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Abstract approved: _____

Alan T. Bakalinsky

Sulfite is a normal, but potentially toxic metabolite, in *S. cerevisiae* and other organisms, produced as an intermediate during reductive sulfate assimilation. *S. cerevisiae* has a basal tolerance to sulfite, presumably due to formation of a non-toxic adduct with acetaldehyde and reduction by sulfite reductase. The present study was undertaken to explore the existence of additional mechanisms for sulfite detoxification in *S. cerevisiae* using genetics and molecular biology methods. Consequently, a novel regulatory pathway for sulfite detoxification was found, in which Ssu1, a plasma membrane protein with 9 or 10 membrane-spanning domains, and no significant similarity to proteins present to date in public databases, is the putative effector. Ssu1p resembles a transporter that may function in sulfite efflux rather than import, since *ssu1* mutants as well as mutants in genes situated upstream of *SSU1* in the pathway were found to be sensitive rather than resistant to sulfite. *SSU1* was placed downstream of *FZF1* based on multicopy suppression analysis. *FZF1* encodes a putative C_2H_2-type zinc finger transcription factor, previously implicated in sulfite tolerance. The *SSU1* promoter-*lacZ* fusion analysis defined Fzf1p as a transcriptional activator of *SSU1*, confirming a role for the Fzf1 protein in transcriptional regulation, previously suggested solely on the basis of sequence analysis. Transcription from the *SSU1* promoter was

lower in an *fzf1* background, and it was strongly activated in wild-type when *FZF1* was expressed in multicopy. Another protein, Ssu3, may be required for Fzf1-induced activation, since transcription from the *SSU1* promoter was lower in an *ssu3* background. The *SSU3* gene was previously related to sulfite tolerance, but its function is unknown. The Fzf1 protein was shown to bind directly and specifically to the *SSU1* promoter. The first zinc finger region appeared to be essential for DNA binding, while deletion of the fourth and the fifth zinc fingers did not affect it. Another gene, *GRR1*, earlier implicated in glucose repression and cell cycle regulation in yeast, was also found to cause sulfite sensitivity when mutated. Based on multicopy suppression analysis, *GRR1* was placed in the pathway upstream of *SSU1* and *FZF1*, and is presumed to be involved in the regulation of both, and in additional routes of sulfite detoxification.

Genetic Aspects of Sulfite Tolerance in *Saccharomyces cerevisiae*

by

Dorina Avram

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CONTRIBUTION OF AUTHORS

Dr. Alan T. Bakalinsky is the principal investigator of the project and was involved in the design, analysis and writing of each manuscript of the thesis. Dr. Mark Leid was involved in the experimental design of the manuscript "The first zinc finger region of *Saccharomyces cerevisiae* transcriptional activator Fzf1 is required for *SSU1* promoter specific binding".

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Chapter 1

Literature Review

1.1. Introduction

The sequencing of the *S. cerevisiae* genome was completed in 1996. As a result, the scientific community of yeast researchers is now directing intensive efforts towards understanding the role of some 3,000 genes whose functions are unknown and whose sequences are not informative (Goffeau *et al.*, 1996). In this effort, analysis of traits related to environmental conditions or traits developed in industrial strains of *S. cerevisiae* may bring some light into understanding certain functions. Tolerance to sulfite is a trait that has been developed in wine strains by exploiting the basal tolerance of *S. cerevisiae* and that has been taken advantage of since ancient times in wine making. Sulfite is a potentially toxic metabolite in the reductive sulfate assimilation pathway (Fig. 1), which is similar in yeast, bacteria and molds. *S. cerevisiae* can tolerate it at concentrations that will kill the others. An important means of protection against sulfite appears to be the formation of an adduct with acetaldehyde (Taylor *et al.*, 1986). Sulfite consumption by sulfite reductase also minimizes its cellular levels (Thomas *et al.*, 1992a). The present study was undertaken to explore the existence of additional mechanisms involved in sulfite tolerance, using genetics and molecular biology methods. In the literature review, the reductive sulfate assimilation in *S. cerevisiae* and its regulation are analyzed since sulfite is a metabolite in this pathway. Then the effects of sulfite on yeast metabolism are reviewed. The next subchapter

analyzes yeast plasma membrane proteins, since one of the cloned genes involved in sulfite tolerance, *SSU1*, encodes a plasma membrane protein, presumably a transporter. The last subchapter describes C_2H_2-type zinc finger proteins, which are transcriptional regulators, since another cloned gene, *FZF1*, was found to be such a zinc finger protein and to regulate the expression of *SSU1*.

1.2. Reductive sulfate assimilation

1.2.1. Sulfite formation in yeast

Sulfite is formed during reductive sulfate assimilation, which in general outline is similar in plants, fungi and bacteria, organisms that can use sulfate as a source for methionine biosynthesis (Fig. 1).

S. cerevisiae demand for sulfur can be satisfied by uptake of inorganic sulfur or sulfur-containing amino acids. Kinetic studies have revealed at least one high-affinity sulfate transport system in yeast (McRead and Din, 1974). Sequencing of the yeast genome unveiled two ORFs that belong to the Sul family of sulfate transporters. One of the ORFs is located on chromosome II, YBR294w (*SFP*) (Feldman *et al.*, 1994), and has similarity to sulfate permease II of *Neurospora crassa* (Cys-14), soy bean Gmak170 nodulin, human colon mucosa protein, and a putative ORF downstream of *E. coli* phosphoribosyl pyrophosphate synthetase gene (Jin *et al.*, 1995). The second ORF is positioned on chromosome XVI, P9723.03c, and has similarity to the first one (Barrel *et al.*, unpublished).

Once inside the cell, sulfate must undergo a series of enzymatic conversions before being incorporated into the sulfur-containing amino acids. Sulfate is first activated by ATP sulfurylase to form adenosine 5'-phosphosulfate (APS), which is then phosphorylated by APS kinase to produce 3'-phosphoadenosine 5'-phosphosulfate

sulfate (out)

↓

sulfate (in)

ATP ⟩

ADP ⟩

adenosine
5'phosphosulfate (APS)

ATP ⟩

ADP ⟩

3'-phosphoadenosine
5'phosphosulfate (PAPS)

reduced thioredoxin

adenosine-3'
5'bisphosphate

oxidized thioredoxin

sulfite

6NADPH ⟩

6NADP⁺ ⟩

sulfide

homoserine

↓

O-acetyl
homoserine

pyruvate
+ NH₄⁺

5-methyltetra-
hydropteroyl
glutamate

cysteine ⇌ cystathionine ⇌ homocysteine

α-ketobutyrate
+NH₄⁺

serine
adenosine

tetrahydro-
pteroylglutamate

S-adenosyl-
homocysteine

methionine

R-CH₃ ⟩

ATP

R ⟩

PP+Pi

S-adenosyl-
methionine (SAM)

Fig. 1. The reductive sulfate assimilation in *Saccharomyces cerevisiae*.

(PAPS). PAPS is then reduced to sulfite and adenosine-3',5'-bisphosphate (PAP) in a thioredoxin-dependent reaction catalyzed by PAPS reductase (Schwenn *et al.*, 1988) (Fig. 1). Inactivation of the two genes for thioredoxin (*TRX1* and *TRX2*) rendered yeast unable to utilize inorganic sulfate as sulfur source (Muller, 1991). Glutaredoxin seems to be unable to substitute for thioredoxin in PAPS reduction in yeast (Muller, 1991), in contrast to the situation in *E. coli* (Tsang, 1981). As a model for the reduction of PAPS, Schwenn *et al.* (1988) suggested that PAPS reductase is first reduced by thioredoxin, then PAPS reacts with the reduced enzyme and eventually PAP and free sulfite are liberated. There have been indications that PAPS in high endogenous concentration is toxic. Mutants of *Salmonella typhimurium* that were unable to metabolize PAPS, would tend to accumulate mutations in genes leading to the formation of PAPS, and those without such additional mutations would die during storage (Gillespe *et al.*, 1968). Thomas *et al.* (1992b) showed that *S. cerevisiae* without PAPS reductase activity are also devoid of sulfate permease activity. This suggests the existence of a tight control of the ratio between the production and removal of PAPS (Hansen, 1995).

The gene encoding ATP sulfurylase in yeast, *MET3*, has been cloned and it encodes a putative polypeptide of 521 amino acids (Cherest *et al.*, 1985). A region of 14 amino acids in the N-terminal part of the yeast ATP sulfurylase is identical to the ATP sulfurylases from *Penicillium crysogenum* and from a bacterial endosymbiont of *Riftia pachyptyla* (Laue and Nelson, 1994; Foster *et al.*, 1994). A 25 amino acid region, 58 amino acids upstream of the carboxyl terminus, is also conserved among the three proteins. Both conserved regions are rich in basic amino acids, indicating potential binding sites for MgATP and sulfate (Foster *et al.*, 1994). APS kinase is a 50 kDa homodimer, encoded by *MET14* gene (Schriek and Schwenn, 1986), whose expression is repressed by methionine (Korch *et al.*, 1991). The structural gene for

PAPS reductase, *MET16*, was also cloned and it encodes an ORF of 256 amino acids (Thomas *et al.*, 1990). PAPS reductase activity and *MET16* transcription are repressed by addition of L-methionine or SAM (Thomas *et al.*, 1990).

It appears that this part of sulfur assimilation pathway in yeast is highly regulated and coordinated, first because of the possible toxicity of accumulated PAPS intermediate, and second because APS synthesis is energetically unfavorable (Hansen, 1995).

1.2.2. Reduction of sulfite

Sulfite reduction to sulfide involves the transfer of six electrons, as expressed in the equation:

$$6 \, NADPH + 6 \, H^+ + SO_3^{2-} \rightarrow 6 \, NADP^+ + 3 \, H_2O + S^{2-}$$
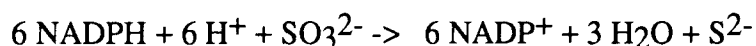
There are six complementation groups ascribed to the enzymatic step of sulfite reduction: *met1, met5, met8, met10, met18* and *met20*. Mutants of each of these groups accumulate intracellular sulfite and none have measurable sulfite reductase activity (Thomas *et al.*, 1992a). The genes complementing *met5* (Mountain *et al.*, 1991), *met8* (Cherest *et al.*, 1990) and *met10* (Hansen *et al.*, 1994) have been cloned. While for the first two the function could not be assigned based on sequence analysis, *MET10* ORF has been found to encode the a subunit of yeast sulfite reductase (Hansen *et al.*, 1994). Met10p has FAD- and NADPH-binding regions which are similar to those of *E.coli* sulfite reductase flavoprotein subunit (Ostrovski *et al.*, 1989) and to several other flavoproteins (Hansen *et al.*, 1994).

met1 and *met20* may be mutant alleles of the ORF encoded by YKR069w (Hansen, 1995), which is localized on chromosome XI (Dujon *et al.*, 1994). This ORF has been found to have 36.6% identity in the 238 amino acid overlap with the *Salmonella typhimurium cysG* ORF (Hansen, 1995), which encodes a uroporphyrinogen III methylase, involved in the biosynthesis of siroheme (Wu *et al.*,

1991). Further supporting evidence for homology comes from the fact that the 5'-noncoding region of YKR069w contains DNA elements homologous to those of other *MET* genes, believed to take part in the regulation of the whole pathway (Hansen, 1995). *MET8, MET5* and *MET18* ORFs' functions are unknown (Hansen *et al.*, 1994).

1.2.3. Assimilation of sulfide into homocysteine and methionine

The condensation of *O*-acetylhomoserine with sulfide to form homocysteine is one of the crucial points in methionine biosynthesis. *O*-acetylhomoserine is derived from homoserine, a precursor of threonine. Homoserine is acetylated by homoserine acetyltransferase, using acetyl-CoA as cosubstrate (Fig. 1). *met2* mutants were found to be defective in this activity (Masselot and Robinchon-Szulmajster, 1975). The *MET2* gene has been cloned (Baroni *et al.*, 1986) and its 5'-untranslated region contains motifs known to be involved in methionine-specific regulation (Thomas *et al.*, 1989).

O-acetylhomoserine sulfhydrylase catalyzes the sulfhydrylation of *O*-acetylhomoserine to form homocysteine (Fig. 1). *met25* mutant was found to lack the enzymatic activity (Masselot and de Robinchon-Szulmajster, 1975) and the gene has been cloned and it encodes an ORF of 444 amino acids (Sangsoda *et al.*, 1985) whose expression was shown to be regulated by methionine (Kerjan *et al.*, 1986).

Homocysteine can also be derived from cysteine and *O*-acetylhomoserine, via the intermediate cystathionine, in the transsulfuration pathway, and it can also be formed via SAM demethylation, followed by hydrolysis of *S*-adenosylhomocysteine (Fig. 1).

Methionine is formed from homocysteine and 5-methyltetrahydropteroyl-glutamate by a transmethylation reaction, catalyzed by homocysteine methyltransferase (Fig. 1). *met6* mutants lack this activity and are unable to utilize homocysteine, cysteine

or any inorganic sulfur compound for methionine biosynthesis (Masselot and Robichon-Szulmajster, 1975).

1.2.4. Cysteine biosynthesis: the transsulfuration pathway

Cysteine biosynthesis in yeast is still controversial, but it is generally agreed that there is a C4 to C3 transsulfuration pathway. In this pathway, cystathionine can be formed by the condensation of homocysteine and serine, catalyzed by cystathionine β-synthetase and by the condensation of cysteine and O-acetylhomoserine catalyzed by cystathionine γ-synthase. Cystathionine can be deaminated and cleaved by cystathionine γ-lyase to form α-ketobutyrate and cysteine and by cystathionine β-lyase to form pyruvate and homocysteine (Fig. 1).

The gene for cystathionine β-synthase, *STR4* (*CYS4*) encodes an ORF of 507 amino acids (Cherest *et al.*, 1993) and was found to have similarity with the rat cystathionine β-synthase gene (Ono *et al.*, 1992). Yeast cystathionine γ-lyase is encoded by *STR1* (*CYS3*) and has similarity with the rat and bacterial enzymes and with O-acetylhomoserine sulfhydrylase (Barton *et al.*, 1993 and Cherest *et al.*, 1993). *STR2* may be the gene for cystathionine γ-synthase (Cherest and Surdin-Kerjan, 1992).

1.2.5. S-adenosylmethionine

S-adenosylmethionine (SAM) is synthesized by the transfer of an adenosyl group from ATP to the sulfur atom of methionine, catalyzed by SAM synthetase. The methyl group of SAM is activated by the positive charge of the adjacent sulfur atom, and SAM is able to donate this group in a vast number of biosyntheses. Demethylation of SAM leaves S-adenosylhomocysteine, which can be further converted to homocysteine. Methylation of homocysteine will reconstitute methionine. This circuit is called the activated methyl cycle, and constitutes an important salvage pathway in yeast through

which organic sulfur can be reused preferentially to *de novo* sulfur assimilation (Hansen, 1995).

There are two isozymes of SAM synthetase in *S. cerevisiae* (Chiang and Cantoni, 1977). The genes encoding them have been cloned and the two polypeptides share 92% identity (Thomas and Surdin-Kerjan, 1987; Thomas *et al.*, 1988). The 5'-noncoding regions of both *SAM* genes contain DNA elements presumably involved in methionine-specific repression (Thomas and Surdin-Kerjan, 1991).

Faroqui *et al.* (1983) showed the existence of labile cytosolic and stable vacuolar SAM pools, leading Thomas and Surdin-Kerjan (1991) to hypothesize that the *SAM1*-encoded enzyme is cytosolic, while the *SAM2*-encoded enzyme has a vacuolar location. Indeed, some *vps* mutants of class "C" are methionine auxotrophs (Jaxquemin-Faure *et al.*, 1994). *VPS33* encodes a protein involved in vacuolar biogenesis and protein sorting, and has been shown to be the same as *MET27*. When mutated, this gene caused the repression of the *MET25* promoter under normally derepressing conditions (Jacquemin-Faure *et al.*, 1994). This suggests the importance of proper compartmentalization of SAM for the regulation of sulfur metabolism.

1.2.6. Regulation of sulfur assimilation

Sulfate uptake and assimilation are repressed by organic sulfur which means that methionine will preferentially be used if both sulfate and methionine are present in the habitat. This would appear to be the preferred route of uptake since inorganic sulfur assimilation requires high energy expenditure. SAM rather than methionine appears to be the compound that represses the genes of sulfur assimilation and methionine biosynthesis. A yeast strain with disruptions in *SAM1* and *SAM2* genes showed no repression for *O*-acetylhomoserine sulfhydrylase when methionine was supplied, whereas the addition of SAM restored the repression (Thomas *et al.*, 1988). Whether

SAM induces the formation of a repressor protein or works as a corepressor with a constitutively expressed repressor is not known. Repression of transcription by addition of methionine and/or SAM has been confirmed for *MET3* (Cherest *et al.*, 1985, Mountain *et al.*, 1991), *MET14* (Korch *et al.*, 1991, Mountain *et al.*, 1991), *MET16* (Thomas *et al.*, 1990), *MET2* (Baroni *et al.*, 1986, Langin *et al.*, 1986), *MET25* (Sangsoda *et al.*, 1985), *MET5* (Mountain *et al.*, 1991) and *MET10* (Hansen *et al.*, 1994).

To date, the products of three genes are known to be involved in the transcriptional regulation of the sulfur metabolic network (Kuras and Thomas, 1995). One, encoded by *CBF1* and belonging to the basic region-helix-loop-helix protein family, recognizes the DNA motif RCACGTG (where R is a purine) (Mellor *et al.*, 1990), which is present in one or two copies upstream of nearly all known sulfur genes. *CBF1* is involved in the transcriptional activation of the sulfur network (Thomas *et al.*, 1989) and also is the *CDE1* element of all the centromeres of *S. cerevisiae* (Hieter *et al.*, 1985). Cbf1p lacks a transcriptional activation domain and it is likely involved in aiding local reconfiguration of the chromatin structure in the transcription initiation region, rather than in direct transcriptional activation (Thomas *et al.*, 1995).

MET4 encodes another known protein mediating transcriptional control of the sulfur network and is a basic region-leucine zipper protein (bZIP) (Thomas *et al.*, 1992b). The *MET25* promoter contains a motif (TGGCAAATG) which is recognized directly or indirectly by Met4p, but this element could not be found in the promoter of other *MET* genes, the expression of which is dependent on Met4p (Thomas *et al.*, 1992b).

Kuras and Thomas (1995) showed that the transcriptional activation function of Met4p is negatively controlled by the elevation of intracellular SAM. They identified two distinct domains of Met4p: an activation domain, located at its N-terminus which

functions in a constitutive manner, and an inhibitory domain, responding to high levels of SAM. Negative transcriptional regulation of the sulfur network therefore consists of a mechanism preventing the Met4 activation function. Met4 contains another distinct functional domain that appears to function as an antagonist of the inhibitory region when intracellular SAM is low, though allowing the activation domain to function (Kuras and Thomas, 1995). The third protein implicated in transcriptional regulation of *MET* genes, Met30, contains WD40 repeats, interacts with Met4p and, impairs SAM-mediated repression of the sulfur network when mutated (Thomas *et al.*, 1995).

1.3. Effects of sulfite on *S. cerevisiae*

Macris and Markakis (1974) and Stratford and Rose (1986) proved that sulfite transport into the yeast cell is pH dependent and only the undissociated sulfurous acid is transported. This form is present at significant concentrations only at low pH. The first authors tried to demonstrate that sulfite is transported by a carrier, while the former brought evidence for simple diffusion and against carrier-mediated transport. The following are the facts that argue for simple diffusion:

- lack of saturability

- inability of potential inhibitors to affect initial rates of transport

- omission of glucose from reaction mixtures had no effect on initial rates of uptake

- inability of 2-deoxyglucose to affect initial rates

- temperature increase to $39^{\circ}C$ did not cause an overall decrease of sulfite uptake.

Analysis of metabolic changes in yeast cells treated with sulfite (5mM) revealed an immediate depletion of intracellular ATP (Schimz and Holzer, 1979). This is believed to be mainly due to the inactivation of glyceraldehyde-3-phosphate

dehydrogenase (Hinze and Holzer, 1986; Maier *et al.*, 1986). As a result the glycolytic pathway is blocked and the two ATPs consumed earlier in the pathway cannot be recovered.

On the other hand, sulfite also reacts with acetaldehyde to form 1-hydroxyethane sulfonate, which is a non-toxic compound (Stratford *et al.*, 1987) and this has been found to be one of the major routes of detoxification (Nordlov, 1985; Stratford *et al.*, 1987; Pilkington and Rose, 1988; Casalone *et al.*, 1992). In the presence of sulfite alcohol dehydrogenase and ethanol formation are inhibited (Maier *et al.*, 1986). Casalone *et al.* (1992) found that yeast mutants resistant to sulfite (below 3.6 mM) produced a higher level of acetaldehyde than did the wild-type, but Xu *et al.* (1994) reported that a resistant strain showed a lower level than the parental one. Probably other mechanisms than acetaldehyde production, involved in sulfite tolerance, are enhanced in the second case. In any case, a sulfite sensitive mutant, which was later shown to have a nonfunctional *GRR1* gene, (Avram and Bakalinsky, 1996) produced a lower level of acetaldehyde than the parental strain (Xu *et al.*, 1994).

In the presence of sulfite glycerol production is also increased. Sulfite reaction with acetaldehyde blocks ethanol formation and regeneration of NAD^+ (Gancedo *et al.*, 1968). Under these conditions, dihydroxyacetone phosphate is reduced to glycerol phosphate to regenerate the oxidized cofactor. Free glycerol is formed following the enzymatic hydrolysis of the phosphate group and consequently, glycerol production is stimulated (Gancedo *et al.*, 1968).

As mentioned before, metabolic and genetic studies suggested that the main routes for sulfite detoxification in yeast cells are the consumption by sulfite reductase (Thomas *et al.*, 1992a) and non-toxic adduct formation with acetaldehyde (Taylor *et al.*, 1986). A new regulatory pathway for sulfite detoxification was found based on multicopy suppression analysis (Avram and Bakalinsky, 1996b). In this pathway,

Ssu1p, a plasma membrane protein, presumably a transporter involved in sulfite extrusion from the cell, is likely the effector. The expression of *SSU1* gene is activated by FZF1p, a five zinc finger transcription factor, which was earlier implicated in sulfite tolerance (Cassalone *et al.*, 1994). Deletion of *SSU1* and *FZF1* genes resulted in sensitivity to sulfite, confirming their involvement in the protection against sulfite. Overexpression of these genes resulted in tolerance to higher level of sulfite, supporting the previous results (Avram and Bakalinsky, 1996a; Avram and Bakalinsky, 1996b). The activation of *SSU1* promoter in an *ssu3* background by multicopy *FZF1* was lower, suggesting that a functional *SSU3* gene may be required for full activation. The *ssu3* mutant was previously shown to be sensitive to sulfite and was only partially suppressed by multicopy *FZF1* (Xu *et al.*, 1994; Avram and Bakalinsky, 1996a). A fourth gene, *GRR1*, earlier implicated in glucose repression and cell cycle regulation, appeared to be also involved in this pathway, being positioned upstream of the above-mentioned genes by multicopy suppression analysis, and probably responsible for their regulation (Avram and Bakalinsky, 1996a; Avram and Bakalinsky, 1996b).

1.4. Transport systems in the plasma membrane of *Saccharomyces cerevisiae*

All eukaryotic cells contain a wide variety of proteins embedded in the plasma and internal membranes, which ensure transmembrane solute transport. A large proportion of these proteins can be grouped into families and share high sequence similarity with proteins found in other organisms, ranging from bacteria to humans. Most transport proteins characterized to date catalyze the uptake of solutes across the plasma membrane or mediate extrusion of different compounds into the medium. Other membrane proteins, localized into intracellular membranes, catalyze efflux from or compartmentalization within the mitochondria, vacuole, peroxisomes or secretory organelles. At least 5% of the total yeast genome encodes plasma membrane proteins

(Garrels, 1995), most of them presumably involved in catalyzing the transport of solutes across membrane. This number does not take into consideration uncharacterized transport proteins which share no significant similarities with known transport systems. Membrane transport proteins are generally classified in three main categories: pumps (ATPases), channels, and facilitators (also named transporters, permeases or carriers) (André, 1995).

1.4.1 ATP-driven transport systems (Primary transport proteins)

ATPases are involved in primary active transport (Fig. 2) (Van Der Rest *et al.*, 1995) which is coupled to ATP hydrolysis, enabling them to operate against (electro-) chemical gradients. In *S. cerevisiae*, there are two types of plasma membrane ATPases: P-type and ABC transporters (André, 1995).

1.4.1.1. P-type ATPases

The principal plasma membrane ATPase, the proton ATPase, Pma1, accounts for 50% of the plasma membrane protein content of exponentially grown cells (Serrano, 1991). This protein forms a covalent acyl phosphate intermediate as part of the reaction cycle and has two forms, E1 and E2, which differ in conformation (Goffeau and Slayman, 1981). This type of enzyme is therefore called E1 E2- or P-type ATPase. The catalytic mechanism of P-type ATPase is distinct from that F-type ATPase of the mitochondria and V-type ATPase of the vacuolar membrane (Van Der Rest *et al.*, 1995). Pma1p hydrolyzes ATP and generates an electro-chemical gradient of protons, Δp, which is used to drive membrane-associated processes, such as solute transport.

Isolation of the *PMA1* gene encoding the plasma membrane ATPase (Serano *et al.*, 1986) has permitted the molecular analysis of the enzyme. In contrast to mitochondrial F-type and vacuolar V-type ATPases, composed of multiple different

subunits, Pma1p only contains one. The protein has 8 to 12 putative membrane-spanning segments with the N and C termini located in the cytoplasm (Serrano, 1991). Amino acid sequence motifs which are highly conserved among P-type ATPases have been detected and mutagenesis of the residues in these regions has established their roles in the ATPase function (Serrano, 1991).

Another P-type plasma membrane ATPases has been described, Pma2 ATPase. It is 90% identical to the Pma1 enzyme, but has distinct enzymatic properties (Schlesser *et al.*, 1988). The high affinity for MgATP may indicate that Pma2p is a glucose-regulated ATPase that plays a role under starvation conditions, when the ATP levels are low (Supply *et al.*, 1993).

Three other subfamilies of P-type ATPases exist in yeast. Subfamily 1 comprises ion pumps related to Ca^{++}-transporting ATPases (Fagan and Saier, 1994). The *PMR1/SSC1* gene encodes a Ca^{++}-pump with a potential Ca^{++}-binding site in its N-terminal region and appears to contribute to calcium homeostasis. The *PMR2/ENA1* gene encodes a protein which has 20% similarity to Pma1 and may be involved in transport of monovalent cations (Na^+, Li^+ and K^+) (Rodriguez-Navaro *et al.*, 1994). Deletion of this gene results in sensitivity to high Na^+ concentrations and elevated pH. The *PMR2/ENA1* gene is a part of a cluster of four contiguous genes, *PMR2/ENA1*, *ENA2*, *ENA3*, and *ENA4*, located on chromosome IV (Rodriguez-Navaro *et al.*, 1994). These genes encode nearly identical proteins and likely arose by duplication.

Sub-family 2 is composed of proteins most closely related to Cu^{++}-pumps, like the human gene products defective in patients with Wilson and Menkes diseases (Fu *et al.*, 1995), while the third subfamily includes proteins of unknown function (Van Der Rest, *et al.*, 1995).

15

Primary Transport Systems

Secondary Transport Systems
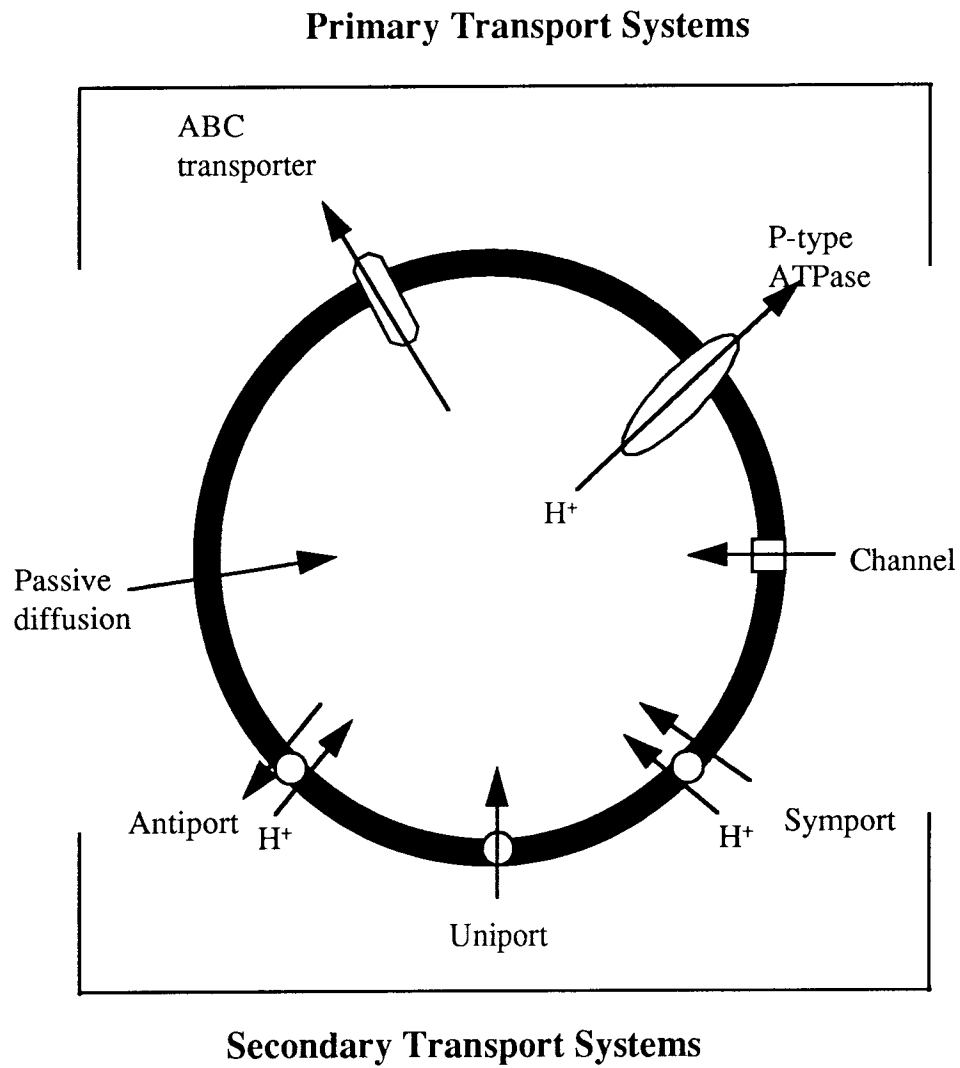
Fig. 2. Transport systems in yeast. Modified from Van der Rest *et al.* (1995).

1.4.1.2. ABC transporters

The members of this family contain a highly conserved domain of about 200 amino acids, the ATP-binding cassette (ABC) or the nucleotide-binding fold (NBF), which confers the ability to bind and hydrolyze ATP (Higgings, 1992). The structural unit of the ABC transporter typically consists of six transmembrane domains (TM6) preceded or followed by the conserved cytosolic ATPase domain. Many ABC transporters have a duplicate of TM6-NBF.

The Ste6 protein, required for the secretion of *a* factor, is a representative of ABC transporters (Kutchler *et al.*, 1992). Other members are the proteins involved in pleiotropic drug resistance, similar to the multiple drug resistance proteins from higher eukaryotes (Balzi and Goffeau, 1994). Others are located in the membranes of intracellular compartments, such as vacuole, peroxisomes and mitochondria and are presumed to mediate solute import in these organelles (Van Der Rest *et al.*, 1995).

1.4.2. Passive and facilitated diffusion across the plasma membrane

1.4.2.1. Passive diffusion

Passive diffusion of solutes also occurs in *S. cerevisiae* and is governed in part by the physical properties of the membrane such as the acyl chain length, degree of saturation of the fatty acids, membrane fluidity and other factors (Van Der Rest *et al.*, 1995). A variety of sugar-alcohols such as arabinitol, erythritol, galactitol, mannitol, ribitol, sorbitol, and xylitol are thought to cross the membrane by passive diffusion only (Canh *et al.*, 1975). Although specific transporter systems have not yet been found for these molecules, the relatively hydrophilic nature of the sugar-alcohols makes it unlikely and the rate of diffusion is very high (Van Der Rest *et al.*, 1995). More-lipophilic compounds such as fatty acids, alkanols and hydrocarbons are more likely to diffuse

into the plasma membrane (Cartwright *et al.*, 1989). Most likely, sulfite is also transported by passive diffusion (Stratford and Rose, 1986).

1.4.2.2. Ion Channels

Ion channels are complexes of membrane proteins that allow the downhill flux of solutes across the plasma membrane by forming an aqueous diffusion pore (Fig. 2). Two properties distinguish them from the transporters that mediate solute-facilitated diffusion:

1. ion flow is extremely fast (more than 10^6 ions/sec.)

2. ion channels are gated; their opening frequency is regulated by changes in membrane potential, by binding of a specific ligand or by mechanical constrains such as membrane stretching (André, 1995).

The predominant plasma membrane channel (Ypk1) is an outward rectifying K^+ channel (i.e. mediating K^+ efflux), activated by membrane depolarization and by high cytoplasmic Ca^{++} concentration. The function of this channel is probably to balance charge displacement during proton-coupled substrate uptake. Gustin *et al.* (1988) have shown that stretching the plasma membrane activates another channel conducting both cations and anions. This mechano-sensitive channel may play a role in osmoregulation.

The predominant vacuolar channel (Yvc1) is a cation selective inward rectifier (i.e. transferring ions from the vacuole into the cytoplasm), conducting Na^+, K^+ and Ca^{++} (Wada *et al.*, 1987) and may play a role in adjusting the cytosolic Ca^{++} concentration from the vacuolar reservoir.

The channels proteins of the MIP family comprise proteins found in bacteria, fungi, plants and animals whose sequences are highly related to that of the Major Intrinsic Protein (MIP) of the bovine lens fiber cell membrane (Gorin *et al.*, 1984). All these proteins have a predicted topology of six transmembrane segments flanked by

hydrophilic termini facing the cytoplasm. They have been implicated in the diffusion of diverse substrates, including ions, water, glycerol, etc. (André, 1995).

Sequencing of the yeast genome has revealed several genes likely to encode ion channels: $GEF1(CLC1)$, having similarity to voltage-gated Cl⁻ channel, and $JO911$ with similarity to voltage-gated K^+ channels (André, 1995). Other proteins have sequence similarity with channel proteins from other organisms (Garrels, 1995), but only functional analysis will prove that they are really involved in processes for which their "relatives" are responsible.

1.4.2.3. Facilitators/Transporters

In the secondary transport, the energy for translocation of the solute is supplied by (electro-) chemical gradients of other solutes, which are often generated by primary transport systems (Fig. 2). Three general categories of secondary transport systems can be distinguished: uniport, symport and antiport (Fig. 2). Transport of a single solute which is facilitated by a carrier protein without the movement of a coupling solute is termed uniport. When transport involves the coupled movement of two (or more) solutes in the same direction, the transport process is referred to as symport. Antiport refers to the coupled movement of solutes in opposite directions (Van Der Rest *et al.*, 1995).

Many solute-transporting proteins identified in bacteria, fungi, plants, and animals possess a common structural topology: a central hydrophobic core of 10-12 membrane spanning domains (with an optional, large cytoplasmic loop between two transmembrane segments) flanked by hydrophilic domains which presumably face the cytoplasm (André, 1995). In contrast to a channel, a transporter is assumed to transfer the solute across the membrane by undergoing reversible conformational changes that expose its solute binding site alternatively on each side of the membrane. Furthermore,

transporters lack both the nucleotide-binding sequence typical of ABC transporters and the conserved amino acid stretches which contribute to the formation of the catalytic site of P-type ATPases. Many facilitators/trasnporters have been identified in yeast and they can be grouped into several families on the basis of sequence similarities (André, 1995). Some of these families belong to the Major Facilitator Super-family (MFS) (Marger and Saier, 1993).

1.4.2.3.1. The *HXT* family of hexose transporters and related proteins

This family of yeast proteins belongs to the MFS and the proteins display similarity to the large family of sugar transporters described in mammalian cells (Glut family) and bacteria (André, 1995). A large variety of high affinity and low affinity glucose transporters have been identified, as well as proteins with strong similarity to the previous ones, but with unknown function (André, 1995; Özcan and Johnston, 1995). Two glucose transporters (Snf3p and Rgt2p) were found to be glucose receptors also, that generate an intracellular glucose signal, suggesting that glucose signaling in yeast is a receptor-mediated process (Özcan *et al.*, 1996).

Proteins involved in transport of other sugars have similarities to Hxts: Gal2, which is both involved in galactose and glucose transport, a transporter for trehalose (Crowe *et al.*, 1991) and one for methylglucoside (Broklehurst *et al.*, 1977). Some proteins, which are not sugar transporters, have also significant similarities to Hxt proteins, such as Pho84, which is an inorganic phosphate transport component repressed by high intracellular Pi levels (Bun Ya *et al.*, 1991), and the Itr1 and Itr2 proteins, involved in inositol transport (Nikawa *et al.*, 1991).

1.4.2.3.2. The AAP family of amino acid permeases

One general amino acid permease has been identified (GAP1), which is sensitive to nitrogen repression (Grenson *et al.*, 1970), along with several more specific transport systems (Grenson, 1992). All these proteins show high similarity and they are assumed to function as H^+-symporters (Grenson, 1992). They are also similar to amino acid permeases from other fungi and bacteria (André, 1995) and distantly related to other transport proteins, such as the animal y(+)-type cationic amino acid transporters that act as cell surface receptors for ecotropic murine retroviruses (ecoR) (Kim *et al.*, 1991), and an amino acid transporter from *Arabidopsis thaliana* (Alexandraki and Tzemia, 1994).

1.4.2.3.3. The MFS drug-resistance proteins and similar proteins

Several yeast membrane proteins have been found to have sequence similarity with the bacterial drug-resistance-conferring MFS proteins (André, 1995). This group includes bacterial antiporters conferring resistance to various compounds by exporting them from the cell. The driving force for the export is provided by the electrochemical H^+ gradient, generated by the respiratory chain across the plasma membrane (Marger and Saier, 1993). Among the yeast proteins, only two, Atr1p and Sge1p, have been characterized. Atr1p confers resistance to amino-triazole and 4-nitroquinoline N-oxide (Kanazawa *et al.*, 1988), while Sge1p confers resistance to crystal violet (Ehrenhofer-Murray *et al.*, 1994).

1.4.2.3.4. The Fur and Fcy families: transporters of purines, pyrimidines and derivatives

This family comprises uracil permease (Fur4) and allantoin permease (Dal4) which share 68% identity. A uridine permease, also similar to these two proteins, has been found (André, 1995). *FCY2* gene encodes a transport system for purine bases

(adenine, guanine and hypoxanthine) and cytosine which do not share similarity with Fur family (Weber *et al.*, 1990).

1.4.2.3.5. The Dal family - carboxylic acid transporters and other transporters of organic compounds

DAL5 gene encodes a nitrogen-repressible permease for ureidosuccinate and allantoate (Greth *et al.*, 1977). Several other genes of unknown function have been found in the yeast genome encoding proteins with significant similarity to Dal5 (André, 1995).

Some characterized transporters do not share high sequence similarity with any other yeast proteins. One is urea permease, encoded by *DUR3* gene (El Berry *et al.*, 1993). Dur3p displays similarity to the Na^+-proline symporter (putP) of *E. coli* and to the mammalian Na^+-nucleoside co-transporter which belongs to the recently defined family of Na^+-solute symporters (SSF) (sodium-solute symporter super-family) (Reizer *et al.*, 1994).

1.4.2.3.6. Transporters of inorganic phosphate and sulfate

An interesting and well characterized system for inorganic ions is the phosphate transport system. One of the components is encoded by the *PHO84* gene and is a derepressible high affinity transporter mediating H^+-phosphate symport. As mentioned before, it has high sequence similarity to the Hxt family (Bun Ya *et al.*, 1991).

Two ORFs which may be involved in sulfate transport have been identified: one located on chromosome II, YBR294w (*SFP*), (Feldman *et al.*, 1994), which has similarity to sulfate permease II of *Neurospora crassa* (Cys-14), soy bean Gmak170 nodulin, human colon mucosa protein and a putative ORF downstream of *E.coli* phosphoribosyl pyrophosphate synthetase gene (Jin *et al.*, 1995), and another on

chromosome XVI, P9723.03c, which has similarity to the first one (Barrel *et al.*, 1995, unpublished).

1.4.2.3.7. Metal transporters

Iron and copper transport systems are interconnected in yeast. Fe(III) reductases, which are plasma membrane proteins, catalyze the reduction of extracellular Fe(III) to Fe(II), which is then taken up by the cell (Klausner and Dancis, 1994). *FET3* gene encodes a high affinity Fe(II) transporter and is a copper-containing ferro-oxidase (Askwitt *et al.*, 1994). *FET4* gene, a multicopy suppressor of *fet3* mutants, encodes a low affinity Fe(II) transporter (Dix *et al.*, 1994). *CTR1* gene encodes a plasma membrane protein required for high affinity copper uptake. *ctr1* mutants are severely deficient in Fe(II) uptake, because the ferro-oxidase, Fet3p, is a copper-dependent enzyme (Askwith *et al.*, 1994).

1.5. C$_2$H$_2$-type zinc finger transcription factors

The first discovery of a zinc metalloprotein that controls transcription of a specific gene was made in 1983 by Hanas *et al.*, who showed that transcription factor TFIIIA from *Xenopus* oocytes, necessary for the transcription of the 5S RNA gene by RNA polymerase III, was a zinc protein. When the nucleotide sequence of the gene became available in 1985, it was noticed that the amino acid sequence of the translated product could be arranged so that a pair of conserved C residues and a pair of conserved H residues separated by a 12- to 13-residue spacer defined a series of 9 repeated amino acid sequences of ca. 30 residues as follows: -C-X$_{2-5}$-C-X$_{12-13}$-H-X$_{3-4}$-H- (Miller *et al.*, 1985) (Fig. 3). Within the X$_{12-13}$ spacers there was an additional pair of conserved residues, an aromatic amino acid, F or Y, and a branched aliphatic amino acid, usually L, so that the conserved sequence becomes -C-X$_3$-F-X$_5$-L-X$_{2-3}$-H- (Fig.

3 and Fig. 4). The pairs of C and H residues appeared to be excellent candidates for ligands to form the tetrahedral zinc complex (Coleman, 1992). Miller *et al.* (1985) called the repeated structure "zinc finger".

The number of transcription factors reported to contain the zinc finger motifs has increased rapidly. Besides the C_2H_2 type pair found in TFIIIA, other structural motifs have been found, such as C_2C_2 ligand pairs which have been divided into two groups. One is the large family of hormone receptors which contain two isolated zinc domains at either end of a DNA-binding helix loop, and the second is the family of yeast transcription factors in which six C residues form a Zn_2C_6 binuclear cluster within their DNA-binding domains (Coleman, 1992).

Because Fzf1p, a transcription factor involved in sulfite tolerance, is a C_2H_2 type zinc finger protein, this group will be described in more detail. 2D NMR and crystallography studies on single zinc fingers of C_2H_2-type were summarized by Coleman (1992). The finger consists of an N-terminal antiparallel β-sheet of 9 to 11 residues, including the β-turn of $C-X_{2-4}-C$ group, followed by an α-helix (Fig. 3 and Fig. 4). The zinc is held in a tetrahedral complex by the two $-S^-$ ligands from the C residues and the N3 nitrogens of the imidazole side chains of the $H-X_{2-5}-H$ sequence. The two conserved aromatic residues, Y (or F) and L, are in contact, forming a hydrophobic interaction, important for stabilizing the distal portion of the finger. This fold of the finger was predicted on theoretical grounds by Berg (1988) prior to the completion of the solution structures.

Determination of the X-ray crystallographic structures of the zinc finger peptides derived from Zif268 (Pavletich and Pabo, 1991) and Gli (Pavletich and Pabo, 1993) complexed with their respective DNA binding sites indicated that the amino acids within the α-helix of the zinc fingers provide the majority of the contacts to the DNA bases in the major groove (Fig. 3 and Fig. 4). In the case of Zif268 all three zinc fingers contact

the DNA in a similar pattern with that observed in the case of single zinc fingers and wrap around the double helix (Pavletich and Pabo, 1991). Conversely, Gli zinc fingers have very different roles in the interaction with the DNA: some make base contacts, some touch phosphates, others do not contact the DNA at all (Pavletich and Pabo, 1993). Fingers 2 to 5 fit in the major groove and wrap around the DNA for a full helical turn. Fingers 4 and 5 appear to be the most important for recognition. These fingers make extensive base contacts in a 9-bp region that is conserved in the binding sites. Finger 2 and 3 make a set of contacts with the DNA backbone, while finger 1 does not contact the DNA, but instead makes extensive protein-protein contacts with finger 2.

In the case of TFIIIA the majority of energetically important contacts required for DNA binding are formed between the first three zinc fingers and base pairs in the box C promoter element (Christensen $et\ al.$, 1991). Mutation analysis indicated a unique property of the α-helix of finger 3 of TFIIIA which is involved in the interaction with the DNA (Zang $et\ al.$, 1995). For other zinc fingers proteins studied so far, it appeared that the α-helix of the finger is situated in the major groove of the DNA so that only the amino acids from the start of the α-helix up to the first zinc-coordinating histidine residue are positioned to form contacts with the DNA. Contrasting, mutagenesis results indicated that the α-helix of finger 3 of TFIIIA is positioned in the major groove of the DNA in such a way that residues along the entire α-helix, including those between the two-zinc coordinating histidine residues, are involved in making direct contacts to the DNA bases (Zang $et\ al.$, 1995) (Fig. 4).

It is clear that these proteins can recognize a diverse set of DNA sequences due to variations of certain key amino acids, though the general pattern of interaction is similar. For example, the $Drosophila$ Hunchback protein recognizes a site that includes the sequence AAAAA (Stanojevic $et\ al.$, 1989), the human Sp1 recognizes a site which includes the sequence GGGGGC (Kadonaga $et\ al.$, 1988), and the human glioblastoma

protein (Gli) (Kinzler *et al.*, 1988; Roberts *et al.*, 1989) recognizes a site that includes

the sequence TGGGTGGTC (Kinzler and Vogelstein, 1990).
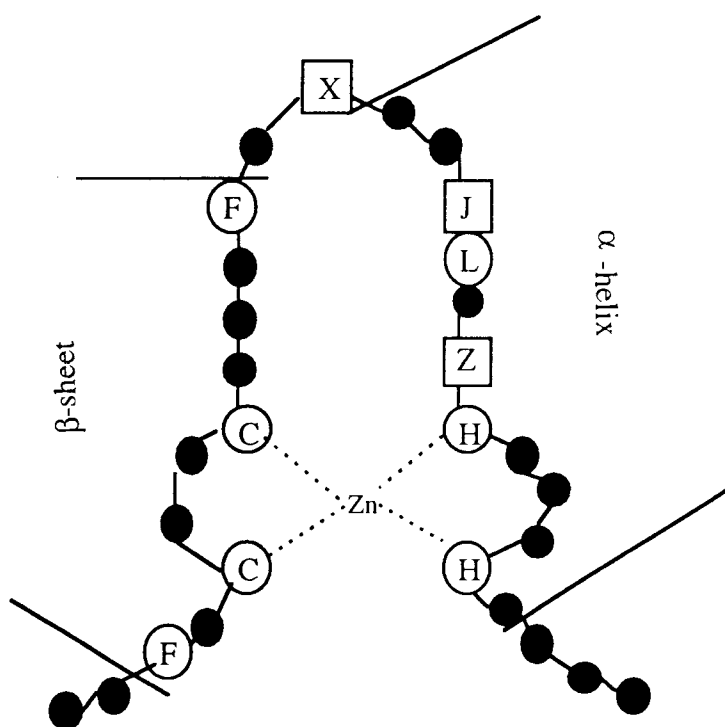
Fig. 3. The C_2H_2-type zinc motif. Circled residues are conserved. DNA-contacting residues, X, J, Z, are boxed. Modified from Klevit (1991).

Protein	Finger	Sequence	
		β-sheet	**α-helix**
human Gli	4	YMCEHEGCRKS___FSN	ASDRAKHQNRT HSNE
	5	YVCKLPGCTKR___YT	DPSSLRKHVKTVHGPD
Zif268	1	YRCPVES CDRR___FS	RSDELTRHIRI _ HTGQ
	2	FQCRI__ CMRN___FS	RSDHLTTHIRT _ HTGE
	3	FACDI__ CGRK___FA	RSDERKRHTKI_ HLRQ
TFIIIA	2	FPCKEEGCEKG ___ FT	SLHHLTRHSLT_ HTGE
	3	FTCDSDGCDLR___ FT	TKANMKKHENRFHNIKI
Fzf1	1	YKCSFDGCEKV ___ YN	RPSLLQQHQNS_HTNQ
	2	YHCDEPGCGKK ___ FI	RPCHLRVHKWT_HSQI
	3	KACTL__ CQKR ___ FV	TNQQLRRHLNS_HERK
	4	LQCPYKS CQKV ___ TS	FNDDLINHMLQ_HHIA
	5	NRCKHSDCQEL___S	PFASVFDLIDHYDHTHAFI
		β-sheet	**α-helix**

Fig. 4. Alignment of Fzf1 zinc fingers with other C2H2 fingers. C and H residues known to interact with the zinc ion are in bold. Residues known to interact with DNA are underlined. Protein- DNA contacts were identified by X-ray crystallography for Gli (Pavletich and Pabo, 1993) and Zif268 (Pavletich and Pabo, 1991), and with mutagenesis for TFIIIA (Zang *et al.*, 1995).

Some zinc fingers seem to be closer relatives to the Zif protein, if the DNA

sequence they bind is taken into consideration, such as Krox-20 (Chavrier *et al.*, 1989),

WT-1 and Sp1 (Pavletich and Pabo, 1993), while others are closer to the Gli protein,

such as Gli3 (Ruppert *et al.*, 1990), tra-1 (Zarkower and Hodgkin, 1992) and ciD

(Orenic *et al.*, 1990). There is an amazing diversity of zinc finger proteins in nature,

though the pattern of the main design is similar. The fact that Gli interacts differently

than Zif, and TFIIIA different than both of them, shows us that the models that we

imagine should not be constrained by rigid rules. The possibilities for zinc finger-DNA

interactions are richer and more complex than one may assume (Pavletich and Pabo,

1993).

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Chapter 2

Multicopy *FZF1* (*SUL1*) suppresses the sulfite sensitivity but not the glucose derepression or aberrant cell morphology of a *grr1* mutant of *Saccharomyces cerevisiae*

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2.1. Abstract

An *ssu2* mutation in *Sacccharomyces cerevisiae*, previously shown to cause sulfite sensitivity, was found to be allelic to *GRR1*, a gene previously implicated in glucose repression. The suppressor *rgt1*, which suppresses the growth defects of *grr1* strains on glucose did not fully suppress the sensitivity on glucose or non-glucose carbon sources, indicating that it is not strictly linked to a defect in glucose metabolism. Because the Cln1 protein was previously shown to be elevated in *grr1* mutants, the effect of *CLN1* overexpression on sulfite sensitivity was investigated. Overexpression in *GRR1* cells resulted in sulfite sensitivity, suggesting a connection between *CLN1* and sulfite metabolism. Multicopy *FZF1*, a putative transcription factor, was found to suppress the sulfite sensitive phenotype of *grr1* strains, but not the glucose derepression or aberrant cell morphology. Multicopy *FZF1* was also found to suppress the sensitivity of a number of other unrelated sulfite-sensitive mutants, but not that of *ssu1* or *met20*, implying that *FZF1* may act through Ssu1p and Met20p. Disruption of *FZF1* resulted in sulfite sensitivity when the construct was introduced in single copy at the *FZF1* locus in a *GRR1* strain, providing evidence that *FZF1* is involved in sulfite metabolism.

2.2. Introduction

Sulfite is a potentially toxic metabolite in *Saccharomyces cerevisiae* and other organisms in which it occurs naturally as an intermediate in the reductive sulfate assimilation pathway or as a product of the catabolism of sulfur-containing amino acids. In yeast, sulfite is formed by reduction of 3'-phosphoadenosine phosphosulfate (PAPS) through the action of PAPS reductase. It is then reduced to hydrogen sulfide in a six-electron transfer catalyzed by sulfite reductase. Hydrogen sulfide condenses with *O*-acetylhomoserine to form homocysteine leading directly to methionine, or to cysteine via

cystathionine (Cherest and Surdin-Kerjan, 1992; Thomas *et al.*, 1992). Catabolic formation in mammals occurs through the oxidation of cysteine leading to β-sulfinyl pyruvate that spontaneously hydrolyzes to form sulfite and pyruvate. The sulfite formed is oxidized to sulfate in mitochondria by sulfite oxidase (Huxtable, 1986).

Because sulfite is toxic to many organisms, those that produce it are presumed to have evolved mechanisms to prevent deleterious reactions from occurring *in vivo*. While yeast has a basal tolerance for sulfite, we previously isolated mutants with heightened sensitivity or resistance in expectation that sulfite-protective mechanisms may be impaired in the former and enhanced in the latter (Xu *et al.*, 1994). The differences in levels of sulfite tolerated among the mutants were found to be small. The resistant mutant *RSU1*, was found to tolerate a three-fold greater concentration of sulfite than wild-type, which in turn was tolerant of a two-fold higher level than the most sensitive mutants. One of the sensitive mutants, *ssu2*, was found to have an elongated cell morphology, enhanced sensitivity to the reducing agents DTT, nitrite, and thiosulfate, to excrete less acetaldehyde, and to contain a reduced amount of glutathione. While the lattter two compounds are reactive with sulfite and represent potential routes of detoxification, a mutant severely deficient in glutathione was found not to be sensitive (Xu *et al.*, 1994). Casalone *et al.*, (1992) reported that a sulfite resistant mutant excreted significantly more acetaldehyde than wild-type, suggesting that the reaction between acetaldehyde and sulfite may be important *in vivo*. Stratford *et al.* (1987) found that exogenous sulfite induced excretion of acetaldehyde by wild-type strains of *S. cerevisiae* and *Saccharomycodes ludwigii*.

Here we show that *SSU2* is the same as *GRR1*, and identify a multicopy suppressor of the sulfite sensitivity, but not the glucose derepression or aberrant cell morphology previously observed in *grr1* strains. *GRR1* encodes a 135 kDa weakly expressed protein that is presumed to play a regulatory role, but whose biochemical function(s) is unknown (Flick and Johnston, 1991). *GRR1* has been implicated in a

variety of functions: glucose repression (Bailey and Woodward, 1984; Flick and Johnston, 1991), glucose transport (Özcan *et al.*, 1994; Vallier *et al.*, 1994), *SUC2* gene expression (Vallier and Carlson, 1991), divalent cation transport (Conklin *et al.*, 1993), turn-over of G_1 cyclins (Barral *et al.*, 1995), and suppression of *bem2* mutations involved in cellular morphogenesis (Kim *et al.*, 1994). We add sensitivity to sulfite to a growing list of phenotypes associated with mutations in *GRR1*, provide evidence for a link between sulfite sensitivity and *CLN1* overexpression, and implicate *FZF1* in sulfite metabolism.

2.3. Materials and Methods

2.3.1. Yeast strains, media, growth conditions, and genetic techniques

Yeast strains are listed in Table 1. Standard yeast genetic techniques were used (Rose *et al.*, 1990). Yeast transformations were performed using the method of Gietz *et al.*, 1992. Yeast media and plates used to test sulfite sensitivity are described elsewhere (Xu *et al.*, 1994). SM is glucose-based synthetic complete medium (SD plus required amino acids and bases at the prescribed concentrations, except for uracil, which was added to a final concentration of 10 μg/ml). Drop-out media are SM lacking the indicated amino acid or base (Rose *et al.*, 1990). Sulfite sensitivity on different carbon sources was determined on: YEP (1% yeast extract, 2% peptone) containing: 2% glucose (YEPD); 2% galactose (YEPgal); 2% maltose (YEPmal); 2% ethanol (YEPE); 3% glycerol (YEPgly); and 0.5% acetate (YEPac). All media containing sulfite were buffered at pH 3.5 with 75 mM L(+)tartaric acid (Xu *et al.*, 1994), with the exception of YEPac, which was buffered at pH 4.8, to avoid a precipitate that formed at the lower pH. 5-fluoro-orotic acid (5-FOA) plates, for selection of *ura3* segregants, were

TABLE 1. Yeast strains.

Strain	Genotype	Source
2757-4d-T4 [a]	*MATa LEU2::pRY181 ura3-52 ade2*	this study
3090-9d	*MATα ura3-52 leu2-3, 112*	this study
3118-19c	*MATα ssu2-6 ura3-52 leu2-3, 112 arg4*	this study
3118-27d	*MATa ssu2-6 ura3-52 leu2-3, 112 arg4 lys9*	this study
3118-27d-T1	*MATa LEU2::pRY181 ssu2-6 ura3-52 arg4 lys9*	this study
3089-1d	*MATa ssu3-7 ura3-52 leu2-3, 112*	this study
3100-5b	*MATa ssu4-11ura3-52 leu2-3, 112 pet*	this study
3149-1a	*MATa LEU2::pRY181 his3 trp1-1 ura3-52 ade2*	this study
3150-14	*MATa LEU2::pRY181ssu2-6 ura3-52*	this study
3154-2b	*MATa grr1::URA3 ade2 leu2-3, 112 lys2-801 ura3-52*	this study
3090-9d-T6	*MATα ura3-52 leu2-3, 112 grr1Δ::URA3*	this study
3090-9d-T6-L1	*MATα ura3-52 leu2-3, 112 grr1Δ6-1*	this study
3090-9d-T4	*MATα ssu1Δ::URA3 leu2-3, 112 ura3-52*	this study
3090-9d-T10	*MATα fzf1Δ99-182::URA3 leu2-3, 112 ura3-52*	this study
3090-9d-T6-L1-T10	*MATα grr1Δ6–1 fzf1Δ99-182::URA3 leu2-3, 112 ura3-52*	this study
3152	*MATa/MATα LEU2::pRY181/leu2-3, 112 ura3-52/ura3-52 ssu2-6 /grr1Δ::URA3 HIS3/his3Δ- 200 ADE2/ade2-101 LYS2/ lys2-801 MET/met arg4 /ARG4*	this study
YM2061	*MATa LEU2::pRY181ura3-52 his3Δ- 200 ade2-101 lys2-801 met*	M. Johnston

TABLE 1 (Continued)

YM3499	*MATa LEU2::pRY181 ura3-52*	M. Johnston
	grr1Δ::URA3 his3Δ-200 ade2-101 lys2-801 met	M. Johnston
YM3502[b]	*MATα LEU2::pRY181 ura3-52 grr1Δ::URA3*	M. Johnston
	his3Δ-200 ade2-101 lys2-801 met	
YM2957	*MATα grr1Δ-1829 ura3-52 his3Δ-200*	M. Johnston
	ade2-101 lys2-801 gal80Δ LEU2::pRY181	
YM3378	*MATa grr1Δ-1829 rgt1-101 ura3-52 his3Δ-200*	M. Johnston
	ade2-101 lys2-801 gal80Δ LEU2::pRY181	
YM3478	*MATa rgt1-101 ura3-52 his3Δ-200 ade2-101*	M. Johnston
	lys2-801 gal80Δ LEU2::pRY181	
YPH499	*MATa ura3-52 his3Δ-200 ade2-101 lys2-801*	C. Mann
	leu2Δ1 trp1Δ63	
CC359-OL2	*MATα leu2 ura3 his3*	Y. Surdin-Kerjan
CC363-20B	*MATa ura3 leu2 met18*	Y. Surdin-Kerjan
CC370-8C	*MATα leu2 ura3 met20*	Y. Surdin-Kerjan

[a] pRY181 contains a *GAL1-lacZ* fusion (Yocum *et al.*, 1984).
[b] Strains YM3502-T1 and YM3502-T2 are YM3502 transformed with YEplac195 and YEplac195::*FZF1*, respectively.

prepared as described (Boeke *et al.*, 1984). An in vivo assay for hydrogen sulfide formation was performed on "BiGGY" agar (Nickerson medium, Difco Laboratories, Detroit, Michigan) according to the manufacturer's instructions.

β-galactosidase activity was assayed as described by Kippert (1995).

2.3.2. Sulfite chemistry

As used in this paper, sulfite is an inclusive term referring to all species and salts of sulfurous acid, including sulfur dioxide, its anhydride. Free sulfite includes all unbound species of sulfurous acid: H_2SO_3, HSO_3^-, and SO_3^{-2}. All species are reactive with carbonyl groups present in ingredients commonly found in microbiological media or produced during fermentation, such as sugars, acetaldehyde, and pyruvate. The resultant sulfonates (bound forms of sulfite) are not inhibitory to yeast growth, but their formation reduces the effective free sulfite concentration. The only species of sulfite that is inhibitory to yeast is undissociated sulfurous acid, H_2SO_3, as it freely traverses the cell membrane whereas the other free forms apparently cannot (Stratford *et al.*, 1987). Because the concentration of the various sulfite species of sulfite is pH-dependent (pKa_1 and pKa_2 are 1.77 and 7.2, respectively (King *et al.*, 1981) media containing sulfite were buffered.

2.3.3. Hydrogen sulfide, sulfite, and protein determinations

Intracellular hydrogen sulfide and sulfite were assayed as fluorescent adducts of monobromobimane (Calbiochem, La Jolla, CA) following separation by HPLC (Fahey and Newton, 1987) as modified by Vetter *et al.*, 1989. Briefly, cells were grown to about 1×10^7 cells/ml in 100 ml of SM-met, pelleted by centrifugation, washed twice in 20 mM HEPES, pH 8.0, and resuspended in 100 µl of the same buffer to which was added 10 µl of 50 mM monobromobimane in acetonitrile. A volume of acid-washed glass beads equal to the cell pellet was added, the mixture was vortexed six times at high

speed in 30 second bursts, and a fresh 110 µl of the HEPES-monobromobimane mixture was added. The liquid fraction was then centrifuged at 12,000 x g for five minutes at 4°C. A portion of the supernatant (10 µl) was removed for protein determination and the remainder was held 10 minutes in the dark at room temperature to allow the monobromobimane to react completely with thiols. Per 110 µl of supernatant, 100 µl of acetonitrile were added, the mixture was incubated at 60° for 10 minutes, and 300 µl of 25 mM methane sulfonic acid were then added. The samples were centrifuged at 12,000 x g at room temperature for five minutes to pellet precipitated protein, the supernatants were filtered through 0.45 µm PTFE membranes (VWR), and held at -80°C until HPLC analysis on a Beckman C18 reversed phase column, #235329, (via Rainin Instrument Co., Inc., Ridgefield, NJ) using an integrator and gradient maker from OMS Tech (Miami, FL), and an Alltech 325 HPLC pump (Alltech Associates, Inc., Deerfield, IL).

Protein was measured by the Bradford method using ovalbumin as a standard (Bradford, 1976).

2.3.4. Subcloning, plasmids, DNA sequencing, and PCR

Standard procedures for the manipulation of plasmid DNA and bacterial transformation were used (Sambrook *et al.*, 1989). Subcloning involved YCplac and YEplac, centromeric and episomal vectors, respectively (Gietz and Sugino, 1988), and pBluescript KS II (Stratagene, La Jolla, CA). pDR1 (Barral *et al.*, 1995) was obtained from C. Mann. pRY181 (Yocum *et al.*, 1984), used to introduce a *GAL1-lacZ* fusion in order to monitor glucose derepression, and pBM2101, which contains a null allele of *GRR1* (Flick and Johnston, 1991) were obtained from M. Johnston. pDIS vectors (personal communication, 1994) were obtained from C. Marcireau.

DNA was sequenced using the dideoxy dye terminator method on an ABI Model 373A sequencer (Applied Biosystems, Inc., Foster City, CA) at the Central Services

Laboratory of the Oregon State University Center for Gene Research and Biotechnology.

Escherichia coli strain DH5α was used in all subcloning experiments (Hanahan, 1983).

PCR was performed using Taq polymerase (Promega, Madison, WA) in an Easycycler (Ericomp, Inc., New Haven, CT) or a Robocycler 40 (Stratagene, La Jolla, CA) thermal cycler.

2.3.5. Cloning of *SSU2*

SSU2 was cloned by complementation of the sulfite sensitivity of mutant 3118-27d (*ssu2-6*) by transformation with a yeast genomic library in the centromeric vector p366 (F. Spencer and P. Hieter, unpublished data, 1989). Transformants were selected on SM-leu and screened on YEPD containing 2 mM sulfite. One positive clone was isolated from 11,323 transformants. The complementing clone, 6-6-2, contained an insert of about 10 kb (Fig. 5). Subclone BBC4 was constructed by deleting an internal 2.5 kb *NruI/SmaI* fragment. Subclone BX1 was derived from BBC4 by deletion of a 1.6 kb *Bam*HI fragment. The remaining subclones were derived from BX1: Delta H3 as a 5.0 kb *Bam*HI/*Hin*dII fragment; Delta HP as a 4.0 kb fragment with a 1.9 kb *Hpa*I deletion spanning all three sites; SH1 as a 2.6 kb *Sac*I/*Hin*dIII fragment; and Delta BG as a fragment with a 3.6 kb *Bgl*II deletion. SH1 was subcloned into YCplac33 cut with *Sac*I and *Hin*dIII, and sequenced at both ends, using YCplac33 primers 5'T T A G G C A C C C C A G G C T T T A C A C T T T 3' and 5'GCTGGCGAAAGGGGGATGTG3'.

Null alleles of *SSU2* were obtained by transforming 2757-4d-T4 and 3090-9d with *Bam*HI-digested pBM206 (Flick and Johnston, 1991), which contained a *grr1::URA3* disruption to direct integration to the *GRR1* locus. The null allele was confirmed by PCR (Sathe *et al.*, 1991) with the *GRR1* primers
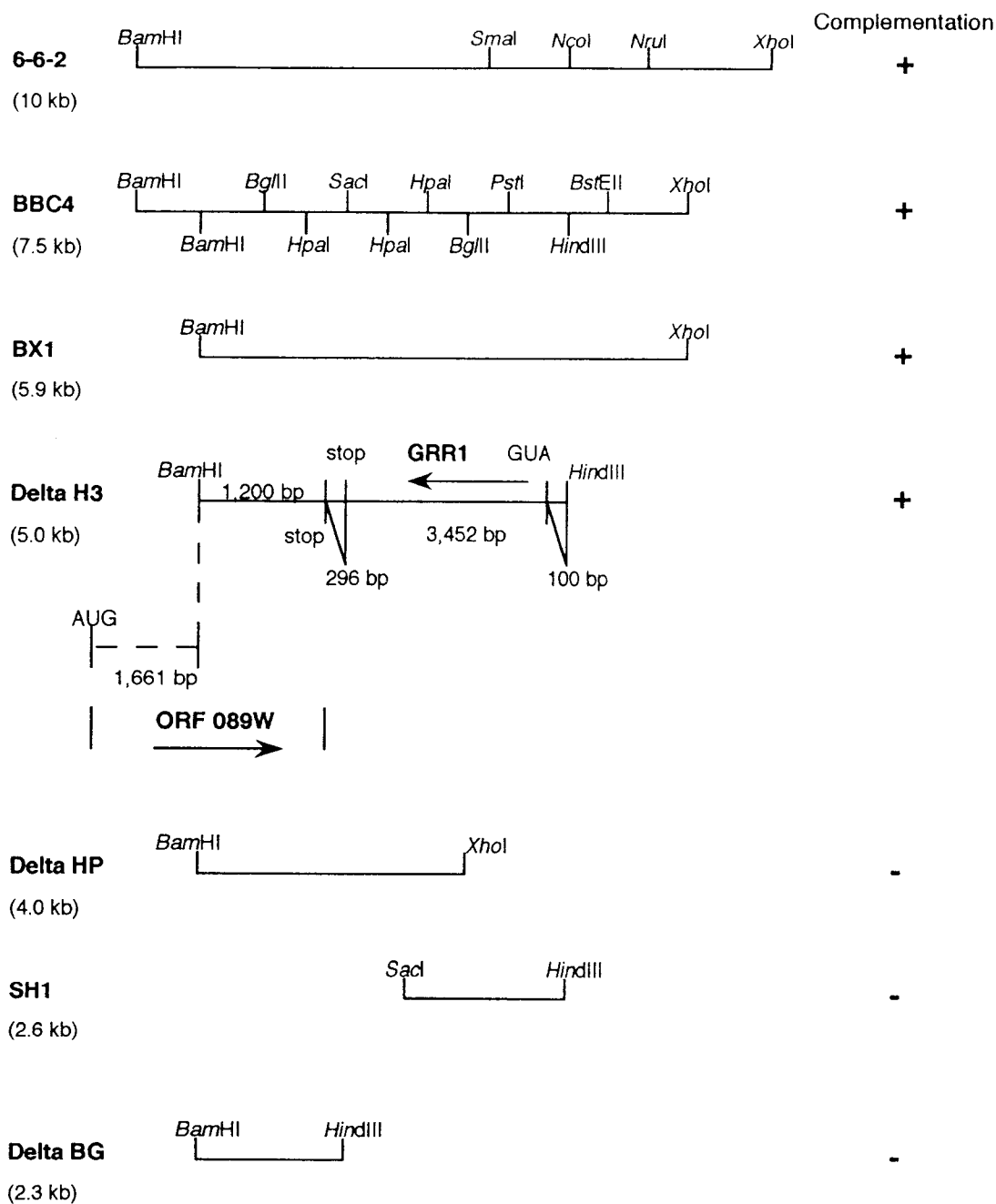
Fig. 5. Restriction map of the primary *SSU2*-containing fragment and derived subclones (not drawn to scale). The primary clone was designated 6-6-2. Subclone BBC4 was derived by removal of a 2.5 *Sma*I/*Nru*I fragment. Subclone BX1 was derived from BBC4 by deletion of a 1.6 kb *Bam*HI fragment. The remaining subclones were derived from BX1: Delta H3 as a 5.0 kb *Bam*HI/*Hind*III fragment.

5'CGAGATATTCAAGGCAGTTC3' and 5'TACAGCACGCAAAGTCCAC3', and

5'CTAAACTCACAAATTAGAGCTTC3', a *URA3* primer.

2.3.6. Cloning of *FZF1*

A yeast genomic library in the episomal vector pGAD (Chien *et al.*, 1991) was screened to isolate multicopy suppressors of the sulfite-sensitivity of 3154-2b (*grr1::URA3*). Three positive clones were isolated from 3,025 transformants plated on SM-leu, containing 1.5 mM sulfite. Two contained vector sequences only. The third contained an insert of about 5 kb which was shown not to be *GRR1* by PCR using the *GRR1* primers given above. The ends of the insert were sequenced using pGAD primers.

Subclones were constructed as follows (Fig.6): *ORF1* was subcloned as a 2.2 kb *Hind*III fragment in the *Hind*III site of YEplac195; *FZF1* was subcloned as a 1.4 kb *Hpa*I/*Ecl*136II fragment in the *Ecl*136II site of YEplac195; and *HXK2* was subcloned as a 2.0 kb *Ecl*136II/*Sma*I fragment in the *Ecl*136II site of YEplac195. The subclones were confirmed by restriction analysis and PCR, using *FZF1* primers: 5'GGACAATAAATACGCTAAAG3' and 5'CACATGAGTAGAGGACGGAA 3'.

2.3.7. Disruption of *FZF1*

FZF1 was disrupted using the one-step gene replacement method (Rothstein, 1983). The 1.4 kb *Hpa*I/*Ecl*136II *FZF1* fragment was subcloned into *Bam*HI/*Sal*I-digested Bluescript KS II that had been pretreated with Klenow polymerase. A *Hind*III fragment, encoding amino acids 99 to 182 was replaced by the disruption cassette of pDIS3, containing *URA3* flanked by two direct repeated sequences, DRS, (C. Marcireau, personal communication, 1994) (Fig. 6). The final construct was digested with *Kpn*I which cuts once in non-coding sequences adjacent to the 5' end of *FZF1* and once in the vector within 30 bp of the 3' end of *FZF1*. The digest was then used to

transform 3090-9d (*GRR1 ura3*) and 3090-9d-T6-L1 (*grr1Δ6–1 ura3*) to yield 3090-9d-T10 (*fzf1Δ99–182::URA3*) and 3090-9d-T6-L1-T10 (*fzf1Δ:99-182:URA3 grr1Δ6–1*), respectively. Integration of the disruption construct at the *FZF1* locus was confirmed by PCR (Sathe *et al.*, 1991) using the two *FZF1* primers and the *URA3* primer given above.
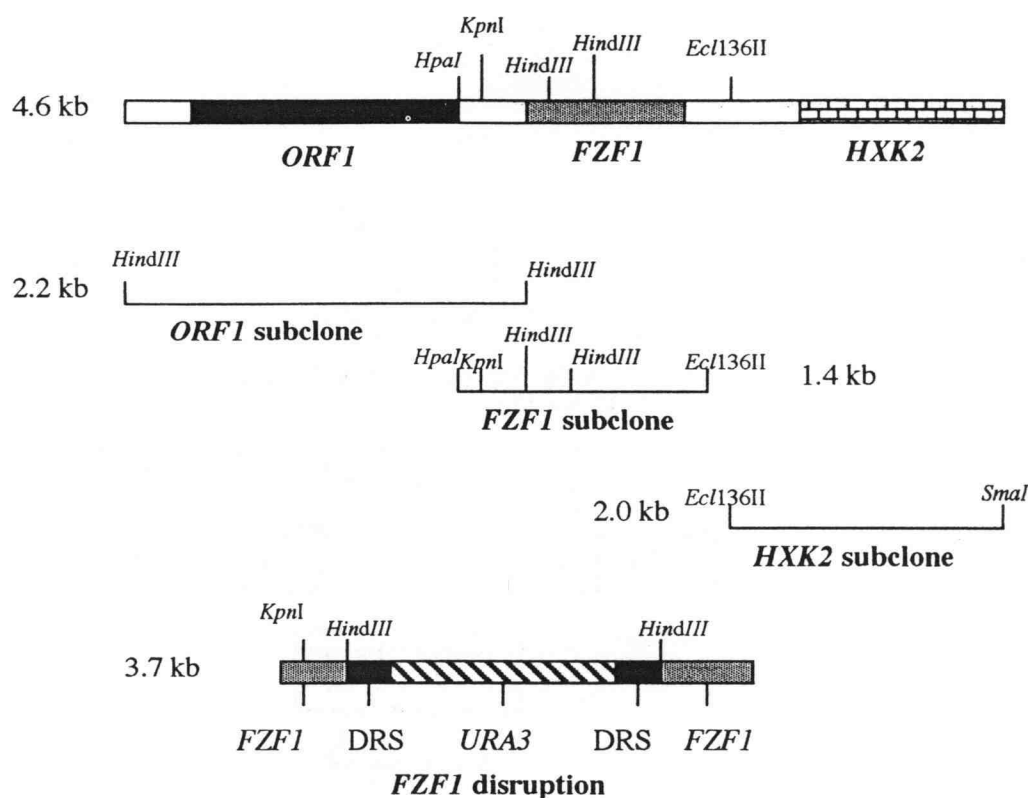

Fig. 6. Restriction map and subcloning of *FZF1*. Unshaded regions in the initial 4.6 kb clone refer to non-coding sequences of *ORF1*, *FZF1*, and *HXK2*. *DRS* in the 3.7 kb subclone refers to a direct repeated sequence from pDIS3. Shaded regions designated *FZF1* in this clone refer to both coding and adjacent non-coding *FZF1* sequences.

2.4. Results

2.4.1. *SSU2* is identical to *GRR1*

A sulfite-sensitive mutant, *ssu2-6*, was previously shown to have an elongated cell morphology, to excrete less acetaldehyde than wild-type, and to have 50% of the wild-type level of glutathione (Xu *et al.*, 1994). The one positive clone obtained by transformation with DNA from a centromeric wild-type genomic library grew on selective media, YEPD containing 2 mM sulfite, and exhibited wild-type cell morphology. The transforming plasmid was rescued from yeast, amplified in *E. coli*, and subcloned (Fig. 5). Subclone Delta H3, the smallest complementing fragment, was presumed to contain the intact gene. Three smaller subclones derived from Delta H3 (Delta BG, Delta HP, and SH1) failed to complement the sulfite sensitivity of *ssu2-6*, indicating that sequences necessary for function had been deleted. The ends of one of these, SH1, were partially sequenced. Analysis of 218 and 231 bp of non-contiguous terminal sequences, showed identity to sequences 412 to 629 and 2931 to 3161, respectively of *GRR1* (Flick and Johnston, 1991). Once *GRR1* sequences were localized, we were able to determine that subclone Delta H3 contained the intact *GRR1* open reading frame and a promoterless 1.2 kb carboxyl terminal fragment of ORF 089W (Galibert *et al.*, 1995).

To confirm that *SSU2* was identical to *GRR1*, 3118-19c (*ssu2-6*) was crossed with YM3502 (*grr1Δ::URA3*) to yield the diploid 3152, which was found to be sensitive to sulfite indicating non-complementation (Fig. 7). To establish allelism, 24 tetrads were dissected of which 12 produced four viable spores (overall spore viability was 85%). All progeny in these 12 tetrads were tested and shown to be sensitive to sulfite, confirming that *ssu2-6* is an allele of *GRR1*. Mutant *ssu2-6* was also found to

Strain	Relevant genotype	Sulfite (mM)						
		0	0.5	0.75	1.0	1.5	2	2.5
3118-19c	*ssu2-6*	+	+	+	+	+/-	-	-
YM2957	*grr1Δ–1829*	+	+	+/-	-	-	-	-
YM3378	*grr1Δ-1829 rgt1-101*	+	+	+	+/-	-	-	-
YM3478	*rgt1-101*	+	+	+	+	+	-	-
3152	*ssu2-6/grr1Δ::URA3*	+	+	+	+	-	-	-
3090-9d-T6-L1	*grr1Δ6-1*	+	+	+	-	-	-	-
YM2061	*GRR1*	+	+	+	+	+	+/-	-
3090-9d	*GRR1*	+	+	+	+	+	+	-
3090-9d-T10	*fzf1Δ 99-182 GRR1*	+	+	+	+	-/+	-	-
3090-9d-T6-L1-T10	*grr1Δ6-1 fzf1Δ 99-182*	+	+	-/+	-	-	-	-

Fig. 7. Growth of *grr1* (*ssu2*) mutants on YEPD containing sulfite. Cells were grown overnight in liquid YEPD, washed once, and resuspended in distilled water to yield 2 x 10⁹ cells/ml. Aliquots of 5 ml (10⁷) cells were plated and scored after 2 days at 30° C. Growth of strain YM 3502, not tabulated above, was identical to that of YM 2957. rgt1-101 was originally isolated as a suppressor of *grr1* (Erickson and Johnston, 1994). + normal growth, - no growth, +/- poorer than normal growth, -/+ very poor growth.

TABLE 2. Expression of β—galactosidase in *GAL1-lacZ* fusion strains grown under inducing or repressing conditions[a].

Strain	Relevant genotype	2% gal	2% gal + 2% glu	Level of repression
3150-14	*ssu2-6 LEU2::GAL1-lacZ*	637 ± 42	145 ± 10	4.4
2757-4d-4T	*SSU2 LEU2::GAL1-lacZ*	592 ± 55	2.8 ± 0.5	211
YM3502-T1	*grr1Δ LEU2::GAL1-lacZ/ YEplac195*	682 ± 39	277 ± 12.8	2.5
YM3502-T2	*grr1Δ LEU2::GAL1-lacZ/ YEplac195::FZF1*	768 ± 44	361 ± 4.2	2.1

Values are means ± standard deviations for three experiments.

[a] β—galactosidase activity is expressed as OD_{420} normalized to cell number (Kippert, 1995).

exhibit glucose derepression, a key phenotype of *grr1* strains, as measured in a *GAL1-lacZ* fusion construct (strain 3150-14 in Table 2).

2.4.2. *grr1* mutants are sensitive to sulfite and are partially defective in hydrogen sulfide formation

A number of *grr1* mutants, isolated or constructed on the basis of their glucose derepression phenotype (Flick and Johnston, 1991) were tested for sensitivity to sulfite. All were found to be sensitive, with the null mutants YM3502 and YM2957 exhibiting the greatest sensitivity (Fig. 7). A *grr1* null mutant was constructed in the same genetic background in which the *ssu2-6* allele had originally been isolated by transforming strain 3090-9d with *Bam*H1-digested pBM206. The null mutant obtained, 3090-9d-T6-L1, was found to be sulfite-sensitive (Fig. 7).

Because an inability to reduce sulfite to hydrogen sulfide via the reductive sulfate assimilation pathway would explain potential accumulation of the compound and sensitivity to exogenous sulfite, a qualitative estimate of intracellular hydrogen sulfide was made on BiGGY agar. This medium allows a qualitative, visual estimate of hydrogen sulfide formation, an obligate intermediate in methionine biosynthesis, by its intracellular precipitation as brown bismuth sulfide. Relative to wild-type, the *grr1Δ* mutants 3090-9d-T6 and 3154-2b exhibited a weak ability to form hydrogen sulfide in vivo on BiGGY agar (data not shown). The fact that *grr1* mutants are not methionine auxotrophs indicates that enzymatic formation of hydrogen sulfide by sulfite reductase is operative, but apparently not at wild-type levels. Measurement of intracellular hydrogen sulfide by reaction with monobromobimane confirmed that significantly less ($p = 0.05$, Student's t-test) was formed in a *grr1Δ* mutant than in a *GRR1* strain: 0.61 ± 0.22 vs. 2.56 ± 0.67 pmoles/µg protein, respectively ($n = 3$). Free sulfite could not be detected in either strain.

2.4.3. The sulfite-sensitivity caused by *grr1* and partially suppressed by *rgt1* is independent of carbon source

grr1 mutants grow poorly on glucose as a result of reduced glucose uptake caused by a significant decrease in expression of genes encoding hexose transporters (Özcan and Johnston, 1995; Özcan *et al.*, 1994). The Grr1 protein is thought to be a negative regulator of the product of the *RGT1* gene, which itself negatively regulates glucose transporters (Erickson and Johnston, 1994). Mutations in *RGT1* suppress the slow growth of *grr1* mutants on glucose (Erickson and Johnston, 1994). In order to determine if the sulfite-sensitivity of *grr1* mutants was related to the defect in transport and resultant slow growth on glucose, *grr1* mutants and a *grr1 rgt1* double mutant (YM3378) were tested for sensitivity during growth on glucose and non-glucose carbon sources. On glucose, the double mutant was more sulfite-tolerant than the *grr1* mutant--

poor growth on 1.0 mM sulfite relative to no growth by the *grr1* strain--although wild-type tolerance was not observed, indicating partial suppression of the sensitivity (Fig.7). On non-glucose carbon sources, where the absolute sulfite levels tolerated varied slightly, *grr1* strains were consistently sensitive relative to wildtype (Table 3). While the *rgt1* strain by itself exhibited mild sensitivity to sulfite when grown on glucose (Fig. 7), and greater sensitivity on acetate and maltose, it partially suppressed the sensitivity of the *grr1* mutant on all the non-glucose carbon sources tested: galactose, maltose, glycerol, ethanol, and acetate (Table 3). Taken together, these data indicate that the sulfite sensitivity of *grr1* is not strictly linked to the growth defect on glucose and that *rgt1* is mediating suppression through a route likely unrelated to its role as a negative regulator of glucose transporters.

An interesting observation was made relative to *RGT1* that is clearly unrelated to sulfite. In the absence of added sulfite, the *rgt1* mutant was found to grow on acetate, to grow very poorly on ethanol, and not to grow at all on glycerol. However, the *rgt1 grr1* double mutant grew on glycerol and grew well on ethanol, indicating that *grr1* was able to suppress the *rgt1*-specific growth defect (Table 3).

The acetate plates (YEPac) were buffered at pH 4.8 rather than at 3.5 to avoid a precipitate that formed at the lower pH. At pH 4.8, wild-type was expected to tolerate an approximate 20-fold greater concentration than in the medium buffered at pH 3.5 for the following reasons. 1) The concentration of the toxic form of sulfite, H_2SO_3, is pH dependent and is 20-times lower at pH 4.8 than at 3.5 ($pKa_1 = 1.77$). 2) Acetate is not known to react with and detoxify sulfite. The observation that wild-type failed to grow in the presence of significantly higher total sulfite in acetate suggests significantly greater sensitivity under these conditions. This conclusion assumes that H_2SO_3 is the only species that freely traverses the cell membrane (Stratford *et al.*, 1987). Acetaldehyde is not an obligate intermediate of acetate catabolism, whereas it is likely formed during metabolism of the other carbon sources tested (Fraenkel, 1982). These

TABLE 3. Growth of *grr1* mutants on different carbon sources containing sulfite[a].

Medium[c]	GRR1	grr1Δ–1829	grr1Δ–1829 rgt1-101	rgt1-101	ssu2-6
YEPgal					
0 mM sulfite	+	+	+	+	+
0.125 "	+	+	+	+	+
0.25 "	+	+	+	+	+
0.5 "	+	-	+	+	-
0.75 "	+	-	-	-	-
1.0 "	-	-	-	-	-
YEPmal					
0 mM sulfite	+	+	+	+	+
0.125 "	+	+	+	+	+
0.25 "	+	+	+	+/-	+
0.5 "	+	-	+	-	-/+
0.75 "	+	-	-	-	-
1.0 "	-	-	-	-	-
YEPE					
0 mM sulfite	+	+	+	-/+	+
0.125 "	+	+	+	-	+
0.25 "	+	+	+	-	+
0.5 "	+	-	-	-	+/-
0.75 "	+	-	-	-	-
1.0 "	-	-	-	-	-

TABLE 3 (Continued)

<u>YEPgly</u>

0 mM sulfite	+	+	+	-	+
0.125 "	+	+	+	-	+
0.25 "	+	-	+	-	-
0.5 "	+	-	-	-	-
0.75 "	-	-	-	-	-

<u>YEPac</u>

0 mM sulfite	+	+	+	+	+
0.125 "	+	+	+	+	+
0.25 "	+	+	+	+	+
0.5 "	+	+	+	+	+
0.75 "	+	-	+	-	+/-
1.0 "	+	-	+	-	-/+
1.5 "	+	-	-	-	-
2.0 "	-	-	-	-	-

---

a + normal growth, - no growth, +/- poorer growth than normal, -/+ very poor growth. Refer to Figure 3 for a visual indication of these scores.

b Growth of strain *grr1Δ*, not tabulated above, was identical to that of *grr1Δ–1829*.

c YEPac plates were buffered at pH 4.8, all others at 3.5.

results suggest that the detoxification reaction that occurs non-enzymatically between sulfite and acetaldehyde--and which does not occur during growth on acetate--is an important mechanism in mediating the basal sulfite tolerance of *Saccharomyces*.

2.4.4. Overexpression of *CLN1* causes a *GRR1* strain to become sulfite-sensitive

Barral *et al.* (1995) showed that in *grr1* strains, Cln1 and Cln2 proteins are stabilized and speculated that this might promote the characteristic change in cell morphology, from round to elongate (Barral *et al.*, 1995). They also showed that overexpression of *CLN1* in a *GRR1* background induced a similar change in cell shape. In order to determine if overexpression of *CLN1* in a *GRR1* strain would cause sulfite sensitivity, 2757-4d-T4 was transformed with a vector containing the *CLN1* gene under the control of the *GAL10* promoter (pDR1) and grown on galactose. Transformants overexpressing *CLN1* became twice as sensitive to sulfite as wild-type, suggesting a connection between the sensitive phenotype and *CLN1* expression (Table 4). Expression of *GAL10*-driven *CLN1* in a *grr1* strain resulted in such slow growth that assessment of sulfite sensitivity was difficult.

TABLE 4. Growth of *GRR1* cells carrying multicopy *CLN1*[a].

Relevant genotype	Sulfite (mM)				
	0	0.5	1	1.5	2.0
GRR1	+	+	+	-	-
GRR1, *GAL10*-driven *CLN1*, YEplac195	+	-	-	-	-
GRR1, *GAL10*-driven *CLN1*, YEplac195::*FZF1*	+	+	+	-	-

The parental strain (*GRR1*) is 2757-4d-T4. *GAL10*-driven *CLN1* was introduced by transformation with plasmid pDR1, and *FZF1* was introduced on plasmid YEplac195::FZF1. All strains were grown on galactose-based SM plates buffered at pH 3.5, under selective conditions (no uracil or leucine) if necessary to maintain plasmids. The apparent increased sensitivity to sulfite on galactose relative to glucose (compare with *GRR1* grown on YEPD, Fig. 7) is an artifact due to a lack of a chemical detoxification reaction between the added sulfite and galactose. In glucose-based media, a portion of the added sulfite is always quenched in a chemical reaction with glucose.
a + growth, - no growth. Refer to Fig. 7 for a visual indication of these scores.

2.4.5. Multicopy *FZF1/SUL1* suppresses the sulfite sensitivity, but not the glucose derepression or aberrant cell morphology of a *grr1* mutant

In order to identify proteins that might be functionally related to Grr1, multicopy genes were sought that would suppress the sulfite sensitivity of a *grr1* mutant. A null *grr1* mutant was transformed with DNA from a multicopy genomic library and a single clone containing an insert of about 4.6 kb was identified. The ends of the suppressing DNA were sequenced and indicated identity with sequences 1292 to 1592 of *HXK2* and sequences 109 to 409 upstream of *ORF1*. The intact DNA contained a part of the *HXK2* gene (lacking the carboxy terminal end), the entire *FZF1* ORF, identified on the basis of restriction analysis and PCR using *FZF1*-specific primers (Breitwieser *et al.*, 1993), and an open reading frame whose identity is unknown, *ORF1* (Fig. 6).

The three fragments were subcloned separately in YEplac195 and tested for suppressing activity. *FZF1*, encoding a putative transcription factor containing five zinc fingers, was the only DNA to suppress the sulfite sensitivity of the *grr1* mutant. Others have shown that substitution of glutamic acid for histidine in position 180 of the Fzf1 protein conferred a sulfite-resistant phenotype (Casalone *et al.*, 1994). *FZF1* failed to suppress the aberrant cell morphology and weak ability to form hydrogen sulfide (data not shown). Furthermore, *FZF1* did not suppress the glucose derepression phenotype of *grr1*. A null mutant of *grr1* carrying an integrated *GAL1-lacZ* fusion construct (YM3502) transformed with multicopy *FZF1* (TM3502-T2) or vector alone (YM3502-T1) exhibited the same level of derepression as a *grr1* strain (*ssu2-6*) (Table 2).

2.4.6. Sulfite sensitivity caused by mutations not allelic to *grr1* and by *CLN1* overexpression is suppressed by multicopy *FZF1*

To determine if multicopy *FZF1*-mediated suppression of sulfite sensitivity was specific to *grr1*, a number of other sulfite-sensitive mutants were examined. The growth of the strains in the presence and absence of multicopy *FZF1* as a function of

sulfite concentration is shown in Table 5. *ssu1*, *ssu3*, and *ssu4* were previously isolated on the basis of their sulfite sensitivity (Xu *et al.*, 1994). *SSU1* has since been cloned and sequenced and does not share significant similarity with any other gene in public data banks, GenBank accession number U20254 (D. Avram and A. T. Bakalinsky, unpublished data, 1995). The sulfite sensitivity of *ssu1* and *met20* was not suppressed by multicopy *FZF1*. However, the sensitivity of *ssu4* and *met18* was suppressed and that of *ssu3* was partially suppressed. The petite character of *ssu4* which co-segregates with its sulfite sensitivity was not suppressed. *met20* mutants are defective, and *met18* mutants are partially defective in sulfite reductase, and both accumulate sulfite intracellularly (Thomas *et al.*, 1992). Interestingly, multicopy *FZF1* in a *GRR1* strain caused a modest increase in its sulfite tolerance (3090-9d in Table 5).

TABLE 5. Suppression of sulfite sensitivity by *FZF1* in high copy number[a].

Strain	Relevant mutation	Sulfite Concentration (mM)				
		0	1	1.5	2	2.5
3090-9d	wild-type	+(+)	+(+)	+(+)	+(+)	+/-(-)
YM3502	*grr1*	+(+)	+(-)	+(-)	+/-(-)	-(-)
3090-9d-T4	*ssu1*	+(+)	-(-)	-(-)	-(-)	-(-)
3089-1d	*ssu3*	+(+)	+(-)	+(-)	-(-)	-(-)
3100-5b	*ssu4*	+(+)	+(-/+)	+(-)	+/-(-)	-(-)
CC370-8C	*met20*	+(+)	+(+)	-(-)	-(-)	-(-)
CC363-20B	*met18*	+(+)	+(-)	+(-)	+(-)	-(-)

The values in parentheses indicate the level of tolerance of strains not carrying multicopy *FZF1* and are preceeded by values for the same strains transformed with the multicopy *FZF1* construct. Cells were grown on SM-ura buffered at pH 3.5. Strains not carrying multicopy *FZF1* carried the vector alone, YEplac195.

[a] + normal growth, - no growth, +/- poorer growth than normal, -/+ very poor growth. Refer to Fig. 7 for a visual indication of these scores.

As noted above, overexpression of *CLN1* in a *GRR1* strain was found to cause sulfite sensitivity (Table 4). Transformation of such a strain (2757-4d-T4/pDR1) with multicopy *FZF1* suppressed the sensitive phenotype.

2.4.7. Disruption of *FZF1* causes sulfite sensitivity in a *GRR1* strain

A deletion allele of *FZF1* was constructed by replacing sequences corresponding to amino acids 99 to 182, which includes the fourth zinc finger and most of the bipartite motif, with the disruption cassette from pDIS3, and integrating the construct at the *FZF1* locus into a *GRR1* strain (Fig. 6). The disruptant strain, 3090-9d-T10, exhibited sensitivity to sulfite, whereas in a *grr1* background (strain 3090-9d-T6-L1-T10), the disruption caused significantly greater sensitivity (Fig. 7).

2.5. Discussion

A mutation causing sulfite sensitivity, *ssu2-6*, was identified as an allele of *GRR1*. *grr1* mutants were also found to be partially deficient in the formation of hydrogen sulfide, but not to the extent that methionine auxotrophy was evident. Sulfite sensitivity was observed during growth on glucose, galactose, maltose, acetate, ethanol, and glycerol, indicating that it is not strictly associated with a defect in glucose metabolism. The suppressor *rgt1*, which restores the transport and derepression defects of *grr1* mutants on glucose was found to partially suppress the sulfite sensitive phenotype on glucose and other carbon sources. Overexpression of *CLN1* in *GRR1* cells resulted in sulfite sensitivity, and an elongated cell morphology characteristic of *grr1* strains. Multicopy *FZF1*, encoding a putative zinc finger protein, was found to suppress the sulfite sensitive phenotype of *grr1* strains, but not the glucose derepression, aberrant cell morphology, or partial deficiency in hydrogen sulfide formation. Multicopy *FZF1* was also found to suppress the sensitivity of a number of

unrelated sulfite-sensitive mutants: a *CLN1*-overexpressing strain, *ssu3*, *ssu4*, and *met18*, but not that of *ssu1* or *met20*. Disruption of *FZF1* resulted in sulfite sensitivity when the construct was introduced in single copy at the *FZF1* locus in a *GRR1* strain.

Different functions have been attributed to Grr1, a weakly expressed, putative regulatory protein of 135 kDa: involvement in glucose repression (Bailey and Woodward, 1984; Flick and Johnston, 1991), positive regulation of glucose transport (Özcan and Johnston, 1995; Özcan *et al.*, 1994; Vallier *et al.*, 1994), *SUC2* gene expression (Vallier and Carlson, 1991), regulation of divalent cation transport (Conklin *et al.*, 1993), involvement in turn-over of G_1 cyclins (Barral *et al.*, 1995), and suppression of *bem2* mutations (Kim *et al.*, 1994). *grr1* mutants grow slowly on glucose apparently due to impaired high affinity glucose uptake (Vallier *et al.*, 1994). The sulfite sensitivity of *grr1* may, in part, be related to this defect. Xu *et al.* (1994) previously found that a *grr1* (*ssu2-6*) mutant excreted a reduced amount of acetaldehyde relative to wild-type during growth on glucose. Because acetaldehyde can react with and detoxify sulfite, it is reasonable that the mutant has a reduced capacity to tolerate added sulfite. Casalone *et al.*, (1992) reported that a sulfite resistant mutant excreted more acetaldehyde than wild-type. The sulfite-sensitivity of *grr1* mutants is likely due in part to distinct consequences of defective glucose uptake. One is slow growth on glucose leading to the reduction in acetaldehyde production. The other is a possible impairment in a glucose-dependent process, such as transport, which may slow or abolish a hypothetical sulfite-efflux pump, or other sulfite-detoxifying pathway. However, the observation that *grr1* mutants are sensitive to sulfite on carbon sources other than glucose, requiring other transporters (i.e., maltose) (Lagunas, 1993), or on which growth defects are restored (galactose) (Flick and Johnston, 1991), or on non-fermentable carbon sources (glycerol and ethanol), indicates that sensitivity is not only caused by growth defects on glucose. The poor growth of *grr1* mutants on glucose is suppressed by a mutation in *RGT1* (Erickson and Johnston, 1994). The Rgt1 protein is

thought to be a negative regulator of glucose transporters and has been proposed to be negatively regulated by Grr1 (Erickson and Johnston, 1994). A slight suppression of sulfite sensitivity was observed in the double mutant *grr1 rgt1*, not only on glucose but on all other carbon sources tested, providing further indirect evidence that the defect in glucose transport cannot account entirely for the sensitive phenotype. These data together with the observation that *grr1* was able to suppress the poor growth and lack of growth of an *rgt1* mutant on ethanol and glycerol, respectively, suggest that *RGT1* may interact with *GRR1* in functions unrelated to glucose metabolism.

grr1 mutants were found to produce a reduced amount of hydrogen sulfide, but were not methionine auxotrophs, indicating a functional sulfite reductase. One explanation is a defective sulfite reductase that produces an adequate but limiting amount of hydrogen sulfide sufficient to avoid methionine auxotrophy, but insufficient to form a prominent precipitate with the bismuth indicator on "BiGGY" agar. It is also possible that flux through the reductive sulfate assimilation pathway is slowed in *grr1* strains, so that intermediates such as hydrogen sulfide do not accumulate significantly.

Barral *et al.* (1995) showed that in *grr1* mutants, Cln1 and Cln2 proteins are stabilized and suggested that this might cause the change in cell morphology. When *CLN1* was overexpressed in a *GRR1* strain, cells became sensitive to sulfite and also acquired the elongated morphology characteristic of *grr1* mutants. These workers postulated that in a *grr1* mutant, the high level of Cln1 and Cln2 proteins would advance the time at which START is executed. The execution of START results in the expression of functions required for DNA replication. Among these, the reduction of ribonucleotides to deoxyribonucleotides requires NADPH as does sulfite reduction to sulfide. Premature execution of START may result in a deficit of NADPH, inadequate for reduction of exogenous sulfite, leading to the observed sensitivity.

We found that multicopy *FZF1*, encoding a putative transcription factor (Breitwieser *et al.*, 1993), suppressed the sulfite sensitivity of *grr1* mutants, but not the

aberrant cell morphology, glucose derepression, or partial deficiency in hydrogen sulfide formation. Casalone *et al.* (1994) found that replacement of histidine with glutamic acid in position 180 of Fzf1 resulted in a dominant sulfite resistant phenotype, characterized by a reduced accumulation of sulfite (Casalone *et al.*, 1992), consistent with diminished uptake or enhanced efflux. The dominant nature of the resistant phenotype suggests possible hyperactivation. Breitwieser *et al.* (1993), who cloned *FZF1* in a search for cell-cycle regulated genes having a five zinc finger motif, proposed that histidine in position 179 is the second histidine involved in tetrahedral coordination of the zinc atom. Pavletich and Pabo (1993) suggested that the fourth zinc finger of Gli protein is important for interactions with DNA. Our finding that disruption of *FZF1* resulted in sensitivity to sulfite suggests that the Fzf1 protein plays a role in sulfite metabolism. The observation that multicopy *FZF1* suppressed the sulfite sensitivity of several sensitive mutants but not that of *ssu1* or *met20* is consistent with a role as a positive regulator of Ssu1p and Met20p. The function of Ssu1p is presently unknown but is the subject of investigation in this laboratory. However, Met20p is required for sulfite reductase activity, and increased sulfite reduction is a reasonable route through which exogenous sulfite can be detoxified.

2.6. Acknowledgements

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Chapter 3

SSU1 encodes a putative transporter with a central role in a network of proteins conferring sulfite tolerance in *Saccharomyces cerevisiae*

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3.1. Abstract

The *SSU1* gene of *S. cerevisiae* was isolated based on its ability to complement a mutation causing sensitivity to sulfite, an intermediate in methionine biosynthesis. The gene encodes a novel deduced protein of 458 amino acids that does not share significant similarity to other proteins in public databases. The predicted protein contains 9 or 10 membrane-spanning domains and resembles the general structure of facilitators/transporters. A confocal microscopy study localized an Ssu1-GFP fusion protein to the plasma membrane. Multicopy *SSU1* conferred about a two-fold higher level of sulfite tolerance to wild-type cells.

Multicopy suppression analysis was undertaken to explore possible relationships among genes previously implicated in sulfite metabolism. Multicopy *SSU1* suppressed the sulfite sensitivity of *grr1*, *fzf1*, and *ssu3* mutants. In contrast, multicopy *GRR1* was unable to suppress the sensitivity of *ssu1*, *fzf1*, and *ssu3* strains. Multicopy *FZF1* suppressed the sulfite sensitivity of a *grr1* mutant and partially suppressed that of *ssu3*, but was unable to suppress that of an *ssu1* mutant. These data suggest a pathway in which *SSU1* acts downstream of *FZF1* and *SSU3*, which in turn are downstream of *GRR1*. In order to examine expression of the sulfite-proximal member of the pathway, *SSU1*, a fusion of the *lacZ* ORF and the *SSU1* promoter was constructed. Full expression of the *SSU1* promoter was found to require functional *FZF1*, *SSU3*, and *GRR1* genes. The promoter was found to be activated by multicopy Fzf1p, confirming a role for *FZF1* in transcriptional regulation, previously suggested solely on the basis of sequence analysis. The activation of *SSU1* promoter in an *ssu3* background by multicopy *FZF1* was lower, suggesting that a functional *SSU3* gene may be required for full activation.

3.2. Introduction

Sulfite is a normal, but potentially toxic metabolite produced as an intermediate during reductive sulfate assimilation in fungi, bacteria and plants. Its toxicity to microorganisms has been widely exploited through use as a preservative in foods, beverages, and pharmaceuticals (Taylor *et al.*, 1986). The deleterious effects of sulfite on plants are evident in regions of the world where it is a significant component of air pollution (Ghisi *et al.*, 1990). The fact that sulfite is also a normal metabolite in a large variety of organisms raises the question of how endogenous toxicity is avoided. Clearly, efficient regulation of the reductive sulfate assimilation pathway would minimize pools of intermediates, sulfite among them, and may be the major form of control. In *S. cerevisiae*, formation of acetaldehyde during fermentation appears to be another means of controlling sulfite levels because the two compounds react to form a stable and non-toxic product, 1-hydroxyethane sulfonate (Taylor *et al.*, 1986).

In order to study how toxicity is avoided, we previously isolated mutants of *S. cerevisiae* in expectation that sensitive mutants may be impaired in protective functions and resistant mutants may have enhanced protection. This analysis identified four genes involved in the sensitive phenotype, *SSU1*, *SSU2*, *SSU3*, *SSU4*, and a single gene in resistance, *RSU1* (Xu *et al.*, 1994). One of the mutations conferring sensitivity (*ssu2*) was found to be an allele of *GRR1* (Avram and Bakalinsky, 1996), which others have implicated in glucose repression (Bailey and Woodward, 1984; Flick and Johnston, 1991; Gamo *et al.*, 1994), glucose transport (Özcan *et al.*, 1994, Özcan Jonston, 1995, Vallier *et al.*, 1994), *SUC2* gene expression (Vallier and Carlson, 1991), divalent cation transport (Conklin *et al.*, 1993), turn-over of G1 cyclins (Barral *et al.*, 1995), suppression of a *bem2* mutation (Kim *et al.*, 1994), and morphological differentiation (Blacketer *et al.*, 1995). While the sulfite-sensitivity of a *grr1* mutant may be partly due to defective glucose metabolism, sensitivity was observed during growth on non-

glucose carbon sources as well. Further, a suppressor of the glucose repression defect, *rgt1*, failed to completely suppress the sensitivity (Avram and Bakalinsky, 1996). We also previously showed that *FZF1* is a multicopy suppressor of the sulfite sensitivity of a *grr1* mutant. *FZF1* encodes a five zinc finger putative transcription factor (Breitwieser *et al.*, 1993) and a particular allele was independently found to confer dominant resistance to sulfite (Casalone *et al.*, 1994).

In the present study, we demonstrate that Ssu1p is a plasma membrane protein and that its expression is activated by Fzf1p in the presence of functional *SSU3*. We also propose potential relationships among four genes involved in protection against sulfite, *SSU1*, *FZF1*, *SSU3*, and *GRR1*.

3.3. Materials and Methods

3.3.1. Yeast strains, media, growth conditions, and genetic techniques

Yeast strains are listed in Table 6. Standard yeast genetic techniques were used (Rose *et al.*, 1990). Yeast transformations were performed using the method of Gietz *et al.*, 1992. Yeast media and plates used to test sulfite sensitivity are described elsewhere (Xu *et al.*, 1994). Drop-out media are SM lacking the indicated amino acid or base (Rose *et al.*, 1990). SM is glucose-based synthetic complete medium (SD plus required amino acids and bases at the prescribed concentrations, except for uracil, which was added to a final concentration of 10 mg/ml). 5-fluoroorotic acid (5-FOA) plates, for selection of *ura3* segregants, were prepared as described (Boeke *et al.*, 1984). β-galactosidase activity was assayed as described by Kippert (1995).

3.3.2. Subcloning, plasmids, DNA sequencing, and PCR

Standard procedures for the manipulation of plasmid DNA and bacterial transformation were used (Sambrook *et al.*, 1989). *Escherichia coli* DH5α (Hanahan,

TABLE 6. Yeast strains.

Strain	Genotype	Source
3090-9d	*MATα ura3-52 leu2-3, 112*	this laboratory
3088-6d	*MATa ssu1-1 ura3-52 leu2-3, 112*	this laboratory
3089-1d	*MATa ssu3-7 ura3-52 leu2-3, 112*	this laboratory
3100-5b	*MATa ssu4-11 ura3-52 leu2-3, 112 pet*	this laboratory
3090-9d-T6	*MATα ura3-52 leu2-3, 112 grr1Δ::URA3*	this laboratory
3090-9d-T6-L1	*MATα ura3-52 leu2-3, 112 grr1Δ6-1*	this laboratory
3090-9d-T4	*MATα ssu1Δ::URA3 leu2-3, 112 ura3-52*	this laboratory
3090-9d-T4-L1	*MATα ssu1Δ leu2-3, 112 ura3-52*	this laboratory
3090-9d-T10	*MATα fzf1Δ99-182::URA3 leu2-3, 112 ura3-52*	this laboratory
3090-9d-T10-L1	*MATα fzf1Δ99-182 leu2-3, 112 ura3-52*	this laboratory

1983) or *E. coli* SURE (Stratagene, La Jolla, CA) were used in all subcloning experiments involving YCplac33, YEplac181 and 195, and YIplac211, centromeric, episomal, and integrating vectors, respectively (Gietz and Sugino, 1988), and pUC19 (New England Biolabs, Beverly, MA). pDIS vectors as well as the "bright" S65T

mutant of *GFP* (Heim *et al.*, 1995) were obtained from C. Marcireau (personal communication, 1994, 1995).

DNA was sequenced using the dideoxy dye terminator method on an ABI Model 373A sequencer (Applied Biosystems, Inc., Foster City, CA) at the Central Services Laboratory of the Oregon State University Center for Gene Research and Biotechnology.

PCR was performed using *Pfu* (Stratagene, La Jolla, CA) and *Taq* polymerases (Promega, Madison, WA) in an Easycycler (Ericomp, Inc., New Haven, CT) or a Robocycler 40 (Stratagene, La Jolla, CA) thermal cycler.

3.3.3. *SSU1* constructs

SSU1 was cloned by complementation of the sulfite sensitivity of an *ssu1-1* mutant (3088-6d) by transformation with a yeast genomic library in the centromeric vector p366 (F. Spencer and P. Hieter, unpublished data, 1989). A single complementing clone, pDA17, contained an insert of about 9.5 kb (Fig.8). A 3.6 kb *Bam*HI fragment from pDA17 was subcloned in YCplac33 (pDA18), and in the integrating vector YIplac211 (pDA19, not shown). The latter construct was cut with *Mlu*I, and integrated into the yeast genome in an *ssu1-1* mutant (3088-6d). Other subclones of pDA17 were generated in YCplac33 digested with the appropriate restriction enzymes to yield pDA21, containing the entire *GLR1* ORF; pDA20, containing the *SSU1* ORF, 1 kb of the 5′ upstream region, and a DNA fragment corresponding to the first 48 N-terminal amino acids of the *GLR1* ORF; pDA22, as a *Sac*I fragment (the 5′ *Sac*I site is within vector sequences not shown in Fig. 8) containing a small ORF encoding 67 amino acids, located upstream of the *SSU1* ORF; and pDA23, containing the *SSU1* ORF.

Complementation

pDA17

BamHI   BamHI   HindIII

+

pDA18

BamHI   SacI   XbaI   HpaI BamHI

small ORF   SSU1 ORF   MluI   part of GLR1 ORF

+

pDA21

Xbal   HindIII

GLR1 ORF

−

pDA20

BamHI   Hpal

small ORF   SSU1 ORF   part of GLR1 ORF

+

pDA22

BamHI   SacI

small ORF

−

pDA23

SacI   BamHI

SSU1 ORF   part of GLR1 ORF

+

pDA24

BamHI   Hpal

DRS-URA3-DRS

−

pDA42

SSU1 ORF fused to GFP ORF

+
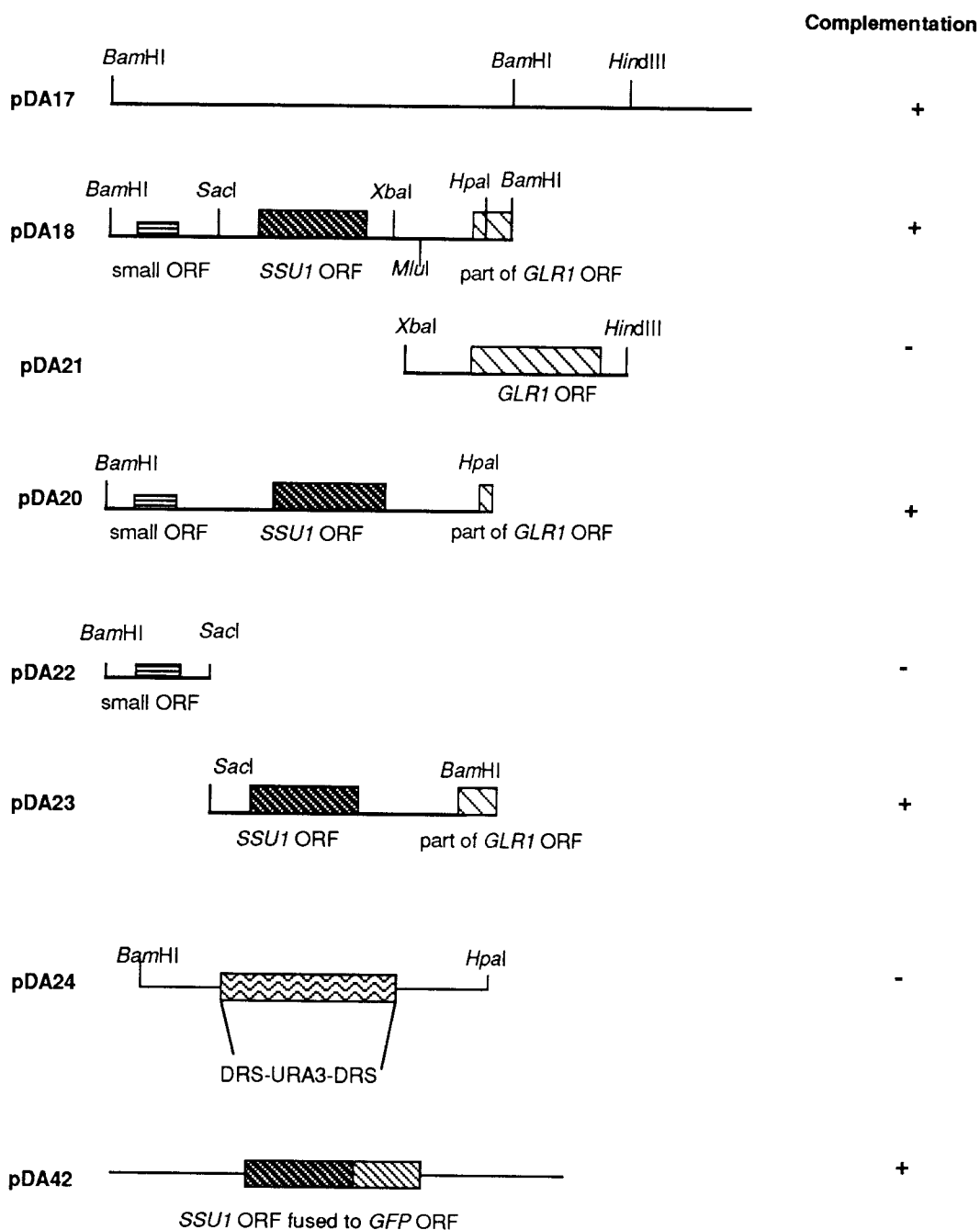
Fig. 8. Restriction map of the primary *SSU1*-containing fragment and derived subclones (not drawn to scale). The primary subclone was designated pDA17. pDA18 contains a 3.6 kb *Bam*H1 fragment from pDA17 cloned in YCplac33 which includes the small ORF, the entire *SSU1* ORF, and one third of the *GLR1* ORF. pDA21 contains the entire *GLR1* ORF. pDA20 contains the small ORF and the *SSU1* ORF, and pDA22 contains only the small ORF. pDA23 contains the *SSU1* ORF, and pDA24, the disrupted *SSU1* ORF (consisting of the *URA3* gene flanked by direct repeats, designated *DRS*). pDA42 contains the *SSU1* ORF fused to the *GFP* ORF.

The *SSU1* gene was disrupted using the one-step gene replacement method (Rothstein, 1983). A *Bam*HI/*Pst*I fragment from pDA20, blunted by treatment with T4 DNA polymerase was subcloned in pUC19, cut with *Sph*I and *Sac*I, and blunted with the same enzyme to yield pDA32 (not shown). The *Sac*I/*Xba*I disruption cassette of pDIS4 (Marcireau, personal communication, 1994) was cloned in pDA32 digested with *Sac*I and *Xba*I, replacing the *SSU1* ORF, except for the last 21 bases of the coding sequence. This construct, pDA24, was linearized with *Dra*I and *Aat*II and used to transform 3090-9d (*SSU1 ura3*) to yield 3090-9d-T4 (*SSU1Δ::URA3*). Integration of the disruption construct at the *SSU1* locus was confirmed by PCR (Sathe *et al.*, 1991), using two *SSU1* primers: 5′-GATATTGGCTGAACAAATTCTCC-3′ and 5′-AGAAGCAAAAGCAGCAAA-3′ and a *URA3* primer (Avram and Bakalinsky, 1996).

In order to obtain an Ssu1-GFP fusion protein, the *GFP* ORF containing the S65T mutation (Heim *et al.*, 1995) from pCM153 (Marcireau, personal communication, 1995) was cloned in YCplac33 as a *Bam*HI/*Sac*I fragment to yield pDA8 (not shown). The *SSU1* ORF and 150 bp upstream region were generated by PCR using the following primers: 5′-ACGCGTCGACACTTATTTCTAATCCTTGTC-3′ and 5′-CGCGGATCCGTAAAATCTAGAGCCGAGTT-3′ containing *Sal*I and *Bam*HI sites to allow in-frame cloning with *GFP* in pDA8 digested with *Bam*HI and *Sal*I, to yield pDA28. The rest of the *SSU1* upstream region was cloned by replacing the 427 bp *Hind*III fragment of pDA28 with a 1.3 bp fragment from pDA19, to generate pDA42 (not shown). pDA42 was used to transform 3088-6d, and 3090-9d-T4-L1, to check for complementation. The construct was then cut with *Bst*XI to delete the *CEN* and *ARS* sequences and was integrated at the *SSU1* locus by digesting with *Sfi*I.

To study the *SSU1* promoter, a 1 kb region upstream of the *SSU1* ORF was generated by PCR, using the following primers: 5′-

A A A A C T G C A G G A T C C A A G G C C G A C A G G - 3 ′ and 5′-
AAAACTGCAGTTTTTTCTTGTACTTGTCTT-3′, and then cloned as a *Pst*I fragment upsteam of the *LacZ* ORF, previously cloned in YIplac181 (and designated pDA1, not shown) to generate pDA6. This construct, as well as pDA1, were integrated at the *URA3* locus in the following strains: wild-type (3090-9d), *fzf1Δ* (3090-9d-T10-L1), *ssu3-7* (3089-1d), *grr1Δ* (3090-9d-T6-L1), *ssu4-1* (3100-5b), and *ssu1Δ* (3090-9d-T4-L1).

3.3.4. Sequence analysis

DNA Strider (Marck, 1988) was used to generate a Kyte-Doolittle hydropathy plot, and PSORT (Nakai and Kanehisa, 1992) and TMpred (Hoffman and Stoffel, 1993) were employed for detecting transmembrane domains.

3.3.5. Confocal microscopy.

Confocal laser scanning microscopy was performed on a Leica TCS 4D microscope, using a 100x/1.4 oil-immersion lens. Images were acquired using the 488-nm excitation line of an argon/krypton ion laser and an FT 490/520 dichroic mirror with a short bandpass filter of 510 nm. Fluorescence of the Ssu1-GFP fusion protein was observed in living cells and the images were processed using Adobe Photoshop. Cells were grown in SM-based selective medium and harvested in log phase. Protoplasts were prepared from log phase cells by treatment with 2 mg/ml zymolyase in 50 mM Tris, pH 7.5, containing 0.9 M sorbitol, and 0.1% β- mercaptoethanol for 1 hour at 37° C. Unwashed cells or protoplasts were adsorbed onto a polylysine-coated coverslip for viewing.

3.4. Results

3.4.1. Isolation of *SSU1* and DNA sequence analysis.

A genomic clone of *SSU1* was isolated from a centromeric library by complementation of the sulfite sensitivity of an *ssu1-1* mutant, 3088-6d (Xu *et al.*, 1994). Approximately 3,460 transformants were selected on SM-leu and screened on YEPD containing 2 mM sulfite. A single complementing clone contained a plasmid with an insert of about 9.5 kb, designated pDA17 (Fig. 8). A complementing 3.6 kb *Bam*HI fragment was subcloned in YCplac33 (pDA18) and in the integrating vector YIplac211 (pDA19) (Fig. 8). The latter construct was integrated into the yeast genome, in an *ssu1-1* mutant (3088-6d). Transformants were able to grow on plates containing 2 mM sulfite on which the mutant was unable to grow. All thirteen tetrads obtained from a cross between one of the transformants (BBI3) and an *SSU1 ura3* strain produced spores resistant to sulfite and which segregated 2+:2- for *URA3*. This confirmed that pDA19 carried the wild-type *SSU1* gene. Sequence analysis showed that pDA18 contained two complete ORFs, one of 67 amino acids and one of 458 amino acids, and a part of of glutathione-oxidoreductase (*GLR1*) ORF, encoding the first 146 N-terminal amino acids which represent about one third of the protein (Fig. 8). Subcloning established that the centrally located ORF of 458 amino acids (YPL092W) possessed complementing activity, and therefore was *SSU1* (Genbank accession no. U20254) (Fig. 9).

The predicted protein sequence of *SSU1* did not share significant similarity with proteins from public databases. Its hydrophobicity plot suggested ten hydrophobic regions (Fig. 10). A search at the PSORT server for predicting protein sorting signals (Nakai and Kanehisa, 1992; Goffeau *et al.*, 1993) indicated that the protein did not have an N-terminal signal sequence. A search at TMpred (Hofmann and Stoffel, 1993)

showed nine or ten transmembrane domains. Two models for transmembrane topology were suggested: the first (strongly preferred) having ten transmembrane domains and an internal amino-terminal fragment, and a second with nine transmembrane domains and an amino terminus external to the plasma membrane. The carboxy-terminal end of the protein (49 amino acids) is hydrophilic and has 10 (20%) serine residues, some being putative phosphorylation sites. TMpred and PSORT suggested a cytoplasmic location for the carboxy terminus.

SSU1p lacks the nucleotide binding sequence typical of ABC (ATP binding cassette) transporters (Higgins, 1992) and the conserved amino acid stretches which form the catalytic site of P-type ATPases (Fagan *et al.*, 1994). Based on its topology (10 transmembrane domains surrounded by hydrophilic regions), Ssu1p may be a facilitator/transporter.

3.4.2. An Ssu1-GFP fusion protein is localized to the plasma membrane.

The *GFP* ORF was fused in frame to the carboxy-terminal end of the *SSU1* ORF. The construct contained the entire 5' upstream region of the *SSU1* gene to insure expression under its own promoter. The *SSU1-GFP* construct complemented the sulfite sensitivity of an *ssu1* null mutant, demonstrating that *SSU1* function was retained (Fig. 8). *ARS* and *CEN* sequences were deleted and the construct was integrated at the *SSU1* locus. Examination of a wild-type strain expressing the Ssu1p-GFP fusion protein by laser scanning confocal microscopy revealed peripheral fluorescence (Fig. 11). Examination of protoplasts expressing the same fusion construct showed the same peripheral fluorescence (picture not shown), confirming a plasma membrane location. In another strain, in which the *GFP* ORF was expressed under the control of the *GAL7* promoter during growth on galactose, fluorescence was observed throughout the cytoplasm (picture not shown).

```
GATCCAAGGCCGACAGGATGGCCAAGATGGAAAGAAGAGAAAGAAATAGACATGCCAAGCAAGGTGAAT
CTGATAGACACAATGCTGTTTCCTTATCAAAGCATTTATTCAGTGGTAAGCGTGGTGTCGGTAAGACAG
ATTTCCGGGGATTTCTCTACTTCTTATTCTTTCTGGGGCATGTACTTTAAAAAAAAATATGAATATAAAA
TGTGGATGTACGTACACGACATTCTAATCTTTGGGGGGCTGGTAGGATTACCATTTACATTTGATTATC
TCTCTCACTATTAGTGGTTTCTTTTTCTTTCTTTCTCACTTTTTCTGTATCTTTTTTTTAAAAAAATTT
TATTTAATCTGTATAATAATAATAAACCGATTTAAATTATCCAACAAGCCGACCCCTCCATGTTCTACT
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CAAGATGACACTTCTACTTTTTTGGTGACACATCATCATGCAACCTATCGAGTCTCCCACGAGGTTGAC
AAATAAGAAATTGTTATCGTTTTTGGCAGCGTATCGTATAAGGCAACAATAGCGATGTCTCCCATCAAT
TGACTGATAAATTCCTGCAAACTATCATGTTATTTTTTTCATCCTTGTGCCGCGTCTGTAGCCGAAAAT
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CTTCTTTTTGCGGGGCTTTGCTTATTTTACCTATTTAACTAGAGGTTCTAAAAGAATAGCCAACCAGCG
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CAATTTAAAAACGTTTTATAGTGTAAGAGAAGACAAGTACAAGAAAAAAATGGTTGCCAATTGGGTACT
                                               M   V   A   N   W   V   L
TGCTCTTACGAGGCAGTTTGACCCCTTCATGTTTATGATGGTCATGGGTGTCGGCATTTCATCGAATAT
 A   L   T   R   Q   F   D   P   F   M   F   M   M   V   M   G   V   G   I   S   S   N   I
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 L   Y   S   F   P   Y   P   A   R   W   L   R   I   C   S   Y   I   M   F   A   I   T   C
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 L   I   F   I   A   V   Q   A   L   Q   I   L   H   L   I   V   Y   I   K   E   K   S   F
CAGAGAATATTTTAATGACTTTTTCAGAAATATGAAGCACAATTTATTTTGGGGTACTTATCCCATGGG
 R   E   Y   F   N   D   F   F   R   N   M   K   H   N   L   F   W   G   T   Y   P   M   G
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 L   V   T   I   I   N   F   L   G   A   L   S   K   A   N   T   T   K   S   P   T   N   A
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 R   N   L   M   I   F   V   Y   V   L   W   W   Y   D   L   A   V   C   L   V   I   A   W
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 G   I   S   F   L   I   W   H   D   Y   Y   P   L   E   G   I   G   N   Y   P   S   Y   N
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 I   K   M   A   S   E   N   M   K   S   V   L   L   L   D   I   I   P   L   V   V   V   T
TTCAAGTTGTGGAACATTCACAATGTCAGAAATATTCTTCCATGCGTTTAATAGAAACATTCAACTGAT
 S   S   C   G   T   F   T   M   S   E   I   F   F   H   A   F   N   R   N   I   Q   L   I
AACGTTGGTCATATGTGCCTTAACGTGGCTGCATGCCATTATCTTCGTCTTCATACTGATTGCGATATA
 T   L   V   I   C   A   L   T   W   L   H   A   I   I   F   V   F   I   L   I   A   I   Y
CTTCTGGAGTCTTTATATTAATAAGATACCACCAATGACACAGGTTTTCACCTTATTCCTGCTTTTGGG
 F   W   S   L   Y   I   N   K   I   P   P   M   T   Q   V   F   T   L   F   L   L   L   G
CCCGATGGGCCAAGGAAGTTTTGGAGTCTTATTGCTTACAGATAATATAAAAAAAATATGCGGGCAAATA
 P   M   G   Q   G   S   F   G   V   L   L   L   T   D   N   I   K   K   Y   A   G   K   Y
TTACCCAACAGATAACATTACAAGAGAACAAGAGATATTGACTATTGCAGTTCCATGGTGTTTCAAAAT
 Y   P   T   D   N   I   T   R   E   Q   E   I   L   T   I   A   V   P   W   C   F   K   I
TCTAGGCATGGTTTCTGCTATGGCATTGCTCGCTATGGGCTATTTTTTCACCGTGATTTCTGTCGTTTC
 L   G   M   V   S   A   M   A   L   L   A   M   G   Y   F   F   T   V   I   S   V   V   S
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 I   L   S   Y   Y   N   K   K   E   I   E   N   E   T   G   K   V   K   R   V   Y   T   F
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 H   K   G   F   W   G   M   T   F   P   M   G   T   M   S   L   G   N   E   E   L   Y   V
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 Q   Y   N   Q   Y   V   P   L   Y   A   F   R   V   L   G   T   I   Y   G   G   V   C   V
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 C   W   S   I   L   C   L   L   C   T   L   H   E   Y   S   K   K   M   L   H   A   A   R
TAAATCTTCATTATTTTCAGAGTCAGGTACGGAAAAGACGACAGTTTCTCCGTATAACAGCATTGAAAG
 K   S   S   L   F   S   E   S   G   T   E   K   T   T   V   S   P   Y   N   S   I   E   S
CGTGGAAGAATCAAACTCGGCTCTAGATTTTACGCGTTTAGCATAAAATTGTTGGTTTTATACGTACAT
 V   E   E   S   N   S   A   L   D   F   T   R   L   A   *
AATTGGCAAGCATTTCATGTAGGAGTCTCAATA
```

Fig. 9. The *SSU1* gene sequence and predicted protein. Underlined amino acids represent the putative transmembrane helices, suggested by a TMpred search (prediction parameters: TM-helix length between 17 and 33 amino acids.
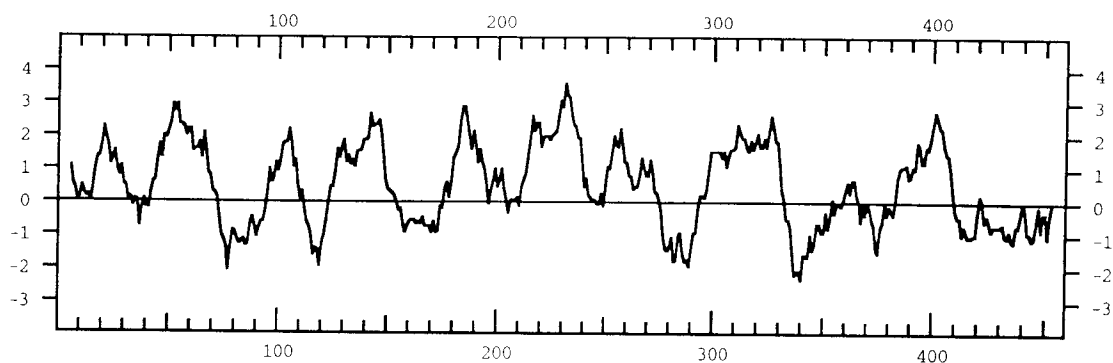
Fig. 10. Kyte-Doolittle hydropathy plot generated by the DNA Strider program for Ssu1p. The area of hydrophobicity is above the horizontal line at y = 0, hydrophilicity beneath. The scale on the X-axis indicates the number of amino acids residues with the amino terminus at left.

3.4.3. An *SSU1* null mutant is viable and sensitive to sulfite

The null mutant constructed by replacing nearly the entire *SSU1* ORF with the *URA3* ORF (pDA24 in Fig. 8) was found to be viable but sensitive to sulfite. Growth requirements for amino acids or bases were not evident, nor was the strain UV- or temperature-sensitive at 37° C. Sporulation of a homozygous *ssu1Δ/ssu1Δ* diploid was qualitatively indistinguishable from that of a wild-type diploid.

3.4.4. Multicopy suppression analysis

Multicopy suppression analysis was undertaken to uncover possible functional relationships among four genes implicated in the sulfite-sensitive phenotype, based on the premise that defects upstream in a regulatory pathway can be suppressed by overexpressed genes downstream, but not vice versa. Overexpression of the three cloned genes *GRR1*, *FZF1*, and *SSU1* was performed reciprocally in each of the single
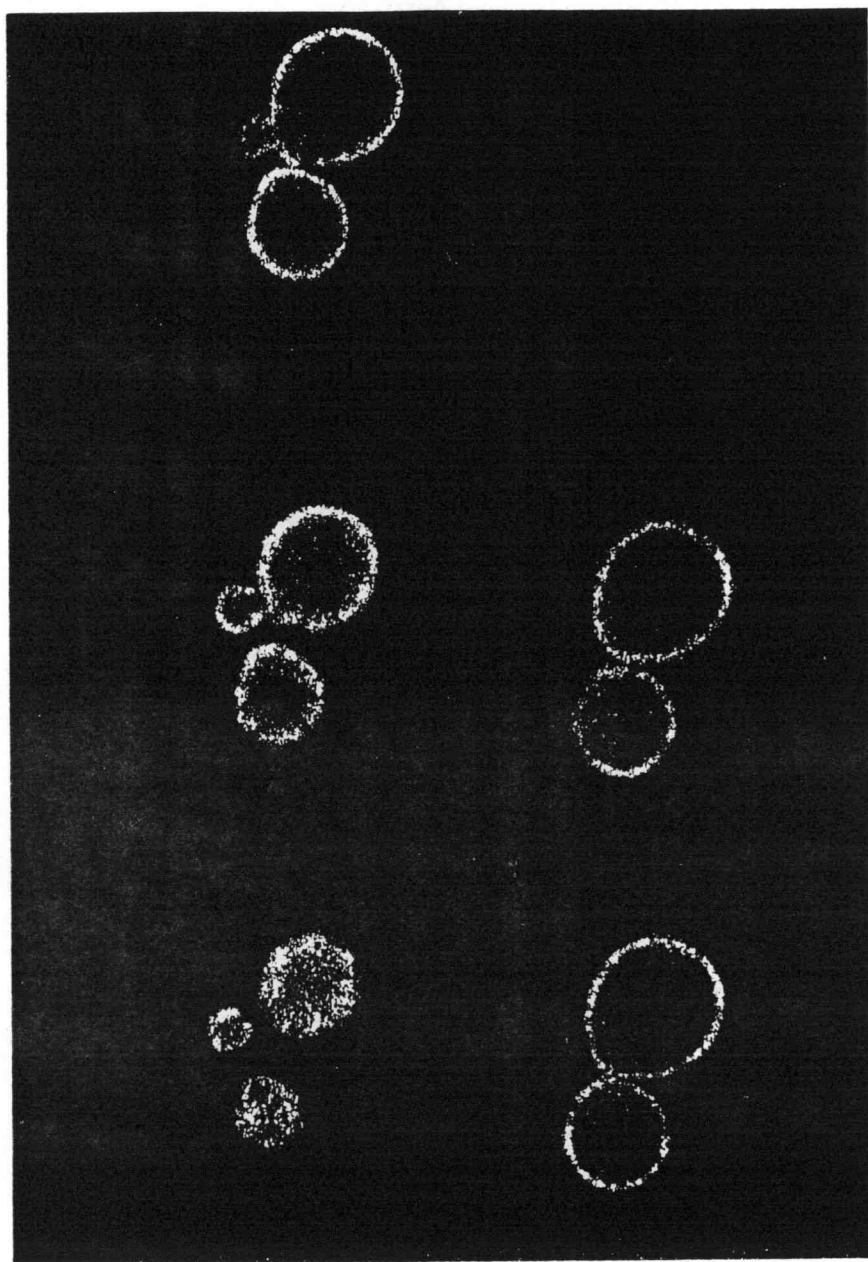
Fig. 11. Localization of the Ssu1-GFP fusion protein. GFP fluorescence was observed in living cells visualized using a Leica TCS 4D confocal microscope with images processed using Adobe Photoshop. The five frames represent serial sections of the same cells.

TABLE 7. Multicopy suppression of the sulfite-sensitivity of various mutants.

Mutant	Multicopy gene	Suppressing activity[a]
grr1Δ	FZF1	+
grr1Δ	SSU1	+
fzf1Δ	GRR1	-
fzf1Δ	SSU1	+
ssu1Δ	GRR1	-
ssu1Δ	FZF1	-
ssu3-7	GRR1	-
ssu3-7	FZF1	+/-
ssu3-7	SSU1	+

[a] Suppressing activity was tested as growth on plates containing 1.5 mM sulfite. "+", "-", and "+/-" indicate wild-type growth, no growth, and slow growth, respectively. On this medium, mutants carrying the vector alone did not grow. In a wild-type background, multicopy SSU1 conferred resistance to 3 mM, while single copy SSU1 permitted growth on no more than 1.5 mM sulfite.

mutants. Because SSU3 has not yet been isolated, its overexpression could not be evaluated. However, multicopy GRR1, FZF1, and SSU1 were tested individually in an ssu3 background.

Analysis of multicopy suppression of GRR1, FZF1, and SSU1 gave internally consistent results suggesting that GRR1 acts upstream of FZF1 which in turn acts upstream of SSU1 (Table 7). Overexpression of the three genes in an ssu3 background placed SSU3 downstream of GRR1 but upstream of SSU1. These results are in agreement with previous observations of multicopy FZF1 suppressing the sulfite-sensitivity of a grr1 mutant and partially suppressing that of an ssu3 mutant (Avram and

Bakalinsky, 1996). Independently, *SSU1* was isolated from a high copy DNA library as a multicopy suppressor of the sulfite-sensitivity of an *fzf1* mutant (data not shown). One interpretation of the partial suppression of an *ssu3* mutant by high copy *FZF1* is that the two may be involved in the same step in the pathway, and overexpression of *FZF1* cannot completely compensate for loss of *SSU3* function. In summary, the overexpression data favor a single pathway in which Ssu1p acts downstream of Fzf1p and Ssu3p, which in turn, act downstream of Grr1p (Fig. 13).

3.4.5. The *SSU1* promoter is activated by Fzf1p which requires a functional *SSU3* gene

Fzf1p is a putative five zinc finger transcription factor (Breitwieser *et al.*, 1993), and in order to test the possibility that it regulates *SSU1* as suggested by the multicopy suppression analysis, an *SSU1* promoterlacZ fusion was constructed. The region (1016 bp) upstream of the first predicted ATG of the *SSU1* ORF was cloned in front of the *lacZ* ORF and integrated at the *URA3* locus in several strains. β-galactosidase activity was 3.7-fold lower in an *fzf1* mutant than in wild-type, suggesting that expression of the *SSU1* promoter may be regulated by Fzf1p (Fig. 12).

β-galactosidase activity was also 2.4-fold lower in an *ssu3* mutant suggesting that SSU3p may also regulate this promoter under the same conditions. In *ssu1* and *ssu4* mutants, the values were not significantly different from that in wild-type. In a *grr1* mutant, activity was 20-fold lower than in wild-type. Grr1p has been implicated in a variety of functions and exerts control over the G1 cyclins and certain proteins involved in glucose transport (Barral *et al.*, 1995; Erickson and Johnston, 1993) but does not appear to be a transcription factor (Flick and Johnston, 1991; Barral *et al.*, 1995). Such a low level of expression in a *grr1* background may be due to the fact that among proteins controlled by Grr1p are some involved in the regulation of *SSU1* promoter. When *FZF1* was expressed in high copy number in wild-type, β-

galactosidase activity increased 27-fold relative to single-copy *FZF1* (61.3 ± 3.8 versus 2.8 ± 0.25). Activation of the *SSU1* promoter appeared to be specific since a *CYC1* promoter *LacZ* fusion (pLGΔ-132) (Guarente and Mason, 1983) was not activated in the presence of multicopy *FZF1* (34.1 ± 4.8 versus 39.5 ± 4.17).
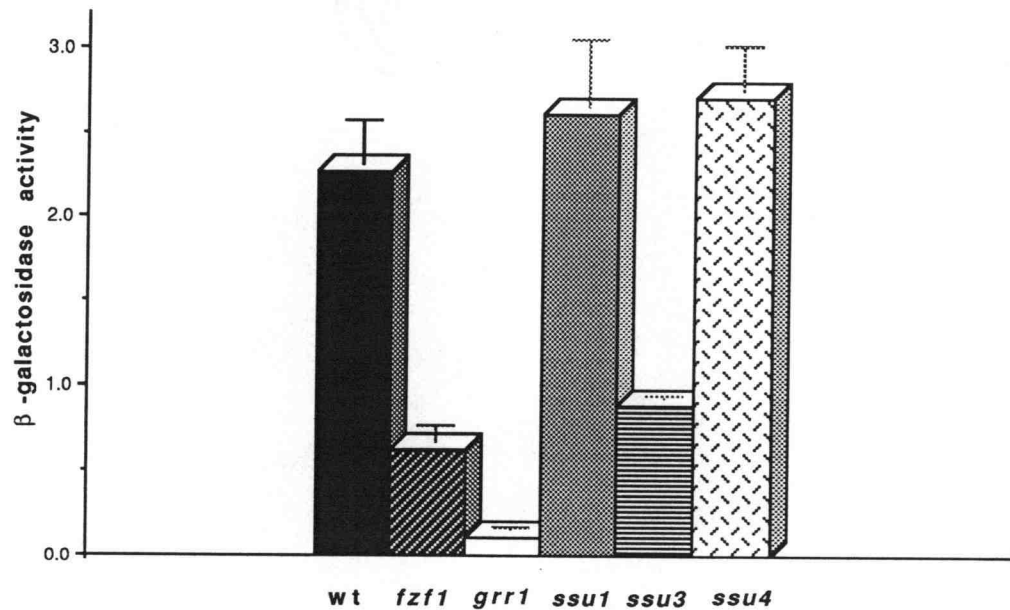
Fig. 12. *SSU1* promoter expression in SM-leu in wild-type and different mutant strains. A 1 kb region upstream of the *SSU1* ORF was generated by PCR and fused to the *lacZ* ORF, previously cloned in YIplac181. The construct was integrated at the *URA3* locus in wildtype (3090-9d), *fzf1Δ* (3090-9d-T10-L1), *ssu3-7* (3089-1d), *grr1Δ* (3090-9d-T6 L1), ssu4-1 (3100-5b), and *ssu1Δ* (3090-9d-T4-L1). β-galactosidase activities were assayed in permeabilized cells (Kippert, 1995). Activities are given in Miller units and are the means of two to three assays of three independent transformants. Error bars are standard deviations.

In an *ssu3* background, multicopy *FZF1* increased β-galactosidase activity only 3.8-fold (3.5 ± 0.1 versus 0.9 ± 0.1), suggesting that a functional *SSU3* gene is required for full activation.

3.5. Discussion

A new gene, *SSU1*, was isolated on the basis of conferring sulfite tolerance to *S. cerevisiae*. It encodes a protein with 9 or 10 transmembrane domains, is located in the plasma membrane, and does not share significant similarity with other proteins in public databases.

About 4% of the yeast genome encodes membrane proteins known to catalyze the transport of small solutes across the membrane or having sequence similarity to other characterized membrane proteins in yeast or other organisms (André, 1995). In addition, a number of uncharacterized proteins have been identified having transmembrane domains, but which do not share significant similarity with known transporters.

Membrane proteins have been classified in three major categories: channels, facilitators/transporters, and pumps (P-type ATPases and ABC transporters) (André, 1995). The predicted topology of Ssu1p (Fig. 9 and Fig. 10) based on a TMpred analysis (Hofmann and Stoffel, 1993), indicates a central hydrophobic core of 10 transmembrane domains, flanked by hydrophilic domains which presumably face the cytoplasm. This structure, together with the fact that Ssu1p lacks the nucleotide binding sequence typical of ABC transporters (Higgins, 1992), and the conserved amino acid stretches which contribute to the formation of the catalytic site of P-type ATPases (Fagan *et al.* 1994), places this protein in the category of facilitators/transporters.

Yeast transporters have been classified in five families based on sequence similarity and function: HXT (hexose) transporters, AAP (amino acid permeases), MFS

drug resistance proteins and similar proteins, transporters of purines, pyrimidines, and derivatives (FUR and FCX family), and carboxylic acid transporters (DAC family), which together form the Major Facilitator Superfamily (MFS) (Marger and Saier, 1993). Several additional facilitator families have not been classified as MFS members: some transporters of organic compounds, transporters of inorganic ions (TRK family of potassium transporters, MEP family of ammonium transporters, phosphate transporters, the SUL family of sulfate transporters), metal transporters (interconnected iron and copper uptake systems, and a family of metal detoxification transporters) (André, 1995). Ssu1p does not share significant similarity with proteins from these families and may belong to a distinct family of transporters with only one representative in *S. cerevisiae*. Progress in sequencing other genomes will likely identify "relatives" of this protein.

Assuming that Ssu1p is a transporter, we presume that it is involved in sulfite efflux rather than uptake because 1) an *SSU1* null mutant is sulfite sensitive rather than resistant; 2) deletions or mutations of the genes upstream in the pathway that appear to regulate or are required for *SSU1* function (*GRR1*, *FZF1*, and *SSU3*) also result in sulfite sensitivity rather than resistance. One exception is a particular mutant allele of *FZF1* which confers resistance, perhaps due to hyperactivation of *SSU1* (Casalone *et al.*, 1994); and 3) biochemical studies of sulfite uptake support passive diffusion of the undissociated form of sulfite, sulfurous acid--H_2SO_3--and have not provided evidence for a sulfite permease (Stratford and Rose, 1986). This latter point is consistent with sulfite being a normal yeast metabolite, but an unusual source of exogenous sulfur. Sulfate is the major inorganic sulfur source.

We envision sulfite detoxification to be a complex phenomenon mediated via different pathways. The primary route may be flux through the reductive sulfate assimilation pathway leading to formation of methionine and cysteine. Production of

acetaldehyde may be another because of its recognized affinity for sulfite and the non-toxic nature of the reaction product, 1-hydroxyethanesulfonate. The present study has uncovered a regulatory pathway involved in sulfite metabolism and its relationship to the aforementioned routes of sulfite detoxification is unclear. Genes in this pathway were ordered based on analysis of multicopy suppression of sulfite sensitivity and point towards a possible role for *SSU1* in sulfite efflux (Fig. 13).

The member of the postulated pathway furthest from the target, Grr1p, has been implicated in the regulation of Cln1p and Cln2p (Barral *et al.*, 1995) and possibly Rgt1p (Erickson and Johnston, 1994, and Barral *et al.*, 1995). Multicopy suppression data suggests that Grr1p may be involved directly or indirectly in the regulation of Fzf1p and possibly Ssu1p. *GRR1* may also be involved in a second pathway, independent of that shown in Fig. 13, because *grr1* mutants are more sulfite-sensitive than the other mutants (Avram and Bakalinsky, 1996). Fzf1p is a five zinc finger protein (Breitwieser *et al.*, 1993) which has been shown independently to be involved in sulfite tolerance (Casalone *et al.*, 1992; Casalone *et al.*, 1994). The multicopy suppression data placed the *FZF1* gene upstream of *SSU1*, and consistent with this placement, the *SSU1* promoter-*lacZ* fusion analysis identified *SSU1* as a target for transcriptional activation by Fzf1p. Ssu3p appears to act upstream of *SSU1*, and is required for Fzf1p-mediated activation of *SSU1*. It is possible that Ssu3p is a transcriptional regulator which cooperates withFzf1p. Cloning of *SSU3* gene is in progress in this laboratory.

Of the four genes in which mutations confer sulfite sensitivity, *GRR1*, *SSU3*, *FZF1*, and *SSU1*, the latter appears to act closest to the target--sulfite-- and its membrane location is consistent with a possible role in sulfite efflux.

GRR1

SSU3 ◄─► FZF1

SSU1

SULFITE DETOXIFICATION
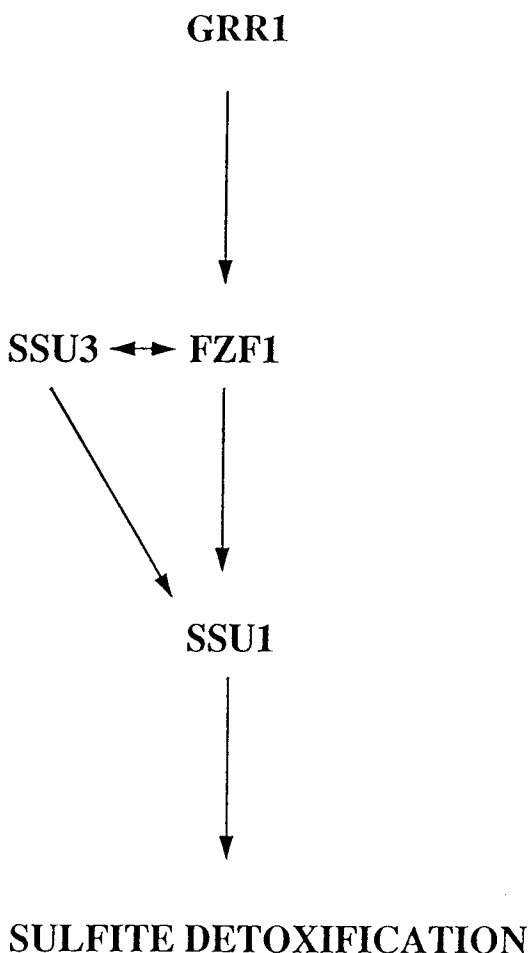
Fig. 13. A regulatory model for sulfite detoxification in *S. cerevisiae*. The sulfite-proximal member of this pathway is Ssu1p, a putative transporter which when mutated causes sulfite sensitivity. Expression of *SSU1* is controlled by Fzf1p, independently shown to be involved in sulfite tolerance. Activation by Fzf1p requires a functional *SSU3* gene, which when mutated also causes sensitivity to sulfite. Expression of the *SSU1* promoter also requires a functional *GRR1* gene. The sulfite-sensitivity of *grr1* is suppressed by multicopy *FZF1* and *SSU1* and thus, Grr1p is presumed to regulate both.

3.6. Acknowledgements

3.7. References

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Chapter 4

The first zinc finger region of *Saccharomyces cerevisiae* transcriptional activator Fzf1 is required for *SSU1* promoter specific binding

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4.1. Abstract

The *FZF1* gene encodes a putative five zinc finger transcription factor involved in sulfite tolerance. Previous multicopy suppression analysis placed *FZF1* upstream of *SSU1*, which encodes a putative transporter also implicated in sulfite detoxification. *SSU1* promoter-*lacZ* fusion analysis identified Fzf1p as a transcriptional activator of *SSU1* gene. Here we define the *SSU1* promoter region involved in the activation by multicopy *FZF1* and show that Fzf1 protein binds directly and specifically to it *in vitro*. We also report that deletion of the first zinc finger and the eleven N-terminal amino acids of Fzf1 protein resulted in the loss of binding, while removal of the fourth and the fifth zinc fingers did not. These results indicate that the first zinc finger region is essential for DNA binding *in vitro*.

4.2. Introduction

Multicopy suppression analysis revealed a novel regulatory pathway for sulfite detoxification in *S. cerevisiae* (Avram and Bakalinsky, 1996, submitted). The putative effector of the pathway, *SSU1*, encodes a transmembrane protein with a possible role in sulfite efflux. *SSU1* was placed in the pathway downstream of *FZF1* gene (Avram and Bakalinsky, 1996, submitted), which encodes a putative transcription factor (Breitweiser *et al.*, 1993), previously implicated in sulfite tolerance (Casalone *et al.*, 1992; Casalone *et al.*, 1994; Avram and Bakalinsky, 1996). Consistent with this placement, the *SSU1* promoter-*lacZ* fusion analysis defined Fzf1p as a transcriptional activator of *SSU1* gene. The level of transcription from the *SSU1* promoter was lower in an *fzf1* mutant than in wild-type, and was strongly activated in wild-type background when *FZF1* was expressed in multicopy (Avram and Bakalinsky, 1996, submitted).

The Fzf1 protein contains five zinc fingers of the C_2H_2-type with striking similarity to the TFIIIA-like zinc finger motif (Breitwieser *et al.*, 1993). Some C_2H_2-

type zinc finger proteins have only one zinc finger (Pieler and Bellefroid, 1994; Cook *et al.*, 1994), while others have as many as 19 contiguous fingers (Cunliffe *et al.*, 1990). In yet other transcription factors, the zinc fingers are grouped together and separated from other groups or individual zinc fingers in the protein (Hoffman *et al.*, 1993; Keller and Maniatis, 1992; Svetlov and Cooper, 1995). In Fzf1 protein the first three zinc fingers are clustered at the NH$_2$-terminus of the protein (amino acids 12 to 94), each separated only by six amino acids, while the last two are isolated. The fourth zinc finger is 61 amino acids downstream from the third and the fifth is separated by 67 amino acids from the fourth. The fourth zinc finger of another five zinc finger protein, glioblastoma protein (Glip), was found to be involved in DNA recognition (Pavlevitch and Pabo, 1993). Casalone *et al.* (1994) obtained a sulfite resistant mutant with a point mutation in the fourth zinc finger of Fzf1p and speculated, based on similarity to Glip, that the point mutation may be responsible for some change in the protein structure leading to alterations in its interaction with DNA.

Here we define by deletion analysis the region of *SSU1* promoter responsible for activation by multicopy *FZF1*. We also show that Fzf1p binds directly and specifically to the *SSU1* promoter *in vitro*, that the first zinc finger region of the protein is essential for binding, and that the last two fingers are not.

4.3. Materials and methods

4.3.1. Cloning, DNA sequencing, and PCR

Standard procedures for manipulation of plasmid DNA and bacterial transformation were used (Sambrook *et al.*, 1989). *Escherichia coli* DH5a (Hanahan, 1983) or *E. coli* SURE (Stratagene, La Jolla, CA) were used in all subcloning experiments. DNA was sequenced using the dideoxy dye terminator

method on an ABI Model 373A sequencer (Applied Biosystems, Inc., Foster City, CA) at the Central Services Laboratory of the Oregon State University Center for Gene Research and Biotechnology. PCR was performed using *Pfu* (Stratagene, La Jolla, CA) and *Taq* polymerases (Promega, Madison, WA) in an Easycycler (Ericomp, Inc., New Haven, CT) or a Robocycler 40 (Stratagene, La Jolla, CA) thermal cycler.

4.3.2. Promoter deletion analysis

The *SSU1* promoter-*lacZ* fusion (pDA6) has been described (Avram and Bakalinsky, 1996, submitted). pDA46 was obtained by digestion of pDA6 with *Bsa*A and *Sph*I, followed by treatment with T4 DNA polymerase, and subsequent ligation (Fig. 14). pDA50 and pDA56 (Fig. 14) were obtained by digestion of pDA6 with *Bsa*A and *Sph*I, followed by treatment with ExoIII, mung bean nuclease, T4 DNA polymerase, and ligase, as recommended by Stratagene (La Jolla, CA) in the ExoIII/Mung Bean Nuclease deletion kit. Constructs were sequenced to determine the extent of the deletions.

All constructs were integrated at the *URA3* locus in *S. cerevisiae* 3090-9d (Avram and Bakalinsky, 1996). The resultant strains were transformed with pDA15 which contains the *FZF1* gene cloned in YEplac181 (Geitz and Sugino, 1988). Yeast transformations were performed using the method of Gietz *et al.* (1992).

4.3.3. β-galactosidase assays

Two ml of minimal medium (Rose *et al.*, 1990) were inoculated with an overnight yeast culture and grown to an OD600 of 1.5 to 2. β-galactosidase activities were assayed in permeabilized cells (Kippert, 1995).

4.3.4. Preparation of purified Fzf1p from bacteria

Purified Fzf1p and the derived mutants were expressed as fusions with 6X His

tags in *E. coli*. In order to construct the Fzf1-6XHis variants, the complete open

reading frame of *FZF1* gene was amplified by PCR using the following

oligonucleotides: FZF1-N-U: 5′-CATGCCATGGCAGATATAGGGAGAACCAAG-3′

and FZF1-B-L: 5′-CGCGGATCCTCGAATAAATCCCAGACGTC-3′. The PCR

products were digested with *Nco*I and *Bam*HI and cloned in *Nco*I/*Bam*HI-digested

pET-24d (Novagen, Madison, WI), to produce pDA57 which was introduced in *E. coli*

HMS174 (DES) to ensure expression from the T7 promoter. LB medium was

inoculated with fresh transformants and cultures were grown to an OD600 of 0.6-1, at

which time expression was induced by addition of IPTG to a final concentration of 0.4

mM. Cultures were grown for an additional three hours. Fzf1-6XHis tagged proteins

were purified in batch, using Ni-NTA-resin, according to the manufacturer's

instructions for purification of cytoplasmic proteins (Qiagen, Chatsworth, CA) .

4.3.5. Construction of Fzf1p mutants

Mutants Δ1-68 and Δ1,2-69 were obtained by PCR using as upper primers

FZF1-38: 5′- CATGCCATGGCCAATCAAAAGCCGTATCAT-3′, and FZF1-68:

5′-CATGCCATGGCACAAATCAAGCCCAAAGC-3′, and FZF1-B-L as the lower

primer. The PCR products were digested with *Nco*I and *Bam*HI and cloned in

*Nco*I/*Bam*HI-digested pET-24d to produce pDA68 and pDA69, respectively.

Mutants Δ4-58 and Δ4,5-59 were obtained taking advantage of the *Hind*III sites

present in *FZF1* and pET-24d. Mutant Δ4-58 has deleted the sequence from K99,

where the first *Hind*III site is located, to A182, where the second site is located, and

mutant Δ4,5-59 has all the amino acids downstream of K99 deleted.

4.3.6. Gel mobility retardation assays

SSU1 promoter fragments: -506 to -315, -506 to -455, -506 to -378 and

-204 to -1 were generated by PCR, using combinations of the following primers:

SSU1-520: 5´-GACACATCATCATGCAA-3´, SSU1-545: 5´-

TTCTTATTTGTCAACCT-3´, SSU1-621: 5´-CAGGAATTTATCAGTCA-3´,

SSU1-700: 5´-GCACTCTCAGATTTTCG-3´, SSU1-Sal1-UP: 5´-

ACGCGTCGACACTTATTTCTAATCCTTGTC-3´, and SSU1-PrmtL-Pst1: 5´-

AAAACTGCAGTTTTTTCTTGTACTTGTCTTC-3´. DNA fragments were labeled

by incorporating [α-32P]dTTP or [α-32P]dCTP in the PCR reactions. The binding

reactions contained, in a total volume of 10 µl, 1-2 ng (10,000 to 20,000 cpm) of

labeled probe, 1X reaction buffer (25 mM Hepes-KOH pH-7.5, 50 mM KCl, 1mM

DTT, 0.1% Nonidet P-40, 10 mM Zn_2SO_4), 8% glycerol, 1 mM EDTA, 1 mg poly

dI-dC, and 1-25 ng Fzf1 protein. After incubation for 30 minutes at room

temperature, the protein-DNA complexes were separated on a native 5%

polyacrylamide gel in 0.5X TBE.

4.4. RESULTS

4.4.1. Deletion analysis of *SSU1* promoter defined a region containing the sequence required for the activation by multicopy *FZF1*

We previously showed that multicopy *FZF1* activates the *SSU1* promoter using

a construct containing the *SSU1* promoter fused to *lacZ*, pDA6 (Avram and Bakalinsky,

1996, submitted). To identify the *SSU1* promoter region through which Fzf1p activates

SSU1 expression, a deletion analysis of pDA6 was performed. Removal of the region

between -1015 and -499 did not influence significantly the ability of multicopy *FZF1* to

activate the promoter (Fig. 14, pDA6, pDA46, and pDA50), while deletion of the

sequence between -499 and -318 resulted in a dramatical loss of activation (Fig. 14, pDA56). Based on this analysis we concluded that the activation sites are located between -499 and -318.

4.4.2. FZF1p protein specifically binds the *SSU1* upstream region implicated in activation

To determine whether Fzf1p activates the *SSU1* promoter by direct binding we performed gel retardation assays. A 191 bp fragment, covering the region between -506
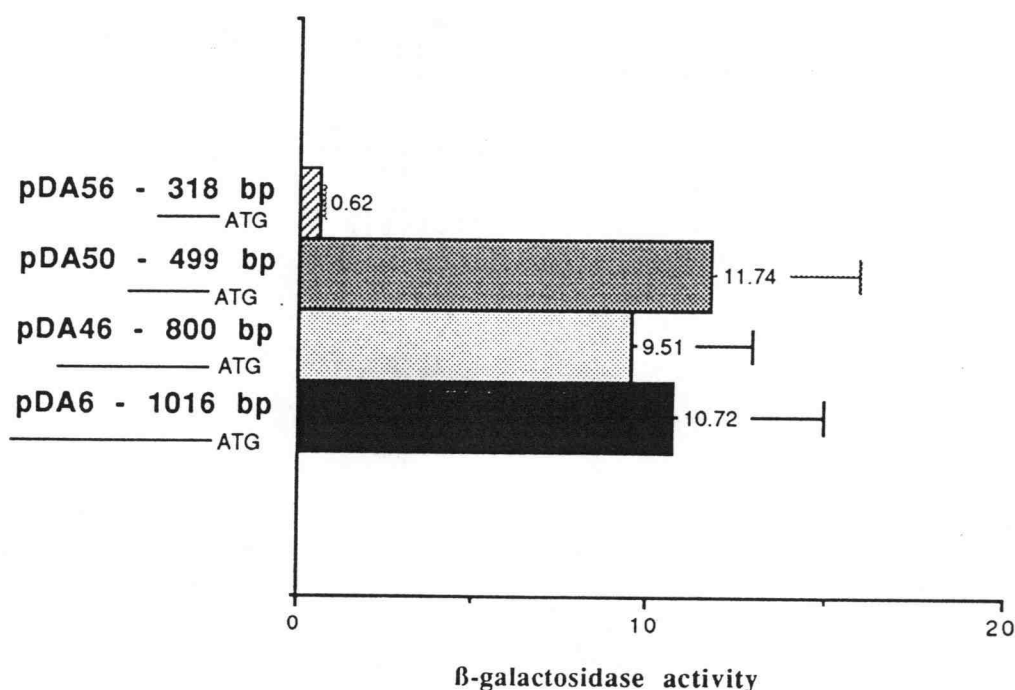
Fig. 14. Deletion analysis of the *SSU1* promoter. The *SSU1* promoter was fused to *lacZ* ORF in pDA6, as described (Avram and Bakalinsky, 1996, submitted). pDA46, pDA50 and pDA56 were constructed as described in Materials an Methods. β-galactosidase acitivities were assayed in permeabilized cells (Kippert, 1995). Activities are given in Miller units and are the means of three to five assays of three independent transformants. Error bars are standard deviations. *FZF1* was expressed in the multicopy vector YEplac181 (Geitz and Sugino, 1988). Control experiments with vector alone gave the following results: pDA6, 0.54 ± 0.07; pDA46, 0.51 ± 0.068; pDA50, 0.71 ± 0.16; and pDA56, 0.72 ± 0.1
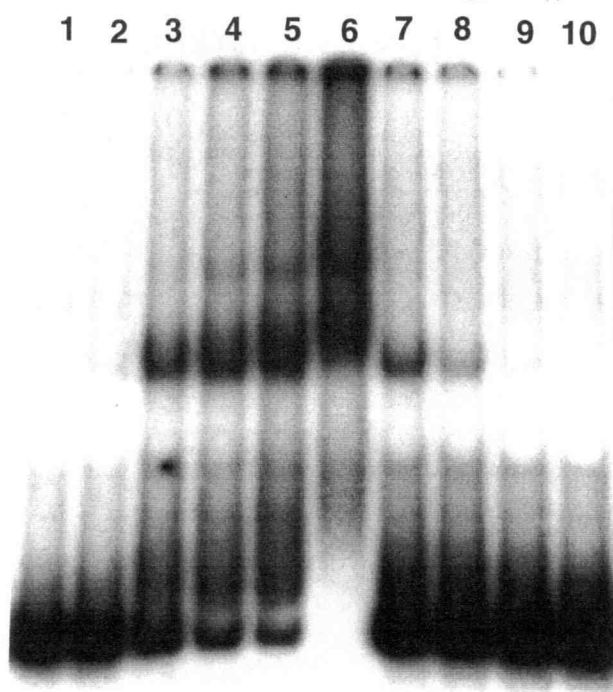
Fig. 15. Gel mobility retardation assays with an *SSU1* promoter fragment from -506 to -315 as labeled probe (1 ng) and Fzf1p purified from *E. coli*. Lane 1, no Fzf1p added. Increasing amounts of Fzf1p were used in lanes 2 through 6 (1, 2.5, 5, 10, and 25 ng, respectively). Poly dI-dC (3 µg) was added as a nonspecific competitor in lane 7. Unlabeled fragment was added as a specific competitor (0.25, 1, and 2 ng, respectively) in lanes 8 through 10. The reactions in lanes 7 through 10 contained 2.5 ng of Fzf1p
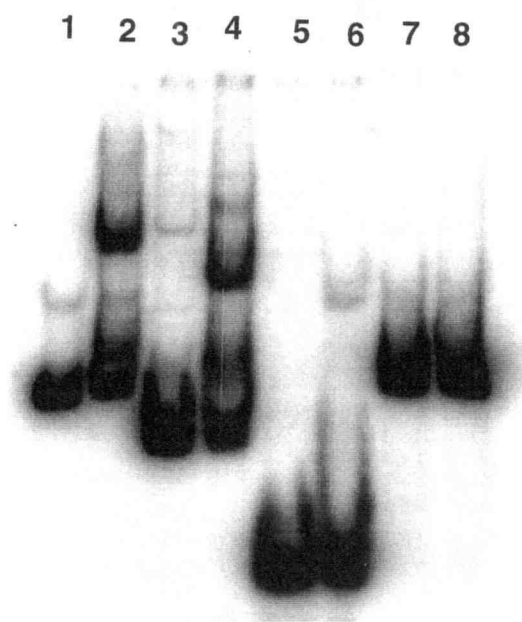
Fig. 16. Gel mobility retardation assays with different *SSU1* promoter fragments as labeled probes and Fzf1p purified from *E. coli*. Reactions loaded in the odd numbered lanes did not contain Fzf1p; those in the even numbered lanes contained approximately 2.5 ng. Lanes 1 and 2, fragment -506 to -315. Lanes 3 and 4, fragment -506 to -378. Lanes 5 and 6, fragment -506 to -455. Lanes 7 and 8, fragment -204 to -1.

and -315, presumed to contain the activation site(s), was used as a probe. Purified

Fzf1p gave rise to one complex when lower amounts of protein were added (Fig. 15,

lines 2 and 3), and to a second complex, when the amount of protein increased 2-, 4-,

and 10-fold (Fig. 15, lanes 4 through 6). Binding of Fzf1p to the fragment containing

the promoter sequence between -506 and -315 was specific, because addition of

unlabeled fragment (Fig. 15, lanes 8 through 10), but not of a non-specific competitor

(Fig. 15, lane 7), competed for Fzf1p. A fragment of the *SSU1* promoter containing

the sequence from -204 to -1, which is downstream of the presumed activating region, was not shifted by Fzf1p (Fig. 16, lanes 7 and 8). A fragment containing the sequence from -506 to -455 also failed to be retarded (Fig. 16, lanes 5 and 6), while a fragment containing the sequence from -506 to -378 was shifted (Fig. 16, lanes 3 and 4). Taken together, these results localized the activating sites between -455 and -378.

4.4.3. Deletion of the first zinc finger region of Fzf1p resulted in the loss of binding, while the fourth and the fifth fingers removal did not affect it

The zinc fingers of Fzf1p are of the C_2H_2-type, with remarkable similarities to the TFIIIA-like zinc finger motif (Breitwieser et al. 1993). The first three zinc fingers are clustered at the NH_2-terminus of the protein, while the last two are isolated. Taking advantage of the *Hind*III sites present in *FZF1* gene, an in frame mutant, Δ4-58, was constructed, in which the whole fourth zinc finger and almost all the region which separates the third and the fourth zinc fingers were deleted. This mutant bound the -506 to -315 promoter fragment (Fig. 17, lane 3) suggesting that the fourth zinc finger is not essential for binding. We also exploited the presence of a *Hind*III site upstream of the 6XHis tag, in pET-24d, and used it to construct an additional in-frame mutant (Δ4,5-59) derived from Δ4-58, in which the fourth and fifth zinc fingers and intervening region (encoding 67 amino acids) were removed, to produce a variant containing the first three zinc fingers and preceeding eleven N-terminal amino acids. This protein still bound the DNA suggesting that the two separated fourth and fifth zinc fingers are not essential for DNA binding (Fig. 17, lane 4). Mutated proteins in which either the first zinc finger and eleven N-terminal amino acids were deleted (Δ1-68), or both the first zinc finger region and the second finger were deleted (Δ1,2-69) failed to bind the probe (Fig. 17, lanes 5 and 6, respectively), indicating that at least the first zinc finger region is essential for binding.
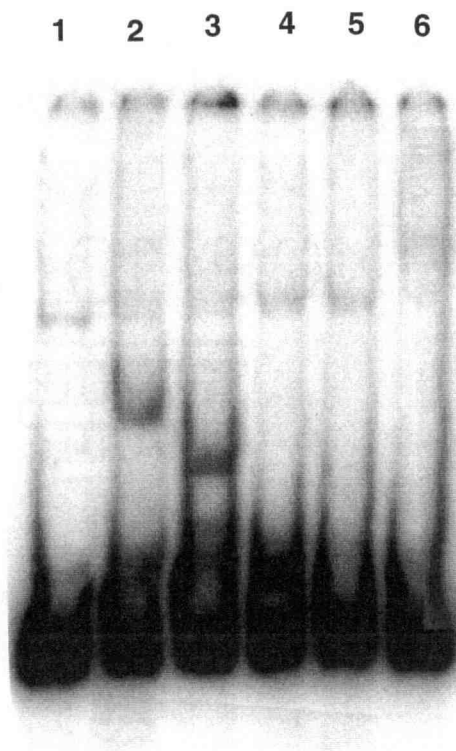
Fig. 17. Gel mobility retardation assays with different mutants of Fzf1p. A labeled *SSU1* promoter fragment from -506 to -315, and 0.5 to 5 ng of protein were used in the binding reactions. Lane 1, no Fzf1p added. Lane 2, wild-type Fzf1p. Lane 3, mutated protein Δ4-58. Lane 4, mutated protein Δ4,5-59. Lane 5, mutated protein Δ1-68. Lane 6, mutated protein Δ1,2-69.

4.5. Discussion

A new regulatory pathway for sulfite tolerance in *S. cerevisiae* was previously uncovered by multicopy suppression analysis. Ssu1p is a putative transporter in this pathway and may play a role in sulfite efflux from the cell (Avram and Bakalinsky, 1996, submitted). *FZF1* was presumed to act upstream of the *SSU1* gene. Consistent with this placement, *SSU1* promoter-*lacZ* fusion analysis showed Fzf1p to be a transcriptional activator of *SSU1* (Avram and Bakalinsky, 1996, submitted). In the present study, *SSU1* promoter deletion analysis defined the region between -499 and

-318 as being responsive to activation by multicopy *FZF1*. Gel mobility retardation assays demonstrated that Fzf1p binds specifically the *SSU1* promoter in the region between -455 and -378. DNase I protection analysis will help to define the response element more precisely.

Fzf1p contains five zinc fingers of the C_2H_2-type with noticeable similarity to the TFIIIA-like zinc finger motif (Breitwieser *et al.*, 1993) which shares the consensus sequence $YxCx_4(2)Cx_2Y/(F)x_5Lx_2Hx_3H$ (Berg, 1990). The number of zinc fingers in different C_2H_2-type zinc finger proteins is variable as is their distribution: some are grouped, while others are isolated (Keller and Maniatis, 1992; Svetlov and Cooper, 1995). The first three zinc fingers of Fzf1p are clustered at the NH_2-terminus, while the last two are isolated. Such an organization of the protein suggests a possible functional division. Casalone *et al.* (1994) obtained a sulfite resistant mutant with a point mutation in the fourth zinc finger of Fzf1p and hypothesized that this mutation may be responsible for an alteration in protein structure with consequences on the interaction with DNA, based on the fact that the fourth zinc finger of the glioblastoma protein (Glip), another five zinc finger protein, was found to be involved in DNA binding (Pavlevitch and Pabo, 1993). Our results show that a mutated Fzf1p, in which the fourth zinc finger was deleted, was still able to bind the *SSU1* promoter *in vitro*. Moreover, an Fzf1p variant missing the region downstream of the third zinc finger, representing about two thirds of the protein, still bound the DNA, suggesting that at least *in vitro* this region is not essential for binding. On the other hand, when the first zinc finger region was deleted, the protein lost the capacity to bind DNA, indicating that this domain is necessary for binding.

There is a great diversity of C_2H_2-type zinc finger proteins in nature, though the pattern of design is similar. In this respect, Pavletich and Pabo (1993) showed by crystallography that in the case of Glip, only fingers four and five make main base

contact with the target sequence, while the first finger region does not have any contact with the DNA. This is quite different from Zif268, where all the zinc fingers were shown to interact with the DNA (Pavletich and Pabo, 1991). In the case of Fzf1p, our data suggest a different situation: the first finger region is essential for binding, and the last two fingers are not.

4.6. Acknowledgements

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Chapter 5

Conclusions and future directions

In an effort to explore additional mechanisms involved in sulfite tolerance in *S. cerevisiae*, a novel regulatory pathway was found by multicopy suppression analysis. Four genes have been identified so far as members of the pathway: *GRR1*, *FZF1*, *SSU1*, and *SSU3*. Mutations in any of the four genes result in sulfite sensitivity, confirming a role in protection against sulfite. Multicopy *SSU1* suppressed the sulfite sensitivity of *grr1*, *fzf1*, and *ssu3* mutants. In contrast, multicopy *GRR1* was unable to suppress the sensitivity of *ssu1*, *fzf1*, and *ssu3* strains. Multicopy *FZF1* suppressed the sulfite sensitivity of a *grr1* mutant and partially suppressed that of *ssu3*, but was unable to suppress that of *ssu1* mutant. These data suggest that *SSU1* acts downstream of *FZF1* and *SSU3*, which in turn are downstream of *GRR1*.

The member of the postulated pathway, furthest from the target, Grr1p, has been implicated in the regulation of Cln1 and Cln2 proteins (Barral *et al.*, 1995), and possibly Rgt1p (Erickson and Johnston, 1994, and Barral *et al.*, 1995). For future studies it would be informative to explore the possibility that Fzf1 and Ssu1 proteins are also regulated by Grr1p by evaluating their levels in *grr1* mutants using *lacZ* fusions and pulse chase analysis.

grr1 mutants are known to grow slowly on glucose, apparently due to impaired high affinity glucose uptake (Vallier *et al.*, 1994). The sulfite sensitivity observed in *grr1* mutants is likely due, in part, to consequences of defective glucose uptake, leading to the reduction in acetaldehyde production. Xu *et al.* (1994) previously found that a

grrl mutant excreted a reduced amount of acetaldehyde. The suppressor *rgtl*, which overcomes the growth defects of *grrl* strains on glucose (Erickson and Johnston, 1994), did not fully suppress the sulfite sensitivity of a *grrl* mutant, indicating that the sensitivity is not strictly linked to a defect in glucose metabolism. Furthermore, *grrl* mutants are sensitive to sulfite on carbon sources other than glucose, requiring other transporters, like maltose (Lagunas, 1993), or on which growth defects are restored (galactose) (Flick and Johnston, 1991), or on non-fermentable carbon sources (glycerol and ethanol). These findings indicate that sensitivity is not caused solely by growth defects on glucose. Another piece of evidence supporting the involvement of mechanisms other than defects in glucose metabolism in sulfite sensitivity of *grrl* mutants is that multicopy *FZF1* suppressed the sulfite sensitive phenotype, but not the glucose derepression (Avram and Bakalinsky, 1996a). It may be informative to explore if low acetaldehyde production on glucose as well as the sensitivity on other carbon sources are recovered when *FZF1* is expressed in multicopy.

The next member of the postulated pathway is the *FZF1* gene which encodes a C_2H_2-type zinc finger transcriptional regulator. Multicopy suppression analysis placed it upstream of *SSU1*. *SSU1* promoter-*lacZ* fusion analysis identified Fzf1p as a transcriptional activator of the *SSU1* gene, confirming a role for *FZF1* in transcriptional regulation, previously suggested solely on the basis of sequence analysis. The activation of the *SSU1* promoter in an *ssu3* background by multicopy *FZF1* was lower, suggesting that a functional *SSU3* gene may be required for full activation. In addition, multicopy *FZF1* only partially suppressed sulfite sensitivity of an *ssu3* mutant, indicating that they may act in parallel (Avram and Bakalinsky, 1996b). Cloning of the *SSU3* gene was unsuccessful, since all the positive clones obtained so far from a centromeric genomic library appeared to be revertants. Cloning *SSU3* from a high copy library and screening at higher concentrations of sulfite may be a solution.

Another approach may be to transform *grr1* or *fzf1* null mutants with a high copy library to identify *SSU3* as a multicopy suppressor of their sulfite sensitivity, and in this way to avoid *ssu3* revertants. Further work might include one hybrid and two hybrid screening which may disclose other proteins involved in the regulation of *SSU1* promoter.

We also proved that Fzf1 protein binds directly and specifically to a fragment of *SSU1* promoter *in vitro*. Deletion of the first zinc finger and the eleven N-terminal amino acids of Fzf1 protein resulted in the loss of binding, while removal of the fourth and the fifth zinc fingers did not affect binding. These results indicated that the first zinc finger region is essential for DNA binding *in vitro*. Substitution of the first zinc finger with zinc fingers from other proteins or with one of the other fingers of Fzf1 may support these results. In addition, site-directed mutagenesis may reveal which amino acids are involved in DNA binding activity. The arginine residue at the N-terminus of the α-helix of finger 1 and the first histidine involved in the tetrahedral complex may be candidates, since basic amino acids are known to preferentially interact with the DNA.

The putative effector of the pathway is Ssu1, a plasma membrane protein with 9 or 10 membrane-spanning domains. This protein does not share significant similarity with proteins present to date in public databases, and resembles the general structure of facilitators/transporters since it lacks the nucleotide binding sequence typical of ABC transporters and the conserved amino acid stretches which contribute to the formation of the catalytic site of P-type ATPases. It may be involved in sulfite efflux rather than uptake because *ssu1* mutants and mutants of the genes situated upstream in the pathway that appear to regulate or are required for *SSU1* function are sensitive to sulfite. One exception is a particular mutant allele of *FZF1* gene which confers resistance, perhaps due to hyperactivation of *SSU1* expression. For future studies it would be useful to determine which transmembrane domains are essential for Ssu1 protein function and

also to mutate the charged amino acids localized in the transmembrane domains which are putative sites for sulfite transport (K112, D136, D183, H226, K354, H410). A two-hybrid screen may reveal other proteins that interact with Ssu1, and contribute to understanding its function in sulfite tolerance.

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