OVEREXPRESSION OF MULTIVALENT TRANSCRIPTION FACTOR, CTCF IN HeLa CELL LINES

by

NURUL WAHIDA BINTI AB. GHANI

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CERTIFICATE

This is to certify that the dissertation entitled **"Overexpression of Multivalent Transcription Factor, CTCF in HeLa Cell Lines"** is the bonafide record of research work done by Ms Nurul Wahida binti Ab. Ghani during the period from July 2008 to October 2008 under my supervision.

Supervisor,

....

Shaharum Shamsuddin *D. Phil. (Oxon)*
Senior Lecturer in Molecular & Cellular Biology
School of Health Sciences
Universiti Sains Malaysia
Health Campus
16150 Kubang Kerian
Kelantan, Malaysia

Date: ....23/12/08............

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PREFACE

Objective of the Research Project

The objectives of this project are to propagate the pCI 7.1-CTCF recombinant constructs in bacteria, followed by miniplasmid preparation of the plasmids using alkaline method, characterization of the construct and to use it in the Conventional Calcium Phosphate transfection experiments.

Background of the Research Project

CTCF is a protein that binds to the boundary elements in a methylation-dependent manner. CTCF is already exist and being expressed in HeLa cell line and will produce protein bands following Western blotting with appropriate primary and secondary antibodies. The use for transforming and transfecting CTCF into the HeLa cells is that so that it can be over expressed during protein assay, after treating with respective antibodies.

The method used in transfecting the gene is by manual method, and not using a kit, that is calcium-phosphate method. Using transfection kits are easier since nowadays there are lots of transfection kits available for expression research, but all the kits are expensive require a big amount of money to purchase it, so the transfection using calcium phosphate method is choosen.

pCI-CTCF is transformed into *Escherichia coli (E.coli)* bacteria, and then transfected into human cervical cancer (HeLa) cell line for overexpression. The overexpression can be detected by protein assay such as Western Blot and the positive results for this overexpression may indicate that the research study is a success and transfection using calcium phosphate method can always be practiced in the research field for its low cost instead of the expensive transfection kits.

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LIST OF SYMBOLS AND ABBREVIATIONS

APS	Ammonium Persulphate
$CaCl_2$	Calcium Chloride
CO_2	Carbon Dioxide
CTSs	CTCF-target Sequences
ddH_2O	Deionized Distilled Water
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
FBS	Foetal Bovine Serum
FW	Formula Weight
g	Gram
HCl	Hydrochloric Acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish Peroxidase
KCl	Potassium Chloride
kDa	kiloDalton
L	Litre

LB	Luria-Bertani
ml	Milliliter
NaCl	Sodium Chloride
Na_2HPO_4	Disodium Hydrogen Phosphate
NaOH	Sodium Hydroxide
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pen-strep	Penicillin-Streptomycin
PVDF	Polyvinylidene Difluoride
rpm	Rotation per Minute
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TAE	Tris-Acetate-EDTA
TBS	Tris Buffered Saline
TEMED	N,N,N',N',-tetramethylethylenediamine
V	Volts
µg	Microgram
µl	Microlitre
^oC	Degree Celcius

ABSTRACT

Transformation of pCI-CTCF plasmid was done after receiving this recombinant construct from Dr. Elena M. Klenova, Gene Regulation Laboratory, University of Essex, UK. The plasmid was propagated in *E.coli* DH5-α competence cell. Following transformation, a transformant containing the recombinant plasmid was selected and cultured overnight in LB Broth. The plasmid was then extracted using a plasmid extraction kit QIAprep Spin Miniprep Kit (QIAGEN, Germany). Purified plasmid was further analysed and characterized. The plasmid was then transfected into HeLa cells using Calcium Phosphate method. Following transfection, the HeLa cells were lysed and the supernatant of the lysed cells was analysed using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) followed by Western Blotting using anti CTCF monoclonal antibody. There is a positive expression of exogenous CTCF detected in the lysate compare to the normal HeLa cell lines control. However due to short period of time for this project, transfection-efficiency was not properly estimated. This work established conventional, cheap and reasonable transfection method for future functional assay works in the lab.

ABSTRAK

Transformasi plasmid pCI-CTCF dijalankan setelah menerima binaan rekombinan ini daripada Dr. Elena M. Klenova, Gene Regulation Laboratory, University of Essex, UK. Plasmid ini dipropagasikan di dalam kompeten sel *E.coli* DH5-α. Diikuti dengan transformasi, transforman yang mengandungi plasmid rekombinan telah dipilih dan dikulturkan semalaman di dalam LB Broth. Plasmid ini kemudiannya diekstrak dengan menggunakan kit pengekstrakan plasmid QIAprep Spin Miniprep Kit (QIAGEN, Germany). Plasmid yang telah dipurifikasikan dianalisis dengan lanjut dan digambarkan sifatnya. Kemudian plasmid ini ditransfek ke dalam sel HeLa dengan menggunakan kaedah Kalsium Fosfat. Selepas transfeksi, sel HeLa dilisiskan dan supernatant dari sel yang dilisiskan itu dianalisa dengan menggunakan Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) diikuti Western Blotting menggunakan anti CTCF monoklonal antibodi. Terdapat ekspresi positif pada eksogenus CTCF yang dikesan di dalam lisat berbanding dengan normal sel HeLa kontrol. Bagaimanapun, disebabkan kesuntukan masa untuk projek ini, kecekapan transfeksi tidak dapat diestimasikan dengan sempurna. Penyelidikan ini berjaya membangunkan kaedah yang murah dan sesuai untuk proses transfeksi bagi kegunaan asai berfungsi di dalam makmal pada masa hadapan.

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Human Cervical Cancer and HeLa Cell Line

Cervical carcinoma is caused mostly by infection with a high-risk group of human papillomaviruses (HPV) (Lorincz *et al.*, 1987, zur Hausen, 1989, Cullen *et al.*, 1991). After high-risk HPV infection, two viral oncogenic proteins, E6 and E7, play a critical role in inducing cervical cancers by interacting with p53 and pRB, respectively and in inactivating these cellular regulatory proteins (Scheffner *et al.*, 1990, Werness *et al.*, 1990, Scheffner *et al.*, 1991). The two viral oncogenic proteins, E6 and E7 are commonly expressed in these carcinoma cells and are required for maintaining cancer malignancy (Santin *et al.*, 1998).

It has been reported that, except for cervical cancers, most cancer development results from *p53* gene mutation (Levine, 1997). p53 mutation is detected in more than 50% of cancer cells, but rarely in cervical cancer cell types (Greenblatt *et al.*, 1994). In most cervical cancers, however, the function of p53 is down-regulated by the E6 protein of HPV 16, whereby E6 binds to p53, resulting in degradation of E6-p53 complexes through the ubiquitin pathway (Hamada *et al.*, 1996, Pim and Banks, 1999, Kessis *et al.*, 1993).

For instance, human cervical cancer cell lines, such as CaSki (HPV 16), SiHa (HPV 16), HeLa (HPV 18) and HeLaS3 (HPV 18) express intact p53 protein (Woong *et al.*, 2002). HeLa cell line that was used in this research project was HeLa cell line. Generally HeLa cell line is known as an immortal cell line used in medical research and it is

commercially available. The cell line was derived from cervical cancer cells taken from Henrietta Lacks, who died from her cancer disease on October 4, 1951. HeLa cell lines are treated as cancer cells, as they are descended from a biopsy taken from a visible lesion on the cervix as part of the diagnosis of Ms. Lacks' cancer.

HeLa cells are termed immortal because they can divide an unlimited number of times in the laboratory cell culture plate as long as fundamental cell survival conditions are met, that is the cells are being maintained and sustained in a suitable environment. There are many strains of HeLa cells as they continue to evolve by being grown in cell cultures, but all HeLa cells are descended from the same tumor cells removed from Ms. Lacks. It has been estimated that the total number of HeLa cells that have been propagated in cell culture far exceeds the number of cells in Henrietta Lacks' body.

1.2 CTCF as a multivalent multifunctional protein

CCCTC-binding factor (CTCF) is a versatile zinc finger protein (11-zinc-finger transcriptional factor) with unusual multiple DNA sequence binding specificity (Klenova *et al.*, 1998) and with diverse regulatory functions and ubiquitously expressed gene upregulated during the S-G_2 stage of the cell cycle. It is an exceptionally highly conserved protein displaying 93% overall identity and 100% identity in the 11-zinc-finger DNA-binding domain between avian and mammalian amino acid sequences (Filippova *et al.*, 1996).

CTCF encodes a nuclear factor containing three major, functionally distinct regions with amino acid sequences that were maintained practically identical throughout vertebrate evolution: a DNA-binding domain (composed of 11 ZFs), and two flanking *trans*-acting transcriptional repressor/activator regions that account for approximately two-thirds of the entire protein.

CTCF is a protein that binds to the boundary elements in a methylation-dependent manner. One of the well characterized examples of such element is the ICR (imprinting control region). It is situated in the 5'-flank of the *H19* gene and 90 kb downstream of *Igf2* gene (Thorvaldsen *et al.*, 1998, Kanduri *et al.*, 2000). This domain, which is maternally unmethylated and paternally methylated, regulate the expression of the maternal allele of the *Igf2* gene (Bartolomei and Tilghman, 1997). The differentially methylated imprinting control region (ICR) upstream of the *H19* gene regulates allelic *Igf2* expression by means of a methylation-sensitive chromatin insulator function (Pant *et al.*, 2004).

Moreover, CTCF was recently found to be a parent of an origin-specific and methylation-sensitive structural and functional component of the chromatin insulator upstream of the *H19* gene (Bell and Felsenfeld, 2000, Hark *et al.*, 2000, Kanduri *et al.*, 2000), thus suggesting a new important role for a CpG-containing subset of CTSs (CTCF-target Sequences) in control of genomic imprinting (Kanduri *et al.*, 2000).

Recent studies on transcriptional control of gene expression have pinpointed the importance of long-range interactions and three-dimensional organization of chromatins within the nucleus. Distal regulatory elements such as enhancers may activate transcription

over long distances; hence, their action must be restricted within appropriate boundaries to prevent illegitimate activation of non-target genes. Insulators are DNA elements with enhancer-blocking and/or chromatin-bordering functions. In vertebrates, the versatile transcription regulator CCCTC-binding factor (CTCF) is the only identified *trans*-acting factor that confers enhancer-blocking insulator activity. CTCF-binding sites were found to be commonly distributed along the vertebrate genomes (Bao *et al.*, 2008).

A study of cohesion mediates transcriptional insulation by CCCTC-binding factors that was done by Wendt *et al.,* 2008 indicate functions of cohesion that act at CTCF-binding sites, which may function as transcriptional insulators or boundary elements in vertebrate genomes and the cohesion itself is just like CTCF, that is widely expressed in mammalian tissues, most of which are predominantly composed of postmitotic cells. CTCF has been described in vertebrates and *Drosophila* and it is conceivable that the main function of CTCF in mammalian genomes is to define binding sites for cohesion, and that cohesion is the molecule that structures DNA in a way that cause insulator and boundary effect (Wendt *et al.*, 2008).

CTCF is a truly multifunctional factor, because depending on the context, distinct 50- to 60-bp-long CTSs bound through combinatorial contributions of ZFs, mediate a variety of distinct functions. In this study, the c-*myc* promoter was employed as a model of the CTCF-binding regulatory target to demonstrate that the presence of posttranslational modifications at the strictly conserved C-terminal serines, which we mapped for the first time, can affect transcriptional activity of the protein. Attempts to investigate possible effects of phosphorylation on CTCF-driven regulation of diverse chromatin insulators

(Allshire and Bickmore, 2000) and of negative nuclear receptor-binding hormone-responsive elements (Awad *et al.*, 1999, Burcin *et al.*, 1997) are currently underway.

It is clear, however, that for better understanding of why under-phosphorylated CTCF partially loses its ability to inhibit cell growth, direct comparison of the whole expression spectrum of target genes, for example by a microarray technology, will be required. Recent review of Ohlsson *et al.*, 2001, established CTCF as a true 'multivalent multifunctional' protein that utilizes different sets of ZFs to form distinct complexes with varying ~50 bp CTCF-target sequences (CTSs) that mediate distinct functions in regulation of gene expression. Binding of targeting sequence elements by CTCF can block the interaction between enhancers and promoters, therefore limiting the activity of enhancers to certain functional domains. Besides acting as enhancer blocking, CTCF can also act as a chromatin barrier by preventing the spread of heterochromatin structures.

The family of nucleic acid-binding C_2H_2 type zinc finger transcription factors is divided into two classes. One class consists of small proteins (Gli1, Krox-20, WT1, Egr-1, and Sp1) with conserved zinc finger clusters of 3 to 5 units, while the other class (ZNF91, ZNF74, ZFP37, CTCF) can contain more than 10 zinc finger clusters. CTCF is a ubiquitously expressed, highly conserved transcription factor that contains 10 C_2H_2- and 1 C_2H-type zinc-finger motifs.

CTCF was first described as a factor that binds to the chicken c-*myc* promoter (Lobanenkov *et al.*, 1990) and to the silencer element of the chicken lysozyme gene (Baniahmad *et al.*, 1990, Burcin *et al.*, 1997). CTCF binds to diverse sequences by

utilizing different combinations of essential zinc finger (Filippova *et al.*, 1996, Burcin *et al.*, 1997). Consequently, a defined DNA recognition sequence cannot readily be recognized. The function of CTCF in gene regulation is also diverse. For example, CTCF binds to the chicken lysozyme silencer 2.4 kilobase pairs upstream from the transcriptional start site (Vostrov *et al.*, 2002).

CTCF-mediated repression may include binding to insulator regions between enhancers and promoters resulting in enhancer blocking. CTCF has been found to directionally block enhancer activation by binding to the insulator element at the 5' end of the chicken β-globin gene locus and similar CTCF-binding sequences were identified in a variety of insulators from diverse vertebrate species, suggesting a widespread role for CTCF in the regulation of enhancer-activated genes (Bell *et al.*, 1999). CTCF binds to the promoter-proximal regions of the chicken c-*myc* gene where it acts either as a transcriptional repressor or activator (Lobanenkov *et al.*, 1990, Klenova *et al.*, 1993, Klenova *et al.*, 1997) as the nuclear factor CTCF was first identified as one of the factors binding to the regulatory regions of the c-*myc* gene (Dunn and Davie, 2003).

In the human and mouse c-*myc*, genes CTCF binds to divergent sequences that coincide with RNA polymerase pausing sites within the transcribed region of the genes (Filippova *et al.*, 1996). CTCF gene may be a candidate tumor suppressor gene, since it localizes to a narrow cancer-specific rearrangements such as in breast cancer patients. It shows that CTCF transcriptional regulation may be important for cell cycle progression, differentiation, apoptosis, and tumorigenesis. Functions of varying CTCF/DNA complexes may be regulated by posttranslational protein modifications (Klenova *et al.*, 2001); by

physical interactions with other multifunctional nuclear proteins, which include, among others, RNA/DNA binding factor YB-1 (Chernukhin *et al.*, 2000); and the repression-associated mSin3A/HDACs (Lutz *et al.*, 2000); and by attenuation of the interactions with DNA *via* specific methylation of CpG pairs involved in recognition of specific CTSs by the protein (Kanduri *et al.*, 2000).

However, despite the fact that CTCF emerged as an important player in networks linking expression domains with epigenetics and cell-growth regulatory processes, there was no investigation of the possible effects, if any, of CTCF on cell growth. Based on Rasko *et al.*, 2001, in different experimental systems, ectopic expression of CTCF does not lead to an acute cell death but results in a severe cell-growth inhibition involving a nearly-complete blockade of DNA replication and cell division. Together, these events lead to a dramatic inhibition of cell clonogenic capacity.

1.3 pCI Mammalian Expression Vector

The mammalian expression vector used in this research project was pCI mammalian expression vector. This vector can be obtained from Promega Corporation, Madison, WI, USA. The pCI mammalian expression vector is designed to promote constitutive expression of cloned DNA inserts in mammalian cells. The major difference between the pCI and pSI mammalian expression vectors is the enhancer/promoter region controlling the expression of the inserted gene. The pCI expression vector contains the human cytomegalovirus (CMV) major immediate-early gene enhancer/promoter region (Table

1.3.1 and Figure 1.3.1). This vector can be used for both transient and stable expression of genes.

For stable expression, the pCI vector must be co-transfected with an expression vector containing a selectable gene for mammalian cells. The pCI vector's CMV enhancer/promoter region enables strong, constitutive expression in many cell types. A β-globin/IgG chimeric intron located downstream of the enhancer/promoter region can further increase expression. The late SV40 polyadenylation signal increases the steady-state level of RNA approximately fivefold more than the early SV40 polyadenylation signal. Also, multiple cloning sites exist for easy insertion of cDNA and it is versatile, where synthesize transcripts in vitro using the T7 RNA polymerase promoter or generate single-stranded DNA in *E.coli* using the fl origin of replication.

pCI Mammalian Expression vector sequence reference points (Whole size 4006 bp)	
Description of Gene (s)/Marker	**Position**
Cytomegalovirus immediate-early enhancer/promoter region	1-742
Chimeric intron	857-989
T7-EEV sequencing primer binding site	1020-1041
T7 RNA Polymerase Promoter (-17 to +2)	1034-1052
T7 promoter transcription start site	1051
Multiple cloning region	1052-1104
SV40 late polyadenylation signal	1111-1332
Phage fl region	1422-1877
Beta-lactamase (AmpR) coding region	2314-3174

Table 1.3.1: pCI mammalian expression vector sequence reference points, based on the pCi vector from Promega Corporation, Madison, WI, USA.

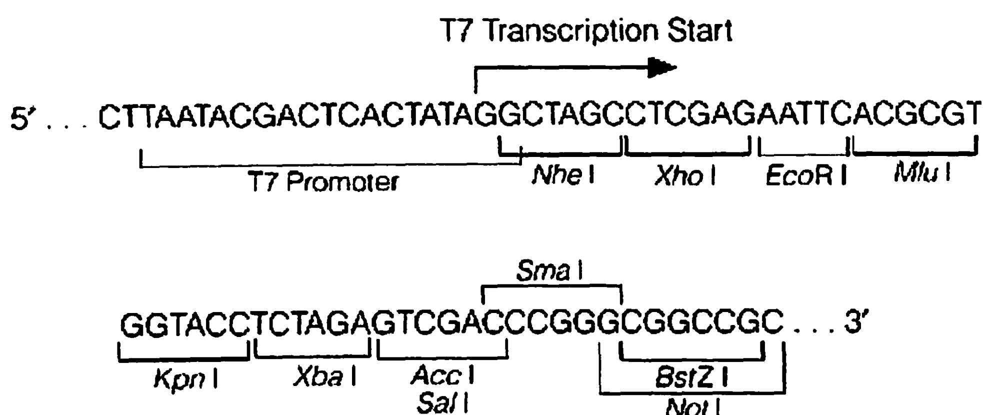

Figure 1.3.1: Multiple cloning region of pCI mammalian expression vector

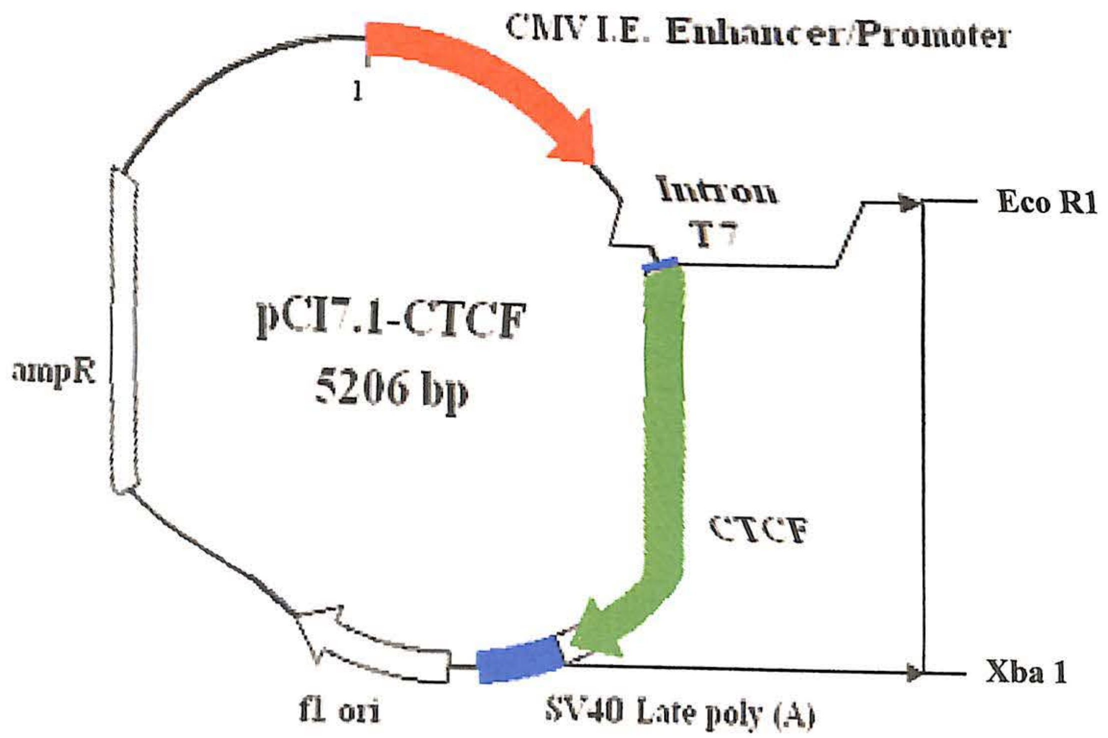

Figure 1.3.2: Schematic presentation of pCI 7.1-CTCF (Shaharum, 2002)

1.4 Calcium Phosphate Transfection

An important technique in biological research is that of expressing exogenous DNA in a variety of mammalian cell backgrounds. Introduction of the plasmid DNA into the cell, however, has been problematic. A variety of techniques for DNA introduction have been employed, including DNA complex formation with calcium phosphate, diethylaminoethyl dextran, or cationic lipids, direct introduction via direct injection, electroporation, microprojectiles, or fusion with liposomes, and use of recombinant viruses. Of these methods, DNA complex formation techniques are the simplest and least expensive, requiring neither the specialized equipment of direct introduction methods nor the generation of a new recombinant virus for each DNA.

In fact, novel transcriptional regulatory elements, as well as various RNA processing and translational signals, have been discovered by using DNA transfection of tissue culture cell. Based on Chen and Okayama, 1987, nearly a dozen transfection techniques have been devised, all of which involve the use of either calcium phosphate to deliver DNA into cells, osmotic shock or treatment with lysosomal inhibitors to enhance the transfection efficiencies and recently, a method involving the use of high-voltage electric pulses to create pores in membranes has been devised for delivering DNA into cells. These transfection methods are quite useful for examining the transient expression of DNA, but they are inefficient for stable transformation.

While the calcium phosphate transfection method is a very efficient means of introducing DNA into cells in many cell systems, it is very inefficient in many others. This