CHAPTER 5

FUNCTIONAL ASSESSMENT OF THREE SITE DIRECTED MUTANTS OF UBIQUITIN IN *Saccharomyces cerevisiae*

5.1. INTRODUCTION

5.1.1. Ubiquitin and its role in biological system

Ubiquitin dependent processes play various roles in cellular physiology. Primarily, Ubiquitin regulates protein turnover in a cell by closely regulating the degradation of specific proteins involved in Regulation of cell cycle (Goebl et al., 1988) and division, differentiation and development (Bowerman et al., 2006), morphogenesis of neuronal networks, modulation of cell surface receptors (Hicke and Riezman, 1996), ion channels and stress effectors (Ozkaynak et al., 1984; Finley et al., 1987), DNA repair (Jentsch et al., 1987), transcriptional regulation (Hochstrasser and Varshavsky, 1990), circadian rhythms, regulation of immune and inflammatory responses (Ciechanover and Iwai, 2004; Varshavsky, 2005), biogenesis of organelles as mentioned in Chapter 1.

Regulating protein degradation is very important cells can quickly eliminate a protein that has served its function. By elimination of a regulatory protein the process controlled by the protein is shutdown immediately.

Ubiquitin takes part in ATP-dependent proteasomal degradation of proteins (Varshavsky, 1997; Weissman, 1997). ATP is required to specifically target the proteins that need to be degraded. Ubiquitin serves as a tag that marks proteins for degradation and does not degrade the proteins directly and is not degraded in the process. Proteins tagged by ubiquitin undergo degradation by the 26S proteasome (Hershko et al., 1984, Baumeisteret et al., 1998; Lee and Goldberg, 1998;

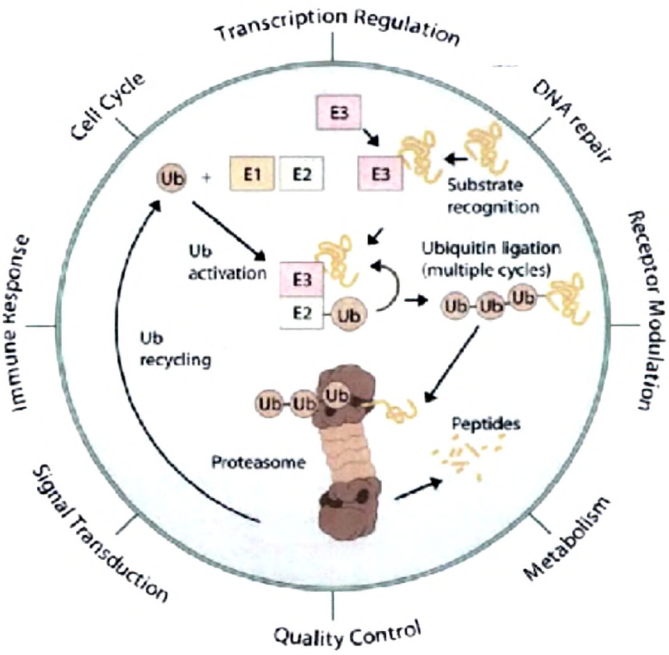


Figure 5.1. Ubiquitin Proteasome System (UPS) and its various biological roles.

Rechsteiner, 1988; Groll and Huber, 2004; Pickart and Cohen, 2004; Wolfand Hilt, 2004; Rechsteiner and Hill, 2005). Proteins destined for degradation are first tagged with 4-5 chains of ubiquitin and these tagged proteins are then recognized and sent into the proteasomal tunnel for degradation. Additionally mutated, denatured and misfolded proteins are recognized specifically and are removed efficiently by this pathway (Hershko and Ciechanover, 1998).

5.1.2. Differential ubiquitination

Depending on the number ubiquitin molecules tagged to a protein, the number of ubiquitins attached to each other in the tagging chain and number of locations on the protein which have been tagged with ubiquitin, ubiquitinations have been classified into *monoubiquitination, polyubiqitination* and *multiubiquitination* (Pickart et al., 2004). In *monoubiquitination* a protein is tagged with a single ubiquitin molecule on a single Lys residue. It is important for the histone function, transcription, endocytosis (Hicke, 1999; Hicke and Dunn, 2003) and membrane trafficking (Hicke et al., 2003). P*olyubiqitination* refers to the modification of a protein with several ubiquitin chains. If more than one Lys on the substrate protein is tagged with polyubiquitin chains then it is referred to as *multiubiquitination.* Ubiuitin has seven Lys residues and all of them are used for chain formation. Lys^{11}, Lys^{29} and Lys^{48} linked polyubiquitin chains target proteins to the proteasome (Hershko et al., 1984, Baumeisteret et al., 1998; Lee and Goldberg, 1998; Rechsteiner, 1988; Groll and Huber, 2004; Pickart and Cohen, 2004; Wolfand Hilt, 2004; Rechsteiner and Hill, 2005). Monoubiquitin chains Lys^{63} target the protein to the signaling, DNA repair repair (Spence et al., 1995; Ulrich et al., 2002), signal transduction and endocytosis (Hicke et al., 2003).

5.1.3. Specific degradation signals on substrate protein for ubiquitination

The ε-amino groups on a Lys residue of substrate protein is attached to ubiquitin thorough its C-terminal carboxyl forming an isopeptide linkage. However, presence of Lys is not sufficient for ubiquitnation of target proteins. Proteins must contain a degradation signal, often referred to as 'degron' motif, which is recognized by the ubiquitination system for attaching the ubiquitin tag (Varshavsky et al., 1996).One such degron may be the N-

terminal residue of a protein (Hershko et al., 1984, Bachmair et.al. 1986). Other degrons include the 'destruction box' motif found in the cyclins (Glotzer et al., 1991) and the sequences rich in Pro (P), Glu (E), Ser (S) and Thr (T) the PEST sequences (varshavsky, 1997). These PEST elements contain phosphorylation sites which tagets the protein for ubiquitination. Apart from phosphorylation of Ser (S) Thr (T) and Tyr (W) (Deshaies, 1999) (Jackson et al., 2000), hydroxylation of Pro (P), glycosylation of Asn , deacetylation of Lys (Brooks and Gu, 2003) and specific protein association and disassociation are the penitential signals which targets protein degradation.

5.1.4. The N-end rule

The N-terminal residue of a protein determines half-life of a protein. The N-end rule classifies N-terminal residues into stabilizing and destabilizing residues (Varshavsky, A. 1996). The destabilizing residues, known as N-degron, include Arg, Lys, His, Phe, Leu, Tyr, Ile, Asp, Glu, Asn and Gln. The stabilizing residues are by default Gly, Val and Met, those residues which do not belong to the above class. The N-end rule pathway is present in all organisms examined, from the bacterium *E. coli* to the eukaryotic mammalian cells. The N-degrons can bear different destabilizing N-terminal residues which are recognized by distinct targeting complexes (Varshavsky et al., 1996).

Proteins with relatively shorter half life are rapidly ubiquitinated and removed from the system while the protein with longer half life are often deubiquitinated after ubiquitination by deubiquitinating (DUB) enzymes.

5.1.5. The ubiquitin N-terminal fusions of β-galactosidase as an assay system for ubiquitin function

A model system was developed to study the specific degradation signal where a N-terminal ubiquitin fusion to a reporter protein was engineered. The start codon ATG that

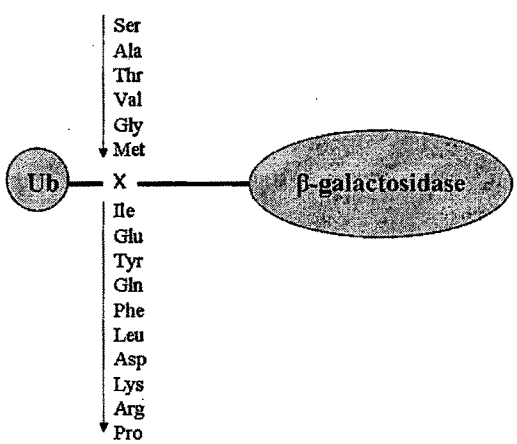


Figure 5.2. Changing amino acid residue of β-galactosidase at ubiquitin β-gal.

specifies the original Met residue of the reporter protein β-galactosidase was converted to array of stabilizing and destabilizing residue by site directed mutagenesis. The enzyme β-galactosidase was manipulated such that only the N-terminal fused ubiquitin can be polyubiquitinated and therefore acts as a reporter to assay the effect of different amino acids as N-terminal residue on the turn over of the protein (Varshavsky, A. 1986). The β-galactosidase molecules engineered with stabilizing N-terminal residue undergo deubiquitination and have longer half-life, where as those with destabilizing N-terminal residues fail to undergo deubiquitination, following the polyubiquitination route to quick degradation (Varshavsky et al., 1996).

5.1.6. Physiological functions and stress response

Ubiquitin is known to be involved in cell cycle control (Goebl et al., 1988), DNA repair (Jentsch et al., 1987), protein synthesis, transcriptional regulation (Hochstrasser and Varshavsky, 1990), and stress responses (Ozkaynak et al., 1984; Finley et al., 1987) as mentioned earlier. Out of four ubiquitin genes present in *Saccharomyces cerevisiae* the polyubiquitin gene UBI4is induced in the stress conditions to rescue the cells. UB14 expression is under the control of the stress response regulatory network (Ozkaynak et al., 1987). In *S. cerevisiae* the UBI4 gene mutants are hypersensitive to stress (Finley et al., 1987). Heat stress sensitivity was the first hypersensitive phenotype to be discovered. The proteins receiving irreparable damage due to temperature rise are selectively removed by ubiquitinating with UBI4 thus rescuing the cells. Second is the starvation sensitivity. Withdrawal of nitrogen source or carbon source or both from the media drives the yeast cells to form spores for survival. The ubi4/ubi4 mutant diploids cannot sporulate under nutritional stress (Ozkaynak et al., 1987). Stress proteins can be induced not only by heat and starvation, but also by a variety of toxic compounds, such as amino acid analogs, toxic antibiotics and mutagen owing to the production of abnormal proteins. These malformed proteins are disposed of by the cellular machinery through ubiquitination, failure of which results in lethality.

The biological role of the residues Glu64, Ser65 and Gln2 in the second β-bulge of ubiquitin has not been understood so far. In Chapters 2, 3 and 4 three site directed mutants UbE64G, UbS65D and UbQ2N have been constructed to study their in the structure of

ubiquitin. The substitutions were chosen so as to maintain the secondary structure undisturbed and hence figure out their significance in determining the overall structure and stability of the molecule. The results established that there was no drastic change in the structure and stability of these molecules due to single residue substitutions. To unravel the importance of the residues in the biological functions of ubiquitin, various functional studies were carried out with the mutants, which include complementation and rescue of UBI4 mutants by ubiquitin variants under stress conditions of exposure to heat, antibiotic and UV. In addition participation of ubiquitin variants in ubiquitination and their ability to determine the half-life of β-galactosidase in ubiquitin-X- β-galactosidase fusions depending on stabilizing or destabilizing nature of N-terminal residue through polyubiquitin chain formation were assessed.

5.2. MATERIALS AND METHODS

5.2.1. Yeast strains, media, and plasmids (Finley et.al., 1987; 1994; Spence et.al., 1995)

Yeast strains **SUB62** (MATα lys2-801 leu2-3,112 ura3-52 his3-A200 trpl-1), **SUB60** (MATα ubi4-A2:: LEU2 lys2-801 leu2-3,112 ura3-52 his3-A200 trpl-1), **SUB280** (MA4Ta lys2-801 leu2-3,112 ura3-52 his3-A200 trpl-1ubil::TRP1 ubi2-A2:ura3 ubi3-Aub2 ubi4-A2::LEU2 [pUB39][pUB100] were used in the study. The pUB39 plasmid is similar to YEp96 but marked with the LYS2 gene. pUB100 carries a Ubil tail expression cassette and **SUB413** was identical to **SUB280** except for the UBK63R mutation.

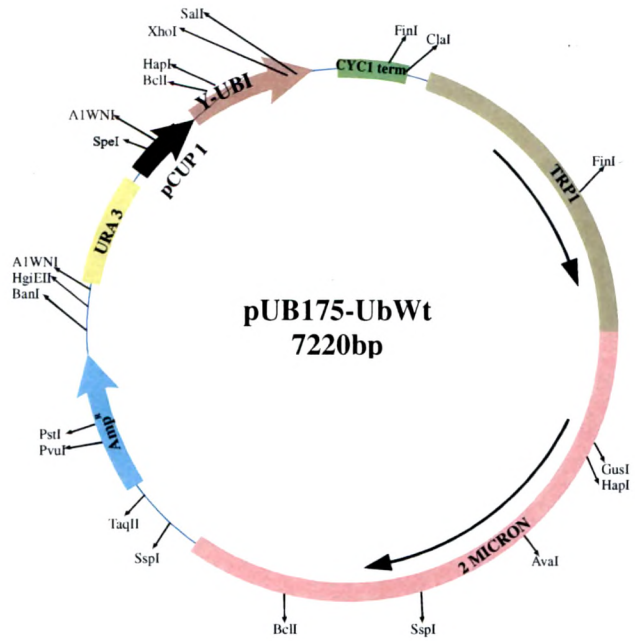
Cultures were grown at 30°C at 200 rpm, except where indicated, in synthetic dextrose medium consisted of 0.67% Hi-media yeast nitrogen base supplemented with uracil, leucine, tryptophan, lysine and histidine as and when required supplemented with 2% glucose as carbon source or 4% galactose as and when required.

Ubiquitin was expressed from a high-copy number yeast episomal vector that is identical to the 2 micron based copper inducible ubiquitin expression plasmid YEp96, pUb39 and pUB175. Ubiquitin overproduction from the CUP1 promoter was induced by the addition of $CuSO_4$ to 100 μM. YEp96, pUb39 and pUB175 are identical in all respects except the axotropic selection. Plasmid pUB175 has both URA3 and TRP1 as selection markers. YEp has only TRP1 and pUB39 has TRP1 inactivated by LYS2 gene. Tagged ubiquitin (c-myc/his-Ubiquitin) was expressed from pUB221. Back bone of pUB221 is identical to pUB17. pUB23 is 2 micron based galactose inducible shuttle vector expressing ubiquitin-X-β-galactosidase fusion protein (Ub-X-β gal) with a selection marker of URA3 gene. The X position was varied with different animo acids depending upon their primary, secondary or tertiary destabilizing categorization.

5.2.2. Plasmid construction (yeast expression vector) (Finley et.al., 1994)

Ubiquitin gene mutation were carried in plasmids YEp96, pUB175 which express a synthetic yeast ubiquitin gene and pUB221 which expresses a c-myc tagged synthetic yeast ubiquitin gene under the CUP1 promoter and is a shuttle vector between *E.coli* and *S.cerevisiae*. Ubiquitin gene amplicons generated by PCR were cloned into the *Bgl* II and *Kpn* I sites of YEp96, pUB175 and pUB221. Screening of mutations for UbE64G and

UbS65D were done with loss of *Sal* I site where as for UbQ2N was done with loss of *Bgl* II site.



The DNA sequence of the *Eco*R I-*Kpn* I insert in Yep 96-UbWt

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

Figure 5.3. Yeast expression vector pUB175-UbWt. The plasmid pUB175-UbWt carries wild type synthetic yeast ubiquitin gene under CUP1 promoter induced by 10-20μM copper sulphate. The plasmid when transformed into *E.coli*, DH5α strain was maintained under a selection pressure of 50μg-100μg/ml of ampicillin. The backbone of the pUB175-UbWt was similar to YEp96 except for URA3 fragment cloned into *Bam* H I site. TRP1 and URA3 are the selection markers and 2μ fragment maintains YEp 96 at 50 copies/cell.

5.2.3. Growth Effects

The cultures of SUB60 cells expressing wild type and mutant forms ubiquitin were grown and their growth was compared with wild type strain for UBI4, SUB62 (Finley et.al., 1994). Freshly grown stationary phase cells were used as inoculum for the growth curve. 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 uracil, leucine, tryptophan, lysine and histidine supplemented with 2% glucose as carbon source as and when required. 100 μM copper sulphate was used as a inducer to check the effects of expression of mutant ubquitin genes on *S.cerevisiae* cells. OD was recorded at intervals of two hours at 600nm (Figure 5.15.)

5.2.4. Complementation assay

Stress hypersensitive Ubl4 mutant, the SUB60 strain of yeast was transformed with YEp96 plasmid carrying wild type or mutated ubiquitin gene under CUP1 promoter and was tested for complementation under stress. SUB60 yeast cells can be complemented and thus rescued from stress, with the expression of wild type ubiquitin gene.

5.2.5. Heat sensitive test

Heat sensitivity test was done to confirm the functional integrity of the mutant ubiquitins (Finley et.al., 1987). SUB60 yeast transformants were grown to log phase with optical density of the cultures reaching a value between 0.5 to 0.6 and serial diluted four fold and plated on SD selection media with and without induction by 100 μM copper sulphate. Plates were incubated at 40°C for variable time periods of 0, 4,8,12 and16 hours and shifted back to 30°C and the colonies were counted.

5.2.6. UV-C sensitivity test

UV-C sensitivity test was performed following the method given by (Spence et.al., 1995). SUB60 yeast transformants grown to mid log phase as mentioned above were serial diluted four fold and plated on SD selection media with and without induction by 100 μM copper sulphate. Plates were exposed to UV-C at 254nm for variable time periods of 0, 10, 20, 30 and 40 seconds. Plates were incubated in dark at 30°C. At the end of seventh day colonies were counted.

5.2.7. Antibiotic sensitivity test

Complementation potential of the ubiquitin variants was tested using antibiotic sensitivity test (Hanna et.al., 2003). SUB60 transformants were grown to log phase with

their optical density values around 0.2 and serially diluted three fold and spotted on YPD media with induction by copper sulphate and cycloheximide (4μg/ml). Plates were incubated for ten days for assessment of growth revival.

5.2.8. N-end rule as degradation signals (Bachmair et.al., 1986; Baker et.al., 1991)

Yeast double transformants of YEp96 and pUB23 were grown to exponential log phase on SD-galactose selection media with and without induction by copper. Cells were pelleted and washed twice with distilled water and resuspended in saline and the optical density was adjusted to 0.5 to 0.6. Protein estimation done by modified Lowry method (Lowry et al., 1951) and β-galactosidase assay was done (Baker et.al., 1991).

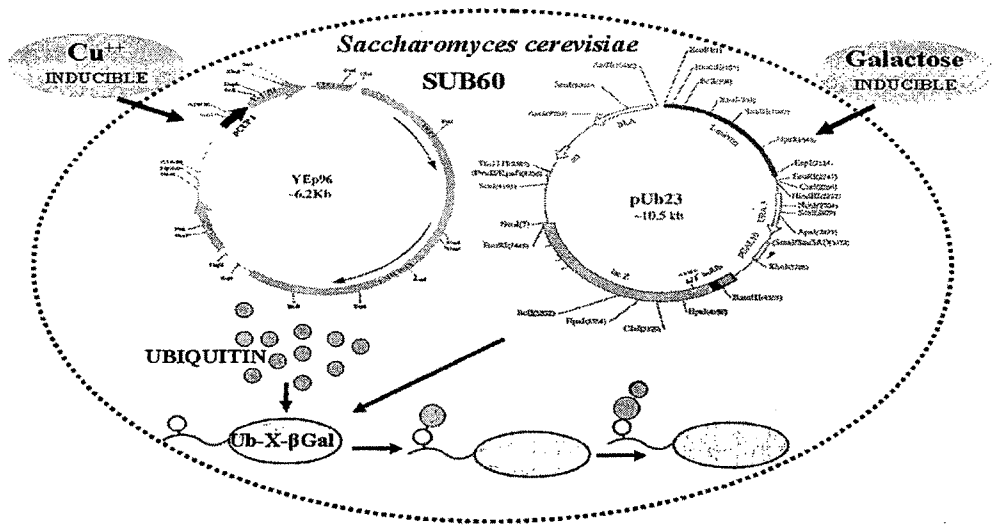


Figure 5.4. Schematic representation of YEp96 and pUB23 yeast double transformants to study the N-end rule as a degradation signal.

5.2.9. Western blot analysis (Ellison et.al., 1987)

Yeast transformants were grown to log phase to optical density 0.5 then the cells were induced with 100μM $CuSo_4$ for 4 hours and then shifted to 40°C for 2 hours. Yeast cells were pelleted and washed twice with distilled water, suspended in normal saline and treated with gel loading buffer (Ellison et.al., 1987) Equal amount of proteins were loaded in each well. Discontinuous SDS-PAGE was performed. Wet transfer of proteins on PVDF membrane was performed for 16 hours at 4°C. Incubation of PVDF membrane for four

hours in 1% BSA in phosphate buffered saline (PBS) pH-7.8 was used for blocking (Sambrook and Russel et .al. 2001). Anti-c-myc-horse radish peroxidase conjugated antibody procured from Roch was used at 1/1000 dilution. Blot was developed with a chromogenic substrate 3,3'-Diaminobenzidine (DAB) in presence of H_2O_2 and $NiCl_2$.

5.2.10. Plan of work

The site directed mutants generated for the ubiquitin (UbE64G, UbS65D and UbQ2N) were cloned in the shuttle vector for yeast and bacteria. These cloned gene were introduced into the UBI4 polyubiquitin gene mutant of *Saccharomyces cerevisiae* (SUB60) and examined fro complementation of phenotype.

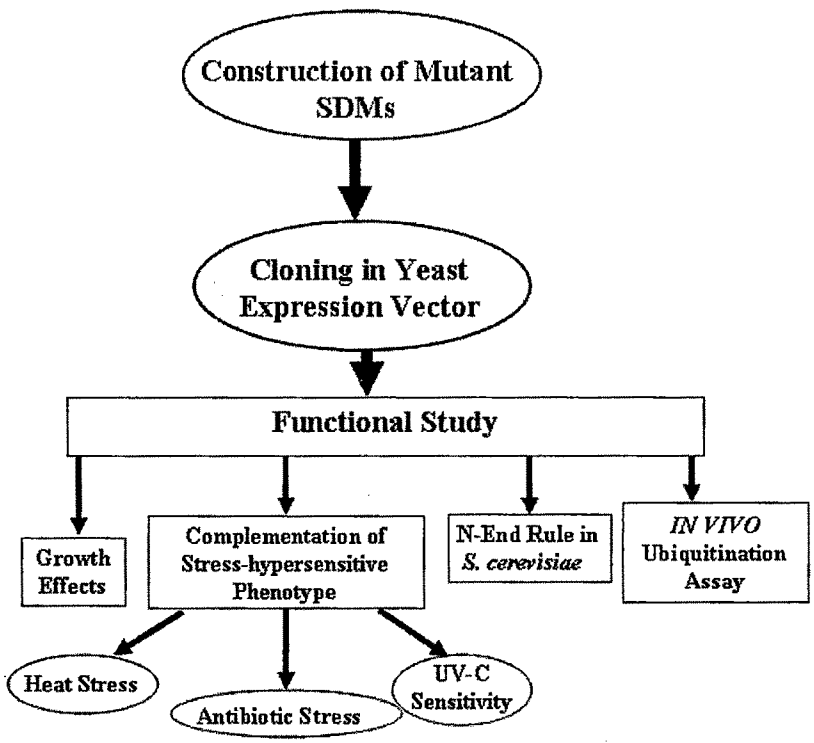


Figure 5.5. Schematic representation of functional complementation studies.

5.2.11. Bacterial strains and media

E.coli DH5α (F, 80dlacZ M15, *endA*1, *recA*1, *hsdR*17 ($r_k^-m_k^+$), *supE44, thi-1, gyrA96, relA1, (lacZYA-argF)U169,*) strain was used. Cultures were grown at 37°C at 200 rpm in Luria broth procured

from hi-media. Selection pressure of 100µg/ml of ampicillin was used for the strain. Bacterial transformation was done by $CaCl_2$ method. DNA preparation for plasmid was done by alkaline lysis method (Sambrook and Russel et al., 2001).

5.2.12. Sequence Analysis

The plasmid with mutant gene was sequenced using dideoxy sequencing method for the detection of the mutation incorporation in the DNA. MWG-Biotech sequenced the DNA samples (Yep96 plasmid back bone) in a single forward reaction using primer sequence 5'GCGCCGACATCATAACGGTTCTGGC'3.

5.3. RESULTS

5.3.1. Construction yeast expression vector carrying UbF45W and UbE64G gene in pUB175 and YEp96 vectors

Yeast bacteria shuttle vector pUB175-UbE64G and YEp96-UbE64G was constructed with UbE64G gene under CUP1 promoter, which can be induced in yeast by 10-100μM copper sulphate. Approximately 350bp long fragment of DNA carrying mutant ubiquitin gene UbE64G was amplified by PCR from pKK223-3-UbE64G plasmid (Figure 5.6.) (Chapter 2). The resultant DNA was subcloned into pUB175 and YEp96 yeast expression vector using sites *Bgl* II and *Kpn* I. Similarly the gene for UbF45W was also cloned. The plasmids pUB175-UbE64G and YEp96-UbE64G was digested with restriction enzymes (*Sal* I) for screening and later it was sequenced for confirmation. Plasmid pUB175-UbF45W was also checked for the incorporation of *Sau*96 I restriction site in the gene and further confirmed by sequencing (Figure 5.5.).

5.3.1.1. PCR amplification of ubiquitin genes generated from pKK223-3

Approximately 350bp long fragment of DNA carrying ubiquitin gene was amplified by PCR from pKK223-3 vectors mentioned chapter 2, 3 and 4 (Figure 5.6.).

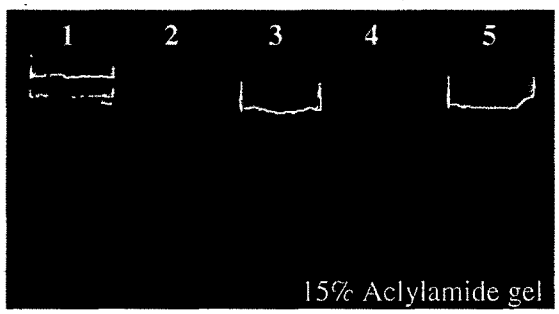


Figure 5.6. PCR amplification of ubiquitin gene. Lane 1. Contains 100bp ladder. Lane 2. and Lane 4. are the reagent blanks. Lane 3. and lane 5. Contain 364bp extension form vector pKK-223-UbF45W and pKK-223-UbE64G respectively using primer combination of FORWARD PRIMER: 5'GCGCCGACATCATAACGGTTCTGGC`3 and REVERSE PRIMER: 5'TCCGGTACCCGCTCAACCACCTCTTAG`3.

5.3.1.2. Construction of pUB175-UbF45W

The ubiquitin gene (≈250bp) was PCR amplified from pKK223-3-UbF45W was clone in the pUB175 vector in *Bgl* II and *Kpn* I site. The gene after the cloning in pUB175 was amplified and checked for the presence of *Sau*96 I (Figure 2.5) site which is introduced during the cloning of UbF45W (Figure 5.7).

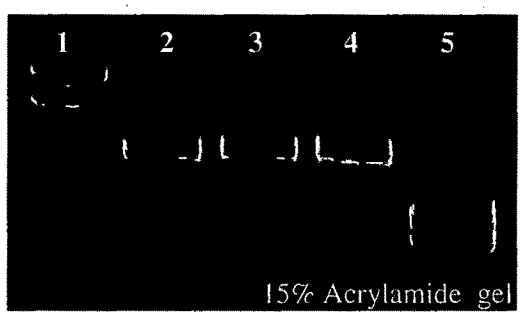
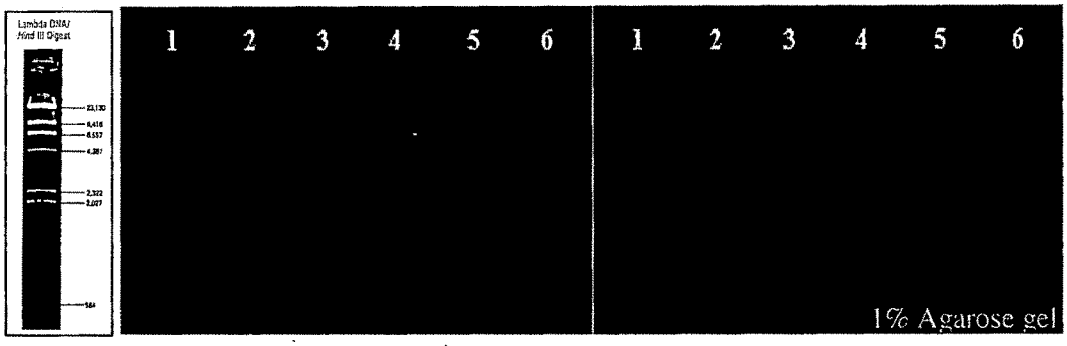


Figure 5.7. Restriction digestion pattern of pUB175-UbF45W. Lane 1. Contains 100bp ladder. Lane 2. and Lane 4. Contain 234bp extension from pUB175-UbWt and pUB175-UbF45W respectively using primer combination of FORWARD PRIMER: 5`ATGCAGATCTTCGT CAAGACGTTAACCGG´3 and REVERSE PRIMER: 5`TC CGGTACCCGCTCAACCAC CTCTTAG´3. Lane 3. and Lane 5. PCR products digested by *Sau*96 I before and after cloning of UbF45W gene. After cloning of the UbF45W gene the in pUB175 the gene develops a new restriction site for *Sau*96 I, which cuts the gene into two fragments of 100bp and 134bp.

5.3.1.3. Construction of pUB175-UbE64G

The ubiquitin gene (≈250bp) was PCR amplified from pKK223-3-UbE64G was clone in the pUB175 vector in *Bgl* II and *Kpn* I site. Cloning of UbE64G in pUB175 vector was confirmed by loss of *Sal* I restriction site and further by sequencing (Figure 5.8.).



(a) (b)

Figure 5.8. Restriction digestion pattern of (a) pUB175-UbF45W and (b) pUB175-UbE64G. Lane 1. Contains λ *Hin*d III Digest marker. Lane 2. Contains undigested plasmid (a) pUB175-UbF45W and (b) pUB175-UbE64G. Lane 3. There are three *Eco*R I sites in the vector and hence *Eco*R I digest shows three fragments of 628bp, 2818bp, 3733bp fragments. Lane 4. Contains *Bgl* II digest. Lane 5. Contains *Sal* I digest with a unique restriction site, giving a single band of 7220bp fragment with pUB175-UbF45W (a), and is lost after subcloning of gene with UbE64G (b). Lane 6. Contains *Kpn* I digest. *Bgl* II and *Kpn* I sites were used for cloning.

5.3.1.4. Construction of YEp96-UbF45W

The ubiquitin gene (≈250bp) was PCR amplified from pKK223-3-UbF45W was clone in the YEp96 vector in *Bgl* II and *Kpn* I site. The gene after the cloning in YEp96 was amplified and checked for the presence of *Sau*96 I (Figure 2.5) site which is introduced during the cloning of UbF45W (Figure 5.9.).

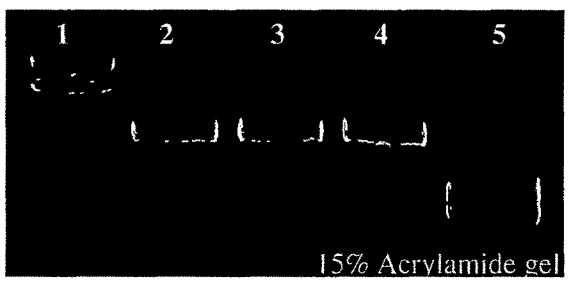
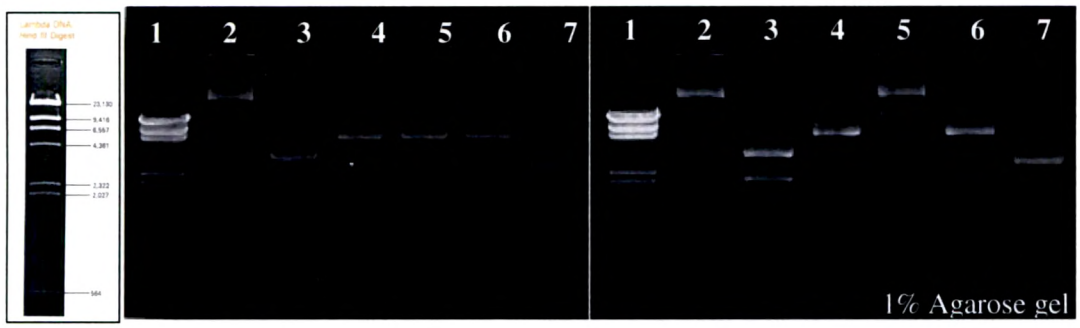


Figure 5.9. Restriction digestion pattern of YEp96-UbF45W. Lane 1. Contains 100bp ladder. Lane 2. and Lane 4. Contains 234bp extension from YEp96-UbWt and YEp96-

UbF45W respectively using primer combination of FORWARD PRIMER: 5`ATGCAGATCTTCGTCAAGACGT TAACCGG´3 and REVERSE PRIMER: 5`TCCGGTACCCGCTCAACCACCTCTTAG´3. Lane 3. and Lane 5. PCR products digested by *Sau*96 I before and after cloning of UbF45W gene. After cloning of the UbF45W gene the in YEp96 the gene develops a new restriction site for gene *Sau*96 I which cuts the gene into two fragments of 100bp and 134bp.

5.3.1.5. Construction of YEp96-UbE64G

The ubiquitin gene (≈250bp) was PCR amplified from pKK223-3-UbE64G was clone in the YEp96 vector in *Bgl* II and *Kpn* I site. Cloning of UbE64G in YEp96 vector was confirmed by loss of *Sal* I restriction site and further by sequencing (Figure 5.10.).

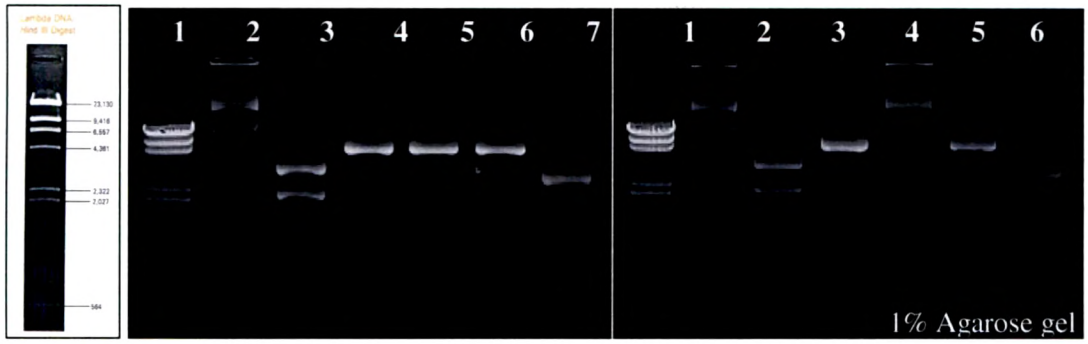


(a) **(b)**

Figure 5.10. Restriction digestion pattern of (a) YEp96-UbWt and (b) YEp96-UbE64G. Lane 1. Contains λ *Hin*d III digest as marker. Lane 2. Contains undigested plasmid (a) YEp96-UbWt and (b) YEp96-UbE64G. Lane 3 There are three *Hind* III sites in the vector and hence the *Hind* III digest shows three fragments of 646bp, 2099bp and 3434bp. Lane 4 contains *Bgl* II digest. Lane 5. Contains *Sal* I digest with a unique restriction site, gives a single band of 6179bp fragment with (a) YEp96-UbWt, (b) and is lost after subcloning of the gene with UbE64G mutation. Lane 6. Contains *Kpn* I digest. *Bgl* II and *Kpn* I sites were used for subcloning. Lane 7. There are three *Eco*R I sites in the vector, and therefore the *Eco*R I digest shows three fragments of 628bp, 2818bp and 2733bp.

5.3.2. Construction yeast expression vector carrying UbS65D mutation in YEp96 vector

YEp96-UbS65D was constructed by subcloning of UbS65D gene PCR amplified from pKK223-3-S65D plasmid (Chapter 2). The subcloning strategy was same as UbE64G gene. The plasmid YEp96-UbS65D was also screened for loss of restriction site for *Sal* I and later it was sequenced for confirmation (Figure 5.11.).



(a) **(b)**

Figure 5.11. Restriction digestion pattern of (a) YEp96-UbWt and (b) YEp96-UbS65D. Lane 1. Contains λ *Hin*d III digest as marker. Lane 2. Contains undigested plasmid (a) YEp96-UbWt and (b) YEp96-UbS65D. Lane 3. There are three *Hind* III sites in the vector and hence the *Hind* III digest shows three fragments of 646bp, 2099bp and 3434bp. Lane 4. Contains *Bgl* II digest. Lane 5. Contains *Sal* I digest with a unique restriction site giving a single band of 6179bp fragment with (a) YEp96-UbWt, (b) and is lost after subcloning of UbS65D gene. Lane 6. Contains *Kpn* I digest. *Bgl* II and *Kpn* I sites were used for subcloning. Lane 7. There are three *Eco*R I sites in the vector so the digest contains three fragments of 628bp, 2818bp and 2733bp.

5.3.3. Construction yeast expression vector carrying UbQ2N mutation in YEp96 vector

YEp96-UbQ2N was constructed by indirect subcloning of UbQ2N gene PCR amplified from pKK223-3-UbQ2N plasmid (Chapter 2). Subcloning of UbQ2N gene directly using the restriction enzyme sites *Bgl* II and *Kpn* I was not possible as the *Bgl* II site was lost while introducing Q2N mutation. Alternative strategy was used to clone UbQ2N gene where the 687bp fragment containing ubiquitin ORF (UbORF) under CUP1 promoter

from the YEp96 backbone (Figure 5.14.) was subcloned in pBSK KS/SK(+)(Figure 5.12.). The directional subcloning of (pBSK-UbORF) (Figure 5.13.) was done between *Bam* HI and *Kpn* I restriction sites. The mutant gene UbQ2N was amplified by PCR from pKK223-3-Q2N plasmid and was subcolned in pBSK-UbORF (pBSK-UbORF) (Figure 5.13.). The clones were screened for loss of *Bgl* II site in the ubiquitin ORF. This mutant ubiquitin ORF is cloned back to YEp96 vector (Figure 5.14.). The plasmids YEp96-UbQ2N was also screened by loss of restriction site for *Bgl* II and later it was sequenced for confirmation.

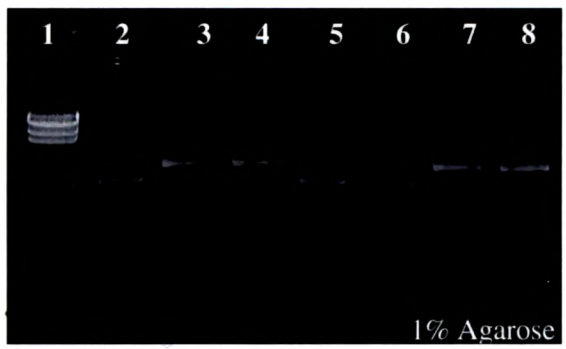
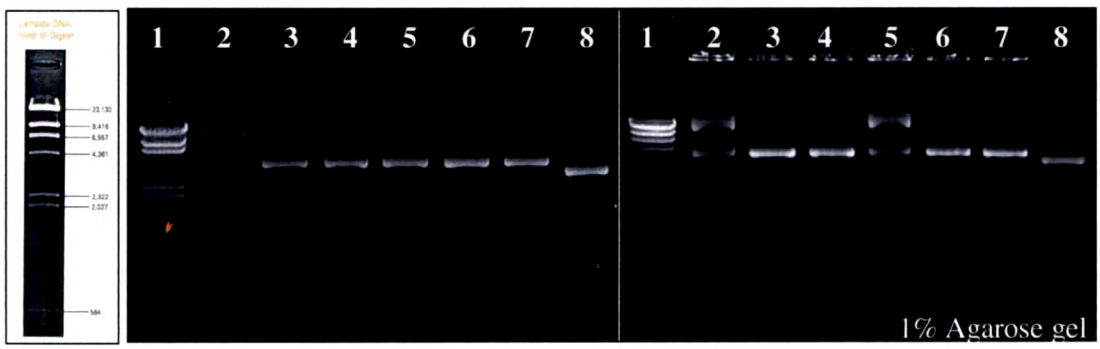
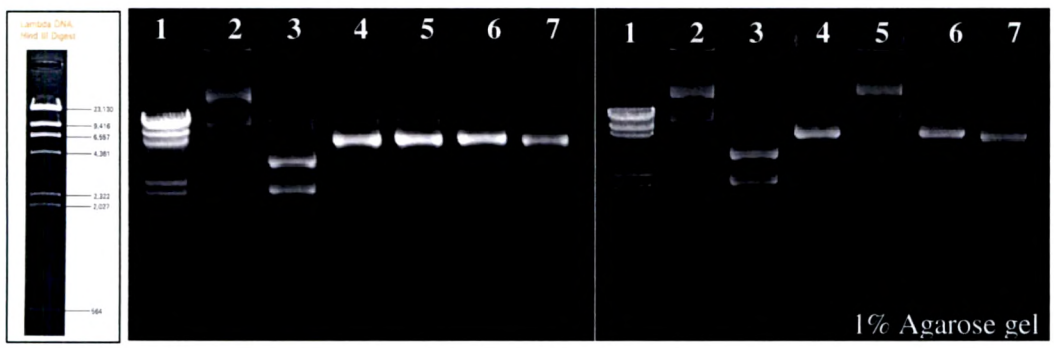


Figure 5.12. Restriction digestion pattern of pBSK KS/SK(+) . Lane 1. Contains λ *Hin*d III digest as marker. Lane 2. Contains undigested plasmid of pBSK KS/SK(+). Lane 3,. Lane 4., Lane 5., Lane 6., and Lane 7. Contains *Eco* RV digest, *Sal* I digest, *Bgl* II digest, *Afl* II digest, *Bam* HI digest and *Kpn* I digest respectively. Restriction sites of *Eco* RV, *Sal* I, *Bam* HI and *Kpn* I are unique to the vector and gives a single band of 2961bp on digestion. *Bgl* II and *Afl* II site are absent in the pBSK KS/SK(+).



(a) **(b)**

Figure 5.13. Restriction digestion pattern of pBSK-KS/SK(+)-Ub(Wt.ORF) and pBSK KS/SK(+)-Ub(Q2N.ORF). Lane 1. Contains λ *Hind* III digest as marker. Lane 2. contains undigested plasmid (a) pBSK-KS/SK(+)-Ub(Wt.ORF) and (b) pBSK KS/SK(+)-Ub(Q2N.ORF). Lane 3. and Lane 4. Contatins *Bam* HI and *Eco* RI respectively digesting the vector to give a single band of 3639 bps. Lane 5. Contains *Bgl* II digest with a unique restriction site, giving a single band of 3639bp fragment with (a) pBSK KS/SK(+)-Ub(Q2N.ORF) and (b) and is lost after subcloning of UbQ2N gene in *Eco* RI-*Kpn* I site. Lane 6. Contains *Kpn* I digest. Lane 7. Contains *Bam* HI-*Kpn* I double digest, which releases 678bp ubiquitin ORF along with CUP1 promoter leaving 2961bp backbone.



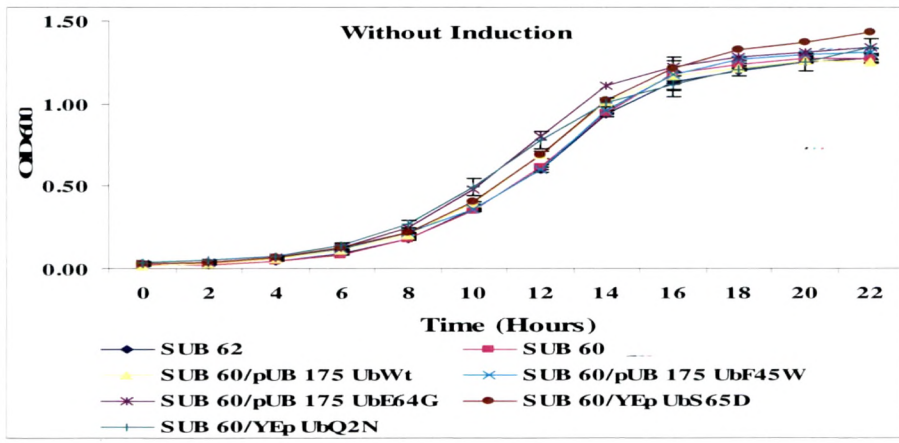
(a) **(b)**

Figure 5.14. Restriction digestion pattern of (a) YEp96-UbWt and (b) YEp96-UbQ2N. Lane 1. contains λ *Hind* III digest as marker. Lane 2. Contains undigested plasmid (a) YEp96-UbWt and (b) YEp96-UbQ2N. Lane 3. There are three *Hind* III sites in the vector and the *Hind* III digest shows three fragments of 646bp, 2099bp and 3434bp. Lane 4. contains *Bam* HI digest. Lane 5. contains *Bgl* II digest with a unique restriction site, giving a single band of 6179bp fragment with (a) YEp96-UbWt, (b) and is lost after subcloning of gene with UbS65D mutation. Lane 6. contains *Kpn* I digest. Lane 7. Contains *Bam* HI-*Kpn* I double digest, which releases 678bp ubiquitin ORF along with CUP1 promoter leaving 5501bp backbone. *Bam* HI and *Kpn* I sites were used for subcloning.

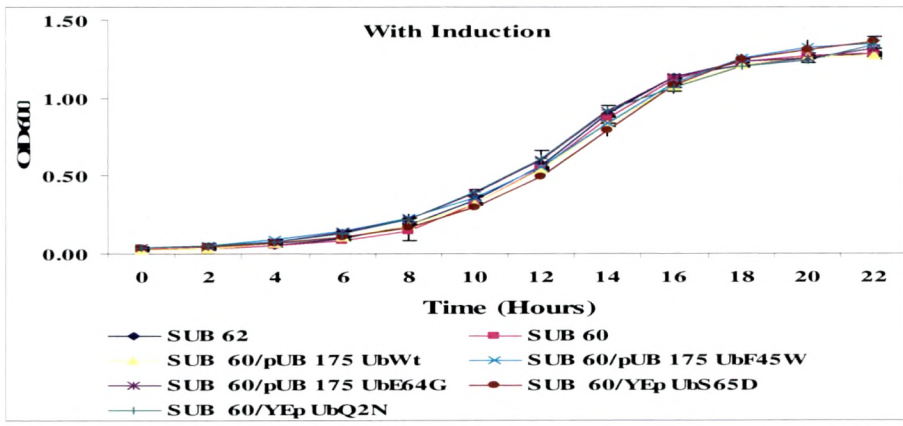
5.3.4. Effects of mutants gene expression on growth of *S. cerevisiae.*

The mutant genes for UbE64G, UbS65Dand UbQ2N were expressed in yeast cells after transformation and by subjecting to auxotropic selection. The genes were under copper sulphate inducible CUP1 promoter. *S. cerevisiae* cells transformed with UbWt and UbF45W were used as controls for growth conditions.

The mutants of ubiquitin may have effect on the growth of yeast cells. As ubiquitin participates in variety of physiological processes, impaired function due to mutation has a bearing on growth rates. However these mutants do not show any altered growth effects compared to UbWt and UbF45W controls under normal conditions (Figure 5.15.).



(a)



(b)

Figure 5.15. Growth curves of *Saccharomyces cerevisiae* (SUB60 and SUB62) and SUB 60 transformant under (a) uninduced and (b) induced conditions.

5.3.5. Complementation of Stress hypersensitive phenotype SUB60 cells

5.3.5.1. UV-C Sensitivity Complementation

The mutations do not have show any effect on the survival of the cells after being exposed to UV-C radiation, implying that the mutations do not have any bearing on UV repair mechanisms (Figure 5.16.).

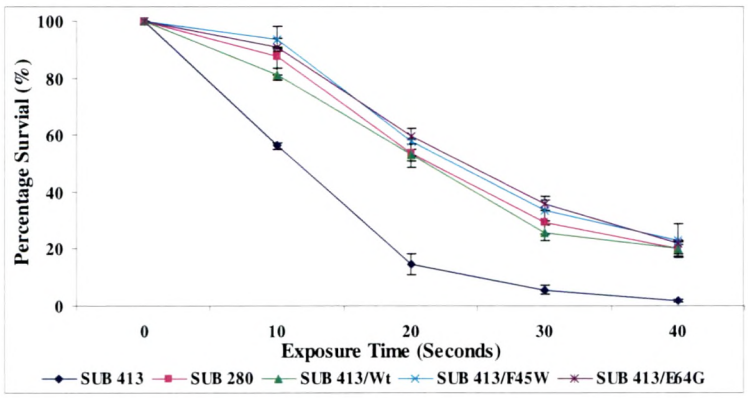
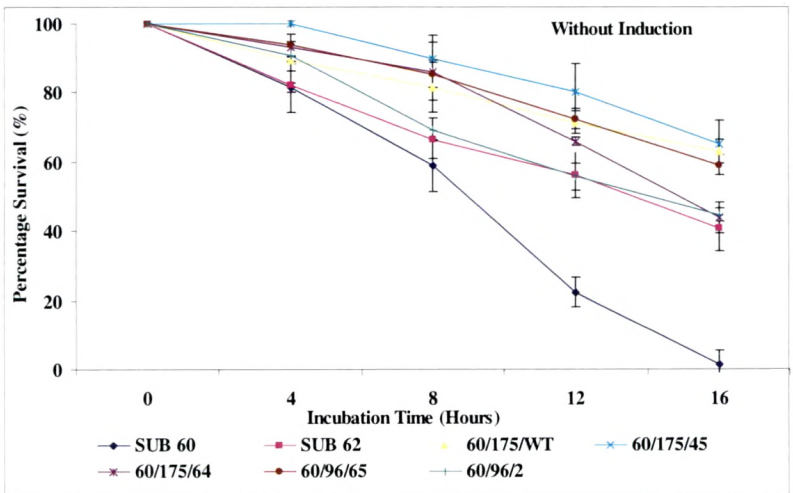


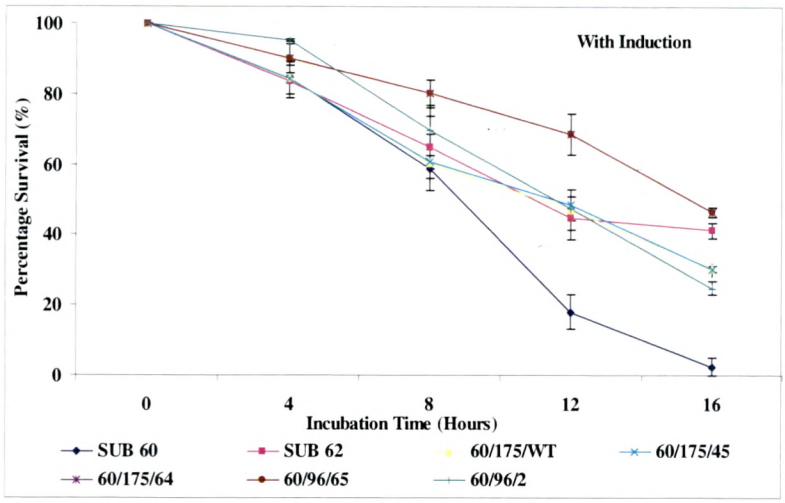
Figure 5.16. UV-C Complementation of *Saccharomyces cerevisiae* (SUB60 and SUB62) and SUB 60 transformant after induction by copper sulphate.

5.3.5.2. Heat Stress Complementation

The mutations do not seem to have any negative effect on the survival rate of the SUB60 cells after heat treatment (Figure 5.17).



(a)



(b)

Figure 5.17. Heat stress complementation of *Saccharomyces cerevisiae* (SUB60 and SUB62) and SUB 60 transformants under (a) uninduced and (b) induced conditions.

5.3.5.3. Antibiotic Sensitivity test

The antibiotic stress complementation analysis with mutant forms of ubiquitin shows that the mutations have drastic effect on survival of SUB60 cells (Figure 5.18.). The β-bulge of ubiquitin seems to play a crucial role in overcoming the antibiotic stress. Alterations in ubiquitin structure produced due to the mutations do not allow the variants to play active role.

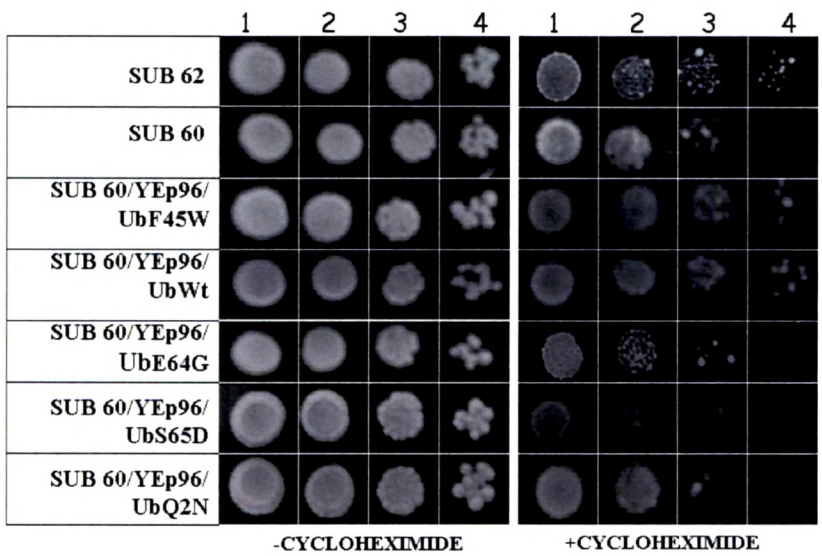
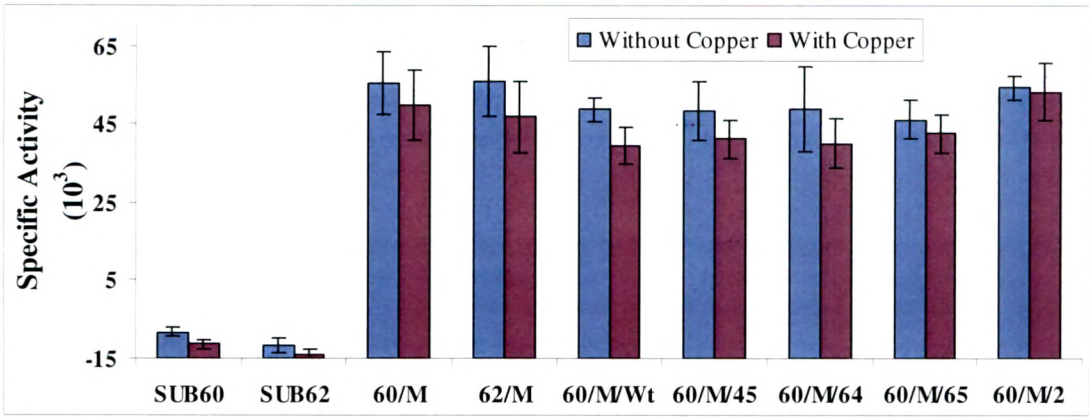


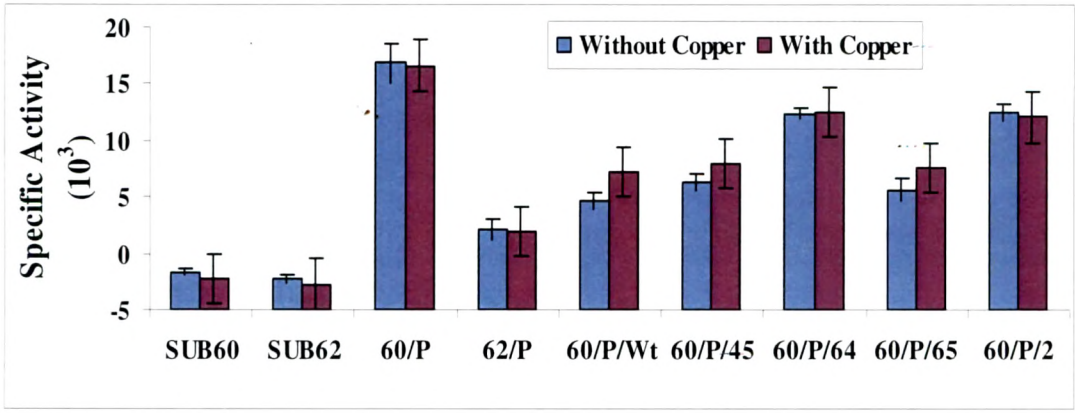
Figure 5.18. Antibiotic stress complementation of *Saccharomyces cerevisiae* (SUB60). Cycloheximide resistance of SUB62, SUB60 cells transformed with UbWt and UbF45W gene. The SUB60 and SUB60 transformed with UbE64G, UbS65D and Q2N fail to grow in presence of cycloheximide. Three fold serial dilutions (2), (3) and (4) spotted on YPD plates containing 100μM of copper and 4μg/ml of cycloheximide.

5.3.6. N-end rule as degradation signal in *Saccharomyces cerevisiae*

Ubl4 gene cluster has 4-5 copies of ubiquitin expressed as a single polypeptide chain, which is processed into ubiquitin molecules post-translationally. SUB60 mutants lacking Ubl4 fail to withstand stress conditions, but grow normally at 30°C. SUB60 mutants lacking UBI4 gene and SUB62 wild type cells were transformed with yeast plasmids (carrying variants of ubiquitin gene as indicated), Yep96-UbWt, Yep96-UbF45W, Yep96-UbE64G, Yep96-UbS65D and Yep96-UbQ2N. Yep96-UbWt, Yep96-UbF45W, Yep96-UbE64G, Yep96-UbS65D and Yep96-UbQ2N are plasmids carrying ubiquitin gene under CUP-1 promoter and have tryptophan auxotrophy for selection. These transformants were co-transformed by pUB23, a plasmid with wild type ubiquitin gene fused to lacZ. According to N-end rule, the stability of a protein is determined by its N-terminal residue. β-galactosidase fusion with stabilizing N-terminal residues like Met, is cleaved by ubiquitin hydrolases releasing a free β-galactosidase with longer half life. Those fusions with destabilizing residues such as Pro are not processed and a polyubiquitin chain is built subsequently on the ubiquitin in the fusion protein, resulting in degradation of the β-galactosidase. The ubiquitin-β-galactosidase fusion is under pGAL10 promoter. β-galactosidase activity assay has been used as a measure of protein stability. Our results show β-galactosidase activity remained more or less unchanged in Yep96-Wt, when Met (stabilizing residue) was present as the N-end residue. β-galactosidase activity showed a decline, when Pro was present as the N-end residue. However, the β-galactosidase activity increased for Pro-β-galactosidase in the mutants UbE64G, UbS65D and UbQ2N compared to UbWt and UbF45W in Ubl4 mutant strain SUB60 (Figure 5.19.)



I



II

Figure 5.19. Effect of UbE64G, UbS65D and UbQ2N mutations on the half-life of proteins in UbI4 background. SUB60, SUB62, SUB60 transformed with YEp96-UbWt (60/Wt), YEp96-UbF45W (60/45), YEp96-UbE64G (60/64), YEp96-UbS65D (60/65) and YEp96-UbQ2N (60/2) expressing the wild type ubiquitin UbWt, UbF45W and three mutants of ubiquitin, namely UbE64G, UbS65D and UbQ2N respectively. These transformant were also cotransformed with 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 transformed by plasmid pUb23 expressing Ub-β-galactosidase fusion with (I) Met (M) (60/M, 62/M) and (II) Pro (P) (60/M, 62/M) as the N-terminal residues. SUB60 and SUB62 were used as controls.

5.3.6. UbE64G and UbS65D polyubiquitination of substrate protein in *Sacchromyces cervisiae*

Polyubiquitnation ability of UbE64G and UbS65D mutant ubiquitins was studied using myc-tagged ubiquitin mutants (Figure 5.23). Tagging with myc was necessary to demarcate the ubiquitin molecules carrying the mutation from basal levels of ubiquitin which is present in the background, as ubiquitin is expressed by more than one gene in the yeast cells. Though, myc-tagged wild type ubiquitin is used for ubiquitination, the resultant polyubiquitin chains are not recognized for degradation by proteasome machinery, giving rise to a characteristic ladder pattern in SDS-PAGE gels.The myc-tagged UbE64G and UbS65D were utilized for polyubiquitination and were not discriminated by ubiquitination machinery (Figure 5.23).

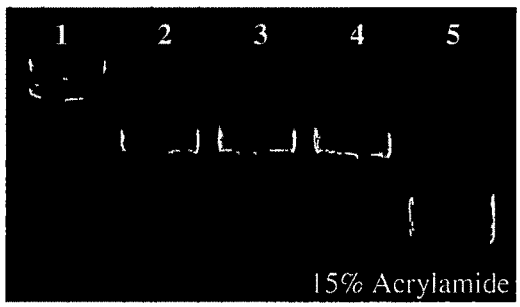
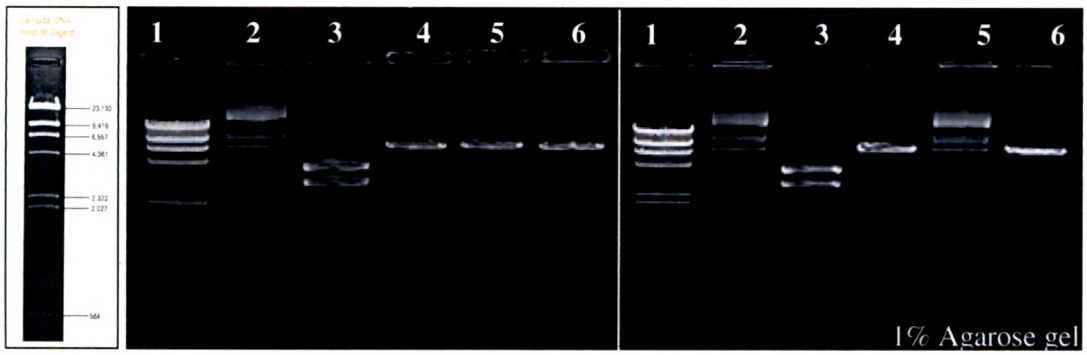
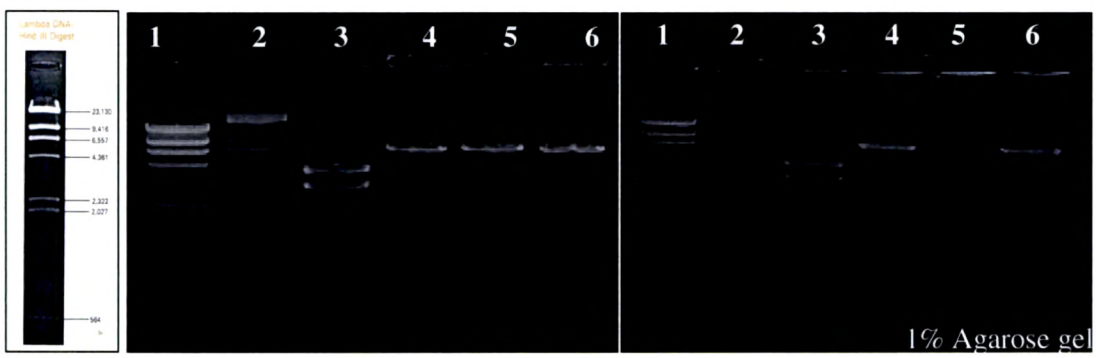


Figure 5.20. Restriction digestion pattern of pUB221/c-myc-UbF45W. Lane 1. Contains 100bp ladder. Lane 2. and Lane 4. Contains 234bp extension from pUB221/c-myc-UbWt and pUB221/c-myc-UbF45W respectively using primer combination of FORWARD PRIMER: 5`ATGCAG ATCTTCGTCAAGACGTTAACCGG ´3 and REVERSE PRIMER: 5`TCCGGTACCCGC TCAACCACCTCTTAG´3. Lane 3. and Lane 5. Digested PCR products of *Sau*96 I before and after cloning of UbF45W gene. After cloning of the UbF45W gene the in pUB221 the gene develops a new restriction site to the gene *Sau*96 I which digests the gene into two fragments of 100bp and 134bp.



(a) (b)

Figure 5.21. Restriction digestion pattern of (a) pUB221/c-myc-UbWt and (b) pUB221/c-myc-UbE64G. Lane 1. Contains λ *Hin*d III Digest marker. Lane 2. Contains undigested plasmid of (a) YEp96-UbWt and (b) pUB221-c-myc-UbE64G. Lane 3. There are three *Eco*R I sites in the vector so which contains *Eco*R I digest shows three fragments of 694bp, 2818bp and 3733bp fragments. Lane 4. Contains *Bgl* II digest. Lane 5. contains *Sal* I digest is a unique restriction sites and give a single band of 7286bp fragment with (a) pUB221/c-myc-UbWt, which is the actual size of the vector and is lost after subcloning of c-myc-UbE64G gene (b). Lane 6. Contains *Kpn* I digest. *Bgl* II and *Kpn* I sites used for subcloning the gene.



(a) (b)

Figure 5.22. Restriction digestion pattern of (a) pUB221/c-myc-UbWt and (b) pUB221/c-myc-UbS65D. Lane 1. Contains λ *Hin*d III digest as marker. Lane 2. Contains undigested plasmid (a) pUB221-c-myc-UbWt and (b) pUB221-c-myc-UbS65D. Lane 3. There are three *Eco*R I sites in the vector and hence *Eco*R I digest contains three fragments of 694bp, 2818bp and 3733bp. Lane 4. Contains *Bgl* II digest.

Lane 5. Contains *Sal* I digest is a unique restriction site, giving a single band of 7286bp fragment with (a) pUB221/c-myc-UbWt and (b) is lost after subcloning of c-myc-UbS65D. Lane 6. Contains *Kpn* I digest. *Bgl* II and *Kpn* I sites were used for subcloning the gene.

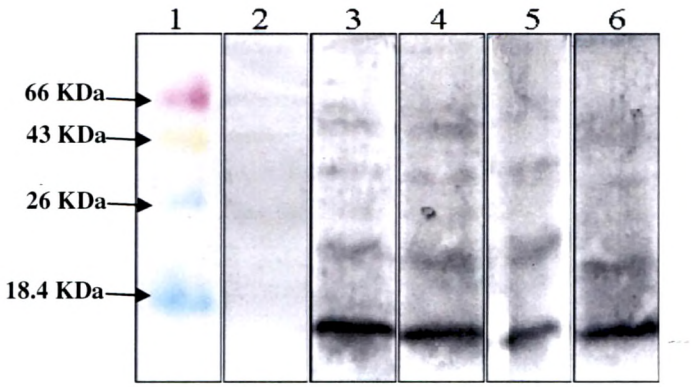


Figure. 5.23. Western blot showing ubiquitination profile. Lane 1. Rain bow molecular weight marker. Lane 2. SUB60 as negative Control. Lane 3. SUB60-UbWt and Lane 4. SUB60-UbF45W as positive control and Lane 5. SUB60-UbE64G Lane 6. SUB60S65D

5.4. DISCUSSION

Proteins showing complete conservation of their sequence through millions of years of evolution are examples of optimal balance of structure and function. Ubiquitin is one such protein, showing no change in sequence from insects to humans and only three substitutions in yeast. A close look at its structure indicates that in the second β-bulge there are certain unusual features. The first residue in the β-bulge has a glutamate in place of glycine, the later being a preferred choice in such structures. Structural homology searches also revealed that close structural homologs of ubiquitin, NEDD8 and Rub1 (Whitby et al., 1998) and ubiquitin like protein from baculovirus *Autographa californica* (Guarino et al., 1990) have glycine at the same position. Interestingly, those structures are identical to ubiquitin in this region. Similarly Ser65 and Gln2 were changed to Asp and Asn respectively, keeping the percentage preferences in β-bulge and the residues in respective positions in homologs in mind.

The changes in structure of the molecules are marginal and have minor effects on the stability of ubiquitin. However, these substitutions appear to be more significant effect on the function of the molecule. The mutant forms of ubiquitin fail to complement and rescue UBI4 deletion mutant SUB60 cells. Interestingly, UbE64G, UbS65D and UbQ2N have extended the half life of unstable substrate proteins with N-terminal Pro residue.

In conclusion, replacing the conserved residues Glu64, Ser65 and Gln2 to Gly, Asp and Asn respectively lead to subtle changes in the structure of the protein, resulting in alterations in its substrate interaction behavior and complementation under antibiotic stress. The wild type protein leads to slower turnover of protein with N-terminal Met residue, compared to the proteins with N-terminal Pro residue. The mutants UbE64G, UbS65D and UbQ2N have reversed behavior, they indeed extended the half life of protein with N-terminal Pro, which should have been removed from the system faster.

This study provides interesting examples of single residue substitutions with subtle structural alteration and with a significantly altered function.