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Note

Comparison of HCMV IE and EF-1α Promoters for the Stable Expression of β-Subunit of Hexosaminidase in CHO Cell Lines

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INTRODUCTION

The ability to use in vitro mutagenesis and transfection techniques to generate stable cell lines that highly express a mutant protein associated with a metabolic disease has been an important technique in linking genotype to clinical phenotype (Özkara and Sandhoff, 2003). One group of metabolic diseases is GM2 gangliosidosis (AB-variant, Tay-Sachs and Sandhoff diseases). These diseases are characterized by the accumulation of GM2 ganglioside, primarily in the lysosome of neuronal tissue, leading to progressive neurodegeneration. Tay-Sachs and Sandhoff diseases are caused by defects in the HEXA gene, which encodes the α-subunit, and the HEXB gene, which encodes the β-subunit of heterodimeric N-acetyl-β-hexosaminidase (Hex) (EC 3.2.1.52), respectively (Gravel *et al.*, 2001; Mahuran, 1999). The AB-variant is associated with defects in the GM2 activator protein, a substrate-specific cofactor for Hex A. The second major Hex isozyme in normal human tissue is Hex B, a homodimer of β-subunits.

In this study, we introduced a cDNA encoding the β-subunit of human Hex (βcDNA) into three different eukaryotic expression vectors containing two different promoters, in order to establish which produces the highest expression levels of Hex B activity and β-protein in permanently transfected CHO cells. Our results indicate that the type of promoter employed can be critical in the establishment of highly expressing cell lines.

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MATERIALS AND METHODS

Construction of Plasmids

Three different mammalian expression vectors were used; pIRES2-EGFP and pcDNA3.1D/V5-His-TOPO contained the HCMV IE promoter, and pEFneo carried the EF-1α promoter.

βcDNA (1747 bp) was generated by PCR using primer-a ($5'$-CACCATGGGCTGTGCGGGCGGGGCTGC-$3'$), which encodes the Kozak sequence with a start codon, and primer-b ($5'$-CCTCCA’ITTT TTACATGTTCTCATG-$3'$), which contains the $3'$ stop codon. To lower the PCR mutation frequency, KOD High Fidelity (HiFi) polymerase (Novagen, Japan), which has a $3' \rightarrow 5'$ exonuclease activity, was used. PCR was performed in a total volume of 50 μL as previously described (Sinici *et al.*, 2004). PCR products were separated on a 1% agarose gel and purified by QIAquick gel extraction kit (Qiagen, Canada).

The generated βcDNA was first cloned into pCR2.1-TOPO vector to facilitate cloning into pIRES2-EGFP vector (Clontech, Canada), according to instructions of the TOPO TA Cloning Kit (Invitrogen, USA) protocol. The βcDNA was excised from pCR2.1-TOPO by digestion with *Xho*I-*Bam*HI. The βcDNA was then ligated into the *Xho*I-*Bam*HI digested pIRES2-EGFP vector. This vector coexpresses enhanced green fluorescent protein (EGFP) and allows monitoring of the transfection and selection efficiencies of cells by using fluorescence microscopy. It places the βcDNA under direct transcriptional control of the HCMV IE promoter.

The βcDNA was next cloned into the pcDNA3.1D/V5-His-TOPO (Invitrogen) vector based on the instructions of the Directional TOPO Expression Kit manual (Invitrogen). The βcDNA in pcDNA3.1D/V5-His-TOPO vector is also under the control of HCMV IE promoter. The pcDNA3.1D/V5-His-TOPO/lacZ vector, encoding β-galactosidase, was cotransfected as a control.

The third construct was prepared using pEFneo vector (made by Dr. Anson) (Anson *et al.*, 1992). Once the βcDNA was inserted, by the digestions of *Bam*HI-*Not*I site derived from pcDNA3.1D/V5-His-TOPO vector carrying the βcDNA, its expression was driven by the EF-1α promoter, i.e., the human protein elongation factor promoter.

The three different constructs were transformed into competent *E. coli* cells by One Shot TOP10 Chemical Transformation Kit (Invitrogen). To check the transformation efficiency, we transformed one vial of One Shot Top10 cells with 10 pg of pUC19 using the manufacturer’s protocol. Clones were isolated by QIAprep Kit (Qiagen).

All constructs were confirmed by restriction digestions and sequencing analyses (ACGT Corp., Toronto).

Cell Culture and Transfection

Chinese hamster ovary (CHO) cells were grown in α-Minimal Essential Media (MEM) with 10% fetal calf serum (FCS), penicillin, and streptomycin at 37°C in 5% CO_2. Ten micrograms of each vector was transfected to the CHO cells (40% confluent) using Superfect Transfection Reagent (Qiagen). After 48 h, the cells were trypsinized and diluted ten-fold in α-MEM plus FCS containing 600 μg/μL neomycin. Following 2 weeks growth in drug-containing media, stably transfected neomycin-resistant cells were harvested, and their Hex activity and β-protein– levels determined. Stably transfected (pIRES2-EGFP), living CHO cells were also monitored for their expression of the EGFP reporter gene using a Leica DMIRB fluorescence microscope (Canada).

Hex Activity Assay

Transfected CHO cells were lysed in 10 mM sodium phosphate buffer, pH 6.0, and 5% glycerol containing a protease inhibitor cocktail (Calbiochem, Canada), by six freeze-thaw cycles. Another set of transfected CHO cells was treated in the same manner, with the additional use of proteasome inhibitors lactacystin and kifunensine (Sigma, USA). Protein from cell lysates was quantitated (Bio-Rad, USA) (Bradford, 1976) and the Hex and β-glucuronidase activity levels were determined using the fluorogenic substrates 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MUG) and 4-methylumbelliferyl beta-glucuronide, respectively (Brown and Mahuran, 1991). For CHO cells transfected with pcDNA3.1D/V5-His-TOPO, β-galactosidase activity in lysates was assayed as a transfection efficiency control (Zhang *et al.*, 2000). All experiments were repeated three times in duplicate.

Western Blot Analysis

Samples of transfected and nontransfected CHO cell lysates, carrying equal β-glucuronidase activities, were analyzed by polyacrylamide gel electrophoresis in sodium dodecylsulfate by Laemmli gel system (12.5% gel) using the Bio-Rad mini-gel system (Laemmli, 1970). Western Blot analyses were performed as previously described (Sinici *et al.*, 2004) and protein bands visualized according to the protocol recommended for the Amersham ECL system (Amersham).

RESULTS

Prior to transfections, the nucleotide sequences and orientation of the βcDNA insert were confirmed in all three constructs (data not shown). After 2 weeks of neomycin selection, nontransfected control cells died, and antibiotic-resistant

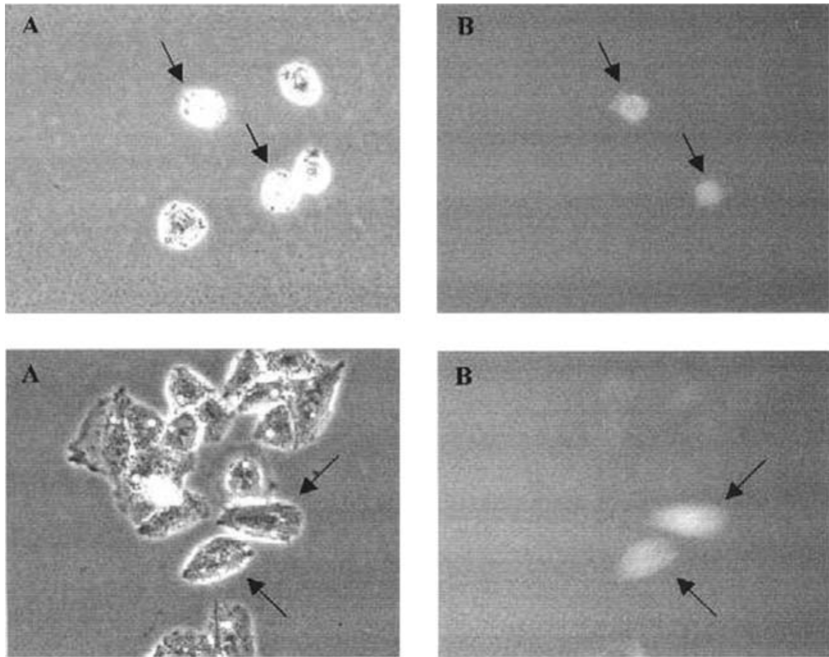

Fig. 1. A: CHO cells were visualized under light microscope. B: EGFP expressions were visualized using fluorescence microscope. *Arrows* indicate the cells having EGFP expressions. *Upper* figures show mock, *lower* figures show βcDNA-inserted pIRES2-EGFP transfected CHO cells.

colonies, transfected with the pIRES2-EGFP vector, expressed the EGFP reporter gene (Fig. 1). In other cells transfected with the pcDNA3.1D/V5-His-TOPO/lacZ construct, significant neutral β-galactosidase activity was observed (Table I). Hex activity levels were then determined (Table I). The resultant specific activities of Hex from the cells transfected with the βcDNA, inserted into either the pIRES2-EGFP or pcDNA3.1D/V5-His-TOPO vectors, were very low, nearly identical to the endogenous levels in mock transfected cell lysates. The inclusion of proteasome inhibitors had no effect on these low levels of Hex activity (data not shown). When the pEFneo construct was used, the human Hex activity was found to be $\sim$1.8 times higher in βcDNA inserted pEFneo transfected cells than in mock transfected cells (Table I).

Extracts from mock and βcDNA-transfected CHO cells were analyzed by Western Blotting using rabbit anti-human Hex B that does not cross-react with endogenous CHO cell Hex (Fig. 2). Although EGFP expression was observed in CHO cells transfected with pIRES2-EGFP, β-subunit proteins (pro β and mature β forms) could not be detected by Western blot analysis. The same lack of detectable β-protein was seen in the pcDNA3.1D/V5-His-TOPO transfected cells. However,

Table I. β-Galactosidase and Hexosaminidase Activities in Mock and βcDNA Transfected CHO Cells

	pIRES2-EGFP transfection		pcDNA3.1D/V5-His-TOPO transfection				pEFneo transfection (Sinici *et al.*, 2004)	
	Hex activiity (nmol/h/μg protein)	Hex activiity (%)	β-galactosidase activity (nmol/h/μg protein)	β-galactosidase activity (%)	Hex activiity (nmol/h/μg protein)	Hex activiity (%)	Hex activiity (nmol/h/μg protein)	Hex activiity (%)
Mock transfected	50.57	100	283.93	100	48.24	100	56.14	100
βcDNA transfected	58.07	114.8	798.77[a]	281.3[a]	51.88	107.5	99.10	176.5

Note. 100% of Hex activity equals the activity found in mock transfected CHO cells.
[a] β-galactosidase activity found in lacZ containing pcDNA3.1D/V5-His-TOPOlacZ construct which was used as a transfection control.

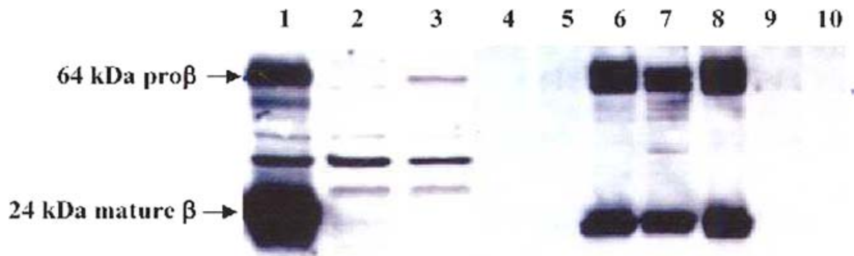

Fig. 2. Western Blot analysis of pEFneo, pIRES2-EGFP, and pcDNA3.1D/V5-His-TOPO transfected CHO cell lysates. The positions of the pro β and mature β protein bands are indicated by *arrows*. *Lane* 1: (+) control; CHO cell lysates transfected with βcDNA inserted pEFneo. *Lane* 2: (−) control; mock-transfected CHO cell lysates. *Lane* 3: CHO cell lysates, transfected with βcDNA inserted pIRES2-EGFP. *Lane 4*: (−) control; mock-transfected CHO cell lysates that were treated with lactacystin. *Lane* 5: (−) control; mock-transfected CHO cell lysates that were treated with kifunensine. *Lanes* 6 and 8: (+) control; CHO cell lysates transfected with βcDNA inserted pEFneo that were treated with lactacystin. *Lane* 7: (+) control; CHO cell lysates transfected with βcDNA inserted pEFneo that were treated with kifunensine. *Lane* 9: CHO cell lysates transfected with βcDNA inserted pcDNA3.1D/V5-His-TOPO that were treated with lactacystin. *Lane* 10: CHO cell lysates transfected with βcDNA inserted pcDNA3.1D/V5-His-TOPO that were treated with kifunensine.

extracts from pEFneo-transfected cells contained both a strong pro β-chain band and the processed mature β-chain band, consistent with a high level of βcDNA expression in these cells (Sinici *et al*., 2004).

DISCUSSION

This study examines the relative effectiveness of the two common promoters in expressing the β-subunit of Hex. In an attempt to get a high level of expression of β-subunits in CHO cells, we inserted the βcDNA into two new generation vectors pIRES2-EGFP and pcDNA3.1D/V5-His-TOPO, each carrying the HCMV IE promoter, and the older pEFneo vector, carrying EF-1α promoter. Interestingly, in neomycin-resistant cells transfected with vectors containing the HCMV IE promoter, no significant β-subunit protein or increase in Hex B activity was detected. Expression of the β-subunit (activity and protein) was observed only when the βcDNA was under the control of the EF-1α promoter.

Others have reported differences in expression of heterogeneous genes under EF-1α and HCMV promoters in certain cell types (Gopalkrishnan *et al*., 1999; Kim *et al*., 2002; Teschendorf *et al*., 2002; Tokushige *et al*., 1997). Chung *et al.* (2002) found that the EF promoter was 200–300 times stronger than the HCMV promoter in driving the expression of the humanized renilla green fluorescent protein (hrGFP) gene. In another report, the EF-1α promoter was found to be 3-fold more active than the HCMV promoter in transfected CHO cells (Kim *et al*., 2002). As well, the expression of cDNAs under the EF-1α promoter was reported

to be unaffected by the passage number or time elapsed between introduction and integration of the plasmid DNA. It is possible that with time, CHO cells may develop methods of suppressing the HCMV IE promoter. It has been suggested that expression driven by the EF-1α promoter would be relatively independent of cell type and difficult to suppress, because of its indispensable housekeeping function in all mammalian cells (Goldman *et al.*, 1996; Kim *et al.*, 1990).

Furth *et al.* (1994) reported, based on histochemical analysis, that the cDNA expression driven by the HCMV IE promoter was highly variable, even in the same cell type. Teschendorf *et al.* (2002) described a scattered or mottled pattern of expression of the green fluorescence protein (GFP) in stable cell lines transfected by vectors carrying HCMV promoter. On the other hand, EF-1α promoter gave rise to a remarkably homogeneous cell population expressing the GFP gene. We observed a similar situation in our neomycin-resistant cells expressing the EGFP reporter gene (Fig. 1). Thus the expression levels driven by the HCMV IE promoter can vary considerably even from cell to cell within a stably transfected cell line.

We conclude that any inexplicable failure to establish cell lines stably expressing a cDNA of interest, such as the β-subunit of Hex, may be due to promoter selection. Certainly, in our case the HCMV IE promoter was not the best choice, and by switching to an expression vector utilizing the human EF-1α promoter, we significantly increased the level of expression of the β-subunit of Hex in CHO cells.

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