CHAPTER II

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MATERIALS

Tissue culture reagents, enzymes and kits, radioisotopes, membranes, X-ray films, chemicals and other assorted reagents were obtained from the following sources:

Amersham-Pharmacia Biotech, UK: X-ray film (Hyperfilm MP and Hyperfilm β-max), Rediprime DNA labelling kit, Rainbow protein molecular weight markers, Hybond N+ nitrocellulose membrane

Bengal Chemicals, India: Ethanol

Boehringer Mannheim GmbH, Germany: Poly dI.dC, T4 polynucleotide kinase, T4 DNA ligase, glycogen

GIBCO BRL Inc, USA: FBS, Grace’s insect cell medium, antibiotic-antimycotic solution, DNA molecular weight markers, Lipofectin

CLONTECH Laboratories, Inc., USA: BacPAK™ baculovirus expression vector system kit

Corning Inc., USA: Tissue culture plasticware

Hi-Media, India: Agar agar, tryptone, yeast extract


New England Biolabs, USA: Restriction enzymes, T4 polynucleotide kinase, Klenow fragment of E. coli DNA polymerase I

Perkin Elmer Inc., USA: dNTPs, Taq DNA polymerase
Pierce, USA: Bicinchonic acid (BCA) kit for protein estimation

Promega Inc., USA: Pure recombinant human Sp1, DNaseI

Qualigens, India: Acetic acid, acetone, ammonium acetate, boric acid, chloroform, glucose, glycerol, HCl, isopropyl alcohol, KCl, KH$_2$PO$_4$, K$_2$HPO$_4$, methanol, NaCl, Na$_2$HPO$_4$, NaH$_2$PO$_4$, NaOH, Tris, trisodium citrate

Rama Biotechnologies, India: Oligonucleotides

Sigma, St. Louis, USA: Acrylamide, APS, β-mercaptoethanol, bisacrylamide, BSA, bromophenol blue, CaCl$_2$, DTT, EDTA, EGTA, EtBr, Ficoll, formic acid, lactalbumin, low gelling temperature agarose, magnesium acetate, MgCl$_2$, neutral red staining solution, NP-40, PMSF, piperidine, Sephadex G-50, sonicated salmon sperm DNA, TEMED, trypan blue, urea, xylene cyanol

United States Biochemicals (USB), USA: Agarose, Sequenase ver. 2.0 sequencing kit, PVP, SDS, Tris-saturated phenol
METHODS

1. Cell and Virus Culture

1.1 Cell line and virus used

Sf9 cell line (Vaughn et al., 1977), an ovarian cell line from the fall armyworm *Spodoptera frugiperda*, was used as the host for the baculovirus AcNPV strain C6 in all studies.

1.2 TNMFH medium

TNMFH medium (Hink, 1970) is Grace's basal insect cell culture medium (Grace, 1962), containing lactalbumin and yeast hydrolysate. It provides basic nutrients to the cell and has a pH of 6.2 buffered with sodium phosphate. To make 1 l of TNMFH medium (incomplete medium- ICM), 46.3 g of Grace's medium was dissolved in ~800 ml of MQ water and the pH adjusted to 6.2 with 10 N KOH. 3.33 g each of lactalbumin and yeast hydrolysate were then added, and the volume made up to 1 l. The medium was sterilized by passing through a sterile 0.22 μm filter (Millipore, USA) in a laminar flow hood.

The cells were maintained in CM (complete medium) i.e. TNMFH supplemented with 10% FBS and 1X antibiotic-antimycotic solution (GIBCO BRL, USA), according to the method described by Summers and Smith (1987).

1.3 Maintenance of Sf9 cells

Cells were maintained at 27°C in a BOD incubator and grown either as monolayer or suspension cultures (O'Reilly et al., 1992). Sf9 cells have a doubling time of 18-24 h at 27°C. The cells were subcultured every 2-3 days when they were >90% confluent.

For monolayer cultures, Sf9 cells were maintained in 25 cm² tissue culture flasks. The cells were dislodged by gentle pipetting and about 1 X 10^6 cells were used as a seed inoculum, in a total volume of 5 ml CM. Only cells with >95% viability, as determined by staining with 10% trypan blue, were used for experiments.

For suspension cultures, Sf9 cells were grown in 100 ml spinfer flasks with constant stirring. The flask were treated with 0.2 N NaOH, 0.2 N HCl and
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distilled water before being sterilized 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 and used for
experiments or viral infection, as described below.

1.4 Virus infection and amplification

For viral infection of monolayer Sf9 cells, 3 X 10^6 or 9 X 10^6 cells were
seeded in CM for 45 minutes in a 25 cm² or 75 cm² tissue culture flask,
respectively. After seeding, the CM was removed and viral inoculum added to
achieve an m.o.i. of 0.1 pfu/cell. Infection was allowed to proceed for an hour,
with gentle intermittent rocking to allow for uniform cell infection. After an
hour, the viral inoculum was removed and 5 ml or 12 ml CM added to the 25
cm² or 75 cm² flask, respectively. The flasks were incubated for 5-7 days at
27°C until all the cells were infected and almost lysed. The cell suspension was
then centrifuged to pellet the cells and the supernatant containing the virus
was used as a virus stock. The viral titre was determined by carrying out a
plaque assay as described (section 1.5).

Virus infection of suspension cultures was carried out essentially as
described above, with a few modifications as follows: The cells were harvested
by centrifugation once they attained a mass of ~1 X 10^6 cells/ml. Viral
inoculum was then added to the cell pellet to achieve an m.o.i. of 0.1 and
swirled for an hour for infection to occur, after which the cell pellet was added
back to the spinner flask along with fresh CM. Virus harvesting was
subsequently carried out as for monolayer cell cultures.

For nuclear extract preparation from virus-infected cells, the same
procedure was followed except that infection was carried out at an m.o.i. of 10,
and the cells were harvested 36-40 h.p.i. and used to prepare nuclear extract
(described in section 2.1).

1.5 Plaque assay

The titre of the amplified virus was determined by carrying out a plaque
assay (Summers and Smith, 1987). Briefly, ~1/5 X 10^6 Sf9 cells were seeded in
a 35 mm tissue culture dish for 45-60 min. Serial dilutions of the virus stock
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were made in CM, from $10^{-1}$ to $10^{-8}$, in duplicate. 100 ml of each viral dilution was added dropwise to each dish and infection allowed to proceed for an hour with gentle intermittent rocking, after which the viral inoculum was removed, and 2 ml of LGT agarose added (a 1:1 mixture of 3% LGT in distilled water and CM) to each dish at ~38°C. After the LGT agarose polymerised, it was overlaid with 1 ml CM and the dishes incubated at 27°C. 3-5 days later the liquid media was removed and 1 ml of neutral red staining solution was added to the agarose overlay [1.2 ml neutral red + 20 ml plaque assay buffer (8.2 g NaCl, 2 g KCl, 1.14 g Na₂HPO₄, 0.2 g KH₂PO₄ in a total volume of 1 lit, pH adjusted to 7.3)]. The plates were incubated for 1 h after which the excess stain was drained off and the dishes incubated again for 12-24 h for the plaques to become clearly visible. The plaques were seen as fuzzy, refractile, occ⁺, negatively stained patches in a background of cells stained red. Recombinant viruses without the polh gene showed occ⁻ plaques. The viral titre (pfu/ml) was calculated according to the following formula:

\[(1 / \text{dilution}) \times (\text{no. of plaques}) \times (1 / \text{ml of inoculum per dish}).\]

1.6 Making recombinant viruses

Three recombinant viruses were constructed by first cloning the Ppolh-driven luciferase gene with intact upstream sequences (pBacMAIuc), an 800 bp upstream deletion (pBacΔIuc), or a deletion substituted by the AcSp sequence (pBacAcSpIuc) into the pBacPAK8 baculovirus transfer vector (CLONTECH Laboratories Inc., USA) (Fig. 4.5 A, Chapter IV). Each plasmid was then transfected into Sf9 cells along with BacPAK6 viral DNA (Bsu361 digest) and recombinant viruses were constructed as described earlier (Chatterji et al., 1996). The procedure is briefly described below:

i. 2 X $10^6$ Sf9 cells were seeded in a 35 mm dish and washed twice with 2 ml of ICM. 1.5 ml of ICM was then added and the dish kept aside.

ii. 500 ng of sterile plasmid, 5 µl of BacPAK6 viral DNA (Bsu361 digest), and 4 µl of Bacfectin were added in a polystyrene tube and the volume made up to 100 µl with sterile distilled water. The components were mixed by
gentle tapping. The Bacfectin-DNA mixture was added slowly to the ICM in the 35 mm dish.

iii. The dish was incubated at 27°C for 5 h after which the transfection mix was aspirated and the cells gently overlaid with 1.5 ml CM.

iv. The dish was then incubated for 72 h before removing the supernatant (containing recombinant virus particles) and using it in a plaque assay.

v. Occ- negative viral plaques were selected, suspended in 200 µl of sterile CM each, vortexed vigorously, and allowed to stand overnight at 4°C for the viral particles to seep into the medium.

vi. The next day, 1 X 10⁵ Sf9 cells were seeded per well in a 96-well tissue culture plate and infected with 45 µl of the viral CM. After an hour the inoculum was removed and 150 µl of fresh CM added.

vii. The plate was incubated at 27°C for 60-65 h after which the supernatant was saved as a source of recombinant virus, and the cells used in dot-blot analysis (described in section 4.3) to determine whether they contained recombinant virus particles. The appropriate heterologous gene, present in the recombinant virus, was used as a radiolabeled probe.

2. DNA-Protein Interactions

2.1 Insect cell nuclear extract preparation

Typically, at least 5 X 10⁷ AcNPV-infected or uninfected Sf9 cells were used for nuclear extract preparation. The following protocol (Hasnain et al., 1996) was followed for 5 X 10⁷ (~50 ml) cells:

i. Cells were pelleted by centrifugation at 4°C for 10 min at 2000 rpm in a SIGMA 2K15 microcentrifuge (rotor 12151).

ii. The supernatant was discarded and the pellet washed twice with 10 ml chilled PBS (8 g NaCl, 0.2 g KCl, 0.2 g KH₂PO₄, 1.15 g Na₂HPO₄ in 1 lit, pH adjusted to 7.4).

iii. The cell pellet was resuspended in 1 ml ice-cold lysis buffer (0.03M Tris-Cl pH 7.5, 0.03M magnesium acetate, 1% NP40), transferred to a
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microcentrifuge tube and incubated on ice for 5 min during which the tube was vortexed thrice for 10 s each.

iv. The nuclei were harvested by centrifugation in a microcentrifuge at 1600 rpm for 3 min at 4°C.

v. The nuclear pellet was suspended in 100-150 µl of ice-cold protein extraction buffer (420 mM NaCl, 10 mM Hepes-KOH pH 7.4, 10 mM MgCl₂, 1 mM EDTA pH 8, 1 mM EGTA pH 8, 1 mM each DTT and PMSF, 25% glycerol) and incubated on ice for 30 min, vortexing every 2-3 min for 10 s.

vi. The microfuge tube was centrifuged for 5 min at 4°C at 11000 rpm in a microcentrifuge and the supernatant, containing DNA-binding proteins, was collected. This was again centrifuged for 30 min under the same conditions, to precipitate histones.

vii. The supernatant was collected, divided into aliquots of 30 µl, and stored frozen at -80°C till required.

viii. When required, the extract was dialyzed against 50 volumes of dialysis buffer (20 mM Hepes-KOH pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA pH 8, 0.5 mM each DTT and PMSF) for 5 hours at 4°C before being aliquoted and frozen.

Protein yields typically ranged from 3-10 mg/ml. Protein estimation was carried out using the BCA kit (Pierce, USA) according to the manufacturer's instructions, with BSA as the standard. HeLa cell nuclear extracts were a kind gift from Dr. Narendra K. Tuteja, ICGEB, New Delhi, India.

2.2 Radiolabelling oligonucleotides

Synthetic oligonucleotides (100 ng per reaction) were end-labelled using bacteriophage T4 polynucleotide kinase (5-10 U per reaction) in a 20 µl reaction volume in the presence of 30 µCi γ[³²P]ATP and a reaction buffer containing 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. The reaction was allowed to proceed for 1 h at 37°C after which the enzyme was inactivated by heating at 55-65°C for 5-10 min. The
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The probe was then purified over a Sephadex G50 column by gravity purification as described below:

A 1 ml syringe was packed with a slurry of Sephadex G50 equilibrated with TE buffer (10mM Tris-Cl, 1mM EDTA, pH 8.0), and washed with 1 ml of TE. The labelling reaction was diluted to 100 μl with TE and loaded onto the column. Fractions of two drops each were collected (~80 μl) in microcentrifuge tubes and scanned using a hand-held Geiger counter (Morgan, series 900, UK). Two distinct peaks of activity were obtained - the first representing the labelled probe and the second the unincorporated radiolabel. The first peak was pooled and the specific activity was checked by Cerenkov or scintillation counting. 1 ng of the probe, corresponding to at least 10⁴ cpm, was used for EMSAs.

The different oligonucleotides used for EMSAs, UV crosslinking and cloning into plasmid vectors are listed in the following table. Mutated bases in the "mutant" oligonucleotides are in boldface.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Sequence (coding strand: 5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>AcSp</td>
<td>TAATGGGGTGTTATGTTACCGCTGCGCATAGTC</td>
</tr>
<tr>
<td>2.</td>
<td>cSp</td>
<td>TGCAGGCTCGCCCCGCCCCGATCGAATAG</td>
</tr>
<tr>
<td>3.</td>
<td>Acm1</td>
<td>TAATGGGGTGTTATGTAATAATGCGCATAGTC</td>
</tr>
<tr>
<td>4.</td>
<td>Acm2</td>
<td>TAATGTTTGTATGTTACCGCTGCGCATAGTC</td>
</tr>
<tr>
<td>5.</td>
<td>Acm3</td>
<td>TAATGGGGTGTCGCTGCCCGCATAGTC</td>
</tr>
<tr>
<td>6.</td>
<td>Acm4</td>
<td>TAATGGGGTGTTATAGTAAXGCCTGCTGCCATAGTC</td>
</tr>
<tr>
<td>7.</td>
<td>Acm5</td>
<td>TAATGGGGTGTTATAGTAATAGTACAGCTGA</td>
</tr>
<tr>
<td>8.</td>
<td>Acm6</td>
<td>TAATTTTTGTATGTTACAGCTGCAATGCGGCATAGTC</td>
</tr>
<tr>
<td>9.</td>
<td>LAcSp</td>
<td>TAATGCGGTTGATATA</td>
</tr>
<tr>
<td>10.</td>
<td>RAcSp</td>
<td>TAGTACCGCTGCGC</td>
</tr>
<tr>
<td>11.</td>
<td>SScSp</td>
<td>ATCGGGGCGGGTTC</td>
</tr>
<tr>
<td>12.</td>
<td>2AcSp</td>
<td>GGTATTTCTACAATGCGCGGGTTTGTGCAAAA</td>
</tr>
<tr>
<td>13.</td>
<td>TFIID</td>
<td>GCAGAGCATATAAGGGTCTAGTTAGGA</td>
</tr>
<tr>
<td>14.</td>
<td>B domain</td>
<td>CTCGCAATAATAATAGTATTTTACTGGTTCTCG</td>
</tr>
</tbody>
</table>
2.3 Electrophoretic Mobility Shift Assay (EMSA)

2.3.1 Sp EMSAs:

Oligonucleotide probes were end-labeled using T4 polynucleotide kinase and $\gamma^{32}$PATP. The binding reaction was carried out entirely at ambient temperature as follows: ~5 µg of nuclear extract (HeLa cell or insect cell) was preincubated for 5-10 minutes with 1 µg of poly dI.dC and binding buffer (10 mM Tris.HCl pH 7.5, 0.7 mM Hepes-KOH pH 7.7, 30 mM KCl, 1 mM EDTA, 50 mM EGTA, 0.8 mM MgCl$_2$, 7 mM DTT, 1 mg/ml BSA, 0.05% NP-40, 10% glycerol). 1 ng of the $\gamma^{32}$P labeled probe was then added and the binding reaction allowed to continue for 20 minutes. For competition experiments, a 400 fold molar excess of the unlabeled competitor was added to the reaction along with the labeled probe. The reaction was then loaded onto a native 8% polyacrylamide gel (75:1 acrylamide:bisacrylamide) and electrophoresed in 0.5X TBE buffer at 200 V for 2.5 h at 4°C after which the gel was covered with plastic wrap, dried at 80°C for 1.5 hours, and subjected to autoradiography at -70°C.

For "supershift" EMSAs α-Sp1, α-Sp3, and α-Sp4 antibodies (a kind gift from Dr. Guntram Suske, Germany), were first incubated with crude nuclear extract for 2 hours at 4°C prior to the addition of the probe. Subsequent steps were carried out as described for Sp EMSAs.

In EMSAs using the pure Sp1 protein, 1 footprinting unit (fpu) of Sp1 (~25 ng protein) was used in the binding reaction instead of Sf9 nuclear extract, without the addition of poly [d(I-C)].

2.3.2 TFIID EMSAs:

In the binding reaction for the TFIID EMSAs, 5 µg of HeLa cell or Sf9 nuclear extract was incubated with 1 ng of radiolabeled TFIID consensus oligonucleotide in the presence of binding buffer (10% glycerol, 16 mM Tris.HCl pH 7.9, 64 mM KCl, 5 mM MgCl$_2$, 8 mM DTT) for 15 minutes at ambient temperature. The DNA-protein complexes were resolved at 250 V at 4°C for 90
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minutes in a 6% (29:1 acrylamide/bisacrylamide) non-denaturing polyacrylamide gel containing 0.05% Nonidet P-40 which was pre-run for 20 mins. The running buffer was 0.5X TBE containing 5 mM MgCl₂ and 0.05% Nonidet P-40. For competition analysis, a 50-fold molar excess of the appropriate cold competitor DNA was added along with the radiolabeled probe in the reaction. The gel was dried and subjected to autoradiography as described above.

2.4 UV crosslinking

The binding reaction was carried out as described for the EMSAs but after incubation with the labeled probes the tubes were exposed to short-wave UV light for half an hour using a hand-held UV monitor (model UVGL-58, UVP, inc. San Gabriel, USA). An equal volume of 2X 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 the reaction, and the DNA-protein complexes were resolved at 100 V in a denaturing polyacrylamide gel (SDS-PAGE) in a Tris-glycine buffer [25 mM Tris, 192 mM glycine (pH 8.3), 0.1% SDS] (Laemmli, 1970). The gel was then dried under vacuum and subjected to autoradiography as for EMSAs.

2.5 DNase I footprinting

A 140 bp PCR product was generated using forward and reverse primers termed FPL and FPR, corresponding to the coding and non-coding strands of AcNPV respectively, containing the AcSp motif approximately in the center of the amplicon. The primer sequences were: FPL-TATGTATCTATCGTATAGAG and FPR-ACACACTCCGAAGAACTACC. 5 ng of pKN603luc was used as the template for PCR. 200 ng of each primer was radiolabeled with T4 polynucleotide kinase as described above (section 2.2) and used separately in a PCR reaction along with an equal amount of unlabeled opposite primer to generate a radiolabeled coding or non-coding strand. The PCR cycling conditions were as follows:

i. 94°C : 3 min
ii. Denaturing 94°C : 30 s
iii. Annealing 50°C : 30 s
iv. Extension 72°C : 1 min

Steps ii to iv. were repeated for 30 cycles, followed by a final extension at 72°C for 7 min.

The PCR products were gel purified and concentrated using butanol to a final volume of about 100 µl. 2 µl was used to measure Cerenkov counts in a scintillation counter. About 25000 cpm was used per reaction. The DNase I reaction was carried out at ambient temperature as follows: the binding reaction was carried out using crude nuclear extract or 4 fpu (~100 ng) of pure recombinant human Sp1 (rhSp1), as per the Sp1 EMSA conditions in a volume of 40 µl followed by the addition of 40 µl of a CaCl₂:MgCl₂ mix (5 mM:10 mM). 30 seconds later, 1.3 µl of 1:3 (or appropriately) diluted DNaseI was added and incubated for 2 minutes, followed by the addition of 90 µl of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS). The DNA was extracted twice with an equal volume of 1:1 phenol:chloroform, glycogen added to a final concentration of 20 mg/ml, precipitated with ethanol at -70°C for 3 hours, washed twice with 70% ethanol and resuspended in 5 µl sequencing loading buffer containing 90% formamide. Control DNase I reactions were carried out under identical conditions except that no protein was added. A+G ladders were generated for both strands using formic acid and piperidine according to the method of Maxam and Gilbert (1977). Equal counts of all reactions were loaded onto an 8% denaturing acrylamide gel containing 1X TBE and 8M urea and resolved by gel electrophoresis at 75 watts for 2 hours, after which the gel was fixed for 10 minutes in 10% acetic acid and 20% methanol, covered with plastic film, dried under vacuum and subjected to autoradiography at -70°C.

3. Molecular Cloning

All DNA manipulations were carried out essentially as described by Sambrook et al (1989).

pKNLuc (with about a 4 kb sequence upstream to Ppolh) was constructed by cloning the 1.9 kb BamHI fragment of the luc gene (deWet et al., 1987).
obtained from the plasmid pAcluc (Hasnain and Nakhai, 1990), into the BamHI site of the transfer vector pVL1393 (Luckow and Summers, 1989) (Fig. 3.1, Chapter III). A 2.77 kb SalI-HindIII fragment (which contains ~766 bp upstream to Ppolh) obtained from pKNluc was ligated at the SalI-HindIII site of pUC18 to obtain the construct pKN603luc (Fig. 3.2, Chapter III).

For the construction of pAJpolluc harboring the polyhedrin promoter, the 92-bp EcoRV-BamHI promoter fragment was obtained from the transfer vector pVL1393 and cloned at the HincII-BamHI site of pUC18. The 1898 bp BamHI fragment of the luc gene was then ligated at the BamHI site, placing it downstream of Ppolh (Fig. 3.3, Chapter III).

pAcSp.pol.luc and pcSp.pol.luc vectors were constructed by cloning the AcSp oligonucleotide and the consensus Sp1 oligonucleotide (cSp) respectively, at the PstI-HindIII site in pAJpolluc (Fig. 4.1 A, Chapter IV). All vectors carrying different AcSp-mutant oligonucleotides upstream of Ppolh were also constructed using the same strategy. The constructs were confirmed by dideoxy sequencing (Sanger et al., 1977) or automated sequencing on an ABI 377 sequencer (PE Biosystems, USA).

pAR1 and pAR2 vectors used for the in vivo mopping experiments were constructed by cloning the AcSp and cSp oligonucleotides respectively at the PstI-HindIII site in pUC19 (Fig. 4.2 A, Chapter IV).

The three plasmids used for constructing recombinant viruses with varying sizes of Ppolh upstream regions were made as follows: pBacMAluc (Fig. 4.5 B, Chapter IV) was constructed by cloning the luc gene at the BamHI site of the pBacPAK8 transfer vector (Fig. 4.5 A, Chapter IV). pAcSp.pol.luc and pAJpolluc were digested with PvuII and SacI to release the promoter-luciferase cassette with and without AcSp oligonucleotide respectively, and end-filled using the Klenow fragment of E. coli DNA polymerase I. The AcSp-containing ~2.16 kb fragment from pAcSp.pol.luc was cloned into the MluI-XhoI sites of pBacPAK8 (after end-filling the digested vector fragment first), while the other ~2.13 kb PvuII-SacI fragment from pAJpolluc was cloned into the MluI-Smal site of pBacPAK8. The clones so obtained were called pBacΔluc (Fig. 4.5 C, Chapter IV) and pBacAcSpluc (Fig. 4.5 D, Chapter IV) respectively. Thus, pBacMAluc contains 4 kb upstream of Ppolh, pBacΔluc contains a deletion of
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the ~800 bp promoter-proximal upstream sequence, and pBacAcSpluc contains the 32 bp AcSp sequence replacing the 800 bp deletion. The respective viruses are termed vMAluc, vAluc and vAcSpluc respectively (Fig. 4.5 E, Chapter IV).

The plasmids containing a further ~300 bp fragment upstream of the 766 bp Ppolh upstream sequence in pKN603luc were constructed by ligating the 300 bp SalI fragment from pKNluc into the SalI site of pKN603luc, placing the fragment upstream of the 766 bp region. Various clones were characterized according to the number and orientation of the inserted SalI fragment (Fig. 4.8 A, Chapter IV).

pARhspluc and pARAcSphspluc are plasmids with the Drosophila heat shock promoter (Phsp70) driving expression of the luciferase gene without and with the AcSp sequence cloned upstream of the promoter respectively (Fig. 4.7 B, Chapter IV). pARhspluc was constructed by first cloning the ~480 bp Xbal-EcoRI Phsp70 fragment from pNNI (Sah et al., 1999) into the NheI-EcoRI sites of pLITMUS38 vector (New England Biolabs, USA) to generate pARhsp. The BamHI luc fragment was then ligated into the BamHI site of pARhsp, generating pARhspluc. pARAcSphspluc was constructed by synthesizing the AcSp oligonucleotide with MluI overhangs, and ligating it into the MluI site of pARhspluc, placing it upstream of Phsp70.

4. Transient Expression Assays

4.1 Transfection of plasmid DNA into Sj9 cells and luciferase assay

The expression of luciferase in Sj9 cells transfected with the reporter plasmids was carried out as described (Habib et al., 1996). Briefly, 2x10^6 cells were seeded/well of a 6-well tissue culture plate, washed thrice with serum-free TNMFH, and left for 90 min at 27°C. Reporter plasmid DNA (20 μg DNA/2x10^6 cells) dissolved in 50 μl water was diluted to 500 μl with serum-free medium. This was filtered into 500 μl serum-free medium containing 15 μl (1 μg/μl) Lipofectin reagent and the mixture was added to the culture wells. The Lipofectin-containing medium was removed after an eight hour incubation at 27°C. Cells were washed thrice with complete medium and infected with AcNPV at a multiplicity of infection of 10 pfu/cell for 1 hour. Complete medium was
added and the cells were incubated at 27°C. At 60 h.p.i. 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 μM luciferin, 14 mM MgCl₂ and 14 mM glycine (pH 7.6). Light emission was monitored with a manual luminometer (model 1250, Bio-Orbit Oy, Turku, Finland) over an integration period of 10 sec. All the transfections were repeated, in duplicate, at least three times. To ascertain that equal amounts of plasmid DNA from the different constructs had entered the insect cells, equal amounts of the reaction mixture after the luciferase assay were dot-blotted on a nylon membrane. This was followed by probing with the *luc* cDNA and densitometric scanning for all transfections, in order to rule out artefacts caused by unequal amounts of transfected DNA entering the cells.

*In vivo* mopping (Habib and Hasnain, 1996) and sensitivity to α-amanitin was monitored on a Lumicount Microplate Luminometer (Packard Instrument Company, USA) according to the manufacturer's instructions. For the *in vivo* mopping experiments, 2 μg of reporter plasmid was used (pAcSp.pol.luc or pCSp.pol.luc) with or without 18 μg of specific competitor (pAR1 or pAR2) or non-specific competitor (pUC19). For detection of sensitivity to α-amanitin, 2 μg of pAcSp.pol.luc or pCSp.pol.luc was used with and without 1 μg/ml of α-amanitin, added 36 h.p.i.

### 4.2 Dot blot analysis

10 μl of cell suspension, in replicates, was kept aside for dot blot analysis, while the remaining cells were used for the luciferase assay. 200 μl of 0.5 N NaOH and 20 μl of 10 M ammonium acetate were added to the cells and mixed thoroughly. The lysed cells were then vacuum blotted onto a nitrocellulose membrane, followed by baking at 80°C for 2 h, hybridized with the radiolabeled *luc* gene and subjected to autoradiography as described (Sambrook et al., 1989). The hybridization signals were then compared by densitometric scanning to ascertain that equal amounts of plasmid DNA were transfected. Various dilutions of the cells were also blotted as and when necessary, in order to arrive at a more accurate comparison.