CLONING AND EXPRESSION OF TRUNCATED CTCF

by

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CERTIFICATE

This is to certify that the dissertation entitled

Cloning and Expression of Truncated CTCF

is the bonafide record of reseach work done by

Mr / Mrs / Ms **Chot San Ngan**

during the period **June 2004** to **March 2005**

under my supervision.

Signature of Supervisor : .....

Name and address of Supervisor : .....

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ABBREVIATION

APP	Amyloid β-Promoter Precursor
APS	Ammonium Persulfate
CTCF	CCCTC Binding Factor
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
ICR	Imprinting Control Region
Igf2	Insulin-like Growth Factor 2
IPTG	Isopropylthiogalactosidase
OMP	Outer Membrane Protein
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
TEMED	N,N,N',N',-tetramethylethylenediamine
YB-1	Y Box Binding Factor

Kloning dan Ekspresi Sebahagian Gen CTCF

ABSTRAK

Gen CTCF terletak pada kromosom 16 di kedudukan 16q21 – q22.3. Gen in mengkodkan satu faktor transkripsi yang dikenali sebagai protein 'zinc finger'. CTCF memainkan peranan penting di dalam sel termasuk mengawal pembahagian sel melalui interaksi dengan protein lain. Terdapat beberapa protein yang berkait rapat dengan CTCF. Contohnya ialah protein yang terlibat di dalam kawalan transkripsi dan pembahagian sel, pemprosesan RNA, transduksi signal, komponen nucleosome dan gen 'tumor suppressor'.

Gen CTCF mengandungi domain terminal-N, domain 'zinc finger' dan domain terminal-C. Kajian secara meluas telah dijalankan untuk mengkaji bahagian penting yang mungkin memainkan peranan di dalam pengikatan DNA dan interaksi antara protein. Kajian yang lepas mendapati sebahagian domain-C mempunyai interaksi secara langsung dengan RNA Polimerase II dan mengawal pelbagai proses sel.

Di dalam projek penyelidikan ini, sebahagian CTCF C-domain telah dihasilkan melalui 'polymerase chain reaction (PCR)'. Produk hasil daripada PCR disubklonkan ke dalam vektor kloning perantara iaitu pTOPO2.1 dan ditransformasi ke dalam *E. Coli DH5α*. Bahagian gen tersebut kemudian dipotong dengan enzim restriksi tertentu lalu diklonkan

ke dalam vektor ekspresi pET16b untuk pengekspresan di dalam *E. coli BL21 (DE3)*. Protein yang terhasil diasingkan dengan SDS-PAGE diikuti dengan Western Blot menggunakan antibodi monoklonal α-Histag. Saiz protein ini adalah lebih kurang 27kDa walaupun secara teori, saiznya adalah 4kDa. Ini adalah dijangkakan dan disebabkan perubahan konformasi protein semasa migrasi pada SDS-PAGE.

ABSTRACT

The CTCF gene maps on chromosome 16 at 16q21 – q22.3. It encodes a transcriptional factor protein CTCF called zinc finger protein. CTCF has a number of functions in the cell including controls of cell proliferation by having interactions with other proteins. There are several proteins associated with CTCF. Among them are proteins involved in transcriptional and cell proliferation control, RNA processing, signal transduction, nucleosome components and tumor suppressors.

CTCF consists of N-terminal domain, zinc finger domain and C-terminal domain. Extensive research have been done to elucidate the important regions that might play important role in both DNA binding and protein-protein interactions. Previous studies has shown there was a region in the C-domain which has direct interaction with large subunit of RNA polymerase II and controlling various cellular process.

In this study, a truncated CTCF from the C-terminal domain was produced by polymerase chain reaction (PCR). The amplified products was then subcloned into the intermediate cloning vector, pTOPO2.1 and transformed into *E. coli* strain *DH5α*. The insert was then cut with respective restriction enzyme to obtain the truncated region and further ligated into pET16b expression vector for its expression in *E. coli* strain *BL21 (DE3)*. Expressed protein was separated using SDS-PAGE followed by Western Blotting using α-Histag monoclonal antibody. The truncated CTCF protein was detected to migrate at the size of

approximately 27kDa despite its theoretical size of 4kDa. This migration was expected and due to anomalous conformational changes during migration.

INTRODUCTION

CTCF was originally identified as a protein that binds to the three repeats of the core sequence CCCTC regularly spaced at 12-13 bp interval within the chicken c-myc promoter. (Lobanenkov *et al.*, 1990). CTCF is a multivalent transcription factor due to its ability to recognize multiple target sites. This widely expressed transcription factor with multiple DNA sequence specificity is localized in chromosome 16 at 16q22 – q22.3. It can bind to a number of different sequences in the human, mouse and avian *myc* promoters and regulates the transcription of several genes, like the human oncogene *c-myc* or the chicken lysozyme gene with different sets of its 11 zinc fingers.

Structure of CTCF

The CTCF protein belongs to the family of the zinc finger proteins because it consists eleven finger-like structure. Its zinc finger domain is composed of ten fingers of the C2H2 class and one of the C2HC class. The eleven zinc finger domain of CTCF spans 1/3 of the full length CTCF protein and different combination of the zinc fingers are used to recognize diverse DNA sequences. In addition to the zinc finger domain, there are two repression domains mapped as N-terminal and C-terminal. The N-terminal domain contains a proline rich fragment, whereas the C-terminal domain carries a high negative charge, although it is not clear whether these characteristics are important for repression (Fillipova *et al.*, 1996).

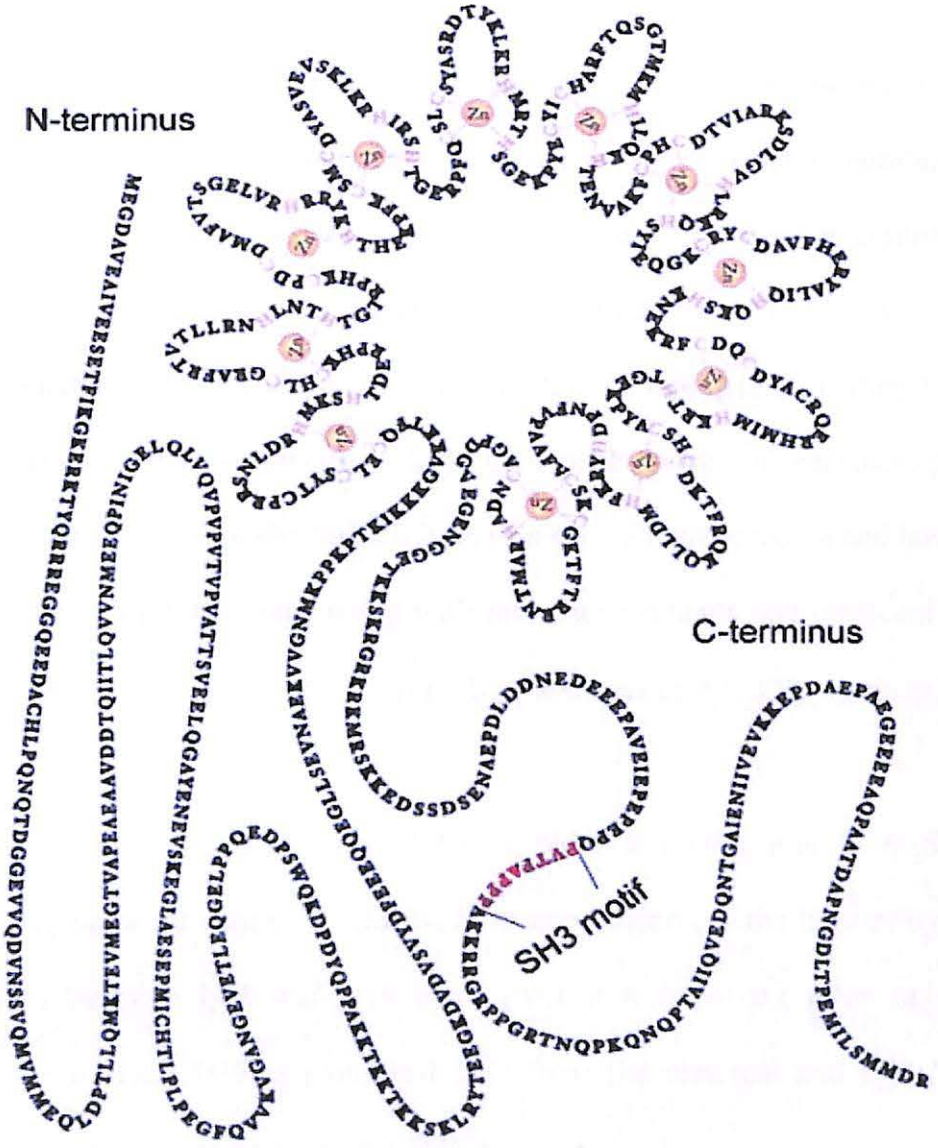

Figure 1 : Structural features of CTCF. The complete amino acid sequence of the wild-type human CTCF protein shows the DNA binding domain, which is composed of ten C2H2-class zinc fingers and one C2HC-class zinc fingers.

Functions of CTCF

CTCF is a unique, highly conserved and ubiquitously expressed transcriptional factor with multiple DNA site specificities. This is because it can use different combination of its zinc fingers to recognize a variety of specific sequences on the DNA to control several important genes. It is able to bind to varying target sequences on the DNA to perform different regulatory roles such as promoter activation or repression, creating hormone-responsive gene silencing elements and functional block of enhancer-promoter interactions. Transcription of the chicken lysozyme gene and the chicken and human *myc* genes is repressed by CTCF interacting with promoter elements and upstream silencer elements of the gene. It activates the amyloid β-protein precusor (APP) promoter.

Recently it has been suggested that CTCF is plays a central role in methylation-dependent chromatin insulation. Its involvement in regulation of the Imprinting Control Region (ICR) between Igf2 and H19 gene gives it a important roles in genomic imprinting. As a result H19 is expressed only from the maternal and Igf2 from the paternal chromosome (Reik and Murrell, 2000).

The zinc fingers are not only capable of binding to DNA but can interacts with protein as well such as YB-1, YY1 and RNP-K proteins to control various cellular process. Protein interactions are very important to all cellular process. The interaction among protein complexes can result in number of effects such as activation or inactivation of certain protein, formation of new binding sites, alteration of protein's kinetic properties and many more.

REVIEW OF LITERATURE

1. CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease.

~ Ohlsson R., Renkawitz R. and Lobanenkov V., Trends in Genetics Vol.17 2001~

The human CTCF gene is localized at chromosome band 16q22, a locus where abnormalities have been observed in various human cancers. The CTCF gene itself has been found re-arranged or mutated in several cancer cell lines and clinical tumour samples. Thus, indirect evidence suggests that CTCF may be a novel tumour suppressor gene.

The zinc fingers of CTCF are capable of binding either DNA or protein, for example with several multifunctional factors such as YB-1, YY1 and RNP-K proteins (V. Lobanenkov, unpublished). Formation of different CTCF-DNA complexes, some of them which are methylation sensitive results in distinct functions such as gene activation, silencing and chromatin insulation. Disrupting the spectrum of target specificities by zinc finger mutations or by abnormal selective methylation of targets is associated with cancer. CTCF emerges, therefore as a central player in networks linking expression domains and cell growth regulation.

Epigenetics is defined as modifications of the genome, heritable during cell division that do not involve a change in the DNA sequence. Since CTCF has been shown to bind specifically in regulating genomic imprinting, mutations in CTCF may impair binding to target sequences and altering the functional spectrum of proteins which may consequently leads to the emergence of tumors and cancers.

2. CTCF C – domain have direct protein interactions with large subunit of RNA Polymerase II which is crucial for initiation of transcription

~ (Shamsuddin, 2002) ~

Besides the zinc finger domain, extensive search has been done to find the regions in the N- and C-domain which might have a role in both DNA binding and protein-protein interactions since these two domains together account for approximately two-thirds of the entire protein.

Previous studies had shown there was a region in the C-domain which have direct protein interaction with large subunit of RNA Polymerase II (Shamsuddin, 2002). Figure 2 shows some of the regions in the C-domain which have been studied. However the exact target sequence that binds to the RNA Polymerase II is still unknown.

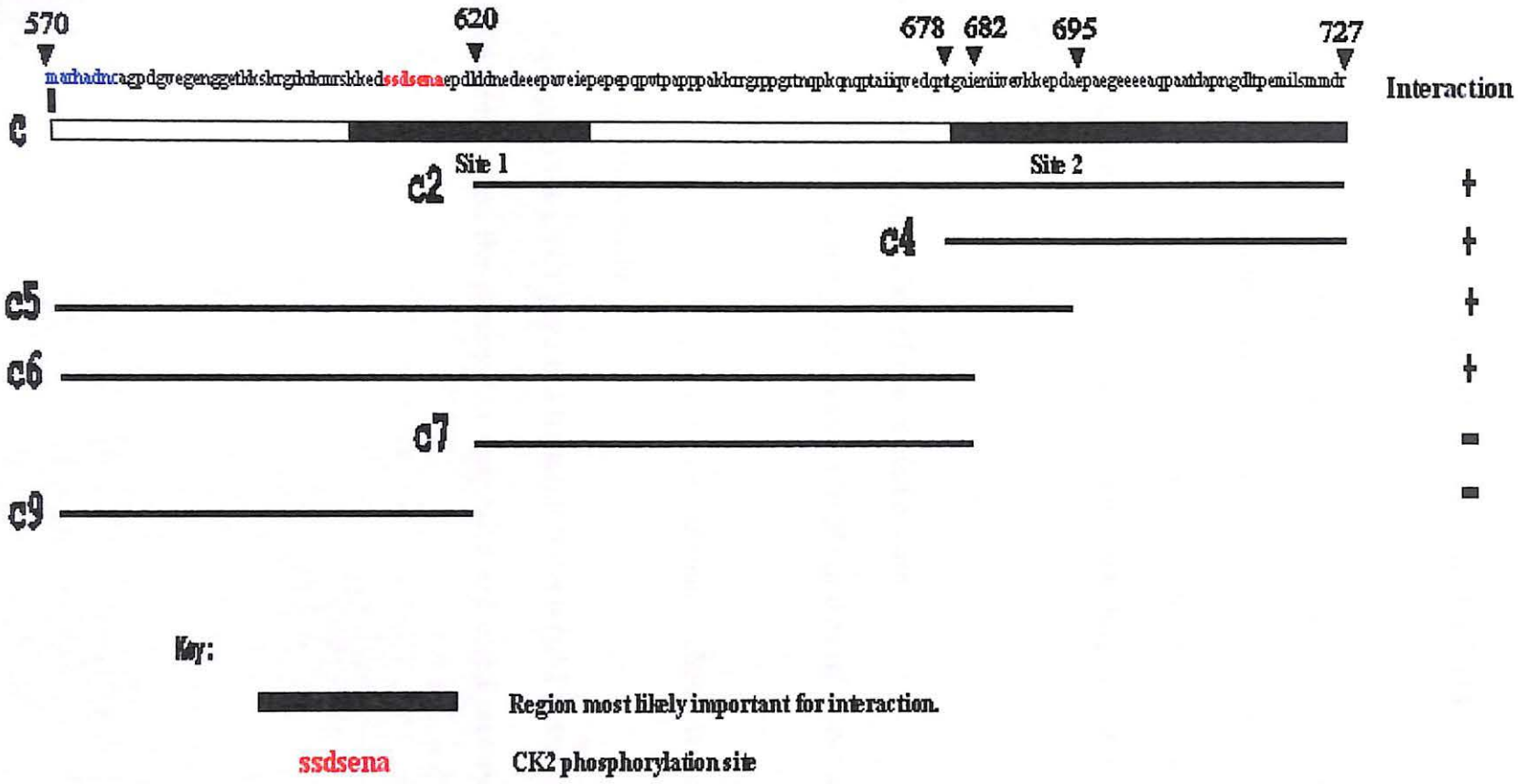

Figure 2 : Regions in the C-domain which have interactions with RNA Polymerase II.

OBJECTIVE OF THE STUDY

CTCF proteins play multiple roles in regulation of various cellular processes. In this study we are focussing on the production of another truncated CTCF that might contain the region crucial for RNA Polymerase II interactions and to gain new knowledge on structure-function of transcription for better understanding of protein folding and protein dynamics.

The main aim of works that have been carried out are :-

1. To design and amplify a truncated CTCF consists of *NdeI* and *AgeI* restriction enzymes sites.
2. To propagate the respective recombinant plasmids using conventional transformation method.
3. To express the CTCF gene in a bacterial systems (pET system).
4. To demonstrate the presence of respective expressed protein by means of SDS-PAGE.

MATERIALS AND METHODS

1. Media preparation

1.1 Preparation of Luria Bertani (LB) agar

LB agar was prepared by dissolving 10g of tryptone, 5g of yeast extract, 10g of sodium chloride and 15g of microbiological agar into 750ml of deionised distilled water. The pH was adjusted up to 7.0 by using 1M of sodium hydroxide and the final volume was made up to 1000ml. It was then autoclaved at 121°C for 15 minutes before pouring into sterile petri plates with 25ml each. The media was cooled down to 40°C before the addition of appropriate antibiotic.

1.2 Preparation of Luria Bertani (LB) broth

LB broth was prepared by dissolving 10g of tryptone, 5g of yeast extract and 10g of sodium chloride into 750ml of deionised distilled water. The pH was adjusted up to 7.0 by using 1M of sodium hydroxide and the final volume was made up to 1000ml. It was then aliquoted 10ml each into universal bottles and autoclaved at 121°C for 15 minutes and stored at room temperature.

1.3 Preparation of Antibiotics

The ampicillin was the antibiotic used in this study for isolation of bacteria host carrying the plasmid with antibiotic resistant gene. It was added into the LB agar or broth with the suitable concentration as recommended. The ampicillin stock was prepared by dissolving

500mg sodium salt of ampicillin in 10ml of deionised distilled water. The resulting ampicillin has concentration of 50 mg/ml. The solution was sterilized by filtering through 0.2 micron membran filter. Concentration of ampicillin used in LB agar and LB broth are 100 µg/ml. The autoclaved media were allowed to cool to 50°C before adding ampicillin to a final concentration 100 µg/ml. Ampicillin plates can be stored up to 2 weeks at 4°C.

1.4 Preparation of 10X Tris Acetate EDTA (TAE) stock

A 10X stock solution of TAE was prepared by dissolving 48.4g of tris-base, 20ml of 0.5M EDTA and 11.42ml glacial acetic acid in deionised distilled water. Deionised distilled water was added to make a final volume of 1 liter and stored at room temperature. A working solution (1X TAE) was prepared by diluting 10ml of stock solution with 990ml deionised distilled water.

1.5 Preparation of 1.0% TAE agarose gel

0.5g of agarose was weighed and placed in a 250ml capacity conical flask. Then 50ml of 1X TAE was added into the flask. The agarose powder was dissolved with brief mixing. It was then boiled for 2 minutes in microwave. Once it was completely dissolved, the flask was allowed to cool to 50°C before addition of 5µl of ethidium bromide (10mg/ml). The solution was mixed well and immediately poured into the agarose gel tray which was pre-assembled with desirable gel comb.

1.6 Preparation Transformation Buffer

The transformation buffer was made up of 50mM of $CaCl_2$ and 10mM Tris Chloride with pH 8.0. It was prepared by mixing 25ml of 1M $CaCl_2$ and 5ml of 1M Tris Chloride in 450ml deionised distilled water. The pH was adjusted to 8.0 using 1M NaOH and top up to 500ml with deionised distilled water before sent to autoclave.

1.7 Preparation of reagents used for SDS-PAGE

1.7.1 Monomer Solution (30.8% Acrylamide, 2.7% Bisacrylamide)

For 200ml preparation, 60g of Acrylamide and 1.6g of Bisacrylamide are added together and top up with deionised distilled water.

1.7.2 4X Running Gel Buffer (1.5M Tris-Cl, pH 8.8)

For 200ml preparation 36.6g of Tris and 150ml of deionised distilled water are added together and the pH adjusted to 8.8 with 5M NaOH. The volume are made to 200ml with deionised distilled water.

1.7.3 4X Stacking Gel Buffer (0.5M Tris-Cl, pH 6.8)

For 200ml preparation 12.0g of Tris and 150ml of deionised distilled water are added together and the pH adjusted to 6.8 with concentrated HCl. The volume are made to 200ml with deionised distilled water.

1.7.4 10% (w/v) Sodium Dodecyl Sulfate

10g SDS are added to 100ml deionised distilled water. It can be stored up to 6 month at room temperature.

1.7.5 10% Ammonium Persulfate

1.0g of ammonium persulfate are added to 10ml of deionised distilled water. It was then aliquoted into small tubes and kept at -20°C until use.

1.7.6 2X Treatment buffer

125µl of 1M Tris-Cl, 400µl of 10% SDS, 2ml of glycerol, 10µl of mercaptoethanol and 1µl of phenol red are added together and top uo to 10ml with deionised distilled water.

1.7.7 Tank Buffer

For 1 liter preparation 14.41g of glycine, 1g of SDS and 3.028g of Tris are added together and top up with deionised distilled water.

1.7.8 TEMED

Commercially available from SIGMA, USA.

1.7.9 Coomassie Brilliant Blue Staining Solution

0.025% (w/v) of Coomassie Brilliant Blue G-250, 40% (v/v) of methanol and 7% (v/v) of acetic acid are added together.

1.7.10 Destaining solution I

It is made up of 40ml methanol and 7ml acetic acid and top up to 100ml with deionised distilled water.

1.7.11 Destaining solution II

It is made up of 50ml methanol and 70ml acetic acid and top up to 1000ml with deionised distilled water.

1.8 Preparation of reagents used for Western Blotting

1.8.1 CAPS transfer buffer (10mM CAPS, 2.5% NaOH)

For 500ml preparation, 10ml of 0.5M CAPS and 12.5ml of methanol are added together and top up with deionised distilled water.

1.8.2 Blocking solution (5% skimmed milk, 0.1% Tween 20, 1X TBS)

5g of 5% skimmed milk, 500µl of 20% Tween 20 and 10ml of 10X TBS are added together and top up with deionised distilled water to 100ml.

1.8.3 Washing buffer (1X TBS, 0.05% Tween 20)

100ml of 10X TBS and 2.5ml of 20% Tween 20 are added together and made up to 1 litre with deionised distilled water.

1.8.4 Antibodies

All antibodies are diluted into blocking solution following the manufacturer's catalogue intstruction.

1.8.5 10X TBS

A 10X of TBS are prepare by dissolving 80g of NaCl, 2g KCl and 30g Tris base in 1 litre deionised distilled water. The pH are calibrated to 7.0 with HCl.

2. Experimental Procedure

2.1 Harvesting of cells

The pET16b~*SH3* in *BL21 (DE3)* is obtained from Dr. Shaharum Shamsuddin's stock culture. This vector has been pre-ligated with the whole C-domain of CTCF by Dr. Shaharum's previous work. The plasmid extraction is done using QIAprep Spin Miniprep kit from Qiagen. The extracted plasmid will be used as template for the PCR amplification of the truncated CTCF.

2.2 Plasmid extraction with alkaline lysis method

A single, isolated colony was picked from a freshly streaked plate. It was inoculated into 10ml of LB broth added with appropriate antibiotic corresponding to that of the plasmid to be extracted. Here we used 10µl of ampicillin. The culture was incubated in the shaker (180 rpm, 37°C) for overnight. The next day, the culture was transferred into a sterile 1.5ml centrifuge tube and centrifuged at 1000rpm for 5 minutes. The supernatant was discarded and the process are repeated twice. Then, 250 µl of P1 solution (with RNase) was added into the pellet and mixed well by vortex. The P1 solution contains glucose that gives osmotic shock to rupture the bacterial cell wall and membrane. It also contains EDTA, which inhibit any nucleases which might present while the RNase will degrade any RNA. This was followed by adding 250µl of P2 solution and the tube was inverted slowly for 3 to 5 times. The P2 solution has a strong alkaline pH (~pH12) that denatures the chromosomal DNA while not disturbing the covalently closed circular plasmid DNA structure. The reaction was incubated at room temperature for 3 to 5 minutes to allow the

cells to lyse completely. Next, 350µl of N3 solution was added into the tube and immediately inverted 5 to 7 times in a slow and steady motion. The N3 solution is an acidic solution of pH5 and it will neutralize the alkaline pH in the reaction. On the other hand, it has a high salt content that precipitate the protein to form the SDS-protein complex. The chromosomal DNA will renature and aggregate along with the protein, forming a visible white precipitate in the tube. The tube was centrifuged at 13,000 rpm for 10 minutes at room temperature. This will pellet down the protein-DNA aggregates while the plasmids will be floating in the supernatant layer. The supernatant was collected into a spin column tube and immediately centrifuged at 13,000 rpm for 1 minute. The silica membrane of the spin column binds to plasmid DNA in the high salt buffer condition. After centrifuged, the flow through was discarded and 500µl of PB buffer (optimal wash buffer) was added and again centrifuged at 13,000rpm for 1 minute. The flow through was discarded and 750µl of PE buffer (wash buffer with 70% ethanol) was added. The tube was incubated at room temperature for 2 to 3 minutes before it was centrifuged again at 12,000 rpm for 1 minute. The flow through was discarded and the column was dried spin again to remove the excess residual buffer. Lastly, the spin column was transferred into a new tube and 50µl of EB buffer was added right at the center of the column. The reaction was incubated at room temperature for 30 minutes and then centrifuged at 13,000 rpm for 1 minutes. The low salt EB buffer will elute the plasmid from the silica membrane and the plasmid was successfully extracted. The plasmid extracted can be store at -20°C. Agarose gel electrophoresis is used to comfirm the purity and amount of DNA.

2.3 Agarose Gel Electrophoresis

The concentration of agarose gel used in this study was 1.0%. This concentration may vary depends on the desired resolution power to visualize the bands. After the agarose gel polymerized, it was transferred to an electrophoresis tank filled with 1X TAE buffer. The DNA sample to be visualized was placed in a new tube and added with 2µl loading buffer. After gentle mixing, the samples were loaded into the wells of the gel. 4µl of the molecular weight marker (100bp or 1kb ladder) were loaded as well. The gel must submerge fully beneath the buffer and electrophoresis was run at 50V for 1 hour 30 minutes. When completed, the DNA bands were visualized under UV transilluminator. The ethidium bromide added to the gel will intercalate with the DNA and upon exposure to UV light, the DNA fluores as orange bands.

2.4 Designing primers of the truncated CTCF.

Primers are typically short, single stranded oligonucleotide (DNA) which are complementary to the other regions of known sequence. Generally, primers used are usually between 18-30 mer (bases) in length and has 35% to 65% GC content. Invert repeated sequence should be avoided as it will prevent hybridization to the template. Forward primer should not be complementary to reverse primer. Besides that, 3' end of the primer should be complementary to the target DNA sequence, 5' end of the primer can have other sequences like restriction enzyme sites, promoter sites, etc. the distance between primers is preferably less than 10kb in length. Here we design our own forward and reverse primers. The primers we are using have 21 bp.

CTCF-F : 5'-ATG-*CAT-ATG*-CGC-TCT-AAG-AAA-3'

CTCF-R : 5'-TC*A-CCG-GT*C-AGG-CTG-AGG-CTC-3'

These primers consists of restriction enzyme sites for *Nde I* and *Age I* (the Italic region).

2.5 Amplification of Truncated CTCF using Polymerase Chain Reaction (PCR).

PCR is a reaction involves repetitively amplifying a small region of DNA molecules so that the particular piece of DNA is present at a greater amount and become more detectable. A prerequisite for amplifying the sequence is to have a known, unique sequence that flank the segment of DNA to be amplified. The PCR reaction is carried out in a thermal cycler, which subjects the DNA to repeated cycles of heating and cooling.

A simple cycle of PCR consists of template DNA denaturation, primer annealing and primer extension. Each primer has its own characteristic annealing temperature. It's length and base composition as well as the reaction buffer ionic strength are taken account.

The melting and annealing temperature of this set of primers are as shown in Table 1.

Table 1 :- Primers Used for PCR Amplification

Primers	Sequence	Tm	Ta
CTCF-F	5'-ATG-*CAT-ATG*-CGC-TCT-AAG-AAA-3'	56.7°C	51.7°C
CTCF-R	5'-TCA-*CCG-GTC*-AGG-CTG-AGG-CTC-3'	68.4°C	63.4°C

After optimization, it was determined that the best annealing temperature was 54.3°C. The preparation of PCR master mix was done by adding the following contents as shown in Table 2. An example of a PCR set up for twelve reactions was shown as below:

Table 2 : Master Mix for PCR Amplification of Truncated CTCF.

Components	**1 reaction (µl)**	**12 reactions (µl)**
Taq DNA polymerase (1unit/ µl)	1	1
Template DNA	2	2
10X PCR buffer $(NH_4)_2SO_4$	2	24
Primers – forward – reverse	0.4 0.4	0.4 0.4
$MgCl_2$ (25mM)	1.2	14.4
dNTPs (10mM)	0.4	4.8
Deionised distilled water	12.6	196.8
Total	20	-

All the components were added in the master mix except the template DNA, *Taq* DNA polymerase and primers. The 240µl of master mix was aliquoted into 10 PCR tubes with 16.2µl each. Then the remaining components were added to make up the final volume of 20µl of PCR reaction per tube. The PCR was carried out in a thermal cycler (Mastercycler 5330 Eppendorf and MJ Research Minicycler) with the following PCR program as shown in Table 3.

Table 3: PCR program used in this study

Steps	Temperature	Time
Step 1 : Pre-heating	94°C	7 minutes
Step 2 : Denaturation	94°C	1 minutes
Step 3 : Primer annealing	54.3°C	1 minutes
Step 4 : Primer extension	72°C	1 minutes
Step 5 : Loop	31 cycles	
Step 6 : Final extension	72°C	7 minutes
Step 7 : Keeping	4°C	Forever

After completing the PCR process, we run the PCR product on the agarose gel for electrophoresis. Then the PCR product was visualized under the UV light. From the electrophoresis result, the truncated CTCF that have been amplified can be detected. The molecular weight band is known by comparing it to the molecular weight marker, λ Hind III.

2.6 Purification of PCR Products.

The PCR products were purified using QIAquick PCR Purification Kit fromQiagen. 100µl of PCR products are put into column before add in 500µl of PB buffer. The column was centrifuged for 1 minutes at 13000rpm. The flow through was discarded and 750µl of PE buffer was added and centrifuged again for 1minutes at 13000rpm. The flow through are discarded and the column are centrifuged again for an additional 1 minutes at maximum speed. Then the column are placed in a clean 1.5ml microcentrifuge tube. 50µl of EB buffer are dispensed directly onto the membrane for complete elution of bound DNA.

2.7 Ligation of Purified PCR Products into pTOPO2.1 Vector.

The purified PCR products were propagated using TOPO-TA Cloning method (Invitrogen 2004) by cloning onto pTOPO2.1 cloning vector. The clone was named pTOPO2.1-*trunCTCF*. The size of the TOPO vector is 3931 base pairs (3.9 kb). With the *trunCTCF* insert, the cloned pTOPO2.1~*trunCTCF* is 4048 base pairs. The diagrammatic representation of the pTOPO2.1- *trunCTCF* is shown in Figure 11.

2.8 Competent cell preparation using calcium chloride method

100ml of *DH5α* glycerol stock was inoculated into 10ml LB plain broth and incubated at 37°C with shaking condition of 180 rpm for overnight. On the next day, 500µl of the overnight culture was taken and inoculated into 50ml LB broth. The culture was incubated in the shaker of 250rpm at 37°C for 2 –3 hours. As the bacterial growth reached an optical density (OD 650nm) of 0.5, the culture was immediately put on ice for 5 minutes to stop the growth. It was then centrifuged at 4000 rpm at 4°C for 10 minutes. The supernatant was discarded and the pellet was gently resuspended with 6ml of ice-cold 100mM magnesium chloride. It was then incubated in ice for 45 minutes. Then the suspension was again centrifuged at 4000 rpm at 4°C for 10 minutes. The supernatant was discarded and the pellet was gently resuspended with 400µl of ice-cold 100mM calcium chloride. Both the chemicals altered the permeability of bacterial cell membrane and making it competence for transformation. The competent cells can be used immediately for transformation. If storage is desired, 60µl of glycerol is added to the competent cell as cryopreservative and aliquoted 50µl each into sterile microcentrifuge

tube for storage at -70 °C. The entire preparation was carried out on ice as the cells were extremely sensitive against heat.

2.9 Transformation into *E.coli* strain *DH5α*

The methodology are as suggested by Maniatis *et al.*, 1982. The suitable volume of ligation mixture or plasmid to be transformed was added into the competent cells and incubated on ice for 20 minutes. Then, heat shock was given to the cells by incubating the tube at 42°C for 30 to 45 seconds. This step is important to increase the permeability of cell by opening up the pores of membrane. Then the tube was immediately placed on ice and kept for 2 minutes. This is another critical step and the transfer to ice should be quickly done as prolong heating would kill the cells. Next, 350µl of fresh LB broth was added into the tube and incubated at 37°C for 1 hour. The LB broth was added to provide nutrient to the cells for recovery from the heat shock. During incubation, any plasmid which successfully transformed into the competent cells would express the antibiotic marker gene for selection of successful transformation later. After incubation, an appropriate amount of cells was inoculated on LB agar containing antibiotics which corresponding to the antibiotic marker gene in plasmid. The plates were incubated overnight at 37°C. The next day, the white colony are choose and inoculated into LB broth for overnight incubation at 37°C in 180rpm shaker. The culture will used for preparation of glycerol stock and plasmid extraction.

2.10 Preparation of glycerol stock

850µl of the overnight culture are put into 1.5ml microcentrifuge tube. 150µl of 100% glycerol are added into the tube and vortex for a few seconds before keep in -20°C. This glycerol stock can be used directly to prepare overnight culture by inoculating 100µl into 10ml LB broth.

2.11 pTOPO2.1~*trunCTCF* extraction

The protocol for plasmid extraction is as described in page 13.

2.12 Digestion of pTOPO2.1~*trunCTCF* with restriction enzymes

The restriction enzymes used in this study were obtained from Fermentas, USA as shown in Table 4.

Table 4: Restriction Enzymes Used in This Study

Restriction enzymes	**Cleavage site**	**Optimal buffer**	**Optimal temperature**
EcoRI	5'...G|AATTC...3' 3'...CTTAA|G...5'	*EcoRI* buffer	37°C
NdeI	5'...CA|TATG...3' 3'...GTAT|AC ...5'	Buffer 1	37°C
AgeI	5'...A|CCGGT ...3' 3'...TGGCC|A ...5'	Buffer 4	37°C

First the pTOPO2.1- *trunCTCF* are digested with *EcoRI* to confirm that the insert have been ligated into the vector. The restriction digestion of DNA was performed in a 0.5ml tube. The reaction mixture was prepared by adding the following reagents as shown in Table 5. The volume was made up to 20µl and mixed well before incubated at 37°C for overnight. Upon completion of digestion, the restriction pattern was studied by loading the sample in agarose gel electrophoresis.

Table 5 : Preparation of the *EcoRI* digestion reaction mix

Contents	Volume
Sterile deionised distilled water	12.0 µl
EcoRI buffer	2.0 µl
EcoRI	1.0 µl
DNA	5.0 µl
Total	20.0 µl

When double digestion was performed, the most appropriate buffer as recommended by the manufacturer was used. The reaction mixture are as shown in Table 6.

Table 6 : Preparation of the double digestion with *NdeI* and *AgeI* reaction mix

Contents	Volume
Sterile deionised distilled water	12.0 µl
Buffer 4	2.0 µl
NdeI	1.0 µl
AgeI	2.0 µl
DNA	5.0 µl
Total	20.0 µl

2.13 Analysis of truncated CTCF by sequencing

The truncated CTCF in pTOPO2.1 was sent for sequencing in the Human Genome Centre in Universiti Sains Malaysia. The sequence are shown in Figure 12.

2.14 Gel elution

After visualization with the UV transilluminator, the desired band of DNA can be eluted from the gel for further manipulation. In this study, the elution was carried out by using QIAquick Gel Extraction Kit from Qiagen. The agarose gel containing the desired DNA was carefully excised with a clean sharp scapel and transferred into a 1.5ml microcentrifuge tube. The gel slice was weighed and 3 volumes of buffer QG was added to 1 volume of gel. Buffer QG will solubilizes the agarose gel slice and provides the appropriate conditions for binding of DNA to the silica membrane. It contains a pH indicator which allows easy determination of the optimal pH for DNA binding. The tube was then incubated at 50°C for 10 minutes until the gel slice has completely dissolved. 1 gel volume of isopropanol was added to the sample and invert mixed. This sample is only needed for DNA fragments <500 bp and >4 kb to increase the yield. To bind DNA, the sample was applied to the column and centrifuged at 13,000rpm for 1 minutes at room temperature. The flow-through was discarded followed by addition of 500µl of buffer QG to the column. This step will remove all traces of agarose. After that 750µl of buffer PE was added and centrifuged at 13,000rpm for 1 minutes. The ethanol containing buffer PE will wash away unwanted primers and impurities such as salts, enzymes, unincorporated nucleotides, agarose, dyes, ethidium bromide, oils and detergents that do not bind to the silica membrane. The flow-through was discarded. The column was then placed into a

clean 1.5ml microcentrifuge tube and 30µl of buffer EB was added to elute the DNA. The eluted DNA is stored at -20°C for further manipulation.

2.15 Ligation of truncated CTCF into pET16b

In ligation reaction, the vector and insert ration are in the ratio 1:3. Ligase buffer, sterile deionised water and T4 DNA ligase are mixed together with the treated vector and DNA insert in a sterile microfuge tube as shown in Table 7. The mixture are then incubated at 4°C for overnight. The ligation mix was then ready for transformation.

Table 7 : Sticky end ligation of restricted vector and insert

Contents	Volume
T4 DNA ligase	1 µl
Ligation buffer	2 µl
DNA Insert	12 µl
Vector	5 µl
Total	20 µl

2.16 Transformation of pET16b~*trunCTCF* into *BL21 (DE3)*

The protocol for transformation is as described in page 20.

2.17 Expression of truncated CTCF proteins using pET system

The truncated CTCF was expressed using expression vector pET16b. The recombinant plasmids were transformed into *E. coli* strains *BL21 (DE3)* and plated on LB plates containing 100mg/ml ampicillin. Freshly transformed colonies were selected and