorn 2007; Gottwein *et al.* 2008) many reports were

not able to detect small RNAs produced during virus infection. Study of Pfeffer et al (Pfeffer *et al.* 2005) stands out in this in which they tried to detect small RNAs during infection of various DNA viruses like human cytomegalovirus, Kaposi Sarcoma Associated Herpes Virus, Epstein-Barr virus and mouse herpes virus 68, as well as the retrovirus human immunodeficiency virus type 1 (HIV- 1) and the RNA viruses yellow fever virus and hepatitis C virus, but they could find only miRNAs encoded by these viruses and no traces of small RNAs against viral genomic sequences. To counter these arguments K.T. Jeang *et al* have found small RNAs against the virus genome of HIV-1 by deep sequencing (ter Brake *et al.* 2008). Earlier it was shown that miRNA pathway is required to maintain the latency of HIV (Huang *et al.* 2007) but knock down of *Dicer*, *DGCR8*, and *drosha* increased HIV production in infected cells (ter Brake *et al.* 2008). Convincing evidences to show small RNAs are produced in mammalian cells because of viral infection, and they get incorporated into RISC to regulate viruses are still awaited.

Other factor that might prompt us to think RNAi could be an antiviral response in mammals is the 'suppressors of RNAi' encoded by mammalian viruses. Nearly forty RNAi suppressors are known till date from plant viruses. Even mammalian viruses are known to encode RNAi suppressors like Tat protein of HIV-1 (Bennasser *et al.* 2005) and vp35 of Ebola Virus (Haasnoot *et al.* 2007). Adeno viruses encode VA1 and VA-II RNAs which saturate Dicer and thus inhibits RNAi in its hosts (Andersson *et al.* 2005). Since dsRNAs are triggers of both RNAi & interferon response pathways and Most of the RNAi suppressors reported act as antagonists of interferon response in mammals. It is difficult to emphasize the importance of RNAi over interferon response in these animals. but a recent report by shnettler et al revealed that RNAi indeed is an antiviral response in mammalian cells and it restricts HIV replication in host cells, they showed it by substituting Tat's RNAi suppressor activity but not interferon inhibition by NS3 protein of Rice hoja blnaca virus (Niblett *et al.* 1978; Schnettler *et al.* 2009). Interferon response itself can modulate RNAi pathway to inhibit viruses. miR122 which is essential for hepatitis C virus replication was down regulated and as many as eight other miRNA which can target viral genomic sequences were up-regulated by IFN-β (Pedersen *et al.* 2007).

It seems logical to see viruses encode suppressors of RNAi because RNAi is an antivirus response. Another recent report by Wu Z et al showed that Herpes simplex virus can inhibit RNAi and suppressing Argonaute2 increased the replication of herpes viruses in cells (Wu *et al.* 2009). So evidences are accumulating to support RNAi as an antiviral response even in mammalian cells. RNAi along with various other kinds of immune responses act in concert to defend against viruses.

1.1.5.2 miRNA and Virus:

However it is accepted that viruses encode miRNAs which regulate both host and viral transcripts. Most of these miRNAs were discovered from herpes viruses. These large dsDNA viruses infect host and have two phases of infection latent and lytic. Viruses are known to express miRNAs in latent phase of infection (Cui *et al.* 2006) miRNAs of herpes viruses are not conserved across herpes viruses or across other viruses indicating the rapid rate of evolution of these viruses. Human Cytomegalo viruses (HCMV) and murine Cytomegalo viruses (MCMV) encode large number of miRNAs *i.e.* nine and seventeen respectively, expressed in lytic cylcle of infection (Pfeffer *et al.* 2005; Buck *et al.* 2007; Dolken *et al.* 2007). Epstein Barr Virus (EBV) encode at least 14 miRNAs in their latent phase of infection and they are homologous to rhesus lymphocryptovirus encoded miRNAs these are the only known conserved viral miRNAs (Pfeffer *et al.* 2004; Cai *et al.* 2005) Apart from Herpes viruses adenoviruses and polyoma viruses are also known to encode miRNAs (Sullivan *et al.* 2005; Sano *et al.* 2006).
Many host encoded miRNAs are known to regulate Viruses. There are at least two cases to supports this where host miRNAs target virus transcripts or virus genome. Primate foamy virus type-1 is restricted by human mir-32 (Lecellier *et al.* 2005) and HIV-1 mRNAs are targeted by many miRNAs like *miR*-28, *miR*-125b, *miR*-150, *miR*-223 and *miR*-382.
Hepatitis-C virus is an interesting example of viruses exploiting host miRNAs for their benefit; they exploit *miR*-122 for their replication (Kumar 2008). In other instance host miRNAs were exploited to manipulate host transcripts itself to

suppress other type of immune responses HIV-1 can replicate more efficiently when dicer and drosha were knocked down, this is due to down regulation of specific miRNAs *miR*-17-5p and *miR*-20a which target histone acetylase PCAF (Muller *et al.* 2007; Triboulet *et al.* 2007; Grassmann *et al.* 2008).

1.1.5.3 RNAi as antiviral response in Insects:

Insects possess only innate immune system unlike higher vertebrates which contain innate and adaptive immune system. With the discovery of *Toll* like receptors which were first found in drosophila and later found in all other animals including humans. Drosophila became a popular model of study of innate immune system in animals. In the last two decades there has been tremendous progress in study of antibacterial, antifungal innate immune responses in insects especially drosophila and surprisingly most of them are very well conserved immune response pathways found in mammals also. So it was thought that study of innate immune responses against viral infections in drosophila or other insects could reveal their importance. Another important factor to study insect immune systems is to understand more about the responses of insects to many pathogens like bacteria, virus etc is due to the fact that, many insects act as vectors to spread these diseases. As a result of research in *Drosophila*, there is evidence for the involvement of many innate immune response pathways in antiviral defense: degradation of viral RNA by RNAi, the *Toll* pathway, *Imd* pathway and cytokine-mediated induction of genes via the *JAK–STAT* (signal transducer and activator of transcription) signaling pathway (Cherry *et al.* 2006).

Initial implications of involvement of RNA mediated silencing in countering virus infection were reported by using Alphaviurses to express a fragment of viral gene in mosquitoes to see increased resistance against virus infection like plants (Gaines *et al.* 1996; Olson *et al.* 1996). Later it was shown that introducing dsRNAs in cultured drosophila cells inhibit accumulation of Folck house viruses (FHV) (Li *et al.* 2002) and in mosquitoes dsRNAs were expressed using inverted repeat expressing viral vectors to see increased resistance to dengue viruses, to which these mosquitoes

act as vectors (Adelman *et al.* 2001; Adelman *et al.* 2002) This strategy was used not only against RNA viruses which could act as substrates to RNAi but dsDNA viruses like Bracovirus as well in Lepidopteran insect *Trichoplusia nimphi* cells (Beck *et al.* 2003). First evidences to show that RNAi is a natural response of insects against viruses were given by Li *et al.* that showed accumulation of small RNAs in virus infected cells. They also showed that mutations of RNAi machinery resulted in increased viral infection and decreased accumulation of small RNAs (Li *et al.* 2002). Ulhirova et al (Uhlirova *et al.* 2003) observed accumulation of small RNAs in *Bombyx mori* after infecting with Sindbis virus. Anitiviral response in *drosophila* is mediated by siRNA pathway rather than miRNA pathway. This was observed by genetic studies in which mutants of siRNA pathway like *r2d2, dcr-2*, and *Argonaute-2* (*Ago-2*) were more susceptible to infection of Flock House Virus (FHV), Cricket Paralysis Virus (CrPV) Sindbis virus (SINV) and Drosophila C virus (DCV) than their counterparts in miRNA pathway like *dcr-1* and *Ago-1* (Galiana-Arnoux *et al.* 2006; van Rij *et al.* 2006; Wang *et al.* 2006). Surprisingly Drosphila X Virus (DXV) and West Nile Viruses (WNV) were restricted by AGO-2 but not DCR-2 (Li *et al.* 2002; Zambon *et al.* 2006). These viruses (DXV and WNV) were inhibited by piRNA pathway components like *piwi*, *aubergine* and *spindle-E*. *piwi* and *aubergine* belong to *piwi* sub family of Argonaute proteins and *Ago-2* belongs to *Ago* sub family of Argonaute proteins in drosophila this shows that different viruses are targeted by different RNAi pathways. This might depend on the mechanism of virus replication, transcription, localization of the virus in cellular sub-compartments or put together, it might depend on the life cycle of these viruses in host cells and the type of the tissue they are infecting. Dicer-2 an important component of RNAi can induce different types of antiviral immune responses either through small RNAs resulting in degradation of viral RNAs or by inducing *vago* after sensing dsRNAs in the host cell. Induction of *vago* results in a response similar to cytokine response in higher animals (Deddouche *et al.* 2008).

1.1.6 RNAi in Insects:

1.1.6.1 Some special features

Among different methods of delivering dsRNAs *i.e* triggers of RNAi into organisms, soaking of the tissue or organism in a solution of dsRNAs and injection of dsRNAs into the body of organisms are very convenient and efficient methods. Many lower organisms like *C.elegans*, can take up dsRNAs just by soaking them in a solution of dsRNAs (Timmons *et al.* 1998). In plants and *C.elegans* it is well established that RNAi can spread systemically. dsRNAs injected to *C.elegans* or introduced by soaking or feeding can induce a broad range silencing throughout the body. In *C.elegans Sid-1*, *fed* (feeding defective) and *rsd* (RNA spreading defective) genes are known to be responsible for uptake and systemic spreading of RNAi (Tijsterman *et al.* 2004). The genes which are responsible for uptake of dsRNAs like *Sid-1* are not required for systemic spread within the body as *Sid-1* can only import dsRNAs but not export out of the cells. Other groups of genes *fed* and *rsd* might help in this process (Winston *et al.* 2002; Saleh *et al.* 2006). These genes have a role in endocytosis and vesicular trafficking in C. *elegans*. Insects also show systemic spreading of silencing but there is lot of diversity among different species in their efficiency. Insects like *Tribolium castaneum,* aphids *Aphis gossypii*, *Rhopalosiphum padi*, *Sitobion avenae* , locust *Schistocerca americana* (Dong *et al.* 2005). Honey bee *Apis mellifera* are known to encode genes homologous to *Sid1* and show very efficient systemic spreading of RNAi. *Sid-1* genes are very much essential in uptake of dsRNAs by cells in organisms wherever they are found but many other organisms are capable of taking up dsRNAs but they are deficient in *sid1, viz,* Paramecium, planarians *Brugia malayi*, *Dugesia japonica* and *Schmidtea mediterranea* (Newmark *et al.* 2003; Orii *et al.* 2003), insects like *Diabrotica virgifera LeConte*, ticks (Baum *et al.* 2007; Patel *et al.* 2007), indicating that *Sid-1* independent mechanisms are also present. Some organisms are not capable of taking up dsRNAs by soaking but they are able to do it by feeding on dsRNAs. Both cases involve uptake of dsRNAs from the environment but the cell types that are exposed to dsRNAs are different. This could be the reason behind these different

observations. Best examples would be *C.elegans* and *C.briggaseae* latter can take up dsRNAs only by feeding or injecting but not by soaking. Many higher animals like insects also exhibit lot of variation in their ability to take up dsRNAs from their environment. Feeding of dsRNAs was success full in insects like *Rhodnius prolixus (Araujo et al. 2006)*, *Ixodes scapulari (Soares et al. 2005)*, *Epiphyas postvittana* (Turner *et al.* 2006). *Spodoptera littura* were unable to trigger RNAi by feeding but injecting these dsRNAs into their hoemocoel triggered efficient RNAi (Rajagopal *et al.* 2002). These differences in uptake of dsRNAs by soaking, feeding and injecting indicate differential ability of various cells types to take up dsRNAs and diversity in their gut environments.

Even Drosophila can also take up dsRNAs by soaking its embryos in a solution of dsRNA and show effects of RNAi but other developmental stages are not capable of doing this (Eaton *et al.* 2002), feeding of Drosophila with dsRNAs to induce RNAi has not been demonstrated yet. Drosophila does not encode any homologues of *Sid-1* or *Sid-1* like gene. S2 and kc167 cells of drosophila which do not express *Sid-1* can take up dsRNAs very efficiently, but not siRNAs. Expression of *sid-1* in drosophila cells can increase their ability to uptake dsRNAs (Feinberg *et al.* 2003) many experiments involving whole genome RNAi screening have used S2 cells and kc167 cells which can uptake dsRNAs without the help of transfection reagents (Ramadan *et al.* 2007). Saleh et al screened whole genome of drosophila for genes affecting the uptake of dsRNAs and could find genes involved in receptor mediated endocytosis and subunits of vascular ATPase are required (Saleh *et al.* 2006).

1.1.6.2 Parental RNAi:

A surprising observation while using RNAi to knock down genes in some insects was to see the targeted genes suppressed even in next generations like *C.elegans* (Fire *et al.* 1998). Bucher et al observed *Tribolium* insects treated with dsRNAs in one generation gave rise to offsprings with suppressed expression of those targeted genes. This suppression was less efficient and faded with time (Bucher *et al.* 2002). Similar effects were seen in *Spodoptera littura* by Rajagopal *et al,* (Rajagopal *et al.* 2002) and in *Hyapora cecropia* by Bettencourt *et al.* (Bettencourt *et al.* 2002).

Although amplification of small RNAs which is known to happen in *C.elegans*, fungi and plants due to the activity of RdRP is not reported in insects, such long lasting effects of RNAi can be seen.

1.1.7 Suppressors of RNAi:

In order to overcome the hosts' immune system, Viruses have evolved various mechanisms. Since RNAi is an antiviral response of the hosts many viruses have developed mechanisms to overcome RNAi targeted against them. Viruses can escape RNAi by expressing specialized proteins to inhibit RNAi, called Suppressors of RNAi, or by adapting methods to replicate and express their genes by restricting themselves in a protective environment where RNAi has limited access.

Suppressors of RNAi: Many viruses are known to encode viral RNAi suppressors (VRS). So far around 41 suppressors have been identified. VSRs Inhibit RNAi at various critical steps of RNAi by employing various strategies. A small list of RNAi suppressors is given in (**Table1.1**)

Most of the plant viruses have VSRs. Some viruses like Geminiviruses encode more than one type of proteins which inhibit RNAi at various steps (Voinnet *et al.* 1999; Lu *et al.* 2004; Vanitharani *et al.* 2004). Suppressors are present in insect viruses also like Cricket paralysis Virus and Flock house virus (Li *et al.* 2002). More than 10 VSRs have been identified in vertebrate viruses so far including HIV, and Ebola virus.

Interestingly some viruses express structured RNAs like Adenovirus VA1 RNA which can inhibit small RNA production. *Red clover necrotic mosaic virus* (RCNMV) is also known to fallow a similar mechanism to inhibit host dicer (Takeda *et al.* 2005).

Most of the known VSRs are RNA binding proteins and they inhibit RNAi by binding to small RNAs produced by RNAi machinery. Such proteins can act as RNAi suppressors in heterologous systems also where they can bind to small RNAs. the small dsRNA binding domains in VSRs are unique and not generally found in any of the host dsRNA binding domains only known exception is Vaccinia virus E3L

protein which has a conserved DSRM domain found in many host proteins like PKR, Dicer etc (Li *et al.* 2004). Usually VSRs identified in a virus are found in all viruses of their family, but they often show significant variation in their sequences. Obbard DJ *et al* have reported that RNAi genes involved in host defense have evolved faster than other RNAi pathway genes so it is expected that even the suppressors of viruses have evolved to counter their host defense (Obbard *et al.* 2006). That is why VSRs found in the same family of viruses show some variation in their sequence which might have accumulated in the process of better adaptation to their hosts. Reviewed in (Li *et al.* 2006).

As we have mentioned VSRs can inhibit RNAi in different ways *viz*, **1**. Suppression of production of siRNAs, **2**.Sequestration of siRNAs **3**.Inhibition of systemic spreading, and **4**.Manipulaltion of specific genes or miRNAs.

Adenoviurs VA1 RNA, FHV B2 can inhibit production of small RNAs. Adenovirus VA1 RNA acts as a substrate to host dicer and saturate all available DICER protein, so that viral RNAs can escape DICER cleavage. FHV-B2 can bind to the double stranded replication intermediates of virus and protect them from Dicer cleavage. Mutating FHV B2 results in increased accumulation of virus specific small RNAs (Chao *et al.* 2005; Lu *et al.* 2005). *Red clover necrotic mosaic virus* (RCNMV) employs another fascinating mechanism to inhibit Dicer. The dsRNA replication intermediates of RCNMV and other viral proteins which form a 'Viral RNA replication complex' together inhibit DCLs of their host plants and in turn inhibit host siRNA and miRNA pathways. Although mechanism and relevance of inhibiting RNAi for virus replication is not clearly understood, it is surprising to see that RCNMV cannot replicate in DCL mutants (Takeda *et al.* 2005). Hc-Pro is another VSR which can suppress production of 21nt small RNAs (Mallory *et al.* 2002; Dunoyer *et al.* 2004).

P19 of tomato bushy stunt virus binds to small RNAs of ~21nts and inhibit their incorporation into RISC in heterologous systems like drosophila, but in their natural hosts they seem to bind to siRNAs and prevent them from spreading to other cells. Many other VSRs are known to bind to smallRNAs directly or indirectly and inhibit their incorporation into RISC *viz.* P21, b2, 2b, NS1 etc (Bucher *et al.* 2004; Li *et al.* 2004; Chao *et al.* 2005; Lu *et al.* 2005; Ye *et al.* 2005).

572.8633
H1277
Fu
TH-16513

572.8633 TH
H1277 Fu

VSRs like P25 and 2b bind to small RNAs and prevent their movement from one cell to other. This prevents systemic spreading of silencing in plants (Voinnet *et al.* 2000; Guo *et al.* 2002). P25 cannot inhibit RNAi in the infected cells but they can prevent cell to cell movement of small RNAs.

AC2 protein of Geminiviruses (*Tomato Leaf Curl Virus* TLCV) is a transcription factor. It can suppress RNAi by inducing expression of several host genes including negative regulator of RNAi (van *et al.* 2002; Dong *et al.* 2003; Trinks *et al.* 2005; Bisaro 2006). AC2 of Tomato golden mosaic virus directly interacts with Adenosine kinase of the host and inhibits DNA methylation in their host. Protection of viruses by inhibiting DNA methylation indicates that viral DNA might be restricted by inducing methylation by the host small-RNA mediated pathway (Li *et al.* 2005; Wang *et al.* 2005). Overexpression or heterologous of VSRs line p19, Hc-Pro, P21 etc which bind to small RNAs, show developmental defects. They are known to bind and inhibit host miRNAs resulting in these problems. Even in virus infected plants inhibition of miRNA results in miss-regulation of RNAi components (Chapman *et al.* 2004; Chen *et al.* 2004; Dunoyer *et al.* 2004).

Table 1.1: A list of Viral Suppressors of RNAi

Virus	VSR	Domain involved & Mode of action
Beet yellows virus	P21	dsRNA binding
Sweet potato chlorotic stunt virus	P22, RNase3	RNase III
Cucumber mosaic virus	2b	dsRNA binding
Potato virus X	P25	
Tobacco etch virus	Hc-Pro	
Tomato bushy stunt virus	P19	dsRNA binding
Rice hoja blanca virus	NS3	
Mungbean yellow mosaic virus-Vigna (MYMV	AC2	DNA binding
Tomato leaf curl virus	C2	DNA binding
Flock house virus, nodamura virus	B2	dsRNA binding
Influenza virus A	NS1	dsRNA binding
HIV-1	Tat	
Primate foamy virus	Tas	
Adenovirus	VA1	Dicer binding
Vaccinia virus	E3L	dsRNA binding
Red clover necrotic mosaic virus *(RCNMV)*	Viral RNA P88,P27	replication complex

1.2 Baculovirus:

1.2.1 History of Baculoviruses:

We all have started our lives by learning to differentiate between the Good & bad and Useful & Harmful things, and generally we have learnt that, whatever is beneficial to humans is 'Good'. If this anthropocentric point of view is genuine then Baculoviruses are the most beneficial viruses known to the mankind. While all other viruses are studied to understand and overcome their harmful effects baculoviruses are studied for their usefulness and applications. They infect a large number of insects most of which are pests to agricultural and horticultural crops. They also infect insects in the wild and widely used as a biological control to limit their population. Their applications do not end there, but the modern genetic and molecular biological tools have exploited them as one of the most preferred expression vectors and there is a growing interest in using them as gene delivery vectors. So far so good baculoviruses are now known to infect silkworms. Silk has an important status in our Indian cultural practices and also in other Asian countries like China, Japan etc. India being one of the major producers of silk has a large population dependent on sericulture. Baculovirus infection incurs severe economic damage to the production of silk. Lack of methods to cure the Baculovirus infection and lack of virus resistant germplasm have left the sericulture industry vulnerable to loss.

On one hand Baculoviruses are studied to understand and increase their virulence to kill insect pests and on the other hand they are studied to identify their Achilles heel so that beneficial insects like silkworms can be engineered to increase resistance to viruses.

Earliest written evidences of Baculovirus infection to silkworms are found Chinese literature but it is only in the early 20th century that we found evidences of occurrence of virus polyhedral bodies and their importance in killing insects' pests and using them as a biological control were appreciated. Bergold G in 1953 discovered the rod shaped virions in the crystalline polyhedral and started the systematic study of Baculoviruses. There were many instances of successful using

of Baculovirus as biological control against insects but with few failures. Such instances increased the importance of understanding of Baculovirus pathology which might lead us in both ways i.e., to increase the virulence and to find out the vulnerable targets to counteract them.

1.2.2 Baculovirus Infection Cycle:

1.2.2.1 Overview:

Most of the insects show periodicity in their life cycle. That may be correlated to availability of food or other resources, which could be seasonal plants or crops. This is an adaptation to escape from adverse environmental conditions for their survival. So Baculoviruses which infect such insects also have developed mechanisms to adapt to the life cycle of their hosts. Baculoviruses have evolved to infect insects through their guts and complete their infection cycle in these insects and produce viral particles which can remain potent in the environment. Some viruses can induce latent infection in their hosts so that they remain in the live host during dormancy and when the host comes out of dormancy in favorable conditions virus can amplify and continue the cycle of infection (Hughes *et al.* 1993; Hughes *et al.* 1997; Burden *et al.* 2002; Burden *et al.* 2006). Although this is not a prevalent feature of baculoviruses very few cases of latent infection are reported. But even those viruses are capable of virulent infection and show all the features of virulent baculoviruses *i.e.* producing viral particles which are stable in the environment and infecting hosts through their gut.

Baculoviruses form "Occlusion bodies" (OB), which are resistant to freezing and dessication but soluble in alkalies. The OBs get dissolved in the alkaline environment of insect gut and releases virus particles called Occlusion Derived Viruses (ODV). These ODVs enter into gut cells and infect them to produce another form of Virus called Budded Viruses (BV). BVs are capable of infecting and spreading into other cells of a host. At very late stages of infection viruses produce ODVs again and get occluded to form Occlusion bodies. OBs are released into the environment after the death of the host. **(Figure1.3)**

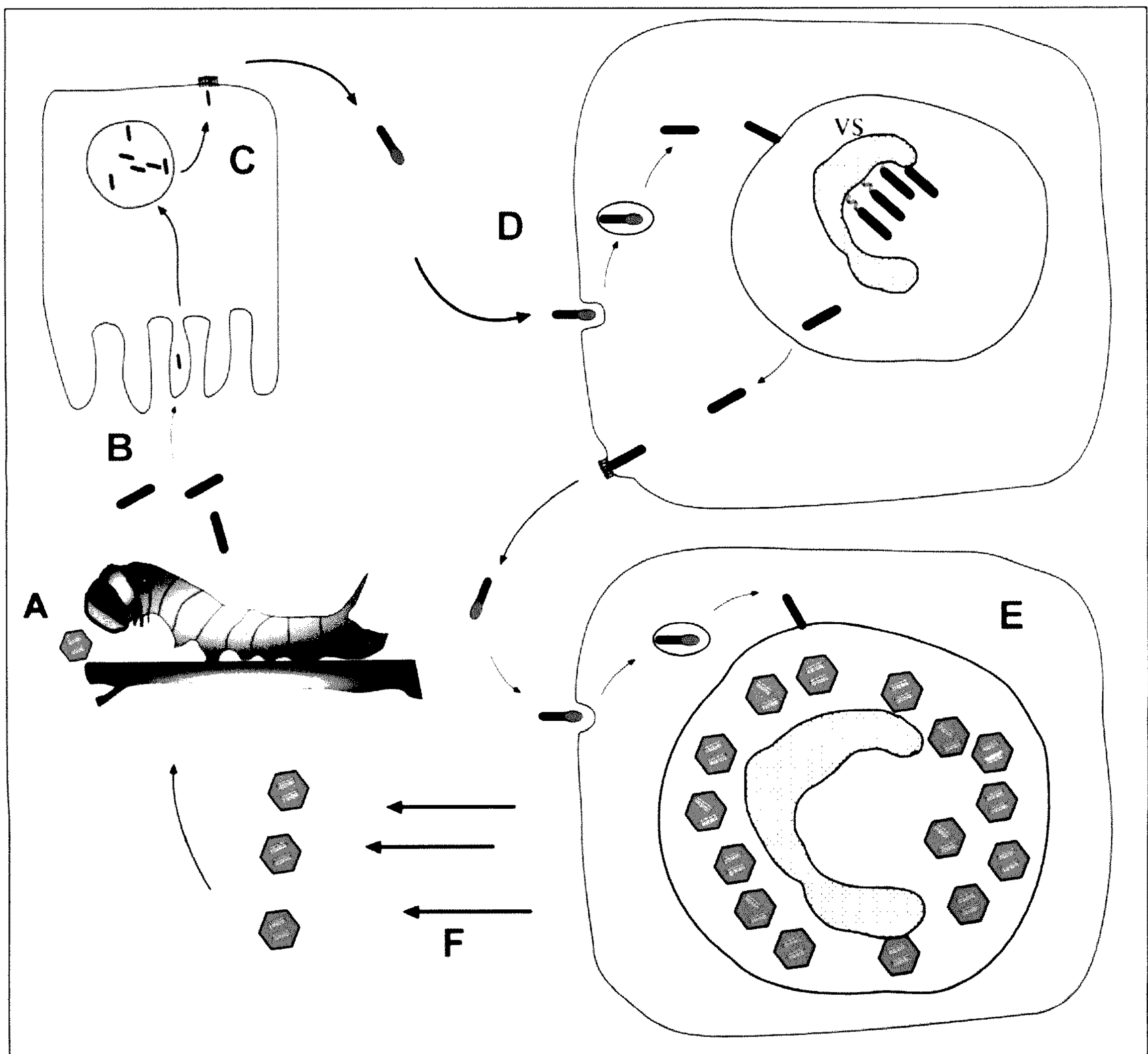


Figure 1.3:Infection cycle of Baculovirus: (adapted from Rohrmann GF. Baculovirus Molecular Biology. Bethesda (MD): National Library of Medicine(US), National Center for Biotechnology Information)

Baculoviruses enter into the host body when they feed on virus contaminated leaves (A). The ODVs enter into the gut epithelial cells of the insect larvae (B) replicate and produce progeny viruses in the form of BVs (C). BVs bud out of the gut epithelial cells from the basal side and infect other cells in the body (D). Within an insect body BVs reproduce and spread infection. In late stages of infection viruses produce proteins required for Occlusion and form ODVs (E). ODVs are released in the environment after the death of the host (F).
ODVs mediate spreading of infection from one individual to other and BVs mediate infection from cell to cell within a host body.

1.2.2.2 Infection through gut: (Entry of Baculovirus into midgut cells through gut)

Baculovirus OBs are ubiquitously present as the number of susceptible hosts and the rate of production of infectious particles is enormously high. Insect larvae generally feed on leaves. Feeding on OB contaminated leaves allows OBs to enter into the gut of the insect larva. The alkaline pH of the gut dissolves the protein matrix of OBs to release ODV contained in them. The calyx proteins or Polyhedral Envelope protein (PEP) of OBs and the carbohydrates in the polyhedral envelope are digested by the proteases and other enzymes of the insect gut. OB protein matrix not only contains structural proteins but many other proteins like metalloproteinase (Enhancin) and other proteinases, but optimal catalytic conditions required by these proteinases are in the acidic range of pH (pH-5). So their role in infecting gut cells may be limited. e*nhancins* are not found in all baculoviruses. In such viruses proteinases of insect or gut bacterium might be exploited to do the same job.

The next barrier that viruses face is peritrophic membrane of insect gut. OBs isolated from infected insects are known to contain proteinases of insect and bacterial origin which can perform efficiently in the alkaline pH of insect gut. These proteases seem to get co-occluded during the ODVs occlusion process. After dissolution of OBs these proteases are also released into insect gut and they help in digesting the peritrophic membrane ((Washburn *et al.* 1995). Mucin is another major component of peritrophic membranewhich gets degraded by viral metalloproteinases (Wang *et al.* 1997). Together these proteinases digest peritrophic membrane of gut and expose gut epithelial cells to ODVs. Another component of ODVs, BV/ODV-E26 has hyaluronan lyase activity which can digest hyaluronan polysaccharides present in the extracellular matrix.

ODVs are known to contain specific set of proteins mediating the entry of virus into midgut cells. Chitin is a part of peritrophic membrane and tracheal cells. Several viral proteins have chitin binding domains (*viz.* Ac145, Ac150, VP91) (Dall *et al.* 2001) and they might help in binding to peritrophic membrane and assist in penetrating through it by localized digestion of that barrier. They might also help in

binding to tracheal cells containing chitinous membrane and facilitate viral entry into those cells.

Other set of genes which are very essential are called *per os* infectivity factors (pif) there are four such genes in baculovirus and all four *pif* proteins are found in ODVs *p74-pif* (Ac138), Ac 22 (*pif-2*), Ac115 (*pif-3*), and Ac119 (*pif-1*) (Faulkner *et al.* 1997; Kikhno *et al.* 2002; Fang *et al.* 2006; Li *et al.* 2007). PIF, PIF-1 and PIF-2 help ODVs to bind specifically to midgut cells (Ohkawa *et al.* 2005). ODVs are known to bind to proteinase sensitive receptors on the surface of midgut epithelial cells. Other ODV proteins having enzymatic activity might assist in entering into such cells. After binding to cells surface receptors ODVs enter into cells by membrane fusion (Granados *et al.* 1981; Horton *et al.* 1993).

1.2.2.3 Entry of Nucleocapsids into nucleus of Midgut epithelial cells and Production of Nucleocapsids.

After entering into gut epithelial cells by membrane fusions nucleocpsids are released into the cells and they enter into nucleus. Exact mechanism of entry is not known but there are indications that whole nucleocapsids enter into nucleus through nuclear pores (Granados *et al.* 1981). The viral DNA which was compactly packed into capsids and bound by several DNA binding proteins gets rid of them and transcribes its genes. As the viral genes start expressing and viral proteins accumulate viral DNA replicates and assembles into new viral capsids and come out of the nucleus along with a bit of nuclear membrane which looks like a vesicle (Gross *et al.* 1993). These nucleocapsids move towards the basal membrane of epithelial cells, and during this transit they lose the nuclear membrane derived vesicles leaving naked nucleocapsids. These naked nucleocapsids continue to move towards the basal membrane, where viral glycoprotein like GP64 or F-protein (Pearson *et al.* 2000) are concentrated and bud out of the cells along with a part of the plasma membrane (Oomens *et al.* 1999). These are called budded viruses (BV). These viruses initiate another cycle of infection by infecting other cells in the body (Monsma *et al.* 1996). Sometimes viruses bypass replication and directly exit

through the basal membrane of epithelial cells into hemolymph where it can infect other cells like tracheal cells, midgut muscle, hemocytes and so on (Granados *et al.* 1981; Engelhard *et al.* 1994; Flipsen *et al.* 1995). It is possible that midgut epithelial cells which are already infected by viruses express proteins like GP64 required for budding out of the plasma membrane and any secondary infection of the same cells by baculoviruses can help them to bud out of the basal membrane without entering into the nucleus again. This could significantly increase the rate of infection of cells.

1.2.2.4 Secondary infection of cells:

The viruses which bud out of the gut epithelial cells are enveloped and contain GP64/F proteins in a crown like structure having spike like outgrowths called peplomeres, these glycoproteins bind to specific proteins or phospholipids on the surface of host cells ((Horton *et al.* 1993; Tani *et al.* 2001)) and internalized by clathrin mediated endocytosis (Long *et al.* 2006). Viral capsids contained in endocytic vesicles fuse with the vesicular membrane, after acidification of the vesicles. This releases capsids into the cytoplasm. Capsids now enter into the nucleus through nuclear pores and start expressing its genes. Products of early expressing genes along with viral DNA and RNA form specialized foci in the nucleus of the infected cells. These foci enlarge as the protein products accumulate to form a "Vorogenic Stroma". Virogenic stroma is visualized as an electron dense region with a few clear spots distributed in them. These spots are thought to be the regions, where viral caspids are assembled after viral DNA is replicated. Formation of virogenic stroma is an essential feature to orchestrate processes like transcription, replication and packaging of viral capsids. After infection of cells G-actin specifically transported into nucleus and converted into F-actin. This actin localization and polymerization is an essential step in infection as it co-ordinates assembly of nucleocspids, viral proteins like PP78/83 (Ac9) and BV/ODV-C42 are involved in this process (Goley *et al.* 2006). BV/ODV-C42 are present in basal side of the capsids, they bind and move along with actin in the cytoplasm while budding. In order to exploit the host cells to the maximum possible extent baculovirues

manipulate hsot metabolism to their convenience. They encode proteins like ecdysteroid UDP-glucosyltransferase (*egt*) to delay molting of their hosts (O'Reilly *et al.* 1989; O'Reilly *et al.* 1990; O'Reilly *et al.* 1998). They also encode a fibroblast growth factor like gene (*Ac-fgf*) which induces motility in infected hosts ((Detvisitsakun *et al.* 2007). During late stages of infection virions move towards the periphery of virogenic stroma and get occluded in the occlusion body proteins which are localized in nucleus to produce ODVs (Miller 1997). The host cells lyse due to accumulation of ODVs in their nuclei and disperse ODVs. These BVs and ODVs can again start another cycle of infection.

As we mentioned ODV are formed only in 'Very Late stages' of infection. But the transition of infection phases from BV to ODV formation is still not completely understood. It could be a random event which starts after accumulation ODV related proteins in the nucleus or it could be a highly regulated process in which virions initiate the process by recruiting specific proteins related to ODV formation.

Some strange behavioral changes are observed in Baculovirus infected insects,like induction of motility in infected larvae. Trans-ovarian transmission of infection of baculoviruses happens due to mild infection in the parents. Newly hatched larvae usually stay in close groups and this may result in spreading of infection from one larva to other. Once they are infected, virus infection induces motility in these larvae which enhances their tendency to move away from the group. This type of behavior helps in spreading the virus infection. Another strange change int the behavior is that, infected larvae move to higher elevation and firmly cling to the leaves (Kamita *et al.* 2005), Baculoviruses encode enzymes like chitinase and cathepsin like proteases which digests the insect body and which looks almost like a tube filled with fluid. Finally when these insects die viruses which have reproduced exorbitantly get dispersed in the environment.

Baculovirus infection can be broadly divided into four phases; Immediate early, Early, Late and Very late phase. During Immediate early phase a set of genes are transcribed which are trans-activators of genes which will be transcribed next and they also help in initiation of viral DNA replication.

Next phase is 'Early phase' in which genes required for viral DNA replication, manipulation of host metabolism in favor of the virus and trans-activators of late genes are expressed. Viral DNA replication begins in this phase. This phase lasts for about 6 hrs after infection. Next phase is late phase, during which heavy DNA replication takes place followed by expression of late genes. Late genes include structural genes involved in BV formation and a few ODV related genes are also expressed. Very Late phase is the last phase in which Structural proteins related to the process of Occlusion, express and accumulate. ODV are formed and finally cells lyse to release OBs.

1.2.3 Taxonomy:

Baculoviruses are amongst the class of viruses showing a very large diversity in their sequence and gene content. Historically Baculoviruses were classified based on morphology of the occlusion derived viruses like Nucleopolyhedrosis viruses (group A) and Granuloviruses (group B) (Bilimoria 1986) Many other criteria like serological properties, virion structure, type of fusion proteins on their surface, pathology and host range were also used, but they were not widely accepted for a generalized classification of baculoviruses.

For classification of viruses it was necessary to have a gene which is found in all baculoviruses and which is evolving along with these viruses. Polyhedrin gene of baculoviruses is conserved across baculoviruses and was considered for classification of baculoviruses. They were divided into four classes depending on the *polh* sequence **1**. Dipteran group **2**. Hymenopteran group **3**. Nucleopolyhedrosis group and **4**. Granuloviruses (Rohrmann 1986). Further phylogeneitc analysis of polh gene revealed two clusters among the nucleopolyhedroviruses and they were called group-I and group-II NPVs, group-II NPVs and GVs encode an envelope fusion protein F-protein which helps viruses to enter into host and Group-I NPVs encode another protein gp64 for the same purpose (Lung *et al.* 2002). Isolation and characterization of CniNPV challenged this

method of classification and a new method of classification was required. With growing information of genomic sequences of baculoviruses the picture is getting clearer and information about conserved genes across baculoviruses is also coming up. So far ~41 Baculovirus genomes are sequenced and by comparing their genome there are about 29 genes which are higly conserved these are called 'core genes'. These core set of genes perform basic functions like replication, transcription and also include essential structural proteins. So using this information of the gene content, gene order and the concatenated sequence of all core genes for phylogenic analysis of baculoviruses revealed that, the classification based on these (mentioned above) criteria followed the classification of their hosts, more closely than other viral morphological characters. This shows that viruses have evolved along with their host species (Jehle *et al.* 2006). According to these criteria baculoviruses are classified into four classes **a**. Alphabaculoviruses **b**. Betabaculoviruses **c**. Gammabaculoviruses and **d**. Deltabaculoviruses.

a. Alphabaculoviruses include all Lepidopteran Baculoviruses including SNPVs and MNPVs, their genome size ranges between 100-180kbs and the type species is AcMNPV.

b.Beta baculoviruses include all lepidopteran specific genus Granuloviruses, genome size is between 100-180kbps and the type species is *Cydia pomonella granulovirus* (CpGV).

c.Gammabaculoviruses include all Hymenoptera-specific baculoviruses, their genome size is between 82-86kbps and type species is *Neodiprion lecontei nucleopolyhedrovirus* (NeleNPV), so far there are no evidences of BV formation in these viruses.

d. Deltabaculoviruses include all Dipteran specific baculoviruses (*Culex nigripalpus nucleopolyhedrovirus* (CuniNPV).

Alpha baculoviruses (lepidopteran baculoviruses) were further classified based on the fusion proteins present in their BVs, Group-I baculoviruses encode gp64 protein and group-II baculovirues lack gp64 and encode an F-protein for the same purpose.

1.2.4 Genome:

1.2.4.1 General features:

Baculoviruses have a large double stranded DNA genome. Viral genomic DNA is supercoiled but the condensation varies with different stages of infection. This difference could be due to binding of different proteins to viral DNA which might be necessary for tuning the expression of genes and also for maintenance of the viral genome itself for packaging and assembly depending on the stages of infection. The genome size varies from 80-180kbps. Smallest baculoviruses are reported from hymenoptera and largest are from GVs, group-I baculoviruses have a genome size of approximately 130kb and group-II viruses range between 130-170kbps. Numbers of orfs are also highly variable in these viruses ranging between 89 in NeseGV and 181 in XecnGV. This difference in size could be mainly due to the repeated orfs and few other genes which are seen only in larger viral genomes like *enhancin* and *ribonucleotide reductase*. Even within a particular type of virus there seems to be lot of variation. AcMNPV which was first isolated 20 years ago showed seven different genotypes (Stiles *et al.* 1998; Cory *et al.* 2005) and twenty four different genotypes were isolated from pine beauty moths (Hodgson *et al.* 2001). *Spodoptera frugiperda* MNPVs also exhibit lot of variation in their genome content. It has been reported that different isolates of SfMNPV contain different numbers of orfs. Theses genotypes vary in their ability to infect their hosts, but most of the times these genomes with variations are co-occluded and such viruses show enhanced infectivity when they infect together, so they are positively selected in the environment. Since genetic variation is an important factor which drives evolution in organisms, such variations in viruses either due to mutations accumulated during replication or by recombination drive evolution of viruses. (Frank 2003; Lopez-Ferber *et al.* 2003; Simon *et al.* 2004)

Orfs are distributed on both strands of Baculovirus genome. Number of orfs located on the same strand on which *polh* gene is located vary from 38% in NeseNPV and 56% in *Cryptophlebia leucotreta* NPV. There is no clustering of genes based on their temporal expression pattern like early genes and late genes. They are

scattered all over the genome and seem to be regulated independently. There are very less non coding regions in baculoviruses and when present they constitute 5'- and/or 3'-utrs and homologous repeat regions (*hr*).
Baculovirus orfs are conventionally numbered by considering *polyhedrin* (*polh*) gene as the first orf and numbering continues in the direction of the coding region of polyhedron gene (Vlak *et al.* 1982). But AcMNPV is an exception and its numbering begins from *ptp1* gene (Ayres *et al.* 1994).
The genome sequencing of 41 baculoviruses and their comparison have revealed that, 29 genes are highly conserved. These core genes constitute minimal set of genes required for viruses. Based on their function they are divided into five groups viz., Replication, Transcription, Packaging and Assembly, Cell cycle arrest, Oral infectivity and genes with Unknown function. Apart from these many other genes are highly conserved in a sub set of baculoviruses . Lepidopteran baculoviruses (both NPVs and GVs) have additional 33 genes conserved in their genome. NPVs and GV again have 15 and 22 genes conserved in them respectively. Apart form these conserved genes a large number of genes have shown lot of variability in their existence across baculoviruses and these genes might be contributing to factors like host range, virulence etc

As mentioned earlier there is lot of variation in the genomes of baculoviruses and occur as different genotypes. Many factors contribute to this other than mutations accumulated over time, like intra- and inter-genomic recombination, transposition and other replication related variations.

1.2.4.2 Hrs:

Most of the Baculovirus genomes contain repeated regions occurring at different locations called 'homogous repeat regions', in general hrs are AT rich regions containing imperfect palindromic repeats. In AcMNPV these *hr*-regions have an EcoR-1 restriction sites and SeMNPVs have BglII sites in them ((Cochran *et al.* 1982; Broer *et al.* 1998). Within a genome variation occurs in the *hr*-regions but they

are quite similar in having some basic sequences. They vary in the number of repeats and their direction also. Surprisingly in some viruses *hr*-regions are highly similar, even when their (viral) overall nucleotide content and amino acid content are not similar. The number of *hrs* per genome also varies considerably. CrleGV has 3 *hrs*, while 17 *hrs* have been reported for *Spodoptera litura* (Splt)NPV, . *hr* domains with palindromic repeats are not found in CpGV genome but there are some palindromic sequences at 13 different locations (Luque *et al.* 2001). Such *hr* regions are not found in some baculoviruses like TnSNPV (Willis *et al.* 2005). But when they are present, they are known to act as origin of replication and transcriptional enhancers (Guarino *et al.* 1986; Guarino *et al.* 1986; Pearson *et al.* 1992; Leisy *et al.* 1993; Pearson *et al.* 1993; Pearson *et al.* 1995). Baculoviruses have origin of replications other than hr regions also and they are found to be much stronger than hr regions (Pearson *et al.* 1995).

1.2.5 Organization of genes in Baculovirus:

Baculovirus genes do not have introns except in *ie-0* gene, so it is easier to predict orfs in Baculovirus genomes. Usually orfs of more than 150bps are considered for annotation. In baculoviruses, orfs are distributed on both strands and there is no specific clustering of these orfs based on their time of expression like immediate early, early phase or late phase. So these genes are regulated independently depending on individual gene organization which includes promoters and other regulatory factors.

1.2.5.1 Promoters of viral genes:

Computational analysis have highlighted some salient features in the promoter regions of viral genes depending on whether they are early or late expressing genes (Marks *et al.* 2006). Viral genes which are expressed in the immediate early phase of infection and early phase are dependent on host RNA polymerase-II. Such early expressing genes have an CAGT motif indicating a transcriptional start site and a

TATA box upstream to it (Blissard *et al.* 1992; Guarino *et al.* 1992; Kogan *et al.* 1995). In case of gp64 gene of OpMNPV and pe38 of AcMNPV a GATA motif is present which can recruit GATA family of zinc finger binding transcription factors (Kogan *et al.* 1994). OpMNPV gp64 gene has an additional CGT motif which is important for its transcription.

Baculovirus late and very late genes are transcribed by a virus encoded RNA polymerase itself but not by host RNA polymerases, but they require some host factors to achieve their optimum expression level. These late and very late promoters contain a canonical (A/T/G)TAAG motif. (Rankin *et al.* 1988; Morris *et al.* 1994; Miller 1997; Yang *et al.* 1998)

Some genes of Baculovirus are expressed in both early and late phases of infection. They contain a late promoter preceding an early promoter. These promoters have a conserved TATAAAG motif enabling them to be transcribed by both host and Viral RNA pol II. Some genes initiate transcription from a common site but terminate at different positions depending on the RNA polymerase they were transcribed with. *gp64* is one such gene exhibiting these features. Early transcripts are shorter than late transcripts (Wu *et al.* 1993; Garrity *et al.* 1997). Some orfs which are located next to each other in same orientation are transcribed from their own promoters but they co-terminate to give rise to transcripts having same 3' ends (Gross *et al.* 1993).

All genes of baculoviruses contain a short unstructured 5'UTR (Scheper *et al.* 1997). 3'ends of all viral transcripts have a poly-A tail but they don't terminate consistently at the same termination signal. Most of the time, they read through to produce variable 3'termini. In baculoviruses a GT rich region upstream to AATAAA motif acts as a good termination signal rather than jest AATAAA (McLauchlan *et al.* 1985; van Oers *et al.* 1999).

1.2.6 Transcription of Baculovirus genes:

1.2.6.1 Early genes:

Baculoviruses being dsDNA viruses have to enter into nucleus to transcribe their genome. During natural infection limited number of viruses enters into a host cell and they have to transcribe their genes in required order to achieve complete infection. Viruses have to compete with the host transcription machinery and they employ several strategies to outcompete them. Viruses are known to have their own transcriptional enhancers which can enhance their transcription significantly with minimum available transcription machinery. These early expressed genes can act as trans-activators to over express other set of genes which might carry on this stepwise cycle to exploit host machinery leading to successful infection. In some viruses few structural proteins (Herpes virus protein vp16) can act as transcriptional activator of specific viral genes leading to their over expression. Such cases are not reported in baculoviruses and moreover it is known that even transfecting viral DNA can lead successful production of viral particles. So such proteins are not essential for Baculovirus to infect their hosts but it is possible that they can increase the efficiency of infection (Burand *et al.* 1980; Theilmann *et al.* 1993).

As soon as the virus enters the host cells viruses employ their transcriptional enhancers and transcribe their immediate early genes.hr regions are known to act as transcriptional enhancers in eukaryotic cells. The immediate early gene ie-1 which is transcribed by host RNA pol-II is a trans-activator protein (Guarino *et al.* 1986; Kovacs *et al.* 1991; Kovacs *et al.* 1992). ie-1 promoters are not very strong promoters but presence of a CAGT motif upstream to TATA box can independently act to promote formation of initial complex of host RNA pol II to increase transcription to a modest level (Pullen *et al.* 1995). This modest amount of ie-1 protein dimers can bind to hr regions specifically and enhance transcription of early phase genes very effectively (Choi *et al.* 1995; Gomi *et al.* 1997). There could be enhancer regions in viral genome other than hr regions also which can loop the viral DNA to facilitate high level of transcription. Deletion of individual hr regions did not

affect transcription in some viruses but in those experiments other *hr* regions were intact. These *hr*s along with other enhancer elements and ie-1 gene can bring about high level of transcription of viral genes. The mechanism of these events is not understood completely. Few other proteins can also act as trans-activators of viral gene expression like *ie0, pe38 and ie2/ie-n* (Wu *et al.* 1993; Yoo *et al.* 1994; Milks *et al.* 2003; Stewart *et al.* 2005). IE1 activates expression of early genes (*p35, gp64, 39K, p143, dnapol, pe38, lef-1, lef-2, lef-3, and ie1*) and is required for DNA replication and transcription of late genes. Most of the immediate early genes, but a few like *ie-1* are shut down in late phase of infection by unknown mechanism.

1.2.6.2 Viral RNA polymerase:

Baculoviruses are the only insect dsDNA viruses replicating in nucleus and encoding their own RNA polymerase. Both host and viral RNA polymerase can transcribe viral genes depending on their promoters and many other factors which are not understood completely.

Early studies of transcription of viral genes observed that even after inhibiting host RNA Polymerase-II by α-amanitin late gene transcription was not inhibited ((Grula *et al.* 1981; Fuchs *et al.* 1983). Then studies of role of late genes viral replication and subsequent isolation of a multi-protein complex (Guarino *et al.* 1998) containing a few late genes observed in the earlier experiments prompted the possibility of an viral encoded RNA polymerase. The late genes implicated to form a RNA polymerase like complex were *lef4, lef8, lef9 and p47*. *Lef 8* and *9* are homologous to the large β and β' subunits of eukaryotic RNA polymerase II (Lu *et al.* 1994). These subunits show lot of variation in their size but the catalytic site is intact. Lef4 is an RNA capping enzyme (Gross *et al.* 1998; Jin *et al.* 1998). P47 showed slight similarity to α-subunit of bacterial RNA polymerase but its function is not yet understood.

Ac38 is an *ADP-ribose pyrophosphatase* type of protein this might be negatively regulating genes by removing 7-methylguanosine diphosphate. This is an essential gene affecting viral transcription but not a subunit of viral RNA polymerase.

1.2.6.3 Late & Very Late Phase genes:

A set of genes expressed after early phase genes are late genes. These two phases (early and late) are separated by replication of viral DNA. The transition between early to late phase is not completely understood yet but several possibilities are proposed. first observation was that since late transcription fallows DNA replication, naked DNA which is transiently not bound by other DNA binding proteins or structural proteins can act as templates to viral RNA polymerase or to factors which promote binding of these RNA polymerase (Keck *et al.* 1990). Secondly, the replication intermediates which are partially double stranded or DNA-RNA hybrids due to okazaki fragments during Replication can also act as templates of Viral RNA polymerases (Brody *et al.* 1995). Thirdly, specific sequence motifs which are present in viral promoters can recruit viral RNA polymerases to bind and transcribe these genes.

Analysis of several late gene promoters from 26 baculovirus genomes revealed the presence of TAAG motif in their promoter region ((Rankin *et al.* 1988; Xing *et al.* 2005), among these TAAG motifs ATAAG and GTAAG motifs were found more frequently than TTAAG. CTAAG motifs are also seen in these genomes but there are no correlations with late genes and they do not act as late gene promoter elements. TAAG motifs were usually located upstream to ATG site. These motifs could not transcribe early genes when introduced in their promoter regions and their distribution in viral genome is not random. This shows a bias in their distribution in the late gene promoter regions (Rohrmann 1986). A region of 18 bps around TAAG motif is known to contain all the basic regulatory elements of a late gene promoter (Morris *et al.* 1994). Many late genes contain multiple TAAG motifs in their promoters but the significance of occurrence in multiple copies or the relevance with respect to neighboring sequences are not clear from the computational analysis, but th flanking regions can influence the amplitude of expression of these genes (Thiem *et al.* 1989). Genes which contain TAAG motifs combined with TATA i.e. “TATATAAG” elements can be transcribed by both host RNA pol-II and viral RNA polymerases.

Very late gene promoters also contain a TAAG motif and its associated neighboring sequence elements (Mans *et al.* 1998). The region between the TAAG motifs and the transcription initiation sites are AT rich and they can significantly enhance transcription of very late genes called 'Burst' sequences. These AT rich region do not contain any conserved sequence, at least no pattern was observed by analyzing these regions computationally. When these burst regions were mutated the timing of expression of late genes was not altered but levels of transcripts decreased, indicating the presence of additional features in these regions outside TAAG motifs, burst sequences and transcription initiation sites.

Late and very late transcripts of baculoviruses contain a 5'-cap. In eukaryotes 5'-capping enzymes interact with the host RNA polymerase directly and add 5'- cap to all transcribed RNAs but viral RNA polymerase does not have a CTD domain and thus cannot utilize the host capping machinery. Guarino *et al* showed Viral RNA polymerases itself can add a 5'-cap to viral transcripts with the help of lef-4 sub unit of viral RNA polymerase (Guarino *et al.* 1998). Few other viral genes, like *RNA triphosphatase (ptp)* (Gross *et al.* 1998; Takagi *et al.* 1998) and a RNA cap *2'O-methyltransferase* family gene (AcORF 69) (Wu *et al.* 2003) might also contribute in various steps of this process. Viral transcripts also have a -3' poly-A tail but they are not cleaved and poly-adenylated by the host machinery. Viral RNA polymerase terminates when it encounters a U-rich region and it can add a poly A tail to these transcripts in template independent manner (Jin *et al.* 2000).

Since viral genes are very compactly arranged without much gap and the viral RNA polymerase does not end at the first termination signal that it encounters transcripts of different sizes are produced. Such transcripts will have same 5'- end but different -3' sequences. There are cases where transcripts of different sizes are produced having different 5'- end but same -3' termini and they are formed in genes which have multiple transcription initiation sites or if the genes are transcribed by different RNA polymerases (as some genes can be transcribed both by viral and host RNA polymerases). Such read through transcription sometimes results in overlapping transcripts. Some cases are known where transcription of an upstream gene inhibits its downstream gene, because of the active transcription of that upstream gene.

When the TAAG motif of the upstream gene was removed to inhibit its transcription the downstream gene transcribed more efficiently ((Gross *et al.* 1993).
Transcription of genes on one strand can affect the transcription of genes on the other strand also. Transcription of very late gene *p10* decreases the expression of orf1629 and when transcription of p10 gene was inhibited by mutating it, transcription of orf 1629 did not decrease. The mechanism of this kind of regulation is not yet understood but it could be either due to prevention transcription on the other strand or due to RNA mediated silencing, which might get triggered as both strands are getting transcribed simultaneously (Ooi *et al.* 1990).
As it is known that expression of some viral genes inhibit other genes, resulting in smooth transition from one phase to another phase of infection and the reasons behind this transition are not completely understood such mechanisms might be used positively by the virus to fine tune the expression levels of viral genes and might use this mechanism to achieve active transcription or to shut down specific genes.

Late and very late genes are expressed in very high levels especially very late genes like VLF-1, p10 and Polyhedrin. Various factors contribute to this extremely high level expression of these genes like 1.Expression of late and very late gene fallows replication of viral DNA which might provide large number of templates for transcription. 2. Shutting off of early and late genes might increase the availability of machinery to transcribe and translate very late genes. 3. Specific sequence elements in the very late promoters can enhance their expression, 4. Protein factors (like vlf-1, lef-2 and pk-1) expressed early or in the late phase can enhance very late gene expression specifically. 5. Genes like p10 can undergo cap independent translation.

Late expression factors are very much essential for the viruses and they are involved in vital processes of viral life cycle. Late expression factors like lef-6, lef-11, and lef-12, lef-5, pp31 & lef10 are involved in regulating other viral genes (Passarelli *et al.* 1993; Gomi *et al.* 1997; Guarino *et al.* 2002; Lin *et al.* 2002). Functions of all

these genes are not clearly understood but their presence is required to achieve optimal expression of other late and very late expressing genes.
Some late expression factors like lef-1, lef-2, lef-3, p143, dnapol, ie-1, ie-2, lef-7 & p35 have role in viral DNA replication (Merrington *et al.* 1996; Miller 1997; Rapp *et al.* 1998; Li *et al.* 1999; Lin *et al.* 2002). Since late genes are transcribed following viral DNA replication all those genes which affect viral DNA replication directly or indirectly affect late and very late gene transcription also.

Host range of baculoviruses is determined by both viral and host genes. Many late expression factors like. *lef-7, p143, ie-2, p35, hcf1, p143, hrf1* and *bro* (Clem *et al.* 1991; Kondo *et al.* 1991; Kamita *et al.* 1993; Lu *et al.* 1995; Lu *et al.* 1996; Thiem *et al.* 1996; Rapp *et al.* 1998) determine the host range of baculoviruses. Introducing these genes from one virus to another is known to widen the host range of recipient viruses. But this effect of increasing host range cannot be generalized because all these above mentioned genes can increase the host range is restricted to only a few viruses but not in all viruses.
When cells are infected with virus, transcription and translation of host cells are shut down. But host factors are very essential in viral transcription and replication. A host encoded protein called polyhedrin promoter binding protein (PPBP) is known to bind to promoters of *polh* and *p10* genes of baculovirus and can increase the transcription of these genes (Burma *et al.* 1994; Jain *et al.* 1996; Ghosh *et al.* 1998). Host actin is another major factor in virus infection, and depolymerisation of actin in infected cells affects late and very late gene transcription (Wei *et al.* 1992; Volkman *et al.* 1996; Miller 1997)

1.2.7 Viral DNA replication:

Baculoviruses are dsDNA viruses which replicate their DNA in the nucleus of the cells. As the virus enters the host cells it produces immediate early and early genes which encode proteins involved in Viral DNA replication. Initially ie1 proteins and hr regions form specific foci in the nucleus of the cell and proteins associated with replication also localize to these spots. Then lef3, DBP and p143 (DNA helicase

assisted by lef3) also localize to these foci. As replication continues these spots grow larger to form a electron dense region called Virogenic stroma (or viral stroma) ((Mainz *et al.* 2002; Nagamine *et al.* 2005; Nagamine *et al.* 2006; Wileman 2007). Subsequently late genes also start expressing and Viral DNA replication continues in specialized regions called 'replication factories' within the viral stroma and accumulate large quantity of Viral DNA.

Once the virus enters into cells and expresses its immediate early and early phase genes which can replicate viral DNA they have to differentiate between the viral DNA and host genomic DNA to start replication. These viral proteins can bind to specific regions in the viral genome called origin of replication. In baculoviruses homologous repeat regions (hr) are known to act as origins of replication ((Pearson *et al.* 1992; Kool *et al.* 1993) along with non hr origins (Lee *et al.* 1992; Pearson *et al.* 1993) like early promoters (Lee *et al.* 1994; Habib *et al.* 2000). It seems, in baculoviruses any sequence that is unwound during replication , transcription or due to binding of some protein factors can allow formation of a replication complex and can act as origin of replication (Okano *et al.* 2006). Study of Baculovirus replication intermediates suggests that they replicate either through rolling circle mechanism or through extensive recombination (Leisy *et al.* 1993; Oppenheimer *et al.* 1997; Okano *et al.* 2007). Since baculoviruses without hr regions exist, early promoters and almost any unwound part of DNA can act as origin of replication and accumulation of replicated viral DNA which is larger than the actual viral genome indicate a specific origin of replication are not important for baculoviruses. Once replication starts by using any of these features it can continue and amplify viral DNA independent of any specific origins of replication.

Genes involved in viral DNA replication were identified by studies on temperature sensitive mutants (Lu *et al.* 1991) and by transient replication assays (Kool *et al.* 1994; Lu *et al.* 1995). So far 6 viral genes are found to be essential and many others can stimulate replication. The essential viral genes include *dnapol*, *helicase (p143)*, *lef-1 (late expression factor-1)*, *lef-2*, *lef-3* and *ie1.*

Ie-1 gene is a trans-activator or other early phase genes and has the ability to bind to hr regions which can form cruciform like structures. This ability of ie-1 proteins to

specifically bind to viral sequences (hr regions), and to recruit RNA polymerase-II to transcribe viral genes might cause unwinding of viral DNA promoting viral DNA replication.

Lef-3 is a single stranded DNA binding protein (Hang *et al.* 1995). in in-vitro experiments lef3 is known to anneal or unwind DNA depending on the redox condition of the reaction ((Mikhailov 2000; Mikhailov *et al.* 2005). It binds to DNA helicase (p143) another essential protein in DNA replication and helps in transporting it into nucleus (Wu *et al.* 1998). Lef-3 might be helping to recruit DNA helicase to partially unwind single stranded viral DNA to promote replication. lef3 has a role in replication independent of recruiting p143 also. It can bind to ssDNA and restrict it from forming secondary structures or annealing to favor replication.

P143 is a DNA helicase which can unwind DNA to facilitate the movement of DNA polymerase (Lu *et al.* 1991). *lef-1* is DNA primase and *lef-2* is a DNA primase associated factor. These two genes together synthesize the RNA primer required for DNA polymerase to continue replication (Evans *et al.* 1997). *dnapol* gene of baculoviruses are related to DNA polymerases type-B family, they have 3'-5'exonuclease activity but 5-3' exonuclease activity was not observed. They are very high fidelity enzymes so they can replicate both leading and lagging strands ((Mikhailov *et al.* 1986; Tomalski *et al.* 1988; Hang *et al.* 1999; McDougal *et al.* 1999). AcMNPV encodes a putative DNA ligase but its role and catalytic abilities are yet to be demonstrated. Although baculoviruses encode key genes involved in replication, they lack few genes like topoisomerase and DNA ligase etc. it is possible that host genes complement for these functions.

1.2.8 Packaging assembly:

Viral capsids or Virions assemble after DNA replication and expression of genes required for assembly and package. This process in baculoviurs is not completely understood. So a possible mechanism is proposed. DNA which replicates and produces fragments larger than actual viral genome undergoes recombination and finally produce covalently closed circular DNA (final viral genome) which is ready for package. Simultaneously viral proteins also accumulate in the viral stroma and they

form a pre-assembly of capsids. Viral DNA binds to specific proteins like p6.9, vlf-1 (McLachlin *et al.* 1994; Vanarsdall *et al.* 2004; Li *et al.* 2005; Vanarsdall *et al.* 2006), Alknaline nuclease (Okano *et al.* 2004; Okano *et al.* 2007) etc, which help in protecting and processing the viral genome and also help in condensing the genome which gets assembled into a capsid that comes out of the nucleus. The assembly happens on the boundary of viral stroma.

To mention another possibility replication might be partitioned. DNA destined to get packaged is co-ordinated with packaging and kept away from recombination events. But the DNA when accumulates to high concentration can act as template for over expression of late and very late genes. In infected cells usually large number of viral DNA gets replicated but only a small fraction is assembled in to virions.

1.2.9 Virus structure:

Baculoviruses exist in two different forms Budded viruses and Occluded viruses. Both BV and ODVs can be produced in the same cell and they contain same nucleocaspids having same genetic composition. BVs mediate infection from one cell to other in the same host body or in cells culture. ODVs are responsible for transmitting viruses from one insect to other and they can infect through the oral route of insects.

1.2.9.1 Nucleocaspids:

Nucleocapsids are tubular structures with a cap like structure at one end. They contain finger like outgrowths in the cap like region (Miller 1997). Viral DNA is packaged in these nucleocapsids and it is highly condensed due to binding of highly basic arginine rich proteins p6.9 in NPVs and VP12 in GVs (Tweeten *et al.* 1980; Wilson *et al.* 1987). VP39 is a major capsid protein (Pearson *et al.* 1988; Thiem *et al.* 1989). P80 and p24 are other structural proteins in the capisds. Basal side of the capsids contain PP78, PP83 and VLF-1. EC27 and C42 (Braunagel *et al.* 2001). GP41 is a tegument protein present between the viron membrane and capsids. This is located on the apical end of the capsid and is essential for efficient nuclear

egress (Whitford *et al.* 1992; Olszewski *et al.* 1997). Ac144 is a cyclin which can regulate the host cellcycle. Ac 144 is present in both ODV and BV derived virions and mutants are diffective in nucleocapsid formation but not in replication of viral DNA. Many other proteins involved in replication and transcription are also found in the assembled nucleocapsids. It is not known whether these proteins are really necessary for optimum infection of the virus or they are packaged in capsids just because they were located in the vicinity of assembly and packaging sites. This also shows that replication, transcription, assembly and packaging are closely regulated and happen in regions like 'virogenic stroma' (Braunagel *et al.* 2003; Deng *et al.* 2007; Perera *et al.* 2007). Capsids usually bind to the membranes from their apical side containing finger like outgrowths. Depending on the stage of infection and many other undetermined factors they form Budded viruses or Occluded viruses.

1.2.9.2 Budded virus:

The viral nucleocapsids come out of the nucleus with a pinch of nuclear membrane which form vesicle like structure around them. These vesicles are associated with host actin and sometimes fuse together to contain multiple nucleocapsids in them (Miller 1997). Vesicles move away from the nucleus towards plasma membrane. During this transit they lose the vesicle like structure around them derived from the nuclear membrane. These naked nucleocaspids move to the plasma membrane where viral encoded fusion proteins are located. In group-I NPVs both GP64 and F-protein are present but in Group-II NPVs and GVs s only F-proteins are found((Pearson *et al.* 2000) and attach to the membrane from their apical side (Volkman 1986; Blissard *et al.* 1989; Whitford *et al.* 1989; Monsma *et al.* 1995; Monsma *et al.* 1996). Nucleocapsids bud out of the cells along with a part of plasma membrane having GP64 protein on their apical sides. GP64 proteins form a crown like structure consisting of spike like outgrowth called peplomeres in the envelope of Budded viruses. Ubiquitin and ubiquitin ligase type proteins (IAP2 IE2, PE38) are also found within the enveloped Budded viruses (Guarino 1990; Haas *et al.* 1996; Imai *et al.* 2003). Viral ubiquitin are not efficient as host ubiquitin so they might help in avoiding faster degradation of selected viral proteins. Many other viral proteins

are also found in BVs like ODV/BV-E26, IE1, V-CATH protease etc (Theilmann *et al.* 1993; Lanier *et al.* 1996).

1.2.9.3 Occlusion derived virus:

Occluded viruses are virion particles contained in a crystalline protein matrix having an envelope structure around it. This form of virus is quite stable in the outer environment and can protect the virion particles for years outside a living host. Extreme environments like high temperature, alkaline pH, and UV can reduce their virulence but ODVs are produced in such a large number that even a small fraction of viruses which survived these extreme conditions can infect other hosts. Until 24hrs PI *i.e.* during Late phase BVs are produced in the infected cells but after late phase of infection BV production decreases and ODV production begins. The reasons behind transition from BV to ODV production are not completely understood. FP25 proteins seem to help in this process. FP25 proteins which usually reside in cytoplasm inhibit nuclear egress of capsids which will otherwise bud out of the cells as BVs (Jarvis *et al.* 1992; Harrison *et al.* 1995). This nuclear retention of capsids favors ODV production. FP25 mutants produce a phenotype called few polyhedral which is commonly found in viruses passaged for very long time. They produce fewer polyhedra compared to wild type. This causes overproduction of BVs and BV related proteins, suppressing ODV related structural proteins in infected cells ((Harrison *et al.* 1996; Katsuma *et al.* 1999; Rosas-Acosta *et al.* 2001).

During late phase micro-vesicle like structures are formed in the inner nuclear membrane and these vesicles produce a trilamellar membrane which envelopes the occluded capsids. Many proteins which get incorporated into envelope of ODVs are produced in this phase of infection, whether production of these proteins supports formation of ODVs is to be investigated but their time of expression matches the time of ODV formation.

ODV compared to BV have more proteins in their envelopes (Deng *et al.* 2007). Proteins found in ODV are either structural proteins, or proteins which help in infection of the host through oral route called *per os* infectivity factors (*pif*). Most of

these proteins have hydrophobic domains which help them to specifically localize into vesicles formed in the inner nuclear membrane. Theses vesicle are further used in enveloping the ODVs (Braunagel *et al.* 2007; Deng *et al.* 2007). Proteins like ODV-E18 (Deng *et al.* 2007; McCarthy *et al.* 2008), ODV-E25 ((Russell *et al.* 1993; Braunagel *et al.* 2003; Deng *et al.* 2007), ODV-E27, ODV-E56, ODV-EC43 (Fang *et al.* 2003) are essential for viruses and BV/ODV-E26 (hyaluran lyase) ((Vigdorovich *et al.* 2007), ODV-E66 ((Hong *et al.* 1994; Hong *et al.* 1997) are necessary but not absolutely essential for ODVs. Other per os infectivity factors are P74, PIF-1, PIF-2, Ac145, Ac-150, PIF-3 (Ohkawa *et al.* 2005; Zhou *et al.* 2005; Deng *et al.* 2007), They are also necessary for ODVs. Mutants of these factors are compromised in infecting insect hosts but may not be very important for production of BVs. Gp41 a tegument protein and *ptp* a protein tyrosine phosphatase are also found in ODVs. Proteins related to DNA replication and transcription are also found in ODVs like dnapol, P143, LEF3, IE-1, F protein (Ac23), BRO (Ac2), Ac68, Ac81, Ac92, Ac96, Ac98, LEF-9, Ac62 etc , they may not absolutely essential to infect the host but might contribute to efficiency of infection. Proteomic studies of CuniNPV ODVs revealed presence of 45 proteins (Perera *et al.* 2007). Envelope of ODV contain more number and quantity of proteins compared to BV envelopes. Although ODV envelope is derived from Nuclear membranes their lipid profile is considerably different. This shows that viruses manipulate the lipid metabolisms and actively recruit specific lipids into their envelopes (Rosas-Acosta *et al.* 2001).

1.2.9.4 Occlusion Bodies:

Along with the virions and the envelope that covers the ODVs Occlusion bodies contain a protein matrix. During very late phase of infection proteins related to Occlusion start accumulating. P10 and polyhedrin (or Granulin) ((Rosas-Acosta *et al.* 2001; Anduleit *et al.* 2005; Perera *et al.* 2006) are the two major protein which constitute the protein matrix, Polh preferably localizes in nucleus whereas p10 forms fibrillar bodies in both nucleus and cytoplasm (Gross *et al.* 1994; Lee *et al.* 1996). Next abundant protein is calyx protein or Polydrin envelope protein (PEP) also called PP34 (Gross *et al.* 1994; Lee *et al.* 1996). PEP and many other

carbohydrates constitute the polyhedron envelope protein they cover the protein matrix of polyhedra (Russell *et al.* 1991). They are resistant to harsh chemical environments and seem to seal the surface of the polyhedral and increase their stability. Mutants of PEP or p10 produce pitted polyhedral with severely compromised stability. These proteins in the polyhedra are degraded in the alkaline pH of insect gut and the protein covers are degraded by proteases facilitating exposure of gut cells to viruses. Occluded polyhedral contain proteases which help in digesting the peritrophic membrane of insect gut and facilitate direct exposure of gut cells to virus. Enahancin (metalloproteinases) (Wang *et al.* 1997; Popham *et al.* 2001) and cathepsin like proteases (Slack *et al.* 1995). It was seen that ODVs produced in cell culture lacked proteinases which are active in insect gut environment (Slack *et al.* 1995). But such proteinases are found in ODVs produced in insect hosts. This indicates that these proteases found in poyhedra or ODVs are derived from insect host or insect gut bacteria but get packaged in ODVs. Polyhedra are resistant to freezing and desiccation but very sensitive to light. Some baculoviruses encode superoxide dismutase genes (SOD) and are found in polyhedral bodies also they might provide protection against free radicals produced in light ((Tomalski *et al.* 1991). Polyhedra are extremely sensitive to ultraviolet (UV) radiation. UV radiation dimerzes pyrimidine residues in viral genomic DNA. Attempts were made to produce UV tolerant baculoviruses by expressing DNA repair enzymes but their resistance was limited to BVs but not in polyhedral bodies exposed to UV outside a host (Petrik *et al.* 2003).

Chapter 2:

Materials

and

General methods

Chapter 2: Materials and General Methods

2.1. Cell lines and Medium

2.1.1 sf9 cells:

Spodoptera frugiperda cell line sf9 derived from pupal oviduct epithelial cells (Vaughn *et al.* 1977) were obtained from cell repository of National Center for cell Science (NCCS) INDIA. Cells were grown in TNM-FH medium supplemented with 10% heat inactivated Fetal Bovine Serum (FBS)(Gibco-invitrogen). Cells were incubated at 27^{0}C.

2.1.2 BmN cells:

Bombyx mori cell line "BmN" is a larval midgut epithelial cell line. We obtained them from cell repository of National Center for cell Science (NCCS) INDIA. Cells were grown in TNM-FH medium supplemented with 10% heat inactivated Fetal Bovine Serum. Cells were incubated at 27^{0}C.

2.1.3 D.mel2 cells:

Drosophila melanogaster cell line *D.mel 2* were obtained from cell repository of National Center for cell Science (NCCS) INDIA. Cells were cultured in Schneider's medium (Gibco–Invitrogen) supplemented with 10% heat inactivated Fetal Bovine Serum. Cells were cultured at 25^{0}C

2.1.4 Medium of culture: Grace's medium, TNM-FH and serum free medium, Schneider's medium, and serum free medium)

Grace's medium (Grace 1962) was purchased from Gibco-invitrogen in powdered form. TNM-FH medium (pH-6.2) was prepared by adding Yeast extract (3.33gm/liter) and Lactalbumin hydrolysate (3.33gm/liter) to the grace's basal medium (Hink 1970). TNM-FH medium was supplemented with 10% heat inactivated FBS, and antibiotic-Anitmycotic solution (Sigma). Sf900 (Gibco-invtrogen) serum free medium was also used for culturing sf9 cells.

Schneider's medium (Gibco-invitrogen) was supplemented with 10% heat inactivated FBS.

2.2. Virus methods:

We adopted most of the methods in this section from "Baculovirus Expression Vectors a laboratory manual"

2.2.1 Wild type virus AcMNPV, BmNPV

Wild type Autographa Californica MultiNucleo Polyhedrosis Virus (AcMNPV) strain C6 was kindly provided by Prof Dr. KVA Ramaiah of Hyderabad Central University (NC_001623). BmNPV virus was procured from RIKEN Japan

2.2.2 Production of recombinant viruses

Recombinant AcMNPV were produced by using reagents and methods provided in the Bac-to-Bac system (Invitrogen)

Recombinant viruses expressing *egfp* and *rfp* vRecEGFP and vRecRFP were produced by cloning Enhanced Green Fluorescent Protein (EGFP) and dsRED into pFastBac-HTb transfer vector (Invitrogen) and then by site specific transposition into Bacmid containing AcMNPV viral DNA, provided in the kit.

Other recombinant viruses 'vRecRed-S' and 'vRecRed-SAS' were produced by cloning *rfp* and other fragments encoding dsRNAs against dsRED gene in two different expression cassettes using the transfer vector pFastBac-Dual (Invitrogen)

2.2.3 General method of infection of sf9/BmN cells with virus:

Virus stock was diluted to required dilution in complete medium and added to cells. These cells were incubated at 27^{0}C in a humidified a chamber. Care was taken not to allow cells to dry and the culture dishes were gently rocked once in every 10-15 minutes. Generally 0.5ml medium was used for a 60mm plate seeded with cells. The volume of virus stock was scaled-up or scaled-down according to the area of

culture dish. After 1 hour of incubation virus stock was replaced with complete medium and incubated at 27^0C.

2.2.4 Amplification of virus stock:

Baculoviruses can be amplified simply by infecting cultured cells and harvesting medium after four to five days of Occluded viruses (Occ+) and five to six days for Occ- viruses we produced large stocks of Budded viruses of the above mentioned type of viruses (AcMNPV and recombinant viruses) by infecting sf9 cells grown to 70-80% confluency with 1ml of virus stock (in case of virus stock with unknown titer) or by infecting cells with an MOI of 0.1 (in case of virus stock with known titer) and harvesting the spent medium after five days for Occ+ virus and after six days for OCC- virus. Virus stock was filtered through a 0.22μ membrane and stored at 4^0C for immediate use (3-4 months) and few aliquots were stored at -70^0C for long term storage.

2.2.5 Concentration of virus stock:

Virus stock produced by inoculating cells and harvesting virus particles in the spent tissue culture medium is in the range of 10^8 to 10^9 pfu per ml. during our experiments we had to infect cells with high MOI and sometimes had to infect high density of cells so the virus stocks were concentrated by ultra centrifugation.

Virus stocks were centrifuged at 10,000 rpm for 15 minutes at 4^0C (Sorval GS-68 rotor) to remove cell debris. And then the supernatant was centrifuged at 48,000g for 2 h at 4^0C (Beckman, L-8 Ultracentrifuge, Beckman Inc.) to get a pellet of virus particles. This pellet was re-suspended in PBS or Medium supplemented with FBS to get a concentrated virus stock

Alternatively virus stocks were centrifuged at 10000rpm for 15minutes at 4^0C (Sorval GS-68) rotor to remove cell debris and the supernatant (approximately 33ml) was loaded into a 38ml polyallomer ultracentrifuge tube (Beckman SW28 tubes) underlayed with a Sucrose cushion (25%sucrose W/V in 5mM NaCl, 10mM EDTA) and centrifuged at 80000 X g for 75 minutes at 4^0C. Supernatant was discarded and a blue tinged pellet of budded virus was re-suspended in required

amount of sterile PBS. The virus stock was filtered through 0.22μ membrane and stored.

2.3. Isolation of viral DNA:

Viral DNA can be isolated by different methods depending on the quantity and quality required for downstream analysis. In our experiments we used 3 different methods to isolate viral DNA from infected cells in culture.

2.3.1 Isolation of viral DNA by Phenol:Chloroform Method

This is the simplest and cheapest method to isolate DNA. We infected cells with virus and after 4-5 days collected those cells along with medium, centrifuged and took supernatant which contains the viral DNA. Equal volume of phenol (pH8.0 saturated with Tris-HCl) was added to the culture supernatant and mixed well by inverting. The resulting emulsion was centrifuged at 12000g for 5 minutes at 4^0C to allow phase separation. Aqueous phase was collected and equal volume of Phenol:Chloroform:iso-amyl alcohol (25:24:1) was added, mixed well by inverting and centrifuged at 12000g for 5 minutes at 4^0C. Again aqueous phase was collected and equal volume of Choloroform:isoamyl alchohol (24:1) was added mixed well by inverting and centrifuged at 12000g for 5 minutes at 4^0C. The aqueous phse was collected carefully and viral DNA was precipitated by adding 2 volumes of absolute ethanol and centrifuging at 12000g for 15 minutes at 4^0C, precipitated DNA was washed with 70% ethanol, air dried and dissolved in 10mM Tris buffer. DNA was stored in aliquots at -20^0C for long term storage and at 4^0C for shorter duration

2.3.2 Isolation of DNA from purified Budded Virus.

We used 10ml of virus stock to concentrate and pellet budded viruses as explained in the earlier section 2.4 (section 2.4 concentration of virus stock) and the pellet was re-suspended in 1ml of disruption buffer (10mM Tris-HCl pH 7.6, 10mM EDTA, 0.25% SDS) and mixed well by pipetting up and down using a cut-off tip. Proteinase K was added to a final concentration of 500μg/ml and digested at 37^0C until the

solution became clear (4-12hrs) and extracted with phenol, Phenol:Chloroform and Chloroform as mentioned in section 3.1 (3.1 Isolation of viral DNA by Phenol:Chloroform method) and precipitated with ethanol and dissolved in 10mM Tris buffer (pH-8). DNA was stored in aliquots at -20^{0}C for long term storage and at 4^{0}C for short term.

2.3.3 Isolation of viral DNA by High-Pure Viral DNA isolation kit.

We used this method to isolate viral DNA for Real Time PCR based estimation of virus titer and for amplification of specific orfs by PCR for in vitro transcription, cloning of complete orf for protein expression etc. Viral DNA was isolated as per the manufacture's recommendations. We collected supernatant (200µl) from infected cells to which 200µl of binding buffer (supplemented with carrier RNA) and 50µl proteinase K was added and incubated at 72^{0}C for 10 minutes. Additional 100µl of binding buffer was added and loaded onto the column, centrifuged (8000g for 1 minute) and flow through was discarded. Columns were washed once with inhibitor removal buffer and twice with wash buffer and centrifuged 8000g for 1 minute. A final spin was given at 12000g to remove residual buffers and viral DNA was eluted with 50µl nuclease free water.

2.4. Estimation of Viral titer:

Several methods are used by researchers to estimate the titer of the virus based on end point dilution, Real Time PCR, Plaque Assay, Antibody based assay and Flow Cytometry mediated assay (O'Reilly *et al.* 1992; Kitts *et al.* 1999; Kwon *et al.* 2002; Shen *et al.* 2002; Lo *et al.* 2004; Fridholm *et al.* 2005; Janakiraman *et al.* 2006; Janakiraman *et al.* 2006).

In our study we used End point dilution method and/or Real time PCR based methods to estimate the titer of the virus.

2.4.1 Estimation of viral titer by end point dilution.

This method estimates the dilution of the virus required to infect 50% of the cultures ($TCID_{50}$) which can be expressed either in terms of $TCID_{50}$/ml or plaque forming units per ml (pfu /ml). sf9 cells ($1X10^5$) were seeded per well of a 96 well tissue culture plate and each row was infected with a virus of particular dilution (10^{-4} to 10^{-10}) and one row was left as uninfected control. The culture plate was incubated at 27^0C in a humidified chamber for 5 days in case of Occ+ virus and 7 days in case of Occ- viruses. After incubation each well was examined for signs of infection under a Microscope and were scored as +ve if cells in a particular well were infected. Theses scores were used to calculate $TCID_{50}$ of the virus stock. The principle behind determining titer in this way is based on study of Reed and munich. This method is widely used for most of the animal viruses. A simplified Micorsoft Excell spread sheet for calculation of titer after scoring the wells for infection described in *Baculovirus Expression Vectors: A Laboratory Manual.* O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1992) Oxford University Press, New York (O'Reilly *et al.* 1992) was also used apart from calculating manually to estimate the viral titer.

2.4.2 Estimation of viral titer by Real Time PCR:

This method is advantageous over other methods like end point dilution or Plaque assay because of the time taken for estimating the titer. This method can be used soon after producing the viral stock without waiting for 6-7 days to infect cells and score for their infection so as to estimate titer.

In this method we designed taqman probe

(AAAAGTCTACGTTCACCACGCGCCAAA) labeled with FAM at 5'end and a quencher TAMRA at the 3'end and primers

realtgp64-F-"CGGCGTGAGTATGATTCTCAAA"

realtgp64-R "ATGAGCAGACACGCAGCTTTT"

to amplify gp64 gene of the virus as mentioned in (Hitchman *et al.* 2007). The copy number of the gp64 gene was determined and converted into viral titer based on a standard curve developed by using Viral DNA of known concentration. Standard DNA of 1ng to 10^{-5}ng were used to produce a standard curve. The viral DNA was isolated using High pure viral DNA isolation kit (Roche) and 0.1ng of Viral DNA of unknown titer was used in Real time PCR reaction to estimate the Ct values which was converted into pfu/ml to get an accurate measure of viral titer (Hitchman *et al.* 2007). PCR reactions were carried out in 384 well plate in a reaction volume of 10µl each. 0.1ng (in 1µl) of template DNA (unknown samples) along with 1.25pM each of forward and reverse primers, 0.2µl of ROX dye, and 5µl of 2X Platinum Quantitative PCR SuperMix-UDG (Invitrogen), and 10pM of Taqman probe was used. Reaction conditions were 95^{0}C enzyme activation, and 40 cycles of 95^{0}C-denaturation and 60^{0}C-anneal/extension all reactions were done in triplicates.

2.4.3 Estimation of virus titer by Plaque assay:

This method was used to estimate viral titer of Wild type AcMNPV, where infection produced plaques in 5 days after infection. 60mm plates were seeded with 1 X 10^{6} cells per plate and incubated at 27^{0}C for 24 hours to allow uniform and healthily growing cells in complete medium. Virus stock was diluted to 10^{-5}, 10^{-6} and 10^{-7} and plates were infected with 0.5ml of virus stock for one hour. Each dilution of virus was infected in two plates each and two wells were not infected and used as uninfected controls. Simultaneously 5% low melting agarose was autoclaved and the melted agarose was cooled down to 60^{0}C and then diluted to 0.5% with complete medium and incubated at 42^{0}C. The medium (medium with virus) was removed completely and the medium (complete medium and 0.5% agarose) equilibrated at 42^{0}C was overlaid on infected cells without allowing cells to dry. Plates were kept in laminar air flow hood until the agarose overlay dried, and then incubated at 27^{0}C for 4-5 days until they developed good plaques which were distinct and of the size 1-3mm in diameter. These plates were stained with 2ml of Neutral Red (50µg/ml) prepared in 0.5% agarose and incubated overnight to see dark plaques clearly against a light red background. Each dilution was done in

triplicates and the number of plaques were counted and averaged. The numbers of plaques were multiplied with dilution factor (10^{-5}, 10^{-6} or 10^{-7}) and by 2 as 0.5ml of virus stock was used to infect cells which give the actual titer of virus.

2.5. Plasmids and vectors

2.5.1 Commercially availbe vectors used in this study

pBluescript-KS (stratagene), pMOS (Amersham pharmacia), pTOPO vectors , pIZT-v5-his, pFastBac-HTa, pFastBac-HTb, pFastBac-HTc, pFast Bac-dual, pAc5.1 ((invitrogen), pET-28a (Novagen), pEGFP-n3, pdsRED1-n1 (BD biosciences) etc

2.5.2 Plasmids constructed in this study:

Many plasmids vectors were constructed for our experiments described in this work including shRNA expression vectors, dsRNA expression vectors, reporter gene expression vectors, Bacterial protein expression vector, Insect gene expression vector, Transfer vectors for Recombinant Viruses. Their names and purposes are mentioned in the text at relevant places.

2.6. shRNA and dsRNA production by plasmid:

2.6.1 Plasmid mediated shRNA expression:

shRNA s can be transcribed inside a cell by cloning specific DNA fragments in an plasmid based expression cassette. Many different RNA polymerase II (RNA pol-II) or RNA polymerase III (RNA pol-III) dependent promoters can be used to transcribe shRNAs. In our study we used heat shock promoters (hsp70) to express shRNAs. Drosophila melanogaster Heat shock promoters (hsp70 promoter) is RNA polymerase II dependent promoter but can be induced by giving heat shock at 37^0C for 30 minutes. We designed siRNAs using bioinformatics tools (url: http://genomics.jp/sidirect) (Naito *et al.* 2004) and synthesized DNA oligos encoding

the siRNAs, separated by a loop region to facilitate hairpin like secondary structure formation in the transcripts. These oligonucleotides were cloned into plasmid vectors under hsp70 promoter. To express shRNAs under hsp70 promoter we cloned those oligonucleotides in plasmids "pHSPshX".

2.6.2 Plasmid mediated dsRNA expression:

dsRNA used to trigger RNAi do not trigger interferon response in insects unlike mammals so dsRNAs can also be effectively used to trigger RNAi. So we used plasmid vector pIZT-V5-His to express dsRNAs under op-ie2 promoter. Viral sequences were obtained from NCBI Entrez database and primers were designed along with suitable restriction sites at their 5' end to amplify specific regions of orf by PCR. The PCR product was cloned into plasmid vector which can transcribe dsRNAs after transfection into cells.

2.7 *In-vitro* transcription to produce dsRNAs:

2.7.1 *In-vitro* transcription:

In order to induce RNAi in insect cells dsRNAs can be effectively used. In order to target a particular gene by dsRNAs we first selected unique regions in that gene so that It does not have sequence homology to any other known genes or ORFs. We designed primers to amplify that particular region and added T7 RNA polymerase binding site towards the 5' end of those selected primers and synthesized them chemically using "Phosphoramidite method". These primers (T7 binding site tagged primers) were then used to amplify specific regions whose products will have T7 RNA polymerase binding sites at their ends. This double stranded DNA (PCR product) was then used as a template and transcribed in-vitro using T7 RNA polymerase enzyme (Megascript or Maxiscript kit, Ambion). The product was treated with DNase1 to remove traces of template. The transcribed product was then heated to 95^{0}C in a boiling water bath and allowed to cool down slowly. The

product was checked by Agarose gel electrophoresis to ensure most of the product was double stranded.

Alternatively PCR primers to amplify specific regions were tagged with different RNA polymerase binding sites like T7 RNA polymerase binding site and T3 RNA polymerase binding site at their 5' ends. The PCR product was transcribed by T7 RNA polymerase and T3 RNA polymerase enzyme in two separate tubes and product was then treated with DNase1 to remove traces of template. The transcribed product were then mixed in equi-molar ratio and heated to 95^{0}C in a boiling water bath and allowed to cool down slowly. The annealed double stranded product was confirmed by agarose gel electrophoresis.

2.7.2 *in-vitro* transcriptions to produce labeled RNA:

We used the same strategy of in-vitro transcription explained in the earlier section but replaced one of the ribo-nucleotides with a mixture of unlabelled and labeled ribo-nucleotides (Cy-3 or Cy-5 labeled). The ratio of modified nucleotide and normal nucleotide was 1:3 (1 part of Cy3 or Cy5 labeled UTP and 3 parts of unlabelled UTP).

2.7.3 Annealing of dsRNAs:

The RNA products obtained after in vitro transcription were mixed in equi-molar ratio if carried out in separate tubes or the same tube which contains both strands of transcripts were heated to 95^{0}C in a boiling water bath and allowed to cool down slowly to room temperature. The product was confirmed to be double stranded by Agarose gel electrophoresis.

2.8. Transfection of plasmid and dsRNA

2.8.1 Transfection of plasmid using Cellfectin reagent:

Sf9 cells were plated to 50% confluency in a suitable tissue culture grade plastic ware and incubated at 27^{0}C in a humidified chamber for 24 hours (as indicated in

Table 2.1). This will allow the cells to grow to approximately 70% confluency. To prepare transfection mixture we mixed required amount of DNA in grace's medium (without serum and antibiotic) and required amount of transfection reagent in grace's medium (without serum and antibiotic) in two separate tubes and incubated for 15 minutes in room temperature. Then we mixed the DNA (DNA + medium) to transfection reagent (transfection reagent + medium) and mixed thoroughly by pipetting in and out to get a 'Transfection mixture'. The mixture was incubated for about 45 minutes in room temperature. Simultaneously cells plated for transfection were washed twice using PBS to remove traces of Serum and antibiotics. The transfection mixture was then overlaid on cells and incubated for 5 hours. We found 5 hours incubation was sufficient to get reasonably good transfection efficiency and low cell toxicity. After 5 hours of incubation we replaced the transfection mixture with complete medium (supplemented with Serum and antibiotics)

Table 2.1: A table showing the number of sf9 cells and quantity of DNA used for transfection in suitable tissue culture grade plastic wares.

Size of culture dish/plate	Number of cells	Plasmid DNA In µg	Ratio of DNA to transfection reagent	Volume of transfection mixture in µl
60mm dish	5×10^7	6.000	1:3	2000
35mm dish/6 well plate	2×10^6	3.000	1:3	800
12 well plate	1×10^6	1.500	1:3	400
24 well plate	5×10^5	0.750	1:3	200
48 well plate	2.5×10^5	0.375	1:3	100
96 well plate	1.25×10^5	0.190	1:3	50

2.8.2 Transfection of dsRNA using trans-messenger reagent:

Sf9 cells were plated to 50% confluency and then incubated for 24 hours to get a confluency of 70% in a humidified chamber in a 24 well plate. At the time of transfection we first mixed 1µl of 'Enhancer R' and 'Buffer EC-R' and then added dsRNA (500ng) to the same mixture. Final volume of this mixture was 100µl. this mixture was vortexed and incubated at room temperature for 10-15 minutes. To this 100µl mixture, 4µl of trans-messenger transfection reagent was added mixed well by vortexing, and again incubated for 10-15 minutes at room temperature to allow 'transfection reagent-dsRNA' complex formation. This mixture was overlaid on cells plated in a 24 well plate and incubated for 3 hours in a humidified chamber at 27^0C. After 3 hours of incubation transfection mixture was replaced by complete medium. The ratio of dsRNA and other transfection reagents can be scaled down or scaled up depending on the number of cells.

2.9. Making of stable dsRNA expressing cells:

In order to induce stable and long lasting RNAi in cells we made stable dsRNA expressing cell lines. We amplified specific DNA fragments by PCR using primers having suitable restriction sites at their 5' termini. These fragments were cloned in both sense and antisense orientation in 'pIZT-V5-His' vector in suitable restriction sites. These two fragments were separated by an intron (2nd intron of *Drosophila melanogaster* gene *White*) cloned between sense and antisense oriented fragments. After transfection this vector can produce a transcript containing both sense and antisense sequence, separated by an intron which will be spliced out to produce two fragments of RNA of both orientation (sense and antisense). These fragments can anneal to form dsRNA in the cell and trigger RNAi. The same vector contains a selectable marker for Zeocin selection. (Figure 3.2 in Chapter 3)

2.9.1 Selection of stable dsRNA expressing lines by transfection and Zeocin selection:

Cells grown to 70-80% confluency in a 12 well plate were transfected with dsRNA expressing plasmid using Cellfectin reagent and were allowed to grow for 48 hours. These cells were then diluted 10 times and 100times and aliquoted into a 96 well plate so that each well has around 100µl. Cells were allowed to settle down and attach to the bottom of the plate for 1 day and zeocin was added at a concentration of 500µg/ml. Zeocin at this concentration can kill cells which do not express the selectable marker gene. Zeiocin containing medium was changed once in every two days for a week or two. After almost 7-14 days the 96 well plate was observed to for wells which have single colony of cells growing in them. These cells were picked and amplified to get a stable clone of sf9 cells. These cells were maintained in zeocin at this concentration for about 30 days and then zeocin was lowered to 100µg/ml.

2.9.2 Selection of stable dsRNA expressing pool of cells by transfection and Zeocin selection:

Cells were grown and transfected as described in the earlier section in 12 well or 6 well plates. After two days zeocin was added at a concentration of 500µg/ml. once in every two days medium was changed along with fresh zeocin. After repeating this for 10-15 days only cells which can express zeocin selection marker will survive as other transiently expressing cells cannot survive for these many days under the pressure of high concentration (500µg/ml) of zeocin. These cells were maintained for about 30-45 days under 500µg/ml of zeocin and then lowered to 100µg/ml to ensure only stable integrants are alive. These cells were amplified and some of them were frozen for long term storage.

2.10 Inhibitors of DNA methyl transferase. (AZdC)

5'aza-2'deoxy-cytidine also known as 4-amino-1-(2-deoxy-beta-d-erythro-pentofuranosyl)-s-triazin-2(1H)-one, or 5-azadeoxy cytidine or Decitabine is an inhibitor of DNA methyl transferases. We made a stock solution of 10mM in tissue culture grade DMSO and a final concentration of 1μM and 10 μM were used in different experiments. Stock solutions were stored at -20^{0}C

2.11 Bacterial expression of BRO gene.

2.11.1 PCR amplification of BRO gene

We designed primers to amplify complete orf of BRO gene of AcMNPV by PCR. Primers

AbroL- GGGAATTCATGGCTCGCGTTAAAATTG and

AbroR-TTGCGGCCGCTTATCCTATTTTCAAATTGTTGC

were designed along with restriction sites at their 5' termini, selected based on the multiple cloning site of vector and sequence of BRO. Viral DNA was isolated from sf9 cells infected with AcMNPV and used a s template to amplify complete orf of BRO (length of BRO is 998nts) using a proof reading DNA polymerase viz. Deep Vent DNA polymerase (NEB). We used 2.5 *p*moles of forward and reverse primers, 2.5mM dNTPs, and 0.3μl of Deep vent DNA polymerase enzyme and 1μl of 10X reaction buffer per 10μl reaction and used annealing temperature at 57^{0}C and extension at 72^{0}C for 1minute to amplify complete ORF of BRO. PCR was done using *Eppendorf Mastercycler epGradient S* instrument. The amplified product was gel eluted using *Quiaquick gel elution* columns (*quiagen*) to avoid nonspecific products.

2.11.2 Cloning of BRO gene in pET-28a vector

The amplified BRO gene and pET-28a vector were digested with restriction enzymes and checked on agarose gel to ensure complete digestion. The digested fragments were run on 1% agarose gel and gel eluted using *Quiaquick gel elution* columns (*quiagen #*). Vector and BRO amplicon were mixed in molar ratio of 1:3 and ligated at 16^{0}C for 14 hours. Ligated mixture was transformed into DH5α Ultra-Competent cells and plated onto LB-Agar plates containing kanamycin 50μg/ml. isolated colonies were selected and inoculated into liquid LB medium containing kanamycin (50μg/ml) and plasmids were isolated. Plasmids were checked on gel to see the difference in size and selected plasmids were confirmed to contain BRO amplicon by PCR Digestion & sequencing.

2.11.3 Expression of BRO gene.

The recombinant plasmid containing BRO gene was transformed into BL21 and BL21-DE3 cells. Transformed cells were grown in liquid LB with kanamycin (50μg/ml) overnight at 37^{0}C to get a primary culture. This primary culture is used as an inoculum to inoculate secondary culture (at 0.1% v/v) and allowed to grow until it reaches an O.D of 0.6 units at 600nm. Then the culture was induced with IPTG and again cultured at 37^{0}C. Similar procedure was followed to grow and induce protein expression at 30^{0}C and 18^{0}C also.

2.12 Purification of protein BRO:

After transforming and growing BL21 and BL21-DE3 cells with recombinant plasmid expressing BRO we collected Fractions of 5ml at 3,4,5 and 6 hours after induction to verify the appropriate time of expression of protein. Similar procedure was followed to collect the culture fractions grown at 30^{0}C and 18^{0}C. Cells were centrifuged at 4^{0}C and the pellet containing bacterial cells was frozen at -70^{0}C until protein was purified.

The bacterial cells collected at these time points were peletted and resuspended in 1ml of 0.1mM Phosphate buffer (pH 7.2) and sonicated to break the cells. Sonicated mixture was centrifuged at 14000g for 15 minutes at 4^0C and both pellet and supernatant were collected. Pellet was re-suspended and boiled in 1X Laemelli buffer (50 mM Tris.HCl pH 6.8, 100 mM DTT, 7% SDS, 0.1% Bromophenol blue, 10% glycerol) for 5 min. supernatant was also mixed with protein loading buffer and subjected to denaturing PAGE. Since protein was found only in the insoluble fraction (pellet) at all three temperatures we purified BRO protein from the insoluble fraction for our future experiments.

The insoluble pellet obtained after induction of expression, sonication and centrifugation at 4^0C from 50ml culture was re-suspended in 5ml of 0.1mM phosphate buffer (pH 7.2) with 6M Urea to get a clear solution. This solution containing the BRO protein along with –His tag was purified using Ni-NTA resin (Qiagen cat no # 30430). The clear solution was mixed with 1ml of Ni-NTA resin and incubated in room temperature for 1hour on a rotary shaker allowing the protein containing -His tag to bind to the Ni-NTA resin. Then the mixture was loaded onto a column with bottom cap attached and later bottom cap was removed and the unbound liquid fraction was collected for further analysis. The Ni-NTA resin in the column was then washed with phosphate buffer (0.1mM pH7.2+ 6M Urea) containing 50mM Imidazole and the flow-through was collected. Similarly the slurry (resin+protein) was washed with Phosphate buffer (0.1mM pH7.2+ 6M Urea) with increasing concentrations of Imidazole i.e., 100mM, 300mM, 500mM and 1M Imidazole, and the flow-through was collected. All the fractions were subjected to SDS-PAGE and we found 300mM Imidazole was sufficient to elute BRO protein bound to Ni-NTA resin. The purified protein was quantified by Bradford method and stored in 0.5ml aliquots at -70^0C.

2.12.1 Quantification of Protein by Bradford method

This method was described by Bradford in 1976. We made serial dilutions of BSA and the proteins samples whose concentration was to be estimated using sterile milli-Q water to a final volume of 150µl and then 50µl of 4 X-Bradford reagent

(Biorad) was added to the same to make it to 200μ. The solutions were mixed properly and incubated for 5 minutes at room temperature. Then absorbance of each sample was recorded by an Elisa reader at a wavelength of 595nm. Absorbance of BSA sample with known concentrations were used to plot a standard curve and then quantities of unknown protein samples were calculated based on the standard curve.

2.13 Raising polyclonal antibodies against BRO.

We used the purified BRO protein containing a -His tag to generate polyclonal antibodies. Antibodies were raised in 3 different ways i.e. in Rabbits, in Mice and in Mice Ascites. Animals (rabbits and mice) were obtained from the Animal house facility of CCMB. After selecting animals, we collected serum (pre-immune sera) before injecting them with proteins.

2.13.1 Raising polyclonal antibodies against BRO in rabbits

Approximately 300μg of purified protein was re-suspended in 500μl of buffer (0.mM phosphate buffer with 6M urea) and mixed with 500μl of Freund's complete adjuvant. The mixture was vortexed and sonicated until it forms an emulsion. This mixture was injected to Rabbits subcutaneously at two different sites. First booster doze was given to these rabbits after 3 weeks with 300μg of protein emulsified with Freund's incomplete adjuvant. Similarly a 2nd booster doze was also given after 2 weeks. After 10 days of 2nd booster animals were bled from the ear marginal vein and checked for presence of antibodies by dot blot taking pre-immune sera as a negative control. Animals were given booster dozes once in a month and were bled to collect 10ml of blood after a week of administering the booster dozes.

2.13.2 Raising polyclonal antibodies against BRO in mice

Mice were injected with 200μg of protein (re-suspended in 150μl of phosphate Buffer (pH7.2) with 6M Urea and emulsified with equal volume of Freund's complete adjuvant). First and second booster dozes were given after 3^{d} and 5th week. A test

bleeding was done in 6th week to check for presence of antibodies. Booster dozes were repeated in 7th and 9th week and animals were bled after 1 week of every booster doze after 7th week.

2.13.3 Raising polyclonal antibodies against BRO in Mice Ascites

Pristane was injected into mice intra-peritoneally to induce ascites and then animals were injected with 200µg of protein (re-suspended in 150µl of phosphate Buffer (pH7.2) with 6M Urea and emulsified with equal volume of Freund's complete adjuvant) booster schedule were given as mentioned in the earlier section and ascetic fluid was collected after a week of every booster dose.

2.14. Isolation of Total RNA

2.14.1 Isolation of RNA

Total RNA from cells was isolated by using Trizol reagent (Invitrogen #). Cells were harvested from the culture flask or dish to which approximately 1ml of Trizol was added per 1X 10^7 cells and vortexed for 40-50 seconds. 200µl of Chloroform was added to every 1ml of Trizol used (ratio 1:5) and mixed by vigorous shaking in hands for 20 seconds and kept for 5 minutes at room temperature to allow phase separation. The upper aqueous layer was collected and RNA was precipitated by adding equal amount of *iso*-Propanol and then by washing with 75%ethanol at 4^0C. RNA pellet was air dried and dissolved in RNase free water.

2.14.2 RNA clean up by RNeasy columns

Isolated total RNA was purified by using RNeasy columns of Qiagen. The purified RNA was re-suspended in 100µl of RNase free water and 350µl of RLT buffer and 250 µ l of absolute ethanol was added and mixed. The mixture was loaded onto a RNeasy column, centrifuged and the flow through was discarded. The column was washed with Buffer RPE twice and flow-through was discarded. RNA was eluted out of the column by 50µl of RNase free water in fresh 1.5ml collection tubes.

2.15. Polymerase chain reaction (PCR)

PCR was used as a general strategy to amplify specific fragments of DNA. We used both Proof reading enzymes like 'Deep Vent DNA polymerase (NEB), '*Pfu*-Turbo' (stratagene) and non proof reading enzyme like *Taq*-polymerase to amplify specific DNA fragments depending on the requirements as mentioned in relevant sections.

2.15.1 PCR using Taq DNA Polymerase

We performed PCR using Taq DNA polymerase using in house purified enzyme as well as commercially available enzymes like 'Ampli-Taq Gold' (perkin elmer). We used 1µl of 10X reaction buffer and 2,5mM dNTP mix, 150mM $MgCl_2$, 1.25pM each of both forward and reverse primers and 3 units of Taq DNA polymerase per every 10µl of reaction. (perkin elmer buffer pack). PCR was performed in a thermal cycler at Denaturing temperatures of 95^0C and extension temperature of 72^0C along (1minute per 1kb of expected product) with suitable annealing temperature depending on the primers used. a final extension of 10 minutes was done at 72^0C at the end of every reaction and product was stored at -20^0C

2.15.2 PCR using proof reading DNA polymerases

We used 'Deep Vent DNA Polymerase' and 'pfu-Turbo DNA Polymerase' for amplifying specific DNA fragments wherever errors during polymerization were to be avoided. Such cases are specially mentioned in relevant sections.

We used 1µl of 10X reaction buffer, 2.5mM dNTP mix, 2.5pM each of forward and reverse primers and 2units of enzymes per every 10µl reaction. PCR was performed in a Thermal cycler using denaturing at 95^0C extension at 72^0C (1minute per 1kb of expected product) and suitable annealing temperature depending on the primers used. A final extension was done at 72^0C at the end of every reaction. The reaction mixture was purified by phenol chloroform or purified using Qiagen PCR

purification kits to get rid of thermo-stable DNA polymerase enzyme and product was stored at -20^{0}C

2.16. Reverse transcription PCR

2.16.1 DNase treatment of RNA

Total RNA isolated by Trizol or any RNA isolation kits usually are contaminated with genomic DNA so DNase treatment was given to remove these contaminants before going for further experiments with this RNA. We used 1µl of Turbo DNA free (ambion) per 5µg of Total RNA isolated (although the DNA concentration varies with different isolations of RNA, this concentration seems to sufficient to remove any traces of DNA from the RNA isolated by us) and incubated at 37^{0}C for 30 minutes to 1 hour in a reaction volume of 20-50µl in presence of suitable amount of buffer. Then 1/10th volume or 5µl of DNase inactivation reagent (whichever was more) was added to the reaction mixture and incubated for 5 minutes at room temperature. The reaction mixture was centrifuged at 14000g and clear supernatant which contains DNA-free RNA was collected and taken for reverse transcription.

2.16.2 Reverse transcription of RNA

We used Reverse transcription PCR for semi-quantitative estimation of transcribed genes and also to clone cDNAs of few genes. We used IMPROM II (promega) and Superscript II (invitrogen) reverse transcriptase kits for this purpose. For RT-PCR 100ng to 1µg of total RNA was digested with DNase-1 (ambion) to remove DNA from total RNA and mixed with 1µl of 10mM dNTPs and 250ng of Oligo-dT or 25ng of random hexamer depending on the transcripts to be analyzed in a 10 µl reaction volume. The sample was incubated at 70°C for 5 minutes, quick chilled on ice for 2 minutes and then following components were added to the reaction: 2µl of 10 X RT buffer, 4µl of 25 mM MgCl2, 2µl of 0.1 M DTT and 1 µl of RNase Inhibitor. Subsequently the reaction tube was incubated at 42°C for 2 minutes and 1 µl of Reverse Transcriptase was added to the reaction followed by incubation at 42°C for

90 minutes and inactivation of enzyme at 70°C for 15 minutes. The resulting first strand cDNA was diluted 5 times and 1-2 µl was used as template for subsequent PCR reactions.

2.17 Cloning of DNA fragments into plasmids

2.17.1 Restriction endonuclease digestion of DNA:

Plasmid DNA isolated by Boiling method or Alkali Lysis method were purified using Phenol:Chloroform method and used for digestion using Restriction enzymes. Plasmids isolated by Qiagen kits were good enough to be digested without further purification. We used approximately 5 units of restriction enzymes per 2µg of plasmid DNA in a suitable buffer and incubated at recommended temperatures in a reaction volume of 20µl. Double digestion of DNA was carried out in compatible buffers as recommended by the manufacturers. Digested product was confirmed by electrophoresis on agarose gel for complete digestion and then the residual active Restriction Enzyme was inactivated either by heat inactivation (if recommended) or by Phenol:Chloroform extraction or by gel elution.

2.17.2 Ligation of DNA (vector and insert)

For cloning of DNA fragments into plasmid vectors we mixed digested vector (~50ng) and inserts in a molar ratio of 1:3and added T4-DNA Ligase (200units) in suitable amount of reaction buffer in a total reaction mixture of 10µl and incubated at 16^0C for 14hrs. 2 to 3µl of ligated mixture was transformed into Ultra-Competent cells or stored at -20^0C.

2.17.3 Preparation of ultra-competent cells

Ultra competent cells prepared from E.coli DH5α and DH10β were used to transform plasmids or ligation mixture while cloning DNA fragments into plasmids. Ultra-competent cells were prepared according to the protocol described by Inoue *et al* (Inoue *et al.* 1990). A single colony of Bacteria was inoculated in 10ml of LB and

incubated at 37^0C overnight to get a primary culture. Then this primary culture was used to inoculate (1%) fresh LB medium and incubated at 18^0C until it reached an OD of 0.6 at a wavelength of 600nm. As soon as it reached OD of 0.6 culture was kept on ice for 15 minutes and centrifuged to collect bacterial pellet at 3000g at 4^0C. The pellet was re-suspended 32ml of in ice cold Inoue buffer (10 mM PIPES pH 6.7, 15 mM CaCl2, 250 mM KCl, 55 mM MnCl2) and incubated on ice for 15minutes and centrifuged again to pellet bacterial cells. This step was repeated once again and finally bacteria were re-suspended in 8ml of Inoue buffer. They were aliquoted 100µl each in 1.5 ml tubes, snap frozen in liquid nitrogen and stored at -70^0C.

2.17.4 Transformation of bacterial cells (ultra-competent cells):

2-3µl of ligation mixture or 1ng of plasmid DNA was used in transforming ultra-competent cells. A vial of ultra-competent cells was thawed on ice and the DNA to be transformed was added and gently tapped to mix it. These cells were incubated on ice for 20 minutes and heat shock was given at 42^0C for 90-120 seconds in a water bath and immediately transferred to ice for 5 minutes. 900µl of LB medium was added to these cells and incubated at 37^0C for an hour. Then cells were plated on a LB-Agar plate containing suitable antibiotic and incubated for 12-14 hours at 37^0C to get isolated colonies. These colonies were then streaked on a fresh LB-Agar plate and analyzed further.

2.17.5 Screening for recombinant Plasmids

Recombinant plasmids were screened and confirmed different methods like colony PCR, isolation of plamsids and comparing the size difference on Agarose gel, PCR amplification of insert from plasmid DNA and Restriction digestion of isolated plasmids.

2.17.6 Colony PCR:

Screening of recombinant clones were done by colony PCR. An isolated colony of bacteria was taken in a 1.5ml tube containing 50µl of sterile water and mixed well

by vortexing for 30 seconds. The resuspended bacteria were boiled for 5 minutes to lyse the cells and denature DNases. Boiled mixture was centrifuged at 14000g for 1 minute and supernatant was collected carefully avoiding the pellet. 5microliter out of this supernatant was used in PCR reaction as template to amplify the insert using either insert specific primers or standard primers present in the vector itself. The product was confirmed on agarose gel for presence or absence of required band and the size of the amplified product.

2.17.7 Isolation of plasmid and PCR using Plasmid as template:

Plasmids were isolated using protocols as described in the section 2.18 and used as a template in PCR reaction. Recombinants were confirmed by electrophoresing on agarose gel of the amplified product.

2.17.8 Isolation of plasmid and Restriction digestion:

Plasmids were isolated as described in section 2.18 and Restriction enzyme digestion of Plasmids were carried out as described in section 2.17.1 and confirmed on agarose gel by electrophoresis

2.18 Isolation of plasmid:

Plasmids were isolated using different methods according to the requirement of downstream applications. Plasmids for screening Clones by PCR were isolated by Alkali lyses or Boiling method. Plasmid for restriction digestion and sequencing plasmids were isolated by Alkali lyses method or Boiling method fallowed by Phenol: Chloroform extraction. For transfection of insect cells plasmids were isolated using Qiagen plasmid purification kits. All three methods are explained in this section.

2.18.1 Isolation of plasmid: by Alkali lyses method.

The method fallowed to isolate high copy number plasmids from 3ml of bacterial culture is explained here. 3ml bacterial culture was centrifuged 5000g for 2minutes

to collect bacterial pellet and the pellet is resuspended in 100µl GTE solution or also called solution-1 (containing 50 mM glucose+25 mM Tris,Cl, pH 8.0+10 mM EDTA Autoclave and store at 4°C) and 200µl of solution-2 (0.2 N NaOH 1% (wt/vol) sodium do-decyl sulfate (SDS) Prepared immediately before use) was added and mixed immediately by inverting it 4-5 times and kept on ice for 5 minutes. 150µl of Ice cold solution-3 (3M potassium acetate pH-5.2) was added to the same and mixed by inverting 4-5 times and kept on ice for 5 minutes. The mixture (lysate) was then centrifuged at 14000g for 15minutes and supernatant was collected. Plasmid DNA present in the supernatant was precipitated by adding equal volume of *iso*-propanol and centrifuging at 14000g for 20 minutes and washing the DNA pellet with 70% ethanol. DNA pellet was dried and resuspended in suitable amount (50µl) of water or 10mM Tris.Cl pH-8.0.

2.18.2 Isolation of Plasmid by Boiling method:

This method was followed to isolate plasmid from 2ml of bacterial culture. The volumes of each solution can be scaled up or scaled down according to the volume of bacterial culture upto 20ml.

2ml of bacterial culture was centrifuged at 5000g for 2 minutes and bacterial pellet was collected. Pellet was resuspended in 500µl of STET buffer (8% (wt/vol) sucrose, 5% (wt/vol) Triton X-100, 50 mM EDTA, 50 mM Tris,Cl, pH 8.0 Filter sterilize and store at 4°C) and 50µl of Lysozyme (10mg/ml) was added and vortexed to mix it well. The whole mixture was boiled at 1000C for 40sec to 1 minute and transferred to ice immediately for 5 minutes. The lysate was centrifuged at 14000g for 15 minutes and supernatant was collected. Plasmid DNA was precipitated by adding 50µl of 3M Sodium acetate ph-5.2 and 500µl of *iso*-propanol and centrifuging at 14000g for 15 minutes. The DNA pellet was washed with 70% ethanol, air dried and resuspended in required amount of sterile milli-Q water or 10mM Tris,Cl pH-8.

2.18.3 Isolation of plasmid by Qiagen plasmid purification system:

For transfection of insect cells with plasmids, Plasmids were isolated using Qiagen's plasmid purification kits (catalogue number 12143) using the protocol recommended by the manufacturer.

2.19 Phenol:Chloroform extranction of nucleic acids.

Nucleic acids (DNA or RNA) were purified to get rid of contaminants like protein, carbohydrates and other endotoxin contaminants by phenol chloroform extraction method.

DNA was diluted to a moderate DNA concentration (more than 10ng/µl) to have a decent volume because in every step of this method some Aqueous phase will be lost which indirectly is losing the DNA itself. The DNA was added with equal volume of Phenol:Chloroform:iso-amyl alchohol mixture in the ratio of (v:v) 25:24:1 (phenol saturated with 0.05M Tris pH-8), vortexed for 20 seconds mix them properly. The mixture was kept at room temperature for 5minutes to allow phase separation and then centrifuged at 12000g for 10 minutes to separate the phases clearly. Aqueous phase was transferred to new tube and mixed with Chloroform:iso-amyl alcohol (ratio v:v::24:1) vortexed and again kept at room temperature for 5 minutes. The mixture was centrifuged again to separate the phases and aqueous phase was carefully transferred to a new tube. DNA was precipitated by adding 1/10 volume of 3M sodium acetate pH5.2 and equal amount (equal to aqueous phase) of iso-propanol and centrifuging at 14000g for 20 minutes in room temperature fallowed by a washing step with 70% ethanol. DNA pellet was air dried and dissolved in required amount of water or Buffer.

2.20 Protein methods:

2.20.1 Denaturing (SDS) discontinuous gel electrophoresis: (Laemmli method):

Denaturing discontinuous poly-acrylamide gels were used to resolve proteins either purified or from the total lysate of cells. This method is described by laemmli 1970 and the protocol followed in this work was adapted from protocols in the books "Current protocols in Molecular Biology" and "Molecular cloning" stacking gel 5% and resolving gel 10% were generally used in 1.5mm thick gel. Protein samples were boiled in loading buffer or laemmli buffer (50 mM Tris.HCl pH 6.8, 100 mM DTT, 7% SDS, 0.1% bromophenol blue, 10% glycerol) for 5 minutes and loaded onto a SDS-Polyacrylamide gel and electrophoresed at 30mA till the proteins cross the stacking gel and enter into resolving gel, and at 40mA thereafter. Depending on the proteins size which has to be observed gel was run for required length of time.

After the run the gel was removed from the glass plates and stained with coomassie brilliant blue stain (0.025% in Acetic-acid:Water:Methanol::V:V:V::10:40:40) for 2-3 hours and destained in destaining solution (Acteic acid:Methanol:Water::10:40:40) by changing it several times until protein bands were clear against a clear background.

In cases where protein was to be transferred onto membranes coomassie staining was avoided but proteins were stained after transferring to membrane using Ponceu-S

2.20.2 Transfer of Proteins to membranes:

Proteins resolved by SDS-PAGE were transferred to a membrane for downstream applications like western blotting. Two methods were used for transferring proteins onto membranes 1.Wet transfer and 2. Dry transfer methods both are explained bellow

2.20.2.1 Wet transfer method:

The gel containing the protein was assembled with the transfer membrane (hybond-P) which was wetted in methanol and equilibrated with transfer buffer in a wet transfer apparatus cassette. The order of assembly was "Sponge - three Whatmann papers – Gel – Membrane - three Whatmann papers-Sponge" all the above mentioned materials like whatmann paper, gel, sponge were equilibrated in transfer buffer (39mM Glycine, 48mM Tris.Cl, 0.037% (w/v) SDS & 20% (v/v) methanol) the cassette was assembled and kept In the transfer apparatus so that gel is towards cathode. Transfer was performed by applying 100V for 1hour under cold conditions. The membranes were dried and stored for future use.

2.20.2.2 Semi-Dry transfer method:

In this method the whatmann papers were equilibrated with transfer buffer and kept on the anode of transfer apparatus. They were cut exactly to the size of the gel. Then the membrane was kept on the whatmann papers fallowed by gel and three more wahtmann papers. Membrane was wetted in methanol and then equilibrated with transfer buffer. A glass rod was rolled on them to remove air bubbles and cathode was kept on the assembly. Proteins were transferred at $0.8mA/cm^2$ for 1 hour. The transferred membranes were dried and stored for future use

2.20.3 Ponceu-S staining of membranes:

Proteins transferred on to the membrane were stained using Ponceu-S stain (0.5gm dissolved in 1ml acetic acid and then diluted to 100ml using water) for 10 *minutes* and destained in water for few minutes with shaking until band were clearly visible against a clear background. This stain was completely destained with water after recording the picture to have an estimate of protein loaded in each well.

2.20.4 Immunodetection of Proteins:

The membrane with proteins transferred on to them were wetted in methanol and equilibrated in TBST buffer (TBST buffer prepared using 12.1 g Trizma-base 40.0 g Sodium chloride Diluted to 5000 ml with distilled water and pH adjusted to 7.6 with

HCl and tween-20 was added to a final concentration 0.1% v/v). Membrane was first blocked with TBST buffer containing 5% non-fat dried milk or Blotto (Santa cruz) for 2hours at room temperature with constant stirring. Membrane was incubated with primary antibody suspended in blocking solution in appropriate dilution at 4^0C for 8-10hours or for 1hour in room temperature with constant rotation. The membrane was washed 2-3 times with TBST buffer and incubated with secondary antibody in appropriate dilution for 1hour in room temperature with constant shaking or rotation. The membrane was again washed with TBST buffer 3-4 times and taken further for detection of antibodies with suitable detection methods. Blots were detected by chemiluminescent methods by using SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to manufacturer's instructions. Luminal and the peroxide reagents were mixed in equal volume and added to the blot (125µl mixture per cm^2 of membrane) and exposed to X-ray film at different time points depending on the intensity of the signal and developed using developer and fixing solutions.

2.21. Silkworm methods:

2.21.1 Silk Worm strains:

We used three strains of *Bombyx mori* strains for our study, they Pure Mysore-5, CSR-2 and APS-8 they were kindly provided by Andhra Pradesh state sericulture department.

2.21.2 Rearing of silkworms:

B.mori eggs which were stored in cold storage were obtained and treated with bleach 0.1% sodium hypochlorite and rinsed with water to remove traces of hypo solution. The eggs were incubated in a humidified chamber until they hatch at 27^0C which took around 8-9 days. Worms were checked twice a day and were transferred onto finely sliced mulberry leaves (*Morus alba .L.*). Worms were fed with fresh leaves thrice a day until they stop feeding and start spinning.

2.21.3 Infection of *B.mori* larvae by injection:

Larvae of 4th or 5th instar stage were used for injecting Budded viruses. Worms were etherized to keep them still and Hamilton syringes were used inject BV stock of required titer in 10µl. Injection was done by injecting the worms longitudinally into the third abdominal segment and the needle was poked inside almost for three segments and virus was released.

Alternatively worms were kept at 4^{0}C until they were still instead of etherizing them and injected with virus stock. Injected worms were again fed with leaves and photographed or cultured for future experiments.

2.21.4 Genomic DNA isolation of *B.mori*:

The larvae of 2nd instar stage were generally used for this purpose but sometimes 4th and 5th instar larvae were also used to isolate genomic DNA. when 2nd instar larva has to be used then we used one larva but when 4th and 5th instar stage larvae have to be used then we used a segment (3^{d} to $6^{th)}$ of the worm avoiding the gut region weighing about 50mg. the tissues were frozen n liquid nitrogen and ground into powder by adding liquid nitrogen again and again. The fine powder of worms were resuspended in 200µl of Buffer A (100mM TrisCl pH 7.5, 100mM EDTA, 100mM NaCl & 0.5% (w/v) SDS) and homogenized in a 1.5ml tube until only cuticle remain and rest of the solution appears clear. Another 200µl of Buffer A and mixed well and incubated at 65^{0}C for 30 minutes. 800µl of LiCl-K.Acetate solution (1part 5M K.Acetate, 2.5 parts 6M LiCl) was added to the above mixture and mixed well by inverting the tube several times and kept on ice for 10 minutes. The mixture was then centrifuged at 14000g for 15 minutes in room temperature and supernatant was collected. 600µl of *iso*-propanol was added to this supernatant and centrifuged at14000g for 15 minutes to precipitate genomic DNA. The DNA pellet was washed with 70% Ethanol, air dried and re-dissolved in 100µl of Water or 10mM Tris.Cl buffer pH-8. In some cases RNase treatment was given and DNA was extracted with Phenol:Chloroform to get rid of RNA.

2.22. Flow Cytometric analysis of DNA content in cells. (cell cycle analysis)

1-2 X 106 Sf9 Cells were taken and washed with cold PBS to remove trances of medium. Cells were re-suspended in 200 µl of cold PBS and added drop by drop to cold 70% ethanol to avoid aggregation of cells in ethanol. These cells were kept overnight in -20^{0}C to fix and permiabilise cells. These cells were stored at 4^{0}C until they were used for Flow cytometric analysis. On the day of flow cytometric analysis cells were centrifuged and re-suspended in propidium iodide staining solution (PI staining master mix- PI 40 µg/ml, RNase 10 µg/ml, PBS 950 µl and 70% ethanol 1ml) and incubated at 37^{0}C for 30 minutes just before analysis. A final concentration of 0.5 X 10^{6} cells/ml was maintained and used for Flow cytometric analysis of DNA content of cells. Cells were infected with recombinant baculoviruses either RFP-recombinant virus or EGFP-recombinant viruses so first cells expressing either RFP or EGFP were counted first and cell cycle analysis was done by analyzing the DNA content in cells using FACS caliber instrument.

2.23 Microscopy: several methods were used for imaging worms and cells. They are indicated in the text with appropriate details. Here we are mentioning different methods used for microscopic imaging.

2.23.1 Imaging of silkworms and complete tissue culture dish containing cells infected with recombinant viruses.

Silkworms injected with EGFP or RFP recombinant viruses were photographed using a leica sterio microscope with appropriate filters for EGFP and RFP. Least magnification was used in order to cover maximum part of the silkworms.

Similarly Cells were infected in tissue culture dishes and infected with recombinant viruses were also imaged using the same microscope.

Images were taken under same exposure and magnification using leicaASD-3D microscope with camera (IC 3D 134881106) (Image capture settings: 16 bit/channel, 3132 X 2325 full frame HQ , colour). Captured images were converted into grey scale images using a scale of '0' to '255' (0-black & 255-white). The pixel

intensities were calculated using software "LAS AF Version; 1.8.1 build 1390" (Leica microsystems CMS Gmbh). This method was used only in relative quantification as this is not an accurate method of quantification of fluorescence from microscopic images (Baghdoyan *et al.* 2004; Budovskaya *et al.* 2008).

2.23.2 Imaging of sf9 cells using live cell imaging system:

Sf9/BmN cells infected with viruses and/or transfected were imaged under live cells imaging system Axiovert 1200 microscope of Zeiss. Incubation conditions of 27^0C and appropriate excitation filters were used in order to capture fluorescence and phase contrast images were also taken using appropriate configurations.

2.23.3 Imaging by confocal microscopes:

Sf9/BmN cells infected and /or transfected were imaged under confocal microscopes. Live cell confocal microscope "LSM5 live' (Zeiss) was used to capture images of sf9 cells in live condition incubated at 27^0C

Chapter-3:

RNAi can suppress viral genes in AcMNPV infected permissive and non-permissive hosts.

Chapter 3: RNAi can suppress viral genes in AcMNPV infected permissive and non-permissive hosts.

3.1 Introduction:

RNAi is triggered by dsRNAs resulting in specific degradation of their complementary transcripts. For experimental purpose, dsRNAs can be introduced into cells by various strategies that can be selected depending on various factors like, cell type in which silencing has to be performed, genes to be targeted and efficiency of RNAi expected etc.,

The potential of RNAi is used for therapeutic applications also. Many efforts have been made to target diseases caused by over expression of genes and a wide range of viral diseases. Selecting a genuine target, choosing appropriate strategy to instigate RNAi and choosing suitable delivery methods have been major challenges in designing an RNAi based drug for such diseases. Even for experimental purpose these are the major issues to be addressed. In order to achieve the best possible levels of silencing, various strategies have to be tried and the strategy showing the best efficiency is used.

RNAi can be triggered by introducing siRNAs and/or dsRNAs (**Figure 3.1**). siRNAs or dsRNAs can be synthesized in vitro and these naked dsRNAs are introduced into cells. Alternatively siRNA and dsRNA encoding DNA fragments can be cloned in plasmid based vectors capable of transcribing the cloned fragments to produce siRNA or dsRNAs.DNA oligonucleotides encoding siRNAs can be cloned in plasmid based vectors separated by a loop sequence, so that a hairpin like transcript is produced and processed into siRNAs by endogenous RNAi machinery. DNA fragments encoding dsRNAs can also be cloned in plasmid based vectors separated by a loop or by an intron. These fragments produce dsRNAs after

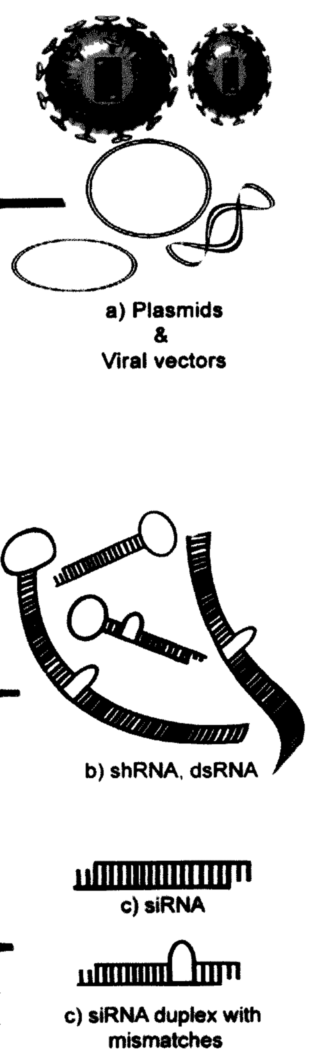


Figure 3.1: Classical miRNA and siRNA pathways & various methods to induce RNAi.

a) RNAi can be induced by using Viral or Plasmid based vectors which enter Nucleus and get transcribed to produce dsRNAs/shRNAs which are precursors of small RNAs.

b) dsRNAs (longer than siRNAs) and shRNA can be transfected into cells. They are processed by Dicer and subsequently get loaded into RISC in the cytoplasm.

c) siRNAs with or without chemcial modifications in the backbone and/or bases, can be transfected into cells. They get loaded into RISC directly and execute their functions.

transcription that can be processed into siRNAs by RNAi machinery of the host cell. One can choose the promoter under which shRNAs/dsRNAs are to be expressed. Choosing an appropriate promoter provides additional advantages in achieving specific temporal and spatial expression and control over quantity of siRNAs produced depending on the strength of the promoter. Viral vectors can also be used to deliver shRNA/dsRNAs. Adenovirus, Adeno-Associated viral vectors, Lentiviral vectors, Herpes virus based vectors, Geminiviruses etc have been used for this purpose. There are reports showing use of baculoviruses as vectors to deliver dsRNAs in mammalian cells. The major advantage of baculoviruses is that they do not show any pathological symptoms in mammalian cells as they cannot replicate in them, but they can enter into these cells and deliver the DNA encoding shRNA/dsRNA.

A major interest of researchers working on RNAi for therapeutic applications is to target viral diseases. Several viruses are successfully inhibited in *in vitro* experiments and in experimental model systems but in vivo trials have met only limited success. With increasing knowledge of RNAi mechanism and discovery of improvised methods of delivery, we have a number of choices to deliver RNAi *in vivo*. However the key factor is to narrow down to most suitable delivery method and most susceptible targets. Our major interest was to suppress Baculovirus (viral genes) in insect cells. So we wanted to study whether RNAi can be used against baculoviruses and if it can be used, which method of delivery will be most suitable.

In this chapter we describe different strategies used to knock down viral genes like naked dsRNAs, transient vector mediated expression of shRNA & dsRNA and stable vector mediated expression of dsRNAs. We have compared the efficiency of all these strategies to knock down genes. Our results show that dsRNAs were more efficient in knocking down viral genes than shRNAs and stable expression of these dsRNAs was better than transient expression of same dsRNAs. We also used baculoviruses to deliver dsRNAs against a reporter gene and found, they can also induce RNAi.

We also performed in vivo RNAi in *Bombyx mori* larvae by injecting dsRNAs and also by delivering dsRNAs using recombinant AcMNPV. We found both these strategies are capable of knocking down genes.

3.2 Materials and methods:

3.2.1 Cells and medium: sf9 cells and BmN cells were used in transient knock down experiments. Both cell lines were cultured in TNM-FH medium supplemented with 10% FBS at 27^{0}C.

3.2.2 Silkworm Strains: *Bombyx mori* strains Pure Mysore (PM), CSR2, APS were used in this study. They were obtained from Andhra Pradesh State Sericulture Department farms. They were reared as described in Section 2.21 earlier.

3.2.3 Designing siRNAs and shRNA encoding DNA oligonucleotides: sequence of AcMNPV genome and orfs were collected from NCBI entrez database. (AcNPV genome accession number NC_001623), sequence of AcMNPV orfs *gp64* (**GeneID:** 1403961) *ie-1* (**GeneID:** 1403980), *p35* (**GeneID:** 1403968). Based on these sequence information siRNA was designed using web based tools (url: http://www.ambion.com/techlib/misc/siRNA_finder.html & http://design.RNAi.jp/). siRNAs whose sequences did not show significant similarity to any sequence of *Drosophila melanogaster* and *Bombyx mori* were selected. DNA oligos corresponding to the selected siRNAs separated by a 7 bp loop sequence were chemically synthesized. Each strand of DNA oligo consisted of sense and anti-sense Strand of siRNA separated by a 7-bp loop region (5'-siRNA-loop-anti sense siRNA-3'), other DNA oligonucleotide was complementary to the former (5'-anti sense siRNA-loop-sense siRNA). On either side of these oligonucleotides suitable restriction sites were added. For each gene two shRNAs were designed.

3.2.4 Cloning of shRNA encoding DNA oligonucleotides in pHSPshX plasmid vector: Two sets of both sense and antisense strands were synthesized

along with compatible restriction sites on either sides. Sense and antisense oligos corresponding to *p35* and *gp64* genes were annealed and cloned under *Heat shock protein-70 (hsp70)* promoter to generate plasmid vectors pHSPsh35, and pHSPsh64 respectively(**Figure 3.2A**).

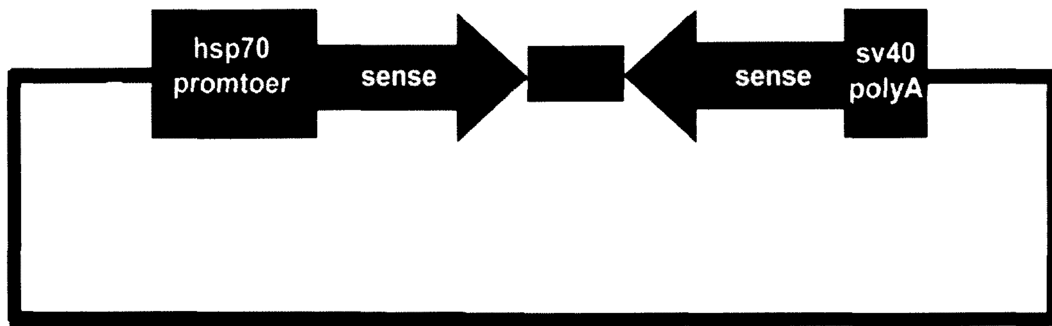
3.2.5 Construction of plasmid vector to produce dsRNAs: PCR primers were designed to amplify unique region of AcMNPV orfs *gp64, p35, p143* and *rfp* and *egfp* genes. Restrictions sites were added to the 5' end of these primers so that they can be cloned in both sense and antisense orientation in vector *pIZT-V5-His* (invitrogen).

In order to construct a vector for dsRNA expression against p35 **pZdsP35,** gene specific fragments of p35 were amplified by PCR using primers along with suitable restriction sites listed in **Table 3.1** each fragment was amplified using two sets of primers one for a fragment in sense orientation and the other for antisense orientation. Both PCR products were cloned in pMOS blue blunt end cloning vector kit and confirmed by sequencing. The cloned fragments were digested out by using Sac1 & Spe1 and Not1 & Xba1 for fragments of sense and antisense orientation and cloned under respective restriction sites in pIZT-V5 His vector. Sequence of 2nd intron of *Drosophila white* gene was obtained from Flybase (flybase id FBgn0003996), both strands of whole intron were chemically synthesized along with Spe1 and Not1 restriction sites at their 5' and 3'ends of the intron sequence respectively.

(sequence of 2nd intron of white gene: Sense strand-
CTAGTGTGAGTTTCTATTCGCAGTCGGCTGATCTGTGTGAAATCTTAATAAAGG
GTCCAATTACCAATTTGAAACTCAGGC, and antiSense strand-
GGCCGCCTGAGTTTCAAATTGGTAATTGGACCCTTTATTAAGATTTCACACAGA
TCAGCCGACTGCGAATAGAAACTCACA)

Synthesized oligonucleotides were annealed and cloned between the sense and antisense oriented fragments of *p35* gene under Spe1 and Not1 restriction sites. **(Figure 3.2B)**

A.



B

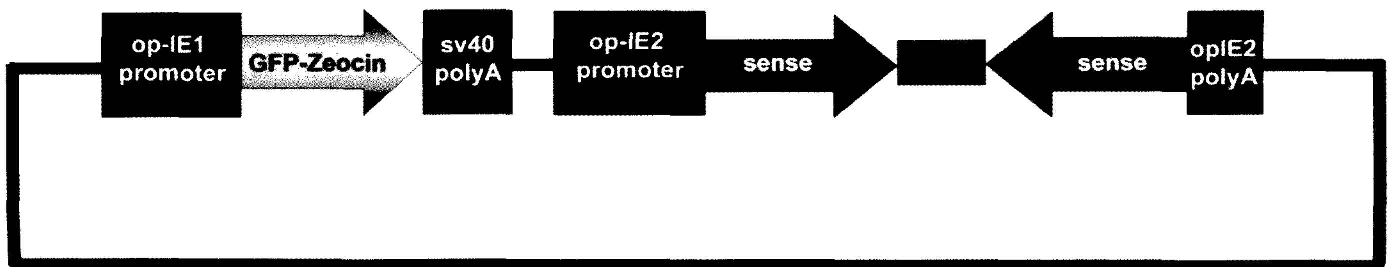


Figure 3.2: A genral map of plasmid vectors constructed for delivering shRNA and dsRNA

A: Map of plasmid vector encoding shRNAs: Figure shows a generalized map of plasmid vector constructed for expressing shRNAs under Drosophila hsp70 promoter. DNA oligonucleotides containing siRNA sequences in both sense and antisense orientation separated by a loop sequence were chemically synthesized, annealed and cloned in plasmid vector under hsp70 promoter.

B:Map of plasmid vector constructed for expressing dsRNAs: Figure shows a generalized map of Plasmid vector constructed for expressing dsRNA under a viral Op-IE2 promoter of OpMNPV in pIZT-V5-His vector (invitrogen). Specific fragments from EGFP, RFP and selected viral genes (*p35, gp64, ie-1*) were amplified by PCR and cloned into pIZT-V5-His vector in apposite orientation. 2nd intron of Drosophila White gene was inserted between these sense and antisense oriented fragments. Finally vectors which express dsRNAs against *p35, ie1, gp64, egfp* and *rfp* were named pZdsP35, pZdsIE-1, pZdsGP64, pZdsEGFP and pZdsRFP respectively.

Similar strategy explained for **pZdsP35** vector was used to construct plasmid vectors **pZdsIE1, pZdsGP64, pZdsEGFP and pZdsRFP** by cloning dsRNA encoding fragments corresponding to ie-1 and gp64 genes of AcMNPV and enhanced green fluorescent protein (EGFP) and Red fluorescent protein (RFP) under same restriction sites. (**Figure 3.2B**) (for primer sequences see **Table 3.1**)

<u>Table 3.1:</u> List of primers used to amplify specific fragments of viral orfs in both sense and antisense orientation.Target genes and the orientation of the fragments after cloning into the dsRNA expression vectors are indicated in 3d and 4th columns.

Name of primer	Sequence	Target Gene	Orientation of fragment in plasmid
acp35sl	GGGAGCTCAACCCGTTCTCATGATGTTT	*p35*	sense
acp35sr	GGACTAGTCAAACTTGCTTTTCAACACG	*p35*	sense
acp35asl	GGTCTAGAAACCCGTTCTCATGATGTTT	*p35*	antisense
acp35asr	AAGCGGCCGCCAAACTTGCTTTTCAACACG	*p35*	antisense
acgp64sl	GGGAGCTCATCTACAAAGAAGGGCGTTG	*gp64*	sense
acgp64sr	GGACTAGTATGGTGGTTATGAGGTTGCT	*gp64*	sense
acgp64asl	GGTCTAGAATCTACAAAGAAGGGCGTTG	*gp64*	antisense
acgp64asr	AAGCGGCCGCATGGTGGTTATGAGGTTGCT	*gp64*	antisense
acie1sl	GGGAGCTCGCTTATCATGCGGAATC	*ie-1*	sense
acie1sr	GGACTAGTGCAAATGTTCGTGTTGTGAT	*ie-1*	sense
acie1asl	GGTCTAGAGCTTATCATGCGGAATCTTT	*ie-1*	antisense
acie1asr	AAGCGGCCGCGCAAATGTTCGTGTTGTGAT	*ie-1*	antisense
egfpsl	AAGAGCTCGCCGACAAGCAGAAGAAC	*egfp*	sense
egfpsr	GAACTAGTGTCCATGCCGAGAGTGAT	*egfp*	sense
egfpasl	GGTCTAGAGCCGACAAGCAGAAGAAC	*egfp*	antisense
egfpasr	AAGCGGCCGCGTCCATGCCGAGAGTGAT	*egfp*	antisense
rfpsl	AAGAGCTCCCCCGTAATGCAGAAGAA	*rfp*	sense
rfpsr	GAACTAGTCTCGTTGTGGGAGGTGAT	*rfp*	sense
rfpasl	GGTCTAGACCCCGTAATGCAGAAGAA	*rfp*	antisense
rfpasr	AAGCGGCCGCCTCGTTGTGGGAGGTGAT	*rfp*	antisense

In our vector mediated knock down experiments, we used **pZsP35, pZdsIE1, pZsGP64, pZsEGFP and pZsRFP** as transfection controls. They contain only the sense oriented fragments of *p35, ie-1, gp64.egfp* and *rfp* respectively.

3.2.6 Making of Stable lines expressing dsRNAs: Plasmid vectors **pZdsP35, pZdsGP64, pZdsIE1, pZdsEGFP and pZdsRFP** produced by above mentioned methods were transfected into sf9 cells and stable lines were produced as per descriptions in the Section 2.9 earlier.

3.2.7 Production of dsRNAs: Sequence information of AcMNPV orfs *gp64, ie-1* & *p35* were collected from NCBI Entrez database. Sequence information of *Red Fluorescent Protein (RFP)* was taken from pdsRED1-N1 vector (BD Biosciences Clontech)and *Enhanced Green Fluorescent Protein(EGFP)* sequence was taken from *pEGFP-N3* vector (BD Biosciences Clontech) (GenBank Accession #: U57609). Primers were designed to amplify specific regions of AcMNPV orfs, *rfp* and *egfp* (for amplifying *p35, gp64* and *ie1* genes primers named Ac136L& Ac136R, Ac129L & Ac129R, and Ac142L & Ac142R were used, and for *rfp* and *egfp* DsRED-T7L & Dsred-T7R, EGFP-T7L and EGFP-T7R were used respectively. see **Table 3.2 and 4.1** for primer sequences sequences). A restriction site and T7-RNA polymerase binding sites were attached to 5'- end of these primers and chemically synthesized. PCR was done to amplify specific regions of *RFP* and *EGFP*. These amplicons were used as templates for in-vitro transcription to produce both sense and antisense RNA in the same tube. These sense and antisense RNAs were annealed to produce dsRNAs (**Figure 3.3**).

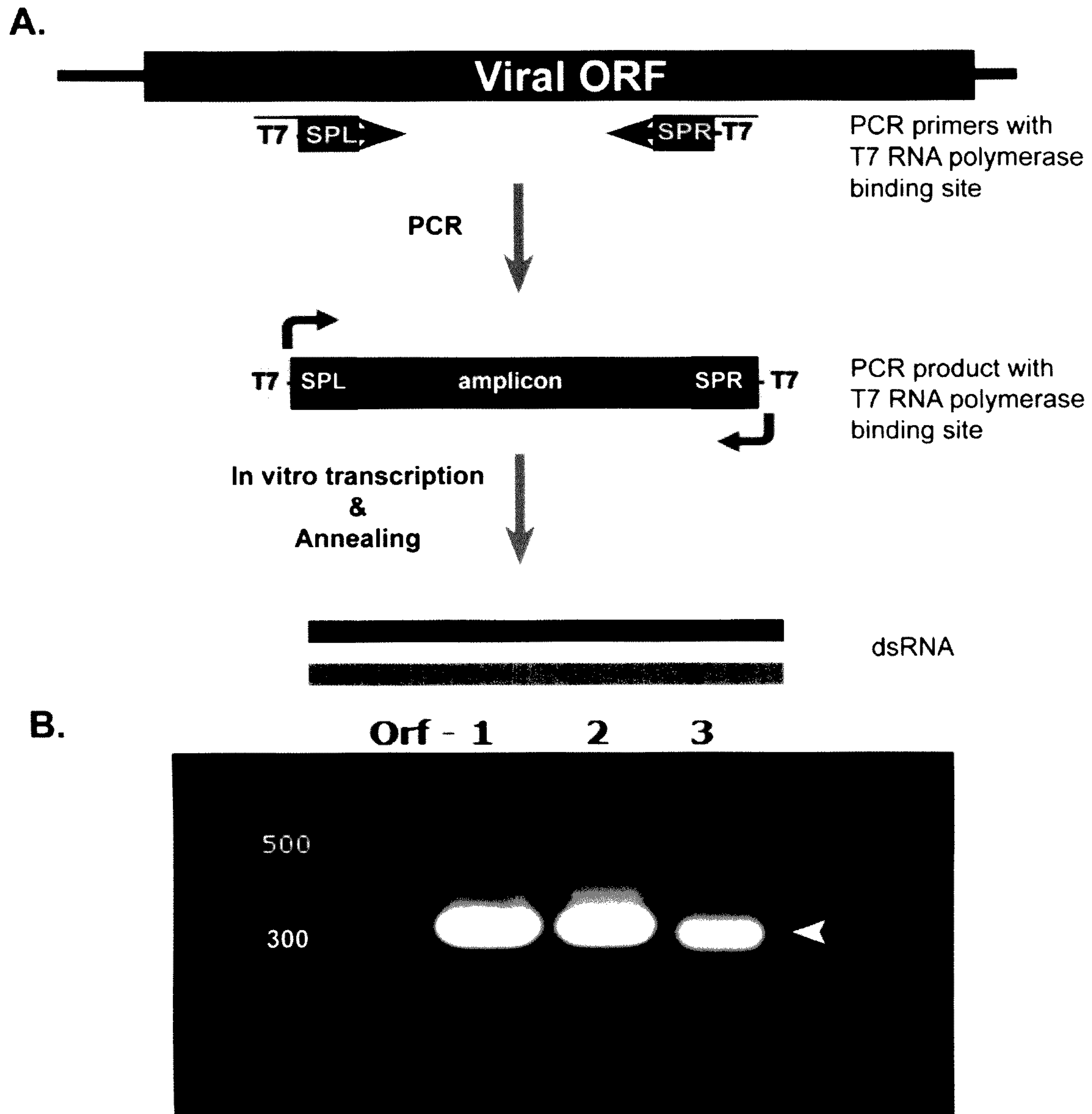


Fig 3.3: Generating dsRNAs by *in vitro* transcription

A. An overview of production of dsRNA: Specific regions of viral orfs were amplified by PCR using primers containing T7 RNA polymerase binding sites at their 5' end. The PCR product (amplicon) was used as a template for in vitro transcription and both sense and antisense strands were produced in the same reaction tube. In vitro transcribed RNA (both sense+antisense) were annealed to ensure dsRNA formation.

B. A 2% agarose gel picture of In vitro transcribed and annealed dsRNA: dsRNAs produced for AcMNPV orfs 1, 2 and 3 are shown in the picture. First lane contains 100bp dsDNA ladder. Arrow head indicates the expected dsRNA product.

Table 3.2: List of primers used for amplifying specific fragments of *egfp* and *rfp..*PCR products were used as templates *in-vitro* transcription to produce dsRNA.

No	Name	Sequence
1	Dsred-T7L	TAATACGACTCACTATAGGGCTCGAGCTCCTCCAAGAACGTCATC
2	Dsred-T7R	TAATACGACTCACTATAGGGATCCGGGTGCTTCACGTACACCTT
3	EGFP-T7L	TAATACGACTCACTATAGGGATCCGAACGGCATCAAGGTGAACT
4	EGFP-T7R	TAATACGACTCACTATAGGGAATTCTGCTCAGGTAGTGGTTGTCG

3.2.8 Transfection of Plasmids and dsRNA: Transfection was done in sf9 cells using Cellfectin or Trans-Messenger Transfection reagent as described in Section 2.8.2 in Chapter 2.

3.2.9 Production of Recombinant AcMNPV: Recombinant viruses were produced using Bac-to-Bac kit (Invitrogen). Four types of recombinant viruses were produced in this study *viz*, *vRec-EGFP*, *vRec-RFP*, *vRecRed-S* and *vRecRed-SAS*. All related fragments were cloned in suitable transfer vectors provided in the kit and recombinant viruses were produced as per the protocol described in section 2.2.2 of Chapter 2

3.2.10 Recombinant AcMNPV expressing RFP (vRec-RFP): pFastBac-b transfer vector provided in the *Bac-to-Bac* kit was used in making this recombinant vector. Sequence information about RFP was collected from *pdsRED1-N1* vector (BD biosciences Clontech). Whole *rfp* coding region was extracted out by digesting pdsRed1-N1 with BamH1 andNot1 restriction enzymes primers along with suitable restriction sites. The digested RFP fragment was cloned in the pFastBac-HTb transfer vector which was also digested using same restriction enzymes. The recombinant transfer vector was then used in making recombinant virus. (**Figure 3.4**)

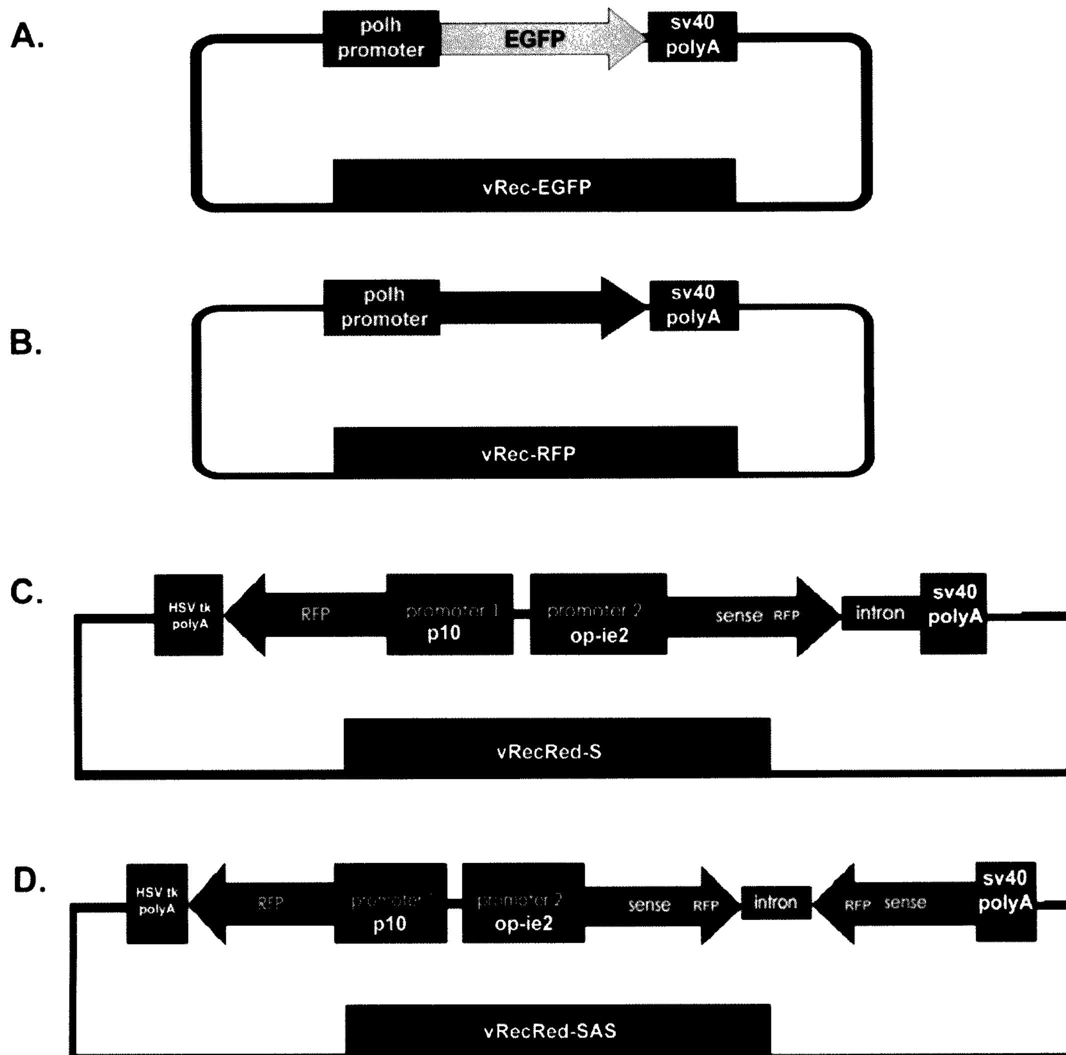


Figure 3.4: Maps of recombinat AcMNPV

A: vRec-EGFP is a recombinant virus containing EGFP cloned under polh promoter.

B: vRec-RFP is a recombinant virus containing RFP cloned under polh promoter

C: vRecRed-S is a recombinant virus containing two expression cassettes one with polh promoter and other with op-ie2 promoter. The p10 promoter expresses RFP and op-ie2 promoter expresses a ~223 fragment of RFP gene.

D: vRecRed-SAS has similar expression cassettes like vRecRed-S but can express ~223bp dsRNAs against RFP gene.

3.2.11 Recombinant AcMNPV expressing EGFP (vRec-EGFP): pFastBac-b transfer vector provided in the Bac-to-Bac kit was used in making this recombinant virus. EGFP sequence information was collected from pEGFP-N3 vector (BD biosciences Clontech) whole EGFP coding region was taken out by digesting pEGFP-N3 vector with BamH1 and Not1 restriction enzymes. This EGFP fragment was cloned in to the transfer vector pFast Bac HTb, which was digested using same restriction enzymes. The transfer vector was then used in making recombinant virus. (**Figure 3.4**)

3.2.12 Recombinant virus for dsRNA production against RFP (*vRecRed-S* and *vRecRed-SAS*): pFastBac-Dual transfer vector provided in the Bac-to-Bac kit was used in making this recombinant virus. pFastbac Dual vector has 2 expression cassettes, one with p10*(Pp10)* promoter and the other with polyhedrin promoter (*Pph*). We cloned all fragments related dsRNA production in the *Pph* promoter driven cassette and *'rfp'* reporter gene was cloned in Pp10 driven cassette. All fragments (details are given bellow) were amplified using primers listed in **Table 3.3** and proof reading enzymes and were cloned using *'pMOS-Blue Blunt end cloning kit'*. Clones were confirmed by sequencing. All fragments were again digested using suitable restriction enzymes and individual fragments were cloned serially to get the final transformation vector. Clones were confirmed by sequencing after cloning each and every fragment into the transformation vector.

Polyhedrin promoter in the pFastBac-Dual vector was replaced with Op-ie2 promoter. Op-ie2 promoter sequence information was collected from pIZT-V5-His vector sequence (invitrogen). PCR primers were designed to amplify complete Promoter region (Op-ie2 promoter) along with restriction sites BstZ1171 and BamH1 added towards 5'end of both primers (see **Table-3.3**). Op-ie2 promoter was amplified and cloned into pMOS Blue blunt end cloning vector kit. Clones were confirmed by sequencing and digested with BstZ1171 and BamH1 restriction enzymes to take out the opIE2 promoter fragment along with sticky restriction sites. pFastBac-Dual vector to replace polyhedrin promoter with Op-ie2 promoter, **(pFastBac-DUAL-IE2)**.

RFP sequence information was collected from pdsRED1-N1 vector (BD biosciencs Clontech). PCR primers were designed to amplify complete RFP coding sequence along with Xho1 and Nsi1 restriction sites towards their 5' and 3' ends of coding region respectively. Amplified RFP was cloned into **pFastBac-DUAL-IE2** transfer vector between Xho1 and Nsi1 sites (**Figure 3.4C**).

In order to clone dsRNA expressing fragments against RFP, We took out the region of dsRNA encoding fragments from **pZdsRFP** vector by digesting with Sac1 and Xba1. The digested fragment was cloned in the pFastBac-DUAL-IE2 between Sac1 and Xba1 restriction sites. This recombinant transfer vector was used to produce recombinant AcMNPV vRecRed-SAS(**Figure 3.4D**) similarly fragments encoding a part of RFP was excised out of pZsRFP with Sac1 and Xba1 enzymes and cloned into pFastBac-Dual-IE2 between same restriction sites. This recombinant transfer vector was used to produce recombinant AcMNPV vRecRed-S (**Figure 3.4C**).

Table 3.3: List of primers used in Recombinant Baculovirus construction.

Name	Sequence	Target
rfpf	ATCTCGAGCGCCACCATGGTGCGCTC	RFP
rfpr	ACATGCATCTACAGGAACAGGTGGTGG	RFP
egfpl	CTCGAGCGCCACCATGGTGAGCAAGG	EGFP
egfpr	ATGCATTACTTGTACAGCTCGTCCATGCCG	EGFP
ie2prL	GATATCTCATGATGATAAACAATGTATGGTGC	OP-ie2 promoter
ie2prR	GGATCCTTCGAACAGATGCTGTTCAACTGT	OP-ie2 promoter

3.2.13 Quantification of Silencing Efficiency by Reverse transcriptase PCR: We performed Reverse transcriptase PCR to estimate the transcript levels to see the knockdown efficiency of RNAi constructs. For fluorescent reporter genes like RFP and EGFP fluorescent images were also taken to indirectly measure the protein levels. We often used recombinant viruses to infect cells and to knockdown

viral genes and used the expression of reporter genes as an indirect measure of viral titer. This was further confirmed by estimating viral titer by Real-Time PCR or End Point Dilution methods.

We isolated total RNA form cells and synthesized cDNA by using Improm II RT-PCR kit as explained in general materials and methods. cDNA was synthesized using random primers provided in the kit and This cDNA was used as a template to amplify specific fragments of genes to be quantified and their levels were compared with an internal control of 18s-rRNA transcript levels. 18s-rRNA specific fragments were amplified using primers (**Sf18sL**-GCGGACTCTTTGTTCGGTTA, **Sf18sR**-GGTGATCCTCCCTGATCTGA).

3.2.14 Estimation of silencing Efficiency by microscopy: In our experiments where EGFP or RFP recombinant viruses were used to analyze the efficiency of silencing, we observed the cells under fluorescence microscope for the presence of EGFP or RFP. Images were taken under same exposure and magnification using LeicaASD-3D microscope with camera (IC 3D 134881106) (Image capture settings: 16 bit/channel, 3132 X 2325 full frame HQ , color). Captured images were converted into grey scale images using a scale of '0' to '255' (0-black & 255-white). The pixel intensities were calculated using software Leica microsystems CMS Gmbh "LAS AF Version; 1.8.1 build 1390". This method was used only in relative quantification (Baghdoyan *et al.* 2004; Budovskaya *et al.* 2008).

3.3 Results and Discussion:

sf9 cells are permissive to AcMNPV infection. Cells infected by AcMNPV are destined to die as they cannot recover or overcome viral infection. AcMNPV infection causes global shut down of transcription followed by global shut down of translation in their permissive hosts like sf9 cells. So our interest was to examine whether genes of AcMNPV which are so virulent to the sf9 cells can be silenced by RNAi in sf9 cells. So we employed different strategies to instigate RNAi in sf9 cells to knock down viral genes. Strategies used in this study to knock down viral genes

are by transient Plasmid mediated shRNA delivery, transient and stable plasmid mediated dsRNA delivery, naked dsRNA transfection and recombinant virus mediated delivery.

3.3.1 Infection of sf9 cells with recombinant Baculoviruses: For studying the virus titer and expression of specific viral genes we generated two recombinant baculoviruses (AcMNPV) *viz,* vRec-EGFP and vRec-RFP. In order to determine the ability of infection of these viruses, we infected sf9 cells with both vRec-EGFP and vRec-RFP and found these viruses can infect sf9 cells. So in many of our experiments we used these recombinant viruses instead of wild type AcMNPV. These recombinant viruses expressing EGFP or RFP under the very late polyhedrin promoter provide the convenience of estimating infection indirectly based on the levels of fluorescence of EGFP or RFP (**Figure 3.5 A and B**).

3.3.2 Infection of *Bombyx mori* larvae and BmN cells with recombinant AcMNPV: *Bombyx mori* is not a natural host of AcMNPV and generally infection of *B.mori* larvae with AcMNPV does not lead to their death. A study of 31 strains of *B.mori* showed that some 14 strains were susceptible to AcMNPV infection and others were resistant. So in order to verify the susceptibility of the *Bombyx mori* strain that we are using in our experiments, we injected *Bombyx mori* larvae belonging to strain Pure Mysore-5 (PM5)with recombinant AcMNPVs vRec-RFP and vRec-EGFP ($1X10^5$pfu/larva) and observed the expression of *rfp* and *egfp* of recombinant viruses under fluorescent microscope. Injected larvae were seen to express RFP and EGFP indicating that these larvae were infected by AcMNPV (**Figure 3.4 E and F**). We also observed that the recombinant AcMNPV spread throughout the body of larvae and expressed EGFP and RFP throughout the body (**Figure3.6**). The fluorescence of EGFP and/or RFP kept on increasing with time of infection (**Figure-3.6**). The infected larvae did not die due to infection but started spinning cocoons with a delay of 24 hours compared to uninfected larvae. These results confirmed that the larvae of *Bombyx mori* PM5 strain are not permissive to AcMNPV infection. They can be infected with AcMNPV but the infection is not lethal

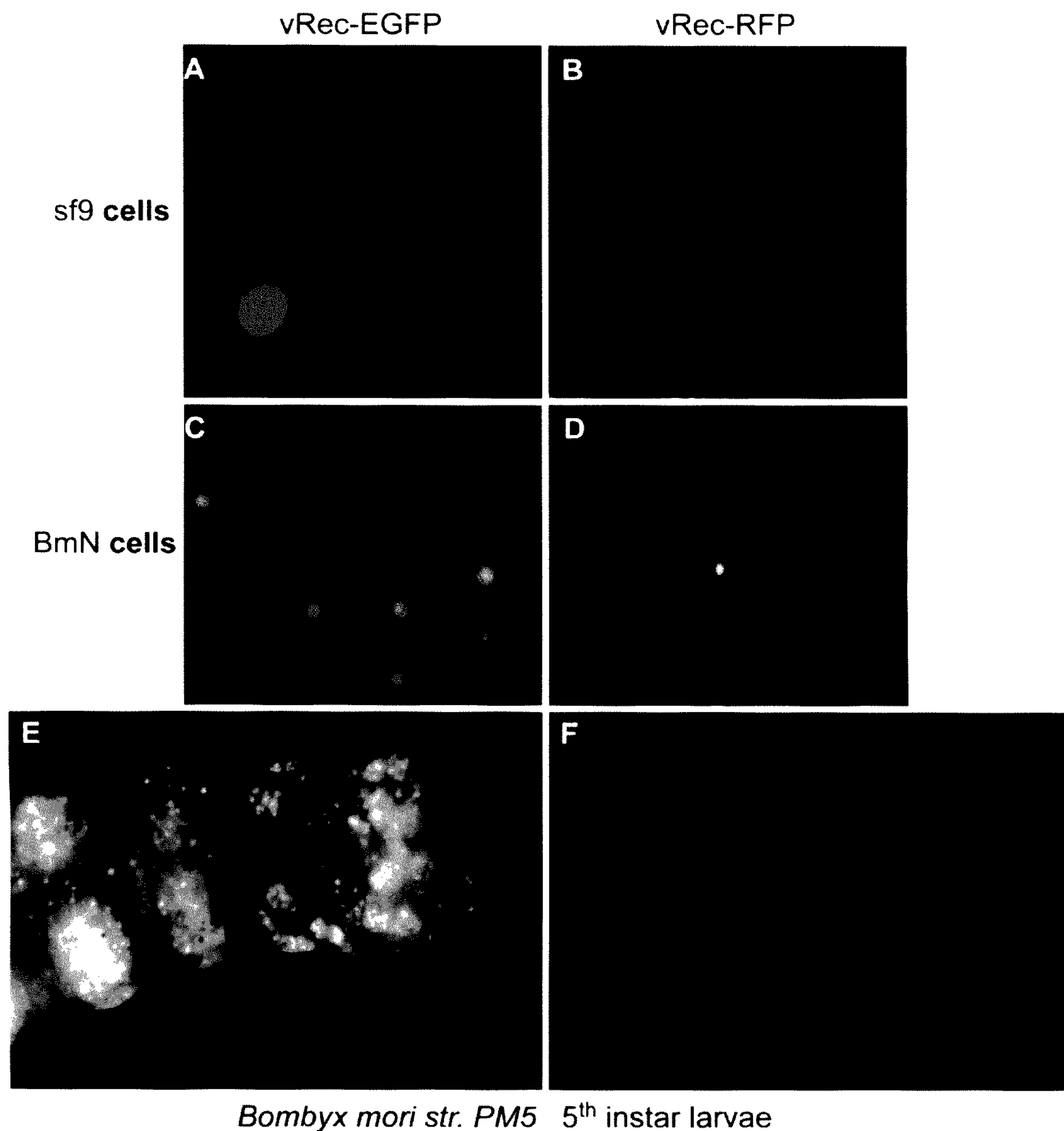


Figure 3.5: Infection of insect cells (sf9, BmN) and larvae (*Bombyx mori*) with recombinant AcMNPVs (vRec-EGFP and vRec-RFP).

Recombinant AcMNPVs, vRec-EGFP and vRecRFP can infect sf9 cells (A & B), BmN cells (C & D) and larvae of *Bombyx mori* strain Pure Mysore-5 (E & F). Picture shows cells infected with virus (MOI-5) and images were taken at 72hrs PI.

5th instar larvae of *Bombyx mori* were injected with 1X10^5 pfu of recombinant virus vRec-EGFP and vRec-RFP and images were taken after 120hrs and 96hrs respectively.

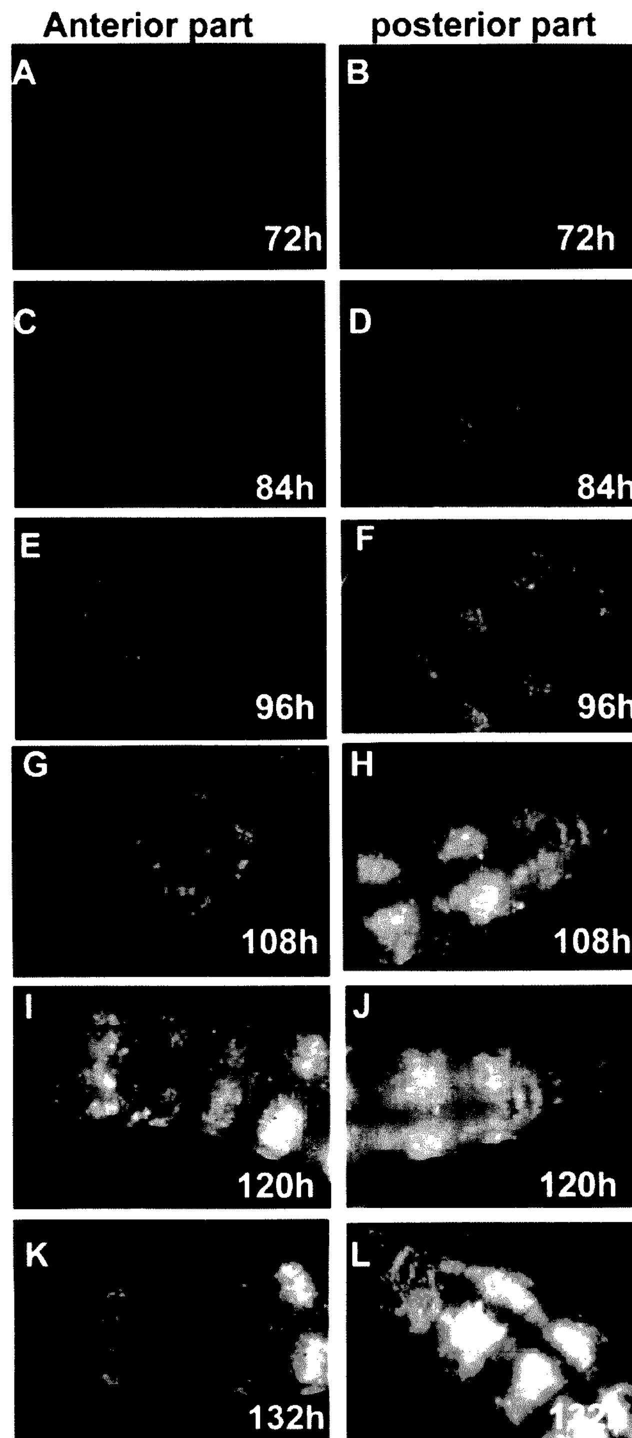


Figure 3.6: Infection of Bombyx larvae with recombinant AcMNPV (vRec-EGFP).

vRec-EGFP was injected into larvae of *Bombyx mori* (strain Pure Mysore-5) with 1X10^5 pfu and observed under fluorescent stereo-microscope, after regular intervals of 72, 84, 96, 108, 120 & 136hrs PI. EGFP fluorescence was visible after ~72hrs and accumulated with time. EGFP expression was seen throughout the body of the larvae. AcMNPV could enter into cells of *Bombyx mori* (PM5) larvae and express viral genes, but infection was not lethal to hosts.

A cell line of *Bombyx mori* called BmN is considered non permissive to AcMNPV infection. A microarray study of expression of AcMNPV genes in BmN cells has shown that all AcMNPV genes including very late genes which require viral factors for their expression (like *polyhedrin* and *p10*) express in BmN cells but with a delay of almost 24 hours (Iwanaga *et al.* 2004). So in order to see to what extent the recombinant AcMNPV (vRecEGFP) can infect BmN cells, we infected *Bombyx mori* cell lines (BmN) (MOI-5, viral titer was estimated in sf9 cells), and found that recombinant AcMNPV can enter BmN cells and express EGFP or RFP under the very late promoter of *polyhedrin* gene (**Figure 3.5 C & D**). Expression of EGFP & RFP was delayed by almost 12 hours in BmN cells compared to sf9 cells infected with the same viruses.

3.3.3 Knockdown of viral genes by vector mediated shRNA delivery: We transfected plasmid vectors which express shRNAs under a drosophila hsp70 promoter into sf9 cells. After 12 and 24hours of transfection, heat shocks were given at 37^0C for 30 minutes followed by infection with wild type AcMNPV (MOI-5) at 36hours post transfection. After 24hours of infection we isolated total RNA and estimated the transcript levels by semi-quantitative reverse transcriptase PCR. Ratios of transcript levels were measured by comparing with internal control of 18s rRNA transcripts (**Figure 3.7 A, B & C**). As shown in the **Figure 3.7** shRNA transfected cells showed lower level of transcripts than control transfected cells. The level of suppression between different shRNAs targeting same gene showed lot of variation. This could be due to the fact that, all siRNAs do not suppress their targets with equal efficiency. Moreover efficiency of RNAi in virus infected cells might also vary according to the level of infection. It is known that viral infected cells exhibit global shut down of transcription and translation of host genes. So It is also possible that RNAi pathway genes are also shut down as a part of this global shut down of transcription and translation. We have observed the knock down efficiency of viral genes after 24hours of infection at which 18s-rRNA transcript levels are fairly uniform and could be used as internal control in semi quantitative estimation. After 48h, 72h and 96hrs we checked the levels of EGFP expression in these shRNA transfected cells and we found there was not much difference in later stages of

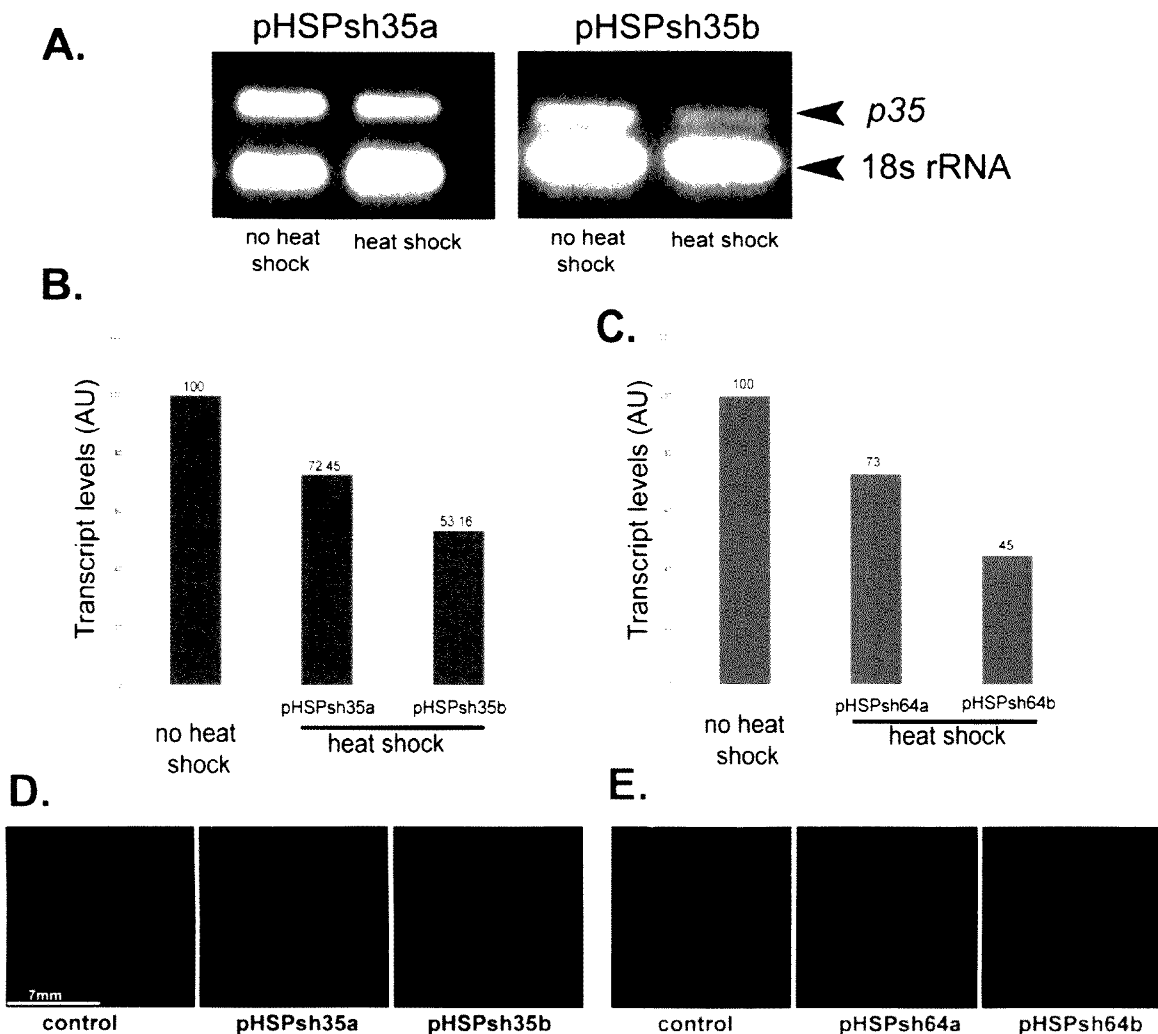


Fig 3.7: shRNA mediated silencing of *p35* and *gp64* genes.

shRNA encoding plasmids pHSPsh35a, pHSPsh35b, pHSPsh64a and pHSPsh64b were transfected into sf9 cells and heat shock was given to induce expression of shRNA. After 36hours of transfection cells were infected with vRec-EGFP(MOI-5).

A. A gel showing RT-PCR products of *p35* transcripts in cells with or without heat shock to induce shRNA expression. 18s rRNA trasncript levels were used as internal controls.

B. A bar digram showing the levels of *p35* transcripts in shRNA transfected cells after 48hours of virus infection. p35 levels in control transfected cells (without heat shock) were taken as 100 and others are represented as ratios of 100.

C. A bar digram showing the levels of *gp64* transcripts in shRNA transfected cells after 48hours of virus infection. *gp64* levels in control transfected cells (without heat shock) were taken as 100 and others are represented as ratios of 100.

D & E. Micorscopic images of cells transfected with shRNA encoding plasmids against *p35* and *gp64* and infected with vRec-EGFP (MOI-5). Images were taken after 72hrs of infection.

infection. This could be either due to loss of silencing triggers i.e., small RNAs and/or the virus infection is so virulent that it overcomes the modest effects of RNAi targeted against its genes by over expressing its genes of by inhibiting RNAi itself. It is also possible that both these factors contribute to the observed silencing effect.

3.3.4 Knockdown of Viral genes by transient vector mediated dsRNA delivery: To test whether transient vector mediated delivery of dsRNAs can trigger RNAi in virus infected cells, plasmids **pZdsP35 & pZdsIE1**expressing dsRNAs against *p35, ie-1* were transfected into sf9 cells and after 24hours of transfection cells were infected with wild type AcMNPV (MOI-5). After 72 hours of viral infection cells were harvested and total RNA was isolated and ratios of transcript levels of *p35* and *ie-1* were measured based on 18srRNA levels as internal control. We found significant reduction in transcript levels of p35 and *ie-1* (**Figure 3.8 A, B & C**) after 72 hours. We observed significant reduction in transcript levels of viral genes even after 72 hours when RNAi was triggered by dsRNAs compared to shRNAs.

In another set of experiments, we transfected sf9 cells with plasmids expressing dsRNAs against *p35* & *ie-1* genes. After 24 hours of transfection cells were infected with virus *vRec-EGFP* (MOI-5). After 72 hours of infection, cells were observed under fluorescent microscope for EGFP fluorescence. Since *p35* and *ie-1* are essential genes for Baculovirus infection, suppression of these viral genes will reduce expression of viral genes and production of progeny viruses in infected cells which can be indirectly visualized by reduction in EGFP fluorescence. Comparison of these two silencing strategies indicates that dsRNA mediated silencing is long lasting and more efficient in silencing viral genes in Baculovirus infected cells.

3.3.5 Knockdown of viral genes by stable expression of dsRNA: Since we observed reasonably good silencing of viral genes by transient transfection itself but the efficiency of silencing goes down with time. As noted earlier one of the reasons could be, loss of silencing triggers (small RNAs) with time so we expected stable expression of these dsRNAs might improve the silencing efficiency. So we made stable dsRNA expressing sf9 cells by transfecting the vectors (**pZdsP35, pZdsGP64, pZdsIE1, pZdsEGFP and pZdsRFP**) which can express dsRNAs -

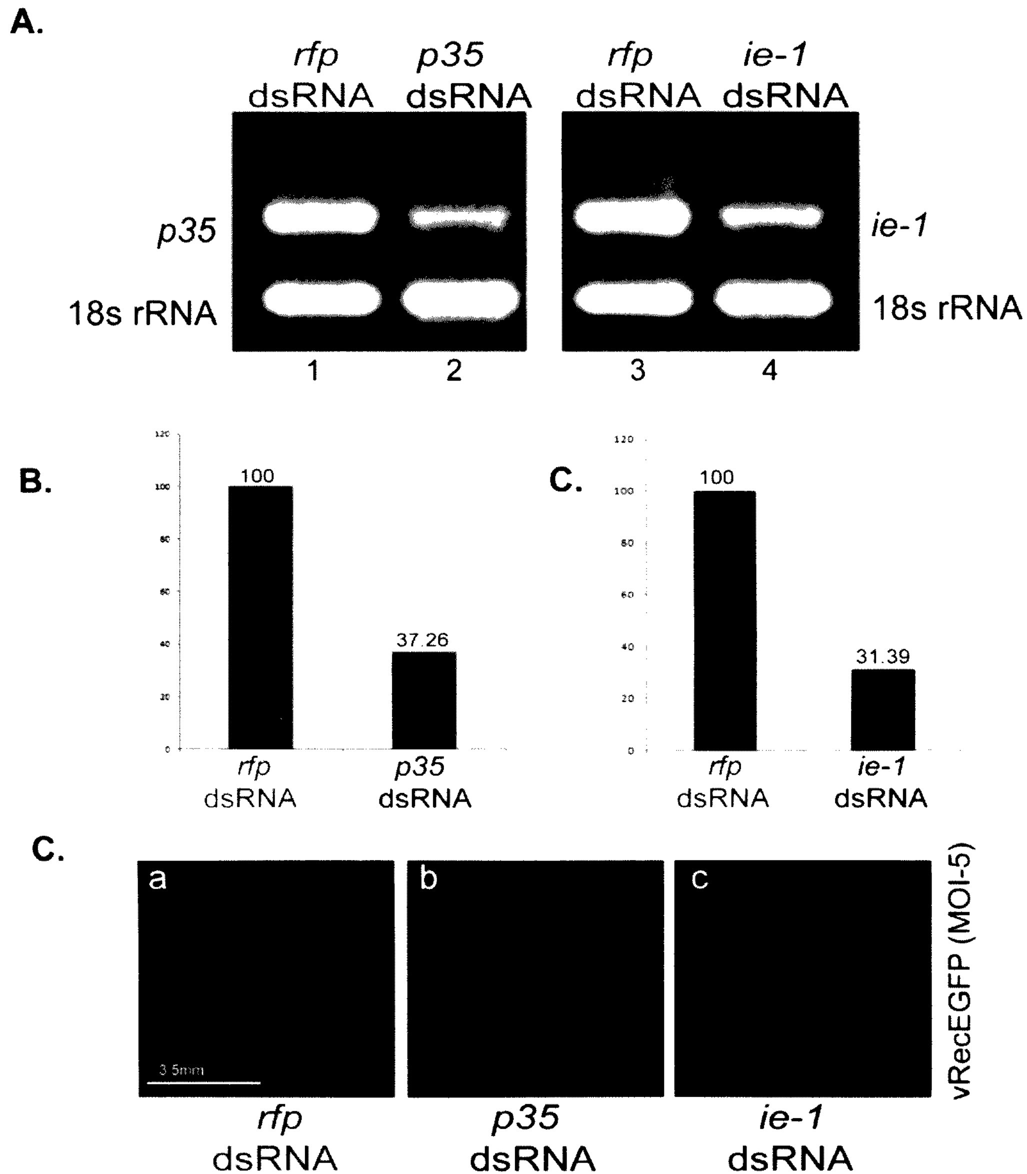


Figure 3.8: Knockdown of viral genes by transient vector mediated dsRNA delivery.

sf9 cells were transfected with pZdsP35, pZdsIE1 and after 24 hours infected with vRec-EGFP (MOI-5). pZdsRFP was used as a negetive control in these experiments.

A. A gel showing RT-PCR products of transcripts of *p35* and *ie-1* in pZdsP35 (lane 2), pZdsIE1 (lane 4) transfected cells after 72 hours of infection. pZdsRFP was used as -ve control (lane 1 & 3).

B and C. Bar diagrams showing the transcript levels of *p35* and *ie-1* respectively. transcript levels in pZdsRFP transfected cells were taken as 100 and levels in pZdsP35 & pZdsIE1 transfected cells are expressed as ratios of 100.

D. Images of sf9 cells transfected with pZdsRFP(a), pZdsP35 (b) & pZdsIE1(c), an dinfetd with vRecEGFP (MOI-5) after 72hrs PI

-against *p35, gp64, ie-1, egfp* and *rfp* and selecting them for zeocin resistance. The zeocin resistant transfected sf9 cells were infected with recombinant *vRec-EGFP* (MOI-5) and after 72 hours of infection cells were observed under fluorescent microscope for EGFP expression. (**Figure 3.9**). Stable expression of dsRNA in cells supports continuous production of small RNAs which is expected to exhibit more efficient silencing than transient transfection experiments expressing the same dsRNAs. This was evident by comparing the suppression in virus production in stable knock down and transient knock down experiments. But even by stably expressing dsRNAs to knock down essential genes of virus we could not achieve complete suppression of virus infection. These results raise the possibility of Baculoviruses overcoming RNAi or due to the limitations in availability of RNAi components and small RNA triggers in infected cells. It is known that level of transcripts of viral genes in infected cells is very high and the concentration of these transcripts themselves might limit the consequences of silencing of these genes by RNAi.

In the experiments using plasmid mediated shRNA or dsRNA delivery to silence viral genes, achieving constitutive expression of dsRNAs throughout the course of infection to support continuous production of siRNAs is a major concern. This is very important factor especially in organisms where small RNAs (triggers of silencing) are not amplified by RNA dependent RNA polymerases. In our experiments we used promoter of *ie2* gene to drive the expression of dsRNAs. *ie2* promoter is a viral promoter which can be transcribed by both RNA polymerase-II of the host and RNA polymerase encoded by virus and ie2 driven viral transcripts were seen in both early and late phases of Baculovirus infection indicating that this promoter is active in both infected and uninfected conditions (Theilmann *et al.* 1992). The *ie2* promoter is not a very strong promoter like Actin5C promoter of drosophila which is widely used in over expression studies. But *ie2* promoter ensures continuous production of dsRNAs throughout viral infection, unlike other host promoters like Actin5C promoter, *hsp70* promoter etc, which could be shut down during late stages of viral infection.

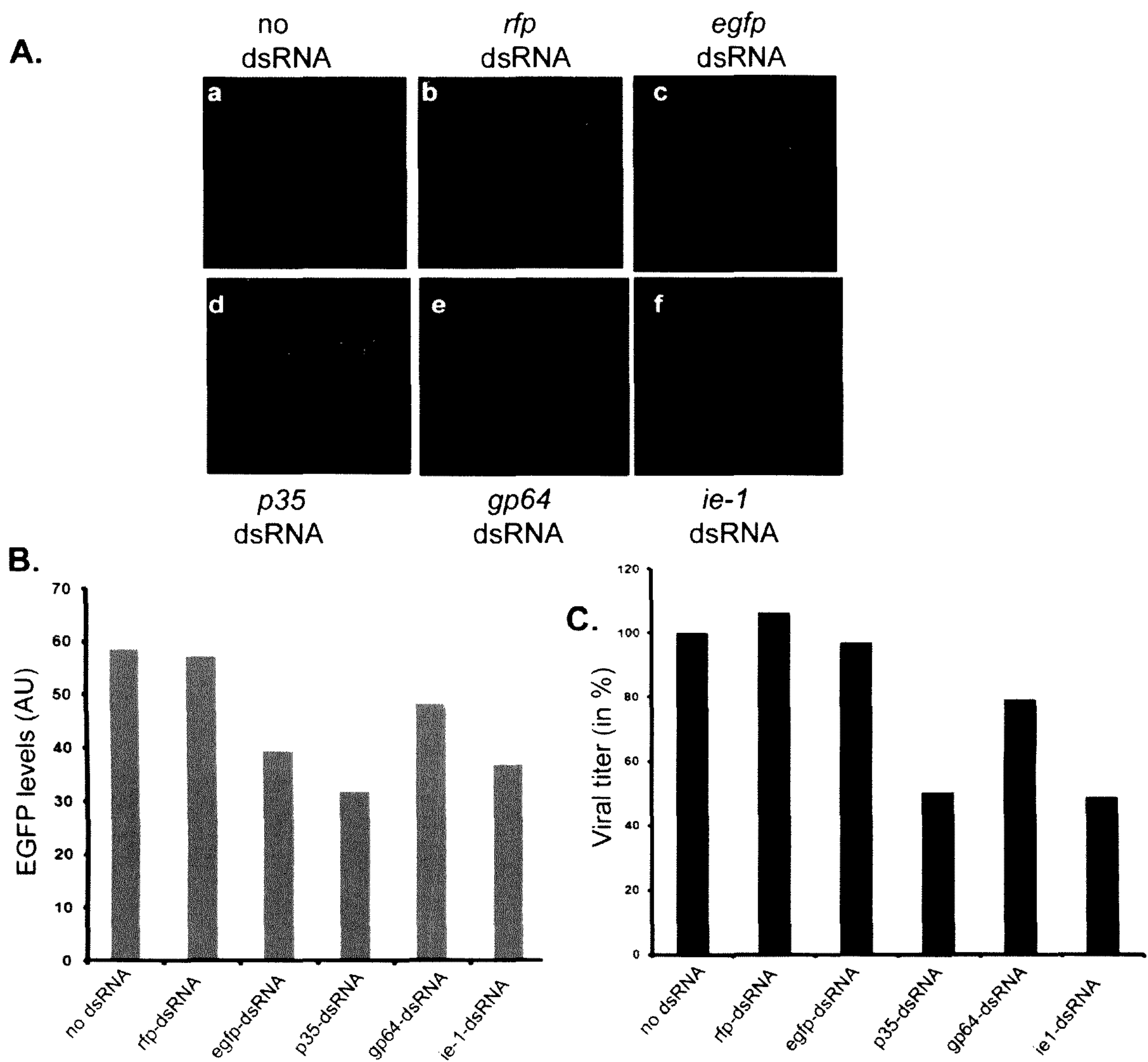


Figure 3.9: Knock down of viral genes by stable expression of dsRNA.

pZdsP35, pZdsGP64, pZdsIE1, pZdsEGFP and pZdsRFP vectors were used to establish stable dsRNA expressing sf9 cells and infected with vRec-EGFP (MOI-5).

A. Fluorescent stereomicrocopic images of cells infected with vRec-EGFP after 72hours. cells that express dsRNA against *rfp* (b) did not show significant silenicng compared to those expressing dsRNAs agaisnt *egfp, p35, gp64 & ie-1* (c-f). (a) are *sf9* cells which do not express any dsRNA.

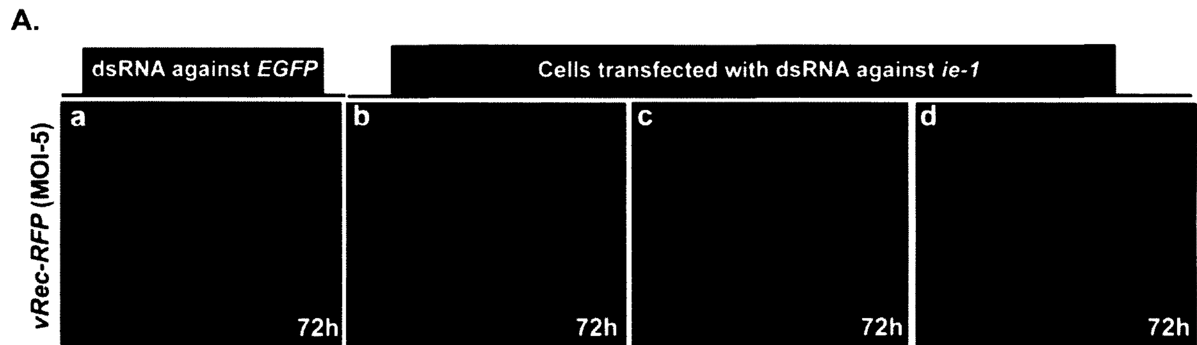
B. A Bar diagram showing the levels of EGFP in cells shown in panel 'A'

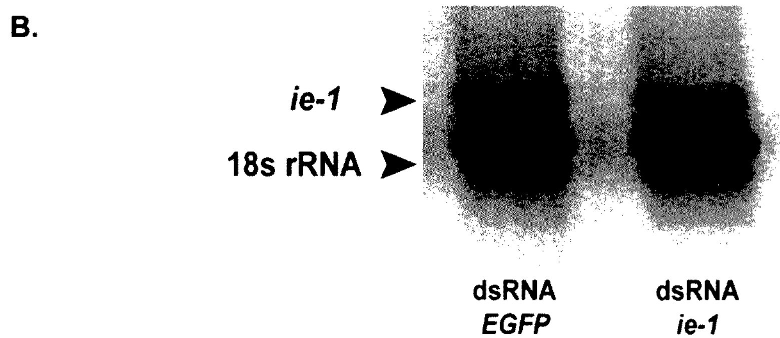
C. A bar diagram showing the viral titer in cells shown in A estimated by end point dilution. viral titer estimated in sf9 cells which do not express any dsRNA (a) were taken as 100 and all other experimantal samples (b-f) were expressed as ratios of 100. viral titer of 'a' was 5.000439×10^7.

3.3.6 Knockdown of viral genes by dsRNA transfection:

In order to see whether viral genes can be suppressed by transfecting naked dsRNAs, We amplified specific region of *ie-1* gene and used this product as a template to produce dsRNAs by in vitro transcription with T7 RNA polymerase (see section 2.7 in Chapter 2). These dsRNAs were transfected into sf9 cells. After 24 hours of transfection cells were infected with recombinant virus *vRec-RFP.* 72 hours after infection cells were visualized under fluorescent microscope (**Figure 3.10A**) for Red fluorescent Protein expression. Fluorescent images show significant reduction in the fluorescence levels which is an indirect indicator of reduced production of viruses (**Figure 3.10C**). A fraction of these cells were harvested and total RNA was isolated from these cells to estimate the level of transcripts by semi quantitative Reverse transcriptase PCR and found significant reduction in the transcript levels(**Figure 3.10B and D**).

Transfection of dsRNAs can support small RNA production only for a limited duration because of their degradation and results in gradual decrease in the efficiency of RNAi. But in spite of these limitations our experiments to knock down genes by stable dsRNA transfected cells and naked dsRNA transfected cells **Figures 3.9 and 3.10**) - show suppression of virus to almost equal levels. This could be due to better transfection efficiency of dsRNAs than plasmids which are much larger in size and have to transcribe inside the cell to produce dsRNAs. Another possibility is that the efficiency is approximately same at the time points at which we are observing, but this might vary at later time points. But such a phenomenon is difficult to measure in Baculovirus infected sf9 cells as the infection is virulent and the expression level and dynamics of viral genes themselves will not remain same. It is also possible that the observed effects are specific (or limited) to the genes we have studied and other genes might show differences in silencing efficiency.





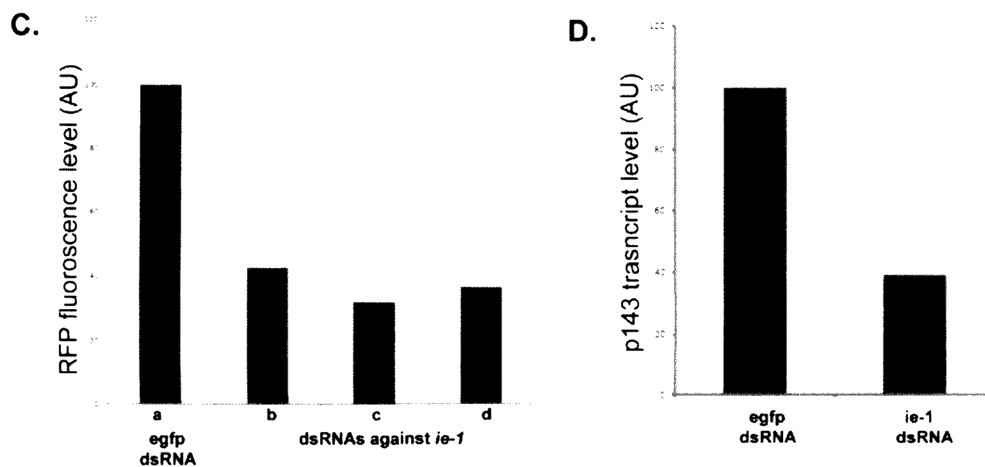


Figure 3.10: Knock down of viral genes by dsRNAs.
dsRNAs against *ie-1* gene were transfected into sf9 cells and infected with vRec-RFP (MOI-5) 24hrs post transfection.

A. Sterio micorscopic images of *sf9* cells transfected with dsRNA agaisnt *ie-1* (b,c & d) and infected with vRec-RFP. dsRNAs generated against EGFP sequence were used as control (a)

B. A gel picture showing the transcript levels of *ie-1* gene in cells transfected with dsRNAs against *egfp* and *ie-1*. after 72 hours of infection.

C. A Bar digram showing the levels of RFP in the cells of a,b,c & d of panel A

D. A Bar digram showing the levels of *ie-1* transcripts in *egfp*-dsRNA and *ie1*-dsRNA transfected cells

In all our experiments we could observe the effect of silencing up to a maximum of 120 hours post infection and we saw the efficiency of suppression of virus which was 60% at 72 hours goes down with time. In very late stages of infection cells start dying and do not give a realistic measure of silencing. Although different methods to induce silencing have shown different levels of suppression at a particular time point, efficiency of silencing goes down with time in all cases.

3.3.7 Knockdown of genes by recombinant baculoviruses:

3.3.7.1 Recombinant AcMNPV mediated silencing in sf9 cells:

RNAi can be induced by using recombinant viral vectors. Viral vectors like Adenonvirus, Adeno-associated virus, Lentivirus, herpes viral vectors and even Baculovirus vectors are used to deliver RNAi into cells. Viral delivery methods are preferred over transfection methods because efficiency of viral transduction is much higher than transfection. The basic feature of viral vectors, used to deliver RNAi is that the virus should not cause severe infection in hosts. This can be achieved by attenuation or by using viruses which can induce only mild infection in host cells. Even Baculovirus based vectors are used to deliver RNAi in mammalian cells which are non-permissive to Baculovirus infection. Baculoviruses can enter into mammalian cells by membrane fusion or endocytosis but they do not replicate in mammalian cells. This character makes Baculovirus an ideal candidate for delivering DNA into mammalian cells.

Our interest was to investigate whether baculoviruses can be used to induce RNAi in sf9 cells that are highly susceptible to AcMNPV infection. So we designed two recombinant viruses vRecRed-S and vRecRed-SAS. Both viruses have two expression cassettes, one of those can express RFP under p10 promoter and the other cassette expresses a fragment of RFP either only in sense orientation or in both sense and antisense orientation producing a double stranded RNA. The virus which produces RFP in sense orientation is vRecRed-S and the one which produces dsRNA against RFP is called vRecRed-SAS (**Figure-3.11**).

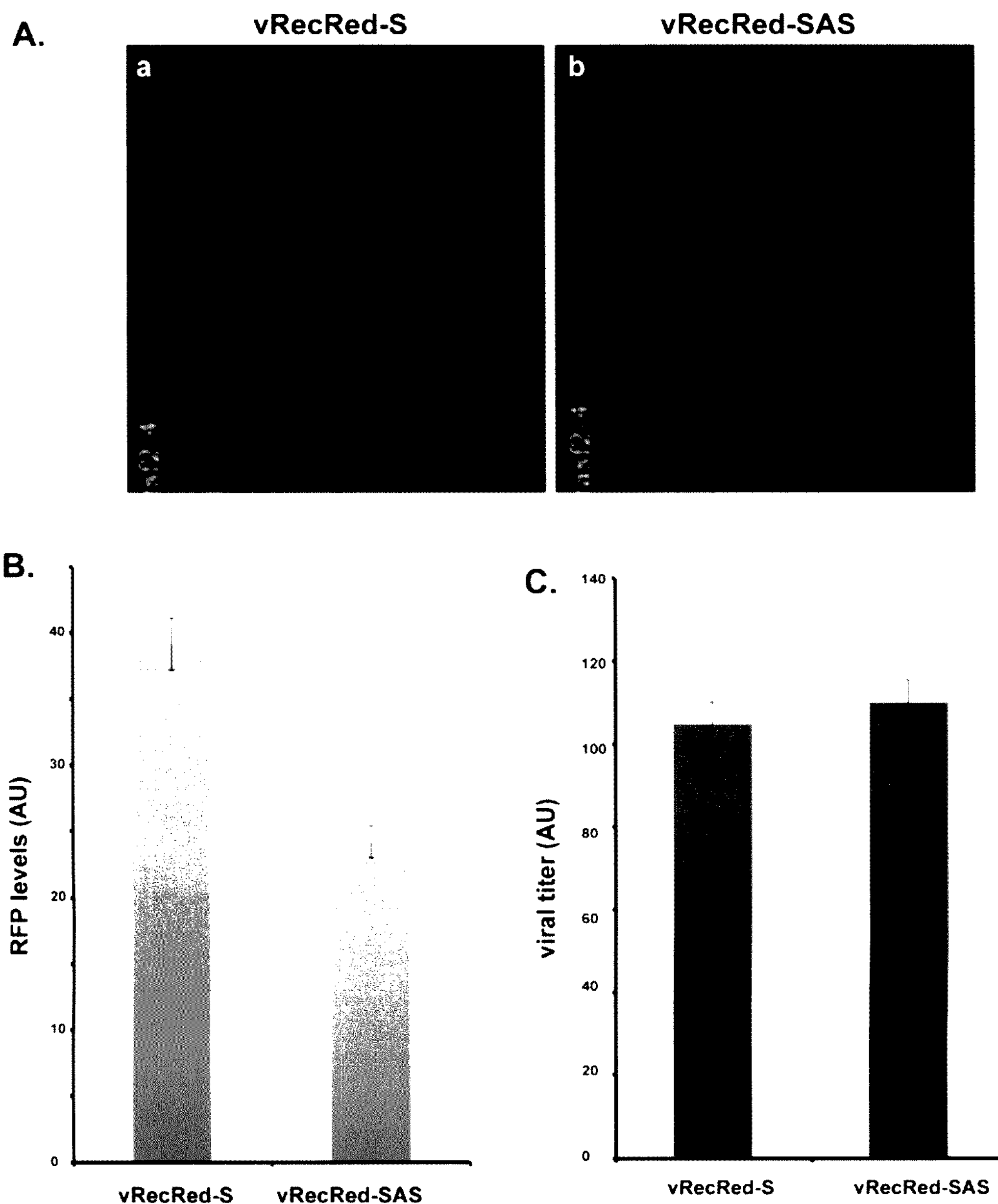


Figure 3.11: Knock down of genes by recombinant AcMNPV mediated dsRNA delivery.

A. Fluorescent stereo microscopic images of sf9 cells infected with vRecRed-S (a) and vRecRed-SAS (b) (MOI-5) after 72hrs of infection. RFP expression was lesser in (b) than (a)

B. A bar digram showing the levels of RFP in cells (a) and (b) of panel A

C. A bar diagram showing the titer of the viruses estimated from cells shown in panel A after 72 hrs of infection with vRecRed-S (a) and vRecRed-SAS (b) (MOI-5)

We infected sf9 cells with both these viruses *viz.* vRecRed-S and vRecRed-SAS (MOI-5), separately and observed under fluorescent microscope after 72hours of infection. We could observe difference in fluorescence of RFP between vRecRed-S and vRecRed-SAS infected cells. To confirm whether the difference in fluorescence is due to specific down regulation of RFP specifically or due to non specific effects of dsRNAs against virus itself, we estimated viral titer by real time PCR in both sets and found no significant difference between them. This shows dsRNAs against RFP do not induce any non specific effects resulting in suppression of virus and whatever suppression in fluorescence was observed was due to specific suppression of RFP only. These results suggest that delivering dsRNAs using recombinant baculoviruses can also trigger RNAi.

3.3.7.2 Recombinant AcMNPV mediated silencing in *Bombyx mori* larvae:

As we have shown in earlier sections that *Bombyx mori* larvae can be infected (transduced) with recombinant AcMNPV we wanted to see whether AcMNPV can be used to deliver RNAi in these larvae. We injected *Bombyx mori* strain PM5 with vRecRed-S and vRecRed-SAS viruses ($1X10^5$pfu/larva) and observed fluorescence at different time points from 24 to 120 hours. We could see suppression of RFP in injected larvae and the effect was visible throughout the body of larvae (**Figure 3.12**).

All organisms have evolved multiple ways to defend themselves against viral infection and RNAi could be one of them. But the role of each of these defense responses varies depending on the virus or host or a combination of both. In highly susceptible hosts like sf9 cells the pathological symptoms are so severe that role of various moderate immune responses are completely masked but A model system which shows slower progress of pathological symptoms can help in analyzing the importance of these different immune responses. It is possible that some of these immune responses might limit the host range of Baculoviruses to their hosts. So we think *Bombyx mori* can serve as a good model system to study general host responses against AcMNPV infection.

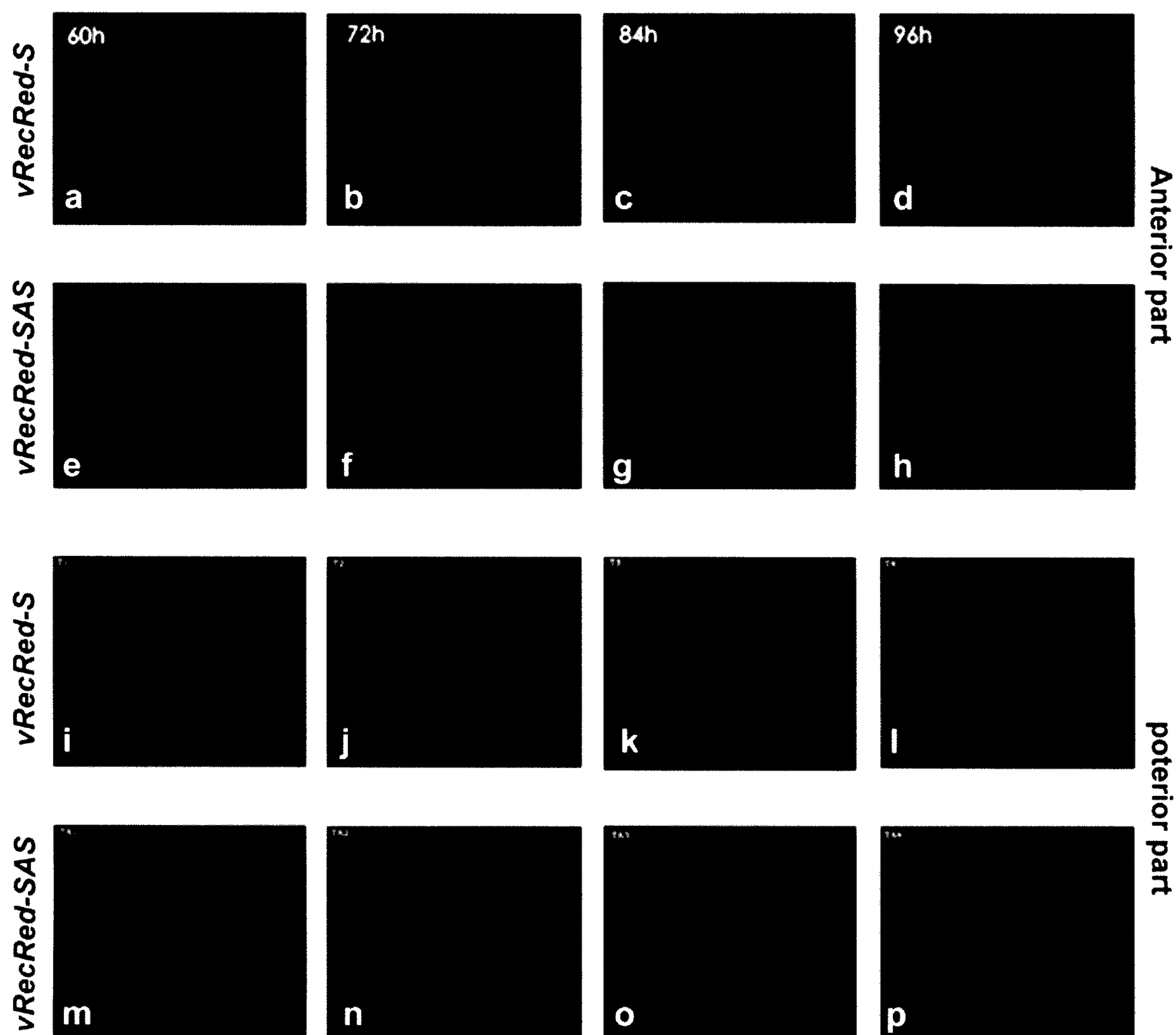


Figure 3.12: Silencing of genes by recombinant AcMNPV medated delivery of dsRNA in nonpermissive hosts.

5th instar larvae of *Bombyx mori* (PM5) were infected with vRecRed-S and vRec-Red-SAS (1 X 10^5 pfu/larva)
Larvae injected with vRecRed-S expressed more RFP (a-d) than those injected with vRecRed-SAS (e-h).

Panels 'a-d' are stereomicrocopic images of anterior part and 'i-l' show the posterior parts of the same larva infected with vRecRed-S. 'e-h' and 'm-p' are infected with vRecRed-SAS. Images were taken at 60, 72, 84 & 96hrs PI.

Chapter-4

RNAi based screening and Functional Genomics of AcMNPV

Chapter 4: RNAi based Screening and functional genomics of AcMNPV

4.1 Introduction:

Since the discovery of RNAi in *C.elegans*, there is a considerable progress in understanding of the mechanism of RNAi. Biochemical and genetic studies of mechanism of RNAi have enabled us to use RNAi as a tool to knock down genes. The sequence specificity of this technique has improved the possibility of designing drugs to diseases which were otherwise thought to be untreatable. Several attempts have been made to target diseases or disorders, which are caused due to over expression of genes. Major challenges in using RNAi for therapies are, to employ optimal delivery methods, to maintain the efficiency of RNAi for longer duration and to knock down specific targets avoiding other non specific side effects. Enormous amount of research has been done to exploit this technique to use more efficiently by employing different strategies to deliver, and to increase the specificity of RNAi. Viral vectors are the most economical methods for *in vivo* delivery but there are severe ethical concerns about using them for humans. For many other organisms like live stock and many other invertebrates the methods of delivery are not really major ethical issues and these animals can be engineered to express small RNAs or dsRNAs endogenously by transgenesis or by other viral delivery methods.

The livestock and other invertebrates like prawn and silkworms are also plagued by many bacterial and viral diseases. Silkworms are economically important insects and of great cultural importance in Asian countries including India. In culture, silkworms often get infected with baculoviruses resulting in huge economic loss to the farmers. Initial signs of success in experiments to suppress AcMNPV in sf9 cells using transient induction of RNAi raised hopes to produce Baculovirus resistant silkworms ((Valdes *et al.* 2003; Flores-Jasso *et al.* 2004; Huang *et al.* 2007). Several attempts have been made to genetically engineer silkworms to develop resistance against baculoviruses, but have ended up in limited success (Isobe *et al.* 2004; Kanginakudru *et al.* 2007; Yang *et al.* 2008). This could be due to

inappropriate strategy employed to target viral genes with RNAi or the targets selected for RNAi mediated knockdown were not really the best candidates to suppress the virus. In some cases even after targeting essential genes like *lef1* and *ie-1*, Virus could not be suppressed completely indicating that all essential genes may not be completely or equally susceptible to RNAi mediated silencing and this did not provide resistance to virus infection (Isobe *et al.* 2004; Kanginakudru *et al.* 2007). By comparing the genome sequences of baculoviruses, 31 core genes have been identified but detailed analysis of their functions and their role in virus infection are still being explored. Based on the studies done in other baculoviruses one can predict the necessity of particular viral genes in causing successful virus infection, but at least in few cases this strategy has not paid off. To quote an example, *bro-d* is an essential gene for BmNPV (Kang *et al.* 1999) but found to be non essential for BV production in AcMNPV (Bideshi *et al.* 2003). Likewise many genes which are essential for one virus in a particular host may not be essential for other viruses infecting the same host or for the same virus infecting other hosts. Therefore Importance of a particular viral gene for its successful infection depends both on the host and virus. In order to target a virus with RNAi, it is necessary to know which genes are essential at least, whether or not the functions of those genes are known. Another limitation of RNAi is that its efficiency depends on the secondary structure, localization and many other ununderstood features of their targets. So even after identifying essential genes it is necessary to know which of those can be best targeted by RNAi.

So In this chapter we have described our experiments to screen whole AcMNPV genome for essential genes. The screening was based on RNAi in which we knocked down each and every orf of AcMNPV by dsRNAs and observed its effect on virus infection and production of progeny viruses. Based on the observation we could point out genes which are more essential than others. We could also find out which of the known essential genes were more susceptible to RNAi, so that they can be particularly targeted in order to increase the resistance of hosts to Baculovirus infection.

4.2 Materials and Methods:

4.2.1 Cells and Medium: sf9 cells of *Spodoptera frugiperda* were used in this study. They were cultured in TNM-FH medium supplemented with 10% heat inactivated BSA at 27^{0}C.

4.2.2Recombinant viruses: A recombinant AcMNPV named vRecRed-S virus was used in this study. Details of the construct and methods used for generating this virus are explained in chapter3 under the section 3.2.12. Virus titer was estimated by endpoint dilution method. Concentration of the virus used in experiments are mentioned in relevant sections of the text

4.2.3 Amplification of orfs and other genes for in vitro transcription: Sequence information of all orfs was collected from NCBI entrez database (Accession number of AcMNPV genome and features NC_001623). Primers were designed to amplify specific region of each of the 156 orfs of AcMNPV based on the sequence of orfs. A T7 RNA polymerase binding site ((TAATACGACTCACTATAGGGAGA) was added towards the 5' end of each primer. A restriction site was inserted between T7 RNA a polymerase binding site and orf specific primer which was not present in the expected amplified product. This restriction site will help in removing the T7 RNA polymerase binding site at the end of amplified product.. Primers were designed to amplify approximately 300bp of each orf. (see **Table 4.1** for sequence of primers designed amplify AcMNPV orfs and **Table 3.2** for *egfp* and *rfp* dsRNAs respectively)

A schematic showing the features of primers designed to PCR amplify and in vitro transcription:

All viral orf specific regions & other genes like *egfp* and *rfp* were amplified using a proof reading enzyme DeepVent DNA polymerase (NEB) (see section 2.15 in Chapter 2 for details of PCR) and the products were verified on Agarose gel for

appropriate size. All products were confirmed by sequencing. Appropriate PCR products were eluted from agarose gel using Qiaprep gel elution kit (Qiagen). Eluted product was concentrated using a vacuum concentrator to have a concentration of ~100ng/µl and stored in -20^{0}C for downstream experiments. The gel eluted product was used a template in *in vitro* transcription reactions to produce dsRNA.

Table 4.1: List of primers and their sequence used to amplify specific regions of each of the 156 orfs of AcMNPV (NCBI Reference Sequence: NC_001623.1) name of the primers include the orf number. (For Ex: AC**1**L and AC**1**R are used to amplify specific regions of orf-1).

No	Name	Sequence
1	AC1L	TAATACGACTCACTATAGGGCTCGAGTAGCGAAGAAGATGTGTGGA
2	AC1R	TAATACGACTCACTATAGGGTCTAGAATACCCAGGGTGTGCATTA
3	AC2L	TAATACGACTCACTATAGGGCTCGAGCCATCAAGGACAAGCAGAT
4	AC2R	TAATACGACTCACTATAGGGTCTAGAGTACGTCTTTTGGCCGGTAA
5	AC3L	TAATACGACTCACTATAGGGCTCGAGATGCAAATCAAAACTGTACTAT
6	AC3R	TAATACGACTCACTATAGGGTCTAGATTATTGTGGTAAGCAATAATTAA
7	AC4L	TAATACGACTCACTATAGGGCTCGAGTCGACGGCATGATTAAAAGC
8	AC4R	TAATACGACTCACTATAGGGTCTAGAGTTCGTTGACGCCTTCCT
9	AC5L	TAATACGACTCACTATAGGGCTCGAGATGTATCGCACGTCAAGAATTA
10	AC5R	TAATACGACTCACTATAGGGTCTAGATTATACGATGCATTCGCCATAC
11	AC6L	TAATACGACTCACTATAGGGCTCGAGTACAATGGCGGGGTTTTG
12	AC6R	TAATACGACTCACTATAGGGTCTAGATTTCACACTTGGCACACGA
13	AC7L	TAATACGACTCACTATAGGGCTCGAGAAGCAACACACTCCGAAGAA
14	AC7R	TAATACGACTCACTATAGGGTCTAGACAAACCAAACGCAACAAGAA
15	AC8L	TAATACGACTCACTATAGGGCTCGAGATTCATACCGTCCCACCATC
16	AC8R	TAATACGACTCACTATAGGGTCTAGATTTCCTTTCCATCCAACGAC
17	AC9L	TAATACGACTCACTATAGGGCTCGAGACTTCTAACGACGAGGGTTG
18	AC9R	TAATACGACTCACTATAGGGTCTAGACCGATGGCTGGACTATCTA
19	AC10L	TAATACGACTCACTATAGGGCTCGAGTTTTATCGCACAAGCCCACT
20	AC10R	TAATACGACTCACTATAGGGTCTAGACACAATCCGTAATCGCAAAC
21	AC11L	TAATACGACTCACTATAGGGCTCGAGCGTTTGAAGAGCGGCAGT

22	AC11R	TAATACGACTCACTATAGGGTCTAGAGCAGCAGTTTCCCGTTTCT
23	AC12L	TAATACGACTCACTATAGGGCTCGAGGGGAGCTGTTATCGGTTG
24	AC12R	TAATACGACTCACTATAGGGTCTAGATCGTCTTGTCTCAGTTCGTTG
25	AC13L	TAATACGACTCACTATAGGGCTCGAGTACCACATAAACGCCCAACA
26	AC13R	TAATACGACTCACTATAGGGTCTAGATTTCCAACAACCCGTGCT
27	AC14L	TAATACGACTCACTATAGGGCTCGAGCGCATTGTGTGAGAGAGG
28	AC14R	TAATACGACTCACTATAGGGTCTAGATGGCGTCGTTGTCGTT
29	AC15L	TAATACGACTCACTATAGGGCTCGAGCGATTATGCGTTGGTGTTTG
30	AC15R	TAATACGACTCACTATAGGGTCTAGATTGCACCTTGTTGCGTAGTT
31	AC16L	TAATACGACTCACTATAGGGCTCGAGACACACACCATCACCTCCA
32	AC16R	TAATACGACTCACTATAGGGTCTAGACGGCCAAACGTCTCCTTAC
33	AC17L	TAATACGACTCACTATAGGGCTCGAGAATGTCAGATGCCGTTCTCC
34	AC17R	TAATACGACTCACTATAGGGTCTAGAATCCACCACGTCCTTCAC
35	AC18L	TAATACGACTCACTATAGGGCTCGAGATGTGTTGGTGTCGTCGTTG
36	AC18R	TAATACGACTCACTATAGGGTCTAGATCGGTGGTCAAGTAGATGTG
37	AC19L	TAATACGACTCACTATAGGGCTCGAGCGACGCAACACGACTACACT
38	AC19R	TAATACGACTCACTATAGGGTCTAGACAATGCAGAAATTAACAAATCC
39	AC20L	TAATACGACTCACTATAGGGCTCGAGATGTGCGACGCCTCGTGC
40	AC20R	TAATACGACTCACTATAGGGTCTAGACTAATCATCATAAACGGGTAATA
41	AC21L	TAATACGACTCACTATAGGGCTCGAGCTACGCAAATACCGAAACCT
42	AC21R	TAATACGACTCACTATAGGGGGATCCGAGACCACTTCCAGCAATCT
43	AC22L	TAATACGACTCACTATAGGGCTCGAGCGGTGCCTTTGTTTGTTAGTG
44	AC22R	TAATACGACTCACTATAGGGTCTAGATGTTGTCTGCCTGCGAGTT
45	AC23L	TAATACGACTCACTATAGGGCTCGAGACACGCTGCTAACGGAAA
46	AC23R	TAATACGACTCACTATAGGGTCTAGATAGTGGCAATCGGCTCAGT
47	AC24L	TAATACGACTCACTATAGGGCTCGAGAAACTGCAATCGACAAAATG
48	AC24R	TAATACGACTCACTATAGGGTCTAGATCGCTGTGTTTTATGAACGAA
49	AC25L	TAATACGACTCACTATAGGGCTCGAGCCTGCGGATTTTGGTTTTC
50	AC25R	TAATACGACTCACTATAGGGTCTAGACGTTGTTGTCACGGTTGATT
51	AC26L	TAATACGACTCACTATAGGGCTCGAGATGGACGACTCTGTTGCCAG
52	AC26R	TAATACGACTCACTATAGGGTCTAGATTAGCTCGTTAAAGTTACGGTC
53	AC27L	TAATACGACTCACTATAGGGCTCGAGCCGTTTTATTTTATCAGCGTGT
54	AC27R	TAATACGACTCACTATAGGGTCTAGACTTGGGTTTGCCTTCTTTCA
55	AC28L	TAATACGACTCACTATAGGGCTCGAGTTCATATTGCGCCTGATTTG
56	AC28R	TAATACGACTCACTATAGGGGGATCCGTCGTAGTCGTCGTAGCC
57	AC29L	TAATACGACTCACTATAGGGCTCGAGATGTTCTCAAGGAATTACAACG

58	AC29R	TAATACGACTCACTATAGGGTCTAGATTAAAAATTGTCTATTCCGTAGT
59	AC30L	TAATACGACTCACTATAGGGCTCGAGCTCCAACCCAAGTGCTACAA
60	AC30R	TAATACGACTCACTATAGGGTCTAGACCAACAAGTCCCCAAACG
61	AC31L	TAATACGACTCACTATAGGGAAGCTTCATTAGCGGCGATGTTC
62	AC31R	TAATACGACTCACTATAGGGTCTAGATCGTCTTTGTCCGTGTGC
63	AC32L	TAATACGACTCACTATAGGGCTCGAGCCTCTATGGCTGACTGCTC
64	AC32R	TAATACGACTCACTATAGGGTCTAGAGGGAGTACCGTCGTTCTTCA
65	AC33L	TAATACGACTCACTATAGGGCTCGAGACCAAGCCCATTTAGGTTCC
66	AC33R	TAATACGACTCACTATAGGGTCTAGATGCTCTGGCGTGTAAAACTG
67	AC34L	TAATACGACTCACTATAGGGCTCGAGCGATACCCACACCACGAAT
68	AC34R	TAATACGACTCACTATAGGGTCTAGATGAAAATCACACCACAACCAG
69	AC35L	TAATACGACTCACTATAGGGCTCGAGATGCAAATATTCATCAAAACATTG
70	AC35R	TAATACGACTCACTATAGGGTCTAGATTAATACCCTCCTCGTAATCG
71	AC36L	TAATACGACTCACTATAGGGCTCGAGTAAGATCAAGCAGCCCGAGT
72	AC36R	TAATACGACTCACTATAGGGTCTAGACTTAGCAGAGGCGCAACAT
73	AC37L	TAATACGACTCACTATAGGGCTCGAGATGCCCCCCAAAAATTGCAC
74	AC37R	TAATACGACTCACTATAGGGTCTAGATTACCATGTTTGCTTCTTGTAAAC
75	AC38L	TAATACGACTCACTATAGGGCTCGAGCAAGGCTGTCCTGCTGTGT
76	AC38R	TAATACGACTCACTATAGGGTCTAGAGTGTTGGGTTTGGCGTTCA
77	AC39L	TAATACGACTCACTATAGGGCTCGAGATGCTGCCAACCGAGAAAC
78	AC39R	TAATACGACTCACTATAGGGTCTAGACAAAACCGCCCTTGTCTG
79	AC40L	TAATACGACTCACTATAGGGCTCGAGCAGCGAGGTTATCAAACGAC
80	AC40R	TAATACGACTCACTATAGGGGGATCCACCATTCCAGAGCCACGATT
81	AC41L	TAATACGACTCACTATAGGGCTCGAGCCACAATGATGAAACGAAC
82	AC41R	TAATACGACTCACTATAGGGTCTAGAGCACAAAATACGGCAACC
83	AC42L	TAATACGACTCACTATAGGGCTCGAGAATCGGAAGAGGCGTATGTG
84	AC42R	TAATACGACTCACTATAGGGTCTAGACACTTTTAATTGGCCCGTGT
85	AC43L	TAATACGACTCACTATAGGGCTCGAGATGAACACCCGATATGCTAC
86	AC43R	TAATACGACTCACTATAGGGTCTAGATTAACGCGACCGTCGTGCA
87	AC44L	TAATACGACTCACTATAGGGCTCGAGCTCGCTACCTCCGCTGT
88	AC44R	TAATACGACTCACTATAGGGTCTAGAGTTTACCGTTTCGTCGCAGTC
89	AC45L	TAATACGACTCACTATAGGGCTCGAGTGATTGCCGTGTTGGTTTA
90	AC45R	TAATACGACTCACTATAGGGTCTAGATTGCTGTAGTGTAATGCGAGTG
91	AC46L	TAATACGACTCACTATAGGGCTCGAGGCAAGAGCAGGGAATACA
92	AC46R	TAATACGACTCACTATAGGGTCTAGAGTTACGCCCACAACCGAAC
93	AC47L	TAATACGACTCACTATAGGGCTCGAGATGATGGAGCGCACAGTCA

94	AC47R	TAATACGACTCACTATAGGGTCTAGATTATTTATTTATTAAACTGTTTAATT
95	AC48L	TAATACGACTCACTATAGGGCTCGAGATGGATGCGCTGCATGGAAT
96	AC48R	TAATACGACTCACTATAGGGTCTAGATTAGCTCGTTTCATTGATGTAAACA
97	AC49L	TAATACGACTCACTATAGGGCTCGAGATGTGATGGCGTTCGTGTT
98	AC49R	TAATACGACTCACTATAGGGTCTAGACGGTTTGCTTTGCTTTGAG
99	AC50L	TAATACGACTCACTATAGGGCTCGAGCCCAAGCAGTCGTGTTTAGT
100	AC50R	TAATACGACTCACTATAGGGTCTAGATCCACTCGGTCAGGTCTTC
101	AC51L	TAATACGACTCACTATAGGGCTCGAGGTCGGTTGTTTCGGATG
102	AC51R	TAATACGACTCACTATAGGGTCTAGACCGTGTTGTAATGGTGCTTT
103	AC52L	TAATACGACTCACTATAGGGCTCGAGATGACGTGGCCTTACTCCAA
104	AC52R	TAATACGACTCACTATAGGGTCTAGATTAATTTTTCATTGTCGCCAAAC
105	AC53L	TAATACGACTCACTATAGGGTCTAGAGCACGACAGAATGCCAGA
106	AC53R	TAATACGACTCACTATAGGGGGATCCAGGGCGTCTTCCTGTGTAA
107	AC54L	TAATACGACTCACTATAGGGCTCGAGATGACGAACGTATGGTTCGC
108	AC54R	TAATACGACTCACTATAGGGTCTAGATTACGTGGACGCGTTACTTT
109	AC55L	TAATACGACTCACTATAGGGCTCGAGTGTATTATCGGCACTTGTTGC
110	AC55R	TAATACGACTCACTATAGGGTCTAGACCGCTTTCACTATCGCTTTC
111	AC56L	TAATACGACTCACTATAGGGCTCGAGATGAAAAAAGTAGCGCTTGG
112	AC56R	TAATACGACTCACTATAGGGTCTAGATCAATATTTTTCACCAATTTGT
113	AC57L	TAATACGACTCACTATAGGGCTCGAGATGCTGCGATCAATCATGCG
114	AC57R	TAATACGACTCACTATAGGGTCTAGATTAACACAGAGGGTAAAATAGGG
115	AC58L	TAATACGACTCACTATAGGGCTCGAGCGCTGACCGTGTTGGTGT
116	AC58R	TAATACGACTCACTATAGGGTCTAGAGGCGTCTCGCACTTTTGG
117	AC59L	TAATACGACTCACTATAGGGCTCGAGATGTTTAGTAGTCGTAAACGG
118	AC59R	TAATACGACTCACTATAGGGTCTAGATTAATAGTTGTAATAATTATCTTCG
119	AC60L	TAATACGACTCACTATAGGGCTCGAGATGTATCAAATTCCCGATATGTT
120	AC60R	TAATACGACTCACTATAGGGTCTAGACTATCTCGTAGGCGGCTTT
121	AC61L	TAATACGACTCACTATAGGGCTCGAGATGTTGCCTAAATATTATCAAAAC
122	AC61R	TAATACGACTCACTATAGGGTCTAGATTAAGCAAGTTCGTCTTCGG
123	AC62L	TAATACGACTCACTATAGGGCTCGAGATTTGCGCTTTACTCGACCT
124	AC62R	TAATACGACTCACTATAGGGTCTAGAAACACGCGACTCTTTTCGT
125	AC63L	TAATACGACTCACTATAGGGCTCGAGCAATGGAGGCGTTGTTTA
126	AC63R	TAATACGACTCACTATAGGGTCTAGATGGCTCGTGAAGACACAGTT
127	AC64L	TAATACGACTCACTATAGGGCTCGAGTGCACGGAGAAGCAAAATACT
128	AC64R	TAATACGACTCACTATAGGGTCTAGAGCCATCTTGATCTCGTTTGG
129	AC65L	TAATACGACTCACTATAGGGCTCGAGCTCTCACGGCTATTTGTCC

130	AC65R	TAATACGACTCACTATAGGGTCTAGACGTATTTGGCCTCCAGTTGT
131	AC66L	TAATACGACTCACTATAGGGCTCGAGTACGCAGCCATCACAAACA
132	AC66R	TAATACGACTCACTATAGGGTCTAGAGTTGCCGTCTTCGCATAAA
133	AC67L	TAATACGACTCACTATAGGGCTCGAGCGGCTCCTCTTTCTCCTC
134	AC67R	TAATACGACTCACTATAGGGTCTAGACTTTCGGCATTTTCATCTCTC
135	AC68L	TAATACGACTCACTATAGGGCTCGAGGCTAAAGAAAGCGAAGCA
136	AC68R	TAATACGACTCACTATAGGGTCTAGATCCAGCAGCATTGAGATTTG
137	AC69L	TAATACGACTCACTATAGGGCTCGAGCTCACCGCTACTTTCTCCA
138	AC69R	TAATACGACTCACTATAGGGTCTAGAGCCGCTCTTATTCTCTCGTT
139	AC70L	TAATACGACTCACTATAGGGCTCGAGCAGCAAGTCGGTGGTTT
140	AC70R	TAATACGACTCACTATAGGGTCTAGAGCCCCGTAATGGTTGTGA
141	AC71L	TAATACGACTCACTATAGGGCTCGAGCAACAATCAAACCGGAGATG
142	AC71R	TAATACGACTCACTATAGGGGGATCCGGTACAAAAACAGCGTGCAA
143	AC72L	TAATACGACTCACTATAGGGCTCGAGATCACAATCCGTGGTCGTTG
144	AC72R	TAATACGACTCACTATAGGGTCTAGACCTGTTGAGTTAGCGGTTCG
145	AC73L	TAATACGACTCACTATAGGGCTCGAGATGACAAAAAAATTATTAACCTAC
146	AC73R	TAATACGACTCACTATAGGGTCTAGATTATTTTACAATATTTATGTATATG
147	AC74L	TAATACGACTCACTATAGGGCTCGAGATGAACACGTCCGTGGACG
148	AC74R	TAATACGACTCACTATAGGGTCTAGATTATTGTACATAATGTTTTATTGTA
149	AC75L	TAATACGACTCACTATAGGGCTCGAGCGTTAGCAAAGAGGTCAGCA
150	AC75R	TAATACGACTCACTATAGGGTCTAGAATATACTCGTCGGCGCAATC
151	AC76L	TAATACGACTCACTATAGGGCTCGAGAAGGTGTCCGTGGTCAAAAC
152	AC76R	TAATACGACTCACTATAGGGTCTAGAGGCAGTTGGTATGCTTCATC
153	AC77L	TAATACGACTCACTATAGGGCTCGAGATGAATTTATATTTGTTGTTGGGC
154	AC77R	TAATACGACTCACTATAGGGTCTAGATCAATCTATTGAGCTGGTATTTT
155	AC78L	TAATACGACTCACTATAGGGCTCGAGACGATGTTCACCTGGAAGAC
156	AC78R	TAATACGACTCACTATAGGGTCTAGACGCATACCCGTCCCTAAC
157	AC79L	TAATACGACTCACTATAGGGCTCGAGATGAATTTGGACGTGCCCTAC
158	AC79R	TAATACGACTCACTATAGGGTCTAGATTAATCAAATTTATTAAAAACAAAAGG
159	AC80L	TAATACGACTCACTATAGGGCTCGAGATGGCGACGACTCTGTACAC
160	AC80R	TAATACGACTCACTATAGGGTCTAGATTACAACTTATTTGCTAACAGGAAT
161	AC81L	TAATACGACTCACTATAGGGCTCGAGCACCGTACAACAAGAGCAA
162	AC81R	TAATACGACTCACTATAGGGTCTAGATAGCACAAACGATGGCTGAA
163	AC82L	TAATACGACTCACTATAGGGCTCGAGCTCCAACGCCTAAGAAACA
164	AC82R	TAATACGACTCACTATAGGGTCTAGACAACATTCGTCGCACAAAA
165	AC83L	TAATACGACTCACTATAGGGCTCGAGCATTACGCCAGACATCATCG

166	AC83R	TAATACGACTCACTATAGGGTCTAGACAGCAGCGTCCGTCTTACT
167	AC84L	TAATACGACTCACTATAGGGCTCGAGCACCAACGCAGACGAACC
168	AC84R	TAATACGACTCACTATAGGGTCTAGAGGCACGGGAACACATTTTAG
169	AC85L	TAATACGACTCACTATAGGGCTCGAGCGGTTGTTTAGTGGGTGACAT
170	AC85R	TAATACGACTCACTATAGGGTCTAGAGCGGCACTTTTCTTTGTAGC
171	AC86L	TAATACGACTCACTATAGGGCTCGAGATGAAGCTGTTAACAATTTTGAT
172	AC86R	TAATACGACTCACTATAGGGTCTAGATTAACACCATTTCCAATATACGT
173	AC87L	TAATACGACTCACTATAGGGCTCGAGGCAAGCAGAAACAACGAA
174	AC87R	TAATACGACTCACTATAGGGTCTAGATTCAAAGTCAAACCCGACA
175	AC88L	TAATACGACTCACTATAGGGCTCGAGATGAATACAAGGGGTTGTGT
176	AC88R	TAATACGACTCACTATAGGGTCTAGATTAACTGTTTACATAACATTCTACT
177	AC89L	TAATACGACTCACTATAGGGCTCGAGACGACGCAGAAGACAACAA
178	AC89R	TAATACGACTCACTATAGGGTCTAGACGATTTTAGCGCCTTTTCAG
179	AC90L	TAATACGACTCACTATAGGGCTCGAGTGAGAAGTCAACCCAATCGTT
180	AC90R	TAATACGACTCACTATAGGGTCTAGAGGCTATTCCTCCACCTGCTT
181	AC91L	TAATACGACTCACTATAGGGCTCGAGTACGGCGATTTTGTGATTGA
182	AC91R	TAATACGACTCACTATAGGGTCTAGAGGCAGTTTGATTTCTTTGCTTT
183	AC92L	TAATACGACTCACTATAGGGAAGCTTCGCCTTCTCCTATTCCTCCT
184	AC92R	TAATACGACTCACTATAGGGTCTAGACGGATACAACGGGCAGTC
185	AC93L	TAATACGACTCACTATAGGGCTCGAGTGCCAGCCCAAGAGTTTTA
186	AC93R	TAATACGACTCACTATAGGGTCTAGACGTAATGTCTGTGCCGTATTT
187	AC94L	TAATACGACTCACTATAGGGCTCGAGACGACCAATGCGACAAGTTT
188	AC94R	TAATACGACTCACTATAGGGTCTAGAGCCACGACGATTTTACTGCT
189	AC95L	TAATACGACTCACTATAGGGCTCGAGTCGTGTTACTTATCGTTTTGCTC
190	AC95R	TAATACGACTCACTATAGGGTCTAGAGCGTTTGGTGATTTGATTCC
191	AC96L	TAATACGACTCACTATAGGGCTCGAGCCCTGTGGATTTGTTTGTG
192	AC96R	TAATACGACTCACTATAGGGTCTAGATCTGAGGCTTTTGAGGTTGA
193	AC97L	TAATACGACTCACTATAGGGCTCGAGTGTTGTTTGGCGCGTATGTA
194	AC97R	TAATACGACTCACTATAGGGTCTAGAGTGATCGGGCACGTTATTTT
195	AC98L	TAATACGACTCACTATAGGGCTCGAGATGGCTACGTGGATTTGTTG
196	AC98R	TAATACGACTCACTATAGGGTCTAGACTATTCATTGTCGCTGTCTTC
197	AC99L	TAATACGACTCACTATAGGGCTCGAGTAACGACGCCATCATCAAAC
198	AC99R	TAATACGACTCACTATAGGGTCTAGATCCAAATCAAACACGACCAC
199	AC100L	TAATACGACTCACTATAGGGCTCGAGCCATCTGTTTCACTCGTTG
200	AC100R	TAATACGACTCACTATAGGGTCTAGATTTGGGCGGCTTTATGTC
201	AC101L	TAATACGACTCACTATAGGGCTCGAGATGGTTTATCGTCGCCGTC

202	AC101R	TAATACGACTCACTATAGGGCTCAGATTAATAGTAGCGTGTTCTGTAAC
203	AC102L	TAATACGACTCACTATAGGGCTCGAGCCGCAACAATACATCAACA
204	AC102R	TAATACGACTCACTATAGGGTCTAGACGACGACTGAACTCCCAAA
205	AC103L	TAATACGACTCACTATAGGGCTCGAGATGATTGCTTCAATAAATGATACCG
206	AC103R	TAATACGACTCACTATAGGGTCTAGATTACTCTGTAACTATCACTTGGATC
207	AC104L	TAATACGACTCACTATAGGGCTCGAGTCAAGCCAACAACACAGACA
208	AC104R	TAATACGACTCACTATAGGGTCTAGATCGAAGCAACACGTACAACA
209	AC105L	TAATACGACTCACTATAGGGCTCGAGCAGGAAGCGGAGAGATTGAG
210	AC105R	TAATACGACTCACTATAGGGTCTAGACGTCTTGACCAGGGAATTGT
211	AC106L	TAATACGACTCACTATAGGGCTCGAGCGTAATGCCCGAAGATGAGA
212	AC106R	TAATACGACTCACTATAGGGGGATCCTCGCCGTAGACAATAAAACC
213	AC107L	TAATACGACTCACTATAGGGCTCGAGATGGACGATTCGATAGATTATA
214	AC107R	TAATACGACTCACTATAGGGTCTAGATCATTGTAATGCTGAAGCATTG
215	AC108L	TAATACGACTCACTATAGGGCTCGAGATGTTTGGAAAATCTAGACAATC
216	AC108R	TAATACGACTCACTATAGGGGGATCCTTACAAAGAAAACAAAGGCAATAA
217	AC109L	TAATACGACTCACTATAGGGCTCGAGATGAAACCGACGGCGGC
218	AC109R	TAATACGACTCACTATAGGGTCTAGATTATATTGTTGCATTTCTATTTCTA
219	AC110L	TAATACGACTCACTATAGGGCTCGAGTGCCCGTTTCAGATTCAAGT
220	AC110R	TAATACGACTCACTATAGGGTCTAGATGGGACATTTGGGGTTTTC
221	AC111L	TAATACGACTCACTATAGGGCTCGAGATGAAATATTTTCTGTCTGCTACC
222	AC111R	TAATACGACTCACTATAGGGTCTAGATTATTTTAATTTGTGAACTCGTACC
223	AC112L	TAATACGACTCACTATAGGGCTCGAGATGGATAATTATTCGGTGCA
224	AC112R	TAATACGACTCACTATAGGGTCTAGATTATTTATATTTGTTTTCTTTGTTAT
225	AC113L	TAATACGACTCACTATAGGGCTCGAGATGACTAAACGACAATTTGCTT
226	AC113R	TAATACGACTCACTATAGGGTCTAGATCAATTTTTGCCAGCACGT
227	AC114L	TAATACGACTCACTATAGGGCTCGAGCGTATTGTTTTGAGCCGTTTT
228	AC114R	TAATACGACTCACTATAGGGTCTAGACAAGGTTTCATCGTGTTGTCC
229	AC115L	TAATACGACTCACTATAGGGCTCGAGACGGCAATGAAAATCTGGTG
230	AC115R	TAATACGACTCACTATAGGGTCTAGACGTCCTGGGGATGTAAACTG
231	AC116L	TAATACGACTCACTATAGGGCTCGAGCGAGGCGTAGATTGTTCATT
232	AC116R	TAATACGACTCACTATAGGGTCTAGATCGGTGTTCCCGTATCGT
233	AC117L	TAATACGACTCACTATAGGGCTCAGAATGTATTTCACGTCCCGCTT
234	AC117R	TAATACGACTCACTATAGGGTCTAGATTAACTGAGTGTGAAAAGCGT
235	AC118L	TAATACGACTCACTATAGGGCTCGAGATGCATCTGACGGCTAATG
236	AC118R	TAATACGACTCACTATAGGGTCTAGATTATACACAATTTTTTTGGTAATAC
237	AC119L	TAATACGACTCACTATAGGGCTCGAGATGCCGTGTTTAGCAATTTT

238	AC119R	TAATACGACTCACTATAGGGTCTAGATACAACGCCACATCTATTCC
239	AC120L	TAATACGACTCACTATAGGGCTCGAGTCAAGATGCGTGGAGATG
240	AC120R	TAATACGACTCACTATAGGGTCTAGAGGACGGCAATACGGAGTTT
241	AC121L	TAATACGACTCACTATAGGGCTCGAGATGAGCATTTTAAAAGTTGTAG
242	AC121R	TAATACGACTCACTATAGGGTCTAGATCATTTGATAGTGTAAATTTTTATT
243	AC122L	TAATACGACTCACTATAGGGCTCGAGATGATGTCATCATCACAAATAATAG
244	AC122R	TAATACGACTCACTATAGGGTCTAGATTAACAATTACTACGGCGCA
245	AC123L	TAATACGACTCACTATAGGGCTCGAGATGAAACTGATTATCCTGCTG
246	AC123R	TAATACGACTCACTATAGGGTCTAGATTACAAATAAATTTTACATTGAATCA
247	AC124L	TAATACGACTCACTATAGGGCTCGAGCAAGTTTGTTGATTTGGTG
248	AC124R	TAATACGACTCACTATAGGGTCTAGAGTCAATTTAGGATGGCCTTG
249	AC125L	TAATACGACTCACTATAGGGCTCGAGAACCACAGAAGCCCATGAAG
250	AC125R	TAATACGACTCACTATAGGGTCTAGAGTACAAAGCGCACACAAACG
251	AC126L	TAATACGACTCACTATAGGGCTCGAGAAGTCGTGGGATTAACAACG
252	AC126R	TAATACGACTCACTATAGGGTCTAGATCAACTTTCTCGCCAACT
253	AC127L	TAATACGACTCACTATAGGGCTCGAGCGAGCAGCAGGTATGGAAAG
254	AC127R	TAATACGACTCACTATAGGGTCTAGATTTGAGGGCGTCGTTTATTC
255	AC128L	TAATACGACTCACTATAGGGCTCGAGCGTCGTCTCAACAAAGTCA
256	AC128R	TAATACGACTCACTATAGGGTCTAGACCGACAAGGCGTAACAAATC
257	AC129L	TAATACGACTCACTATAGGGCTCGAGGAGACGGACTACAACGAAAA
258	AC129R	TAATACGACTCACTATAGGGTCTAGATGTTATTCTGCCGCTTCACC
259	AC130L	TAATACGACTCACTATAGGGCTCGAGCCAAAGTCATGTCACCTTTTCT
260	AC130R	TAATACGACTCACTATAGGGTCTAGACTCTCCTGAATCGCACACA
261	AC131L	TAATACGACTCACTATAGGGCTCGAGATGAACTTTTGGGCCACGTT
262	AC131R	TAATACGACTCACTATAGGGTCTAGATTATTGAACGTTTAGCACGTTGTT
263	AC132L	TAATACGACTCACTATAGGGCTCGAGTTGGATCGACACGGACTACA
264	AC132R	TAATACGACTCACTATAGGGTCTAGACGAGGTCTGCAATGAGGAG
265	AC133L	TAATACGACTCACTATAGGGCTCGAGTCCGACAAAACACCAACAAA
266	AC133R	TAATACGACTCACTATAGGGTCTAGATTCACGAATGCGACAATAAAA
267	AC134L	TAATACGACTCACTATAGGGCTCGAGATGTTTGCGTCGTTGACCT
268	AC134R	TAATACGACTCACTATAGGGTCTAGATGCCGTTGGTTTCTTTGAC
269	AC135L	TAATACGACTCACTATAGGGCTCGAGCTGCTGTCTTTTGTTCGGTTC
270	AC135R	TAATACGACTCACTATAGGGTCTAGATGACTTGTTGCGATTGTGC
271	AC136L	TAATACGACTCACTATAGGGCTCGAGTGCGCGACAGAATAAAATCA
272	AC136R	TAATACGACTCACTATAGGGTCTAGATGAGCAAACGGCACAATAAC
273	AC137L	TAATACGACTCACTATAGGGCTCGAGCGCGTTTGTGCATATTTTAG

274	AC137R	TAATACGACTCACTATAGGGTCTAGAGGTAACAGCCATTCGTTTTC
275	AC138L	TAATACGACTCACTATAGGGCTCGAGATGTCAAAGCCTAACGTTTTG
276	AC138R	TAATACGACTCACTATAGGGTCTAGATTACTTGGAACTGCGTTTACC
277	AC139L	TAATACGACTCACTATAGGGCTCGAGCCGAAACCCAAGTTCAGTC
278	AC139R	TAATACGACTCACTATAGGGTCTAGAGCGATTCCCACCATCTAA
279	AC140L	TAATACGACTCACTATAGGGCTCGAGAGCACAGCAGCATTGTAAG
280	AC140R	TAATACGACTCACTATAGGGTCTAGATCGTCCAACGGCTTTTC
281	AC141L	TAATACGACTCACTATAGGGCTCGAGATGGAAACCTTATCTATAGAGATA
282	AC141R	TAATACGACTCACTATAGGGTCTAGATTATACTATCCGGCGCGC
283	AC142L	TAATACGACTCACTATAGGGCTCGAGCATCCGTTCCGACTGTATC
284	AC142R	TAATACGACTCACTATAGGGTCTAGAGCGGGGTTTTCTTCAATCAC
285	AC143L	TAATACGACTCACTATAGGGCTCGAGTGCGTCATCGCCATATTC
286	AC143R	TAATACGACTCACTATAGGGTCTAGATACGCGCTTTCAATCTCTTT
287	AC144L	TAATACGACTCACTATAGGGCTCGAGCATCCGTTCCGACTGTATCT
288	AC144R	TAATACGACTCACTATAGGGTCTAGACTCATAGCGGGGTTTTCTTC
289	AC145L	TAATACGACTCACTATAGGGCTCGAGATGTTCTTGACCATCTTGGC
290	AC145R	TAATACGACTCACTATAGGGTCTAGACTACAACATTTGCCTTTGAGG
291	AC146L	TAATACGACTCACTATAGGGCTCGAGACTGAAACCAACGACACAAGC
292	AC146R	TAATACGACTCACTATAGGGTCTAGATGCTAAAATCGCTCAAATCCA
293	AC147L	TAATACGACTCACTATAGGGCTCGAGATGAATCAAATTCATCTAAAGTGTC
294	AC147R	TAATACGACTCACTATAGGGTCTAGATCATAGTAACAAGTTTCTATACATGC
295	AC148L	TAATACGACTCACTATAGGGCTCGAGCCCTAATGGCGAACACGATA
296	AC148R	TAATACGACTCACTATAGGGTCTAGAGCGTTACCACAAATCCCAAC
297	AC149L	TAATACGACTCACTATAGGGCTCGAGAACCAACCATCGGCAACT
298	AC149R	TAATACGACTCACTATAGGGTCTAGATTCAAACGGCTTCACTTCAC
299	AC150L	TAATACGACTCACTATAGGGCTCGAGTCAACGCCATCAATAACACC
300	AC150R	TAATACGACTCACTATAGGGTCTAGAGCCAACTAAATCACCAAACGA
301	AC151L	TAATACGACTCACTATAGGGCTCGAGATGAATATTATAATTAACATGTTGG
302	AC151R	TAATACGACTCACTATAGGGTCTAGATCACAGTAAAGGTTTTGCAAAAC
303	AC152L	TAATACGACTCACTATAGGGCTCGAGATGTTAAAACCCAACATGTTAAT
304	AC152R	TAATACGACTCACTATAGGGTCTAGATTAGTTTTGGTTAGCGGTACA
305	AC153L	TAATACGACTCACTATAGGGCTCGAGCCCGAAGAAGAAGTAGAGGTT
306	AC153R	TAATACGACTCACTATAGGGTCTAGAAGTCCGTGGTGTGATTGT
307	AC154L	TAATACGACTCACTATAGGGCTCGAGATGAATTTTTTTGCAATGCAAAA
308	AC154R	TAATACGACTCACTATAGGGTCTAGACTAGGCTGTGAGGCTTGAAC
309	AC155L	TAATACGACTCACTATAGGGCTCGAGCATAAACCGCCGAGACAGA

310	AC155R	TAATACGACTCACTATAGGGTCTAGAGCACAATGGACAGCACACA
311	AC156L	TAATACGACTCACTATAGGGCTCGAGATGGATAGTAGTAATTGTATTAAAA
312	AC156R	TAATACGACTCACTATAGGGTCTAGATTAAATTTTTATTATGCAAGAATTG

4.2.4 Generation of dsRNA by *in vitro* transcription: We used 'Megascript in-vitro transcription kit' to generate RNA from DNA template. The purified PCR products were used as template in *in vitro* transcription. Approximately 2pmoles of template was used in each reaction containing 1.5µl of 10X reaction buffer, 1.5µl each of 75mM ribonucleotide solutions (ATP, CTP,GTP & UTP), 1.5µl of T7 RNA polymerase and appropriate volume of Water to round up the volume to 15µl. the reaction mixture was incubated at 37^0C for 12hours. The product was confirmed by 2% Agarose gel electrophoresis. The RNA product was treated with TurboDNA free DNase1 to remove traces of DNA template. RNA was precipitated by adding $1/10^{th}$ volume of 3M Sodium Acetate (pH-5.2) and equal volume of iso-propanol and centrifuging for 20minutes at 14000g at 25^0C. Pellet was washed with 75% ice cold ethanol and air dried. RNA pellet was dissolved in RNase free water (DEPC treated and Double autoclaved MI.Q. biocel grade water).

To ensure the formation of dsRNA from the in vitro transcribed RNA, the tubes containing both sense and antisense strands were heated to 95^0C for 5minutes and allowed to cool slowly in a water bath or heat block. Formation of double stranded RNA was confirmed by electrophoresis of the dsRNA on 2% agarose gel.

4.2.5 Transfection of dsRNA into sf9 cells: Trans-messenger transfection reagent (qiagen) was used to transfect dsRNAs into sf9 cells. Sf9 cells (2.5 X 10^5per well) were seeded in 48 well plates and incubated at 27^0C in a humidified chamber until they grow to a confluency of 70% then cells were transfected with 250ng of dsRNA according manufacturer's protocol as described in section 2.8.2 in Chapter 2.

4.2.6 Infection of transfected sf9 cells with vRecRed-S: virus titer was estimated and required amount of virus was resuspended in complete medium and

overlaid on sf9 cells. A volume of 0.5ml of virus stock was used to infect cells in a 60mm dish. The volume was increased or decreased according to the culture area (for details of virus infection see section 2.2.3). Cells were overlaid with virus suspension for one hour and replaced with complete medium. For our screening experiments described in this chapter we infected dsRNA transfected cells with an MOI of 5.

4.2.7 Estimation of viral Titer: viral DNA was isolated from infected sf9 cells and viral titer was estimated by real time PCR based method. Details of the method are mentioned in section 2.4 of general materials and methods.

4.2.8 Microscopy and imaging of infected cells: vRecRed-S infected cells were observed under the Leica stereo microscope in which whole well of infected cells can be visualized. An excitation wavelength of 558nm was used to visualize expression of RFP expressed by vRecRed-S infected cells. Images were captured using digital camera through Leica ASD-3D system.

4.2.9 Analysis of images: Images of a broad area of culture dish containing cells was captured under same exposure and magnification using LeicaASD-3D microscope with camera (IC 3D 134881106) (Image capture settings: 16 bit/channel, 3132 X 2325 full frame HQ , color). Captured images were converted into grey scale images using a scale of '0' to '255' (0-black & 255-white). The pixels were calculated using image analysis software "LAS AF Version; 1.8.1 build 1390" (Leica microsystems CMS Gmbh). This method was used only in relative quantification as this is not an accurate method for absolute quantification of fluorescence from microscopic images(Baghdoyan *et al.* 2004; Budovskaya *et al.* 2008). The pixel values were used in analyzing the differences in expression and plotted in the bar diagram in the form of arbitrary units. Usually cells transfected with dsRNAs designed against EGFP were considered as controls, Fluorescence of these control cells was taken as 100 and all others were represented as ratios of 100.

4.3 Results and discussion:

To investigate the essential genes of AcMNPV, We generated dsRNAs against each of the 156 orfs to knock down each one of them and observed its effects on total virus production.

4.3.1 dsRNAs generated against viral orfs, *egfp* & *rfp* are not toxic to cells: A major concern in RNAi experiments is to achieve specificity to desired targets and to avoid other nonspecific effects due to sequence complementarity or due to sequence content.

Non specific effects due to sequence similarity can be seen in small RNAs because perfect complementarity is not an essential criterion for siRNAs to act. It is known that siRNAs can regulate their target mRNAs with partial complementarities and suppress them like miRNAs. Even siRNA like activity can be seen with partially complementarity to their targets. Nonspecific effects due to sequence content are also reported. Federov *et. al* reported that siRNAs with particular domains cause cells death without inducing interferon response (Fedorov *et al.* 2006). dsRNAs are known to be potent inducers of interferon response in mammalian cells. So in mammalian cells smaller RNAs (siRNA) are used which can induce RNAi avoiding the induction of interferon response. But there are few reports showing induction of interferon response even by siRNAs also, therefore it is always necessary and safe to check for any nonspecific response before using such siRNAs or dsRNAs for knockdown experiments. Insect cells lack interferon like immune response and this reduces at least one possibility of nonspecific response but other non-sequence specific toxic effects cannot be ruled out. The dsRNAs which are transfected into cells are processed by endogenous dicer and pools of small RNAs are produced. Since we don't have any control over the sequence of small RNA produced inside a cell and there are always chances of getting nonspecific effects.

In order to see whether these dsRNAs produced by in-vitro transcription induce any non specific toxic effects in host cells, we transfected each of these dsRNAs (dsRNAs designed against 156 viral orfs, *egfp* & *rfp*) into sf9 cells and incubated

them for 72 hours (**Figure 4.1**). After 72 hours, we observed them under microscope for cell death or severe changes in morphology but could not see any significant difference between untransfected (no dsRNA and no transfection reagent), mock transfected (no dsRNA but transfection reagent) and transfected cells (dsRNA with transfection reagent). A representative picture of cells is shown in **Figure 4.1** which shows results of cells transfected with one of the 156 dsRNAs. The results showed that none of these dsRNAs were toxic to sf9 cells and did not induce cell death. This was true at least for the dsRNA concentration that we used for transfection. This ensured that whatever effects that we are going to see in our future experiments will be specific to the dsRNAs we generated.

4.3.2 Analysis of off target /nonspecific activity of dsRNAs designed against *egfp* and *rfp*:

RNAi based screening for essential genes of virus: AcMNPV infection to sf9 cells always leads to cell death within 5 days of infection. So far there are no reports showing rescue of sf9 cells from AcMNPV infection. AMNPV has 156 predicted orfs and all of them are not really essential for completing its infection cycle. Therefore it was necessary to identify which of these genes are more important for the virus than others.

Analysis of off target /nonspecific activity of dsRNAs designed against *egfp* and *rfp*: Lately there are reports from Westenberg *et al* ((Westenberg *et al.* 2005) and kim *et al* (Kim *et al.* 2007) showing that siRNAs and dsRNAs induce nonspecific resistance to white spot syndrome virus infection. They found that siRNAs and dsRNAs designed for *egfp* could also induce resistance to virus infection like siRNAs and dsRNAs designed against viral genes. Kim et al also showed that dsRNAs against viral genes induced more resistance than *egfp* dsRNAs.

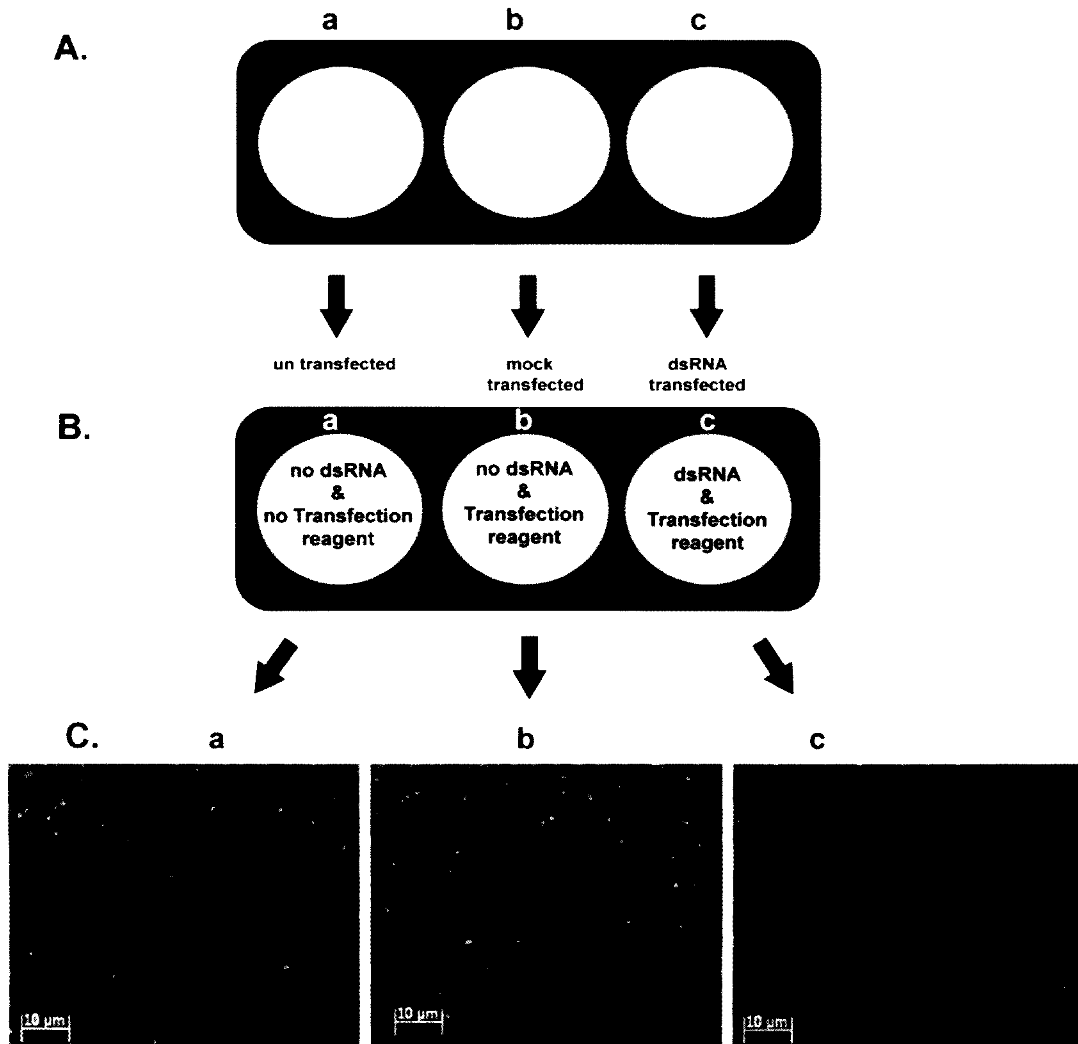


Figure 4.1:Analysis of dsRNA toxicity in *sf9* cells

sf9 cells were transfected with dsRNAs corresponding to all 156 viral orfs, *egfp* and *rfp* (C) seperately. After 72 hours cells were observed under microscope for excessive cell death or severe change in morphology of cells.

No significant difference was found in untransfected (a), mock transfected (b) and dsRNA transfected cells (c) with respect to morphology or cell death.

So there seems to be a convergence of sequence specific RNAi and other innate immune responses that are probably enhanced by dsRNAs (Robalino *et al.* 2005; Shekhar *et al.* 2009). So we wanted to verify whether such non sequence specific induction of immune response happens in our experimental set up.

We transfected dsRNAs designed against *egfp* and *rfp* with Trans-messenger transfection reagent into sf9 cells. After 24hours of transfection these cells were infected with vRecRed-S virus (MOI-5). After 72 hours these cells were observed for fluorescence of RFP under microscope. (**Figure 4.2A**) we could not find any significant difference in RFP fluorescence between mock transfected cells (no dsRNA) and egfp dsRNAs transfected cells whereas cells transfected with dsRNA against RFP showed significant reduction in fluorescence (**Figure 4.2 B and C**).

We harvested these cells, centrifuged and collected the supernatant containing BV of vRecRed-S and estimated the titer of the virus in all three sets of experiments. We could not see any significant difference in the titer of the virus between untransfected and those transfected with dsRNA against *egfp* and *rfp* (**Figure 4.2 D**). These results show that dsRNA against *egfp* and *rfp* do not affect Budded virus production but dsRNAs designed against *rfp* can silence *rfp* gene expression without affecting virus infection and BV production. These results also support that dsRNAs against *egfp* do not show any off target nonspecific effects and does not induce any nonspecific resistance to AcMNPV (vRecRed-S) infection insf9 cells hence they can be used as a control for other RNAi based screening experiments by viral orf specific dsRNAs transfection. dsRNAs against *rfp* were also very specific to their target, they knocked down only *rfp* in the recombinant virus without suppressing the virus infection as such again ruling out the possibility of induction of various immune responses.

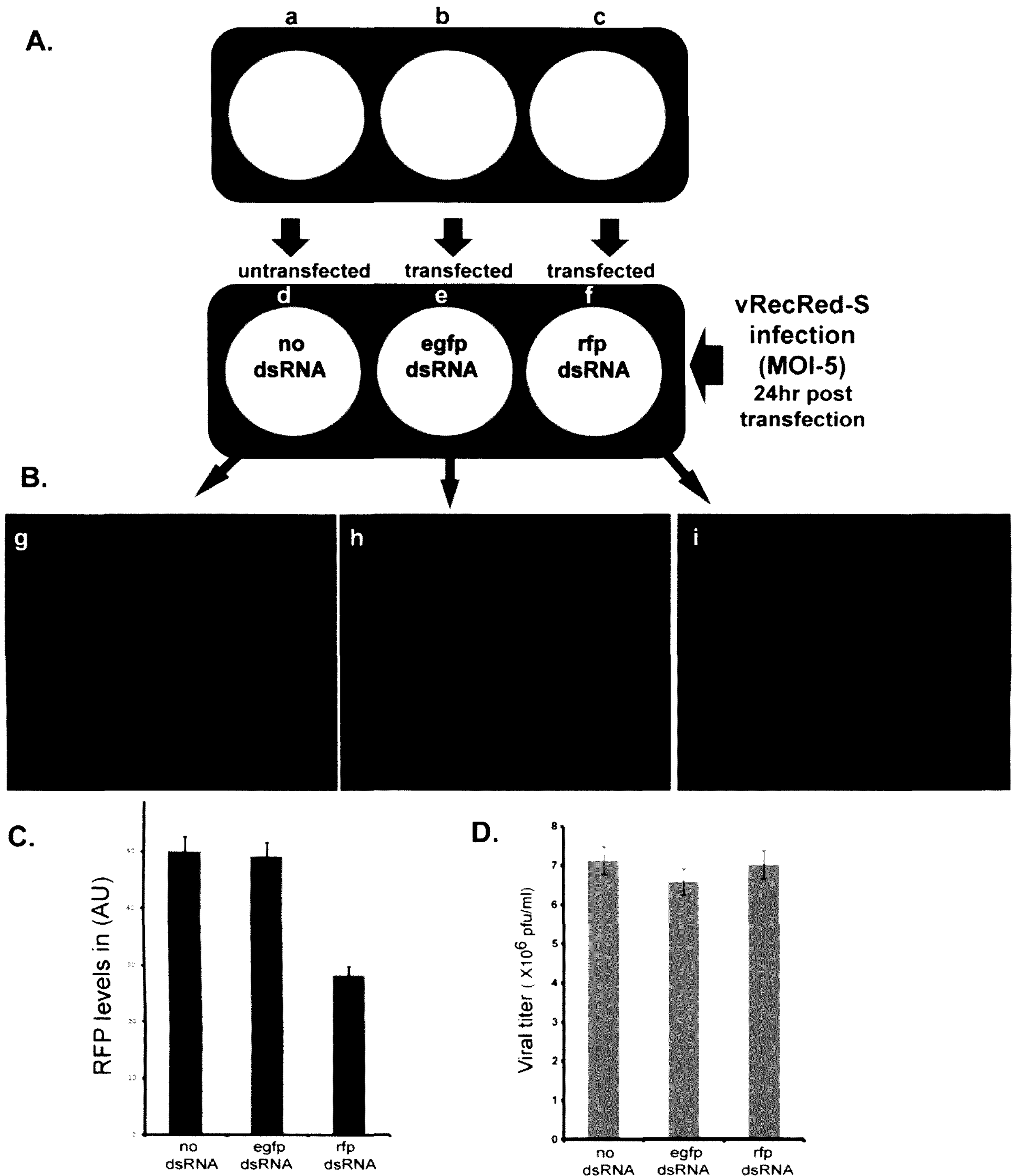


Figure 4.2: Analysis of non specific effects of dsRNAs on viral titer.

A. An overview of the experiment: cells were transfected with dsRNA of EGFP and RFP (b and c) or no dsRNA (a), and infected with vRecRed-S (d,e & f) (MOI-5). After 72 hours of infection cells were observed under fluorescent microscope for RFP expression.

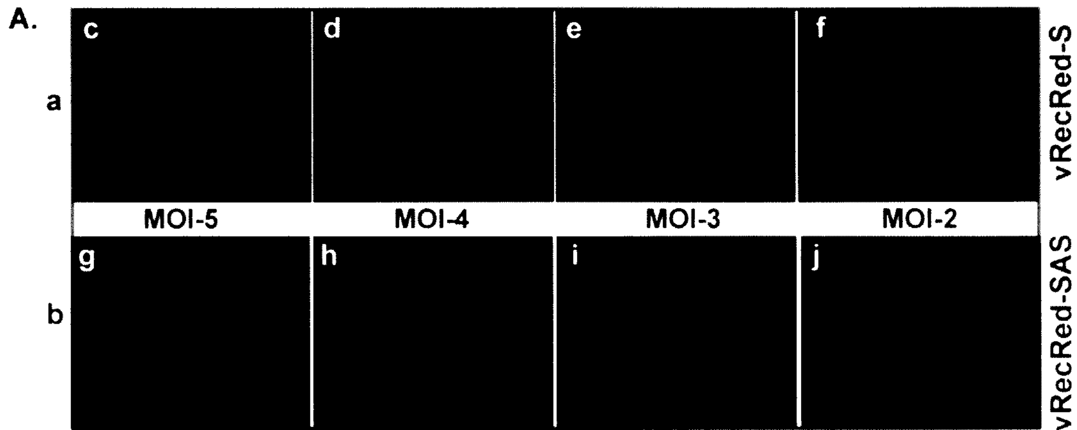
B. Fluorescent streomicroscopic images of Cells showing RFP expression after 72hrs PI. RFP-dsRNA transfected cells (i) express lesser RFP than EGFP-dsRNA transfected (h) and untransfected (g) cells.

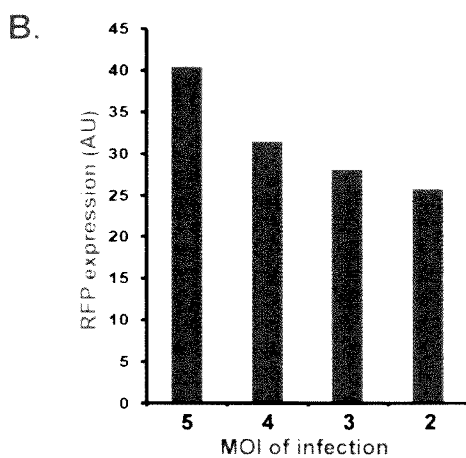
C. A bar diagram showing the levels of expression of RFP in cells shown in panel B (g,h & i).

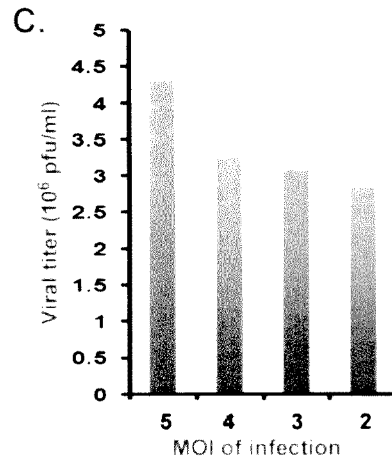
D. A bar diagram showing the titer of virus in cells shown in panel B (g,h & i)

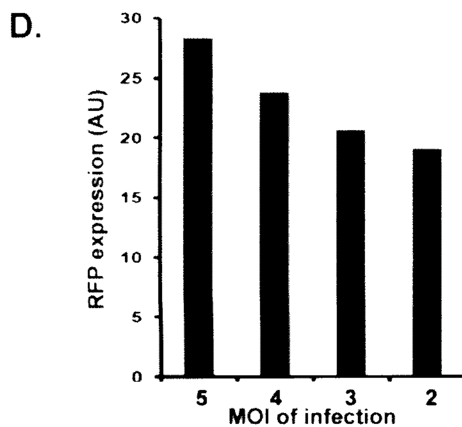
4.3.3 Fluorescence of RFP and viral titer are correlated: The vRecRed-S virus was used in our screening experiments because it is convenient to observe the cells infected with this recombinant virus and the RFP expressed under the very late p10 promoter can be used as an indirect measure of viral titer. In order to draw a positive correlation between fluorescence of RFP in vRecRed-S infected cells and the amount of virus produced in those cells, we infected sf9 cells with different dilutions of virus and observed for fluorescence after 72hrs (**Figure 4.3 A c-f & B**). Simultaneously viral titer was estimated in these cells (**Figure 4.3 C**) comparing the fluorescence and the viral titer we found significant correlation between these two parameters. So fluorescence can be used as an indirect measure of viral titer, at least in those experiments where the expression of RFP is not specifically inhibited, as in the case of RFP-dsRNA transfected cells (**Figure 4.2**). Similarly we found correlation in fluorescence and viral titer in vRecRed-SAS infected sf9 cells also (**Figure 4.3A g-j, D & E**)

In our experiments mentioned in this section and in the next section, we captured images of cells infected with vRecRed-S virus by using a stereomicroscope. This covered most of the area of a 24 well plate containing cells (~$8X10^5$ cells per each well). Images were captured using same conditions of exposure & magnification. And the fluorescence was estimated using image analysis software "LAS AF Version; 1.8.1 build 1390" (Leica microsystems CMS Gmbh). This method of quantification is not a very accurate method for absolute estimation but we used it to measure only relative differences in fluorescent intensity. The low magnification and the large no of cells captured in each image gave us very consistent results and a good correlation between the estimated fluorescence and viral titer was seen (**Figure 4.3**). So it can be used as a reliable method to measure viral titer in large scale screening experiments where estimation of viral titer by other sensitive and reliable methods may be difficult to perform (Baghdoyan *et al.* 2004; Budovskaya *et al.* 2008). The only possibility of getting false positive results by this method is observed in those cases where RFP expression under *p10* promoter is specifically affected. An example of this type can be seen in **Figure 4.2**.









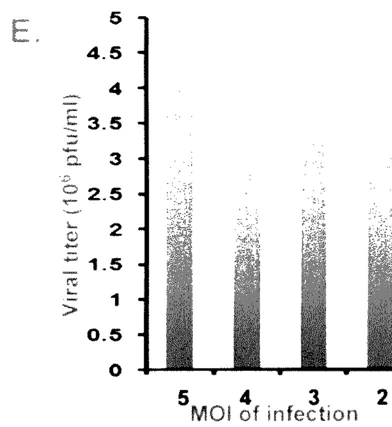


Figure 4.3: Infection of sf9 cells with vRecRed-S and vRecRed-SAS show correlation between RFP levels and viral titer.
sf9 cells were infected with vRecRed-S and vRecRed-SAS viruses with MOI of 5, 4, 3 & 2 and observed after 72hours.

A. Fluorescent stereo microscopic pictures of sf9 cells infected with viruses (vRecRed-S and vRecRed-SAS) at an MOI of 5, 4, 3 & 2.

B & D. A bar diagram showing RFP expression levels in virus infected cells shown in rows (a) and (b) of panel A with vRecRed-S and vRecRed-SAS respectively.

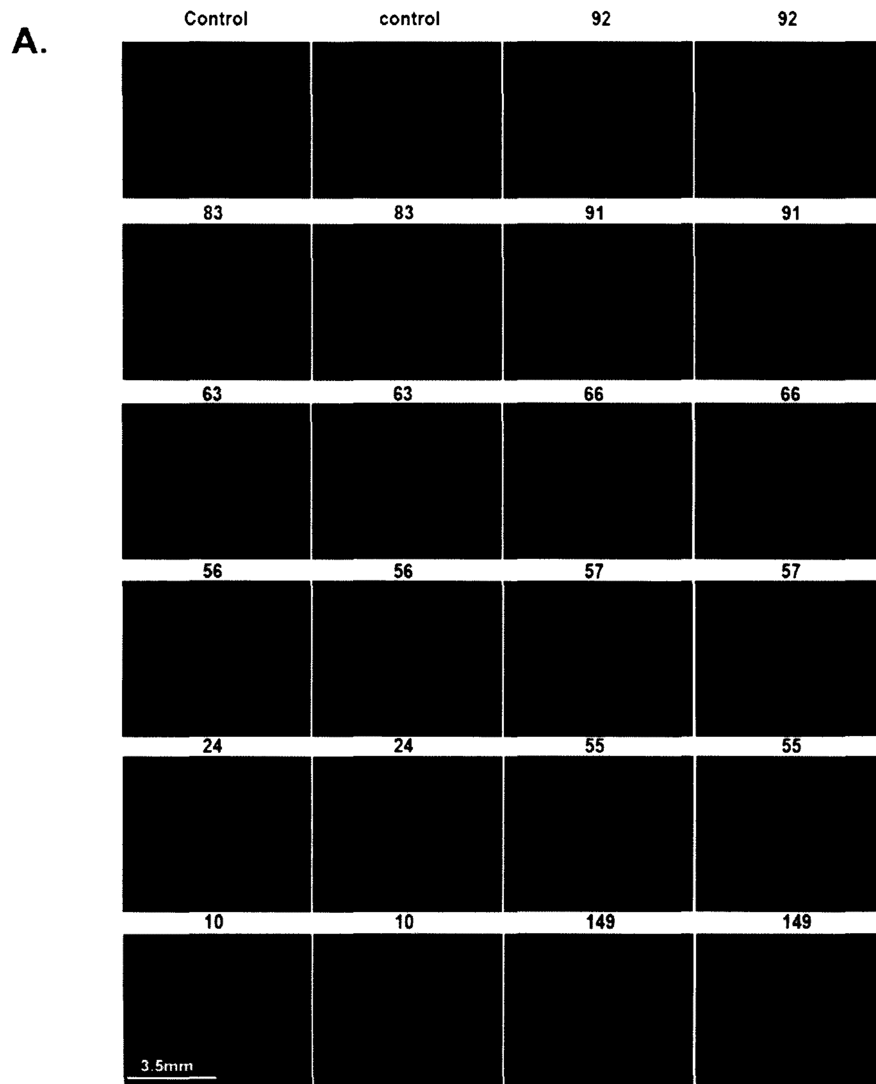
C & E. A bar diagram showing viral titer estimated from cells (c-j) shown in panel A

4.3.4 RNAi mediated screening for essential genes of AcMNPV in sf9 cells: To identify essential genes of AcMNPV we transfected dsRNAs generated against each orf of AcMNPV with trans-messenger transfection reagent. After 24 hours of transfection we infected them with vRecRed-S virus (MOI-5). These cells were observed under fluorescence microscope for RFP expression after 72hours.

Based on the level of expression of RFP in dsRNAs transfected cells compared to the control (*egfp* dsRNA transfected cells) we classified them into three categories. First category consists of orfs showing more than 60% suppression (**Table 4.2**), Second category shows suppression in the range of 40-60% (**Table 4.3**) and a third category which show less than 40% suppression.

Figure 4.4 shows images of a few randomly selected orfs, which belong to the first group i.e., orfs showing more than 60% suppression. The levels of suppression due to silencing of other orfs are indicated in the **Tables 4.2** and **4.3.**

A.



B.

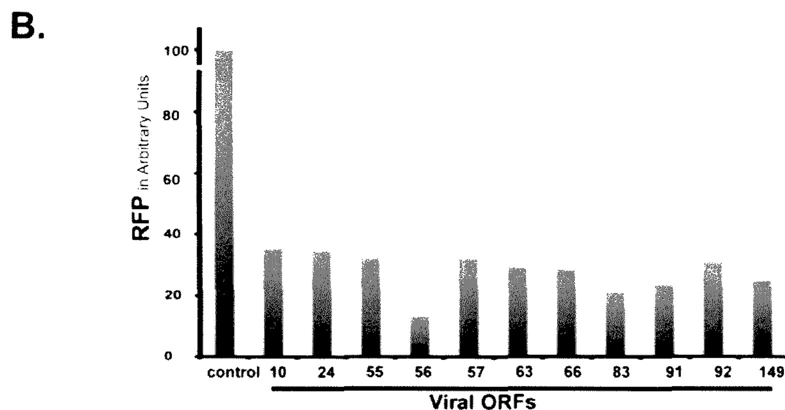


Figure 4.4: Viral orfs suppressing RFP by more than 60%.

A. Fluorescent stereo microscopic pictures of cells transfected with dsRNA against 11 selected viral orfs (indicated above each panel) first 2 panels (control) were transfected with dsRNAs specific to *egfp* and taken as control. Each orf is represented by Two images.

B. A bar diagram showing the Expression levels of RFP in the cells shown in 'A'. RFP levels in *egfp*-dsRNA transfected cells were taken as 100 and all others are represented as ratios of 100.

Table 4.2: List of genes/orfs showing more than 60% suppression.

Orfs are arranged in the order of the suppression of RFP expressed by recombinant virus vRecRed-S. orfs highlighted in green are core genes of Baculoviruses. Orfs highly conserved in Lepidopteran baculoviruses are highlighted in pink (known function) and orfs with unknown functions are highlighted in orange color. Other orfs are not highly conserved across all baculoviruses but are found in AcMNPV.

Product Name	Locus	orf /Locus tag	Suppres-sion of RFP in %
AcOrf-55 peptide	AcOrf-55	ACNVgp056	86.7
late expression factor 3	Ac-lef3	ACNVgp068	84.3
AcOrf-68 peptide	AcOrf-68	ACNVgp069	79.9
telokin-like protein-20	Ac-TLP	ACNVgp083	78.9
AcOrf-96 peptide	AcOrf-96	ACNVgp097	77.8
late expression factor 4	Ac-lef4	ACNVgp091	76.3
late expression factor 1	Ac-lef1	ACNVgp014	75.4
early gene transactivator	Ac-IE-1	ACNVgp149	75.0
polynucleotide kinase/ligase	Ac-PNK/PNL	ACNVgp087	71.4
DNA-dependant DNA-polymerase	Ac-DNA-pol	ACNVgp066	71.3
late expression factor 9; putative DNA-dependant RNA-polymerase beta' subunit	Ac-lef9	ACNVgp063	70.7
protein kinase	Ac-pk-2	ACNVgp124	69.3
apoptosis inhibitor; annihilator	Ac-35K/p35	ACNVgp136	69.1
AcOrf-91 peptide	AcOrf-91	ACNVgp092	68.8
AcOrf-121 peptide	AcOrf-121	ACNVgp122	68.0
AcOrf-56 peptide	AcOrf-56	ACNVgp057	67.8
ecdysteroid UDP-glucosyl transferase	Ac-egt	ACNVgp015	67.7
viral capsid associated protein	AcOrf-54	ACNVgp055	67.0
protein kinase interacting protein	Ac-pkip	ACNVgp024	65.4
late expression factor 5	Ac-lef5	ACNVgp100	64.9

Table 4.2: continued,

Product Name	Locus	orf /Locus tag	Suppres-sion of RFP in %
AcOrf-111 peptide	AcOrf-111	ACNVgp112	64.7
viral capsid associated protein	Ac-ORF1629	ACNVgp009	64.4
protein kinase	Ac-pk-1	ACNVgp010	64.1
major viral capsid protein; vp39	Ac-vp39	ACNVgp090	62.7
AcOrf-58 peptide	AcOrf-58	ACNVgp059	62.5
AcOrf-85 peptide	AcOrf-85	ACNVgp086	62.3
AcOrf-120 peptide	AcOrf-120	ACNVgp121	62.1
p15	Ac-p15	ACNVgp088	61.9
late expression factor 7	Ac-lef7	ACNVgp126	60.8
ssDNA binding protein	AcOrf-25	ACNVgp025	60.4
AcOrf-149 peptide	AcOrf-149	ACNVgp151	60.0

Table 4.3: List of genes showing 40-60% suppression:

Orfs are arranged in the order of the suppression of RFP expressed by recombinant virus vRecRed-S. orfs highlighted in green are core genes of Baculoviruses. Orfs highly conserved in Lepidopteran baculoviruses are highlighted in pink (known function) and orfs with unknown functions are highlighted in orange. Other orfs are not highly conserved across all baculoviruses but are found in AcMNPV.

Product Name	Locus	ORF/Locus_tag	Suppression of RFP in %
cg30	Ac-cg30	ACNVgp089	59.9
AcOrf-124 peptide	AcOrf-124	ACNVgp125	59.3
late expression factor 2	Ac-lef2	ACNVgp006	58.7
FP protein	Ac-FP	ACNVgp062	57.6
AcOrf-11 peptide	AcOrf-11	ACNVgp011	57.3
AcOrf-119 peptide	AcOrf-119	ACNVgp120	56.7
AcOrf-84 peptide	AcOrf-84	ACNVgp085	56.2
viral capsid associated protein	Ac-p95	ACNVgp084	55.2
AcOrf-102	AcOrf-102	ACNVgp103	55.1
AcOrf-66 peptide	AcOrf-66	ACNVgp067	55.1
baculovirus repeated ORF	Ac-bro	ACNVgp002	54.8
AcOrf-59 peptide	AcOrf-59	ACNVgp060	54.1
38K	Ac-38K	ACNVgp099	53.6
putative methyl transferase	AcOrf-69	ACNVgp070	53.2
late expression factor 6	Ac-lef6	ACNVgp028	52.7
very late expression factor 1	Ac-vlf-1	ACNVgp078	52.6
late expression factor 8; putative DNA-dependant RNA-polymerase beta subunit	Ac-lef8	ACNVgp050	52.3
AcOrf-60 peptide	AcOrf-60	ACNVgp061	51.6

Table 4.3: continued,

Product Name	Locus	ORF/Locus_tag	Suppression of RFP in %
helicase	Ac-helicase	ACNVgp096	50.8
AcOrf-93 peptide	AcOrf-93	ACNVgp094	49.6
chitinase	Ac-chitinase	ACNVgp127	49.6
p48	Ac-p48	ACNVgp104	49.2
AcOrf-18 peptide	AcOrf-18	ACNVgp018	49.0
AcOrf-16 peptide	AcOrf-16	ACNVgp016	48.3
nuclear matrix associated phosphoprotein	Ac-39K/pp31	ACNVgp036	48.1
p40	Ac-p40	ACNVgp102	47.9
late expression factor 10	Ac-lef10	ACNVgp054	47.5
AcOrf-17 peptide	AcOrf-17	ACNVgp017	46.9
ORF603 peptide	Ac-ORF603	ACNVgp007	46.7
occlusion-derived virus glycoprotein	Ac-gp41	ACNVgp081	46.6
AcOrf-26 peptide	AcOrf-26	ACNVgp026	46.3
AcOrf-92 peptide	AcOrf-92	ACNVgp093	46.1
late expression factor 11	Ac-lef11	ACNVgp037	43.9
AcOrf-97 peptide	AcOrf-97	ACNVgp098	43.8
fusolin; spindle body protein	Ac-gp37	ACNVgp065	42.5
protein tyrosine phosphatase	Ac-ptp	ACNVgp001	42.5
AcOrf-12 peptide	AcOrf-12	ACNVgp012	42.3
major DNA binding protein; basic protein	Ac-p6.9	ACNVgp101	42.2
fibroblast growth factor	Ac-fgf	ACNVgp032	41.1
AcOrf-13 peptide	AcOrf-13	ACNVgp013	41.0
AcOrf-63 peptide	AcOrf-63	ACNVgp064	40.4

Through this screening method we could pick up candidates which were already proven to be essential. This justifies our method of screening is indeed accurate and works. In addition we have also identified genes whose functions are not known but are essential for virus. This RNAi based screening has its own limitations and the non specific silencing of more than one viral gene cannot be ruled out. But this is not practically possible to verify the cross reactivity of each RNAi experiment with microarray and proteomics, which themselves have several limitations. So these limitations of RNAi have to be accepted and/or compromised in order to narrow down to candidates with a few false positive results in a large scale screening experiment. Even in our experiments, we would like to validate the results of this screening by more sensitive methods like real time PCR & ELISA which are under process. Here we describe our results obtained mainly on the basis of methods described in this chapter.

The results of our RNAi based screening are summarized below. We have focused on the essential genes which are already known in this section because it supports our experimental strategy indeed works and effects observed by knocking down other uncharacterized orfs can be considered as genuine candidates, although further validation of such candidates is necessary.

4.3.4.1 Baculovirus core genes in the screening:

Based on the available genome sequence and earlier literature some 31 genes were found to be highly conserved across baculoviruses. These are considered core genes and some of them are very essential for the virus and perform various basic functions like replication, transcription, packaging and assembly etc. Through our screening method we found 15 of these core genes were able to suppress virus infection by more than 50% and 3 others were marginally less than 50% (genes highlighted in green color in **Table 4.2 and 4.3**). Function of some of these core genes are not yet identified but through our screening, we found orf69 (locus tag-ACNVgp069) and orf 97 (locus tag-ACNVgp097) as very essential genes and their silencing resulted in almost 79.9% and 77.8% suppression of recombinant gene

respectively. Another core gene with unknown function is orf-93 (locus tag-ACNVgp093) showed ~46% suppression.

4.3.4.2 Conserved genes in Lepidopteran baculoviruses other than core genes:

Other than core genes which are found in all known baculoviruses, few other genes are highly conserved only in Lepidopteran baculoviruses also but genes performing similar functions or showing very low sequence similarity to these genes are found in other baculoviruses. These genes might have accumulated changes (mutations) to adapt to their environment and diverged so much that they can no more be grouped under same class. There are about 33 genes which are conserved in Lepidopteran baculoviruses and some of those genes are involved in viral structure, Replication, transcription and in interacting with host to manipulate their metabolism in favor of viruses. Nearly 14 genes of this category were identified in our screening (genes/orfs highlighted in pink color in **Table 4.2 and 4.3**). Functions of about 15 genes out of 33 core genes are not yet known but 5 such genes were selected in our screening (**Table 4.2 and 4.3** highlighted in orange color).

4.3.4.3 Other novel and uncharacterized genes:

Other genes like ACNVgp24, ACNVgp124, ACNVgp126, ACNVgp136 etc are known to be essential for virus infection or at least known to enhance the rate of viral infection such genes are sown in white box in **Table 4.1 and 4.2**

Functions of many AcMNPV genes were predicted based on the comparative genomics studies of highly related viruses. In some cases, essential genes of one virus are found to be nonessential for other viruses, even though they share very high sequence similarity in the functional domains of their proteins. We have found a few genes such as (orf2) in our screening which were earlier thought to be non-essential. We found reasonably good suppression of the virus when they were suppressed by RNAi. One possibility could be nonspecific effects of the dsRNAs targeting that gene. But they can of course be utilized as far as the purpose is to suppress/silence the virus.

4.4 Conclusions:

In conclusion, we would like to mention such RNAi based screening of AcMNPV orfs can be used to screen essential gens of the virus and was justified by picking up many known essential genes of the virus. It revealed some candidates which were earlier thought to be non-essential. Many other orfs whose functions are not known were also picked by us. Based on their efficiency of suppression. They can very weel be used as candidates for targeting viruses. From the available literature we know some of the viral genes are essential which were studied either by deletions or by disrupting those genes by insertion. But it is not known to what extent these essential genes can be targeted by RNAi, in order to suppress the virus. Our method of screening by RNAi itself addressed such aspects and revealed genes which are both essential and susceptible to RNAi mediated suppression, which can be targeted alone or in combination by RNAi to achieve the best possible suppression.

Chapter-5

RNAi based AcMNPV Genome wide screening for Putative Suppressors of RNAi.

Chapter 5: RNAi based AcMNPV genome wide screening for putative Suppressors of RNAi.

5.1 Introduction:

Baculovirus infection of permissive hosts usually results in their death. Very rarely latent and mild infections of baculoviruses are also found. It is evident that Baculoviruses can somehow overcome the general antiviral defense responses of the host and many other Baculovirus genes can manipulate and alter the host metabolism in their favor. Viral genes like ecdysteroid UDP-glucosyltransferase (*egt*), *Ac-fgf, p35 etc*, are known to help virus to manipulate host metabolism. *egt* can delay the molting of larvae so that virus can spread to more cells and produce more ODVs (O'Reilly *et al.* 1989; O'Reilly *et al.* 1990; O'Reilly *et al.* 1998). Programmed cell death is a mechanism by which infected host cells restrict spreading of infection, a viral gene *p35* and other Inhibitor of Apoptosis (IAP) inhibits apoptosis in virus infected cells so that virus can complete its cycle of infection more efficiently. SOD is another viral gene which helps the virus to counter the reactive oxygen species mediated defense of hosts (Tomalski *et al.* 1991; Bergin *et al.* 2005; Katsuma *et al.* 2008). Studies from *Drosophila* and mosquitoes have shown that even insects use RNAi as an antiviral immune response (Li *et al.* 2002; Uhlirova *et al.* 2003; Galiana-Arnoux *et al.* 2006; Deddouche *et al.* 2008). To counter this RNAi mediated host immune response viruses have evolved various mechanisms to escape. Some viruses encode proteins which can inhibit RNAi called suppressors of RNAi, and some viruses transcribe highly structured RNAs which also act as substrates to host RNAi machinery and saturate them so that they cannot degrade essential transcripts or genomic RNA of the virus. Some viruses avoid RNAi by restricting themselves in sub cellular compartments, where access to RNAi machinery is limited.

Since hosts cannot recover from Baculovirus infection it seems obvious that baculoviruses have evolved mechanisms to escape from most, if not all types of immune responses. In this chapter we are describing our experiments performed to

verify whether baculoviruses can escape RNAi and which of the viral genes might contribute to overcome RNAi mediated immune response.

5.2 Material and methods:

All materials used in this chapter and methods fallowed are explained in Materials and Methods section of chapter 4.

5.3 Results and discussion:

Based on the results of chapter 3 and 4 it was evident that Baculoviruses could not be knocked down completely by RNAi. Although there was some reduction in the viral gene expression and viral titer at least in the initial phases of infection, their expression levels and titer revert back to normal or close to normal levels in later stages of infection (Isobe *et al.* 2004). Similar results have been reported elsewhere also where suppression of virus ws observed for a short duration which can only post pone the inevitable death, and it was observed that baculoviruses can overcome RNAi targeted against them.

Baculoviruses might be overcoming RNAi by various mechanisms. Baculoviruses severely infect their natural / permissive hosts and usually lead to death. Upon infection they inhibit host's transcription, translation and manipulate their metabolic pathways to preferentially transcribe and translate viral genes. During this process they might be suppressing genes involved in RNAi pathway in addition to many other immune response genes resulting in suppression of RNAi as the infection progresses. Baculoviruses might encode some genes which can specifically suppress RNAi (suppressors of RNAi) like many other viruses (for detailed description of RNAi suppressors see section 1.1.7 in Chapter 1) and they probably accumulate gradually to overcome RNAi only in later stages of infection. Baculoviruses are known to reside in the nucleus where they replicate and transcribe their genes. Viral DNA replicates with a very high rate and accumulate in very large copies in each cell. This provides large number of templates for transcription which results in accumulation of enormous amount of transcripts. Generally Promoters of most of the late and very late genes are very strong and

they also contribute to the high amount of transcripts found in infected cells. Such a high quantity of transcripts might be too large for the host RNAi machinery to degrade especially under severe pathological conditions. All these factors might be contributing their bit to overcome/escape the RNAi mediated antiviral response of the hosts.

In this chapter we have investigated whether any of the Baculovirus orfs (genes) are capable of inhibiting RNAi. We performed an RNAi based screening of all 156 orfs of AcMNPV to find putative RNAi suppressors.

5.3.1 Generation of Recombinant Sensor viruses: (vRecRed-S and vRecRed-SAS)

We generated two recombinant viruses, vRecRed-S and vRecRed-SAS. Both viruses have two expression cassettes, one of which expresses RFP under *p10* promoter and the second cassette has an *op-ie2* promoter which can express any gene / fragment cloned under it. vRecRed-S expresses a ~300bp fragment of RFP in sense orientation by its *op-ie2* promoter in addition to RFP. Whereas vRecRed-SAS can express the same ~300bp fragment of RFP in both sense and antisense orientation producing dsRNAs. As a result of production of dsRNAs against RFP, cells infected with vReRed-SAS virus express lesser levels of RFP than those infected with vRecRed-S and this is due to RNAi (see **Figure 3.11** in Chapter 3).

As explained in chapter 4 we have knocked down each one of the 156 orfs of AcMNPV and observed its effect on RFP gene expression. Silencing of each orf suppressed virus production to different levels depending on the importance of that gene for virus production, as indicated in **Table 4.1 and 4.2**. So we expected vRecRed-SAS would also show similar levels of suppression for each of these orfs. vRecRed-SAS expresses lesser levels of RFP due to RNAi triggered by dsRNAs encoded in the virus itself, but when we use this virus for screening of essential genes like we did by using vRecRed-S, we expect suppression of RFP in similar ratios, But if we find a difference in levels of RFP suppression then it is logical to think that that particular orf is affecting the expression of RFP (which in this case is

regulated by RNAi) in addition to its role in virus infection. So we designed a strategy to fish out genes which can inhibit RNAi using vRecRed-SAS and vRecRed-s viruses. The outline of the strategy is explained bellow (**Figure 5.1**)

5.3.2 Outline of Experimental strategy for screening of RNAi suppressors:

We planned to transfect cells with dsRNAs against each orf and after 24hours of transfection these cells were infected with vRecRed-S and vRecRec-SAS viruses (MOI-5). After 72 hrs cells will be observed under fluorescence microscope and images will be analyzed. (**Figure 5.1A**) If silencing of an orf per se 'X' (in this case 'X' can be any AcMNPV orf (orf 1-156) inhibits the expression of RFP by ~50% in vRecRed-S infected cells. We expect ~50% reduction in cells infected with vRecRed-SAS also (**Figure 5.1B**). But if the suppression of RFP in vRecRed-SAS infected cells is more than vRecRed-S infected cells (**Figure 5.1C**), then the orf-X must be having some influence on expression of RFP in addition to its role in virus production. Since the RFP expression in vRecRed-SAS infected cells is regulated by RNAi and silencing of orf-X further reduces the RFP levels indicates that orf-X when present (not silenced by dsRNA induced RNAi) was inhibiting RNAi (although partially) and by silencing its (orf-X) expression, the inhibition was removed and RNAi could now act more efficiently. This can be visualized by further decrease in RFP expression in vRecRed-SAS infected cells than vRecRed-S infected cells (**Figure 5.1C**).

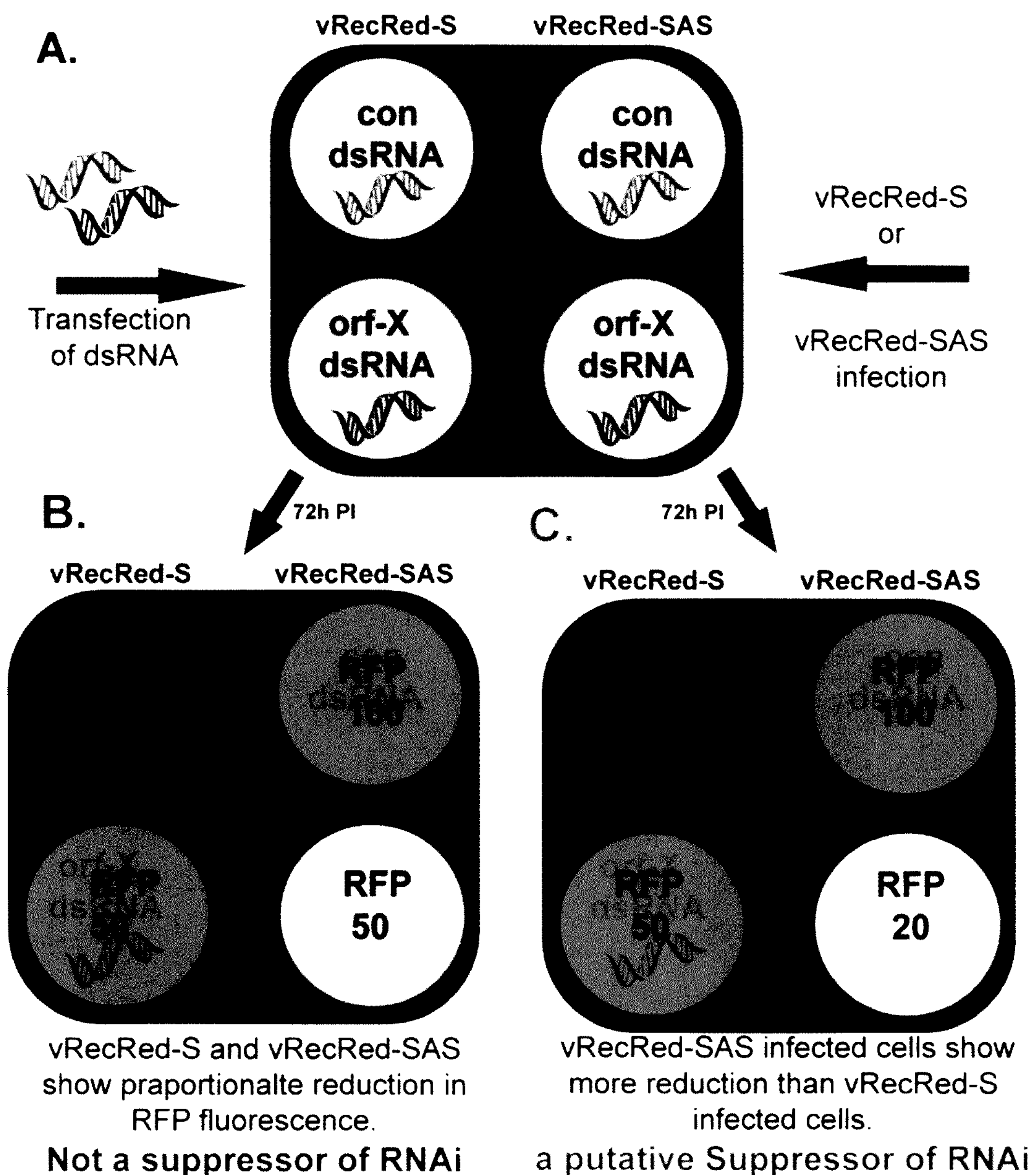


Figure 5.1: Experimental outline of RNAi based screening for putative RNAi suppressors. (orf-X can be any AcMNPV orf 1-156 and numbers indicated in circles are levels of RFP expression in arbitrary units)

A. sf9 cells transfected with dsRNAs (*egfp*-dsRNA in control and orf specific dsRNAs in orf-X)and after 24hours of transfection, cells were infected with vRecRed-S and vRecRed-SAS (MOI-5) in seperate wells. 72hours post infection cells were observed for RFP expression. we expect 2 possible outcomes in these experiments as indicated in B & C.

B. shows one of the two possible outcomes. both vRecRed-S and vRecRed-SAS infected cells show praportionate reduction in the levels of RFP (from 100 to 50 units) and viral titer. orfs fallowing this pattern can not be suppressors of RNAi.

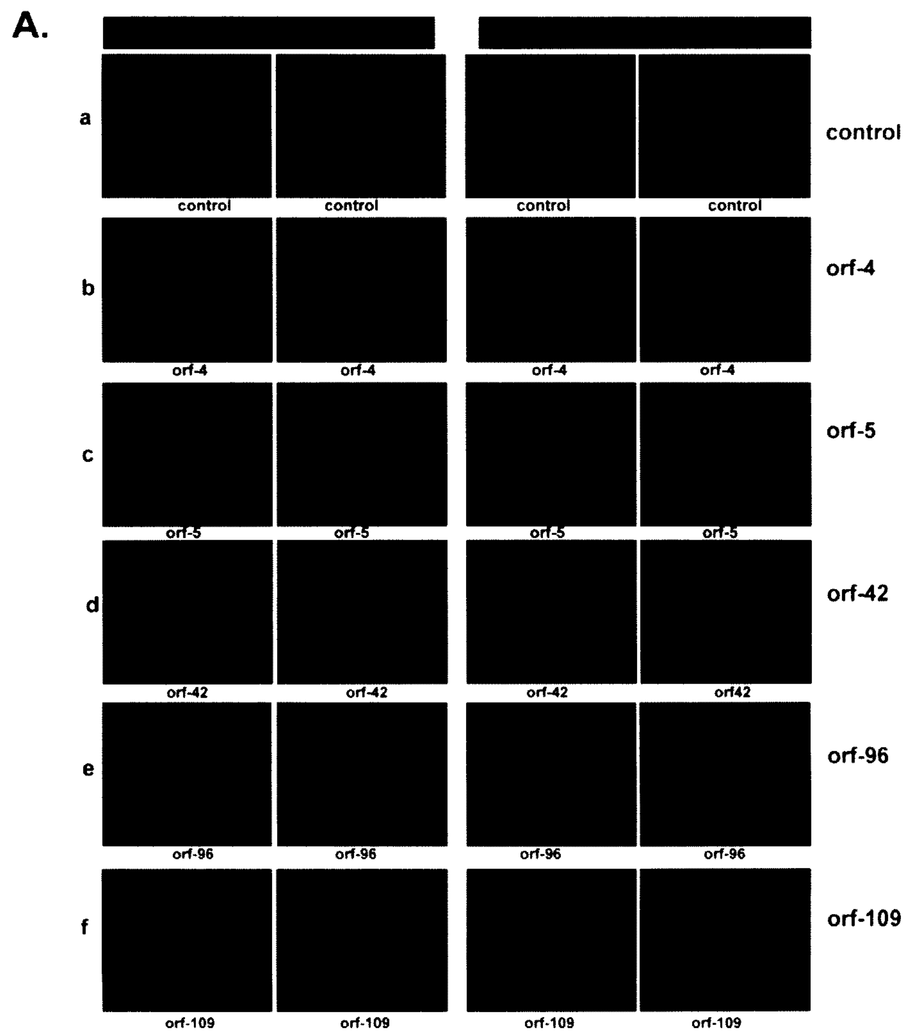
C. shows the other possible outcome. cells infected with vRecRed-SAS show considerable reduction in RFP levels (from 100 to 20 untis) compared to vRecRed-S infected cells (from 100 to 50), without affecting the viral titer. viral orfs following this pattern are putative RNAi suppressors

5.3.3 Putative candidates of RNAi suppressors in AcMNPV

Accordingly we screened all 156 orfs to find out putative Suppresors of RNAi. We transfected dsRNAs corresponding to each orf into sf9 cells and after 24 hours cells were infected with vRecRed-S and vRecRed-SAS viruses in separate wells. After 72 hours cells were observed under microscope for expression of RFP and images were captured and analyzed using image analysis software 'LAS AF Version; 1.8.1 build 1390' (Leica microsystems CMS Gmbh). Viral ORFs showing more than 20% differences were picked up as putative RNAi suppressors (**Table 5.1**) (**Figure 5.2A & B**). The table lists a few candidate genes. Their functions/ probable functions based on the literature are also indicated.

The difference between vRecRed-S and vRecRed-SAS viruses is that the latter expresses dsRNAs. It is possible that expression of dsRNAs might trigger many other immune responses in addition to RNAi. This type of induction of various immune responses may not be happening with vRecRed-S virus. In order to counteract such immune responses (other than RNAi) viruses might encode some genes capable of suppressing them also.

In our screening we have knocked down each and every orf of Baculovirus and in this process we might have silenced some of the genes which are necessary to inhibit immune responses triggered by dsRNAs other than RNAi. Even such genes will be picked up by our method of screening giving a few false positives. But this screening has definitely narrowed down to fewer candidates for validating an RNAi suppressor like activity.



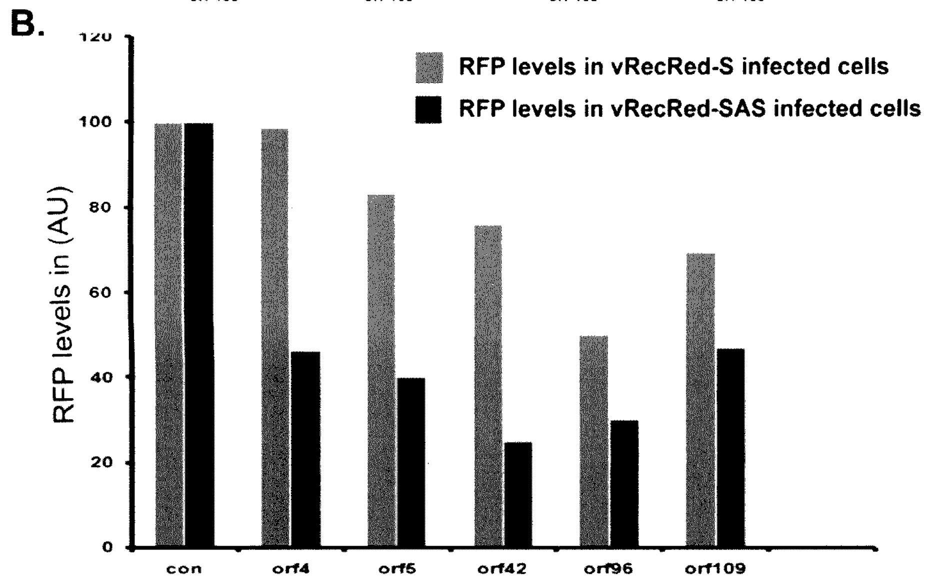


Figure 5.2: Putative RNAi suppressors obtained by RNAi based screening for RNAi suppressors (5 selected orfs).

A. Microscopic images of sf9 cells transfected with viral orf specific dsRNAs and infected with vRecRed-S and vRecRed-SAS viruses (MOI-5). EGFP-dsRNA transfected sf9 cells which were taken as control (lane a) and cells transfected with dsRNAs corresponding to orf 4, 5, 42, 96 & 109 respectively, were obtained from RNAi based screening (lanes (b-f).

B. A bar diagram showing the levels of RFP in the cells shown in panel A. RFP levels of 'control' were taken as 100 and levels of other orfs are represented as ratios of 100. Blue columns show RFP levels in vRecRed-S infected cells and Red columns show RFP levels in vRecRed-SAS infected cells.

Other important output of this screening is that some of these candidates may not be suppressors of RNAi but they definitely play a role in inhibiting other dsRNA induced immune response pathways if there are any. By comparing the viral titer in vRecRed-S and vRecRed-SAS infected cells this possibility can be ruled out. Cells which show difference in expression of RFP but not in the viral titer will be genuine candidates of RNAi suppressor. Those ORFs which suppress expression of RFP more than vRecRed-S virus and reduces viral titer also are suppressors of other dsRNA induced immune responses.

Conclusions: To summarize our results, the RNAi based screening for 'Suppressors of RNAi' have narrowed down to fewer putative genes. Since none of the baculoviral genes have sequence similarity to any of the known suppressors, it is difficult to predict the suppressors of RNAi. the other ways of screening for RNAi suppressors is by expressing specific proteins (orfs) in RNAi sensor lines and observe the effects on a genes suppressed by RNAi (give citation) but number of orfs in Baculovirus genome are relatively high (156 orfs) and it makes it very labor intensive and we are not aware of any such work being undertaken. So our strategy of screening using sensor viruses has narrowed down to fewer putative suppressors which are currently being validated by us. The added advantage of our screening is that if the candidates that we picked are not suppressors of RNAi then they must be inhibitors of some dsRNA triggered immune responses.

Table 5.1: A List of orfs which show more than 20% difference in levels of RFP expression between vRecRed-S and vRecRed-SAS infected cells after knocking down specific viral orfs (3d column).

Product Name	Locus	Locus_tag	% difference in RFP levels
AcOrf-34 peptide	AcOrf-34	ACNVgp034	74.6
AcOrf-5 peptide	AcOrf-5	ACNVgp005	53.0
global transactivator-like protein	Ac-GTA	ACNVgp042	50.0
occlusion-derived virus envelope protein	Ac-odv-e66	ACNVgp046	49.3
AcOrf-41 peptide/lef12	AcOrf-41	ACNVgp041	47.3
AcOrf-4 peptide	AcOrf-4	ACNVgp004	43.5
AcOrf-45 peptide	AcOrf-45	ACNVgp045	42.2
HE65	Ac-HE65	ACNVgp106	36.3
AcOrf-51 peptide	AcOrf-51	ACNVgp051	34.5
proliferating cell nuclear antigen	Ac-pcna	ACNVgp049	33.7
viral capsid associated protein	Ac-vp80	ACNVgp105	33.6
AcOrf-52 peptide	AcOrf-52	ACNVgp052	27.5
AcOrf-107 peptide	AcOrf-107	ACNVgp108	27.3
AcOrf-22 peptide	AcOrf-22	ACNVgp022	27.2
p26	Ac-p26	ACNVgp137	26.8
AcOrf-47 peptide	AcOrf-47	ACNVgp047	25.8
helicase	Ac-helicase	ACNVgp096	25.6
AcOrf-97 peptide	AcOrf-97	ACNVgp098	25.4
conotoxin-like peptide	Ac-ctx	ACNVgp003	24.9
AcOrf-76 peptide	AcOrf-76	ACNVgp077	24.3
AcOrf-81 peptide	AcOrf-81	ACNVgp082	24.0
AcOrf-48 peptide	AcOrf-48	ACNVgp048	23.4
AcOrf-53 peptide	AcOrf-53	ACNVgp053	23.3
AcOrf-108 peptide	AcOrf-108	ACNVgp109	22.7

Product Name	Locus	Locus_tag	% difference in RFP levels
occlusion-derived virus envelope protein; p25	Ac-odv-e25	ACNVgp095	21.7
AcOrf-106 peptide	AcOrf-106	ACNVgp107	21.7
putative early gene transactivator	Ac-IE-01	ACNVgp142	21.5
AcOrf-43 peptide	AcOrf-43	ACNVgp043	21.4
late expression factor 11	Ac-lef11	ACNVgp037	21.0
fibrous body protein	Ac-p10	ACNVgp138	20.7
AcOrf-72 peptide	AcOrf-72	ACNVgp073	20.7
AcOrf-44 peptide	AcOrf-44	ACNVgp044	20.0

Chapter-6

Role of *bro* in AcMNPV infection and Transcription: A Microarray based study.

Chapter 6: Role of *bro* in AcMNPV infection and Transcription: A Microarray based study.

6.1 Introduction:

Baculoviruses are dsDNA virus, which generally infect insects. They have a covalently closed circular DNA genome and they exist episomally wihtut integrating into their host genome. As soon as the virus enters into a host cell, it enters into the nucleus and performs all basic essential activities like replication, transcription and viral genome packaging. Their considerably large genome size and a large number of genes, puts them along with some of the very big viruses. Having a simple dsDNA genome and encoding a large number of genes including their own DNA polymerase, RNA polymerase, and many transcriptional activators, apoptotic inhibitors and genes that are capable of manipulating the host genome *etc.* makes them very interesting specimens for studying viral pathogenesis. Probably having such a decent number and variety of genes makes them one of the most difficult viruses to crack. dsDNA viruses in general contain many multi-gene families in their genome which might have been acquired from their host. There are a few multi-gene families in viruses which are not found in any of the host genomes. Baculoviruses have one such gene family in their genome and are found in most if not all of the known baculoviruses. That gene family is called Baculovirus repeated orf also called "BRO" family. To put it in simple words bro is a very complex family of genes which exhibits lot of variation in their occurrence, number, length and sequence, each one of which are major determinants of functional aspects of any gene. Bro family genes are found in Lepidopteran NPVs and GVs and homologues are found in the dipterans, but not hymenopteran NPV genomes. Even in the most closely related Lepidopteran nucleopolyhdero viruses, number of bro genes varies. AcMNPV has only one *bro* (orf-2) in its genome but the most closely realted viruses like OpMNPV has three (Ahrens *et al.* 1997), and LdMNPV has as many as seventeen genes (Kuzio *et al.* 1999). Two other closely realted viruses of AcMNPV lack bro genes like Anagrapha falcifera MNPV (Federici *et al.* 1997) and Rachiplusia ou MNPV (Harrison *et al.* 1999). Other than baculoviruses they are

found in members of entomopoxvirus and entomoiridovirus also. Homologs of *bro* are found in double-stranded DNA phage, prokaryotic class II transposons (Bideshi *et al.* 2003).

The bro genes contain a characteristic highly conserved N-terminal domain which can bind to DNA and a less conserved C- terminal domain, mainly found in baculoviruses. bro genes can be broadly devided into 3 categories group I, II and III (Kuzio *et al.* 1999). AcMNPV bro belongs to group I. *bro* genes of BmNPV are found to interact with laminin-b a homolog of drosophila β1-Laminin (Kang *et al.* 2003). These are extracellular glycoproteins found on the surface of the cells but bro does not appear to have any role in facilitating the entry of the virus into cells by interacting with laminin as they are not associated with ODVs. But there are some intracellular proteins like LBP/p40 which can bind to laminin and they are nown to associate with ribosomes and regulate translation of genes. LBP/p40 is found in nucleus also and regulates nucleosome organization of the host. Although there is no homology between *bro* and LBP/p40 there is lot functional similarity between them, both of them can interact with laminins, found in both nucleus and cytoplasm and associate with nucleosomes. Zemskov *et al* reported that *bro-a, bro-c* and *bro-d* are found in the fractions of histones H1 purified from BmN cells. Many such observations highlight their possible role in virus infected cells where they might play a role in regulating host DNA replication by influencing the chromatin organization of the host genome. This was supported by other observation in which BROs are found interact with dsDNA, without any sequence specificity (Zemskov *et al.* 2000; Iyer *et al.* 2002). BRO-d of BmNPV is shown to interact with CRM1 for their export into cytoplasm by which it can interfere with host mRNA export like many other viruses do in the infected cells to inhibit host translation.

In BmNPV *bro* genes are essential for the virus to cause effective infection but in AcMNPV their disruption did not show any significant effect on budded virus production. But they were compromised in ODV formation. So in cell culture *bro* did not affect virus infection and BV production. But they found (Bideshi *et al.* 2003) disruption of bro genes at different locations affected the virus production to different extent. Kang *et al* (Kang *et al.* 1999) reported that all 5 bro genes of

BmNPV are not essential for its infection. But their functions are not redundant but they complement each other.

Despite their conservation across many viruses their exact role in virus infection is not yet clearly understood. In an interesting finding BmNPV viruses which are capable of only minimal amount of replication in sf9 cells could infect and replicate in sf9 cells when their *bro-d* gene was replaced by *bro* of AcMNPV. But the BmNPV bro could not show similar effects when they were introduced into AcMNPV genome in infecting BmN cells. The AcMNPV *bro* gene which is essential for BmNPV to infect sf9 cells was not essential for AcMNPV in same *sf9* cells and bro genes which were essential for BmNPV in infecting BmN cells could not make AcMNPVs to infect the same BmN cells. So the importance of *bro* appears to depend on both virus and their host.

Surprisingly In our RNAi based screening for essential genes of AcMNPV when we silenced the Bro gene by using dsRNAs we found significant suppression of recombinant virus production unlike the observations of Bideshi *et al.* This discrepancy is possible because the *bro* genes disrupted by Bideshi *et al* (Bideshi *et al.* 2003) may not be completely compromising its functions. Evidences can be seen in their results where two different insertions inhibited ODV production to different extents. It is also possible that *bro* like genes are present in some of the host species and they might complement the function of *bro* which was disrupted by insertion. So far there are no reports of homologues of *bro* genes in any insect species and coincidentally all Lepidopteran baculoviruses which infect such insects whose genome sequence is known contain bro genes and are essential for their infection (ex: Bombyx mori and BmNPV), since the sequence of *Spodoptera frugiperda* and *Trichoplusia nimphi* are not known this possibility cannot be ruled out. But RNAi mediated suppression of *bro* gene, does not discriminate between *bro* like genes of host and the virus and can suppress both of them (if they are present) and might have resulted in suppression of the virus. We would also like to mention about another possibility that, the dsRNA we used to knock down BRO,

might have suppressed other essential genes resulting in suppression of the virus. Therefore at this moment functional relevance of *bro* cannot be decided with certainty.

To take it little further we were interested to see whether *bro* gene is really essential and whether it can regulate other viral genes during infection. As Bideshi *et al* (Bideshi *et al.* 2003)has reported that disruption of bro gene affects ODV formation, we wanted to investigate whether genes involved in ODV formation were specifically regulated by bro or they regulate other genes as well supporting our observations. Keeping the properties and functions of *bro,* like their ability to interact with laminins, nucleosomes, and binding to DNA *etc* in mind, we expected it to regulate expression of many viral genes directly or indirectly, and all such possibilities can be tested to understand viral genes being regulated by *bro* by using a microarray chip.

6.2 Materials and methods:

6.2.1 Cells medium and virus: sf9 cells were cultured in TNM-FH medium supplanted with 10% FBS and incubated at 27^{0}C

6.2.2 Transfection of dsRNA and sample collection: dsRNAs were synthesized in vitro by in-vitro transcription and transfected into sf9c cells using Trans-messenger transfection reagent details of the methods followed are explained in section 2.8.2 of Chapter 2.

6.2.3 RNA isolation and purification: total RNA was isolated using Trizol reagent digested with DNase to remove traces of DNA and purified with RNeasy columns (Qiagen) according to manufacturer's protocol.

6.2.4 RNA Quality Control: Total RNA integrity was assessed using RNA 6000 Nano Lab Chip on the 2100 Bioanalyzer (Agilent, Palo Alto, CA) following the manufacturer's protocol. Total RNA purity was assessed by the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Nanodrop technologies, Rockland, USA). Total RNA with OD260/OD280>1.8 and OD260/OD230≥1.3 was used for microarray experiments. We considered RNA to be good quality when the rRNA 28S/18S ratios

were greater than or equal to 1.5, with the rRNA contribution being 30% or more and an RNA integrity number (RIN) was ≥7.0

6.2.5 Labeling and microarray hybridization: Poly (A)-tails were added to the 3'-end of RNA by using A-plus Poly (A) polymerase tailing kit (Epicentre Biotechnologies).Then the samples were labeled using Agilent Quick Amp Kit PLUS (Part number: 5190-0442). Five hundred nanograms each of the samples were incubated with reverse trancription mix at 42°C and converted to double stranded cDNA primed by oligodT with a T7 polymerase promoter. The cleaned up double stranded cDNA were used as template for aRNA generation. aRNA was generated by *in vitro* transcription and the dye Cy3 CTP(Agilent) was incorporated during this step. The cDNA synthesis and *in vitro* transcription steps were carried out at 40°C. Labeled aRNA was cleaned up and quality assessed for yields and specific activity.

6.2.6 Hybridization and scanning: The labeled aRNA samples were hybridized on to a Custom Baculovirus Gene Expression Array 8X15K (AMADID: 20511). 600 ng of cy3 labeled samples were fragmented and hybridized. Fragmentation of labeled aRNA and hybridization were done using the Gene Expression Hybridization kit of Agilent (Part Number 5188-5242). Hybridization was carried out in Agilent's Surehyb Chambers at 65° C for 16 hours. The hybridized slides were washed using Agilent Gene Expression wash buffers (Part No: 5188-5327) and scanned using the Agilent Microarray Scanner G Model G2565BA at 5 micron resolution.

6.2.7 Feature Extraction: Data extraction from Images was done using Feature Extraction software v 10.5 of Agilent.

6.2.8 Microarray Data Analysis: Feature extracted data was analyzed using GeneSpring GX v 10.0.2 software from Agilent. Normalization of the data was done in GeneSpring GX using the percentile shift and median normalization. Genes with greater than 1.3 and 1.5 fold difference among the groups were identified. Genes were clustered using hierarchical clustering to identify gene expression patterns.

6.2.9 Semi quantitative Estimation of viral transcripts by RT-PCR: Total RNA was isolated from infected sf9 cells at particular time points and reverse transcribed to produce cDNA. cDNA was used as a template to amplify both orf

specific fragments and 18s rRNA as internal control. PCR products were electrophresed on an 2% Agarose gel and Expression levels of viral orfs were measured by semi quantitative estimation taking 18s rRNA as internal control.

6.2.10 Estimation of BRO protein levels by Western blot: BRO protein was cloned and expressed in bacteria. Polyclonal antibodies were raised in both Mouse and Rabbit in the Animal maintenance facility of CCMB. (see section 2.12) Cells were harvested after dsRNA transfection and viral infection at particular time points. Cells were centrifuged and palette was resuspended in laemmli buffer, boiled for 15 minutes and electrophoresed in 10% Acrylamide. Separated proteins were transferred onto Hybond-P membrane by wet transfer method and stained with Ponceu-S to estimate the quantity of protein in each lane. The blot was incubated with 1:1000 dilution of BRO mouse antiserum, washed and again incubated with HRP conjugated secondary antibody. Antibodies were detected by chemiluminescent methods by using SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to manufacturer's instructions and exposed X-ray films and signal were recorded. Proteins levels of BRO were estimated based on equal loading of total proteins in corresponding lanes

6.3 Results and discussion:

6.3.1 Silencing of AcMNPV bro gene by RNAi: in order to silence *bro* gene in AcMNPV infected cells, We transfected dsRNAs corresponding to bro genes into sf9 cells and after 24 hours infected them with wild type AcMNPV C6 with an MOI of 5. Cells were harvested after 12, 24, 48 and 72hours PI and RNA was isolated. Suppression of bro transcripts was confirmed by semi-quantitative RT-PCR by comparing the transcripts of bro with transcripts of 18s rRNA used as an internal control (**Figure 6.1A**). We also verified whether decrease in bro transcripts leads to decrease in protein levels of BRO in infected cells by western blot (**Figure 6.1B**) and confirmed that protein levels were reduced corresponding to their transcripts.

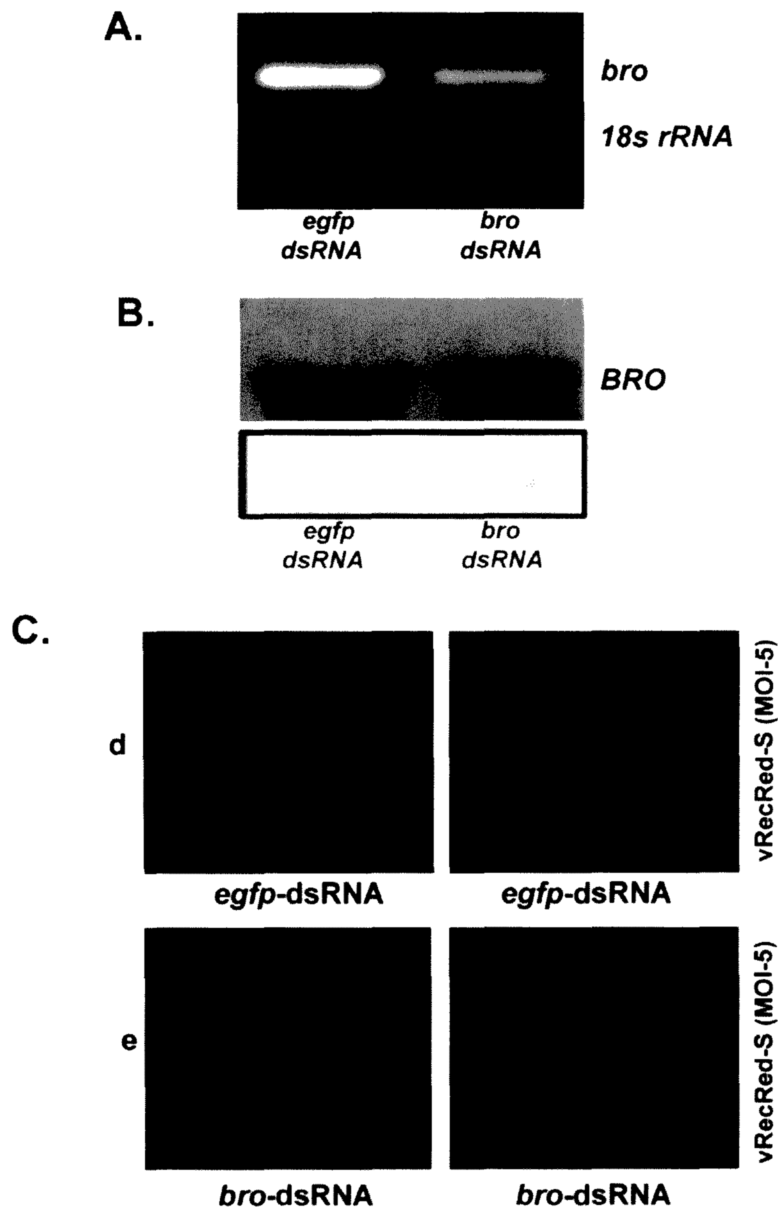


Figure 6.1: Shows results confirming the knock down of *bro* in dsRNA transfected samples.

A. A gel picture showing products of semi-quantitative RT-PCR of *bro* transcripts in *egfp*-dsRNA transfected cells and *bro*-dsRNA transfected cells after 12 hours of infection with AcMNPV-C6.

B. A western blot showing levels of BRO protein in *egfp*-dsRNA transfected cells (control) and *bro*-dsRNA transfected cells after 12 hours of infection with AcMNPV-C6

C. Fluorescent stereo microscopic images of cells transfected with *egfp*-dsRNA (Row-d) and *bro*-dsRNA (Row-e) after 72 hours of infection with vRecRed-S (MOI-5). 54% suppression in the levels of RFP was observed.

6.3.2 Microarray analysis of expression of AcMNPV orfs:

To investigate the role of bro in AcMNPV infection in sf9 cells we analyzed the expression profile of all 156 orfs of AcMNPV at different time points after infection. We transfected sf9 cells with dsRNA designed against EGFP and *bro* genes in separate wells and infected them with wild type AcMNPV-C6 virus with an MOI of 5. Cells transfected with dsRNAs against EGFP were taken as "Control" and used to compare the transcriptional profile of *bro* silenced cells. Cells were harvested after regular time intervals i.e., 12, 24, 48 and 72hrs after infection and Total RNA was isolated, labeled and hybridized to a microarray chip containing probes unique to each of the 156 orfs of AcMNPV.

6.3.4 Analysis of microarray results: we compared the expression profile of 156 orfs in Control (EGFP-dsRNA transfected cells) and *bro* silenced cells at each time point i.e., after 12, 24, 48 and 72hours and Genes exhibiting more than 1.5 fold and 1.3 fold difference were selected. To validate the microarray data we performed semiquantitative RT-PCR to estimate the transcript levels for 10 randomly selected samples and found consistent results with microarray.

The number of orfs up-regulated or down-regulated at any given time points are mentioned in the Table 6.1, and expression profile of all 156 orfs compared to control cells were represented in a scattered plot. The fold difference in the expression level of each orf is expressed in terms of "Log_2" (**Figure 6.2**).

Table 6.1: Number of genes up-regulated and down-regulated in *bro* silenced cells by 1.5 fold and 1.3 fold.

	1.5 fold difference		**1.3 fold difference**	
	Up regulated	**Down regulated**	**Up regulated**	**Down regulated**
At 12hrs PI	1	38	5	72
At 24hrs PI	4	13	20	29
At 48hrs PI	0	3	16	14
At 72hrs PI	0	6	2	9

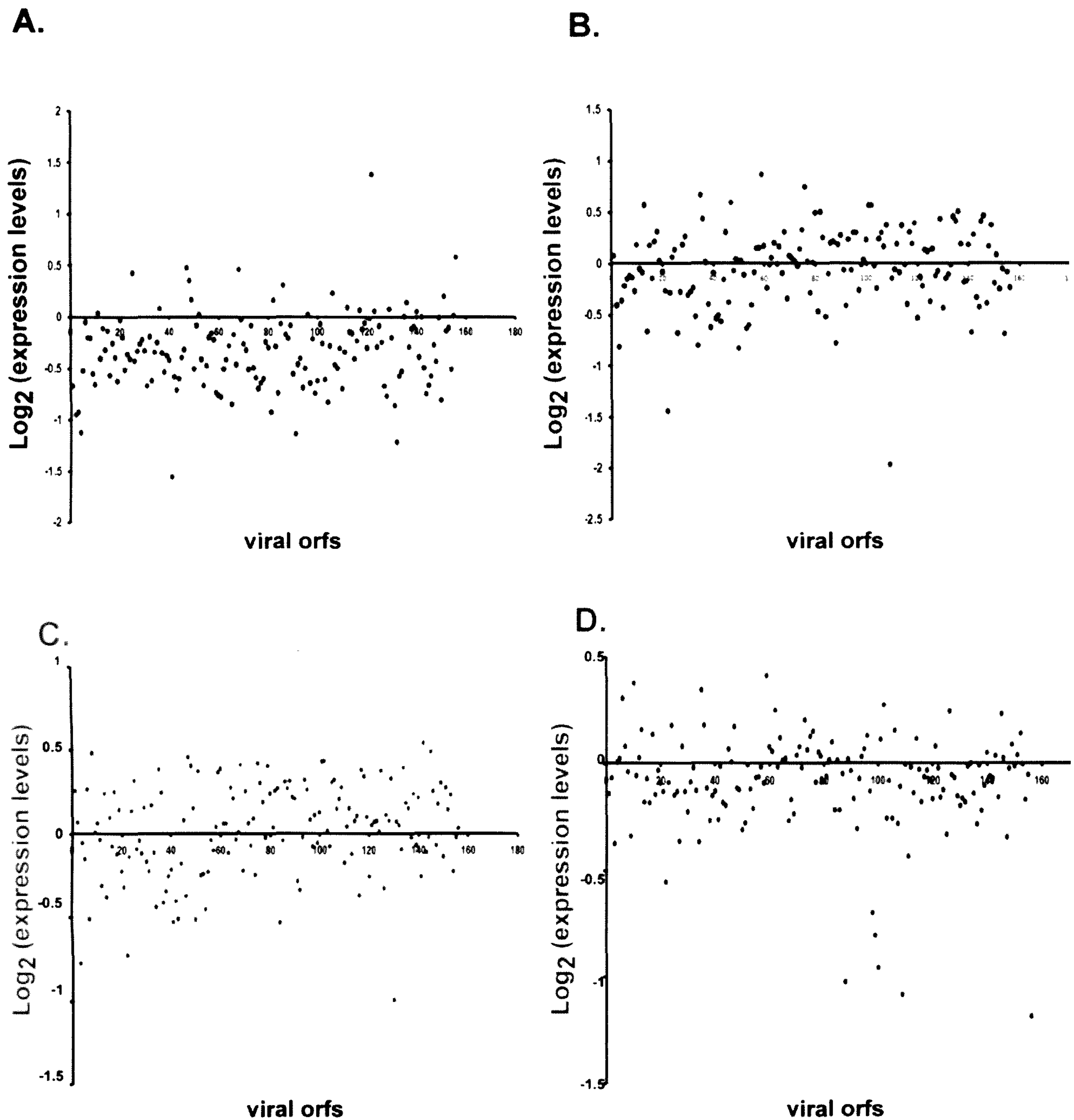


Figure 6.2: Differential gene expression profiling of all 156 AcMNPV orfs, in BRO knock down cells infected with virus AcMNPV-C6 (MOI-5) compared to control cells infected with same virus (MOI-5). control cells were transfected with dsRNAs against EGFP and BRO knock down cells were transfected with dsRNAs against *bro*.

A-D. A scattered plot analysis of fold change in the levels of expression of all 156 orfs of AcMNPV at 12h, 24h, 48h & 72hrs PI (A, B, C & D rspectively).
X-axis shows the nunber of orfs (for ex. '1' corresponds to orf ACNVgp001). Y-axis shows the fold change in the expression levels of orfs.

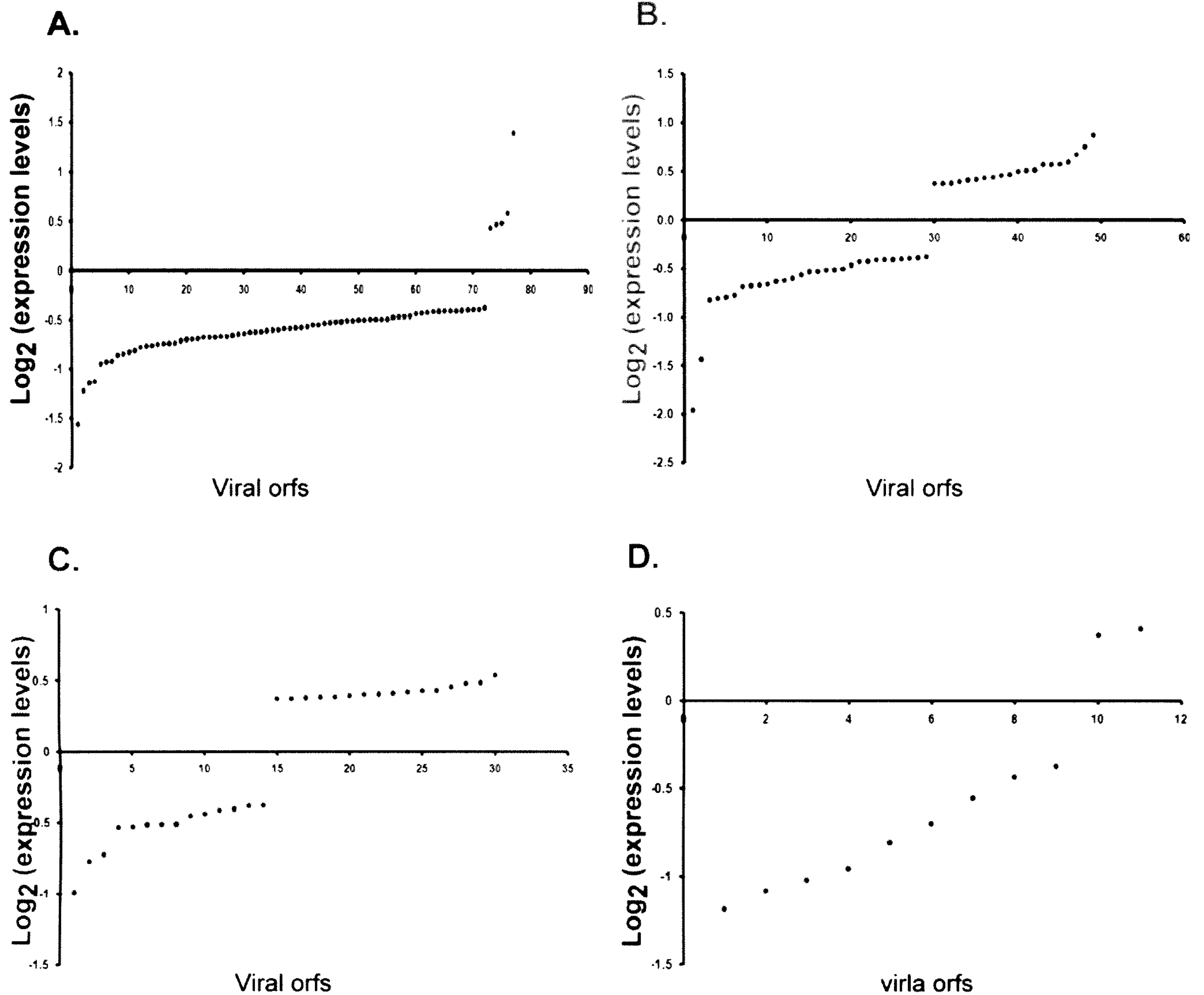


Figure 6.3: Differential gene expression profiling of AcMNPV orfs in BRO knock down cells infected with virus AcMNPV-C6 (MOI-5) compared to control cells infected with same virus (MOI-5). Control cells were transfected with dsRNAs designed against *egfp* and BRO knock down cells were transfected with dsRNAs against *bro*.

A-D. A scattered plot analysis of orfs showing more than 1.3 fold (Log2) difference in expression at 12, 24, 48 & 72 hrs PI (Panels A, B, C & D rspectively).
X-axis shows the total nunber of orfs out of 156 showing more than 1.3 fold (log2) difference. Y-axis shows the fold change (log 2) in the expression levels of orfs.

AcMNPV *bro* expresses at delayed early phase of infection *i.e.,* around 6hr PI, and its expression depends upon viral factors and is known to regulate many other viral genes related to ODV formation. So it was expected that genes related to ODV formation will be affected by silencing bro. in consent with the earlier reports, we did find genes related to ODV formation were specifically down-regulated and in addition we found evidences to support our notion that *bro* performs other essential roles and does indeed affect virus production in addition to ODV formation.

6.3.4.1 Down-regulated genes:

By analysis of the expression profile of orfs whose functions are known we could find genes related to particular pathways were specifically affected. Based on their functions and their role in particular aspects of viral pathogenesis we grouped these genes into seven broad categories. **1**. Genes involved in replication and nucleic acid metabolism, **2**. Genes involved in transcription, **3**. Packaging and assembly **4**.ODV and BV associated proteins, **5**. *per-os* infectivity factors **6**. Genes affecting host metabolism, and **7**. Genes with unknown functions.

1. Genes involved in Replication and Nucleic acid metabolism: (Ac33, Ac77, Ac38 , Ac42 and Ac14):

Ac14 is DNA primase that is very essential for DNA replication, Ac33-A polynucleotide kinase, Ac38 is a Nudix protein and homologues are capable of decapping viral mRNAs, ADP-ribose pyrophosphatase [ADPRase) deletions of Ac38 severely affect BV production in AcMNPV reducing it to almost 1% of the wild type. Ac77 is a lambda integrase type of protein also called Very late factor-1 [Vlf-1). It is a very essential gene for the virus and it is known to have an indispensable role in DNA recombination, viral nucleocapsid packaging, hyper activation of other late genes etc.

Down regulation of genes involved in replication, results in reduced virus replication and production of progeny viruses both BVs and ODVs. Silencing of Bro gene severely affects genes which are very important for BV formation like Ac38,

supporting our earlier results (Figure 6.1C). It is known that replication of viral DNA is an important prerequisite for efficient late and very late gene expression which could be a rate limiting step in the formation of ODVs supporting earlier evidences of reduced ODV production.

2. Genes involved in transcription: genes expressed in the Late and very late phases of infection are transcribed by an RNA polymerase encoded by the virus itself. Two subunits of a functional viral RNA polymerase were down-regulated in bro compromised virus infected cells. Ac50 also called Lef-8 is the catalytic domain of the viral RNA polymerase and Ac99 also called lef-5 acts as an initiation factor for transcription. Down-regulation of viral RNA polymerase subunits will decrease viral late and very late transcripts and affect both the ODV and BV formation.

3. Genes involved in packaging and assembly: Ac68, Ac80, Ac77 and Ac53 are essential proteins of Baculovirus involved in proper packaging of viral DNA and proteins into functional capsids, down regulation of Ac53 affects capsid assembly but not the viral DNA replication.

4. ODV and BV associated proteins: Proteins form an integral part of the BV and ODV structure. Some proteins are found associated with ODV and BVs not just as a structural components but they might also get incorporated in them during the process of their assembly. Many genes like Ac103, Ac108, Ac96, and Ac16 were down regulated. All these genes are not auxiliary proteins but are indispensable for ODV and/or BV formation. Ac87 and Ac129 are other capsid associated proteins but thought to be nonessential for viruses, but their deletions take more time to kill their hosts.

5. *per os* infectivity factors: these are the factors which are important for the virus to infect their host through their gut. Mutants of these genes produce BV normally like their wild type counter parts but the polyhedral bodies produced could not infect

insect hosts like wild type viruses. pif genes like Ac22, Ac145, Ac150 were down regulated.

6. Genes affecting host metabolism: viruses which encode genes to manipulate the host metabolism are very much essential to efficiently complete the viral life cycle. Genes involved in inhibition of apoptosis (IAP-2), homologues of juvenile hormone esterase, Ac144 a multifunctional cyclin which regulates host cell cycle, were down-regulated and this compromises the production of BV and to a greater extent ODV. Ac31 is a superoxide dismutase gene which helps in maintaining the stability of polyhedral bodies against radiation and might also help in confronting the host Reactive oxygen species mediated antiviral response.

7. Genes with unknown function: Ac152 Ac116, Ac91, Ac140, Ac68, Ac154, Ac97, and Ac87 are other genes which were found down-regulated. These genes are reported to be nonessential but it might be due to the way these genes were studied and they might have an essential role that is not addressed properly in cell culture or other *in vivo* models.

To support the specificity of regulation of BRO and the RNAi mediated suppression of BRO we observed the expression profile of orfs which appear to be fused in AcMNPV like Ac106-Ac107 found only Acgp107 was down-regulated. It is proposed that the genes which function together in protein levels, do not lose their function even if their orfs get fused and such fused genes will not be lost during evolution. But our results suggest that such genes will be regulated separately and bro can influence the expression of only one orf out of those two.

6.3.4.2 Up-regulated genes:

compared to the number of down-regulated genes, genes which are up-regulated are very less. When we analyzed the gene expression profile by keeping 1.5 fold increases as cut off values we found only 5 genes, but the number of genes up-regulated by more than 1.3 fold were significantly high. At 12 hours and 72 hours very few genes were found up-regulated but at 24 and 48 the numbers of genes

were 20 and 16 respectively. Indicating that, some processes undergoing at 24-48 hours are generally down-regulated by Bro in normal situations.

By looking at the known and predicted function of such up-regulated genes it appears that most of the early phase genes have to be down-regulated in order to proceed to later stages of infection and bro could be one of those factors influencing this process. This is also the time when most of the host transcription and translation is shut down so genes which are dependent upon host transcription machinery will be specifically down-regulated in normal situations so we expected that genes of early phase will continue their expression which appears to be up-regulated when compared to control cells.

Here we have mentioned about few such candidates that we observed in our microarray analysis.

Based on their functions, up-regulated genes were divided into six broad categories like **1**. Genes involved in early gene *trans*-activation, **2**. Genes involved in DNA damage response and formation of Virogenic stroma, **3**. Structural proteins **4**. Genes involved in host manipulation, **5**. Structural proteins & **6**. Genes with unknown functions.

1. Genes involved in early gene *trans*-activation: as noted earlier, early gene transcription reduces with progress in infection. There are various mechanisms of achieving it, either by inhibiting polymerases transcribing early genes or by inhibiting the trans-activators of early genes. Ac142, Ac121 is an early genes *trans*-activator which continues to express in *bro* knock down cells. Acgp149 is a immediate early gene which acts as a *trans*-activator to transcribe early and late genes and helps is initiation of DNA replication by binding to origins of replication by facilitating binding of DNA polymerase and other accessory proteins involved in DNA replication.

2. Genes related DNA replication and damage response: Interestingly a significant number of genes up- regulated were DNA binding proteins, proteins involved in DNA damage response, DNA recombination and involved in formation of Virogenic Stroma like Ac25, Ac36, Ac45, Ac27, Ac67(lef-3), and Ac133

Another consistently up-regulated gene was P6.9 which is a highly basic protein which helps in compaction of viral DNA to facilitate capsid assembly.

3. Structural proteins: Very few structural proteins associated with ODVs &BVs were up-regulated like Acgp145, Acgp146, Ac8, Acgp129 etc.

4. Genes involved in host gene manipulation: Ac35 is an Ubiquitin like protein and probably inhibits or takes part in ubiquitin mediated protein degradation. Ac59 is a non essential gene for BV formation but larvae infected with mutant viruses affect the mobility of larvae. Usually baculoviruses induce motility in larvae and this behavioral change helps in better dispersal of viral particles.

5. Genes with unknown function: few genes with unknown function were up-regulated like Ac134, Ac149, Ac79, Ac13, Ac75 and Ac81. Ac75 and Ac81 appear to be essential for the virus but rest of them were reported to nonessential.

6.4 Summary: To summarize our observations, we found genes involved in replication, transcription, ODV and BV associated proteins and proteins involved in efficiently infecting insect hosts, were down-regulated. Many genes which were usually suppressed during normal infection to allow the infection to proceed towards late and very late phases remained up-regulated in *bro* knock down cells. Genes involved in binding to DNA and having role in DNA damage response were specifically up-regulated. The observation of such specific suppression and up-regulation of particular genes involved in virus infection suggest that the effect on BV production during dsRNA based suppression of BRO s not a nonspecific effect. Unlike the earlier report *bro* indeed plays role I both BV and ODV production.

Chapter-7

Role of AZdC a DNA methyltransferase inhibitor, on Baculovirus infection in sf9 cells.

Title: Role of AZdC a DNA methyltransferase inhibitor, on Baculovirus infection in sf9 cells.

7.1 Introduction:

Epigenetic regulation of the Genome relies on molecular marks like DNA methylation and histone modifications. DNA methylation is found both in prokaryotes and eukaryotes (Colot *et al.* 1999; Ng *et al.* 1999). In prokaryotes they appear to act like a defense mechanism against invasive genetic elements such as transposons. However in eukaryotes, DNA methylation can be correlated to many cellular processes like inhibition of transcription initiation (Busslinger *et al.* 1983), inhibition of transcriptional elongation, a mediator of genetic imprinting (Lloyd 2000), inhibition of transposons (Bird 2002), inhibition of homologous recombination, chromosome maintenance, X chromosome inactivation (Norris *et al.* 1994) etc. There is lot of variation in the content of methylated cytosine across species. Insects contain as little as 0–1% methyl cytosine, whereas it is 5% in mammals and birds, 10% in fish & amphibians and more than 30% in some plants. *Drosophila melanogaster* was earlier thought to lack DNA methylation (Urieli-Shoval *et al.* 1982). Based on these observations of methyl cytosine content, it was hypothesized that increased complexity due to increase in gene number was one of the causes of evolutionary transition from invertebrates to vertebrates and this increased gene number which results in increased transcriptional noise were otherwise reduced by DNA methylation (Bird 1995). Then Jablonka and Regev came up with another hypothesis that DNA methylation has a major role in regulating repetitive sequences like transposons (Jablanka *et al.* 1995) and their effect on genes is only an indirect or nonspecific effect (Yoder *et al.* 1997; Walsh *et al.* 1999), but again Bird *et al* carried a different notion, and they reported about genomes which contain methylated gene sequences and unmethylated transposable elements. They also reported that selfish genetic elements were not restricted completely due to DNA methylation (Bird 1997) and highlighting the importance of DNA methylation as one of the major player in regulation of genes and genomes.

DNA methylation is widely studied in vertebrate systems and in-depth mechanistic understanding of the process of DNA methylation and regulation comes from these model systems. The enzymes which methylate DNA are called DNA methyl transferases (Dnmt) (Goll *et al.* 2005). So far four DNA methyl transferases (Dnmt) are identified *viz*, *Dnmt1, Dnmt2, Dnmt3a* and *Dnmt3b*. Based on their amino acid sequences they are categorized into 3 major classes Dnmt1, 2 and 3 (Bestor 2000). Dnmts have two common features, a DNA recognizing motif and catalytic motif. Depending on the variable sequences of their domains their functions differ (Kumar *et al.* 1994). *Dnmt*s mainly carry out two types of DNA methylation *viz,* Maintenance and de novo methylation (Riggs 1975). Maintenance methylation happens after DNA replication on symmetric regions like CpG or CpNpG. maintenance methylation is a process of copying the methylation pattern on the parent strand of DNA onto a newly synthesized daughter strand. *De novo* methylation happens on DNA sequences which were not methylated earlier and they are seen in both symmetric and non-symmetric sequences (Ponger *et al.* 2005). Dnmt1 belongs to the class of maintenance Dnmts, which copy the methyaltion pattern of parent DNA strand to newly synthesized daughter strand (Yoder *et al.* 1997). Dnmt3a and 3b preferably bind and methylate completely unmethylated DNA and hence belong to the class of de novo methyl transferases (Okano *et al.* 1999). Their activity is targeted to specific sites (sequences) of DNA with the help of transcription factors and/or chromatin modifying factors (Burgers *et al.* 2002). Dnmt2 are the most highly conserved *Dnmt*s among eukaryotes from protozoa to mammals (Lyko *et al.* 2006). Dnmt2 contains only a catalytic domain and lack extended N-terminal regulatory domains found in other Dnmts. Dnmt2 shows very week de novo methylation activity and can methylate sequences other than CpG. They are known to methylate $tRNA^{(asp)}$ but the importance of this dual activity is not yet clearly understood (Dong *et al.* 2001; Goll *et al.* 2006; Jeltsch *et al.* 2006). Catalytic activity of Dnmt2 has been shown in protozoa (Fisher *et al.* 2004), Drosophila (Kunert *et al.* 2003), Mouse (Mund *et al.* 2004) and humans (Hermann *et al.* 2003). In Drosphila majority of methyl cytosine (mC) is found in CpA & CpT dinucleotides than CpG (Lyko *et al.* 2000) and Dnmt2 alone is necessary and

sufficient to maintain DNA methylation in *Drosophila*. Its (*Dnmt2*) deletions showed complete loss of methyaltion, and over expression resulted in hypermethylation. Expression of other Dnmts in dorsophila proved to be toxic to cells. This shows that even though level of DNA methylation is vey less, DNA methylation is important for regulation of the genes or genomes in insects like drosophila. Further studies have revealed that other dipteran members also show DNA methylation like *Drosophila pseudoobscura* & *Anopheles gambiae* (Marhold *et al.* 2004).

Many other insect species also contain methylated DNA but they exhibit lot of variation in the DNA methyaltion machinery compared to dipterans. Honey bees contain all 3 classes of DNA methyl transferases and all of them have retained their catalytic activity. They show extensive methylation of CpG nucleotides than non CpG sequences in their genome. Another insect *Mamestra brassicae* genome is also highly methyalted at CpG sequences. Level of methylated Cytosine content is as high as 10% in these insects (Mandrioli *et al.* 2003). Genome sequencing of *Bombyx mori, Tribolium castaneum*, *Aedes albopticus, Culex tritaeniorhynchus,* revealed the presence of Dnmts in their genome *Bombyx mori* contains 2 *Dnmt*s in its genome *Dnmt1 and Dnmt2* (Patel *et al.* 1987). But a detailed investigation of levels of CpG and non CpG methylation in these species is still awaited. Other insects species which are known to conatian methylated DNA are homopterans, including *Megoura viciae, Planococcus lilacius, Pseudococcus calceolariae, P. obscurus*, and *Myzus persicae*; orthopterans, including *Locusta migratoria, Eyprepocnemis ploras, Pyrgomorpha conica, Gryllotalpa fossor*, and *Baeticaustalata*.

Most of the organisms are infected by viruses and many of them integrate into their hosts' genome. Genes of such integrated viruses are regulated by the host factors involved in host gene regulation like transcription factors, chromatin modifiers etc. integrated viral genomes get packaged in nucleosomes like their host genome and are regulated by all those factors which affect their hosts' gene expression like chromatin modifications, DNA methylation etc. In contrast to this, many dsDNA viruses exist episomally without integrating into their host genome. Even these viruses are also bound by several host proteins, like histones and assemble into a

nucleosome like structures (Deshmane *et al.* 1989; Bock *et al.* 1994). Expression of such viral genes is also sensitive to chromatin modification and DNA methylation status.

Baculoviruses being dsDNA viruses exist episomally and replicate in the host nucleus. Since nucleus contains genomic DNA and all related chromatin modifying factors, it was expected that viral DNA might also get packaged into nucleosome like structure and at least a few viral genes are sensitive to chromatin modifications. DNA methylation is also an important regulatory mechanism and expected to regulate viral gene expression. Since the transition between different phases of Baculovirus infection are not clearly understood, we wanted to investigate whether chromatin and DNA modifications that have a major role in regulating host genes assist in regulating expression of viral genes and also in transition between different phases of infection.

Recently Peng Y *et al* reported that a Histone deacetylase inhibitor Sodium butyrate (NaB) could inhibit expression of recombinant gene expressed under p10 promoter (Peng *et al.* 2007) but surprisingly another Histone decetylase inhibitor Trichostatin A (TSA) did not inhibit the recombinant gene expression. Further they found both NaB and TSA treatment did not affect viral replication. These results showed that histone decetylases do not contribute to Baculovirus late gene expression and production of viruses is not affected by inhibition of histone deacetylases. Surprisingly when recombinant baculovirus (AcMNPV) was used to express recombinant genes in mammalian cells, treating these cells by NaB or TSA increased the expression of recombinant gene expressed under a mammalian promoter, highlighting existence of different mechanisms of insect and mammalian cells for regulation of baculovirus genes (Kenoutis *et al.* 2006).

5-Azacytidine (AZC) and 5'-Aza-2'-deoxycytidine (AZdC) are potent inhibitors of DNA methyl transferases (Dnmts). Both are nucleoside analogues which can be utilized by cellular DNA and RNA polymerases during polymerization. AZC or AZdC is a prodrug that could be activated into nucleoside tri-phosphate and can be used by both DNA and RNA polymerases and get incorporated into both DNA and RNA. Azacytidine or Aza-deoxy-cytidine after activation gets incorporated into DNA at the

positions of methylated Cytosine by DNA polymerase. The Dnmts which bind to such modified DNA (AZC containing DNA) are inactivated due to covalent bond formation between Azacytidine or Aza-deoxy-cytidine and Dnmts. This results in inhibition of DNA methyltrasnferases (Dnmt1, Dnmt2, Dnmt3a & Dnmt3b) and tRNA methyl transferases (Dnmt2) (Lee *et al.* 1976; Lu *et al.* 1980). De novo Dnmts like Dnmt2 Dnmt3a and Dnmt3b can be inactivated very quickly because they act on unmethylated template during replication to convert them into methylated DNA. Any Dnmt which once binds to such modified DNA (AZC or AZdC incorporated DNA) gets inactivated irreversibly. Dnmt1 which is a maintenance-Dnmt can act only on hemi-methylated DNA after replication, is not inhibited very quickly by this method. However Dnmt1 can bind to AZC or AZdC that is incorporated in hemi-methylated DNA after replication and get irreversibly inactivated. Usually it takes at least 2 generations of replication to completely inactivate Dnmt1 depending on the concentration of AZC or AZdC used. So in order to inhibit Dnmts by AZC or AZdC DNA replication is essential. Methylation plays an important role in synthesis of ribosomal RNA also and inhibition of ribosomal RNA affects protein synthesis (Weiss *et al.* 1974; Glazer *et al.* 1980). So Azacytidine treatment results in inhibition of DNA, RNA and also protein synthesis. AZdC can be utilized only by DNA polymerases and get incorporated into DNA, inhibiting only DNA synthesis (Li *et al.* 1970). This results in inhibition of Dnmts which can act only on DNA template but not RNA.

Eukaryotic species which exhibit DNA methylation have been shown to methylate the genome of the viruses infecting them. (Willis *et al.* 1980; Wagner *et al.* 1985; Karlin *et al.* 1995). Some of these viruses encode their own DNA methyl trasnferases (Kaur *et al.* 1995). Methylation of the viral genome is essential and seems to regulate the production of viruses at least in few cases. The exact mechanism of regulation is not clearly understood. They might be doing it by inhibiting replication of viral genome or through inhibition of some crucial genes. The mechanisms might be different for different viruses also. Generally those Viruses whose genomes are methylated can be suppressed / inhibited by DNA methyl transferase inhibitors like AZC or AZdC. The first case reported was of Frog

Virus 3 whose replication was inhibited by AZC treatment.(Goorha *et al.* 1984). However most of the reports concerning viral DNA methylation are from viruses infecting vertebrates. Insects exhibit lot of variation in DNA methylation with respect to both the levels of methylation and in the genetic components required for DNA methylation. So the viruses infecting such hosts are also expected to show significant variation and diversity in levels of methylation of their genome and in responding to inhibitors of Dnmts.

In an interesting report Bigot *et al* (Bigot *et al.* 2000) observed DNA methylation of insect Ascoviruses and iridovirus genomes [*Spodoptera frugiperda ascovirus*(SfAV1), *Heliothis virescensascovirus* (HvAV3) and iridovirus, Mosquito (IV type 3), lepidopteran (IV type 6) and isopod (IV type 31)]. In mammalian ascoviruses CpG methyaltion was as high as 76% but in insect ascoviruses (sfAV1 and HvAV3) methylation was only 2-5%. DpAV4 (a Wasp ascovirus) has much higher methylcytosine content than other insect viruses. In vertebrate (in this case a mammalian virus) viruses and DpAv4 (wasp ascovirus) CpG methylation was very abundant unlike in other insect viruses where CpT and CpC methylation were more abundant. This could be due to the fact that vertebrates and wasps have all three classes of Dnmts unlike other insects which have only Dnmt2 or both Dnmt1 & Dnmt2 which can methylate preferably CpC and CpT than CpG.

So it will be interesting to investigate whether DNA methylation has any role in baculovirus replication and/or viral gene expression in their insect hosts, which themselves exhibit very limited DNA methylation. Efforts in this direction have limited scope as the DNA methylation components are not characterized in *Spodoptera frugiperda* (*sf9* cells), but their nearest relative *Bombyx mori* is reported to have only two Dnmts, *Dnmt1* and *Dnmt2* in their genome. Two very old papers have addressed the role of DNA methylation in Baculovirus gene expression. Kneel *et al* (Knebel *et al.* 1985) reported that in vitro methylated promoter sequence of p10 gene inhibits expression of *p10* gene in transient assays, but it is possible that these sequences which were specifically methylated *in vitro* are not methylated in their natural hosts at all and whatever effect they saw might be due to the inability of binding of transcription factors to promoters containing such an alien pattern of DNA

methylation. The regions which they specifically methylated in vitro were CpG nucleotides which may not be methylated in these insects as they may not contain all Dnmts which can preferably methylate CpG. Other report by Bach *et al* showed that Dnmt inhibitors (AZC or AZdC) inhibit expression of recombinant protein cloned under p10 promoter (Bach *et al.* 1995). Baculoviruses are used to deliver recombinant DNA into mammalian cells, which have a completely functional DNA methylation apparatus, but AZC or AZdC treatment did not affect expression of recombinant gene delivered by baculoviral vectors, unlike Histone deacetylase inhibitors as mentioned earlier (Kitajima *et al.* 2006).

So In this chapter we describe our experiments to see whether DNA methylation has any role in Baculovirus infection, replication and gene expression in insect cells (sf9), and tried to point out the steps or phases of infection which might be affected by inhibiting DNA methylation.

7.2 Materials and Methods:

7.2.1 Cells and medium: sf9 cells were cultured in TNM-FH medium supplemented with 10% fetal bovine serum at 27^{0}C.

7.2.2Recombinant viruses and infection: recombinant AcMNPVs of EGFP and RFP (vRec-RFP and vRec-EGFP) were constructed as explained in Chapter 2 and Chapter 3 earlier Sf9 cells were infected by viruses by adding known concentration of viruses in complete medium and incubated for 1 hour in humidified incubator at 27^{0}C. After one hour virus suspension was replaced by complete medium and incubated until 72 hours at which downstream experiments were carried out as mentioned in the text.

7.2.3 Treatment of sf9 cells with AZdC: 5'-AZA-2-deoxycytidine (Calbiochem) was dissolved in DMSO to make a stock solution of 10mM/ml and stored at -20^{0}C. for usage in cell culture experiments an appropriate volume of AZdC stock solution was mixed with complete medium (TNM-FH supplemented with

10% FBS) to get a final concentrations of 1μM and 10μM. this AZdC containing medium was stored in $4^{0}C$ and consumed before 24 hours of preparation.
In order to treat sf9 cells with AZdC, Sf9 cells were seeded in tissue culture dishes and the culture medium was replaced by AZdC containing medium (1μM or 10μM). Spent medium was replaced with fresh AZdC containing medium after every 24 hours of incubation depending on the duration of experiments.

7.2.4 Analysis of Baculovirus replication: We used Real time PCR to estimate the replicated viral DNA products. Viral DNA was isolated from virus infected sf9 cells using HighPure Viral DNA isolation kit (Roche) and 0.01ng of DNA was used as a template for PCR reaction. The copy numbers were estimated based on a standard curve obtained by using viral DNA of known concentrations. Details of Viral DNA isolation and RealTime PCR are explained in section-2.3 and 2.4 in Chapter 2.

7.2.5 Microscopy and analysis of images: In our experiments where we used recombinant viruses containing RFP or EGFP (vRecRFP or vRec-EGFP), we observed them under fluorescence microscope for expression of RFP or EGFP as an indirect measure of viral titer. Usually this strategy works except conditions in which the p10 gene promoter which is used to express RFP or EGFP is specifically inhibited. Images of a broad area of culture dish was captured under same exposure and magnification using Leica-ASD-3D microscope with camera (IC 3D 134881106) (Image capture settings: 16 bit/channel, 3132 X 2325 full frame HQ , color). Captured images were converted into grey scale images using a scale of '0' to '255' (0-black & 255-white). The pixels were calculated using software Leica microsystems CMS Gmbh "LAS AF Version; 1.8.1 build 1390". This method was used only in relative quantification as this is not an accurate method of quantification of fluorescence from microscopic images. The pixel values were plotted in the bar diagram in the form of arbitrary units.

7.3 Results and discussion:

Effect of AZdC on Baculovirus infection/ AZdC inhibits expression of recombinant gene: In order to investigate whether DNA methylation has any potential role in Baculovirus life cycle, we infected sf9 cells with Recombinant AcMNPV (vRec-RFP) at an MOI of 5. Infected cells were treated with 1µM and 10 µM AZdC and incubated (along with AZdC) for 72 hours. After 72 hours cells were observed under fluorescence microscope for analyzing the expression of RFP. We found that (**Figure 7.1**) control cells treated only with DMSO (solvent for AZdC), showed less fluorescence compared to AZdC treated cells. We found similar results with vRec-EGFP also (data not shown). We observed even 1µM AZdC showed significant inhibition of florescence, Therefore in all our future experiments we have used 1µM AZdC. This concentration is much less than 60µM, which was used in the earlier report by Bach *et al* (Kitajima *et al.* 2006). This extremely low concentration of AZdC will reduce the non specific side effects of AZdC. The reduced Expression of fluorescence could be due to specific inhibition of P10 promoter under which RFP gene is cloned or it might be due to inhibition of virus replication and expression of viral genes. As we have mentioned earlier (in Chapter 1) that expression of late and very late genes follow viral DNA replication and the level of their expression strictly depends on the level of viral DNA replication.

Effect of AZdC treatment at different phases of virus infection: To ascertain whether DNA methylation specifically inhibits the very late promoter of p10 gene or it inhibits the virus replication which in turn inhibits the expression of late genes. We infected sf9 cells with vRec-EGFP virus (MOI-5) and these infected cells were treated with 1µM AZdC at different time points corresponding to different phases of virus infection. In one well of sf9 cells infected with vRec-EGFP, AZdC treatment was given after 0hrs of infection (immediately after infection) and continued till 72hrs. Similarly for other wells treatment was begun at 2hrs, 6hrs, 12hrs, 24hrs, 36hrs and 48hrs after infection and continued till 72hrs. After 72 hrs cells were observed under fluorescence microscope to analyze expression level of EGFP (**Figure 7.2A**). We found that Expression of EGFP was significantly suppressed in

cells treated with AZdC from 0, 2, 6 and 12 hours. Cells which were treated from 24 hours onwards showed moderate levels of EGFP expression which was still less than control cells. After 48 hours AZdC treatment did not really affect the expression of EGFP and the level of EGFP expression was almost equal to control cells (not treated with AZdC). An important trend in suppression of EGFP levels was observed (**Figure 7.2B**) that, In cells treated with AZdC before 12 hours inhibition of EGFP expression was very high, whereas at 24 hours level of inhibition was between 12 and 48 hours time points. If AZdC treatment was specifically inhibiting p10 promoter, then EGFP levels should have been lower in cells treated at 48 hrs PI and also in 24hrs PI compared to control cells (no AZdC but only DMSO treated) but the levels of EGFP in cells treated after 48 hours were almost equal to control cells indicating that AZdC does not inhibit p10 promoter specifically. The observed trend of inhibition of EGFP reveals that AZdC treatment if given at the time of viral DNA replication or before replication, results in significant reduction of EGFP expression. Since we know that expression level of p10 gene like other viral late and very late phase genes is interlinked to level of DNA replication, it is possible that AZdC somehow inhibits viral DNA replication and in turn suppresses late and very late gene expression. Other possibility could be that AZdC inhibits one or more early expressing genes which might be regulating replication and/or the late gene transcription.

Fig 7.1A:

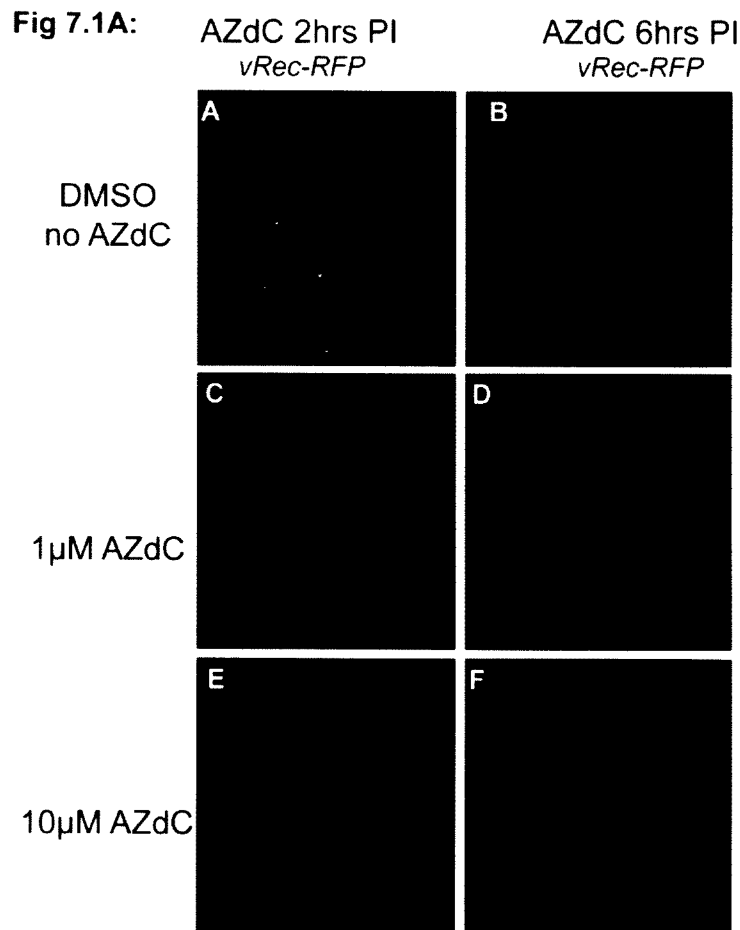


Fig 7.1B:

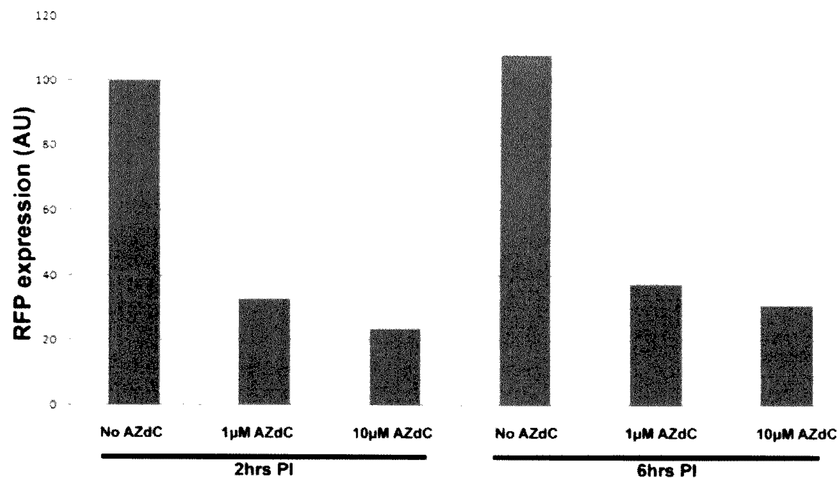
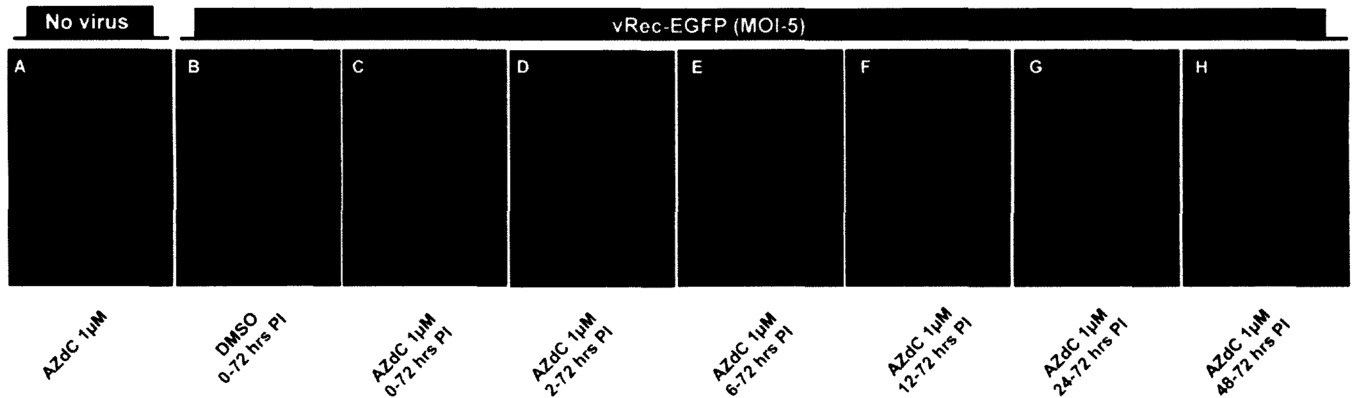


Fig 7.1: AZdC inhibits expression of recombinant gene:

Fig 7.1A: sf9 cells were infected with recombinant virus vRec-RFP (MOI-5) (A-F). Infected cells were treated with 1μM AZdC after 2hrs (C) and 6hrs (D) of infection. after 72hrs AZdC treated cells (C & D) express significantly less amount of RFP compared to only DMSO treated cells. Similarly viurs infected sf9 cells were treated with 10μM AZdC after 2hrs (E) and 6hrs (F) of infection. After 72 hrs expression of RFP was severely inhibited and the levels were less than 1μM AZdC treated cells. Suppression of RFP was more in cells treated with AZdC at 2hrs PI (C & E) than those at 6hrs PI (D & F).

Fig 7.1B: Bar diagram showing the levels of RFP in Arbitrary units (AU). RFP expession levels were estimated in cells infected with vRec-RFP (MOI-5) and treated with AZdC after 2hr and 6hrs PI.

A.



B.

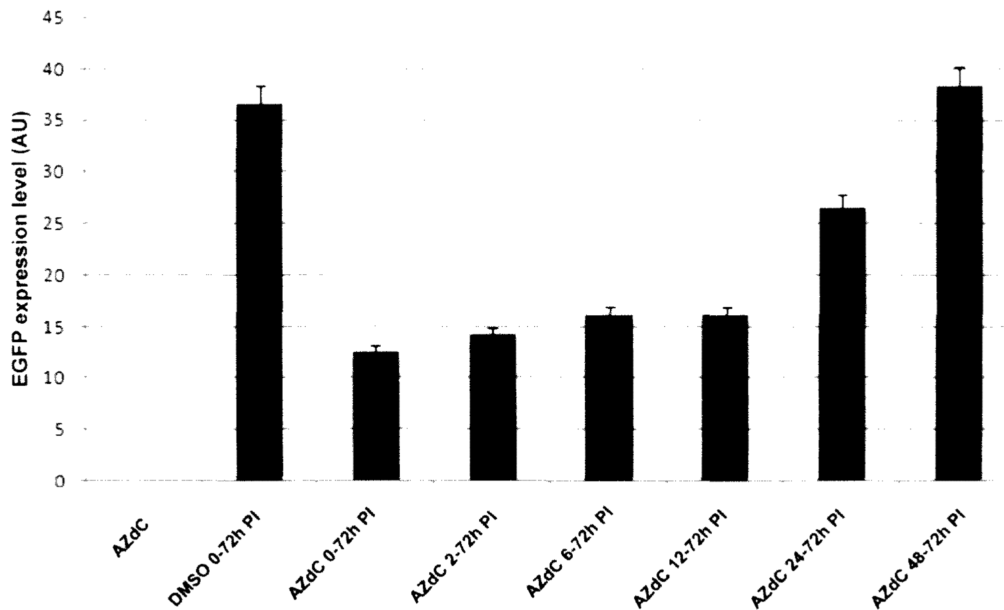


Fig 7.2: AZdC treatment during early phases of infection inhibits recombinant gene expression expressed under viral promoter.

A: *vRec-EGFP* infected *sf9* cells (MOI-5) were treated with 1µM AZdC starting from 0,2,6,12,24 & 48hrs to 72hrs PI. EGFP expression was lesser in cells treated with AZdC at early phases of infection (C,D,E & F) than at later phases.

B: A bar diagram showing expression levels of EGFP in vRec-EGFP infected cells, treated with 1µM AZdC at different time points

Effect of AZdC on Replication of vRec-EGFP in sf9 cells: To verify the mechanism of inhibition of EGFP by AZdC during early hours of infection but not in the later stages we estimated the level of Viral DNA replication in AZdC treated cells at particular time intervals as per the previous section. We harvested cells from each well of the experiment mentioned in the previous section and isolated total DNA and estimated viral replication products by real time PCR. The levels of viral DNA replication (**Figure 7.3A**) indicate that AZdC inhibits viral DNA replication significantly in cells treated at 12hrs or before 12hrs of infection. Replication was moderately inhibited in cells treated at 24 hrs but there was no significant inhibition in cells treated at 48hours after infection. These results confirm that inhibition of EGFP expression was indeed due to inhibition of replication. The time periods at which viral DNA is replicated at a rapid rate were more sensitive to AZdC treatment and later time points at which rate of replication is less and/or becomes irrelevant for regulating the expression of very late genes which are already close to their peak levels of expression were not affected significantly.

In order to verify our methods to estimate the levels of EGFP/RFP expression in cells by fluorescence microscopy as an indirect measure of viral titer, we compared the results obtained by fluorescence estimation (**Fig 7.2B**) and estimation of Viral DNA by Real time PCR (**Fig 7.3A**). The 'fluorescence' and 'DNA copy number' values obtained from virus infected sf9 cells treated with DMSO (no AZdC), were taken as 100 and all other corresponding values obtained from cells treated with AZdC from different time points of infection are represented as ratios of 100 (percentage) (**Fig 7.3B**). Both levels of fluorescence and Viral DNA replication showed a similar trend with respect to AZdC treatment at different phases of infection (**Fig 7.3B**).

Fig 7.3A:

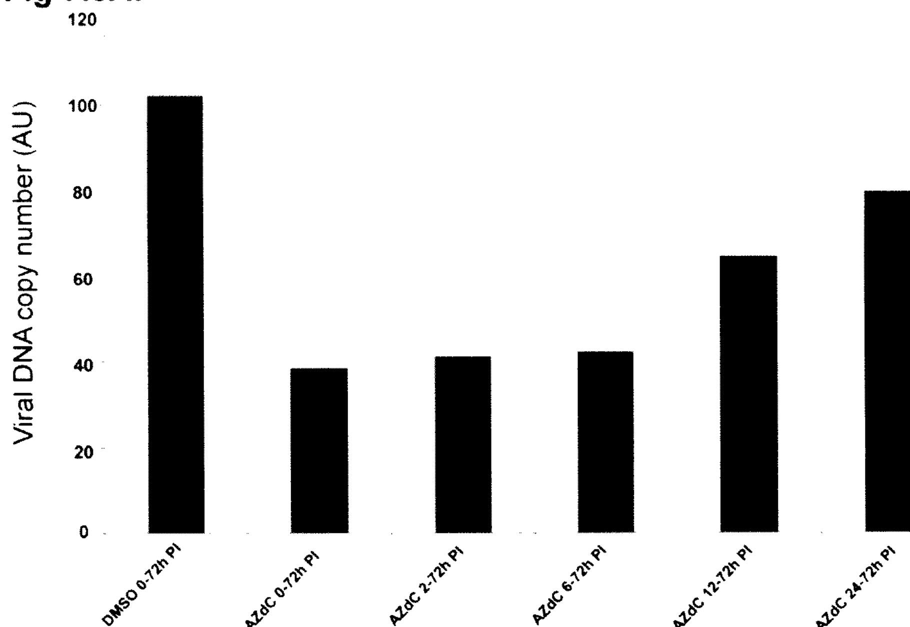


Fig 7.3B:

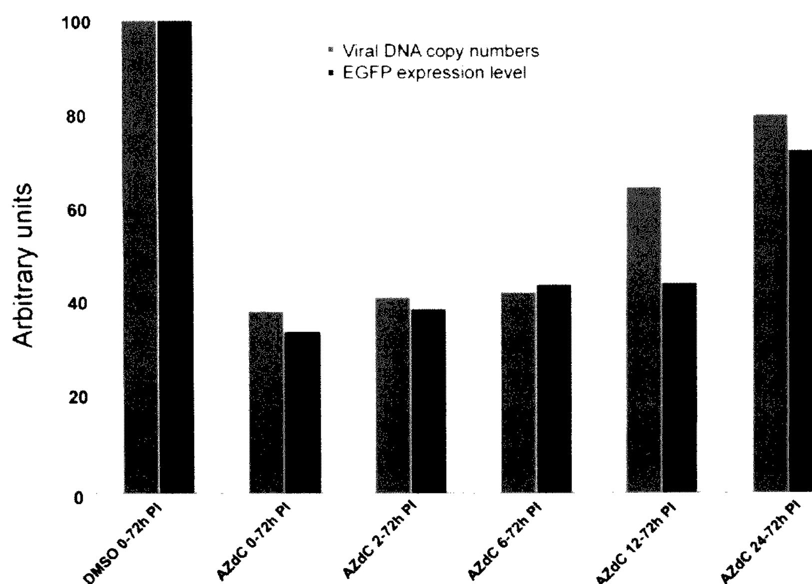


Fig 7.3: AZdC inhibits replication of Baculoviurs vRec-EGFP

Fig 7.3A: sf9 cells were infected with vRec-EGFP (MOI-5) and treated with 1μM AZdC from 0, 2, 6,12 & 24 hrs to 72hrs PI. and viral DNA copy numbers were estimated and plotted by taking viral DNA from DMSO treated samples as 100.

Fig 7.3B: A graph showing the comparison of Viral DNA copy number (fig 7.3A) and the levels of EGFP (fig 7.2B) estimated from virus infected cells treated with AZdC at different time points after infection. In both cases corresponding values (viral DNA copy number and EGFP levels) obtained from DMSO treated cells were considered as 100 and all other corresponding values are plotted as a ratio of 100. A similar trend in the levels of EGFP and Viral DNA copy number is shown.

Conclusions:

To summarize our results, We found that Dnmt inhibitor AZdC affects expression of genes expressed under baculoviral promoters and we found the effect of AZdC is not specifically directed towards inhibiting those late phase genes. We also found that AZdC does not inhibit very late *Polyhedrin* promoter if treated in late phases of infection but can inhibit severely in early phases of infection. This implies that AZdC might be affecting some early events of viral infection which directly and/or indirectly regulate viral late gene promoters. We could also point out that AZdC affects Baculovirus DNA replication in sf9 cells if treated during the time points when rapid viral DNA replication happens. since the viral DNA replication and late & very late gene transcription are tightly linked, any suppression of viral replication by AZdC affects late and very late gene transcription in general.

It was interesting to observe these effects in insect cells which have very limited levels of DNA methylation, and DNA methylation might have role in regulating Baculovirus DNA replication and infection like many vertebrate and wasp DNA viruses.

References:

Adelman, Z. N., C. D. Blair, et al. (2001). "Sindbis virus-induced silencing of dengue viruses in mosquitoes." Insect Mol Biol **10**(3): 265-73.

Adelman, Z. N., I. Sanchez-Vargas, et al. (2002). "RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome." J Virol **76**(24): 12925-33.

Ahrens, C. H., R. L. Russell, et al. (1997). "The sequence of the Orgyia pseudotsugata multinucleocapsid nuclear polyhedrosis virus genome." Virology **229**(2): 381-99.

Allen, E., Z. Xie, et al. (2005). "microRNA-directed phasing during trans-acting siRNA biogenesis in plants." Cell **121**(2): 207-21.

Andersson, M. G., P. C. Haasnoot, et al. (2005). "Suppression of RNA interference by adenovirus virus-associated RNA." J Virol **79**(15): 9556-65.

Anduleit, K., G. Sutton, et al. (2005). "Crystal lattice as biological phenotype for insect viruses." Protein Sci **14**(10): 2741-3.

Araujo, R. N., A. Santos, et al. (2006). "RNA interference of the salivary gland nitrophorin 2 in the triatomine bug Rhodnius prolixus (Hemiptera: Reduviidae) by dsRNA ingestion or injection." Insect Biochem Mol Biol **36**(9): 683-93.

Ayres, M. D., S. C. Howard, et al. (1994). "The complete DNA sequence of Autographa californica nuclear polyhedrosis virus." Virology **202**(2): 586-605.

Bach, C., A. Cramer, et al. (1995). "Effect of methyltransferase inhibitors on the regulation of baculovirus protein synthesis." J Gen Virol **76 (Pt 4)**: 1025-32.

Bagga, S., J. Bracht, et al. (2005). "Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation." Cell **122**(4): 553-63.

Baghdoyan, S., Y. Roupioz, et al. (2004). "Quantitative analysis of highly parallel transfection in cell microarrays." Nucleic Acids Res **32**(9): e77.

Baulcombe, D. C., G. R. Saunders, et al. (1986). "Expression of biologically active viral satellite RNA from the nuclear genome of transformed plants." Nature **321**(6068): 446-449.

Baum, J. A., T. Bogaert, et al. (2007). "Control of coleopteran insect pests through RNA interference." Nat Biotechnol **25**(11): 1322-6.

Beck, M. and M. R. Strand (2003). "RNA interference silences Microplitis demolitor bracovirus genes and implicates glc1.8 in disruption of adhesion in infected host cells." Virology **314**(2): 521-35.

Bennasser, Y., S. Y. Le, et al. (2005). "Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing." Immunity **22**(5): 607-19.

Bergin, D., E. P. Reeves, et al. (2005). "Superoxide production in Galleria mellonella hemocytes: identification of proteins homologous to the NADPH oxidase complex of human neutrophils." Infect Immun **73**(7): 4161-70.

Bestor, T. H. (2000). "The DNA methyltransferases of mammals." Hum Mol Genet **9**(16): 2395-402.

Bettencourt, R., O. Terenius, et al. (2002). "Hemolin gene silencing by ds-RNA injected into Cecropia pupae is lethal to next generation embryos." Insect Mol Biol **11**(3): 267-71.

Bideshi, D. K., S. Renault, et al. (2003). "Phylogenetic analysis and possible function of bro-like genes, a multigene family widespread among large double-stranded DNA viruses of invertebrates and bacteria." J Gen Virol **84**(Pt 9): 2531-44.

Bigot, Y., K. Stasiak, et al. (2000). "Characterization of repetitive DNA regions and methylated DNA in ascovirus genomes." J Gen Virol **81**(Pt 12): 3073-82.

Bilimoria, S. (1986). " *In* The Biology of Baculoviruses. pp. 37–59. (Granados & Federici Ed.) CRC Press, Boca Raton, FL."
Bird, A. (1997). "Does DNA methylation control transposition of selfish elements in the germline?" Trends Genet **13**(12): 469-72.
Bird, A. (2002). "DNA methylation patterns and epigenetic memory." Genes Dev **16**(1): 6-21.
Bird, A. P. (1995). "Gene number, noise reduction and biological complexity." Trends Genet **11**(3): 94-100.
Bisaro, D. M. (2006). "Silencing suppression by geminivirus proteins." Virology **344**(1): 158-68.
Blissard, G. W., P. H. Kogan, et al. (1992). "A synthetic early promoter from a baculovirus: roles of the TATA box and conserved start site CAGT sequence in basal levels of transcription." Virology **190**(2): 783-93.
Blissard, G. W. and G. F. Rohrmann (1989). "Location, sequence, transcriptional mapping, and temporal expression of the gp64 envelope glycoprotein gene of the Orgyia pseudotsugata multicapsid nuclear polyhedrosis virus." Virology **170**(2): 537-55.
Bock, C. T., P. Schranz, et al. (1994). "Hepatitis B virus genome is organized into nucleosomes in the nucleus of the infected cell." Virus Genes **8**(3): 215-29.
Braunagel, S. C., P. A. Guidry, et al. (2001). "Identification of BV/ODV-C42, an Autographa californica nucleopolyhedrovirus orf101-encoded structural protein detected in infected-cell complexes with ODV-EC27 and p78/83." J Virol **75**(24): 12331-8.
Braunagel, S. C., W. K. Russell, et al. (2003). "Determination of the protein composition of the occlusion-derived virus of Autographa californica nucleopolyhedrovirus." Proc Natl Acad Sci U S A **100**(17): 9797-802.
Braunagel, S. C. and M. D. Summers (2007). "Molecular biology of the baculovirus occlusion-derived virus envelope." Curr Drug Targets **8**(10): 1084-95.
Brody, E. N., G. A. Kassavetis, et al. (1995). "Old phage, new insights: two recently recognized mechanisms of transcriptional regulation in bacteriophage T4 development." FEMS Microbiol Lett **128**(1): 1-8.
Broer, R., J. G. Heldens, et al. (1998). "Specificity of multiple homologous genomic regions in Spodoptera exigua nucleopolyhedrovirus DNA replication." J Gen Virol **79 (Pt 6)**: 1563-72.
Bucher, E., H. Hemmes, et al. (2004). "The influenza A virus NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants." J Gen Virol **85**(Pt 4): 983-91.
Bucher, G., J. Scholten, et al. (2002). "Parental RNAi in Tribolium (Coleoptera)." Curr Biol **12**(3): R85-6.
Buck, A. H., J. Santoyo-Lopez, et al. (2007). "Discrete clusters of virus-encoded micrornas are associated with complementary strands of the genome and the 7.2-kilobase stable intron in murine cytomegalovirus." J Virol **81**(24): 13761-70.
Budovskaya, Y. V., K. Wu, et al. (2008). "An elt-3/elt-5/elt-6 GATA transcription circuit guides aging in C. elegans." Cell **134**(2): 291-303.
Burand, J. P., M. D. Summers, et al. (1980). "Transfection with baculovirus DNA." Virology **101**(1): 286-90.
Burden, J. P., C. M. Griffiths, et al. (2002). "Vertical transmission of sublethal granulovirus infection in the Indian meal moth, Plodia interpunctella." Mol Ecol **11**(3): 547-55.
Burden, J. P., R. D. Possee, et al. (2006). "Phenotypic and genotypic characterisation of persistent baculovirus infections in populations of the cabbage moth (Mamestra brassicae) within the British Isles." Arch Virol **151**(4): 635-49.
Burgers, W. A., F. Fuks, et al. (2002). "DNA methyltransferases get connected to chromatin." Trends Genet **18**(6): 275-7.

Burma, S., B. Mukherjee, et al. (1994). "An unusual 30-kDa protein binding to the polyhedrin gene promoter of Autographa californica nuclear polyhedrosis virus." J Biol Chem **269**(4): 2750-7.
Busslinger, M., J. Hurst, et al. (1983). "DNA methylation and the regulation of globin gene expression." Cell **34**(1): 197-206.
Cai, X., S. Lu, et al. (2005). "Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells." Proc Natl Acad Sci U S A **102**(15): 5570-5.
Chao, J. A., J. H. Lee, et al. (2005). "Dual modes of RNA-silencing suppression by Flock House virus protein B2." Nat Struct Mol Biol **12**(11): 952-7.
Chapman, E. J., A. I. Prokhnevsky, et al. (2004). "Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step." Genes Dev **18**(10): 1179-86.
Chen, J., W. X. Li, et al. (2004). "Viral virulence protein suppresses RNA silencing-mediated defense but upregulates the role of microrna in host gene expression." Plant Cell **16**(5): 1302-13.
Cherry, S. and N. Silverman (2006). "Host-pathogen interactions in drosophila: new tricks from an old friend." Nat Immunol **7**(9): 911-7.
Choi, J. and L. A. Guarino (1995). "The baculovirus transactivator IE1 binds to viral enhancer elements in the absence of insect cell factors." J Virol **69**(7): 4548-51.
Chung, W. J., K. Okamura, et al. (2008). "Endogenous RNA interference provides a somatic defense against Drosophila transposons." Curr Biol **18**(11): 795-802.
Clem, R. J., M. Fechheimer, et al. (1991). "Prevention of apoptosis by a baculovirus gene during infection of insect cells." Science **254**(5036): 1388-90.
Cochran, M. A., E. B. Carstens, et al. (1982). "Molecular Cloning and Physical Mapping of Restriction Endonuclease Fragments of Autographa californica Nuclear Polyhedrosis Virus DNA." J Virol **41**(3): 940-946.
Colot, V. and J. L. Rossignol (1999). "Eukaryotic DNA methylation as an evolutionary device." Bioessays **21**(5): 402-11.
Cory, J. S., B. M. Green, et al. (2005). "Genotypic and phenotypic diversity of a baculovirus population within an individual insect host." J Invertebr Pathol **89**(2): 101-11.
Cui, C., A. Griffiths, et al. (2006). "Prediction and identification of herpes simplex virus 1-encoded microRNAs." J Virol **80**(11): 5499-508.
Cullen, B. R. (2006). "Is RNA interference involved in intrinsic antiviral immunity in mammals?" Nat Immunol **7**(6): 563-7.
Czech, B., C. D. Malone, et al. (2008). "An endogenous small interfering RNA pathway in Drosophila." Nature **453**(7196): 798-802.
Dall, D., T. Luque, et al. (2001). "Insect-virus relationships: sifting by informatics." Bioessays **23**(2): 184-93.
Deddouche, S., N. Matt, et al. (2008). "The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in drosophila." Nat Immunol **9**(12): 1425-32.
Deng, F., R. Wang, et al. (2007). "Proteomics analysis of Helicoverpa armigera single nucleocapsid nucleopolyhedrovirus identified two new occlusion-derived virus-associated proteins, HA44 and HA100." J Virol **81**(17): 9377-85.
Deshmane, S. L. and N. W. Fraser (1989). "During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure." J Virol **63**(2): 943-7.
Detvisitsakun, C., E. L. Cain, et al. (2007). "The Autographa californica M nucleopolyhedrovirus fibroblast growth factor accelerates host mortality." Virology **365**(1): 70-8.
Dolken, L., J. Perot, et al. (2007). "Mouse cytomegalovirus microRNAs dominate the cellular small RNA profile during lytic infection and show features of posttranscriptional regulation." J Virol **81**(24): 13771-82.

Dong, A., J. A. Yoder, et al. (2001). "Structure of human DNMT2, an enigmatic DNA methyltransferase homolog that displays denaturant-resistant binding to DNA." Nucleic Acids Res **29**(2): 439-48.
Dong, X., R. van Wezel, et al. (2003). "Functional characterization of the nuclear localization signal for a suppressor of posttranscriptional gene silencing." J Virol **77**(12): 7026-33.
Dong, Y. and M. Friedrich (2005). "Nymphal RNAi: systemic RNAi mediated gene knockdown in juvenile grasshopper." BMC Biotechnol **5**: 25.
Du, T. and P. D. Zamore (2005). "microPrimer: the biogenesis and function of microRNA." Development **132**(21): 4645-52.
Dunoyer, P., C. H. Lecellier, et al. (2004). "Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing." Plant Cell **16**(5): 1235-50.
Dykxhoorn, D. M. (2007). "MicroRNAs in viral replication and pathogenesis." DNA Cell Biol **26**(4): 239-49.
Eaton, B. A., R. D. Fetter, et al. (2002). "Dynactin is necessary for synapse stabilization." Neuron **34**(5): 729-41.
Engelhard, E. K., L. N. Kam-Morgan, et al. (1994). "The insect tracheal system: a conduit for the systemic spread of Autographa californica M nuclear polyhedrosis virus." Proc Natl Acad Sci U S A **91**(8): 3224-7.
Eulalio, A., I. Behm-Ansmant, et al. (2007). "P-body formation is a consequence, not the cause, of RNA-mediated gene silencing." Mol Cell Biol **27**(11): 3970-81.
Evans, J. T., D. J. Leisy, et al. (1997). "Characterization of the interaction between the baculovirus replication factors LEF-1 and LEF-2." J Virol **71**(4): 3114-9.
Fang, M., Y. Nie, et al. (2006). "Open reading frame 132 of Helicoverpa armigera nucleopolyhedrovirus encodes a functional per os infectivity factor (PIF-2)." J Gen Virol **87**(Pt 9): 2563-9.
Fang, M., H. Wang, et al. (2003). "Open reading frame 94 of Helicoverpa armigera single nucleocapsid nucleopolyhedrovirus encodes a novel conserved occlusion-derived virion protein, ODV-EC43." J Gen Virol **84**(Pt 11): 3021-7.
Faulkner, P., J. Kuzio, et al. (1997). "Analysis of p74, a PDV envelope protein of Autographa californica nucleopolyhedrovirus required for occlusion body infectivity in vivo." J Gen Virol **78 (Pt 12)**: 3091-100.
Federici, B. A. and R. H. Hice (1997). "Organization and molecular characterization of genes in the polyhedrin region of the Anagrapha falcifera multinucleocapsid NPV." Arch Virol **142**(2): 333-48.
Fedorov, Y., E. M. Anderson, et al. (2006). "Off-target effects by siRNA can induce toxic phenotype." RNA **12**(7): 1188-96.
Feinberg, E. H. and C. P. Hunter (2003). "Transport of dsRNA into cells by the transmembrane protein SID-1." Science **301**(5639): 1545-7.
Fire, A., S. Xu, et al. (1998). "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans." Nature **391**(6669): 806-11.
Fisher, O., R. Siman-Tov, et al. (2004). "Characterization of cytosine methylated regions and 5-cytosine DNA methyltransferase (Ehmeth) in the protozoan parasite Entamoeba histolytica." Nucleic Acids Res **32**(1): 287-97.
Flipsen, J. T., J. W. Martens, et al. (1995). "Passage of Autographa californica nuclear polyhedrosis virus through the midgut epithelium of Spodoptera exigua larvae." Virology **208**(1): 328-35.
Flores-Jasso, C. F., V. J. Valdes, et al. (2004). "Silencing structural and nonstructural genes in baculovirus by RNA interference." Virus Res **102**(1): 75-84.
Frank, S. A. (2003). "Viral genetics: deadly partnerships." Nature **425**(6955): 251-2.

Fridholm, H. and E. Everitt (2005). "Rapid and reproducible infectivity end-point titration of virulent phage in a microplate system." J Virol Methods **128**(1-2): 67-71.
Fuchs, L. Y., M. S. Woods, et al. (1983). "Viral Transcription During Autographa californica Nuclear Polyhedrosis Virus Infection: a Novel RNA Polymerase Induced in Infected Spodoptera frugiperda Cells." J Virol **48**(3): 641-646.
Gaines, P. J., K. E. Olson, et al. (1996). "Pathogen-derived resistance to dengue type 2 virus in mosquito cells by expression of the premembrane coding region of the viral genome." J Virol **70**(4): 2132-7.
Galiana-Arnoux, D., C. Dostert, et al. (2006). "Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila." Nat Immunol **7**(6): 590-7.
Garrity, D. B., M. J. Chang, et al. (1997). "Late promoter selection in the baculovirus gp64 envelope fusion protein gene." Virology **231**(2): 167-81.
Ghildiyal, M., H. Seitz, et al. (2008). "Endogenous siRNAs derived from transposons and mRNAs in Drosophila somatic cells." Science **320**(5879): 1077-81.
Ghosh, S., A. Jain, et al. (1998). "The host factor polyhedrin promoter binding protein (PPBP) is involved in transcription from the baculovirus polyhedrin gene promoter." J Virol **72**(9): 7484-93.
Glazer, R. I., A. L. Peale, et al. (1980). "The effect of 5-azacytidine and dihydro-5-azacytidine on nuclear ribosomal RNA and poly(A) RNA synthesis in L1210 cells in vitro." Mol Pharmacol **17**(1): 111-7.
Goley, E. D., T. Ohkawa, et al. (2006). "Dynamic nuclear actin assembly by Arp2/3 complex and a baculovirus WASP-like protein." Science **314**(5798): 464-7.
Goll, M. G. and T. H. Bestor (2005). "Eukaryotic cytosine methyltransferases." Annu Rev Biochem **74**: 481-514.
Goll, M. G., F. Kirpekar, et al. (2006). "Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2." Science **311**(5759): 395-8.
Gomi, S., C. E. Zhou, et al. (1997). "Deletion analysis of four of eighteen late gene expression factor gene homologues of the baculovirus, BmNPV." Virology **230**(1): 35-47.
Goorha, R., A. Granoff, et al. (1984). "The role of DNA methylation in virus replication: inhibition of frog virus 3 replication by 5-azacytidine." Virology **138**(1): 94-102.
Gottwein, E. and B. R. Cullen (2008). "Viral and cellular microRNAs as determinants of viral pathogenesis and immunity." Cell Host Microbe **3**(6): 375-87.
Grace, T. D. (1962). "Establishment of four strains of cells from insect tissues grown in vitro." Nature **195**: 788-9.
Granados, R. R. and K. A. Lawler (1981). "In vivo pathway of Autographa californica baculovirus invasion and infection." Virology **108**(2): 297-308.
Grassmann, R. and K. T. Jeang (2008). "The roles of microRNAs in mammalian virus infection." Biochim Biophys Acta **1779**(11): 706-11.
Grewal, S. I. and S. C. Elgin (2007). "Transcription and RNA interference in the formation of heterochromatin." Nature **447**(7143): 399-406.
Gross, C. H. and G. F. Rohrmann (1993). "Analysis of the role of 5' promoter elements and 3' flanking sequences on the expression of a baculovirus polyhedron envelope protein gene." Virology **192**(1): 273-81.
Gross, C. H., R. L. Russell, et al. (1994). "Orgyia pseudotsugata baculovirus p10 and polyhedron envelope protein genes: analysis of their relative expression levels and role in polyhedron structure." J Gen Virol **75 (Pt 5)**: 1115-23.
Gross, C. H. and S. Shuman (1998). "Characterization of a baculovirus-encoded RNA 5'-triphosphatase." J Virol **72**(9): 7057-63.

Gross, C. H. and S. Shuman (1998). "RNA 5'-triphosphatase, nucleoside triphosphatase, and guanylyltransferase activities of baculovirus LEF-4 protein." J Virol **72**(12): 10020-8.
Gross, C. H., G. M. Wolgamot, et al. (1993). "A baculovirus encoded 16-kDa glycoprotein localizes near the nuclear membrane of infected cells." Virology **192**(1): 386-90.
Grula, M. A., P. L. Buller, et al. (1981). "alpha-Amanitin-Resistant Viral RNA Synthesis in Nuclei Isolated from Nuclear Polyhedrosis Virus-Infected Heliothis zea Larvae and Spodoptera frugiperda Cells." J Virol **38**(3): 916-921.
Guarino, L. A. (1990). "Identification of a viral gene encoding a ubiquitin-like protein." Proc Natl Acad Sci U S A **87**(1): 409-13.
Guarino, L. A., M. A. Gonzalez, et al. (1986). "Complete Sequence and Enhancer Function of the Homologous DNA Regions of Autographa californica Nuclear Polyhedrosis Virus." J Virol **60**(1): 224-229.
Guarino, L. A., J. Jin, et al. (1998). "Guanylyltransferase activity of the LEF-4 subunit of baculovirus RNA polymerase." J Virol **72**(12): 10003-10.
Guarino, L. A., T. A. Mistretta, et al. (2002). "Baculovirus lef-12 is not required for viral replication." J Virol **76**(23): 12032-43.
Guarino, L. A. and M. Smith (1992). "Regulation of delayed-early gene transcription by dual TATA boxes." J Virol **66**(6): 3733-9.
Guarino, L. A. and M. D. Summers (1986). "Functional mapping of a trans-activating gene required for expression of a baculovirus delayed-early gene." J Virol **57**(2): 563-71.
Guarino, L. A. and M. D. Summers (1986). "Interspersed Homologous DNA of Autographa californica Nuclear Polyhedrosis Virus Enhances Delayed-Early Gene Expression." J Virol **60**(1): 215-223.
Guarino, L. A., B. Xu, et al. (1998). "A virus-encoded RNA polymerase purified from baculovirus-infected cells." J Virol **72**(10): 7985-91.
Guil, S. and M. Esteller (2009). "DNA methylomes, histone codes and miRNAs: tying it all together." Int J Biochem Cell Biol **41**(1): 87-95.
Guo, H. S. and S. W. Ding (2002). "A viral protein inhibits the long range signaling activity of the gene silencing signal." EMBO J **21**(3): 398-407.
Guo, S. and K. J. Kemphues (1995). "par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed." Cell **81**(4): 611-20.
Haas, A. L., D. J. Katzung, et al. (1996). "Functional characterization of the ubiquitin variant encoded by the baculovirus Autographa californica." Biochemistry **35**(17): 5385-94.
Haasnoot, J., W. de Vries, et al. (2007). "The Ebola virus VP35 protein is a suppressor of RNA silencing." PLoS Pathog **3**(6): e86.
Habib, S. and S. E. Hasnain (2000). "Differential activity of two non-hr origins during replication of the baculovirus Autographa californica nuclear polyhedrosis virus genome." J Virol **74**(11): 5182-9.
Hang, X., W. Dong, et al. (1995). "The lef-3 gene of Autographa californica nuclear polyhedrosis virus encodes a single-stranded DNA-binding protein." J Virol **69**(6): 3924-8.
Hang, X. and L. A. Guarino (1999). "Purification of Autographa californica nucleopolyhedrovirus DNA polymerase from infected insect cells." J Gen Virol **80 (Pt 9)**: 2519-26.
Harrison, B. D., M. A. Mayo, et al. (1987). "Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA." Nature **328**(6133): 799-802.
Harrison, R. L. and B. C. Bonning (1999). "The nucleopolyhedroviruses of Rachiplusia ou and Anagrapha falcifera are isolates of the same virus." J Gen Virol **80 (Pt 10)**: 2793-8.

Harrison, R. L., D. L. Jarvis, et al. (1996). "The role of the AcMNPV 25K gene, "FP25," in baculovirus polh and p10 expression." Virology **226**(1): 34-46.
Harrison, R. L. and M. D. Summers (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." J Gen Virol **76 (Pt 6)**: 1451-9.
Henderson, I. R. and S. E. Jacobsen (2007). "Epigenetic inheritance in plants." Nature **447**(7143): 418-24.
Hermann, A., S. Schmitt, et al. (2003). "The human Dnmt2 has residual DNA-(cytosine-C5) methyltransferase activity." J Biol Chem **278**(34): 31717-21.
Hink, W. F. (1970). "Established insect cell line from the cabbage looper, Trichoplusia ni." Nature **226**(5244): 466-7.
Hitchman, R. B., E. A. Siaterli, et al. (2007). "Quantitative real-time PCR for rapid and accurate titration of recombinant baculovirus particles." Biotechnol Bioeng **96**(4): 810-4.
Hodgson, D. J., A. J. Vanbergen, et al. (2001). "Phenotypic variation between naturally coexisting genotypes of a lepidopteran baculovirus." Evol. Ecol. Res. **3**: 687-701.
Hong, T., S. C. Braunagel, et al. (1994). "Transcription, translation, and cellular localization of PDV-E66: a structural protein of the PDV envelope of Autographa californica nuclear polyhedrosis virus." Virology **204**(1): 210-22.
Hong, T., M. D. Summers, et al. (1997). "N-terminal sequences from Autographa californica nuclear polyhedrosis virus envelope proteins ODV-E66 and ODV-E25 are sufficient to direct reporter proteins to the nuclear envelope, intranuclear microvesicles and the envelope of occlusion derived virus." Proc Natl Acad Sci U S A **94**(8): 4050-5.
Horton, H. M. and J. P. Burand (1993). "Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion." J Virol **67**(4): 1860-8.
Huang, J., F. Wang, et al. (2007). "Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes." Nat Med **13**(10): 1241-7.
Huang, Y., F. Deng, et al. (2007). "Baculovirus-mediated gene silencing in insect cells using intracellularly produced long double-stranded RNA." J Biotechnol **128**(2): 226-36.
Hughes, D. S., R. D. Possee, et al. (1993). "Activation and detection of a latent baculovirus resembling Mamestra brassicae nuclear polyhedrosis virus in M. brassicae insects." Virology **194**(2): 608-15.
Hughes, D. S., R. D. Possee, et al. (1997). "Evidence for the presence of a low-level, persistent baculovirus infection of Mamestra brassicae insects." J Gen Virol **78 (Pt 7)**: 1801-5.
Imai, N., N. Matsuda, et al. (2003). "Ubiquitin ligase activities of Bombyx mori nucleopolyhedrovirus RING finger proteins." J Virol **77**(2): 923-30.
Inoue, H., H. Nojima, et al. (1990). "High efficiency transformation of Escherichia coli with plasmids." Gene **96**(1): 23-8.
Isobe, R., K. Kojima, et al. (2004). "Use of RNAi technology to confer enhanced resistance to BmNPV on transgenic silkworms." Arch Virol **149**(10): 1931-40.
Iwanaga, M., K. Takaya, et al. (2004). "Expression profiling of baculovirus genes in permissive and nonpermissive cell lines." Biochem Biophys Res Commun **323**(2): 599-614.
Iyer, L. M., E. V. Koonin, et al. (2002). "Extensive domain shuffling in transcription regulators of DNA viruses and implications for the origin of fungal APSES transcription factors." Genome Biol **3**(3): RESEARCH0012.
Jablanka, E. and A. Regev (1995). "Gene number, methylation and biological complexity." Trends Genet **11**(10): 383-4.

Jain, A. and S. E. Hasnain (1996). "A 30-kDa host protein binds to two very-late baculovirus promoters." Eur J Biochem **239**(2): 384-90.

Janakiraman, V., W. F. Forrest, et al. (2006). "A rapid method for estimation of baculovirus titer based on viable cell size." J Virol Methods **132**(1-2): 48-58.

Janakiraman, V., W. F. Forrest, et al. (2006). "Estimation of baculovirus titer based on viable cell size." Nat Protoc **1**(5): 2271-6.

Jarvis, D. L., D. A. Bohlmeyer, et al. (1992). "Enhancement of polyhedrin nuclear localization during baculovirus infection." J Virol **66**(12): 6903-11.

Jehle, J. A., G. W. Blissard, et al. (2006). "On the classification and nomenclature of baculoviruses: a proposal for revision." Arch Virol **151**(7): 1257-66.

Jeltsch, A., W. Nellen, et al. (2006). "Two substrates are better than one: dual specificities for Dnmt2 methyltransferases." Trends Biochem Sci **31**(6): 306-8.

Jin, J., W. Dong, et al. (1998). "The LEF-4 subunit of baculovirus RNA polymerase has RNA 5'-triphosphatase and ATPase activities." J Virol **72**(12): 10011-9.

Jin, J. and L. A. Guarino (2000). "3'-end formation of baculovirus late RNAs." J Virol **74**(19): 8930-7.

Kamita, S. G. and S. Maeda (1993). "Inhibition of Bombyx mori nuclear polyhedrosis virus (NPV) replication by the putative DNA helicase gene of Autographa californica NPV." J Virol **67**(10): 6239-45.

Kamita, S. G., K. Nagasaka, et al. (2005). "A baculovirus-encoded protein tyrosine phosphatase gene induces enhanced locomotory activity in a lepidopteran host." Proc Natl Acad Sci U S A **102**(7): 2584-9.

Kang, W., M. Suzuki, et al. (1999). "Characterization of baculovirus repeated open reading frames (bro) in Bombyx mori nucleopolyhedrovirus." J Virol **73**(12): 10339-45.

Kang, W. K., N. Imai, et al. (2003). "Interaction of Bombyx mori nucleopolyhedrovirus BRO-A and host cell protein laminin." Arch Virol **148**(1): 99-113.

Kanginakudru, S., C. Royer, et al. (2007). "Targeting ie-1 gene by RNAi induces baculoviral resistance in lepidopteran cell lines and in transgenic silkworms." Insect Mol Biol **16**(5): 635-44.

Karlin, S. and C. Burge (1995). "Dinucleotide relative abundance extremes: a genomic signature." Trends Genet **11**(7): 283-90.

Katsuma, S., S. Kawaoka, et al. (2008). "Genome-wide survey for baculoviral host homologs using the Bombyx genome sequence." Insect Biochem Mol Biol **38**(12): 1080-6.

Katsuma, S., Y. Noguchi, et al. (1999). "Characterization of the 25K FP gene of the baculovirus Bombyx mori nucleopolyhedrovirus: implications for post-mortem host degradation." J Gen Virol **80 (Pt 3)**: 783-91.

Kaur, K., J. Rohozinski, et al. (1995). "Identification and characterization of the frog virus 3 DNA methyltransferase gene." J Gen Virol **76 (Pt 8)**: 1937-43.

Kawahara, Y. and T. Imanishi (2007). "A genome-wide survey of changes in protein evolutionary rates across four closely related species of Saccharomyces sensu stricto group." BMC Evol Biol **7**: 9.

Kawahara, Y., B. Zinshteyn, et al. (2007). "Redirection of silencing targets by adenosine-to-inosine editing of miRNAs." Science **315**(5815): 1137-40.

Kawamura, Y., K. Saito, et al. (2008). "Drosophila endogenous small RNAs bind to Argonaute 2 in somatic cells." Nature **453**(7196): 793-7.

Keck, J. G., C. J. Baldick, Jr., et al. (1990). "Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late trans-activator genes." Cell **61**(5): 801-9.

Kenoutis, C., R. C. Efrose, et al. (2006). "Baculovirus-mediated gene delivery into Mammalian cells does not alter their transcriptional and differentiating potential but is accompanied by early viral gene expression." J Virol **80**(8): 4135-46.
Kikhno, I., S. Gutierrez, et al. (2002). "Characterization of pif, a gene required for the per os infectivity of Spodoptera littoralis nucleopolyhedrovirus." J Gen Virol **83**(Pt 12): 3013-22.
Kim, C. S., Z. Kosuke, et al. (2007). "Protection of shrimp (Penaeus chinensis) against white spot syndrome virus (WSSV) challenge by double-stranded RNA." Fish Shellfish Immunol **23**(1): 242-6.
Kitajima, M., H. Hamazaki, et al. (2006). "Characterization of baculovirus Autographa californica multiple nuclear polyhedrosis virus infection in mammalian cells." Biochem Biophys Res Commun **343**(2): 378-84.
Kitts, P. A. and G. Green (1999). "An immunological assay for determination of baculovirus titers in 48 hours." Anal Biochem **268**(2): 173-8.
Knebel, D., H. Lubbert, et al. (1985). "The promoter of the late p10 gene in the insect nuclear polyhedrosis virus Autographa californica: activation by viral gene products and sensitivity to DNA methylation." EMBO J **4**(5): 1301-6.
Kogan, P. H. and G. W. Blissard (1994). "A baculovirus gp64 early promoter is activated by host transcription factor binding to CACGTG and GATA elements." J Virol **68**(2): 813-22.
Kogan, P. H., X. Chen, et al. (1995). "Overlapping TATA-dependent and TATA-independent early promoter activities in the baculovirus gp64 envelope fusion protein gene." J Virol **69**(3): 1452-61.
Kondo, A. and S. Maeda (1991). "Host range expansion by recombination of the baculoviruses Bombyx mori nuclear polyhedrosis virus and Autographa californica nuclear polyhedrosis virus." J Virol **65**(7): 3625-32.
Kool, M., C. H. Ahrens, et al. (1994). "Identification of genes involved in DNA replication of the Autographa californica baculovirus." Proc Natl Acad Sci U S A **91**(23): 11212-6.
Kool, M., P. M. van den Berg, et al. (1993). "Location of two putative origins of DNA replication of Autographa californica nuclear polyhedrosis virus." Virology **192**(1): 94-101.
Kovacs, G. R., J. Choi, et al. (1992). "Functional dissection of the Autographa californica nuclear polyhedrosis virus immediate-early 1 transcriptional regulatory protein." J Virol **66**(12): 7429-37.
Kovacs, G. R., L. A. Guarino, et al. (1991). "Identification of spliced baculovirus RNAs expressed late in infection." Virology **185**(2): 633-43.
Kumar, A. (2008). "RNA interference: a multifaceted innate antiviral defense." Retrovirology **5**: 17.
Kumar, S., X. Cheng, et al. (1994). "The DNA (cytosine-5) methyltransferases." Nucleic Acids Res **22**(1): 1-10.
Kunert, N., J. Marhold, et al. (2003). "A Dnmt2-like protein mediates DNA methylation in Drosophila." Development **130**(21): 5083-90.
Kuzio, J., M. N. Pearson, et al. (1999). "Sequence and analysis of the genome of a baculovirus pathogenic for Lymantria dispar." Virology **253**(1): 17-34.
Kwon, M. S., T. Dojima, et al. (2002). "Development of an antibody-based assay for determination of baculovirus titers in 10 hours." Biotechnol Prog **18**(3): 647-51.
Lanier, L. M., J. M. Slack, et al. (1996). "Actin binding and proteolysis by the baculovirus AcMNPV: the role of virion-associated V-CATH." Virology **216**(2): 380-8.
Lecellier, C. H., P. Dunoyer, et al. (2005). "A cellular microRNA mediates antiviral defense in human cells." Science **308**(5721): 557-60.

Lee, H. and P. J. Krell (1994). "Reiterated DNA fragments in defective genomes of Autographa californica nuclear polyhedrosis virus are competent for AcMNPV-dependent DNA replication." Virology 202(1): 418-29.
Lee, H. Y. and P. J. Krell (1992). "Generation and analysis of defective genomes of Autographa californica nuclear polyhedrosis virus." J Virol 66(7): 4339-47.
Lee, S. Y., A. Poloumienko, et al. (1996). "A common pathway for p10 and calyx proteins in progressive stages of polyhedron envelope assembly in AcMNPV-infected Spodoptera frugiperda larvae." Arch Virol 141(7): 1247-58.
Lee, T. T. and M. R. Karon (1976). "Inhibition of protein synthesis in 5-azacytidine-treated HeLa cells." Biochem Pharmacol 25(15): 1737-42.
Leisy, D. J. and G. F. Rohrmann (1993). "Characterization of the replication of plasmids containing hr sequences in baculovirus-infected Spodoptera frugiperda cells." Virology 196(2): 722-30.
Li, F. and S. W. Ding (2006). "Virus counterdefense: diverse strategies for evading the RNA-silencing immunity." Annu Rev Microbiol 60: 503-31.
Li, H., W. X. Li, et al. (2002). "Induction and suppression of RNA silencing by an animal virus." Science 296(5571): 1319-21.
Li, H. W. and S. W. Ding (2005). "Antiviral silencing in animals." FEBS Lett 579(26): 5965-73.
Li, L., S. H. Harwood, et al. (1999). "Identification of additional genes that influence baculovirus late gene expression." Virology 255(1): 9-19.
Li, L. H., E. J. Olin, et al. (1970). "Cytotoxicity and mode of action of 5-azacytidine on L1210 leukemia." Cancer Res 30(11): 2760-9.
Li, W. X., H. Li, et al. (2004). "Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing." Proc Natl Acad Sci U S A 101(5): 1350-5.
Li, X., J. Song, et al. (2007). "The N-terminal hydrophobic sequence of Autographa californica nucleopolyhedrovirus PIF-3 is essential for oral infection." Arch Virol 152(10): 1851-8.
Li, Y., J. Wang, et al. (2005). "vlf-1 deletion brought AcMNPV to defect in nucleocapsid formation." Virus Genes 31(3): 275-84.
Lim, L. P., N. C. Lau, et al. (2005). "Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs." Nature 433(7027): 769-73.
Lin, G. and G. W. Blissard (2002). "Analysis of an Autographa californica multicapsid nucleopolyhedrovirus lef-6-null virus: LEF-6 is not essential for viral replication but appears to accelerate late gene transcription." J Virol 76(11): 5503-14.
Lindbo, J. A., L. Silva-Rosales, et al. (1993). "Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance." Plant Cell 5(12): 1749-1759.
Lippman, Z. and R. Martienssen (2004). "The role of RNA interference in heterochromatic silencing." Nature 431(7006): 364-70.
Lloyd, V. (2000). "Parental imprinting in Drosophila." Genetica 109(1-2): 35-44.
Lo, H. R. and Y. C. Chao (2004). "Rapid titer determination of baculovirus by quantitative real-time polymerase chain reaction." Biotechnol Prog 20(1): 354-60.
Long, G., X. Pan, et al. (2006). "Functional entry of baculovirus into insect and mammalian cells is dependent on clathrin-mediated endocytosis." J Virol 80(17): 8830-3.
Lopez-Ferber, M., O. Simon, et al. (2003). "Defective or effective? Mutualistic interactions between virus genotypes." Proc Biol Sci 270(1530): 2249-55.
Lu, A. and E. B. Carstens (1991). "Nucleotide sequence of a gene essential for viral DNA replication in the baculovirus Autographa californica nuclear polyhedrosis virus." Virology 181(1): 336-47.

Lu, A. and L. K. Miller (1994). "Identification of three late expression factor genes within the 33.8- to 43.4-map-unit region of Autographa californica nuclear polyhedrosis virus." J Virol **68**(10): 6710-8.
Lu, A. and L. K. Miller (1995). "Differential requirements for baculovirus late expression factor genes in two cell lines." J Virol **69**(10): 6265-72.
Lu, A. and L. K. Miller (1995). "The roles of eighteen baculovirus late expression factor genes in transcription and DNA replication." J Virol **69**(2): 975-82.
Lu, A. and L. K. Miller (1996). "Species-specific effects of the hcf-1 gene on baculovirus virulence." J Virol **70**(8): 5123-30.
Lu, L. J. and K. Randerath (1980). "Mechanism of 5-azacytidine-induced transfer RNA cytosine-5-methyltransferase deficiency." Cancer Res **40**(8 Pt 1): 2701-5.
Lu, R., A. Folimonov, et al. (2004). "Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome." Proc Natl Acad Sci U S A **101**(44): 15742-7.
Lu, R., M. Maduro, et al. (2005). "Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans." Nature **436**(7053): 1040-3.
Luciano, D. J., H. Mirsky, et al. (2004). "RNA editing of a miRNA precursor." RNA **10**(8): 1174-7.
Lung, O., M. Westenberg, et al. (2002). "Pseudotyping Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV): F proteins from group II NPVs are functionally analogous to AcMNPV GP64." J Virol **76**(11): 5729-36.
Luque, T., R. Finch, et al. (2001). "The complete sequence of the Cydia pomonella granulovirus genome." J Gen Virol **82**(Pt 10): 2531-47.
Lyko, F., C. Beisel, et al. (2006). "Epigenetic regulation in Drosophila." Curr Top Microbiol Immunol **310**: 23-44.
Lyko, F., B. H. Ramsahoye, et al. (2000). "DNA methylation in Drosophila melanogaster." Nature **408**(6812): 538-40.
Mainz, D., I. Quadt, et al. (2002). "Nuclear IE2 structures are related to viral DNA replication sites during baculovirus infection." J Virol **76**(10): 5198-207.
Mallory, A. C., B. J. Reinhart, et al. (2002). "A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco." Proc Natl Acad Sci U S A **99**(23): 15228-33.
Mandrioli, M. and N. Volpi (2003). "The genome of the lepidopteran Mamestra brassicae has a vertebrate-like content of methyl-cytosine." Genetica **119**(2): 187-91.
Mans, R. M. and D. Knebel-Morsdorf (1998). "In vitro transcription of pe38/polyhedrin hybrid promoters reveals sequences essential for recognition by the baculovirus-induced RNA polymerase and for the strength of very late viral promoters." J Virol **72**(4): 2991-8.
Marhold, J., N. Rothe, et al. (2004). "Conservation of DNA methylation in dipteran insects." Insect Mol Biol **13**(2): 117-23.
Marks, H., X. Y. Ren, et al. (2006). "In silico identification of putative promoter motifs of White Spot Syndrome Virus." BMC Bioinformatics **7**: 309.
Matzke, M. A., M. Primig, et al. (1989). "Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants." EMBO J **8**(3): 643-649.
McCarthy, C. B. and D. A. Theilmann (2008). "AcMNPV ac143 (odv-e18) is essential for mediating budded virus production and is the 30th baculovirus core gene." Virology **375**(1): 277-91.
McDougal, V. V. and L. A. Guarino (1999). "Autographa californica nuclear polyhedrosis virus DNA polymerase: measurements of processivity and strand displacement." J Virol **73**(6): 4908-18.
McLachlin, J. R. and L. K. Miller (1994). "Identification and characterization of vlf-1, a baculovirus gene involved in very late gene expression." J Virol **68**(12): 7746-56.

McLauchlan, J., D. Gaffney, et al. (1985). "The consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for efficient formation of mRNA 3' termini." Nucleic Acids Res **13**(4): 1347-68.
Mello, C. C. and D. Conte, Jr. (2004). "Revealing the world of RNA interference." Nature **431**(7006): 338-42.
Merrington, C. L., P. A. Kitts, et al. (1996). "An Autographa californica nucleopolyhedrovirus lef-2 mutant: consequences for DNA replication and very late gene expression." Virology **217**(1): 338-48.
Mikhailov, V. S. (2000). "Helix-destabilizing properties of the baculovirus single-stranded DNA-binding protein (LEF-3)." Virology **270**(1): 180-9.
Mikhailov, V. S., K. A. Marlyev, et al. (1986). "Characterization of 3'----5' exonuclease associated with DNA polymerase of silkworm nuclear polyhedrosis virus." Nucleic Acids Res **14**(9): 3841-57.
Mikhailov, V. S., K. Okano, et al. (2005). "The redox state of the baculovirus single-stranded DNA-binding protein LEF-3 regulates its DNA binding, unwinding, and annealing activities." J Biol Chem **280**(33): 29444-53.
Milks, M. L., J. O. Washburn, et al. (2003). "Deletion of pe38 attenuates AcMNPV genome replication, budded virus production, and virulence in heliothis virescens." Virology **310**(2): 224-34.
Miller, L. K. *e.* (1997). "*In* The Baculoviruses (Ed. Miller, .K.) Plenum Press, New York."
Moazed, D. (2009). "Small RNAs in transcriptional gene silencing and genome defence." Nature **457**(7228): 413-20.
Monsma, S. A. and G. W. Blissard (1995). "Identification of a membrane fusion domain and an oligomerization domain in the baculovirus GP64 envelope fusion protein." J Virol **69**(4): 2583-95.
Monsma, S. A., A. G. Oomens, et al. (1996). "The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection." J Virol **70**(7): 4607-16.
Morris, T. D. and L. K. Miller (1994). "Mutational analysis of a baculovirus major late promoter." Gene **140**(2): 147-53.
Muller, S. and J. L. Imler (2007). "Dicing with viruses: microRNAs as antiviral factors." Immunity **27**(1): 1-3.
Mund, C., T. Musch, et al. (2004). "Comparative analysis of DNA methylation patterns in transgenic Drosophila overexpressing mouse DNA methyltransferases." Biochem J **378**(Pt 3): 763-8.
Nagamine, T., Y. Kawasaki, et al. (2005). "Focal distribution of baculovirus IE1 triggered by its binding to the hr DNA elements." J Virol **79**(1): 39-46.
Nagamine, T., Y. Kawasaki, et al. (2006). "Induction of a subnuclear structure by the simultaneous expression of baculovirus proteins, IE1, LEF3, and P143 in the presence of hr." Virology **352**(2): 400-7.
Naito, Y., T. Yamada, et al. (2004). "siDirect: highly effective, target-specific siRNA design software for mammalian RNA interference." Nucleic Acids Res **32**(Web Server issue): W124-9.
Napoli, C., C. Lemieux, et al. (1990). "Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans." Plant Cell **2**(4): 279-289.
Newmark, P. A., P. W. Reddien, et al. (2003). "Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in planarians." Proc Natl Acad Sci U S A **100 Suppl 1**: 11861-5.
Ng, H. H. and A. Bird (1999). "DNA methylation and chromatin modification." Curr Opin Genet Dev **9**(2): 158-63.

Niblett, C. L., E. Dickson, et al. (1978). "Cross protection among four viroids." Virology **91**(1): 198-203.
Norris, D. P., D. Patel, et al. (1994). "Evidence that random and imprinted Xist expression is controlled by preemptive methylation." Cell **77**(1): 41-51.
O'Reilly, D. R., R. S. Hails, et al. (1998). "The impact of host developmental status on baculovirus replication." J Invertebr Pathol **72**(3): 269-75.
O'Reilly, D. R. and L. K. Miller (1989). "A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase." Science **245**(4922): 1110-2.
O'Reilly, D. R. and L. K. Miller (1990). "Regulation of expression of a baculovirus ecdysteroid UDPglucosyltransferase gene." J Virol **64**(3): 1321-8.
O'Reilly, D. R., L. K. Miller, et al. (1992). "Baculovirus Expression Vectors: A Laboratory Manual. pp. 1-347. W. H. Freeman/Oxford University Press, New York."
Obbard, D. J., F. M. Jiggins, et al. (2006). "Natural selection drives extremely rapid evolution in antiviral RNAi genes." Curr Biol **16**(6): 580-5.
Ohkawa, T., J. O. Washburn, et al. (2005). "Specific binding of Autographa californica M nucleopolyhedrovirus occlusion-derived virus to midgut cells of Heliothis virescens larvae is mediated by products of pif genes Ac119 and Ac022 but not by Ac115." J Virol **79**(24): 15258-64.
Okamura, K., S. Balla, et al. (2008). "Two distinct mechanisms generate endogenous siRNAs from bidirectional transcription in Drosophila melanogaster." Nat Struct Mol Biol **15**(6): 581-90.
Okamura, K., W. J. Chung, et al. (2008). "The Drosophila hairpin RNA pathway generates endogenous short interfering RNAs." Nature **453**(7196): 803-6.
Okano, K., A. L. Vanarsdall, et al. (2006). "Conserved molecular systems of the Baculoviridae." Virology **344**(1): 77-87.
Okano, K., A. L. Vanarsdall, et al. (2004). "Characterization of a baculovirus lacking the alkaline nuclease gene." J Virol **78**(19): 10650-6.
Okano, K., A. L. Vanarsdall, et al. (2007). "A baculovirus alkaline nuclease knockout construct produces fragmented DNA and aberrant capsids." Virology **359**(1): 46-54.
Okano, M., D. W. Bell, et al. (1999). "DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development." Cell **99**(3): 247-57.
Olsen, P. H. and V. Ambros (1999). "The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation." Dev Biol **216**(2): 671-80.
Olson, K. E., S. Higgs, et al. (1996). "Genetically engineered resistance to dengue-2 virus transmission in mosquitoes." Science **272**(5263): 884-6.
Olszewski, J. and L. K. Miller (1997). "A role for baculovirus GP41 in budded virus production." Virology **233**(2): 292-301.
Ooi, B. G. and L. K. Miller (1990). "Transcription of the baculovirus polyhedrin gene reduces the levels of an antisense transcript initiated downstream." J Virol **64**(6): 3126-9.
Oomens, A. G. and G. W. Blissard (1999). "Requirement for GP64 to drive efficient budding of Autographa californica multicapsid nucleopolyhedrovirus." Virology **254**(2): 297-314.
Oppenheimer, D. I. and L. E. Volkman (1997). "Evidence for rolling circle replication of Autographa californica M nucleopolyhedrovirus genomic DNA." Arch Virol **142**(10): 2107-13.
Orii, H., M. Mochii, et al. (2003). "A simple "soaking method" for RNA interference in the planarian Dugesia japonica." Dev Genes Evol **213**(3): 138-41.
Pal-Bhadra, M., U. Bhadra, et al. (1997). "Cosuppression in Drosophila: gene silencing of Alcohol dehydrogenase by white-Adh transgenes is Polycomb dependent." Cell **90**(3): 479-90.

Passarelli, A. L. and L. K. Miller (1993). "Identification of genes encoding late expression factors located between 56.0 and 65.4 map units of the Autographa californica nuclear polyhedrosis virus genome." Virology **197**(2): 704-14.
Patel, A., M. K. Fondrk, et al. (2007). "The making of a queen: TOR pathway is a key player in diphenic caste development." PLoS ONE **2**(6): e509.
Patel, C. V. and K. P. Gopinathan (1987). "Determination of trace amounts of 5-methylcytosine in DNA by reverse-phase high-performance liquid chromatography." Anal Biochem **164**(1): 164-9.
Pearson, M., R. Bjornson, et al. (1992). "The Autographa californica baculovirus genome: evidence for multiple replication origins." Science **257**(5075): 1382-4.
Pearson, M. N., R. M. Bjornson, et al. (1993). "Identification and characterization of a putative origin of DNA replication in the genome of a baculovirus pathogenic for Orgyia pseudotsugata." Virology **197**(2): 715-25.
Pearson, M. N., C. Groten, et al. (2000). "Identification of the lymantria dispar nucleopolyhedrovirus envelope fusion protein provides evidence for a phylogenetic division of the Baculoviridae." J Virol **74**(13): 6126-31.
Pearson, M. N. and G. F. Rohrmann (1995). "Lymantria dispar nuclear polyhedrosis virus homologous regions: characterization of their ability to function as replication origins." J Virol **69**(1): 213-21.
Pearson, M. N., R. L. Russell, et al. (1988). "p39, a major baculovirus structural protein: immunocytochemical characterization and genetic location." Virology **167**(2): 407-13.
Pedersen, I. M., G. Cheng, et al. (2007). "Interferon modulation of cellular microRNAs as an antiviral mechanism." Nature **449**(7164): 919-22.
Peng, Y., J. Song, et al. (2007). "The histone deacetylase inhibitor sodium butyrate inhibits baculovirus-mediated transgene expression in Sf9 cells." J Biotechnol **131**(2): 180-7.
Perera, O., T. B. Green, et al. (2007). "Proteins associated with Culex nigripalpus nucleopolyhedrovirus occluded virions." J Virol **81**(9): 4585-90.
Perera, O. P., S. M. Valles, et al. (2006). "Molecular analysis of an occlusion body protein from Culex nigripalpus nucleopolyhedrovirus (CuniNPV)." J Invertebr Pathol **91**(1): 35-42.
Petrik, D. T., A. Iseli, et al. (2003). "Improving baculovirus resistance to UV inactivation: increased virulence resulting from expression of a DNA repair enzyme." J Invertebr Pathol **82**(1): 50-6.
Pfeffer, S., A. Sewer, et al. (2005). "Identification of microRNAs of the herpesvirus family." Nat Methods **2**(4): 269-76.
Pfeffer, S., M. Zavolan, et al. (2004). "Identification of virus-encoded microRNAs." Science **304**(5671): 734-6.
Pillai, R. S., S. N. Bhattacharyya, et al. (2005). "Inhibition of translational initiation by Let-7 MicroRNA in human cells." Science **309**(5740): 1573-6.
Ponger, L. and W. H. Li (2005). "Evolutionary diversification of DNA methyltransferases in eukaryotic genomes." Mol Biol Evol **22**(4): 1119-28.
Popham, H. J., D. S. Bischoff, et al. (2001). "Both Lymantria dispar nucleopolyhedrovirus enhancin genes contribute to viral potency." J Virol **75**(18): 8639-48.
Pullen, S. S. and P. D. Friesen (1995). "The CAGT motif functions as an initiator element during early transcription of the baculovirus transregulator ie-1." J Virol **69**(6): 3575-83.
Rajagopal, R., S. Sivakumar, et al. (2002). "Silencing of midgut aminopeptidase N of Spodoptera litura by double-stranded RNA establishes its role as Bacillus thuringiensis toxin receptor." J Biol Chem **277**(49): 46849-51.

Ramadan, N., I. Flockhart, et al. (2007). "Design and implementation of high-throughput RNAi screens in cultured Drosophila cells." Nat Protoc **2**(9): 2245-64.
Rankin, C., B. G. Ooi, et al. (1988). "Eight base pairs encompassing the transcriptional start point are the major determinant for baculovirus polyhedrin gene expression." Gene **70**(1): 39-49.
Rapp, J. C., J. A. Wilson, et al. (1998). "Nineteen baculovirus open reading frames, including LEF-12, support late gene expression." J Virol **72**(12): 10197-206.
Rehwinkel, J., P. Natalin, et al. (2006). "Genome-wide analysis of mRNAs regulated by Drosha and Argonaute proteins in Drosophila melanogaster." Mol Cell Biol **26**(8): 2965-75.
Riggs, A. D. (1975). "X inactivation, differentiation, and DNA methylation." Cytogenet Cell Genet **14**(1): 9-25.
Robalino, J., T. Bartlett, et al. (2005). "Double-stranded RNA induces sequence-specific antiviral silencing in addition to nonspecific immunity in a marine shrimp: convergence of RNA interference and innate immunity in the invertebrate antiviral response?" J Virol **79**(21): 13561-71.
Rohrmann, G. F. (1986). "Polyhedrin structure." J Gen Virol **67 (Pt 8)**: 1499-513.
Romano, N. and G. Macino (1992). "Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences." Mol Microbiol **6**(22): 3343-53.
Rosas-Acosta, G., S. C. Braunagel, et al. (2001). "Effects of deletion and overexpression of the Autographa californica nuclear polyhedrosis virus FP25K gene on synthesis of two occlusion-derived virus envelope proteins and their transport into virus-induced intranuclear membranes." J Virol **75**(22): 10829-42.
Russell, R. L., M. N. Pearson, et al. (1991). "Immunoelectron microscopic examination of Orgyia pseudotsugata multicapsid nuclear polyhedrosis virus-infected Lymantria dispar cells: time course and localization of major polyhedron-associated proteins." J Gen Virol **72 (Pt 2)**: 275-83.
Russell, R. L. and G. F. Rohrmann (1993). "A 25-kDa protein is associated with the envelopes of occluded baculovirus virions." Virology **195**(2): 532-40.
Saleh, M. C., R. P. van Rij, et al. (2006). "The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing." Nat Cell Biol **8**(8): 793-802.
Sandberg, R., J. R. Neilson, et al. (2008). "Proliferating cells express mRNAs with shortened 3' untranslated regions and fewer microRNA target sites." Science **320**(5883): 1643-7.
Sano, M., Y. Kato, et al. (2006). "Sequence-specific interference by small RNAs derived from adenovirus VAI RNA." FEBS Lett **580**(6): 1553-64.
Scheper, G. C., R. G. Vries, et al. (1997). "Translational properties of the untranslated regions of the p10 messenger RNA of Autographa californica multicapsid nucleopolyhedrovirus." J Gen Virol **78 (Pt 3)**: 687-96.
Schnettler, E., W. de Vries, et al. (2009). "The NS3 protein of rice hoja blanca virus complements the RNAi suppressor function of HIV-1 Tat." EMBO Rep **10**(3): 258-63.
Schott, D. H., D. K. Cureton, et al. (2005). "An antiviral role for the RNA interference machinery in Caenorhabditis elegans." Proc Natl Acad Sci U S A **102**(51): 18420-4.
Shekhar, M. S. and Y. Lu (2009). "Application of nucleic-acid-based therapeutics for viral infections in shrimp aquaculture." Mar Biotechnol (NY) **11**(1): 1-9.
Shen, C. F., J. Meghrous, et al. (2002). "Quantitation of baculovirus particles by flow cytometry." J Virol Methods **105**(2): 321-30.

Simon, O., T. Williams, et al. (2004). "Genetic structure of a Spodoptera frugiperda nucleopolyhedrovirus population: high prevalence of deletion genotypes." Appl Environ Microbiol **70**(9): 5579-88.
Slack, J. M., J. Kuzio, et al. (1995). "Characterization of v-cath, a cathepsin L-like proteinase expressed by the baculovirus Autographa californica multiple nuclear polyhedrosis virus." J Gen Virol **76 (Pt 5)**: 1091-8.
Smith, C. J., C. F. Watson, et al. (1990). "Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants." Mol Gen Genet **224**(3): 477-81.
Soares, C. A., C. M. Lima, et al. (2005). "Capillary feeding of specific dsRNA induces silencing of the isac gene in nymphal Ixodes scapularis ticks." Insect Mol Biol **14**(4): 443-52.
Stewart, T. M., I. Huijskens, et al. (2005). "The Autographa californica multiple nucleopolyhedrovirus ie0-ie1 gene complex is essential for wild-type virus replication, but either IE0 or IE1 can support virus growth." J Virol **79**(8): 4619-29.
Stiles, B. and S. Himmerich (1998). "Autographa californica NPV isolates: restriction endonuclease analysis and comparative biological activity." J Invertebr Pathol **72**(2): 174-7.
Sullivan, C. S., A. T. Grundhoff, et al. (2005). "SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells." Nature **435**(7042): 682-6.
Takagi, T., G. S. Taylor, et al. (1998). "A protein tyrosine phosphatase-like protein from baculovirus has RNA 5'-triphosphatase and diphosphatase activities." Proc Natl Acad Sci U S A **95**(17): 9808-12.
Takeda, A., M. Tsukuda, et al. (2005). "A plant RNA virus suppresses RNA silencing through viral RNA replication." EMBO J **24**(17): 3147-57.
Tam, O. H., A. A. Aravin, et al. (2008). "Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes." Nature **453**(7194): 534-8.
Tani, H., M. Nishijima, et al. (2001). "Characterization of cell-surface determinants important for baculovirus infection." Virology **279**(1): 343-53.
ter Brake, O., J. Haasnoot, et al. (2008). "ESF-EMBO symposium: antiviral applications of RNA interference." Retrovirology **5**: 81.
Theilmann, D. A. and S. Stewart (1992). "Molecular analysis of the trans-activating IE-2 gene of Orgyia pseudotsugata multicapsid nuclear polyhedrosis virus." Virology **187**(1): 84-96.
Theilmann, D. A. and S. Stewart (1993). "Analysis of the Orgyia pseudotsugata multicapsid nuclear polyhedrosis virus trans-activators IE-1 and IE-2 using monoclonal antibodies." J Gen Virol **74 (Pt 9)**: 1819-26.
Thiem, S. M., X. Du, et al. (1996). "Identification of baculovirus gene that promotes Autographa californica nuclear polyhedrosis virus replication in a nonpermissive insect cell line." J Virol **70**(4): 2221-9.
Thiem, S. M. and L. K. Miller (1989). "Identification, sequence, and transcriptional mapping of the major capsid protein gene of the baculovirus Autographa californica nuclear polyhedrosis virus." J Virol **63**(5): 2008-18.
Tijsterman, M., R. C. May, et al. (2004). "Genes required for systemic RNA interference in Caenorhabditis elegans." Curr Biol **14**(2): 111-6.
Timmons, L. and A. Fire (1998). "Specific interference by ingested dsRNA." Nature **395**(6705): 854.
Tomalski, M. D., R. Eldridge, et al. (1991). "A baculovirus homolog of a Cu/Zn superoxide dismutase gene." Virology **184**(1): 149-61.

Tomalski, M. D., J. G. Wu, et al. (1988). "The location, sequence, transcription, and regulation of a baculovirus DNA polymerase gene." Virology **167**(2): 591-600.
Triboulet, R., B. Mari, et al. (2007). "Suppression of microRNA-silencing pathway by HIV-1 during virus replication." Science **315**(5818): 1579-82.
Trinks, D., R. Rajeswaran, et al. (2005). "Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes." J Virol **79**(4): 2517-27.
Turner, C. T., M. W. Davy, et al. (2006). "RNA interference in the light brown apple moth, Epiphyas postvittana (Walker) induced by double-stranded RNA feeding." Insect Mol Biol **15**(3): 383-91.
Tweeten, K. A., L. A. Bulla, et al. (1980). "Characterization of an Extremely Basic Protein Derived from Granulosis Virus Nucleocapsids." J Virol **33**(2): 866-876.
Uhlirova, M., B. D. Foy, et al. (2003). "Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of Broad-Complex in insect metamorphosis." Proc Natl Acad Sci U S A **100**(26): 15607-12.
Urieli-Shoval, S., Y. Gruenbaum, et al. (1982). "The absence of detectable methylated bases in Drosophila melanogaster DNA." FEBS Lett **146**(1): 148-52.
Valdes, V. J., A. Sampieri, et al. (2003). "Using double-stranded RNA to prevent in vitro and in vivo viral infections by recombinant baculovirus." J Biol Chem **278**(21): 19317-24.
van der Krol, A. R., L. A. Mur, et al. (1990). "Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression." Plant Cell **2**(4): 291-9.
van Oers, M. M., J. M. Vlak, et al. (1999). "Role of the 3' untranslated region of baculovirus p10 mRNA in high-level expression of foreign genes." J Gen Virol **80 (Pt 8)**: 2253-62.
van Rij, R. P., M. C. Saleh, et al. (2006). "The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster." Genes Dev **20**(21): 2985-95.
van, W. R., X. Dong, et al. (2002). "Mutation of three cysteine residues in Tomato yellow leaf curl virus-China C2 protein causes dysfunction in pathogenesis and posttranscriptional gene-silencing suppression." Mol Plant Microbe Interact **15**(3): 203-8.
Vanarsdall, A. L., K. Okano, et al. (2004). "Characterization of a baculovirus with a deletion of vlf-1." Virology **326**(1): 191-201.
Vanarsdall, A. L., K. Okano, et al. (2006). "Characterization of the role of very late expression factor 1 in baculovirus capsid structure and DNA processing." J Virol **80**(4): 1724-33.
Vanitharani, R., P. Chellappan, et al. (2004). "Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing." J Virol **78**(17): 9487-98.
Vaughn, J. L., R. H. Goodwin, et al. (1977). "The establishment of two cell lines from the insect Spodoptera frugiperda (Lepidoptera; Noctuidae)." In Vitro **13**(4): 213-7.
Vazquez, F., H. Vaucheret, et al. (2004). "Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs." Mol Cell **16**(1): 69-79.
Vigdorovich, V., A. D. Miller, et al. (2007). "Ability of hyaluronidase 2 to degrade extracellular hyaluronan is not required for its function as a receptor for jaagsiekte sheep retrovirus." J Virol **81**(7): 3124-9.
Vlak, J. M. and G. E. Smith (1982). "Orientation of the Genome of Autographa californica Nuclear Polyhedrosis Virus: a Proposal." J Virol **41**(3): 1118-1121.
Voinnet, O., C. Lederer, et al. (2000). "A viral movement protein prevents spread of the gene silencing signal in Nicotiana benthamiana." Cell **103**(1): 157-67.

Voinnet, O., Y. M. Pinto, et al. (1999). "Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants." Proc Natl Acad Sci U S A **96**(24): 14147-52.
Volkman, L., K. Storm, et al. (1996). "Overexpression of actin in AcMNPV-infected cells interferes with polyhedrin synthesis and polyhedra formation." Virology **225**(2): 369-76.
Volkman, L. E. (1986). "The 64K envelope protein of budded Autographa californica nuclear polyhedrosis virus." Curr Top Microbiol Immunol **131**: 103-18.
Wagner, H., D. Simon, et al. (1985). "Methylation pattern of fish lymphocystis disease virus DNA." J Virol **53**(3): 1005-7.
Walsh, C. P. and T. H. Bestor (1999). "Cytosine methylation and mammalian development." Genes Dev **13**(1): 26-34.
Wang, H., K. J. Buckley, et al. (2005). "Adenosine kinase inhibition and suppression of RNA silencing by geminivirus AL2 and L2 proteins." J Virol **79**(12): 7410-8.
Wang, P. and R. R. Granados (1997). "An intestinal mucin is the target substrate for a baculovirus enhancin." Proc Natl Acad Sci U S A **94**(13): 6977-82.
Wang, X. H., R. Aliyari, et al. (2006). "RNA interference directs innate immunity against viruses in adult Drosophila." Science **312**(5772): 452-4.
Washburn, J. O., B. A. Kirkpatrick, et al. (1995). "Comparative pathogenesis of Autographa californica M nuclear polyhedrosis virus in larvae of Trichoplusia ni and Heliothis virescens." Virology **209**(2): 561-8.
Watanabe, T., Y. Totoki, et al. (2008). "Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes." Nature **453**(7194): 539-43.
Wei, N. and L. E. Volkman (1992). "Hyperexpression of baculovirus polyhedrin and p10 is inversely correlated with actin synthesis." Virology **191**(1): 42-8.
Weiss, J. W. and H. C. Pitot (1974). "Inhibition of ribosomal ribonucleic acid maturation by 5-azacytidine and 8-azaguanine in Novikoff hepatoma cells." Arch Biochem Biophys **160**(1): 119-29.
Westenberg, M., B. Heinhuis, et al. (2005). "siRNA injection induces sequence-independent protection in Penaeus monodon against white spot syndrome virus." Virus Res **114**(1-2): 133-9.
Whitford, M. and P. Faulkner (1992). "Nucleotide sequence and transcriptional analysis of a gene encoding gp41, a structural glycoprotein of the baculovirus Autographa californica nuclear polyhedrosis virus." J Virol **66**(8): 4763-8.
Whitford, M., S. Stewart, et al. (1989). "Identification and sequence analysis of a gene encoding gp67, an abundant envelope glycoprotein of the baculovirus Autographa californica nuclear polyhedrosis virus." J Virol **63**(3): 1393-9.
Wileman, T. (2007). "Aggresomes and pericentriolar sites of virus assembly: cellular defense or viral design?" Annu Rev Microbiol **61**: 149-67.
Wilkins, C., R. Dishongh, et al. (2005). "RNA interference is an antiviral defence mechanism in Caenorhabditis elegans." Nature **436**(7053): 1044-7.
Willis, D. B. and A. Granoff (1980). "Frog virus 3 DNA is heavily methylated at CpG sequences." Virology **107**(1): 250-7.
Willis, L. G., R. Seipp, et al. (2005). "Sequence analysis of the complete genome of Trichoplusia ni single nucleopolyhedrovirus and the identification of a baculoviral photolyase gene." Virology **338**(2): 209-26.
Wilson, M. E., T. H. Mainprize, et al. (1987). "Location, transcription, and sequence of a baculovirus gene encoding a small arginine-rich polypeptide." J Virol **61**(3): 661-6.
Winston, W. M., C. Molodowitch, et al. (2002). "Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1." Science **295**(5564): 2456-9.

Wu, X. and L. A. Guarino (2003). "Autographa californica nucleopolyhedrovirus orf69 encodes an RNA cap (nucleoside-2'-O)-methyltransferase." J Virol **77**(6): 3430-40.

Wu, X., S. Stewart, et al. (1993). "Alternative transcriptional initiation as a novel mechanism for regulating expression of a baculovirus trans activator." J Virol **67**(10): 5833-42.

Wu, Y. and E. B. Carstens (1998). "A baculovirus single-stranded DNA binding protein, LEF-3, mediates the nuclear localization of the putative helicase P143." Virology **247**(1): 32-40.

Wu, Z., Y. Zhu, et al. (2009). "Herpes Simplex Virus Type 1 Suppresses RNA-Induced Gene Silencing in Mammalian Cells." J Virol.

Xing, K., R. Deng, et al. (2005). "Analysis and prediction of baculovirus promoter sequences." Virus Res **113**(1): 64-71.

Yang, H., W. Fan, et al. (2008). "Transgenic breeding of anti-Bombyx mori L. nuclear polyhedrosis virus silkworm Bombyx mori." Acta Biochim Biophys Sin (Shanghai) **40**(10): 873-6.

Yang, S. and L. K. Miller (1998). "Expression and mutational analysis of the baculovirus very late factor 1 (vlf-1) gene." Virology **245**(1): 99-109.

Ye, K. and D. J. Patel (2005). "RNA silencing suppressor p21 of Beet yellows virus forms an RNA binding octameric ring structure." Structure **13**(9): 1375-84.

Yoder, J. A., N. S. Soman, et al. (1997). "DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe." J Mol Biol **270**(3): 385-95.

Yoder, J. A., C. P. Walsh, et al. (1997). "Cytosine methylation and the ecology of intragenomic parasites." Trends Genet **13**(8): 335-40.

Yoo, S. and L. A. Guarino (1994). "The Autographa californica nuclear polyhedrosis virus ie2 gene encodes a transcriptional regulator." Virology **202**(2): 746-53.

Zambon, R. A., V. N. Vakharia, et al. (2006). "RNAi is an antiviral immune response against a dsRNA virus in Drosophila melanogaster." Cell Microbiol **8**(5): 880-9.

Zemskov, E. A., W. Kang, et al. (2000). "Evidence for nucleic acid binding ability and nucleosome association of Bombyx mori nucleopolyhedrovirus BRO proteins." J Virol **74**(15): 6784-9.

Zhou, W., L. Yao, et al. (2005). "The function of envelope protein P74 from Autographa californica multiple nucleopolyhedrovirus in primary infection to host." Virus Genes **30**(2): 139-50.