CHAPTER THREE

Galactose Metabolism in Yeast—Structure and Regulation of the Leloir Pathway Enzymes and the Genes Encoding Them

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Abstract

The enzymes of the Leloir pathway catalyze the conversion of galactose to a more metabolically useful version, glucose-6-phosphate. This pathway is required as galactose itself cannot be used for glycolysis directly. In most organisms, including the yeast *Saccharomyces cerevisiae*, five enzymes are required to catalyze this conversion: a galactose mutarotase, a galactokinase, a galactose-1-phosphate uridyltransferase, a UDP-galactose-4-epimerase, and a phosphoglucomutase. In yeast, the genes encoding these enzymes are tightly controlled at the level of transcription and are only transcribed under specific sets of conditions. In the presence of glucose, the genes encoding the Leloir pathway enzymes (often called the *GAL* genes) are repressed through the action of a transcriptional repressor Mig1p. In the presence of galactose, but in the absence of glucose, the concerted actions of three other proteins Gal4p, Gal80p, and Gal3p, and two small molecules (galactose and ATP) enable the rapid and high-level activation of the *GAL* genes. The precise molecular mechanism of the *GAL* genetic switch is controversial. Recent work on solving the three-dimensional structures of the various *GAL* enzymes proteins and the *GAL* transcriptional switch proteins affords a unique opportunity to delve into the precise, and potentially unambiguous, molecular mechanism of a highly exploited transcriptional circuit. Understanding the details of the transcriptional and metabolic events that occur in this pathway can be used as a paradigm for understanding the integration of metabolism and transcriptional control more generally, and will assist our understanding of fundamental biochemical processes and how these might be exploited.

Key Words: Galactose, Gal3p, Gal4p, Gal80p, Gene regulation, Transcriptional control, Transcriptional memory, Yeast. © 2008 Elsevier Inc.

1. Introduction

Glucose is a metabolically important monosaccharide that can be used by cells as both a source of energy and as a metabolic intermediate. Glucose is catabolized during glycolysis, and this pathway cleaves the six-carbon glucose molecule ($C_6H_{12}O_6$) into two molecules of pyruvate ($C_3H_3O_3^-$). Concomitant with this oxidation is the production of two molecules of ATP, which can be subsequently used in the myriad of energy requiring processes that the cell depends upon. Glucose is also available to a cell in a variety of other forms. Such as alternative hexose sugars, for example, galactose that is found in diary products and in a variety of fruits, and as disaccharides, for example, lactose that is composed of β-D-galactose and β-D-glucose molecules bonded through a β-1–4 glycosidic linkage.

Like glucose, the sugar galactose is a molecule composed of six carbon atoms (see Fig. 3.1) and differs from glucose only in the stereochemistry of

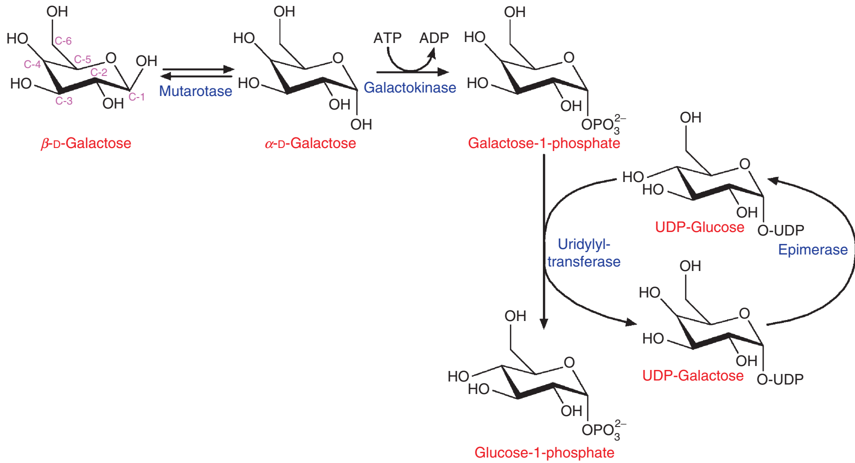

Figure 3.1 **The Leloir pathway. The enzymes of this pathway promote the conversion of β-D-galactose into glucose-1-phosphate which can subsequently be used in glycolysis.**

one carbon atom, that is located at position C-4. Before it can enter glycolysis, however, galactose must be converted into a derivative of glucose. The enzymes of the Leloir pathway are responsible for this conversion (Caputto *et al.*, 1949). This pathway is named after Luis Leloir (1906–1987) who was awarded the 1970 Nobel Prize in Chemistry in part for his contribution to the understanding of galactose metabolism. To convert galactose to glucose, the sugar is phosphorylated by galactokinase to produce galactose-1-phosphate. The galactokinase enzyme will, however, only convert the α-form of the sugar in this reaction. The α and β anomers of the sugar differ in the stereochemistry of the C-1 carbon atom (see Fig. 3.1). Therefore, the initial step of the Leloir pathway is the conversion of β-D-galactose into α-D-galactose by a mutarotase enzyme. Once in the form of galactose-1-phosphate, galactose is then exchanged with the glucose group from UDP-glucose to create UDP-galactose and release glucose-1-phosphate. An epimerase enzyme changes the stereochemistry of C-4 in UDP-galactose, creating UDP-glucose. In the next round of the transfer reaction, glucose is released in the form of glucose-1-phosphate. Once released, glucose-1-phosphate is converted to glucose-6-phosphate and can enter glycolysis to generate energy. In humans, the mutation of the kinase, the transferase, or the epimerase can result in clinical deficiencies in galactose metabolism known as galactosemias (Holden *et al.*, 2004; Timson, 2006). Galactosemias range in severity depending on the nature of the genetic change, and are usually treated through avoidance of galactose in the diet, primarily from lactose in dairy products.

In this chapter, we will concentrate on the Leloir pathway enzymes from yeast and discuss, in some detail, how the genes encoding these enzymes are regulated at a transcriptional level. The genes encoding the Leloir pathway enzymes in yeast (the *GAL* genes) are tightly regulated by the availability of sugars and other carbon sources to cells. This property has been exploited for the production of heterologous proteins in yeast (Schultz *et al.*, 1987) and the separable functions of RNA polymerase II transcription factors—as exemplified by the DNA-binding and activation domains of Gal4p—form the basis of the widely used two-hybrid screen (Fields and Song, 1989). Therefore, understanding the precise molecular details of the regulation of the *GAL* genes is not only important for the understanding of a transcriptional paradigm, but also provides new insights into how these processes may be exploited (Timson, 2007).

2. Leloir Pathway Enzymes in Yeast

2.1. Mutarotase

Most organisms contain a mutarotase enzyme that is responsible for maintaining the equilibrium between the α- and β-anomers of galactose. The interconversion of the anomers can occur readily in aqueous solution until an equilibrium mixture is formed (Pigman and Anet, 1972). Although the anomers will interconvert in water, the rate of interconversion in the cytoplasm would not appear to be sufficient to provide for the needs of metabolic pathways (Bouffard *et al.*, 1994; Frey, 1996). Indeed, the conditions prevailing in the cytoplasm may be such that little spontaneous interconversion of the galactose anomers occurs (Cayley *et al.*, 1991). The *E. coli* (Bouffard *et al.*, 1994) and human (Timson and Reece, 2003b) forms of the enzyme are relatively well characterized. In contrast to the prokaryotic and higher eukaryotic counterparts, the mutarotase in budding yeast is found in the same polypeptide chain as the UDP-galactose-4-epimerase function—Gal10p. This dual activity appears to be restricted to *Saccharomyces cerevisiae* and other closely related yeast species. Co-localization of these activities does not occur in more distantly related fungi such as *Hypocrea jecorina* or *Neurospora crassa* (Seiboth *et al.*, 2002). Indeed, the mutarotase and epimerase activities of Gal10p function wholly independently of each other (Scott and Timson, 2007). That is, the steady state kinetic parameters of epimerase activity are not affected by simultaneous activity of the mutarotase active site. The absence of reciprocal kinetic effects between the active sites suggest that they act independently and do not influence, or regulate, each other.

Yeast Gal10p is a 699 amino acid (78 kDa) protein that exists as a homodimer in solution (Majumdar *et al.*, 2004; Scott and Timson, 2007).

The amino-terminal portion of the protein, which also encompasses the dimerization interface, contains the epimerase active site (see Section 2.4 below), while the carboxyl-terminal portion of the protein contains the mutarotase activity. The three-dimensional structure of Gal10p in complex with NAD^+, UDP-glucose, and β-D-galactose has been solved using X-ray crystallography to a resolution of 1.85 Å (Thoden and Holden, 2005). Overall, the protein has approximate dimensions of $91 \times 135 \times 108$ Å and adopts a structure that is almost V-shaped (Fig. 3.2). The significance of this structure, if any, is currently unknown. The selective advantage of having the first and last enzymes of a metabolic pathway (Fig. 3.1) contained within a single polypeptide chain is not obvious. Given the unusual shape of the Gal10p dimer (Fig. 3.2), it is tempting to speculate, however, that either another protein binds within the V-shaped structure and/or some type of substrate channelling may occur *in vivo*.

With regard to the mutarotase activity, the Gal10p polypeptide chain extending from amino acids 361–699 adopts a β-sandwich motif that harbors the binding site for galactose. It has been suggested that the catalytic mechanism of galactose mutarotase proceeds through the abstraction of the proton from the C-1 hydroxyl group of the sugar by an active site base and the donation of a proton to the C-5 ring oxygen by an active site acid, thereby leading to ring opening (Hucho and Wallenfels, 1971). Subsequent

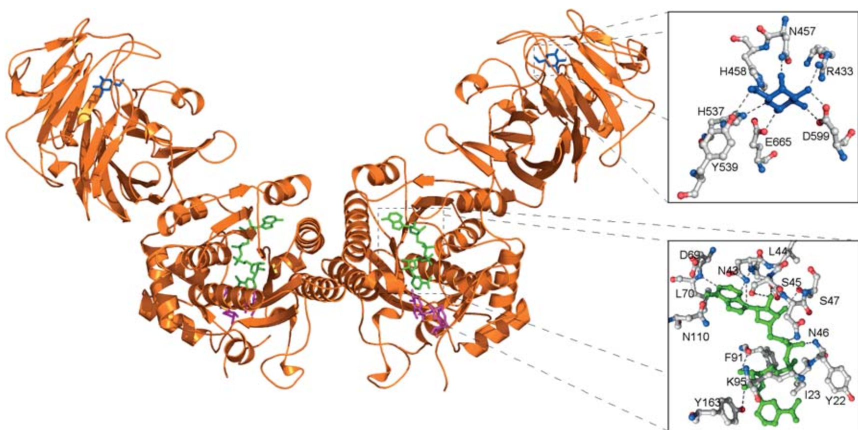

Figure 3.2 The structure of yeast Gal10p. In *Saccharomyces cerevisiae*, Gal10p is a dimeric bifunctional protein bearing both a galactose mutarotase activity and UDP-galactose-4-epimerase activity. The protein is shown as a ribbon cartoon (generated from the PDB file 1Z45) with its substrates in stick model form (Thoden and Holden, 2005). The galactose is shown in blue, NAD in green, and UDP-glucose in magenta. The insets show potential hydrogen bond interactions between the protein and galactose (top) and NAD (bottom).

rotation of 180° about the C-1–C-2 bond, followed by abstraction of a proton from the C-5 oxygen and donation of a proton back to the C-1 oxygen generates the product (Holden *et al.*, 2003). In the case of Gal10p, Asp-599 may serve as the active site base to abstract the C-1 hydroxyl hydrogen, and His-537 may serve as the active site acid to protonate the C-5 ring oxygen (see Fig. 3.2).

2.2. Galactokinase

The phosphorylation of galactose is a stereospecific reaction, with only the α-form of the sugar being a suitable substrate for galactokinase. The catalytic mechanism of galactokinase would appear to depend upon the source of the enzyme. For the enzyme isolated from *E. coli*, it appears that the reaction mechanism is random with either ATP or galactose being able to bind to the enzyme first (Gulbinsky and Cleland, 1968). The human, rat, and yeast enzymes, however, have a reported reaction mechanism that is ordered with ATP binding first (Timson and Reece, 2002, 2003a; Walker and Khan, 1968). The reaction mechanism of plant galactokinase is also ordered but with the binding of galactose, rather than of ATP, being the first step (Dey, 1983; Foglietti and Percheron, 1976). It is not clear if these apparent mechanistic differences represent real changes in the way each enzyme functions or reflect the methodology used to quantify the catalytic mechanism.

In yeast, the galactokinase is encoded by the *GAL1* gene. The enzyme is composed of 528 amino acids (58 kDa). The three-dimensional structure of galactokinase from yeast, Gal1p, has been solved recently in the presence of galactose and a non-hydrolyzable ATP analog (Thoden *et al.*, 2005). Overall, the enzyme, of approximate dimensions of $\sim 72 \times 49 \times 66$ Å, folds into two approximately equal sized domains (see Fig. 3.3). The active site is wedged between these two domains. The N-terminal domain is dominated by a six-stranded mixed β-sheet flanked on one side by an α-helix and on the other side by four α-helices. There are two four-stranded antiparallel β-sheets in the C-terminal domain in addition to 10 α-helices. The specificity of the enzyme for galactose is predominately controlled by Asp-62 (Fig. 3.3), which has the potential to contact the hydroxyl groups at carbons 3 and 4 of galactose and it is at C-4 that galactose differs from glucose. The mutation of this residue to alanine, leucine, phenylalanine, or leucine resulted in a protein that was unable to phosphorylate galactose but retained a weak ability to phosphorylate glucose (Sellick and Reece, 2006).

2.3. Transferase

The reversible transfer of a uridine phosphate moiety from UDP-glucose to galactose-1-phosphate is catalyzed by the third enzyme of the Leloir pathway, galactose-1-phosphate uridylyltransferase. This enzyme belongs to the

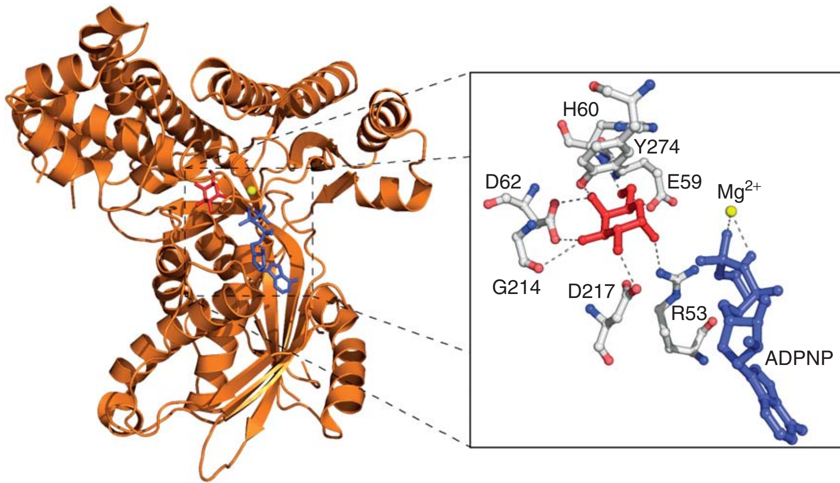

Figure 3.3 The structure of yeast Gal1p. The protein is shown as a ribbon cartoon (generated from the PDB file 2AJ4) with its substrates in stick model form (Thoden *et al.*, 2005). The galactose is shown in red, the non-hydrolyzable ATP analog shown in blue, and the magnesium ion as a yellow sphere. The inset shows potential hydrogen bond interactions between the protein and galactose.

histidine triad (HIT) superfamily of nucleotide binding proteins (Brenner *et al.*, 1999) that contain derivations of the consensus sequence -His-ϕ-His-ϕ-His-ϕ-ϕ- (where ϕ represents any hydrophobic amino acid). The mechanism of action of the transferase proceeds through a covalently bound intermediate. It has been suggested that UDP-glucose binds to the enzyme to allow the formation of an uridylylated enzyme intermediate. In the case of the *E. coli* enzyme, His-166 is the residue that is transiently modified and represents the active site base that attacks the α-phosphate of the incoming UDP-glucose substrate (Kim *et al.*, 1990; Wedekind *et al.*, 1996). The association of galactose-1-phosphate with the active site then allows the transfer of the uridine phosphate moiety to generate UDP-galactose. Nucleophilic attack on the α-phosphate of the uridylyl-enzyme intermediate by either galactose-1-phosphate or glucose-1-phosphate results in the transfer of the uridine phosphate moiety back to recreate the UDP-sugar (Holden *et al.*, 2003).

The yeast galactose-1-phosphate uridylyltransferase is encoded by the *GAL7* gene. Gal7p contains 366 amino acids (42 kDa). Amino acids 314–320 of Gal7p (HMHFYPP) contain similarity to the HIT consensus sequence. The structure of this enzyme has not been solved, but the protein is $\sim$50% identical to its *E. coli* counterpart which has been extensively analyzed at both a structural (Fig. 3.4) and biochemical level (Ruzicka *et al.*, 1995). The structure of *E. coli* galactose-1-phosphate uridylyltransferase indicates that the enzyme is a dimer

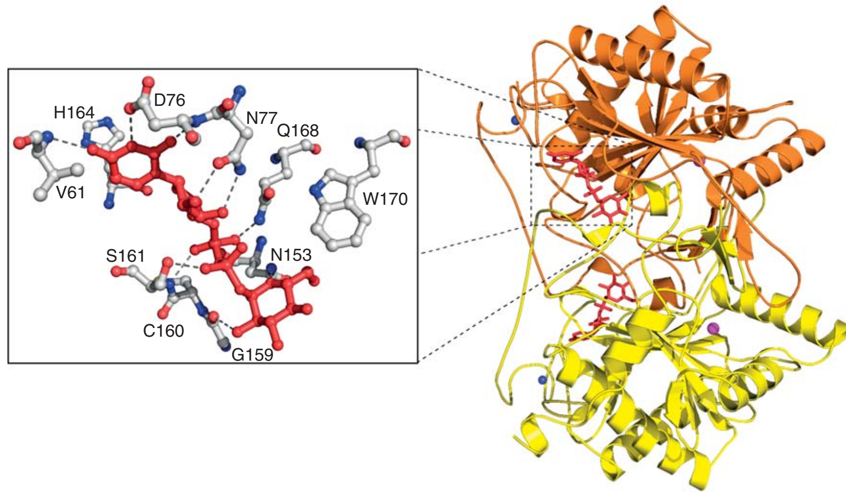

Figure 3.4 The structure of *E. coli* galatose-1-phosphate uridylyl-transferase complexed with UDP-glucose. The protein is dimeric and is shown as a ribbon cartoon (generated from the PDB file 1GUQ) with its substrates in stick model form (Thoden *et al.*, 1997; Wedekind *et al.*, 1995). The UDP-glucose is shown in red, and the Zn^{2+} (blue) and Fe^{3+} (magenta) ions are shown as spheres.

(Wedekind *et al.*, 1995), and the yeast enzyme is also found to be dimeric (Segawa and Fukasawa, 1979). Using a version of Gal7p that was tagged with the green fluorescent protein, it has been noted that the localization of galactose-1-phosphate uridylyltransferase in the cytoplasm of yeast was not uniform (Christacos *et al.*, 2000). That is, the fusion protein was found to localize to ~1–3 "spots" within the cytoplasm of most cells. In addition, these spots were only found to occur when Gal1p and Gal10p were also coproduced in the cell. This may suggest that the Gal1p-Gal7p-Gal10p enzymes may co-localize within the cytoplasm. Taken together, these data suggest that it is possible (although it has not been proven) that the Leloir pathway enzymes in yeast form a multi-enzyme complex (or metabolon) similar to that seen in other pathways (Abadjieva *et al.*, 2001; Kindzelskii *et al.*, 2004; Ovadi, 1988). The significance of such a complex is not clear, but substrate shuttling between the Leloir pathway enzymes would have the advantage of sequestering galactose-1-phosphate, which is toxic to both yeasts and mammals (Tsakiris *et al.*, 2002).

2.4. Epimerase

For the completion of the Leloir pathway, UDP-galactose is converted back to UDP-glucose by the action of an epimerase enzyme. The enzymatic function requires NAD^+ as a cofactor. NAD^+ is irreversibly, but non-covalently,

bound to the active site (Wilson and Hogness, 1964, 1969). The first step in the conversion process is an abstraction of the 4-hydroxyl proton by an enzymatic base and an abstraction of a hydride from the C-4 position of the sugar to the C-4 position on NAD^+ to form NADH (Allard *et al.*, 2001). The resulting 4′-ketopyranoase rotates in the active site prior to transfer of the hydride from NADH to the C-4 of the sugar and reprotonation of the C-4 oxygen (Thoden and Holden, 2005).

As stated above, the epimerase in yeast (Gal10p) is encoded on the same polypeptide as the mutarotase. The first 356 amino acids of Gal10p fold into the classical bilobal topology found in other UDP-galactose-4-epimerases (Thoden and Holden, 2005). This portion of the protein contains the binding sites for UDP-glucose (see Fig. 3.2). In the crystal structure, the UDP-glucose moiety was found to be not well ordered. In contrast, the NAD^+ moiety is held by 25 hydrogen bond interactions with the protein or solvent. This type of extensive hydrogen bond network is typical for the UDP-galactose-4-epimerases that bind the dinucleotide cofactor irreversibly (Thoden and Holden, 2005).

2.5. Human disease models

Galactosemias are a group of diseases caused by aberrant galactose metabolism (Timson, 2007). Galactosemia refers to a spectrum of disorders that are generally characterized by increases in the concentration of galactose in the blood and the development of childhood cataracts. Mutations in each of the Leloir pathway enzymes (except galactose mutarotase) have been identified which give rise to galactosemias. There are three relatively clinically distinct types of galactosemia:

1. Type I galactosemia is caused by mutations in galactose-1-phosphate transferase (encoded by the human *GALT* gene). This is the most common form of galactosemia with over 130 different mutations known that give rise to the diseased state (Elsas and Lai, 1998). Usually, *GALT* defects cause no symptoms at birth, but jaundice, diarrhea, and vomiting soon develop and the affected baby fails to gain weight. If not detected immediately, type I galactosemia can result in liver disease, cataracts, mental retardation, and death.
2. Type II galactosemia is caused by mutations in galactokinase (encoded by the human *GALK1* gene). The main symptom of patients with mutations in *GALK1* is the formation of childhood cataracts and the disease is generally less severe than either type I or type III galactosemia (Holden *et al.*, 2004).
3. Type III galactosemia is caused by mutations in UDP-galactose-4-epimerase (encoded by the human *GALE* gene). The disease was considered to exist in two forms—a severe, or generalized, form and a milder

peripheral form. The disease should, perhaps, be considered as a continuum disorder with a variety of potential phenotypes arising from different mutations (Timson, 2006). Untreated sufferers of the generalized form have low (or zero) *GALE* activity in all tissues and typically develop cataracts within the first few months of life; these are followed by liver, kidney, and brain damage (Timson, 2006).

Current treatment for all types of galactosemia is the restriction of dietary galactose and its precursors such as lactose. For type II galactosemia, this can be effective. However, it is less so in the case of generalized type III galactosemia because galactose cannot be completely eliminated from the diet as *GALE* is responsible not only for the interconversion of UDP-galactose and UDP-glucose but also for the interconversion of UDP-*N*-acetylgalactosamine and UDP-*N*-acetylglucosamine (Timson, 2006).

The Leloir pathway enzymes of yeast have been used extensively as models to investigate disease-causing mutations of their human counterparts, or as a host for the purification of mutant proteins (Daude *et al.*, 1995; Riehman *et al.*, 2001; Slepak *et al.*, 2005; Wells and Fridovich-Keil, 1996). In a number of cases, the severity of the human disease has been correlated with the severity of the drop in enzymatic activity of the purified protein (Timson, 2005, 2006; Timson and Reece, 2003a).

3. The Regulation of *GAL* Gene Expression

3.1. The yeast *GAL* genes and the proteins that control their expression

The transcription of the genes of the Leloir pathway in yeast is controlled by the *GAL* genetic switch. This switch, and the transcriptional principles that underlie it, are often considered as a paradigm for eukaryotic gene expression. The genes regulated by galactose in yeast include *GAL1*, *GAL2*, *GAL3*, *GAL5*, *GAL7*, *GAL10*, *GAL80*, and *MEL1* (Lohr *et al.*, 1995). *GAL1*, *GAL7*, and *GAL10* encode structural enzymes of the Leloir pathway (see Section 2 above) and form a cluster of similarly regulated genes located on chromosome II (Schmid *et al.*, 2006). Expression from this cluster is almost undetectable when yeast are grown on carbon sources such as glucose or glycerol, but expression levels of each of these *GAL* genes increase by around 1000-fold when induced by growth on galactose (Lohr *et al.*, 1995).

The *GAL* switch can be described as existing in three distinct states depending on the source of carbon that is available to the cells:

1. *Repressed*: In carbon sources such as glucose, catabolite repression ensures that *GAL* gene expression does not occur even if galactose is available to

the cell. The repression of the *GAL* genes by glucose is largely controlled by the repressor protein Mig1p (Nehlin *et al.*, 1991), although deletion of the *MIG1* gene does not completely relieve glucose repression indicating that other factors are also involved. Mig1p is involved in a glucose repression pathway with Hxk2p (Ahuatzi *et al.*, 2007) and interacts with the general corepressor complex Tup1p–Cyc8p (Papamichos-Chronakis *et al.*, 2004). Tup1p appears to inhibit transcription by multiple redundant mechanisms, including promoting the formation of a repressive chromatin state and interaction with the transcription machinery (Edmondson *et al.*, 1996; Wahi *et al.*, 1998; Zhang and Reese, 2004).
2. *Uninduced*: When grown on medium containing glycerol or raffinose, the *GAL* genes are not expressed, but are "poised" for rapid activation if galactose becomes available.
3. *Induced*: The *GAL* structural genes are actively transcribed when galactose is the preferred carbon source. The transcription of *GAL1*, *GAL2*, *GAL7*, and *GAL10* is induced by more than 1000-fold when galactose is the preferred carbon source (Johnston, 1987; Johnston *et al.*, 1994). In the induced state, the mRNA of each of the *GAL* genes represents between 1 and 2.5% of the total mRNA within the cell (St John and Davis, 1981). The induction of *GAL* gene expression is rapid, occurring within 30 min of adding galactose to the culture.

Unlike the other *GAL* genes, the expression of *GAL3*, *GAL80*, and *MEL1* occurs at a low level even under repressing conditions; *MEL1* encodes an enzyme which converts melibiose (a glucose–galactose disaccharide) to its constituent sugars and thus may be needed before the induction of the other *GAL* genes occurs, while basal expression of *GAL3* and *GAL80* is required as they form part of the induction mechanism (Lohr *et al.*, 1995).

In this chapter, we will consider the mechanism by which the *GAL1*, *GAL10*, and *GAL7* gene cluster is regulated by the *GAL* genetic switch. The switch is composed of three proteins: a transcriptional activator (Gal4p), a transcriptional inhibitor (Gal80p), and a transcriptional inducer/ligand sensor (Gal3p).

3.2. *GAL* gene expression in *K. lactis*

Whereas *S. cerevisiae* may have evolved to utilize glucose and galactose as the major carbon sources, the related milk yeast *Kluyveromyces lactis* has adapted to utilize the milk sugar lactose. This means that in addition to the *GAL* metabolic genes described above, the lactose utilization genes *LAC12* and *LAC4*, encoding a lactose permease and β-galactosidase, respectively, are also regulated by the *K. lactis GAL* genetic switch. In *K. lactis*, transcription of the *GAL/LAC* genes is repressed in most, but not all strains, in the

presence of glucose by KlMig1p. In addition, catabolite (glucose) repression in *K. lactis* is weaker than that seen in *S. cerevisiae* (Breunig, 1989). Overall, *K. lactis* utilizes galactose in a very similar way to *S. cerevisiae*. The transcriptional activator, KlGal4p, shares significant homology to ScGal4p in the DNA-binding, oligomerization, and activation domains but low overall similarity (28% amino acid identity and 57% similarity over their entire length). It binds as a homodimer to sequences in the *GAL* promoters and can functionally substitute for ScGal4p in *S. cerevisiae* (Salmeron and Johnston, 1986). The transcriptional inhibitor, KlGal80p, is highly related to ScGal80p (58% amino acid identity and 82% similarity) and will inhibit transcriptional activation of either version of Gal4p (Zenke *et al.*, 1993). KlGal80p has been demonstrated to be a phosphoprotein which is hyperphosphorylated under non-inducing conditions (Zenke *et al.*, 1999). Upon switching to inducing conditions, where galactose is the sole carbon source, KlGal80p is hypophosphorylated. It has been suggested that this phosphorylation may be involved in the regulation of KlGal4p activity (Zenke *et al.*, 1999). Conversely, there is no evidence to suggest that ScGal80p is phosphorylated. Interestingly, KlGal80p is also phosphorylated when expressed in *S. cerevisiae* under non-inducing conditions (Zenke *et al.*, 1999). The major difference in the genetic switch itself is that there is no ScGal3p homolog in *K. lactis*, instead KlGal1p is a bifunctional galactokinase and transcriptional inducer. KlGal1p is able to complement both an *Scgal3* (ligand sensor deficient) and an *Scgal1* (galactokinase deficient) mutation but neither ScGal3p nor ScGal1p can complement for a *Klgal1* deletion unless KlGal80p is also substituted for ScGal80p. The mechanisms of transcriptional activation of the *GAL* genes in the two yeasts also appear to differ. It has been demonstrated that *in vitro*, the *S. cerevisiae* proteins can form a tripartite complex (Platt and Reece, 1998); however, Anders *et al.* (2006) demonstrated that the binding sites for KlGal4p and KlGal1p on KlGal80p overlap and that transcriptional regulation occurs through competition between the transcriptional activator and inducer. The same authors also demonstrated that the interaction between KlGal80p and KlGal1p occurs in the nucleus, whereas in *S. cerevisiae* it has been suggested that ScGal3p sequesters ScGal80p out of the nucleus into the cytoplasm where they interact (Peng and Hopper, 2000, 2002).

3.3. Components of the *GAL* genetic switch

3.3.1. Gal4p

Gal4p is an 881 amino acid (97 kDa) protein that functions as the transcriptional activator of the *GAL* genes. It is a member of the $Zn(II)_2Cys_6$ binuclear cluster family of proteins (Macpherson *et al.*, 2006). The DNA-binding and dimerization domain of Gal4p is located within the N-terminal 100 amino acids of the protein and recognizes the DNA binding site

5′-CGG(N_{11})CCG-3′. The crystal structure of the DNA binding and dimerization domain of Gal4p bound to its cognate site has been solved and shows that the protein binds as a dimer, and that the DNA-binding domain from the two subunits bind in the major groove of the DNA and on opposite sides of the molecule (see Fig. 3.5; Marmorstein *et al.*, 1992). *In vivo*, Gal4p is constitutively bound to its upstream activator sequence (UAS_{GAL}) upstream of the core TATA-box promoter elements in *GAL* gene promoters (Lohr *et al.*, 1995)—although less binding is observed when yeast are grown in the presence of glucose as the *GAL4* gene is itself subject to glucose repression (Griggs and Johnston, 1991).

Gal4p contains an acidic activation domain, so-called due to its preponderance of acidic amino acids, which is located at the C-terminal end of the protein (amino acids 768–881) and is required for recruitment of the

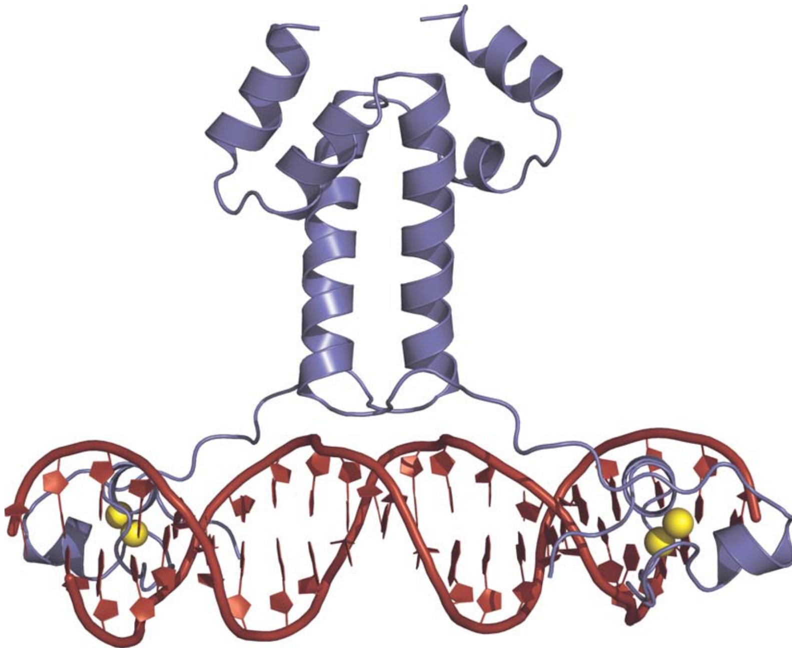

Figure 3.5 The Gal4p-DNA complex. This image represents the crystal structure of the DNA binding and dimerization domain of Gal4p (amino acids 1-100 - PDB code 3COQ) in complex with DNA (Hong et al., 2008). The DNA is shown in red and the protein in blue. The two zinc ions in each monomer required for the structural integrity of the Zn(II)2Cys6 binuclear cluster are shown as yellow spheres. The Zn(II)2Cys6 binuclear clusters interact directly with the CGG triplets within the DNA binding site, while the dimerization domain extends away from the DNA.

transcriptional machinery. The acidic nature of this region is important for its function (Gill *et al.*, 1990), but a mix of acidic and hydrophobic amino acids would appear to be important (Ruden, 1992). Under inducing conditions, the activation domain of Gal4p has been shown to interact with TBP and TFIIB (Ansari *et al.*, 1998; Wu *et al.*, 1996), the Gal11p component of the RNA polymerase II Mediator complex (Jeong *et al.*, 2001; Reeves and Hahn, 2005), and the Tra1p subunit of SAGA (Bhaumik *et al.*, 2004). SAGA, the Spt/Ada/GCN5/acetyltransferase co-activator-histone-modifying complex, is critical to *GAL* gene transcription and is probably recruited first to the gene promoters by Gal4p upon induction (Bhaumik and Green, 2001; Bhaumik *et al.*, 2004; Larschan and Winston, 2001). The disruption of SAGA, by the deletion of *SPT10*, prevents the recruitment of RNA polymerase II to the *GAL* promoters. Once recruited to a gene, SAGA (via interactions with its Spt3p subunit) in turn recruits TBP and the Srb8–Srb11 (Mediator) complex (Bryant and Ptashne, 2003; Dudley *et al.*, 1999; Larschan and Winston, 2001), leading to stable pre-initiation complex formation and RNA polymerase II recruitment, and so allowing transcription to occur.

The activation domain of Gal4p has additionally been shown to co-localize with the Gal80p interaction site and it is the 30 amino acids at the extreme carboxyl terminus that are recognized by Gal80p (Ma and Ptashne, 1987). Indeed, amino acids 869–873 have been demonstrated to cross-link with both Gal80p and components of SAGA and Mediator, among others (Reeves and Hahn, 2005). This, and other evidence (Carrozza *et al.*, 2002; Wu *et al.*, 1996), indicates that Gal80p fulfills its role as a transcriptional inhibitor by blocking the surface by which Gal4p interacts with the transcriptional machinery.

Gal4p is subject to phosphorylation and ubiquitination, and some of these posttranslational modifications may play a regulatory role in controlling Gal4p activity. Gal4p migrates as three distinct forms on SDS-PAGE, representing different phosphorylation states. Under repressing conditions, Gal4p migrates as two distinct forms (Gal4p I and II forms), while the third larger form (Gal4p III) is detectable only under activating conditions, and corresponds to the transcriptionally active version of Gal4p (Muratani *et al.*, 2005; Sadowski *et al.*, 1991). Several sites for Gal4p phosphorylation have been defined, including serine residues 691, 696, 699, and 837 (Sadowski *et al.*, 1996), with Gal4p phosphorylation being mediated by Kin28 and Cdk8/Srb10, cyclin-dependent kinases associated with the RNA Pol II holoenzyme (Hirst *et al.*, 1999). Most of these phosphorylation events appear to occur as a result of activation, rather than as a cause of it (discussed in Leverentz and Reece, 2006). However, phosphorylation on serine 699 (mediated by Cdk8/Srb10) is required for full *GAL* gene activation, with the S699A (non-phosphorylated) mutant

only giving around 20% of the activity of the wild-type protein (Sadowski *et al.*, 1996).

The transcriptionally active phosphorylated form of Gal4p (form III) has been demonstrated to have a half-life of only 5 min, compared to ~20 min for the transcriptionally inactive forms of Gal4p (Muratani *et al.*, 2005). Muratani *et al.* (2005) reported that Gal4p is targeted for proteasome-mediated decay by two different F-box ubiquitin ligases, with Grr1p being responsible for Gal4p turnover under repressing conditions, while Dsg1p targets Gal4p for destruction during *GAL* gene activation. In cells deleted for *dsg1*, translation of *GAL* mRNA is abolished, and it is likely that Dsg1p-mediated destruction of active Gal4p has a role in coordinating transcription-coupled events such as mRNA processing (Muratani *et al.*, 2005).

While F-box ubiquitin ligases such as Grr1p and Dsg1p work by adding polyubiquitin chains to their target proteins, that signal the modified protein for destruction by the proteasome, mono-ubiquitination of Gal4p (by an as-yet unknown E3 ligase) has been reported to protect it from destabilization by proteosomal ATPases (Ferdous *et al.*, 2001). The 19S proteasome is recruited to gene promoters such as those of the *GAL* genes (Gonzalez *et al.*, 2002) and has a non-proteolytic role in regulating elongation by RNA Pol II (Ferdous *et al.*, 2001), and may play other roles such as in stabilizing interactions between SAGA and the components of the transcription initiation machinery (Lee *et al.*, 2005). It is possible that mono-ubiquitination of Gal4p has evolved as a protective measure against unwanted functions of the proteasome at the gene promoter, such as the ATPase activity of the proteasome stripping off the bound activator from the DNA before transcription initiation can occur.

3.3.2. Gal80p

Gal80p is a 435 amino acid (48 kDa) protein that functions as an inhibitor of transcription of the *GAL* genes. Gal80p binds directly to the activation domain of Gal4p (Lue *et al.*, 1987), and is thought to mask the activation domain of Gal4p and restrict the interaction between Gal4p and SAGA (Carrozza *et al.*, 2002). The crystal structure of Gal80p from *K. lactis* has been solved to a resolution of 2.1 Å (Thoden *et al.*, 2007). The protein was found to be exclusively a dimer in solution (Fig. 3.6A). It consists of two identical subunits and each monomer consists of two domains. The N-terminal domain, together with an additional α-helix from the C-terminal domain, contains a classic Rossmann fold which creates a cleft in the protein. The Rossmann fold motif is found in proteins that bind nucleotides, especially the cofactor NAD, and the archetypal structure consists of three or more β-sheets linked by two α-helices. In the case of KlGal80p, six parallel β-sheets are flanked on each side by three α-helices, one of which is from the C-terminal domain of the protein. The C-terminal domain contains a large nine-stranded mixed β-sheet and it is

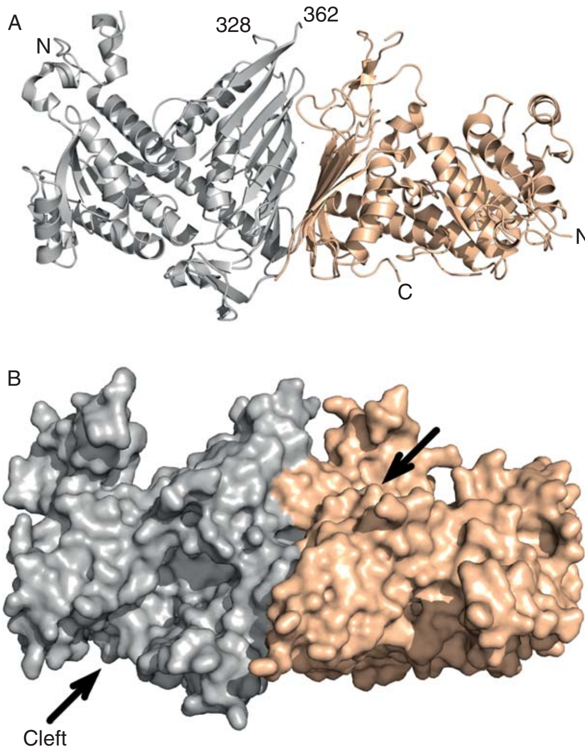

Figure 3.6 The molecular structure of Gal80p from *Kluveryomyces lactis*. (A) A ribbon model of the Gal80p dimer (generated using the PDB code 2NVW). One monomer of the dimer is shown in gray and the other in wheat. The amino- and carboxyl-terminal ends of one monomer are indicated. Residues 328 and 362 are highlighted to show that the polypeptide chain between these residues was not visible in the structure. (B) A space-filling model of the Gal80p dimer. Arrows indicate the cleft in each monomer to which Gal4p has been proposed to bind (Thoden *et al.*, 2007).

this region that is responsible for dimer formation. Dimerization results in the Rossmann fold of each monomer being located on opposite sides of the protein. When viewed as a dimer in a space-filling model (Fig. 3.6B), there is also a distinct groove that runs diagonally across the surface of the protein and it is within this groove that the openings to both clefts reside. There are two short loops regions (Asn-247 to Gly-248 and Gly-311 to Ser-316) and two larger regions (Gly-328 to Glu-361 and Gly-395 to Lys-413) that are not visible in either subunit of the protein. These regions are all located on the same side of the dimer.

The structure of KlGal80p is highly similar to the glucose–fructose oxidoreductase (GFOR) from *Zymomonas mobilis* (Kingston *et al.*, 1996). GFOR has a dual enzymatic role to both catalyze the reduction of fructose

to produce sorbitol and for the oxidation of glucose to produce glucono-lactone. Comparison of the α-carbon chains of KlGal80p and GFOR monomers reveals a root-mean-square deviation of 2.0 Å over 307 structurally equivalent amino acids (Thoden *et al*., 2007). This is particularly remarkable given the amino acid identity between the two proteins is only ~13%, and the fact that GFOR is a tetramer. It is a tetramer of four identical subunits and each subunit contains one tightly, but not covalently, bound NAD(P) molecule. Hydrogen is transferred from glucose to fructose via NAD(P) in a ping-pong mechanism whereby one substrate is converted to a product and released before the other substrate binds (Kingston *et al*., 1996). NAD(P) is not released during the cycle. The major structural difference between KlGal80p and GFOR is that the latter possesses an amino terminal extension of 31 amino acids that are not present in KlGal80p. The removal of these amino terminal residues from GFOR results in the formation of a dimeric protein (Louise Ryan and RJR, unpublished observations). The dinucleotide binding properties of the Rossmann fold in oxidoreductase enzymes have led to the suggestion that that Gal80p may also bind NAD(P) and that this could represent a previously undiscovered level of regulation of the *GAL* genetic switch. From the structure of KlGal80p (Thoden *et al*., 2007), however, two issues would suggest that it is probably unlikely that KlGal80p binds NAD(P). First, the protein crystallized in the absence of the dinucleotide and attempts to grow crystals in the presence of dinucleotide were unsuccessful (James Thoden and Hazel Holden, unpublished observations). Second, Gal80p does not possess one of the signature sequences of a Rossmann fold often found in oxidoreductase enzymes (Gly-X-Gly-X-X-Ala/Gly). KlGal80p contains a three amino acid insertion in this region of the protein, which results in a markedly different conformation in the Rossmann fold. In KlGal80p, this cleft is also considerably wider (~14 Å at the mouth of the cleft) than the oxidoreductase enzymes (~6 Å). In oxidoreductases, the second glycine of the sequence motif packs against the phosphoryl groups of the NAD(P), whereas in KlGal80p, the second glycine of the signature sequence is replaced with a threonine. Given that the side chain of threonine is too bulky to lie against the phosphoryl groups of NAD(P) and that the typical lysine residue that hydrogen bonds to the nicotinamide ribose is replaced with a tryptophan, it is unlikely that it binds NAD(P), at least in the orientation observed in other family members.

One major unanswered question arises from the structure of Gal80p—why is the inhibitor of the *GAL* genetic switch homologous to an oxidoreductase? The likely reason is that Gal80p has evolved from an oxidoreductase. In this way, the enzyme scaffold of the oxidoreductase has been hijacked to form a transcriptional regulator. It is not known what type of oxidoreductase Gal80p may have evolved from and therefore what substrates would have been bound, and it is possible that Gal80p has completely lost the ability to bind the substrates and cofactor of its predecessor.

3.3.3. Gal3p

The third protein involved in control of the *GAL* system is Gal3p—the inducer or ligand sensor. Gal3p interacts with Gal80p in a galactose- and ATP-dependent manner, and this interaction results in activated transcription by Gal4p. Gal3p, therefore, acts as the sensor and transducer of the galactose signal in the induction of Gal4p-activated genes (Suzuki-Fujimoto *et al.*, 1996; Yano and Fukasawa, 1997). Gal3p shows a large degree of homology with the previously discussed galactokinase, Gal1p—see Section 2.2. Gal1p and Gal3p share ~70% amino acid identity and >90% similarity over their entire length. Despite this similarity, Gal3p has no galactokinase activity itself. However it can be imparted with, albeit weak, galactokinase activity by the addition of two amino acids (a serine and an alanine immediately following amino acid 164) which are not normally present in Gal3p (Platt *et al.*, 2000). This underscores the functional similarities between the proteins.

In the absence of Gal3p, however, *GAL* gene induction still occurs but with a significant delay in the onset of expression in the order of 3–4 days (Bhat *et al.*, 1990). This is through the action of Gal1p, which can substitute for Gal3p function in gene expression because it can act as a bifunctional galactokinase/transcription inducer. A yeast strain deleted for both the *GAL1* and *GAL3* gene can never induce the expression of the other *GAL* genes (Broach, 1979), and the introduction of 7–10 additional plasmid-borne copies of the *GAL1* gene significantly reduces the delay in induction to 4.5 h (Bhat *et al.*, 1990).

The high level of similarity between ScGal1p and Gal3p has allowed the crystal structure of ScGal1p (discussed in Section 2.2) to be used to construct a homology model for Gal3p (Thoden *et al.*, 2005; see Fig. 3.7). The structure of ScGal1p has only been solved in the presence of both ligands, galactose and the non-hydrolyzable ATP analog AMP–PNP, therefore the homology model which is based on this structure represents ScGal3p in the state where it is competent for interaction with ScGal80p. To date, it has not been possible to crystallize ScGal1p in the absence of ligands which led to the suggestion that in the absence of ligands the N- and C-terminal domains are flexible with respect to each other, whereas following binding of the ligands a more stable conformation is adopted which forms the binding site for ScGal80p. From the homology model, it was not obvious how and where ScGal3p would interact with ScGal80p. In an effort to understand this interaction, mutations that affect the ScGal3p–ScGal80p interaction were mapped onto the homology model (Fig. 3.7). The mutations fall into two types; non-inducible and constitutive mutations. The non-inducible mutations are defective for their interaction with ScGal80p and when these were mapped, they were poorly defined which might indicate that they result in defects in either the structure or function of the protein. The constitutive mutations, on the other hand, have a reduced requirement for galactose and ATP to interact with ScGal80p;

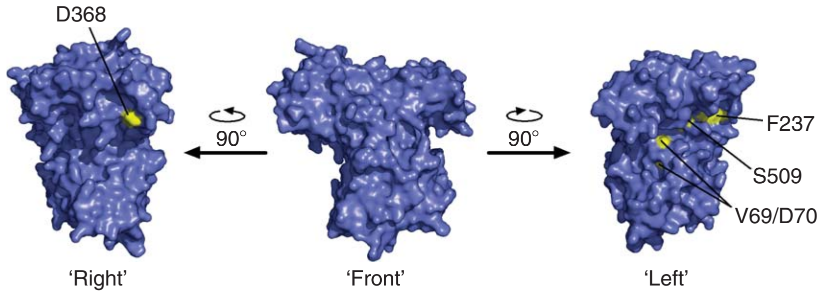

Figure 3.7 The interactions between members of the *GAL* genetic switch. A surface rendered homology model of Gal3p based on the structure of ScGal1p (Thoden *et al.*, 2005) showing the potential interaction between Gal80p and Gal3p. The front of the molecule is shown in the approximate same orientation as in Fig. 3.3. The constitutive mutations are highlighted in yellow on models that have been rotated 90° around the vertical axis, either clockwise or counterclockwise.

hence these mutations represent a gain of function rather than a loss of function and are therefore inherently more interesting. *In vivo* these mutations result in increased transcriptional activation in the absence of ligands, and *in vitro* these mutant proteins are able to interact with ScGal80p in the absence of galactose, although some require ATP. Nine constitutive Gal3p mutations have been previously identified: L50P, V69E/D70V, V203I, F237Y, D368V, V396A, F414L, S509P/L/D, and K510E (Blank *et al.*, 1997). When mapped onto the homology model of ScGal3p (Fig. 3.7), all the mutations except F414L lie on the interface between the N- and C-terminal domains of the protein. Of the mutations that lie on the interface, four (residues 69/70, 237, 509, and 510) are within 16 Å of each other along a groove on one side between the two domains. Two mutations (residues 203 and 368) lie on the opposite face of the protein on the interface and the final two mutations (residues 50 and 396) are buried in the middle of the protein in the C-terminal domain but still residing on the interface between the two domains. Constitutive mutations for KlGal1p have also been identified (Menezes *et al.*, 2003; Vollenbroich *et al.*, 1999) and, although the equivalent residues in the *S. cerevisiae* protein are not always conserved, those that were conserved were mapped onto the model (Thoden *et al.*, 2005). The KlGal1p mutations are much more diverse and the majority fall into two distinct categories. The first (equivalent residues Asp-293 and Asn-294) fall at the interface of the N- and C-terminal domains of Gal3p in a region that is disordered in the ScGal3p model. Other constitutive KlGal1p mutants (equivalent residues Ser-44, Phe-94, and Cys-152) occur close to the nucleotide-binding site and may mimic the state of the protein when the nucleotide is bound. The final two KlGal1p constitutive mutants that do not fall into either category (equivalent residues

Leu-78 and Leu-394) occur at locations distant to either the N- and C-terminal interface or the ligand binding sites. The mechanism by which these mutations may act is not known.

Based on the homology model for ScGal3p (Fig. 3.7) and the positions of the constitutive mutations, a model for the galactose and ATP-dependent interaction with ScGal80p was proposed. It was suggested that in the absence of galactose and ATP, the N- and C-terminal domains of Gal3p are flexible, as has also been suggested for ScGal1p, with respect to each other (Thoden *et al.*, 2005). Upon binding of the ligands, a more rigid structure is adopted, and the interface between the two structural domains of the protein forms the binding site for ScGal80p. It is predicted that ScGal80p contacts ScGal3p at multiple points around the interface between the domains of ScGal3p. An alternative model suggested by Menezes *et al.* (2003) predicts that parts of Gal80p may fit into the groove between the two domains. However, this theory was based on a homology model of KlGal1p based on the structures of four GHMP family members (Diphosphomevalonate decarboxylase (MDD) from *S. cerevisiae*; homoserine kinase (HSK) from *M. jannaschii*; mevalonate kinase (MVK) from *M. jannaschii*; phosphomevalonate kinase (PMK) from *S. pneumoniae*), which are poorly conserved (MDD, 9% identity; HSK, 9%; MVK, 17%; PMK, 6%). This, together with the crystal structures of ScGal1p and KlGal80p (Figs. 3.3 and 3.6, respectively) make this interaction unlikely.

The extraordinary levels of similarity between ScGal3p and ScGal1p strongly suggest that ScGal3p evolved from the galactokinase so why is there a need for a separate ligand sensor? In *K. lactis*, the galactokinase KlGal1p acts as both the galactokinase and the ligand sensor/transcriptional inducer (Meyer *et al.*, 1991) and, similarly, ScGal1p can also fulfill both of these roles in *S. cerevisiae* in the absence of ScGal3p, although not as efficiently with a significant lag-time in the response (Bhat *et al.*, 1990). The answer to this lies in the regulation of transcription. By having an inducer protein, it allows the *GAL* genes in *S. cerevisiae* to be tightly regulated with very low basal levels of transcription which are induced 1000-fold in the presence of galactose (Johnston *et al.*, 1994). This means that ScGal1p is one of the most tightly regulated genes in the genome. By contrast, ScGal3p has a higher basal level of transcription but is only upregulated three- to fivefold under inducing conditions (Bajwa *et al.*, 1988). How then did *S. cerevisiae* evolve to have a separate galactokinase and ligand sensor? The origins of this can be traced to a whole genome duplication about 100 million years ago (Wolfe and Shields, 1997). Hittinger and Carroll (2007) have proposed a model in which the presence of two identical copies of an ancestral bifunctional galactokinase/inducer allowed one copy to accumulate mutations which resulted in the loss of its galactokinase activity and most of the Gal4p binding sites in the promoter of its gene yielding a weakly inducible regulatory protein. The presence of the other copy of the gene would

prevent these mutations affecting the fitness of the yeast. Subsequently, this would allow the second copy to evolve to become a more tightly regulated galactokinase without affecting the yeasts ability to rapidly respond to the presence of galactose. This hypothesis would explain why induction by ScGal1p is so weak, since ScGal3p has a higher basal level of transcription with a low level of induction in the presence of galactose, whereas transcription of ScGal1p is tightly controlled by ScGal4p. This creates a feedback loop that, in the absence of Gal3p, requires leaky expression from the *GAL1* promoter for induction of transcription and explains the three- to four-day lag phase upon switching of *Scgal3* deletion mutants to galactose as the sole carbon source. Increasing the copy number of the *GAL1* gene and hence the basal levels of the protein reduces the lag time from 3–4 days to 4.5 h therefore highlighting this point. KlGal1p transcription is also controlled by KlGal4p and therefore would be subject to a feedback loop but the basal level of transcription of the *K. lactis GAL* genes is much higher and therefore there is sufficient KlGal1p present in the cell to efficiently induce transcription without a significant lag time.

3.4. Interactions between the *GAL* genetic switch proteins

The regulation of the transcriptional activity of the *GAL* genetic switch is dependent on protein–protein interactions—in particular those between Gal4p and Gal80p and between Gal80p and Gal3p. The co-localization of the activation domain of Gal4p and its site of interaction with Gal80p (Ma and Ptashne, 1987) make structural analysis of this interaction particularly important to gain insights into the nature of an acidic activation domain. Several mutational analyses of ScGal80p have been performed (Melcher, 2005; Pilauri *et al.*, 2005) and recent studies have identified mutations that result in protein that is defective in either Gal4p or Gal3p binding (Pilauri *et al.*, 2005). These mutations are particularly interesting since loss of only one function indicates that the protein is still viable. Given the amino acid sequence homology between *S. cerevisiae* and *K. lactis* Gal80p (58% amino acid identity and 82% similarity), these mutations were mapped onto the KlGal80p structure (Thoden *et al.*, 2007).

3.4.1. The Gal80p–Gal4p interaction

Those mutations that give rise to defective Gal4p binding to the ScGal80p and that are visible in the KlGal80p model are located at positions Gly-153, Gly-184, Arg-190, Asp-261, His-262, Gly-283, and Leu-320 (Fig. 3.8; Pilauri *et al.*, 2005). Five of these are located at the dimer interface (Arg-190, Asp-261, His-262, Gly-283, and Leu-320) and could potentially result in disruption of the dimer. The mutations at Gly-153 and Gly-184, however, are particularly interesting because they are separated by 17 Å and lie on either side of a large cleft formed by the C-terminal end of the β-sheet

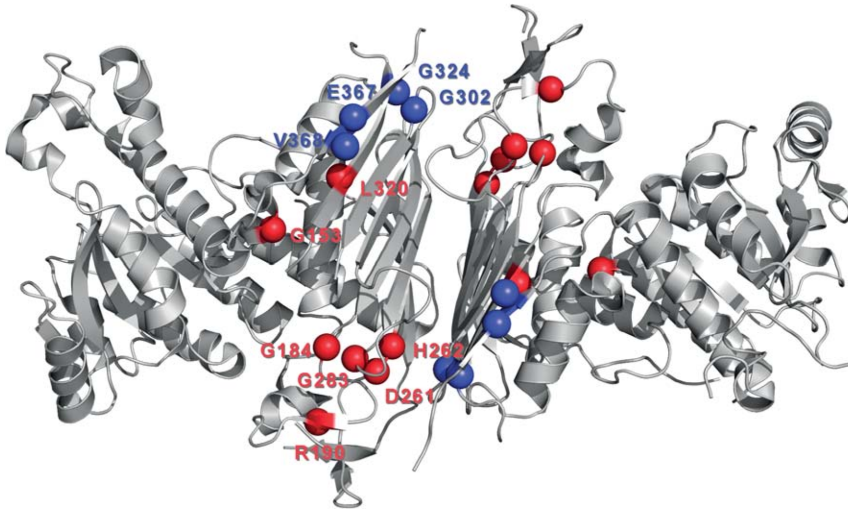

Figure 3.8 Mutations in Gal80p that result in either defective Gal4p or Gal3p binding. The positions of the mutations in Gal80p that are known to give rise to defective Gal4p binding are highlighted by the red spheres. The positions of the mutations in ScGal80p that are known to give rise to defective Gal3p binding are mapped onto the Gal80p model (Fig. 3.6B) and are shown in blue. For the sake of clarity, only those mutations in one subunit of the dimer are labeled.

in the Rossmann fold and an α-helix defined by Ser-211 to Ile-222. Two additional mutations, A309T and G310D, that have been identified in ScGal80p that result in defective Gal4p-binding cannot be mapped onto the structure because the corresponding amino acids in KlGal80p, namely Ala-310 and Gly-311, reside in a disordered surface loop of six residues that was not visible in the crystal structure. This loop connects two antiparallel *β*-strands and is situated at the top of the cleft. In GFOR, there is a three-residue deletion in the loop, which folds in toward the nicotinamide ring of the NAD(P), which presumable stabilizes its structure allowing it to be seen in the crystal structure (Kingston *et al.*, 1996). As has already been mentioned, this cleft in GFOR and similarly related enzymes is the binding site for NAD(P); however, the cleft in KlGal80p is much wider with no apparent salt bridges to close the gap and the stereochemistry of the amino acids inside the cleft would seem to preclude the binding of NAD(P). Given the clustering around the cleft of four of the mutants that were defective only in the interaction with Gal4p, and the three-dimensional characteristics of the cleft, it has been suggested that this region forms the binding site for Gal4p. In order to further predict the interaction site, Thoden *et al.* (2007) used the latest prediction algorithms for peptides to define the secondary structure of the 30 C-terminal amino acids of KlGal4p which

are the equivalent to the C-terminal 30 residues of ScGal4p that have been experimentally demonstrated to be the amino acids recognized by ScGal80p (Ma and Ptashne, 1987). Using this method, these amino acids were predicted to form an α-helix; however, other studies, in marked contrast, have suggested that the activation domain of ScGal4p is β-sheet at low pH and is essentially unstructured at physiological pH (Leuther *et al.*, 1993; Van Hoy *et al.*, 1993). In an α-helix, the hydrogen bonding capacity of the backbone carbonyl groups and amide nitrogens is mostly satisfied. On the other hand, in a β-hairpin motif, for example, the backbone hydrogen bonding pattern would not be completely satisfied if it were to bind into the type of cleft observed in Gal80p which is devoid of β-sheet. On the basis of both secondary structural predictions, and the nature of the Gal80p putative binding cleft, it was predicted that the C-terminal 30 residues of KlGal4p most likely bind into the KlGal80p cleft as an α-helix. It is also conceivable, however, that the C-terminal end of KlGal4p may bind over the top of the cleft in the grove that runs around the protein. Co-crystallization experiments with Gal80p and a peptide representing the C-terminus of KlGal4p are in progress to address this issue. In either scenario, it is conceivable that binding of Gal4p in or over the cleft may prevent NAD(P) from entering the cleft. It is possible, however, that NAD(P) binding would inhibit binding of Gal4p if it was bound in the cleft, although current observations would suggest that this is not the case.

3.4.2. The Gal80p–Gal3p/Gal1p interaction

From the homology model of ScGal3p built on the crystal structure of ScGal1p, a number of constitutive mutants were mapped onto the model to give insights into areas in which ScGal3p may interact with ScGal80p. As already discussed (Section 3.3.3), this showed that the mutations mapped close to the interface between the N- and C-terminal domains of ScGal3p, which therefore may be the area of interaction with ScGal80p. This was repeated with the structure of KlGal80p to indicate the interaction site where ScGal3p binds using the Gal3p-defective ScGal80p mutations. The mutations in ScGal80p that are defective in only Gal3p binding correspond to Gly-302, Gly-324, Glu-367, and Val-368 in KlGal80p (Fig. 3.8; Pilauri *et al.*, 2005). When mapped onto the structure of KlGal80p, their mutations are clustered and map to the structure at the edge of the mixed β-sheet in the C-terminal domain. These residues are located near a large disordered region between Gly-328 and Glu-362 which is not visible in the crystal structure which makes it impossible to model Gal3p onto this site. It has been suggested that these residues mark the binding surface for Gal3p and that the disordered region in KlGal80p becomes ordered upon binding of the ligand sensor (Thoden *et al.*, 2007). It had been proposed that the binding site of ScGal3p on ScGal80p may overlap with the dimerization domain of ScGal80p such that binding of

ScGal3p competes with dimerization of ScGal80p (Anders *et al.*, 2006; Thoden *et al.*, 2007). Additionally, Timson *et al.* (2002) demonstrated using gel filtration and protein cross-linking that ScGal80p and ScGal3p interact to form a 1:1 complex with one ScGal80p monomer interacting with one ScGal3p monomer. While the proposed interaction site for KlGal1p on KlGal80p is close to the dimerization domain, both light scattering and gel filtration experiments have demonstrated that KlGal80p is dimeric following binding of KlGal1p (Thoden *et al.*, 2007); therefore, it is a distinct possibility that the mechanism of binding is different in *S. cerevisiae* and *K. lactis*.

3.5. Mechanism of *GAL* gene expression

The molecular mechanism by which the activation of the *GAL* genes occurs has been the subject of much debate. In *S. cerevisiae*, two somewhat conflicting models for its molecular mode of action have been proposed (Fig. 3.9). It has been suggested that the induction of the *GAL* genes occurs via the association of a tripartite complex formed between ScGal4p, ScGal80p, and ScGal3p resulting in a conformational change in ScGal80p

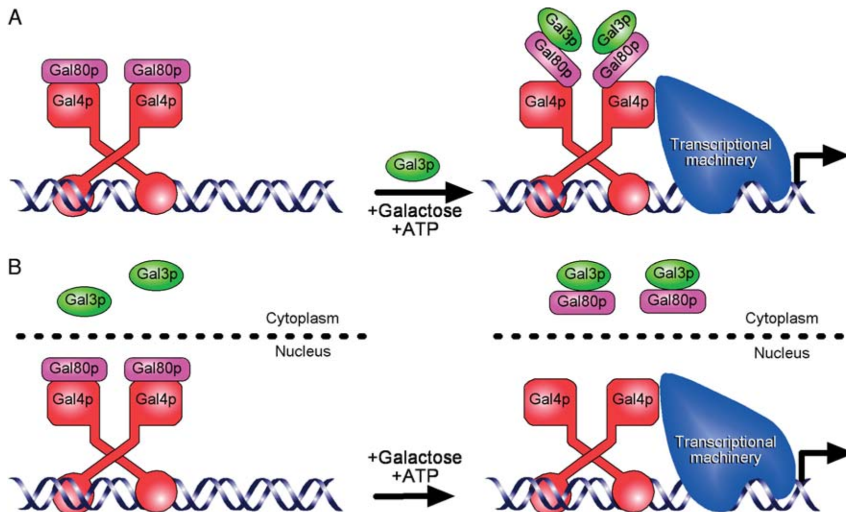

Figure 3.9 Models for *GAL* gene activation. (A) A non-dissociation model in which activation occurs via the formation of a tripartite complex of Gal4p, Gal80p, and Gal3p in the presence of galactose and ATP. A conformational change in Gal80p enables Gal4p-mediated recruitment of the transcriptional machinery. (B) A dissociation model in which the interaction between Gal3p and Gal80p results in a reduction in the nuclear concentration of Gal80p, thereby, enabling Gal4p to interact with the transcriptional machinery.

and thus relieving its inhibitory effects (Platt and Reece, 1998). In favor of this model (Fig. 3.9A) are the observations that (1) ScGal4p purified from yeast, grown either in the presence or absence of galactose, is associated with ScGal80p (Parthun and Jaehning, 1992); (2) artificially constructed ScGal80p molecules that contain an activation domain can regulate transcription in the presence and absence of galactose (Leuther and Johnston, 1992); (3) *in vitro,* the three proteins can be seen to assemble in a gel-shift assay (Platt and Reece, 1998); and (4) using fluorescence resonance energy transfer (FRET), ScGal4p and ScGal80p did not dissociate from each other in the presence or absence of galactose (Bhaumik *et al.*, 2004).

Other evidence, however, suggests that this model is incorrect and that ScGal80p dissociates from ScGal4p and interacts with ScGal3p in the cytoplasm of yeast cells (Peng and Hopper, 2000). This would result in the freeing of ScGal4p from the inhibitory effects of ScGal80p and enabling transcriptional activation to occur (Peng and Hopper, 2002). The dissociation model (Fig. 3.9B) is supported by data indicating that (1) ScGal3p is predominately, and perhaps exclusively, cytoplasmic (Peng and Hopper, 2000), (2) the expression of a myristoylated version of ScGal3p (which is targeted to the plasma membrane of the cell) does not unduly impair the induction of the *GAL* genes (Peng and Hopper, 2002), and (3) in chromatin immunoprecipitation experiments (Peng and Hopper, 2002) and pull-down assays (Sil *et al.*, 1999), the ScGal4p–ScGal80p complex is somewhat weakened (although perhaps not completely dissociated) when cells are grown in the presence of galactose. To date, no definitive evidence has been published to conclusively disprove either model.

In *K. lactis*, the mechanism is thought to be different from that of *S. cerevisiae*. It has been proposed by Anders *et al.* (2006) that the KlGal4p and KlGal1p binding sites are overlapping so that only one interaction can occur at a time, and that this interaction occurs exclusively in the nucleus with KlGal1p able to shuttle between the cytoplasm and nucleus. In all of these models, the ability of the inducer/ligand sensor to interact with Gal80p in a galactose-dependent manner is essential for the transcriptional induction of the *GAL* genes. Consequently, understanding this interaction is a key to understanding the transcriptional activation in response to galactose.

3.6. Regulation of *GAL* gene expression in other yeasts

Regulation of the *GAL* gene expression in other yeasts beyond *S. cerevisiae* and *K. lactis* is less well characterized. Orthologs of Gal4p have been identified in other yeasts but this is usually based on similarities in the DNA-binding domain of the proteins. The genome of the yeast *Candida albicans* encodes a Gal4p homolog (termed CaGal4p) which had 86% similarity with ScGal4p in the DNA-binding domain, but shows no significant similarity in the activation domain. This protein was presumed to be

the transcriptional activator of the *C. albicans GAL* genes until a study by Martchenko *et al.* (2007b) showed that deletion of this gene had no effect on *GAL* gene activation. Instead, they identified a homolog of *S. cerevisiae* Ste12p, CaCph1p, which was responsible for *GAL10* expression (Martchenko *et al.*, 2007a). It acts through the second of two regulatory sites, which represent an enhancer and a galactose/dextrose-responsive element. Analysis of genomic data from *ascomycota* (sac fungi) showed that close relatives of *S. cerevisiae* contain classic tandem Gal4p-binding sites and lack Cph1p binding sites, whereas *C. albicans* and its close relatives are vice versa. This suggests that there are significant differences in the regulation of the *GAL* genes in other yeasts. The differences in regulation may be due to the different roles for which galactose is used in different yeast strains. Galactose in *C. albicans*, for instance, also plays important roles in adhesion and biofilm formation, which are required for the pathogenic nature of this yeast and are absent in *S. cerevisiae* (Jin *et al.*, 2004).

A number of genes in other yeast have been identified as candidates for Gal80p homologs, although these tend to have low similarity (e.g., the *C. albicans* candidate has 40% similarity) (Martchenko *et al.*, 2007b). It is possible that these genes represent genuine Gal80p homologs; however, it is equally possible that they are simply close structural relatives of Gal80p, such as an oxidoreductase, that has no capacity for transcriptional regulation. This is particularly likely in yeast strains such as *C. albicans* where transcriptional regulation does not appear to be controlled by a Gal4p homolog. To date, no candidates for Gal3p homologs have been identified in any other yeast strains. It may be that, as in the case of *K. lactis*, the Gal1p homolog is a bifunctional galactokinase and transcriptional inducer; however, in the absence of a Gal80p homolog, there would presumably be no requirement for a Gal3p-type transcriptional inducer.

Although there are differences in the regulation of the *GAL* genes, the genomic organization of the *GAL* genes in *ascomycota* shows clustering of *GAL1*, *GAL7*, and *GAL10* in each example studied. These include the *Saccharomyces*, *Kluyveromyces*, *Debaryomyces*, *Candida*, and *Schizosaccharomyces* species (Martchenko *et al.*, 2007b). In contrast to *ascomycota*, the *GAL* genes of filamentous fungi, such as *Aspergillus nidulans*, *H. jecorina*, and *N. crassa*, are not clustered. They also have a high basal level of expression during growth on all carbon sources, including glucose, indicating some major differences in the regulation of the *GAL* genes between yeasts and filamentous fungi (Seiboth *et al.*, 2002, 2004). Analysis of galactose metabolism in *H. jecorina* revealed that *HjGAL1* and *HjGAL7*, but interestingly not *HjGAL10*, are further induced by both galactose and L-arabinose. *HjGAL10* also lacks the C-terminal mutarotase domain present in the yeast protein (Seiboth *et al.*, 2002). A search of the *H. jecorina* genome showed that this fungus does not possess an *ScGAL3* ortholog. Therefore, it was initially assumed that regulation of the *GAL* genes in *H. jecorina*

followed the model of the *K. lactis GAL/LAC* regulon; however, HjGal1p was completely dispensable for basal as well as induced transcription (Hartl *et al.*, 2007).

4. Chromatin Re-Localization and Transcriptional Memory in *GAL* Gene Regulation

A number of inducible *S. cerevisiae* genes have been observed to re-localize to the nuclear periphery upon activation, via an interaction between the chromatin and the nuclear pore complex (Brickner and Walter, 2004; Cabal *et al.*, 2006; Casolari *et al.*, 2004; Dieppois *et al.*, 2006; Schmid *et al.*, 2006; Taddei *et al.*, 2006). This has generated much interest as previously the nuclear periphery was instead associated with gene silencing, for instance at telomere ends (Taddei *et al.*, 2004) and silent mating type loci (Andrulis *et al.*, 1998). Research has aimed to elucidate whether the gene re-localization events have a regulatory role in gene expression.

4.1. Localization of the induced *GAL* genes to the nuclear periphery

The *GAL* gene cluster located on chromosome II in *S. cerevisiae* consists of the *GAL1*, *GAL10*, and *GAL7* genes. Cabal *et al.* (2006) tagged this locus with a fluorescent marker in order to track chromatin movement in three dimensions. They demonstrated that the *GAL* gene cluster displayed marked differences in dynamic mobility between the repressed and activated states. Essentially, the *GAL* gene cluster was randomly positioned within the nucleus when the genes were not being transcribed, but became confined to the nuclear envelope upon activation. Although *GAL* gene re-localization was not observed in all cells examined (71% displayed re-localization), RNA fluorescence *in situ* hybridization experiments utilizing a *lacZ* reporter revealed that only those *GAL* genes that re-localized to the nuclear periphery were actively transcribed (Cabal *et al.*, 2006). Is *GAL* gene re-localization required for proper activation, or is it simply some effect of a co-transcriptional process that results in efficient export of mRNA? Several of the molecular factors involved in the process of re-localization have been identified, giving some vital clues as to the nature of this process.

Previously, interactions between the nuclear pore complex and a wide range of *S. cerevisiae* genes had been reported (Casolari *et al.*, 2004). The nuclear pore complex is involved in regulating mRNA export and interacts

with components of the mRNA export machinery. Furthermore, some of these same mRNA export factors interact with the transcriptional machinery and may function in transcription-coupled mRNA export (Vinciguerra and Stutz, 2004). For example, Sac3p, part of the Sac3p–Thp1p–Cdc31p mRNA export complex, interacts with the Ada2p subunit of transcriptional co-activator SAGA via the adaptor protein Sus1p (Rodriguez-Navarro *et al.*, 2004). The Spt7p subunit of SAGA may also interact with Mlp1p and Mlp2p (Luthra *et al.*, 2007), filamentous proteins associated with the nuclear basket and which help in the anchoring of mRNA export factors (such as Sac3p) to the nuclear pore complex (Green *et al.*, 2003; Kosova *et al.*, 2000).

Galactose-induced *GAL* gene re-localization was found to be diminished following deletion of Nup1p (nuclear pore complex component), Sac3p, Sus1p, or Ada2p, suggesting that the genes were being physically tethered to the nuclear periphery via interactions between the nuclear pore complex, mRNA export factors, and SAGA (Cabal *et al.*, 2006). However, deletion of Nup1p or Ada2p did not affect induced *GAL1* mRNA levels, suggesting that gene re-localization was not involved in regulating gene expression (although Sus1p and Sac3p deletion did markedly reduce *GAL1* mRNA levels, although this may be due to effects on mRNA export) (Cabal *et al.*, 2006). The removal of Mex67p, another mRNA export factor that interacts with Sac3p and the TREX mRNA export complex (Fischer *et al.*, 2002; Zenklusen *et al.*, 2001), from cells was found to abolish induced re-localization for both the *GAL10* and *HSP104* genes. The deletion of *MEX67* did not, however, appear to affect mRNA expression levels of these genes (Dieppois *et al.*, 2006). Hence, *GAL* gene re-localization appears to be due to interactions between gene, transcription factors, mRNA processing factors, and the nuclear pore complex. *GAL* gene re-localization is not necessary for gene activation and, instead, appears to be a consequence of downstream mRNA processing events. It is yet to be determined whether gene re-localization to the nuclear periphery plays a regulatory role in these downstream events.

In contrast, proximity to the nuclear periphery has been reported to regulate expression of the *INO1* and *HXK1* genes, as demonstrated by artificially tethering the genes to the nuclear membrane (Brickner *et al.*, 2007; Taddei *et al.*, 2006). It has been proposed that the gene re-localization step somehow optimizes mRNA export rates (Cabal *et al.*, 2006; Schmid *et al.*, 2006). Alternatively, it is possible that *GAL* gene re-localization is simply a product of coupling transcription to downstream activities such as mRNA export, and is not in itself a regulatory device. Several contradictions as to which factors are necessary for the gene–nuclear periphery interaction can be found in the literature. For example, Schmid *et al.* (2006) show that the *GAL1* gene displays a galactose-dependent interaction with the Nup2p component of nuclear pore complex even in the absence of SAGA; instead, this interaction was dependent upon the presence of UAS_{GAL} and TATA-box

sites in the *GAL1* promoter together with the Gal4p activator protein. Meanwhile, Cabal *et al.* (2006) had reported that Nup2p was non-essential for *GAL* gene re-localization. It is possible that such discrepancies are indicative of one or more different pathways by which gene–nuclear periphery interactions can occur. Clearly, more work needs to be done here. A model based on current understanding is presented (Fig. 3.10).

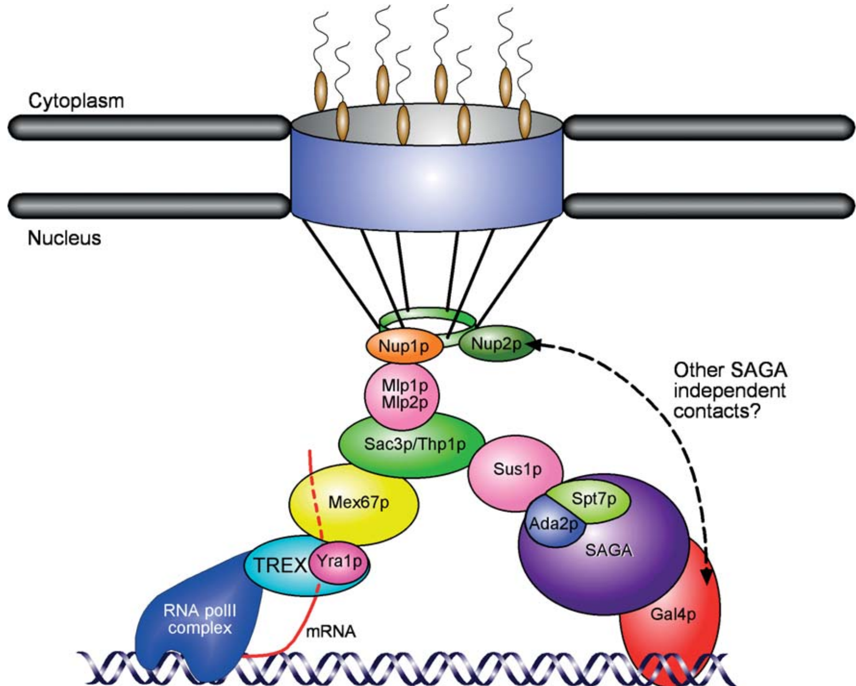

Figure 3.10 Galactose-induced re-localization of the *GAL* gene cluster and interaction between the genes and the nuclear periphery may be mediated as follows. The nuclear pore complex is a multi-protein channel which spans the nuclear membrane. On the nuclear side, the nuclear pore complex forms a basket-like structure (Fahrenkrog *et al.*, 1998). Mlp1p and Mlp2p are associated with the nuclear basket and help in the anchoring of mRNA export factors such as Sac3p to the nuclear pore complex (Green *et al.*, 2003; Kosova *et al.*, 2000). The Nup1p nuclear pore complex component may tether genes via interactions between the Ada2p component of SAGA, the Sac3p component of the Sac3p–Thp1p–Cdc31p mRNA export complex, and the adaptor protein Sus1p (Cabal *et al.*, 2006). SAGA may also interact with Mlp1p and Mlp2p via its Spt7p subunit (Luthra *et al.*, 2007). Another nuclear pore complex component, Nup2p, may interact with the *GAL* gene promoter in a SAGA-independent way via the DNA bound transcription factor Gal4p (Schmid *et al.*, 2006). Mex67, another mRNA export factor which interacts with both Sac3p and the Yra1p component of the mRNA export complex TREX (Fischer *et al.*, 2002; Zenklusen *et al.*, 2001), may also be involved in tethering genes to the nuclear periphery (Dieppois *et al.*, 2006).

4.2. Transcriptional memory

The expression of the *GAL* genes has been reported to display a type of transcriptional memory, where the rate of transcriptional activation is higher in cells which have previously experienced galactose compared to those that have not (Brickner *et al.*, 2007; Kundu *et al.*, 2007). *GAL* transcriptional memory was first demonstrated by Kundu *et al.* (2007), who grew yeast cells with galactose, treated them with glucose to repress *GAL* gene activity, and then induced the *GAL* genes again with galactose. On the initial induction, *GAL1* transcript was detectable within 20 min, taking over an hour to reach maximal levels, while the maximum was attained within 5 min following repression and re-induction (Kundu *et al.*, 2007). Furthermore, this effect persisted for at least one cell generation, and was dependent upon chromatin remodelling enzyme SWI/SNF (Kundu *et al.*, 2007), suggesting an epigenetic mechanism for regulating gene expression in yeast, perhaps by modifying chromatin structure.

Intriguingly, Brickner *et al.* (2007) proposed that *GAL* transcriptional memory was propagated by a mechanism involving the galactose-induced interaction between the *GAL* locus and the nuclear pore complex. While galactose-induced re-localization of the *GAL* locus to the nuclear periphery has not been found to influence gene expression levels (Cabal *et al.*, 2006; Dieppois *et al.*, 2006), *GAL1* was found to be retained at the periphery even after gene repression by glucose administration (Brickner *et al.*, 2007). Furthermore, *GAL1* nuclear periphery localization persisted for several generations' growth in glucose media (Brickner *et al.*, 2007).

Both *GAL1* transcriptional memory and the persistent interaction between the gene and the nuclear periphery after transcriptional shutoff seemed to require histone variant H2A.Z (Brickner *et al.*, 2007). H2A.Z, which replaces histone H2A in a significant proportion of nucleosomes and is highly conserved among eukaryotes, is found at the promoters of many repressed genes in yeast, and also functions in preventing the spread of silencing heterochromatin at genes near telomeres and silent mating-type cassette loci (Guillemette *et al.*, 2005; Meneghini *et al.*, 2003). However, although H2A.Z appeared to be required for the persistent perinuclear localization and rapid reactivation of *GAL1*, it does not necessarily follow that gene re-localization confers transcriptional memory. The loss of H2A.Z had previously been reported to cause a reduction in the rate of *GAL1* gene activation, although this effect was not dramatic, and was not related to previous exposures to galactose (Gligoris *et al.*, 2007). Hence H2A.Z may be involved in a general mechanism for keeping repressed genes in a primed state for activation, which is not related to any form of gene memory. Indeed, it is difficult to see how an alternative histone composition at the gene promoter could be preserved following histone disassembly during replication. No mechanism has been reported for the propagation of any

such histone code (a point argued in Ptashne, 2007). It is more likely that a *trans*-acting factor retained in the cell during cell division is functioning to somehow restore the primed state of the previously induced gene.

Zacharioudakis *et al.* (2007) used heterokaryon assays to show that transcriptional memory at the *GAL* gene cluster was not associated with chromatin and was instead due to the inheritance of a cytoplasmic factor, namely the galactokinase Gal1p. Gal1p can substitute for the homologous protein Gal3p in galactose sensing and inducing, although Gal1p is expressed at extremely low levels under repressing conditions so induction by this protein normally takes days rather than minutes as for induction by Gal3p (see Section 3.3.3 above). However, Gal1p protein levels increase by around a 1000-fold following galactose induction, and it seems that enough Gal1p survives in the cytoplasm after gene repression and cell division to enable rapid *GAL* gene activation on future exposures to galactose (Zacharioudakis *et al.*, 2007). In the absence of Gal1p, expression of Gal3p from the *GAL1* promoter also enabled rapid reactivation of the *GAL* genes, providing more evidence that the transcriptional memory was a result of positive feedback by *trans*-acting cytoplasmic factors rather than by chromatin modifications and gene localization events (Zacharioudakis *et al.*, 2007). Presumably, the increased concentration of the inducer (Gal1p or Gal3p) allows a more rapid alleviation on Gal4p from the effects of the Gal80p repressor protein. However, it is not clear how the *GAL* locus remains tethered to the nuclear periphery after several generations of inactivity, as was previously observed (Brickner *et al.*, 2007). Over-expression of Gal3p is known to cause constitutive *GAL* gene expression, even in the absence of galactose (Bhat and Hopper, 1992). Is the level of Gal1p which persists after gene repression and cell replication sufficient for *GAL* gene induction, and therefore the chromatin–nuclear pore interaction, even in the absence of galactose? What is the involvement of H2A.Z? This area of research is relatively young, and the research cited here largely represents a work in progress. Further examination of the involvement of chromatin modifications, gene–nuclear pore interactions and *trans*-acting factors on *GAL* gene regulation would be useful here.

The above can be summarized as follows (Fig. 3.10): After galactose induction, the *GAL* gene cluster becomes transcriptionally active and, at the same time, becomes localized to the nuclear periphery. The *GAL* genes are tethered to the periphery by physical interaction with the nuclear pore complex, which may also involve SAGA and mRNA export factors. However, no effect on gene regulation from this event has been found, and it may simply represent an effect of coupling transcription with downstream processes. After transcriptional shutoff, the *GAL* gene cluster is retained at the periphery, possibly by interaction between the nuclear pore complex and the histone variant H2A.Z. While the presence of H2A.Z may contribute to rapid activation of the *GAL* genes, it is most

likely that the observed transcriptional memory at *GAL* loci is due to sustained levels of the Gal1p inducer in the cytoplasm.

5. Conclusions and Future Directions

The analysis of galactose metabolism in yeast remains an important field of study. Stretching back over 50 years, firstly the genetic analysis of the *GAL* genetic switch, followed by more recent biochemical and structural work have lead to an unprecedented level of knowledge about this system. However, it would be foolish to assume that our knowledge is complete. For example, the very recent discovery of a new (and potentially contradictory) mechanism for a ''cellular memory'' of exposure to galactose clearly shows that the system still has huge potential for uncovering fundamental processes in transcriptional control.

In recent years, and predominately through the efforts of Hazel Holden's laboratory, we have learned much about the structures and mechanisms of the individual Leloir pathway enzymes. High-resolution structures are now available for the yeast mutarotase, galactokinase, and UDP-galactose-4-epimerase. The structure of the yeast mutarotase (Gal7p) is not currently known, but its similarity to orthologous enzymes whose structures have been solved gives a reasonable indication as to the likely structure of Gal7p itself. However, much less is known about how (or indeed if) the individual enzymes work together as a concerted pathway for, perhaps, the more efficient channeling of metabolic intermediates between the enzymes of the pathway. Do the Leloir pathway enzymes form a complex (or metabolon) *in vivo*? Work needs to be done to address this, and determine what role any potential metabolon may play in regulating metabolic flux.

The structural work on the *GAL* genetic switch has yielded even more important information. The implied structure of Gal3p, based on its extraordinary levels of similarity to Gal1p (the galactokinase), and the structure of Gal80p have allowed, for the first time, structural models of the transcriptional control proteins to be confidently built. In the future, work must focus on the complexes between the *GAL* switch proteins. In particular, high-resolution analysis of the Gal4p–Gal80p complex and the Gal80p–Gal3p complex will be required to fully dissect the intricate details of this important and exquisitely controlled transcriptional circuit.

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