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Galactose Metabolism in *Saccharomyces cerevisiae*

David J. Timson

School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast, BT9 7BL, UK

Correspondence: **d.timson@qub.ac.uk**

ABSTRACT

Galactose is metabolised to the more metabolically useful glucose 6-phosphate by the enzymes of the Leloir pathway. This pathway is necessary as the initial enzymes of glycolysis are unable to recognise galactose. In most organisms, including *Saccharomyces cerevisiae*, five enzymes are required to catalyse the conversion: galactose mutarotase, galactokinase, galactose 1-phosphate uridyltransferase, UDP-galactose 4-epimerase and phosphoglucomutase. The pathway has attracted interest in *S. cerevisiae* as it is under very strict genetic control and thus provides an excellent model for the study of gene expression in eukaryotes. In the presence of glucose the genes encoding the Leloir pathway enzymes (the *GAL* genes) are completely repressed through the action of a transcription factor Mig1p. Only in the presence of galactose and the absence of glucose do the concerted actions of Gal4p, Gal80p and Gal3p enable the rapid and high level activation of the *GAL* genes. The exact mechanism of action of these three proteins is controversial. Galactose metabolism in *S. cerevisiae* is also of interest because it can be exploited both in the laboratory (for high level expression of heterologous proteins and in the yeast two hybrid screen) and industrially (increasing flux through the Leloir pathway in order to make more efficient use of feedstocks with high galactose content). Recent work on the structures of the various proteins, their mechanisms of action and attempts to gain an integrated understanding of transcriptional and metabolic events will assist our understanding of both the fundamental biochemical processes and how these might be exploited commercially.

Keywords: galactokinase, galactose mutarotase, galactose 1-phosphate uridyltransferase, *GAL* genes, Leloir pathway, UDP-galactose 4-epimerase

CONTENTS

INTRODUCTION

The central pathways of carbohydrate metabolism have evolved to process the hexose monosaccharide glucose. Many of the enzymes of the glycolytic pathway are so specific for glucose that other sugars, even other hexoses, are not processed at any appreciable rate. To overcome this problem, there are number of short pathways which convert other common sugars (e.g. galactose and fructose) into glycolytic intermediates. Galactose is metabolised by the enzymes of the Leloir pathway (Frey 1996). This pathway, which was named after the Nobel Prize-winning Argentinean biochemist Louis Leloir (Cabib 1970), requires five enzymes to convert galactose to glucose 6-phosphate (**Fig. 1; Table 1**). In mammals, mutations in some of these enzymes can result in the genetic disease galactosemia (Leslie 2003; Holden *et al*. 2004; Timson 2006). In higher plants, enzymes from the pathway are required for the synthesis of galactose containing components of the cell wall (Dormann and Benning 1998; Seifert *et al*. 2002; Barber *et al,* 2006)

In the budding yeast *Saccharomyces cerevisiae*, these five enzyme activities are provided by five proteins – Gal1p, Gal7p, Gal10p Pgm1p and Pgm2p. Gal1p is a galactokinase and catalyses the stereospecific phosphorylation of α-D-galactose to give α-D-galactose 1-phosphate (Howard and Heinrich 1965; Schell and Wilson 1977). This compound reacts with UDP-glucose to give D-glucose 1-phosphate and UDP-galactose in a reaction catalysed by galactose 1-phosphate uridyltransferase, Gal7p (Segawa and Fukasawa 1979). UDP-glucose is regenerated from UDP-galactose by the action of UDP-galactose 4-epimerase which is encoded by Gal10p (Fukasawa *et al*. 1980). This protein also en-

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Review

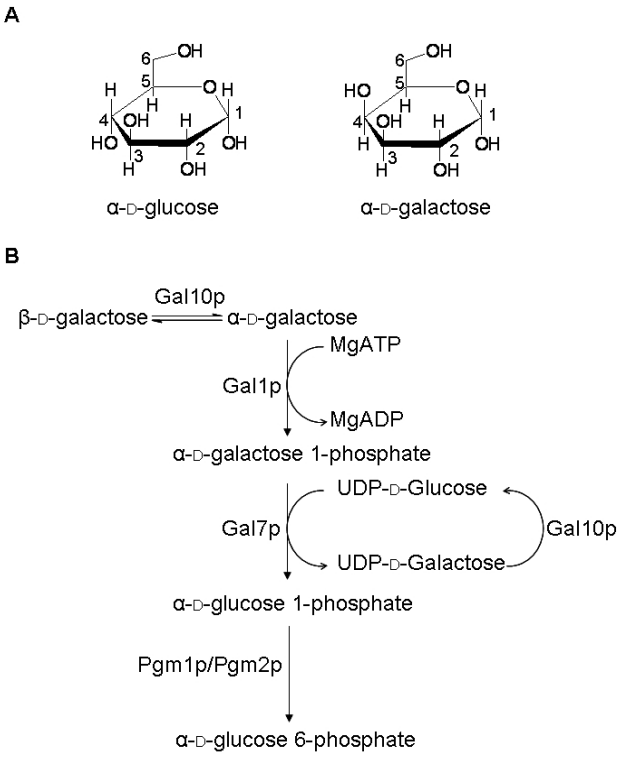

Fig. 1 (**A**) The pyranose ring structures of D-glucose and D-galactose shown as Haworth projections. Note that these compounds differ only in the configuration about carbon-4. Both sugars are shown as the α-anomers. The β-anomers are identical, except that the configuration at carbon-1 is reversed. The numbers around the rings refer to the convention for numbering the carbon atoms. Note that, in the pyranose ring forms, carbons 1 through 5 are chiral centres. (**B**) The Leloir pathway of galactose metabolism. The names of the yeast proteins which catalyse the various reactions are given.

codes galactose mutarotase activity which catalyses the attainment of equilibrium between α- and β-galactose (Majumdar *et al*. 2004). This dual activity is an oddity of *S. cerevisiae* and some other yeast species. In both bacteria and higher eukaryotes, the two enzyme activities are provided by two separate proteins. The final stage in the pathway is the isomerisation of glucose 1-phosphate to glucose 6-phosphate catalysed by phosphoglucomutase.

In *S. cerevisae* there are two phosphoglucomutase isoforms, Pgm1p and Pgm2p. About 80% of the total activity is provided by Pgm2p (Tsoi and Douglas 1964). Phosphoglucomutase is not exclusive to the Leloir pathway; it also plays a role in glycogen metabolism.

Over the years the metabolism of galactose in yeast has attracted considerable attention. It is not just the metabolic pathway itself which has been of interest. The control of the expression of the genes encoding the enzymes of the Leloir pathway (the *GAL* genes) was one of the first eukaryotic gene expression systems to be studied in any detail. Indeed, to this day, this still represents a genetically and biochemically amenable system for elucidating mechanisms of gene expression that extend far beyond the regulation of sugar metabolism in a single celled organism. In addition, in recent years, the organism has been exploited as a model system to study the effects of disease-causing mutations in the Leloir pathway enzymes.

CONTROL OF *GAL* GENE EXPRESSION

S. cerevisiae's preferred carbon and energy source is glucose. If this sugar is present, even at low levels compared to alternatives, it will be metabolised exclusively. The protein Mig1p is responsible for repressing the expression of the *GAL* genes (and others) in the presence of glucose. Two other transcription factors, Mig2p and Nrg1p may be able to substitute partially for Mig1p (Lutfiyya *et al*. 1998; Wu and Trumbly 1998; Zhou and Winston 2001). However, the role of these proteins compared to Mig1p appears to be minor in wild type yeast. Mig1p does not, directly, bind to glucose. Indeed the precise mechanism for sensing the presence of glucose is not known. Mig1p is a two cysteine/two histidine zinc finger DNA binding protein which binds at many sites in the genome, including upstream from the *GAL* genes. These sites are close to, or in some cases overlap with, the binding sites for a transcriptional activator, Gal4p (Frolova *et al*. 1999). Gal4p binds specifically at several sites in the yeast genome, all upstream from the various *GAL* genes. One hypothesis to explain the action of Mig1p is that the binding of Mig1p and Gal4p at these sites is mutually exclusive, possibly due to steric hindrance (Nehlin *et al*. 1991). However, recent work suggests that Mig1p can remain bound upstream of *GAL* genes, even when those genes are transcriptionally active (Papamichos-Chronakis *et al*. 2004).

Table 1 Genes and proteins important in galactose metabolism in *S. cerevisiae*. * Not an enzyme.

Protein	Function	Gene (common aliases)[a]	Systematic gene name	Enzyme commission (EC) number	Protein databank accession number
Gal1p	Galactokinase. Can also substitute for Gal3p	*GAL1*	YBR020W	2.7.1.6	2AJ4
Gal2p	Galactose permease	*GAL2*	YLR081W	*	
Gal3p	Ligand sensor in the *GAL* genetic switch. No kinase activity	*GAL3*	YDR009W	*	
Gal4p	Transcription factor (activator) in the *GAL* genetic switch	*GAL4 (GAL81)*	YPL248C	*	1D66 (DNA binding domain plus DNA); 1AW6 (DNA binding domain); 1HBW (Dimerisation domain)
Lap3p	Aminopeptidase; Bleomycin hydrolase	*LAP3 (GAL6)*	YNL239W	3.4.22.40	1GCB
Gal7p	Galactose 1-phosphate uridyltransferase	*GAL7*	YBR018C	2.7.7.12	
Gal10p	Galactose mutarotase and UDP-galactose 4-epimerase	*GAL10*	YBR019C	5.1.3.2 and 5.1.3.3.	1Z45
Gal80p	Transcription factor (repressor) in the *GAL* genetic switch	*GAL80*	YML051W	*	2NVW[b]
Mig1p	Transcription factor which represses numerous systems including the *GAL* genes in the presence of glucose	*MIG1*	YGL035C	*	
Pgm1p	Phosphoglucomutase (minor isoform)	*PGM1*	YKL127W	5.4.2.2	
Pgm2p	Phosphoglucomutase (major isoform)	*PGM2 (GAL5)*	YMR105C	5.4.2.2	
Cyc8p	Transcriptional co-repressor; acts in complex with Tup1p	*CYC8 (SSN6)*	YBR112C	*	
Tup1p	General transcriptional repressor; acts with Cyc8p	*TUP1*	YCR084C	*	1ERJ (WD40 domain)
Snf1p	Protein kinase, phosphorylates Mig1p	*SNF1*	YDR477W	2.7.11.1	2EUE (kinase domain); 2FH9 (kinase domain dimer)

Mig1p also represses the transcription of the *GAL* genes by interacting with the general transcriptional co-repressor complex Ssn6p-Tup1p (Keleher *et al*. 1992; Treitel and Carlson 1995). This complex recruits the histone deacetylases Hda1p, Rpd3p, Hos1p and Hos2p (Wu *et al*. 2001; Davie *et al*. 2003; Malave and Dent 2006) which maintain the chromatin in its deacetylated, compact, transcriptionally inert form. Furthermore, the localisation and phosphorylation state of Mig1p is dependant upon the concentration of glucose within the cell. In high glucose concentrations, the protein is dephosphorylated and located within the nucleus. When glucose concentrations fall, the AMP-activated protein kinase Snf1p phosphorylates Mig1p (at serines 108, 278 and 311) (Ostling and Ronne 1998; Treitel *et al*. 1998), an event which causes dissociation from the Ssn6p-Tup1p complex (Papamichos-Chronakis *et al*. 2004) and transport out of the nucleus into the cytoplasm (De Vit *et al*. 1997). Currently, it is unclear if there is a protein which measures, directly, the concentration of glucose in the yeast cell. Although there are several proteins (e.g. hexose transporters, glycolytic enzymes) which interact with this sugar none have been shown to have a role in signalling to the Mig1p-mediated system. It is possible that the cell detects glucose levels indirectly, for example through the ratio of the concentrations of ATP and ADP (or AMP). In this light it may be important that the Snf1p kinase is structurally and functionally related to mammalian AMP-dependent kinases (Woods *et al*. 1994). Although mammalian AMP-dependent kinase is allosterically activated by micromolar concentrations of AMP (Ferrer *et al*. 1985), purified Snf1p appears not to be (Wilson *et al*. 1996). However, *in vivo*, the system responds to the AMP:ATP ratio and it is assumed that this is sensed by either an upstream kinase or one of the regulatory subunits associated with Snf1p (Wilson *et al*. 1996). In mammals the γ-subunit of the AMP-dependent kinase complex confers AMP sensitivity. The yeast homologue is Snf4p. However, key residues in the mammalian γ-subunit are not present in Snf4p and when a histidine residue in the mammalian protein was changed to glycine (the equivalent residue in Snf4p), AMP sensitivity was lost (Adams *et al*. 2004). This shows that there are clear differences between the yeast and mammalian systems, despite both being involved in regulating the cell's response to glucose concentrations.

The absence of glucose is necessary, but not sufficient, for the induction of galactose metabolising enzymes. The presence of galactose is also required and this is sensed and responded to by a genetic switch containing three main components: Gal4p (a transcriptional activator), Gal80p (a transcriptional repressor) and Gal3p which is believed to act as a direct sensor of cellular galactose concentration. Gal4p binds to conserved sequences upstream of the *GAL* genes known as the upstream activating sequences (UAS_{GAL}). The protein is a dimer and each monomer can be divided into three main regions – an N-terminal two-Zn^{2+} six cysteine DNA binding and dimerisation domain, a central section of unknown function and a C-terminal transcriptional activation domain. The N- and C-termini can be fused together to produce a protein which is an active transcriptional activator both *in vitro* and *in vivo* (Ding and Johnston 1997). The structures of the DNA binding and dimerisation regions are known (**Fig. 2**), but the rest of the protein has yet to be characterised structurally (Marmorstein *et al*. 1992; Hidalgo *et al*. 2001). Indeed it is likely that the activation domain, which contains a high proportion of negatively charged residues, may be essentially unstructured in the absence of a binding partner (Ansari *et al*. 1998). Such unstructured regions are common in biological switches. Specificity requires that numerous contacts are made between proteins involved in a switch. Generally speaking this will have a favourable enthalpy change (ΔH highly negative). When unstructured polypeptide chains bind to their targets, they suffer a considerable loss of conformational freedom which is reflected in a large decrease in entropy (ΔS). This energetically unfavourable consequence of binding largely balances out the enthalpy change resulting in an overall free energy change (ΔG) close to zero and, therefore, an equilibrium constant close to one. Thus the presence of an unstructured region in the binding site between two proteins can result in a highly specific, but readily reversible interaction (Dyson and Wright 2002). This reversibility can be critical in achieving sensitivity to changing concentrations of metabolites and, critically, enables to system to be easily switched off when it is no longer required.

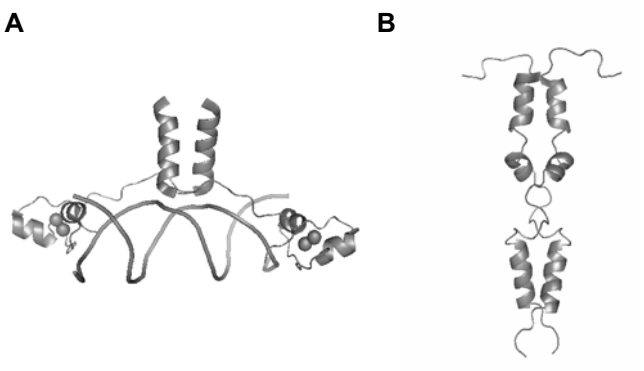

Fig. 2 The structures of (**A**) the DNA-binding domain bound to DNA as a dimer and (**B**) the dimerisation domain of Gal4p visualised using the program PyMol (http://www.pymol.org). The spheres in (**A**) represent Zn^{2+} ions. These figures are derived from the PDB entries 1D66 and 1HBW (Marmorstein *et al.* 1992; Hidalgo *et al.* 2001).

Gal4p has a number of targets. When the *GAL* genetic switch is on, it interacts through its C-terminal domain, with the general transcription factors Gal11p and TFIID subunits (Sua7p, Taf4p, Taf6p, Taf12p) along with subunits of the chromatin remodelling SAGA complex (Ada2p, Tra1p, Taf4p, Taf6p, Taf12p; note that these last three polypeptides are considered to be part of both TFIID and SAGA) and possibly TATA-binding protein (TBP, Spt15p) (Wu *et al*. 1996; Xie *et al*. 2000; Hidalgo *et al*. 2001; Jeong *et al*. 2001; Larschan and Winston 2001; Klein *et al*. 2003; Bhaumik *et al*. 2004). The exact binding partners and the order in which they bind remain to be determined. Thus its mechanism of action as a transcriptional activator is two-fold: it recruits proteins which will relax the structure of the chromatin and it begins the process of assembly of an active transcription complex upstream of each of the *GAL* genes. The end result of these two events is the recruitment of RNA polymerase II, which is able to gain access to the relaxed chromatin and transcribe the genes. Gal4p is only fully activated when phsophorylated at serines 691, 696 and 699. Of these, serine 699 appears to be the most important (Sadowski *et al*. 1996).

In the repressed state, Gal4p interacts with Gal80p again through the C-terminal domain. Indeed the interactions with either Gal80p or general transcription factors are likely to be mutually exclusive. Interaction with Gal80p completely represses Gal4p's transcriptional activity. The structure of Gal80p from *S. cerevisiae* has not yet been solved. However, the protein from the very closely related yeast *Kluyveromyces lactis* has been solved recently (Thoden *et al*. 2007). This structure (**Fig. 3**) reveals that, surprisingly, the protein has high structural similarity to glucose-fructose oxidoreductase. However, Gal80p does not appear to have any $NAD(P)^+$-binding or dehydrogenase activity. Although the protein does contain a region which conforms to the overall Rossmann fold, there are a number of insertions and substitutions in the amino acid sequence which partly occupy the space where the nucleotide would bind (Thoden *et al*. 2007). Whether any other small molecules bind at this site remains to be determined. The oligomeric state of Gal80p *in vivo* and how this is controlled is uncertain. Since Gal4p binds to DNA as a dimer, it seems reasonable that at least one Gal80p molecule will interact with each Gal4p monomer. Furthermore, Gal80p can be cross-linked *in vitro* to form

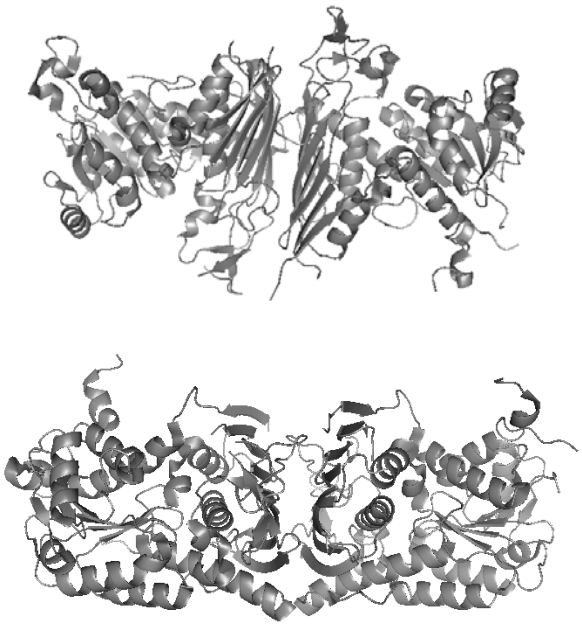

Fig. 3 The structure of the *K. lactis* Gal80p dimer derived from PDB entry 2NVW (Thoden *et al.* 2007). The two views are related by an approximate 90° rotation about a horizontal axis. Note the extensive interface between the two monomers which is composed almost entirely of β-sheet.

dimers and dimers could also be detected in a modified yeast two hybrid assay (Melcher and Xu 2001). Native gel shift assays suggested a very high affinity interaction (K_d < 10^{-9} M) but a short half life (<1 min). These dimers are likely to remain intact when Gal80p interacts with Gal4p resulting in a Gal4p:Gal80p stoichiometry of 2:2. Indeed, Gal80p-Gal4p interaction appears to increase the half life of the Gal80p homodimer and increase the affinity of Gal4p for DNA. Transient associations between Gal80p dimers were also observed and these higher order oligomers may play a role in strongly repressed *GAL* promoters where there are more than one UAS_{GAL} (Melcher and Xu 2001). The transient nature of isolated Gal80p dimers was also observed in gel filtration experiments. The protein either eluted as a monomer (Yun *et al*. 1991) or failed to elute as a discrete peak (Timson *et al*. 2002). The homologous protein from *K. lactis* also failed to elute as a discrete peak, unless millimolar concentrations of either citrate or EDTA were added. Under these conditions, the protein behaved like a dimer (Anders *et al*. 2006). Interestingly, the experiments of Melcher and Xu were carried out in the presence of EDTA which may explain why dimers were so readily observed in their experiments (Melcher and Xu 2001). The crystal structure of *K. lactis* Gal80p reveals a large interface (4400 Å^2) between the two monomers in the homodimer (Thoden *et al*. 2007). This presumably accounts for the very high affinity observed *in vitro*. Since, by definition, the structure seen in the crystals must be a long lived one, it is likely that the structure represents a situation similar to that seen when Gal80p dimers bind to Gal4p even though no citrate or EDTA was included in the crystallisation solutions. Presumably the process of crystallisation "locks" the protein into this conformation.

The repressive action of Gal80p is relieved by Gal3p. This relief of repression requires two small molecules – galactose and ATP – which are also the substrates for the Leloir pathway enzyme galactokinase. It is believed (although it has never been formally proven) that Gal3p interacts directly with both these ligands and that this interaction results in a conformational change which enables Gal3p to interact with Gal80p (Yano and Fukasawa 1997). Gal3p has a high level of sequence similarity to the yeast galactokinase, Gal1p, and this protein can also substitute for Gal3p in the *GAL* genetic switch (Bhat and Hopper 1992). In *K. lactis* there is only one protein, Gal1p, which carries out both functions (Meyer *et al*. 1991). *S. cerevisiae* Gal3p has no galactokinase activity (Platt *et al*. 2000). The structure of Gal3p is not yet known, but homology models based on the structure of Gal1p (see below) have been built (Thoden *et al*. 2005).

Although it is well established that a galactose and ATP-dependent interaction between Gal80p and Gal3p is responsible for the activation of the *GAL* genetic switch, the cellular location of this interaction is controversial. Initially, it was believed that the interaction occurred within the nucleus and this hypothesis was supported by the detection of a quaternary DNA-Gal4p-Gal80p-Gal3p complex by native gel electrophoresis (Platt and Reece 1998). This evidence is consistent with a model in which, in the repressed state Gal4p and Gal80p are in a DNA-bound complex. In the presence of the activating ligands, Gal3p adds to this complex and, through a series of conformational changes, relieves Gal80p's repression of Gal4p. In a variant of this model Gal3p causes the complete dissociation of Gal80p from Gal4p in the presence of galactose and ATP. It should be noted that if a multi-protein complex does form on the DNA it is quite likely that, in the transcriptionally active form, the Gal4p-Gal80p interaction is likely to be weakened and this might result in dissociation in some *in vitro* experiments. This is especially the case in protocols such as GST pull downs or co-immunoprecipitation where the procedure requires the isolation of a complex at equilibrium. However, subsequent experiments suggested that Gal3p was excluded from the nucleus in both the presence and absence of galactose. In contrast Gal80p was shown to be located in both the nucleus and the cytoplasm (Peng and Hopper 2000; Peng and Hopper 2002). The absence of a Gal80p-Gal4p interaction *in vivo* under inducing conditions was also suggested by chromatin immunoprecipitation (ChIP) experiments (Peng and Hopper 2002). This lead to a model being proposed in which Gal80p is able to move freely between the nucleus and the cytoplasm. In the presence of galactose and ATP it binds to Gal3p in the cytoplasm and becomes trapped in this compartment. Once it is trapped in the cytoplasm it is unable to interact with Gal4p in the nucleus and thus transcription can occur (Peng and Hopper 2002). Recent experiments have tended to point back towards a model where key changes take place in the nucleus and not the cytoplasm. Fluorescence resonance energy transfer has been observed *in vivo* between Gal4p and Gal80p labelled with different variants of green fluorescent protein (GFP), regardless of whether galactose is present, or not (Bhaumik *et al*. 2004). Since this effect is only observed over the Förster distance (typically 5-6 nm), the two proteins must remain bound together for it to occur. This suggests that galactose does not cause complete Gal4p-Gal80p dissociation and that at least some Gal3p must be in the nucleus in order to transduce the signal.

The availability of partial structures for Gal4p, the *K. lactis* Gal80p structure and homology models for Gal3p, means that we are moving towards being able to describe the molecular details of the *GAL* genetic switch. However, to do this it will be necessary to resolve some of the uncertainties described here.

TRANSPORT OF GALACTOSE INTO THE YEAST CELL

In order for galactose to be metabolised by *S. cerevisiae* it must first be transported into the cell. Yeast cells have at least 19 hexose transporters in their membranes – Hxt1p to Hxt17p, Mal11p and Gal2p (Ozcan and Johnston 1999; Wieczorke *et al*. 1999). All of these are integral membrane proteins. They all act as channels facilitating diffusion of hexoses down a concentration gradient rather than pumps which use energy to drive molecules across the membrane. However not all the hexose transporters are expressed under the same conditions. Some (e.g. Hxt1p) are expressed in conditions of high glucose concentrations whereas others (e.g. Hxt2p and Hxt4p) are expressed at lower concentra-

tions of the sugar. Some of the sequences may be pseudogenes. Those expressed in high glucose concentrations tend to have lower affinities for glucose than those expressed in low glucose concentrations. This enables the yeast cell to grow in a wide range of different glucose concentrations (micromolar to molar) while still taking up appropriate concentrations of the sugar into the cell (Ozcan and Johnston 1995; Ozcan and Johnston 1999). Mal11p is high affinity maltose transporter, which is induced in the presence of maltose (Cheng and Michels 1991). Gal2p is one of the *GAL* genes and is a high affinity galactose transporter (Tschopp *et al*. 1986; Huibregtse *et al*. 1993). It is expressed in the presence of galactose and repressed (through the Mig1p system described above) in the presence of glucose. In addition to these mechanisms, the protein is rapidly ubiquitinated, endocytosed and degraded if the yeast cells are shifted from an environment rich in galactose to one where glucose predominates (Horak and Wolf 1997). However, it is not highly specific for galactose and can also transport glucose (Reifenberger *et al*. 1997; Maier *et al*. 2002). Furthermore, it is likely that when the organism is growing in galactose, the sugar is transported into the cell by both Gal2p and the less selective hexose transporters of the Hxt family.

Gal2p is predicted to have 11 transmembrane segments and the tenth segment contains two aromatic residues (Tyr446 and Trp455) responsible for discriminating in favour of galactose (Nishizawa *et al*. 1995; Kasahara and Maeda 1998). Replacement of Tyr446 with a phenylalanine residue reduced the transport of galactose to less than 20% of wild type levels, but increased the transport of glucose (Kasahara and Maeda 1998). In addition to these two residues in the tenth putative transmembrane segment, Tyr352 and Phe504 (in segments 7 and twelve respectively) must be bulky hydrophobic residues in order for the protein to function as a galactose transporter (Kasahara and Kasahara 2000a). Double site-directed mutagenesis experiments suggest that these two residues interact with each other (Kasahara and Kasahara 2000b). As yet, no residues have been unequivocally identified as interacting directly with galactose. The results of the various site-directed mutagenesis experiments targeting hydrophobic residues could infer either that these residues interact with the sugar, or that they help create a structural environment for the interaction of other residues with galactose. Ideally, this question would be answered by solving the structure of Gal2p in complex with galactose. Of course, the solution of structures of integral membrane proteins is difficult and even if this was achieved it would only show a "snap-shot" of the channel in action as presumably the sugar must interact with a number of different residues as it passes through the protein.

THE ENZYMES OF GALACTOSE METABOLISM IN YEAST

Galactose mutarotase (Gal10p)

Hexose sugars in the six-membered, pyranose ring configuration have five chiral centres at carbons 1 to 5 (**Fig. 1A**). The chiral centres at carbons 2 to 4 determine the identity of the sugar. For example, the two possible stereoisomers at carbon 4 are glucose and galactose. Carbon 5 determines whether the sugar is a D- or a L-hexose and carbon 1 (which is only a chiral centre in the ring form) determines whether the molecule is the α- or β-anomer. Unlike the other chiral centres the α- and β-anomers can be interconverted, through the straight chain form of the sugar, in aqueous solution. This reaction is catalysed by both acids and bases and the equilibrium constant is usually close to unity (ie there is not much energetic difference between the two configurations). Thus, there are four possible pyranose configurations of galactose – α-L, β-L, α-D and β-D. In common with most other monosaccharides, only the D-form is usually found in living systems. Furthermore some enzymes, such as galactokinase, are highly specific for one configuration (the α-D form in the case of galactokinase). Although the α- and β-anomers inter-convert at a measurable rate in aqueous solution, this rate does not appear to be great enough to supply the rest of the Leloir pathway with sufficient α-D-galactose and, for this reason, most organisms express a galactose mutarotase (aldose 1-epimerase) to catalyse this interconversion or mutarotation. In the case of *S. cerevisiae*, this enzyme activity is encoded by the C-terminal half of Gal10p.

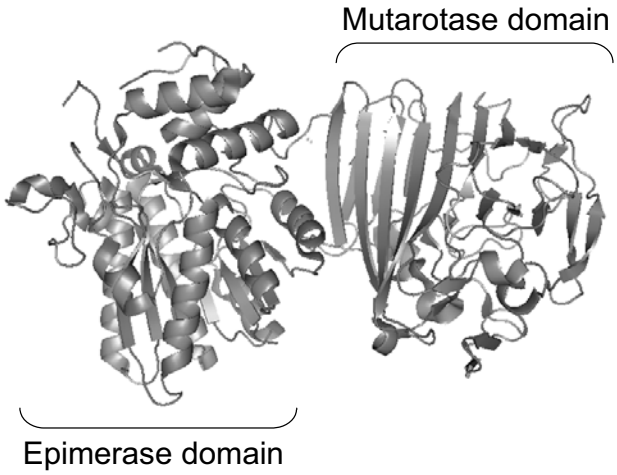

Fig. 4 The structure of *S. cerevisiae* Gal10p showing the mutarotase and epimerase domains (PDB entry 1Z45 (Thoden and Holden 2005)). The protein forms a stable homodimer through contacts which are entirely located in the epimerase domain.

In common with galactose mutarotase enzymes purified from other species, the yeast enzyme is characterised by a high value of the specificity constant, k_{cat}/K_m, when galactose is the substrate (68,000 $l.mol^{-1}.s^{-1}$) (Scott and Timson 2007). It appears to be more selective for galactose over glucose than the enzyme from some other species: the ratio of the specificity constants for the yeast enzyme is approximately 90 compared to 4 for the human enzyme (Timson and Reece 2003). Given that the enzyme is only expressed in the presence of galactose and the absence of glucose, it seems unlikely that it plays are major role in maintaining the anomeric equilibrium of glucose, or any other monosaccharide. Indeed there are at least two other mutarotase-like sequences in the yeast genome (YHR210c and YNR071c) (Goffeau *et al*. 1996). Of these, YHR210c is more likely to be involved in catalysing anomeric interconversions as YNR071c lacks a critical residue in the active site. Thus it is YHR210c which is most likely to be account for the mutarotase activity observed in extracts from yeast cells grown in 2% (w/v) glucose (Brahma and Bhattacharyya 2004).

The overall structure of the galactose mutarotase domain of Gal10p is very similar to that of other mutarotases being largely composed of β-sheets with only a small proportion of α-helix (**Fig. 4**) (Thoden and Holden 2005). The active sites of galactose mutarotases also show a high degree of structural similarity and they are all believed to share the same mechanism of action. No co-factors are required to bring about catalysis. Instead, the reaction is initiated by the abstraction of a proton from the hydroxyl group attached to carbon-1 of the sugar by a glutamate residue acting as an active site Brønstead base. A histidine residue, acting a Brønstead acid donates a proton to the oxygen of the pyranose ring and the combination of these two events is to cause breakage of the ring. Reversing these events results in the ring reforming, but the enzyme is not stereospecific for this action and so either the α- or β-anomer may be formed and thus an equilibrium mixture of products are formed.

Galactokinase (Gal1p)

Galactokinases belong to the GHMP (galactokinase, homoserine kinase, mevalonate kinase and phosphomevalonate kinase) family of enzymes (Bork *et al*. 1993; Timson 2007).

The structure of Gal1p is similar to other galactokinases consisting of two domains separated by a cleft in which is found the substrate binding site (**Fig. 5**) (Thoden *et al*. 2005). This substrate binding site includes an aspartate residue which is positioned such that it could act as a Brønstead base in the mechanism of the enzyme. If this were so, this aspartate (which may be stabilised in its ionised form by an adjacent asparagine residue) would abstract a proton from the C-1 hydroxyl of galactose. This would convert the poorly nucleophilic hydroxyl group into the strongly nucleophilic alkoxide ion which then attacks the γ-phosphorus of the ATP. This would induce bond breakage between the β- and γ-phosphates and transfer the γ-phosphate to C-1 of the galactose. A similar mechanism may also occur in mevalonate kinase (Fu *et al*. 2002). However, in homoserine kinase, there is no residue in a position to act as an active site base. In this enzyme it is postulated that the enzyme effects catalysis through transition state stabilisation (Krishna *et al*. 2001).

If an active site base is required for the galactokinase reaction, it would be expected that pH would have an effect on the turnover number, k_{cat}. In the pH range 6.0 to 9.5 no such effect was observed (Timson and Reece 2002). Of course, there may be effects outside this range (which could not be studied due to the insolubility of the enzyme at higher and lower pH values). Nevertheless, this does open up the possibility that in galactokinase, like homoserine kinase, transition state stabilisation may be an important, possibly predominating, contributor to catalysis. The exact definition of enzyme mechanisms is difficult, if not impossible, in the absence of any means of reliably tracking electron movements through active sites. Nevertheless, further experimental studies (such as mutagenesis of key active site residues including the putative base and the adjacent arginine residue) may throw some light on this controversy.

Kinetically the enzyme appears to follow an ordered, ternary complex mechanism in which ATP is the first substrate to bind (Timson and Reece 2002). The similarity between Gal1p and Gal3p means that this protein probably also follows a similar mechanism when it carries out its role as a transcriptional inducer. In both cases, it is likely that ATP binds first causing a conformational change which enables the binding of galactose. When both molecules have bound the protein can carry out either catalysis (in the case of Gal1p) or Gal80p binding (in the case of Gal3p or Gal1p when it acts in transcription). Interestingly, few changes have been observed between the active site conformations of galactokinases with different ligands bound although it should be noted that a ligand-free form of the enzyme has yet to be reported. Of course, it may be that crystal packing effects force the enzyme into a conformation which represents only one of its conformational possibilities in solution.

Galactose 1-phosphate uridyltransferase (Gal7p)

The structure of *S. cerevisiae* Gal7p has not yet been solved. However, the structure of the *Escherichia coli* enzyme has been determined (**Fig. 6**) (Wedekind *et al*. 1995; Wedekind *et al*. 1996). The *E. coli* enzyme, like the *S. cerevisae* one, is a dimer which binds one Zn^{2+} and one Fe^{3+} ion per monomer. The role of both these metal ions is structural rather than catalytic. Mutagenesis of the ion binding sites showed that while a functional, occupied Zn^{2+}-binding site is essential to retain activity, loss of function at the Fe^{3+}-binding site can be tolerated with loss of only about half the specific activity of the enzyme (Geeganage and Frey 1999).

The enzyme is a member of the histidine triad (HIT) family of proteins which are named after the motif HΦHΦHΦΦ (where Φ represents any hydrophobic amino acid) (Brenner 2002). Its mechanism is a substituted enzyme (or "ping-pong") one in which an active histidine reacts with UDP-glucose to form a covalent histidine-UMP adduct and glucose 1-phosphate. This latter compound must leave the active site before the reaction can proceed. Its place is taken by galactose 1-phosphate and the reaction is completed by the transfer of the UMP moiety from the histidine to the sugar phosphate to form UDP-galactose (Hester and Raushel 1987).

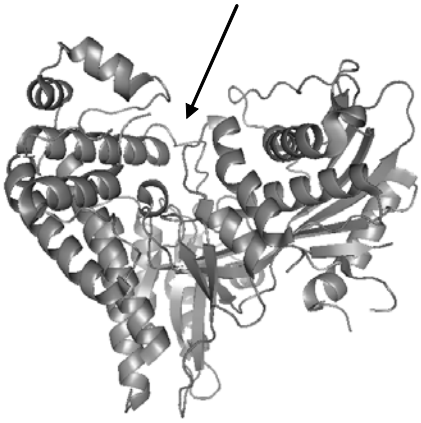

Fig. 5 The structure of *S. cerevisiae* Gal1p (PDB entry 2AJ4 (Thoden *et al.* 2005)). The structure is highly similar to other members of the GHMP family (Timson 2007). The substrate binding cleft is marked with an arrow.

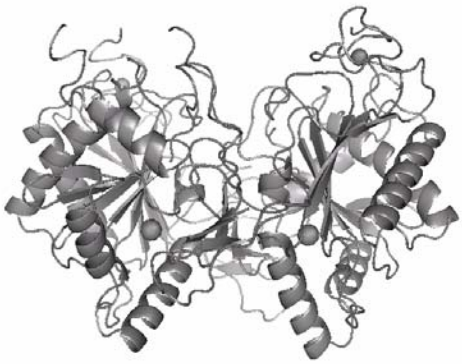

Fig. 6 The structure of the homodimeric *E. coli* galactose 1-phosphate uridyltransferase (PDB entry 1HXQ (Wedekind *et al.* 1995, 1996)). The spheres represent the positions of the metal ions (see text). The structure of *S. cerevisiae* Gal7p is expected to be similar.

UDP-galactose 4-epimerase (Gal10p)

The net result of the pathway so far is to produce one molecule of glucose 1-phosphate and one of UDP-galactose. For the pathway to continue functioning it is necessary that the UDP-galactose is not permitted to accumulate and is recycled to UDP-glucose instead. This transformation is catalysed by UDP-galactose 4-epimerase, which in *S. cerevisiae* is encoded by the N-terminal half of Gal10p. The structure of this part of the protein (**Fig. 4**) is very similar to that of other UDP-galactose 4-epimerases being largely composed of α-helices (Thoden and Holden 2005). The enzyme is a member of the short-chain dehydrogenase/reductase (SDR) family of enzymes (Kallberg *et al*. 2002). It catalyses the epimerisation reaction using a tightly bound NAD^+ molecule as an essential cofactor. This molecule oxidises the hydroxyl group attached to carbon-4 of the sugar moiety of UDP-galactose. The result of this is to convert the hydroxyl group into a ketone which is then reduced back to the alcohol. The reduction reaction is not stereospecific and can occur at either side of the carbonyl group, resulting in a mixture of configurations at carbon-4 in the products. This lack of stereospecificity is thought to be caused by the enzyme's relatively loose grip on the sugar moiety compared to the UDP. The enzyme makes many, specific contacts with the UDP, but few with the sugar. Consistent with this, 5'-uridine monophosphate (5'-UMP) is an inhibitor of the enzyme both *in vitro* and *in vivo* (Nayar *et al*. 2004). Consequently the sugar has considerable mobility in the active site compared to the UDP. Indeed, this may be one reason why the

pathway has evolved to include a pair of UDP sugars, which are involved in the stage of the pathway which alters the configuration at carbon-4 (Frey 1996).

The existence of a polypeptide containing two enzyme activities from the Leloir pathway appears to be unique to *S. cerevisae* and a few other yeasts (*K. fragilis*, *K. lactis*, *Pachysolen tannophilus* and *Schizosaccharomyces pombe*) (Brahma and Bhattacharyya 2004). Bacteria, plants and animals have separate mutarotase and epimerase enzymes as do some other fungi, for example *Hypocrea jecorina* and *Neurospora crassa* (Seiboth *et al*. 2002). Although there are many well-documented cases of enzyme activities from a pathway being encoded by the same polypeptide, these are usually activities with occur adjacent to each other in the sequence of reactions which make up the pathway. Such an arrangement permits the products of one enzyme-catalysed reaction to be passed directly to the next enzyme in the pathway. There are clear advantages here in both the speed and efficiency of transfer and in sequestering unstable or toxic intermediates. It is rare to see, as in Gal10p, two non-adjacent activities encoded on the same polypeptide chain. There are no substrates and products in common between the two enzymes and the substrates of one reaction do not affect the kinetics of the other (under steady state conditions) – i.e. galactose is not an allosteric modifer of the epimerase activity and UDP-galactose has no influence on mutarotase activity (Scott and Timson 2007).

Another way of achieving the same results as having multiple activities encoded by the same polypeptide is for the enzymes of the pathway to associate into a supra-molecular complex, or metabolon. Although often ignored in undergraduate textbooks, metabolons have been detected in most of the common pathways of intermediary metabolism, including the Krebs tricarboxylic (citric) acid cycle and glycolysis (Robinson *et al*. 1987; Ovadi 1988; Mitchell 1996). It is, therefore, entirely possible that the same situation occurs in the Leloir pathway. Indeed there is some evidence of association *in vivo* between the enzymes in *S. cerevisiae*. A green fluorescent protein (GFP) tagged Gal7p was observed to localise to discrete spots in the yeast cytoplasm. Yeast which had the *GAL1* or *GAL10* gene deleted failed to demonstrate this localisation of Gal7p. Interestingly, yeast transformed with a gene encoding a human galactose 1-phosphate uridyltransferase-GFP fusion showed similar, discrete fluorescent spots (Christacos *et al*. 2000). No direct interaction between these proteins has yet been demonstrated *in vitro* or *in vivo*. However, the idea that Gal1p, Gal7p and Gal10p interact (either directly or via another protein) to form a metabolon is surely more probable than a model in which β-galactose is first converted to its α-anomer and then released from Gal10p to diffuse through the crowded cytoplasm until it finds Gal1p, with most of the atoms due to return later to Gal10p for the epimerase reaction. Such an arrangement would have a further advantage. Galactose 1-phosphate, which is toxic to both yeast and mammals (Slepak *et al*. 2005), would not be released into the bulk cytoplasm but would be passed from one active site to the next.

Phosphoglucomutase (Pgm1p and Pgm2p)

The final enzyme of the Leloir pathway catalyses the isomerisation of glucose 1-phosphate to glucose 6-phosphate. In *S. cerevisiae* there are two enzymes with phosphoglucomutase activity, Pgm1p and Pgm2p. Analysis of deletion mutations showed that Pgm2p provides most of the activity required for the Leloir pathway (Tsoi and Douglas 1964). Yeast carrying deletion or loss of function mutations in either *PGM1* or *PGM2* are viable and can metabolise galactose; loss of both genes results in an inability to grow on galactose. Although Pgm2p is induced in the presence of galactose, it is expressed at a relatively high basal level (compared to the other *GAL* genes) in the presence of glucose (Oh and Hopper 1990). This may be because the enzyme is also required for glycogen and trehalose metabolism which can occur when either glucose or galactose is the main carbon source.

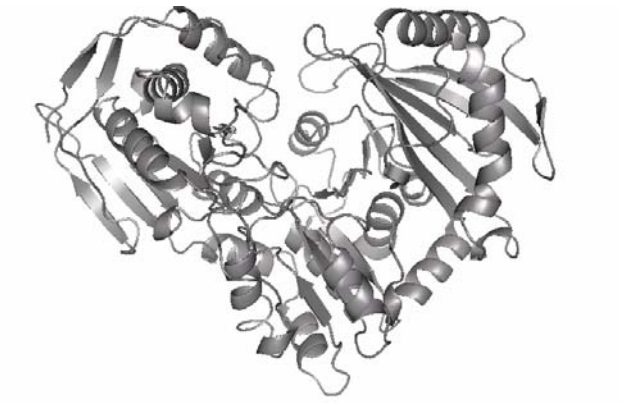

Fig. 7 The structure of a monomer of rabbit muscle phosphoglucomutase (PDB entry 1LXT (Liu *et al.* 1997)). The structures of *S. cerevisiae* Pgm1p and Pgm2p are expected to be similar.

There is no crystal structure of either Pgm1p or Pgm2p. However the enzyme has been crystallised from a number of different sources, including rabbit (Dai *et al*. 1992; Liu *et al*. 1997). The enzyme is a monomer (**Fig. 7**) and the catalytic cycle involves the stable phosphorylation of a serine residue (Ser-116 in the rabbit enzyme) in the active site. Glucose 1-phosphate binds to the phosphorylated enzyme and the phosphate is then transferred from the enzyme to the sugar to give glucose 1,6-*bis*phosphate. The *bis*phosphorylated intermediate must be reorientated in the active site so that that phosphate at position 1 can be transferred to the enzyme thus restoring the active site to its original state and permitting the release of glucose 6-phosphate (Ray and Roscelli 1964). If the other enzymes of the Leloir pathway do form a metabolon, it will be especially interesting to see if Pgm2p (or Pgm1p) form part of the complex given this enzyme's role in several different pathways.

Bleomycin hydrolase (Lap3p/Gal6p)

Bleomycin is a DNA-binding glycopeptide which, due to its cytotoxicity has been used in the treatment of human cancers. However, it can be hydrolysed and inactivated by a cysteine protease of unknown physiological function, bleomycin hydrolase. The *S. cerevisae* homologue of this protein, Lap3p, was originally named Gal6p as it is regulated by the *GAL* genetic switch in response to galactose (Zheng *et al*. 1997). In addition to acting a protease, Lap3p/Gal6p also binds to the UAS_{GAL} leading to speculation that it might play a role in *GAL* gene regulation (Xu and Johnston 1994). Deletion of the gene does lead to increased (2.5-fold) levels of *GAL* gene induction (Zheng *et al*. 1997). The protein is a hexamer and its crystal structure reveals a ring-shaped structure with a central channel (Joshua-Tor *et al*. 1995). The protease active sites are located on the inside of this channel which also contains some 60 exposed lysine residues suggesting that this may be the site of DNA binding. Interestingly the protein has a variety of protease activities – aminopeptidase, carboxypeptidase and endopeptidase. It can also act as a peptide ligase (Zheng *et al*. 1998). In the crystal structure, the C-termini of the six subunits of the hexamer are located close to the protease active sites (Joshua-Tor *et al*. 1995). Protelytic processing of these C-termini appears to determine which of the protein's enzymatic activeties are exhibited (Zheng *et al*. 1998). The role of this protein in galactose metabolism remains unclear. Although it is possible that the DNA-binding activity of the protein provides another layer of *GAL* gene regulation, it is not clear why the expression of a protease should be regulated by the presence of a sugar or if this protease activity contributes to galactose metabolism in any way. It is possible that the DNA binding and protease activities are not required for the same physiological processes, but this seems unlikely given

that the gene is upregulated in the presence of the sugar. One possibility is that Las6p/Gal6p acts to degrade excess *GAL* gene products. Indeed the similarity of the structure to that of the proteosome has been commented on (Joshua-Tor *et al*. 1995; Zheng *et al*. 1998).

Alternative pathways of galactose metabolism

In mammals there are some strong suggestions that galactose can be metabolised through pathways other than the Leloir pathway. Patients with type I galactosemia (galactose 1-phosphate uridyltransferase deficiency) and cells derived from these patients along with mice completely deleted for this enzymatic activity all retain a residual ability to process galactose. The compounds formed include carbon dioxide, an oxidised form of the sugar galactonate, a reduced form galactitol and UDP-glucose (Berry *et al*. 2001; Segal *et al*. 2006; Wehrli *et al*. 2007). Whether these compounds are formed through the action of an undiscovered pathway or through the action of enzymes which show low activity towards galactose (and its derivatives) in addition to their normal substrates remains to be seen. That the products are not identical in different tissues (Wehrli *et al*. 2007), suggests that it may be the latter possibility. Similar alternative pathways may exist in *S. cerevisiae* (Lai and Klapa 2004), but to date there has been little interest in investigating them.

LINKAGES BETWEEN GENE EXPRESSION AND METABOLISM

Of course, the processes of gene expression, galactose transport and metabolism do not occur in isolation; they are closely linked. For example, in order for galactose to activate the *GAL* genetic switch, it must first be transported into the cell. Presumably this occurs via one of the non-specific hexose transporters as there will be little, or no, Gal2p available under repressing conditions. In wild type yeast Gal2p, like the other *GAL* genes is rapidly and highly expressed under inducing conditions. High concentrations of the permease in the cell membrane probably contribute to the largely binary nature of the *GAL* genetic switch by bringing substantial concentrations of galactose into the cell to saturate Gal3p. Deletion of the *GAL2* gene results in a more linear response by the genetic switch to increasing concentrations of the sugar (Hawkins and Smolke 2006).

Clearly these linkages between gene expression and the metabolic pathway are complex and several attempts have been made to model the system *in silico*. As with all metabolic models, one of the challenges is knowing which subprocesses will have significant effects and which can be safely eliminated from the model. Considering too many factors can result in a model which is unwieldy and overly sensitive to inaccurate estimates of unimportant parameters. However, failure to consider a factor can result in biologically misleading results. By including estimates for the time course of new protein production as well as binding kinetics, one study showed that it was possible for a feedback loop to be established in *GAL* gene expression (Smidtas *et al*. 2006). In this loop, the first events to occur are the binding of galactose to Gal3p and the subsequent activation of the switch. Over a longer time scale, new Gal3p (and other switch components) are produced. This could result in a system which senses the relative rather than absolute concentrations of galactose and thus maintains the ability of the switch to react to changes in galactose concentration. It has also been proposed that the nesting of multiple feedback loops (two positives ones mediated by Gal2p and Gal3p and one negative mediated by Gal80p) in the *GAL* genetic switch enables the system to "remember" previous events (Acar *et al*. 2005). Experimental verification of this model showed that yeast grown on galactose concentrations between 0.7 mM and 19 mM expressed the *GAL* genes to a level which depended not only on the current galactose concentration but also on that which the cells had been exposed to previously (Acar *et al*. 2005).

Modelling has also been used to help explain the mechanism of Gal1p acting as a transcriptional activator. It is well established that, while Gal1p can substitute for Gal3p, the time taken for *GAL* gene induction is substantially increased. One possible mechanism for this is that stochastic reductions in Gal80p concentrations may permit transient expression of Gal1p at sufficient levels for it to then act as a ligand sensor and transcriptional activator. The model (Bhat and Venkatesh 2005) shows that such a mechanism is possible with the concentrations of Gal1p and Gal80p present in yeast cells. It may be that *S. cerevisiae* has evolved a separate ligand sensor to overcome the possible problem of accidental induction caused by fluctuations in Gal80p concentration (Bhat and Venkatesh 2005). A recent model has attempted to integrate both the events occurring in the *GAL* genetic switch, in galactose transport, the Leloir pathway and glycolysis (Demir and Aksan Kurnaz 2006). The model correctly predicts the selectivity of yeast grown on mixtures of glucose and galactose, and the ultimate metabolic fate of these sugars. Models are only as good as the predictions they make and the ultimate utility of these models will be shown by whether they help or hinder our understanding of the systems biology of galactose metabolism.

EXPLOITATION OF GALACTOSE METABOLISM IN YEAST

The metabolism of sugars by *S. cerevisiae* has been used in biotechnologies (bread making, brewing) since the dawn of civilisation. Recently galactose metabolism has also been investigated for possible industrial exploitation. There is interest in increasing the flux through the Leloir pathway because some feedstocks for ethanol production (e.g. lingocellulose, cheese whey and molasses) contain the sugar (van Maris *et al*. 2006). Furthermore, most feedstocks contain mixtures of sugars including glucose at concentrations which repress the *GAL* genes in wild type yeast. Consumption of galactose and flux through the pathway can be increased by deleting the negative regulators of *GAL* gene induction (Mig1p and Gal80p) and, interestingly, Gal6p (Ostergaard *et al*. 2000). The effect of these deletions is to increase the amount of ethanol produced per unit biomass under fermentative conditions. However, the amount of biomass was not affected meaning that this change would not be useful for applications where biomass is desired end product (e.g. single cell protein). Upregulation of the *PGM2* gene resulted in an increase in galactose uptake into the cell and overall flux through the pathway (Bro *et al*. 2005). Another important carbohydrate constituent of many feedstocks is the pentose monosaccharide L-arabinose. Although this sugar is structurally related to D-galactose, it is not metabolised by *S. cerevisiae* probably because Gal1p does not catalyse its phosphorylation (Sellick and Reece 2006). One solution to this problem is to introduce bacterial L-arabinose metabolising genes into the yeast (Becker and Boles 2003). This approach works because Gal2p is able to transport the pentose sugar into the cell. Further metabolism of L-arabinose is catalysed by enzymes of the pentose phosphate pathway, however (Becker and Boles 2003).

The binary nature of the *GAL* genetic switch and the high level of expression of the genes following induction make it an attractive system for exploitation in the regulated expression of recombinant proteins. Many systems are now available commercially and are in use in both academic laboratories and industrial processes. Gal4p will, given appropriate promoter sequences, function as a transcriptional activator in higher eukaryotes and the proteins of the *GAL* genetic switch have also been exploited in these organisms (Suster *et al*. 2004). The yeast two-hybrid screen (Chien *et al*. 1991) relies on the independent folding and function of the DNA binding and activation domains of Gal4p. Together these domains are sufficient to direct activated transcription and there is no requirement for them to be part of the same polypeptide chain. Fusing the two domains to interacting

partners is often sufficient to bring them into close enough proximity that the non-covalent complex can bind specifically to UAS_{GAL} and activate transcription. Although the assay has a number of well-documented problems (especially a high background of false-positives) it has found widespread applications in the detection of new protein-protein interactions, the mapping of interaction domains within proteins and in global interaction screens.

The Leloir pathway in *S. cerevisiae* has also been used as a model system for studying the human genetic disease galactosemia. This disease is caused by mutations in the Leloir pathway enzymes. *S. cerevisae* offers some advantages for these experiments. The ease of genetic manipulation means that strains can be constructed lacking one or more *GAL* gene and these strains then complemented with plasmids containing wild type or mutant human genes. The use of diploid yeast means that the effects of heterozygosity can be studied – something which is difficult using either transfected human cell lines or isolated proteins. (Note that the dimeric nature of the uridyltransferase and the epimerase mean that three possible dimers are possible in heterozygotes – two different homodimers and a heterodimer. Reliable methods for dissociating and reforming dimers of these proteins *in vitro* or *in vivo* have yet to be developed. Recombinant expression in heterozygous yeast overcomes this problem as all possible dimers will be synthesised *in vivo*.) The organism's short doubling time means that the effects of the mutations can be studied on cells grown on galactose as the only carbon source over many generations. Relatively simple experiments such are comparisons of doubling times can be carried out and the proteins can be isolated if necessary for *in vitro* work. The system has been used successfully to characterise mutations in both the uridyltransferase and epimerase enzymes (Quimby *et al*. 1997; Riehman *et al*. 2001; Christacos and Fridovich-Keil 2002; Wasilenko *et al*. 2005). If there are alternative pathways of galactose metabolism in *S. cerevisiae* (see above), these may need to be taken into account in the future interpretation of these kinds of experiments.

FUTURE PERSPECTIVES AND CHALLENGES

The study of the galactose metabolism in yeast remains an important field. Although we have learned a lot over the last 25 years about the mechanisms of gene induction in the *GAL* genetic switch – much of which is applicable to a wide range of eukaryotic gene expression systems – many facts remain to be clarified. The precise mechanism of action remains controversial and more details are required about the nature of the interactions between the various components in atomic detail. Perhaps most importantly, the unambiguous identification of the general transcription factors which Gal4p interacts with is required along with details of the affinities and time courses of these interactions. We have learned much about the structures and mechanisms of the individual enzymes, but much less about how they work together as pathway. More practical and theoretical studies on the control of flux through the pathway are required and the question of whether, or not, the enzymes form a metabolon needs to be tackled conclusively. The acquisition of this information will have clear scientific and industrial benefits. A better understanding of how galactose metabolism in yeast works as an integrated system will enhance our ability to exploit it both in industrial biotechnology and in the research laboratory.

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