

Transmembrane segments 1, 5, 7 and 8 are required for high-affinity glucose transport by *Saccharomyces cerevisiae* Hxt2 transporter

Toshiko KASAHARA and Michihiro KASAHARA¹

Laboratory of Biophysics, School of Medicine, Teikyo University, Hachioji, Tokyo 192-0395, Japan

Hxt2 is a high-affinity facilitative glucose transporter of *Saccharomyces cerevisiae* and belongs to the major facilitator superfamily. Hxt1 shares $\approx 70\%$ amino acid identity with Hxt2 in its transmembrane segments (TMs) and inter-TM loops, but transports D-glucose with an affinity about one-tenth of that of Hxt2. To determine which TMs of Hxt2 are important for high-affinity glucose transport, we constructed chimaeras of Hxt2 and Hxt1 by randomly replacing each of the 12 TMs of Hxt2 with the corresponding segment of Hxt1, for a total of 4096 different transporters. Among $> 20\,000$ yeast transformants screened, 39 different clones were selected by plate assays of high-affinity glucose-transport activity and sequenced. With only two exceptions, the selected chimaeras contained Hxt2 TMs

1, 5, 7 and 8. We then constructed chimaeras corresponding to all 16 possible combinations of Hxt2 TMs 1, 5, 7 and 8. Only one chimaera, namely that containing all four Hxt2 TMs, exhibited transport activity comparable with that of Hxt2. The K_m and V_{max} values for D-glucose transport, and the substrate specificity of this chimaera were almost identical with those of Hxt2. These results indicate that TMs 1, 5, 7 and 8 are necessary for exhibiting high-affinity glucose-transport activity of Hxt2.

Key words: chimaera, glucose transporter, saturation mutagenesis, substrate recognition, yeast.

INTRODUCTION

The movement of substrates across biological membranes is mediated by a variety of membrane proteins known as transporters [1]. Although substantial progress has been made in the structural analysis of these proteins, which function as channels [2,3], pores [4–6] or pumps [7], no atomic structures are yet available for any facilitative or ion-coupled active transporter. The major facilitator superfamily (MFS) is one of the largest classes of evolutionarily related membrane transporters [8]. The glucose transporters of the MFS include those of prokaryotes as well as those of single-celled and multicellular eukaryotes, examples being GalP of *Escherichia coli* [9], Hxt2 of *Saccharomyces cerevisiae* [10] and Glut1 of animals [11]. The MFS also includes bacterial lactose permeases, such as LacY of *E. coli* [12], and bacterial tetracycline antiporters, such as TetA(B) of *E. coli* [13]. Many site-directed biophysical and biochemical studies of these proteins have led to the development of helix-packing models that provide detailed information on the structure and function of each transporter [12–14]. In addition to these models, a recent characterization of the three-dimensional structure of OxIT, a member of the MFS in *Oxalobacter formigenes*, revealed 12 transmembrane segments (TMs) arranged around a central cavity in a symmetrical manner [15]. These findings have led to the development of a view applicable to a wide variety of transporters, which includes the notion that multiple TMs contribute to the structural basis of substrate recognition.

The yeast *S. cerevisiae* is able to utilize glucose over a wide concentration range, and genes for 20 different hexose-transporter-related proteins belonging to the MFS (Hxt1–Hxt17, Gal2, Snf3 and Rgt2) have been identified in its genome [16]. Alignment of the amino acid sequences of these 20 proteins reveals conservation of the regions that form the 12 putative TMs. Hxt2 is a major high-affinity facilitative glucose transporter and consists of 541 amino acids (Figure 1), whereas Hxt1 is a low-

affinity facilitative glucose transporter and consists of 570 amino acids. The numbers of amino acids in each putative TM and inter-TM loop of Hxt2 are identical with those in the corresponding regions of Hxt1, and the two proteins share $\approx 70\%$ sequence identity in these regions. In contrast, the N- and C-terminal regions, which are both predicted to be located on the cytoplasmic side of the membrane, differ substantially in size and sequence between Hxt2 and Hxt1.

We have investigated which TMs of Hxt2 are important for its high substrate affinity by a new approach, designated ‘TM shuffling’. We thus randomly replaced, at the DNA level, all 12 TMs of Hxt2 with the corresponding segments of Hxt1. Clones encoding transporters with a high affinity for glucose were selected by plating on carbon-source-limited agar. Our results demonstrate that TMs 1, 5, 7 and 8 of Hxt2 are necessary for high-affinity glucose transport.

MATERIALS AND METHODS

Construction of cassette vectors

As a first step to TM shuffling for Hxt2 and Hxt1, we constructed the plasmids Hxt2mnx-pVT and Hxt1mnx-pVT to allow expression of Hxt2 and Hxt1 respectively under the control of the *ADHI* promoter in the multicopy plasmid pVT102-U (YEp *URA3 bla*) [17]. The *HXT2* sequence, which contains an *EcoRI* site at nucleotides 7–12 of the open reading frame, was modified to create (i) a *ClaI* site immediately downstream of the termination codon (TAAGAGAT \rightarrow TAATCGAT), (ii) a *MroI* site in the nucleotide sequence corresponding to the N-terminal end region of TM4 (ATTATCTCTGGT \rightarrow ATTATCTCCGGA), (iii) an *NheI* site in the nucleotide sequence encoding the loop between TM6 and TM7 (GCTTCT \rightarrow GCTAGC) and (iv) an *XhoI* site in the nucleotide sequence for the loop between TM9

Abbreviations used: MFS, major facilitator superfamily; TM, transmembrane segment.

¹ To whom correspondence should be addressed (e-mail kasahara@main.teikyo-u.ac.jp).

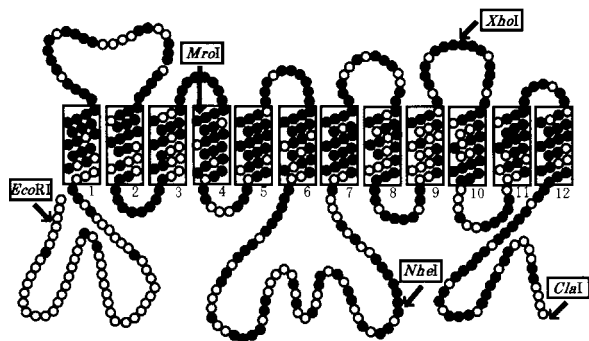


Figure 1 Schematic representation of the Hxt2 transporter

The structure of Hxt2 is based on a model of 12 TMs [32]. Each circle represents one amino acid, with filled circles indicating residues common to both Hxt2 and Hxt1. Hxt2 and Hxt1 comprise 541 and 570 residues respectively, with the difference in size being due to the different lengths of the N-terminal and C-terminal tails. Hxt1 thus contains nine additional residues in its N-terminal tail and an additional 20 residues in its C-terminal tail. Restriction-enzyme sites for *EcoRI*, *MroI*, *NheI*, *XhoI* and *ClaI* used for the construction of Hxt2–Hxt1 chimaeras are indicated; *MroI*, *NheI* and *XhoI* sites were located in Hxt1 at the exact corresponding positions.

and TM10 (CCATCTTCC → CCCTCGAGC). The creation of these sites did not affect the encoded amino acid sequence. The *EcoRI*–*ClaI* fragment of the modified *HXT2* sequence was then ligated to the multicloning site of pVT102-U to generate Hxt2mnx-pVT. The *HXT1* sequence was modified (i) to match the reading frame to that of *HXT2* by changing the nucleotides at the start of the open reading frame from ATGAATCAACT to ATGTCTGAATTC, creating a new *EcoRI* site and resulting in a change in the encoded amino acids from Met-Asn-Ser-Thr to Met-Ser-Glu-Phe; (ii) to create a *ClaI* site immediately downstream of the termination codon (TAAACTAA → TAATCGAT); (iii) to create a *MroI* site in the nucleotide sequence corresponding to the N-terminal end region of TM4 (ATTATCTCTGGT → ATTATCTCCGGA); (iv) to create a *NheI* site in the nucleotide sequence encoding the loop between TM6 and TM7 (GCATCT → GCTAGC); and (v) to create an *XhoI* site in the nucleotide sequence for the loop between TM9 and TM10 (CCATCTTCA → CCCTCGAGT). Again, the creation of the new restriction sites did not affect the encoded amino acids. The *EcoRI*–*ClaI* fragment of the modified *HXT1* sequence was then ligated to the multicloning site of pVT102-U to yield Hxt1mnx-pVT. Hxt2mnx-pVT or Hxt1mnx-pVT was introduced separately into KY73 (*MATα hxt1Δ::HIS3::Δhxt4 hxt5::LEU2 hxt2Δ::HIS3 hxt3Δ::LEU2::Δhxt6 hxt7Δ::HIS3 gal2Δ::DR ura3-52 MAL2 SUC2 MEL*) [18].

TM shuffling

Both *HXT2* and *HXT1* were modified so as to be divisible into four regions by restriction-enzyme digestion as described above: (i) from *EcoRI* to *MroI* sites (TMs 1–3), (ii) from *MroI* to *NheI* sites (TMs 4–6), (iii) from *NheI* to *XhoI* sites (TMs 7–9) and (iv) from *XhoI* to *ClaI* sites (TMs 10–12). TM shuffling was performed to replace randomly each TM of Hxt2 with the corresponding segment of Hxt1. The nucleotide sequences encoding each TM of Hxt2 or Hxt1 were first amplified by PCR (Figure 2) with *ExTaq* polymerase (TAKARA, Otsu, Japan) and the following protocol: an initial denaturation at 94 °C for 4 min; 25–30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min. The *HXT2* sequences were amplified with

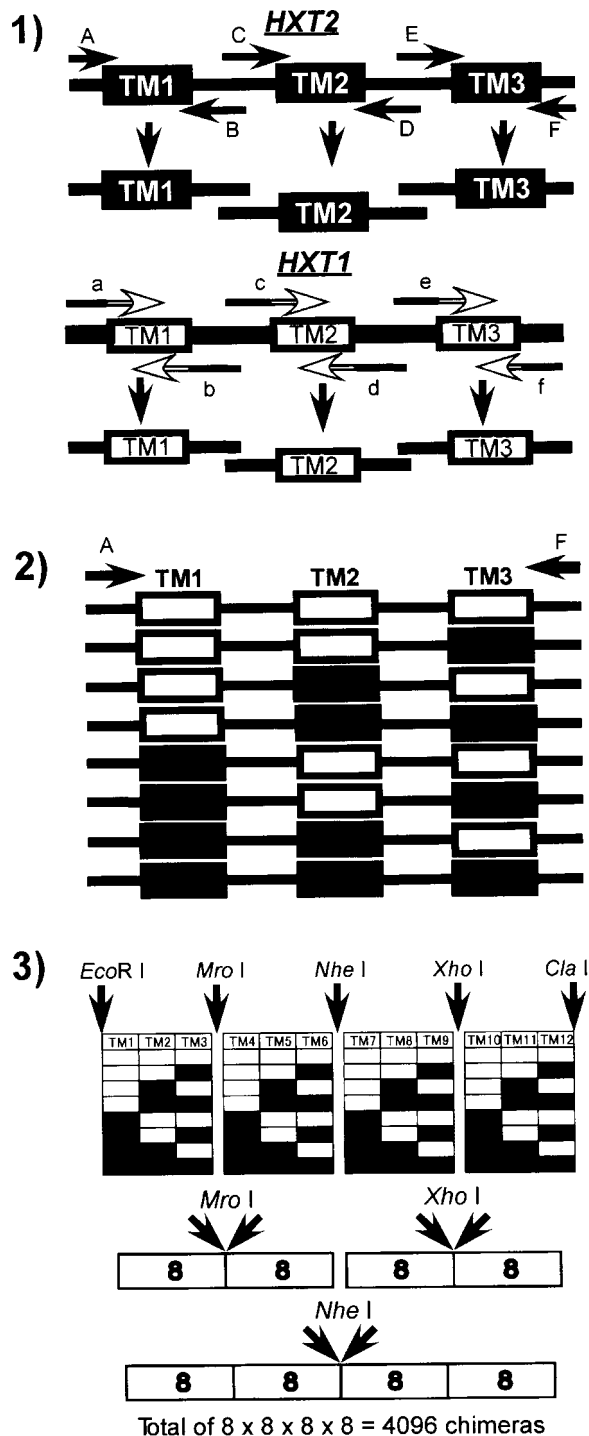


Figure 2 TM shuffling strategy

The strategy for replacing each TM of Hxt2 with the corresponding segment of Hxt1 comprised three steps. Panel (1) The nucleotide sequence encoding each TM of Hxt2 or Hxt1 was amplified by PCR as described in the Materials and methods section. Primers (A–F and a–f for TM1–TM3 of *HXT2* and *HXT1* respectively) were designed to overlap at the middle of each loop to allow the joining of adjacent PCR products. For amplification of the nucleotide sequence encoding each TM of Hxt1, PCR primers were designed to contain the nucleotide sequences for Hxt2 loop regions. Panel (2) The PCR products corresponding to each consecutive group of three Hxt1-derived TMs and Hxt2-derived TMs were mixed and randomly joined by PCR (with primers A and F for TM1–TM3), yielding 8 (2 × 2 × 2) different combinations of TMs. Panel (3) The adjacent sets of PCR products, each encoding three TMs, were connected by consecutive ligation first at *MroI* or *XhoI* sites and then at the *NheI* site. The resulting products encoding 4096 (8 × 8 × 8) different chimaeras were introduced into Hxt2mnx-pVT in place of *HXT2* with the use of *EcoRI* and *ClaI*.

primers corresponding to the adjacent loop regions (Hxt2-derived TMs), whereas the *HXT1* sequences were amplified with primers containing sequences for the adjacent loop regions of Hxt2 at their 5' ends and sequences for the TMs of Hxt1 at their 3' ends (Hxt1-derived TMs). The various primers were designed to overlap by seven to ten nucleotides at the middle of each loop region in order to allow the joining of neighbouring PCR products, with the exception of the loops between TMs 3 and 4, TMs 6 and 7, and TMs 9 and 10, for which the *MroI*, *NheI*, and *XhoI* sites were used for joining. In the second step, PCR products encoding each group of three adjacent Hxt2-derived TMs and Hxt1-derived TMs were mixed and joined by PCR (Figure 2), yielding four sets of eight random combinations of three TMs. PCR was performed with *Pfu* polymerase (Stratagene) and an amplification protocol of 94 °C for 4 min; 25 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min; and 72 °C for 7 min. In the third step, these four sets of PCR products were connected first with the use of *MroI* and *XhoI* and then with *NheI* (Figure 2). The resulting final products were used to replace *HXT2* in Hxt2mnx-pVT with the use of *EcoRI* and *ClaI*, thereby generating Hxt2rnd-pVT.

Plate selection

Clones with high-affinity glucose-transport activity were selected after incubation of Hxt2rnd-pVT-transformed cells for 3 or 4 days at 30 °C on either glucose-limited (glucose, 1 mg/ml) or mannose-limited (mannose, 5 mg/ml) agar plates containing a synthetic medium supplemented with adenine and amino acids, but not with uracil (S0.1D and S0.5Man plates respectively) [19]. KY73 cells were not able to grow on S2D plates (glucose, 20 mg/ml) in which glucose was the only carbon source. The introduction of *HXT2* or *HXT1* into KY73 cells allowed them to grow on S2D plates; however, only *HXT2*-expressing cells were able to grow on S0.1D plates. We also found that S0.5Man plates could be used for the selection of cells harbouring *HXT2*. With the use of S0.1D and S0.5Man plates we were thus able to select clones possessing high-affinity glucose-transport activity. It turned out that clones with high V_{\max} values similar to Hxt2 were picked up with this selection method (see the Results section). The total number of transformants was counted in parallel by plating a portion of the yeast cells on S2Mal plates (maltose, 20 mg/ml). The modified nucleotide sequences of all clones selected in the present study were verified by sequencing with an automated DNA sequencer (model 310; Applied Biosystems).

Transport assay

Cells harbouring plasmids were grown to exponential phase [attenuance (D_{650}) 0.3–0.4] at 30 °C in the synthetic medium used for plate selection containing 2% (w/v) maltose as the carbon source. Glucose transport in the cells was measured at 30 °C for 5 s as described in [20,21]. Transport activities measured at D- ^{14}C glucose concentrations of 0.1 or 20 mM were expressed as pmol of glucose/5 s per 10^7 cells and were corrected for the background activity determined either in the presence of 0.5 mM HgCl_2 or with 0.1 or 20 mM L- ^{14}C glucose. In some experiments, transport activity was calculated as a percentage of that obtained with cells expressing wild-type Hxt2.

Other assays

A crude membrane fraction was prepared from cells as described in [22], and immunoblot analysis of this fraction was performed

with rabbit polyclonal antibodies specific for the C-terminal region of Hxt2 [20] and with ^{125}I -labelled Protein A (Amersham). The intensity of bands corresponding to immune complexes was measured with imaging plates (BAS1800II; Fuji Film) [22]. Cell number was determined with a particle counter (Z2; Coulter). Protein concentration was measured with bicinchoninic acid ('BCA'; Pierce).

RESULTS

With the exclusion of the N- and C-terminal tails, Hxt2 and Hxt1 share $\approx 70\%$ amino acid sequence identity (Figure 1). However, Hxt2 is a high-affinity glucose transporter, whereas Hxt1 is a low-affinity glucose transporter. The K_m and V_{\max} of Hxt2 expressed in KY73 yeast cells for D-glucose transport were 3.1 mM and 1200 pmol/5 s per 10^7 cells, whereas the corresponding values for Hxt1 were 44 mM and 2800 pmol/5 s per 10^7 cells (Table 1).

To delineate the regions of Hxt2 essential for high-affinity glucose-transport activity with the use of TM shuffling, we constructed a comprehensive series of 4096 (2^{12}) chimaeras by replacing each TM of Hxt2 with the corresponding segment of Hxt1. A total of 218 out of 22 500 transformants was found to exhibit high-affinity glucose-transport activity by plate selection. Of the 218 selected clones, 48 clones were randomly picked up, sequenced and found to encode 39 different chimaeric proteins (Figure 3). The numbers of Hxt1-derived TMs at each TM position for these 39 chimaeras were 1 (TM1), 9 (TM2), 25 (TM3), 9 (TM4), 0 (TM5), 5 (TM6), 1 (TM7), 1 (TM8), 13 (TM9), 20 (TM10), 21 (TM11) and 9 (TM12). Hxt1-derived TMs supported high-affinity glucose-transport activity at all TM positions with the exception of TM5, with TM positions 1, 7 and 8 also showing a high preference for Hxt2-derived segments. These results indicated that TMs 1, 5, 7 and 8 of Hxt2 are important for high-affinity glucose-transport activity.

We examined further the contribution of each of these four Hxt2-derived TMs to high-affinity glucose transport by constructing 16 chimaeric transporters containing the various possible combinations of Hxt2-derived TMs at these four positions, with the remainder of the TMs being derived from Hxt1. Only one chimaera, namely that containing all four Hxt2-derived TMs (C1578), was selected by plate assay (Table 1); the other 15 chimaeras did not grow on either S0.1D or S0.5Man plates. We prepared a crude membrane fraction from cells expressing each of these 16 chimaeric transporters and examined the extent of transporter expression by immunoblot analysis with antibodies to the C-terminal region of Hxt2. All 16 chimaeras were detected as a predominant immunoreactive band of 47 kDa, at a position corresponding to that of wild-type Hxt2. We also measured the glucose transport activities of these 16 chimaeras with 20 mM D-glucose as substrate and then normalized the activities either by cell number or by the amount of immunoreactive transporter protein (Table 1). In both instances, the activity of the C1578 chimaera was almost identical with that of wild-type Hxt2. The chimaeras C57, C58, C157, C158, C178 and C578 exhibited the highest transport activities after C1578, but the activities of these proteins did not exceed 40% (when normalized by cell number) or 30% (normalized by the amount of immunoreactive transporter protein) of that of Hxt2 (Table 1). With this glucose concentration, Hxt1 exhibited almost the same transport activity as Hxt2 and C1578. This fact indicates that the low activities of the other chimaeras are not due to the low affinity that may be introduced by Hxt1-derived TMs. The measurement of K_m and V_{\max} values showed that two (C17 and C57) of the chimaeras having two Hxt2-derived TMs possessed high-affinity and

Table 1 Characterization of the 16 chimaeras containing all possible combinations of Hxt2-derived TMs 1, 5, 7 and 8

Chimaera C0 was constructed by replacing all of the TMs of Hxt2 with those of Hxt1. The other chimaeras (C1–C1578) were generated by replacing one or more of TMs 1, 5, 7 and 8 of C0 with the corresponding Hxt2-derived segments that are indicated as crosses. KY73 cells expressing each of the 16 chimaeras were subjected to plate assays with S0.1D or S0.5Man plates. Cell growth or no growth after incubation for 3 or 4 days at 30 °C is indicated by (+) and (–) respectively. KY73 cells expressing Hxt1, Hxt2 or each of 16 chimaeras were grown to exponential phase at 30 °C in S2Mal synthetic medium. Glucose-transport activity was then measured for 5 s at 30 °C with 0.1 or 20 mM D-glucose as substrate. Transport activities were normalized by cell number (means \pm S.E.M., $n \geq 3$) or normalized by the mean level of transporter expression as determined by quantitative analysis of immunoblot from four experiments. The K_m and V_{max} values (means \pm S.E.M., $n \geq 3$) were determined with 1–100 mM D-glucose as substrate as described [21]. Abbreviations: nd, not determined; Ab, antibody.

Clone	Hxt2-derived TM				Plate		Glucose transport (20 mM)		Glucose transport (0.1 mM)		K_m (mM)	V_{max} (pmol/5 s per 10^7 cells)
	TM1	TM5	TM7	TM8	S0.1D	S0.5Man	(pmol/5 s per 10^7 cells)	Normalized by Ab	(pmol/5 s per 10^7 cells)	Normalized by Ab		
C0					–	–	11 \pm 4	0.01	–0.3 \pm 0.1	–0.01	nd	nd
C1	×				–	–	8 \pm 5	0.01	–0.4 \pm 0.3	–0.01	nd	nd
C5		×			–	–	–3 \pm 11	0.00	–0.1 \pm 0.2	0.00	nd	nd
C7			×		–	–	20 \pm 10	0.02	–0.2 \pm 0.1	0.00	nd	nd
C8				×	–	–	19 \pm 6	0.02	–0.4 \pm 0.1	–0.01	nd	nd
C15	×	×			–	–	37 \pm 10	0.04	–0.1 \pm 0.1	0.00	nd	nd
C17	×		×		–	–	103 \pm 10	0.06	4.5 \pm 0.3	0.06	4.7 \pm 1.2	210 \pm 40
C18	×			×	–	–	41 \pm 7	0.06	0.3 \pm 0.2	0.01	nd	nd
C57		×	×		–	–	230 \pm 18	0.15	12.9 \pm 0.4	0.17	3.9 \pm 0.2	540 \pm 10
C58		×		×	–	–	117 \pm 12	0.09	2.0 \pm 0.1	0.03	nd	nd
C78			×	×	–	–	5 \pm 7	0.00	0.9 \pm 0.2	0.01	nd	nd
C157	×	×	×		–	–	180 \pm 24	0.24	2.2 \pm 0.2	0.06	11 \pm 2	220 \pm 80
C158	×	×		×	–	–	225 \pm 12	0.29	4.3 \pm 0.2	0.11	11 \pm 1	440 \pm 50
C178	×		×	×	–	–	124 \pm 19	0.14	5.4 \pm 0.7	0.12	1.1 \pm 0.1	150 \pm 30
C578		×	×	×	–	–	229 \pm 28	0.24	12.8 \pm 1.2	0.27	4.1 \pm 0.3	410 \pm 70
C1578	×	×	×	×	+	+	678 \pm 59	0.82	26.0 \pm 1.0	0.63	6.2 \pm 0.2	1200 \pm 110
Hxt2	×	×	×	×	+	+	715 \pm 59	1.00	35.4 \pm 1.0	1.00	3.1 \pm 0.3	1200 \pm 90
Hxt1					–	–	693 \pm 39		9.4 \pm 0.5		44 \pm 3	2800 \pm 270

Clone	TM1	TM2	TM3	TM4	TM5	TM6	TM7	TM8	TM9	TM10	TM11	TM12
A1	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A2	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A4	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A5	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A6	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A7	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A8	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A9	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A10	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A11	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A13	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A14	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A15	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A16	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
A18	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
ALL6	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
ALL7	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
ALL8	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
ALL9	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
ALL10	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
ALL17	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
ALL18	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A2	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A3	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A5	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A6	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A8	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A11	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A13	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A14	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A16	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A17	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A19	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A21	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A29	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5A38	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5AA1	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5AA4	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5AA7	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
5AA13	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched	hatched
Hxt2(%)	97	77	36	77	100	87	97	97	67	49	46	77

Figure 3 Shuffled TMs in chimaeric transporters with high-affinity glucose-transport activity

The distribution of Hxt2-derived (hatched boxes) and Hxt1-derived (open boxes) TMs is shown for the 39 different Hxt2–Hxt1 chimaeras that exhibited high-affinity glucose-transport activity and whose sequence was determined. The percentage of Hxt2-derived TMs at each TM position for the 39 chimaeras is shown.

low-capacity transport, and all the chimaeras having three Hxt2-derived TMs possessed high-affinity and low-capacity transport. Only C1578 exhibited transport activity almost identical with Hxt2. With 0.1 mM glucose, similar results were obtained. The transport activities of C17, C57, C58, C157, C158, C178 and C578 did not exceed 40% of that of Hxt2 (when normalized by cell number), while C1578 showed > 70%. About a 2-fold difference in the affinity between Hxt2 and C1578 contributes to the apparent decrease of transport in C1578 at this concentration. The rest of the clones exhibited null activity. The substrate specificities (Figure 4) of C1578 were also similar to those of Hxt2. In contrast, the transport activities of C1578 with D-glucose, 2-deoxy-D-glucose, D-fructose and D-mannose were markedly smaller than those of Hxt1.

DISCUSSION

Various helix-packing models of MFS members have indicated that the substrate pathway is formed by multiple TMs and that the pathway is sufficiently narrow to allow substrate selectivity [12–15]. Localized mutagenesis has been customarily applied to delineate the mechanism of substrate recognition by these transporters, with residue changes often resulting in loss of function of the proteins. In addition, the results of such studies have been assumed to indicate the local effects of replacement of an amino acid. However, substitution of a single residue has been shown to affect overall protein conformation [23], as well as transporter biogenesis, including vesicular transport [24].

To circumvent these problems, we have adopted a comprehensive approach involving the construction of chimaeras between transporter paralogues. This approach offers the potential to identify regions of the transporters that are responsible for

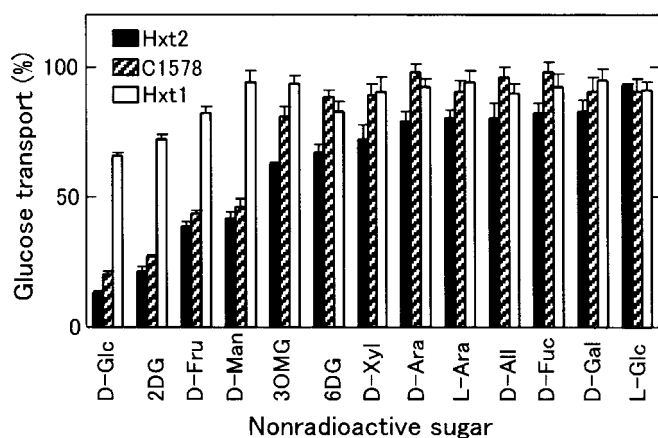


Figure 4 Substrate specificities of Hxt2, Hxt1 and C1578

The transport activities of Hxt2, Hxt1, and C1578 were measured for 5 s with 0.1 mM D- 14 C]glucose as substrate in the presence of a 200-fold excess of the indicated non-radioactive sugars. Data are means from three experiments and are expressed relative to the transport activity determined in the presence of 20 mM sorbitol (which was used to adjust osmolality). The glucose transport activity of Hxt2 in the presence of sorbitol was 32.9 ± 4.1 pmol/5 s per 10^7 cells ($n = 6$); background activity estimated from transport in the presence of 0.5 mM HgCl₂ did not exceed 4% of that activity. Data are means \pm S.E.M. Abbreviations: 2DG, 2-deoxy-D-glucose; 3OMG, 3-O-methyl-D-glucose; 6DG, 6-deoxy-D-glucose; All, allose.

differences in properties between the paralogues. We have previously created a battery of chimaeras between Hxt2 and Gal2 in a systematic manner with the use of homologous recombination in *E. coli* and other comprehensive approaches. Our results identified two aromatic amino acids in TM10 of these transporters that are required for substrate recognition [20,25,26]. We further showed that replacement of these two amino acids of Hxt2 with the corresponding residues of Gal2 changed Hxt2 to transport D-galactose [26].

We have now introduced the strategy of TM shuffling. One result of the high level of homology between Hxt2 and Hxt1 is that, with the exception of TMs 10 and 11, the amino acids adjacent to the TMs are identical in the two proteins, which thus facilitated replacement of the TMs of Hxt2 with those of Hxt1. This difference at TMs 10 and 11 turned out not to be a problem, since these segments were found not to be determinants of substrate affinity.

Our results obtained by the use of TM shuffling revealed that TMs 1, 5, 7 and 8 of Hxt2 are important determinants of high-affinity glucose-transport activity. To confirm the importance of these TMs, we constructed chimaeras containing all the possible combinations of the four Hxt2-derived TMs. Screening of these 16 chimaeras for high-affinity glucose-transport activity by plate assays resulted in the selection of C1578, which showed virtually identical transport properties with those of Hxt2. As a first step for the determination of amino acid residues responsible for high-affinity transport, four amino acid residues in TM5 of C1578 that are different between Hxt1 and Hxt2 (Figure 1) were randomly replaced with those of Hxt1. The results indicated that Leu²⁰¹ was essential and either Cys¹⁹⁵ or Phe¹⁹⁸ was necessary for high affinity transport (T. Kasahara and M. Kasahara, unpublished work). Similar experiments on TMs 1, 7 and 8 are in progress.

It should be noted that TMs required for the low-affinity transport activity of Hxt1 are not necessarily limited to TMs 1, 5, 7 and 8, since all chimaeras showing low activities exhibited high-affinity and low-capacity transport. Also, the fact that C0 that possesses all Hxt1-derived TMs exhibited a markedly low activity as compared with Hxt1 (Table 1) may indicate that a

combination of inter-TM loops and adjacent TMs is necessary. Several amino acids in inter-TM loops between TMs 1 and 2, 2 and 3, 7 and 8 or 9 and 10 are implicated as important for transport activity [14,27–29]. These amino acids are, however, conserved between Hxt2 and Hxt1. Further efforts are required to clarify this matter.

In a recent study of two-dimensional crystals of OxlT at 6.5 Å (1 Å = 0.1 nm) [15], it is discussed that its 12 TMs are divided into three groups: a peripheral group comprising TMs 3, 6, 9 and 12 which does not appear to contribute to the substrate transport pathway; a second group comprising TMs 2, 5, 8 and 11 which faces the central substrate transport pathway along most of the span of the membrane; and a third group comprising TMs 1, 4, 7 and 10 that participates in the transport pathway either on the cytoplasmic side (TMs 4 and 10) or on the periplasmic side (TMs 1 and 7) of the membrane. This TM topology model matches well the helix-packing model of TetA(B) [13], except for the positions of the peripheral group. In our previous studies of Hxt2 and Gal2, we showed that TM10 plays an essential role in substrate recognition [30]. Our present study has revealed the importance of TMs 1, 5, 7 and 8 for high-affinity glucose transport by Hxt2. All of these five TMs are predicted to face the substrate pathway, although the positions of the peripheral group in the TetA(B) model is not consistent with our data. We found that TMs7 and 12 interact with each other [31], while they are far apart in the model.

In conclusion, our present data indicate that at least four TMs, namely 1, 5, 7 and 8, are required for exhibiting the high-affinity glucose-transport activity of Hxt2. These segments may co-ordinate the transmembrane passage of glucose and contribute to high-affinity substrate recognition.

We thank A. L. Kruckeberg (University of Amsterdam, The Netherlands) for yeast strain KY73. This work was supported by grants from Ministry of Education, Culture, Sports, Science and Technology of Japan and from Teikyo University.

REFERENCES

- 1 Busch, W. and Saier, Jr, M. H. (2002) The transporter classification (TC) system, 2002. *Crit. Rev. Biochem. Mol. Biol.* **37**, 287–337
- 2 Doyle, D. A. Cabral, J. M., Plietzer, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T. and MacKinnon, R. (1998) The structure of the potassium channel: molecular basis of K⁺ conduction and selectivity. *Science* **280**, 69–77
- 3 Chang, G., Spencer, R. H., Lee, A. T., Barclay, M. T. and Rees, D. C. (1998) Structure of the MscL homolog from *Mycobacterium tuberculosis*: a gated mechanosensitive ion channel. *Science* **282**, 2220–2225
- 4 Schirmer, T., Keller, T. A., Wang, Y.-F. and Rosenbusch, J. P. (1995) Structural basis for sugar translocation through maltoporin channels at 3.1 Å resolution. *Science* **267**, 512–514
- 5 Murata, K., Mitsuoka, K., Hirai, T., Walz, T., Agre, P., Heymann, J. B., Engel, A. and Fujiyoshi, Y. (2000) Structural determinants of water permeation through aquaporin-1. *Nature (London)* **407**, 599–605
- 6 Fu, D., Libson, A., Miercke, L. J. W., Weitzman, C., Nollert, P., Krucinski, J. and Stroud, R. M. (2000) Structure of a glycerol-conducting channel and the basis for its selectivity. *Science* **290**, 481–486
- 7 Toyoshima, C., Nakasako, M., Nomura, H. and Ogawa, H. (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. *Nature (London)* **405**, 647–655
- 8 Pao, S. S., Paulsen, I. T. and Saier, M. H., Jr. (1998) Major facilitator superfamily. *Microbiol. Mol. Biol. Rev.* **62**, 1–34
- 9 Henderson, P. J. and Maiden, M. C. (1990) Homologous sugar transport proteins in *Escherichia coli* and their relatives in both prokaryotes and eukaryotes. *Philos. Trans. R. Soc. London B Biol. Sci.* **326**, 391–410
- 10 Kruckeberg, A. L. and Bisson, L. F. (1990) The Hxt2 gene of *Saccharomyces cerevisiae* is required for high-affinity glucose transport. *Mol. Cell. Biol.* **10**, 5903–5913
- 11 Mueckler, M., Caruso, C., Baldwin, S. A., Panico, M., Blench, I., Morris, H. R., Allard, W. J., Lienhard, G. E. and Lodish, H. F. (1985) Sequence and structure of a human glucose transporter. *Science* **229**, 941–945

- 12 Sorgen, P. L., Hu, Y., Guan, L., Kaback, H. R. and Girvin, M. E. (2002) An approach to membrane protein structure without crystals. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 14037–14040
- 13 Tamura, N., Konishi, S., Iwaki, S., Kimura-Someya, T., Nada, S. and Yamaguchi, A. (2001) Complete cysteine-scanning mutagenesis and site-directed chemical modification of the Tn10-encoded metal-tetracycline/H⁺ antiporter. *J. Biol. Chem.* **276**, 20330–20339
- 14 Hruz, P. W. and Mueckler, M. M. (2001) Structural analysis of the GLUT1 facilitative glucose transporter (Review). *Mol. Membr. Biol.* **18**, 183–193
- 15 Hirai, T., Heymann, J. A., Shi, D., Sarker, R., Maloney, P. C. and Subramaniam, S. (2002) Three-dimensional structure of a bacterial oxalate transporter. *Nat. Struct. Biol.* **9**, 597–600
- 16 Boles, E. and Hollenberg, C. P. (1997) The molecular genetics of hexose transport in yeasts. *FEMS Microbiol. Rev.* **21**, 85–111
- 17 Vernet, T., Dignard, D. and Thomas, D. Y. (1987) A family of yeast expression vectors containing the phage f1 intergenic region. *Gene* **52**, 225–233
- 18 Ye, L., Kruckeberg, A. L., Berden, J. A. and Van Dam, K. (1999) Growth and glucose repression are controlled by glucose transport in *Saccharomyces cerevisiae* cells containing only one glucose transporter. *J. Bacteriol.* **181**, 4673–4675
- 19 Kaiser, C., Michaelis, S. and Mitchell, A. (1994) *Methods in Yeast Genetics*, pp. 208–209, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 20 Nishizawa, K., Shimoda, E. and Kasahara, M. (1995) Substrate recognition domain of the Gal2 galactose transporter in yeast *Saccharomyces cerevisiae* as revealed by chimeric galactose–glucose transporters. *J. Biol. Chem.* **270**, 2423–2426
- 21 Kasahara, T. and Kasahara, M. (2000) Three aromatic amino acid residues critical for galactose transport in yeast Gal2 transporter. *J. Biol. Chem.* **275**, 4422–4428
- 22 Kasahara, T. and Kasahara, M. (1996) Expression of the rat GLUT1 glucose transporter in the yeast *Saccharomyces cerevisiae*. *Biochem. J.* **315**, 177–182
- 23 Kawabe, T. and Yamaguchi, A. (1999) Transmembrane remote conformational suppression of the Gly-332 mutation of the Tn10-encoded metal-tetracycline/H⁺ antiporter. *FEBS Lett.* **457**, 169–173
- 24 Wright, E. M., Turk, E. and Martin, M. G. (2002) Molecular basis for glucose–galactose malabsorption. *Cell. Biochem. Biophys.* **36**, 115–121
- 25 Kasahara, M., Shimoda, E. and Maeda, M. (1996) Transmembrane segment 10 is important for substrate recognition in Gal2 and Hxt2 sugar transporters in the yeast *Saccharomyces cerevisiae*. *FEBS Lett.* **389**, 174–178
- 26 Kasahara, M., Shimoda, E. and Maeda, M. (1997) Amino acid residues responsible for galactose recognition in yeast Gal2 transporter. *J. Biol. Chem.* **272**, 16721–16724
- 27 Barrett, M. P., Walmsley, A. R. and Gould, G. W. (1999) Structure and function of facilitative sugar transporters. *Curr. Opin. Cell Biol.* **11**, 496–502
- 28 Will, A., Grassl, R., Erdmenger, J., Caspari, T. and Tanner, W. (1998) Alteration of substrate affinities and specificities of the *Chlorella* hexose/H⁺ symporters by mutations and construction of chimaeras. *J. Biol. Chem.* **273**, 11456–11462
- 29 Doege, H., Schurmann, A., Ohnimus, H., Monser, V., Holman, G. D., Joost, H. G. (1998) Serine-294 and threonine-295 in the exofacial loop domain between helices 7 and 8 of glucose transporters (GLUT) are involved in the conformational alterations during the transport process. *Biochem. J.* **329**, 289–293
- 30 Kasahara, M. and Maeda, M. (1998) Contribution to substrate recognition of two aromatic amino acid residues in putative transmembrane segments 10 of the yeast sugar transporters Gal2 and Hxt2. *J. Biol. Chem.* **273**, 29106–29112
- 31 Kasahara, T. and Kasahara, M. (2000) Interaction between the critical aromatic amino acid residues Tyr³⁵² and Phe⁵⁰⁴ in the yeast Gal2 transporter. *FEBS Lett.* **471**, 103–107
- 32 Baldwin, S. A. (1993) Mammalian passive glucose transporters: members of an ubiquitous family of active and passive transport proteins. *Biochim. Biophys. Acta* **1154**, 17–49

Received 3 January 2003/17 February 2003; accepted 25 February 2003

Published as BJ Immediate Publication 25 February 2003, DOI 10.1042/BJ20030044