Glucose-transport-deficient mutants of *Schizosaccharomyces pombe*: phenotype, genetics and use for genetic complementation

Birgitta Milbradt and Milan Höfer

Author for correspondence: Milan Höfer. Tel: +49 228 735545. Fax: +49 228 735504.

Botanisches Institut der
Universität, Kirschallee 1,
D-53115 Bonn, Germany

Glucose-transport-deficient mutants of *Schizosaccharomyces pombe* were obtained by treatment of wild-type cells (972h⁻) with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, and by selection of resulting mutants on gluconate medium containing 0.05% 2-deoxy-D-glucose (2DG). One mutant, designated YGS-B22, was unable to grow on D-glucose and/or D-fructose as a carbon source (Glc/Fru⁻), and was resistant to 2DG; hence, none of the three sugars was taken up by the mutant cells. The hexokinase activity in the wild-type and the mutant cells was equal. Genetic purification of YGS-B22 by back-crossing with a leucine-auxotrophic mutant and the wild-type resulted in two strains: YGS-4, with reduced 2DG resistance, and YGS-5, which had lost 2DG-resistance. YGS-5 grew in D-glucose-containing media, albeit very slowly. No measurable sugar uptake was detectable in either of the two mutants within the 1 h test interval. Tetrad analyses proved a Mendelian segregation of growth on D-glucose and leucine auxotrophy. However, 2DG resistance did not co-segregate with the Glc/Fru⁻ phenotype, indicating that the transport deficiency and 2DG resistance characters are not encoded on the same genomic locus. Using a genomic bank of *Sch. pombe*, two transformants, YGS-5-G7 and YGS-5-G12, were found which had regained the wild-type growth and transport phenotype by complementation. Correspondingly, both D-glucose uptake and 2DG accumulation were restored in the transformed strains. Restriction analysis and Northern blots suggested that the G7 genomic fragment and the 4.1 kb *Sa*II restriction fragment of the G12 genomic fragment both contain a complete structural symporter gene.

Keywords: yeast, glucose transport mutants, genetic complementation, glucose symporter gene, *Schizosaccharomyces pombe*

INTRODUCTION

The fission yeast *Schizosaccharomyces pombe* takes up hexoses in symport with H⁺ (Höfer & Nassar, 1987). The transport across the plasma membrane is rate-limiting for utilization of both D-glucose and D-fructose. Hence, only non-metabolizable glucose analogues, such as 2-deoxy-D-glucose (2DG) and glucosamine, are significantly accumulated in the cytosol. The glucose symporter of *Sch. pombe* displays higher affinity to 2DG ($K_T = 2$ mM) than to D-glucose ($K_T = 15$ mM), which is the richest carbon source

for cell growth. The maximum transport velocity for D-glucose [$V_T = 90$ nmol min⁻¹ (mg dry wt)⁻¹] is, however, about three times higher than that for 2DG (Höfer & Nassar, 1987).

The challenging goal in membrane transport research is the isolation of a particular transport protein and its functional reconstitution in artificial phospholipid vesicles. The main problem of this strategy is the identification of the transport protein in a mixture of solubilized membrane proteins, especially if the protein of interest belongs to a constitutive transport system. However, this problem can be circumvented by identifying the gene which encodes the transport protein, and by its *in vitro* expression. Our strategy in cloning the glucose symporter has therefore been to screen a genomic

Abbreviation: 2DG, 2-deoxy-D-glucose.

This paper is dedicated to Augustin Betz, colleague and scholar, on the occasion of his 75th birthday.

gene bank of *Sch. pombe* in a yeast/bacterial shuttle vector (Beach *et al.*, 1982) for the transport structural gene by genetic complementation (Rose, 1987).

This report deals with isolation and genetic characterization of *Sch. pombe* mutants deficient in glucose transport and their use in identifying genomic DNA fragment(s) encoding the glucose symporter. The isolation of glucose-transport-deficient mutants was made possible by an earlier finding that D-gluconate, as the sole carbon source, is taken up in *Sch. pombe* by a specific transport system different from that for D-glucose (Hoever *et al.*, 1992). Preliminary results of this work have been published (Milbradt & Höfer, 1991; Milbradt *et al.*, 1993).

METHODS

Organism and growth. The mutants described were obtained from the wild-type strain of the fission yeast *Schizosaccharomyces pombe* 972h⁻ (NCYC 1827h⁻). The strains used for crossing experiments were: the wild-type strain 975⁺ (NCYC 1828h⁺) and the auxotrophic mutant *leu1-32b*⁺. For genetic complementation a mutant deficient in the early meiotic phase, *mat2-B102 lys1-131*, was used. All four strains were kindly provided by P. Munz, University of Bern, Switzerland. For mutagenesis, transport experiments, hexokinase assay, transformation, and plasmid preparation, the particular yeast strain was grown aerobically in synthetic minimal medium (MM) at 30 °C (Gutz *et al.*, 1974) containing 4.7% (w/v) D-gluconate (potassium salt) as sole carbon source (MMG). When required, the liquid growth media were supplemented with 0.5 g 2-deoxy-D-glucose l⁻¹ (MMG + 2DG) and/or 10 mg leucine l⁻¹ (MMG + L). Prior to autoclaving, the pH of the media was adjusted to 4.5. Cells were harvested in the mid-exponential phase. To prepare solid minimal media, 2% (w/v) agar was added (MMA). The first step of screening of mutants and transformants was performed at 30 °C on three different solid screening media: MMAG medium supplemented with 0.5 g 2DG l⁻¹ (MMAG + 2DG) and MMA media in which 3% (w/v) D-glucose (MMAGl) and/or 3% (w/v) D-fructose (MMAFr) substituted D-gluconate as the carbon source. Sporulation was induced by incubation of yeast cells on malt extract agar plates (1/2 MEAG: malt extract, 15 g l⁻¹; D-gluconate, 23.5 g l⁻¹; and agar, 20 g l⁻¹; pH 5.9) at 25 °C. Spore germination was carried out at 30 °C on yeast extract agar plates (YEAGGl: yeast extract, 5 g l⁻¹; D-gluconate, 47 g l⁻¹; D-glucose, 6 g l⁻¹; and agar, 20 g l⁻¹; pH 5.9) enriched with the appropriate supplements (leucine or lysine) where required; the supplement of D-glucose was necessary to initiate germination. Another rich medium used was YEAG, with the same composition as YEAGGl but without D-glucose.

Plasmids were propagated in *Escherichia coli* DH5α [*endA1 hsdR17* (r_k⁻, m_k⁺) *supE44 recA1 gyrA96 relA1 deoR Δ(lacZYA-argF)U196 thi-1 φ80dlacZΔM15 F'*] grown in Luria broth (Lennox, 1955) in the presence of 100 mg ampicillin l⁻¹. The strain was kindly supplied by B. Persson, University of Umeå, Sweden.

Mutagenesis. Wild-type cells of *Sch. pombe* 972h⁻ were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (3 mg ml⁻¹) for 20 min in 0.2 M potassium acetate buffer (pH 5) at 30 °C. Mutant enrichment was achieved by a modification of the procedure of Megnet (1965a) in which the culture media contained 0.5 mg 2DG ml⁻¹ and the incubation time in batch culture (containing gluconate) was extended to 2.5 d. Culture aliquots were plated

on selection plates (MMAG + 2DG) to form colonies (10–15 d growth at 30 °C) which were further tested for potential transport-deficient phenotype by replica plating on the screening plates (3 d growth at 30 °C).

Transport experiments. The uptake of D-[¹⁴C]glucose and D-[¹⁴C]fructose was measured in supernatants of reaction suspensions as described by Milbradt & Höfer (1990). The accumulation of 2-deoxy-D-[³H]glucose was determined by analysis of hot water cell extracts as reported by Niemietz *et al.* (1981).

Enzyme assay. The hexose-ATP-kinase activity was assayed by the procedure used earlier (Mahlberg *et al.*, 1985). The cell-free extracts were prepared as described by Ciriacy (1976); the hexose 6-phosphate was determined enzymically (Lang & Michal, 1974).

Genetic methods. Mutants were crossed with the auxotrophic *Sch. pombe* strain *leu1-32b*⁺ to introduce a Leu⁻ marker for transformation experiments. The resulting double mutants were back-crossed three times with the wild-type 975h⁺ using free-spore analysis as described by Gyax & Thuriaux (1984). After each crossing the mating type of the progeny was also determined. Tetrad analysis was performed by micro-manipulation of the asci (Gutz *et al.*, 1974). Stable diploid cells for genetic complementation were obtained by crossing the transport-deficient mutants with the *mat2-B102 lys1-131* strain and identifying diploid colonies by Phloxin B (Gutz *et al.*, 1974).

Transformation. *E. coli* DH5α cells were transformed according to the methods of Maniatis *et al.* (1982). *Sch. pombe* cells were transformed by the method of Ito *et al.* (1983) and Heyer *et al.* (1986) except that 50 µg salmon sperm DNA was added together with the DNA to be transformed.

Complementation and plasmid recovery. Recombinant plasmids complementing the mutant deficiency were identified by transformation of mutant cells with a genomic gene bank, *CV13*, which was kindly supplied by P. Nurse, University of Oxford, UK. The *CV13* genomic library was constructed by partial digestion of *Sch. pombe* nuclear DNA with *Sal*3A and ligation in the *Bam*HI restriction site of the yeast/bacterial shuttle vector YEpl3 (Beach *et al.*, 1982). Leu⁺ transformants were detected on MMAG plates. All transformants were tested for their ability to grow on D-glucose and D-fructose by replica plating on the screening plates. Plasmids from complemented transformants with wild-type phenotype were recovered by extracting total DNA as described by Moreno *et al.* (1991) and transforming *E. coli* DH5α.

Restriction analysis. DNA manipulation was performed using standard methods (Maniatis *et al.*, 1982). Plasmid DNA was purified by equilibrium centrifugation in caesium chloride/ethidium bromide density gradients. Genomic DNA inserts were excised and further digested by treatment with appropriate endonucleases. DNA fragments were analysed after denaturation at 68 °C by electrophoresis in 0.8% agarose gels in Tris/acetate buffer (pH 8). Enzymic reactions were carried out according to the protocols of the enzyme manufacturer (Boehringer). When required, DNA fragments were purified using the Quiaex-Kit obtained from Diagen.

RNA analysis. Total yeast RNA was extracted as described by Moreno *et al.* (1991). Samples of the total RNA preparations were resolved in 1% (w/v) agarose gels containing formaldehyde and transferred to nitrocellulose membranes according to Maniatis *et al.* (1982). For hybridization and detection of hybridization products the non-radioactive DNA labelling kit of Boehringer was used.

Chemicals. 2DG was from Calbiochem, *N*-methyl-*N'*-nitro-*N*-

Table 1. Growth phenotype of the wild-type and the 2DG-resistant colonies

MMAGI, MMAFr, MMAG and MMAG + 2DG: screening agar plates with D-glucose, D-fructose, D-gluconate and D-gluconate + 2DG as carbon source, respectively. WT, wild-type; wanted, predicted phenotype of mutant required. For further details see Methods.

Group	MMAGI	MMAFr	MMAG	MMAG + 2DG	Colonies
WT	++++	++++	+++	—	
Wanted	—	—	+++	+++	
I	—	—	+++	+++	26
II	—	—	+++	+	7
III	—	—	+++	—	2

nitrosoguanidine from Boehringer, and the radiolabelled monosaccharides from Amersham-Buchler. All other compounds were of reagent grade, from Merck.

Table 2. Hexose-ATP-kinase activity of the wild-type and the mutant YGS-B22

For analytical procedures see Methods.

Substrate	Hexose-ATP-kinase [nmol min ⁻¹ (mg protein) ⁻¹]	
	Wild-type	YGS-B22
D-Glucose	322 ± 55	345 ± 23
D-Fructose	510 ± 48	523 ± 18
D-Gluconate	23 ± 4	20 ± 4

RESULTS AND DISCUSSION

Mutagenesis and screening of mutants

In four independent mutagenesis experiments, 1642 2DG-resistant (2DG^R) colonies were isolated. The mutants were tested for their potential transport-deficiency phenotype by growth on screening plates containing either D-gluconate (MMAG), D-gluconate + 0.05 % 2DG (MMAG + 2DG), D-glucose (MMAGI) or D-fructose (MMAFr) as carbon source (Table 1). Twenty-six mutants displayed resistance to 2DG when grown on D-gluconate and no growth with either D-glucose or D-fructose. To eliminate metabolic mutants, mostly deficient in the hexose-phosphorylation capacity, which frequently occur after selection with 2DG (Megnet, 1965b), both the activity of the hexose-ATP-kinase and the ability of the cells to take up hexoses were measured. Nine mutants of group I in Table 1 exhibited hexose-ATP-kinase activity which was comparable to that of wild-type cells, as shown for YGS-B22 in Table 2.

Fig. 1. Hexose transport in *Sch. pombe* wild-type and the mutant YGS-B22. (a) Uptake of D-glucose and D-fructose determined as sugar consumption from cell suspensions; (b) intracellular accumulation of 2DG (the broken line corresponds to the external 2DG concentration). Initial external hexose concentration $[(\text{Hexose})_o]$, 5 mM; filled symbols, wild-type cells; open symbols, YGS-B22 cells; circles, D-glucose; triangles, D-fructose; squares, 2DG.

The two colonies of group III of Table 1 displayed a rather unusual phenotype: no growth on either D-glucose or D-fructose, and yet high sensitivity to 2DG (2DG^S) when grown on D-gluconate. One of them, *GPD*, was further biochemically characterized: its hexose-ATP-kinase activity and transport capacity were significantly higher than those of the wild-type; however, the glucose-6-phosphate dehydrogenase activity was virtually eliminated (unpublished results). Hence, the *GPD* mutant was a glucose-6-phosphate-dehydrogenase-deficient mutant. Its failure to grow on D-glucose was due to its inability to synthesize pentose phosphates via the oxidative branch of the pentose phosphate cycle. The reductive part of the pentose phosphate cycle is obviously not effective enough to supply wild-type cells with pentose phosphates for nucleotide synthesis, as manifested by their slow growth on glycerol and lack of growth on ethanol (data not shown).

Phenotypic characteristics

The nine mutants of group I with unaffected hexose-ATP-kinase activity met the criteria for glucose transport deficiency: all were unable to take up either D-glucose or D-fructose, as shown for the mutant *YGS-B22* in Fig. 1(a). Hence, none of the mutants accumulated 2DG, a non-metabolizable analogue of D-glucose (Fig. 1b). Of these transport-deficient mutants, strain *YGS-B22* was chosen for further genetic analysis.

Genetic analysis of the *YGS* (yeast glucose symporter) mutants

The transport-deficient mutant *YGS-B22* was first crossed with the auxotrophic mutant *Sch. pombe* (*leu1-32h⁺*) in order to introduce a marker for complementation experi-

Table 3. Phenotypes of wild-type *Sch. pombe* and the two glucose-transport-deficient mutants used for complementation

Glc, D-glucose; Fru, D-fructose; Glt, D-gluconate. All three strains have the *h⁻* mating type.

Strain	Growth	2DG ^R / 2DG ^S	Leucine phenotype
Wild-type 972h ⁻	Glc/Fru ⁺ Glt ⁺	2DG ^S	Leu ⁺
Mutant <i>YGS-4</i>	Glc/Fru ⁻ Glt ⁺	2DG ^R	Leu ⁻
Mutant <i>YGS-5</i>	Glc/Fru ⁻ Glt ⁺	2DG ^S	Leu ⁻

ments; this double mutant was designated *YGS-B25*. Furthermore, to eliminate unwanted (and undefined) mutations, the *YGS-B25* mutant was further back-crossed three times with the wild-type strain 975h⁺, and, after each crossing, selected for leucine auxotrophy and transport-deficiency by free spore-analysis on screening plates (see Methods). The back-crossing experiments revealed that resistance to 2DG is not necessarily linked to transport deficiency. Consequently, two back-crossed strains were isolated: *YGS-4*, which was 2DG-resistant (2DG^R) and *YGS-5* which was 2DG-sensitive (2DG^S): see Table 3. The two strains did not take up either D-glucose (Fig. 2a) or D-fructose (not shown), nor did they accumulate 2DG; however, there was a very slow but measurable uptake of 2DG in strain *YGS-5* (Fig. 2b).

Tetrad analysis was performed following the crossing of both strains *YGS-4* and *YGS-5* with the wild-type *Sch. pombe* 975h⁺. Spores from 55 asci were separated and screened on screening plates with or without D-glucose/D-fructose, and with or without leucine. Both the

Fig. 2. Hexose transport in *Sch. pombe* wild-type, the mutant *YGS-5* and the complemented strains *YGS-5-G7* and *YGS-5-G12*. (a) Uptake of D-glucose (15 mM) determined as glucose consumption from cell suspensions; (b) intracellular accumulation of 2DG (2 mM, the broken line corresponds to the external 2DG concentration). Circles, wild-type cells; filled triangles, *YGS-5* cells; open triangles, *YGS-4* cells; squares, *YGS-5-G7* cells; virtually identical results were obtained with *YGS-5-G12* cell suspensions (not shown).

Fig. 3. Phenotype of the clones *YGS-4-G12* (1) and *YGS-5-G12* (2) as compared with wild-type *Sch. pombe* 972h⁻ (WT). (a) Growth on glucose; (b) growth on gluconate; (c) growth on gluconate + 2DG. Virtually identical results were obtained with clones *YGS-4-G7* and *YGS-5-G7* (not shown).

transport deficiency and the leucine auxotrophy segregated according to Mendel's law. However, the segregation of the 2DG resistance of strain *YGS-4* was not

2:2 but random (2 Glc/Fru⁺ 2DG^S:1 Glc/Fru⁻ 2DG^S:1 Glc/Fru⁻ 2DG^R). This confirms the conclusion from the free-spore analysis that 2DG-resistance and transport deficiency are not co-segregated.

Stable diploid cells for complementation experiments were obtained by crossing strain *YGS-4* with the mutant deficient in the early meiotic phase, *mat2-B102 lys1-131*. Of a population of 109 diploid colonies tested, all exhibited the wild-type growth phenotype. Thus, the genetic analysis of the two D-glucose-transport-deficient mutant strains, *YGS-4* and *YGS-5*, derived from *Sch. pombe* 972h⁻ proved that the mutation was nuclear, monogenic and recessive.

Molecular cloning of the glucose symporter gene

By complementation of strain *YGS-5* with a genomic gene bank of *Sch. pombe*, *CV13* (Beach *et al.*, 1982), two clones, *YGS-5-G7* and *YGS-5-G12*, were isolated which had regained the wild-type growth and transport phenotype (Fig. 3). Correspondingly, the uptake of D-glucose (Fig. 2a) and the accumulation of 2DG (Fig. 2b) were restored. The stability test proved that the complementation was due to the function of the genomic DNA inserts G7 and G12 in YEp13. In addition, back-transformation experiments confirmed that each of the two DNA inserts contained the complementing information.

Fig. 4. Restriction analysis (a) and a scheme (b) of the plasmid YEp13-G7. In (a), fragment separation was carried out in a 0.7% agarose gel after 4 d growth at 30 °C; 1, *Bam*HI/*Sph*I; 2, *Bam*HI/*Sal*I (the lower band is a doublet); 3, *Sph*I; 4, *Bam*HI; 5, λ DNA digested with *Hind*III.

Fig. 5. Restriction analysis (a) and a scheme (b) of the plasmid YEp13-G12. In (a), fragment separation was carried out in a 0.7% agarose gel after 4 d growth at 30 °C; 1, *Bam*HI/*Sal*I; 2, *Sal*I (the lower band is a doublet); 3, *Bam*HI; 4, *Hind*III; 5, λ DNA digested with *Hind*III.

The sizes of the two genomic DNA inserts, 4 kb (G7) and 7.5 kb (G12) within the *Bam*HI and *Sal*I restriction sites, were determined by restriction analysis of the plasmids YEp13-G7 (Fig. 4) and YEp13-G12 (Fig. 5), respectively. The genomic insert in YEp13-G12 contained an additional *Sal*I restriction site, giving two fragments of approximately 3.4 and 4.1 kb (Fig. 5). Two additional restriction sites, *Eco*RI and *Xba*I, were found both in G7 (4 kb) and the 4.1 kb fragment of G12, suggesting that the two DNA fragments are conformable.

Subclones of the two genomic DNA inserts, G7 (4 kb) and the 4.1 kb fragment of G12, were used as probes in Northern blots with total *Sch. pombe* RNA to determine the size of the glucose symporter mRNA transcript. The two probes detected signals of 3.7 kb in both the wild-type and the complemented strains (unpublished results), strongly supporting their proposed conformity. These results provide substantial evidence that with the two DNA fragments, the complete structural symporter gene has been cloned. Sequencing of the two fragments is in progress.

Two other clones, *YGS-4-G7* and *YGS-4-G12*, were isolated by transforming strain *YGS-4* with YEp13-G7 and YEp13-G12, following their propagation in *E. coli* DH5 α . These two clones did not grow on D-glucose, although they regained the 2DG sensitivity of the wild-type (see Fig. 3). This indicates that the mutant *YGS-4* exhibits, in addition to a mutation in the structural symporter gene, another mutation in a different locus on

the *Sch. pombe* genome. Whether or not the glucose symporter gene is under genetic regulation will be further investigated.

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