**CONTRIBUTION TO SUBSTRATE RECOGNITION OF TWO AROMATIC AMINO ACID RESIDUES IN PUTATIVE TRANSMEMBRANE SEGMENT 10 OF THE YEAST SUGAR TRANSPORTERS GAL2 AND HXT2**

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The comprehensive study of chimeras between the Gal2 galactose transporter and the Hxt2 glucose transporter of *Saccharomyces cerevisiae* has shown that Tyr<sup>446</sup> is essential and Trp<sup>455</sup> is important for galactose recognition by Gal2. Consistent with this finding, replacement of the corresponding Phe<sup>431</sup> and Tyr<sup>440</sup> residues of Hxt2 with Tyr and Trp, respectively, allowed Hxt2 to transport galactose, suggesting that the two amino acid residues in putative transmembrane segment 10 play a definite role in galactose recognition. (Kasahara, M., Shimoda, E., and Maeda, M. (1997) J. Biol. Chem. 272, 16721–16724). Replacement of Trp<sup>455</sup> of Gal2 with any of the other 19 amino acids was shown to reduce galactose transport activity to between 0 and <20% of that of wild-type Gal2. The role of Phe<sup>431</sup> in Hxt2 was similarly studied. Other than Phe, only Tyr at position 431 was able to support glucose transport activity, at the reduced level of <20%. In contrast, replacement of Tyr<sup>440</sup> of Hxt2 with other amino acids revealed that most replacements, with the exception of Pro and charged amino acids, supported glucose transport activity. The importance of residue 431 in sugar recognition was more pronounced in a modified Hxt2 in which Tyr<sup>440</sup> was replaced with Trp. Glucose transport was supported only by the aromatic amino acids Phe, Tyr, and Trp at position 431, and galactose transport was supported only by Tyr. These results suggest that an aromatic amino acid located in the middle of transmembrane segment 10 (Tyr<sup>446</sup> in Gal2 and Phe<sup>431</sup> in Hxt2) plays a critical role in substrate recognition in the yeast sugar transporter family to which Gal2 and Hxt2 belong.

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†† The abbreviations used are: TM, transmembrane; PCR, polymerase chain reaction.
The nucleotide sequences of GAL2 and HXT2 were modified to encode Gal2(X-W), Gal2(Y-X), Hxt2(F-X), Hxt2(X-W), and Hxt2(Y-X) transporters as described under "Experimental Procedures." The original codons are UAU(Y446) in GAL2 and UUC(F451) and UAC(Y446) in HXT2.

### Table I

| Codon usage for mutation of GAL2 and HXT2 |
|------------------------------------------|
| **X446 Codon** | **X446 Codon** | **X446 Codon** | **Y446 Codon** | **Y446 Codon** | **Y446 Codon** |
| GGG | GGG | GGG | GGG | GGG | GGG |
| ACC | ACC | ACC | ACC | ACC | ACC |
| GUC | GAG | ACG | GGG | GGA | GGG |
| CUG | CGU | UUG | UGG | UGG | UGG |
| AUG | AUG | AUG | AUG | AUG | AUG |
| CGU | CUG | UGU | UGU | UGU | UGU |
| AGU | AGA | AGC | AGG | AGG | AGG |
| GAG | GAA | GAC | GAG | GAG | GAG |
| UGK | UGA | UGC | UGG | UGG | UGG |
| CGK | CGA | CGC | CGG | CGG | CGG |
| AUG | AUG | AUG | AUG | AUG | AUG |
| CUG | CUA | CUC | CUG | CUG | CUG |
| UAG | UAA | UAG | UAA | UAA | UAA |
| AGA | AGC | AGT | AGA | AGA | AGA |
| GAG | GAA | GAC | GAG | GAG | GAG |
| UGK | UGA | UGC | UGG | UGG | UGG |
| CGK | CGA | CGC | CGG | CGG | CGG |
| AUG | AUG | AUG | AUG | AUG | AUG |
| CUG | CUA | CUC | CUG | CUG | CUG |
| UAG | UAA | UAG | UAA | UAA | UAA |
| AGA | AGC | AGT | AGA | AGA | AGA |
| GAG | GAA | GAC | GAG | GAG | GAG |

With that of HXT2, yielding HXT2-pTV3e. Both GAL2-pTV3e and HXT2-pTV3e were further modified to create five restriction enzyme sites (Sac I, Mlu I, Spe I, Stu I, and Nol I) in the distal half of each gene (6), yielding GAL2-pTV3e and HXT2-pTV3e, respectively (the Stu I site in GAL2 and the Nol I site in HXT2 are preexisting sites).

**Replacement of Specific Residues in Gal2 or Hxt2—Residues Tyr446 and Trp450** of Gal2 and the corresponding Phe431 and Tyr440 of Hxt2 were targeted for by each of the other 19 amino acids. The nucleotide sequence of Gal2 coding for Trp450 was randomly modified by PCR with a degenerate primer. A forward primer 5'-CACG TAAAAGC CCCG CTCTA AAGGT GCCGG TA and a degenerate reverse primer 5'-TTCGA CTTGA CGCGT AGTGG GAATG were washed with H2O and disrupted with glass beads. The homogenates were used for long PCR (Ex Taq, Takara). Twenty-five cycles of 95 °C for 30 s, 50 °C for 2 min and 72 °C for 1 min were performed with a thermocycler (model 2400, Perkin-Elmer). The PCR products were digested with Sac I and Mlu I and introduced into the corresponding position of GAL2-pTV3e. A total of 40 clones was sequenced, and 14 clones harboring the various Gal2 or Hxt2 plasmids was measured at 30 °C for 5 s (7). Transport of galactose and glucose (5–7) in place of LBY416 or added a mixture of protease inhibitors consisting of 5 mM EDTA, pepstatin A (2 μg/ml), leupeptin (2 μg/ml), aprotinin (100 units/ml), and 2 mM phenylmethylsulfonyl fluoride (final concentrations) to the homogenization solution. Neither approach appeared to affect the amounts of these transporters.

**RESULTS**

**Effects of Substrate Concentration on Galactose and Glucose Transport by Gal2(Y-X) Transporters**—Our previous studies with systematic series of chimeras of Gal2 and Hxt2 revealed that Tyr446 in Gal2 is essential for the differential recognition of galactose and glucose (5–7). To characterize further the role of Tyr446 in substrate recognition, we measured transport of galactose or glucose by Gal2(X-W) transporters at a substrate concentration (10 mM) 100 times that used previously (Fig. 1). Even at this high substrate concentration, substantial galactose transport was mediated by many of the Gal2(X-W) transporters (Fig. 1B). Gal2(Y-W) and Gal2(F-W) showed 12% of the galactose transport activity of Gal2, whereas the other Gal2(X-W) transporters showed no significant activity (Fig. 1A). Glucose transport at the high substrate concentration was mediated by many of the Gal2(X-W) transporters (Fig. 1B). Gal2(Y-W) and Gal2(F-W) were most active, with Gly, Ala, Val, Leu, Met, Cys, Ser, Thr, and Asn substitutions also conferring transport activity at the high glucose concentration. Comparison of glucose transport activities between substrate concentrations of 0.1 and 10 mM indicates that the transporters with glucose transport activities as high as 20% at 10 mM glucose possess 3 values of 100 mM. The differences in transport activity were not due to differences in the extent of expression of Gal2(X-W) transporters, as revealed by immunoblot analysis of cell homogenates (Fig. 2A).

**Galactose and Glucose Transport by Gal2(Y-X) Transporters**—We have previously shown that Trp450 of Gal2 is important for the differential recognition of galactose and glucose (7). The replacement of Trp450 with any of the other 19 amino acids homgeneous was subjected to SDS-gel electrophoresis and blotted onto a polyvinylidene difluoride membrane (Immobilon P², Millipore), followed by incubation with 125I-protein A (IM144, Amersham Pharmacia Biotech) overnight. Autoradiography was performed with imaging plates (BAS2900, Fuji Film). Under the present conditions, a linear relation of amounts of protein and radioactivity was observed. These antibodies reacted with Gal2 or Hxt2, but not with other homologous proteins revealed by the yeast genome sequence (5, 6). To evaluate the possible degradation of modified Hxt2 transporters in vivo and in vitro, we used the protease-deficient strain BJ3505 (MATa pep4::HIS3 prb1-Δ1.6R his3 (by trp1 can1)) in place of LBY416 or added a mixture of protease inhibitors consisting of 5 mM EDTA, pepstatin A (2 μg/ml), leupeptin (2 μg/ml), aprotinin (100 units/ml), and 2 mM phenylmethylsulfonyl fluoride (final concentrations) to the homogenization solution. Ne
markedly reduced galactose transport to <20% of that of Gal2, although significant activity was exhibited by transporters containing Tyr, Cys, Thr, Ile, or Met at this position (Fig. 3). In glucose transport assays, Gal2(Y-W), or wild-type Gal2, showed only 28% of the activity of Hxt2; this may be mainly due to Tyr446 since replacement of this residue with Phe increased the glucose transport activity to 60% of that of Hxt2 (Fig. 1B).

Aromatic Amino Acids in Yeast Gal2 and Hxt2 Transporters

Importance of Phe\textsuperscript{431} and Tyr\textsuperscript{440} of Hxt2 for Glucose Transport—Our previous study (7) and the results described above indicate that Tyr\textsuperscript{446} in Gal2 is essential for the recognition of galactose. Since Gal2 and Hxt2 share an amino acid sequence identity of ~70% (5), it is reasonable to hypothesize that Phe\textsuperscript{431} of Hxt2, corresponding to Tyr\textsuperscript{446} of Gal2, may be important for recognition of glucose. Transport of glucose or galactose in cells expressing Hxt2(X-Y) transporters was measured at a substrate concentration of 0.1 mM (Fig. 4A). The only transporter other than Hxt2(F-Y), or wild-type Hxt2, that exhibited significant glucose transport activity was Hxt2(Y-Y), which showed an activity of 17% of that of Hxt2. In contrast, none of the other Gal2(Y-X) transporters supported significant activity. The expression levels were similar for each of the Gal2(Y-X) transporters (Fig. 2B).
Hxt2(X-Y) transporters supported galactose transport. Some of the Hxt2(X-Y) transporters were expressed at a low level, as revealed by immunoblot analysis (Fig. 5A). Transporters containing Pro or charged amino acids at position 431 tended to show a reduced level of expression, suggesting that a proper conformation of the protein is necessary for it to maintain its integrity, although the possibility of protein degradation during preparation can not be excluded at present (see “Experimental Procedures” and “Discussion”).

To study the role of Tyr 440 of Hxt2, we measured glucose transport in cells expressing Hxt2(F-X) transporters (Fig. 4B). In contrast to Hxt2(X-Y) transporters, many Hxt2(F-X) transporters showed high glucose transport activities. However, no significant glucose transport activity was apparent with transporters containing Pro or the charged amino acids Asp, Glu, His, Lys, or Arg at position 440. None of the Hxt2(F-X) transporters exhibited galactose transport activity. Since the expression of transporters containing these charged amino acids was markedly reduced (Fig. 5B), the results obtained with the corresponding cells were inconclusive.

**Galactose Transport by Doubly Modified Hxt2(X-W) and Hxt2(Y-X) Transporters**—In the previous study (7), to confirm that Tyr 446 and Trp 455 of Gal2 are important for galactose recognition, we replaced the corresponding amino acids of Hxt2, Phe 431, and Tyr 440, with Tyr and Trp, respectively. The resulting Hxt2(Y-W) transporter mediated galactose transport in addition to glucose transport. In the present study, we constructed the Hxt2(X-W) series of transporters to determine whether amino acids other than Tyr at position of 431 of Hxt2 can support galactose transport. Hxt2(Y-W) was the only transporter in the Hxt2(X-W) series that exhibited galactose transport activity (Fig. 6A). This apparently essential role for Tyr 441 in galactose transport by doubly modified Hxt2 suggests that, in wild-type Hxt2, Phe 431 together with Tyr 440 acts to admit glucose and reject galactose. Hxt2(Y-W) showed a $K_m$ for galactose of 43 mM, which is about eight times that of Gal2 and almost identical to that of Hxt2–8, which possesses a Gal2-derived TM10 (6). For glucose transport, the importance of aromatic amino acids at position 431 was apparent, with only Hxt2(X-W) transporters containing Phe, Tyr, or Trp exhibiting glucose transport activity.

Hxt2(Y-X) transporters behaved differently (Fig. 6B). For galactose transport, Trp, Met, Cys, Thr, and Leu were effective, but the aromatic amino acids Phe and Tyr were not. Thus, the combination of Tyr 431 and Trp 440 present in Gal2 was also most effective in conferring galactose transport activity on the background of Hxt2. Glucose transport in cells expressing Hxt2(Y-X) transporters was at most 25% of that in cells expressing Hxt2, a value similar to that obtained with cells expressing Gal2. Glucose transport at this reduced level was observed with Hxt2(Y-X) transporters containing a wide range of amino acids, including Trp, Phe, Tyr, Cys, Thr, Met, Leu, and Ile. Taken together, our data indicate that the combination of Phe 431 and Tyr 440 is most accepting of glucose and rejects galactose efficiently. The extent of expression of Hxt2(Y-I) and Hxt2(Y-D) was low, indicating that no definitive conclusions can be drawn for these mutants (Fig. 7B).

**DISCUSSION**

In this study, we have demonstrated the importance of two aromatic amino acid residues in putative TM10 for substrate recognition by Gal2 and Hxt2. Hereafter, the aromatic amino acid situated in the middle of TM10, Tyr 446 in Gal2 and Phe 431 in Hxt2, is referred to as the middle aromatic site (Fig. 8), and
that situated at the cytoplasmic end of TM10, Trp^{455} in Gal2 and Tyr^{440} in Hxt2, is referred to as the cytoplasmic aromatic site.

We previously showed (7) that Tyr^{446} of Gal2 is essential for the differential recognition of galactose and glucose on the basis that none of 19 other amino acids was able to replace it for galactose transport. However, it remained possible that the apparent inability of other amino acids to support galactose transport may have been overcome by higher concentrations of substrate. In the present study, to determine whether the role of the middle aromatic site is merely to contribute to the affinity for galactose, we compared galactose transport at substrate concentrations of 0.1 and 10 mM. Our observation that, even at the high galactose concentration, substantial galactose transport was mediated only by Gal2(Y-W), with Gal2(F-W) showing a small amount of activity, is consistent with the idea that Tyr at the middle aromatic site is not merely a contributor to the affinity for galactose, but rather is exclusively required for the selection of galactose. In contrast, for glucose transport, Tyr at the middle aromatic site of Gal2 was not essential. The observation that other amino acid residues were able to replace it, especially at the high substrate concentration, indicates that the role of this Tyr residue in glucose transport is to increase the affinity for glucose. Glucose transport in cells expressing Hxt2(X-Y) transporters was supported by Phe and, to a much lesser extent, by Tyr. A similar tendency was apparent with Hxt2(X-W) transporters, in which the requirement for an aromatic amino acid showed an order of preference of Phe ≫ Tyr > Trp, as well as with Gal2(X-W) transporters, which showed the same rank order of preference at the low glucose concentration. These observations suggest that the glucose recognition process is similar in both transporters. The apparently different roles of the middle aromatic site in galactose and glucose transport might be indicative of multiple functions of the amino acid at this site (see below).

With regard to the cytoplasmic aromatic site, galactose transport by Gal2(Y-X) transporters was preferentially supported by Trp, with several other amino acids, including Tyr > Cys = Met = Thr > Ile, also conferring activity. Glucose transport by Hxt2(F-X) transporters was supported by most amino acids, excluding Pro and charged residues. Similar patterns of glucose transport activities were observed with Hxt2(Y-X) and Gal2(Y-X) transporters, although Tyr at the middle aromatic site appeared to reduce the activities of these series. These results indicated that the interaction of the cytoplasmic site with glucose may be similar in Gal2 and Hxt2. It should be mentioned that the variable level of expression of Hxt2(F-X) and Hxt2(Y-X) transporters revealed by immunoblot analysis may not necessarily reflect the amounts of the transporters in intact cells, since the expression level of Gal2(Y-X) transporters was not variable and yet they showed a pattern of glucose transport activity similar to those of the Hxt2(F-X) and Hxt2(Y-X) transporters.

A total of 18 closely related sugar transporters has been identified in S. cerevisiae, the cluster I of sugar permease homologs (4) that is equivalent to the hexose transporter family (3), excluding Snf3 and Rgt2, which appear to be glucose sensor (11). Of these 18 homologs, only Gal2 contains Tyr at the middle aromatic site and Trp at the cytoplasmic site. All the other 17 homologs, including Hxt4, Hxt12, and Hxt14, show that the middle aromatic site in galactose and glucose transport might be indicative of multiple functions of the amino acid at this site (see below).

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galactose transporter and that the other 17 transporters are glucose transporters. If this is the case, low affinity galactose transport noted in previous studies (2) may be attributable to one or more of the glucose transporters carrying galactose with low affinity. This notion is also consistent with the selection of galactose transport-positive clones from gal2 mutants (7) and the selection of glucose transport-positive clones from strains carrying gal2 and multiple hxt mutations in the presence of antimycin (12).

Several functions for the two aromatic sites in galactose transport by Gal2 have been proposed (7), including roles in steric hindrance, hydrogen bonding, a stacking effect with the sugar, and structural changes in other amino acids that interact directly with the sugar. In addition, the possibility that the two amino acids function independently has also been suggested. Recent structural studies on porins (13, 14) may be worth mentioning in this respect. The three-dimensional structures of maltoporin and the sucrose-specific porin SrcY analyzed by x-ray crystallography indicate that maltose and sucrose passes through the corresponding porins via a relay of aromatic amino acids. Since most galactose and glucose molecules are in the form of β- and α-pyranose in aqueous solution (15), differential recognition of galactose and glucose requires differentiation between α- and β-anomers and C4 epimers.

Crystallographic studies of binding proteins in the periplasm of bacteria (16) and of lectins (17), in addition to the porin studies, have shown that the binding of sugars to these proteins is mediated by hydrogen bonding to various amino acids and H2O and stacking of the sugars with aromatic amino acids. Thus, further insight into the molecular mechanism of substrate recognition by Gal2 and Hxt2 should be provided by identification of the amino acid residues that presumably form hydrogen bonds with galactose and glucose located at the middle site. In addition, it will be important to determine whether other aromatic amino acids in these transporters contribute to substrate binding.

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