CHAPTER 6

IN VITRO EVOLUTION OF UBIQUITIN

6.1 INTRODUCTION

Evolution is a very slow process happening over millions of years. Evolutionary changes in the protein sequences result from slow accumulation of mutations which are retained in the gene. These mutations can be positive or negative depending upon their contribution to evolution. Positive mutations are beneficial with their ability to explore and manage the more complex biological functions and regulatory processes taking the organism a step ahead in evolution. On other hand the negative mutations might be either inefficient or lethal. These changes are thus lost in the evolutionary progression. However, certain proteins do not tolerate changes in their sequence and remain conserved through out in the course of evolution. The conserved amino acid residues in protein sequences either contribute to the structural integrity of the proteins or affect the function of the proteins. Ubiquitin is an excellent example for sequence conservation of protein.

6.1.1. Ubiquitin: The Conserved protein

Ubiquitin represents one of the most conserved proteins in eukaryotes. Yeast, plant and animal ubiquitins differ only by substitutions in three positions in protein sequence (Gavialnes et al., 1975, Watson et al., 1978, Schlesinger et al., 1975 and Schlesinger and goldsteiner., 1975, Wilkinson et al., 1986, Vierstra et al., 1986). Strong sequence conservation suggests that the vast majority of amino acids that make up ubiquitin are essential as apparently any mutations that have occurred over evolutionary history have been removed by natural selection. Ubiquitin plays key role in protein degradation (Ciechanover et al., 1980 and Hershko et al.,1980) and various other cellular phenomena (Wilkinson et al., 2000, Pickart et al., 2001 and Shih et al.,200) explaining why mutations are not permitted in Nature. The remarkable conservation of ubiquitin along with its smaller size, points to constraints imposed on its sequence by folding, stability and functional interaction with other proteins. A natural variant of ubiquitin from *Autografa californica* displays only 75% identity with ubiquitin protein sequence. This isoform of ubiquitin attenuated the formation of polyubiquitin chain (Hass et al., 1996). This observation suggests the potential of ubiquitin variants to interfere with essential cellular phenomena leading to cell death. In *Saccharomyces cerevisiae* ubiquitin is expressed from four distinct loci (Varshavsky et al., 1987) three of which UBI1, UBI2 and UBI3 code for ubiquitin which is released into the

cytosolic pool. This ubiquitin is required for the normal cellular growth. The fourth ubiquitin gene, UBI4, contains five consecutive ubiquitin-coding repeats in a head-to-tail arrangement encoding a polyubiquitin precursor protein which is processed to mature ubiquitin (Ozkaynak et al., 1984 and 1987). Polyubiquitin gene is an essential component of the stress response system and its function under stress helps the cell to cope up in difficult conditions to maintain life. Null phenotype of ubiquitin is lethal but *S.cerevisiae* can survive if the polyubiquitin gene is deleted from the system. The UbI4 mutant of *S.cerevisiae* is hypersensitive to stress conditions such as high temperature, starvation, UV repair and exposure to amino acid analogs (Finley et al., 1987).

In Chapters 2, 3 and 4 the approach of site directed mutagenesis was used to investigate structure-function relationships in ubiquitin. Site directed mutagenesis may be effective in revealing the importance of a given residue. However, it would not be helpful either in highlighting which are the most crucial residues or arriving at desirable phenotypes. Random mutagenesis turns out to be an alternative tool to address the above questions.

6.1.2. Random Mutagenesis

Random mutagenesis is a powerful technique for developing proteins and enzymes with novel properties which have altered stability and specificity for their targets. This technique often yields unique mutations that could not have been predicted. In addition, further improvements can be expected by repeating the mutagenesis and selection (screening) processes in a manner resembling evolution. Hence, the approach is called **in vitro evolution.** The mutation frequency and mutation incorporation of in vitro evolution may not be identical to evolutionary changes observed in Nature, but the consequences are an accelerated reproduction of natural evolutionary process.

There are various methods in use for random mutagenesis. Random mutagenesis through a bacterial mutator strain is a very useful method. The most popular mutator strain is *E.coli* XL1- red, which lacks three genes of primary DNA repair pathway, namely MutS, MutD and MutT, resulting in a random mutation rate ~5000 fold higher than wild type. The protocol for using the mutator strain is much simpler than error prone PCR and a ligation step is unnecessary. However, the mutation frequency is low under standard conditions (0.5

mutations per kilo base) and a culturing period longer than 24 hours is often required for introducing multiple mutations.

Mutating agents like UV and chemicals causing deamination, alkylation and incorporation of base analog mutagens are effective tools to generate mutants. The major limitation with the technique is its randomness. It can introduce mutations in those parts of the DNA or gene where mutation is not required. Selectively targeting a gene for generating mutations is a limitation.

Random mutagenesis of gene can also be carried out by using error prone polynucleotide chain reaction (PCR) (Arnheim, 1992) based technique. DNA shuffling is another very efficient technique but it cannot be used for ubiquitin because of its smaller size.

The most commonly used random mutagenesis method is **error prone PCR,** which introduces random mutation during PCR by reducing the fidelity of DNA polymerase. The fidelity of DNA polymerase can be reduced by adding Mn ions or introducing Mg ions and biasing the dNTPs concentration. Use of compromised DNA polymerase causes incorporation of incorrect nucleotides during PCR reaction yielding randomly mutated products. To convert the product to a suitable form for transformation of a host strain, at least three steps are required. They are - 1) digestion of the product with restriction enzymes, 2) separation and isolation of the resultant fragment by electrophoresis and 3) ligation into suitable vector. In addition amplification of circular DNA can be used for direct transformation of *E.coli* and/ or yeast, yielding recircularized template DNA. This process has a limitation of randomly targeting the other parts of the circular DNA which may be important in replication and maintenance of the plasmid. Therefore the amplified ligated product can be used directly to transform a host strain and can be screened for complementation of phenotype.

6.2 MATERIALS AND METHOD

6.2.1. Yeast strains, media and plasmids

Strains SUB62 (MATα lys2-801 leu2-3,112 ura3-52 his3-A200 trpl-1) and SUB60 (MATα ubi4-A2:: LEU2 lys2-801 leu2-3,112 ura3-52 his3-A200 trpl-1)

Cultures were grown at 30°C at 200 rpm, except where indicated in synthetic dextrose medium consisted of 0.67% Hi-media yeast nitrogen base supplemented with histidine, leucine, tryptophan, lysine and uracil as and when required with 2% glucose as carbon source.

Ubiquitin is expressed from a high copy number yeast episomal plasmid that is identical to the TRPI copper inducible ubiquitin expression plasmid YEp 96. YEp 96 is a 2 μm based shuttle vector between *E.coli* and *S.cerevisiae*. The ubiquitin gene is expressed from CUP1 promoter induced by the addition of 100 μM $CuSO_4$.

6.2.2. Plasmid construction (yeast expression vector)

All ubiquitin gene mutations were carried out in plasmids derived from YEp 96 (Figure 6.1). Amplicons of ubiquitin gene generated by error prone PCR were cloned into the *Bgl* II and *Kpn* I sites of YEp96.

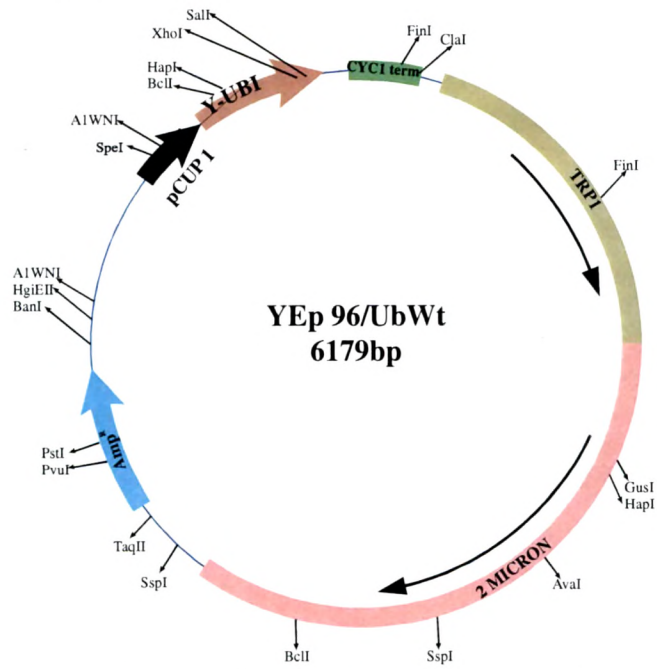


Figure 6.1. YEp96-UbWt vector map

The DNA sequence of the *Eco* R I - *Kpn* I synthetic yeast ubiquitin gene insert in YEp 96/UbWt

GAATTC ATTATGC AGATCT.....Yeast-UB....GGT GGT TGA GGTACC
*Eco R*I Met *Bgl* II Gly Gly *Kpn* I

Plasmid pUB221 is identical to YEp96-UbWt with respect to its 2 μm based copper inducible CUP1 promoter shuttle vector expressing c-myc tagged ubiquitin with a selection marker of both URA3 and TRP1. pUB23 is 2 μm based galactose inducible shuttle vector expressing ubiquitin β-galactosidase fusion protein (Ub-X-β gal) with a selection marker of URA3 gene. The X position represents different amino acid residues, which have already been classified as primary, secondary or tertiary destabilizing categorization according to N-end rule.

6.2.3. Error prone PCR

Error prone PCR was performed using the Taq DNA polymerase with 5'-3' polymerase and 5'-3' exonuclease activity and no proof reading activity. Standard PCR reaction system contains 10mM Tris–HCl buffer (pH 9 at 25°C), 1.5mM $MgCl_2$, 50mM KCl, 0.01% gelatin, 250μM dNTPs each. The error conditions were set up by adding the following chemicals in separate reactions: 10mM $MgCl_2$, 0.5mM $MnCl_2$, 1mM dATP, 1mM dGTP and 1mM dATP + 1mM dGTP, to reduce the fidelity of Taq polymerase and increase the rate of incorporation of mutations during PCR reaction. The forward primer 5'ATGCAGATCTTCGTCAAGACGTTAACCGG3' and reverse primer 5'TCCGGTACCC GCTCAACCACCTCTTAG'3 were used to generate 240bp amplicons in different error prone conditions and cloned in TRPI copper inducible Ub expression plasmid YEp 96 at *Bgl* II and *Kpn* I sites.

6.2.4. Mutant screening

The YEp 96 plasmid was constructed expressing the ubiquitin under CUP1 promoter. Stress-hypersensitive SUB60 strain of *S.cerevisiae* lacking UBI4 gene, was transformed with above chimeric constructs and tested for complementation of heat sensitive phenotype.

6.2.5. Heat sensitive phenotype

Yeast transformants were grown to exponential log phase and streaked on SD selection media with and without induction by copper. They were incubated at 40°C for 16 hours and shifted back to 30°C.

6.2.6. Plan of work

Ubiquitin gene has been subjected to error prone PCR to generate various random mutants of the gene which were cloned back into the shuttle vector and introduced into the host strain of *S.cerevisiae* SUB60, which is a polyubiquitin (UBI4) gene mutant. The mutants were screened for failure of complementation of stress hypersensitive phenotype (Figure 6.2).

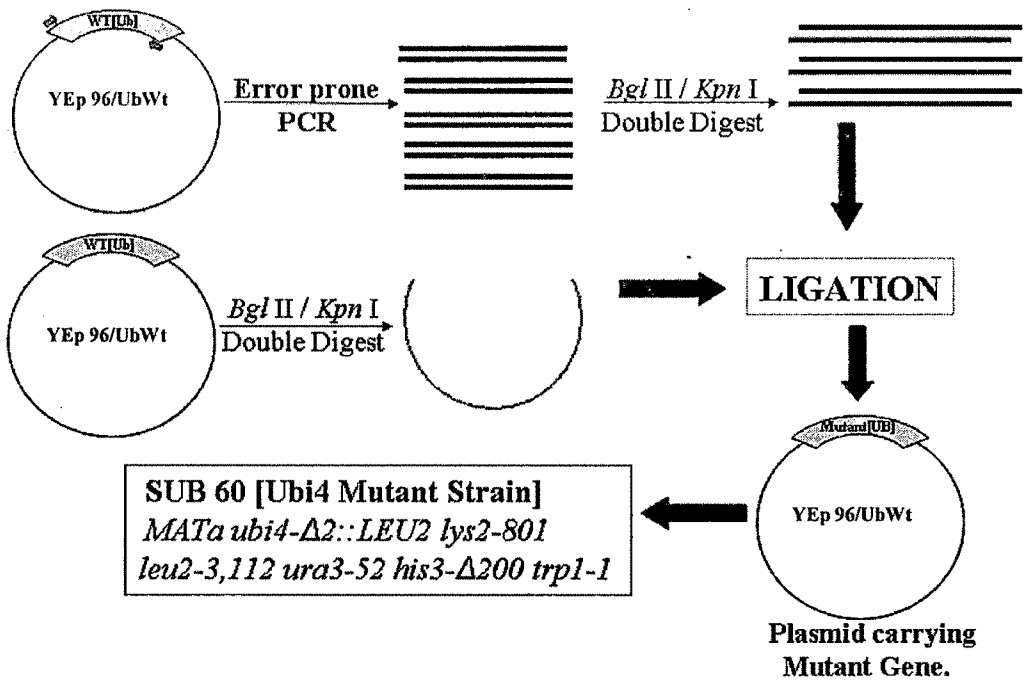


Figure 6.2. Strategies for generation of error prone mutants and their screening.

6.2.7. Functional analysis using antibiotic sensitivity test, efficiency of degradation based on N-end residue and Western blot analysis for polynucleotide chain formation

Antibiotic sensitivity test, efficiency of degradation based on N-end residue and Western blot analysis for polynucleotide chain formation were performed according to the method described in Material and Methods section of Chapter 5.

6.2.8. Sequence Analysis

The plasmid with mutant gene was sequenced using Sanger's dideoxy sequencing method for the detection of incorporation the mutation in the DNA. MWG-Biotech sequenced the DNA samples (YEp plasmid backbone) in a single forward reaction using primer sequence 5'GCG CCGACATCATAACGGTT CTGGC'3.

6.2.9. Bacterial strains and media

DH5α (F', 80 d lacZ M15, *endA*1, *recA*1, *hsdR*17 (r_k^-, m_k^+), *supE44, thi-1, gyrA96, relA1,(lacZYA-argF)U169,*) strain of *E.coli* culture were grown at 37°C at 200 rpm in nutrient rich Luria broth medium procured from Hi-media. Selection pressure of 100μg/ml of ampicilin was used for the strain. Bacterial transformation was done by $CaCl_2$ method. Plasmid DNA preparation was done by alkaline lysis method (Sambrook and Russel et al., 2001).

6.2.10. Construction of Bacterial expression vector

The *UbEP42* mutant gene was cloned as PCR amplicon in pKK223-3 in place of *UbF45W* (Pharmacia Biotech) (Searle et al., 2003). The mutated cassette was inserted between the *Eco* RI and *Hind* III restriction sites of pKK223-3, and the clones were screened by the loss of enzyme site *Xho* I and *Sau*96 I. Mutation was further confirmed by DNA sequencing.

6.2.11. Gene expression and purification

The expression of UbEP42 protein and its purification were carried out following the protocol described in Material and Methods section (2.2.2. and 2.2.3.) of Chapter 2.

6.3 RESULTS

6.3.1. Plasmids construct YEp96-UbWt (yeast expression vector)

All ubiquitin gene mutations were carried in plasmid YEp96-UbWt, which expresses a synthetic yeast ubiquitin gene under the CUP1 promoter (Figure 6.3). Mutated ubiquitin genes were cloned back into *Bgl* II and *Kpn* I sites of YEp96-UbWt.

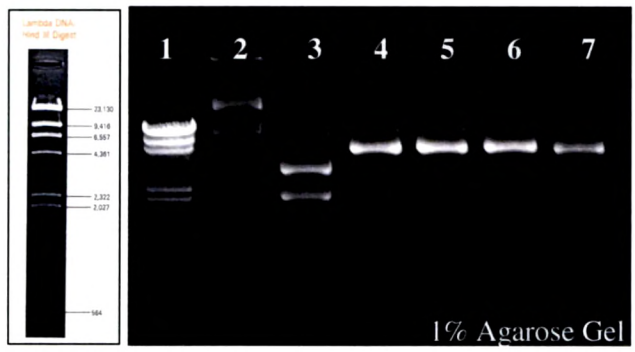


Figure 6.3. YEp 96-UbWt digestion pattern. Lane 1. Contains λ *Hin*d III digest marker. Lane 2. Contains YEp96-UbWt undigested plasmid. Lane 3. *Hind* III digest of the vector with three *Hind* III sites showing three fragments of 646bp, 2099bp and 3434bp. Lanes 4., Lane 5. and Lane 6. Show *Bam* HI digest, *Bgl* II digest and *Kpn* I digest respectively. *Kpn* I and *Bgl* II sites were used for cloning error prone amplicons. All these restriction sites are unique and give a single band of 6179bp fragment, which is the actual size of the vector. In lane 7, *Bam* HI, *Kpn* I double digest shows release of 678bp ubiquitin ORF along with CUP1 promoter and 5501bp backbone.

6.3.2. Amplicons generated under various error prone conditions

PCR products obtained under error prone conditions were of the same size (~ 240 bp) as the amplicons obtained under standard conditions (Figure 6.4). The PCR products were digested with *Kpn* I and *Bgl* II sites and were cloned into the YEp96-UbWt plasmid replacing the wild type ubiquitin gene. Plasmid DNA was extracted from all the transformants and retransformed into SUB60 strain of *S.cerevisiae*. These yeast transformants were screened for failure of **stress hypersensitive phenotype** complementation, using heat sensitivity test. Yeast transfromants which failed to show

complementation in the heat sensitivity test were selected. The results are presented in Table 6.1.

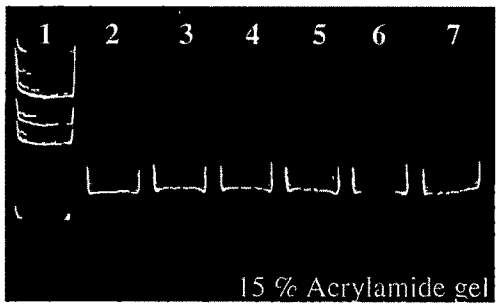


Figure 6.4. PCR amplicons under different error prone conditions. Lane 1. Contains 100 bp ladder. Lane 2. Contains amplicons of gene under standard conditions. Lanes 3., Lane 4., Lane5., Lane 6. and Lane 7. Contain amplicons of the gene under error prone conditions 10mM $MgCl_2$, 0.5mM $MnCl_2$, 1mM dATP, 1mM dGTP and 1mM dATP/dGTP respectively.

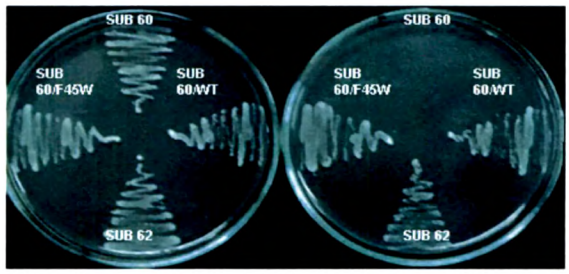
Table 6.1. Yeast transformants Colony number were failed to show stress hypersensitive phenotype complementation

Error prone PCR	Colony number (Bacterial transformant)	Heat sensitive mutants (Yeast transformant)
$MgCl_2$ [8.5 mM]	1,2,3,14,15,16,21,22,23,24,25,26,27,28, 29,30,31,32,33,34.	26,30,34.
$MnCl_2$ [0.5 mM]	6,7,8,9,10,11,12,13,42,43,44,45,46.	6,9,11,42.
dATP [1mM]	35,54,55,56,57,58,59,61,62,63,64,65,66, 67,68.	64,68.
dGTP [1mM]	36,37,38,39,40,41,47,48,49,50,51	37,38,40.

6.3.3. Screening of error prone mutants for loss of stress hypersensitive phenotype (Heat Stress) complementation

SUB60 is a UBI4 mutant. UBI4 is induced under stress condition and rescues the cell. Petri plates were transferred to 40^0C to give heat stress for 16 hours and then again incubated back at 30^0C which is a favorable temperature for the growth of yeast cells. SUB60 fails to revive back after the stress, while wild type strain SUB62 does. However

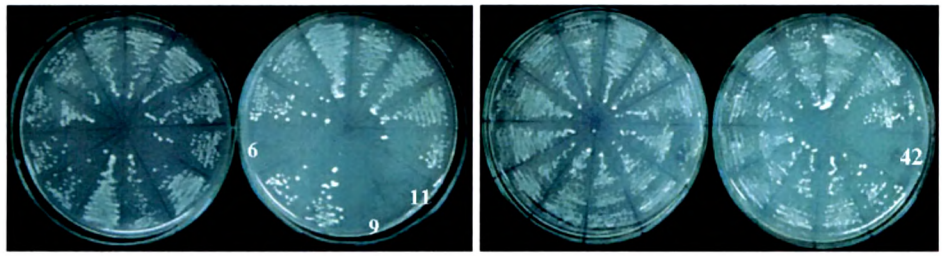
SUB60 transformed with YEp96-UbWt and YEp96-UbF45W complements the stress sensitive phenotype of SUB60 and grow normally. The yeast transformant failing to complement the stress sensitive phenotype shows complete lethal phenotype as shown in Figure 6.5.



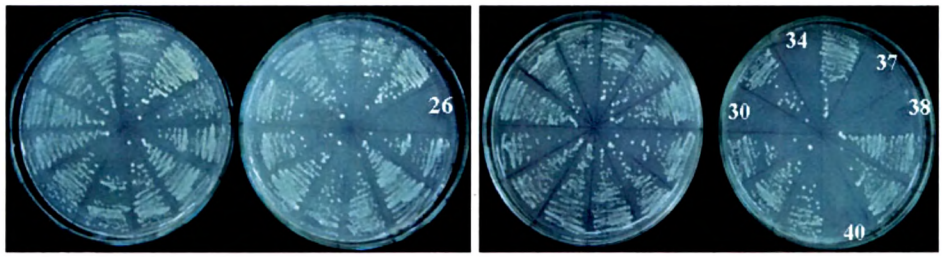
Without heat stress at 30^0C Heat stress at 40^oC

Figure 6.5. Screening of mutants by heat complementation in SUB60 yeast cells.

A. Heat sensitive colonies 6, 9, 11 and 42 obtained with error prone PCR in $MnCl_2$



B. Heat sensitive colonies 26, 30, 34 and 38, 40 obtained with error prone PCR in $MgCl_2$ and in dGTP



C. Heat sensitive colonies 64 and 68 obtained with error prone PCR in 1mM dATP

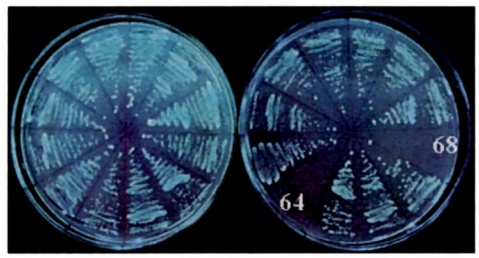


Figure 6.6. Failure of complementation of stress hypersensitive phenotype by mutated ubiquitin gene in yeast cell transformants. Mutagenesis under different error prone conditions (A) $MnCl_2$, (B) $MgCl_2$ and dGTP, (C) dATP.

Error Prone variants were generated by incorporating 0.5mM $MnCl_2$, 10mM $MgCl_2$, 1mM dGTP and 1mM dATP in the reaction which reduce the fidelity of Taq polymerase and there by increase the rate of mutation during PCR reaction. SUB60 transformed colony No. 6, 9, 11, and 42 ($MnCl_2$) colony No.26, 30 and 34 ($MgCl_2$,) colony No.37, 38 and 40 (dGTP) colony No. 64 and 68 (dATP) fail to revive back after the stress (Figure 6.6).

Plasmids were isolated from the above twelve bacterial transformants (Figure 6.7) and the presence of ubiquitin gene was confirmed using PCR (Figure 6.8). Colony no No.37, 38 and 40 did not yielded PCR suggesting either they are false positive clones or there is alteration in the primer binding or primer extending site.

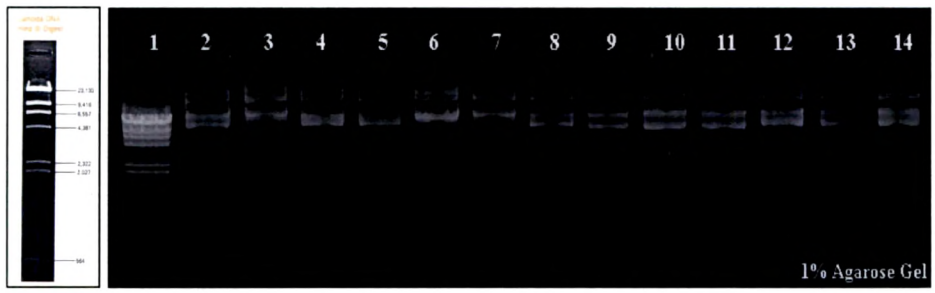


Figure 6.7. λ *Hind* III Digest (DNA Marker) Lane 1. YEp96-UbWt in lane 2. and plasmids of all the mutants (colony No. 6, 9, 11, 26, 30, 34, 37, 38, 40, 42, 64 and 68) were loaded and resolved in lane 3 to 14 respectively on 1% agarose.

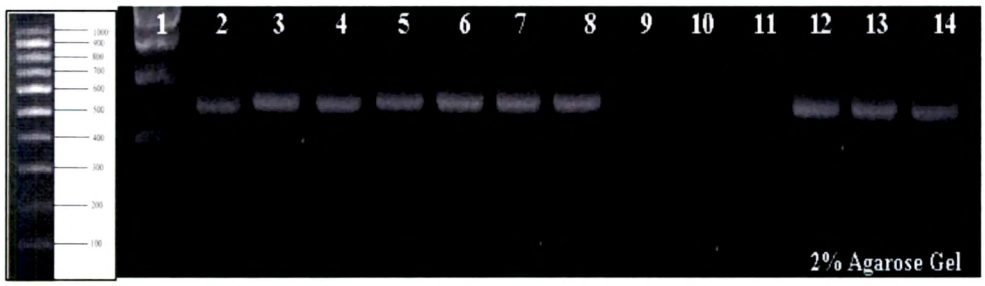


Figure 6.8. The 100 bp ladder (DNA Marker) in lane 1, PCR of YEp96-UbWt in lane 2 and PCR products of the ubiquitin gene of mutants (Colony No. 6, 9, 11, 26, 30, 34, 37, 38, 40, 42, 64 and 68) were in lane 3 to 14 respectively on 2 % agarose.

6.3.4. Isolation of a dosage dependent lethal mutant of ubiquitin (UbEP42) in *Saccharomyces cerevisiae*

Copper sulphate at 100μM concentration was used for inducing expression of ubiquitin in the twelve yeast transformants. Interestingly one of the transformants failed to grow on 100μM $CuSO_4$. The mutant, referred to as EP42, also conferred a concentration dependent lethality to the SUB60 yeast cells. SUB62 and SUB60 transformed with wild type ubiquitin gene shows no effect of copper sulphate. On other hand SUB62 transformed with YEp96-UbEP42 and EP42 transformed with YEp96-UbWt were rescued even with 200μM $CuSO_4$ which is other wise lethal (Figure 6.9).

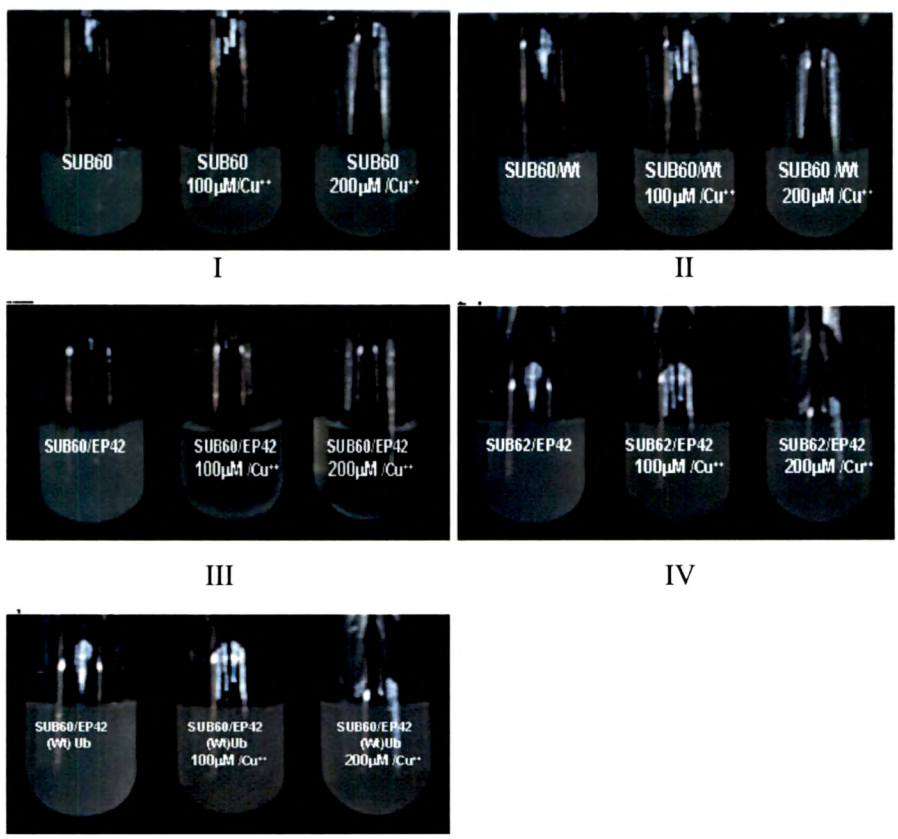


Figure 6.9. Culture tubes with UBI4 mutant of *S.cerevisiae* (SUB60) (I, II and III) with 0 μM, 100μM and 200μM $CuSO_4$, transformed with plasmid carrying YEp96-UbWt [II] and YEp96-EP42Ub [III] the lethal form of ubiquitin gene under CUP1 promoter, which is induced by Cu^{2+} at 30°C (permissive temperature). *S.cerevisiae* (SUB62) (IV) with 0 μM, 100 μM and 200μM $CuSO_4$ transformed with plasmid carrying YEp96-EP42Ub. *S.cerevisiae* (SUB60-EP42) [V] the lethal form of ubiquitin gene under CUP1 promoter with 0 μM, 100 μM and 200μM $CuSO_4$ cotransformed with YEp96-UbWt, which is induced by Cu^{2+} at 30°C (permissive temperature).

6.3.5. Sequence analysis of UbEP42 gene

The majority of ubiquitin's essential residues involve the C-terminal tail and Lys29, Lys48, and Lys63 known sites of polyubiquitin chain formation in vivo. Sequence analysis of UbEP42 reveals that the mutations do not include any of the lysines or C-terminal tail due to which the function can be altered (Figure 6.10). There are seven bases in the original sequence where substitution mutations have occurred. Out of these three are silent or neutral mutations resulting in replacement by synonymous codons and the other four mutations led to replacement of amino acid residues (Table 6.2.).

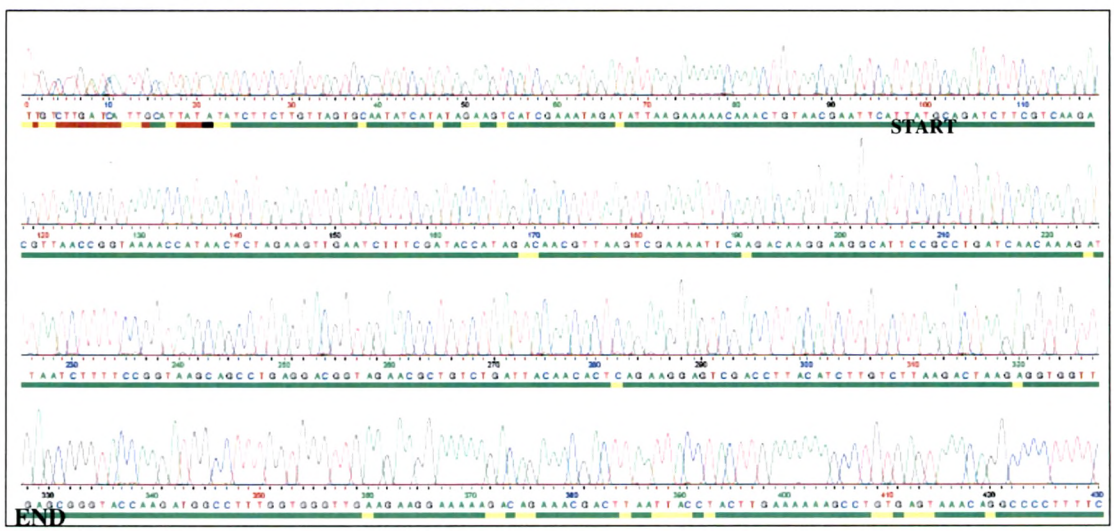


Figure 6.10. Electrophoretogram of UbEP42 gene in YEp96-UbEP42 vector. Start and end arrow indicated the start and end of the gene.

Table 6.2. Silent or neutral mutations in UbEP42.

Codon	Residue position	Amino acid	Secondary structure involved
TCC / TTC	20	Ser / Phe	3rd residue of a type I turn
ATC / ATA	23	Ile / Ile	---
CCA / CCG	37	Pro / Pro	----
TTG / TTA	43	Leu / Leu	----
GCC / TCC	46	Ala / Ser	2nd residue of type III turn
CTC / CCT	50	Leu / Pro	β-sheet
ATT / ACT	61	Ile / Thr	Between two turns in the turn rich region

6.3.6. Stress-hypersensitive Phenotype complementation

The ubiquitin genes UBI1, UBI2 and UBI3 maintain the basal levels of ubiquitin in the cell for normal functioning of cells. Under stress conditions UBI4 is needed to rescue the cells and therefore SUB60 yeast cells lack UBI4 polyubiquitin gene. UBI4 supports the survival under stress condition like heat shock, starvation, UV damage, amino acid analogs and antibiotics. SUB60 cells transformed with YEp96-UbEP42 could not rescue the cells under any of the stressed conditions heat stress, starvation stress or antibiotic stress.

6.3.7. Heat Stress Complementation

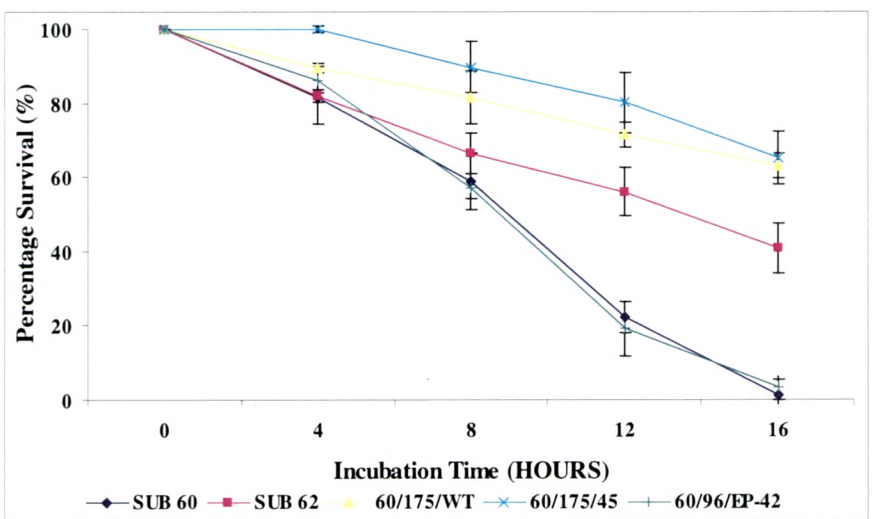


Figure 6.11. Percentage survival of SUB60, SUB62, SUB60/UbWt, SUB60/UbF45W and SUB60/UbEP42 yeast cells at differential time of incubation 0, 4, 8, 12 and 16 hours of incubation.

UbEP42 transformants of SUB60 cells fail to revive after 12 and 16 hour of incubation at 40°C behaving similar to untransformed SUB60 cells. The controls SUB62 and SUB60 transformed with gene for UbWt or UbF45W could withstand heat stress (Figure 6.11). Similarly the stress hypersensitive phenotypes could not be complemented under antibiotic stress.

6.3.8. Antibiotic Sensitivity test

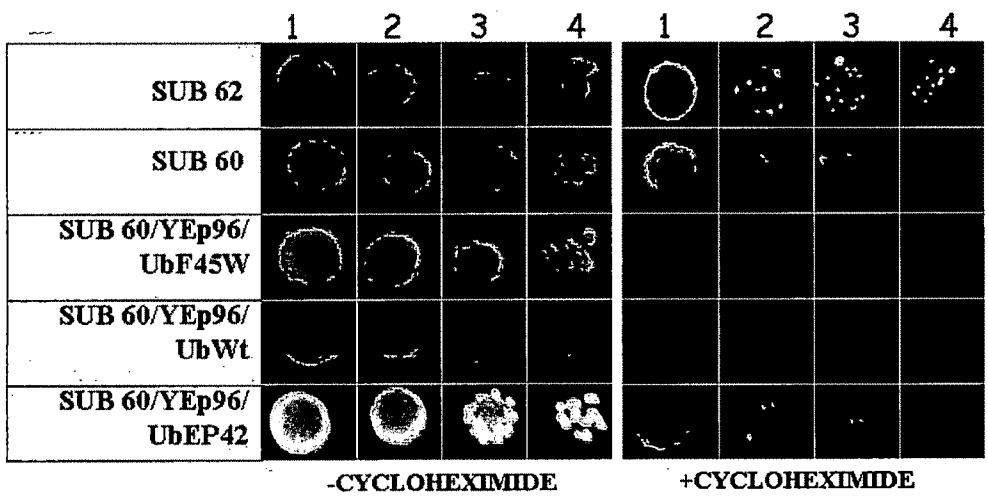


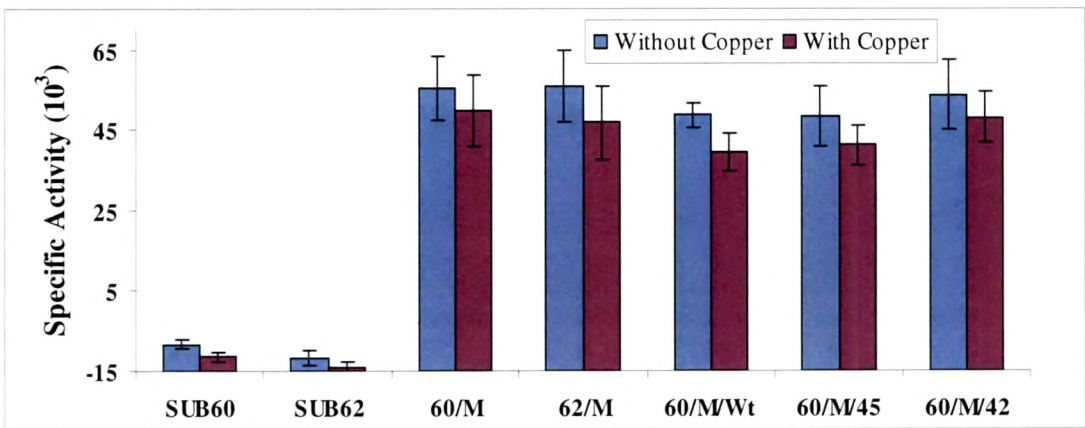
Figure 6.12. Antibiotic stress complementation of *Saccharomyces cerevisiae* (SUB60). Cycloheximide resistance of SUB62, SUB60 cells transformed with UbWt and UbF45W gene. The SUB60 and SUB60 transformed with UbEP42 fail to grow in presence of cycloheximide. Three fold serial dilutions (2), (3) and (4) spotted on YPD plates containing 100μM of copper and 4μg/ml of cycloheximide.

UBEP42 fails to complement SUB60 cells subjected to antibiotic stress (Figure 6.12). Either one or more of the amino acid residue substitutions are occurring directly in the region/s of the protein important for its function or due to structural alteration resulting from substitutions the protein is functionally impaired.

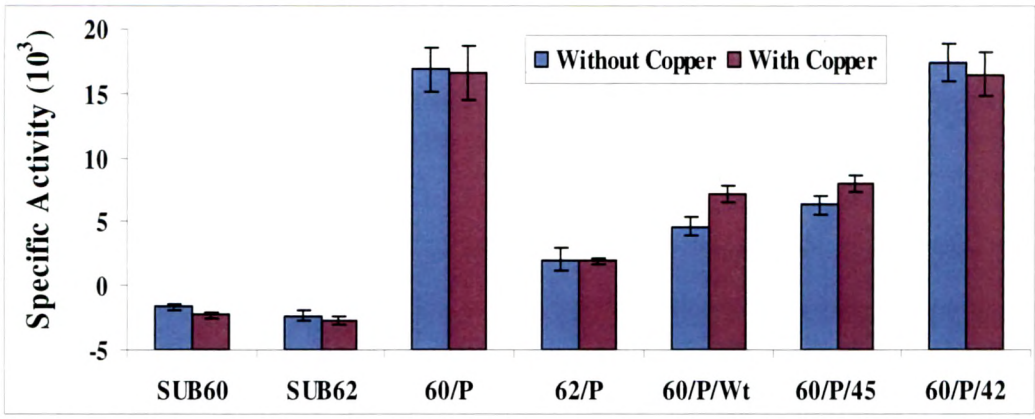
6.3.9. N-end rule as degradation signals in *Saccharomyces cerevisiae*

The sequencing analysis revealed that the majority of mutations probably affect the hydrophobic patch or the pocket in the vicinity of Ile44, which is important for protein degradation function of ubiquitin.

N-end rule describes that the stability of a protein is determined by its N-terminal residue. β-galactosidase fusion with stabilizing N-terminal residues like Met(M), is cleaved by deubiquitinating enzyme releasing a free Met-β-galactosidase which leads to a longer half life. Those fusions with destabilizing residues like Pro are not deubiquitinated and the subsequent ubiquitination in the fusion protein, results in degradation of the Ubiquitin-Pro-β-galactosidase. The ubiquitin-X-β-galactosidase fusion is under pGAL10 promoter, where X is the N-terminal residue. Thus β-galactosidase activity assay is a measure of protein stability. Results indicate Met-β-galactosidase activity remained more or less unchanged, when Met (highly stabilizing residue) was present as the N-end residue with UbEP42. However, the β-galactosidase activity increased with Pro-β-galactosidase in the UbEP42 mutant, as compared to SUB60 transformed by gene for UbWt and UbF45W. On the other hand β-galactosidase activity for Pro-β-galactosidase in the UbEP42 mutant is comparable to SUB60 transformed with pUB23 (Ubiquitin-Pro-β-galactosidase) alone. This indicated again the loss of function for UbEP42 in vivo. Hence, EP42 fails to execute N-end rule in *S.cerevisiae.*



(I)



(II)

Figure 6.13. Effect of UbEP42 on the half-life of proteins in UbI4 background. SUB60, SUB62, SUB60 transformed by plasmids YEp96-UbWt, YEp96-UbF45Wand YEp96-UbEP42 expressing the three forms of ubiquitin, namely wildtype UbWt, UbF45Wand UbEP42. These cells were also transformed by pUb23 expressing Ub-β-galactosidase fusion with (I) Met(M) and (II) Pro(P) as the N-terminal residues. SUB60 and SUB62 were also cells were co-transformed by plasmid pUb23 expressing Ub-β-galactosidase fusion with (I) Met(M) and (II) Pro(P) as the N-terminal residues. SUB60 and SUB62 were used as controls.

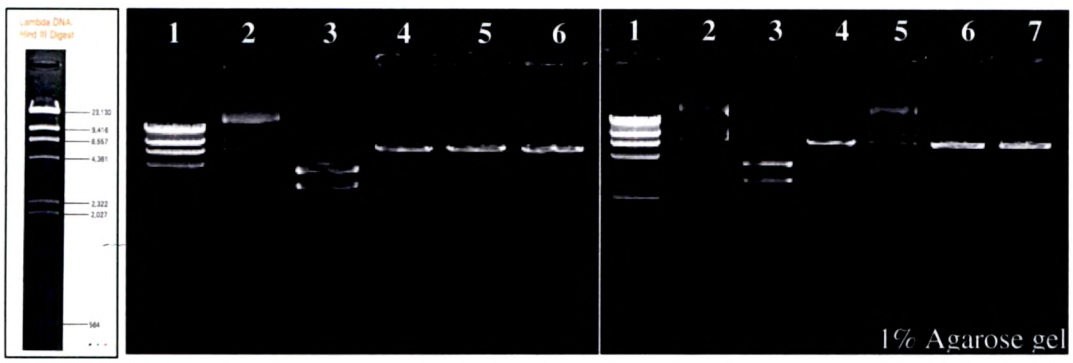
As the sequence analysis already showed that the mutation in the gene does not effect any functional site reported till date thus the loss of function can be attributed to structural defects which affect the molecule in a subtle way or direct participation of the residues in the interaction with other proteins, which calls for better understanding of molecular interactions.

High level of Pro-β-galactosidase activity in SUB60 with UbEP42 indicated two possibilities: either UbEP42 is not recognized and thus not used by ubiquitination system or it is recognized and bound by components of ubiquitination system, where it acts as an inhibitor for protein degradation (Figure 6.13).

6.3.10. UbEP42 polyubiquitination of substrate protein in *Sacchromyces cervisiae*

To investigate the above fact UbEP42 was tagged with c-myc to follow its ubiquitination profile in SUB60 yeast cells. UbWt and UbF45W were used as controls.

Tagged ubiquitin is correctly conjugated in vivo to protein and can be covalently extended into the multimeric ubiquitin chains, which is required for the degradation of protein. However, conjugation of tagged ubiquitin inhibits proteolysis. The amino-terminal region of ubiquitin is important in protease-substrate recognition and hence the protein degradation (Ellison et al., 1991).



(a) (b)

Figure 6.14. Restriction digestion pattern of (a) pUB221/c-myc-UbWt and (b) pUB221/c-myc-UbEP42). Lane 1. Contains λ *Hin*d III Digest marker. Lane 2. Contains undigested plasmid (a) pUB221/c-myc-UbWt and (b) pUB221/c-myc-UbEP42). Lane 3. There are three *Eco*R I sites in the vector and so *Eco*R I digest shows three fragments of 694bp, 2818bp and 3733bp. Lane 4. contains *Bgl* II digest. Lane 5. Contains (a) *Xho* I digest is a unique restriction sites and give a single band of 7286bp fragment with pUB221/c-myc-UbWt and (b) is lost after subcloning of c-myc-UbEP42 gene Lane 6. Contains *Kpn* I digest. *Bgl* II and *Kpn* I sites were used for subcloning of the gene.

Immunoblot analysis of c-myc tagged UbEP42 indicated that UbEP42 is used for ubiquitination of proteins suggesting that the conjugation is not affected for the protein. The probable function affected was the selective and regulated degradation of proteins. This result also explains the lethal effect of UbEP42 over expression. The over expression of UbEP42 far exceeds the basal levels of wild type ubiquitin in SUB60. UbEP42 ubiquitinated products of protein accumulate in the system on subsequent nonremoval of the products from system resulted in lethality.

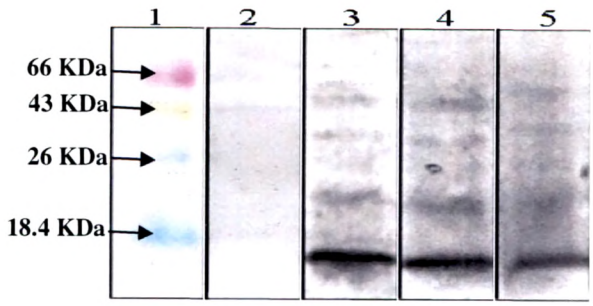
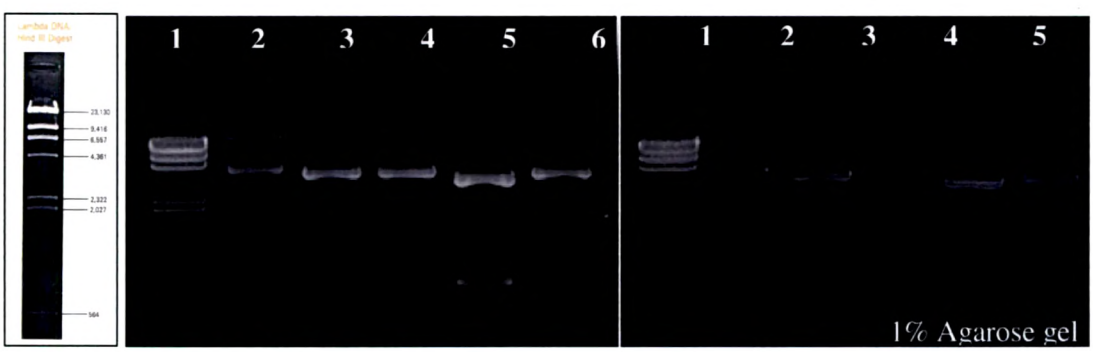


Figure 6.15. Western blot showing ubiquitination profile. Lane 1. Rain bow molecular weight marker. Lane 2. SUB60 as negative Control. Lane 3. SUB60/UbWt and Lane 4. SUB60/UbF45W as positive control and Lane 5. SUB60/UbEP42.

6.3.11. Construction, expression and purification of UbEP42 gene in bacterial expression system pKK 223-3

Complementation failure of UbEP42 in different assays and the dose dependent arrest of cells provoked the quest to understand the consequences of mutations on the structure function relationships of UbEP42. To study the effects of mutation on the protein structure the protein has to be expressed and purified from bacterial system, where the possibility for polyubiquitin chain formation does not exist. The gene was subcloned into pKK223-3 bacterial expression vector using sites *Eco* RI and *Hind* III. The plasmids pKK223-3/ UbEP42 were screened for loss of restriction site for *Xho* I and later it was sequenced for confirmation.



(a) **(b)**

Figure 6.16. Restriction digestion pattern (a) pKK223-3-UbWt and (b) pKK223-3-UbEP42. Lane 1. Contains λ *Hind* III digest marker. Lane 2. Contains (a) undigested plasmid of pKK223-3-UbWt and (b) pKK223-3-UbEP42. Lane 3. Contains *Eco*R I digest with a unique restriction site. Lane 4. (a) Contains *Xho* I digest and gives a single band with pKK223-3-UbWt and (b) the site is lost after subcloning of UbEP42 gene. Lane 5. Contains *Sal* I digest which has two sites and hence releases a 700bp fragment. Lane 6. Contains *Hind* III digest with a unique restriction site. *Eco* RI and *Hind* III sites were used for subcloning of the gene.

The pKK223-3-UbEP42 was used to express the protein successfully in bacterial expression system. Purification was attempted as mentioned in earlier in chapter 2. The protein could not be purified as the protein is heat sensitive. The third step in the purification procedure is treatment of lysate at 85°C for 15 minute. And at this step the protein was lost suggesting that the mutations had turned the protein heat labile. A probable disturbance in the hydrophobic core generated due to replacements of Ala46Ser, Leu50Pro and Ile61Thr residues could be responsible for the above effect.

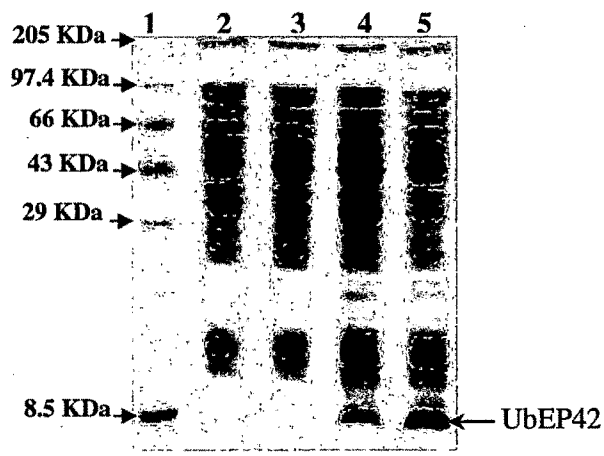


Figure 6.17. UbEP42 expression gel profile. Lane 1. Shows the protein molecular weight marker. Lane 2. and Lane 3. Show protein profile of JM109 cells without and with IPTG induction respectively. Lane 4. and Lane 5. Show protein profile of JM109/ pKK223-3-UbEP42 transformed cells without and with IPTG induction respectively.

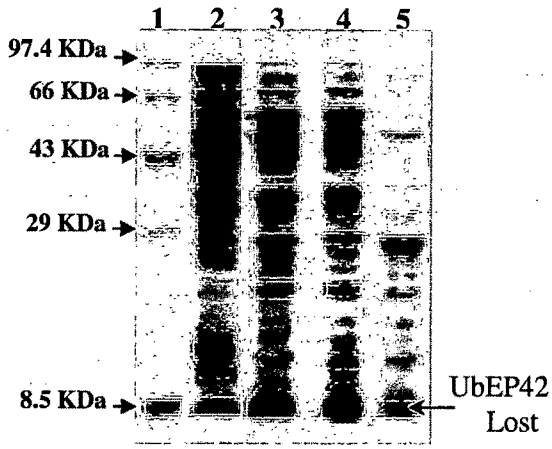


Figure 6.18. UbEP42 expression and purification gel profile. Lane 1. Shows the protein molecular weight marker. Lane 2. Shows protein profile of JM109/ pKK223-3-UbEP42 transformed cells with IPTG induction. Lanes 3., Lanes 4. and Lanes 5. Show cell lysate, 0.2% PEI treated supernatant and heat treated supernatant respectively.

6.4. DISCUSSION

The mutant ubiquitin UbEP42 has four mutations conferring dosage dependent lethal phenotype to the host cell. These mutations reduce the thermal stability of the protein considerably. The mutations Ser20 to Phe and Ala46 to Ser have occurred in type I and type III turns respectively. These two are surface residues and hence the substitution of a hydrophilic residue Ser by a hydrophobic residue Phe may have a drastic effect on the structure of the molecule. Formation of a hydrogen bond between the ε-amino group of Lys48 and Ala46 was reported in the wild type ubiquitin earlier, which may be affected with the substitution in the mutant. The third mutation Leu50 to Pro occurring in the β-sheet may be significant as well since the side chain of Leu is buried in the interior of the protein. Moreover, Pro being restricted in geometry due to its torsion angle ϕ, introduces kink in the protein backbone. Even though, substitution of Ile61 by Thr is not a drastic change, Ile61 is one of the first residues to be protected from H-D exchange during refolding of ubiquitin (Briggs and Roder, 1992). Further, the side chain of Ile is also buried in a hydrophobic pocket Ala46 and Lue67 of the wild type native molecule. Thr being polar may not show same preference (Figure 6.19.). Indeed our results indicate that the thermal stability of

UbEP42 is much reduced compared to wild type ubiquitin. Structural characterization of the mutant protein will give a better picture in this regard.

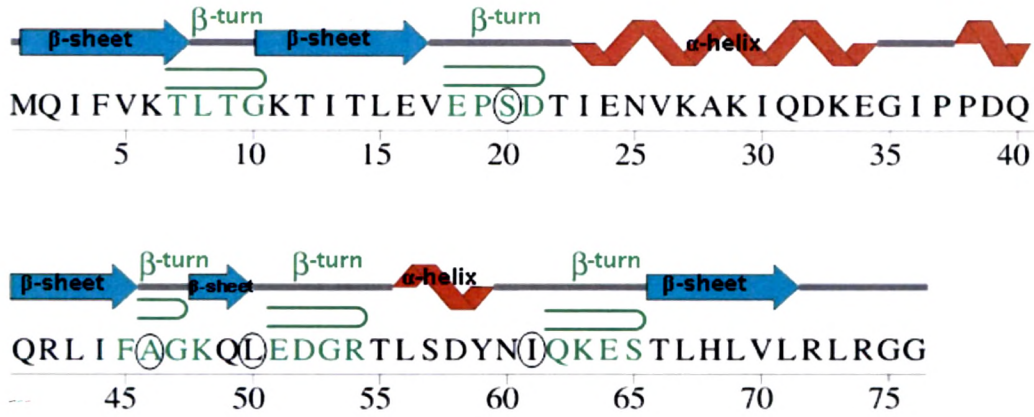


Figure 6.19. Shows the position of four mutations S20F, A46S, L50P and I61T accompanied with the secondary structural features of ubiquitin.

Ubiquitin is known for its structural stability entirely contributed by its globular hydrophobic core. Two distinct clusters in the globular core domain centered around Phe4 and Leu8/Ile44/Val70 form a hydrophobic patch. Observations demonstrate that Ile44 is critical for multiple functions of ubiquitin and suggest that Ile44 may interact with different proteins to facilitate either proteasome degradation or endocytosis while Phe4 and adjacent residues are important for only the endocytic role of ubiquitin but do not function in proteasome degradation. Mutation at Ile44 hydrophobic patch although does not interfere with ubiquitin conjugation but the subsequent downstream recognition and signaling pathway is affected in proteasome mediated proteolysis (Mould et al., 2001).

Hydrophobic patch present around the Ile44 is contributed by Leu8 and Val70. In the vicinity of this patch a hydrophobic pocket is present which is made up of Ala46, Ile61 and Leu67 (Vijay-Kumar et al., 1985). Ala46 interacts with Ile61 and Leu67, which are buried in the hydrophobic core. Leu50 is also a buried hydrophobic residue. A probable disturbance in hydrophobic effect is generated due to replacements of Ala46Ser, Leu50Pro and Ile61Thr which has destabilized the structure of ubiquitin and adversely affected its function.

UbEP42 can be used normally by the cell for polyubiquitination. Though, UbEP42 failed to complement SUB60 cells under antibiotic stress. It was observed that the mutant extended the half-life of Ubiquitin-Pro-β-galactosidase, implying that the polyubiquitin chain of UbEP42 is not recognized by proteasomes for degradation. The protein also could not rescue the cells from heat stress, probably due to its thermal instability.

Studying the four mutations individually and in combinations may reveal more information about the roles of individual residues.

Ubiquitin of *S.cerevisiae* is almost identical to human ubiquitin in its sequence and structure. The results indicate possibilities of either the same combination of mutations in ubiquitin or some other mutations generated by *in vitro* evolution of human ubiquitin gene are likely to give rise to dosage dependent lethal effects in human cells as well. Fine tuning the expression of such a dosage dependent mutation in tissue specific manner can have profound medical implications.