EXPRESSION ACTIVITY OF SUPER CORE PROMOTER BETA AND ITS HYBRIDS IN CHO-K1 CELLS

By

Tharatree Srichan

A Thesis Submitted in Partial Fulfillment of the Requirement for the Degree

MASTER OF PHARMACY

Program of Biopharmaceutical Sciences

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การแสดงออกของซุปเปอร์คอร์โปรโมเตอร์บีต้าและลูกผสมในเซลล์เพาะเลี้ยง CHO-K1

โดย
นายธาราธีร์ ศรีจันทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชศาสตร์ชีวภาพ
บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
ปีการศึกษา 2554

The Graduate School, Silpakorn University has approved and accredited the thesis title of “Expression Activity of Super Core Promoter Beta and Its Hybrids in CHO-K1 Cells” by Tharatree Srichan in partial fulfillment of the requirements for the degree of Master of Pharmacy, program of Biopharmaceutical Sciences.

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Recombinant gene promoter optimization is one of the most efficient methods which is able to increase recombinant protein production using mammalian cell culture. This study therefore is to develop a core promoter by introducing transcription factor II B recognition element (BRE) into the super core promoter (SCP). The result presented that reporter gene expression under the control of a SCP containing BRE (SCP beta) was higher than under the control of the SCP in Chinese hamster ovary (CHO) cells by 49% and 35% increase in serum supplemented and serum depleted cell culture, respectively. Furthermore, the study of hybrid enhancer/SCP beta activity demonstrated that different enhancer type generated various promoter strengths. It varied from non-effect in hybrid human EF-1α/SCP beta, about 11-fold increase in hamster β-actin/SCP beta, about 21-fold increase in hamster GADD 153/SCP beta, to about 287-fold increase in CMV IE/SCP beta by comparison with SCP beta activity. The similar trend is also observed in serum-free medium. Altogether, it could be concluded that BRE consensus sequence, locating at position -38 to -32 (upstream of TATA-box) of the SCP up-regulates transcriptional expression of gene. The hybrid hamster β-actin/SCP beta, the hybrid hamster GADD 153/SCP beta and the hybrid CMV IE/SCP beta could increase transgene expression in both cells cultured with completed medium and serum-free medium. This information would lead an idea to advance promoter activity for biological research use and improve therapeutic protein production efficiency.

Program of Biopharmaceutical Sciences Graduate School, Silpakorn University Academic Year 2011
Student's signature.....
Thesis advisors' signature 1. …… 2. ……..……...

50355201 : สาขาวิชาเภสัชศาสตร์ชีวภาพ
คำสำคัญ : ซุปเปอร์คอร์โปรโมเตอร์ / โปรโมเตอร์ลูกผสม / เซลล์เพาะเลี้ยง CHO

ธาราธีร์ ศรีจันทร์ : การแสดงออกของซุปเปอร์คอร์โปรโมเตอร์บีต้าและลูกผสมในเซลล์เพาะเลี้ยง CHO-K1. อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ภก. ผศ. ดร. วิสิฐ ตั้งเคียงศิริสิน และ ภญ. ผศ. ดร. สิริพรรณ ลิ้มศิริชัยกุล. 93 หน้า.

การเพิ่มฤทธิ์โปรโมเตอร์ของ recombinant gene เป็นวิธีที่มีประสิทธิภาพวิธีหนึ่งในการเพิ่มศักยภาพการผลิต recombinant protein ด้วยเซลล์เพาะเลี้ยงของสัตว์เลี้ยงลูกด้วยน้ำนมได้ การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อปรับปรุงคอร์โปรโมเตอร์ด้วยการใส่ transcription factor II B recognition element (BRE) ให้กับซุปเปอร์คอร์โปรโมเตอร์ (SCP) ผลพบว่าซุปเปอร์คอร์โปรโมเตอร์ที่มี BRE (SCP beta) มีฤทธิ์ทำให้ยีนรายงานผลแสดงออกในเซลล์ CHO ได้มากกว่าซุปเปอร์คอร์โปรโมเตอร์ (SCP) โดยมีผลทำให้การแสดงออกของยีนรายงานผลเพิ่มมากขึ้นประมาณร้อยละ 49 ในเซลล์ที่เลี้ยงในอาหารแบบมีซีรั่ม และประมาณร้อยละ 35 ในเซลล์ที่เลี้ยงในอาหารแบบปราศจากซีรั่ม และการศึกษาฤทธิ์ของซุปเปอร์คอร์โปรโมเตอร์บีต้าลูกผสมพบว่าชนิดของ enhancer มีผลต่อฤทธิ์ของโปรโมเตอร์ลูกผสมแตกต่างกัน โดย human EF-1α/SCP beta มีฤทธิ์ไม่แตกต่างจาก SCP beta แต่ hamster β-actin/SCP beta, hamster GADD 153/SCP beta และ CMV IE/SCP beta มีฤทธิ์เพิ่มขึ้นประมาณ 11, 22 และ 287 เท่าในเซลล์ CHO ที่เลี้ยงด้วยอาหารแบบมีซีรั่มตามลำดับ และผลการทดลองเป็นไปในทิศทางเดียวกันเมื่อทดสอบในเซลล์ที่เลี้ยงแบบปราศจากซีรั่ม จากผลการทดลองทั้งหมดพอจะสรุปได้ว่า BRE ที่ตำแหน่ง -38 ถึง -32 (ทางด้าน 5' ของ TATA-box) ของซุปเปอร์คอร์โปรโมเตอร์มีผลเพิ่มการแสดงออกของยีนในระดับ transcription และโปรโมเตอร์ลูกผสม hamster β-actin/SCP beta, hamster GADD 153/SCP beta และ CMV IE/SCP beta มีฤทธิ์ทำให้ transgene แสดงออกในเซลล์ CHO เพิ่มมากขึ้นทั้งแบบที่เลี้ยงในอาหารที่มีซีรั่มและแบบปราศจากซีรั่ม ข้อมูลจากการศึกษาในครั้งนี้คงจะช่วยก่อให้เกิดแนวคิดในการพัฒนาคุณสมบัติของโปรโมเตอร์เพื่อใช้ในการศึกษาวิจัยเกี่ยวกับการแสดงออกของยีน รวมทั้งการเพิ่มประสิทธิภาพในการผลิตโปรตีนที่มีคุณสมบัติในการรักษาโรคต่อไปในอนาคต

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รายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ 1. ……... 2. ……..

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This thesis is aimed to create the new generation of promoters for mammalian expression vector. It is a little study, but could not be accomplished without the great support by my graduate advisors, Asst. Prof. Dr. Wisit Tangkeangsirisin and Asst. Prof. Dr. Siripan Limsirichaikul. I'm much appreciated. I cannot forget to thank you to all of them. I would also like to thank Prof. Dr. Prasert Auewarakul for material supplement. I would like to present my sincere gratitude to the member of thesis committee, Dr. Piyanuch Jongsamak, Dr. Krit Thirapanmethee and Dr. Perayot Pamonsinlapatham who devoted their time to suggest me to write this report. Without their guidance and reviews, this thesis cannot complete. I always keep in mine about people who gave me knowledge and many tools. This thesis has been finished because of many helps from laboratory members, Miss Sirikul Dangmanee, Miss Siripun Tipluechar and Miss Arporn Kaeopardtana. I also thank to my best friend Mr. Niti Osirisakul who always stand by me even when I lost my mind. Finally, I would like to mention to my smiling mother, Mrs. Ploi Srichan who stands behind this success and raises me up whenever I fall.

Tharatree Srichan

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CHAPTER 1

INTRODUCTION

1. Statements and Significance of the Problem

Advancement in biotechnology led to produce many therapeutic biologics. Most of these drugs are efficient for prevention or treatment severe diseases including coronary artery diseases, auto-immune disease and cancers (Robert et al. 2011).

The biologics are classified into recombinant proteins, monoclonal antibodies, vaccines, toxins, blood products and others. The recombinant proteins are the major group (Matasci et al. 2008). Monoclonal antibody is a significant class of biologics. Monoclonal antibodies are produced by both recombinant DNA technology and hybridoma technology. Muromonab (OKT3) is the first approved for transplant rejection (Stephanopoulos 1993). Other therapeutic monoclonal antibodies can protect viral infections, relieve autoimmune diseases and cure certain type of cancers (Miller et al. 2007). Erythropoietin is the first recombinant human protein product that approved to treat anemia in chronic renal failure. Tissue plasminogen activator is one of long time used recombinant protein that has been approved for the prevention of pulmonary embolism and heart attack. Therapeutic recombinant proteins are continuously required at high level; according to its high potency, unique property and specificity of action although recombinant proteins are relatively expensive. Erythropoetin valued more than 1 billion dollars annually (Wurm 2004). The monoclonal antibodies for therapeutic use earned approximately 11 billion in 2004 (Pai, Sutherland and Maynard 2009). Nevertheless, there are many recombinant proteins that have a therapeutic potency. Present intensive studies of biotechnology worldwide will increase approval biologics in the near future.

However, some patients cannot access these agents because of the product deficiency and/or its high cost. The problem is resulted from research and development which consumes high investment and manufacturing process consists of many difficult performances, but low yield. License free for local supplier and developments of biological technology seem like a future hope for the patient in developing country.

1.1 Therapeutic Recombinant Protein Production

Production of therapeutic recombinant protein fundamentally includes genetic engineering, protein expression in production host and product recovery.

1.1.1 Genetic Engineering

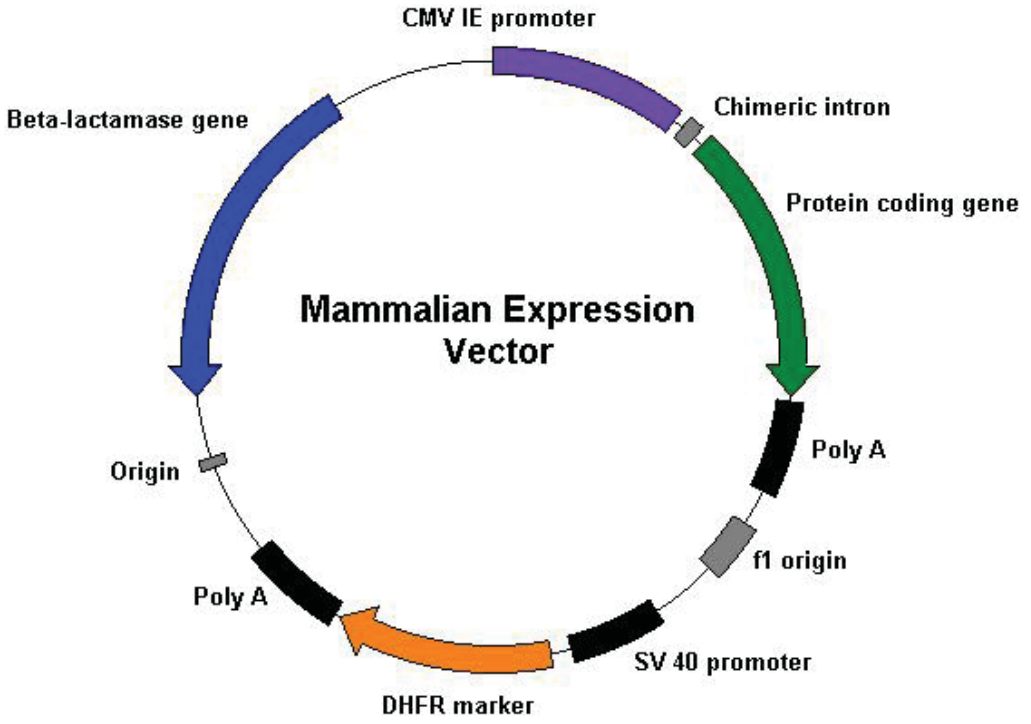
Genetic engineering is the first step of recombinant protein research and development to produce the target protein. The complementary DNA is first synthesized using mRNA as template. Sometimes, the coding sequence is modified to optimize protein function as well as to express in the host system, properly. The synthetic DNA fragment is preferably inserted into an expression vector (circular double stand DNA). The vector consists of several genes and DNA cassettes, for examples, a promoter of recombinant DNA, recombinant protein-coding sequences and mammalian selectable marker gene (Figure 1). Some plasmid DNA consists of every feature described, but typical plasmid does not contain every item. Prokaryotic expression plasmid and eukaryotic expression vector contains a difference DNA sequence in the several features, especially regulatory elements and translation codons (Lynch 2006).

1.1.2 Expression of Recombinant Protein in Production Host

This step includes recombinant gene transfer and cultivation. Bacterium is generally preferred as a primary production host because it can be manipulated easily and provide high recombinant protein yield. Yeast is generally the second choice if more host properties are required. However, about 65% of therapeutic recombinant proteins are expressed in mammalian cells. Although mammalian cell is hard to manipulate, it provides proper protein folding, post-translational modification and protein assembly. The most preferable mammalian host is Chinese hamster ovary (CHO) cell. Baby hamster kidney (BHK) cells, mouse myeloma (NS0) cells and human embryo kidney (HEK-293) cells are the alternative choices (Wurm 2004).

1.1.3 Product Recovery and Purification

Recombinant protein is normally designed as a secretory protein that makes it easy to be harvested directly from culture medium. Often, expression of intracellularly protein is necessary to preserve its active form. This protein could be collected by cell disruption. All products are purified to eliminate adventitious contaminants. There are several methods for purification process, for example, precipitation, ultrafiltration and chromatographic methods (Wang et al. 2005).



Feature

Poly A : polyadenylation signal

f1 origin : origin of bacteriophage f1 DNA replication

DHFR marker : dihydrofolate reductase gene

Origin : plasmid origin of replication

Figure 1 Schematic represents the features in a typical mammalian expression vector.

1.2 Development of Bioprocess for Recombinant Protein Production Using Mammalian Host

The demand of therapeutic protein is increasing, but product amount is not enough for supply (Bishop and Lawson 2004). Thus, productivity is needed to be developed rapidly. There are several approaches for this purpose as follows.

1.2.1 Promoter Activity Optimization

Promoter optimization is one of the most potential methods to enhance the recombinant protein production in CHO cell. Cytomegalovirus immediate early (CMV IE) promoter is one of the most extensive used (Primrose and Twyman 2006). Unfortunately, The CMV promoter activity in CHO cell is lower than that in other cells such as HeLa cell (Cheng et al. 2004). Optimization of promoter that is good activity in CHO cell would be advantage. A synthetic promoter could be applied to over saturated level of transgene expression. For example, a hybrid cytomegalovirus immediate early (CMV IE) enhancer/super core promoter is stronger than the wild type CMV IE promoter in HeLa cell (Juven-Gershon, Cheng and Kadonaga 2006). This activity is increased by initiator (Inr), motif ten element (MTE) and downstream promoter element (DPE) addition at the super core promoter. However, the most potent core promoter in mammalian expression plasmid lacks transcription factor II B recognition element (BRE) which was reported that transcription factor II B is required by RNA polymerase II to initiate gene transcription at core promoter (Kostrewa et al. 2009). Typically, promoter activity depends on type of *cis*-acting element, location and number of responsive elements aligning promoter/enhancer (Brown and Brown 2002).

1.2.2 Gene Coding Protein Codon Modification

Abundance of each type of transfer ribonucleic acid (tRNA) in the cell is difference. Gene with codons that recognized by enrich cognate tRNA generally express at high level. Changing rare tRNA codon to abundance tRNA codon would help protein expression at translation step (Makrides 1999). Green fluorescent protein gene was demonstrated that codon optimization (native codon to mammalian codon) increase both the expression of the gene and fluorescent intensity (Zolotukhin et al. 1996 ; Yang, Cheng and Kain 1996). However, the expression of codon-optimized gene also depends on cell type.

1.2.3 Host Cell Engineering

Recently, host cell engineering was focused on cell proliferation control, enhancement cellular viability, increase of protein secretion capacity and modulation of post-translational modification (Matasci et al. 2008). Chinese hamster ovary cell is the most preferable mammalian host (Ratledge and Kristiansen 2006). It was engineered for suspension cultivation with growing ability in bioreactor at high density. It is also transformed to culture in serum free medium. At the maximum level of production, the stable CHO cells could yield the recombinant protein about 100 mg per 10^6 cells day (Fouser et al. 1992).

1.2.4 DNA Transfer

Packaging plasmid DNA into a cell line could be preformed either chemical-based transfection or physical-based transfection. Calcium phosphate, liposome, polymer and protein are an example of chemical-based carriers while an electroporation is a popular method of physical-based transfection. Although there are several methods for DNA transfer but it is hardly to determine what method is the best for transfer plasmid DNA into the mammalian cells. Direct comparison of these carriers or developing a new tool would perform to optimize the transfection efficiency**,** especially, in bioreactor platform which is required to produce high protein yield (Haldankar et al. 2006).

1.2.5 Stable Cell Selection

Stable cell selection is generally performed using antibiotic resistant maker (neomycin, hygromycin and puromycin) but the selection of the stable CHO cell generally uses dihydrofolate reductase (DHFR) marker because it shows the high degree of selectivity. This method could be preformed by exposing the cells to methotrexate. Transfected cells with recombinant gene expression are selected in first round. The clones with highly expressed protein of interest are picked up in second or third round. There are many selectable markers for a stable cell selection. However, this process generally takes times. Nevertheless, the stable cells usually have lower amount of recombinant protein yield than transiently transfected cells because of gene silencing. Thus, development of selectable stringency would help to increase protein yield by decreasing selection period and rushing to production process before gene silencing occur (McBurney et al 2002 ; Chen et al. 2011).

1.2.6 Cell Culture Medium Formulation

Medium formulation is one of the most popular methods because the cost of medium supplementation with serum is very expensive. Purification of product from medium containing serum is also very difficult to eliminate dangerous contamination (virus and prion). Serum-free medium enriched of essential nutrients and growth hormones has been being optimized to achieve high yield protein and lower production cost. However, serum contains many essential substrates for cell such as growth factors, hormones, lipids, vitamins and other trace elements (Stoll et al. 1995). Formulation might mimic these components. Nevertheless, glucose and glutamate that are used as carbon and nitrogen sources might be replaced by other substrates to delay the accumulation of lactate and ammonium in the medium (Kim et al. 2006).

Altogether, promoter activity optimization and cell culture medium formulation are the most reported potential methods. The optimized promoter directly increases recombinant gene expression while serum-free medium drecrease production cost and sequentially reduces protein purification process. However, others methods should be developed to elevate production efficacy that would help to increase supply and lower therapeutic recombinant protein production cost.

2. The Focus of This Research

This study focuses on promoter activity optimization. There are two types that have been developed; synthetic promoter and hybrid promoter. The super core promoter (SCP) is one of the most interesting synthetic promoters. It was created by Juven-Gershon and colleagues by incorporating four core promoter motifs into a single promoter (Juven-Gershon et al. 2006). However, it lacks transcription factor II B recognition element (BRE) that recognized by transcription factor II B (TF IIB) which is required by RNA polymerase II to initiate transcription of gene. This study therefore introduced the BRE into the SCP and named super core promoter beta (SCP beta). After that, four hybrid enhancer/super core promoter beta promoters were constructed to increase the promoter activity using housekeeping gene's enhancer. All promoter constructs were designed to increase the expression activity in CHO-K1 cells. The promoter activities were quantified in both CHO-K1 cell cultured in completed medium and serum-free medium to evaluate the potency for industrial use.

3. Goal and Objectives

The study aims to increase the promoter activity on reporter gene expression in CHO cells. First, to optimize the core promoter activity, a super core promoter beta (SCP beta) will be constructed by introducing the transcription factor II B recognition element into the super core promoter (SCP). Second, to potentiate the promoter activity, a hybrid human elongation factor-1α/SCP beta, a hybrid hamster β-actin/SCP beta, a hybrid hamster growth arrest and DNA damage inducible 153/SCP beta and a hybrid cytomegalovirus immediate early/SCP beta will be generated. All promoter constructs will be tested their activity in transiently transfected CHO-K1 cells cultured in serum-supplemented medium and serum-depleted medium. The SCP beta activity will be compared with its prototype. The hybrid enhancer/SCP beta activity will be analyzed to determine the strongest promoter.

4. The Research Hypothesis

The super core promoter activity on reporter gene expression in CHO cells could be enhanced by adding of transcription factor II B recognition element (BRE) into the SCP. The new series of super core promoter (SCP beta) activity could be potentiated by linkage house keeping's gene enhancer such as the human elongation factor-1α enhancer extended through intron1, the hamster β-actin enhnancer/proximal promoter, the hamster growth arrest and DNA damage inducible 153 enhnancer/ proximal promoter and the cytomegalovirus immediate early enhancer upstream of the core promoter.

CHAPTER 2

LITERATURE REVIEWS

1. Regulation of Protein-coding Gene Expression

The gene expression process composes of two major steps including transcription and translation. Transcription generates messenger ribonucleic acid (mRNA) and translation generates polypeptide using the messenger RNA (mRNA) as a template. The template is polymerized by RNA polymerase II. Gene expression is strongly regulated to maintain cell homeostasis. Most genes is controlled in several stages of gene expression process including activation of gene's promoter, processing of the transcription, transportation of mRNA from nucleus to cytoplasm and translation of mRNA. Generally, "Eukaryotic gene expression is usually controlled at the level of initiation of transcription" (Lewin 2008). Without activation of transcription initiation, the following steps cannot be processed. This chapter therefore provides detail of gene transcription, focusing on transcription initiation.

1.1 Transcription of Protein Coding Gene

Gene transcription is the first process of gene expression which produces mRNA. Transcription process composes of three main step; transcription initiation, transcription elongation and transcription termination.

1.1.1 Transcription Initiation

Transcription initiation requires a multiple initially transcribing complex (ITC), so call pre-initiation complex (PIC). The complex composes of DNA which named promoter/enhancer and many classes of transcription factor. Promoter is a DNA region which locates immediately upstream of protein coding sequence. Its length is generally definited not more than 1 Kbp (Figure 2) that is classified into two subsidiary regions, proximal promoter and core promoter. Proximal promoter is catched with transcriptional activator or specific DNA sequence binding proteins which induce recruitment of basal transcription factors which allows ITC formation on core promoter with an essential activity for initiation of gene transcription (Maston, Evans and Green 2006).

(A) Promoter and Enhancer

Promoter is a region of DNA which facilitates the transcription of gene. Enhancer is a region of DNA which enhances transcription level of gene. Enhancer supports transcriptional activation (enhancer usually locates upstream of proximal promoter but may locate at 3' untranslated region of the gene). Silencer serves to repress transcription, insulator blocks transcription effect of neighboring genes, locus control region plays a role in gene activation, proximal promoter serve the recognition elements for activators that synergistically stimulate transcription. A core promoter is a docking site for pre-initiation complex assembly with defines major transcription start site (Maston et al. 2006)

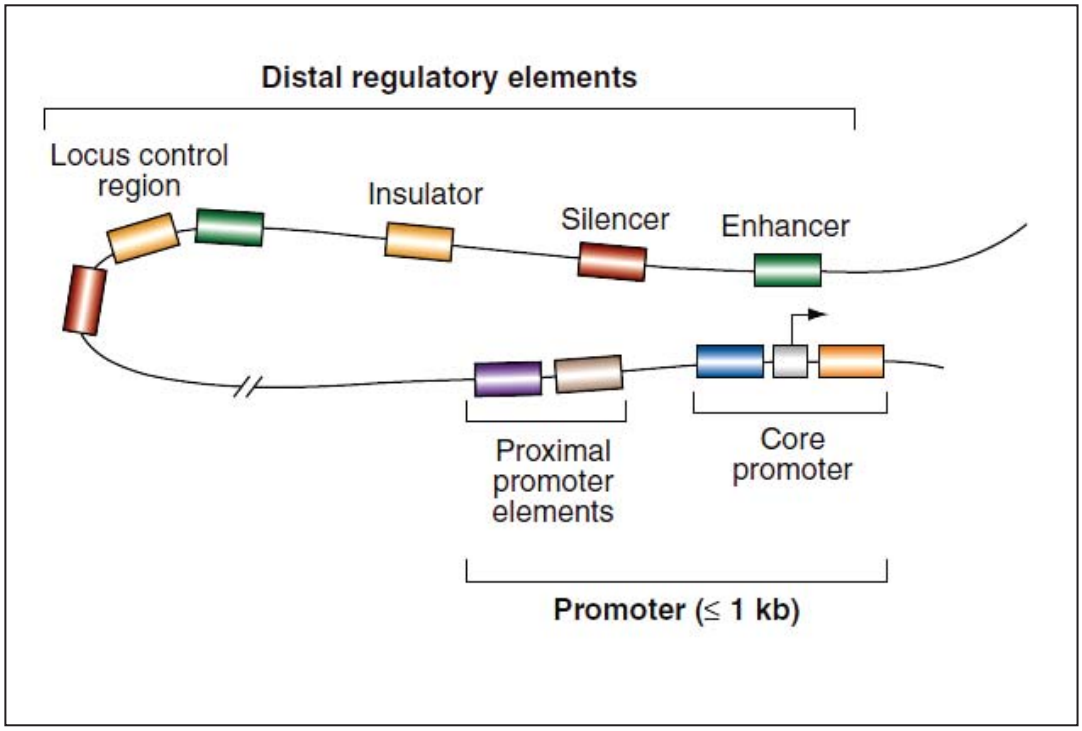


Figure 2 Schematic represents common distal regulatory elenments that locate at distance region of gene and promoter.

Source : Glenn A. Maston, Sara K. Evans and Michael R. Green, "Transcriptional Regulatory Elements in the Human Genome," The Annual Review of Genomics and Human Genetics 7 (2006) : 31.

(B) Transcription Factor

Transcription factor refers to protein with gene transcription effect. There are many types of animal transcription factor (Table 1). Three thousand types were assumed for human (Lander et al. 2001). Generally, they could be basically classified into three groups. The first group plays a role in many genes. They are chromatin remodelling and modification factors. The second group acts as sequence-specific DNA binding protein that mediates gene transcription activation or repression. The final class is basal transcription factor including TFIIB and TFIID that work together with RNA polymerase to generate RNA synthesis (Levine and Tjian 2003).

Transcription factor II B (TFIIB) is one of the transcription factors that play a role in transcription initiation. TFIIB recognizes transcription factor II B responsive element (BRE) that locates either upstream or downstream of TATA-box. The consensus sequence of the BRE locating immediately upstream of the TATA element (BREu) has no similarity to the sequence of BRE locating downstream of the TATA-box (BREd).The BREu consensus sequence is 5'-G/C-G/C-G/A-C-G-C-C-3' (G/C means guanine or cytosine) (Lagrange et al. 1998). The BREd sequence is 5'-G/A-T-T/G/A-T/G-G/T-T/G-T/G-3' (T/G/A means thymine or guanine or adenine) (Deng and Robert 2005). The BREd has deviant much more than the BREu. Both BREu and BREd were reported that it provided a positive effect on *in vitro* transcription, but it decrease effect of GAL4-AH transcriptional activator (Evans, Fairley and Roberts 2001; Deng and Robert 2005). However, recente study found that TFIIB is required by RNA polymeras II to initiate gene transcription. Mutations of TFIIB decreases *in vitro* transcription expression level in yeast model because it is unable to open the promoter. Nevertheless, adenine at position -8 and initiator (Inr) are also important for transcription start site selection (Kostrewa et al. 2009).

Moreover, it was reported that TFIIB combines promoter recognition by recruitment of RNA polymerase II. TFIIB-RNA polymerase II complex is essential to open promoter and selects transcription start site. The TFIIB regulates transcription rate via the B-linker. Therefore, the TFIIB is the rate-limiting step on transcription initiation of gene (Wiesler and Weinzierl 2011).

Table 1 Transcription factors and their recognition elements

Transcription factors	**Responsive elements**	**Consensus sequences 5' end to 3' end**	**Actions**
TFIID	Initiator	TCAGTT	TSS scan
TBP	TATA_box	TATAAAA	PIC
TFIID	MTE	CGAGCCGAGC	PIC
TFIID	DPE	GGTTG	PIC
SP1	SP1	CCCGCCCC	Stimulate
CREB1	CRE	TGACGTCA	Stimulate
NF-κB	NF-κB	GGGAATTTCC	Stimulate
AP1	AP1	TGACTCA	Stimulate
CEBPA	CAAT	TTTCGCAAT	Stimulate
EST	EST	GGAA/T	Vary
Myb	Myb	GGCGGTTG	Stimulate
Myc	Myc	CGCACGTGGC	Stimulate
GATA	GATA	AGATAAGA	Stimulate
Methylation factors	CpG island	GC scattering	Controversial

Nucleotide sequences of responsive elements in this table are mammalian consensus sequences (Portales-Casamar et al. 2009).

(C) Transcription Initiation Mechanism

Chromatin forms a compact structure (solenoid structure) in background phase. This compact structure does not transcriptionally active, but alteration of the structure by chromatin remodeling and modification factor was a dynamic mechanism (Mellor 2005). The demodeling stage of chromatin allows basal and/or specific transcription factors bind to its responsive elements that mediate ITC formation, immediately. Basically, promoter/enhancer remodeling allows DNA binding domain (DBD) of activators to bind its recognition responsive elements. In another site of activator, activation domain (AD) was purposed to interact with co-activator and basal transcription factors. Some basal transcription factors directly bind its responsive element such as TATA binding protein. These interactions recruit other transcription factors (including mediator) and induce a pre-initiation complex (PIC) formation (Maston et al. 2006). The recruitment directly brings RNA polymerase II to the core promoter (Figure 3) which requires transcriptional factor II B to scan the major transcription start site (TSS) locating in initiator (Inr) that resulting activates mRNA synthesis (Kostrewa et al. 2009).

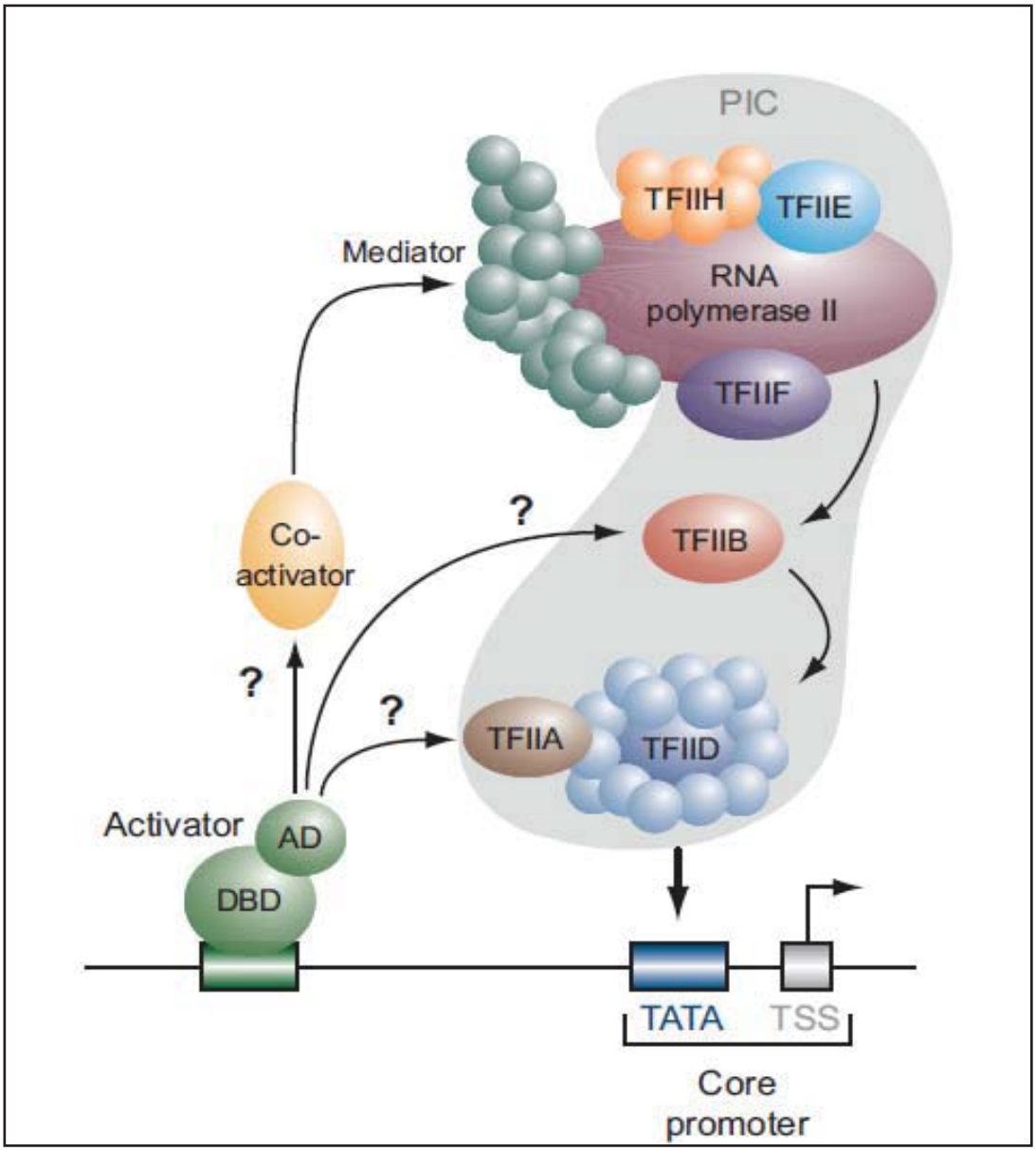


Figure 3 Complexation of preinitiation complex that initiates mRNA synthesis.

Source : Glenn A. Maston, Sara K. Evans and Michael R. Green, "Transcriptional Regulatory Elements in the Human Genome," The Annual Review of Genomics and Human Genetics 7 (2006) : 32.

1.1.2 Transcription Elongation

Transcription elongation is polymerized by RNA polymerase II. This process starts immediately after the promoter escapes which is illustrated in Figure 4. Initial transcribing complex (ITC) composes of RNA polymerase II, TFIIB, TBP and template DNA at least. Polymerization occurs within the ITC. The first 4 nucleotides are synthesized in the unwinding bubble and are stablized by switch 2 domain of RNA polymerase II and TFIIB. ITC stress is increased after RNA is added the fifth nucleotide. The stress probably contributes the escape of promoter and release TFIIB from the ITC. Then, the ITC has been changed to elongation complex that drives polymerization along DNA template with unwinded and re-annealed the template (Saunders, Core and Lis 2006).

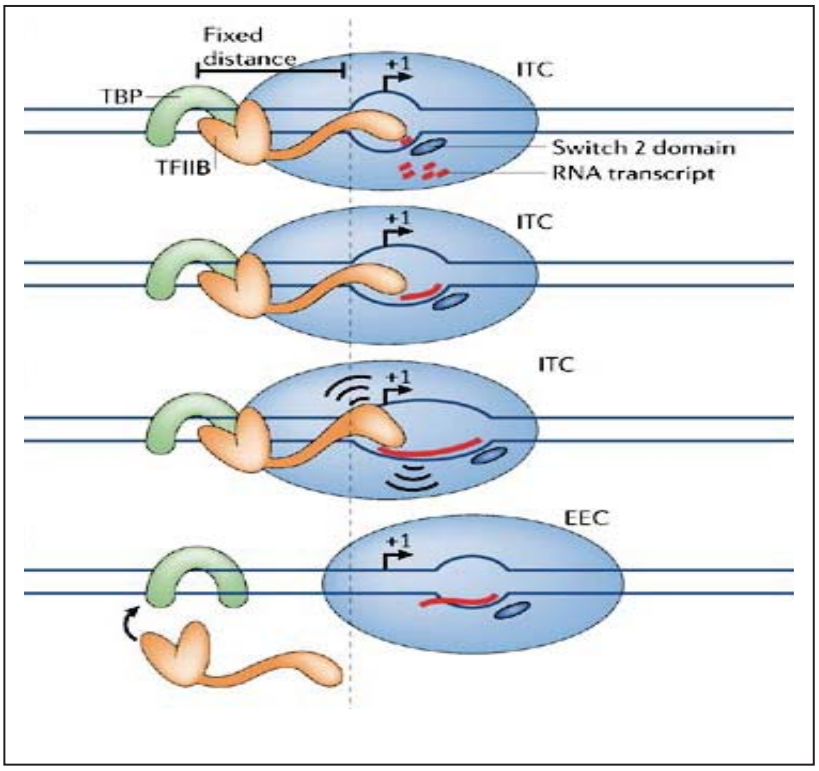


Figure 4 Schematic represents RNA synthesis and promoter escape.

Blue ellipse : RNA polymerase II. EEC : early-elongation complex.

Source : Abbie Saunders, Leighton J. Core and John T. Lis, "Breaking barriers to transcription elongation," Nature Reviews Molecular Cell Biology 7 (2006) : 561.

1.1.3 Transcription Termination

Transcription is terminated by cleavage at nearing polyadenylic acid sequences. An intact polyadenylation signal is necessary for transcription termination of protein-coding genes (Whitelaw and Proudfoot 1986 ; Connelly and Manley 1988 ; Richard and Manley 2009). Clevage and polyadenylation occur at the 10 to 30 nucleotides long from a conserved AAUAAA sequence of mRNA. The sequence is recognized by clevage and polyadenylation factor (Figure 5). RNA polymerase II is finally released from the DNA template to terminate transcription. The 3' end of mRNA is protected from exonuclease by poly (A) binding protein that also corrects polyadenylic acid synthesis. The mRNA is completely modified before export to cytoplasm. The mRNA is further used as a template to translate into protein.

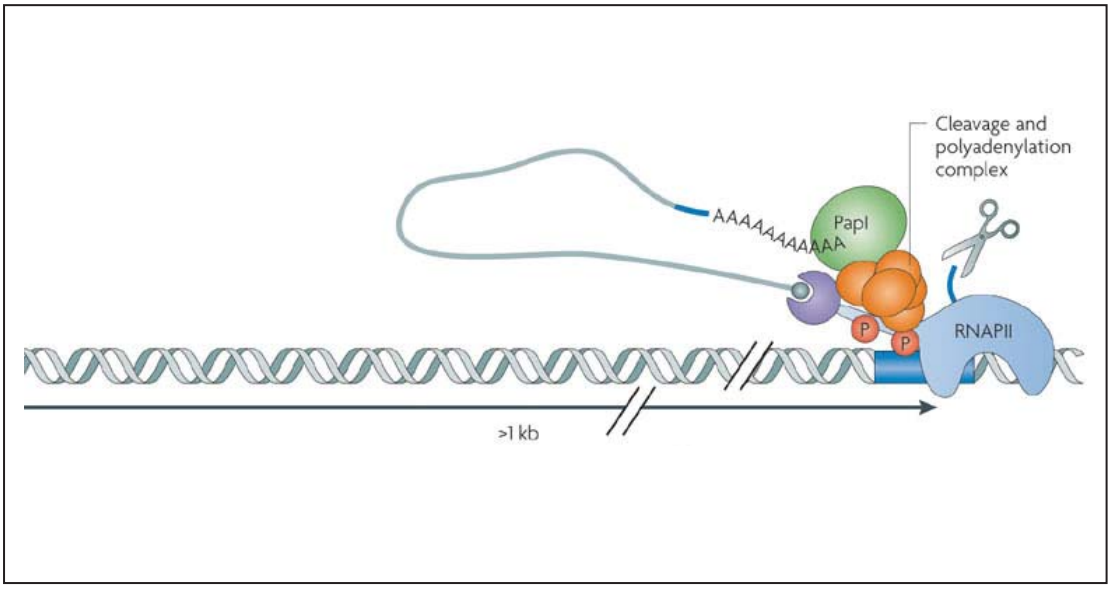


Figure 5 Schematic represents the transcription termination.

Source : Alain Jacquier, “The complex eukaryotic transcriptome: unexpected pervasive transcription and novel small RNAs,” Nature Reviews Genetics 10 (2009) : 840.

In summary, the crucial step of gene transcription is transcription initiation. Pre-initiation complex is essential to drive transcription of gene that allows the downstream process. Thereby, the core promoter is important in gene expression.

2. Commonly Used Promoters in Molecular Biotechnology

The most commonly used promoter for transgene expression is TATA containing promoter that interacts with TBP and locate RNA polymerase II to initiate the transcription. There are many applicable promoters (Table 2) that could be selected for specific usage.

Table 2 Commonly used promoter in molecular cloning

Promoter names	Applications
Cytomegalovirus immediate-early (CMV IE)	Recombinant gene
Simian virus 40 early (SV40)	Selectable gene
Human phosphoglycerate kinase 1 (PGK)	Selectable gene
Human thymidine kinase (hTK)	Reporter gene
Hybrid CMV/chicken β-actin promoter (CAG)	Stem cell lineage
Long terminal repeat of Rous Sarcoma virus (RSV-LTR)	Transgenic mice
Human Ubiquitin C (UBC)	Transgenic mice
Metallothionein I (MT-I)	Inducible promoter
Tetracycline-responsive element (TRE)	Inducible promoter

The strongest promoter in several cell lines is the cytomegalovirus immediate early (CMV IE) promoter (Boshart, et al. 1985). The promoter is extensively used to drive transgene expression. It contains a TATA-box consensus sequence, four CRE responsive elements and four NF-κB responsive elements but lacks initiator motif (Lang, Fickenscher and Stamminger 1992). The CMV IE promoter highly expresses in differential cell but stem cell linage. Transgene expression under the control of the CMV IE promoter in stable cell was usually droped because of gene silencing (Brooks et al. 2004). The mechanism of gene silencing has not been elucidated. However, the CMV IE promoter is generally used to drive recombinant protein expression in CHO cells.

3. New Generation of Used Promoter in Molecular Biotechnology

Previous reports suggested that the human elongation factor 1α promoter extended though intron 1 (Sinici et al. 2006) and the hamster β-actin promoter potentialy expressed in CHO cell (Estes and Zhang 2004). Hamster growth arrest and DNA damage inducible 153 (GADD 153) promoter is another interesting promoter because its activity in serum free cell culture is stronger than the expression in complete medium (Boer, Gray and Sunstrom 2004). The hamster GADD 153 promoter might be a potential promoter in serum-free cell culture. The recombinant protein production cost would be decreased if serum-free cell culture provides high protein yield. Nevertheless, the recombinant protein can be purified easily because the product is low risk of viral and prion contamination.

3.1 Human Elongation Factor-1α Promoter

Human elongation factor-1α (EF-1α) promoter extended through intron1 was isolated and characterized. EFP1 and EFP2 cis-element are adjacent in 5' flanking region of the proximal promoter. Intron1 contains of seven constitutive SP1 responsive elements and AP1. Both the 5' flanking region and the first intron are essential to initiate transcription of the gene (Wakabayashi-Ito and Nagata 1994). The intron 1 was also reported that it elevated cytomegalovirus immediate early (CMV IE) promoter activity by combination with the two promoters. The hybrid between CMV IE promoter and human EF-1α intron1 was effective in CHO and HeLa cells (Kim et al. 2002).

Moreover, the human EF-1α was reported that it contained a great potential to drive transgene expression at every stage of mouse ES cell differentiation when compared to CMV IE, CAG and PGK promoter. However, the human EF-1α promoter lost its ability in neuronal cells (Hong et al. 2007).

3.2 Hamster β-actin Promoter

The hamster β-actin promoter was studied in CHO cells. It has a low level of sequence homology to human and chicken promoter. The hamster beta-actin promoter showed GFP expression higher than CMV promoter, human elongation factor-1α, hamster GAPDH and hamster ribosomal protein 21 (rpS 21) promoter in CHO cells (Estes and Zhang 2004). However, responsive element on the hamster β-actin promoter/enhancer has not been elucidated. Generally, the β-actin promoter drives gene expression in proliferative phase.

3.3 Hamster Growth Arrest and DNA Damage Inducible153 Promoter

Hamster growth arrest and DNA damage inducible 153 (GADD 153) promoter was charaterized from DNA of CHO cells. A hogness box, an inverted CAAT box, AP1 site and seven constitutive SP1 recognition site locate in 420 bp 5' flanking region of the gene. An unusually long strech of altering CpG island is also observed. The promoter was stimulated to express by DNA damage substances such as hydrogen peroxid and UV irradiation (Luethy et al. 1990).

The activity of the hamster GADD 153 promoter was tested in serum free CHO cell culture. The highest activity was detected at G1 phase of cell cycle while the CMV promoter decreases gene expression in G1 phase. The hamster GADD153 promoter was purposed for recombinant protein production under protein-free culture condition (Boer et al. 2004).

3.4 Super Core Promoter

Super core promoter (SCP) is synthetic core promoter created by Tamar Juven-Gershon and colleagues. Designation based on incorporate of multiple core promoter motifs from different gene promoter into a single promoter (Juven-Gershon et al. 2006). It contains four transcription responsive elements; TATA-box, initiator (Inr), motif ten element (MTE) and downstream promoter element (DPE). TATA-box was derived from CMV IE promoter. Inr was designed base on AdML and *Drosophila melanogaster* G retrotransposon core promoter. MTE and DPE were mimiced from *D. melanogaster* Tollo and *D. melanogaster* G promoter (Figure 6).

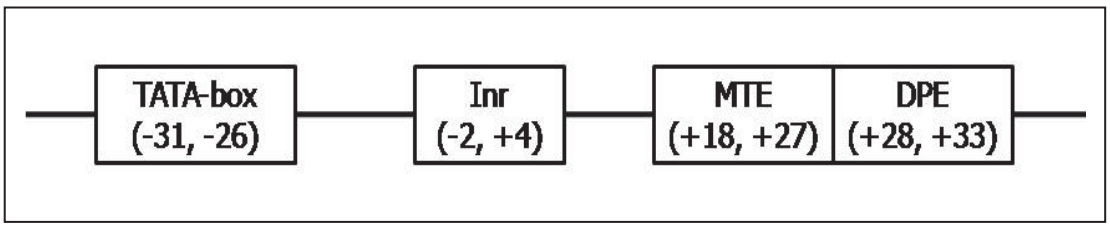


Figure 6 Diagram represents the super core promoter motifs

The SCP activity was tested both *in vitro* transcription analysis and transiently transfected HeLa cells. The SCP exhibits activity higher than CMV IE and AdML core promoter. In single round transcription analysis, 40% template DNA was used under the control of SCP while template DNA under the control of CMV IE and AdML core promoter was used 15% and 6% respectively. The data showed that

transcription factor II D plays a central role on pre-initiation complex formation. The study presented that Inr, MTE and DPE are bound strongly with transcription factor II D (TFIID). TATA-box is bound strongly with TBP and weakly interacted with TFIID. SCP is formed pre-initiation complex faster than CMV IE and AdML core promoter. Therefore, the super activity of the SCP causes by its effective interaction with TFIID. Moreover, SCP also increases enhancer driven gene transcription. Demonstration presented that it increased downstream SV40 enhancer and CMV IE enhancer in HeLa cells but others cells have not been tested (Juven-Gershon et al. 2006).

4. Chinese Hamster Ovary Cell Line

Chinese hamster ovary (CHO) cell was initially isolated from an ovary of adult Chinese hamster (*Cricetulus griseus*). The cell was transformed to cell line for culture that is sufficientely to research (Tjio and Puck, 1958). Nowaday, it becomes a popularly mammalian host for therapeutic recombinant protein production in industry.

CHO cell has been used as a mammalian cell model in numberous biological research descriplines. It was used in a study of mammalian genetics, cell cycle, toxicology and recombinant protein production (Trill, Shatzman and Ganguly 1995). CHO cells grow as adherent cell that have a monolayer and an epithelial-like morphology. The cells could be adapted to culture in suspension. They can grow in high density of cell in bioreactor. Nevertheless, the cells could be modified to grow in serum-free medium (Kim et al. 2006). There are many therapeutic biologics that produce from CHO cells. Most of these products are the protein that requires post-translational modification for folding its active form. The examples of these products are tissue plasminogen activator, erythropoietin, factor VIII, luteinizing hormone, interferon-β, anti-IgE mAb, etc.

CHO-K1 cell is one of the transformant that derived from the original CHO cell (Figure 7). The CHO-K1 cell contains a chromosome number lower than the origin. It is deficient of gene that needed to proline synthesis (Kao and Puck 1967). CHO-K1 cell culture therefore requires proline enrich medium such as F-12 nutrient mixture (Ham) medium that available on commercial. Generally, it is efficient to plating and its doubling time is about 12 to 15 hours. Fortunately, this nutrient sensitive CHO cell is advantage when stably transfected cell is necessary. However, Poliovirus 2, Modoc virus and Botton Willow virus was reported as viral resistance in CHO-K1 cell.

In summary, CHO cell is the first choice of mammalian host for therapeutic recombinant protein production because it provides high protein yield. Thus, the development of recombinant protein producibility based on CHO cell would increase therapeutic recombinant protein supply and provide accessibility for patients. Nevertheless, CHO-K1 cell is a popular host for transfection and biotechnological research because it grows very rapidly and easy to manipulate.

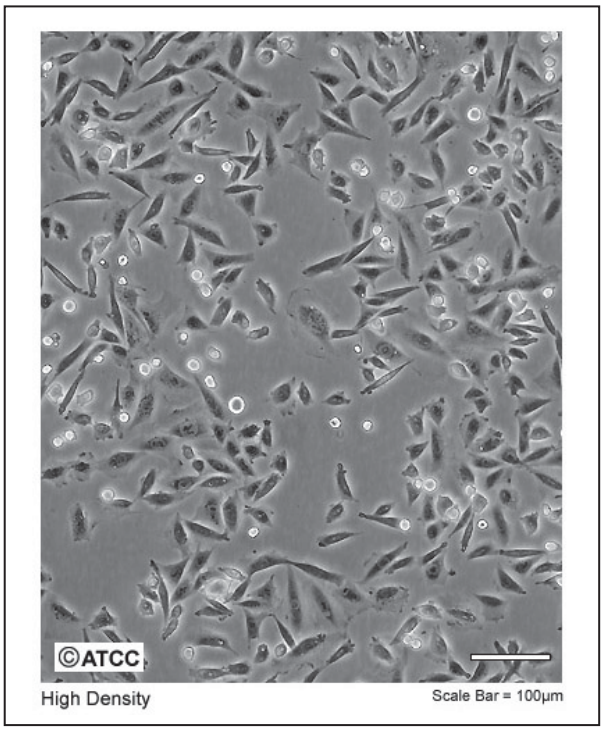


Figure 7 Photograph of CHO-K1 cells.

Source : American type cell collection. CHO-K1 [Online], accessed 10 May 2010. Available from http://www.atcc.org/Attachments/1768.jpg.

5. Conceptual Framework of the Study

Transcription initiation of gene is under the control of many transcription factors existing in the cell (in the nucleus). These transcription factors work via interaction with its responsive elements aligning on an enhancer/promoter. If its responsive element does not available, it is not active. Transcription factor II B function at the core promoter to initiate transcription of gene. Transcriptional activators stimulate the transcription by binding its regulatory element on enhancer/proximal promoter (Figure 8).

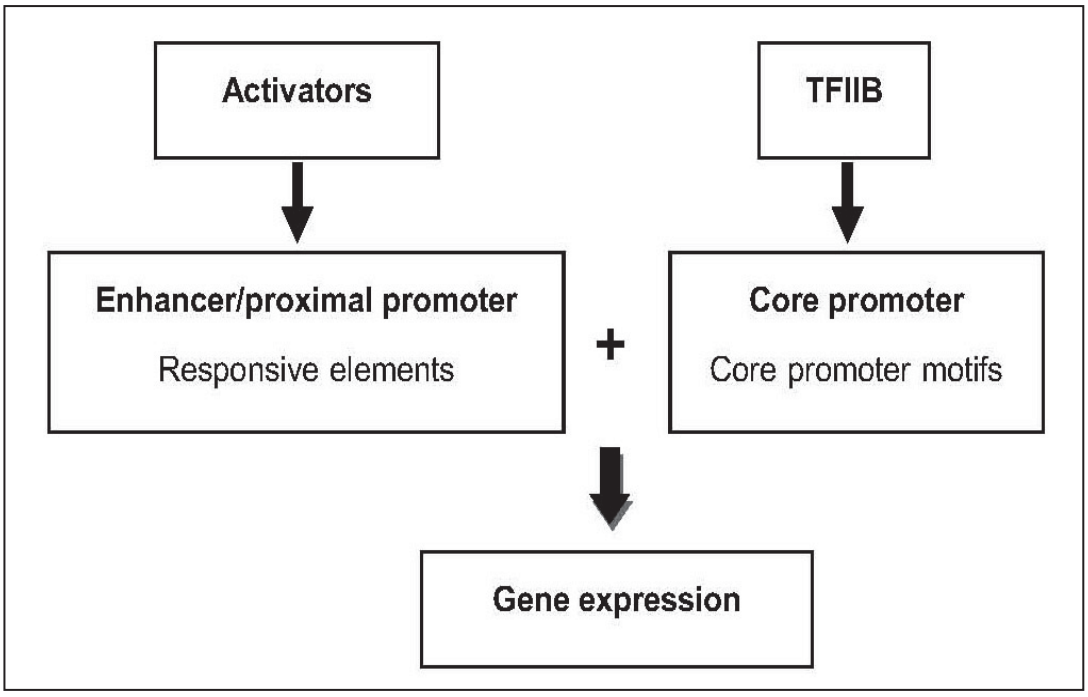


Figure 8 Diagram represents the conceptual framework of this study.

CHAPTER 3

MATERIALS AND METHODS

This chapter presents all experiments that were performed in this study. Ordinary step could present in flowchart form at Figure 9. The details of specific methods were described in each subsection.

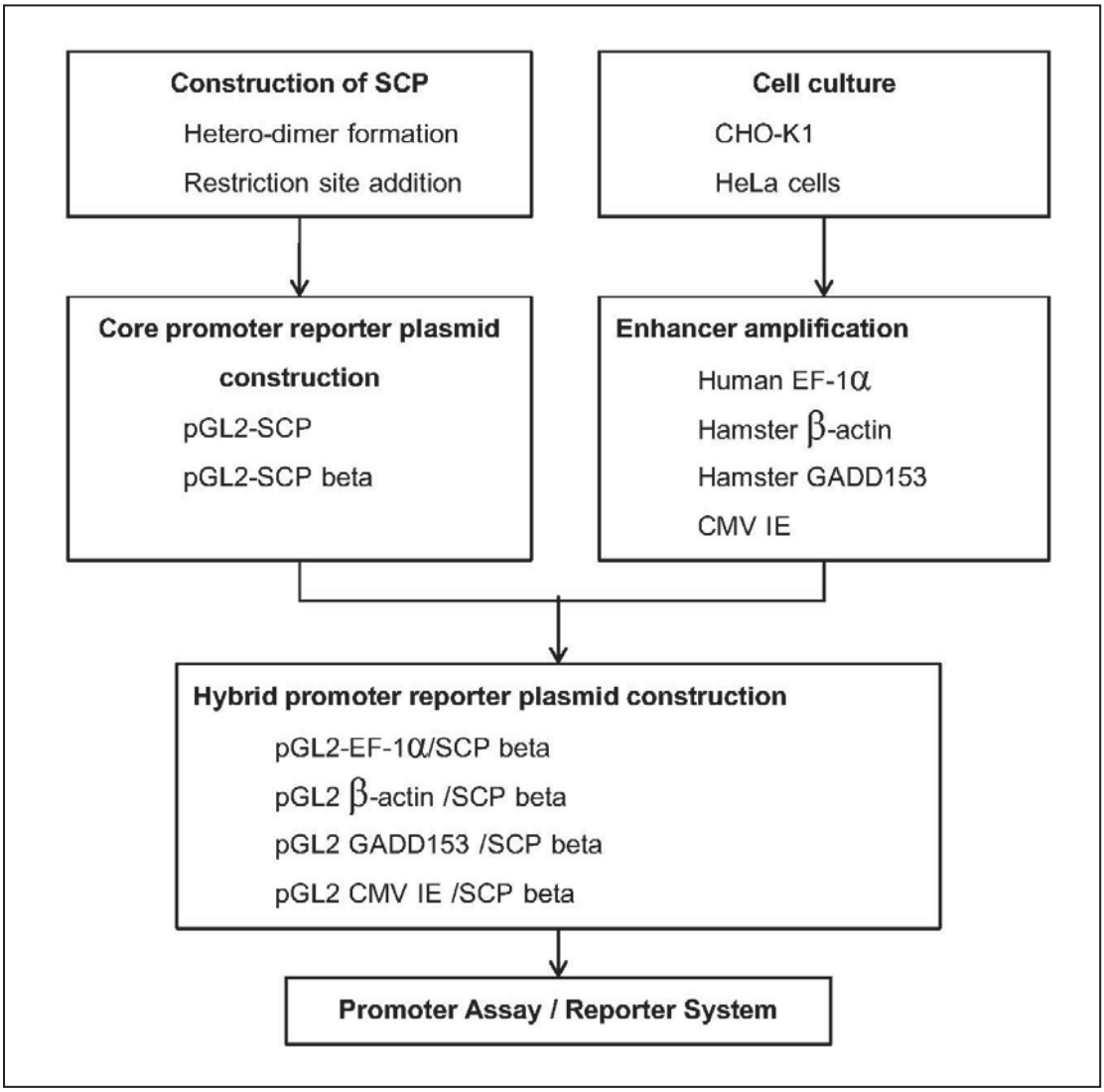


Figure 9 Flowchart of the experiment

1. Bacterial Culture

1.1 Bacterial Medium Preparation

Luria Bertani (LB) broth was formalated as described in Table 10 (Appendix). The medium was autoclaved at 121°C for 15 min. The broth was left for cooling down to room temperature. Ready-to-use LB broth was kept in cold room and warmed up to room temperature before opened inside laminar air flow cabinet. Amplicillin was added to bring a final concentration at 100 µg/mL if a selective broth was needed.

LB agar plate was prepared by dissolving 16 g LB agar powder in 400 mL distilled water. Sterilization was fulfilled using autoclave. The melted LB agar bottle was put on a bench to let temperature down (40-50°C). Antibiotic was added before pouring plate if selective agar plat was needed. The plates were left to solidify in laminar air flow cabinet. The LB agar plates were kept in cold room until use.

1.2 Growth of Bacterial Cultures

Escherichia coli strain DH5α (Invitrogen, Carlsbad, CA) was cultured in LB broth with 150 to 200 rpm shaking in bacterial incubator adjusting 37°C for 16 to 18 hours. *E. coli* (DH5α) was used as plasmid amplify host. *E.coli* containing pGL2-basic or pCMVβ was cultured in LB broth plus ampicillin. *E.coli* containing pEGFP-C2 was cultured in LB broth plus kanamycin.

1.3 Electro-completent Cell Preparation

The electro-completent cell was prepared for plasmid transformation. Briefly, *E. coli* (DH5α) was cultured in LB broth for 18 hr. The cell was centrifuged at 11,000 xg and re-suspended with 10% glycerol three times. The product was re-suspended in 10% glycerol and aliquoted (50 µL /tube). The completent cell was rapidly frozen and kept at -80°C before use.

1.4 Preservation of Bacteria

E.coli (DH5α) containing plasmid of interest was maintained for several uses. The single colony was picked up and cultured in LB broth plus selective antibiotics. The *E.coli* was centrifuged at 2,700 xg, 4°C for 5 min. The liquid phase was discarded as much as possible. The cells were resuspended in 25% (v/v) glycerol in LB broth supplemented with selective antibiotics. The cell suspension was aliquoted into microcentrifuge tubes (1 mL/tube) and kept at -80°C in freezer.

2. The Maintenance of Cells Lines

2.1 Cell Culture Medium Preparation

CHO-K1 culture medium was prepared by mixing 10.6 g F-12 nutrient mixture (Ham) medium powder (Gibco, Auckland, NZ) in autoclave distilled water with 1.5 g sodium bicarbonate. The pH of medium was adjusted to 7.4 with 1 N sodium hydroxide. The solution was filled to 1,000 mL with sterile distilled water. Sterilization was performed using filtratation through 0.22 μm cellulose acetate membrane. The medium was kept at 4°C until use.

HeLa culture medium was prepared by mixing 9.6 g minimum essential medium (MEM) powder (Gibco, Auckland, NZ) in autoclave distilled water with 2.2 g sodium bicarbonate. Further performance steps were followed to previously procedure.

Heat inactivated fetal bovine serum (FBS) was prepared by thawing the frozen serum at room temperature and further heat at 56°C for 30 minutes in water bath. The heat inactivated serum was rapidly transfered into ice box, further aliquoted (40 mL/tube) and kept at -20 °C in freezer before use.

2.2 Phosphate Buffer Saline Preparation

Phosphate buffer saline (PBS) solution was prepared follow to Table 11 (Appendix). The pH value was adjusted to 7.4 with NaOH solution. Sterilization was performed using autoclave at 121°C for 15 min. The buffer was put for cooling down and kept in room temperature before use.

2.3 Thawing Cell

Frozen cells were rapidly warmed by rolling in hands. The suspension cell was put into 10 mL PBS and futher centrifuge at 200 xg, 4 $^{\circ}$C for 5 min. Supernatant was removed and the cell pellet was re-suspended with completed medium. The cell culture was incubated in CO_2 incubator. Culture medium was replaced after the cell attachment (at 24 hrs after thawing). Subculturing was performed before the confluency of the cell was more than 80% area of cell culture flask surface.

2.4 Cell Culture

CHO-K1 cells were obtained from the American Type Culture Collection (ATCC, Virginia, USA). Propagation was followed the recommendation of ATCC. Briefly, the cells were seeded onto T75 cm^2 cell culture flask and cultured in F-12 nutrient mixture (Ham) medium supplemented with 10% (v/v) heat inactivated FBS and 2 mM L-

glutamine (completed medium). The cells were cultured at 37 °C, 5% CO_2 and 100% relative humidity (RH) in cell culture incubator. Subculturing was performed every 2 days. The cell was de-attached with Trypsin-EDTA. The suspension cell was aliquoted in ratio 1:4. The complete medium was added and the cells were further cultured.

HeLa cells were also obtained form ATCC. The cells were cultured in minimum essential medium (MEM) supplemented with 10% (v/v) heat inactivated FBS and 2 mM L-glutamine. The cells were incubated in the CO_2 incubator. Subcultivation was performed every 3 days in ratio 1:3. Aseptic techniques were performed follow to the culture of animal: a manual of basic technique (Freshney 2010).

2.5 Cryopreservation of Cell

Growing cells were trypsinized and pelleted. The cell pellet was re-suspended with completed medium. The stocking cell was prepared by mixing 245 µL fresh medium, 500 µL FBS, 10 µL dimetyl sulfoxide (DMSO) and 245 µL suspension cell in the completed medium. The suspension was aliquoted into cryopreservation tube and put in ice box. The tube was rapidly transfered to freezer adjusting to -20°C and left for about 6 hrs. The frozen cell was kept at -80°C which can survive for unless a year. For long time storage, frozen tubes were kept in liquid nitrogen (approximatly -196°C).

3. Core Promoter Synthesis

3.1 Hetero-dimer Formation

The synthesis was performed based on hetero-dimer formation of primers using polymerase chain reaction (PCR) technique. Schematic representation of super core promoter beta (SCP beta) formation is presented in Figure 10. All primers used in this study were obtained from Wardmedic (Bangkok, Thailand). The DNA sequences of primers are presented in Table 3. The primer properties were analyzed using OligoAnalyzer (version 3.1). The PCR reactions were performed using KOD DNA polymerase with proof-reading property (TOYOBO, Japan). The PCR reaction components and the thermal cycles were performed as presented in Table 4 and Table 5, respectively. Total thermal cycles were 25. Then, the restriction endonuclease recognitionsites were linked to the SCP beta. The primers, containing *Xho*I site on forward primer and *Hind*III site on reverse primers, were designed and used for amplification. These restriction sites will assist the cloning process into the pGL2-Basic

vector. Super core promoter (SCP) synthesis was also performed by PCR technique using SCP beta as a template DNA, but the forward preimer was deleted BRE.

Table 3 DNA sequences of primers that were used for core promoter synthesis.

Primer name	**DNA sequence (5' end to 3' end)**
SCP beta forward	CCC GGG CGC CTA TAT AAG CAG AGC TCG TTT AGT GAA CCG TCA GTT C
SCP beta reverse	ACA ACC GCT CGG CTC GAC GTC TCC AGG CGA ACT GAC GGT TCA CTA AA
SCP forward	GGG GCT CGA GTA TAT AAG CAG AGC TCG TT
*Xho*I forward	GGG GCT CGA GGG GCG CCT ATA TAA GCA
ATG reverse	GGG GAA GCT TTC ATG GTG GCA CAA CCG CTC GGC T

Table 4 PCR reaction component for SCP beta synthesis

Chemicals	**Volume (μL)**
Sterile distilled water	18.80
10x KOD PCR buffer	2.50
10 mM dNTP mix	1.00
100 mM SCP beta forward primer	1.25
100 mM SCP beta reverse primer	1.25
5 U/μL KOD DNA polymerase	0.20

Table 5 PCR thermal cycle for SCP beta synthesis

Period	**Temperature (°C)**	**Time**	**Cycle**
Denaturing	94	30 sec	25
Annealing	56	30 sec	
Elongation	72	40 sec	
Extension	72	10 min	1

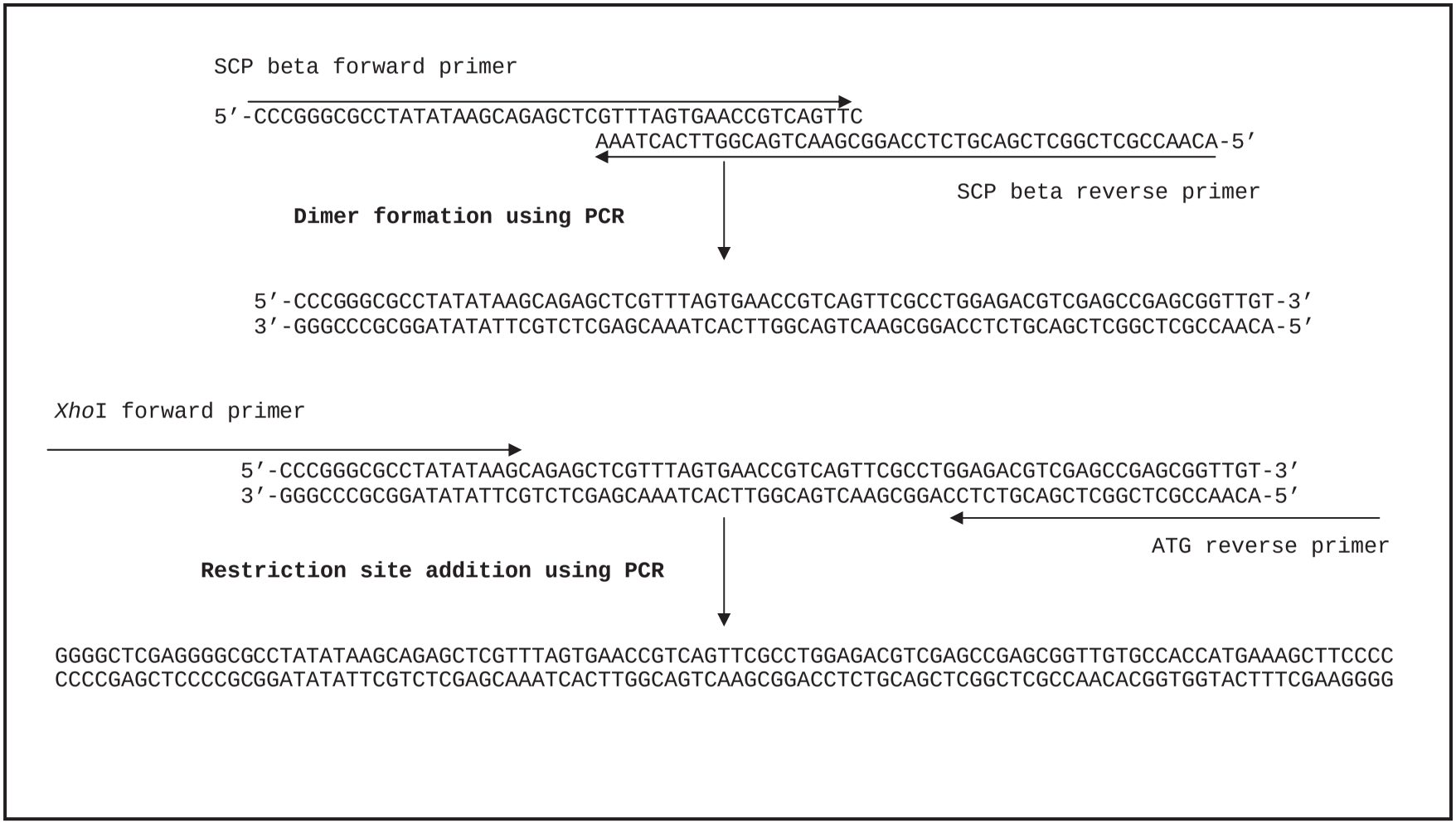


Figure 10 Schematic representation of SCP beta synthesis process

3.2 Synthetic Core Promoter Analysis

The PCR products were resolved in 3% agarose gel in Tris-acetate EDTA (TAE) buffer pH 8.0 (Table 12, Appendix) at 90 volts for 90 minutes using electrophoresis apparatus. The gel was visualized under UV transluminator after staining with ethidium bromide. The DNA band was captured by Syngene document program.

3.3 Restriction Enzyme Mapping of Super Core Promoter Beta

Restriction enzyme map of the SCP beta fragment was predicted using New Enland BioLabs cutter (NEBcutter version 2.0; the on-line DNA restriction mapper tool). The endonuclease restriction sites are presented in Figure 11.

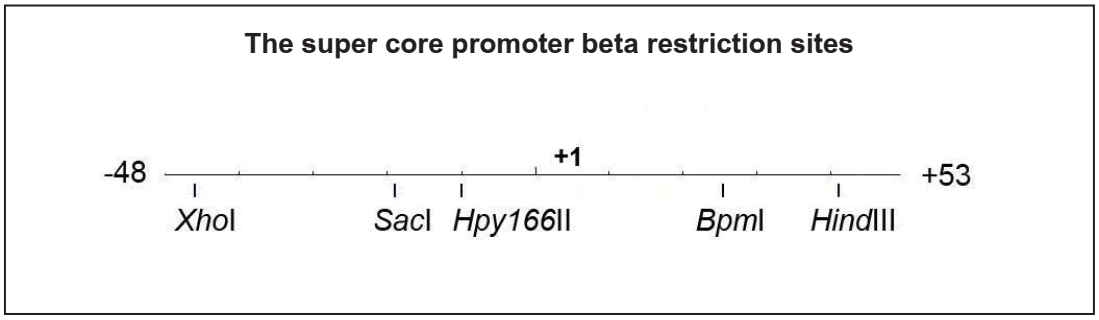


Figure 11 Restriction endonuclease recognition sites align on the SCP beta.
The positions of the sites were relative to adenine in Inr as +1.

4. Core Promoter Reporter Plasmid Constructions

4.1 Synthetic Core Promoter Fragment Preparation

The SCP beta and the SCP DNA fragment were digested by *Xho*I first. The reaction composed of 16 µL sterile distilled water, 1 µL PCR product, 10x H buffer (Takara, Otsu, JP) and 1 µL *Xho*I enzyme. The reaction was activated at 37 °C for 2 hours. The product was purified using NucleoSpin® extraction kit (Macherey-Nagel, Duren, DE). The purified product was futher cut with *Hind*III restriction endonuclease. The reaction composed of 15 µL PCR product cut with *Xho*I, 2 µL of 10x buffer 2 0.2 µL of 100x bovine serum albumin (BSA), 0.2 µL *Hind*III enzyme (New England BioLabs, Massachusetts, USA) and 3 µL sterile distilled water. The reaction was activated at 37 °C for an hour. The final product was repeatly purified using NucleoSpin® extraction kit.

4.2 PCR Product Purification

Quality of insert fragment is required to accomphish ligation. Therefore, the PCR products were separated using agarose gel electrophoresis and futher purified with NucleoSpin® extraction kit. The procedure was followed the manufacturer's instruction manual which the exception of final step that was eluted with 20 µL sterile distilled water. The product was kept at -20°C before used.

4.3 Plasmid Backbone Preparation

A promoterless/enhancerless pGL2-Basic vector (Promega, Wisconsin, USA) was isolated from *E.coli* strain DH5α. The isolation was performed using NucleoSpin® plasmid kit. Briefly, three milliliters of overnight *E. coli* containing plasmid of desire was harvested by centrifugation. The others step was followed the manufacturer instruction except the collection step that eluted with 40 µL sterile distilled water instead of elution buffer. The product was quantitated by UV-spectophotometry. The plasmid was kept at -20°C in freezer before use.

The pGL2-Basic vector (Figure 12) was cut with *Xho*I and *Hind*III endonuclease as previously described in the core promoter fragment preparation. The product was analyzed using agarose gel electrophoresis. Linearized plasmid backbone was extracted from the agarose gel using NucleoSpin® extraction kit. The procedure was performed as PCR product purification.

4.4 Core Promoter/Plasmid Ligation

The synthetic core promoter (the SCP beta and the SCP) was inserted into pGL2-Basic vector at polycloning sites as *Xho*I/*Hind*III DNA fragment to flank the core promoter upstream of firefly luciferase gene (Figure 13). Ligation was accomplished using T4 DNA ligase (Fermentas, Maryland, USA) The reaction composed of 2.0 µL plasmid backbone, 2.5 µL prepared PCR product, 5.0 µL of 2x ligation buffer and 0.5 µL T4 DNA ligase. The mixture was put in thermal cycler adjusting 22°C for 2 hrs.

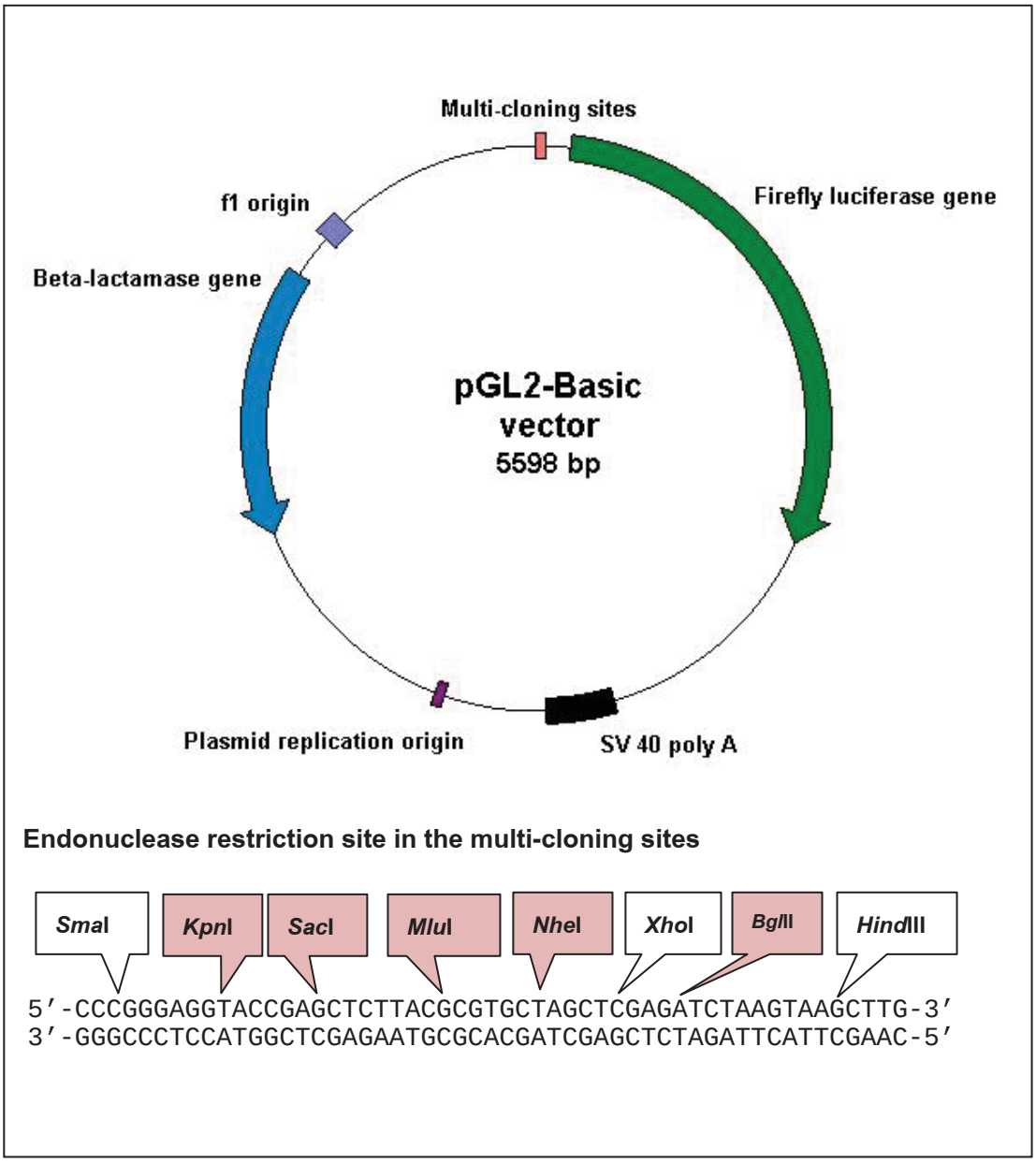


Figure 12 Schematic representation of pGL2-Basic vector map

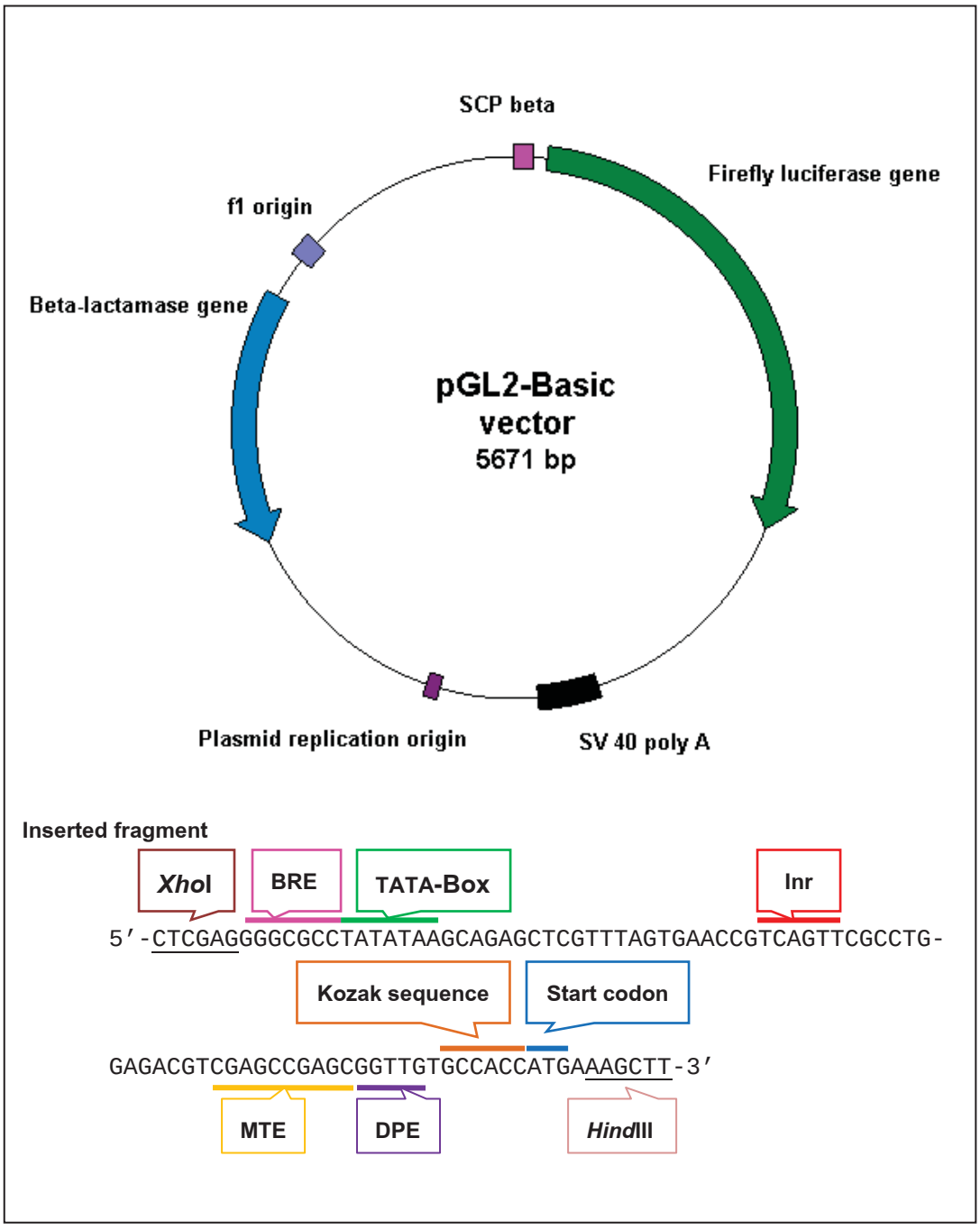


Figure 13 Schematic representation of pGL2-SCP beta vector map

4.5 Plasmid Constructs Amplification

Plasmid constructs were transformed into *E.coli* strain DH5α. Transformation was performed using electroporation. Briefly, Two microliter of plasmid construct was mixed with 50 µL electro-complepent *E.coli* (DH5a). The mixture was transfered into 1 mm gap-cuvette and put on ice for 2 min. The completent cell was eletroporated at 1,500 volts. Then, the transformed bacteria were cultured with LB broth for an hour before spreading on ampicillin selective agar plate. The transformed cells were further incubated overnight in bacterial incubator adjusting to 37°C.

4.6 Bacterial Colony PCR Selection

A single bacteria harboring antibiotic colony was picked up. It was re-suspended in 20 µL sterile water. Ten microliter of bacterial suspension was pipetted into antibiotic LB broth. Five microliter of suspension bacterium was used as DNA template for PCR. The PCR reaction consisted of 5 µL *E. coli* suspension, 2.5 µL 10x PCR buffer, 25 mM $MgCl_2$, 10 mM d NTPs, 20 mM forward primer, 20 mM reverse primer and 0.2 µL Taq DNA Polymerase (1U/µl). Thermal cycles were followed the cycle for flanking restriction endonuclease recognition site. Agarose gel electrophoresis was employed to analyze the bacteria containing plasmid of interest. A desired colony was amplified by culture in ampicillin selective LB broth for others performance.

4.7 Core Promoter Reporter Plasmid Analysis

Nucleotide sequences of the super core promoter beta (SCP beta) and the super core promoter (SCP) were analyzed using DNA sequencing analysis. The used primers are presented in Table 6. This performace was serviced by First Base Company (First Base, SG).

Table 6 DNA sequences of primers used in promoter sequencing

Primer name	DNA sequence (5’ end to 3’ end)	Tm
GL primer1	TGT ATC TTA TGG TAC TGT AAC TG	57 °C
GL primer2	CTT TAT GTT TTT GGC GTC TTC CA	59 °C

5. Hybrid Enhancer/SCP beta Constructions

5.1 Genomic DNA Isolation

Human and hamster genomic DNA was isolated from HeLa and CHO-K1 cell line, respectively, The growing cells at 80% confluence area of T25 cm^2 cell culture flask were harvested by trypsinization and further isolated for genomic DNA using ultra clean blood DNA isolation kit (MOBIO Laboratories, Carlsbad, CA). The isolation was performed follow to the manufacturer's instruction. Briefly, the suspension cell was added 900 μl of solution G1. The tube was inverted twice and incubated at room temperature for 5 minutes. The suspension cell was centrifuged at 6,800 xg for a minute. The supernatant was discarded. The pellet cell was suspended by vortexing. The suspended cell was added 300 μl of solution G2. The suspension was mixed by pipetting. RNase A solution was put into the lysed cell. The tube was mixed by inverting and vortex at low speed. The tube was added 100 μl of solution G3 and immediately vortex on high speed for 15 seconds. The tube was further centrifuged at 6,800 xg for 3 minutes. The supernatant was transferred to a collection tube. The tube was added 300 μL of isopropanol and incubated at room temperature for 3 minutes. Centrifugation at 6,800 xg for a minute was performed to precipitate the genomic DNA. The liquid phase was discarded. The pellet DNA was washed with 300 μl of 70% ethanol. Centrifugation was performed. The liquid phase was discarded. The pellet DNA was added 100 μl of solution G4 and dissolved by heat in a 65 °C water bath for 10 minute. The genomic DNA was kept at -20°C for enhancer/proximal promoter amplification.

5.2 Enhancer/proximal Promoter Amplification

The enhancer/proximal promoters were amplified for hybrid promoter construction. HeLa genomic DNA was employed for human EF-1α enhancer extended though intron1 (GenBank No. J04617.1) amplification. Hamster β-actin enhancer/ proximal promoter (GenBank No. U201114.1) and hamster GADD 153 enhancer/ proximal promoter (GenBank No. J05613.1) were amplified from CHO-K1 genomic DNA. The CMV IE enhancer (GenBank No. X03922.1) was copied from pEGFP-C2 vector (Clontech Laboratories, Palo Alto, CA). Oligonucleotide primer sequences are shown in Table 7.

Table 7 DNA sequences of primer for enhancer/proximal promoter amplification

Name	DNA sequence (5'end to 3' end)	Melting temperature (Tm) (°C)	Expected target size (bp)
EF-1α forward	GGC GAA GGT ACA CCC TAA TCT CAA T	56	1,266
EF-1α reverse	TCC AAA AGC TCG AGA ACT AAT CGA		
β-actin forward	TCT CTT TTG TGG GAA CCA CAG AGT	60	1,449
β-actin reverse	GCC ACT CGA GCC ATA AAA GGC AA		
GADD153 forward	ACA GGA ATT CTG GCG TGC AGT	57	758
GADD153 reverse	CCC CCT CGA GGG GCC CGT CCC CTC TTT TG		
CMV IE forward	ACC GTA TTA CCG CCA TGC AT	55	555
CMV IE reverse	GGG GCT CGA GTC CCA CCG TAC ACG CCT A		

PCR reaction consisted of 18.10µL sterile distilled water, 2.50 µL 10x PCR buffer, 1.00 µL 10 mM dNTP, 0.50 µL 20 mM forward primer, 0.50 µL 20 mM reverse primer, 0.50 µL genomic DNA (100-500 ng), 1.25 µL DMSO and 0.20 µL 1 U/µL KOD DNA polymerase. PCR thermal cycles was performed as follow, pre-denaturing at 94°C for 3 min, denaturing at 94°C for 30 sec, annealing at 3 degree lower calculated Tm for 30 sec, elongation at 72°C for 40 sec per 1 kb of the amplicon and extension at 72°C for 10 min. Total cycles was 25.

5.3 Amplicon Analysis

The PCR products were analyzed by gel electrophoresis. The DNA fragments were separated in ~1% (w/v) agarose soaking TAE buffer using a horizontral eletrophoresis apparatus adjusted at 90 volts for 40 min. The band of interest was isolated from agarose gel and further purified for subsequent confirmation by restriction enzyme mapping. The hybrid promoters were predicted for restriction endonuclease recognition site using NEB web cutter 2.0 (Figure 14 to 17).

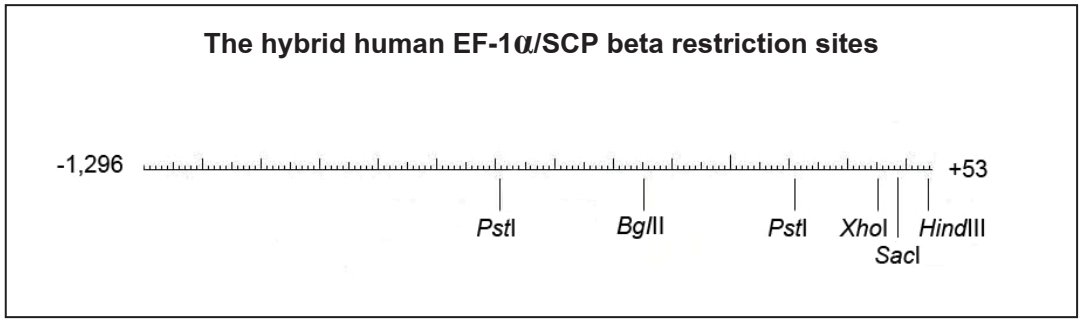


Figure 14 Restriction endonuclease recognition sites align on the EF-1α/SCP beta. The positions of the sites were relative to adenine in Inr as +1.

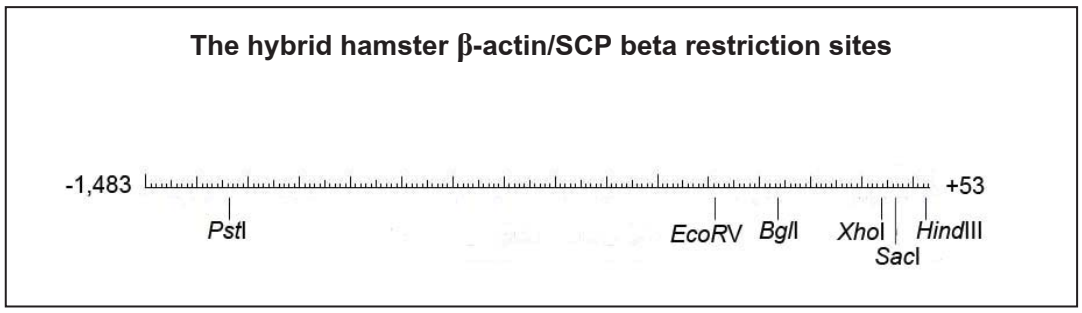


Figure 15 Restriction endonuclease recognition sites align on the β-actin/SCP beta. The positions of the sites were relative to adenine in Inr as +1.

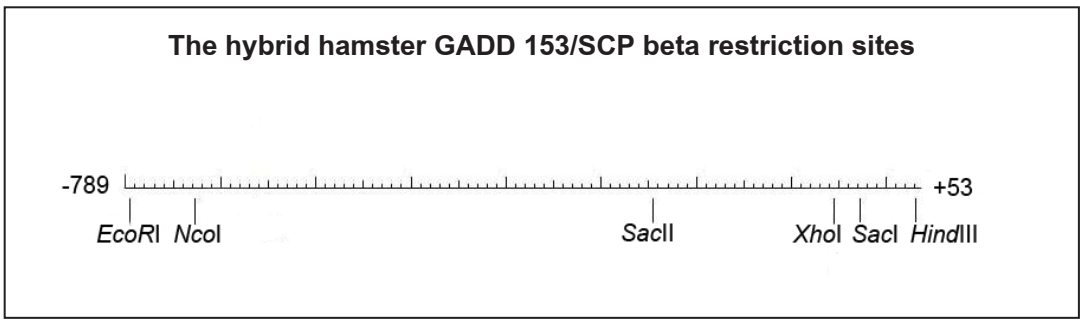


Figure 16 Restriction endonuclease recognition sites align on the GADD153/SCP beta. The positions of the sites were relative to adenine in Inr as +1.

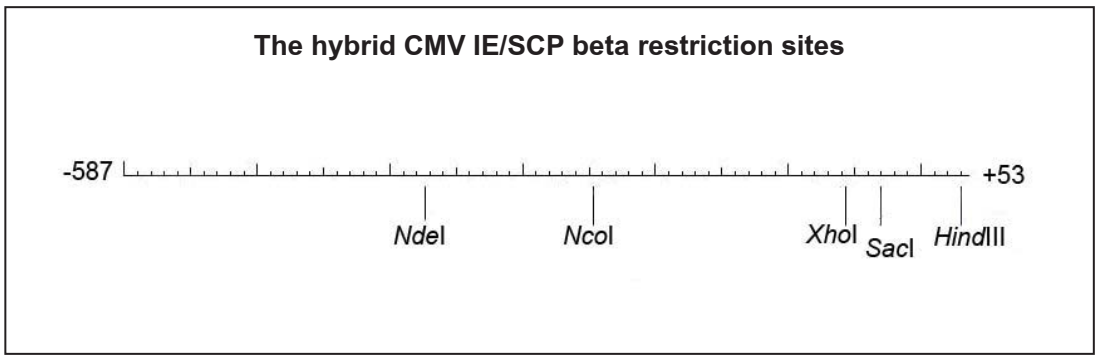


Figure 17 Restriction endonuclease recognition sites align on the CMV IE/SCP beta. The positions of the sites were relative to adenine in Inr as +1.

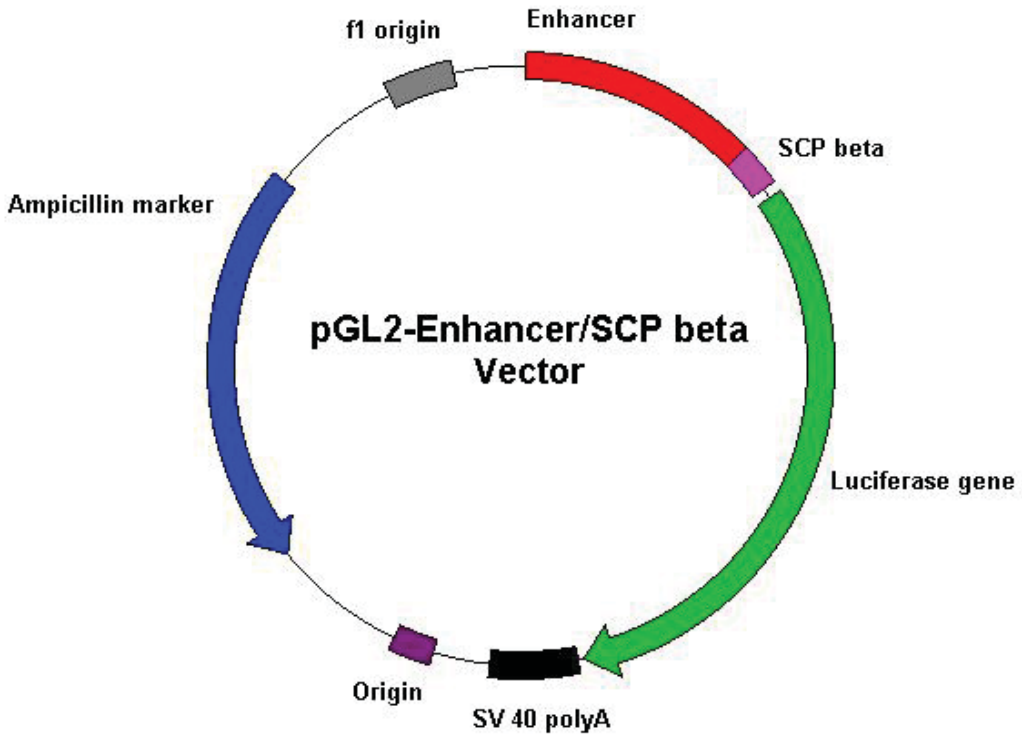
5.4 Hybrid Promoter Reporter Plasmid Construction

Each enhancer was inserted into pGL2-SCP beta at *Sma*I and *Xho*I restriction site upstream of the SCP beta. The performances (PCR product purification, plasmid DNA preparation, ligation and transformation) were followed as the previousely described protocols. Bacterial colony containing plasmid of interest was selected by restriction enzyme mapping technique.

Each plasmid constructs were cut with *BamH*I and a unique endonuclease restriction enzyme that cut within the hybrid promoter region. The reactions were performed as the recommendation of the company (New Enland BioLabs, Massachusetts, USA).

5.5 Hybrid Promoter Verification

The hybrid promoter was designed to locate upstream of firefly luciferase gene (Figure 18). The hybrid promoter reporter plasmid constructs were verified using DNA sequencing analysis by First Base Company (First Base, SG). The used primers were GL primer1 and GL primer 2 (Table 6).



Length of plasmid constructs

Length of pGL2-EF-1α/SCB beta is 6894 bp.

Length of pGL2-β-actin/SCB beta is 7081 bp.

Length of pGL2-GADD 153/SCB beta is 6390 bp.

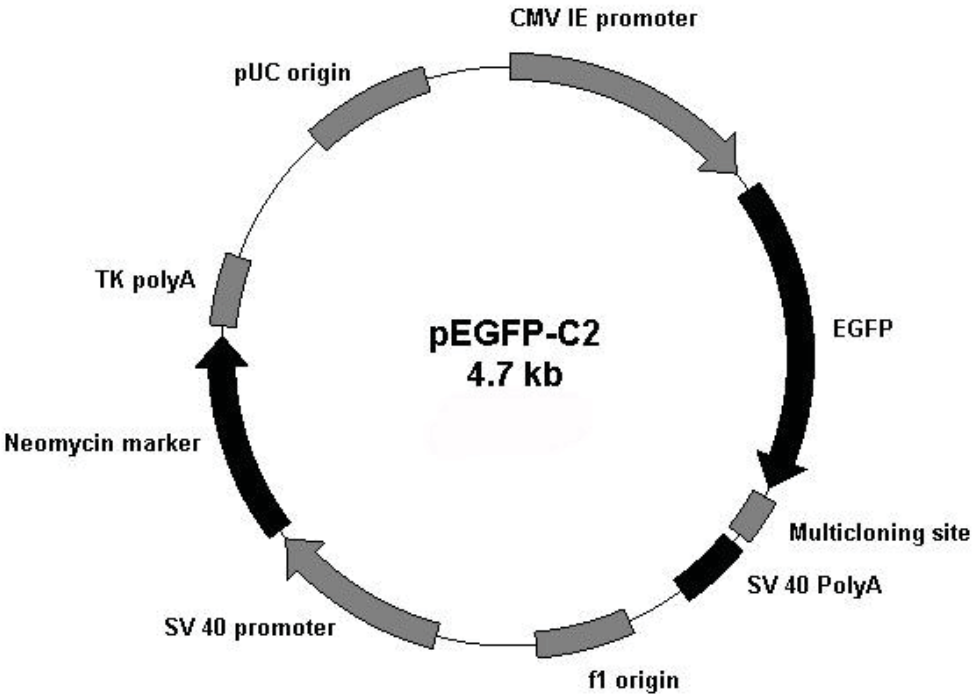
Length of pGL2-CMV IE/SCB beta is 6185 bp.

Figure 18 Schematic representation of pGL2-Enhancer/SCP beta plasmid map.

6. Transfection Optimization

Transfection protocol was optimized by using an enhanced green fluorescent protein expression vector to mornitor transfection efficiency. Map of pEGFP-C2 plasmid vector is shown in Figure 19 (Clonetech, California, USA).

Twenty four hours prior to transfection, CHO-K1 cells were de-attached with trypsin-EDTA and further seeded onto twelve-well plates (Corning Costar, New York, USA) at a density of 2.0 x 10^5 cells per well in complete medium. Circular pEGFP-C2 plasmid vectors were complexed with Fugene® 6 transfection reagent. This complexation step was left for 25 min at room temperature. DNA/liposome concentration that use to optimize transfection efficiency are 2:3, 1:3 and 1:6 µg/µL.The growing cells were washed three times with PBS. Two hundred microliter F-12 nutrient mixture (Ham) medium was added. The DNA/liposome complex was gently droped onto the cells. The plate was rocked slowly for 5 min. Transfection plate was incubated in a cell culture incubator for 5 hr. Completed medium was added to 1 mL per well and further continued culture. To determine transfection efficiency, Expression of GFP was tracted under an inverted fluorescent microscope, Nikon eclipse TE-2000 (Nikon, Tokyo, JP) at 24, 36, 48 and 72 hrs after transfection. The transfected cells were taken photograps using Nikon digital camera (Nikon, Tokyo, JP). This optimized condition was used for transfecting the plasmid constructs into CHO cells for promoter study.



Endonuclease restriction site in the multi-cloning sites

*Eag*I *Xho*I *Hind*III *Pst*I
5′-TCCGGCCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGAC-
*Bgl*II *Sac*I *Eco*RI *Sal*I

*Kpn*I *Apa*I *BamH*I Stop codon
GGTACCGCGGGCCCGGGATCCACCGGATCTAGATAACTGATCA-3′
*Sac*II *Sma*I *Xba*I *Bcl*I

Figure 19 Schematic representation of pEGFP-C2 vector map

7. Promoter Activity Assay

7.1 Plasmid DNA Quality

Quality of plasmid DNA is a crucial factor effected transfection efficiency. Thus, the quality was determined using UV-spectrophotometry before use to transfection. The plasmid DNA was measured an absorbance at wavelength 260 nm and 280 nm. The concentration of plasmid was calculated based the absorbance at 260 nm. Purity of plasmid was estimated as a ratio of the absorbance at 260/280 nm. The ratio of good DNA should be 1.8 to 2.0.

7.2 Packaging Plasmid DNA into CHO-K1 Cells

Twenty four hours prior transfection, CHO-K1 cells were prepared in twelve-well plates as described in transfection optimization step. The plasmid containing promoter construct was co-transfected with β-galactosidase expressing plasmid (Figure 20) to normalize transfection efficiency. Each plasmid DNA/ liposome complex mixture consisted of 0.4 µg plasmid construct, 0.2 µg pCMVβ reference plasmid (Clontech, California, USA) and 3.6 µL Fugene® 6 transfection reagent (Roche, Indiana,USA) in F-12 nutrient medium (Ham). The procedure was followed the optimized transfection protocol. After transfection, cells were rehabilitated in completed medium for 12 hours.

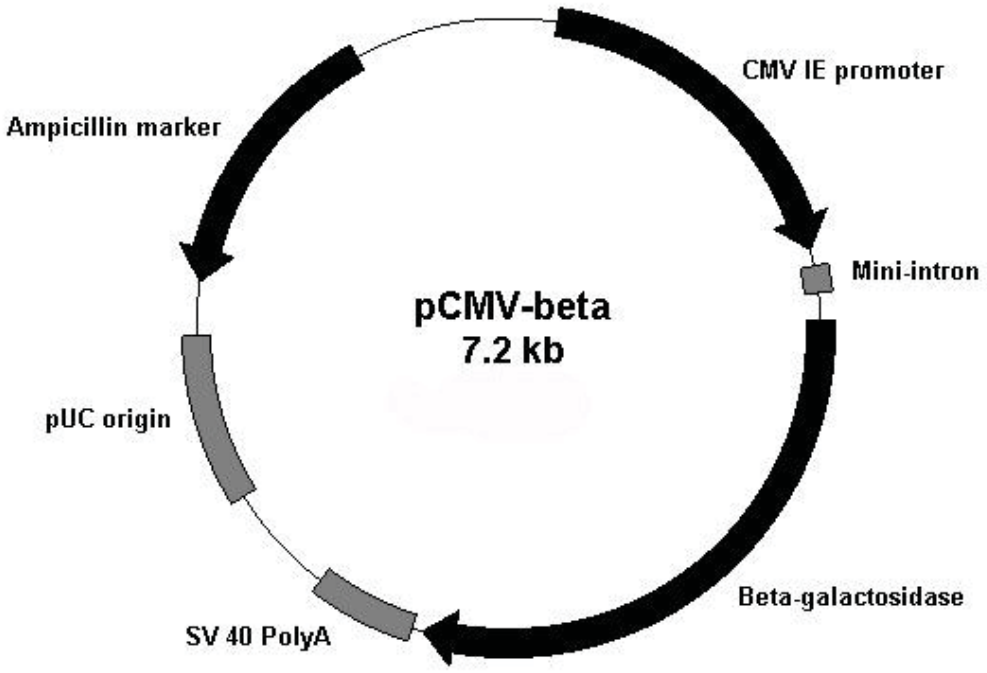


Figure 20 Schematic representation of β-galactosidase expressing plasmid (pCMVβ)

7.3 Transient Transfection Test

Twelve hours after transfection, cell culture medium was removed from transiently transfected CHO-K1 cells. F-12 nutrient medium (Ham) supplemented with 2 mM L-glutamine and 10% (v/v) FBS or serum-depleted medium was added and the cells were further cultured. The cells were harvested for the reporter proteins after culture for 24 hours. Whole cell lysate procedure was followed instruction of luciferase kit (Promega, Wisconsin, USA). In brief, transfected cells were washed three times with PBS. The cells were lysed with 100 µL reporter lysis buffer (Table 13, Appendix). The cells were scrapped and transferred into microcentrifuge tube. Freeze- thaw technique was performed by freezing the cell at -80°C for 20 minutes and thawing at 37°C. The cell lysate were centrifuged at 11,000 xg at 4°C for 5 min. The supernatant was collected and put on ice. The promoter activity assay was immediately performed.

7.4 Luciferase Assay

Reporter protein expression levels were determined the activity of constructed promoter. Firefly luciferase chemical reaction was performed. First, fifty microliter of luciferase assay reagent (Promega, Wisconsin, USA) was dispensed in 96 well solid white (flate bottom). Second, ten microliter of cell lysate was added and rapidly mixed. Then, the luminescence was generated as the chemical reaction in Figure 21. The luminescence was immediately quantified a relative luminescent unit within a minute under fusion™ luminescent based-microplate reader (Packard BioScience, Connecticut, USA). The reading time was adjusted at 10 seconds. The half-life of luminescence and correlation between reporter protein and luminescent unit were preliminary performed to ensure the quality of assay.

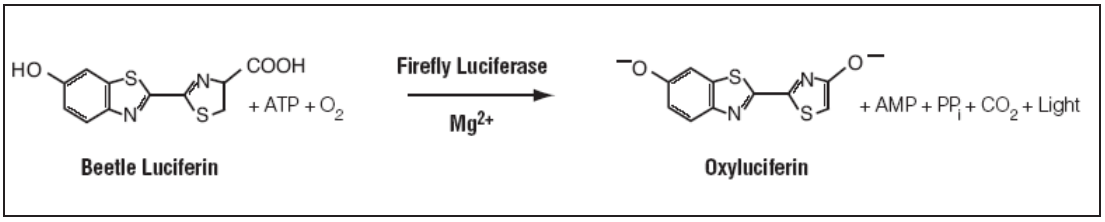


Figure 21 Firefly luciferase catalyzes chemiluminescent reaction.

Source : Simon T.M. Allard, Bioluminescent Reporter Genes [Online], accessed 30 June 2011. Available from http://www.promega.com/resources/articles/pubhub/enotes/bioluminescent-reporter-genes.

7.5 β-galactosidase Assay

To normalize the transfection efficiencies between testing group, β-galactosidase in the same cell lysate was determined based on its activity to convert the colorless *p*-nitrophenyl-β-D-galactopyranoside (PNPG) (Sigma, Missouri, USA) to yellow *p*-nitrophenol (Figure 22). Ten microliter of the cell lysate in the same group was mixed with β-galactosidase assay solution. Chemicals component of the assay solution consisted of 283.92 mg sodium hydrogen phosphate, 14.91 mg potassium chloride, 275.98 mg sodium phosphate monohydrate, 4.92 mg magnesium sulfate, 20 mg PNPG, 70.3 µL β-mercaptoethanol and distilled water (final volume was 10 mL). The reaction was performed at 37°C by adding substrate to start the reaction. Twenty five to fourty minutes after the activation, 150 µL stop reaction solution (1 M sodium carbonate) was rapidly added. The activity determination was accomplished using the fusion™ microplate reader. The absorbant value of converted substrate was measured at wavelength 420 nm.

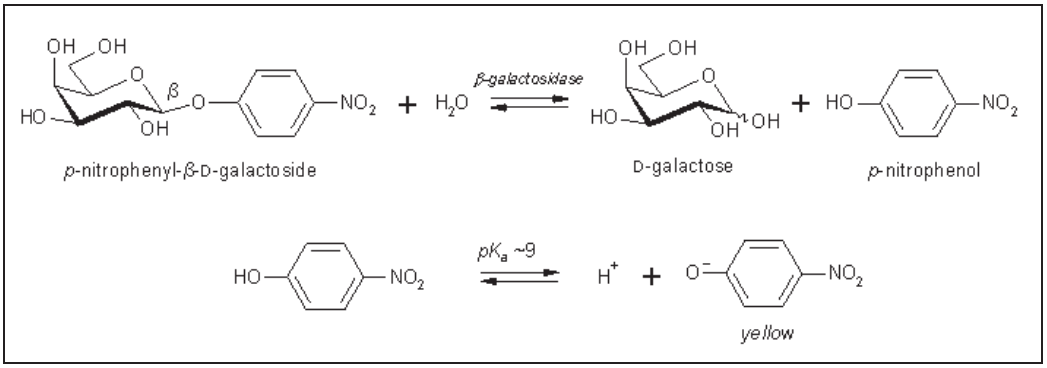


Figure 22 Chemical reaction of β-galactosidase

Source : Tom Taylor and Jerry Stahlberg, Enzyme kinetics [Online], accessed 30 June 2011. Available from http://xray.bmc.uu.se/Courses/KE7001per4/Labs/enz_kinetics_lab.html.

Luciferase activity was normalized as follows.

$$\text{Normalized activity} = \frac{\text{Luminescent unit}}{\text{Aabsorbance}}$$

7.6 Statistic Consideration

The promoter activity was analyzed to determine its potency. Firstly, the super core promoter activity was compared to a promoterless/enhancerless activity using one-tailed student *t*-test (to ensure the effectiveness of the control). Secondly, the super core promoter beta activity was compared to the SCP activity using one-tailed student *t*-test (to determine the effect of BRE). Thirdly, the effect of ehancer on hybrid promoter activity was analyzed with analysis of variance (ANOVA) and further compared between groups using multiple comparisons (Post hoc test). The calculations were performed with the statistical package for the social sciences (SPSS) computer program (version 15). Significant difference was considered when p-value < 0.05.

CHAPTER 4

RESULTS AND DISCUSSION

1. Core Promoter

1.1 Super Core Promoter Synthesis

The synthetic core promoter was created for general utilization. The core promoter was synthesized by hetero-dimer formation of the primers containing 19 priming bases. The resulting DNA fragment has a 74 bp-long. The SCP beta DNA fragment was synthesized by PCR using the core promoter as a template DNA. The SCP and SCP beta fragment has a 95 bp-long and 102 bp-long, respectively. The DNA band of interest was visualized under the UV transilluminator after separation by agarose gel electrophoresis (Figure 23).

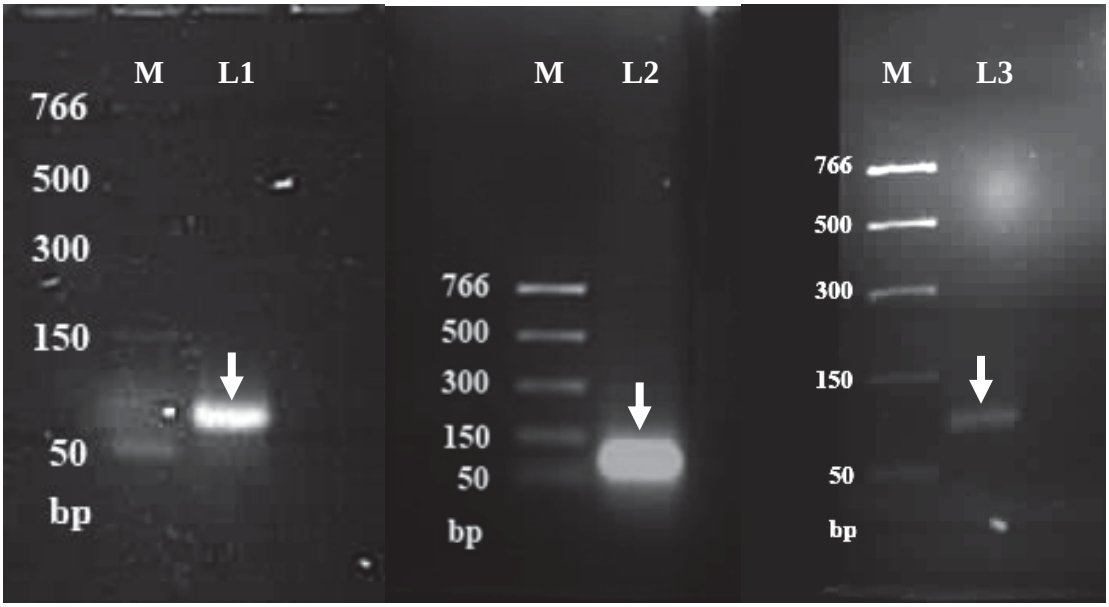


Figure 23 Electrophoresis visualized core promoter fragments. The DNA fragments were resolved on 3.0% agarose gel stained with ethidium bromide. M denotes 50 bp DNA ladder. L1, L2 and L3 represent hetero-dimer fragment, super core promoter beta and super core promoter, respectively.

1.2 Super Core Promoter Beta Structure

The synthetic super core promoter beta (SCP beta) was verified by DNA sequencing analysis (Figure 24). The SCP beta consists of 5 core promoter motifs including TFIIB responsive element (BRE), TATA-box, initiator (Inr), motif ten element (MTE) and downstream promoter element (DPE) (Figure 25). Full length of SCP beta DNA sequence is presented in Appendix. The SCP was also synthesized for using as the core promoter control. The DNA sequencing analysis showed that the SCP contains the same DNA sequences of the SCP beta, but no BRE.

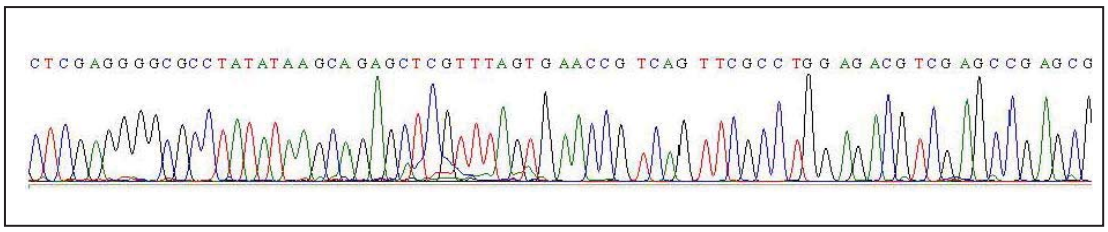


Figure 24 DNA sequencing analysis shows nucleotide sequences of the SCP beta.

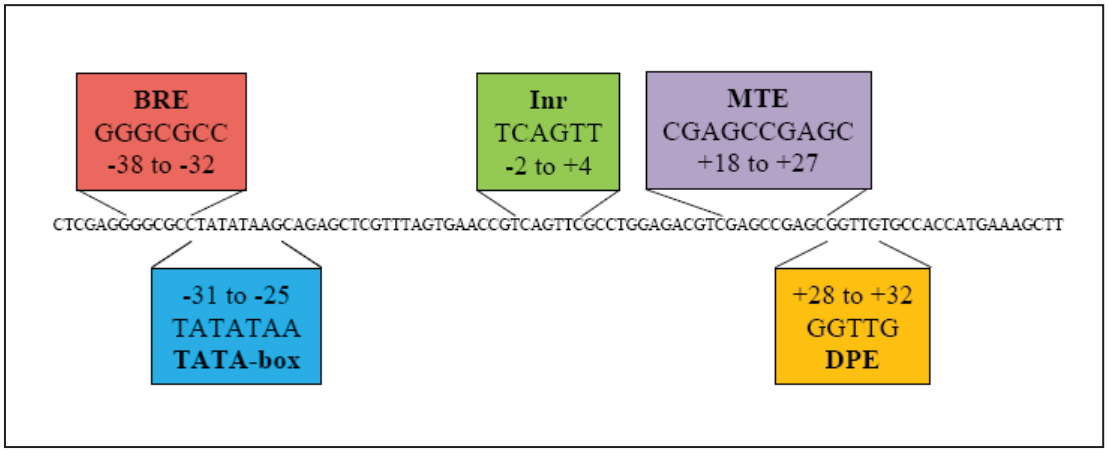


Figure 25 Super core promoter beta structure (Kozac sequence and ATG translation strat codon were linked downstream of the SCP beta). Positions of nucleotide are relative to adenine in initiator as +1.

2. Hybrid Enhancer/Super Core Promoter Beta Constructions

2.1 Enhancer and Proximal Promoter Amplification

Four enhancer and proximal promoters were amplified for construction of a hybrid enhancer/super core promoter beta. Oligonucleotides (Table 7) were used as

primers in PCR reactions. It could not accomplish a single band of interesting DNA, except CMV IE enhancer. However, they could be amplified the desired enhancer/ proximal promoter fragments. The human elongation factor-1α (EF-1α), the hamster β-actin (β-actin), the hamster growth arrest and DNA damage inducible 153 (GADD 153) and the cytomegalovirus immediate early (CMV IE) amplicon length is 1266, 1449, 758 and 553 bp, respectively (Figure 26).

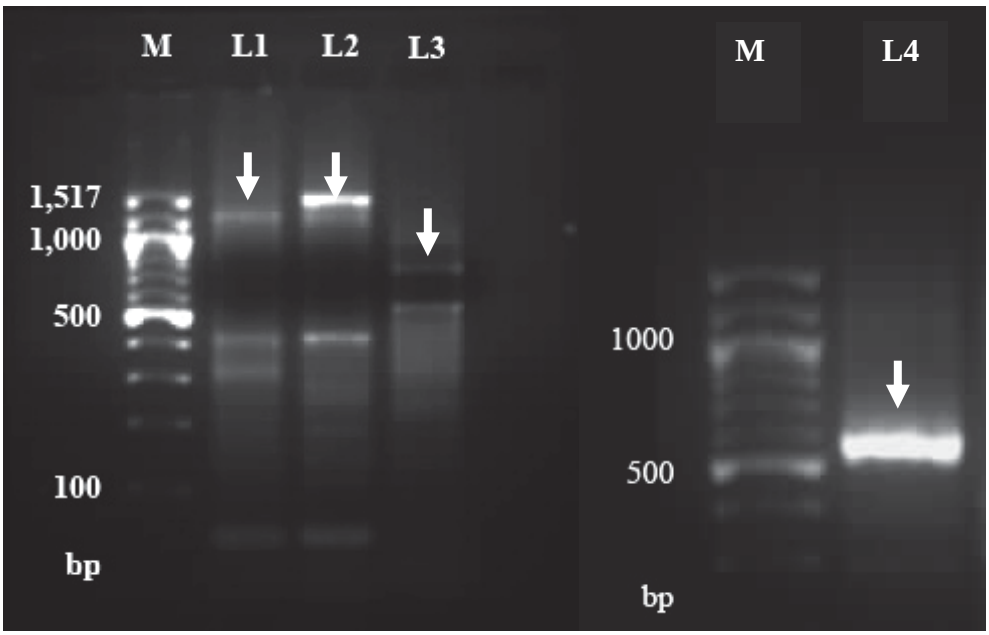


Figure 26 Electrophoresis visualized the enhancer amplicons. The DNA fragments were resolved on 1.0% agarose gel stained with ethidium bromide. M denotes 50 bp DNA ladder. L1, L2, L3 and L4 represent human EF-1α enhancer extended though intron1, hamster β-actin enhancer/proximal promoter, hamster GADD 153 enhancer/proximal promoter and CMV IE enhancer, respectively.

2.2 Analysis of Hybrid Promoter Reporter Plasmid Construction

All hybrid enhancer/super core promoter beta plasmid constructs were cut at the unique restriction site within the enhancer and the specific site within the plasmid backbone. The products were separated using 1% (w/v) agarose gel. The lengths of the DNA fragments are presented in Table 8. The gel electrophoresis is showed in Figure 27.

Table 8 Length of plasmid fragments after cutting with restriction endonucleases

Plasmid name	Restriction enzymes		DNA fragment (bp)	
	No.1	No.2	Small	Large
pGL2-GADD 153/SCP beta	*BamH*I	*Nco*I	2,933	3,453
pGL2- β-actin/SCP beta	*BamH*I	*Pst*I	3,024	4,059
pGL2- EF-1α/SCP beta	*BamH*I	*Pst*I	2,926	3,463
pGL2- CMV IE/SCP beta	*BamH*I	*Nco*I	2,974	3,213

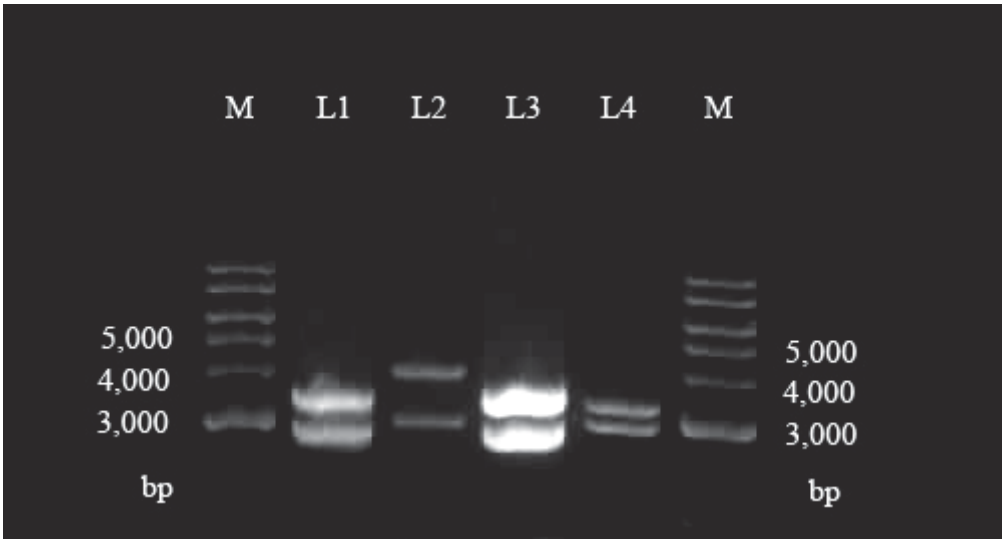


Figure 27 Electrophoresis visualized the restriction enzyme map. The DNA fragments were resolved on 1 % agarose gel stained with ethidium bromide. M denotes 1 Kbp DNA ladder. L1, L2, L3 and L4 represent the pGL2-GADD 153/SCP beta, pGL2-β-actin/SCP beta, pGL2-EF-1α/SCP beta and pGL2-CMV IE/SCP beta, respectively.

2.3 Hybrid Enhancer/SCP Beta Structure

The hybrid promoter construction was projected to enhancer the super core promoter beta activity in CHO-K1 cells. The DNA responsive elements alining on the enhancer/proximal promoter were predicted using JASPAR on-line tool. The output showed that four hybrid promoter constructs contain different responsive elements on enhancer/proximal promoters. The hybrid human EF-1α/SCP beta contains of seven constitutive SP1 responsive elements, TATA–boxes which lacates on enhancer and an AP1 resposive element at proximal promoter. The hybrid hamster β-actin/SCP beta contains three EST1, three SP1, three Myb, one GATA1 and one CAAT responsive element. The hybrid hamster GADD 153/SCP beta contains of seven constitutive SP1, AP1, converted CAAT responsive element, CpG Islands (distribute over enhancer region, but not show) and a predicted Ahr responsive element. The hybrid CMV IE/SCP beta contains of four CRE, four NF-κB responsive elements and one constitutive SP1 responsive element. Notably, a constitutive SP1 responsive element consists in all hybrid promoters. The locations of each element are presented in Figure 28.

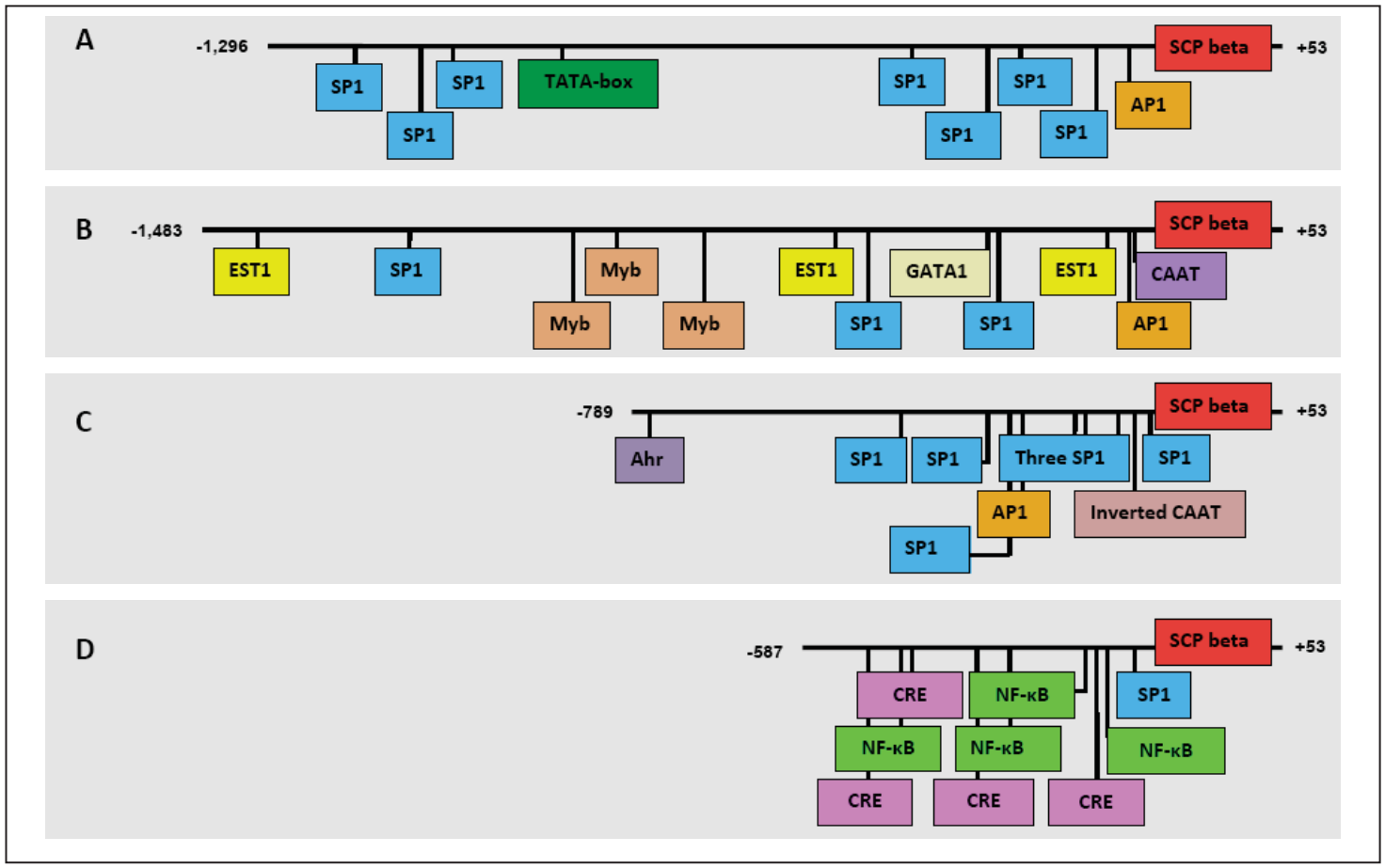


Figure 28 Diagram presents the structure of the human EF-1α/SCP beta (A), the hybrid hamster β-actin/SCP beta (B), the hybrid hamster GADD 153/SCP beta (C) and the hybrid CMV IE/SCP beta (D). Positions of nucleotides were relative to adenine in initiator as +1. Full length of DNA sequences are presented in Appendix.

3. Cell Culture and Transfection Optimization

3.1 CHO-K1 Cell Culture

CHO-K1 cells were cultured in F-12 nutrient mixture (Ham) medium supplemented with 10% v/v heat inactivated FBS and 2 mM L-glutamine. The cells grew up rapidly. The morphology of CHO-K1 cell at intermediate and high confluency is presented in Figure 29. The cell morphology was similar to the appearance in the photo from ATCC (Figure 7). Thus, the cell culture medium and culture condition was considered to be optimized.

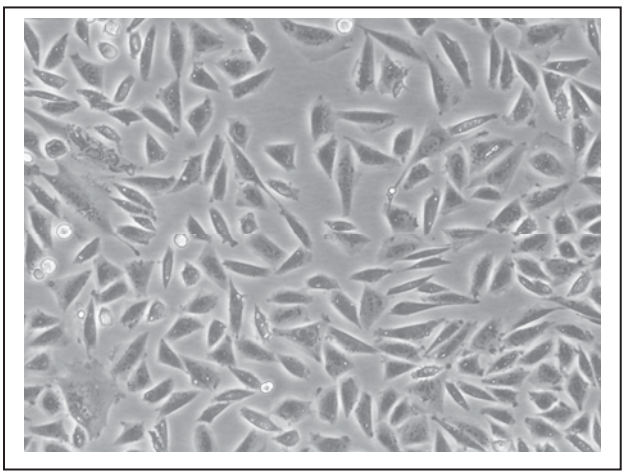


Figure 29 The CHO-K1 cells were growing at intermediate confluency.

3.2 Transfection Optimization in CHO-K1 cell

Transfection efficiency is one of the crucial factors affecting transient model quality. CHO-K1 cell transfection protocol was therefore optimized. The enhanced green fluorescent protein expression plasmid (pEGFP-C2) was employed for mornitering the transfection result. Green fluorescent protein expressing cells were photographed at 24, 36 and 48 hours after transfection (Figure 30-32). At 72 hours after transfection, cell confluency was too high and the cell number is uncountable in practice. By observation, the expression of EGFP under the control of CMV IE promoter closely saturated around 36 hours after transfection. Therefore, percentage of transfection efficiency was calculated at 36 hours after transfection. The result of each transfection groups are showed in Table 9.

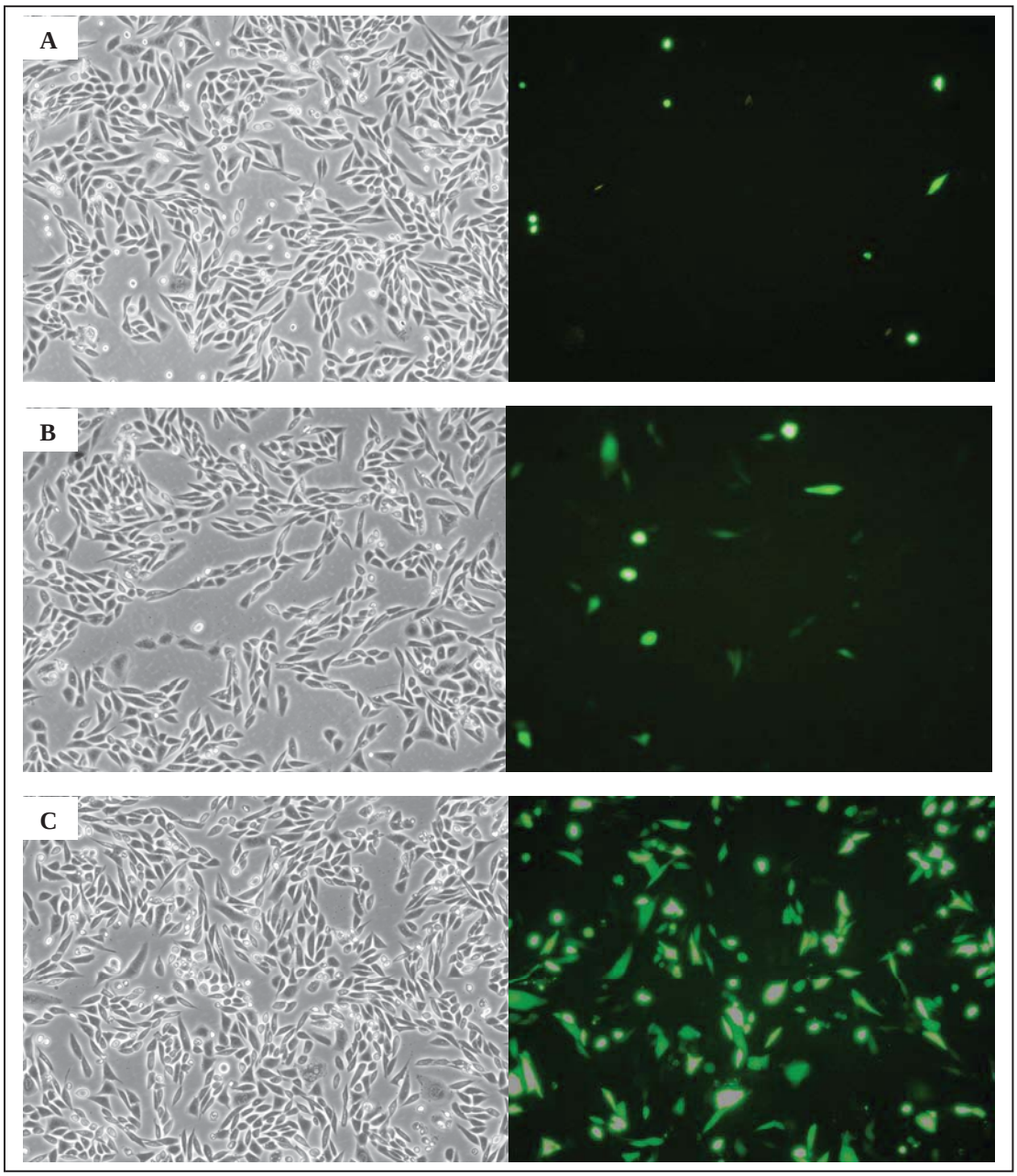


Figure 30 CHO-K1cells were transfected with pEGFP-C2 plasmid. The photos were photographed under inverted microscope at 24 hours after transfection. The pictures on the left were captured under light. The pictures on the right were cuptured under fluorescent light; A : 2 μg plasmid DNA per 3 μL liposome, concentration, B : 1 μg plasmid DNA per 3 μL liposome, C : 1 μg plasmid DNA per 6 μL liposome.

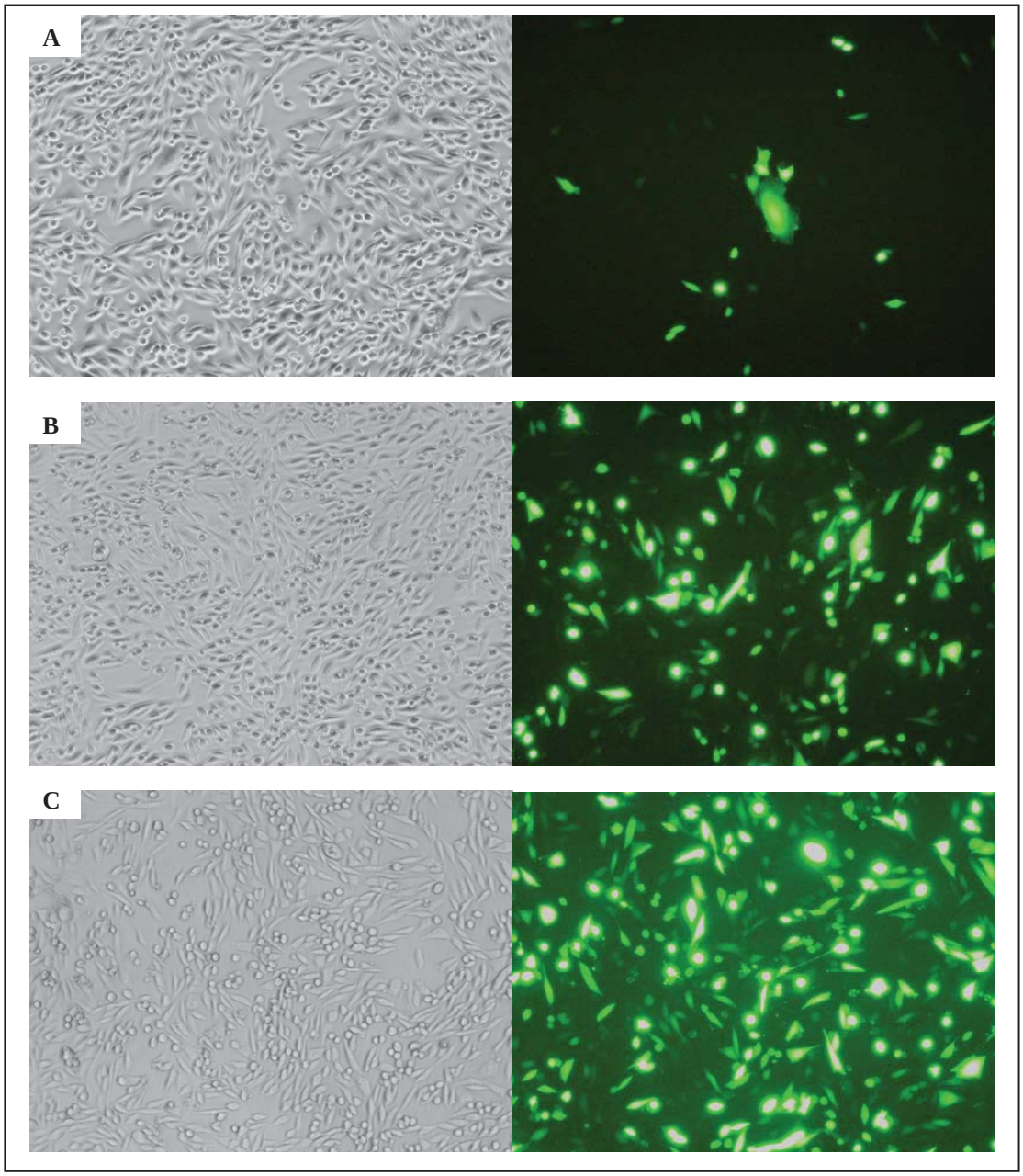


Figure 31 CHO-K1cells were transfected with pEGFP-C2 plasmid. The photos were photographed under inverted microscope at 36 hours after transfection. The pictures on the left were captured under light. The pictures on the right were cuptured under fluorescent light; A : 2 μg plasmid DNA per 3 μL liposome, concentration, B : 1 μg plasmid DNA per 3 μL liposome, C : 1 μg plasmid DNA per 6 μL liposome.

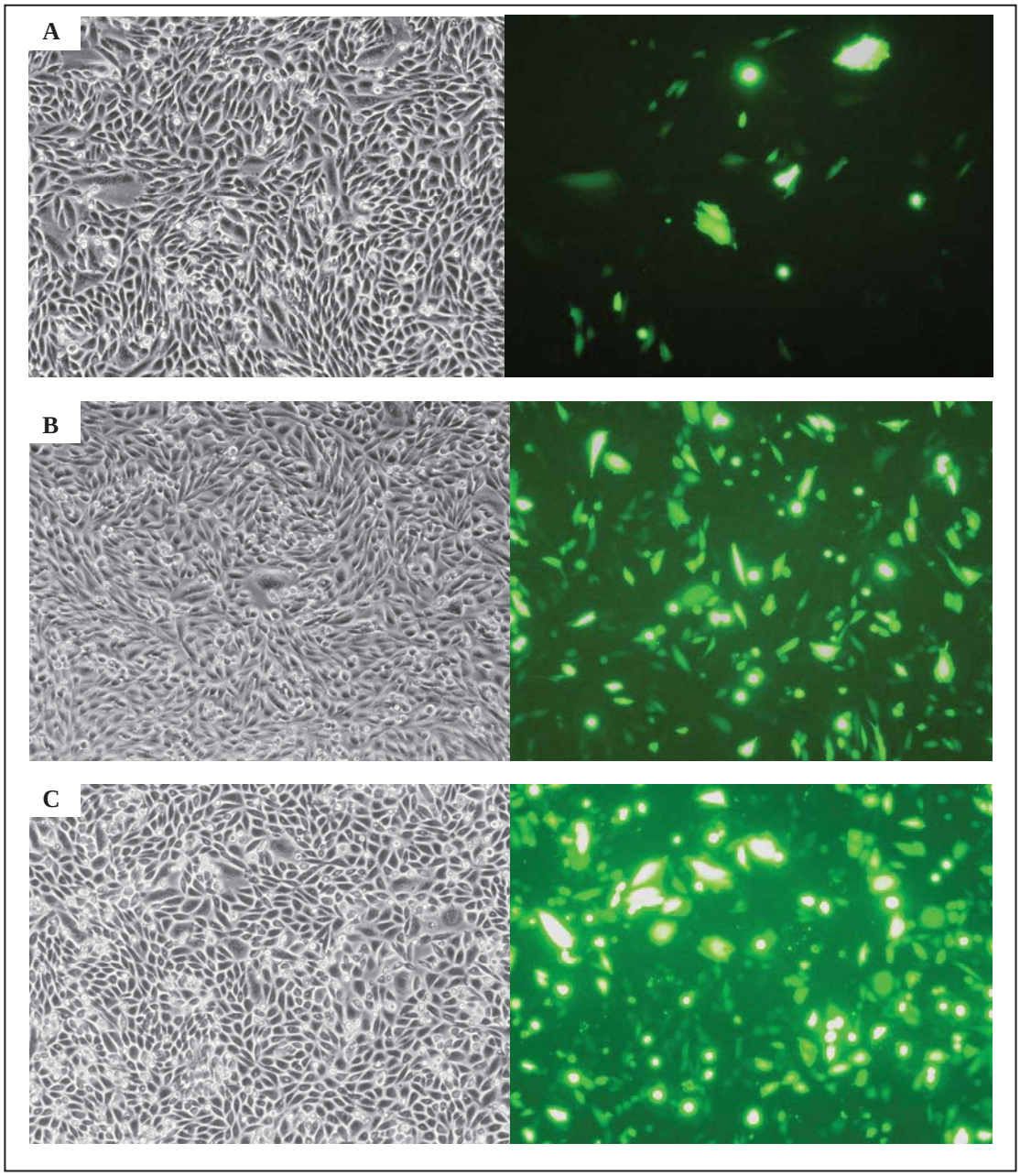


Figure 32 CHO-K1cells were transfected with pEGFP-C2 plasmid. The photos were photographed under inverted microscope at 48 hours after transfection. The pictures on the left were captured under light. The pictures on the right were cuptured under fluorescent light; A : 2 μg plasmid DNA per 3 μL liposome, concentration, B : 1 μg plasmid DNA per 3 μL liposome, C : 1 μg plasmid DNA per 6 μL liposome.

Table 9 Efficiency of transfection plasmid into CHO-K1 cells using Fugene® 6 reagent

Plasmid / Liposome (µg/µL)	**Cell number (GFP expressing/ total cell)**			**% transfection**
	Sample 1	**Sample 2**	**Sample 3**	
2 : 3	7/243	15/247	10/250	4±2
1 : 3	97/253	82/237	89/245	36±2
1 : 6	120/230	103/215	107/208	51±2

These data demonstrated that transfection efficiency was directly increased to low concentration of plasmid DNA/liposom. The 1:6 (w/v) gave the highest efficiency. Approximately 51% transfection was considered to be an effective transfection protocol for this study.

4. Luciferase Assay

The luminescence of luciferase reaction is stable for a short time. This study therefore preliminary tested the half life of the luminescence to evaluate the possibility of manual measurement. The reaction is nearly constant for several minutes. The data presented that the luminescence decreased as exponential curve. The half life of the luminescence was about 8 minutes (Figure 33). Therefore, it was possible to quantify the luciferase activity using this method that could measure within a minute. The firefly luciferase assay was also tested the correlation between reporter protein and luminescent unit. The data showed that the correlation between luficerase and luminescent intensity was linear regression (Figure 34) similar to the previous report (Lembert 1996).

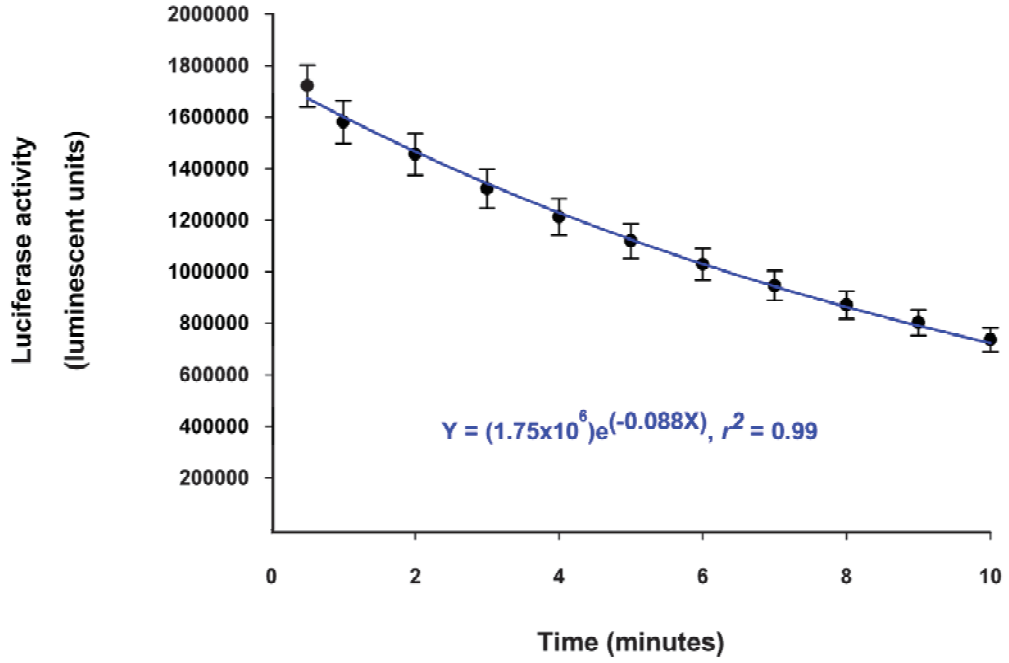


Figure 33 Effect of time on luciferase activity. Each bar represents luminescent unit means ± S.E (n=3).

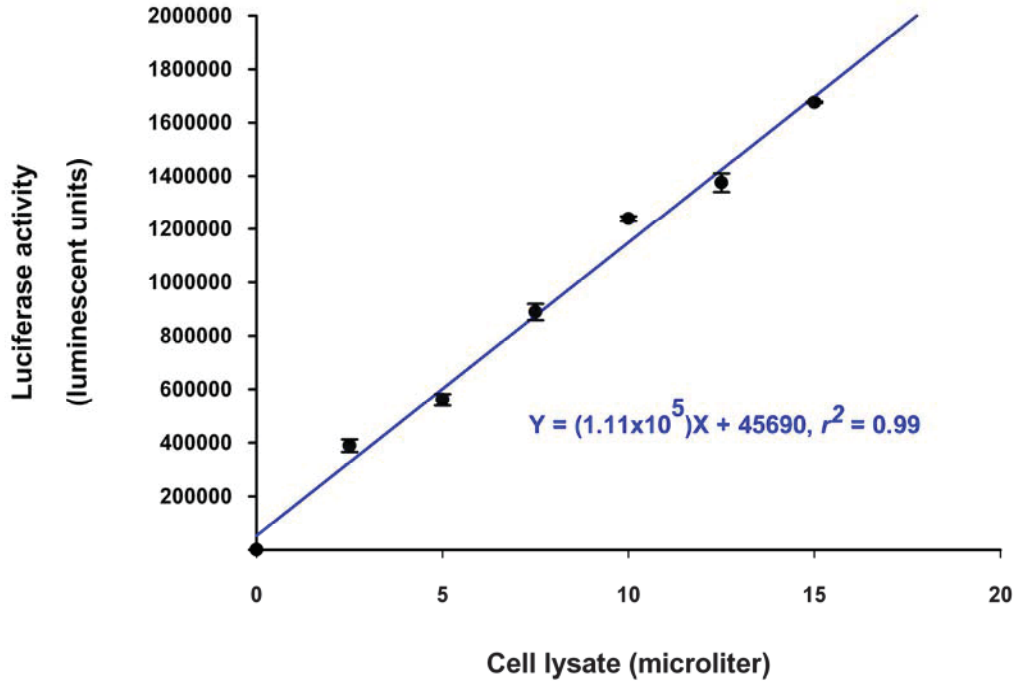


Figure 34 Linear regression of luciferase activity. Each bar represents luminescent unit means ± S.E (n=3).

5. Promoter Activity

The super core promoter beta activity was tested for evaluating new series of SCP. The core promoter function was tested without enhancer and proximal promoter. The hybrid promoters were screened for new potential promoter. All promoters; promoterless, SCP, SCP beta, hybrid human EF-1α/SCP beta, hybrid hamster β-actin/SCP beta, hybrid hamster GADD 153/SCP beta and hybrid CMV IE/SCP beta were exhibited the activity in transiently transfected CHO-K1 cells. Experiments in serum-supplemented cell culture (general condition) and serum-free cell culture (industrial preferable condition) were performed simultaneously. The firefly luciferase activities (express under the control of the promoters) were quantified and the relative luminescent units were recorded (Table 14 to Table 19, Appendix). The galactosidase activities were determined by measuring the absorbance of converted substrate. The galactosidase activities were used as the internal control for normalizing transfection efficiency.

The results showed that all promoters be able to drive firefly luciferase expression. They acted as constitutive promoter in CHO-K1 cells, but various strengths. In serum-supplemented condition, promoterless was the weakest promoter. The SCP, SCP beta, hybrid human EF-1α/SCP beta, hybrid hamster β-actin/SCP beta, hybrid hamster GADD 153/SCP beta and hybrid CMV IE/SCP beta activity was about 5, 8, 7, 85, 169 and 2688 fold higher than promoterless activity, respectively (Table 20, Appendix). In serum-free condition, Promoterless also showed the weakest activity and was used as a control. The SCP, SCP beta, hybrid human EF-1α/SCP beta, hybrid hamster β-actin/SCP beta, hybrid hamster GADD 153/SCP beta and hybrid CMV IE/SCP beta activity was about 4, 5, 5, 79, 180 and 2495 fold higher than promoterless activity, respectively (Table 21, Appendix).

Although a small vector may be transferred into the cell easier than a big vector, the size of plasmid vector might affect the promoter activity test. If the query interfere this evaluation, the promoter constructs existing in the biggest vector should exhibit the lowest activity. Unexpectedly, the hybrid hamster β-actin/SCP beta locating in the biggest vector did not the weakest promoter. Therefore, the size of vector did not affect in this study.

5.1 Effect of BRE on Core Promoter Activity

The SCP activity was compared with promoterless/enhancerless activity first. The result showed that the SCP activity was higher than the promoterless activity (p-value < 0.05, one-tailed student t-test). Thus, the SCP was confirmed the effective core promoter (Juven-Gershon et al. 2006). The SCP beta activity was compared with its prototype (the SCP) to evaluate the effect of BRE on the core promoter function. The result revealed that BRE up-regulate the reporter gene expression by 49% in transiently transfected CHO-K1 cells cultured in medium supplemented with serum and 35% in the cells cultured in serum-free medium (Figure 35) (p-value < 0.05, one-tailed student t-test, Table 22-23, Appendix).

In previous reports, transcription factor II B was reported either positive or negative effect on transcription initiation of protein coding gene. Thierry Lagrange and colleagues found that consensus sequence 5'-G/C-G/C-G/A-C-G-C-C-3' (G/C means guanine or cytosine) locating immediate upstream of TATA-box is recognized by TFIIB and supports transcriptional activation in *in vitro* transcription. Their experiment also showed that TATA-box, initiator and downstream promoter element (which interact with transcription factor II D) involved in transcription initiation of gene (Lagrange et al. 1998). Despite, the next study presented that BRE acts to repress basal transcription in crude nuclear extract and transiently transfected human embryonic kidney 293 cells when it is located between GAL4-AH responsive element and TATA-box (Evans et al. 2001). In the last 6 years, Wensheng Deng and Stefan G.E. Roberts discovered a different DNA sequence of BRE that locate downstream of TATA-box. They demonstrated that the BRE had a positive effect on transcriptional expression in *in vitro* transcription and in transient transfected HEK 293 cells but it interrupted transcriptional stimulation of GAL4-AH activator (Deng and Roberts 2005).

However, this study demonstrated that the BRE consensus sequence as follows 5'-GGGCGCC-3' locating upstream of TATA-box of the SCP increased transcriptional expression of gene in CHO-K1 cells. There are two possible mechanisms to explain this result. First, BRE (without distal promoter) had a positive effect on transcriptional activation because it supported RNA polymerase II function to open transcription start site and led to initiate gene transcription (Kostrewa et al. 2009). Second, CHO-K1 nuclear context specifically activated transcription of gene via BRE.

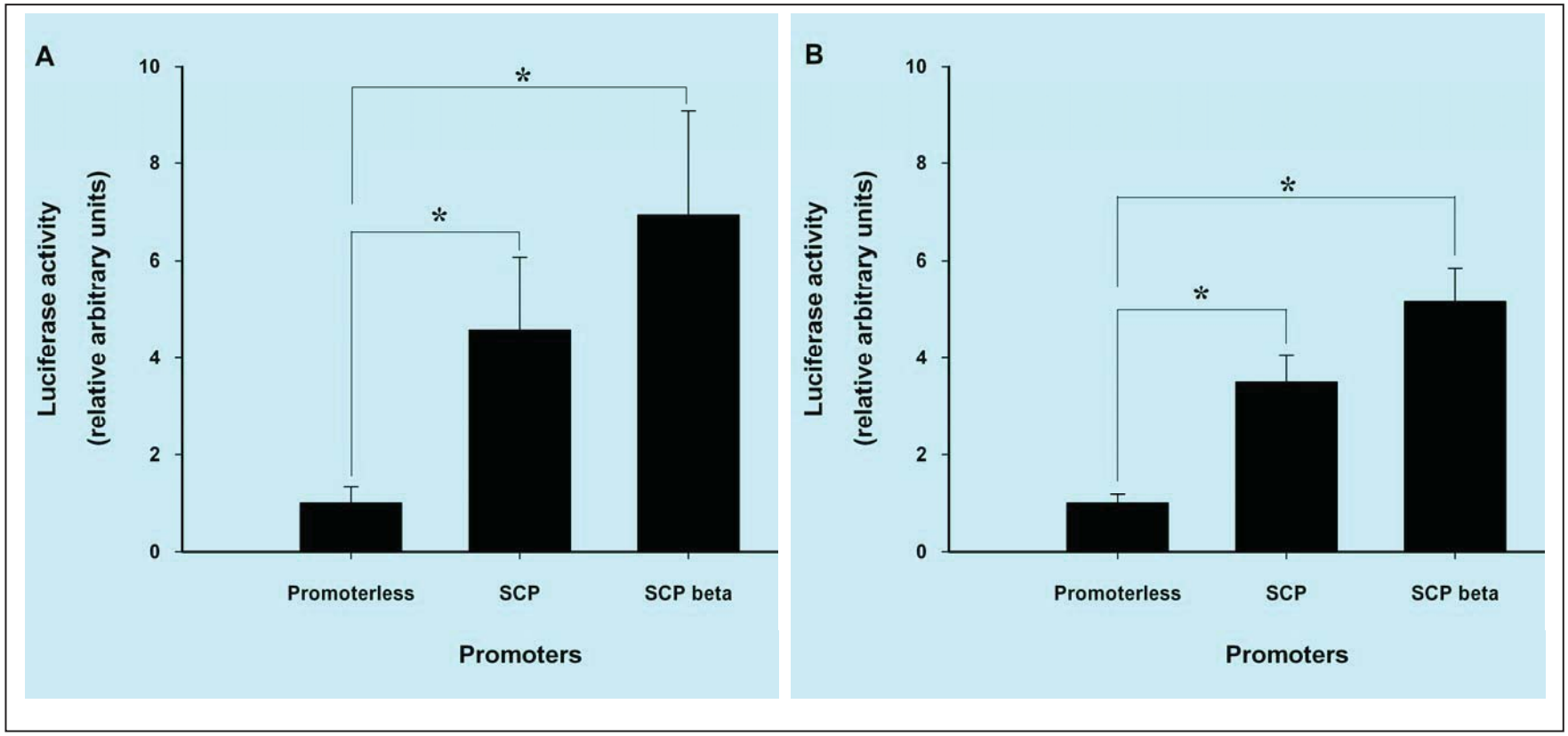


Figure 35 Core promoter activity was exhibited in transiently transfected CHO-K1 cells. A : The cells were cultured in completed medium. B : The cells were cultured in serum-free medium. Each bar represents mean ± S.E.

* denotes *p*-value < 0.05 student *t*-test (compare with promoterless activity)

Nevertheless, this evidence agreed with the fact that TFIIB acts as a rate-limiting step of the transcription initiation by controling the abortive transcription rate and recruitment of RNA polymerase II (Wiesler and Weinzierl 2011). Altogether, it could be considered that BRE provided a positive effect on transcription of gene. Although BRE interrupted transcriptional activation of GAL4-AH activator but it enhanced the function of TATA-box, initiator (Inr), motif ten element (MTE) and the downstream promoter element (DPE) which promotes transcription initiation of protein-coding gene (Juven-Gershon et al. 2006).

However, this study tested only the BREu. Introduction the BREd into the SCP has not been performed. Nevertheless, the nucleotide on the position -8 of SCP is guanine. Although it dose not abolish transcriptional activation, substitution it with adenine would increase the core promoter activity and need to be tested. Moreover, the distance between BRE and TATA-box would be varied to optimize the location of BRE that enhances core promoter activity.

5.2 Effect of Enhancer on Hybrid Promoter Activity

To design new high potency promoters, four hybrid promoters were constructed by linking housekeeping gene's enhancer upstream to the SCP beta. Activity of these hybrid promoters were evaluated in transiently transfected CHO-K1 cells. The result showed that enhancer affected hybrid promoter activity (p-value < 0.05, ANOVA, Table 24, Appendix).The enhancers generated various strengths. The hybrid EF-1α/SCP beta was the weakest promoter and did not differ from the SCP beta. The hybrid β-actin/SCP beta, the hybrid GADD 153/SCP beta and the CMV IE/SCP beta activity increased about 11, 22 and 336 fold higher than SCP beta activity in completed cell culture, respectively (Figure 36, A).

This experiment demonstrated that the SCP beta activity is increased by introducing housekeeping gene's enhancer/proximal promoter containing responsive elements which recognized by activators that would recruit TATA binding protein (TBP), transcription factor II D (TFIID), transcription factor II B (TFIIB) and RNA II polymerase to form pre-initiation complex and drive gene transcription (Liu et al. 2009). However, it should be mentioned that human responsive element DNA might not be active in a hamster cellular context. The hybrid human EF-1α/SCP beta is the example. Its enhancer is a human DNA sequence which contains seven constitutive SP1 responsive elements and an AP1 responsive element, but it did not alter promoter activity.

Nevertheless, this is the first time revealed that the hamster GADD 153 enhancer/proximal promoter was stronger than hamster β-actin enhancer/ proximal promoter. It may be explained by the fact that SP1 responsive elements is adjacent to core promoter provided the stimulation much more than the EST1 and Myb responsive elements distributing over enhancer fragment (Tornoe et al. 2002). Furthermore, the strongest promoter in this study is the hybrid CMV IE/SCP beta. The SCP beta activity was extremely increased when it was introduced the enhancer with four cyclic AMP responsive elements (CRE), four NF-κB responsive elements and a constitutive SP1 responsive element. This extreme stimulation would be due to the activation of cyclic AMP responsive binding protein (CREBP). It is one of the most active transcriptional factors (Schlabach et al. 2010). The transcriptional activation on the CMV IE enhancer was triggered by NF-κB, also (He and Weber 2004).

To evaluate utility of the hybrid promoters for industrial cell culture, the promoter activity was therefore tested in serum-free CHO-K1 cell culture. The result showed that the human EF-1α/SCP beta yielded the lowest level of the reporter activity and it did not differ from the SCP beta (*p*-value > 0.05, Post hoc test, Table 25, Appendix). The hamster β-actin/SCP beta, the hamster GADD 153/SCP beta and the CMV IE/SCP beta increased the reporter protein level about 15, 33 and 463 fold higher than the SCP beta, respectively (Figure 36, B). These results supported that the CMV IE/SCP beta had the greatest potency to drive transgenic expression in serum-free condition. Although the CMV IE promoter was reported that its activity decreased while the hamster GADD 153 promoter activity increased in serum-free cell culture, the CMV IE enhancer still remained the strongest enhancer in CHO cells cultured with serum-free medium (Bore et al. 2004).

However, this study has not optimized the distance between proximal promoter and SCP beta as well as number of responsive element aligning on enhancer. Probably, truncated proximal promoter may provide positive effect, but should not be too short. Moreover, increase the number of responsive element possibly enhances the transcriptional activation. Unless these suggessions take cost, new generation of the hybrid enhancer/SCP beta could be created using synthetic enhancer such as F10 enhancer (Schlabach et al. 2010).

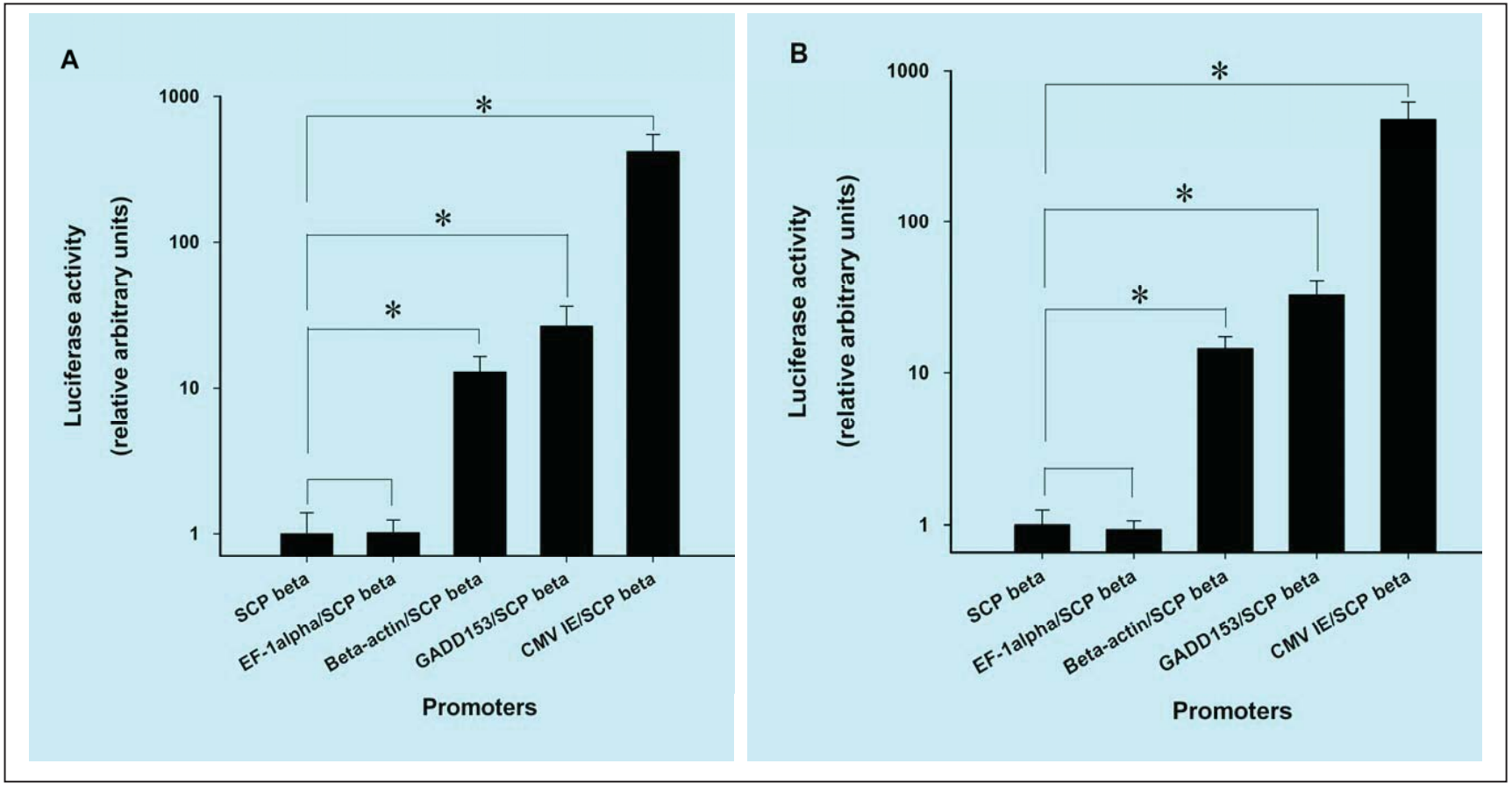


Figure 36 Promoter activity was exhibited in transiently transfected CHO-K1 cells which cultured with 10% serum-supplemented medium (A) and cultured in serum-free medium (B). Each bar represents luciferase activity mean ± s.d of three experiments (n=9).

* denotes *p*-value < 0.05 multiple comparison (Post hoc test).

5.3 Effect of Serum on Promoter Activity

The luciferase expression under the control of the SCP, the SCP beta, the human EF-1α/SCP beta and the CMV IE/SCP beta slightly decreased in serum-free condition. Otherwises, the hybrid hamster GADD 153/SCP beta and hybrid hamster β-actin/SCP beta activity did not significantly decrease (Figure 37). Shortly, the hybrid CMV IE/SCP beta activity decreased about 28% that might be because of depletion of serum growth factor stimulating NF-κB pathway (Baldwin et al. 1991). This study supported the fact that serum provided a positive effect on promoter activity as well as gene expression (Durocher, Perret and Kamen 2002).

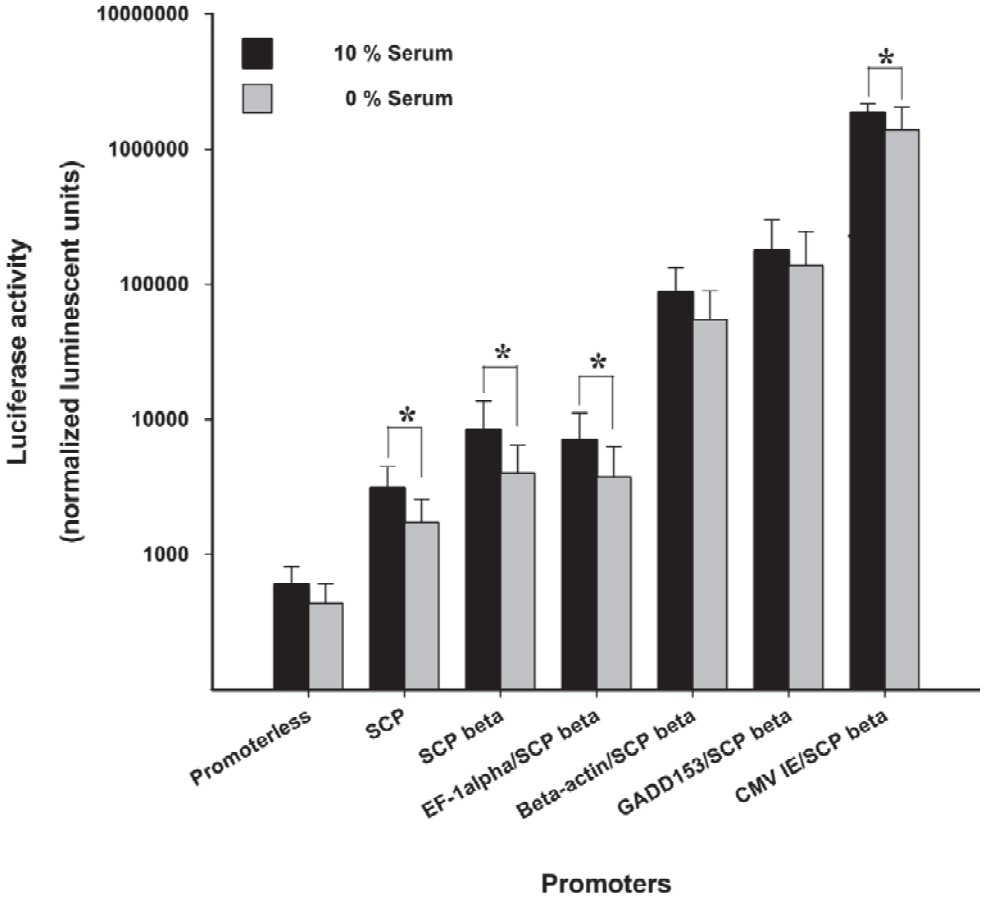


Figure 37 Luciferase expression under the control of promoter constructs. Histogram was plotted with normalized luminescent unit means of three experiments. * denotes *p*-value < 0.05 one-tailed student *t*-test.

CHAPTER 5
CONCLUSIONS

The promoter is an important feature of mammalian expression vector. The promoter with a greater activity would improve recombinant protein production efficiency. The recent study therefore created both synthetic promoter and hybrid promoters to optimize promoter activity.

To improve the promoter activity, the super core promoter was optimized by introducing the transcription factor II B responsive element (BRE) upstream of TATA-box. The BRE was included because RNA polymerase II required the TFIIB transcription factor to initiate protein-coding gene transcription. The result presented that the SCP containing BRE (SCP beta) was stronger than its prototype. This study indicated that BRE increases the SCP activity on firefly luciferase gene expression in CHO-K1 cells. To create new applicable promoters, the four hybrid enhancer/SCP beta promoters were introduced in this study. The results revealed that the SCP beta activity could be increased by adding the housekeeping's gene enhancer/ proximal promoter. The effect of enhancers varied from non-responsive that detected in the human EF-1α/SCP beta, intermediate active which observed in the hamster β-actin/SCP beta and the hamster GADD 153/SCP beta to extreme stimulative which found out in the CMV IE/SCP beta. Nevertheless, this study suggested that fetal bovine serum had a positive effect to the SCP, SCP beta and the hybrid CMV IE/SCP beta activity. In serum-free cell culture, these promoter activities were decreased. However, the hybrid CMV IE/SCP beta was the strongest promoter in CHO-K1 cells both in the serum-supplemented culture and serum-free culture.

In summary, the SCP beta robustly supports transcriptional initiation of expression of protein-coding gene. The hybrid CMV IE/SCP beta could achieve transgene expression in both CHO-K1 cell culture supplemented with serum and depleted serum. Altogether, the information from this study would lead an idea for synthetic promoter design to increase the power of the useful expression vector which would advance therapeutic recombinant protein production.

BIBLIOGRAPHY

Baldwin, Albert S. et al. "Induction of NF-κB DNA-Binding Activity during the Go-to-G1Transition in Mouse Fibroblasts." Molecular and Cellular Biology 11 (1991) : 4943-4951.

Bishop, Paul, and Jeff Lawson. "Recombinant Biologics for Treatment of Bleeding Disorders." Nature Reviews Drug Discovery 3 (2004) : 684-694.

Boer, Leonore De, Peter P. Gray, and Noelle-Ann Sunstrom. "Enhanced Productivity of G1 Phase Chinese hamster Ovary Cells Using the GADD 153 Promoter." Biotechnology Letters 26 (2004) : 61–65.

Boshart, Michael et al. "A Very Strong Enhancer Is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus." Cell 41 (1985) : 521-530.

Brooks, Alan R. et al. "Transcriptional Silencing Is Associated with Extensive Methylation of the CMV Promoter Following Adenoviral Gene Delivery to Muscle." The Journal of Gene Medicine 4 (2004) : 395-404.

Brown, William M., and Philip M. Brown. Transcription. New York : Taylor & Francis Inc., 2002.

Chen, Weiye et al. "Establishment of a Stable CHO Cell line with High Level Expression of Recombinant Porcine IFN-β." Cytokine 54 (2011) : 324–329.

Cheng, Tong et al. "A Rapid and Efficient Method to Express Target Genes in Mammalian Cells by Baculovirus." World Journal of Gastroenterology 10 (2004) : 1612-1618.

Connelly, Sheila, and James L. Manley. "A Functional mRNA Polyadenylation Signal is Required for Transcription Termination by RNA Polymerase II." Genes & Development 2 (1988) : 440-452.

Deng, Wensheng, and Stefan G.E. Roberts. "A Core Promoter Element Downstream of the TATA Box that is Recognized by TFIIB." Gene & Development 19 (2005) : 2418–2423.

Durocher, Yves, Sylvie Perret, and Amine Kamen. "High-level and High-throughput Reconbinant Protein Production by Transient Transfection of Suspension-growing Human 293-EBNA1 Cells." Nucleic Acids Research 30 (2002) : 1-9.

Estes, Scott D., and Weiqun Zhang. "Novel β-actin Promoters and Uses Thereof." World Intellectual Property Organization U.S patent no. 60/480768, 2004.

Evans, Rachel, Jennifer A. Fairley, and Stefan G.E. Roberts. "Activator-mediated Disruption of Sequence-specific DNA Contacts by the General Transcription Factor TFIIB." Genes & Development 15 (2001) : 2945–2949.

Fouser, La et al. "High Level Expression of a Chimeric Anti-gangiinside GD2 Antibody: Genumic Kappa Sequences Improve Expression in COS and CHO Cells." Biotechnology 10 (1992) : 1121-1127.

Freshney, Ian R. Culture of Animal Cells. 5th ed. New Jersey : John Wiley & Sons, 2010.

Haldankar, Raj et al. "Serum-free Suspension Large-scale Transient Transfected of CHO Cells in Wave Bioreactors." Molecular Biotechnology 34 (2006) : 191-199.

He, Bin, and Georg F. Weber. "Synergistic Activation of the CMV Promoter by NF-κB P50 and PKG." Biochemical and Biophysical Research Communications 321 (2004) : 13–20.

Hong, Sunghoi et al. "Functional Analysis of Various Promoters in Lentiviral Vectors at Different Stages of In Vitro Differentiation of Mouse Embryonic Stem Cells." Molecular Therapy 15 (2007) : 1630–1639.

Jacquier, Alain. "The Complex Eukaryotic Transcriptome: Unexpected Pervasive Transcription and Novel Small RNAs." Nature Reviews Genetics 10 (2009) : 833-844.

Juven-Gershon, Tamar, Susan Cheng, and James T. Kadonaga. "Rational Design of a Super Core Promoter that Enhancers Gene Expression." Nature Methods 3 (2006) : 917-922.

Kao, Fa-Ten, and Theodore T. Puck. "Genetics of Somatic Mammalian Cells. IV. Properties of Chinese Hamster Cell Mutants with Respect to the Requirement for Proline." Genetics 55 (1967) : 513-524.

Kim, Do Yun et al. "Development of Serum-free Media for a Recombinant CHO Cell Line Producing Recombinant Antibody." Enzyme and Microbial Technology 39 (2006) : 426–433.

Kim, Seon-Young et al. "The Human Elongation Factor 1alpha (EF-1α) First Intron Highly Enhances Expression of Foreign Genes from the Murine Cytomegalovirus Promoter." Journal of Biotechnology 93 (2002) : 183–187.

Kostrewa, Dirk et al. "RNA Polymerase II–TFIIB Structure and Mechanism of Transcription Initiation." Nature 462 (2009) : 323-330.

Lagrange, Thierry et al. "New Core Promoter Element in RNA Polymerase II-dependent Transcription: Sequence-specific DNA Binding by Transcription Factor II B." Genes & Development 12 (1998) : 34–44.

Lander, Eric S. et al. "Initial Sequencing and Analysis of the Human Genome." Nature 409 (2001) : 860-921.

Lang, Dieter, Helmut Fickenscher, and Thomas Stamminger. "Analysis of Proteins Binding to the Proximal Promoter Region of the Human Cytomegalovirus IE-1/2 Enhancer/promoter Reveals Both Consensus and Aberrant Recognition Sequences for Transcription Factors Sp1 and CREB." Nucleic Acids Research 20 (1992) : 3287-3295.

Lembert, Nicolas. "Firefly Luciferase Can Use L-luciferin to Produce Light." Biochemical Journal 317 (1996) : 273-277.

Levine, Michael, and Robert Tjian. "Transcription Regulation and Animal Diversity." Nature 424 (2003) : 147-151.

Lewin, Benjamin. Genes IX. 9th ed. Massachusetts : Jones and Bartlett Publishers, 2008.

Liu, Wei-Li et al. "Structures of Three Distinct Activator-TFIID Complexes." Genes & Development 23 (2009) : 1510–1521.

Luethy, Jennifer D. et al. "Isolation and Characterization of the Hamster GADD 153 Gene." The Journal of Biological Chemistry 265 (1990) : 16521-16526.

Lynch, Michael. "The Origins of Eukaryotic Gene Structure." Molecular Biology and Evolution 23 (2006) : 450–468.

Makrides, Savvas C. "Components of Vectors for Gene Transfer and Expression in Mammalian Cells." Protein Expression and Purification 17 (1999) : 183–202.

Maston, Glenn A., Sara K. Evans, and Michael R. Green. "Transcriptional Regulatory Elements in the Human Genome." The Annual Review of Genomics and Human Genetics 7 (2006) : 29-59.

Matasci, Mattia et al. "Recombinant Therapeutic Protein Production in Cultivated Mammalian Cells: Current Status and Future Prospects." Drug Discovery Today: Technologies Protein therapeutics 5 (2008) : 37-42.

Mcburney, Michael W. et al. "Evidence for Repeat-Induced Gene Silencing in Cultured Mammalian Cells: Inactivation of Tandem Repeats of Transfected Genes." Experimental Cell Research 274 (2002) : 1–8.

Mellor, Jane. "The Dynamics of Chromatin Remodeling at Promoters." Molecular Cell 19 (2005) : 147–157.

Miller, Kathy et al. "Paclitaxel Plus Bevacizumab Versus Paclitaxel Alone for Metastatic Breast Cancer." The New England Journal of Medicine 357 (2007) : 2666-2676.

Pai, Jennifer C., Amie N. Sutherland, and Ennifer A. Maynard. "Progress Towards Recombinant Anti-Infective Antibodies." Recent Patents on Anti-Infective Drug Discovery 4 (2009) : 1-17.

Portales-Casamar, Elodie et al. "JASPAR 2010: the greatly expanded open-access database of transcription factor binding profiles." Nucleic Acids Research 38 (2009) : 105-110.

Primrose, Sandy B., and Richard M. Twyman. Principles of Gene Manipulation and Genomics. 7th ed. Massachusetts : Blackwell Publishing, 2006.

Ratledge, Colin, and Bjorn Kristiansen. Basic Biotechnology. 3rd ed. Cambridge : Cambridge University press, 2006.

Richard, Patricia, and James L. Manley. "Transcription termination by nuclear RNA polymerases." Genes & Development 23 (2009) : 1247–1269.

Robert, Caroline et al. "Ipilimumab plus Dacarbazine for Previously Untreated Metastatic Melanoma." The New England Journal of Medicine 364 (2011) : 2517-2526.

Saunders, Abbie, Leighton J. Core, and John T. Lis. "Breaking Barriers to Transcription Elongation." Nature Reviews Molecular Cell Biology 7 (2006) : 557-567.

Schlabach, Michael R. et al. "Synthetic Design of Strong Promoters." Proceedings of the National Academy of Sciences 107 (2009) : 2539-2543.

Sinici, Incilay et al. "Comparison of HCMV IE and EF-1α Promoters for the Stable Expression of β-Subunit of Hexosaminidase in CHO Cell Lines." Biochemical Genetics 44 (2006) : 173-180.

Stephanopoulos, Gregory. Bioprocessing. 2nd ed. New York : VCH Puhlishers Inc., 1993.

Stoll, Thibaud S. et al. "Systematic Improvement of a Chemically-defined Protein-free Medium for Hybridoma Growth and Monoclonal Antibody Production." Journal of Biotechnology 45 (1996) : 111-123.

Tjio, Joe-Hin, and Theodore T. Puck. "Genetics of Somatic Mammalian Cells II. Chromosomal Constitution of Cells in Tissue Culture." The Journal of Experimental Medicine 108 (1958) : 259-268.

Tornoe, Jens et al. "Generation of a Synthetic Mammalian Promoter Library by Modification of Sequences Spacing Transcription Factor Binding Sites." Gene 297 (2002) : 21–32.

Trill, John J., Allan R. Shatzman, and Subinay Ganguly. "Production of Monoclonal Antibodies in COS and CHO cells." Current Opinion in Biotechnology 6 (1995) : 553-560.

Wakabayashi-Ito, Noriko, and Shigekazu Nagata. "Characterization of the Regulatory Elements in the Promoteorf the Human Elongation Factor-lalpha Gene." The Journal of Biological Chemistry 269 (1994) : 29831-29837.

Wang, Jing et al. "Purification and Characterization of a Single-chain Chimeric Anti-p185 Antibody Expressed by CHO–GS system." Protein Expression and Purification 41 (2005) : 68–76.

Whitelaw, Emma, and Nick Proudfoot. "α-Thalassaemia Caused by a Poly(A) Site Mutation Reveals that Transcriptional Termination is Linked to 3' end Processing in the Human α2 Gobin Gene." The EMBO Journal 5 (1986) : 2915-2922.

Wiesler, Simone C., and Robert O. J. Weinzierl. "The Linker Domain of Basal Transcription Factor TFIIB Controls Distinct Recruitment and Transcription Stimulation Functions." Nucleic Acids Research 39 (2011) : 464–474.

Wurm, Florian M. "Production of Recombinant Protein Therapeutics in Cultivated Mammalian Cells." Nature Biotechnology 22 (2004) : 1393-1398.

Yang, Te-Tuan, Linzhao Cheng, and Steven R. Kain. "Optimized Codon Usage and Chromophore Mutations Provide Enhanced Sensitivity with the Green Fluorescent Protein." Nucleic Acids Research 24 (1996) : 4592–4593.

Zolotukhin, Sergei et al. "A Humanized Green Fluorescent Protein cDNA Adapted for High-Level Expression in Mammalian Cells." Journal of Virology 70 (1996) : 4646–4654.

APPENDIX

LIST OF ABBREVIATIONS

A	Adenine
AdML	Adeno major late promoter
AMP	Adenosine monophosphate
Amp	Ampicillin
ATCC	American type culture collection
ATP	Adenosine triphosphate
Av	Average
bp	Base pair
BRE	Transcription factor II B recognition element
C	Cytosine
CHO	Chinese hamster ovary
cm	Centimeter
CMV IE	Cytomegalovirus immediate early
CRE	Cyclic AMP responsive element
dATP	Deoxyadenosine triphosphate
DHFR	Dihydrofolate reductase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPE	Downstream promoter element
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EF-1α	Elongation factor-1alpha
EGFP	Enhance green fluorescent protein
FBS	Fetal bovine serum
G	Guanine
g	Gram
GADD	Growth arrest and DNA damage inducible
gDNA	Genomic deoxyribonucleic acid
GFP	Green fluorescent protein
HeLa	HeLa cell line

hr	Hour
Inr	Initiator
ITC	Initial transcribing complex
k	kilo
L	Liter
LB	Luria Bertani
Luc	Luciferase
M	Molarity
mg	milli gram
min	Minute
mL	milli liter
mM	milli molar
mRNA	messenger ribonucleic acid
MTE	Motif ten element
N	Normality
NCBI	National Center for Biotechnology Information
ng	Nano gram
No.	Number
OD	Optical density
ORF	Open reading frem
Ori	Origin of replication
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
PIC	Pre-initiation complex
PNPG	*p*-nitrophenyl-beta-D-galactopyranoside
RH	Relative humidity
RLU	Relative luminescent unit
RNA	Ribonucleic acid
RnaseA	Ribonuclease A
rpm	Revolution per minute
S.D	Standard deviation
S.E	Standard error

SCP	Super core promoter
SDS	Sodium dodecyl sulphate
sec	Second
SPSS	Statistical package for the social sciences
SV40	Simian virus 40 promoter
T	Thymine
TBE	Tris-borate-EDTA
TF	Transcription factor
Tm	Melting temperature
Tris	Tris (hydroxymethyl) aminomethane
TSS	Transcription start site
UV	Ultra-violet light
Vol	Volume
W	Weight
β-gal	Beta-galactosidase
%	Percentage
*	Asterisk
±	Plus per minus
°C	Degree Celsius
µg	micro gram
µL	micro liter

LIST OF DEFINITIONS

CMV IE promoter	Human cytomegalovirus immediate early 1 promoter
Completed medium	F-12 nutrient mixture (Ham) medium supplemented with 10% (v/v) heat inactivated fetal bovine serum and 2 mM L-glutamine
Position of nucleotide	The positions of nucleotides were relative to adenine in initiator as +1.
Room temperature	25 °C by approximately
Serum-free medium	F-12 nutrient mixture (Ham) medium supplemented with 2 mM L-glutamine

Medium and Buffer Formula

Table 10 Luria Bertani (LB) Broth Formula

Chemicals	Amount
Tryptone	4.00 g
Sodium chloride	4.00 g
Yeast extract	2.00 g
Distilled water	400 mL

Table 11 Phosphate Buffer Saline (PBS) Formula

Chemicals	Amount
Sodium chloride	8.00 g
Potassium chloride	0.20 g
Potassium phosphate monobasic	0.12 g
Sodium phosphate dibasic	0.80 g
Distilled water q.s to	1,000 mL

Table 12 Tris-Acetate EDTA (TAE) Buffer

Chemicals	Amount
Tris base	4.84 g
Glacial acetic acid	1.14 mL
0.5 M EDTA (pH 8.0)	2.00 mL
Distilled water q.s to	1,000 mL

Table 13 Luciferase Cell Culture Lysis Reagent

Chemicals	Final concentration
Tris-phosphate (pH 7.8)	25 mM
Dithiothreitol (DTT)	2 mM
1,2-diaminocyclohexane-N,N,N´,N´-tetraacetic acid	2 mM
Glycerol	10 %
Triton® X-100	1 %

DNA SEQUENCE

1. Super core promoter beta DNA sequence

```
...|....| ....|....| ....|....| ....|....| ....|.... ....|....|
      -40        -30        -20         -10          +1       10
GGGGCTCGA GGGGCGCCTA TATAAGCAGA GCTCGTTTAG TGAACCGTC AGTTCGCCTG

....|....| ....|....| ....|....| ....|....| ...
         20         30         40         50
GAGACGTCGA GCCGAGCGGT TGTGCCACCA TGAAAGCTTC CCC
```

2. Hybrid human EF-1α/SCP beta DNA sequence

```
.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
    -1290      -1280      -1270      -1260      -1250      -1240
GGCGAAG GTACACCCTA ATCTCAATAC AACCTTTGGA GCTAAGCCAG CAATGGTAGA GGG

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
    -1230      -1220      -1210      -1200      -1190      -1180
AAGATTC TGCACGTCCC TTCCAGGCGG CCTCCCCGTC ACCACCCCCC CCAACCCGCC CCG

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
    -1170      -1160      -1150      -1140      -1130      -1120
ACCGGAG CTGAGAGTAA TTCATACAAA AGGACTCGCC CCTGCCTTGG GGAATCCCAG GGA

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
    -1110      -1100      -1090      -1080      -1070      -1060
CCGTCGT TAAACTCCCA CTAACGTAGA ACCCAGAGAT CGCTGCGTTC CCGCCCCCTC ACC

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
    -1050      -1040      -1030      -1020      -1010       -1000
CGCCCGC TCTCGTCATC ACTGAGGTGG AGAAGAGCAT GCGTGAGGCT CCGGTGCCCG TCA

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -990       -980       -970       -960       -950       -940
GTGGGCA GAGCGCACAT CGCCCACAGT CCCCGAGAAG TTGGGGGGAG GGGTCGGCAA TTG

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -930       -920       -910       -900       -890       -880
AACCGGT GCCTAGAGAA GGTGGCGCGG GGTAAACTGG GAAAGTGATG TCGTGTACTG GCT

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -870       -860       -850       -840       -830       -820
CCGCCTT TTTCCCGAGG GTGGGGGAGA ACCGTATATA AGTGCAGTAG TCGCCGTGAA CGT

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -810       -800       -790       -780       -770       -760
TCTTTTT CGCAACGGGT TTGCCGCCAG AACACAGGTA AGTGCCGTGT GTGGTTCCCG CGG

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -750       -740       -730       -720       -710       -700
GCCTGGC CTCTTTACGG GTTATGGCCC TTGCGTGCCT TGAATTACTT CCACGCCCCT GGC

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -690       -680       -670       -660       -650       -640
TGCAGTA CGTGATTCTT GATCCCGAGC TTCGGGTTGG AAGTGGGTGG GAGAGTTCGA GGC
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.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -630       -620       -610       -600       -590       -580
CTTGCGC TTAAGGAGCC CCTTCGCCTC GTGCTTGAGT TGAGGCCTGG CCTGGGCGCT GGG

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -570       -560       -550       -540       -530       -520
GCCGCCG CGTGCGAATC TGGTGGCACC TTCGCGCCTG TCTCGCTGCT TTCGATAAGT CTC

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -510       -500       -490       -480       -470       -460
TAGCCAT TTAAAATTTT TGATGACCTG CTGCGACGCT TTTTTTCTGG CAAGATAGTC TTG

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -450       -440       -430       -420       -410       -400
TAAATGC GGGCCAAGAT CTGCACACTG GTATTTCGGT TTTTGGGGCC GCGGGCGGCG ACG

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -390       -380       -370       -360       -350       -340
GGGCCCG TGCGTCCCAG CGCACATGTT CGGCGAGGCG GGGCCTGCGA GCGCGGCCAC CGA

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -330       -320       -310       -300       -290       -280
GAATCGG ACGGGGGTAG TCTCAAGCTG GCCGGCCTGC TCTGGTGCCT GGCCTCGCGC CGC

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -270       -260       -250       -240       -230       -220
CGTGTAT CGCCCCGCCC TGGGCGGCAA GGCTGGCCCG GTCGGCACCA GTTGCGTGAG CGG

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -210       -200       -190       -180       -170       -160
AAAGATG GCCGCTTCCC GGCCCTGCTG CAGGGAGCTC AAAATGGAGG ACGCGGCGCT CGG

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -150       -140       -130       -120       -110       -100
GAGAGCG GGCGGGTGAG TCACCCACAC AAAGGAAAAG GGCCTTTCCG TCCTCAGCCG TCG

.|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...
     -90        -80        -70        -60        -50        -40
CTTCATG TGACTCCACG GAGTACCGGG CGCCGTCCAG GCACCTCGAT TAGTTCTCGA GGG

.|....| ....|....| ....|....| ....|.... ....|....| ....|....| ....
     -30        -20        -10         +1        10         20
GCGCCTA TATAAGCAGA GCTCGTTTAG TGAACCGTC AGTTCGCCTG GAGACGTCGA GCCG

|....| ....|....| ....|....
     30         40
AGCGGT TGTGCCACCA TGAAAGCTT
```

3. Hybrid hamster β-actin/SCP beta DNA sequence

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...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
 -1480      -1470      -1460      -1450      -1440      -1430
TCTC TTTTGTGGGA ACCACAGAGT AGCCCTGAAC GTGGGGTGTG CTTCCAGTAT ACTCTG

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
 -1420      -1410      -1400      -1390      -1380      -1370
GGGT CACCCTTTCC ATACTGGAGG CCTCTGCAAC TTCAAAATGC TCTGCTACCA ACCTAG

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
 -1360      -1350      -1340      -1330      -1320      -1310
CACA AGGAAGTTGG TCCAGCCTCC CCACGCAGGG CCACTGCTGC AGTCCATATA TGGACT

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
 -1300      -1290      -1280      -1270      -1260      -1250
AAGC CTTCCTTGGT TTCAACACCT ACACTCACTG AGCCCTACTA TGTGTATGCA GAGCCG

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
 -1240      -1230      -1220      -1210      -1200      -1190
AGAC AGGCCCGAGC ATCTCATCTG AAGCACCCTT CTTGCCTAAA TTCAGTTTTC TGTCAC

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
 -1180      -1170      -1160      -1150      -1140      -1130
TTTC TCCCAGGAGG TGTGTGTCCC TCTAAGCTAA GCCAGGGGTC CCTCACCCCT GCCCCA

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
 -1120      -1110      -1100      -1090      -1080      -1070
CTCC CATCCCTAGT GTAGGTATCA GCTGAAGAGC TTCCTGAGCA GAACACTCTT GGGTGC

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
 -1060      -1050      -1040      -1030      -1020      -1010
TGAC ATTTTGATAA ATAGGCCCAT GTTTAGGAGA GCAGGGGTCC GGGGGCGGGA GATCTT

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -1000      -990       -980       -970       -960       -950
CTCT GGTGGATTGA GGGCTCCAAG AACTACTCTT TGAGCACGCT GCCCCTCCCA GAGTCC

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -940       -930       -920       -910       -900       -890
CCAC AGCCTCCAGA TGGACTAGAA CACAGTTCGG CTGTGGCTGC ACATAACTAA CAGAGG

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -880       -870       -860       -850       -840       -830
ATAG ATGGTGGGTC CCAGCCCAAC AGTGCCTGGC AATCACCCAG AGCCACCAGC TAACGG

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -820       -810       -800       -790       -780       -770
CCTT GGCTTAGTTT TTTGCCTGGG TGTGATCAGG CAGCCCTCCA AAACTGCCCG GACTCC

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -760       -750       -740       -730       -720       -710
ATGA CAAGTTTTGC TTGTTCTATA GAGCACAGTT CCTTTCTAGG TCTGGGGCAA GGGACA

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -700       -690       -680       -670       -660       -650
TCGG GAGACATCTT CCTGCAACAG CTCCAGTCAC TGGACCACCA GGCTCGCCCT GTCTTT

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -640       -630       -620       -610       -600       -590
GGTG TGTGGCCCTG AGTCTCCTAA GTGGCCCAAA CCTGTGAAGA CCCCTCCAAC CACAGT
```

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...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -580       -570       -560       -550       -540       -530
TTTG CTTCTAAATT GTACCCCAAC ACACCTAGCA AATTGAAACC CCACCAGAAG TCCCCC

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -520       -510       -500       -490       -480       -470
AGAT CTGGCTTTCC GGCTATTGCT GGCAAGGGGG AGTGACTCCC GGCCCATTCA ATCCAG

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -460       -450       -440       -430       -420       -410
GCCC CGCCGTGTTC CTCAAACAAG AAGCCACGTA AACATAAACC GAGCCTCCAT GCTGAC

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -400       -390       -380       -370       -360       -350
CCTT GCCCATCGAG GTACTCAATG TTCACGTGAT ATCCACACCC AGAGGGTCCT GGGGTG

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -340       -330       -320       -310       -300       -290
GGTG CATGAGCCCC AGAATGCAGG CTTGATAACC GAGACCCTGA ATCGGGCAGT GTCCAC

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -280       -270       -260       -250       -240       -230
AAGG GCGGAGGCCC AGTCATGCAT GTTCGGGCCT ATGGGGCCAG CACCCAACGC CAAAAC

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -220       -210       -200       -190       -180       -170
TCTC CATCCTCTTC CTCAATCTCG GCTTTCTCTC TCTCTCTCTT TTTTTTTTTT TTTTTT

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -160       -150       -140       -130       -120       -110
TTTT TTTTTCCAAA AGGAGGGGAG AGGGGGTAAA AAAATGCTGC ACTGTGCGGC TAGGCC

...| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
  -100       -90        -80        -70        -60        -50
GGTG AGTGAGCGGC GCGGAGCCAA TCAGCGCTCG CCGTTCCGAA AGTTGCCTTT TATGGC

...| ....|....| ....|....| ....|....| ....|.... ....|....| ....|..
  -40        -30        -20        -10          +1       10
TCGA GGGGCGCCTA TATAAGCAGA GCTCGTTTAG TGAACCGTC AGTTCGCCTG GAGACGT

..| ....|....| ....|....| ....|....
  20         30         40
CGA GCCGAGCGGT TGTGCCACCA TGAAAGCTT
```

4. Hybrid hamster GADD 153/SCP beta DNA sequence

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....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        -780       -770       -760       -750       -740       -730
ACAGGAATTC TGGCGTGCAG TGGGCGACTC AGAAACGCCC AAAGGTGCTC CCCCGAGACA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        -720       -710       -700       -690       -680       -670
AGCGGTNGGA CTACCATGGA AATGCCAGGA CCTCAGCCTC TGGGAAGCGT TAGGAGGTTA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        -660       -650       -640       -630       -620       -610
AAAGAGATGA GATCCCTTCT AAAGGGCTGG AGAAGATGTC AGTCCAGGTA GGACTAATGG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        -600       -590       -580       -570       -560       -550
AAACTTTATG CGGTTCCAGG GGCCTCGGNN CGACTGAGCT GGGAGGGGCC GGGAAGCTGG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        -540       -530       -520       -510       -500       -490
GAGTCTGGAT GGAGGACGAA GTTGGAGGTG ATGGGAGGTG GGTGGGCAGA CCGCAGCTCC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        -480       -470       -460       -450       -440       -430
TGGGCAGACA AGTTCAGGAA GGACAGCCGT TGGGGCCGTT GGATACTGGG AGCTGGCGCT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        -420       -410       -400       -390       -380       -370
CCGCCCTCTT CCCTCTCATC CCCCCACCGC GCCTCCCACC ACCGTCGGCG GCCCCTGCGC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        -360       -350       -340       -330       -320       -310
GTGCGCGCGC GCAGACACCG GTTGCCAAAC ATTGCATCAT CCCCGCCCCC CGTCATCCCT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        -300       -290       -280       -270       -260       -250
CCCTCGCCGC ACTCTCCTTC GCCCGCCCGC GCGCGCGCGC GCGCGCGCGN GCGCATGACT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        -240       -230       -220       -210       -200       -190
CACTCACCTC CTCCGCGGAG CCTCGTGACC CAAAGCCACT TCCGGGTCAA GACAACGTAG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        -180       -170       -160       -150       -140       -130
CTCTCCAGCC AGAGGGCGGG GCGGAGGCGG GGGCCGAGGG GGCTCCTGAG TGGCGGATGT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        -120       -110       -100        -90        -80        -70
AGGGGTGGGG CGGAGTCAGT GCCAGCGTGC CGCTTTCTGA TTGGCAGGCT CCTGGGTCCC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        -60        -50        -40        -30        -20        -10
GCCCCCCAAA AGAGGGGACG GGCCCCTCGA GGGGCGCCTA TATAAGCAGA GCTCGTTTAG

....|.... ....|....| ....|....| ....|....| ....|....| ....|....
         +1         10         20         30         40
TGAACCGTC AGTTCGCCTG GAGACGTCGA GCCGAGCGGT TGTGCCACCA TGAAAGCTT
```

5. Hybrid CMV IE/SCP beta DNA sequence

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..|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..
      -580       -570       -560       -550       -540       -530
ACCGTATT ACCGCCATGC ATGGTCATTA GTTCATAGCC CATATATGGA GTTCCGCGTT AC

..|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..
      -520       -510       -500       -490       -480       -470
ATAACTTA CGGTAAATGG CCCGCCTGGC TGACCGCCCA ACGACCCCCG CCCATTGACG TC

..|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..
      -460       -450       -440       -430       -420       -410
AATAATGA CGTATGTTCC CATAGTAACG CCAATAGGGA CTTTCCATTG ACGTCAATGG GT

..|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..
      -400       -390       -380       -370       -360       -350
GGAGTATT TACGGTAAAC TGCCCACTTG GCAGTACATC AAGTGTATCA TATGCCAAGT AC

..|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..
      -340       -330       -320       -310       -300       -290
GCCCCCTA TTGACGTCAA TGACGGTAAA TGGCCCGCCT GGCATTATGC CCAGTACATG AC

..|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..
      -280       -270       -260       -250       -240       -230
CTTATGGG ACTTTCCTAC TTGGCAGTAC ATCTACGTAT TAGTCATCGC TATTACCATG GT

..|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..
      -220       -210       -200       -190       -180       -170
GATGCGGT TTTGGCAGTA CATCAATGGG CGTGGATAGC GGTTTGACTC ACGGGGATTT CC

..|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..
      -160       -150       -140       -130       -120       -110
AAGTCTCC ACCCCATTGA CGTCAATGGG AGTTTGTTTT GGCACCAAAA TCAACGGGAC TT

..|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..
      -100       -90        -80        -70        -60        -50
TCCAAAAT GTCGTAACAA CTCCGCCCCA TTGACGCAAA TGGGCGGTAG GCGTGTACGG TG

..|....| ....|....| ....|....| ....|....| ....|.... ....|....| ...
      -40        -30        -20        -10          +1       10
GGACTCGA GGGGCGCCTA TATAAGCAGA GCTCGTTTAG TGAACCGTC AGTTCGCCTG GAG

.|....| ....|....| ....|....| ....|...
      20         30         40
ACGTCGA GCCGAGCGGT TGTGCCACCA TGAAAGCTT
```

Table 14 Data record in first experiment of promoter activity assay (serum supplemented condition)

Relative luminescent unit							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
sample 1	508	1919	3749	3281	33532	81483	1664276
sample 2	423	1904	4469	3656	60178	104736	1674733
sample 3	394	1382	3354	3046	64885	103463	1961517
Absorbance of converted PNPG substrate							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
sample 1	0.400	0.502	0.595	0.602	0.762	0.656	0.531
sample 2	0.437	0.419	0.696	0.676	0.812	0.765	0.558
sample 3	0.637	0.372	0.589	0.659	0.839	0.663	0.553
Promoter activity fold							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
Av ± S.E	1.0±0.3	4.6±1.5	6.9±2.1	5.8±1.6	78.7±45.2	163.8±79.6	3767.7±1732.6

Table 15 Data record in first experiment of promoter activity assay (serum-free condition)

Relative luminescent unit							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
sample 1	426	2356	6685	5518	61454	102839	1544410
sample 2	517	3490	7506	7074	62133	108000	1714774
sample 3	478	3408	7784	4345	59077	76422	1574971
Absorbance of converted PNPG substrate							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
sample 1	0.41	0.459	0.863	0.703	0.711	0.664	0.492
sample 2	0.413	0.447	0.852	0.594	0.601	0.565	0.506
sample 3	0.403	0.410	0.915	0.652	0.595	0.473	0.470
Promoter activity fold							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
Av ± S.E	1.0±0.2	3.5±0.6	5.1±0.7	4.8±0.7	73.6±22.1	173.9±25.7	3086.0±592.5

Table 16 Data record in second experiment of promoter activity assay (serum supplemented condition

Relative luminescent unit							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
sample 1	767	4050	11657	13168	156783	372967	2319914
sample 2	953	4224	10504	13905	147681	319542	2319971
sample 3	896	5380	19952	9030	134896	324366	1993830
Absorbance of converted PNPG substrate							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
sample 1	0.223	0.234	0.451	0.526	0.421	0.437	0.427
sample 2	0.25	0.229	0.433	0.425	0.415	0.454	0.416
sample 3	0.216	0.199	0.307	0.398	0.425	0.489	0.372
Promoter activity fold							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
Av ± S.E	1.0±0.1	6.1±1.0	7.2±0.2	7.6±1.9	83.2±0.6	146.0±8.7	2851.2±158.6

Table 17 Data record in second experiment of promoter activity assay (serum-free condition)

Relative luminescent unit							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCPbeta	CMV IE/SCP beta
sample 1	324	881	1642	1892	27933	58307	1017167
sample 2	339	950	2158	1615	23222	62573	1029969
sample 3	321	1037	2022	1988	33160	65284	944317
Absorbance of converted PNPG substrate							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
sample 1	0.481	0.397	0.555	0.588	0.611	0.512	0.498
sample 2	0.440	0.402	0.533	0.506	0.549	0.538	0.530
sample 3	0.598	0.468	0.651	0.658	0.631	0.603	0.475
Promoter activity fold							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
Av ± S.E	1.0±0.2	3.6±0.5	4.5±0.8	3.9±0.8	64.5±4.0	111.4±24.5	1981.6±325.5

Table 18 Data record in third experiment of promoter activity assay (serum supplemented condition)

Relative luminescent unit							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
sample 1	351	1621	3130	2510	36297	87096	961692
sample 2	360	1479	3005	2530	38708	60348	791332
sample 3	352	1456	3303	1899	33833	74769	873038
Absorbance of converted PNPG substrate							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
sample 1	0.313	0.385	0.541	0.478	0.49	0.688	0.502
sample 2	0.256	0.356	0.583	0.596	0.459	0.498	0.297
sample 3	0.355	0.371	0.721	0.483	0.505	0.558	0.376
Promoter activity fold							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
Av ± S.E	1.0±0.1	5.5±0.9	9.8±5.1	7.1±1.6	92.7±15.9	197.6±45.5	1444.9±144.6

Table 19 Data record in third experiment of promoter activity assay (serum-free condition)

Relative luminescent unit							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
sample 1	554	3313	8445	7783	110763	304391	2185403
sample 2	820	2616	6988	6523	89237	285588	2304974
sample 3	517	2246	5502	6930	97847	243038	2312274
Absorbance of converted PNPG substrate							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
sample 1	0.248	0.232	0.445	0.484	0.440	0.472	0.405
sample 2	0.235	0.221	0.382	0.371	0.377	0.427	0.373
sample 3	0.254	0.217	0.462	0.500	0.406	0.417	0.371
Promoter activity fold							
Promoter	Promoterless	SCP	SCP beta	EF-1α/SCP beta	β-actin/SCP beta	GADD153/SCP beta	CMV IE/SCP beta
Av ± S.E	1.0±0.3	5.0±1.5	6.5±1.7	6.3±1.2	99.6±27.7	255.5±55.3	2416.2±645.5

Table 20 Promoter activity in transient transfected CHO-K1 cells with cultured in completed medium (summary)

Experiment		Promoter activity (arbitrary fold)						
Time	Sample	Promoterless	SCP	SCP beta	EF-1α/ SCP beta	β-actin/ SCP beta	GADD153/ SCP beta	CMV IE/ SCP beta
1	1	1.0	3.0	5.0	4.3	34.7	97.8	2467.9
	2	1.0	4.7	6.6	5.6	76.6	141.4	3100.6
	3	1.0	6.0	9.2	7.5	125.0	252.3	5734.7
2	4	1.0	4.9	7.5	7.6	83.2	149.1	3021.1
	5	1.0	6.2	7.0	9.5	82.6	152.7	2707.2
	6	1.0	7.0	7.2	5.6	83.7	136.2	2825.2
3	7	1.0	5.0	7.5	7.3	108.3	248.1	1579.6
	8	1.0	4.8	6.4	8.6	93.4	184.6	1463.0
	9	1.0	6.5	15.7	5.5	76.5	159.9	1292.1
Av ± S.D		1.0±0.2*	5.4±1.2	8.0±3.1	6.8±1.7	84.9±24.8	169.1±51.4	2687.9±1337

* denotes an average and S.D which was calculated within group.

Table 21 Promoter activity in transient transfected CHO-K1 cells with cultured in serum-free medium (summary)

Experiment		Promoter activity (arbitrary fold)						
Time	Sample	Promoterless	SCP	SCP beta	EF-1α/ SCP beta	β-actin/ SCP beta	GADD153/ SCP beta	CMV IE/ SCP beta
1	1	1.0	3.3	4.4	4.8	67.9	169.1	3032.2
	2	1.0	3.1	5.3	4.1	54.9	151.0	2522.3
	3	1.0	4.1	5.8	5.6	97.9	201.7	3703.6
2	4	1.0	3.8	5.2	4.7	66.1	112.9	1708.3
	5	1.0	3.0	3.7	3.0	60.0	86.2	1894.7
	6	1.0	4.0	4.6	4.0	67.6	135.1	2341.7
3	7	1.0	6.4	8.5	7.2	112.7	288.7	2415.6
	8	1.0	3.4	5.2	5.0	67.8	191.7	1771.0
	9	1.0	5.1	5.9	6.8	118.4	286.3	3062.0
Av ± S.D		1.0±0.2*	4.0±1.1	5.4±1.3	5.0±1.3	79.2±23.8	180.3±70.7	2494.6±671.3

* denotes an average and S.D which was calculated within group.

Table 22 Statistic comparion between SCP beta activity and SCP activity in CHO-K1 with cultured in completed medium

Group Statistics					
Activity	Promoter	Number	Mean	Std. Deviation	Std. Error Mean
	SCP	9	5.3656	1.21407	0.40469
	SCP beta	9	8.0011	3.08714	1.02905

Independent Samples Test										
Activity	Levene's Test for Equality of Variances			t-test for Equality of Means						
	Equal variances	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Upper	Lower
	assumed	1.653	0.217	-2.383	16	0.030	-2.63556	1.10576	-4.97967	-0.29144
	not assumed			-2.383	10.417	0.037	-2.63556	1.10576	-5.08605	-0.18506

Table 23 Statistic comparion between SCP beta activity and SCP activity in CHO-K1 with cultured in serum-free medium

Group Statistics

	Promoter	Number	Mean	Std. Deviation	Std. Error Mean
Activity	SCP	9	4.00222	1.108194	0.369398
	SCP beta	9	5.38667	1.354954	0.451651

Independent Samples Test

	Levene's Test for Equality of Variances			t-test for Equality of Means						
Activity	Equal variances	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Upper	Lower
	assumed	0.043	0.838	-2.373	6	0.031	-1.384444	0.583476	-2.621358	-0.147531
	not assumed			-2.373	5.394	0.031	-1.384444	0.583476	-2.625325	-0.143564

Table 24 Statistic comparison of enhancer effect on hybrid promoter activity (serum supplemented condition)

ANOVA; Activity	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	49,611,980.499	4	12402995.125	34.609	0.000
Within Groups	14,335,124.396	40	358,378.110		
Total	63,947,104.895	44			

Multiple comparison of hybrid promoter activity (Post Hoc Tests; Tamhane), Dependent Variable: Activity

					95% Confidence Interval	
(I) Promoter	(J) Promoter	Mean Difference (I-J)	Std. Error	Sig.	Upper Bound	Lower Bound
SCP beta	EF-1alpha/SCP beta	1.18333	1.17213	0.982	-2.7950	5.1617
	Beta-actin/SCP beta	-76.87333(*)	8.31559	0.000	-108.2822	-45.4645
	GADD153/SCP beta	-161.13333(*)	17.14811	0.000	-226.4154	-95.8513
	CMV IE/SCP beta	-2679.93778(*)	445.79989	0.003	-4,381.2606	-978.6149
EF-1alpha/SCP beta	SCP beta	-1.18333	1.17213	0.982	-5.1617	2.7950
	Beta-actin/SCP beta	-78.05667(*)	8.27074	0.000	-109.5215	-46.5919
	GADD153/SCP beta	-162.31667(*)	17.12641	0.000	-227.6289	-97.0045
	CMV IE/SCP beta	-2681.12111(*)	445.79906	0.003	-4,382.4451	-979.7971
Beta-actin/SCP beta	SCP beta	76.87333(*)	8.31559	0.000	45.4645	108.2822
	EF-1alpha/SCP beta	78.05667(*)	8.27074	0.000	46.5919	109.5215
	GADD153/SCP beta	-84.26000(*)	19.00234	0.009	-149.7455	-18.7745
	CMV IE/SCP beta	-2603.06444(*)	445.87507	0.004	-4,304.2799	-901.8490
GADD153/SCP beta	SCP beta	161.13333(*)	17.14811	0.000	95.8513	226.4154
	EF-1alpha/SCP beta	162.31667(*)	17.12641	0.000	97.0045	227.6289
	Beta-actin/SCP beta	84.26000(*)	19.00234	0.009	18.7745	149.7455
	CMV IE/SCP beta	-2518.80444(*)	446.12721	0.005	-4,219.6630	-817.9459
CMV IE/SCP beta	SCP beta	2679.93778(*)	445.79989	0.003	978.6149	4,381.2606
	EF-1alpha/SCP beta	2681.12111(*)	445.79906	0.003	979.7971	4,382.4451
	Beta-actin/SCP beta	2603.06444(*)	445.87507	0.004	901.8490	4,304.2799
	GADD153/SCP beta	2518.80444(*)	446.12721	0.005	817.9459	4,219.6630

*. The mean difference is significant at the .05 level.

Table 25 Statistic comparison of enhancer effect on hybrid promoter activity (serum-free condition)

ANOVA; Activity	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	42599903.04708	4	10649975.76177	116.73267	0.000
Within Groups	3649355.6456	40	91233.89114		
Total	46249258.69268	44			

Multiple comparison of hybrid promoter activity (Post Hoc Tests; Tamhane), Dependent Variable: Activity

(I) Promoter	(J) Promoter	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Upper Bound	Lower Bound
SCP beta	EF-1alpha/SCP beta	0.356667	0.635363	1.000	-1.70263	2.41596
	Beta-actin/SCP beta	-73.857778(*)	7.947919	0.000	-104.12281	43.59275
	GADD153/SCP beta	-174.904444(*)	23.577815	0.001	-264.86298	-84.94591
	CMV IE/SCP beta	-2489.214444(*)	223.755716	0.000	-3,343.14250	-1,635.28639
EF-1alpha/SCP beta	SCP beta	-0.356667	0.635363	1.000	-2.41596	1.70263
	Beta-actin/SCP beta	-74.214444(*)	7.947649	0.000	-104.47985	-43.94904
	GADD153/SCP beta	-175.261111(*)	23.577724	0.001	-265.21978	-85.30245
	CMV IE/SCP beta	-2489.571111(*)	223.755707	0.000	-3,343.49918	-1,635.64304
Beta-actin/SCP beta	SCP beta	73.857778(*)	7.947919	0.000	43.59275	104.12281
	EF-1alpha/SCP beta	74.214444(*)	7.947649	0.000	43.94904	104.47985
	GADD153/SCP beta	-101.046667(*)	24.873174	0.024	-190.28934	-11.80399
	CMV IE/SCP beta	-2415.356667(*)	223.895918	0.000	-3,269.08559	-1,561.62775
GADD153/SCP beta	SCP beta	174.904444(*)	23.577815	0.001	84.94591	264.86298
	EF-1alpha/SCP beta	175.261111(*)	23.577724	0.001	85.30245	265.21978
	Beta-actin/SCP beta	101.046667(*)	24.873174	0.024	11.80399	190.28934
	CMV IE/SCP beta	-2314.310000(*)	224.993613	0.000	-3,166.59168	-1,462.02832
CMV IE/SCP beta	SCP beta	2489.214444(*)	223.755716	0.000	1,635.28639	3,343.14250
	EF-1alpha/SCP beta	2489.571111(*)	223.755707	0.000	1,635.64304	3,343.49918
	Beta-actin/SCP beta	2415.356667(*)	223.895918	0.000	1,561.62775	3,269.08559
	GADD153/SCP beta	2314.310000(*)	224.993613	0.000	1,462.02832	3,166.59168

*. The mean difference is significant at the .05 level

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