Analysis of heterologous gene expression from the *KIMAL21-KIMAL22* bi-directional promoter using cyan and yellow fluorescent proteins

By

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Abstract

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The main components of any heterologous protein expression system are a suitable host organism and an active genetic promoter. The dairy yeast *Kluyveromyces lactis* efficiently expresses and secretes heterologous gene products; however, the number of genetic promoters developed for use in this system is limited. *Kluyveromyces lactis* strain CBS 1065 expresses large quantities of maltase late in fermentation. The maltase (*KlMAL22*) gene is divergently transcribed from an intergenic region along with the maltose permease (*KlMAL21*) gene. This intergenic region was identified as a potential candidate promoter for heterologous protein expression.

To fully characterize the promoter, oligonucleotide primers were designed from the maltase and maltose permease genes and were used to amplify the 1069 bp promoter from *K. lactis*. Basal promoter elements and putative transcription factor binding sites were identified by sequence analysis. Several sites of interest were identified including MIG1 glucose repressor protein consensus sites and an upstream activator sequence ($KlUAS_{MAL}$).

To explore regulation of the *KlMAL21-KlMAL22* promoter, expression of the fluorescent proteins CFP and YFP from the *KlMAL22* and *KlMAL21* orientations of the promoter, respectively, were analyzed. *Kluyveromyces lactis* cells were transformed with three expression plasmids. Each plasmid contained a different promoter variant. The first construct, pREX-IC, contained a wild-type promoter. The second variant, pREX-IC-ΔMig, contained the promoter with a putative MIG1 binding site removed. The third construct,

pREX-IC-ΔPos, contained the promoter with a portion of the putative *KlUAS*$_{MAL}$ removed. Fluorescent protein expression was compared between the three expression plasmids during growth on glucose, galactose or glycerol as the sole carbon source.

Expression of both fluorescent proteins from the native promoter was repressed during growth on glucose and galactose and was induced during growth in glycerol. Deletion of the putative MIG1 binding site did not relieve repression during growth in glucose. It did, however, decrease expression of both CFP and YFP during growth in glycerol. Disruption of the *KlUAS*$_{MAL}$ greatly reduced expression of both CFP and YFP during growth in all carbon sources. The results of this study demonstrate that the *KlMAL21-KlMAL22* promoter can be utilized to express heterologous proteins from both orientations. Expression from the promoter can be down-regulated by galactose. Therefore, the production of heterologous proteins from the *KlMAL2* bi-directional promoter can be regulated by the judicious selection of carbon source.

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List of Abbreviations

3'	three prime
5'	five prime
ABF1	autonomously replicating sequence (ARS) binding factor
ATP	adenosine triphosphate
ADP	adenosine diphosphate
AREA	activator of nitrogen-regulated genes from *Aspergillus nidulans*
bp	base pair
CBS	Centraalbureau voor Schimmelcultures
CFP	cyan fluorescent protein
CIP	calf-intestinal alkaline phosphatase
dCTP	deoxycytosine triphosphate
CYC8	general repressor of transcription
dH_2O	distilled water
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
FACS	fluorescence-activated cell sorter
FITC	fluorescein isothiocyanate
GCN4	general amino acid control activator
GFP	green fluorescent protein
GLN3	activator of nitrogen-regulated genes from *S. cerevisiae*
GRC1	glucose regulation protein
H. polymorpha	*Hansenula polymorpha*
HAP2/3/4	global regulator of respiratory genes
HIS4	histidinol dehydrogenase
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
K. lactis	*Kluyveromyces lactis*
kb	kilobase pair
M	molar
MAL	genes of the maltose utilization pathway
MIG1	global glucose repressor protein
ml	millilitre
mM	millimolar
N-terminal	amino-terminal
ng	nanogram
NIT2	activator of nitrogen-regulated genes from *Neurospora crassa*
P. pastoris	*Pichia pastoris*
PCR	polymerase chain reaction
pmol	picomole
RAP1	repressor/activator protein

RFI	relative fluorescence intensity
RNase	ribonuclease
S. cerevisiae	*Saccharomyces cerevisiae*
SUC2	invertase (β-fructofuranosidase)
SV40	simian vacuolating virus No. 40
TAE	tris acetate EDTA
TCA	tricarboxylic acid cycle
TE	tris EDTA
TUP1	general repressor of transcription
UAS	upstream activator sequence
µg	microgram
µl	microlitre
µm	micromolar
YFP	yellow fluorescent protein

Introduction

1.1 Gene Expression in Yeast

The expression of foreign (heterologous) proteins in a host organism is an important process for the production of biopharmaceuticals and commercially important enzymes. Some proteins are very difficult to purify or are expressed in only small quantities in their native organisms. The development of suitable heterologous protein expression systems offers a solution to these problems. The components of any expression system include a host organism, a promoter of transcription and an optimized culture system. Model bacterial species, such as *Escherichia coli*, have been used as production hosts. Bacteria, however, often exhibit alternate prokaryotic codon biases, improper protein folding and cannot perform many post-translational modifications necessary for the proper functioning of eukaryotic proteins. For this reason many researchers have turned their attention to eukaryotic expression systems provided by yeast and filamentous fungi.

Yeasts have been used commercially for heterologous expression of many proteins such as insulin, hepatitis B surface antigen (HbsAg) and human epidermal growth factor (EDF) (Hitzeman *et al.*, 1981; Stepien *et al.*, 1983; Brake *et al.*, 1984). While yeasts generally offer advantages over bacterial expression systems, such as proper protein folding and maturation of prohormones, N- and O-linked glycosylation and disulfide bond formation, they also remain attractive when compared to other eukaryotic expression systems. Yeasts are amenable to genetic manipulation, are easy to culture and are inexpensive to grow to large volumes (Buckholz *et al.*, 1991; Gellissen *et al.*, 1992; Romanos *et al.*, 1992; Eckart *et al.*, 1996; Dominguez *et al.*, 1998; Cereghino *et al.*, 2000).

Saccharomyces cerevisiae has been widely used to produce heterologous proteins, mostly because of the significant amount of knowledge that is available to researchers due to its history of use in baking and brewing. In the past 15 years, yeasts considered "non-conventional" have emerged as inviting hosts for the production of foreign proteins. The dairy yeast *Kluyveromyces lactis* has attracted much attention because of some significant advantages it offers over the standard yeast *S. cerevisiae*.

1.2 *Kluyveromyces lactis* as a Host Organism for Heterologous Protein Expression

The "conventional" yeast *S. cerevisiae* has been used in the production of foods for many centuries and has been exploited in the past few decades for the production of a variety of biochemical compounds. *Kluyveromyces lactis* shares many characteristics with *S. cerevisiae* that make it suitable for heterologous protein production, but also exhibits some uniquely superior properties.

Kluyveromyces lactis efficiently secretes heterologous proteins into its surrounding environment. The secretory apparatus of certain *K. lactis* strains has been evolutionally linked to a "killer" phenotype. These killer *K. lactis* strains express an exotoxin encoded by a set of transferable, linear, cytoplasmic plasmids known as pGKL1 and pGKL2 (Stark *et al.*, 1990). It is probable that *K. lactis* has developed an active secretory system out of necessity, since the killer phenotype requires the effective secretion of the toxin into the surrounding environment (Stark *et al.*, 1986; Stark *et al.*, 1990). Indeed the *K. lactis* killer toxin signal sequence has been used to direct efficient expression of the *Thermotoga maritime* xylanase to the extracellular environment (Bergquist *et al.*, 2002).

While some *S. cerevisiae* strains also have a killer phenotype associated with expression of an exotoxin, the conventional yeast has exhibited relatively low product yield and inefficient secretion of expressed heterologous protein (Magliani *et al.*, 1997; Dominguez *et al.*, 1998). During production of recombinant prochymosin, extracellular secretion of the recombinant product driven by the SUC2 yeast secretion signal represented only 10% of the total expression product (Smith *et al.*, 1985). Only after several rounds of mutagenesis and selective screening were secretion yields of up to 85% of total recombinant prochymosin generated (Smith *et al.*, 1985). In contrast, prochymosin expressed in *K. lactis* is secreted prior to any rounds of mutation and selection, at efficiencies of greater than 95% (van den Berg *et al.*, 1990). These efficiencies were observed using several different secretion signals including the native chymosin secretion signal, the *K. lactis* α-factor secretion signal, and the *Aspergillus awamori* amyloglucosidase secretion signal (van den Berg *et al.*, 1990). Several studies have determined that most of the expressed protein in *S. cerevisiae* was either associated with the cell wall or retained in the periplasmic space (van den Berg *et al.*, 1990; Buckholz *et al.*, 1991; Romanos *et al.*, 1992; Dominguez *et al.*, 1998).

As well as inefficient product secretion, *S. cerevisiae* has been shown to express low quality products. *Saccharomyces cerevisiae* expressing prochymosin did not properly introduce one or more of the three disulfide bonds required for the native molecule (Smith *et al.*, 1985). Prochymosin expressed in *K. lactis*, however, is expressed with correctly formed disulfide bonds (van den Berg *et al.*, 1990). Vacuolar proteases released into the culture medium can cause the truncation of expression products (Heim *et al.*, 1994; Hinnen *et al.*, 1995; Van Den Hazel *et al.*, 1996). The disruption of some vacuolar proteases,

however, can cause a reduction in cell proliferation of *S. cerevisiae* during late-growth stages and periods of nitrogen starvation, an undesirable characteristic in high-performance heterologous protein expression systems (Chen *et al.*, 2000). Even in *S. cerevisiae* strains deficient in major vacuolar proteases, expression of recombinant human parathyroid hormone and human serum albumin by *S. cerevisiae* is confounded by proteolytic cleavage mediated by cell-bound proteases (Chung *et al.*, 1998; Kang *et al.*, 2000). The digestion of heterologous proteins also seems to increase in *S. cerevisiae* strains secreting expression products at a high level, a definite problem when moving from laboratory-scale shake cultures to large-scale production fermentation (Stevens *et al.*, 1986). *Kluyveromyces lactis*, however, produces low levels of vacuolar and extracellular proteases, thus decreasing the proteolytic cleavage of expression products and allowing the easy isolation of recombinant proteins (van den Berg *et al.*, 1990; Hollenberg *et al.*, 1997).

Another drawback to using *S. cerevisiae* for the production of recombinant proteins is that it exhibits ethanol fermentation under aerobic conditions. This phenomenon is known as the Crabtree effect and *S. cerevisiae* is considered the benchmark Crabtree-positive organism (Crabtree, 1929; De Deken, 1966). *Kluyveromyces lactis*, on the other hand, is Crabtree-negative and regulates its carbon metabolism with dominance of respiration over fermentation, even in anaerobic conditions (Breunig *et al.*, 2000; Gonzalez-Siso *et al.*, 2000). The Crabtree effect has significant implications for heterologous protein production. Crabtree-positive yeasts exhibit reduced ATP production and lower biomass yield since pyruvate that has been decarboxylated and reduced to ethanol is not available to the TCA cycle (Breunig *et al.*, 2000). Reduced biomass results

in a lower yield of the expression product. Crabtree-negative yeasts are not limited by this characteristic and thus are more amenable for large-scale heterologous protein expression.

As with *S. cerevisiae*, *K. lactis* is generally recognized as safe (GRAS) by the American Food and Drug Administration (FDA). *Kluyveromyces lactis* is easily cultured and has been used to express heterologous proteins such as extracellular prochymosin and recombinant human serum albumin (HSA) in fermentation volumes up to 41,000 litres (van den Berg *et al.*, 1990; Fleer *et al.*, 1991). *Kluyveromyces lactis* thus offers an alternative expression platform to the conventional yeast *S. cerevisiae*. Many properties of *K. lactis*, such as efficient secretion apparatus, low levels of endogenous proteases and Crabtree-negative status make it often a more attractive choice than *S. cerevisiae* as a protein expression host.

1.3 Promoters

Many factors contribute to the optimal production of high-value protein. Expression host issues aside, a highly active constitutive or regulated promoter is necessary for high performance protein expression. Many promoters have been developed for protein expression in *S. cerevisiae* (Table 1). As discussed at length above, *S. cerevisiae* is not necessarily the best-suited yeast for the production of heterologous protein. Several alternative non-conventional yeasts are also available as expression hosts. The methyltrophs *Pichia pastoris* and *Hansenula polymorpha* are able to grow on methanol as a sole carbon source. Because of this researchers have been able to harness highly expressive alcohol oxidase gene promoters (*AOX1* and *AOX2* in *P. pastoris* and *MOX* in *H.*

Table 1. A selection of homologous promoters used for the expression of heterologous proteins in *S. cerevisiae* and *K. lactis*.

Species	Promoter	Gene	Reference
S. cerevisiae	*ADH1*	alcohol dehydrogenase	(Hollenberg *et al.*, 1997)
	ADH2	alcohol dehydrogenase	(Romanos *et al.*, 1992)
	ARG3	ornithine carbamyltransferase	(Hollenberg *et al.*, 1997)
	CUP1	copper ion binding	(Romanos *et al.*, 1992)
	ENO1	phosphopyruvate hydratase	(Hollenberg *et al.*, 1997)
	GAL1	galactokinase	(Romanos *et al.*, 1992)
	GAL7	galactose-1-phosphate uridyl transferase	(Wesolowski-Louvel *et al.*, 1996)
	GAL10	UDP-glucose 4-epimerase	(Hollenberg *et al.*, 1997)
	GAP	general amino acid permease	(Romanos *et al.*, 1992)
	PGK	3-phosphoglycerate kinase	(Romanos *et al.*, 1992)
	PHO5	acid phosphatase	(Romanos *et al.*, 1992)
	MFα	α-factor mating pheromone	(Hollenberg *et al.*, 1997)
K. lactis	*LAC4*	β-galactosidase	(Dominguez *et al.*, 1998)
	PHO5	acid phosphatase	(Ferminan *et al.*, 1998)
	AAC	ADP/ATP carrier	(Mustilli *et al.*, 1999)
	ADH4	Alcohol Dehydrogenase	(Saliola *et al.*, 1999)

polymorpha) for the purposes of heterologous protein production (Ellis *et al.*, 1985; Ledeboer *et al.*, 1985; Koutz *et al.*, 1989; Hollenberg *et al.*, 1997).

Kluyveromyces lactis has been used to produce foreign proteins for over a decade, although there is limited published information on genetic promoters useful for this purpose. Four native promoters, the *LAC4* β-galactosidase promoter, the *PHO5* repressible acid phosphatase promoter, and most recently the *AAC K. lactis* ADP/ATP carrier and alcohol dehydrogenase (*ADH4*) promoters have been identified and used for protein production in *K. lactis* (Table 1). The *LAC4* promoter is the most intensively studied and has been used in the production of a number of proteins including *S. cerevisiae* invertase, *E. coli* β-galactosidase, bovine pancreatic trypsin inhibitor (BPTI), *T. maritime* xylanase (XYNA), a single-chain Fv (svFv) of the antibody 4B2, and *Arxula adeninivorans* glucoamylase (Bui *et al.*, 1996; Hsieh *et al.*, 1998; Bergquist *et al.*, 2002; Panuwatsuk *et al.*, 2002; Panuwatsuk *et al.*, 2003; Robin *et al.*, 2003). Interestingly enough, more heterologous promoters from *S. cerevisiae* have been used in *K. lactis* than have endogenous *K. lactis* promoters. The *S. cerevisiae PHO5*, *PGK*, *GAP*, *MFα1* and *GAL1* promoters have all been used to drive gene expression in *K. lactis* (Fleer *et al.*, 1991; Bui *et al.*, 1996; Hsieh *et al.*, 1998; Morlino *et al.*, 1999; Bao *et al.*, 2001; Lorberg *et al.*, 2003). Although the two yeasts are closely related, endogenous promoters tend to be more effective than the introduced promoters (van den Berg *et al.*, 1990; Romanos *et al.*, 1992). Thus, it is more desirable to develop protein expression systems based upon active promoters identified from the chosen host organism than to rely on heterologous promoters.

1.4 Carbon Catabolite Regulation

Understanding the mechanisms that regulate a promoter is essential for utilizing a promoter for controlled protein production. One of the most well defined regulatory mechanisms in yeast is the activation or repression of genes associated with carbon assimilation. Many yeasts, including *S. cerevisiae* and *K. lactis*, can utilize a number of carbon sources but prefer glucose or fructose (Gancedo, 1998). The *K. lactis LAC4* promoter is activated by galactose and repressed in the presence of glucose. When growing in the presence of these sugars, the pathways involved in alternative carbon source utilization are shut down or dampened and synthesized at basal levels. This process is known as carbon catabolite repression, catabolite repression or glucose repression (Gancedo, 1998). Most glucose repression pathways in *S. cerevisiae* involve MIG1, a zinc-finger DNA-binding protein that recognizes binding sites within the promoters of many glucose repressed genes, such as *GAL*, *SUC2*, and *MAL* (Wang *et al.*, 1997; Gancedo, 1998). *Saccharomyces cerevisiae* MIG1 recruits a complex containing TUP1 and CYC8, which are thought to either modify chromatin condensation or interfere directly with transcriptional machinery (Wang *et al.*, 1997; Gancedo, 1998). *Kluyveromyces lactis* contains a MIG1 homologue, which acts upon many of the same genes as its *S. cerevisiae* counterpart (Cassart *et al.*, 1995; Dong *et al.*, 1997; Gancedo, 1998).

While most genes expressed for alternative carbon source metabolism are repressed by MIG1, each is normally associated with a specific positive transcriptional activator; hence, two aspects of regulated gene expression, release from repression and positive activation, are required for optimal expression. Glucose repression is globally mediated by the MIG1 pathway and the transcriptional activation of alternative pathways is induced by

positive regulators that act specifically upon a particular pathway. For example, the *S. cerevisiae* ScHAP2/3/4 complex is responsible for activating transcription of those proteins involved in metabolism of non-fermentable carbon sources, such as ethanol or glycerol (Gancedo, 1998; Breunig *et al.*, 2000), ScCAT8 is responsible for de-repression of genes involved in gluconeogenesis (Hedges *et al.*, 1995) and ScGAL4 is a transcriptional activator of the galactose and melibiose metabolic enzymes of the *ScGAL* gene family (Johnston, 1987). Many of the enzymes involved in carbon utilization of *S. cerevisiae* have analogs that share amino acid sequence similarity in *K. lactis*. While pathways in *S. cerevisiae* exhibit some redundancy, many analogous pathways in *K. lactis* do not; herein lies one of the drawbacks of modeling *K. lactis* regulatory pathways after those in *S. cerevisiae*. Several examples of those proteins that exhibit redundancy in *S. cerevisiae* but not in *K. lactis* include the SNF1 protein kinase complex, GAL regulatory proteins, and the glucose sensing transmembrane proteins (Meyer *et al.*, 1991; Breunig *et al.*, 2000; Betina *et al.*, 2001).

1.5 The MAL Locus

Maltose is a fermentable disaccharide that consists of two glucose units joined by an α-1,4 bond. Maltose is hydrolyzed into its component glucose units by the action of enzymes known as α-glucosidases, or maltases. The *MAL* loci have been extensively studied in *S. cerevisiae* with most investigation concentrating on the *MAL6* locus (Barnett, 1976; Needleman *et al.*, 1984; Dubin *et al.*, 1986; Hong *et al.*, 1986; Charron *et al.*, 1989; Ni *et al.*, 1990; Needleman, 1991; Levine *et al.*, 1992; Yao *et al.*, 1994; Medintz *et al.*, 1996; Wang *et al.*, 1997; Ferreira *et al.*, 2000; Hu *et al.*, 2000; Jiang *et al.*, 2000; Brondijk

et al., 2001). Only recently have *MAL* loci been uncovered and studied in other yeasts, such as *Candida albicans* (Geber *et al.*, 1992; Backen *et al.*, 2000), *Hansenula polymorpha* (Liiv *et al.*, 2001) and *Kluyveromyces lactis* (Current Study; Dominguez *et al.*, 1998).

Five unlinked, telomeric, *ScMAL* loci (*ScMAL1*, *ScMAL2*, *ScMAL3*, *ScMAL4*, and *ScMAL6*) in *S. cerevisiae* allow the yeast to break down maltose and use its component glucose molecules (Barnett, 1976; Hong *et al.*, 1986; Needleman, 1991). Each locus contains at least three genes involved in maltose metabolism, *ScMALx1* encoding maltose permease, *ScMALx2* encoding maltase, and *ScMALx3* encoding a positive regulatory activator, where *x* represents the locus number (Needleman *et al.*, 1984; Hong *et al.*, 1986; Needleman, 1991; Yao *et al.*, 1994). Each locus is organized such that *ScMALx2* (maltase) and *ScMALx1* (maltose permease) are divergently transcribed from a common intergenic region (Figure 1). The *ScMALx3* gene (encoding a self-activating positive activator which also engages the *ScMALx1/x2* promoter) is found upstream of the *ScMALx2-ScMALx1* region in the opposite orientation to the *ScMALx1* gene (Figure 1).

In *S. cerevisiae*, *ScMAL6* gene transcription is induced by maltose through the ScMAL63 positive activator and glucose repressed through the action of the ScMIG1 repressor protein (Ni *et al.*, 1990; Levine *et al.*, 1992; Yao *et al.*, 1994; Hu *et al.*, 1995; Wang *et al.*, 1997; Hu *et al.*, 2000). In the presence of glucose, ScMIG1 binds to two promoter sites, D (proximal to *ScMAL61*) and B (proximal to *ScMAL62*), which are primarily responsible for repression of the *ScMAL* gene to which they are proximal; ScMIG1 also binds upstream of the *ScMAL63* promoter (Figure 1) (Ni *et al.*, 1990; Hu *et al.*, 1995; Wang *et al.*, 1997; Hu *et al.*, 2000). When glucose is depleted and maltose is available as a substrate, it is thought that ScMIG1 is phosphorylated by ScSNF1 protein

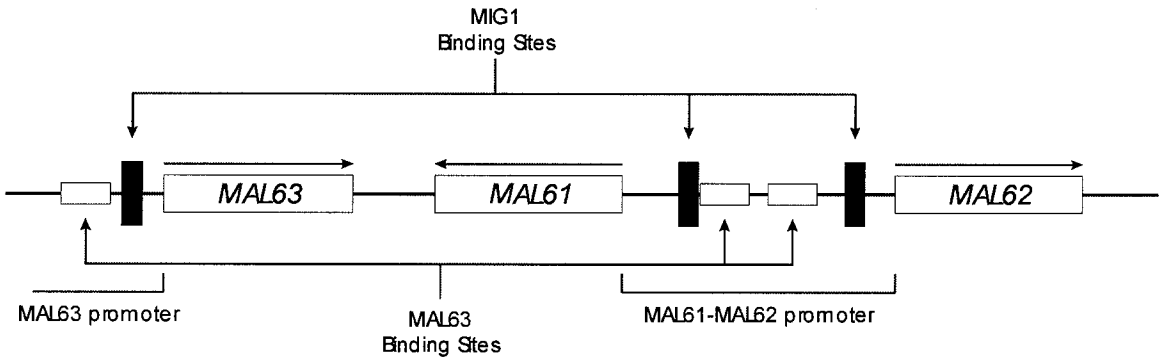


Figure 1. Organization of the *S. cerevisiae MAL6* locus. White boxes denote MAL63 positive activator binding sites. Black shaded boxes denote MIG1 repressor protein binding sites. Arrows above open reading frames indicate the direction of transcription.

kinase and removed from the nucleus (Gancedo, 1998). The removal of ScMIG1 from the *ScMAL* gene promoters allows the ScMAL63 transcriptional activator to bind to sites along the *ScMAL62-ScMAL61* bidirectional promoter, which results in the induction of *ScMAL6* gene transcription (Sirenko *et al.*, 1995; Wang *et al.*, 1997; Gancedo, 1998).

The *ScMALx1* gene encodes maltose permease, a high affinity proton/maltose symporter that shuttles maltose and turanose into the yeast cell (Hong *et al.*, 1986; Chang *et al.*, 1989; Cheng *et al.*, 1989; Cheng *et al.*, 1991; Needleman, 1991). *ScMALx2* encodes the maltase protein, which is an enzyme of the α-glucosidase family of enzymes (Hong *et al.*, 1986; Needleman, 1991; Krasikov *et al.*, 2001). Maltase catalyzes the hydrolysis of a broad array of α-glucosides, such as maltose, sucrose, and turanose, into their component monosaccharides (Needleman, 1991; Krasikov *et al.*, 2001). A trans-acting positive activator, containing an N-terminal zinc-finger and belonging to the same family of zinc cluster proteins as GAL4, is encoded by the *ScMALx3* genes (Chang *et al.*, 1988; Kim *et al.*, 1988; Needleman, 1991; Gancedo, 1998). The *ScMAL3* and *ScMAL6* loci both contain duplications of their respective *ScMALx3* genes and these duplications are designated *ScMALx4* (Dubin *et al.*, 1986; Charron *et al.*, 1989; Needleman, 1991).

1.6 Reporters of Gene Expression

In the study of protein expression it is necessary to have a reporter protein that can be easily assayed. Most quantitative reporter proteins require some protein purification or cell fixing steps. Green fluorescent protein (GFP), a 27 kDa protein originally isolated from the jellyfish *Aequorea victoria* (Shimomura *et al.*, 1962) has been used as a reporter

of gene expression. The use of GFP and its variants has advantages over the usual enzymatic reporters, such as β-galactosidase (*lacZ*), luciferase or chloramphenicol acetyl transferase (CAT) because GFP requires no cofactors or substrates and therefore does not require the disruption or fixation of cells to report activity (Chalfie *et al.*, 1994; Albano *et al.*, 1998; Cormack, 1998). This means that GFP signal can be recorded directly from living cells at a population level in a spectrofluorimeter or luminometer, or at the single cell level with flow cytometry or fluorescence activated cell sorting (FACS). While GFP and other fluorescent proteins have mostly been used as a tool for visualization of cellular localization, the use of GFP as a reporter of transcriptional activity is becoming more commonplace.

Several studies have used fluorescent proteins as reporters for protein expression. Fluorescence of GFP has been used to evaluate promoter strength in *Agrobacterium tumefaciens* (Tang *et al.*, 1999) and the fungi *Phanerochaete chrysosporium* (Ma *et al.*, 2001), to monitor MetArg-proinsulin production in *E. coli* (Dabrowski *et al.*, 1999), as a real-time quantitative reporter during *E. coli* fermentation (Albano *et al.*, 1998; DeLisa *et al.*, 1999) and to study the expression of heterologous protein in insect larvae (Cha *et al.*, 1997). The effect of inducing the *GAL1* and *GAL4* promoter in *S. cerevisiae* has been studied with FACS and with the use of on-line and off-line fluorescence intensity sensors (Niedenthal *et al.*, 1996; Li *et al.*, 2000).

Multiple colour variants of GFP are useful as reporters since they allow the simultaneous monitoring of expression within the same cell – simply by varying the wavelength of the excitation energy and by detection of different emission energies. For example; cyan fluorescent protein (CFP) has an excitation major peak at 434 nm and a

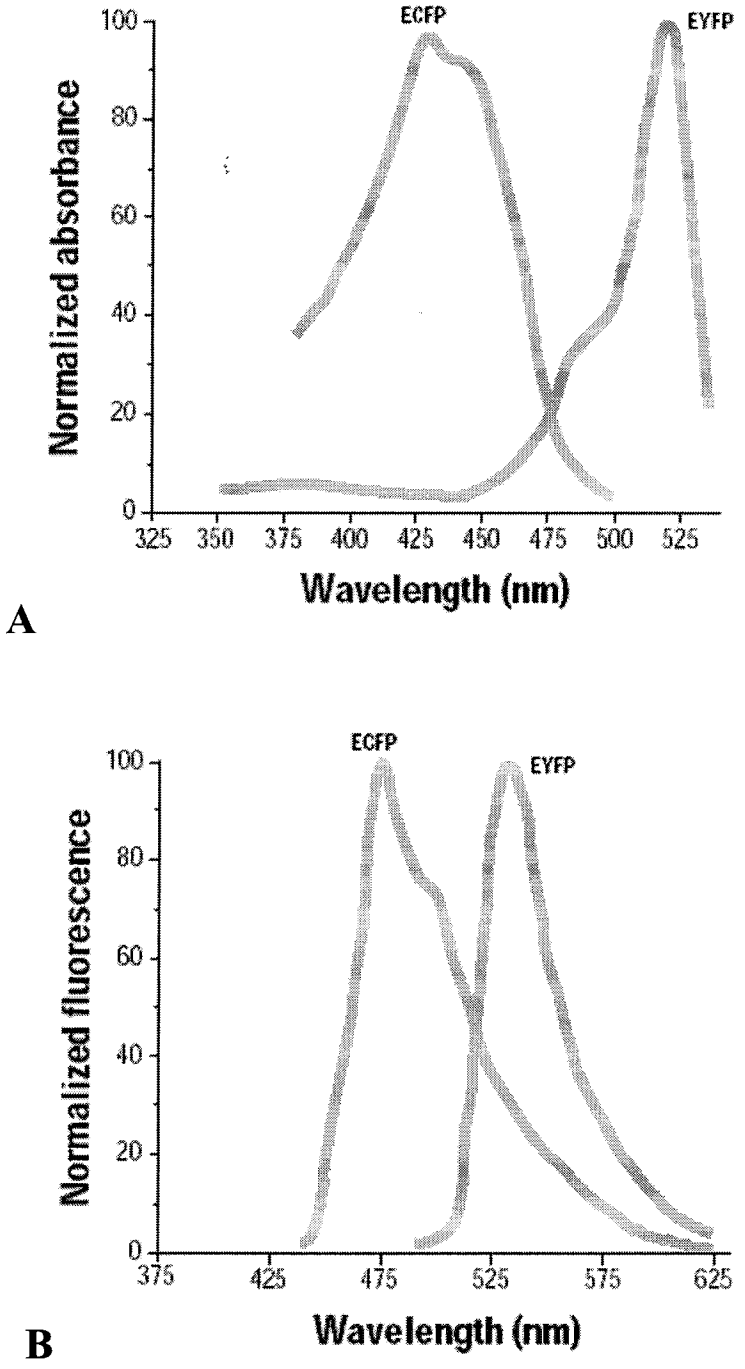


Figure 2. Fluorescence excitation (A) and emission (B) spectra of cyan and yellow fluorescent proteins (from Angres *et al.*, 1999).

minor peak at 453 nm, while yellow fluorescent protein (YFP) has an excitation peak at 514 nm (Figure 2) (Angres *et al.*, 1999; Patterson *et al.*, 2001). On the monitoring side, CFP has a major emission peak at 477 nm and a minor emission peak at 501 nm, while YFP has a single, major emission peak at 527 nm (Figure 2) (Angres *et al.*, 1999; Patterson *et al.*, 2001). The differences in excitation and emission spectra of CFP and YFP allow the simultaneous expression and subsequent analysis of both reporters using appropriate filter sets that isolate fluorescence from each chromophore. Using this dual-reporter system, relative changes in the expression levels of both fluorescent proteins can be quantified simultaneously.

1.7 Current Study and Research Objectives

The specific objective of this study was to identify, clone and functionally characterize the *KlMAL21-KlMAL22* promoter and to explore potential directionality of regulatory elements embedded within this bi-directional promoter. Few native promoters have been developed for heterologous protein expression in *K. lactis*. While some *S. cerevisiae* promoters have been used in *K. lactis* it is desirable to utilize endogenous promoters because they tend to be more active than heterologous promoters. The highly active promoter is the cornerstone of an efficient protein expression system. In conjunction with an appropriate expression host, the promoter dictates under what conditions the protein will be expressed and in what quantities. The *MAL* locus in *K. lactis* was chosen as a candidate for use in heterologous protein expression because it has been shown to secrete endogenous maltase in high amounts into its surrounding environment. Both the ability of the *KlMAL21-KlMAL22* bi-directional promoter to express heterologous proteins and the effect of promoter alterations on gene regulation were examined using the fluorescent reporter proteins, CFP and YFP.

Materials and Methods

2.1 Strains and Culture Conditions

Escherichia coli electromax DH10B (F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*Δ M15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*Δ139 Δ(*ara, leu*)7697 *gal*U *gal*K λ^- *rps*L (Str^r) *nup*G λ^- *ton*A) competent cells were used for all DNA cloning steps (Invitrogen, Carlsbad, CA). Bacterial cultures were routinely grown in 2YT liquid media (1.6% (w/v) tryptone, 1.0% (w/v) yeast extract, 5.0% (w/v) NaCl) overnight at 37°C (Sambrook *et al.*, 2001).

Kluyveromyces lactis strain Centraalbureau voor Schimmelcultures (CBS) 1065 (syn. CBS 2980, K+, pKD1°) was obtained from Extreme Biotech Inc. (MA) and yeast cultures were grown in YP liquid media (1.0% (w/v) yeast extract, 2.0% (w/v) tryptone peptone), supplemented with 2.0% (w/v or v/v) of the required carbon source (D = glucose, G = glycerol, A = galactose). For yeast agar plates, 1.5% (w/v) granulated Agar (Difco, Detroit) was added. Yeast cultures were grown for fluorescence assessment in Synthetic Complete (SC) media containing 6.7% (w/v) Yeast Nitrogen Base (YNB) with amino acids and ammonium sulfate (Difco, Detroit), supplemented with 2.0% (w/v or v/v) of the required carbon source. The YNB was prepared in 750 ml of dH_2O and sterilized by autoclaving at 121°C for 15 minutes. A 50 ml volume of 0.2 μm filter-sterilized 32% (w/v or v/v) carbon source was then added aseptically to the sterile YNB to yield 800 ml of SC media.

2.2 Yeast Genomic DNA Isolation

Yeast genomic DNA was isolated using a glass bead disruption method (Hoffman, 1997). A single yeast colony from a fresh streak plate was transferred by sterile loop to 5 ml of YPD and incubated overnight at 30°C with shaking at 250 rpm. The cells were collected by centrifugation at 2500 rpm for 10 minutes, resuspended in 0.5 ml sterile double distilled water (ddH_2O) and transferred to a sterile 1.5 ml micro-centrifuge tube. The cells were again collected by a centrifugation at 10,000g for 5 seconds and the supernatant removed. Yeast cells were resuspended in a 200 µl volume of yeast breaking buffer (2.0% (v/v) Triton X-100; 1.0% (v/v) sodium dodecyl sulphate (SDS); 100 mM NaCl; 10 mM Tris-HCl, pH 8.0; 1mM EDTA, pH 8.0). To facilitate cell disruption, 200 µl of 425-600 µm diameter acid-washed glass beads (Sigma, St. Louis) was added to the suspension. The suspension was extracted with 200 µl of 25:24:1 (v/v/v) phenol:chloroform:isoamyl alcohol with vortexing at the highest setting for 4 minutes. After vortexing, 200 µl of TE (pH 7.4) was added to the suspension and the cellular and protein debris was pelleted by centrifugation at 10,000g for 5 minutes. The aqueous (upper) phase was transferred to a sterile 1.5 ml micro-centrifuge tube and the nucleic acids were precipitated by the addition of 1 ml of 95% (v/v) ethanol. Samples were incubated at -20°C for 5 minutes and then centrifuged at 10,000g for 5 minutes. The supernatant was subsequently removed and the pellet was resuspended in 400 µl of TE (100-mM Tris-HCl pH 7.4, 10-mM EDTA pH 8.0). RNA contamination was removed by adding 3 µl of 10 µg/ml DNase-free RNase A (Sigma, St. Louis) and incubating the suspension for 5 minutes at 37°C. Following the incubation, DNA was precipitated by the addition of 10 µl of 4M ammonium acetate and 1 ml of 95% (v/v) ethanol. The sample was then incubated at -

20°C for 1 hour and the precipitated DNA was collected by centrifugation at 10,000g. The supernatant was removed and the isolated genomic DNA pellet was resuspended in 100 µl of TE (pH 7.4) and stored at -20°C until use.

2.3 PCR Amplification

All PCR amplifications of the *KlMAL2* promoter were carried out under the following conditions. The *KlMAL2* promoter was amplified from CBS 1065 genomic DNA in a 20 µl total reaction volume containing 50 pmol of each primer (Table 2), 200 µM of each dNTP, 1 µl (approximately 200 ng) of genomic DNA as template, 2.5 units (U) of high-fidelity KlenTaq Mix (Sigma, St. Louis) and 2 µl 10X Klentaq PCR buffer. Amplification reactions were carried out in a DNA Thermal Cycler (Perkin Elmer, Wellesley, MA) set to 5 cycles of denaturation at 95°C for 60 seconds, annealing at 60°C for 60 seconds and elongation at 72°C for 120 seconds, followed by 30 cycles of denaturation at 95°C for 60 seconds, annealing at 65°C for 60 seconds, and elongation at 72°C for 120 seconds. The reaction was completed by an extension at 72°C for 10 minutes.

2.4 Restriction and Modifying Enzyme Reactions

All restriction digests were carried out using enzymes supplied by New England Biolabs (NEB, Beverly, MA). Digests were carried out according to the manufacturer's instructions by combining 2 µl of the appropriate 10X buffer, 5-10 µl (0.5-1.0 µg) of DNA in TE (pH 7.4), and 10-20 units of restriction enzyme, depending on supplied

Table 2. PCR primer sequences. Novel restriction sites are underlined and identified in each primer. Introduced mismatches are highlighted in grey and the original sequence is given below each introduced sequence.

Template DNA	Primer Name	Primer Sequence
Genomic	Permease-F	CAGCTTTACTGTTTTGTTTGAAGTGAATAAAGATCTTT
K. lactis	Maltase-R	CTGTTTGGGGGTCTCGTAAGATCTTGGTAA
	MPFP-F	GGGTTTT CTTAAG GCTAGC GGATCC TTGCCA CGCTAGAACTATGTTGTCGA *Afl*II *Nhe*I *Bam*HI
	MPFP-R	GGGTTTT GCGGCCGC AGATCT GGATCC ACTTGATGTTTTTTTGCGCTTAACGTTT *Not*I *Bgl*II *Bam*HI
	MalP3-F	CCCTCGAGAAAATTTCTCTATTACTTTTAGTTGAAACTACTAACTTTTTG
	MalP3-R	GTAATAGAAATTTTCTCGAGGGAAAAAAATGGAG
	Mal-Mig-F	ACGACAACCAGCATCAAAAACATGGTGAAGACGGAAAATCTCTG AA AT TGGGG
	Mal-Mig-R	TGTTTTTGATGCTGGTTGTCGTCATACATCGGAAGAAAATCCG CCCCA AT TT
	Mal-Mig-Diag-F	TGACGACAACCAGCATCAAAAAC
	Mal-Diag-F	TGAAAACAAATATGGGGAAAAACATG
	Mal-Diag-R	GGGAAACGGAAAAATGGAGAAA
No Template	Synthetic Linker	*Pac*I *Bsu*36I *Sac*II GGGGTTTGCATGCTTAATTAATCGATAGGCCTGAGGCGTACGCCTCAGGCTAGCCGCGGAGATCTTAAGAAACCC *Sph*I *Bsu*36I *Nhe*I *Afl*II

concentration, made up to a final volume of 20 µl with sterile dH_2O. Restriction digests of plasmids for cloning purposes were incubated at 37°C for 1 hour and digests of genomic DNA for southern analysis were incubated at 37°C overnight. Calf-intestinal alkaline phosphatase (CIP) (NEB, Beverly, MA) was used to dephosphorylate vector DNA in all non-directional cloning steps. Restriction enzyme digests were halted by heat-inactivating the enzyme at 65°C for 30 minutes. Each reaction was cooled to room temperature and 10 U of CIP was added directly to the digest reaction. The dephosphorylation reaction was incubated at 37°C for 1 hour and the product DNA was gel purified by separation through a 1.0% (w/v) agarose gel.

2.5 Ligation Reactions

All ligation reactions were conducted using T4-DNA ligase and 10X ligation buffer (Promega, Madison, WI). Ligation reactions were prepared by combining 1 µl of 10X ligation buffer, 50 ng of pGEM-T (Promega, Madison, WI) or digested, purified plasmid, and 3 U of T4 DNA Ligase, made up to a final volume of 10 µl (150 to 300 ng) of gel purified insert DNA in TE (pH 7.4). Ligation reactions were incubated for 3 hours at 15°C and subsequently stored at 4°C until they were used for the transformation of electro-competent *E. coli*.

2.6 Promoter Sequencing and Analysis

All PCR fragments were ligated into pGEM-T prior to sequencing (Promega, Madison, WI). Promoter sequencing was performed in triplicate for each DNA fragment by the Centre for Biomedical Research, University of Victoria, using M13-F and M13-R primers.

Putative transcription factor binding sites were found within the native *KlMAL2* promoter and each altered promoter using MatInspector V2.2, an on-line program accessed through the TRAnscription Databases and Analysis Tools (TRADAT), http://www.itba.mi.cnr.it/cgi-bin/tradat/tradat_launcher (Quandt *et al.*, 1995). Minimum core and matrix similarity scores were set at 0.80 each and sequences were compared against all fungal matrices to identify putative transcription factor binding sites.

2.7 Construction of Expression Plasmids

2.7.1 Fluorescent Protein Expression Plasmid Construction

The maltase promoter was amplified by high-fidelity PCR and then cloned into pGEM-T generating a plasmid termed pMP. PCR amplification of the *KlMAL2* promoter introduced novel restriction sites in the regions flanking the maltase promoter and was mediated by the MPFP primer set (Table 2). The novel restriction sites facilitated the introduction of the sequence coding for the GFP colour variants on either side of the promoter. A fragment of DNA holding the CFP gene was isolated from the plasmid pECFP-N1 (Clontech, Palo Alto, CA), by a *Bgl*II/*Not*I restriction digest, and then ligated into a similarly digested pMP, resulting in a plasmid termed pMPCP. Cloning of the CFP

gene was verified by *Bgl*II/*Not*I restriction digest. The YFP gene was directionally cloned from the plasmid pEYFP-N1 (Clontech, Palo Alto, CA) into the *Nhe*I/*Afl*II sites upstream of the promoter in the same manner as the CFP gene. Successful cloning of the YFP gene was confirmed by *Nhe*I/*Afl*II restriction digest. The plasmid holding the maltase promoter flanked by genes encoding both fluorescent proteins was termed pMPCYP.

In order to bring the translational start site of the fluorescent protein genes as close as possible to the native translation start sites of the maltase and maltose permease genes a *Bam*HI re-section was performed. Plasmid pMPCYP was digested with *Bam*HI, dephosphorylated with CIP and the plasmid vector fragment was gel purified. A non-dephosphorylated gel purified promoter fragment was then ligated into the dephosphorylated plasmid fragment. The *Bam*HI re-sectioned plasmid was termed pMPCYP-B. This portion of the expression plasmid is removable and thus different promoter variants, flanked by *Bam*HI restriction sites can be moved into and out of the plasmid pMPCYP-B.

In order to permit selection of the expression plasmids following transformation into *K. lactis*, a kanamycin/neomycin (kan^r/neo^r) resistance marker from pECFP-N1 (Clontech, Palo Alto, CA) was inserted into the plasmid. The kan^r marker is driven by the SV40 early promoter and codes for neomycin phosphotransferase conferring resistance to G418 (Geneticin) in *K. lactis* (Sreekrishna *et al.*, 1984). To facilitate cloning of the kan^r marker a synthetic linker was inserted between the *Sph*I and *Afl*II restriction sites of pMPCYP-B (Table 2) to yield plasmid pMPCYP-LB. The kan^r fragment was isolated from pECFP-N1 by a *Bsu*36I restriction digest and ligated into similar restriction sites in the linker of pMPCYP-LB. The new plasmid was termed pMPCYP-LBK.

The final step of the fluorescent expression cassette construction was to insert the pKD1 form B self-replicating plasmid from *Kluyveromyces lactis* var. *drosophilarum*. The addition of pKD1 to the expression plasmids allows the plasmids to replicate autonomously in the host cells. The yeast self-replicating plasmid pKD1 was isolated by an *Eco*RI digest from pCWK2celB4/5 (from P. Bergquist, Macquarie University, Australia) and cloned into pBlueScriptII (pBS2). The orientation of pKD1 in pBS2 was verified so that the native pBS2 *Sac*I site and the *Sac*I site adjacent the *Eco*RI site of the insert were on opposite sides of pKD1. The pKD1 fragment was cloned into the *Sac*I restriction site of pMPCYP-LBK. The final fluorescent protein expression cassette is shown in Figure 3, and was termed pREX-IC (p**R**eplicative **EX**pression – **I**ntra**C**ellular).

2.7.2 Construction of Promoter Variants

Modification of the *KlMAL21-KlMAL22* promoter was performed by two-stage PCR mutagenesis. The first stage consists of two reactions, one using an MPFP-F / Mutagenesis-R primer set and the other using a Mutagenesis-F / MPFP-R primer set. The Mutagenesis primers MalP3-F, MalP3-R, Mal-Mig-F, and Mal-Mig-R are shown in Table 2.

The PCR products from the first-stage reaction were separated by electrophoresis through a 1.0% (w/v) agarose gel. Agarose gel fragments containing the amplified DNA were extracted into 20 µl of sterile ddH_2O using QIAquick gel extraction columns (QIAGEN, Hilden) according to the manufacturers instructions and mixed together in a 1:1 (v:v) ratio. The resulting mixture was diluted to 500 µl, boiled for 5 minutes and used as template DNA for the second-stage PCR. MPFP primers were used to amplify the final, full-length, mutated *KlMAL2* promoter.

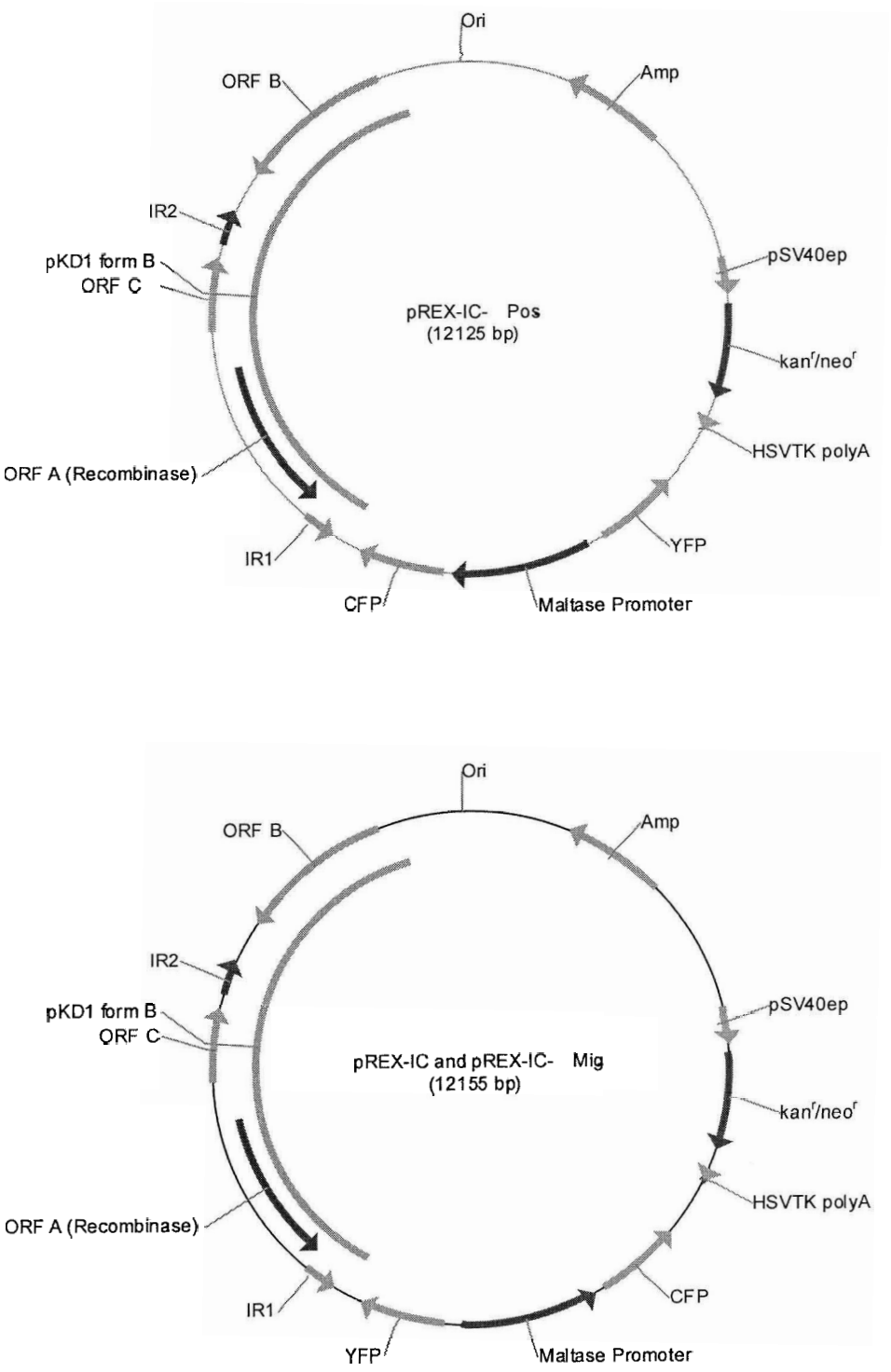


Figure 3. Schematic representation of the pREX-IC, pREX-IC-ΔMig and pREX-IC-ΔPos expression plasmids. Open reading frames and regions of importance are shown. The direction of each element is shown and the direction of the *KlMAL2* promoter is shown in relation to the native *KlMAL22* gene.

Two altered promoters were created by two-stage PCR as an initial test of the regulatory system of the *KlMAL2* regulon. The first promoter alteration was engineered using the Mal-Mig primer set (Table 2) and consists of substitutions in the region from 671 to 657 base pairs (bp) upstream of the *KlMAL22* coding region (Figure 4). The PCR primers MalP3-F and MalP3-R (Table 2) were used to generate the second alteration, consisting of a deletion at 411 to 441 bp upstream of the *KlMAL22* ATG start codon (Figure 4). The altered promoters were first cloned into pGEM-T and their sequences verified. They were subsequently transferred to pMPCYP by replacement of the original promoter via flanking *Bam*HI sites. The orientation of each promoter in pMPCYP relative to the fluorescent protein genes was confirmed to be the same as that of the native promoter by a *Kpn*I/*Afl*II double restriction digest. A fragment containing the promoter and the genes for both fluorescent proteins was isolated from pMPCYP by a *Not*I restriction digest and was then ligated into a similarly digested pREX-IC plasmid. The resulting plasmid was named either pREX-IC-ΔMig or pREX-IC-ΔPos corresponding to a deletion of a MIG1 or a positive activator binding site accordingly (Figure 3).

2.8 Transformation of *E. coli* and *K. lactis*

Escherichia coli DH10B cells were transformed by electroporation according to Sambrook *et al.* (2001). A modified electroporation procedure was used to transform *K. lactis* CBS 1065 cells (Sanchez *et al.*, 1993). To obtain electro-competent cells, fresh *K. lactis* CBS 1065 cells were grown on YPD agar plates for two days at 30°C. A 5 ml starter culture of liquid YPD was inoculated with a single colony from the fresh streak plate and incubated overnight at 30°C with shaking at 250 rpm. A 100 ml volume of liquid YPD

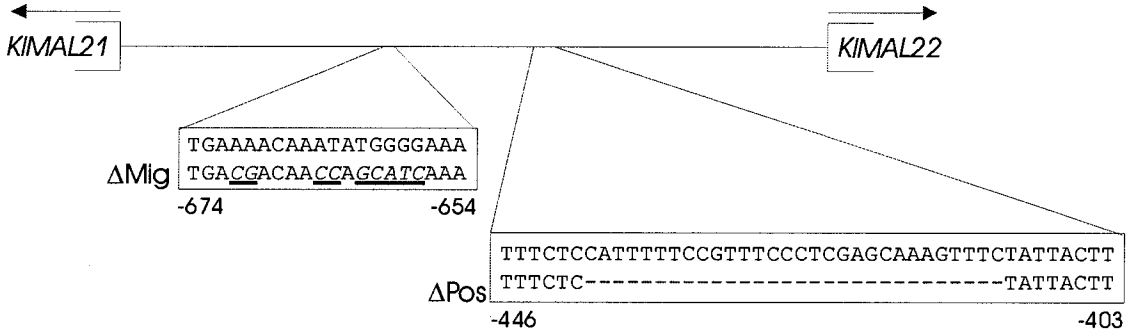


Figure 4. Nucleotide sequences of ΔMig and ΔPos promoter variants. Changes in the *KlMAL21-KlMAL22* promoter sequence are shown below the native promoter sequence. Nucleotide positions are given relative to the *KlMAL22* translation start site and the native promoter sequence. Arrows indicate the direction of gene transcription.

was inoculated with 25 µl of the starter culture and incubated at 30°C with shaking at 200 rpm until the cells reached mid-log phase (OD_{600} 0.6-1.0). Cells were then collected by centrifugation at 4°C, washed once with one half volume of sterile ice-cold electroporation buffer (EB, 10 mM Tris-HCl pH 7.5, 270 mM sucrose, 1 mM $MgCl_2$) and harvested again. The cells were resuspended in 30 ml of liquid YPD supplemented with 25 mM DTT and 20 mM HEPES (pH 8.0) and incubated for 30 minutes with gentle agitation at 30°C. Cells were collected once more, washed with an equal volume of EB and then resuspended in 1 ml of 15% (v/v) glycerol. Cells were dispensed in 45 µl aliquots and stored at -70°C until use.

Before transformation an aliquot of electro-competent *K. lactis* cells was pelleted by centrifugation at 10,000g for 10 seconds. The 15% glycerol supernatant was removed and the cells were resuspended in 45 µl of ice-cold EB. Plasmid DNA (1 µg) along with 2 µg of homogenized and boiled salmon sperm DNA (type III, Sigma, St. Louis) in 10 µl of dH_2O were added to a 45 µl suspension of electro-competent *K. lactis* cells and incubated on ice for 15 minutes. The suspension was transferred to a 0.2 cm gap electroporation cuvette (BTX, Holliston, MA) and electroporation was performed using an Electro Cell Manipulator 600 (BTX, Holliston, MA) with the following parameters: voltage = 1.0 kV, resistance = 186 Ω and capacitance fixed at 50 µF. Liquid YPD was added to the cuvette to a final volume of 1 ml immediately after pulse delivery and the cells were subsequently transferred to a 15 ml glass test tube. The treated cells were incubated at 30°C overnight with shaking at 150 rpm.

To select for cells expressing the antibiotic resistance marker, the yeast cells were collected by centrifugation, resuspended in 200 µl of liquid YPD and 100 µl aliquots were spread onto YPD plates supplemented with 25 µg/ml G418 (Invitrogen, Carlsbad, CA). The plates were incubated at 30°C and putative transformants appeared after 2-4 days.

2.9 Confirmation of *K. lactis* Transformants

Transformants were confirmed by replicate streaking putative transformants alongside wild-type *K. lactis* onto fresh YPD plates supplemented with either 25 µg/ml, 100 µg/ml or 200 µg/ml G418. Positive transformants grew on all concentrations of G418, while wild-type cells showed no growth.

To confirm that the yeast colonies growing on G418 supplemented plates indeed carried the introduced plasmid and were not spontaneously resistant to the antibiotic, total yeast DNA was isolated from 10 ml overnight cultures of liquid YPD for each putative transformant. Presumably the total DNA of positive transformants would also carry plasmid DNA capable of conferring *E. coli* DH10B cells with ampicillin resistance. Hence, this test was performed for each putative yeast transformant. Plasmid DNA isolated from the *E. coli* cells was then subjected to i) *Bam*HI, ii) *Kpn*I, and iii) *Kpn*I/*Pst*I restriction digests to confirm that the restriction patterns of the isolated plasmids matched the patterns of the original plasmid used in the yeast transformations.

A diagnostic PCR was used to confirm promoter variants in positive transformants. Mal-Diag-F and MalP3-R identified the ΔPos promoter variant, while Mal-Mig-Diag-F and Mal-Diag-R identified the ΔMig promoter variant. A positive PCR for each promoter

variant was visualized as a 265 bp fragment when separated by electrophoresis through a 1.5% (w/v) agarose gel.

2.10 Determination of Plasmid Segregational and Structural Stability

The segregational and structural stability of the expression plasmids were monitored during the growth of the cultures in the absence of continued selection. Segregational stability was determined by plating aliquots of each culture onto non-selective YPD agar and selective YPD agar supplemented with 25 μg/ml G418. The percentage of cells that grew on selective media as compared to non-selective media indicated the segregational stability of the plasmid.

Structural stability was determined by extracting total DNA from yeast cells collected from each culture. *Escherichia coli* cells were transformed with yeast plasmid DNA and transformants were cultured overnight to obtain large quantities of expression plasmid DNA. The DNA isolated from the overnight cultures was digested with i) *Bam*HI, ii) *Kpn*I, and iii) *Kpn*I/*Pst*I and the restriction pattern was compared to the restriction pattern of the original expression plasmid. A plasmid was considered to be structurally stable when the restriction pattern did not change throughout the culture run.

2.11 Expression of Fluorescent Proteins

To evaluate the expression of fluorescent proteins, *K. lactis* cultures were grown in 100 ml cultures of liquid YPD for up to 96 hours. Aliquots (4 ml) were removed from the cultures at 24, 48, 72, and 96 hour sample intervals. Cells were harvested from the culture

media by centrifugation at 500g for 10 minutes. These cells were then resuspended in 4 ml of sterile ddH_2O and kept on ice out of direct light until fluorescence intensity could be measured.

The OD_{590} of each sample was measured and then each sample was placed in a 4 ml disposable methyl acrylate spectrofluorometer cuvette of 1 cm path length with 4 optical sides (VWR, Mississauga, Ont). Each sample was assayed for YFP and CFP fluorescence in a PTI QM-2 spectrofluorimeter (Photon Technologies Inc., Lawrenceville, NJ). Cells were assayed for YFP and CFP using the excitation wavelengths of 513 nm and 435 nm, respectively. Emission spectra were obtained for YFP from 520 to 560 nm and for CFP from 465 to 505 nm. The slit width for both excitation and emission monochromators was set at 2 nm and YFP was always assayed first to avoid any overlap of the emission and excitation spectra.

Relative fluorescent intensity (RFI) was calculated using the following formula:

$$\text{RFI} = \frac{(\text{Fluorescence of Transformant})}{OD_{590} \text{ of Transformant}} - \frac{(\text{Fluorescence of Control})}{OD_{590} \text{ of Control}}$$

RFI values were converted to percentage of maximum RFI by dividing a given RFI by the maximum RFI measured for the particular fluorescent protein. For the purposes of showing the percentage of maximum RFI, any negative RFI values were assumed to be due to instrument noise and were set to zero.

2.12 Southern Analysis

To confirm the presence of the introduced plasmid at each sampling interval, total DNA obtained from each culture at each time interval sampled was digested with *Bam*HI and separated by electrophoresis on a 1.0% (w/v) agarose gel and transferred to Genescreen Plus membrane (NEN Life Science Products, Boston, MA) following the protocol given by Sambrook *et al.* (2001). To obtain ^{32}P-dCTP-labelled probe DNA corresponding to the maltase promoter, genomic DNA was amplified from *K. lactis* CBS 1065 using MPFP-F and MPFP-R primers and purified using a QIAquick PCR purification column (Qiagen, Hilden). Blots were hybridized with the probe DNA overnight and washed according to Sambrook *et al.* (2001). To obtain autoradiographs, each blot was exposed to a Fuji-film phosphor screen (Fujifilm, Tokyo) for 6 hours and then scanned using a Storm 860 blot imaging system (Amersham Biosciences, Buckinghamshire, UK).

2.13 Fluorescence Microscopy

Photographs of fluorescing cells were taken under a Zeiss Universal epi-fluorescence microscope at the UVic Advanced Imaging Lab. Aliquots of culture were pelleted for 5 seconds at 10,000g and resuspended in sterile ddH_2O. Small volumes (10 µl) of 1:100 dilutions were placed on a microscope slide and viewed under a total magnification of 160x. Fluorescence was visualized through a fluorescein isothiocyanate (FITC) filter set which excites from 450 to 490 nm and allows emission beyond 520 nm. This filter set is close enough to the excitation and emission spectra of YFP to allow visualization of YFP expressing cells. Photos were taken with a Nikon Coolpix 990 digital camera.

Results and Discussion

3.1 Discovery and Analysis of the *K. lactis* CBS 1065 Maltase Promoter

The discovery of a highly efficient fungal promoter is often the result of careful observation of the natural processes or strategies employed by these extremely versatile and adaptable organisms. Inducible genes that are highly expressed are particularly useful for biotechnology applications and may be uncovered by comparing the profile of expressed genes during normal growth with the profile of expressed genes during growth in varying nutrients or stress conditions. Differences in growth conditions can appear even as ambient conditions change during fermentations as there can be a gradual change in nutrient availability, pH and more. Such was the case late in the fermentation process of *K. lactis* strain CBS 1065 where an accumulation of a single unknown polypeptide corresponded to the depletion of glucose during the stationary phase of fermentation (Williams and Hintz, personal communication). The identity of the protein was unknown but since the protein occurred in such high amounts the promoter driving its expression became a candidate for heterologous protein expression. N-terminal analysis identified the unknown protein as a homolog of the *S. cerevisiae* maltase (ScMALx2) proteins (Williams and Hintz, personal communication). Degenerate PCR primers were used to amplify a section of the *K. lactis* maltase gene and the amplification product was used to identify clones containing the gene from a *K. lactis* genomic λ-DNA library (Williams and Hintz, personal communication). One clone in particular, pE6, contained some coding region sequence of the maltase gene and approximately 500 bases of upstream promoter. Subsequent to this discovery, the *K.*

lactis strain CBS 2359 maltase (*MAL22*) and maltose permease (*MAL21*) sequences were submitted to GenBank by San Vincent *et al.* (Accession #AJ007636, 1998).

For the purpose of isolating the *KlMAL* promoter from *K. lactis* strain CBS 1065, a PCR primer set, Permease-F and Maltase-R, were designed from the sequence flanking the maltase promoter from *MAL21* and *MAL22* genes respectively (Table 2). Using these primers an approximately 1 kb fragment was amplified from *K. lactis* strain CBS 1065 genomic DNA and fully sequenced. The amplified sequence represented the entire intergenic region and was found to be a 1069 bp bi-directional / bi-functional promoter for both the maltose permease (*KlMAL21*) and maltase (*KlMAL22*) genes. Further to sequence determination, the promoter was analyzed for the presence of basal promoter elements and putative transcription factor binding sites. To analyze regulated protein expression from the *KlMAL21-KlMAL22* bi-directional promoter three expression plasmids were constructed using CFP and YFP as reporter genes (Figure 3).

All three plasmids consisted of the same basic elements including the kan^r/neo^r marker that conferred G418 resistance to cells containing the plasmid, the pKD1 self-replicating plasmid that allowed the expression plasmids to replicate outside of the yeast genome, and the two fluorescent protein variants. The only difference between the three plasmids was the promoter variant. The plasmid pREX-IC contained the native *KlMAL21-KlMAL22* bi-directional promoter. The plasmid pREX-IC-ΔMig contained mutations in the promoter in the region between 671 and 657 bp upstream of the *KlMAL22* translation start site. This mutation effectively removed a putative MIG1 repressor protein binding site from the promoter. The plasmid pREX-IC-ΔPos contained a promoter with a deletion of the sequence from 411 to 441 bp upstream of the *KlMAL22* coding region. The deletion

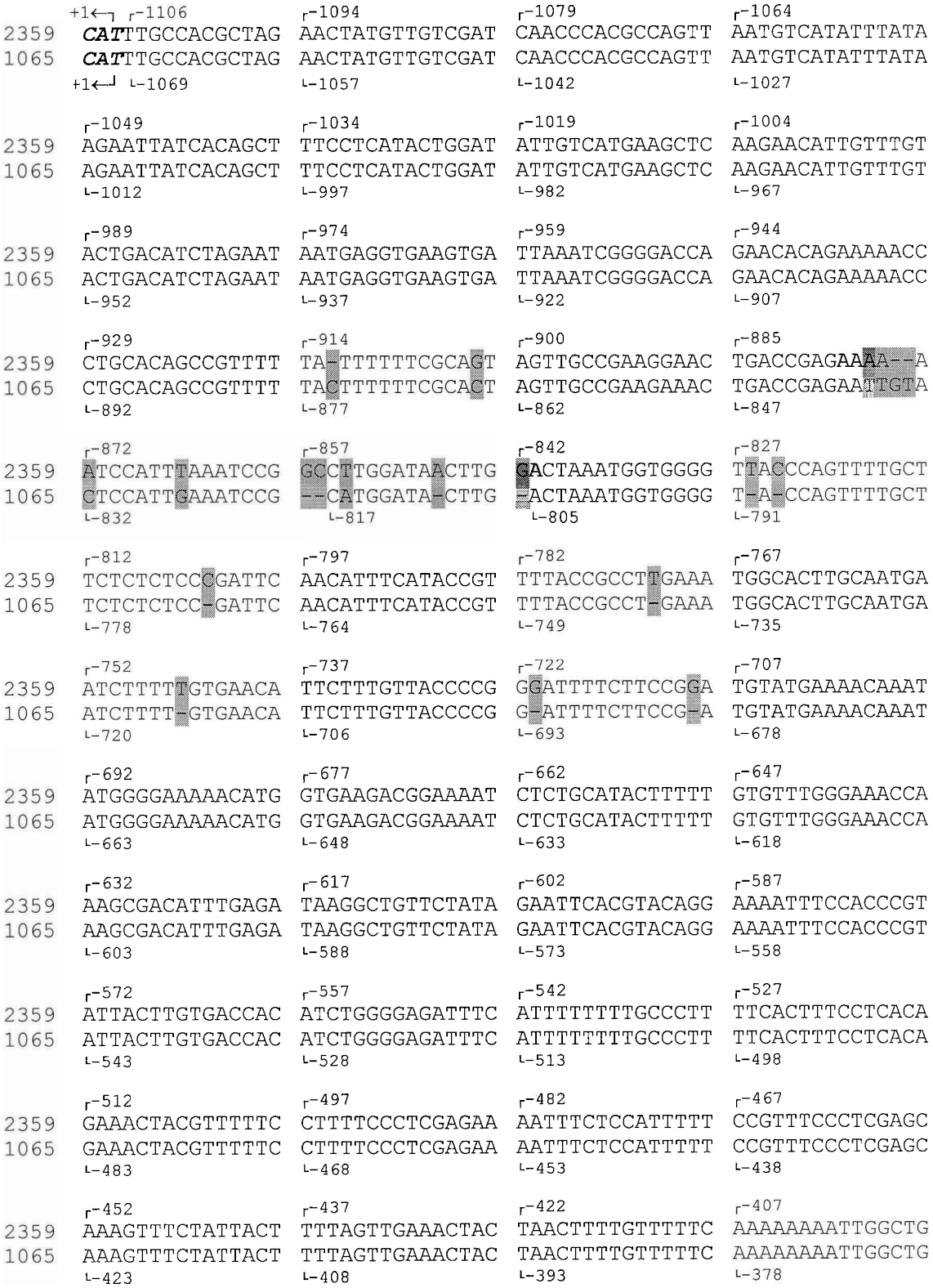
removed a 30 bp region that corresponded to a portion of a putative upstream activator sequence (*UAS*). It was hypothesized that mutation of the putative MIG1 binding site would relieve repression of the promoter during growth on glucose (repressing conditions) and that deletion of the putative UAS_{MAL} region would cause a reduction in expression during growth in the absence of glucose (de-repressing conditions).

3.1.1 Strain Differences Found in the KlMAL21-KlMAL22 Promoter

An alignment of the *K. lactis* maltase promoter sequence from #AJ007636 and the *KlMAL2* promoter sequence from *K. lactis* CBS 1065 is shown in Figure 5. Aside from small, single bp differences several significant differences in these sequences have been found. It is important to note that the promoter region presented by San Vincent *et al.* was isolated from *K. lactis* strain CBS 2359, while the current study *KlMAL2* promoter was isolated from strain CBS 1065. Variations between the promoters of these isolates may represent interstrain differences and may be significant in the differential regulation of the promoter. Variations in the promoter sequence between strains CBS 2359 and CBS 1065 may in part explain differences, if any are discovered, in maltase expression levels. The *KlGAL4* promoters of *K. lactis* strain JA6 and CBS 2359 differ by two nucleotides. When the *KlGAL4* promoter from strain JA6 was replaced with the promoter from strain CBS 2359 glucose repressed *KlGAL4* gene expression was alleviated and expression was only weakly repressible by glucose (Zachariae *et al.*, 1993a; Zachariae *et al.*, 1993b).

Four adenine residues 875 bp upstream of the maltase coding region in the #AJ007636 sequence have been replaced by the sequence 'TTGTAC' in the *KlMAL2* promoter sequence. A 'CA' in the *KlMAL2* promoter replaces the 'GCCT' sequence found at 857 bp upstream of the translation start site in the San Vincent *et al.* promoter. At 106

←maltose permease (*KlMAL21*)



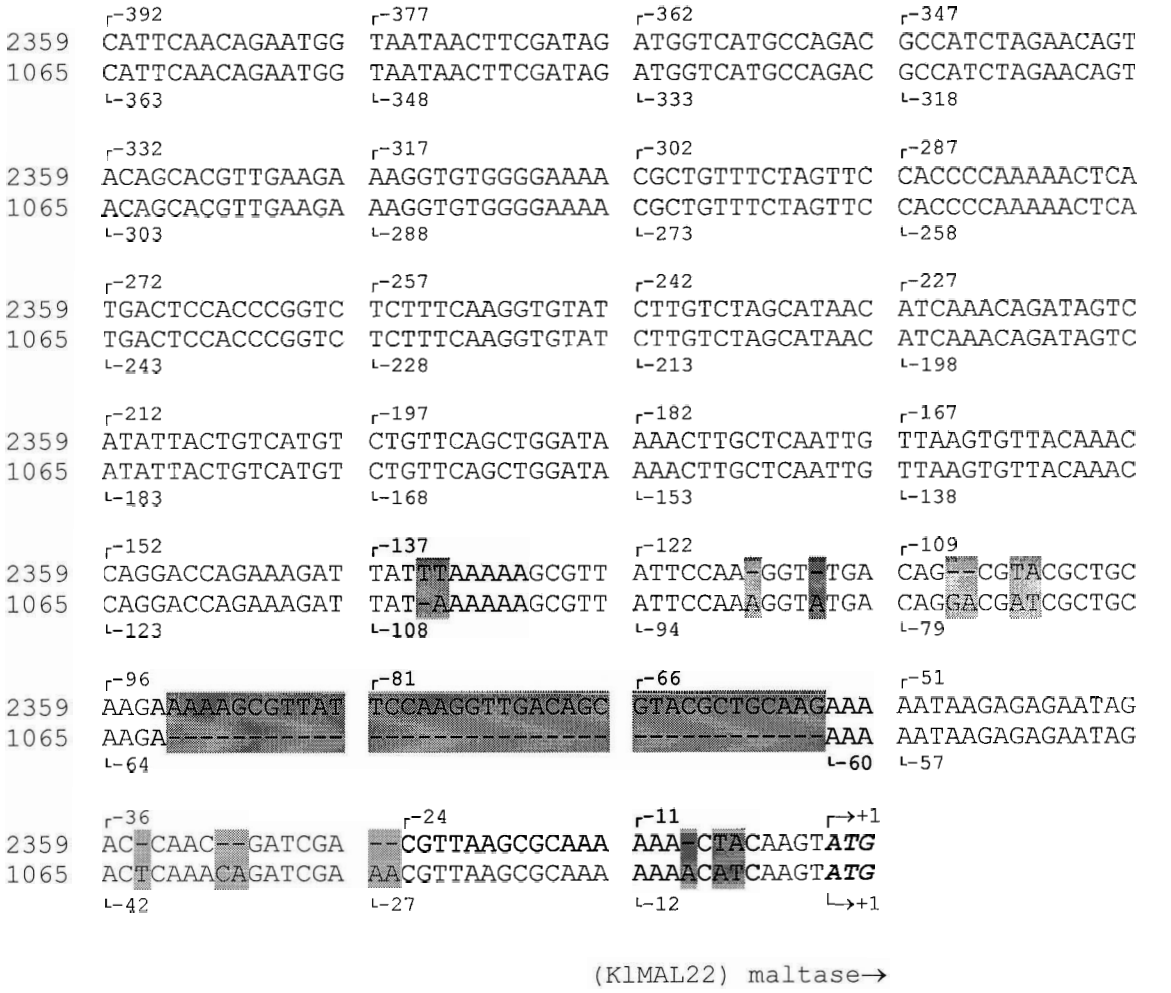


Figure 5. Sequence alignment of homologous MAL locus intergenic sequences. The upper sequence is *K. lactis* strain CBS 2359 sequence reported by San Vincente *et al.* (Accession #AJ007636). The lower sequence is the *K. lactis* strain CBS 1065 maltase promoter. Differences are indicated as shaded boxes. Distance relative to the maltase translation start site is given for each sequence.

bp upstream, a 'CGTA' sequence is found in the #AJ007636 intergenic region and a 'GACGAT' is located at the corresponding position in the *KlMAL2* promoter. The largest difference between the two intergenic sequences is a 38 bp direct repeat that occurs from 92 bp to 55 bp upstream from the maltase gene coding region in the #AJ007636 sequence. A repeat of the sequence 'AAAAAGCGTTATTCCAAGGTTGACAGCGTACGCTGCA AG' is not present in the *KlMAL2* promoter sequence (Figure 5). The sequence 'GGTTACC' located at 831 bp upstream of the coding region in the #AJ007636 sequence is missing a T residue in the corresponding CBS 1065 *KlMAL2* promoter sequence. This deletion changes the sequence 'GGTTACC' to the sequence 'GGTACC', which is the recognition site for the restriction enzyme *Kpn*I. The two strains could therefore be easily distinguished from one another by a *Kpn*I restriction digest of the *MAL2* promoter.

3.1.2 Core Promoter Elements

The sequence and orientation of the maltase (*KlMAL22*) and maltose permease (*KlMAL21*) promoters are shown in Figures 6 and 7 respectively. Putative TATA boxes are located in the maltase promoter at positions 182 and 108 bp upstream of the ATG start codon and in the maltose permease promoter at 57 bp upstream of the ATG start codon (Table 3a and 3b). The 'TATAA' sequences at 108 bp upstream of the *KlMAL22* gene and 57 bp upstream of the *KlMAL21* gene represent the closest match to the TATA consensus sequence. In contrast to higher eukaryotes, yeast promoters may contain more than one sequence homologous to the TATA consensus (Mellor, 1989). The TATA box of higher eukaryotes is generally located 25-30 bases upstream of the mRNA initiation site. *Kluyveromyces lactis* and *S. cerevisiae* TATA boxes, however, are found at variable distances from initiation sites. The *K. lactis LAC4* promoter contains three TATA-like

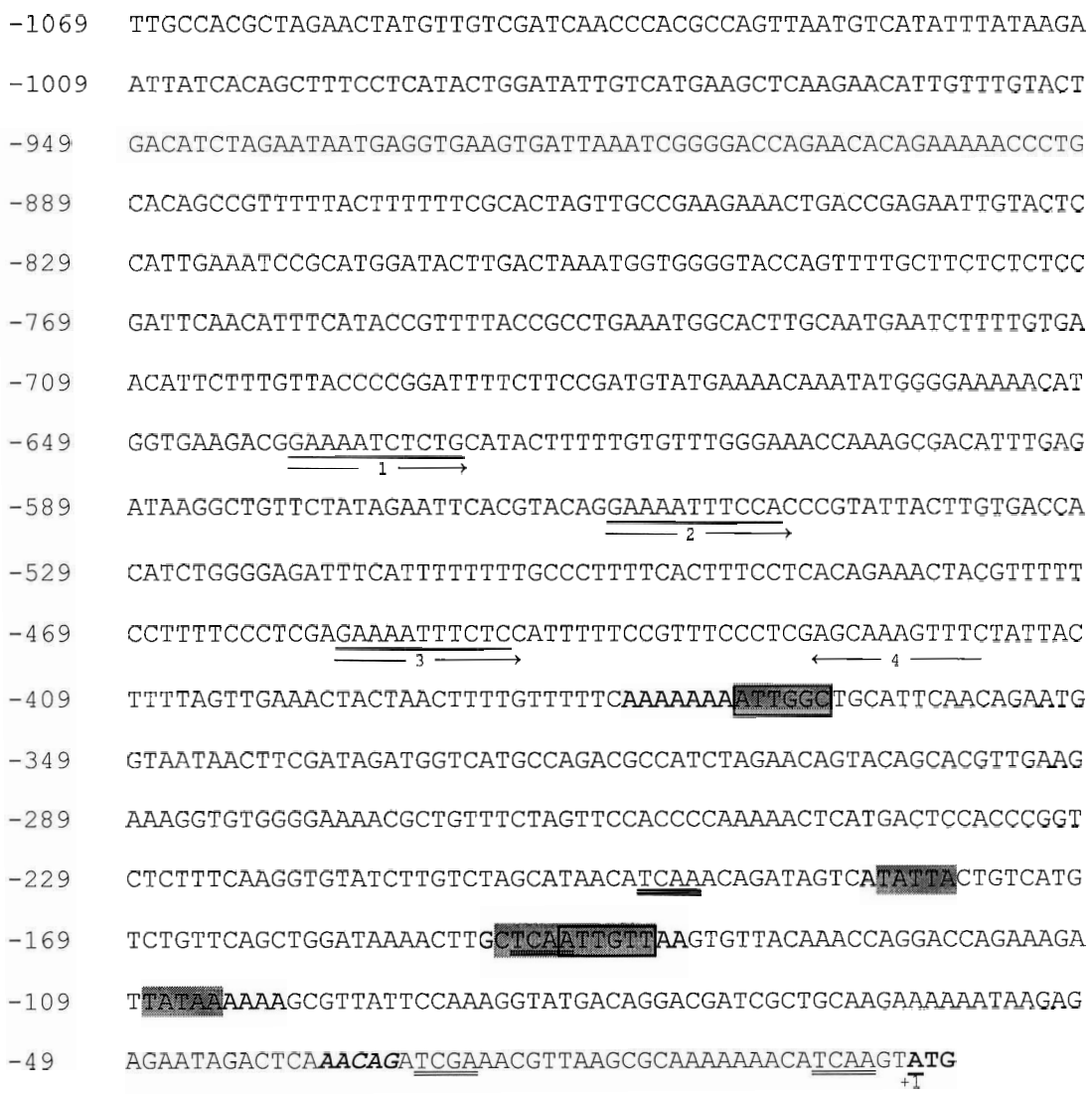


Figure 6. Kluyveromyces lactis maltase promoter (KlMAL22) sequence. The translation start site is shown in bold. A putative RRYRR transcription start site is shown in bold italics and putative TC(G/A)A transcription start sites are double underlined. Putative TATA and CAAT boxes are highlighted. Boxed sequences indicate the CAAT consensus sequence is the reverse complement. Regions homologous to the *S. cerevisiae* UAS_{MAL} consensus sequence are shown underlined (a solid line indicates that the consensus is matched in the 5`→3`direction and a dashed line indicates that the consensus is matched in the opposite orientation).

-1069 ACTTGATGTTTTTTTGCGCTTAACGTTTCGATCTGTTTGAGTCTATTCTCTCTTATTTTT

-1009 TCTTGCAGCGATCGTCCTGTCATACCTTTGGAATAACGCTTTTTTATAATCTTTCTGGTC

-949 CTGGTTTGTAACACTTAACAATTGAGCAAGTTTTATCCAGCTGAACAGACATGACAGTAA

-889 TATGACTATCTGTTTGATGTTATGCTAGACAAGATACACCTTGAAAGAGACCGGGTGGAG

-829 TCATGAGTTTTTGGGGGTGGAACTAGAAACAGCGTTTTCCCCACACCTTTCTTCAACGTGC

-769 TGTACTGTTCTAGATGGCGTCTGGCATGACCATCTATCGAAGTTATTACCATTCTGTTGA

-709 ATGCAGCCAATTTTTTTTGAAAAACAAAAGTTAGTAGTTTCAACTAAAAGTAATAGAAAC

-649 TTTGCTCGAGGGAAACGGAAAAATGGAGAAATTTTCTCGAGGGAAAAGGAAAAACGTAGT
4 → 3 →

-589 TTCTGTGAGGAAAGTGAAAAGGGCAAAAAAAATGAAATCTCCCCAGATGTGGTCACAAGT

-529 AATACGGGTGGAAATTTTCCTGTACGTGAATTCTATAGAACAGCCTTATCTCAAATGTCG
2 →

-469 CTTTGGTTTCCCAAACACAAAAAGTATGCAGAGATTTTCCGTCTTCACCATGTTTTTCCC
← 1

-409 CATATTTGTTTTCATACATCGGAAGAAAATCCGGGGTAACAAAGAATGTTCACAAAAGAT

-349 TCATTGCAAGTGCCATTTCAGGCGGTAAAACGGTATGAAATGTTGAATCGGAGAGAGAAG

-289 CAAAACTGGTACCCCACCATTTAGTCAAGTATCCATGCGGATTTCAATGGAGTACAATTC

-229 TCGGTCAGTTTCTTCGGCAACTAGTGCGAAAAAAGTAAAAACGGCTGTGCAGGGTTTTTC

-169 TGTGTTCTGGTCCCCGATTTAATCACTTCACCTCATTATTCTAGATGTCAGTACAAACAA

-109 TGTTCTTGAGCTTCATGACAATATCCAGTATGAGGAAAGCTGTGATAATTCTTATAAATA

-49 TGACATTAACTGGCGTGGGTTGATC***GACAACA***TAGTTCTAGCGTGGCAA**ATG**
+1

Figure 7. Kluyveromyces lactis maltose permease promoter (KlMAL21) sequence (reverse complement of the maltase promoter sequence). The translation start site is shown in bold. A putative RRYRR transcription start site is shown in bold italics and putative TC(G/A)A transcription start site is double underlined. Putative TATA and CAAT boxes are highlighted. Regions homologous to the *S. cerevisiae* UAS_{MAL} consensus sequence are shown underlined (a solid line indicates that the consensus is matched in the 5`→3`direction and a dashed line indicates that the consensus is matched in the opposite orientation).

Table 3. Putative promoter motifs found in the *K. lactis* a) maltase promoter and b) maltose permease promoter. *Putative CAAT box is present in the opposite orientation was the gene and the sequence given is the reverse complement.

a) *KlMAL22* promoter

Motif	Sequence (5`-3`)	Location 5' of the translation start site (in bp)
RRYRR-Like Initiation Site	AACAG	37
TC(G/A)A Initiation Site	TCAA	6
	TCGA	31
	TCAA	145
TATA-like Box	TATAA	108
	TATTA	182
CAAT Box	AACAAT*	132
	GACAAT	146
	GCCAAT*	365

b) *KlMAL21* promoter

Motif	Sequence (5`-3`)	Location 5' of the translation start site (in bp)
RRYRR-Like Initiation Site	AACAG	23
TC(G/A)A Initiation Site	TCGA	26
TATA-like Box	TATAA	57
CAAT Box	GACAAT	93
	AACAAT	114
	TACAAT	237
	TTCAAT	247

sequences at 230, 170 and 142 bp from the translation start site giving rise to transcripts of variable length (Leonardo *et al.*, 1987). While these sequences are not all necessary for correct gene function, they each have a functional priority when contributing to gene expression (Ficca *et al.*, 1989).

Hahn *et al.* (1985) noted the presence of two transcription initiation consensus sequences in a majority of *S. cerevisiae* promoters. The first and most common sequence is a purine rich RRYRR consensus. The second common motif is a TC(G/A)A consensus sequence and is predominant in promoters where the TATA box is located more than 50 bp upstream of the initiation site. Analysis of the *KlMAL22* promoter revealed putative TC(G/A)A sites located 6, 31 and 145 bp upstream of the translation start site and a putative RRYRR motif at 37 bp upstream of the ATG start codon (Figure 6 and Table 3a). The putative initiation sites located at 6 and 31 bp upstream of the *KlMAL22* promoter are both over 50 bp from the putative TATA box and closely resemble the TC(G/A)A consensus sequence. The *KlMAL21* promoter contains a RRYRR motif at 23 bp upstream of the coding region and a TC(G/A)A sequences 26 bp upstream of the translation start site (Figure 7 and Table 3b). Transcription of the *ScMAL62* and the *ScMAL61* genes begin at TGTA, and TTGA motifs, respectively (Hong *et al.*, 1987). By analogy it is most likely that the TC(G/A)A sequences at 6 and 31 bp upstream of the *KlMAL22* gene and 26 bp upstream of the *KlMAL21* gene are the functioning transcription initiation sites.

All of the putative TATA-like boxes of the maltase and maltose permease promoters are within 31 to 102 bp of the closest putative initiation site. Variation in the distance between TATA-box and transcription start site is not unusual in yeast. Functional TATA sequences are generally located 60 to 120 bp upstream of transcription start sites in

S. cerevisiae (Hahn *et al.*, 1985). S1 nuclease mapping determined that the *K. lactis LAC4* promoter contains strong transcriptional start sites located between 69 to 130 bp downstream of the putative TATA boxes (Breunig *et al.*, 1984; Leonardo *et al.*, 1987).

Putative CCAAT boxes were identified in the *KlMAL22* promoter at 132, 146 and 365 bp upstream of the ATG start codon and in the *KlMAL21* promoter at 93, 114, 237 and 247 bp upstream of the coding region (Table 3a and 3b). While TATA sequences are direction dependent, CAAT motifs can function at varying distances upstream from transcriptional start sites and in either orientation (Mantovani, 1999). Two of the putative CAAT-like sequences in the *KlMAL22* promoter are present in the opposite orientation to the *KlMAL22* coding region (Figure 6 and Table 3a).

Mapping functional core promoter elements is necessary when studying the effect of sequence alterations on promoter activity. It is important to avoid core promoter regions while making specific changes to the promoter so that the effects caused by manipulating core elements do not complicate results.

3.1.3 The K. lactis UAS_{MAL}

Promoter regions may share common core motifs, however, more complex regulatory sequence elements known as upstream activator sequences (UAS) in yeast and enhancers in higher eukaryotes are present in many eukaryotic promoters. A region mid-way between the *KlMAL21* and *KlMAL22* genes shared homology with an upstream activator sequence from the *ScMAL61-ScMAL62* intergenic region, named the $ScUAS_{MAL}$ (Figure 6 and 7). The *S. cerevisiae* positive activator ScMAL63 binds to the $ScUAS_{MAL}$ and activates transcription of the *ScMAL6* genes in the presence of maltose (Ni *et al.*, 1990; Levine *et al.*, 1992).

Four regions within the *S. cerevisiae ScMAL6* promoter consisting of 11 bp repeats of the consensus 5'-GAAA $^{A}/_{T}$ TTT $^{C}/_{A}$ GC-3' make up the $ScUAS_{MAL}$ (Hong *et al.*, 1987; Levine *et al.*, 1992; Yao *et al.*, 1994; Sirenko *et al.*, 1995). A multiple sequence alignment between the *ScMAL6* and the *KlMAL2* intergenic regions reveals sites similar to the 11-bp repeats of the $ScUAS_{MAL}$. These sites span from 640 to 415 bp upstream of the *KlMAL22* gene (Figures 5 and 6). The $KlUAS_{MAL}$ sites 2 and 3 are imperfect palindromes and match the consensus sequence closely in both directions (Figures 5 and 6). Sites 1 and 4, however, match the consensus sequence closely in only one direction (Figures 5 and 6).

3.1.4 Transcription Factor Binding Site Analysis of the KlMAL2 Promoter

The putative transcription factor binding sites found in the #AJ007636 *MAL2* and the *KlMAL2* promoters were compared (Table 4). The selected binding sites all play a role in regulating metabolic pathways and had both a similarity and a matrix score of greater than 0.80. Each transcription factor binding site is assigned a nucleotide distribution matrix that identifies the conservation of each position within the matrix (Quandt *et al.*, 1995). An array of values, termed the consensus index vector (C_i vector), is associated with each nucleotide distribution matrix (Quandt *et al.*, 1995). The lower the C_i value, the less conserved that nucleotide position is. A C_i value of 0 indicates a position with equal conservation of all nucleotides while a C_i value of 100 indicates that a position has complete conservation of one nucleotide. The core region of a binding site is defined by four consecutive nucleotides with the highest C_i sum (Quandt *et al.*, 1995). A core similarity score is determined by dividing the sum of the matrix values for base *b* at each position *j* by the sum of the maximum matrix value for each position. The matrix similarity score is slightly more complex than the core similarity score. The matrix similarity is

Table 4. Selected transcription factor binding sites found by MatInspector 2.2. (Quandt *et al.*, 1995). RE value indicates the number of matches expected in a random sequence of 1000 bp. The position given is x-bases upstream of the maltase start codon. (+) indicates that the consensus sequence from 5' to 3' in the direction of the maltase (*KlMAL22*) gene and (-) to the maltose permease (*KlMAL21*) gene. The MIG1p binding site that was removed in the plasmid pREX-IC-ΔMig is shown in bold italics.

Transcription Factor	**RE value**	**Core similarity ≥ 0.80, matrix similarity ≥ 0.80**			
		San Vincent *et al.* Position	**San Vincent *et al.* Similarity Score**	**Current Study Position**	**Current Study Similarity Score**
ABF1	**0.17**	466 (-)	0.825	437 (-)	0.814
		359 (+)	0.857	333 (+)	0.857
GCN4	**0.22**	802 (-)	0.833	816 (+)	0.819
		-	-	769 (+)	0.833
		757 (+)	0.909	725 (+)	0.909
		274 (+)	0.921	245 (+)	0.921
		-	-	43 (-)	0.814
GCR1	**0.38**	716 (+)	0.805	686 (+)	0.805
HAP2/3/4	**0.18**	649 (-)	0.821	620 (-)	0.821
		39 (+)	0.862	-	-
MIG1	**0.09**	962 (+)	0.863	925 (+)	0.863
		841 (+)	0.910	805 (+)	0.922
		728 (-)	0.904	697 (-)	0.969
		700 (+)	0.900	***671 (+)***	***0.900***
		563 (+)	0.844	534 (+)	0.844
		320 (+)	0.874	291 (+)	0.874
NIT2	**2.53**	1043 (+)	0.967	1006 (+)	0.967
		1023 (-)	0.950	986 (-)	0.950
		852 (-)	0.950	816 (-)	0.950
		620 (-)	0.995	591 (-)	0.995
		368 (-)	0.967	339 (-)	0.967
		244 (+)	0.980	215 (+)	0.980
		220 (-)	0.965	191 (-)	0.965
		187 (-)	0.950	158 (-)	0.950
RAP1	**0.03**	1079 (+)	0.810	1042 (+)	0.810

weighted such that mismatches occurring at more conserved regions cause greater reductions in the overall similarity score than do mismatches occurring at less conserved regions (Quandt *et al.*, 1995).

One drawback to this transcription factor search is that the similarity matrix used is based on *S. cerevisiae* and consequently some binding sites may have slight differences in their consensus sequence when compared to their *K. lactis* counterparts. Since *S. cerevisiae* and *K. lactis* are so closely related, it is likely that many of the same transcription factors and their respective binding sites are found in both species. Indeed it does appear that many *K. lactis* homologs exist for specific *S. cerevisiae* transcription factors (Bergkamp-Steffens *et al.*, 1992; Goncalves *et al.*, 1992; Cassart *et al.*, 1997; Dong *et al.*, 1997; Bourgarel *et al.*, 1999; Lamas-Maceiras *et al.*, 1999)

ABF1 and HAP2/3/4

Autonomously replicating sequence (ARS) binding factor 1 (ABF1) binding sites are often present in the 5'-flanking regions of yeast genes and also in ARS sites and are implicated in DNA replication as well as the induction of transcription (Brand *et al.*, 1987; Goncalves *et al.*, 1992). In *K. lactis,* KlABF1 is required for the rapid induction of mitochondrial genes during the switch from glucose to non-fermentable carbon sources. A carbon source responsive regulatory complex known as HAP2/3/4 enhances binding of ABF1 and is responsible for induction of mitochondrial genes under non-fermentive conditions (Forsburg *et al.*, 1989; Goncalves *et al.*, 1992; de Winde *et al.*, 1993; Mulder *et al.*, 1995; Bourgarel *et al.*, 1999). Both ABF1 and the HAP2/3/4 complex are involved in inducing transcription of genes required during the transition from fermentation to respiration.

Putative binding sites for ABF1 in the *KlMAL2* intergenic region are located at 333 and 437 bp upstream of the *KlMAL22* coding region (Table 4). In addition, a putative HAP2/3/4 consensus sequence can be found 620 bp upstream of the *KlMAL22* gene (Table 4). One possible explanation for the presence of these sites is that in the absence of glucose (de-repressing conditions) these transcription factors mediate a global response by activating genes required for the metabolism of non-glucose carbon sources.

In *S. cerevisiae* the ScHAP2/3/4 complex interacts specifically with the CAAT sequence motif and this interaction is related to de-repression of genes involved in the respiratory pathway during the diauxic shift between glucose and alternative, usually non-fermentable, carbon sources (Ramil *et al.*, 1998; Lodi *et al.*, 2002). In contrast, the KlHAP2/3/4 complex does not necessarily act through these CAAT motifs (Ramil *et al.*, 1998). Since *K. lactis* prefers respiration to fermentation, even while growing in anaerobic environments or on non-fermentable sugars, it might be expected that the pathways involved in alternative carbon source metabolism would be regulated in a different manner. The presence of HAP2/3/4 binding sites may maintain active transcription of the genes necessary for the metabolism of fermentable carbon sources, even when respiration is being favoured.

RAP1 and GCR1

Putative binding sites at 1042 bp upstream of the *KlMAL22* coding region for repressor/activator protein 1 (RAP1) and 686 bp upstream for glucose regulation-1 protein (GCR1) exist in the *KlMAL2* promoter region (Table 4). In *S. cerevisiae*, expression of glycolytic and ribosomal genes is regulated by these two transcription factors (Deminoff *et al.*, 2001). Both KlRAP1 and KlGCR1 homologs are present in *K.*

lactis and KlRAP1 is capable of binding to the same *cis*-acting element as ScRAP1 (Haw *et al.*, 2001). Introduction of the *KlGCR1* gene can restore normal growth to a *S. cerevisiae ΔGCR1* mutant, indicating that the two genes share a similar function (Haw *et al.*, 2001).

RAP1 performs both repressor and activator functions, depending on its context. As an activator, it is thought that RAP1 prevents the formation, or causes the dissociation of nucleosomes that are in close proximity to RAP1 binding sites (Morse, 2000; Yu *et al.*, 2003). In fact, RAP1 can recruit histone acetylase (HAT) complexes to target genes (Yu *et al.*, 2003). As a result, RAP1 may block nucleosome formation at the promoter and allow GCR1 to activate transcription.

GCN4 and NIT2

The general amino acid control activator GCN4 and nitrogen regulator protein NIT2 are both involved in the activation of genes involved in protein synthesis and nitrogen metabolism during periods of amino acid and nitrogen starvation (Marzluf 1997; Lamas-Maceiras, Cerdan et al. 1999; Hinnebusch and Natarajan 2002). Putative GCN4 binding sites occur at 43, 245, 725, 769 and 816 bp upstream of the *KlMAL22* coding region (Table 4). Amino acids are derived from intermediates of the pentose phosphate pathway, the citric acid cycle and glycolysis. For example, the synthesis of histidine requires ribose-5-phosphate, a product of the pentose phosphate pathway, ATP, glutamine and glutamate (Lehninger *et al.*, 1993). The *HIS4* gene of yeast encodes for a trifunctional protein responsible for three steps of the histidine synthesis pathway (Keesey *et al.*, 1979). The transcriptional activator GCN4 induces the expression of the *S. cerevisiae HIS4* gene and GCN4 consensus sites have been found to exist in the *KlHIS4* promoter (Lamas-

Maceiras *et al.*, 1999). During amino acid synthesis glucose molecules are converted to ribose-5-phosphate instead of proceeding through glycolysis for the production of energy. It may be that GCN4 also induces the transcription of glucose producing genes in order to supply the necessary sugar backbone for amino acid biosynthesis and to keep glucose levels at a normal level within the cell.

Sequence analysis also identified possible NIT2 binding sites at 158, 191, 215, 339, 591, 816, 986, and 1006 bp upstream of the *KlMAL22* ATG start codon. The NIT2 binding sites located at 158, 191 and 215 bp as well as those located at 986 and 1006 bp upstream of the *KlMAL22* translation start site are the most likely. NIT2 binding sites are strongest when they exist as a pair (or more) within 30 bp of one another (Marzluf, 1997). NIT2 binding sites represent a family of response elements that mediate nitrogen catabolite de-repression. AREA from *Aspergillus*, NIT2 from *Neurospora* and GLN3 from *Saccharomyces* are all global activating factors that recognize GATA-type sites (Marzluf, 1997). Nitrogen is supplied to intermediates by glutamine and glutamate during histidine biosynthesis (Lehninger *et al.*, 1993). Glutamine and glutamate are the nitrogen sources of preference in fungi and when they are unavailable alternative nitrogen sources must be used. Replenishing glutamine reserves within the cell requires α-ketoglutarate and another nitrogen source, usually ammonia. NIT2 may be a global activator causing the de-repression of genes involved in the production of glucose in order to replenish the α-ketoglutarate used in the production of glutamine.

Since all amino acids require a carbon backbone provided by some intermediate of glucose metabolism, it may be that GCN4 and NIT2 activate the *KlMAL2* promoter during either amino acid or nitrogen starvation in order to scavenge sources of carbon present in

the surrounding environment. A functional genomic analysis of *S. cerevisiae* has shown that levels of *MAL31* and *SUC2* mRNA are at least two-fold higher after 33 hours of growth in low-nitrogen media than after the same time of growth in high-nitrogen media even when glucose is in abundant supply (Backhus *et al.*, 2001). Also, glycogen and trehalose metabolism is activated in *S. cerevisiae* when the nitrogen source is depleted during growth in a nitrogen limiting environment (Parrou *et al.*, 1999)

MIG1

The *KlMAL2* genes are responsible for the metabolism of maltose into its component glucose molecules. During conditions when glucose is abundant the yeast conserves energy by turning off the genes responsible for the assimilation of alternative carbon sources. This repression is mediated by the action of the global repressor protein MIG1. Transcription of the *MAL6* locus in *S. cerevisiae* is repressed during growth in glucose by MIG1 and it is expected that a similar mechanism is working in the *K. lactis MAL2* locus. Thus, MIG1 binding sites are likely the most important negative regulatory elements found in the maltase promoter, especially when considering the use of promoters for the expression of heterologous proteins. If a protein expression system is required to perform efficiently during growth on undefined media, glucose repression must be relieved. Identifying important MIG1 binding sites and removing them would relieve repression of the promoter even when glucose is present in the growth media.

The zinc finger protein MIG1 is responsible for glucose repression in *S. cerevisiae* and binds to a GC box consensus sequence of (G/C)(C/T)GGGG with an associated with a 5'-AT rich region. The *K. lactis* homolog of MIG1, KlMIG1, can restore *ΔMIG* phenotypes in *S. cerevisiae* and mediates glucose repression of the *KlGAL1*

galactokinase gene (Dong *et al.*, 1997). Putative binding sites for the global glucose repression factor MIG1 are found at 291, 534, 671, 697, 805, and 925 bp upstream of the *KlMAL22* translation start site (Table 4). Two MIG1 binding sites were identified by Hu *et al.* (1995) in the *ScMAL61-ScMAL62* intergenic region. The first site (B) is located on the *ScMAL61* proximal side of the $ScUAS_{MAL}$ and the second site (D) is located on the opposite side of the $ScUAS_{MAL}$, proximal to *ScMAL62* (Hu *et al.*, 1995; Wang *et al.*, 1997). The putative *K. lactis* MIG1 binding site located at 671 bp upstream of the KlMAL22 gene is in a similar position to site B of *S. cerevisiae*. ScMIG1 binds strongly to both sites B and D. Deletion experiments have shown that site B is responsible for glucose repression of *ScMAL61*, while site D contributes to glucose repression of *ScMAL62* (Hu *et al.*, 1995). That is not to say that each site does not contribute slightly to the repression of genes distal to the sequence. A slight bi-directionality of the ScMIG1 binding sites is seen in the *ScMAL61-ScMAL62* regulon. In the absence of the proximal site, the maltase and maltose permease genes are still glucose repressed, though not to the same degree (Hu *et al.*, 1995).

3.2 Construction of Promoter Variants and Transformation of *K. lactis*

In order to characterize important regulatory regions within a promoter a mutational analysis was performed. Deletion mutants of the promoter were generated to assess the importance of a given DNA sequence within the promoter. It was expected that removal of a negative regulatory region would result in increased reporter gene expression from the promoter when the cells were grown in rich, complete medium. Conversely, it is expected

that when a positive regulatory element is removed expression from the promoter would decrease.

To examine the regulation of the *KlMAL21-KlMAL22* bi-directional promoter three expression plasmids, pREX-IC, pREX-IC-ΔMig and pREX-IC-ΔPos were constructed (Figure 3). The reporter genes CFP and YFP were expressed from the *KlMAL22* and *KlMAL21* promoters respectively. Fluorescent protein levels were compared between cells grown on different carbon sources and between plasmids containing different promoter variants. To determine whether a specific DNA sequence was important for the regulation of the promoter, the sequence in question was subjected to mutagenesis or deleted completely. The subsequent change in fluorescent protein expression was then assayed.

Each of the three expression plasmids contained a different variation of the *K. lactis* maltase bi-directional promoter. The first plasmid, pREX-IC, consisted of the native promoter and was constructed to study regulation of the promoter during growth on glucose, galactose or glycerol as the sole carbon sources. This plasmid was also used as a control against which the expression levels generated by the two subsequent promoter variants were compared. The promoter variants were constructed to study the importance of putative regulatory elements. The two putative DNA elements chosen for mutation were identified through promoter region sequence analysis and represent homologs of the most significant negative and positive regulating elements of the *S. cerevisiae* maltase locus.

A putative binding site for the carbon catabolite repressor protein MIG1 and a portion of the putative $KlUAS_{MAL}$ positive regulatory element were chosen because of the significance each region has for the regulation of the maltase locus. Since MIG1 is a global repressor of metabolic pathways involved in the utilization of alternate carbon sources, it

was theorized that the MIG1 binding sites constitute the most important repressor elements of the maltase promoter. In *S. cerevisiae* the action of the MIG1 repressor protein is countered by the binding of the positive activator ScMAL63 to the $ScUAS_{MAL}$. Therefore, the putative $KlUAS_{MAL}$ represents the most significant positive regulatory element of the *KlMAL21-KlMAL22* promoter. To determine if these putative binding sites were indeed responsible for regulating the promoter, each was either altered such that it would be unrecognizable to its corresponding transcription factor or deleted altogether from the promoter.

The first altered promoter, pREX-IC-ΔMig, contained an alteration of a putative MIG1 binding site proximal to and on the *KlMAL21* side of the putative $KlUAS_{MAL}$ (Table 4). The region between 671 to 657 bp upstream of the *KlMAL22* start site was engineered to remove this putative MIG1 binding site (Figure 4). A binding site (site B) for ScMIG1 is located proximal to and on the *ScMAL61* side of the $ScUAS_{MAL}$. This site B has been shown to strongly bind to ScMIG1 in gel-shift assays (Hu *et al.*, 1995). The engineered region in the current study was considered analogous to site B of the *ScMAL61-ScMAL62* promoter. This putative site was altered so that its sequence no longer resembled the MIG1 binding consensus sequence. It was theorized that effectively removing this recognition sequence would relieve glucose repression of the promoter.

The second altered promoter, pREX-IC-ΔPos, contained a deletion from 411 to 441 bp upstream of the *KlMAL22* gene (Figure 4). The region of the promoter spanning from 640 to 415 bp upstream of the *KlMAL22* gene shares homology with the *ScMAL61-ScMAL62* intergenic region $ScUAS_{MAL}$ (Figure 6 and 7). The $ScUAS_{MAL}$ includes four repeated elements (sites 1-4) that constitute a binding site for the positive activator

ScMAL63 (Yao *et al.*, 1994). The deleted 30 bp region in pREX-IC-ΔPos corresponds to site 4 of the $ScUAS_{MAL}$. It was hypothesized that deletion of this region would reduce the activity of the *KlMAL21-KlMAL22* promoter in both orientations.

3.2.1 Transformation of K. lactis

Kluyveromyces lactis cells were transformed by electroporation with one of three expression plasmids, pREX-IC, pREX-IC-ΔMig or pREX-IC-ΔPos. Putative transformants for each promoter construct appeared approximately 2-4 days after being plated onto selective media with a transformation efficiency between 10-100 transformants per μg of transforming DNA. Transformants initially selected on primary 25 μg/ml G418 YPD plates were subsequently transferred to secondary plates, supplemented with either 100 μg/ml or 200 μg/ml G418, to verify survivability. While higher G418 concentrations increased the incubation time required for the appearance of transformant colonies, no wild-type *K. lactis* survived on any of the secondary selective plates. The SV40 early promoter drove expression of the kan^r marker conferring resistance to G418. The SV40 early promoter has been utilized in other yeasts including *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*; however, no cases of this promoter being successfully used in *K. lactis* could be found (Jones *et al.*, 1988; Axelrod *et al.*, 1990; Tokunaga *et al.*, 1993).

Positive transformants were verified by DNA hybridization (Figure 8) (Southern, 1975). The appearance of a 1075 bp DNA fragment in putative transformants confirmed the presence of the expression plasmid. An initial test of plasmid stability and structural stability was performed using a pREX-IC transformant. Liquid cultures of the transformant were grown at 30°C and cells remained viable on 25 μg/ml G418 selective media at percentages of 95%, 46% and 8% after 24, 48 and 72 hours respectively. Plasmid DNA

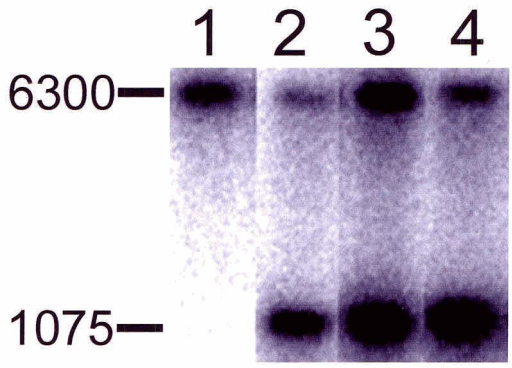


Figure 8. Confirmation of K. lactis transformations by DNA hybridization analysis. Total genomic DNA was isolated from wild-type *K. lactis* strain CBS 1065 (lane 1) and from transformants of each expression plasmid: pREX-IC (lane 2), pREX-IC-ΔMig (lane 3), pREX-IC-ΔPos (lane 4). The isolated DNA was digested with *Bam*HI and hybridized with ^{32}P-dCTP labeled PCR product of the *KlMAL2* promoter. The size of each band was calculated by its relative position to known *Hin*dIII fragments of λ-DNA.

was isolated after 96 hours and analyzed for plasmid structural stability by diagnostic restriction digest. Restriction digest patterns were identical between the re-isolated plasmid pREX-IC and the original transforming DNA indicating that the plasmids were structurally stable.

During the growth of cells expressing fluorescent protein, DNA hybridization was performed on total chromosomal and plasmid DNA isolated from each culture after 96 hours of growth to confirm that the cells retained the expression plasmids. Again, the appearance of a 1075 bp DNA fragment confirmed the presence of the expression plasmid in all transformants after 96 hours of growth (Figure 9). The lack of fluorescent protein expression from these constructs therefore would not merely reflect loss of plasmid but would correctly represent a reduction in promoter activity. It must be noted, however, that Figure 9 only indicates the presence of the plasmid after 96 hours of growth. Equal volumes, but not equal quantities, of total yeast DNA extract were loaded onto each gel. Consequently, no determination of plasmid copy number after 96 hours of growth could be made from this autoradiograph.

3.3 Expression of Fluorescent Proteins in *K. lactis*

To examine the effect of different carbon sources on the bi-directional maltase promoter expression of fluorescent proteins from the *KlMAL21* and *KlMAL22* promoters was analyzed. It was hypothesized that expression from both the *KlMAL21* and *KlMAL22* promoters would be repressed during growth in glucose and galactose and induced during growth in glycerol. The effect that the promoter alterations had on the expression of the

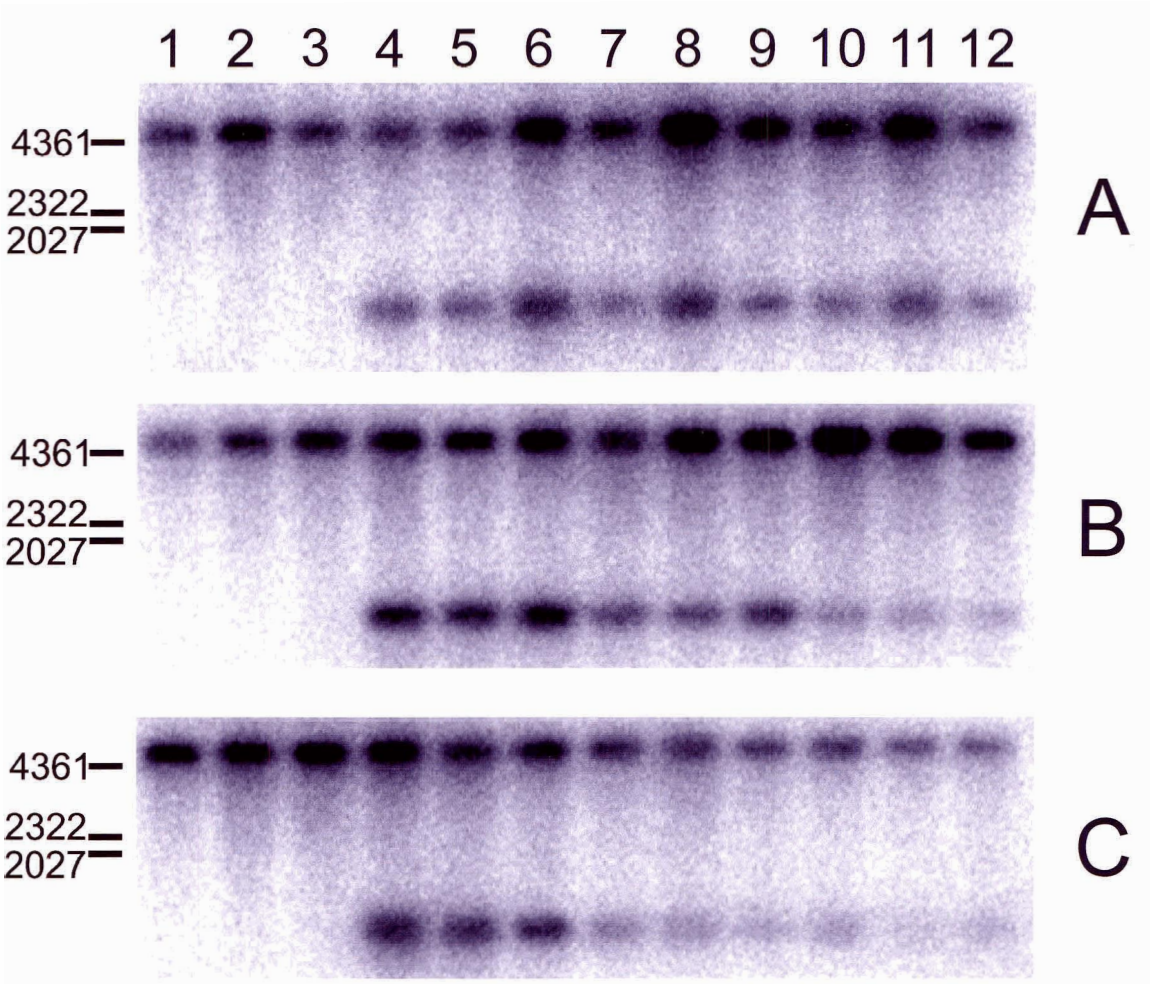


Figure 9. DNA hybridization analysis of K. lactis transformants after 96 hours of growth. Total genomic DNA was isolated from each transformant, digested with *Bam*HI and hybridized with ^{32}P-dCTP labeled PCR product of the *KlMAL2* promoter. Wild-type cultures 1, 2 and 3 (lanes 1, 2, 3), pREX-IC cultures 1, 2 and 3 (lanes 4, 5, 6), pREX-IC-ΔMig cultures 1, 2 and 3 (lanes 7, 8, 9) and pREX-IC-ΔPos cultures 1, 2 and 3 (lanes 10, 11 and 12). Cells were grown in A) glucose, B) glycerol and C) Galactose. The size of each band was calculated by its relative position to known *Hin*dIII fragments of λ-DNA.

fluorescent proteins was also analyzed during growth in glucose, galactose and glycerol. It was hypothesized that alteration of the putative MIG1 binding site at 671 to 657 bp upstream of the *KlMAL22* translation start site would result in increased fluorescent protein expression during growth on glucose. It was also hypothesized that the deletion of site 4 of the putative $KlUAS_{MAL}$ between 411 to 441 bp upstream of the *KlMAL22* gene would result in a reduction in fluorescent protein expression during growth on glycerol.

A starter culture was initially grown in repressing media containing glucose and used to inoculate 100 ml cultures containing either glucose, glycerol or galactose as the sole carbon source. Levels of fluorescence were measured after 24, 48, 72, and 96 hours after culture inoculation. RFI levels can be seen for each carbon source and sample time in Table 5. RFI values can only be compared across different carbon sources with the same fluorescent protein. The quantum yield of CFP is approximately two-thirds of the quantum yield of YFP and therefore the fluorescent intensity generated by equal amounts of CFP will be less than the fluorescent intensity of YFP (Patterson *et al.*, 2001). Therefore, it is only useful to compare RFI levels of one fluorescent protein among different carbon sources or promoter variants and not to the RFI levels measured for the other fluorescent protein.

3.3.1 The Native Promoter, pREX-IC

When glycerol was provided as the sole carbon source expression of both CFP (*KlMAL22*) and YFP (*KlMAL21*) was induced (Table 5 and Figures 9 and 10). After 48 hours both CFP and YFP levels were 10 times higher when the cells were induced with glycerol as compared to induction with either glucose or galactose (Table 5). Expression of YFP in glycerol peaked at 48 hours and was greater than 10 times higher than YFP

Table 5 Relative Fluorescent Intensity (RFI) of CFP and YFP for each promoter variant grown on either glucose, glycerol or galactose, for 24, 48, 72, and 96 hours.

Promoter Variant	Time (Hrs)	Carbon Source	CFP RFI (*KlMAL22*) ($x10^3$)	Standard Error ($x10^3$)	YFP RFI (*KlMAL21*) ($x10^3$)	Standard Error ($x10^3$)
pREX-IC	24	Glucose	-6.9	.5	6.3	1.0
		Glycerol	10.7	1.1	129	5.9
		Galactose	.8	.4	4.4	.7
	48	Glucose	1.6	.2	14.6	.8
		Glycerol	13.4	1.5	150	5.1
		Galactose	.7	.7	4.6	.6
	72	Glucose	6.1	2.4	12.2	3.5
		Glycerol	28.7	.6	131	2.8
		Galactose	3.7	.3	5.1	1.1
	96	Glucose	19.1	2.5	127	5.0
		Glycerol	19.8	2.0	116	5.4
		Galactose	14.0	1.1	12.6	1.8
pREX-IC-ΔMig	24	Glucose	-.2	.5	1.5	1.3
		Glycerol	7.2	.5	83.9	2.6
		Galactose	1.5	1.3	4.4	2.1
	48	Glucose	2.3	1.2	11.5	1.3
		Glycerol	13.0	2.7	85.2	6.7
		Galactose	.5	.7	1.2	1.6
	72	Glucose	7.6	.9	37.5	9.8
		Glycerol	24.5	1.0	90.0	6.7
		Galactose	1.1	1.3	.2	2.9
	96	Glucose	8.3	1.6	71.5	7.5
		Glycerol	15.6	.7	80.5	2.9
		Galactose	7.0	.3	8.4	1.6
pREX-IC-ΔPos	24	Glucose	-2.5	.4	-4.7	.7
		Glycerol	4.8	1.0	16.4	1.2
		Galactose	.6	.4	1.3	1.2
	48	Glucose	1.2	1.2	5.6	4.0
		Glycerol	2.7	1.1	26.8	1.2
		Galactose	3.3	1.0	3.3	1.2
	72	Glucose	4.5	2.2	14.4	4.2
		Glycerol	12.8	1.2	30.3	2.2
		Galactose	1.1	.5	2.6	.9
	96	Glucose	4.7	2.7	61.1	3.0
		Glycerol	7.3	.6	21.8	1.8
		Galactose	6.7	1.3	14.4	2.3

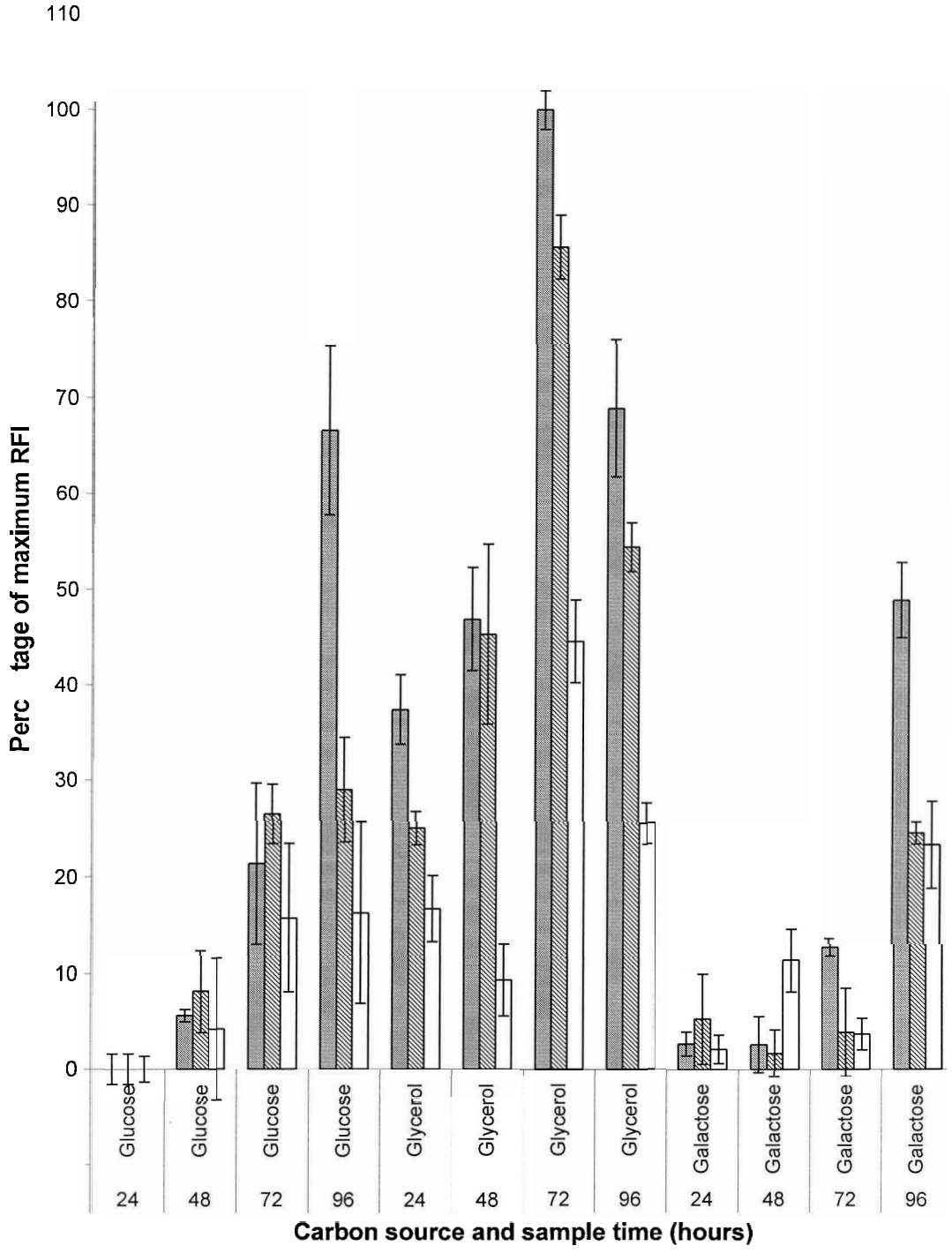


Figure 10. Relative Fluorescent Intensity (RFI) of CFP for *K. lactis* cultures grown on glucose, glycerol or galactose. RFI values are shown as a percentage of the maximum measured RFI. Each culture was sampled at 24, 48, 72 and 96 hours. Grey columns = pREX-IC; Slatted columns = pREX-IC-ΔMig; White columns = pREX-IC-ΔPos. Standard error bars are given.

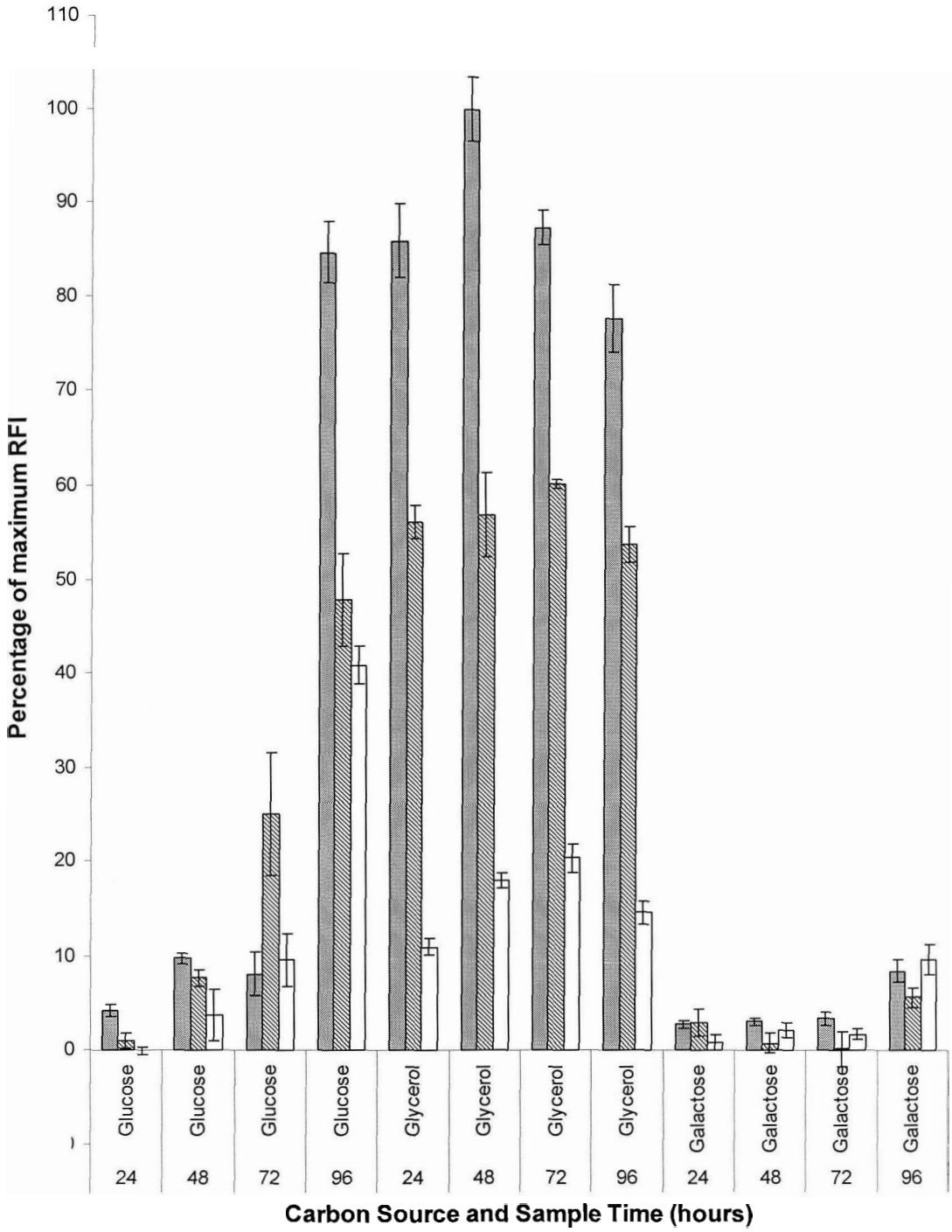


Figure 11. Relative Fluorescent Intensity (RFI) of YFP for *K. lactis* cultures grown on glucose, glycerol or galactose. RFI values are shown as a percentage of the maximum measured RFI. Each culture was sampled at 24, 48, 72 and 96 hours. Grey columns = pREX-IC; Slatted columns = pREX-IC-ΔMig; White columns = pREX-IC-ΔPos. Standard error bars are given.

expression in glucose and over 30 times higher than YFP expression in galactose (Table 5 and Figure 11). After 96 hours the expression of YFP in glucose increased to levels comparable to YFP expression in glycerol. The most likely explanation for this dramatic increase in YFP expression is that the glucose in the media became depleted and expression was no longer repressed by glucose.

Expression of YFP in galactose doubled after 96 hours of growth (Table 5). *Kluyveromyces lactis* may utilizes glucose more efficiently than galactose, however, this is not likely since the growth curves for the cultures grown in glucose and galactose were similar (data not shown). *Kluyveromyces lactis* has evolved as a dairy yeast and is well adapted to grow on the disaccharide lactose, composed of the subunits glucose and galactose (Schaffrath *et al.*, 2000). The mechanism of galactose repression may be longer lasting than the mechanism of glucose repression.

In contrast to YFP expression, peak CFP expression in glycerol was not observed until after 72 hours of growth (Table 5 and Figure 10). After 96 hours CFP expression in glucose had increased over 10 times relative to its levels after 48 hours and was comparable to CFP expression in glycerol at the same sampling time (Table 5 and Figure 10). CFP expression in glucose exhibited a greater than 10 times increase after 96 hours of growth when compared to CFP expression levels after 48 hours of growth (Table 5 and Figure 10).

In *S. cerevisiae*, maltase expression is relieved from repression during growth in galactose (Needleman, 1991; Wang *et al.*, 1997). When *S. cerevisiae* is grown in galactose, ScMAL62 levels are around 10 times higher than those observed when grown in glucose. In the current study almost the opposite is seen. Expression levels of CFP from the *KlMAL22* promoter are similar when grown in glucose or galactose. The expression of

YFP from the *KlMAL21* promoter does not reach the same levels in galactose as in glucose. After 96 hours of growth, YFP expression levels in glucose were 10-times higher than compared to galactose. Thus, galactose appears to strongly suppress expression from both the *KlMAL21* and *KlMAL22* promoters. Since the carbon source must become depleted as fermentation progresses, it seems that galactose maintains repressive conditions even when present in small quantities.

Kluyveromyces lactis cells expressing YFP from the native *KlMAL21* promoter were visualized under an epi-fluorescence microscope using the FITC filter set. Yeast cells were harvested from a wild-type CBS 1065 culture grown in glucose, a pREX-IC transformed culture grown in glucose and a pREX-IC transformed culture grown in glycerol. Cells were harvested from each culture after 72 hours of growth, diluted by 100x in dH_2O and visualized with excitation wavelengths from 450 to 490 nm and emission wavelengths greater than 520 nm. As can be seen in Figure 12, background autofluorescence was observed in wild-type cells, some spot fluorescence was observed in cells grown in glucose, and an abundance of fluorescence was observed in cells grown in glycerol. It may be possible to examine the percentage of cells retaining the plasmid by simply studying the relative numbers of fluorescing cells to non-fluorescing cells.

3.3.2 Altered Promoter, pREX-IC-ΔMig

To examine if removal of a putative MIG1 repressor protein binding site would relieve repression of the maltase promoter during growth in glucose, the region between 671 to 657 bp upstream of the *KlMAL22* translation start site was altered by PCR mediated mutagenesis (Table 4). This region is analogous to a ScMIG1 binding site (site B) in the *S. cerevisiae MAL61-MAL62* promoter. Site B is located proximal to and on the *ScMAL61*

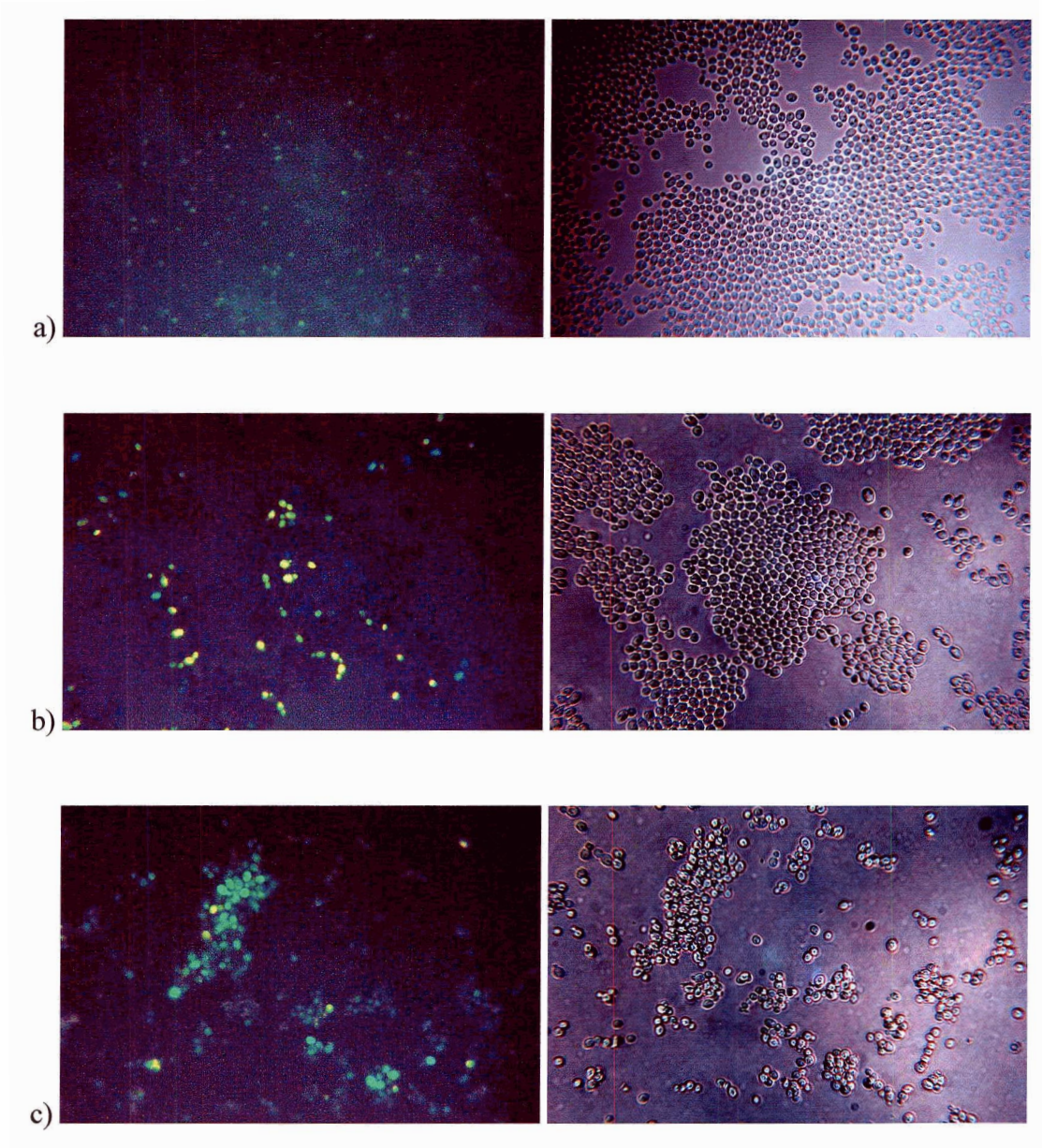


Figure 12. Epi-fluorescence photomicrograph of K. lactis cells expressing YFP visualized using a Zeiss universal epi-fluorescence microscope at 160x magnification. A FITC filter set was used to isolate YFP fluorescence. Cells were photographed after 72 hours of growth under the following conditions: a) wild-type *K. lactis* in glucose, b) pREX-IC transformed *K. lactis* in glucose, c) pREX-IC transformed *K. lactis* in glycerol.

side of the $ScUAS_{MAL}$. The homologous site in the *KlMAL21-KlMAL22* promoter was altered so that its sequence no longer resembled the MIG1 binding consensus sequence (Figure 4).

In the presence of glucose there was little or no difference in the expression of CFP between the native promoter and the pREX-IC-ΔMig promoter until 96 hours (Table 5 and Figure 10). After 96 hours of growth the native promoter continued to increase in expression while the pREX-IC-ΔMig promoter remained at the same expression level observed at 72 hours (Table 5 and Figure 10). The MIG1 binding site alteration also affected the expression of YFP from the *KlMAL22* promoter. After 72 hours of growth in glucose, the pREX-IC-ΔMig YFP expression levels tripled when compared to the 48 hour levels (Table 5). Though the increase of YFP expression in glucose from 72 to 96 hours was more dramatic for the native promoter, the pREX-IC-ΔMig promoter seemed to become relieved sooner. The MIG1 binding site located at 671 to 657 bp upstream of the *KlMAL22* gene may be more involved in regulation of the *KlMAL21* gene than in regulation of the *KlMAL22* gene. Removal of this MIG1 binding site may relieve repression of the *KlMAL21* promoter somewhat, however, other MIG1 binding sites within the promoter may contribute to *KlMAL21* repression.

Deletion of the putative MIG1 binding site caused the expression of YFP in glycerol to be reduced by about one third, when compared to the native promoter (Table 5 and Figure 11). Expression of CFP was not reduced to the same extent by the deletion. CFP levels observed for the pREX-IC-ΔMig promoter after 72 hours were reduced by less than one quarter when compared to native promoter expression levels (Table 5 and Figure 10). YFP expression in galactose remained repressed with the pREX-IC-ΔMig alteration,

while the level of CFP expressed in galactose by the pREX-IC-ΔMig altered promoter was at most half of CFP expression by the native, unaltered promoter (Table 5 and Figure 10).

In an experiment that explored MIG1-dependent and MIG1-independent glucose regulation of the *ScMAL61-ScMAL62* promoter Hu *et al.* (1995) expressed *lacZ* from both the *ScMAL62* and *ScMAL61* orientations of the promoter. In a wild-type strain of *S. cerevisiae*, the deletion of the MIG1 binding site proximal to *ScMAL61* (site B) did not relieve glucose repression of either the *ScMAL61* or *ScMAL62* orientations of the promoter. The deletion caused expression from the *ScMAL62* promoter to drop to about 70% of its wild-type levels during growth in glycerol. Similarly, in the current study, expression of CFP from the pREX-IC-ΔMig promoter after 72 hours was reduced slightly to 85% of the CFP levels expressed by the wild-type promoter when cells were grown in glycerol (Table 5 and Figure 10). Expression of CFP from the pREX-IC-ΔMig promoter during growth in glucose exhibited no difference when compared CFP expression from the wild-type promoter for sampling points up to 72 hours (Table 5 and Figure 10). After 72 hours of growth on glucose, however, expression of YFP from the pREX-IC-ΔMig promoter was three times the expression levels of the wild-type promoter at the same sampling point (Table 5 and Figure 11). No expression of CFP or YFP was observed for up to 72 hours of growth in galactose (Table 5, Figure 10 and Figure 11). After 96 hours of growth in galactose, deletion of the putative *KlMAL2* MIG1 protein binding site B caused a reduction of CFP expression by one half, when compared to the wild-type promoter.

In general, the alteration of this putative MIG1 binding site in the *KlMAL21-KlMAL22* promoter caused a drop in expression from both orientations of the promoter

when grown in glycerol. The proximity of this site to the identified *KlUAS$_{MAL}$* putative site 1 may affect the binding properties of positive acting regulatory elements.

3.3.3 Altered Promoter, pREX-IC-ΔPos

A portion of the *KlMAL21-KlMAL22* intergenic region from 411 to 441 bp upstream of the *KlMAL22* gene was deleted (Figure 4). This 30 bp region corresponds to site 4 of the *ScUAS$_{MAL}$* (Yao *et al.*, 1994). The four binding sites identified in the *ScMAL61-ScMAL62* intergenic region constitute a binding site for the *trans*-acting ScMAL63 positive activator. These sites, however, do not contribute equally to the induction of the *ScMAL61* or *ScMAL62* genes (Hong *et al.*, 1987; Yao *et al.*, 1994). For example, a deletion of site 1, 2 or 3 alone did not affect induction of the *ScMAL61* or *ScMAL62* genes (Hong *et al.*, 1987; Yao *et al.*, 1994). Removal of sites 3 and 4 together caused not only a reduction in the inducibility of both genes by maltose, but also a reduction in *ScMAL61* expression to only 28% of wild-type expression levels and a reduction in *ScMAL62* expression to 53% of wild-type expression (Yao *et al.*, 1994). Similarly, in the current study, a reduction to less than 20% of wild-type *KlMAL21* promoter activity and a reduction to less than 45% of wild-type *KlMAL22* promoter activity was observed after 72 hours when site 4 of the *KlUAS$_{MAL}$* was removed (Table 5).

Deletion of this region severely affected the activity of the promoter in both orientations during growth on glycerol. When grown in glycerol, YFP expression was reduced to less than one quarter of wild-type promoter levels, for all sampling times (Table 5 and Figure 11). CFP expression was reduced by more than half of its wild-type promoter levels (Table 5 and Figure 10). When grown in glucose, CFP expression from the pREX-IC-ΔPos promoter was not noticeably reduced when compared to wild-type levels until

after 96 hours, when fluorescence expressed by the wild-type *KlMAL22* promoter saw a tripling of its 72 hour levels while, pREX-IC-ΔPos levels remained unchanged from its 72 hour glucose expression levels (Table 5 and Figure 10).

YFP expression from the pREX-IC-ΔPos promoter was essentially repressed in galactose and neither of the altered promoter constructs relieved galactose repression of the *KlMAL21* promoter (Table 5 and Figure 11). CFP levels in galactose after 96 hours were double expression levels after 48 hours. However, expression generated by the pREX-IC-ΔPos plasmid was still less than half of the CFP levels expressed by the native promoter after 96 hours (Table 5 and Figure 10).

Summary and Conclusions

Several native and foreign promoters have been used to drive heterologous protein production in *K. lactis* (van den Berg *et al.*, 1990; Fleer *et al.*, 1991; Bui *et al.*, 1996; Hsieh *et al.*, 1998; Morlino *et al.*, 1999; Bergquist *et al.*, 2002; Panuwatsuk *et al.*, 2002). Promoter strength is a cornerstone of any successful heterologous protein expression system. In cooperation with an appropriate expression host, a strong promoter can be used to produce large amounts of high-quality recombinant proteins. The *K. lactis KlMAL21-KlMAL22* intergenic region is an active promoter and regulatory region that has shown promise as the driving force behind a protein expression system. Endogenous promoters in general outperform promoters from other organisms. It is therefore important to develop a variety of regulated promoters to be matched with specific protein production systems or growth regimes. Alternate carbon sources can then be utilized to regulate the timing of the expression of high value proteins.

The *KlMAL21-KlMAL22* was in many respects typical of other highly expressed fungal promoters. The *KlMAL21-KlMAL22* bi-directional promoter contained TATA-like sequences at 108 bp upstream of the *KlMAL22* gene and 57 bp upstream of the *KlMAL21* coding region. Transcription initiation sites matching the TC(G/A)A consensus sequence were identified at 6 and 31 bp upstream of the *KlMAL22* coding region and 26 bp upstream of the *KlMAL21* gene. Many putative transcription factor binding sites were identified within the promoter including sites for ABF1, HAP2/3/4, RAP1, GCR1, GCN4, NIT2 and of particular interest MIG1. A region from 640 to 415 bp upstream of the *KlMAL22* gene

included four regions homologous to the *S. cerevisiae* UAS_{MAL} providing a unique target for the development of promoter variants.

To test the promoter variants, the *KlMAL21* and *KlMAL22* genes flanking the intergenic region were replaced with two colour variants of the green fluorescent protein (GFP). *KlMAL21* was replaced with the gene encoding yellow fluorescent protein (YFP) and *KlMAL22* was replaced with the gene encoding cyan fluorescent protein (CFP). To establish a baseline for carbon catabolite regulated expression from the *KlMAL21-KlMAL22* promoter, expression of each fluorescent protein from the native promoter was analyzed during growth of *K. lactis* on the sole carbon sources of glucose, glycerol or galactose. Expression of both CFP and YFP was repressed throughout fermentation when grown on galactose and during the early phases of fermentation when grown on glucose. Late in culturing, however, cells growing on glucose did start to express both CFP and YFP as the glucose resource was presumably exhausted and the promoter was no longer repressed. During growth on glycerol expression of CFP and YFP from the wild-type promoter was 10 times higher than during growth on glucose.

A putative MIG1 repressor protein binding site at 671 to 657 bp upstream of the *KlMAL22* translation start site was altered by PCR mediated mutagenesis so that it no longer resembled the MIG1 consensus sequence. It was expected that deletion of this putative binding site would relieve repression of the promoter during growth on glucose. Alteration of this site, however, did not alleviate glucose repression in either orientation. It did cause a decrease in CFP and YFP expression during growth on glycerol and glucose. It is possible that one or several of the other putative MIG1 binding sites identified regulate the *KlMAL21-KlMAL22* promoter during growth on glucose. It is important to identify

which DNA elements within the promoter are responsible for repression during growth on glucose when developing an efficient heterologous protein expression system. In many cases the most inexpensive and richest media for yeast growth is undefined and would most likely contain glucose. *Kluyveromyces lactis* grows more quickly on glucose than on glycerol media. Therefore if glucose is used as a carbon source during heterologous protein expression maximum biomass is achieved earlier, reducing necessary fermentation time and ultimately increasing the overall efficiency of the expression system.

Deletion of a region that showed sequence homology to the *S. cerevisiae* UAS_{MAL} caused a dramatic decrease in expression of both YFP and CFP during growth in glycerol. In *S. cerevisiae*, the *ScMAL61-ScMAL62* promoter is regulated by the *trans*-activator ScMAL63. It is unknown whether a homolog of ScMAL63 exists in *K. lactis*, however, the results of this study show that the region from 411 to 441 bp upstream of the *KlMAL22* translation start site is important for the induction of expression from both the *KlMAL21* and *KlMAL22* promoters. It is necessary to identify positive regulator elements within the promoter so that these sequences can be avoided when modifying the promoter to remove negative regulatory elements. This region could also be used to "fish out" and identify binding proteins that regulate protein expression.

During regular growth on the preferred carbon source, i.e. glucose, the expression of genes that utilize alternate carbon sources is efficiently shut down. This was true for the regulation of the maltase and maltose permease promoter. Glucose repressed activity of the *KlMAL21-KlMAL22* promoters and this repression was relieved only when glucose was presumably exhausted from the media. Glycerol did not repress these promoters whereas galactose completely repressed these promoters. Most heterologous protein expression in

K. lactis has been achieved using the *KlLAC4* promoter which is induced in the presence of galactose (Leonardo *et al.*, 1987; Godecke *et al.*, 1991). The *KlMAL2* promoter now offers an alternative expression system whereby expression can now be down-regulated by galactose. The expression of heterologous proteins from the *KlMAL2* bi-directional promoter can be regulated by the judicious selection of carbon source.

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Appendix I: Future Studies

This study presents an initial framework for the regulation of the maltase / maltose permease pathway in *K. lactis* and provides a powerful tool for the study of protein expression from bi-directional promoter regions. Much more exploration into the regulation of this interesting system is necessary to understand exactly how maltose utilization is governed when primary carbon sources are available or unavailable to the cell. As well, the data presented in these experiments suggest that a galactose repression system may be at work in *K. lactis*. It is not unimaginable that *K. lactis* growing on lactose is well adapted to use both galactose and glucose as primary carbon sources and has evolved to repress alternative carbon utilization pathways when either of those two sugars are present.

There are many remaining questions as to how the *KlMAL21-KlMAL22* intergenic region regulates the activity of *KlMAL21* and *KlMAL22* gene expression. While these experiments have explored two small regions of the promoter by deletion analysis, much more remains to be studied. A linker scanning mutagenesis experiment of the entire intergenic region would be useful in identifying important regulatory regions. Once regions that affected protein expression had been identified those regions could be altered to de-repress the promoters and enhance protein expression.

A homolog of the ScMAL63 positive activator protein needs to be found, or confirmed to be absent from *K. lactis*. Amplification of a *KlMALx3* gene was attempted using degenerate PCR primers designed from the *ScMALx3* and *ScMALx4* genes but was not successful. Though a homologous *KlMALx3* gene has not yet been identified in *K. lactis* this does not mean it does not exist. The arrangement or location of the gene may be

different than that of *S. cerevisiae*. In *S. cerevisiae*, the *ScMAL63* gene, or in general the *ScMALx3* gene, is located upstream of the *ScMAL61* gene. In *K. lactis*, a gene encoding a repressible acid phosphatase is located just upstream and in the opposite orientation of the *KlMAL21* gene (Ferminan *et al.*, 1997). A region of approximately 750 bp separates the ends of the two genes. The presence of the *KlPHO5* gene means that if a *K. lactis* positive activator homolog exists, the organization of the *KlMAL2* locus is different from that of the *ScMAL* loci. It remains a possibility that no positive activator exists for the *K. lactis* maltase regulon. Preliminary experiments using the fluorescent protein system have suggested that expression during growth on maltose is not induced in *K. lactis* and that expression levels are lower when grown on maltose, than when compared to growth on glycerol. However, this must be pursued further. If a positive activator does exist, one orientation of the promoter could be used to drive expression of the activator, while the other orientation could be used to express a protein of interest. This construct would essentially be self-activating and would allow maximal production of protein under the proper inducing or de-repressing conditions.

Another use for this promoter construct is to use one orientation of the promoter to drive expression of a heterologous protein of interest and use the other orientation to express a fluorescent protein. An expression profile could then be generated for each protein during fermentation and the levels of fluorescent protein could be correlated with the levels of the other protein of interest. Subsequently, any batch culture could be sampled and analyzed for fluorescence during its growth phase. An in-line fluorescence monitor could be used during fermentation and would allow the precise determination of the time of peak expression without invasive sampling.