Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2010; 35 (4) ; 340–343.

Technical Report [Yöntem Bildirisi]

Yayın tarihi 15 Aralık, 2010 © TurkJBiochem.com

[Published online 15 December, 2010]

Establishment of Green Fluorescent Protein Expressing CHO Cells by Stable Transfection Using Activated Dendrimers and G418 Selection

[Aktive Edilmiş Dendrimerler ve G418 Ayrımı ile Yapılan Kalıcı Transfeksiyonla Yeşil Flöresan Protein Ekspresse Eden CHO Hücrelerinin Yapılması]

Başak Çeltikçi[1],
Nuhan Puralı[2],
H. Asuman Özkara[1]

Hacettepe University Faculty of Medicine, Departments of Biochemistry[1] and Biophysics[2], 06100 ANKARA, Turkey

Yazışma Adresi
[Correspondence Address]

H. Asuman Özkara

Hacettepe University
Faculty of Medicine
Department of Medical Biochemistry
06100 Ankara, Turkey
Tel: (312) 3051652/118
E-ail: ozkara@hacettepe.edu.tr

Date Registered: 18 March 2010; Date Accepted: 13 May 2010

[Kayıt Tarihi : 18 Mart 2010; Kabul Tarihi : 13 Mayıs 2010]

ABSTRACT

Background. Stable transfection of Green Fluorescent Protein (GFP) has been widely used as a marker for both monitoring protein expression and direct observations of cellular dynamics. Despite the proven utility of GFP as a marker, the appropriate conditions of stable transfection, like selection of optimal concentration of G418 and the effect of passage number on fluorescence emission time has not been fully described for stable transfection of kanamycin/neomycin resistant pIRES-EGFP to Chinese Hamster Ovary (CHO) cells by activated dendrimers.

Methods. To address the issues and the establishment of GFP expressing CHO cells for further experiments, we transfected the cells with pIRES2-EGFP by using activated dendrimers. Transfected cells were selected by eluding the reminder population by exposing to G418 (500 µg/ml for 3 weeks). Selected transfected cell strains were imaged by using confocal microscopy.

Results. After 48 hours following transfection, 5% of cells were transiently transfected and the emitted green fluorescence faded within 4 days. Following G418 treatment for 3 weeks, 10% of the survived cells expressed GFP with a fluorescence intensity several folds larger than that in the control cells. GFP expressing cells had complex morphology which is different from the typical fusiform shape of CHO cells. A part of stably transfected cells showed some important changes in their morphology even though no green fluorescence was detected.

Conclusions. This study demonstrates that fluorescent proteins can succesfully be expressed in mammalian cells for multiple purposes. Our future goal is to obtain stable GFP expression in different cell lines to be used for different experimental purposes.

Key words: geen fuorescent potein, CHO cells, stable transfection, activated dendrimers

ÖZET

Arka plan: Yeşil flöresan protein (GFP), protein ekspresyonu ve hücre dinamiklerinin direk gösterimi için yaygın olarak kullanılan bir belirleyicidir. GFP'nin bir belirleyici olarak yararı ispatlanmış olmasına rağmen, kanamisin/neomisine dirençli pIRES-EGFP vektörünün "Chinese Hamster Ovary (CHO)" hücrelerine aktive edilmiş dendrimerler yoluyla kalıcı transfeksiyonu için uygun koşullar, G418 le ayırım için gereken G418 konsantrasyonu ve pasaj sayısının flöresan ışıma zamanına olan etkisi gibi, tam olarak tanımlanmamıştır.

Yöntem: Bu duruma açıklık getirmek ve daha sonraki deneylerde kullanmak üzere GFP ekspresse eden CHO hücreleri elde etmek için, bu hücreleri aktive edilmiş dendrimerler kullanarak pIRES2-EGFP vektörü ile transfekte ettik. Bu transfekte hücreler 500 µg/ml G418 ile 3 hafta boyunca seçildi. Seçilmiş transfekte hücreler konfokal mikroskopi ile görüntülendi.

Sonuçlar: Transfeksiyonun yapılışından 48 saat sonra, hücrelerin %5'i geçici olarak transfekte oldu ve flöresansları 4 gün içinde soldu. Üç hafta boyunca G418 uygulamasının ardından, kalıcı olarak transfekte olan hücreler yaşayanların %10'u oldu ve kontrol hücrelere göre ekspresse edilen GFP çok daha fazla idi. GFP ekspresse eden hücreler, normal fusiform şekilli CHO hücrelerinden farklı karmaşık bir morfoloji gösterdiler. Kalıcı transfekte olan bazı hücreler şekil değişikliği göstermelerine rağmen, bunlarda ışıma saptanamadı.

Tartışma: Bu çalışma flöresan proteinlerin değişik amaçlarla kullanımı için memeli hücrelerinde başarı ile ekspresse edilebileceğini göstermektedir. Daha sonraki hedefimiz değişik deneylerde kullanmak üzere GFP ekspresse eden farklı hücre dizileri elde etmektir.

Anahtar sözcükler: yşil fuoresan potein, CHO hücreleri, kalıcı transfeksiyon, aktive edilmiş dendrimerler

Introduction

Fluorescently labeled mammalian cell cultures are very frequently used *in vitro* experimental models. The model enables a method to follow the related biochemical event specifically by recording the emitted fluorescent signal. GFP is one of the molecules that emit fluorescent light which has firstly been isolated from the jellyfish, *Aquorea Victoria*. It can emit green light maximally at 475 nm when excited at 396 nm wavelength. Expression of heterolog gene or fusion protein genes can be followed through the insertion of the GFP gene into mammalian cells via stable or transient transfection methods. For this reason, GFP is used as a gene expression "marker". It is a convenient reporter molecule to monitor gene and protein expression. The utility of this protein in experimental biology has already been defined in various cell lines [1].

Transient gene expression is used for generating usable quantities of a protein from cultured cells for several experimental purposes. Though transient transfection is a comparatively fast method however, GFP or the expression of the accompanying protein level may vary significantly from cell to cell. Further, stable transfection helps us to get a homogeneous and durable gene expression at about the same level [2]. Stable transfection process takes a longer time, however all cells express the same amount of GFP and the accompanying protein, and it is possible to obtain new cell populations expressing GFP by simply cell passage since the inserted gene is inherited to the daughter cells.

Mammalian cell lines vary widely in their ability to be transfected with recombinant DNA, so using a reporter gene, such as GFP efficiency of the transfection might be monitored by using an automated flow cytometry method [3]. Cell lines like CHO has commonly be used in transfection experiments since they take up DNA and express recombinant proteins very efficiently [4].

The fluorescent life time of GFP is about 3.1 ns. The fluorescent life time for enhanced GFP (EGFP) on the other hand varies between 2.7-3.4 ns. This amount of time is important for the imaging techniques. For this reason, GFP variants with longer radiation times such as EGFP are sought to be obtained. In stable transfection, the cells that have obtained EGFP cells produce this gene by transferring it through generations and thus allow the metabolic events in the living cell to be observed under a confocal microscope. Thus, in normal and pathological situations could be monitored some important cellular activities such as, mitoses, cell organelles, metastatic ability of cancer cells, secretion paths and expression of some specific proteins [4-10].

The aims of this study are firstly to optimize the conditions of stable transfection of pIRES2-EGFP in CHO cells, and secondly to demonstrate (long term) changes in GFP expression level after removal of G418.

Materials and Methods

Cell Culture

CHO cells were grown in MEMα cell media supplemented with 10% fetal bovine serum. Cells were plated at 70% confluence in T25 flasks. The cells were incubated at 37˚C in air containing 5% carbon dioxide. [11,12]

Transformation

pIRES2-EGFP (Clontech Laboratories) vector has been transferred into DH5 alpha competent cells by using a chemical method. Transformed competent cells have been produced by 12-16 hour incubation in LB-agar plaques containing 50 mg/ml kanamycin. The colonies multiplied in 500 ml LB medium which was continuously shaked at 300 rpm for 12-16 hours in 37°C [4-10]. pIRES2-EGFP vector was isolated from the transformed and reproduced cells by using QIAprep Maxipreparation kit.

Transfection

pIRES2-EGFP, an expression vector containing EGFP coding region, was transfected into CHO cells by using activated dendrimers in Qiagen superfect transfection reagent. 5µg of purified DNA was used for transfection, and G418 was added to the cell media 48 hours after the transfection at a final concentration of 500 µg/ml for 3 weeks. Media was changed once in every other days. At the end of 3 weeks of G418 exposure, FACS method was employed for seperation of the GFP expressing cells.

Confocal microscopy

T25 flasks were placed onto an inverted microscope (Zeiss 200M) equipped with a laser scanner (LSM Pascal) [13, 14]. 488 nm laser line was used for excitation and the emitted fluorescence was collected through a LP filter at 505 nm. 3-D projection images were constructed from stack of focal image sections.

Results

CHO cells were transfected with pIRES2-EGFP and then selected in G418. After 48 hours following transfection, 5% of cells were transiently transfected (Figure 1B) and their high-level fluorescence faded within 4 days. Following G418 treatment for 3 weeks, untransfected CHO cells did not survive in G418, suggesting that all cells within the transfected pools contained at least one copy of a transcriptionally active kanamycin/neomycin resistance gene. 10% of the stably transfected cells expressed GFP (Figure 2B). GFP expressing cells had a complex morphology which is different from the usual fusiform shape of CHO cells. Even some stably transfected surviving cells showed some similar changes in their morphology though no fluorescence was detected (Figure 2C). Unfortunately, transfected cells could not be sorted by FACS method, due to the low yield in the efficiency of stable transfection.

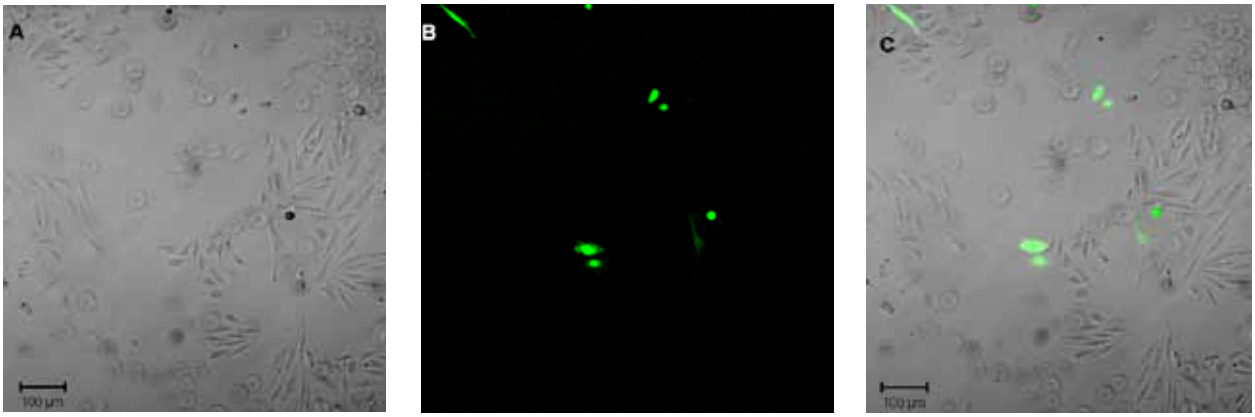

Figure 1: Examples of transient GFP transfected CHO cells. Bright field (A) and fluorosencent (B) pictures of the sample merged into a single picture (C).

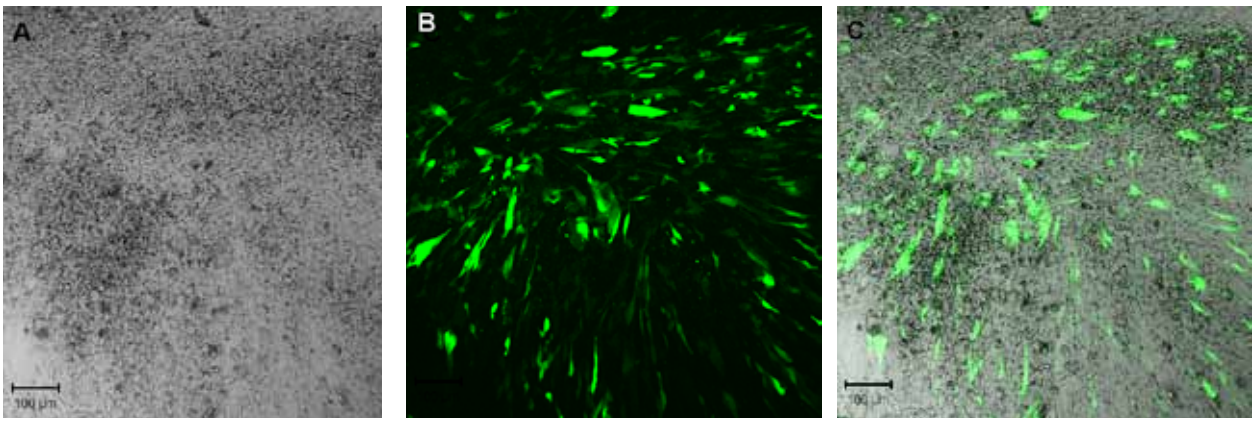

Figure 2: Examples of stable GFP transfected CHO cells. Bright field (A) and fluorosencent (B) pictures of the sample merged into a single picture (C). GFP transfected cells are green.

Discussion

Mammalian cell culture has become important system for the production of proteins for many applications because of their capacity for proper protein folding, assembly and post-translational modification. However, it is an expensive process with low yields and long development times. Due to the growing need for proteins produced in mammalian cell lines there is a requirement to increase culture yields and decrease the time taken to select a suitable producing cell line. GFP is one of the most frequently used selection method. However, it needs be supported by some additional selection methods such as G418 for kanamycin/neomycin resistant gene containing plasmids or selection methods based on flow cytometry and cell sorting [15]. We have used both of the methods and had success with G418 selection.

The density of GFP positive cells after transient and stable transfection is 5 and 10%, respectively. The result is similar to those obtained by using some other transfection methods [1]. The role of activated-dendrimers which have been used for transfection is important for rapid and efficient transfection. Dendrimers are some branched molecules and their branches radiate from a central core and terminate at charged amino groups which can interact with negatively charged phosphate groups of nucleic acids and DNA assembles to form compact structures which may bind to the cell membrane and be taken up by nonspecific endocytosis [16]. The pH of the reagent buffers leads to a pH change in endosome and causes a subsequent inhibition of endosomal nucleases, which ensures the stability of activated dendrimer–DNA complexes. This ensured consistent transfection-complex formation and reproducible transfection results.

The mean fluoresence emission of GFP expressing cells are larger than that in non-expressing control cells and it has not faded away during selection and after selection period as shown by the other studies.

In conclusion, we showed that GFP can successfully be expressed transiently and stably in CHO cells, as a reporter gene. Following selection process in 500 ug/ml of G418, in the absence of selective growth conditions, GFP expression is adequate for identification, isolation and monitoring of stable transfection events in eukaryotic cells. GFP transfected CHO cell lines produced in the present work will be used for further experiments.

References

[1] Gubin AN, Reddy B, Njoroge JM, Miller JL (1997). Long-Term, Stable Expression of Green Fluorescent Protein in Mammalian Cells. BBRC 236:347–350.

[2] Sitton G, Hansgate A, Srienc F (2006). Transient gene expression in CHO cells monitored with automated flow cytometry. Cytotechnology 52:13–24.

[3] Parham JH, Iannone MA, Overton LK, Hutchins JT (1998). Optimization of transient gene expression in mammalian cells and potential for scale-up using flow electroporation. Cytotechnology 28:147–155.

[4] Wadsworth P, Rusan MN, Tulu US, Fagerstrom C (2005). Stable expression of fluorescently tagged proteins for studies of mitosis in mammalian cells. Nature Methods 2(12): 981-987.

[5] Presley JF (2005). Imaging the secretory pathway: the past and future impact of live cell optical techniques. BBA 1744:259-272.

[6] Haraguci T (2002). Live cell imaging: approaching for studies protein dynamics in living cells. Cell Struct Func 27:33-334.

[7] Xu Y, Sun HC, Tian B, Li Y, Chen J, Chen J, Gao DM, Xue O (2004). Establisment of GFP-expressing hepatocellular carcinoma cell lines with different metastatic potential: relevant models for in vivo monitoring of metastasis and anjiogenesis. J Can Res Clin Oncol 130:375-382.

[8] Scruggs AW, Flores CL, Wachter R, Woodbury NW (2005). Development and characterization of GFP mutants with altered lifetimes. Biochemistry 44:13377-13384.

[9] Beiberich E, MacKinnon S, Silva J, Li DD, Tencomnao T, Irwin L, Kapitonov D, Yu RK (2002). Regulation of ganglioside biosynthesis by enzyme complex formation of glycosyltransferases. Biochemistry 41:11479-11487.

[10] Heim R, Cubitt AB, Tsien RY (1995). Improved green fluorescent. Nature 373:664.

[11] Özkara HA, Sandhoff K (2003). Characterization of two Turkish beta-hexosaminidase mutations causing Tay-Sachs disease. Brain Dev 25(3):191-194.

[12] Sinici İ, Zarghooni M, Tropak MB, Mahuran D, Özkara HA (2006). Comparison of HCMV IE and EF-1 Promoters for the Stable Expression of beta-Subunit of Hexosaminidase in CHO Cell Lines. Biochem Genet 44(3-4):168-175.

[13] Cohn PM (1999). Confocal microscopy. Methods Enzymol 307: 1-662.

[14] Puralı N (2005). Structure and function relationship in the abdominal stretch receptor organs of the crayfish. J Comp Neurol 488:369-83.

[15] Browne SM, Al-Rubeai M (2007). Selection methods for high-producing mammalian cell lines. TRENDS in Biotech. 25(9):425-432.

[16] Tang MX, Redemann CT, Szoka FC (1996). In vitro gene delivery by degraded polyamidoamine dendrimers. Bioconjugate Chem. 7:703-714.