CHAPTER 2
MATERIALS AND METHODS

1. SEQUENCE ANALYSES
   1.1 Sequences
      1.1.1 AcNPV gene sequences
      1.1.2 Heterologous gene sequences
   1.2 Analyses of translation initiation codon context pattern
      1.2.1 Alignment of AcNPV genes sequence:
      1.2.2 Computation of percent base usage matrix
      1.2.3 Computation of AcNPV consensus comparison with
           vertebrate sequences
      1.2.4 Weight matrix approach to evaluate translation
           initiation codon context (TICC)
      1.2.5 Scatter and regression analysis of TICC and level of
           heterologous gene expression
   1.3 Analyses of codon usage pattern
      1.3.1 Computation of codon usage pattern
      1.3.2 Correspond analysis
      1.3.3 Scatter and regression analysis of codon usage and
           heterologous gene
      1.3.4 Analysis of RNA secondary structure

2. CONSTRUCTION OF LUCIFERASE BASED TRANSIENT EXPRESSION
   VECTOR AND GENERATION OF MUTATED luc GENE EXPRESSION
   CONSTRUCTS
   2.1 Isolation of plasmid vector and clones
   2.2 Digestion of DNA
   2.3 Purification of digested DNA fragments by spin elution
   2.4 Ligation of DNA fragment
   2.5 Transformation of DH5α cells
   2.6 Labeling of DNA
   2.7 Colony Hybridization
   2.8 Southern Hybridization
   2.9 In-Gel Hybridization
   2.10 PCR primer directed mutation of 5'end of luc gene
   2.11 Construction and screening of mutant clone

3. INSECT CELL TISSUE CULTURE
   3.1 Insect cells and Baculovirus
   3.2 TNM-FH Medium
   3.3 Insect Cell Culture
   3.4 Monolayer Cultures
   3.5 Infection of Insect Cells
   3.6 Suspension cultures for virus amplification
   3.7 Plaque assay for virus titration

4. LUCIFERASE REPORTER BASED TRANSIENT EXPRESSION ASSAY
   4.1 Lipofectin-mediated Transfection of Plasmid DNA Constructs
   4.2 Luciferase assay
   4.3 Dot-Blot analysis of Transfected plasmid DNA
MATERIALS:

A. Sequence Analyses

For sequence analyses, following hardware and software were utilized

APPLE MACINTOSH, USA: Qudra 650
DIGITAL CORPORATION, USA: µVAX II
DNASTAR, USA: Laser Gene Sequence Analyses Package for Macintosh computers.
INTELLIGENETICS, USA: PC/GENE Sequence Analyses Package for IBM compatible computers.
TORCH COMPUTER, Cambridge: IBM compatible PC/AT 486.
UNIVERSITY OF WISCONSIN, USA: Genetics Computer Group Sequence Analyses Package for VAX/VMS computers.

Sequences were obtained form:
GENBANK, USA: GenBank Database on CD ROM, release 72.
EBI, UK: EMBL Database.

B. DNA Cloning and Insect Cell Culture

Cell culture media, chemicals, enzyme, oligonucleotide and radioisotopes were obtained from the following manufacturers:

AMERSHAM, United Kingdom: γ-32P-ATP, α-35S-dATP, α-32P-dCTP, Multiprime DNA labeling kit, Hybond N+ Nylon membrane.
AMRESCO, USA: Acrylamide.
**Materials and Methods**

**BOEHRINGER MANNHEIM, Germany:** ATP, Bacteriophage T4 DNA ligase, Bacteriophage T4 polynucleotide kinase, deoxy-ribonucleosides, Foetal calf serum, Luciferin.

**CORNING, USA:** Tissue Culture Flasks (25cm^2 and 75cm^2)

**DIFCO, USA:** Bacto-agar, Bacto-tryptone, Lactalbumin hydrolysate, Yeast extract.

**FLOW LABORATORIES, UK:** Neutral red solution.

**GIBCO BRL, USA:** Grace's insect cell culture medium, Lipofectin.

**GRINER, Germany:** Tissue Culture Flasks (25cm^2 and 75cm^2), Petriplates (35mm^2)

**MERCK, Germany:** Ethanol.

**Millipore, USA:** Filters for sterilization Whatmann papers

**PHARMACIA, Sweden:** Sephadex G-50.

**PROMEGA, USA:** Restriction enzymes.

**SIGMA, USA:** Acrylamide, Agarose, Amphotericin B, Ampicillin, bis Acrylamide, Bovine serum albumin, Bromophenol blue, EDTA, Ethidium Bromide, Ficoll, Foetal bovine serum, gentamycin, Formaldehyde, HEPES, Lysozyme, Potassium Hydroxide, LGT agarose, Sodium bicarbonate, Sodium dodecyle sulphate, Sonicated salmon sperm DNA, TEMED, Trypan blue, PVP, Xylene Cyanol.

**NEW ENGLAND BIOLABS, USA:** Bacteriophage λDNA, Bacteriophage φX 174 phage DNA, Restriction enzymes.

**QUALIGENS, India:** Acetic Acid, Na Acetate, Boric acid, Chloroform, Glycerol, glycine, HCl, H_2O_2, Isoamyl alcohol, KCl, KH_2PO_4, methanol.
Mg(CH$_3$COO)$_2$, MgCl$_2$, NaCl, Na$_2$CO$_3$, Na$_2$HPO$_4$, NaOH, NH$_4$OH, Phenol, Tris.

RAMA BIOTECHNOLOGIES, India: Oligonucleotides

UNITED STATES BIOCHEMICALS, USA: USB Sequenase sequencing kit (version 2.0).

METHODS:

1. SEQUENCE ANALYSES

1.1 Sequences

1.1.1 AcNPV gene sequences

Sequences of AcNPV genes coding for known viral proteins and heterologous genes were retrieved from GenBank. Those of AcNPV were: 25k (Accession No. M22537); 34.8k (D00583); 35k (M16821); 39k (M37122); 94k (M16821); cap (M22978); cg30 (M33604); core (M15370); dnapm (M20744); dnare (M57687); etl (M20718); etm (M20718); ets (M20718); egt (M22619); gp41 (M86592); ie-1 M21884; ie-n (M59422); p10 (M10023); p74 (M31301); p80 (M94914); pe-38 (M62488); polh (K01149); sod (M68862); ub (M30305) and v-cath (M67451). Later, when the complete genome sequence of AcNPV had become available (Ayers et al., 1994) in EMBL database (Accession no L222858) it was used to extend and validate the findings.

1.1.2 Heterologous gene sequences

Those of heterologous origins were: luciferase (M15077; Hasnain and Nakhai, 1990), beta subunit of human chorionic gonadotropin (J00117; Nakhai et al., 1992), alpha subunit of human chorionic gonadotropin (sequence available within lab.; Nakhai et al., 1991), Adenovirus (type II)
DNA polymerase (J01917; Watson and Hay, 1990), Dengue virus envelope protein (M29095; Deubel et al., 1991), Influenza virus type II (M55469; St. Angelo et al., 1987), basic preproattacin (X17619; Gunne et al., 1990), Rift Valley fever virus RNA-M segment (M11157; Schmaljohn et al., 1989), Rabies glycoprotein G (X71879; Prehaud et al., 1989), Vesicular stomatitis virus glycoprotein (V01214; Bailey et al., 1989), Hepatitis B surface antigen (M54898; Lanford et al., 1989). The reference within parenthesis succeeding the accession number refers to the report describing their expression in BEVS.

1.2 Analyses of translation initiation codon context pattern

1.2.1 Alignment of AcNPV genes sequence:
The first step towards identification of translation initiation codon context pattern was alignment of nucleotide sequences containing the translation initiation sites of AcNPV genes. A twenty base pair long nucleotide sequence ranging from -10 to +10 base (with reference to 'A' of initiation codon ,ATG, as +1,) of each AcNPV gene was extracted using ASSEMBLE program of UWGCG software package for VAX/VMS machine (Devereaux et al., 1984). The positions of the translation initiation sites in individual AcNPV genes were retrieved from the feature table (FT) field of the retrieved sequence file.

1.2.2 Computation of percent base usage matrix:
Using the aligned AcNPV gene sequences, base usage profile was computed. This step was done using a computer program called CONSENSUS available as part of UWGCG software package for VAX/VMS
machine (Devereaux et al., 1984). The program computes the base usage frequency at each position in nucleotide sequence string and outputs it as percent base usage table.

1.2.3 Computation of AcNPV consensus comparison with vertebrate sequences
Based on the percent based usage matrix the consensus translation initiation codon context was computed using Caveners 75/50 consensus rule (Cavener and Ray, 1991). According to this rule, a capital letter is used to denote the consensus base that is used either more than 75% of time or more than twice of the next prevalent base. In case a base is highest used but it does fall short of the previous condition it is denoted by a small letter. If a second highest used base is very close to the first highest base it is denoted by a small letter succeeding the small letter denoting the highest used base, with a "/" separating the two. A near random utilization of all bases is denoted by a small "n". The computed AcNPV consensus was compared to vertebrate consensus sequence, that was computed originally by Kozak (1987) and also by Cavener and Ray(1991), and the Drosophila consensus computed by Cavener and Ray (1991).

1.2.4 Weight matrix approach to evaluate translation initiation codon context (TICC)
A quantitative method based on weight matrix approach, for rating the quality of match for translation initiation codon context of an individual gene to the AcNPV pattern, was developed. A software program, called
FITCONSENSUS, based on similar algorithm was available within GCG package. This program was used to evaluate the match quality value for translation initiation codon context of individual gene using AcNPV base usage pattern as reference. The match quality value was calculated by first selecting values associated with given bases of the test sequence at each position from the weight table (in this case the percent base usage table computed for AcNPV context). All these selected values for each position was used to compute an average value that represented the quality of match to the base usage profile on which the consensus is based. This average value was called Match Quality value. To restrict the numbers of reported match by program FITCONSENSUS a condition was imposed that only those match would be reported that has ATG at a position where they are 100% used.

1.2.5 Scatter and regression analysis of TICC and level of heterologous gene expression

Scatter and regression analysis was carried out to demonstrate the association of match quality value of translation initiation codon context for heterologous genes and their expression level in BEVS. A sample of heterologous genes (mentioned previously) that were placed under identical transcriptional control late viral polh gene promoter and were expressed in baculovirus infected Spodoptera frugiperda cell line, were used for this purpose. The analyses were carried out using scatter and regression function of Origin scientific graphics system of windows 3.1. The expression values of
heterologous gene were log transformed and used as dependent variable where as match quality values were used as independent variable.

1.3 Analyses of codon usage pattern
1.3.1 Computation of codon usage pattern
Codon usage pattern for the various genes was computed by using the program CODONFREQUENCY of UWGCG's GCG software package (Devereaux et al., 1984). The CODONFREQUENCY counts codons and writes their frequencies into codon frequency tables. It counts the codons from ranges within a gene sequences or from existing codon frequency tables. The output table is a file with the sum of all the observations of each of the 64 possible codons. In addition to the simple frequency count the CODONFREQUENCY also normalize the codon observations to a frequency per thousand and to a fraction for each codon within its synonymous family. All homologous or heterologous genes were used to compute the individual codon usage pattern. All the AcNPV genes were used collectively to compute the overall AcNPV codon usage profile pattern. The two highly expressed AcNPV genes polh and p10 were used to compute the pattern for the highly expressed AcNPV genes.

1.3.2 Correspond analysis
The codon usage of the genes were compared with each other by a program called CORRESPOND, available in UWGCG sequence analysis software package (Devereaux et al., 1984). This method is based on procedure originally proposed by Grantham and coworker (1981). This method computes a parameter called D-squared statistic value or simply D-squared
value which is an average of the frequency difference observed between codons of the two codon usage tables. The frequencies compared in this method are the number of incidents of the codon in question divided by the total number of codons specifying that amino acid or terminator in each table. If an amino acid is not used at all in one of the tables, its codons contribute nothing to the sum of squares. The statistic gets smaller as the patterns of codon usage become more similar. Hence a lower D-squared statistic value indicates similar codon usage.

1.3.3 Scatter and regression analysis of codon usage and heterologous gene
To find out the relationship between D square values of heterologous genes and their expression level in BEVS the scatter and regression analysis was used. In this analysis the same set of heterologous genes were used as were used for the analysis of influence of translation initiation codon context on heterologous gene expression. The analyses were similarly carried out using scatter and regression function of Origin scientific graphics software system of Windows 3.1. The expression values of heterologous gene were log transformed and as dependent variable where as D square values were used as independent variable. The analysis was carried out with two sets of D-square values - one computed using overall AcNPV codon usage profile as reference while the other using the highly expressed AcNPV gene as reference.

1.3.4 Analysis of RNA secondary structure
Secondary structure analysis was carried out by RNAFOLD program of PC/Gene software package of MS-DOS based IBM compatible computers. The secondary structure was computed using Turner's thermodynamic parameters and other default options. Since the size of most of gene is often larger than what a computer can compute, only first 300 base long sequence stretch of coding sequence was used for computation. Since these methods show one of the many possible secondary structure at same energy level, the energy level associated with these structures was taken as a parameter for the inherent secondary structure forming potential.

2. CONSTRUCTION OF LUCIFERASE BASED TRANSIENT EXPRESSION VECTOR AND GENERATION OF MUTATED luc GENE EXPRESSION CONSTRUCTS

2.1 Isolation of plasmid vector and clones

Transformed E. coli clones containing the plasmid constructs were used as the source of plasmid DNA. The plasmid DNA was isolated using alkaline-SDS lysis protocols (Sambrook et al., 1989).

For mini scale preparation 3 ml of overnight culture of transformed E. coli cells was used. The cells were harvested in a microfuge at 12000 rpm for 30 sec in a microfuge tube. The harvested cells were resuspended in 100 µl of TEG buffer [50mM Tris, 10mM EDTA, Glucose] followed by addition of 200 µl lysis solution [0.2M NaOH, 1% SDS] and incubation at room temperature for 5 min. The alkaline pH of the cell lysate was neutralized
Materials and Methods

using a neutralizing solution [3M Na Acetate, pH 5.2]. The neutralized lysate was incubated on ice for 20 min for effective precipitation of chromosomal DNA and much of associated protein. Precipitated protein and chromosomal DNA was separated from plasmid DNA present in aqueous phase by centrifugation at 12000 rpm for 20 min. The supernatant containing the aqueous phase was retained and extracted of remaining protein by Phenol: Chloroform: Isoamyl alcohol [25:24:1] extraction. The nucleic acid containing the plasmid DNA was extracted out of aqueous solution by ethanol precipitation. The ethanol precipitation essential involved addition of 2.5 volume of cold ethanol to the aqueous solution containing DNA and chilling the final solution at -70°C for 30 min.

For large scale preparation QIAGEN purification kit was used. Cells were grown overnight in 500ml LB cultures. Cells were harvested at 6000xg in a Sorvall GS 3 rotor. Subsequently the cell pellet was resuspended in 10ml P1 buffer [100 μg/ml RNase A, 50 mM Tris/HCl, 10mM EDTA, pH 8.0] followed by addition of another 10ml of P2 buffer [0.2M NaOH, 1% SDS] and incubation at room temperature for 5 min. After incubation, 10ml of P3 buffer [3M K Acetate, pH 5.5] was added and the solutions were mixed immediately. The mixed solution was incubated on ice for 20 min followed by a centrifugation at 30,000xg at 4°C for 30 min. The supernatant was removed promptly and passed through a QBT solution [750mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100] equilibrated QIAGEN tip-500. The plasmid DNA present in the supernatant now binds the resin packed in the tip. The tip was then washed with 60 ml of QC wash buffer [1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0] followed by plasmid DNA elution using
15ml of QF buffer [1.25 M NaCl, 50 mM Tris/HCl, 15% ethanol, pH 8.5]. The eluted DNA was precipitated by adding 0.7 volume isopropanol at room temperature and the DNA pellet was washed with 70% ethanol. The quality of plasmid isolated by this technique is quite good and it is suitable for animal cell transfection experiments.

2.2 Digestion of DNA

All plasmid DNA were digested using appropriate restriction endonuclease enzyme(s) in a compatible commercial buffer for 5 to 6 hours at the recommended temperature. The reaction was terminated by heat inactivation at 65°C for 20 minutes.

2.2.1 For construction of *luc* based transient expression vector pAR*luc*

The parent vector pKN*luc* was digested with *XbaI* and *BglII* restriction endonuclease in a commercial buffer. This digestion reaction gave two fragments one of 9.6 kb (the vector containing a small 5′*luc* fragment) and other of 1.8 kb (containing most of the *luc* gene including its 3′end and all of MCS. The large fragment was used as the vector backbone for the transient expression vector construction.

The insert (containing the complementary part of the *luc* gene) was prepared using pAcluc plasmid. pAcluc was digested with *BamHI* and *XbaI* for generating the mutant TICC sequence.

2.2.2 For characterization of pAR*luc*:
The construct pAR/luc was characterized by digesting the plasmid by BglII, BamHI, Smal.

2.2.3 For generation of luc mutant constructs pAR/lucAC and pAR/lucKz
The parent vector pAR/luc was digested with Xbal and Bam HI restriction endonuclease. This restriction digestion reaction generated two fragments one of 11.25 kb (the vector containing a large 3’luc fragment) and other of 138 bp (containing the 5’end of luc gene). The large fragment was used as vector backbone for introducing different translation initiation codon context sequence at the 5’end of luc gene.

The insert (containing the complementary 5’ part of the luc gene with mutation in its translation initiation codon context) was prepared by digesting PCR amplified product (See Site specific mutation using PCR) with Sau3A and Xbal.

2.2.4 For characterization of pAR/lucAC and pAR/lucKz:
The construct pAR/lucAC and pAR/lucKz was characterized by digesting the plasmid with BamHI and Xbal restriction enzyme. The BamHI did not cut the mutant construct where as Xbal leniearized the plasmid.

2.3 Purification of digested DNA fragments by spin elution
After digestion, the inserts and the vectors were first fractionated by electrophoresis on a 0.8% agarose gel in TAE buffer (Tris Acetate and 2 mM EDTA). The agarose gel piece containing the desired DNA fragment was cut
and the DNA was eluted using spin elution technique. In this technique the finely sliced agarose gel pieces were put on a siliconised glass-wool plug in an eppendorf vial which had a small pore in its bottom. The vial was put on another intact vial and was centrifuged at 12,000 rpm in a microfuge for 10 minutes. The liquid collected in the second tube contained the eluted DNA.

2.4 Ligation of DNA fragment

All ligation reactions were carried out using Bacteriphage T4 DNA ligase in presence of 1X Boehringer Mannhiem ligase buffer (containing 20 mM Tris-Cl (pH 7.6), 5 mM MgCl₂, 5 mM DTT, 1 mM ATP) along with the vector and insert fragments. The reaction was carried out for 16 h at 16°C in a volume of 20 µl.

2.5 Transformation of DH5α cells

The ligation reaction mixture was used directly to transform competent DH5α E.coli cells (Sambrook et al., 1989). The transformed cells were plated on LB agar [LB + 15% bacto-agar] plates containing 50 µg/ml ampicillin. The plates were incubated at 37°C for 10-12 h following which the positive colonies (bacterial colonies bearing the plasmids with the insert of choice) were identified by colony hybridization.

2.6 Labeling of DNA

2.6.1 Radiolabeling DNA fragments

50 ng of DNA fragment was labeled by random priming using a multiprime DNA labeling kit (Amersham, UK). DNA was denatured by boiling for 5 min
Materials and Methods

and chilled on ice for 10 min. Random hexanucleotide primers, dNTPs without dCTP in a concentrated buffer solution [containing Tris-Cl (pH 7.8), MgCl₂, and β-mercaptoethanol], ³²P-α-dCTP (30 μCi), and 2 units of Klenow fragment of *E. coli* DNA polymerase I were added and the reaction volume made up with water to 50 μl. The reaction was carried out by incubating the reaction mix at 37°C for 30 min. The Klenow enzyme was inactivated by heating at 65°C for 5 min and the probe was purified by spun-column chromatography.

For spun-column chromatography, a 1 ml syringe was packed with a slurry of Sephadex G-50 equilibrated with TE buffer. The column was placed in a 1.5 ml microcentrifuge tube and spun for exactly 1 min at 1,600 rpm in a microcentrifuge to pack the column tightly. The volume of the labeling reaction was made up to 100 μl with TE buffer and loaded onto the column which was again spun for 1 min at 1,600 rpm. The unincorporated nucleotides were retained in the column while the labeled probe eluted out. The specific activity of the probe was determined by scintillation counting.

2.6.2 Radiolabeling synthetic oligonucleotides

Synthetic oligo-deoxy-ribonucleotides or simply oligonucleotides were labeled by bacteriophage T4 Polynucleotide kinase (2 units per reaction) using γ-³²P-ATP (30 μCi), in presence of 50 mM Tris-Cl (pH 7.6), 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine-HCl, and 0.1 mM EDTA (pH 8.0), in a total volume of 20 μl (Sambrook et al., 1989). Around 100 ng of oligonucleotides were used per reaction. The reaction was incubated at 37°C for 60 min.
Subsequently, the enzyme was inactivated by heating at 65°C for 5 min and the probe was purified using a sephadex G-50 column by gravity purification.

### 2.6.3 Gravity purification of radiolabeled oligonucleotide

A 1 ml syringe was packed with a slurry of Sephadex G-50 equilibrated with TE buffer [10 mM Tris-Cl (pH 8.0), 1mM EDTA (pH 8.0)], (Sambrook et al., 1989). Afterwards the column was washed with 1 ml of TE buffer. The labeling reaction mix was diluted to a total volume of 100 μl with TE and loaded onto a column. Once the loaded sample entered the column bed, the upper reservoir of the column was replenished with TE buffer so that it did not run dry. Fractions of two drops per tube (60-80 μl) were collected into microcentrifuge tubes and scanned using a hand-held mini monitor (Morgan, series 900, UK). Two distinct peaks of activity were obtained- the first representing the labeled DNA and the second representing unincorporated nucleotides. The fractions containing labeled DNA were pooled together and the specific activity of the probe was determined by scintillation counting in a Beta emission counter.

### 2.7 Colony Hybridization

The bacterial colonies obtained after transformation of DH5α were transferred onto a nylon membrane (Hybond N+, Amersham, UK) and grown for 16 hours at 37°C. Subsequently the nylon membrane was treated as follows:
Materials and Methods

- The nylon membrane was first wet with SDS by keeping the membrane (colonies facing upwards) on a piece of polythene sheet wet with 10% SDS.

- The cells of bacterial colonies were lysed and their DNA was denatured for 5 min by keeping the nylon membrane (colonies facing upwards) on a piece of polythene sheet wet with 0.5 N NaOH.

- The alkali was then neutralized by keeping the membrane on a piece of polythene sheet (with the colonies facing upwards) wet with 0.5 M Tris-Cl (pH 7.5) and 1.5 M NaCl.

  The filter was air dried and the DNA released for the lysed bacterial colonies immobilized by baking at 80°C for 2 h before proceeding for prehybridization and hybridization steps.

  The blot was prehybridized at 55°C in prehybridization buffer containing 6X SSC (pH 7.0), 5X Denhardt's solution, 200 μg/ml sonicated salmon sperm DNA, and 0.05% SDS (Sambrook et al., 1989).

  20x SSC was prepared by dissolving 175.3 g of sodium chloride and 88.2 g of sodium citrate in 1 litre water with pH adjusted to 7.0.

  50X Denhardt's solution was prepared by dissolving 5 g each of Ficoll, PVP, and BSA in 500 ml water.
DNA probe (50 ng, $10^6$ cpm) was added after 3 h of prehybridization and the blot was hybridized at $55^0\text{C}$ for 16 h.

The membrane was washed as follows:

A. When probed with radiolabeled DNA probe

- 2X SSC - 2 X 10 min at RT
- 0.2X SSC with 0.1% SDS - 2 X 10 min at $55^0\text{C}$
- 0.1X SSC with 0.1% SDS - 1 X 10 min at $65^0\text{C}$

B. When probed with radiolabeled oligonucleotide probe

- 2X SSC - 2 X 10 min at RT
- 2X SSC - 1 X 2 min at $55^0\text{C}$

The membrane was then dried, covered with plastic wrap, and subjected to autoradiography. Positive clones were thus identified using labeled luc cDNA or oligonucleotide fragment.

2.8 Southern Hybridization

After electrophoresis, the gel was transferred to a glass baking dish (Borosil, India). The DNA was denatured by soaking the gel for 30 min in a denaturation solution (1.5 M NaCl and 0.5 M NaOH) with constant gentle agitation. The gel was briefly rinsed in deionized water and then neutralized by soaking for 30 min in several volumes of 0.5 M Tris-Cl (pH 7.4), 1.5 M NaCl at RT. The gel was again rinsed in deionized water and blotted onto a
Materials and Methods

nylon membrane (Hybond N+, Amersham, UK) using 20X SSC for 16 h. The membrane was then neutralized in 6X SSC for 2-3 min, air dried and baked at 80°C for 2 h. This was followed by prehybridization and hybridization with labeled luc gene fragment for 16 h at 55°C. The blot was washed as described earlier and exposed overnight to Hyperfim, MP (Amersham, UK) at -70°C.

2.9 In-Gel Hybridization

The digested DNA fragments were separated by electrophoresis in a 0.8% agarose gel run in 1X TBE for in-gel hybridization analyses (Ehtesham and Hasnain, 1991). After electrophoresis, the gel was vacuum dried and directly hybridized with radiolabeled luc fragment without any prehybridization step. Hybridization followed by gel washing was carried out exactly as described previously followed by autoradiography.

2.10 PCR primer directed mutation of 5'end of luc gene

PCR amplification was carried out using 1μM each of primers Up (Ac: 5' GGAAGATCTTTGTTAAAATGGAAGACG-3'; Kz: 5'- GGAAGATCTTGCGCGATGGAAGACGC-3') and Dp (5'-GATGTTTCACCTCGATATGTGC-3') in a reaction mix containing 1.5 mM MgCl2, 0.2 mM dNTP's (Pharmacia Chemicals, Sweden), 0.5 unit of Taq DNA polymerase (Amersham, UK), pACluc as target DNA. The cycling parameters were 95°C for 1 min, 48°C for 1 min, 72°C for 1 min, for 30 cycles. Followed by a 5 min 72°C extension cycle. The amplified product was fractionated on 1.2% agarose gel. A 154 bp
product carrying the 5' region of luc with a modified ATG context was eluted from the gel.

**2.11 Construction and screening of mutant clone**

The purified PCR amplified product was digested with Sau3A and XbaI restriction enzyme and ligated to pARluc vector at the BamHI and the XbaI site. The competent DH5α cells were transformed with the ligation reaction mix. The transformed colonies were screened for recombinants using colony hybridization as described previously. The end-labeled 5' oligos primer (Up) containing mutant sequence was used for colony hybridization exactly according to previously described protocol. After a 16 h hybridization at 55°C, the filter was washed twice at RT for 10 min each with 2X SSC, and once for 10 min at 55°C with (2X SSC + 0.1% SDS). The putative mutant clones were identified by autoradiography by exposing the hybridized filter to Hyperfilm MP (Amersham, UK) at -70°C.

Plasmid DNA was isolated from a putative mutant and the region corresponding to the polh gene promoter was sequenced to confirm the mutation using the primer which lies immediately upstream to -92. Dideoxy sequencing reaction was performed using the Sequenase version 2.0 kit (United States Biochemicals, USA) and the prescribed protocols (Sanger et al., 1977).

**3. INSECT CELL TISSUE CULTURE**

**3.1 Insect cells and Baculovirus**
Materials and Methods

The E2 strain of the baculovirus AcNPV or *Autographa californica* Nuclear Polyhedrosis Virus was used to carry out all our experiments. Insect cell lines, such as Sf9 and Sf21, derived for *Spodoptera frugiperda* species of the lepidoptera class was used to serve as host to the baculovirus.

3.2 TNM-FH Medium

TNM-FH medium is a popular medium to propagate insect cell lines such as Sf9 and Sf21 (Vaughn et al., 1977). It provides all the basic nutrients required by these insect cells. The medium is prepared using Grace's basal insect cell culture medium (Grace, 1962) containing lactalbumin hydrolysate and yeastolate (Hink, 1970). It has a pH of 6.2 buffered with sodium phosphate. To prepare one liter of TNM-FH, first, 46.3 g of Grace's medium was dissolved in 800 ml of distilled water, 0.35 g of NaHCO₃ was added and the pH adjusted to 6.2 using 10M KOH. After adjusting the pH, 3.33 g of yeast extract and 3.33 g lactalbumin hydrolysate were added. Finally, the volume was made up to 1 liter. The medium was then prefiltressed through 0.8µ and 0.45µ filter followed by final sterilization by passing through a sterile 0.22 µ filter (Millipore, USA).

3.3 Insect Cell Culture

*Sf9* cell line (Vaughn et al., 1977) which serves as the host for AcNPV (strain E2) was maintained in complete medium (CM: TNM-FH medium supplemented with 10% fetal bovine serum, 50 µg/ml gentamycin and 5 µg/ml amphotericin B) as described by Summers and Smith (1987).
Cells were maintained at 27°C in complete medium. Cells were grown either as a monolayer or in suspension cultures (O'Reilly et al., 1992). Sf9 cells have a doubling time of 18-24 h at 27°C and were subcultured twice a week when they were 90% confluent.

3.4 Monolayer Cultures
Cells were maintained in 25 cm² tissue culture flasks. The cells were dislodged by washing the surface by gentle pipetting. For each subculture, about 2 million cells were seeded in a 25 cm² flask in 5 ml of CM. The viability of the cells was checked by staining with 10% v/v trypan blue (non-viable cells stain blue). Cells with viability greater than 95% were used for experiments.

3.5 Infection of Insect Cells
For viral infection Sf9 cells were seeded at 3 X 10⁶ cells or 9 X 10⁶ cells per 25 cm² or 75 cm² flasks for 30 min in TNM-FH medium. After 30 min, TNM-FH was removed and AcNPV diluted in CM at an MOI of 10 pfu/cell was added to each flask. Total volume of viral inoculum was 1 ml/25 cm² or 3 ml/75 cm² flask. The flasks were shaken gently at intervals for ~60 min to allow for virus attachment and entry. The viral inoculum was removed after 1 h and 5 ml or 15 ml CM was added to 25 cm² or 75 cm² flasks, respectively.

3.6 Suspension cultures for virus amplification
For suspension culture, Sf9 cells were grown in 100 ml spinner flasks (Corning, USA). These flask contained a suspended magnetic bar which
keeps the insect cells suspended as it rotates sitting on a magnetic stirrer. Before use, these flasks were cleaned, first, by treating it with 0.2 N NaOH, followed by 0.2 N HCl and later by distilled water. After cleaning, these flask were sterilized by autoclaving. Before inoculating cells the sterility of the flask is checked by filling insect cell medium prior to the inoculation.

Cells were inoculated in these spinner flask at a density of 0.5 X 10^6 cells/ml and grown for 3-4 days at 27°C until a cell density of 1 X 10^6 cells/ml was attained. Cells were then infected with AcNPV for amplification of the virus.

For viral amplification, cells from the spinner flask were harvested by pelleting at 1100 rpm for 15 min at 4°C in a Hemle centrifuge (AD 8.9 rotor). The cells were then infected with the virus by swirling them periodically for 1 h in an inoculum of AcNPV such that an MOI of 0.1 pfu/cell was reached. After infection cells were transferred back into the spinner flasks, fresh medium was added and the infection allowed to proceed for 6-7 days until all the cells were infected and close to lysis. The cell suspension was centrifuged again to pellet the cells and the supernatant containing amplified virus was serially diluted from 10^{-1} to 10^{-8} in complete medium. 100 µl of the viral dilutions were added drop by drop to each petri dish.

The titer of the amplified virus in the medium was determined by serial dilutions of the virus stock in a plaque assay.
Materials and Methods

3.7 Plaque assay for virus titration

The titre of the amplified virus was determined using a plaque assay (Summers and Smith, 1987). 1.5 ml of Sf9 cells at 1.2 X 10^6 cells/ml and >99% cell viability were seeded in a 35mm dish (Corning, USA) in incomplete medium. The seeded cells were infected with log dilutions of amplified virus stock and incubated at RT for about 60 min with gentle intermittent shaking. After 1 h the viral inoculum was removed and 2 ml of low gelling temperature (LGT) overlay [1:1 ratio of 3% LGT agarose in ddW and CM] was added. 1 ml of CM was added to each dish after the agarose was polymerized and these were then incubated at 27°C. 3-5 days later, the liquid media was removed from top of the agarose overlay and 1 ml of neutral red staining solution [1.2 ml neutral red + 20 ml plaque assay buffer (8.2 g NaCl, 2.0 g KCl, 1.14 g Na_2HPO_4, 0.2 g KH_2PO_4 in a total volume of 1 l and pH adjusted to 7.3)] was added. The plates were incubated for 1 h after which the stain was drained off and the plates were kept inverted for ~12-16 h for the plaques to become well formed. The virus titre (pfu/ml) was calculated using the following formula:

\[
\text{No. of plaques} = \frac{\text{Dilution} \times \text{Inoculum per plate}}{\text{Inoculum per plate}}
\]

No. of plaques

Dilution X Inoculum per plate
4. LUCIFERASE REPORTER BASED TRANSIENT EXPRESSION ASSAY

4.1 Lipofectin-mediated Transfection of Plasmid DNA Constructs

A sensitive luc gene based transient expression system for baculovirus infected insect cells (Hasnain and Nakhai 1990; Hasnain et al., 1994) was used to study the influence of translation initiation codon context. A Lipofectin-mediated transfection of insect cells were carried out with plasmid DNA carrying the luciferase reporter gene placed under different translation initiation codon context sequence. This technique makes use of cationic liposomal preparation Lipofectin -a synthetic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium chloride with the phospholipid dioleoyl phosphatidyl ethanolamine in a 1:1 ratio- can spontaneously interact with DNA or RNA (Felgner et al., 1987) and form liposomes. In insect cells, it is routinely used to carry out co-transfection of viral and plasmid DNA for recombinant viral production (O'Reilly et al., 1992). Light emission of cells transfected with the luciferase reporter plasmids and infected with AcNPV, as measured by luminometry, was used as an index of luc expression. The cDNA encoding luciferase cloned from the firefly Photinus pyralis in 1985 (deWet et al., 1985) is now emerging as the gene of choice for in vitro and in vivo reporting of transcriptional activity in eukaryotic cells. Luciferase reporter enzyme generates luminescence through mono-oxygenation of luciferin, utilizing ATP and O2 as co-substrates. The light output generated is linearly proportional to the amount of luciferase, hence the luminescence correlates directly to expression of the luc reporter gene in transfected cells.
Sf9 cells propagated in complete medium were seeded at a density of 2 X 10^6 cells/well in a 6-well (35 mm) tissue culture plate, washed three times with serum free TNM-FH, and left for two hours at 27°C. 10 µg of plasmid DNA was transfected using lipofectin (Gibco BRL, USA). For transfection, highly pure reporter plasmid DNA (purified on a Qiagen column) was dissolved in 35 µl water, and 15 µl (1 mg/ml) Lipofectin was separately diluted to 500 µl in serum-free TNMFH, mixed slowly (drop by drop) and added to the culture wells. These cells were incubated for 8 h at 27°C, cells were washed twice with complete medium, before infecting them with AcNPV for 1 h. The infected cell were the incubated in complete medium at 27°C.

4.2 Luciferase assay

At 65 h pi, cells were dislodged in 400 µl medium, diluted to 600 µl with 0.1 M Tris acetate (pH 7.75), 2 mM EDTA buffer and assayed for luciferase in a buffer containing 40 mM luciferin, 14 mM MgCl₂ and 14 mM glycine (pH 7.6). Firefly luciferase catalyzes the oxidation of D(-) luciferin in the presence of ATP-Mg²⁺ and O₂ to generate oxyluciferin and light (Gould and Subramani, 1988; Karp et al., 1992). Exogenous ATP was not added since luc expression in live cells was being measured. Light emission in mV was monitored using a luminometer (Model 1250, Bio-Orbit Oy, Turku, Finland) over an integration period of 10 s. All transient expression assays were carried out using 10 µg of reporter plasmid construct DNA and AcNPV infection at an MOI of 10 pfu/cell.

4.3 Dot-Blot analysis of Transfected plasmid DNA
Materials and Methods

After luc assay, transfected Sf9 cells (~2 X 10^6) were pelleted down and lysed using 500 µl of 0.5 N NaOH. The cell lysis step was followed by neutralization step by adding 10 M ammonium acetate. The 100 µl and 200µl aliquots of lysed cells were then vacuum blotted on a Hybond N+ membrane (Amersham, UK). The membrane was air-dried and baked at 80°C for 2 h. The blot was pre-hybridized with salmon sperm DNA for 4 h and hybridized with 32P-labeled pUC DNA probe at 55°C for 16 h. The filter was then washed and autoradiographed using Hyperfilm-MP (Amersham, UK).