Journal of Industrial Microbiology & Biotechnology (1998) 21, 289–291
© 1998 Society for Industrial Microbiology 1367-5435/98/$12.00
http://www.stockton-press.co.uk/jim

Simultaneous synthesis of luciferase and human nerve growth factor in caterpillars infected with a recombinant baculovirus

X-W Zhang, T Sun, X Liu and X-N Huang

Food Engineering Research Center of State Education Commission, Zhongshan University, Guangzhou 51025, People's Republic of China

Human nerve growth factor (hNGF) gene was proliferated with human leucocyte DNA as template by PCR. Then a fusion gene coding hNGF and luciferase (Luc) cDNAs was inserted into transfer vector pSXIVVI$^+$X3/3 with the control of Syn XIV promoter. Luc and hNGF were simultaneously synthesized in *Spodoptera* larvae upon infection with a recombinant baculovirus, TnNPV-Luc-NGF-OCC$^+$. Densitometric scanning of SDS-PAGE revealed that ~18% of the total Coomassie blue-stainable protein of the infected larvae was represented by Luc protein, while the hNGF level was ~8%. Both proteins were similar to their authentic counterparts in terms of immunoreactivity.

Keywords: hNGF; Luc; PCR; baculovirus system; transfer vector; gene expression

Introduction

Nerve growth factor (NGF) is an important protein involved in the development and maintenance of sensory, sympathetic and central neurons [4,5]. It mediates many biological responses including axonal outgrowth, survival of the developing and adult nervous system, and neural regeneration following nerve injury [15]. NGF is probably used to treat Alzheimer disease, pathological changes of peripheral nerve system and bone marrow injury [6]. NGF is rich in the submaxillary gland of mature male mouse and purified easily, while there is little human NGF in the body tissue and purification is difficult [17].

The baculovirus expression vector system has been widely used to express a variety of heterologous genes in insect cells and caterpillars [7–9]. A number of established cell lines are available in the baculovirus expression vector system, and these give good yields of synthesized foreign protein. The insect larval system, however, offers an exciting alternative because of the low costs involved in mass scale production (rearing and maintenance of larvae) as compared to tissue-cultured cells. Furthermore, expression in whole insect larvae may provide specialized cell types (such as secretory cells) and specialized post-translational modification enzymes which may be valuable for the expression of some genes [3].

In this report, we describe the simultaneous synthesis of two unrelated and differently destined proteins, luciferase (Luc) and human nerve growth factor (hNGF), in *Spodoptera* larvae infected with a genetically engineered baculovirus carrying the genes encoding the respective proteins.

Correspondence: X-W Zhang, Food Engineering Research Center of State Education Commission, Zhongshan University, Guangzhou 510275, People's Republic of China
Received 16 August 1998; accepted 14 October 1998

Materials and methods

Cell lines, plasmid and virus

Luc cDNA was from pDR102 [10]. Baculovirus transfer vector pSXIVVI$^+$X3 [16] was used for the expression plasmid. *E. coli* DH5α was used for plasmid propagation and cloning purposes. Multiple nucleocapsid nuclear polyhedrosis virus Trichoplusia ni (TnNPV) was introduced from NERC, Institute of Virology, Oxford, UK, and its recombinant parent virus TnNPV-SVI$^-$ G DNA(gal$^+$OCC$^-$) containing the Syn XIV promoter was from the Research Institute of Entomology, Zhongshan University, Guangzhou, China. Routine molecular biology tools including PCR kit, restriction enzymes and other enzymes were obtained from Promega (Beijing, China).

DNA manipulation and construction of recombinant virus

Plasmid DNA manipulation, co-transfection, isolation, purification and characterization of virus were carried out as described [11,13]. To obtain the final hNGF clone, we utilized PCR amplification. Extraction of human leucocyte cDNA and PCR amplification were as described [11]. According to the previous report [14], we used a 5′ PCR primer containing a *Bam*HI site (5′-GGGGATCCTCATCATCCCATCCCATC-3′) and a 3′ primer with a *Sal*I site (5′-CTGTCGACTTAGGCTCTTCTCACAGCCT-3′). The PCR was performed at 94°C for 1 min, 56°C for 1 min, and then 72°C for 2 min for 28 cycles using the healthy human leucocyte genomes as the template. The sequencing was performed by dideoxynucleotide chain termination [12] with the sequencing kit following the manufacturer's instructions.

Detection and characterization of Luc and hNGF

Four instar of larvae were fed with diet coated with recombinant virus polyhedrin. Larvae were harvested for 4 or 8 days post-infection (p.i.) and assayed for Luc and hNGF. Total cell extracts of virus-infected caterpillars were prepared [3] and assayed for Luc using an X-ray film fogging

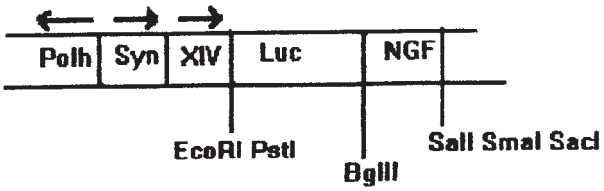

Figure 1 Partial physical map of the transfer vector pSXIVVI$^+$X3-Luc-NGF. Polh—Polyhedrin promoter, Syn—Synthesis promoter, XIV—XIV promoter, Luc—luciferase, NGF—nerve growth factor.

assay [3]. Alternatively, larvae were bled by cutting off the prolegs, and the hemolymph collected. Both the hemolymph and the body tissue were assayed separately for Luc and hNGF by SDS-PAGE and radioimmunoassay (RIA) [1,2]. For RIA, a monoclonal antibody specific to hNGF (Beijing Institute of Radiation Medicine, Beijing, China) was used.

Western blot analysis

A Western blot of larval protein extracts separated by SDS-PAGE was probed with an appropriate dilution of a polyclonal antiserum against Luc, or with a monoclonal antibody against hNGF. Membranes were either incubated in sheep anti-mouse IgG-HRP (horse radish peroxidase, Amersham, UK) or in goat anti-rabbit IgG-HRP (Beijing Institute of Radiation Medicine, Beijing, China). The enzymatic activity was revaled by colour development with freshly prepared 3,3′-diamino-benzidine solution (DAB).

Results and discussion

Construction of transfer vector carrying Luc and hNGF cDNAs and characterization of recombinant baculovirus

The *Pst*I + *Bam*HI fragment carrying the cDNA encoding Luc from pDR102 was subcloned into the *Pst*I + *Bgl*II site of transfer vector plasmid pSXIVVI$^+$X3/3. The recombinant plasmid was designated as pSXIVVI$^+$X3/3-Luc. PCR-amplified *Bam*HI + *Sal*I fragment carrying hNGF cDNA was cloned into the *Bgl*II + *Sal*I site of pSXIVVI$^+$X3/3-Luc, ie the downstream of Luc gene, and the cloned plasmid pSXIVVI$^+$X3/3-Luc-NGF carrying the fusion gene Luc-NGF was obtained. The ligation mix was transformed (*E. coli* DH5α), thus recombinant plasmid pSXIVVI$^+$X3/3-Luc-NGF was generated. The sequencing analysis revealed that the sequences of the fusion gene were as expected (data not shown). The partial physical map of the transfer vector, pSXIVVI$^+$X3/3-Luc-NGF, used for generating recombinant virus, is illustrated in Figure 1.

The recombinant baculovirus was constructed by *in vivo* recombination [7] between the recombinant plasmid and TnNPV-SVI$^-$G DNA. The recombinant baculovirus designated TnNPV-Luc-NGF-OCC$^+$ was isolated and plaque purified [9,13]. The presence of both Luc as well as hNGF genes in TnNPV-Luc-NGF-OCC$^+$ was confirmed by Southern hybridization and restriction enzymes analysis (data not shown).

Expression of recombinant proteins in infected larvae

Analysis of hemolymph and body tissue of *Spodoptera* larvae, infected with recombinant virus TnNPV-Luc-NGF-OCC$^+$, revealed the synthesis of both gene products, Luc and hNGF. Luc was identified on the basis of its cross-reactivity with a Luc-specified rabbit anti-Luc polyclonal serum on a Western blot (Figure 2a). Recombinant Luc comigrated with the standard firefly Luc protein (Boehringer-Mannheim, Germany) at an apparent molecular weight of ~62 kDa. Recombinant hNGF, synthesized in infected larvae, was also identified by its cross-reactivity with a mono-specific mouse anti-hNGF monoclonal antibody on a Western blot. The recombinant hNGF, with an apparent molecular weight of~12 kDa, reacted with a mono-specific antibody (Figure 2b), proving that the recombinant hNGF was similar to native hNGF. Densitometric scanning of SDS-PAGE revealed that ~18% of the total Coomassie blue-stainable protein of the infected larvae was represented by Luc protein, while the hNGF level was ~8% (data not shown).

Densitometric scanning of X-ray film, fogged due to the light emitted by enzymatically active Luc, revealed that while most (~95%) activity was located in body tissue, a small fraction (~5%) was present in the hemolymph (Figure 3a). RIA confirmed the presence of recombinant hNGF in the infected larvae and indicated that the synthesis

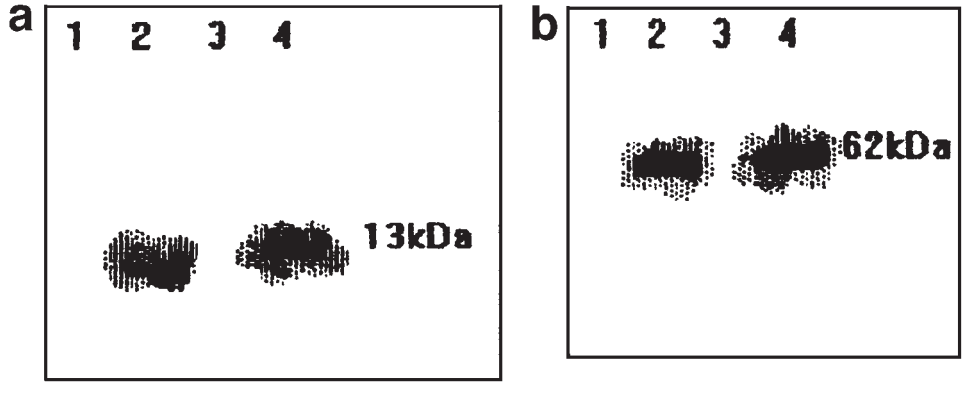

Figure 2 Western blot of larval samples extracted 4 days p.i. (a) A Luc-specific rabbit anti-Luc polyclonal serum was used. (b) The blot was probed with a monoclonal antibody against hNGF. (a) and (b) Lane 1, TnNPV-infected larvae; lanes 2,3, TnNPV-Luc-NGF-OCC$^+$-infected larvae (2, body tissue; 3, hemolymph); lane 4, commercial preparation of firefly Luc (>2 μg) and standard hNGF (>5 μg) for (a) and (b), respectively.

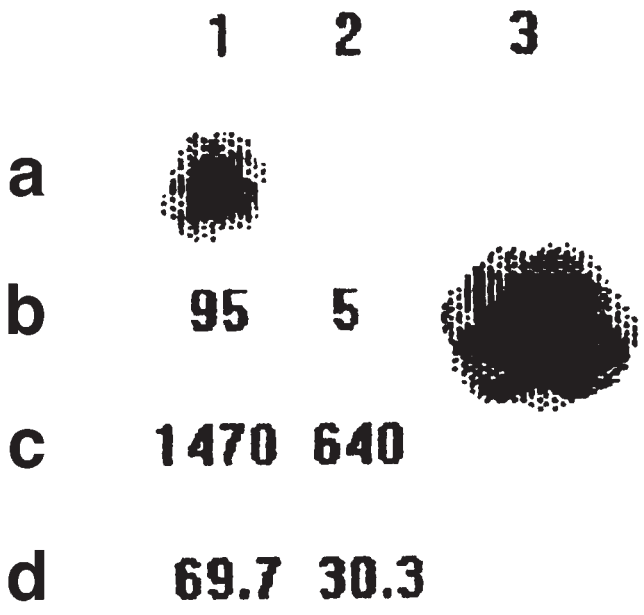

Figure 3 X-ray film fogging assay of luciferase and radioimmunoassay of hNGF synthesized in the TnNPV-Luc-NGF-OCC$^+$-infected larva 4 days p.i. (a) Lanes 1,2, TnNPV-Luc-NGF-OCC$^+$-infected larva (1, body tissue; 2, hemolymph); lane 3 of panel (b) shows standard Luc (1 μg). (c) hNGF (ng) synthesized in larva (lane 1, body tissue; 2, hemolymph). (b and d) Lanes 1 and 2 give the relative percentage of recombinant proteins in corresponding lanes of (a) and (c), respectively.

of hNGF increased with the time of infection, starting from 2110 ng hNGF per larva (640 and 1470 ng, respectively, in hemolymph and body tissue) at 4 days p.i. (Figure 3c), and increasing to 3120 ng per larva (930 and 2190 ng, respectively, in hemolymph and body tissue) at 8 days p.i.

The majority of recombinant proteins were retained in body tissue. SDS-PAGE and Western blots revaled the presence of recombinant proteins in the hemolymph as well. The hemolymph contains hemocytes, a cell type which is indeed infected with baculovirus. Previous studies indicate that the fat body of larvae synthesizes and releases protein into the hemolymph and also takes up protein from the hemolymph. Other tissues, such as midgut, epidermis, pericardial cells and hemocytes also contribute to hemolymph proteins [3]. The presence of recombinant proteins in the hemolymph, could therefore be explained on these lines.

A large majority of the recombinant virus-infected larvae did not molt into pupa, and remained in the larval stage till their death due to the arrest of metamorphosis caused by the viral *egt* gene [9]. The prolonged lifespan of infected larvae by 5–6 days is of special relevance for harvesting of recombinant proteins from larvae. Most, if not all, foreign proteins synthesized during the larval stage naturally have sufficient time to undergo proper post-translational modifications.

The results presented above demonstrate that the cDNAs encoding luciferase and human NGF can be expressed simultaneously in an occluded recombinant baculovirus expression system with the control of synthetic and XIV promoters. Specifically, the recombinant proteins can be produced in caterpillars and this is valuable for the mass-scale production of such proteins at low cost.

References

1 Dicou E. 1992. Expression of recombinant human nerve growth factor in *Escherichia coli*. Neurochem Int 20:129–134.
2 Fujimori K, S Fukuzono, N Kotomura, N Kuno and N Shimizu. 1992. Overproduction of biologically-active human nerve growth factor in *E. coli*. Biosci Biotechnol Biochem 56: 1985–1990.
3 Hasnain SE and B Nakhai. 1990. Expression of the gene encoding firefly luciferase in insect cells using a baculovirus vector. Gene 91: 135–138.
4 Hu GL and KE Neet. 1988. Expression of the cDNA for mouse beta-nerve growth factor protein in *E. coli*. Gene 70: 57–65.
5 Levi-Montalcini R. 1987. The nerve growth factor: thirty-five years later. EMBO J 5: 1145–1154.
6 Lindsay RM, SJ Wiegand and CA Altar. 1994. Neurotrophic factor: from molecular to man. TINS 17: 182–186.
7 Luckow VA and MD Summers. 1988. Trends in the development of baculovirus vectors. Biotechnology 6: 45–55.
8 Miller LK. 1988. Baculovirus as gene expression vectors. Annu Rev Microbiol 42: 177–199.
9 O'Reilly DR, LK Miller and VA Luckow. 1992. Baculovirus Expression Vectors—A Laboratory Manual. WH Freeeman and Company, New York.
10 Riggs CD and MJ Chrispeels. 1987. Luciferase reporter gene cassettes for plant gene expression studies. Nucleic Acid Res 15: 8115–8120.
11 Sambrook J, EF Fritsch and T Maniatis. 1989. Molecular Cloning. A Laboratory Manual, 2nd edn. CSHL Press, Cold Spring Harbor, NY.
12 Sanger FS, S Nicklen and A Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467.
13 Summers MD and GE Smith. 1987. A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures. Texas Agric Exp Stn Bull 1555, College Station, TX, USA.
14 Ullrich A, A Gray and C Berman. 1983. Human beta-nerve growth factor gene sequence highly homologous to that of mouse. Nature 303: 821–826.
15 Vissavajjhala P and AH Ross. 1990. Purification and characterization of the recombinant extracellular domain of human nerve growth factor receptor expressed in a baculovirus system. J Biol Chem 265: 4746–4752.
16 Wang X, BG Ooi and LK Miller. 1991. Baculovirus vectors for multiple gene expression and for occluded virus production. Gene 100: 131–137.
17 Yu P, JY Long, Q Zhao, C Liu and HX Wang. 1997. Cloning and sequencing of human nerve growth factor gene. Lett Biotechnol 8: 64–65.