Transmembrane segment 10 is important for substrate recognition in Gal2 and Hxt2 sugar transporters in the yeast *Saccharomyces cerevisiae*

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Abstract A systematic series of chimeras between Gal2 galactose transporter and Hxt2 glucose transporter in yeast was produced to delineate the essential domain for substrate recognition. A domain of 101 amino acids close to the COOH-terminus that has been previously identified as the critical substrate recognition region was further divided into four subdomains, by introducing five restriction enzyme sites at exactly corresponding locations of both genes without changing coding amino acids. When each of all possible 16 modified genes was expressed, all the galactose transport-active chimeras were found to possess Gal2-derived transmembrane segment (TM) 10. Of the 35 amino acids in the TM10 region, only 12 differ between Gal2 and Hxt2, indicating that these 12 amino acids include the critical residue(s) responsible for the differential recognition of galactose and glucose in these transporters.

Key words: Sugar transport; Substrate recognition; Galactose; Glucose; Chimera; *Saccharomyces cerevisiae*

1. Introduction

A variety of membrane transporters of prokaryotes and eukaryotes belong to a transporter superfamily termed the major facilitator superfamily (MFS) [1] which has been divided into 5 or 17 families [1–3]. The largest of these is known as the Glut family and consists of various membrane transporters for monosaccharides and unknown substances [3–6], although the delineation of the family is still to be determined. The single organism in which the largest numbers of transporters in this family have been found is the yeast *Saccharomyces cerevisiae*, where more than 15 have been described and others are anticipated [3]. Gal2 transporter belongs to the Glut family and is a major high affinity galactose transporter in this organism that also transports glucose with almost the same affinity [6,7]. Hxt2 is homologous to Gal2 with 71% amino acid identity in the middle part of the sequence (excluding variable amino- and carboxyl-terminal regions) and is a major glucose transporter in *S. cerevisiae*, with an affinity for glucose similar to the Gal2 transporter; it does not, however, transport galactose. Thus, it seems worth while to compare these two transporters for the study of substrate recognition. Most recent work has employed localized mutagenesis as a tool to determine the substrate recognition site [5], results of which have been based on mutations on sporadic sites and are mostly negative in nature. Moreover, it has been difficult with localized mutagenesis to state whether those mutations directly changed the substrate recognition sites or changed it indirectly through conformational changes of the surrounding structures.

In a previous study [6], we employed a systematic approach using chimeras of these two transporters, in which three series of chimeras were created with the use of the *Escherichia coli recA* recombination system. We identified a 101 amino acid domain that lies close to the COOH-terminus, the replacement of which in Hxt2 with the corresponding domain of Gal2 changed the substrate specificity of Hxt2 to that of Gal2. The present study was designed to further delimit the domain. With the use of chimeras made by systematically replacing four subdomains of the 101 amino acid domain, we localized the domain related to the differential recognition of galactose and glucose to a 35 amino acid segment in transmembrane segment (TM) 10 region, where only 12 out of 35 amino acids are different between Gal2 and Hxt2.

2. Materials and methods

GAL2 [8] was introduced into a multicloning site of pTV3 (YEp *TRP1 bla*) [9], which was then modified: (1) to disrupt an *Eco*RI site, (2) to create a *Cla*I site immediately following the termination codon of *GAL2* and (3) to create a new *Eco*RI site after the initiation codon of *GAL2*, yielding GAL2-pTV3e [6]. *HXT2* [10] was modified to create a *Cla*I site immediately following the termination codon of *HXT2* and replaced as a cassette with *GAL2* in GAL2-pTV3e by using two restriction enzyme sites: an *Eco*RI site extending from the 7th to 12th nucleotide of the *HXT2* coding sequence and the *Cla*I site, which yielded HXT2-pTV3e. These plasmids were introduced into *S. cerevisiae*, LBY416 (*MATα hxt2::LEU2 snf3::HIS3 gal2 lys2 ade2 trp1 his3 leu2 ura3*) [6,10]. The amino acid sequences of Gal2 and Hxt2 were aligned (malign, ODEN Package) [11], which is shown together with nucleotide sequences (Fig. 1). To introduce five new restriction enzyme sites into *GAL2* and *HXT2*, *Spe*I and *Stu*I sites in pTV3 were disrupted by blunting with T4 DNA polymerase. Using PCR, a *Spe*I site in *GAL2* was modified from ACTAGT to ACTGGT and a *Nco*I site in TM9 from CCATGG to CTATGG, while keeping the coding amino acids the same. Five restriction enzyme sites were introduced by PCR into the corresponding locations of both *GAL2* and *HXT2*, without changing the coding amino acids: *Sac*I between TM9 and TM10, *Mlu*I between TM10 and TM11, *Spe*I between TM11 and TM12, *Stu*I at the beginning part of the carboxyl-terminal tail and *Nco*I at the middle part of the carboxyl-terminal tail (Fig. 1). The *Stu*I site in *GAL2* and the *Nco*I site in *HXT2* are existing ones. All 16 possible combinations of the four subdomains were made by cutting both genes with these restriction enzymes and introducing the fragment between the *Sac*I and *Nco*I sites of modified *HXT2* (Fig. 2). These chimeric *HXT2* genes were called *HXT2-0* to *HXT2-15* (Fig. 2) and LBY416 cells possessing each of them were called Hxt2-0 to Hxt2-15 cells, respectively. Both strands of the nucleotide sequences of these genes were sequenced between the *Sac*I and *Cla*I sites and confirmed to be error-free.

For transport assays, the cells were cultured at 30°C to an early log phase (OD_{650}=0.2–0.4) in a synthetic medium [12] supplemented with uracil, adenine and amino acids except for tryptophan with 2% galactose as a carbon source. Transport of D-[^{14}C]galactose and D-[^{14}C]glucose in yeast cells, immunoblotting of yeast homogenates

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Abbreviations: TM, transmembrane segment; PCR, polymerase chain reaction

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and measurement of protein concentration were performed as described previously [6].

3. Results and discussion

Three series of chimeras between Gal2 and Hxt2 have been made by the use of the *recA* recombination system of *Escherichia coli* and measurement of galactose transport has clearly indicated that a Gal2 domain of 101 amino acids that includes TMs 10–12 and the beginning half of the COOH-terminal hydrophilic tail is important for the recognition of galactose [6]. For further delineation of the substrate recognition region, this domain was subdivided into four subdomains by introducing five restriction enzyme sites (Fig. 1). All 16 possible combinations of subdomains were made and connected

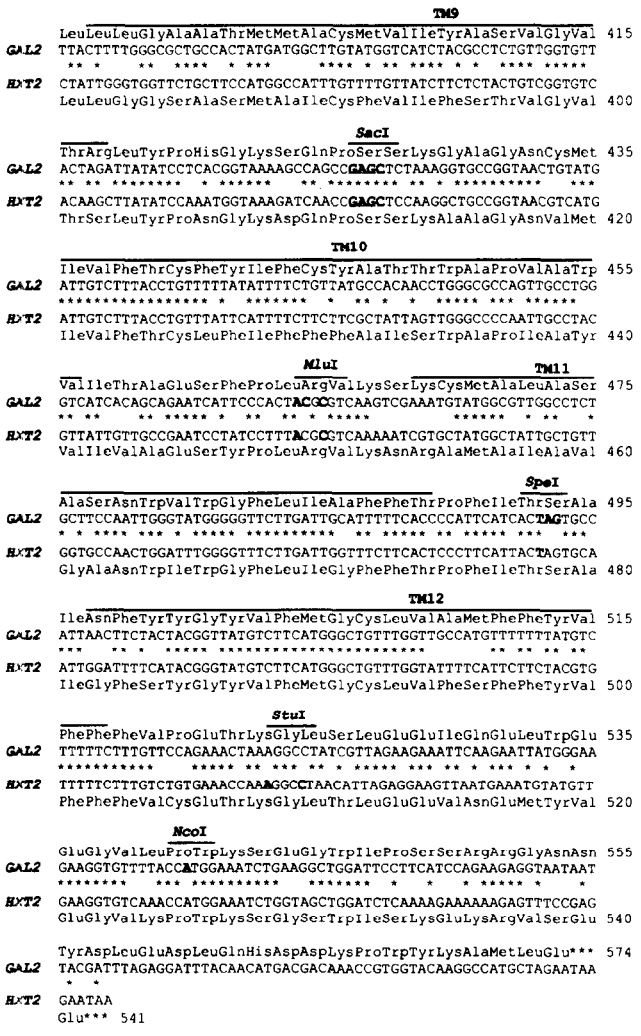

Fig. 1. Fig. 1. Introduction of restriction enzyme sites into *GAL2* and *HXT2*. Gal2 and Hxt2 are aligned [11] and putative TM9 to TM12 [5] are shown by horizontal bars. Identical nucleotides between *GAL2* and *HXT2* are indicated by asterisks. The two genes are modified to create five restriction enzyme sites at exactly the corresponding places, without changing coding amino acids. Restriction enzyme sites created are *Sac*I between TM9 and TM10, *Mlu*I between TM10 and TM11, *Spe*I between TM11 and TM12, *Stu*I at the beginning part of the COOH-terminal tail and *Nco*I at the middle part of the COOH-terminal tail. Replaced nucleotides are shown in bold. Two restriction enzyme sites (*Stu*I in *GAL2* and *Nco*I in HXT2) are non-modified original ones. The numbering of amino acid residue is shown on the right.

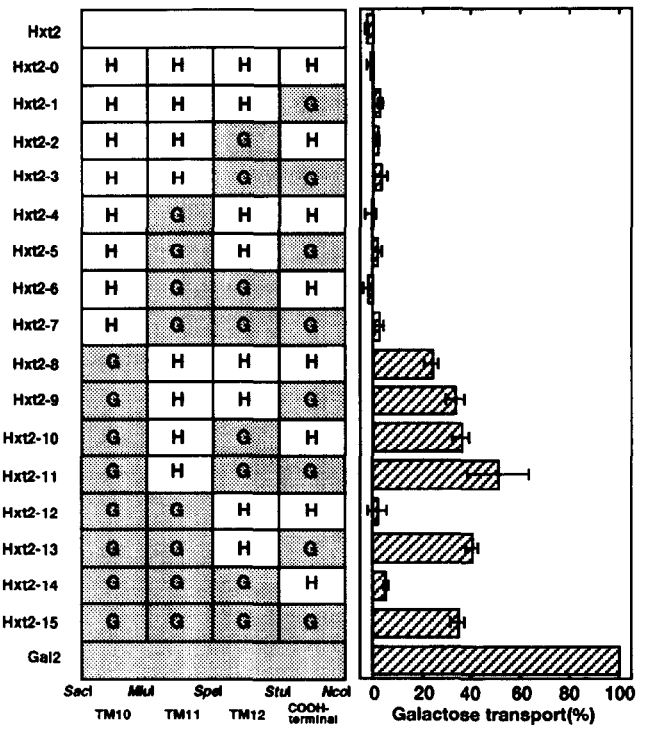

Fig. 2. Systematic production of chimeras in the 101 amino acid region and galactose transport in these chimeras. Chimeras between Gal2 and Hxt2 were produced using five restriction enzyme sites as described in Fig. 1 and named as shown on the left. G and H indicate that the subdomain shown is derived from Gal2 and Hxt2, respectively. Galactose transport activities in these chimeras are measured as described in Section 2. Transport activities are expressed relative to that in Gal2 cells and the average and SEM of three experiments are shown. Note that the transporters Hxt2-0 to Hxt2-15 possess indicated subdomains in the 101 amino acid region and all the rest of the transporter is derived from Hxt2. Hxt2-8 transporter, for example, consists of TM10 derived from Gal2 and the rest of the area including that not shown in the panel is from Hxt2.

between the *Sac*I and *Nco*I sites of modified *HXT2* (Fig. 2). LBY416 cells harboring each of these 16 modified *HXT2* were called Hxt2-0 to Hxt2-15 cells and subjected to transport assays. These cells exhibited various extents of galactose transport activity, but a conspicuous feature was that all the galactose transport-active cells possessed transporters consisting of the Gal2-derived TM10 (Hxt2-8, 9, 10, 11, 13 and 15 cells) (Fig. 2). Two exceptional cases were observed in which transporters contained the Gal2-derived TM10 but were inactive in galactose transport (Hxt2-12 and 14 cells). Galactose transport in all cells having the Hxt2-derived TM10 was found to be inactive (Hxt2-0 to 7 cells). The possibility that some of the modified transporters may not be produced or may be sensitive to proteolytic degradation was checked by immunoblotting, and the production of all 16 transporters was confirmed (Fig. 3). Hxt2-12 and Hxt2-14 transporters were expressed as much as the other transporters. Transport of glucose in these 16 cells was measured in the same manner as galactose transport. All the cells except Hxt2-12 and Hxt2-14 cells showed glucose transport, although the extent of glucose transport was not well correlated with that of galactose transport (data not shown). These results indicate that the region containing TM10 is important for the differential recognition of galactose and glucose. It may well be that Hxt2-12 and

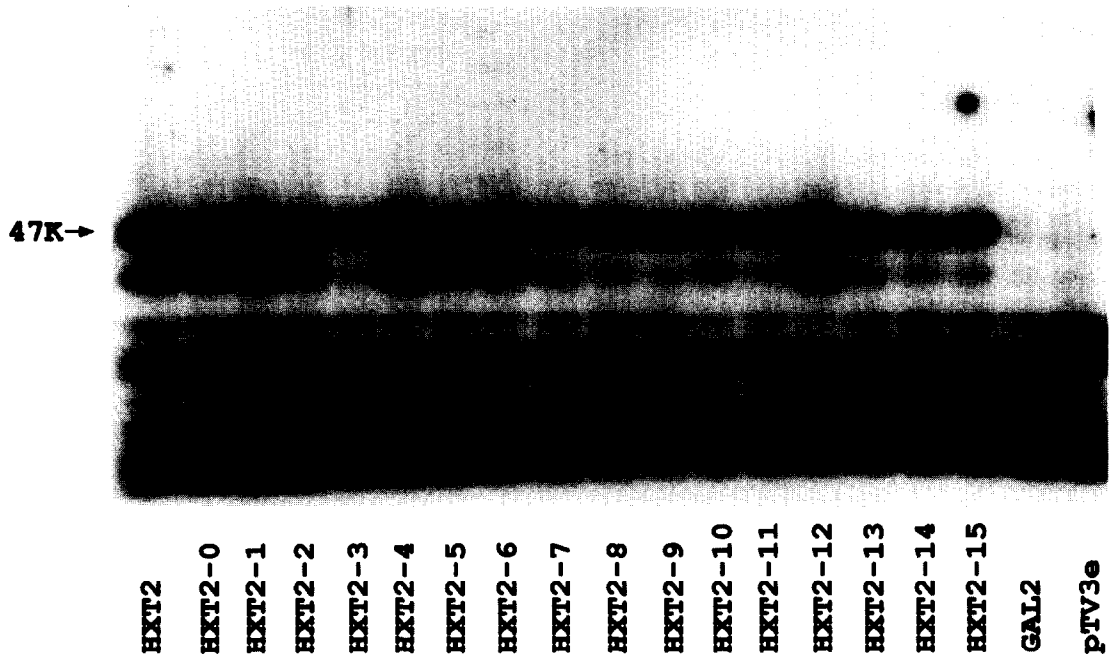

Fig. 3. Expression of chimeric transporters detected by immunoblotting. Cells harboring *HXT2*, *GAL2*, chimeric transporter genes *HXT2-0* to *HXT2-15* or vector only (pTV3e) were cultured in the same conditions as for transport assays. Cell homogenates (20 μg each) were immunoblotted with antibody to Hxt2 COOH-terminus. Autoradiography of [^{125}I]protein A (IM144, Amersham) was performed with imaging plates (BAS2000, Fuji Film). The 47 kDa band indicates transporters having the Hxt2 COOH tail. The amount of the lower molecular weight band seen below the 47 kDa band increased after repeated freeze-thawing.

Hxt2-14 are either inactive in monosaccharide transport or are not expressed in the plasma membrane, and thus do not contribute to galactose transport in yeast cells.

The Hxt2-8 transporter that possesses the smallest Gal2 region of TM10 alone was used for further characterization. Kinetic parameters of galactose transport in Hxt2-8 cells were compared with those of Gal2 cells. A K_m of 41 ± 2 mM (mean ± SEM, n=3) was observed in Hxt2-8 cells, which was about 8-fold higher than that of 5.3 ± 0.3 mM (n=3) in Gal2 cells, while V_{max} of both cells was practically the same: 0.69 ± 0.03 nmol/10^7/5 s in Hxt2-8 cells and 0.97 ± 0.1 nmol/10^7/5 s in Gal2 cells. These results explain the relatively low galactose transport activity in Hxt2-8 cells under the conditions as in Fig. 2 in which 0.1 mM galactose was used as the substrate. Substrate specificity of Hxt2-8 and Gal2 cells is shown in Fig. 4. The addition of a 200-fold excess amount of non-radioactive sugars inhibited galactose transport to varying extents. The order of inhibition was almost the same between Gal2 and Hxt2-8 cells. The extent of inhibition, however, was stronger in the former cells. This may be due to a decrease in the affinity for galactose in the Hxt2-8 transporter. Decrease in the affinity for galactose and similar substrate specificity in Hxt2-8 are consistent with the idea that a major galactose recognition domain lies in TM10, but other region(s) also contributes to enhancing the binding affinity for galactose. In fact, HXT2-15 cells showed an intermediate K_m of 23 mM.

Our previous study [6] clearly showed that the 101 amino acid region containing TM10–12 and the beginning half of the COOH-terminal hydrophilic part is important for the differential recognition of galactose and glucose. The present study further narrowed down that domain to the TM10 region consisting of 35 amino acids (Fig. 5). The alignment of Gal2 and Hxt2 indicated that only 12 out of 35 amino acids are different between the two transporters. It is highly probable that a few of these 12 amino acids are responsible for the differential recognition of galactose and glucose. The previous and present studies, both of which utilized chimeras, adopted a general principle of collecting all data comprehensively, covering unbiased information on all the areas. Although many studies

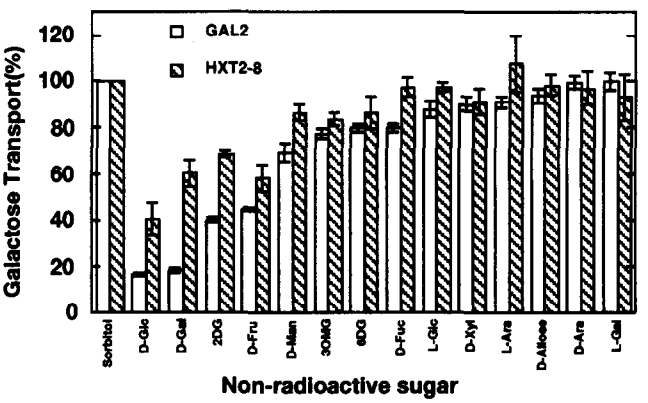

Fig. 4. Substrate specificity of Gal2 and Hxt2-8. The substrate specificity of Gal2 and Hxt2-8 possessing the smallest region of Gal2 in the present study was studied by adding a 200-fold excess of indicated non-radioactive sugar. The initial rate of galactose transport at 0.1 mM in the presence of each non-radioactive sugar was measured for 5 s. The average and SEM of three experiments are expressed relative to the value in the presence of sorbitol that was used for adjusting osmolality. Abbreviations: Glc, glucose; Gal, galactose; 2DG, 2-deoxy-D-glucose; Fru, fructose; Man, mannose; 3OMG, 3-*O*-methyl-D-glucose; 6DG, 6-deoxy-D-glucose; Fuc, fucose; Xyl, xylose; Ara, arabinose.

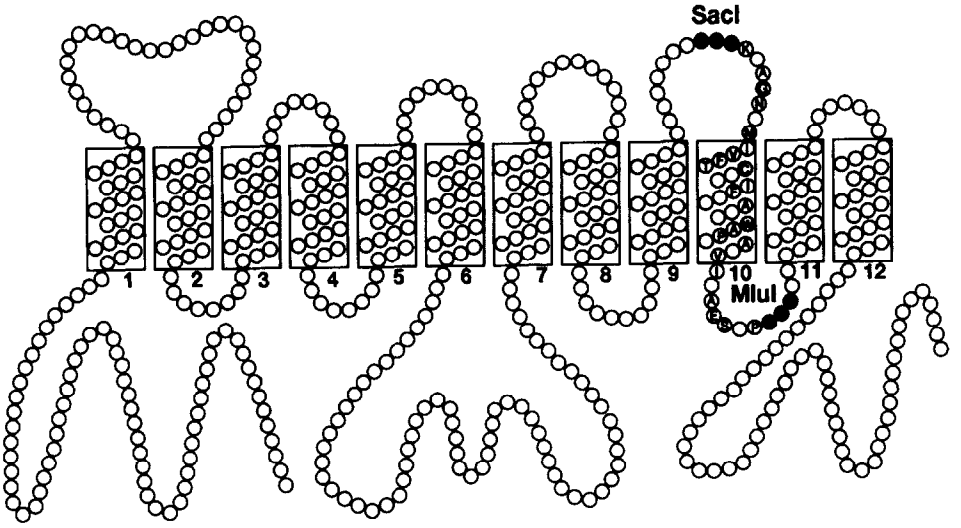

Fig. 5. Schematic representation of the Gal2 transporter. In the chimeras used in this study, the TM10 region is coded by a DNA fragment bordered by restriction enzymes, *Sac*I and *Mlu*I. Of 35 amino acids in this region, 12 are different between Gal2 and Hxt2 and 23 are identical as shown by the one letter amino acid code. It is noteworthy that when TM10 is depicted as forming an α-helix, these identical amino acids are situated at one side of the helix and different amino acids on the opposite side; this might be suitable for making almost the same hydrophilic pore with different substrate specificity.

have pointed out two substrate binding sites in Glut family transporters, one for an outward-facing sugar-binding site and the other for an inward-facing [5], our results have indicated only one in the 101 amino acid region. The possibility that this region might contain two substrate binding sites could be raised, although the region seems too short to accommodate two sites. In addition, the fact that only one recognition region emerged can be interpreted as indicating that the 35 amino acid region is the direct interaction site with galactose or glucose, but not the place indirectly affecting substrate recognition that takes place in the other region that should have been identified in our comprehensive search.

Previous studies have pointed out two functionally important amino acids in TM10 region. Tamori et al. [13] observed that substitution of $Pro^{385} \rightarrow Ile$ of Glut1, corresponding to Pro^{452} of Gal2, caused inhibition of glucose transport and suggested that conformational flexibility of the proline in TM10 is important. Garcia et al. [14] observed in *Xenopus* oocytes that the $Trp^{388} \rightarrow Gly$ or $Trp^{388} \rightarrow Leu$ mutation caused a decreased level of Glut1, decreased targeting to the plasma membrane and a modest reduction in the intrinsic activity, whereas with the $Trp^{388} \rightarrow Leu$ mutation Oka and coworkers [15,16] noted a decrease in 3-*O*-methylglucose transport in Chinese hamster ovary cells under zero-trans influx or equilibrium exchange but no apparent decrease under zero-trans efflux and they suggested a decrease in the rate of interconversion between the inward-facing and outward-facing conformations of the transporter. Trp^{388} has been proposed as a candidate for the binding site of forskolin [17]. With the $Trp^{388} \rightarrow Leu$ substitution, however, photolabelling of a forskolin analogue was inhibited by 70% in COS cells [18] and only by 30% in Chinese hamster cells [15]. These data on the effects of Pro^{385} or Trp^{388} substitution were not all consistent, but indicated that changes in the TM10 region affect several aspects of glucose transport in Glut1. Our data are consistent with this notion and clearly indicate that TM10 is related to substrate recognition. It is of considerable interest whether the site for differential recognition of galactose and glucose contains these two amino acids in the TM10 region.

Among Glut family transporters, the number of those transporting galactose is limited, and their substrate specificity is so broad that many other monosaccharides including glucose are accepted. They include GalP in *Escherichia coli* [19], STP1 in *Arabidopsis thaliana* [20,21], HUP2 in *Chlorella kessleri* [22] and SGTP1 in *Schistosoma mansoni* [23]. The alignment of amino acid sequences indicates that no single amino acid is conserved in the TM10 region among these transporters suggesting that more than two amino acids are related to differential recognition of galactose and glucose or that substrate recognition sites may not be conserved among these transporters. In this respect, the recent report by Will and Tanner [24] is of considerable interest. They made six chimeras between HUP1 and HUP2 transporters of *Chlorella kessleri* and claimed that the first external loop is important for substrate recognition and binding. Although properties of these chimeras did not solely reflect either of the original transporters, their data together with ours seem to support the notion that different members of the Glut family transporters recognize substrate at different sites. One of the possibilities Will and Tanner have raised, that the first extracellular loop and the last extracellular loop may corporate in galactose binding, is not supported in our study, since our study indicates the importance of TM10, but not the other regions. In addition, it should be noted that the difference between galactose and glucose is minor: epimeric at the C-4 carbon and a possible difference in α- or β-anomers as the transporter-discernible form. Thus, the other portion of monosaccharides might be recognized in the other parts of the transporters. To clarify this matter will require similar chimera studies using other pairs of sugars such as glucose and mannose.

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