Modifications of β-glucuronidase as a reporter gene for protein sorting in saccharomyces cerevisiae

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Modifications of β-Glucuronidase As a Reporter Gene for Protein Sorting in *Saccharomyces cerevisiae*

by

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DEDICATION

This dissertation is dedicated to all the members of the Leung's Family.

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ABBREVIATIONS

Amp = Ampicillin

A_{260} = Absorbance at 260nm

A_{600} = Absorbance at 600nm

bp = Base pair

CPY = Carboxypeptidase Y

dNTP = Deoxyribonucleoside triphosphate

ddNTP = Dideoxyribonucleoside triphosphate

DTT = Dithiothreitol

EDTA = Ethylenediaminetetra-acetic acid

EtBr = Ethidium bromide

*Exo*III = Exonuclease III

GUS = β-Glucuronidase

IPTG = Isopropylthiogalactoside

Kb = Kilobase

kD = Kilodalton

LMP = Low melting point

MUG = 4-Methylumbelliferyl glucuronide

PAGE = Polyacrylamide gel electrophoresis

PEG = Polyethylene glycol

RNase = Ribonuclease

SDS = Sodium dodecyl sulphate

ssDNA = Single-stranded DNA

Tris = Tris(hydroxymethyl)methylamine[2-amino-2-(hydroxymethyl)propane-1,3-diol, (tris)]

ABSTRACT

Several carboxypeptidase Y-β-glucuronidase (CPY-GUS) plasmids were expressed in yeast. The CPY-GUS fusion proteins were inactivated during passage through the secretory pathway. This work was done in an attempt to recover GUS activity in yeast. CPY-GUS plasmids were transformed to protease-deficient yeast strains so as to test whether the low GUS activity was due to the degradation by intracellular proteases. The results failed to show this because of the problems associated with the western blots. Tunicamycin, a drug that inhibits glycosylation, was added to the yeast culture in order to recover GUS activity. The results show that a short term treatment could increase GUS activity by about 10%. In a long term treatment, tunicamycin was found to affect the normal growth of yeast adversely and reduce the GUS activity by 90%. Site-directed *in vitro* mutagenesis was employed to remove the two cryptic N-glycosylation sites within GUS. Two single mutants (160Mu and 161Mu) and one double mutant (160+161)Mu were created. They were cloned in *E. coli* TG2 and their enzymatic activities were tested. The results show that all the mutated GUS (160Mu, 161Mu and (160+161Mu)) had higher enzymatic activities than the wild type GUS.

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Chapter I

INTRODUCTION

1.1 Protein Targeting

One of the crucial functions of cells is protein production. With thousands of different proteins produced in each cell, specific mechanisms (protein targeting) must be present in order to transport the newly made proteins to the correct compartment so as to avoid biochemical confusions that would result from a random mix of proteins.

In eukaryotes, there are several transport pathways. The secretory pathway transports proteins through a series of membranous organelles from the ER (endoplasmic reticulum) to the Golgi apparatus and hence to the lysosomes or vacuoles or plasma membrane. These events are associated with glycosylation, sorting and folding of the transported proteins (Burgess and Kelly, 1987). Other pathways exist for proteins that are destined for organelles, such as mitochondria, chlorplasts, peroxisomes and the nucleus.

The localization of these newly synthesized polypeptides into their respective sub-cellular compartments is thought to be mediated by signals within proteins which are recognized by specific receptors (Horwich *et al.,* 1985).

Proteins that destined for the nucleus or mitochondria are synthesized completely in the cytoplasm before transfer begins. Nuclear proteins have a short, bacic sequence within the protein that ensures delivery to the nuclear matrix and retention within (Dingwall and Laskey, 1986). Most mitochondrial

proteins are synthesized as soluble higher molecular weight precursors, which possess an amino-terminal extension or transit peptide. The transit peptide is cleaved from the mature protein during or after translocation into the mitochondria (Gellar *et al.*, 1983; Douglas *et al.*, 1984; Hurt *et al.*, 1984). Proteins following the secretory pathway are synthesized in the cytoplasm and co-translationally translocated across the ER membrane under the control of a generally hydrophobic signal peptide (Blobel and Dobberstein, 1975). This is aided by two protein complexes, the signal recognition particle (SRP) (Walter and Blobel, 1981) and the SRP-receptor or docking protein (Meyer *et al.*, 1982). During translocation, most signal peptides are removed by signal peptidase (Blobel and Dobberstein, 1975) and the core asparagine-linked carbohydrate units are added within the lumen of the ER (Kiely *et al.*, 1976; Rothman and Lodish, 1977). Most proteins are exported from the ER to the Golgi, but those soluble proteins that function in the lumen of the ER have a carboxy-terminal tetrapeptide KDEL or HDEL in mammalian cells or yeasts respectively (Munro and Pelham, 1987; Pelham *et al.*, 1988). Those proteins, that are destined to vacuoles, plasma membranes or secretory vesicles, are then transported to the Golgi where they may be further phosphorylated and/or glycosylated by glycosyl transferases and mannosidases (Hirschberg and Snider, 1987). The function of the carbohydrate in many cases is poorly understood. Glycosylation may be important for sorting, e.g. the targeting of proteins to the lysosomes is mediated by a receptor protein that recognizes a mannose phosphate. It is not essential for secretion because this still occurs in the presence of drugs, such as tunicamycin, which block glycosylation. For some proteins, however, glycosylation may help to stabilize an optimum conformation that increases the efficiency of secretion (Ferro-Novick *et al.*, 1984).

1.2 Yeast: An Eukaryotic Model to Study Protein Targeting

The basic structure of the yeast secretory pathway has been shown to be conserved with respect to the secretory pathways of higher eukaryotes (Schekman, 1985). Yeast is then an ideal eukaryotic model to study protein targeting. Novick and Schekman (1983) and Stevens *et al.,* (1982) have demonstrated that yeast secretory, plasma membrane, and vacuolar proteins transit at least certain portions of the secretory pathway en route to their ultimate subcellular compartments. The study of vacuolar proteins transport provides a way to elucidate the targeting and sorting signals of the secretory pathway. Proteins or peptides that lack any apparent delivery signals other than an amino-terminal signal peptide can be secreted from yeast and mammalian cells (Johnson *et al.,* 1987; Kelly, 1985; Valls *et al.,* 1987: Wiemken *et al.,* 1987). This suggests that secretion occurs by a default mechanism. Proteins which are diverted from the secretory pathway to other cellular destinations therefore must contain active sorting information in their amino acid sequence or in their structure that is recognized during transit through the cell.

1.3 A Yeast Vacuolar Protein: CPY

The yeast vacuole is a lysosome-like organelle as it sequesters a number of glycoprotein hydrolases, e.g. carboxypeptidase Y (CPY), proteinase A, proteinase B, alkaline phosphatase and ribonuclease A. Among them, CPY has been studied in some detail. It is synthesized as an inactive precursor protein (Hasilik and Tanner, 1978a). CPY is a mannoprotein and initially undergoes dolichol-mediated core glycosylation (in which four asparagine-linked

oligosaccharide chains are added) in the ER, which leads to the formation of a precursor form of molecular weight equivalent to 67kD (Hasilik and Tanner, 1978b). Subsequently, addition of α1-3- and α1-2-linked mannose residues to asparagine-linked core carbohydrate and phosphorylation at the 6-position of mannose takes place in the Golgi complex (Rungo, 1988). The resulting proCPY has a molecular weight of 69kD which is ready to be delivered to the vacuole (Stevens *et al.,* 1982). Sorting of proCPY or other vacuolar proteins is thought to take place on the trans side of the Golgi complex (Steven et 1982; Johnson *et al.,* 1987). Just before or upon arrival in the vacuole, the inactive proCPY is activated by the cleavage of the amino-terminal 8kD propeptide by proteinase A and proteinase B (Hemming *et al.,* 1981; Mechler *et al.,* 1987).

1.4 Use of a Reporter Gene to Study Protein Targeting

When the signal peptide for proteins that enter the secretory pathway are compared, they are usually between 20 to 40 amino acids long and comprise a stretch of basic residues at the extreme N-terminus followed by a block of at least nine hydrophobic residues (von Heijne, 1985). On the other hand, the transit sequences of the proteins destined for mitochondria are extremely variable and tend to have a high number of basic and hydrophobic residues but few acidic residues. They also have the potential to form amphiphilic α-helices (von Heijne, 1986), but there is no obvious homology in terms of their amino acid sequences (Watson, 1984). This means that it has not been feasible to identify unequivocal targeting signals by simple analysis of the primary amino acid sequences or by directly mutating suspected sequences. An alternative approach to the identification of targeting or sorting information is to a use reporter gene fusion system, in which the region coding for the putative signal

is fused to a heterologous coding sequence. The heterologous coding sequence (reporter gene) should encode a protein that has a readily assayable function and which contains no internal topogenic sequences.

Initial studies fused the yeast secretory protein invertase signal sequence to the *E. coli* β-galactosidase coding sequence. The fusion protein was transported to the ER indicating that the signal sequence was functional but the protein was not secreted (Emr *et al.*, 1984). It appears that β-galactosidase contains a signal that causes retention within the ER and other studies have shown that it also contains a nuclear targeting signal (Kalderon *et al.*, 1984b). Hence, it is not an ideal reporter gene.

The other reporter gene used to identify the signal that targets carboxypeptidase Y (CPY) to vacuoles was yeast invertase (Johnson *et al.*, 1987). The coding sequence of invertase (lacking signal peptide) was fused to amino-terminal segments of CPY. The results showed that the N-terminal 50 amino acids of CPY were quite sufficient to direct delivery of a CPY-invertase hybrid protein to vacuoles. They also found that the first 20 amino acids constituted the N-terminal signal peptide that directs translocation of CPY to the ER and the remaining 30 amino acids contain the vacuolar targeting signal. Deletion of this vacuolar sorting signal from wild type CPY leaded to missorting and eventual secretion of CPY. These results were further supported by Valls *et al.*, (1987) who used the same invertase gene fusion system. In addition, they discovered that the N-terminal region of the CPY propeptide near amino acid 24 was required for the targeting of CPY to the vacuole. A lot of valuable information about protein sorting was obtained from this invertase gene fusion system but it has some disadvantages. It is

heavily glycosylated in a heterogenous fashion. Therefore, no clear bands will be obtained in western blots of cell extracts with anti-invertase antibody. In addition, it is an endogenous enzyme found both as secreted and cytoplasmic forms (Perlman and Halvorson, 1981; Carlson and Botstein, 1982). To avoid confusion of results, the host strains should have a complete deletion of the chromosomal copy of *SUC2* and contain no other unlinked invertase structural genes. Thus, tedious strain construction must be carried out before experimental work can begin.

1.5 Beta-Glucuronidase

The *E. coli* β-glucuronidase (GUS) has a monomer molecular weight of about 68.2 kD (Jefferson *et al.*, 1986). The behaviour of the native enzyme on gel filtration columns indicates that it is probably a tetramer. GUS is very stable to many detergents, and can tolerate widely varying ionic conditions. It is most active in the presence of thiol reducing agents such as beta-mercaptoethanol or dithiothreitol (DTT). GUS has no cofactors nor any ionic requirements, however, it is inhibited by copper (II) and zinc (II) ions. Thus, it is necessary to include EDTA in the assay buffer (Stoeber, 1961). GUS can be assayed at any physiological pH, with an optimum between 5.2 and 8.0. The enzyme is about 50% as active at pH4.3. (Jefferson, 1985; Jefferson *et al.*, 1986). GUS is reasonably resistant to thermal inactivation with a half-life at 55°C of about two hours. It is an acid hydrolase that catalyzes the cleavage of a wide variety of beta-glucuronides (Stoeber, 1961). Substrates of GUS are generally water soluble (Paigen, 1979) and many are available, including those for spectrophotometric (p-nitrophenyl glucuronidase), or fluorometric (4-methy umbelliferyl glucuronide (MUG)) and for histochemical

(5-bromo-4-chloro-3-indolyl-glucuronide) analysis.

GUS is encoded by the *uid*A locus (Novel and Novel, 1973). The GUS gene has been cloned and sequenced (Jefferson *et al.*, 1986). The coding region of the gene is 1809 base pairs long. GUS was used initially as a gene fusion marker in *E. coli* and in *Caenorhabditis* (Jefferson *et al.*, 1987b). There is little or no detectable β-glucuronidase activity in *Drosophila melanogaster*, *Saccharomyces cerevisiae*, certain strains of *Caenorhabditis elegans* (Jefferson *et al.*, 1987a); *Dictyostelium discoidum* (Jefferson *et al.*, 1986) and in almost any higher plant (Jefferson *et al.*, 1987). The enzyme can tolerate a large amino-terminal addition without loss of activity (Jefferson *et al.*, 1987; Jefferson, 1987a) and be translocated across chloroplast membranes with high efficiency (Kavanagh *et al.*, 1988).

1.6 Study of Protein Targeting by a GUS fusion system

As β-glucuronidase can tolerate a large amino-terminal additions without loss of activity and be transported across chloroplast membranes with high efficiency, so construction of translational fusions is possible and may be a valuable method for assaying the behaviour of transit or signal peptides either in transgenic systems or *in vitro* (Jefferson, 1987a). Indeed, Iturriaga (1989) and Haider (1989) did try to use this system to study protein targeting to the ER in tobacco plants and to the vacuole in yeasts, respectively.

1.7 Inactivation of GUS in Secretory Pathways of Cells

Although the GUS-fusion system has been used in the study of protein targeting, and worked excellently with the targeting of chlorophyll a/b proteins to chloroplasts (Kavanagh *et al.*, 1988) , it was found to be inactivated

when used for protein targeting with the secretory pathway. Haider (1989) found GUS activity was reduced by one-third in yeasts compared with that in *E. coli* . Datta *et al.,* (1986) found no GUS activity at all when the cathepsin-like protease from *Dictyostelium* was fused to GUS. In addition, Iturriaga *et al.,* (1989) observed that when the putative signal peptide of patatin was fused to GUS, very little activity was found.

1.8 Attempts to Recover GUS Activities

Iturriaga *et al.,* (1989) realized that the inactivation of GUS in plants may be due to the glycosylation of the two cryptic glycosylation sites, NLS at position 358 to 360 and NIS at position 423 to 425. If GUS was directed to the ER by the putative signal peptide, then these sites may have been N-glycosylated by oligosaccharide transferase, which is only found on the lumenal side of the ER (Hirschberg and Snider, 1987). These post-translational modifications could inhibit the enzymatic activity of GUS (Iturriaga *et al.,* 1989) and may cause difficulty in determining the actual amount of GUS activity. Therefore, Iturriaga *et al.,* (1989) used tunicamycin, a drug that inhibits glycosylation, to treat their plant cell cultures, GUS activity was found to be recovered.

1.9 Site-directed *in vitro* Mutagenesis

An alternative approach to recover the GUS activity is to mutate the potential N-glycosylation sites by site-directed mutagenesis.

1.10 Aims of the Work

1. Use of tunicamycin to inhibit the glycosylation of β-glucuronidase (GUS) so as to recover the GUS activity in yeasts.

2. Use of site-directed *in vitro* mutagenesis to remove the N-linked glycosylation sites of GUS and test the change of the GUS activity.

Chapter II

MATERIALS AND METHODS

2.1 Materials

All materials and biological reagents were from the Sigma Chemical Company Ltd, Poole, Dorset, unless otherwise stated.

Restriction endonucleases, T4 DNA ligase, X-gal, agarose, LMP agarose were from Bethesda Research Labs (U.K.) Ltd, Cambridge, U.K.; Boeringer Corporation (London) Ltd, Lewes, East Sussex; or New England Biolabs, CP Labs, Bishops Stortford, Herts, U.K..

Klenow DNA polymerase I was from Northumbria Biological Ltd, Cramlington, Northumberland, U.K..

Caesium chloride, sodium chloride and potassium chloride were from Koch-light Ltd, Haverhill, Suffolk, U.K..

Bactoagar, Bactopeptone and Yeast nitrogen base without amino acids were from Difco Labs, Detroit, Michigan, U.S.A..

Yeast extract was from Sterlin Ltd, Teddington, U.K..

Glass beads were from BDH Chemicals Ltd, Poole, Dorset, U.K..

3MM paper was from Whatman Ltd, Maidstone, Kent, U.K..

Nitrocellulose filters were from Schleicher and Schuell, Postfach-4, D-3354, Dassel.

High vacuum grease was from Dow Corning S.A., Senefte, Belgium.

Polaroid 667 film was from Polaroid (U.K.) Ltd, St. Albans, Hertfordshire.

BBL trypticase peptone was from Becton Dickinson and Co, Cockeysville, M.D., U.S.A..

2.1.1 Yeast Strains

All strains used throughout this work were *Saccharomyces cerevisiae.*

MD40-4C α *ura2, trp1, leu2-3, leu2-112, his3-11, his3-15.*

YHH19 α *leu, his, ura3*Δ*5.*

YHH32 α *pra1::URA3 prb1*Δ*A5 his 3-11,15 leu2-3,112 ura3*Δ*5*

CL-2 ABYS106 *pra1-1 prb1-1 prc1-1 cps1-3 his3 ura3 leu2*

2.1.2 Bacterial Strains

The bacterial strain used in the course of this work was *E. coli* .

TG2 Δ(*lac, pro*) *sup*E, *thi rec*A, srl::Tn10^{TcR}, *hsd*Δ5 (r^-, m^-), F'(*tra* D36, *pro* AB^+, *lac*I^q, *lac*Z ΔM15.

2.1.3 Plasmids

pUC18: (Vieira and Messing, 1982), Amp^R

pDUB2505: CPY-GUS in pEMBLYe31

pDUB2512: CPY-GUS in YCp50

pDUB2525: CPY-GUS (no ATG-GUS) in pEMBLYe31

2.1.4 Glassware and Other Equipment

All solutions, glassware, plasticware and other equipment used in DNA manipulation, growth and maintenance of yeast and bacterial cultures were autoclaved at 15p.s.i. for 15 minutes or filter sterilized through a 0.25μm filter before use.

Glass beads were prepared by soaking in 4% nitric acid for an hour, washing extensively with sterile distilled water and then dried.

2.1.5 Growth Media

YPD Medium (complete medium)

10g yeast extract
20g bacto-peptone
20g glucose
(1.5% Bacto-agar)
per 1 litre of distilled water

Ymin (Yeast minimal medium)

6.7g yeast nitrogen base without amino acids
20g glucose
20mg amino acid as required
(1.5% Bacto-agar)
per 1 litre of distilled water

Amino acids were dissolved in distilled water at a concentration of 5mg/ml and autoclaved.

L-Broth

10g tryptone

5g yeast extract

5g sodium chloride

(1.5% Bacto-agar)

per 1 litre of distilled water

2x YT medium

16g tryptone

10g yeast extract

5g sodium chloride

(1.5% Bacto-agar)

per 1 litre of distilled water

2.1.6 Other Media or Buffers

SOB medium

20g tryptone

5g yeast extract

0.6g NaCl

0.5g KCl

10mM magnesium chloride

10mM magnesium sulphate

per 1 litre of distilled water

Transformation buffer 1

12g RbCl

9.9g $MnCl_2.4H_2O$

30ml of 1M potassium acetate, pH7.5

1.5g $CaCl_2.2H_2O$

150g Glycerol

Adjust to pH5.8 by 0.2M acetic acid and volume to 1 litre

and then filter sterilized

Transformation buffer 2

20ml of 0.5M MOP, pH6.8

1.2g RbCl

11.0g $CaCl_2.2H_2O$

150g Glycerol

Adjust to pH6.8 by NaOH and volume to 1 litre

and then filter sterilized

100x Denhardt's solution

2g Ficoll

2g Polyvinlpyrrolidone

2g Bovine serum albumin

per 100ml of distilled water and filter sterilized

30x SSC

263g NaCl

132.3g trisodium citrate

Adjust to pH7.0 with NaOH and 1 litre

Antibiotics

Ampicillin (Amp)

Stock solution: 25mg/ml

Final concentration: 50μg/ml

Chloramphenicol (Cm)

Stock solution: 12.5mg/ml

Final concentration: 25μg/ml

2.2 Growth Conditions

2.2.1 Yeast

Yeasts were grown in YPD as a complete medium or YMM as a minimal medium at 30°C. Liquid cultures were agitated on a rotary shaker to ensure good aeration.

2.2.2 Bacteria

Bacteria were usually grown at 37°C on L-agar or 2X YT agar plates. TG2, which is the host of the phage M13, was grown on glucose/minimal medium to select for the maintainance of F-factor. Liquid cultures were agitated on a rotary shaker to ensure good aeration.

2.3 General Techniques

2.3.1 Enzymatic Manipulation of DNA

10x stocks of restriction buffers were made as described in (Maniatis, 1982). DNA was digested with restriction enzyme (1-2 units), 1/10 volume 10x restriction buffer and the volume made up with distilled water. Plasmid and bacteriophage DNA were incubated overnight at 37°C. The reaction was terminated with a stop dye.

2.3.2 Ligation of DNA Fragments

Ligation was performed by mixing isolated DNA fragments (digested by the appropriate restriction enzymes) and linearized plasmid vector DNA (digested with the appropriate restriction enzymes) in presence of T4 DNA ligase enzyme and 10x ligation buffer (660mM Tris-HCl, pH7.6, 50mM $MgCl_2$, 50mM DTT, 10mM ATP pH7.0). The reaction mixture was normally composed of 15μl (6.0μg) of the DNA to be cloned, 10μl (2.0μg) of the vector DNA, 3μl of the 10X ligation buffer, 1-2 units of T4 DNA ligase and sterile distilled water to 30μl. The reaction was carried out at 15^{o}C overnight.

2.3.3 Phenol-Chloroform Extraction of DNA

Samples were deproteinized by the addition of 1 volume of redistilled phenol equilibrated with TE buffer (10mM Tris, 1mM ethylenediaminetetracetic acid (EDTA)), pH8.0. The phases were mixed by vortexing for 10 seconds and then separated by centrifugation at 13000x g in a microfuge (MSE Microcentaur) for 3 minutes. The aqueous phase was re-extracted with 1 volume of phenol:chloroform:isoamylalcohol (25:24:1) followed by 1 volume chloroform:isoamylalcohol (24:1) to remove traces of remaining phenol.

2.3.4 Precipitation of DNA with Ethanol

0.1 volume of 3M sodium acetate, pH6.0 and 2.5 volume of 100% ethanol were added to the DNA solution, mixed by vortexing and stored at -80^{o}C for 30 minutes. In mini-preparations of plasmid DNA, potassium acetate was already present in the DNA solution the sodium acetate was excluded in the ethanol precipitation step. Precipitates were collected by centrifugation at 13000x g for 10 minutes in a microfuge, or at 12000x g for 20 minutes at 4^{o}C in the Sorvall

RC-5B centrifuge. Pellets were washed in 70% ethanol, dried in a vacuum desiccator and resuspended in sterile distilled water or TE buffer.

2.3.5 Recovery of DNA from LMP Agarose Gel

Preparation of CETAB Mixes

200ml of butanol was mixed with 200ml of sterile distilled water. The mixture was shaken thoroughly and the phases separated. To the upper butanol layer, 1g of CETAB was added. The lower butanol saturated aqueous layer was then added and the whole vigorously and repeatedly shaken. The mixture was then kept at room temperature overnight to allow the two phases to separate. Each phase was then kept in separate bottles at 37^{o}C.

Recovery of DNA Fragments

The overnight restricted DNA was electrophoresced in 1% LMP agarose gel. Under ultraviolet light, the DNA fragment of the correct size was cut out with a sterile sharp razor blade as an agarose block, which was then centrifuged for 30 seconds in an Eppendorf tube. The agarose was melted at 65-70^{o}C for 15 to 30 minutes. The melted agarose was vortexed for 5 to 10 minutes. One volume of CETAB containing butanol saturated aqueous phase and another volume of CETAB containing water saturated butanol were added. The mixture was vortexed extensively for 5 minutes and followed by centrifugation for 3 minutes. The upper butanol phase was transferred into a clean Eppendorf tube while the lower aqueous phase was re-extracted with a half volume of fresh CETAB-butanol and added to the first butanol phase. One quarter of the volume 0.3M sodium acetate pH7.0 was added to the pooled butanol phases and vortexed thoroughly for a minute and then centrifuged for 3 minutes. The lower

aqueous phase was collected, chloroform extracted and ethanol precipitated. The vacuum dried DNA was resuspended in a small volume of TE buffer (10-20μl).

2.3.6 Dialysis of DNA Solutions

Dialysis tubing was prepared by boiling for 5 minutes in a solution of 5mM EDTA and 200mM sodium bicarbonate, followed by 5 washes in distilled water and boiling for 5 minutes in distilled water. The tubing was stored at 4^{o}C with 0.02% sodium azide and washed well with distilled water before use. The DNA solution was placed inside of the tubing avoiding air bubbles and each end secured with a clip. The samples were dialysed against 2 litres of TE buffer at 4^{o}C with stirring for 24 hours and the buffer changed at least 2 times.

2.3.7 Agarose Gel Electrophoresis

0.8% (w/v) agarose was dissolved in 200ml of 1x Alexs buffer (0.04M Tris-acetate, 1mM EDTA pH7.7) in maxi-gel or 100ml of 1x TBE buffer (108g Tris, 55g boric acid, 7.4g Na_2EDTA per litre) by heating in a microwave oven for 2 minutes. When the agarose had cooled to above hand temperature, 3μg/ml EtBr were added and poured into the mould. After setting, the agarose gel was immersed into a tank containing 1x Alecs buffer in maxi-gel or 1x TBE buffer in mini-gel. DNA samples were loaded into wells and electrophoresed for 4 hours (maxi-gel) or 2 hours (mini-gel) at 100V. After electrophoresis, the gels were visualized under long wave ultraviolet light (302nm) and photographed using a red filter and Polaroid 667 film.

2.4 Small Scale Preparation of Plasmid DNA

1.5ml of an overnight culture was taken and spun down in an eppendorf tube for 1 minute. The supernatant was discarded while the pellet was kept and dried slightly by aspiration. 100μl of ice cold solution 1 (50mM glucose, 10mM EDTA, 25mM Tris-HCl, pH8.0 and 0.2% lysozyme) was added and then kept at room temperature for 5 minutes. 200μl of freshly prepared alkaline solution (0.2N NaOH and 1% SDS) was added and mixed by inverting several times. After standing on ice for 5 minutes, 150μl of ice cold 5M potassium acetate, pH4.8 was added. The eppendorf tube was briefly inverted and then stored on ice for 5 minutes. The eppendorf tube was centrifuged for 5 minutes, the supernatant was transferred to a fresh tube. A half volume of phenol and a half volume of chloroform were added and then vortexed for 15 seconds and centrifuged for 3 minutes. The upper aqueous layer was transferred to a fresh tube and 2 volumes of 100% ethanol were added and stood for 10 minutes at room temperature and then centrifuged for 5 minutes. The pellet was kept and washed with 1ml of 70% ethanol and then centrifuged for 5 minutes. The pellet was dried by a vacuum desiccator and then resuspended in 50μl of TE buffer. 1μl of 20mg/ml RNase was added and mixed well.

2.5 Large Scale Preparation of Plasmid DNA

10ml of L-broth was inoculated with a single bacterial colony and grown up overnight with ampicillin selection (5mg/ml). 2ml overnight culture was added to 100ml L-broth plus ampicillin selection and allowed to grow up overnight. Plasmids were amplified once the culture had reached an OD_{600} of O.6 with 170μg/ml of chloramphenicol. Pellets were harvested by centrifugation at

8000rpm for 5 minutes at 4°C, washed with 25mM Tris pH8.0, 10mM EDTA without resuspending, and resuspended in 2ml of 50mM Tris pH8.0, 50mM EDTA, 15% (w/v) sucrose (15% sucrose $T_{50}E_{50}$). The cell suspension was frozen at −80°C for 10 minutes and then thawed at room temperature. 6ml of 15% sucrose $T_{50}E_{50}$ and 0.8ml of lysozyme (10mg/ml) in 15% sucrose $T_{50}E_{50}$ were added and incubated at 37°C for 10 minutes. After that, the tubes were stood on ice for 15 minutes. 2.4ml of 5M potassium acetate, pH4.8 was added, mixed gently and stood on ice for 15 minutes. 1.2ml of 10% (w/v) SDS was added and mixed gently at room temperature for 10 minutes and then stood on ice for an hour. The tubes were centrifuged at 18000rpm for 20 minutes at 4°C. The supernatant was kept. Sodium chloride was added at the rate of 36g per litre of supernatant. 50% (w/v) polyethylene glycol 6000 was added to give a final concentration of 10% (w/v) and then left for 2 hours at 4°C. The pellets were collected by centrifuging at 6000rpm for 20 minutes and resuspended in 3ml of TE buffer. After 90μl of EtBr (10mg/ml) was added, the weight of the sample was weighed. For each gram of sample, 0.94g of caesium chloride was added. The solution was then loaded into a quickseal centrifuge tube (Beckman) using a syringe and needle. The tubes were balanced, heat-sealed and centrifuged at 44000rpm for 24 hours at 15 minutes. After centrifugation, the tube was carefully removed from the rotor and observed under UV illumination. Usually two bands were clearly visible, the upper band coresponding to chromosomal DNA and the more highly fluorescent lower band corresponding to plasmid DNA. The lower band was removed from the gradient and an equal volume of isopropanol saturated with caesium chloride was added to remove the EtBr. Once the sample was clean (bottom layer), it was dialysed against TE buffer (section 2.3.6) and an aliquot was taken to check the purity

and concentration of the DNA sample.

2.6 Small Scale Preparation ssDNA

5ml of culture was poured into five 1.5ml eppendorf tubes, which were then centrifuged for 5 minutes. The supernatants were transferred to five fresh tubes, being careful not to carry over any cells. 200μl PEG/NaCl (20% polyethylene glycol 6000, 2.5M sodium chloride) were added and shaked and then left to stand for 15 minutes at room temperature. The tubes were centrifuged for 5 minutes. The supernatant was discarded. The tubes were centrifuged again for 2 minutes. All traces of PEG were removed by a drawn-out pipette. 10μl of TE buffer were added to each tube and vortexed for 30 seconds to resuspend the pellet. The samples were phenol: chloroform extracted (section 2.3.3). 500μl of diethyl ether were added to remove traces of phenol and vortexed for 15 seconds. The upper ether layer was removed and discarded. The samples were then ethanol precipitated (section 2.3.4) The pellets were resuspended in TE buffer in a total volume of 100μl and centrifuged for 5 minutes to remove all the protein precipitates.

2.6.1 Determination of Concentration of ssDNA

The ssDNA sample was measured at 260nm by adding a 1μl aliquot to 1ml of TE buffer in a quartz cuvette. 1 O.D. of single-stranded DNA is equivalent to 40μg/ml. The samples were ethanol precipitated again and resuspended in TE buffer to give 1μg/μl.

2.7 Large Scale Preparation of ssDNA

2.7.1 Preparation of 1ml Phage Stock

A single TG2 colony was picked from a glucose/minimal medium plate to 10ml 2x YT medium, grown overnight, shaken at 37°C. One drop of the overnight culture was added to 20ml of fresh medium, and shaken at 37°C for 3 hours. 100μl of the 3 hour culture and a recombinant plaque were inoculated in 1ml 2x YT medium in a 10ml sterile culture tube which was incubated for 4 hours with shaking at 37°C. The culture was transferred to a microcentrifuge tube and centrifuged for 5 minutes at ambient temperature. The supernatant was poured into a fresh tube and kept overnight at 4°C. An overnight culture of TG2 was set up for the next stage.

2.7.2 Growth of 100ml Phage Culture

1ml of overnight TG2 culture was inoculated into 100ml 2x YT medium and shaken at 37°C to and OD_{550} of 0.3. 1ml phage supernatant (from section 2.7.1) above was added to the 100ml culture and then grown for 4 hours with shaking, at 37°C. The culture was transferred to clean and sterile centrifuge tubes and centrifuged at 5000x g for 30 minutes at 4°C . The supernatant was transferred to clean and sterile centrifuge tubes. 0.2 volumes of 20% w/v PEG in 2.5M NaCl were added to the supernatant, mixed well and then left to stand for 1 hour at 4°C . It was followed by centrifugation at 5000x g for 20 minutes. The pellet was kept and centrifuged again at 5000x g for 5 minutes. All the remaining PEG/NaCl was removed with a drawn-out Pasteur pipette. The pellet was resuspended in 500μl TE buffer and transferred to a microcentrifuge tube. Any remaining cells were removed by centrifuging for 5

minutes. The supernatant was transferred to a fresh microcentrifuge tube. 200μl of PEG/NaCl was added to microcentrifuge tube, mixed well and then left to stand for 15 minutes at ambient temperature before centrifuging for 5 minutes. The supernatant was discarded and the pellet was centrifuged for 2 minutes. All remaining traces of PEG were removed by a drawn-out Pasteur pipette. The pellet was resuspended in 500μl TE buffer.

2.7.3 Purification of ssDNA

The viral ssDNA prepared from the above section 2.7.2 was purified by phenol (twice) and diethyl ether (three times) extraction as described in section 2.6. Two chloroform extractions were followed to remove any traces of PEG and phenol. The sample was ethanol precipitated as described in section 2.3.4. The concentration of the ssDNA was determined as decribed in section 2.6.1.

2.8 Preparation of TG2 competent Cells and Transformation

2.8.1 Preparation of *E. coli* TG2 Competent Cells

A colony was picked from a glucose/minimal medium plate, inoculated into 30ml of SOB and incubated overnight at 37^{o}C with moderate agitation. 8ml of the overnight culture were added to a 1 litre flask containing 200ml SOB which was incubated at 37^{o}C with moderate agitation to OD_{550} about 0.3. The culture was collected in four 50ml sterile centrifuge tubes, chilled rapidly in an ice-salt-water bath and left on ice for 15 minutes. The cells were pelleted by centrifugation at 3000x g for 5 minutes at 4^{o}C, resuspended gently by mild vortexing of each in 16ml transformation buffer 1 (16ml per 50ml of initial culture) and incubated on ice for 15 minutes. The cells were pelleted again at

3000x g for 5 minutes at 4°C and resuspended in 4ml of transformation buffer 2 per 50ml initial culture. The cell suspension was poured into eppendorf tubes and quickly frozen by dropping into liquid nitrogen and then placed at –80°C.

2.8.2 Transformation Reaction (for M13 recombinants)

300μl of competent cells and 20μl of DNA were put into chilled tubes which were rolled gently for a few minutes on ice. The cells were incubated on ice for 45 minutes, heat-shocked at 42°C for 45 seconds and placed on ice for 5 minutes before plating out. While the cells were on ice, the following mixture for each tube was prepared: 10μl of 100mM IPTG, 2μl of 10% X-gal in dimethylformamide, 200μl of log phase *E. coli* (TG2) cells. The X-gal and IPTG were prepared freshly and kept on ice. After 5 minutes, the tubes were placed at room temperature and the mixture of IPTG, X-gal, log phase cells and 3ml of molten top agar, kept at 50°C were added and mixed by rolling. The whole mixture was poured immediately onto a prewarmed (37°C) 2x YT agar plates. The plates were inverted after setting and incubated at 37°C overnight. The wild-type M13 would give blue plaques while the mutants would form clear plaques. The clear plaques were picked up as an agar plug by sterile Pasteur pipettes and transferred to 5ml 2x YT medium with 50μl of a fresh overnight culture of TG2 cells. They were incubated with shaking at 37°C for 5 hours. The cultures then were used for minipreparation of plasmid DNA, i.e. M13 and singled-stranded (ss) template DNA preparation.

2.9 Preparation of Yeast Competent Cells and Transformation

The modified method of Ito *et al.*, (1983) was performed for preparation of yeast competent cells and DNA transformation. 10ml of YPD medium was

inoculated with a single yeast colony and left to grow at 30°C until an $O.D._{600}$ was reached 0.4-0.5. The cells were harvested by centrifugation at 3500rpm for 5 minutes in a benchtop centrifuge, washed once with sterile distilled water, then once with TE buffer. The pellet was resupended in 10ml of 0.1M lithium acetate and incubated for 60 minutes at 30°C statically. The cells were pelleted again and then resupended in 0.4ml of 0.1M lithium acetate. For transformation of DNA, 1μg (10μl) of DNA was added to 50μl of freshly prepared competent cells in an eppendorf tube and incubated at 30°C for 30 minutes. The cells were then shaken and 0.6ml of 40% PEG-4000 in 10mM Tris-HCl pH7.6 was added, mixed well by repeating pipetting up and down and incubated for an hour at 30°C before being heat-shocked at 42°C for 5 minutes and standing immediately on ice for further 5 minutes. The cells were centrifuged for 10 seconds at low speed (about 8000rpm) in a MSE microfuge. The supernatant was discarded and the pellet containing transformed cells was resuspended gently in 200μl of sterile distilled water. 100μl of the resuspended cells was added to the selective yeast minimal agar plates and spread by an ethanol-flamed glass rod. After the suspension was dried and absorbed into the agar, the plates were inverted and incubated at 30°C for 4-7 days.

2.10 Oligonucleotide-directed Mutagenesis

2.10.1 Designs of the Mutagenic Oligonucleotides

The 5' to 3' DNA sequence of the GUS gene around the two potential N-glycosylation sites is shown in figure 1. The synthetic mutagenized oligonucleotides were designed to mutate the serine to alanine of the two N-glycosylation sites by changing the first base, theonine, of codon of serine to

guanine.

(a) First potential N-glycosylation site

```
5'-GCTGTCGGCTTTAACCTCTCTTTAGGCATTGGTT-3'  (Wildtype GUS)
               AsnLeuSer

        3'-GAAATTGGAGCGAAATCCGTA-5'    (Oligonucleotide 160)
```

(b) Second potential N-glycosylation site

```
5'-CAAGTGCACGGGAATATTTCGCCACTGGCGGAAG-3'  (Wildtype GUS)
               AsnIleSer

        3'-GCCCTTATAACGCGGTGACC-5'     (Oligonucleotide 161)
```

Figure 1: DNA sequences of the β-glucuronidase gene around the two potential N-glycosylation sites and the two synthetic mutagenized oligonucleotides (note: the mismatched bases are underlined).

2.10.2 5'-Phosphorylation of Oligonucleotide

The mutagenic oligonucleotide was 5'-phosphorylated before annealing to the single-stranded template DNA for the oligonucleotide-directed mutagenesis reaction. 2.5μl of oligonucleotide stock solution (5 OD units/ml), 3.0μl of 10x kinase buffer, 25.0μl of water and 2.0 units of T4 polynucleotide kinase were added to an eppendorf tube and mixed by gently pipetting up and down. It was incubated at 37^{o}C for 15 minutes and then heated at 70^{o}C for 10 minutes to inactivate the enzyme. It was used immediately or stored at –20^{o}C.

2.10.3 Annealing Mutant Oligonucleotide to ssDNA Template

5.0μl of single-stranded DNA template (1μg/μl), 2.5μl of phosphorylated mutant oligonucleotide, 3.5μl of buffer 1 and 6.0μl of water were added to an eppendorf tube and placed in a 70^{o}C water bath for 3 minutes, and then incubated at 37^{o}C for 30 minutes. The reaction was placed on ice before doing the synthesis and ligation of the mutant DNA strand.

2.10.4 Synthesis and Ligation of Mutant DNA Strand

5.0μl of $MgCl_2$ solution, 19.0μl of Nucleotide mix 1, 6.0μl of water, 6.0 units of Klenow fragment and 6.0 units of T4 DNA ligase were added and mixed by pipetting up and down. The tube was placed in a 16^{o}C water bath and left overnight. 1μl of the reaction mix was removed for analysis on an agarose gel (stored at –20^{o}C).

2.10.5 Removal of Single-stranded (non-mutant) DNA

The reaction mix was mixed with 170μl of water and 30μl of 5M NaCl which were added to the top half of the disposable centrifugal filter unit

supplied by the Amersham system which was centrifuged at 500x g in a bench top centrifuge for 10 minutes at room temperature. 100μl of 500mM NaCl was added and spun for 10 minutes to wash through any remaining replicative form DNA. The lower tube which contained the sample was removed and ethanol precipitated by adding 28μl of 3M sodium acetate and 700μl of –20°C ethanol and placing at –80°C for 30 minutes. After centrifuging for 15 minutes, the pellet was washed with 1ml –20°C ethanol, dried in a vacuum desiccator and resuspended in 10μl of buffer 2.

2.10.6 Nicking of the Non-mutant Strand of DNA using *NciI*

65μl of buffer 3 and 5 units of *NciI* were added to the resuspended sample and incubated at 37°C for 90 minutes. 10μl of the reaction mix were removed for analysis on an agarose gel (stored at –20°C).

2.10.7 Digestion of Non-mutant Strand using ExoIII

12μl of 500mM NaCl, 10μl of buffer 4 and 2μl of exonuclease III were added to the reaction mix and incubated at 37°C for 30 minutes. The enzyme was inactivated by heating at 70°C for 15 minutes. 15μl of the reaction mix were removed for analysis on an agarose gel (stored at –20°C).

2.10.8 Repolymerization and Ligation of the Gapped DNA

13μl of the nucleotide mix 2, 5μl of $MgCl_2$, 3 units of DNA polymerase I and 2 units of T4 DNA ligase were added and incubated for 3 hours. Again 15μl of the reaction mix were removed for analysed on an agarose gel (stored at –20°C).

2.10.9 Ammonium Acetate Precipitation

The DNA sample was purified by adding 1 volume of 4M ammonium acetate pH5.4 and 4 volumes of 100% ethanol at room temperature. The mixture was left at room temperature for 10 minutes and centrifuged for 10 minutes. The pellet was washed with 500μl of 70% ethanol and centrifuged for 5 minutes, dried in a vacuum desiccator and resuspended in 100μl of TE buffer.

2.11 Analysis of Mutant Progeny

2.11.1 Preparation of Phage Stocks

100ml of 2x YT were inoculated with 1ml of an overnight culture of *E. coli* TG2. 1.5ml aliquots were dispensed into sterile 10ml culture tubes. Each tube was inoculated with a plaque using a sterile Pasteur pipette. The tubes were shaked at 37^{o}C for 5 hours and transferred to eppendorf tubes and then centrifuged for 5 minutes. The supernatant was collected and added with 200μl of PEG/NaCl. Having shaken, the tubes were left to stand for 15 minutes, centrifuged for 5 minutes. The supernatant was discarded and the tubes were centrifuged for 2 miuntes. All traces of PEG were removed with a drawn out Pasteur pipette. The viral pellet was resuspended in 100μl of TE buffer and stored at 4^{o}C.

2.11.2 Preparation of Labelled Probe

1.5μl of oligonucleotide (2.5 OD_{260} units/ml), 3μl of 10x kinase buffer (omit ATP), 30μCi[γ-^{32}P]ATP, 22μl of water and 2 units of polynucleotide kinase were mixed and incubated at 37^{o}C for 30 minutes. The reaction mix was diluted with 3ml 6x SSC and filtered through a 0.45μm filter unit and washed through

the filter with 1ml 6x SSC. The labelled probe was then stored at -20^oC freezer.

2.11.3 Dot Blot Hybridization

$2\mu l$ of phage stock were spotted onto a dry sheet of nitrocellulose. Having air-dried, the nitrocellulose membrance was baked at 80^oC for 2 hours in a vacuum oven. The filter was prehybridized with 10ml of (6x SSC, 10x Denhardt's solution, 0.2% SDS) at 67^oC for 1 hour in a hybridization bag and then rinsed in 50ml of 6x SSC for 1 minute. The filter was placed into a hybridization bag with 4ml of labelled probe from procedure 2 above and hybridize at 67^oC for 30 minutes and then cooled to room temperature over 30 minutes. The filter was washed three times at ambient temperature in 100ml of 6x SSC for 5 minutes. The filter was covered with clean film and autoradiographed at -70^oC with preflashed film and an intensifying screen, for 1-4 hours, or at ambient temperature overnight with unpreflashed film. The filter was removed from the cassette and washed at 5^oC below the calculated Td (where Td is the temperature at which the perfectly-matched DNAs remained hybridized while the mismatched DNA dissociated which is estimated by 'Wallace rule' and is equal to [4 x number of G, C base pairs + 2 x number of A, T base pairs of the mutant oligonucleotide]) with 50ml of 6x SSC for 5 minutes. The washed filter was autoradiographed as before. If the washing step did not give the required discrimination between the mutant and wild type phage dots, then the filter could be washed with higher temperature e.g. 2^oC below the calculated Td, until the mutant phage could be distinguished.

2.12 DNA Sequencing

DNA was sequenced by the method of Sanger *et al.* (1977) using the BRL

sequencing kit and manufacturer's instructions. 10mM stocks of dNTPs were diluted in double distilled deionized water to a final concentration of 0.5mM and used to prepare the nucleotide mixes. For the annealing reaction, 1μl of template DNA (1μg/μl) was mixed with 8.4μl of distilled water, 2μl of 20mer oligonucleotide and 1μl of 10x polymerase reaction buffer. The mixture was heated to 100^{o}C for 5 minutes and allowed to cool to room temperature very slowly. 1μl of 0.1M DTT, 1μl of ^{35}S-dATP and 1μl of DNA polymerase I (Klenow fragment) were added to each template/primer mix. An equal volume of the appropriate nucleotide and ddNTP solutions were mixed and 2μl aliquots were added to the correct A, C, G or T tubes and mixed by pipetting up and down several times and then incubated at 30^{o}C for 20 minutes. 1μl of dATP chase was added to each tube and incubated for a further 15 minutes. 5μl of stop dyes (0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue, 10mM EDTA, 95% (v/v) deionized formamide) were added and samples stored at –20^{o}C until required. Before loading, samples were boiled at 100^{o}C for 3 minutes and loaded directly onto a DNA sequencing gel.

2.13 Preparation of PAGE in DNA Sequencing

Two glass sequencing plates were washed and one side of each, cleaned 3 times with 100% ethanol and twice with "repelcote". 0.4mm spacers were arranged on the larger glass plate and the smaller glass plate placed on top, ensuring that clean surfaces were innermost. The plates were taped together. A solution of 38% acrylamide and 2% bis-acrylamide was prepared and stored at 4^{o}C. 10ml of this solution was added to 21g of urea and 10ml of 10x TBE buffer and the final volume made up to 50ml with distilled water. The acrylamide solution was degassed, 0.4ml of 10% ammonium persulphate solution and

10μl of TEMED (N,N,N',N'-tetramethyl-ethylene diamine) was added and the mixture poured between the glass plates using a 100ml syringe. A comb was inserted and the gel allowed to set overnight, covered with cling film. Once the gel was set the tape was removed form the base of the gel and the comb removed. Gels were set up vertically in a sequencing tank with the base submerged in a reservoir of 1x TBE buffer and the top sealed with high vacuum grease to a second reservoir of buffer. An aluminium sheet was clamped across one glass plate to aid heat distribution and the wells were cleared of urea by flushing with buffer. The gel was pre-electrophoreised at 50mA until the voltage reached 1500V. Samples were boiled and loaded in the order A, C, G, T for each template and the gel electrophoreised at 50mA (1500V) for about 4 hours until the xylene cyanol reached the base of the gel. The gel was removed from the apparatus and the two plates separated. A sheet of Whatman 3MM paper was placed on top of the acrylamide gel and lifted. The position of samples were marked and the gel covered with cling film. The gel was dried under vacuum at 80^{o}C for at least 3 hours. The dried gel was autoradiographed by placing a unsentized Fuji RX X-ray film and exposed for about a week. 5' to 3' sequences were read directly from the autoradiograph.

2.14 Preparation of Crude Extracts

For yeast cells, 10ml of cell cultures were harvested by centrifugation in a benchtop centrifuge for 10 minutes at 3500rpm. For bacterial cells, 1.5ml of the cell cultures were transferred to an Eppendorf tube and centrifuged in a MSE microfuge for 5 minutes at 12000rpm. The pellet was kept on ice before being resuspended in 200μl of extraction or sample buffer. The suspension was then transferred to an Eppendorf tube, pelleted lightly and an equal volume of glass

beads was added to the cell pellet. The cells were vortexed for 5 x 5 seconds, cooling on ice between vortexing. This was followed by centrifugation in a MSE microfuge for 3 minutes at 12000rpm. The supernatant was kept on ice before further analysis.

2.15 Determination of β-Glucuronidase (GUS) Activity

β-glucuronidase activity was assayed flurometrically as described by Jefferson *et al.*, (1987a). GUS was extracted from GUS extraction buffer (50mM sodium phosphate pH7.0, 10mM EDTA, 0.1% Triton X-100 and 10mM β-mercaptoethanol). 200μl of extracts was added to 200μl of assay buffer containing 2mM 4-methyl umbelliferyl glucuronide (MUG) in GUS extraction buffer. The blank was made by mixing 200μl of GUS extraction buffer and 200μl of assay buffer. The mixtures were incubated at 37^{o}C for 60 minutes. The reactions were stopped by adding 1ml of 0.2M sodium carbonate. The addition of sodium carbonate served the dual purposes of stopping the enzyme reaction and developing the fluorescence of MU, which was about seven times as intense at alkaline pH. The fluorescence was measured with an excitation at 365nm and emission at 455nm using a Bairo-Atomic spectrofluorimeter.

2.16 SDS Polyacrylamide Gel Electrophoresis

2.16.1 Acrylamide Stock

30g acrylamide

0.8g N',N'-methylene-bisacrylamide

per 100ml of sterile distilled water

2.16.2 10% SDS Polyacrylamide Gel

20.04ml of 30% acrylamide stock

22.5ml of 1M Tris-HCl, pH8.8

16.08ml of distilled water

Degassed

0.6ml of 10% SDS

180μl of 10% ammonium persulfate

15μl of TEMED

Mixed and then poured into the gel mould

2.16.3 Stacking Gel

6.7ml of 30% acrylamide stock

25ml of 0.25M Tris-HCl, pH6.8

15.79ml of distilled water

Degassed

0.5ml of 10% SDS

1.5ml of 10% ammonium persulfate

12.5μl of TEMED

Mixed and then poured into the gel mould

2.16.4 Polyacrylamide Gel Electrophoresis Buffer (PAGE)

41g glycine

30g Tris

10g SDS

per 1 litre of distilled water

2.16.5 Laemmli Sample buffer

0.05M Tris-HCl pH6.8

2.5% SDS

0.0125% Bromophenol blue

141g glycerol

0.05M DTT

2.16.6 PAGE Staining Solution

1 litre of methanol

140ml of acetic acid

1.9g of kenacid blue R250

Make up to 2 litre by adding distilled water

2.16.7 PAGE Destaining Solution

50% methanol

7% acetic acid

43% distilled water

2.16.8 Preparation of Protein Samples for SDS-PAGE

Up to 50μl of protein sample could be loaded per track. The sample was then boiled for 5 minutes before loading to the well of the gel. The gel was electrophoresed at 50V overnight.

2.16.9 Staining and Destaining

After electrophoresis, the gel was removed from the plates and stained overnight in the staining solution. The gel was then destained in the destaining

solution for another day until a clear background was seen. The gel was then soaked in solution containing 30% methanol and 3% glycerol overnight before being dried in the vacuum gel drier.

2.17 Preparation of IgG Fraction from Crude Antiserum

50% saturated ammonium sulphate solution was added slowly to the antiserum with continuous stirring and then left for 2 hours. The precipitated protein was recovered by centrifugation at 5000x g for 10 minutes. The supernatant was discarded and the precipitate resuspended in 5mM sodium phosphate, pH6.5 at one-fifth of the original volume. This IgG fraction was dialysed against the same buffer with two changes overnight. The protein was then clarified by centrifugation at 5000x g for 5 minutes. The purified IgG was stored at -20^{o}C .

2.18 Western Blot

The western blot was carried out with the Bio-Rad Semi-Dry electrophoresis method. After electrophoresis, the gel was removed from the plates and trimmed to the size that containing the samples. Six pieces of 3MM filter papers, one piece of nitrocellulose membrane and one piece of dialysis membrane of the same size of the trimmed gel were cut. The graphite plates were washed with distilled water. The followings were placed in sequence from the anodic plates: two pieces of filter paper soaked in anode buffer number 1 (0.3M Tris. HCl pH9.4, 20% methanol, 0.1% SDS), one piece of filter paper soaked in anode buffer number 2 (25mM Tris.HCl pH10.4, 20% methanol, 0.1% SDS), one piece of nitrocellulose membrane soaked in distilled water, sample containing gel, one piece of filter paper soaked in cathode buffer (25mM Tris.HCl pH9.4, 40mM

6-amino-n-hexanoic acid, 20% methanol, 0.1% SDS), one piece of dialysis membrane soaked in distilled water and two pieces of filter paper soaked in cathode buffer. The cathode lid plate was then plated on the top of these sandwishes and electrophoresed for one hour at 0.8mA per cm^2 of the gel. After that, the nitrocellulose membrane was removed for immunological detection of the immobilized proteins. The method of Johnson *et al.*, (1984) was used as follows: the nitrocellulose membrane was incubated for an hour at 40°C with agitation in 50ml of blotting buffer (5% w/v non-fat dried milk dissolved in 1x Tris-Salt buffer (20mM Tris-HCl, 0.9% sodium chloride pH7.2)). It is then washed three times with 50ml each of blotting buffer for 10 minutes. The membrane was incubated in 50ml of the blotting buffer with 25μl of the primary antibody in serum or purified IgG fraction of crude antiserum with agitation for 3 hours at 40°C. Three washes with 50ml of blotting buffer for 10 minutes each were followed before incubated the membrane in the same buffer containing 20μl of anti-rabbit goat antibody. The membrane was then washed three more times in blotting buffer and once quickly with 1x Tris-salt. The membrane was stained with 4-chloro-1-napthol as follows: 50mg of 4-chloro-1-napthol in 17ml of methanol was added to a solution containing 5ml of 1M Tris-HCl pH7.2, 1g NaCl and 78ml of water. After the mixture was poured to the membrane, 100μl of hydrogen peroxide was added and mixed. The reaction was carried out in dark for about 15 minutes. As the bands were visible, the reaction was stopped by washing with 1x Tris-salt and distilled water.

Chapter III

RESULTS

3.1 Introduction

The following experiments were performed to investigate the use of β-glucuronidase in *Saccharomyces cerevisiae* for the study of vacuolar protein targeting. Several carboxypeptidase Y-β-glucuronidase (CPY-GUS) fusions had been made previously (Haider, 1989). This work seeks to use and modify these constructs in order to generate an efficient reporter gene system.

3.2 Tunicamycin Treatment

Tunicamycin is a drug that inhibits the formation of high mannose side chains on dolichol phosphate, thereby inhibiting the N-glycosylation of proteins in the lumen of the ER. There are two potential N-glycosylation sites within the GUS sequence, the glycosylation of which inhibits the catalytic activity of GUS (Iturriaga, 1989). Tunicamycin was therefore used to inhibit glycosylation in an attempt to recover GUS activity. Yeast strain, YHH19 containing CPY-GUS plasmids pDUB2505 or pDUB2523 were grown in YPD medium overnight, with agitation at 30°C. The overnight cultures were treated with tunicamycin at a concentration of 10μg/ml for two and a half hours. The yeast cells were harvested and tested for GUS activity. The results are presented in Table 1. By Bradford protein assay (Bradford, 1976), it was found that the protein concentrations of the tunicamycin treated or untreated yeasts were similar. It indicates that the tunicamycin treatment had no significant effects on the crude

protein production within two and a half hours. The GUS activity was found to be increased by 7.28% and 10% in yeasts containing pDUB2505 and pDUB2523 respectively.

Yeast with	Tunicamycin	P.C. ($mgml^{-1}$)	Emission (E)	GUS Activity ($Emlmg^{-1}$)
pDUB2505	+	2.80	17.33	6.19
pDUB2505	-	3.12	18.00	5.77
pDUB2523	+	3.10	2.30	0.74
pDUB2523	-	3.12	2.10	0.67

Table 1: The β-glucuronidase activities of yeasts with different plasmids after two and a half hours tunicamycin treatment, where P.C. = protein concentration of crude extracts.

Another experiment was performed by treating yeasts with pDUB2523 with the same concentration of tunicamycin for 16 hours. The results are shown in the Table 2. By Bradford protein assay, it was found that the protein concentration of the tunicamycin treated samples decreased by 80%. The GUS activity was found to be decreased by 90% when comparing with the non-tunicamycin treated sample.

Yeasts with	Tunicamycin	Protein Conc. ($mgml^{-1}$)	GUS Activity ($Emlmg^{-1}$)
pDUB2523	+	0.83	0.65
pDUB2523	-	3.84	7.85

Table 2: The GUS activities of yeasts with pDUB2523 after 16 hours of tunicamycin treatment.

3.3 Transformation of GUS into Protease-deficient Strains

GUS is a foreign protein in yeast, thus it may be degraded by intracellular proteases found in the vacuole to which it is directed. GUS is known to be quite resistant to most protease action (Jefferson, 1987a). To investigate if the low level of GUS activity is due to proteolytic activity, the CPY-GUS plasmids pDUB2505, pDUB2512 and pDUB2523 were transformed into protease-deficient strains, YHH32 and CL-2 ABYS106. The control was made by transforming all these plasmids to a normal strain, YHH19.

Large scale preparations of these plasmids DNA were performed as described in the section 2.5. The prepared plasmid DNAs were transformed into yeast strain YHH19, YHH32 and CL-2 ABYS106 as described in section 2.9. The transformed cells were plated out onto selective Ymin agar plates. Table 3 lists the conditions of transformations and the amino acids used in the selective Ymin media.

Transformed colonies were re-streaked onto fresh selective Ymin agar plates to confirm the purity of the culture. One single colony was isolated and grown under selection in 10ml of Ymin media overnight at 30^{o}C with agitation. The cells were harvested. The crude cell extracts were prepared as described in section 2.14 and analyzed by SDS-PAGE and western blots. Because the non-transformed yeast strains YHH19, YHH32 and CL-2 ABYS106 could grow on selective Ymin media, so a non-transformed wild-type yeast strain MD40-4C was used as a control in SDS-PAGE and western blots.

Yeast strains	Transformed plasmids	Amino acids in Ymin media
YHH19	pDUB2505	his ura
YHH19	pDUB2523	his ura
YHH19	pDUB2512	his leu
YHH32	pDUB2505	his
YHH32	pDUB2523	his
CL-2 ABYS106	pDUB2525	his ura
CL-2 ABYS106	pDUB2512	his ura
CL-2 ABYS106	pDUB2523	his leu

Table 3: Conditions of transformations and the amino acids used in selective Ymin agar plates and media, (where his=histidine; ura=uridine and leu=leucine).

3.3.1 SDS-PAGE

The result of SDS-PAGE is shown in figure 2. It indicates that all transformants, except the wild-type yeast strain MD40-4C, expressed GUS protein.

Figure 2: SDS-PAGE of crude cell extracts of transformed yeasts (Lane 1: Molecular weight marker; Lane 2: GUS standard from Sigma; Lane 3: MD40-4C; Lane 4: YHH19 t.w. pDUB2505; Lane 5: CL-2 ABYS106 t.w. pDUB2505; Lane 6: YHH19 t.w. pDUB2512; Lane 7: CL-2 ABYS106 t.w. pDUB2512; Lane 8: YHH19 t.w. pDUB2523; Lane 9: CL-2 ABYS106 t.w. pDUB2523; Lane 10: YHH32 t.w. pDUB2523). Note: t.w. means 'transformed with'.

3.3.2 Western Blot

The yeast crude extracts were run in 10% SDS-PAGE, and then western-blotted with crude rabbit anti-GUS antibodies. The results are shown in figure 3. It indicates that normal strain YHH19 transformed with pDUB2512 (lane 6) expressed a large amount of GUS while the transformed protease deficient strain CL-ABYS106 (lane 7) expressed a little amount of GUS. However, the other transformants and the GUS standard (lane 2) loaded with a quantity of one microgram does not show very clear or any GUS bands.

The weak detection of GUS in western blots may be due to the low specificity of the crude anti-GUS antiserum, so the crude antiserum was purified as described in section 2.17. The collected IgG fraction was used again to blot against the same yeast crude extracts. However, similar results were obtained.

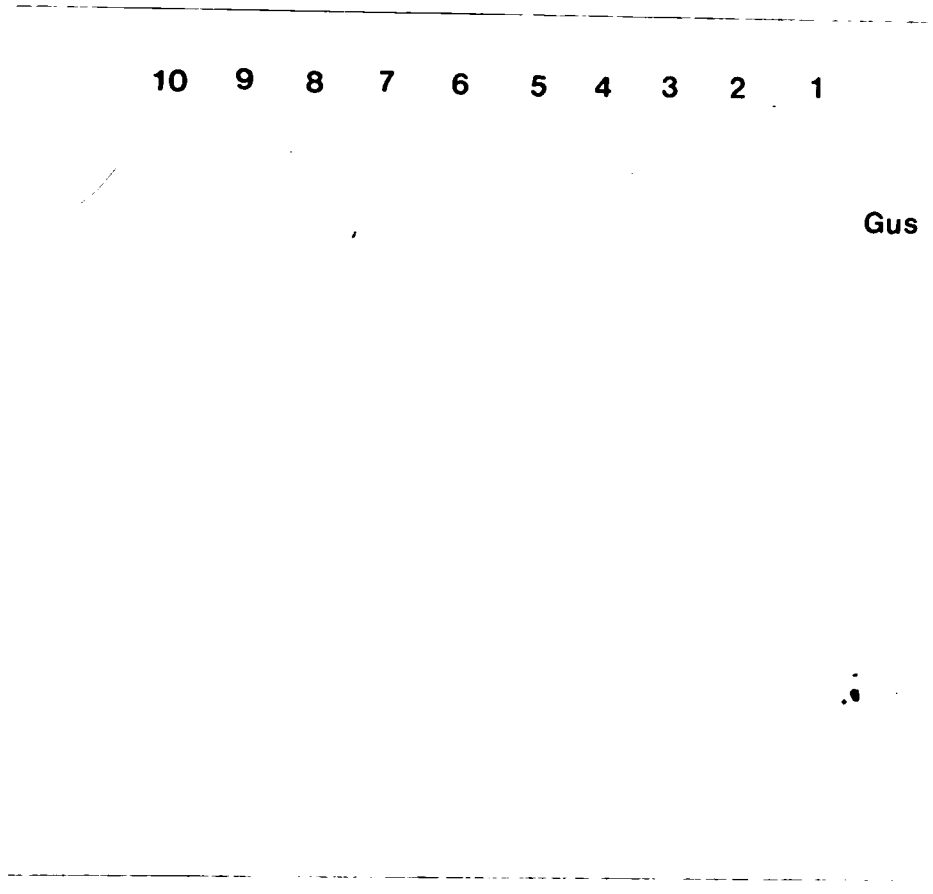

Figure 3: Western blots of crude cell extracts of transformed yeasts against anti-GUS antibody. (Lane 1: Molecular weight marker; Lane 2: GUS standard from Sigma; Lane 3: MD40-4C; Lane 4: YHH19 t.w. pDUB2505; Lane 5: CL-2 ABYS106 t.w. pDUB2505; Lane 6: YHH19 t.w. pDUB2512; Lane 7: CL-2 ABYS106 t.w. pDUB2512; Lane 8: YHH19 t.w. pDUB2523; Lane 9: CL-2 ABYS106 t.w. pDUB2523; Lane 10: YHH32 t.w. pDUB2523). Note: t.w. means 'transformed with'.

3.4 Site-directed *in vitro* Mutagenesis

The second approach to recover the GUS activity was to mutate the two potential N-glycosylation sites in GUS with site-directed mutagenesis using mutagenized synthetic oligonucleotides.

3.4.1 Isolation of GUS Gene

pDUB2511 encoding a *Bam*HI-*Eco*RI full length GUS gene was digested with *Bam*HI and *Eco*RI. The fragments were separated by 1% LMP agarose gel electrophoresis and recovered by CETAB method (section 2.3.5). The purified fragments was ligated with *Bam*HI and *Eco*RI restricted M13mp19 and then transformed into TG2. Twenty white transformants were isolated and grown in 10ml 2x YT cultures. 1.5ml of the cultures were used for the minipreparation of the plasmid DNA which was restricted with *Bam*HI and *Eco*RI to confirm the presence of the GUS fragment. The results are shown in figure 4. Sample 4 and 11 were selected for single-stranded template DNA preparations. The DNA sequence of sample 11 was then determined by an automatic DNA sequencer. The sequence is shown in figure 5. The M13-GUS single-stranded DNA was treated as wild type GUS and ready for site-directed mutagenesis.

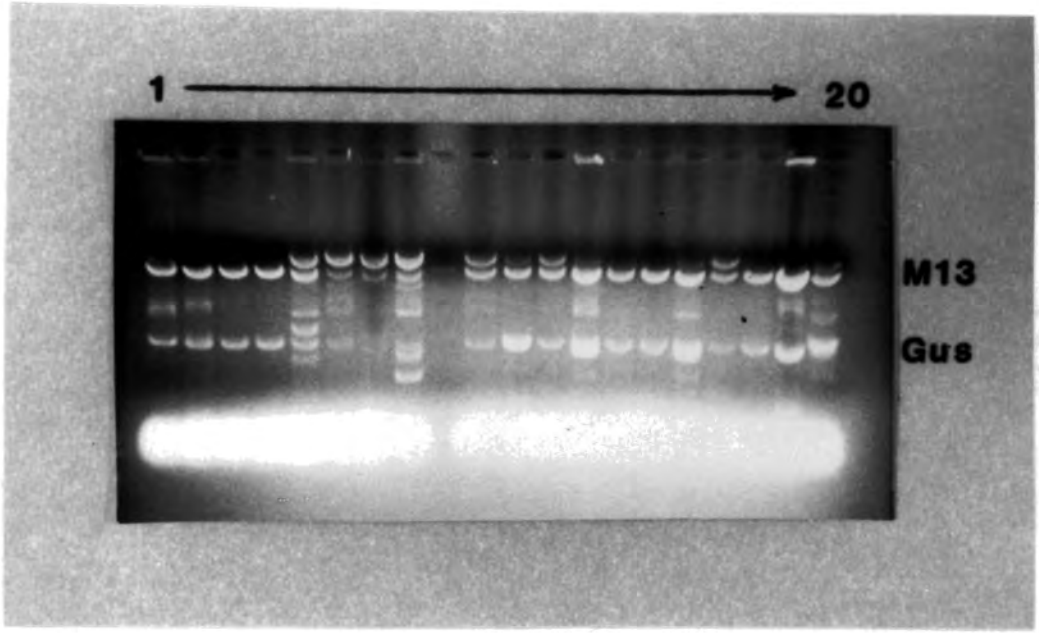

Figure 4: Agarose gel electrophoresis of minipreps. of twenty transformants digested with *Bam*HI and *Eco*RI.

?AC?C????GATCTAGTAACATAGATGACACCGCGCGCGATAATTTATCCTAGTTTGCGC

GCTATATTTTGTTTTCTATCGCGTATTAAATGTATAATTGCGGGACTCTAATCATAAAAA

CCCATCTCATAAATAACGTCATGCATTACATGTTAATTATTACATGCTTAACGTAATTCA

ACAGAAATTATATGATAATCATCGCAAGACCGGCAACAGGATTCAATCTTAAGAAACTTT

ATTGCCAAATGTTTGAACGATCGGGGAAATTCGAGCTCGGTAGCAATTCCCGAGGCTGTA

GCCGACGATGGTGCGCCAGGAGAGTTGTTGATTCATTGTTTGCCTCCCTGCTGCGGTTTT

TCACCGAAGTTCATGCCAGTTCCAGCGTTTTTGCAGCAAGAAAAGCCGGCCGACTTCGGT

TTTGCGGGTCGCGAGT?AAAGATCCC?TTTCTTTGTTTA?CCGGCCAACGCGCAAATATG

CC

Figure 5: The DNA sequence of transformant 11 determined by an automatic DNA sequencer. It shows the presence of the GUS insert.

3.4.2 Analysis of *in vitro* Mutagenesis Reaction

The designs of the synthetic oligonucleotides are shown in section 2.10.1. The site directed mutagenesis reactions were done by following the instruction manual of Amersham International plc. Each stage of the *in vitro* mutagenesis protocol could be examined by using samples which were taken at each step of the reaction. These were stored at -20°C and could then be analyzed by 0.8% gel electrophoresis (figure 6). Sample 1 was collected after the extension and ligation reaction and showed the presence of covalently closed circular DNA, with a smear of material above it. There was also a single-stranded DNA band consisting of unconverted template. Sample 2 was collected after the filtration and nicking and showed to have a nicked DNA band. Sample 3 was collected after the exonuclease III digestion. The DNA band showed increased mobility relative to sample 2, and was smeared due to exonuclease digestion from the site of the nick. Sample 4 showed the DNA after the final repolymerization stage. A clear close circular DNA band could be observed.

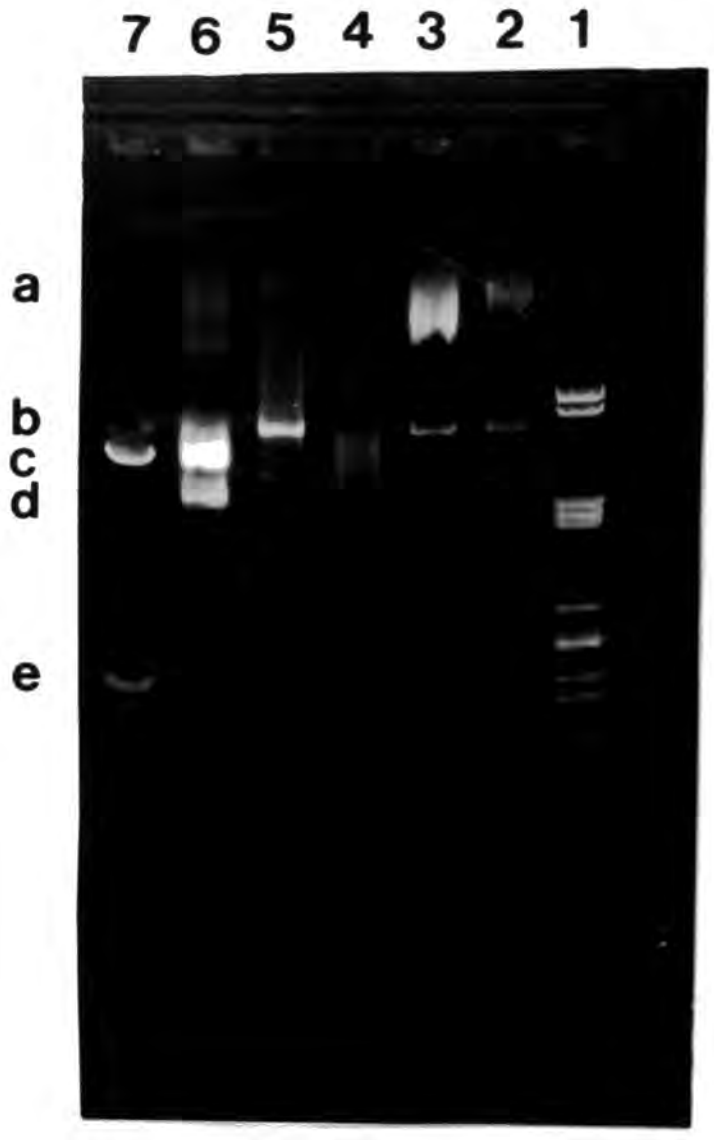

Figure 6: Agarose gel electrophoresis of samples taken from stages of the *in vitro* mutagenesis reactions. Lane 1: bacteriophage λ *Pst*I marker; Lane 2: Sample 1 after extension and ligation; Lane 3: Sample 2 after filtration and nicking; Lane 4: Sample 3 after exonuclease III digestion; Lane 5: Sample 4 after repolymerization; Lane 6: single-stranded DNA template; Lane 7: Miniprep of M13-GUS with *Bam*HI and *Eco*RI digestion. a: nicked DNA; b: covalently closed circular DNA; c: *Bam*HI and *Eco*RI digested linear M13 double-stranded DNA; d: single-stranded DNA of M13-GUS; e: double-stranded GUS DNA.

3.4.3 Removal of the First N-glycosylation site in GUS

The wild type GUS gene isolated from pDUB2511 was mutated by oligonucleotide 160 to eliminate the first N-glycosylation site. The reaction was analyzed as described in section 3.3.2. The mutated GUS gene was named as 160Mu. 160Mu was then transformed into TG2. Thirty one transformants were selected randomly and grown in 5ml 2x YT media. Phage stocks were prepared as described in section 2.11.1 and used in dot blot hybridization screening with labelled oligonucleotide 160 (section 2.11.3). Referring to the composition of nucleotides in the oligonucleotide 160 in section 2.10.1, the Td is equal to 60^{o}C , so the washing temperatures used in second and third washes of the nitrocellulose membrane were 55^{o}C and 58^{o}C , respectively. Wildtype single-stranded GUS DNA was used as controls. The results are shown in figure 7. Sample 9, 13, 22, 26 and 27 were found to be mutants.

In order to confirm that the desired mutation was made, single-stranded DNA was prepared (figure 8) and sequenced by the dideoxynucletide chain termination reaction as described in section 2.12. Since oligonucleotide 161 was used as the primer, the DNA sequence of the opposite strand was obtained (figure 9). In the corresponding position of the serine codon in the first N-glycosylation site, adenine was found to be changed to cytosine. Therefore, T-A was changed to G-C and alanine would be formed at this position of the GUS protein instead of serine.

Minipreparation of DNA of the samples were also carried out and digested with *Bam*HI and *Eco*RI and then electrophoresced in 0.8% agarose gel to confirm the presence of GUS insert (see figure 10).

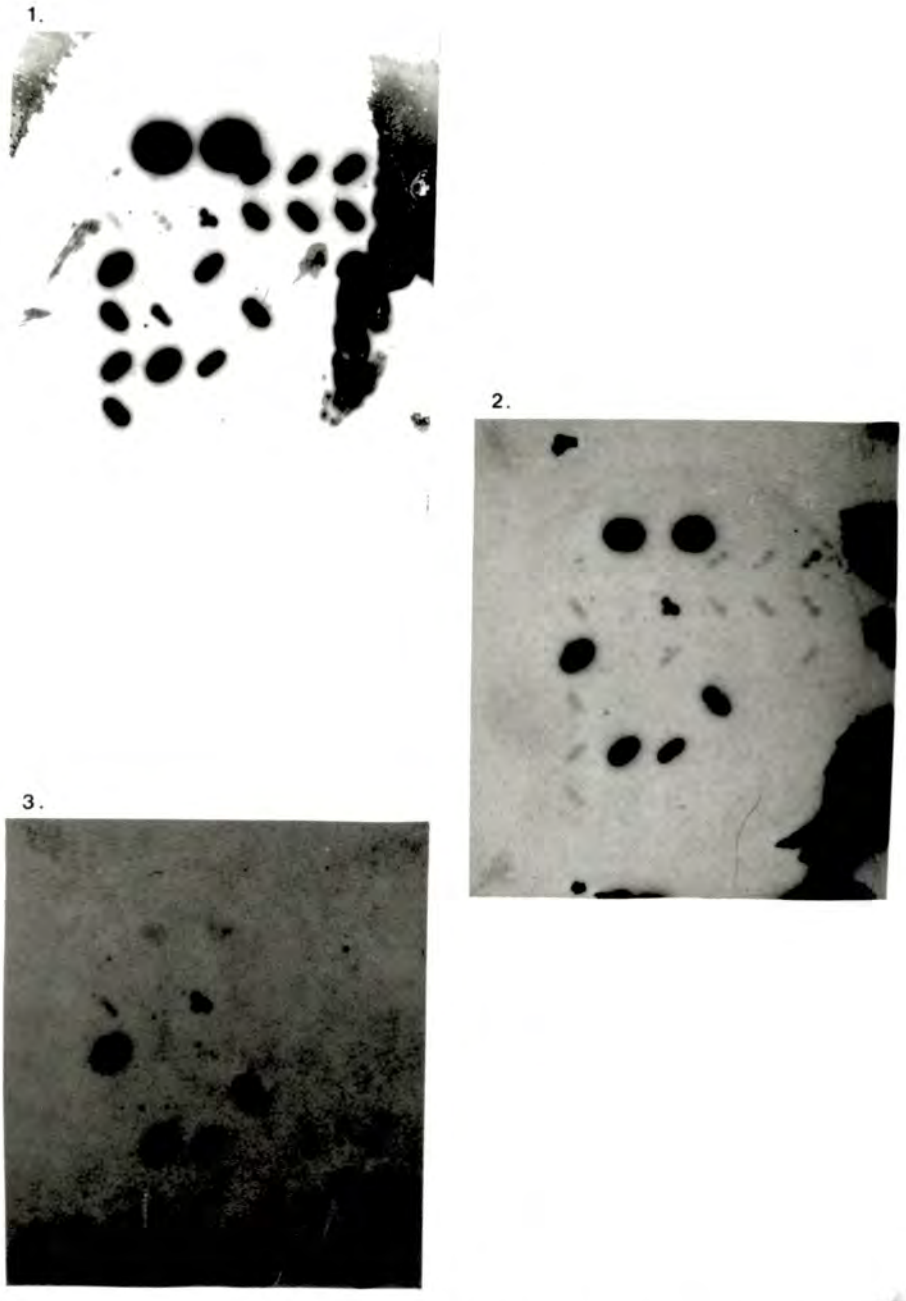

Figure 7: Dot blot hybridization screening of mutants with oligonucleotide 160. The first row consists of two controls of wild type GUS while the rest are tested samples. 1: End-labelled oligonucleotide 160 hybridized to the nitrocellulose bound DNA at 67^{o}C , the nitrocellulose membrane was then washed with 50ml of 6x SSC at room temperature for 5 minutes; 2: Second wash with 50ml of 6x SSC at 55^{o}C for 5 minutes; 3: Third wash with 50ml of 6x SSC at 58^{o}C for 5 minutes. The washing temperatures were calculated using the Wallace rule (see text for detail).

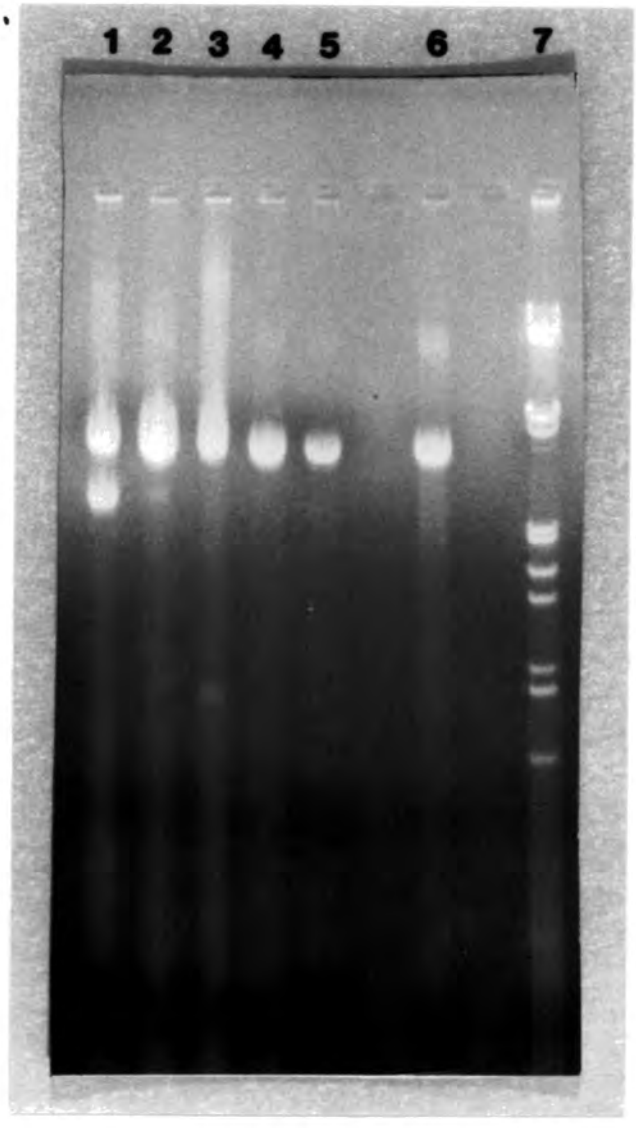

Figure 8: Agarose gel electrophoresis of M13-GUS single-stranded DNA of 5 samples of 160Mu. Lane 1: sample 27; Lane 2: sample 26; Lane 3: sample 22; Lane 4: sample 13; Lane 5: sample 9; Lane 6: wild type GUS as control; Lane 7: bacteriophage λ *Pst*I /*Hin*dIII.

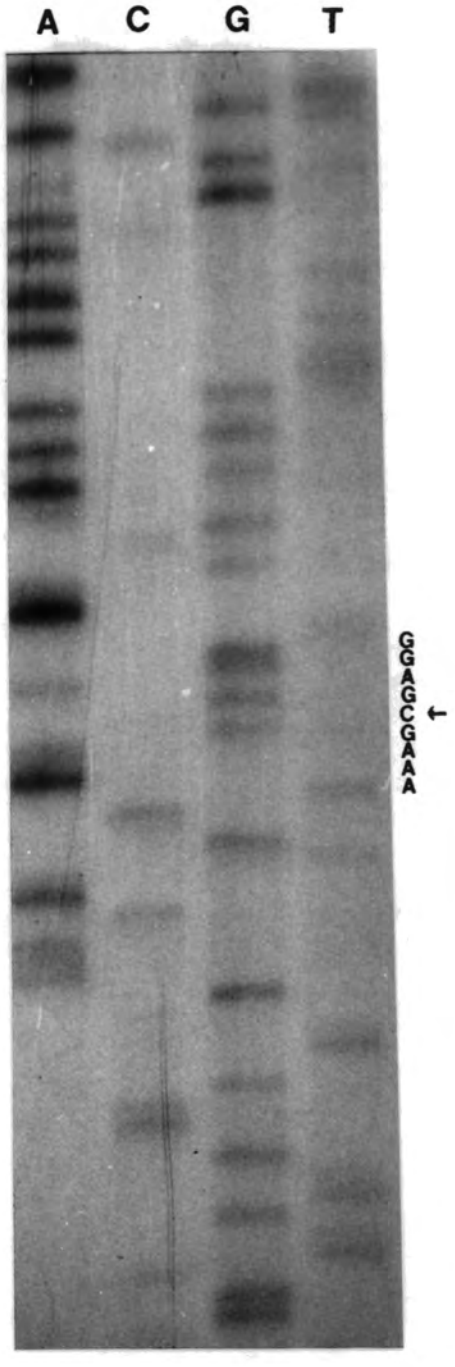

Figure 9: A DNA sequence of 160Mu around the mutated site.

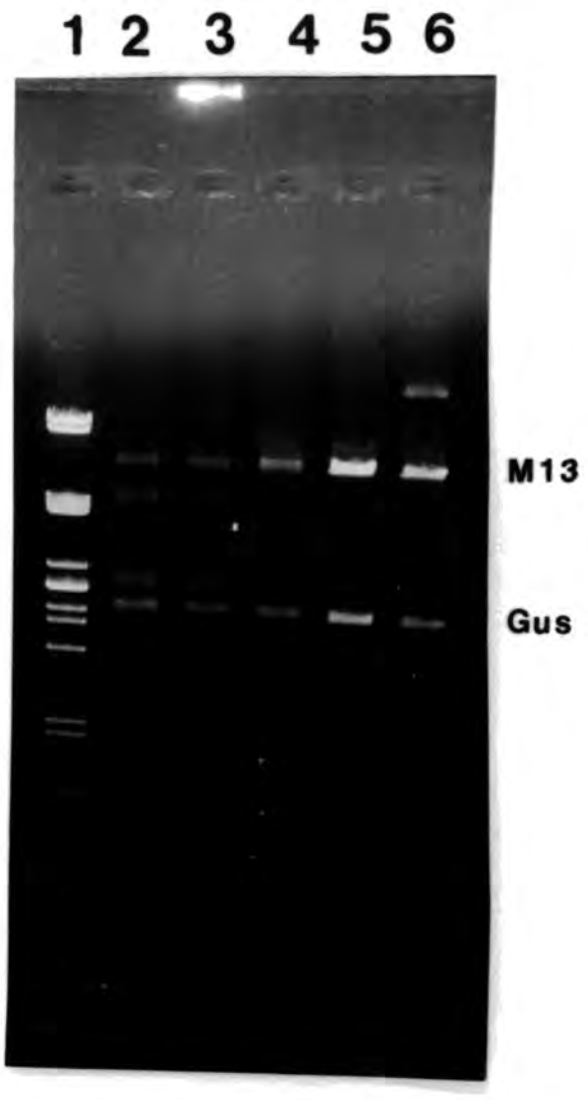

Figure 10: Agarose gel electrophoresis of miniprep of DNA digested with *Eco*RI and *Bam*HI. Lane 1: bacteriophage λ *Pst*I marker; Lane 2: sample 9; Lane 3: sample 13; Lane 4: sample 22; Lane 5: sample 26; Lane 6: sample 27.

3.4.4 Removal of the Second N-glycosylation Site in GUS

The second potential site for N-glycosylation of the GUS gene was mutated by oligonucleotide 161 as described in section 2.10. The mutated GUS DNA was named as 161Mu and transformed into *E. coli* TG2 as described in section 2.8.1. Thirty three transformants were selected randomly and grown in 5ml 2x YT media at 37°C overnight. Single-stranded template DNAs were prepared as described and used in the dot blot hybridization using labelled oligonucleotide 161 as a probe and wild type single-stranded GUS DNA as controls. The Td of the oligonucleotide 161 was calculated from Wallace rule and found to be 64°C. The second and third washing temperatures of the nitrocellulose membrane were then 59°C and 62°C, respectively. The results are shown in figure 11. Twenty-one colonies were found to be the desired mutants. Samples 5, 10, 15 and 22 were selected randomly to grow up for the large scale preparation of single-stranded DNA as described in section 2.7, and plasmid DNA (section 2.5). The purity of the prepared single-stranded DNA were tested by 0.8% agarose gel electrophoresis. The results are shown in figure 12. 10μl of the prepared plasmid DNA samples were double-digested with *Bam*HI and *Eco*RI and electrophoresced in 0.8% agarose gel. The result is shown in figure 13.

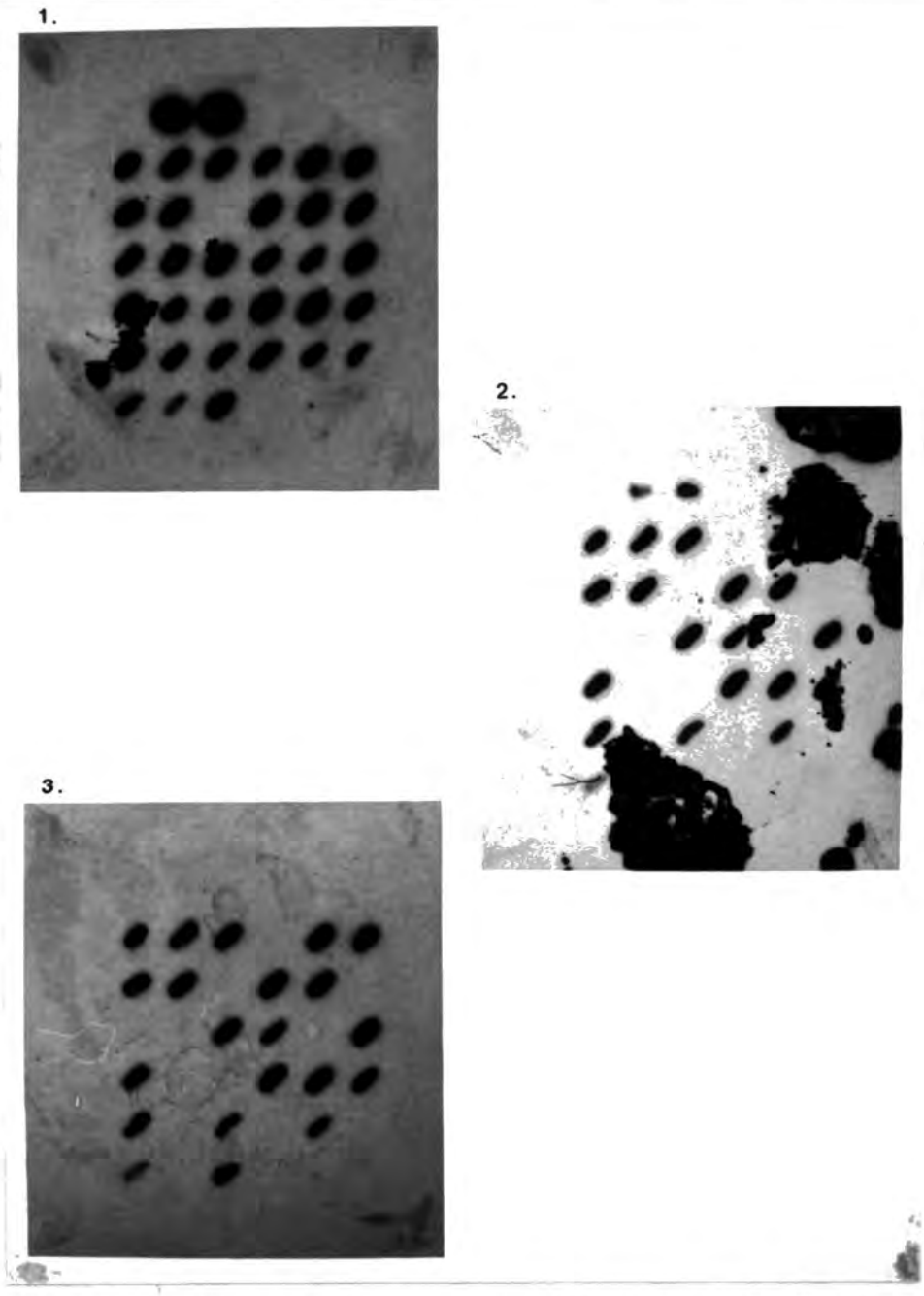

Figure 11: Dot blot hybridization screening of mutants with oligonucleotide 161. The first row consists of two controls of wild type GUS while the rest are tested samples. 1: The end-labelled oligonucleotide 161 hybridized to the nitrocellulose bound DNA at 67°C , the nitrocellulose membrane was then washed with 50ml 6x SSC at room temperature for 5 minutes; 2: Second wash with 50ml 6x SSC at 59°C for 5 minutes; Third wash with 50ml 6x SSC at 62°C for 5 minutes. The washing temperature were calculated using the Wallace rule (see text for detail).

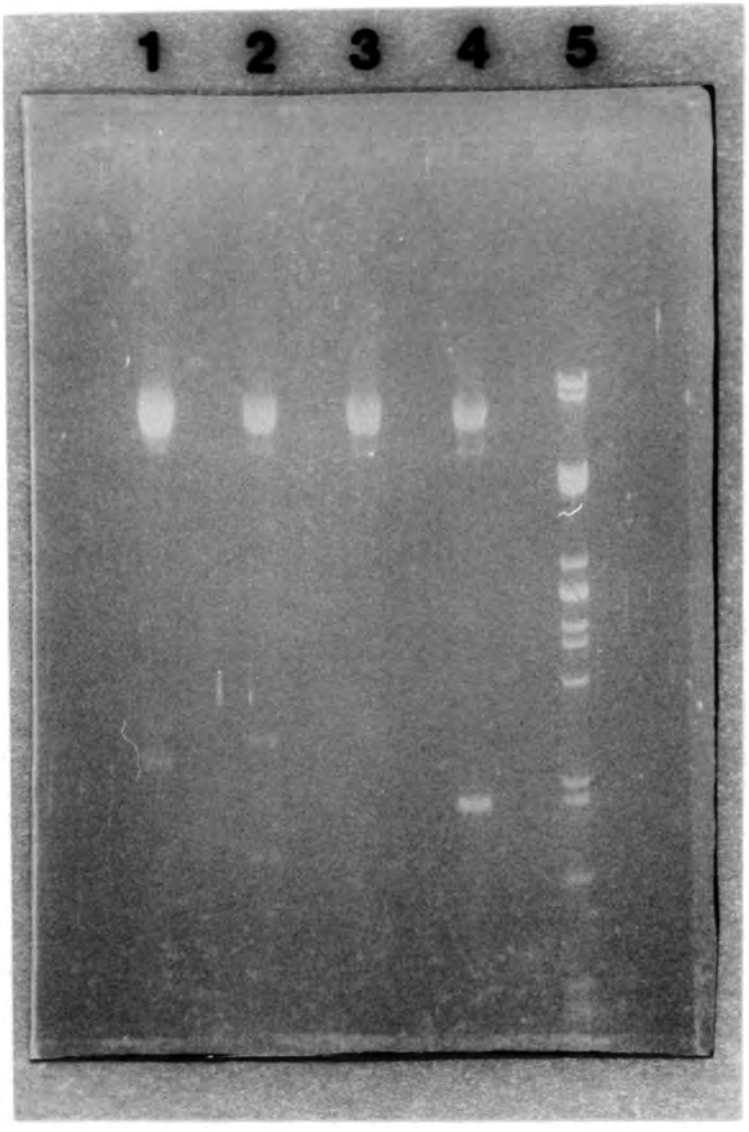

Figure 12: Agarose gel electrophoresis of single-stranded DNA of 161Mu. Lane 1: sample 22; Lane 2: sample 15; Lane 3: sample 10; Lane 3: sample 5; Lane 5: bacteriophage λ *Pst*I marker.

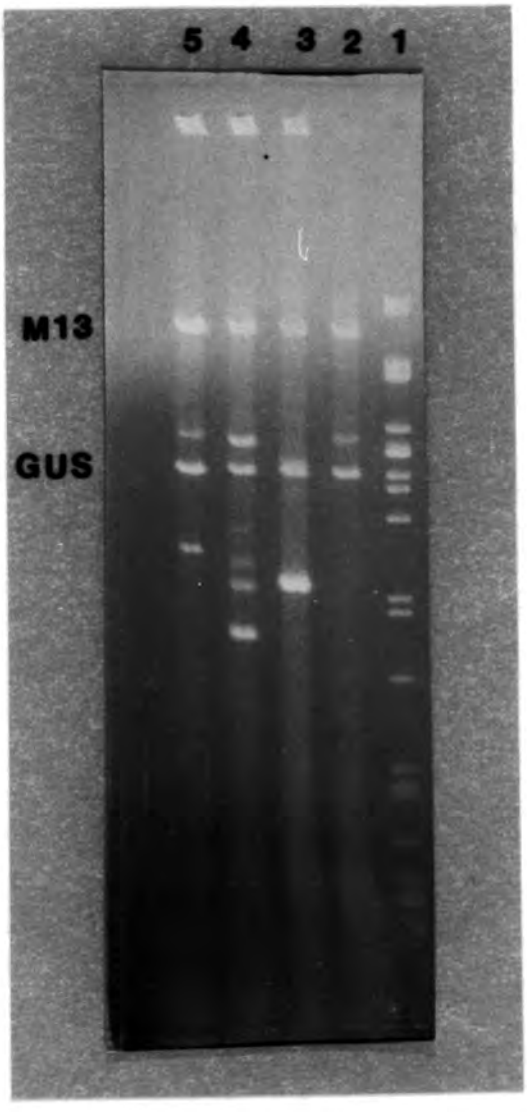

Figure 13: Agarose gel electrophoresis of miniprep of 161Mu digested with *Eco*RI and *Bam*HI. Lane 1: bacteriophage λ *Pst*I marker; Lane 2: sample 5; Lane 3: sample 10; Lane 4: sample 15; Lane 5: sample 22.

3.4.5 Removal of Both N-glycosylation Sites in GUS

Sample 13 of the single-mutant, 160Mu was selected and mutated by oligonucleotide 161 to form a double mutant. Therefore, the two possible N-glycosylation sites were removed in this double mutant. The method used was the same as described in section 2.10. The double-mutated DNAs were transformed into *E. coli* TG2. Thirty six transformants were selected randomly and grown up in 5ml 2 x YT media. The phage stock prepared (section 2.11.1) were used in the dot blot hybridization using labelled oligonucleotide 161 as a probe and single-stranded DNA of sample 13 of 160Mu was used as control. The washing conditions in the hybridization screening were the same as in section 3.4.4. The results are shown in figure 14. Ten colonies were found to be the desired double mutants. Sample 29, 30, 33 and 34 were selected randomly for the large scale preparation of single-stranded DNA and plasmid DNA. The purity of the prepared single-stranded DNA were tested by electrophorescing on 0.8% agarose gel. The results are shown in figure 15. *Eco*RI and *Bam*HI double digestion and then gel electrophoresis of the plasmid DNA samples were also performed to test for the presence of the GUS gene (figure 16).

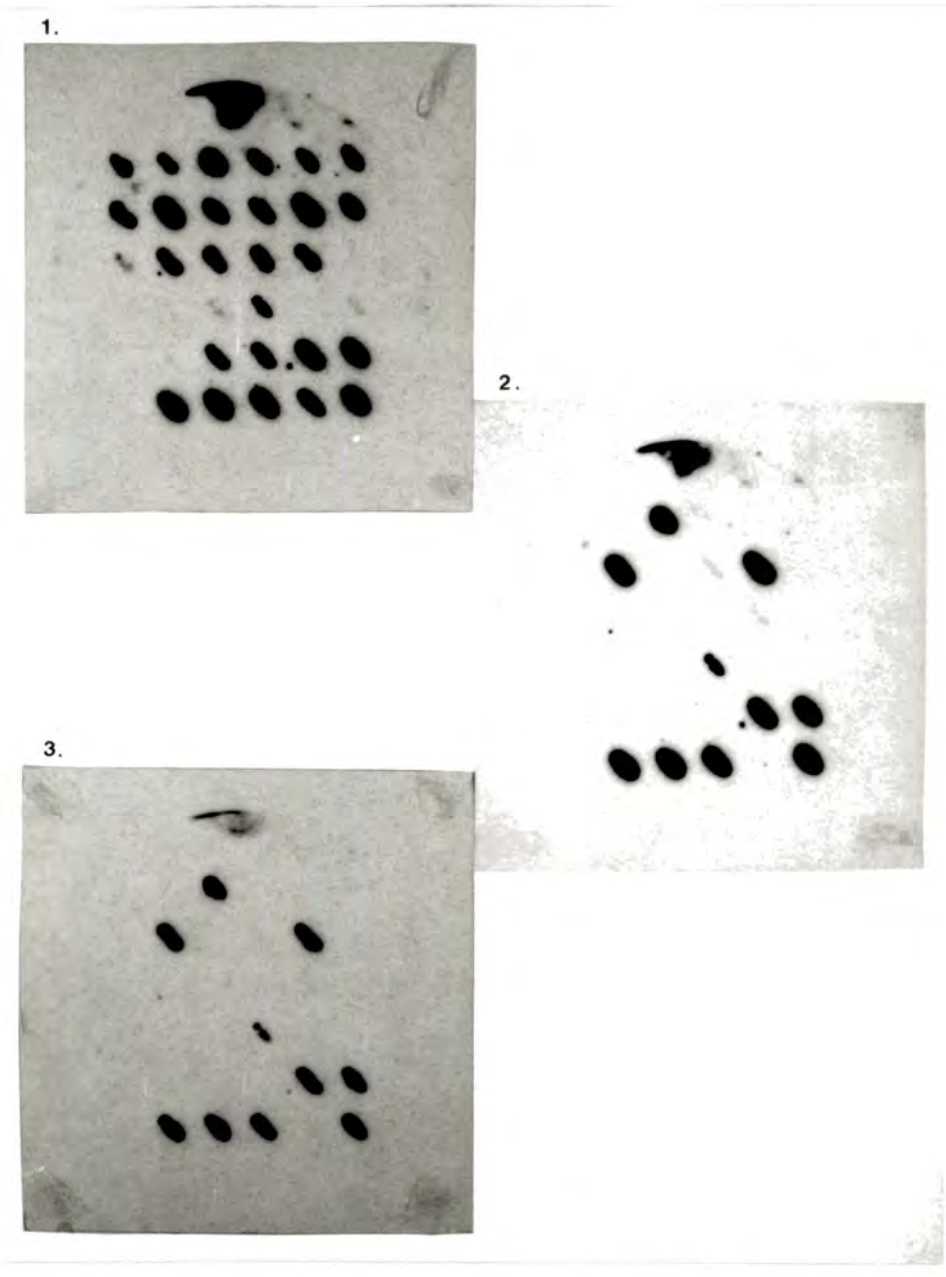

Figure 14: Dot blot hybridization screening of mutants with oligonucleotide 161. The first row consists of a control of 160Mu while the rest are tested samples. 1: End-labelled oligonucleotide 161 hybridized to the nitrocellulose bound DNA at 67°C , the nitrocellulose membrane was then washed with 50ml of 6x SSC room temperature for 5 minutes; 2: Second wash with 50ml of 6x SSC at 59°C for 5 minutes; 3: Third wash with 50ml of 6x SSC at 62°C for 5 minutes. The washing temperatures were calculated from Wallace rule (see text for detail).

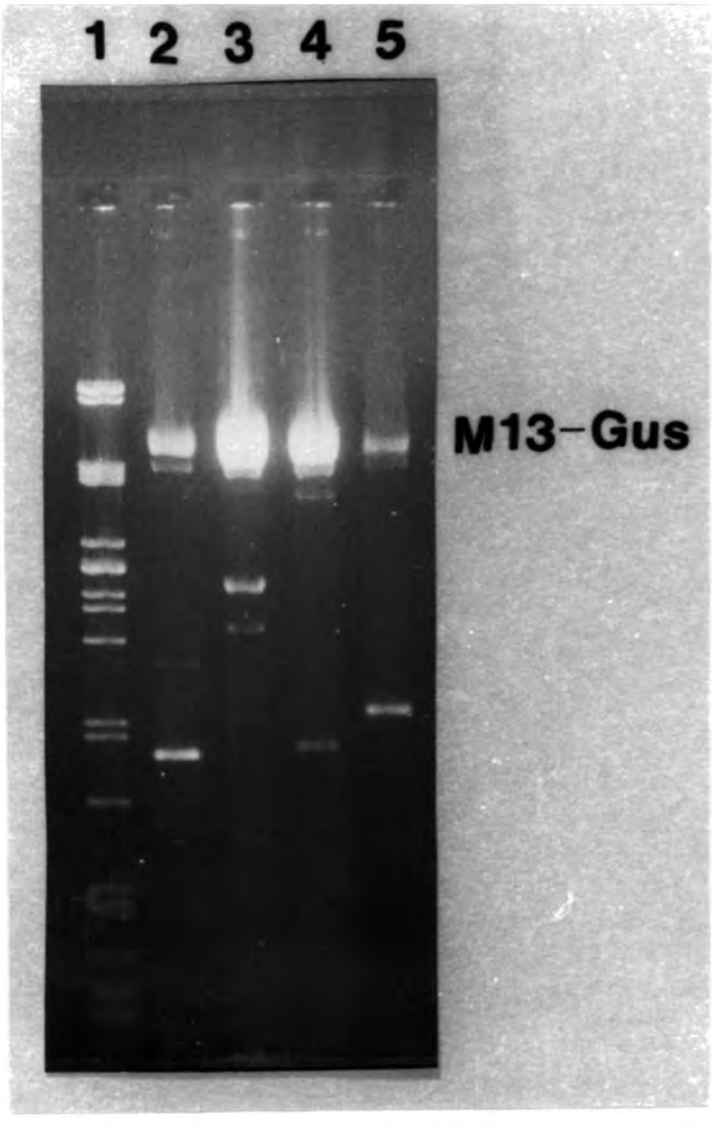

Figure 15: Agarose gel electrophoresis of the single-stranded DNA of (160+161)Mu. Lane 1: bacteriophage λ *PstI* marker; Lane 2: sample 29; Lane 3: sample 30; Lane 4: sample 33; Lane 5: sample 34.

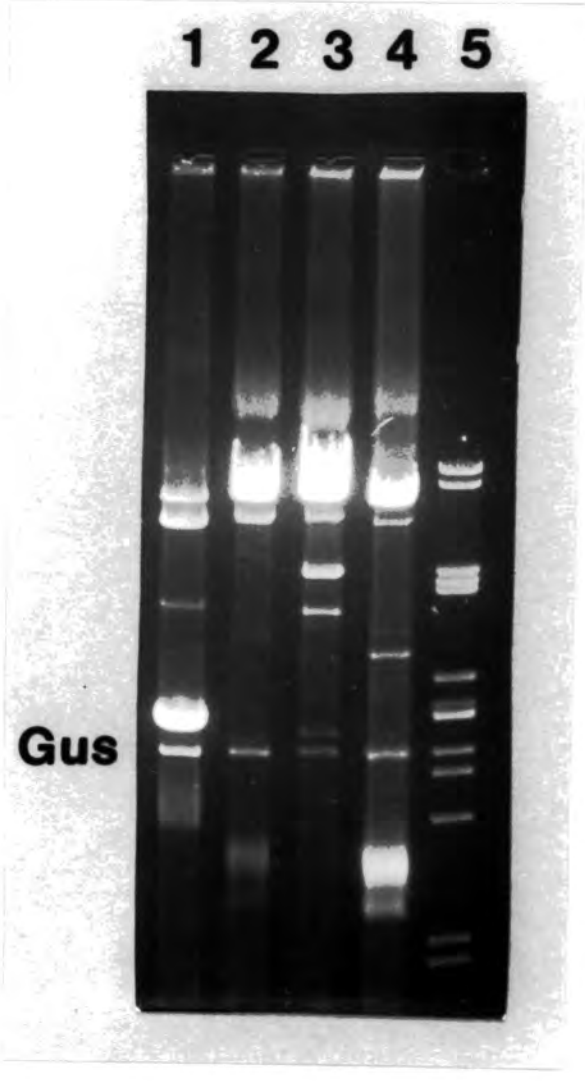

Figure 16: Agarose gel electrophoresis of miniprep of (160+161)Mu digested with *Eco*RI and *Bam*HI. Lane 1: sample 34; Lane 2: sample 33; Lane 3: sample 30; Lane 4: sample 29; Lane 5: bacteriophage λ *Pst*I marker

3.5 Cloning of Mutated GUS Genes into pUC18

1μg of the pUC18 vector DNA was digested with *Bam*HI and *Eco*RI. The restriction enzymes were removed from the digested fragments by phenol-chloroform extraction. The purified DNA was concentrated by ethanol precipitation and resuspended in 10μl of TE buffer.

The mutated GUS genes in M13 plasmids were digested with *Bam*HI and *Eco*RI. The GUS fragments were separated and isolated in 1% LMP agarose gel electrophoresis and then recovered by CETAB method.

The mutated GUS fragments were ligated separately with restricted pUC18 vector DNA and then transformed into *E. coli* TG2. The transformed cells were grown on selective L-ampicillin plates. The white transformed colonies bearing inserted GUS fragments were re-streaked onto L-ampicillin plates to confirm the purity of the sub-culture.

The colonies were screened for the correct *Bam*HI-*Eco*RI insert by digestion of the miniprep plasmid DNA (section 2.4) with these two enzymes in a double digest. The results are shown in figure 17 to 19.

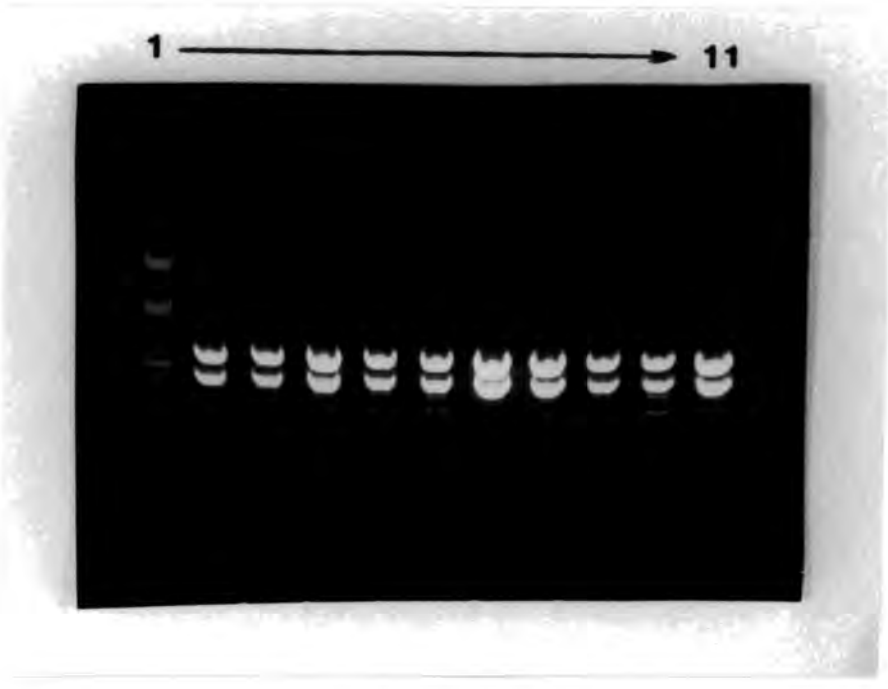

Figure 17: Agarose gel electrophoresis of miniprep of 160Mu GUS cloned in pUC18 digested with *Bam*HI and *Eco*RI. Lane 1: bacteriophage λ *Pst*I marker; Lane 2-11: 160Mu GUS cloned in pUC18. The fast running bands are GUS while the slow running bands are pUC18. Note that all the tested samples have GUS inserts.

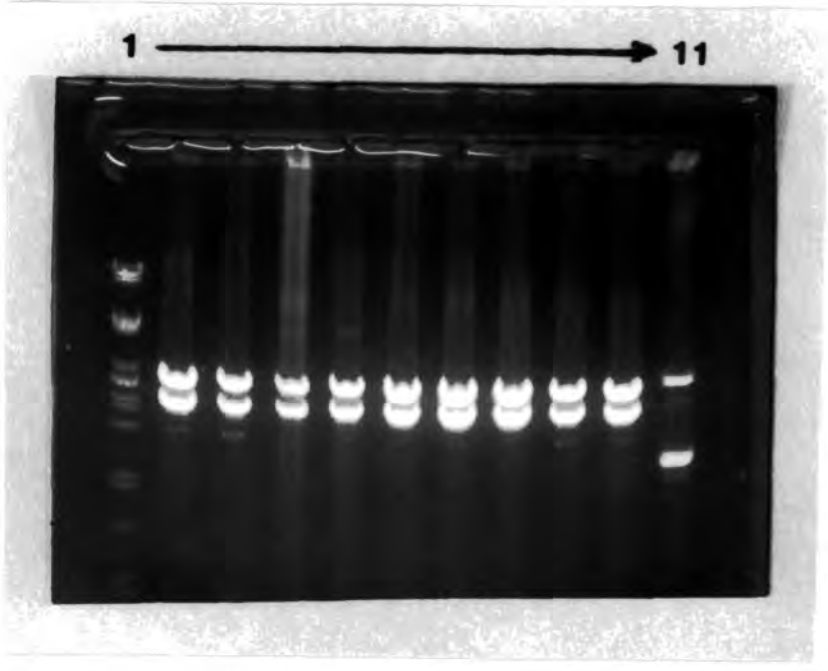

Figure 18: Agarose gel electrophoresis of miniprep of 161Mu GUS cloned in pUC18 digested with *Bam*HI and *Eco*RI. Lane 1: bacteriophage λ ***Pst*I** marker; Lane 2-11: 161Mu GUS cloned in pUC18. The fast running bands are GUS while the slow running bands are pUC18. Lane 2 to 10 show the presence of GUS inserts.

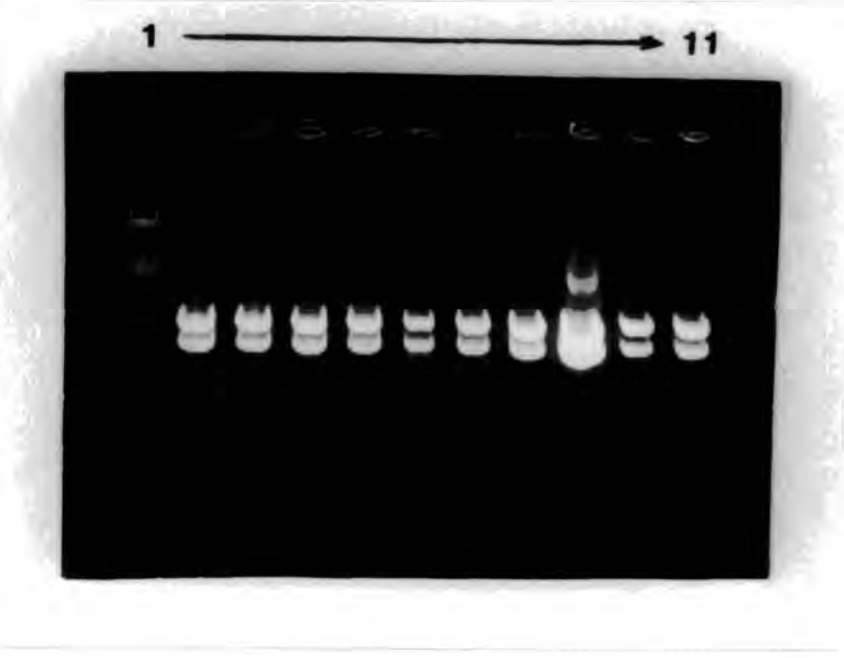

Figure 19: Agarose gel electrophoresis of miniprep of (160+161)Mu GUS cloned in pUC18 digested with *Bam*HI and *Eco*RI. Lane 1: bacteriophage λ *Pst*I marker; Lane 2-11: (160+161)Mu GUS cloned in pUC18. The fast running bands are GUS while the slow running bands are pUC18. Note that all the tested samples have GUS inserts.

3.6 Transformation of pDUB2511 into *E. coli* TG2

In order to test the effect of the mutations on β-glucuronidase activity, pDUB2511 containing the wild type GUS gene was also transformed into the host *E. coli* TG2 as a reference. The method was the same as described above. The white transformed colonies were confirmed as having GUS inserts by a double digestion of the miniprepared plasmid DNA with *Bam*HI and *Eco*RI. The results shown in figure 20 prove that all ten randomly chosen colonies had GUS inserts.

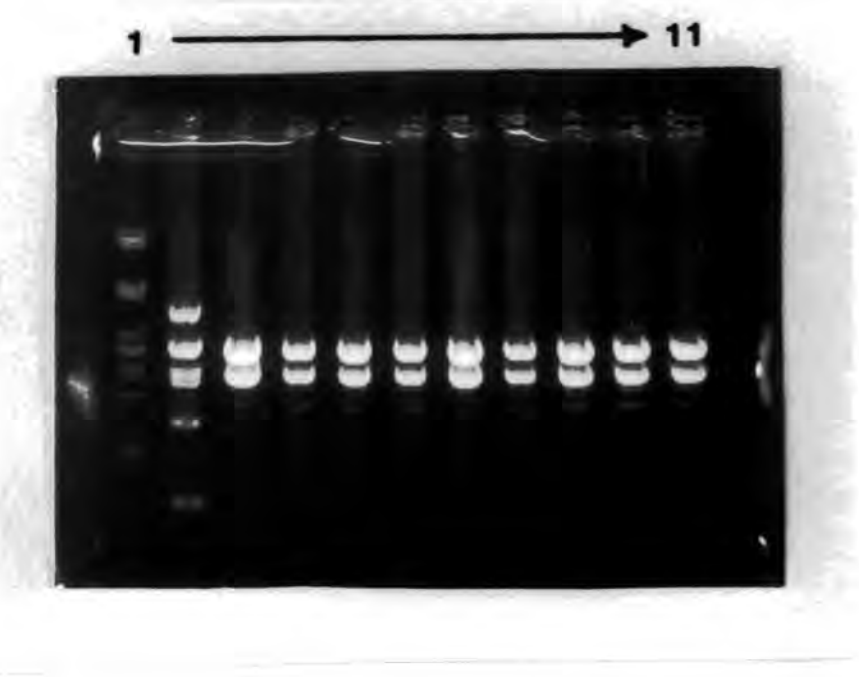

Figure 20: Agarose gel electrophoresis of miniprep of pDUB2511 containing wildtype GUS transformed in TG2 digested with *Bam*HI and *Eco*RI. Lane 1: bacteriophage λ *Pst*I marker; Lane 2-11: tested samples The fast running bands are GUS while the slow running bands are pUC19. Lane 3 to 11 show the presence of GUS inserts.

3.7 Determination of β-Glucuronidase activity

The enzymatic activities of the wild type and mutated GUS were determined as described in section 2.16. The control was the pUC18 transformed *E. coli* TG2 cell extract. The results are presented in table 4 to 9 and figure 21 and 22. The results shown in figure 22 indicate that *E. coli* TG2 had some background GUS activity. The enzymatic activities of the mutated GUS, including two single mutants and one double mutant, were higher than that of the wild type GUS.

Control	Protein Conc ($mgml^{-1}$)	Emission (E)	GUS Activities ($Emlmg^{-1}$)
1	0.78	12.7	16.21
2	0.66	5.6	8.46
3	0.69	2.6	3.78

Table 4: The GUS activities of TG2 transformed with pUC18 (control)

Wildtype	Protein Conc ($mgml^{-1}$)	Emission (E)	GUS Activities ($Emlmg^{-1}$)
1	0.62	29.5	47.29
2	0.54	22.0	40.55
3	0.46	30.0	65.39
4	0.61	24.0	39.48
5	0.60	23.0	38.45
6	0.62	21.7	34.96
7	0.61	29.3	48.30
8	0.65	22.3	34.08
9	0.57	26.7	46.59

Table 5: The enzymatic activities of 9 samples of wildtype GUS

160Mu	Protein Conc ($mgml^{-1}$)	Emission (E)	GUS Activities ($Emlmg^{-1}$)
1	0.57	22.7	39.95
2	0.44	26.3	59.38
3	0.50	30.0	60.18
4	0.52	22.7	43.25
5	0.45	24.5	54.41
6	0.61	22.7	37.49
7	0.40	25.7	63.83
8	0.78	24.3	31.03
9	0.66	27.0	41.07

Table 6: The GUS activities of 9 samples of 160Mu

161Mu	Protein Conc ($mgml^{-1}$)	Emission (E)	GUS Activities ($Emlmg^{-1}$)
1	0.59	35.0	59.74
2	0.41	29.0	69.91
3	0.52	47.0	89.56
4	0.51	46.5	92.03
5	0.45	20.3	44.90
6	0.51	27.7	54.50
7	0.47	39.5	84.19
8	0.46	21.3	46.18
9	0.58	45.5	78.56

Table 7: The GUS activities of 9 samples of 161Mu

(160+161)Mu	Protein Conc ($mgml^{-1}$)	Emission (E)	GUS Activities ($Emlmg^{-1}$)
1	0.45	23.3	51.96
2	0.61	47.0	77.32
3	0.60	37.0	61.48
4	0.46	24.0	52.66
5	0.51	31.8	62.71
6	0.51	55.0	107.81
7	0.47	46.5	99.62
8	0.52	37.5	71.96
9	0.49	23.7	48.80

Table 8: The GUS activities of 9 samples of (160+161)Mu

Conditions	Average GUS Activities ($Emlmg^{-1}$)	Standard Deviation ($Emlmg^{-1}$)
Control	9.48	5.13
Wildtype	43.90	9.05
160Mu	47.84	11.07
161Mu	68.84	17.24
(160+161)Mu	70.48	19.90

Table 9: Comparisons of GUS activities between wildtype and mutated GUS

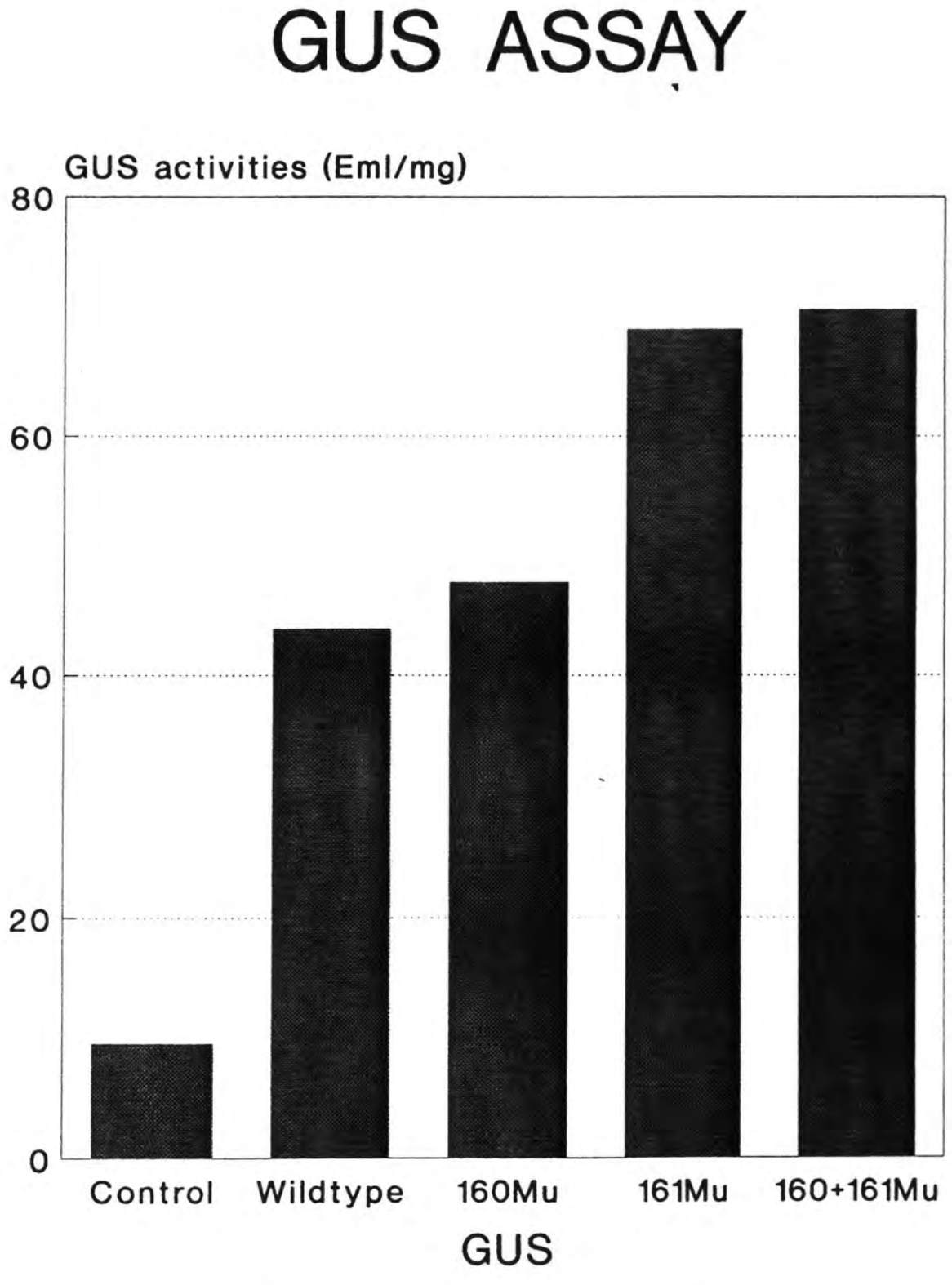

Figure 21: A bar chart shows the GUS activities of wild type and mutated GUS.

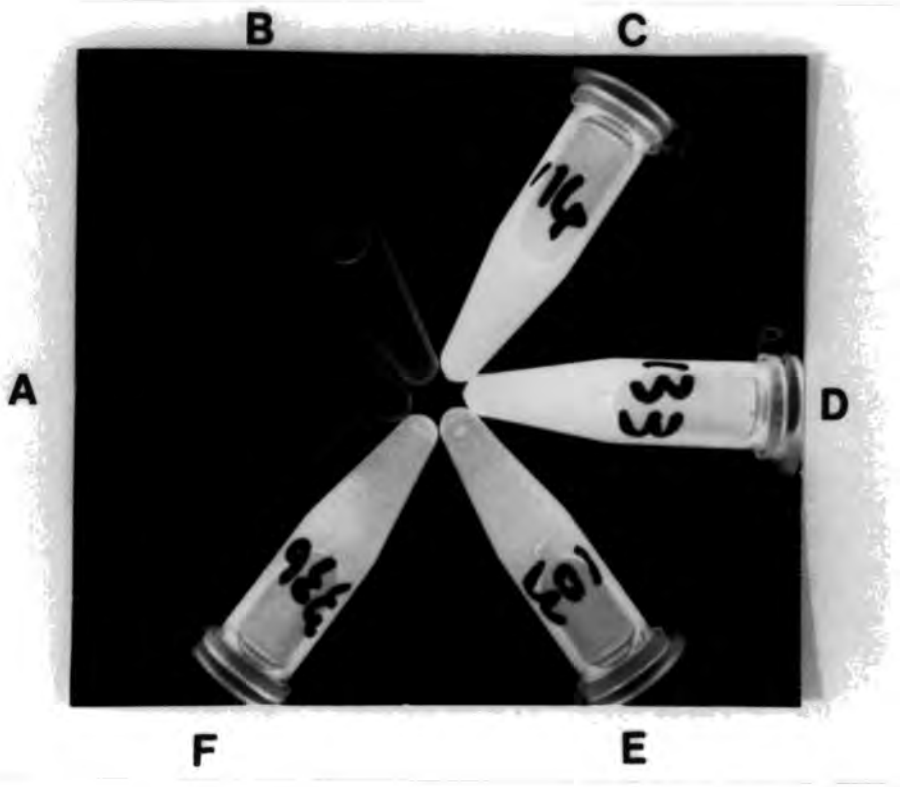

Figure 22: GUS reaction mixtures of A: blank; B: control (*E. coli* TG2 transformed with pUC18); C: pDUB2511; D: 160Mu; E: 161Mu; F: (160+161)Mu, exposed under U.V. light.

Chapter IV

DISCUSSION

4.1 Transformation of GUS into Protease-deficient Strains

Unsatisfactory results were obtained. Although GUS was shown to be expressed in all transformed yeast strains (see figure 2). the amounts of GUS produced in protease-deficient yeast strains YHH32 and CL-2 ABYS106 were less than that produced by normal YHH19 as shown by western blot. This might be due to several reasons: (i) the transfer of proteins to nitrocellulose membrane was not efficient. The standard GUS in lane two loaded with a quantity of one microgram is barely detectable; (ii) differences in the amount of the protein loaded into the wells; (iii) poor anti-GUS antibody; (iv) mutations may occur as the transformation protocol from Ito *et al.*, (1983) has mentioned the possibility of mutations of yeasts with this method. It is difficult to decide between these possibilities as the western blots were never reproducible.

4.2 Tunicamycin treatment

Haider (1989) used the CPY-GUS fusion system to show *in vitro* vacuolar protein targeting. Although most of the GUS was found in the vacuole, the enzymatic activity was found to be reduced by 80%. This is consistent with the observation of Iturriaga *et al.*, (1989) who used GUS as a reporter gene to study protein targeting in plants. They found that GUS in the endomembrane system had very low levels of GUS activity, although large amounts of GUS protein were formed. Iturriaga *et al.* (1989) realized that GUS had two cryptic

protein were formed. Iturriaga *et al.* (1989) realized that GUS had two cryptic glycosylation sites which were potential substrates of the oligosaccharide transferase in the lumen of the ER. These glycosylations could possibly inhibit the enzymatic activity by altering the secondary structure at the active site or by inhibiting the assembly of tetramers which were the enzymatically active components of GUS (Jefferson *et al.*, 1986).

Tunicamycin, a drug that inhibits glycosylations was used to to recover GUS activity. When the yeast cells were treated with tunicamycin in a concentration of 10μg/ml for a short period, two and a half hours, the GUS activity was found to be increased by 7-10%. However, when the period of tunicamycin treatment was increased to 16 hours, the protein concentration of the tunicamycin treated yeasts decreased by 80%. This indicates that tunicamycin did affect the gross protein synthesis of yeasts. Compared with the results of the Iturriaga *et al.* (1989) in which the tobacco plant cell cultures were treated with this concentration of tunicamycin, but for the even longer period of 24 hours, there was no effect on the gross protein synthesis. Yeasts being faster growing might be more sensitive to tunicamycin. In addition, correctly glycosylated proteins are essential for the normal functioning and survival of the cells.

If glycosylation is responsible for the reduction in GUS activity with the CPY-GUS system, then this experiment did not show it. A far greater stimulation by tunicamycin would have been expected.

4.3 Site-directed Mutagenesis

Although a shorter tunicamycin treatment could be used to recover the enzymatic activity of GUS and may increase the sensitivity of the targeting power of the CPY-GUS fusion system in yeast, it will inevitably complicate the process. In the long term, a more direct way to recover GUS activity is to remove the putative N-linked glycosylation sites by site-directed mutagenesis, thus allowing for a direct comparison of steady-state levels of translocated and cytosolic GUS enzyme.

However, the mutations of these N-glycosylation sites may also affect the secondary structure of GUS and possibly reduce the GUS activity. Hence, the design of the mutations was quite critical. Possible N-glycosylation sites are those with the amino acid sequence of NXS/T. In β-glucuronidase, the first N-glycosylation site is NLS at 358-360 and the second one is NIS at 423-425. In order to remove them, either asparagine or serine should be substituted by a suitable amino acid by changing the genetic codes. To do this, the following factors were considered: (a) the difference in size and charge between the present and replaced amino acids, (b) the number of bases needed to be changed in order to obtain the wanted amino acid. Table 10 compared the possibility of the other amino acids to replace asparagine or serine.

In order to conserve the conformation of the protein, all amino acids have large differences in molecular mass comparing with asparagine or serine were not considered, for example, isoleucine, phenylalanine, proline, tyrosine and tryptophan. In addition, all the charged amino acids, for instance, arginine, asparatic acid, glutamic acid, lysine and histidine were not the likely candidates to replace the uncharged asparagine or serine. Cysteine forms disulphide bonds

Amino acids	Genetic code	No. of bases to be changed from		Side Chain	
		Asparagine	Serine	Size (MW)	Nature
Alanine	GCX	2	1	15	nonpolar
Arginine	CGX or AGA/G	2	2	101	basic
Asparagine	AAT/C	0	1	58	polar
Aspartic acid	GAT/C	2	2	62	acidic
Cysteine	TGT/C	2	2	47	polar
Glycine	GGX	2	2	1	polar
Glutamic acid	GAA/G	2	2	72	acidic
Glutamine	CAA/G	2	2	72	polar
Histidine	CAT/C	1	2	82	basic
Isoleucine	ATT/C/A	1	1	57	nonpolar
Leucine	CTX or TTA/G	2	2	57	nonpolar
Lysine	AAA/G	1	2	73	basic
Methionine	ATG	2	3	75	nonpolar
Phenylalanine	TTT/C	2	1	91	nonpolar
Proline	CCX	2	1	42	nonpolar
Serine	TCX or ACT/C	2	0	31	polar
Threonine	ACX	1	1	45	polar
Tryptophan	TGG	3	2	132	nonpolar
Tyrosine	TAT/C	1	1	107	polar
Valine	GTX	2	2	43	nonpolar

Table 10: Comparison of amino acids to replace asparagine or serine of the N-glycosylation sites of GUS. (A: adenine, C: cytosine, G: guanine, T: thymidine, X: any nucleotides).

in the protein which may change the conformation or activity of the protein. Therefore, it was also eliminated as a possible choice.

The length of the synthetic oligonucleotide used increases with the number of the mis-matched bases. However, longer synthetic oligonucleotide may decrease the efficiency of the annealing step of the mutagenesis reaction, possibly due to the secondary structure of the template DNA. Hence, the number of the base change was reduced to minimum so as to reduce the length of the synthetic oligonucleotide used. Therefore, all amino acids that to be obtained by changes of two or three bases were also not considered.

Then, the remaining possible amino acid that may be used to substitute asparagine is threonine because it only require a change of the second base from A to C and has a similar size as asparagine. However, threonine tends to be phosphorylated through the transfer of phosphate group from ATP catalyzed by protein kinase. Such phosphorylation would change the protein conformation and activity of the protein (Alberts *et al.,* 1983). It seems that asparagine is not suitable to substitute by the other amino acids.

Referring to the possibility of the replacing other amino acids for serine, the most likely choice was alanine because it could be achieved by changing the first base of the serine codon from T to G. In addition, alanine has a similar size as serine although it is slightly less polar than serine when the hydroxyl group was replaced by a hydrogen atom. Thus, two synthetic mutagenic oligonucleotides were designed and made to replace serine in the two N-glycosylation sites with alanine.

The mutations were done stepwise. It has ane advantage of observing the effects of mutation to specific sites on the enzymatic activity of GUS. Wild type

GUS was isolated from pDUB2511 and mutated with oligonucleotide 160 or 161 to produce the mutated GUS 160Mu or 161Mu respectively. The mutated GUS 160Mu was then mutated again with oligonucleotide 161 to produce a double mutant. All the mutated GUS genes were cloned into pUC18 and inserted in correct orientation with *lacZ* promoter of pUC18. The GUS-pUC18 plasmids were transformed into *E. coli* TG2. The enzymatic activity of the mutated GUS was tested. The results show that the activities of all the mutated GUS are not only retained but also higher than that of the wild type GUS. It seems that the mutations can modify the GUS activity. When the GUS activities of the mutated GUS are compared, both 161Mu and (160+161)Mu are much higher than that of 160Mu. On the other hand, the GUS activity of (160+161)Mu is slightly higher than that of 161Mu; and the GUS activity of 160Mu is slightly higher than that of wildtype. These observations show that the substitution of alanine for serine at position 425 enhances GUS activity. The reason for the increase in enzymatic activities in mutated GUS may be due to the fact that the less polar alanine changes the conformation of GUS which might expose the active site to the substrate more easily. These experiments also show that neither of the serine residue at position 360 nor that at position 425 in the wild-type GUS are at the active site. The control was the *E. coli* TG2 transformed with intact pUC18. It shows that some background GUS activity but far below that of the wild type or mutated GUS.

4.4 Future works

1. As the mutated β-glucuronidase 161Mu and the (160+161)Mu have not been sequenced to confirm directly the desired change to the GUS gene have been made. When the appropriate primers are available, this final confirmation can be done.

2. The mutated GUS genes can be fused to a signal sequence, for example, the signal sequence of carboxypeptidase Y (CPY) which is then then cloned into the yeast-bacteria shuttle vector. The CPY-GUS containing shuttle vector would be transformed into yeasts. Under the control of a suitable promoter, the expressed GUS activity would be assayed and compared with the wildtype GUS. Since yeasts are known to have low GUS background, it could be used to confirm the evidence of Iturriaga *et al.* (1989) that the decreased GUS activity is due to solely the glycosylation of one or two of cryptic N-glycosylation sites or other unknown factors.

3. Site-directed mutagenesis may be needed to mutate and remove the translational start codon, ATG, of GUS β-glucuronidase , so that any chances of independent translation of the GUS can be eliminated. Hence, all the GUS produced is under the control of the amino-terminal fused signal sequence of CPY. In addition, any appearance of GUS activity in the cytosol, periplasmic space or cell surface should be due to the mistargeting of the signal or sorting functions of the cells.

4.5 Potential Uses of the CPY-GUS system

This CPY-GUS fusion system can be used to study the vacuolar protein targeting and sorting in yeasts. It has the same function of screening vacuolar

protein targeting (*vpt*) mutants as the CPY-invertase fusion system developed by Bankaitis *et al.*, 1986, but it has an advantage of the absence of GUS in yeast, and thus eliminating the tedious construction of the host strains to remove any background activity of the reporter protein. If mutants occur that mislocalized the CPY-GUS fusion protein to the periplasmic space, GUS activity will appear at the cell surface. Such *vpt* mutants will give rise to blue colonies while the wild type strains with CPY-GUS hybrid protein will form white colonies on X-gluc containing plates. The screened mutants can then be characterized for the defects in sorting and processing of the CPY. Genetic crosses of the characterized mutants with the newly isolated mutants will isolate complementation groups. The complementary strains will form white colonies amongst the blue non-complementary colonies (Haider, 1989). This gene fusion approach in theory could be applied to any vacuolar protein whether its biochemical activity was known or not and the identification of mutants is independent on their secretion of the active CPY (Robinson *et al.*, 1989).

SUMMARY

1. CPY-GUS plasmids were transformed into protease-deficient strains, YHH32 and CL-2 ABYS106 so as to test whether the low GUS activity in yeast was due to the degradation by intracellular proteases found in the vacuole to which it it directed. Although SDS-PAGE shows the presence of GUS in the transformed yeast strains, the amount of GUS produced in the transformed protease-deficient strains were less than that produced by transformed normal YHH19 as shown by western blot. The reasons for these might be due to (i) the inefficiency of transfer of proteins to nitrocellulose membrane by the electroblot; (ii) difference in the amount of proteins loaded into the wells; (iii) poor anti-GUS antibody; (iv) mutations of the yeast during transformation.

2. Tunicamycin was used in an attempt to increase GUS activity by blocking glycosylation which causes the inactivation of GUS. A short-term treatment of tunicamycin at a concentration of $10\mu gml^{-1}$ increased the GUS activity by about 10%. A long-term treatment with the same concentration of tunicamycin not only could not recover the GUS activity but it also interfered with the normal growth of yeast.

3. Site-directed mutagenesis was employed to remove N-glycosylation sites in GUS. Mutated GUS 160Mu, 161Mu and (160+161)Mu were created. The enzymatic activity of these mutated GUS was found to be higher than that of the wild type GUS when tested in the bacterium *E. coli* TG2.

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