USE OF LUCIFERASE AS A REPORTER
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4.0 Introduction

In BEVS the detection and identification of recombinant viruses is a very difficult, tedious and time consuming step as only a small fraction (0.1-1%) of the viral progenies carry a replacement of the viral polyhedrin gene with the foreign gene. These recombinant viruses, due to the loss of the polyhedrin gene, exhibit an occlusion negative phenotype which is visibly detected in light microscope, while the wild type viruses exhibit an occlusion positive phenotype. Quite often one encounters a false negative or a false positive, necessitating 3-4 rounds of plaque purification. Alternatives to the plaque purification strategy have been proposed which include immunological detection (Capone, 1989; Chen et al., 1991), use of bacterial β galactosidase gene as a reporter (Pennock et al., 1984; Summers and Smith, 1987) etc. The problem with the former method is the limitation of antibodies available for a given foreign gene. In the β galactosidase selection method the plaques are stained with x-gal and after overnight color development reaction blue plaques are picked up (Pennock et al., 1984; Summers and Smith, 1987). Although this method is more general and does not have the limitations encountered in the antibody screening method, the problem, however, is the diffusion of the color into the neighbouring wild type plaques thereby generating false positives. Keeping in mind the simplicity and sensitivity of the luc assay, we decided to develop a dual expression vector system where the luc gene could be introduced in the viral genome along with a foreign gene of interest, to facilitate the purification of recombinant virus.
Reporter genes, which code for enzymes with readily detectable activities, are commonly used for measurement of gene expression regulation and are excellent tools for molecular genetic analyses. In mammalian cells, the gene coding for chloramphenicol acetyl transferase (cat) is most commonly used. However cat-based assays require a laborious, multistep procedure for quantitation of any gene activity. In the past few years luciferase (luc) which catalyzes the light-producing chemical reaction of bioluminescent organisms, has gained wide popularity as a reporter gene for both animal and plant systems due to the extreme sensitivity with which luc activity is measured (luc is roughly 100 fold more sensitive than cat reporter). Another major advantage of firefly luciferase in genetic experiments is the rapidity of its assay. The light intensity of the luminescence reaction can be measured immediately upon addition of the substrates to the cell extracts containing the luciferase.

Luciferase is a single polypeptide (62 kDa) enzyme which requires no post-translational modifications for enzymatic activity. The only extensively characterized insect luc, isolated from the firefly Photinus pyralis (Photinus luciferin: oxygen 4-oxidoreductase (decarboxylating, ATP-hydrolyzing), EC 1.13.12.7) in the presence of ATP and luciferin, forms an enzyme-bound luciferyl-adenylate complex which undergoes oxidative decarboxylation leading to the production of CO₂, oxyluciferin, AMP and light (deWet et al., 1987). The oxyluciferin emits a photon as the excited state converts to the ground state. The emitted light, which has an absorption maxima at 560 nm, can be measured directly in a luminometer or by exposure to x-ray film.
Luc assay being very sensitive, its gene has been widely used as a reporter in eukaryotic cells.

In this chapter, we first describe the cloning of luc gene in a baculovirus vector and the standardization of its expression in Spodoptera frugiperda insect cells. A cDNA encoding the firefly luciferase was cloned downstream to the polyhedrin gene promoter of Autographa californica nuclear polyhedrosis virus and expressed in Spodoptera frugiperda clone 9 cells. These cells have zero background activity for luc and those infected with recombinant AcNPV can be assayed for luc by a simple, rapid, sensitive, inexpensive and non-isotopic method. Finally, having demonstrated the efficiency of the luc enzyme as a reporter, luc was then cloned in a dual expression vector, along with βhCG gene for use in selecting recombinant virus. Sf9 cells infected with the dual recombinant virus were first checked for luc expression which then, as expected, also expressed βhCG. The recombinant βhCG was identical in all respects to the one expressed alone (3.1.6).

4.1 Results and discussion
4.1.1 Plasmid construction: The source of luc was the plasmid pDO432. This plasmid has the complete coding sequence for the intronless firefly Photinus pyralis luc gene flanked by the cauliflower mosaic virus 35 S gene promoter at the 5' end and the nopaline synthase gene poly-adenylation sequence at the 3' end, to enable it to be expressed in plants (Ow et al., 1986). The complete luc coding sequence was obtained as a 1892-bp fragment after digestion of pDO432·DNA with BamHI. This fragment
was cloned into the *BamHI* site of the baculovirus vector pAc373 (Fig. 25). Recombinant plasmid which had the *luc* in the correct orientation with respect to the polyhedrin promoter was characterized by restriction enzyme digestion and termed as pAcluc (Fig. 26). Orientation of *luc* gene with respect to the polyhedrin gene promoter in plasmid pAcluc, was checked with *EcoRV* digestion which released a 1.4 kbp fragment if the insert was in correct orientation (Fig. 26 lane 1). This was further confirmed after *in-gel* hybridization (Ehtesham and Hasnain, 1991) of the same gel (lane 1 panel B). pAcluc was also digested with *BamHI*, *XbaI+EcoRV* and *PvuII* (lanes 2-4). *BamHI* digestion released a 1892 bp *luc* fragment (lane 2), while double digestion with *XbaI+EcoRV* released three fragments - 10300 bp, 1290 bp and a 228 bp (not shown in the gel). Of these, the 1290 bp fragment containing the *luc* gene, hybridized with the nick translated *luc* probe (panel B, lane 3). *PvuII* does not cut the *luc* insert but has 4 sites in the vector; digestion with *PvuII* (lane 4) generates 4 bands of 7020 bp, 2600 bp, 1500 bp and 700 bp of which only the 7020 bp fragment (containing the insert) is detected by hybridization. In addition to the expected bands in panel B, there is an extra band in all the lanes (on top) which depicts a small percentage of undigested DNA. The method of detection, *in-gel* hybridization, is extremely sensitive and is, therefore, able to pick up a small concentration of DNA not visible on the EtBr stained gel.

These results clearly demonstrate that the *luc* gene is under the viral polyhedrin gene promoter in a correct orientation. Recombinant pAcluc, carrying *luc* in the correct orientation with
Fig. 25. Strategy for the construction of recombinant plasmid, pAcluc. The cDNA coding for luc was cloned into the BamHI site of pAc373, under the polyhedrin gene promoter.

Fig. 26. Characterization of pAcluc by restriction enzyme digestion (panel A) followed by in-gel hybridization analysis (panel B). pAcluc DNA was isolated and digested with restriction enzymes, EcoRV, BamHI, XbaI+EcoRV and PvuII, shown respectively in lanes 1-4; fractionated on 1% agarose gel, dried under vacuum and probed in-gel with a nick translated 1892 bp BamHI fragment of pD0432. HindIII digest of λ DNA was used as size markers and are indicated in (kb) on the left margin.
Transformation of competent E. coli DH5α cells
Isolate and purify the recombinant plasmid
respect to the polyhedrin gene promoter was purified and used for generating recombinant vAcluc.

4.1.2 Transfection and isolation of recombinant virus, vAcluc: Sf9 cells were co-transfected with a mixture of pAcluc and wt AcNPV DNA by the calcium phosphate method as described earlier (2.2.4). Recombinant virus carrying the luc gene was purified by plaque purification.

4.1.2.1 Purification and characterization of DNA and RNA from vAcluc virus infected Sf9 cells: Viral DNA and RNA from infected cells were isolated according to Summers and Smith, (1987) and Bustos et al., (1988), respectively and the purified nucleic acids were characterized by Southern and northern analyses (Sambrook et al., 1989). Southern hybridization (Fig. 27) of total cellular DNA isolated from infected cells at 72 h pi confirmed the integration of luc at the polyhedrin locus of the AcNPV genome in a correct orientation relative to the polyhedrin gene promoter (Fig. 28). vAcluc DNA was digested with EcoRV enzyme and 1.4 kbp luc fragment hybridized with the nick translated 1.8 kbp luc probe, which proved the integration of the luc gene in the viral genome. Based on the above results a restriction map of vAcluc was constructed (Fig. 28). The transcription start point and the polyadenylation site of the viral polyhedrin gene is denoted as tspP and pasP respectively. The initiating methionine of luc is at +80 and the TAA termination codon is at +1730 with respect to BamHI cloning site (arbitrarily designated as +1). In order to determine whether
Fig. 27. Southern blot analysis of total cellular DNA isolated from vAcluc infected cells, wt AcNPV and pD0432, fractionated on 0.7\% agarose gel, transferred to nylon membrane and probed with 1.8 kbp BamHI fragment of pD0432 (Ow et al., 1986). vAcluc4 and vAcluc6 represent two different isolates.

Fig. 28. Partial restriction map of plasmid pAcluc. A 1.8-kb BamHI fragment of pD0432 containing the intron-less \textit{P. pyralis} luc gene (cDNA) was cloned into pAc373 vector to construct the recombinant transfer vector pAcluc. vAcluc6, is one of the three different isolates characterized, and this was used for infection at all times, unless otherwise indicated. The integration of luc at the ployhedrin locus in a correct orientation relative to the polyhedrin gene promoter is shown. The BamHI site in pAc373 was designated as +1. Distance in nt is given in parentheses. The tsp for polyhedrin and luc (tsp\textsuperscript{P}, tsp\textsuperscript{L}) and the pas for polyhedrin and luc (pas\textsuperscript{P}, pas\textsuperscript{L}) are indicated.
**luc** is being transcribed from the polyhedrin promoter, northern blot analysis of total RNA isolated from infected Sf9 cells at 54 h pi, was carried out. Two well defined mRNA species corresponding to \( \sim 1.8 \) kbp and \( \sim 2.7 \) kbp were present in vAc**luc**-infected cells (Fig. 29), but not in AcNPV or mock-infected cells. The presence of two transcripts, suggests initiation and/or termination of transcription from the tsp\(^p\)-pas\(^p\) (polyhedrin) and tsp\(^l\)-pas\(^l\) (**luc** gene) (Fig. 28). The polyhedrin tsp is about 40 nt upstream to the BamHI cloning site in pAc373 (Luckow and Summers, 1988b). The **luc** fragment also has its own transcription initiation and termination signals (deWet et al., 1987) which can easily explain the presence of \( \sim 1.8 \)-kbp transcript. On the other hand, if the polyhedrin terminator is utilized the mRNA will be \( \sim 2.7 \) kbp in length. The levels of the two **luc** transcripts are identical which indicates that initiation of transcription from the polyhedrin promoter proceeds without any bias for homologous or heterologous tsp and perhaps shows no bias for recognition of the termination signals either. It is noteworthy that translation of one or both of the **luc** mRNAs will still result in a single **luc** polypeptide.

4.1.3 Assay for **luc** activity: Luc enzyme requires luciferin, ATP and oxygen as substrates. The absolute requirement for ATP is characteristic for firefly luc (deWet et al., 1985). Our luc assay was designed keeping in mind the fact that enough ATP is available within the intracellular pool, and luciferin can easily permeate the cytoplasmic membrane (deWet et al., 1987). The enzymatically active **luc** produced in Sf9 cells (Fig. 30)
Fig. 29. Northern blot analysis of total RNA in infected cells. RNA was isolated from cells infected with AcNPV, and vAcluc and from mock infected cells, denatured with formamide, fractionated on 1.4% agarose gel, transferred to nylon membrane and probed with a nick translated 1.8-kbp BamHI fragment of pDO432 containing the luc gene. HindIII digest of λ DNA was used as size markers, indicated (in kb) on the left margin. The luc specific transcripts are clearly absent in AcNPV or mock-infected cells.
Fig. 30. Assay for luc activity. Sf9 cells were infected with AcNPV or three different vAcluc isolates, vAcluc3, vAcluc4, and vAcluc6, separately in a 24-well plate at an moi>0.5. At 72 h pi, 200 cells from each infections were transferred to a 96-well plate. Luc reaction was started by the addition of luciferin (16µM, 80µM or 200µM final concentrations) to each well after which a Kodak OG-1 film was placed under the plate. After 30 min of incubation in the dark at RT the film was developed manually using Kodak chemicals. Fogging of film was due to the light emitted by the enzymatically active luc.
apparently requires much less luciferin (16 μM) as opposed to 217 μM in mammalian cells, or 400 μM in plant cells (Ow et al., 1986). AcNPV-infected cells did not exhibit any detectable endogenous luc activity, thus indicating the absence of background luc in Sf9 cells. The commonly used assay (deWet et al., 1985) though very sensitive, measures the peak intensity of the resulting flash of light while simultaneously generating a background noise produced by the luminometer, thereby decreasing the signal-to-noise ratio.

In earlier reports of luc synthesized in mammalian cells it was shown that luc was several orders of magnitude more sensitive than commonly used reporter enzymes, such as β-galactosidase (Rodriguez et al., 1988) or cat (deWet et al., 1987; Wood, 1990). Moreover, its synthesis could be monitored, using a luminometer, from as low as 5x10⁶ cells. We could initially (θ 0.5 moi) detect the presence of luc in 200 cells (Fig. 30). However, the enzyme activity could be easily monitored from a single cell using a higher titre of the virus (θ 10-20 moi). Comparison of the Sf9 synthesized luc with commercially available (Sigma Chemical Co. USA) luc indicated that at about 60 h pi, luc levels reached ∼150 μg/10⁶ cells. It is, therefore, apparent that insect cells are making fairly large amounts of this enzyme. A more detailed analysis of luc synthesized in Sf9 cells at different times pi is shown in Fig. 31. Cell samples were infected in quadruplicate for periods of 18, 24, 48 and 72 h pi with vAcluc, and the relative levels of luc were quantified by scanning of the corresponding fogs (Fig. 31, inset) in the x-ray film with a video-densitometer (Bio-Rad VD 620) and integration.
Fig. 31. Time course of luc synthesis in insect cells infected with vAcLUC. Cells were infected with vAcLUC at an moi>0.5 and aliquots of infected cells were transferred to four well, for each time point, in a 96 well plate. Luc synthesis was monitored at 18, 24, 48 and 72 h pi, after the addition of luciferin (16μM). The actual fogging of the film as a result of luc activity, at different times after infection, is shown as photographs of 4-well clusters above the graph. The relative levels of luc activity were obtained by scanning the actual fogs for each time point using a video-densitometer (BioRad VD620) and integrating the corresponding peak areas.
of peak areas. Approximately 48 h pi, luc activity started peaking with a maximum at 72 h, which is typical for genes under the polyhedrin gene promoter. The fogging of x-ray film by the flash of light, produced as a result of luc activity, is a very simple assay for luc and this makes it an attractive reporter enzyme.

4.1.4 Cellular localization of luc: An attempt was made to localize the luc produced in infected cell by assaying for luc in vAcluc-infected cell supernatant and pellet (Fig. 32). Mock infected cells (Fig. 32 A) or infected cell supernatant (Fig. 32 D) did not register any fogging of x-ray film. Luc synthesized in Sf9 cells is, therefore, not secreted into the culture medium but retained within the cell (Fig. 32 C). Keller et al., (1987) demonstrated the peroxisomal targeting of a recombinant luc synthesized in mammalian cells and subsequently showed that the peroxisomal sorting domain was present within the last 12 aa at the C-terminal end (Gould et al., 1987). Recently, Miyazawa et al., (1989) have identified the targeting signal for another peroxisomal enzyme, rat-liver acyl-coenzyme A oxidase. Interestingly, a highly conserved ser-lys-leu motif which is part of the cell-sorting domain, is also present at the C-terminal end of the firefly luc and few other peroxisomal enzymes.

Firefly luc, unlike the bacterial luciferase (lux), is a single subunit enzyme and can tolerate N-terminal fusions as is evident from the expression of a luc fusion protein in E.coli (deWet et al., 1985). The simple, rapid, sensitive and inexpensive assay described in this chapter makes luc a superior
Fig. 32. Localization of luc produced in Sf9 cells. Aliquots of 100 mock-infected cells (wells A) or vAcluc-infected cells (moi > 0.5) were harvested about 60 h pi and transferred in duplicate to a 96-well plate (wells B). Alternatively, aliquots were centrifuged to pellet cells and the supernatant was transferred to other wells (wells D). The cell pellet was washed twice and suspended in fresh culture media (wells C). Luc was assayed after the addition of luciferin (16 μM) to all the wells. ATP, the other substrate for luc, is already present in the cells (endogenously within the intracellular pool) in all the wells except in wells D (cell supernatant). Exogenous ATP (6 mM) was, therefore, added (wells D) to ensure the availability of this substrate for luc activity.
reporter enzyme for understanding the molecular signals involved in processing, targeting and stability of heterologous proteins in insect cells. These cells are increasingly being used as host for expression of secretory glycoproteins. Although the general conclusion from previous studies that these cells cannot process immature N-linked oligosaccharide to an endo-β-N-acetyl-D-glucosaminidase H-resistant form has been refuted (Jarvis and Summers, 1989), much is yet to be known about the secretory pathway of these cells. Luc synthesized in Sf9 cells does not enter the secretory pathway and it is retained intracellularly. This should provide an excellent model system for understanding the precise nature of the protein-sorting domain and the accompanying molecular machinery involved in the movement of proteins between organelles (Kelly, 1987). This can also aid in the construction of new transfer vectors, utilising a multiple expression strategy, where the expression of a foreign gene could be readily detected after luc expression has first been easily demonstrated. These results are described in the following pages.

4.1.5 pVCβhCG-luc plasmid construction: Simplicity and sensitivity of the luc assay led us to use this reporter gene in a dual expression vector-pVC3 (3.1.4). In this vector, the βhCG gene was previously cloned in the BamHI site (3.1.4), under the control of one of the two copies of the polyhedrin gene promoter. The other polyhedrin promoter cassette was used to direct the transcription of luc in order to develop a vector system where the recombinant viruses could be easily detected and purified.
The cDNA fragment coding for \textit{luc} was obtained after digesting the plasmid \textit{pAcluc} (4.1.1) with \textit{BamHI}, purified after electrophoresis in low melting point agarose, and was ligated to \textit{BglII} cleaved \textit{pVC\beta hCG} DNA (3.1.4). Similar strategy (3.1.1) was used to remove the vector background. Digestion of the ligation mixture with \textit{BglII} prior to transformation linearized the vector DNA which transformed \textit{E.coli} with much reduced efficiency, while the recombinant plasmid will not be linearized and would thus, help to remove the background. The recombinant plasmid which had the \textit{luc} gene in the correct orientation with respect to the polyhedrin gene promoter was termed as \textit{pVC\beta hCG-luc} (Fig. 33).

4.1.5.1 Construction of recombinant baculovirus transfer vector containing \textit{\beta hCG-luc} gene: The 1892-bp cDNA coding for \textit{luc} was cloned into the unique \textit{BglII} site of the recombinant baculovirus transfer vector, \textit{pVC\beta hCG}, such that it was placed under the transcriptional control of viral polyhedrin gene promoter, to construct the dual expression recombinant vector, \textit{pVC\beta hCG-luc}. Based on \textit{\beta hCG}, \textit{luc} and polyhedrin gene sequence available in the literature (Fiddes and Goodman, 1979, 1980; deWet et al., 1985; Summers and Smith, 1987) a partial physical map of \textit{pVC\beta hCG-luc} was constructed. The transcription start point and the polyadenylation site of the viral polyhedrin gene was denoted as \textit{tspP} and \textit{pasP} respectively. One copy of the polyhedrin promoter is \textasciitilde 705 bp upstream from the \textit{BamHI} cloning site and the other is about 100 bp upstream from the \textit{BglII} cloning site, in the other orientation. \textit{\beta hCG} as well as \textit{luc} genes with their own initiation and termination signals will produce \textit{luc} and \textit{\beta hCG} transcripts.
Fig. 33. Strategy for the construction of recombinant plasmid, pVCG\textit{\$\text{\textasciicircum}$hCG$\text{- luc}. The \textit{luc} cDNA was cloned at the unique BglII cloning site in the recombinant vector pVCG\textit{\$\text{\textasciicircum}$hCG$}, downstream to the polyhedrin gene promoter.
Bam HI digestion

Bam HI digested

Sticky end ligation

T₄ DNA ligase

Bgl II digestion to remove the nonrecombinants

Transform into TG-1 cells
which upon translation should result in a single βhCG and luc polypeptides, respectively. The recombinant plasmid was characterized by multiple restriction enzyme digestions (Fig. 34, panel A) and Southern hybridization (panel B). There is one PstI site in βhCG cDNA (at 218 bp, with respect to the BamHI cloning site) and none on the vector or the luc gene. As naturally expected, PstI digestion linearized the plasmid (Fig. 34 A, B lane 1). Orientation of the luc gene with respect to polyhedrin gene promoter in the plasmid pVCβhCG, was checked by EcoRV digestion which released a 1513 bp fragment if the insert was in the correct orientation (Fig. 34 A lane 2). This was further confirmed after Southern blot of the same gel (Fig. 34 B, lane 2). pVCβhCG-luc was also digested with XbaI, HincII and SmaI (lane 3-5). XbaI digestion linearized the plasmid (lane 3). HincII has multiple sites in the luc gene and its digestion, released a large number of fragments (panel A, lane 4) of which the one containing the luc gene was picked up by Southern analysis. Digestion with SmaI, which has a site each in βhCG (487 bp from BamHI cloning site) as well as luc (1889 bp from BglII cloning site) genes and none on the vector, released a 3176 bp fragment (panel A, lane 5), which hybridized with the nick translated luc probe (panel B, lane 5). These results clearly demonstrated that the luc gene was under the transcriptional control of viral polyhedrin gene promoter. Transcription from the two oppositely oriented polyhedrin promoters should now result in authentic βhCG and luc mRNAs (Fig. 35).
Fig. 34. Characterization of pVCβhCG-luc by restriction enzyme digestion (panel A) followed by in-gel hybridization (panel B). pVCβhCG-luc DNA was isolated and digested with restriction enzymes, PstI, EcoRV, XbaI, HincII and SmaI, shown in lanes 1-5, respectively, fractionated on 1% agarose gel, dried under vacuum and probed in-gel with a nick translated 1892 bp BamHI fragment of pAcluc containing the luc gene. HindIII digest of λ DNA and HaeIII digest of φX DNA was used as size markers and are indicated (in kb) on the left margin.

Fig. 35. Physical map of the transfer vector pVCβhCG-luc showing restriction sites in βhCG as well as luc cloned at the polyhedrin locus. The transcription start point and the polyadenylation site of the viral polyhedrin gene is denoted as tspβ and pasβ respectively.
4.1.6 Transfection, isolation, purification and characterization of vAcβhCG-luc virus: The strategy for transfection and isolation of recombinant baculovirus carrying βhCG-luc gene was similar to that of βhCG virus (3.1.5) including the two rounds of dot blot hybridization (Fig. 36). Recombinant vAcβhCG-luc was isolated, plaque purified and confirmed by Southern hybridization of DNA isolated from vAcβhCG-luc infected Sf9 cells using 32P-labeled BglII fragment of βhCG as well as luc as a probe. The recombinant virus was amplified and purified using a 10-50% sucrose cushion and virus titre determined by plaque assay (10^8 PFU/ml).

The logic of constructing vAcβhCG-luc was to exploit the very simple assay for the detection of luc activity, which would then help in isolating the recombinant viruses from the mixture of wt viruses. This, thereby circumvents the tedious procedure of plaque purification. Accordingly, the co-transfection supernatant was serially diluted (10^4 to 10^8) and was used for plaque assay. Fifth day pi, 4 μM luciferin was added on top of the agar overlay and the plate was exposed to OG-1 x-ray film. Fogging of x-ray film was registered for each recombinant plaque which carried the βhCG-luc gene (Fig. 37). Only the isolated plaques which were free of polyhedrin contamination were picked up and used for recombinant virus amplification.

To confirm the integration of βhCG-luc gene in the viral genome, total DNA isolated from vAcβhCG-luc infected cells was digested with restriction enzymes for hybridization analysis. In vAcβhCG-luc genome PstI sites flank the polyhedrin gene locus, besides having unique site within the βhCG gene. A single EcoRV
Fig. 36. Dot blot hybridization of cells infected with the co-transfection supernatant. DNA was isolated from the infected cells on the fifth day pi, blotted on nitrocellulose membrane and probed with nick translated 1892 bp BamHI fragment of pAcluc containing the luc gene. Panels a and b represent $10^{-5}$ and $10^{-6}$ dilutions of the co-transfection supernatants.

Fig. 37. Plaque assay of the co-transfection supernatant. On the fifth day pi 4 \( \mu \text{M} \) luciferin was added to each plate and exposed to OG-1 x-ray film o/n.
site is present in the vector (100 bp upstream to the BglII cloning site) as well as in the luc gene (1418 bp from BglII cloning site). Therefore, digestion of vAcβhCG-luc with these two enzymes will release two fragments of ~5.483 and 0.925 kbp when probed with βhCG fragment and ~6.81 and 1.518 kbp when probed with luc fragment (Fig. 38 A & B). These results confirmed the integration of βhCG as well as the luc gene at the polyhedrin locus of AcNPV genome and in a correct orientation relative to the polyhedrin gene promoters.

4.1.7 Detection and characterization of recombinant proteins: Culture supernatant and the cell pellet of vAcβhCG-luc-infected Sf9 cells were analyzed for the presence of i) luc activity by the film fogging assay, and ii) the beta subunit of hCG by radioimmunoassay (Chakrabarti et al., 1989), using a monoclonal antibody against hCG (Gupta et al., 1985). The biological activity of this gonadotropin was further determined, as previously described (3.1.6).

4.1.7.1 Expression and biological activity of recombinant proteins: vAcβhCG-luc, was plaque purified and used to infect Sf9 cells seeded in a 24-well plate. The vAcβhCG-luc infected Sf9 cell were analyzed for the presence of luc activity by fogging of x-ray film by the photon emitted as a result of an enzymatically active luc. This is a very simple assay for luc. Luc activity was indeed detected in the vAcβhCG-luc infected cell pellet (Fig. 39 A). This was expected since we previously showed (4.1.4) that luc is not secreted but is localized intracellularly. Having
Fig. 38. Hybridization analysis of vAcβhCG-luc DNA. Total DNA was isolated, third day pi from Sf9 cells infected with AcNPV. vAcβhCG and vAcβhCG-luc, digested with PstI+EcoRV, fractionated on 1.0% agarose gel and hybridized in-gel, with a nick translated 539 bp BglII fragment of pBS2 containing the βhCG gene (panel A) or with a 1892 bp BamHI fragment of pAcluc containing the luc gene (panel B). In panel A, vAcβhCG was used as a positive control.
1: vAcβhCG
2: AcNPV
3: vAcβhCG-luc

1: vAcβhCG-luc
2: AcNPV
Fig.39. Time course of (A) luc and (B) βhCG synthesis in insect cells infected with vAcβhCG-luc. Cells were infected with vAcβhCG-luc at an moi>0.5 and βhCG. luc expression was monitored at 24, 36, 48 and 72 h pi, by a subunit specific radioimmunoassay and luc assay (2.2.14; 2.2.13).
Hour

pi

72  48  36  24

[Diagram with symbols for each hour]
shown luc activity, the presence of the other protein –βhCG was checked since the genes for both of these are present in the recombinant virus vAcβhCG-luc. For this at different times pi the medium was assayed for the presence of β subunit of hCG by RIA. Near zero back ground values were observed, once again, in the culture media from uninfected or wt AcNPV infected cells. In contrast, beta subunit of hCG was present in the medium of cells infected with vAcβhCG-luc. The time-course of hCG secreted in the medium and luc synthesized in the cell pellet of vAcβhCG-luc infected cells is shown in Fig. 39 (A & B). The levels of β subunit of hCG increased with time and reached a maximum of 10.023 μg/2x10^6 cells/ml at 72 h pi -- an observation typical for genes under the transcriptional control of the very late polyhedrin gene promoter (Luckow and Summers, 1988a). In case of luc, the activity was very low at 24 h pi and increased with time and reached a maximum at 72 h pi.

4.1.7.2 Synthesis of human chorionic gonadotropin: Sf9 cells were infected with vAcβhCG-luc and the culture medium was incubated with standard αhCG and the extent of αβ dimer formation was estimated by a rat testicular radioreceptor assay and a mouse Leydig cell bioassay. Mock infected Sf9 cells (Fig. 40) or those infected with wild type virus did not exhibit biological activity either alone or in combination with externally added purified αhCG standard, in a mouse Leydig cell assay or receptor binding assay. Although βhCG was synthesized in vAcβhCG-luc infected cells (Fig. 39), this, however, did not exhibit bioactivity, unless annealed in-vitro to purified αhCG. Both assays,
Fig. 40. Biological activity of expressed βhCG. Culture supernatant were incubated with a large molar excess of purified standard αhCG for 16 h at 27°C and the αβ dimer was quantitated using a radioreceptor assay and a Leydig cell bioassay as described.
Receptor Binding assay

Mouse Leydig cell assay
therefore, clearly demonstrate the presence of biologically active βhCG in infected cell supernatant, which could associate with standard αhCG to generate the hormonal activity.

4.1.7.3 Immunoreactivity of Sf9 synthesized βhCG and luc protein: Sf9 cells seeded in 24 well plate were infected with vAcβhCG-luc virus at moi>10. The medium was assayed for the presence of secreted βhCG while the cell pellet was assayed for the presence of luc protein.

βhCG synthesized in Sf9 cells infected with vAcβhCG-luc is immunoreactive (Fig. 41, A) but shows a slight difference in its electrophoretic mobility compared to the authentic βhCG purified from urine of pregnant women. The observation that Sf9-synthesized βhCG and native βhCG are identical in terms of bioactivity, strongly suggests that the hormone made in this system is apparently glycosylated such that there is no impairment of function.

Luc synthesized in Sf9 cells infected with vAcβhCG-luc is also immunoreactive (Fig. 41, B) and shows identical electrophoretic mobility compared to the authentic luc used from commercial sources. The observation that Sf9 synthesized luc and commercially available luc are identical in terms of their electrophoretic mobility, and the fact that Sf9 synthesized luc is capable of its enzymatic activity strongly suggests that the protein made in this system are such that there is no impairment of its function and are identical to their authentic counterparts.

It is very interesting to note that in this dual expression
Fig. 41. Western blot analysis of total protein isolated from infected cells. Total protein isolated from infected cells at 48 and 72 h pi were fractionated on 12.5% PAGE, transferred to nitrocellulose membrane and probed with monoclonal anti-βhCG (A) and polyclonal anti-luciferase (B) antibodies.
vector system, where βhCG and luc were expressed simultaneously, luc was a nonsecretory protein and was destined to stay intracellularly while the other protein, βhCG, was a secretory protein and was transported out of the cell. As is evident, BEVS is capable of correctly processing two different proteins and transport them to their final destinations. Luc is a very interesting reporter for it facilitates the otherwise tedious process of detecting recombinant viruses and greatly expedites plaque purification. This is made possible by scoring plaques using the very sensitive luc assay. A plaque which is positive for expression of luciferase will necessarily carry the second gene of interest which is co-expressed. In the absence of any report about the instability of the dual gene cassette as a function of viral passage, it may be reasonable to expect that this strategy will offer an easy method, to pick up recombinant viruses.