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Firefly luciferase, synthesized to very high levels in caterpillars infected with a recombinant baculovirus, can also be used as an efficient reporter enzyme in vivo

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Trichoplusia ni and *Spodoptera littoralis* larvae were infected with a recombinant AcNPV, having the viral polyhedrin gene replaced with the cDNA encoding firefly luciferase. Both *S. littoralis* and *T. ni* synthesized very high levels of luciferase representing $\geqslant 25\%$ and $\geqslant 15\%$, respectively of the total Coomassie blue stainable protein. Luciferase was apparently not secreted into the hemolymph but was contained within the body tissue. Expression in *S. littoralis* larvae suggests that luciferase can be an excellent reporter enzyme to study virus infection, dissemination and expression in different tissues, host range determination, insect physiology and also to monitor the release of recombinant virus in the environment when used as a biocide.

Luciferase expression; Baculovirus vector; Reporter enzyme; Targeting; Biocide; *Spodoptera littoralis*; *Trichoplusia ni*

1. INTRODUCTION

The use of insect larvae as host for baculovirus mediated expression is a fascinating possibility for very large-scale production of foreign proteins [1-3], because they are easily mass-cultured at very low cost and show extraordinarily rapid and efficient growth, with about a 10^4-fold increase in body weight from the time of hatching to sixth instar. The efficiency of protein synthesis is extremely high during the larval stage, as several milligrams of a specific protein can be synthesized within a day. Pest insects such as *Spodoptera frugiperda* and *Trichoplusia ni* have been cultured for scientific studies and for mass-scale production of baculovirus pesticides [2]. However, not many reports exist on the use of insect larval system as host for large-scale synthesis of foreign protein. A systematic evaluation of virus infectivity, dose and routes of infection, level of gene expression and purification of product have not been carried out for the known baculovirus-larval systems.

Reporter genes, not present in the host system, are excellent tools for molecular genetic analyses. The gene coding for luciferase, the enzyme that catalyzes light producing chemical reaction of bioluminescent organisms, is one such widely used reporter gene [4,5] whose expression can be measured directly in a luminometer or by exposure to X-ray film or by direct counting in a scintillation counter. In this communication we report the expression of Luc in *Trichoplusia ni* and *Spodoptera littoralis* larvae using vAc*luc*, a recombinant AcNPV containing *luc* under the viral polyhedrin gene promoter. These larvae have zero background Luc activity and after infection with vAc*luc* synthesize very high levels of this enzyme. Luc can also be used as an excellent reporter to study infection of larvae by a baculovirus, dissemination of the virus, and the expression of foreign genes and targeting to specialized organs.

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Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; Luc, luciferase enzyme; *luc*, the gene coding for Luc; p.i., post infection

2. MATERIALS AND METHODS

2.1. *Infection of larvae with vAc*luc

Trichoplusia ni and *Spodoptera littoralis* larval cultures were reared on castor (*Ricinus communis*) leaves and were maintained at $26 \pm 2°C$ with $65 \pm 10\%$ relative humidity. The recombinant baculovirus vAc*luc* was purified from infected *Spodoptera frugiperda* (Sf9) cells as described [6]. Same-age and similar-weight early fifth instar larvae were selected for infection with a high titre (10^6 PFU/*S. littoralis* larvae and 10^5 PFU/*T. ni* larvae) by injecting vAc*luc* directly into the larval hemolymph. Larvae were harvested about 3-4 days p.i. and assayed for Luc activity.

2.2. *Assay and Characterization of expressed protein*

The larvae were weighed and individuals were selected at random and homogenised in 200 μl of phosphate buffered saline (50 mM, pH 7.3) containing 2 mM of phenylmethylsulphonyl fluoride and the extract was assayed for Luc at 25°C in 100 μl volumes of reaction mixture containing 14 mM $MgCl_2$ 14 mM glycine buffer (pH 7.7), 0.6 mM ATP and 16 μM of luciferin, unless stated otherwise, in 96-well plates.

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The plate was placed on top of KODAK OG-100 film and incubated for 30 s at room temperature to register fogging of the X-ray film by the emitted light. For characterization of Luc, aliquots of larval extract [3] were prepared and analysed by polyacrylamide gel electrophoresis. The gel was scanned using a Hoefer Scientific Systems Transmittance/Reflectance Scanning Densitometer model GS 300.

2.3. *Targeting of Luc in infected larvae*

Infected *T. ni* larvae were bled three days after infection by cutting off the prolegs and the haemolymph was withdrawn. Both hemolymph and the body tissue were assayed, separately for Luc activity as described earlier.

3. RESULTS AND DISCUSSION

3.1. *Expression of luciferase in infected larvae*

The physical map of the transfer vector pAc*luc* [6] and the construction of the recombinant virus is shown in Fig. 1. *T. ni* and *S. littoralis* larvae infected with vAc*luc* showed a high level of *luc* expression which could be visually detected or recorded as black fogs after 30 s exposure to X-ray film (Figs 2a and b). Luc enzyme requires luciferin, ATP and oxygen as substrates. The molar amounts of luciferin required by Luc made in different hosts varies from 16 μM in Sf9 cells [6], 217 μM in mammalian cells to 400 μM in plants [7]. However, the in vitro assay, using Luc synthesized in larvae, naturally does not have problems of permeability of luciferin [4] and therefore, even 0.4 μM luciferin was found to be effective (Fig. 3). This, however, does not imply that sub-saturating levels of luciferin are sufficient for an optimal response in view of the fact that the X-ray film assay is non-linear. The absolute requirement for ATP, a characteristic for firefly Luc [4], is also much lower (Fig. 3) in our in vitro assay.

Polyacrylamide gel electrophoresis and densitometry scanning of the stained gel (Fig. 4) further confirmed that Luc synthesized in larvae of both *S. littoralis* and *T. ni* constituted a major band of ~61 kDa, the size expected for Luc. This protein band is not present in mock infected or AcNPV infected *S. littoralis* and *T. ni* larvae (data not shown). However, the expression was about twofold higher in the former than the latter, representing $\geqslant$25% and $\geqslant$15% respectively, of the total Coomassie blue stainable protein. Polyacrylamide gel electrophoretic comparison of vAc*luc* infected larval extract (data not shown) with commercially available Luc (Sigma Chemical Co., USA) revealed that these values roughly correspond to ~0.6 mg and ~1.3

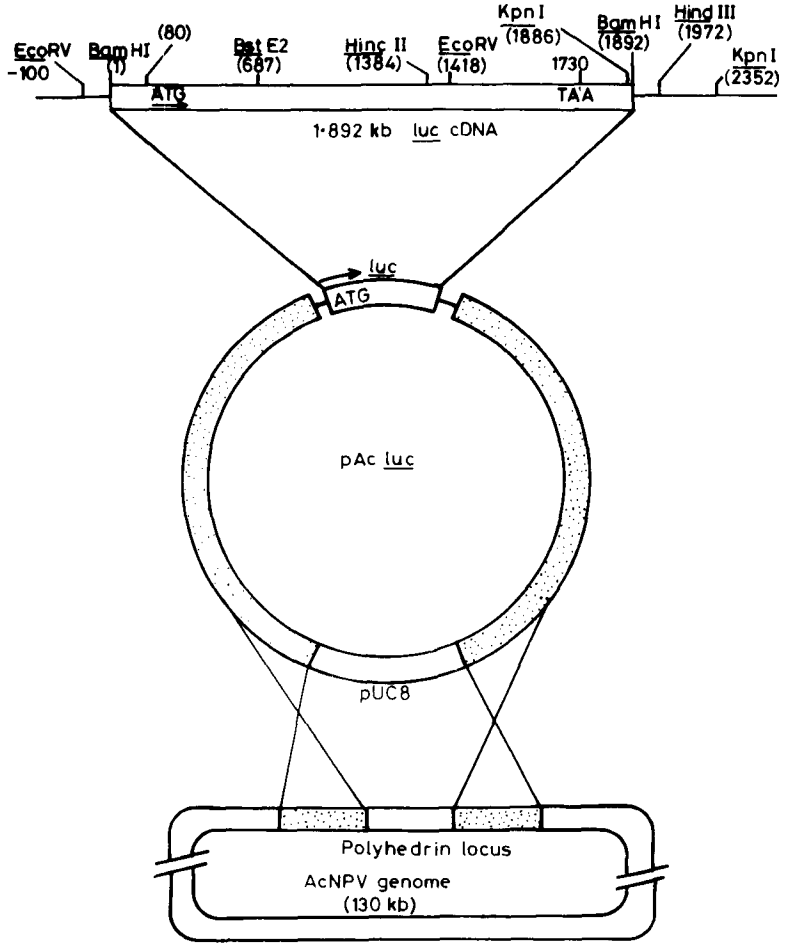

Fig. 1. Physical map of transfer vector pAc*luc* showing restriction sites in *luc* and homologous recombination with AcNPV DNA at the polyhedrin locus.

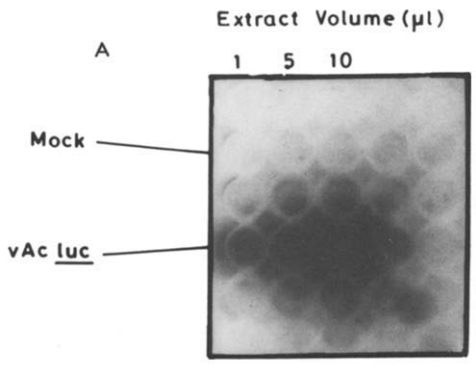

Fig. 2a. Detection of Luc expression in the larval homogenate of *T. ni* infected with vAc*luc*. Fogging of the X-ray film was due to the light emitted as a result of Luc activity. As low as 1 µl of infected larval extract contained enough enzymatically active Luc (lower panel) but there was no activity in mock infected larval extract (upper panel).

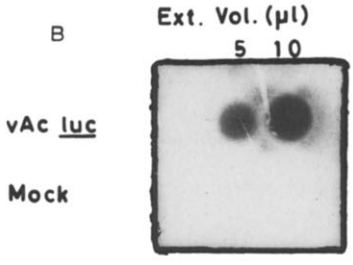

Fig. 2b. Expression of Luc in *S. littoralis*. The Luc assay well was exposed to the X-ray film for just a few seconds to prevent overexposure.

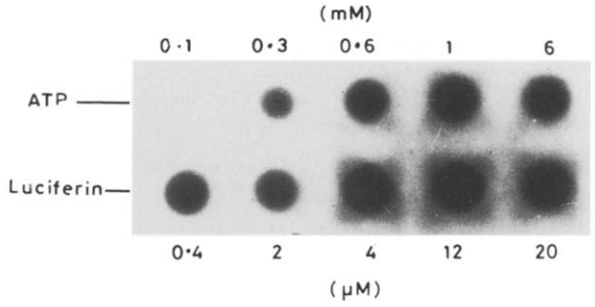

Fig. 3. Assay for Luc as a function of luciferin and ATP concentrations. Luc was assayed as described in section 2. The ATP concentration was varied (upper panel) from 0.1 to 6 mM, keeping all other assay parameters, including luciferin concentration (16 µM) as in section 2. The luciferin concentration was varied (lower panel) from 0.4 to 20 µM, while all other assay parameters including ATP concentration (0.6 mM) remained as in section 2.

mg per infected *T. ni* and *S. littoralis* larvae, respectively. While the enzyme activity seemingly correlates well with the high level of protein, it is difficult to estimate the exact ratio of active vs inactive enzyme in these overproducing systems. It is important to mention the gradual decline of Luc activity as a function of storage time of larval extract at −20°C. Similarly, a distinct loss of enzymatically active Luc was also evident when caterpillars were harvested close to or immediately after death (i.e. 4–5 days p.i.). It is pertinent to mention that new improved baculovirus transfer vectors allow expression of foreign genes to levels close to the viral polyhedrin level [8,9] which is much higher than the presently used pAc373 vector. Luc enzyme has recently found applications [10] in determination of ATP [11], detecting microbes in clinical diagnosis [12], bioluminescence enhanced immunoassays for Western and Southern blotting [13], etc., besides being used as a reporter [4,5,14]. Luc, available commercially, is purified from the lantern organ of firefly which makes it an expensive enzyme. The high levels of enzymatically active Luc synthesized in caterpillars makes the purification of this enzyme from infected larvae a commercially viable proposition.

3.2. *Luc as a reporter to study infection of larvae*

The observation that caterpillars of *S. littoralis* are infected by AcNPV is interesting and is considerably significant because larvae of *S. littoralis* can now be exploited for the expression of genes by infecting them with suitable AcNPV based recombinant virus which is extensively used in the baculovirus expression vector system. Luc is certainly several orders of magnitude more sensitive than other commonly used reporter enzymes like CAT [4] and the assay is more rapid and less expensive. The easy detection of Luc in insect larvae compared to another recently described reporter, juvenile hormone esterase [15], makes this a very attrac-

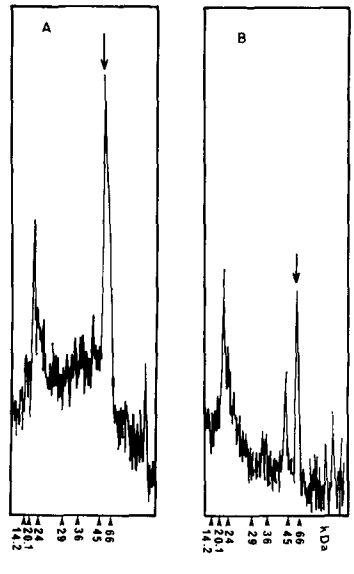

Fig. 4. Densitometry scan of stained polyacrylamide gel. Homogenates of vAc*luc*-infected *S. littoralis* larva (A) and *T. ni* larva (B) were fractionated on 12.5% polyacrylamide gel, stained with Coomassie blue and scanned as described in section 2. The protein peak corresponding to ~61 kDa in size (marked with an arrow), represents Luc which was absent in mock infected or wild type AcNPV virus infected larvae (scan not shown).

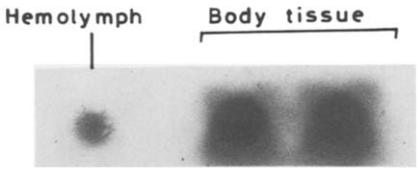

Fig. 5. Assay for Luc in the hemolymph and total body tissue. Hemolymph from all the segments of an infected larva was drawn out and the larva was then homogenised as before. Luc was assayed both with hemolymph and the larval homogenate.

tive reporter enzyme for screening a large number of baculoviruses for their ability to infect a range of lepidopteran insect species. In the absence of a vector system based on the homologous *Spodoptera littoralis* nuclear polyhedrosis virus (SlNPV), AcNPV can be used. This opens the feasibility of converting a notorious plant pest *S. littoralis*, which affects numerous species of plants including many cash crops in the tropics, into living protein factories [16]. Additionally, AcNPV can be even used as a biocide for controlling this pest. With *luc* integrated in the viral genome it will also be possible to follow the stability of these biocides when released in the environment [2,15,17,18].

3.3. *Targeting of expressed protein in infected larvae*

An attempt was made to localize Luc expressed in insect larvae. Luc, a non-secretory enzyme, was expressed and retained in the body tissue of larvae as evident from a high degree of fogging of the X-ray film when an assay was performed on the extract from body tissue and the hemolymph (Fig. 5). There was some enzymatic activity in case of hemolymph also, which could have possibly appeared due to injuries to the body tissue while bleeding the larvae. It is now known that luciferase is specifically targeted to peroxisomes in fireflies, mammalian cells, plants and even yeast [5] where the high oxygen tension ensures efficient availability of substrates for this enzyme. We previously reported the intracellular localization of this enzyme in infected Sf9 cells [6] and most, if not all, of the Luc synthesized in larvae is certainly retained within the body tissue and is not secreted into the hemolymph. Therefore, this could provide a good model system for understanding the precise nature of the protein sorting domain and the accompanying molecular machinery involved in the movement of proteins between eukaryotic organelles [19]. Such findings may also be useful in constructing new baculovirus transfer vectors where the expression of a foreign gene could be targeted to specific organelles, thereby possibly avoiding the problems of stability and storage of over-expressed proteins [2]. Luc can also serve as a very sensitive reporter to study physiology, biochemistry and endocrinology of insects.

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REFERENCES

[1] Maeda, S. (1989) Annu. Rev. Entomol. 34, 351-372.
[2] Miller, L.K. (1989) BioEssays 11, 91-95.
[3] Emery, V.C. and Bishop, D.H.L. (1987) Protein Eng. 1, 359-366.
[4] Gould, S.J. and Subramani, S. (1988) Anal. Biochem. 175, 5-13.
[5] Schauer, A.T. (1988) Trends Biotechnol. 6, 23-27.
[6] Hasnain, S.E. and Nakhai, B. (1990) Gene 91, 135-138.
[7] Ow, D.W., Wood, K.V., DeLuca, M., DeWet, J., Helinski, D.R. and Howel, S.H. (1986) Science 234, 856-859.
[8] Matsuura, Y., Possee, R.D., Overton, H.A. and Bishop, D.H.L. (1987) J. Gen. Virol. 68, 1233-1250.
[9] Luckow, V.A. and Summers, M.D. (1988) Virology 167, 56-71.
[10] DeLuca, M.A. and McElroy, W.D. (eds) Methods Enzymol. (1986) Volume 133.
[11] Lundin, A., Rickardson, A. and Thorpe, A. (1976) Anal. Biochem. 75, 611-620.
[12] Hanna, B.A. (1986) Methods Enzymol. 133, 22-27.
[13] Geiger, R., Hauber, R. and Miske, W. (1989) Mol. Cell. Probes 3, 309-328.
[14] Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A. and Felgner, P.L. (1990) Science 247, 1465-1468.
[15] Hammock, B.D., Bonnig, B.C., Posse, R.D., Hanzlik, J.N. and Maeda, S. (1990) Nature (London) 344, 458-461.
[16] Medin, J.A., Hunt, L., Gathy, K., Evans, R.K. and Coleman, M.S. (1990) Proc. Natl. Acad. Sci. USA 87, 2760-2764.
[17] Maeda, S. (1989) Biochem. Biophys. Res. Commun. 165, 1177-1183.
[18] Bishop, D.H.L. (1989) Pestic. Sci. 27, 173-189.
[19] Kelly, R.B. (1987) Nature 326, 14-15.