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Expression of Enhanced Green Fluorescent Protein from Stably Transformed *Drosophila melanogaster* S2 Cells

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Abstract Recombinant plasmids harboring a heterologous gene coding for the enhanced green fluorescent protein (EGFP) were transfected and expressed in *Drosophila melanogaster* S2 cells. A stable transformation of polyclonal cell populations expressing EGFP were isolated after 4 weeks of selection with hygromycin B. The recombinant EGFP expressed in transformed S2 cells consisted of a molecular weight of 27 kDa. EGFP expression was also confirmed by fluorometric measurement. The maximum EGFP concentration was about 9.3 mg/l. The present findings demonstrate not only the successful stable expression of EGFP in *Drosophila* S2 cells, but also the use of EGFP as a reporter to analyze gene expression, with its potential of a *Drosophila* cell-expression system for recombinant protein production being an alternative to a baculovirus-insect cell expression system.

Key words: Enhanced green fluorescent protein, stable expression, *Drosophila melanogaster* S2 cells

The Schneider line-2 cell (S2 cell) of *D. melanogaster* was established from primary cultures of 20–24 h embryos [12]. Stable transformation of S2 cells have several advantages. First of all, S2 cells are suitable for achieving an efficient expression of heterologous gene products, allowing stable insertion of as much as several hundred gene copies in the chromosomal DNA [2, 5]. Secondly, S2 cells grow readily in suspension to high cell densities when left at room temperature. Finally, S2 cells are easily maintained as they do not require trypsin treatment for splitting nor a CO_2 supply.

The heterologous gene coding green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*, has recently become very useful as a reporter for gene expression [1, 9]. The native GFP is a 238 amino acid polypeptide, which undergoes an intramolecular cyclization to produce a brightly fluorescent molecule. This protein does not require any substrates, cofactors, or enzymes for its fluorescence, making this protein unique in that it can be detected in living cells in real time. Since this reporter does not require any staining techniques, it may be a better alternative over the enzyme and antibody based methods to monitor gene expression in single cells. EGFP, a red-shift GFP variant, contains a chromophore mutation to improve the detection of GFP in transfected mammalian cells [13].

GFP has been expressed in mammalian, plant, and baculoviral expression systems [3, 7, 13]. Recently, we described the expression of GFP in *D. melanogaster* S2 cells [8]. To extend our work, we examined the stable expression of EGFP in cultured *Drosophila* S2 cells. In this study, we conducted a thorough evaluation of a *Drosophila* cell-expression system by testing the stable expression of EGFP in *D. melanogaster* S2 cells.

D. melanogaster Schneider 2 (S2) cells were grown at 27°C in Nunc (T-25, Rockilde, Denmark) flasks in M3 medium (Shields and Sang M3 insect medium; Sigma, St. Louis, MI, U.S.A.) containing 10% IMS (insect medium supplement, Sigma). The plasmid pAc5CPPA (7.3 kb) contains a strong distal promoter and polyadenylation sequences of the *Drosophila* actin 5C gene [4]. The selection plasmid pCoHygro (Invitrogen, Carlsbad, U.S.A.), which contains the bacterial hygromycin B phosphotransferase gene that is under the control of the constitutive *Drosophila* Copia 5' LTR promoter, was used for stable transformation. The plasmid containing EGFP was pEGFP (Clontech, Palo Alto, U.S.A.). *E. coli* JM109 was used as the primary host for constructing and propagating plasmids. *E. coli* cells were routinely grown and maintained in LB medium (1% bacto-tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.3) containing 50 μg of ampicillin per ml, with agitation at 37°C. DNA restriction enzymes were purchased from Promega (Madison, U.S.A.) or Takara (Shiga, Japan), in which reactions were carried out according to the manufacturer's instructions.

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A 0.72-kb *Bam*HI-*Eco*RI fragment of pEGFP (Clontech) was inserted into pBacPAK9 (Clontech) to yield pBacPAK9-EGFP. The pAc5CPPA-EGFP was constructed by inserting a *Bam*HI-*Sac*I fragment of pBacPAK9-EGFP between the *Bam*HI and *Sac*I sites of pAc5CPPA (Fig. 1). A proper orientation and reading frame of the gene insertion in the recombinant plasmids of pAc5CPPA-EGFP were confirmed by both restriction enzyme mapping and nucleotide sequencing.

Exponentially growing S2 cells were cotransfected with plasmids pAc5CPPA-EGFP and pCoHygro (a ratio of 19:1) by using the lipofectin method. To make the transfection medium, plasmid DNA and lipofectin reagent (Gibco BRL, Rockville, U.S.A.) were diluted with IMS-free M3 medium separately, and then they were mixed together in a ratio of 1:5. This transfection medium was incubated at room temperature for 15 min and it was transferred into 6-well plates which were preseeded 2 h earlier with S2 cells in IMS-free M3 medium. After a 24 h

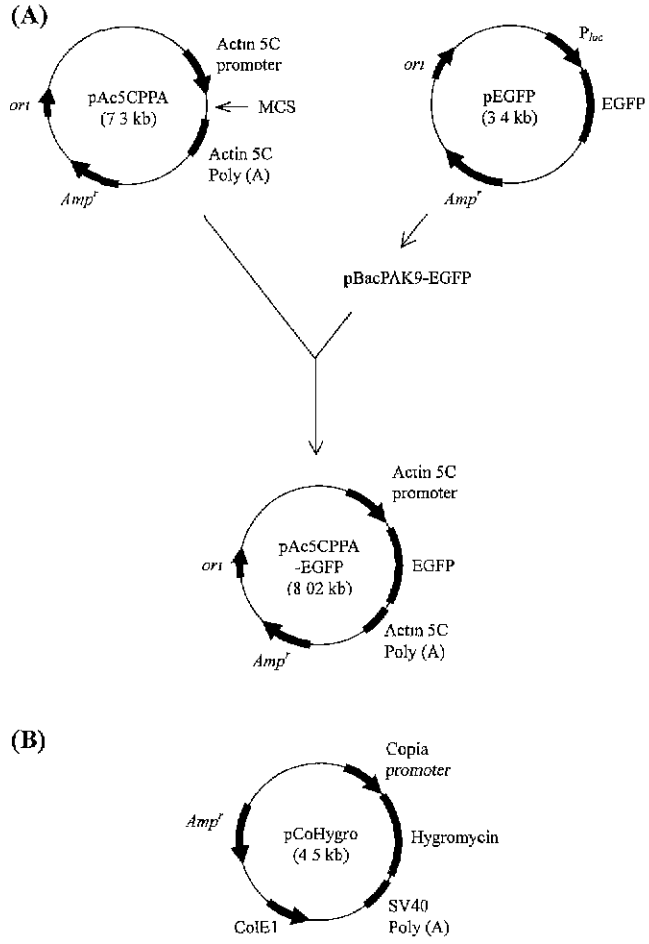

Fig. 1. Schematic representation of plasmids used in this work. (A) Construction of expression plasmid, pAc5CPPA-EGFP. pAc5CPPA-EGFP was constructed by inserting a *Bam*HI-*Sac*I fragment of pBacPAK9-EGFP between the *Bam*HI and *Sac*I sites of pAc5CPPA. (B) pCoHygro.

incubation period, the medium was changed to remove the lipofectin, while the cells were incubated for another 5 days in a M3 medium containing 10% IMS without the drug selection. Then, the cells were centrifuged and resuspended in a selective M3 medium containing 10% IMS and 300 μg hygromycin B/ml. The selective medium was replaced every 5 days, and stably transformed polyclonal cell populations were isolated after 4 weeks of selection with hygromycin B. Hygromycin B was maintained routinely in the media at all times after the selection process.

Stably transformed S2 cells expressing EGFP were grown at 27°C in Nunc (T-25) flasks in 5 ml of M3 medium containing 10% IMS and 300 μg hygromycin B/ml. For static cultures of S2 cells expressing EGFP, multiple T-flask cultures were grown simultaneously. Two of the T-flasks were used to analyze cell growth and EGFP concentration each day. The cultures were then centrifuged at 3,000 rpm for 5 min to separate the cells. The supernatant was used for gene expression analysis for identifying extracellular recombinant proteins. In order to identify intracellular recombinant proteins, the cell fraction was rocked for 1 h in a lysis buffer [11] and subjected to 3 freeze-thaw cycles of incubation in a -70°C freezer with subsequent incubation process in a 37°C water bath. After centrifugation of the cell extracts at 14,000 rpm for 15 min to remove cell debris, the supernatant was used to analyze gene expression.

The EGFP fluorescence intensity [10] was determined by measuring the light emission at 515 nm with excitation at 460 nm using a fluorometer (Turner Digital Fluorometer, Model 450; Barnstead/Thermolyne, Dubuque, U.S.A.). The EGFP concentration was estimated roughly by converting the intensity of fluorescence into a EGFP concentration using a calibration curve that is determined from standard GFP (Clontech).

Protein samples were analyzed by SDS-PAGE as described by Laemmli [6]. The electrophoresed EGFP proteins on the gel were transferred onto the nitrocellulose, incubated with rabbit anti-GFP polyclonal antibody (1:1000 v/v, Clontech), and probed with a goat anti-rabbit IgG alkaline phosphatase conjugate (1:1000 v/v). After washing, BM purple AP substrate solution (Boehringer Mannheim, Mannheim, Germany) was added and the reaction was quenched with distilled water. The standard GFP was obtained from Clontech.

The stable S2 cells carrying pAc5CPPA-EGFP expressed recombinant protein with a molecular weight of about 27,000 (Fig. 2). In Western blot analysis with rabbit anti-GFP polyclonal antibody, this protein was presumptively identified as a recombinant EGFP, since its molecular size matched that (27 kDa) of the standard GFP. This immunoreactive band was absent from the medium fraction of transfected S2 cells and from both the cellular

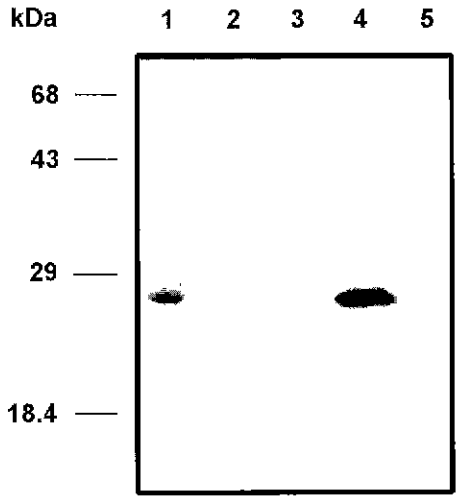

Fig. 2. Western blot analysis of EGFP from S2 cells.
1) Standard GFP, 2) cellular fraction of non-transfected cells, 3) medium fraction of non-transfected cells, 4) cellular fraction of stable S2 cells expressing EGFP, and 5) medium fraction of stable S2 cells expressing EGFP. Numbers on the left indicate the molecular mass markers (kDa).

and medium fractions of non-transfected S2 cells as well. This indicates that the expression of EGFP is a result of the integration of pAc5CPPA-EGFP in the S2 cell genome.

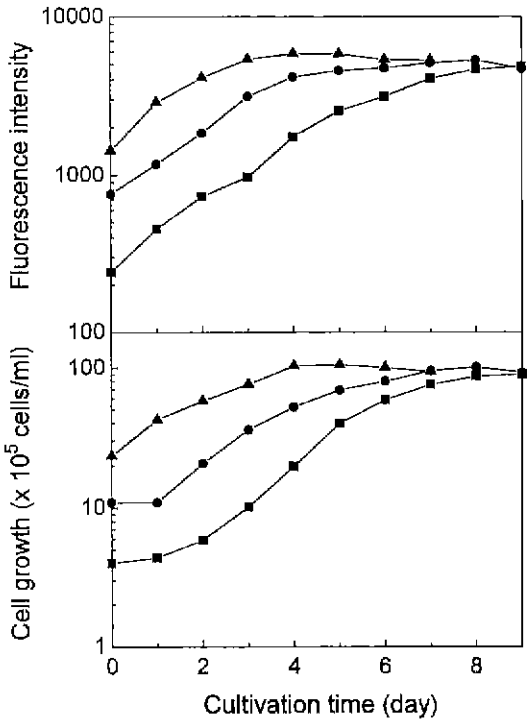

Fig. 3. Profiles of cell growth and EGFP expression in T-flask cultures of stable S2 cells.
EGFP expression is given in terms of fluorescence intensity. Initial cell concentration: ■, 4×10^5 cells/ml, ●, 1.1×10^6 cells/ml; ▲, 2.3×10^6 cells/ml.

EGFP expression was also confirmed by fluorometric measurement. Figure 3 shows the changes in the time course of cell growth and EGFP expression in T-flask cultures of stable S2 cells. Increasing the initial cell density from 4×10^5 cells/ml to 2.3×10^6 cells/ml reduces the lag phase of S2 cell growth in T-flasks. The maximum cell density was about 1×10^7 cells/ml. The level of EGFP expression reached a maximum fluorescence intensity of 5.840 after 4 days of incubation with an initial cell density of 2.3×10^6 cells/ml.

In our experiments, the maximum EGFP concentration was estimated to be about 9.3 mg/l from the peak intensity (5,840) of fluorescence, by referring to the calibration curve that was determined from the standard GFP. The level of EGFP expression was similar to the level (9 mg/l) of previous findings on recombinant GFP from stably transformed S2 cells [8]. This indicates that the intensity of fluorescence for recombinant EGFP expression did not increase markedly. However, our level of EGFP expression is higher than that (1 mg/l) reported for the expression of GFP in *Trichoplusia ni* (High Five) cells using a baculovirus expression vector system [7]. Our result shows that the *Drosophila* cell-expression system expresses EGFP efficiently and that this system may be used as an alternative to the baculovirus-insect cell expression system.

Interestingly enough, stably transformed polyclonal S2 cells were recovered from a single transfection after only 4 weeks of hygromycin selection. Therefore, the time required for constructing transformed S2 cells is reasonably comparable to the baculovirus-insect cell expression system, in which recombinant baculoviruses are obtained after about 3–4 weeks by using a commercial kit (Invitrogen).

In conclusion, the stable expression of EGFP in *D. melanogaster* S2 cells was used to evaluate the *Drosophila* cell-expression system. The maximum level of EGFP expression was about 9.3 mg/l, which is higher than the level reported for the expression of GFP in *T. ni* (High Five) cells using a baculovirus expression system. Our findings show the successful expression of EGFP in *Drosophila* S2 cells, the use of EGFP as a reporter to analyze gene expression, and a potential of the *Drosophila* cell-expression system for recombinant protein production to be used as an alternative to a baculovirus-insect cell expression system.

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