5. SUMMARY AND CONCLUSIONS

In this study, a eukaryotic type serine/threonine kinase (pk1) from SpltNPV-I genome was identified and characterized for its functional role. The role of analogous AcNPV pk1 in regulating its polh was also explored. The summary of the experimental results obtained in this study is given below:

Dot blot hybridization of Xhol genomic DNA library of SpltNPV-I in pKS+ indicated the presence of pk1 ORF in the clone of Xhol-W fragment (Fig. 3.1). The putative pk1 ORF from SpltNPV-I encodes for a polypeptide of 272 amino acids (Fig. 3.5) of predicted molecular mass of 32.3 kDa and isoelectric point 8.04. The putative ORF expressed as a glycoprotein having 5% carbohydrate in insect cells and as an apoprotein in E. coli (Fig. 3.9). Both SpltNPV-I pk1 and AcNPV pk1 are nuclear protein.

Nucleotide sequence deduced amino acid sequence of SpltNPV-I pk1 indicated the presence of I-XI consensus subdomains of Hanks type kinase (Hanks et al., 1988) in SpltNPV-I pk1, along with two bromodomains (aa 17-33 and 53-71) (Fig. 3.5). SDS-PAGE (12%) analysis of proteins in the cell free extracts of E. coli (Fig. 3.9A) and Sf9 (Fig. 3.9B) indicated the hyper-expression of a protein corresponding to predicted ~32 kD molecular weight of pk1.

Whole cell and nuclear extracts of SpltNPV-I infected NIV-HA-197 cells upon Immunoblot detection suggested the localization of viral pk1 in the infected nuclei (Fig. 3.11).

Auto- and substrate (myelin basic protein, MBP and Histone H1) phosphorylation assays of pk1 indicated that the protein has both phosphorylating activities (Fig. 3.12A) but both these phosphorylation activities were not exhibited by pk1 mutant K50M (Fig. 3.12B).
Thin layer chromatographic separation and identification of amino acids of auto-
phosphorylated pk1 and phosphorylated HistoneH1 indicated the phosphorylations
of their serine and threonine residues but not of tyrosine residue (Fig. 3.13A).
Autophosphorylation of pk1 at serine and threonine were further confirmed by anti-
phosphoserine and anti-phosphothreonine immunoblots (Fig. 3.13B).

The Lineweaver Burk double reciprocal plot (Fig. 3.15D) confirmed the higher $v_{max}$ of
MBP (0.714 pmole/min/mg) than H1 (0.526 pmole/min/mg), however, $K_{mS}$ for both
the substrates is 3 $\mu$M.

Generation of a transcript of 187 nt with SpltNPV-I polh promoter containing SspI-
BamHI fragment from pCBT4.lacZ DNA (Behera et al., 1997) was observed in vitro transcription assays (Fig. 3.16B).

The dose dependent inhibition of in vitro transcription from polh promoter was
observed with the use of anti-pk1 antibody, and these inhibitions were reversed on
addition of increasing amount of recombinant pk1.

Anti-pk1 antibody immuno-precipitated micrococcal nuclease treated DNA digest of
SpltNPV-I infected cell extract was specifically amplified by primers set
encompassing the polh promoter. However, no PCR amplification was obtained with
primer set encompassing the late gene promoter of ubiquitin.

The precise nucleotide sequence within the 100 bp region of polli promoter region
with which pk1 interacted in vivo, was identified by electrophoretic mobility shift
assays (EMSAs). Recombinant pk1 formed complex with polh promoter DNA
domain, W (35bp, -31 to -65 nt upstream sequences of translation initiation codon) as
shown in the figure 3.19. Mutations of SpltNPV-I pentanucleotide sequence AATTG
or octanucleotide motif TAAGTAAT in ‘W’ domain showed that immediate up and
downstream sequences of polh TAAG motif are important for interaction with pk1
(Fig. 3.20A).
The apparent dissociation constant ($K_d$) for the specific interaction of domain W and pk1 was estimated to be $5.5 \times 10^{-11}$ (Fig. 3.20C).

The 400nt long in vitro generated short pk1 transcript cleaved by E1 into two fragments of sizes 87 nt and 313 nt and while that by E2 into 250 nt and 150 nt fragments as expected in in vitro DNAzyme cleavage reactions. These DNAzymes did not cleave the full length polh mRNA in in vitro reactions (Fig. 3.22).

The pk1 mRNA levels in Sf9 cells were found to be reduced on transfecting the cells with pk1 specific DNAzymes E1 and E2. The decrease in pk1 mRNA level in E1 transfected cells was more than that in E2 transfected cells at all the three used concentrations (1, 3 and 5 nmoles/5x10^5 cells) (Fig. 3.23 A, C; Fig. 3.27). Interestingly polh mRNA levels in pk1 specific DNAzyme (E1 or E2) transfected cells were also reduced (Fig. 3.23 B, D; Fig. 3.27).

Western blot analysis of proteins from pk1 specific DNAzymes E1 and E2 transfected Sf9 cells showed the suppression in expression of both pk1 and polh. The extent of inhibitions of pk1 and polh protein expressions by pk1 specific DNAzymes is comparable with that of their mRNAs.

Recombinant C-terminal GFP tagged AcNPV pk1 expresssd in Sf9 transfected cells was visualized microscopically under UV light (Fig. 3.28).

Eletrophoretic mobility shift assays (EMSAs) indicated that mobility of 12 mer (5'-TAAATAAAGTATT -3') AcNPV polh promoter region retards by both purified pk1 and nuclear extract of AcNPV infected Sf9 cells. Supershift of the pk1 bound promoter DNA was also observed with anti-pk1 antibody when purified pk1 or AcNPV infected nuclear extract was used, as shown in the figure 3.29.

SDS-PAGE analysis of DNA affinity purified RNA polymerase complex showed 32 kD AcNPV pk1 is its major component when probed with anti-pk1 antibody (Figs. 3.30 and 3.31).
Incubation of RNA polymerase complex in kinase buffer with [γ-32P]ATP indicated the phosphorylation of 102 kD protein of the complex and Autophosphorylation of pk1. Phosphorylation of 102 kD protein and autophosphorylation were progressively inhibited on addition of increasing amount of pk1 antibody (Fig. 3.32).

The following conclusions may be drawn from above results:

1. An ORF of 819 bp encoding serine/threonine kinase (pk1) is present in SplitNPV-I viral genome. This ORF encode for a polypeptide of 272 amino acids with a predicted mol. wt. of 32.3 kD.
2. The pk1 gene of SplitNPV-I has maximum homology with SeNPV pk1 gene at both nucleotide and amino acid sequence levels.
3. Nucleotide sequence deduced amino acid sequence of SplitNPV-I pk1 shows the presence of putative Hank’s type kinase subdomains (I-XI), two bromodomains, and a N-linked glycosylation site were found in amino acid sequence
4. The putative pk1 ORF expresses a ~ 31 kD apoprotein in E.coli and ~ 32.3 kD glycoprotein in Sf9 cells.
5. The carbohydrate content (5%) in the recombinant protein expressed in insect cells accounts for the increase in mol. wt. of pk1 protein due to glycosylation, also indicated by PAS staining.
6. The virus encoded pk1 is a nuclear protein.
7. SplitNPV-I pk1 exhibits both the auto- and substrate (myelin basic protein, MBP and Histone H1) phosphorylation activities in in vitro kinase assays.
8. The pk1 auto-phosphorylates at serine and threonine amino acid residues, and also phosphorylate its substrate (HistoneH1) only at serine and threonine.
9. The SspI-BamHI fragment of pCBT4.lacZ DNA (Behera et al., 1997) generates a transcript of 187 nt from its polh promoter in in vitro transcription assays.
10. The in vitro transcription from *polh* promoter is inhibited with the use of anti-pk1 antibody in a dose dependent manner, and these inhibitions reverses on addition of recombinant pk1.

11. The pk1 binds with *polh* promoter region both in vivo and in vitro conditions with a strong binding affinity ($K_d \sim 5.5 \times 10^{-11}$).

12. The very late gene transcription complex from nuclei of AcNPV infected cells shows the presence of pk1 and a 102 kD *lef*-8 like protein in addition to other proteins.

13. The AcNPV pk1 phosphorylate *lef*-8 like 102 kD protein of transcription initiation complex as its native substrate.

14. The AcNPV pk1 is also a nuclear protein and binds to *polh* promoter with strong affinity.

15. The AcNPV pk1 specific DNAzymes E1 and E2 cleave the in vitro synthesized short pk1 transcript at their specific sites in in vitro reactions.

16. Even though E1 and E2 does not cleave *polh* transcript they silence both mRNA and protein expressions of polyhedrin gene.

17. Both SpltNPV-I and AcNPV coded serine/threonine kinases act astranscription factor in baculovirus very late gene transcription by binding to the promoter region and phosphorylating the other transcription factor *lef*-8 like protein.