Chapter-6

Role of *bro* in AcMNPV infection and Transcription:

A Microarray based study.
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6.1 Introduction:

Baculoviruses are dsDNA virus, which generally infect insects. They have a covalently closed circular DNA genome and they exist episomally without 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 divided 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 known 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 nimphii 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°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 transcriptase mix at 42°C and converted to double stranded cDNA primed by oligo dT 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
electrophoresed 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 “Log2” (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.

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<th>1.5 fold difference</th>
<th>1.3 fold difference</th>
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<tr>
<td></td>
<td>Up regulated</td>
<td>Down regulated</td>
</tr>
<tr>
<td>At 12hrs PI</td>
<td>1</td>
<td>38</td>
</tr>
<tr>
<td>At 24hrs PI</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>At 48hrs PI</td>
<td>0</td>
<td>3</td>
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<tr>
<td>At 72hrs PI</td>
<td>0</td>
<td>6</td>
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**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 respectively). X-axis shows the number 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 respectively). X-axis shows the total number 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.


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.