CHAPTER II

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Cell culture media, chemicals, enzymes, and radioisotopes were obtained from the following companies:

**AMERSHAM plc, United Kingdom:** $^{32}$P-$\gamma$ATP, $^{35}$S-dATP, $^{32}$P-$\alpha$dCTP, multiprime DNA labeling kit, Rainbow protein molecular weight markers

**AMRESCO INC., USA:** ampicillin, kanamycin, lysozyme

**BANGALORE GENEI, India:** restriction enzymes

**BENGAL CHEMICALS, India:** ethanol

**BOEHRINGER MANNHEIM GmbH, Germany:** ATP, deoxynucleotides, luciferase, luciferin, poly[d(I-C)], T4 DNA ligase, T4 polynucleotide kinase, T7 primer (5'-TAATACGACTCACTATAGGG-3')

**DIFCO, USA:** bactoagar, bacto-tryptone, lactalbumin hydrolysate, yeastolate

**FLOW LABORATORIES, Scotland:** neutral red staining solution

**GIBCO BRL, USA:** Grace's basal insect cell culture medium, 1 Kb DNA molecular size ladder, Lipofectin

**NESTLE, India:** non-fat dry milk

**NEW ENGLAND BIOLABS, USA:** DNA polymerase I-Klenow fragment, $\lambda$ DNA, $\phi$X174 phage DNA, restriction enzymes
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PHARMACIA, Sweden: Sephadex G-50

PROMEGA CORPORATION INC., USA: purified TBP, purified human Sp1

QUALIGENS, India: boric acid, CH₃COOH, CH₃COONa, chloroform, CuSO₄, diethyl ether, glycerol, glycine, HCl, H₂O₂, isoamyl alcohol, isopropanol, KCl, KH₂PO₄, methanol, Mg(CH₃COO)₂, MgCl₂, NaCl, Na₂CO₃, Na₂HPO₄, NaOH, Na-K-tartarate, NH₄OH, NH₄OAc, phenol, Tris

REAGENT BANK, NII: goat anti-rabbit IgG-HRP

SIGMA, St. Louis, USA: acrylamide, agarose, ammonium persulphate, amphotericin B, bisacrylamide, bovine serum albumin, bromophenol blue, CaCl₂, citric acid, DAB, DTT, EDTA, EGTA, EtBr, Ficoll, foetal bovine serum, Folin & Ciocalteu's phenol reagent, gentamycin, HEPES, KOH, low gelling temperature agarose, β-mercaptoethanol, mineral oil, NaHCO₃, NaH₂PO₄, Nonidet P-40, PEG-8000, PMSF, PVP, SDS, sonicated salmon sperm DNA, spermidine, sucrose, TEMED, trypan blue, urea, xylene cyanol

UNITED STATES BIOCHEMICALS, USA: Sequenase version 2.0 sequencing kit, universal M13 forward sequencing primer

Oligonucleotides: Most of the oligonucleotides used in this study were kind gifts from Dr. Altaf Lal, Chief, Malaria Immunology, Division of Parasitic Diseases, Centers for Disease Control, Atlanta, USA. The remaining oligonucleotides were commercially obtained from Rama Biotechnologies, India.
Methods

1. Insect Cell Tissue Culture

1.1 Cell Line and Virus

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).

1.2 TNM-FH Medium

TNM-FH medium (Hink, 1970) is Grace's basal insect cell culture medium (Grace, 1962) containing lactalbumin hydrolysate and yeastolate. It provides basic nutrients to the insect cells and has a pH of 6.2 buffered with sodium phosphate. To make 1 l of TNM-FH, 46.3 g of Grace's medium was dissolved in 700 ml of distilled water, 0.35 g of NaHCO₃ was added and the pH adjusted to 6.2 with 10 M KOH. 3.33 g each of lactalbumin hydrolysate and yeastolate were then added and the volume was made up to 1 l. The medium was sterilized by passing through a sterile 0.22 μ filter (Millipore, USA).

Cells were maintained at 27°C in complete medium and 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 upon gaining >90% confluency.

1.3 Monolayer Cultures

Cells were maintained in 25 cm² tissue culture flasks (Corning, USA). The cells were dislodged by washing the surface by gentle pipetting. For each subculture, about 2 x 10⁶ 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). Only cells with viability greater than 95% were used for experiments.
1.4 Infection of Insect Cells

During viral infection for preparation of nuclear extract, Sf9 cells were seeded at 3 x 10^6 cells or 9 x 10^6 cells per 25 cm² or 75 cm² flasks, respectively for 30 min in TNM-FH medium. After 30 min, TNM-FH was removed and AcNPV stock, diluted in CM to achieve 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 rocked 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. The flasks were incubated for 51 h at 27°C and the infected cells were used for nuclear extract preparation.

1.5 Suspension Cultures

Sf9 cells were grown in 100 ml spinner flasks (Corning, USA) which have a suspended magnetic bar for stirring. The flasks were treated successively with 0.2 N NaOH, 0.2 N HCl and distilled water before sterilizing them by autoclaving. Cells were inoculated at a density of ~0.5 x 10^6 cells/ml and grown for 3-4 days at 27°C till a cell density of ~1 x 10^6 cells/ml was attained. Cells were then harvested or infected with AcNPV for amplification of the virus. For viral amplification, cells from the spinner flask were harvested by pelleting at 1000 rpm for 15 min at 4°C in a Hermle AD 8.9 rotor. The cells were then infected by swirling them periodically for 1 h in an inoculum of AcNPV such that an MOI of 0.1 pfu/cell was reached. Infected 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 were approaching lysis. The cell suspension was centrifuged again to pellet the cells and the supernatant (complete medium containing AcNPV) was used as the virus stock.

The concentration of the amplified virus in the medium was determined by serial dilutions of the virus stock in a plaque assay.
1.6 Plaque Assay

The titre of the amplified virus was determined using a plaque assay (Summers and Smith, 1987). Briefly, 1.5 ml of Sf9 cells at 1.2 x 10^6 cells/ml and >99% cell viability were seeded in a 35 mm dish (Corning, USA) in complete medium. The amplified virus stock 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 dishes were incubated at RT for about 60 min with gentle intermittent rocking. 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 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: [1/dilution] x number of plaques x [1/(ml inoculum/plate)].

2. DNA-PROTEIN INTERACTIONS

2.1 Insect Cell Nuclear Extract Preparation

Typically, 10^8 cells were infected with AcNPV at a multiplicity of infection of 10 pfu/cell (O'Reilly et al., 1992). Nuclear protein extract was prepared (Hasnain et al., 1995) from uninfected cells and infected cells 51 h pi (a time point when the baculovirus very late promoters are maximally active). All operations were carried out at 4°C; pre-chilled solutions, micro-centrifuge tips, microcentrifuge tubes, and ultracentrifuge tubes were used. Cells were harvested by centrifugation at 800 rpm for 15 min in a HERMLE AD8.9 rotor. The cells were washed (to remove traces of serum-containing medium) by resuspension in 50 mM PBS (pH 7.3). The cells were repelleted and suspended in 2 ml lysis buffer [1% Nonidet P-40, 30 mM Tris-Cl (pH 7.5), 10 mM Mg(CH_3COO)_2] and incubated, with three vortexings of 10 s
each, for 5 min. The suspension was transferred to microcentrifuge tubes and the nuclei harvested by centrifugation at 1,600 rpm for 3 min. The crude nuclear preparation was then resuspended in 3 ml of 2 M sucrose buffer [10 mM HEPES-NaOH (pH 7.5), 15 mM KCl, 2 M sucrose, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT] and layered onto a 10 ml cushion of the same buffer and centrifuged in a Beckman SW41 rotor at 28,100 rpm for 60 min (Marzluff, 1990). The ultra purification step eliminated most of the nuclease activity present in late infected insect cells (Glocker et al., 1992). The purified nuclei were resuspended in a minimum volume of protein extraction buffer [10 mM HEPES-NaOH (pH 7.5), 420 mM NaCl, 1.5 mM MgCl₂, 25% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT] (Dignam et al., 1983), and stirred gently for 30 min. The nuclear pellet was pelleted by centrifugation at 11,000 rpm in a microcentrifuge for 15 min and the supernatant was aliquoted and stored at -70°C. Typically, 1-1.5 mg protein was obtained from 100 million cells.

2.2 Determination of Protein Concentration

Concentration of the nuclear protein extract was determined by the method of Lowry (1951). The following reagents were prepared:

Reagent 1: 2% Na₂CO₃ in 0.1 N NaOH
Reagent 2: 2.7% Na-K-tartarate in reagent 1
Reagent 3: 1% CuSO₄ in water
Reagent 4: 1 part of reagent 2 mixed with 1 part of reagent 3
Reagent 5: 1 part of reagent 4 with 50 parts of reagent 1
Reagent 6: 1 N Folin & Ciocalteu's phenol reagent (2 N stock diluted with water)
Reagent 7: Protein standard- 1 mg/ml BSA (fraction V)

For estimation of protein concentration, 1 ml of reagent 5 was added to 100 µl protein (5 µl protein extract diluted to 100 µl with 0.9% saline). The solution was vortexed and kept at RT. After 10 min, 100 µl of reagent 6 was added to each tube dropwise while stirring. The mixture was allowed to stand at RT for 30 min while
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protected from light. The OD was read against blank (100 µl saline to which 1 ml reagent 5 and 100 µl reagent 6 had been added) at 650 nm. The concentration of the nuclear protein extract was determined from a standard curve prepared using increasing concentrations of BSA.

2.3 Radio labeling Oligonucleotides

Synthetic oligonucleotides (100 ng per reaction) were labeled by bacteriophage T4 polynucleotide kinase (2 units per reaction) using $^{32}$P-$\gamma$ATP (30 µCi), in a 20 µl reaction buffer containing 50 mM Tris-Cl (pH 7.6), 10 mM MgCl$_2$, 5 mM DTT, 0.1 mM spermidine-HCl, and 0.1 mM EDTA (pH 8.0) (Sambrook et al., 1989). The reaction was incubated at 37°C for 60 min. The enzyme was inactivated by heating at 65°C for 5 min and the probe was purified by gravity purification.

For gravity purification, a 1 ml syringe was packed with a slurry of Sephadex G-50 equilibrated with TE buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0)], (Sambrook et al., 1989). The column was washed with 1 ml of TE buffer. The labeling reaction was diluted to a total volume of 100 µl with TE and loaded onto a column. Immediately after the sample entered the column, the column was replenished with TE buffer so that it did not run dry. Fractions of two drops per tube (~80 µl) were collected into microcentrifuge tubes and scanned using a handheld 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.

2.4 Electrophoretic Mobility Shift Assays and Competition Studies

1 ng of labeled oligonucleotide (~$10^4$ cpm) was added to the nuclear extract (Sf9, HeLa or pea) in a microcentrifuge tube in a 20 µl final volume of reaction buffer (10 mM HEPES-NaOH (pH 7.5), 200 mM NaCl, 0.5 mM DTT and 1 µg poly[d(I-C)]) (Chodosh, 1988a; Pharmacia, 1990; Stone et al., 1991). The HeLa and the pea
nuclear & chloroplast extracts were a kind gift from Dr. N. Tuteja, ICGEB, New Delhi. The components were mixed gently by tapping the bottom of the tube, centrifuged briefly, and incubated at 25°C for 15 min. 20 μl of loading dye [20 mM HEPES-NaOH (pH 7.5), 0.04% bromophenol blue, 10% glycerol] was added to each reaction, mixed gently, and centrifuged briefly. The DNA-protein complex was resolved by electrophoresis at 4°C in a 5% (29:1 acrylamide-bisacrylamide) polyacrylamide gel in a low-ionic strength TAE buffer [7 mM Tris-Cl (pH 7.5), 3 mM CH₃COONa, 1 mM EDTA], (Pharmacia, 1990). Constant pH between the electrode chambers was maintained by recirculation of the buffer during electrophoresis using peristaltic pumps. After electrophoresis, the gel was transferred to a 3 mm thick Whatmann chromatography paper, covered with plastic wrap and dried under vacuum. The DNA-protein complex was visualized after autoradiography at -70°C. For competition studies, appropriate amount of unlabeled, double stranded DNA or oligonucleotide was added to the reaction mixture.

For TFIID binding studies, 1 μg of crude extract or 20 ng of the purified protein were incubated for 15 min at RT with 1 ng of end-labeled oligonucleotide in presence of TFIID binding buffer [4x binding buffer- 40% glycerol, 80 mM Tris-Cl (pH 7.9), 320 mM KCl, 40 mM MgCl₂, 8 mM DTT] without any poly[d(I-C)]. The reactions were electrophoresed at 4°C for 90 min (half life of TFIID in a running gel is ~90-120 min) in 0.5x TBE gel running buffer [0.045 M Tris-borate, 0.001 M EDTA, 5 mM MgCl₂ and 0.05% Nonidet P-40] on a 6% non-denaturing gel containing 0.05% Nonidet P-40 which was pre-run for 20 min.

For Sp1 binding studies, the crude nuclear extract or purified Sp1 was incubated for 5 min at RT in 5x binding buffer [20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-Cl (pH 7.5), 0.25 mg/ml poly [dI-dC],poly [dI-dC]] followed by the addition of ³²P-labeled oligonucleotide and incubated for 20 min. The reaction mixture also included 2 μg of BSA in a total volume of 20 μl. The DNA-protein complex was resolved under Sp1-specific conditions at 4°C in a 4% (29:1 acrylamide/bisacrylamide) non-denaturing
polyacrylamide gel in 0.5x TBE buffer at 100 V for 3 h. The gel was then dried and exposed overnight to Hyperfilm MP (Amersham, UK) at -70°C.

2.5 UV Crosslinking Analysis of DNA-protein Complex

The binding reaction was carried out as described above in a microcentrifuge tube with its mouth sealed with Saran wrap held in place with a strip of Parafilm (Sigma, St. Louis, USA). After the reaction was over, the tube was placed on ice and irradiated from a distance of 1 cm with a hand-held UV monitor of 254 nm wavelength for 30 min (Chodosh, 1988b; Hughes et al., 1991). An equal volume of SDS sample buffer [0.0625 M Tris-Cl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue] was added to each tube and boiled for 5 min at 100°C. The samples were electrophoresed on a 15% SDS-polyacrylamide (29:1 acrylamide-bisacrylamide) gel in a Tris-glycine buffer [25 mM Tris, 192 mM glycine (pH 8.3), 0.1 % SDS], (Hughes et al., 1991). After electrophoresis, the gel was dried and autoradiographed as described above.

2.6 Western Blot Analysis

Western analysis was carried out using standard protocols (Gallagher et al., 1991; Burnette, 1981). 100 μg of crude nuclear extracts or 60 ng of purified protein were electrophoresed on a 15% SDS-PAGE (Laemmli, 1970) and transferred to a nylon membrane (Hybond C-extra, Amersham, UK) at 300 mA for 2 h at 4°C [Transfer buffer- 25 mM Tris, 190 mM glycine, 20% methanol]. The membrane was blocked with 1% non-fat dry milk for 1 h and then incubated with 1:3000 dilution of anti-TBP antisera (a kind gift of Dr. Robert G. Roeder) for 1 h. Goat anti-rabbit IgG-HRP conjugate (procured from the NII Reagent Bank) was used as the second antibody and was stained with H₂O₂ (0.03%) and DAB [50 μg/ml in PBS].
3. IN VIVO ANALYSES OF POLYHEDRIN PROMOTER MUTATIONS AND CONTEXT SEQUENCES

3.1 MOLECULAR CLONING

3.1.1 PLASMID DNA CONSTRUCTS

3.1.1a Unmutated and mutated promoter-reporter plasmid constructs

All DNA manipulations were carried out according to Sambrook et al. (1989). All ligation reactions were carried out using T4 DNA ligase in 1x ligase buffer [20 mM Tris-Cl (pH 7.6), 5 mM MgCl₂, 5 mM DTT, 1 mM ATP]. The ligation reaction was carried out at 16°C for ~16 h in a total volume of 20 μl. The ligation reaction mixture was transformed into competent E. coli DH5α (Gibco-BRL) cells (Sambrook et al., 1989) and 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.

Different complementary oligonucleotides carrying various mutations in the hexa- and octanucleotide motifs, as detailed in Chapter IV, Table I (page 61), were chemically synthesized. The oligos were synthesized in such a manner that they had HindIII-SalI overhangs at either end to facilitate cloning. The complementary oligonucleotides were annealed and cloned at the HindIII-SalI site of pAJluc (Fig. 4.1, panel A, page 62) to generate the respective promoter mutant plasmids. All promoter mutations and promoter-reporter orientations were confirmed by dideoxy sequencing (Sanger et al., 1977, Chapter IV) using the universal M13 forward sequencing primer and the Sequenase version 2.0 kit (United States Biochemicals, USA) with its prescribed protocols and reagents. The plasmid construct, pAJ pol1uc, harboring the unmutated polyhedrin promoter was constructed as described in Chapter IV (Fig. 4.2, page 63)
3.1.1b Plasmid constructs with various lengths of polyhedrin promoter upstream sequences

pKNluc (with a ~4 Kb sequence upstream to the polyhedrin promoter) was constructed by cloning the 1.8 Kb BamHI fragment of the luc gene as described in Chapter V (Fig. 5.2, panel A, page 88) at the BamHI site of the transfer vector pVL1393. A 2.77 Kb SalI-HindIII fragment (which contains ~766 bp sequence upstream to the polyhedrin promoter, 92 bp polyhedrin promoter and 1.8 Kb luc gene fragment) obtained from pKNluc was ligated at the SalI-HindIII sites of pUC18 (Fig. 5.3, panel A, page 89) to obtain the construct pKN603luc.

3.1.1c Phagemid construct for site-specific mutation of the hexanucleotide motif

A plasmid construct, pAJpBS603luc, was generated for carrying out site-directed mutagenesis of the hexanucleotide motif AATAAA keeping the 766 bp sequence upstream to the polyhedrin promoter intact. For this the 2.77 Kb SalI-HindIII fragment from pKN603luc was cloned at the SalI-HindIII sites of the 3.0 Kb phagemid pBS+ (Fig. 5.5, panel A, page 93).

3.1.2 Colony Hybridization

The bacterial colonies obtained after CaCl₂-mediated transformation of the ligation mix were transferred onto a nylon membrane (Hybond N+, Amersham plc, UK) and grown overnight at 37°C. The following day, the filter was treated as follows by keeping it on a piece of polythene sheet with the colonies facing upwards: The DNA was denatured twice for 2 min each with 0.5 N NaOH. The alkali was neutralized with 1 M Tris-Cl (pH 7.5) for 5 min followed by a 5 min neutralization with 0.5 M Tris-Cl (pH 7.5) and 1.5 M NaCl. The filter was air dried and the DNA immobilized by baking at 80°C for 2 h before proceeding for prehybridization and hybridization.

The blot was prehybridized at 55°C on a rotary shaker in a 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). (175.3 g of NaCl and 88.2 g of sodium...
citrate was dissolved in 1 l water and the pH adjusted to 7.0 with 100 mM citric acid to prepare 20x SSC. 10 g each of Ficoll, PVP, and BSA was dissolved in 500 ml water to prepare 50x Denhardt’s solution. DNA probe (50 ng, 10⁶ cpm) was added after 4 h of prehybridization and the blot was hybridized at, 55°C for 16 h. The membrane was washed as follows:

- 2x SSC - 2 x 10 min at RT
- 0.2x SSC with 0.1% SDS - 2 x 10 min at 55°C
- 0.1x SSC with 0.1% SDS - 1 x 10 min at 65°C

The membrane was then dried, covered with Saran wrap (Dow Chemicals, USA) and subjected to autoradiography. Positive clones were thus identified using labeled luc cDNA or polh promoter fragment.

### 3.1.3 Radio labeling 1.8 Kb Luciferase cDNA and the 92 bp Polyhedrin Promoter Fragment

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 and chilled on ice for 10 min. Random hexanucleotide primers, dNTPs (-dCTP) in a concentrated buffer solution [containing Tris-Cl (pH 7.8), MgCl₂, and β-mercaptoethanol], ³²P-αdCTP (30 μCi) and Klenow fragment of E. coli DNA polymerase I (2 units per reaction) were added and the reaction volume made up with water to 50 μl. The reaction mixture was incubated at 37°C for 30 min. The 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 centrifuged 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
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column while the labeled probe eluted out. The specific activity of the probe was determined by scintillation counting.

3.1.4 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 nylon membrane (Hybond N+, Amersham plc, UK) using 20x SSC for 16 h (Southern, 1975). 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 92-bp polh promoter fragment for 16 h at 55°C. The blot was washed as described earlier and exposed overnight to Hyperfim, MP (Amersham plc, UK) at -70°C.

3.1.5 In-Gel Hybridization

The DNA fragments were processed for in-gel analyses (Ehtesham and Hasnain, 1991) by electrophoresis in a 1.2 % EtBr stained agarose gel run in 1x TBE (Sambrook et al., 1989). 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.

3.2 OLIGONUCLEOTIDE-MEDIATED SITE-DIRECTED MUTAGENESIS

3.2.1 Preparation of Single Stranded Template DNA

The recombinant construct, pAJpBS603luc, was transformed into competent E. coli TG1 cells. The following protocol (Sambrook et al., 1989) was used to prepare single-stranded template DNA from the recombinant clone:
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1. A single colony of the recombinant clone (pAJpBS603luc) was grown overnight at 37°C with shaking in 1 ml of LB [10 g of bacto-tryptone, 5 g of yeast extract and 10 g of NaCl were dissolved in 800 ml ddW, pH adjusted to 7.2 and the volume adjusted to 1 l] with ampicillin at a concentration of 50 µg/ml.

2. 1% of the primary culture was inoculated in 2 ml superbroth [Composition for 1 l: 32 g bacto-tryptone, 20 g yeast extract, 5 g NaCl and 5 ml of 1 N NaOH] with ampicillin at a concentration of 20 µg/ml. The culture was grown at 37°C with vigorous shaking at ~200 rpm till the OD$_{600}$ was ~0.2-0.4.

3. After ~3 h, 30 µl of the "helper phage" (M13KO7) stock (3 X 10$^{11}$ pfu/ml) was added at an MOI=20 to each culture and the culture was left undisturbed at RT for 15 min (for phage attachment).

4. The infection was carried on at 37°C for 6-8 h with vigorous shaking (~250 rpm).

5. The culture was spun at 12,000 rpm in a microfuge at RT for 5 min. The supernatant was spun again and proceeded for single stranded DNA precipitation with 300 µl of (20% PEG 8000 + 3.5 M NH$_4$OAc).

6. The precipitation was done for 15 min on ice and the ssDNA was pelleted at 12,000 rpm in a microfuge for 10 min. The pellet was respun to remove traces of residual liquid.

7. The DNA pellet was resuspended in 200 µl of TE [10 mM Tris-Cl (pH 7.5), 0.1 mM EDTA].

8. The ssDNA was extracted once with buffered phenol and twice with diethyl ether and was then precipitated overnight at -20°C with 0.15 M NaCl and 2 volumes of ethanol. The precipitated DNA pellet was washed once in 70% alcohol and resuspended in 20 µl ddW.

9. Five µl of the template DNA was sequenced with the T7 primer to determine the authenticity and quality of the DNA using the dideoxy sequencing protocol as prescribed by Sequenase version 2.0 (United States Biochemicals, USA).
3.2.2 Site-Directed Mutagenesis

The protocol followed was a modification of the standard site-directed mutagenesis procedure (Kunkel, 1985).

1. Phosphorylation of the primer:
5 pmoles of AL628, a 24-mer oligonucleotide spanning the region to be mutated (sequence CATCTCGCACCCCCCTAAGTATTT replacing CATCTCGCAAATAAATAAGTATTT in the promoter) was phosphorylated using 10 units of T4 polynucleotide kinase, 0.5 mM ATP and 1x polynucleotide kinase buffer [50 mM Tris-Cl (pH 7.6), 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine-HCl, 0.1 mM EDTA] in a total volume of 5 µl at 37°C for 30 min. The reaction was terminated for 5 min at 65°C.

2. Annealing and Elongation:
The phosphorylated primer was annealed to ~0.2 pmoles of ssDNA template (~5 µl) in 1x solution A [5x solution- 0.4 M Tris-Cl (pH 7.5), 0.5 M NaCl, 0.2 M MgCl₂]. The reaction mixture was denatured at 65°C for 5 min followed by slow annealing at RT for 30 min and then on ice for 30 min.
Following the annealing reaction, 1x solution B [10x solution- 0.2 M Tris-Cl (pH 7.5), 0.1 M MgCl₂, 0.05 M DTT] with 0.5 mM ATP and 0.25 mM dAGCT in 50 mM Tris-Cl (pH 7.5) was added and the elongation reaction was incubated on ice for 30 min in presence of 2.5 units of Klenow enzyme (this incubation helps to stabilize the mismatched oligo by elongating it along the template). Another 5 units of Klenow and 0.5 units of T4 DNA ligase were added and the incubation was done at RT (20°C) for 2 h. After 2 h a further addition of 5 units of Klenow and 0.5 units of ligase and RT incubation for 2 h was done.

3.2.3 Colony Hybridization for Mutant Clone Identification
The reaction mix was transformed into competent E. coli TG1 cells and the colonies obtained were picked and transferred on to a nylon membrane (Hybond
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N+, Amersham plc, UK). AL628 oligo (CATCTCGCACCCCCCTAAGTATT) was end-labeled as described earlier and was used for colony hybridization exactly as previously. After a 16 h hybridization at 55°C, the filter was washed twice at RT for 10 min each with 2x SSC, once for 10 min at 55°C with (2x SSC + 0.1% SDS) and for another 10 min at 55°C with (1x SSC + 0.1% SDS). The filter was exposed overnight to Hyperfilm MP (Amersham plc, UK) at -70°C. The putative mutant clones were identified by autoradiography, plasmid DNA was isolated (Sambrook et al., 1989), retransformed into E. coli TG1 competent cells and proceeded for secondary screening again with labeled AL628 oligo. Nearly 90-95% colonies represented mutant colonies this time.

3.2.4 Confirmation of the Mutant Clone, pAJpBsmH603luc

Plasmid DNA was isolated from a putative mutant and the region corresponding to the polyhedrin promoter was sequenced to confirm the mutation (Chapter IV) using the 20 mer AL626 (ACAAACTAATATCACAAACT) primer which lies immediately upstream to -92. Dideoxy sequencing reaction (Sanger et al., 1977) was performed using the Sequenase version 2.0 kit (United States Biochemicals, USA) and the prescribed protocols.

3.3 TRANSIENT EXPRESSION ASSAY WITH LUCIFERASE AS REPORTER

3.3.1 Lipofectin-Mediated Transfection of Plasmid DNA

A sensitive transient expression system for baculovirus infected insect cells with Lipofectin-mediated transfection of plasmid DNA carrying the luciferase reporter was used to study the polyhedrin promoter of AcNPV. The cationic liposomal preparation Lipofectin, (which employs DOTMA, a synthetic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium chloride with the phospholipid dioleoyl phosphatidylethanolamine in a 1:1 ratio) can form liposomes that spontaneously interact with DNA or RNA (Felgner et al., 1987). 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. Because light output is
linearly proportional to the amount of luciferase, luminescence correlates directly to expression of the *luc* reporter gene in transfected cells.

*Sf9* cells cultured in CM were seeded as 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 2 h at 27°C. 20 μg of plasmid DNA was transfected using Lipofectin (Gibco BRL, USA). For transfection, reporter plasmid DNA (purified on a Qiagen column, Qiagen, USA according to prescribed protocols) was dissolved in 35 μl water, and 15 μl (1 mg/ml) Lipofectin was separately diluted to 500 μl in serum-free TNM-FH, mixed and added to the culture wells. After 8 h incubation at 27°C, cells were washed twice with CM, infected with *AcNPV* for 1 h and incubated in complete medium at 27°C.

### 3.3.2 Luciferase Assay

At 60 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 20 μg of reporter plasmid DNA and *AcNPV* infection at an MOI of 10 pfu/cell.

### 3.3.3 Dot-Blot Analysis of Transfected Plasmid DNA

*Sf9* cells from a 35 mm culture dish (~2 X 10^6 cells/dish) were dislodged in 500 μl medium. Replicates of 10 μl of cell suspension were removed from the wells for dot-blot analysis while the rest were used for luc assay. For blotting, 200 μl of 0.5 N NaOH was added to the cells and mixed followed by the addition, with thorough mixing, of 10 M ammonium acetate. The lysed cells were then vacuum blotted on
a Hybond N+ membrane (Amersham plc, 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 $^{32}$P-labeled luc probe at 55°C for 16 h. The filter was then washed and autoradiographed using Hyperfilm-MP (Amersham plc, UK).