DESIGN, CONSTRUCTION AND EXPRESSION OF A SYNTHETIC GENE FOR METAL BINDING PROTEINS

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ABSTRACT

DESIGN, CONSTRUCTION AND EXPRESSION OF A SYNTHETIC GENE FOR METAL BINDING PROTEINS

Construction of synthetic genes is today the most elegant way to optimize the heterologous expression of a recombinant protein. The availability of sequences of entire genome has significantly increased the number of protein targets which many of them will need to be overexpressed in cells other than the original source of DNA. Gene synthesis often provides a fast and economically efficient approach. In this study we have optimized a two-step polymerase chain reaction (2-step PCR) method for the fast and extremely accurate synthesis of a 186 bp *CUP1* gene encoding yeast *Saccharomyces cerevisiae* copper metallothionein. A total of the six overlapping oligonucleotides ranged from 43 to 49 in length, designed with the unique restriction sites, were assembled in a single step PCR. The assembly was then further amplified by second PCR to produce a synthetic gene which has been cloned into the pET28a(+) vector to allow the expression of CUP1 gene in *E. coli* BL21 (DE3) host cell. In order to compare the difference in expression level of the gene with optimized codon usage for *E. coli*, *CUP1*gene was redesigned according to codon bias of host cell. A significant increase of expression level of codon optimized gene was obtained compared to original sequence of *CUP1* gene of copper metallothionein in yeast Saccharomyces cerevisiae.

ÖZET

METAL BAĞLAYAN PROTEİNLER İÇİN SENTETİK GEN TASARIMI, YAPIMI VE İFADELENMESİ

Sentetik gen yapımı, günümüzde rekombinant proteinlerin farklı türlerde ifadelenmesini uygun hale getiren en mükemmel yoldur. Genomdaki tüm sekanslara ulaşılabilirlik, orjinal kaynağının dışında ifadelendirilmesine ihtiyaç duyulan protein hedeflerinin sayısını önemli derecede arttırmıştır. Gen sentezi bunun için hızlı ve ekonomik bir yöntemdir. Bu çalışmada 186 baz çifti uzunluğundaki *CUP1* geni tarafından kodlanan *Saccharomyces cerevisiae*' ye ait bakır bağlayan proteininin hızlı ve eksiksiz sentezi için iki basamaklı polimeraz zincir reaksiyonu yöntemi oluşturulmuştur. Bunun için birbirleriyle belli bölgelerde örtüşen ve özel restriksiyon bölgeleri eklenmiş 43 ila 49 baz uzunluğunda altı adet oligomer ilk basamakta polimeraz zincir reaksiyonu ile birleştirilmiştir. Birleştirilen ürün ikinci basamak polimeraz zincir reaksiyonu ile çoğaltıldıktan sonra pET28a(+) vektörüne klonlanmıştır. Klonlanan genin ifadelenmesi *E. coli* BL21 (DE3) hücrelerinde gerçekleştirilmiştir. *E. coli* ile *S. cerevisiae* hücreleri arasında kodon kullanım farklılığından dolayı ortaya çıkan protein ifadelenmesinin yetersiz olması problemi orjinal gen dizisinin *E. coli* hücrelerinin kodon kullanımına uyarlanmasıyla aşılmıştır. Bu aşamadan sonra başarılı bir şekilde ifadelenen protein, His Select® HF nikel afinite kolon kromatografisi ile saflaştırılmıştır.

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ABBREVIATIONS

bp	Base pair
Cu-MT	Copper metallothionein
DNA	Deoxyribonucleic acid
DTT	Dithiothretiol
E. coli	*Escherichia coli*
IPTG	isopropyl thio-β-D-galactoside
kDa	kilo Dalton
MT	Metallothionein
nt	Nucleotide
PAGE	Polyacrylamide gel electrophoresis
PCR	Polyacrylamide gel electrophoresis
S. cerevisiae	*Saccharomyces cerevisiae*
X-Gal	5-bromo-4chloro-3indolyl-β-D-galactopyranoside

CHAPTER 1

INTRODUCTION

1.1. Metallothioneins

Organisms are often exposed to changes in environmental factors like nutrition, osmotic pressure, metal ions. They have evolved homeostatic control mechanisms to grow and develop optimally under these conditions (Okuyama et al. 1999).

With the production of metallothioneins (MTs), higher organisms show respond to the presence of heavy metals (Sousa et al. 1998). Metallothioneins is common name for superfamily of ubiquitous low molecular weight (6-7 kDa) metal binding proteins or polypeptides (Kagi and Kojima 1987; Kagi and Schaffer 1988; Romero-Isart and Vasak 2002). They were first detected in 1957 as cadmium-binding proteins in equine renal cortex (Waalkes and John 1996). Metallothioneins occurs through the animal kingdom and also found in higher plants, eukaryotic microorganisms, and some prokaryotes (Romero-Isart and Vasak 2002). As the name implies, in order to provide the basis of high-affinity binding of certain metals, metallothioneins contain great number of thiol groups, owing to their high cysteine content.

1.1.1. Metal Binding and Structure of Metallothioneins

Metallothionein structure is characterized by a high content of cysteine (up to 30% of the total amino acid residues) serine and glycine and also lacks aromatic amino acids and histidine. Cysteinyl residues are dispersing 61-residue chain with a series of

Cys-Xaa-Cys, Cys-Xaa-Xaa-Cys, Cys-Xaa-Xaa-Xaa-Cys units (Cys: Cysteine Xaa: other amino acid) (Waalkes and John 1996). This arrangement confers the capacity to binding high number of metal atoms. The metal ions are organized in one or two metal-thiolate cluster(s), with thiolate ligands acting both as a terminal and bridging (Dabrio et al. 2002). All cysteinyl sulfurs participate in metal ions so there are no disulfid bridges in metallothioneins (Waalkes and John 1996).

General structures almost similar and exhibit a monomeric dumbbell-shaped protein with seven metal ions located in two separate metal thiolate clusters. The metal ions are co-ordinated by both bridging and terminal thiolate ligands in both clusters (Dabrio et al. 2002).

Briefly, general characteristics of metallothioneins can be summarized as listed in Table 1.1.

Table 1.1. Distinctive features for classification as metallothionein
(Source: Waalkes and John 1996)

Low-molecular weight. (6000-7000 kDa)
High cysteine content.
Metal clusters with bridging thiolate ligands.
High metal affinity and content.
Lack of aromatic amino acids.
Optical properties characteristics of metal-thiolate bonds.
Highly ordered and conserved cysteine arrangement.
Inducible by carious chemicals and physical treatments, including metals.
Typically polymorphic.

1.1.2. Biological Role of Metallothioneins

Metallothioneins thought to take place in various biological processes like homeostasis of essential metals such as zinc (Zn) and copper (Cu) or detoxification of toxic metals like cadmium (Cd) and mercury (Hg) (Dabrio et al. 2002).

They also play role as a donor of zinc and copper when several apoenzymes require zinc or copper as cofactor (Waalkes and John 1996).

1.1.3. Quantification of Metallothioneins

Quantification of metallothioneins can be done by using three different steps (Table 1.2.). Since metallothioneins localized in the soluble portion of the cell, estimation of the metallothionein content is carried out after sample preparation by homogenization and centrifugation to achieve a cytosolic fraction (Waalkes and John 1996). After then, separation of metallothionein from other proteins can be carried out by various of methods like; liquid chromatography and treatment with acid, heat and /or solvent.

Quantification of the metallothioneins then can be successfully completed by direct or indirect measurement that can be carried out by determination of metal or thiol contents of the purified or semipurified metallothionein protein (Waalkes and John 1996). Moreover, electroanalytical techniques, UV-Vis spectrophotometers, metal saturation assays or immunological methods can be successfully applied to reach to goal.

Based on the redox properties of the complexes formed by thiol groups with metal ions contained in protein, the electroanalytical methods for quantification metallothioneins can be used. The saturation capability of the metallothioneins could be used for quantification methods such as metal affinity assays. Based on the colorimetric properties of different reagents in combination with mercaptans, spectrophotometric determinations may be used (Dabrio et al. 2002).

1.1.3.1. Experimental Problems Related to Metallothioneins

Methodologies for metallothionein determination are performed under experimental conditions keeping the purified proteins as a metal complex form and at neutral pH value because of the stability constants for the various metals are pH dependent. Also it is important to be aware of changes in protein structure due to potential loss of the metals. (Dabrio et al. 2002)

Determination of metallothioneins has some difficulties that make quantification of metallothioneins impractical. Most common problems considered in the current protocols are oxidation and polymorphism of the metallothioneins which related to the nature of the proteins. The presence of free sulphur groups results in high sensitivity to oxidation under aerobic conditions when metallothioneins are not fully saturated with metals. To avoid the formation of disulphide bonds, an oxygen-free environment recommended and reducing agent, such as 2-mercaptoethanol or dithiothretiol (DTT), can be used to crack the disulphide bonds (Dabrio et al. 2002).

Table 1.2. Steps in metallothionein quantification
(Source: Dabrio et al. 2002)

Sample preparation
Homogenization or cellular disruption Centrifugation Metal saturation
Purification or Semipurification of MT
Acid treatment Heat treatment Solvent extraction Chromatography Electrophoresis
Quantification
Indirectly (metal content) Directly (immunological)

1.2. Yeast Copper Metallothionein (Cu-Mt)

In this study we were chosen the copper metallothionein from the yeast *Saccharomyces cerevisiae* because this protein has been very well characterized.

Copper (Cu) is an essential element required by all organisms; but also it is highly toxic at elevated levels. It functions as an important cofactor for variety of enzymes such as cytochrome *c* dismutase, Cu or Zn -superoxide dismutase, lysl oxidase. Furthermore, copper can take part in feton-like reactions that can generate excessively reactive hydroxyl radicals which cause cellular damage such as the oxidation of proteins, cleavage of the DNA and RNA molecules, and membrane damage. So it is important that organisms have to emphasize sufficient mechanisms for uptake and detoxification of Cu ions to drive the essential biochemical processes while preventing its accumulation to toxic levels. Regulation of the Cu homeostatic machinery requires the ability of the Cu ion sensors to detect excess level of Cu and respond by properly regulating the expression of Cu homeostasis genes in order to maintain the balance between essential and toxic levels (Pena et al. 1998).

In the yeast *Saccharomyces cerevisiae*, copper metallothionein (Cu-MT), which is encoded by *CUP1* gene locus, mediates the resistance to toxic effects of copper (Berka et al. 1988). Biosynthesis of Cu-MT is regulated at the level of transcriptional induction in response to high environmental copper levels. Even thought yeast Cu-MT is able to bind to variety of metals *in vivo* and *in vitro*, copper is the only inducer of *CUP1* gene transcription (Karin et al. 1984).

1.2.1. *CUP1* Locus of *Saccharomyces cerevisiae*

The copper resistance in yeast *S. cerevisiae* is mediated by the function of the *CUP1* locus, which is located on chromosome VIII, 42 centimorgans distal to the centromere (Butt and Ecker 1987). While copper-sensitive strains ($cupI^{S}$) contain a single functional copy of *CUP1* locus, the copper-resistance (*CUP1*^{r}) yeast strains

contain multiple several copies of *CUP1*, tandemly amplified as an approximately 2-kilobase (kb) DNA segment. The basic repeat contains two transcription units that one of them is 246 amino acid protein which is named as protein X. The function of the protein X has not been fully identified yet. The smaller unit encodes the, 61 amino acid, a low molecular-weight cystein rich, yeast copper metallothionein (Karin et al. 1984). Expression of the Cu-MT gene from the *CUP1* locus in *S. cerevisiae* is mediated by the *ACE1* transcriptional activator when the copper ions in the environment. *ACE1* contains a Cu-thiolate polynuclear cluster which provides the necessary conformation for DNA binding and activation of transcription from *CUP1* when high level of metal ions present in the environment (Sayers et al. 1999).

1.2.2. Nucleotide Sequence of the Yeast Copper Metallothionein

186-nucleotide open reading frame of the *CUP1* locus encodes 61 amino acid protein molecule that contains 12 cysteine and 8 serine residues. Thus, cysteine residues consist 20% of total amino acid residues in yeast Cu-MT.

ATG	TTC	AGC	GAA	TTA	ATT	AAC	TTC	CAA	AAT	GAA	GGT	CAT	GAG	TGC
MET	*Phe*	*Ser*	*Glu*	*Leu*	*Ile*	*Asn*	*Phe*	*Gln*	*Asn*	*Glu*	*Gly*	*His*	*Glu*	*Cys*
CAA	TGC	CAA	TGT	GGT	AGC	TGC	AAA	AAT	AAT	GAA	CAA	TGC	CAA	AAA
Gln	*Cys*	*Gln*	*Cys*	*Gly*	*Ser*	*Cys*	*Lys*	*Asn*	*Asn*	*Glu*	*Gln*	*Cys*	*Gln*	*Lys*
TCA	TGT	AGC	TGT	CCA	ACG	GGG	TGT	AAC	AGC	GAC	GAC	AAA	TGC	CCT
Ser	*Cys*	*Ser*	*Cys*	*Pro*	*Thr*	*Gly*	*Cys*	*Asn*	*Ser*	*Asp*	*Asp*	*Lys*	*Cys*	*Pro*
TGC	GGT	AAC	AAG	TCT	GAA	GAA	ACC	AAG	AAG	TCA	TGC	TGC	TCT	GGG
Cys	*Gly*	*Asn*	*Lys*	*Ser*	*Glu*	*Glu*	*Thr*	*Lys*	*Lys*	*Ser*	*Cys*	*Cys*	*Ser*	*Gly*
AAA	TGA													
Lys	**													

Figure 1.1. DNA sequence of yeast copper metallothionein

1.2.3. Yeast Copper Metallothionein is Related to Vertebrate Metallothionein

Recent studies indicate that yeast Cu-MT is similar to mammalian MT. It is low molecular weight and cysteine rich protein and holds as much as 90% of the total cellular copper in yeast. The coding region for Cu-MT is mostly G+C rich while the controlling sequences of the gene are 70% A+T rich. Presence of two phenylalanine codons in the gene at positions 1 and 7 of the polypeptide is another surprising observation. Because none of the mammalian MTs studied before contain phenylalanine. The importance of the phenylalanine in the metal binding process has unknown (Butt et al. 1984).

1.2.4. Differences to Other Metallothioneins

However, several features of the yeast Cu-MT are similar to higher eukaryotic MTs, there are some remarkable differences appear. The difference in the amino acid composition between yeast Cu-MT and human MT II have shown in Table 1.3. While lysine and serine are present in nearly similar amounts, the yeast Cu-MT contains 20% cysteine content as compared to 32% present in the human MT. Also yeast Cu-MT contains 10% glutamic acid which makes the protein negatively charged and result slower mobility on polyacrylamide gel electrophoresis (Butt et al. 1984). Existence of two phenylalanine residues in the yeast Cu-MT is the most surprising finding while MTs from higher eukaryotes lack of aromatic amino acids.

Table 1.3. Amino acid comparison composition of yeast and human MT
(Source: Butt et al. 1984)

Amino acid	Percentage (%)	
	Yeast Cu-MT	**Human MT II**
Alanine	0	11.5
Cysteine	20.0	32.8
Aspartic acid	3.3	3.3
Glutamic acid	10.0	1.6
Phenylalanine	3.3	0
Glycine	8.3	8.2
Histidine	1.6	0
Isoleucine	1.6	0
Lysine	11.6	11.5
Leucine	1.6	1.3
Methionine	1.6	1.6
Asparagine	10.0	1.6
Proline	3.3	1.6
Glutamine	8.3	1.6
Arginine	0	0
Serine	13.3	13.1
Threonine	3.3	4.9
Valine	0	4.9
Tryptophane	0	0
Tyrosine	0	0
MOLECULAR WEIGHT (Da)	**6573.18**	**5888.68**

The tandem arrangement of cysteine residues in vertebrate MT is thought to play a basic role in clustering of the metals in the protein. Figure 1.2. shows a comparison of the arrangement of cysteine residues in yeast Cu-MT to human MT. In the yeast Cu-MT, as in human MT, many of the cysteine residues are flanked by serine and glycine. The clusters of cysteine in the yeast Cu-MT are not similar in the case of in human MT. (eg., Cys-Ser-Cys or Cys-Lys-Cys) but one remarkable homology is precisely conserved at the amino acid sequence Lys-Lys-Ser-Cys-Cys-Ser. As a conclusion, the yeast Cu-MT binds to copper through thiolate metal clusters similar to those in the MTs of higher eucaryotes (Butt et al. 1984).

Figure 1.2. Comparison of cysteine residues in yeast Cu-MT and Human MT
(Source: Butt et al. 1984)

1.2.5. Purification Studies of Yeast Copper Metallothionein

Purification studies of the *CUP1* locus product on yeast revealed that the predicted amino acid sequence lacks first eight amino acids (Wright et al. 1987). Metal binding studies indicated that truncated Cu-MT contains eight Cu ions bound to the 12 cysteines. Also overexpression of truncated form in *E. coli* was shown that protein contains eight Cu ions per molecule (Sayers et al. 1999). Structural and functional studies of the amino terminus have showed that 8-residue peptide did not appear to participate the tertiary fold of the protein and the properties of the 61–residue molecule were not different from the truncated protein (Wright et al. 1987).

Berka et al. also purified 61-residue peptide successfully at bacterial expression system. The bacterial expression system has an advantage because of the synthesis of Cu-MT was not depend on copper. They were also shown that the purified protein was capable to bound cadmium and zinc by using displacement experiments (Berka et al. 1988).

In this study we planned to construct yeast Cu-MT by using synthetic gene technology, overexpression of constructed protein in bacterial expression system and then purification of overexpressed protein successfully.

1.3. Synthetic Gene

Gene synthesis is the procedure of synthesizing an artificially designed gene into a physical DNA sequence. Synthetic genes provide an immediate and easy path from virtual sequences to physical DNA. This method offers a cost-effective way for research that wouldn't be possible or feasible relying on existing DNA sequences. Since, protein and DNA sequences have become easily obtain electronically through databases than physically from library clones, direct synthesis of genes is rapidly becoming the most efficient way to make functional genetic constructs and enables applications such as codon optimization, making RNAi resistant genes and protein engineering (Villalobos et al. 2006).

At late 1970's, synthetic genes have been made by oligonucleotide assembly and today several methods are currently used (Denis-Quanquin et al. 2007). These methods can represented as oligonucleotide ligation, the *Fok I* method, self priming PCR (Hoover and Lubkowski 2002). However, all these methods requires phosphorylated oligonucleotides and polyacrylamide gel purified oligonucleotides for the best result which made them costly for construction of the genes (Young and Dong 2004). The more attractive and simple method is called assembly polymerase chain reaction (PCR). This method involves short oligonucleotides that overlap with each other.

1.3.1. Construction of Synthetic Genes by Two-Step Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is used not only for the cloning and manipulation existing DNA sequences but also it makes possible to create a new DNA fragment consisting of a nucleic acid sequence that is completely designated by investigator. The two step PCR method is based on early observations by Mullis et al. in which multiple overlapping oligonucleotides could be used to generate synthetic DNA through several sequential rounds of Klenow based PCR amplification (Dillon and Rosen 1990).

The principles of the method can be summarized in Figure 1.3. Before the two sequential PCR reactions, researcher has to design the construct and determine the nucleic acid sequence of the desired synthetic gene. Then oligonucleotides that span the length of the gene designed and synthesized. Generally, synthesized oligonucleotides should contain overlaps that are between 15 and 30 nucleotide (nt) long. The direction of the designed oligonuleotides should be same as Figure 1.3.A. The outer oligonucleotides must correspond to opposite strands and be positioned thereby they will extend inward toward each other over the gene (Dillon and Rosen 1990).

The length and nucleotide number of the each oligonucleotide may change according to the size of the gene to be generated. Often, they should be between 60 and 125 nt long (Dillon and Rosen 1990).

After the oligonucleotides have been obtained, the first step of the method, which generates a template DNA for the second step, is done by mixing the overlapping oligonucleotides in a standard PCR reaction. How overlapping oligonucleotides would be extended through the first a few cycles shown at Figure 1.3.B. Since only small numbers of full-length DNA fragments are produced at the first step PCR, a second step is used for the amplification of the full-length product. Therefore, the assembled genes from the first step are use as templates in second step using outer primers as illustrated in Figure 1.3.C. The sequence of the outer primers should contain restriction sites for cloning studies (Dillon and Rosen 1990).This method provides sufficient amount of DNA for cloning into desired vectors.

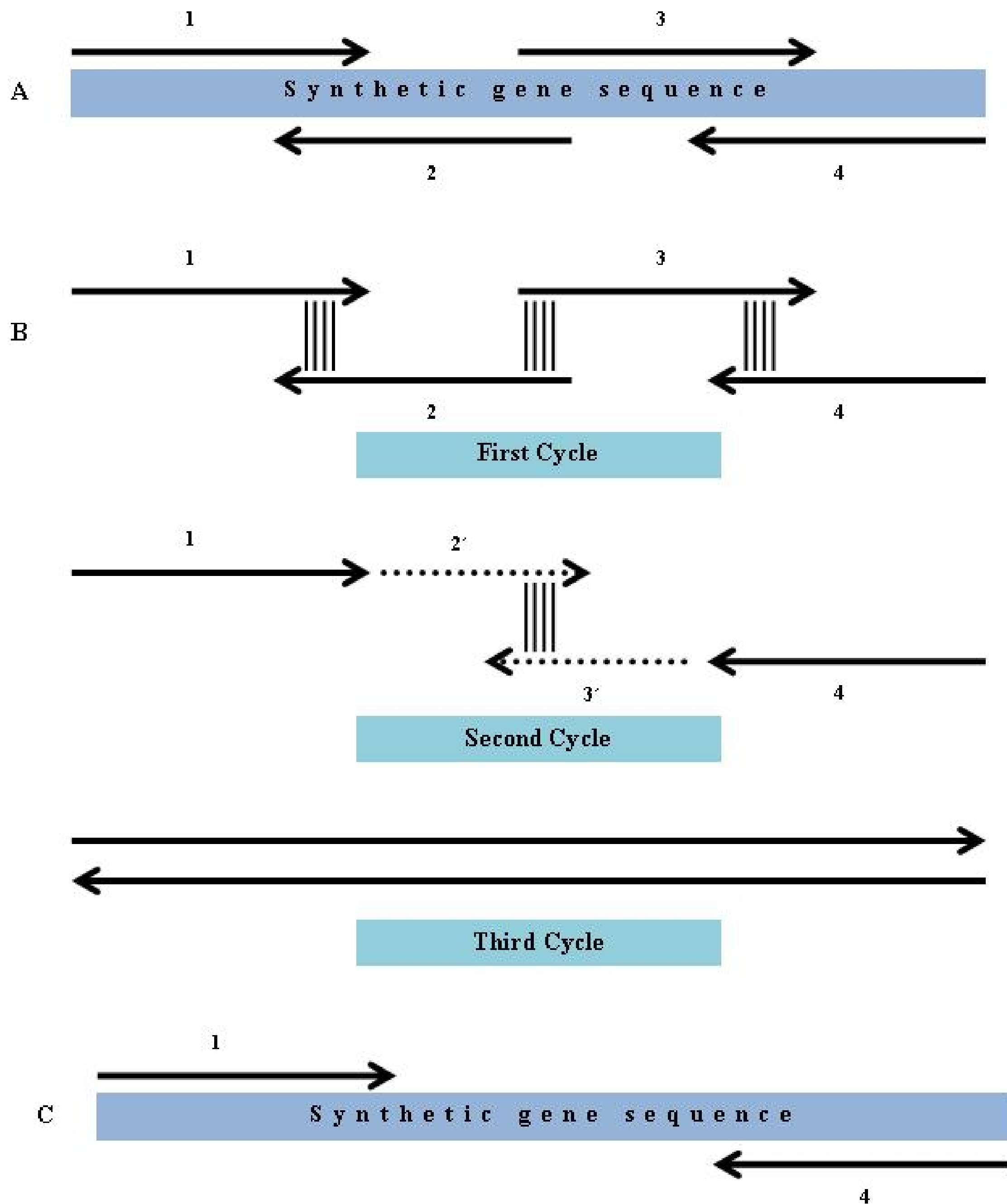

Figure 1.3. Schematic view of the two-step PCR method for construction synthetic gene. (A) Schematic of design and orientation of overlapping oligonucleotides for first PCR reaction. (B) Diagram of oligonucleotide extensions during initial cycles of the first PCR. (C) Schematic of design and orientation of flanking primers used in second PCR reaction (Source: Dillon and Rosen 1990)

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

A detailed list of commonly used chemicals, buffers, solutions and their compositions are presented in Appendix A.

The bacterial strains used in this study were: *E. coli* DH5α *E. coli* BL21(DE3). *E. coli* DH5α strains were used for construction of all plasmids and blue-white colony selection studies. The BL21(DE3) strains were used in the expression studies that driven by the T7 promoter.

Plasmids that were chosen for this study; pBSIIKS + (Stratagene) vector were chosen for subcloning studies. pET28a(+) (Novagen) plasmid was chosen for the expression studies. This plasmid gives additional advantage in terms of purification steps via His-tag (Figure 2.1.).

All of the DNA manipulations and electrophoresis procedures were carried out according to Sambrook et al. (Sambrook et al. 1989). Also all restriction enzymes, T4 DNA ligase, Pfu DNA polymerase, and solutions for PCR were obtained Fermentas.

Nondenaturing polyacrylamide gel electrophoresis (Nondenaturing-PAGE) studies were carried out according to Walker, J.M. (Walker 1994). and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) studies were carried out according to Laemmli, U.K. (Laemmli 1970).

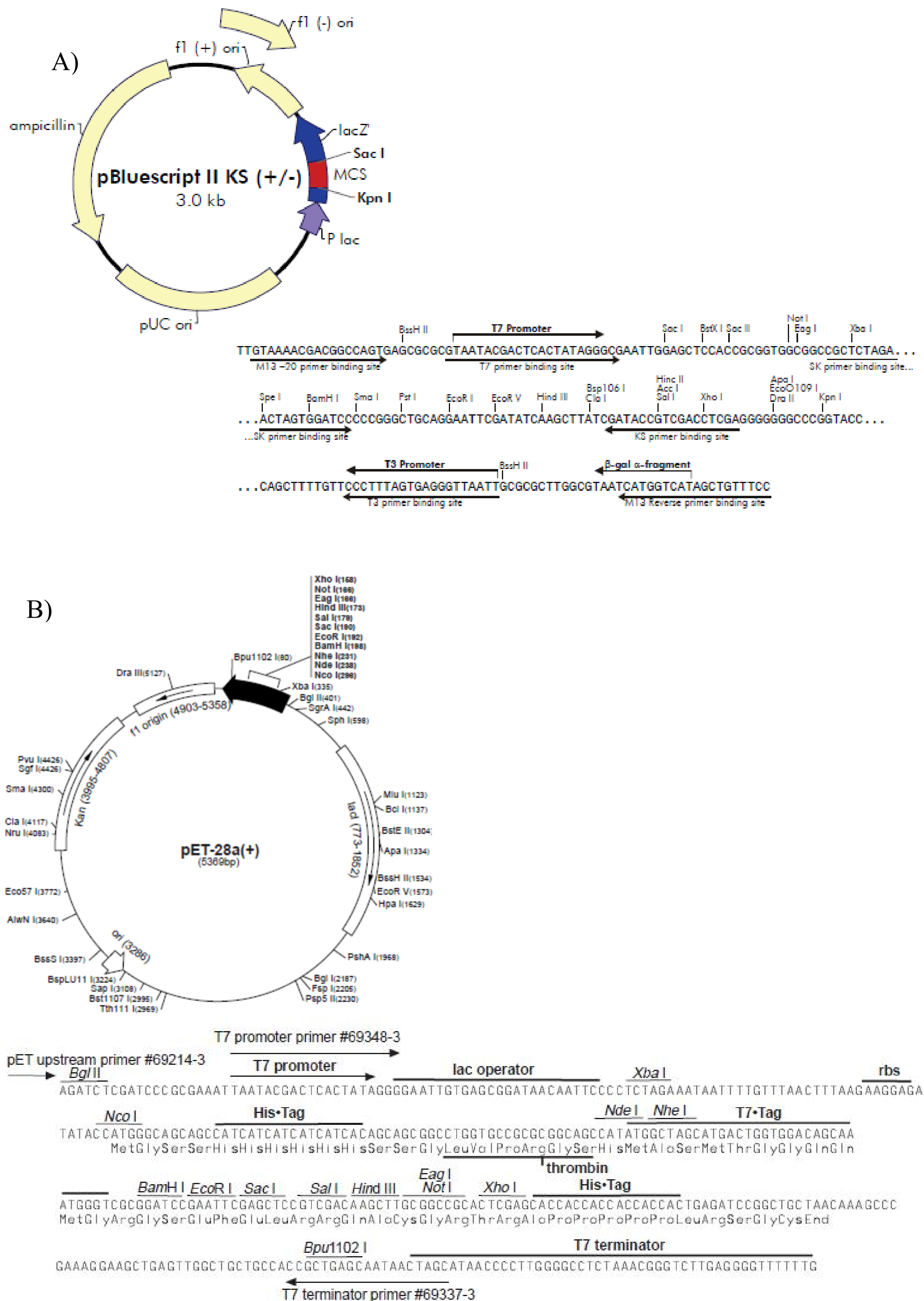

Figure 2.1. A) pBSIIK(+) vector and sequence of multiple cloning site (Source: http://www.stratagene.com/manuals/212205.pdf , accessed July 22, 2009) B) pET28a(+) expression vector and multiple cloning site and expression region(Source:http://www.merckbiosciences.co.uk/docs/docs/PROT/TB074.pdf, accessed July 22, 2009)

2.2. Methods

2.2.1. Design and Construction of the Synthetic Yeast Copper Metallothionein Gene

Amino acid sequence for yeast Cu-MT was obtained from www.yeastgenome.org. The sequence of the yeast Cu-MT is shown in Figure 2.2.

```
  1   ATGTTCAGCG  AATTAATTAA  CTTCCAAAAT  GAAGGTCATG  AGTGCCAATG
 51   CCAATGTGGT  AGCTGCAAAA  ATAATGAACA  ATGCCAAAAA  TCATGTAGCT
101   GCCCAACGGG  GTGTAACAGC  GACGACAAAT  GCCCCTGCGG  TAACAAGTCT
151   GAAGAAACCA  AGAAGTCATG  CTGCTCTGGG  AAATGA
```

Figure 2.2. Amino acid sequence of yeast copper metallothionein.

In order to construct the synthetic yeast Cu-MT gene, six separate overlapping oligonucleotide fragments were designed manually and unique restiction site *Nde I* at the 5′ and *Hind III* at the 3′ was introduced to simplify subcloning. Oligonucleotides ranging from 43-49 nucleotides in length were synthesized. (MWG Oligo Synthesis, Germany) For construction purpose, these oligonucleotides were designed with regions of complementary overlap regions (13-18 bases in length) with neighboring oligonucleotides. High G/C content regions selected as overlapping regions. The oligonucleotides used in the construction and PCR amplification of yeast Cu-MT gene are listed in Figure 2.4. In addition, an *E. coli* codon optimized yeast Cu-MT gene designed and constructed at ShineGene Molecular Biotech, Inc. Figure 2.3. shown optimized gene sequence of yeast Cu-MT gene for *E. coli* with restriction sites.

```
  1   ATGTTTAGCG  AACTGATTAA  CTTTCAGAAC  GAGGGACATG  AATGTCAATG
 51   CCAATGCGGC  TCCTGCAAAA  ACAACGAACA  GTGCCAAAAA  AGCTGTAGTT
101   GTCCAACCGG  TTGTAACTCT  GATGACAAAT  GTCCATGTGG  TAACAAATCA
151   GAAGAAACCA  AGAAAAGCTG  CTGCTCCGGC  AAATGA
```

Figure 2.3. Optimized yeast Cu-MT gene sequence for *E. coli* codon usage

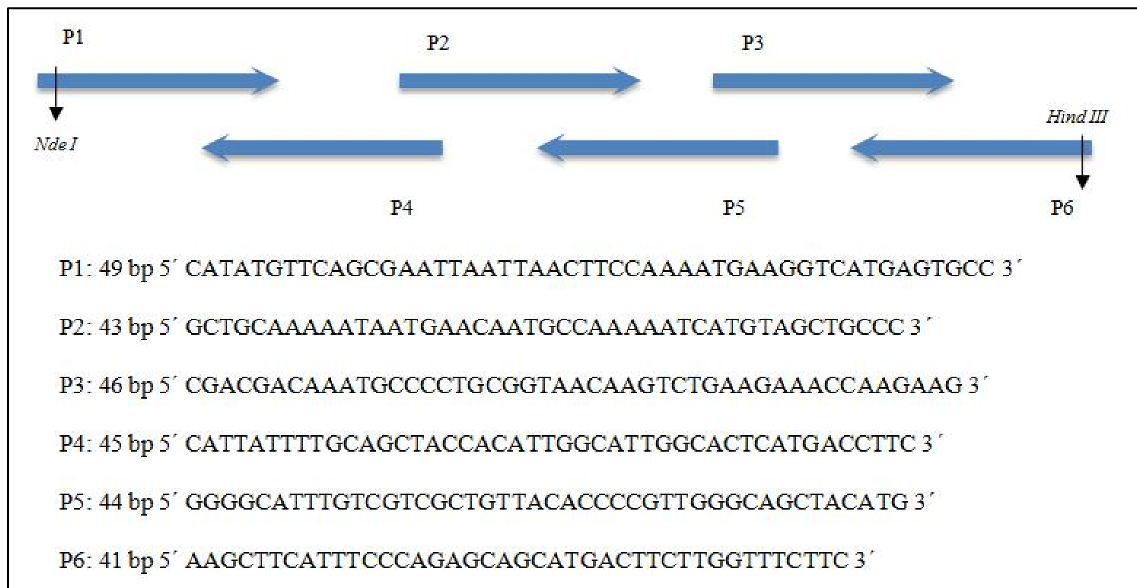

Figure 2.4. List of designed oligonucleotides.

Two-step PCR method was used for the construction of the synthetic yeast Cu-MT gene. Template DNA corresponding to the full-length synthetic gene was generated using the complete set of six overlapping oligonucleotides in a single PCR. Non-phosphorylated oligonucleotides (each 50 pmol), dNTPs (10mM), *Pfu* DNA polymerase (2.5 u/ µl) and PCR reaction buffer were mixed together in a 50 µl sample. Since only a small amount of full-length gene fragments are produced at the end of the first step, a second PCR was used for the amplification of the full-length product using outer primers. Therefore, the assembled genes from the initial PCR were used as templates in a second PCR using the outer primers (P1 and P6, Figure 2.4). Templates (2 µl of the first PCR reaction), dNTPs (10mM), primers (each 50 pmol), *Pfu* DNA polymerase (2.5 u/ µl), and PCR reaction buffer were mixed together in a 50 µl sample. Both reactions were carried out in an Applied Biosystems thermal cycler for 30 cycles.

Each cycle comprised denaturation, annealing, extension and final extension conditions of 95 ºC for 1 min, 57 ºC 1 min, 72 ºC for 1 min and 72 ºC for 5 min was applied for each reaction. An initial denaturation step of 95 ºC 5 min was applied for the first run for each PCR reaction. Polynucleotide products from both the first and second PCR were analyzed using ethidium bromide stained 1% Agarose gel electrophoresis.

2.2.2. Cloning and DNA Sequencing

The PCR products of second step were excised from the 1% Agarose gel with a blade and purified with the Fermentas gel extraction kit. The purified DNA fragments were first cloned into the *Sma*I site of pBSIIKS + (Stratagene). The ligation products were used to transform *E. coli* DH5α competent cells and Blue-White colony selection was performed on LB agar supplemented with 100 μg/ml ampicillin, isopropyl thio-β-D-galactoside (IPTG) and 5-bromo-4chloro-3indolyl-β-D-galactopyranoside (X-Gal). The white colonies that harboring the yeast Cu-MT gene fragment was incubated for plasmid isolation. (Gene Jet™ Plasmid Mini Prep Kit, Fermentas) Then isolated plasmids were screened for the presence of insert by restriction analysis with *Nde*I and *Hind*III (Fermentas). Positive clones were sequenced (IYTE Biotechnology Center) to check for fidelity.

Plasmids which had correct nucleotide sequence were digested with *Nde*I and HindIII enzymes and fragments purified from 1% Agarose gel and then ligated to pET28a(+) (Novagen) expression vector. The ligation products were transformed into *E. coli* BL21(DE3) competent cells and selected on LB plates with 30 μg/ml kanamycin. The plasmids isolated from selected colonies were screened by restriction digest analysis. Plasmids containing the gene of interests were sequenced in both forward and reverse directions.

2.2.3. Gene Expression and Isolation of the Recombinant Yeast Copper Metallothionein Protein From *E. Coli*

Expression plasmids containing eror-free inserts directly transformed into competent *E. coli* BL21(DE3) cells. Transformed cells were then spread on LB-agar plates containing 30 μg/ml kanamycin. Overnight colonies were then used to inoculate 20 ml LB cultures containing 30 μg/ml kanamycin that were incubated overnight at 37 ºC at 200 rpm. The entire 20 ml culture were then added to 200 ml medium containing

30 μg/ml kanamycin and incubated at 37 ºC with shaking at 200 rpm to an OD_{600} of approximately 0.6-0.8. Expression of the synthetic yeast Cu-MT was induced with 0.1 mM IPTG at a final concentration for 1 h. Also, after IPTG induction, growth media was supplemented with 0,5 mM CuSO4 for the stability of protein.

Cells were then harvested by low-speed centrifugation at 5000 rpm for 10 min (Hettich Universal 30 RF). Cell pellets were resuspended in 20mM Tris-HCl buffer (pH 7.0) and disrupted by sonication for about 2 min (Bandelin Sonopuls UW 2070). Cell debris was removed by centrifugation at 10.000 g for 20min at +4 ºC (Sigma 6K 15 Ultracentrifuge). The supernatant was used for further SDS-PAGE analysis and protein purification.

2.2.4. Detection of Recombinant Yeast Copper Metallothionien Protein

Synthetic yeast Cu-MT was detected by SDS-PAGE (4% stacking and 15% resolving gels) (Laemmli 1970) and non-denaturing-PAGE (4% stacking and 12% resolving gels) (Walker 1994).

With non-denaturing gels, the soluble cellular extract of the overexpressed cells was loaded to freshly prepared gel after carboxymethylation to alkylate sulphydryl groups of the protein according to Berka et al. (Berka et al. 1988). For that reason, 200 μl of IPTG induced and non-induced cells were taken after expression studies. Cell pellets form 200 μl IPTG induced and non-induced cultures were suspended in 25 μl of lysis buffer consisting 25 mM Tris-HCl [pH 8.0], 10 mM ethylenediaminetetraacetic acid [EDTA], 50 mM Glucose, and 1 mg of lysozyme per ml and solution incubated 15 min on ice. After the incubation the lysed cells were mixed for 30 s with 25 μl of a solution consisting of 25 mM dithiothreitol (DTT), 100 mM NaCl, 200 mM $MgCl_2$ and 0.8% (vol/vol) Triton X-100. Then 5 μl of freshly prepared 3 M solution of sodium iodoacedic acid (Sigma-Aldrich) in 100 mM Tris-HCl (pH 8.6) was added and the samples were incubated in the dark for 3 h at 25 ºC. Prior to electrophoresis on non-denaturing 15% polyacrylamide gels, each sample were mixed with 35 μl of loading dye containing 125 mM Tris-HCl (pH 6.8), 2.5% β-mercaptoethanol, 25% glycerol, and 0.005% bromophenol blue. Electrophoresis was carried out at 65 W constant current

until the blue stain had run out of the gel. After electrophoresis the gel was stained with coomassie blue as a standard procedure.

Also, expression of recombinant yeast Cu-MT protein was evaluated using 15% SDS-PAGE. Supernatants that prepared at isolation step for each induced and non-induced cells were mixed with sample buffer and heated on boiling water for 4 min. The gel stained with coomassie blue after the electrophoresis.

2.2.5. Purification of Recombinant Yeast Copper Metallothionein Protein Expressed in Pet28a (+) Expression Vector

The purification procedure was carried out at 4 ºC using low pressure liquid chromatography system (Pro Team LC™ 320, Teledyne Isco) and all buffers were degassed. Histidine residues that coming from the selected expression vector give an advantage in purification step via bounding the affinity gel that was used in our studies.

Isolation of the recombinant protein was performed as indicated previously. Supernatant were loaded on His Select® HF Nickel Affinity Gel (Sigma) Column. After loading the sample the column was first washed with 50 ml washing buffer including 50 mM sodium phosphate buffer (NaP) pH7.0, 0.3 M NaCl to remove unwanted proteins. Later on, recombinant yeast Cu-MT protein which bounds to the His Select® HF Nickel Affinity Gel was eluted with elution buffer consisting of 50 mM NaP buffer pH 7.0, 0.1 M NaCl, 250 mM imidazole (AppliChem). Eluted proteins were fractioned 20 drop of each collection tubes.

Collected fractions were then analyzed on 15% SDS and %12 non-denaturing PAGE.

CHAPTER 3

RESULTS

3.1. Construction, Cloning and Sequencing the Synthetic Yeast Copper Metallothionein

In this study, a synthetic gene for yeast Cu-MT gene was designed according to published sequence of *CUP1* locus. In the procedure of construction of the gene both strands of the sequence were divided into overlapping oligos of 41-49 bases in length with complementary overlaps of 15-22 nucleotides. In addition, the restriction sites were generated at the beginning and the end position of the gene to facilitate cloning the gene in a proper plasmid.

The initial assembly reaction has involved the construction of the full-length gene from a mixture of the six oligomers. An aliquot of this assembly reaction mixture was then used as a template for the amplification process, in which only the two outermost primers of the assembly were used at a concentration of 1 μl each. Optimum yields of the PCR products were obtained with using *Pfu* DNA polymerase which exhibits a 3´-5´ proofreading exonuclease activity. Analysis of the two PCRs on 1% agarose gels has revealed the presence of the 186 bp expected product of the gene. (Figure 3.1.)

The synthetic DNA products, which purified on agarose gel, were blunt end ligated into *Sma*I cut pBSIIKS+ vector. Ligation products were transformed *E. coli* DH5 α competent cells with heat-shock method and screened for insertion on LB agar plates containing ampicilin, IPTG, and X-gal. The white colonies, which harboring synthetic yeast Cu-MT inserted pBS II KS + vector, selected for plasmid isolation. Isolated plasmids were used then in restriction analysis and sequencing studies. Figure

3.2. shows restriction analysis of yeast Cu-MT inserted pBSIIKS+ vectors digested with *Nde*I and *Hind*III restriction enzymes.

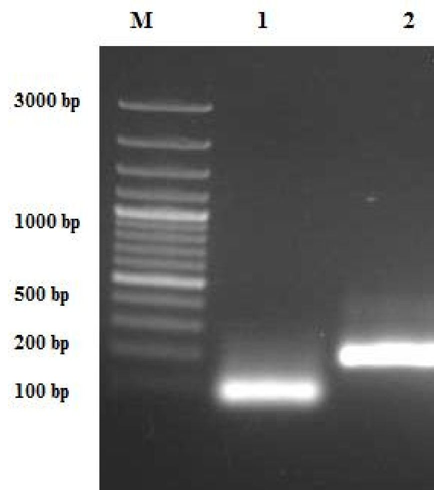

Figure 3.1. Analysis of synthetic Yeast Cu-MT gene products by 1% agarose gel electrophoresis. (Lane M: O′ Gene Ruler™ 100 bp DNA Ladder Plus; Lane 1: Products of the first step PCR in construction of the synthetic Yeast Cu-MT gene; Lane 2: Products of the second step PCR in construction of the synthetic Yeast Cu-MT gene, using outer primers)

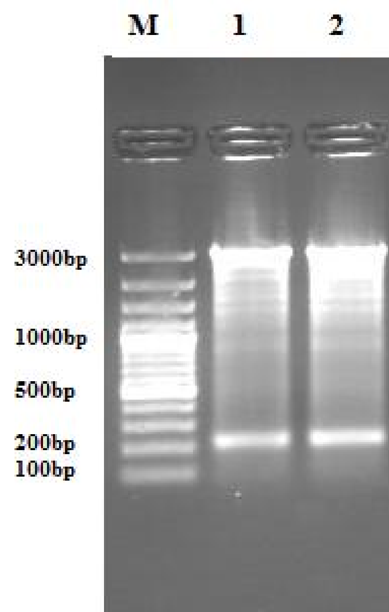

Figure 3.2. Digestion analysis of isolated pBSII K+ from white colonies with restriction enzymes *Nde*I and *Hind*III (Lane M: O′ Gene Ruler™ 100 bp DNA Ladder Plus; Lane 1 and 2: digested Yeast Cu-MT/pBs II K + vectors)

Two of the isolated plasmids were sequenced with using T7 primer of the plasmid used for cloning purposes and results confirmed that our synthetic gene for yeast Cu-MT protein was successfully constructed. Sequence results are presented on Appendix C and nucleodite blast results presented on Figure 3.3.

Yeast Cu-MT (1) - pBS K +

```
Score =  344 bits (186),  Expect = 3e-99
Identities = 186/186 (100%), Gaps = 0/186 (0%)

Query  45   TCATTTCCCAGAGCAGCATGACTTCTTGGTTTCTTCAGACTTGTTACCGCAGGGGCATTT  104
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  186  TCATTTCCCAGAGCAGCATGACTTCTTGGTTTCTTCAGACTTGTTACCGCAGGGGCATTT  127

Query  105  GTCGTCGCTGTTACACCCCGTTGGGCAGCTACATGATTTTTGGCATTGTTCATTATTTTT  164
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126  GTCGTCGCTGTTACACCCCGTTGGGCAGCTACATGATTTTTGGCATTGTTCATTATTTTT  67

Query  165  GCAGCTACCACATTGGCATTGGCACTCATGACCTTCATTTTGGAAGTTAATTAATTCGCT  224
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  66   GCAGCTACCACATTGGCATTGGCACTCATGACCTTCATTTTGGAAGTTAATTAATTCGCT  7

Query  225  GAACAT  230
            ||||||
Sbjct  6    GAACAT  1
```

Yeast Cu-MT (2) - pBS K +

```
Score =  344 bits (186),  Expect = 3e-99
Identities = 186/186 (100%), Gaps = 0/186 (0%)

Query  55   TCATTTCCCAGAGCAGCATGACTTCTTGGTTTCTTCAGACTTGTTACCGCAGGGGCATTT  114
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  186  TCATTTCCCAGAGCAGCATGACTTCTTGGTTTCTTCAGACTTGTTACCGCAGGGGCATTT  127

Query  115  GTCGTCGCTGTTACACCCCGTTGGGCAGCTACATGATTTTTGGCATTGTTCATTATTTTT  174
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126  GTCGTCGCTGTTACACCCCGTTGGGCAGCTACATGATTTTTGGCATTGTTCATTATTTTT  67

Query  175  GCAGCTACCACATTGGCATTGGCACTCATGACCTTCATTTTGGAAGTTAATTAATTCGCT  234
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  66   GCAGCTACCACATTGGCATTGGCACTCATGACCTTCATTTTGGAAGTTAATTAATTCGCT  7

Query  235  GAACAT  240
            ||||||
Sbjct  6    GAACAT  1
```

Figure 3.3. Nucleotide blast results of Yeast Cu-MT inserted pBS K + vector

The synthetic yeast Cu-MT gene fragment was digested with *Nde*I and *Hind*III from the pBSIIKS+vector. Then, the expression vector, pET28a(+), was constructed by fusing the *Nde*I/*Hind*III fragment of the synthetic yeast Cu-MT gene to the pET28a(+) vector by ligation. The ligation sample was further transformed into the expression host *E. coli* BL21(DE3) competent cell. The transformed cells were grown in LB agar plate supplemented with 30 mg/ml kanamycin. The resulting colonies were screened by

restriction analysis (Figure 3.4.) and pET28a(+) vectors which had yeast Cu-MT fragment were sequenced and used further expression studies. Sequencing results for positive clones are presented in Appendix C and nucleotide blast results are shown in Figure 3.5.

Figure 3.4. Restriction analysis of isolated pET28a(+) vectors from selected colonies (Lane M1: O´ Gene Ruler™ 100 bp DNA Ladder Plus; Lane M2: O´ Gene Ruler™ 1 kb DNA Ladder Plus; Lane 5 and 7: pET28a(+) vectors with Yeast Cu-MT gene)

Yeast Cu-MT Pet28 a (+) F (5)

```
Score =  344 bits (186),  Expect = 3e-99
Identities = 186/186 (100%), Gaps = 0/186 (0%)

Query  61   ATGTTCAGCGAATTAATTAACTTCCAAAATGAAGGTCATGAGTGCCAATGCCAATGTGGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGTTCAGCGAATTAATTAACTTCCAAAATGAAGGTCATGAGTGCCAATGCCAATGTGGT  60

Query  121  AGCTGCAAAAAATAATGAACAATGCCAAAAATCATGTAGCTGCCCAACGGGGTGTAACAGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AGCTGCAAAAAATAATGAACAATGCCAAAAATCATGTAGCTGCCCAACGGGGTGTAACAGC  120

Query  181  GACGACAAATGCCCCTGCGGTAACAAGTCTGAAGAAACCAAGAAGTCATGCTGCTCTGGG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GACGACAAATGCCCCTGCGGTAACAAGTCTGAAGAAACCAAGAAGTCATGCTGCTCTGGG  180

Query  241  AAATGA  246
            ||||||
Sbjct  181  AAATGA  186
```

Figure3.5. Nucleotide blast results of yeast Cu-MT inserted pET28a(+) expression vector.

(cont. on next page)

Yeast Cu-MT Pet28 a (+) R (5)

```
Score =  344 bits (186),  Expect = 3e-99
Identities = 186/186 (100%), Gaps = 0/186 (0%)

Query  123   TCATTTCCCAGAGCAGCATGACTTCTTGGTTTCTTCAGACTTGTTACCGCAGGGGCATTT  182
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  186   TCATTTCCCAGAGCAGCATGACTTCTTGGTTTCTTCAGACTTGTTACCGCAGGGGCATTT  127

Query  183   GTCGTCGCTGTTACACCCCGTTGGGCAGCTACATGATTTTTGGCATTGTTCATTATTTTT  242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126   GTCGTCGCTGTTACACCCCGTTGGGCAGCTACATGATTTTTGGCATTGTTCATTATTTTT  67

Query  243   GCAGCTACCACATTGGCATTGGCACTCATGACCTTCATTTTGGAAGTTAATTAATTCGCT  302
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  66    GCAGCTACCACATTGGCATTGGCACTCATGACCTTCATTTTGGAAGTTAATTAATTCGCT  7

Query  303   GAACAT  308
             ||||||
Sbjct  6     GAACAT  1
```

Yeast Cu-MT Pet28 a (+) F (7)

```
Score =  344 bits (186),  Expect = 3e-99
Identities = 186/186 (100%), Gaps = 0/186 (0%)

Query  60    ATGTTCAGCGAATTAATTAACTTCCAAAATGAAGGTCATGAGTGCCAATGCCAATGTGGT  119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     ATGTTCAGCGAATTAATTAACTTCCAAAATGAAGGTCATGAGTGCCAATGCCAATGTGGT  60

Query  120   AGCTGCAAAAATAATGAACAATGCCAAAAATCATGTAGCTGCCCAACGGGGTGTAACAGC  179
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    AGCTGCAAAAATAATGAACAATGCCAAAAATCATGTAGCTGCCCAACGGGGTGTAACAGC  120

Query  180   GACGACAAATGCCCCTGCGGTAACAAGTCTGAAGAAACCAAGAAGTCATGCTGCTCTGGG  239
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   GACGACAAATGCCCCTGCGGTAACAAGTCTGAAGAAACCAAGAAGTCATGCTGCTCTGGG  180

Query  240   AAATGA  245
             ||||||
Sbjct  181   AAATGA  186
```

Yeast Cu-MT Pet28 a (+) R (7)

```
Score =  344 bits (186),  Expect = 3e-99
Identities = 186/186 (100%), Gaps = 0/186 (0%)

Query  121   TCATTTCCCAGAGCAGCATGACTTCTTGGTTTCTTCAGACTTGTTACCGCAGGGGCATTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  186   TCATTTCCCAGAGCAGCATGACTTCTTGGTTTCTTCAGACTTGTTACCGCAGGGGCATTT  127

Query  181   GTCGTCGCTGTTACACCCCGTTGGGCAGCTACATGATTTTTGGCATTGTTCATTATTTTT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126   GTCGTCGCTGTTACACCCCGTTGGGCAGCTACATGATTTTTGGCATTGTTCATTATTTTT  67

Query  241   GCAGCTACCACATTGGCATTGGCACTCATGACCTTCATTTTGGAAGTTAATTAATTCGCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  66    GCAGCTACCACATTGGCATTGGCACTCATGACCTTCATTTTGGAAGTTAATTAATTCGCT  7

Query  301   GAACAT  306
             ||||||
Sbjct  6     GAACAT  1
```

Figure3.5. (Cont.) Nucleotide blast results of yeast Cu-MT inserted pET28a(+) expression vector.

3.2. Expression and Detection of Yeast Copper Metallothionein

The yeast Cu-MT gene was inserted immediately downstream of the T7 promoter and His-tag region of the plasmid. The constructed yeast Cu-MT in pET28a(+) vector was confirmed by DNA sequencing for the correct alignment of the gene. Then, the synthetic gene for yeast Cu-MT protein was transformed into the host of *E. coli* BL21(DE3). As a result of induction of expression of the gene by IPTG in the pET28a(+) expression vector, E. coli BL21 (DE3) host has resulted in the production of a ~6.5 kDa protein for yeast Cu-MT. Also, as a negative control for the expression experiment was carried out with the cells of *E.coli* without IPTG induced.

Because of the codon usage of *E. coli* is different from the yeast *S. cerevisiae,* the expression level of designed gene seems to be poor. For this reason, the sequence of the yeast Cu-MT according to codon usage of E. coli was redesigned at ShineGene Molecular Biotech, Inc. The newly synthesized gene cloned to pET28a(+) expression vector at *Nde*I and *Hind*III sites and transformed to the E. coli BL21(DE3) host cell. Then, resulting colonies were used in expression studies same as stated before. For the stability of the recombinant protein 0.5 mM $CuSO_4$ was added to culture medium. After then, cellular extracts were analyzed on 15% SDS-PAGE as shown in Figure 3.6. In addition, carboxymethylated proteins were analyzed on 12% non-denaturing PAGE. The existence of the recombinant protein was confirmed with IPTG induced cells in compare to non induced cells (Figure 3.7.).

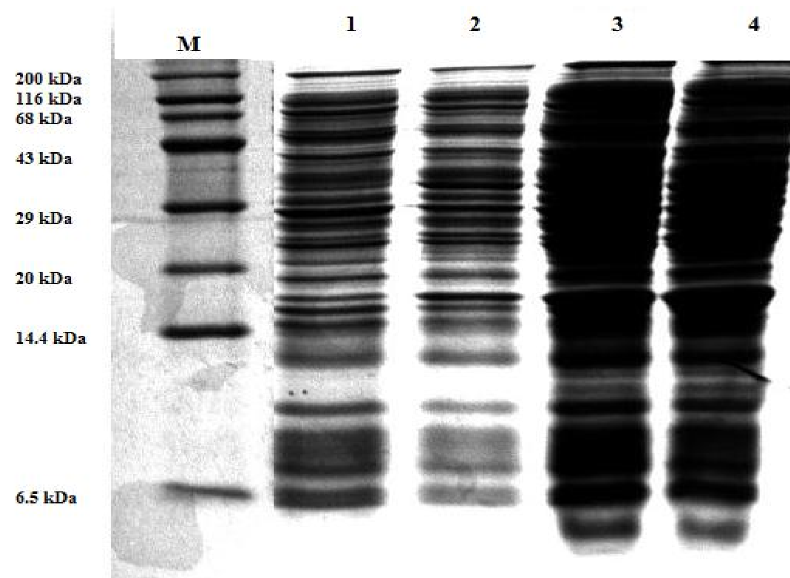

Figure 3.6. 15% SDS-PAGE analysis of expressed synthetic Yeast Cu-MT and Yeast Cu-MT (op.) proteins. Lane M, molecular mass marker; Lanes 1 and 2 synthetic Yeast Cu-MT (op.) and Yeast Cu-MT genes in pET28a(+) expression vector, respectively, non-induced; Lane 3 and 4, synthetic Yeast Cu-MT (op.) and Yeast Cu-MT genes in pET28a(+) expression vector induced by 1mM IPTG, respectively.

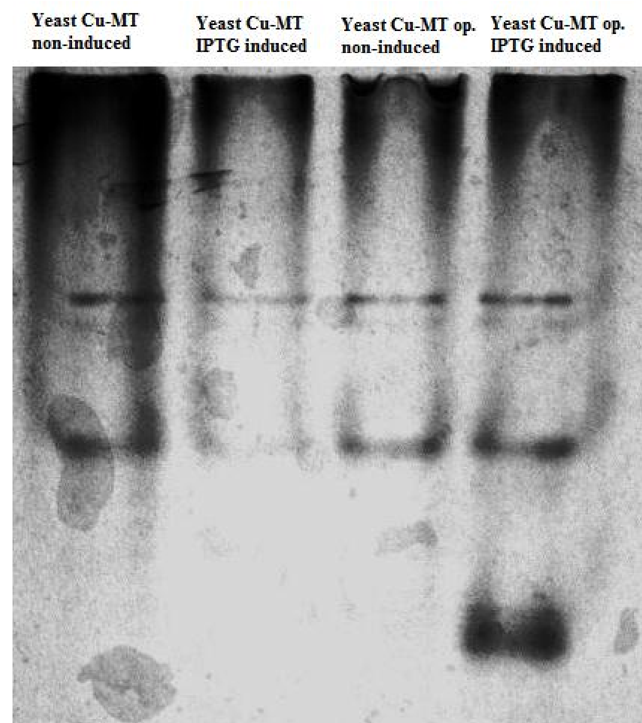

Figure 3.7. 12% Nondenaturing-PAGE analysis of expressed synthetic Yeast Cu-MT and Yeast Cu-MT (op.) proteins after carboxymethylation.

3.3. Purification of Recombinant Yeast Copper Metallothionein

The existence of the His-tag® on the recombinant protein provides an effective one-step purification of the recombinant yeast Cu-MT protein by His Select® HF Nickel Affinity Gel Column. Elution of the protein from the affinity column was accomplishhed by elution buffer (50 mM NaP buffer pH 7.0, 0.1 M NaCl, 250 mM imidazole) at 4ºC. Elution profile of the recombinant protein at 280 nm is shown in Figure 3.8. Protein elution has started to come out of column at fraction number 10 and continued until fraction number 38. Selected fractions from peak were analyzed on 15% SDS-PAGE and no expected bands observed (Figure 3.9.). Expected bands were supposed to be around molecular weight of 6 KDa. Unfortunately, with no reasonable explanation no any band has been seen in 15% SDS-PAGE analysis of selected fractions. The yeast Cu-MT protein may be so susceptible to air oxidation because of many Cys residues in the sequence. The studies may be repeated under Ar gas in the future.

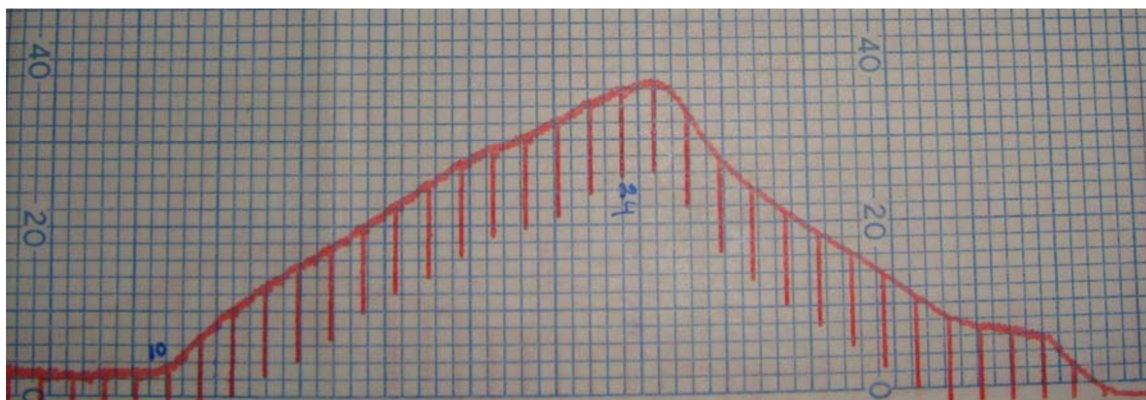

Figure 3.8. Elution profile of the His Select® HF Nickel Affinity Gel Column

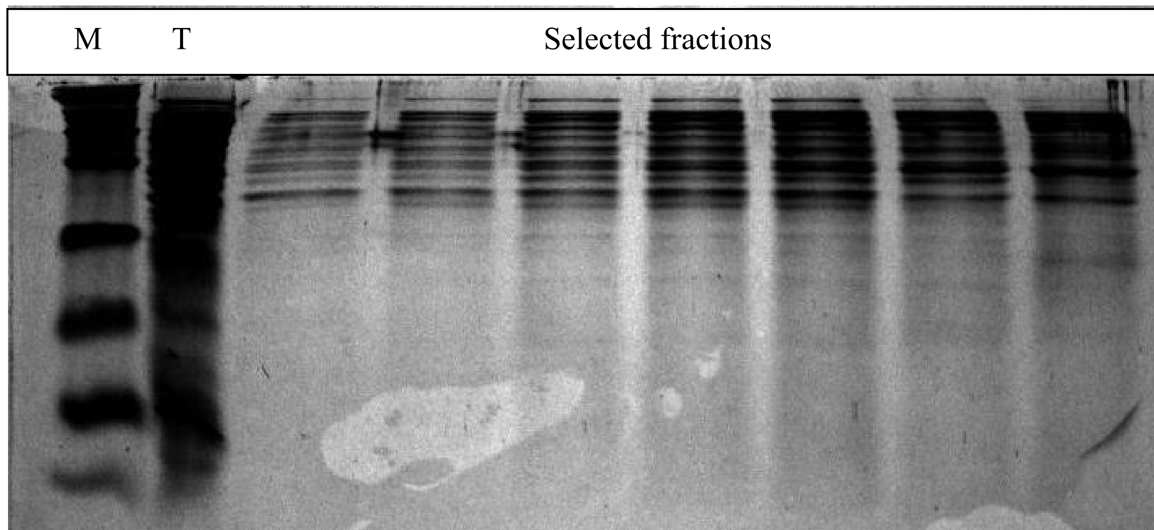

Figure 3.9. 15% SDS-PAGE analysis of selected fractions. M: molecular mass markers top the bottom 200, 116, 68, 43, 29, 14.4, and 6.5 kDa T: material loaded on the column.

CHAPTER 4

DISCUSSION

We have described a PCR-based gene synthesis method for the fast and accurate construction of the 186 base pair yeast Cu-MT gene. The two-step PCR method was used here to produce a synthetic gene for yeast Cu-MT protein. This method has been applied in the construction of variety of genes, gene libraries and plasmids. The method described here is the fastest and economical approach according to the other synthetic gene construction methods. Construction of the gene can be completed within only one day in terms of time. The flexibility of the method, in which complementary oligos were mixed together to generate a synthetic product in a two step reaction is impressive. It has been reported that there is no practical limit on the number of the oligos which may be mixed together so that genes in much bigger size could be synthesized successfully (Mehrnejad et al. 2008). However, the oligos with longer size used in this method have to be purified before the experiment to eliminate inborn mutation during the construction.

The most time-consuming step of the synthesis is the removal of inborn mutations by subsequent subcloning steps. The major parameter for overall success of the method is determined by the fidelity of the polymerase used in the PCR reaction. According to this we set out the PCR conditions in order to allow the exclusive use of *Pfu* DNA polymerase which exhibits the highest fidelity of any thermostable DNA polymerase. Under our optimized conditions, construction of the full-length gene has been made successfully.

Another goal in the development of synthetic gene for yeast Cu-MT protein was to allow high-level expression in an *E. coli* host. Heterologous expression of the full length and truncated form of the yeast Cu-MT in *E. coli* has been reported earlier (Berka et al. 1988; Sayers et al. 1993). In our study, production of recombinant yeast Cu-MT protein was carried out in *E. coli* BL21 (DE3) under control of the T7 promoter. For the stability of the recombinant protein $CuSO_4$ was added in to expression medium.

SDS PAGE results of the IPTG induced *E. coli* BL21 (DE3) host indicates low expression level of the synthetic gene for yeast Cu-MT protein constructed from original sequence. In order to solve this problem a synthetic gene for yeast Cu-MT protein with preferred codon usage of *E. coli* was designed for efficient expression of recombinant yeast Cu-MT in *E. coli*.

Air sensitivity and the unusual nucleotide sequence of the yeast Cu-MT protein require indirect detection and quantification. With the blocking the sulfhydryl groups by carboxymethylation, yeast Cu-MT could be detected on non-denaturing PAGE and visualized by Coomassie blue.

Previous reports have indicated that Cu-MT can be purified by conventional gel-filtration chromatography (Winge et al., 1985; Berka et al., 1988). The final steps of these procedures include either gel-permeation HPLC (Wright et al., 1987) or reverse-phase HPLC prior to amino acid analysis (Berka et al., 1988). Regardless of the source of the protein and the method of purification, detection and determination of the homogeneity of the Cu containing MT has been proven to be difficult (Vasak, 1991). In our study we tried to purify recombinant protein using as an advantage of histidine tags that pET28a(+) expression system has. The system provides an effective one-step purification of the recombinant protein by His Select® HF Nickel Affinity Gel Column. As it was mentioned above the recombinant protein is sensitive to the air and can be easily oxidized. This may be the reason that no protein bands were observed after purification trials. As reported previous studies this problem may be solved if purification studies carried out under argone (Ar) gas (Sayers et al. 1993).

CHAPTER 5

CONCLUSION

We designed special oligomers for construction of synthetic gene for yeast Cu-MT protein from the *CUP1* locus of *Sacchaomyces cerevisiae*. The described procedure might be a cost-effective method for the production of the desired peptides and proteins. *pfu* DNA polymerase which exhibits proofreading exonuclease activity was used at two-step PCR method. As a result, construction of the synthetic yeast cooper metallothionein gene both from original nucleotide sequence and optimized nucleotide sequence for *E. coli* codon bias have been accomplished successfully.

According to expression studies, high level expression of the constructed yeast copper metallothionein gene was obtained with codon optimized gene according to the usage of *E. coli* compared to original sequence of *CUP1* gene in yeast *Saccharomyces cerevisiae.*

Purification of the expressed protein was the main problem in our study. Unusual characteristics of the yeast metallothionein protein may give rise to degradation of the expressed protein during purification step. This may be solved by using different purification methods in future.

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APPENDIX A

COMPOSITION of BUFFERS and STOCK SOLUTIONS

1. 50x TAE Electrophoresis Buffer, per 1 L
   - 242 g Tris base
   - 37.2 g Na_2EDTA (H_20)
   - 57.1 ml Glacial acetic acid
   - dH_2O to 1 L

2. Ethidium Bromide (10 mg/ml)
   - 0.2 g Ethidium bromide in 20 ml dH_2O

3. 10mM dNTP
   - 10mM each dATP, dTTP, dCTP, dGTP

4. LB Broth, per liter
   - 10 g Tryptone
   - 5 g Yeast Extract
   - 5 g NaCl
   - dH_2O up to 1 L

5. SOC Medium, per 100 ml
   - 2 g Tryptone
   - 0.5 g Yeast Extract
   - 1 ml 1M NaCL
   - 0.25 ml 1M KCL
   - 1 ml 2M Mg^{2+} Stock
   - 1 ml 2m Glucose
   - dH_2O up to 100 ml

6. Ampicillin (100 mg/ml)

- 0.1 g amphicilin in 1 ml dH_2O

7. Kanamycin (30 mg/ml)

- 0.3 g kanamycin in 1 ml dH_2O

8. X-GAL Stock Solution (30mg/ml)

- 3% x-gal in di-methyl formimide

9. IPTG Stock Solution (1M)

- Dissolve 2.83 g IPTG in 8 mL dH_2O. Bring to 10 mL with dH_2O.

APPENDIX B

REAGENTS and GEL PREPARATION FOR SDS-PAGE and NON-DENATURING PAGE

SDS-PAGE

Stock Solutions

A. 30% Acrylamide Mixture

- 29.2g acrylamide
- 0.8g N'N'-bis-methylene-acrylamide

Make up to 100 ml with distilled water. Filter and store at 4ºC in the dark for at least one month.

B. 1.5M Tris-HCl, pH 8.8

- 18.15g Tris Base
- ~80ml distilled water

Dissolve Tris base in distilled water, adjust to pH 8.8 with HCl. Make up to 100ml with distillled water and store at 4ºC.

C. 0.5M Tris-HCl, pH 6.8

- 6g Tris Base
- ~80ml distilled water

Dissolve Tris base in distilled water, adjust top H 6.8 with HCl. Make up to 100ml with distilled water and store at 4ºC.

D. 10% SDS

Dissolve 10g SDS in 90ml water with gentle stirring and bring to 100 ml with distilled water.

E. Sample Buffer

- 3.8 ml deionized water
- 1.0ml 0.5M Tris-HCl, pH 6.8
- 0.8ml Glycerol
- 1.6ml 10% (w/v) SDS
- 0.4ml 2-mercaptoethanol
- 0.4ml 1% (w/v) bromophenol blue

F. 5X Running Buffer

- 15g Tris Base
- 72g Glycine
- 5g SDS

Dissolve Tris base, glycine and SDS in ~800ml deionized water and make uo tp 1L with water. Store at 4ºC. For electrophoretic run, dilute 5X stock solution to 1X with deionized water.

G. 10% Ammonium persulfate (APS)

Dissolve 0.1g APS in 1ml deionized water. This solution should be prepared fresh daily.

H. Colloidal Coomassie Staining Solution

Dissolve 40g ammonium sulfate in ~300ml water, add 8ml 85% o-phosphoric acid and add 0.5g Coomassie Brilliant Blue G-250. Make up to 400ml with water, add 100ml methanol to 500ml total volume. Store at 4ºC.

I. Neutralization Buffer

0.1M, pH 6.5 Tris-phosphate in deionized water.

J. Destaining Solution

25% (v/v) methanol solution.

K. Fixation Solution

20% (w/v) Ammonium sulfate in deionized water.

Gel Preparation

- Separating Gel

Table B.1. Preparation of 15% SDS-PAGE separating gel (for 5ml)

Deionized water	1.18ml
1.5M Tris-HCl, pH 8.8	1.25ml
10% SDS	50μl
Acrylamide/Bis (30% Stock)	2.5ml
10% APS	25μl
TEMED	2.5μl

- Stacking Gel

Table C.2. Preparation of 4% SDS-PAGE stacking gel (for 5ml)

Deionized water	3.05ml
0.5M Tris-HCl, pH 6.8	1.25ml
10% SDS	50μl
Acrylamide/Bis (30% Stock)	665μl
10% APS	25μl
TEMED	5μl

NON-DENATURING PAGE

Stock Solutions

A. 30% Acrylamide Mixture

- 29.2g acrylamide
- 0.8g N'N'-bis-methylene-acrylamide

Make up to 100 ml with distilled water. Filter and store at 4ºC in the dark for at least one month.

B. 1.5M Tris-HCl, pH 8.8

- 18.15g Tris Base
- ~80ml distilled water

Dissolve Tris base in distilled water, adjust to pH 8.8 with HCl. Make up to 100ml with distillled water and store at 4ºC.

C. 0.5M Tris-HCl, pH 6.8

- 6g Tris Base
- ~80ml distilled water

Dissolve Tris base in distilled water, adjust top H 6.8 with HCl. Make up to 100ml with distilled water and store at 4ºC.

D. Sample Buffer

- 3.8 ml deionized water
- 1.0ml 0.5M Tris-HCl, pH 6.8
- 0.8ml Glycerol
- 0.4ml 1% (w/v) bromophenol blue

E. 5X Running Buffer

- 15g Tris Base
- 72g Glycine

Dissolve Tris base, glycine and SDS in ~800ml deionized water and make uo tp 1L with water. Store at 4ºC. For electrophoretic run, dilute 5X stock solution to 1X with deionized water.

F. 10% Ammonium persulfate (APS)

Dissolve 0.1g APS in 1ml deionized water. This solution should be prepared fresh daily.

G. Colloidal Coomassie Staining Solution

Dissolve 40g ammonium sulfate in ~300ml water, add 8ml 85% o-phosphoric acid and add 0.5g Coomassie Brilliant Blue G-250. Make up to 400ml with water, add 100ml methanol to 500ml total volume. Store at 4ºC.

H. Neutralization Buffer

0.1M, pH 6.5 Tris-phosphate in deionized water.

I. Destaining Solution

25% (v/v) methanol solution.

J. Fixation Solution

20% (w/v) Ammonium sulfate in deionized water.

Gel Preparation

- Separating Gel

Table C.3. Preparation of 12% NONDENATURING-PAGE separating gel (for 5ml)

Deionized water	1.725ml
1.5M Tris-HCl, pH 8.8	1.25ml
Acrylamide/Bis (30% Stock)	2ml
10% APS	25µl
TEMED	2.5µl

- Stacking Gel

Table C.4. Preparation of 4% NONDENATURING-PAGE stacking gel (for 5ml)

Deionized water	3.55ml
0.5M Tris-HCl, pH 6.8	1.25ml
Acrylamide/Bis (30% Stock)	665µl
10% APS	25µl
TEMED	5µl

APPENDIX C

SEQUENCE RESULTS

Sequence primers

T7 primer AATACGACTCACTATAG were used for sequencing yeast Cu-MT inserted pBS II K +
5′ TGTTAGCAGCCGGATCTCAG 3′ , 5′ GGAGATATACCATGG GCAGC 3′ were used for sequencing optimized and non optimized Cu-MT inserted pET28a(+)

Sequence results for yeast Cu-MT inserted pBS II K +

Yeast Cu-MT (1) - pBS K +

GTAATGCCGGTGCGGCCGCTCTAGACTAGTGGATCCCCCAAGCTTCATTTCC
CAGAGCAGCATGACTTCTTGGTTTCTTCAGACTTGTTACCGCAGGGGCATTT
GTCGTCGCTGTTACACCCCGTTGGGCAGCTACATGATTTTTGGCATTGTTCA
TTATTTTTGCAGCTACCACATTGGCATTGGCACTCATGACCTTCATTTTGGAA
GTTAATTAATTCGCTGAACATATGGGCTGCAGGAATTCGATATCAAGCTTAT
CGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTA
GTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTG
TGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGGAGCATA
AAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCG
TTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATT
AATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTT
CCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGC
GGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA
TAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAAC
CGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACG
AGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGA
CTATAAAGATACCAGGCGTTT

Yeast Cu-MT (3) - pBS K +

TAATAAATTTTATCCTATGCGGGCGGCGCTCTAGACTAGTGGATCCCCCAAG
CTTCATTTCCCAGAGCAGCATGACTTCTTGGTTTCTTCAGACTTGTTACCGCA
GGGGCATTTGTCGTCGCTGTTACACCCCGTTGGGCAGCTACATGATTTTTGG
CATTGTTCATTATTTTTGCAGCTACCACATTGGCATTGGCACTCATGACCTTC
ATTTTGGAAGTTAATTAATTCGCTGAACATGGGCTGCAGGAATTCGATATCA
AGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTT
CCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTT
TCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGG
AGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTA
ATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGC
TGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGC
GCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCG
GCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATC
AGGGGATAACGCAGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAG
GAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCT
GACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGAC
AGGACTATAAAGAATACCAGGGCGTTTCCCCCTGGAAG

Yeast Cu-MT Pet28 a (+) F (5)

GGCATAGGAAAGAAATAAAGTGTGCTGGTCGGCGCAGCTTGATATCGATTC
CTGCAGCCCATGTTCAGCGAATTAATTAACTTCCAAAATGAAGGTCATGAGT
GCCAATGCCAATGTGGTAGCTGCAAAAATAATGAACAATGCCAAAAATCAT
GTAGCTGCCCAACGGGGTGTAACAGCGACGACAAATGCCCCTGCGGTAACA
AGTCTGAAGAAACCAAGAAGTCATGCTGCTCTGGGAAATGAAGCTTGTCGA
CGGAGCTCGAATTCGGATCCGCGACCCATTTGCTGTCCACCAGTCATGCTAG
CCATATGGCTGCCGCGCGGCACCAGGCCGCTGCTGTGATGATGATGATGAT
GGCTGCTGCCCATGGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTA
GAGGGGAATTGTTATCCGCTCACAATTCCCCTATAGTGAGTCGTATTAATTT
CGCGGGATCGAGATCTCGATCCTCTACGCCGGACGCATCGTGGCCGGCATC
ACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGAT
GGGGAAGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTTCGGCGTG
GGTATGGTGGCAGGCCCCGTGGCCGGGGGACTGTTGGGCGCCATCTCCTTG
CATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTCAACCTACTACTGG
GCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGAGATCCCGGAC
ACCATCGAATGGCGCAAAACCTTTCGCCGGTATGGCATGATAGCGCCCCGG
AAGAGAGTCAATTTCAGGGTGGGTGAATGGTGGAAACCCAGGTAACCGTTA
TATACGG

Yeast Cu-MT Pet28 a (+) R (5)

GACTAGGGTTCGATAAGAAATCATCCAGCAGCGGCCTGGTGCCGCGCGGCA
GCCATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGCGGATCCGAAT
TCGAGCTCCGTCGACAAGCTTCATTTCCCAGAGCAGCATGACTTCTTGGTTT
CTTCAGACTTGTTACCGCAGGGGCATTTGTCGTCGCTGTTACACCCCGTTGG
GCAGCTACATGATTTTTGGCATTGTTCATTATTTTTGCAGCTACCACATTGGC
ATTGGCACTCATGACCTTCATTTTGGAAGTTAATTAATTCGCTGAACATGGG
CTGCAGGAATTCGATATCAAGCTTGCGGCCGCACTCGAGCACCACCACCAC
CACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCT
GCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGG
GTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATAAGGGAAT
GGGACGCCCCCCTGTAGCGGCGCAATTAAGCGCGGCCGGGTGGTGGTGGGT
TACACTCAGCGTGGACCGGGTACACTTTGCCAGCGCCCCTAACGCCCGCTTC
CTTTTCGCTTTTTTTCCCCTTTCCTTTTTCTTCGCCCACCGTTTCCGCCCGGGT
TTTTCCCCCCGTCCAAGCTTCTTAAAATTCGGGGGGGGCTCCCCTTTTTAGG
GGGTTCCCCGCAATTTTAATGGGCGTTTTACCGGGAACCCTGCGGAACCCCC
CAAAAAAAAACTTTGAATATAAGGGGGGGGAAGGGGGTGTCCACCACAAA
GAGAGGAGGACCCATCCGCCCCCCCTCAAAAAAAAAAAGAGTGTGTTTTTT
CTCCCCCCCCTCTTTTAGAAGAGATTGTGAGAAGATACACACACCAATTTCT
TCTTTTATATAAAAGAGAGGAGAAGATCATTTTGTTGTGGTTCATCTAATCA
GTAGCGAGGGAGCATACGCAGCACGACATCACAAACCACGCAGCAGTCTAC
TATCACGGCTGCTGCGTGTACT

Yeast Cu-MT Pet28 a (+) F (7)

AAAAGGGAAATGAAAGTGTGTCTGAGTGCGGCGCAGCTTGATATCGATTCC
TGCAGCCCATGTTCAGCGAATTAATTAACTTCCAAAATGAAGGTCATGAGT
GCCAATGCCAATGTGGTAGCTGCAAAAATAATGAACAATGCCAAAAATCAT
GTAGCTGCCCAACGGGGTGTAACAGCGACGACAAATGCCCCTGCGGTAACA
AGTCTGAAGAAACCAAGAAGTCATGCTGCTCTGGGAAATGAAGCTTGTCGA
CGGAGCTCGAATTCGGATCCGCGACCCATTTGCTGTCCACCAGTCATGCTAT
CCATATGAATGCTGCGCGGCACCAAGCCACGGCCCCGATGATGATTATAAA
AAAAGATGAATTGCTCCCCTCCTTCCCCATTTTAATCCCTTTATAAATAAAA
AAAAAATTTTATCTGGGCGGAAAAAGTTTATTTTCCTTCACCCCCCCATTCT
CATGATTGGGAAGGTAGTAATTTATGGGGGGGGGGGGGGAAAAATAAAT
CTTTCCCCCCCCCCCCCCCACAGGGGTGTGGGGGGGTTGGGGGGGGGGGCC
CCGATCTCCCCCCGGAGGGGGGAGCGGGGTGGGGGGGTTTATTCGTTGTC
TCCCCCCCCCTTTCCCCCCACACCAATAGTAGGGGAGAGGGGGTTCAGGGC
CCCCCGCACTCCTGGGCTCGCTCCCGAGTCACCCTTGCGGGGGACACTGTGA
AGAACCAGGCTGGGGGGGGCGGAGCAGAGGCGTCGCGTAGGGTGATTGAG
TACGAGCACTGATACCCCGAGTGGCCACCACCGTGTGCAGGGTCTGCGACT
TCCAAAAATCATTTTTCTCCTTTTTCATTACTAGATTAATGCTTTCAATTTAG
GTTGCTCCCCTTGTGCTGTTTGCAGCGATGTAGAAGGATGAGCAAGAAGAT
GCGTTAGGCCAGCATCTCCGGCGCCCGCACCTGTATGATACAGGCGGGGGG
CCTTACATGAATATCAGCTTACCGTTTTTTTTCTCTTGCTCGAATGGAAGGG
AATTTGCTGTGTCAGCCTTGTTCA

Yeast Cu-MT Pet28 a (+) R (7)

CCTAGGGGAAGTGACAGTTCAATCCAGCAGCGGCTGGTGCCGCGCGGCAGC
CATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGCGGATCCGAATTC
GAGCTCCGTCGACAAGCTTCATTTCCCAGAGCAGCATGACTTCTTGGTTTCT
TCAGACTTGTTACCGCAGGGGCATTTGTCGTCGCTGTTACACCCCGTTGGGC
AGCTACATGATTTTTGGCATTGTTCATTATTTTTGCAGCTACCACATTGGCAT
TGGCACTCATGACCTTCATTTTGGAAGTTAATTAATTCGCTGAACATGGGCT
GCAGGAATTCGATATCAAGCTTGCGGCCGCACTCGAGCACCACCACCACCA
CCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGC
TGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGT
CTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATTGGCGAATGG
GACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGC
AGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCT
TCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGG
GGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAA
AACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGG
TTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTAATAGTGGACTCTTGTTCC
AAACTGGAACAACACTCAACCCTATCTCGGTCTAATTCTTTTTGAA

Yeast Cu-MT Pet28 a (+) F (op)

AACTTTTTTTGTTAATTTTTGGTCTGATCGGCCGCAGCTTTCATTTGCCGGAG
CAGCAGCTTTTCTTGGTTTCTTCTGATTTGTTACCACATGGACATTTGTCATC
AGAGTTACAACCGGTTGGACAACTACAGCTTTTTTGGCACTGTTCGTTGTTT
TTGCAGGAGCCGCATTGGCATTGACATTCATGTCCCTCGTTCTGAAAGTTAA
TCAGTTCGCTAAACATCATATGGCTGCCGCGCGGCACCAGGCCGCTGCTGTG
ATGATGATGATGATGGCTGCTGCCCATGGTATATCTCCTTCTTAAAGTTAAA
CAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAATTCCCCTATAGTG
AGTCGTATTAATTTCGCGGGATCGAGATCTCGATCCTCTACGCCGGACGCAT
CGTGGCCGGCATCACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGC
CGACATCACCGATGGGGAAGATCGGGCTCGCCACTTCGGGCTCATGAGCGC
TTGTTTCGGCGTGGGTATGGTGGCAGGCCCCGTGGCCGGGGGACTGTTGGG
CGCCATCTCCTTGCATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTC
AACCTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGT
CGAGATCCCGGACACCATCGAATGGCGCAAAACCTTTCGCGGTATGGCATG
ATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATGTGAAACCAGTAAC
GTTATACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGC
GTGGTGAACCAGGCCAGCCACGTTTCTTGGCGAAAACCGCGGGAAAAA
GGTGGAAAGGCGG

Yeast Cu-MT Pet28 a (+) R (op.)

TGCCACAAATTTTAAAACCCTCCTATCCGCAGCGGCCTGGTGCCGCGCGGCA
GCCATATGATGTTTAGCGAACTGATTAACTTTCAGAATGAGGGACATGAAT
GTCAATGCCAATGCGGCTCCTGCAAAAACAACGAACAGTGCCAAAAAAGCT
GTAGTTGTCCAACCGGTTGTAACTCTGATGACAAATGTCCATGTGGTAACAA
ATCAGAAGAAACCAAGAAAAGCTGCTGCTCCGGCAAATGAAAGCTTGCGGC
CGCACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGC
CCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATA
ACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGG
AACTATATCCGGATTGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGC
GCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCC
CTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGG
CTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGT
GCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTA
GTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCAC
GTTCTTTAATAGTGGACTCTTGTTCCAAACTGGGAACAACACTCAACCCTAT
CTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGG
TTAAAAATGAGCTGATTTAAACAAAAATTTACGCGGAATTTTAACAAAATA
TTAAC