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NOTES

Cloning of a Gene Encoding Choline Transport in *Saccharomyces cerevisiae*

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By genetic complementation in a yeast choline transport mutant from a yeast gene library, we isolated plasmids encoding choline transport. The cloned plasmids contained a common 4.0-kilobase DNA fragment and also complemented an ethanolamine transport defect. The cloned sequence present in the yeast genome was possibly unique.

The yeast *Saccharomyces cerevisiae* possesses the CDPcholine pathway and can utilize extracellular choline for the synthesis of phosphatidylcholine. Hosaka and Yamashita have shown that the entry of choline into yeast cells is mediated by a specific transport system, and mutants defective in this process have been isolated (3). The transport system is dependent on metabolic energy and exhibits a high affinity for choline. Competition studies have shown that the choline transport system also mediates the entry of *N*-methylethanolamine, *N*,*N*-dimethylethanolamine, and β-methylcholine, but unambiguous data have not been obtained that show whether ethanolamine is incorporated through the same transport system. Furthermore, although the enzymes of the CDPcholine pathway are constitutive, the choline transport system is highly regulated and inducible and serves as a regulatory step in the CDPcholine pathway (4). As a first step in clarifying the expression of the gene encoding choline transport, we attempted to clone the gene in *S. cerevisiae* by using a genetic complementation method.

The choline transport mutant (strain 426 α *ctr*) used for gene cloning was obtained by UV mutagenesis of wild-type X2180-1B cells (Yeast Genetic Stock Center, University of California), followed by selection for 2-hydroxyethylhydrazine resistance (6). 2-Hydroxyethylhydrazine inhibits yeast phospholipid methylation after its entry into cells through the choline transport system (6). Therefore, a defect in choline transport renders cells resistant to 2-hydroxyethylhydrazine. When the wild-type choline transport gene (*CTR*) is introduced into the mutant, cells are transformed to the 2-hydroxyethylhydrazine-sensitive phenotype, Thus, the selection of transformants with this inhibitor is difficult. To facilitate the positive selection of transformants, we introduced the *ctr* mutation into strain 172 α *ise* (11), a mutant involved in the regulation of the phosphatidylethanolamine methylation pathway. Although the methyltransferases in the wild-type strain are repressed by the coexistence of *myo*-inositol and choline, the enzymes in the *ise* mutant are repressed by *myo*-inositol alone (11, 12). Therefore, the *ise* mutant will grow on a low-inositol medium but not on a high-inositol medium. However, choline supplementation will restore growth by stimulating the supply of phosphatidyl choline via the CDPcholine pathway. We constructed a double mutant that carried the *ctr* mutation in the *ise* background. The strain failed to grow on a choline-supplemented high-inositol medium. We expected that the choline transport gene could be cloned by positive selection of transformants growing on a choline-supplemented high-inositol medium.

Choline transport mutant 426 (α *ctr*) was backcrossed with wild-type strain X2180-1A (Yeast Genetic Stock Center, University of California) to remove any possible secondary mutations. The *ctr* mutant was crossed with strain 172 (α *ise*) and then with strain AH22 (a *leu2 his4*; provided by N. Gunge, Central Research Laboratories, Mitsubishi Chemical Industries) by standard genetic methods to construct cloning host strain D308-14D (α *ctr ise leu2 his4*). A yeast gene library was constructed by ligating a partial *Sau*3AI digest of wild-type X2180-1B genomic DNA into the *Bam*HI site of the yeast episomal plasmid YEp13, which contains the yeast *LEU2* gene as a selectable marker (2), as described previously (7). D308-14D cells were treated with Zymolyase 60000 (Kirin Brewery, Tokyo, Japan), and the resultant spheroplasts were transformed with the gene library by the method of Beggs (1). Transformants were selected on minimum medium containing choline, *myo*-inositol, and L-histidine at concentrations of 20 μg/ml each and were examined for choline transport activity by the replica-printing method previously described for the *myo*-inositol transport assay (8). Three clones showing choline transport activity were obtained, one of which was a revertant. Approximately one-half of the cells of the two nonrevertant clones simultaneously lost the Leu^+ phenotype and choline transport activity after they were cultured for 11 generations in a nonselective medium. Therefore, we concluded that the Leu^+ phenotype and choline transport activity in these clones were linked to a nonintegrated plasmid which was mitotically rather unstable under nonselective conditions.

Plasmids were isolated from these two clones and then used to transform *Escherichia coli* ML4901 (5) (Nal^r F^- *galK2 galT22 hsdR lacY1 metB1 relA supE44*; provided by M. Inoue and S. Mitsuhashi, Gunma University). Two different plasmids (designated as pCT12 and pCT18) were obtained from ampicillin-resistant *E. coli* cells which complemented the *ctr* and *leu2* mutations simultaneously in yeast strain D308-14D. Restriction endonuclease analysis showed

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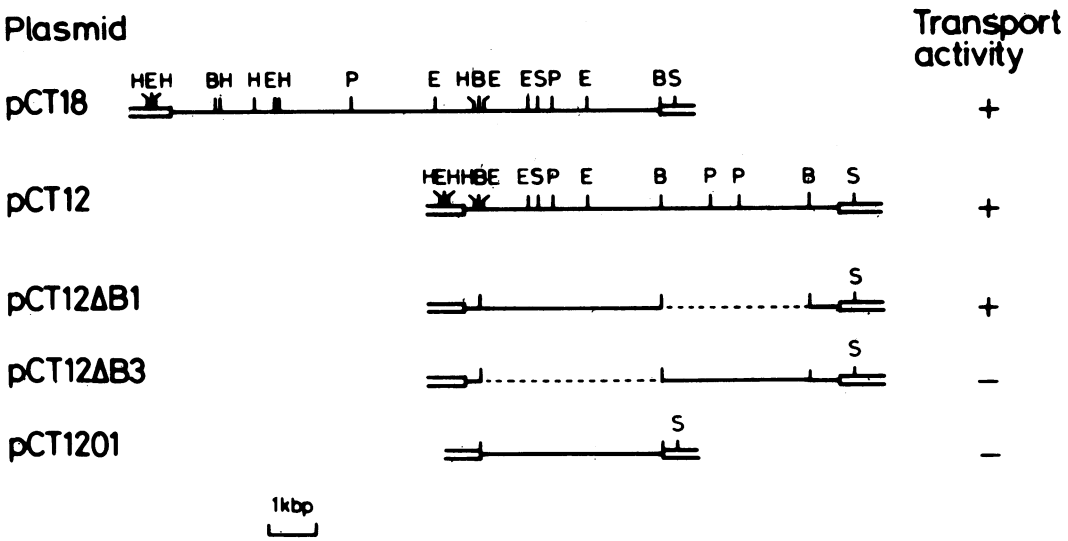

FIG. 1. Restriction endonuclease maps for pCT12, pCT18, and deletion derivatives. The boxes represent the YEp13 vector, and the lines between them represent inserted DNA. -----, Deleted DNA sequence. The locations of the sites for restriction endonucleases *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Sal*I (S), and *Pst*I (P) are shown. kbp, Kilobase pair. The choline transport activity of the transformant carrying each plasmid, determined by the replica-printing method (8), is indicated on the right by + (activity) or – (no activity).

that the pCT12 and pCT18 plasmids possessed a common 4.0-kilobase-pair DNA sequence (Fig. 1). This was further confirmed by Southern blot hybridization with the 3.7-kilobase *Bam*HI fragment of pCT18 as a probe (Fig. 2B, lanes 5 and 6). Various deletion derivatives of the pCT12 plasmid were constructed, and the choline transport of the transformants was examined by the replica-printing method (Fig. 1). The results of the examination supported the hypothesis that the *CTR* gene is located to the left of the middle *Bam*HI site in the pCT12 insert. A closer comparison of choline transport activity revealed that the pCT12ΔB1 transformants were much less active than the pCT18 transformant (Table 1). Deletion of the left extreme portion of the pCT12 insert abolished the activity (pCT1201). A portion of the pCT18 sequence lying to the left of the left end of the pCT12 insert probably is required for full expression of the *CTR* gene.

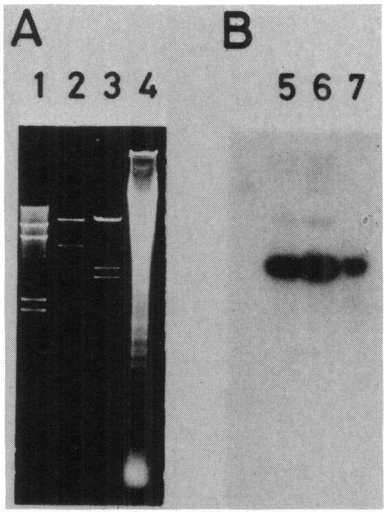

FIG. 2. Southern blot analysis of cloned plasmids and genomic DNA. Plasmid DNA of pCT12 and pCT18 and genomic DNA from wild-type strain X2180 cells were digested with restriction endonuclease *Bam*HI and fractionated by electrophoresis on a 0.7% agarose gel. The DNA fragments were transferred to a nylon membrane filter (Poll Biodyne A) and hybridized to the pCT18 3.7-kilobase *Bam*HI fragment which had been labeled with ^{32}P by nick translation. (A) Ethidium bromide-stained agarose gel. (B) Autoradiogram of the Southern blot hybridized with ^{32}P-labeled probe. *Hin*dIII digests of λ DNA were used as size markers (lane 1), and other lanes contained *Bam*HI digests of pCT18 (lanes 2 and 5), pCT12 (lanes 3 and 6), and genomic DNA from X2180-1B (lanes 4 and 7).

S. cerevisiae possesses the CDPethanolamine pathway, which synthesizes phosphatidylethanolamine from ethanolamine (10). Since ethanolamine is structurally related to choline, it was of interest to know whether ethanolamine and choline transports were mediated by the same transport system. As shown in Table 1, mutant cells were deficient in both choline and ethanolamine transports. Of the plasmids, pCT18 was the most effective, pCT12 and pCT12ΔB1 were much less effective, and pCT12ΔB3 and pCT1202 were totally inactive. These results strongly suggest that a single gene is responsible for both choline and ethanolamine trans-

TABLE 1. Choline and ethanolamine transport activities

Strain and plasmid	Transport activity (mmol of choline or ethanolamine transported/min per mg of protein)[a]	
	Choline	Ethanolamine
X2180-1B (wild)	7.51	19.4
D308-14D (mutant)		
None	0.06	0.78
pCT18	3.56	18.2
pCT12	0.85	1.44
pCT12ΔB1	0.96	2.12
pCT12ΔB3	0.01	0.79
pCT1201	0.15	0.78

[a] Choline transport activity was assayed as described previously (3). Ethanolamine transport activity was assayed in a similar manner, except that 2 μmol of [1,2-^{14}C]ethanolamine was used instead of [*methyl*-^{14}C]choline as the substrate.

port and that the CDPcholine and CDPethanolamine pathways share the same transport system in *S. cerevisiae*.

To confirm that the cloned sequences were derived from the yeast genomic DNA, we carried out a blotting experiment in which wild-type yeast genomic DNA and the pCT12 and pCT18 plasmids were digested with *Bam*HI, electrophoresed, and then subjected to Southern blotting (9). The ^{32}P-labeled 3.7-kilobase *Bam*HI fragment of pCT18 obtained by nick translation was hybridized, and autoradiography was used to visualize the resulting hybrids. A single band was present in the *Bam*HI digest, which comigrated with the 3.7-kilobase fragments of pCT12 and pCT18 plasmids, (Fig. 2B, lane 7), indicating that the cloned sequences were derived from the genomic DNA. The data also suggest that the choline transport gene is present in the yeast genome as a unique sequence.

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