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New strategy for rapid isolation of stable cell lines from DNA-transformed insect cells using fluorescence activated cell-sorting

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ABSTRACT

A stably transformed insect cell expression system is superior to a baculovirus expression system, since the expression system is sustained and there is no cell lysis, but the isolation of cell lines producing recombinant proteins is time-consuming and laborious. In this study, we developed a technique for the rapid isolation and efficient cultivation of sorted cells in a 24 well deep plate Bioshaker, utilizing the fluorescence activated cell-sorting (FACS) method. TnpXme11 cells, which stably expressed GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase2 (GGT2), were transfected with a plasmid vector for the expression of a molecular chaperone (TnpXme11-hCNX6 cell line). The expression levels of GGT2 and the molecular chaperone fused with HcRed were analyzed by FACS. Two cell lines were established by single and double sorting. Sorting of the top 10% of the TnpXme11-hCNX6 cell population with the highest fluorescence yielded the TnpXme11-hCNX6-1 cell line. TnpXme11-hCNX6-2 cells were created in a similar fashion, as mentioned above, by a second sorting of the top 10% of the TnpXme11-hCNX6-1 cell population with the highest fluorescence. The cells thus isolated produced approximately 2-fold higher extracellular activity than that before cell sorting. This procedure can be accomplished in only 2 weeks, including transfection, isolation and analysis of high protein-producing cells, and is a breakthrough strategy for the rapid isolation of a recombinant, stable insect cell line.

1. Introduction

Insect cells are often used for recombinant protein production, due to their capacity for post-translational modification (Altmann et al., 1999). The baculovirus expression system (BES) is a well known system that utilizes insect cells, and only a few weeks are needed to construct recombinant baculoviruses and express recombinant proteins by using the bacmid system (Luckow et al., 1993). The BmNPV bacmid (Motohashi et al., 2005) was recently developed, which accelerated the rapid expression of recombinant proteins in silkworm larvae as well as silkworm-derived cell lines.

As an alternative, a stably transformed insect cell expression system is suitable for the expression of recombinant proteins (Douris et al., 2006; McCarroll and King, 1997). Protein expression in the stably transformed cell system is often higher than that in the BES, as the cell lines can be cultured for a longer period of time, as compared with BES, where the expression period is only 72–80 h post-infection. The stably transformed cell system is free from lytic cell death and causes less stress on the secretory pathway from the baculovirus infection. Moreover, living cells produce more correctly folded-proteins, as compared with BES. However, the isolation of cells producing recombinant proteins requires several months of labor. Therefore, a key step in isolating cells for protein production is the rapid selection of cells that achieve the highest expression level.

Fluorescence activated cell-sorting (FACS) technology allows the rapid isolation of polyclonal cell populations with the highest expression levels of recombinant proteins (Rieseberg et al., 2001; Sleiman et al., 2008). FACS technology can easily screen a million cells in a brief time, and its high-through-put screening allows the isolation of cell populations with high expression levels. Green fluorescent protein (GFP) has been used for monitoring protein expression and selecting cells expressing recombinant proteins. Yuk et al. reported the rapid selection of populations of CHO cells that could maintain a high level of recombinant protein expression, using GFP (Yuk et al., 2002).

In this study, we tried to isolate populations of insect cells expressing a high level of GFP-fused protein and to establish a rapid screening method using the cell sorter and the cultivation of a stably transformed expression cell line.

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2. Materials and methods

2.1. Cell lines, media and cultivation

The Tn-5B1-4 and TnpXme11-hCNX6 cell lines were reported previously (Kato et al., 2005). The TnpXme11-hCNX6 cell line stably expresses the GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase 2 (β3GnT2) fusion protein (GGT2). Tn-5B1-4 and TnpXme11-hCNX6 cells were cultivated in Express Five medium (Invitrogen, San Diego, CA, USA), supplemented with 1% antibiotic–antimycotic (Invitrogen) and 30 mM glutamine.

Cells were cultivated in a 24 well deep plate Bioshaker (M·BR-024, TAITEC, Saitama, Japan) with working volumes of 1, 2, 4, and 8 ml per well. The agitation rates and the temperature were controlled at 200, 400, and 600 rpm and 27 °C, respectively.

2.2. Plasmid construction

Molecular chaperones were expressed under the control of the actin promoter. The genes encoding calnexin (CNX), calreticulin (CRT), ERp57, immunoglobulin heavy chain binding protein (BiP, GRP78) and heat shock protein 70 (Hsp70) were amplified by PCR, using the primers shown in Table 1 and Human Brain Quick-Clone cDNA (Clontech, Palo Alto, CA, USA) as the template. Each amplified fragment was cleaved with SalI and then inserted into the pHcRed1 vector (Clontech). Each chaperone gene fused with the HcRed1 gene was amplified by PCR, using the primers shown in Table 1, and then inserted into the HindIII–EcoRI sites in pIB/His-V5. The resulting plasmids were named pIB/Bip-HcRed, pIB/ERp57-HcRed, pIB/CNX-HcRed, pIB/CRT-HcRed, and pIB/Hsp70-HcRed.

2.3. FACS and isolation of cell populations

FACS and isolation of cell populations were performed using an EPICS ALTRA cell sorter (Beckman Coulter, Inc., Fullerton, CA, USA), equipped with an argon laser (488 nm) and a helium-neon laser (532 nm). GFP_{uv} and HcRed fluorescence were collected with 525 nm and 675 nm bandpass filters, respectively. The top 10% of cells with high-fluorescent intensity were sorted into a tube, using phosphate-buffered saline (PBS, pH 7.0) as a running buffer. The sorted cells were then washed with Express Five medium and cultivated using a 24 deep well plate Bioshaker.

2.4. Fluorescence microscopy

Cell suspensions were collected from 4 to 6 days culture medium and immediately observed using a confocal laser scanning microscope (TCS-LS, Leica Microsystem, Heidelberg, Germany), equipped with argon (488 nm) and helium-neon (633 nm) lasers.

Table 1
Primers used in this study.

	5′–3′
Bip(F)	TATGTCGAC ATGAAGCTCTCCCTGGTGGCCGCGATG
Bip(R)	TATGTCGACCTACAACTCATCTTTTTCTGCAGTATCCTCTTCACC
CNX(F)	TATGTCGACATGGAAGGGAAGTGGTTGCTGTGTATGTTA
CNX(R)	TATGTCGACCTACAGCTCGTCCTTGGCCTGGCCGGGGAC
ERp57(F)	TATGTCGACATGCGCCTCCGCCGCCTAGCGCTGTTC
ERp57(R)	TATGTCGACTTAGAGATCCTCCTGTGCCTTCTTCTTCTTCTTGGG
Hsp70(F)	TATGTCGACATGGCCAAAGCCGCGGCGATCGGCATC
Hsp70(R)	TATGTCGACCTAATCTACCTCCTCAATGGTGGGGCCTGACCCAGA
Red(R)	TATAAGCTTCTACAGCTCGTCCTTGTTGGCCTTCTCGGGCAGGTCGCT
Red(Hsp-R)	TATAAGCTTTCAGTTGGCCTTCTCGGGCAGGTCGCT

2.5. SDS-PAGE

To detect the expression of the recombinant proteins, cell supernatants and lysates were subjected to SDS-PAGE on a 10 or 12% (w/v) polyacrylamide gel, using the Mini-protean II system (Bio-Rad Hercules, CA, USA). The cell lysates were prepared in lysis buffer (50 mM Tris–HCl, pH 8.0, 1% Triton X-100). For the detection of the fluorescent GGT2 fusion protein on the SDS-PAGE gel, samples were suspended in sample buffer (Aoki et al., 1996) and the fluorescent bands of the GGT2 fusion protein were detected with a Molecular Imager FX (Bio-Rad).

2.6. β3GnT assay and protein concentration measurement

The β3GnT activity was measured in 50 mM Tris–HCl (pH 8.0), 15 mM $MnCl_2$, 19 mM UDP-GlcNAc, 22 mM Galβ1-4 GlcNAcβ-*p*NP and 5 μl enzyme solution (total volume 25 μl). The reaction was started by the addition of the β3GnT sample. At each sampling time, 5 μl of the reaction mixture was added to 195 μl of distilled water, and the solution was boiled for 5 min. After filtration with a 0.45 μm nitrocellulose filter (Millipore, Bedford, Massachusetts, USA), the filtrates were analyzed by HPLC on a Mightysil RP-18 (H) GP 150–4.6 (Kanto Chem. Co. Inc.) column. The reaction products were eluted with 10% methanol and were detected at an absorbance of 300 nm. HPLC was performed at 40 °C, with a flow rate of 1.0 ml per min. One unit of enzyme activity is defined as the amount of enzyme capable of catalyzing the transfer of 1 μmol of GlcNAc per minute. Protein concentrations were determined by the Bradford method (Bio-Rad).

3. Results

3.1. Cultivation of insect cells using a 24-well deep plate Bioshaker

To cultivate the cell populations rapidly isolated by FACS, a 24-well deep plate Bioshaker was used for the suspension cultivation of Tn-5B1-4 cells. Shaking speeds and culture volumes were investigated. The cells precipitated when cultured in a shaker at 200 rpm in culture volumes of 1, 2, 4 and 8 ml, and the cells also did not grow at 600 rpm in culture volumes of 1 and 2 ml (data not shown). However, cell growth was confirmed at 400 rpm in culture volumes of 1 and 2 ml, but not in culture volumes of 4 and 8 ml (data not shown).

3.2. Coexpression of GGT2 fusion protein with each chaperone

Previously, Tn-5B1-4 cells stably expressing the GGT2 fusion protein (TnpXme11 cells) were isolated (Kato et al., 2004). A plasmid for the expression of each chaperone was constructed and then transfected into the TnpXme11 cells. In this study, five chaperones (Bip, ERp57, CNX, CRT, and Hsp70) were studied, and HcRed was fused at the C-terminus of each chaperone. Bip, ERp57 and CRT each have an endoplasmic reticulum (ER) retention signal at the C-terminus. To maintain the three chaperones in the ER, the ER retention signal (-KDEL) was fused at the C-terminus of HcRed. The transfected cells were cultivated in the presence of 100 μg/ml Blasticidin for 3 days. After 3 days of cultivation in a static culture, the cells were cultivated for 6 days in a suspension culture, using a 100-ml shaker flask with a working volume of 20 ml, and then fluorescence observations and β3GnT assays were performed. The GFP_{uv} and HcRed fluorescence intensities were observed in TnpXme11 cells transfected with each plasmid for the expression of the chaperones (data not shown). As compared to TnpXme11 cells, the intra- and extracellular β3GnT activities increased in all chaperone expression plasmid-transfected cells (Fig. 1). The intracellular activity in TnpXme11 cells coexpressing Bip was enhanced