MATERIALS AND METHODS

1. Reporter gene expression and study of virus replication and transcription
2. Analyses of DNA-Protein interactions at the polyhedrin promoter
MATERIALS

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

AMERSHAM, United Kingdom: $\gamma^{32}\text{P-ATP}$, $\alpha^{32}\text{P-dCTP}$, $^{125}\text{I}$, multiprime DNA labeling kit

BOEHRINGER MANNHEIM, Germany: dATP, bacteriophage T4 Polynucleotide kinase, $\text{Bam HI}$, $\text{Bgl II}$, foetal calf serum, Klenow fragment of $\text{E. coli DNA polymerase I}$, luciferase, luciferin, poly[d(l-C)], Proteinase K, RNase

DIFCO, USA: lactalbumin hydrolysate, yeastolate

GIBCO BRL, USA: Grace’s insect cell culture medium

NESTLE, India: non fat dry milk

PHARMACIA, Sweden: Sephadex G-50

REAGENT BANK, NII: Anti-βhCG antibodies

SIGMA, USA: acrylamide, actinomycin D, amphotericin B, bisacrylamide, BSA, bromophenol blue, citric acid, DEPC, DTT, EDTA, EGTA, Ficoll, Folin & Ciocalteu’s phenol reagent, formaldehyde, formamide, gentamycin, Hepes, KOH, β-mercaptoethanol, MOPS, NaHCO$_3$, Nonidet P-40, PMSF, PVP, SDS, sonicated salmon sperm DNA, spermidine, trypan blue

QUALIGENS, India: ammonium acetate, CH$_3$COOH, CH$_3$COONa, C$_4$H$_4$KNaO$_6$, CuSO$_4$, chloroform, ethanol, glycerol, glycine, HCl, isoamyl alcohol, methanol, Mg(CH$_3$COO)$_2$, MgCl$_2$, NaCl, Na$_2$CO$_3$, NaH$_2$PO$_4$,
Na₂HPO₄, NaOH, NH₄OH, phenol, sodium citrate, Tris

**Oligonucleotides:** All the oligonucleotides used in this study were the kind gift of Dr. Altaf Lal, Chief, Malaria Immunology, Division of Parasitic Diseases, Center for Disease Control, Atlanta, USA.
METHODS

1. Reporter gene expression and study of virus replication and transcription

*Insect cell culture*

The cell lines used in this study were derived from *Spodoptera frugiperda* (Sf9 and Sf21; Vaughn et al., 1977), *Bombyx mori* (BmN and Bm5; Maeda, 1989) and *Trichoplusia ni* (TN368; Hink, 1970). Cells were maintained in serum-supplemented TNM-FH medium. TNM-FH medium (Hink, 1970) is Grace's basal insect cell culture medium (Grace, 1962) supplemented with lactalbumin hydrolysate and yeastolate. TNM-FH supplies basic nutrients (salts, sugars, amino acids, organic acids, vitamins, and trace elements) and has a pH of 6.2 buffered with sodium phosphate. To make 1 liter 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 added and the volume adjusted to 1 litre. The medium was sterilized by passing through a sterile 0.22 μm filter (Millipore, USA). Cells were maintained at 27°C in a "complete medium" (prepared by adding 10% foetal calf serum, 50 μg/ml gentamycin, and 5 μg/ml amphotericin B to TNM-FH medium), (O'Reilly et al., 1992). Cells were grown as a monolayer (O'Reilly et al., 1992) in 25 cm² tissue culture flasks (Corning, USA). These cells have a doubling time of about 18-24 h at 27°C and were subcultured twice a week. Cells were subcultured when they were 90% confluent. The cells were washed off the surface of the flask by gentle pipeting. ~2 X 10⁶ cells were seeded in a fresh 25 cm² flask in 5 ml of "complete medium".

Viability of cells was checked by staining with 10% v/v trypan
blue - non-viable cells stain blue. Only those batches of cells with $\geq 98\%$ viability were used for experimental purposes.

**Infection of cells**

Densities of viable cells were determined by four replicate counts in a hemocytometer. Equal number of cells from the five cell lines were infected with the same volume of recombinant virus $vAc\beta hCG\text{-}luc$ (Jha et al., 1992) from the same stock at a multiplicity of infection of 10 plaque forming units/cell. $1 \times 10^5$ cells were seeded in a 96-well plate (Corning, USA) or $2 \times 10^6$ cells were seeded in a 6-well plate (Corning, USA). After 15-20 min (to allow attachment of cells), the medium was removed and an appropriate volume of viral inoculum was added (50 $\mu$l for 96-well plates and 200 $\mu$l for 6-well plates). After 1 h, the viral inoculum was removed and the cells were repeatedly washed with medium to remove any traces of residual virus. The cells were incubated in fresh medium (200 $\mu$l for 96-well plates and 2 ml for 6-well plates) for 72 h unless otherwise mentioned. Mock-infected cells received identical treatment except that no virus was added. Cells were photographed at different time points post infection at 400X magnification in a Nikon Optiphot-2 microscope using a white filter with no staining.

**Detection of luciferase and $\beta hCG$**

$10^5$ cells from each cell line were infected with $vAc\beta hCG\text{-}luc$ and expression of luciferase was quantitated, at 72 h p.i., by the LUC assay (Gould and Subramani, 1988, Jha et al., 1992). The cells were dislodged and to 96 $\mu$l of the cell suspension in a 96-well plate, 4.2 $\mu$l of assay buffer (containing 14 mM MgCl$_2$, 14 mM glycine, 0.6 mM ATP, and 16 $\mu$M luciferin) was added and the plate exposed to KODAK OG-100 film for 30 min.
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Culture supernatant from 2×10^6 infected cells was analyzed, at 72 h p.i., for the presence of βhCG by a radioimmunoassay (Salahuddin and Singh, 1983; Jha et al., 1992) using a monoclonal antibody specific to the β subunit of hCG. An antibody dilution giving 30-40% binding of ^125^I-βhCG (40-60 μCi/μg, 10,000 cpm) in the absence of the competing hormone was incubated with 1.25, 2.5, 5, 10, 20, 40, and 40 ng/ml of standard βhCG and culture supernatant dilutions, 40% normal horse serum, and tracer. Incubation was carried out for 16 h at 4°C and immune complexes were precipitated using 66.6% w/v ammonium acetate and the amount of radioactivity in the complex was quantitated by scintillation counting.

**Radiolabeling luc and βhCG**

luc was obtained as a 1.8 kb Bam HI fragment from the transfer vector pAcluc (Hasnain and Nakhai, 1990) while βhCG was obtained as a 539 bp Bgl II fragment from the vector PBS2 (Nakhai et al., 1992). 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-HCl (pH 7.8), MgCl₂, and β-mercaptoethanol], ^32^P-α-dCTP (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 10 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 [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)], (Sambrook et al., 1989). 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.

**Dot blot analysis of viral DNA**

10⁵ cells infected with vAcbCG-luc were lysed at the indicated time points, the DNA dot blotted onto nitrocellulose (Summers and Smith, 1987), and probed with radiolabeled luc fragment. At the indicated time point, the medium was removed from the wells and the cells lysed by the addition of 200 μl of 0.5 N NaOH. The alkali was neutralized by adding 20 μl of 10 N ammonium acetate. The lysate was transferred onto a nitrocellulose membrane (Amersham, UK) using a vacuum dot blot apparatus (Millipore, USA). The blot 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 0.5% 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:

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X SSC</td>
<td>2 X 10 min at room temperature</td>
</tr>
<tr>
<td>0.2X SSC with 0.5% SDS</td>
<td>2 X 10 min at 55°C</td>
</tr>
<tr>
<td>0.1X SSC with 0.5% SDS</td>
<td>1 X 10 min at 65°C</td>
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</tbody>
</table>
The membrane was then dried, covered with plastic wrap, and subjected to autoradiography.

**Analysis of reporter mRNA**

Total RNA was isolated from $2 \times 10^6$ infected cells at $72 \, \text{h pi}$ as described by Hasnain *et al.* (1994). All operations were carried out at $4^\circ\text{C}$ using pre-chilled solutions prepared in DEPC-treated water. Cells were lysed by vigorous vortexing in a 1:1 mixture of lysis buffer containing 30 mM Tris-HCl (pH 9.0), 0.3 M NaCl, 10 mM magnesium acetate, and 0.075% DTT. The lysate was then extracted with phenol:chloroform (24:1) at 11,000 rpm in a microcentrifuge for 15 min. SDS and EDTA were added to the aqueous phase to final concentrations of 0.1% and 20 mM, respectively. The aqueous phase was once again extracted with phenol:chloroform:isoamyl alcohol (25:24:1) at 11,000 rpm in a microcentrifuge for 15 min. RNA was precipitated with an equal volume of 100% ethanol and 1/10 th volume of 3 M sodium acetate (pH 5.2) at $-70^\circ\text{C}$ for 30 min. The RNA was stored as a dry pellet at $-20^\circ\text{C}$. Before proceeding for electrophoresis, the pellet was dissolved in 10 $\mu\text{l}$ of DEPC-treated water and a 1 $\mu\text{l}$ aliquot was used for spectrophotometric quantitation.

10 $\mu\text{g}$ of the RNA sample was incubated with gel-running buffer (containing formaldehyde and formamide) at $65^\circ\text{C}$ for 15 min, chilled on ice, and separated for in-gel analyses (Ehtesham and Hasnain, 1991) by electrophoresis in a 1.4 % denaturing agarose gel run in 0.2 M MOPS, (Sambrook *et al.*, 1989). After electrophoresis, the gel was vacuum dried and directly hybridized with radiolabeled *luc* fragment without any prehybridization step. Hybridization was carried out at $55^\circ\text{C}$ for 16 h in a buffer containing 6X SSC (pH 7.0), 5X Denhardt's solution, 200 $\mu\text{g/ml}$ sonicated salmon sperm DNA, 0.5% SDS, and DNA probe (50ng, $10^6$ cpm). The gel was washed as follows:
2X SSC - 2 X 5 min at room temperature
0.2X SSC with 0.1% SDS - 2 X 20 min at 55°C

The gel was covered with plastic wrap and subjected to autoradiography. The same gel was stripped at 65°C in 0.2X SSC and 0.1% SDS and reprobed with a constitutive ribosomal DNA probe to reflect the amounts of RNA loaded in each well.

For dot-blot analysis, 5 μg of RNA in 10 μl of DEPC-treated water was mixed with 20 μl of 100% formamide, 7 μl of 37% formaldehyde, and 2 μl of 20X SSC. The mixture was incubated for 15 min at 65°C and chilled on ice. Two volumes of 20X SSC was added to the sample which was transferred onto a nitrocellulose membrane (Amersham, UK) using a vacuum dot blot apparatus (Millipore, USA). The blot was air dried and the RNA 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 0.5% SDS (Sambrook et al., 1989). 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 5 min at room temperature
0.2X SSC with 0.1% SDS - 2 X 20 min at 55°C

The membrane was then dried, covered with plastic wrap, and subjected to autoradiography. The membrane was stripped at 65°C in 0.2X SSC and 0.1% SDS and reprobed with a constitutive ribosomal DNA probe to reflect the amounts of RNA loaded in each slot.

**mRNA stability**

Actinomycin D was added at 10 μg/ml to 2X10⁶ cells at 60 h p.i. (Guzo et al., 1992). The stability of reporter mRNA was analyzed by probing dot blots of total cellular RNAs with radiolabeled luc or βhCG at 0, 3, 6
and 9 h after the addition of inhibitor.

2. Analyses of DNA - Protein interactions at the polyhedrin promoter

*Nuclear protein extraction*

All centrifugations were carried out in a refrigerated microcentrifuge at 4°C; between steps samples were placed on ice. 10^7 cells were harvested from a single 25 cm² tissue culture flask -the cells were dislodged in 1.5 ml of cold phosphate buffered saline (PBS). The suspension was transferred to a microcentrifuge tube and the cells were centrifuged at 1,600 rpm for 3 min. The cells were resuspended in cold lysis buffer [1 % Nonidet P-40, 30 mM Tris-HCl (pH 7.5), 10 mM Mg acetate] and incubated on ice, with three vortexings of 10 s each, for 5 min. The nuclei were harvested by centrifugation at 1,600 rpm for 3 min. The nuclear pellet was resuspended in 100 μl of cold 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] and incubated on ice (Dignam et al., 1983) with intermittent vortexings of 10 s each. After 30 min the samples were centrifuged at 11,000 rpm for 15 min and the supernatant (containing DNA-binding proteins) was stored at -70°C. We typically obtained yields of 100 μg protein from 10 million cells.

*Determination of protein concentration*

Protein concentration was determined by the method of Lowry et al. (1951). The following reagents were made:

Reagent 1: 2% Na₂CO₃ in 0.1 N NaOH
Reagent 2: 2.7% C₄H₄KNaO₆ in reagent 1
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Reagent 3: 1% CuSO₄ in H₂O
Reagent 4: 1 part of reagent 2 mixed with 1 part of reagent 3
Reagent 5: 1 part of reagent 4 mixed with 50 parts of reagent 1
Reagent 6: 1 N Folin & Ciocalteu's phenol reagent (2 N stock diluted with H₂O)

The nuclear extract was diluted in 0.9% NaCl to give a total volume of 100 µl (5 µl protein and 95 µl saline). To this was added 1 ml of reagent 5, vortexed, and incubated at room temperature for 10 min. 100 µl of Reagent 6 was added to each tube in a dropwise manner while vortexing and kept at room temperature in dark for 30 min. A₆₅₀nm was then measured. 100 µl of 0.9 % NaCl and 1 ml of reagent 5 was taken as blank. Appropriate dilutions of a 1mg/ml solution of bovine serum albumin were taken as standard.

Radiolabeling promoter DNA

Synthetic oligonucleotides (100 ng per reaction) were labeled by bacteriophage T4 Polynucleotide kinase (2 units per reaction) using γ-³²P-ATP (30 µCi), in the presence of 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine-HCl, and 0.1 mM EDTA (pH 8.0), in total volume of 20 µl (Sambrook et al., 1989). The reaction was started by the addition of the enzyme and the reaction mixture was incubated at 37°C for 60 min. The enzyme was inactivated by heating at 65°C for 10 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-HCl (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 and loaded onto the column. Immediately after the sample entered the column, the column was replenished with TE buffer.
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so that the column did not run dry. Fractions (2 drops per tube) were collected into microcentrifuge tubes and scanned using a hand-held minimonitor (Morgan, series 900). 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.

**Gel retardations and competition studies**

1 ng of labeled dsDNA or 0.5 ng of labeled ssDNA (~10^4 cpm) was incubated with 2 µg of nuclear extract in a microcentrifuge tube in the presence of 10 mM Hepes-NaOH (pH 7.5), 200 mM NaCl, 0.5 mM DTT and 1 µg poly[d(I-C)] in a final volume of 20 µl (Chodosh, 1988a; Stone, 1991). 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-HCl (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 Whatman chromatography paper, covered with plastic wrap and dried under vacuum. The DNA-protein complex was visualized after autoradiography at -70°C. For competition experiments, an appropriate amount of unlabeled, double-stranded or single-stranded DNA was added to the reaction mixture.
**UV Cross-linking of DNA-protein complexes**

The binding reaction was carried out as described above in a microcentrifuge tube with the cap snipped off and sealed with plastic wrap held in place with a strip of Parafilm. After 15 min the tube was placed on ice and irradiated from 1 cm directly above by inverting a hand-held UV transilluminator of 254 nm wavelength (UVP) for 30 min (Chodosh, 1988b; Hughes, 1991). An equal volume of SDS sample buffer [0.0625 M Tris-HCl (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, 1991). After electrophoresis, the gel was dried and autoradiographed as described above.

**Southwestern analysis**

All operations were carried out at 4°C. Nuclear extract (50 μg of protein) was fractionated on a 15% SDS-polyacrylamide gel (as described above, except that the protein sample was not boiled after addition of SDS sample buffer) and electrophoretically transferred to a nitrocellulose membrane in a buffer containing 25 mM Tris, 190 mM glycine, and 20% methanol for 16 h at 30 mA (Bowen et al., 1980; Miskimins et al., 1985; Philippe, 1994). The protein on the filter was renatured by incubating with blocking buffer [2% nonfat dry milk, 1% BSA, 10 mM Hepes-NaOH (pH 7.5), 0.1 mM EDTA, 200 mM NaCl, 50 mM MgCl₂, 16 μg/ml sonicated salmon sperm DNA] for 2 h on a rotary shaker. The filter was then placed in a hybridization bag with binding buffer (blocking buffer with 0.2% nonfat dry milk) containing labeled probe (≈10⁶ cpm/ml). Hybridization was performed with constant shaking for 16 h. The filter was briefly rinsed in blocking buffer...
without skim milk or bovine serum albumin, dried, covered with plastic wrap, and subjected to autoradiography.

**Proteinase K and RNase treatment**

2 μg of nuclear extract was treated with 2 μg of Proteinase K or 2 μg of RNase at 37°C for 1 h (Young et al., 1991) and assayed for PPBP activity by gel retardation.